Phagocytosis of Apoptotic Cells by Macrophages Is Impaired in Atherosclerosis

Dorien M. Schrijvers, Guido R.Y. De Meyer, Mark M. Kockx, Arnold G. Herman, Wim Martinet

Objective—Apoptotic cell death has been demonstrated in advanced human atherosclerotic plaques. Apoptotic cells (ACs) should be rapidly removed by macrophages, otherwise secondary necrosis occurs, which in turn elicits inflammatory responses and plaque progression. Therefore, we investigated the efficiency of phagocytosis of ACs by macrophages in atherosclerosis.

Methods and Results—Human endarterectomy specimens and human tonsils were costained for CD68 (macrophages) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) (apoptosis). Free and phagocytized ACs were counted in both tissues. The ratio of free versus phagocytized AC was 19-times higher in human atherosclerotic plaques as compared with human tonsils, indicating a severe defect in clearance of AC. Impaired phagocytosis of AC was also detected in plaques from cholesterol-fed rabbits and did not further change with plaque progression. In vitro experiments with J774 or peritoneal mouse macrophages showed that several factors caused impaired phagocytosis of AC including cytoplasmic overload of macrophages with indigestible material (beads), free radical attack, and competitive inhibition among oxidized red blood cells, oxidized low-density lipoprotein and ACs for the same receptor(s) on the macrophage.

Conclusion—Our data demonstrate that phagocytosis of ACs is impaired in atherosclerotic plaques, which is at least partly attributed to oxidative stress and cytoplasmic saturation with indigestible material. (Arterioscler Thromb Vasc Biol. 2005;25:1256-1261.)

Key Words: atherosclerosis • apoptosis • macrophages • oxidative stress • phagocytosis

Apoptotic cell death is an important pathophysiological mechanism in many diseases. With regard to the cardiovascular system, an increased apoptosis rate occurs in advanced human atherosclerotic plaques.1–3 All cell types present in atherosclerotic plaques, including endothelial cells, smooth muscle cells (SMCs), lymphocytes, and macrophages, are known to undergo apoptosis. Despite many efforts in determining the potential mechanisms of apoptosis, the significance of apoptotic cell death in atherosclerosis remains unclear. SMC apoptosis can be harmful because it may weaken the fibrous cap of the plaque. In contrast, apoptosis of macrophages and T lymphocytes may be beneficial because removal of these cells from the plaque could attenuate the inflammatory response and decrease the synthesis of matrix metalloproteinases. However, loss of macrophages also decreases the uptake of apoptotic bodies. Inefficient removal of apoptotic cells (ACs) by macrophages may contribute to the formation of a necrotic core through induction of postapoptotic necrosis and accumulation of necrotic debris. Moreover, cells undergoing secondary necrosis release potentially toxic components from the cytoplasm which may trigger inflammatory responses.4,5 Macrophages also secrete pro-inflammatory mediators by the ingestion of ACs undergoing secondary necrosis, but not by intact ACs.6 Apart from SMC loss, decreased synthesis of collagen and plaque destabilization, apoptosis is also an important determinant of atherothrombosis. ACs rapidly expose phosphatidylserine on their surface, which is a potent substrate for the generation of thrombin and activation of the coagulant cascade.7 Furthermore, phosphatidylserine containing membrane microparticles with significant tissue factor activity are shed from AC, which may lead to an increased risk of thrombosis.8 Free AC may therefore contribute to large thrombi after plaque rupture, which in turn could result in vascular occlusion, acute ischemic events, and infarction.

In view of the aforementioned findings, we questioned whether cells undergoing apoptosis are efficiently removed in human atherosclerosis and, if not, which factors contribute to impaired phagocytosis of ACs.

