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Early Events in Apoptosis Induction in Polymorphonuclear Leukocytes

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1. Introduction

Different white blood cells are involved in immune responses to pathogens, environmental stress, alterations in energy and nutrition supply as well as traumata. To find an adequate answer to the myriad of exogenous and endogenous noxes is a high challenge for the human immune system. Chronic inflammatory diseases like rheumatoid arthritis, arteriosclerosis, inflammatory bowel disease, and many others are associated with a disturbed regulation of immune functions (Peng, 2006; Zernecke & Weber, 2010). Despite numerous worldwide investigations regulatory aspects are only scarcely understood in innate and acquired immunity.

Polymorphonuclear leukocytes (PMNs, also called neutrophils) are among the first cells that are rapidly recruited to infected and/or injured tissue. Their infiltration into inflammatory sites is highly regulated and supported by adhesion molecules, cytokines, and extracellular matrix components in both blood vessel wall and adjacent tissue (Muller, 2002; Taylor & Gallo, 2006). These cells, by releasing special proteins and generating reactive metabolites, contribute to pathogen defense, regulation of the inflammatory process, and to tissue injury.

Unperturbed or slightly activated PMNs die by apoptotic cell death (Walker et al., 2005; Erwig & Henson, 2007). The rapid clearance of apoptotic PMNs by macrophages is crucial for efficient resolution of inflammation including the activation of different anti-inflammatory mechanisms that stop the recruitment of novel immune cells, deactivate pro-inflammatory cytokines, depress pro-inflammatory and anti-apoptotic pathways, and promote tissue repair. Apoptosis is induced either by release of mitochondrial constituents or by signalling via death receptors. In these pathways, different initiator and executioner caspases are activated that induce the degradation of molecules of the cytoskeleton, DNA, and others. In the result, numerous apoptotic vesicles will be formed without the release of internal constituents.

Apoptosis induction is mainly counterregulated by signals from phosphoinositide 3-kinase and protein kinase B that suppress caspase activation (Simon, 2003). In PMNs, the anti-apoptotic pathway is activated when the cells phagocytose foreign microorganisms or become attached to other cells or materials. This pathway ensures the functional responsibility of PMNs at inflammatory sites.

Both apoptotic cells/vesicles or cells, in which the anti-apoptotic pathway is activated, can undergo a necrosis that represents a cell death accompanied by cell and organelle swelling, plasma membrane rupture, and release of cytoplasmic content. Besides uncontrolled necrosis due to physical stress, necrotic cell death may be induced and regulated by signalling pathways (Degterev & Yuan, 2008). The uptake of necrotic PMNs by macrophages is associated with the release of pro-inflammatory mediators that further promote the inflammatory process (Vandivier et al., 2006). Thus, the interplay between macrophages and apoptotic/necrotic cells, mostly with PMNs, considerably determines the fate of an inflammation. This scheme, although very simplified, is also supported by the fact that PMN apoptosis is highly delayed and dysregulated in patients with severe sepsis (Jiminez et al., 1997; Fanning et al., 1999).

Special products of apoptotic PMNs apparently contribute to induction of anti-inflammatory signaling pathways in macrophages. Among them, PMN-derived chloramines like taurine chloramine and monochloramine are known to dampen the activation of NFκB in pro-inflammatory cells (Kontny et al., 2003; Ogino et al., 2005). Moreover, the 5-lipoxygenase of PMNs is involved in the transcellular synthesis of lipoxins that are able to stop the invasion of unperturbed PMNs to inflammatory sites (Serhan et al., 2008). However, fine mechanisms of their involvement in regulation of inflammatory processes are only poorly understood.

Thus, the induction of apoptosis in PMNs is an important prerequisite for the successful resolution of immune responses. From this background, we give here an overview about flow cytometry approaches to study early events in activation of PMNs and in apoptosis induction in these cells. These methods enable determination of specific properties of a large number of individual cells in a very short time. Because it is impossible to consider flow cytometry analysis for all PMN constituents, we will focus here on the analysis of those components that are unique to PMNs and highly necessary for specific functions of this type of immune cells.

2. Early activation of PMNs

Recruitment of PMNs from peripheral blood is mediated by selectins and integrins (Springer, 1994). After firm adhesion to the endothelium, PMNs invade into the inflamed tissue. Several chemotactic factors form a gradient for the directed movement of PMNs to the inflammatory loci. The bacterial product fMet-Leu-Phe, soluble immune complexes, leukotriene B_4 and the cytokine interleukin 8 (IL-8) are important chemoattractant agents (Lin et al., 2004). PMNs express specific receptors to these chemotactic factors. Receptor activation by these agents causes local changes in the contractility of cytoskeleton components and favors a directed movement of the cells. As PMNs have to pave their way through the tightly packed vessel wall and adjacent regions filled with different extracellular matrix components, these cells express and release also special proteases helping to degrade tissue and matrix components. Among these proteases are collagenase, gelatinase, different other matrix metalloproteases and the specific PMN serine proteases proteinase 3, cathepsin G, and elastase.

