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The myeloid expressed EF-hand proteins display a diverse pattern of lipid raft association

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Abstract EF-hand proteins are known to translocate to membranes, suggesting that they are involved in signaling events located in the cell membrane. Many proteins involved in signaling events associate cholesterol rich membrane domains, so called lipid rafts, which serve as platforms for controlled protein–protein interaction. Here, we demonstrate that the myeloid expressed EF-hand proteins can be distinguished into three classes with respect to their membrane association. Grancalcin, a myeloid expressed penta EF-hand protein, is constitutively located in lipid rafts. S100A9 (MRP14) and S100A8 (MRP8) are translocated into detergent resistant lipid structures only after calcium activation of the neutrophils. However, the S100A9/A8 membrane association is cholesterol and sphingolipid independent. On the other hand, the association of S100A12 (EN-RAGE) and S100A6 (calcyclin) with membranes is detergent sensitive. These diverse affinities to lipid structures of the myeloid expressed EF-hand proteins most likely reflect their different functions in neutrophils.
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1. Introduction

The calcium binding EF-hand domain is a common motif characterizing a large family of proteins specifically expressed in various cell types. Due to their calcium binding properties, they are thought to play a pivotal role in a variety of signaling pathways [1]. Furthermore, they are known for their ability to translocate to membranes in a calcium-dependent manner [2]. Selective recruitment of proteins from the cytoplasm to the membrane can regulate signaling events, such as those downstream of growth factor addition, ion channel activation, or changes in cell adhesion. Lipid–lipid interactions can organize membranes into detergent-resistant domains, so called lipid rafts. These domains have been proposed as platforms for the controlled interaction of substrates and signaling enzymes. Many signaling proteins are known to be organized in lipid rafts, including heterotrimeric G proteins and members of the Src family of protein tyrosine kinases, which are dually acylated [3].

In myeloid cells changes in $[Ca^{2+}]_i$ have been associated with multiple functions, including activation of cellular kinases and phosphatases, degranulation, phagosome–lysosome fusion, regulation of cytoskeletal binding proteins, transcriptional control, and modulation of surface receptors. The S100 proteins S100A9 (MRP14), and S100A8 (MRP8), S100A12, S100A6 (calcyclin) and the penta EF-hand protein grancalcin, all members of the calcium binding EF-hand protein family, are expressed in a myeloid specific manner. They are translocated in a calcium-dependent manner to the cell membrane [4–7], although the sequences of these proteins do neither encode a signal peptide nor any other sequence known to mediate membrane association. Nevertheless, the calcium-dependent membrane association suggests that they may be involved in the calcium-dependent signaling processes. However, nothing is known about their localization in lipid rafts.

Here, we report that the EF-hand proteins show a diverse and protein-specific pattern of membrane and lipid raft association, which most likely reflects a functional diversity of these proteins in granulocytes.

2. Materials and methods

2.1. Chemicals and antibodies

Preparation of anti-human S100A9, S100A8 and S100A12 antibodies was described earlier [8,9]. Anti-human S100A6 antibody was generously provided by C.W. Heizmann, Zürich. Human grancalcin was recombinantly expressed as his-tagged protein in *Escherichia coli* using pQE-32 (Qiagen, Düsseldorf, Germany) as expression vector. His-tagged grancalcin was purified by Ni–NTA affinity column following the manufacturer's instructions (Qiagen, Düsseldorf). Purified his-tagged grancalcin was used to immunize rabbits and IgG specific antibodies were finally isolated by protein G Sepharose affinity purification of rabbit serum. The flotillin-1 antibody was purchased from BD Biosciences Pharmingen (USA).

All stimulating agents were purchased from Sigma (St. Louis, USA).