Materials and Methods

Tissue Specimens

The present study has been approved by the Review Board of the University of Antwerp. Human carotid endarterectomy specimens...
(n = 10) were obtained from patients (71 ± 3 years, 70% men) with a carotid stenosis of >70%. According to the adapted American Heart Association classification scheme, thin fibrous cap atheroma alternated with fibrous cap atheroma and intimal xanthoma in the same specimen. Human noninflamed hyperplastic tonsils (n = 8) were obtained from patients (28 ± 9 years, 50% men) undergoing tonsillectomies. Male New Zealand White rabbits (2.8 to 3.5 kg) were fed a diet supplemented with 0.2% cholesterol for 20 to 40 weeks to induce plaques in the aorta. Specimens were fixed in 4% formalin and paraffin-embedded. In the germinal centers of tonsils, self-reactive or low-affinity lymphocytes are eliminated by apoptosis and rapidly removed by neighboring phagocytes. The striking starry-sky pattern in the follicles reflected phagocytosis of apoptotic bodies. Uptake of ACs was determined by counting the number of free and ingested ACs in an entire section of plaques and tonsils. ACs were considered free when they were not surrounded by macrophage cytoplasm. Because binding of ACs to macrophages does not automatically result in uptake, bound cells were always considered not ingested. All counts were performed by 2 independent observers, including a trained pathologist.

**Immunohistochemistry and TUNEL**

Immunohistochemical analysis of macrophages was conducted by an indirect peroxidase antibody conjugate method using anti-CD68 monoclonal antibody (human plaques, clone PG-M1; DAKO) or anti-rabbit macrophage monoclonal antibody (rabbit plaques, clone RAM11; DAKO) combined with an anti-cleaved caspase-3 polyclonal antibody (clone 67341A; Pharmingen). For the detection of oligonucleosomal DNA cleavage, a stringent TUNEL (terminal deoxynucleotidyl transferase end labeling) technique was used.

**Low-Density Lipoprotein Isolation, Aggregation, and Oxidation**

Human blood samples from fasting normolipidemic healthy volunteers were centrifuged (1360g, 10 minutes) and plasma was adjusted to a density of 1.24 g/mL with potassium bromide (381.6 mg/mL). A gradient was formed by layering the density-adjusted plasma underneath phosphate-buffered saline (PBS). Plasma lipoproteins were separated by ultracentrifugation. Low-density lipoprotein (LDL) was isolated and dialyzed against EDTA-containing PBS to remove remaining KBr. Oxidation of LDL (200 µg/mL) was performed in PBS by using CuCl2 (10 µmol/L, 16 hours, 37°C). The reaction was terminated by adding 200 µmol/L EDTA and kept on ice for 1 hour. Finally, the oxidized LDL solution was dialyzed for 24 hours against EDTA-containing PBS to remove Cu2+ ions. Aggregated LDL (agLDL) was prepared by vortexing LDL for 4 minutes. The purity and oxidation of LDL were checked on agarose gel (Hydragel Lipo-gradient) and oxidation of LDL were checked on agarose gel (Hydragel Lipo). The murine macrophage cell line J774A.1 and the human monocyte cell line U937 (American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 U/mL polymyxin B, and 50 µg/mL gentamicin. Mouse peritoneal macrophages were isolated from fasting adult female Swiss CD1 mice by peritoneal lavage with PBS containing 10 U/mL heparin 3 days after the injection of thioglycolate (100 mg; Sigma). Cells were suspended in RPMI 1640 supplemented with 10% fetal bovine serum and incubated at 37°C for 3 hours. Thereafter, nonadherent cells were washed away. After overnight incubation, RPMI 1640 medium was replaced with DMEM supplemented with antibiotics to perform the phagocytosis assay.

To generate ACs, U937 monocytes were incubated with etoposide (50 µmol/L, 4 hours), resulting in 77 ± 1% annexin V-positive cells and complete cleavage of caspase-3 but without propidium iodide incorporation. These ACs were washed to remove nonincorporated etoposide and labeled with the fluorescent marker 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA, 10 µmol/L; Molecular Probes, Eugene, Ore) according to the manufacturer’s instructions.

Erythrocytes (red blood cells [RBCs]) from fresh human blood were washed twice with PBS and resuspended at 20% hematocrit in PBS containing 0.1% glucose. This preparation was stored at 4°C and used as control RBC. Oxidized RBCs (4% hematocrit in PBS) were produced for 90 minutes at 37°C with a mixture of 0.2 mmol/L CuSO4 and 5 mmol/L ascorbate. Subsequently, the oxidized RBCs were washed twice with PBS containing 0.2% EDTA and twice with PBS. Oxidized RBCs (hematocrit 1%) were incubated with macrophages for 1 hour during exposure to ACs.

