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Dopaminergic inhibition of human neutrophils is exerted through D1-like receptors and affected by bacterial infection

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Abstract

Dopamine (DA) affects immune functions in healthy subjects (HS) and during disease by acting on D1-like (D1 and D5) and D2-like (D2, D3 and D4) dopaminergic receptors (DR); however, its effects on human polymorphonuclear leukocytes (PMN) are still poorly defined. We investigated DR expression in human PMN and the ability of DA to affect cell migration and reactive oxygen species (ROS) production. Experiments were performed on cells from HS and from patients (Pts) with bacterial infections as well, during the acute phase and after recovery. Some experiments were also performed in mice knockout (KO) for the *DRD5* gene. PMN from HS express both D1-like and D2-like DR, and exposure to DA results in inhibition of activation-induced morphological changes, migration and ROS production which depend on the activation of D1-like DR. In agreement with these findings, DA inhibited migration of PMN obtained from wild-type mice, but not from DRD5KO mice. In Pts with bacterial infections, during the

Abbreviations: CNS, central nervous system; DA, dopamine; DR, dopaminergic receptors; DRD5KO, DRD5 knockout mice; HPLC-ED, high-performance liquid chromatography-multielectrode electrochemical detection; HS, healthy subjects; MS, multiple sclerosis; PD, Parkinson's disease; PMN, polymorphonuclear leukocytes; Pts, patients; ROS, reactive oxygen species; SEM, scanning electron microscopy; TEM, transmission electron microscopy; WT, wild-type mice.

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febrile phase D1-like DRD5 on PMN were downregulated and DA failed to affect PMN migration. Both D1-like DRD5 expression and DA-induced inhibition of PMN migration were however restored after recovery. Dopaminergic inhibition of human PMN is a novel mechanism which is likely to play a key role in the regulation of innate immunity. Evidence obtained in Pts with bacterial infections provides novel clues for the therapeutic modulation of PMN during infectious disease.

KEYWORDS
inflammation, infective disease, neutrophils

INTRODUCTION

The idea that the immune system might be affected by dopamine (DA) [1] is supported by the identification of dopaminergic receptors (DR) on different immune cells [2–5] and by the ability of immune cells to synthesize, store and release DA [6–8].

The role of catecholamines (CA) as modulators of immune cell functions is well documented [1, 9] and a role for the dysregulation of CA pathways was postulated in several neurological and autoimmune diseases, as well as in cancer [1, 10–13]. In particular, the ability of DA to affect adaptive immunity was widely investigated both in healthy individuals [5, 9, 12, 14] and in different pathological conditions [11, 15–17]. On the contrary, there are relatively few data about the possible modulation by dopaminergic pathways of innate immunity and only few reports are available showing the ability of DA to affect innate immunity cells, for example macrophages [18], dendritic cells [19, 20] and monocytes [21].

Among innate immune cells, polymorphonuclear leukocytes (PMN) represent the first cell type which can leave the microcirculation and enter inflamed tissues during an inflammatory response [22–26]. PMN can produce DA [8] and express DR on their surface [2], but whether DA or other DR ligands can interfere with their functional responses, as well as the DR subtypes involved, remains so far unclear.

Data from animal models and humans show that DA can induce morpho-functional changes in vitro and modulate PMN apoptosis in different conditions [27–29] while for example PMN from Parkinson's Disease (PD) patients (Pts), in comparison to healthy controls, had decreased mRNA levels of all the D_2-like DR and increased D_1-like DR D_5 and tyrosine hydroxylase (TH), the key rate limiting enzyme in the synthesis of CA [30]. We and others have shown that PMN are able to produce DA [8] and express DR on their surfaces [2].

In animal models, circumstantial evidence suggests that dopaminergic agents may influence the number of PMN during anaphylactic shock: more in detail, DA antagonists (e.g., chlorpromazine and pimozide) reduce cell count, while apomorphine (acting as DR agonist) increases cell number [28]. Dopaminergic agonists were also reported to reduce the Th17-induced response and ovalbumin antigen-induced activation of PMN in a mouse model of airway inflammation [29]. In vitro studies show that DA inhibits reactive oxygen species (ROS) generation, superoxide anion production and cell migration in human PMN [31–34], but the possible involvement of DR in such effects was not investigated.

The main aim of the present study was to investigate the presence of the different DR in human PMN and the ability of DA to affect functional responses in human PMN. Data obtained from in vitro studies were validated in an animal model, by investigating PMN migration in DRD5-knockout (KO) mice. Finally, to obtain information about the possible clinical relevance of the observed effects, we investigated DR expression on PMN from Pts during bacterial infections and, in the same clinical conditions, the ability of DA to affect a key function for PMN such as migration.

METHODS

Test substances

The Dopaminergic ligands DA-Hydrochloride (code: H8502), SKF-38393 (code: D047), Pramipexol (code: A1237), SCH-23390 (code: D054), Haloperidol (code: H1512), fMLP (code: F3506), cacodylate buffer (code: 20840), Tween20 (code: P5927), Mayer's Haematoxylin Solution (code: MHS16-500ML), Dextran (code: 31392), paraformaldehyde (PFA, code: 200-001-8), bovine serum albumin (BSA, code: A2153), KH2PO4, Na2EDTA, octansolfonic acid sodium salt and H3PO4 were purchased by SIGMA Aldrich; the filter for migration assay (code SSWP01300) by Merck SpA; Isopropanol (code: 20880,32) and Xylene (code: 28975,325) by BDH Prolabo; acetonitrile

by VWR; Ficoll-Paque Plus (code: GEH17144003) by GE Healthcare; C-DCFH-DA (code: C29938) by Molecular probes.

Whole blood, PMN isolation and cell culture for the in vitro study

Whole blood from healthy donors was obtained from the local blood bank (Ospedale di Circolo, Fondazione Macchi). A part of the obtained sample was used for the evaluation of cell surface DR expression in PMN by flow cytometry according to the experimental procedure described below, while the remaining sample was used to isolate PMN by standard density-gradient centrifugation as previously described [35] and published online (dx.doi.org/10.17504/protocols.io.bpxxmppn; dx.doi.org/10.17504/protocols.io.bpxymppw).

Briefly, blood was allowed to sediment on dextran at 37°C for 30 min. Supernatant was recovered and PMN were isolated by Ficoll-Paque Plus density-gradient centrifugation. Erythrocytes were eliminated by hypotonic lysis for 10 min in distilled water with added (g/L): 8.25 NH_4Cl, 1.00 $KHCO_3$, 0.04 EDTA. Cells were then washed three times in 0.15 M NaCl. Purity and viability of PMN preparations were always ≥95% and no platelets or erythrocytes could be detected by either light microscopic examination or flow cytometric analysis (morphological parameters, side-scatter [SSC] and forward-scatter [FSC]).

DR expression and DA intracellular content

Cell surface DR expression by flow cytometry

Cell surface DR membrane expression was evaluated by flow cytometry on cells that were cultured for 30 min under resting and activated conditions (Figure S1). This procedure was performed on buffy coats from healthy subjects (HS) obtained by the local blood bank. In the second part of the study, we performed the assay on whole blood of Pts and HS in the ex vivo clinical part of the study. For both conditions, six aliquots of 50 µl of whole blood were prepared: five were used for DR staining and one was used for staining with Alexa-Fluor 647-conjugated donkey anti-rabbit secondary antibody (Ab2°) alone (DAR-AF647, Biolegend), as a negative control to evaluate the level of background fluorescence. The aliquots were then diluted with 950 µl of RPMI and incubated under resting or activated (fMLP 0.1 µM) conditions for 30 min (37°C, in a moist atmosphere of 5% CO_2). At the end of the incubation, whole blood was centrifuged at 600 g for 5 min at room temperature (RT), supernatants were removed and 3 ml of a lysis solution (composition [g/L]: NH_4Cl [8.248], $KHCO_3$ [1.0] EDTA [0.0368]) were added to each aliquot to remove the erythrocytes. Incubation was performed at RT for 5 min, during which samples were gently vortexed. Samples were then centrifuged; supernatant was removed, and cells were washed one time in 1 ml of PBS (pH 7.4) supplemented with 0.5% BSA (PBS/BSA) and finally resuspended in 100 µl PBS/BSA. Resting and fMLP-activated granulocytes were then stained for 30 min on ice with the following rabbit polyclonal primary antibodies (Ab1°) directed against each of the human DRs (dilution 1:100): D1, D3, D5 (Merck, Milan, Italy), and D2, D4 (LifeSpan BioSciences). All these five Ab1° recognize an extracellular epitope of each DR and were titrated to determine the optimal working dilution. Moreover, any unspecific binding of Ab1° to Fc Receptors expressed on human granulocytes such as CD16, CD32 and CD64 were reduced by using a commercially available FcR blocking solution (Human TruStain FcX™, Biolegend) according to the manufacturer's instructions. To this end, 5 µl of this solution, containing specialized human IgGs which prevent unwanted FcR-involving binding without interfering with Ab1°-mediated specific staining, was added to 100 µl cell suspensions before staining with Ab1° and samples were incubated for 10 min at RT. At the end of incubation with the Ab1° anti-DRs, cells were then washed with PBS/BSA and pellets incubated with 100 µl DAR-AF647 (final dilution 1:400) for 30 min on ice in the dark. Finally, the samples were washed with 1 ml PBS/BSA, centrifuged, resuspended in 300–400 µl PBS/BSA and kept on ice until flow cytometric analysis. Acquisition was performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy) with BD FACSDiva software (version 6.1.3). Granulocytes were identified by their classical FSC and SSC properties, and at least 20 000 granulocytes from each sample were collected in the gate. Data were analysed using BD FACSDiva software (version 6.1.3) and the results were finally expressed both as percentage (%) of DR positive cells in the granulocyte gate and ratio of the median fluorescence intensity (MFI) corresponding to specific staining for each DR over MFI corresponding to a negative control (DAR-AF647 alone).

