# phage surface induced modulation of inflammatory responses

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**Abstract**

Chronic inflammation responses hamper the tissue engineering. immune system has main function in the regeneration and maintenance of all tissue, the immune reaction to an implant begins by the innate immune cells including macrophages which can eventually lead to accept or reject of the implant. to avoid adverse immune reactions, current strategies use of immunomodulatory biomaterials rather than inert materials. The present study aimed to introduce as biomaterial is capable of modulating macrophage responses. Macrophages cultured on top of four surfaces then analysis morphological characteristics, cellular outgrowth and function. In addition, measured the key cytokine/chemokine markers of macrophage polarization in each sample. The results of our study pointed out that phage nano-structure can modulate polarization of macrophages toward anti-inflammatory phenotype over time. In addition, the combination of well-characterized RGD peptide motif embedded in bacteriophages can stimulate macrophages to gain regenerative M2-like phenotype more effectively and it may introduce an Immuno-modulating biomaterial for tissue engineering applications.

ANTI-INFLAMMATORYIMMUNO-MODULATING

**Introduction:**

the interface between the implant and the inflammation responses of the body, determined by innate and adaptive immune responses which decide on the successful for regeneration of tissue(Anderson, Rodriguez, & Chang, 2008).

Shortly after implantation, scaffolds are extensively infiltrated by immune cells, macrophages are key cell in the early interaction between the biomaterial and immune system. these cells are really important to regulate chronic inflammation related by biomaterial. (Gurtner, Werner, Barrandon, & Longaker, 2008; Wynn, Chawla, & Pollard, 2013; Zhou & Groth, 2018). They can modulate the immune response via their signaling. macrophages resolution of the inflammation, due to their ability to shift from a pro-inflammatory polarization state (M1) towards a regenerative profile (M2)(Mosser & Edwards, 2008). Macrophages as an important source of chemokines and inflammatory molecules such as interleukin-6 and tumor necrosis factor alpha (TNF-α) are involved in initial cellular responses in damaged tissue. After the initial responses, for a successful tissue regeneration, macrophage characteristics should go toward the anti-inflammatory state of the M2 macrophages, which exert their effects through the secretion of cytokines such as interleukin 10 and transforming growth factor beta (TGF- β)(Wynn & Vannella, 2016).

immunomodulation scaffolds avoid unwanted immune responses to implant. Over the past years, researchers have been able to develop of modulation scaffold by incorporation of various anti-inflammatory biomaterial (Zhou & Groth, 2018). From the discovery of the bacteriophage to the present, Phages are being considered

as tools for biomedical science(Twort, 1915). Several researches have illustrated that filamentous phages as ECM-mimicking nanofibers could enhance cell adhesion, proliferation, and differentiation. (Merzlyak, Indrakanti, & Lee, 2009; Wang, Wang, Li, & Mao, 2013; Wang et al., 2014). Bacteriophages are bacterial viruses that naturally come in contact with mammalian immune cells through microbiome (Reyes, Semenkovich, Whiteson, Rohwer, & Gordon, 2012). Phages can interact with immune cells after phagocytosis or direct contact with immune cell receptors. Both protein and nucleic acid structure of the phage can stimulate different pathogen recognition receptors of immune cells and turn on the relevant signaling pathways (Carroll-Portillo & Lin, 2019). In addition, phage-immune system interaction influences immune responses to environmental stimuli that leads to anti-inflammatory condition (Van Belleghem, Dabrowska, Vaneechoutte, Barr, & Bollyky, 2019). We hypothesize that alter immune cell response. It is known that the type of scaffold material exerted on macrophage cell can directly affect their phenotype and their cytokine profile(Badylak, Valentin, Ravindra, McCabe, & Stewart-Akers, 2008). The aim of the present study is to suggestion a novel application of phage for an Immuno-modulating biomaterial production. So, their bioactivity and immunostimulatory properties in encountering with macrophages were assessed in in-vitro condition.

## Materials and methods

### Large-scale amplification of and preparation coated surfaces

phage was grown and purified following standard biochemical protocol. An amount of 500 mL E. coli TG1 culture was grown in 2ytx media to mid-log phase and infected with 1 mL wild-type bacteriophage ($10^{12}$ PFU/ mL). The culture was incubated at 37°C with shaking for five to six hours and centrifuged at 8000g for 30 minutes to remove bacterial cells; and then, the virus was collected by subsequent centrifuging at 20000 g for 150 minutes. The resultant pellet was suspended in 500 µL PBS and concentration of the isolated bacteriophage was determined spectrophotometrically using an extinction coefficient of 3.84 $cm^2$/mg at 269 nm.

