Early cell interactions with magnesium-based materials

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Abstract

Magnesium (Mg) and its alloys are biodegradable materials with known advantageous mechanical and physical properties for orthopaedic applications. The influence of degrading products (extracts) on cell activity is the next step to be considered for the acceptance of magnesium-based materials in medical applications. After implantation of a biomaterial, the cells of the immune system promote an inflammatory reaction in the tissue that surrounds the implant material. Macrophages are the principal cell players, and their capacity to release cytokines defines the cross-talk between different cell types and finally the acceptance of the biomaterials by the body. During the biodegradation process, the materials release high concentrations of Mg. This element, in its ion form (Mg^{2+}), strongly influences the cells' activities but its specific role in cytokine release from macrophages is yet unclear.

The central point of this work is to explore the response of macrophages to degradation products of Mg-based material. Besides, the specific effect of Mg^{2+} on macrophage activity was investigated by stimulating the cells with Mg salt in the form of $MgCl_2$.

Macrophages were exposed to the degradation products of pure Mg, Mg-10Gd and Mg-2Ag, both alone and in co-culture with osteoblasts. For the macrophage monoculture, an inflammatory *in vitro* model was established exposing the cells to a temperature of 39°C. This model was also used for the evaluation of macrophage behaviour in different Mg^{2+} concentrations. The cells' response to the extracts and $MgCl_2$ were explored by analysing the key cytokines involved in the key steps of the inflammatory reaction.

Macrophages in this study are derived from circulating monocytes in blood. After differentiation into macrophages, two different subpopulations can be distinguished. These cell populations are broadly classified as M1 and M2 macrophages.

The specific influences of the extracts on macrophage differentiation and M1/M2 formation were investigated as well. Freshly isolated monocytes were exposed to the extracts during and after differentiation and the formation of M1/M2 populations. The cells were analysed for surface marker protein expression. Results obtained in the inflammatory *in vitro* model show that macrophages cultured with and without material extracts preserve the cells' natural behaviour in aseptic inflammatory conditions. Such a response was also evident in the co-culture. When in contact with osteoblasts, macrophages down-regulate cytokines involved in the amplification of the inflammatory reaction. The extracts tested promoted this, inducing the release of target molecules involved in bone remodelling. It could be shown in both, mono and co-culture conditions that the total extract compositions of Mg-10Gd and Mg-2Ag influenced cytokine release via different pathways.

The results obtained with fresh monocytes indicate that Mg-2Ag extracts play a role in M2 macrophage polarization. Experiments performed with different concentrations of $MgCl_2$ showed that Mg^{2+} influences the release of cytokines in a time-dependent manner.

The work presented here demonstrates the bioactivity property of Mg-based materials at molecular level and that such influence prevents chronic inflammatory events after implantation of this kind of material in the body.

Zusammenfassung

Magnesium (Mg) und Magnesiumlegierungen sind biologisch resorbierbare Materialien mit äußerst vorteilhaften mechanischen und physikalischen Eigenschaften für orthopädische Anwendungen. Der nächste Schritt für die Akzeptanz von Magnesiumwerkstoffen in der Medizin ist die Untersuchung des Einflusses der Abbauprodukte (Extrakte) auf Zellaktivitäten in dem umgebenden Gewebe.

Nach der Implantation des Biomaterials reagiert das umgebende Gewebe gewöhnlich mit einer Entzündung auf das eingebrachte Material. Die Zellen des Immunsystems, allen voran die Makrophagen, setzten dabei Zytokinen frei und triggern so die Entzündungsreaktion. Diese Zytokinfreisetzung definiert die Wechselwirkung zwischen verschiedenen Zelltypen und damit sind die Makrophagen vorrangig verantwortlich für die finale Akzeptanz der Biomaterialien im Körper. Während des Abbauprozesses der Materialien wird eine hohe Konzentration von Mg freigesetzt. Mg^{2+} beeinflusst stark die Zellaktivität, wobei seine spezifische Rolle bei der Freisetzung von Zytokinen bisher unklar ist.

Zentraler Punkt dieser Arbeit ist es daher, die Reaktion von Makrophagen auf die Abbauprodukte von Mg-Materialien zu erforschen. Zusätzlich wurde die spezifische Rolle von Mg^{2+} auf die Aktivität von Makrophagen untersucht. Hierfür wurden Zellen mit Mg-Salz in Form von $MgCl_2$ stimuliert. Makrophagen wurden den Abbauprodukten von Mg, Mg-10Gd und Mg-2Ag in einer Reinkultur oder in einer Co-Kultur mit Osteoblasten ausgesetzt und analysiert. Für die Makrophagen in Reinkultur wurde ein in vitro Entzündungsmodell etabliert, beidem die Zellen einer Temperatur von 39°C ausgesetzt wurden. Das Modell wurde außerdem für die Untersuchung der Makrophagen reaktion auf verschiedene Mg^{2+} Konzentrationen verwendet. Die Zellereaktionen auf die Extrakte und $MgCl_2$ wurde erforscht, indem wichtige Zytokine, die an der Verstärkung-bzw der Herunterregulation der Entzündungsreaktion und an der Zellrekrutierung beteiligt sind, analysiert wurden. Die Makrophagen in dieser Arbeit wurden aus im Blut zirkulierenden Monozyten gewonnen. Nach der Differenzierung zu Makrophagen, können zwei verschiedene Zellsubpopulationen entstehen. Diese werden allgemein als M1 und M2 bezeichnet. Der spezifische Einfluss der Extrakte auf die Makrophagen-Differenzierung und die Bildung von M1/M2 Subpopulationen wurde ebenfalls untersucht. Frisch isolierte Monozyten wurden während und nach der Differenzierung und der Bildung von M1/M2 -Subpopulationen verschiedenen Extrakten ausgesetzt. Ihr Einfluss auf die Zellen wurde anhand der Expression von Oberflächenmarker proteinen untersucht. Es konnte anhand des Entzündungs in vitro Modells gezeigt werden, dass Makrophagen, die mit und ohne Material-Extrakten kultiviert wurden, ihr natürliches Zellverhalten des aseptischen Entzündungszustandes beibehalten. Dieses Verhalten zeigt sich auch in Co-Kultur. In Kontakt mit Osteoblasten, regulieren Makrophagen die an der Verstärkung der Entzündungsreaktion beteiligten Zytokine herunter. Die getesteten Extrakte unterstützen dies, indem sie die Freisetzung von Targetmolekülen, die am Knochenumbau beteiligt sind, induzieren. Sowohl in der Mono-Kultur als auch in der Co-Kulturkonnte gezeigt werden, dass die jeweilige Extrakt zusammensetzung von Mg-10Gd und Mg-2Ag die Zytokin-Freisetzung unterschiedlich beeinflusst. In Versuchen mit frisch aus dem Blut gewonnenen Monozyten konnte gezeigt werden, dass Mg-2Ag Extrakte bei der M2-Makrophagen Polarisation eine Rolle spielen. Experimente, die mit verschiedenen Konzentrationen von $MgCl_2$ durchgeführt wurden, ergaben, dass der Einfluss der Mg^{2+} Ionen

auf die Freisetzung von Zytokinen zeitabhängig ist. Die vorliegende Arbeit beleuchtet die bioaktiven Eigenschaften von Mg-Materialien auf molekularer Zellebene und die Ergebnisse legen nahe, dass diese die Entzündung nach der Implantation des Materials in den Körper günstig beeinflussen und einen chronischen Verlauf verhindern kann.

Content

1 Introduction

Biomaterials have been used to repair or replace damaged human tissue since ancient Egypt [1]. Such materials were improvised and were not able to improve the body's functionality. Despite their long history, the development of biomaterials for defined clinical applications has developed considerably only in the last 50 years [2]. According to the market researcher company "Markets and Markets" (M&M; www.marketsandmarkets.com),the global market for biomaterials is expected to be worth $88.4 billion by 2017. More than 50% of the total market is led by the production of orthopaedic devices. The aging of the population and increasing awareness among aging individuals have resulted in a high incidence of orthopaedic surgery. This increase promotes the incredible impact of biomaterial production for the musculoskeletal system.

After tissue healing, in some cases, orthopaedic fixations require a secondary surgery to remove the implants. Such a disadvantage increases the costs and the morbidity attributed to secondary surgeries. These reasons drive the researcher toward the development of biodegradable materials, which can completely degrade during new bone formation.

Biodegradable polymers were introduced into clinics in 1984 [3], [4]. Since the first internal fixation it was observed that the implantation of such materials has resulted in increased inflammatory reactions [5], [6]. Moreover, the mechanical properties of the polymers do not fully satisfy the requirements of mechanical strength for load-bearing applications. In contrast to polymers, biodegradable metals meet the physical property demands of a biodegradable material for orthopaedic applications.

Among the different biodegradable metals available for suitable clinical applications (i.e., based on magnesium (Mg), iron (Fe), zinc (Zn), and strontium (Sr)), Mg and its alloys are considered the most suitable BMs for orthopaedic use [7], [8]. *In vivo* animal experiments show that the materials enhanced new bone formation and are well tolerated, with no systemic inflammatory reactions [9], [10], [11]. Such interesting results have led to the production of screws, which are available on the market. However, Mg and its alloys are still not available for routine clinical use. Most of the concerns reference the unclear tissue responses towards degradable products.

After implantation, many cells of many types interact in a complex exchange of biological signals that drive the final acceptance of the materials into the body [5], [12]. Macrophages are regarded as the principal cells involved in tissue reactions against implanted biomaterial [13]. This cell type is mainly known for its capacity to promote inflammatory reactions in response to infections. However, their important role during the phases of wound healing has been recently

recognized [14]. Particularly interesting are emerging studies that indicate a clear influence of macrophages on bone cell activity [15]–[17].

In the present thesis, for the first time, macrophage behaviour in response to the total composition of degradable products in Mg materials was investigated. The investigation was performed using pure Mg material and particularly focused on cellular reactions to Mg-2Ag and Mg-10Gd, which are regarded as promising alloys for orthopaedic implantations.

2 State of the art

2.1 Magnesium-based material in orthopaedic applications

Metals are regarded as the better solution for load-bearing applications thanks to their physical and mechanical properties [18]. Despite concerns of corrosion in the body environment, metallic implants based on stainless steel, cobalt chromium (Co-Cr) and titanium have been used for decades in routine clinical applications[19], [20]. However, their use is associated with some disadvantages. The significant mismatch of the elastic modulus of these materials with that of cortical bone results in peri-implant bone resorption (i.e., stress shielding phenomena) [19]. Such events lead to bone loss, with consequent decreases in implant stability [21]. Moreover, secondary surgery might be necessary for the removal of the implant after satisfactory tissue healing. This necessity increases both costs and patient morbidity. These restrictions are bypassed with the possibility of using magnesium (Mg)-based materials in routine clinical use [7], [22], [23]. Mg is an element that, in metal form, can completely degrade under physiological conditions. Compared to the other metals, Mg has physical properties that more closely resemble bone; therefore it is more suitable as a biomaterial for orthopaedic use [24], [25].

The idea of using materials based on Mg as a temporary implant is a story more than 200 years in the making [26]. Patients have been successfully treated in general, orthopaedic and cardiovascular surgeries [26]. Since these early experiments, it was observed that the fast degradation could not satisfy the required initial mechanical stability of a medical implant. The basic idea of a successful biodegradable material requires that it should be integrated into the host compartment and should be gradually substituted with the new growing tissue (Figure 1).

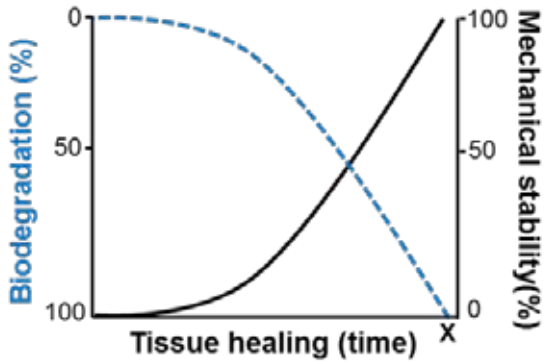


Figure 1 **Ideal biodegradable implant performances.** The relationship between mechanical stability, biodegradation and tissue healing of an ideal biodegradable implant.The present graph is a modified version of Fig.1 of reference [25]. With permission of Elsevier.

The combination of Mg with other elements (Mg alloy) is a method that has been used since the early experiments to increase the degradation resistance of the materials [27], [28]. Poor knowledge of the degradation process and the difficulties involved in implementing the alloying technique have discouraged their use in clinical applications [24]. Today, methods for the production of Mg and its alloys have been considerably improved thanks to interest in transportation industry for light-weight metals. The chance to produce Mg materials again attracted the attention of researchers in the biomaterial field, who are now attempting to propose and investigate suitable Mg alloys for orthopaedic use [24], [29].

In an aqueous environment, magnesium and its alloy degrade via an electrochemical reaction that produces magnesium hydroxide and hydrogen gas [24]. The gas formed accumulates in the tissue surrounding the implants, with unclear implications for bone healing [11], [9]. Moreover, the kinetics and properties of the degradation process under physiological conditions is still unclear. To improve the information in this field, material scientists and surgeons have focussed their attention on the determination of parameters that influence the material degradation process *in vitro* and the associated performances of the materials *in vivo* [11], [30], [31], [32], [33]. The first animal experiments using Mg biomaterial in the musculoskeletal applications took place in 1900 [26]. Since then, Mg has been evaluated in a total of 50 animal studies, and by 2014, more than 23 Mg alloys and pure Mg had been investigated [34]. *In vivo* observations have demonstrated the proper biological performance of the materials analysed, with no systemic inflammation and adequate support of tissue healing, and have indicated the promotion of new bone formation compared to biodegradable polymers (Figure 2) [9], [11].

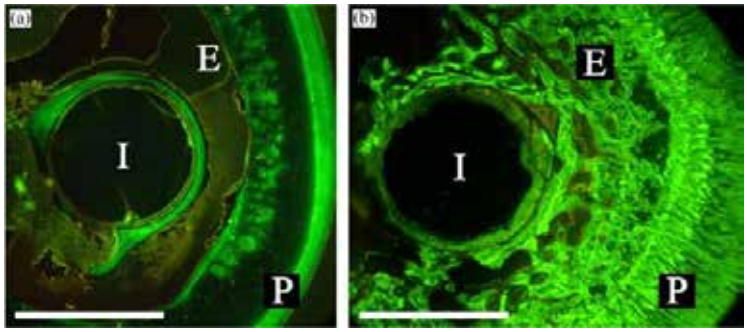


Figure 2 **The bioactivity property of Mg alloys.** The images show induced bone formation with Mg alloy (b) compared to a polymer control (a); I= residual implant; P= periosteal bone formation; E= endosteal bone formation. Scale bar: 1.5 mm [9].

The correlation between *in vitro* and *in vivo* material' s corrosion results have been calculated [34]. The outcomes obtained demonstrate that material degradation in vivo is slower than in in vitro conditions [34], [22]. Such a mismatch is mainly attributed to the interactions of the materials with protein and cell components, which constitute the *in vivo* microenvironment [34]. To clarify the impact of the complex *in vivo* condition on the biodegradation process, recent studies have focussed their work using complex solutions (i.e., blood, cell culture media) and cell culture conditions for immersion tests (5% CO2, 20% O2, 37°C, 95% rH) [35]–[37]. The results obtained from these works clearly demonstrate the specific roles of the cells on the formation of the materials' corrosion layer.

Most of the experiments performed using cells have aimed to consider only their effects on the degradation process. The biological impact evaluations of degradation products on cells has been almost restricted to cytotoxicity testing, which provides mainly information on cell viability percentages [29], [38], [39], [32]. This lack of information can be attributed to the difficulties involved in the use of the materials and their degradable products (i.e., extracts) in cell culture procedures [40]. Methods for *in vitro* cell testing with biomaterials have been described in the International Organization for Standardization (ISO) 10993 procedures. In particular, the ISO 10993-12 provides information concerning the preparation of the biomaterial samples and the extracts while the ISO 10993-5 describes methodology for the determination of the biological response of mammalian cells *in vitro* [41], [42]. Following ISO 10993-12 procedures, extracts preparation results in a basic solution with a high osmotic pressure (~ 0.400 Osmol/kg)[1]. The exposure of cells to such a solution promotes their death due to osmotic shock, regardless of which cell types and alloys are used for the experiments. As a direct consequence, all of the material tested would result in toxicity and would not be suitable for further investigations of cellular responses. Materials that interact with leaving tissue that is left after a damage (biomaterial)are artificial components that could, by definition, negatively impact cell behaviour [43]. Considering this basic idea, it is clear that the ISO guideline has been designed for the establishment of procedures that aim to evaluate only the adverse effects of a material on cell viability. Furthermore, ISO procedures referred only to the use of cell line.

Pilot experiments have shown that the biological evaluation of different Mg alloys can be further investigated by mixing (diluting) the pure extracts (obtained following ISO 10993-12 procedures) with cell culture medium [44]. In particular, a 10-fold dilution (1:10) was suggested [44]. The use of a diluted solution is intended to evaluate the biological effects of different Mg alloy extracts by bypassing the limitation of the high osmolality pressure [44]. Even if such an

[1]The osmolarity value of standard culture medium is ~0.300 Osmol/kg

alternative were proposed, little information is available concerning the biology of the cellular response toward Mg-based biodegradable products [45], [46], [47]. These works clearly demonstrate that the degradation products of Mg materials influence cellular activity at the gene level, promoting bone cell maturation and differentiation. Such information refers to the effects of Mg-based material without the addition of alloying elements and only on bone-lineage cells. After biomaterial implantation, several different cell types interact. The roles of Mg extracts and its alloys on the other cells that surround the biomaterial (i.e. cells of the immune system) are still in question.

2.1.1 Role of magnesium and its alloying elements in the body

The choice of the alloying elements for the preparation of biomaterials is an undergoing process. For an element to be suitable, its biosafety must first be ensured; it must also enhance the mechanical properties of the material. Among the several elements considered for the production of the alloys (i.e., aluminium (Al), calcium (Ca), zinc (Zi), and iron (Fe)), gadolinium (Gd) and silver (Ag) seem to satisfy these demands [48], [24], [28], [49]. In particular, Gd is considered a good element based on the final performance of the material, while Ag is interesting mainly from a biological point of view due its known antibacterial properties [48], [50]. During the biodegradation process, implanted Mg-based materials alloyed with Gd and Ag release the three elements into the microenvironment. The Mg element is present naturally in the body, while Gd and Ag can be considered xenobiotic materials (chemical substances found in an organism but naturally not produced by the organism itself). The following sections summarize the specific effects of Mg, Ag and Gd at the systemic and cellular levels.

2.1.2 Magnesium

Mg is naturally present in the body. It is introduced in the body through the diet, and physiological conditions require its consumption in a range of 320-420 mg per day [51]–[56]. The balance between intestinal absorption and renal excretion regulates Mg^{2+} homeostasis [57]. Almost 99% of the Mg present in the body as a whole is located in the bone. The last 1% is found in the cells and in serum. Serum Mg is categorized in three different states: ionized (55-70%), complexed (5-15%) and protein-bound (20-30%) [51]. Among these three fractions, ionized magnesium has the greatest biological activity; the physiologic blood plasma Mg^{2+}concentration ranges between 0.65 and 0.95 mmol/L [51]. Lower concentrations of serum magnesium lead to the condition of hypomagnesaemia, which can result in pathological

consequences that impact renal and intestinal functionality. The hypomagnesemia can be associated with improper dietary routines and the use of alcohol, diuretics and chemotherapeutic agents [51]–[53]. Moreover, hypomagnesaemia is a common condition in hospitalized patients; the routine analysis of total Mg serum concentration has been suggested as an essential examination for patient care [56]. Hypomagnesaemia leads to several pathologic disorders, such as cardiovascular diseases, atherosclerosis, osteoporosis and obesity [58]–[60]. Most of these diseases have a common inflammatory stress component, and Mg-depleted diet-fed animal experiments have clearly demonstrated the specific correlation between lower Mg concentrations and immune reactions [61]–[63]. These studies describe exacerbate systemic inflammatory responses to bacterial infection and high plasma concentrations of inflammatory signals (i.e. tumour necrosis factor alpha (TNFα) and interleukin (IL)-6) compared to the normal-diet animal control group. Such evidence has also been reported in human studies, which have shown a clear inverse correlation between plasma concentrations of C-reactive protein (CRP) (which rises in the blood when inflammation is present) and Mg intake [64].

The specific correlation between the extracellular Mg^{2+} concentration and the release of inflammatory cytokines has also been clearly observed in *in vitro* conditions. The group of J. Maier showed that a co-culture of human umbilical vein endothelial cells (HUVECs) and U937 cells (a monocyte cell lineage) at concentrations lower than 1 mM of $MgSO_4$ led to increased levels of inflammatory markers (i.e., IL-1) and the associated inhibition of HUVEC proliferation[65]. Interestingly, in other experiments, the same group showed that solo- and co-cultured HUVECs with U937 cells stimulated with 1, 2, 5 and 10 mM of Mg^{2+}resulted in increased HUVEC proliferation. Moreover, an attenuated response to lipopolysaccharide (LPS) was observed at the higher [Mg^{2+}], suggesting a specific correlation between the down-regulation of the inflammatory condition and the increasing concentration of the element [66]. Such an indication was further confirmed by Jun Sugimoto et al., who showed that culturing TLR-activated macrophages with $MgSO_4$ (2.5 mM) induced an increasing intracellular concentration of Mg and a corresponding inhibition of NF-kB activity (the intracellular signals that induce the NF-kB activity are explained in detail in Paragraph 2.2; Figure 4) [67]. In this work, it was clearly demonstrated that the mechanism by which Mg exerts its action on NF-kB involved increasing the basal level of IkB [68]. In addition to the interesting explanation that justified how Mg^{2+} influenced inflammatory cytokine release, in the work, no explanation was provided concerning the mechanism that led to the intracellular Mg^{2+} accumulation. Intracellular Mg^{2+} is present in a total concentration range from 17 to 20 mM and is distributed in the different organelles forming the cell structure (i.e., the nucleus, the mitochondria and the endo/sarcoplasmic reticulum) [69]. In the cytosol, Mg^{2+} is mainly present in the form of a

complex with adenosine triphosphate (ATP) [69]. The abundance of ATP inside the cells and its binding affinity for Mg^{2+} results in a cytosolic *free* [Mg^{2+}] between 0.25-1 mM [70]. The influx and efflux of Mg^{2+} across the cell membrane appears to be mediated through channels (Mg^{2+} accumulation) and exchangers (Mg^{2+} extrusion) (Table 1) [69], [71], [72].

Table 1 **Transporter involved in Mg^{2+} accumulation and extrusion across cell membranes**. This table was completed according to the reference [69], [71]-[73]-

Transporter	Function
Mg^{2+} accumulation	
Transient receptor potential melastatin 6 (TRPM6)	Intestinal and renal Mg^{2+} absorption
Transient receptor potential melastatin 7 (TRPM7)	Transcellular Mg^{2+} transporter, cells adhesion, viability, immune response
Solute carrier family 41, member 1 (SLC41A1)	General transporter for divalent cation
Solute carrier family 41, member 2 (SLC41A2)	General transporter for divalent cation (not for Ca^{2+})
Magnesium transporter 1 (MagT1)	Mg^{2+} specific transporter
Ancient conserved Domain Protein 2 (ACDP2)	General transporter for divalent cation (not for Ca^{2+})
Mg^{2+} extrusion	
Ca^{2+}/Mg^{2+} antiporter	Exchanges Ca^{2+} for Mg^{2+}
$Mn^{2+/}Mg^{2+}$ antiporter	Exchanges Mn^{2+} for Mg^{2+}
Cl^{-} - Mg^{2+} cotransporter	Cotransport Cl^{-} and Mg^{2+}
$Sr^{2+/}Mg^{2+}$ antiporter	Exchange Sr^{2+} and Mg^{2+}
$[HCO_3]$ – Mg^{2+} cotransporter	Cotransport $[HCO_3]$ and Mg^{2+}
Mg^{2+}/Mg^{2+} antiporter	Exchanges
Na^{+}/Mg^{2+} antiporter	Exchange Na^{+} and Mg^{2+}

Among the different transporters, TRPM7 is particularly regarded as the mediator for Mg^{2+} accumulation in the cells of the immune system [71], [74], [75]. The TRPM7 protein consists of six transmembrane segments and contains a Mg-permeable pore specifically located between the fifth and the sixth segments [76]. The protein is fused to a serine/threonine kinase domain (protein kinase C (PKC) family) in the region of the COOH terminus; for this reason, TRPM7 is also known as a “chanzyme” (channel plus enzyme) [71], [76], [77].

Inside cells, the PKC can be present also in the cytosolic form and was demonstrated to be a component involved in the activation of intracellular signals that lead to Mg^{2+}accumulation inside the cells [69], [77]. PKC belongs to a family of isoenzymes that promote the TLR pathways, resulting in the activation of mitogen-activated protein kinases (MAPKs) (TLR signalling is explained in detail in Paragraph 2.2, Figure 4) [78], [79]. PCK mediates Mg^{2+} accumulation through the MAPKs ERK1/2 and p38 [80]. In fact, it has been observed that the pharmacological inhibition of these proteins results in the interruption of Mg^{2+} influx [80].It is not known if the increased [Mg^{2+}] in TLR-activated macrophages is specifically mediated by PKC phosphorylation of TRPM7 or if cytosolic PKC mediates the activation of other receptors. Even if these aspects need to be clarified, there is clear evidence that suggests that TRPM7 plays a specific role in macrophage activities. Interesting studies have shown that TRPM7 inhibition leads to decreased activation of ERK1 and ERK2 in hepatic cells and that in IL-4-stimulated macrophages, the specific influx of Mg^{2+}mediated by TRPM7 induces the cells toward M2 polarization (see Paragraph 2.2 for more details about M2) [75], [81]. Even though it remains to be clarified whether free cytosolic PKC can promote the entrance of Mg^{2+} through other receptors (i.e., MagT1). Never the less thanks to the information available, it can be speculated that in TLR-induced macrophages TRPM7 contributes to the increase of the intracellular Mg^{2+} content. This event, as a consequence, would lead the anti-inflammatory effects observed by Jun Sugimoto et al.. The Figure 3 summarizes the role Mg^{2+} plays in cytokine release.

In addition to food, Mg is also largely found in the crust of the earth in natural combinations with other elements, including aluminium (Al) nickel (Ni), iron (Fe), copper (Cu), beryllium (Be), silicon (Si), and strontium (Sr) [24]. These impurities are also present during material casting (the manufacturing process in which a liquid material is poured in to a mould and then solidified) and are influencing factors for the degradation process [24]. Up until now, there has been no information on the biological effect of the total composition of Mg on macrophage activity. This lack is because Mg-containing materials have only recently been reconsidered as potential biomaterials. Moreover, there are conceptual and methodological problems involved in the quantification of Mg (in its ion form) and the impurities once they are released into fluids during the degradation process.

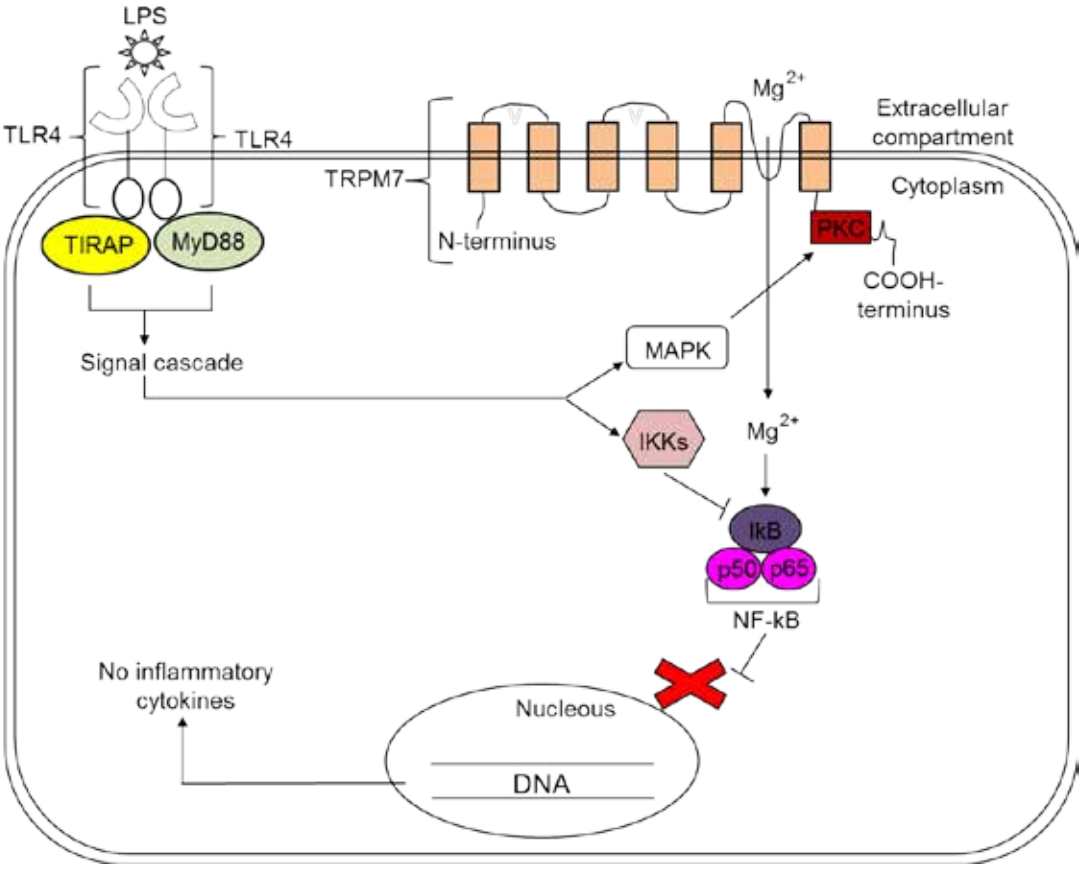


Figure 3 **Anti-inflammatory effects of Mg^{2+}**. TLRs induce the signal cascade that leads to the activation of MAPK and IKKs. MAPK promotes the activation of TRPM7 channels to the phosphorylation of the PKC domain and the consequent entrance of Mg^{2+}. Once in the cells, Mg^{2+} blocks the degradation of IKb, which is mediated by IKKs. Due to the stabilization of IkB, NF-Kb is blocked in the cytoplasm; therefore, there is no inflammatory cytokine transcriptional activity. The influence of Mg^{2+} on cytokine release is illustrated according to the references [67], [69], [77]-[80].

2.1.3 Silver

Silver is used as an incorporation element in cosmetics, antibiotics, and wound dressings and is an additional component in medical devices (i.e., bone cements, hygiene textile cate), mainly thanks to its antibacterial properties. In bacteria and fungi, silver is absorbed though pinocytosis. Once in the cells, Ag^{+} can promote the denaturation of essential enzymes (i.e., RNAses; DNAses), an action that mediates known antimicrobial effects.

Due is large usage, silver is absorbed into the human body through several pathways, such as inhalation, ingestion, dermal contact and intraparenteral insertion of medical devices. Silver

can be accumulated in liver, kidney, bone, and skin and can be excreted through urine, bile and the nails [82], [83]. Ag is biologically active in its ion form (Ag^+), and chronic absorption of Ag^+ results in a typical blue discoloration of the epidermis known as “Argyrya”. In addition to the undesirable cosmetic problem, Argyrya is an uncommon and irreversible condition that is not associated with carcinogenicity. It is a widely manifested condition, and for this reason, several have reported cases that documented the difficulties involved in its diagnostic recognition [84]–[87].