Here we will focus on flow cytometry approaches for binding of IL-8 to PMNs and for the expression of PMN specific serine proteases on the surface of PMNs.

2.1 Binding of IL-8 to PMNs

The cytokine IL-8 (also called CXCL8 according to the chemokine nomenclature) is released at inflammatory sites from fibroblasts, monocytes, endothelial and epithelial cells. It is a strong chemoattractant to PMNs (Rajarathnam et al., 1994). Besides the receptor binding site (the ELR motif near the NH_2-terminus) on IL-8, there are positive charged epitopes on its surface involved in binding to sulfated glycosaminoglycans (Kuschert et al., 1999; Lortat-Jacob et al., 2002; Pichert et al., 2012). Around inflammatory loci, IL-8 is mostly fixed to sulfated extracellular matrix components forming, thus, a gradient for the invading PMNs.

Neutrophils have two kinds of G protein-coupled receptors for IL-8 called CXCR1 and CXCR2 (Stillie et al., 2009). Receptor activation causes phosphorylation of Akt, calcium influx, formation of F-actin, and cytoskeletal rearrangement. These events are important for chemotactic movement. Several factors are known to modulate the binding of IL-8 to its receptors. Alpha-1 antitrypsin and IL-8 form a complex that is unable to interact with CXCR1 (Bergin et al., 2010). Truncation of amino acids at the NH_2-terminus by matrix metalloproteases, CD13, cathepsin L, or proteinase 3 creates a series of natural isoforms of IL-8. Some of them have a higher biological activity than the original IL-8 protein with 77 amino acid residues (Padrines et al., 1994; Mortier et al., 2011).

Oligomerization of chemokines affects also their interaction with receptors. While both monomeric and dimeric IL-8 forms were capable of inducing cell recruitment, the dimeric form induced a stronger migration in a mouse lung model (Das et al., 2010). Binding to sulfated glycosaminoglycans promotes oligomerization of IL-8 (Hoogewerf et al., 1997).

The binding of recombinant IL-8 to PMNs is shown in Fig. 1.

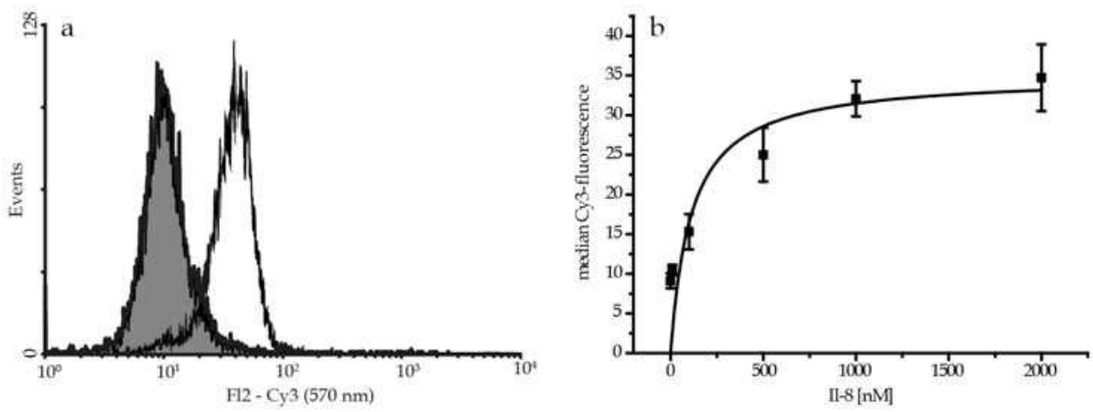

Fig. 1. Binding of IL-8 on the surface of freshly isolated PMNs. PMNs (2 x 10^6) were incubated with varying IL-8 concentrations for 60 min, followed by washing steps to remove unbound protein. After incubation of cells with the primary anti-human IL-8 rabbit antibody (4 µg/ml) for 1 h, PMNs were washed again and the secondary Cy3-conjugated goat anti-rabbit antibody (1.5 µg/ml) was added for 30 min in the dark. A representative example of fluorescence distribution in the presence of 1 µM IL-8 is shown in (a). The control is given in grey. Using median values from three independent measurements a binding curve was created with a K_d value of (112.7 ± 94.3) nM (b)

2.2 Detection of serine proteases on the surface of PMNs

In invading PMNs, the serine proteases proteinase 3, cathepsin G, and elastase are involved in microbicidal activity, penetration of cells through endothelium and adjacent connective tissue, and processing of various cytokines (Meyer-Hoffert, 2009; Kessenbrock et al., 2011). Although all three proteases have striking structural similarities (Korkmaz et al., 2008), there are clear differences in their functional response at inflammatory loci (Fleddermann et al., 2011). Unlike elastase, proteinase 3 and cathepsin G are already released from resting or slightly activated PMNs. While proteinase 3 binds heavily to cell surface epitopes, cathepsin G interacts preferentially with sulfated glycosaminoglycans. Proteinase 3 is apparently involved in both the infiltration of unperturbed PMNs into inflammatory sites and in cell necrosis, while cathepsin G plays most likely an important role in the degradation of specific components of the extracellular matrix during PMN invasion. In contrast, elastase probably contributes to shedding of surface molecules on macrophages helping to induce a pro-inflammatory feature in these cells (Pham, 2006). Examples for the incubation of resting PMNs with proteinase 3, cathepsin G, and elastase are given in Fig. 2.