RNA isolation and real-time polymerase chain reaction for DR

DR mRNA expression was evaluated by culturing PMNs at the concentration of 1×10^6 cells/ml in RPMI 1640 alone (resting) or with fMLP (0.1 µM; activated) at 37°C for 3 h. After incubation, cells were centrifuged (400 g,

5 min, 20°C), and pellets were separated and stored at −80°C for subsequent analysis according to our previous study [11].

Briefly, total mRNA was extracted from 1×10^6 cells by PerfectPure RNA Cell Kit™ (5 Prime GmbH) and quantity and quality of RNA extracted was estimated by spectrophotometry with the 260/280 nm ratio. Total RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions and real-time polymerase chain reaction (PCR) was performed (StepOne® System; Applied Biosystems) using assay-on-demand kits. Gene expression level in each sample was represented as $2^{-\Delta Ct}$ where $\Delta Ct = (Ct\ [sample] - Ct\ [housekeeping\ gene])$. Relative expression was determined by normalization to 18S cDNA (analysed by StepOne software™ 2.2.2 – Applied Biosystems). Primers (Applied Biosystems) used are shown in Table 1.

Immunocytochemical characterization of DR expression

PMN (8×10^4 cells/ml) were seeded on rounded glass coverslips (12 mm, treated with 0.1% gelatin) in RPMI-1640 medium and incubated for 30 min at 37°C, under resting conditions or after activation with fMLP 0.1 μM. After the incubation period, cells were fixed with 4% PFA in PBS and then incubated for 30 min with a blocking/permeabilizing solution made of PBS containing 2% BSA and 0.1% Tween20 (both from Sigma-Aldrich). The presence of the different DR was assessed using the following rabbit polyclonal Ab1°: DR D1 (1:100 dilution) and DR D5 (1:100 dilution) from Merck. Incubation with suitable Ab2° conjugated with AlexaFluor 488 (1:200 dilution, Thermo Fisher Scientific) was performed for 1 h in a dark humid chamber at RT. Nuclei were counterstained with 4′,6′-diamino-2-phenylindole (DAPI, Sigma-Aldrich). All the Ab were diluted in blocking solution. In control samples, Ab1° antibodies were omitted and cells were treated with BSA/Tween20 containing PBS. Coverslips were mounted in CitiFluor (Electron Microscopy Sciences) and slides were observed under an Eclipse Nikon microscope.

Assay of intracellular DA

DA intracellular content was measured by high-performance liquid chromatography with multielectrode electrochemical detection (HPLC-ED) [9]. The system consisted of a dual-piston pump (model LC10ADVP, Shimadzu), a C18-3 mm reverse phase column (Xbridge Shields, Waters) and pre-column, an autosampler (model SIL9A, Shimadzu) and a multielectrode electrochemical detector (Coularray 5600A; ESA) with a 4-channel sensor cell (model 6210; ESA).

The mobile phase was composed by ultrapure water–acetonitrile (92:8 vol/vol), 0.049 M KH_2PO_4, 0.349 mM Na_2EDTA and 0.627 mM octansolfonic acid sodium salt as the counter ion. The pH was adjusted to 3.62 with H_3PO_4. Freshly prepared mobile phase was filtered (0.45 μm) and degassed under vacuum for 10 min. The flow rate during the analysis was 0.7 ml/min.

The multielectrode detector operated in the oxidation-reduction mode, with the potentials of four electrodes set at 100, 200, 300 and −200 mV.

The chromatograms were collected, stored, and processed with the application software Coularray for Windows (Version 3.1, ESA). DA in the samples was quantified using the peak height of a standard curve generated by injecting known samples (from 3 fmol to 3 pmol), and values were finally expressed as pmol/10^6 cells.

Intracellular cAMP measurement

Isolated PMN were resuspended in RPMI 1640 medium supplemented with 10% heath-inactivated foetal calf serum and 2 mM glutamine at the final concentration of 10^6 cell/ml, then incubated at 37°C in a moist atmosphere of 5% CO_2. Cells were cultured, under resting or activated conditions, alone or in the presence of the different DR ligands.

After the different incubation periods, PMN cAMP levels were determined with a direct enzyme immunoassay kit (R&D Systems, Mckinley Place, NE) as described by the manufacturer. Briefly, the reaction was terminated by snap-freezing in liquid nitrogen and the cells were stored at −80°C. The optical density was determined using a spectrophotometer set to 450 nm. Values were quantified by using the ABS values of a standard curve and expressed as pmol/10^6 cells.

PMN apoptosis and functional assays

Cell apoptosis

The concentration-range of DA used through the study was chosen according to data of receptor affinity and on data referred to the plasma levels achieved after infusion or on DA levels measured in vivo in different conditions [36–38].

The effect of DA (concentration range: 1 nM–1 μM) on cell viability and apoptosis was evaluated on isolated PMNs resuspended in RPMI medium (1×10^6/ml, cultured for 30 min/3 h at 37°C). Immediately after culture,

TABLE 1 Real-time PCR primers

Gene	UniGene ID	Interrogated sequence *RefSeq/ GenBank mRNA*	Detected coding transcripts	Amplicon context sequence	Chromosome location	Amplicon length	Annealing temperature (°C)	Efficiency (%)
DRD1	Hs.2624	NC_000005.9 NG_011802.1 NT_023133.13	ENST00000329144 ENST00000393752	GACAGGAAACAGGC AGTGAGGATACGAACA GAGAAGTCCCTTCCACCAC CAGCCCAGTCCCGTCCA TGGCAGAGGTGTTCAGAG TCCTCATCTTCCTAAG AGAAAGCACATCA	5:174870011-174870124	84	60	100
DRD2	Hs.73893	NC_000011.9 NG_008841.1 NT_033899.8	ENST00000346454 ENST00000362072 ENST00000544518 ENST00000542968 ENST00000538967 ENST00000355319	AGCAGGGTGACAATGAAGGGCACG TAGAAGGAGACGATGG AGGAGTAGACCACGAAGG CCGGGTTGGCAATGAT GCACTCGTTCTGGTCTG CGTTATTGAGTCCGAAGAGGAG	11:113286246-113287609	83	60	103
DRD3	Hs.121478	NC_000003.11 NG_008842.1 NT_005612.16	ENST00000460779 ENST00000467632 ENST00000295881 ENST00000383673 ENST00000281274	TTAAGGATGCTGGCTGT ACACATCATGACATCCAG GGTGACAAAAACATCAC AGCAAATGCGGCTGAAAT TCCAGACTCCACCTGTCA CCTCCAGGTATACCAC CCAGGGCATCACCAA GGTGGCCACCAGCAAGTC TGCCACAGCCAGGCTCACTA CTAAGTAGT	3:113878626-113890646	136	60	100
DRD4	Hs.99922	NC_000011.9 NG_021241.1 NT_009237.18	ENST00000176183	GCTGTGCTGGACGC CCTTCTTCGTGGTGCA CATCACGCAGGCGCTGTG TCCTGCCTGCTCCGTGC CCCCGCGGCTGGTCAG CGCCGTCACCTGGC TGGGCTACGTCAACAGCG CCCTCAACCCCGTCATCT ACACTGTCTTCAACGCCG AGTTCCGCAACGTCTTCCGCA	11:640411-640580	140	60	98
DRD5	Hs.380681	NC_000004.11 NG_012024.1 NT_006316.16	ENST00000304374	TTTTAAACAGCAGGTTGTG TGTGTGTGCAGTGATGTG GTGGGAGCACAGCTTT CCTGGGTCTGGATTCC CGTGGCTTTGTGCTTA	4:9785451-9785585	105	60	97

(Continues)

TABLE 1 (Continued)

Gene	UniGene ID	Interrogated sequence *RefSeq/GenBank mRNA*	Detected coding transcripts	Amplicon context sequence	Chromosome location	Amplicon length	Annealing temperature (°C)	Efficiency (%)
				TGTCATTTCTTCTCTCTG				
				TGCTGGTGGGGGCCT				
				CTTTACCATAGCTTAAG				
RPS18	Hs.627414	NC_000006.11	ENST00000454021	GTGGAACGTGTGAT	6:33243742-33243838	67	60	98
		NT_007592.15	ENST00000486781	CACCATTATGCAGAA				
		NT_113891.2	ENST00000484321	TCCACGCCAGTACAAGATC				
		NT_167245.1	ENST00000211372	CCAGACTGGTTCTTGAACAG				
		NT_167247.1	ENST00000477055	ACAGAAGGATGTAAAG				
		NT_167248.1	ENST00000476288	GATGGAAAATACA				
		NT_167249.1	ENST00000439602					
			ENST00000474973					
			ENST00000457341					
			ENST00000494232					
			ENST00000434122					

samples were centrifuged (600 g for 5 min at RT) to remove supernatant and washed with 1 ml of PBS. Apoptosis was evaluated by using a FITC Annexin V Detection Kit I (Becton Dickinson) according to the manufacturer's instructions. Briefly, the cells were resuspended in 100 µl of Annexin V Binding Buffer provided in the kit and stained with 5 µl of FITC-conjugated Annexin V (ANX-FITC) and 5 µl of Propidium Iodide Staining Solution (PI) in the dark for 15 min. After incubation, 250 µl of Binding Buffer were added and samples were analysed by BD FACSCanto II Flow Cytometer (Becton Dickinson Italy). PMN were identified based on FSC and SSC properties, and at least 15 000 cells from each sample were collected in the gate. Viable (ANX−/PI−), early apoptotic (ANX+/PI−) and late/necrotic (ANX+/PI+) PMNs were identified on a biparametric plot ANX-FITC versus PI with a log scale and the percentages (%) were calculated by using BD FACSDiva software (version 6.1.3).