### Macrophage preparation and characterization

Twenty female BALB/c mice were obtained at six to eight weeks of age from the Pasteur Institute of Iran, and maintained in the animal laboratory in accordance with the Ethical Commission of Tarbiat Modares University guidelines. For preparation of peritoneal macrophages, 3 ml of 4% w/v thioglycollate medium was injected into the peritoneal cavity of the BALB/c mice. After four days, the macrophages were harvested by injecting; and subsequently, harvesting of fresh cold DMEM from the peritoneal cavity near the fat region of the lower abdominal area took place. Harvested peritoneal fluid was centrifuged for five minutes at 350×g and the resultant cell pellets were seeded (to $1\times10^6$ cells/ ml) 4 or 24 well plates for downstream analysis and incubated in a humidified incubator (37@C and 5% CO2). The medium of newly isolated macrophages was changed after six hours for separating the non-adhesive cells. Phenotypic analysis of isolated cells was performed by flow cytometry for CD14+ and CD11b+ markers (Figure S2, Supporting Information ) as described previously (Gonçalves & Mosser, 2015).

### Cell viability assay

To investigate the biocompatibility of a selected concentration of (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for examining the viability of peritoneal macrophages seeded onto and gelatin-coated and control plates. Two or seven days after culture of cells in a 96 well plate ($1 \times 10^4$ cells/well), MTT solution (20 µL, 5 mg/ml) was added to cell culture media and plates were incubated at 37°C. After four hours, the medium was removed and 200 µL dimethyl sulfoxide (Sigma-Aldrich, USA) was added to each well for eluting the formazan crystals, and optical density was measured at 490 nm with a microplate reader (BioTek, USA).

### Scanning electron microscopy

Next, the morphology of macrophages from each experimental group was observed using SEM in order to evaluate the induced morphological changes. Briefly, cells were cultured on precoated tissue culture grade coverslips and after 2 and 7 days the coverslips were air-dried. Then, the samples were gold-coated and after that they were visualized using a scanning electron microscope (KYKY-EM3200, 26KV).

### RNA preparation, cDNA synthesis, and qRT-PCR

Macrophages of experimental groups were harvested for RNA extraction after two or seven days in culture. Total RNA was extracted from freshly harvested macrophages using RiboEX (GeneAll) according to the manufacturer's recommendations. After Dnase treatment (Thermo), RNA samples were subjected to cDNA synthesis and qRT-PCR. $RT^2$ SYBR Green High ROX Master mix was used for qRT-PCR and data were quantified using [?]CT method.

### Cytokine measurement

Supernatants of the macrophages in experimental groups were collected after two and seven days and stored at -20@C. The presence of IL-6, TNF-α, IL-10 and TGF- β cytokines were assessed using ELISA kits (eBioscience) following the manufacturer's instructions. Each sample was dispensed in triplicate. The optical density of each well was determined at 450 nm.

### NO production

NO production was measured according to the accumulation of NO2 in culture supernatants after 2 and 7 days culture using the Griess reagent, as previously described (Edwards, Zhang, Frauwirth, & Mosser, 2006). Briefly, 100 μL of Griess reagent was mixed with equal volumes of culture supernatants from each experimental group for 10 minutes at room temperature. Then, the absorbance at 540 nm was measured using a microplate reader. Standard curve was established using a graded solution of NO2. Results were presented as mean values from three separate samples.

Determination of intracellular ROS

The accumulation of intracellular ROS in each experimental group was evaluated by using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). This molecule de-acetylates after entry into the cells and then oxidizes with intracellular ROS to form fluorescently reactive DCF(Eruslanov & Kusmartsev, 2010). To determine ROS production, the experimental groups were incubated with DCFH-DA (10 μM) in serum-free culture media for 45 minutes at 37@C, washed twice with PBS, and finally, analyzed by flow cytometry (BD FACSCanto II, BD Bioscience, San Diego, CA, USA).

### Statistical analysis

Differences between the two experimental groups were estimated by using Student's t-test. For more than two groups, significance was estimated by using one-way analysis of variance (ANOVA). Statistical analyses were performed by a GraphPad Prism (Version 6). Data were presented as mean ± standard error of means. P ? 0.05 was considered statistically significant.

## Results

### Different coatings change cellular morphology and viability

To determine the effects of different coatings on cellular morphology and viability, primary mouse macrophages were placed directly on the gelatin-coated and control plates. Cell morphology on each surface was observed and analyzed after two and seven days using PKH dye labeling and scanning electron microscopy (SEM) as shown in**Figure 1** A and B, Fluorescent microscope images and SEM micrographs showed that the macrophages represented the more pronounced outgrowth and well-spread morphology on the containing surfaces, respectively. It should be noted that as compared to other coated surfaces, macrophages seeded

on control plates occupied the least area. Macrophages on the gelatin surface also exhibited a well-spread morphology but their cell sizes were smaller than those of **Figure 1C** ). To investigate the effects of different coatings on the survival and viability of primary macrophages, cells were subjected to MTT assay on the second and seventh days. Results of MTT assay suggested that mouse macrophages in each experimental group displayed a similar viability pattern, and the survival rate was not significantly different among non-coated or coated surfaces at any point of time (**Figure 1D** ).