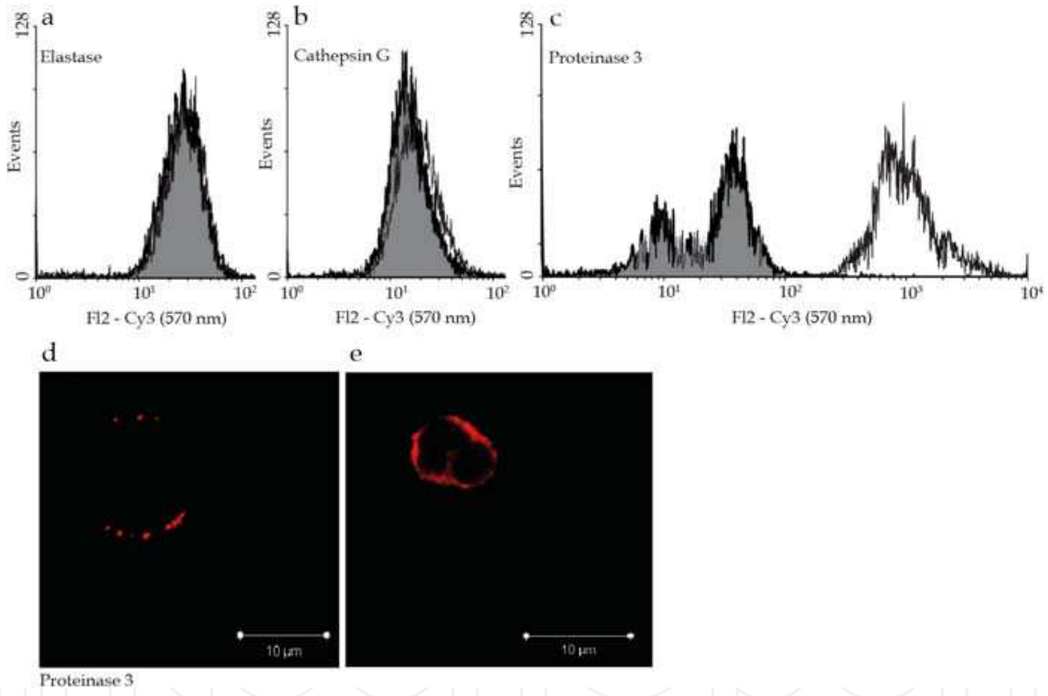

Fig. 2. Binding of externally added serine proteases on the surface of freshly isolated PMNs. Cells (2 x 10^6) were incubated with elastase (0.5 µM), cathepsin G (1.06 µM) and proteinase 3 (1.09 µM) for 1 h. After removal of unbound proteases by washing steps, PMNs were incubated with anti-neutrophil elastase rabbit antibody (134 µg/ml), anti-cathepsin G rabbit antibody (474 µg/ml) or mouse monoclonal proteinase 3 antibody (4 µg/ml) for 60 min. After washing and resuspension, Cy3-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1.5 µg/ml) were added and incubated for 30 min in the dark (a-c). Flow cytometry distribution curves with externally added proteases are given in white. Controls are highlighted in grey. Representative confocal fluorescence microscopy images of freshly isolated PMNs treated with Cy3-labeled antibodies against proteinase 3 without (d) or after addition of proteinase 3 (e). Representative data from four measurements are given

As shown in Fig. 2, the difference in the amount of elastase and cathepsin G on the PMN surface is very small after protease addition. However, a drastic shift in fluorescence yield is observed in case of proteinase 3, indicating the existence of a high number of potential binding sites.

3. Alterations in PMNs during apoptosis induction

In contrast to other cell types, systems to resist an apoptosis induction such as glutathione-dependent antioxidant enzymes are expressed to a minor degree in PMNs (Kinnula et al., 2002). In non-affected tissue, PMNs are known to undergo a spontaneous apoptosis (Payne et al., 1994). This type of cell death can be simulated *in vitro* keeping the cells at 37 °C under sterile conditions for several days. Phorbol 12-myristate 13-acetate (PMA) or the calcium ionophore ionomycin accelerate this process and enable investigation of PMN apoptosis within few hours. PMNs activated by 50 nM PMA are characterized by enhanced hydrogen peroxide levels, reduced cell size, condensed nuclei, and enhanced DNA fragmentation (Lundqvist-Gustafsson & Bengtsson, 1999). A late event in apoptosis induction is the breakdown of the original cell into smaller apoptotic vesicles. Their rapid removal by macrophages prevents the release of toxic components due to secondary necrosis of these apoptotic bodies.