Phagocytosis of AC by macrophages was studied by flow cytometry. To distinguish binding of apoptotic cells from uptake, macrophages were washed 3 times with cold PBS and briefly treated with a solution of 0.05% trypsin and 0.02% EDTA. SIN1A (lissodamine HCl, 200 µmol/L) was used to investigate the effect of oxidative stress on phagocytosis.

Washed human platelets were incubated with macrophages (2 × 10^8 platelets/10^6 macrophages) for 18 hours. Alternatively, macrophages were incubated with fluorescent beads (1.0 µm carboxylate-modified yellow–green fluorospheres; Molecular Probes; 50 beads/cell) for 4 hours or 24 hours.

Viability of macrophages was evaluated using neutral red assays.

In some experiments, macrophages were incubated with 250 µg/mL oxidized LDL or 50 to 200 µg/mL agLDL. To assess foam cell formation, cells were fixed with paraformaldehyde (1%, 2 minutes), followed by incubation for 90 minutes at 37°C in air at room temperature, and stained with oil red O. Cell death was determined by flow cytometry using an annexin V–fluorescein isothiocyanate and propidium iodide staining kit (BD Biosciences). All data were analyzed using Cell Quest Pro software (BD Biosciences).

**Confocal Microscopy**

Macrophages were fluorescently labeled (red) and coincubated with fluorescently labeled blood platelets (green) or beads for 20 hours or 4 to 24 hours, respectively. Thereafter, cells were washed 3 times with cold PBS and briefly treated with a solution of 0.05% trypsin and 0.02% EDTA. Finally, coverslips were formalin-fixed and mounted on a microscope slide. Dual-channel images were taken with a confocal laser scanning microscope (LSM510; Zeiss). Individual macrophages were isolated from Z stacks with the extract region feature and further analyzed using the ortho and gallery displays of the LSM510 imaging software.

**Statistical Analysis**

Results are expressed as mean ± SEM. In all experiments, n represents the number of experiments, each of them performed at least in duplicate. All analyses were performed using SPSS software. To compare the percentage of free versus phagocytized ACs the percentage of macrophages engulfing one or more ACs in tonsils versus atherosclerotic plaques, the percentage phagocytosis after preload with beads and the percentage of TUNEL-positive cells in both tissues, the Mann–Whitney test was used. Phagocytosis (mean fluorescence of macrophages after engulfment of labeled particles) under different atherosclerosis related conditions was evaluated with one-way analysis of variance, followed by the Dunnett test. If variances were unequal, as assessed with the Levene’s test, data were logarithmically transformed.

**Results**

ACs were counted in carotid endarterectomy specimens (Figure 1A) and data were compared with human hyperplastic tonsils (Figure 1B), in which a high turnover of B lymphocytes and consequently a high apoptosis rate occur. The frequency of apoptosis in atherosclerotic plaques versus tonsils was similar (4 ± 2% and 3 ± 2%, respectively), but the number of macrophages was much higher in atherosclerotic...
plaques (Figure 1C) as compared with the germinal centers of tonsils (Figure 1D). TUNEL-positive ACs were present in the entire plaque, especially in regions around the necrotic core. The ratio of free AC versus phagocytized AC was 19-times higher in atherosclerotic plaques, indicating a severe impairment in uptake of ACs (Figure 1G). In plaques, only 26% of the AC was phagocytized by macrophages versus 87% in tonsils. Moreover, macrophages in tonsils were able to engulf more than one apoptotic cell, whereas in atherosclerotic plaques the majority of phagocytic macrophages engulfed only one apoptotic cell (Figure 1H). Other in situ markers for apoptosis such as caspase activation were not suitable for evaluating efficiency of phagocytosis. Tonsils immunostained for cleaved caspase-3 showed a large number of AC outside of macrophages, indicating that caspase cleavage can occur even before dying cells are internalized by macrophages (Figure 1E and 1F), as previously described.11