**Different surfaces alter gene expression and cytokine secretion of M1-M2 macrophage markers**

In this study, to further confirm the effects of different coatings on macrophage characteristics and paracrine secretion, the gene expression and protein secretion of master regulatory cytokines related to macrophage polarization were determined in either two or seven days after culture. To examine the effects of different surfaces on macrophage characteristics, we conducted qRT-PCR and ELISA assays on the second and seventh days after culture for IL-6, IL-10, TGF- β and TNF-α cytokines. Gene expression and cytokine production of IL-6 and TNF-α were upregulated in macrophages of containing surfaces in two days; but after seven days, the expression of TNF-α showed a downtrend as compared to control surface (**Figure 2 A and B** ). Accordingly, levels of IL-10 and TGF- β gene expression and cytokine secretion were much higher in containing surfaces as compared to the control surface in both 2 and 7 days (**Figure 2C, D** ). Furthermore, macrophages from gelatin surface showed the same gene expression and cytokine production patterns as control surface.

To confirm the macrophage polarization pattern, primary murine macrophages in each experimental group were either treated or not treated with LPS. 2 days later, the expression of IL-10, IL-22, CCL22, CXCL10, TNF-α, TGF- β, genes. According to the results, non-LPS treated macrophages cultured on containing surfaces showed higher levels of anti-inflammatory gene expression and cytokine production as compared to gelatin and control surfaces (except for TNF-α). Interestingly, exposure to LPS could not increase inflammatory cytokines of macrophages in **Fig. 2E** ).

**Different surfaces alter gene expressions of iNOS and ARG1, NO secretion**

To investigate the role of selected surfaces on polarization state of macrophages cultured cells from each experimental group were analyzed for ARG1 and iNOS gene expression and NO secretion. Our results demonstrated that the transcript level of ARG1 in the phage containing surfaces was higher than the control surface at 2 days. During the 7 days culture of cells, the gene expression of ARG1 was significantly upregulated only in the cells cultured on surface compared to control surface (**Figure 3A** ). In addition, iNOS gene expression was significantly upregulated in the containing and Gelatin surfaces compared to their control counterparts after 2 days. While, the expression of iNOS was significantly downregulated after 7 days in . The results of qRT-PCR for iNOS gene expression were confirmed by the results of NO production in the culture supernatant of macrophages from each group (**Figure 3C** ). For determining the M1/M2 balance of cultured macrophages, the ARG1/iNOS mRNA expression ratio was measured. There was a significant increase in the ratio of ARG1/iNOS mRNA.

**Different surfaces alter cellular redox potential in macrophages**

The redox potential of cultured macrophages was assessed using the determination of intracellular ROS production. Here, culturing of macrophages on surfaces dramatically decreased the intracellular ROS level as compared to control surface in both 2 and 7 days (**Figure 4** ).

**Different surfaces alter phagocytosis and efferocytosis of cultured primary macrophages**

To define the functional effects of selected surfaces on macrophage phagocytosis and efferocytosis, we analyzed the uptake of labeled yeasts and apoptotic cells by cultured cells. In this study, the capacity of macrophages for internalization of yeasts and apoptotic cells was evaluated by both fluorescent microscopy and flow cytometry. We found significantly increased uptake of apoptotic cells by macrophages cultured on on the second and seventh days after culture. However, macrophages from control and gelatin surfaces exhibited increased level of phagocytic activity in two and seven days (**Figure 5A, B** ).

**Discussion:**

after implantation graft, Inflammation is initiated. Prolonged primary inflammation can lead to tissue damage which can prevent regeneration. The M2 macrophage (or anti-inflammatory macrophages) has been shown to lead to more reduce tissue damage and increased ability to regenerated (Brown, Ratner, Goodman, Amar, & Badylak, 2012) (Badylak et al., 2008). Bacteriophages can stimulate immune cells and modulate both innate and adaptive immune responses(Van Belleghem et al., 2019). Based on Bacteriophage-immune system interaction, the or production of modulation scaffold that has not been considered. In The present study, we showed that the is biomaterial can modulate macrophage responses. For this purpose, we examined nteraction with macrophages as a representative of tissue resident immune cells in vitro. we showed change macrophage response and alter cytokine profile and polarization.