Cell migration

Cell migration was measured via the Boyden chamber assay as previously described [35] and published online (dx.doi.org/10.17504/protocols.io.bhrmj546), as per the following experimental design. The chemotactic peptide fMLP (0.1 µM) was placed in the bottom compartment of the chamber, and a 3-mm-pore cellulose nitrate filter (Millipore Corporation) was placed between the two compartments. PMNs were resuspended at 1×10^6/ml in RPMI medium with 0.5% BSA before being placed in the top compartment of the chamber. In experiments with DA or dopaminergic agents, drugs were added on the top of the chamber: DA was used in a concentration range from 1 nM to 1 µM; while the selective D1- and D2-like DR agonists and antagonists SKF-38393, pramipexole, SCH-23390 and haloperidol were purchased by Sigma; the appropriate concentrations of the different ligands were selected according to data from literature and are indicated in the Results section and in the corresponding figures. After an incubation period of 1.5 h at 37°C, the filter was recovered, dehydrated, fixed, and finally stained with haematoxylin/eosin. Finally, migration was measured and quantified microscopically, measuring the distance (in µm) from the filter's surface to the leading front of cells.

ROS generation

Intracellular ROS levels were assessed via the redox sensitive dye C-DCDHF-DA as previously described [35] and published online (dx.doi.org/10.17504/protocols.io.biywkfxe). Briefly, freshly isolated PMN were resuspended

at the concentration of 1×10^6 cells/ml in HBSS medium and incubated for 1 h with 2 μmol/L C-DCFH-DA at 37°C in the dark. Cells were then washed twice with HBSS and centrifuged (400 g, 20°C, 5 min). Measurement of fluorescence was performed by means of a spectrofluorometer (Perkin-Elmer LS-50B, Perkin-Elmer Instruments), with excitation wavelength of 488 nm and fluorescence emission of 525 nm. ROS levels were assayed under resting conditions and after activation with fMLP (0.1 μM).

The ability of DA or dopaminergic agents to affect resting ROS generation by adding the substances into the cuvettes after a resting collection period of 60 s. In experiments with fMLP-activated cells, dopaminergic antagonists were added at 60 s, DA or dopaminergic agonists at 120 s and fMLP at 180 s. ROS generation were always monitored up to 30 min and finally data were analysed and expressed considering the area under the curve of the whole measurement intervals (after the first resting 60 s, the 29 min analysis).

Light microscopy and transmission electron microscopy examination

Neutrophils (5×10^6 cells/ml) were resuspended in RPMI 1640 medium and incubated for 30 min under resting conditions or after fMLP activation and DA or other dopaminergic agents were added according to the same experimental design used for the functional studies.

At the end of the incubation period, the collected pellets were fixed with glutaraldehyde 4% in Na-cacodylate buffer (0.1 M; pH 7.2), washed and post-fixed for 20 min with 1% osmic acid in cacodylate buffer. After standard dehydration in ethanol scale, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultrotome (Leica). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin) and were observed with a light microscope (Eclipse Nikon). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol).

For the ultrastructural analysis of PMNs, scanning electron microscopy (SEM) was used. PMN were resuspended at the concentration of 1×10^6 cells/ml in RPMI medium and incubated for 3 h in resting conditions or in the presence of 0.1 μM fMLP. After incubation, cells were harvested and processed as previously described [35]. Samples were coated with 10 nm of pure gold in a vacuum sputter coater Emitech K550 (Emitech Ltd.) and images were collected in a direct mode using a Philips XL30SEM-FEG scanning electron microscope (SEMXL30FEG).

Animal model

Cell migration in wild-type mice and DRD5KO mice

The ability of DA to affect fMLP-induced migration was investigated in wild-type (WT) and DRD5KO C57BL/6 mice (7–11 weeks old). Animals were sacrificed and bone marrow was obtained from femurs and tibias. Afterwards, cells were homogenized, washed, and neutrophils were purified using a neutrophil Isolation kit (Miltenyi Biotech) according to the manufacturer's instructions. Purity of the obtained neutrophils was analysed by flow cytometry and determined as the percentage of $CD11b^+$ $Ly\text{-}6G^+$; with this procedure, the measured purity was always ≥80%. For migration assays, 5×10^5 neutrophils were deposited inside the top chambers of 5 μm-membrane Transwell® plates. fMLP (5 μM, dissolved in RPMI medium containing 5% BSA) was added to the bottom chamber and the migration of neutrophils was measured as the number of $CD11b^+Ly\text{-}6G^+$ cells reaching the bottom chamber after 90 min of incubation (37°C and 5% CO_2) using the 123count eBeads kit (eBioscience). In some experiments, neutrophils were treated with DA (1 μM) for 3 min just before adding cells in the top chamber of the transwells.

PMN frequency in the spleen

Three female WT (8 weeks old) and DRD5KO (12 weeks old) mice were sacrificed and dissected. After cutting of the right atrium, 50 μl of blood was collected into BD Microtainer tubes (Becton, Dickinson & Company) and the spleen, right femur and tibia were removed and treated as described before [20]. Briefly, the spleens were mechanically disaggregated using 70 μm cell strainer (Falcon, Corning Incorporated) and the bone marrow from tibia and femur was obtained after cutting both ends of each bone and flushing 1 ml of PBS inside the bones using 25G × 5/8″ syringe (BD). Thereafter, erythrocytes were removed after ACK lysis-treatment both in the spleen and bone marrow-derived cells and in blood as well.

Cells derived from blood, spleen and bone marrow were resuspended in 200 μl, 10 ml and 5 ml of PBS, respectively. Thereafter, 200 μl from each sample was used to determine the absolute count of neutrophils by flow cytometry according to 123count eBeads (eBioscience, Thermo Fischer Scientific) guidelines. Before counting, cells were stained with α-CD11b conjugated to Allophycocyanin (clone M1/70) and α-Ly-6G conjugated to Phycoerythrin-Cyanine7 (clone 1A8), both antibodies were purchased from Biolegend.

DR expression and functional studies in PMN from Pts and HS

Expression of DR on PMN of Pts with acute infections and effect of DA on cell functions

To retrieve information on the possible clinical relevance of data obtained from the in vitro and animal models, in a preliminary ex vivo clinical pilot study, we enrolled a group of six Pts and six HS. Ethics approval statement and Pt consent statement are reported.

Study design

After inclusion in the study, Pts with suspected bacterial infection and HS were visited in the morning (8:00–10:00 AM) and after a fasting night. A blood sample (5 ml) was withdrawn for routine analyses; an additional blood sample (5 ml; Visit 1) was used for DR expression measurement and for in vitro functional assays. Only for Pts, after 72 h of antibiotic therapy and positive clinical signs of infection resolution, a second blood sample (Visit 2) was withdrawn to assess parameters measured at Visit 1.

Inclusion criteria

Inclusion criteria for all participants (Pts and HS): 18 < age (years) < 60; confirmation of bacterial infection was obtained for all Pts included in the study by means of clinical examination and biochemical analysis including antibiogram. More in detail, for Pts, a diagnosis of suspected bacterial infection according to the Centers for Disease Control/NHSN Surveillance Definitions for Specific Types of Infections (https://www.cdc.gov/nhsn/pdfs/pscmanual/17pscnosinfdef_current.pdf) and a need for systemic antibiotic therapy.

Exclusion criteria

Exclusion criteria for all participants: infection with human immunodeficiency virus, hepatitis B virus or hepatitis C virus; other underlying causes of immunodeficiency, such as solid organ transplantation, autoimmune, malignant, or other diseases; systemic glucocorticoid treatment, systemic antibiotic treatment, antidepressant treatment or treatment with dopaminergic drugs up to 3 months before enrolment. Additional exclusion criteria only for HS: documented bacterial infections up to 3 months before inclusion in the study. Clinical characteristics of Pts at enrolment are described in Table 2.

Statistical analysis

Data are reported as mean ± standard deviation (SD) of the mean, or median ± 25th and 75th percentile, as appropriate, and n, with n indicating the number of observations. Gaussian distribution was analysed by means of D'Agostino-Pearson omnibus normality test. Mann–Whitney U, Wilcoxon and Kruskal–Wallis tests were used to assess differences in the distribution of non-parametric data. Paired and unpaired t-tests were used, as appropriate, to compare means and SDs for parametric data. Statistical significance for correlations was set at $p < 0.05$. Calculations were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, www.graphpad.com).