Similar to human plaques, phagocytosis of apoptotic cells was significantly impaired in rabbit plaques. Moreover, impaired phagocytosis remained constant in time (73±4% [20 weeks], 74±9% [26 weeks], 76±4% [32 weeks], and 77±3% [40 weeks])free apoptotic cells; Figure IA and IB, available online at http://atvb.ahajournals.org). Confocal microscopy indicated that blood platelets, once phagocytized, were rapidly degraded by macrophages (not shown). Moreover, preincubation of macrophages with ACs for 20 hours before a second load of ACs did not affect phagocytosis of the latter (Figure IV). In contrast, preloading macrophages for 4 or 24 hours with indigestible beads led to accumulation of inert material (Figure III, available online at http://atvb.ahajournals.org) and induced a marked decrease in phagocytosis of AC (Figure 2). To investigate whether an upload of macrophages with beads affects uptake of extracellular material different from ACs, macrophages were preloaded with beads for 4 hours and subsequently incubated with a second set of beads or heat-killed Escherichia coli. Uptake of beads or E. coli was not affected by preloading macrophages with beads (Figure 2).

Figure 1. Phagocytosis efficiency of ACs in human tonsils and advanced human atherosclerotic plaques. A and B, Immunohistochemical detection of CD68 (macrophages, blue) combined with TUNEL (AC, red) in atherosclerotic plaques (A) and tonsils (B). Arrows in (B) show sequestration of ACs in macrophages. Despite presence of many CD68-positive macrophages in (A), most ACs in the plaque remain unremoved (arrows). C and D, Immunohistochemical staining for CD68 (macrophages, blue) in human plaques (C) and tonsils (D); nc indicates necrotic core; gc, germinal center. E and F, Immunohistochemical detection of cleaved caspase-3 (AC, red) and CD68 (macrophages, blue) in atherosclerotic plaques (E) and tonsils (F). Arrows show cleaved caspase-3 positive AC either free or sequestered in macrophages. G, Quantification of free and phagocytized ACs in tonsils (open bars) and atherosclerotic plaques (solid bars) after immunostaining of both tissues for CD68 combined with TUNEL. H, Quantification of macrophages engulfing one or more AC in tonsils compared with atherosclerotic plaques. *P<0.05; ***P<0.001 vs tonsil. Bar=10 μm (A,B,E,F) or 200 μm (C,D).

Figure 2. Phagocytosis of ACs, beads, or E. coli after preincubation of J774A.1 macrophages with indigestible beads for 4 hours. ***P<0.001 vs control macrophages incubated without beads (n=3).
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In addition to cytoplasmic overload of macrophages, the influence of oxidative stress on phagocytosis was analyzed. Treatment of macrophages with the peroxynitrite donor SIN1A (200 μmol/L) resulted in a marked decrease in phagocytosis of ACs, whereas uptake of beads or E. coli was not affected (Figure 3). Treatment of ACs or E. coli with SIN1A also inhibited their phagocytosis, whereas uptake of SIN1A treated beads remained unaltered. Furthermore, when oxidized LDL (250 μg/mL) or oxidized RBCs (hematocrit 1%) were added to macrophages in combination with ACs, phagocytosis of ACs was decreased (Figure 4). To exclude the possibility that this impairment in phagocytosis results from toxic effects of oxidized lipids, neutral red viability assays were performed. Only prolonged exposures to RBCs, oxidized RBCs (20 hours), or oxidized LDL (up to 5 hours) induced cell death of macrophages (69±1%, 53±5% and 76±1%, respectively).

Discussion

Rapid recognition and clearance of ACs by phagocytes is essential for normal development and prevention of inflammation and disease.18,19 In patients with systemic lupus erythematosus,20 chronic obstructive pulmonary disease,21 or cystic fibrosis,22 phagocytosis of ACs is decreased. In systemic lupus erythematosus, impairment of serum complement activation and reduced expression of CD44 on apoptotic polymorphonuclear neutrophils seem to render reduced phagocytosis of apoptotic polymorphonuclear neutrophils. Alternatively, antiphospholipid antibodies, present in 20% to 40% of patients with systemic lupus erythematosus, may opsonize ACs so that macrophages incite inflammation or autoimmunity.23 Defective clearance of ACs in cystic fibrosis is caused by elastase-mediated cleavage of the phosphatidylserine receptor and contributes to ongoing airway inflammation. In the present study, we demonstrate that also in advanced human and experimental atherosclerotic plaques, phagocytosis of AC is inefficient. Despite similar apoptosis frequencies, the ratio of free versus phagocytized AC was 19-times higher in human atherosclerotic plaques compared with tonsils. Moreover, in tonsils macrophages can engulf several apoptotic bodies at the same time to ensure effective and swift clearance, whereas in atherosclerotic plaques, the majority of phagocytic macrophages engulfed one single apoptotic cell. Examination of rabbit plaques not only confirmed our findings in human plaques but also showed that during plaque progression impaired clearance of apoptotic cells did not further change. Although impaired phagocytosis has been proposed as a mechanism that contributes to necrotic core formation and persistent inflammation in atherosclerosis,24,25 quantitative evidence did not exist so far.

