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EFFECTS OF DOPAMINERGIC PATHWAYS ON HUMAN NEUTROPHIL

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Summary

The existence of a bidirectional communication between the immune system and the central nervous system was postulated some years ago by different researchers. More recently some evidence supports the notion that immune system can be affected by dopamine (DA).

DA is a neurotransmitter of the central nervous system that exerts its effects through the activation of the five dopaminergic receptors (DR). DA can affect some functions of the cells of the immune system and this topic was widely investigated on the cells of adaptive immunity.

Therefore, we decided to focus our attention on the different cell populations of the innate immunity and to explore the data present in literature about the evidence of the existence of a dopaminergic regulation of these cells.

The first part of the thesis is a description of dopamine and of the dopaminergic system, with reference to interactions with the immune system, in particular the innate immunity. Moreover, in the last part of this first chapter are mentioned some diseases involving the innate immunity in which the role of dopaminergic pathway was postulated and in some case demonstrated.

The second chapter is devoted to the characterization from the physiological point of view of the other major actors of the work, neutrophils (PMN). Also in this case, at the end of the chapter there is a section dedicated to the relevance of PMN in diseases in which the immune component is relevant.

The third chapter represents the main results of my PhD project, based on the investigation of the role and relevance of the dopaminergic system in human neutrophils. The aim of this PhD research program was in fact, to characterize the presence of DR and if dopaminergic agent can affect some pivotal function of neutrophil in a receptor-dependent manner.

Finally, a last chapter resumed the other projects that I have followed during the three year of my PhD course. The two attached files represent the results of some of them, that were conclude and published.

Chapter 1

The data collected in this chapter will be included in the preparation of a review that I'm writing for the submission to an International peer-reviewed journal.

Dopamine and innate immunity

Dopamine (DA) is a neurotransmitter of the central nervous system (CNS) involved in the control of several key functions. The first idea about a possible relationship between CNS and immune system appear in the eighties (Devoino et al., 1988; Roszman & Brooks 1985), but only some years later, this concept was sustained by specific experimental studies and reviews investigating this issue are elegantly reported by Basu and coworkers (Basu et al., 2000). Further studies strengthened this aspect were published reporting the evidence that immune cells are able to release DA and are equipped with the enzymes necessary for the synthesis, storage and metabolism of DA (Basu et al., 1993; Bergquist et al., 1994; Marino et al., 1999; Cosentino et al., 2000) and by the identification of dopaminergic receptors (DR) on the different immune cells (Sarkar et al., 2010; Kustrimovic et al., 2014). On the basis of all these evidence, DA is now defined as "NeuroImmunotransmitter" thanks to its ability to modulate the functions of different cells of the immune system (Levite 2015).

In the last decades, several lines of evidences have elucidated the key role of DA outside the CNS and in particular in the adaptive immunity (Levite 2015; Pacheco et al., 2014), but only sporadically evidence are present about the ability of DA and dopaminergic agents to affect the functions of the cells of the innate branches of immunity.

1.1. Physiology and pharmacology of dopaminergic pathways

DA is one of the main neurotransmitters in the central nervous system (CNS). DA is synthesized from the precursor l-dihydroxyphenylalanine (l-DOPA) and in turn can be converted to epinephrine or norepinephrine (Figure 1).

DA present in human tissues can originate not only from biosynthetic pathways but also from many types of food (Kulma and Szopa 2007*)*. DA in the periphery, introduced with food or as a drug, is unable to cross the blood–brain barrier (BBB). In the CNS, DA is found in different areas, such as substantia nigra, ventral tegmental area (VTA), amygdala, nucleus accumbens, and prefrontal cortex (Feldman et al., 1997). Main dopaminergic pathways in the mammalian brain include: the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibolar (Anden et al., 1964; Dahlstroem and Fuxe 1964). They are involved in the control of several key functions, like: behavior, movement (Cenci 2007), reward (Dayan 2009), cognition and emotional response (Wise, 2008). In the periphery, established physiological functions of DA include the control of cardiovascular system and the ability to influence kidney functions, controlling the renin-angiotensin-aldosterone system (Missale et al., 1998). DA plays also an important role in the homeostasis of sodium and in maintaining the renal functions (Zeng and Jose 2007; Zhang et al., 2011).

Indeed, DA together with other inotropes, is the first vasopressor used in the case of sepsis or septic shock that occurs during an overwhelming immune response to bacterial infections that trigger an inflammatory response throughout the body (Zhang and Chen 2016). Nevertheless, several studies indicate that the use of DA is associated with an increased risk of cardiovascular accidents and death (Martin et al., 1993, Hollenberg 2007, Xu and Peter 2011, Shenoy et al., 2011, De Backer et al., 2012, Avni et al., 2015, Ventura et al., 2015). The D_2-like selective agonist bromocriptine has a clinical application in the treatment of systemic hypertension (Frishman and Hotchkiss 1996) and type 2 diabetes (Defronzo 2011). The most relevant problem related to the clinical use of DA is the lack of an oral form inasmuch dopaminergic agonists are not able to cross the blood brain barrier.

1.2. Dopaminergic receptors

DA exerts its effects through the interaction with the dopaminergic receptors (DR), which are 7-transmembrane, G protein-coupled receptors. DR are classified into D_1-like (D_1 and D_5, previously known as D_{1a} and D_{1b}), located both pre - and post-synaptically and D_2-like (D_2, D_3 and D_4), that are mainly post-synaptic (Andersen et al., 1990; Sibley et al., 1993; Jaber et al., 1996; Beaulieu et al., 2011). D_1-family activate $G_{\alpha s/olf}$ proteins to stimulate cyclic adenosine monophosphate (cAMP) production by adenyl cyclase (AC), whereas D_2-class stimulate $G_{\alpha i/o}$ proteins, which inhibit AC, resulting in a drop of the levels of cAMP (Beaulieu et al., 2015). There are several others mechanisms involved in the signal transduction of DR: D_2-like receptors positively modulate K^+ channel currents, as well as Na^+/H^+ exchanger in many cells types (Missale et al., 1998). Activation of DR D_2 in CHO cells potentiates the release of calcium-evoked arachidonic acid (Piomelli et al., 1991). In general, it can be assumed that the D_1-like receptors stimulate an increase in intracellular calcium levels while D_2-like receptor, particularly DR D_3 and DR D_4, appears to decrease them (Missale et al., 1998).

The DR D_2 receptor exists in two different isoforms: short (S) and long (L), generated by the alternative splicing of an exon (Missale et al., 1998; Lindgren et al., 2003). The DR D_2L has 29 additional aminoacids in the third intracellular loop, resulting in a different affinity for G proteins (Usiello et al., 2000). From literature is known that the DR D_2 is the target of several antipsychotic drugs. In particular, it was shown that the action of one of the most common of these drugs, haloperidol, is mediated by the long isoform, as mice deficient for D_2L, lack the cataleptic effect due to haloperidol. The short form, localized at pre-synaptic level, seems implicated in mechanisms of interference of the DR D_1 signaling (Usiello et al., 2000). These data have been in part contradicted because it has been shown that the aminoacid insert in the D_2L is not correlated with the DR D_2 changes induced by the treatment with antipsychotic drugs (Sedaghat et al., 2006). These two variants of DR D_2 receptor

show not only functional differences, but also distinctive distribution in the brain: D_2L is mainly present on the neurons of striatum and nucleus accumbens, whereas the short form in mesencephalon and hypothalamus (Kahn et al., 1998).

Another important point regarding DR, is the possibility that they can be present as oligomers with other DR or with other classes of receptors (Perrault et al., 2014). The most well-known allosteric interaction between receptors is between the adenosine A_2A receptor (A_2AR) and DR D_2 (Fuxe et al., 2005; Casadò-Anguera et al., 2016). This heteromer has multiple and unique biochemical properties, including functional and ligand selectivity and is responsible for the depressant effects of adenosine analogues and on the contrary, for the psychostimulant effects of selective adenosine A_2AR antagonists and the non-selective antagonist (like caffeine). It is also implicated in several neuropsychiatric disorders (Bonaventura et al., 2015; Ferrè et al., 2016).

Other studies have evaluated the formation of complexes between the DR D_2 and the 5-HT receptors $5\text{-}HT_{2A}R$ and $5HT_{1A}R$, because the mutual influence between these two classes of receptors could be very useful to develop new antipsychotic drugs in the treatment of schizophrenia (Albizu et al., 2011; Łukasiewicz et al., 2016). For several functions in the brain mediated by DA, there is a concomitant stimulation DR D_1 and DR D_2 which form an heteromer, not only in pathological conditions, but also under physiological conditions. Even if some evidence is against the existence of such complex in the adult animals (Frederick et al., 2015), D_1-D_2 DR complex leads to the release of intracellular calcium which results in the activation in the striatal neutrons of CaMKIIa and BDNF that have been shown to be involved in synaptic plasticity (Hasby et al., 2011). D_1-D_2 heteromer is required also for evokes the sensitization to cocaine (Capper-Loup et al., 2002) and have been proposed as relevant to the pathophysiology and treatment of depression, anxiety-like behaviors and schizophrenia (Shen et al., 2015).

Regarding other DR heteromers we have only sporadic information; the D_1-D_3 DR, was suggested to have therapeutic implications as a pharmacological target in Parkinson's disease (Ferrè et al., 2010). D_2-D_3 DR is considered as target for antipsychotics (Maggio and Millan 2010) and the D_2-D_5 DR heteromer, similarly to D_1-D_2 DR, may have a role in calcium signaling, with the activation of CaMKII, involved in drug addiction and schizophrenia (So et al., 2009).

1.3. Dopamine in plant and food

The role of catecholamines (CA) in plants is poorly documented, but from the few works present in literature, it is clear that they are involved in many aspects of growth and development.

As is the case in animals in which CA stimulate the mobilization of glycogen stores, in plants they regulate the metabolism of carbohydrate reserve (starch). The ways through which CA are metabolized in plant and animals are different; in plants, we do not find the vanillyl mandelic and homovanillic acid, but the methylated derivatives, such as normetanephrine. In plants CA also serve as a substrate for the synthesis of other compounds. For example, DA is an intermediate for the synthesis of alkaloids, such as papaverine and morphine (Kulma and Szopa 2007).

The physiological action of CA in animals is mediated by their interaction with receptors coupled to G proteins, whereas in plants DR have been not identified. There is only one study in the literature in which the plants were transfected with human DR (Skyricz et al., 2005).

High concentrations of DA were found in the yellow (42 μg/g) and red banana (55 μg/g) and in avocado (4 μg/g). The highest levels of DA are found in the leaves of the mature potatoes (2-7 μg/g), while very low levels were found in the leaves and tubers of young or senescent plants (<0.5 μg/g; Kulma and Szopa 2007). In thirty varieties of fruits and vegetables, the content of DA, serotonin,

epinephrine and norepinephrine was measured by means of a radio-enzymatic assays (Feldman et al., 1987), and it was shown that: fruits that contain the highest concentrations of DA in the pulp are bananas, and in particular the red bananas (336 µmol/kg), followed by yellow (275 µmol/kg) and plantain (36 µmol/kg). Meanwhile, avocado, cocoa bean powder, broccoli and bruxelle sprouts containing moderate amounts (7 µmol/kg) of DA. Finally, there are fruits and vegetables such as tomatoes, kiwi, pineapple and peanuts, which contain very low levels, less than 7 µmol/kg (Feldman et al., 1987). It was also shown that CA are differently distributed in the parts of the plant: indeed, examining the various parts of the bananas, the highest concentration of DA is at the level of the peel (Kanazawa and Sakakibara 2000).

Also in a study performed in our laboratory, we found that DA is present in in little amount (µg/g) in the dry extract of several plants as for example in: Hypericum Perforatum (St. John's Wort, 20.6), Lippia Citriodora (verbena, 2.46), Timus Vulgare (thyme, 1.74), Pimpinella Saxifraga (burnet-saxifrage, 1.65), Melissa Officinalis (lemon balm, 0.70), Menta piperita (peppermint, 0.83), Salvia Officinalis (sage, 0.63). The highest levels (mg/g) were founded in Menta piperita (1.91) and Pimpinella saxifraga (0.22; unpublished data).

DA was identified to be a powerful water-soluble antioxidant, with a power comparable to glutathione or butylated hydroxytoluene, which is an additive used in the food industry. At the moment, there are not strong evidence about the antioxidant effect *in vivo*, however it is clear the function in mediating the photosynthetic process of oxygen reduction (Allen 2003).

Furthermore, for plants CA are also important for resistance to pathogens, even if, it is unknown whether directly, or by activating other defense systems. They also favor the growth and development of the plant by means of an interaction with plant hormones, resulting in an increase

in the speed of flowering. Finally, CA influence the metabolism of sugar, drastically reducing the growth at 4° C (Szopa et al., 2001).

1.4. Dopaminergic regulation of the immune response

Dopaminergic modulation of the immune response was widely investigated in several comprehensive reviews (Basu and Dasgupta 2000; Pacheco et al., 2009; Sarkar et al., 2010; Levite 2012; Sarkar et al., 2013; Cosentino and Marino 2013; Prado et al., 2012; Levite 2016). DA is involved in the CNS-immune system interplay as well as in communication among immune cells. Immune cells themselves produce DA, which may act as autocrine/paracrine mediator on immune cells as well as on neighboring cells (Bergquist and Silberring 1998; Cosentino et al., 1999; Marino et al., 1999; Cosentino et al., 2000; Cosentino et al., 2002a and 2002b, Cosentino et al., 2005). The effects of DA have been extensively studied on the adaptive immune response and in particular on T lymphocytes. Prominent examples of DA-induced modulation of T cells include: (i) the ability of DA to induce T cell quiescence by up-regulating lung Krüppel-like factor-2 expression through the inhibition of ERK1/ERK2 phosphorylation (Sarkar et al., 2006), (ii) the inhibitory loop subserved by endogenous DA in human CD4+CD25^{high} regulatory T lymphocytes, a specialized subset of T cells playing a key role in the maintenance of immune homeostasis (Cosentino et al., 2007), and (iii) the influence exerted by DA secreted from dendritic cells on the naive CD4+ T cells differentiation (Nakano et al., 2009). The relevance of dopaminergic modulation of the adaptive immune response in disease conditions has been documented for multiple sclerosis (MS) (Zaffaroni et al., 2008; Cosentino et al., 2012; Cosentino and Marino 2013; Marino and Cosentino, 2016) and rheumatoid arthritis (RA) (Capellino et al., 2010; Nakano et al., 2011), and preliminary evidence has been recently provided for Parkinson's disease (PD) (González et al., 2013; Kustrimovic et al., 2014).

In comparison, the role of DA in the modulation of the innate immune response has received little attention so far, with the notable exception of a study conducted by Gaskill and colleagues in which it has been examined the regulation of the production of cytokines and chemokines by human macrophages exposed to elevated levels of DA (Gaskill et al., 2012), as well as the dopaminergic regulation of dendritic cells and its relevance in the priming and differentiation from naïve T cells into effector cells (Pacheco et al., 2009).

1.5. The innate immune system - Generalities and link with dopaminergic pathways

The primary function of the innate immune system is usually considered the defense of the host from infections by other organisms. In recent years, evidence has accumulated regarding its key role in noninfectious diseases like atherosclerosis (Chávez-Sánchez et al., 2014; Courties et al., 2014), cancer (Marcus et al., 2014, van den Boorn & Hartmann 2013), autoimmune diseases such as multiple sclerosis and other demyelinating diseases (Hernandez-Pedro et al., 2013, Mayo et al., 2012), systemic sclerosis (O'Reilly 2014), autoimmune uveitis (Rosenbaum & Kim 2013), lupus erythematosus (Aringer et al., 2013), neurodegenerative diseases (Boutajangout & Wisniewski 2013), gastrointestinal diseases such as inflammatory bowel disease (Levine & Segal 2013), obesity (Lumeng 2013), diabetes (Lee 2014) and liver inflammation (Meli et al., 2014, Liaskou et al., 2012). Innate immunity is also involved in preeclampsia (Perez-Sepulveda et al., 2014), organ transplantation (Farrar et al., 2013), and possibly even in psychiatric diseases (Jones & Thomsen 2013).

Agents of innate immunity, besides anatomical barriers such as skin and mucosal surfaces, include effector molecules such as the complement system and antibacterial peptides, as well as several types of cells. The complement system is a proteolytic cascade which acts as a first-line host defense against pathogenic infections, selectively recognizing foreign pathogens and damaged self-cells

(Noris & Remuzzi 2013, Degn & Thiel 2013). **Antibacterial peptides** are cationic peptides with antibiotic and immunomodulating properties (table 1; Boman 2003, Ganz 2003, Zanetti 2004). **Innate immune cells** include: granulocytes (neutrophils, eosinophils, basophils, mast cells), monocytes/macrophages, dendritic cells, natural killer (NK) cells, γδ T lymphocytes, and innate lymphoid cells (ILC). In the CNS, innate immune responses are mediated by resident microglia, also comprising perivascular and juxtavascular subsets, and astrocytes.

Innate immune system cells express **pattern recognition receptors (PRR)**, which recognize both exogenous pathogen-associated molecular patterns (PAMP) and endogenous damage-associated molecular patterns (DAMP). PRR include: toll-like receptors (TLR, "toll" meaning in German "amazing" or "great"), NOD-like receptors (NLR, NOD meaning Nucleotide-binding Oligomerization Domain), C-type lectin receptors (CLR), RIG-I-like receptors (RLR, RIG-I meaning Retinoic acid-Inducible Gene 1), and AIM2-like receptors (ALR, AIM-2 standing for "absent in melanoma 2", which is a protein contributing to the defense against bacterial and viral DNA). PRR may also include formyl peptide receptors (FPR, binding N-formyl peptides derived from the degradation of bacterial or host cells) and scavenger receptors (binding oxidized or acetylated low-density lipoprotein) (Kawai & Akira 2010, Takeuchi & Akira 2010, Saxena & Yeretssian 2014). Examples of PAMP and DAMP interacting with PRR are provided in table 2 and table 3, respectively. Among PRR, TLR and NKR are the most extensively characterized. In humans, 10 functional TLR have been identified (12 in mice, with TLR1–TLR9 being conserved in both species), which recognize microbial membrane components such as lipids, lipoproteins and proteins (TLR1, TLR2, TLR4, TLR5, TLR6, expressed on cell surfaces) and microbial nucleic acids (TLR3, TLR7, TLR8 and TLR9, expressed exclusively in the endoplasmic reticulum, endosomes, lysosomes and endolysosomes) (Kawai & Akira 2010). As for NLR, the 22 human receptors are divided into five subfamilies by their N-terminal effector domains which confer unique functional characteristics. NLRA (CIITA) are transcriptional regulators of MHC class II antigen

presentation. NLRB (NAIP) proteins contribute to host defense and cell survival. NLRC include NOD1 (NLRC1) and NOD2 (NLRC2), the first NLR to be identified, which sense bacterial peptidoglycan and are key players in tissue homeostasis and host defense against bacterial pathogens. The pyrin domains (PYD) containing NLRP subfamily (NLRP 1-14) have a role in inducing the inflammasome. The NLRX subfamily so far comprises only NLRX1, which may affect mitochondrial activity, however its precise role remains to be established (Saxena & Yeretssian 2014). Currently in literature there are not papers in which the possible influence of the dopaminergic system on activity and functions of PRR is analyzed.

1.5.1. Complement

In vitro, the complement factor C5a, which has several harmful effects during sepsis, blunted PC12 cell production of noradrenaline and dopamine, possibly inducing apoptosis in these cells, suggesting that blockade of C5a receptors might represent a promising complement-blocking strategy in the clinical setting of sepsis (Flierl et al., 2008). In a prospective randomized trial in patients undergoing coronary artery bypass grafting, the D_1-like DR agonist fenoldopam reduced the release in blood of the complement component C3a (but not of C4a and C5a) and of of IL-6 and IL-8 (but not of IL-10, IL-12, and tumor necrosis factor α), suggesting that fenoldopam may induce a partial attenuation of the inflammatory response (Adluri et al., 2010).

1.5.2 Antibacterial peptides

No evidence exists so far regarding any direct connection between dopaminergic pathways and antibacterial peptides. Nonetheless, it has been shown that some antibacterial peptides may display cytoprotective properties at least *in vitro* on dopamine-producing cells like the neuroblastoma SH-

SY5Y cells (Nam et al., 2013; Kim et al., 2014). From an energy-based conformational modeling, it should be considered that DA at high concentrations (10-100 μM) displays significant affinity for β_2-adrenoceptors (Katritch et al., 2009), which in turn may affect the expression of several antibacterial peptides in many epithelia (reviewed in Scanzano and Cosentino, 2015). DA therefore might contribute to the modulation of antibacterial peptide production also through adrenergic mechanisms. It might then be possible that although the endogenous DA is present in the range of nM, in particular situations or specific microenvironments, such as at level of cells or in the niche of a tumor, it can achieve much higher levels.

1.5.3. Cells of innate immunity and dopamine

Neutrophils

Neutrophils are the first line of host defence against a wide range of infectious pathogens. They exert their role in host defence through the secretion of cytokines, proteases, ROS generation and neutrophil extracellular trap (NET) formation (Kumar and Sharma 2010).

In the past, these cells were considered as playing a role only in the early stages of acute infection, given their short lifespan, but at present it is demonstrated that they can survive more than the postulated few hours and that they exert a more complex role in the communication with other cells of both the innate and adaptive immune system (Mantovani et al., 2011; Amulic et al., 2012; Kolaczkowska and Kubes 2013, Pinoli et al 2016). Neutrophils are involved not only in the generation but also in the maintenance of *in loco* inflammation (Kruger et al., 2015). The existence of DR on human PMN was initially reported in 1999 by Sookhai and co-workers, who identified, by means of immunohistochemistry, the presence of the D_1-like DR D_1 (Sookhai et al., 1999). The presence of the D_2-like DR D_2, D_3 and D_4 was subsequently reported (Pereira et al., 2003; Boneberg et al., 2006). Chen

and colleagues, by means of Q-PCR, and McKenna and colleagues, using flow cytometry (although without the use of an isotype control) documented the presence on human PMN of all five DR, both at mRNA and membrane protein levels (Chen et al., 2014; McKenna et al., 2002). In particular, it was observed that mRNA levels of DR were expressed with the following order of magnitude, $D_4>D_3>D_1>D_5>>D_2$ (Chen et al., 2014). On the contrary, McKenna found that DR D_5 was the highest expressed, whereas DR D_1 the lowest (McKenna et al., 2002).

Functional studies performed in *in vitro* conditions, usually report inhibitory effects of DA on several PMN functions as for example the inhibition of fMLP-stimulated superoxide anion production by human PMN (Yamazaki et al., 1989). DA has also been reported to attenuate CD11b/CD18 expression in these cells, with consequently diminished ability of human PMN adherence to the endothelium, as well as a decrease in the production of reactive oxygen species and superoxide anions, cell migration and phagocytic activity (Wenisch et al., 1996; Sookhai et al., 2000; Matsuoka 1990; Trabold et al., 2007).

A study about the morpho-functional changes that occur in neutrophils following treatment with DA also showed that DA increases *in vitro* apoptosis in PMN both in healthy controls and in patients with systemic inflammatory response syndrome (SIRS) (Sookhai et al., 1999).

Is interesting to note that dopaminergic agents can also modify the number of neutrophils during anaphylactic shock; in fact, the use of DA antagonists, such as chlorpromazine and pimozide, significantly inhibited the neutrophil count, while agonists like apomorphine, lead to an increased number of neutrophils, which is typical of conditions occurring during anaphylactic shock (Altenburg et al., 1995). In a paper published last year, there was case report of a patient with Parkinson's disease, in which the treatment with L-DOPA, a precursor in the synthesis of DA, lead to a neutropenia, as well as a reduction of D_2-like DR and an increase of DR D_5 (Cordano et al., 2015).

Eosinophils

Eosinophils are associated with allergy and asthma (Rothenberg and Hogan 2006) and exert a protective role against parasites, as demonstrated by the discovery of eosinophilic granules content on helminth surface (Cadman et al., 2014).

These cells express on their membrane all the five DR (McKenna et al., 2002). Aside from this data, only a few studies are reported in literature about the ability of DA to modulate eosinophil count. The first report comes from an *in vitro* study in rats and dates back to 1979; they reported that treatment with L-DOPA and apomorphine result in a biphasic effect on eosinophil counts. At high concentrations of the drug, eosinophil count decreases, whereas at low concentrations, the scenario is exactly the opposite (Podolec et al., 1979). A study on eosinophilic myocarditis in human shows that in one of the patients studied, there is a correlation between peripheral eosinophilia and eosinophilic myocarditis after intravenous therapy with DA (Takkenberg et al., 2004).

Basophil

Basophils are less than 1% of total blood leukocytes and are considered the most important cell lines that protect against infections of parasites (Karasuyama and Yamanishi 2014). They produce cytokines involved in the cross-talk with the adaptive immunity, such as IL-4 (Karasuyama et al., 2011).

To our knowledge, no data are present in literature about the presence of DR on these cells as well as a possible role of DA as modulator of basophil functions.

Mast cells

These cells, mainly present in the airways, were firstly described in the 1878 by Paul Ehrlich. Precisely because of their ubiquity and abundance, they are involved in most of the inflammatory processes

of the respiratory system (Erjefält et al., 2014). In mice, the bone-marrow derived mast cells contain DA and express the rate-limiting enzyme TH, necessary for the biosynthesis of DA (Rönnberg et al., 2012). Several dopaminergic agents showed a dose-dependent inhibition of degranulation in the cell line RBL-2H3, but this effect seemed not to be related to DR (Seol et al., 2004). This is in contrast with what was found by Mori and colleagues, who have shown that treatment with DA induces mouse bone marrow-derived mast cell degranulation, and that the use of a D_1-like antagonist reveres this effect (Mori et al., 2013).

Monocyte/Macrophage and microglia

Monocytes and macrophages, together with dendritic cells, represent the mononuclear phagocyte system, which plays a key role maintaining tissue integrity during development. Mononuclear phagocytes are also critical in tissue restoration after injury, as well as in the initiation and resolution of innate and adaptive immunity response. Due to their heterogeneity, human monocytes are divided in subsets based on the different stages of differentiation, size and activation. They are characterized by the expression of CD14, the LPS-receptor, and by another marker, the CD16. Monocytes of peripheral blood are usually considered as classical (CD14++CD16-), intermediate (CD14++CD16+) and lastly non classical monocytes (CD14++CD16++; Williams et al., 2012).

