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Use of Primary Macrophages for Searching Novel Immunocorrectors

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Abstract

In this mini-review, the role of macrophage phenotypes in atherogenesis is considered. Recent studies on distribution of M1 and M2 macrophages in different types of atherosclerotic lesions indicate that macrophages exhibit a high degree of plasticity of phenotype in response to various conditions in microenvironment. The effect of the accumulation of cholesterol, a key event in atherogenesis, on the macrophage phenotype is also discussed. The article presents the results of transcriptome analysis of cholesterol-loaded macrophages revealing genes involved in immune response whose expression rate has changed the most. It turned out that the interaction of macrophages with modified LDL leads to higher expression levels of pro-inflammatory marker TNF- α and anti-inflammatory marker CCL18. Phenotypic profile of macrophage activation could

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

be a good target for testing of novel anti-atherogenic immunocorrectors. A number of anti-atherogenic drugs were tested as potential immuno-correctors using primary macrophage-based model.

Keywords

Atherosclerosis; macrophages; phenotype; transcriptome; activation; modified LDL; immunocorrectors; macrophage-based test

INTRODUCTION

The concept of two main types of macrophage activation is the most popular in scientific community [1, 2]. First type (M1), or the classically activated macrophages, is the result of exposure to pro-inflammatory stimuli such as interferon-gamma (IFN-gamma) or lipopolysaccharide (LPS). M1 is characterized by secretion of reactive oxygen species (ROS) and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin (IL) -1, -6, -12, and also, expression of Fc-gamma receptor 1, 2, 3 [3]. The second type (M2), or alternatively activated macrophages can be obtained by exposing the initial cell to anti-inflammatory cytokines such as IL-4, -10, -13, and transforming growth factor beta or other inflammatory mediators such as glucocorticoids [4]. M2 macrophages express anti-inflammatory cytokines: IL-1ra, IL-10, chemokine (C-C motif) ligand 18 (CCL18) and markers such as haptoglobin receptor CD163, mannose receptor (CD206) and stabilin-1 [4–6].

Other phenotypes may be present in an atherosclerotic lesion such as M(Hb) and Mhem [7]. These macrophages show the inability to accumulate lipids. In vitro M(Hb) and Mhem are obtained by exposure of initial cells to haptoglobin-hemoglobin complexes and heme, respectively. M4 population is obtained by adding chemokine (C-X-C motif) ligand 4 (CXCL4) [8].

MACROPHAGE PHENOTYPES IN ATHEROSCLEROTIC LESIONS

Atherosclerotic plaques usually represent all main morphologic types of lesion. Therefore, conditions in the plaque are not homogeneous. Phenotype of macrophages can be modulated by many external factors, cytokines, cholesterol crystals, modified low density lipoprotein (LDL), fatty acids, immune complexes. The presence of pro-inflammatory M1 macrophages in atherosclerotic lesions has been demonstrated several decades ago, but alternatively activated macrophages M2 were found relatively recently [9]. CD68 and mannose receptor (MR) positive M2 macrophages were first found in the peripheral areas of the atherosclerotic lesions, as well as in more stable areas. CD68+ MR+ macrophages were localized in areas with high level of IL-4. CD68+ MR+ macrophages had fewer and smaller lipid drops compared with CD68 + MR-macrophages [10].

Boyle *et al.* have found macrophages expressing high levels of CD163 and low levels of human leukocyte antigen - antigen D related (HLA-DR). These cells were located in the areas of hemorrhagic lesions. This phenotype was called HA-mac [7]. These cells show

largely anti-atherogenic functions involved in suppressing oxidative stress. Functional features of HA-mac are similar to functional features of Mhem macrophages, exhibiting strong gems-dependent phosphorylation and activation of transcription factor 1. Activation of transcription factor 1 initiates a cascade of liver X receptor (LXR) - α / ATP-binding cassette transporter ABCA1 / Apolipoprotein E (ApoE), preventing foam cells formation [11]. M (Hb) macrophages are characterized by expression of surface markers of M2 macrophages: MR and CD163 [12]. Neovascularization was often found in atherosclerotic plaques resulting in infiltration of erythrocytes followed by accumulation of iron in the lesion. CD68+ MR+ macrophages were found in areas with high iron content which indicates a role of these cells in iron metabolism [13]. M4 macrophages have been found in atherosclerotic lesions by measuring the expression of matrix metalloproteinase (MMP) 7 and the calcium-binding protein S100A8 [14]. M4 macrophages express MMP12, MP, as well as some pro-inflammatory cytokines: IL-6 and TNF- α . M4 macrophages do not express the receptor of the hemoglobin-haptoglobin CD163.

