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DOPAMINERGIC MODULATION OF PHENOTYPICAL AND FUNCTIONAL CHARACTERISTICS OF HUMAN T LYMPHOCYTES: PERSPECTIVES FOR NONCONVENTIONAL IMMUNOMODULATION

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Table of contents

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Abbreviations

α-syn	alpha-synuclein
5-HT	serotonin
6-OHDA	6-hydroxydopamine
7-AAD	7-aminoactinomycin
A	adrenaline
AAAD	aromatic amino acid decarboxylase
Ab/Abs	antibodies
AC	adenylyl cyclise
ADH	antidiuretic hormone
ADHD	attention deficit hyperactivity disorder
Ag	antigen
ANF	atrial natriuretic factor
AP-1	activator protein 1
APC	antigen presenting cell
AR	adrenoceptors
BBB	brain blood barrier
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BM	bone marrow
CA	catecholamine
cAMP	cyclic adenosine monophosphate
CD	Crohn's disease
CHF	congestive heart failure
CIA	collagen-induced arthritis
CNS	central nervous system
COMT	catechol-O-methyl transferase
Con A	concanvalin A
COPD	chronic obstructive pulmonary disease
CPD670	Cell Proliferation Dye eFluor670
CREB	cAMP-responsive element-binding protein
CSF	cerebro spinal fluid
CTLA-4	cytotoxic T-lymphocyte-associated protein 4

DA	dopamine (IUPAC: 4- (2-aminoethyl)benzene-1,2-diol)
DβH	dopamine β-hydroxylase
DAT	dopamine transporter
DDIs	DOPA-decarboxylase inhibitors
DLB	dementia with Lewy bodies
DOPA	dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DR	dopamine receptors
EA	early apoptotic cells
EAE	experimental autoimmune encephalomyelitis
EMT	extraneuronal monoamine transporter
FBS	heat-inactivated foetal bovine serum
FSC	forward scatter
GABA	γ-aminobutyric acid
GAT	GABA transporter
GDP	guanosine 5'- diphosphate
GH	growth hormone
GI	growth index
GITR	glucocorticoid-induced TNFR-related protein
GM-CFU	granulocyte/macrophage-colony-forming unit
GnRH	gonadotropin-releasing hormone
GTP	guanosine 5'-triphosphate
GPCRs	G protein coupled receptors
GTS	Gilles de la Tourette syndrome
HEV	high endothelial venules
HPLC	high performance liquid chromatographic method
HS	healthy subjects
HSC	hematopoietic stem cell
HVA	homovanillic acid
HVEM	herpes virus entry mediator
IBD	inflammatory bowel diseases
ICAM-1	Intercellular Adhesion Molecule 1
ICOS	inducible T cell co-stimulator

IGF-1	insulin-like growth factor-1
ION	inonomycin
LA	late apoptotic cells
LB	Lewy bodies
L-DOPA	levodopa
LFA	lymphocyte function associated antigen
MAO	monoamineoxidase enzyme(MAO-A,-B two distinct isoforms)
MDMA	3,4-methylenedioxymethamphetamine
MHC	major histocompatibility complex
MO-DCs	monocyte-derived DCs
MPP+	1-methyl-4-phenylpyridinium (positively charged MPTP)
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, neurotoxin
MS	multiple sclerosis
NA	noradrenaline
NAT	noradrenaline transporter
NFAT	nuclear factor of activated T cells
OCD	obsessive-compulsive disorder
OCT	organic cation transporter
PCNA	proliferating cell nuclear antigen
PCP	phencyclidine
PD	Parkinson's disease
PD-dn	drug naïve PD patients who had never been treated
PD-dt	drug treated PD patients (currently on dopaminergic therapy)
PD-L1	programmed cell death 1 ligand
PEGAR	PE-goat anti-rabbit
PET	positron emission tomography
PFC	prefrontal cortex
PHA	mitogen phytohemaglutinin
PKA	protein kinase A
PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophils
PNS	peripheral nervous system
PRIF	prolactin-release inhibiting factor

PWM	pokeweed mitogen
RA	rheumatoid arthritis
RT	room temperature
SERT	serotonin transporter
SIRS	systemic inflammatory response syndrome
SLE	systemic lupus erythematosus
SNpc	substantia nigra pars compacta
SNS	sympathetic nerve system
SSC	side scatter
STAT	signal transducer and activator of transcription factor
T_{CM}	T central memory
TCR	T Cell Receptor
T_{EM}	T effector memory
TEMRA	terminally differentiated central memory subset
TH	tyrosine hydroxylase
Th	T helper cell lineages (Th1/Th2/Th17)
TIM	Type 1 Trans-membrane Glycoprotein
Tn	T naïve
Treg	T regulatory cells
TSH	thyrotropin
TTd	tetanus toxoid
UC	ulcerative colitis
VMAT	vesicular membrane transporter
VTA	ventral tegmental area
VLA-4	Very Late Antigen 4
WT	wild type

Abstract

Dopamine (DA) besides its action in the nervous system, plays an important role in immune cells interactions. Emerging role of DA as a regulator of CD4+ T cells physiology is important since dysregulation of different T cell subsets, showing abnormal cell numbers, functions, expression of dopamine receptors (DR) and/or response to DA, could contribute to the onset and development of some immune-related disorders. Thus, directly and indirectly acting dopaminergic therapeutics, currently used in approved clinical indications, could represent an attractive source of non-conventional agents for the modulation of CD4+ T cell functions.

The aim of the present work was to develop *in vitro* methods to investigate the effects of dopaminergic agents, currently used in the pharmacotherapy, on the functional responses of CD4+ T cells, namely: (i) CD4+ T naïve (Tn), T central memory (T_{CM}) and T effector memory (T_{EM}) cells, and their responses to recall antigen (Ag); (ii) CD4+ T regulatory cells (Treg), and their suppressive effects on T effector cells (Teff) and (iii) CD4+ T naïve cells, and their ability to differentiate towards different T helper (Th) lineages (Th1/Th2/Th17).

In cultured CD4+ T cells, our results have shown higher expression of DR in apoptotic cells in comparison to viable cells and stimulation-induced DR upregulation of all DR on viable cells. Addition of high concentrations of DA and L-DOPA (100 μM) have shown profound effect on survival of CD4+ T cells. Interestingly, based on preliminary experiments, our *ex vivo* data have shown trend of proliferating cells expressing DR in higher percentages that still need to be validated in subsequent studies on more subjects. So far, *in vitro* tested concentrations of dopaminergic agonists have not shown any major effects on proliferation of CD4+ T cells.

In addition, through the use of flow cytometric analysis, expression of DR was examined on human: CD4+ naïve T lymphocytes (CD3+CD4+CD45RA+CCR7+), T_{CM} (CD3+CD4+CD45RA-CCR7+), T_{EM} (CD3+CD4+CD45RA-CCR7-), Treg cells (CD4+CD25highCD127low), and also frequency of different Th subsets: Th1 (CD4+CXCR3+CCR4-CCR6-), Th2 (CD4+CXCR3-CCR4+CCR6-), Th17 (CD4+CXCR3-CCR4-CCR6+) and Th1/Th17 (CD4+CXCR3+CCR4-CCR6+) were analysed. DR expression of all five DR was confirmed on each subset, present in a different extension potentially represents an opportunity to develop targeted immunomodulating strategies.

Validated and developed *in vitro* method to test functional response of memory CD4+ T cells towards recall Ag have potential relevance for a wide range of different fields of T cell biology research in health and disease. Additionally, obtained preliminary results have confirmed *in vitro* experimental conditions likely appropriate to study commitment of naïve CD4+ T cells and factors mimicking specific polarisation routes (Th1/Th2/Th17), which are T subsets important in onset and development of some dopamine-related disorders.

Further, *in vitro* methods have shown CD4+CD25high T cell-dependent inhibition of CD4+ T effector lymphocyte proliferation. Treg cells also suppressed production of IFN-γ and TNF-α from Teff cells. In addition, effects of DA and L-DOPA treatments seems to suppress Treg suppressive capacity in healthy subjects and in the group of Parkinson's disease (PD) patients who had never been treated (PD-dn), but not in PD patients that were on dopaminergic therapy (PD-dt).

Available evidence supports the possibility to repurpose dopaminergic agents as modulators of dopaminergic pathways, shifting the balance towards beneficial outcomes in some pathological conditions, such as PD. Over the last decades, an impressive number of studies in the animal model of immune diseases and in the clinical setting supported this evidence, and strongly required futher testing. The development of therapeutic protocols needs to take into account that DR exists in multiple subtypes and their patterns of expression, and that fuctional relevance differs among immune cells - and may even depend on the functional status (e.g. resting/activated) of specific cells.

Proposed *in vitro* methods examined and characterised the various CD4+ T cell lineages, providing both the conceptual as well as the experimental framework for more in-depth investigation of dopaminergic pathways modulating CD4+ T cell function.

I. Introduction - Physiopharmacology of dopamine

1.1. Physiopharmacology of dopamine

1.1.1. Physiology of dopamine

Dopamine was synthesized for the first time in 1910 by George Barger and James Ewens in London. It was named DA because it has a monoamine structure, containing nitrogen formed from ammonia by replacement of one of the hydrogen atoms by hydrocarbon radicals. Later on, the molecule itself was isolated and described as a neurotransmitter of the brain by Arvid Carlsson. For this discovery, together with Paul Greengard who described cellular signalling mechanisms by DA, these two researchers won the Nobel Prize for Medicine in 2000.

Dopamine is known today as one of the principal catecholamine neurotransmitters in the central nervous system (CNS). It has a large variety of actions and it is involved in control of several key functions such as brain reward, motivation and positive reinforcement (Bressan and Crippa, 2005). Overproduction in the "pleasure center" of the brain leads to addiction. Natural pleasurable and rewarding stimuli increase DA released in the nucleus accumbens area of the basal ("deep") forebrain. Some drugs (e.g. amphetamine, cocaine), with a high potential for abuse and addiction, also increase DA release by acting directly on dopaminergic neurons within the reward system, while others (e.g. alcohol, opiates) increase DA indirectly, via effects of other neurotransmitter systems. Dopamine in the brain regulates locomotor activity and movement (Cenci, 2007), attention span, emotional response, behaviour, cognition function, pain perception (Potvin et al., 2009) and neuroendocrine secretion (Missale et al., 1998). In order to understand the function of DA, it is important to consider sites of action in both CNS and peripheral tissues, since it has been shown that DA has a significant physiological role in the cardiovascular, renal, hormonal and gastrointestinal systems (Dayan, 2009).

Hereafter, the current knowledge on physiopharmacology of DA will firstly be reviewed, together with DR's distribution in CNS, as well as in peripheral tissues, discussing key roles and available evidence that DA has in selected pathological conditions, and the opportunity to repurpose dopaminergic agents as modulators of dopaminergic pathways shifting the balance towards beneficial outcomes.

Indeed, any directly and indirectly acting dopaminergic therapeutics currently used in approved clinical indications could represent an attractive source of non-conventional agents for the modulation of the described pathological processes.

1.1.2. ***Dopamine synthesis***

DA is a monoamine, classified as a catecholamine (CA) and a member of a group of neurotransmitters called "biogenic amines" together with noradrenaline (NA), adrenaline (A) and serotonin (5-HT). It originates from a group of catecholamines (CAs), chemical compounds containing a catechol moiety with a 3,4-dihydroxyphenyl group (catechol core of the molecule is a benzene ring with two adjacent hydroxyl groups) and an amine side chain, together define the main functional groups and activity of the molecule.

Dopamine, together with other CAs, is produced from non-essential amino acid, tyrosine. The enzyme dopamine β-hydroxylase (DβH) synthesizes NA from DA, and phenyletanolamine N-metyltransferase converts NA to A. Chemical structure and biosynthetic pathway is shown in **Figure 1**.

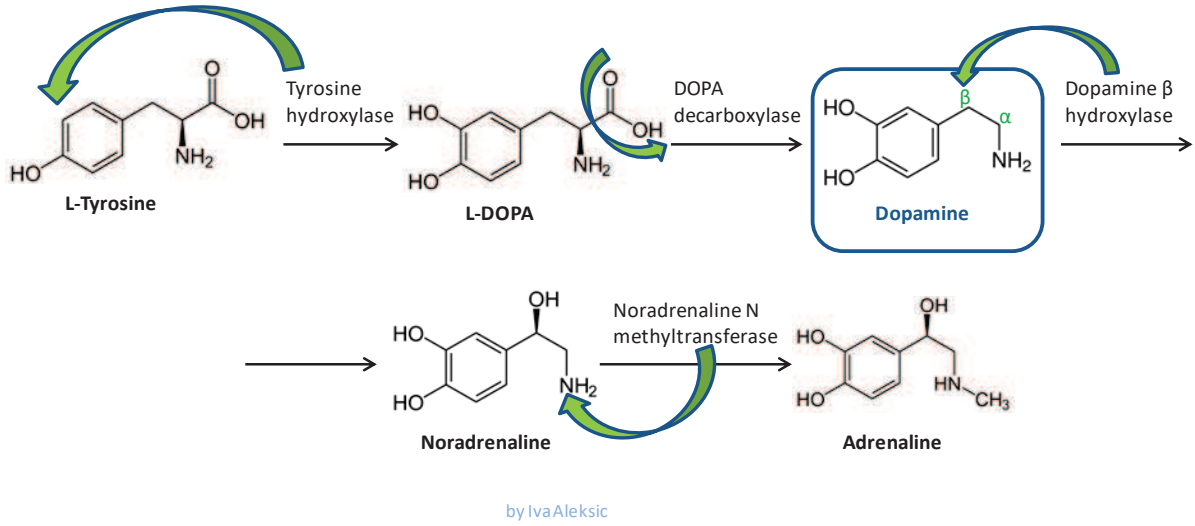


Figure 1. Dopamine synthesis

Dopamine is synthesized from its metabolic precursor L-tyrosine, a nonessential aromatic amino acid that is synthesized from phenylalanine. In DA-producing cells, the rate-limiting step in CA biosynthesis is the oxidation of L-tyrosine to (S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid (L-DOPA) by tyrosine hydroxylase (TH). This compound is subsequently metabolised by aromatic amino acid decarboxylase (AAAD) to produce cytosolic DA (Weihe et al., 2006). Tyrosine hydroxylase is a cytosolic enzyme that is present only in cells that are producing CAs. Tyrosine hydroxylase is a selective enzyme in the way that is not proceding derivatives of indol as substrates, part of other metabolic pathway leading to synthesis of 5-HT. This reaction is the key point of controlling the process of synthesis of NA,

since NA itself is inhibiting the TH enzyme, thus regulating the final rate of neurotransmitter production (rate limiting step reaction) which is indeed the faster way of synthesis regulation compared to synthesis of enzyme molecule *de novo*. The next step is decarboxylation of DOPA (dihydroxyphenylalanine) to DA by aromatic L-amino acid decarboxylase, also a cytosolic enzyme, that is present in a wide range of cells (not only CAs producing cells), that acts non-specifically, and does catalitical decarboxylation of amino acids such as L-hystidine and L-tryptofan, precursors of histamine and serotonin.

Dopamine is converted to NA in the vesicles by DβH and in adrenal medulla; it is further converted to A.

1.1.3. ***Dopamine store and reuptake***

Synthesized DA is normally stored into acidic vesicles. Cytosolic DA, that is either *de novo* synthesized or captured, is mobilised and stored toward intracellular vesicles, mediated by type 1 and type 2 vesicular monoamine transporters (VMATs, -1 and -2) (Masson et al., 1999; Mignini et al., 2006).

If not stored in the cytosol vesicles, DA can either be oxidised by monoamine oxidases (MAO) enzyme (Mignini et al., 2009) or in some cells, DA can adopt the third fate and be further processed by DβH to yield NA (Alaniz et al., 1999).

Dopamine can be also auto-oxidised to quinone and hydrogen peroxide when it is released into the neutral pH cytoplasm. The release is usually avoided, but it could occur when the system of vesicles is damaged.

1.1.3.1. ***Dopamine active transporter***

Neurotransmitters are packaged into vesicles in presynpatic neurons. The membrane and vesicular transport systems for monoamines (DA, NA and 5-HT) are involved in the regulation of synaptic communication (Masson et al., 1999). The action of CAs released in the synapse is terminated mainly by reuptake of the transmitter into presynaptic neurons or postsynaptic cells.

Dopamine active transporter (DAT) consists of 620-amino acids, organised in 12 transmembrane domains, with cytoplasmic amino- and carboxy-termini (Giros et al., 1991). It is 80 kD glycoprotein that belongs to the large neurotransmitter Na^+/Cl^- dependent proteins

family. This family includes other transporters specific for defined amine transmitters: noradrenaline transporter (NAT), serotonin transporter (SERT), GABA transporter (GAT) and glycine transporter (Torres et al., 2003). The uptake process of membrane DAT is active (requires energy) and the transporter itself is saturable since it obeys Michaelis-Menten kinetics. Mechanisms of transport for both DA and NA act as co-transporters of Na^+, Cl^- and the amine in question, using the electrochemical gradient for Na^+ as a driving force.

Under physiological conditions, DA reuptake from the extracellular space of the synaptic cleft is controlling half-life of DA and mostly depends on the presence and activity of DA transporter. The primary function of DAT is the reuptake of DA, terminating its actions, although DAT also weakly interacts with NA. Although present on presynaptic neurons at the neurosynapatic junction, DAT is also present in abundance along the neurons, away from the synaptic cleft, suggesting that DAT may play a role in the clearance of excess DA in the vicinity of neurons. Physiologically, DAT is involved in the various functions that are attributed to the dopaminergic system, including mood, behaviour, reward, and cognition. The evidence of DAT biological role is shown by the severe cognitive deficits, motor abnormalities, and hyperactivity of mice with no dopamine transporters (Gainetdinov et al., 1999) with characteristics have striking similarities to the symptoms of attention deficit hyperactivity disorder (ADHD). The half-life of DA in the extracellular spaces of the brain is prolonged considerably in DAT knockout mice, which are shown to be hyperactive and have sleep disorders.

1.1.3.2. ***Vesicular monoamine transporter and extraneuronal monoamine transporter***

The packaging of DA, available for synaptic transmission, into vesicles, occurs through the vesicular monoamine transporter (VMAT-1 and VMAT-2) (Masson et al., 1999; Mignini et al., 2006). VMAT is H^+ dependent, so the transport is driven by the proton gradient between the cytosol and the vesicle content. The neuronal membrane DAT, EMT, and VMAT-2, are the same in all CA neurons and differ in pharmacological properties, chromosomal localisations and numbers of amino acids (**Table 1**). Extraneuronal uptake is performed by the extra neuronal monoamine transporter (EMT, also known as the organic cation transporter (OCT)), which belongs to a large and widely distributed family of organic cation transporters. The organic cation transporters 1, 2, and 3 (OCT1-3, namely SLC22A1-3) mediate the facilitated

transport of diverse organic captions, drugs and toxins, having an important role in the clearance of xenobiotics. OCTs have also been implicated in the elimination of endogenous compounds, such as biogenic amine neurotransmitters: tyrosine-derived catecholamines (dopamine, epinephrine, and norepinephrine), serotonin (5-hydroxytryptamine), and histamine (Jonker and Schinkel, 2004). Among them, OCT3 was identified as the uptake-2 transporter in diverse tissues such as the kidney, heart, vascular system, and central nervous system (Zwart et al, 2001), whereas the other two non-neuronal catecholamine transporters (OCT1 and OCT2), are mainly localised to the liver, kidneys, and intestine (Eisenhofer et al., 1997).

Generally, transporter systems are considered as potential pharmacologic targets for neuropsychiatric drugs (Gether et al., 2006). Their inhibition, by increasing extracellular and synaptic concentrations and lifespan of neurotransmitter may be advantageous from a therapeutic point of view and, in the case of DA, represents an important mechanism of action of substances abuse (Fritz et al., 1998; Giros and Caron, 1993). VMAT-2 is present in all monoaminergic neurons and is less sensitive than DAT to pharmacological alterations (Vander Borght et al.,1995; Narendran et al., 2012). Drugs that interact with DAT include cocaine and its analogues and amphetamines. DAT is also important system through which toxic substances, such as neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), can enter into dopaminergic neurons (Javitch et al., 1985; Miller et al., 1999).

Mounting evidence indicates that DAT and VMAT-2 transporters are probably the most specific markers of dopaminergic neurons. The DAT is located primarily in the brain in dopaminergic neurons, and thus is the unique marker (Kuhar, 1998). The highest concentration of DATs are found in the basal ganglia, corresponding to the amount of DA nerve terminals in this brain region. Expression of DAT and VMAT-2 markers in brain can predict vulnerability of neurons and it has been shown early alterations in Parkinson's disease (Miller et al., 1999). On the contrary, no association between the tissue density of DAT and the stage of parkinsonism degeneration and in schizophrenia was found (Fujiwara et al., 1997).

Table 1. Characteristics of DA system transporters

Transporter	Location	Other substrates besides DA	Inhibitors
Neuronal (DAT)	Neuronal membrane	*Amphetamine* (Fleckenstein et al., 2007) *Phenmetrazine* (Solis et al., 2016)	*Cocaine* (Ferris et al., 2012) *Atypical benztropine-like DAT inhibitors* (e.g. benztropine, modafinil, and vanoxerine) (Schmitt et al., 2013)
Extraneuronal (EMT)	Non-neuronal cell membrane	*Noradrenaline* *Serotonin* *Histamine* *MPP+* (Ryan et al., 2014) *Amphetamine* (Schmitt et al., 2013)	*Cocaine* (Ferris et al., 2012) *MDMA** (Biezonski et al., 2013) *PCP** (Cagniard et al., 2014) *Corticosterone and cortisol* (Hayer-Zillgen et al., 2002)
Vesicular (VMAT)	Synaptic vesicle membrane	*Serotonin* (Amphoux et al., 2006) *Histamine* (Amphoux et al., 2006) *Noradrenaline* (Amphoux et al., 2006) *Adrenaline* *MPP+* (Ryan et al., 2014) *Amphetamine* (Schmitt et al., 2013)	*Reserpine* (Chaudhry et al., 2008; Bernstein et al., 2014) *Tetrabenazine* (Chaudhry et al., 2008; Bernstein et al., 2014)

Notes: *MDMA - 3,4-Methylenedioxymethamphetamine; PCP - phencyclidine, MPP+ (1-methyl-4-phenylpyridinium) is a positively charged molecule of MPTP neurotoxin

DAT ligands have traditionally been divided into two categories: cocaine-like inhibitors and amphetamine-like substrates (**Table 1**). Whereas cocaine-like inhibitors block monoamine uptake by the DAT but are not translocated across the membrane, amphetamine-like substrates are actively translocated and trigger the DAT-mediated release of DA by reversal of the translocation cycle (Schmitt et al., 2013). Finally, both inhibitors and substrates increase extraneuronal DA levels.

Interestingly, it has been shown that presynaptic human DA transporter interact with alpha-synuclein (α-syn), a protein highly enriched in presynaptic terminals, which mutations have been implicated in the expression of familial forms of Parkinson's disease, thereby accelerating cellular DA uptake and DA-induced cellular apoptosis (Lee et al., 2001).

1.4. ***Degradation of dopamine***

Circulating DA is degraded by the two main catecholamine-metabolising enzymes that are located intracellularly, so uptake into cells necessarily precedes metabolic degradation.

Dopamine and other CAs are metabolised through two distinct, although partially interacting pathways, including MAO and the catechol-O-methyl-transferase (COMT) enzymes (Nagatsua and Sawadab, 2009). Monoamineoxidase enzyme, which exists in two distinct isoforms, MAO-A and MAO-B, occurs within cells, bound to the surface membrane of mitochondria. It is abundant in sympathetic nerve terminals but is also expressed in many other tissues, such as liver and intestinal epithelium (Ramonet et al., 2003; Billett, 2004). Monoamineoxidase enzyme converts catecholamines to their corresponding aldehydes, potentially neurotoxic, and it is thought to play a role in certain neurodegenerative disorders. Monoamineoxidase enzyme can also oxidise other monoamines, including NA and 5-HT. It is inhibited by various drugs, such as amphetamine. Within sympathetic neurons, MAO controls the content of DA and NA, and the releasable store of NA increases if the enzyme is inhibited. About 25% of a dose of DA is metabolised to NA within the adrenergic nerve terminals.

The second major pathway for CA metabolism involves methylation of one of the catechol hydroxyl groups by COMT to give a methoxy derivative. COMT is absent from noradrenergic neurons, but it is present in the adrenal medulla and many other cells and tissues. CAs are widely distributed throughout the body, and are predominantly metabolised in the liver, kidneys, and plasma by MAO and COMT enzymes into inactive compounds, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) (**Figure 2**). Dopamine is excreted primarily in the urine, principally as HVA and its sulfate and glucuronide conjugates as DOPAC.

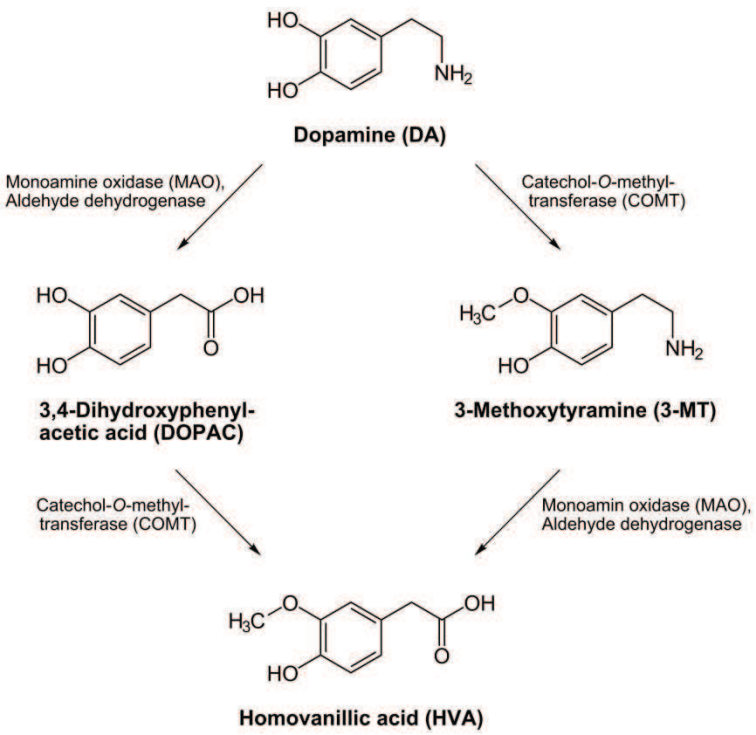


Figure 2. Metabolic pathways of DA degradation
(redrawn from the Wikipedia: https://it.wikipedia.org/wiki/Dopammina)

*1.2. **Dopamine receptors***

Dopamine exerts its effects binding DR expressed on the cell surface, which further activates second messenger systems inside the cell causing changes in excitability, metabolism and gene expression. Until the publication of the dopamine D_2 receptor sequence in 1988 (Bunzow et al, 1988), it was believed that only D_1 and D_2 dopamine receptors exist. Subsequent gene cloning studies revealed 5 different genes coding 5 dopamine receptor subtypes identified as D_1, D_2, D_3, D_4, and D_5 DR (Missale et al, 1998).

These receptors belong to the family of hepta-spanning transmembrane guanine nucleotide-binding proteins (G protein) coupled receptors (GPCRs). So-called large, heterotrimeric G proteins consist of three subunits, namely: α, β and γ. When the endogenous ligand binds to the receptor, guanosine 5'-triphosphate (GTP) is bound to the α subunit in exchange for guanosine 5'- diphosphate (GDP), and the α subunit is then released from the β subunit. The α subunit that has been activated in this way is then inactivated by dephosphorylation of GTP to GDP (intrinsic GTPase) and can thus be re-associated with the β-γ subunits. Numerous peptide hormones use cyclic adenosine monophosphate (cAMP) as the second messenger in such a way that, mediated by a stimulating G protein (Gs), adenylyl

cyclise (AC) is activated and thus more cAMP is formed. cAMP activates protein kinase A (PKA), which phosphorylates, among others, enzymes and transport molecules. cAMP can also be involved in gene expression via PKA and phosphorylation of a cAMP-responsive element-binding protein (CREB). cAMP is converted to noncyclic AMP by intracellular phosphodiesterases and the signal thus turns off.

Based on the genomic structure (sequence homology), signal transduction machinery and pharmacological properties DR have been classified into two subgroups: D1-like and D2-like families (reviewed Civelli et al., 1993; Missale et al., 1998; Sibley and Monsma, 1992). D_1 DR and D_5 DR are two forms of the D1-like group receptors, coupled with the Gαs class of G proteins, leading to an increase in intracellular cAMP formation. On the other hand, DR D_2, (DR D_{2L} and DR D_{2S}, see below for more details) D_3 and D_4 form the D2-like group couple with the Gαi class of G proteins, leading to a decrease in intracellular cAMP formation (Sibley et al., 1993; Seeman and Van Tol, 1994).

The genomic organization of the DR supports the concept that they derive from the divergence of two gene families that mainly differ in the absence or the presence of introns in their coding sequences. D1-like receptors genes do not contain introns in their amino acid coding regions, in both D_1 and D_5 subtypes in mammals (Civelli et al., 1993; O'Dowd, 1993). In contrast, the protein coding regions of the D2-like receptors are interrupted by introns and different receptor variants (isoforms) have been identified as a result of alternative splicing (Civelli et al., 1993; Sibley and Monsma, 1992; Missale et al., 1998). The DR D_2 coding region contains six introns. So far two alternatively spliced transcripts of the DR D_2 gene code are identified, for two different DR D_2 isoforms known as the "long" and "short" forms, $D_{2(443)}$ and $D_{2(414)}$ respectively. When compared, long DR D_2 has 29 more amino acids, which are located in the putative third intracellular loop of the receptor, a region involved in the coupling of the receptor to G-proteins. These two receptor isoforms exhibit largely similar pharmacological characteristics but differ in G-protein coupling affinities (Liu et al., 1996).

The short isoform of the DR D_2 is placed pre-synaptically, and has modulatory function, while, long DR D_2 isoform, is a classic post-synaptic receptor and transmit information in an excitatory or an inhibitory fashion. Splice variants of the DR D_3 receptor and polymorphic variants of the DR D_4 receptor have also been identified (Missale et al., 1998). The DR D_4 gene displays polymorphisms, that has been identified in the length of the third intracellular loop, implicated in the interaction with the G-protein (Petronis et al., 1998). Sunahara et al. reported the cloning of a gene encoding a protein with strong homology to the

cloned DR D_1, identify as DR D_5, that binds drugs with a pharmacological profile similar to that of the DR D_1, but displays a 10-fold higher affinity for the endogenous agonist, DA (Sunahara et al., 1991). Pharmacologically, DR D_1 and DR D_5 are indistinguishable by their ability to bind the numerous antagonists and can only be discerned by their differing affinity for DA and other D1-like agonists. For as yet unknown reasons, the affinity of DR D_5 for agonists is up to 10 times higher to that of DR D_1 (Weinshank et al., 1991). The function of the DR D_5 remains elusive largely due to the fact that no D_5-selective drugs are available.

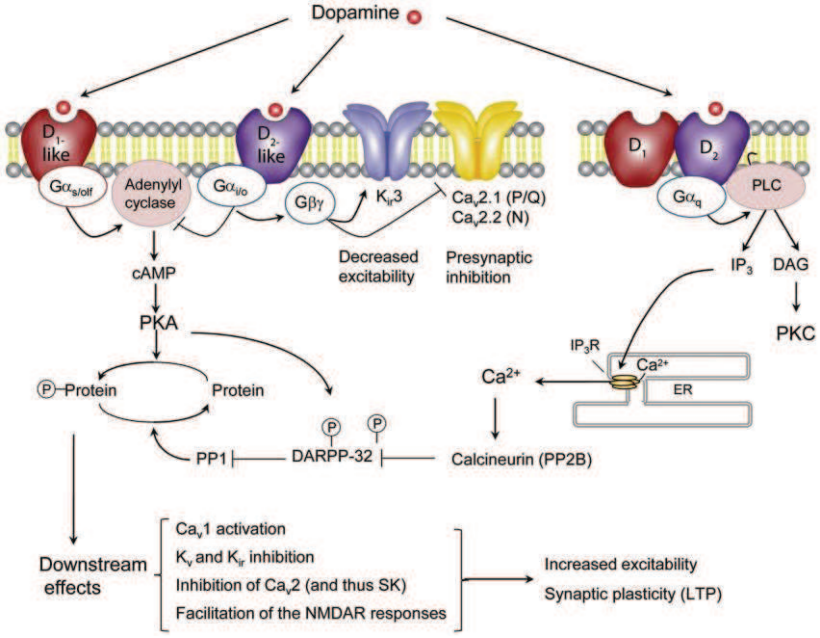


Figure 3. Dopamine receptors signalling
(reproduced with permission from Savica and Benarroch, 2014)

Model of DR activation and signalling throughout the sequential activation of G proteins and specific enzyme or channel effectors might be too simplistic to explain the functional flexibility of DR (Savica and Benarroch, 2014) (**Figure 3**). Dopamine signalling should rather be reconsidered in the way that it is not limited only to activation or inhibition of adenylyl cyclase, but that DR regulate multiple signalling pathways by interacting with various G proteins and G protein-independent mechanisms (reviewed in Beaulieu and Gainetidinov, 2011). Recent studies have shown that DR has complex function involving dimers (homo- and/or heterodimers) or even higher order of oligomers (Vischer et al., 2011). Some antiparkinsonian agents – such as the preferential high-efficacy DR D_3 versus DR D_2 receptor agonists, pramipexole and ropinirole – show amplified potency at D_3/D_2 heterodimers versus constituent monomers, and others in contrast, such as the D_3/D_2 receptor agonist pergolide, show no difference (Maggio et al., 2009). Protein-protein interaction among GPCRs and

downstream DA signalling is regulated by specific and finely orchestrated actions of GPCR kinases isoforms, playing a critical role in the modulation of receptor pharmacology and functions upon activation by an agonist (Gurevich et al., 2016). Those forms have different pharmacological, signalling and trafficking properties from their single constituent receptors (Fuxe et al., 2008).

Since heterodimers represent novel receptor entities working as unique functional units, heterodimerization increases heterogeneity within DR subtypes, regarding different combinatorial possibilities. The discovery of DR heterodimers with atypical properties opens a new horizon to the development of promising targets for bifunctional compounds selectively. Possible targets could involve action on the: entire complex, allosteric ligands, that could interact with one co-receptor, modify the function of other co-receptor, or small molecules that can disrupt heterodimeric complexes. For example, the recent discovery of DR D_2 signalling heterogeneity has led to a reconsideration of the mechanism(s) of action of some antipsychotic drugs used in the treatment of schizophrenia (Urs et al., 2012). One of first attempts to develop "biased drugs" involved new compounds binding to the DR D_2, acting as partial agonists for arrestin 3 (β-arrestin2) (Allen et al., 2011).

Evidence accumulating through the study of DR signalling in the last ten years has pointed to a further degree of complexity within these receptor families. This differential coupling of DR allows that DA might promote distinct cellular effects in two different kinds of cells expressing the same DR. Furthermore, differential expression of DR on different cells also contributes to DA exerts distinct effects in those cells. According to this idea and to the fact that there are a differential expression and differential coupling of DR in distinct neurons, DA may play different roles in the distinct zones of the nervous system (Sidhu, 1998). Taken together, complexity and differential coupling of DR might represent a new approach needed to be taken into account in the development of innovative drugs for the treatment of a variety of DA-related disorders.

1.2.1. ***Dopaminergic pathways in the central nervous system***

Although role of DA as a neurotransmitter in the brain was characterised in 50-ies of the last century, a detailed analysis of its neural distribution became possible after developing the fluorescence technique based on the formation of fluorescent derivatives of CAs in the tissues that were previously treated with formaldehyde (Falck et al., 1962). Detailed maps of

dopaminergic pathways were identified in the laboratory animals, and similar basic characteristics have been confirmed, later on in the human brain. The central dopaminergic neuron system is comprised of three major pathways, identified in the mammalian brain (Anden et al., 1964; Dahlstroem and Fuxe, 1964) (**Figure 4**).

(1) *Dorsal* (or upper) *pathway*, also called the ***nigrostriatal pathway***, originates in *substantia nigra pars compacta (SNpc) (A9 region),* projects to the *basal ganglia* and *striatum*, and is involved in extrapyramidal motor function. Dopaminergic neurons of SNpc projects primarily to the striatum, as a major origin of the dopaminergic innervation. The major function of the striatum is the regulation of posture and muscle tonus. Under physiological conditions, the extrapyramidal system processes information coming from the cortex to the striatum and returns it back to the cortex through the thalamus. Nigral cell loss results in the depletion of striatal DA, and decreased nigrostriatal input leads to an increase of inhibitory output from the globus pallidus interna to the thalamus and, indirectly, to the cortex, thereby repressing the initiation of movements and leading to motor manifestations characteristic for Parkinson's disease (Shulman et al., 2011) (**Figure 5**).

(2) *Ventral* (lower) *pathway*, also called the ***mesolimbic/mesocortical pathway,*** projects from the *ventral tegmental area* (VTA) (*A10 region*) to the prefrontal cortex (PFC) considered to be crucial for cognitive function and motivation (Willner and Scheel-Kruger, 1991). The PFC is a crucial target area for the action of antipsychotic drugs such as neuroleptics, which impair DA neurotransmission. Mesolimbocortical DA plays role in: i) reward and reinforcement mechanisms (shown by the observation that psychostimulants and drugs of abuse elicits DA release in the mesolimbic areas); ii) learning and iii) memory.

(3) ***Tubular- infundibular pathway*** originates in the hypothalamus (*A12 region*), projects to the hypophysis, and is involved in neuroendocrine regulation.

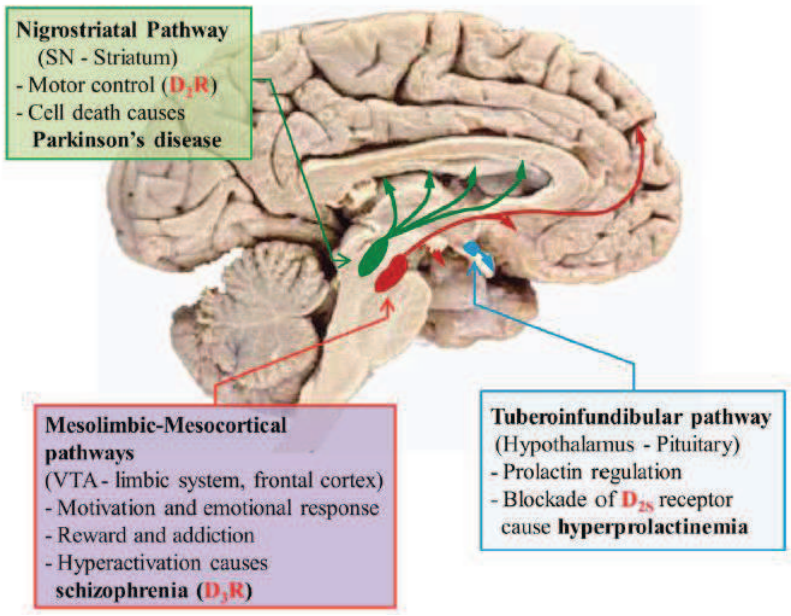


Figure 4. Three major dopaminergic pathways in the brain.
(reproduced with permission from Cho et al., 2010).

Dopamine is important for the normal function of the nervous system, that is shown by several brain impairments and diseases that are shown to be significantly correlated with/to abnormalities in DA levels and/or in the expression, function of DR and dopaminergic signalling.

1.2.2. Dopamine receptors in the central nervous system

In the CNS, DA receptors are widely expressed and are involved in a range of physiological functions. DR subtypes show different topographic segregation within the CNS (Strange 1993). DR D_1 are abundant in the basal ganglia, nucleus accumbens, and cerebral cortex, DR D_2 have highest concentrations in the basal ganglia and anterior pituitary, DR D_3 in the ventral striatum (nucleus accumbens, islands of Calleja, olfactory tubercle), DR D_4 show the highest density in the frontal cortex, hippocampus and amygdala, and DR D_5 are mainly located in the hippocampus and thalamus. The precise distribution of diverse DR subtypes in the brain is summarised in **Table 2**.

Table 2. Dopamine receptor distribution in the human brain

Family	Receptor	Type of receptors	Transduction mechanisms (effectors)	Tissue distribution	Physiological functions
D1 like	DR D_1	G_s-coupled	Increase of cAMP Activating AC	Basal ganglia, striatum, nucleus accumbens and cerebral cortex, retina	Control of locomotor activity; reward and reinforcement mechanisms; learning and memory
	DR D_5			Hyppocampus and hypothalamus	Cognitive functions
D2 like	DR D_2	G_i/G_0 coupled	Decrease of cAMP Inhibiting AC	Striatum, substantia nigra (SNpc), basal ganglia, the anterior pituitary gland	Control of locomotor activity; reward and reinforcement mechanisms; learning and memory
	DR D_3			Ventral striatum (nucleus accumbens, islands of Calleja, olfactory tubercle), hypothalamus	Control of locomotor activity; cognitive functions
	DR D_4			Frontal cortex, hippocampus and amygdale, midbrain, medula	Cognitive functions

(based on Beaulieu and Gainetidinov 2011; Cosentino and Marino, 2013)

The DR D_1 is the most widespread DR in the brain and is expressed at higher levels than any other DR (Dearry et al., 1990). Its mRNA and protein have been found in the striatum, the nucleus accumbens, and the olfactory tubercle, but also have been detected in the limbic system, hypothalamus, and thalamus. Lesion studies in animal models shown that localisation of DR D_1 can be primarily presynaptic (in the substantia nigra, on afferent projections), but also postsynaptic (in the caudate-putamen) (Joyce and Marshall, 1987).

The distribution of the DR D_2 is similar to that of the DR D_1, being localised primarily in the mesencephalon, where dopaminergic cells contribute to the innervation of the cerebral cortex, striatum, and limbic regions (Weiner et al., 1991). DR D_2 are abundant in regions of the

hypothalamus that innervate the pituitary, and are located throughout the midbrain, found localised on DA neurons themselves (Autelitano et al., 1989; Sesack et al., 1994).

The DR D_2 has nanomolar affinity for DA and is located both pre-synaptically (short isoform of the DR D_2, mostly involved in autoreceptor functions and has modulatory function) and postsynaptically (long DR D_2 isoform, that transmit information in an excitatory or an inhibitory fashion) (Usiello et al., 2000; De Mei et al., 2009). These splice variants of the DR D_2 (D_{2L} and D_{2S}) have different neuronal distributions, and therefore, the varying roles (Usiello et al., 2000; De Mei et al., 2009). Pharmacological and genetic evidence suggests that DR D_3 exert some relatively minor modulatory influences on D_{2S} autoreceptor's role, thereby complementing the presynaptic regulation phasic release of DA, regulating the neuronal firing rate and synthesis of DA (De Mei et al., 2009). Koulchitsky and co-workers suggested that the alteration of the main frequency of the rat ventral tegmental area (VTA) rhythm induced by the action of quinpirole on D_2 autoreceptors disturbs the ability of this region to interact with its input or output regions, hence the altered locomotor behaviour (Koulchitsky et al., 2016).

Enrichment of DR D_3 is observed in the nucleus accumbens and islands of Calleja (Meador-Woodruff et al., 1994), at moderate levels in the basal ganglia, and at slightly lower densities in the substantia nigra, hippocampus, and the amygdaloid (Lahti et al., 1995). The DR D_4 seems to occur at 10- to 100-fold lower densities than the DR D_1 and DR D_2 in the striatum (Schlachter et al., 1997; Patel et al., 1996).

In general, these receptors can be differently distributed among neurons and according to this they are divided into two groups, referred to as a postsynaptic receptor (locate on dendrites or cell body, axons or nerve terminals) and presynaptic autoreceptors locate on the same neuron. Autoreceptors are sensitive to the transmitter that is secreted from the neuron on which these receptors are located. Terminal autoreceptors are involved in the control of transmitter release, and somatodendritic autoreceptors are involved in the control of transmitter synthesis. Activation of autoreceptors by released DA is thought to be one of the principal mechanisms responsible for regulation of dopaminergic neuronal function. Autoreceptors are present on most parts of a DA neuron and are responsive to both terminal and dendritic DA release. Stimulation of DA autoreceptors in the somatodendritic region slows the firing rate of DA neurons (impulse-regulating autoreceptors) and according to findings, inhibits subsequent dendritic DA release (Cragg and Greenfield, 1997), while stimulation of autoreceptors located on DA nerve terminals results in an inhibition of DA synthesis and/or release (release and synthesis- regulating autoreceptors). Dopamine autoreceptors are more sensitive to the effects

of DA than postsynaptic DA receptors. In general, all DA autoreceptors can be classified as D2-like receptors. Taking all above mentioned, it might be rational to reconsider autoreceptor-selective agonist as a useful pharmacological tool.

1.2.3. ***Behavioural role of dopamine receptors***

To date, knowledge of the functional role of specific DR on various functions or behaviours varies because of the availability of specific pharmacological tools, DR agonist, and antagonist. Generally, agonists increase DA function, thus increasing motor activity, while antagonists have the opposite effect. The systemic administration of the DR D_1 partial agonist (SKF 38393) in rats increases grooming and sniffing but does not significantly increase locomotion or other stereotypical behaviour (Jackson and Westlind-Danielsson, 1994). In the brain, the biological significance of some specific classes of DA receptors has been well-documented with the help of transgenic mice deficient in the expression of a specific class of DR. Disruption of the DR D_1 gene showed locomotor hyperactivity in mice (Xu et al., 1994), and inactivation of DR D_2 gene produced almost the opposite phenotype. These DR D_2 deficient mice were akinetic and bradykinetic with significantly reduced spontaneous movement (Baik et al., 1995).

A recent study of Moraga-Amaro et al., 2016 indicated that DR D_5 deficiency resulted in impaired spatial memory without provoking depression-like symptoms. This study represents the first genetic evidence pointing the involvement of DR D_5 in memory, linking the same receptor with hippocampal synaptic plasticity. The same authors also demonstrate a selective reduction in the expression of the NMDAR subunit NR2B observed in the hippocampus of D5RKO mice, suggesting collaboration between DR D5 and glutamatergic pathways, providing a useful tool for future therapies for disorders involving alterations in memory and the dopaminergic system (Moraga-Amaro et al., 2016).

Early studies with a putative selective DR D_1 agonist, SKF 38393, including seven patients with idiopathic PD, administered orally, alone or in combination with levodopa (L-DOPA) was not effective in reversing symptoms of PD (Braun et al., 1987). Later on, it has been shown, that SKF 38393 is only a partial DR D_1 agonist, that has limited penetration through the blood-brain barrier and a short duration of action which, at least partially, might explain why it did not induce any beneficial effects in patients with PD. First full developed DR D_1 agonist A-77636 was shown to be a highly potent, long-lasting, and together with

dihydrexidine (selective, full DR D_1 agonists) have been shown to reverse MPTP-induced motor deficits in primates (Schneider et al., 1994).

Increased dopaminergic transmission in the CNS leads to a behaviourally aroused state, referred to as psychomotor activity. This stands for DA, non-selective DA agonists, and also for the central stimulants (cocaine and amphetamine). In experimental animals, enhanced dopaminergic transmission is observed as an increase in locomotion (Beninger, 1983). Treatments that decrease DA signalling cause a specific sedation that is often comparative with motor disturbances such as parkinsonism or catalepsy (Johnels, 1982). Regarding the role in behaviour and motor stimulation and increased locomotion the best characterised, so far, are DR D_2 receptors (Missale et al., 1998), while DR D_1 function is less understood. Decline in DR D_1 receptors in the prefrontal cortex as a function of age (Suhara et al., 1991) is significant as drugs targeted at this receptor subtype have proven to be beneficial for the amelioration of age-related memory deficits. In aged monkeys, acute administration of either low doses of a partial DR D_1 agonist (SKF 38393) or selective, full DR D_1 agonists (dihydrexydine, A77636, SKF 81297) has improved spatial working memory performance (Arnsten et al., 1994). These cognitive-enhancing effects of DR D_1 agonists are believed to be due to enhanced signalling via DR D_1 receptors in PFC and notably, in all cases, were blocked by pretreatment with the DR D_1 antagonist SCH 23390. This principle has been used to develop a novel treatment regimen targeted to enhance cognitive function in DA-deficient states (Castner et al., 2000). Specifically, repeated intermittent low-dose treatment with the selective full DR D_1 agonist (ABT-431) produced a pronounced and enduring enhancement of spatial working memory performance in both endogenous (aging) and pharmacologically (chronic haloperidol treatment) induced DA-deficient states (Castner and Goldman-Rakic, 2004).

Overall, the findings from the non-human primate models of prefrontal DA deficiency and cognitive dysfunction indicate that the DR D_1 is a key target for developing novel compounds for the alleviation of cognitive deficits in DA dysfunctional states such as schizophrenia. The data so far, are suggesting that in monkey repeated, yet intermittent, DR D_1 agonist treatment produces a robust and sustained enhancement of cognition should prove to be a useful therapeutic strategy for the treatment of cognitive dysfunction in conditions where DR D_1 receptor signalling in PFC is suboptimal, as is likely the case in both PD and schizophrenia (Goldman-Rakic et al., 2004).

Interest in the DR D_3 is related to its selective localisation in limbic areas of the brain. Accumulating evidence suggests high comorbidity of depression with PD. Recent data, obtained in rodents, has demonstrated that brain dopamine and its mesolimbic projections have a role in the induction of depressive-like symptoms (Tye et al., 2013). Pharmacological studies performed in both rodents and humans, support antidepressant effects of high-affinity DR D_3 agonist pramipexole, classically used for the treatment of PD symptoms (Breuer et al., 2009; Chernoloz et al., 2012). One of the recent studies provided evidence that D3RKO knockout mice (lacking DR D3) developed symptoms similar to depression and anxiety, pointing that DR D3 itself mediates the antidepressant effect, which deficiency results in chronic depression (Moraga-Amaro et al., 2014).

1.3. ***Endocrine roles of dopamine***

1.3.1. ***Regulation of prolactin release***

Prolactin is formed in the anterior lobe of the pituitary gland and is important hormone for the: i) stimulation of growth and differentiation of the mammary gland, ii) inhibition of pulsatile, but not the basal, release of the gonadotropins such as luteinising hormone (LH) and follicle-stimulating hormone (FSH), iii) induction of the milk production during lactation period, iv) inhibition of cellular glucose uptake and interestingly v) inhibition of the cellular immune defences. Dopamine inhibits prolactin release. As prolactin increases DA metabolism in the hypothalamus, it inhibits its own release (negative feedback regulation mechanism). Excess prolactin can be caused by administration of antidopaminergic drugs or hormone-producing tumours (Torre and Falorni, 2007).

1.3.2. ***Regulation of female sex hormones release***

A lack of estrogens and progestogens is frequently the result of a decreased GnRH (gonadotropin-releasing hormone) release in severe psychological or physical stress. The GnRH release can also be reduced through the influence of the neurotransmitters NA, DA, 5-HT, and endorphins. It is relatively common for a reduction in gonadotropin release to be due to raised prolactin secretion, for example, as a result of the absence of inhibition of pituitary secretion of prolactin or a prolactin-producing pituitary tumour. Gonadotropin release can be inhibited by dopaminergic drugs that cause a rise in prolactin secretion (Ben-Jonathan and Hnasko, 2001).

1.3.3. ***Growth hormone release***

Growth hormone (GH) is a stress hormone that inhibits the uptake of glucose in fat and muscle cells and like these raises the concentration of glucose and free fatty acids. It also stimulates the enteric absorption of calcium and phosphates as well as the renal excretion of calcium. This peptide hormone stimulates growth, cell reproduction, and cell regeneration promotes T-cell proliferation, interleukin 2 (IL-2) production and the activity of natural killer cells, cytotoxic T cells, and macrophages (Jeay et al., 2002). In this way, it strengthens the immune defence. Dopamine can stimulate the release of GH (Jaffe and Barkan, 1992).

1.3.4. ***Dopamine and thyroid gland***

Formation and release of T_3 and T_4 as well as the growth of the thyroid gland are stimulated by thyrotropin (TSH) from the anterior pituitary. Its release is, in turn, stimulated by thyroliberin from the hypothalamus. Stress and estrogens increase TSH release, while glucocorticoids, somatostatin, and DA inhibit it (Haugen, 2009).

1.3.5. ***Regulation of kidney function***

Dopamine stimulates the release of the antidiuretic hormone (ADH), which is formed in the hypothalamus and is transported to the posterior lobe of the pituitary gland via the axons. ADH promotes water reabsorption in the distal tubules and in the collecting duct of the kidney. ADH stimulates the tubular absorption of Na^+ and urea and high concentration of ADH leads to vasoconstriction. An important stimulus for the release of the mineralocorticoid aldosterone is angiotensin II, which is formed in increased amounts via the renin–angiotensin system when the renal perfusion pressure is reduced. Aldosterone release is decreased by DA and the atrial natriuretic factor (Wu et al., 2001).