Ilya Mechnikov introduced the term macrophage (from Greek, ‘‘large eaters’’), based to their ability to engulf microorganism and damaged tissues (Zalkind 2001). They originate from monocytes, which leave the bloodstream and undergo to morpho-functional changes in response to several differentiation factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF; Parihar et al., 2010).

Several studies indicate that human monocytes express DR both at mRNA and protein level (McKenna et al., 2002; Watanabe et al., 2006; Coley et al., 2015). DA and dopaminergic agents can affect several

functions of these cells; for example, DA is able to decrease LPS-induced proliferation of human monocytes (Bergquist et al., 2000), while the D_1-like agonist SKF-38393 increases CD14+CD16+ monocyte chemokinesis (Coley et al., 2015). DA is an important endogenous regulator of the life of these cells, indeed for example the oxidative burst is under an endogenous dopaminergic tone (Carvalho-Freitas et al., 2007, 2008, 2011). Monocytic cell lines, such as RAW 264.7 (mouse cells line), contains DA (Brown et al., 2003), and, like human cell line U937, present into the cytoplasm L-DOPA decarboxylase, the enzyme that converts L-DOPA into DA (Kokkinou et al., 2009). Moreover, RAW 264.7 cells are able to produce DA: stimulation with LPS induces an increase of DA levels within 48 h (Brown et al., 2003). In addition, it has been shown that DA at high concentrations (millimolar range), increased the apoptosis of RAW 264.7 cells and decreased the proliferation (Brown et al., 2003). DA is also able to mediate the cytokine production (Gaskill et al., 2012). Haskò and colleagues confirmed that treating mouse macrophages with DA, LPS-induced IL-12p40 production (cytokine secreted primarily by antigen-presenting cell that has a key role in determining the type of immune response to antigens) is suppressed, even if this mechanism is mediated by adrenergic receptors (Haskò et al., 2002). DA and dopaminergic agonists are able to interfere with the production of TNF-α and nitric oxide (NO) in mice (Haskò et al., 1996; Chi et al., 2003), DA can also modulate the expression of surface markers in guinea pigs, such as the Fc-gamma receptor, important for the defense, whose expression is reduced following treatment with dopaminergic agents (Gomez et al., 1999).

In viral infection, it seems that DA play a negative role. In fact, primary monocyte-derived macrophages (MDM) inoculated with HIV in the presence of DA, are more susceptible to virus infection, indeed increase the viral replication (Gaskill et al., 2009). This DA-dependent effect was confirmed by means of a pan-DR antagonist, flupenthixol, which abrogated the activation of DR (Gaskill et al., 2014). The same results were obtained during treatment with methamphetamine. In this case researchers observed an increase in the activity of reverse transcriptase of the virus in

human macrophages that is blocked after treatment with SCH23390 and SKF83566, two D_1-like DR antagonist (Liang et al., 2008).

A specialized population of macrophages resident at the level of CNS is **microglia**. Originally described in 1932 by del Rio-Hortega, these cells show a unique phenotype different from both glia and neurons (Block et al., 2005, Kettenmann et al., 2011). Upon activation, they change their morphology from ramified to amoeboid, increase their phagocytic activity and increase their release of pro-inflammatory mediators (Pannell et al., 2014, Nayak et al., 2014). Microglia, as well as for the shape, is divided into subpopulations according to anatomical location: perivascular microglia, mainly within the basal lamina of a blood vessel and juxtavascular microglia, externally in contact with the basal lamina of the blood vessel (Gehrmann et al., 1995).

Unregulated activation of microglia can lead to neuroinflammation resulting in the neurodegeneration that occurs in several diseases (Block and Hong 2005) like Alzheimer's (Meda et al., 1995; Rogers et al., 2007; Li et al., 2014; Bodea et al., 2014) and Parkinson's disease (Qian and Flood 2008; Mastroeni et al., 2009; Tanaka et al., 2013; Wang et al., 2015), as well as multiple sclerosis (Li et al., 1996; Duffy et al., 2016; Raine 2016).

The evidence about the dopaminergic regulation of microglia are reviewed in the manuscript of Gaskill and colleagues (Gaskill et al., 2013) and it was shown that cultured murine and rat glial cells expressed all five DR (Färber et al., 2005; Huck et al., 2015). Human elderly microglia were found to express all DR with the exception of DR D_1, based on RT-PCR and immunohistochemistry studies (Mastroeni et al., 2009). DA can also influence the functions of these cells attenuating the release of nitric oxide in microglia derived from mice and rats (Chang and Liu 2000; Färber et al., 2005). Moreover, DA significantly stimulated both human, mice and rats' microglial chemotaxis (Färber et al., 2005; Mastroeni et al., 2009).

Astrocytes

Astrocytes are one of the most abundant glial cells in the nervous tissue (Kimelberg and Nedelgaard 2010). They are divided in two principal subtypes: protoplasmic, mostly found in the grey matter, and fibrous astrocytes in the white matter (Sofroniew and Vinters 2010). The most common marker used in their identification in immunohistochemistry is the glial fibrillary acid protein (GFAP), even if they show a local and regional variability due to the action of different signaling molecules (Sofroniew and Vinters 2010). Among the most important functions of these cells is the regulation of cerebral blood flow. These cells also play an important role in the formation and elimination of synapses (Chung et al., 2015). They have an important role not only in physiological, but also in pathological conditions such as Rett syndrome, fragile X mental retardation, Alexander's disease and possibly in Down syndrome (Molofsky et al., 2012). This emerging role in neural circuit maturity is also linked to the development of several psychiatric and neurological disorders resulting from synaptic deficit (Seifert et al., 2006; Molofsky et al., 2012; Clarke and Barres 2013; Moraga-Amaro et al., 2014; Koiama 2015; Pekny et al., 2016).

Several paper demonstrate the presence of DR on astrocytes: DR D_2 receptor was described on monkey and rat astrocytes (Bal et al., 1994; Khan et al., 2001). In rat astrocytes, it was found that a non-toxic concentration of DA action on monoamine oxidase (MAO) and produces reactive oxygen species that influence the intracellular calcium signaling (Vaarmann et al., 2010). The same increase in Ca^{2+} levels occurs utilizing a D_1-like DR agonist and this effect is prevented with a dopaminergic antagonist (Liu et al., 2009; Zhang et al., 2009). Moreover, DA stimulate cAMP production in cultured striatal astrocytes of rat. This effect is mimicked by D_1-like agonist SKF-38393 (Zanassi et al., 1999).

Dendritic cells

Dendritic cells (DC) are antigen-presenting cells of immune system and their name comes from protrusions, similar to dendrites of neurons that grow on their surface during maturation. They are divided into two major subgroups: myeloid dendritic cells, similar to monocyte, and plasmacytoid dendritic cells, which look like plasma cells (De Kleer et al., 2014). DC possess high phagocytic activity as immature cells and high cytokine producing ability as mature cells. They are able to migrate into the lymphoid organs and regulate T cell responses both in the steady-state and during infection (Mellman and Steinman 2001; De Kleer et al., 2014). DC are important to T cells activation and DA seems to play an important role in this context (Levite 2015). DR are expressed on human monocyte-derived DC (Nakano et al., 2008) and these cells also store DA in compartments close to the plasma membrane (Nakano et al., 2009). Moreover, mouse bone marrow-derived DC express TH (Prado et al., 2012). DR expression can be modulated by different kind of stimuli. For example, Prado and colleagues found that both immature and mature DC of mice express on their surface all the DR with the only exception of DR D_4, while treatment with LPS induces a significant down-regulation of DR D_5, suggesting a role of this receptor in the maturation's process of these cells (Prado et al., 2012).

Most agents acting on DR and presenting ability to interfere with DC cell functions are presently used in therapy. Haloperidol, a D_2-like receptor antagonist (a typical antipsychotic drug), is able to suppress murine DC maturation and attenuate the secretion of IL-12p40, which is an important marker of cell maturation (Matsumoto et al., 2015). In human monocyte-derived DC, risperidone, an atypical antipsychotic drug acting preferentially on DR D_2, but also on serotonin receptors, was able to modulate the pro-inflammatory cytokine production of mature DC (Chen et al., 2012).

Natural Killer cells

Natural Killer (NK) cells, play an important role in innate immunity and their primary role is the killing of pathogens (origin of the name) and protection of the organism against external invasion (Vivier et al., 2008). They show the ability to eliminate cancer cells and virus-infected ones (Moretta et al., 2014), thanks to the secretion of a large amounts of cytokines, like granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, interferon (IFN)-γ, and chemokines such as CC chemokine ligand (CCL) 3, macrophage inflammatory protein (MIP)-1, CCL4 and CCL5 (Walzer et al., 2005).

DR are expressed on NK (McKenna et al., 2002; Zhao et al., 2013; Boneberg et al., 2006) and treatment of NK cells derived from mouse spleen with SKF-38393 (D_1-like DR agonist) enhanced NK cytotoxicity, whereas quinpirole, a D_2-like DR agonist, weakened the cytotoxic activity (Zhao et al., 2013). Spleen-derived NK of rats with a hyperactive dopaminergic system had a reduced killing capacity compared to those of hypodopaminergic rats (Teunis et al., 2004). The suppression of immune status observed after the treatment with morphine (Bayer et al., 1990; Saurer et al., 2004), was antagonized with 7-OHDPAT, a D_2-like agonist (Saurer et al., 2004). In addition, in human peripheral-blood derived NK, it was shown that drugs acting as dopaminergic antagonists are able to inhibits NK cell responses (Theorell et al., 2014; Won et al., 1995).

γδ T lymphocytes

γδ T cells represent less than 5% of circulating T lymphocytes in peripheral blood. These cells play an important role in fighting infections and cancer and also have implications in autoimmunity (Su et al., 2013; Paul et al., 2015). They are thought to be a link between the innate and the adaptive immune system and are able to stimulate the maturation of dendritic cells (Ismaili et al., 2002). They produce pro-inflammatory mediators such as IFN-γ and TNF-α (Duhindan et al., 1997), as well as anti-inflammatory cytokine such as IL-10 (Rhodes et al., 2008) and IL-17 (Martin et al., 2009). At present

no data are available about a possible presence of DR on these cells or about the ability of dopaminergic agents to modulate their functions.

1.6. Dopamine and innate immunity: pathogenic and therapeutic relevance

This last section of the chapter provides a brief overview of the contribution of the dopaminergic system in some important diseases in which the role of immune system is considered to play a relevant role.

Cancer - Cancer is one of the leading causes of death in the world and many studies are aimed at finding new targets for the development of innovative treatments that also limit the major side effects of the treatments. Indeed, despite remarkable achievements especially in biotechnology, anticancer treatment is still based on nonspecific, nonselective cytotoxic chemotherapy, which damages healthy tissues and compromises anticancer immune response. Even biological drugs, e.g. anti-vascular endothelial growth factor (VEGF) agents, are burdened with significant side effects, such as hypertension, hepatic and renal damage, neutropenia and thrombocytopenia (Chen and Cleck, 2009; Gressett and Shah, 2009; Pirker et al., 2008). There is therefore growing interest in developing combined chemoimmunotherapy, to reinforce immunity thereby maintaining durable and effective antitumor response.

Innate immunity is the first line of defense, but the same mechanisms used to counter the non-self cells can be potentially harmful and favor the growth of cancer. Innate immune cells have a dual role in cancer initiation and progression: they can prevent the growth of the tumor or support its development by increasing the level of inflammation. Among the innate immune cells which have a prominent role in the tumorigenesis process, it is necessary to remember the tumor-associated macrophages and neutrophils, as well as dendritic cells NK cells and γδ T cells, which can act as pro-

or anti-tumoral agents (Liu and Zeng 2012; Hagerling et al., 2015; Woo et al., 2015). For example, neutrophils in the tumor show a double phenotype: N1 neutrophils are anti-tumor, with a hypersegmented nucleus, pro-apoptotic and anti-angiogenic properties, while on the contrary, N2 present the opposite tasks, leading to immunosuppression, bypassing apoptosis and promoting angiogenesis. This different polarization is associated with the hypoxic state, typical of the tumor, as well as to factors such as TGF-β and G-CSF (Coffelt et al., 2016). Similarly to neutrophils, macrophages also have the same functional dichotomy, on one hand, by stimulating angiogenesis and increasing tumor cell invasion, motility and intravasation, on the other hand exerting an immuno-stimulatory role that opposes the development of cancer cells (Noy and Pollard 2014). NK exhibit a natural cytotoxicity against tumor cells, releasing cytoplasmic granules, in the absence of pre-immunization. It was also observed that patients with an elevated level of tumor infiltrating NK (TINK), were associated with a favorable outcome (Cheng et al., 2013). It was instead demonstrated that DC, which are defined as natural adjuvants for their peculiarities to coordinate innate and adaptive immunity, are used in vaccines for certain types of cancer, inducing tumor-specific effector T cells that can reduce the tumor mass (Palucka and Banchereau 2012; Anguille et al., 2014).

It has been documented that DA by acting on DR D_2 inhibits angiogenesis (i.e. the development of new blood vessels that allows rapid expansion and progression of cancer) by suppressing the action of VEGF-A (Basu et al., 2001; Sarkar et al., 2013) and might have a role as an antiangiogenic agent for the treatment of breast and colon cancer, enhancing the efficacies of commonly used anticancer drugs (Sarkar et al., 2008). The role of DR, in particular DR D_2 and DR D_4, has also been studied in corticotroph ectopic tumors; it was demonstrated that the treatment of some subgroups of these tumor with cabergoline, a DR agonist, could be useful in the control of cortisol secretion, preventing the exacerbation due to glucocorticoids excess (Pivonello et al., 2007).

In immature and abnormal tumor blood vessels in malignant colon and prostate tumor, there is a leak of sympathetic innervation and endogenous DA (Chakroborty et al 2011), indeed the ablation of peripheral dopaminergic nerves stimulates the malignant tumor growth (Basu et al 2004). Conversely, the administration of exogenous DA that acts on pericytes and endothelial cells, induces a normalization of the vessel morphology (Chakroborty et al 2011). Even from clinical studies it was shown that for these antiangiogenic peculiar characteristics, the low cost, the limited side effects, DA may be an ideal alternative to anti-VEGF-A drug (Banerjee et al 2015).

It was also demonstrated that dopaminergic agents exert an antitumoral effect against melanoma (Wick 1982), and improved the efficacy of cyclophosphamide while reducing its hematotoxicity (Lakshmi et al., 2005). The action of DA and D_1-like agonist on this receptor suppressed cell viability, inhibited invasion and induced apoptosis in cell lines of breast cancer (Borcherding et al 2015). Bromocriptine, a D_2-like DR agonist, shows a high selectivity for the leukemic cell and can have a synergistic effect with the treatments already in use (Liberante et al 2015).

HIV - HIV is a virus that attacks the cells of immune system, primarily CD4+ T cells and macrophages. There are two main types: HIV-1, the most common type found in the worldwide; and HIV-2, mainly found in Western Africa.

Several lines of evidences suggest the participation of innate immunity in the early phases of HIV infections. Between innate immune cells, macrophages are the primary target for HIV in the CNS. Several paper have demonstrated that DA has a very strong impact on the function of these cells. It seems that in drug abuser the increase of DA level is correlated with an enhancement of HIV virus entry into macrophages (Carbone et al., 1989; Gaskill et al., 2014). This effect requires the activation of DR, found on the surface of monocyte-derived-macrophages (Gaskill et al., 2009; Gaskill et al., 2012). This activation is abrogated with the use of the pan DR antagonist flupenthixol (Gaskill et al.,

2014). Coley and colleagues demonstrated that CD14+CD16+ monocytes had the mRNA of all five DR and that they are functional, as showed by the up-regulation of Erk2. Moreover, the treatment with DA, as well as SKF-38393, a D_1-like DR agonist, increased migration in a chemotaxis assay and transmigration across an in vitro BBB model (Coley et al., 2015). It was also demonstrated that both infected and non-infected monocytes can cross the BBB, the first mediating HIV entry in the brain, the second increasing the level of inflammation which exacerbates the HIV associated neurological disorders (HAND) (Williams et al., 2012-2013-2014).

DC play a pivotal role as sentinels that alert other cells by the secretion of a series of cytokines, even if some evidence highlighted a contribute in HIV infection and progression (Manches et al., 2014). Epidemiologic data strongly support the role of NK cells to mediate the antiviral control through the activation of killer immunoglobulin-like receptors (KIRs) (Carrington and Alter 2012). To counter the initial replication of the virus and allow acquired immunity to take action, innate immunity plays an important role and in particular it has been observed that DC producing IFN-α, activate T cells and play themselves an antiviral role (Borrow and Bhardwaj 2008). Effectors of antiviral innate cells can contribute to the control of viremia and modulate the response of the adaptive immunity to HIV-1 (Altfeld et al 2015).

The virus disrupts the central dopaminergic pathway also acting on the dopamine transporter (DAT). With a computational model it was demonstrated that inducing a mutation in two aminoacids, the inhibition of DAT due to the HIV-1 protein transactivator of transcription (tat) is attenuated (Midde et al., 2015). tat is a regulatory protein essential for the replication of the virus; it was indeed demonstrated that mutant tat could inhibit the activity of the reverse transcription of HIV (Lin et al., 2015). tat is also able to enhances Parkinson's-like behavior in the rats (Liu et al., 2014).

Jacobs observed that in their population the polymorphisms present in DR D_1 and DR D_2 SNPs is associate with substance abuse and differed within racial groups (Jacobs et al., 2013). Treating a primary culture of human astrocytes with methamphetamine, leads to a decrease in the levels of DR D_2, as well as dopamine transporter expression (Samikkannu et al., 2015). It was demonstrated that cocaine facilitates the entry of HIV in macrophages and microglial cells and promotes astrogliosis via astrocyte activation and proliferation (Cai et al., 2016). Moreover, methamphetamine increase the activity of HIV and suppressed TLR-9-mediated anti-HIV activity in human blood monocyte-derived macrophages with a D_1-like DR-mediated effect (Liang et al., 2008; Cen et al., 2013). The synergistic negative effect of the virus and methamphetamine is also evident at epigenetic level, with a with a characteristic pathway of methylation of the host DNA (Desplats et al., 2014). Cocaine instead, down-regulating miR-155 and 20a expression in monocytes-derived DC, increases DC-SIGN expression that may increase the susceptibility to HIV infection (Napuri et al., 2013).

HIV infection is also associated with psychiatric diseases (Lundberg et al., 2013; Yehia et al., 2014; Jallow et al., 2016; Kagee et al., 2016).

Psychiatric disorders - A mental disorder, is diagnosed as a behavioral or mental pattern that can cause suffering or a poor ability to function in ordinary life. One adult on twenty experiences a condition of mental illness such as schizophrenia or bipolar disorder (NAMI, National Alliance on Mental Illness, https://www.nami.org/Learn-More/Mental-Health-Conditions). They may be persistent, relapsing and remitting, or occur as a single episode. The causes are often unknown.

Emerging studies analyzing and characterizing of the role of innate immunity in psychiatric diseases (Kraneveld et al., 2014) show that inflammation can influence several aspects of CNS function, like neurotransmitter metabolism and neuroendocrine function which can lead to behavioral changes in humans (Irwin and Miller 2007). It has been shown that neuroinflammation due to the secretion of

pro-inflammatory cytokines, such as TNF-α, is correlated with diseases such as depression, post-traumatic syndrome and bipolar disorder (Jones and Thomsen 2013). The role of inflammation in the course of depression is reviewed by Miller and Raison (2016). It was demonstrated that the pro-inflammatory cytokines of patients with depression or suicidality lead to the dysregulation of kynurenine pathway, which is correlated with a worsening of the severity of the symptoms (Bay-Richter et al., 2015). Patients with depression present signs of inflammation in the brain and also in the peripheral blood (Felger and Lotrich 2013), with increased levels of pro-inflammatory cytokines, such as IL-1β, TNF and IL-6 (Miller et al., 2009). The exogenous administration of cytokines such as IFN-α induce depression (Capuron et al., 2009) and leads to alterations also at the level of monoamines (Felger and Lotrich 2013). Increased levels of cytokines such as IL-6, IL-2, IL-8 and IFN-γ are also found in bipolar disorder (Muneer 2016). Another potential bio-marker associated with this pathology is the activation of microglia (Watkins et al., 2014).

In several psychiatric disorders, including schizophrenia, there is dysregulation of DA and dopaminergic pathways (Brisch et al., 2014; Yamaguchi et al., 2015; Howes et al., 2016). Certain gene variants in genes encoding for the DR have been shown to represent a risk or in some cases, protection factors of psychiatric diseases such as schizophrenia (Hoenicka et al 2007). Among the most studied genes in this field is certainly DR D2; the expression of the gene of this receptor is altered, not only in schizophrenia, but also in the post-traumatic stress syndrome (Noble 2003). However, these changes do not take place only on the DR genes, but also at the epigenetic level (Abdolmaleky et al 2008). In the case of schizophrenia, the combination of the alteration in gene expression and other factors results in a dysregulation of dopaminergic system, with higher release of DA and more DR D2 (Seeman and Kapur 2000). The available drugs used for the treatment of schizophrenia act by blocking the DR D2 (Harrison 2000; Moncrieff et al 2009; Howes et al 2012), but some studies suggest that dopaminergic drugs induce important side effects and may be supplanted

by drugs affecting the glutamatergic system (Weinberger 2007). The contribute of dopaminergic system is also featured in bipolar disorder (Cousins et al., 2009) and depression (Brown and Gershon 1993; Dunlop and Nemeroff 2007).

Infections - Infection is the invasion of an organism by disease-causing agents, their multiplication, and the reaction of host tissues to these organisms and the toxins that they produce. They are caused by infectious agents like viruses, bacteria, nematodes, arthropods, fungi or helminths.

The innate immune response is fundamental against infections, indeed chemokines released from infected tissues recruit innate immune cells. This process triggers the phagocytic activity and the production of various cytokines, giving the necessary time to acquired immunity to take action (Tosi 2005; Koyama et al. 2008).

In several studies, it has been demonstrated the dopaminergic system undergoes profound changes in the course of infection. Infection with *Toxoplasma gondii* induces significant changes in infected human and animal's behavior (Flegr 2007-2013; Webster 2007; Vyas 2015). The importance of the DA was confirmed by the use of dopaminergic antagonists that have blocked and prevented the change in the behavior of animals (Martin et al 2015). Moreover, *Toxoplasma gondii* possess the TH, the rate-limiting enzyme in the synthesis of DA and the cell encysted with parasite present high level of DA (McConkey et al 2013). Although the innate immune cells may play a role in the effects mediated by the dopaminergic system, it has not yet been studied. This could be seen as a paradox considering that the innate immune cells play a primary role in fighting infection, with frontline neutrophils and macrophages.

Multiple sclerosis - Multiple sclerosis (MS) is a chronic demyelinating disease induced by an autoimmune response against constituents of the central nervous system. It affects about 2.4 million individuals worldwide and is clinically characterized by progressive loss of neurological functions due

to the destruction of the sheath axonal myelin in different areas of the brain and spinal cord (Pacheco et al 2014). The etiology of MS is still under investigation and appears to involve both genetic and environmental factors, but it is not yet clear what triggers and determines the development of pathology. IFN-β has been used for a long time as a first-line drug for MS (Dubois et al., 2003; Rog and Mottershead 2006). Aside from conventional therapy, it has been shown that patients respond well to complementary and alternative medicines, especially herbal remedies, which address many of the symptoms of the disease for which conventional drugs do not pose a remedy, such as anxiety, gastrointestinal disorders, migraine, depression and genital-urinary infections (Loraschi et al., 2016).

Numerous studies have been performed to evaluate the immunological dysfunction at the base of MS, which is usually considered a $CD4^+$ T cell-mediated disease, engaging in particular the Th_1 and Th_{17} subsets (Korn et al., 2007; Bettelli et al., 2008). Only recently the role of innate immunity in this disease has been reevaluated (Fernandez et al., 2010; Hernandez-Pedro et al., 2013): for example, DC accumulated in the CNS in the early phases of inflammation in experimental autoimmune encephalomyelitis (EAE), the murine model of MS (Bailey et al., 2007), moreover, in MS patients, DC show an activated phenotype with an increased expression of activation markers (Gandhi et al., 2010). Human macrophages and microglia display a double role, protecting from tissue damages but simultaneously producing pro-inflammatory mediators which can exacerbate the pathology (Kawanokuchi et al., 2008; Vogel et al., 2013), as well as disrupt the myelin sheet (Bauer et al., 1994). Even NK have this dichotomy in their function (Sakuishi et al., 2010): they show an *in vitro* cytotoxicity towards microglia and astrocytes during inflammation, but they are also able to protect and repair CNS through the production of brain-derived neurotrophic factor and neurotrophin-3 in murine model of EAE (Hammarberg et al., 2000). Furthermore, NK function is associated with MS activity, indeed their depletion exacerbates the disease in EAE (Chanvillard et al., 2013). Mast cell in the meninges contribute to the early development of EAE in rodent model (Sayed et al., 2010) and

accumulate in plaque (Olsson 1974). γδ T cells increased in MS patients and are present in MS lesions (Selmaj et al., 1991).

It has been established that in the course of MS there is a dysregulation of the dopaminergic system (Cosentino and Marino 2013). According to data obtained from studies on human cells, the dopaminergic pathway appears to be extensively involved in the pathogenesis of MS (Cosentino and Marino 2013). In particular, Zaffaroni highlighted that peripheral blood mononuclear cells (PBMC) from MS patients treated with IFN-β (one of the first line drug in this disease), showed an increased synthesis of DA and a progressive upregulation of DR D_5 (Zaffaroni et al 2008). It was also demonstrated that after treatment with IFN-β, the mRNA level of DR D_5 of circulating lymphocytes of MS patients is restored and that DR D_5 levels can represent an early marker of response to IFN-β in these patients (Cosentino et al., 2014).

In MS patients, neutrophils display a pro-inflammatory phenotype and infiltrate the central nervous system in the early stages of the disease (Hernandez-Pedro et al., 2013; Naegele et al., 2012; Hertwig et al., 2016). DA could directly affect the motility, migration and adhesion of these cells, favoring the passage of neutrophils in the central nervous system (Sookhai et al 2000) and at brain level could contribute to the inflammatory response associated with the severity of symptoms, producing reactive oxygen species (van Horssen et al 2011; Steinbach et al 2013).