RESULTS

In vitro studies

Flow cytofluorometric analysis, mRNA levels and immunocytochemical analysis of DR in human PMN

All DR are expressed on human PMN and their expression can be modulated by activating stimuli both at mRNA expression and membrane level. Whole blood was analysed, by means of flow cytometry, for the presence of the five DR on PMN membrane. The representative flow

TABLE 2 Pts clinical characteristics at enrolment (V1)

Pt code	Sex, age (year)	Infection	Comorbidities	Fever (°C)	Therapy
001	M (40)	Pharyngitis	Asthma; ictus	>39	Ampicillin/sulbactam – moxifloxacin
002	F (69)	Pneumonia	Multinodular goitre	>38	Levofloxacin + ceftriaxone
003	F (30)	Gastroenteritis	None	>38	None
004	M (55)	Urinary infection	Subtotal colectomy	37.8	Cefotaxime
005	F (70)	Soft tissue infection	Infiltrating ductal carcinoma (more than 20 years ago)	38	Dalvabancin
006	F (43)	Urinary infection	Cervical hernia	39	Ceftriaxone

Abbreviation: Pts, patients.

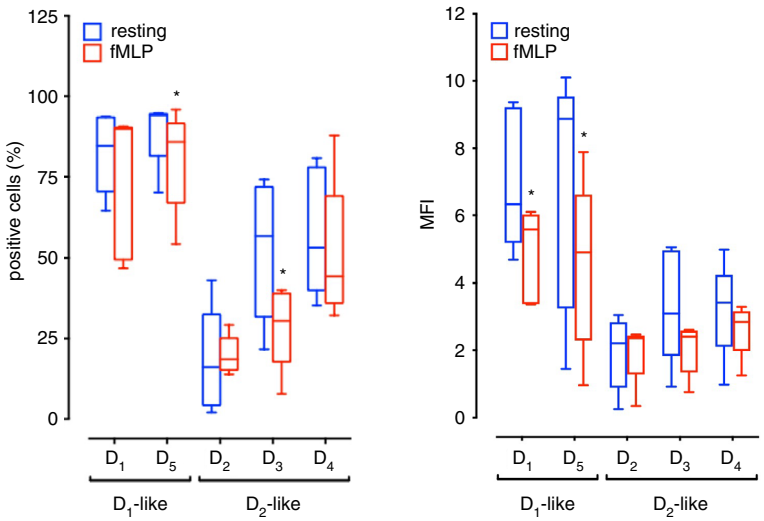

FIGURE 1 Human polymorphonuclear leukocytes express on their surface dopaminergic receptors which are regulated upon activation. * represents the statistical value of the analysis and represent a p value < 0.05

TABLE 3 DR positive cells expressed as percentage of total cells in $n = 5$ different samples

	Resting		fMLP		
	Mean	**SD**	**Mean**	**SD**	***p* vs. resting**
D_1-like					
D_1	82.5	12.3	73.9	22.3	ns
D_5	89.2	10.7	80.5	15.8	0.041
D_2-like					
D_2	17.9	16.1	19.9	5.9	ns
D_3	52.8	21.5	28.8	12.8	0.019
D_4	57.8	19.6	50.9	21.7	ns

Note: Data refer to results shown in Figure 1.
Abbreviations: DR, dopaminergic receptor; ns, not significant.
$p > 0.05$.

cytofluorometric analysis of whole blood in Figure S1 shows expression of all DR on human PMNs.

Figure 1 and Table 3 show that the highest frequency of expression occurs for the D_1-like DR (82%–89%) and the lowest for the D_2-like DR (18%–58%; panels c and d). Activation with fMLP reduces the expression of all DRs, although this reduction was statistically significant only when comparing the percentage of positive cells for D5 and D3 DRs (left) and MFI for D1 and D5 DRs (right). Table 3 reports the differences between values measured under resting and activated conditions.

Figure 2 shows the expression in PMN of detectable mRNA levels for all the DRs measured both as absolute values (left panel) and ratio of DR mRNA levels between fMLP-stimulated and resting cells. The order of magnitude was: $D_4 > D_5 > D_1 = D_3 >> D_2$. Activation with fMLP increased DRD_1 and decreased DRD_5 and all the D_2-like DRs.

Finally, the presence of both DRD1 and D5 (D1-like family) on PMN was investigated immunocytochemically. As shown in Figure 3, immunocytochemistry shows that DRD1 (panel a) was expressed in most PMN under resting conditions and after activation with fMLP (0.1 μM, 30 min; right), while DRD5 (panel b) was expressed under resting conditions (left) and this expression seemed to be weakened by cell activation (right) in agreement with results obtained by flow cytometry.

Endogenous DA in cultured PMN, cell viability and apoptosis

PMN produce endogenous DA and this production is affected by cell activation, and exogenous DA in the micromolar range does not affect PMN apoptosis. The relevance of dopaminergic regulation of human PMN was investigated on some key functions of these cells. The presence of DA in PMN was established by HPLC analysis showing that cell pellet and cell medium contain a significant DA amount both under resting and activated conditions (fMLP 0.1 μM; 3 h). Intracellular content significantly increases after activation and values were respectively 0.21 ± 0.12 pmol/10^6 cells under resting and 1.02 ± 0.41 pmol/10^6 cells after activation ($n = 7$; $p < 0.05$); DA in culture medium was unaffected by cell activation (resting: 0.05 ± 0.03 pM and activated: 0.04 ± 0.03 pM, $n = 7$, $p > 0.05$).

The ability of DA to affect cell viability was measured by means of flow cytometry. In particular, viable (ANX−/PI−), early apoptotic (ANX+/PI−) and late/necrotic (ANX+/PI+) cells were analysed by propidium iodide staining. DA treatment (concentration range: 1 nM − 1 μM), for either 30 min or 3 h, did not affect the percentage of viable, early, and late apoptotic or necrotic PMNs (Table 4).

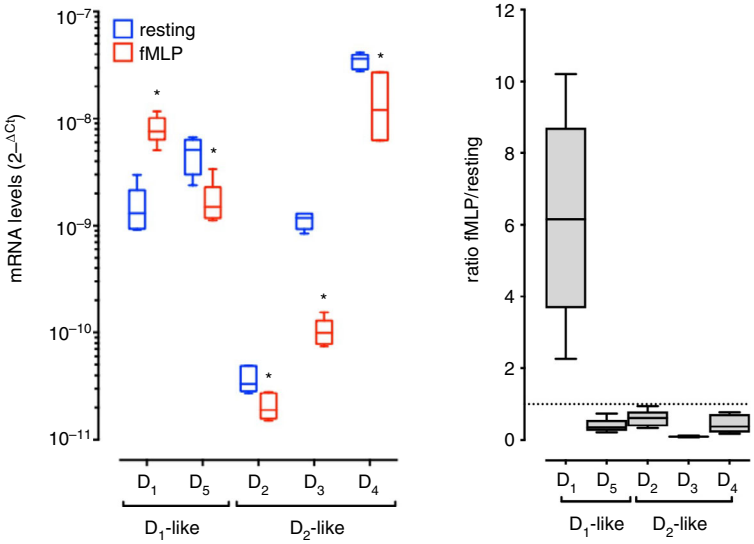

FIGURE 2 mRNA levels of dopaminergic receptors on human polymorphonuclear leukocytes. * represents the statistical value of the analysis and represent a p value < 0.05

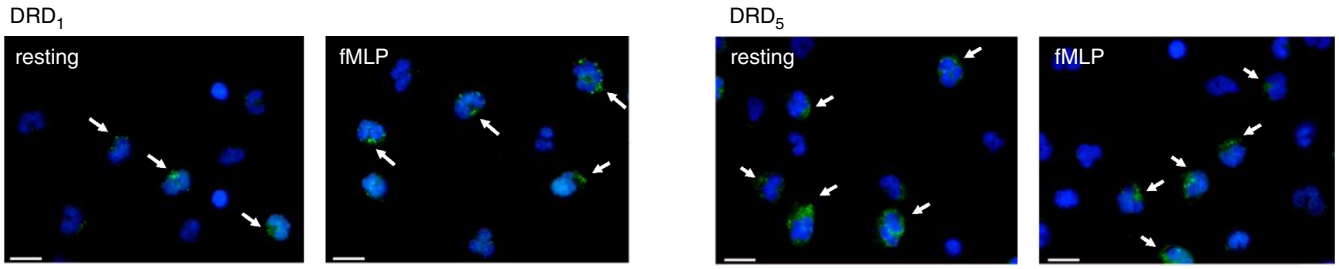

FIGURE 3 Immunostaining of D1-like dopaminergic receptors on human polymorphonuclear leukocytes. The arrow indicates cells that are modified by the treatment

TABLE 4 Effect of DA on cell viability and apoptosis in in vitro studies

	30 min (% of positive cells)				3 h (% of positive cells)			
	ANX−/PI−	ANX+/PI−	ANX+/PI+	ANX−/PI+	ANX−/PI−	ANX+/PI−	ANX+/PI+	ANX−/PI+
Control	94.26 ± 2.00	5.33 ± 2.40	0.77 ± 0.28	0.46 ± 0.23	92.37 ± 1.37	6.92 ± 1.07	0.56 ± 0.30	0.15 ± 0.07
DA 1 nM	93.73 ± 1.70	4.98 ± 2.05	0.72 ± 0.26	0.56 ± 0.35	92.60 ± 1.70	6.33 ± 1.70	0.89 ± 0.14	0.19 ± 0.08
DA 10 nM	92.85 ± 2.67	5.72 ± 3.10	0.84 ± 0.14	0.58 ± 0.44	91.49 ± 2.69	7.46 ± 2.47	0.86 ± 0.29	0.19 ± 0.07
DA 0.1 μM	93.50 ± 2.25	5.14 ± 2.64	0.88 ± 0.30	0.48 ± 0.31	92.21 ± 1.55	6.71 ± 1.33	0.90 ± 0.26	0.19 ± 0.05
DA 1 μM	93.61 ± 3.07	5.23 ± 2.99	0.60 ± 0.14	0.56 ± 0.22	91.55 ± 1.12	7.26 ± 1.03	0.99 ± 0.21	0.20 ± 0.09

Note: Effect of DA on PMN viability (ANX−/PI−), early apoptosis (ANX+/PI−), late apoptosis (ANX+/PI+) and necrosis (ANX−/PI+) measured by flow cytometry and identified on a biparametric plot ANX-FITC vs. PI with a log scale and the percentages (%) were calculated using BD FACSDiva software. PMN were cultured for 30 min or 3 h alone or in the presence of increasing concentrations of DA. PMNs were identified based on FSC and SSC properties, and at least 15 000 cells from each sample were collected in the gate. Data are expressed as % of total cells and are represented as means ± SEM of three separate experiments. Friedman test statistical analysis was performed for each incubation time.
Abbreviations: DA, dopamine; FSC, forward-scatter; PMN, polymorphonuclear leukocytes; SSC, side-scatter.

