CLEARANCE OF DYING AUTOPHAGIC CELLS OF DIFFERENT ORIGIN BY HUMAN PROFESSIONAL AND NON-PROFESSIONAL PHAGOCYTES

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by

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INTRODUCTION

Molecular elements of the apopto-phagocytic synapse

Dynamic interrelationship between dying cells and engulfing cells exists with many overlapping molecular elements which constitute the apopto-phagocytic system, often called as the third synapse. These elements can be categorized into the following functional categories: phagocytosis receptors, cell surface molecules, bridging molecules, signal transducers, engulfment proteins, effector molecules, transcription factors, inflammatory regulators and cytokines. Molecular changes during phagocytosis occur on both sides of the synapse. Such changes on the surface of dying cells involve elimination, modification or translocation of molecules which already have well established roles in living cells. Furthermore, an intimate relationship between the dying cells of different origin and the inflammatory response exists clearly suggesting sharing of elements with the innate immune response as well.

Clearance of apoptotic cells by professional and non-professional phagocytes

Clearance of apoptotic cells by professional phagocytes has been a well described phenomenon so far. In this regards, studies on mice having no macrophages have helped increase the knowledge regarding the function of professional phagocytes. These mice could develop normally and without excess apoptotic cells in tissues having relied only upon non-professional phagocytes for efficient clearance of dying cells. Therefore, macrophages have been considered important in the clearance of dying cells in the circulation and in places where the apoptotic rate is so high that the non-macrophage system can not cope with the high load of corpses. Although now a recognized phenomenon, less is known about the clearance of apoptotic cells by non-professional phagocytes.

On the side of the dying cells, the surface changes “flag” their recognition and removal which are unanimously recognized by both professional and non-professional phagocytes include, but are not limited to: exposure of specific carbohydrates binding to phagocyte lectins, thrombospondin, collectins or complement products (bridging molecules); loss of phospholipid asymmetry and surface exposure of PS; $\alpha_V\beta_3$, $\alpha_V\beta_5$, CD36 in fibroblasts, $\alpha_V\beta_3$ kidney mesangial cells; SR-B1 (scavenger receptor) in testis Sertoli and ovarian thecal cells, lectins on endothelial cells (receptors for target interaction). It has become obvious that there are no unique molecular tools available for the non-macrophage system to ingest apoptotic cells; they just take advantage of the same molecular repertoire which is used by the

professionals. However, during engulfment of apoptotic cells it has been observed that non-professional phagocytes are slower, recognize late stage apoptotic cells more and digest the corpses slower than professional ones.

Non-macrophage cellular systems, such as epithelial cells can regulate the function of other cells locally through paracrine actions coupled to the production of the pro-inflammatory cytokines IL-1β, IL-8, TNF-α and MIP-2 as it has been shown in mammary epithelial cells stimulated by LPS. Apoptotic cells, unlike necrotic cells, do not provide pro-inflammatory signals for these cells and can downregulate the expression of pro-inflammatory cytokines induced by LPS.

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Autophagic cell death and the lack of information how these cells are cleared from tissues

Besides the widely known apoptosis or type I 'programmed cell death' (PCD) and necrosis, cells can also undergo anoikis and autophagic cell death. Anoikis is induced in cells after their detachment from the extracellular matrix switching on apoptotic signaling pathways. Type II PCD or autophagic cell death has been described as a process having dual role: one being a degradative mechanism for long-lived proteins and damaged organelles through the auto-phago-lysosomal pathway and therefore a survival pathway preventing or delaying apoptosis and the other being a possibility of self-destruction for cells.

MCF-7 cells can be induced to undergo autophagy upon being fed with 3% charcoal-stripped FCS (DCC) containing medium and treatment with the anti-estrogen, tamoxifen(TAM) (Bursch et al. 1996). To our present knowledge the final fate and clearance mechanism of cells either dying through autophagy (killed by autophagy) or by other mechanisms with associated autophagy have not been clarified. While cell surface changes and markers for phagocytic recognition of cells undergoing apoptosis have been extensively researched, no such surface changes have been implicated for engulfment of autophagic cells. Furthermore, while the inflammatory response has

been well studied during clearance of apoptotic and necrotic cells, nothing is known about the response induced in phagocytes during engulfment of autophagic dying cells. Different danger signals (bacteria, uric acid and ATP) have been shown to induce Nod-like receptors (NLRs) and the inflammasome in mammalian cells. How autophagic dying cells affect the production of inflammatory cytokines and relate to the known inflammatory pathways has not been clarified yet. The purpose of the studies presented here is to answer these questions.