J. Laurant Stöger *et al.* studied the localization of M1 and M2 macrophages in various types of lesions. Both macrophage phenotypes were found in atherosclerotic lesions. Both phenotypes were present in higher amount in unstable lesions compared with stable plaques. M1 macrophages predominated in rupture prone plaque shoulder areas. No significant differences between the amount of M1 and M2 cells were observed in the fibrous cap. Lots of M2 macrophages were found in the adventitia. Areas of internal hemorrhages were strongly stained by CD163-antibodies. Remarkable is that, lipid-loaded macrophages expressed markers of both M1 and M2 macrophages [15].

Cho KY *et al.* attempted to identify the relationship between the vulnerability of atherosclerotic lesions and the ratio of M1 and M2 macrophages in the plaques. In this study, subjects were divided into 2 groups: with symptomatic atherosclerosis (patients suffered acute ischemic attack), and asymptomatic. It was found that lesions of symptomatic group had high content of M1 macrophages, whereas asymptomatic lesions of the group had increased amount of M2 macrophages. M1 macrophages were exclusively found only in the lesions of symptomatic patients. Thus, the ratio M1 and M2 macrophages in lesions can be described as a marker of plaque stability [16].

LDL METABOLISM

Native LDLs are recognized by the LDL receptor (LDLR). LDLs are transported to the lysosome by endocytosis where lysosomal acid lipase hydrolyzes cholesterol esters to free cholesterol. The free cholesterol is then transported to the endoplasmic reticulum for esterification by cholesterol acyltransferase (ACAT) [17, 18]. High amount of free cholesterol in the endoplasmic reticulum initiates a signaling cascade that leads to a decrease in the expression of LDLR. Such regulation of LDLR prevents formation of foam cells. ApoB-containing lipoproteins also containing ApoE can cause cholesterol accumulation through interaction of ApoE with ApoE-receptors (such as Low density lipoprotein receptor-related protein 1 (LRP1) and VLDL-receptor) that are not regulated by the amount of intracellular cholesterol. Uptake of native LDL by pinocytosis also promotes the formation of foam cells. Modifications in apoB-containing lipoproteins cause significant accumulation

of cholesterol. Aggregation of LDL leads to their uptake by phagocytosis [19, 20]. Oxidation or glycolysis of LDL particles increases their internalization through a number of receptors that are not regulated by intracellular cholesterol levels: CD36, scavenger receptor A (SRA), lectin-like receptors (LOX) and toll-like receptors (TLR) [21, 22]. Cholesterol esters are accumulated in cytoplasmic lipid droplets where neutral cholesterol esterase hydrolyses cholesterol esters to free cholesterol followed by new esterification by ACAT.

Processes of cholesterol accumulation and efflux may change macrophage phenotype. Accumulation of free cholesterol causes pro-inflammatory activation of macrophages resulting in the endoplasmic reticulum stress [23]. This process also promotes calcium leak into the cytosol [24]. Accumulation of lipid droplets was shown to cause activation of TLR4, whereas cholesterol crystals lead to inflammasome activation [25, 26].

Activation of TLR4 can lead to activation of NF- κ B, ERK, c-Jun N-terminal kinase (JNK) and interferon response followed by different effects. Activation of IL-4 receptor induces activation of the signal transducer and activator of transcription 6 (STAT6), which can inhibit signaling of TLR4 [27]. Intersections between different signaling pathways are likely to change the phenotype of macrophages.

da Silva RF *et al.* have studied the ability of cholesterol-loaded macrophages being activated in pro- and anti-inflammatory directions. Formation of foam cells was initiated by acetylated LDL. Pro-inflammatory stimulated cholesterol-laden macrophages expressed a lower level of inflammation markers as compared to control macrophages. However, there were no differences in anti-inflammatory response of cholesterol-laden macrophages and control cells [28].