Cell migration

Cell migration is a key process through which PMN migrate into tissues and occurs both under physiological conditions and after release into the tissues of proinflammatory stimuli that act as chemoattractants for cells. The results show that DA and dopaminergic ligands profoundly affect this key function. More in detail, treatment with DA, at all tested concentrations (1 nM − 1 μM), was unable to affect spontaneous migration (Figure 4). On the contrary, migration induced by activating stimuli such as fMLP (which, as expected, induced a significant increase of PMN migration; Figure 4a) was significantly prevented in a concentration-dependent way by pretreatment with (Figure 4a).

To establish which DR were involved in this effect, we investigated the ability of D1-like and D2-like DR selective

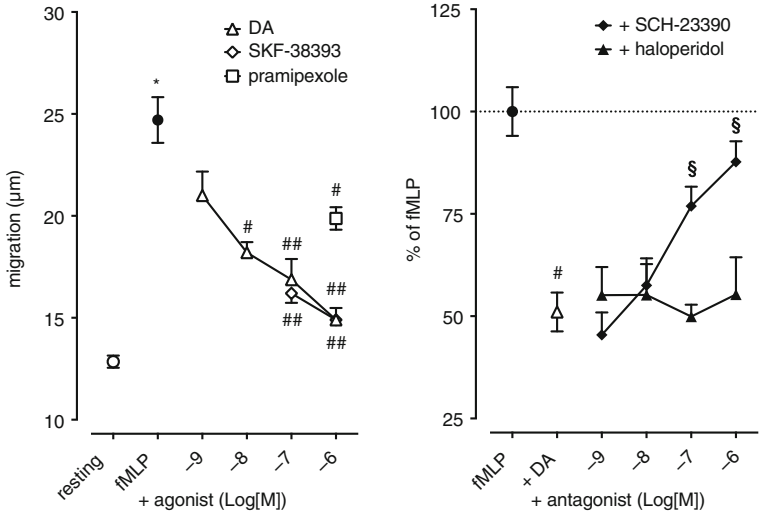

FIGURE 4 Effect of dopamine and dopaminergic agents on polymorphonuclear leukocytes migration. * is *p* value vs resting while # vs activated conditions

antagonists to prevent dopaminergic inhibition of fMLP-induced migration. As shown in Figure 4, the effect of DA on fMLP-induced migration was unaffected by the D_2-like antagonist haloperidol (1 nM − 1 µM) while it was concentration-dependently reverted by the D_1-like antagonist SCH-23390 (Figure 4b); remarkably, haloperidol, per se, did not affect spontaneous migration (14.4 ± 1.0 µm in cells alone vs. 12.4 ± 0.5 µm in cells co-incubated with haloperidol, $n = 5$, $p > 0.05$), nor fMLP-induced migration (21.5 ± 3.5 µm in cells treated with fMLP vs. 22.3 ± 4.9 µm in cells treated with fMLP in the presence of haloperidol, $n = 3$, $p > 0.05$), nor did SCH-23390 on spontaneous migration (SCH-23390 1 µM: 12.3 ± 2.5 µm vs. 12.3 ± 0.8 µm, $n = 5$; $p > 0.05$) or on fMLP-induced migration (26.7 ± 4.5 µm in cells treated with fMLP vs. 23.2 ± 2.3 µm in cells treated with fMLP in the presence of SCH-23390, $n = 3$, $p > 0.05$).

Treatment with D_2-like agonist pramipexole (1 µM), did not influence spontaneous cell migration (12.9 ± 1.4 µm; $n = 4$; $p > 0.05$ vs. resting) and only slightly prevented fMLP-induced migration (panel a), while the D_1-like agonist SKF-38393 was unable to affect spontaneous migration ($p > 0.05$ for all tested concentrations), but significantly prevented fMLP-induced migration (panel a) and this effect was superimposable to the effect of DA. The effect of SKF-38393 was completely reverted by pre-treatment with SCH-23390 (27.6 ± 6.1 µm; $p < 0.05$ vs. SKF-38393 + fMLP) but not with haloperidol (21.6 ± 1.3 µm; $p > 0.05$ vs. SKF-38393 + fMLP).

ROS production

ROS generation is a mean for PMN to respond to infectious or inflammatory stimuli and neutralize pathogens [22]. DA and dopaminergic ligands can interfere with this function and, interestingly, no effects were observed in cells under resting conditions while activation of D1-like DR induced a

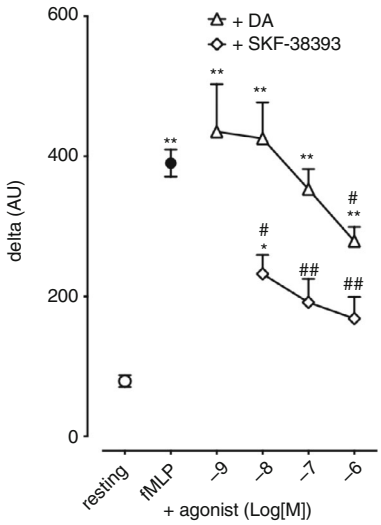

FIGURE 5 Dopaminergic modulation of reactive oxygen species production in human polymorphonuclear leukocytes. * is *p* value vs resting while # vs activated conditions

profound inhibition of cell activation. DA up to 1 µM did not affect resting ROS production, while significantly preventing fMLP-induced ROS production in a concentration-dependent manner (Figure 5). The effect of DA on fMLP-induced ROS generation was mimicked by the D_1-like agonist SKF-38393 (which per se did not affect spontaneous ROS generation), while the D_2-like agonist pramipexole (1 µM) did not influence either spontaneous (129.38 ± 56.68 Δ; $n = 4$, $p > 0.05$ vs. R), or fMLP-induced ROS production (265.6 ± 64.6 Δ, $n = 4$, $p > 0.05$ vs. fMLP alone).

cAMP production in cultured PMN

The increase in cAMP was directly related to D1-like DR activation while, on the contrary, cAMP inhibition was the consequence of D2-like DR activation. We previously

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showed DA can activate cAMP increase in human lymphocytes, and in the present study we used the same experimental strategy [39]. As shown in Figure 6, cAMP levels were very low in both resting and fMLP-activated PMN up to 5 h (Figure 6, left). DA and SKF-38393, but not pramipexole, significantly increased cAMP production and such an increase was time dependent, with the strongest increase after a 5 h incubation (Figure 6, left). fMLP significantly prevented the increase in cAMP induced by both DA and SKF-38393, without affecting cAMP levels induced by pramipexole (Figure 6, right). In particular, the time-dependent cAMP increase was completely abolished in the case of DA and strongly reduced in the case of SKF-38393 (Figure 6, right).

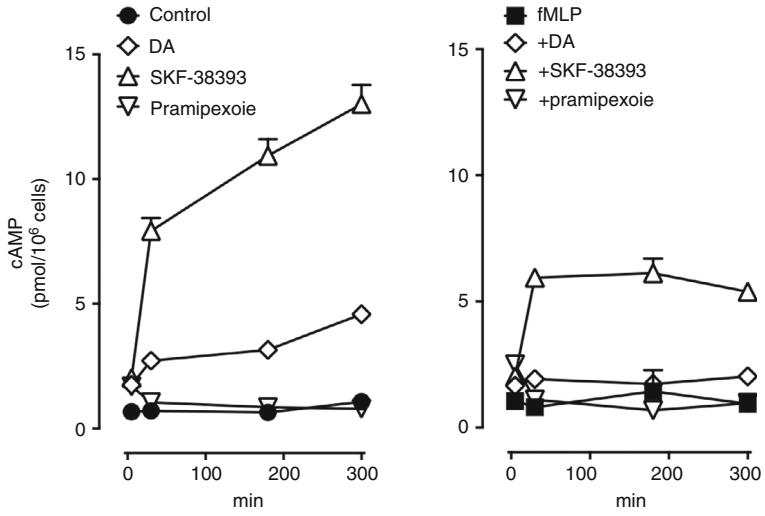

FIGURE 6 Dopaminergic modulation of human polymorphonuclear leukocytes functions is exerted through activation of D1-like dopaminergic receptors coupled to intracellular cAMP increase.