MATERIALS AND METHODS

Cells and treatments

MCF-7 and Human-monocyte derived macrophages were culture and/or isolated using standard culturing conditions. The monocytes were differentiated to macrophages over a 5 day period by a standard protocol using MCSF. Tamoxifen, 3-methyladenine (3-MA), recommbinant annexin-V and caspase-1 inhibitor (zYVAD-FMK) were used at concentrations of 1μM, 10mM 2 days prior to peaking autophagy, 10μg per 10^5 cells and 50μM 30min before and throughout a phagocytosis assay, respectively. PolyHEMA-coated dishes were made for induction of anoikis.

Assays of cell death and autophagy

Cell death was assessed by the Annexin-V-FITC/PI Apoptosis Detection Kit (MBL, Woburn, MA) on BD FACS according to manufacturer's recommendations. Autophagy was assessed by detection of autophagic vacuoles with 0.05 mM monodansylcadaverin (MDC) according to the method of Biederbick et al. 1995.

Electron microscopy

Standardized protocols were used for sample fixation in sodium cacodylate-buffered and glutaraldehyde/saccharose solution and postfixed in OsO_4 solution, then dehydrated in ethanol gradient, embedded, stained with uranyl acetate and lead citrate and examined in Philips CM 10 microscope at 80 kV.

Antibodies and immunoblotting

Custom made rat anti-human LC3 polyclonal antibody was prepared by Eurogentec (Seraing, Belgium). NuPAGE 12% Bis-Tris polyacrylamide gel (Invitrogen) and transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA; pore size 0.45 μm). Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% non-fat dry milk (BioRad) for 1 hour. After blocking, membranes were probed overnight at 4°C with a primary rat anti-LC3 polyclonal antibody in antibody dilution buffer (TBS-T containing 1% non-fat dry milk), followed by 1 hour incubation with a rabbit anti-rat peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) for 1 hour at room temperature. Peroxidase activity was detected with SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Phagocytosis assay

Dying cells (day 4, 3%DCC+TAM treated (*de novo* autophagic); day 6, 10%FCS+PolyHEMA (anoikic-autophagic); UV-induced apoptotic MCF-7 cells) pre-stained with 12.5μM CFDA (green) were fed to macrophages or living MCF-7 cells also pre-stained with 7.5μM CFDA (red) in a 5:1 ratio over 1, 12 or 24 hours and serum-free conditions. Phagocytic binding at 4^{o}C was subtracted during flow cytomtric analysis of percent phagocytosis from the 37^{o}C (binding+engulfment). Inhibition of autophagy with 10mM 3-MA and 10μg per 10^5 cells recombinant annexin-V ocurred as described above.

RNA preparation and TaqMan Real-Time-PCR

Macrophages or MCF-7 cells engulfing *de novo* autophagic or anoikic-autophagic MCF-7 cells were used for analysis after washing away the dying cells. Their total RNA isolated with TRIzol Reagent (Life Technologies) was used to make cDNA using RT-PCR technology and High-Capacity cDNA Archive Kit (Applied Biosystems) which was further used in a custom made TaqMan Low Density Array (TLDA) (Applied Biosystems) for studying 95 genes involved in phagocytosis according to manufacturer's protocol. Two replicates per target gene and for each biological sample two parallel analysis were carried out. Relative quantities (RQ or fold changes) were determined using the equation where relative quantity equals $2^{-\Delta\Delta Ct}$ (ΔCt represents the threshold cycle (Ct) of the target minus that of 18S rRNA)

Cytokine release assay

The supernatants from macrophages engulfing autophagic dying cells were collected and analyzed for the presence of IL-8, IL-6, IL-1β, IL-10, TNF-α, IL-12p70 using the Human Inflammation BD Cytometric Bead Array (CBA) (BD Biosciences). IL-1β inhibition studies with the caspase-1 inhibitor (zYVAD-FMK) was carried as described above.