Various experimental models have shown that Ag exhibits anti-inflammatory properties [88]–[90]. The use of silver in the form of nitrate and nanocrystal reduces the inflammatory conditions of disorders such as cystitis and allergic contact dermatitis. In an experimental rat model of ulcerative colitis, it was clearly demonstrated that the administration of nanocrystalline silver (either via intracolonic injection or orally) inhibited the release of specific inflammatory markers (matrix metalloproteinase (MMP-9), TNFα, IL-1β, and IL-12) [91]. Interestingly, the suppression of such molecular signals was also observed in RAW264.7 cells (mouse macrophages) cultured with Mg-based material alloyed with Ag [92].

2.1.4 Gadolinium

Gadolinium is a rare earth element that, in clinical applications, is used as a component of a contrast agent [93]. The risks and benefits of its use in the clinic are questionable [94], [95]. Most concerns have referred to the high incidence of nephrogenic systemic fibrosis (NSF) in dialyzed patients treated with gadolinium-based solutions [96]. Information concerning the role of Gd at the cellular and molecular levels is contradictory: if on the one hand it is observed that Gd can induce the release of proteins that enhance inflammatory reactions(such as IL-1β), on the other hand, the treatment of ischaemia-injured rats with $GdCl_3$solution can reduce myocardial inflammation [97], [98]. With these contrasting results, the specific role of Gd in immune cell reactions is still an open question.

2.2 Immune response to biomaterials

Subsequent to damage, the body responds with a complex reaction named inflammation. This process is a protective response of the immune system that aims to restore tissue integrity. Inflammation is characterized by the concomitant presence of five key signals: redness, warmth, swelling, pain and temporary loss of function of the afflicted tissue. The inflammatory condition trigged after the implantation of a biomaterial is specifically named a foreign body reaction

(FBR) [12]. The injury to vascularized tissue during an implantation procedure results in the material coming into contact with bodily fluids and leads to the adsorption of a protein layer on the material surface [99], [100]. Blood protein deposition on a biomaterial surface is described as “provisional matrix formation” and provides the basic biochemical structure for the following steps of cell adhesion and initiated events in FBRs [12]. The proteins that are absorbed on the biomaterial´s surface mainly include fibrinogen, complement fragments and albumin [101], [102]. Upon binding such proteins, cells of the immune system produce molecular signals that lead to the recruitment and activation of cells involved in tissue repair (i.e., fibroblasts) to the implant site. These cells deposit collagen matrix and encapsulate the material in a fibrous tissue layer [12]. In the absence of sepsis, the inflammation caused by the successful implantation of a medical device diminishes with time. This event is described as a phenomenon of tolerance and represents the results of the compromise between encapsulation of the implant and new tissue formation (in case of permanent implants) or complete resorption of the biomaterial (in case of degradable implants) [103]. If the inflammation is inappropriately extended overtime, cells then release molecular signals that inhibit tissue healing. This event leads to the phenomenon of allograft rejection, which consists of tissue destruction with consequent loss of the mechanical stability of the biomaterial (stress cracking) [104], [105], [106]. The scientist Ilya Metchnikoff (1845-1916) was the first to document the early cells’ reaction toward biomaterials [107]. In a pioneering experiment, he inserted a splinter into the body of a fish larva. Thanks to its transparent body, he observed the migration and ingestion activities of the cells against the splinter. The cells observed were described according to their size and were called microphages (smaller cells, today known as “neutrophils”) and macrophages (larger cells) using as the suffix the reek term “phage”, meaning “ingestion, eating” [11]. Neutrophils predominate during the first several days following the injury and then are replaced by macrophages as the predominant cell type [12].

Macrophages are called antigen-presenting cells (APCs) and are responsible for the initial recognition and processing of antigen (innate immunity), which leads to the subsequent activation of B and T lymphocytes (adaptive immunity). In the absence of an infection, cell debris and biodegradable products of the material activate Toll-like receptors (TLRs). TLRs constitute one of the most important class of receptors expressed in APCs and are responsible for the recognition of potentially dangerous agents. Until now, it eleven TLR isoforms have been identified in humans and are classified in a progressive numeration (TLR1-TLR11) according to their capacity to recognize different damage signals [108], [109]. TLRs are integrin membrane glycoproteins consisting of an extracellular region containing a leucine-rich motif (LRR) and a conserved intracellular portion of~200 amino acids. Because the cytoplasmic portion of the

protein shows high similarity to that of the IL-1 receptor family, that region of the protein is named the Toll/IL-1receptor (TIR) domain [109]. The LRR motif of the external portion of the protein is typically 22–29 residues in length and contains hydrophobic residues spaced at different intervals, forming a typical “horseshoe” structure [109], [110]. Thus far, the ligands that signal through the TLRs include microbiological agents, (also named pathogen-associated molecular patterns (PAMPs)) or intracellular host proteins that are released during necrosis (i.e., heat shock proteins (HSPs)) (also named damage-associated molecular patterns (DAMPs)) [109].

Among the different TLRs, of particular interest is the specific role of TLR2, which is regarded as the receptor involved in the recognition of DAMPs and non-biological agents [109], [111], [112]. In fact, it was observed that polymeric alkane structures are able to activate the TLR2 signalling pathway, suggesting a specific role of TLR2 on FBR [111]. Signalling through TLRs requires the processes of homo- (in case of TLR4) or heterodimerization (as in case of TLR2, which can dimerize with TLR1 or TLR6) [113]. TLR2 stimulation requires the phosphorylation of two adapter proteins, the protein myeloid differentiation primary-response protein 88 (MyD88) and the toll-interleukin 1 receptor adaptor protein (TIRAP). These proteins, in turn, recruit IL-1R-associated kinase (IRAK)4 and IRAK1. These two proteins are sequentially phosphorylated and dissociated from MyD88, which results in activation of tumour necrosis factor receptor-associated factor 6 (TRAF6) [108], [109]. TRAF6 activates transforming growth factor-β-activated protein kinase 1 (TAK1), which, in turn, promotes the activation of the mitogen-activated protein (MAP) kinase signalling and the IKK complex. IKK leads the recruitment into the nucleus of the transcriptional nuclear factor (NF)-kB [113], [114]. NF-kB resides in latent form in the cytoplasm complexed to its inhibitor(I) kappa B. The phosphorylation of I kappa B, operated by the complex IKK and enabling its transcription activity for several inflammatory genes (i.e., IL-1β, TNFα) [12], [115], [116], [117]–[119], [120]. NF-kB expression is activated in a multitude of signalling pathways involved in the immune response. Thus, NF-kB down-regulation is considered the key step for the resolution of an inflammatory condition [117], [118], [121], [122]. Figure 3 summarizes the complex signal pathways trigged after TLR2 stimulation.

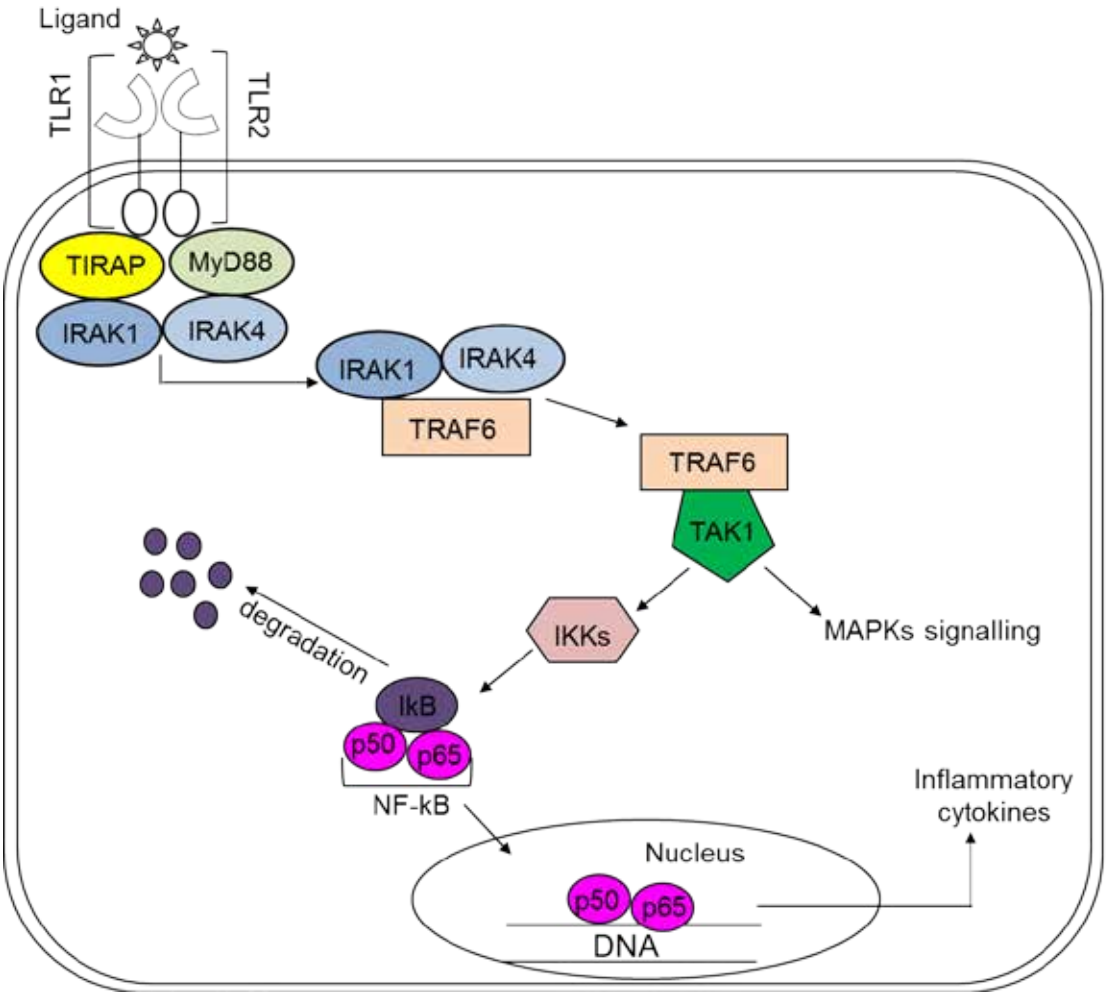


Figure 4 **TLR2 signalling**. After dimerization with TLR1, the adapter proteins TIRAP and MyD88 induce the phosphorylation of IRAK1 and IRAK4. Activation of the TRAF6 protein following the activation of TAK1. TAK1 promotes the activation of the MAPK signalling pathway and of the transcriptional factor NF-kB. Once in the nucleus, NF-kB induces the gene expression of inflammatory genes. The illustration was completed according to the references [108]–[110], [114].

Since the early minutes that follow the implantation of the biomaterial, the implant site is characterized by a dynamic microenvironment thanks to the constant recruitment of macrophages from the blood [123], [124]. Chemokines are a group of cytokines responsible for the homing of immune cells from the blood to damaged tissue [125], [126]. This group is constituted by almost 100 amino acid motifs classified into four subfamilies (CXC, CC, CX3C, C) according to the location and number of the cysteine residues at the N-terminus of the molecules [125]. Among them, the chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1) (known also as CCL-2) are specifically regarded as the key factors for the recruitment of macrophages to damaged sites [125]–[129]. Once at the tissue, macrophages up-regulate

the expression of the intercellular adhesion molecule 1 (ICAM-1) (known also as CD54) thanks to IL-1β and TNFα signalling [130]. ICAM-1 is a transmembrane glycoprotein constitutively present in several cell types (i.e., leukocytes, fibroblasts) that enhances the interaction between the cell and the provisional matrix of biomaterial binding the integrins LFA-1 and Mac-1 [131]. *ICAM-1* gene expression is promoted by the activation of intracellular signal transduction pathways involving PKC, NF-kB, mitogen-activated protein (MAP) kinases (ERK, p38, JNK), the levels of which decrease after cells are exposed to anti-inflammatory factors (i.e., transforming growth factor β (TGF β) and IL-10). This information suggests that, even if constitutively present on the cell membrane surface, *ICAM-1* expression is strongly dependent on the microenvironment [130], [132], [133].

When in contact with biomaterial, macrophages can fuse together, forming a multinucleated cell called a foreign body giant cell (FBGC) [12], [13], [115], [116]. This phenomenon is typical of the FBR and occurs due the inability of the macrophages to digest particles > 10 μm in size [13]. In *vivo* experiments have clearly shown that the molecules MCP-1and osteopontin (OPN) influence FBGC formation [134], [135]. In particular, it was observed that the subcutaneous implantation of a biodegradable alginate-based scaffold in mice that lack MCP-1 expression results in reduced FBGC formation [134]. Other research groups have observed that, in OPN knockout mice, the implantation of poly (vinyl alcohol) sponges leads to a decrease in FBGCs in the area surrounding the implant compared to wild-type mice [135]. Even if FBGC formation can be reproduced in *in vitro* conditions (by stimulating the cells with IL-4, granulocyte-macrophage colony-stimulating factor(GM-CSF) and IL-13), the mechanism and the molecules involved in the cell fusion are unclear [12], [135], [136]. Interestingly, a specific role of the receptor CD36 has been proposed [137]. CD36 is a scavenger receptor that binds endogenous and pathogen ligands and has been studied mainly in the context of atherosclerosis pathologies due to its capacity to recognize oxidized LDL (oxLDL) [138]–[141]. Helming and al have shown that, after stimulation with IL-4 and GM-CSF, macrophage fusion is mediated thanks to CD36 recognition of phosphatidylserine(PS), a phospholipid membrane component [137].

Both morphological cell variants (macrophages and FBGCs) are observed at the tissue/material interface [12], [13], [142], [143]. The specific roles of macrophages/FBGCs with respect to final implant performance are controversial. The adherence of the cells on biomaterial surfaces and the release of bio-react intermediates are associated with the phenomena of host tissue destruction and biomaterial degradation [12], [105], [115]. Nevertheless, *in vivo* studies have shown that macrophages and FBGCs surrounding the implants release cytokines involved in the down-regulation of inflammation and tissue healing (i.e., IL-10 and transforming growth factor beta (TGF-β)) [120], [144]–[146].

In response to the microenvironment, macrophages form different cell subpopulations, which are mainly distinguished as two large groups called M1 and M2 macrophages [14], [147]–[153]. Depending on different stimuli, macrophages can also be classified in further subclasses according the specific expression of receptors in answer to defined stimuli (i.e., M1a, M2b, M2c), [148], [150], [151]. The complex mechanism that leads to M1 and M2 generation is defined as the polarization phase [148] (Figure 5).

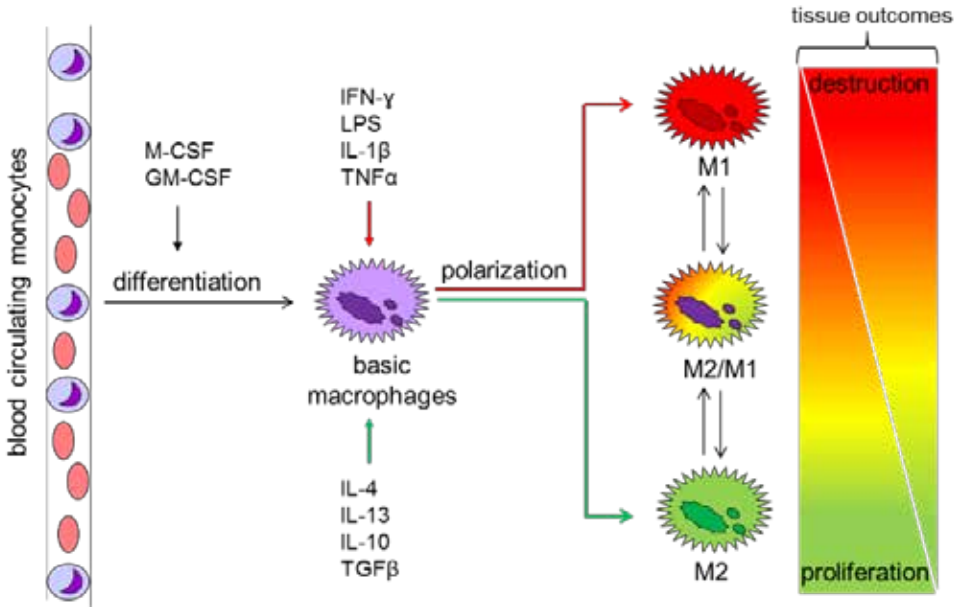


Figure 5 **Macrophage differentiation and polarization.** According to defined stimuli present in the microenvironment, macrophages can be polarized intoM1 and M2 subpopulations. The two phenotypes influence final tissue responses during an inflammatory process. The polarization of the cells into M1 leads to destruction (chronic inflammation), while M2 induces proliferation (resolution of the inflammation). This figure has been based on the references [14], [147], [148], [153].

M1 macrophages (“classically activated”) are responsible for chronic inflammation and tissue destruction, while M2 macrophages (“alternatively activated”) are associated with tissue healing (Figure 5). Recent studies have demonstrated that a high M2/M1 ratio leads to decreased inflammation toward biomaterials and that the polarization phase is a fundamental process for the final outcome of medical devices [154], [155].

An interesting aspect of macrophages is that they can switch from one phenotype to another, forming a heterogeneous cell population [149], [156]. The concept behind the identification of the two different macrophage cell type populations (and their subclasses) is one of the main and central issues in the field of the immunology. It should be noted that the establishment of the

M1/M2 macrophage classifications comes from the pre-genomic era, when few markers had been identified as responsible for the macrophage activation [150]. The cumulative data obtained up to this point clearly show that macrophages can easily change their genetic and molecular expression profiles according to the microenvironment [14], [147], [148], [157], [158]. Therefore, the definition of M1/M2 macrophages is no longer intended to denote a strict discrimination of two different cell subpopulations but rather a “temporary status” [150], [156], [159]. The final result is the dynamic release of cytokines, which could justify the wide range of molecules detected in an implant’s surroundings [14], [116], [120], [147], [148], [150], [153], [159], [160], [161], [162]. Table 2 summarizes the most important molecules released by macrophages and their biological activities.

Table 2 **Macrophage cytokine production.** For each protein, the full name is reported in the list of the abbreviations

Abbreviation	Biological function	References
C5/Ca	C5a and C5a are leukocyte chemoattractants, forming the provisional matrix of implanted materials	[12], [116], [163]
I-309	Chemokine (C-C motif) ligand 1 (CCL1) is involved in the chemotaxis of monocytes, NK cells, immature B cells and dendritic cells	[164], [125], [126]
sIcam-1	Soluble form of ICAM-1 that specifically promotes the transmigration of cells from the blood through the endothelial layer	[165]
IL-16	Promotes the chemotaxis of T lymphocytes	[166], [167]
IP-10	Also known as C-X-C motif chemokine 10 (CXCL10) and is secreted in response to IFN-γ	[125], [126], [168]
I-TAC	Produced in response to IFN-ɣ stimulation and its promoting factor for lymphocyte recruitment	[125], [126], [169]
MCP-1	Also known as chemokine (C-C motif) ligand 2 (CCL2) and is particularly known for macrophage homing	[125], [127], [129], [169]
MIF	Ligand for the CXC chemokine receptors and is mainly released in cells in carcinogenic conditions	[170]
MIP 1α/β	Also known as CCL3 and is a CC chemokine involved in the acute inflammatory state of leukocyte recruitment and activation	[125], [126], [169]

Serpin E1	Also known plasminogen activator inhibitor-1 (PAI-1) and is an important factor in inflammation-induced macrophage migration	[125], [126], [169]
RANTES	Chemokine expressed by many hematopoietic and non-hematopoietic cell types that plays an important role in homing and migration of effectors and memory T cells during the acute phase of inflammation	[125], [126], [171]
SDF-1	Lymphocyte chemoattractant induced by proinflammatory stimuli, such as LPS, TNF, or IL-1	[172], [173]
IL-8	Chemokine mainly associated with neutrophil and macrophage recruitment	[125], [127], [174]
IL-27	Amplifies the inflammatory signals in synergy with IFN-ɣ and promotes T lymphocyte activities	[175], [176]
IL-12p70	Involved in the differentiation of naive T cells into Th1 cells and reduces IL-4-mediated IFN-γ suppression	[177], [178]
GM-CSF	Promotes the proliferation and differentiation of cells of the myeloid lineage	[150], [179], [180]
M-CSF	Promotes the proliferation and differentiation of cells of the myeloid lineage	[14], [147], [148], [150]
IL-2	Promotes leukocyte differentiation and activity and is essential for T cell maintenance during inflammation	[181]
IL-5	Haematopoietic factor that is responsible for eosinophil growth and differentiation	[182], [183]
CD40L	Promotes M1 macrophage activation	[184], [185]
GROα	Activator and chemoattractant for T lymphocytes	[186], [187]
IL-6	One of the most important mediators of fever initiating PGE_2synthesis in the hypothalamus	[188]–[190]
IFN-ɣ	Strong macrophage activator that is generally considered the bridge between innate and acquired immunity	[14], [153], [159], [160]
IL-1α/β	Pro-inflammatory cytokine, one of the first cytokines released in answer to a damage signal	[191]–[195]
TNF-α	Promotes the acute phase of inflammation and is released by M1 macrophages	[14], [152], [196]
sTrem-1	Amplifies cellular TLR signalling	[197], [198]

IL-17/IL-17E	Amplifies inflammation, promoting the release of several factors, such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, and TNF-α from several cell types (i.e.,fibroblasts, endothelial cells, etc.)	[180], [199]
IL-23	Induces matrix metalloprotease9 (MMP9) upregulation and promotes angiogenesis	[150], [180]
IL-32α	Pro-inflammatory cytokine increased in response to IL-6 signalling	[200], [201]
IL-13	Promotes alternative activation of macrophages into M2 cells in synergy with IL-4	[14], [147], [151]
IL-4	Promotes alternative activation of macrophages into M2 cells and inhibits classical activation of macrophages into M1 cells. IL-4 induces M2 differentiation in synergy with M-CSF	[147], [148], [152]
IL-1Ra	Natural inhibitor of the pro-inflammatory effect of IL1β	[202]–[204]
IL-10	Down-regulates T1 cytokines by blocking NF-κB activity	[205]–[207]

The cytokines are pleiotropic molecules once they are released in the microenvironment, they can promote effects of:

- Synergy: two different or the same cytokines induce a final biological impact greater than the response that would arise with the action of a single protein;
- Antagonism: the biological outcome of the action of one cytokine can be minimized with another cytokine;
- Redundancy: different cytokines promote the same biological effect.

An inflammatory condition that leads to the elimination of a foreign agent, preserving the host tissue integrity (physiologic inflammation), is strongly determined by the dynamic balance between groups of cytokines that amplify and down-regulate the inflammatory reaction [14], [151], [153], [160], [196]. The groups of cytokines that promote these two events are generically identified as inflammatory and anti-inflammatory cytokines [196]. Imbalance between these two classes of proteins leads to damage to the host tissue and triggers immune diseases such as rheumatoid arthritis (RA) [202]. In this case, for example, the pathology is specifically attributed to the ratio of the production of IL-β and its antagonist, IL-Ra [202]. The inflammatory cytokine IL-1β is one of the first cytokines identified as a key responsible factor in both physiologic and pathologic inflammatory conditions [188], [191], [195]. Physiologic inflammation requires the release of a 100-fold greater concentration of IL-1Ra with respect to IL-1β in the plasma [202]. IL-1Ra is a protein that binds the same target receptors as IL-1β, but with low affinity. This aspect explains the required higher concentration of IL-1Ra with respect IL-1β in physiological

conditions [202]. It has been observed that IL-1Ra subministration attenuates inflammatory symptoms; a recombinant version of IL-1Ra (also known as “Anakinra”) has been successfully used as a therapeutic agent against immune disorders [208]. When the inflammatory status condition is pronged overtime (days), the constant overproduction of inflammatory markers leads to increased body temperature [188]–[190]. Physiological body temperature is normally maintained within a range from 36.0°C to 37.5°C, and an increase up to 41°C is known as a febrile condition [188], [189]. Fever is generally considered a “cardinal signal” of an inflammatory condition and arises when the inflammatory cytokines (i.e., IL-1β and TNFα, which are also called endogenous pyrogens) promote the elevation of prostaglandin E (PGE) in the cerebrospinal fluid, with a consequent increase of the body temperature’s hypothalamic set point [188], [189], [209]–[211]. These events enhance the release of thyroid hormones, glucocorticoids and catecholamines, with consequent vasoconstriction and shivering. Altogether, these processes induce an increase in the body’s temperature and the establishment of a fever [189], [211]. The specific role of fever is still unclear; nevertheless, it is considered part of the physiologic response against foreign agents [188]–[190], [210].

While the interest concerning the macrophage responses toward biomaterials is still growing, the dynamic mechanisms resulting in the release of the different groups of cytokines and how such phenomena could influence macrophage polarization during an FBR are still unclear.

Macrophage activities can be investigated through the analysis of the cytokines released in cell culture and through the identification of macrophage sub-phenotypes. In studies that aim to identify the molecular mechanisms involved in the rapid genetic profile changes during the differentiation and polarization phases, the use of primary cells is preferred compared to cell lines. In fact, due their malignant condition, cells line partially lose the capacity to undergo differentiation even when exposed to inflammatory stimuli. *In vitro* macrophage differentiation and polarization require the use of “artificial stimuli”, which means molecular signals not naturally produced in the tissue. The use of such drugs is conventionally suggested in standard operating procedures for the culture of defined cell types and promotes the activation of pathways (i.e., NF-kB, MAPKs) that leads to the differentiation of myeloid leukaemia cell lines into macrophage cell types [188]. The use of such molecules partially indicates the use of primary cells as a preferable target for the *in vitro* reproduction and analysis of the differentiation and polarization phases. Independent of this limitation, cell lines are commonly used to investigate cytokines and gene expression thanks to their strong correlation with primary cells [212]–[216]. Due to the well-known molecular signals involved in the activation of macrophages using septic or aseptic stimuli, it is obvious that cell reactions can be described by referring to a

defined agent in a well-defined microenvironment. This is not the case using biomaterial; in the particular case of biodegradable Mg-based material, no studies with macrophage cell type have been reported.

3 Motivation and objectives

Biodegradable magnesium-based materials are promising biomaterials for orthopaedic applications. After implantation, the tissue shows appropriate responses that indicate the enhancement of new bone formation. These observations refer only to *in vivo* animal models; the reactions toward the degradation products of the materials at the cell molecular level are unknown.

The FBR occurs immediately after the implantation of a biomaterial, with the consequent release of molecular signals into the microenvironment. In ideal conditions, the acceptance of the biomaterial into the body is based on the balance between the production of cytokines that promote inflammation (inflammatory cytokines) and that protect the tissue (anti-inflammatory cytokines). Macrophages are the principal source of cytokines in the FBR.

The present thesis intends to explore, for the first time, the specific reactions of macrophages on the degradation products of pure Mg, Mg-2Ag and Mg-10Gd. In particular, the work aims to investigate the indicated phenomena of tolerance though the influence of extracts in the release of key factors for FBR and their roles in the macrophage differentiation and polarization processes. To achieve these purposes, the research was divided into three steps:

1) Cytokine release and gene profiles, which describe the basic macrophage activities
2) Modulation of key inflammatory factors in a co-culture model of macrophages/osteoblasts
3) M1 and M2 macrophage differentiation and polarization

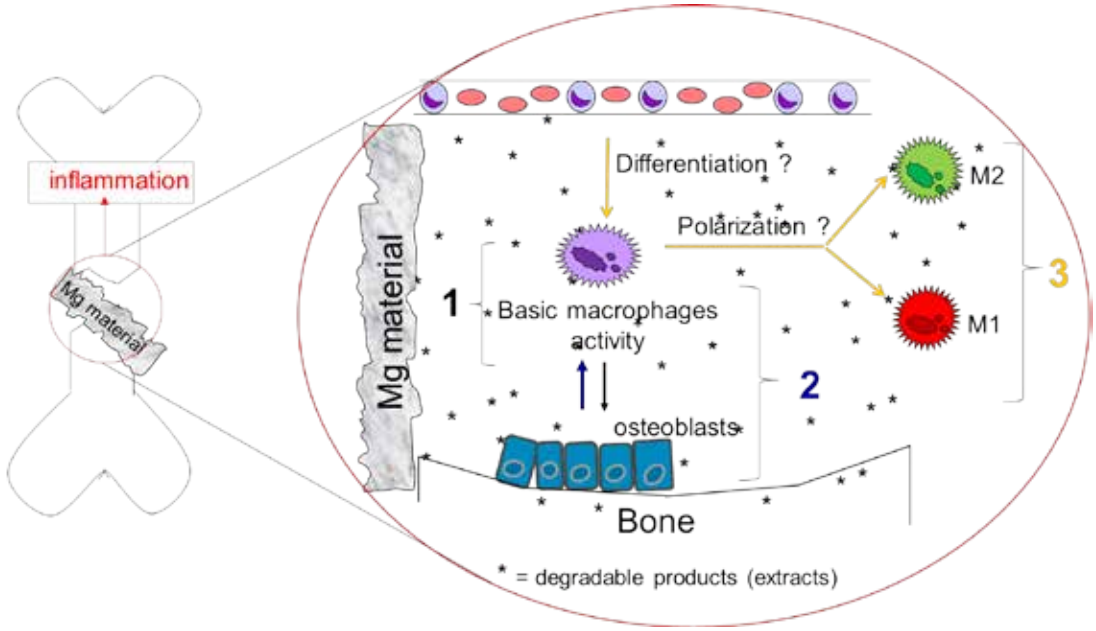


Figure 6 **Aim of the thesis**. Representation of the three essential points of the work in a broad overview.

4 Materials and Methods

4.1 Extract preparation and characterization

Pure magnesium (Mg, 99.95%) and magnesium with 10wt% gadolinium (Mg-10Gd) alloys were prepared by permanent mould gravity casting, while Magnesium with 2 wt% silver (Mg-2Ag) alloy was produced via permanent mould direct chill casting (Helmholtz Zentrum Geesthacht, Germany). Discs of 1.5-mm thickness were then cut, singularly weighed and packaged for gamma sterilization. A total dosage of 29 kGy was administered (BBF Sterilisationsservice GmbH, Kernen-Rommelshausen, Germany). Sterilized samples were incubated with RPMI 1640 medium (Sigma-Aldrich, Steinheim Germany) supplemented with 10% of foetal bovine serum (FBS; PAA Cell Culture Company, Linz, Austria) for 72 h with 5% CO_2 at 37°C according to the EN ISO standards 10993:5 and 10993:12 [41], [42]. The ratio of specimen weight to medium volume was 0.2 g/mL. The resulting extracts were centrifuged (1200 rpm, 5 min, room temperature) and filtered using a membrane filter (0.2 µm; Merck KGaA, Darmstadt, Germany). The total concentrations of elements released after the biodegradation process were measured using an inductively coupled plasma mass spectrometry (ICP-MS) technique. This method is based on mass spectrometry analysis and works by ionizing chemical compounds to generate charged molecules and measuring the ratios between their masses and charges. For each measurement, 0.5 mL of sample was used.

The values of pH and osmolality were determined using a SENTRON ARGUS X pH-meter (Fisher Scientific GMBH, Schwerte, Germany) and an Osmomat 030 (Gonotec, Berlin, Germany), respectively. The volumes of analysed extracts were all 50 µL.

