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Hydrophilic titanium surface-induced macrophage modulation promotes pro-osteogenic signalling.

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ABSTRACT

Objectives: As biomaterial-induced modulation of mediators of the immune response may be a potential therapeutic approach to enhance wound healing events, the aim of this study was to delineate the effects of titanium surface modification on macrophage phenotype and function.

Material and methods: Rodent bone marrow derived macrophages were polarised into M1 and M2 phenotypes and cultured on micro-rough (SLA) and hydrophilic modified SLA (modSLA) titanium discs. Macrophage phenotype and cytokine secretion were subsequently assessed by immunostaining and ELISA, respectively. Osteoblast gene expression in response to culture in the M1 and M2 macrophage conditioned media was also evaluated over 7 days by RT-PCR.

Results: M1-macrophage culture on the modSLA surface promoted an M2-like phenotype as demonstrated by marked CD163 protein expression, Arg1 gene expression and the secretion of cytokines that significantly up-regulated in osteoblasts the expression of genes associated with the TGFß/BMP signalling pathway and osteogenesis. In comparison, M2-macrophage culture on SLA surface promoted an inflammatory phenotype and cytokine profile that was not conducive for osteogenic gene expression.

Conclusions: Macrophages are able to alter or switch their phenotype according to the signals received from the biomaterial surface. A hydrophilic micro-rough titanium surface topography elicits a macrophage phenotype associated with reduced inflammation.

INTRODUCTION

Clinical studies have shown that the rate and the degree of osseointegration around endosseous titanium implants can be significantly improved by the modification of their surface topography and chemistry (Wennerberg & Albrektsson 2009). In attempting to understand the biological mechanisms associated with surface modification, considerable emphasis has been placed on the response of the osteoblast and its precursors. However, bone forming cells are relatively late arrivals at the surface-tissue interface and cells associated with the early inflammatory response such as macrophages are more likely to play a critical role in determining the resultant wound healing outcome as they will define the nature of the local micro-environment via autocrine and paracrine cell signalling.

This relationship between the inflammatory response and the skeletal system is well established (Takayanagi 2007; Lorenzo & Choi 2005) and recent research has now focused on understanding how the surface properties of implanted biomaterials can modulate the behaviour of inflammatory response mediators such as macrophages, including their secretion of cytokines and chemokines, and associated paracrine biological roles such as bone formation. The inflammatory phase of the healing process starts within minutes after implant placement surgery (Enoch & Leaper 2008) and macrophages are one of the main cells that control the host's response to foreign bodies such as titanium implants (Anderson et al. 2008) by releasing a wide array of molecules that can regulate the function of target cells (Cruse & Lewis 2010) . This secretory profile differs according to the macrophage phenotype and can thus orchestrate different phases of the host's immune response. Classically activated M1 macrophages enhance the inflammatory response (Vega & Cobri 2006) while alternatively activated M2 macrophages initiate the resolution of inflammation and enhance repair (Gordon 2003) but notably, macrophages can also change their phenotype and thus their secretory profile according to changes in their microenvironment and interaction with biomaterials (Stout et al. 2005; Anderson & Jones 2007; Kou & Babensee 2011).

Titanium surface induced modulation of the macrophage secretory profile has been demonstrated to affect both the osteoblastic differentiation of mesenchymal stem cells (Omar et al. 2011) and the osteogenic activity of osteoprogenitor cells (Lee et al. 2012). In the present study we've examined the effect of titanium surface topography and chemistry on macrophage phenotype using commercially pure titanium discs with either a large-grit blasted and acid-etched micro-rough surface (SLA), or a hydrophilic-modified SLA (modSLA) surface. Hydrophilicity and topographical surface modification of titanium have been shown *in vitro* to down-regulate macrophage inflammatory cytokine gene and protein expression (Hamlet & Ivanovski 2011; Hamlet et al. 2012; Alfarsi et al. 2014). Moreover, *in*

vivo, transcriptional changes during the osseointegration of titanium implants have shown that the initial expression of immuno-inflammatory response associated genes during the early stages of osseointegration are subsequently replaced temporally with the expression of genes associated with osteogenesis amongst others (Ivanovski et al. 2011).