RESULTS AND DISCUSSION

Induction of autophagy in MCF-7 cells by tamoxifen treatment and through anoikis

MCF-7 cell when cultured in 3% DCC medium followed by tamoxifen treatment undergo autophagic or type II PCD peaking at day 4 (Bursch et al. 2000). We have discovered that these cells when plated on poly-HEMA in 10% FCS undergo anoikis which switched to autophagy at day 6 of incubation as revealed by monodansylcadaverine staining, elevated LC3II expression and electron microscopy. Different treament regimens (3% DCC+TAM, 10% FCS+poly-HEMA, 3% DCC+poly-HEMA, 3% DCC+TAM+poly-HEMA) affected the time course but not the maximal degree of the autophagic response (about 70% of MCF-7 cells were affected in each case). The percent of dying cells as assessed by annexin-V and/or PI positivity in anoikic-autophagic cells was twice as much than in case of *de novo* autophagy, death occurred earlier and peaked not in association with the peaking of autophagy

Based on several indirect evidences it has been proposed that active cell death is induced through autophagy in tamoxifen treated MCF-7 cells (Bursch et al. 1996); one of the evidences is the inhibition of both autophagy and cell death by 3-MA, a specifical inhibitor of autophagy. On the other hand, it is still unknown how anoikis-initiated death response can switch on autophagy and why does anoikis accelerate tamoxifen-induced autophagy? Examples for the induction of apoptosis with the help of autophagy regulators, such as the *p53*-induced *dram* and the calpain-generated cleavage product of *atg5*, have been described. It is also possible that elements of the apoptosis machinery, while killing cells, may initiate autophagy to fasten self elimination even before phagocytosis; in our case loss of integrin-dependent survival pathways may lead to induction of death effectors which in turn converge onto mediators of autophagy, such as *Atg5*, *Atg7*, and *beclin-1*.

Clearance of cells dying through (*de novo*) and with (anoikic-) autophagy

Macrophages acting as professional and non-professional phagocytes, respectively, could engulf dying *de novo* autophagic (less efficiently - at the 24th hour of co-incubation about 15% of them contained these cells) and anoikic-autophagic (more efficiently – at the same time 32% contained the dying cells). MCF-7 cells, however, engulfed dying *de novo* autophagic cells much more efficiently than either anoikic-autophagic or apoptotic (UV irradiated) ones, reaching an average phagocytosis frequency of 32% at the same time point. These data were confirmed as well by counting under a confocal microscope.

Since in our experiments 3-MA inhibited both macrophage and MCF-7 cell mediated phagocytosis of dying *de novo* autophagic cells but not of anoikic-autophagic ones, it can be suggested that molecular events linked to autophagy and death are involved in specific surface changes which are important in subsequent recognition and elimination of these dying cells. Therefore, we have described clearance of autophagic dying cells in two kinds of paradigm; one in which cells died mainly through autophagy, generating specific cell surface changes for recognition and engulfment, and another, where cell death occurred with concomitant autophagy which is not the main inducer of death and does not contribute to specific cell surface changes for removal by phagocytes.

Macrophages and non-professional phagocytes use distinct mechanisms for engulfment

Dying cells present on their surface several "eat-me" signals facilitating their engulfment one such being the externalized PS. Recombinant annexin-V, which binds PS exposed on the surface of dying cells, could block the engulfment of dying *de novo* autophagic cells by MCF-7 cells; this clearly suggests that whatever specific changes are induced by autophagy on the surface of these dying cells is directly or indirectly linked to PS. During development, autophagy in dying cells may be crucial to generate sufficient ATP for PS exposure as demonstrated in PCD associated with morphogenesis although the need for autophagy-dependent ATP production for triggering PS exposure was observed only in a three-dimensional culture setting. Uptake of the *de novo* autophagic cells by macrophages could not be inhibited by recombinant annexin-V indicating that PS-independent cell surface changes are also induced in cells dying through autophagy.