The pure extracts of Mg, Mg-10Gd and Mg-2Ag were subsequently diluted (1:10) with cell culture RPMI medium to decrease their osmolality to a value of 0.300, which is the value of the cell culture medium control solution. Prior dilution of extracts before their use with the cells was applied for all of the experiments performed in this work.

4.2 Cell culture systems and *in vitro* investigations

4.2.1 Cell culture and in vitro inflammatory model

The U937 cell line was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% of FBS under standard cell culture conditions (5% CO_2; 37°C)

up to a density of $5x10^5$ cells/mL. Subsequently, the cells were stimulated with phorbol 12 myristate acetate (PMA) (5 nM/mL) for 24 h to induce their differentiation into macrophage cell types. Non-adherent cells were removed, and while adherent cells were washed with phosphate-buffered saline buffer (PBS; 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4-$2H_2O$, 0.00176 M KH_2PO_4, pH 7.4; all chemicals were purchased Sigma-Aldrich, Steinheim Germany), and fresh medium was added. The cell culture was performed for a further 3 days. The adherent DiffU937 cells were subsequently collected from the cell culture flask using 5% Trypsin-EDTA (Invitrogen, Schwerte, Germany) and were seeded in 12-well plates at a density of $5x10^5$ cells/mL, with a total of 3 mL per well.

As described in Paragraph 2.2.1, higher temperature is a key signal of an inflammatory condition. Considering that fact, the inflammatory microenvironment was reproduced by exposing the cells to the higher temperature of 39°C. The standard temperature of 37°C was used as a control. An inflammatory reaction is a time-dependent event (Paragraph 2.2). To investigate cell activity with respect to this parameter, the model was finally established by exposing the cells to the different temperatures for 1 and 3 days: representative time points of early and late inflammation, respectively. The cells were exposed to the diluted extracts in the described conditions as represented in Figure 7.

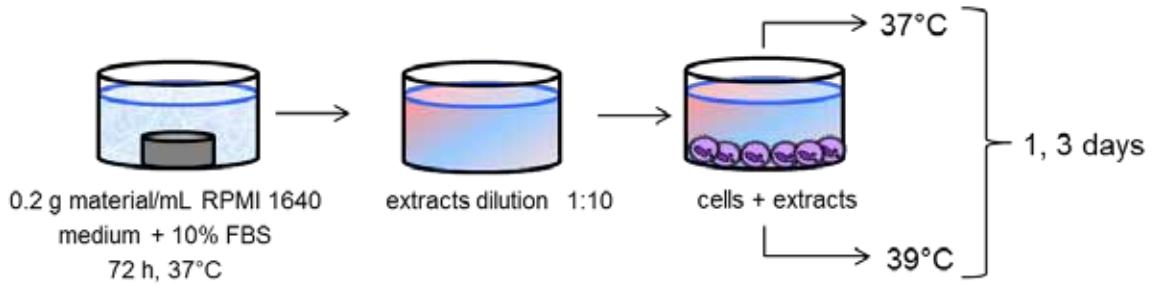


Figure 7 **Scheme of the extract preparation and cell stimulation.** After extract preparation in cell culture medium for 3 days, the obtained solutions were diluted with cell culture medium (1:10) to decrease the osmolality pressure. Subsequently, the cells were incubated at the temperatures of 37°C and 39°C for 1 and 3 days.

Mg in the form of $MgCl_2$ in solution was also used to elucidate the influence of Mg^{2+} on cytokine production. The stock solution was prepared at the concentration of 100 mM (Sigma-Aldrich, Steinheim Germany) in double-distilled water (ddH_2O). The cells were cultured with 1, 5 and 10 mM Mg^{2+} in an inflammatory *in vitro* model.

4.2.2 Saos-2 solo-culture and Saos-2/DiffU937´ co-culture

To determine the effect of the extracts on the cross-talk between macrophages and osteoblasts, a co-culture was established. For these experiments, the osteosarcoma cell lines Saos-2 (ECACC, Salisbury, United Kingdom) and DiffU937 were used.

The co-culture model was established after analysis of Saos-2 cells in solo-culture. Saos-2 cells were first cultured with McCoy's 5A medium (Life Technologies, Darmstadt, Germany), (standard medium used for Saos-2 cells) and RPMI (standard medium used for U937 cells) at 37°C. After 3 days of cell culture, their viability and growth were verified using CASY technology (see Paragraph 4.2.4). Once confirmed with the use of RPMI medium, the activities of Saos-2 cells toward Mg-10Gd and Mg-2Ag extracts were investigated at the protein level. For these experiments, lipopolysaccharide (LPS; from Escherichia coli 026:B6; 0.1 µg/mL; Sigma, Germany) stimulation was used as an internal inflammatory positive control.

The Saos-2 cells were then used for a co-culture model with DiffU937 cells. DiffU937 cells were first cultured alone in 12-well plates ($1.500x10^6$ per well) for 24 h, the necessary time to allow their adherence on the plate surface. After 24 h, all medium was removed, and 3 mL of RPMI medium containing $150x10^3$ Saos-2 cells (ECACC, Salisbury, United Kingdom) was simply added to the DiffU937 culture. The co-culture was performed for a total 3 days at 37°C with and without Mg-10Gd and Mg-2Ag extracts. Investigations of the cells in solo- and co-culture conditions were performed at the protein level.

4.2.3 Primary monocyte isolation and stimulation

Human monocytes were obtained from buffy coats (DRK-Blutspendedienst NSTOB, Hannover, Germany) of six healthy donors. Buffy coats were diluted (1:4) with sterilized PBS. Subsequently, 25 mL of the total blood obtained was transferred in 50 mL tubes containing 15 mL of Ficoll (Biocoll, Biochrom AG, Berlin, Germany), followed by a centrifugation step with no brake (400xg) for 30 min at room temperature. The PBMCs were obtained by collecting the cells seeded at the interface between the plasma and the Ficoll (known as the "white ring") (Figure 8). Cells were subsequently incubated withCD14 MicroBeads (Miltenyi, Berlin, Germany) for 20 min at +4°C. Cells expressing the CD14 receptor (monocytes) were selectively isolated using an autoMACS Pro Separator (Miltenyi, Berlin, Germany). The fresh monocytes obtained were cultured in 48-well culture plates (Falcon, USA) at a density of $5X10^5$/0.5 mL using CellGro®serum-free medium (CellGenix, Freiburg, Germany) at 37°C.

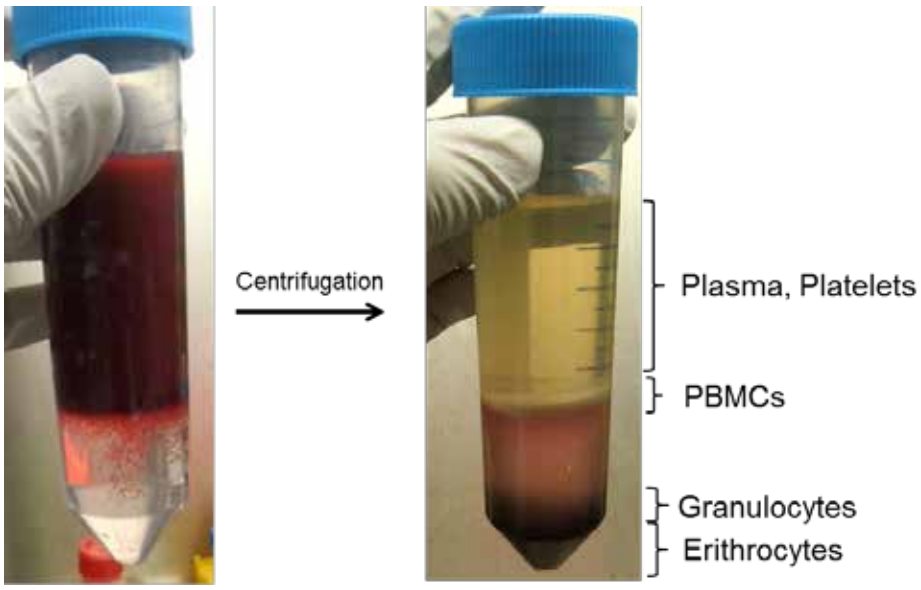


Figure 8 **Separation of blood components using Ficoll**. After centrifugation, cells with densities lower than Ficoll (PBMCs) are visible at the top. Cells with higher densities then Ficoll are at the bottom.

For the M1 and M2 macrophage experiments after cell culture with the extracts, two different protocols were used. Protocol 1 was performed aiming to investigate cytokine release from M1 and M2 macrophages after the exposure of the cells to the extracts. Protocol 2 was used in an attempt to verify whether the extracts could influence monocyte differentiation into macrophages and the M1/M2 polarization phase.

Protocol 1: the cells were stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF; 80 ng/mL) and macrophage colony-stimulating factor (M-CSF, 10 ng/mL) for 5 days to induce their polarization into M1 and M2 macrophage phenotypes. Stimuli were purchased from Miltenyi, Berlin, Germany. After 5 days, the cell culture medium was changed, and the cells were exposed to the material extracts and LPS (0.1 µg/mL)/interferon gamma (IFN-ɣ) (PeproTech, USA; 200 U/mL) for 24 h at 37°C.

Protocol 2: the phases of differentiation and polarization were reproduced in separate steps. The differentiation of freshly isolated monocytes into basic macrophages was promoted by stimulating the cells with M-CSF (10 ng/mL) for a total of 5 days. Basic macrophage polarization into respective M1 and M2 phenotypes was achieved by adding interferon gamma (IFN-ɣ; 200 U/mL) and interleukin-4 (IL-4; CellGenix, Freiburg, Germany; 1000 U/mL) into the cell culture medium on day 3. Figure 9 illustrates the cell stimulation and the exposure to the extracts.

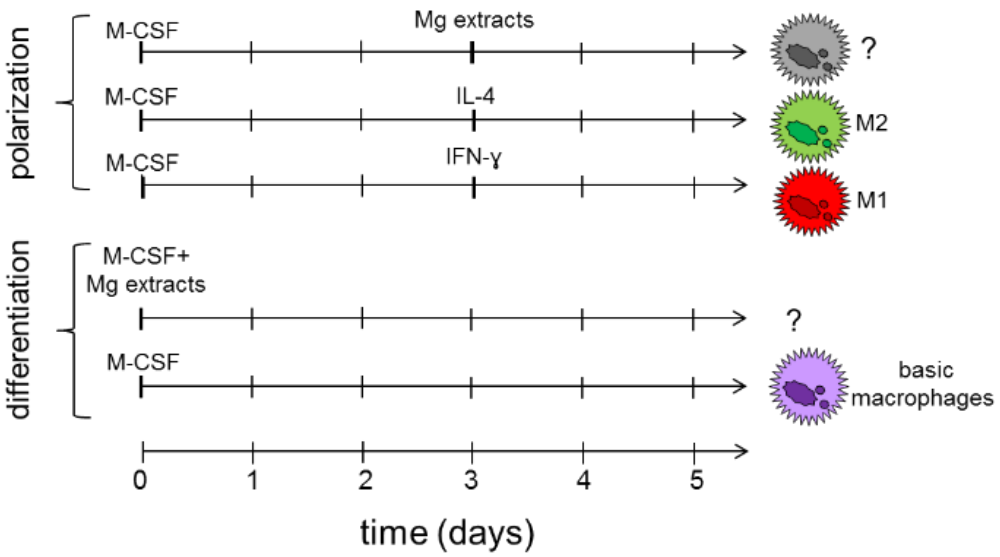


Figure 9 **Macrophage differentiation and polarization phases *in vitro*.** Fresh M-CSF-stimulated monocytes were cultivated with and without (control) the extracts for 5 days. For the polarization phase, the extracts were added after 3 days of basic macrophage cultivation. IFN-γ and IL-4 were used as positive controls for polarized M1/M2 macrophages, respectively.

The capacity of the cells to respond to inflammatory stimulation after extract exposure was also investigated. In a separate experiment, fresh monocytes were differentiated and polarized with and without extracts, as previously described. After 5 days, the stimuli and the extracts were removed, and fresh medium was added. Stimulation with LPS (100 ng/mL) was performed for an additional 24 h.

Cell culture with primary cells was performed using serum-free media, while the extracts were prepared using RPMI 1640 medium + 10% FBS (Paragraph 4.1). In an attempt to verify the influence of the different media used, RPMI medium + 10% FBS without the extracts (named “MOCK”) was used as a negative control and was added to the cell culture conditions in a 10-fold dilution with CellGro® medium (replicating the conditions used with the extracts).

4.2.4 Counting of the cells

The cells were counted using either the classic Burker chamber method or the cell counter CASY® Model TT (Roche, Mannheim, Germany). CASY technology is an automated method for the determination of cell density and viability. The measurements are based on the fact that the cell membrane is an electric insulator; therefore, when the cell is intact, a current cannot pass through it (Figure 10). The difference in the applied current going in and the current coming out

is automatically determined by the instrumentation and refers to the diameter of the targeted cells. For each cell type used, the instrument was programmed following the manufacturer's protocol.

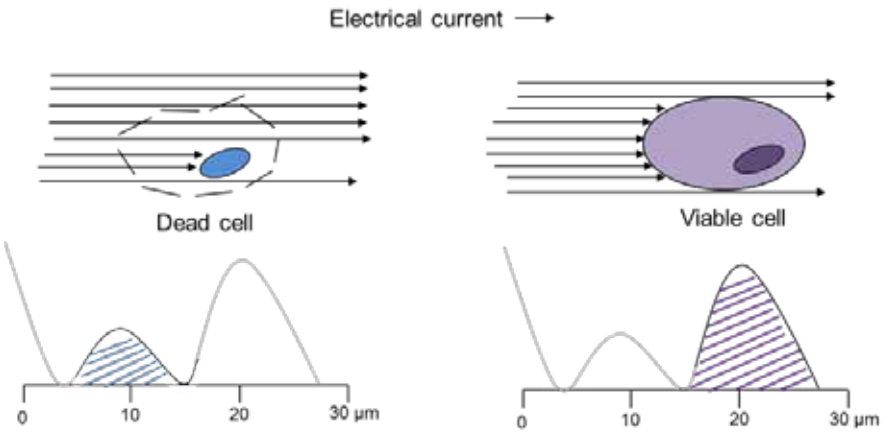


Figure 10 **Principle of CASY measurements**. The difference in the electric impulses into and out of a culture defines the cell density and viability according to cell size. This figure was reproduced from the instrument manual.

4.2.5 Live/dead staining

Cell viability was also checked using a Viability/Cytotoxicity Kit for mammalian cells (Eugene, Oregon, USA). The test is based on the chemical reaction with calcein AM and Ethidiumhomodimer-1 (EthD-1) and a cellular enzymatic reaction. Live cells are distinguished by intracellular esterase activity, which converts the non fluorescent cell-permanent calcein AM to the intensely fluorescent calcein. The reaction produces a green fluorescence in live cells. EthD-1 is absorbed when the cell membrane is compromised. The interaction between with EthD-1 and DNA produces a red fluorescent colour, an indication of dead cells. The test was performed according to the manufacturer's protocol. Cells were washed with PBS and incubated for 20 minutes with 2 mL of staining solution (5 mL PBS + 2 µL Calcein AM + 5 µL Ethidiumhomodimer-1). The staining solution was then removed, and new PBS was added. The cells were visualized using fluorescence microscopy (Nikon GmbH, Dusseldorf, Germany). The light value intensities of live (green) and dead (red) cells were obtained using NIS-Elements Microscope Imaging Software 3.2 (Nikon GmbH, Dusseldorf, Germany).

4.2.6 Cytokine Proteome Profiler Array

The qualitative method “Proteome Profiler Array” (Human cytokine array panel A; R&D, USA) was used for the simultaneous identification of 36 extracellular signal molecules (markers). Proteins analysed with this methods were classified in a total of 6 groups and subsequently were named according their biological activities (Table 3).

Table 3 **Cytokines detected with the array panel.** The 36 cytokines detected with the array panel were classified in 6 groups according their common biological effects on inflammatory reactions and macrophage activity. The specific biological function of each protein is explained in Table 2.

Cytokines	Name group	Biological activities
C57Ca, I-309, Sicam-1, IL-16, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, IL-8	Chemotaxis	Promotion of cell adhesion on the endothelium and following recruitment into the tissue
IL-27, IL-12p70, G-CSF, GM-CSF, IL-12, IL-5	M1 factors	Factor involved in M1 macrophage phenotype polarization
CD40L, GROα	Lymphocytes activators (lymph activ)	Protein involved in the acquired immune response
IL-6, IFN-ɣ, IL-1α, IL-1β, TNF-α, sTREM-1, IL-17, IL-17E, IL-23, IL-32α	Inflammatory cytokines	Amplification of inflammation with consequent tissue destruction
IL-13, IL-4	M2 factors	Factor involved in M2 macrophage phenotype polarization
IL-1Ra, IL-10	Anti-inflammatory cytokines	Inhibition of inflammatory reactions and tissue proliferation

This qualitative method consists of captured antibodies spotted on a nitrocellulose membrane. Each membrane contains 36 different captured antibodies printed in duplicate. The procedures were followed according to the manufacturer’s protocols. A volume of 15 µL of

detection antibody cocktail was added to the supernatant (0.5 mL), and samples were incubated for 1 h at room temperature. The cytokine detection antibody complex obtained was then incubated with the nitrocellulose membrane overnight. Unbound proteins were removed by subsequent washing steps, while bounded cytokines were incubated with streptavidin conjugated to horseradish peroxidase (streptavidin-HRP) and chemiluminescent detection reagents for 30 minutes in the dark. The light produced at each spot was detected using a ChemiDoc MP System (BioRad, Germany). The spots were visualized using UV trans illumination light (n=3 set exposed time of 0.500 sec) and acquired as an image using Image Lab software version 4.1 (Biorad, Germany). The intensity light value of each spot was automatically obtained using the program. Further calculations were performed using Excel. The average signal (pixel density) of a pair of duplicate spots representing each cytokine was subtracted from the average the signal background. As each cytokine is spotted in duplicate, no standard deviation was calculated. The use of this method was considered essential as a “screening” for the identification of essential proteins produced after cell treatments. Of the 36 cytokines investigated, all were associated with inflammation. The capacity of the cell to produce or not such molecular signals *in vitro* was considered the initial point for the selection and further quantification of defined cytokine groups.

4.2.7 Enzyme-linked immunosorbent assay (ELISA)

Quantitative measurements of IL-1β, TNFα IL-10, MCP-1, IL-8, IL-1Ra and OPN were performed using the sandwich ELISA test method. Cytokines were carefully selected due their recognized impact on macrophage activities during FBR. The cytokine OPN was measured using the OPN detection ELISA kit from Cloud-Clone Corp (Houston, USA), while measurements for all other cytokines were performed using a DuoSet ELISA (R&D; Germany).

The sandwich ELISA is an indirect method based on the quantification of an analyte between two layers of antibodies called capture and detection antibodies. Each assay was performed according to the manufacturer’s protocol. For each cytokine, the specific antibody (capture antibody) was diluted in PBS to the indicated concentration and incubated overnight in a 96-well polystyrene microplate. For each well, a volume of 100 µl was used. Then, unbound antibodies were removed with several washing steps. A solution containing FBS (300 µl/well) was used to block non-specific binding to the plate surface. After 20 min of incubation and washing steps, 100 µl/well of sample and standard provided in the kit were incubated for 2 h at room temperature.

The tests used for the determination of the selected cytokines have the limitation of determining the maximum concentration in the range of 250-300 pg/mL. Therefore, it was necessary to establish suitable dilution factors for each cytokine and condition investigated. For the cytokines released by DiffU937 cells (either alone or in co-culture), it was necessary to perform a 1:10 dilution of the sample for the determination of IL-1β, TNFα, IL-10, IL-1Ra and a dilution of 1:1000 for the detection of MCP-1, IL-8 and OPN. In the case of primary macrophages, the dilutions were strongly dependent on the donors. No dilution factor was necessary for cytokines produced by Saos-2 cells in solo-culture. After incubation, the secondary antibody (detection antibody) and streptavidin (both100 µl/well) were added to detect the cytokine target. The incubation times for the detection antibody and streptavidin were 2 h and 20 min, respectively. Washing was performed between the steps. Finally, the tetramethylbenzidine (TMB) substrate solution (100 µl/well) was added. The resulting blue colour developed in proportion to the amount of the analyte present in the sample. Colour development was stopped using a provided solution of 2 N H_2SO_4 (50 µl/well), which turned the colour in the wells to yellow. Finally, the measurements were performed at 450 nm optical density (OD) using a Tecan Sunrise ELISA reader (TECAN Deutschland GmbH, Crailsheim, Germany). The test was performed in biological and technical triplicate for each cytokine tested.

The final concentration was obtained with the interpolation of the OD value of the analyte with the linear regression of the standard. Both linear regression and interpolation were calculated using the program excel according to the following formulas:

Linear regression:	Interpolation
$y = a + b(x)$	$x = [(y - a)/b]\ fd$

Where:

a= intercept point of the regression line and the y axis

b= slope of the regression line

x= pg/mL

y= OD value

fd= dilution factor

Increased and decreased protein levels were also calculated by comparing the control (cells cultured only with medium) with DiffU937 cells exposed to their the extracts or the $MgCl_2$ solution, Calculations were performed only in case where the differences between the control and the stimulated cells (samples) were statistically significant (p= 0.05) using the following formula:

(CV control – CV sample) / (CV control)

Where:
CV= cytokine value

From the results obtained, only value differences > 0.1 were reported in the tables

4.2.8 Real-time qualitative polymerase chain reaction (RT-qPCR)

Analyses of the influences of the extract materials at the gene level were performed using the RT-qPCR technique .First, RNA was extracted using an RNeasy Mini Kit (250) following the manufacturer's manual. The RNA concentration was obtained by measuring the absorbance at 260 nm (A_{260}). Additionally, the ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) was used to verify the purity. The measurements were performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Bonn, Germany).

The reverse transcription of RNA into complementary DNA (cDNA) was performed using an Omniscript RT Kit (Qiagen, Hilden, Germany) as indicated:

10x Buffer RT	2 µL
dNTP mix	2 µL
RNase inhibitor	1 µL
Omniscript Reverse transcriptase	1 µL
RNA template	100 ng
RNase-free water	variable
Final volume reaction	20 µL

The reaction was promoted by incubating the samples at 37°C for 60 min using a thermal cycler (Eppendorf Mastercycler, Hamburg, Germany). The cDNA obtained was diluted 1:10 with ddH_2O and stored at -80°C until use.

The RT-qPCR was performed using a SsoFastEvaGreen Supermix kit (Bio-Rad Laboratories GmbH, Munchen, Germany), which employs the dye SYBR Green as a fluorescent reporter of gene amplification. Reactions were performed as indicated:

SsoFast EvaGreen supermix	16.25 µL
Forward primer	1.63 µL
Reverse primer	1.63 µL
cDNA	4.0 µL
ddH_2O	9.0 µL
Total final volume	32.5µL

From this final volume, 10 µL/well was used. The reaction was performed using a CFX96 Touch real-time PCR detection system with CFX Manager Software (Bio Rad, Munich, Germany; version 3.1). The RT-qPCR was programmed according to the following steps:

Step	**Duration**	**Temperature**	**Cycle**
Initial duration	30 sec	95°C	1
Denaturation	15 sec	95°C	60
Primer-annealing	15 sec	60°C	
Elongation	30 sec	72°C	1
Denaturation	30 sec	95°C	1
Melt curve	5 sec	35-95°C	0.5°C/step
Hold	∞	4°C	1

Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany) and were used at a concentration of 20µmol/L. Table 4 summarizes the sequences of the target *CD36, TLR2, ICAM1, NF-kB; IL-1β, OPN* and housekeeping *(Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH); Beta-2-Microglobulin (B2M*) genes. The gene targets were chosen according their specific roles in FBR (Paragraph 2.2).

Table 4 **Primer sequences of the genes targeted**

Gene	**Forward (5'-3')**	**Reverse (5'-3')**
Target genes		
CD36	GCCAAGGAAAATGTAACCCAGG	GCCTCTGTTCCAACTGATAGTGA
TLR2	GGGTTGAAGCACTGGACAAT	CTTCCTTGGAGAGGCTGATG
ICAM-1	TCTGTGTCCCCCTCAAAAGTC	GGGGTCTCTATGCCCAACAA
NF-kB	CTGGAAGCACGAATGACAGA	CCTTCTGCTTGCAAATAGGC
IL-1β	CAGCTACGAATCTCCGACCAC	GGCAGGGAACCAGCATCTTC
OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
Reference gene		
GAPDH	GTCGGAGTCAACGGATTTG	TGGGTGGAATCATATTGGAA
B2M	TGCTGTCTCCATGTTTGATGTAATCT	TCTCTGCTCCCCCACCTCTAAGT

The relative quantity (ΔCt) for any gene of interest (GOI) was automatically calculated using CFX Manager Software and according to the formula:

$$\text{Relative quantity}_{\text{sample (GOI)}} = E_{GOI}^{\text{cq (control) – Cq (sample)}}$$

Where:

- E = Efficiency of primer and probe set. This efficiency is calculated with the formula (% Efficiency * 0.01) + 1, where 100% efficiency = 2
- Cq (control) = Average Cq for the control sample
- Cq (sample) = Average Cq for any samples with a GOI

4.2.9 Fluorescence-activated cell sorting (FACS)

FACS measurements were performed to investigate the expression of specific markers in primary M1/M2 macrophage subpopulations. The basic principle of the FACS is the analysis of the fluorescence and light scatter proprieties of cells.

Adherent macrophages were harvested using a pipette to gently scrape the cells from the well plates. Once pelleted, the supernatant was aspirated, and the cells were suspended with 0.5 mL of staining buffer (PBS + 2% heat-inactivated FBS (56°C; 30 minutes)containing 10-15 µL of conjugated mouse anti-human antibodies (BD Pharmingen, Germany) against cluster differentiation (CD)11b (AP), CD14 (Pacific Blue), CD64 (FITC) and CD163 (PE), which were added to each sample. The CDs chosen are the target surface proteins of myeloid-lineage cells (CD11b) and M1 (CD14, CD64) and M2 (CD163) macrophages. The CD markers used to identify the macrophage subpopulations were selected according to the literature [14], [147], [148], [150], [151], [153], [180]. After incubation for 15 minutes at 4°C, the cells were centrifuged (250xg; 5 min, 4°C) and washed using the staining buffer. In the final step, the cells were fixed with 0.25% of paraformaldehyde (PFA) in FACS-buffer for 30 min at room temperature. The measurements were performed using a BD LSR II flow cytometer, and the results obtained were analysed using FlowJo 7.6.5 software.

4.3 Statistical analysis

Statistics were performed using Origin 9 software. Normal distribution was calculated based on the standard deviation. Statistical analysis was conducted using a one-way ANOVA followed by Bonferroni's. The Bonferroni correction is a multiple comparison test based on the idea to verify the family wise error rate (FWER) for each test performed considering *m* hypothesis. In the present work *m* hypothesis verified were the correlation between treatment 37°C *vs* 39°C and control (DiffU937) *vs* cells cultured with extracts. Statistics for qPCR were obtained using CFX Manager Software 3.0.

5 Results

5.1 Establishment of the inflammatory *in vitro* model

The differentiation of U937 cells (which are in a pro-monocyte stage) into macrophage cell types was the essential point for the purpose of this work. The cell stimulation and culture were performed as described in paragraph 4.2.1. As expected, cells stimulated with PMA change their morphology. The cells with and without (control) PMA stimulation are presented in Figure 11. The pictures of cells were taken after a total of 4 days of cell culture and clearly show that, compared to the control, the PMA-stimulated cells were bigger in size and had acquired a well-defined spherical shape. Considering these observations, the U937 cells stimulated with PMA were named DifferentiatedU937 (DiffU937) cells.

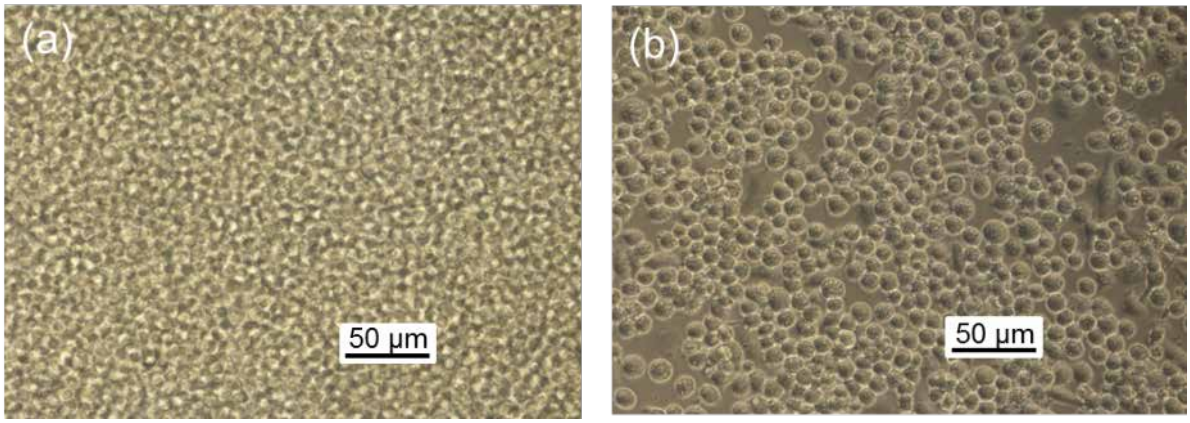


Figure 11 **U937 cell differentiation into macrophage cell types.** U937 cells before (a) and after (b) PMA stimulation.

Besides the shape, macrophages status is defined by the ability of the cells to produce a wide range of inflammatory cytokines. In an attempt to confirm that stimulation with PMA induces DiffU937 cells into macrophage cell types, the production of inflammatory cytokines was measured using the proteome profile array (Paragraph 4.8). The results obtained are presented in Figure 12. In coherence with previous observations, DiffU937 cells released an increased spectrum of cytokines compared to unstimulated cells. Particularly evident was the up-regulation of the chemokines group. The only exception was the protein MIF, which was down-regulated after differentiation.