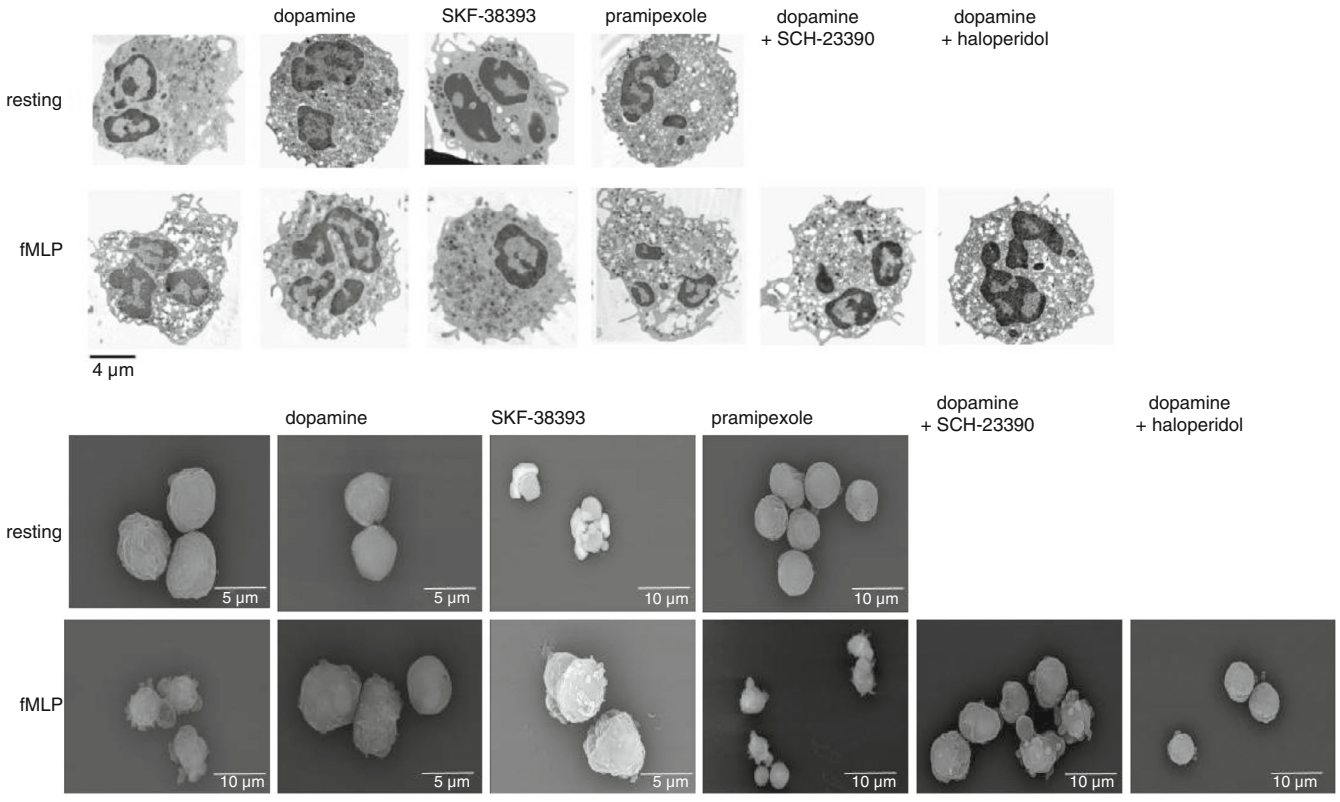

FIGURE 7 Transmission electron microscopy and scanning electron microscopy analysis of changes induced by dopaminergic agents on polymorphonuclear leukocytes morphology.

Ultrastructural analysis and SEM

Qualitative transmission electron microscopy and SEM analysis confirmed the functional data showing the presence of DR and a significant change in morphology following D1-like DR activation. As shown in Figure 7, unstimulated PMN showed a typical morphology which was unaffected by DA, SKF-38383 and pramipexole (Figure 7, lines 1–2). Activation with fMLP profoundly affected cell morphology (line 2). Pre-treatment with DA and SKF-38393, before activation with fMLP, partially prevented the effects induced by fMLP, while pramipexole had no effects on fMLP-induced changes. Since DA and other DR agonists alone did not affect the morphology of resting PMN, no experiments were performed with DA + SCH-23390 or haloperidol under resting conditions while we investigated the ability of DA to prevent fMLP-induced morphological changes alone or after pre-treatment with DR antagonists. As shown in Figure 7 (lines 1 and 2), pre-treatment with SCH-23390 counteracted the effects of DA on the morphological changes induced by fMLP while, on the contrary, pre-treatment with haloperidol did not exert noticeable effects (line 2).

SEM analysis (Figure 7, lines 3–4) showed no qualitative major morphological differences between PMN alone or in the presence of dopaminergic agonists (line 3); stimulation with fMLP induced morphological changes (line 4). The presence of DA or SKF-38393, before fMLP activation, prevented fMLP-induced changes while pramipexole exerted no effects. SCH-23390, but not haloperidol, prevented the effects of DA (line 4).

Ex vivo studies on DRD5KO mice

PMN frequency in bone marrow, blood and spleen in WT and KO mice

The involvement of the D1-like DR family in the effects of DA on PMN was investigated in a murine model KO

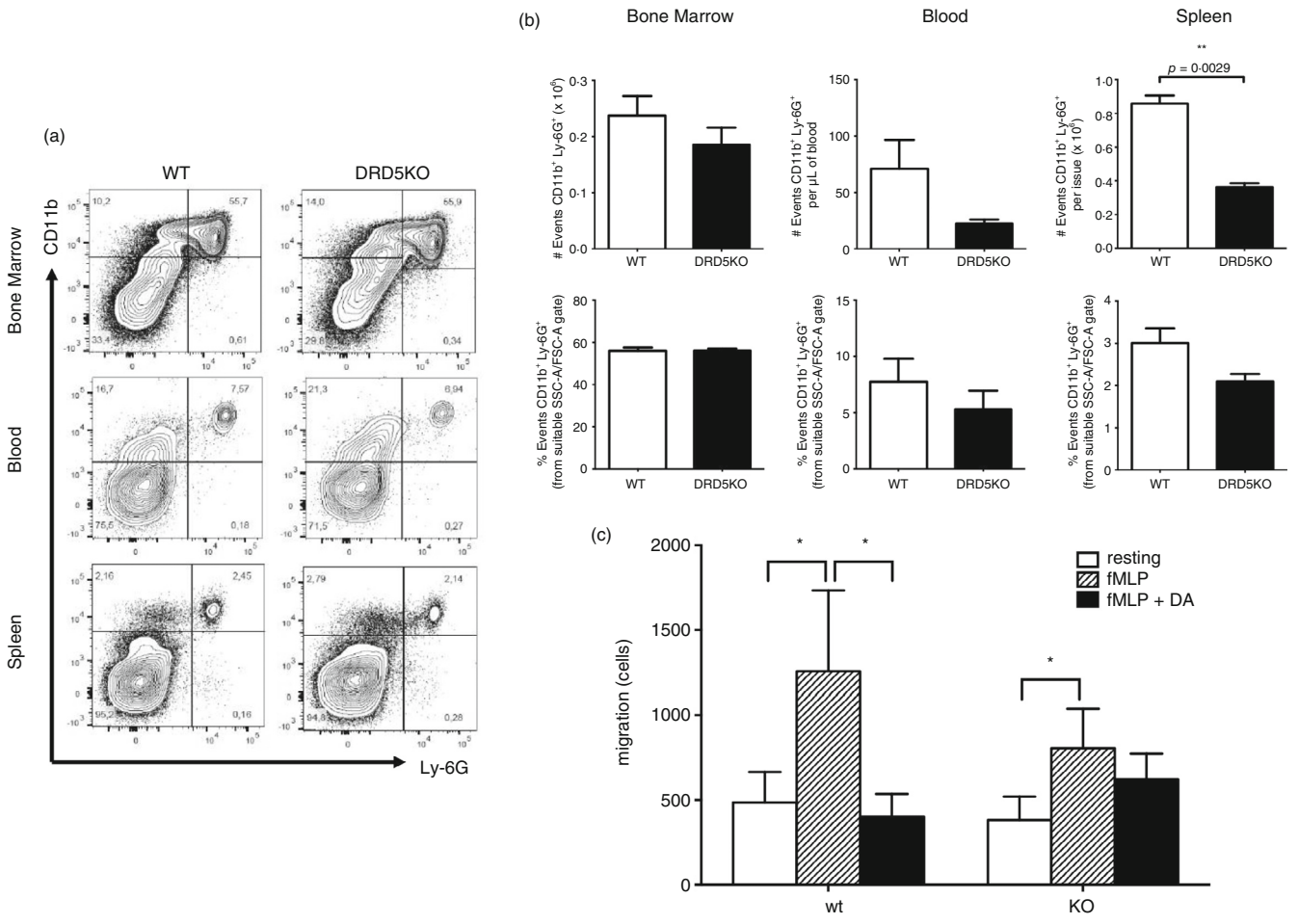

FIGURE 8 DRD5 deficiency results in reduced polymorphonuclear leukocytes (PMN) in the spleen of and failure of dopamine to inhibit fMLP-induced PMN migration in DRD5KO mice. Panel a is the gating strategy, while panel C represent the values obtianed in cell migration

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for DRD5 (DRD5KO). PMN counts in bone marrow, blood, and spleen of DRD5KO mice showed significantly lower cell numbers only in the spleen (Figure 8a,b).