Monocyte appear to play a central role in MS, contributing to the breakdown of the blood-brain barrier and facilitating the trafficking of T cells within the CNS (Waschbisch et al 2011).

Rheumatoid arthritis - Rheumatoid arthritis (RA) is an autoimmune disease in which the main characteristic is the irreversible joint destruction associated with progressive disability (McInnes and Schett 2011). It can occur at any age, but is most common between 40 and 60 years and the frequency is higher in women than men (Heidari 2011).

Innate immunity is critically in the pathogenesis of RA. Macrophages infiltrating in the synovial tissues have a pivotal role, indeed therapies including methotrexate and cytokine inhibitors decrease the production of cytokines produced primarily by macrophages (Arend 2001). As in tumors, even in RA macrophages display a pro-inflammatory phenotype, with reduced expression of pro-apoptotic genes and increased production of pro-inflammatory mediators, like IL-1β (Gierut et al., 2010). A little but significantly increased number of DC, in comparison to healthy control, was identified in peripheral blood of RA patients (Gierut et al., 2010) and in synovial fluids, where they can present the antigen to the local cells, contributing to disease progression (Lutzky et al., 2007). Neutrophils, as well as NK, are prevalently found in synovial fluid of RA patients (Falgarone et al., 2005). They participate to the disruption of joint (Kaplan 2013) and contribute to the development of the pathology, releasing NET and producing pro-inflammatory cytokines (Wright et al., 2014). The cross-talk with other immune cells leads to the release of cytokines, among which, TNF-α has the pivotal role in the onset of RA and also in the NK-dependent DC maturation (Shegarfi et al., 2012). On the contrary, NK cells can act as immune regulator against activated T cells and macrophages through their cytotoxic effects (Ahern and Brennan 2011). γδ T cell may contribute to chronic synovitis in RA not eliminating activated macrophages (Arend 2001).

The dopaminergic system has a strong impact on RA (Pacheco et al., 2014). It was demonstrated that synovial fibroblasts express DR and this expression increased in patients with RA (Capellino et al., 2014). Moreover, they present DA transporter, and tyrosine hydroxylase (Capellino et al., 2010). Nakano and colleagues showed that DA released by DC induces the production of IL-17 (IL-6-dependent) by T cell that leads to the differentiation of Th_{17} that exacerbated cartilage destruction in RA mouse model, and this effect is prevented by the D_1-like DR antagonist SCH-23390 (Nakano et al., 2011; Nakashioya et al., 2011).

Haloperidol, a well-known and used D_2-like DR antagonist, showed an anti-RA effect, mitigating the effects due to oxidative stress in adult female albino rats (Fahmy Wahba et al., 2015). On the contrary, it was demonstrated that cabergoline, a DR agonist, reduced the levels of prolactin in ten female rats with active RA, improving RA (Mobini et al., 2011). Despite even the innate immune cells are involved in the dopaminergic pathway and have an important role in this disease, in literature there are no specific studies that evaluate the relationship between dopaminergic system and innate immune cells in the specific context of RA.

1.7. Conclusions and perspectives

In conclusion, the data present in literature about the presence of DR on the cells of the innate branch of immunity and the ability of DA to interfere with some functions of these cells, suggests that the communication between immune system and CNS is more complex than suggested by our current models. In addition, it can be assumed that these connections, when altered or in cases of pathological activations by different stimuli, such as for example infective or inflammatory stimuli, can be involved in the genesis of some diseases.

This new vision can offer new opportunities for further studies aimed to clarify whether these findings can help to promote new therapeutic strategies in counteracting cell activation and tissues invasion, known to be the first step in most part of immune-mediated diseases, including inflammatory diseases of the CNS. In particular, it is known that infiltration of leukocytes among the CNS, retrieved from the bloodstream by chemotactic factors and cytokines, is a crucial step involved in the destruction of the blood–brain barrier (Barthelmes et al., 2015). The data present in literature about a potential anti-inflammatory effect of DA and dopaminergic agents give us new information about

the relevance of the role of a dysregulation of dopaminergic pathways in the genesis of some diseases.

It is also known that, actually, efforts are devoted to the investigation of new therapeutic strategies aimed at the reduction of the side effects of some drugs. We currently use dopaminergic agonists to treat Parkinson's disease, attention deficit/hyperactivity disorder, prolactinoma, restless legs syndrome, schizophrenia, cancer, bipolar disorders or as antiemetics. This long list suggests, that dopaminergic drugs can be safely used and on the basis of the data resumed in the present review, that they can be used as non-conventional therapeutics as addition to conventional drugs.

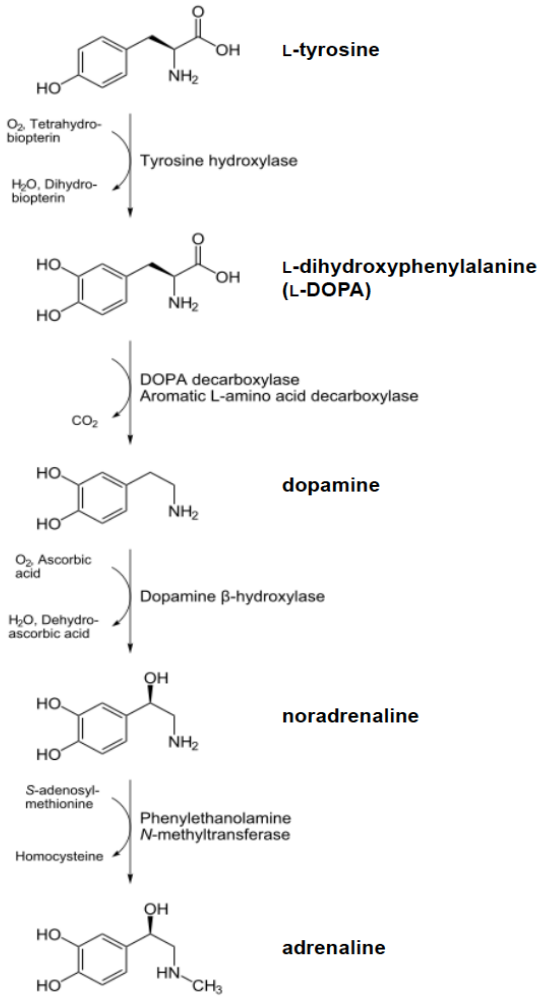


Figure 1. Biosynthetic pathway of the catecholamines dopamine, noradrenaline and adrenaline from the aminoacid tyrosine. The synthesizing enzymes are shown to the right of each arrow, while enzyme cofactors are shown to the left (reproduced from the Wikimedia Commons—http://commons.wikimedia. org).

Table 1. Human antimicrobial peptides (based on Boman 2003, Ganz 2003, Zanetti 2004).

Name		Distribution
α-Defensins		
	HNP1-3	granulocytes (spleen, thymus, lung?)
	HNP4	Granulocytes
	HNP5	Paneth cells of the intestine, genital tract
β-Defensins		
	hBD-1	skin, lung, gut (epithelial cells)
	hBD-2	skin, lung, gut (epithelial cells)
	hBD-3	skin, lung, tonsils
	hBD-4	testis, gastric antrum
Cathelicidin family		LL-37 (proFALL, hCAP18)
		granulocytes, lung, skin, testis, gut, lymphocytes
Saposin family	Granulysin	cytolytic T cells, NK cells

Abbreviations: HNP, human neutrophil peptide; hBD, human beta defensin; LL-37, 37-residue peptide whose N-terminal sequence is LL; FALL, 39-residue peptide whose N-terminal sequence is FALL; hCAP, human cationic antimicrobial protein.

Table 2. PAMP detection by TLR and other PRR (reproduced with permission from Kawai & Akira 2011).

Species	PAMP	TLR Usage	PRR Involved in Recognition
Bacteria, mycobacteria	LPS	TLR4	
	lipoproteins, LTA, PGN, lipoarabinomannan	TLR2/1, TLR2/6	NOD1, NOD2, NALP3, NALP1
	flagellin	TLR5	IPAF, NAIP5
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI, IFI16
	RNA	TLR3, TLR7, TLR8	RIG-I, MDA5, NALP3
	structural protein	TLR2, TLR4	
Fungus	zymosan, β-glucan	TLR2, TLR6	Dectin-1, NALP3
	Mannan	TLR2, TLR4	
	DNA	TLR9	
	RNA	TLR7	
Parasites	tGPI-mutin (*Trypanosoma*)	TLR2	
	glycoinositolphospholipids (*Trypanosoma*)	TLR4	
	DNA	TLR9	
	hemozoin (*Plasmodium*)	TLR9	NALP3
	profilin-like molecule (*Toxoplasma gondii*)	TLR11	

Table 3. DAMP interaction with TLR and NLR and their physiological effects (reproduced with permission from Tolle & Standiford 2013).

DAMP	Receptor	Effect
ECM components		
Fibronectin	TLR4	Increase NF-*k*B, promote leukotriene synthesis and PMN migration, activate the adaptive immune system
Hyaluronan	TLR4±TLR2, NLR3	Induce proinflammatory cytokines, activate DCs and macrophages
Heparan sulphate	TLR4	Increase TNFα expression, activate DCs
Stress-response molecules		
Heat shock proteins	TLR2, TLR4	Induce cytokines and protein kinases, activate PMNs
HMGB1	TLR2, TLR4	Induce NF-*k*B nuclear translocation and cytokine expression
Nucleic acids	TLR3, TLR7, TLR9	Induce cytokine expression, activate DCs, stimulate recruitment of leukocytes and PMNs
microRNA	TLR8	Induce NF-*k*B and cytokines expression
Immunomodulatory proteins		
β-Defensins	TLR1/TLR2, TLR4	Induce NF-*k*B and cytokine expression, activate DCs and monocytes
Surfactant protein A	TLR4±TLR2	Reduce NF-*k*B and cytokine expression
Surfactant protein D	TLR2, TLR4	Inhibit cytokine production and recruitment of PMNs
Others		
Uric acid	NLR3	Increase IL-1β expression

Chapter 2

Polymorphonuclear leukocytes

At the end of nineteenth century, examining the leukocyte subpopulations, Paul Ehrlich discovered the polymorphonuclear leukocyte (PMN) subset, which he named neutrophils. In humans, this leukocyte subset constitutes the 50-70% of the entire leukocyte pool. The size of PMN is relatively homogeneous, between 9 and 12 µm. The name neutrophil is derived from the polymorphic nature of their nucleus, constituted from two to five lobes joined with a hair-like filament, and by the lack of affinity toward basic and acid dyes (Amulic et al., 2012).

Neutrophils originate from the bone marrow in a process called granulocytopoiesis, in response to the stimulus of granulocyte or granulocyte macrophage colony stimulating factor (G- and GM-CSF). It was demonstrated that animals deficient in these cytokine can increase neutrophil production in response to inflammatory stimuli in a process called emergency granulopoiesis (Basu and Dasgupta 2000). Bone marrow of healthy individuals produces around 10^{11} cells daily and this number can increase during inflammation or infections (Athens et al., 1961).

Many studies show that neutrophils can be found among the various subpopulations with different phenotypes and functions both in physiological and pathological conditions (Tsuda et al., 2004; Denny et al., 2010; Pillay et al., 2012). In addition to division of neutrophils into the N1 and N2 phenotype, with regard to the associated neutrophils to tumors (discussed in the section "neutrophils and disease"), in the review by Beyrau and colleagues there are schematized markers that they express, as Olfactomedin-4, CD177, CD54, CD182 and CD184, which are associated with different subsets (Beyrau et al., 2012).

PMN are among the first cell types that leave the bloodstream and enter into the inflamed tissues to defend against infectious pathogens such as bacteria, fungi, and protozoa (Kumar and Sharma 2010). They are able to produce different kind of molecules such as reactive oxygen species, cytokines and

proteases that allow to eliminate the non-self material (Witko-Sarsat et al., 2000). All proteins and proteases are contained in intracellular granules. These granules are formed in different stages of cell development and are distinct in primary or azurophilic granules, formed while they are promyelocytes. These granules can contain myeloperoxidase, elastase, proteinase-3, cathepsin G, azurocidin and bactericidal permeability increasing protein. In the secondary or specific granules, developed in the metamyelocyte phase, there are several collagenases, including the important matrix metalloproteinases (MMP)-8 and lactoferrin. Immediately after the development of secondary granules, tertiary granules or gelatinases develop. These granules contain MMP-9 and degrade of type V collagen and the extracellular matrix (Sheshachalam et al 2014). A fourth type of granule, called secretory vesicles, appears once the neutrophil is mature. They may have an endocytic origin, containing plasma proteins such as albumin.

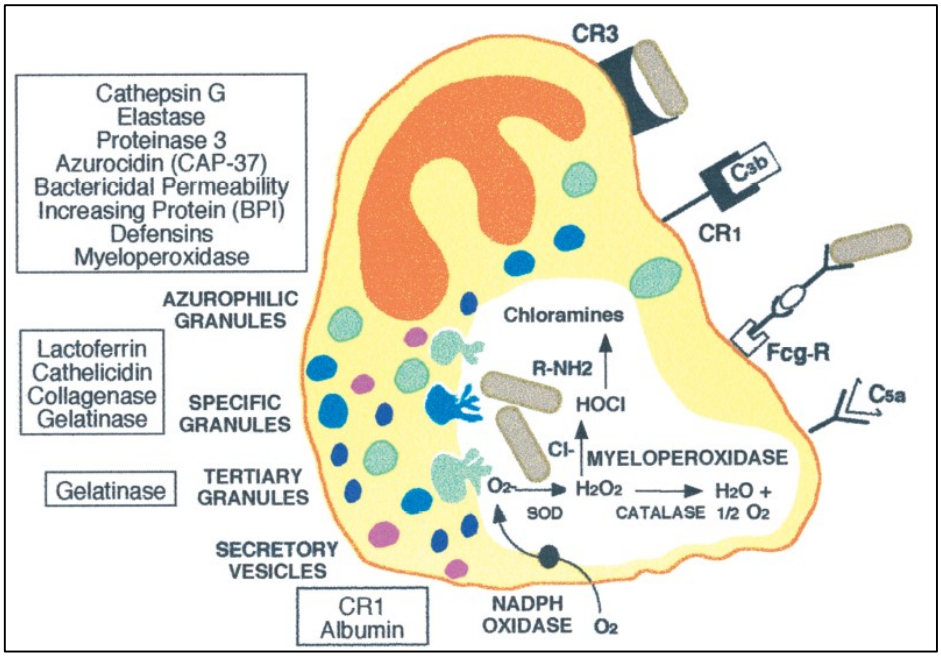


Fig 1. Witko-Sarsat et al., 2000 - Neutrophil effector mechanisms

Beside proteins and proteases, neutrophils produce a variety of different cytokines, such as tumor necrosis factor (TNF)-α, and chemokines. The most known and well-characterized chemokine is interleukin (IL)-8, a prototype of the C-X-C family, first described as a potent neutrophil

chemoattractant and activator (Baggiolini et al., 1994). It is expressed in response to LPS, mitogens such as PHA and cytokines, such as TNF-α, by a variety of cells including T lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells, and neutrophils. Interestingly PMN are not only able to produce this chemokine, but are the primary cellular target of IL-8 (Baggiolini et al., 1994; Zeilhofer and Schorr 2000). In PMN, IL-8 is stored in specific vesicles that seem to be associated with the endoplasmic reticulum (Pellmè et al., 2006).

Neutrophils express a large number of receptors on their surface, including G-protein-coupled chemokine receptors, Fc-receptors (Futosi et al., 2013), receptors for adhesion molecules such as selectin ligands and integrin like Mac-1 (CD11b/CD18; Van Spriel et al., 2001), fMLP receptor (Allen et al., 1992), Toll-like receptors (Prince et al., 2011) and other cytokines and innate immune receptors.

Under physiological conditions, neutrophils circulating in the blood stream are considered to be in a resting state. During the presence of proinflammatory or infective stimuli, they become “activated”, resulting in morphological and functional changes. They are initially “primed”, or partially activated, and when they encounter a pathogen, they become fully activated enhancing the oxidative burst, degranulation, phagocytic capabilities (Naegele et al., 2012). Full activation also enhances the production of neutrophil extracellular traps (NET); this latter consists of proteases and DNA (Mayadas et al., 2014). In our laboratory, we have extensively characterized by means of scanning and transmission electron microscopy, how the morphology of human neutrophil dramatically changes after activation with fMLP. The surface of stimulated neutrophils is uneven and rich with protrusions and the vesicular content increases upon activation.

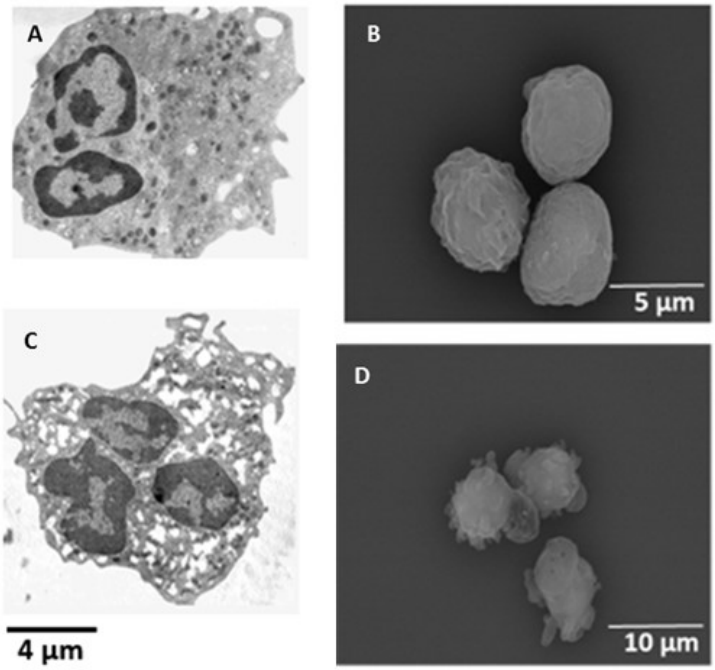


Figure 2: TEM (A, C) and SEM (B and D) images of human neutrophils isolated with standard procedures (Pinoli et al., 2016) and cultured alone (panels A, B) or in the presence of 0.1 µM fMLP (C and D). (Unpublished data of our laboratory)

In these conditions, PMN are activated and able to trigger a feedback amplification mechanism of their functions by autocrine and paracrine mechanisms that act also at the level of granules and proteases (Nemeth and Mocsai 2016).

At the intracellular level, stimulation triggers a cascade of events mediated by different pathways. In particular, in neutrophils the most prominent signals after the activation of G-protein coupled receptors (GPCR) are mediated by calcium (Futosi et al., 2013; Mocsai et al., 2015). Activation of phospholipase C (PLC) enzymes and consequently the production of inositol phosphate (IP) and diacylglycerol (DAG) lead to a release of calcium from intracellular organelles. The emptying of these stores induces the opening of the store-operated calcium entry (SOCE) channel (Nacacche 2013). The other major signaling pathway used upon neutrophil activation is linked to the stimulation of adenylate cyclase, increasing the concentration of cyclic adenosine monophosphate (cAMP). This

activates Protein Kinase A (PKA), which subsequently induces different intracellular signaling including the modulation of gene transcription (Mayer et al., 2013).

The intracellular cascade due to a chemoattractant signal is a very complex and delicate step, which induces a polarization of PMN with rearrangement of the internal architecture of the cells, which allows it to move towards the chemical gradient (Mocsai et al., 2015). Recent evidence emphasizes that the process by which neutrophils migrate to the site of inflammation is not one-way. Usually, once these cells execute their defensive function against pathogens, they undergo apoptosis, but this pattern is not followed by all neutrophils. Indeed, de Oliveira and Nourshargh excellently reviewed that neutrophils are able to return into the bloodstream by means of a sort of "backward migration" (de Oliveira et al., 2016; Nourshargh et al., 2016). This phenomenon was observed *in vivo* in the well-known animal model Zebrafish (Starnes and Huttenlocher 2012; Henry et al., 2013), in a rat model of glomerular capillary injury (Huges et al., 1997) and also in human neutrophils (Woodfin et al., 2011; Hamza and Irimia 2015). If this could have some relevance in the pathological field is still debated.

2.1. Neutrophil recruitment

Recruitment is a process that happens within minutes, in which neutrophils from the bloodstream arrive at the site of injury (Williams et al., 2011). The first event is the change in the endothelium, which up-regulates adhesion molecules, like P- and E-selectin that bind their ligands, including P-selectin glycoprotein ligand 1 (PSGL1), capturing the "free" neutrophils (Ley et al., 2007). L-selectin also mediates rolling on the endothelium, as well as lymphocyte function-associated antigen 1 (LFA1) that binds intercellular adhesion molecule 1 (ICAM-1) and ICAM-2 on inflamed endothelium (Ding et al., 1999). Moreover, LFA-1, together with MAC1 (also known as CD11b/C18 β_2-integrin), plays an

important role for firm adhesion of neutrophils binding ICAM-1 and it enables the cells to stop the rolling (Amulic et al., 2012; Mocsai et al., 2015).

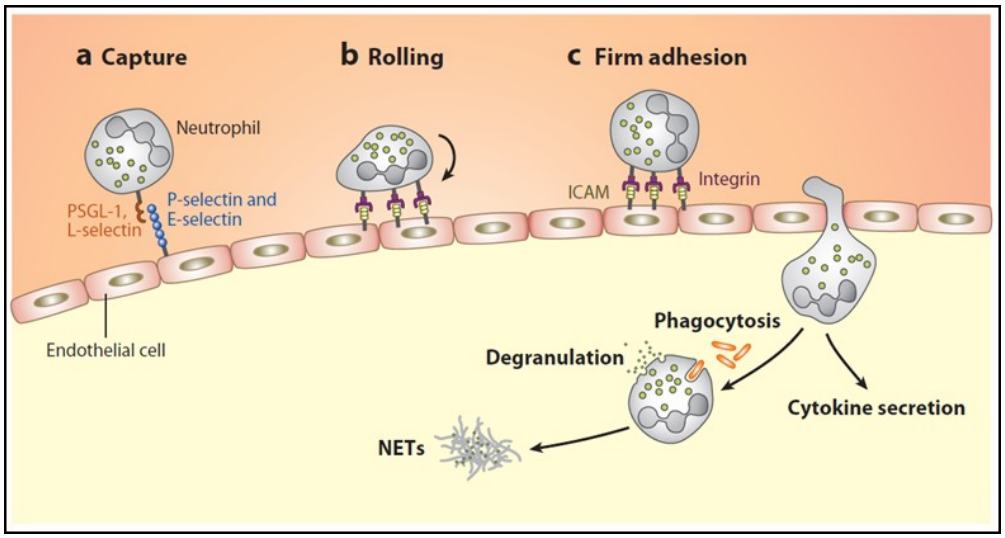


Figure 2: Amulic et al., 2012 - Neutrophil recruitment to sites of inflammation.

Neutrophils, then are able to migrate through the endothelium where encounter pro-inflammatory cytokines, like tumor necrosis factor (TNF)-α, that induces neutrophils priming and lead to activate the ability to release granules and activate the respiratory burst, necessary to fight the pathogen (Kolaczkowska and Kubes 2013).

2.3. Neutrophil lifespan

In the literature it is usually reported that neutrophils are characterized by a short life, and it is widely accepted that they die soon after their entry into the bloodstream in the absence of inflammation or infection, due to a spontaneous apoptotic program. In the last few years it was determined that the neutrophil lifespan can be prolonged for several days by inflammatory signals, although most of these data come from studies in mice (Geering and Simon 2011). During this additional period, neutrophils release inflammatory mediators and contribute to the orchestration of the inflammatory response (Witko-Sarsat et al., 2000; Mantovani et al., 2011).

Pillay and his collaborators demonstrated that human neutrophils under homeostatic conditions, labelled *in vivo* with $^{2}H_2O$, showed an average of circulatory neutrophil lifespan of 5.4 days (Pillay et al., 2010). In a previous study conducted in our laboratory, we showed that human PMN can be cultured up to 24 h and were able to respond to stimulation and releasing Ca^{++} from the intracellular stores (Marino et al., 2006). We confirmed these evidence in a paper of last year in which we demonstrated that neutrophils survive up to 24 h and are able to explicate their pivotal function such ad migration, ROS generation and cytokine production (Pinoli et al., 2016).

During the first part of my PhD research program, part of my projects was devoted to the investigation of the physiology of these cells, both under physiological conditions or during specific diseases such as atherosclerosis; in this period, I was involved in a study performed on patients undergoing endarterectomy for the presence of carotid plaques and my contribute in the study was acknowledged (Marino et al., 2015).

In addition, part of the first year of my PhD project, as above described, was dedicated to the investigation of the ability of neutrophils to survive for long time in culture (file attached 1: Pinoli et al., 2016).

2.4. Neutrophils and disease

For long time the role of neutrophils in the different immune-mediated pathologies, as well as autoimmune diseases, was neglected, most likely because they were regarded as cells with a short lifespan (Mocsai et al., 2015).

In the past decade, the idea that neutrophils play a role only in the first phase of diseases was replaced by a new and interesting vision that considers these cells as actively involved in different kind of diseases. The following paragraphs describe three of the main diseases where recently it was

demonstrated that neutrophils play a key role: atherosclerosis, multiple sclerosis and cancer. The first two diseases were widely investigated in the Center of Medical Pharmacology where I'm working. In particular regarding atherosclerosis, in our lab the pivotal role of neutrophil both in the initiation and in the progression of the disease was widely characterized.