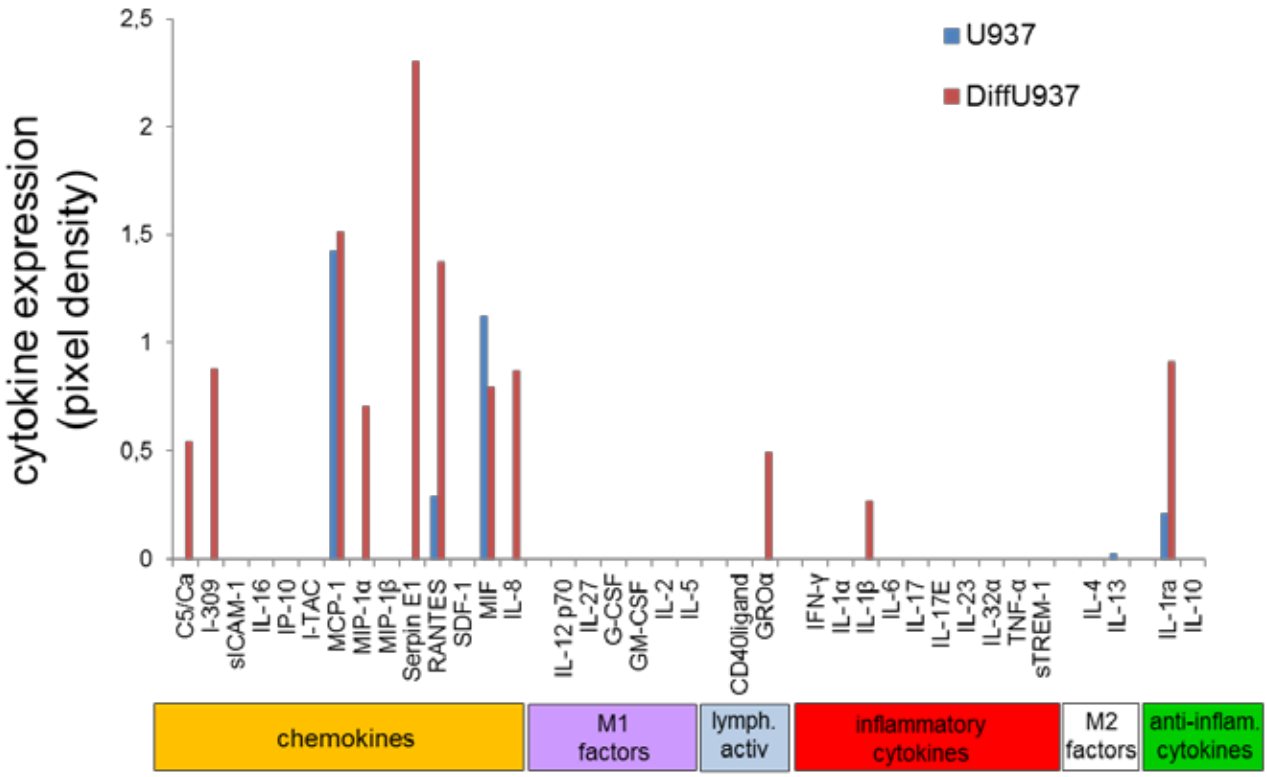


Figure 12 **Cytokine production.** Overview of the cytokines produced before (U937, blue) and after (DiffU937, red) stimulation with PMA. The 36 cytokines analysed were classified in six groups according to their biological function.

An inflammatory *in vitro* model was then established by culturing the cells at the higher temperature of 39°C for 1 and 3 days (Paragraph 4.2.1). The first step was to observe whether the higher temperature could influence cell viability; therefore, live/dead staining was performed, and the results are presented in Figure 13.

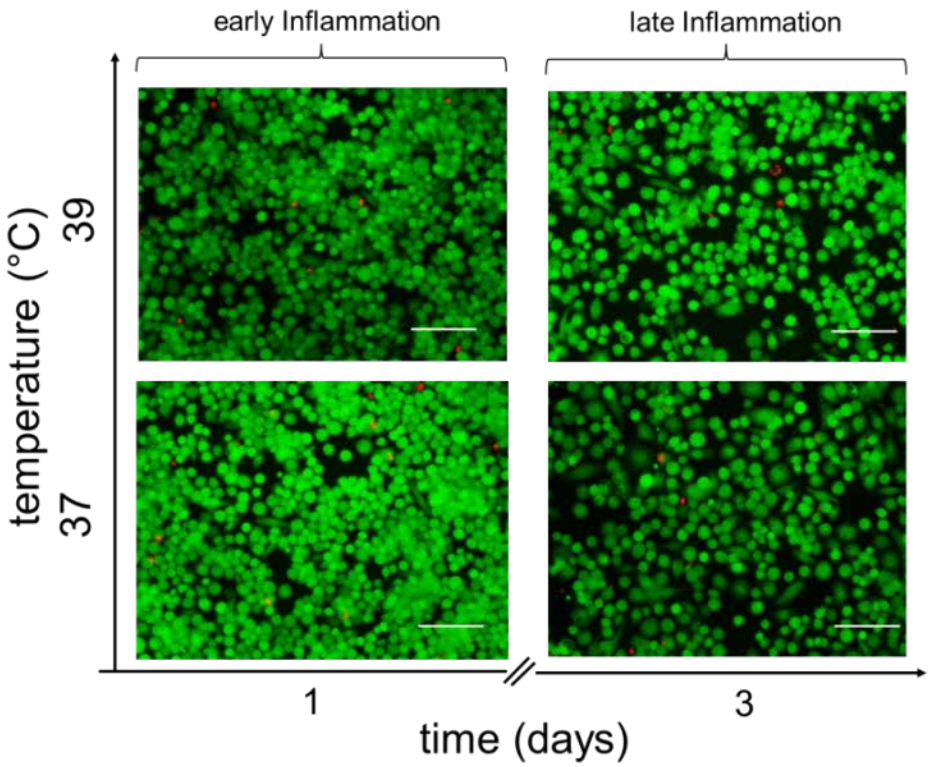


Figure 13 **Live/dead staining**. Viability of the cells cultured after 1 (early inflammation) and 3 days (late inflammation) at 37°C (control) and 39°C. Scale bar is 100µm.

As observed, no differences in cell viability when they were cultured at 39°C were noted after 1 and 3 days compared with the cells cultured at the standard temperature of 37°C.

After validation of the use of the different temperature condition, the next step aimed to investigate the influence of the mentioned condition on cytokine release; therefore, all cytokine protein levels were measured again using the ELISA method.

As previously mentioned (Paragraph 2.2), the inflammatory cytokines IL-1β and TNFα are the principal cytokines released in the FBR and are involved in the amplification of the inflammatory reaction (Paragraph 2.2). As presented in Figure 14, surprisingly, inflammatory cytokine release was downregulated at 39°C. In particular, the degrees of IL-1βand TNFα down-regulation were significant after 1 and 3 days, respectively ($p < 0.001$ = ***).

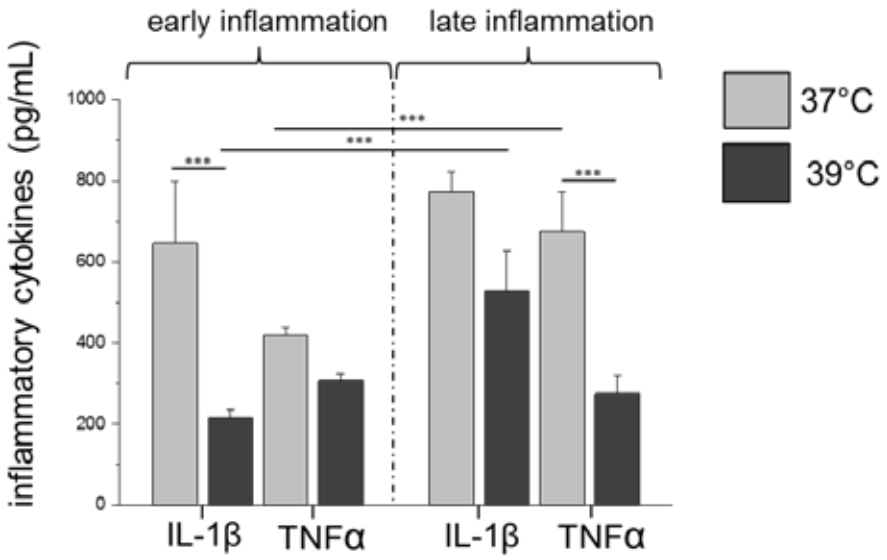


Figure 14 **Inflammatory cytokine production.** Release of IL-1β and TNFα in DIffU937 cells after they were cultured at 37°C and 39°Cfor 1 (early inflammation) and 3 days (late inflammation). Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

Is also interesting to note that targeted proteins were released differently according to the time factor: IL-1β was significantly up-regulated after 3 days in cells cultured at 39°C, while TNFα was increasingly released only after cells were exposed to the standard temperature condition.

In the tissue microenvironment, the pyrogenic and destructive powers of the inflammatory cytokines are minimized thanks to the release of anti-inflammatory cytokines. The proteins IL-1Ra and IL-10 were the two key markers of this process that were analysed. IL-1Ra was chosen as an inhibitory factor of IL-1β, while IL-10 was chosen as a marker directly involved in new tissue proliferation (Paragraph 2.2). As presented in Figure 15, the two targeted cytokines were released at comparable levels in cells cultured at standard and higher temperatures after 1 day. At day 3, IL-10 was significantly up-regulated at 39°Cwith respect to 37°C ($p < 0.001$ = ***). No differences were observed in the production of IL-1Ra after 3 days of cell culture at the different temperature conditions.

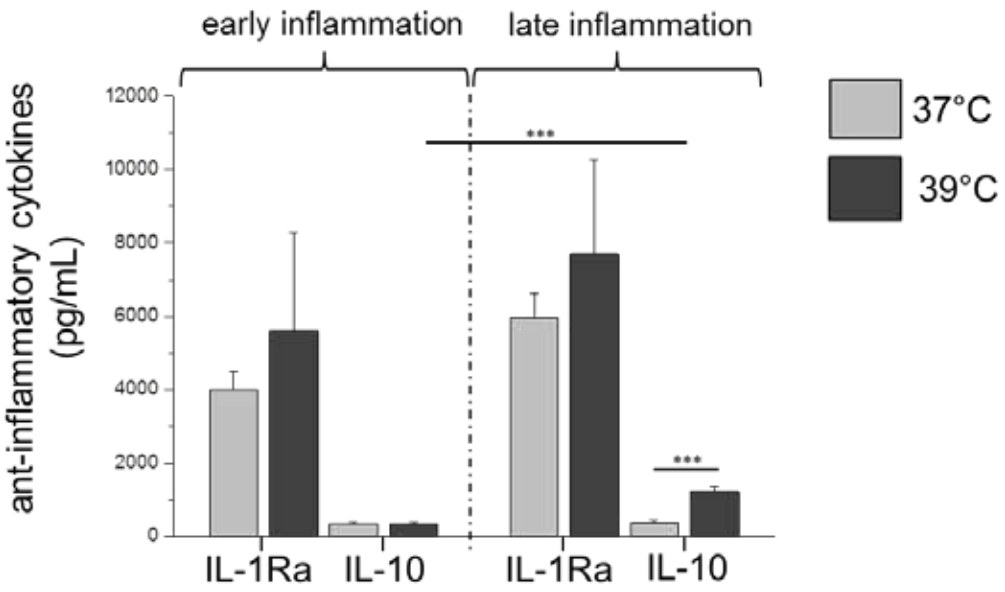


Figure 15 **Anti-Inflammatory cytokine production**. Release of IL-1Ra and IL-10 in DIffU937 cells after they were cultured at 37°C and 39°C for 1 (early inflammation) and 3 days (late inflammation). Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

Considering the time factor, significantly increased IL-10 expression was observed in DiffU937 cells cultured at 39°C. *In vivo,* the dynamic microenvironment is supported thanks to the constant cells turn over; the chemokines MCP-1 and IL-8 are the principal players of this process (Paragraph 2.2). This aspect was also evident in the proposed inflammatory *in vitro* model (Figure 16).

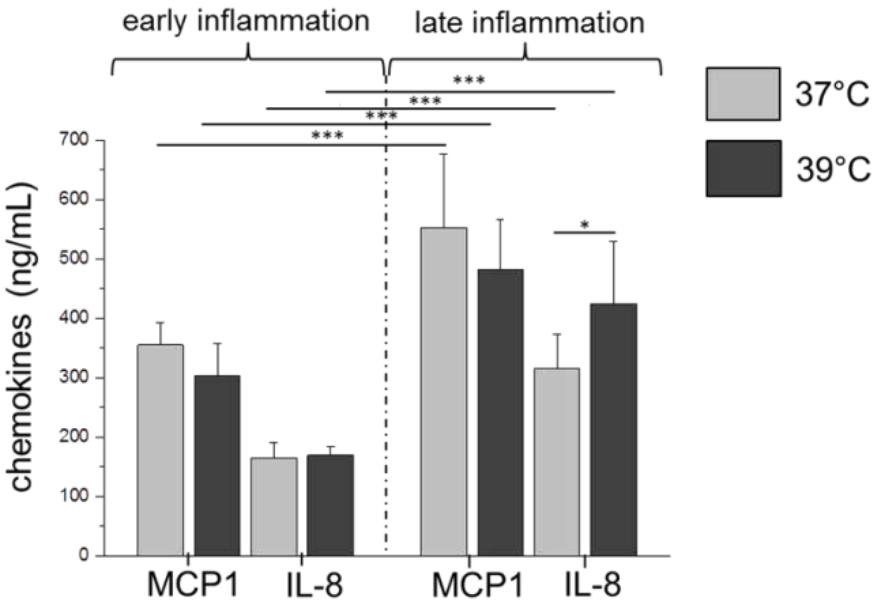


Figure 16 **Chemokine production.** Release of MCP-1 andIL-8in DiffU937 cells after they were cultured at 37°C and 39°C for 1 (early inflammation) and 3 days (late inflammation). Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

As expected, DiffU937 cells released chemokines mainly according to the time factor. In fact, significant up-regulations (p< 0.001 =***) of MCP-1 and IL-8 were clearly observed after 3 days at standard and higher temperature conditions. It is interesting to note that while there were no differences in either chemokine with respect to the temperatures at day 1, there was a significantly increased release of IL-8 (p< 0.05 = *****) at 39°C compared to 37°C. These results would suggest a different mechanism in the release of the two targeted proteins according to the parameters investigated.

The fusion of macrophage cell types is a typical event of the FBR once the cells are recruited into damaged tissue. As described in Paragraph 2.2, the increase of OPN is associated with an inhibition of FBGC formation. The marker OPN is also generally expressed in activated macrophage cell types. In this work, the marker was investigated to observe the modulations of the protein only in response to time and temperature conditions (Figure 17).

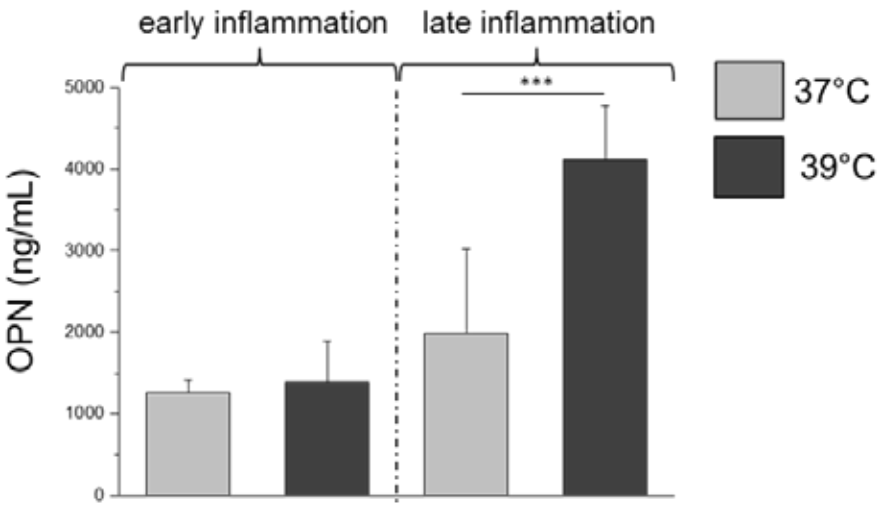


Figure 17 **OPN production.** Release of OPN in DiffU937 cells after they were cultured at 37°C and 39°C for 1 (early inflammation) and 3 days (late inflammation). Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p< 0.05$ = *, $p< 0.01$ = **, $p< 0.001$ = ***).

As expected, the protein was released in all of the parameters considered in the inflammatory *in vitro* model (Figure 17). It was clearly observed that OPN production was comparable at 37°C and 39°C at early inflammation, while in the late inflammatory phase, the marker was significantly released in the febrile condition compared to the standard temperature.

5.2 Culture of macrophage cell types with $MgCl_2$

Despite the material composition, Mg is the essential element released in the microenvironment as consequence of material degradation. As previously described (Paragraph 2.1.2), concentrations higher than 1 mM decrease the release of inflammatory cytokines, promoting IkB stability. Because the influence of increasing [Mg^{2+}] on macrophage-mediated cytokine release was unclear, the first approach was to observe the modulation of the investigated molecular targets (inflammatory cytokines (I), anti-inflammatory cytokines (II), chemokines (III) OPN (IV) after cells were exposed to 1, 5 and 10 mM of $MgCl_2$.

(I) Inflammatory cytokines

As displayed in Figure 18, the cells significantly decreased their release of IL-1β at 39°C compared 37°C after they were cultured with 1 or 10 mM of $MgCl_2$ at early and late inflammation ($p< 0.001$), respectively. After 1 day of cell culture with 5 mM of $MgCl_2$, TNFα production was

clearly down-regulated in the cells exposed to higher temperature compared to cells exposed to standard temperature. This trend, with respect to the temperature condition, was also observed at late inflammation. At this time point, the cytokine release decreased significantly in cells cultured with 1 and 5 mM of $MgCl_2$ (p< 0.001). Surprisingly, it was noted that the protein was strongly produced after they cells were cultured with 10 mM of $MgCl_2$ (p< 0.001).

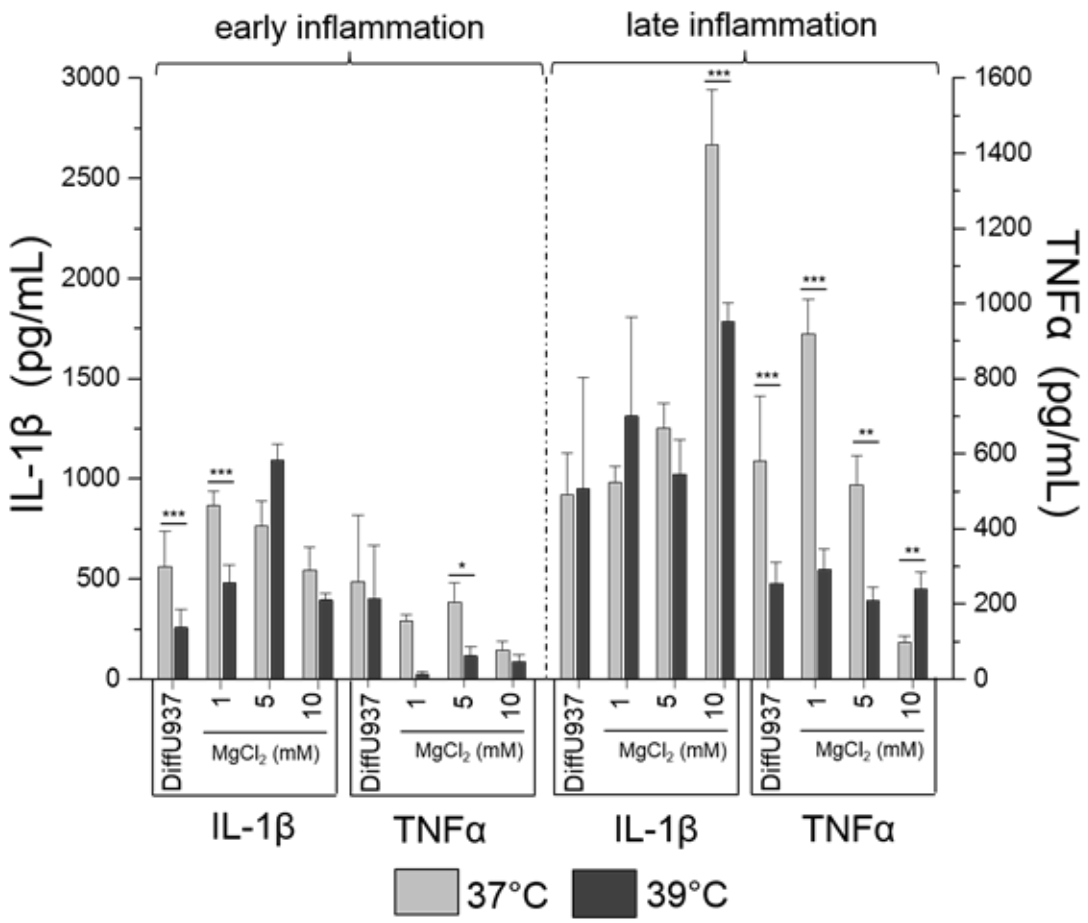


Figure 18 **Inflammatory cytokine concentrations after cell stimulation with $MgCl_2$.** IL-1β and TNFα release after exposure to 1, 5 and 10 mM $MgCl_2$ for 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Cells cultured at standard temperature (37°C) serve as the controls. Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant difference between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

(II) Anti-inflammatory cytokines

In the reproduced inflammatory condition of higher temperature, the release of the cytokine IL-1Ra was generally increased compared to the physiologic temperature of 37°C (Figure 19).

At early inflammation, this trend was statistically significant in cells cultured with the concentrations of 1 and 5 mM $MgCl_2$ (p< 0.01; p< 0.05). At late inflammation, the high regulation of IL-1Ra production was significant for all three different concentrations tested (p< 0.05; p< 0.001). The results obtained from IL-10 measurements show that, similarly to IL-1Ra, macrophages exposed to the temperature of 39°Chad a tendency to increase their release of the protein compared to the cells exposed to the 37°C temperature. This trend was noted in both early and late inflammatory phases. A statistically significant up-regulation was obtained in case of cells cultured after 3 days with the concentration of 10 mM $MgCl_2$.

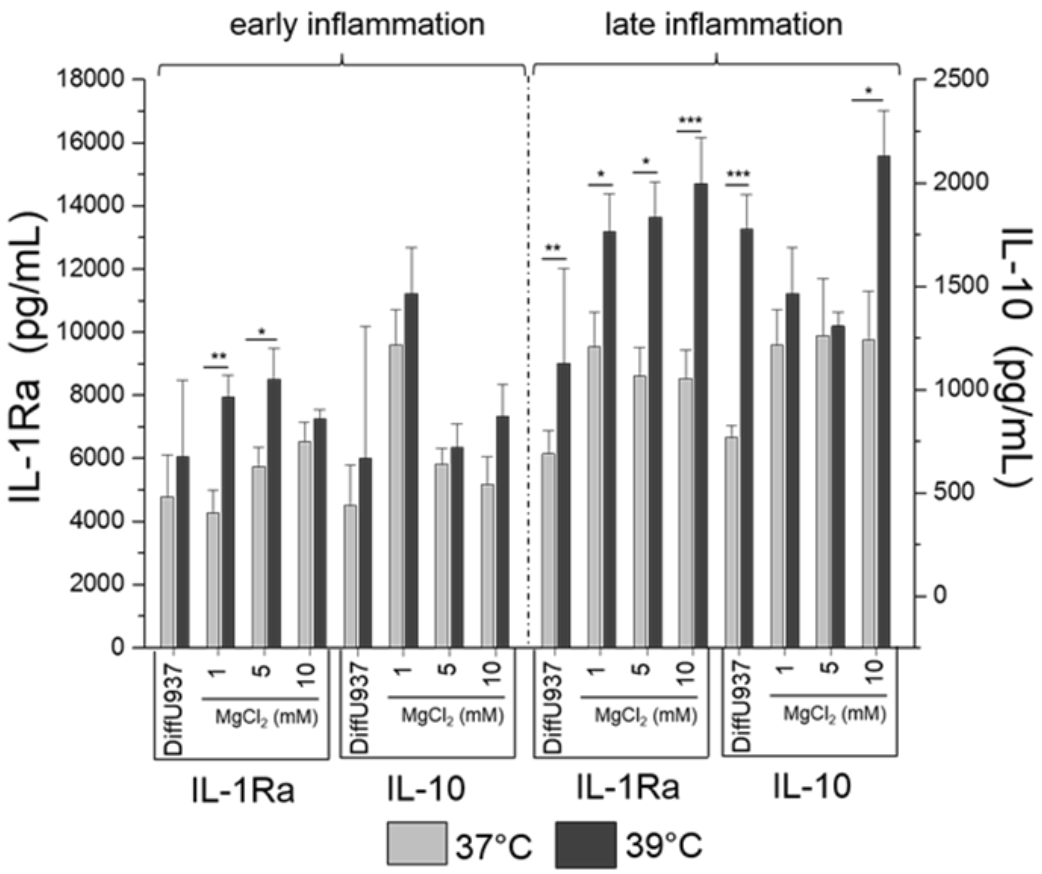


Figure 19 **Anti-inflammatory cytokines after stimulation of cells with $MgCl_2$.** IL-1Ra and IL-10 release after stimulation with $MgCl_2$ for 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Cells cultured at standard temperature (37°C) served as the controls. Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

The physiologic inflammatory reaction requires a balance between inflammatory and anti-inflammatory cytokine activities. Such a status of equilibrium can be represented by the IL-

1Ra/IL-1β ratio (Paragraph 2.2). The ratio in the production of these two molecules was calculated to observe the balance in the production of these two markers in the inflammatory *in vitro* model (control) and the influence of increasing Mg^{2+} concentration in such an equilibrium. The results obtained in the calculation of the ratio between IL-1Ra and IL-1β are displayed in Figure 20. Upon comparing the two temperature conditions, the anti-inflammatory cytokine was highly up-regulated with respect to the inflammatory cytokine after 1 day of cell culture with 1 mM of $MgCl_2$ at 39°C ($p < 0.001$). This trend was also clearly evident at late inflammation in cells cultured with 1 mM of $MgCl_2$ at 39°C ($p < 0.01$). In the other conditions measured, the production of IL-1Ra with respect to IL-1β was comparable at both the standard and higher temperatures.

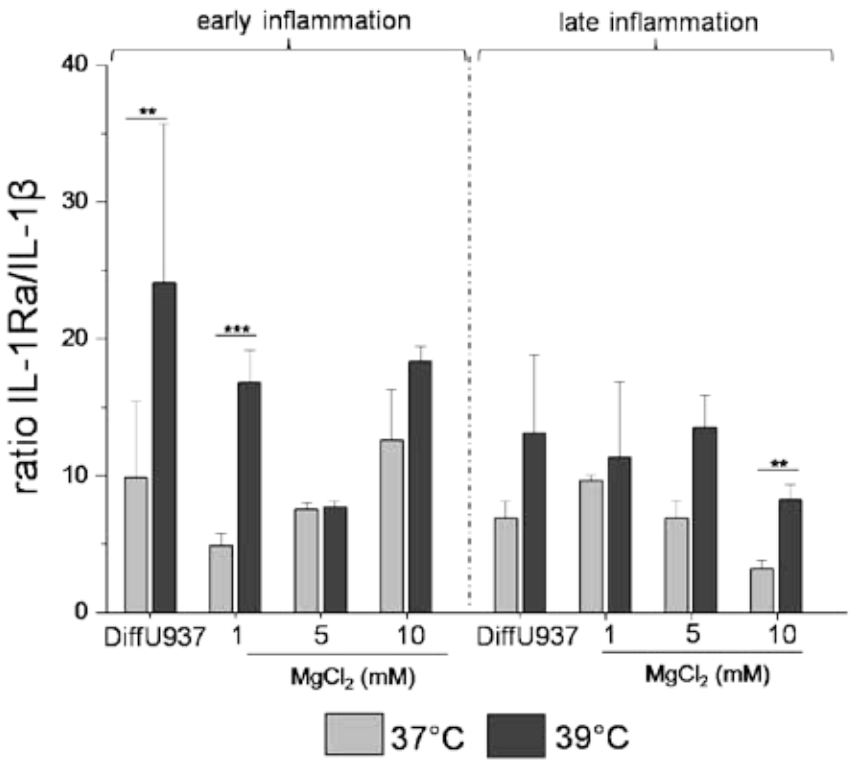


Figure 20 **IL-1Ra /IL-1β ratio in DiffU937 cells stimulated with $MgCl_2$.** IL-1Ra production with respect to IL-1β after exposure to $MgCl_2$ (1, 5, 10 mM) for 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

(III) Chemokines

As presented in Figure 21, the MCP-1 results obtained show that the cells cultured with 1 and 5 mM of $MgCl_2$ had the clear tendency to down-regulate the release of the protein at 39°C compared to 37°C after 1 and 3 days of cell culture.

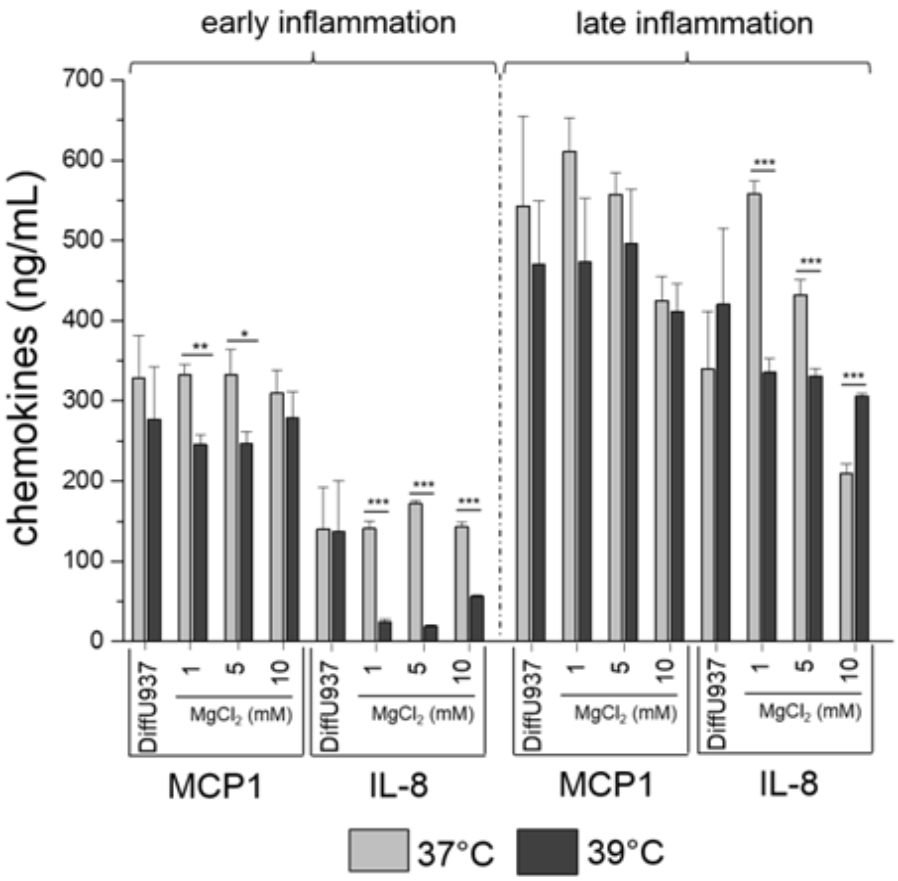


Figure 21 **Chemokines in the culture medium after stimulation of cells with $MgCl_2$.** IL-1Ra and IL-10 release after stimulation with $MgCl_2$ for 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Cells cultured at the standard temperature (37°C) are the controls. Bars represent means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

Statistical significance between the two temperature conditions was obtained only in the early inflammatory phase (p< 0.01; p< 0.05). For IL-8, protein release levels at early and late inflammation were comparable in cells cultured with 1 and 5 mM of $MgCl_2$. After 1 and 3 days, in fact, the decrease in protein production was clearly observed in cells cultured at the high temperature compared to cells cultured at the standard temperature (p < 0.001). The results

obtained with cells cultured with the highest $MgCl_2$ concentration were interesting, as there was an opposite regulation of the protein release with respect to the two different temperature conditions. In the early inflammatory phase, IL-8 was down-regulated at 39°C ($p < 0.001$), while in the late inflammatory phase, the protein was clearly upregulated at the higher temperature compared to the standard temperature of 37°C ($p < 0.001$).

(IV) OPN release

As presented in Figure 22, the protein release was downregulated at 39°C compared to 37°C in early inflammation after stimulation of DiffU937 cells with 5 and 10 mM $MgCl_2$ ($p < 0.01$; $p < 0.05$). At late inflammation, a significant increase of OPN release was noted in DIffU937 cells at the higher temperature with respect to standard temperature ($p < 0.01$). No differences were observed with respect to the different temperatures and $MgCl_2$ concentrations.