2.2. Preparation of granulocytes

Granulocytes were isolated from human buffy coats as described elsewhere [10]. Briefly, the whole blood sample was diluted with complete spinner medium and overlaid on Biocoll (1.077 mg/ml). After centrifugation, the erythrocytes in the cell pellet were lysed in ice cold water and the remaining granulocytes were washed in phosphate buffered saline. Finally, the granulocytes were incubated in Hanks' buffers saline (HBSS). Where indicated cells were treated for 10 min at 37 °C with 5 μM nocodazole (NOCOD), 10 μM cytochalasin D (CytD), 50 μM phospholipase A2 inhibitor AACOCF3 (PLA2I) or 1 μM PMA either in the presence of 5 μM A23187 or 2 mM EDTA. Methyl-β-cyclodextrin (MCD): cells were pre-treated for 30 min at 37 °C with 10 μM MCD to deplete cholesterol. After stimulation, cells were collected by centrifugation and resuspended in 2-(*N*-morpholino)ethanesulfate buffer (25 mM MES, pH 6.5; 150 mM NaCl; protease inhibitors) with 1% Triton X-100.

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2.3. Lipid raft fractionation using a sucrose gradient

Granulocytes were incubated in MES-buffer (25 mM MES, pH 6.5; 150 mM NaCl; protease inhibitors) containing 1% Triton X-100 at 4 °C for 20 min and then homogenized using a loose-fitting Dounce homogenizer (10 strokes). The homogenates (corresponding to 3×10^7 cells) were then adjusted to 40% sucrose by the addition of an equal volume of 80% sucrose solution prepared in the above buffer, but lacking Triton X-100, placed in the bottom of ultracentrifuge tubes, and then overlaid with a discontinuous sucrose gradient of 4 ml 30% (w/v) sucrose and 4 ml 5% (w/v) sucrose, both prepared in MES buffer lacking Triton X-100. The samples were centrifuged at 35 000 rpm ($200\,000 \times g$) in an SW41 rotor (Beckman Instruments) for 16–20 h, fractionated into 1 ml fractions sequentially from the top of the gradient, and concentrated by precipitation with trichloroacetic acid.

The pellets were dissolved in denaturing Lämmeli loading buffer. Equal quantities of each fraction were loaded onto an SDS–PAGE. Western blots were performed according to standard procedures and developed using the ECL Plus^{TM} Western Blotting Detection Reagents from Amersham Biosciences.

2.4. EGFP-fusion protein expression

Grancalcin and S100A9 were cloned into pEGFP-C1, a vector that allows the eukaryotic expression of fluorescent GFP-fusion proteins (BD Bioscience Clontech, USA). The EGFP-hgrancalcin and EGFP-hS100A9 constructs were transfected into HEK293 cells via electroporation. Expression of fusion proteins was verified by Western blot analysis (data not shown).

3. Results

First, we investigated the association of the penta EF-hand protein grancalcin with detergent resistant lipid structures. Lipid rafts containing fractions were identified by the presence of flotillin-1, a marker protein for granulocytic lipid rafts. We observed that grancalcin is localized in lipid rafts independent of calcium (Fig. 1). Grancalcin is also detected in the pellet of the sucrose gradient confirming its association with cytoskeletal proteins [11]. Furthermore, fractionating the lipid rafts containing fractions a second time via a discontinuous sucrose gradient grancalcin is still present in the low density, cholesterol rich fractions as flotillin-1 (Fig. 2), suggesting that grancalcin can be considered as integral part of granulocytic lipid rafts. This finding was confirmed by the addition of MCD, a cholesterol and sphingolipid-depleting agent [12] that is commonly used to disrupt lipid rafts. Pre-treating the granulocytes with MCD grancalcin disappeared from the lipid raft fractions, indicating that this protein is indeed located in granulocytic lipid rafts (Fig. 1). To further confirm the subcellular localization of grancalcin, HEK293 cells were transfected with an EGFP-grancalcin encoding vector. The grancalcin fusion protein is localized around or in the nucleus. Additionally, fluorescent spots, which may represent fusion