1.3.6. ***Dopamine and hematopoiesis***

The direct evidence of the involvement of DA in hematopoietic regulation has been proposed. Exogenous administrated DA stimulates erythropoiesis and platelet production in both normal and tumour-bearing mice (Lahiri et al., 1990). Significant uptake of DA by bone marrow (BM) cells have been shown *in vivo* and *in vitro* in murine, and this uptake was found to be specific since DA receptor antagonists inhibited this uptake (Basu et al., 1993). Until

today, huge advancement has been made in characterising the different cell types that are important for maintenance of hematopoetic stem cells (HSC). Anatomical studies have shown that sympathoadrenergic innervation has role in BM hematopoiesis that occurs through the adrenoceptors (AR) and DR expressed on hematopoietic cells affecting their migration, proliferation and survival ability (Lymperi et al., 2010; Wang and Wagers, 2011; Mendelson and Frenette, 2014).

The first record of adrenergic modulation of hematopoiesis *in vivo* has been shown after syngeneic BM transplantation in mice, when chemical sympathectomy mimicked by the α_1-adrenoceptor antagonist prazosin, increases the number of peripheral blood leukocytes (Maestroni et al., 1992). The regulation of hematopoietic system is achieved trough three steps: i) at the cellular level of bone marrow stroma, ii) at the humoral level by cytokines, and iii) by CAs and other neuroendocrine factors.

It seems that AR agonists, like the sympathetic neurotransmitter NA, inhibit myelopoiesis and this effect might be of clinical relevance. An early summary of the facts was performed in normal mice (Maestroni and Conti, 1994) showing that prazosin can enhance myelopoiesis and platelet formation, while α_1-adrenergic agonist, directly inhibits *in vitro* growth of granulocyte/macrophage-colony-forming unit (GM-CFU). In following studies Maestroni (1995) emphasised the ability of α-AR antagonists to augment myelopoiesis and platelets production while declining lymphopoiesis, in both normal mice as well as after BM transplantation.

The effects of NA and DA in the BM launch the issue regarding their physiological relevance, especially when it comes to the origin of these catecholamines at the BM level. It was suggested that sympathetic nerve endings and hematopoietic cells are the main source of bone marrow NA, as well as DA and A, but also immune cells themselves are a valuable source of CAs (Maestroni et al., 1998). By use of a high performance liquid chromatographic method (HPLC), Marino et al. (1997) measured endogenous catecholamines in BM from normal, 6-OHDA- treated and pargyline-treated mice. Noradrenaline levels were lower after sympathetic denervation with 6-OHDA and higher after irreversible MAO inhibition with pargyline, while A and DA were not affected under either condition (Marino et al., 1997). Thus, it seems that NA in the bone marrow originates mainly from sympathetic nerve endings. Among the CAs, a substantial amount of DA was detected in bone marrow, that is only a minor part, if any of neurogenic origin, and at the low level present in BM could be considered as a biosynthetic precursor of NA (Marino et al., 1997).

Interestingly, in murine hosts, NA and DA showed a rhythmicity of levels in BM with peak values observed during the night (Maestroni et al., 1998). Scheiermann et al., 2013 suggested that daily rhythmicity is an important regulator of specific immune system functions of BM catecholamines. Marino et al., 1999 have shown endogenous production of CAs by immune cells.

To show DA regulation of bone marrow hematopoiesis, Spiegel et al. (2007) showed, by the means of flow cytometry, that human CD34+ cells expressed both DR D_3 and DR D_5 on their surfaces. Amusingly, dopaminergic agonists augmented the polarisation, motility, clonogenic progenitor content and engraftment potential of these cells (Spiegel et al., 2007).

It has been recently established that activation of sympathoadrenergic system is a link between chronic stress and inflammatory response (Heidt et al., 2014), so the neutrophil/lymphocyte ratio can represent a negative prognostic marker in a numerous critical conditions, such as cardiovascular disease (Guasti et al., 2011; Bhat et al., 2013) and even cancer (Templeton et al., 2014), offering opportunity for therapeutic intervention. Results obtained so far in preclinical models would already support to the various extent the clinical evaluation of dopaminergic agonists (Spiegel et al., 2007) for HSC transplantation, as well as dopaminergic agonists (Sarkar et al., 2014) to protect against the adverse effects of cytotoxic agents on BM.

1.4. Peripheral tissues producing dopamine

Besides of its action in the CNS, DA exerts its function in the periphery, primarily as a precursor of NA and A. Dopamine is main and independent transmitter of most autonomic sympathetic postganglionic fibers, having an important effect on various physiological functions in organs and tissues including: the vascular beds, the heart, the gastrointestinal tract, and renal physiology acting as an endocrine hormone, but also in regulation of olfaction and retinal processes. Moreover, a number of studies showed DA components in the immune system, suggested that DA plays a key role on neural-immune interactions acting as an important modulator of peripheral physiologic functions (Basu and Dasgupta, 2000; Besser et al., 2005; Ilani et al., 2004; Sarkar et al., 2006; Watanabe et al., 2006).

Dopamine may arrive into the bloodstream from several different sources. An important peripheral source of DA and other neurotransmitters is a peripheral nervous system (PNS) and its sympatho-adrenergic termini that can release both DA and/or NA. Noradrenaline is the main

neurotransmitter released by the sympathetic nerve system (SNS) and thereby the main neurotransmitter responsible for SNS-mediated regulation of immunity. Several lines of evidence suggest that DA may be stored in, and release from sympathetic nerve terminals, acting as a transmitter at this level, outside the CNS (Bell, 1998; Bencsics et al., 1997). The major primary and secondary lymphoid organs (thymus, spleen, lymph nodes and intestinal Peyer's patches) are extensively supplied by dopaminergic terminals, where SNS seems to play an important role in the regulation of T cell-mediated responses (Mignini et al, 2009). The presence of adrenergic and DR on immune cells provide channels for noradrenergic signalling to lymphocytes and macrophages by sympathetic nerves (Madden et al., 1995). Epithelial cells in the gut are an important source of a gastrointestinal DA described as an endogenous gastroprotective element, acting through DR D_1 receptors (Rasheed and Alghasham, 2012).

1.4.1. ***Role of peripheral dopamine in metabolic control***

Dopamine was found to modulate regulation of glucose homeostasis and body weight by influencing the endocrine pancreatic hormone levels, in more specific, it inhibits insulin secretion in both animals and humans (Quickel et al., 1971; Leblanc et al., 1977). It has been recently confirmed that DA and selective DR D_2 agonist inhibit insulin exocytosis (Rubí et al., 2005). Regulating the pancreatic endocrine function, DA also modulates the effects of insulin action on adipocytes. Dopamine action in the central DA pathways is a putative additional component in mediating metabolic homeostasis in the human body. In the brain, at the basal ganglia level, DA participates in the signalling of the rewarding effects of food intake, in a similar way described as a mechanism of drug abuse (Volkow et al., 2008). Behavioural addiction-like syndrome and compulsive food seeking were shown in a DR D_2 striatal knockdown rats (Johnson and Kenny, 2010). Briefly, treatment with bromocriptine (D2-like receptor agonist) reduces hyperphagia and adiposity in animals with diet-induced obesity (Davis et al., 2009). Moreover, antipsychotic antagonists of DR D2-like receptors, such as clozapine, have been shown to increase insulin secretion in isolated rat pancreatic islets (Melkersson and Jansson, 2007). Consequently, schizophrenic patients are at risk of obesity, insulin resistance, impaired glucose tolerance and hypertension (Newcomer, 2007).

1.4.2. ***Dopamine – activates multiple receptors in the periphery***

Catecholamines plasma levels are defined by release from autonomic nerve endings and through suprarenal glands presenting the main source of CAs. The physiological concentration of DA, as an endogenous catecholamine ranges between 10^{-10} M and 10^{-11} M. Among this physiological range, it is expected that DA acts preferentially on DR, affecting several important functions and features of human effector cells. In addition, it is important to consider that among DR each receptor displays different affinities for endogenous DA: DR DR_3 > DR D_5 > DR D_4 > DR D_2 > DR D_1 [Ki (nM) = 27, 228, 450, 1705, 2340, respectively] and so stimulatory effects of different DR on different cell and tissue may provoke different physiological responses (Sunahara et al., 1991; Van Tol et al., 1991). Thus, low levels of DA, e.g. 50 nM, would stimulate mainly DR D_3, while moderate DA levels, e.g. 300 nM, would stimulate DR D_5 as well. It is likely that, by stimulating multiple DR, higher DA levels promote complex effects in different circulating cells types and tissues expressing this receptor subtype (Pacheco et al., 2014). Binding studies have shown that DA also has affinities for different AR (Xhaard et al., 2006). Origin of the specificity of binding in adrenergic versus dopamine receptors was confirmed by distinct morphological features of the receptors, such as: i) an unusually long third intracellular loop (α_2 and D2-like receptors), ii) a long carboxyl-terminal segment (α_1-ARs, β-ARs and D1-like receptors), but also iii) specificity for coupling certain G-proteins ($G_{i/0}$ for α_2-ARs and D2-like receptors and G_s for β-ARs and D1-like receptors) (Xhaard et al., 2006). This observation suggests that the ligand-based pharmacological classification does not reflect the evolutionary history of the ARs and DR. In addition, *in vivo* has shown the interplay between DA and ARs, where DA activated adrenoreceptors in the preotic area of Japanese quail (Cornil et al., 2002) and re-uptake of DA has been reported at adrenergic neurons in adult male Sprague-Dawley rats (Pan et al., 2004).

Structure-activity relationship study, comparing DA with A and NA, reveals that by removing the hydroxyl group from the side chain of NA, DA molecule has less affinity for α- and β- adrenergic receptor, and higher affinity for dopaminergic receptors. The β-hydroxyl group is found only in NA and not in DA, thus is expected to form unique interactions with ARs (Xhaard et al., 2006). Since *S*-enantiomer of NA binds to the ARs with an affinity similar to DA in contrast to the tight binding of the *R*-enantiomer, it might be assumed that chirality of this group is key to NA selectivity (Nyrönen et al., 2001). So far, no amino acid has been identified that could account for specific interactions of β-hydroxyl of NA with α_1-ARs or α_2-

ARs. In the β-ARs, the main candidate for interaction with the β-hydroxyl group on ligands is asparagine at the position N6.55 (Wieland et al., 1996), but asparagine is also found at the equivalent position in the D1-like type receptors activated by DA lacking the β-hydroxyl group. Adrenaline, having a methyl group attached to the positively charged nitrogen, binds with a higher affinity to the ARs in comparison to NA (Nyrönen et al., 2001).

Knowledge of peripheral DR function is mainly derived from observations of DA hydrochloride administered intravenously, widely used in the treatment of various shock states and congestive heart failure. In congestive heart failure, decrease in cardiac output triggers a series of compensatory actions: fluid retention, vasoconstriction, an increase in peripheral vascular resistance, tissue hypoxia and an increase in the levels of circulating CAs. The state of shock leads to a strong activation of the SNS resulting in a massive increase of circulating CAs. When endogenous CA release fails to stabilise cardiovascular parameters, therapeutic CAs are frequently administered (Flierl et al., 2008). Administration of CAs becomes the choice of last resort to stabilise cardiovascular functions in the critically ill patient (Annane et al., 2005).

The pharmacological low dose of DA stimulates mainly DR, while higher doses stimulate both β_1-adrenergic and DR as well (**Table 3**). High therapeutic doses stimulate also α -adrenergic receptors (α_1, α_2) (Smit, 1989).

Both D1-like and D2-like receptors are located at various sites within the cardiac, vascular, and renal vascular bed (Lokhandwala and Amenta, 1991). Low doses of DA are widely used for its specific effects on renal function, suggested to be beneficial. Dopamine is able to improve negative circulatory events by renal vasodilatation (via DR D_1), the decrease of renal vascular resistance and to improve urine output trough the increase of the kidney blood flow. However, a meta-analysis of multiple studies fails to demonstrate that DA can prevent acute renal failure or reduce mortality (Kellum and Decker, 2001; Friedrich et al., 2005). At low concentrations, the primary cardiovascular effect of DA is stimulation of vascular DR D_1 leading to vasodilatation. Therefore, DA is particularly useful in the management of states of low cardiac output associated with a compromised renal function such as cardiogenic shock. At higher, moderate concentrations, the β_1-adrenergic-mediated response occurs and a selective increase of force of myocardial contraction without a significant effect on heart rate. High doses of DA, like all CAs (which activate the β_1-AR), can induce rhythm disturbances. In addition, at high concentrations, DA stimulates α_1-AR, leading to vasoconstriction.

Table 3. Degree of different receptor stimulation and major effects mediated by DA based on different dosing rate

Dopamine infusion rate (clinically relevant doses)	**Receptor subtype**	**Location**	**Contribution to therapeutic effect**	**Theoretical concentrations of DA in plasma in steady state (nM)**
Lower DA infusion rates (0,5-4 μg/min/kg)	D1–like receptors (DR D_1)	Kidney Vascular smooth muscle in renal, mesenteric, and coronary arteries	Increased renal blood flow, natriuresis, urine output and a decrease of fluid retention (edema). Relaxation of vascular smooth muscle.	2 - 522*
	D2–like receptors	Neurons Kidney	Inhibited release of NA and aldosterone secretion, vasodilatation and sodium excretion (Smit, 1989; Girbes et al., 1992)	
Intermediate DA doses (4-10 μg/min/kg)	β_1 - AR	Heart	Positive inotropic effect, increased heart rate, cardiac output, all beneficial in congestive heart failure (Amenta et al., 1993).	16 -1300*
Infusion rates 10-20 μg/min/kg	α_1/α_2 - AR	Smooth muscle Blood vessels Gastrointestinal tract Liver	Systemic vasoconstriction and increase in blood pressure (Girbes & Hoogenberg, 1998).	40- 2600*

		Pancreatic islets Nerve terminals	May increase risk of tachyarrhythmias The decrease of insulin secretion. Relaxation of GIT. Glycogenolysis. Adrenergic and cholinergic decrease release	

Notes: * Theoretical DA concentrations in the human plasma in the steady state (C_{ss}, *nM)* during prolonged continuous infusion were estimated from the equation $C_{ss} = \upsilon_{inf}/CL_{tot}$ (where υ_{inf} represents the infusion rate ($\mu g \cdot kg^{-1} \cdot min^{-1}$), and CL_{tot} represents DA total clearance, expressed in units ($ml \cdot kg^{-1} \cdot min^{-1}$) used from DA pharmacokinetics parameters presented in **Table 4**.

Of note is, that plasma DA concentrations in patients receiving DA infusion at identical rates, despite a homogeneous population of healthy male subjects (n = 9) and weight-based dosing, may vary profoundly (MacGregor et al., 2000). Variability in plasma DA concentrations was 10- to 75-fold intersubject, thus DA dosing based on body weight does not yield predictable blood concentrations, but rather shows marked intraindividual and interindividual variability in DA distribution and/or metabolism (MacGregor et al., 2000).

In cardiology, DA is the drug commonly used to prevent renal failure and treat moderate hypotension in the critically ill patients (Oberbeck, 2006). Dopamine is approved by Food and drug administration (FDA) since 1974 and is in use for more than 40 years with a favourable therapeutic index. On the basis of inter-individual variation in pharmacodynamics (**Table 4**), DA requires careful monitoring of the achievement of the desired hemodynamic profile. For this reason, the use of DA infusion is often restricted to intensive care units and operating rooms that are equipped with appropriate hemodynamic surveillance. Summary of established clinical use of DA in hospital cardiovascular unit is: to correct hemodynamic imbalances present in shock syndrome due to myocardial infarction, endotoxic septicemias, renal failure, open heart surgery and chronic cardiac decompensation.

Table 4. Pharmacokinetics parameters of the clinically used intravenous DA

Absorption	Usual dosage: 5-10 μg/min/kg Onset: 5 min (adults)
Distribution	Vd: 0.8-2.45 L/kg
Metabolism	Metabolised in liver, kidney, and plasma by MAO and COMT Metabolites: NA (active), inactive metabolites
Elimination	Half-life: 2 -20min Total body clearance: 50 – 1600 mL/kg/min Excretion: Urine (80%)

(data from Johnston et al., 2004; Lehtonen et., 2004)

*1.5.**Dopaminergic pathways involvement in pathological conditions***

Dopamine is particularly important in relation to neuropharmacology, because it is involved in several common disorders of brain function, with a number of neurological or psychiatric disorders such as: Parkinson's disease, schizophrenia (Hoenicka et al., 2007; Strange, 1993), migraine, drug dependence, mania, depression, and Gilles de la Tourette syndrome, ADHD, as well as in certain endocrine disorders (**Table 5**). Many of the drugs used clinically to treat these conditions work by influencing DA transmission. Due to the extensive and important role that DA plays in the nervous system, the imbalance on the capture/release of DA and/or DR expression have been extensively studied.

Table 5. Role of DA in certain pathophysiological conditions

Dysfunction of the dopaminergic system might be expressed by: Impaired DA levels in CNS and/or at the periphery Expression of dopaminergic receptors Impaired dopaminergic signalling	
Autoimmune disease	Multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), inflammatory bowel diseases (IBD)
Neurological/neuropsychiatric disorders	Parkinson's disease, Huntington's disease Alzheimer's disease, schizophrenia, bipolar disorder, mania, hypersexuality, depression, social phobia, anxiety disorder, obsessive-

	compulsive disorder**,** drug abuse/use/addiction, alcohol dependence**,** migraine, attention deficit hyperactive disorder, Gilles de laTourette syndrome
Cancer	breast and colon cancer (Sarkar et al., 2008), gastric cancer (Chakroborty et al., 2008)

Dopamine–DR interaction is responsible for different cellular responses depending on the target cell and subtype of the receptor. Abnormalities of intracellular response can occur if the number of receptors is reduced (e.g. down-regulation in persistently high concentrations of DA), or the receptor's affinity for DA is reduced, or coupling to the intracellular signalling cascade is impaired**.**

1.5.1. *Neurological and neuropsychiatric disorders*

Dysfunction of dopaminergic neurotransmission in the CNS has been shown in a variety of neurological and neuropsychiatric disorders. These include PD, schizophrenia, ADHD, drug abuse, addiction, alcohol dependence, social phobia, obsessive-compulsive disorder (OCD) and Tourette's syndrome. Some disorders, such as hypersociality, bipolar disorder, mania and hypersexuality are related to an increase in DA, but also in the conditions, as cancer and stress are found increased levels of circulating DA are found (**Table 5**). Interestingly, in some disease, there have been shown impaired DA levels/receptors/signalling and abnormalities in DR expression on lymphocytes, and some other important immune functions are, sometimes in significant correlation with the severity of the disease. So far, no define evidence exists supporting primary dysfunction of dopaminergic pathways in psychiatric conditions. Neuroleptic drugs, which are used in psychosis and schizophrenia are mainly (but not only) DR antagonists. Neurolepsis is "*an altered state of consciousness, as induced by a neuroleptic agent, characterised by quiescence, reduced motor activity, decreased anxiety, and indifference to the surroundings*" (http://medical-dictionary.thefreedictionary.com/neurolepsis).

1.5.1.1.**Parkinson's disease**

In 1960, a ground-breaking study of Ehringer and Hornykiewicz discovered significant reduction of DA levels in the striatum of idiopathic Parkinson's patients (Ehringer and

Hornykiewicz, 1960). Today, PD is considered to be an aging-related neurodegenerative disorder mainly characterised by loss of dopaminergic neurons in SN in the locus coeruleus. Unfortunately the main course of disease onset still remains ill defined. So far, defined risk factors are age, hereditary disposition, trauma (e.g., in boxers), inflammation (encephalitis), impaired circulation (atherosclerosis), tumours and poisoning (especially by CO, manganese, and MPTP). Parkinson's disease symptoms become manifest when about 50-60 % of the DA-containing neurons in the SN and 70-80 % of striatal DA are lost. Disease-related symptoms are suppression of voluntary movements becoming slow and rigid, muscle tremor, hypokinesia (difficulty initiating movement), resting tremor, rigid facial expression, micrographia (abnormally small handwriting). Many other disturbances occur, such as increased salivation, depression, and dementia, due to additional lesions and death of neurons in the nucleus of the median raphe of the locus coeruleus, or lesions of the vagus nerve.

In the mammalian brain, DA is present at the highest levels within the striatum, which major function is regulation of posture and muscle tonus. The striatal neurons are partly activated and partly inhibited by DA from the SNpc, and also activated via cholinergic neurons. An imbalance between inhibitory and activating influences has a harmful effect on motor functions: too strong an inhibition of the thalamic nuclei has a hypokinetic, too little has a hyperkinetic effect. When dopaminergic neurons degenerate in the nigro-striatal dopaminergic tract, and the inhibitory influence of DA on the striatum is diminished, the consequence is an increased activity of excitatory cholinergic neurons (**Figure 5**).

Molecular mechanisms leading to degeneration of SNpc neurons are not fully understood. A number of evidence indicate some factors important for the final degeneration, but relationships among them are not well characterised. Major factors have been identified as mitochondrial impairment, ubiquitin-proteasome dysfunction, altered calcium homeostasis and oxidative stress (Banerjee et al., 2009; Surmeier et al., 2010).

Degeneration of nigrostriatal pathway and ensuing deficit in brain DA remains at present the most prominent alteration in PD (Dauer and Przedborski, 2003). The appearance of intracytoplasmic inclusions, Lewy bodies (LB) is another pathological hallmark of disease (Shults, 2006). Lewy bodies consist mainly of α-synuclein protein, which is encoded by the causative gene of fPD/PARK1 in familial Parkinson's disease.

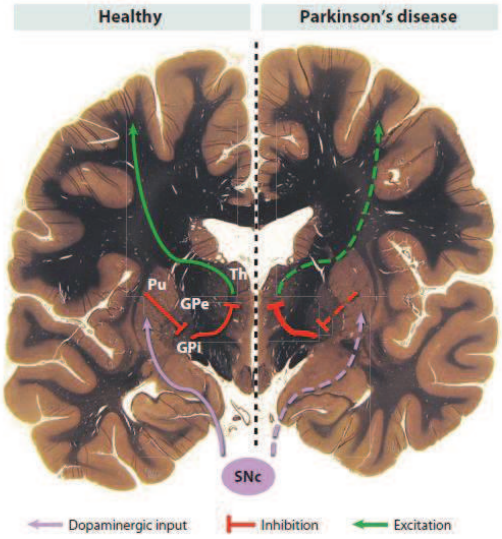


Figure 5. Anatomy and physiology of Parkinson's disease (PD) motor manifestations. A simplified schematic of the neuronal circuits involving the basal ganglia, thalamus, and cortex and their derangement in PD. For simplicity, only the direct pathway is shown. It normally functions to facilitate movements (*left*), but in PD the output is attenuated (*right*). The midbrain substantia nigra pars compacta (SNpc) provides dopaminergic input to the putamen, which is excitatory to the direct pathway. The putamen inhibits (*red*) the globus pallidus interna, which subsequently inhibits the thalamus. The thalamus projects excitatory input (*green*) to the motor cortex. In PD, degeneration within the SNc leads to net increased inhibition of the thalamocortical projection. The indirect pathway (*not shown*), including the globus pallidus externa and subthalamic nucleus, is inhibited by SNc dopaminergic input and normally functions to repress movements, but its activity is enhanced in PD. (Reproduced with permission from Shulman et al., 2011)

Whatever insult initially provokes neurodegeneration, studies of toxic PD models suggest that one of the major hypotheses regarding the pathogenesis of the disease is misfolding and aggregation of proteins as instrumental in the death of SNpc dopaminergic neurons (Auluck et al., 2002; Luk et al., 2012). The abnormal deposition of protein in brain tissue is a feature of several age-related neurodegenerative diseases such as dementia with Lewy bodies (DLB). Neurodegenerative diseases with LB are generally referred as synucleinopathies. Although the composition and location (i.e., intra- or extracellular) of protein aggregates differ from disease to disease, this common feature suggests that protein deposition *per se* is toxic to neurons. Little is known about the factors that might affect the propagation of α-syn pathology. Recent research suggests that neuroinflammation plays an important role in promoting of the prion-like behaviour of misfolded α-syn and that the same mechanisms contribute to inflammation in the olfactory bulb and gastrointestinal tract and promote the initial misfolding and aggregation of α-syn that lead to PD neuropathology (Lema Tomé et al., 2013). The same group propose that neuroinflammation and α-syn propagation

may be targeting mechanism for a novel antiinflammatory therapies that could slow disease progression (Lema Tomé et al., 2013).

Nigral dopaminergic neurons are particularly susceptible to oxidative stress because of their exposure to a high oxidative load: first of all, the metabolism of DA gives rise to various molecules that can act as endogenous toxins and start the formation of oxygen reactive species (ROS). Normally, these species are eliminated by intracellular antioxidant systems, which might be impaired by aging or by specific alterations owing to the PD pathogenesis (Alberio and Fasano, 2011).

Recent evidence increasingly points to another prominent neuropathological feature in PD patients' brains, the presence of a glial response (Sanchez-Guajardo et al., 2013) in all areas of the brain where signs of neurodegeneration can be found (Przedborski, 2010). The initial observation that activated microglia were detectable in brains of PD patients at autopsy came 25 years ago (McGeer et al., 1988). Since then, numerous studies, both in humans and animal models of parkinsonism, have implicated inflammatory processes in the development and progression of nigral dopaminergic neuron death. Several reviews of the subject of neuroinflammation have clearly demonstrated glial reaction in pathological situations of the CNS can play either a beneficial or detrimental role (Wyss-Coray and Mucke, 2002; McGeer and McGeer, 2004; Przedborski 2007). Microglia cells contribution to chronic inflammation in PD and their toxicity towards dopaminergic neurons is confirmed *in vitro* studies, but also in animal models of PD (McGeer and McGeer, 2008).

In recent years serious attention has been given to the potential impact of the immune system in pathogenesis of PD. Attention has been dedicated to changes in cellular immune responses in the peripheral immune system of PD patients, since there is a growing body of evidence that immune cells infiltrate the brain from peripheral compartment and that these cells are responsible for consequent changes of levels of neuroprotective or neurotoxic substances such as cytokines and reactive molecules (Nagatsu and Sawada, 2006).

Mounting evidence supports the fact that the peripheral immune system actively patrols the CNS and contributes to the functional integrity (Ransohoff et al., 2003). Under physiological conditions, the entry of immune system cells in the CNS parenchyma is restricted primariily by the brain blood barrier (BBB). Importantly, some immune cells involved in immunosurveillance may infiltrate into the cerebro spinal fluid (CSF) and patrol CNS. CSF flows into the subarachnoid space and drains into cervical lymph nodes, enabling peripheral immune cells to recognise and respond to CNS Ags in the absence or presence of inflammation

(Hatterer et al., 2008; Laman and Weller, 2013). Recently proposed, possible mechanisms, that lead to the development of the disease are neuroinflammatory processes in the brain of PD patients (reviewed in Hirsch and Hunot, 2009; Tansey and Goldberg, 2010). Recently, it has been shown that CD4+ T cells infiltrate brain and mediated dopaminergic toxicity in murine models of PD, as well as, human brain specimens examined post mortem (Benner et al., 2008; Brochard et al., 2009). In a recent immunohistochemical analysis of several leukocyte markers in the SN, Brochard and co-workers reported no B cells or natural killer cells and higher densities of CD8+ and CD4+ T cells in the brains of patients with PD than in healthy individuals. These cells were in close contact with blood vessels (suggesting migration from the bloodstream) and near to melanised dopaminergic neurons (suggesting an interaction between the lymphocytes and the dopaminergic neurons) (Brochard et al., 2009). Recent studies have shown that peripheral T cells that infiltrate into the brain play a fundamental role in neurodegeneration in PD (Reynolds et al., 2010). T cells with a pro-inflammatory phenotype (Th1, Th17) contribute to the destruction of dopaminergic neurons. Conversely, other T cell subsets, such as Treg and Th2 cells, could contribute to microglial switch towards M2-like anti-inflammatory phenotype (releasing neurotrophic factors e.g., insulin-like growth factor-1, IGF-1) promoting neuronal protection (Appel, 2009; Reynolds et al., 2010).

Infiltrating T cells can control the neurodegenerative process by the production of different molecules, acting on microglia cells and modulating their phenotype and function. The crosstalk of diverse population and phenotypes of CD4+ cells and activated microglial cells depends on activation status of infiltrating T lymphocytes that are able to promote neuroprotection or neurotoxicity, suggesting that an immunologic mechanism may be important in the development of PD, but it is uncertain whether immunological changes are primary or secondary events (Baba et al., 2005). During MPTP-induced PD, $CD4^+$ T cells that infiltrate in the SN produce high levels of cytokines that synergistically act to promote microglia inflammatory M1-like phenotype (Barcia et al., 2012). This pro-inflammatory phenotype is characterised by the secretion of inflammatory factors mediating neurotoxicity, such as TNF-α, IL-1β, superoxide anions, and other neurotoxins and cytokines (Appel, 2009; Klegeris and McGeer, 2000).

Several researchers have demonstrated that pathological features of PD can also be detected outside CNS, increasing the possibility that PD may, in fact, be a generalised disease (Lema Tomé et al, 2013). One direct consequence of this concept has been to prompt scientists to scrutinise non-CNS tissues, including blood and other body fluids, from PD patients for

hints of problems in oxidative metabolism. Several of these studies have reported significant alterations in the measured parameters in PD blood and CSF (Buhmann et al, 2004; Prigione et al, 2006).

Since inflammation and inflammatory mediators significantly contribute to the neurodegenerative process of PD, strategies targeted towards central and peripheral inflammation may, therefore, result in significant neuroprotective effects with unanticipated therapeutic relevance (Przedborski, 2010). In the case of PD, epidemiological studies have shown that the use of nonsteroidal anti-inflammatory drugs decreased the risk of developing PD (Chen et al, 2003). Involvement of peripheral adaptive immunity in neurodegeneration might provide novel perspectives in the pathogenesis of PD as well as in innovative therapeutic strategies.

*1.5.1.2.**Parkinson's disease therapy***

Since the pathological process in neurodegenerative diseases causes irreversible neuronal death, it appears very unpromising territory for pharmacological intervention and drug therapy has rather little to offer. So far, no treatment has been shown to slow or stop the progression of PD. Therefore, the treatment is symptomatic.

Dopamine replacement therapy with L-DOPA (dopamine precursor) in PD has shown need to the clinical utility of several other dopamine ergot agonists including: bromocriptine, lysuride, piribedil, pergolide, cabergoline and also some non-ergoline, newer generation DA agonist: pramipexole, ropinirole, rotigotine, and other similar compounds (Millan, 2010), but also apomorphine that act by direct stimulation of DR. Dopamine agonists bind to postsynaptic DR and mimic the action of DA in the synaptic cleft (Deleu et al., 2002).

They exert their action by directly activating DR, bypassing the presynaptic synthesis of DA. The activation of D2-like receptors (especially DR D_3) is important for antiparkinsonian effects of DA agonists, although concurrent D1-like and D2-like stimulation is required to produce optimal physiological and behavioural effects (Jankovic and Aguilar, 2008). Some commonly used DA agonists in the clinical practice are ropinirole, pramipexole, and rotigotine.

Pharmacological modulation of dopaminergic pathways can be obtained also by targeting: DA synthesis, storage, release, uptake, and metabolism with indirectly acting drugs (**Table 6**).

Although currently available PD therapies both delay disability and prolong life expectancy, none has been proven to significantly alter the ongoing neurodegenerative process (Shulman et al., 2011).

Table 6. Pharmacological targets and examples for the modulation of dopaminergic pathways by indirectly acting agents

Dopaminergic pathway:	**Target**	**Example of drug**	**Main effect/indication**
DA synthesis enzymes	TH	α-methyl-p-tyrosine	Enzyme inhibitor; treatment of pheochromocytoma and treatment-resistant hypertension
	DOPA decarboxylase	benserazide, carbidopa	Enzyme inhibitors, unable to cross the blood–brain barrier; treatment of PD in association with L-DOPA
	Dopamine-β-hydroxylase	disulfiram	Enzyme inhibitor; treatment of chronic alcohol dependence
Storage and release	VMAT	reserpine	Transporter inhibitor; treatment of hypertension and psychosis
		tetrabenazine	Transporter inhibitor; treatment of hyperkinetic movement disorders
Reuptake	DAT	benztropine	Transporter inhibitor; treatment of PD
	NET	tricyclic antidepressants (desipramine, imipramine, amitriptyline)	Transporter inhibitors; Treatment of depression
		noradrenaline reuptake inhibitors (atomoxetine,	Transporter inhibitors; treatment of depression and

		reboxetine)	other psychiatric syndromes
		serotonin-noradrenaline reuptake inhibitors (venlafaxine, duloxetine, sibutramine)	Transporter inhibitors; treatment of depression, anxiety disorder, chronic pain syndromes
		noradrenaline-dopamine reuptake inhibitors (amineptine, bupropion, methylphenidate)	Treatment of depression and other psychiatric syndromes
Metabolism	MAO	nonselective MAO inhibitors (nialamide, tranylcypromine)	Treatment of depression and anxiety
		selective MAO-A inhibitors (moclobemide, toloxatone)	Treatment of depression and anxiety
		selective MAO-B inhibitors (selegiline, rasagiline)	Treatment of PD
	COMT	tolcapone entacapone	Treatment of PD

(data adapted from Cosentino and Marino, 2013)

1.5.1.2.1. ***Levodopa in PD therapy***

Although PD is characterised by a loss of neurons that contain and release DA, oral or intravenous DA is not effective because it presents charged amino acid that does not pass the BBB. L-DOPA, as a precursor of DA, passes through the BBB and it is metabolised to DA in dopaminergic neurons hence supplying a source of DA to the brain (Olanow et al., 2001). L-DOPA is the cornerstone of PD therapy, sometimes also called "golden standard" and the most efficacious antiparkinsonian medication in moderate and advanced disease, as it provides relatively rapid symptomatic benefits and significantly improves patient quality of life.

L-DOPA is generally well tolerated with few initial side effects. Unfortunately, the therapeutic benefit of L-DOPA (maximal benefit usually lasts 3–5 years) is diminished by the unacceptable motor and psychological side effects that occur in many patients after several

years of treatment. In the severe stage of PD, very few nerve terminals remain where occurs conversion of L-DOPA to DA, and the effects of treatment consequently diminish. L-DOPA treatment over years leads to variability and fluctuation in response that patients sense like "on" and "off" periods. The causes of these fluctuations still are not defined. Most likely, postsynaptic DR changes are important, however variable pharmacokinetic of L-DOPA are equally involved. Another form of motor fluctuation is uncontrolled abnormal movements called dyskinesias. Some clinical and experimental data had concerned DR D_1 receptor subtype stimulation in the genesis of L-DOPA-induced dyskinesias, but experimental findings obtained with selective DR D_1 and DR D_2 high-efficacy agonists showed that neither dyskinesias nor antiparkinsonian effects could be ascribed solely to activity at DR D_1 or DR D_2 receptors (Boyce et al., 1990). Probably a synergistic effect between the two receptors appears to mediate these responses (Grondin et al., 1999). Controlled-release L-DOPA preparations provide fewer fluctuations in plasma than standard preparations. In this way, a smoother therapeutic response is achieved, although the onset of action is slower. For most individuals, treatment with L-DOPA reduces the symptoms of slowness, stiffness, and tremor.

Further on, L-DOPA and DA autoxidation gives rise to quinones, semiquinones and H_2O_2, which can be easily reduced in the presence of ferrous iron to hydroxyl radicals (Asanuma et al., 2003). Since it has been shown that L-DOPA is potentially neurotoxic, it was difficult to evaluate whether motor complications and dyskinesias are caused by the progression of the disease or exposure to L-DOPA. The controversy stems from the observation that L-DOPA increases DA metabolism, augmenting production of free radical species. It has been also shown that DA initiate apoptosis of neurons *in vitro* and exert toxic effects on various cultured cell lines (Offen et al., 1996), hence it seemed justified to speculate that the treatment with L-DOPA, as a DA precursor may, augment neuronal damage and provoke inflammatory changes in the SNpc and striatum by formation of free radical as well.

However, it has been shown, that long-term L-DOPA treatment, at large, cumulative doses, is not toxic to human SNpc neurons and does not lead to the development of the symptoms of PD (Rajput et al., 1997). *In vitro* experiments showed that L-DOPA generates oxyradicals and the formation of unnatural neurotoxic metabolites, such as 6-OHDA as other possible mechanisms for causing neuronal degeneration (Maharaj et al, 2005) or could lead to the excess formation of naturally-occurring, however neurotoxic intermediaries in the dopa-quinone-melanin pathway. Recent reports suggest that peripheral neuropathy is more common in patients with PD than age-matched controls (Toth et al., 2010). Possible reasons for this

association include peripheral nerve involvement by the pathology of PD or L-DOPA-induced metabolic derangements. The latter may include cobalamin deficiency and/or elevation of methylmalonic acid and homocysteine, which may be neurotoxic (Toth et al., 2010). Nonetheless, the evidence is lacking for L-DOPA toxicity in the treatment of PD patients (Olanow et al., 2004).

In vitro assays have demonstrated both toxic and protective effects of L-DOPA on dopaminergic cells, while in vivo studies have not provided convincing data. Colamartino et al., have demonstrated the protective effect of both L-DOPA and carbidopa on neuroblastoma cells in vitro. They have evaluated in vitro: i) modulation of DNA damage in the presence of oxidative stress, ii) direct scavenging activity of L-DOPA and carbidopa and iii) the expression of genes that were involved in cellular oxidative metabolism in the PBLs of healthy donors affected by different concentrations of L-DOPA and carbidopa confirming the antioxidant capacity of L-DOPA and carbidopa and their ability to protect DNA against oxidative-induced damage (Colamartino et al., 2015).

Because L-DOPA has antioxidant effects in the striatum (Camp et al., 2000), there has also been a rationale to explore a beneficial effect in vivo. Another interesting study aimed to examine in vivo effect of chronic treatment with L-DOPA and DA on murine lymphocyte proliferation and cytokine production/release, such as IFN-y and IL-4 (designate Th1/Th2 cell subsets) and to ascertain whether these effects were mediated through direct stimulation of DR (Carr et al., 2003). *In vivo* treatment with L-DOPA for 5 days resulted in an increase in the proliferative response to ConA of splenic lymphocytes while cell supernatant concentrations of IL-4 and IFN-γ were not significantly altered (Carr et al., 2003). However, the number of IFN-γ, but not IL-4 producing cells was significantly reduced by L-DOPA and this effect was replicated by infusion of DA, suggesting that DA may have a direct role in regulating immune responses through down-regulation of IFN-γ (Carr et al., 2003). In conclusion, peripheral *in vivo* administration of DA or L-DOPA, has regulatory effects on T lymphocyte function since it augment the proliferative capacity of T lymphocytes in response to mitogenic stimuli and the reduction of numbers of IFN-γ-producing cells in the spleen, through stimulation of DR D_2, since concomitant administration of DR D_2 antagonist reversed these effect (Carr et al., 2003).

To investigate if treatment with L-DOPA/benserazide exerts immunomodulatory potential of T-cell accumulated in the post-ischemic brain, Kuric and Ruscher subjected male Sprague–Dawley rats to transient occlusion of the middle cerebral artery and initiated with treatment on day 2 post-stroke. One week after intervention, distribution of T-cell populations

was analysed and it have been shown that treatment had significantly reduced CD3+CD8+ cytotoxic T-cells in the ischemic hemisphere together with reduced levels of T-cell-associated cytokine IL-5, while other T cell populations (CD3+, CD3+CD4+, CD3+CD4+CD25+) were unchanged compared with vehicle-treated rats (Kuric and Ruscher, 2014). Post-stroke treatment with L-DOPA/benserazide significantly downregulated the expression ICAM-1 levels on endothelial cells, which correlated with a reduced number of infiltrating cytotoxic T cells, suggesting that DA might act as a potential immunomodulator by attenuating inflammation in the post-ischemic brain (Kuric and Ruscher, 2014).

1.5.1.2.2. ***Catechol-O-methyl transferase inhibitors***

To prevent the peripheral metabolic breakdown of most of an administered dose of L-DOPA, it is frequently combined with DOPA-decarboxylase inhibitors (DDIs) and COMT inhibitors. In the US, the licensed DOPA-decarboxylase inhibitor is carbidopa, whereas in Europe benserazide is used. This drug combination allows more L-DOPA to reach the brain, prolong the duration of action of L-DOPA and prevents the peripheral accumulation of L-DOPA metabolites (Olanow et al., 2001).

COMT inhibitors, dose-dependently inhibit the formation of the major metabolite of L-DOPA (3-*O*-methyldopa). They are added to the therapy, as adjuncts to L-DOPA in patients with end-of-dose fluctuations and when DA agonists are not tolerated. Main representatives, entacapone, and tolcapone cause potent, selective and reversible inhibition of soluble COMT in the liver, kidney, small intestine and red blood cells (Kaakkola, 2000). While, entacapone is a peripherally-acting COMT inhibitor with no effect on central enzyme activity, tolcapone also inhibits *O*-methylation in the brain (Nissinen et al., 1992). Tolcapone causes severe hepatic failure, therefore its use has been restricted and is indicated only for patients whose symptoms are not adequately controlled, with appropriate monitoring of liver function (Borges, 2003).

1.5.1.2.3. ***Monoamine oxidase B inhibitors***

MAO-B inhibitors increase DA availability by inhibiting the degradation of DA by MAO-B (Youdim and Bakhle, 2006). When it is inhibited, the action of DA is prolonged in the brain, and the symptoms of PD are improved. Selegiline, MAO-B inhibitor, appears to have neuroprotective properties as it slows progression of PD, however, there is no firm evidence for

these now. It is effective as monotherapy for symptomatic relief or as an adjunctive agent. Inhibition of MAO-B prevents DA metabolism and the subsequent formation of oxygen species. These drugs also have a mild antidepressant effect. Side effects include heartburn, nausea, dry mouth, insomnia, and dizziness, as well as: confusion, nightmares, hallucinations, and headache that occur less frequently.

1.5.1.2.4. ***Ergot dopamine agonists***

The first generation of DA agonists were ergot derivatives with a different pharmacological profile from L-DOPA. Ergot derivatives had a longer half-life than L-DOPA and a differential affinity primarily to D1-like and D2-like DR. The ergolines, including bromocriptine, lysuride, cabergoline, and pergolide, are commonly available DA agonists which are structurally similar semisynthetic ergoline derivatives, and are generally considered to be full agonists.

Bromocriptine is an agonist of DR D_2 receptors (De Leeuw Van Weenen et al., 2010) and various types of serotonergic receptors. Both bromocriptine and cabergoline, acting as a DR D_2 agonists, have shown efficacy in the treatment of pituitary tumours, hyperprolactinemia, and related conditions (Colao et al., 2006). Bromocriptine has been used in the treatment of type 2 diabetes (Scranton and Cincotta, 2010).

Bromocriptine and the recently withdrawn pergolide may rarely cause retroperitoneal, pulmonary and pericardial fibrosis, but also cardiac valvulopathies (Elangbam, 2010).

1.5.1.2.5. ***Non-ergoline dopamine agonist***

After the remarkable success of the use of the DA precursor and indirect DR agonist in patients with PD (Birkmayer and Hornykiewicz, 1961), a number of highly effective compounds that activate DR have been developed. Nevertheless, it should be noted that none of these DA agonists can be compared in efficacy to L-DOPA as the first choice in PD treatment.

The most of the currently used non-ergot DA agonists have entered the clinic more recently and include pramipexole, ropinirole, rotigotine, naxagolide, and piribedil. Besides two orally prescribed DA agonist, pramipexole and ropinirole, rotigotine is used as a transdermal

patch, containing an active ingredient that is released gradually when is applied to the skin. Initially, they were introduced as an adjunct to L-DOPA chronic treatment in patients exhibiting motor complications and dyskinesias (Oertel and Quinn, 1997). Introduce of DA agonist reduce around a 20-30% of the L-DOPA dose, leading to significant improvement of L-DOPA treatment complications. There is evidence that DA agonists may provide symptomatic benefit but also be a neuroprotective and thereby slow progression of PD (Whone et al., 2003).

Although DA agonists treatment causes motor fluctuations less frequently than L-DOPA, it is more likely that this drugs will cause a number of other side effects: nausea, vomiting, dry mouth, dizziness, hallucinations, somnolence, orthostatic hypotension and lower extremity oedema. These particularly occur in patients over 70 and those with baseline cognitive deficits. In some individuals, DA agonists cause confusion, hallucinations, or even psychosis. Sleep attacks, drowsiness, or sedation is sometimes a significant side effect that may occur with all of the DA agonists. Behavioural side effects occur in 5-10% of patients and often reflect a disorder of "impulse control". These behavioural changes are often compulsive and include gambling, shopping, and binge eating, as well as increased sexual behaviours and can be resolve once the dose of the DA agonist is reduced or discontinued (Weintraub, 2008).

1.5.1.2.5.1. Pramipexole

Pramipexole has high selectivity for interacting with dopamine D2-like subfamily receptors, in particular, DR D_3 and a very low affinity for adrenergic or serotonergic receptors (5-HT_{2A} and 5-HT_{2B} receptors), as well as no affinity for D1-like receptors. Preferential affinity for the DR D_3 receptor subtype, according to preclinical studies, could contribute additional efficacy for treatment of both motor and psychiatric syndromes in PD (Piercey, 1998). High selectivity for DR D_3 provides safe drug profile, so pramipexole does not carry increased risk for valvular heart disease or pulmonary and retroperitoneal fibrosis, usually seen with long-term use of the ergot-derived DA agonists. Immediate-release pramipexole dihydrochloride is indicated for the treatment of signs and symptoms of idiopathic PD. It is administered alone or in combination with L-DOPA, during the entire progress of the disease, up to an advanced stage.

The first clues suggestive of beneficial effects of DA agonists on neurons came from a study in animal models. Whole-animal and cell culture studies suggest that pramipexole might provide neuroprotective effects through the decrease of DA metabolism, antioxidant effects,

and stimulation of trophic activity. In fact, all DA agonists are shown to have antioxidant properties *in vivo* and *in vitro* and these effects might be mediated by direct action on mitochondrial membrane potential and the inhibition of apoptosis (Schapira, 2002). However, a therapeutic benefit is not sustained, and it is possible to treat patients for only several years with DA agonists alone. Rather is considered preferable to use a combination of DA agonists and L-DOPA.

Studies with pramipexole have demonstrated a number of potentially protective actions against oxidative stress and the influence on dopaminergic neurons of various experimental toxins, including 6-OHDA and MPTP (Ferger et al., 2000). The mechanisms contributing to the protective actions of pramipexole have not been defined, although activation of the DR D_3 was suggested and blocking the cascade of apoptosis (Deigner et al., 2000). These effects appeared to be derived from the enhanced expression of Bcl-2 protein in neuronal dendritic processes or to other actions unrelated to the dopaminergic actions of the drug (Ferger et al., 2000; Deigner et al., 2000).

1.5.1.2.6. ***Other medications***

Other medications used in antiparkinsonian treatment can act by modulation of ganglia neurotransmission or affect receptors other than dopaminergic. Commonly used are apomorphine, amantadine, and anticholinergic medications.

1.5.1.2.6.1. ***Apomorphine***

Subcutaneous apomorphine is currently the only non-oral formulation of a DA agonist available. Apomorphine is a liposoluble molecule, moderately soluble in water and rapidly oxidised by light and air. Oral intake of the drug leads to nephrotoxicity since the high therapeutic doses are needed to overcome the extensive first-pass hepatic metabolism. Subcutaneous administration of apomorphine avoids first-pass metabolism and allows low dosages which are free of renal toxicity (Gancher et al., 1991). It acts as a potent nonselective DR D_1 and DR D_2 receptor agonist. Structurally it is a non-narcotic, synthetic morphine derivative, structurally related to DA. Apomorphine has been reported as a strong antiparkinsonian drug (Antonini and Tolosa, 2009; Garcia Ruiz et al., 2008). It is used as add-on rescue therapy for patients who have advanced PD and a wide spectrum of complications

not controlled by optimal oral dopaminergic therapy (Riley and Lang, 1993). Common side effects are gastrointestinal, including severe nausea and vomiting at the recommended doses. Psychiatric side effects include hallucinations and confusion and slowing down of cognitive performance has been reported as well (Schellekens et al., 2010). Cardiovascular side effects have included syncope, and have been reported in very rare cases.

1.5.1.2.6.2. ***Amantadine***

Amantadine is an antiviral drug, originally licensed for the prophylactic or symptomatic treatment of influenza A (Dolin et al., 1982). It has been used for decades as an antiparkinsonian agent, in particular for idiopathic PD (Lang and Blair, 1989), but also to treat extrapyramidal reactions, in particular, uncontrolled muscle movements caused by some medicines and for post-therapeutic neuralgia. Although most PD patients experience symptomatic improvement upon treatment, the exact mechanism of action remains elusive. The mechanism of action of the drug is probably a reflection of an increase dopaminergic transmission by augmentation of synthesis and release of DA, with possible inhibition of DA uptake and in addition it has mild antimuscarinic activity (Kulisevsky and Tolosa, 1990). It is well absorbed and widely distributed. In practice, amantadine may be used as a monotherapy or as an add-on to L-DOPA/peripheral decarboxylase inhibitor combination or DA receptor agonists in early and advanced PD.

Amantadine is used to reduce symptoms of fatigue and tremor in patients with early PD, but benefits are short-lived. Side effects include difficulty concentrating, confusion, insomnia, nightmares, agitation, headache and hallucinations.

1.5.1.3. ***Psychosis***

The term psychosis is very broad and includes state from relatively normal aberrant experiences through to the complex and catatonic expressions of schizophrenia and bipolar disorder. Clinical research has focused on applying brain research to understand the etiology, as well as to improve treatment, prognosis, and progression. Although direct evidence is lacking, it seems that when DA levels increase in the thinking areas of the brain, hallucinations start to occur in hearing, sensing, tasting and smell, but also delusions, disordered thinking, unusual speech or behaviour can occur. At the more extreme, this results in schizophrenia,

characterised by the loss of contact with reality and social dysfunction. The symptoms of schizophrenia are classified in categories as positive (delusions, hallucinations, thought disorder), negative (flat affect, poverty of thought, amotivation, social withdrawal), cognitive (distractibility, impaired working memory, impaired executive function), and mood (mania, depression) sensations (Wallwork et al., 2012).

Since the mechanism of pathogenesis is not very well understood, schizophrenia is a model of disease that relies on the basis of responsiveness to the treatment of known drugs. In fact, pharmacological studies indicate a strong correlation between the potency of antipsychotic drugs and blockade of the DR D_2 (Kapur et al., 2000; Seeman, 2010). Notably, all clinically approved antipsychotics are DR D_2 antagonist.

Originally described as neuroleptics, antipsychotics are effective in treating "positive" symptoms (particularly hallucinations and delusions). Unfortunately, the drugs may not be as helpful with other symptoms, such as reduced motivation and emotional expressiveness. "Positive" symptoms are linked to increased DA, especially in basal forebrain areas and are known for the presence of abnormal experiences and behaviour, disordered thought and speech, hallucinations (usually auditory) and delusions (often paranoid). "Negative" symptoms are linked to reduced DA, especially in the frontal and prefrontal cortex, the absence of normal experiences and behaviour, emotional blunting, anhedonia, apathy, social withdrawal and poverty of speech. Thus, antagonist drugs that act directly on DR D_2 and reduce DA activity in the brain are effective in the treatment of positive symptoms.

Antipsychotics are divided into first-generation or typical antipsychotics and second-generation or atypical antipsychotics:

1) typical antipsychotics are potent DR D_2 and D_3 antagonists

2) atypical antipsychotics target DR D_4 receptors (Van Tol et al., 1991) and cause a blockade of $5HT_{2A}$/$5HT_{2C}$/$5HT_{1A}$ serotonin receptors.

First antipsychotics (haloperidol and chlorpromazine) also known as neuroleptics have some side-effects that patients may experience as short-term side-effects including drowsiness, restlessness, muscle spasms, dry mouth, tremor, and blurred vision, but the also important long-term side effect is described as tardive dyskinesia. Neuroleptic antipsychotics can cause Parkinson-like side-effects, which are referred to as "motor side-effects" or extrapyramidal symptoms linked to reduced DA in the dorsal system (Tandon and Jibson, 2002). In some patients occur unpleasant subjective reactions to medication: feelings of restlessness (akathisia), emptiness, anhedonia, and apathy referred to as "mental side-effects" or

"neuroleptic-induced dysphoria" linked to reduced DA in the ventral system (Gerlach and Larsen, 1999).