It is important to note that apoptotic bodies from different cell types may expose different ligands to act as "eat-me" signals, so that we cannot rule out the possibility that recognition of apoptotic lymphocytes (the predominant cell type undergoing apoptosis in tonsil) may occur differently as compared with recognition of apoptotic bodies (macrophages and SMCs) in human plaques.26 However, in both tissues apoptotic cells are found in the vicinity of macrophages, which rules out a possible geographic problem. Moreover, atherosclerotic plaques are highly inflamed tissue and contain large areas of macrophage infiltration, whereas in tonsils usually no signs of inflammation are detected. Therefore, it is not the quantity of macrophages that is important for phagocytosis, but their functionality. From a technical point of view, comparison of very early lesions (adaptive intimal thickening and fatty streaks) with advanced lesions was not feasible because cell death is hardly detectable in early lesions. Only advanced human and experimental plaques are characterized by high levels of apoptosis, mainly in regions with macrophage infiltration.3,10 Importantly, the TUNEL technique was used to define AC in all tissues. Other techniques such as staining for cleaved caspase-3 are not recommendable, because caspase cleavage can occur even before dying cells are internalized by macrophages.11

According to our in vitro data, several factors may cause impaired phagocytosis of AC in atherosclerotic plaques including cytoplasmic overload of macrophages, oxidative stress, and competitive inhibition of oxidized RBCs, oxidized LDL, and ACs for the same receptor(s) on the macrophage. Foam cell formation as a result of lipid uptake or platelet phagocytosis is a common feature of advanced atherosclerotic plaques. The cytoplasm of macrophage-derived foam
cells is often crammed with large lipid droplets. Therefore, it is tempting to speculate that these macrophages are no longer able to engulf AC (“full is full”). To investigate this hypothesis, macrophages were incubated with an excess of platelets or aggregated LDL.16 Macrophages in vitro, however, rarely reach the same degree of foam cell formation as seen in human plaques, caused by rapid digestion of phagocytosed material so that phagocytosis of platelets or aggregated LDL by macrophages did not induce cytoplasmic overload or a decline in AC uptake. As an alternative, beads were added to macrophages, which resulted in the accumulation of indigestible material in the cytoplasm and a nearly complete inhibition of phagocytosis of AC. Möller et al27 reported that uptake of indigestible, rigid particles by macrophages induces cellular “stiffening” so that changes in cellular shape, which are needed to form pseudopodia for phagocytosis are inhibited. It is important to note that cells have a variety of mechanisms to internalize particles and solutes, including pinocytosis (uptake of fluid), receptor-mediated endocytosis (particles <0.5 μm), and phagocytosis (particles >0.5 μm). Pinocytosis and receptor-mediated endocytosis share a clathrin-based mechanism and occur independently of actin polymerization. In contrast, phagocytosis occurs by an actin-dependent mechanism and independent of clathrin.28 Preloading macrophages with beads inhibited phagocytosis of AC but did not affect uptake of E. coli (receptor-mediated endocytosis) or a second set of beads (possibly through emperiploise14).

In addition to foam cell formation, increased oxidative stress is a hallmark of advanced atherosclerotic plaques.29,30 Although several reactive oxygen species can be found in human plaques, peroxynitrite (ONOO−) plays a central role in the pathophysiology of atherosclerosis because it induces protein nitrosylation and oxidative DNA damage. Here we demonstrate that SIN1A inhibits uptake of ACs by macrophages. In vitro, SIN1A is a spontaneous donor of NO− and O2−, which quickly combine to form ONOO−. Treatment of macrophages with SIN1A before phagocytosis significantly inhibited uptake of ACs, whereas uptake of E. coli or beads was unaltered. However, SIN1A treatment of ACs or E. coli but not beads decreased their uptake by macrophages, suggesting that oxidative stress could modify cellular structures so that their uptake is diminished.31 Alternatively, phosphatidylserine or other factors present on macrophages or AC required for phagocytosis of AC may be sensitive to oxidative treatment.

