J Mater Sci: Mater Med (2007) 18:2233–2236
DOI 10.1007/s10856-007-3014-9

# Immune evaluation of biomaterials in TNF-α and IL-1β at mRNA level

**Tingting Ding · Jiao Sun · Ping Zhang**

Received: 29 March 2006 / Accepted: 26 March 2007 / Published online: 20 August 2007


**Abstract**
*Objective* To examine biomaterial biocompatibility and improve current methods of immunological evaluation TNF-α and IL1-β were used as indicators at mRNA levels.
*Methods* Rat peritoneal macrophages were stimulated with different biomaterials and expression of TNF-α and IL1-β was measured by RT-PCR and compared within different groups.
*Results* Macrophages that were stimulated with PTFE produced significantly more TNF-α and IL1-β than unstimulated macrophages ($p < 0.001$). The PLGA, NPG, β-TCP, and CPC groups also induced moderate TNF-α and IL1-β expression ($p < 0.01$).
*Conclusions* TNF-α and IL1-β are sensitive indicators of immune stimulation that can help to monitor the levels of cellular activation induced by different biomaterials. Our findings showed that β-TCP and CPC had a good biocompability, and CPC was the most biocompatible of all the biomaterials tested. While PTFE and NPG still had side effect because of producing pro-inflammatory cytokines in vitro once implanted.

T. Ding · J. Sun (✉)
Ninth People's Hospital School of Medicine, Shanghai Jiao tong University/Shanghai Biomaterials Research & Testing Center, Shanghai 200023, China
e-mail: Jiaosun59@yahoo.com

P. Zhang
School of Life Science, East China Normal University, Shanghai 200062, China

## Introduction

Biomaterials can serve as substitutes for tissues and organs, at times even enhancing their function. Researchers have shown that there is an important interaction between biomaterials and the immune system. The recognition of biomaterials as foreign antigen stimulates defense reactions in the body that include inflammation, antibody expression, complements activation, and cytokine release [1, 2]. Thus, it is important to develop an effective immune technology to evaluate the biocompatibility of biomaterials. Until now, no immune standards or guides have been publicized regarding the evaluation of biomaterials. While the ISO is applying to draft out related guideline files, more researches are required before estimate guidelines can be decided upon. In this study we aim to further evaluate biocompatibility of biomaterials in an immune manner. Many immune studies of the biocompatibility of biomaterials, including measurement of immunoglobulin and complement production, or utilizing E Rosette Test [3], are conducted at cellular or histological level. Immune reactions that are mounted against biomaterials can occur at either the cellular or molecular level, with the latter being more sensitive than the former. Thus, in order to assure the safe application of biomaterials, anti-biomaterial immune responses must be examined at molecular level.

Since biocompatibility is reduced when biomaterials induce higher amounts of cytokine expression, cytokine induction can be used to evaluate the intensity of anti-biomaterial immune reactions [4]. Most research on biomaterials had focused on the relationship between expression of a specific cytokine and biocompatibility of one biomaterial. In this study, we used RT-PCR to measure TNF-α and IL1-β expression of macrophages at the molecular level in response to multiple biomaterials with

different biocompatibilities, including Polyvinyl chloride (PVC) containing 8% organic tin, a positive control material with cellular toxicity.

Rat peritoneal macrophages are unable to express TNF-$\alpha$ and IL1-$\beta$ in the absence of LPS in vitro [5], so biomaterials were characterized as having immune effects if they could induce these cytokines without LPS. We also analyzed whether each biomaterial could augment LPS-induced cytokine expression, in order to more thoroughly evaluate the biocompatibility of each biomaterial.

## Materials and methods

### Animals

Experiments were carried out in young (2 months, 200–250 g), healthy, male SD rats. Animals were purchased from the Shanghai Xipu'er-bikai Experimental Animal Company, housed in wire-mesh cages at 22 °C, and maintained on food and water with a 12 h/12 h light/dark cycle for 1–2 weeks prior to the experiments.