The second generation of antipsychotics, target receptors other than DR D_2, such as the serotonin 5-HT_{2A}, but also $5HT_{2C}$/$5HT_{1A}$ having a lower incidence of side effects, but still possessing antagonistic activity at DR D_2 (Nord and Farde, 2011). Clinically, atypical antipsychotics evoke less severe extrapyramidal side effects because of the lower blockade of DR in the basal ganglia. Recent studies indicate that atypical forms are more likely to cause metabolic alterations, but a drug-associated obesity and type 2 diabetes are observed with both typical and atypical antipsychotics (Lindenmayer et al., 2003). This observation suggests that DR D_4 or serotonin receptor antagonism might be a contributing factor(s) in metabolic syndrome since it has been shown that DR D_4 are expressed in human pancreatic islets (Rubí et al., 2005).

In view of the role of prefrontal dopamine DR D_1 in cognition, cognitive symptoms, in particular, might respond well to treatment with DR D_1 agonist (Castner et al., 2000; Goldman-Rakic et al., 2004). Also, negative symptoms such as emotional indifference and social withdrawal may be amenable to treatment with DR D_1 agonist, as such symptoms might be the result of the hypodopaminergic function in the brain (Fink-Jensen, 2000).

Neuroanatomical, physiological and behaviour data suggest that DA agonist, ropinirole, and pramipexole, exhibit high affinity for cerebral DR D_3 receptor subtype. Use of these medications in PD has been complicated by the side effects characterised as pathologic behavioural patterns (gambling, hyperphagia, excessive hobbying, hypersexuality) shown in patients having no history of such disorders (Kelley et al., 2012). This receptor specificity may have relevance to increase rates of described pathological behaviours, since DR D_3 is particularly expressed in limbic areas, where the DA levels have shown to be increased by use of addictive drugs. Pathological behaviour has not been generally observed in patients taking bromocriptine, likely reflecting bromocriptine lacking affinity for DR D_3 (Montastruc et al., 2003), suggesting that DR D_3 receptor might represent a therapeutic target for new atypical antipsychotic drugs and OCDs. Various observations also suggest that DR D_3 might be implicated in schizophrenia (Schwartz et al., 2000). Behavioural abnormalities are shown to be mediated by DR D_3 (locomotor hyperactivity in mice mediated by glutamate/NMDA receptor blockade), and since glutamate/NMDA transmission is deficient in schizophrenia, DR D_3 receptor-selective antagonist may be useful as a novel antipsychotic drug (Sokoloff et al., 2006).

1.5.1.4. ***Drug abuse/use/addiction***

A large number of drugs act by altering the synthesis, storage, release, transport, or metabolism of endogenous ligands such as neurotransmitters. For instance, there are many examples of drugs that act on neuroeffector junctions by altering neurotransmitter synthesis, storage, release into the synaptic cleft, and subsequent removal. Cocaine blocks reuptake of DA, leading to increased concentration in the synaptic cleft. Mechanisms of cocaine dependence were described by Dackis and O'Brien (2001) by positive reinforcement where acute subjective effects of cocaine are intensely pleasurable and negative reinforcement, described as unpleasant rebound effects due to DA depletion (depressed mood, anhedonia, apathy, lethargy) are reversed by further drug use. Drugs that increase DA levels in the brain (acting indirectly on different components of dopaminergic pathways) are i) L-DOPA - used to treat symptoms of PD, ii) MAO inhibitors - prevent the breakdown of DA (and other monoamines, noradrenaline, and serotonin) and iii) psychostimulants - amphetamine and cocaine.

1.5.2. ***Autoimmune diseases***

In autoimmune diseases, DA levels are altered and this change also affects deregulation of dopaminergic components expressed in immune cells association in inflamed tissues of patients in the different stages of the development and progression diseases (reviewed by Pacheco et al., 2014).

1.5.2.1. ***Multiple Sclerosis***

Multiple sclerosis is one of the most common autoimmune diseases that represents a major cause of disability in both young and older populations (Frohman et al., 2006; Nylander and Hafler, 2012). Genetic and environmental factors have been proposed to be involved in the pathophysiology of the disease. Key pathological features of MS include: progressive loss of neurological function, increased BBB permeability and infiltrated lymphocytes into the CNS, crhonic glial activation and destruction of the axonal myelin sheath in several areas of the brain and spinal cord (Chastain et al., 2011).

Until recently, the contribution of T cells to the CNS function was largely ignored, since only a few cells were found in CNS parenchyma in healthy individuals (Smolders et al., 2013). However, evidence of an interactive communication between the CNS and peripheral immune system cells are accumulating (Zipp and Aktas, 2006; Ferrari and Tarelli 2011). Human CD4+ T cells that patrol the CSF and perivascular space for detrimental Ags are shown to have central memory phenotype: CCR7+, L-selectin+, CD27+ and activation marker CD69+ (Kivisakk et al., 2003). In MS or in infectious encephalitis, upon severe immune attack (by self- of non-self-reactive T cells, respectively), damage takes place locally, leading to conclusion that CNS is primarily damaged by the immune system (Ellwardt and Zipp, 2014).

Multiple sclerosis is regarded as an autoimmune process mediated by myelin-specific CD4+ T helper cells. In MS, myelin-reactive CD4+ T effector cells cross the BBB, enter the CNS and interact with resident cells to promote inflammation and promote further demyelination, leading to neurodegeneration (Kasper and Shoemaker, 2010). In the pathogenesis of MS, Th1 cells secrete the proinflammatory cytokines TNF-α and IFN-γ (Hemmer et al., 2006), while Th17 cells produce IL-17 (Tzartos et al., 2008). Self-reactive T cells differentiation toward inflammatory Th17 phenotype, recently described as a novel subset (Harrington et al., 2006), distinct from Th1 and Th2 cells, have been shown to contribute to the development of autoimmunity (Bailey et al., 2007).

A recent report shows that DA can decrease IL-17 and IFN-γ production by PBMCs both in patients with relapsing–remitting MS and in healthy controls (Melnikov et al., 2016). According to their work: i) the number of circulating Th17 cells was augmented during MS relapses, ii) cultured PBMCs from patients in relapse release more IFN-γ and TNF-α than cells from patients in remission or cells from healthy controls and iii) *in vitro* treatment of PBMCs with DA reduced the production of both proinflammatory cytokines in all groups (Melnikov et al., 2016). Further, strengthening evidence for a potential benefit of dopaminergic agents in MS is summarised in recent reviews, briefly: i) in untreated patients, the expression and activity of D1-like DR (possibly not D2-like DR) on circulating PBMCs is reduced, and ii) in treated patients, immunomodulatory drugs, such as IFN-β, restore the functional responsiveness of DR on lymphocytes and shift the balance of DR in lymphocytes from predominantly D2-like (in cells of untreated patients) towards mostly D1-like. Since D1-like DR mediate most DA-dependent inhibition of human T cell proliferation and cytotoxicity, whereas D2-like DR can induce T cell proliferation and adhesion, increased expression of D1-like DR would, therefore,

be expected to be beneficial in MS (Cosentino and Marino, 2013; Marino and Cosentino, 2016).

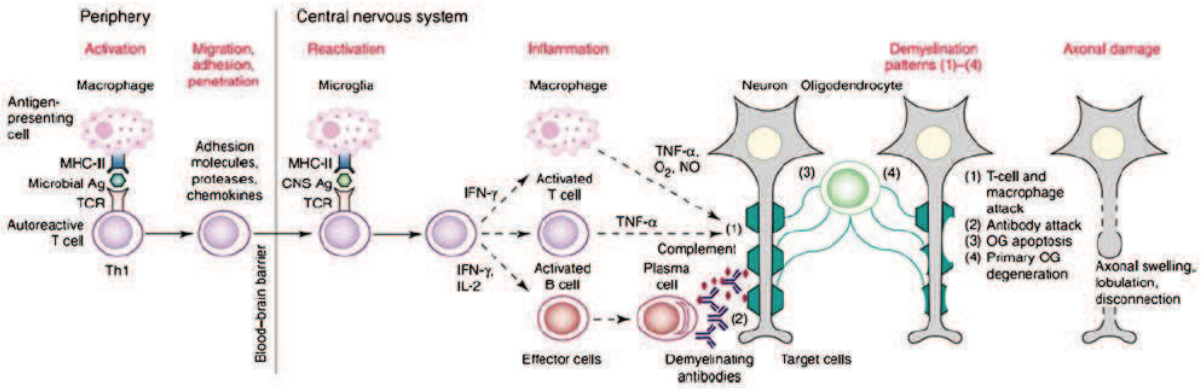


Figure 6. The putative mechanism of pathogenesis and progression in MS. T cells with adopted pro-inflammatory phenotype after activation are infiltrating to CNS from the periphery and passing BBB mediated by adhesion molecules and chemokines. In the CNS these cells are once again activated by microglial cells bearing the same Ags. This way activated T cells start to produce and secrete pro-inflammatory cytokines IFN-γ or IL-2, which induce inflammation by consequent activation of macrophages, B cells, and other T cells. Macrophages and T cells attack the myelin sheath of oligodendrocytes by cytotoxic molecules that they produce, mainly by TNF-α, O_2 and NO. Upon differentiation into plasma cells, B cells produce demyelinating antibodies that can activate macrophages, and initiate the complement cascade, that forms membrane attack complex and causes pore formation in myelin membranes, finally leading to demyelination which occurs by four different pathological. Reproduced with permission from Neuhaus et al., (2003).

1.5.2.2. ***Studies supporting modulation of dopaminergic pathways in multiple sclerosis***

So far, there is no cure for MS and treatment aim at slowing disease progression and reduce relapse rates. It relies mainly on immunosuppressive therapeutics, such as IFN-β (Kremenchutzky et al., 2007), although the mechanism of its action awaits clarification.

Evidence in humans and animal model of MS support the relevance of both sympathoadrenergic and dopaminergic pathways, therefore both systems could be considered in a common, integrated context to understand and exploit better the therapeutic potential of drugs acting on both systems at the same time.

Bromocriptine, a dopaminergic D2-like receptor agonist, showed improvement of clinical course in experimental autoimmune encephalomyelitis (EAE, animals model of MS disease) (Dijkstra et al., 1994). In MS patients study, the same dopaminergic agent was tested in in a pilot study, where the majority of patients complete the study showed disease

progression (Bissay et al., 1994). Mechanism of bromocriptine action may be explained in terms of the ability of this drug to reduce pituitary secretion of prolactin (Riskind et al., 1991). Prolactin has been reported to have a stimulatory role on immune function and is shown to be elevated in MS (Kira et al., 1991). Indeed prolactin might promote autoimmunity, and also hyperprolactinemia has been recorded in several autoimmune diseases (Orbach and Shoenfeld, 2007).

Besides bromocriptine, amantadine is the only drug that directly affects dopaminergic pathways and has been used for exhaustion in MS. Amantadine is not a pure dopaminergic agent, as it affects also noradrenergic and serotonergic pathways, blocks MAO-A and NMDA receptors, and may also increase beta-endorphin/beta-lipotropin levels (Huber et al., 1999). Evidence for the clinical effectiveness of amantadine suggests that the improvements in fatigue MS were small, while the impact on patients' functioning and quality of life remained undetermined (Pucci et al., 2007).

Although the clinical experience with dopaminergic drugs in MS are very limited, emerging evidence point to dopaminergic pathways in immune cells as potential therapeutic targets.

1.5.2.3. ***Rheumatoid arthritis***

Accumulating evidence concerning aetiology of RA involves a complex interplay among environmental triggers and suggests that the disease develops in genetically predisposed individuals. Rheumatoid arthritis is characterised by certain clinical features: synovial inflammation and hyperplasia ("swelling"), autoantibody production (rheumatoid factor and anti–citrullinated protein antibody), cartilage and bone destruction ("deformity"), and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders.

The SNS has been proposed to be involved in the pathogenesis of RA since it has been shown in experimental animals that developed collagen-induced arthritis (CIA) is less severe in sympathectomised mice than in animals with intact SNS (Härle et al., 2008), suggesting a pro-inflammatory role of SNS. Further, an adoptive transfer of Treg in this animals revealed that this cells might have a significant impact on disease severity (Härle et al., 2008).

Importantly, during RA synovial TH+ leukocytes, that have been found in RA patients but not in healthy controls, produce DA and NA, independently of SNS function (Capellino et al., 2010). By production of CAs that have strong anti-inflammatory effects, those TH+ leukocytes obtained from synovial tissues of RA patients showed *in vitro* and *in vivo* that these cells start to replace sympathetic nerve fibers around the onset of disease (Capellino et al., 2010).

Nakano et al., found that DA present in DCs in the synovial tissue of RA patients, and significantly increased in RA synovial fluid (Nakano et al., 2011). In the human RA synovial/SCID mouse chimera model, the selective D2-like receptor antagonist significantly induced accumulation of IL-6+ and IL-17+ T cells, and exacerbated cartilage destruction. Treatment with SCH-23390, selective DR D_1 antagonist, strongly suppressed these responses and diseases severity. These findings suggest that DA released by DCs may act, via D1-like receptors, to elevate the IL-6–dependent Th-17 production by CD4+ T cells and causes aggravation of RA synovial inflammation (Nakano et al., 2011).

A study in DBA/1 mice, immunised with type II collagen develop CIA showed that treatment of arthritic mice with the SCH-23390 suppressed CIA severity (Nakashioya et al., 2011). Nevertheless, the treatment did not affect serum levels of antibodies to type II collagen or the splenic Th1/Th17 differentiation in the treated animals. Co-administration of other selective DR D_1 agonist A68930 abrogated the *in vivo* anti-arthritic effect and has shown suppression of osteoclastogenesis when macrophages were isolated from the bone marrow and stimulated *in vitro*.

Taken together, elevated DA levels in RA synovial fluid play an important role in RA, and blocking of D1-like receptors could represent a potentially novel approach that can be of benefit in RA treatment.

1.5.2.4. ***Studies supporting modulation of dopaminergic pathways in rheumatoid arthritis***

Observations gained from animal models, together with the preclinical studies of DR antagonists implied studies in patients. Several clinical trials evaluated the effect of bromocriptine on RA disease activity. Clinical therapeutic trials using bromocriptine have shown efficacy in RA treatment (McMurray, 2001) inducing immunosuppression and improvement in morning stiffness and swollen/painful joints. These clinical observations give

support to the use of bromocriptine, as a non-standard primary or adjunctive therapy to traditional approaches, in the treatment of RA. Cabergoline exhibits a higher affinity for D2 – like receptors, with less severe side effects and more convenient dosing schedule, and has much less tendency to cause nausea than bromocriptine. In summary, treatment of active RA by administration of bromocriptine (McMurray, 2001) or cabergoline (Mobini et al., 2011), both well known as a DR D_2 and DR D_3 agonists, suppress immune parameters and significantly reduces RA disease activity.

1.5.3. ***Role of dopamine in cancer***

At present, very few studies deal with the role of DA in cancer, mainly are studies that are concerned about the role of DA in angiogenesis in tumour tissue. Angiogenesis is essential for the development of the embryo, tissue repair, and reproductive functions in the adult, but also this is a process of new blood vessel formation that is critical for the growth and progression of malignant tumours (Dvorak, 2005). Although angiogenesis is a balanced phenomenon between proangiogenic and antiangiogenic factors in normal physiological processes, in pathological conditions like cancer this balance is lost, thereby leading to the formation of abnormal blood vessels with increased permeability (Dvorak, 2005). Nowadays it is established that antiangiogenic therapy can slow down the growth and progression of malignant tumours (Dvorak, 2005).

Accordingly, there is considerable interest in identifying antiangiogenic molecules and their mechanism of actions so that newer therapies can be designed to effectively target tumour angiogenesis.

More than two decades ago DA was suggested as a novel anti-tumour agent against e.g. melanoma (Wick, 1982), however, its possible mechanism(s) of action remained not so clearly defined (FitzGerald and Wick, 1983). Dopamine possibly controls cell survival and proliferation, in a cell-type specific manner and exerts a paradoxical two-way outcome: i) in nontransformed cells, DA promotes cell proliferation and survival, and in tumour cell lines DA exhibits predominantly antiproliferative effects (Rubi and Maechler, 2010). In addition, DA might also protect against apoptosis (Nair and Olanow, 2008).

Adjunctive therapy of DA enhances the efficacy of anticancer drugs on breast and colon tumours in mice animal models (Sarkar et al., 2008), which is probably a consequence of an inhibitory role on tumour neo-vessel formation through the control of endothelial progenitor

mobilisation from bone marrow (Chakroborty et al., 2008). The inhibitory effect on cancer growth is confirmed by both DA and SKF-38393 (selective DR D_1 and DR D_5 receptor partial agonist) inhibiting the growth of human meningioma cells *in vitro* (Schrell et al., 1990). Fascinatingly, it was recently shown that DA also inhibits tumour angiogenesis and growth of human colon cancer, not causing hypertension, hematological, renal and hepatic toxicities in normal and tumour bearing animals (Sarkar et al., 2015). Also, D2-like receptor agonist, bromocriptine inhibited proliferation of human small lung cancer cells (Ishibashi et al., 1994). Collectively, the important role of DA in the regulation of metabolic effects (decrease of insulin release (Rubí et al., 2005) and immunomodulatory effect (by increase migration and proliferation from bone marrow (Chakroborty et al., 2008) may underlie tumour-protective effects of DA.

It has been documented that DA by acting upon DR D_2 inhibits angiogenesis by suppressing the action of vascular permeability factor/vascular endothelial growth factor-A (VPF/VEGF) (Basu et al., 2001; Chakroborty et al., 2008). In view of these findings, endogenous DA in lymphocytes provides in principle these malignant tumour cells with a source of antiangiogenic mediators which could be released upon appropriate pharmacological treatment, e.g. with type I IFNs (Cosentino et al., 2005) or with reserpine-like drugs (Cosentino et al., 2007). It cannot be excluded that DA as an antitumour agent may also act through downregulation of Treg, which maintain tolerance towards tumour cells (Hiura et al., 2005). Interestingly, it was reported that activation of IFN-Type I receptors effectively induces DA release from activated lymphocytes (Cosentino et al., 2005), an effect which occurs also *in vivo*, in humans (Zaffaroni et al., 2008). Treg play a key role in immune evasion mechanisms employed by cancer. Treg are actively recruited and induced by tumours to block immune priming, effector function and memory response, which can inhibit the efficacy of therapeutic cancer vaccines. It is therefore highly provocative that DA can effectively inhibit human Treg function, at least *in vitro* (Cosentino et al., 2007). Treg are critical for the maintenance of immune homeostasis and are often found at elevated frequencies in blood and tumours of patients, and for many cancers, a high density of Treg correlates with poor disease outcome (De Leeuw et al., 2013).

An improved understanding of the fundamentals and complexities of Treg – DA interplay may enable the selective modulation of this cells and valuable pharmacological target for the cancer treatment.

1.6. **Use of dopamine agonists as a "tool" to modulate dopaminergic system**

1.6.1. ***Pharmacological properties of dopamine agonists in clinical use***

Table 7 summarises the potency of some DR agonists with current marketing approval for PD to inhibit DR D_1, D_2, or D_3 binding in human putaminal tissue (Gerlach et al., 2003). Dopamine exhibited a low affinity for the D_1 receptor. The second generation of non-ergot DR agonists, pramipexole, and ropinirole, displayed no affinity for D_1 receptors even at high concentrations (up to 10^4 M).

Table 7. Pharmacological properties of DA agonists with current marketing approval for PD

	D_2/D_3 receptor affinity	**D_1 receptor affinity**	**NA receptor affinity**	**5-HT_{2B} receptor affinity**	**Half-life (h)**
Dopamine	$D_3>D_2$	low	+	-	2 min
Ergot agonists					
α-dihydroergocriptine	$D_2> D_3$	+	n.d.	n.d.	n.d.
Bromocriptine	$D_2> D_3$	+	+	+/-	3-6
Cabergoline	$D_3>D_2$	low	+	+	65
Lisuride	$D_2 = D_3$	+	+	+ (antagonist)	2-3
Pergolide	$D_3>D_2$	+	+	+	15-20
Non-ergot agonists					
(-)Apomorphine	$D_2>D_3$	+	-	-	0,5
Piribedil	$D_3>D_2$	-	+/-	-	20
Pramipexole	$D_3>>D_2$	-	+/-	-	10
Ropinirole	$D_3>>D_2$	-	-	-	6
Rotigotine	$D_3>D_2$	+	-	-	5-7 *
Quinpirole	D_2 low D_3 – n.d.	n.d.	n.d.	n.d.	n.d.

Notes: *(transdermal application), n.d. not determined

(data modified from Antonini et al., 2009; Gerlach et al., 2003)

DA itself shows actions on DR D_1 and D_2 receptors but appears to have the greatest affinity for the DR D_3 receptor subtype.

Apomorphine is active on DR D_1 and D_2 and to some extent on DR D_3 receptors.

Lisuride is used as an antiparkinson drug, and has been described as an agonist of DR D_5 receptors, a partial agonist of all other DR, and also is an antagonist at α_2-AR and $5HT_{-2B}$ receptors.

Cabergoline is a primary agonist at DR D_1 and D_5 receptors, a partial agonist at D2-like receptors, and also an antagonist at α_2-AR.

Bromocriptine has multi-receptor and complex action among them, acting as a full agonist at DR D_2, a partial agonist at DR D_3, an antagonist at DR D_4, also a full/partial agonist at D1-like DR and antagonist at α_2-AR.

Pergolide is a full agonist at DR D_2, and a partial agonist at DR D_3 and D_4 (Cosentino and Marino, 2013).

Receptor profiles of DA agonists corresponding to their clinical actions are not clear since none of these compounds are specific for only one receptor subtype. Consequently, DR agonist overall, can be at least considered as either D1-like (D_1 and D_5 receptors) and D2-like (D_2, D_3, and D_4 receptors) specific.

DR agonists are currently used in the treatment of restless leg syndrome, hyperprolactinemia, and PD, while antagonists are mainly used as antipsychotics and antiemetics (**Table 8**).

Table 8. Dopamine receptor agonists that have been used clinically

Dopamine agonist	**Major Clinical Application**	**References**
Apomorphine	PD, erectile dysfunction	Carson, 2007; Garcia et al., 2008; Antonini and Tolosa, 2009
Bromocriptine	PD, pituitary tumours, hyperprolactinemia, type 2 diabetes	De Leeuw Van Weenen et al., 2010; Shirasaki et al., 2010; Colao et al., 2006; Scranton and Cincotta, 2010
Cabergoline	Pituitary tumours, hyperprolactinemia	Colao et al., 2003; Freda et al., 2004
Fenoldopam	Hypertension	Murphy et al., 2001; Sarafidis et al., 2012
Pramipexole	PD, restless legs syndrome,	Zintzaras et al., 2010; Aiken, 2007

	bipolar disorder, depression	
Piribedil	PD	Rascol et al., 2006
Pergolide	PD	Elangbam, 2010
Ropinirole	PD, restless legs syndrome	Zintzaras et al., 2010; Aiken, 2007
Rotigotine	PD, bipolar disorder and depression	Aiken, 2007

(data adapted from Beaulieu and Gainetidinov, 2011)

1.6.2. **Therapeutic potential of drugs acting on dopaminergic system**

According to available literature, there are some abnormalities in DA levels in the brain and in the periphery associated several neurological/neuropsychiatric, autoimmune and cancer diseases (**Table 5**). Recent papers stress that in these pathologies, abnormalities in expression of DR in lymphocytes, and/or in immune functions might also be alerted. Based on these relatively small number of publications, it can be hypothesised that when the DA levels in the brain and periphery are changed, the immune system is also influenced.

A range of DA agonists is in various stages of preclinical and clinical development and the reports of their efficacy await confirmation. The overall activity of these drugs is governed by their affinity, efficacy, and selectivity with respect to different types of DR, and intensive research has been devoted to developing drugs with the most desirable properties for specific clinical indications.

The pharmacological targeting of DR has proven to be a very effective approach to affect deficient functions in above-mentioned pathological conditions (**Table 8**). Several DA agents are already in clinical use for non-immune/non-oncological/non-autoimmune indications and have a usually favorable risk-benefit. DR agonists are currently used mainly in the treatment of PD, restless leg syndrome, and hyperprolactinemia, while antagonists are mainly used as antipsychotics and antiemetics.

Pharmacological modulation of DA pathways can be gain also by use of indirectly acting agents. All the steps involved in DA synthesis, storage, and release, uptake and metabolism represent the target of several drugs already in use for non-immune indications (e.g. cardiovascular, neurologic, neuropsychiatric) (Cosentino and Marino, 2013). The established use of such DA drugs and the extensive available clinical experience would allow

the straightforward translation of the present results "from bench to bedside", through the development of well-designed clinical trial protocols.

Upon this background, new mounting evidence should be documented through *in vitro* experimental approach using DA agents to potentiate the beneficial immune response through the action on specific DR (D1-like and/or D2-like family) and to pave the way to further clinical trials of DA agents as add-on medications in conventional treatments. The most appropriate agent among the many that are currently available should be chosen in the way to achieve modulation of targeted, specific immune cell function, which have been shown to hold a key function in defining pathological diseases. So far developed research direction(s), may help in the repurposing of established drugs for the novel, original and critical indications, with the potential to significantly increase the therapeutic efficacy of current conventional, already established and in use, treatments.

The immunoregulatory effects of DA are becoming more evident, but the actual role of DA and DR in the regulation of pathologic conditions, as well as in specific immune responses, demands further studies. From a pharmacologic standpoint, the availability of several DA agonists coupled to their potential application *in vitro*, in stimulation/inhibition of functional activities of immune cells, appears provocative. Nonetheless, a clear understanding of the pharmacology, *in vivo* effects and potential side effects of these agents requires careful interpretation and clarification.

Table 9. Arguments for taking in account DA agonist, and not DA antagonist

Focus of presented work is on DA agonists for several strong reasons:
1. Preliminary evidence (*in vitro*/animal models/clinical trials) have shown specific modulation of functional immune response among key immune cells involved in certain pathological conditions.
2. Dopamine agonists are usually with a very complex binding properties for either D1-like or D2-like family receptors and so far obtained pharmacological agonist and antagonist effects on physiological cell function(s) represent a useful tool to clarify the extent of DA modulation involvement in important immunological response(s).
3. The molecular and pharmacological heterogeneity of DR potentially represents an opportunity to develop targeted immunomodulating strategies, towards D1-like or D2-like receptors-specific agonist, targeting DR in the brain or in the periphery, and consequently leading to a pharmacological challenge in producing selective drugs that may be of use in the

DA-related pathologies.
4. The range of different DA agonists already in clinical use represent a good starting point for a further understanding of potential ways of immunomodulatory response and possibly drug repurposing.
5. Antagonist are not part of our focus, since this pharmacological drug group (in the first line D2-like antagonist, used like neuroleptics/antipsyhotics) has a lot of side effects, in comparison to DA agonist that have safer pharmacological profiles, display a greater therapeutical index and usually favorable risk-benefit ratio.

Dopaminergic modulation of CD4+ T cells

1.7.1. Introduction to immunity

The physiological function of immune system is to protect the host from foreign infection, but also from injury. The collective and coordinated response of the cells and molecules elicited by foreign substances is called immune response. A wide range of mechanisms is involved in the normal protection of the human body from infection and foreign substances, which are capable of causing tissue injury and eventually disease. Studies of the response to infectious agents, inflammation process (such as occur in autoimmune or neuroinflammatory diseases), transplanted organs and tumour immunology, helped shape modern immunology and the study of the immune system as a model system in molecular cell biology have yielded dramatic advances in our understanding of the mechanisms of immunity.

Immune system comprehends two types of responses: early response, known as innate immunity, and late, adaptive or acquired immunity. Adaptive immunity is specific, specialised and has prodigious memory cells, which increase future responses. It is divided into two types of responses, humoral and cell-mediate immune response. The humoral immune response is the main defence mechanism against extracellular microbes and their toxins. Most recognisable cells of humoral immune response are B lymphocytes, that after the encounter of foreign Ag transform into plasma cells and produce specific Ab. The cell-mediated immune response is defending human organism against viruses and some bacteria. Hallmark cells are T lymphocytes, that are able to recognise intracellular Ags of microbes and destroy these microbes or all infected cell (Abbas et al., 2005).

1.7.2. Biology of the T lymphocyte immune response

T lymphocytes originate from stem cells in the bone marrow, mature in the thymus through complex stages of developmental selection upon maturation and finally acquire phenotype and functional characteristics of mature cells, upon which leave the thymus, enter the bloodstream and populated the peripheral lymphoid organs as naïve T cells ready to be activated after the recognition of an Ag. T cells have restricted specificity for Ag; they recognise only peptide Ag that are attached to host proteins that are encoded by genes of major histocompatibility complex (MHC) and that are expressed on the surface of other cells, hence, T cells respond to cell surface-associated but not soluble Ag. T cell-mediated immunity is an adaptive process of developing Ag-specific T lymphocytes to eliminate pathogenic treat or

development of malignant cells. T cell-mediated immunity can also involve abnormal recognition of self-Ag, leading to autoimmune inflammatory diseases.

The most important marker of T cells is a unique, surface T cells receptor (TCR), and invariant proteins CD3 and ζ chain, that together shape the TCR complex. Antigen presentation consist of coordinated reaction between TCR and MHC molecules expressed on APCs (so-called "signal 1") and co-stimulation mediated by CD28 and CTLA 4 (cytotoxic T-lymphocyte-associated protein 4) molecules expressed on T cells and B7 family of molecules (CD80 and CD86) expressed on APCs ("signal 2"), during which T cell becomes activated. Recognition of Ag by the immune system evokes a coordinate number of changes in lymphocyte subsets allow them (*i*) to eliminate or neutralise potential harmful agents and (*ii*) to respond more rapidly and appropriately after renewed Ag encounter, a process referred to as immunological memory.

Naïve T cells (that have not yet encountered foreign Ag), circulate through the blood and the lymphatic system and reside in secondary lymphoid organs. Presentation of Ag to naïve T lymphocytes lead to activation and subsequent complicated patterns of differentiation and clonal expansion of Ag-specific lymphocyte pool that will give rise towards short-lived effector T cells that will combat the infection. At the end of the immune response, most of the effector cells die by apoptosis, and an eventually small portion of these cells will become memory T lymphocytes (Zhu et al., 2010). In response to the activation, T cells start to produce and secrete diverse cytokines to promote further proliferation and differentiation and to potentiate effector function of other immune cells, such as macrophages, DC, and NK cells (Levite, 2012).

To conclude, pursuant to their activation, T lymphocytes can be discriminated into three subsets: unprimed, naïve T cells (which have not yet encountered Ag), effector T cells (with specialised functions), and memory T cells (long-lived cell capable of being reactivated in a case if an infection is reoccurred).

1.7.3. Polarisation of naïve T cells

Upon activation, signals from the TCR and co-receptors alter the pattern of gene transcription for proliferation and differentiation into effector T helper cells (Th1, Th2, or Th17 cells). The effector activity of the T cell is accomplished through the cytokines produced by surounding cells. TCR binding to Ag and initial T-cell activation involves a cascade of

signalling events that include the transcription factors NF kB, nuclear factor of activated T cells (NFAT), and activator protein 1 (AP-1). The production of IL-2 in response to T-cell activation is important for the initial proliferation and differentiation of the T cell.

All Th subsets are produced from a non-committed population of precursor T-cells which are polarised upon contact with APC. Besides the presence of specific cytokines produced by the innate immune system responding to microbial and parasitic Ag, or allergens, the differentiation of naïve CD4+ T cells into effector T helper cells requires the engagement of TCR and costimulatory molecules. Depending on the signal brought by APCs and surrounding microenvironment naïve $CD4^+$ T cells can differentiate into various effectors cell populations with specialised functions: Th1, Th2, and Th17 cells. These Th subsets secrete a defined, and largely non-overlapping, subset of cytokines acting on distinct target cell populations.

Non-polarised, naïve Th0 precursor can differentiate into Th1 after activation in the presence of IL-12 (Moser and Murphy, 2000) and IL-18 (Rodriguez-Galan et al., 2005), both derived from DCs or by IFN-γ derived from NK. Importantly, recent studies using $IL\text{-}12^{-/-}$ knockout mice have shown that stimulation of T cells with IL-18 and anti-CD3 failed to provoke IFN-γ producing cells, but markedly upregulated IL-12Rβ2 expression, suggest that IL-18 cannot drive Th1 cells alone, but it is an important cytokine to enhance IL-12 signalling and to promote Th1 development (Chang et al., 2000). Although IL- 12 appears to be a key player in Th1 development, Th1 cell polarisation shown to occur in $IL12^{-/-}$ mice indicates that also other mechanisms and pathways exist. DC recognising DNA, RNA, or bacterial structures, such as lipopolysaccharide (LPS), but also viruses as intracellular parasites, have been shown to promote differentiation towards Th1 phenotype (Amsen et al., 2004). Th1 cells thus direct cell-mediated inflammatory reactions to control intracellular pathogen infections effectively (**Figure 7**).

Differentiation of T cells towards strong Th2 phenotype occurs in answer to parasitic helminths, fungal products, toxins such as cholera and allergens, which cause chronic T cell stimulation, often without any innate immune response or macrophage activation, in the presence of IL-4 derived from B cells or lymphoid DC (Kapsenberg, 2003). Further, while IL-4 is an important differentiation factor for Th2 cells, IL-4 production by non-T cells is not absolutely required for Th2 cell differentiation (Schmitz et al., 1994). Recently, other cytokines such as IL-33 (Schmitz et al., 2005) and IL-21 (Wurster et al., 2002) have been shown to potently induce Th2 cytokine production and downregulate Th1 responses. Thus, investigators

seek to identify alternative mechanisms that can direct differentiation of CD4+ T cells into Th1 and Th2 lineages (Reizis and Leder, 2002).

Recent evidence showed that former dogma that IL-12-driven Th1 response as a critical contributor to inflammation has to be revised since it has been found that IL-23 induced production of CD4+ T cells that secrete proinflammatory cytokine IL-17A (Palmer and Weaver, 2010). These cells were characterised as a separate Th subset called Th17. Initially, it has been described that result of Ag stimulation of DC results in IL-23 secretion, which induces T cell production of IL-17 in a T cell receptor-independent manner (Kolls and Lindén, 2004). Bettelli et al., demonstrated in transgenic mice, that IL-23 is not the differentiation factor for the generation of Th17 cells since the IL-23 receptor is not expressed on naïve T cells, and it was not possible to generate *de novo* IL-17-producing T cells from sorted naïve T cells. Instead, IL-6 and TGF-β together induce the differentiation of pathogenic Th17 cells from naïve T cells (Bettelli et al., 2006). However, IL-23 stabilises differentiating Th17 cells and leads to the further maturation of Th17 cells (Zhou et al., 2007) further supporting its importance for full and sustained differentiation of Th17 cells.

It seems that TGF-β is absolutely required to induce RORγt, but its function is inhibited by high concentration of TGF-β, suggesting a biphasic effect of this cytokine. TGF-β is required to induce expression of IL-23R, since it is not present originally on naïve T cells. Only when additional cytokines: IL-6, IL-23 or IL- 21 are present, RORγt is relieved from inhibition and naïve T cells can begin transcribing IL-17 (Manel et al., 2008). At a molecular level, it seems that the differentiation conditions of mouse and human Th17 cells do not appear to be different (Korn et al., 2009). Interestingly, IL-17 cannot amplify Th17 cells because IL-17 does not act as a growth or differentiation factor for the Th17 lineage (Korn et al., 2009).

The effector cytokines that are subsequently produced by Th1 and Th2 cells (such as key cytokines IFN-γ and IL-4, respectively) can potentially feed back to amplify or Th1 or Th2 cells and further enhance differentiation of the respective T cell subset.

Th17 cells have been recognised as a lineage separate from Th1 and Th2 cells, and also differentiation of Th17 cells is inhibited by factors, both IFN-γ and IL-4, that support Th1/Th2 differentiation (Harrington et al., 2005; Park et al., 2005).

To conclude, since it has been shown that INF-γ produced by Th17 inhibits Th2 developmental pathway, IL-4 produced by Th2 inhibits Th1 development, both INF-γ and IL-4 inhibit Th17 polarisation ruth, and also IL-17 inhibits Th1, appear that all effector CD4+ T

cells exert certain suppressive activities holding the balance in immune system homeostasis (Corthay, 2009).

1.7.4. Maturate and developed T helper subsets

Th1 cells mainly produce IFN-γ, and to a lesser extent IL-2 and IL-12, TNF-α, lymphotoxine α (LTα) and lymphotoxin, that are all involved in enhanced pro-inflammatory cell-mediated immunity (Zhu and Paul, 2008). Th1 cells stimulate anti-microbicidal and cytotoxic effector functions, activate macrophages and recruit and activate NK cells, CD8+ T lymphocytes, and stimulated B cells to differentiate into plasma cells. Th1 cells drive a cellular immune response to fight viruses and other intracellular pathogens, but also to eliminate cancerous cells.

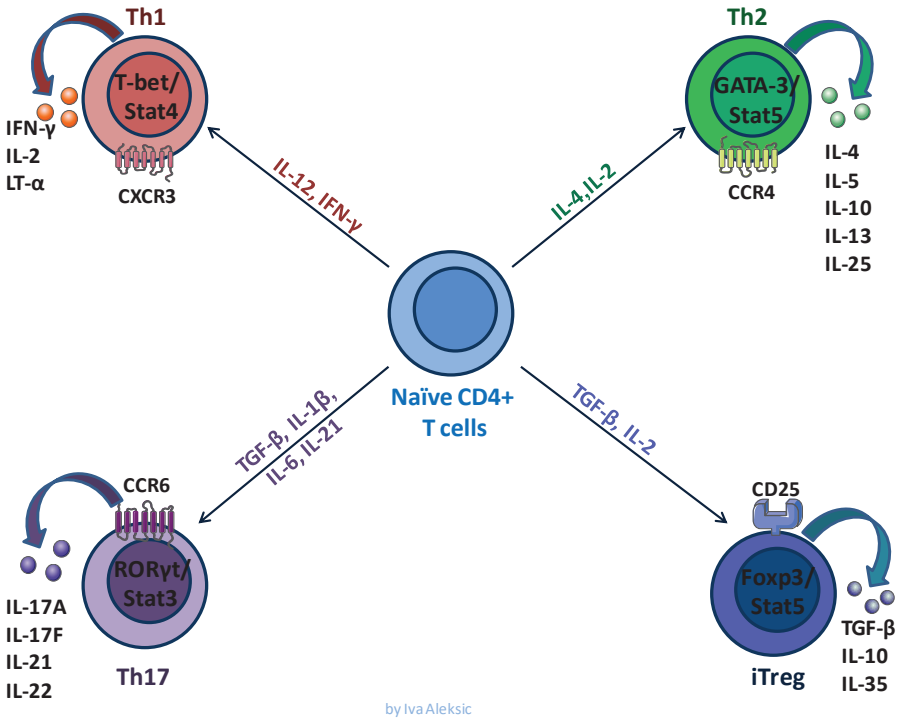


Figure 7. Differentiation of naïve $CD4^+$ T cells into diverse subset of effector cells, their characteristic cytokines production and specific transcription factors.

Th2 cells basically promote non-inflammatory immediate immune responses through the production of cytokines that include IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann and Coffman, 1989; Fiorentino et al., 1989; Le Gros et al., 1990). Th2 cells drive a "humoral immunity" pathway and up-regulate Ab production to fight extracellular organisms. This type

of response is particularly important for host defence or resistance against parasitic infections. Overactive Th2 cells recruit eosinophils and maintain their function, but also cause macrophages to become alternatively activated. In contrast to Th1 cells, Th2 cells direct and enhance B cell activation and Ab production (particularly IgE) to promote allergic reactions and eosinophilic inflammation important in the induction of asthma.

Mature Th17 cells produce mainly IL-17, which is best known for its participation in the recruitment and survival of polymorphonuclear neutrophils (PMN) (Schwarzenberger et al., 2000; Kolls and Lindén, 2004; Yu and Gaffen, 2008). Once secreted, IL-17 in the bone marrow seem to induce stromal/ fibroblast expression of both G-CSF and stem cell factor, an effect that increases neutrophils differentiation and production, and by directly blocking neutrophil apoptosis promotes greater circulating PMN numbers (Schwarzenberger et al., 2000). In macrophages, IL-17 induces TNF-α, IL-1β and IL-6 production (Jovanovic et al., 1998). IL-17 further contributes to PMN influx by inducing endothelial cells CXC chemokine release and NO production, which may increase vascular permeability (Kolls and Lindén, 2004; Miljkovic et al., 2003). Th17 cells mediate responses against extracellular bacteria, which cause acute inflammation such as Streptococcus, and fungi (Weaver et al., 2006). It has been confirmed that patients lacking these cells have frequent Candida and Staphylococcus infections, and in additionally, they are implicated in the induction of many organ-specific autoimmune diseases (Zhu and Paul, 2008).

The differentiation of naïve Th cells towards Th1, Th2, and Th17 cells is regulated by the master transcriptional regulator factors T-box expressed in T-cells (T-bet), GATA-binding protein-3 (GATA-3) and orphan nuclear receptor RORγt (RORγt) (Korn et al., 2009), respectively (**Table 10**). The loss of the IL-12Rβ chain is thought to be a marker for Th2 commitment, while the transcription factor GATA-3 is lost in Th1 cells. These functionally distinct cytokine-producing T cell lineages can be also distinguished by expression of master transcription factors. Evidence suggests that signal transducer and activator of transcription 4 (STAT4) regulated IL-12Rβ expression and T-bet expression are associated with the development of Th1 cells (Nishikomori et al., 2002) while GATA-3, STAT5, and c-Maf are associated with the development of Th2 responses (Mathew et al., 2001; Zhu et al., 2006; Chakir et al., 2003). Recent experimental data suggests that naïve T cells tend toward a Th2-like development through GATA-3 (which down-regulates the STAT4/ IL-12Rβ pathway) unless T-bet is activated (Usui et al., 2006). Once T-bet is activated, GATA-3 is downregulated

suggesting that the role of T-bet is to control GATA-3 levels rather than to positively regulate the IFN‑γ gene as originally proposed (Usui et al., 2006).

Table 10. Signature characteristic molecules of different CD4+ T cell subsets

CD4+ Th designation	Cytokines produced	Cellular targets	Transcription factor	Proximal regulators	Homing receptors
Th1	IL-2, IL-3, IL-15, IFN-γ, TNF-α, TNF-β, GM-CFS	B cells, macrophages, NK cells	T-bet, STAT4	IL-12, IFN-γ	CXCR3, CCR5
Th2	IL-4, IL-5, IL-6, IL-13, IL-21, TNF-β, GM-CFS	B cells, mast cells, eosinophils	GATA-3, STAT5	IL-4, IL-25, IL-33	CCR4, CCR3
Th17	IL-17A, IL-22, IL-21	Neutrophils, macrophages, endothelial cells	RORγt, STAT3	IL-6, IL-1β, TGF-β, IL-21, IL-23	CCR6, CCR4

(Data adapted from Sallusto et al, 2012; Miossec and Kolls, 2012)

The driving factors that dictate the quality of the immune response are recognised as quantitative events that affect the specification, differentiation and commitment of Th cells. *In vitro* approaches have been of essential importance in the elucidation of the different quantitative factors determining the development and commitment of Th cells. New discoveries in terms of previously unknown cytokines, expanded numbers of potential helper T cell subsets and the influence of identified factors and pathways triggered by different pathogens.

1.7.5. Phenotypical and functional charactherisation of different T cell subsets

It was originally believed that chemokine receptor expression could reliably distinguish the different subsets, and although there is some functional division, it is becoming clear that *in vivo* this association is less well defined. So far, there are only a few cell surface markers that can reliably differentiate between Th1 Th2 and Th17 cell populations (**Table 11**).

Table 11. T helper cells related chemokine markers and transcriptional factors

	Receptor	Description/Function	References
Th1	CXCR3 (CD183)	Receptor for IP-10, Mig, and I-Tac	Bonecchi et al., 1998
	CCR5 (CD195)	Receptor for RANTES, MIP-1α, β	Bonecchi et al., 1998
	STAT-4	Transcription factor that regulates IL‑12Rβ expression and drives Th1 development	Nishikomori et al., 2002
	T-bet	Transcription factor overexpressed in Th1 cell populations may inhibit GATA-3 levels to promote Th1 development	Usui et al., 2006; Chakir et al., 2003
Th2	CXCR4 (CD184)	Receptor for CXCL12, co-receptor for HIV-1	Galli et al., 1998
	CCR3	Chemokine receptor for MIP-1α, MIP-1β, RANTES, MCP-2, 3, and 4, and eotaxin 1, 2, and 3	Sallusto et al., 1998; Bonecchi et al., 1998
	STAT6	Transcription factor was shown to regulate Th2 recruitment and effector function as well as eosinophilia	Mathew et al., 2001
	GATA-3	Transcription factor upregulated in developing Th2 cells, enhances IL-4, IL-5, and IL-13 production. Downregulates IFN-γ production	Zhu et al., 2006; Chakir et al., 2003
	CCR4 (CD194)	Receptor expressed on Th2 cells, skin-homing T cells and IL-17-producing cells	Acosta-Rodriguez et al., 2007
	CCR6	Receptor expressed on T cells (regulatory and	Acosta-Rodriguez et

Th17		memory), B cells and DC, shown to be involved in mucosal humoral immunity, allergic asthma and intestinal T-cell homing, particularly at epithelial sites	al., 2007; Cook et al., 2000; Singh et al., 2008
	RORγt	Transcription factor that (together with Runx1) regulates IL-17 transcription	Zhang et al., 2008; Korn et al., 2009

(The table was drawn based on the compilation of findings revealed by several research groups worldwide)

1.7.6. Plasticity and commitment of helper T cells

Although once thought to be permanently polarised and committed stable lineages, nowadays it is apparent that conversion between these cellular phenotypes can occur depending on a range of altering genetic factors and micro-environmental signals that are able to promote flexibility in the programmes of this cells, so these effector T cell subsets can rapidly react in order to promote the most effective type of immune response and to allow flexibility in T cell programmes. Even though the cytokine profile may initially not be entirely polarised, with differentiating T cells producing a combination of both Th1 and Th2 cytokines, chronic stimulation leads to unequivocal, terminally differentiated phenotypes. In fact, a large body of evidence suggests that there is a conversion between Th1, Th2 and Th17 populations under defined activation conditions (Abbas et al., 1996; Zhu and Paul, 2008; O'Garra et al., 2011). *In vitro* has been demonstrated that Th17 cells are very unstable and that they are able to change their cytokine production from IL-17 to INF-γ in chronic immune responses (Annunziato et al., 2007; Hirota et al., 2011).

In cases where the immune response is strongly fixed towards one phenotype, the prevalent cytokine imbalance has been associated with disease pathogenesis. For example, a dominant Th2 response has been associated with atopic dermatitis, asthma, and the outgrowth of a number of cancers (Pellegrini et al., 1996; Nakazawa et al., 1997), a dominant Th1 response has been described for sarcoidosis, tuberculosis, and CIA (Mauri et al., 1996), while a Th17 response is shown to be important in chronic inflammatory diseases such as psoriasis, RA, ankylosing spondylitis, Crohn's disease, MS, chronic obstructive pulmonary disease (COPD) and other conditions (Miossec and Kolls, 2012).

1.7.7. T helper subsets role in disease pathology

Inappropriate or poorly controlled effector T cells can cause host pathology and are particularly deleterious when directed against self or ubiquitous environmental or commensal Ags, which cannot be effectively cleared. In this setting, persistent effector T cell responses drive chronic inflammatory disorders such as autoimmunity and allergy. Effector T cell responses are therefore normally under stringent regulatory control.

An overactive Th1 pathway is aggressive and can generate organ-specific autoimmune disease. The overactivation of Th2 pathway leads to allergy and IgE-related disorders. It has been recently reviewed that specific Th17 subpopulation might, along with Th1, contribute to neurotoxicity, possibly through the secretion of IL-17 and/or release of a cytolytic enzyme (granzyme B) and directly injure DA neurons by signalling through the Fas/FasL system (Appel et al, 2009). Increasing evidence shows that IL-17 family members play an active role in inflammatory diseases, autoimmune diseases, and cancer.

1.7.8. T regulatory cells subset

CD4+CD25+ regulatory T lymphocytes are specialised T cells that play a crucial role in the control of immune homeostasis, contributing to the maintenance of immune homeostasis and immune responses to foreign and self-antigens setting up and maintenance immune tolerance (Sakaguchi 2004; Sakaguchi et al. 1995). The role of Treg implies critical involvement in immunologic diseases, tumour immunity, and transplantation tolerance. Mice in which Treg cells are absent or depleted are more prone to development of several autoimmune and inflammatory diseases (Ochs et al., 2007; Brunkow et al., 2001) and numerous studies in animal models of autoimmunity showed that the disease could be reversed by the adoptive transfer of Treg cells (reviewed in Sakaguchi et al., 2006).

Treg suppress the function of other T effector cells by the employment of several mechanisms: (i) cell-cell contact (Shevach, 2006), and/or (ii) production of their specific cytokines, including TGF-β and IL-10 (Zhu et al., 2008). Activated Treg produce IL-10 and TGF-β, powerful immunosuppressants that are able to inhibit Th1 cellular immunity and Th2 mediated antibody production and therefore induce tolerance, hence, Treg have been identified as important mediators in the Th1/Th2 balance.

So far, several types of Treg cells have been defined; such as "naturally occurring" ones that originate directly from the thymus in contrast to other types of T regulatory cells such as Tr1 and Th3, which likely develop from conventional CD4+CD25- T lymphocytes (Teffs) in the periphery (**Table 12**) (Rutella and Lemoli, 2004; Thompson and Powrie, 2004; Piccirillo and Thornton, 2004; Milojevic et al., 2008).

Table 12. Key characteristics of Treg subsets.

	Phenotype	**Associated markers**	**Role**	**Mechanism of suppression**
Natural Treg	CD4+CD25high CD127low	CTLA4+, GIRT+FoxP3+ CD127low	Suppression of autoreactive T cells	Contact-dependent, granzyme B-dependent, produce TGF-β
Tr1	CD4+CD25-	CD45RBlow, FoxP3-	Mucosal immunity, inflammatory response	Through IL-10 cytokine production
Th3	CD4+CD25+	CD45RBlow, FoxP3+	Mucosal immunity, inflammatory response	Through TGF-β cytokine production

(data adapted from Milojevic et al., 2008)

A characteristic marker of Treg cells is the transcription factor FoxP3. In humans, the Treg are a heterogeneous population, in which not all CD25+ cells are Treg (Baecher-Allan et al., 2005; Taams et al., 2001). Studies revealed that only those CD4+ cells that expressed very high levels of CD25, representing approximately 2–3% of total CD4 T cells, demonstrate the *in vitro* suppressive activity similar to that described in murine cells, while those who are expressing low-to-intermediate levels of CD25 do not exhibit suppressive activity directly in the *in vitro* experiments (Baecher-Allan et al., 2005).

Treg express a high level of IL-2Rα (CD25) constitutively, and this notion highlights the crucial role of IL-2 in the development, survival and homeostasis (Fontenot et al., 2005; Malek and Castro, 2010), since it has been shown that deficiency of IL-2 or IL-2R may result in defects in Treg. IL-2 is able to activate STAT5, a transcription factor that binds to the Foxp3 promoter, induce Foxp3 expression and hence contribute to Treg development (Yao et al.,

2007; Burchill et al., 2007). Since Treg cells cannot produce significant amounts of IL-2, unlike activated effector CD4+ T cells, they are fully dependent on the IL-2 production by other cells *in vivo*, or exogenously added IL-2 *in vitro* conditions (Rubtsov et al., 2010).

Nonetheless, Treg express several other surface markers of activation, including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced tumour necrosis factor receptor (GITR) (reviewed in Piccirillo and Thornton, 2004). It is very difficult to distinguish activated CD4+ T cells from Treg on the basis of expression of surface molecules, since those molecules are not constitutively expressed in peripheral, naïve T eff cells but their expression is induced after activation.

Recently, it has been described that CD127 expression is down-regulated in the Treg cells, inversely correlating with the expression of FoxP3 marker (Liu et al., 2006). CD127 is part of the heterodimeric IL-7 receptor that is composed of CD127 and the common γ chain, which is also shared by other cytokine receptors (IL-2R, IL-4R, IL-9R, IL-15R, and IL-21R). Studies have shown that IL-7R plays an important role in the proliferation and differentiation of mature T cells, and *in vitro* experiments show that the expression of CD127 is down-regulated following T cell activation (Hofmeister et al., 1999; Fitzgerald et al., 2001). It has been shown that FoxP3 interacts with the CD127 promoter and might contribute to reduced expression of CD127 in Treg (Liu et al., 2006).

To conclude, a population of Treg cells with a highly suppressive function can be identified based on a combination of surface markers such as CD4, CD25, and CD127.