Cytoplasmic cholesterol esters are removed by two processes. Firstly, removal of free cholesterol from the cytoplasmic membrane stimulates transport of free cholesterol obtained by the neutral cholesterol esterase from ACAT to plasma membrane [29, 30]. Secondly, cytoplasmic cholesterol esters are packaged into autophagosomes that can fuse with lysosomes where cholesterol esters are hydrolyzed by acid lipase to free cholesterol and transported to the plasma membrane [30, 31]. Efflux of free cholesterol to HDL also includes a number of mechanisms preventing formation of foam cells. Exogenous free lipid ApoA or ApoE-1 produced by macrophages interacts with ABCA1, leading to the stimulation of efflux of free cholesterol and phospholipids. ABCA1 plays a major role in the removal of cytoplasmic cholesterol esters through autophagy. ABCG1, SR-BI, and diffusion are involved in cholesterol efflux [32–35]. ABCA1 and ABCG1 deficiency cause inflammatory activation of macrophages in mice [17, 18, 21, 22]. Conversely, inflammation prevents cholesterol efflux in macrophages [36].

We tried to find genes activated or repressed by the accumulation of cholesterol under the influence of modified LDL. Native or modified LDL were added in primary culture of human macrophages and incubated for 1 day. Then, the transcriptome was analyzed to find out which genes had changed expression levels because of the accumulation of lipids. EdgeR program, part of the statistical analysis system R, was used [37]. One-factor model was selected. The presence or absence of accumulation of lipid by macrophages was the

factor. EnrichR was used to assess the function of the identified genes. EnrichR includes information of the differential expression of genes and their role in biological processes, signaling pathways and diseases [38]. The greatest changes in gene expression were observed in groups of genes involved in immune response reactions (Table 1).

We hypothesized that high changes in expression of genes involved in immune response reactions may be due to the accumulation of intracellular lipids caused by modified LDL. The ability of modified LDL to change the phenotype of macrophages was studied by measurement of expression levels of pro-inflammatory marker TNF- α and anti-inflammatory marker CCL18 in human macrophages. Native LDLs isolated from serum of healthy donors were added in primary culture of macrophages. Atherogenic LDLs were isolated from blood of patients with cardiovascular diseases. Native LDL did not cause intracellular accumulation of cholesterol in macrophages, while atherogenic LDL induced 1.5–2-fold increase in the intracellular cholesterol level. Macrophages were incubated with LDLs for 24 hours. The results of TNF- α and CCL18 expression are presented in Fig. (1). Native non-atherogenic LDL had no significant effect on TNF- α expression level. Native LDL caused expression of CCL18. Atherogenic LDL caused a 2.5-fold increase of expression of pro-inflammatory marker TNF- α , and 1.5-fold increase of expression of anti-inflammatory marker CCL18 as compared with native LDL. Probably, the accumulation of cholesterol in cells can increase the expression of both markers. However, the data does not exactly determine what kind of phenotype of macrophages atherogenic LDL will generate. A result of heterogeneous population of macrophages incubated with atherogenic LDL is possible. Thus, atherogenic LDL causes macrophage activation in proinflammatory and anti-inflammatory directions. This result correlates well with the researches in situ testifying to the expression of proinflammatory and anti-inflammatory markers in atherosclerotic lesions.

SEARCHING FOR NEW IMMUNOCORRECTORS

Phenotypic profile of macrophage activation could be a good target for testing of novel anti-atherogenic immunocorrectors. Primary monocyte-derived macrophage cell model was used to test the abilities of a number of anti-atherogenic drugs to change phenotype of macrophages. Monocytes were isolated from whole blood of healthy donors by density gradient followed by selection of CD14⁺ cells by magnetic separation. Cells were cultured for 7 days with or without interferon-gamma. Vezugen [39], Allicor [40], Cellex [41], Cardiohealth [42] and SkQ1 [43] were added at the concentrations from 10^{-5} to 10^{-2} mkg/ml and the cells were incubated for 24 hours. Table 2 shows the effect of drugs on the relative expression of TNF- α . The control gene expression level (without drugs or interferon-gamma) was taken as 100%. Vezugen, Allicor and Cardio-health had no effect. 10^{-3} mkg/ml of Cellex caused decreasing of TNF- α expression level in naïve unpolarized (M0) macrophages. 10^{-4} mkg/ml of SkQ1 caused decreasing of TNF- α expression level in both M0- and M1-macrophages.

Earlier, we found dramatic individual differences in the ability of circulating human monocytes being activated by pro- and anti-inflammatory stimuli. It was also found that monocytes isolated from the blood of patients with atherosclerosis have a lower susceptibility to pro- and anti-inflammatory stimuli compared to monocytes from healthy

donors [44]. Chronic inflammation accompanying arteriosclerosis may result from increased activation of monocytes (and then macrophages). Depolarization of monocytes and / or macrophages may be used for prevention of atherosclerosis. In future, we are planning to develop new cell-based models directed not only on the macrophage immune response but atherogenicity too. Multiplying of these cell models will allow for finding new anti-atherogenic substances as well as new properties of existing drugs.