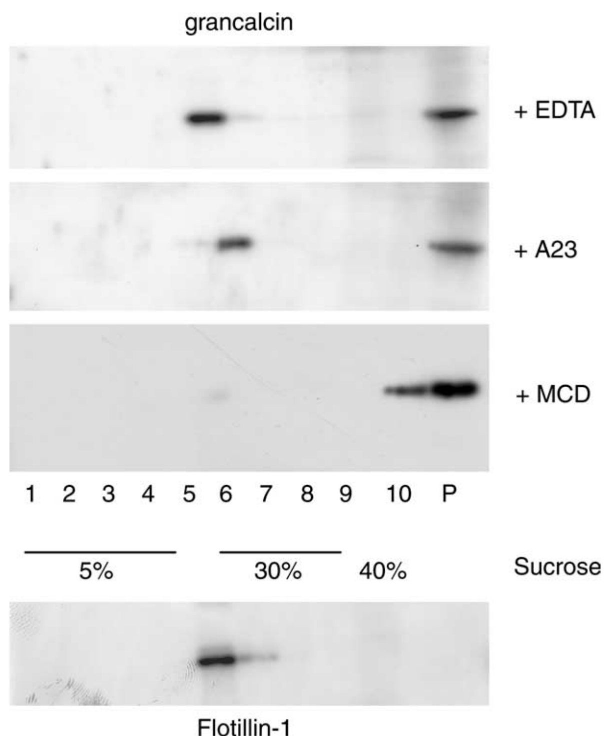

Fig. 1. Granulocytes were incubated with A23187 or with EDTA in HBSS; MCD: cells were pre-incubated with MCD for 30 min at 37 °C. Subsequently, the cells were lysed with 1% Triton X-100, fractionated (1–10; p, pellet) via a sucrose gradient and analyzed by Western blotting using the anti-human grancalcin antibody as probe. The presence of flotillin-1 as a lipid raft marker protein is shown below.

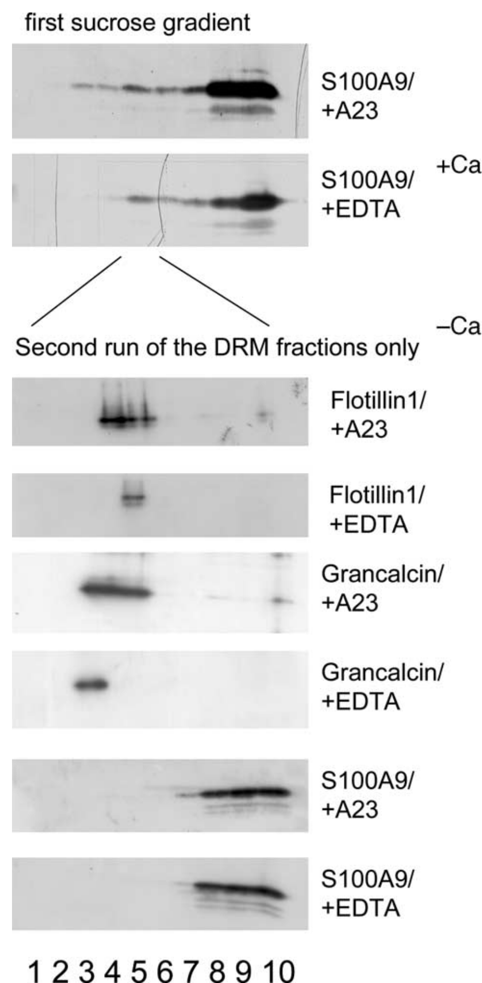

Fig. 2. The lipid raft containing detergent resistant membrane (DRM) fractions derived from A23187 treated and EDTA treated granulocytes were subjected to a second sucrose gradient and subsequently analyzed by Western blotting for the presence of grancalcin, S100A9 and flotillin-1. Whereas both grancalcin and flotillin-1 remain in the lipid raft fractions, S100A9 is no longer associated with detergent resistant lipid structures.