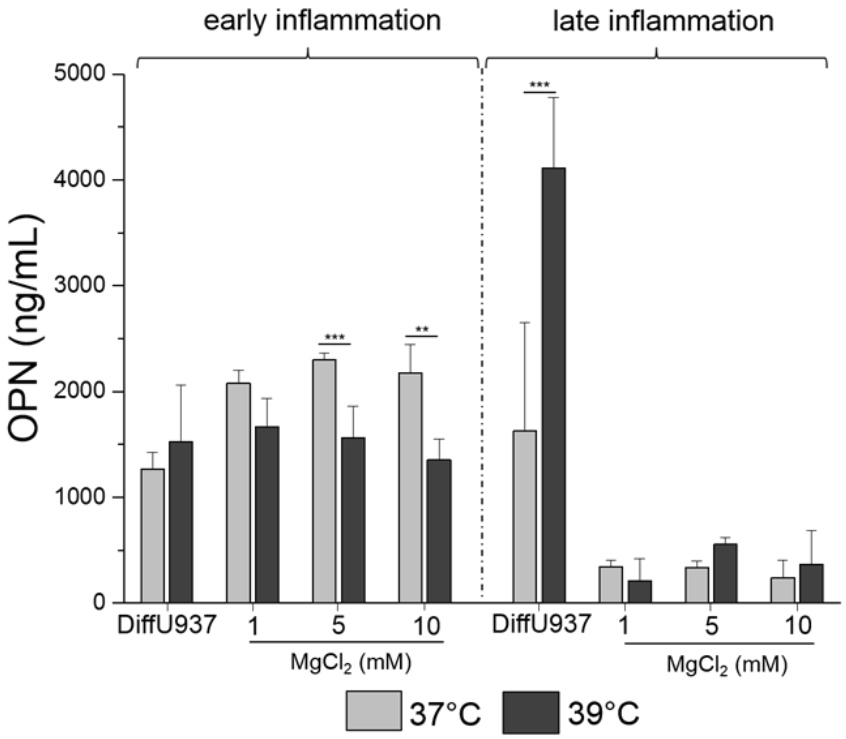


Figure 22 **OPN release after cell stimulation with $MgCl_2$.** Protein production after cell stimulation with $MgCl_2$ for 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Cells cultured at the standard temperature (37°C) are the controls. Bars represent the means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

5.3 Stimulation of the cells with the extracts

The first goal was to characterize the extract solutions in terms of their osmolality and pH values and their element contents. Table 5 shows that after dilution, all three extracts had the same osmolality as RPMI cell culture medium (control) and basic pH values.

Table 5 **Extract compositions**. Concentrations of the total Mg, Ag and Gd released after dilution of the pure extracts

	Mg	**Mg-2Ag**	**Mg-10Gd**	**RPMI medium**
Osmo (Osmol/kg)	0.300	0.300	0.303	0.300
pH	7.9	8	8.1	7.5
Mg (mM)	1.15	1.32	6.15	0.45
Gd (mM)	–	–	3.62×10^{-3}	–
Ag (mM)	–	2.13×10^{-3}	–	–

The Mg-10Gd extract solution had a higher concentration of total Mg compared to the Mg-2Ag and Mg extracts. The Gd and Ag elements were released on the order of µM.

To verify whether the use of the extracts at temperatures higher than 39°C influences cell viability, live/dead staining was performed. The results displayed in Figure 23 show that the viabilities of cells exposed to the degradable products of Mg, Mg-10Gd and Mg-2Ag were comparable.

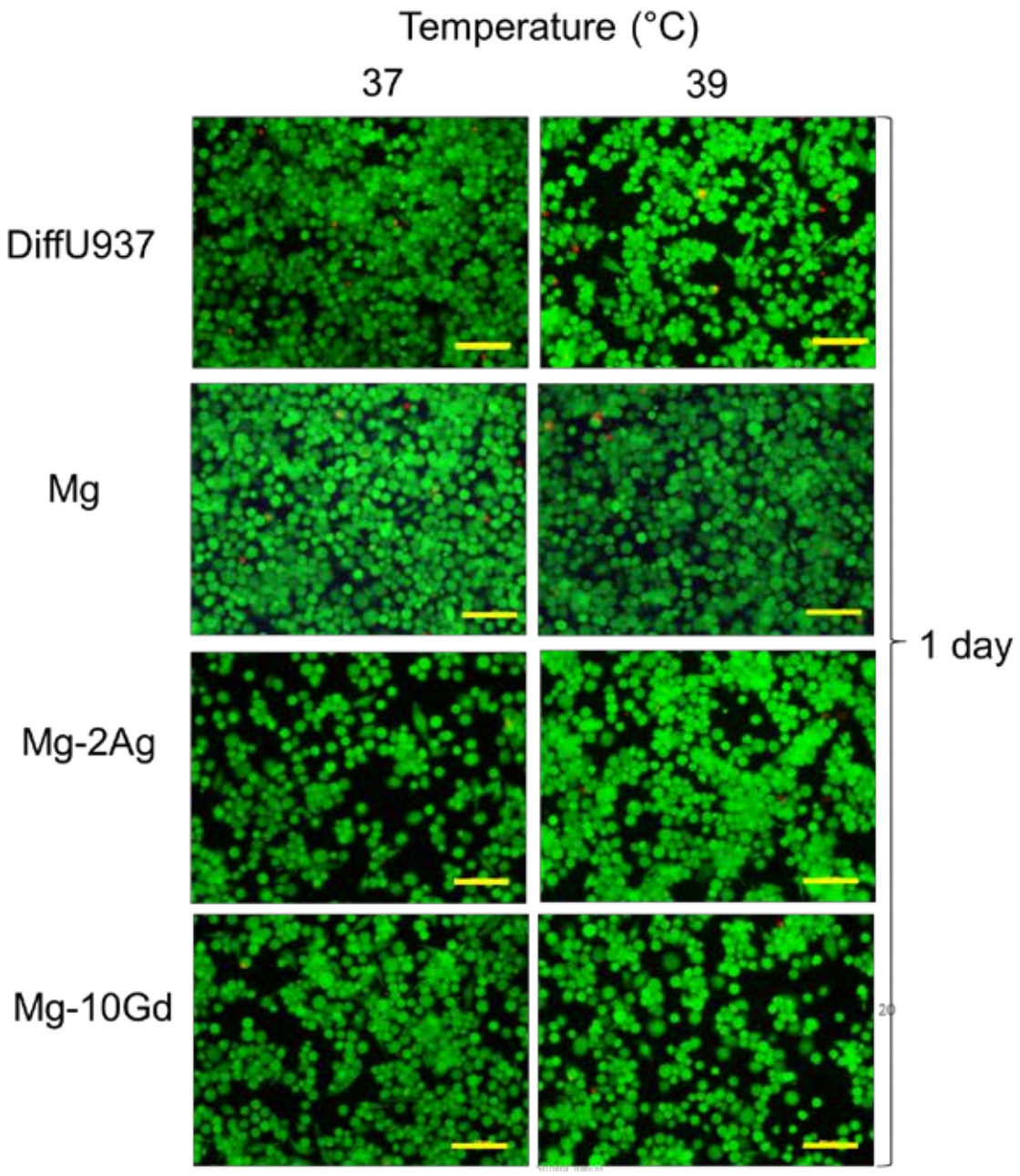


Figure 23 **Live/dead staining after 1 day**. The pictures show the viabilities of the cells at the higher temperature of 39°C compared with the standard temperature of 37°C with and without exposure to Mg, Mg-10Gd and Mg-2Ag.Scale bar: 100 µm.

The viabilities of the cells exposed to the extracts were also maintained after 3 days at standard and higher temperatures. These observations validate the use of the inflammatory in vitro model for the evaluation of the biological responses of DiffU937 cells toward the extracts. The live/dead staining results are presented in Figure 24.

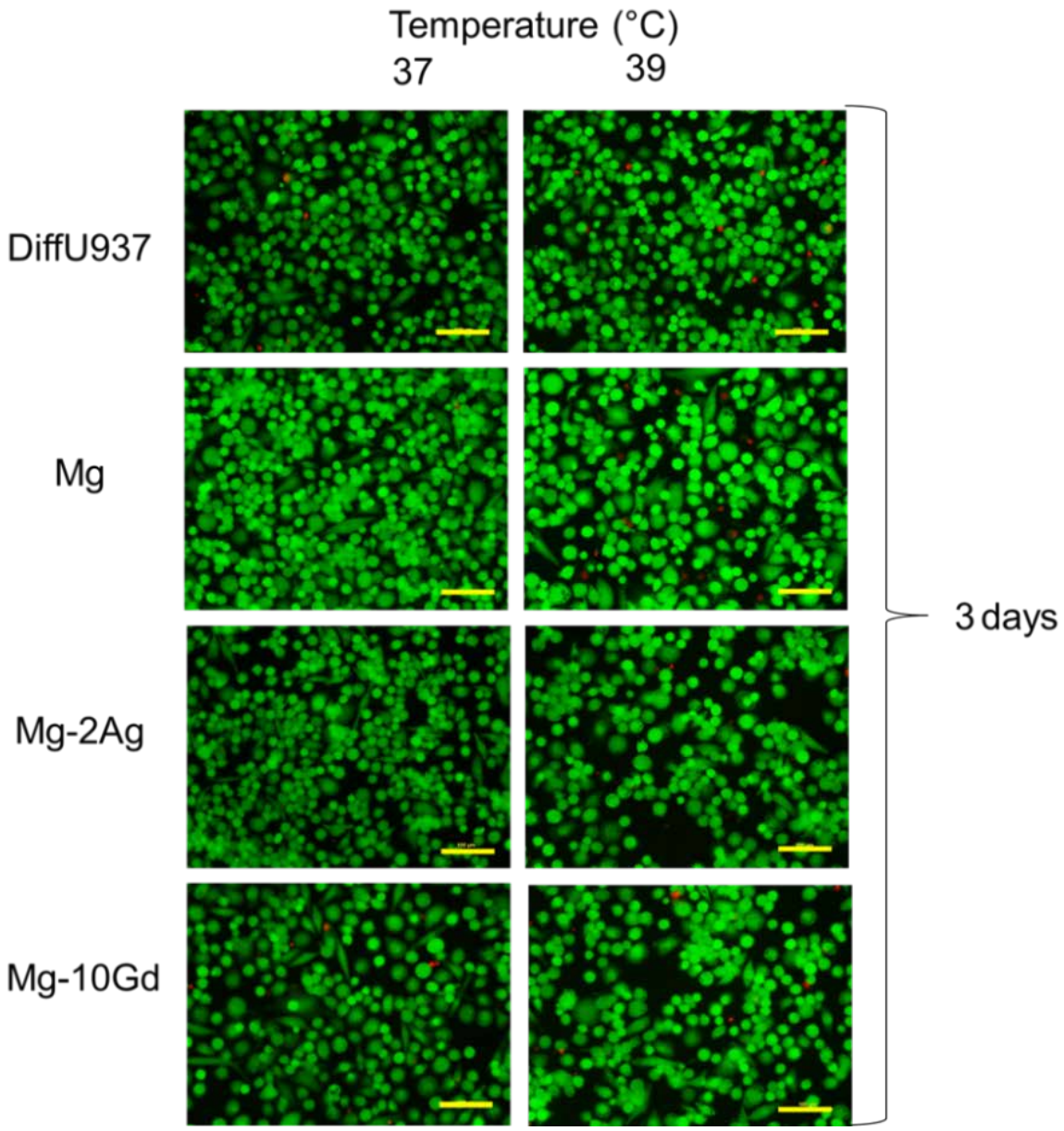


Figure 24 **Live/dead staining after 3 days**. The pictures show the viabilities of the cells at the higher temperature of 39°C compared with the standard temperature of 37°C with and without exposure to Mg, Mg-10Gd and Mg-2Ag. Scale bar: 100 µm.

5.3.1 Protein release in the inflammatory process

The influence of the total composition of Mg, Mg-2Ag and Mg-10Gd degradation products on DiffU937 cell behaviour was further investigated. In agreement with previous experiments, the specific influences of the extracts on amplification, down-regulation of the inflammatory reaction and cell turnover, were analysed with the detection of three corresponding groups of

cytokines:(I) inflammatory cytokines, (II) anti-inflammatory cytokines, and (III) chemokines. The OPN marker of the FBGC formation was also measured (IV). The results presented are described by comparing cytokine production in cells cultured with (DiffU937Ex) and without (control, DiffU937) extracts with respect to the temperature factor at the time points of 1 and 3 days. Quantifications of the proteins for each time, temperature and extract material tested were also performed using the ELISA test.

(I) Inflammatory cytokines

The inflammatory cytokines analysed were IL-1β and TNFα (Figure 25). Results obtained in the inflammatory *in vitro* model showed that in the control, the inflammatory cytokines were significantly downregulated ($p < 0.001$)when 37°C and 39°C were compared (in agreement with the results displayed in Figure 14). As expected, such effects are observed in the early and late inflammatory phases for both cytokines measured.

In DiffU937Ex cells, the production of IL-1β and TNFα at standard and higher temperatures was differently regulated. Compared to 37°C, the IL-1β protein level decreased significantly at 39°C after exposure of the cells to Mg-2Ag (early and late inflammatory phases) and Mg-10Gd (late inflammatory phase) extracts ($p < 0.001$). In all of the other conditions, cytokine release was comparable with respect to the two different temperature conditions.

In the early inflammatory phase, the TNFα protein level decreased significantly compared to the control ($p < 0.001$) in DiffU937 cells cultured with the Mg extracts at 39°C versus 37°C ($p < 0.05$). No significant differences in cells stimulated with Mg-2Ag and Mg-10Gd extracts were observed between the standard and higher temperatures. In the late inflammatory phase, the TNFα levels decreased significantly ($p < 0.001$) in DiffU937 and in DiffU937Ex cells.

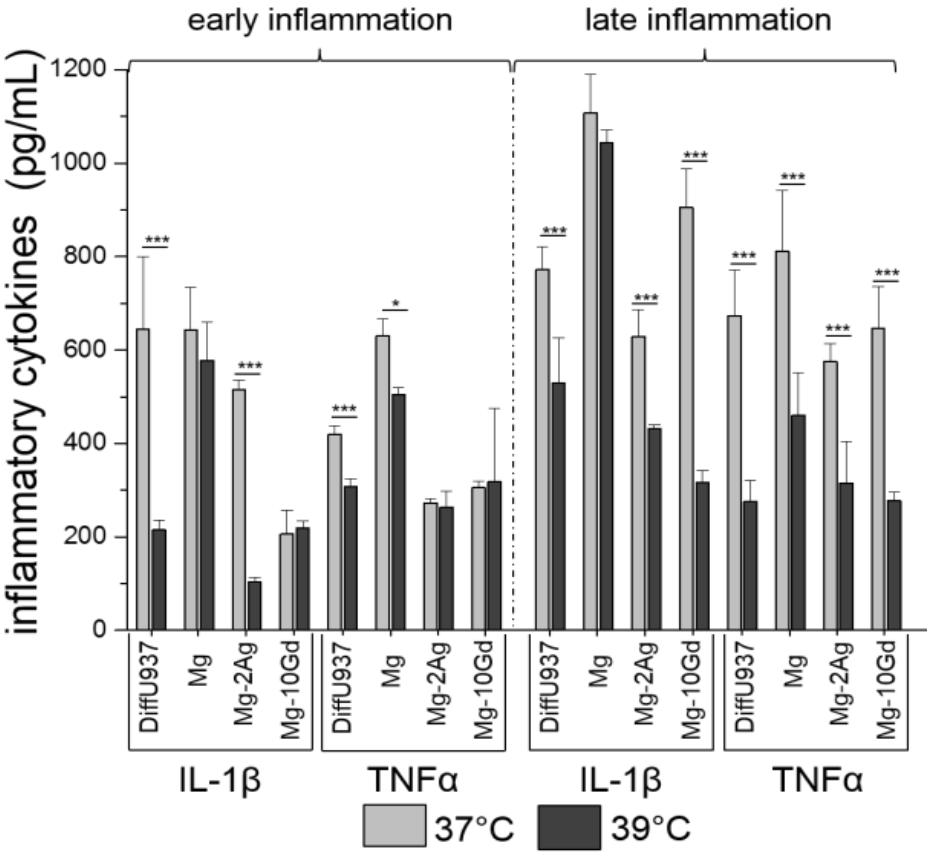


Figure 25 **Inflammatory cytokine release in the inflammatory *in vitro* model.** Release of the cytokinesIL-1β and TNFα after 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars) in DiffU937 cells and cells incubated with the extracts. Bars represent the means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

(II) Anti-inflammatory cytokines

The release of the anti-inflammatory cytokines IL-1Ra and IL-10 with and without exposure to the extracts is presented in Figure 26.

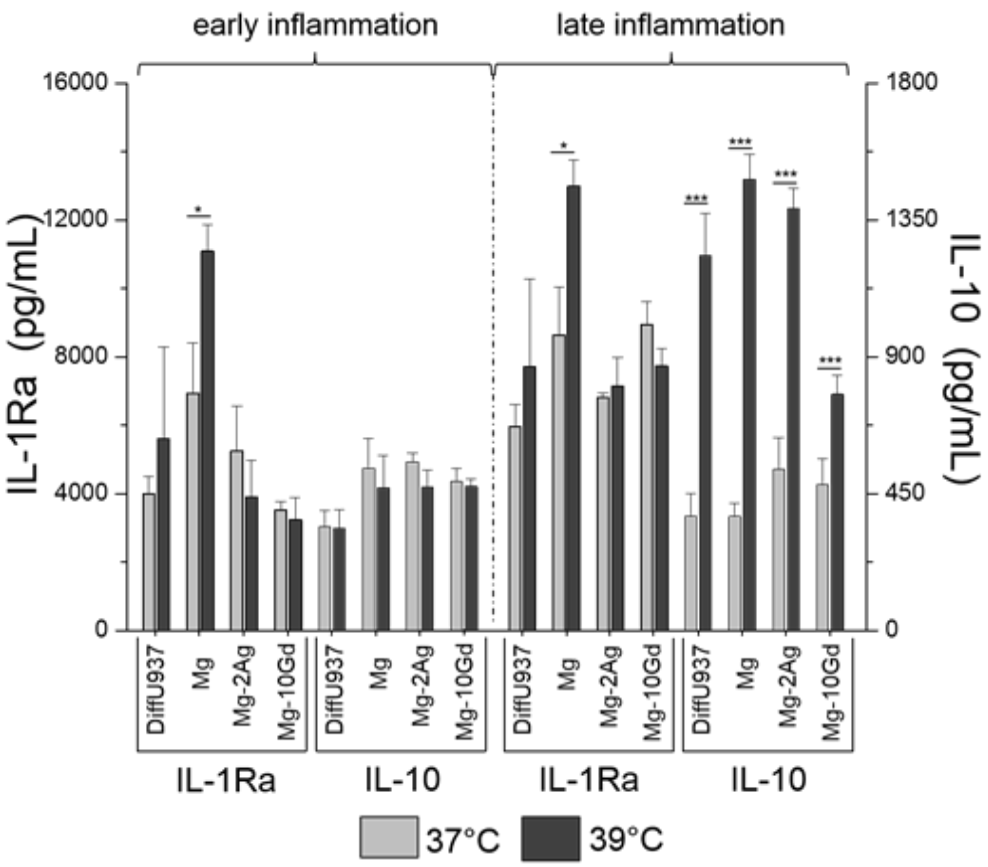


Figure 26 **Anti-Inflammatory cytokine release in the inflammatory *in vitro* model.** Release of the cytokinesIL-10 and IL-1Ra after 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars) in DiffU937 and DiffU937Ex cells. Bars represent the means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

In the early and late inflammatory phases, IL-1Ra production at 37°C and 39°Cwas comparable in DiffU937 cells and in cells stimulated with Mg-2Ag and Mg-10Gd. The only exception was noted in cells cultured with Mg extracts. In this case, protein release was significantly increased at 39°C compared to 37°C ($p < 0.05$) after 1 and 3 days of cell culture.DiffU937 and DiffU937Ex cells released IL-10 in a comparable manner at standard and higher temperature conditions after 1 day. After 3 days, surprisingly, IL-10 was strongly released at 39°C compared to 37°C ($p < 0.001$) in DiffU937 and DiffU937Ex cells.

Considering the importance of the release of IL-1Ra with respect to IL-1β (Paragraph 2.2), the ratio between the two cytokines was calculated, and the results are presented in Figure 27.

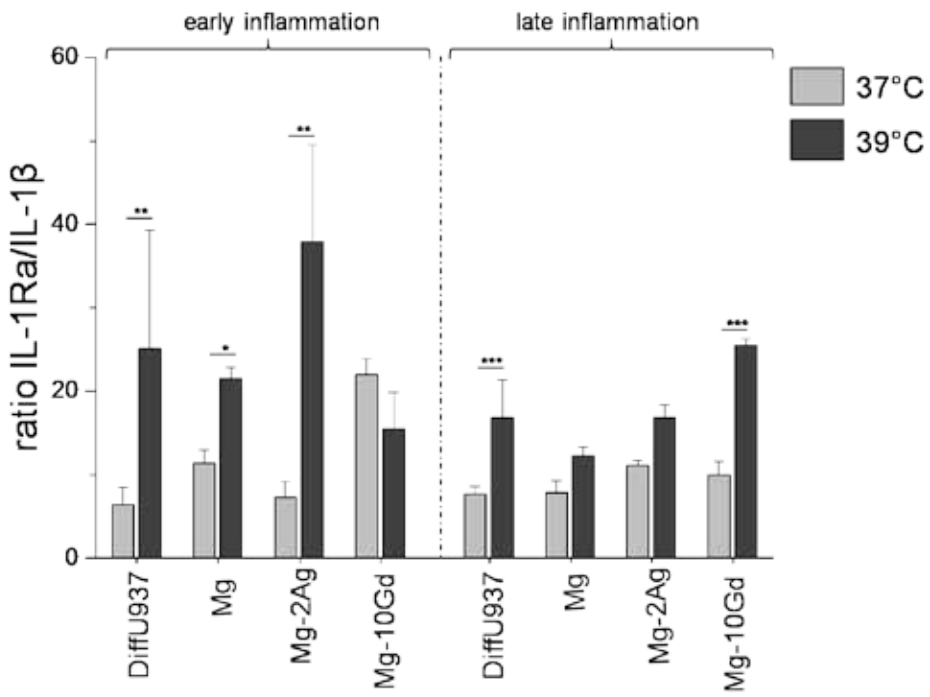


Figure 27 **IL-1Ra /IL-1β ratio in DiffU937 and DiffU937Ex cells.** IL-1Ra production with respect to IL-1β after exposure to or no exposure to Mg extract alloys for 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Bars represent the means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

The graph representing the ratio IL-1Ra/IL-1β shows that IL-1Ra was significantly released in DiffU937 cells cultured at 39°C compared to cells stimulated at 37°Cin the early inflammatory phase ($p < 0.01$, $p < 0.001$). In DiffU937Ex cells, such upregulation was noted in cells exposed to Mg and Mg-2Ag extracts after 1 day and in cells exposed to Mg-10Gd after 3 days.

(III) Chemokines

The chemokines MCP-1 and IL-8 were measured in an attempt to analyse the influence of the extracts on the signals involved in cell recruitment (Figure 28).

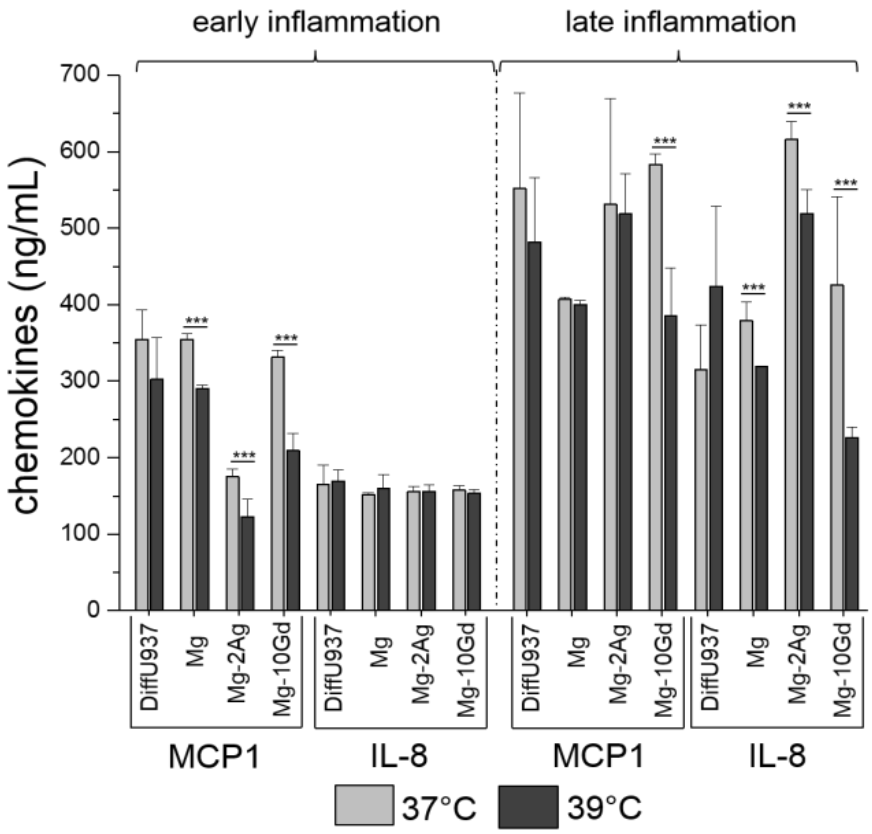


Figure 28 **Chemokine release in the inflammatory *in vitro* model.** Release of the chemokines MCP-1 and IL-8 after 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars) in DiffU937 and DiffU937Ex cells**.** Bars represent the means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

In the early inflammatory phase, surprisingly, MCP1 production decreased significantly (p< 0.001) at the higher temperature with respect to the standard temperature condition in DiffU937Ex cells. The results obtained in the late inflammatory phase showed a different impact of the extracts on protein release. After 3 days of cell culture with Mg and Mg-2Ag extracts, MCP-1 production levels were comparable at 37°C and 39°C. An exception to this trend was noted in cells stimulated with Mg-10Gd extracts. In that case, cells significantly decreased cytokine release at the high temperature (p< 0.001). After 1 day, IL-8 release was comparable in DiffU937 and DiffU937Ex cells with respect to the temperature conditions. After 3 days, protein production decreased (p< 0.001) at 39°C, while in the control, release was independent of the temperature factor.

(IV) OPN release

As described in Paragraph 2.2, OPN is a key protein target involved in FBGC formation; therefore, it was considered an interesting target for the purpose of this work. The results obtained with OPN are illustrated in Figure 29. High temperature and stimulation with the extracts did not influence OPN protein production in the early inflammatory phase. In the late inflammatory phase, protein release increased significantly in DiffU937 cells ($p < 0.001$) at 39°C compared to 37°C. This trend was observed after cells were cultured with Pure Mg and Mg-10Gd extracts. In cells exposed to Mg-2Ag extracts, protein production was strongly attenuated ($p < 0.001$) at high temperature compared to the standard temperature condition.

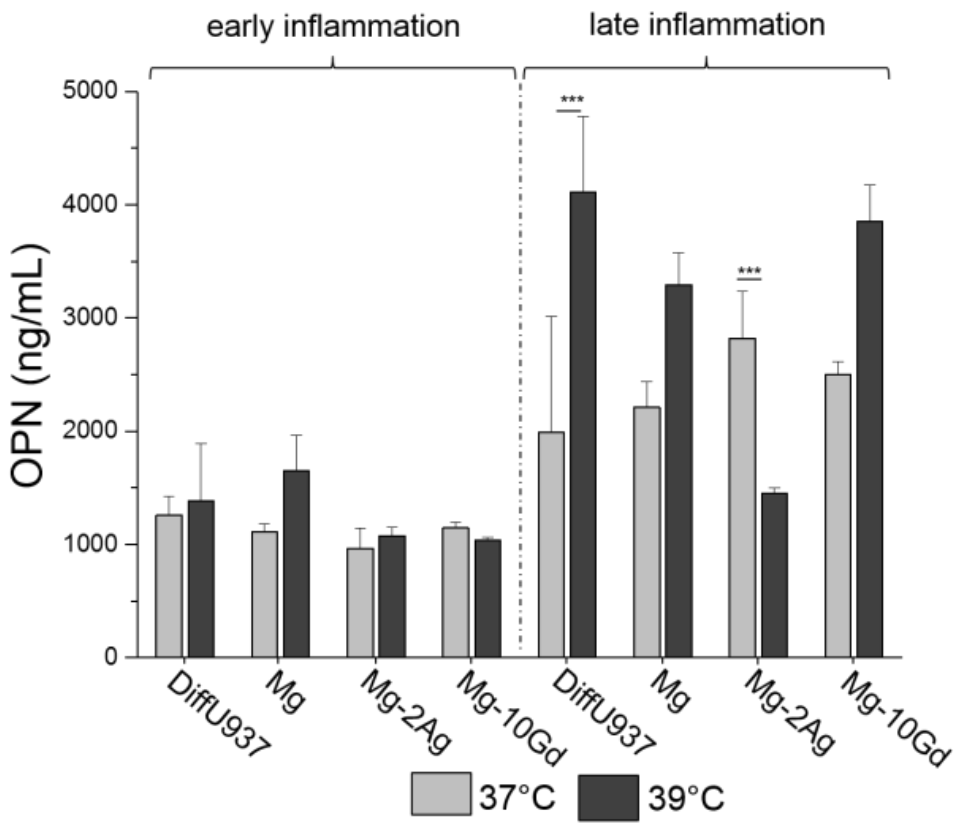


Figure 29 **OPN release**. Protein production in DiffU937 and DiffU937Ex cells after 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Cells cultured at standard temperature (37°C) are the controls. Bars represent the means ± standard deviations (SDs) of independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

5.3.2 Gene expression

Considering the protein results obtained, further investigations were performed at the gene level. The effects of the extracts on cell behaviour were explored with analyses of *NF-kB* and *IL-1β*, two genes that strongly influence macrophage responses in inflammatory conditions (Paragraph 2.2). To clarify the results obtained at the protein level, *OPN* gene expression was analysed. The results obtained after 1 and 3 days of cell stimulation are presented in Figure 30.