TABLE 5 Clinical parameters of patients (Pts) before (V1) and after (V2) the pharmacological treatment

	V1	V2	*p*
Sex: 3 F /2 M			
Age (years): 55 ± 14			
Leukocytes (10^9/L)	10.14 ± 2.09	7.14 ± 1.18	0.040
Erythrocytes (10^9/L)	4.66 ± 0.40	4.36 ± 0.45	ns
Haemoglobin (g/dl)	10.76 ± 4.32	12.10 ± 2.23	ns
Haematocrit (%)	99.50 ± 5.60	160.30 ± 47.0	ns
Platelets (10^9/L)	211.60 ± 42.72	239.0 ± 71.16	ns
CRP (mg/L)	182.40 ± 76.85	55.54 ± 52.14	0.034
CK (U/L)	125.30 ± 54.20	133.0 ± 70.80	ns
Creatinine (mg/dl)	0.92 ± 0.12	0.88 ± 0.04	ns
Urea (mg/dl)	35.17 ± 10.15	35.67 ± 2.08	ns
Bilirubin (mg/dl)	10.50 ± 7.30	11.40 ± 3.30	ns
AST (U/L)	37.20 ± 25.39	24.75 ± 12.55	ns
ALT (U/L)	30.50 ± 23.07	34.50 ± 25.68	ns
Alfa amilase (U/L)	34.67 ± 24.24	59.0 ± 29.70	ns
LDH (U/L)	213.20 ± 46.05	178.80 ± 42.17	ns
Na^+ (mmol/L)	140.0 ± 3.03	140.0 ± 3.54	ns
K^+ (mmol/L)	3.72 ± 0.36	3.97 ± 0.44	ns
Ca^{++} (mg/dl)	8.70 ± 0.45	6.98 ± 2.93	ns

Note: Data are expressed as mean ± SD. Statistical analysis was performed using Wilcoxon test.
Abbreviation: ns, not significant.

Effect of DA on fMLP-induced cell migration

The ability of DA to affect PMN migration was investigated in DRD5KO mice. The ability to spontaneously migrate in cells obtained by whole blood of both WT and DRD5KO mice was superimposable; similarly, no differences were observed in fMLP-induced migration. On the contrary, the ability of DA (1 μM, the same concentration showed to be active in human PMN) to prevent fMLP-induced migration was observed in PMN from WT mice but not from DRD5KO mice (Figure 8c).

Ex vivo studies on Pts and HS

We enrolled six Pts and six HS; as shown in Table 2, one of the six enrolled Pts was dropped from the study because clinical symptoms disappeared before therapy was started. Biochemical data, reported in Table 5, shows Pts have reduced leukocyte counts and CRP levels at V2 in comparison with V1 confirming that Pts were in acute phase of infection at enrolment, while the reduction of CRP and leukocyte count observed at V2 confirms the resolution of the infection (Table 5).

Flow cytofluorometric expression of DR in PMNs of Pts and HS

In fresh whole blood from Pts and HS, we investigated the membrane expression of DR on PMN by means of flow cytometry, showing that DR expression is affected

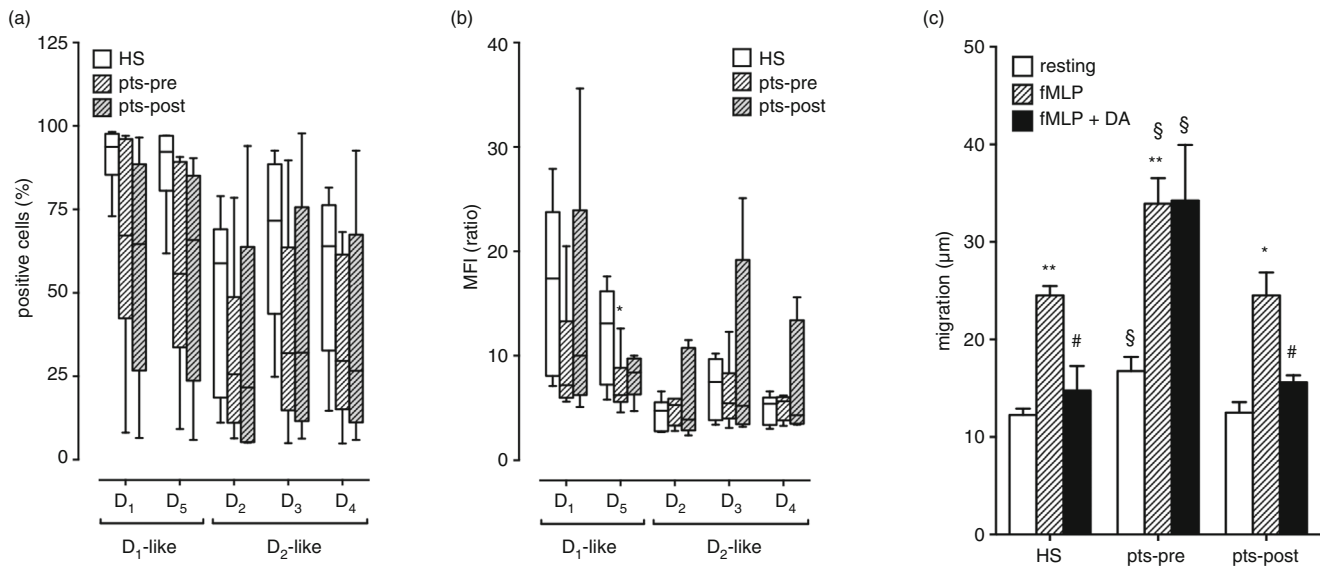

FIGURE 9 Polymorphonuclear leukocytes from patients with acute infections express dopaminergic receptors to a similar extent as cells from healthy subjects, however, dopaminergic inhibition of fMLP-induced migration is suppressed in the acute phase and restored after recovery. *is *p* value vs resting while # vs activated conditions

TABLE 6 ROS generation in HS and Pts

		Pt ($n = 5$)	
	HS ($n = 6$)	V1	V2
Resting	49.4 ± 24.8	81.0 ± 60.0	74.3 ± 65.3
DA	48.0 ± 24.8	68.6 ± 49.4	66.3 ± 51.3
fMLP	196.8 ± 97.6**	216.2 ± 140.5	238.9 ± 207.9
DA + fMLP	135.3 ± 78.7*$	178.1 ± 155.9	207.1 ± 195.8

Note: Effect of treatment with DA on ROS generation measured in isolated PMN from HS and Pt before (V1) and after (V2) antibiotic treatment. Data expressed as mean ± SD. Statistical analysis was performed using Mann–Whitney or Wilcoxon test.

Abbreviations: DA, dopamine; HS, healthy subjects; PMN, polymorphonuclear leukocytes; Pt, patient; ROS, reactive oxygen species.

*$p < 0.05$; **$p < 0.01$ vs. respective R; $^{\$}p < 0.05$ vs. respective fMLP.

by clinical conditions. As shown in Figure 9a,b, all DR were expressed on cells of both HS and Pts. In comparison with HS, Pts at V1 show lower DRD5 expression both as percentage of positive cells (panel a) or MFI (panel b). Such differences in PMN from Pts disappeared at V2.

Effect of treatment with DA on cell migration and ROS generation

We investigated the ability of DA to affect key PMN functions such as migration and ROS generation in Pts during bacterial infections (untreated, V1) and after antibiotic therapy (V2), applying the same experimental design used for the in vitro study. Our results (Figure 9c) show that cell migration was higher in Pts at V1 in comparison with HS and decreased at V2 after recovery.

Activation of PMN with fMLP induced a strong increase in migration and this effect was observed both in HS and in Pts (at both V1 and V2). Pre-treatment with DA prevented fMLP-induced migration in HS but not in Pts at V1, during the acute phase of infection, while at V2 the sensitivity of PMN towards the inhibitory effects of DA on fMLP-induced migration was restored.

In contrast with migration, ROS generation did not differ between Pts at both visits and HS (Table 6). DA was able to prevent fMLP-induced increase in ROS generation in HS (Table 6), while this effect, although present, was never significant in Pts at both visits (Table 6).

DISCUSSION

The present study provides extensive experimental evidence indicating the expression, on cell membranes and at the mRNA levels, of all DR types in human PMN, and for the first time, to the best of our knowledge, a detailed analysis of the effects of cell activation on DR expression and function. Treatment with DA profoundly affects PMN functions, mainly involving the D_1-like DR family, as suggested by the ability of SCH-23390 (a D_1-like DR antagonist) to revert, and of SKF-38393 (a D_1-like DR agonist) to mimic, the effects induced by treatment with DA. The key role of DA in the modulation of PMN functions, and specifically the involvement of the D1-like DR family in the observed effects is also supported by preliminary data from DRD5KO mice, in which treatment with DA was unable to prevent fMLP-induced PMN migration, while this effect was maintained in cells from WT mice. The concentration range of DA used in this study is in line with DA affinity for DR as well as with levels of this neurotransmitter in in vivo conditions. Data from literature show that DA levels are dramatically different in distinct tissues, ranging from 1 nM to 10 pM in blood to 1–10 μM in tissues such as bone marrow, small intestine, kidney, etc. Moreover, several disease conditions can modify DA concentration in tissues and organs [37]. Finally, data obtained in the ex vivo study in PMN from Pts with bacterial infections, which showed a different expression pattern for membrane DR and a different profile in the response to DA, in comparison with cells from HS, corroborated the involvement of the D1-like DR family in dopaminergic modulation of PMN functions in health and disease.