Atherosclerosis is characterized by intense chronic inflammation of the large and medium-sized arteries, characterized by an intense immunological activity. The role of PMN in atherosclerosis only emerged from studies of the last decade (Weber et al., 2008). A 34,000 subject meta-analysis provided clarification of the role of neutrophils as a prognostic risk factor in patients with acute coronary syndrome (Guasti et al., 2011). Among inflammatory cells, PMN contribute to the destabilization of plaque in atherosclerotic process (Marino et al., 2009). Most of their functions depend on the mobilization of cytoplasmic granules and secretory vesicles (Soehnlein et al., 2009, Borregaard et al., 2007). At the level of atherosclerotic plaque, neutrophils represent a minority among the inflammatory cells, but their number increases rapidly after coronary injury (Naruko et al., 2002). The stability of the fibrous cap, consisting of smooth muscle cells and proteins of the extracellular matrix such as collagen and elastin, depends on the balance between synthesis and catabolism of extracellular matrix components. The contribution of neutrophils to plaque destabilization was investigated by Soehnlein and co-workers, who showed the potential interference mechanism in the matrix synthesis mediated by neutrophils (Soehnlein, 2012). In the plaques there were also found several markers of degranulation of neutrophils as α1-antitrypsin, elastase, myeloperoxidase and α-defensins (Leclercq et al., 2007). It was also demonstrated that neutrophils are present in carotid plaque specimens and that the cells present in tissue are able to produce proinflammatory mediators (Marino et al., 2015). In particular, it was shown that both circulating and intra-plaque PMN produce IL-8, VEGF and elastase, all known to be involved in the phase of plaque formation and stabilization (Marino et al., 2015). Interestingly in our lab, it was also shown that

isolated neutrophils from subjects at high cardiovascular risk (according to the ATPIII guidelines classification of cardiovascular risk; JAMA 2001) shows a profile that is quite different with respect to healthy subjects both at phenotype and functional level (Guasti et al., 2006; Guasti et al., 2008; Marino et al., 2007); during these studies it was shown that these conditions can be reverted by pharmacological treatment with statins, suggesting that the activated profile is not irreversible and that possible it can be prevented. In particular, it was shown that isolated neutrophils from venous blood of high-risk subjects (that do not present clinical symptoms of diseases but only at least 3 of the classical risk factors, including high LDL levels), before drug treatment, showed increased production of IL-8 both in basal conditions and after stimulation with fMLP. Similarly, these cells produced increased levels of reactive oxygen species. As a result of the administration of statins (lipid-lowering drugs typically prescribed for these conditions), these values were lowered, reaching values similar to that measured in cells of healthy controls (Guasti et al., 2006; Guasti et al., 2008). Not only functions but also phenotype of these cells were different with respect to healthy subjects and similarly the phenotype shows a similar pattern to healthy subjects after treatment (Marino et al., 2007). In addition, neutrophils are found to be involved also in the stable phase of the disease (Marino et al., 2009). Overall these evidence allow to highlight the important role of neutrophils in atherosclerosis, both in the early stages of plaque formation, and in the subsequent destabilization leading to its rupture.

Multiple Sclerosis (MS) is a progressive, irreversible autoimmune disorder of the central nervous system (CNS) and the role of PMN in this pathology has not been examined extensively. The data present in literature are also contradictory (Ziaber et al., 1998). MS is characterized by inflammation, demyelination and axonal loss (Cosentino and Marino, 2013), and is the most common inflammatory demyelinating CNS disease representing a major cause of disability in both young and older populations (Keegan and Noseworthy, 2002; Frohman et al., 2006; Noseworthy et al., 2000; Nylander

and Hafler 2012). The etiology of MS is still debated, and both genetic and environmental agents are likely to play a prominent role, as suggested by the clear gender difference in MS cases, with females being more susceptible than males by about 2-3:1 (Voskuhl and Gold, 2012). No cure exists and treatments focus on slowing disease progression, reducing relapse and managing symptoms. Current treatment for MS relies mainly on immunomodulatory and immunosuppressive therapeutic strategies (Frohman et al., 2006; Miller and Rhoades 2012) and among available options, IFN-β has represented for many years one of the first-line choice (Kremenchutzky et al., 2007).

In recent years, the role and contribution of neutrophil to MS has been investigated (Rumble et al., 2015). It was demonstrated that PMN in MS patients show a "primed" phenotype with a change in surface level of cytokines, increased degranulation, increased ROS production and cell migration rate in response to pro-inflammatory stimuli (Ferretti et al., 2006; Naegele et al., 2012; Hertwig et al., 2016). Mast cells secreting pro-inflammatory molecules, e.g. the TNF-α, attract neutrophils from the periphery, which enter into the CNS through the compromised blood brain barrier (BBB), where they produce pro-inflammatory cytokines and reactive oxygen species that contribute to axonal damage (Sayed et al., 2010). A report of Carlson and colleagues support this idea; they have shown that the elimination of PMN (in animal model of MS, EAE) prevents the destruction of the BBB and mitigates the clinical and histological features of the disease, although there are peripherally activated T cells that can direct applied to the brain to destroy the myelin sheath (Carlson et al., 2008).

Cancer is one of the leading causes of death in the world. Many studies are aimed at finding new targets for the development of innovative treatments, or at limiting the major side effects of the treatments. The tumor environment is characterized by intense inflammation and thus the immune cells play an important role. In particular, tumor-associated neutrophils (TAN) represent a double-edged sword, with one group of TAN attacking cancer cells, while another may favor the development and progression of the tumor, stimulating angiogenesis by secreting important factors such as VEGF

and IL-8 (Heryanto et al., 2004; Albini et al., 2005; Galdiero et al., 2013). Moreover, PMN promote the degradation of the extracellular matrix through the production of metalloproteases (MMP)-8 and 9, as well as proteolytic enzyme as elastase (Granot and Jablonska 2015). The role of neutrophil extracellular traps (NET), is still debated, even if it seems that NET contribute to the formation of metastasis trapping of circulating tumor cells at distant metastatic sites (Kim and Bae 2016).

These pro- and anti-tumoral "properties" of PMN depend on the microenvironment in which they are found; for example, factors such as transforming growth factor (TGF)-β and interferon (IFN)-β promote the switch between a pro- or anti-tumor phenotype of TAN (Sagiv et al., 2015). Which is the precise role of neutrophils in cancer, it is still unclear. In some cases, it seems that they favor the growth of the tumor (Hattar et al., 2014), while in others neutrophils exert an immunostimulatory function adapted to counter the progression of the disease (Eruslanov et al., 2014).

Chapter 3

The data collected in this chapter report the main results of my PhD project and will be included in the preparation of a manuscript that I am writing for the submission to an International peer-reviewed journal.

Dopamine and neutrophils

The idea that the immune system might be affected by DA (Basu and Dasgupta 2000), was strengthened by the identification of DR on different immune cells (McKenna et al., 2002; Sarkar et al., 2010; Levite et al., 2012; Kustrimovic et al., 2014), as well as by the evidence that immune cells are able to release DA and are equipped with the enzymes necessary for the synthesis, storage and degradation of DA (Basu et al., 1993; Bergquist et al., 1994; Marino et al., 1999; Cosentino et al., 2000; Cosentino & Marino 2013). On the basis of this evidence, DA has been defined by some authors as "NeuroImmunotransmitter", due to its ability to modulate the functions of different cells of the immune system (Levite 2015).

The ability of DA to affect adaptive immunity was widely investigated both in healthy individuals (Cosentino et al., 2007) and in different immune-mediated diseases, such as multiple sclerosis (Zaffaroni et al., 2008; Cosentino et al., 2014) or Parkinson Disease (PD; Phani et al., 2012). On the contrary, there are relatively few studies about the possible dopaminergic modulation of innate immunity. Recently, it was shown that the polymorphonuclear leukocytes (PMN) of PD patients, 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 TH (Cordano et al., 2015).

PMN seems to be able to produce DA (Cosentino et al., 1999) and express DR on their surface (McKenna et al., 2002), but it is not clear whether DA or other DR ligands, through the interaction with their receptors, are able to interfere with the functions of these cells.

In vitro studies showed that in human PMN, DA inhibits reactive oxygen species (ROS), superoxide anion production and cell migration (Wenisch et al., 1996; Sookhai et al., 2000; Matsuoka 1990; Trabold et al., 2007), or that incubation with DA induces apoptosis (Sookhai et al., 1999), but the possible involvement of DR in the observed effects was not investigated.

The main aim of the present study was the investigation by means of flow cytometry and real time PCR, of the presence of DR on human PMN both under resting conditions and after activation of cells with fMLP, the chemotactic the N-formyl oligopeptide that is released by bacteria. In addition, we have investigated the ability of DA to modulate some PMN functions such as migration, ROS and IL-8 production and through the use of selective ligands for both receptors family if the DA-induced effects were receptor-mediated. To complete the picture, the involvement of DR was investigated by means of selective D_1-like and D_2-like ligands. Finally, by means of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) we have also investigated if the effects induced by DA (and dopaminergic ligands) were also reflected by morphological changes.

METHODS

PMN isolation and cell culture

Experiments were performed on whole blood of healthy donors obtained from the local blood bank (Ospedale di Circolo, Fondazione Macchi, Varese, Italy). PMN were isolated by standard density-gradient centrifugation as previously described (Scanzano et al., 2015). 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).

The effect of DA (1 nM – 1 µM) on PMN functions was tested by culturing cells in RPMI 1640 (Euroclone S.p.A, Italy) at the concentration of 1×10^6 cells/mL in resting condition or after activation of cells with fMLP (0.1 µM) at 37°C for different times according to the specific experimental procedures as below described. In addition, some experiments were performed using selective D_1- and D_2-like agonists and antagonists and appropriate concentrations were selected according to the data of literature. After incubation, cells were centrifuged (400 g, 5 min, 20°C), and pellets and supernatants were separated and analyzed immediately or stored at −80°C for subsequent analysis.

Flow Cytometry for DR

For flow cytometric evaluation of DR, aliquots of 50 µL of whole blood from each sample were added to 950 µL of RPMI and incubated under resting or activated (fMLP 0.1 µM) conditions for 30 min at 37°C, with 5 % of CO_2. For both conditions, six aliquots 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 (DR-AF647, cod. 406414 Biolegend-Campoverde, Italy) alone, as a negative control. At the end of the incubation, the whole blood aliquots were centrifuged at 600 g for 5 min at room temperature (RT) and were added with 3 mL of a lysis solution containing (g/L) NH_4Cl (8.248), $KHCO_3$ (1.0) and EDTA (0.0368) in order to remove the erythrocytes. Incubation was performed at RT for 5 min, during which samples were gently vortexed. Samples were then centrifuged, supernatants were removed and cells were washed one time in 1 mL of PBS (pH 7.4) supplemented with 1% BSA (PBS/BSA) and finally resuspended in 100 µL PBS/BSA. The DR staining protocol consisted of two steps. In the first step, each aliquot was subjected to indirect immune fluorescence staining by using rabbit polyclonal primary antibodies (Ab 1°) directed against each of the human D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) DR. To this end, cells were before treated

with 5 µL of Human TruStain FcX-Fc Blocking Solution (cod. 422302, Biolegend-Campoverde) to prevent unspecific binding of Ab 1° to human Fc receptors (FcRs) expressed on granulocytes. After 10 min of incubation at RT and without wash, the cells were then labeled with 10 µL of each anti-DR Ab1° (final dilution 1:100) for 30 min on ice. After one washing with PBS 1X, the pellets were resuspended in 100 µL of PBS 1X added with DAR-AF647 Ab (final dilution 1:400) and incubated for 30 min in ice in the dark. At the end, the samples were washed with 1 mL of PBS and resuspended in 400 µL of PBS and kept in ice until analysis. Acquisition was then performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, 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 samples were collected in the gate. Data were analyzed using BD FACSDiva software (version 6.1.3) and the results were finally expressed as percentage (%) of DR positive cells in the granulocyte gate or ratio of the mean fluorescence intensity (MFI) ratio between the value of MFI and the value of isotypic control.

PMN culture for DA DR Real Time PCR assay

The effect of IL-8 and fMLP on DR mRNA expression was evaluated culturing PMN at the concentration of 1×10^6 cells/mL in RPMI 1640 alone (resting) or in presence of fMLP (0.1 µM) 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.

RNA isolation and Real-Time Polymerase Chain Reaction for DR

Total mRNA was extracted from 1×10^7 cells by Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany) 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, Foster City, CA, USA) according to the manufacturer's instructions and Real-time PCR was performed (ABI prism 7000 apparatus; Applied Biosystems) using assay-on-demand kits. Threshold cycle values (Ct1) for the genes of interest were calculated, normalized to 18S RNA (Ct2) (housekeeping) content, and finally expressed as $2^{-\Delta Ct}$, where ΔCt = Ct2 − Ct1. Primers (Applied Biosystems) are shown in supplemental table S1.

Dopamine intracellular content assay

DA intracellular content was measured by high-performance liquid chromatography with multielectrode electrochemical detection (HPLC-ED). After 3 h of culture under standard conditions, $1x10^6$ cells were harvested, centrifuged, and processed as previously described (Cosentino et al., 2000). Finally, 30 µL of recovered supernatant was injected into the chromatographic system. The chromatograms were collected, stored, and processed with the application software Coularray for Windows (ESA). DA in the samples was quantified by using the peak heights of a standard curve generated by injecting known samples (3 fmol to 3 pmol), and values were finally normalized for protein content and expressed as pg/mg of protein (pg/mg).

Cell apoptosis

Immediately after culture, samples were centrifuged at 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, Milan, Italy) 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 the incubation, 250 µL of Binding Buffer were added and samples were analyzed by BD FACSCanto II Flow Cytometer (Becton Dickinson Italy, Milano, Italy) and data were analyzed using BD FACSDiva software (version 6.1.3). PMN were identified based on FSC and SSC properties, and at least 15.000 events were collected from each samples. Viable (ANX-/PI-), early apoptotic (ANX+/PI-) and late/necrotic (ANX+/PI+) PMN were identified on a biparametric plot ANX-FITC vs PI with a log scale.

Cell migration

Cell migration was measured by use of the Boyden chamber assay (Maio et al., 2011). The chemotactic peptide fMLP (0.1 µM; Sigma, St Louis, Missouri, USA) was placed in the bottom compartment of the chamber, and a 3-mm-pore cellulose nitrate filter (Millipore Corporation, Bedford, Massachusetts, USA) was placed between the two compartments. In experiments with DA or dopaminergic agents, drugs were added on the top of the chamber, while fMLP, when present, was added in the lower compartment. After an incubation period of 1.5 h at 37°C, the filter was recovered, dehydrated, fixed, and finally stained with hematoxylin and eosin. Finally, migration was measured and quantified microscopically measuring the distance (in µm) from the surface of the filter to the leading front of cells.

ROS generation

Intracellular ROS levels were assessed by use of the redox sensitive dye C-DCDHF-DA (Molecular Probes, Eugene, OR, USA) as previously described (Scanzano et al., 2015). Briefly, freshly isolated PMN were resuspended at the concentration of $1x10^6$ 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 spectrofluorimeter (Perkin-Elmer LS-50B, Perkin-Elmer Instruments, Bridgeport, CT, USA), with excitation wavelength of 488 nm. Fluorescence emission was collected at 525 nm. ROS levels were assayed under resting conditions and after stimulation with fMLP 0.1 µM.

In order to evaluate if DA or dopaminergic agents affect resting ROS generation, substances were added to the cells after a resting period of 60 s. In experiments with fMLP-stimulated cells, fMLP was added to the cells at 180 s, DA or dopaminergic agonists at 120 s and dopaminergic antagonists at 60 s. ROS generation were monitored up to 30 min and finally expressed as difference (Δ) between resting values measured at 60 s and levels measured after 30 min monitoring.

IL-8 assays

To evaluate if DA was able to modulate IL-8 production, cells were incubated for 3 h in presence of DA, under resting conditions or after stimulation with fMLP (0.1 µM). IL-8 levels in supernatants of PMN cultures were quantified using a sandwich-type enzyme-linked immunoadsorbent assay (Invitrogen ELISA kit; Life Technologies). The limit of detection was 1 pg/mL.

Light microscopy and Transmission Electron Microscopy of cell morphology

Isolated neutrophils were resuspended at the concentration of $5x10^6$ cells/mL in RPMI medium and incubated for 3 h under resting conditions or after stimulation with fMLP 0.1 µM. In different experiments, DA or other dopaminergic agents were added. After the incubation, the collected pellets were fixed with glutharaldeid 4% in Na-cacodylate buffer (0.1 M; pH 7.2).

Pellets were washed in Na-cacodylate buffer and post-fixed for 20 min 1% osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol scale, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultrotome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin) and were observed with a light microscope (Eclipse Nikon, Amsterdam, Netherlands). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol Tokyo, Japan).

Scanning electron microscopy (SEM) of cultured PMN

Ultrastructural analysis of PMN was performed by means of SEM analysis. To this end, PMN were resuspended at the concentration of 1 x 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 (Scanzano et al., 2015). Samples were then subjected to critical point drying in CO_2, coated with 10 nm of pure gold in a vacuum sputter coater Emitech K550 (Emitech Ltd., Ashford, UK) and images were collected in a direct mode using a Philips XL 30 SEM-FEG scanning electron microscope (SEM XL 30 FEG, FEI Company, Eindhoven, Netherlands).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM) or median ± 25^{th} and 75^{th} percentile, as appropriate, and n, with n indicating the number of observations. Parametric continuous variables were compared by means of Student's t test. Analysis of the statistical significance of the differences was determined by Student's t test or by one-way analysis of variance followed by Dunnett or Bonferroni post-test, as appropriate. 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, San Diego, CA, USA, www.graphpad.com).

RESULTS

Flow cytofluorimetric expression of DR on human PMN

Whole blood was analyzed for the presence of the five DR on PMN membrane. As shown in figure 1, cytofluorimetric analysis showed that all DR are expressed on PMN (Figure 1, panel A). D_1-like DR were expressed on average by 82-89% of PMN, with an average ratio of MFI of 7, while D_2-like DR were expressed by 18-58%, with a ratio of MFI between 1.9 and 3.3 (Figure 1, panel B and C). Stimulation with fMLP (0.1 µM) decreased the percentage of cells expressing the D_1-like DR D_5 and the D_2-like DR D_3, as well as the ratio of MFI of the D_1-like DR D_1 (Figure 1, panel B and C).

Real time PCR expression of DR on human isolated PMN

Figure 2 shows that PMN also expressed detectable mRNA levels of all the DR, with the following order of magnitude: $D_4 > D_5 > D_1 = D_3 >> D_2$. Stimulation with fMLP (0.1 µM) induced a 6-fold increase of DR D_1 and decreased DR D_5 by 2.5 times, as well as all the D_2-like DR, respectively by 1.7 (D_2), 10.8 (D_3), and 2.3 times (D_4) (Figure 2, panel A and B).

Endogenous DA in cultured PMN

Analysis of cell pellet and cell medium by means of HPLC showed that PMN contain a significant amount of DA. In particular, DA content in PMN cultured for 3 h under resting conditions was (n = 8; mean ± SEM) 2.3±2.2 pg/mg of protein and 0.8±1.1 pg/mL in the culture medium; stimulation of cells for 3 h with fMLP (0.1 μM) did not affect DA production or medium content (6.0±6.8 pg/mg of protein and 0.5±0.6 pg/mL, respectively for cell and medium content; $P>0.05$ vs respective resting conditions).

Cell viability and apoptosis

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 analyzed by propidium iodide staining. As shown in table 1, incubation with DA in the 1 nM - 1 μM concentration range for either 30 min or 3 h did not affect the percentage of viable, early and late apoptotic PMN, with the only exception of DA 1 μM which slightly increased late apoptotic cells and slightly reduced viable and early apoptotic cells (less than 1%), but only after 3 h of incubation and DA 0.1 μM that is able to affect late/necrosis stage after 3 h (Table 1).

Cell migration

Cell migration, is a key process through which PMN migrate into tissue and occur both under physiological conditions (spontaneous migration) or after release into the tissue of proinflammatory stimuli that acts as chemoattractant for cells. DA, at all the concentration tested (1 nM – 1 μM), was unable to affect spontaneous migration (data not shown). On the contrary, migration induced by

proinflammatory stimuli such as fMLP (that, as expected, induces a significant increase of PMN migration), was significantly reduced by DA and this effect is concentration-dependently as clearly shown in figure 3, panel A.

The ability of DA to reduce the fMLP-induced migration was unaffected by the D_2-like antagonist haloperidol (figure 3, panel B), which, per se did not affect nor spontaneous migration (14.4±1.0 µm *vs* 12.4±0.5 µm, n = 5, P>0.05), neither fMLP-induced migration (9.0±4.3 µm *vs* 9.8±5.5 µm, respectively, n = 3, P>0.05). On the contrary, the D_1-like antagonist SCH-23390, concentration-dependently revert the inhibitory effect exerted by 1 µM DA on fMLP-induced migration (figure 3, panel B), although did not affect neither PMN spontaneous migration (14.4±1.1 µm *vs* 12.4±0.5 µm, n = 5; P>0.05), nor fMLP-induced migration (9.3±3.7 µm *vs* 9.8±5.5 µm, respectively, n = 3, P>0.05).

The D_1-like agonist SKF-38393 (0.1 µM), similarly to DA, did not affect spontaneous migration (data not shown), but significantly reduced the fMLP-induced migration (figure 3, panel A) and this effect is superimposable to the DA-induced effects. The effects of SKF-38393 were completely reverted by SCH-23390 (1 µM; 13.9±5.6 *vs* 14.3±3.0, 13.9±5.6 *vs* 3.1±0.9 n = 5, P>0.05 *vs* fMLP and P<0.05 *vs* SKF-38393, respectively) and only partially by haloperidol (1 µM; 8.5±2.1 *vs* 14.3±3.0, 8.5±2.1 *vs* 3.1±0.9 n = 5, P<0.01 *vs* fMLP and P<0.01 *vs* SKF-38393, respectively).

The D_2-like agonist pramipexole (1 µM), did not influence spontaneous cell migration (data not shown) and only slightly reduces the fMLP-induced PMN migration (figure 3, panel A).

ROS production

ROS generation represent a mechanism through which neutrophils respond to infective or inflammatory stimuli and provide to neutralize pathogen. DA up to 1 µM did not affect resting ROS production (measured as delta (Δ)-FI [30 min- 60 s]; data not shown). On the contrary, DA

significantly reverted the fMLP-induced ROS production in a concentration-dependent manner (figure 4).

The effect of DA on fMLP-induced ROS generation was mimicked by the D_1-like agonist SKF-38393 (that per se not influence spontaneous migration), while the D_2-like agonist pramipexole (1 µM), did not influence neither spontaneous (data not shown), nor fMLP-induced ROS production (figure 4).

IL-8 production

IL-8 is a chemokine produced by PMN that plays a key role in the inflammatory response induced by these cells and act as chemoattractant. Different kind of proinflammatory stimuli are able to increase the PMN production of this chemokine.

IL-8 resting production in PMN cultured for 3 h was 267.4±129.6 pg/mL and DA 1 µM was unable to affect this production (241.5±125.2 pg/mL; $P>0.05$). As expected, stimulation with fMLP significantly increased IL-8 production with respect to resting values (1189.9±293.3 pg/mL, $P<0.05$ *vs* resting); the fMLP-induced increase of IL-8 was unaffected by the preincubation with DA (1055.8±290.6; $P>0.05$ *vs* fMLP alone).

Ultrastructural analysis (TEM)

TEM analysis of cell structure is a classical method employed to study the changes occurring at morphological levels during pathological conditions or after stimulation of cells with different kind of compounds.

Unstimulated cultured neutrophils showed a typical morphology that was unaffected by the incubation of cells for 30 min with DA and dopaminergic agents (figure 5, upper panels): roundish in

shape, cytoplasm filled with multilobular nuclei characterized by dispersed chromatin, cytoplasm occupied with well-distributed organelles and granules. As expected, the stimulation with the proinflammatory peptide fMLP profoundly affect cell morphology (lower panel): PMN showed irregular profile and massive degranulation. The preincubation with DA revert the effects on morphology induced by fMLP and similarly a reversion of the fMLP-induced changes can be observed after preincubation with the D_1-like agonist SKF-38393 (1 μM). On the contrary, preincubation with the D_2-like agonist pramipexole (1 μM) did not revert the structure induced by fMLP.

Finally, the involvement of DR on DA-induced morphological changes was investigated with the preincubation (before DA) with the D1-like antagonist SCH-23390 (10 μM) and D_2-like antagonist haloperidol (10 μM). As clearly shown, DA-induced effects were reverted by the D_1-like antagonist but not by the D_2-like antagonist.

Cell morphology (SEM)

There were no major morphological differences between PMN alone and in the presence of dopaminergic ligands (figure 6, upper panels); the stimulation with fMLP induced changes clearly represented as several membrane tubulovesicular extensions typical of activated PMN (lower panels). The culture of cells in the presence of DA (1 μM) or SKF-38393 (0.1 μM) (before fMLP) shows images of cells with roundish shape clearly distinguishable from the shape of activated cells, and more similar to non-activated cells, while pramipexole did not affect the morphological changes induced by fMLP. Finally, SCH-23390, but not haloperidol, prevent the DA-induced effects.

DISCUSSION

The main finding of the study presented in this chapter is that human neutrophils express all the dopaminergic receptors, both at mRNA and at surface membrane level and that this expression can be modulated by the proinflammatory stimulus fMLP. In addition, we have shown that DA profoundly affect neutrophil functions and this effect is receptor-mediated, involving for the most part of the observed effects the D_1-like receptor type, as suggested by the ability of SCH-23390 (D_1-like antagonist) to revert and of SKF-38393 (D_1-like agonist) to mimics the DA-induced effects, although a possible contribute of D_2-like DR cannot be excluded (considering the effect of pramipexole on cell migration).

Recently, the involvement of PMN in immune-mediated diseases was postulated not only for inflammatory syndromes like atherosclerosis, inflammatory bowel disease, etc., but also for immune-mediated diseases affecting the central nervous system (CNS) as for example in multiple sclerosis (Naegele et al., 2012). Interestingly, some immune-mediated disease affecting the CNS present a dysregulation in the catecholaminergic pathways, including the dopaminergic pathways (e.g. multiple sclerosis, Parkinson diseases; Cosentino and Marino, 2013; Gatto et al., 1996; Kustrimovic et al., 2016).

It is now known that nervous and immune systems are closely inter-connected, however, the evidence in literature regarding the modulation of PMN by the dopaminergic system are still very little. It was previously shown that PMN contain trace of DA (Cosentino e al., 1999) and in the present study we confirm this evidence. In addition, in the present study we have investigated if this content can be affected by fMLP and our results suggests that DA content was unaffected during stimulation of cells with fMLP.