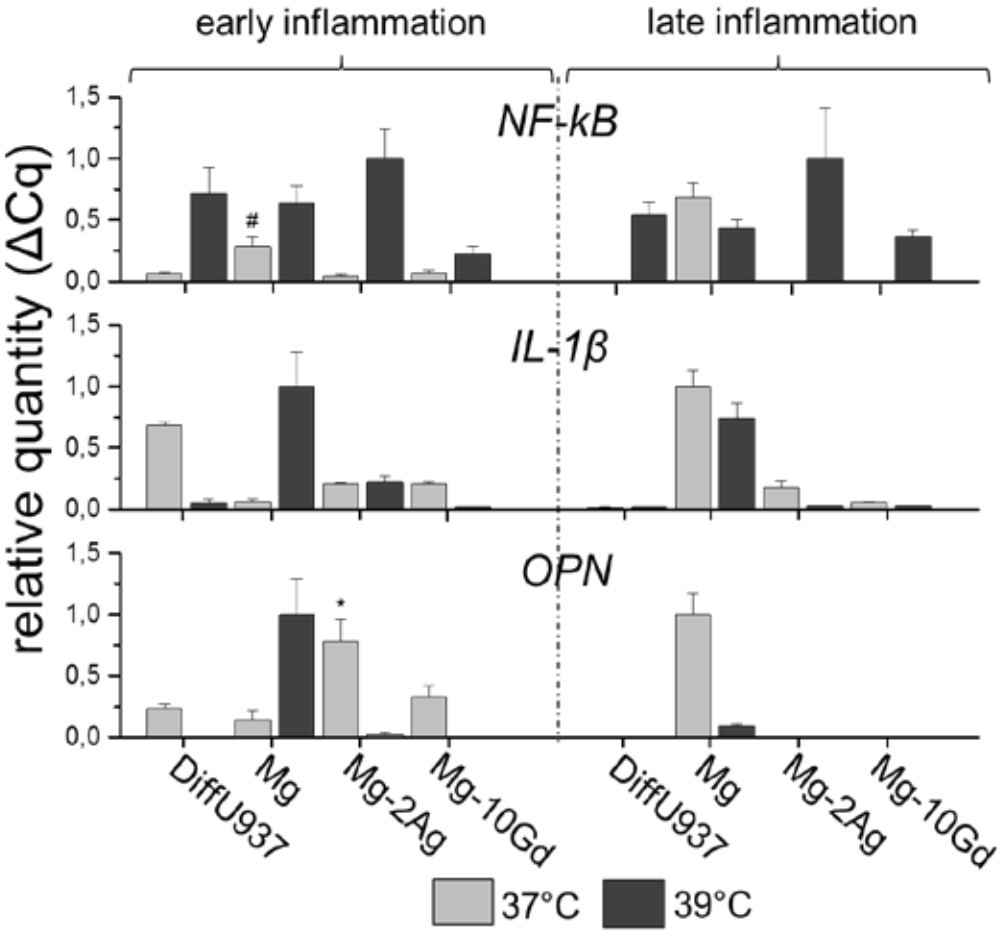


Figure 30 **Semi-quantitative analysis of inflammatory gene expression**. Gene expression of NF-kB, IL-1β, and OPN in DiffU937 and DiffU937Ex cells after 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Gene expression was normalized to the expression of GAPDH and B2M. Significant differences between the unstimulated cells (control, DiffU937) and cells cultured in the indicated conditions are presented by asterisks or hash marks ($p < 0.05$=*; $p < 0.01$= #).

Expression of the transcription factor gene *NF-kB* was up-regulated at 39°C compared to 37°C in DiffU937 and DiffU937Ex cells in early and late inflammatory phases. Interestingly, the gene was expressed at standard temperature only after exposure of the cells to Mg extracts ($p< 0.01$). *IL-1β* was clearly observed to be highly expressed in DiffU937 cells at 37°C and in cells cultured with Mg extracts at 39°C ($p< 0.01$) in the early inflammatory phase. The effects of the Mg extracts on the targeted gene were also noted in late inflammation. At this time point, *IL-1β* expression was highly regulated in standard and high temperature conditions.

OPN was expressed in the control and DiffU937Ex cells in the early inflammatory phase. Interestingly, the gene was highly regulated in cells exposed to Mg extracts and cultured at 39°C and in cells cultured with Mg-2Ag extracts at 37°C. As observed in Figure 20, the strong effect on gene expression was also clearly observed in late inflammation, but in cells stimulated at 37°C. The up-regulation gene expression was observed in cells cultured with Mg-2Ag extract for 1 day at 37°C.

The influences of the extracts on macrophage activity were further investigated considering the expression of genes that strongly influence the capacity of the cells to recognize signals of damage in aseptic conditions. As described in Paragraph 2.2, TLR2 is one of the principal receptors involved in this process and was consequently chosen as the target for the evaluation of the bioactivity properties of the extracts (Figure 31). The TLR2 gene is clearly expressed after 1 day of cell exposure to 39°C. This observation was evident in both DiffU937 and DiffU937Ex cells. Compared to the control, the target gene was significantly expressed after cell exposure to Mg-2Ag extracts ($p< 0.05$). After 3 days, the gene was up-regulated in cells cultured with Mg ($p < 0.01$) and Mg-10Gd extracts ($p< 0.05$) at standard temperature with respect to the control condition.

Another important event considered was the predisposition of the macrophages to form FBGCs. This event reacquires the ability of the cells to adhere with the substrate and the expression of proteins of membrane that enhance the fusion. ICAM-1 and CD36 are specifically regarded as markers for these events (Paragraph 2.2). The modulation in the expression of the two surface receptors was investigated at the gene level, and the results are displayed in Figure 31.The surface marker *CD36* gene was highly regulated in DiffU937 cells and in cells exposed to Mg-2Ag and Mg-10Gd extracts after 1 day of cell culture at 37°C. At the same time point and temperature condition, gene expression decreased significantly with respect to the control in cells stimulated with Mg extracts. In the late inflammatory phase, the *CD36* gene was detectable only after cell exposure to Mg extracts at 37°C and 39°C. ICAM-1 was generally down-regulated at 37°C in DiffU937Ex cells compared to unstimulated cells. The decrease in its expression was significant in cells exposed to Mg extracts ($p< 0.01$), an observation that was true for the early

inflammatory phase. In the late inflammatory phase, the gene was expressed in DiffU937 and DiffU937Ex cells at 39°C. At 37°C, the gene was detectable only in cells exposed to degradation products of Mg.

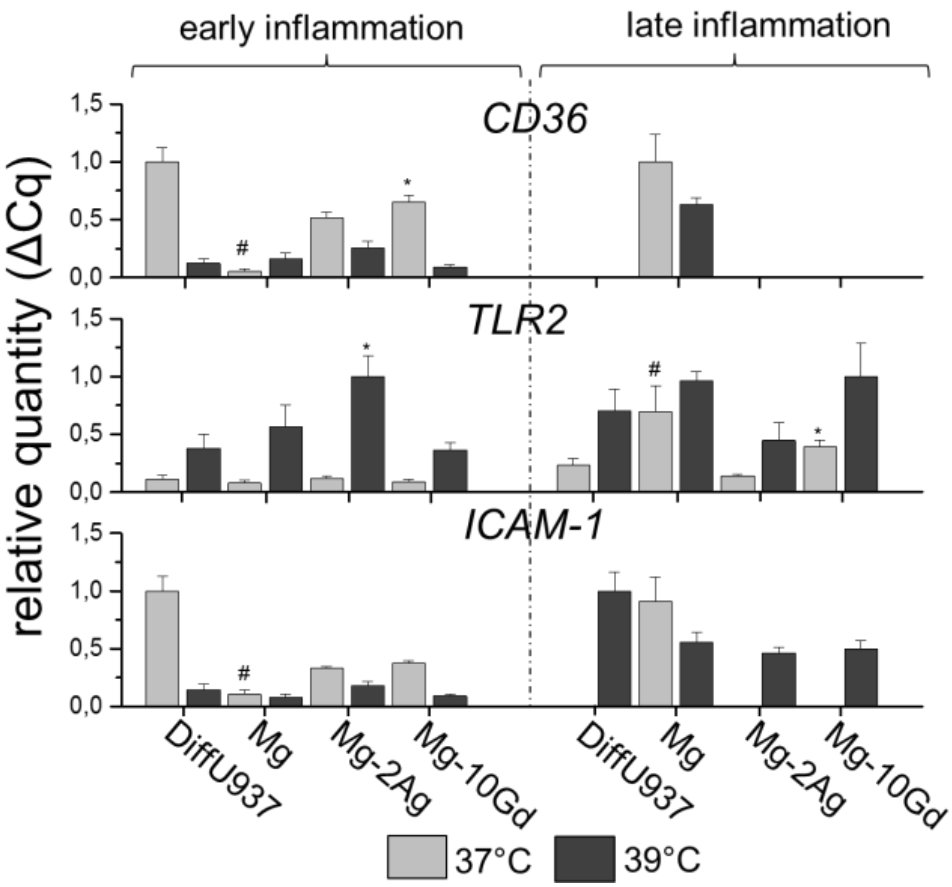


Figure 31 **Semi-quantitative analysis of receptor gene expression.** Gene expression of *CD36*, *TLR2* and *ICAM-1* in DiffU937 and DiffU937Ex cells after 1 (early inflammation) and 3 days (late inflammation) at 37°C (grey bars) and 39°C (black bars). Gene expression was normalized to the expression of *GAPDH* and *B2M*. Significant differences between the unstimulated cells (control, DiffU937) and the cells cultured in the indicated conditions are presented by asterisks or hash marks ($p < 0.05$=*; $p < 0.01$= #).

5.3.3 pH values in the inflammatory in vitro model

As described in Paragraph 2.1, the degradation products obtained following ISO 10933-12 yield a solution with a basic pH (Table 3). It is well known that inflammation is associated with a local acidic environment and that the low pH is associated with macrophage activities [217]. Therefore, extracellular pH value was detected in the proposed inflammatory *in vitro* model. These measurements aimed to understand the contribution of the macrophage activity to the

modulation of the pH in the extracellular microenvironment. Additionally, pH monitoring at the chosen time points and temperature conditions also aimed to verify whether the alkalinity of the medium was directly responsible for results obtained at protein and gene levels. The extracellular pH values obtained are displayed in Figure 21. The results of the measurements performed after 1 day show that inDiffU937 and in DiffU937Ex cells, the extracellular microenvironments had comparable pH values in cells cultured at 37°C and 39°C. Nevertheless, the tendency towards increased alkalinity was noted in the cell culture media of Mg-2Ag and Mg-10Gd extracts. After 3 days, the medium of DiffU937 cells cultured in the higher temperature condition was significantly acidic ($p < 0.001$) compared to the cells cultured at the standard temperature. In DiffU937Ex cells, the pH values were comparable at 37°C and 39°C.

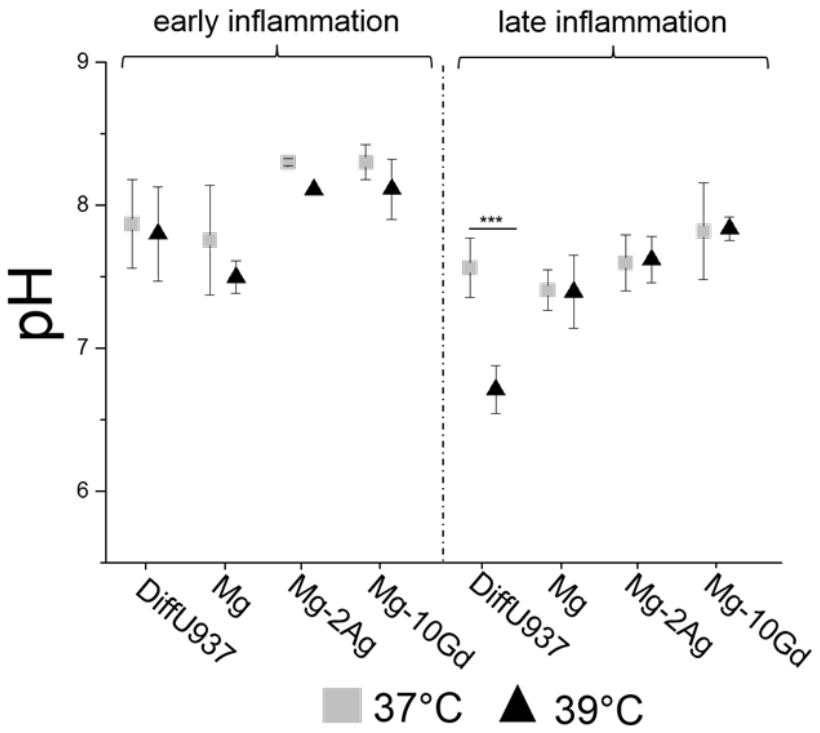


Figure 32 **pH values in the inflammatory *in vitro* model.** The measurements of acidity or alkalinity of the supernatants in DiffU937 and DiffU937Ex cells were performed after 1 (early inflammation) and 3 (late inflammation) days at standard (37°C) and febrile (39°C) temperatures. Triangles and squares symbols represent means ± standard deviations (SDs) of six independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

5.4 Saos-2 solo *vs* DiffU937/Saos-2 co-culture

The aim of the following experiments was to elucidate the cross-talk between macrophages and the bone cell lineage. To achieve this purpose, a co-culture model of DiffU937/Saos-2 cells was established, and the influence of Mg alloy extracts on such interactions was verified.

5.4.1 Saos-2 cell solo-culture

In an attempt to establish the co-culture model, the Saos-2 cell solo-culture was first performed. According to standard protocols, the suggested cell culture media for the U937 and the Saos-2 cell line were RPMI and McCoy's 5A, respectively. In an attempt to observe whether the use of RPMI medium could affect cell behaviour, viability and cell growth were measured after 3 days at 37°C. Measurements were performed using CASY (Paragraph 4.3.1).

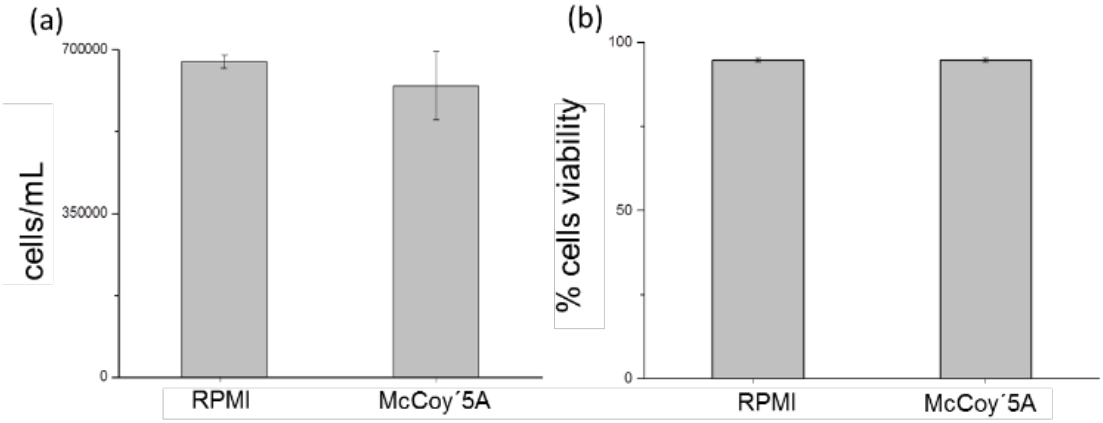


Figure 33 **Saos-2 growth and viability using different media**. Cell growth (a) and viability (b) after 3 days of culture in RPMI and McCoy's 5A media at 37°C. Bars represent the means ± standard deviations (SDs) of three independent measurements.

As observed in Figure 33, the viability and growth of Saos-2 cells in RPMI and McCoy's 5A media were comparable. Considering the results obtained, further experiments were performed using only RPMI medium. In the following investigations, the cells were exposed to Mg-2Ag and Mg-10Gd extracts. In this case, the cells were additionally stimulated with the septic stimulus LPS and were then cultured at 37°C. Because tissue remodelling is part of the final process of the inflammatory reaction (resolution phase), the cells were cultured only at 37°C, but with the addition of LPS (which mimics an infection). The ideas behind these experiments were: 1) to observe the specific contributions of the extracts on cell behaviour and 2) to verify whether the

osteoblast cell type actively contributes to cytokines release in the presence of an inflammatory stimulus. To address point 1, cell viability and growth were measured. The results of the cell growth assay are presented in Figure 34(a) and show that the number of Saos-2 cells increased significantly ($p < 0.001$) in cultures stimulated with LPS compared to unstimulated cultures. A similar trend was noted in cells exposed to Mg-10Gd extracts with and without the septic inflammatory stimulus ($p < 0.01$). After exposure of Saos-2 to Mg-2Ag extracts, cell growth was comparable in LPS-stimulated and LPS-unstimulated cells. Cell viability is displayed in Figure 24 (b). Surprisingly, in Saos-2 cells, the percentage of viable cells was significantly high ($p < 0.001$) after stimulation with LPS compared to the cells cultured only with medium. Considering the effect of the extracts, is clearly observed that cell viability was comparable with and without inflammatory stimulation.

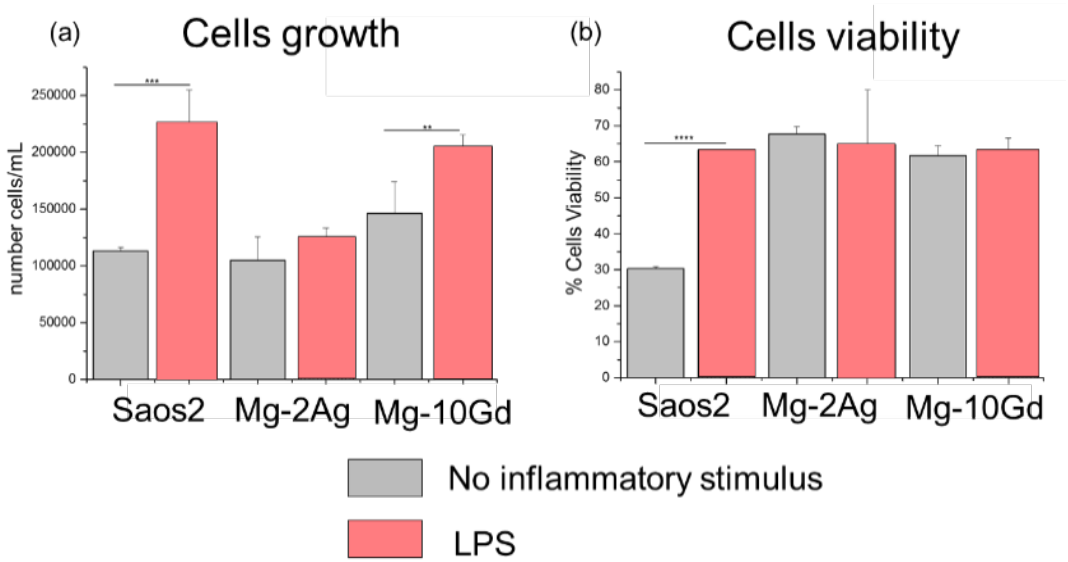


Figure 34 **Saos-2 behaviour with and without LPS stimulation and exposure to Mg-2Ag and Mg-10Gd extracts**. Cell growth (a) and viability (b) after 3 days with and without LPS stimulation in cells cultured with and without Mh-2Ag and Mg-10Gd extracts. Bars represent the means ± standard deviations (SDs) of three independent measurements.

Inflammatory and anti-inflammatory cytokine release was also checked to address point 2. The measurements were performed using an ELISA test and are presented in Table 7; no releases of targeted cytokines were detected in the described conditions

Table 6 **Saos-2 cytokine analysis**

		IL-1β	TNFα	IL-1Ra	IL-10	MCP1	IL-8
+LPS	Mg-2Ag	–	–	–	–	–	–
	Mg-10Gd	–	–	–	–	–	–
	Saos-2	–	–	–	–	–	–
	Mg-2Ag	–	–	–	–	–	–
	Mg-10Gd	–	–	–	–	–	–
	Saos-2	–	–	–	–	–	–

inflammatory cytokines anti-inflammatory cytokines
chemokines

5.4.2 DiffU937/Saos-2 co-culture

DiffU937/Saos-2 co-culture was performed for 3 days at 37°C. Solo cultures of Saos-2 and DiffU937 cells were used as controls.

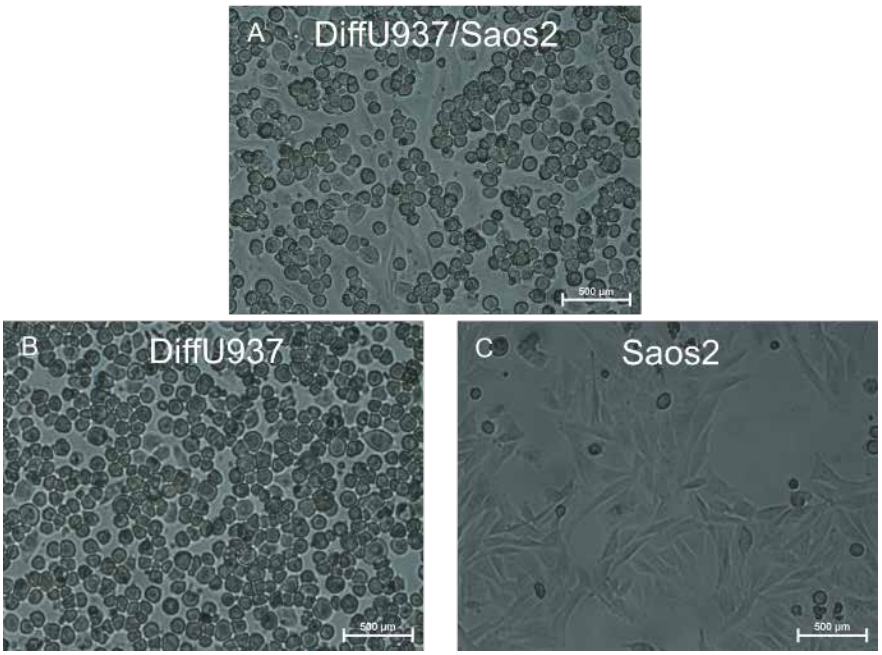


Figure 35 **DiffU937/Saos-2 cells in solo and co-cultures.** DiffU937 and Saos-2 cells (A). Solo-cultures of DiffU937 (B) and Saos-2 cells (C).

As clearly shown in Figure 35, cell growth in the presence of DiffU937 cells resulted in a final mono-layer of DiffU937/Saos-2 cells after 3 days of cell culture. Upon microscopically comparing the solo and co-culture conditions, cell growth showed no morphological differences.

In the following step, cytokine release was measured using the array panel method. The array method was considered the preferable method of investigation due to the wide range of proteins that were simultaneously detectable (Figure 36). As expected, DiffU937 solo-culture expressed a wide range of cytokines, while Saos-2 cells only released the MIF protein in the microenvironment. Surprisingly, in co-culture conditions protein production was generally down-regulated compared to DiffU937 solo-culture. In cases of complement protein C5, GM-CSF, IL-1β and TNFα, signals were detectable only in DiffU937 solo-culture and not in the co-culture conditions. Interestingly, IL-6 was the only detected cytokine whose expression was strongly increased in the DiffU937/Saos-2 co-culture condition.

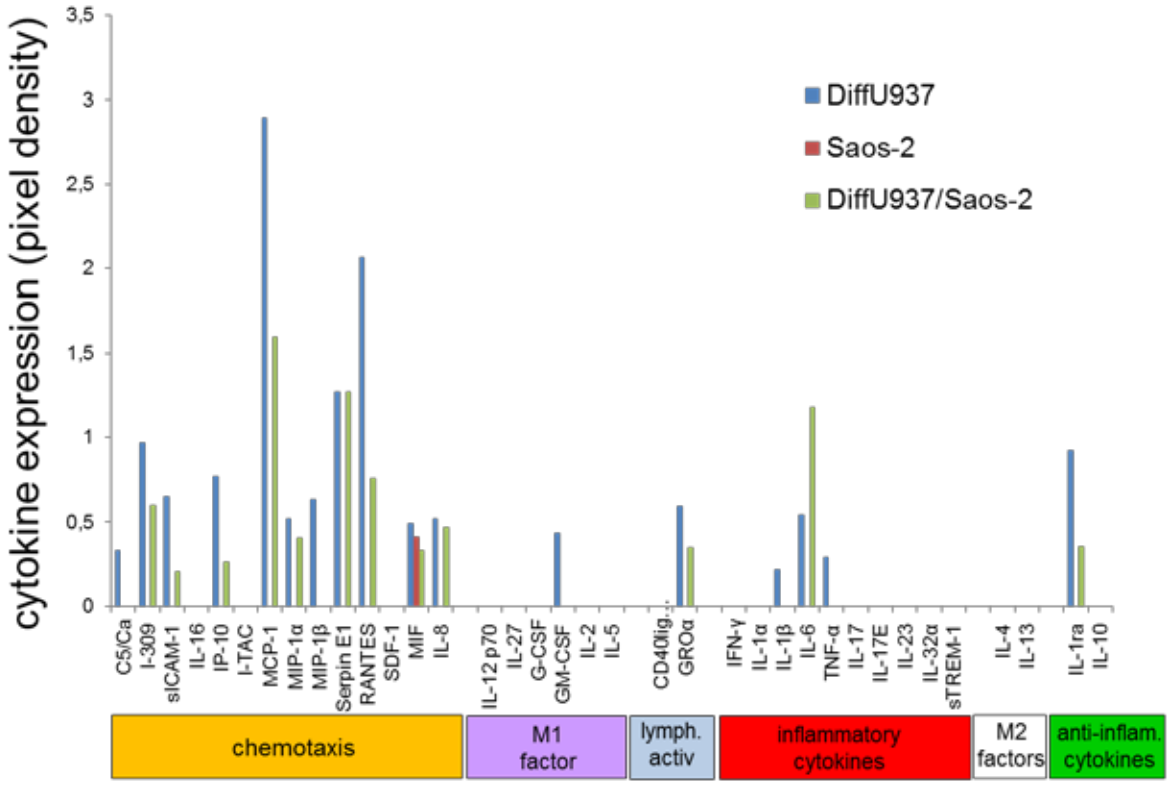


Figure 36 **Array panel of cytokine release in solo and DiffU937/Saos-2 cell co-culture.** Inflammatory cytokines produced in the DiffU937/Saos-2 cell co-culture (green), theSaos-2 solo-culture (red) and the DiffU937 solo-culture (blue) after 3 days at 37°C.

In addition to the inflammatory response in the co-culture itself, the cells' responses following exposure to Mg-2Ag and Mg-10Gd extracts were of special interest. The understanding of the cell cross-talk was based on observations of IL-1β and IL-10 release, two cytokines that induce tissue destruction and remodelling events, respectively (Figure 37).

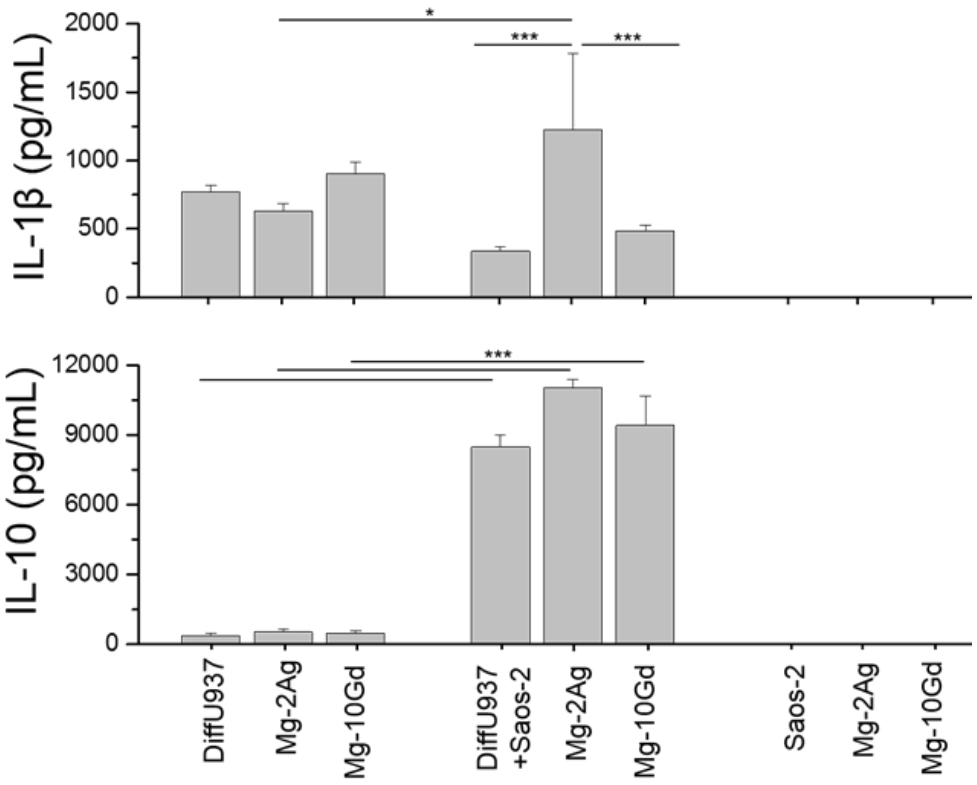


Figure 37 **Cytokine production in solo and DiffU937/Saos-2 cell co-culture.** Release of the proteins IL-1β and IL-10 after 3 days at 37°C. Bars represent the means ± standard deviations (SDs) of three independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

The idea behind these measurements is to verify whether the extracts could promote either amplification or inhibition of the inflammatory response through the modulation of these two key factors. Protein detection was performed using ELISA tests in solo-Saos-2 and DiffU937 cultures and in co-culture conditions after 3 days. As shown in Figure 37, in solo DiffU937 culture, inflammatory cytokine IL-1β production was comparable in the control and in cells exposed to the extracts. In co-culture conditions, the release of the targeted protein was significantly increased in cells exposed to Mg-2Ag extracts compared to control (DiffU937/Saos-2) and Mg-10Gd-stimulated cells ($p < 0.001$). Furthermore, a significant increase in IL-1β production was noted when comparing DiffU937 and DiffU937/Saos-2 cells stimulated with Mg-

2Ag ($p < 0.05$). Anti-inflammatory cytokine IL-10production in DiffU937 solo-culture was comparable in cells cultured with and without extracts. As expected, in the co-culture condition, protein release increased significantly ($p < 0.001$) compared to the DiffU937 solo-culture condition. Is interesting to note that in DiffU937/Saos-2 cells exposed to Mg-2Ag extracts, there is a clear tendency towards increasing IL-10 release compared to cells cultured only with medium.

5.5 Primary M1/M2 macrophage behaviour with extracts

As discussed in Paragraph 2.2.1, specific reproduction *in vitro* and the analysis of M1 and M2 responses require the use of primary cells. The experiments presented in this section aimed to investigate two different aspects of M1/M2 macrophage interactions with the extracts: 1) the capacity of the cells to modulate cytokine release after polarization; and 2) the influence of the degradation products on macrophage differentiation and polarization phases. The experiments that follow were performed attempting to explore the first point.

5.5.1 Cytokine release

The M1 and M2 macrophage responses toward the extracts were analysed through the detection of inflammatory and anti-inflammatory cytokines and chemokines. For each group of proteins, targeted proteins were chosen in agreement with the inflammatory *in vitro* experiments. M1 and M2 polarization and exposure to the extracts described in this section were performed as described in protocol 1 in Paragraph 4.2.3. For these experiments, attention was particularly focused on cell reactions towards Mg-2Ag and Mg-10Gd.

The inflammatory cytokine TNFα was first measured, and the results are shown in Figure 38.