Dopaminergic regulation of innate immunity: 'state-of-the-art'

The ability of DA to affect innate immune cells in general, and PMN in particular was recently reviewed [40]. The key role of PMN in human pathology encompasses not only inflammatory syndromes but also immune-mediated diseases at large, including those affecting the central nervous system (CNS) [41]. Interestingly, some immune-mediated diseases affecting the CNS present a dysregulation in dopaminergic pathways [10, 11, 42]. Similarly, a role of dopaminergic pathways was postulated in other kinds of conditions outside the CNS, such as visceral obesity [43] or airways inflammation [29]. A close interconnection between the nervous and immune systems is now largely recognized, and the ability of DA to affect the adaptive branch of immunity is well established [4, 44]. On the contrary, data regarding dopaminergic modulation of PMN functions and in general of innate immunity is still limited [21, 43].

DR expression and DA content in human PMN

So far, the presence of DR on human PMN was only sporadically investigated and, in some cases, reported data are contradictory [2, 27, 45–47]. Our results showed the expression of both mRNA and proteins for all DR in human PMN, with highest mRNA expression values for DR D_4 and lowest for DR D_2. On the contrary, considering protein membrane expression, we found the highest values for D_1-like DR and the lowest values for D_2-like DR.

Activation of PMN affected the expression of some DR subtypes both at the mRNA and protein levels. We observed a significant increase in mRNA expression for DR D_1 and a reduction for DR D_5 as well as for the whole D_2-like DR family. On the contrary, membrane receptor expression decreased after activation for all DR.

Activation-induced DR changes were however minor, and the effect of DA on activated PMN clearly indicates the occurrence of functional DR on activated PMN as well as their physiological relevance. D1-like DR expression is also supported by results of immunocytochemistry. Finally, the present study confirms our previous data showing that DA [8] is present in PMN and, for the first time, shows that cell activation leads to a significantly increase in intracellular DA content.

The ability of DA, depending on used concentrations, to affect immune cell functions and the finding that cell content can change depending on the surrounding conditions sheds new light on the immunomodulatory role of DA. Many clinical conditions affect intracellular CA content as shown for example in PBMC of multiple sclerosis (MS) Pts and that treatment with drugs such as interferon beta is able to modulate this content [15]. The present findings extend current knowledge about the ability of immune cells to produce and utilize CA and in particular DA.

Preferential involvement of D1-like DR in PMN functional responses

We established that DA exerts profound effects on the functions of activated PMN, without any proapoptotic effects, although evidence from literature shows increased apoptosis in presence of high concentrations of DA [38, 48]. In our experiments, DA affects many key PMN functions, such as a ROS production and cell migration as reported in previous studies [31–34] supporting the hypothesis of DA acting as a neuroimmunetransmitter, affecting innate immune response through activation of its receptors. Interestingly, our data show also that DA and selective DR agonists can interfere with these functions through the activation of D1-like DR, while dopaminergic agents acting on D2-like DR were unable to exert any effects. Considering our results on cell viability and apoptosis, we can exclude a reduction of cell viability to be the cause of the inhibitory effects exerted by DA on PMN functions.

Investigating which kind of receptors were involved in the above-described effects, we started by showing DA and the D1-like DR agonist SKF38393, but not pramipexole (a D2-like DR agonist), were able to stimulate cAMP, suggesting the D1-like DR family to play a major role. Likewise, involvement of the D_1-like DR family was suggested by the ability of the D1-like DR antagonist SCH-23390, but not of haloperidol (a D_2-like DR antagonist) to revert DA-induced effects. Nevertheless, we cannot discriminate whether the observed effects are DR D1 or DR D5 mediated, since current pharmacological tools do not allow a clear-cut discrimination between these receptors.

The possible clinical relevance of such effects, which can be considered as anti-inflammatory, is also supported by current knowledge about the inflammasome activity [49] as well as by the notion that inflammatory conditions can decrease DA synthesis and storage in neurons, leading to depressive conditions [50].

The relevance of DR in regulating innate immunity is also sustained by data showing that DA dysregulation is involved for example in visceral obesity [43] or more in general that DA represents a key signal connecting monocytes/macrophages, adipocytes and sympathetic nerve terminals [40]. DA is, therefore, likely to be involved in the genesis of several diseases including metabolic diseases or depression in the CNS as well as in peripheral tissues [51, 52].

The relevance of DR dysregulation for example is established in adaptive immune cells in CNS diseases where DRD5 mRNA expression in isolated regulatory T lymphocytes are predictive of MS in Pts with clinically isolated syndrome [53] and in PBMC are predictive of MS progression [16]. Moreover, in PD D1-like DR membrane expression in T lymphocytes is downregulated in comparison to cells from HS [11].

Ex vivo studies in KO mice

Data obtained from the ex vivo animal model support the hypothesis of a direct involvement of DR-operated pathways in the observed ability of DA to affect PMN functions and of a predominant involvement of the D_1-like DR family (in particular DR D5) in agreement with other studies showing a dysregulation of DR in different human diseases such as MS or PD [11, 15, 18, 19, 21, 53].

In PMN isolated from the bone marrow of DRD5KO mice we showed that DA lacked the ability to prevent fMLP-induced migration while, on the contrary, this ability was preserved in cells from WT mice; additionally, the key functional role of dopaminergic pathways was suggested also by the reduced PMN count in spleens from DRD5KO mice. These data are in line with those obtained in the in vitro part of this study performed on human PMN and underlines the relevance of dopaminergic pathways, in particular those operated by D1-like DR, in the regulation of PMN.

Ex vivo study in Pts with bacterial infections

Interestingly, data obtained in the in vitro study and in the animal model were supported by results of experiments on PMNs from Pts with acute bacterial infections, where we showed the expression of DRD5 to be lower in Pts with acute disease, while such differences disappeared upon recovery after antibiotic treatment. The recovery of clinical symptoms related to infective status is sustained by data on CRP and leukocyte count, which were superimposable at V2 with data obtained in HS. We would be tempted to interpret this set of results as suggestive for the occurrence of a temporary deactivation of the physiological dopaminergic inhibition of PMN function during bacterial infections, when PMN are required to be more responsive and to fully exert their anti-bacterial potential. On the contrary, upon recovery the dopaminergic inhibitory tone is re-established as part of a general anti-inflammatory programme in order to limit excessive inflammation and eventual tissue damage. DA dysregulation indeed occurs in several human diseases in cells of adaptive immunity [11, 15, 53], as well as of innate immunity. Interestingly, studies on animal models show that infective conditions reduce DAβ hydroxylase activity [54] and that viral disease such as HIV infection can affect DA pathways [55].

Limitations of the study

Although this study, thanks to in vitro and in vivo preclinical investigations and to an explorative clinical part on Pts, gets new light on the immunomodulatory role of DA, it is important to remind that the study on Pts was performed only in an acute condition and only during an infective status (six Pts) and with ex vivo experiments. An investigation in chronic immune-mediated diseases of the effect of DA on PMN functions could help to clarify if dopaminergic pathways can be compromised or if this picture is characteristic only of acute infection.

CONCLUSIONS

The potential clinical implications of dopaminergic modulation of PMN are of the utmost importance, considering the wide range of available drugs acting through activation/inhibition of DR and used in different kinds of diseases; DR are also differently expressed on tumoral cells, thus pointing at dopaminergic drugs as potential therapeutic strategies in different types of cancer [13, 40, 56]. DA itself is notably a widely used therapeutic drug, especially in intensive care units, therefore understanding the immunomodulatory role of these key neurotransmitters is of extreme relevance considering the critical conditions of Pts that are typically treated with this drug or more in general the wide use of drugs acting on DR (e.g., antiparkinson drugs) used in the clinical setting. In this regard, it is important to note that the range of concentrations used in the clinical setting can have negative effects on immune functions as reported in preterm infants [57] or in other clinical conditions of systemic infection [58].

Finally, the present results on the dopaminergic modulation of human PMN functions suggest a new vision on the role of dopaminergic dysregulation in the genesis of some infective and immune-mediated disorders and represents a new opportunity for further studies aimed at clarifying whether these findings can help in promoting new therapies, counteracting PMN activation and tissues invasion, which are known to be the first step in the host defence against pathogens but also contribute to tissue damage during chronic inflammation.

AUTHOR CONTRIBUTIONS

Study conception and design: Franca Marino and Marco Cosentino. *Acquisition of data*: Monica Pinoli and Alessandra Luini (human PMN functional assays), Emanuela Rasini (flow cytometry assays), Laura Pulze and Magda de Eguileor (immunocytochemistry and fluorescence microscopy), Daniela Dalla Gasperina and Paolo Grossi (clinical assessment of patients), Massimiliano Legnaro (real-time PCR assays), Marco Ferrari (HPLC assays), Terenzio Congiu (electron microscopy assays), Rodrigo Pacheco and Francisco Osorio-Barrios (in vivo and ex vivo animal models). *Analysis and interpretation of data*: Franca Marino, Stefano Martini and Marco Cosentino (all data), Emanuela Rasini (flow cytometry assays), Laura Pulze and Magda de Eguileor (immunocytochemistry and fluorescence microscopy), Rodrigo Pacheco and Francisco Osorio-Barrios (in vivo and ex vivo animal

models). 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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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

DATA AVAILABILITY STATEMENT

All collected data for this study will be made available upon request; all applied protocols are also available.

ETHICS STATEMENT

This study was approved by local Ethics Committee (protocol number: 0051885 submitted on 12 May 2016, with final approval on 19 July 2016).

PATIENT CONSENT STATEMENT

The study was performed on subjects (both Pts and HS) enrolled by 'Clinic of Infective and Tropical Diseases' of the 'Ospedale di Circolo e Fondazione Macchi', (ASST Sette Laghi – Varese, University of Insubria, VA, Italy); all subjects (HS and Pts) gave an informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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