Presently, the presence of DR on human PMN was only sporadically investigated and in some cases, data reported are contradictory (McKenna et al., 2001, Sookhai et al., 1999, Pereira et al., 2003, Boneberg et al., 2006, Chen et al., 2014). We have investigated the membrane receptor expression (by means of flow cytometry) and mRNA content (by means of real time PCR) both under resting conditions and during activation with fMLP. To our knowledge, this was the first study performed with this aim and our results show that all the DR are expressed both at mRNA and protein levels, but with different extent. In particular, DR D_4 present the highest mRNA expression while DR D_2 the lowest. On the contrary, considering protein membrane expression, we show higher values for the D_1-like (D_1 and D_5) and lower for the D_2-like (D_2, D_3 and D_4). In addition, the stimulation of PMN with different kind of stimuli affected the expression of some subtypes both at mRNA and protein levels. In particular, stimulation with fMLP induces a significant increase in mRNA expression for DR D_1 (6 time over resting values) while the expression of DR D_3 and DR D_4 was decreased (respectively, 10 and 2.3 times). On the contrary, membrane receptor expression measured by flow cytometry shows that only DR D_1 was significantly affected by stimulation with fMLP and in particular, we observed a strong decrease in expression with no significant changes occurring for the other receptors. So far, real time PCR and flow cytometry present opposite results regarding the modulation of the expression of some receptors. A possible explanation of this apparent discrepancy can be given considering that membrane receptor expression was measured after 30 min of stimulation while mRNA expression after 3 hours; therefore, the increased expression at mRNA level can be due at the increased turnover to reconstitute the receptor loosed.

We cannot exclude that this differences can be due at the presence of other cell subsets (eosinophils, macrophages or other) that cannot be completely excluded with dextran-separation in the case of isolated neutrophils for the mRNA assay and by the presence of eosinophils and basophils in the gate of neutrophils in the case of flow cytometry. It is documented in fact that the

methods used conventionally for the isolation of neutrophils are not free of contamination (Kuhns et al., 2016).

In any case, discrepancy between mRNA (by means of real time PCR) and protein expression (with flow cytometry or western blot analysis) is not so uncommon. For example, we have previously shown that TH expression in human peripheral blood mononuclear cells was quite different after stimulation with phytohaemagglutinin and that mRNA expression reflect this stimulation while membrane expression remains unchanged (Reguzzoni et al 2002). In order to better clarify our observations in the present study, further methods to assess protein levels of DR, such as western blot and immunohistochemistry, could provide useful evidence about the differences observed.

The data showing that DR not only are expressed on PMN, but that the expression can be modulated by fMLP are of extreme relevance considering that previous studies have reported the ability of DA to affect some PMN functions. For example, it has been shown that DA reduces the activity of PMN, such as a production of ROS, expression of CD11b/18 and migration (Wenisch et al., 1996; Sookhai et al., 2000; Matsuoka, 1990; Trabold et al., 2007), but in all these studies the involvement of receptors was not investigated.

In the present study, for the first time, it was demonstrated that the effects exerted by DA on neutrophil functions were receptor-mediated. We found that DA *per se* did not affect PMN functions or morphology under resting conditions. Otherwise, in fMLP-activated PMN, both migration and ROS production were reduced in the presence of DA; similarly, the morphological changes observed after stimulation with fMLP were reverted by the presence in the culture medium of DA. The receptor-mediated effect of the observed changes is suggested by the ability of the D_1-like antagonist SCH-23390 and the lack of haloperidol (D_2-like antagonist) to revert the DA-induced effects. In addition, the involvement of D_1-like DR was sustained by the data showing that

the D_1-like agonist SKF-38393 mimicked the effects exerted by DA on both migration, ROS production and morphology. Nevertheless, we cannot discriminate if the observed effects are DR D_1 or DR D_5 mediated.

In addition, in line with functional studies, we show that cell morphology reflects the functional responses: DA and dopaminergic agonists are unable to affect cell morphology in the resting state. On the contrary, both DA and SKF-38393 are able to revert the morphological changes induced by fMLP, while pramipexole cannot. Similarly, the effects of DA on fMLP induced morphological changes is blocked by SCH-23390 and not by haloperidol. So far, morphological approaches with TEM and SEM confirm that the DA-induced effects are receptor-mediated and involved specifically the D_1-like subtypes.

The inability of DA to modulate both resting and stimulated production of IL-8 in human PMN is quite surprising considering the pivotal role of this chemokine as chemoattractant and proinflammatory mediator (Baggiolini 2001). Interestingly we have previously found that adrenaline and noradrenaline, the other two catecholamines, although profoundly affecting neutrophils functions were unable to modulate IL-8 production (Scanzano et al., 2015). This suggests that at least proinflammatory chemokines production is not under catecholaminergic control. Further investigations, specifically dedicated to the role of catecholamines on the possible modulation of cytokines and chemokines production in these cells of the innate immunity will be considered.

Another interesting finding is that DA was unable to affect cell viability and did not exert proapoptotic effects, although in literature a proapoptotic effect of DA is widely described (Cosentino et al., 2004; Goldstein et al., 2014). This evidence is of extreme relevance because, at least in this model, we can therefore exclude that the profound inhibitory effects by DA observed on some functions can be ascribed at a reduction of cell viability.

In conclusion, the present results about the dopaminergic modulation of human PMN functions can be suggestive of a new vision of the role of dopaminergic dysregulation in the genesis of some immune-mediated diseases. It can represent new opportunity for further studies aimed to clarify whether these findings can help to promote new therapeutic strategies in counteracting PMN activation and tissues invasion, known to be the first step in most part of immune-mediated diseases, including inflammatory diseases of the CNS.

Table 1 Real-Time PCR conditions.

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	GACAGGAAACAGGCAGTGAGGATACGAACAGAGA AGTCCCTTCCACCACCAGCCCAGTCCCGTCCATGGC AGAGGTGTTCAGAGTCCTCATCTTCCTAAGAGAAAG CACATCA	5:174870011-174870124	84	60	100
DRD2	Hs.73893	NC_000011.9 NG_008841.1 NT_033899.8	ENST00000346454 ENST00000362072 ENST00000544518 ENST00000542968 ENST00000538967 ENST00000355319	AGCAGGGTGACAATGAAGGGCACGTAGAAGGAGA CGATGGAGGAGTAGACCACGAAGGCCGGGTTGGCA ATGATGCACTCGTTCTGGTCTGCGTTATTGAGTCCG AAGAGGAG	11:113286246-113287609	83	60	103
DRD3	Hs.121478	NC_000003.11 NG_008842.1 NT_005612.16	ENST00000460779 ENST00000467632 ENST00000295881 ENST00000383673 ENST00000281274	TTAAGGATGCTGGCTGTACACATCATGACATCCAGG GTGACAAAAACATCACAGCAAATGCGGCTGAAATTC CAGACTCCACCTGTCACCTCCAGGTATACCACCCAG GGCATCACCAAGGTGGCCACCAGCAAGTCTGCCACA GCCAGGCTCACTA CTAAGTAGT	3:113878626-113890646	136	60	100
DRD4	Hs.99922	NC_000011.9 NG_021241.1 NT_009237.18	ENST00000176183	GCTGTGCTGGACGCCCTTCTTCGTGGTGCACATCAC GCAGGCGCTGTGTCCTGCCTGCTCCGTGCCCCCGCG GCTGGTCAGCGCCGTCACCTGGCTGGGCTACGTCAA	11:640411-640580	140	60	98

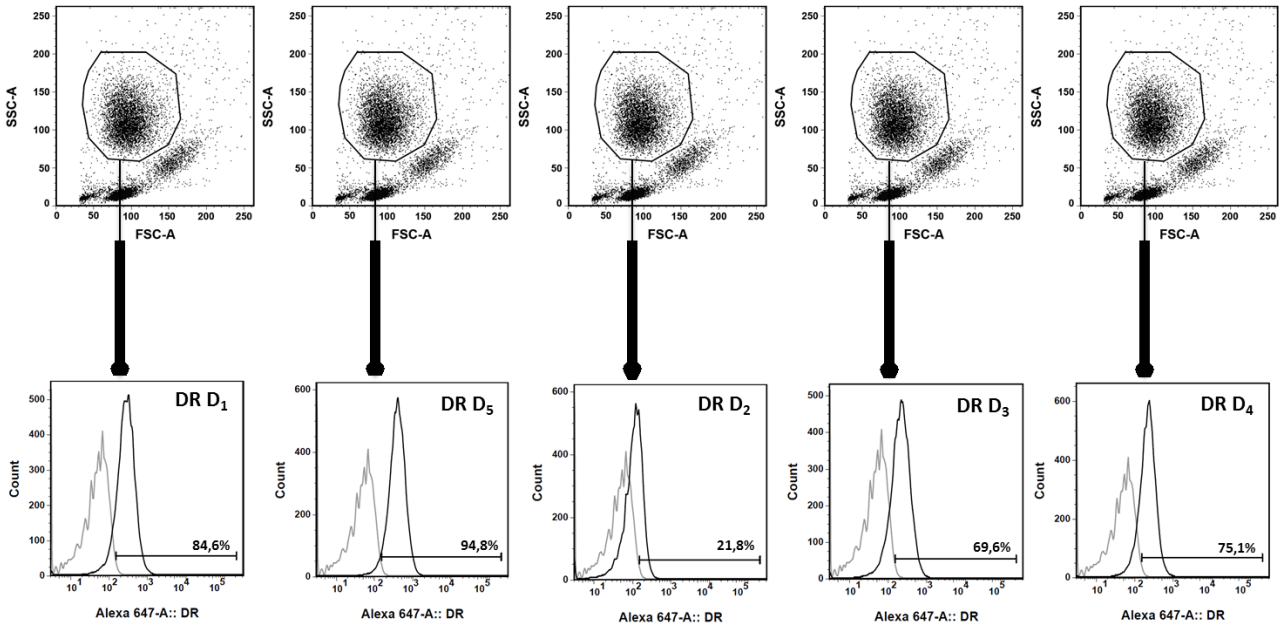
				CAGCGCCCTCAACCCCGTCATCTACACTGTCTTCAAC GCCGAGTTCCGCAACGTCTTCCGCA				
DRD5	Hs.380681	NC_000004.11 NG_012024.1 NT_006316.16	ENST00000304374	TTTTAAACAGCAGGTTGTGTGTGTGCAGTGATGT GGTGGGAGCACAGCTTTCCTGGGTCTGGATTCCCGT GGCTTTGTGCTTATGTCATTTCTTCTCTCTGTGCTGG TGGGGGCCTCTTTACCATAGCTTAAG	4:9785451-9785585	105	60	97
RPS18	Hs.627414	NC_000006.11 NT_007592.15 NT_113891.2 NT_167245.1 NT_167247.1 NT_167248.1 NT_167249.1	ENST00000454021 ENST00000486781 ENST00000484321 ENST00000211372 ENST00000477055 ENST00000476288 ENST00000439602 ENST00000474973 ENST00000457341 ENST00000494232 ENST00000434122	GTGGAACGTGTGATCACCATTATGCAGAATCCACGC CAGTACAAGATCCCAGACTGGTTCTTGAACAGACAG AAGGATGTAAAGGATGGAAAATACA	6:33243742-33243838	67	60	98

	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

Table 2. Effect of DA on PMN viability (ANX-/PI-), early apoptosis (ANX+/PI-) and late apoptosis (ANX+/PI+) measured by flow cytometry. PMN were cultured for 30 min or 3 h alone or in the presence of DA. Data are expressed as % of total cells and are represented as means±SEM of 3 separate experiments. * = $P<0.05$, ** = $P<0.01$ vs respective control and # = $P<0.05$ vs respective condition at 30 min.

FIGURE AND LEGENDS

Panel A



Panels B and C

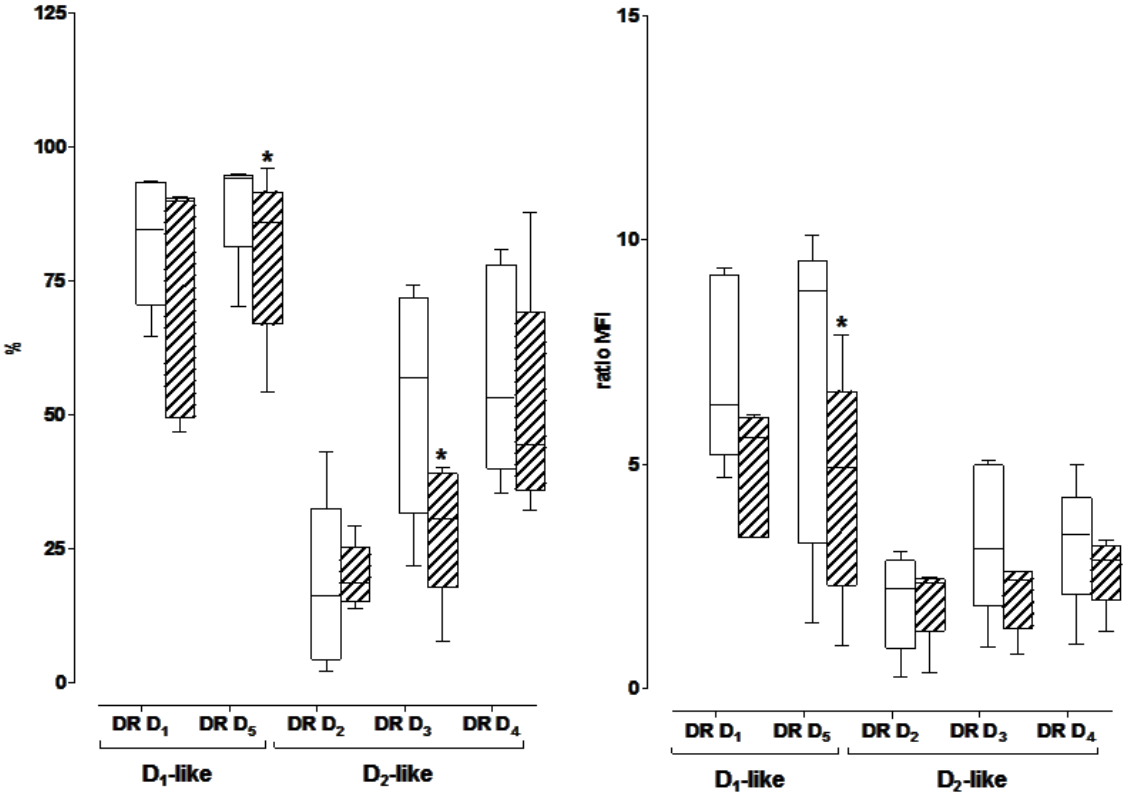


Figure 1. Flow cytometric assay of DR

Panel A: representative flow cytofluorimetric analysis of the DR expression on PMN.

Flow cytometric assay of DR on PMN under resting condition and after stimulation for 30 min with fMLP 0.1 µM. Data are represented as median ± 25th and 75th percentile of 5 separate experiments and are expressed as % of positive cells (panel B) or ratio of MFI (panel C). * = P<0.05 *vs* respective resting.

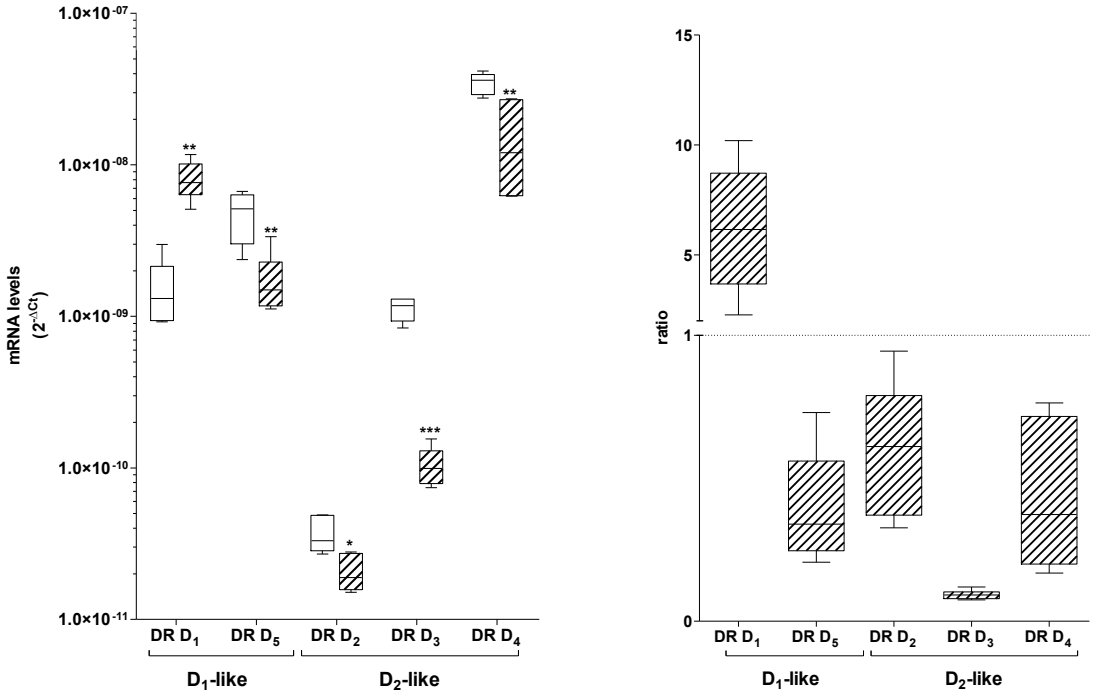


Figure 2. Real time PCR evaluation of DR on PMN

mRNA expression of DR on human PMN cultured for 3 h under resting conditions (empty bars) and after stimulation with fMLP (0.1 µM; hatched bars). mRNA level of DR expressed as $2^{-\Delta Ct}$ (left panel) or ratio of values to respective resting obtained after stimulation with fMLP (right panel) and represented as median ± 25th and 75th percentile of 6 separate experiments. * = P <0.05, ** = P<0.001 and *** = P<0.0001 *vs* respective resting conditions.

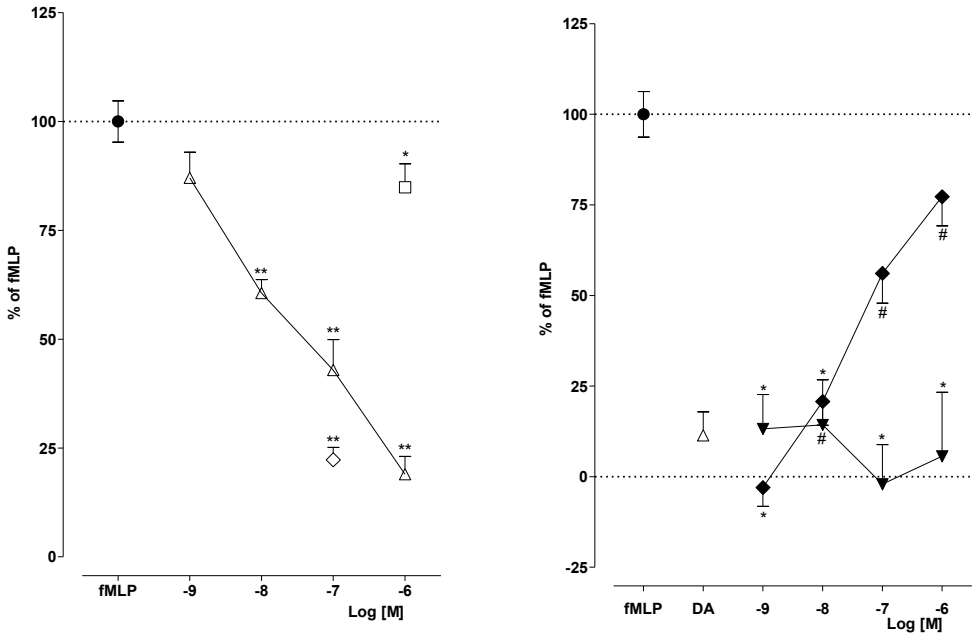


Figure 3. Effect of stimulation with dopaminergic ligands on PMN migration

Left panel: Effect of treatment with DA (empty triangles), SKF-38393 (empty diamonds) and pramipexole (empty square) on fMLP-induced (filled circle; 0.1 µM) cell migration.

Right panel: Effect of addition of with D_1-like antagonist SCH-23390 (filled diamonds) and D_2-like antagonist haloperidol (filled triangles) on DA-induced (empty triangle) inhibition of fMLP-induced (filled circle) cell migration. In both panels, data are expressed as percentage of fMLP-induced effects and represented as means±SEM of 3 - 8 separate experiments. * = P<0.05, ** = P<0.001 *vs* fMLP; # = P<0.05 *vs* DA 1 µM.

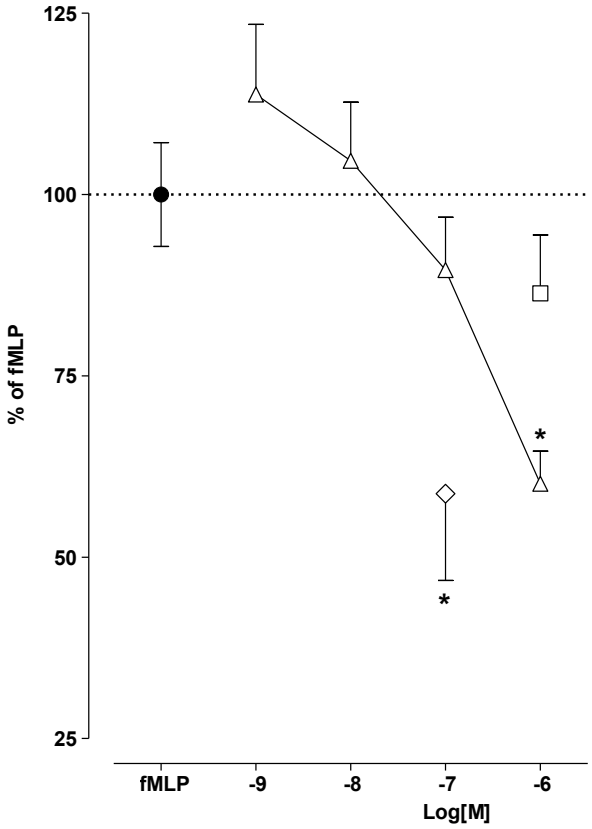


Figure 4. Effect of dopaminergic agents on ROS generation by human PMN.

Effect of treatment with DA (empty triangles), SKF-38393 (empty diamonds) and pramipexole (empty square) on fMLP-induced (filled circle; 0.1 µM) ROS production. Data are expressed as percentage of fMLP-induced effects and represented as means±SEM of 4 - 13 separate experiments. * = P<0.05 *vs* fMLP alone.

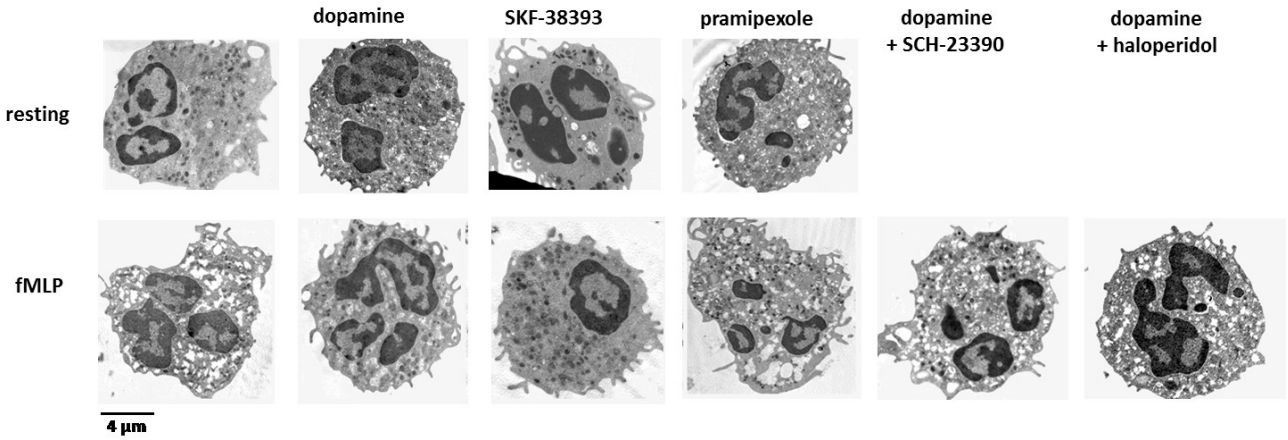


Figure 5. TEM analysis of PMN incubated for 30 min under resting conditions alone, or in presence of dopaminergic agents

Cells present the typical morphology of untreated conditions: cells are roundish with the typical spatial organization of the nuclei in central position and cytoplasm occupied with well distributed organelles and granules (upper panels). PMN were treated with fMLP alone (0.1 µM), or in the presence of DA (1 µM), SKF-38393 (0.1 µM) and pramipexole (1 µM). The treatment with fMLP show a typical morphology of activated cells: cell presents an irregular profile and massive degranulation, while the treatment with DA and the D_1-like agonist SKF-38393 revert the effect induced by fMLP. On the contrary, the morphology remains unchanged in the presence of the D_2-like agonist pramipexole. DA-induced effects were reverted by the D_1-like antagonist SCH-23390 (1 µM) and not by the D_2-like antagonist haloperidol (1 µM).

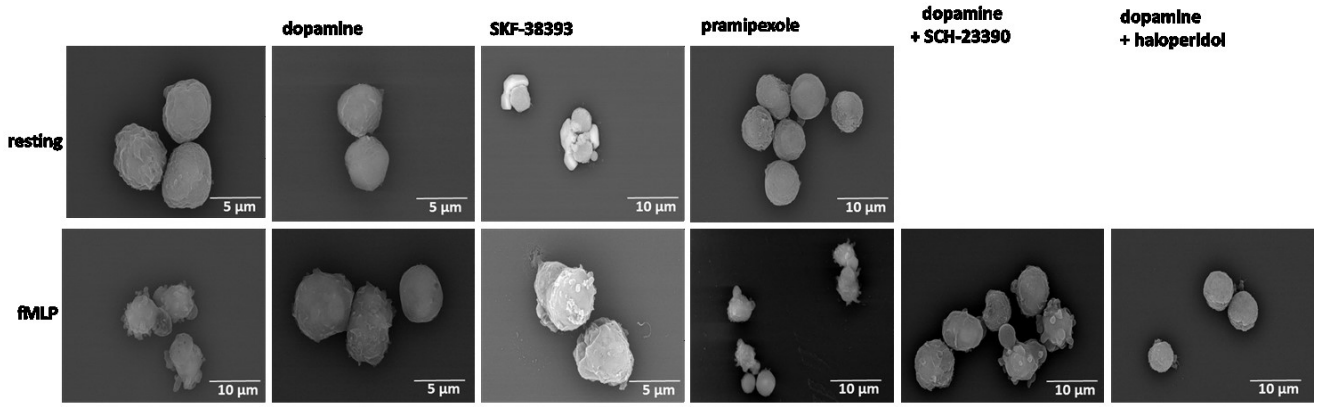


Figure 6. SEM analysis of PMN

SEM analysis of PMN incubated for 3 h. Under resting conditions (upper panels), cells are roundish and the presence of DA or SKF-38393 and pramipexole did not affect cell morphology (panels C and D), whereas the incubation with fMLP alone (0.1 µM; panel E), show a typical morphology of activated cells, with an irregular profile. DA (1 µM) was able to reduce the effect of fMLP (panel F), reverting the phenotype almost to control levels. DA-induced effects were reverted by the D_1-like antagonist SCH-23390 (1 µM; panel I) and not by haloperidol (1 µM; panel L)

Conclusion of PhD thesis

During PhD, my studies were focused on the investigation of the relationship between the dopaminergic system and a particular cell population of innate immune system, neutrophils.