During wound healing it is essential to have an initial M1-mediated pro-inflammatory response prior to the activation of repair via the M2 phenotype. Prolonged or uncontrolled M1 mediated inflammation leads to poor reparative outcomes in response to implanted biomaterials (Badylak et al. 2008). An attenuation of the inflammatory process and / or an accelerated transition to a reparative micro-environment would therefore provide a plausible biological mechanism to explain the earlier bone formation and ultimately earlier osseointegration seen around surface-modified titanium implants clinically (Lang et al. 2011). The aim of the present study therefore was to evaluate the effects of biomaterial-induced immunomodulation on macrophage phenotype and subsequent paracrine gene expression by osteoblasts.

MATERIAL AND METHODS

Titanium

Grade II commercially pure titanium discs (1mm thick, 15 mm diameter, Institut Straumann AG, Basel, Switzerland) with either a micro-rough (SLA), or hydrophilic-modified micro-rough (modSLA) surface were used in the study. Micro-rough surface modification was obtained by blasting a machined titanium surface with 250 – 500μm corundum grit followed by dual acid etching with hot hydrochloric and sulphuric acids. The hydrophilic surface is achieved by rinsing the SLA disks under N_2 protection and storing in isotonic saline.

Cell Culture

Rodent macrophages: Monocytes collected by flushing bone marrow from the femur with αMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin – streptomycin (P/S) were incubated overnight in media with 10ng/ml MCSF. Non-adherent cells were collected and cultured in medium containing 30ng/ml MCSF for a further 4 days before differentiation into either M1 or M2 macrophages by incubation with either 100ng/ml LPS or 20ng/ml IL4 respectively. M1 and M2 phenotype was confirmed by analysis of IL1 and IL10 cytokine secretion respectively by ELISA (Supplementary data Figure S1a & b).

Human macrophages: Monocytes (THP-1, ATCC, Manassas, USA) were allowed to proliferate in RPMI-1640 (Invitrogen, VIC, Australia) supplemented with 10% fetal bovine

serum (Invitrogen, VIC, Australia) and 1% penicillin/streptomycin (Invitrogen, VIC, Australia) at 37^0C in a 5% CO_2 atmosphere. The monocytes were subsequently induced to differentiate into macrophages by incubation with 100ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, NSW, Australia) for two days followed by further two days' incubation without PMA.

Human osteoblasts: hFOB (ATCC, Manassas, USA) were cultured in a 1:1 mixture of Ham's F-12 and Dulbecco's Modified Eagle Medium (DMEM/F-12) without phenol red and supplemented with 10% fetal bovine serum (Invitrogen, VIC, Australia) and 300μg/ml Geneticin (Invitrogen, VIC, Australia) at 34^0C in a 5% CO_2 atmosphere to induce osteoblast proliferation. The temperature was then raised to 37^oC for a week to facilitate differentiation. The cells were then detached with 0.25% Trypsin/EDTA (Invitrogen, VIC, Australia) pending co-culture with the macrophages.

Macrophage Response to Titanium Surfaces

Phenotype Marker Expression: M1 and M2 rat macrophages were cultured separately on SLA or modSLA surfaces ($5x10^4$ cells per disc) in tissue culture plates in DMEM (Gibco, Grand Island, NY) supplemented with 1% P/S, 10% FBS and 30ng/ml MCSF at 37^0C in a humidified 5% CO_2 atmosphere for up to 7 days. Titanium discs were subsequently removed and fixed in 4% paraformaldehyde. The discs were then washed twice with PBS and blocked with 1% BSA in PBS-0.1% Tween-20 (PBST) for 1 hour at room temperature. The macrophages were probed with rabbit polyclonal anti-rat Integrin αX IgG (CD11c, M1 phenotype marker) and mouse monoclonal anti-rat CD163 (M2 phenotype marker) antibodies diluted 1:250 in 1% BSA in PBS at 4^0C overnight (sc-30137and sc-58965 respectively, Santa Cruz Biotechnology). After washing x3 with PBST for 5 minutes, the discs were incubated with secondary PE-goat anti-rabbit IgG and FITC-goat anti mouse IgG diluted 1:50 in PBS at room temperature for 1 hour. The discs were washed x3 with PBS, counterstained with DAPI and mounted on microscope slides using anti-fade gold (Cell signalling technologies, Merk Millipore, VIC, Australia). Fluorescent microscopy (Zeiss M2) was used to image the discs that were subsequently analysed using the ZEN (Zeiss, North Ryde, NSW, Australia) and Image J (http://imagej.net/) software packages.