Infiltration of LDL and accumulation of RBCs after intraplaque hemorrhage followed by their oxidation into oxidized LDL and oxidized RBCs, respectively, are considered major events during the progression of atherosclerosis.32,33 In the present study, we demonstrate that both oxidized LDL and oxidized RBCs significantly inhibited phagocytosis of ACs by macrophages. Recently, autoantibodies to oxidation-specific epitopes of oxidized LDL have been isolated that bind to intact oxidized LDL, as well as to its lipid and protein moieties but not to those of native LDL. These antibodies also recognize phosphatidylcholine moieties on ACs and inhibit their phagocytosis, suggesting that common epitopes exist on AC and oxidized LDL.34 However, binding and phagocytosis of oxidized RBCs are inhibited by the addition of oxidized LDL.35 All these findings indicate that oxidized LDL, oxidized RBCs, and ACs compete for the same receptor on macrophages. Furthermore, uptake of oxidized LDL in the presence of ACs promotes inflammation through release of IL-6 and MCP-1,26 so that a persisting state of inflammation may occur at sites where oxidized LDL, macrophages, and AC coexist. Miller et al37 showed that macrophages exposed to minimally modified LDL become less efficient phagocytes but acquire a higher rate of oxidized LDL uptake. Considering phagocytosis of ACs as an anti-inflammatory process and extensive uptake of oxidized LDL as a pro-atherogenic event, one could predict a net pro-atherogenic role of minimally modified LDL on macrophages. Because minimally modified LDL binds to CD14,37 which is a prerequisite for phagocytosis of apoptotic lymphocytes,38 it is conceivable that CD14 depletion causes impaired phagocytosis in atherosclerotic lesions. However, each of the individual receptors mediating uptake of AC by macrophages accounts for only 30% to 50% of AC uptake, suggesting cooperation between various receptors and ligands on AC.

In conclusion, we have shown that phagocytosis of ACs is severely impaired in advanced atherosclerotic plaques. Reduced uptake of AC may lead to the accumulation of proinflammatory necrotic debris (such as oxidized lipids), plaque instability, and thrombogenesis.39 Several factors present in atherosclerotic plaques, such as accumulation of indigestible material in the macrophage cytoplasm, oxidative stress, and presence of oxidized LDL or oxidized RBCs may contribute to the impairment of phagocytosis. Because atherosclerotic plaques are very complex, we do not exclude that other factors are involved. In this regard, it has been recently proposed that lipid-loaded macrophages are defective in phagocytosis caused by cholesterol-induced changes in membrane structure and function or caused by changes in phagocytosis-related signaling pathways.40

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Figure 1 Phagocytosis efficiency of AC in atherosclerotic plaques of cholesterol-fed (0.3%) rabbits at 20, 26, 32 and 40 weeks. Histogram shows quantification of free (solid bars) and phagocytized (open bars) AC after immunostaining of tissues for RAM-11 combined with TUNEL. *** p<0.001 versus free apoptotic cells
Figure II  Oil red O staining of J774 macrophages after platelet phagocytosis or uptake of agLDL. (A) control macrophages (B) macrophages incubated with platelets for 20 h (C) macrophages incubated with agLDL for 20 h. Bar=20 µm
Figure III Confocal laser scanning microscopy of J774 macrophages (red) after incubation with beads (yellow) for 4 h (A) or 24 h (B).
Figure IV Flow cytometric analysis of phagocytosis of AC by J774 macrophages after platelet uptake (A), preload with 200 µg/ml agLDL (B) or uptake of AC (C) for 20 h. Graphs show control macrophages (black histogram), macrophages incubated with AC for 1 h (open histogram) and macrophages pre-treated with platelets (PLT), agLDL or AC (grey histogram). Inserts show the percentage phagocytosis of labeled AC that are engulfed by macrophages. Data were analyzed using a Mann-Whitney test (n=3).