The dopaminergic system is involved in different and fundamental functions both in the CNS and at the peripheral level. It has been shown that imbalances in this system lead to the onset of different pathologies such as Parkinson's disease, in which the dopaminergic system is hyporeactive or schizophrenia, in which this system is hyperreactive (Temlett 1996), as well as in autoimmune diseases like multiple sclerosis (Cosentino and Marino 2013).

Between innate immune cells, neutrophils are among the first cell that can leave the microcirculation and enter into the inflamed tissues (Beck et al., 2004; Kruger et al., 2015).

Actually, all the five DR are identified on their surface. *In vitro* studies showed that, in human PMN, DA inhibits ROS, superoxide anion production and cell migration (Wenisch et al., 1996; Sookhai et al., 2000; Matsuoka 1990), or that incubation with DA induces apoptosis (Sookhai et al., 1999), but if these effects are the results of DA DR activation was not yet investigated.

We examined the data present in literature about this topic (chapter 1) and on the basis of them, we investigated the influence exerted by the dopaminergic pathway on neutrophil functions. After a brief overview on neutrophil physiology and relevance in health and disease (chapter 2), we evaluated different approaches, first examining the presence of DR on human neutrophils, and secondly, the ability of dopamine to influence some key functions of these cells, namely cell migration and reactive oxygen species production and this part represent the main focus of my PhD project (chapter 3). We have confirmed the presence on neutrophil surface of all five DR (investigated by means of flow cytometry) and also the mRNA level for the receptors (measured using of real time PCR).

In contrast with the data of Sookhai (et al., 1999), we found that DA up to 1 µM determines only a slight increase in PMN apoptosis and only after 3 h of incubation, suggesting that this result cannot interfere with our main observations. The main functions that we have explored were migration, that allows the movement of neutrophils from the bloodstream to the tissues, the production of reactive oxygen species, required to eliminate the pathogens during infection and finally the production of IL-8, considering that is the most abundant chemokine produced by neutrophils (Baggiolini et al., 1994). We have found that DA is able to inhibit the migration and the production of ROS induced by an inflammatory stimulus. These results are in line with previous observations (Wenisch et al., 1996), although with some important differences: Wenisch and coworkers showed that the production of ROS was decreased by DA, however, they work on whole blood and not on isolated cells (so they cannot exclude the influence of the other immune cells on the observed effect). Other researchers (Sookahi et al., 2000; Matsuoka et al., 1990), performed similar assays, respectively transmigration through endothelium and chemotaxis, with extremely high concentrations of DA (10-100 µM) and this can reflect a nonspecific effect on other receptors.

We aimed therefore to investigate if the observed effects on neutrophil functions were receptor-mediated. So far, we have tested if the effect exerted by dopamine were reverted by SCH-23390 (D_1-like DR antagonist) and haloperidol (D_2-like DR antagonist). The data obtained are suggestive of a prevalent role of the D_1-like DR family. To better understand this specific issue, on the basis of the above mentioned results, we have also tested if the DA-induced effects were mimicked by a D_1-like DR selective agonist, SKF-38393, and a D_2-like DR agonist, pramipexole. The data on both migration and ROS generation suggest that for the most part, the observed effects are D_1-like DR mediated, although an involvement of the D_2-like DR cannot be excluded, as suggested by the ability of pramipexole to partially reduced the fMLP-induced migration (slightly than SKF-38393). Finally, we have investigated if the functional effects are reflected also on the morphology. It is indeed well

known that stimulation of neutrophils with pro-inflammatory agents (fMLP, LPS and other), induces dramatically changes in their morphology that can be highlighted with different technical approaches. By means of an original approach linking pharmacological manipulation with transmission electron microscopy (TEM) and scanning electron microscopy (SEM), we have shown that the effects of DA on neutrophils result in profound morphological changes and that these effects are receptor-mediated. So, by means of TEM and SEM, we confirmed that what we observed in the functional, is also reflected on the morphology of neutrophils.

The results of my PhD program about the dopaminergic modulation of human PMN functions can represent a new opportunity for further studies. It is indeed possible that these findings can help to promote new therapeutic strategies in counteracting PMN activation and tissues invasion, known to be the first step in most part of immune-mediated diseases, including inflammatory diseases of the CNS.

The ability of D_1-like DR agents to reduce the activation of neutrophils in several functions, could be used for the developing of new drugs, or for new indication of existing drugs, with specific indication for most important and common neurodegenerative diseases. In fact, as discussed in the section "neutrophils and diseases", increasing new evidence highlights the relevance of these cells in various diseases in which they were for long time ignored.

The last months of my PhD project were therefore devoted to achieve results in this context in order to deeply investigate during the post-doc, in particular regarding specific diseases. From the literature it is known that DA is an inhibitor of angiogenesis (Basu et al., 2001, 2004). Therefore, considering the data obtained during my PhD course, showing that the dopaminergic system profoundly inhibits some neutrophil functions, we want to investigate if DA can affect also the ability of neutrophils to modulate angiogenesis, issue characterized at present only in cancer and not yet investigated in other diseases or physiological conditions (wound healing or tissue growth).

So one of my aims for my post-doc projects will be the characterization of the involvement of neutrophils in angiogenesis and the ability of DA to modulate this process. We hope with this study to fill the gap in the knowledge about the role of neutrophils in the process of angiogenesis both from a physiological and pathological point of view. In addition, clarify the contribute of dopaminergic pathway in this process can help to find new therapeutic approaches (with existing and known-safe drugs) in the treatment of different kind of diseases in which angiogenesis is known to play a role.

Chapter 4

Other projects followed during the PhD course

Approaches to investigate the role of drugs or novel compounds to affect angiogenesis

Angiogenesis is the development of new blood vessels from already existing ones and is a fundamental process in many physiological conditions such as normal tissue growth, embryonic development, wound healing, menstruation, as well as pathological, like for example tumor and atherosclerosis (Carmeliet, 2003). The constituents of blood vessels are:

- endothelial cells, in direct contact with the blood;
- sub-endothelial pericytes;
- smooth muscle cells;
- fibroblasts;
- basal membrane;
- extracellular matrix.

Angiogenesis is rapidly activated in response to hypoxic or ischemic conditions, or vascular relaxation (e.g. mediated nitric oxide).

In the first stage of angiogenesis occurs a "destabilization" of pre-existing vessels due to an increase of vascular permeability and a loss of the connections between the endothelial cells. The second stage involves the migration and proliferation of endothelial cells in the tissue where the formation of new vessels is necessary. In the course of this phase various proteolytic enzymes are involved, to alter the density of the extracellular matrix in order to facilitate the migration of endothelial cells. The third stage corresponds to the differentiation of endothelial cells and is characterized by arrest of cell proliferation and formation of primitive capillaries. The last stage is characterized by the recruiting of support peri-endothelial cells, such as pericytes and smooth muscle cells. (Bussolino et al., 1997).

During the last years the fundamental role of Vascular Endothelial Growth Factor (VEGF) in the pathophysiological regulation of angiogenesis has been highlighted. In support of the priority role of VEGF in angiogenesis is the characterization of many of its biological effects: mitogenic action on endothelial cells, stimulation of the expression of metalloproteinases and formation of endothelial fenestrations, increase of vascular permeability and interaction with the cells of immune system, including neutrophils. Moreover, VEGF plays a pivotal role in the activation of the angiogenic switch leading to a growth of several tumors and in the subsequent process of metastasis (Bergers and Benjamin 2003).

Neutrophils seem to have an important role in this mechanism, especially for the production of VEGF, one of the most powerful pro-angiogenic molecules (Heryanto et al., 2004; Pinoli et al., 2016).

All of this evidence of the link between angiogenesis and neutrophils are performed in the oncological field, in which neutrophils (at least initially), had been largely ignored (Albini et al., 2005). They have a significant impact on the tumoral micro-environment, via the production of cytokines, chemokines and ROS, which affect the recall and activation of inflammatory cells (Gregory & Houghton, 2011).

Very interesting are the studies regarding the role of angiogenesis in multiple sclerosis. In the paper by Girolamo and colleagues, they outline the dual role of angiogenesis, both in MS and in the animal model, the acute autoimmune encephalomyelitis. If there is an increase in inflammation in the early stages of the disease, in the degenerative phase leads to a neurodegeneration (Girolamo et al., 2014). The fact that angiogenesis could be used as a biomarker for the progression of the disease is not new as it had already been highlight previously in review of Kirk and colleagues (Kirk et al., 2004). Among the various agents able to modulate angiogenesis, DA can also be included. It has in fact been widely shown that DA is able to inhibit angiogenesis (Basu et al., 2001; Chakroborty et al., 2004; Basu et al., 2004; Sarkar et al., 2013; Sarkar et al., 2015). The use of dopaminergic antagonists, specifically for the D_2 DR, facilitates the healing of wounds in which the blood supply and nutrients are essential

(Shome et al., 2011, 2012). All this evidence suggests that DA and dopaminergic agents can be investigated for novel therapeutic possibilities capitalizing on the ability of DA to modulate angiogenesis.

In line with the primary aim of my PhD project, that regard the influence of DA on neutrophil functions, I have participated during my permanence in the labs of the Center of Medical Pharmacology at different projects that can be related to the investigation of this issue. In particular, considering the key role of DA in angiogenesis (Basu et al., 2001) and considering the involvement of neutrophil in the process of angiogenesis (Bruno et al., 2014), I spent part of the time of my PhD course in learning how to use in vitro models in the characterization of the ability of specific compounds to modulate the process of angiogenesis. In this period, I contributed to the characterization *in vitro*, of some neo-synthesized peptides; in a specific project, my contribute was devoted to the investigation of a neo-peptide (the cyclic RGD peptide) to affect the formation of neo-vessel on human umbilical venous endothelial cells (HUVEC; the most common cells used in vitro to study this process), in particular performing the angiogenesis assays to clarify if the peptides possessed pro or anti-angiogenic properties. Two of these works on the cyclic RGD peptide, has already been published (attached file 2: Fanelli et al., 2014; and my contribution acknowledged in Zanella et al., 2015), while the paper with the characterization of two other peptides is still in process.

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ATTACHED FILE 1

Production of proinflammatory mediators by human neutrophils during long-term culture

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Abstract:

Neutrophils, usually considered short-living cells, play a key role in several inflammatory processes contributing to disease generation and progression. The present study was devised to investigate the changes occurring during long-term culture in neutrophil functions and morphology. Neutrophils were obtained from venous blood of healthy donors and cultured up to 24 h. Levels of interleukin (IL)-8, vascular endothelial growth factor (VEGF) and elastase were analysed by real time PCR and ELISA under resting and after stimulation with fMLP, LPS and IL-8. Apoptosis was measured by flow cytometry, migration by means of microscopic evaluation, reactive oxygen species (ROS) production by means of spectrofluorometry and cell morphology using optical microscopy and transmission electron microscopy. After 24 h cell number and viability was reduced with respect to 3 h of culture and number of cells in early and late apoptosis were increased. No appreciable differences were found between mRNA levels for IL-8, VEGF and elastase at the two times. Similarly, elastase protein production was unchanged while on the contrary, IL-8 and VEGF protein levels were higher after 24 h. Resting and stimulated migration were unchanged up to 24 h. Values measured for spontaneous ROS generation were superimposable for the two times, fMLP-induced ROS generation was reduced at 24 h and LPS failed to increases ROS generation after 24 h. Cell morphology was preserved up to 24 h. These results indicate that neutrophils can be studied ex vivo even in long-term colture, although time-length of the culture affects some of their functional properties.

Keywords: Neutrophils, proinflamamtory mediators, migration, reactive oxygen species, transmission electron microscopy

Introduction

Polymorphonuclear leukocytes (PMN) are the major cellular arm of the innate immune system. They are among the first cells that incorporate microorganisms and damaged tissues through endocytosis, production of reactive oxygen species (ROS) and a series of proteolytic enzymes such as elastase [1, 2]. At the sites of inflammation, where the signals produced by bacteria and host cells are abundant, the initial response of neutrophils is the secretion of pro-inflammatory cytokines [3-5] and the most abundant of these is interleukin (IL)-8, which acts also as a chemoattractant factor to recruit more neutrophils at the site of injury [6].

Neutrophils are characterized by a short life, indeed they undergo to spontaneous apoptotic process to maintain the cellular homeostasis [7, 8]. However, the lifespan is prolonged for several days by inflammatory signals. During this additional period, they release inflammatory mediators and contribute to the orchestration of the inflammatory response [9] but at present, no additional information's are available about possible functional changes occurring during this prolonged time.

Recently, the notion that these cells are short-living cells has been challenged by the results of Pillay and his collaborators, which demonstrated that in humans, in vivo labelling with 2H2O under homeostatic conditions, showed an average of circulatory neutrophil lifespan of 5.4 days [10]. Similarly, in a previous study conducted in our laboratory, we showed that human PMN can be coltured up to 24 h and were able to respond to stimulation and releasing Ca++ from the intracellular stores [11].

In recent years, the role and relevance of neutrophils in health and disease has been revised and their contribution to disease progression in several pathologies has been increasingly defined [12]. For example, it was shown that neutrophils play a key role in inflammatory diseases affecting the CNS [13, 14], like multiple sclerosis [15], or that they can contribute to pathological angiogenesis [16]

through the production of key angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8 [17, 18]. On the basis of all these evidences, usually in vitro and ex vivo experiments on isolated PMN are conducted only for few hours. So far, it is difficult for example to mimics in vitro what happens in tissue after neutrophil invasion or to explore the long-term effects of specific drugs on their functions.

In the present study, we have investigated the changes occurring during long-term colture in circulating human neutrophils. To this end, we considered two different times: 3 hours (h, short term) and 24 h (long-term), and we have investigated cell viability, migration, ROS production and the production of three key proinflammatory mediators: IL-8, VEGF and elastase. In addition, by means of Transmission Electron Microscopy (TEM) and optical microscopy, we have analysed cell morphology.

Materials and methods

Neutrophils isolation

Experiments were performed on buffy coats obtained by the local blood bank (Ospedale di Circolo, Fondazione Macchi, Varese, Italy). Neutrophils were isolated by standard density-gradient centrifugation as previously described [19]. Finally, cells were examined at light microscopy and no platelets or erythrocytes could be detected. Cell purity was assessed always either by light microscopic examination or by flow cytometric analysis (morphological parameters, SSC and FSC). Experiments were performed only in the conditions in which purity was higher than 95%.

Cell culture

Neutrophils were resuspended at the concentration of 1×107 cells/ml in RPMI 1640 with 10% of fetal bovine serum under standard conditions as previously described [20] and incubated alone (resting) or in the presence of N-formyl-Met-Leu-Phe (fMLP; 0.1 µM; Sigma-Aldrich, Milano), IL-8 (10 ng/ml; Sigma-Aldrich, Milano) or Lypopolisaccharide (LPS; 1 µg/ml; Sigma-Aldrich, Milano) at 37°C for 3 h and 24 h. fMLP is a chemotactic peptide acting on membrane receptors that induces neutrophils activation [21]. IL-8 represents a key neutrophil product and represents also a physiological activator of these cells [22, 23]. LPS is known to induce an inflammatory response and to stimulate the production of proinflammatory mediators [24]. After incubation, cells were centrifuged (400 g, 5 min, 20°C) and pellets and supernatants were collected and stored at -80°C for subsequent analysis.

Cell viability and apoptosis

Immediately after colture, samples were centrifuged at 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, Milan, Italy) according to the manufacturer's instructions. Briefly, the cells were resuspended in 100 µl of Annexin V Binding Buffer and stained with 5 µl of FITC-conjugated Annexin V (ANX-FITC) and 5 µl of Propidium Iodide Staining Solution (PI) for 15 min in the dark. After incubation, 250 µl of Binding Buffer were added, samples were analyzed by BD FACSCanto II Flow Cytometer (Becton Dickinson Italy, Milano, Italy) and data were collected and elaborated using BD FACSDiva software (version 6.1.3). Neutrophils were identified based on forward-scatter (FSC) and side-scatter (SSC) properties, and at least 15.000 events were collected from each sample. Viable (ANX-/PI-), early apoptotic (ANX+/PI-) and late/necrotic (ANX+/PI+) neutrophils were identified on a biparametric plot ANX-FITC vs PI with a log scale.

RNA isolation and real-time polymerase chain reaction

Total mRNA was extracted from 1×106 cells by Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany) and the amount of RNA extracted was estimated by spectrophotometry at 260 nm. Total RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and Real-time PCR was performed (ABI prism 7000 apparatus; Applied Biosystems) using assay-on-demand kits. Threshold cycle values (Ct1) for the genes of interest were calculated, normalized to 18S RNA (Ct2) (housekeeping) content, and finally expressed as 2-ΔCt, where ΔCt = Ct2-Ct1. Primers (Applied Biosystems) are shown in Table 1.

Cell migration

Cell migration was measured by the use of Boyden Chamber assay as previously described [19] and migration was quantified by means of optical microscope measuring the distance (μm) from the surface of the filter to the leading front of cells. Migration was measured after 1.5 h of colture according standard procedures and our previous results [19] and after 24 h of colture in order to evaluate if long-term incubation affects this parameter.

ROS generation

Intracellular ROS levels were assessed by use of the redox sensitive dye C-DCDHF-DA (Molecular Probes, Eugene, OR, USA) as previously described [25]. Briefly, freshly isolated, or coltured neutrophils were suspended at the concentration of 1×106 cells/ml in HBSS medium and incubated with 2 μmol/L C-DCFH-DA (1 h, 37°C in the dark). Cells were then washed twice with HBSS by centrifugation (400 g, 20°C, 5 min) and fluorescence was detected by means of a spectrofluorimeter (Perkin-Elmer LS-50B, Perkin-Elmer Instruments, Bridgeport, CT, USA), with excitation wavelength of

488 nm. Fluorescence emission was collected at 525 nm and intracellular ROS levels were finally expressed as difference (Δ) between resting values measured at 60 s and levels measured after 30 min monitoring. ROS levels were assayed under resting conditions and after stimulation with 0.1 μM fMLP or 1 μg/ml LPS.

IL-8, VEGF and elastase production

IL-8, VEGF and elastase protein levels in supernatants (obtained from cell coltured as above described) were quantified using a sandwich-type enzyme-linked immunoadsorbent assay (QuantikineELISA; R&D System). The limits of detection were 1 pg/ml for VEGF and IL-8 and 1 ng/ml for elastase.

Light microscopy and transmission electron microscopy of cell morphology

Isolated neutrophils were resuspended at the concentration of 5×106 cells/ml in RPMI medium and incubated for 3 and 24 h under resting conditions. After the incubation, the collected pellets were fixed with glutharaldeid 4% in 0.1 M Na-cacodylate buffer (pH 7.2).

Pellets were washed in 0.1 M Na-cacodylate buffer (pH 7.2) and post-fixed for 20 min 1% osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol scale, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultrotome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin) and were observed with a light microscope (Eclipse Nikon, Amsterdam, Netherlands). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol Tokyo, Japan).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM), with n indicating the number of observations. Parametric continuous variables were compared by means of Student's t test. Analysis of the correlation between functional responses of neutrophils was performed by linear regression analysis (for continuous variables) and statistical significance for correlations was set at $P<0.05$. Calculations were performed using commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Results

Cell viability and apoptosis

Colture of neutrophils up to 24 h, as expected, reduced the number of living cells (ANX-/PI-) with respect to 3 h of colture and increased the number of cells in early stage of apoptosis (ANX+/PI-) (Table 2). Cells in late stage of apoptosis (ANX+/PI+) were higher at 24 h with respect to 3 h, even if these values were never higher than 20% (Table 2).

No differences in the number of living, early apoptotic and late apoptotic cells were found between resting conditions or stimulation with both IL-8 and fMLP. On the contrary, stimulation for 24 h with LPS shows an increased number of living cells (ANX-/PI-) with respect to unstimulated cells or cells stimulated with fMLP or IL-8 and a reduced number of early apoptotic cells (ANX+/PI-) with respect to cells stimulated with fMLP or LPS (Table 2).

IL-8, VEGF and Elastase mRNA expression and production

IL-8: mRNA levels under resting conditions (empty columns) were similar at both 3 and 24 h of colture, while resting protein production was higher after 24 h (Figure 1, upper panel). Both fMLP (left

hatched columns) and LPS (filled columns) increased IL-8 mRNA and protein production. mRNA and protein levels were increased at both 3 and 24 h of colture after stimulation with fMLP and LPS and no differences were found in the values measured at the two timing (Figure 1, upper panel, left). On the contrary, protein production after stimulation with both stimuli was significantly higher at 24 h compared to 3 h (Figure 1, upper panel, right).

VEGF: mRNA and protein levels of VEGF under resting condition (empty columns) were higher at 24 h compared to 3 h (Figure 1, middle panel). All the stimuli increased VEGF mRNA after 3 h of colture while, on the contrary, values measured after 24 h of stimulation in all conditions were not significantly different with respect to resting values. Comparison between the two-time period showed that the values measured after stimulation were lower with respect to the values reached after 3 h, although the statistical significance was reached only for the stimulation with fMLP (left hatched columns) (Figure 1, middle panel).

Considering protein production, stimulation with fMLP and LPS, but not with IL-8 (right hatched columns) induced a significant increase of VEGF production after both 3 and 24 h of incubation. Comparison between the two timing showed that the values measured after 24 h were higher than values measured after 3 h of incubation (Figure 1, middle panel).

Elastase: Resting elastase mRNA levels were similar after 3 and 24 h of colture (Figure 1, lower panel). Treatment of cells with either fMLP (left hatched columns) and LPS (filled columns) increased elastase mRNA levels both at 3 and 24 h. IL-8 (right hatched columns) significantly increased mRNA levels only at 24 h.

Protein production was never affected by all the stimuli at both times of observations (Figure 1, lower panel).

Cell migration

Spontaneous cell migration was similar after 1.5 or 24 h colture (18.3±1.4 and 22.9±1.1 μm, respectively; $P>0.05$). As expected, stimulation with fMLP induced always a significant increase of neutrophil migration (1.5 h = 26.0±2.2; $P<0.05$; 24 h = 32.8±1.5; $P<0.01$ vs respective resting conditions) and no differences were observed between values measured at the two times ($P>0.05$).

ROS generation

In PMN cultured for 1 and 24 h spontaneous ROS generation was unchanged. As expected, stimulation with fMLP induced a significant increase of ROS production, both after 1 and 24 h of colture, although the values measured after 24 h were lower with respect to values measured after 1 h (Table 3). In addition, stimulation with LPS, significantly increased ROS generation after 1 h while did not significantly affect ROS generation after 24 h incubation (Table 3).

Cell morphology

Optical microscopic observations showed that cells coltured for 3 h, presented generally round shape, while on the contrary, 24 h coltured cells displayed the presence of different phenotypes (Figure 2 A and 2D).

Electron microscopic observations of neutrophils coltured for 3 h and 24 h (Figure 2B, 2C, 2E, 2F) showed the typical morphology of neutrophils under resting status: round shape and cytoplasm filled with nuclei, dispersed chromatin, well distributed organelles, granules in the cytoplasm, preserved cell membrane structure. No appreciable differences were observed at the two times of incubation (Figure 2B, 2C, 2E, 2F).

Discussion

The role of neutrophils in the initiation and progression of inflammatory process is well known, but for long time the assumption was that these cells survive only few hours in tissues after leaving the circulation, even if the lifespan can be little prolonged during infection or inflammation [7-9].

In the present study, we investigated the ability of human neutrophils to survive in culture up to 24 h. As expected, during culture (after 24 h) the number of apoptotic cells significantly increased although the number of cells in the late stage of apoptosis after 24 h was not higher than 20%. Interestingly, we have shown that the number of apoptotic cells depends not only from the time of culture, but is also stimuli-sensitive. In fact, although cell viability after 1 h incubation was unchanged, on the contrary, 24 h of culture significantly reduced the number of living cells and increased the number of cells in early and late stage of apoptosis. Interestingly, after stimulation with LPS, the percentage of living cells was higher with respect to unstimulated cells or cells stimulated with fMLP or LPS. Similarly, the number of cells in early stage of apoptosis was reduced in presence of LPS. Interestingly, in general in all conditions tested, the number of cells in late apoptosis is never higher than 20%. Furthermore, the preserved morphology clearly evident both with optical microscopy and TEM is in line with these data.

We have also shown that migration, which represents a key step in the ability of these cells to invade tissues, was unchanged up to 24 h. Similarly, ROS generation involved in pathogen aggression and killing [26], was sustained after 24 h of culture with a significant reduction only in the stimulated values.

Interestingly, for ROS generation we showed that the stimuli employed exert different effects, as shown for apoptosis, in short-term culture with respect to long-term culture. In fact, increased ROS generation in fMLP-stimulated cells were sustained, if lower, after 24 h, while on the contrary LPS-

induced increases ROS generation was not present after 24 h of culture, suggesting that these two kinds of proinflammatory stimuli can differently affects the short or long-term cell colture. This observation, together with the data of different effects of LPS on apoptosis (reduced apoptotic cells during LPS stimulation at 24 h), suggests that, although proinflammatory agents could affects neutrophil functions, the results can be different when the stimulation occur for long (chronic) or short time (acute).