CONCLUSION

Probably macrophage phenotypes cannot be divided into specific subsets in atherosclerotic lesions. Phenotypes are likely to be formed by the influence of the microenvironment and activation of specific intracellular signaling pathways in response to external changes. Macrophage phenotype could change rapidly in response to changes in the environment and in the intracellular signaling pathways initiated by interactions with lipids (or various types of modified lipids). Thus, macrophages should be considered as cells exhibiting a wide range of phenotypes and functions.

There is no doubt about the role of innate immunity in atherogenesis. The correlation between proinflammatory activated macrophages and the development of unstable atherosclerotic lesions was observed, whereas anti-inflammatory activated macrophages were associated with reparative features and stable lesions. Transcriptome analysis of cholesterol-loaded macrophages showed that the greatest changes in gene expression were observed in groups of genes involved in immune response reactions.

It was also observed that the accumulation of cholesterol is associated with increased pro- and anti-inflammatory activation of macrophages. The substances reducing macrophage response to the pro- and anti-inflammatory stimuli may find use in the prevention of atherosclerosis. Phenotypic profile of macrophage activation is a good target for testing of novel anti-atherogenic immunocorrectors.

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Native Human Low Density Lipoprotein, Medium Oxidized LDL and Acetylated LDL were obtained from Kalen Biomedical (Germantown, MD, USA 20874).

Appendix 1.: Description of the drugs

Allicor

Manufacturer: Inat-Pharma (Russia).

Composition: 1 Tablet contains 300 mg of garlic powder.

Pharmacological effects: hypocholesterolemic, antiagregatine, fibrinolytic, hypotensive. Reduces cholesterol and triglycerides in the plasma for hyperlipidemia, slows the development of atherosclerosis, promotes the resorption of existing plaques, reduce blood

sugar and blood pressure, inhibits platelet aggregation, normalizes the increased blood clotting, promotes lysis of fresh thrombus.

Indications: atherosclerosis, hypertension, myocardial period, diabetes, migraine, impotence, decreased immunity, pregnancy; prevention of myocardial infarction and stroke; postoperative complications in patients with vascular disease, flu and colds.

Contraindications: Hypersensitivity to the drug.

Side effects: None known.

SkQ1.

Manufacturer: Lomonosov Moscow State University (Russia).

Composition: SkQ1 is dissolved in 50% aqueous propylene glycol. The three most important segments of the molecule SkQ1 are Plastohinol, a powerful natural antioxidant carrying electrons from the chloroplasts of plants; C10, transports SkQ1 molecule in the cell membrane; Triphenylphosphonium, positively charged group delivering the components in the mitochondria.

Pharmacological effects: SkQ1 blocks and reduces the amount of free radicals formed by cells and thus prevents apoptosis induced mitochondrial reactive oxygen species.

Indications: SkQ1 part of the eye drops Vizomitin (Antioxidant, keratoprotektornoe agent for the treatment of early age-related cataract and the syndrome of “dry eye”), as well as part of the MitoVitan serum.

Contraindications: Hypersensitivity to the drug.

Side effects: Allergic reactions.

Vezugen.

Producer: JSC “pharm” (Russia).

Composition: Peptide complex AC-2-(lysine, glutamic acid, aspartic acid). Other ingredients: microcrystalline cellulose, sugar, beet sugar, lactose, starch, Tween-80.

Pharmacological effects: Peptide complex AC-2 has directed tissue-specific effects on the vascular wall. Vezugen promotes normalization of the functional state of vessels, regulates metabolism in the cells of the vascular wall, improves the condition of the vessel walls and normalizes lipid metabolism.

Indications: general and cerebral arteriosclerosis; Hypertension; coronary heart disease; endarteritis; of varicose veins of the lower extremities; systemic and local microcirculation disorders; vascular encephalopathy; hypercholesterolemia; vascular dystonia; psycho-emotional stress; effects of acute stroke; the impact of various factors on the extreme.

Vezugen also used for the prevention of vascular disease in the elderly.

Contraindications: Individual intolerance to the components of dietary supplements, pregnancy, breast-feeding.

Side effects: None known.

Cellex.

Producer: JSC “Pharm-Sintez” (Russia).