We compare sensitive flow cytometry approaches for the detection of changes in the vitality status of PMNs during spontaneous or PMA-mediated apoptosis. PMA was used at very low nanomolar concentrations in order to better visualize early changes during apoptosis induction. Here we present data about forward and sideward scattering of PMNs, binding of fluorescence-labeled annexin V to phosphatidylserine epitopes and the intercalation of propidium iodide into DNA as well as measurement of the integrity of mitochondria using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1).

3.1 Forward and sideward scattering

While the value of the forward scatter depends on the cell size, the sideward scatter is a measure of the cell granularity. In flow cytometry analysis, the first parameter is usually plotted on a linear, the later one on a logarithmic scale.

The assessment of both kinds of scattering already provides a lot of information about the apoptotic process (Fig. 3). After apoptosis induction, a slight increase in the cell size takes place as visualized by a shift in the forward scattering values (Fig. 3b). The granularity remains unaffected. This alteration can be interpreted as a round-up of cells due to the progressive loss of linkages of the actin cytoskeleton with the plasma membrane. An early event in apoptosis induction in PMNs is the degradation of the corresponding link proteins (Kondo et al., 1997). Additionally, parts of the endoplasmic reticulum of the cells will be incorporated into the plasma membrane (Franz et al., 2007).

At later stages of apoptosis (Fig. 3c), enhanced forward scattering values vanished and smaller cell sizes became more prominent. At the same time a broader distribution of sideward scattering values was observed. This indicates the formation of polymorphic apoptotic bodies. The appearance of small apoptotic bodies (low forward and sideward scattering values) can also be seen during later apoptosis. Their appearance is a clear sign for the breakdown of apoptotic cells into smaller vesicles at later stages of apoptosis.

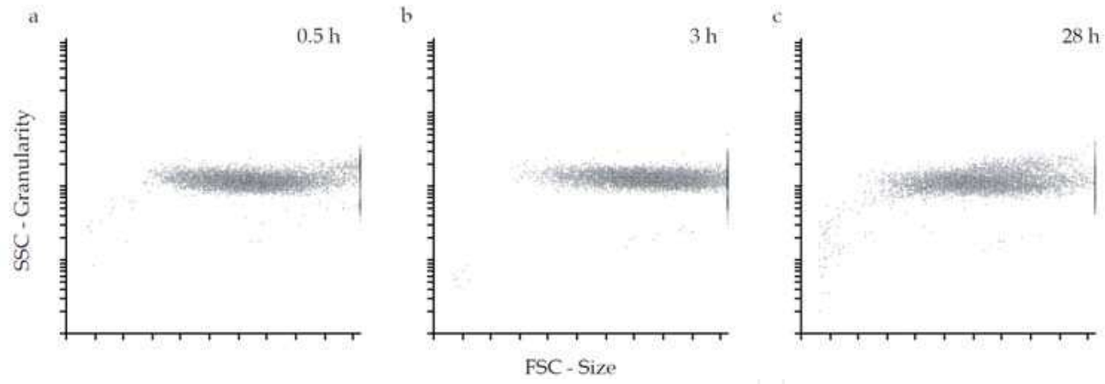

Fig. 3. Changes in cell size and granularity upon spontaneous apoptosis of PMNs. Cells (10^6) were cultivated at 37 °C for 0.5 h (a), 3 h (b), and 28 h (c), respectively. Changes in cell size and granularity were analyzed by flow cytometry using forward scattering (FSC) and sideward scattering (SSC). One representative example from four different cell preparations is shown

3.2 Binding of annexin V and uptake of propidium iodide

This approach is often used to assess the vitality status in a cell population. Vital cells do neither bind annexin V nor incorporate propidium iodide. Apoptotic cells are also unable to take up propidium iodide, but they express phosphatidylserine epitopes on the outer leaflet of the plasma membrane that are able to bind fluorescently labeled annexin V. In necrotic cells, propidium iodide permeates through the damaged plasma membrane and becomes highly fluorescent due to intercalation into DNA.

An example for analysis with both dyes in spontaneous and PMA-induced apoptosis in PMNs is given in Fig. 4.