This means that macrophages, as compared to MCF-7 cells, must have additional recognition and signaling system(s) available for the initiation of efficient phagocytosis of these cells. A handful of proteins have been shown to undergo internalization from the plasma membrane following the induction of autophagy, however, no specific cell surface changes have been described which would make autophagic cells edible for phagocytes. Recently, calreticulin appearing on the surface of the dying cells was found to initiate clearance of apoptotic cells and the authors have suggested that calreticulin acts together with PS for achieving optimal phagocytic clearance of cells. Further studies are required to determine whether these alternative "eat me" signals or others are exposed on the surface of cells dying through autophagy.

The anoikic-autophagic cells were very efficiently eaten, actually more efficiently than *de novo* autophagic ones, by macrophages in a PS-independent manner. Since this was not related to the appearance of autophagy (could not be inhibited by 3 -methyladenine) the PS-independent

recognition and signaling system in this case must be different from the one utilized by cells dying through autophagy.

Expression profile of apopto-phagocytic genes in macrophages and MCF-7 cells

Quantitative gene expression analysis of macrophages and MCF-7 cells was carried out to gain insight into the potential molecular mechanisms mediating phagocytosis by these cells. The genes analyzed on a TaqMan Low Density Array to determine their level of expression can be categorized into the following functional categories: phagocytosis receptors, cell surface molecules, bridging molecules: signal transducers, engulfment proteins, effector molecules, transcription factors, inflammatory regulators and cytokines. These genes have been selected to cover most of the so far described molecular elements which may participate in the clearance of dead cells. The basal expression levels were determined in relation to the abundant 1 8S RNA (average of three repeated experiments) fell. The data from the gene expression profiling clearly show that both macrophages and MCF-7 cells are well equipped for phagocytosis of dying cells. In macrophages, almost all studied genes of the various functional groups were expressed and the following ones have very high basal expression levels: calreticulin, the scavenger receptors CD68 and MSR1, the low density lipoprotein-related protein 1 (LRP1), integrins α_x and β_2 (phagocytosis receptors), annexin A5, the C1QA bridging molecule and TGFβ. Interestingly, many of the studied and expressed phagocytic genes were also expressed in MCF-7 cells. The exceptions, with very low expression levels in comparison to macrophages, include the C1Q receptor 1, MSR1, the oxidized low density lipoprotein 1 receptor (OLR1), integrin α_x among the phagocytosis receptors, the adenosine receptor A3 (ADORA3), the formyl peptide receptor like 1 (FPRL1), the bridging molecules C1QA and ICAM3, the inflammatory regulators caspase 1 and 5, NALP12 and the cytokines IL6, IL10, IL12B, IL23A. Calreticulin, annexin A5 and calpain 1 showed high basal expression in these cells.

Clearance of autophagic and anoikic-autophagic cells initiates distinct transcriptional responses in macrophages and MCF-7 cells

The time point chosen for gene expression profiling of macrophages and MCF-7 cells while they were consuming dying cells as compared to their resting state was 12 hours. Change of mRNA level was considered significant when the average value of relative expression repeatedly (at least in two biological samples, both analyzed in two parallel runs) exceeded 2.5 times the average expression level in controls, either increasing or decreasing (+ or -). The engulfment of *de novo* autophagic cells elicited different response in the two types of phagocytes. In macrophages, the

asialoglycoprotein receptor 1 (ASGR1), adenosine receptor 2A (ADORA2A), the bridging molecule PTX3, phospholipase IPLA27, the engulfment molecules GULP1 (which was not expressed in the resting cells) and RAC1, the interferon regulatory factors (IRFs) 1 and 8, the cytokines IL-6, IL12B, IL23A and TNFα were induced. ADORA3, the platelet activating factor receptor (PTAFR), the bridging molecules C3 and ICAM 3, the inflammatory regulators BIRC1 and NALP12 were downregulated in macrophages eating *de novo* autophagic cells. MCF-7 cells showed increased expression of annexin-1 and the engulfment molecule ELMO1. Both phagocytes upregulated OLR1, IRF4 and IL6 to a significant degree in response to the dying *de novo* autophagic cells.