### Preparation of material extracts

Extracts of the positive control material, Polyvinyl chloride containing 8% organic tin(PVC, Shanghai Biomaterials Research & Testing Center), and the test materials, American NPG alloy (Shanghai Medical Instruments Co., Ltd), $\beta$-Tricalcium Phosphate ($\beta$-TCP, Shanghai Bio-Lu Biomaterials Co., Ltd), Calcium Phosphate Cement (CPC, East China University of Science &Technology), Poly-Lactic-co-Glycolic acid (PLGA, Institute of Macromolecular Science of Fudan University), and Polytetrafluoroethylene (PTFE, Shanghai Plastics Research Institute) were prepared in RPMI-1640 medium (GIBCO, Scotland, UK) according to ISO 10993-12 guidance (1 g/5 mL of medium for 72 h at 37 °C) [6]. Tissue culture polystyrenes (TCPS, Corning, USA) was used as the negative material.

### Culture of rat peritoneal macrophages

The SD rats were injected with starchy broth for two days. Peritoneal macrophages were sterile harvested by flushing the peritoneum with 30 mL cold D-Hank's buffer. Cells were centrifuged at 200 × G for 10 min and resuspended in RPMI-1640 containing 10% heat-activated fetal calf serum. Cells were counted and viability was determined by 0.2% trypan blue exclusion. Only cell preparations with a 95% viability or greater were used for experiments. Cells were suspended at a final concentration of $2.0 \times 10^6$ cells/ml per well in RPMI-1640 and incubated at 37 °C in 5% $CO_2$. Cells were allowed to adhere for 2 h, washed with HBSS three times, and cultured for 24 h.

### Cell induction by biomaterials

Unstimulated macrophages were detached from LPS-stimulated macrophages (the final concentration of LPS was 1.0 μg/mL), and the two cell groups were separately induced with a range of biomaterial extracts for 2 h and harvested.

### Measurement of TNF-$\alpha$ and IL-1$\beta$ mRNA by semi-quantitative RT-PCR

#### *RNA isolation*

Total RNA from each group of cells was isolated by acid guanidium thiocyanate-phenol-chloroform extraction. RNA quality was confirmed by electrophoresis on a 1% agarose-formaldehyde ethidium bromide gel and visualization of the 28S and 18S ribosomal RNA bands.

#### *cDNA synthesis[7]*

Reverse transcription were conducted by heating a solution of 2.0 μg total RNA and Oligo (dT) for 5 min at 70 °C, and quenching on ice. M-MLV, dNTP, and RNase inhibitors were added to make a total reaction volume of 20 μL, and incubated at 37 °C for 1 h. The reaction was terminated by heating at 95 °C for 5 min and quenching on ice.

#### *PCR reaction*

The PCR amplification protocol consisted of denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s, and a final extension at 72 °C for 5 min. For RT-PCR, the sequences of the TNF-$\alpha$ primers were as follows: 5-GCCACCAC GCTCTTCTGT-3 (sense primer) and 5-GGCAGCCTT GTCCCTTGA-3 (anti-sense primer). The sequences of the IL1-$\beta$ primers were as follows: 5-TCTGTGACTCGTGGG ATGAT-3 (sense primer) and 5-CTTCTTTGGGTATTG TTTGG-3 (anti-sense primer). The predicted size of the amplified TNF-$\alpha$ and IL1-$\beta$ DNA products were 316 and 320 base pairs, respectively.

The PCR products were identified by 1.2% agarose ethidium bromide gel electrophoresis. GPDHA was used as an internal standard.

### Statistical analysis

Statistical analysis was performed using SPSS 12.0 software. Descriptive data were expressed as the arithmetic

mean plus or minus the standard error of the mean. The non-parametric tests were applied to detect differences between groups. $p < 0.05$ was considered statistically significant in all evaluations.

## Results

### Cytokines expression by macrophages not stimulated with LPS

Cytokines release induced by various biomaterials in macrophages that were not stimulated with LPS is shown in Figs. 1, 2.

LPS-unstimulated macrophages were unable to express TNF-α and IL-1β in response to the negative materials, but expressed low levels of cytokine in response to the other biomaterials. When LPS-unstimulated macrophages were induced with positive material and PTFE, cytokine expression increased significantly compared with negative control ($p < 0.01$). NPG also induced moderate cytokine expression ($p < 0.05$). But PLGA, β-TCP, and CPC induced no detectable expression ($p > 0.05$). (Table 1).

### Cytokines expression by LPS-stimulated macrophages

Cytokine expression by LPS-stimulated macrophages is shown in Fig 3–4.

