CONFIDENTIAL CONFIDENTIAL CONFIDENTIAL

REPORT ON IN-VITRO RESPONSE OF INFLAMMATORY CELLS TO TU TITANMED DENTAL IMPLANTS

REF.: D.d.t. TITANMED S.r.l.

Our ref.: 1501100437

Written by:

Dr. Marco MORRA .....

Dr.a Clara CASSINELLI .....

Dr. Daniele BOLLATI .....

copies #: 1

sent to:

Text of this report is stored on disc at NBR

CONFIDENTIAL CONFIDENTIAL CONFIDENTIAL

Aim of the work

The aim of the work was the evaluation, by a RT-PCR approach, of the response of inflammatory cells to Titanmed dental implants. In particular, the goal was to measure expression of several key-genes involved in inflammation by cultured macrophages, using a recently developed *in vitro* protocol to assay surface-adherent endotoxins. Tests were performed on two different set of packaged implants

Materials and Methods

Samples tested in this work were the following, as described in the relevant accompanying document :

Titanmed titan implant D 4 mm x 10 mm, TU, n. 12 samples

The 12 samples were divided into two sub groups, as *per* delivery note:

Group A: 6 implants subjected to a new cleaning and sterilization cycle

Group B: 6 implants from a comparatively fresh lot

The following samples were used for testing:

4 implants for group A

4 implants for group B

One implant from group B was subjected to a further cleaning cycle in or clean room and used as a control. The remaining samples were kept in their package, in case of need of more controls.

All samples were fully packaged and sterile, all packages were sealed. Packages were opened just before the test, under a laminar flow hood, in our cell culture lab.

Gene expression measurement through RT-PCR was performed to evaluate the amount of adherent endotoxin. Test were performed through the evaluation of the expression by J774A-1 macrophages of a few key-genes involved in the inflammatory reponse to endotoxin: Interleukin 1 (IL-1), interleukin 6 (IL-6) e Tumor Necrosis Factor alfa (TNFα), MCP-1, COX-2 and MCSF.

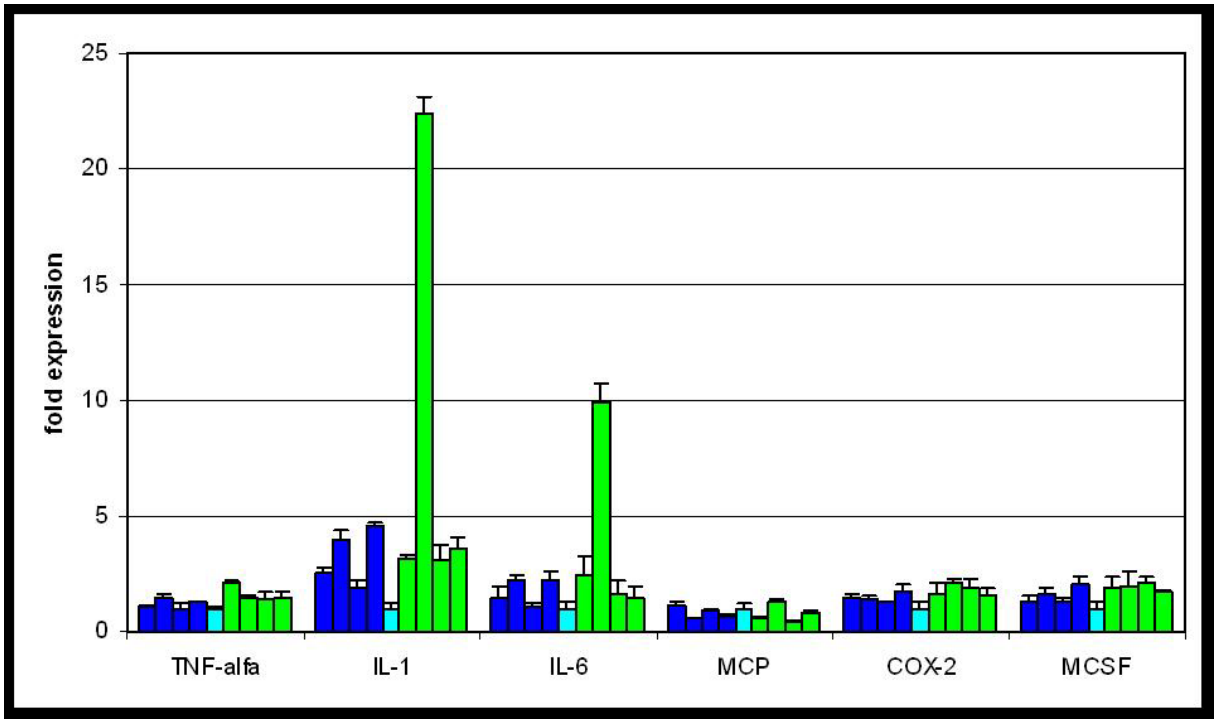
The analytical method was the following: we have recently shown that the level of adherent endotoxins, independently from any surface topography contribution, controls the short time (4 h) expression of quoted genes by the mentioned cell line. Based on this finding, adherent endotoxin