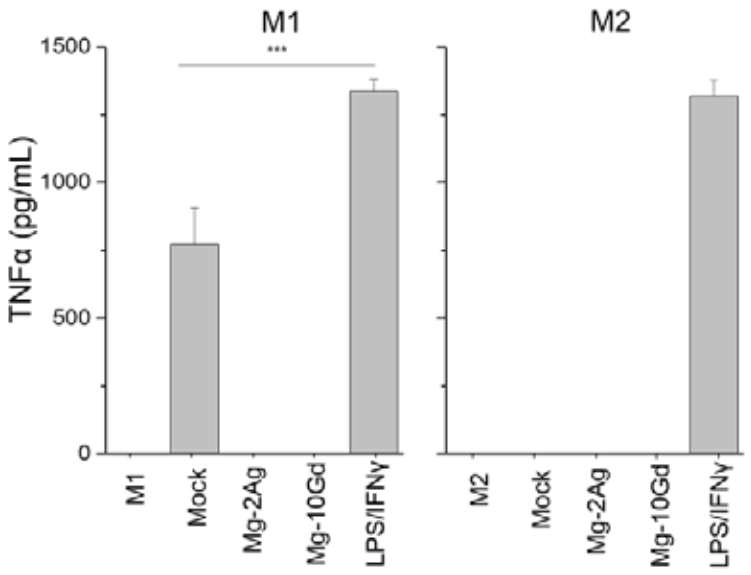


Figure 38 **TNFα production in M1 and M2 primary macrophages.** Protein detection after 24 h of primary macrophage M1/M2 culture with without Mg-2Ag and Mg-10Gd extracts. Cell culture medium + 10% FBS (MOCK) and LPS/IFN-ɣ stimulation were used as negative and positive controls, respectively. Bars represent the means ± standard deviations (SDs) of three independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

In M1 macrophages, the targeted cytokine was clearly released in cells cultured with MOCK medium (negative control; paragraph 4.2.3) and in cells stimulated with LPS/IFN-ɣ. TNFα production was significantly highly regulated in the positive compared to the negative control ($p < 0.001$). In M2 macrophages, inflammatory cytokine production was observed only in the positive control; surprisingly, no interference of the negative control was noted.

Anti-inflammatory cytokine measurements are displayed in Figure 39. For the anti-inflammatory cytokine IL-10, interference of the MOCK control was observed in both M1 and M2 macrophage phenotypes. Protein release also increased in cells stimulated with LPS/IFN-ɣ and in a significantly manner ($p < 0.001$) compared with protein production in cells cultured with the negative control ($p < 0.001$).

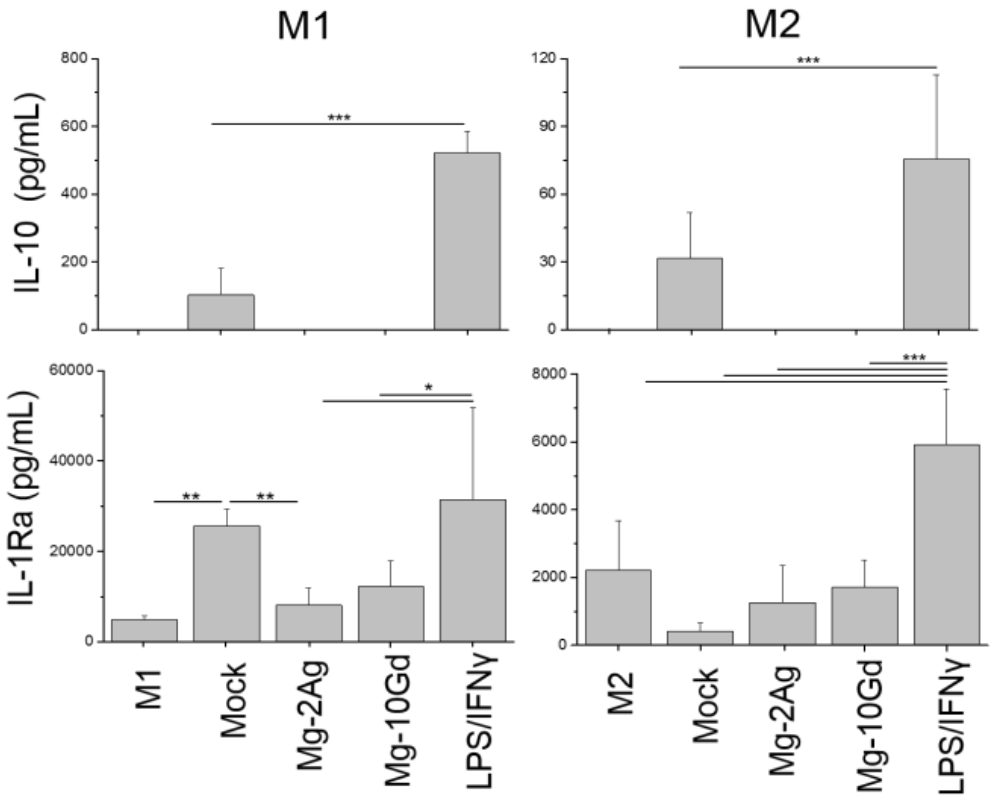


Figure 39 **Anti-inflammatory cytokine production in M1/M2 macrophages.** Detection of IL-1Ra and IL-10 after stimulation of primary M1/M2 macrophages with and without Mg-2Ag and Mg-10Gd. Cell culture medium + 10% FBS (MOCK) and LPS/IFN-ɣ stimulation were used as negative and positive controls, respectively. Bars represent the means ± standard deviations (SDs) of three independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

The second anti-inflammatory cytokine investigated was IL-1Ra. The protein was detected in all of the described conditions. In M1 macrophages, IL-1Ra was strongly produced after cells were exposed to the Mock control ($p < 0.01$) compared to unstimulated cells and Mg-2Ag-stimulated cells. Protein levels increased in the positive control compared to cells exposed to the extracts. Interestingly, M2 cells showed a different response to the stimuli in fact, IL-1Ra production was significantly released ($p < 0.001$) only in LPS/IFN-ɣ cells compared to the other conditions.

The capacity of primary M1/M2 macrophages to produce IL-8 and MCP-1 *in vitro* was verified (Figure 40).

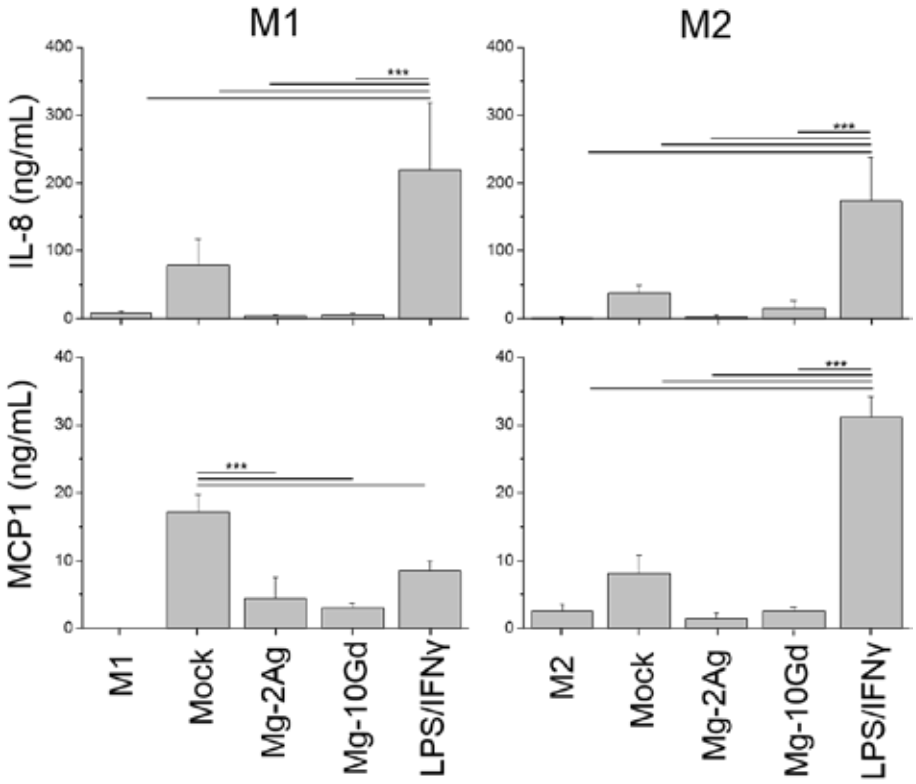


Figure 40 **Chemokine production in M1/M2 macrophages.** Detection ofIL-8 and MCP1 after stimulation of primary M1/M2 macrophages with and without Mg-2Ag and Mg-10Gd. Cell culture medium + 10% FBS (MOCK) and LPS/IFN-ɣ stimulation were used as negative and positive controls, respectively. Bars represent the means ± standard deviations (SDs) of three independent measurements. Stars indicate statistically significant differences between two groups (p< 0.05 = *, p< 0.01 = **, p< 0.001 = ***).

The M1 and M2 macrophages produced IL-8 mainly after cells were exposed to the positive and negative controls. Comparing the two cell culture conditions, protein was highly released in cells stimulated with LPS/IFN-ɣ (p< 0.001). In the case of MCP1, the two macrophage subpopulations differentially modulated the release of the protein in answer to the agents added in the cell cultures. The M1 cells released the targeted chemokine in the controls and in response to Mg-2Ag and Mg-10Gdextracts. Compared to these conditions, MCP1 is strongly released in the Mock control (p< 0.001). Interestingly, the protein was not detected in cells cultured only with medium. In M2 cells, chemokine production was observed in all of the measured conditions. Significant and higher MCP1 production was clearly noted in the positive control (p< 0.001).

5.5.2 M1/M2 Macrophage polarization and differentiation

The following analysis was performed for the investigation of the second point explained in the brief introduction to this section (Paragraph 4.2.3, protocol 2). Figure 41 summarizes the results of the reproduced differentiation phase before and after LPS stimulation.

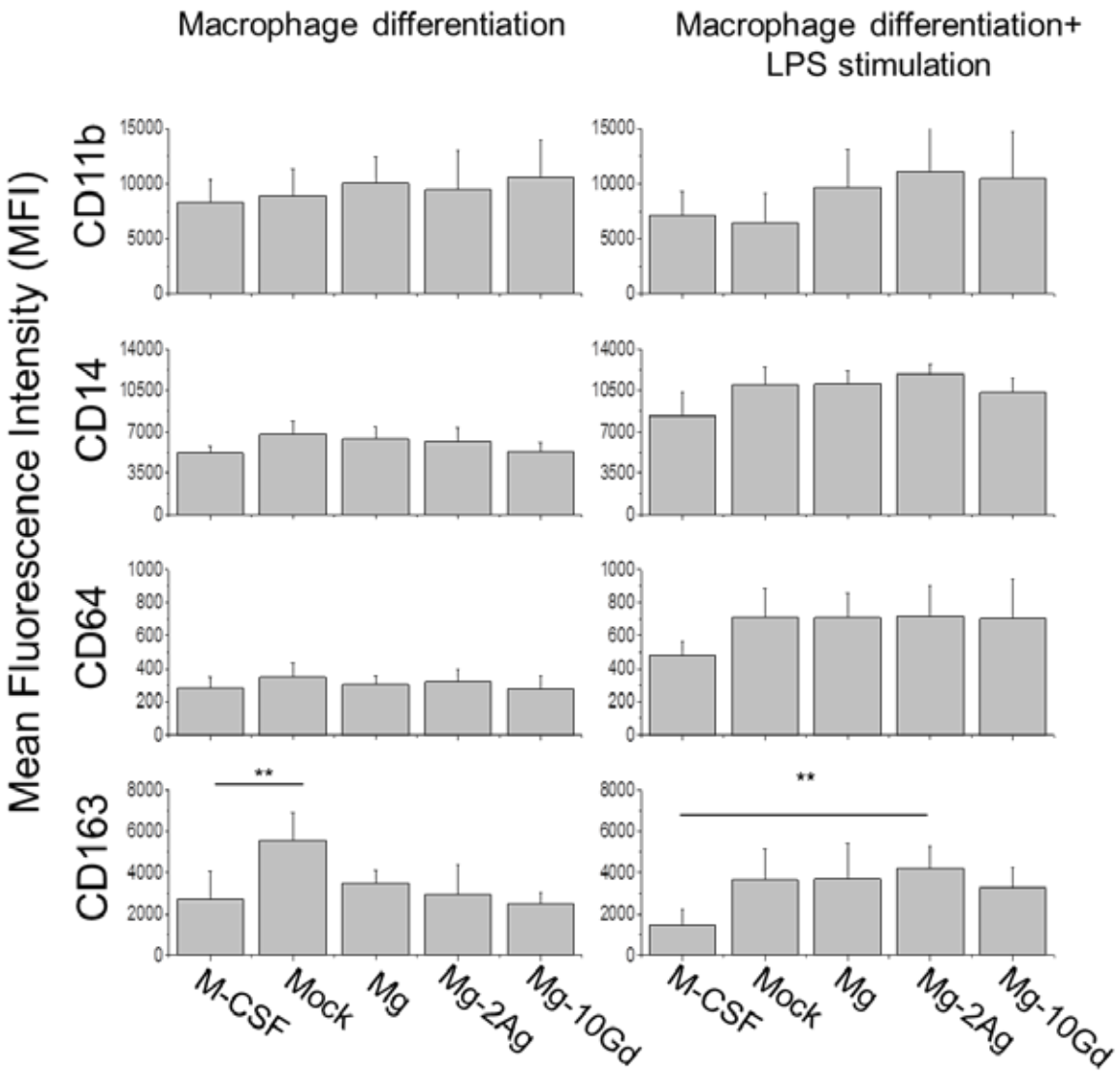


Figure 41 **Characterization markers on differentiated and LPS-stimulated macrophages.** CD expression after treatment of primary monocytes (M-CSF cells) with and without Mg, Mg-2Ag and Mg-10Gd extracts. M-CSF cells are considered the positive control. Cell culture medium + 10% FBS (Mock) is the negative control. Bars represent the means ± standard deviations (SDs) of three independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

The macrophage subpopulations were investigated through the analysis of key CDs, which are characterization markers for myeloid lineage cells (CD11b) and M1 (CD14; CD64) and M2 (CD163) macrophages. For these experiments, the Mg, Mg-2Ag and Mg-10Gd extracts were used.

It was generally observed that the Mock medium interfered strongly with the expression of the investigated CDs. CD11b, CD14 and CD64 detection showed that the regulation of their expression levels was comparable to the positive control for monocyte differentiation into macrophages (M-CSF cells). An exception was observed with CD163 expression. In that case, significantly increased CD163 expression was observed in the Mock control compared to the M-CSF cells ($p < 0.01$). Interestingly, this finding was true only for the unstimulated cells; in fact, after LPS stimulation, the significant increase in protein with respect to the positive control was observed only in cells stimulated with Mg-2Ag extracts ($p < 0.01$).

In the next step, CD expression analysis was performed after the reproduced polarization phase (Figure 42). In these experiments, cells cultured with the addition of IFN-ɣ are considered the positive control for M1 polarization, and cells stimulated with IL-4 are considered the positive control for M2.First, it was clearly noted that the negative control induced the expression of all four surface proteins investigated in unstimulated and LPS-stimulated cells. CD11b was significantly up-regulated in unstimulated M1 positive control cells compared to all of the other conditions tested ($p < 0.001$). After stimulation with LPS, the targeted CD was statistically significant in M2 polarized cells compared to Mock and the M1 positive control ($p < 0.001$; $p < 0.01$). As expected, the proteins CD14 and CD64 were clearly expressed in cells cultured with IFN-ɣ in unstimulated and LPS-stimulated cells. The increase in CD14 expression was statistically significant compared to the cells exposed to IL-4 ($p < 0.01$; $p < 0.05$) before and after stimulation with the septic stimulus. Strong expression of the CD64 marker was statistically significant compared to all of the conditions analysed in unstimulated cells ($p < 0.001$) and with respect to LPS-stimulated cells cultured with IL-4 ($p < 0.001$). Surprisingly, The CD163 marker was comparably expressed in cells exposed to IFN-ɣ and IL-4 before and after stimulation with LPS. Moreover, it was clearly noted that the Mock control in unstimulated cells significantly promoted the expression of the surface marker compared to IFN-ɣ-stimulated cells ($p < 0.01$). In LPS-stimulated cells, the increased expression was statistically significant in M1/M2-positive controls and in cells exposed to Mg-10Gd extracts ($p < 0.05$).

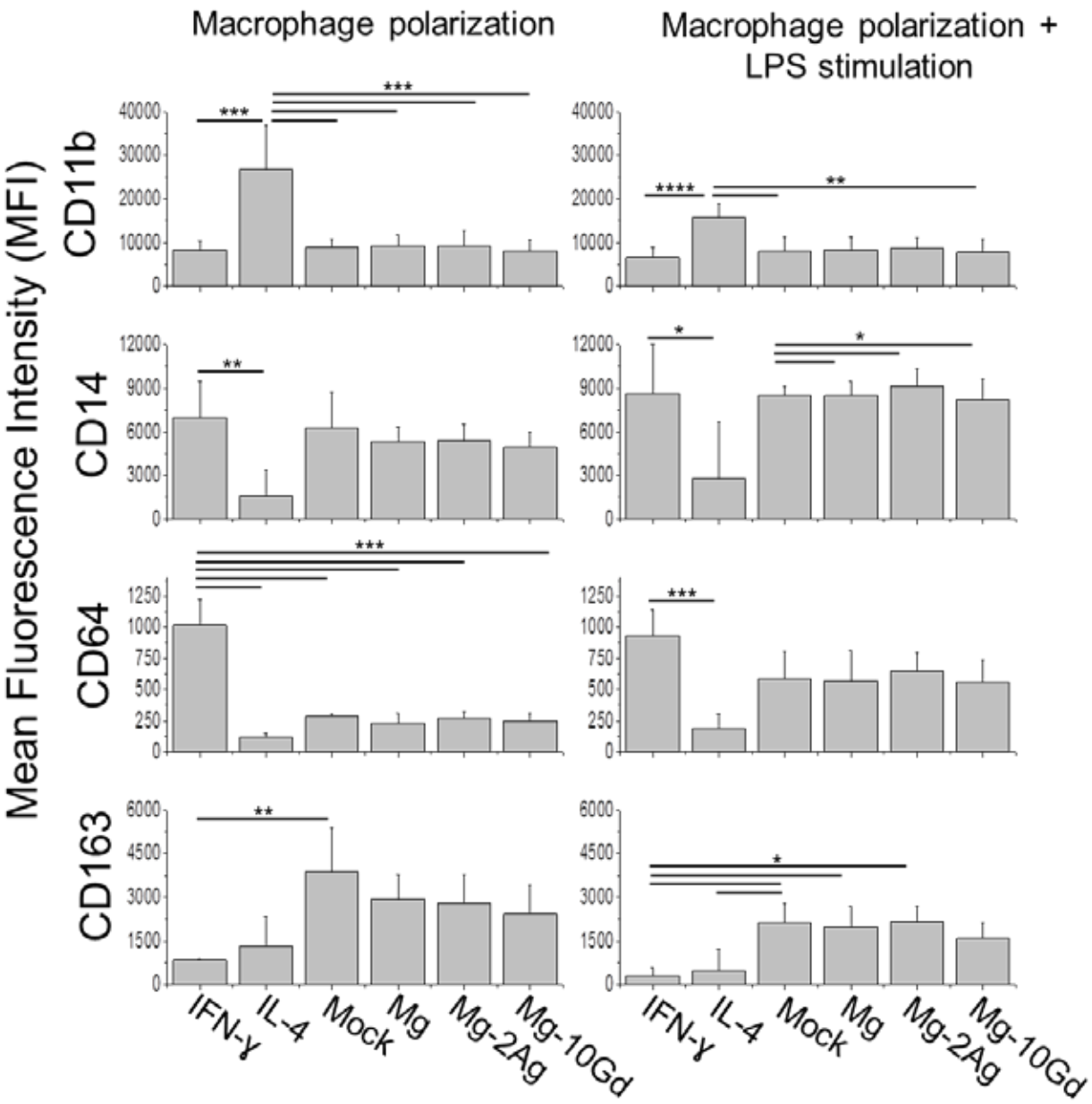


Figure 42 **Characterization of markers on polarized and LPS-stimulated macrophages.** CD expression after exposure of primary monocytes (M-CSF cells) with and without Mg, Mg-2Ag and Mg-10Gd extracts. Cells stimulated with IFN-ɣ and IL-4 are considered the positive control. Cell culture medium + 10% FBS (Mock) is the negative control. Bars represent the means ± standard deviations (SDs) of three independent measurements. Stars indicate statistically significant differences between two groups ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

6 Discussion

Magnesium (Mg)-based materials are promising biomaterials for orthopaedic applications thanks to their advantageous biodegradable and mechanical properties [7], [22], [29]. Suitable biodegradable material for medical applications requires initial mechanical stability and proper material resorption with respect to bone healing [24], [218]. Alloying magnesium with Gd and Ag satisfies such demands [48], [28]. Evaluation of the initial cell interactions towards biodegradable products is an essential step for the translation of Mg-based materials into clinical applications. Macrophages are cells of the innate immune system regarded as responsible for both material performance and bone tissue remodelling [15], [13]. In the present work, the influences of Mg, Mg-2Ag and Mg-10Gd degradation products (extracts) on macrophage activity were analysed.

The first important step of this work was to reproduce the inflammatory microenvironment *in vitro*. While macrophages are stimulated with PAMP or DAMP stimuli in most of the work reported in the literature, comparatively few papers describe the role of temperature on macrophage activities [188], [209], [219], [220]. Therefore, it was considered scientifically interesting to propose a model in which the inflammatory condition is reproduced by culturing macrophage cell types to a temperature of 39°C. Moreover, this method was chosen considering two additional aspects: 1) in the absence of an infection after an implantation of a biomaterial, the microenvironment is “ideally” aseptic; and 2) thanks to the use of only increased temperature, unpredictable chemical reactions between the extracts and the use of additional inflammatory stimuli can be avoided. Another aspect considered in this work is the time factor. As inflammation is a dynamic process, the specific influences of the extracts and *in vitro* hypermagnesaemia condition on cytokine release were analysed after 1 and 3 days, which were considered arbitrary time points of early and late inflammation.

To establish the culture method, the U987 cell line was first differentiated into macrophage cell types and was subsequently cultured at the standard and higher temperatures of 37°C and 39°C, respectively. Differentiation was successfully reproduced *in vitro* by stimulating the cells with PMA. This agent induces PKC activation and consequently triggers NF-kB signalling (the NF-kB pathway is summarized in Figure 4), which results in observed increases in the production of signals involved in the amplification of the inflammatory process (Figure 12). Once the cells were differentiated, the influences of the higher temperature on viability and cytokine release were observed. As illustrated in Figure 13, cell viability was comparable at 37°C and 39°C after culturing the cells at 39°C for 1 and 3 days. Macrophage cell type responses to the chosen time and temperature factors were further investigated at the protein level. The first

cytokines investigated were IL-1β and TNF-α, which both influence FBR and increase the body's temperature (Paragraph 2.2). As presented in Figure 14, the production of the two cytokines was down-regulated in cells cultured at 39°C compared to cells cultured at 37°C. This tendency was clearly observed in the early and late inflammatory phases. The obtained results beg the following question: "Because febrile temperature is an inflammatory signal, why are the inflammatory cytokines downregulated?" Fever is generally considered to be a physiologically beneficial response of the host against infection [188], [190], [210], [221]. In agreement with the results obtained in the inflammatory *in vitro* model presented in this work, Fairchild et all demonstrated the clear decrease in inflammatory cytokine IL-1β, TNFα and IL-6 levels in LPS-stimulated primary macrophages cultured at a higher temperature [219]. Results obtained in this work and information available from the literature would suggest that at higher temperature, macrophages decrease their expression of inflammatory cytokines to protect the tissue against the potential damage of an exacerbated amplification of inflammatory signals. This hypothesis is also supported when considering the release of the anti-inflammatory cytokines IL-1Ra and IL-10. As observed in Figure 15, at the higher temperature, the production of the two proteins was comparable to the standard temperature condition. It is also interesting that in the late inflammatory phase, IL-10 release was increased at 39°C compared to 37°C. IL-10 is an anti-inflammatory cytokine that is regarded as one the molecules specifically involved in tissue remodelling [205]–[207]. Therefore, the results obtained would suggest that the higher temperature induces the resolution phase of the inflammation. The molecular mechanism that leads to the release of inflammatory cytokines in a febrile condition is quite intricate. The increase in temperature induces the release of a group of stress-inducible proteins called heat shock proteins (HSPs) [222]–[224]. Their production at the transcriptional level is regulated by the transcriptional factor HSF1, which is maintained in an inhibitory condition at 37°C [225], [226]. Higher temperature induces HSF1 activation and HSP production [223], [224], [227]. In particular, It has been shown that LPS-stimulated macrophages from heat-treated mice secrete higher levels of HSP70 compared to the control [228]. The specific role of HSP70 in cytokine release is controversial [222]–[224], [229]–[231]. While some works have shown that HSP70 induces increased inflammatory cytokine release, the possible anti-inflammatory effects of this protein have also been reviewed [222]. Because HSP70 was not measured in that work, the protein production can be only speculated based on IL-8 production. Singh et al. demonstrated that the HSF-1 factor binds to IL-8 promoter regions and that it can directly regulate IL-8 gene expression [232]. Therefore, IL-8 production indicates the activity of the HSF-1 factor and, in consequence, HSP70 production. Moreover, in the work of Coaxum et al., it was clearly demonstrated that PKC promotes the intracellular production of HSP70 independent of HSF-1

and that the protein exhibits a protective role in rat myocytes [233]. Because PMA was used for the differentiation of the U937 cells and is a PKC activator, this observation would further support the hypothesis that HSP70 is produced in DiffU937 cells and that this event can also be independent of the temperature condition. Considering all of this information together, it can be speculated that at the 39°C condition, DiffU937 cells produce HSP70 thanks to both PKC and HSF-1 activation. The possible overexpression of the protein could be the basic reason for the reduced expression of inflammatory cytokines and the increased IL-10 production in the late inflammatory phase. This hypothesis is based on the fact that it has been established that increased HSP70 expression inhibits PKC activity and, in consequence, IKKs [234]. The second point refers to IL-10 production. It has been reviewed that HSP70 induces the promotion of IL-10 release [222]. As IL-10 levels increase at 39°C compared to 37°C after 3 days (Figure 10), this change would be the consequence of HSP70 accumulation in the febrile condition compared to the standard cell culture temperature. Indirect proof of this hypothesis is the significantly increased release of IL-8 at 39°C compared to 37°C after 3 days (Figure 16). Together, the information can be used speculate that the decreased levels of inflammatory cytokines at 39°C compared to 37°C is attributed to HSP70 overexpression at the higher temperature. Figure 43 summarizes the hypothetical mechanism in which macrophages regulated cytokine production at the febrile temperature compared to the standard temperature condition. Whether the precise mechanism needs to be defined, the results obtained and the information presented in the literature show that the physiological response of the cells at the higher temperature functions to protect the tissue from damage, decreasing IL-1β and TNFα levels and promoting the production of the anti-inflammatory cytokines IL-1Ra and IL-10. Another important point to discuss is chemokine release. As observed in Figure 16, MCP-1 and IL-8 production is mainly influenced by the time factor. In fact, it can be clearly observed that while their release was comparable at standard and high-temperature conditions, the two chemokines were clearly up-regulated after 3 days of cell culture. Because MCP-1 and IL-8 are essential for the recruitment of macrophages to damaged tissue, the results obtained would suggest that constant cell turnover is a required condition for the physiological inflammatory response.

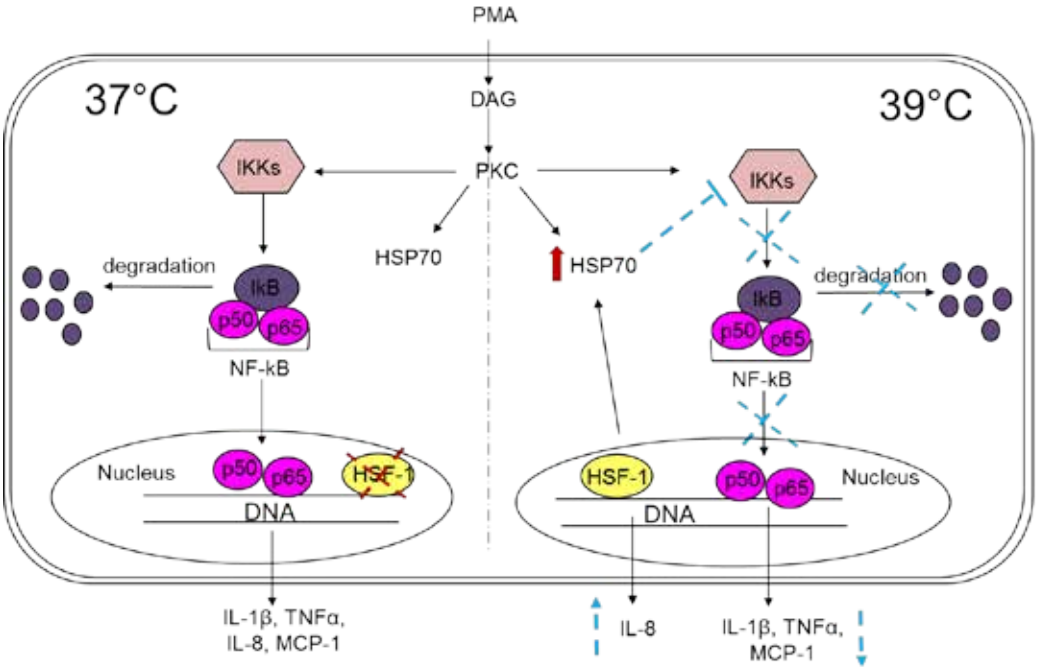


Figure 43 **Speculative model for the production of cytokines at 37°C and 39°C in DiffU937 cells.** PMA is an ester, a polar molecule that can pass through the membrane thanks to transporter proteins. Here, it activates the protein kinase C (PKC) activator, diacylglycerol (DAG).PKC produces HSP70 and promotes the activation of the IKK complex, with consequent activation of NF-kB and the production of signals that promote inflammation. At 39°C, the transcriptional factor HSF-1 is activated due to the higher temperature. HSF-1 produces HSP70 and binds a domain of the IL-8 gene, whose expression is also increased at febrile conditions. HSP70 overproduction inhibits NF-kB activity, with consequent decreases in inflammatory signals. At 37°C, HSF-1 is inhibited, and NF-kB can migrate into the nucleus. This illustration was completed according to the references [78], [198], [200], [225], [226].

A central point of this work is to evaluate the biological responses of macrophage cell types toward the extracts. As explained in Paragraph 2.2, in an ideal condition, the implantation of a biomaterial should result in a gradual decrease of the inflammatory reaction (the phenomenon of tolerance). To understand whether the degradation products of the material could interfere with natural cytokine production, attention was first focussed on observing the molecular production in cells exposed to the extracts (DiffU937Ex cells) with respect to the temperature condition. As displayed in the Figure 25, in DiffU937Ex cells cultured at 39°C, the inflammatory cytokine release levels were comparable or decreased with respect to DiffU937Ex cells cultured at 37°C. The trend for the release of anti-inflammatory cytokines was also comparable at different temperatures in DiffU937 and DiffU937Ex cells (Figure 26). These observations suggest that the natural protective response of the cells against an inappropriate amplification of

inflammatory signals is preserved after cells are exposed to the biomaterial degradation products. This hypothesis was also confirmed upon considering the ratio of IL-1Ra/IL-1β (Paragraph 2.2). As presented in Figure 27, there was a clear up-regulation of IL-1Ra with respect to IL-1β at 39°C compared to 37°C in DiffU937 and DiffU937Ex.