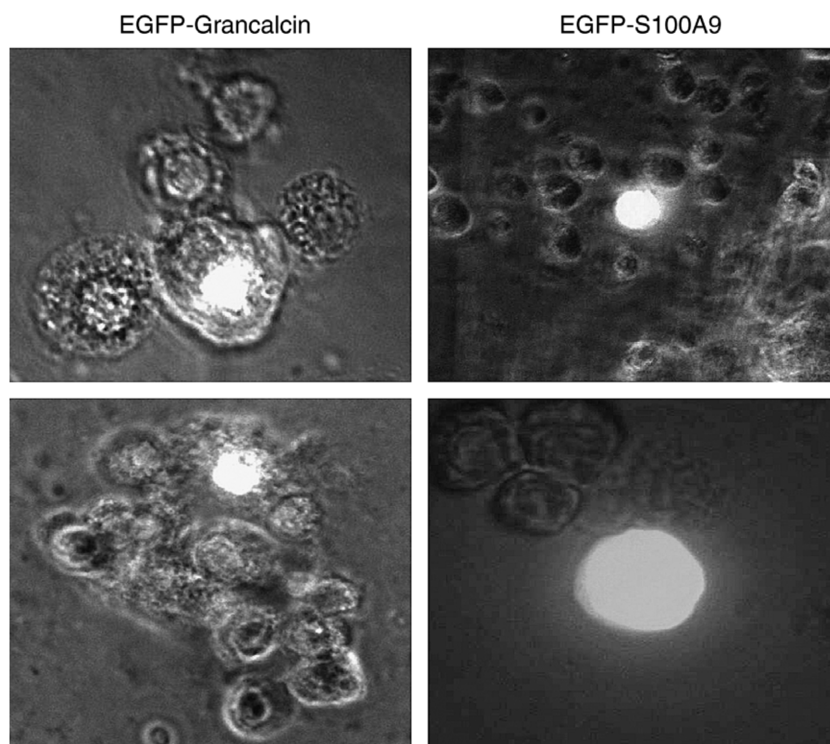

Fig. 3. HEK293 cells were transiently transfected with pEGFP-hgrancalcin (grancalcin) and pEGFP-hS100A9 (S100A9). The expression of the fusion proteins was confirmed by Western blotting (data not shown). The S100A9 fusion protein is distributed over the whole cell body, whereas the EGFP-grancalcin protein is located around the cell nucleus. The fluorescent spots visible at the EGFP-hgrancalcin transfected cells may represent EGFP-hgrancalcin protein in lipid rafts.

protein located in lipid rafts, are also distributed over the cell body. The EGFP-S100A9 fusion protein, however, seems to be distributed over the whole cell body (Fig. 3).

In contrast to grancalcin, the membrane association of S100A6 (calcyclin) and S100A12 is apparently detergent sensitive. Both proteins are exclusively present in the high density fractions, which contain the cytosolic and detergent soluble proteins independent of calcium. They are also not found in the pellet, indicating that they do not interact with cytoskeletal proteins in the presence of Triton X-100 (Fig. 4).

S100A9 and S100A8 are found in lipid structures during phagocyte activation. In resident cells both S100A9 (Fig. 5) and S100A8 (data not shown) were found in the cytoskeletal pellet as well in the high density fractions after treatment with Triton X-100, but not in the low density, lipid rich fractions. Only after an A23187 induced increase of $[Ca^{2+}]_i$ both proteins were detected in the low density, lipid containing fractions (Fig. 5). However, they do not exclusively associate with typical lipid rafts, since both proteins are also present in low density fractions other than the flotillin-1 containing lipid raft fractions. To investigate whether actin or tubulin filaments are involved in this translocation, the cells were incubated with cytochalasin or NOCOD as indicated. NOCOD inhibits the calcium-dependent redistribution of S100A9/A8, whereas cytochalasin does not. The complex of S100A9/A8 binds arachidonic acid in a calcium-dependent manner [13]. To investigate the possibility whether membrane association could be mediated by arachidonic acid during phagocyte activation, we inhibited the phospholipase A2-dependent release of arachidonic acid from cellular lipids by AACOCF3. However, treatment of the granulocytes with the phospholipase A2 inhibitor does not prevent the calcium-dependent shift of S100A8/A9 into lipid rich fractions (Fig. 5). Next, the flotillin containing fractions were isolated and a second sucrose fractionation of this lipid raft containing fractions was performed. Now the S100A9 and S100A8 were detected in the high sucrose fractions, suggesting that the association with the lipid structures is quite loose (Fig. 2). Interestingly, upon treatment with MCD, S100A9/A8 does not shift into other fractions, indicating that their calcium induced membrane association is not cholesterol and sphingolipid-dependent (Fig. 5).