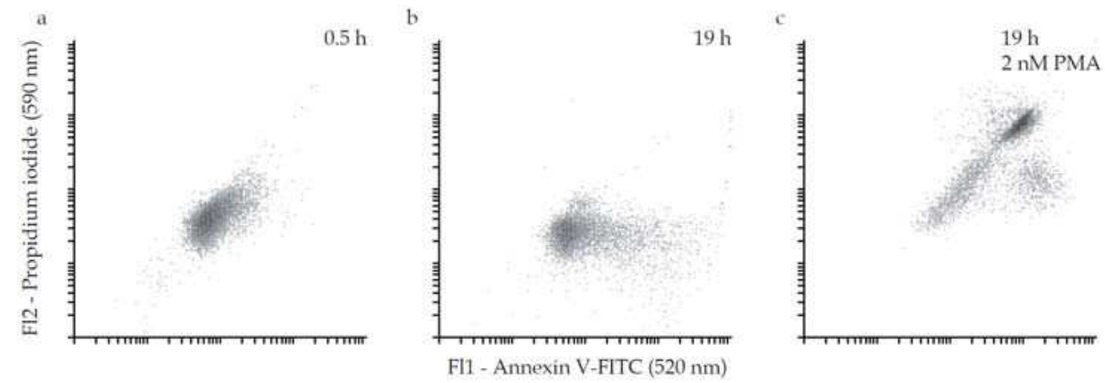

Fig. 4. Detection of apoptosis and necrosis in PMNs by annexin V and propidium iodide. PMNs (10^6) were cultivated at 37 °C for 0.5 h (a), or 19 h (b,c). In (c), cells were additionally activated by 2 nM PMA. Afterwards, PMNs were stained with fluorescently labeled annexin V (annexin V-FITC) and propidium iodide to detect apoptosis and necrosis, respectively. One representative example from four different experiments is shown

Freshly isolated PMNs did neither show any externalization of phosphatidylserine nor an uptake of propidium iodide (Fig. 4a). At prolonged incubation, part of the cells became

annexin V-positive, but they did not incorporate propidium iodide (Fig. 4b). Thus, an apoptosis induction was observed. In contrast, the prolonged incubation of PMNs with PMA yielded double-positive cells with enhanced values in both channels (Fig. 4c). From these data necrotic processes in these PMNs can be assumed.

Although this method is very convenient, there are two main problems necessary to consider in its application. An accumulation of phosphatidylserine at the outer leaflet of the plasma membrane is observed in dying cells, where these epitopes serve as a matrix for a number of proteins like annexin 1, thrombospondin, and β_2-glycoprotein 1 and as a signal for cell clearance by macrophages (Lauber et al., 2004; Walker et al., 2005). In apoptotic cells, myeloperoxidase binds also to phosphatidylserine epitopes (Leßig et al., 2007; Flemmig et al., 2008). It is generally assumed that the phosphatidylserine exposure on the cell surface is associated with activation of scramblases and inhibition of translocases (Yoshida et al., 2005). In apoptotic PMNs, there are caspase-dependent and -independent mechanisms in the appearance of annexin V-positive epitopes (Blink et al., 2004; Chen et al., 2006). On the other hand, a transfer of phosphatidylserine from internal granule stores to the cell surface cannot be ruled out during apoptosis (Mirnikjoo et al., 2009).

The second problem concerns changes in the cell size and the breakdown of cells into smaller vesicles during apoptosis. These alterations are already illustrated in forward and sideward scattering (see the previous section). Thus at later stages of apoptosis, the appearance of smaller vesicles and cell debris would affect all fluorescence measurements. It cannot be excluded that these smaller vesicles, even though they are fluorescent, appear in the same gate where originally vital unperturbed cells were found.

3.3 Changes in mitochondria

Neutrophils possess only a few mitochondria that are functionally different in contrast to mitochondria of other cells. Their participation in ATP synthesis and cytochrome *c* content is limited, but they maintain a membrane potential (van Raam et al., 2006). Cellular stress is

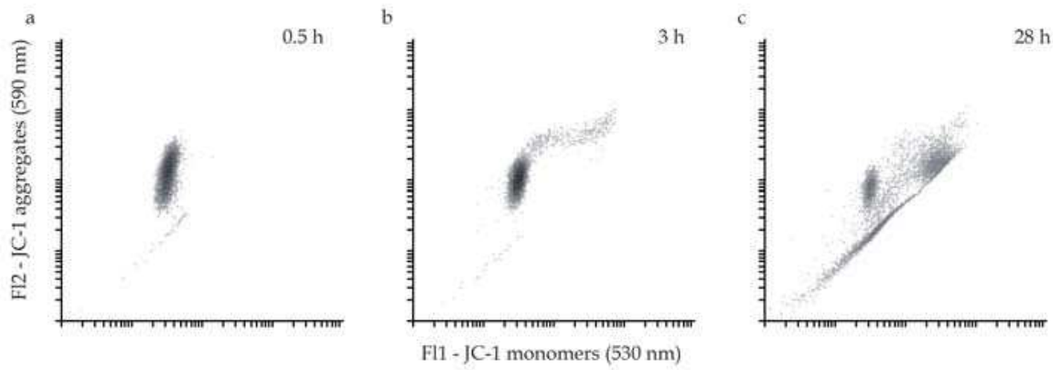

Fig. 5. Application of the dye JC-1 to detect early apoptotic events in PMNs. PMNs (10^6) were cultivated at 37 °C for 0.5 h (a), 3 h (b), or 28 h (c). Afterwards, cells were incubated with JC-1 (0.77 μM) for 10 min. After washing steps, the fluorescence of JC-1 monomers (channel 1) and aggregates (channel 2) was analyzed. Control experiments (not shown) were performed using the mitochondrial membrane ionophore valinomycin. One representative example from five different cell preparations is shown