TNF-α and IL-1β levels increased when LPS-stimulated macrophages came into contact with all the materials. The positive and PTFE materials induced high cytokine expression ($p < 0.001$), while PLGA, NPG, β-TCP and CPC all induced moderate cytokine expression ($p < 0.01$). These results show that different biomaterials induce varying degrees of immune responses. (Table 2)

## Discussions

Cytokines expression is involved in multiple immune reactions. Low levels of TNF-α can arise during inflammatory and anti-tumor responses, and IL-1β is a multi-functional factor involved in both inflammation and healing. Cytokines expression is normally strongly regulated by the immune system. Pathogenic responses can

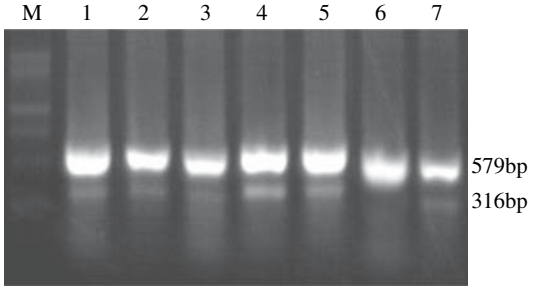


**Fig. 1** TNF-α expression of unstimulated-macrophages

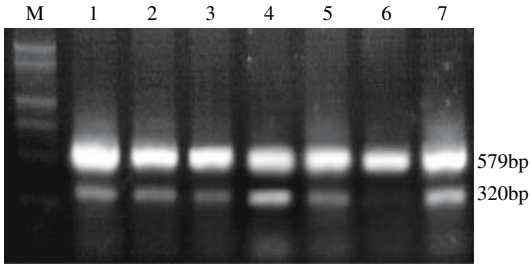


**Fig. 2** IL-1β expression of unstimulated macrophages 1-NPG, 2-β-TCP, 3-CPC, 4-PTFE, 5-PLGA, 6-Negative, 7-Positive, M-DL2000 marker, 579bp-GADPH

**Table 1** Cytokine of LPS-unstimulated macrophages inducted by different biomaterials

| Group | TNF-α/GAPDH | IL-1β/GAPDH |
|---|---|---|
| 1 NPG | 0.24819 ± 0.04361* | 0.22745 ± 0.01486* |
| 2 β-TCP | 0.19961 ± 0.01225 | 0.20135 ± 0.02272 |
| 3 CPC | 0.20364 ± 0.04367 | 0.19452 ± 0.01542 |
| 4 PTFE | 0.25043 ± 0.03322** | 0.31699 ± 0.03047** |
| 5 PLGA | 0.22769 ± 0.03035 | 0.24401 ± 0.03344* |
| 6 Negative | 0.16386 ± 0.00596 | 0.16826 ± 0.01211 |
| 7 Positive | 0.32113 ± 0.03830** | 0.27646 ± 0.02245** |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

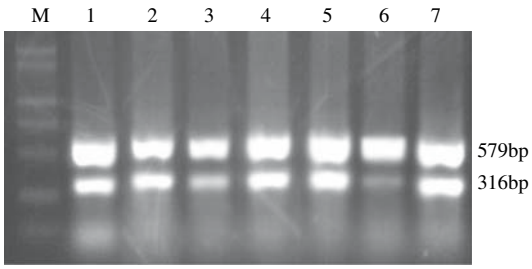


**Fig. 3** TNF-α expression of stimulated macrophages

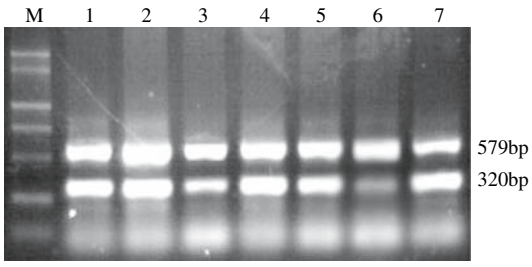


**Fig. 4** IL-1β expression of stimulated macrophages 1-NPG, 2-β-TCP, 3-CPC, 4-PTFE, 5-PLGA, 6-Negative, 7-Positive, M-DL2000 marker, 579bp-GADPH