4. Discussion

According to our experiments, the EF-hand proteins can be classified into three classes: first, proteins that clearly integrate into lipid rafts as grancalcin. Secondly, those that are not associated with lipid rafts and whose interaction with membraneous and cytoskeletal structures is detergent sensitive. S100A12 and S100A6 proteins belong to this class of EF-hand proteins. Thirdly, proteins whose interaction with membranes is detergent resistant, but not restricted to lipid rafts. They only associate under cell-activating conditions. S100A9 and A8 are examples for this class of EF-hand proteins.

Grancalcin has been reported to bind to the actin bundling protein L-plastin. Here, we additionally demonstrate that this protein is constitutively present in neutrophilic lipid rafts. Following activation of phagocytes with inflammatory stimuli such as formyl-methionine–leucine–phenylalanine or immune

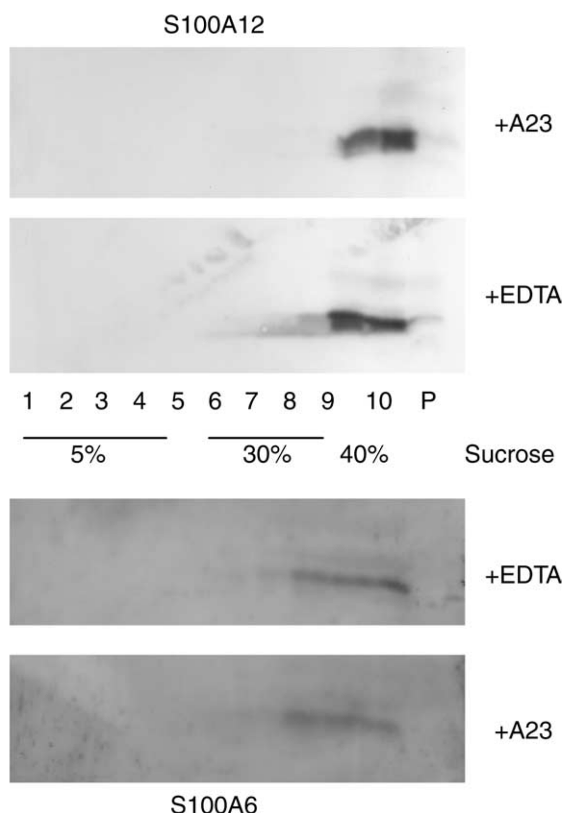

Fig. 4. Western blot of the sucrose gradient fractions probed with anti-human S100A12 and anti-human S100A6 (calcyclin), respectively, indicating that both proteins are localized in the high density fractions after Triton X-100 treatment. Cells were incubated either with A23187 or with EDTA, respectively.