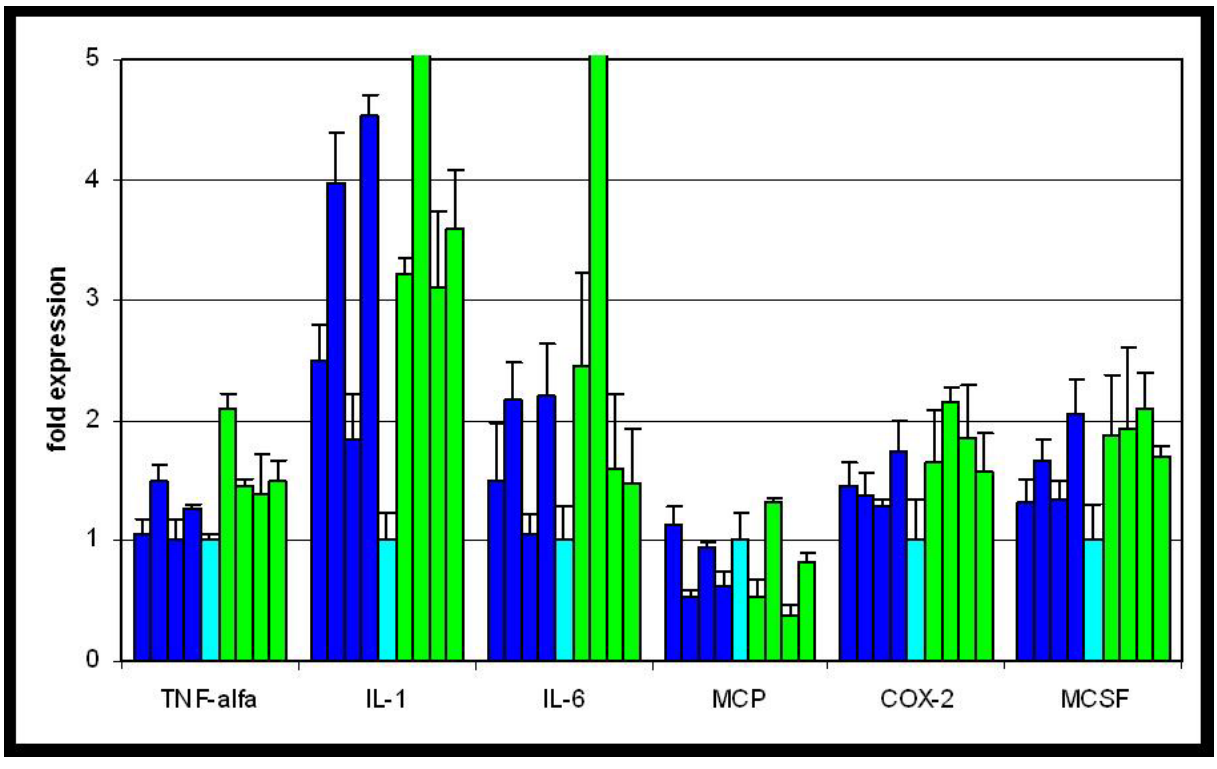
on implant surfaces can be measured by monitoring the short time transcriptional response of J774A-1 macrophages on the test surfaces. Measurements were performed as follows: A suspension of 1.17±0.12 x 10^5 J774A1 cells, cultured in DMEM containing L-glutamine (Gibco, INVITROGEN S.r.l), and 20% Fetal Bovine Serum (FBS Gibco, INVITROGEN S.r.l), penicillin and streptomycin was introduced into sterile 12-well polystyrene culture plates (12-well multiwell plates, Cell Star, Greiner One™) containing the samples. Analysis of gene expression was carried out using real time reverse transcription PCR (qRT-PCR). Total RNA was extracted after 4 h, using the MagMax Total RNA Isolation Kit (Applied Biosystems). The quality of the RNA was assessed by checking that the A260/A280 absorbance ratio was between 1.6 and 2.0. The extracted RNA was subsequently reverse transcribed to give cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription kit.

Relative quantification of the genes was obtained using Taq Man probes specific for each gene under test and GAPDH as the reference gene. The amplification reactions were carried out in duplicate in a StepOne thermocycler (Applied Biosystems) in accordance with the manufacturer's instructions. To obtain the gene expression graphs, data were normalised using the StepOne software in accordance with the ΔCt standard method.

Results

Results of gene expression measurements are reported in the graphs in the next page, that show the same data using two different scales. For each gene, the four blue bars show results obtained on group A samples, the azure bar shows results from the control, the four green bars show results from group B samples. In particular data show fold expression of a given gene over expression on the control sample, taken as 1. While graphs report the error bar obtained in the present experiment, our validation protocol shows that differences of +/- 0.5 fold expression can occur when the same sample is tested in the same experiment. Thus, to accept a difference as "significant", variations higher than +/- 0.5 fold expression should be observed.





Results are satisfactory for the tested samples. Considering the most significant genes, that is IL-1 and IL-6, fold expression over the control is about three as an average, this can be considered satisfactory. No major differences between groups are observed,

except that a single sample from group B shows significant overexpression of IL-1 and IL-6. It is possible that this single sample was inadvertently contaminated by an operator or some foreign endotoxin someway, or that the relevant packaging material was not adequately clean. Shortly, this result could suggest that cleaning and packaging practice is indeed, in general, satisfactory, yet some time, for some reason, something wrong happens. Further enforcement of good practice could yield completely satisfactory results. In general, however, present data show significant improvements over those obtained in our previous test, as described in our report 1501100363.

Conclusions

In conclusion, present data indicate that tested implants are, in general, satisfactory and do not elicit major overexpression of pro-inflammatory genes by J774A-1 macrophages, suggesting a very low amount of adherent endotoxin. A single sample from group B showed, however, significant ILs overexpression, probably due to some occasional contamination.