Macrophages eating anoikic-autophagic cells upregulated C1Q receptor 1, ADORA1 and ADORA2A, (while downregulating several phagocytosis receptor and surface molecules including CD68, MSR1, LRP1, integrins, ADORA3. annexin-1 and the platelet/endothelial cell adhesion molecule), the bridging molecules C4B and MFGE8 (while downregulating C2, C3 and ICAM3), the signaling molecule protein kinase 2, the engulfment protein RAC1 (while downregulating ELMO 1), DNASE1 (while downregulating DNASE2), the IRF 4 (while downregulating IRF8), the pro-inflammatory caspase 5 (while downregulating NALP12) and the cytokine IL6. MCF-7 cells responded to anoikic-autophagic cells by up regulating the AXL receptor tyrosine kinase.

The question may arise whether the elevated mRNA levels observed in engulfing cells could come from the eaten cells. This is very unlikely, since the overlapping up regulated genes in two different types of phagocytes eating the same kind of dead cells do not show systematic “cross-contamination”. In addition, analysis of the gene expression changes in the *de novo* autophagic (Day 4) and anoikic-autophagic (Day 6) cells (the two kinds of dying cells used as targets of phagocytosis) as compared to untreated MCF-7 cells clearly shows that the genes being upregulated in either macrophages or the engulfing MCF-7 cells are not upregulated in the two kinds of dying cells.The exceptions are ASGR1 and IL6 in both kinds of dying cells, and OLR1 in *de novo* autophagic cells. However, other upregulated genes in dying cells (ITGAX, ICAM3, ALOX12) do not have increased levels in the engulfing phagocytes, which again argue against the possible influence of cross-contamination on the results.

Clearance of autophagic and anoikic-autophagic cells evokes pro-inflammatory response in macrophages

Macrophages pre-stimulated for 30 minutes with LPS and then challenged to engulf *de novo* or anoikic-autophagic cells for 1 hour had increased secretion of the pro-inflammatory cytokines IL-1β (only in *de novo* but not anoikic-autophagic cells), IL-6, TNFα and IL-8 and the anti-inflammatory cytokine IL-10. This response could be prevented only by the *de novo* autophagic cells, an exception being IL-1β in which case the *de novo* autophagic cells induced secretion did not differ much from the LPS pre-stimulated one. The IL-1β response was rather rapid at protein secretion and gene expression levels, ocurring mostly in the first few hours of the assay. This response was specifically related to the autophagic death since inhibition of of autophagy with 3-MA blocked it. In addition, studies with supernatants from *de novo* autophagic cells showed that absence of contact between these cells and the macrophages fail to induce IL-1β secretion. Caspase-1 inhibition showing dependency of the pro-IL-1β cleavage, completely blocked *de novo* autophagic cells' induced IL-1β secretion by macrophages.

SUMMARY

Clearance of dying cells is of great importance for maintaining balanced tissue homeostasis in mammalian organisms. Failure to do so may lead to inflammation and autoimmune diseases. Although we know quite a lot about the elimination of apoptotic and necrotic cells, there are still open questions on how cells dying through or with autophagy get eliminated.

MCF-7 cells undergo autophagic death upon tamoxifen treatment; this we consider here death through autophagy or *de novo* autophagy, since death is trigered by autophagy. When plated on non-adhesive substratum these cells die by anoikis while inducing autophagy - what is considered here death with autophagy (cells' death is not caused by autophagy) or anoikic-autophagy to distinguish it from the *de novo* one. In each case autophagy was revealed by monodansylcadaverine staining, elevated LC3II protein levels and electron microscopy examination.

In living cells autophagy takes place usually unnoticed to the neighbours. However, its co-ocurrance with cell death may contribute to the clearance of these dying cells by recruited phagocytes. Both *de novo* and anoikis-derived autophagic dying cells were engulfed by human macrophages and MCF-7 cells. Inhibition of autophagy by 3-methyladenine (3-MA) abolished engulfment of cells dying through *de novo* autophagy, but not those dying through anoikis with autophagy. Blocking exposure of phosphatidylserine on both dying cell types inhibited phagocytosis by MCF-7 but not by macrophages. This means that when autophagy induces cell death it also contributes to the clearance of dying cells.