According to immunofluorescence and scanning electron microscopy analysis, the most dramatic increase was observed in the cell number and contact areas of cultured macrophages on structural support for adhesion that facilitate more cell proliferation and migration(X. Li, Dai, Shen, & Gao, 2017; Yang et al., 2017) (Richbourg, Peppas, & Sikavitsas, 2019). Previous reports demonstrated that RGD peptide plays a critical role in the spread of cells through focal adhesion [46]. In addition, our findings indicated that the combination with RGD peptide are both non-toxic and biocompatible.

Since macrophages are one of the key regulators of tissue remodeling, their response on candidate scaffolds may be an important indicator of successful tissue engineering. It has been well documented that macrophages contribute to modulate immune responses through their paracrine secretion (Corliss, Azimi, Munson, Peirce, & Murfee, 2016). During the early stages of normal wound healing, M1 macrophages infiltrate the wound to promote inflammation and to stimulate the wound healing process. M2 macrophages begin to accumulate around the third or fourth day after injury, while the level of M1 macrophages decreases. In tissue remodeling processes, M2 macrophages generate in several ways including direct shift of M1 type to M2 type macrophages, polarization of newly migrating macrophages toward M2 phenotype, and proliferation of other M2 macrophages (Yu, Tutwiler, & Spiller, 2015). In this study, we showed that the especially when combined with RGD, had the ability to reprogram naive peritoneal macrophages toward M2-like phenotype. Additionally, our results demonstrated that stimulate the secretion of TNF-α, IL-6, TGF- β and IL-10 at 2 days after culture. Also, we observed a time-dependent increase in M2 macrophage markers (IL-10 and TGF- β (Makita, Hizukuri, Yamashiro, Murakawa, & Hayashi, 2015)) and decrease in M1 macrophage marker (TNF-α (Shapouri-Moghaddam et al., 2018)). In addition, cytokine analysis at both gene expression and protein level showed polarization of macrophages to M2 phenotype after interaction.

Moreover, M2 macrophages are metabolically different from M1 macrophages and the metabolic patterns of each are directly related to their tissue modulating function. It has been accepted that the Inducible nitric oxide synthase (iNOS) gene and NO are highly expressed in M1 macrophages, while the upregulation of arginase1 (ARG1) was observed in M2 macrophages (Jin, Liu, & Nelin, 2015).Notably, L-arginine metabolic pathway of macrophages determines the polarization status toward M1 or M2 phenotype (Rath, Müller, Kropf, Closs, & Munder, 2014). It has been widely accepted that NO is a key molecule produced by M1 macrophages to exert their role in immune defense. High level production of NO is generated from the oxidation of L-arginine. ARG1 is responsible for another metabolic pathway for L-arginine in macrophages, and this pathway produces L-ornithine for the biosynthesis of polyamine and collagen. These products help M2 macrophages to improve tissue regeneration (Z. Li et al., 2012; Rőszer, 2015). It is noteworthy to mention that the competition between ARG1 and NOS enzymes determine the M1/M2 phenotypic shift in macrophages. We found that the ratio of ARG1/iNOS transcript levels in the

It has been proposed that redox potential can play a complex role in the determination of macrophage cellular fate (Tan et al., 2016). A previous study demonstrated that ROS level is closely related to essential signaling pathways, which regulate M1 macrophage polarization (Lee et al., 2014). It is interesting to note that interactions between ROS and NO are responsible for the regulation of cellular inflammatory conditions (McNeill et al., 2015).

Furthermore, macrophages are responsible for the clearance of microbial pathogens (phagocytosis) and apoptotic cells (efferocytosis) (Korns, Frasch, Fernandez-Boyanapalli, Henson, & Bratton, 2011). Therefore, to ask if selected surfaces in the current study could alter the Functional properties of macrophages, we analyzed the phagocytic and efferocytic activity of each experimental group. In particular, proper efferocytosis by macrophages is an essential step in the regulation of tissue remodeling. With regard to this, alternatively activated M2 macrophages have enhanced efferocytic capability and therefore, contributed to the tissue regeneration process. In contrast, M1 macrophages are known to have low efferocytic properties (Korns et al., 2011; Sun et al., 2015).

**Conclusion:**

Biomaterials are most important part of strategies for regenerative medicine, In the process of tissue regeneration, modulation of immune and reduce anti-inflammatory responses are one of the most essential strategies to achieve desired tissue remodeling responses. Here, we demonstrated that s toward regenerative M2-like macrophages, promoting tissue remodeling especially through their secreted factors. effectively than In conclusion, we believe that the can be introduced as Immuno-modulating biomaterial for successful tissue regeneration and open new avenues for future in vivo tissue engineering studies.

## Conflict of interest

All the Authors declare no conflict of interest.

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