On the basis of all these observations, we can hypothesize that, neutrophils, after the migration into inflamed tissue, through the in loco production of inflammatory mediators, can contribute to the orchestration and maintenance of the inflammatory status typically found in all the immune-mediated diseases. Indeed, in line with this hypothesis, the production of key mediators such as IL-8, VEGF and elastase was maintained and was higher after prolonged time of incubation and after exposition to proinflammatory stimuli. These data are in line with our previous observations in atherosclerotic patients, in which we have shown, that not only circulating cells but also resident cells were able to produce proinflammatory mediators [27] suggesting that neutrophils can contribute not only to the generation, but also to the perpetration in loco of inflammatory status.

Considering that VEGF and IL-8 are potent angiogenic factor regulating vascular growth, function, and homeostasis, as well as permeability and vasodilatation [22], the observation that neutrophils are able to produce these mediators also after long time suggest a key contribute in processes characterized by intense neutrophil infiltration and neovessel formation such as in tumours and atherosclerosis [28, 29].

The presence of the proteolytic enzyme elastase within inflamed tissues is well established and elastase is considered a notable marker and inducer of inflammation [30]. To our knowledge, the present result represents the first detailed characterization of the modifications in elastase mRNA

levels following exposure of human neutrophils to proinflammatory and activating stimuli such as fMLP, LPS and IL-8.

In this study, we perform for the first time the ultrafine morphology of human neutrophils after short or long time of colture. TEM evaluation clearly showed that cell architecture was preserved suggesting that these cells are able to carry out their functions even after leaving the bloodstream from long-time.

In conclusion, the present study provided for the first time a detailed characterization of the functions of human neutrophils in response to proinflammatory stimuli, showing that these cells even after prolonged times of colture, are able to maintain the fundamental characteristics in order to perform their key functions in pathologic processes. In addition, we have shown, for the first time, that long-term colture could differently modulate some functions depending of the stimuli employed. Finally, the present study provided a detailed characterization of the human neutrophils in response to proinflammatory stimuli, showing that these cells are viable and responsive even after prolonged periods of colture, up to 24 h. However, time-length of the colture is likely to affect many responses in a function-specific fashion.

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Figure legends

Figure 1. IL-8 (upper panel), VEGF (middle panel) and elastase (lower panel) mRNA expression and protein production in human neutrophils in resting conditions (empty columns) and after stimulation with fMLP (0.1 µM, left hatched columns), LPS (1 µg/mL, filled middle columns) and IL-8 (10 ng/mL, right hatched columns). Data are means ± SEM of n = 5-11 separate experiments. * = P<0.05 and ** = P<0.01 vs resting; # = P<0.05 and ## = P<0.01 vs 3 h.

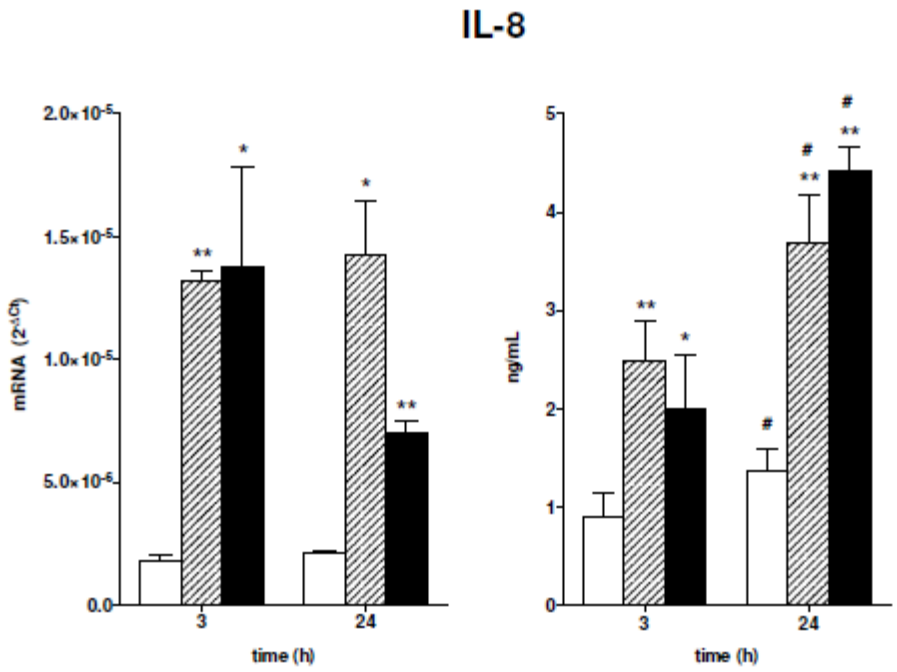

Figure 1 – upper panel

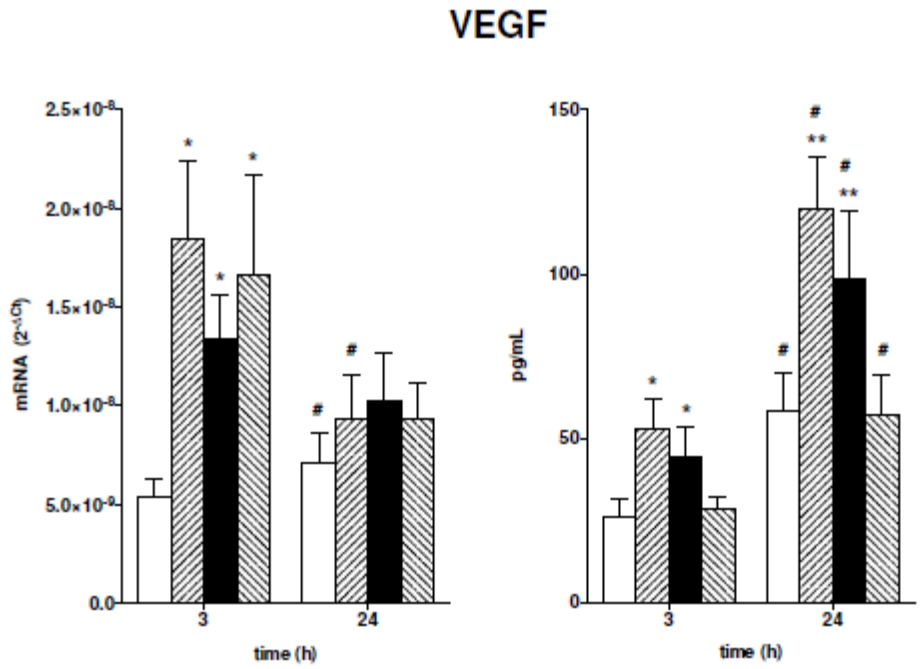

Figure 1 – middle panel

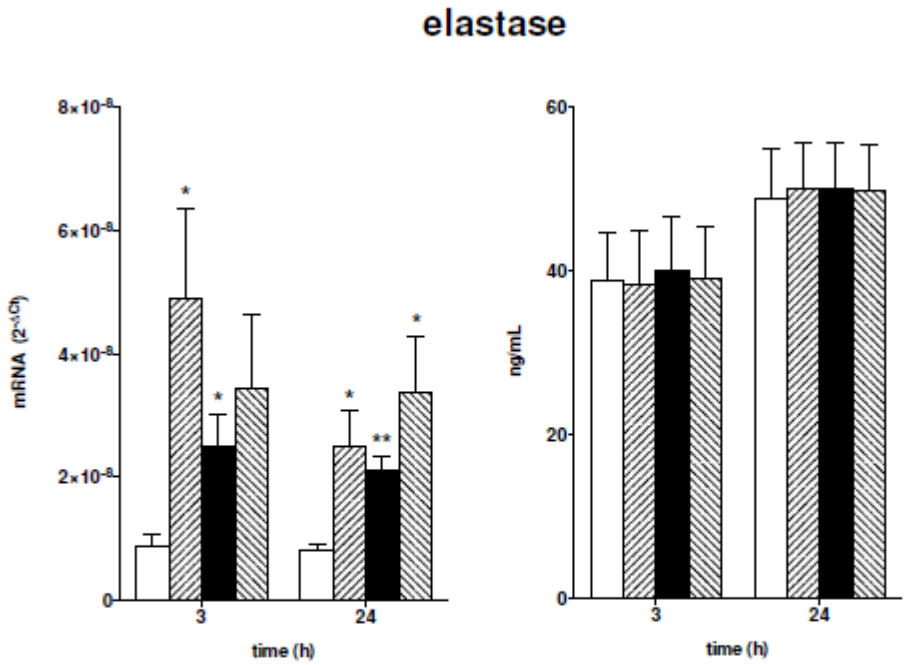

Figure 1 – lower panel

Figure 2. Semithin (A, D) and thin (B, C, E, F) sections of isolated human neutrophils cultured for 3 h (A, B, C) and 24 h (D, E, F). TEM analysis shows that no differences can be appreciate comparing the phenotypes of resting neutrophils at the two times of culture. All the cells are roundish with the typical spatial organization of the nuclei in central position. The cytoplasm is filled with numerous granules and cell membrane appears intact. **Scale bars**: B = 1.5 µm; C = 0.7 µm; E = 1.5 µm; F = 0.7 µm.

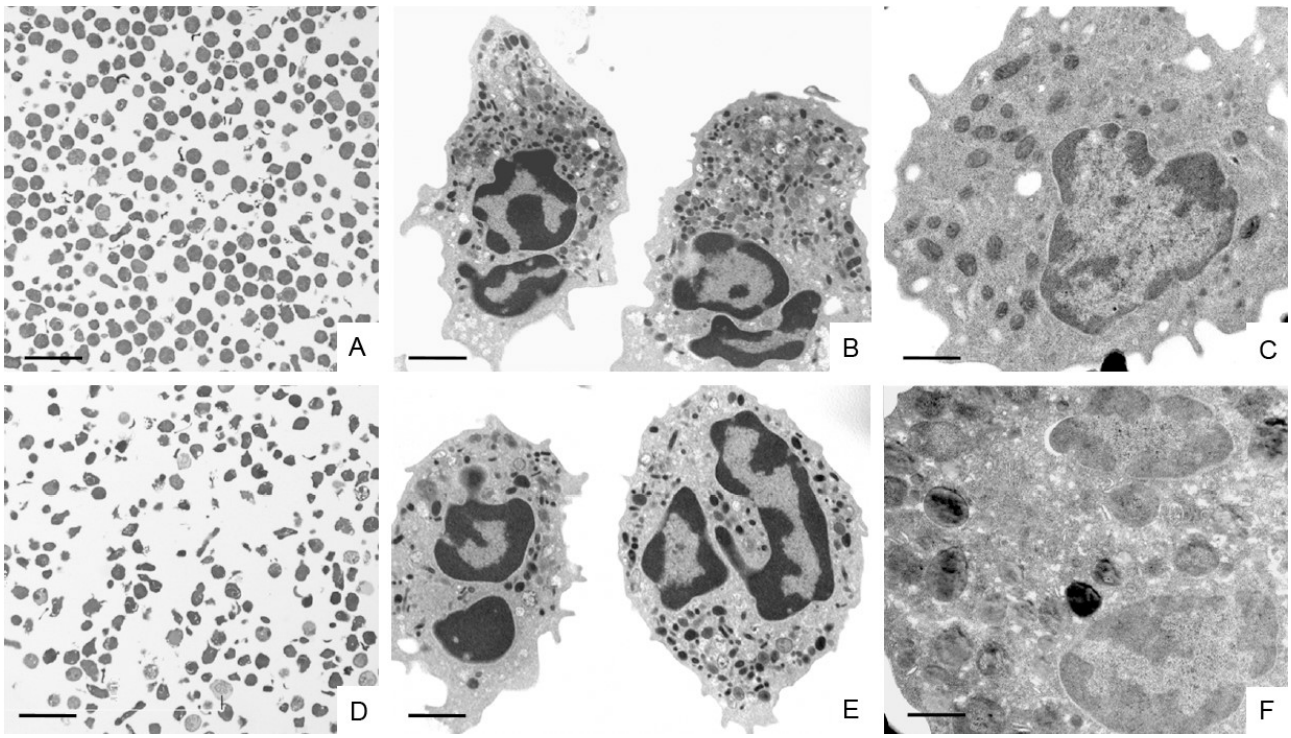

Table 1. Gene sequences assayed by real-time PCR

Gene	UniGene ID	Interrogated Sequence	Translated Protein	Exon Boundary	Assay Location	Amplicon Length	Annealing temperature (°C)	Efficiency (%)
IL-8	Hs. 00174103_m1	NM_000584.3	NP_000575. 1	1-2	222	101	60	100.02
VEGF	Hs. 00900055_m1	NM_001025366. 2	NP_001020 537.2	3-4	1352	59	60	99.9
elastase	Hs. 00357734_m1	NM_001972.2	NP_001963. 1	3-4	402	66	60	100.06
18S rRNA	X03205.1	N.A.	N.A.	N.A.	N.A.	187	60	98.80

Data from the NCBI Reference Sequence Database (RefSeq, http://www.ncbi.nlm.nih.gov/refseq/).

Table 2. Cell viability (ANX-/PI-), early apoptosis (ANX+/PI-) and late apoptosis (ANX+/PI+) measured by means of cytofluorimetric evaluation with a biparametric plot ANX-FITC vs PI

	3 h			**24 h**		
	ANX-/PI-	**ANX+/PI-**	**ANX+/PI+**	**ANX-/PI-**	**ANX+/PI-**	**ANX+/PI+**
resting	94.73±2.00	4.70±2.08	0.42±0.17	33.77±9.78$^{***,\$}$	47.42±8.01**	18.11±7.41**
fMLP (0.1 µM)	94.99±1.49	4.17±1.40	0.57±0.25	42.11±25.44$^{*,\$}$	43.58±21.82$^{*,\$}$	13.76±6.84*
IL-8 (10 ng/ml)	94.79±2.14	4.46±2.01	0.48±0.14	35.12±10.48$^{**,\$}$	46.17±8.37$^{**,\$}$	18.11±7.34**
LPS (1 µg/ml)	94.10±1.70	3.64±1.09	1.19±0.50	60.74±16.76$^{*,\#}$	27.77±14.20$^{*,\#}$	10.59±3.25

PMN were cultured for 3 or 24 h under resting conditions or in the presence of 0.1 µM fMLP, 10 ng/ml IL-8 or 1 µg/ml LPS. Data are expressed as % of total cells and are means ± SE of 5 separate experiments. *** = P<0.0001, ** = P<0.001 and * = P<0.05 vs 3 h; # = P<0.05 vs respective resting. $ = P<0.05 vs LPS 24 h.

Table 3. ROS generation in PMN cultured for 1 or 24 h under resting conditions or after stimulation with 0.1 μM fMLP or 1 μg/ml LPS

	1 h	**24 h**
resting	74.5±6.4	56.9±8.3
fMLP	298.8±30.9**	136.2±20.5**,#
LPS	90.1±12.2*	55.7±11.5

Data are expressed as Δ variations (30 min FI-60 s FI) and are represented as means ± SE of 5-16 separate experiments. * = P<0.01 and ** = P<0.0001 vs respective resting conditions. # = P<0.0001 vs 1 h fMLP. For more details, see Method's section.

ATTACHED FILE 2

Effects of a novel cyclic RGD peptidomimetic on cell proliferation, migration and angiogenic activity in human endothelial cells

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Abstract

Background: Cyclic RGD peptidomimetics containing a bifunctional diketopiperazine scaffold are a novel class of high-affinity ligands for the integrins □V□3 and □V□5. Since integrins are a promising target for the modulation of normal and pathological angiogenesis, the present study aimed at characterizing the ability of the RGD peptidomimetic cyclo[DKP-RGD] 1 proliferation, migration and network formation in human umbilical vein endothelial cells (HUVEC).

Methods: Cell viability was assessed by flow cytometry and annexin V (ANX)/propidium iodide (PI) staining. Cell proliferation was evaluated by the ELISA measurement of bromodeoxyuridine (BrdU) incorporation. Network formation by HUVEC cultured in Matrigel-coated plates was evaluated by optical microscopy and image analysis. Integrin subunit mRNA expression was assessed by real time-PCR and Akt phosphorylation by western blot analysis.

Results: Cyclo[DKP-RGD] 1 does not affect cell viability and proliferation either in resting conditions or in the presence of the pro-angiogenic growth factors VEGF, EGF, FGF, and IGF-I. Addition of cyclo[DKP-RGD] 1 however significantly decreased network formation induced by pro-angiogenic growth factors or by IL-8. Cyclo[DKP-RGD] 1 did not affect mRNA levels of αV, β3 or β5 integrin subunits, however it significantly reduced the phosphorylation of Akt.

Conclusions: Cyclo[DKP-RGD] 1 can be a potential modulator of angiogenesis induced by different growth factors, possibly devoid of the adverse effects of cytotoxic RGD peptidomimetic analogues.

Key words: RGD peptidomimetics; integrins; angiogenesis; human umbilical vein endothelial cells; interleukin-8.

INTRODUCTION

Angiogenesis, the growth of new blood vessels as sprouts or offshoots of the pre-existing microvasculature, is a physiological event occurring in the development of organisms, wound healing and the reproductive cycle, but it is also involved in pathologic processes such as inflammation, tumour growth and metastasis [1]. Angiogenesis can be stimulated by a large number of pro-angiogenic cytokines, such as vascular endothelial growth factor (VEGF), tumour necrosis factor α (TNF-α), basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8) [2;3].

Among the proteins involved in the angiogenic process, integrins play an important role by promoting endothelial cell attachment and migration on the surrounding extracellular matrix, cell to cell interaction and intracellular signal transduction [4]. Integrins are heterodimeric proteins composed of two non covalently associated α and β transmembrane glycoproteins; 18 α and 8 β subunits that give rise to 24 possible distinct integrin proteins [5;6]. Across their extracellular α/β subunit interface containing the metal ion-dependent adhesion site (MIDAS), integrins recognize and bind protein ligands through contiguous tripeptide sequences, the majority of which are present within flexible loop regions and contain an acidic residue [7]. Several integrins, including α_V, $\beta_5\alpha_1$ and $\alpha_{IIb}\beta_3$ integrins, recognize the Arg-Gly-Asp (RGD) sequence in endogenous ligands. The context of the ligand RGD sequence (flanking residues, three dimensional presentation) and individual features of the integrin binding pockets determine the recognition specificity and efficacy. These observations prompted many research groups to investigate the use of conformationally constrained cyclic RGD peptides and peptidomimetics as active and selective integrin ligands [8; 9]. One of these, Cilengitide, namely cyclo-[Arg-Gly-Asp-D-Phe-N(Me)-Val] is currently in phase III clinical trials as an angiogenesis inhibitor for patients with glioblastoma multiforme alone [10] or in combination with other antiblastic drugs [11]. Recently, RGD compounds have been proposed also as targeting ligands for integrins in order to better characterize tumor neovascularisation [12]. Notwithstanding these results, the mechanism

of RGD ligands in the inhibition of angiogenesis is not yet fully understood, as significant cross-talk exists in the regulation of angiogenesis between integrin operated pathways and, for instance, VEGF receptor pathways [13], and on these bases it has been proposed that agents able to inhibit multiple pathways would have important therapeutic potential [14].

Recently, some of us reported a new class of cyclic RGD peptidomimetics containing a bifunctional diketopiperazine (DKP) scaffold, showing a low nanomolar affinity for integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ [15;16]. The present study is aimed at characterizing the ability of the cyclic RGD peptidomimetic cyclo[DKP-RGD] 1 (Figure 1) to affect cell viability, proliferation, migration and capillary network formation in human umbilical vein endothelial cells (HUVEC). In addition, the effect of cyclo[DKP-RGD] 1 on mRNA expression of the integrin subunits α_V, β_3 and β_5, and on the phosphorylation of Akt, a serine/threonine-specific protein kinase that plays a key role in the regulation of vascular homeostasis and angiogenesis [17] was also investigated.

MATERIALS AND METHODS

Reagents

The peptidomimetic cyclo[DKP-RGD] 1 was prepared according to a published procedure [16]. Annexin V-FITC Apoptosis Detection Kit I was purchased by Biosciences (BD, Italy). RNA extraction was performed using Quiazol reagent (Qiagen, Italy) and the quantitative real time RT-PCR reaction were performed using Quantitatec reverse transcription kit (Qiagen, Italy) and Quantitec sybr green pcr kit (Qiagen, Italy). The amount of proteins for western blot analysis were performed by the BCA protein Assay Kit (Pierce Protein Biology, Rockford, IL, USA). Anti-AKT and anti-pAKT, primary antibodies were purchased from Cell Signalling (Cell signalling, Italy) and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for western blot were purchased from Upstate

Biotechnology (Upstate Biotechnology, USA). The detection of the western blot membrane was performed by using ECL plus Western Blotting Detection System purchased from Amersham (Amersham, GE Healthcare Life Science, MI, Italy). Propidium iodide (PI) solution was purchased from Miltenyi (Miltenyi Biotec S.r.l., Bologna, Italy). Matrigel Basement Membrane Matrix (10 mg/ml) for the network formation assay was purchased from BD (Becton Dickinson Italy, Milan, Italy). Cell proliferation Biotrak Ver for the proliferation assay was purchased from GEhealthcare (GeHealthcare, Uppsala, Sweeden). Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell (PromoCell Gmbh, Germany). The EndoGRO™ VEGF Complete Media Kit composed of EndoGRO Basal Medium (SCME-BM) plus fetal bovine serum (FBS), L-glutamine, heparin sulphate, rh-VEGF, rh-EGF, rh-FGF2, rh-IGF-I and ascorbic acid, and the MF-Membrane filters (3.0 μm) for the cell migration assay were purchased from Millipore (Millipore S.p.A., MI, Italy). Recombinant Human CXCL8/IL-8 was purchased from R&D (R&D System, US, Europe).

Cell cultures

HUVEC were cultured in a medium supplemented with FBS (2%), L-glutamine (10 mM), heparin sulphate (0.75 U/ml), VEGF (5 ng/ml), EGF (5 ng/ml), FGF2 (5 ng/ml), IGF-I (15 ng/ml) and ascorbic acid (50 μg/ml) at 37°C, in a moist atmosphere of 5% CO2. HUVEC were used for the experiments between passage 2 and 8. All the experiments were conducted under two different conditions: basal conditions (resting) i.e. cell cultured in EndoGRO basal medium alone, and stimulated conditions, i.e. with the addition of VEGF (5 ng/ml), EGF (5 ng/ml), FGF2 (5 ng/ml), IGF-I (15 ng/ml) together with 10% FBS. In viability, proliferation and migration assays, cells were used after overnight culture in EndoGRO basal medium alone (starvation).

Cell viability

Cell viability assay was performed by flow cytometry. Briefly, after treatment HUVEC were detached with a trypsin solution, centrifuged at 600 g for 5 min at room temperature and the supernatant was finally removed. The cell pellet was resuspended in 100 µL Binding Buffer 1X with the addition of 5 µL annexin V (ANX)-FITC and 5 µL PI, and finally incubated for 15 min at room temperature in the dark. Samples were stored on ice and analyzed without washing. Acquisition was performed on a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) and data were analyzed using BD FACSDiva software (version 6.1.3). HUVEC were identified on the basis of forward-scatter (FSC) and side-scatter (SSC) properties, and a minimum of 15000 cells for each sample was collected in the gate. Viable, apoptotic and necrotic HUVEC were identified on a biparametric plot ANX-FITC vs PI. Data were finally expressed as % viable (ANX-/PI-), early apoptotic cells (ANX+/PI-), late apoptotic/necrotic cells (ANX+/PI+) and necrotic cells (ANX-/PI+).

Proliferation assay

To assess HUVEC proliferation, 1x104 cells were seeded in duplicate in a 96-well plate and cultured for 24 h without or with cyclo[DKP-RGD] 1 at different concentrations. Proliferation was then measured by a colorimetric immunoassay, based on the ELISA measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis. The absorbance (ABS) of the samples was determined by means of a spectrophotometer (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) with wavelength set at 450 nm, and finally expressed as the difference between BrdU positive and negative samples, expressed as Optical Density (O.D.).

Cell migration assay

Cell migration was measured by means of a Boyden chamber assay. Briefly, 1x105 HUVEC were seeded in the top well of the Boyden chamber, cyclo[DKP-RGD] 1 was added in the bottom or in the

top compartment, and a 3 µm-pore cellulose nitrate filter was placed between the two compartments. Stimulated migration was assessed by putting VEGF, EGF, IGF-I, and FGF2 in the bottom chamber. After an incubation period of 5 h at 37°C, the filter was recovered, dehydrated, fixed, and finally stained with hematoxylin. Migration into the filter was quantified by measuring the distance (in µm) from the surface of the filter to the leading front of cells using an optical microscope (Axiolab, Carl Zeiss S.p.A. Milan, Italy).

Angiogenesis assay

To assess angiogenic activity, HUVEC 2.5x104 cells were seeded in a 24-well plate coated with 100 µl/well of Matrigel previously polymerized for 1 h at 37°C. Cells were then incubated for 5 h at 37°C in a moist atmosphere of 5% CO2 without or with cyclo[DKP-RGD] 1 under either resting or stimulated conditions. In some experiments IL-8 (10 nM) was used as pro-angiogenic stimulus. Network formation was evaluated by phase-contrast microscopy using a fluorescence microscope (Axiovert 40CFL, Carl Zeiss S.p.A. Milan, Italy). Network formation was finally quantified in terms of mean number of loops per field as topological parameters and the total length of the branches. For the purpose of the analysis, loops were defined as any complete ring formed by HUVEC, while open ramifications were considered as branches. The total branch length (pixels) and the number of loops were quantified using the ImageJ image analysis software (http://rsbweb.nih.gov/ij/).

Real time PCR

Cells were treated for 5 h in the presence or absence of 1 µM cyclo[DKP-RGD] 1 in different growth conditions, as previously described. At the end of the treatment, RNA extraction was performed using the Qiazol lysis reagent. Primers were designed by using the “Primer3 input” software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/primer3_www.cgi) and the specificity of each primer was controlled by the BLAST software (http://blast.ncbi.nlm.nih.gov) (Table 1). Real time PCR

was performed as previously reported [18] At the end of the reaction, a melting curve analysis was carried out to check for the presence of primer-dimers. Comparison of the expression of each gene was determined by using GAPDH as housekeeping gene. Each run was analyzed in duplicate and data are finally expressed as $2^{-\Delta ct}$.