Based on the appropriate phenotypes (e.g. expression of CD25, CD127, CD45RA and FoxP3 markers), proliferation status in the physiological state, cytokine secreting capacity and *in vitro* suppressive activity, CD4+ Treg cells were divided into 3 distinct subsets: resting (naïve) Treg cells (nTreg, CD45RA+FoxP3lo), activated Treg (aTreg, CD45RA-FoxP3lo) and third subtype of cytokine-secreting CD45RA-FoxP3lo non-Treg cells (Miyara et al., 2009). Both nTreg and aTreg were suppressive *in vitro*, and the third subset has shown cytokine production without suppressive activity *in vitro*, suggesting that functional analysis of FoxP3+ subsets is essential for assessing immunological state in a variety of physiological and pathological immune responses (Miyara et al., 2009).

The microenvironment plays a crucial role in the differentiation of classical Treg cells, but also in their expansion and function. The cytokines that have a major role in promoting the activities of Treg include TGF-β, as a critical differentiation factor for the generation of Treg cells (Chen et al., 2003) and IL-2 as a critical factor for induction and expansion of Foxp3+

Treg (La Cava, 2008). Cytokines that promote Th17 responses significantly counteract the activation and functionality of the Treg (La Cava, 2008).

1.7.9. Therapeutic approach using T regulatory cells

Since the discovery of Treg cells, intense investigation has been conducted aimed at determining how they protect an organism and whether defects in their number and/or function contribute to the development of various pathologies.

Deficiencies in Treg number and function lead to exacerbated lesions or accelerated disease progression in animal models of multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis (Atassi and Casali, 2008; Cosentino et al., 2012).

A new study, in a mouse model of multiple sclerosis, shows that widely used IFN-β treatment induces upregulation of transcription factor FoxA1 and a new population of FoxA1+ regulatory T cells (Delgoffe and Vignali, 2014), which suppress conventional T cells via cell surface expression of programmed cell death 1 ligand (PD-L1). CNS-infiltrating autoreactive T cells are suppressed by FoxA1+ Treg cells upon activation in the presence of neurons and/or IFN-β treatment and inflammatory milieu via PD-L1–PD-1 interaction, which limits proliferation by inhibiting Akt and p38 phosphorylation or promotes cell death by inducing active caspase-3. The induction and function of FoxA1+ Treg cells results in ameliorated disease in animals (Delgoffe and Vignali, 2014). FoxA1+ Treg subset was found in patients with MS who responded to IFN-β therapy (Liu et al., 2014).

Treg seem to be key neuroprotective immunomodulators in acute experimental stroke (Kuric and Ruscher, 2014) as well as in animal models of neuronal injury, possibly through modulation of microglial oxidative stress and inflammation (Reynolds et al., 2007). Comprehensive evaluation of the relevance of the dopaminergic modulation of CD4+ T cells, in particular, Treg subset in PD is burning concern. Preliminary evidence in patients with neurodegenerative disease indicates the occurrence of specific functional alterations affecting the Treg subset (Saunders et al., 2012). Kuric and Ruscher, 2014 provide evidence data that DA can act as a potential immunomodulator by attenuating inflammation in the post-ischemic brain (Kuric and Ruscher, 2014). Since Treg function has been reported to profoundly affect neurodegenerative processes and dopaminergic pathways are identified in Treg cells, Treg cell subset should be reconsidered as a potential target for novel and selective neuroprotective strategies.

Despite their essential role in maintaining the integrity of the host, Treg may act as detrimental players in the process of cancer development and progression in some types of malignancies. Treg cells act to diminish anti-tumour immunity by suppressing the effector functions of a variety of immune cells, including Th1 cells, CD8+ T cells, NK cells and tumour-infiltrating DCs. High levels of Treg have been found in many malignant disorders including lung, pancreas, and breast cancers. Treg may also prevent anti-tumour immune responses, leading to the increase in mortality. So far employed immunotherapies of cancer include (i) antibody blockade of inhibitory molecules (Hodi and Dranoff, 2010), (ii) adoptive T cell transfer (Galluzzi, et al., 2012), and (iii) autologous cell-based vaccines (Kantoff et al., 2010). The majority of current cancer immunotherapies predominantly rely on the ability of CD8+ T cells to fight against tumours, but it should be noticed that Treg cells as well could be a potential target.

As understanding of regulatory T cell populations is constantly growing, convincing evidence does support the hope that *in vitro* expanded Treg, with the potential to be modulated and then, as an adoptive cell transferred, could serve as a therapy and improvement of the certain diseases (neuroinflammatory, autoimmune or cancer progression).

1.7.10. T naïve/memory cell subset

Naïve T cells, are the most homogenous representatives of CD4+ subsets, circulating in the blood, expressing CCR7, a chemokine receptor involved in T-cell homing into lymphoid tissue, L-selectin (CD62L) and LFA-1 (leukocyte function antigen-1), that are mediating the rolling, adhesion and extravasation of the cells in peripheral lymph nodes and mucosal lymphoid organs. Naïve T cells are characterised by CD45RA molecule expression.

Unlike naïve T cells that live for few months or effector cells that disappear at the end of the immune response, memory T cells may survive in lymphoid organs and peripheral tissues for years. In order to understand the memory T cells function, efforts have made to define the properties that distinguish the naïve, effector and memory T cells, including their phenotype, distribution, and lifespan. It has been also shown that not all memory cells have the equal life span. The short-lived activated memory cells mediate early memory response, while later memory is controlled by long-lived resting cells (Tough and Sprent, 1995).

The memory lymphocytes CD4+ T cell subset is easily activated and capable of being reactivated if the same Ag is reoccurred, performing immediate effector functions in peripheral

tissues or undergo activation and clonal expansion in lymphoid organs to mount a secondary immune response. Almost two decades ago Sallusto et al., have proposed division of memory cells into two distinct yet interconnetcted subsets, T central memory and T effector memory cells (Sallusto et al., 1999). Initially, division into T_{CM} and T_{EM} was based on two diverse criteria: absence/presence of immediate effector function and the expression of peculiar homing capacity dictated by specific receptors allowing cells to migrate to secondary lymphoid organs or into non-lymphoid tissues (Sallusto et al., 1999). In the human peripheral blood, the expression pattern of the lymph node–homing receptors, CD62L and CCR7 is linked to the functional status of memory CD4+. Reactive memory is mediated by T_{CM} cells that home to secondary lymphoid organs, have little or no effector function, but are able to readily proliferate and differentiate into effector cells in response to Ag stimulation. On the other hand, protective memory is mediated by T_{EM} that has the ability to migrate to non-lymphoid organs, such as inflamed peripheral tissues and exhibit immediate effector function.

The T effector memory cells express CCR7 and CD62L, residing in lymphoid organs and producing IL-2 upon stimulation. Some of these have been found to migrate into certain inflammation sites, depending on the expression of chemokine receptors such as CCR4, CCR6 and CXCR3. T_{EM} cells that lack CCR7 and have low CD62L expression have intensive effector functions. Upon stimulation, they produce IFN-γ and IL-4 (Sallusto et al., 1999). So far, data support the notion that the two subsets are located in distinct tissues, with T_{CM} cells in lymph node, spleen and blood, and T_{EM} cells in spleen, blood and nonlymphoid tissues (Reinhardt et al., 2001).

Generation of memory cells is still intriguing issue. So far, the classical view of the generation of memory cells (“linear” pathway) and revised model for memory generation called, the “intersecting” pathway model has been described.

Following Ag stimulation, naïve T cells become activated, begin to proliferate and differentiate into Teff cells, which ultimately undergo activation-induced cell death. After Ag clearance, a proportion of Ag-primed T cells persists as a long-lived memory T cells. So, memory T cells are assumed to arise sequentially from Teff cells, through a so-called “linear” pathway.

On the other hand, Moulton and Farber (2006) have proposed a revised model for memory generation called, the “intersecting” pathway model. This model takes into account proliferative turnover, acquisition of effector function and survival. In the Ag-dependent pathway, Ag-stimulated naïve CD4+ T cells upregulate CD25 and downregulate IL-7 receptor

expression, resulting in rapid IL-2-driven proliferation and the acquisition of effector function that increases with Ag exposure. In the Ag-independent pathway, naïve T cells (CD25lowIL-7Rhigh) undergo slow proliferative turnover driven by host factors such as IL-7, with increased turnover driving differentiation to memory-phenotype cells with effector capacity. The intersection of the effector differentiation (Ag-dependent) and memory differentiation (Ag-independent) pathways occurs by Ag removal, resulting in the rapid down-regulation of CD25 and upregulation of IL-7 receptor, forming "pre-memory" T cells. These cells respond to homeostatic survival factors and differentiate into a stable memory T-cell population (Moulton and Farber, 2006).

Subsets of T_{CM} and T_{EM} with distinct functional programs can be identified according to the expression of surface molecules. The first markers used to separate memory T cells into diverse subpopulations were co-stimulatory molecules CD27 and CD28. Further, it has been discovered that human T_{CM} cells are CD45R0+ memory cells that constitutively express CCR7 and CD62L, receptors required for cell extravasation through high endothelial venules (HEV) and migration to secondary lymphoid organs (Forster et al., 1999). But it was shown that T naïve cells also express CCR7 and CD62L, so functional test have shown that T_{CM} cells have higher sensitivity to Ag stimulation, are less dependent on co-stimulation, and up-regulate CD40L to a greater extent, and following TCR triggering, produce mainly IL-2. After the phase of initial proliferation, they efficiently differentiate into Teff cells that produce large amounts of characteristic cytokines such as, IFN-γ or IL-4. On the other hand, human T_{EM} lost the constitutive expression of CCR7, and display characteristic sets of chemokine receptors and adhesion molecules, required for homing to inflamed tissues. Further, T_{EM} cells are characterised by fast effector function, following Ag stimulation, mirrored in the production of IFN-γ, IL-4, and IL-5 cytokines (Sallusto et al., 2004). Within the tissues, however, T_{CM} and T_{EM} show typical patterns of distributions. T_{CM} are augmented in lymph nodes and tonsils, whereas lung, liver, and gut contain greater proportions of T_{EM} (Campbell et al., 2001).

More recent studies demonstrated that T_{CM} cells differentiate to Teff cells expressing receptors for inflammatory chemokines and producing large amounts of cytokines in response to homeostatic cytokines (Geginat et al., 2001). In response to IL-7 and IL-15, CXCR3+ T_{CM} will differentiate to Th1, whereas most CCR4+ T_{CM} will differentiate to Th2, consistent with the notion that these subsets are pre-committed.

Combined expression of adhesion molecules and chemokine receptors on T_{CM} or T_{EM} will allow tissue-specific migration. Thus, for example, the simultaneous expression of CLA

and CCR4 identifies skin homing T cells (Campbell et al., 1999), whereas the expression of α4β7 and CCR9 is characteristic of gut-homing T cells (Zabel et al., 1999).

There has been disagreement about what happens to CD4+ T memory cells upon stimulation *in vitro*. According to some authors when stimulated *in vitro*, memory T cells show low-activation threshold and vigorous proliferation (Sallusto et al., 2004), while some other authors claim that CD8+ T cells require only a short period (6–24 h) of interaction with Ag to undergo an Ag-independent period of programmed expansion and differentiation, and CD4+ T cells may require several days of interaction with Ag for optimal activation and expansion (Ravkov and Williams, 2009). Though both T_{CM} and T_{EM} have a high responsiveness to Ag stimulation, the expansion potential decreases from T_{CM} to T_{EM} (Geginat et al., 2003). The reduced proliferative capacity correlates with a decrease in telomere length and with an increased propensity to undergo apoptosis.

It has been shown that percentage distribution of each of memory subsets within the CD4+ T cells can vary depending on previous experience. Hence, in Ag-primed individuals, tetanus toxoid (TTd)-specific CD4+ T cells can be detected in circulating T_{CM} and T_{EM} up to 10 years after Ag stimulation, and in the case of booster immunisation their frequencies increase in both subsets (Sallusto et al., 1999). Further, HIV- specific T cells largely belong to the T_{EM} subset, whereas CMV-specific T cells are predominantly found in a subset called TEMRA (terminally differentiated central memory subset) (Champagne et al., 2001).

1.7.11. Dopaminergic modulation of peripheral CD4+ T lymphocytes

Although it is well established that sympathoadrenergic system plays an active role in the cross-talk between the nervous and the immune system (Friedman and Irwin, 1997; Elenkov et al., 2000), up to several years ago, it was generally accepted that CAs were produced exclusively by adrenal gland and neurons. Catecholaminergic modulation of the immune response was assumed to be mediated by CAs released by nerve terminals directly in lymphoid tissues and/or by adrenal cells in the bloodstream to act on immunocompetent cells expressing dopaminergic (Ricci and Amenta, 1994) and adrenergic receptors (Khan et al., 1986). Interactions between the nervous and immune systems occur through the hypothalamic–pituitary axis and through sympathetic/parasympathetic innervations of primary and secondary lymphoid organs. Immune system cells activity is regulated by interactions of the receptors on their surface and appropriate endogenous mediators, such as neurotransmitters, neuropeptides,

hormones, and cytokines. These “messengers” molecules produced by immune cells are actually functioning as mediators of the neuro-endocrine-immune network. The ability of CD4+ cells of the immune system, to receive signals from the nervous as well as the endocrine system is dependent on the expression of receptors for neurotransmitters and neurohormones.

In the past 20 years, DA has been increasingly acknowledged as a key transmitter in mediating bidirectional communication between the nervous system and the immune system. Numerous studies support the immunomodulating role of DA, wich is a key molecule bridging this two systems (Basu and Dasgupta, 2000; Sarkar et al., 2010). DA induces direct and potent effects on immune cells depending on i) concentration, ii) different state of cell activation and specific immune cell type/subtype, and iii) specific DR subtype/s and level of expressed on immune cell surface but also at the level of mRNA for these receptor proteins (Levite, 2016).

Besides its action on the nervous system, DA plays a role in immune cell interactions. It has been shown that T cells synthesize DA through the tyrosine-hydroxylase/DOPA-decarboxylase pathway, and express DR and DA transporter (DAT) on their plasma membrane (Cosentino et al., 2007; Sarkar et al., 2010). Latest studies have shown that immune system cells can be regulated by DA acting on DR present on the surface of T and B cells, eosinophils, monocytes, macrophages and microglia, DC and NK cells, and also neutrophils (Pacheco et al., 2009). The presence of DR on immune cells and dopaminergic pathways demonstrated to regulate crucial human immune cells functions, support the importance of dopaminergic regulations in the immune response in physiological and pathological conditions.

A) Expression of dopamine receptors on different T cell subsets

Dopamine receptors have been found in cells of the innate and adaptive immune response, such as DCs, NK cells, macrophages/monocytes, granulocytes (Prado et al., 2012) and also B cells, CD4+ and CD8+ T cells (Besser et al., 2005; Sarkar et al., 2006; Watanabe et al., 2006; Nakano et al., 2009, Kustrimovic et al., 2014). So far, studies performed with human T cells suggested that both D1-like (DR D_1, DR D_5) and D2-like (DR D_2, DR D_3 and DR D_4) receptor types contribute to the regulation of T cell functions (Sarkar et al., 2006; Levite, 2016). Various CD4+ T cell subsets have shown to express different arrangements of DR (Levite et al., 2001; McKenna et al., 2002; Ilani et al., 2004; Besser at al., 2005; Sarkar et al., 2006; Watanabe et al., 2006; Cosentino et al., 2007; Nakano et al., 2009; Pacheco et al., 2009;

Prado et al., 2013; Kustrimovic et al., 2014; Kustrimovic et al., 2016) offering different possibilities for modulation and manipulation of dopaminergic pathways on these cells. Both, human Teff and Treg expressed D1-like and D2-like receptors on their surface (Cosentino et al., 2007).

Kustrimovic et al., 2014 recently demonstrated the cell surface expression of all the 5 DR types in human CD4+ T cell subsets, namely: naïve, T_{CM} and T_{EM} cells with a different expression patterns showing that naïve T cells express more D1-like than D2-like receptors, which on the contrary were higher expressed in T_{CM} and T_{EM} cells. The same group of authors investigate also the changes in DR expression during the *in vitro* activation and shown that expression of D1-like and receptors increased D2-like receptors was markedly elevated by 71-84% and 55-97%, respectively. Another interesting finding of this study was that DR expression is higher in apoptotic than in resting viable cells, suggesting DR involvement in the apoptotic process of T cells (Kustrimovic et al., 2014).

B) Dopamine and dopamine analogues-induced effects on T cell function

Dopaminergic receptors expressed on various subsets of T cells can be activated by either DA or selective agonist, leading to a potent DA-induced effect on T cell function.

Studies showing pro-apoptotic effects of DA typically used concentrations of 10–500 μM (Bergquist et al., 1997) or even 1 mM (Del Rio and Velez-Pardo, 2000). In agreement with those findings, in previous studies DA at concentration 100–500 μM induced a concentration-dependent increase in the percentage of apoptotic cells (Cosentino et al., 2004). On the other hand, it was reported that in human PBMC DA decreases oxidative metabolism and apoptosis, possibly through activation of D1-like DR-dependent mechanisms, which results in a reduction of intracellular ROS levels and subsequent inhibition of apoptosis (Cosentino et al., 2004).

Bergquist et al., 1997 claimed that B cells, but not T cells are sensitive to DA concentration 10 nM, which is in disagreement with later studies conducted by Besser et al., 2005 and Levite, 2012 claiming that DA induce direct and very potent effects on T cells at low concentration of 10 nM, such as triggering beta1 integrin-mediated T cell adhesion to fibronectin, important and critical function for trafficking and extravasation of T cells across blood vessels and tissue barriers.

Further, it have been shown that DA at the very high concentration 10 - 100 μM significantly inhibited the proliferation and production of cytokines (IFN-γ and IL-4) by PBMC in response to mitogens Concanavalin A (Con A) and PWM (Pokeweed Mitogen), pointing that at this high concentration DA is having negative effects on these cells by elevating the synthesis of the apoptotic markers Bcl-2/Bax and Fas/FasL by elevating the level of apoptosis (by ~ 2.8-fold) (Bergquist et al., 1997). In 2000, the same group confirmed that this high concentration of DA (10 - 100 μM) also inhibited the LPS-induced binding of NF-κB to the promoter of TNF-α, inhibiting production of this pro-inflammatory cytokine, and proving that lower concentration of DA (1 μM – 1 nM) did not induce such inhibitory effects (Bergquist et al., 2000).

Levite et al., 2001 have shown T cell increased adhesion to fibronectin (via DR D_2 or DR D_3 selective agonists).

Ghosh et al., 2003 revealed that human T cells *in vitro* stimulated with anti-CD3 and treated with 3-5 ng/mL DA significantly inhibited proliferation of these cells but also cytokine production of IL-2, IL-4, and IFN-γ. Pharmacological study with antagonists revealed that DA inhibited T cells activated with anti-CD3 Ab, through the DR D_2 and DR D_3.

Besser et al., 2005 demonstrated that in T cells obtained from healthy individuals, DA or selective DR D_2 and DR D_3 agonist may induce T cell selective cytokine production: IL-10 and TNF-α, respectively via DR D_2 and DR D_3. These findings suggested that DA has the ability to trigger selective secretion of either IL-10 (anti-inflammatory) or either TNF-α (pro-inflammatory) cytokine, without affecting the secretion of IFN-γ and IL-4.

Kipnis et al., 2004 revealed that Treg cell exposure to DA *in vitro*, before their systemic injection into mice (an animal model of neuronal survival), reduced their suppressive activity *in vivo*. The same authors found that mouse Treg express functional D1-like receptors and that DA binding can suppress the suppressive activity of Treg on Teff cells (Kipnis et al., 2004). In consecutive studies was shown D1-like receptor-dependent activation on Treg leads to suppression of their suppressive function, and their ability to suppress Teff cells, and finally, Teff remained activated (Cosentino et al., 2007). This way DA can indirectly affect Teff cells activation status and functional response. Pharmacological studies performed with the different antagonist, shown that reserpine-induced suppression of Treg function was due to DR D_1, and not DR D_5 stimulation. Nakano et al., 2008 studied the effects of dopaminergic analogues on the interaction between monocyte-derived DCs (MO-DCs) and allogeneic CD4+ T cells from

healthy volunteers and revealed that D1-like receptor blockade reduced, and D2-like receptor blockade increased IL-17 secretion by the T cells. The same group revealed that interaction between MO-DCs and naïve CD4+ T cells induces the release of DA from MO-DCs, which causes Th2 differentiation and polarisation. They also reported that this effect was completely blocked by the pre-treatment with selective D1-like receptor antagonist (Nakano et al., 2009). Collectively, they concluded that MO-DCs contain DA that can release upon Ag-specific interaction with naïve CD4+ T cells, and that released DA can induce Th2 polarisation.

Furthermore, it has been revealed that in DCs from DR D_5 knockout mice, was impaired LPS-induced IL-23 and IL-12 cytokines production, and consequently, activation and proliferation of Ag-specific CD4+ T cells was attenuated (Prado et al., 2012). *In vivo* studies, revealed the role of DR D_5 in a murine model of MS by transfer of DR D_5-deficient DCs into wild-type recipients showing significant reduction of the percentage of Th17 cells infiltrating the CNS, no effects on Th1 cell subsets (Prado et al., 2012; Pacheco et al., 2014).

Another interesting *in vivo* study addressing role of DA and its D1-like receptor in cutaneous immune response in Th subsets polarisation, was conducted by Mori et al., 2013. They showed by the administration of SCH 23390 (D1-like receptor antagonist) into a murine model of Th1-type contact hypersensitivity and of Th2-type atopic dermatitis, that Th2 response was suppressed, and that treatment did not affect Th1 contact hypersensitivity (Mori et al., 2013). This data was also confirmed by real-time RT-PCR method that revealed that mice treated with the same antagonist had higher levels of IFN-γ and lower IL-4 mRNA in the skin (Mori et al., 2013). On the contrary, DA promoted Th2 cell differentiation and mast cell degranulation, without affecting Th1 cell function (Mori et al., 2013).

C) **Clinical relevance for dopaminergic modulation of CD4+ T cells in immune-mediated diseases**

The presence of DR on CD4+ T cells membrane contributes to modulation, development, and initiation of immune responses under physiological conditions and in pathologies such as autoimmunity, neuroinflammation and cancer (Pacheco et al, 2009; Pacheco et al., 2010; Pacheco et al., 2014; Levite, 2016). The emerging role of DA as a regulator of CD4+ T cells physiology and its consequent involvement, in the regulation of immune response is important since the alterations in the DA-mediated regulation of immunity could contribute to the onset and development of immune-related disorders. It has been shown that in some autoimmune and neurological diseases, T cells have abnormal expression of DR

and/or response/production of DA. It is important to have in mind issue that alterations in the DA-mediated immune regulatory mechanisms could contribute to the onset and progression of immune-related disorders, since the DA agents could serve as new therapies in this conditions or an old one, with a safe pharmacological profile can be repurposed.

Recent studies have shown that peripheral T cells are recruited to the CNS parenchyma, and are having a fundamental role in Parkinson's disease pathology (Brochard et al., 2009; González et al., 2013; Reynolds et al., 2010). In the process of neuroinflammation, endothelial cells up-regulate expression of adhesion molecules and allow peripherally activated T cells to penetrate into the CNS parenchyma, where infiltrated CD4+ T cells interact with microglia and cause switch towards pro-inflammatory M1-like phenotype (Barcia et al., 2012). In parallel, activated microglia increase IFN-γ production by Th1 effector T cells, which coordinate pathogen killing. Collectively, this evidence indicate dysregulation of adaptive immunity, greatly contributes to neurodegenerative disease pathogenesis by modulating microglial responses and may provide an attractive therapeutic target for immunomodulatory interventions.

Several other studies described the occurrence of peculiar modifications of peripheral immunity in PD, such as fewer CD4+CD25+ T cells, increased ratios of IFN-γ-producing to IL-4-producing T cells and decreased CD4+/CD8+ T-cell ratios (Baba et al., 2005), and also decreased CD4+ T lymphocytes (Bas et al., 2001; Stevens et al., 2012). Total numbers of lymphocytes in PD cohorts have been shown to be diminished by 17%, and CD3+ T cells were diminished by 22% (Bas et al., 2001). Among CD3+ T cells, numbers of CD4+ T cells were diminished by 31% whereas numbers of CD8+ T cells were not significantly changed. A greater loss of naïve CD4+ T cells (CD45RA+) was observed (Bas et al., 2001). A selective loss of CD4+CD45RA+ cells was also observed in diseases such as MS, suggesting a common immunological abnormality in such neurological disorder (Fiszer et al., 1994; Crucian et al., 1995). CD4+ T cells were also identified in the brain in both, human PD patients and MPTP treated mice. Data obtained in the animal model shown that CD4+ T cells are key players in the detrimential process of dopaminergic cell death (Brochard et al., 2009). Saunders et al., recently reported that PD patients have increased effector/memory CD4+ T cells and decreased CD31+ and α4β7+ CD4+ T cells, which are associated with progressive motor dysfunction, suggesting a direct relationship between chronic immune stimulation and PD neuropathology and disease severity, strengthening the idea that in PD the lead actors among adaptive immune

system cells are CD4+ T lymphocytes (Saunders et al., 2012). The same group has also shown impaired abilities of Treg from PD to suppress effector T cell function, suggesting that Treg dysfunction is linked to PD pathobiology (Saunders et al., 2012).

Our most recent study examined effects of dopaminergic substitution therapy and DA on CD4+ T naïve and memory lymphocytes in PD patients and in healthy subjects, showing that there is an excessive association between DR expression on T lymphocytes and motor dysfunction, assessed by UPDRS Part III score (Kustrimovic et al., 2016). Collectively, in total and CD4+ T naïve cells, expression of D1-like DR decreased, while in T memory cells D2-like increase with increasing UPDRS Part III score (Kustrimovic et al., 2016). In second part, *in vitro* effects of α-syn were assessed on both CD4+ naïve and memory cells, showing an increased CD4+ T memory cells, to a possibly different extent in PD patients in comparison to healthy subjects (HS), and also expression of DR was affected by the presence of α-syn (Kustrimovic et al., 2016). This finding further support involvement of peripheral adaptive immunity in PD.

Evidence from both animal studies and clinical trials studies suggest that manipulation of various cell components of the adaptive immune response may provide considerable neuronal protection.

Saussez et al., 2014 have shown, based on a pioneering study, that *in vitro* addition of DA (10 nM) increases spontaneous, chemotactic and towards autologous cancer migration of T cells of new patients with head and neck cancer. Such DA-induced effect on T cells from cancer patients should give hope for the future beneficial effects that should be examined in other types of cancer as well. Basu et al., 2001 showed that normal human T cells and human Jurkat T leukaemia cells expressed both D1-like and D2-like receptors, but with a different function. They have shown that activation of DR expressed on normal activated T cells leads to inhibition of proliferation, but not of the proliferation of malignant T cells.

Treg are critical for the maintenance of immune homeostasis and are often found at elevated frequencies in blood and tumours of patients, and for many cancers, a high density of Treg correlates with poor disease outcome (DeLeeuw et al., 2013). Treg are actively recruited and induced by tumours to block immune priming, effector function and memory response, which can inhibit the efficacy of therapeutic cancer vaccines. It has been show that DA can effectively inhibit human Treg function, *in vitro* (Cosentino et al., 2007), so it can be highly provocative to hypothesise the role of DA as anti tumour-agent (Hiura et al., 2005).

An improved understanding of the fundamentals and complexities of Treg – DA interplay may enable the selective modulation of this cells and valuable pharmacological target for the cancer treatment.

II. AIMS OF THE EXPERIMENTAL PROGRAM

General aims

The principal aim of the study was to develop and validate *in vitro* methods devised to investigate the effects of dopaminergic agents, currently used in the pharmacotherapy, on the functional responses of CD4+ T lymphocyte subsets. The specific responses were investigated in relation to functional conditions and to the expression and functional responsiveness of intrinsic dopaminergic pathways. The molecular and pharmacological heterogeneity of DR potentially represents an opportunity to develop targeted immunomodulating strategies.

The aim of the present work was to use *in vitro* models to investigate the role of DA pathways in CD4+ T lymphocytes, namely: (i) CD4+ T naïve, T central memory and T effector memory cells, and their responses to recall Ag; (ii) Treg, and their suppressive effects on Teff and (iii) CD4+ T naïve cells, and their ability to differentiate towards different T helper lineages (Th1/Th2/Th17).

III. MATERIAL, METHODS AND RESULTS

3.1. Introduction

Having in mind available literature data and hence observing the lack of data regarding the expression of DR on specific subsets of CD4+ T cells, the first goal set was to develop reliable cytometric methods in order to investigate DR expression on various subsets of CD4+ T cells (**Figure 8**).

In the first part of our experiments we have shown expression of all five DR on CD3+CD4+ circulating T lymphocytes isolated from the venous blood of healthy subjects, followed by detailed examination of expression of each DR on Tn, $T_{CM,}$ $T_{EM,}$ Treg, Th1/Th2/Th17 (**section 3.2., 3.6., 3.7. and 3.8., respectively**).

The most basic approach demanded introduction of apoptosis assay, which allowed us to define DR expression on CD4+ T cells undergoing apoptotic process *in vitro* culture conditions. The effects of different concentrations of dopaminergic agents, namely: DA, L-DOPA and pramipexole were examined in the context of the CD4+ T cell susceptibility to apoptotic process (**section 3.3.**).

Since the function of proliferation of CD4+ T cells is shown to be an important process in the homeostasis of T cells, and also in some pathogenic processes (such as cancer development and progression), we have developed and validated a method to examine DR expression on proliferating cells. The next step was to examine the effects of dopaminergic agents (DA, L-DOPA and pramipexole) on the proliferative capacity of these cells (**section 3.4**.).

Functional responses of CD4+ T naïve and memory subsets were investigated by the *in vitro* model that showed a change in frequencies of Tn, T_{CM} and T_{EM}, evoked by the common recall Ag tetanus toxoid (TTd) (**section 3.5.**).

The *in vitro* method was set up to examine Treg-induced inhibition of Teff proliferation that was developed and validated both in buffy coat samples and healthy controls. Furthermore, the secretion of cytokines (IL-10 and TGF-β) by Treg alone, and Treg cells induced suppression of production of cytokines IFN-γ and TNF-α by Teff cells was characterised, and additionally, effects of SKF 38393 and pramipexole on cytokine production was examined. Finally, the relevance of the *in vitro* dopaminergic modulation of DA and L-DOPA was examined on function of CD4+CD25+ enriched population of T cells alone or co-cultivated with Teff cells obtained from healthy subject, and in a small sample group of patients suffering from Parkinson's disease group that was divided into: (i) patients who have never been treated

with antiparkinson drugs and (ii) patients that are on dopaminergic replacement therapy (**section 3.6.**). The Parkinson's disease group was used as a convenient model of the disease where DA and peripheral immune system interplay is strongly implicated.

Lastly, the final aim proposed was developing and validation of *in vitro* method for examination the regulation of Th differentiation process and exploitation of the role of dopaminergic modulation on polarisation and differentiation process of Th1, Th2 and Th17 subset in healthy subjects (**section 3.7.**).

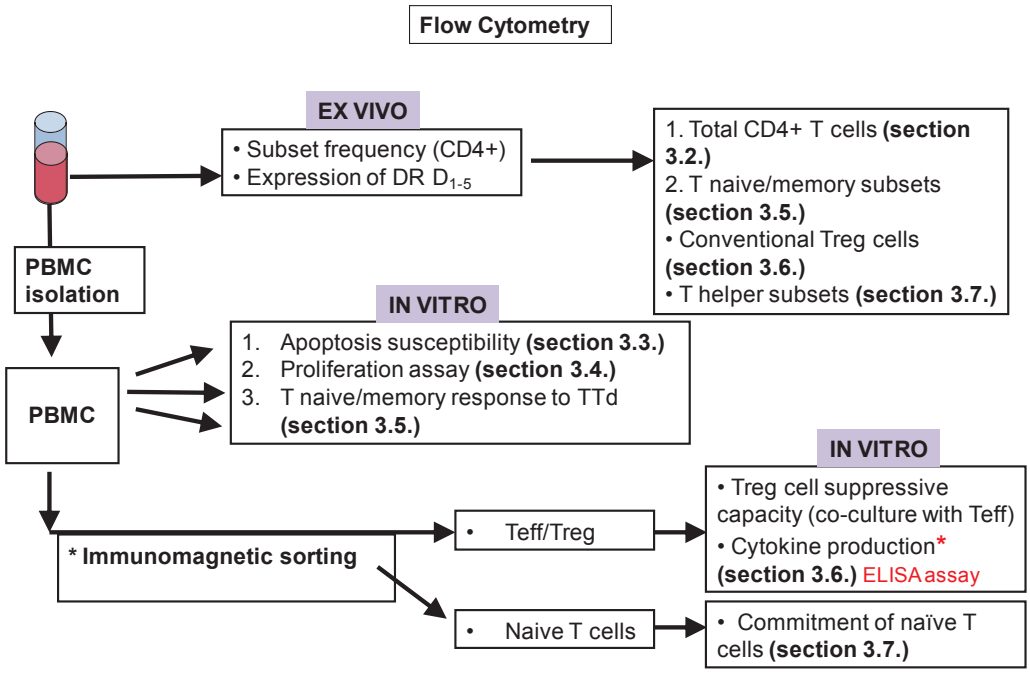


Figure 8. Schematic picture summarising the experimental models that were used and cell population obtained by different methods and approaches.

3.1.1. Chemicals, reagents and antibodies

Rabbit polyclonal antibodies (ab) IgG anti-human DR D_1 (cod. 324390), DR D_3 (cod. 324402) and DR D_5 (cod. 324408) were from Calbiochem-Inalco, Italy. Rabbit polyclonal ab anti-human DR D_2 (cod. LS-C22924) and DR D_4 (cod. LS-C22938) were obtained from LifeSpan-Space Import Exp, Italy. Goat anti-rabbit ab IgG conjugated with phycoerythrin (PE) was obtained from R&D System, Space Import Exp, Italy. PerCPCy5.5-conjugated mouse IgG anti-human CD3 (CD3 PerCPCy5.5) (cod. 317336, clone OKT3; mouse IgG2a, k) and FITC-conjugated mouse IgG anti-human CD45RA (CD45RA FITC, cod. 304106, clone HI100; Mouse IgG2b, k) were obtained from Biolegend– Campoverde, Italy. APC-Cy7-conjugated mouse IgG anti- human CD4 (CD4 APCCy7, cod. 557871, clone RPA-T4; mouse IgG1, k), PE

Cy7 conjugated mouse IgG anti-human CD4 (CD4 PECy7, cod. 557852, clone SK3), Alexa Fluor 647-conjugated rat IgG anti- human CCR7 (CD197) (CCR7 AF647, cod. 557736, clone 3D12; rat IgG2a, k), APC conjugated mouse IgG anti human CD3 (CD3-APC, cod. 555342, clone HIT3a), PE Cy7 conjugated mouse IgG anti-human CD25 (CD25 PECy7, cod. 557742, clone 2A3), AlexaFluor647 conjugated mouse IgG anti-human CD127 (CD127 AlexaFluor647, cod. 558598, clone HIL-7R-M21), AlexaFluor448 conjugated mouse IgG anti-human CD183 or CXCR3 (CD183 AlexaFluor448, cod. 558047, clone 1C6/CXCR3), PE Cy7 conjugated mouse IgG anti-human CD194 or CCR4 (CD194 PECy7, cod. 561034, clone 1G1) and 7-AAD (7-amino-actinomycin) were all purchased from Becton Dickinson, Pharmingen, Italy. PerCPCy5.5-conjugated mouse IgG anti-human CCR6 (CD196 PerCPCy5.5) (cod. 353405, clone G034E3) was purchased from Biolegend– Campoverde, Italy.

Purified mouse ab anti-human CD3 (cod. 555330, clone UCHT1) and purified mouse ab anti-human CD28 (cod. 555726, clone CD28.2) were obtained from Becton Dickinson, Italy. Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma, Italy. RPMI 1640, heat-inactivated foetal bovine serum (FBS), glutamine, and penicillin/streptomycin were obtained from Euroclone, Italy. Ficoll-Paque Plus was from Pharmacia Biotech (Uppsala, Sweden). Cell Proliferation Dye eFluor670 (CPD670) (cod. 65-0840) was purchased from eBioscience-Prodotti Gianni. Dopamine hydrochloride (cod. H8502), L-DOPA (3,4-dihydroxy-L-phenylalanine, cod. D9628), (±)-SKF-38393 hydrochloride (cod. D047), pramipexole dihydrochloride (cod. A1237), mitogen phytohemaglutinin (PHA) and recombinant IL-2 (cod. 0208AF12) were all purchased from Sigma Aldrich, Saint Louis, MO, USA. Human anti-INF-γ antibody (cod. 130-095-743), anti-IL-4 antibody (cod. 130-095-753), IL-1β (cod. 130-093-895), IL-4 (cod. 130-095-373), IL-6 (cod. 130-095-365), TGF-β (cod. 130-095-067) and IL-12 (cod. 130-096-704) were all purchased from Miltenyi Biotec, Bergisch Gladbach, Germany.

Dynalbeads CD4 Positive Isolation Kit (cod. 11331D) and TGF-β (KAC1688) ELISA kit were obtained from Invitrogen, Life Technologies AS, Norway. Human INF-γ (cod. EHIFNG), TNF-α (EH3THFA), and IL-10 (EHIL10) ELISA kits were all purchased from Thermo Scientific, Rockford, USA. Human CD4+CD25+ Regulatory T cell Isolation Kit (cod. 130-091-301) and human naïve CD4+ T cell Isolation Kit (cod.130-094-131) were purchased from Miltenyi Biotec, Bergisch Gladbach, Germany. Human Th1/Th2/Th17 Phenotyping Kit (cod. 560751) containing: Th1/Th2/Th17 cocktail (CD4PerCP-Cy5.5 clone SK3; IL-17A PE clone N49-653; IFN-γ FITC clone B27 and IL-4 APC clone MP4-25D2), BD Cytofix Buffer,

BD Perm/Wash buffer and BD GolgiStop Protein Transport Inhibitor was purchased from Becton Dickinson, Italy. PMA (phorbol 12-myristate 13-acetate, cod. P8139), ionomycine (Calcium Ionophore, cat.nub. I3909) and tetanus toxin, from Clostridium tetani (TTd, cod. T3194) were all purchased from Sigma Aldrich, Saint Louis, MO, USA.

3.1.2. Subjects enrolled in the study

The present study is part of a project ”Dopaminergic modulation of CD4+ T cells: relevance for neurodegeneration and neuroprotection in Parkinson’s disease – the dopaminergic neuro-immune connection” which is aimed at assessing the pattern of expression and the functional role of DR on circulating lymphocytes in healthy subjects and in patients with Parkinson’s disease. PD patients were enrolled among patients attending the Centre for PD and Movement Disorders of the Neurological Service at the Ospedale di Circolo, Varese, the Interdepartmental Research Center for Parkinson’s Disease of the Neurological Institute “C. Mondino”, Pavia and Divisione di Neurologia, Ospedale Maggiore, Novara, Italy. The healthy subjects were mainly spouses and caregivers of the enrolled PD patients. PD was diagnosed according to the UKPDS Brain Bank Criteria. Any patients and controls with a history of autoimmune or inflammatory disorders and/or receiving chronic immunosuppressive treatment were excluded. All enrolled subjects were submitted to a complete examination.

The study protocol was approved by the Ethics Committee of the Ospedale di Circolo, Fondazione Macchi, Varese and Neurological Institute “C. Mondino”, Pavia. All the participants signed a written informed consent form before enrolment. The study was performed according to the Declaration of Helsinki, and to the relevant ethical guidelines for research on humans.

3.2. Expression of DR in CD4+ T lymphocytes in whole blood

To our best knowledge, so far only a few studies dealing with DR expression on human immune cells were undertaken. McKenna et al., 2002 have identified DR on human lymphocytes by flow cytometry, showing that T lymphocytes and monocytes had low expression of DR (frequency was on average 1–5 %). DR D_3 and DR D_5 were found in most individuals, DR D_2 and D_4 had a more variable expression, and DR D_1 was not detected. Subsequently, Besser et al., 2005 reported the occurrence of D2-like DR on human T cells by usage of flow cytometry, showing expression of DR D_2 and D_3 on 9–10% of these cells. Sarkar et al., 2006 documented the membrane expression of DR D_4 by means of western blot analysis, while Brito-Melo et al., 2012 analysed the expression of DR D_2 (2–10 %) and DR D_4 (2–16 %) on CD4+ T cells.

Aim

The aim of study was to develop and validate reliable flow cytometric protocol to examine the expression of DR on circulating CD4+ T lymphocytes in whole blood.

Subjects enrolled in the study

Peripheral venous blood samples were collected from healthy volunteers (n=30) between 8:00 a.m. and 10 a.m. and placed in universal tubes containing preservative-free heparin (215IU/mL).

3.2.1. DR staining in whole blood

The DR staining protocol consisted of two steps of an indirect immune fluorescence labelling procedure (primary Ab + secondary Ab labelled with PE). Briefly, in the first step, each aliquot was stained for one of the five membrane DR by using rabbit polyclonal IgG directed against human D1-like (D_1 and D_5) and D2-like (D_3, D_4, and D_5) DR. In the second step, a cocktail of the following Abs was added to each aliquot, according to manufactures recommendations: pre-titrated PerCPCy5.5-conjugated mouse anti-human CD3 and APC-Cy7-conjugated mouse anti-human CD4.

- Each sample was prepared as 100 µL of a whole blood added to BD tube.
- In order to remove the erythrocytes, 3 mL of a lysis buffer (containing NH_4Cl 8.248 g/L, $KHCO_3$ 1.0 g/L and EDTA 0.0368 g/L) were added to each sample.
- Incubation was performed at room temperature (RT) for 5 min, during which samples were gently vortexed.
- Samples were then centrifuged at 1200 g for 5 min at RT, supernatants were removed and cells were washed with 1 mL of PBS (pH 7.4) supplemented with 1 % BSA (PBS/BSA).
- Finally, pellets were resuspended in 50 µL PBS/BSA.
- From each subject 7 aliquots were prepared as follow: 5 were used for DR staining, 1 was used as control for the secondary PE-goat anti-rabbit (PEGAR) Ab, and 1 was used as a negative control (no Ab).
- Samples was added with the primary anti-DR Ab (final dilution 1:100).
- Samples were incubated for 30 minutes on ice in the dark.
- Cells were washed once with PBS/BSA at 1200 g for 5 min at RT.
- Pellets were resuspended in 200 µl of PBS/BSA and 10 µl of PEGAR Ab was added, following incubation for 30 min on ice in the dark.
- After incubation, samples were washed with 1 mL of PBS/BSA (1200 g for 5 min at RT) and resuspended in 50 µL PBS/BSA.
- A cocktail of anti-human CD3 and anti-human CD4 were added to all samples according to manufacturer's recommendation, and incubated for 30 min in dark at RT.
- Washing was performed with 1 mL of PBS/BSA (1200 g for 5 min at RT).
- Finally, samples were resuspended in 350 µL PBS and kept on ice until analysis.

Acquisition was performed on a BD FACSCanto II flowcytometer (BectonDickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3). Lymphocytes were identified by their classical forward scatter (FSC), side scatter (SSC) signals and a minimum of 20.000 lymphocytes from each sample were collected in the gate. Data were analysed with FlowJo software (version 8.3.2.). The results were finally expressed as a percentage of positive cells (%) (Kustrimovic et al., 2014).

3.2.2. Analysis of obtained results

CD3+CD4+ T lymphocytes expressing DR were identified by means of the following gating strategy: first, lymphocytes were gated on the scatter dot plot in the typical region of low SSC and FSC, CD3 and CD4 double positive cells were then identified among gated lymphocytes, and DR+ cells were finally enumerated (**Fig. 9**).

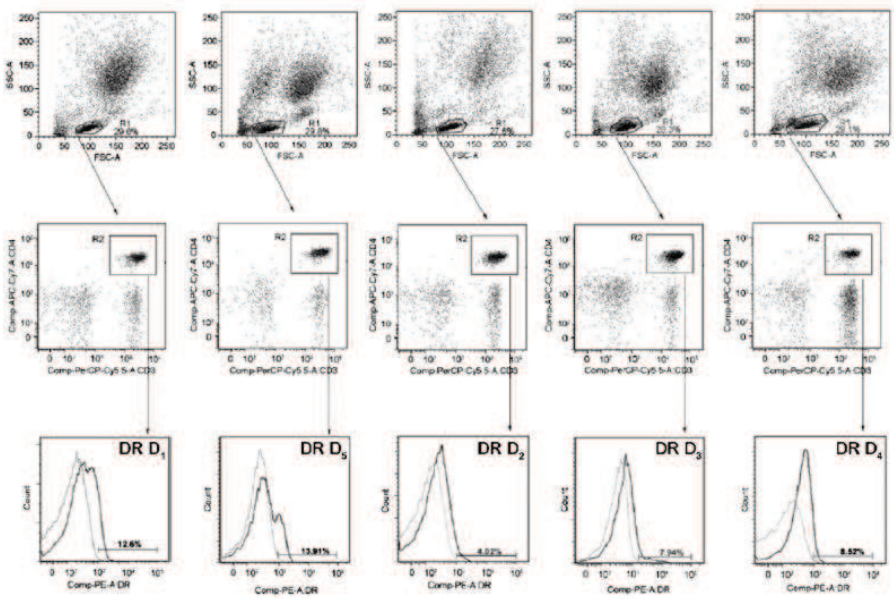


Figure 9. Gating strategy employed to identify DR+CD3+CD4+ T lymphocytes. Total lymphocytes were identified on a biparametric dot plot SSC vs FSC (upper panel, region 1 [R1]), to exclude monocytes, granulocytes and debris. From the cells in R1, CD3+CD4+ T cells were selected by using the CD3 vs CD4 dot plot (middle panel, R2). Finally, DR expression was assessed by a single-parameter histogram (lower panel), where the black line represents DR+ cells and the gray line represents the negative control (cells stained with only the secondary PEGAR ab).

The obtained results showed that CD3+CD4+ T lymphocytes expressed all the five DR (**Fig. 10**), with subsequent frequency: D1-like DR D_1 were expressed by 8.3±0.6% of CD3+CD4+ T cells and D_5 by 11.9±1.0%, while the D2-like DR D_2 by 4.8±0.5%, D_3 by 5.7 ±0.5 %, and D_4 by 6.8±0.8 %. D1-like DR D_1 and D_5 frequencies were significantly higher than those of the D2-like DR D_2, DR D_3 and D_4. Frequency of the CD3+CD4+ cells that are expressing DR D_1 and D_5 were significantly different among themselves (**Fig. 10**).

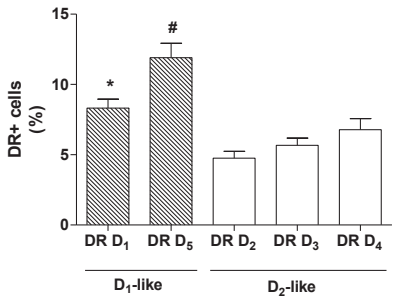


Figure 10. Expression of DR on CD3+CD4+ T cells. DR expression is presented as percentage of total CD3+CD4+ T cells. Results are presented as mean±SEM of 30 subjects. *, $P<0.05$ vs. DR D_5 and DR D_4, $P<0.01$ vs. DR D_3, $P<0.001$ vs. DR D_2; #, $P<0.001$ vs. DR D_2, DR D_3 and DR D_4.

Conclusions, implications and future perspectives

The results of this study have shown the expression of all the five DR on human CD3+CD4+ T lymphocytes to a different extent, thus opening the possibility of exploring in more detail the patterns of DR expression among different CD4+ T cell subsets, as well as the relationship with the functional status of these cells, *ex vivo* and *in vitro*.

Given the obtained results, it seems feasible that the relative prevalence of D1-like over D2-like DR in CD3+CD4+ T cells may imply that effects of DA on these cells are mediated mainly through D1-like DR.

3.3. Effect of dopamine and dopaminergic agonists on T cell susceptibility to apoptosis

Apoptotic mechanism has an important role in the fine-tuning of the immune response. Dopamine itself can exert toxic or protective effects on lymphocytes under specific conditions, by modulating their death or survival.

Studies showing pro-apoptotic effects of DA typically used concentrations of 10–500 μM (Bergquist et al., 1997) or even 1 mM (Del Rio and Velez-Pardo, 2001). In agreement with those findings, in previous studies DA at concentration 100–500 μM induced a concentration-dependent increase in the percentage of apoptotic cells (Cosentino et al., 2004). On the other hand, it was reported that in human PBMC, DA decreases oxidative metabolism and apoptosis, possibly through activation of D1-like DR-dependent mechanisms, which results in the reduction of intracellular ROS levels, and the subsequent inhibition of apoptosis (Cosentino et al., 2004).

Aim

For this part of the study, two specific aims were set:

(i) define the level of expression of DR on CD4+ T cells that are undergoing apoptotic process

(ii) validate and develop an *in vitro* experimental model to obtain the effects of different concentrations of DA, L-DOPA and pramipexole on CD4+ T cells and their susceptibility to apoptosis.

Subjects enrolled in the study

PBMC were isolated either from Buffy coat samples (n=6) or from venous blood from healthy volunteers (n=13).

3.3.1. Method of PBMC cultivation and evaluation of effects of DA, L-DOPA and pramipexole on apoptosis

Flow cytometry method with usage of 7-aminoactinomycin (7-AAD) colour allowed evaluation of DR expression on live, early and late apoptotic cells after 48 h of PBMC cultivation.

3.3.1.1. Separation and purification of PBMC by Ficoll-Hypaque method

PBMC isolation was performed from 20-25 mL of whole blood samples by using Ficoll-Paque Plus density (1.077 g/mL) gradient centrifugation for purifying lymphocytes.

Isolation of PBMC by Ficoll-Plaque gradient (Boyum, 1974) resulted in enrichment of mononuclear cells with less than 5% contamination of neutrophil granulocytes.

- All solutions used in this procedure were gradually equilibrated to RT before being used in the assay.
- Fresh heparinised blood samples were placed into 50 mL conical tubes and mixed with an equal volume of PBS.
- 3 mL of Ficoll-Hypaque solution was placed in 15 mL conical tube and 12 mL of diluted blood samples were carefully layered on the Ficoll-Hypaque.
- Samples were centrifuged for 40 min, 400 g, at RT (without a break).
- After careful centrifugation, the mononuclear lymphocyte cell layer (that appears as a white, cloudy band between the plasma and the Ficoll-Hypaque layers) was collected and transferred to a new tube.
- Isolated PBMC were washed with 10 mL of 2% FBS/PBS (600 g, 10 min, RT).
- After centrifugation supernatants were removed, 3 mL of lysis buffer ($NH_4Cl/KHCO_3$/EDTA) was added in order to remove any residual erythrocytes.
- Samples were centrifuged at 100 g, in order to remove remaining platelets, for 5 min at the RT.
- Supernatant was removed and pellet was washed in 10 mL of 2% FBS/PBS (600 g, 10 min, RT).
- Supernatants were removed and obtained pellets were resuspended in 10 mL of 10% FBS/RPMI 1640 and prepared for counting.

- Preparation of cell suspension for cell counting:
- Identical volumes of Turk solution and cell suspension (10 µL + 10 µL) were placed in tubes, mixed well and left for 10 seconds to allow colour to penetrate inside the cells.
- 10 µL of mixture was placed into Burker chamber and number of cells were counted on microscope.

For counting cells, the following formula was used: N cells x 2 x V x 10^4, where:

N = mean of 3 quadrants of counted cells,

2 = dilution factor of Turk solution,

V = volume of sample,

10^4 = volume of Burker chamber.

The typical PBMC suspension preparation obtained by this method always contained at least 80% of lymphocytes, as confirmed by flow cytometry (**Fig. 11**). Cell viability, assessed by the trypan blue exclusion test, was always >99 %.

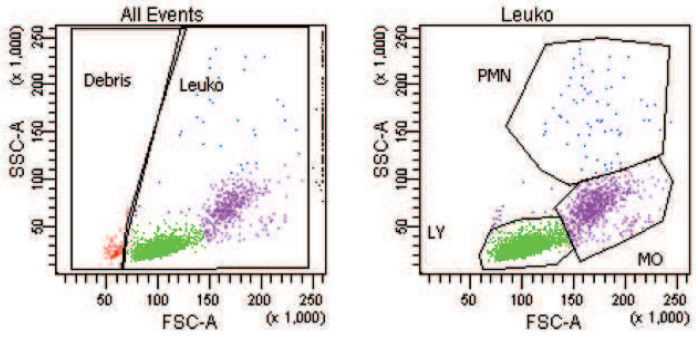


Figure 11. Purity of separated PBMC

3.3.1.2. PBMC culture and staining of DR on viable and apoptotic cells

- After PBMC isolation, cells were washed and resuspended at the final concentration of 1 x 10^6 cells in 1 mL of RPMI/10%FBS for subsequent culture for 48 h at 37°C in a moist atmosphere of 5 % CO_2, alone, or in the presence of anti-CD3/anti-CD28 Ab (0.1 µg/mL) as activators that were added at the beginning of cell culture.
- After 48 h cells were collected, washed twice with 10 mL of PBS/2%BSA, and centrifuged at 1200 g for 10 min at RT. Finally, samples were resuspended in at least 1 mL PBS/2%BSA for DR staining.