sensitively reflected at the mitochondrial level leading to a rupture in processes maintaining the mitochondrial potential. In PMNs, pro-apoptotic proteins such as Smac/DIABLO, HtrA2/Omi and cytochrome *c* are released from mitochondria (Maianski et al., 2004). Early changes in mitochondrial integrity can be easily measured by the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (also called J-aggregate forming cationic dye, JC-1) (Cossarizza et al., 1993). This dye emits a red fluorescence when it accumulates in aggregated form in intact mitochondria. In apoptotic cells with damaged mitochondria, JC-1 is distributed over the whole cell cytoplasm and emits a green fluorescence.

We provide here an example for the application of this dye to PMNs undergoing a spontaneous apoptosis (Fig. 5). In freshly isolated vital PMNs with intact mitochondria, the dye is mainly incorporated in aggregated form with a dominating emission at 590 nm (Fig. 5a). The onset of apoptosis in PMNs was observable by increasing monomer fluorescence (Fig. 5b). At later times, nearly all cells showed a strong fluorescence of JC-1 monomers while the number of JC-1 aggregates was diminished (Fig. 5c).

3.4 Further systems for analysis of apoptosis induction

There are further flow cytometry approaches to detect changes in PMN vitality. As later stages of apoptosis are associated with progressive DNA fragmentation, the appearance of nicks in the DNA can be identified by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The investigation of PMN apoptosis in granulocyte preparations by the TUNEL assay can be affected by eosinophils that yield false positive results (Kern et al., 2000).

Active caspase-3 can be visualized in PMNs by enzyme inhibitors bearing a fluorescence label. This approach has been used to investigate effects of nicotinamide on PMN apoptosis (Fernandes et al., 2011).

4. Generation of oxidants during PMN apoptosis

PMNs are equipped with special enzymes for generation of a large amount of oxidants that are involved in antimicrobial activity, regulation of immune functions and apoptosis induction. These enzymes are NADPH oxidase (Shatwell & Segal, 1996) and myeloperoxidase (Klebanoff, 2005). While the first enzyme generates superoxide anion radicals that further dismutate to hydrogen peroxide, the heme protein myeloperoxidase uses H_2O_2 to oxidize (pseudo)halides to the corresponding (pseudo)hypohalous acids. Under physiological relevant conditions, the production of hypochlorous acid (HOCl) and hypothiocyanite ($^-$OSCN) is important (van Dalen et al., 1997). As myeloperoxidase is strongly expressed in PMNs (and to a lesser extent in monocytes) there is a great interest to understand specific functions of this enzyme during immune reactions of PMNs (Arnhold & Flemmig, 2010).

In PMNs, reactive oxygen species (ROS) are important for apoptosis induction. Apoptosis is delayed in NADPH oxidase deficiency (Lundqvist-Gustafsson & Bengtsson, 1999) and by scavenging of H_2O_2 by glutathione or catalase (Kasahara et al., 1997; Yamamoto et al., 2002). Interestingly, oxidants down-regulate phosphoinositide 3-kinase γ activity and inhibit actin

polymerization during PMN apoptosis (Xu et al., 2010). Myeloperoxidase deficiency suppresses also PMN apoptosis (Tsurubuchi et al., 2001; Milla et al., 2004). Several myeloperoxidase products such as hypothiocyanite, monochloramine and taurine chloramine are potent inducers of apoptosis (Emerson et al., 2005; Lloyd et al., 2008; Ogino et al., 2009).

Here we focused our attention on two sensitive flow cytometry approaches for oxidant generation in close association with apoptosis induction in phorbol ester- and IL-8-activated PMNs. While dihydrorhodamine 123 detects very sensitively but non-specifically the oxidative activity in PMNs (Bizyukin et al., 1995), the combination of the dyes 2-[6-(4'-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) and 2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) allows to visualize specifically the generation of the myeloperoxidase product HOCl in PMNs (Setsukinai et al., 2003). Structures of these dyes are given in Fig. 6.