Composition in 1 ml: active substance: polypeptides from hog brain of embryos based on 0,9–2,4 mg of total protein (nominal total protein content - 1.65 mg per 1 ml of substance); excipients: 3.75 mg of glycine, 0.1 M disodium hydrogen phosphate solution, 5.85 mg of sodium chloride, 0.005 mg of Polysorbate 80, purified water.

Pharmacological effects: The presence of tissue-specific signaling proteins and polypeptides leads to neuroreparation. The drug activates the secondary neuroprotection by stimulating synaptogenesis processes of autophagy recovery signals. Tissue-specific and systemic restorative effect was found as well as the restoration of the regenerative and reparative potential of the brain cells reducing the number of damaged cells and the severity of perifocal edema in the penumbra, the restoration of microcirculation and perfusion. Recovers and regulates stimulation of different compartments of central nervous system. The therapeutic effect usually develops within 3–5 days after the start of administration.

Indications: Cerebrovascular diseases.

Contraindications: Epilepsy; Manic psychosis; age of 18 years (due to the lack of clinical data).

Side effects: allergic reactions.

CardioHealth.

CardioHealth is lant complex from the leaves of the European Olive, standardized to oleuropein content (4 mg), Potentilla goose and Andrographis paniculate. CardioHealth has antihypertensive and moderate hypoglycemic and hypolipidemic effects.

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Fig. (1).

Effect of LDL on cytokine gene expression. Monocytes were isolated from whole blood of healthy donors by density gradient followed by selection of CD14⁺ cells by magnetic separation. Cells were cultured for 7 days. Native or atherogenic LDL was added at a concentration of 100 $\mu\text{g} / \text{ml}$ and the cells were incubated for 24 hours. RNA was isolated and gene expression was measured by RT-PCR technique. The figures show the relative expression of the genes TNF- α (n=14) and CCL18 (n=7). As 1, the control gene expression (without LDL) was taken. * Significant differences from native LDL (T-Test).

Table 1. Ontology of genes whose activity changes in the accumulation of intracellular cholesterol by human macrophages.