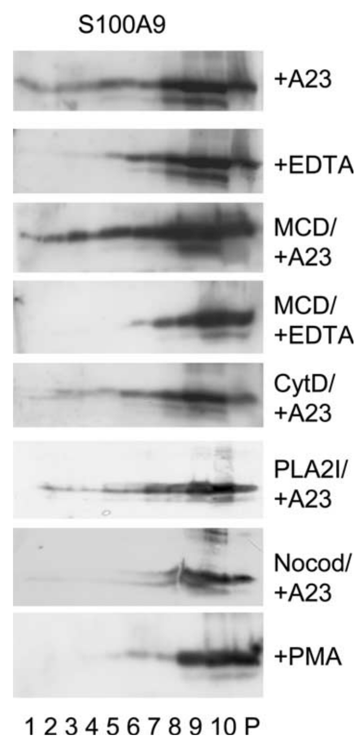

Fig. 5. Western blot of the sucrose gradient fractions (1–10; p, pellet) probed with anti-human S100A9. Before lysing cells with Triton X-100, granulocytes were treated with nocodazole (NOCOD), cytochalasin D (CytD), phospholipase A2 inhibitor AACOCF3 (PLA2I), and PMA (PMA) either in the presence of A23187 or in the presence of EDTA as described in Section 2. MCD: cells were pre-treated with MCD to deplete cholesterol. Probing the Western blot with the anti-human S100A8 antibody revealed a pattern identical to that of S100A9 (data not shown).

complexes that bind to Fc γ receptors, L-plastin is phosphorylated at Ser5, and this in turn leads to integrin activation and subsequent increased adhesion [11]. It is known that these processes are mediated by proteins organized in lipid rafts. Grancalcin is able to interact with L-plastin. Cell stimulation leads to a cytoplasmic Ca^{2+} rise with two consequences for L-plastin: (1) its actin-bundling activity is inhibited, at least in in vitro experiments [11]; and (2) the L-plastin–grancalcin complex dissociates. Grancalcin might thus regulate adherence and migration of neutrophils through interaction with L-plastin [11]. The localization of grancalcin in lipid rafts grancalcin is in accordance with these findings.

Furthermore, granulocytes are devoid of the typical marker protein for lipid rafts, namely caveolin [14]. Thus, we propose to use grancalcin like flotillin as a novel marker protein for granulocytic lipid rafts.

S100A12 and S100A6 do not firmly associate with lipid structures, since after treatment of the cells with the detergent Triton X-100 both proteins are exclusively found in the high density fractions containing the cytosolic and detergent soluble proteins. However, it has been shown that both translocate to the membrane upon stimulation [4,7]. Thus, their association is either loose or mediated by detergent sensitive protein–protein contacts. Moreover, in contrast to the other EF-hand proteins both proteins could not be detected in the cytoskeletal fraction after treatment with the detergent. S100A12 (EN-RAGE) has been reported to present an inflammatory marker protein [8]. However, the molecular mechanism by which this protein is secreted or with which protein it intracellularly interacts is not understood so far.

S100A6 (calcyclin) is predominantly known to be expressed in the central nervous system and epithelial cells [15]. Its presence in neutrophils has been recently documented, but nothing is known about its specific role in theses cells [16]. S100A6 translocation was found to be dependent on actin-stress fibers. Interestingly, recent experiments suggest that different S100 proteins utilize distinct translocation pathways, which might guide them to certain subcellular compartments in order to perform their physiological tasks in the same cellular environment [17].

The most diverse behavior with respect to the association to the lipid rafts is shown for the S100A8 and S100A9 proteins. Both are known to form heterodimers. In summary, the S100A9/A8 membrane association is calcium-dependent, detergent resistant and lipid raft independent. The calcium-dependent association with membranes and the cytoskeleton is detergent resistant as shown in this study. However, it is remarkable that both proteins are not exclusively located in the typical lipid raft containing fractions. Instead, both proteins are also found in the chlolesterol-free, but lipid-rich fractions as confirmed by the cholesterol depletion experiment, suggesting that they bind in a detergent resistant manner to other lipid structures than to the cholesterol and sphingolipid rich lipid rafts. In this context, it is worth mentioning that the early

concept of a uniform lipid raft structure is changing. Instead, the rafts in cells appear to be heterogeneous both in terms of their protein and their lipid content [18] and our data indicate that S100A8/A9 may associate to lipid structures other than the typical cholesterol and sphingolipid rich rafts. Furthermore, tubulin dissociating agents seem to inhibit this translocation. This agrees well with the finding that secretion of S100A8/A9 occurs via a tubulin-dependent mechanism [19].