**Table 2** Cytokine of LPS-stimulated macrophages inducted by different biomaterials

| Group | TNF-α/GAPDH | IL-1β/GAPDH |
|---|---|---|
| 1 NPG | 0.72597 ± 0.04534*** | 0.69825 ± 0.13251** |
| 2 β-TCP | 0.50361 ± 0.02037** | 0.81463 ± 0.1070** |
| 3 CPC | 0.55171 ± 0.04841** | 0.62511 ± 0.10788** |
| 4 PTFE | 0.74174 ± 0.05017*** | 0.90423 ± 0.09151*** |
| 5 PLGA | 0.63957 ± 0.04874** | 0.86643 ± 0.02143** |
| 6 Negative | 0.35369 ± 0.03526 | 0.27256 ± 0.04564 |
| 7 Positive | 0.64542 ± 0.04357*** | 1.58420 ± 0.14574*** |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

result in disregulated cytokine expression, during which cytokines and receptors are either absent or over-expressed [8]. Thus, cytokine detection is an important indicator of immune responses, and cytokine expression must be in a standard range when clinical application of biomaterials.

LPS-unstimulated macrophages that were incubated with the negative material, TCPS, were unable to express TNF-α and IL-1β at mRNA level. PTFE induced LPS-unstimulated macrophages to express cytokine at a low level, and would therefore be likely to produce an inflammatory state within the body. The other biomaterials, including the positive one, were able to induce high expression of cytokine at various degrees. So both TNF-α and IL-1β production can be clearly served as good immune indicators of the biocompatibility of different biomaterials.

The biodegradable material, PLGA, has good tissue biocompatibility and osteoconductivity, and PTFE is thought to provide hemocompatibility because of its chemical inertia and inability to absorb blood cells and plasma [9]. However, RT-PCR results showed that PTFE and PLGA can stimulate high levels of TNF-α and IL-1β ($p < 0.001$). So the two materials may have strong immune effects because their cross-linked macromolecule structures augment their molecular weight. The metal, NPG, also induced moderate cytokine expression, likely because metal ion release has the ability to activate ambient cells [10]. At the mRNA level, β-TCP and CPC also induced cytokine expression that was greater than the negative control, though these materials are often used to repair bone defects because they have good biocompatibility, stable capability, and re-modeling properties.

In our previous studies, ELISA assays were used to measure TNF-α and IL-1β production by macrophages in vitro. PTFE and PLGA could stimulate cytokines high expressing using ELISA. No TNF-α and IL-1β production were detection in response to β-TCP and CPC, however. Since RT-PCR is a more sensitive method of immune activation, this may serve as the best method to evaluate the immune compatibility of biomaterials.

## Conclusions

Our results indicate that cytokine are produced as part of the immune reaction induced by biomaterials. TNF-α and IL-1β expression share an obvious relationship, and prove to be good indicators of the biocompatibility of biomaterials. Our findings indicate that β-TCP and CPC have good biocompability correspondingly. However, PTFE and NPG could induce immune effects because of pro-inflammatory cytokine production.

**Acknowledgements** This research was partially supported by the Shanghai Biomaterials Research and Testing Center and the School of Life Science, East China Normal University, Shanghai. The authors acknowledge Prof. Jiao Sun and Associate Prof. Ping Zhang for their assistance in the research. Supported by grants from National Natural Science Foundation of China (30470479), Shanghai Leading Academic Discipline found (T0202), and Shanghai Sci-Tech Committee Foundation (0452nm043).

## References

1. G. BASTA, P. SARCHIELLI and G. LUCA et al, *Transpl. Immunol.* **13**(4) (2004) 289
2. J. HOFFMANN, P. SIMON and A. K. ZIMMERMANN et al, *Biomaterials* **26**(25) (2005) 5240
3. M. B. GORBET and M. V. SEFTON, *Biomaterials* **25**(26) (2004) 5681
4. M. HUMMEL, S. CZERLINSKI and N. FRIEDEL et al, *Crit. Care. Med.* **22**(3) (2003) 448
5. S. ZEUKE, A. J. ULMER and S. KUSUMOTO et al. *Cardiovasc Res* **56** (2002) 126
6. G. DONATELLA and C. GAGRIELA, *Biomaterials* **21** (2000) 1789
7. B. S. JHUN, Q. JIN and Y. T. OH et al, Biochem. Biophys. Res. Commun. **318** (2004) 372
8. G. G. NERISERNERI, M. BODDI and P. A. MODESTI et al, *Circ Res.* **92** 2003 1359
9. C. B. FELDER, N. VORLAENDER and GANDER B et al, *Vaccine* **19**(7–8) (2001) 706
10. M. K. LEI and X. M. ZHU, Biomaterials **22**(4) (2001) 641