From each sample, 7 aliquots of 100 µL were prepared: 5 were used for staining of each DR, one was used as a control of secondary Ab and one as a negative control. DR staining was performed as previously detailed in section 2.4.

- In each experiment, a sample of 1×10^6 PBMC was labelled with mouse anti-human CD4-APC-Cy7, added according to the manufacturer's recommendations.
- Samples were incubated for 30 min at RT in the dark.
- After incubation, the cells were washed with 1 mL PBS/BSA, and the pellets were resuspended in 100 µL of PBS/BSA.
- 5 µL of 7-AAD was added to each sample and left for incubation for 5 min on ice in the dark.
- After incubation, 250 µL of PBS/2%BSA was added and then samples were immediately analysed.

Viable, early apoptotic (EA) and late apoptotic (LA) CD4+ T cells were identified by 3-color flow cytometric analysis according to an established method (Lecoueur et al., 1997).

3.3.1.3. PBMC culture and DA effect on apoptotic cells

- PBMC, isolated from buffy coat samples or fresh blood of healthy subjects, were resuspended at the final number of 0.5 x 10^6 PBMC cells per well in RPMI/10 % FBS for subsequent culture for 48 h at 37 °C in a moist atmosphere of 5 % CO_2 in the presence/absence of anti-CD3/anti-CD28 Ab (0.1 µg/mL) activators.
- Different concentrations of DA, L-DOPA and pramipexole were added at the beginning of cell culture.
- After 48 h, the cells were collected, washed twice with 1 mL of PBS/BSA, centrifuged at 1200 g for 10 min at RT and resuspended.
- The samples were incubated with mouse anti-human CD4-APC-Cy7 for 30 minutes at the RT in the dark.
- After incubation samples were washed with 1 mL PBS/BSA and pellets were resuspended in 100 µL of PBS/BSA.
- In each sample, 5 µL of 7-AAD was added and incubated for 5 min on ice in the dark.

- After incubation, 250 µL of PBS/BSA was added to the samples, which were immediately analysed.

Viable, EA and LA CD4+ T cells were identified by 3-color flow cytometric analysis according to established methods (Lecoeur et al., 1997).

3.3.2. Analysis of obtained results

3.3.2.1. Expression of DR on cultured CD4+ T cells

In order to distinguish viable and apoptotic CD4+ T cells, a 3-color flow cytometric method was applied to identify three different subpopulations by an already established method (Lecoueur et al., 1997). The following gating strategy was used: first, lymphocytes were identified on the dot plot in the typical region of low SSC and FSC; then, $CD4^+$ positive cells were identified among gated lymphocytes. By the expression of the 7AAD marker, the following populations were defined within the defined population of $CD4^+$ T cells: viable, EA and LA (**Fig. 12**).

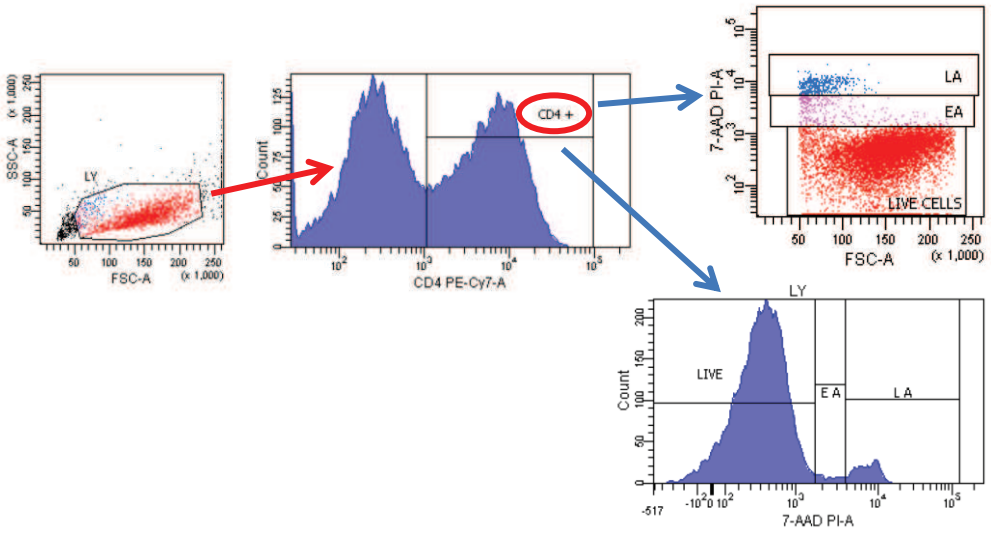


Fig. 12 Gating strategy used to identify viable and apoptotic CD4+ T cells in human PBMC after 48 h of culture. Total lymphocytes were identified on a biparametric dot plot SSC vs. FSC (left panel, region LY). From the cells in the LY region, CD4+ T cells were selected by using the FSC vs. CD4 dot plot (middle panel). Finally, 7-AAD expression was assessed by a single-parameter histogram (lower panel), and CD4+T cell subset was further divided on: viable, EA and LA cells.

The frequency of all DR was examined on such a defined cell population (live, EA and LA). Results showed that all five examined DR were significantly higher in EA and LA cells in

comparison to viable cells, and the frequency of D1-like DR D_1 and D_5 were also significantly higher in LA cells, in comparison to EA cells. However, stimulation with anti-CD3/anti-CD28 Ab significantly increased the expression of all DR on viable cells, without affecting DR expression on EA or LA cells (**Fig 13**).

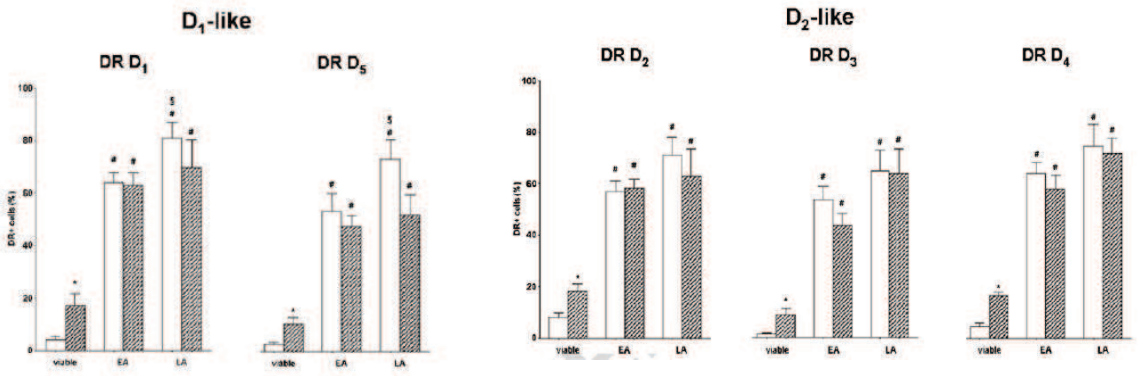


Fig. 13 Expression of D1-like (left panel) and D2-like (right panel) receptor subtypes on: viable, EA and LA CD4+ T cells cultured in resting conditions (open columns) and in the presence of soluble anti-CD3/anti-CD28 Ab (0.1 μg/mL) (dashed columns). Results are presented as a mean±SEM of 13 healthy subjects. *, P<0.01 vs. resting; #, P<0.01 vs. viable cells; §, P<0.01 vs. EA cells.

3.3.2.2. Effects of DA and dopaminergic agents on CD4+ T cell apoptosis

Preliminary experiments were carried out on buffy coat samples in order to establish the best experimental settings. Resting and activated cells were cultivated in standard conditions. Anti-CD3 and anti-CD28 Ab were used as activators in order to mimic conditions the most similar to physiological ones.

The difference between resting and activated conditions were obtained among late apoptotic (LA) cells, without treatment, (*,=P<0.05) and in the presence of 1 μM DA (**,=P<0.01) (**Fig. 14**).

Treatment with DA at concentrations 1μM and 100μM did not influence the percentages of live or LA CD4+ T cells, in neither resting nor activated conditions. The results revealed significance influenced by the DA treatment only in activated EA cells, at the concentration of 100 μM, when compared to activated control conditions (b, P<0.01) (**Fig. 14**).

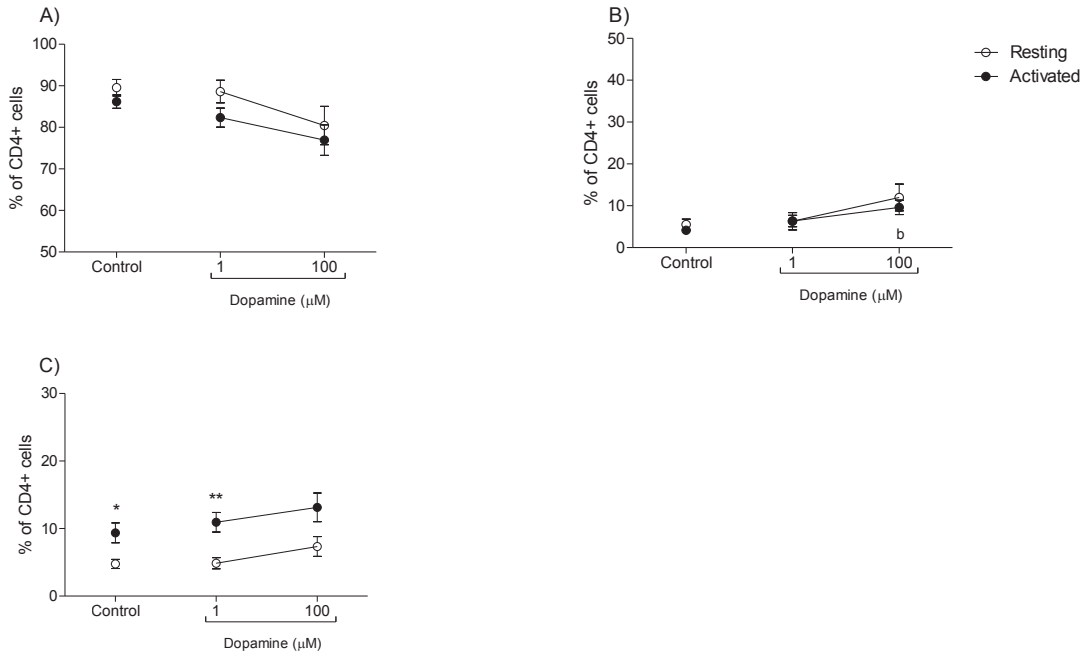


Fig. 14 Effect of different dopamine concentrations on: A) live cells, B) EA and C) LA cells, expressed as a percentage of total CD4+ T cells in human PBMC after 48 h culture. Results are presented as a mean±SEM of BC samples (n=5). *, P<0.05 and **, P< 0.01 vs. resting conditions. b, P<0.01 vs. activated control conditions.

The obtained results of experiments performed on buffy coat samples helped to develop and validate a method, as well as standardise culture conditions for following experiments with peripheral blood of healthy subjects.

A curve was drawn employing concentrations of DA ranging from 1 to 100 μM. The preliminary results, obtained from the fresh blood of 5 healthy subjects, revealed significant differences between resting and activated cultivated conditions, in each cell population examined: live, EA and LA cell groups. Namely, activation seems to reduce the percentage of live cells, while increasing the percentages of EA and LA cells (**Fig. 15**).

Furthermore, the results have shown that DA has a pro-apoptotic effect at concentrations of 100 μM, by decreasing the percentage of live, and increasing the percentage of EA cells (**Fig. 15**).

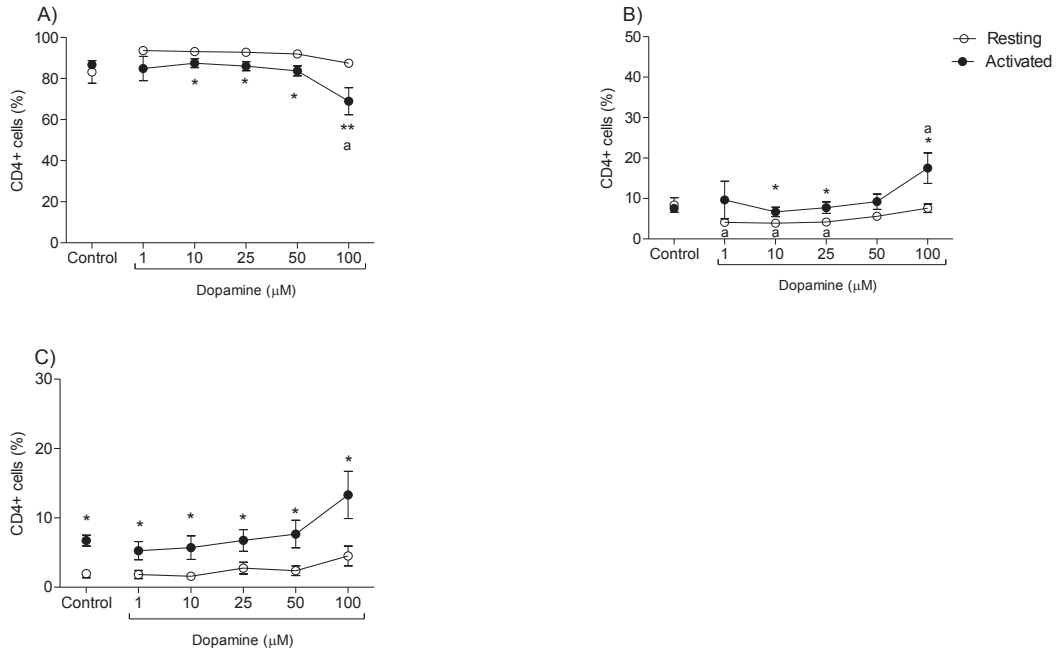


Fig. 15 Effect of different concentrations of DA on: A) live cells, B) early apoptotic and C) late apoptotic cells, expressed as a percentage of total CD4+ T cells in human PBMC after 48 h culture. Results are presented as a mean±SEM of HS (n=5). *, P<0.05 and **, P< 0.01 vs. resting. a, P<0.05 vs. control.

A higher concentration of DA (100 μM) was found to be toxic for PBMC *in vitro* in our experimental settings. On the basis of these results, DA was added at the maximum concentration of 50 μM for comparative experiments between buffy coat samples and healthy subjects PBMC proliferation test (**Fig**. **15**).

A further effect of L-DOPA was examined, including concentrations of L-DOPA ranging from 1 to 100 μM. In resting conditions, treatment with L-DOPA in highest concentrations reduced the percentage of live cells and increased EA and LA, implying a pro-apoptotic effect of L-DOPA (**Fig**. **16**).

On the other hand, activation in control conditions (without the treatment) increased only the percentage of LA. It seems that activation reversed the pro-apoptotic effect of L-DOPA, since it decreased the percentage of live cells and increased the percentages of EA and late apoptotic cells (**Fig. 16**).

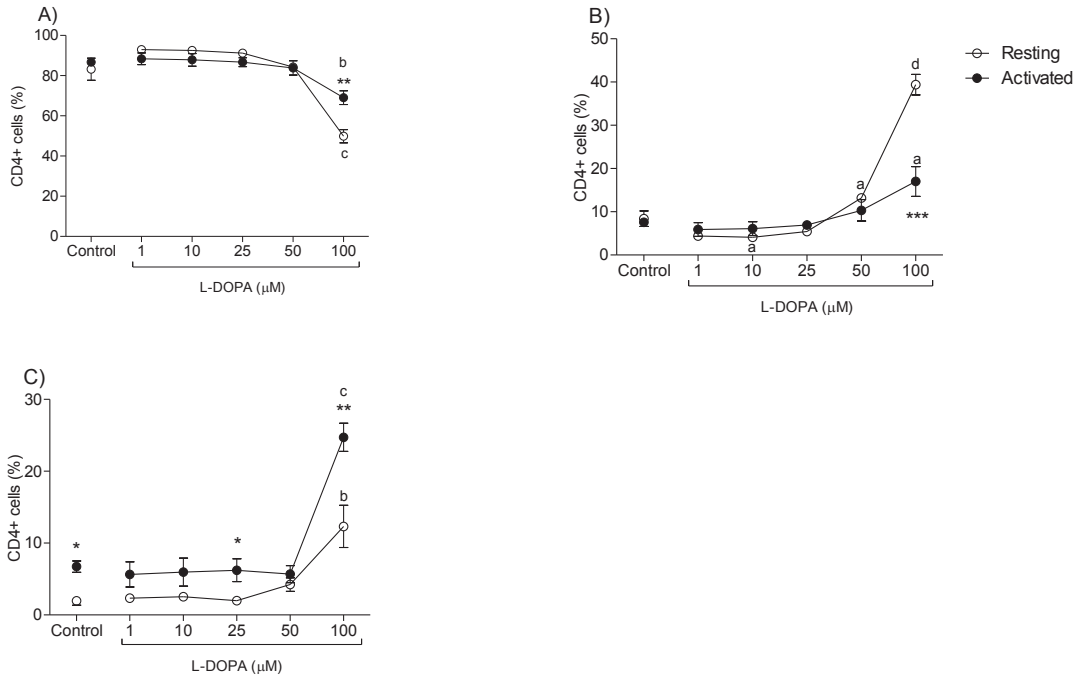


Fig. 16 Effect of different concentrations of L-DOPA on: A) live cells, B) early apoptotic and C) late apoptotic cells, expressed as a % of total CD4+ T cells in human PBMC after 48 h culture. Results are presented as a mean±SEM of HS (n=5). *, P<0.05; **, P< 0.01 and ***, P<0.001 vs. resting conditions. a, P<0.05; b, P<0.01; c, P<0.001 and d, P<0.0001 vs. corresponding control conditions.

So far, treatment with pramipexole did not exert any significant effect on apoptotic process of CD4+ T cells (**Fig. 17**). As expected, activation led to an increase in the percentage of LA cell population in control conditions.

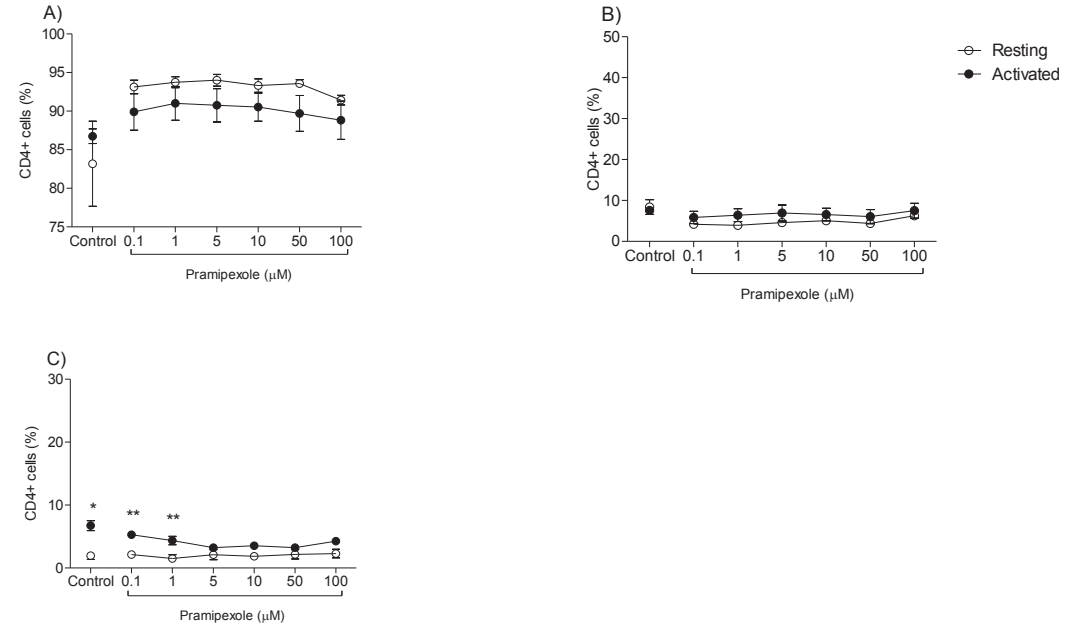


Fig. 17 Effect of different concentrations of pramipexole on: A) live cells, B) early apoptotic and C) late apoptotic cells, expressed as a percentage of total CD4+ T cells in human PBMC after 48 h

culture. Results are presented as a mean±SEM of HS (n=5). *, P<0.05 and **, P< 0.01 vs. resting conditions.

Conclusions, implications and future perspectives

Results have shown a higher expression of DR in apoptotic cells (both EA and LA) in comparison to viable cells after 48 h of cultivation. Activation with anti-CD3/anti-CD28 Ab led to an increase in the percentage of CD4+ T cells that are expressing DR in viable cells, without altering apoptotic cells. High expression of DR in apoptotic cells and stimulation-induced DR increase in cultured CD4+ T cells suggests the involvement of DR in the apoptotic process and further supports the involvement of DR in the functional regulation of activated cells, requiring further investigations to assess the role of DR subtypes in the modulation of specific responses (Kustrimovic et al., 2014).

Furthermore, the addition of high concentrations of DA (100 μM) and L-DOPA (100 μM) profoundly affect survival and death of activated CD4+ T cells. On the other hand, pramipexole did not affect CD4+ T cell viability at all.

Interestingly, it can be proposed that in resting cells dopaminergic pathways participate mainly in apoptotic processes (as suggested by the high proportion of apoptotic cells expressing DR), while their functional relevance increases in activated cells (in line with stimulation-induced upregulation of DR in viable cells).

Further *in vitro* findings are necessary in order to add knowledge about the sensitivity of CD4+ T cell, to DA and other dopaminergic agents presently in clinical use.

3.4. DA effect on CD4+ T cells proliferation and DR expression

Saha et al., have shown that physiological concentrations of DA (53.9 pM) may inhibit the proliferation of human CD4+ T cells through the activation of D1-like receptors, in both healthy individuals (Saha et al., 2001a) and lung carcinoma patients (Saha et al., 2001b).

On the other hand, Giorelli et al., 2005 reported diminished mRNA and protein levels of D1-like DR D_5, but not of D2-like DR D_3, in PBMCs from untreated relapsing-remitting multiple sclerosis patients, and showed *in vitro* that DA (1 μg/mL) reduced T cell proliferation in PBMC from healthy subjects, but not from MS patients.

Aims

The aim was to explore expression of DR on proliferating and non-proliferating cells and to understand if proliferation of peripheral CD4+ T cell is affected by DA and dopaminergic agents.

Subjects enrolled in the study

In the first part of the study, whose aim was to show DR expression on proliferating cells *in vitro,* PBMC were isolated from buffy coat samples (n = 3-4).

The second part of the experiments were dedicated to examinining the effects of DA, L-DOPA and pramipexole on proliferation of CD4+ T cells. PBMC were isolated from venous blood obtained from buffy coat samples (n = 4) and healthy subjects (n=5).

3.4.1. PBMC isolation, CPD staining and cultivation

PBMC isolation was performed from 20-25 mL of whole blood samples by using Ficoll-Paque Plus density (1.077 g/mL) gradient centrifugation for purifying lymphocytes as previously described. Before putting PBMC in the culture, cells were stained for proliferation test with Cell Proliferation Dye eFluor® 670.

Cell staining with Cell Proliferation Dye eFluor® 670 (CPD)

- Cells were washed with 10 mL of PBS, at 400 g, 10 min, RT.
- Pellets were resuspended in 1 mL of 0.1% FBS/PBS.
- Dye colour was added to the cell suspension at the final concentration 2.5 μM.
- Cells were incubated at RT for 8 min, protected from light.
- The reaction was stopped by addition of equal volume of pre-warmed FBS and incubation at 37°C for 10 min.
- Samples were washed 3 times with RPMI/10% FBS, at 400 g, 5 min.
- Supernatants were removed, and pellets were resuspended and prepared for cell counting with Trypane blue.

After CPD staining, PBMC were washed, counted and adjusted to the final concentration of $1x10^6$ cells in 1 mL of RPMI/10 % FBS. Cells were cultured for 4 days at 37 °C in 48 well plate, in a moist atmosphere of 5 % CO_2, with anti-CD3/anti-CD28 Ab (0.1 μg/mL) added at the beginning of cell culture. All treatments, DA, L-DOPA or pramipexole, were added at the beginning of cell culture. After 4 days cells were collected, washed twice with 1 mL of PBS/BSA at 1200 g for 10 min at RT. Supernatants were removed and pellets were resuspended for two steps labelling with DR.

From each subject 7 aliquots were prepared: 5 were used for DR staining, 1 was used as a control for the secondary PEGAR Ab, and 1 was used as negative control (no Ab). The staining protocol consisted of two steps of indirect labelling procedure (primary Ab for one of the five DR + secondary Ab labelled with PE):

- Each aliquot was labelled with rabbit anti-human D1-like (D_1 and D_5) or anti-human D2-like (D_3, D_4, and D_5) DR (dilution 1:100)
- Samples were incubated for 30 min on ice in the dark.
- After incubation, cells were washed with PBS/BSA (1200 g for 5 min at RT).
- Samples were resuspended in 200 μl of PBS/BSA and 10 μl of PEGAR Ab was added, following incubation for 30 min on ice in the dark.
- After incubation, samples were washed and resuspended in 50 μL PBS/BSA.
- APC-Cy7- conjugated mouse anti-human CD4 was added to each aliquot, according to manufacturer's recommendations.
- Samples were incubated for 30 min in dark at RT.

- After incubation, samples were washed with 1 mL of PBS/BSA (1200 g for 5 min at RT).
- Samples were resuspended in 350 µL PBS and kept on ice until analysis.

The acquisition was then performed on a BD FACSCanto II flow cytometer (BectonDickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3). Lymphocytes were identified by their classical FSC, SSC signals and a minimum of 20.000 lymphocytes from each sample were collected in the gate. Data were analysed with FlowJo software (version 8.3.2). The results were expressed as a percentage of positive cells (%).

3.4.2. Analysis of results obtained

3.4.2.1. Expression of DR on proliferating CD4+ T cells

In order to distinguish proliferating and non-proliferating CD4+ T cells, flow cytometric method was applied to identify these two different subpopulations by 2-color flow cytometric analysis. Following gating strategy was used: first, lymphocytes (LY) were gated on the dot plot in the typical region of low SSC and FSC, CD4+ positive cells were then identified among gated lymphocytes. On the basis of expression of CPD marker, within the defined $CD4^+$ T cell population, proliferating and non-proliferating cells were identified (**Fig. 18**).

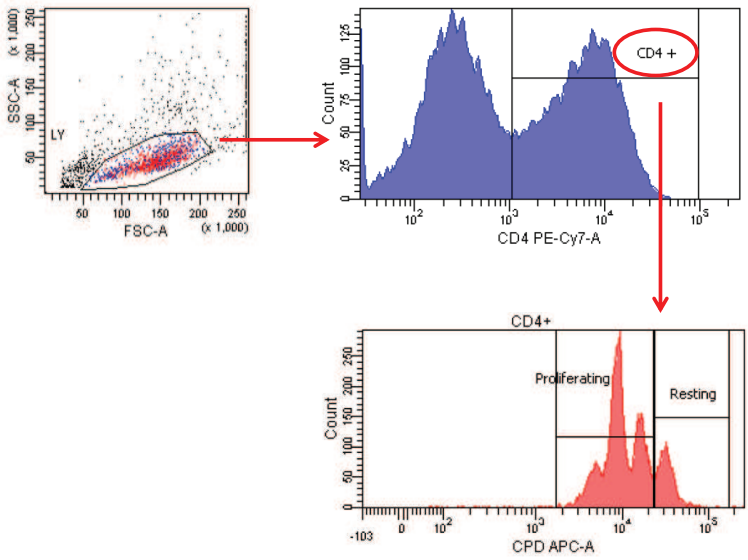


Fig. 18 Gating strategy used to identify proliferating and non-proliferating (resting) CD4+ T cells in human PBMC after 4 days of culture. Total lymphocytes were identified on a biparametric dot plot SSC vs. FSC (left panel, region LY) and from the cells in the LY region, CD4+ T cells were selected using the FSC vs. CD4 dot plot (right, upper panel). Finally, CPD expression was assessed by a single-

parameter histogram (lower panel), and CD4+T cell subset was further divided on proliferating and resting (non-proliferating) cells.

DR expression on proliferating cells confirmed expression of all five DR, although present in different extension: D1-like DR D_1 were expressed by 11.7±0.8% of total CD4+ T cells and DR D_5 by 14.3±6.0%, while the D2-like DR D_2 by 9.4±3.5%, D_3 by 4.7 ±2.1 %, and D_4 by 10.8±2.1 % (**Figure 19**).

Among non-proliferating cells, data have shown that CD4+ T lymphocytes expressed all the five DR. However, expression of each of DR was always less than 5%, with following frequency: D1-like DR D_1 were expressed by 4.1±1.0% of total CD4+ T cells and D_5 by 2.7±0.6%, while the D2-like DR D_2 by 3.4±0.6%, D_3 by 3.1±1.1%, and D_4 by 4.8±0.6% (**Figure 19**).

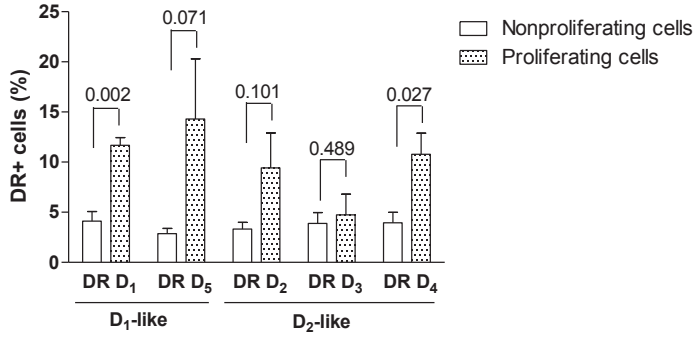


Fig. 19 Expression of DR on CD4+ non-proliferating and proliferating cells cultivated for 4 days under standard conditions, in the presence of anti-CD3/anti-CD28 (0.1 μg/mL). Results are presented as a mean±SEM of buffy coat samples (n=3-4).

3.4.2.2. Effects of DA and dopaminergic agents on CD4+ T cell proliferation

Based on obtained results regarding apoptosis and influence of DA and L-DOPA on cell viability (**section 3.5.2**.), further concentrations of DA and DA agents were chosen for proliferation assay.

Comparing levels of proliferation of activated CD4+ T cells isolated from buffy coats samples and healthy subjects, no major differences were obtained so far. DA, L-DOPA and pramipexole added in different concentrations, did not affect proliferation of CD4+ T cells, neither in the buffy coat samples or in fresh blood samples obtained from healthy subjects (**Figure 20**). Interestingly, pramipexole treatment at concentrations 0.1 and 1 μM decrease of

CD4+ T cell proliferation in healthy subjects in comparison to CD4+ T cell obtained from buffy coats (**Fig**. **20**).

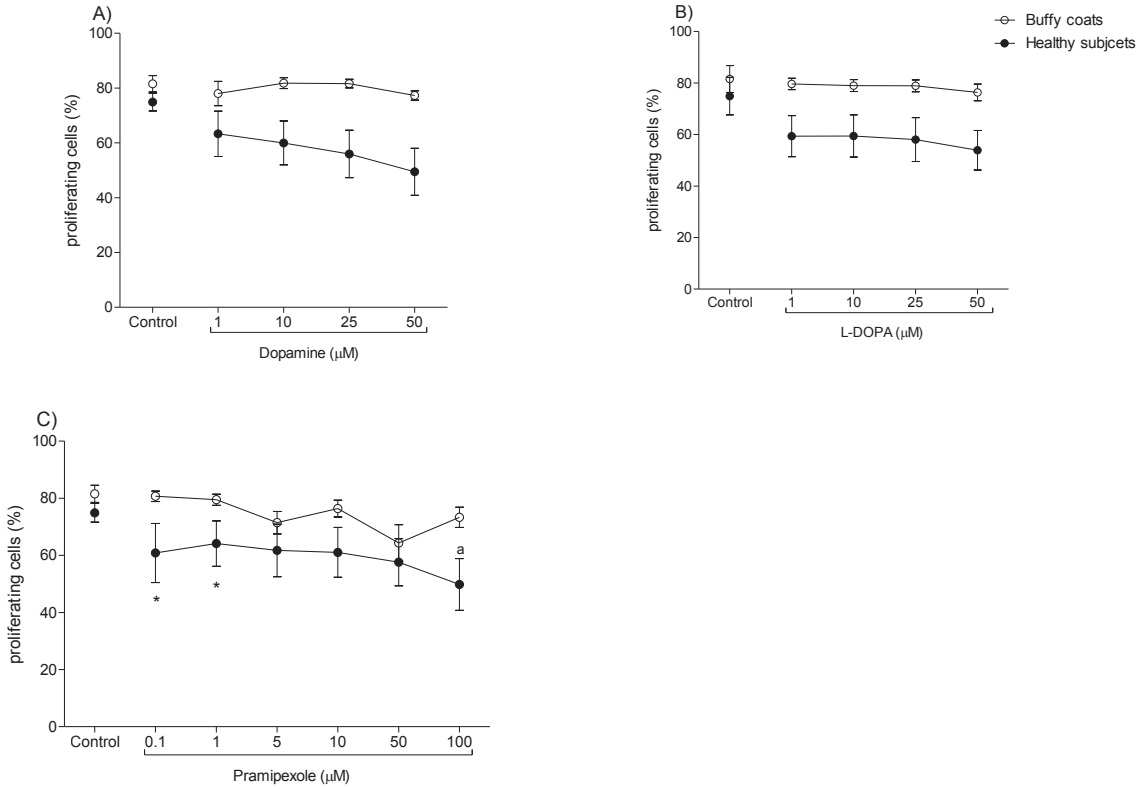


Fig. 20 Effect of DA (A), L-DOPA (B) and pramipexole (C) on the proliferation of CD4+ T cells isolated from buffy coat samples (n=4, empty circles) or healthy subjects (n=5, filled circles) and cultivated under standard conditions, in the presence of anti-CD3/anti-CD28 (0.1 μg/mL). Results are presented as a mean±SEM. *, P <0.05 vs. buffy coat samples a, P<0.05 vs. control conditions.

Conclusions, implications and future perspectives

Our data have shown that proliferating and non-proliferating CD4+ T lymphocytes expressed all the five DR, although in different expression levels. Interestingly, there was a trend of proliferating cells expressing DR in higher percentages, but these results were exploratory (n=3-4) and part of an investigation that is still ongoing in order to increase the number of samples.

So far, tested concentrations of dopaminergic agonists, including DA itself, have not shown any major effects on proliferation of CD4+ T cells.

3.5. Functional response of CD4+ T naïve and memory subsets

Upon stimulation by the presentation of novel Ags by dendritic cells, T lymphocytes become activated and go through the clonal expansion process creating Ag-specific lymphocyte pool. The differentiation of these cells leads to the clonal expansion of both "effector" cells, which immediately fight the foreign pathogen, and "memory" cells. Memory is a peculiar feature of the acquired immune system, which persists for a long time and is capable of reactivation in a subsequent encounter with same Ag. The pool of memory cells CD4+ T cells is heterogenic, phenotypically and functionally. According to the localisation and expression of specific markers, memory T cells are divided into central and effector memory cells T cells, T_{CM} and T_{EM} respectively. The T_{CM} cells express CCR7 and CD62L and produce IL-2 upon stimulation. T effector memory cells do not express CCR7 marker and have a low CD62L expression, and produce IFN-γ and IL-4 upon stimulation (Sallusto et al., 1999). The established model propose that T_{CM} mediate reactive memory, by homing to T cell areas of secondary lymphoid organs and readily proliferating and differentiating into effector cells upon antigenic stimulation, while T_{EM} afford protective memory, by migrating to inflamed peripheral tissues and displaying immediate effector function (Lanzavecchia and Sallusto 2000; Sallusto et al., 2004).

Immunological memory and specific memory CD4+ T cells provides the basis for successful protection against a variety of pathogens and successful vaccines applications (MacLeod et al., 2009). Since some of the activated cells die following the first response, the remaining memory cells are present at higher frequencies than the original naïve T cell. This higher frequency of memory, Ag-specific cells increases the likelihood of detection of re-infection quickly. Second, the difference between naïve and memory cells is that memory cells are able to make effector responses more rapidly than primary responding cells (Swain et al., 2006).

In vitro effects of a common recall Ag, such as TTd on naïve and memory CD4+ T cell frequencies and a qualitative response evoked by this Ag, were used as a well-established system to explore the ability of the peripheral immune system to recognise it.

Aims

1) The aim of this part of the study was to examine the expression of DR on peripheral CD4+ T subsets: Tn, $T_{CM,}$ and T_{EM} by means of a novel flow cytometric method.

2) To investigate the role of DA pathways in CD4+ Tn, $T_{CM,}$ and T_{EM} cells, and their responses to recall Ag tetanus toxoid.

Subjects enrolled in study

Whole blood samples were obtained from adult healthy donors (n = 30) and were used for this part of the study.

3.5.1. DR staining on CD4+ T naïve, T_{CM} and T_{EM} lymphocytes in whole blood

Phenotyping of DR on Tn, T_{CM} and T_{EM} memory CD4+ T cells was performed by a 5-color flow cytometric analysis by use of a two-step protocol which allowed the identification of all the five DR on different cell subsets. A method for DR staining in whole blood was performed as already established DR staining method in our laboratory (Kustrimovic et al., 2014).

- Each sample was prepared as 100 μL of a whole fresh blood added to BD tube.
- From each subject 7 aliquots were prepared as follow: 5 were used for DR staining, 1 was used as a control for the secondary PEGAR Ab, and 1 was used as negative control (no Ab).
- In order to remove the erythrocytes, 3 mL of a lysis buffer (containing NH_4Cl 8.248 g/L, $KHCO_3$ 1.0 g/L and EDTA 0.0368 g/L) were added to each sample.
- Incubation was performed at RT for 5 min, during which samples were gently vortexed.
- Samples were then centrifuged at 1200 g for 5 min at RT, supernatants were removed and cells were washed one time with 1 mL of PBS (pH 7.4) supplemented with 1 % BSA (PBS/BSA).
- Pellets were resuspended in 50 μL PBS/BSA.

Primary anti-DR Ab (final dilution 1:100) was added to the samples and incubated for 30 min on ice in the dark.

- Cells were washed once with PBS/BSA at 1200 g for 5 min at RT.
- Pellets were resuspended in 200 µL of PBS/BSA and 10 µL of PEGAR Ab was added.
- Samples were incubated for 30 min on ice in the dark.
- After incubation, samples were washed with 1 mL of PBS/BSA (1200 g for 5 min at RT) and resuspended in 50 µL PBS/BSA.
- A cocktail of pre-titrated PerCPCy5.5-conjugated mouse anti-human CD3, FITC-conjugated mouse anti-human CD45RA, APC-Cy7- conjugated mouse anti-human CD4, and AlexaFluor 647- conjugated mouse anti-human CCR7 was added to all samples.

Samples were incubated for 30 min in dark at RT, after which were washed with 1 mL of PBS/BSA (1200 g for 5 min at RT).

- Finally, samples were resuspended in 350 µL PBS and kept on ice until analysis.

The acquisition was then performed on a BD FACSCanto II flow cytometer (BectonDickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3). Lymphocytes were identified by their classical FSC and SSC signals and a minimum of 20.000 lymphocytes from each sample were collected in the gate. Data were analysed with FlowJo software (version 8.3.2).

The results were finally expressed as a percentage of positive cells (%) identified as T lymphocytes, CD4+ T lymphocytes, and the following CD4+ T lymphocyte subsets: naïve (CD3+CD4+CD45RA+CCR7+), T central memory (CD3+CD4+CD45RA-CCR7+), and T effector memory (CD3+CD4+CD45RA-CCR7-) cells.

3.5.2. T naïve/memory cell subsets characterisation and *in vitro* responses

PBMC were isolated from the peripheral blood of healthy subjects by density gradient centrifugation as previously described (**section 3.3.1.1.**).

- Prepared samples of isolated PBMC were resuspended at the final concentration of $0.5x10^6$ cells in 500 µL of RPMI/10%FBS for subsequent culture: 48 h at 37°C in a moist atmosphere of 5% CO_2.
- Cells were cultivated with/without anti-CD3/anti-CD28 ab (0.1 µg/mL), and in the presence/absence of TTd (3 µg/mL) added at the beginning of cell culture.
- After 48 h, cells were harvested and washed in PBS/1%BSA.

- Supernatants were removed and the pellet was resuspended and prepared for flow cytometric analysis of CD4+ Tn/T_{CM}/T_{EM} cells (by staining CD3/CD4/CD45RA/CCR7) subsets (**section 3.5.1.**).

3.5.3. Analysis of obtained results

3.5.3.1. Expression of DR in CD4+ Tn, T_{CM} and T_{EM} lymphocytes

To identify CD4+ T naïve/memory cell subsets, the following gating strategy was applied: first, lymphocytes were gated on the scatter dot plot in the typical region of low SSC and FSC; CD3 and CD4 double positive cells were then identified among gated lymphocytes, by use of a biparametric dot plot CD45RA vs CCR7 were further identified T naïve (CD3+CD4+ CD45RA+CCR7+), T_{CM} (CD3+CD4+CD45RA-CCR7+), and T_{EM} (CD3+CD4+CD45RA-CCR7-). DR expression was assessed by a single-parameter histogram in desired subpopulations of CD4+ T cells (**Fig**. **21**).

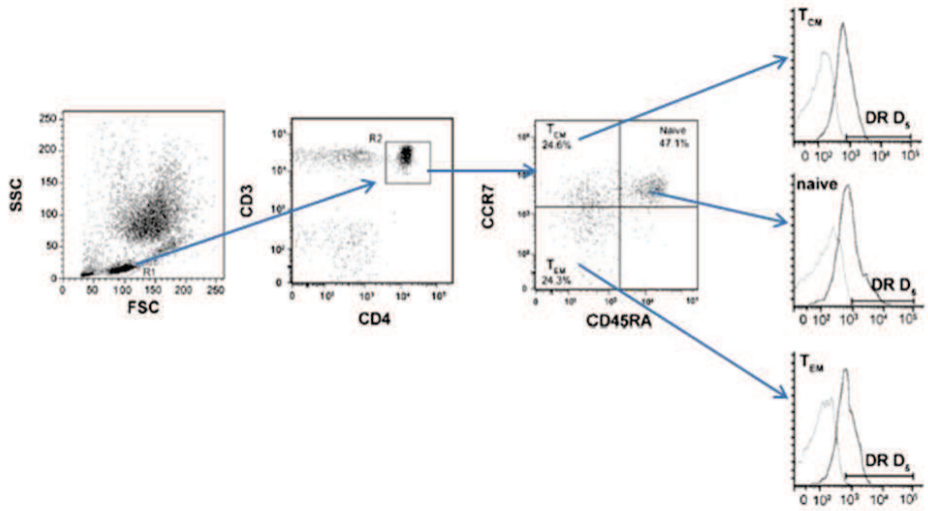


Fig. 21 Gating strategy used to identify CD4+ T naïve, T_{CM} and T_{EM} cells. Total lymphocytes were identified on a biparametric dot plot SSC vs FSC (R1) (left panel). CD3+CD4+ T cells in R1 were selected by using the CD3 vs CD4 dot plot (R2), further specific subsets of CD4+ T cell subsets in R2 were identified by a biparametric dot plot CD45RA vs CCR7. DR expression was assessed by a single-parameter histogram, where dark lines represent DR+ cells and light lines represent negative controls (cells stained with only secondary PEGAR ab), showing the analysis of DR D_5 in a representative sample (right panels).

The frequencies of total CD4+ T cells and defined subsets: Tn, T_{CM} and T_{EM} included in the study are summarised in **table 13**.

Table 13. Frequencies of CD4+ T naïve/memory subsets

Lymphocyte subsets		mean±SEM
CD4+	**% of CD3+**	67.6±1.8
T naïve	**% of total CD3+CD4+**	37.2±1.4
T_{CM}	**% of total CD3+CD4+**	24.8 ±0.9
T_{EM}	**% of total CD3+CD4+**	27.9±1.0

DR+ cells were enumerated in Tn, T_{CM}, and T_{EM} cells. Expression of DR D_1, DR D_5, and DR D_3, was significantly higher in Tn cells compared to T_{CM} and T_{EM} cells (**, P<0.0001), the same trend occurred in the case of DR D_4 (*, P<0.01) and DR D2 (#, P<0.05), but with the different significance. The differences between T_{CM} and T_{EM} expression of each DR were not observed (**Fig**. **22**).

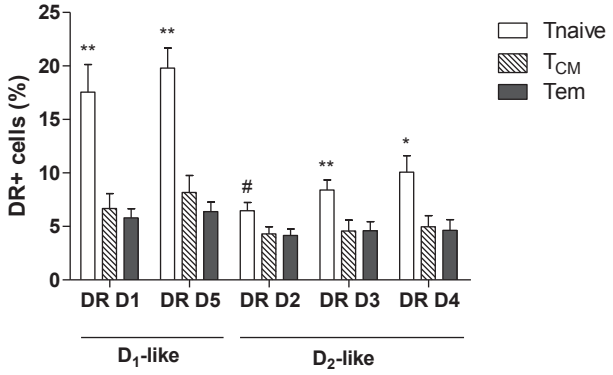


Fig. 22 Comparison of the expression of individual DR among naïve T cells, T_{CM} and T_{EM} cells. Results are presented as mean±SEM of 30 HS. **, P<0.0001 vs. T_{CM} and T_{EM}; *, P<0.01 vs. T_{CM} and T_{EM} and #, P<0.05 vs. T_{CM} and T_{EM}.

3.5.3.2. Effects of TTd on the frequency of CD4+ T naïve and memory subsets

Incubation of PBMC for 48 h with recall Ag TTd (3 μg/mL) decreased Tn and increased T_{EM} cells (**Figure 23**). Co-incubation with either the D1-like DR agonist SKF-38393 (1 μM), or the D2-like DR agonists pramipexole (1 μM), did not affect T naïve/memory cell frequency and did not modify the effects of TTd in PBMC isolated from HS (data not shown).

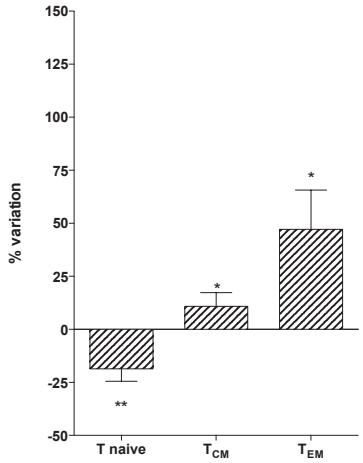


Figure 23. Effects of TTd in PBMC obtained from healthy subjects. Data are expressed as percentage variation with respect to control conditions (without TTd), and are means±SEM of n = 6-8 separate experiments each performed in duplicate. *, P<0.05 and **, P<0.01.

These results are part of our manuscript entitled "Dopaminergic receptors on CD4+ T naïve and memory lymphocytes correlate with motor impairment in patients with Parkinson's disease" Kustrimovic N, Rasini E, Legnaro M, Bombelli R, Aleksic I, Blandini F, Comi C, Mauri M, Minafra B, Sanchez-Guajardo V, Marino F, Cosentino M. (2016) Sci Rep. Sep 22;6:33738 (**Appendix 1.**).

Conclusions, implications and future perspectives

Ex vivo results have shown the expression of all the five DR on human Tn, $T_{CM,}$ and T_{EM} cells, to a different extent. Tn cells express higher levels of all the five different DR receptors, in comparison to T_{CM} or T_{EM} cell subsets. Obtained findings have opened the possibility to explore in more details relationship with the functional status of these cells, *ex vivo* and *in vitro*.

So far, validated and developed *in vitro* method, could be used as an assay to test the function of memory CD4+ T cells towards recall Ag, and might potentially have relevance for a wide range of different fields of T cell biology research in health and disease.

3.6. T regulatory cell function

Regulatory CD4+ T cells are suppressor cells that suppress other immune cells by various mechanisms (Sakaguchi et al., 2008). Characteristic markers of Treg cells are transcription factor Foxp3 and CD25. Treg cells are defined as T cells in charge of suppressing potentially deleterious activities of T helper cells.

Recently, has been shown that in the human CD4+CD25high T lymphocyte fraction, both D1-like and D2-like DR, as well as several α- and β-AR subtypes and TH are constitutively expressed, and substantial amounts of DA, NA and A can be found (Cosentino et al., 2007). Endogenous DA release results in down-regulation of CD4+CD25high T cell-dependent inhibition of CD4+ T effector lymphocyte proliferation, possibly through an autocrine/ paracrine loop involving DR D_5 pathways and resulting in down-regulation of the regulatory function (Cosentino et al., 2007).

Aims

Several specific aims have been set:

1) to assess DR expression on Treg cells obtained from peripheral blood of healthy subjects by the flow cytometry method,

2) to examine *in vitro* Treg-induced inhibition of Teff proliferation,

3) to characterise secretion of cytokines (IL-10 and TGF-β) by Treg alone, and to characterise Treg induced suppression of production of cytokines IFN-γ and TNF-α, by Teff cells, and to characterise effects of SKF 38393 (1 μM) and pramipexole (1 μM) on cytokine production

4) to examine the relevance of the *in vitro* dopaminergic modulation of dopamine (1 μM) and L-DOPA (1 μM) on the function of CD4+CD25+ enriched population of T cells.

Subjects enrolled in study

1) For the first part of *ex vivo* DR expression explorative study, 32 healthy subjects have been enrolled.

2) To investigate *in vitro* Teff proliferation and Treg-induced inhibition preliminary experiments were with cells isolated from buffy coat samples (n=7). On the basis of this set of experiments, further study was focused on Treg and Teff cells obtained from HS (n=10).

3) To characterise of *in vitro* cytokine production by Teff and Treg cells, our study enrolled healthy subjects (n=5).

4) Aiming to explore effects of DA (1μM) and L-DOPA (1μM) on suppressive potential of Treg cells on Teff proliferation, Treg and Teff cells obtained from HS (n=7), PD patients on dopaminergic therapy (n = 10) and patients who had never been treated with antiparkinson drugs (n = 5).

3.6.1. Immunofluorescent staining of DR on T regulatory cells in whole blood

- 100 μL of peripheral venous blood sample was added to FACS-tubes.
- 3 mL of lysis solution was added in order to remove erythrocytes.
- Samples were incubated for 5 min at RT and vortexed gently.
- Cells were centrifuged at 1200 g for 5 min RT and supernatants were carefully removed and additionally washed with 1 mL of PBS/1%BSA.
- The ellet was resuspended in:
- 50 μL PBS/BSA + anti-DA receptors Ab [final dilution 1:100],
- 50 μL PBS/BSA (as a negative control)
- Samples were incubated for 30 min on ice and subsequently washed with 1 mL of PBS/1%BSA.
- Pellet was resuspended with 200 μL PBS/BSA, and 10 μL of PEGAR Ab was added.
- Samples were incubated for 30 min in ice and wash with 1 mL of PBS/1%BSA.
- The pellet was resuspended in 50 μL PBS/1%BSA.
- A cocktail of the following antibodies was added: CD4APCCy5, CD25PECy7 and CD127PerCPCy5.5 according to manufacturer's recommendations.
- Samples were incubated for 20 min at RT in the dark after what were washed with 1 mL of PBS/1%BSA.
- Finally, the pellet was resuspended in 350 μL PBS and samples were kept on ice until flow cytometric acquisition.

By usage of this method CD4+ T cell subsets was identified as conventional Treg (CD4+CD25highCD127low) and subsequently, expression of five different subtypes of DR was examined.

The acquisition was performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3). Identification of lymphocytes was assessed by FSC and SSC signals and a minimum of 20.000 lymphocytes from each sample were collected in the gate. Data were analysed with FlowJo software (version 8.3.2). The results were expressed as a percentage of CD4+ cells (%).

Separation of T effector and T regulatory cells

Separation of PBMC by Ficoll-Hypaque method was performed initially as previously reported (**section 3.3.1.1.**).

The isolation of $CD4^+CD25^+$ regulatory T cells was performed in two-step procedure with human CD4+CD25+ Regulatory T cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions.