Western blot analysis

Cells grown in 60-mm dishes were treated for 5 h with 1 µM cyclo[DKP-RGD] 1. The cells were then rinsed twice in ice-cold PBS and 200 µl of the cell lysis buffer (composition: 50 mM Tris-HCl pH 7.4, 1% v/v NP40, 0.25% w/v sodium deoxycholate, 1 mM phenylmethylsulphonyl-fluoride, 1 mM Na3VO4, 1 mM EDTA, 30 mM sodium pyrophosphate, 1 mM NaF, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin and 1 mg/ml microcystin) was added to the dishes. After scraping, cells were sonicated for 10 s, centrifuged at 12000 g for 5 min at 4°C and the amount of proteins in the supernatant was measured using the BCA protein assay. For western blot analysis, 20 µg of proteins were separated by 10% SDS–PAGE at 150 V for 2 h and blotted onto 0.22 mm nitrocellulose membranes at 90 mA for 16 h. The membranes were first blocked for 2 h in TRIS buffered saline solution (TBST, composition: TRIS 10 mM, NaCl 150 mM, 0.1% Tween 20) plus 5% low fat dry milk (TBSTM) and then incubated with the appropriate antibody diluted 1:1000 in TBSTM, for 16 h at 4°C under gentle agitation. The membranes were rinsed three times in TBST and then incubated for 2 h at 21°C with the secondary antibody diluted 1:10000 in TBSTM. Membranes were then rinsed three times in TBST and luminescence was detected by using the appropriate kit, and densitometric analysis was performed as previously reported [18].

Statistical analysis

Data are shown as means±standard deviation (SD) unless otherwise indicated. Statistical significance of the differences was assessed by two-tailed Student's t test for paired data or by One-way analysis

of variance followed by Dunnett's Multiple Comparison Test as appropriate. Calculations were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Viability and apoptosis

Viable cells, measured after 24 h, were 81.7±6.0% in basal conditions and 90.2±3.7% in the presence of VEGF, EGF, IGF-I, and FGF2 (n = 4, P = 0.066 vs basal conditions). Early apoptotic cells were, respectively, 10.8±2.0% and 6.5±3.4% (n = 4, P = 0.117), late apoptotic/necrotic cells were 5.4±3.5% and 1.7±0.3% (n = 4, P = 0.082) and necrotic cells were 2.5±1.5% and 1.6±0.8% (n = 4, P = 0.430). The presence of cyclo[DKP-RGD] 1 in the 1x10-12-1x10-6 M did not affect the percentage of viable, early apoptotic, late apoptotic/necrotic or necrotic cells to any significant extent in either experimental conditions (with cyclo[DKP-RGD] 1 1x10-6 M, viable cells: 85.4±3.4% and 86.8±9.2%; early apoptotic cells: 10.5±3.1% and 9.1±7.7%; late apoptotic/necrotic cells: 2.7±0.6% and 2.4±0.9%; necrotic cells: 1.8±0.8% and 1.9±1.1%; in all the cases, n = 4 and P>0.05 vs control).

Proliferation

HUVEC proliferation in basal conditions was 0.25±0.18 O.D. and increased up to 1.85±0.50 O.D. in the presence of VEGF, EGF, IGF-I, and FGF2 (n = 3-6, P<0.05). Cell incubation with cyclo[DKP-RGD] 1 up to 1x10-5 M did not significantly affect either basal or stimulated proliferation (data not shown).

Migration

Spontaneous migration of HUVEC was 25.2±9.5 μm and increased by 87.8±53.7%, up to 44.9±13.4 μm in the presence of VEGF, EGF, IGF-I, and FGF2 in the bottom chamber (n = 17, P<0.001 vs basal

conditions). When cyclo[DKP-RGD] 1 was added in the top chamber, i.e. together with HUVEC, spontaneous migration was increased and stimulated migration was decreased, while when it was added in the bottom chamber both spontaneous and stimulated migration were increased (Figure 2).

Angiogenesis

HUVEC under basal conditions did not show any significant network formation. Addition of VEGF, EGF, IGF-I, and FGF2 induced a significant network formation, which was even higher when cells were treated with IL-8 (Figure 3).

Coincubation with cyclo[DKP-RGD] 1 did not significantly affect angiogenesis of HUVEC under basal conditions (Figure 3b-d), however it significantly and profoundly decreased the effect of VEGF, EGF, IGF-I, and FGF2 (Figure 3 and Figure 4, panel A) as well as the effect of IL-8 (Figure 3 and Figure 4, panel B).

Expression of mRNA for αv, β3 and β5 integrin subunits

HUVEC expressed comparable amounts of the mRNA for αv, β3 and β5 integrin subunits in both basal conditions and after treatment with VEGF, EGF, IGF-I, and FGF2, and coincubation with 1x10-6 M cyclo[DKP-RGD] 1 did not affect mRNA expression of any of the subunits in either experimental conditions (Table 2).

Akt phosphorylation

Treatment of HUVEC with 1x10-6 M cyclo[DKP-RGD] 1 in basal conditions reduced phosphorylated Akt, from 16241.7±1763.3 to 8702.7±2008.7 optical density arbitrary units, down to 53.2±7.9% of control (n = 3, P = 0.001), without however any significant effect in the presence of VEGF, EGF, IGF-I,

and FGF2 (15406.0±1218.8 to 15174.7±663.9 optical density arbitrary units, n = 3, P = 0.735) (Figure 5).

DISCUSSION

HUVEC represent a valid in vitro model which provides seminal insights into the cellular and molecular events leading to neovascularization in response to inflammation and hypoxia in cancer, ischemic events, and in embryogenesis [19]. As anticipated in the introduction, integrins are key actors in angiogenesis and vascular homeostasis, acting as promoters of endothelial cell-matrix interactions [20]. It has been recognized that pharmacological inhibition of the αvβ3 subtype suppresses angiogenesis in many experimental models and αvβ3 antagonists (i.e. antibodies, peptides and peptidomimetics) are being developed as antiangiogenic drugs [21]. It is known that integrins αvβ3 and αvβ5 are expressed on HUVEC [22]; as a consequence, these cells represent a suitable model to study the effects of agents acting on such targets. In the present study we used HUVEC to test the ability of the peptidomimetic integrin ligand cyclo[DKP-RGD] 1 to affect the key steps of the angiogenic process by evaluating its effects on proliferation, migration and capillary-like network formation. Some of us previously showed that cyclo[DKP-RGD] 1 inhibits vitronectin binding to αvβ3 and αvβ5 integrins with IC50 of 4.5±1.1 nM and 149±25 nM respectively [16].

In our experiments, the effects of cyclo[DKP-RGD] 1 on HUVEC activity were tested in resting conditions as well as in the presence of a culture medium enriched with growth factors known to promote angiogenesis such as VEGF, EGF, IGF-I and FGF2 or after addition of the pro-inflammatory chemokine IL-8, which has a key role in the regulation of pathological angiogenesis [2;3]. According to our results, cyclo[DKP-RGD] 1 is indeed able to strongly inhibit angiogenesis, as indicated by the reduction of network formation (vide infra), and this occurs without affecting cell viability, apoptosis

or proliferation. Most anti-angiogenic compounds acting through the inhibition of integrin function, such as cilengitide, exhibit cytotoxic activity in the same or very close concentration range [23]. In our experimental conditions, our compound did not affect cell viability and apoptosis or cell proliferation, suggesting that its antiangiogenic activity is likely independent from cytotoxicity. This latter observation deserves further consideration because angiogenesis represents a key step in some pathological conditions beyond tumour growth. For example atheromatous plaque vulnerability is closely related to neoangiogenesis [24]; in this latter case a cytotoxic effect exerted by an antiangiogenic compound could represent a risk for adverse effects. On the other hand, the lack of cytotoxic effects by cyclo[DKP-RGD] 1 was also observed in several different cell-lines such as ovarian carcinoma IGROV-1 or SKOV3, human pancreatic carcinoma PANC-1 and MIA-PaCa2, human osteosarcoma U2-OS [25], and can be considered therefore as a general feature of our compound. Whether this lack of cytotoxicity might be suggestive of reduced toxicity and increased tolerability in vivo in different pathological conditions needs to be assessed in specific studies.

Investigation of the specific mechanisms responsible for the antiangiogenic effects of cyclo[DKP-RGD] 1 was beyond the purpose of the present study; nonetheless, according to our results, this compound did not affect the mRNA levels for the integrin subunits v, 3 and 5, which are the main targets of its action, but it effectively inhibited the phosphorylation of Akt, a serine/threonine-specific protein kinase that plays a key role in the regulation of vascular homeostasis and angiogenesis [17]. The fact that the inhibition of Akt phosphorylation is only detected under basal conditions may be explained considering that, in the presence of growth factors converging on the same intracellular signalling pathway, the inhibitory effect exerted by cyclo[DKP-RGD] 1 is probably overcome. Inhibition of Akt phosphorylation by cyclo[DKP-RGD] 1 is likely the results of disruption of proper endothelial cell–extracellular matrix attachment, due to integrin engagement by cyclo[DKP-RGD] 1. Indeed, it has already been reported that antagonists against αvβ3 or αvβ5 integrin interfere with angiogenesis

induced by several growth factors: for instance, αvβ3 integrin associates with VEGF and platelet-derived growth factor (PDGF) receptors and potentiates VEGF or PDGF signaling, respectively [26].

Disruption of integrin functions may possibly explain also the effects of cyclo[DKP-RGD] 1 on HUVEC migration. Indeed, in the presence of cyclo[DKP-RGD] 1 migration was increased in resting conditions but it was decreased in stimulated conditions when the compound was added in the top compartment of the Boyden chamber, together with the cells, while it was increased in both resting and stimulated conditions when the compound was added in the bottom compartment. As a temptative explanation, we propose that increased migration results from the direct inhibitory effect of cyclo[DKP-RGD] 1 on integrins αvβ3 and αvβ5, resulting in reduced cell anchorage to surfaces. On the other side, the slight decrease of stimulated migration and the reduced increase of spontaneous migration when cyclo[DKP-RGD] 1 was added in the same compartment in which the cells were placed might imply also a slight chemoattractant effect of this compound, which would therefore not only increase cell random migration through decreased integrin-mediated attachment to the surfaces, but also attract the cells along its concentration gradient. The in vivo relevance of such effect, where no concentration gradient is expected to occur, is however questionable. Remarkably, the effect exerted by cyclo[DKP-RGD] 1 was apparently bell-shaped, with a peak at about 1x10-9 M (which however was not observed in the angiogenesis assay). Whether this finding implies different modes of action depending on the extension of integrin engagement on the cell surface, it should be established in specific experiments. Disruption of integrin function could therefore explain both the increased migration and the anti-angiogenic activity exerted by cyclo[DKP-RGD] 1. A similar effect was observed by Mrksich and co-workers [27], who promoted cell migration on self-assembled monolayers containing immobilized cyclic RGD by addition of exogenous linear RGD ligands [27].

In our experiments, cyclo[DKP-RGD] 1 effectively inhibited angiogenesis induced by the growth factors VEGF, EGF, IGF-I and FGF2, as well as by IL-8. All these proangiogenic agents act through

distinct membrane receptors [28;29] which result in the activation of extensively overlapping intracellular cascades finally activating common effector molecules, such as NF-κB or HIF-1 [28]. In addition, recent evidences indicate that direct interactions may occur between integrin activated pathways and signalling from VEGF receptors [30] and EGF receptors [31]. Collectively, in the light of such observations, our results support the ability of cyclo[DKP-RGD] 1 to block common mechanisms, resulting in the effective inhibition of angiogenesis triggered by multiple agents. Angiogenesis is a process that occurs not only in cancer, but also in many other critical diseases such as atherosclerosis [32], and the relevance of cyclo[DKP-RGD] 1-induced effects in such conditions needs careful assessment.

In conclusion, the data of the present study show that the novel compound cyclo[DKP-RGD] 1, an αvβ3 and αvβ5 integrin ligand, effectively inhibits angiogenic processes in HUVEC, possibly through mechanisms involving reduced Akt phosphorylation and disruption of integrin-mediated adhesion, without affecting their viability and proliferation. We propose therefore this compound as a candidate modulator of angiogenesis occurring in different conditions, possibly devoid of the adverse effects of cytotoxic analogues. Further studies clarifying the in vivo activity of cyclo[DKP-RGD] 1, including a complete toxicological assessment, as well as a thorough investigation of the intracellular pathways involved its effects are currently underway in order to evaluate its possible potential applications as a novel pharmacotherapeutic compound.

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Author's contribution

Roberto Fanelli and Laura Schembri = study design, performing all in vitro experiments and data handling

Umberto Piarulli = Study design and manuscript preparation

Monica Pinoli = in vitro experiments on morphogenesis and data handling

Emanuela Rasini = flow cytometry analysis and data handling

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Marco Cosentino = Study design, data handling, manuscript preparation and revision

Franca Marino = Study design, data handling, manuscript preparation and revision

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Table 1. Sequences of the primers and PCR products size.

Gene	Ref. sequence	Sequence	Product size
α_v	NM_002210	Forward: actggcttaagagagggctgtg Reverse: tgccttacaaaaatcgctga	110
β_3	NM_000212	Forward: agacactcccacttggcatc Reverse: tcctcaggaaaggtccaatg	123
β_5	NM_002213	Forward: agcctatctccacgcacact Reverse: cctcggagaaggaaacatca	91
GAPDH	NM_001289746.1	Forward: caactgtgaggaggggagatt Reverse: cagcaagagcacaagaggaag	97

Table 2. Real time PCR analysis of the expression of mRNA for the integrin subunits αv, β3 and β5 in HUVEC cultured for 5 h in basal conditions and with VEGF, EGF, IGF, and FGF, alone (control) or in the presence of 1 μM cyclo[DKP-RGD] 1. Data are means±SD of 3 separate experiments.

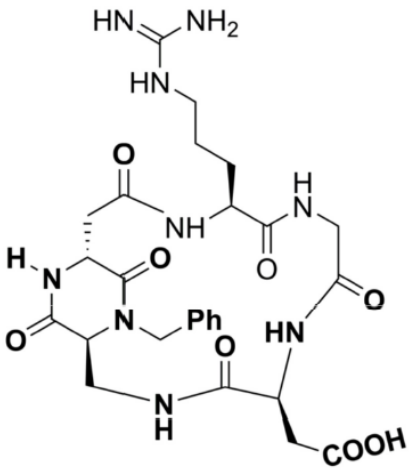
A Basal conditions

subunit	control		+ *cyclo*[DKP-RGD] 1	
	$2^{-\Delta ct} x10^2$	$2^{-\Delta ct} x10^2$	*ratio vs control*	*P vs control*
α_v	6.65±6.41	6.59±5.24	1.32±0.57	0.944
β_3	0.89±0.87	0.91±0.76	1.08±0.27	0.928
β_5	1.36±1.18	1.52±1.34	1.08±0.07	0.225

B. With VEGF, EGF, IGF, and FGF

subunit	control		+ *cyclo*[DKP-RGD] 1	
	$2^{-\Delta ct} x10^2$	$2^{-\Delta ct} x10^2$	*ratio vs control*	*P vs control*
α_v	13.31±12.80	15.09±10.66	1.28±0.42	0.494
β_3	1.15±1.07	1.41±1.07	1.43±0.35	0.288
β_5	1.23±1.25	1.31±1.13	1.34±0.46	0.461

FIGURE LEGENDS



***cyclo*[DKP-RGD] 1**

Figure 1. Structure of the peptidomimetic cyclo[DKP-RGD] 1.

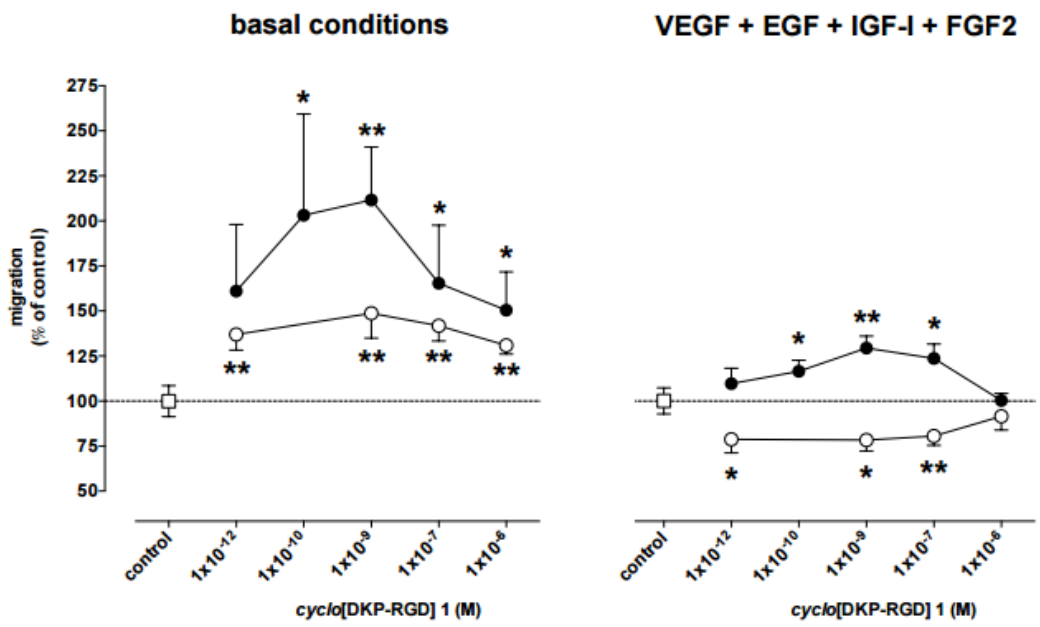


Figure 2. Effect of cyclo[DKP-RGD] 1 on HUVEC migration in the Boyden chamber assay. Cells were placed in the top compartment. Empty circles: cyclo[DKP-RGD] 1 placed in the top compartment. Filled circles: cyclo[DKP-RGD] 1 placed in the bottom compartment. Data are means±SEM of 5-17 separate experiments. * = P<0.05 and ** = P<0.01 vs respective control.

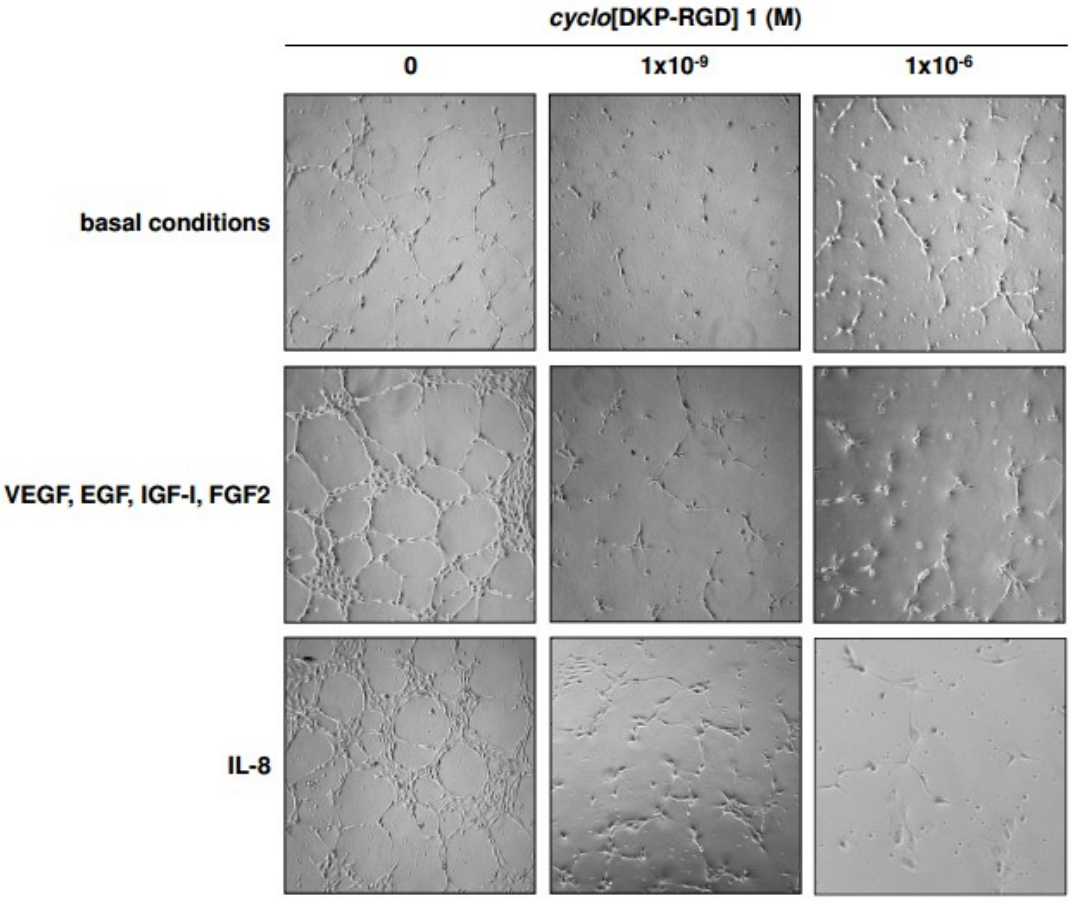


Figure 3. Representative phase contrast photomicrographs of HUVEC plated on Matrigel in basal conditions or in the presence of VEGF, EGF, IGF-I, and FGF2 or IL-8, without and with cyclo[DKP-RGD] 1 at different concentrations.

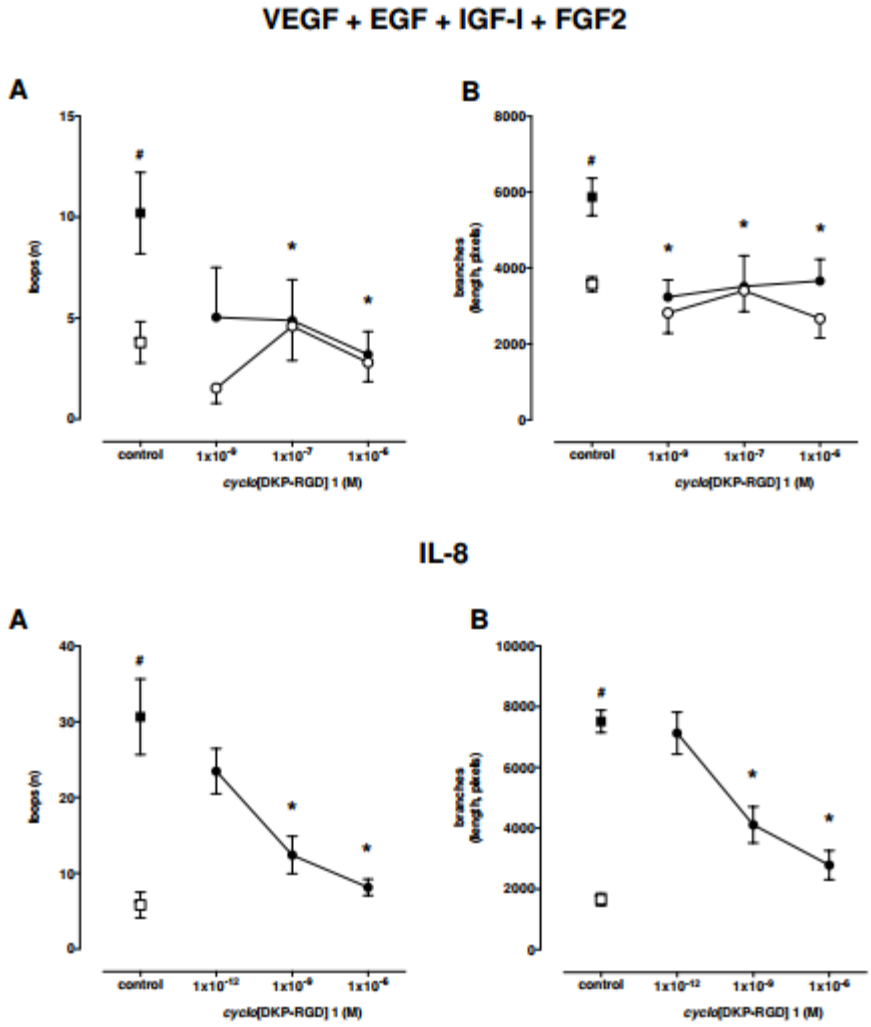


Figure 4. Effect of cyclo[DKP-RGD] 1 on HUVEC angiogenesis induced by VEGF, EGF, IGF-I, and FGF2 (upper panels) or IL-8 (lower panels). Angiogenesis was evaluated as both number of loops (A) and length of branches (B). Empty symbols: basal conditions; filled symbols: stimulated conditions. Data are means±SEM of 3-5 separate experiments. # = P<0.01 vs basal conditions, * = P<0.01 vs respective control.

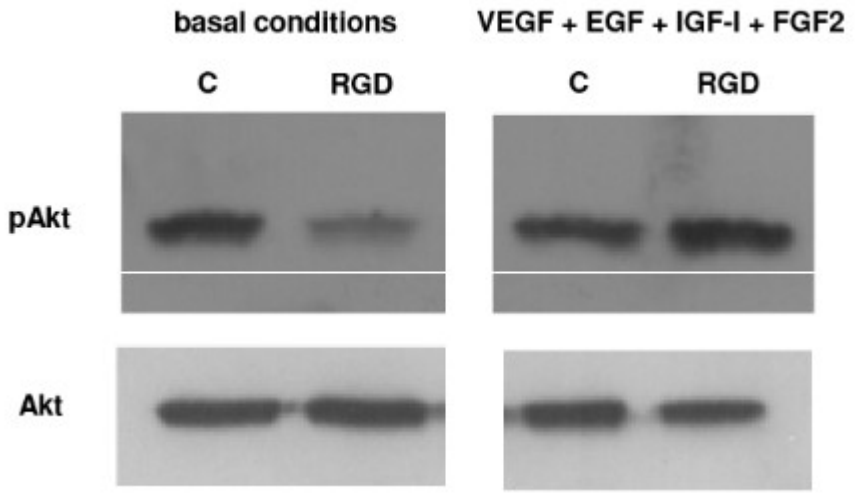


Figure 5. Western blot analysis of Akt phosphorylation in HUVEC cultured for 5 h in basal conditions and with VEGF, EGF, IGF-I, and FGF2, alone (control, C) or in the presence of 1 μM cyclo[DKP-RGD] 1 (RGD). Data are from one representative of 3 separate experiments.