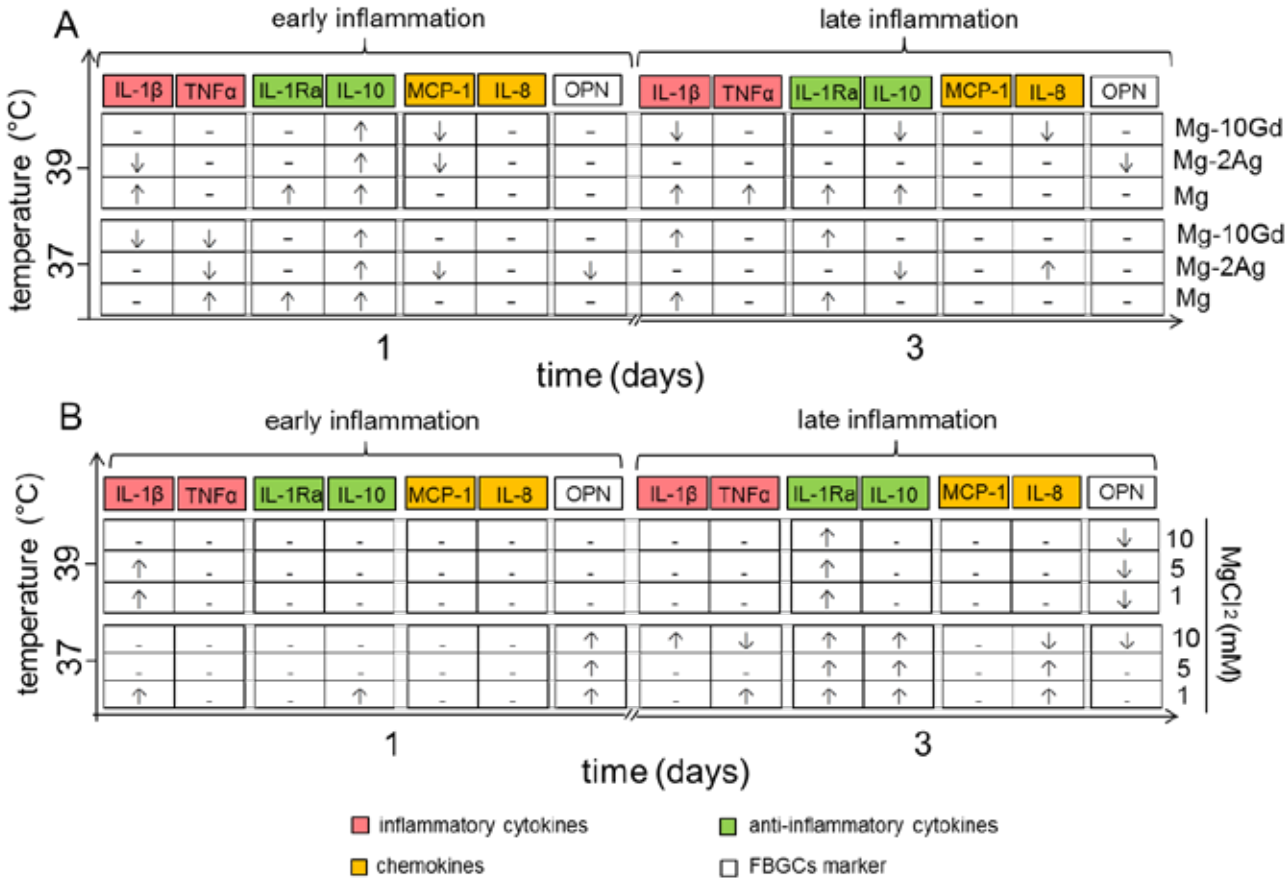
It was interesting to note the specific influences of the extracts on chemokine release (Figure 28). In fact, MCP1 and IL-8 were down-regulated in DiffU937Ex cells in the early and late inflammatory phases. The Mg-10Gd extract in particular seemed to play a specific role in the decreased MCP-1 level at the higher temperature. Independent of the time condition, in fact, the protein was decreased significantly at 39°C compared to 37°C. Another molecule specifically involved in this event is OPN, whose increasing release is associated with a reduction of FBGC formation [12], [236], [237], [135], [238]. Figure 29 shows the release of OPN in the inflammatory *in vitro* model in DiffU937 and DiffU937Ex cells. The results obtained indicate that the modulation for the release of the molecule occurs mainly with respect to the time factor. In fact, it was clearly observed that OPN production was increased in DiffU937 cells exposed to the hyperthermia condition only after 3 days of cell culture. Such a trend was also observed in DiffU937Ex cells exposed to Mg and Mg-10Gd extracts but not in cells exposed to Mg-2Ag extracts. In this case, in fact, OPN release was decreased at 39°C compared to 37°C. All of this evidence suggests that the extracts differentially modulate the release of the molecules investigated.

To further understand the specific influence of each extract solution on the release of the molecular signals, further analyses were performed, comparing protein production in DiffU937Ex cells with respect to DiffU937 cells. In particular, the increases and decreases of the targeted cytokines were calculated as explained in Paragraph 4.2.7 for each time and temperature condition. Table 7 summarizes the results obtained.

The results displayed in Table 7A show that, compared to DiffU937 cells, the Mg-10Gd extract decreased MCP-1 and IL-8 release in cells cultured at 39°C in the early and late inflammatory phases, respectively. The specific inhibition of Gd on cell recruitment has already been documented in the literature in an *in vivo* ischaemia-reperfusion rat model [98]. In this work, it was observed that pre-treatment with $GdCl_3$ solution resulted in clear decreases in circulating monocytes and neutrophils. However, such an effect was not attributed to MCP-1 and IL-8 because no reductions in their production levels were observed [98]. As suggested by the authors, the work was limited in that it investigated only at the tissue level, and the phenomenon was unclear. At the cell and molecular level, this event can be explained by the fact that gadolinium is an inhibitor of Ca^{2+} influx through the store-operated channel (SOC) [239]. Among the large amount proteins that constitute the SOC group, the TRP family is

particularly interesting in the specific case of macrophage cell types cultured at a higher temperature [240].

Table 7 **Release of cytokines in DiffU937Ex (A) and DiffU937 cells exposed to $MgCl_2$ solution (B) compared to DiffU937 cells.** Significant increases and decreases in chemokines (MCP1; IL-8), inflammatory cytokines (IL 1β; TNFα), anti-inflammatory cytokines (IL-1Ra, IL-10) and osteopontin (OPN) production were observed with respect to DiffU937 cells cultured without extract solution for 1 (early inflammation) and 3 days (late inflammation) at 37°C and 39°C. Statistical increases (↑) and decreases (↓) are reported.



Compared to other channels, the TRP family is sensitive to changes in temperature, and the mechanism that results in channel opening/closing is unknown [241]. H.S. Yamamoto et al. demonstrated that the influx of Ca^{2+} through TRPM2 is strongly related to chemokine production in macrophage cell types [242]. Considering this information and the results obtained, the hypothesis is that Gd, in the total composition of Mg-10Gd extracts, decreases the production of chemokines inhibiting Ca^{2+} influx through TRMP2.

Another important effect of Mg-10Gd extracts is IL-1β release. This effect is biologically significant considering the time factor at 37°C, while the temperature is an influencing factor only in the late inflammatory phase. The influence of Gd on IL-1β release has already been reported in the literature [97]. To understand the specific correlation between this element and protein release, it is necessary to briefly explain the complex mechanism that leads to IL-1β production. IL-1β synthesis results after a complex mechanism that starts with NF-kB activation and the resulting expression of the *IL-1β* gene [121], [243]. Subsequently, the cytokine is produced in an inactivated form called pro-IL-1β [193], [195]. The mature form of the cytokine is produced thanks to the cleavage of its precursor, a reaction that is promoted thanks to the activity of the enzyme caspase-1. This enzyme is activated by a multiprotein platform called an inflammasome [191], [195], [244], [245], [246]; NLRP3 (NOD-like receptor family, pyrin domain containing 3) is one of the proteins forming this complex [246]. Schmidt-Lauber et al. described the capacity of free Gd to activate NLRP3 [97]. In particular, the work showed an increase in IL-1β release corresponding to concentrations of free Gd up to 2.5 μM [97]. The Gd concentration in the Mg-10Gd extracts is 3.6 μM Gd (Table 3), which is comparable with the findings of Schmidt-Lauber et al. This mechanism would justify the results obtained at 37°C and in the late phase of inflammation in the *in vitro* model. Even where Gd would promote the inappropriate increase in IL-1β production, it is interesting to note that at the same time and temperature conditions, IL-1Ra was also increased significantly (Table 7A). This result suggests that a potentially dangerous effect of IL-1β would be minimized thanks to the activity of its antagonists. Such an event is influenced by the temperature condition. In fact, it was clearly observed that at the higher temperature, the protein was decreased in the late inflammatory phase with respect to the control. To clarify the specific contribution of the total composition of Mg-10Gd extracts on this pathway, *IL-1β* gene expression was analysed. As displayed in Figure 30, it was clearly observed that in the late inflammatory phase, *IL-1β* was not expressed in either the control or the cells exposed to the Mg-10Gd extracts. These results confirm that the influence of Gd on protein release occurs at the post-transcriptional level and demonstrates that higher temperature is an influencing factor of this event. It can be speculated that temperature influences the specific event of protein release. As previously described, IL-1β required several passages before the production of its biologically active form; nevertheless, it must be quickly released in response to a damage signal. The cells produce a “standard background” of the mature form even when there is no signal of damage and it is accumulated in vesicles inside the cells [195], [247]. Thanks to this system, the cells are able to rapidly release IL-1β in the case of unpredictable damage signals.It was reviewed that there are at list five different mechanism of control for the release of IL-1β [247]. Considering all of this information and the results obtained,

it could be assumed that in the specific condition of the inflammatory temperature of 39°C, Gd in the form of Mg-10Gd extracts inhibits IL-1β release. Behind the complex molecular mechanism, the results obtained in the inflammatory *in vitro* model suggest that Gd, in the total composition of Mg-10Gd, functions as a cushion for the macrophage cell response.

Figure 37 summarizes the essential steps for the IL-1β pathway and the role of Gd according to the information available and the data obtained.

In addition to the specific release of a single molecule, the complexity of the immune response needs to be analysed considering the production of proteins that lead to the same final effect on the tissue, as is the case with the inflammatory cytokines IL-1β and TNFα, which are routinely checked and detected in FBR [5], [12], [104], [163], [215], [248], [249]. As shown in Table 7A, these two molecules are oppositely regulated by Mg and Mg-2Ag extracts in the early inflammatory phase, even where the contents of the total Mg in the solution are comparable (1.15 and 1.32 mM, respectively (Table 5)). In particular, the Mg-2Ag extracts decrease the inflammatory signals independent of the temperature condition compared to the control, while the Mg extracts promote the release of pyrogen signals. Moreover, the influence of Mg extracts is also observed in the late inflammatory phase, suggesting that this effect is independent of the time factor. Peng et al. partially analysed the biological impact of the Mg-Ag alloy system [92]. In their experiments, it was clearly shown that decreases in inflammatory markers (such as IL-1α and nitric oxide (NO)) corresponded to increases in Ag^{+} ion concentrations. The higher concentration tested was 1.27×10^{-6} mM. Compared to the mentioned work, the total silver concentration in the Mg-2Ag extract solution was three units higher (2.13×10^{-3}mM) (Table 5).

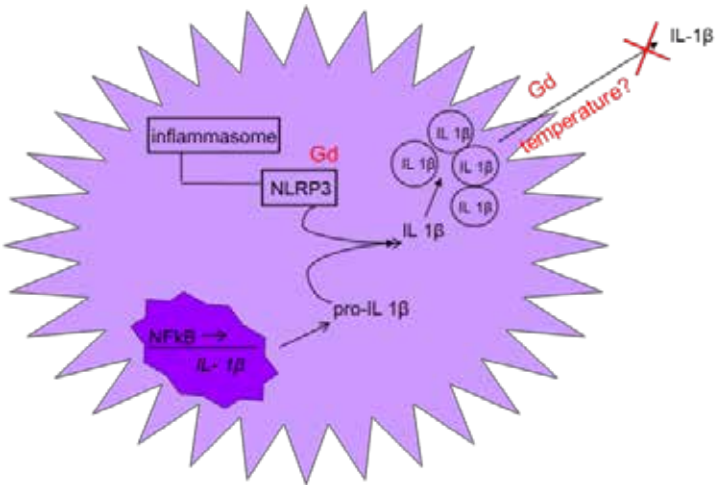


Figure 44 **IL-1β production pathway and the role of Gd in the Mg-10Gd composition.** The influence of Gd is associated with the activation of pro-IL-1β into the mature form IL-1β. Gd also influences the release of the cytokine according to temperature.

The analysis performed at the gene level underlined the different effects of Mg and Mg-2Ag extracts on cell behaviour. The receptor and inflammatory marker genes are illustrated in Figures 30 and 31. The opposite gene regulations of the two extracts were mainly evident at 37°C in the late inflammatory phase. Compared to DiffU937 cells, all of the markers analysed were upregulated only in cells exposed to Mg extracts. Putting all of the information together, it is reasonable to assume that the effects observed referred to the specific role of the Ag^+ content in the total composition of the Mg-2Ag extracts. In particular, Ag^+ content strongly minimizes the release and amplification of inflammatory signals, which should also be considered in the results obtained with OPN. In addition to its role in FBGCs, this protein appears to be constitutively expressed in immune cells, and its production is strongly increased in answer to inflammatory cytokine production and LPS stimulation [117], [237], [250]–[252]. Currently, the specific mechanism that leads to the release of OPN is unknown, but it is well recognized that the protein controls several immune functions, particularly adhesion, macrophage migration and NF-kB activation [238], [251], [253]. It is interesting to note in Table 7A that, in terms of the modulation of OPN at the protein level, molecular release is significantly decreased only in cells exposed to Mg-2Ag. As the gene expression disagrees with the results obtained in the protein analysis, it can be assumed that the specific modulation of Ag^+ in OPN production occurs at the post-transcriptional level. As the OPN signalling mechanism is unknown, the specific intracellular mechanism by which Ag^+ modulates the protein release is also still an open question. In addition, considering the condition in which the cells would be prepared for fusion, the specific influence of the extracts on such a process is still in question, as there are no clear differences observed in marker and ICAM expression compared to the control in the inflammatory *in vitro* model. As observed in Figure 31, the expression levels of those markers are clearly observed in DiffU937 cells exposed to Mg extracts, suggesting that the cells would be ready for the fusion.

As previously described, Mg extracts induce significant increases in inflammatory cytokine regulation (Table 7A). It is interesting to observe that such results were also associated with the induction of higher anti-inflammatory cytokine production. In particular, the higher production of IL-1β corresponded to an increased release of IL-1Ra compared to the control for each time point and temperature condition considered. These results would indicate that the cells minimize the power of the inflammatory signals and protect the tissue against an adverse reaction. At comparable a concentration of Mg content (~1 mM), $MgCl_2$ up-regulated IL-1β release at 37°C and 39°C after 1 day (Table 7B). No significant increase in IL-1Ra was detected at the same time point. Without its antagonist, the increased release of the inflammatory protein would promote a potentially dangerous inflammatory reaction. Upon observing the significant

modulation of the other protein targets after the cells were exposed to Mg extract and $MgCl_2$,it appears clear that the two solutions led to different cell responses, even at comparable Mg concentrations. Such differences could be explained by the fact that the Mg present in the extracts is in undefined status. In particular, questionable still the chemical reaction between the degradation products and complex medium solution. Considering that, it is reasonable to assume that even if the two solutions have comparable concentrations of total Mg, they could have different concentrations of Mg^{2+} free due chemical reactions with those impurities in liquid solution. Because the biological effect of Mg^{2+} on cellular activities is based on the regulation of its influx and efflux mechanisms, different extracellular Mg^{2+} concentrations lead to differences in cellular Mg^{2+}homeostasis, and, consequently, distinct cellular responses [54], [72]–[74], [80], [254]. As described in Paragraph 2.1.2, the cells regulate Mg^{2+} homoeostasis due to the activity of channels and exchangers, which are necessary for the maintenance of the electrochemical potential of Mg^{2+} inside the cells (~50 mM under resting conditions) [77]. Mg^{2+} homeostasis is a complex mechanism consisting of a series of intracellular events resulting in responses to metabolic stimuli [69], [73], [77], [255]. As explained by Romani, it is strongly debated if the regulation of Mg^{2+}intake is dependent on a *sensor* mechanism. In this case, ion efflux and influx would be regulated according to extracellular and intracellular free Mg^{2+} [77]. As described in Paragraph 2.1.2, the information concerning of the anti-inflammatory effect of Mg^{2+} (at the cellular level) refers to concentrations greater than 1 mM [65], [67], [256]. As the Mg^{2+} concentration in plasma is ~1.2 to 1.4 mM [77], it can be supposed that this represents a threshold concentration of external Mg^{2+}. Considering the hypothesis of the “sensor” it can be speculated that the inflammatory *in vitro* model proposed in this work shows that $[Mg^{2+}] > 1$ mM would induce the accumulation in the cells and the consequent observed effects on cytokines release. This hypothesis is supported by the fact that, in the model proposed by Sugimoto, the stabilization of IkB is a consequence of intracellular Mg^{2+}accumulation after exposure of cells to a $[Mg^{2+}]$ of 2.5 mM [67]. Moreover, in the proposed model, increasing Mg^{2+}concentration shows comparable final cellular outcomes. These effects are mainly observed considering the release of OPN, IL-1Ra and IL-10 (Table 7B). It is also important to consider the non-significant results obtained with respect to the controls. In fact, as cytokine production levels are not modulated despite the different Mg^{2+} concentrations, it can be assumed that at concentrations higher than 1 mM, the Mg^{2+} would promote a comparable effect because the free Mg^{2+} level would be comparable at the intracellular level.

Results obtained until now have demonstrated that the total compositions of Mg-2Ag, Mg-10Gd and Mg extracts promote the physiological resolution of inflammation, protecting the

tissue against an exacerbated inflammatory reaction. In particular, such a final outcome is due to the differential modulation of cytokine production.

In real conditions, the complex exchange of molecular signals is strongly dependent on the interactions between different cell types. As Mg-based materials are proposed as materials for orthopaedic implants, part of this work evaluated the influence of the extracts on the cross-talk between macrophage cell types and osteoblast cells. In particular, attention was focussed on the specific roles of Mg-2Ag and Mg-10Gd extract in a co-culture model of DiffU937/Saos-2 cells. The interaction between the immune system and bone cells has only recently been recognized, a field of immunology that is called osteoimmunology [16]. As monocytes are the precursors of osteoclasts, most of the works presented in the literature have focused on the signals involved in osteoclastogenesis and in the contribution of osteoblast cell types to such events [16], [257]–[260]. The work performed in this thesis demonstrates that the interaction between macrophage cell types and osteoblast-derived cells results in the inhibition of the inflammatory reaction. As observed in Figure 36, in the co-culture condition, all of the cytokines detectable using the array method were down-regulated compared to the solo DiffU937 culture. These observations confirm the role of macrophage cell types in the process of bone repair, as previously only hypothesized [15], [17]. Particularly interesting is that among the 36 cytokines detected using the array panel method, only IL-6 was upregulated in the co-culture condition. IL-6 is a pleiotropic protein involved in the inhibition of osteoclast differentiation [261]. Because IL-6 is not produced in the Saos-2 solo-culture condition, it is possible to assume that the interaction between macrophages and osteoblast cell types enhances tissue remodelling through the inhibition of osteoclast differentiation. Saos-2 cells are not able to produce any of the cytokines investigated in this work, even if they are exposed to LPS stimulation (Table 6). Therefore, it is clear that the inflammation cushion results not only in simple modulation between different groups of cytokines but also in the complete genetic reprogramming of DiffU937 cells. These hypotheses are also based on the fact that all of the molecules detected with the array were down-regulated. This observation was also made in cells cultured with Mg-10Gd and Mg-2Ag extracts, where the attention was focussed on the release of IL-1β and IL-10, hallmarks for the promotion and inhibition of bone resorption, respectively [258], [151], [182]. The results of these experiments are presented in Figure 37 and show a significant increase in the release of the cytokine IL-10 in the co-culture condition compared to DiffU937 and Saos-2 solo-cultures, with and without extract stimulation. This observation indicates that the extracts enhance the release of this cytokine and the consequent tissue remodelling. In case of cell cultures with Mg-10Gd extracts, this hypothesis is in agreement, considering the decrease in IL-1β levels in the co-culture condition compared to unstimulated DiffU937/Saos-2 cells. In

contrast, Mg-2Ag promotes the release of the inflammatory cytokine. This result suggests that, compared to Mg-10Gd, Mg-2Ag extracts have the potential to compromise the inhibition of osteoclast activity during bone remodelling. The results obtained concerning Saos-2 cell growth and viability when exposed to the extract and stimulated with LPS are questionable (Figure 34 a, b). It was clearly observed that the viability of Saos-2 cells stimulated with only LPS in medium was comparable with the viability of cells stimulated with the extracts. Nevertheless, there are clear differences between in the growth of Saos-2 cells stimulated with extracts and the controls (Saos-2; Saos-2 + LPS). This unclear aspect should be further investigated.

Up to this point, the information accumulated has referenced experiments performed using cell lines. The malignant condition of such lines partially compromises the “plasticity” property of the monocyte-macrophage lineage and the natural polarization in the M1/M2 subpopulation in answer to defined stimuli (Paragraph 2.2). Thus, the M1/M2 macrophage subpopulations were reproduced *in vitro* using primary cells. The investigations performed aimed to explore M1/M2 cytokine release in response to treatment with the extracts and the influence of the degradation products on the macrophage differentiation and polarization phases. To achieve these purposes, two different protocols were used (Paragraph 4.2.3). In all of the experiments performed with primary cells, FBS had a clear influence. Such interfere was evident considering the results obtained with the Mock control, in which cytokine release and CD expression were promoted. FBS is a cocktail of growth factors and is a standard component used in addition to cell culture medium for the promotion of cell growth and proliferation [264]. The exact serum composition (either derived from animals [FBS] or humans [HuS] was not defined, and possible differences between providers or from lot to lot arise concerning its use with fresh isolated primary monocytes [264]-[266]. The experiments performed in this work confirm that the serum interferes with primary macrophage cell behaviour and that its use is a limiting factor in extract testing with primary macrophage cell types. Extract preparation was performed following the standard procedure ISO 10933-5 -Paragraph 4.2.2, which clearly suggests the use of a serum component in the extraction vehicle: “*Culture medium with serum is the preferred extraction vehicle”*. Despite that, significantly increased expression of the CD163 marker was observed in cells exposed to Mg-2Ag extracts compared to the positive control (M-CSF) after cells were stimulated with LPS (Figure 41). The increase in CD163 expression was also obtained in polarized cells compared to IFN-ɣ-stimulated cells (Figure 42). CD163 is a scavenger receptor particularly expressed in macrophages and has anti-inflammatory properties [147], [267], [268]. These results would suggest that the modulation of the marker expression is independent of the FBS interference. This hypothesis is based on the fact that a significant increase in protein expression is detected only with Mg-2Ag extracts, even when all of the extracts tested and the

negative control contain the same percentage of FBS in the cell culture (1%). The increase of CD163 expression in cells exposed to Mg-2Ag extracts after LPS stimulation indicates the capacity of the cells to moderate inflammation in answer to a stimulus of damage. Such an indication should be further investigated, bypassing the limitation of the FBS interference.

7 Conclusions and outlook

Magnesium-based materials are promising biomaterial for orthopaedic applications thanks to their biodegradable and mechanicals properties. Up until now, the biological inflammatory evaluation of such materials was mainly based on *in vivo* investigations. Such an approach strongly limits the identification of the molecular mechanisms involved in the final tissue reactions toward the degradation products of the materials. The present work demonstrates, for the first time at the cellular and molecular level, that the degradation products of Mg, Mg-10Gd and Mg-2Ag would promote the phenomenon of tolerance by preserving the natural protecting activity of the macrophages. All of the extracts influenced the release of chemokines, with possible influence on cellular recruitment into damaged tissue. Even when the three materials shared common final effects, the research presented in this thesis demonstrates that each material extract interferes with protein release and production following different pathways. A specific role of the Mg-2Ag extract on M2 macrophage polarization can be suggested. Figure 45 illustrates the macrophage's behaviour in response to interactions with the extracts in a final overview.

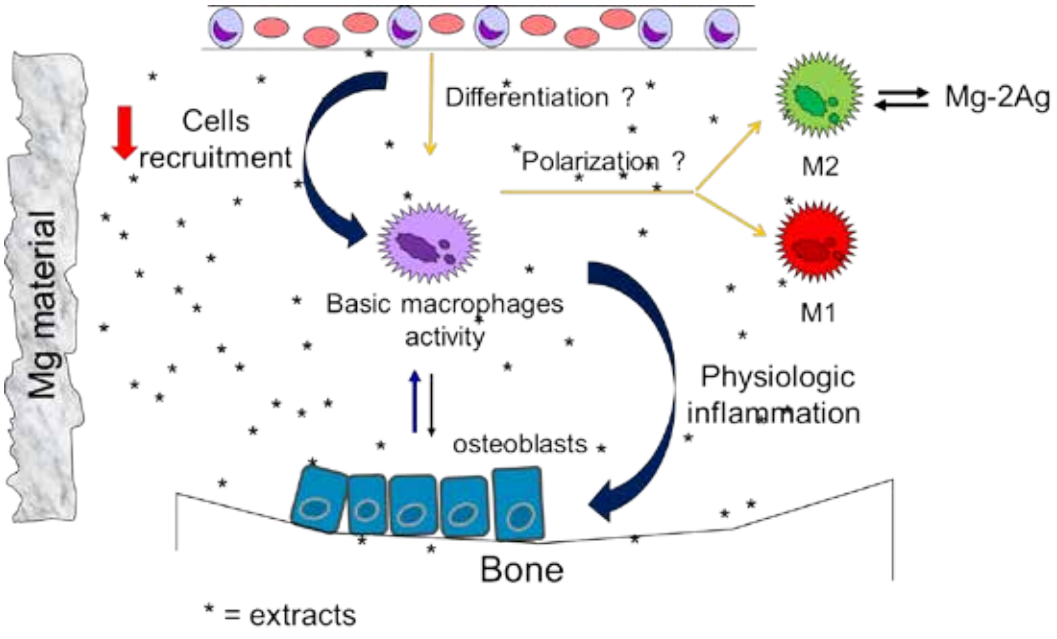


Figure 45 **Impact of the extracts on macrophage cell activity**. In response to the extract, macrophages promote a physiological inflammatory reaction and reduce cellular recruitment to the damaged tissue.

The work performed demonstrates the methodological limitations for the biological evaluation of materials using primary cells. Such limitations are attributed to the interference of the FBS component, which is specifically required to follow the standard operating

procedure. To understand the specific impacts of the elements forming the extract on cellular metabolism, it is strongly suggested to drive future research in the cell electrophysiology area. Particularly interesting would be the analysis of the efflux and influx of Mg^{2+} inside the cells after exposure to the extracts. Despite the material's composition, its degradation results in a solution with a basic pH. It would be interesting to investigate Mg^{2+} cell homeostasis considering this parameter. Moreover, as this work demonstrates that temperature modulates cellular activities after exposure to the extracts, it would be interesting to investigate its influence on Mg^{2+} metabolism in combination with a basic pH condition. Another important point to mention is that most of the work in the biomaterial area lacks information concerning the complex molecular networks that become involved once the cells come into contact with the material. It would be interesting to further analyse the influence of the extracts on the cellular cross-talk, considering the complex final effects of the cytokine network. The basis to do so was laid it out by this thesis.

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9 Symbols and abbreviations

Acronym	Name
(CD)	Cluster differentiation
C5a	Complement component 5a
cDNA	Complementary deoxyribonucleic acid
DiffU937	Differentiated U937 cells
DiffU937MgE	Differentiated U937 cells exposed to magnesium extracts
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating facto
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GROα	Growth-regulated alpha protein
HMGB1	High mobility group box 1
I-309/CCL1	Chemokine (C-C motif) ligand 1
ICAM1	Intercellular adhesion molecule 1
ICP-MS	Inductively coupled plasma mass spectrometry
IFN-γ	Interferon gamma
IP-10	Interferon gamma-induced protein 10
ISO	International standard organization
I-TAC	Interferon–inducible T cell alpha chemoattractant
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LPS	Lipopolysaccharide
MCP1	Monocyte chemoattractant protein-1

MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein 1alpha
MIP-1β	Macrophage inflammatory protein 1beta
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NF-KB	Nuclear factor kappa B
OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor-1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PMA	Phorbol 12 myristate 13 acetate
qRT-PCR	Real-time polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acids
SDF-1	Stromal cell-derived factor
sICAM-1	Soluble intercellular adhesion molecule-1
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
TLR2	Toll-like receptor 2
TNFα	Tumour necrosis factor alpha
UndiffU937	Undifferentiated U937 cells

10 List of figures and tables

11 Risk and safety statement

Following is a list of potentially hazardous chemicals with the respective hazard and precautionary statements, as introduced by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Compound	**Chemical Abstracts Service No.**	**Hazard statements**	**GHS hazard**	**Precautionary statements**
$MgCl_2$	7786-30-3	H319	GHS07	P305; P351; P338
PMA	16561-29-8	H315	GHS07	P264;P280; P302 + 352; P332+313; P362
Tetramethylbenzidine	DY999	H315 H319; H312; H332; H335; H360	GHS07; GHS08	P202; P261; P264; P270; P280; P281
Sulfuric acid	DY994	H315; H312; H315	GHS07; GHS08	P301; P302;P262
H_2O_2	DY999	H302-H318	GHS05; GHS07	P280; P301; P312; P330; P305; P351; P338; P310; P280

GHS precautionary statements

H302	Harmful if swallowed
H312	Harmful in contact with skin
H315	Causes skin irritation
H318	Causes serious eye damage
H319	Causes serious eye irritation
H332	Harmful if inhaled
H335	May cause respiratory irritation

GHS precautionary statements

P202	Do not handle until all safety precautions have been read and understood
P261	Avoid breathing dust/fume/gas/mist/vapours/spray
P262	Do not get in eyes, on skin, or on clothing
P264	Wash hands thoroughly after handling
P270	Do not eat, drink or smoke when using this product
P280	Wear protective gloves/protective clothing/eye protection/face protection
P281	Use of personal protective equipment is required.
P301	If swallowed
P302	If on skin
P305	If in eyes
P310	Immediately call a POISON CENTER or doctor/physician
P312	Call a POISON CENTER or doctor/physician if you feel unwell
P313	Get medical advice/attention
P330	Rinse mouth
P332	If skin irritation occurs
P338	Remove contact lenses, if present and easy to do. Continue rinsing
P351	Rinse cautiously with water for several minutes
P352	Wash with plenty of soap and water
P362	Take off contaminated clothing

12 Declaration

I hereby declare on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids. I hereby declare that I have not previously applied or pursued for a doctorate

Signed:

02.11.2015, Geesthacht

Conference Paper

M. D. Costantino, B.J.C.Luthringer, R. Willumeit-Römer Inflammatory Response to Magnesium Based Biodegradable Implant Materials, European Cells and Materials Vol. 28. Suppl. 3, 2014 (page 24) ISSN 1473-2262 ▪

Conferences contribution:

- Study of initial cell behaviour on magnesium alloys under inflammatory conditions. Magnesium in Translational Medicine, Slovak Republic, May 2014 (*Oral presentation)*
- Magnesium based biodegradable implants and inflammation. MSE 2014; Darmstadt, Germany (*Oral presentation)*
- Inflammatory response to magnesium based biodegradable implant materials 6th International Symposium on Biodegradable Metals, Maratea, 2014 (*Oral presentation)*
- Inflammatory response to magnesium based biodegradable implants materials ESB 2014, Liverpool (UK) August 2014(Poster)
- Molecular pathways involved in the immune response on magnesium alloys 7thInternational Symposium on Biodegradable Metals, Carovigno, 2015 (*Oral presentation)*

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