PMA elicits a strong oxidative burst reaction. The S100A8/A9 complex has been described as a stimulatory cofactor for the phagocytic NADPH oxidase [20]. The membrane bound NADPH oxidase subunits are present in lipid rafts [21]. Stimulation of the cells with the phorbol ester PMA alone does not result in detergent resistant membrane (DRM) association of S100A8/A9. This strengthens the observation that calcium is essential for the DRM S100A9/A8 association and further suggests that the S100A8/A9 mediated stimulation of the NADPH oxidase activity is most likely mediated by detergent sensitive protein–protein interaction with the subunits of the NADPH oxidase and is not dependent on a calcium-dependent membrane localization of the S100 proteins.

Taken together, our findings provide new biochemical evidence that the family of EF-hand and S100 proteins play diverse roles in the membrane of granulocytes.

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References

[1] Donato, R. (2003) Microsc. Res. Tech. 15, 540–551.
[2] Donato, R. (2001) Int. J. Biochem. Cell Biol. 33, 637–668.
[3] Dykstra, M., Cherukuri, A., Sohn, H.W., Tzeng, S.J. and Pierce, S.K. (2003) Annu. Rev. Immunol. 21, 457–481.
[4] Stradal, T.B. and Gimona, M. (1999) J. Biol. Chem. 274, 31593–31596.
[5] Boyhan, A., Casimir, C.M., French, J.K., Teahan, C.G. and Segal, A.W. (1992) J. Biol. Chem. 15, 2928–2933.
[6] van den Bos, C., Roth, J., Koch, H.G., Hartmann, M. and Sorg, C. (1996) J. Immunol. 156, 1247–1254.
[7] Vogl, T., Propper, C., Hartmann, M., Strey, A., Strupat, K., van den Bos, C., Sorg, C. and Roth, J. (1999) J. Biol. Chem. 274, 25291–25296.
[8] Foell, D., Kane, D., Bresnihan, B., Vogl, T., Nacken, W., Sorg, C., Fitzgerald, O. and Roth, J. (2003) Rheumatology (Oxford) 42, 1383–1389.
[9] Roth, J., Burwinkel, F., van den Bos, C., Goebeler, M., Vollmer, E. and Sorg, C. (1993) Blood 82, 1875–1883.
[10] Feige, U., Overwien, B. and Sorg, C. (1982) J. Immunol. Meth. 54, 309–315.
[11] Lollike, K., Johnsen, A.H., Durussel, I., Borregaard, N. and Cox, J.A. (2001) J. Biol. Chem. 276, 17762–17769.
[12] Ottico, E., Prinetti, A., Prioni, S., Giannotta, C., Basso, L., Chigorno, V. and Sonnino, S. (2003) J. Lipid Res. 44, 2142–2151.
[13] Kerkhoff, C., Klempt, M., Kaever, V. and Sorg, C. (1999) J. Biol. Chem. 274, 32672–32679.
[14] Sengelov, H., Voldstedl, M., Vinten, J. and Borregaard, N. (1998) J. Leukoc. Biol. 63, 563–566.
[15] Lesniak, W., Swart, G.W., Bloemers, H.P. and Kuznicki, J. (2000) Acta Neurobiol. Exp. (Wars) 60, 569–575.
[16] Konig, S., Zeller, M., Peter-Katalinic, J., Roth, J., Sorg, C. and Vogl, T. (2001) J. Am. Soc. Mass Spectrom. 12, 1180–1185.
[17] Hsieh, H.L., Schafer, B.W., Cox, J.A. and Heizmann, C.W. (2002) J. Cell Sci. 115, 3149–3158.
[18] Pike, L.J. (2004) Lipid rafts: heterogeneity on the high seas. Biochem. J. 378 (Pt 2), 281–292, Review.
[19] Rammes, A., Roth, J., Goebeler, M., Klempt, M., Hartmann, M. and Sorg, C. (1997) J. Biol. Chem. 272, 9496–9502.
[20] Doussiere, J., Bouzidi, F. and Vignais, P.V. (2002) Eur. J. Biochem. 269, 3246–3255.
[21] Shao, D., Segal, A.W. and Dekker, L.V. (2003) FEBS Lett. 550, 101–106.