Magnetic labelling of non-CD4+ cells

- A defined number of PBMC was passed through 30-μm-nylon mesh in order to remove cell clusters that may clog the columns.
- The cell suspension was centrifuged at 400 g, 10 min, RT.
- The cell pellet was resuspended in Milteny Buffer (PBS/0.5% BSA/EDTA 2 mM).
- 10 μL (for 10^7 cells) CD4+ T cells Biotin-Antibody Cocktail, consisting of biotin-conjugated monoclonal anti-human antibodies, CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a was added into samples and mixed well and incubated for 10 min, in dark, at +4°C.
- 20 μL (for 10^7 cells) Anti-biotin MicroBeads was added into samples mixed well and incubated for 15 min, in dark, at +4°C.
- Samples were washed with 10 mL of Miltenyi Buffer and obtained pellet was resuspended in 500 μL of Miltenyi Buffer.

Magnetic separation: depletion of non-CD4+ cells

- LD column was placed in the magnetic field of a suitable MACS Separator.
- The column was prepared by rinsing with 3 mL of Miltenyi Buffer.
- The cell suspension was applied onto the column.
- After the cell suspension has passed the column was additionally washed three more times with 3 mL of Miltenyi Buffer.
- The effluent was collected and consisted of unlabelled pre-enriched CD4+ cell fraction.

Magnetic labelling of CD4+ CD25+T regulatory cells

- The obtained effluent was centrifuged at 300 g, 10 min, RT.
- Supernatant was removed and 10 μL (for 10^7 cells) of CD25 MicroBeads was added.
- Samples were mixed well and incubated for 15 min, in dark, at +4°C, after which samples were washed in 10 mL of Miltenyi Buffer.
- Samples were resuspended in 500 μL of Miltenyi Buffer.

Magnetic separation: Positive selection of CD4+ CD25+ T regulatory cells

- MS column was placed in the magnetic field of a suitable MACS Separator.
- The column was prepared by rinsing with 500 μL of Miltenyi Buffer.
- The cell suspension was applied onto the column and after it has passed the column was additionally washed 3 times with 2 mL of Miltenyi Buffer.
- Flow-through containing T effector cells (unlabelled for CD25) was collected.
- The column was removed from the separator and placed it in a suitable collection tube.
- 1 mL of Miltenyi Buffer was pipetted onto the column and immediately flushed out by firmly pushing the plunger into the column. The cells that have been flushed out were CD25 labelled cells (T regulatory cells).

- In order to make sure that collection of cells was complete, the last step was repeated a second time.

Both Treg and Teff viability more than 99% assessed by the Trypan Blue exclusion test.

The purity of separated Teff and Treg was checked by flow cytometry. Briefly, $1x10^6$ of isolated PBMC, Teff, and Treg cells were taken and incubated with anti CD4-APC-Cy7, CD25-PR and CD127-AF647 Ab for 20 min in dark at RT. After the incubation samples were washed and resuspended in 350 µL of PBS and left on the ice. The acquisition was then performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3).

Results have shown that CD4+CD25highCD127low cells were present in separated populations as following percentages: 7.1±0.2% in Teff cells (**Fig. 24**), in Treg subpopulation 76.1±3.2% have shown significant enrichment and in PBMC was only 7.9±1.6% (**Fig. 25**).

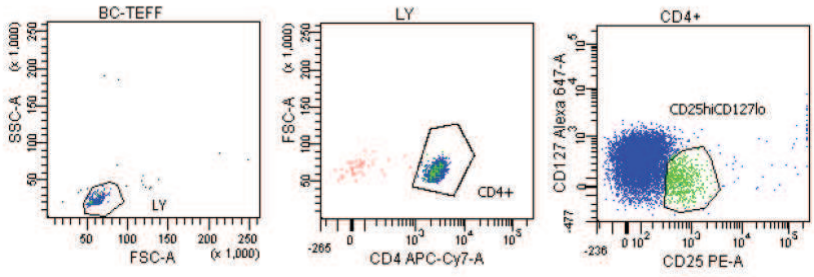


Figure 24. Purity of separated T effector CD4+ cells

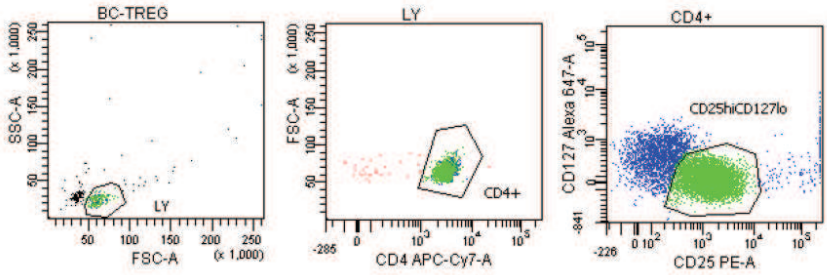


Figure 25. Purity of separated T regulatory CD4+ cells

T effector cell staining with Cell Proliferation Dye eFluor® 670 (CPD)

- A desirable number of cells was washed with PBS 400 g, 10 min, RT.
- Supernatants were carefully removed.

- Pellets were resuspended in 1 mL of 0.1% FBS/PBS working solution.
- Dye colour was added to cell suspension (final concentration 2.5 μM).
- Cells were incubated at RT for 8 min, protected from light.
- The reaction was stopped by addition of equal volume of pre-warmed FBS and incubated for 10 min at 37°C for colour efflux.
- Samples were washed 3 times 400 g, 5 min, with RPMI/10%FBS.
- Supernatants were removed, and pellets were resuspended and prepared for cell counting with Trypan blue colour.

This way Teff cells were prepared for the following cultivation.

In vitro Treg-Teff cell co-culture

Teff and Treg cells were obtained by magnetic separation as previously described. Both types of cells were plated alone or in co-culture (different Teff:Treg ratios) and cultivated under standard conditions, in RPMI 1640 medium supplemented with 10% heath-inactivated FBS, 2mM glutamine and 100 U/mL penicillin/streptomycin, at the final concentration 1 x 10^5 cells/mL in the 96-well plate for 4 days, with/ without PHA (5 μg/mL) and IL-2 (40 ng/mL) that were added at the beginning of cell culture. Where specified, DA (1 μM), L-DOPA (1 μM), D1-like DR agonist (SKF 38393, 1 μM) and D2-like DR agonist (pramipexole, 1 μM) were added at the beginning of cell culture.

After 4 days, cells were collected and prepared for the further FACS Flow analysis.

3.6.2. Flow cytometric analysis of Treg suppression capacity after *in vitro* cultivation

Proliferation assay was quantified, by already established and validated method by flow cytometry, by usage of CPD dye enabling identification of Teff cells that are in proliferation, and thus indirectly enabling a measure of Treg suppressive effects on Teff cells proliferation. Briefly, at the end of cell culture, cells were collected and centrifuged: 1200 g, 5 min, RT, and pellets were resuspended in 350 μL of PBS. Samples were kept on ice prior to flow cytometry acquisition.

3.6.3. Quantification of cytokines by ELISA test

Both Teff and Treg cell subpopulations were obtained by magnetic separation as previously described. For subsequent *in vitro* tests both type of cells was resuspended in RPMI 1640 medium supplemented with 10% heath-inactivated FBS, 2mM glutamine and 100 U/mL penicillin/streptomycin. 1 x 10^4 cells of Teff or Treg cells were plated alone or in co-culture (Teff:Treg ratio = 1:1) and cultivated in standard conditions, RPMI/10% FBS for 48 h at 37°C in a moist atmosphere of 5% CO_2, with or without PHA (5 μg/mL) and IL-2 (40ng/mL). In order to explore the effects of D1-like DR agonist (SKF 38393, 1μM) and D2-like DR agonist (pramipexole, 1μM) on the production of INF-γ and TNF-α cytokines by Teff and IL-10 and TGF-β cytokines by Treg, supernatants from 48 h conditioned cultures were analysed by ELISA. Results were compared with the cytokine secretion from the same culture without treatments.

3.6.4. Analysis of obtained results

In order to define Treg cells in the peripheral blood of healthy subjects, flow cytometric method was applied using the specifically created gating strategy. First, lymphocytes (LY) were gated on the dot plot in the typical region of low SSC and FSC, CD4+ positive T cells were then identified among gated lymphocytes. By the expression of the CD25 and CD127 markers, within the defined $CD4^+$ T cells, the population of CD25highCD127low cells was defined (often referred as conventional Treg) (**Fig. 26**).

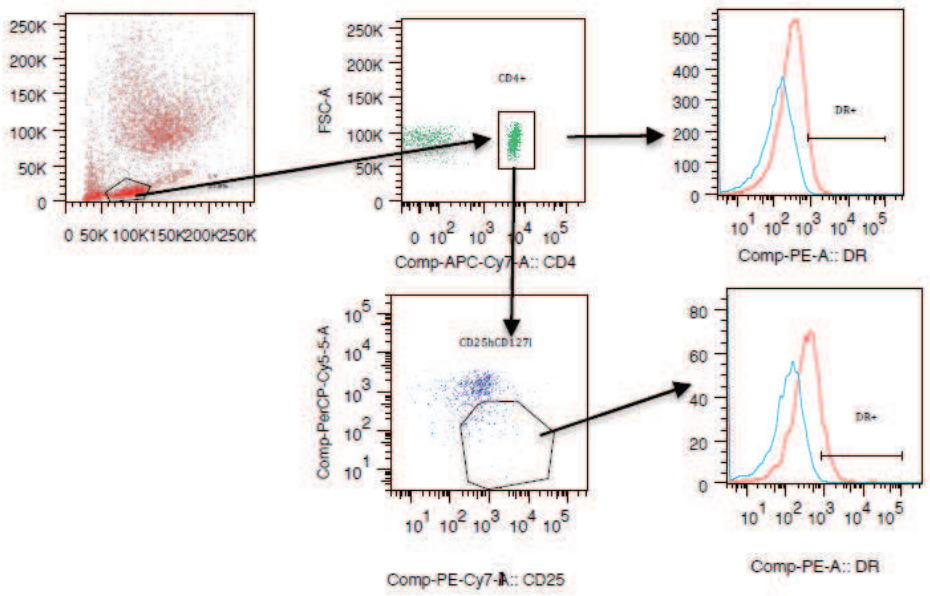


Fig. 26 Gating strategy used to identify DR expression on total CD4+ T cells and conventional Treg (CD4+CD25highCD127low) in the peripheral blood of healthy subjects. Total lymphocytes were identified on a biparametric dot plot SSC vs. FSC (upper left, region LY), to exclude monocytes, granulocytes, and debris. From the cells in the LY region, CD4+ T cells were selected by using the FSC vs. CD4 dot plot (upper middle panel). CD4+T cell subset was further divided on biparametric dot plot CD127 vs. CD25 and cells defined as CD25highCD127low were selected. DR expression on total CD4+ T cells (upper, right panel) or on CD25highCD127low cells (lower, right panel) was assessed by a single-parameter histogram, where red lines represent DR^+ cells and blue lines represent negative controls (cells stained with only secondary PEGAR ab).

3.6.4.1. DR expression on T regulatory cells

Results have shown percentages of total CD4+ T cells (51.0±7.9) and conventional Treg CD4+CD25highCD127low cells as 9.3±0.5 (mean±SEM). Further, conventional Treg ($CD4^+CD25^{high}CD127^{low}$) expressed all five examined DR in following percentages: the D1-like receptors, DR D_1 14.8±2.0% and DR D_5 by 14.4±1.9%, and the D2-like receptors: DR D_2 12.5±1.9%, DR D_3 12.0±1.8% and DR D_4 13.7±1.8% of conventional Treg (**Fig. 27**). So far results have not revealed any difference in the percentages of conventional Treg expressing different subtypes of DR.

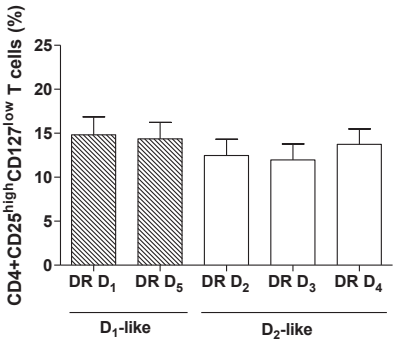
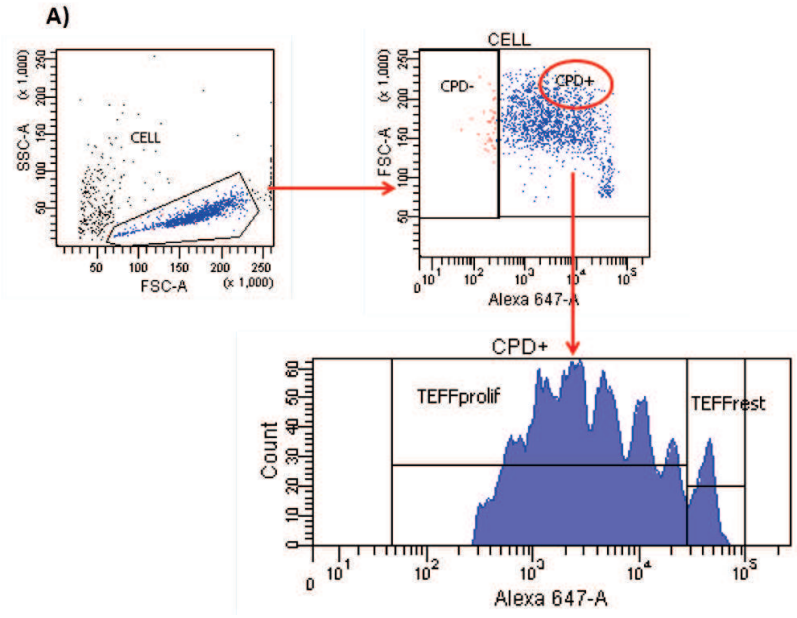


Figure 27 Percentage of CD4+CD25highCD127low T cells in the peripheral blood of HS that are expressing different subtypes of DR. Results are presented as mean±SEM of 32 subjects.

3.6.4.2. Proliferation and inhibition assay

In order to discrete proliferating and resting Teff cells and Treg-induced inhibition, 2-color flow cytometric method was applied. Proliferation assay was quantified, by already established and validated method by flow cytometry, by usage of CPD dye enabling identification of Teff cells that are in proliferation (**Fig 28A**), and thus indirectly enabling a measure of Treg suppressive effects on T effector cells proliferation (**Fig 28B**).

Following gating strategy was used: first, lymphocytes were identified on the dot plot in the typical region of low SSC and FSC, and then CPD^+ positive cells were identified among gated lymphocytes. By the expression of the CPD colour, within the previously defined population of $CD4^+$ T cells, proliferating and resting Teff cells were examined (**Fig. 28**).



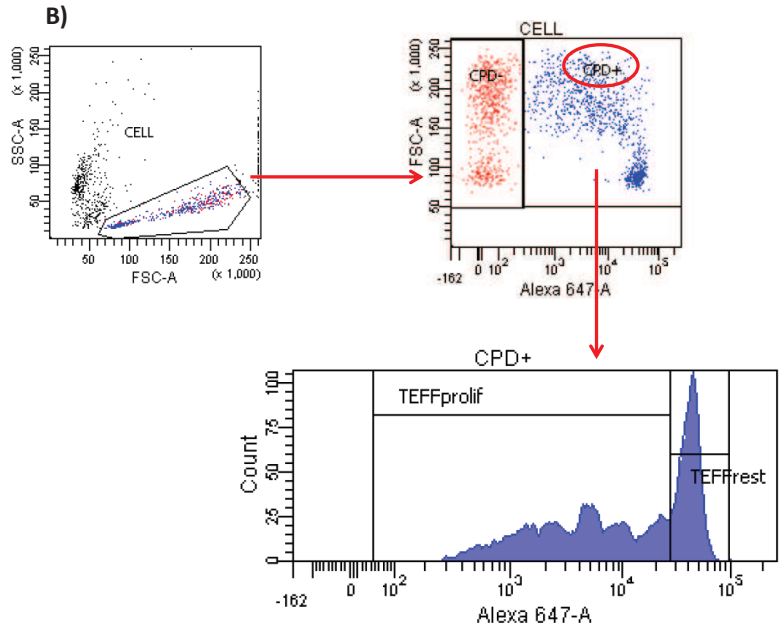


Fig. 28 Gating strategy used to identify proliferating and resting Teff cells alone (A) or in the presence of Treg cells (ratio 1:1) (B) obtained from HS, after 4 days of cultivation in standard conditions. Total lymphocytes were identified on a biparametric dot plot SSC vs. FSC (left panels, region LY). From the cells in the LY region, CPD+ cells were selected by using the FSC vs. CPD dot plot (right, upper panels). Finally, proliferating and resting Teff cells were assessed by a single-parameter histogram (right, lower panels).

The first part of a study aimed to develop and validate an *in vitro* models to investigate and characterise suppressive effect of Treg cells on Teff proliferation. In this regard were performed experiments of Teff-Treg co-culture with different ratios of Treg cells. Preliminary experiments were performed on cells obtained from buffy coat samples. On the basis of this results, showing the suppressive capacity of Treg on Teff proliferation and that this effect is dose dependent (**Figure 29A**) further experiments on peripheral blood from healthy subjects were performed.

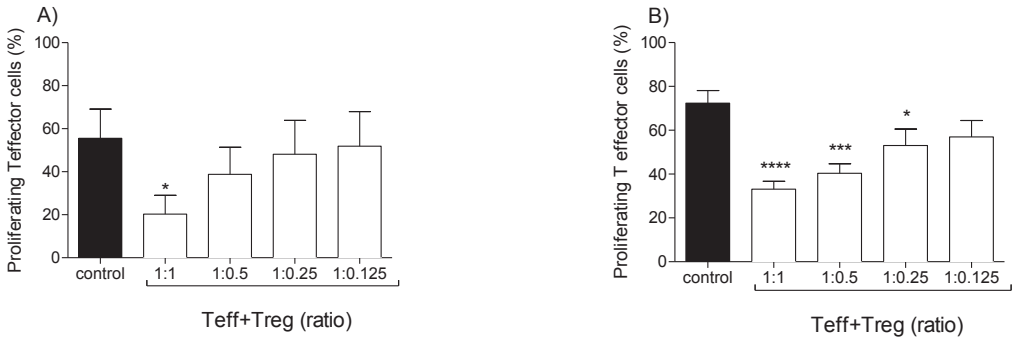


Figure 29 Curve of inhibition of Teff proliferation by Treg in buffy coat samples (n=7, A) and healthy subjects (n=9, B) cultivated in the presence of PHA (5 µg/mL) and IL-2 (40ng/mL). Black bar represents Teff cultured alone, while open bar represents co-culture of Treg and Teff in different ratios. Results are presented as a mean±SEM. *, P<0.05, ***, P<0.0001 vs. control.

Obtained results showed that Teff cells, obtained from peripheral blood of healthy subjects when cultivated alone, proliferate at 74.58±13.24% (**Figure 29B**), while in co-culture this proliferation was significantly diminished in presence of Treg cells at ratio 1:1, 1:0.5 and 1:0.25 (33.16±3.52%; $p<0.0001$, 40.40±4.33%; $p<0.0001$, 53.05±7.48%; $p<0.05$, respectively) (**Figure 29B**).

3.6.4.3. Effects of DA and L-DOPA on Treg-dependent suppression on Teff proliferation

The ability of DA (1 μM) or L-DOPA (1 μM) to influence the suppressive potential of Treg cells was tested. Co-culture assays were performed as described above in cells obtained from 9 healthy subjects. In HS Teff proliferation was suppressed in the presence of Treg cells, however, DA and L-DOPA have restored proliferation of Teff cells trough inhibition of suppressive capacity of Treg cells (**Figure 30**).

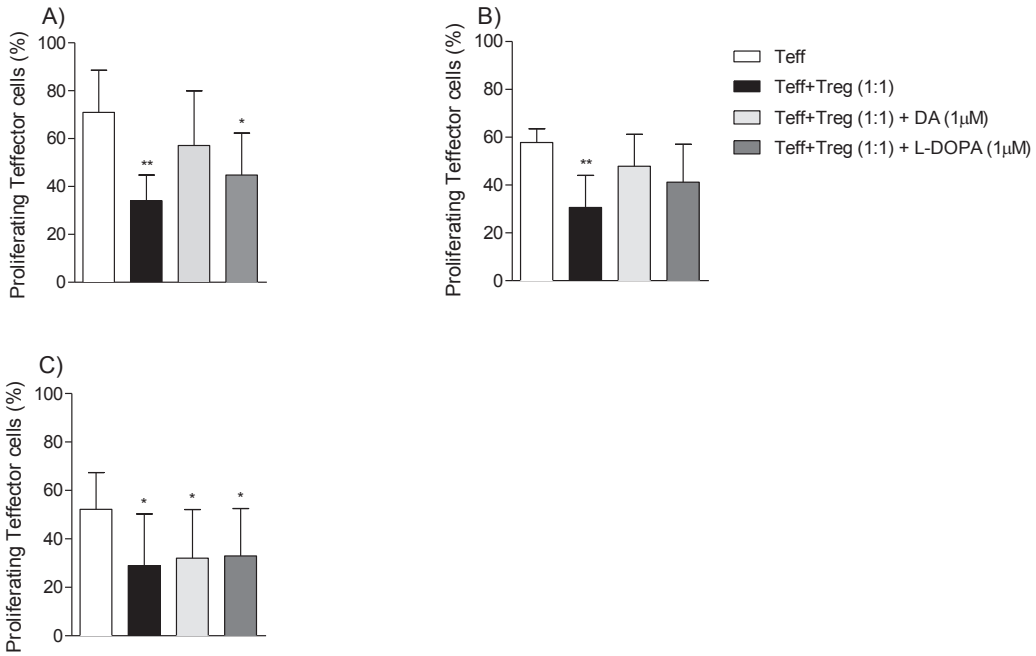


Figure 30 Percentage of proliferating Teff cells isolated from HS (n=7, panel A), patients who had never been treated with antiparkinson drugs (n=5, panel B) and PD patients on dopaminergic therapy (n=10, panel C) cultivated under standard conditions in the presence of PHA (5 μg/mL) and IL-2 (40 ng/mL), alone (open bar), or in co-culture with Treg cells at ratio 1:1 (black bar) or in the presence of DA (light grey bars) or L-DOPA (dark grey bars) for 96 h. Results are presented as a mean±SEM. *, $P<0.05$, **, $P<0.01$ vs. Teff cells.

3.6.4.4. Dopaminergic modulation of cytokine production of Teff and Treg cell subsets

A) Production of proinflammatory cytokines by Teff cells

After preliminary experiments performed (on 3-4 replicates) on non-activated Teff cells (data not shown), where we have shown only trace amounts of produced cytokines or even under detection level, the most reliable experimental design was considered to be established, and the same experimental conditions and design were used in all future experiments where Teff cells were always activated with PHA (5 μg/mL), alone (as a control) or in co-culture with Treg cells always in ratio 1:1, in all cytokine production experiments.

Results obtained in a group of healthy subjects have shown that activated Teff cells produce 34.8±9.1 pg/mL of IFN-γ, and have reduced production on to 4.4±1.1 pg/mL in the presence of the same number of Treg (**Figure 31, left**). Further analysis has revealed that when cultivated alone Teff cells, in the presence of SKF 38393 or pramipexole, produced 23.4±9.0 pg/mL and 23.5±7.1 pg/mL, respectively. Teff cells when co-cultivated with the Treg cells in the presence of SKF 38393 or pramipexole, reduced their production to 6.0±1.1 pg/mL and 8.2±1.2 pg/mL, respectively suggesting that neither of employed agonist nor D1-like nor D2-like DR agonist, have influenced suppressor potential of Treg cells (**Figure 31**).

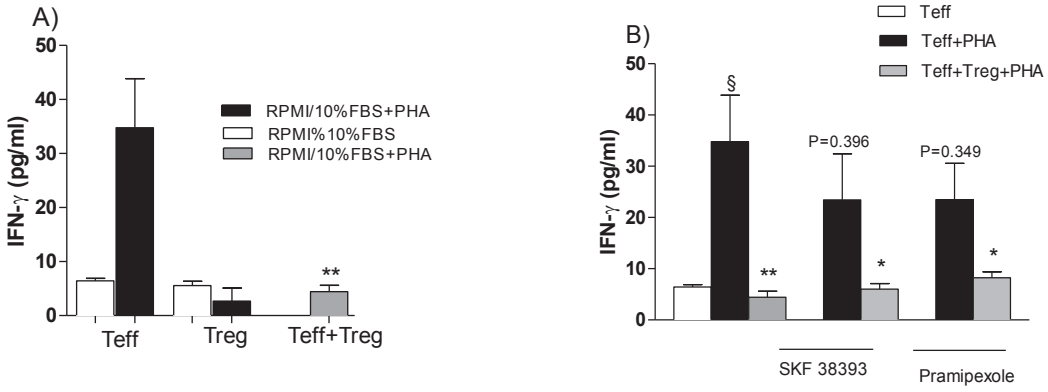


Figure 31 Production of INF-γ by resting (open bars) or activated with PHA (5 μg/mL) (black bars) Teff or Treg cells cultivated alone, or in the co-culture (grey bars) under standard conditions (panel A) and in the presence of D1-like DR agonist, SKF 38393 (1μM) or D2-like DR agonist, pramipexole (1μM) (panel B) for 48h in HS. Results are presented as a mean±SEM of 6 subjects. *, P<0.05 and **, P<0.01 vs. Teff+PHA; $, P<0.0001 vs. activated Teff alone.

Further, results have shown that activated Teff cells alone produce 247.2±19.1 pg/mL of TNF-α, while when cultivated in the presence of Treg (1:1 ratio) with PHA (5μg/mL), production of TNF-α was profoundly reduced to the 11.8±4.8 pg/mL (**Figure 32, left**). Teff cells cultivated in the presence of SKF 38393 or pramipexole produce lower quantities of examined cytokine, 109.0±43.3 pg/mL and 102.0±37.6 pg/mL, respectively. Nevertheless, either of applied DR agonists was not able to interfere with the suppressive potential of Treg cells (**Figure 32**).

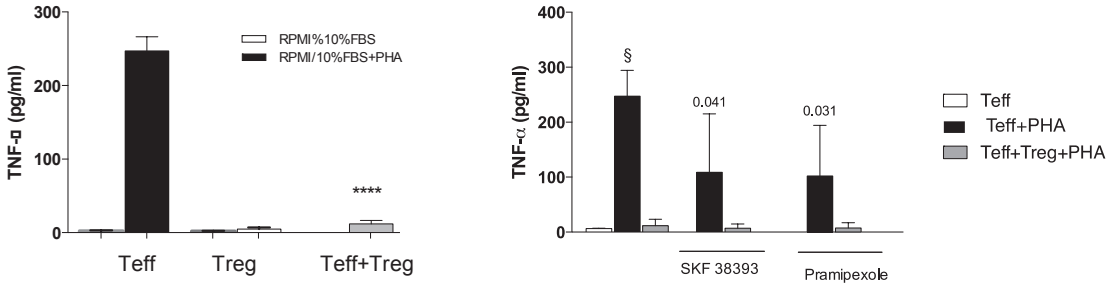


Figure 32 Production of TNF-α by resting (open bars) or activated with PHA (5 μg/mL) (black bars) Teff or Treg cells cultivated alone or in the co-culture (grey bars) under standard conditions (left panel) and in the presence of D1-like DR agonist, SKF 38393 (1μM) or D2-like DR agonist, pramipexole (1μM) (right panel) for 48h in HS. Results are presented as a mean±SEM of 6 subjects. ****, P<0.0001 vs. Teff+PHA; $, P<0.0001 vs. Teff alone.

B) Production of anti-inflammatory cytokines by Treg cells

Obtained results have shown that activated Treg cells produce 17.81±6.78 pg/mL of IL-10 when cultivated under standard conditions. Neither D1-like or D2-like DR agonist have influenced IL-10 production potential of Treg cells (**Fig**. **33**).

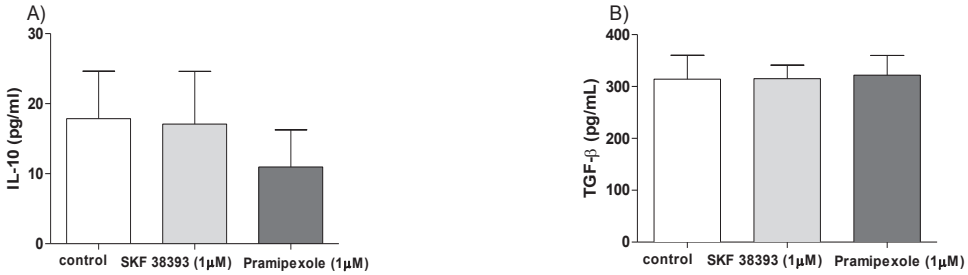


Figure 33 Production of IL-10 (panel A) and TGF-β (panel B) by activated Treg cells cultivated in standard conditions with addition of PHA (5 μg/mL) (open bars), in the presence of D1-like DR agonist, SKF 38393 (1 μM) (light grey bars) or D2-like DR agonist, pramipexole (1 μM) (dark grey bars) after 48h in HS (n=6). Results are presented as a mean±SEM.

Further, the capability of Treg cells to produce TGF-β was tested. Obtained results have shown that Treg cells produce TGF-β in high amounts, 314.0±18.8 pg/mL when cultivated in standard conditions (RPMI/10%FBS) in the presence of activators. Obtained results have shown that neither of DR agonist applied did not influence the production of TGF-β by Treg cells (**Fig. 33**).

Conclusions, implications and future perspectives

Presented *in vitro* method was developed and validated through numerous pilot experiments conducted on buffy coat samples in which data showed suppressive Treg cell capacity. Further experiments were done with fresh blood from healthy subjects and have shown the same inhibitory capacity of Treg cells (at the ratios of 1:0.5 and 1:0.25 Treg cell dilutions).

In addition, effects of DA and L-DOPA treatments seems to suppress Treg suppressive capacity in healthy subjects, since Teff cell proliferation is restored in comparison to co-culture control conditions (Treg + Teff cells, 1:1). Interestingly, the same effect of DA and L-DOPA as in healthy subjects was observed in PD-dn, but not in PD-dt group. Results suggest that dopaminergic agents influenced the suppressive capacity of Treg cells in healthy subjects and drug naïve PD patients, but not in patients that are on dopaminergic replacement therapy.

Treg cells suppressed production of IFN-γ and TNF-α from Teff cells. Treatment with SKF 38393 and pramipexole did not influence the suppressive capacity of Treg. Neither IL-10 or TGF-β production from Treg cells was influenced by SKF 38393 and pramipexole treatments.

Due to the simplicity of Treg suppressive function assay, numerous variables including type of activation, cell number, and degree of proliferation can be manipulated within a single experiment. One of the weaknesses of *in vitro* Treg suppression assay is that Teff cells are sometimes hypo-proliferative, even in response to PHA stimulation (Thornton et al., 2004). In our conditions, the activation status of Teff cells is usually checked by flow cytometry method, based on the characteristically morphological phenotype of activated cells, and samples that were not fully activated were excluded from further procedures.

3.7. Model of commitment of naïve CD4+ T cells

Naïve CD4+ T cells may acquire diverse phenotypes depending on stimulation of surrounding microenvironment (Zhu et al., 2010). The critical determinants for T cell differentiation are a network of cytokines involved in different phases of the complex process that these cells are going through. Cytokines are important for induction, priming phase of Th differentiation, expansion of different T cell subsets (growth and survival factors), autocrine positive/negative feedback loop and stabilisation of the population and finally lineage plasticity.

Th17 cells have been recognised as a lineage separate from Th1 and Th2 cells, and also differentiation of Th17 cells is inhibited by factors, both IFN-γ and IL-4, that support Th1/Th2 differentiation (Harrington et al., 2005; Park et al., 2005).

Aims

Several specific aims were proposed for this part of the study:

1) In first part of study, *ex vivo* phenotypic characterisation of Th1, Th2, and Th17 cell subsets was performed by flow cytometry analysis and DR expression was assessed on each Th defined subpopulations,

2) To develop and validate reliable *in vitro* Th1/Th2/Th17 polarisation and cultivation method (buffy coat samples),

3) To explore the role of dopaminergic modulation on polarisation and differentiation process of Th1, Th2 and Th17 subset in healthy subjects.

Subjects enrolled in study

1) *Ex vivo* determination of DR expression in different Th subsets was done on peripheral blood samples from healthy subjects (n=38).

2) For preliminary *in vitro* experiments, naïve CD4+ T cells were isolated from buffy coat samples (n = 4), and followed by further experiments performed on cells isolated from peripheral blood of healthy subjects (n =4).

3.7.1. Frequency of CD4+ Th1, Th2 and Th17 T cell subsets in peripheral blood

Standard protocol for isolation of PBMC was used (**section 3.3.1.1.**).

From PBMC fraction, human naïve CD4+ T cells were isolated using the naïve CD4+ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's protocol.

The frequency of different Th subsets (Th1/Th2/Th17) among CD4+ T lymphocytes was analysed by a 4-color flow cytometric analysis. In this panel, CD4 was used as lineage marker and for identification of T cell subsets, three chemokine receptors were used: CXCR3 (CD183), CCR4 (CD194) and CCR6 (CD196), as markers for Th1, Th2 and Th17 subsets, respectively. T helper subsets were defined as Th1 (CXCR3+CCR4-CCR6-), Th2 (CXCR3-CCR4+CCR6-), Th17 (CXCR3-CCR4-CCR6+) and Th1/Th17 (CXCR3+CCR4-CCR6+).

Flow cytometry staining was performed directly from whole blood, obtaining approximately 1 x 10^6 cells from 100µl of whole blood sample.

- 100 µL of peripheral venous blood sample was added to FACS-tubes.
- A cocktail of the following antibodies was added: CD4 APCCy5, CCR4 PECy7, CXCR3 AF488 and CCR6 PerCP-Cy5.5 according to manufacturer's recommendations.
- Samples were incubated for 30 min at RT in the dark.
- 3 mL of lysis solution was added in order to remove erythrocytes.
- Samples were incubated for 5 min at RT and vortexed gently.
- Cells were centrifuged at 400 x g for 5 min RT and supernatants were carefully removed and additionally washed with 1 mL of PBS/1%BSA.
- Pellets were resuspended in 350 µL PBS and samples were kept on ice until flow cytometric acquisition.

Expression of DR on CD4+ Th1, Th2, and Th17 cell subsets

Immunophenotyping of DR on CD4+ lymphocytes on different subsets of T helper cells was performed by two-step, 5 – color flow cytometric analysis from the whole blood.

- Each sample was prepared as 100 µL of a whole fresh blood added to BD tube.
- A cocktail of the following antibodies was added: CD4 APCCy5, CCR4 PECy7, CXCR3 AF488 and CCR6 PerCP-Cy5.5 according to manufacturer's recommendations.
- Samples were incubated for 30 min at RT in the dark.

- 3 mL of lysis solution was added in order to remove erythrocytes.
- Samples were incubated for 5 min at RT and vortexed gently.
- Cells were centrifuged at 400 g for 5 min RT and supernatants were carefully removed and additionally washed with 1 mL of PBS/1%BSA.
- The pellet was resuspended in:
- 50 μL PBS/BSA + anti-DA receptors Ab [final dilution 1:100],
- 50 μL PBS/BSA (as a negative control) and
- Samples were incubated for 30 min on ice and subsequently washed with 1 mL of PBS/1%BSA.
- The pellet was resuspended with 200 μL PBS/BSA, and 10 μL of PEGAR Ab was added.
- Samples were incubated for 30 min in ice and wash with 1 mL of PBS/1%BSA.
- Finally, the pellet was resuspended in 350 μL PBS and samples were kept on ice until flow cytometric acquisition.

Flow cytometric analysis was performed on BD FACSCanto II, and a minimum of 20 000 cells was analysed from each sample. The results were finally expressed as percentage of positive cells (%).

Purification and sorting of naïve CD4+ T cell isolation for in vitro cultivation

For the optimal performance, it was important to obtain single-cell suspension before magnetic labelling thus, cells were passed through 30 μm nylon mesh to remove cell clumps.

- PBMC number was determined.
- Cells were centrifuged at 600 g, 10 min, RT and supernatants were removed.
- Cells were resuspended in Miltenyi Buffer (PBS pH 7.2, free of Ca^{2+} and Mg^{2+}, containing 0,5% BSA and 2 mM EDTA).
- Naïve CD4+ T cell biotin-Ab cocktail was added, mixed well and incubated 5 min, at +4 °C.
- Incubation was stopped by adding ice-cold Miltenyi buffer.
- Naïve CD4+ T cell MicroBeads cocktail was added, mixed well and incubated for another 10 min, at +4 °C.

- Meanwhile, LS Miltenyi Biotec column was prepared by rinsing with 3 mL of Miltenyi Buffer.
- After incubation, the cell suspension was directly applied onto the column and flow-through containing unlabeled, enriched naïve CD4+ T cells were collected.
- The column was washed additionally three times with 2 mL of Miltenyi Buffer and all unlabeled cells passed through were collected.
- Cells were centrifuged 600 g, 10 min, at RT and resuspended in culture medium to determine cell number and viability by Trypan blue exclusion.

After immunomagnetic sorting purity of separated naïve CD4+ T lymphocyte, population was checked by flow cytometry.

Briefly, $1x10^6$ of isolated, naïve CD4+ T lymphocyte cells were taken and incubated with anti CD3-PerCPCy5.5, CD4-PECy7, CD45RA-FITC and CCR7-AF647 Ab for 30 min, in dark, at RT. After the incubation samples were washed and resuspended in 350 µL of PBS and left on the ice. The acquisition was then performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3).

Identification of lymphocytes was assessed by FSC and SSC signals, for a minimum of 20.000 lymphocytes from each sample collected in the gate and data were analysed with FlowJo software (version 8.3.2).

To exclude debris and the potential presence of other cells, lymphocyte cells were gated, then CD3+CD4+ double positive T cells were identified and finally, naïve CD4+ T cells were assessed as an enriched CCR7+CD45RA+ cells population (**Fig. 34**). The purity of isolated naïve CD4+ T cells was more than 95% assessed by flow cytometry identified as naïve T cells subset (CD3+CD4+CD45RA+CCR7+).

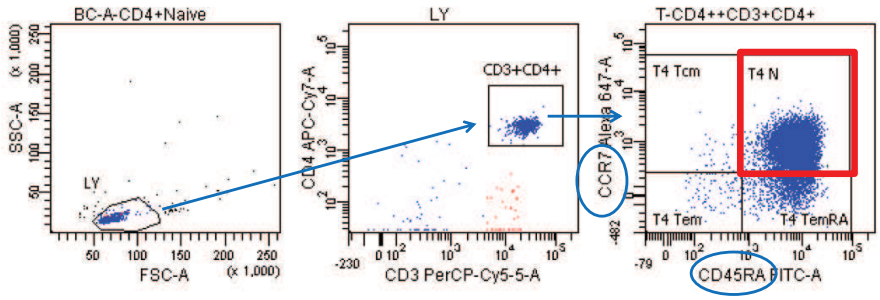


Figure 34 Purity of separated naïve CD4+ T lymphocyte population

In vitro cell culture and T cell differentiation assay

Separated naïve CD4+ T cells were cultured in U-bottomed 96-well plates primed with anti-CD3/CD28 Ab, with/without DA or L-DOPA, added at the beginning of cell culture, under different polarising conditions (**Table 14**). Cells were placed in the incubator for 4 days, at 37 °C in a moist atmosphere of 5% CO_2 with a minimum disturbance. After 4 days of priming, cells were observed under a light microscope to confirm clusters of T cell activation. Samples were gently pipetted to break up clumps and washed at 600 g, 5min, RT. Supernatants were very carefully aspirated and replenished with fresh medium supplemented with human recombinant IL-2 (10 ng/mL or 2 ng/mL, as indicated in the table), and left for another 3 days to obtain preferably Th cell subsets expansion.

Table 14. Polarisation condition of 7 days *in vitro* naïve CD4+ T cell differentiation

	Th0	**Th1**	**Th2**	**Th17**
Day 0	□	IL-12 (10 ng/mL) anti-IL-4 Ab (10 μg/mL)	IL-4 (10 ng/mL) anti-INF-γ Ab (10 μg/mL)	IL-1β (10 ng/mL) IL-6 (50 ng/mL) TGF-β (5 ng/mL) anti-INF-γ anti-IL4 Ab (both 10 μg/mL)
Day 4	IL-2 (10 ng/mL)	IL-2 (10 ng/mL)	IL-2 (10 ng/mL)	IL-2 (2 ng/mL)

Intracellular cytokine staining and flow cytometry acquisition

- After 7 days, cells were collected, washed (600 g for 5 min at RT) and counted to obtained growth index (GI) in each polarisation route (**Table 15**).
- Growth index was expressed as a ratio, a final number of cells after 7 days of culture divided with initial number of cells, and viability of cells was expressed as a % at day 7.
- Cells were counted with Trypan and adjusted to concentration of 1 x 10^6 cells/mL for each sample.

- Each sample was additionally stimulated with: PMA (50 ng/mL), Ionomycine (Calcium Ionophore, 1μg/mL) and BD GolgiStop Protein Transport Inhibitor for 5h, at 37°C in a moist atmosphere of 5% CO_2, prior to intracellular cytokine staining.
- After 5 h of incubation, cells were washed and cell number in each sample was adjusted for analysis of intracellular cytokine expression.
- Cells were stained with Human Th1/Th2/Th17 Phenotyping Kit according to the manufacturer's protocol.

Table 15. Different Th cell subsets GI and viability after 7 days of cell culture

Th0		**Th1**		**Th2**		**Th17**	
GI	**viability**	**GI**	**Viability**	**GI**	**viability**	**GI**	**viability**
2.64±0.65	90.0±6.4	2.33±0.87	89.3±5.5	1.82±0.51	89.3±6.2	1.72±0.32	90.0±6.4

Intracellular cytokine staining of cells

- Cells were centrifuged at 1200 g, 5 min at RT.
- Supernatant was removed and cells were washed once again with 1 mL of 2%FBS/PBS.
- After removing the supernatants, pellets were vortexed (to avoid cell aggregation) and resuspended in 1 mL of cold BD Cytofix Buffer and incubated at RT for 15 min.
- After incubation, samples were centrifuged at 1200 g, 5 min, RT.
- Samples were washed with 1 mL of 2%FBS/PBS, 1200 g, 5 min, RT and pellet were resuspended in 1 mL of 2%FBS/PBS and left overnight at +4 °C.
- The day after, samples were centrifuged (1400 g for 5 min at RT) and the buffer was removed.
- Samples were resuspended in 1 mL of 1X BD Perm/Wash buffer and incubated at RT for 15 min, after which were centrifuged at 1400 g, 5 min, RT and supernatant were carefully removed.
- Fixed and permeabilised cells were resuspended in 50 μL of BD Perm/Wash buffer and 20 μL of Ab-cocktail (human Th1/Th2/Th17 cocktail containing: CD4PerCP-Cy5.5 clone SK3; IL-17A PE clone N49-653; IFN-γ FITC clone B27 and

IL-4 APC clone MP4-25D2) or isotype control (Ig Isotype control, human CD4 PerCP-Cy5.5) were added and incubated 20 min, at RT in the dark.

- Samples were washed with 1 mL of Perm/Wash solution, and finally resuspended in 350 µl of 2%FBS/PBS (stain buffer) and kept on ice prior to flow cytometric analysis.

The acquisition was performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with BD FACSDiva software (version 6.1.3). Total CD4+ T cells were identified on a biparametric dot plot SSC vs FSC to exclude possible presence of debris, and a minimum of 20.000 lymphocytes from each sample collected in the gate and data were analysed with FlowJo software (version 8.3.2).

3.7.2. Analysis of obtained results

3.7.2.1. Flow cytometric analysis of CD4+ T helper subsets

In order to define different subsets of T helper cells in the peripheral blood of healthy subjects, flow cytometric method was applied using the specifically created gating strategy. First, lymphocytes were gated on the dot plot in the typical region of low SSC and FSC, CD4+ positive cells were then identified among gated lymphocytes. By the expression of the CXCR3 and CCR4 markers, within the defined population of CD4+ T cells, following populations were defined: preTh1 and preTh2 subsets, respectively. PreTh1 cells were defined as CD4+CXCR3+CCR4-, while preTh2 were defined as CD4+CXCR3-CCR4+. Among the previously defined CXCR3+CCR4- preTh1 cells, two following subsets were defined as Th1/Th17 (CXCR3+CCR4-CCR6+) and Th1 (CXCR3+CCR4-CCR6-); and among CXCR3-CCR4+ preTh2 cells, other two subsets were defined as Th17 (CXCR3-CCR4+ CCR6+) and Th2 (CXCR3-CCR4+ CCR6-) (**Fig. 35**).

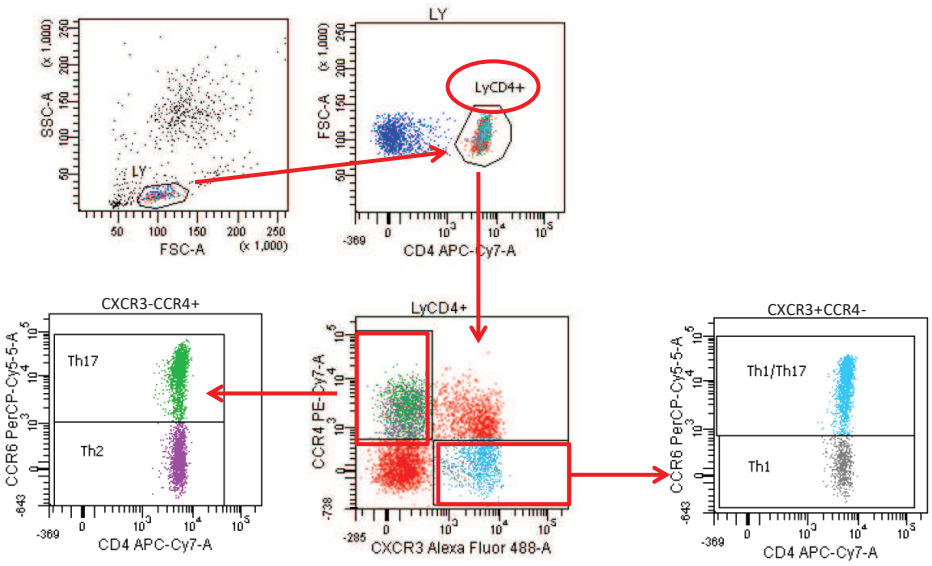


Fig. 35 Gating strategy used to identify specific Th cell subsets in the peripheral blood. Total lymphocytes were identified on a biparametric dot plot SSC vs. FSC (left panel, region LY), to exclude monocytes, granulocytes and debris. From the cells in the LY region, CD4+ cells were selected by using the FSC vs. CD4 dot plot (right, upper panel). Further specific subsets of CD4+ T cell subsets were identified by a biparametric dot plot CCR4 vs CXCR3 (down middle panel). Finally, additional, specific Th cell subsets were further identified on the basis of CCR6 Th17 specific marker.

3.7.2.2. DR expression on Th1, Th2, Th17 and Th1/Th17 cells

The frequencies of total CD4+ T cells and defined subsets: Th1, Th2, Th17, and Th1/Th17 CD4+ T cell included in the study, are presented in **Table 16**.

Table 16. Frequencies of CD4+ T helper cell subsets

Lymphocyte subsets		mean±SEM
CD4+	% of total Ly	47.0±1.3
Th1	% of CD4+	14.5±1.0
Th2	% of CD4+	7.7±0.7
Th17	% of CD4+	9.2±0.7
Th1/Th17	% of CD4+	11.9±0.9

Four different subsets of CD4+ T helper cells were identified by means of the above-described gating strategy. In defined subsets of T helper cells was sought to define the percentage of cells that are expressing diverse DR subtypes (**Fig**. **36**).

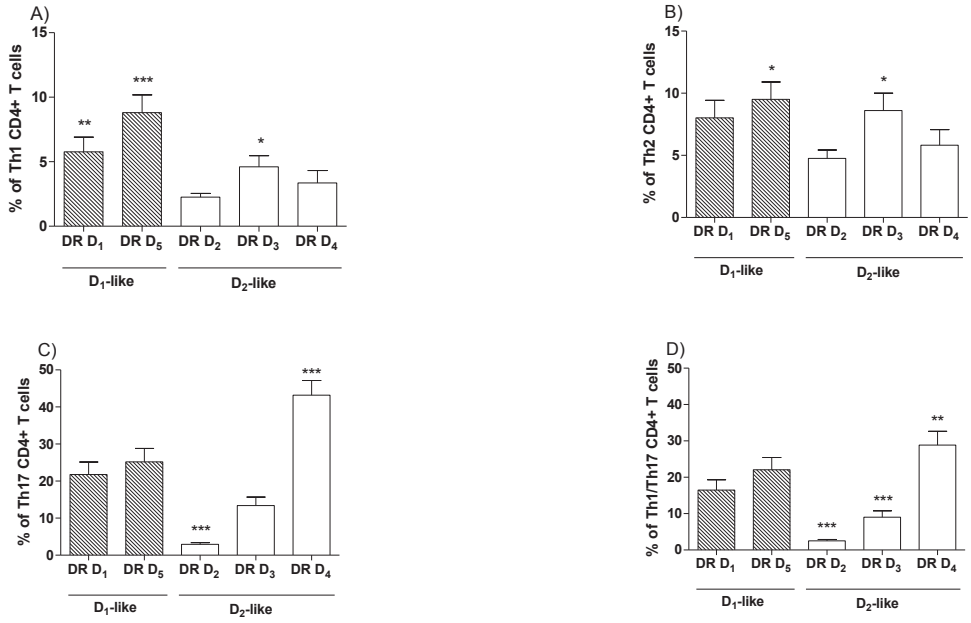


Figure 36 Expression of DR on Th1 (**, P<0.01 vs DR D_2 and DR D_4; ***, P<0.001 vs DR D_2 and DR D_3; *, P<0.05 vs DR D_2 and DR D_5) (A), Th2 (*, P<0.05 vs DR D_2 and DR D_4; *, P<0.05 vs DR D_2 and DR D_4) (B), Th17 (***, P<0.001 vs all other receptor subtypes) (C) and Th1/Th17 (D) cells subsets in the peripheral blood of HS. Results are presented as mean±SEM of 38 subjects.

DR expression on Th1 cells confirmed expression of all five DR (**Fig. 36A**), although present in different extension: D1-like DR D_1 were expressed by 5.8±1.1% of total CD4+ T cells and DR D_5 by 8.8±1.4%, while the D2-like DR D_2 by 2.3±0.3%, D_3 by 4.6 ±0.9 %, and D_4 by 3.4±1.0 % (**Figure 36A**). Among Th1 subset, DR D_1 expression was significantly higher (*,P<0.01) from DR D_2 and DR D_4; DR D_5 expression was significantly higher (***,P<0.001) than DR D_2 and DR D_3 and expression of DR D_3 was significantly higher (*,P<0.05) than DR D_2.

DR expression on Th2 cells confirmed expression of all five DR (**Fig. 36B**). D1-like DR D_1 were expressed by 8.0±1.4% of total CD4+ T cells and DR D_5 by 9.5±1.4%, while the D2-like DR D_2 by 4.7±0.6%, D_3 by 8.6 ±1.4 %, and D_4 by 5.8±1.2 % (**Figure 36B**). Among Th2 subset, DR D_3 and DR D_5 expressions were increased significantly (*,P<0.05) when compared to expression of DR D_2 and DR D_4.

DR expression on Th17 cells confirmed expression of all five DR (**Fig. 36C**). D1-like DR D_1 were expressed by 21.8±3.4% of total CD4+ T cells and DR D_5 by 25.2±3.6%, while the D2-like DR D_2 by 2.9±0.5%, D_3 by 13.4 ±2.3 %, and D_4 by 43.2±3.9 % (**Figure 36C**). Among Th17 subset, DR D_2 was expressed at the lowest percentage of Th17 cells while DR D_4

were expressed at the highest percentage of this CD4+ subset compared to others DR examined.

On Th1/Th17 cells, D1-like DR D_1 were expressed by 16.5±2.8% of total CD4+ T cells and DR D_5 by 22.0±3.4%, while the D2-like DR D_2 by 2.5±0.4%, D_3 by 9.0 ±1.8 %, and D_4 by 28.9±3.8 % (**Figure 36D**). Among Th1/Th17 subset, DR D_2 and DR D_3 expressions were lower (***,P<0.001) than all other receptors, while DR D_4 expression was significantly higher (**,P<0.01) compared to the expression of DR D_1.

3.7.2.3. *In vitro* CD4+ T naïve cell commitment

Further expansion of this method included *in vitro* polarisation of isolated naïve CD4+ T cells towards Th1, Th2 and Th17 subpopulation. Preliminary experiments were done on naïve CD4+ T cells obtained from 4 buffy coat samples (**Figure 37**).