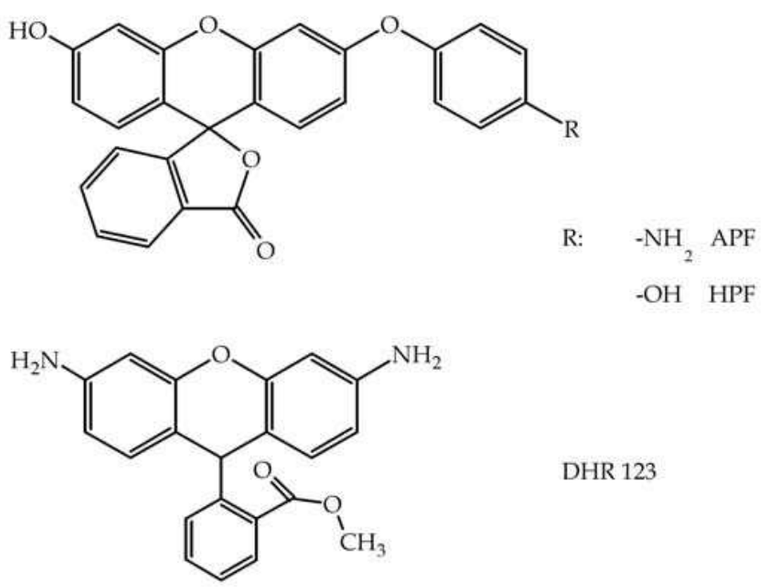

Fig. 6. Chemical structures of dyes applied for oxidant detection in PMNs

4.1 Dihydrorhodamine 123

The non-fluorescent compound dihydrorhodamine 123 is converted intracellularly into the fluorescent rhodamine 123 by several oxidants including superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, hypochlorous acid, but not singlet oxygen (Bizyukin et al., 1995). This selection makes dihydrorhodamine 123 a suitable flow cytometric dye to follow the oxidative activity in neutrophils and other cells. The involvement of given enzymes in oxidant production can be determined by specific enzyme inhibitors.

Here we give an example for the application of dihydrorhodamine 123 to measure the oxidant generation in PMNs activated by the chemokine IL-8 (Fig. 7). In the presence of IL-8 significant higher fluorescence values were observed indicating a more promoted formation of oxidants in stimulated PMNs.

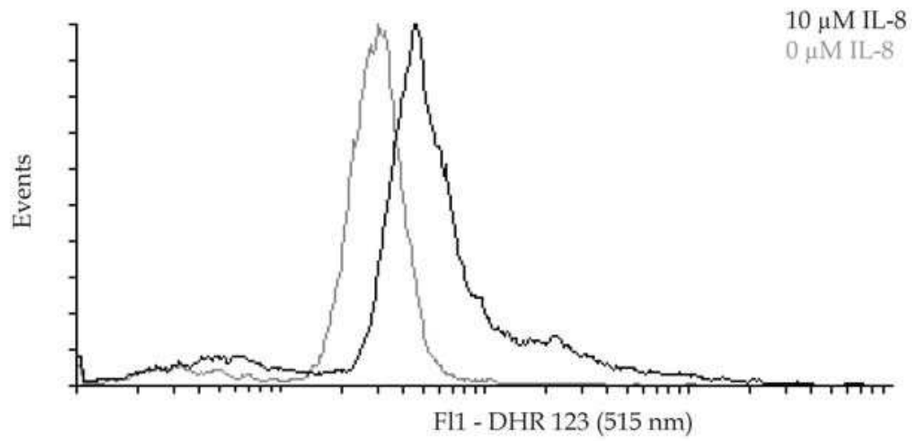

Fig. 7. Application of DHR 123 to detect the oxidative activity in PMNs. PMNs (2 x 10^6) were cultivated for 1 h at 37°C either in the presence (black) or in the absence (grey) of 10 µM IL-8. Afterwards they were stained with 10 µM DHR 123 and analyzed by flow cytometry. One representative example from four different experiments is shown

4.2 The APF/HPF system

In APF and HPF, the fluorescence is quenched by protection of the phenolic hydroxy group at the 6'-position of fluorescein with an electron-rich aromatic ring. The initial reaction with oxidants starts in both dyes by an attack on the aryloxyphenol group resulting in the cleavage of this group and the formation of highly emitting fluorescein. Hydroxyl radicals and peroxynitrite are able to oxidize both HPF and APF. HOCl activates only APF but not HPF. All other biologically relevant oxidants are insensitive against APF and HPF. This oxidant profile allows the application of APF and HPF to detect specifically the formation of HOCl in activated neutrophils (Setsukinai et al., 2003).

We successfully applied APF to visualize the production of HOCl in non-stimulated and PMA-activated neutrophils (Fig. 8). A significant stronger shift in the fluorescence intensity distribution of APF indicates a higher HOCl production in the stimulated cells. In both non-stimulated and PMA-stimulated PMNs the application of the MPO inhibitor 4-aminobenzoic acid hydrazide (4-ABAH) strongly inhibited the HOCl production as can be seen by much lower APF fluorescence values.

4.3 Further systems for oxidant detection

Among other unspecific dyes suitable for flow cytometric analysis of ROS generation in PMNs, we will mention only 2,7-dichlorofluorescein diacetate (Walrand et al., 2003). This non-fluorescent compound is converted by several oxidants into a fluorescent derivative. This system is often used to assess unspecific ROS formation in activated cells.