Gene expression profiling of 95 genes involved in the apopto-phagocytic system by TaqMan Low Density Array (TLDA) showed that while both types of phagocytes expressed full repertoire of the phosphatidylserine recognition and signaling pathway, macrophages could evolve during engulfment of *de novo* autophagic cells the potential of calreticulin-mediated recognition, tethering, tickling and engulfment processes. Different sets of genes (e.g. the phagocytosis receptors for asialoglycoprotein and oxidized LDL, the bridging molecule PTX3, the engulfment molecules GULP1 and RAC1) were upregulated in macrophages engulfing *de novo* autophagic as compared to anoikis-derived autophagic dying cells (e.g. the bridging molecule MFGE8, the engulfment gene RAC1). In MCF-7 cells, *de novo* autophagic cells induced the oxidized LDL receptor and ELMO1 engulfment gene, while anoikic-autophagic ones lead to elevated AXL receptor.

LPS-induced production of pro-inflammatory cytokines in macrophages could be prevented by the dying autophagic cells similarly to anoikic-autophagic and apoptotic cells. However, we also observed that phagocytosis of cells dying through autophagy leads to a pro-inflammatory response in macrophages characterized by the induction and secretion of IL-1β as well as IL-6, TNFα, IL-8 and the anti-inflammatory cytokine IL-10. The IL-1β secretion could be inhibited by preventing autophagy with 3-MA or blocking caspase-1 activation. The results suggest that inside macrophages cells dying through autophagy can activate NLR family protein(s) during the phagocytosis process.

Our data show that cells dying through autophagy and those committing anoikis with autophagy may engage overlapping but distinct sets of clearance mechanisms in professional and non-professional phagocytes. Better understanding of the molecular mechanism of these phenomena may lead to more rational design of autophagy-based therapeutic interventions.

This thesis is built upon the following publications:

Petrovski G., G. Zahuczky, G. Majai, and L. Fésüs. 2007 Phagocytosis of cells dying through autophagy evokes a pro-inflammatory response in macrophages. *Autophagy* (accepted for publication)

IF: 6.708

Petrovski, G., G.Zahuczky, K.Katona, G.Vereb, W.Martinet, Z.Nemes, W.Bursch, and L.Fesus. 2007. Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. *Cell Death. Diffentiation.* 14:1117-1128.

IF: 7.785

Majai, G.*, G.Petrovski*, and L.Fesus. 2006. Inflammation and the apopto-phagocytic system. *Immunology Letters.* 104:94-101. (* contributed equally)

IF: 2.301

Other publications:

Nemes Z., G. Petrovski, and L. Fésüs. 2005. Tools for the detection of protein transglutamination. A*nalytical Biochemistry.* 342:1-10.

IF: 2.670

Nemes Z., E .Csősz, G. Petrovski, and L. Fésüs. 2005. Structure-function relationship of transglutaminases-a contemporary view. *Progress in Experimental Tumor Research* 2005. 38:19-36.

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Posters:

First author of posters on the following meetings:

Petrovski G., Májai G., Zahuczky G., Katona K., Vereb G., Martinet W. and Fésüs L. Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. Induction of pro-inflammatory cytokines during engulfment. Keystone Symposia, Monterey, CA, April, 15-20,2007.

Petrovski G., Májai G., Zahuczky G., Katona K., Vereb G., Martinet W. and Fésüs L. Clearance of dying autophagic cells by macrophages induces a pro-inflammatory response. 1st Workshop on the "The Immune Response Against Dying Tumor Cells" (ECDO), Villejuif, France, December 15-17, 2006.

Petrovski, G., Nemes Z. and L. Fesus. Transglutaminase 2 restores anoikis and enhances phagocytosis of dead cells after proteasome inhibition. *ECDO*, Chania, Crete, Greece, September 17-20, 2004

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Co-author:

Zahuczky G., Májai G., Petrovski G. and Fésüs L.. Gene induction patterns of apopto-phagocytic genes in human macrophages. ECDO, Chia, Sardinia, Italy, September 29-October 4, 2006.

Nemes, Z., Petrovski, G., Devreese, B., Samyn, B., Fésüs L. Integrin signal replacement by transglutaminase 2 in anoikis. 30th FEBS Congress & 9th IUBMB Conference, Budapest, Hungary, July 2-7, 2005.

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