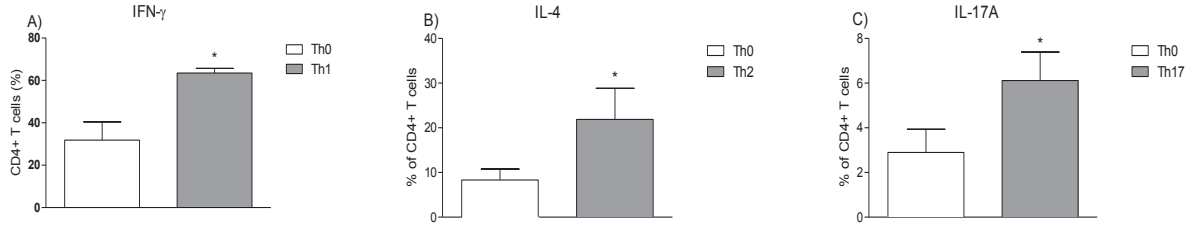


Fig. 37 Polarisation of naïve CD4+ T cells isolated from BC samples (n=4) by *in vitro* priming and expansion for 7 days under specific cultivation conditions, towards Th1 (panel A), Th2 (panel B) and Th17 (panel C). Percentage of CD4+ cells that are producing specific cytokine was assessed by intracellular staining after stimulation with PMA and ionomycin prior to intracellular staining procedure.

Treatment of CD4+ T naïve cells with IL-12+ neutralising anti-IL-4 Ab gave rise to IFN-γ+ cells in comparison to Th0 conditions (63.5±2.2% vs. 31.9±8.6%, P<0.05). In the presence of IL-4 and anti-IFN-γ Ab, expression of IL-4+ cells increased (21.9 ±7.0% vs. 8.3 ±2.5%, P<0.05) compared to Th0 conditions. Percentage of IL-17A+ cells, raised in the presence of IL-1β, IL-6, TGF-β and anti- IFN-γ Ab and anti-IL-4 Ab, and was 6.1±1.3% vs. 2.9 ±1.0% (P<0.05) control conditions.

Obtained preliminary results have confirmed that experimental conditions are likely appropriate to study lineage-specific differentiation of CD4+ T naïve cells and the effects of dopaminergic agents.

3.7.2.4. Dopaminergic modulation of CD4+ T naïve cell commitment

So far, experiments with dopaminergic agents, DA (1μM) and L-DOPA (1μM), did not show any effects on Th1 differentiation route of CD4+ T naïve cells development. Nevertheless, the trend of increase in INF-γ+CD4+ T cells under Th1 polarising conditions was observed in all experimental conditions compared to control, Th0 polarisation conditions (*,P<0.05 and **,P<0.01 vs. Th0 conditions) (**Figure 38**).

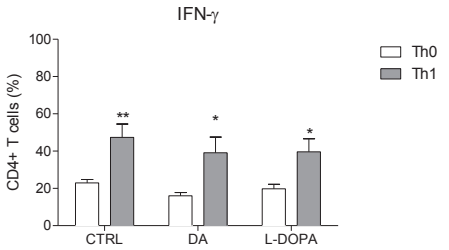


Fig. 38 Effects of DA (1 μM) and L-DOPA (1 μM) treatments on Th1 polarisation route. Naive CD4+ T cells were isolated from peripheral blood of HS (n=4). Polarisation towards Th1 cells was obtained in the presence of IL-12 (10 ng/mL) and anti-IL-4 Ab (10 μg/mL) with addition of IL-2 (10 ng/mL) and anti-CD3 and anti-CD28 Ab. Percentage of CD4+ T cells that are producing specific cytokine was assessed by intracellular staining after stimulation with PMA and ionomycin prior to intracellular staining procedure. Results are presented as mean±SEM of 4 subjects. *, P<0.05 and **, P<0.01 vs. Th0 control conditions.

IL-4+ cell expression was 13.2 ±10.9% in basal conditions, and no significance was observed so far compared to DA (10.5 ±3.6%) or L-DOPA (10.1 ±3.5%) treatments (**Fig. 39**).

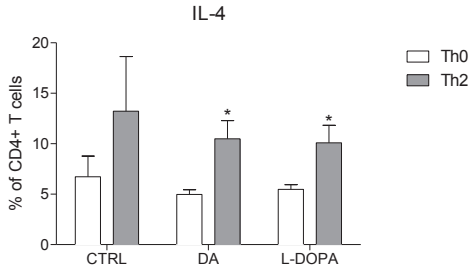


Figure 39 Effects of DA (1 μM) and L-DOPA (1 μM) treatments on Th2 polarisation route. Naive CD4+ T cells were isolated from peripheral blood of HS (n=4). Polarisation towards Th2 cells was obtained in the presence of IL-4 (10 ng/mL) and anti-IFN-γ Ab (10 μg/mL) with addition of IL-2 (10 ng/mL) and anti-CD3 and anti-CD28 Ab. Percentage of CD4+ T cells that are producing specific cytokine was assessed by intracellular staining after stimulation with PMA and ionomycin prior to intracellular staining procedure. Results are presented as mean±SEM of 4 subjects. *, P<0.05 vs. Th0 control conditions.

Basal expression of Th17+ cells was under 5% (3.9 ±1.3%), and the treatments were ineffective DA (3.4 ±1.7%), and L-DOPA (3.9 ±1.6%) (**Fig. 40**).

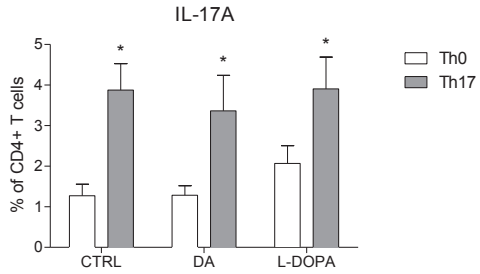


Figure 40 Effects of DA (1 μM) and L-DOPA (1 μM) treatments on Th17 polarisation route. Naive CD4+ T cells were isolated from peripheral blood of HS (n=4). Polarisation towards Th17 cells was obtained in the presence of IL-1β (10 ng/mL) IL-6 (50 ng/mL) TGF-β (5 ng/mL) and neutralising antibodies: anti-INF-γ anti-IL4 Ab (both 10 μg/mL); with addition of IL-2 (2 ng/mL) and anti-CD3 and anti-CD28 Ab. Percentage of CD4+ T cells that were producing specific cytokine was assessed by intracellular staining after stimulation with PMA and ionomycin prior to intracellular staining procedure. Results are presented as mean±SEM of 4 subjects. *, $P<0.05$ vs. Th0 control conditions.

Conclusions, implications and future perspectives

So far, *ex vivo* results have shown the expression of all the five DR on defined T helper subsets: Th1, Th2, Th17 and Th1/Th17, although to a different extent this notion is opening the possibility to explore relationship with the functional status of these cells in more detail.

Conditions needed for the optimal *in vitro* Th1 and Th2 priming are well described in the literature, and are considering addition of IL-12 and IFN-γ or IL-2 and IL-4, respectively (O'Garra et al., 2011; Zhuet al., 2010). Different efforts have been made in identification of human Th17 cells, since the factors regulating mechanisms driving the differentiation of Th17 cell and their function are unclear. Recent report indicates that in humans, RORγt expression in Th17 cells and naïve CD4+ T cells polarisation conditions are induced by IL-1β, and further enhanced by IL-6, and are suppressed by TGF-β and IL-12 (Acosta-Rodriguez et al., 2007). In our hands, optimal *in vitro* condition mimicking the Th17 polarisation route was obtained by IL-1β, IL-6 and TGF-β cytokine combination, and was not suppressed by IL-2. One must have in mind that these results are just preliminary and that this part of investigation is still ongoing in order to increase number of samples.

IV. DISCUSSION AND CONCLUSIONS

Discussion and conclusions

The rationale of the presented research plan included the comprehensive evaluation of the relevance of the dopaminergic modulation of phenotypical and functional characteristics of human CD4+ T cell subsets. For each of the explored CD4+ T cell subsets, the first step was characterisation of the "dopaminergic phenotype" of circulating CD4+ T lymphocyte subsets (*ex vivo*) that served as a base line for the evidence of existence of different DR, followed by the application of *in vitro* functional experiments for each specific subset.

The principal aim of the study was to develop and validate experimental *in vitro* methods devised to investigate the effects of DA agents on the functional responses of CD4+ T lymphocyte and the role of DA pathways in CD4+ T lymphocytes, namely: (i) CD4+ T naïve (Tn), T central memory (T_{CM}) and T effector memory (T_{EM}) cells, and their responses to recall Ag; (ii) CD4+ T regulatory cells (Treg), and their suppressive effects on T effector cells (Teff) and (iii) CD4+ T naïve cells, and their ability to differentiate towards different T helper (Th) lineages (Th1/Th2/Th17).

For each set of experiments and applied and developed methods, preliminary experiments were carried out on buffy coat samples in order to establish the best experimental settings. The obtained results of experiments performed on buffy coat samples helped to develop and validate a method, and standardise culture conditions for following experiments with peripheral blood of healthy subjects. As specified, for each experiment, cells were cultivated in standard conditions, and when it was needed in both resting and activated conditions, anti-CD3 and anti-CD28 Ab were used as activators in order to mimic conditions most similar to the physiological condition. The number of samples for each experiment was obtained from a minimum of 5 subjects, whenever possible.

 Results obtained from the *ex vivo* experiments have shown the expression of all the five DR on a different CD4+ human T lymphocytes subpopulations, to a different extent, opening the possibility to explore in more detail the patterns of DR expression among different CD4+ T cell subsets, and the relationship with the functional status of these cells *in vitro*. The presence of DR on immune cells and dopaminergic pathways demonstrated regulation of crucial human immune functions such as cell apoptosis or proliferation. So far, studies performed with human T cells suggested that both D1-like (DR D_1, DR D_5) and D2-like (DR D_2, DR D_3 and DR D_4) receptor types contribute to the regulation of T cell functions, and various CD4+ T cell subsets have shown to express different arrangements of DR offering different possibilities for

modulation and manipulation of dopaminergic pathways on these cells (Levite et al., 2001; Ilani et al., 2004; Besser at al., 2005; Sarkar et al., 2006; Watanabe et al., 2006; Cosentino et al., 2007; Nakano et al., 2009; Pacheco et al., 2009; Prado et al., 2013; Kustrimovic et al., 2014; Levite, 2016; Kustrimovic et al., 2016). Both human Teff and Treg expressed D1-like and D2-like receptors on their surface (Cosentino et al., 2007).

 The most interesting observation in relation to the presented data was that among human lymphocytes, CD4+CD25+ Treg cells exhibit a peculiar sensitivity to the effects of DA, which is supported by previously published data by our group stating that endogenous DA subserves an autocrine/paracrine regulatory loop (Cosentino et al., 2007). Our data suggested that dopaminergic agents (DA and L-DOPA, 1 μM) have significant effects on suppressive capacity of Treg cells in HS. Furthermore, the results suggest that DA and L-DOPA also have a significant influence on the suppressive capacity of Treg cells in PD-dn, but not in PD-dt. Saunders et al. have also shown impaired abilities of Treg isolated from PD to suppress Teff cell function, which is in the line with our findings, and suggests that Treg disfunction is linked to PD pathobiology (Saunders et al., 2012).

 Dopaminergic modulation of Treg function has been reported to profoundly affect neurodegenerative processes in animal models of neuronal injury (Kipnis et al., 2004; Reynolds et al., 2007). Kipnis et al., 2004 revealed that Treg cell exposure to DA *in vitro*, before their systemic injection into mice (animal model of neuronal survival), reduced their suppressive activity *in vivo*. The same authors found that mouse Treg express functional D1-like receptors and that DA binding can suppress the suppressive activity of Treg on Teff cells (Kipnis et al., 2004). The mechanism, by which Treg suppress metabolic function in effector cells, includes the induction of apoptosis by competition for and deprivation of IL-2 (Stone et al., 2009). In consequtive studies, it was shown that D1-like receptor dependent activation on Treg leads to suppression of their suppressive function, and their ability to suppress Teff cells, and finally Teffs remained activated (Cosentino et al., 2007; Nakano et al., 2008). In this manner DA can indirectly affect Teff cells activation status and functional response. Pharmacological studies performed with different antagonists showed that reserpine-induced suppression of Treg function was due to DR D_1, and not DR D_5 stimulation.

 In the present study, we performed an analysis of Ag-specific CD4+ T cell responses in humans, creating an *in vitro* model in which we used the exposure of PBMC to acute Ag exposure (48h), in particular the common recall Ag, tetanus toxoid. This model was used as a well established system to explore the ability of the peripheral immune system to recognise

TTd Ag and to investigate qualitative memory T cell, more specifically, both responses of naïve and memory CD4+ T cell frequencies evoked by an Ag. Our results provide a complete characterisation of memory CD4 T cell responses against TTd, indicating that the functional heterogeneity of memory CD4 T cells is modulated by the TTd Ag exposure. *In vitro*, TTd treatment increased frequency of CD4+ T memory cells, showing that our method is validated and developed to offer a base line model for application in the further investigation of naïve and memory CD4+ T cell subset, as shown in PD patients in comparison to HS (Kustrimovic et al., 2016). In the future, this model could be applied in similar pathologies where peripheral adaptive immune cell response is involved in an ongoing neuroinflammation process in the CNS, such as MS. To this end, our recently published data examined effects of dopaminergic substitution therapy and dopamine on CD4+ T naïve and memory lymphocytes in PD patients and in healthy subjects, showing that there is excessive association between DR expression on T lymphocytes and motor dysfunction, assessed by UPDRS Part III score (Kustrimovic et al., 2016). Collectively, in total and CD4+ T naïve cells, expression of D1-like DR decreased, while in T memory cells D2-like increase with increasing score (Kustrimovic et al., 2016). In the second part, *in vitro* effects of α-syn were assessed on both CD4+ naïve and memory cells, showing an increase in CD4+ T memory cells, to a possibly different extent in PD patients in comparison to HS, and also to a different extent of DR affection within specific subset patterns (Kustrimovic et al., 2016). This finding further supports the involvement of peripheral adaptive immunity in PD. Interestingly, so far we did not observe any effects of SKF 38393 or pramipexole on the frequency of naïve/memory T cells (Kustrimovic et al., 2016).

 Nakano et al., 2008 studied the effects of dopaminergic analogues on the interaction between monocyte-derived DCs (MO-DCs) and allogeneic, naïve CD4+ T cells from healthy volunteers and revealed that D1-like receptor blockade reduced, and D2-like receptor blockade increased IL-17 secretion by the T cells, showing that release of DA from MO-DCs, causes Th2 differentiation and polarisation (Nakano et al., 2009). Collectively, the conclusion was that MO-DCs contain DA that can release upon Ag-specific interaction with naïve CD4+ T cells, and that released DA can induce Th2 polarisation. Furthermore, it has been revealed that in DCs from DR D_5 knockout mice, LPS-induced IL-23 and IL-12 cytokines production was impaired, and consequently activation and proliferation of Ag-specific CD4+ T cells was attenuated (Prado et al., 2012). Further *in vivo* studies revealed the role of DR D_5 in murine model of MS, showing significant reduction of the percentage of Th17 cells infiltrating the CNS, and no effects on Th1 cell subsets (Prado et al., 2012; Pacheco et al., 2014). Another

interesting *in vivo* study addressed the role of DA through its D1-like receptor in cutanous immune response in Th subsets polarisation, where it was shown that DA promote Th2-cell differentiation and mast cell degranuation, without effecting Th1 cell function (Mori et al., 2013). Considering this background, our goal was to develop and validate an *in vitro* method for the polarisation of human naïve CD4+ T cell and their commitment. Different efforts have been made in identification of human Th17 cells, since the factors regulating mechanisms driving the differentiation of Th17 cells and their functions are unclear. A recent report indicates that in humans, RORγt expression in Th17 cells and naïve CD4+ T cells polarisation conditions are induced by IL-1β, and further enhanced by IL-6, and on the other hand are suppressed by TGF-β, IL-2 and IL-12 (Acosta-Rodriguez et al., 2007; La Cava et al., 2008). In our experience, optimal *in vitro* condition mimicking the Th17 polarisation route was obtained by IL-1β, IL-6 and TGF-β cytokine combination, and was not suppressed by IL-2. So far, our *in vitro* data have shown no significant effect of DA or L-DOPA on the polarisation process of defined T helper subsets: Th1, Th2 and Th17. Nevertheless, it must be emphasised that our results are very preliminary, and this component of the investigation is still ongoing in order to increase the number of samples - so further *in vitro* findings are necessary in order to carefully add knowledge to this complex issue.

 It has been shown that peripheral T lymphocytes are more prone to apoptotic process, thus decrease number of CD4 + T cells may be used to follow up progression of the PD disease. So far, our *in vitro* results suggest that dopaminergic agents show that DA and L-DOPA concentration in range (1-50 μM) did not have any significant influence on CD4+ T cell viability, while much higher concentrations of both agens (100 μM) induced non-specific effects in T cells, which were likely toxic and killed them. Thus, very high concentrations can't be used for induction of specific and beneficial DA effects, and should also be avoided in further experimental procedures. Our data is rather consistent with a Bergquist et al., 1997 study that claims that B cells, and not T cells, are sensitive to the DA concentration (10 nM), than later studies by Besser et al., 2005 and Levite, 2012 claiming that DA induce direct and very potent effects on T cells at low concentrations (of 10 nM). Bergquist et al., 1997 have also pointed out that, at high concentration, dopamine has negative effects on PBMC by elevating the synthesis of the apoptotic markers (Bcl-2/Bax and Fas/FasL) and finaly elevating the level of apoptosis (by ~ 2.8-fold) (Bergquist et al., 1997).

 Most of the studies demonstrate DA as a negative regulator of T cell proliferation in both healthy subjects and pathological conditions (Saha et al., 2001). Bergquist et al, 1997 also

revealed that DA significantly inhibited the proliferation and production of cytokines (IFN-γ and IL-4) at the very high concentration (10 –100 μM) by PBMC in response to mitogens (ConA and PWM) (Bergquist et al., 1997). In 2000, the same group confirmed that these high concentrations of DA (10 –100 μM) also inhibited the LPS-induced binding of NF-κB to the promoter of TNF-α (thus inhibiting production of this pro-inflammatory cytokine), and that lower concentrations of DA (1 μM– 10 nM) did not induce such inhibitory effects (Bergquist et al., 2000). Ghosh et al., 2003 revealed that human T cells *in vitro* stimulated with anti-CD3 and treated with 3-5 ng/mL DA significantly inhibited proliferation of these cells. A pharmacological study with antagonists revealed that DA inhibited T cells activated with anti-CD3 Ab, through the DR D_2 and DR D_3. So far, our data did not show any significant effect of dopaminergic agents on CD4+ T cell proliferation. However, the concentrations of DA used in those *in vitro* experiments were similar to those that we applied, and also to the physiological plasma level of this neurotransmitter, but the method to obtained proliferation of T cells was assessed by incorporation of 3H thymidine, and after a 3-day culture, while our method involved the use of a CPD marker which was measured after 4 days of cell culture.

The molecular and pharmacological heterogeneity of DR potentially represents an opportunity to develop targeted immunomodulating strategies. The main working hypothesis was that different subsets expressed different patterns of DR, which may offer possibilities for immuno-pharmacological manipulations, and the possibility to repurpose dopaminergic agents currently used in the pharmacotherapy of various diseases.

Recently, (Chen et al., 2013; Gonzalez et al., 2013) demonstrated that genetic deficiency of the highest affinity DR, the DR D3, in murine model of PD attenuates neuroinflammation and subsequent neurodegeneration, induced by acute intoxication with MPTP. Based on these findings Elgueta et al., 2017 show that treatment of MPTP-intoxicated mice with DR D3-selective antagonist, PG01037 attenuated loss of dopaminergic neurons in the nigrostriatal pathway and resulted in significant improvement of locomotor impairment. Further analyses of PG01037 therapeutic potential show that it could be mediated by the induction of an intermediate M1/M2-like phenotype in astrocytes, which could transmit an anti-inflammatory signal to microglial cells (Elgueta et al., 2017). Taken together, they concluded that these findings can contribute to a better knowledge of the physiopathology of PD, but they also provide the clues for new therapeutic approaches for the treatment of this neurodegenerative disorder.

In certain pathophysiological conditions, a dysfunction of dopaminergic system might be expressed by: (i) impaired DA levels in CNS and/or at the periphery, (ii) abnormal DA production, (iii) abnormal DR expression and/or response to DA, and possibly (iv) impaired dopaminergic signalling, hence testing different concentrations of DA analogues *in vitro* that potentially affect different T cell subsets that are expressing different patterns of DR might even vary in healthy individuals and cells obtained from individuals that are suffering from certain pathological conditions (in the first line model of Parkinson's disease).

An understanding of the distinct families of molecules present in different T cell subsets actually provides the tools for distinguishing these cell types in both diagnosis and therapy, as well as understanding the molecular basis for T cell functions, such as: proliferation, apoptosis, cytokine production, suppressive, anti-inflammatory or neuroprotective functions, etc. These are of great importance as a prognostic marker if the association between DR expression on T lymphocytes is shown to be associated with a disease's symptoms or severity during the course of lifetime (Kustrimovic et al., 2016).

Understanding functional modifications occurring in peripheral immunity in healthy individuals, but also during different pathological processes, and above all the contribution of dopaminergic pathways in different dopamine-related diseases conditions will lead to a better understanding of physiopathology, and will provide a better exploitation of currently available dopaminergic drugs, possibly also to the development of innovative pharmacological approaches.

Dopamine-induced effects on T cells are very sensitive, and the most important factors that are dictating the outcomes could be attributed to chosen activators and activation conditions of T cells, applied concentrations of DA or agonist, specific subset of CD4+ T cells that DA interacts with, and finally, the particular type of DR that will be activated by DA.

Table 17. Summarising experimental methods, results and effects of dopaminergic agonist

Assay	**DR expression results**	**Effects of dopaminergic agonist (all are *in vitro* methods)**					
In vitro apoptotic assay of CD4+ T cells cultivated as PBMC (48h) (Flow cytometry)	High expression of DR in apoptotic cells and stimulation-induced ↑ % of all examined DR	Effects on CD4+ T cell viability					
		DA (only 100 μM) +++		**L-DOPA** (only 100 μM) +++		**Pramipexole** (0,1-100 μM) –	
In vitro proliferating assay of CD4+ T cells cultivated as PBMC (96h) (Flow cytometry)	Both proliferating and non-proliferating CD4+ T lymphocytes expressed all the five DR Proliferating cells express higher percentages of DR	Effects on CD4+ T cell proliferation					
		DA (1-50 μM) –		**L-DOPA** (1-50 μM) –		**Pramipexole** (0,1-100 μM) –	
Functional responses of T naïve/memory subset (48h) (Flow cytometry)	<u>*Ex vivo*</u>*:* 1. the expression of all the five DR on human T naïve, T_{CM} and T_{EM} cells 2. T naïve cells express higher levels of DR than T_{CM} or T_{EM} subsets <u>*In vitro*</u>*:* developed method to test function of CD4+ T cell memory towards recall Ag	Effects on a frequencies of human T naïve, T_{CM} and T_{EM} cells					
		SKF 38393 (1 μM) –			**Pramipexole** (1 μM) –		
Treg cell functions (96h) (Flow cytometry and ELISA assay)	<u>*Ex vivo*</u>*:* conventional Treg ($CD4^+CD25^{high}CD127^{low}$) expressed all	Effects on Treg suppressive capacity in Teff/Treg co-culture (1:1)					
		HS group		PD-dn group		PD-dt group	
		DA (1 μM) +	L-DOPA (1 μM) +	DA (1 μM) +	L-DOPA (1 μM) +	DA (1 μM) –	L-DOPA (1 μM) –

<table>
<tr><td rowspan="6"></td><td rowspan="6">five DRs

<u>In vitro</u>: suppressive Treg cell capacity in co-culture with Teff cells in all groups</td><td colspan="4">Effects on Teff cytokine production in Teff/Treg co-culture (1:1) in HS</td></tr>
<tr><td colspan="2">INF-γ</td><td colspan="2">TNF-α</td></tr>
<tr><td>SKF 38393 (1 μM) —</td><td>Pramipexole (1 μM) —</td><td>SKF 38393 (1 μM) —</td><td>Pramipexole (1 μM) —</td></tr>
<tr><td colspan="4">Effects on Treg cytokine production (cultivated alone) in HS</td></tr>
<tr><td colspan="2">IL-10</td><td colspan="2">TGF-β</td></tr>
<tr><td>SKF 38393 (1 μM) —</td><td>Pramipexole (1 μM) —</td><td>SKF 38393 (1 μM) —</td><td>Pramipexole (1 μM) —</td></tr>
<tr><td>Comitmment of naïve T cells (7 days) (Flow cytometry)</td><td><u>Ex vivo</u>: expression of all the five DR on defined T helper subsets: Th1, Th2, Th17 and Th1/Th17
<u>In vitro</u>: set up of optimal condition mimicking for each specific polarisation route (Th1, Th2 and Th17)</td><td colspan="2">Effects on a lineage-specific differentiation of CD4+ T naïve cells</td><td colspan="2"></td></tr>
<tr><td colspan="2">DA (1 μM)
–</td><td colspan="2">L-DOPA (1 μM)
–</td></tr>
</table>

Summary of the results:

1. Results have shown the expression of all the five DR on human CD3+CD4+ T lymphocytes to a different extent. Relative prevalence of D1-like over D2-like DR in CD3+CD4+ T cells may imply that effects of DA on these cells are mediated mainly through D1-like DR.

2. High expression of DR in cells undergoing apoptotic process and stimulation-induced DR increase in cultured CD4+ T cells suggests the involvement of DR in the apoptotic process, and further supports the involvement of DR in the functional regulation of activated cells, requiring further investigations to assess the role of DR subtypes in the modulation of specific responses.

3. Possibly, in resting cells dopaminergic pathways participate mainly in apoptotic processes (as suggested by the high proportion of apoptotic cells expressing DR), while their functional relevance increases in activated cells (in line with stimulation-induced upregulation of DR in viable cells).

4. *Ex vivo* data have shown that proliferating and non-proliferating CD4+ T lymphocytes expressed all the five DR, in different expression levels. Interestingly, a trend of proliferating cells expressing DR in higher percentages was shown. Tested concentrations of dopaminergic agonists have not shown any major effects on proliferation of CD4+ T cells.

5. All the five DR were shown to be expressed *ex vivo* on each of the following human T naïve, T_{CM} and T_{EM} subsets. T naïve cells expressed higher levels of DR than T_{CM} or T_{EM} subsets.

6. We have developed and validated an *in vitro* method to test the functional response and balance of frequency of naïve/memory CD4+ T cell in response to a common recall Ag (TTd). Furthermore, treatments with SKF 38393 and pramipexole did not show any effect on the frequencies of human T naïve, T_{CM} or T_{EM} cells.

7. Obtained results from *ex vivo* experiments have shown that conventional Treg ($CD4^{+}CD25^{high}CD127^{low}$) expressed all five DR.

8. Presented *in vitro*, a method was developed and validated through numerous pilot experiments on buffy coat samples in which suppressive Treg cell capacity was shown. Experiments done with fresh blood from HS have shown the same inhibitory capacity of Treg cells.

9. Effects of DA and L-DOPA treatments suppress Treg suppressive capacity in HS, since Teff cell proliferation is restored in comparison to co-culture control conditions (Treg + Teff cells, 1:1). The same effect of DA and L-DOPA as in HS, was observed in PD-dn, but not in

in PD-dt group, suggesting that dopaminergic agents influence the suppressive capacity of Treg cells in HS and PD-dn, but not in PD-dt.

10. Treg cells suppressed the production of IFN-γ and TNF-α from Teff cells. Treatment with SKF 38393 and pramipexole did not influence suppressive capacity of Treg. Neither IL-10 nor TGF-β production by Treg cells was influenced by SKF 38393 and pramipexole treatments.

11. *Ex vivo* data have shown the expression of all the five DR on defined T helper subsets: Th1, Th2, Th17 and Th1/Th17; to a different extent this notion is opening the possibility of exploring in more detail the relationship with the functional status of these cells.

12. We have developed and validated an *in vitro* method optimal condition mimicking specific polarisation routes (Th1/Th2/Th17). Obtained preliminary results have confirmed that experimental conditions are likely appropriate to study lineage-specific differentiation of CD4+ T naïve cells and the effects of dopaminergic agents.

13. So far, experiments with dopaminergic agents (DA and L-DOPA) did not show any effects on Th1/Th2 or Th17 differentiation route of CD4+ T naïve cells development. These results are very preliminary and are part of an ongoing investigation, in order to increase number of samples.

The results of the presented data have shown that CD4+ T cells play a relevant role in the PD pathological condition, and in particular intrinsic dopaminergic pathways in Treg cells may represent a target for pharmacotherapeutic intervention. Further *in vitro* findings are still necessary in order to add knowledge about the sensitivity of CD4+ T cell to DA and other dopaminergic agents presently in clinical use.

Furthermore, it seems quite inconsistent that, to the best of our knowledge, no studies regarding possible immunomodulating effects of dopaminergic agents currently employed for the symptomatic treatment of PD have so far been explored. This is clearly another large area where thorough research may provide unpredictable findings, possibily contributing to a better understanding of the mechanism(s) of action of dopaminergic agents currently used in pharmacotherapy.

In both directions of our research approach (*ex vivo* examination of CD4+ T cell phenotype and *in vitro* models develop for characterisation and testing different CD4+ T cell subsets functions), by combining this two diverse experimental approaches, we fulfilled the expectations to generate and validate new methods to better describe the whole picture of CD4+ T cell subsets function and phenotype. Of course, one must consider that models have

limitations and that these models need further validation, but they could be a good starting point to uncover dopaminergic pathways in healthy subjects, as well as alterations present in dopamine-related diseases.

For the comparison of immune responses *in vitro* and *in vivo*, it is fundamental to understand how so far described *in vitro* functional responses of CD4+ T cells can be translated to the *in vivo* system. Careful analysis of *in vitro* data obtained so far should be translated into the *in vivo* system, and obtained responses to different concentrations of dopaminergic agonist *in vitro* should be used for predicting the effects of dopaminergic agents in biological systems, providing a better understanding of dopamine modulation of different CD4+ T cell subsets *in vivo*.

V. FUTURE PERSPECTIVES

Despite all discrepancies of the *in vitro* data experiments that different studies bring to us, our general knowledge about dopaminergic effects on T cells is growing. Some essential experiments are missing in this specific area, and to this end should bring new findings to the genuine *in vitro* and *in vivo* predictions, thus some questions remains opened.

In my opinion, the experiments that need to be performed in the future are listed below:

(i) Interestingly, the expression of DR on proliferating and, at the same time non-proliferating cells have shown a trend of higher expression of all DRs on proliferating in comparison to non-proliferating CD4+ T cells. These data are part of preliminary experiments (n=3-4), so further experiments are needed to examine this specific, possible effect of dopaminergic agonist on CD4+ T proliferating cells.

(ii) As dopamine is the physiological neurotransmitter, its own effects are more precious than those of artificial agonists (such as SKF 3839), so I propose that, all the future experiments should always be started from the basic settlement (e.g. knowing that DA concentration of 10 nM will give a specific physiological response of CD4+ T cell in our experimental settings and conditions.

(iii) Testing the effects of dopamine agonist (in first instance L-DOPA and pramipexole) that are currently used in the clinical practice, and comparing their effects to the one of dopamine, will add new insights to the body of growing evidence and will clear the direction for new therapeutical approaches.

(iv) Dopamine-induced effect which was confirmed/shown so far should be completed with sufficient experimentation showing that specific effects are exerted by specific DR subtypes on the basis of an antagonists study using highly selective DR antagonist.

VI. Appendix 1

Attached file 1: ***Dopaminergic receptors on CD4+ T naive and memory lymphocytes correlate with motor impairment in patients with Parkinson's disease***. *Kustrimovic N, Rasini E, Bombelli R,* ***Aleksic I****, Blandini F, Comi C, Mauri M, Minafra B, Riboldazzi G, Sanchez-Guajardo V, Marino F, Cosentino M. Sci Rep. 2016;6:33738.*

Abstract

My contribution to the presented manuscript entitled “Dopaminergic receptors on CD4+ T naïve and memory lymphocytes correlate with motor impairment in patients with Parkinson’s disease” published online on 22 September, 2016 (doi: 10.1038/srep33738) in Scientific Reports, involved developing a research program on the dopaminergic modulation of CD4+ T lymphocytes as part of my work for the PhD Course in Clinical and Experimental Medicine and Medical Humanities, University of Insubria (XXIX cycle).

As the extent of the experimental work done so far is beyond the scope of my thesis and manuscript (listed below), it is expected that additional manuscripts will be published in the nearest future.

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Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease

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Parkinson's disease (PD) is characterized by loss of dopaminergic neurons in substantia nigra pars compacta, α-synuclein (α-syn)-rich intraneuronal inclusions (Lewy bodies), and microglial activation. Emerging evidence suggests that CD4+ T lymphocytes contribute to neuroinflammation in PD. Since the mainstay of PD treatment is dopaminergic substitution therapy and dopamine is an established transmitter connecting nervous and immune systems, we examined CD4+ T naive and memory lymphocytes in PD patients and in healthy subjects (HS), with specific regard to dopaminergic receptor (DR) expression. In addition, the *in vitro* effects of α-syn were assessed on CD4+ T naive and memory cells. Results showed extensive association between DR expression in T lymphocytes and motor dysfunction, as assessed by UPDRS Part III score. In total and CD4+ T naive cells expression of D_1-like DR decrease, while in T memory cells D_2-like DR increase with increasing score. *In vitro*, α-syn increased CD4+ T memory cells, possibly to a different extent in PD patients and in HS, and affected DR expression with cell subset-specific patterns. The present results support the involvement of peripheral adaptive immunity in PD, and may contribute to develop novel immunotherapies for PD, as well as to better use of current dopaminergic antiparkinson drugs.

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting an estimated 7 to 10 million people worldwide and resulting in both motor and cognitive disturbances[1–3]. The main neuropathological features of PD are the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, the appearance of intraneuronal inclusions called Lewy bodies, and the occurrence of microglial activation. Microglial cells in particular are key players in neuroinflammation and neurodegeneration, and peripheral adaptive immunity has been recently proposed as a major determinant in the regulation of microglial function during neurodegenerative disease[4–7].

Over the last 15 years, several studies described the occurrence of peculiar modifications of peripheral immunity in PD, such as decreased CD4+ /CD8+ T-cell ratios, fewer CD4+ CD25+ T cells and increased ratios of interferon (IFN)-γ-producing to interleukin (IL)-4-producing T cells[8], as well as decreased CD4+ T lymphocytes and CD19+ B cells[9,10]. Both CD8+ and CD4+ T cells (but not B cells) were identified in the brain in both postmortem human PD specimens and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, and evidence obtained in the animal model pointed to CD4+ T cells as main determinants of T cell-mediated dopaminergic cell death[11]. Remarkably, Saunders *et al.*[12] recently reported that PD patients have increased effector/memory CD4+ T cells and decreased CD31+ and α4β7+ CD4+ T cells, which are associated with progressive motor dysfunction, suggesting a direct relationship between chronic immune stimulation and

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	HS	PD	P
n	28	53	
Gender (F/M)	11/17	17/36	0.625
Age (years)	68.8 ± 8.1	69.7 ± 9.5	0.545[a]
UPDRS Part II (score)[b]		7.2 ± 3.5	
1–10 (n)		35	
11–20 (n)		6	
UPDRS Part III (score)[c]		14.0 ± 6.0	
1–10 (n)		18	
11–20 (n)		27	
>20 (n)		7	
H&Y scale (stage)		1.6 ± 0.6	
1.0 (n)		21	
1.5–2.0 (n)		25	
2.5–3.0 (n)		4	

Table 1. Comparison between HS and PD patients. Data are means ± SD unless otherwise indicated. **Notes:** a = by Mann-Whitney *U* test; b = data missing for 1 PD-dn and 11 PD-dt; c = data missing for 1 PD-dn patient.

PD neuropathology and disease severity, as well as strengthening the idea that in PD the lead actors among adaptive immune system cells are CD4+ T lymphocytes.

No therapies are currently available for the neurodegenerative processes underlying PD, and symptomatic treatments rely on the dopamine (DA) precursor L-DOPA as well on dopaminergic agonists and on other indirect dopaminergic agents. Dopaminergic drugs relieve to some extent the loss of brain dopaminergic neurons occurring in PD, although, as disease progresses, both nonmotor and motor symptoms emerge that are resistant to dopaminergic medications[13]. Interestingly DA, besides its role as brain neurotransmitter, is also an established transmitter connecting the nervous and the immune system, as well as immune cells and peripheral tissues[14–16]. DA affects both innate and adaptive immune system cells, and immune cells produce DA, which may act as autocrine/paracrine mediator on immune cells themselves and on neighboring cells[17–22]. Among T lymphocytes, CD4+ T cells may represent a major target for DA. DA subserves an (auto)inhibitory loop in human CD4+ $CD25^{high}$ regulatory T lymphocytes, a specialized T cell subset playing a key role in the control of immune homeostasis[23], and dendritic cells-derived DA affects the differentiation of naive CD4+ T cells[24]. The effects of DA are exerted through 5 different dopaminergic receptors (DR) grouped into the D_1-like (D_1 and D_5) and the D_2-like (D_2, D_3 and D_4)[25,26]. Immune cells express all DR and in particular CD4+ T cells express both D_1-like and D_2-like DR[23,27], with CD4+ naive T cells expressing more D_1-like than D_2-like DR, which on the contrary are increased in memory T cells[28]. Despite extensive evidence supporting the involvement of CD4+ T cells (and in particular of memory T cells) in PD pathogenesis and progression[8,10–12], no information exists on DR expression on CD4+ T cells in PD patients. Nonetheless, a recent investigation in the MPTP mouse model of PD suggested that DR D_3 expressed on CD4+ T cells are critical for T cell-dependent microglial activation, which finally results in neurodegeneration[29].

The present study, examined CD4+ T cells, as well as naive and memory CD4+ T cell subsets, in PD patients and in healthy subjects, with specific regard to DR expression. Correlations of CD4+ T cell and cell subsets, as well as their respective expression of DR, were investigated with demographic and clinical features of the subjects. Patients on dopaminergic agents were compared with recently diagnosed patients who never received dopaminergic medications. In addition, in preliminary experiments the *in vitro* effects of α-synuclein (α-syn) were assessed on CD4+ T naive and memory cells. α-syn is a protein expressed in brain and in peripheral tissues. It is the main component of Lewy bodies and it may contribute to the pathogenesis of PD through different concurrent mechanisms, including direct activation of microglial cells as well as possibly by acting as an antigen itself, triggering the adaptive immune response in the periphery[30–32]. For these reasons, the effects of α-syn on CD4+ T naive and memory cells were also compared with those of a common recall antigen like tetanus toxoid (TTd).

Results

Subjects. We enrolled 53 PD patients and 28 HS (Table 1). Patients comprised 16 subjects who had been never treated with antiparkinson drugs before enrollment, and were therefore drug naive (PD-dn). In comparison to patients on antiparkinson drugs (PD-dt), PD-dn were younger and had on average lower H&Y stage, as well as UPDRS Part III score (Table 2). Plasma dopamine was 3.2 ± 5.7 nM in HS, 2.7 ± 3.3 nM in PD-dn patients (P = 0.779 vs. HS), and 8.0 ± 9.8 nM in PD-dt patients (P = 0.003 vs. HS).

Complete blood counts of PD patients and HS were all within normal limits (Table 3), however PD patients had less total lymphocytes, both in terms of absolute number (on average, about 17% less) and as percentage of white blood cells (−3.5%).

Complete blood count did not differ between PD-dn and PD-dt patients (Table 4), with the only exception of percentage of basophils, which were slightly lower in PD-dt.

T lymphocytes and CD4+ T naive and memory subsets. Reduction of lymphocytes in PD patients was accounted for essentially by T cells (Table 3). In particular, CD4+ T cells were about 21% less in PD patients

	PD-dn	PD-dt	P
n	16	37	
Gender (F/M)	7/9	10/27	0.337
Age (years)	65.6 ± 10.8	71.4 ± 8.4	**0.041**[a]
UPDRS Part II (score)	6.9 ± 4.7[b]	7.5 ± 2.6[b]	0.606[d]
UPDRS Part III (score)	9.9 ± 5.9[d]	15.6 ± 5.3	**0.002**[a]
H&Y scale (stage)	1.2 ± 0.4	1.8 ± 0.5	**<0.001**[a]
LED (mg/day)		459.4 ± 247.4	
Drugs			
L-DOPA (n)		27[e]	
DA agonists (n)		27[f]	
pramipexole (n)		19	
ropinirole (n)		4	
rotigotine (n)		4	
Rasagiline (n)		19	

Table 2. Comparison between PD-dn and PD-dt. Data are means ± SD unless otherwise indicated. **Notes:** a = by Student's *t* test; b = data missing for 1 PD-dn and 11 PD-dt; c = data missing for 1 PD-dn patient; d = by Mann–Whitney *U* test; e = 8 taking L-DOPA alone, and 19 taking L-DOPA+ DA agents; f = 10 taking DA agonists alone (4) or with rasagiline (6), and 17 taking DA agonists+ L-DOPA, without (6) or with rasagiline (11).

	units	range	HS	PD	P
RBC	10^{12}/L	4.50–6.00	4.9 ± 0.4	4.7 ± 0.4	0.100[a]
hemoglobin	g/dL	13.0–17.5	14.4 ± 1.1	14.2 ± 1.2	0.472[a]
hematocrit	%	42.0–54.0	43.4 ± 3.6	42.6 ± 3.3	0.439[a]
MCH	pg	27.0–32.0	29.8 ± 1.8	30.1 ± 2.3	0.253[a]
MCHC	g/dL	32.0–36.0	33.7 ± 2.8	33.4 ± 1.7	0.780[a]
Platelets	10^9/L	150–450	238.2 ± 50.1	242.8 ± 69.1	0.853[a]
WBC	10^9/L	4.30–11.00	6.9 ± 1.6	6.6 ± 1.7	0.310[a]
lymphocytes	10^9/L	1.50–5.50	2.12 ± 0.73	1.76 ± 0.48	**0.027**[b]
	%	10.0–45.0	30.8 ± 7.4	27.3 ± 6.8	**0.050**[a]
monocytes	10^9/L	0.2–1.1	0.5 ± 0.1	0.5 ± 0.2	0.620[b]
	%	2.0–12.0	7.5 ± 1.9	7.5 ± 2.2	0.991[b]
neutrophils	10^9/L	1.50–5.50	4.1 ± 1.1	4.1 ± 1.4	0.781[b]
	%	40.0–80.0	58.9 ± 8.0	62.4 ± 7.2	0.120[a]
eosinophils	10^9/L	0.0–0.8	0.2 ± 0.3	0.2 ± 0.1	0.679[a]
	%	0.0–7.0	2.3 ± 1.6	2.3 ± 1.6	0.838[a]
basophils	10^9/L	0.0–0.2	0.0 ± 0.0	0.0 ± 0.0	0.793[a]
	%	0.0–1.6	0.5 ± 0.2	0.5 ± 0.4	0.469[a]
lymphocyte subsets					
CD3+	10^6/L		1515.0 ± 651.7	1241.0 ± 366.2	0.109[a]
	% of total lymph		70.8 ± 9.4	69.6 ± 8.7	0.549[a]
CD4+	10^6/L		1012.0 ± 439.1	797.4 ± 263.3	**0.035**[a]
	% of CD3+		67.3 ± 10.8	64.7 ± 10.8	0.419[a]
CD8+	10^6/L		334.0 ± 162.8	274.9 ± 151.5	0.134[b]
	% of CD3+		23.4 ± 9.3	22.0 ± 9.7	0.562[b]
CD4+ /CD8+	ratio		3.8 ± 2.9	4.0 ± 3.4	0.979[a]

Table 3. Complete blood count, comparison between HS and PD patients. Data are means ± SD unless otherwise indicated. Notes: Abbreviations: RBC, red blood cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells. a = by Mann–Whitney *U* test; b = by Student's *t* test.

in comparison to HS, even if the CD4+ /CD8+ ratio did not change in patients, likely due the overall reduction of T lymphocytes. T lymphocyte subsets did not differ in PD-dn and PD-dt patients in terms of absolute counts, even if in PD-dn patients CD3+ T cells as percentage of total lymphocytes were less (−5.4%) and CD4+ T cells as percentage of CD3+ T cells were more (+6.4%) (Table 4).

To identify CD4+ T cell subsets, the gating strategy included the use of a biparametric dot plot CD45RA vs. CCR7, that allowed the identification of T naive (CD3+ CD4+ CD45RA+ CCR7+), T_{CM} (CD3+ CD4+

	units	range	PD-dn	PD-dt	P
RBC	10^{12}/L	4.50–6.00	4.8 ± 0.4	4.7 ± 0.4	0.250[a]
hemoglobin	g/dL	13.0–17.5	14.4 ± 0.9	14.1 ± 1.3	0.456[a]
hematocrit	%	42.0–54.0	43.3 ± 2.9	42.2 ± 3.4	0.324[a]
MCH	pg	27.0–32.0	29.9 ± 1.7	30.2 ± 2.5	0.317[a]
MCHC	g/dL	32.0–36.0	33.3 ± 1.6	33.5 ± 1.8	0.847[a]
Platelets	10^9/L	150–450	239.3 ± 48.8	244.4 ± 76.8	0.859[a]
WBC	10^9/L	4.30–11.00	6.2 ± 1.6	6.7 ± 1.7	0.400[a]
lymphocytes	10^9/L	1.50–5.50	1.63 ± 0.41	1.81 ± 0.50	0.222[b]
	%	10.0–45.0	27.1 ± 6.6	27.5 ± 6.9	0.841[b]
monocytes	10^9/L	0.2–1.1	0.5 ± 0.2	0.5 ± 0.1	0.750[a]
	%	2.0–12.0	8.0 ± 2.6	7.2 ± 2.0	0.221[b]
neutrophils	10^9/L	1.50–5.50	3.9 ± 1.4	4.2 ± 1.4	0.315[a]
	%	40.0–80.0	61.6 ± 7.7	62.7 ± 7.1	0.532[a]
eosinophils	10^9/L	0.0–0.8	0.2 ± 0.1	0.2 ± 0.1	0.668[a]
	%	0.0–7.0	2.6 ± 2.0	2.2 ± 1.5	0.581[a]
basophils	10^9/L	0.0–0.2	0.0 ± 0.0	0.0 ± 0.0	0.378[a]
	%	0.0–1.6	0.7 ± 0.4	0.4 ± 0.3	**0.019**[a]
lymphocyte subsets					
CD3+	10^6/L		1115.9 ± 302.0	1295.0 ± 381.8	0.143[a]
	% of total lymph		65.9 ± 9.5	71.3 ± 7.9	**0.037**[b]
CD4+	10^6/L		757.3 ± 208.3	814.8 ± 284.7	0.672[a]
	% of CD3+		69.2 ± 12.2	62.8 ± 9.6	**0.046**[b]
CD8+	10^6/L		220.7 ± 134.6	299.7 ± 154.1	0.071[a]
	% of CD3+		19.5 ± 9.4	23.1 ± 9.8	0.220[b]
CD4+ /CD8+	ratio		5.3 ± 5.3	3.4 ± 1.8	0.053[b]

Table 4. Complete blood count, comparison between PD-dn and PD-dt. Data are means ± SD unless otherwise indicated. Notes: Abbreviations: RBC, red blood cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells. a = by Mann–Whitney *U* test; b = by Student's *t* test.

CD45RA-CCR7+), and T_{EM} (CD3+ CD4+ CD45RA-CCR7-)[28]. Among CD4+ T cells, PD patients showed decreased number of T naive cells (Fig. 1a). CD4+ T_{CM} and T_{EM} absolute numbers were not different in PD patients and in HS, however T_{EM} as percentage of total CD4+ T cells were increased in PD patients (+3.3% on average) (Fig. 1c). PD-dn and PD-dt patients did not differ in either absolute number or percentage of T naive, T_{CM} and T_{EM} (Fig. 1b,d).

DR expression on CD4+ T cells. DR expression was assessed at both mRNA level (in total CD4+ T cells) and membrane protein level (in total CD4+ T cells and in T naive and memory subsets). In comparison to cells from HS, CD4+ T cells from PD patients had lower mRNA levels of the D_1-like DR D_5 and of the D_2-like DR D_3 and D_4, and higher mRNA levels of the D_2-like DR D_3, while D_1-like DR D_1 mRNA levels were not different between cells from PD patients and HS (Fig. 2a). Interestingly, both the D_1-like DR D_5 and the D_2-like DR D_2 mRNA levels were lower in cells from PD-dn patients compared to cells from PD-dt patients (Fig. 2b).

Flow cytometric analysis of DR expression on CD4+ T cells revealed that in PD patients DR D_5+ CD4+ T cells were decreased in terms of both absolute number and percentage of total CD4+ T cells (Fig. 2c,e), and that PD-dn patients had lower number and percentage of DR D_1+ and D_3+ CD4+ T cells in comparison to PD-dt subjects (Fig. 2d,f).

DR expression on CD4+ T naive and memory cells. In comparison to HS, PD patients had less D_1-like DR D_1+ and D_5+, as well as less D_2-like DR D_2+ and D_3+ T naive cells, both in terms of absolute numbers and, for DR D_3+ cells, also of percentage of total CD4+ T cells (Fig. 3a,c). PD-dn patients had less DR D_1+ T naive cells in comparison to PD-dt patients, in terms of both absolute numbers and percentage of total CD4+ T cells (Fig. 3b,d).

No difference was found in DR expression in T_{CM} and T_{EM} between PD patients and HS, except for DR D_3+ T_{CM} which were higher in PD patients in terms of percentage of total CD4+ T cells, and for DR D_4+ T_{EM} which were higher in PD patients in terms of both absolute numbers and percentage of total CD4+ T cells (see Supplementary Fig. S1 and S2). DR expression on T_{CM} or T_{EM} did not differ between PD-dn and PD-dt patients (see Supplementary Fig. S1 and S2).

Correlations between CD4+ T cells and demographic and clinical features of HS and PD patients. Age exerts major effects on lymphocyte function[33], and in particular T naive cells may be reduced in elderly subjects[34]. In agreement with these findings, in HS both the absolute number as well as the percentage

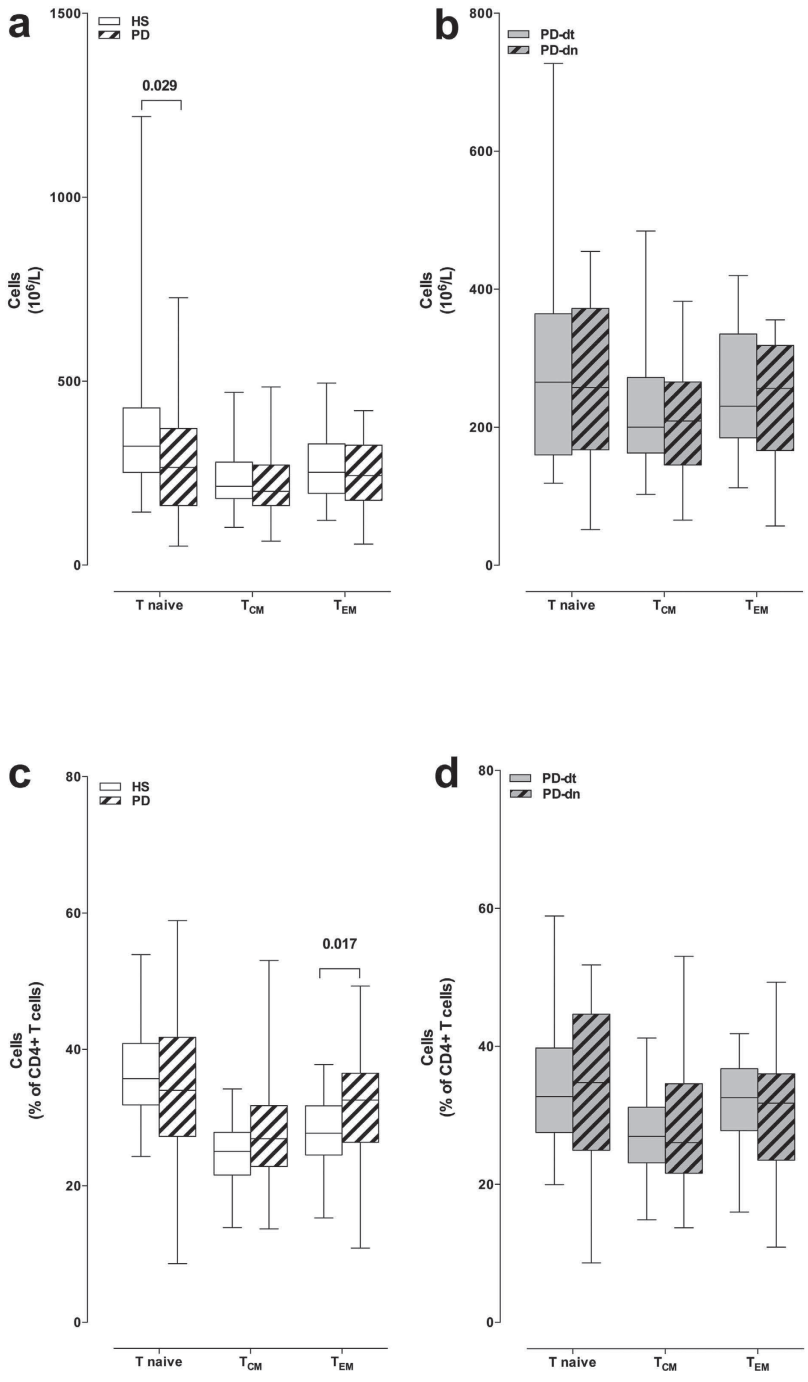

Figure 1. CD4+ T naive and memory cells in HS and PD patients. Cells are shown as absolute numbers (panels **a,b**) and as percentage of total CD4+ T cells (**c,d**). Data are shown as medians with 25°–75° percentiles (boxes) and min-max values (whiskers). Comparisons are shown between HS and PD patients as a whole (**a,c**) and between drug naive (PD-dn) and drug treated (PD-dt) patients (**b,d**). Differences were analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate. P values less than 0.05 are indicated in the graphs.

of CD4+ T naive cells negatively correlated with age (−0.395 (−0.026 to −0.670), P = 0.037, and −0.472 (−0.120 to −0.718), P = 0.011). No correlation on the contrary was found between age and the immune profile in PD patients, either as a whole or in PD-dn and PD-dt patients.