Besides the APF/HPF method for specific detection of HOCl generation in PMNs, a few further systems have recently been described to visualize the intracellular formation of HOCl. These novel approaches include the use of sulfonaphthoaminophenyl fluorescein (Shepherd et al., 2007), a rhodamin-hydroxamic acid-based system (Yang et al., 2009), and a thiol analogue of hydroxymethyltetramethylrhodamine (Kenmoku et al., 2007).

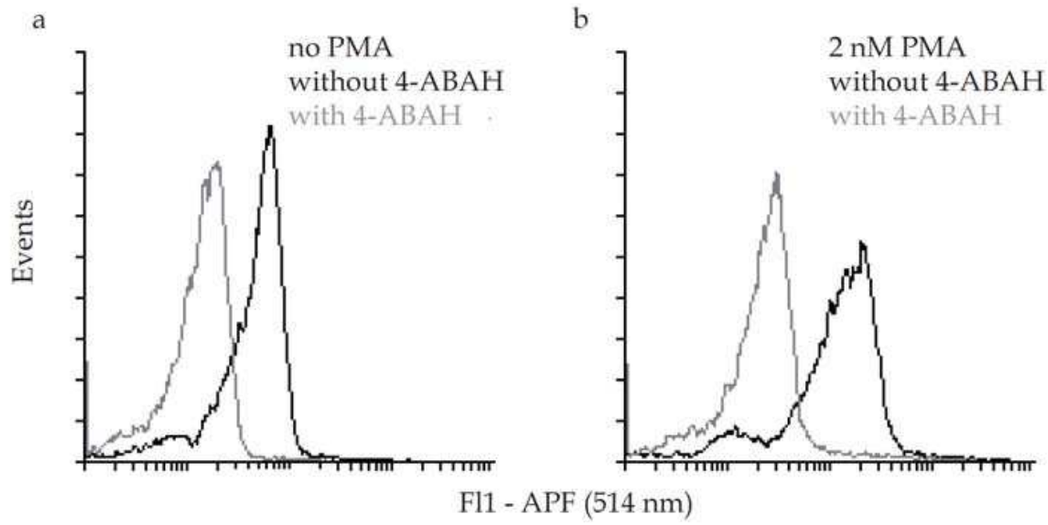

Fig. 8. Detection of HOCl generation in non-stimulated and PMA-stimulated PMNs. PMNs (10^6) were cultivated for 3 h either in the absence (a) or presence (b) of 2 nM PMA. In order to analyze the formation of the myeloperoxidase metabolite HOCl, APF (5 μM) was present during the incubation. In some cases, the myeloperoxidase inhibitor 4-aminobenzoic acid hydrazide (4-ABAH) was also included in the incubation cocktail (grey). One representative example of five different experiments is shown

5. Concluding remarks

Polymorphonuclear leukocytes are key players in innate and acquired immune response. Upon invasion to inflammatory sites, they sense their local environment and contribute to regulation of the further inflammatory process. For successful resolution of inflammation, the initiation of apoptotic cell death in PMNs and the subsequent fast removal of apoptotic bodies by macrophages are crucial. Despite numerous investigations, fine mechanisms of apoptosis induction in PMNs and factors modulating apoptotic pathways are only poorly understood.

Flow cytometry approaches are an important tool in this challenging field. We summarized here advantages and limitations of a number of widely applied methods, but focused also our attention on newly developed approaches for oxidant detection. The myeloperoxidase-hydrogen peroxide-halide system is unique to PMNs and its products are apparently involved in regulation of immune responses. Thus, novel methods for detection of hypochlorous acid generation such as the APF/HPF system are highly necessary for further investigation of modulation of myeloperoxidase activity.

We also directed our focus on enforcement of PMN apoptosis by low-concentrated PMA. This approach allows the convenient study of early changes during cell activation. In granulocytes, mitochondria are mainly responsible for providing pro-apoptotic factors that initiate caspase activation. The application of the dye JC-1 is useful to follow the loss of mitochondria integrity. Mechanisms leading to the appearance of phosphatidylserine epitopes remain puzzling. In this regard, distortions of enzyme reactions maintaining the phospholipid asymmetry and fusion events with internal granule stores are under

discussion. Last but not least, the importance of oxidant generation is acknowledged for apoptosis induction in PMNs however, their fine mechanisms are poorly understood.

Detailed knowledge about PMN functions is also necessary to better understand the role of these immune cells in the pathogenesis of diseases and for introducing new therapies helping to terminate inflammatory processes.

6. Acknowledgements

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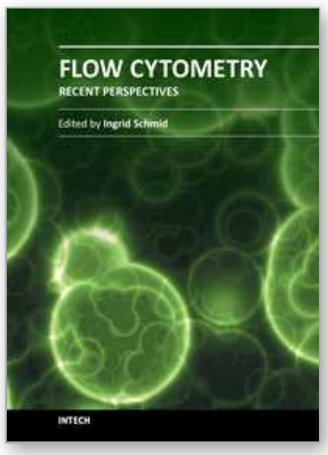

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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