Term	Overlap	Genes
Inflammatory response (GO: 0006954)	40/376	CCL13; CEBPA; CIITA; NOTCH1; NCF1; AK7; IRG1; CRHBP; HRH1; MMP25; CLEC7A; STAB1; SCN9A; ADORA1; SPPI1; C3AR1; BLNK; AOX1; LTA4H; IL6R; APOL3; CCL24; CAMK1D; CCL20; SPHK1; PLA2G4C; SERPINF2; IL18; CYBB; FOS; HCK; TPST1; TLR8; SDC1; TLR7; PRKCQ; SIGLEC1; TLR5; S100A9; S100A8
Cell chemotaxis (GO: 0060326)	23/155	CCL24; CCL13; NRPI1; EDN1; TGFβ2; CXADR; CCL20; HGF; ARHGEF16; PDGFB; PPBP; CORO1A; CXCL16; NR4A1; HRH1; CXCL12; DPYSL3; SPPI1; EPHB1; S100A9; IL6R; BCAR1; S100A8
Taxis (GO: 0042330)	29/263	NRPI1; CCL13; PDGFB; CORO1A; CXCL16; HRH1; DPYSL3; SPPI1; C3AR1; SLIT1; RAC2; EPHB1; IL6R; CMKLR1; CCL24; EDN1; TGFβ2; CXADR; CCL20; HGF; ARHGEF16; PLAUR; PPBP; L1CAM; NR4A1; CXCL12; S100A9; S100A8; BCAR1
Chemotaxis (GO: 0006935)	29/263	NRPI1; CCL13; PDGFB; CORO1A; CXCL16; HRH1; DPYSL3; SPPI1; C3AR1; SLIT1; RAC2; EPHB1; IL6R; CMKLR1; CCL24; EDN1; TGFβ2; CXADR; CCL20; HGF; ARHGEF16; PLAUR; PPBP; L1CAM; NR4A1; CXCL12; S100A9; BCAR1; S100A8
Positive regulation of response to external stimulus (GO: 0032103)	24/201	CCL24; NRPI1; EDN1; CAMK1D; ITGA2; SERPINE1; IL18; PDGFB; IRG1; GPRC5B; NLRP12; CXCL12; ADORA2B; ALOX5AP; RAC2; C3AR1; TLR7; S100A9; IL6R; S100A8; SASH1; TGM2; IDO1; CMKLR1
Sterol metabolic process (GO: 0016125)	17/119	SREBF1; CEBPA; CUBN; CETP; HMGCS1; PRKAG2; APOBR; VLDLR; CYP7B1; EBP; LIPC; C14ORF1; APOC1; CYP11B1; APOE; APOLI1; FDFIT1
Monocarboxylic acid metabolic process (GO: 0032787)	42/473	SLC22A4; ACADVL; NCF1; HPGD; PRKAG2; ACSM4; ENO2; HIF1A; IRG1; CYP26B1; LDHA; LIPC; AMDHD1; THEM5; CYP11B1; LTA4H; PTGDS; ACS1; ABCD1; PDK1; HSD3B7; EDN1; ACSL1; SPHK1; AKR1C1; PLA2G4C; ACSL6; ACSL4; IGFI; ELOVL7; CYP7B1; KMO; BCAN; POR; PC; P4HA1; ACOX2; SCD; ALDH1A2; ALOX5AP; ACSBG1; IDO1
Positive regulation of monooxygenase activity (GO: 0032770)	6/221	POR; GCHI; NPR3; CALM3; APOE; HIF1A
Regulation of response to wounding (GO: 1903034)	32/347	SERPINE1; PDGFB; METRNL; IRG1; ZFP36; C8G; UBASH3B; ADORA1; SPPI1; CD36; APOE; TGM2; CCL24; EDN1; ITGA2; SERPINF2; IL18; PLAUR; NR1H3; KLF4; HCK; GPRC5B; NLRP12; ADORA2B; CD109; ALOX5AP; TLR7; PRKCQ; S100A9; S100A8; IDO1; NFE2L2
Positive regulation of cell migration (GO: 0030335)	27/280	NRPI1; NOTCH1; SERPINE1; PDGFB; HIF1A; CORO1A; AQP1; CXCL16; P2RY6; C3AR1; RAC2; IL6R; SASH1; CMKLR1; CCL24; EDN1; TGFβ2; CAMK1D; CCL20; SPHK1; HGF; ITGA2; IGFI; CXCL12; SNAIL; ROR2; BCAR1
Positive regulation of cell motility (GO: 2000147)	27/287	NRPI1; NOTCH1; SERPINE1; PDGFB; HIF1A; CORO1A; AQP1; CXCL16; P2RY6; C3AR1; RAC2; IL6R; SASH1; CMKLR1; CCL24; EDN1; TGFβ2; CAMK1D; CCL20; SPHK1; HGF; ITGA2; IGFI; CXCL12; SNAIL; ROR2; BCAR1

Table 2.

Effect of drugs Vezugen, Allikor, Tsellex, Cardiohealth and SkQ-1 on the expression of TNF- α by human macrophages. The drugs were added in culture of M0- or M1-macrophages for 24 hours at the concentrations from 10^{-5} to 10^{-2} mg/ml. As 100%, the TNF- α gene expression by M0-macrophages was taken. Dashes indicate cytotoxicity.

Drug concentration in cell culture (mg/ml)		0 (Control)	10^{-5}	10^{-4}	10^{-3}	10^{-2}
		Relative expression of TNF- α , %				
Vezugen n=2	M0	100	94 $\pm$ 2	87 $\pm$ 4	91 $\pm$ 3	92 $\pm$ 2
	M1	180 $\pm$ 14	176 $\pm$ 18	177 $\pm$ 2	175 $\pm$ 17	170 $\pm$ 2
Allikor n=2	M0	100	91 $\pm$ 1	83 $\pm$ 2	92 $\pm$ 1	90 $\pm$ 1
	M1	177 $\pm$ 16	179 $\pm$ 28	171 $\pm$ 20	173 $\pm$ 21	175 $\pm$ 17
Cellex n=3	M0	100	95 $\pm$ 6	88 $\pm$ 6	73 $\pm$ 7 *	-
	M1	180 $\pm$ 9 (16)	187 $\pm$ 21	184 $\pm$ 16	139 $\pm$ 27	-
Cardiohealth n=3	M0	100	92 $\pm$ 4	87 $\pm$ 6	93 $\pm$ 7	93 $\pm$ 1
	M1	188 $\pm$ 23	172 $\pm$ 14	179 $\pm$ 2	174 $\pm$ 10	186 $\pm$ 9
SkQ1 n=3	M0	100	77 $\pm$ 9	26 $\pm$ 6 *	-	-
	M1	201 $\pm$ 10	168 $\pm$ 2	30 $\pm$ 1 *	-	-

* Significant differences from the control (T-Test).