The relationship between disease severity and CD4+ T cells was assessed by dividing PD patients into 3 groups according either to the UPDRS Part III score or the H&Y stage (Table 1), thereafter comparing each group with HS and analyzing the linear trend throughout the groups by means of ANOVA. No relationship was found between CD4+ T naive or memory cells and the UPDRS Part III score or the H&Y stage, with the only exception of a positive linear trend in the percentage of T_{CM} cells and UPDRS Part III (see Supplementary Fig. S3).

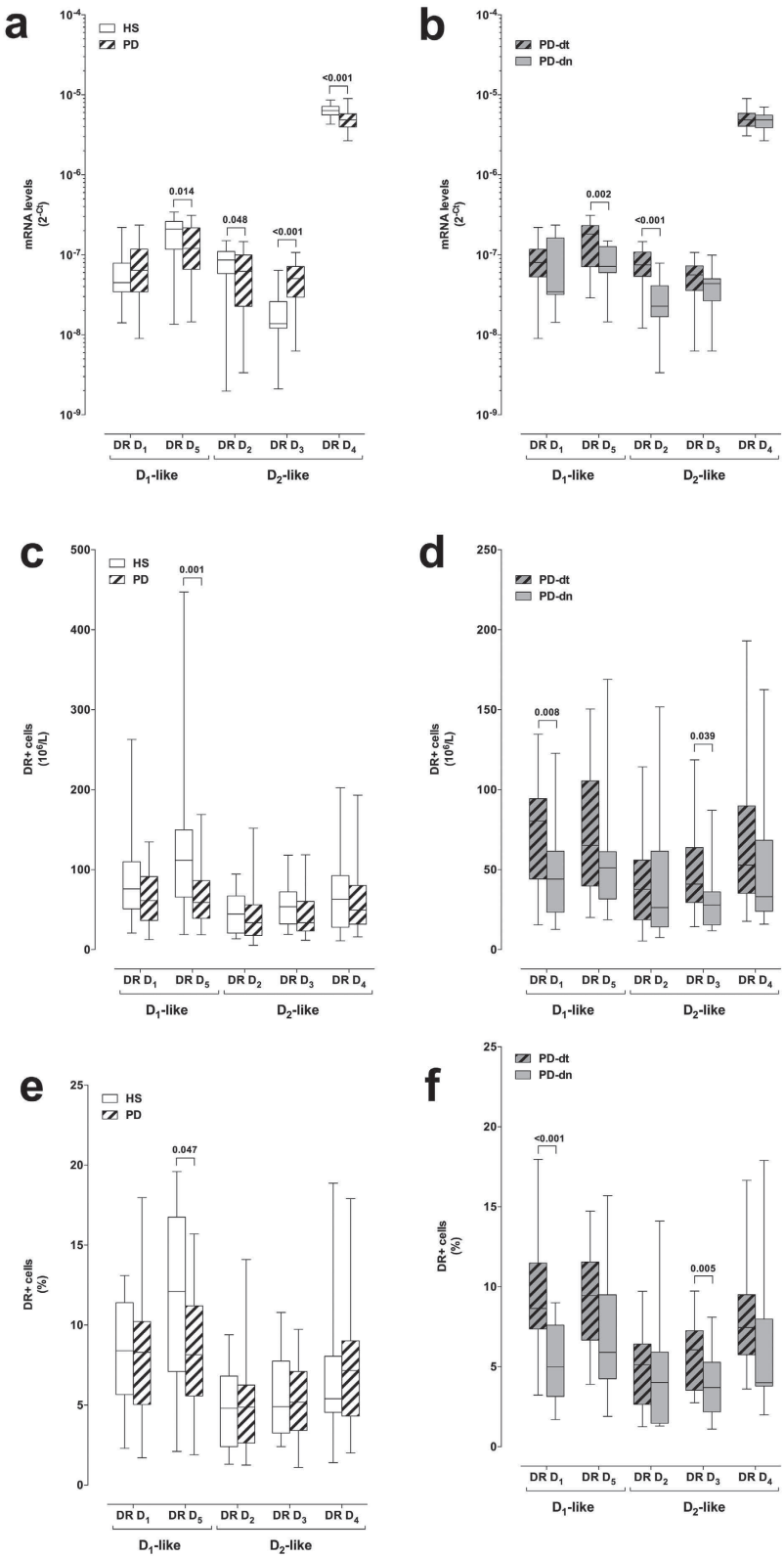

Figure 2. DR expression on CD4+ T cells from HS and from PD patients. DR expression is shown as mRNA levels (panels **a,b**) and as protein expression on the membranes of CD4+ T cells, expressed as absolute numbers of DR+ cells (**c,d**) and as percentage of total CD4+ T cells (**e,f**). Comparisons are shown between HS and PD patients as a whole (**a,c,e**) and between drug naive (PD-dn) and drug treated (PD-dt) patients (**b,d,f**). Data are shown as medians with 25°–75° percentiles (boxes) and min-max values (whiskers). Differences were analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate. P values less than 0.05 are indicated in the graphs.

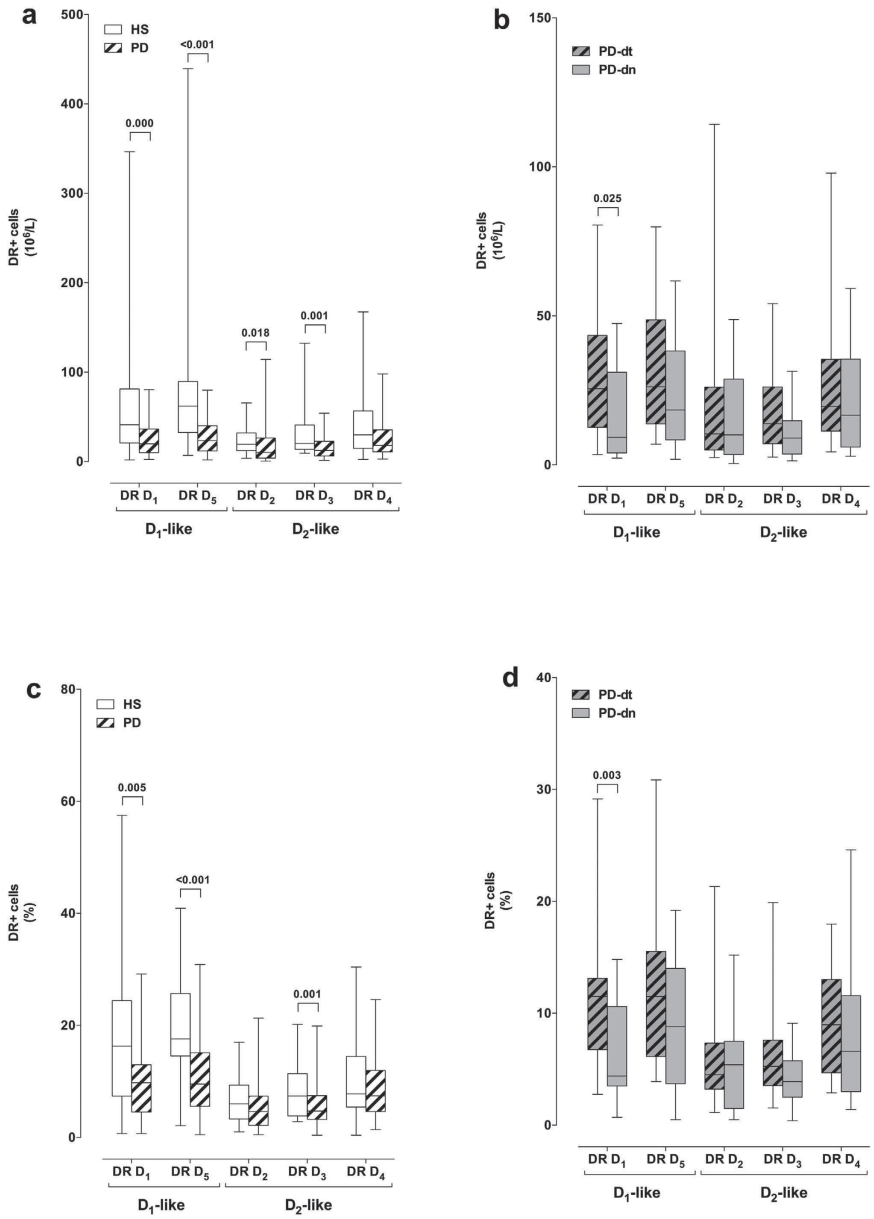

Figure 3. DR expression on CD4+ T naive cells from HS and from PD patients. DR+ cells are shown as absolute numbers (panels **a,b**) and as percentage of total CD4+ cells (**c,d**). Data are shown as medians with 25°–75° percentiles (boxes) and min-max values (whiskers). Comparisons are shown between HS and PD patients as a whole (**a,c**) and between drug naive (PD-dn) and drug treated (PD-dt) patients (**b,d**). Differences were analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate. P values less than 0.05 are indicated in the graphs.

In PD-dt patients, no relationship was found between either CD4+ T cells as a whole or CD4+ T naive or memory cells and disease duration or LED (data not shown).

Correlations between DR expression on CD4+ T cells and demographic and clinical features of HS and PD patients. DR mRNA levels in CD4+ T cells showed extensive correlations with the UPDRS Part III score (Fig. 4a). The D_1-like DR D_5 mRNA levels decreased with increasing UPDRS Part III scores. In comparison to CD4+ T cells from HS, in cells from PD patients DR D_5 mRNA levels were lower in the > 20 UPDRS Part III score group. The D_1-like DR D_1 and the D_2-like DR D_2, D_3 and D_4 mRNA levels did not show linear trends over the UPDRS Part III score, however DR D_3 mRNA levels were higher than those in HS in the 1–10 and 11–20 UPDRS Part III score groups, while DR D_4 mRNA levels were lower in all the UPDRS Part III score groups (Fig. 4a). Some correlations were also observed between DR mRNA levels and H&Y stage, as DR D_3 mRNA levels were higher than those in HS in the H&Y 1 and 2 stage groups, while DR D_4 was lower in H&Y stage 2 (see Supplementary Fig. S4).

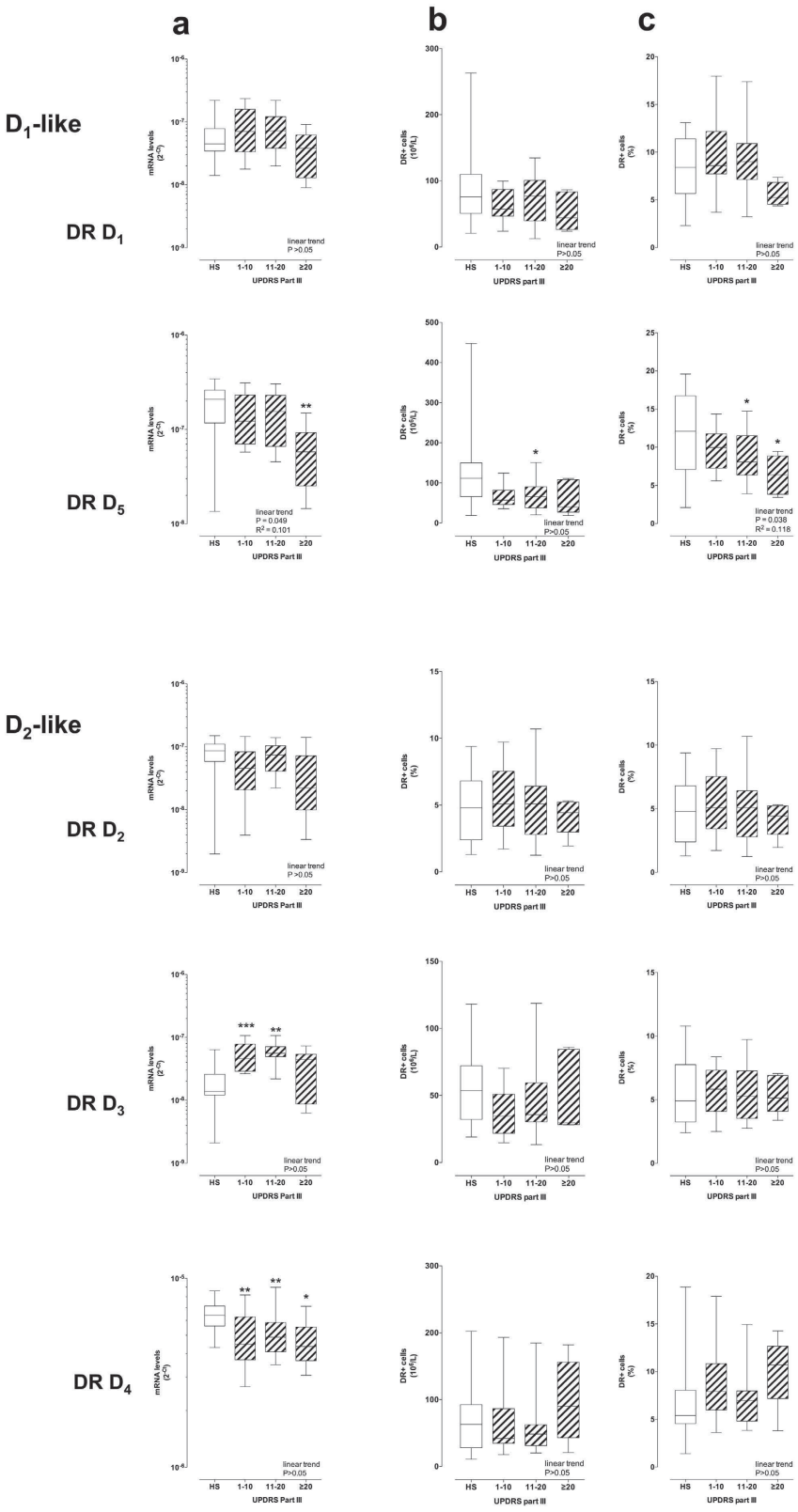

Figure 4. DR expression on CD4+ T cells and UPDRS-III score. DR expression is shown as mRNA levels (panel **a**) and as protein expression on the membranes of CD4+ T cells, expressed as absolute numbers of DR+ cells (**b**) and as percentage of total CD4+ T cells (**c**) Data are medians with 25°–75° percentiles (boxes) and min-max values (whiskers). Differences in DR expression between HS and PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, as appropriate, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = $P < 0.05$ and ** = $P < 0.01$. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

Similar correlations with the UPDRS Part III score were observed for DR expression on CD4+ T cell membranes, in the case of DR D_5 (Fig. 4b,c). Correlations with the H&Y stage included DR D_5 expression lower than that in HS in the H&Y 2 and 3 stage groups, and DR D_3 expression lower than that in HS in the H&Y 3 stage groups (see Supplementary Fig. S5).

D_1-like DR D_1 and D_5 expression on cell membranes negatively correlated with the UPDRS Part III score in CD4+ T naive cells, while D_2-like DR D_2, D_3 and D_4 didn't show any major change (Fig. 5). On the contrary, D_2-like DR D_2 and D_4 increased with the UPDRS Part III score in both CD4+ T_{CM} and T_{EM} cells, while D1-like DR did not change either in T_{CM} or T_{EM} (Fig. 5 and Supplementary Fig. S6). Only minor correlations were observed between DR expression and H&Y stage: D_1-like DR D_1 and D_5 and D_2-like DR D_3 were reduced in T naive from PD patients in comparison to cells from HS, and D_2-like DR D_2 were increased in T_{CM} (see Supplementary Fig. S7-S9).

No correlations were observed between DR mRNA levels and protein expression on CD4+ T cells or DR protein expression in CD4+ T naive and memory cells and age of HS or PD patients, or LED in PD-dt patients. However, in comparison to PD-dt patients treated with L-DOPA and dopamine agonists, those treated with L-DOPA alone had lower mRNA levels of DR D_1 ($6.0 \pm 4.1 \times 10^{-8}$ vs. $10.8 \pm 5.4 \times 10^{-8}$, $P = 0.035$), D_5 ($9.7 \pm 6.9 \times 10^{-8}$ vs. $20.6 \pm 7.5 \times 10^{-8}$, $P = 0.003$), and D_2 ($6.0 \pm 4.4 \times 10^{-8}$ vs. $9.4 \pm 3.3 \times 10^{-8}$, $P = 0.050$), as well as less percentage of CD4+ T cells which were DR D_1+ ($7.2 \pm 2.5\%$ vs. $10.3 \pm 3.5\%$, $P = 0.025$) or DR D_3+ ($4.5 \pm 1.5\%$ vs. $6.4 \pm 1.9\%$, $P = 0.017$), and of CD4+ T naive cells which were DR D_3+ ($3.5 \pm 1.2\%$ vs. 7.9 ± 4.6 &, $P = 0.009$). Patients treated with L-DOPA alone had also higher UPDRS Part III score (19.0 ± 4.3 vs. 14.8 ± 5.0, $P = 0.028$) but similar H&Y stage (1.8 ± 0.7 vs. 1.8 ± 0.5, $P = 0.933$).

***In vitro* responses of CD4+ T naive and memory cells to TTd and to α-syn.** The effect of α-syn on the frequency of CD4+ T naive and memory cells was tested on PBMC obtained from a group of 8 HS (F/M = 4/4, age = 58.1 ± 14.5 years) and 6 PD patients (F/M = 2/4, age = 76.7 ± 7.0 years, UPDRS Part III = 20.5 ± 3.1, H&Y = 1.8 ± 0.8) all treated with l-DOPA without (n = 4) and with DA agents (n = 2, in both cases rasagiline, in one case also ropinirole), with LED = 551.7 ± 140.1 mg/day.

Incubation of PBMC for 48 h with TTd resulted in reduced CD4+ T naive and increased T_{CM} and T_{EM} in both HS and PD patients, however the increase in T_{CM} and T_{EM} was higher in PD patients (Fig. 6a, left). Incubation of PBMC for 48 h with either monomeric or fibrillar α-syn resulted in reduced CD4+ T naive cells and increased T_{EM} cells in both HS and PD, however in PD patients fibrillar α-syn also increased T_{CM} and induced a more pronounced reduction of T naive cells than in HS (Fig. 6a).

Both monomeric and fibrillar α-syn induced several changes in the expression of DR on CD4+ T naive and memory cells (Fig. 6b). In particular, monomeric α-syn increased DR D_5 and D_2 in T naive cells, and DR D_2 in T_{CM}, while fibrillar α-syn increased DR D_1, D_2 and D_4 in T_{CM} and DR D_1 and D_4 in T_{EM}.

Co-incubation of PBMC with either DA, the D_1-like DR agonist SKF-38393, or the D_2-like DR agonists 7-OH-DPAT and PD-168,077 did not affect the frequency of CD4+ T naive and memory cells (see Supplementary Table S3,). SKF-38393 (1 μM) or the D_2-like DR agonist pramipexole (1 μM) did not modify the effects of monomeric and fibrillar α-syn in PBMC of either HS and of PD patients (data not shown).

Discussion

The main result of our study is the evidence supporting an extensive association between DR expression in T lymphocytes and motor dysfunction, as assessed by the UPDRS Part III score, which is commonly used to measure disease severity in the clinical setting[35]. Specifically, in total CD4+ T cells as well as in CD4+ T naive cells the expression of D_1-like DR D_1 and D_5 decrease with increasing UPDRS Part III score. On the contrary, D_2-like DR show changes only at the mRNA level in total CD4+ T cells, do not exhibit major changes in CD4+ T naive cells, but show a clear trend to increased expression with increasing UPDRS Part III score in T_{CM} and in T_{EM}. This is the first study showing a connection between PD severity and DR expression on CD4+ T cells, suggesting that dopaminergic pathways in peripheral immune cells are actively involved in PD. In addition, we provided preliminary evidence that α-syn might affect CD4+ T memory cells, possibly to a different extent in PD patients in comparison to HS.

Our results are in line with previous studies showing decreased CD4+ T lymphocytes in PD patients[9,10], and in particular with Saunders *et al.*[12], who recently reported that in PD patients increased effector/memory CD4+ T cells correlated with increased motor dysfunction. In our study PD patients had decreased absolute count of CD4+ T naive cells, increased percentage of T_{EM} cells, and T_{CM} not different from those in HS. Indeed, our flow cytometric strategy[28] allowed to distinguish between T_{CM}, which mediate reactive memory by homing to T cell areas of secondary lymphoid organs, and T_{EM}, which afford protective memory, by migrating to inflamed peripheral tissues and displaying immediate effector function[36,37]. At apparent variance with the study by Saunders *et al.*[12], who reported that in PD patients effector/memory CD4+ T cells increased with the UPDRS Part III score, we did not identify any clear correlation between T naive/memory cells and the UPDRS Part III score. Saunders *et al.*[12] however identified T memory cells by using CD45RO expression, and found increased CD4+ T memory cells only in PD patients with UPDRS Part III score ≥31, while in our study we enrolled only 7 patients with a score above 20, the highest score being 24, and nonetheless we identified increased T_{EM} in PD patients, possibly also thanks to the specific flow cytometry staining strategy which included the expression of CD45RA and CCR7, and allowed to distinguish between T_{EM} and T_{CM}[28]. It remains however to be established whether the enhanced peripheral T memory function occurring in PD patients is mainly T_{EM}, in line with the possibility that peripheral immune activation in PD has at least in part a protective role.

Concerning the general peripheral immune profile, we also observed reduced CD4+ T naive cells with increasing age in HS but not in PD patients. Reduction of T naive cells in elderly subjects is well described and is believed to result from thymic involution in combination with ongoing differentiation of T naive cells into

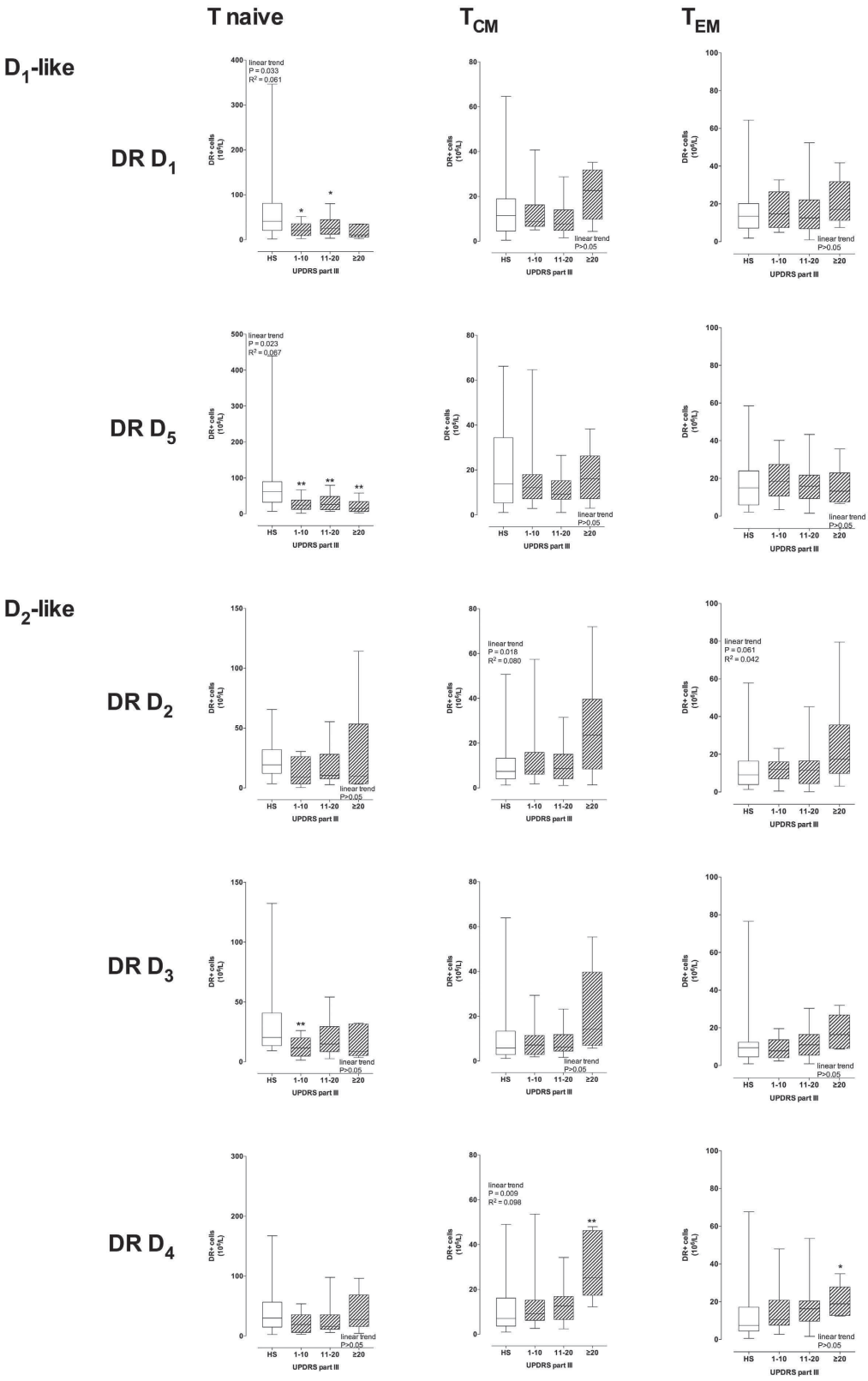

Figure 5. DR expression in CD4+ T naïve, T_{CM} and T_{EM} and UPDRS-III score. DR expression is shown as protein expression on the membranes of CD4+ T naïve (left), T_{CM} (center) and T_{EM} (right) cells, expressed as absolute numbers of DR+ cells. Data are medians with 25°–75° percentiles (boxes) and min-max values (whiskers). Differences between DR levels in HS and in PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = $P < 0.05$ and ** = $P < 0.01$. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

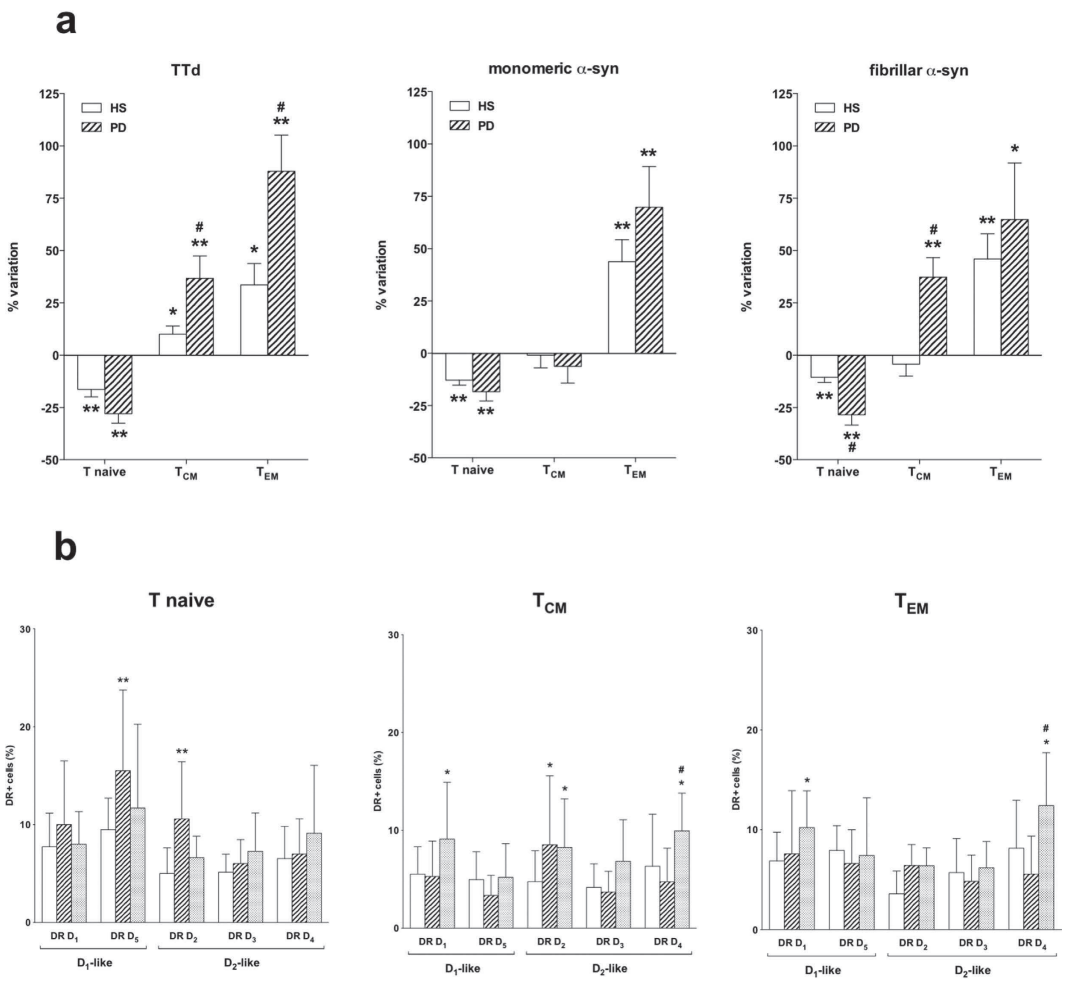

Figure 6. Effect of TTd, monomeric, and fibrillar α-syn on the frequency of CD4+ T naive and memory subsets. Panel (**a**) Effects of TTd (left), monomeric (middle), and fibrillar α-syn (right) in cells from HS (open columns) and PD patients (hatched columns). Data are expressed as percentage variation with respect to control conditions (without TTd or α-syn), and are means ± SEM of n = 6–8 separate experiments each performed in duplicate. * = P < 0.05 and ** = P < 0.01 vs. control conditions, and # = P < 0.01 vs. HS. Panel (**b**) monomeric (hatched columns), and fibrillar α-syn (shaded columns) on DR expression in T naive (left), T_{CM} (center) and T_{EM} cells (right) from 5 HS. Data are means ± SEM. * = P < 0.05 and ** = P < 0.01 vs control (open columns); # = P < 0.01 vs monomeric α-syn.

antigen-experienced memory/effector cells[34]. In PD patients, the absence of correlations between T naive cell count and age, together with the reduced number of T naive cells in comparison to HS, is indeed in agreement with the hypothesis that PD is associated with increased peripheral immune exposure to antigens. A contribution by dysregulated thymic T cells development cannot be discarded, however, also in view of the lack of studies on thymic function during PD.

Although many immune cell subsets are dysregulated in PD, the key role of CD4+ T cells in the pathogenesis of the disease is supported by their presence, together with CD8+ T cells, in the brain in both postmortem human PD specimens and in the MPTP mouse model of PD, and evidence obtained in the animal model indicate that CD4+ T cells are determinants of T cell-mediated dopaminergic cell death[11]. Moreover, a recent study in MPTP-treated mice showed that CD4+ T cells are necessary for MPTP-induced neurodegeneration and that D_2-like DR D_3 expressed on T cells favor their activation and acquisition of the Th1 inflammatory phenotype[29].

Our results show that mRNA expression of several DR are dysregulated in CD4+ T cells from PD patients: in particular, in comparison to cells from HS, in cells from PD patients mRNA for the D_1-like DR D_5 and for the D_2-like DR D_3 and D_4 are decreased, and mRNA for the D_2-like DR D_3 is increased (Fig. 2a,b). Flow cytometry analysis of DR expression on CD4+ T cell membranes provides however a more homogeneous picture (Fig. 2c–f), with D_1-like DR D_5 clearly reduced by 30–49% in cells from PD patients. Interestingly, reduction of D_1-like DR was evident for both DR D_1 and D_5 in CD4+ T naive cells (Fig. 3), while no difference occurred in T_{CM} or T_{EM} cells (see Supplementary Fig. S1 and S2).

Little information is available on the physiopharmacology of D_1-like DR-operated pathways in T cells. D_1-like DR D_5 likely mediate the inhibitory effects of dopamine on proliferation and cytotoxycity of human CD4+ and

CD8+ T cells[38], however they also play a role in the inhibition of human CD4+ $CD25^{high}$ regulatory T cells, thus resulting in a "suppression of the suppressors"[23]. Interestingly, *in vitro* in human naive CD4+ T cells, dopamine via D_1-like DR shifted T-cell differentiation towards Th2, in response to stimulation with anti-CD3 and anti-CD28 mAb[24]. Reduced D_1-like DR on CD4+ T cells in PD patients might thus lead to several effects, such as increased CD4+ $CD25^{high}$ regulatory T cell function and increased Th1/Th2 balance. Saunders *et al.*[12] however, reported impaired function of CD4+ $CD25^{high}$ regulatory T cells from PD patients, thus suggesting that reduced D_1-like DR may have no direct effects on this specialized cell subset. Indeed, our preliminary unpublished data from another protocol, included in this same research program and aimed at investigating DR expression on CD4+ T helper subsets, likely suggest that PD patients have a Th1-biased peripheral immune profile. This observation is in agreement with the previously reported increased ratios of IFN-γ-producing to IL-4-producing T cells in PD patients[8], as well as with the role of D_1-like DR on human CD4+ T naive cells which, according to Nakano *et al.*[24], shift T-cell differentiation towards Th2. It can thus be suggested that reduced D_1-like DR on CD4+ T naive cells in PD patients impair their ability to differentiate towards Th2, promoting a Th1-biased proinflammatory profile.

D_1-like DR on CD4+ T lymphocytes, which are generally reduced in PD, also display a close correlation with PD patients motor dysfunction, as assessed by the UPDRS Part III score. Indeed, DR D_5 expression diminishes with increased UPDRS Part III score, both at the mRNA level as well as in terms of percentage of CD4+ T cells which express the specific receptors (Fig. 4), a behavior which is evident also in CD4+ T naive cells, for both DR D_1 and D_5, but not in T_{CM} of T_{EM} (Fig. 5). By contrast, CD4+ T_{CM} and T_{EM} cells generally display a linear trend towards increased D_2-like DR (Fig. 5 and Supplementary Fig. S6). Such close association with the UPDRS Part III score was not always parallel with the H&Y stage. In particular, in CD4+ T naive cells DR D_1 and D_5 decreased, and in T_{CM} and T_{EM} DR D_2 and D_4 increased with increasing UPDRS Part III score (Fig. 5 and Supplementary Fig. S6) but not with increasing H&Y scale stage (Supplementary Fig. S7–9). A likely explanation is that only one of the 7 subjects with UPDRS Part III > 20 is included among the 4 subjects with H&Y 2.5–3.0 (Table 2). Whether UPDRS Part III scale profiles the underlying immune dysfunction occurring in PD patients better than the H&Y scale remains to be established. Unfortunately, the only other study correlating CD4+ T cells and motor dysfunction in PD considered just the UPDRS Part III score[12].

PD patients with more severe motor dysfunction (score > 20) have T_{CM} cells expressing 131–134% more DR D_2 and 112–126% more DR D_4 than cells from HS, and 64–100% more DR D_2 64–105% more DR D_4 in comparison to cells from PD patients with score 1–10. The picture is similar with T_{EM}, as PD patients with score > 20 have T_{EM} cells expressing 93–112% more DR D_2 and 48–71% more DR D_4 than cells from HS, and 71–111% more DR D_2 and 22–38% more DR D_4 in comparison to cells from PD patients with score 1–10. As a whole, it appears therefore that, with increasing motor dysfunction, D_1-like DR decrease on CD4+ T lymphocytes and in particular on CD4+ T naive cells, while D_2-like DR, increase specifically on CD4+ T_{CM} and also on T_{EM} cells.

As discussed above, reduced D_1-like DR on CD4+ T naive cells may promote a Th1-biased proinflammatory profile, and the present results suggest that such trend increases with increasing motor dysfunction. There is on the contrary paucity of data regarding the role of D_2-like DR on T lymphocytes, even if Levite *et al.*[39] showed that activation of either DR D_2 or D_3 might induce T cell proliferation and adhesion. Of potential relevance for the present results, it was recently reported that, in the MPTP mouse model of PD, D_2-like DR D_3 expressed on CD4+ T cells are critical for T cell-dependent microglial activation[29]. If the same applied to PD patients and to D_2-like DR-operated pathways as whole (as in PD patients DR D_2 and D_4, but not DR D_3, correlated with motor impairment), increased D_2-like DR in the more advanced stages of the disease might imply increased activation of the peripheral immune system, in turn triggering central neuroinflammation leading to neurodegeneration and disease progression. Nonetheless, such findings should be interpreted cautiously since studies also exist showing that at least stimulation of the D_2-like DR D_4 may result in quiescence of human T cells[40]. It is therefore necessary to clarify the role of individual DR in the modulation of memory T lymphocytes and in their relationship with microglia in PD. In addition, the eventual role of antiparkinson treatments on DR expression on T lymphocytes needs careful consideration, as discussed hereafter.

Comparison between PD-dn and PD-dt patients did not reveal any major differences in the peripheral immune profile. In particular, absolute numbers of CD3+ and CD4+ T cells were not different, although percentage CD3+ T cells were slightly higher in PD-dt patients while percentage CD4+ T cells were slightly higher in PD-dn patients (Table 4), and T naive, T_{CM} and T_{EM} were similar in the two patient populations (Fig. 1b,d). A remarkable difference was however found in D_1-like DR D_1 expression in CD4+ T cells and in particular in T naive cells (Figs 2,3), as PD-dt patients had higher expression of DR D_1 in comparison to PD-dn. This difference might be of interest as PD-dn patients have on their T naive cells only 22–35% DR D_1 in comparison to HS T naive cells, while PD-dt patients have on their T naive cells 41–63% DR D_1 in comparison to HS T naive cells. Whether this is an effect of dopaminergic antiparkinson treatments cannot be established on the basis of the present results, also taking into account that no relationship was found between PD duration and/or LED and the immune profile or DR expression in CD4+ T cells and cell subsets. The hypothesis should be nonetheless taken into account, since - as above discussed - D_1-like DR on human CD4+ T naive cells may shift T-cell differentiation towards Th2[24]. Provided that this role of D_1-like DR on T naive cells has any clinical relevance, it might be predicted that in the study which we are presently performing, aimed at investigating DR expression on CD4+ T helper subsets in PD patients, we will find less Th1 cells in PD-dt patients in comparison to PD-dn patients.

α-Syn is the major component of Lewy bodies and a key factor in PD pathogenesis. Pathological α-syn released by degenerating neurons activates microglia to a proinflammatory profile[32], and directs cell migration[41]. Efflux of α-syn from the brain to peripheral blood has been reported in mice and possibly in PD patients[42], and it has been hypothesized that it might prime T cells that, in turn, would enter the brain and sustain microglia activation and neurodegeneration[43]. It has also been suggested that the presence of aberrant forms of α-syn in the periphery may represent a possible means for exposure as a neoantigen and subsequent activation of the adaptive immune system[44].

In our study, we performed preliminary experiments aimed at assessing the effects of different forms of α-syn on CD4+ T naive and memory cells, in comparison to a well established recall antigen like TTd. We tested both monomeric and fibrillar α-syn since accelerated fibril formation by certain variants of α-syn are associated to PD pathogenesis[45,46]. As expected, TTd reduced the frequency of T naive cells while increasing T_{CM} and T_{EM}. The effect on T_{CM} and T_{EM} was however more pronounced in cells from PD patients, possibly in line with the activated profile of peripheral immune system in PD. Interestingly, both monomeric and fibrillar α-syn induced a response which was qualitatively similar to the one evoked by TTd. Both PD patients and HS responded to the same extent to α-syn, with the only exception of fibrillar α-syn, which increased T_{CM} cells in PD patients but not in HS.

Available evidence of course does not allow to conclude that the responses to α-syn are actually due to recognition of the protein by T memory cells, although the response pattern is similar to that induced by TTd, and the ability of the peripheral immune system to recognize α-syn is also supported by the occurrence of specific antibodies in the serum of PD patients and HS[47–49]. Nonetheless, the ability of fibrillar α-syn to increase T_{CM} is suggestive, as these cells mediate reactive memory, by homing to T cell areas of secondary lymphoid organs and readily proliferating and differentiating to effector cells upon antigenic stimulation[36,37].

It is also remarkable that incubation with α-syn affected DR expression on CD4+ T cells, and that in particular fibrillar α-syn induced increased expression of DR D_4 in both T_{CM} and T_{EM}, a finding which resembles increased D_2-like DR in T_{CM} and T_{EM} of PD patients with more severe motor dysfunction. Whether increased expression of DR corresponds to increased responsiveness, and which consequences might be implied for the pro/antiinflammatory balance of peripheral (and possibly also central) immunity need to be carefully considered.

Concluding remarks and perspectives. It is noteworthy that in the present study we were unable to find any association between dopaminergic substitution treatments and the peripheral immune profile. Possible explanations include that L-DOPA may undergo conversion to dopamine only in the brain, and that dopaminergic receptor agonists are usually D_2-like DR selective (pramipexole, ropinirole). Rotigotine is the only dopaminergic agonist currently used in PD that has comparable affinity for D_2-like DR and at least for the D_1-like D_5, however our study enrolled only four subjects on rotigotine (out of a total of 53). Our study included also a group of newly diagnosed PD patients who never received dopaminergic treatments. The main difference between newly diagnosed and antiparkinson-treated subjects consisted in an even lower expression of D_1-like DR D_1 on total CD4+ T cells as well as in T naive cells in newly diagnosed patients, however it remains to be established whether the increased D_1-like DR D_1 expression in antiparkinson-treated patients is actually due to antiparkinson drugs and/or to other factors. In order to clarify this issue, we have already started a longitudinal study on a larger sample of drug naïve PD patients, who will be tested before and after pharmacological treatment.

Anyway, from a general point of view it is possible to conclude that dopaminergic substitution treatments have only minor, if any, impact on the peripheral immune system of PD patients, which on the other side shows profound differences in comparison to that of HS. In particular, specific differences related to dopaminergic pathways in immune cells definitely support the notion of a chronic peripheral immune activation in PD patients, which may affect disease severity. Immunotherapy is being increasingly regarded as an attractive strategy even in PD[44], and it is therefore a priority to unravel the peripheral immune dysregulation occurring in PD patients, to plan adequate immunotherapeutic interventions. In addition, since antiparkinson therapy still lies mainly (if not only) on dopaminergic substitution therapy, detailed understanding of the role of dopaminergic pathways in the immune system might possibly allow a more appropriate use of available drugs, simply by better exploitation of their immunomodulating potential[14–16,50].

Materials and Methods

Subjects. Peripheral venous blood samples were collected from patients with idiopathic PD[51], either drug naive (PD-dn, i.e. PD patients who never received L-DOPA, DA agonists and/or other antiparkinson drugs) or on antiparkinson drug treatment (PD-dt), and from age- and sex-matched healthy subjects (HS). PD was diagnosed according to the United Kingdom Parkinson's Disease Society Brain Bank Criteria. Patients and controls with a history of autoimmune or inflammatory disorders and those receiving chronic immunosuppressive treatment were excluded.

Participants were recruited through the Centre for Parkinson's Disease and Movement Disorders of the Neurological Service at the Ospedale di Circolo of Varese, the Interdepartmental Research Center for Parkinson's Disease of the Neurological Institute "C. Mondino" of Pavia, and the Movement Disorders Center of the University of Piemonte Orientale, Divisione di Neurologia, Ospedale Maggiore of Novara, Italy. Healthy subjects were spouses and caregivers of enrolled PD patients. The Ethics Committees of Ospedale di Circolo of Varese and Neurological Institute "C. Mondino" of Pavia approved the protocol and all the participants signed a written informed consent before enrollment. The study was performed according to the Declaration of Helsinki and to the relevant ethical guidelines for research on humans.

After enrollment, subjects were submitted to a complete examination. PD patients were staged according to the criteria of Hoehn and Yahr (H&Y)[52] and evaluated by means of the Unified Parkinson's Disease Rating Scale (UPDRS) Part III[53]. UPDRS Part II score was also assessed whenever possible. Data on patients and healthy controls were collected using standard data forms, which included demographics, diagnostic features, family history, primary diagnosis, PD features, UPDRS Part III score, and Hoehn and Yahr (H&Y) stage. Antiparkinson drug doses were recorded at the time of enrollment and L-DOPA equivalent doses (LED) were calculated according to established guidelines[54].

Withdrawal of 30 ml venous blood was performed after a fasting night, between 8:00 a.m. and 10:00 a.m., in EDTA-coated tubes (BD Vacutainer). Tubes were subsequently coded and stored at room temperature until processing, which occurred within 24 hours after collection. Complete blood cell count with differential analysis was conducted on separate blood samples collected in EDTA-coated tubes (BD Vacutainer). Serum levels of

dopamine were assayed by high-performance liquid chromatography with multielectrode electrochemical detection (HPLC-ED) according to a previously described method[23].

Reagents. Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma, Italy. RPMI 1640, heat-inactivated fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were obtained from Euroclone, Italy. Ficoll-Paque Plus was from Pharmacia Biotech (Uppsala, Sweden). Purified mouse ab anti-human CD3 (code 555330, clone UCHT1, Mouse IgG1, κ) and purified mouse ab anti-human CD28 (code 555726, clone CD28.2, Mouse IgG1, κ) were obtained from Becton Dickinson, Italy. (±)SKF-38,393 hydrochloride (cod. D047), R(+)7-OH-DPAT hydrobromide (code H168), PD-168,077 maleate (code P233), pramipexol dihydrochloride (code A1237), and dopamine hydrochloride (code H8502) were all from Sigma, Italy. Human recombinant α-synuclein and its fibrillar form were a kind gift from Dr. Lars Kjær and Dr. Daniel Otzen (iNANO - Interdisciplinary Nanoscience Center, Aarhus University, Aarhus, Denmark), and were prepared as published before[55].

Flow cytometric analysis of naive and memory subsets of CD4+ T cells and of DR expression in whole blood. Analysis of CD4+ T naive and memory subsets and of DR expression was performed according to previously established method[28]. Briefly, 100 μl aliquots of whole blood were prepared and erythrocytes were removed by means of a lysis buffer ((g/L) NH4Cl 8.248, KHCO3 1.0, EDTA 0.0368). Samples were then centrifuged, supernatants were removed and cells were washed in PBS (pH 7.4) supplemented with 1% BSA (PBS/BSA) and resuspended in PBS/BSA. Total leukocytes were counted by means of a hemocytometer and cell viability, determined by the Trypan blue exclusion test, was always > 99%.

From each subject 7 aliquots of 100 μL were prepared: 5 were used for DR staining, 1 was used as control for the secondary PE-goat anti-rabbit (PEGAR) ab, and 1 was used as negative control (no ab). The staining protocol consisted of two steps. During the first step each aliquot was stained for one of the five DR by an indirect labeling procedure (primary ab + secondary ab labeled with PE). During the second step all the aliquots were incubated with a cocktail of anti-human CD3, CD4, CD45RA and CCR7 ab for the identification of T lymphocytes, CD4+ T lymphocytes and the following CD4+ T lymphocyte subsets: naive (CD3+ CD4+ CD45RA+ CCR7+), central memory (T_{CM}, CD3+ CD4+ CD45RA-CCR7+), and effector memory (T_{EM}, CD3+ CD4+ CD45RA-CCR7-). The complete list of ab used in the study is shown in Supplementary Table S1.

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 FlowJo software (version 8.3.2). The results were finally expressed as absolute numbers (10^6/ml) as well as percentage of positive cells (%).

Isolation of peripheral blood mononuclear cells (PBMC). PBMC were isolated from whole blood by using Ficoll-Paque Plus density gradient centrifugation. Cells were resuspended and, if necessary, any residual contaminating erythrocytes were lysed by addition of 5 mL of lysis buffer, followed by incubation for 5 min, during which samples were gently vortexed, and centrifugation at 100 g for 10 min at RT. Cells were washed twice in PBS by addition of 15 ml of PBS and centrifugation at 300 g and 10 min at RT, and resuspended at the final concentration of 10×10^6 cells in 10 ml of RPMI/10% FBS for subsequent culture. Typical PBMC preparations contained at least 80% lymphocytes, as assessed by flow cytometry. Cell viability, assessed by the Trypan blue exclusion test was always > 99%.

Real-time PCR assay of DR mRNA in CD4+ T cells. CD4+ T cells were isolated from PBMC by immunomagnetic sorting using Dynalbeads CD4 Positive Isolation kit (Life Technologies, code 11145D). Real-time PCR of DR mRNA was performed according to a previously reported method with modifications[56]. Briefly, to isolate RNA, at least 50000 CD4+ T cells were resuspended in *PerfectPure RNA lysis buffer* (5 Prime Gmbh, Hamburg, Germany), total RNA was extracted by *PerfectPure RNA Cell Kit*™ (5 Prime Gmbh), and the amount of extracted RNA was estimated by spectrophotometry at λ = 260 nm. Total mRNA obtained from CD4+ T cells was reverse-transcribed using a random primer, high-capacity cDNA RT kit (Applied Biosystems). cDNA was then amplified with *SsoAdvanced*™ *Universal Probes Supermix* (BIORAD) for the analysis of DR D_2, DR D_3, and DR D_5 gene expression, and with *SsoAdvanced*™ *Universal SYBR*® *Green Supermix* (BIORAD) for analysis of DR D_1, and DR D_4 gene expression. cDNA was assayed on StepOne® System (Applied Biosystems). Real-time PCR conditions are shown in Supplementary Table S2.

Linearity of real-time PCR assays were tested by constructing standard curves by use of serial 10-fold dilutions of a standard calibrator cDNA for each gene, and regression coefficients (r^2) were always > 0.999; a melting curve was also performed to check for specificity of DR D_1 (melting temperature = 83.5 °C) and DR D_4 (melting temperature = 90 °C). Gene expression level in a given sample was represented as $2^{-\Delta Ct}$ where ΔCt = [Ct (sample) - Ct (housekeeping gene)]. Relative expression was determined by normalization to 18 S cDNA. Analysis of the data were performed by StepOne software™ 2.2.2- Applied Biosystems).

Frequency of CD4+ T naive and memory subsets in cultured PBMC. Isolated PBMC were cultured in RPMI/10% FBS for 48 h at 37 °C in a moist atmosphere of 5% CO_2, without or with anti-CD3/anti-CD28 ab (0.1 μg/ml). Tetanus toxoid (TTd, 3 μg/ml), monomeric or fibrillar α-syn (both 500 nM) were added at the beginning of cell culture. Cells were finally harvested and stained for flow cytometric analysis of naive and memory subsets of CD4+ T cells, as described in section regarding flow cytometric analysis of naive and memory subsets of CD4+ T cells.

Statistical analysis. Distribution of the values was assessed by the D'Agostino & Pearson normality test. Statistical significance of the differences between HS and PD patients and between PD-dn and PD-dt patients was then analyzed by means of two-tailed Student's *t* test or by the Mann-Whitney test, as appropriate, for continuous variables, and by the Fisher's exact test for categorical variables. Correlations among continuous variables were assessed by Pearson or Spearman correlation analysis. Differences between HS and PD patients categorized for UPDRS Part III score or H&Y stage were analyzed by ordinary one-way ANOVA or by the Kruskal-Wallis test, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, and trend analysis in PD patients was performed by ANOVA post test for linear trend. Calculations were performed using commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

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Author Contributions

Study conception and design: M.C., F.M. and F.B. Acquisition of data: N.K., E.R., M.L., R.B., I.A., B.M. and G.R. Analysis and interpretation of data: N.K., M.C., F.B., C.C., M.M., V.S.-G. and F.M. 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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