Gene Expression: The gene expression of M1 (INOS) and M2 (ARG1) phenotype markers by macrophages at days 1, 4 and 7 of culture was assessed by real-time PCR. Total RNA was purified using TRIZOL reagent (Invitrogen) and RNeasy Plus Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. M-MuLV Reverse Transcriptase was used to prepare complementary DNA and real-time PCR analysis was performed using KAPA SYBR

FAST Master Mix (Kapa Biosystems, Boston, MA) and gene-specific primers (Supplementary Table 1).

Cytokine Secretion: A multiplex ELISA (Bio-Plex Pro, Bio-Rad, Gladesville, New South Wales) was used to determine the concentrations of seven chemokines and cytokines (IL1α, IL1β, TNFα, MCP1, IL10, MIP2 and RANTES) in the macrophage culture media as per the manufacturer's instructions.

Macrophage – Osteoblast Co-culture

Primary rat calvariae osteoblasts (R-OST-583, Lonza, Waverley, VIC) were allowed to attach to 24-well tissue culture plates ($5x10^4$ cells/well) for 24 hrs. Culture media previously collected at days 1, 4 and 7 from M1 and M2 macrophages cultured on the two different titanium surfaces was then used as a conditioned media for 72 hr osteoblast co-culture. The expression of six osteogenic genes (ALP, COL1A1, BMP2, OCN and RUNX2) was subsequently assessed by real time PCR.

Signalling Pathway Analysis: Human macrophages (1×10^5 cells) seeded onto titanium discs were incubated separately for a day to mimic the *in vivo* temporal sequence of events where macrophages initially interact with the titanium surface without any osteoblast influence. Osteoblasts (5×10^4 cells) seeded onto 0.4μm pore size permeable transwell membranes (Corning, VIC, Australia) were then introduced to the co-culture system. This system prevents direct cell contact while allowing for media exchange. The culture media used was a mixture of 45% RMPI-1640, 45% DMEM/F-12 and 10% fetal bovine serum (Invitrogen, VIC, Australia). Co-culture transwell plates with triplicate discs were incubated at 37^0C in a 5% CO_2 atmosphere for up to 7 days.

TGFß/BMP signalling in the osteoblasts was subsequently assessed following extraction of total RNA (RT^2 qPCR-Grade Isolation Kit, SABiosciences, VIC, Australia) after one, three and seven days of culture. cDNA was then prepared (RT^2 First Strand Kit, SABiosciences, VIC, Australia) and the expression of eighty-four genes related to TGFß/BMP-mediated signal transduction assessed using the Human TGFß/BMP Signalling Pathway RT^2 Profiler™ PCR Array (SABiosciences, VIC, Australia). The relative expression of the target genes was normalized against a panel of up to five reference genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) using the ΔΔCt method and mean fold-change differences in gene expression for each pair-wise comparison between the titanium surfaces (modSLA c.f. SLA) calculated. The pair-wise gene expression data were analysed using the student's t-test and a p-value <0.05 was considered statistically significant.

Statistical analysis

All experiments were carried out in triplicate. Previous studies by our group using the same calvarial defect model to assess the effects of titanium surface characteristics on macrophage phenotype and osseous healing (Lee et al. 2017), showed this was sufficient to demonstrate statistically significant differences between mean values.

Phenotype Marker Expression: The CD11c and CD163 fluorescence signals were normalized per macrophage from the total number of cells (DAPI fluorescence) counted in each field of view. Three randomly chosen fields of view were assessed per titanium surface at each observation time point. The significance of any change in cell number or signal intensity over the time of culture compared to that at day 1 was subsequently determined using a two-way ANOVA and subsequent post-hoc analysis with Bonferroni correction for multiple comparisons.

Cytokine secretion: The significance of any differences in the mean levels of cytokines (IL1α, IL1β, TNFα, MCP1, IL10, MIP2 and RANTES) secreted by the M1 and M2 macrophages following culture on the two titanium surfaces (SLA and modSLA) for 1, 4 and 7 days, were determined using a two-way ANOVA and subsequent post-hoc analysis with Bonferroni correction for multiple comparisons (GraphPad Prism 7, La Jolla, CA).

Macrophage – Osteoblast Co-culture: The significance of any differences in the mean expression of the osteogenic genes: COL1, ALP, BMP2, BSP, RUNX2 and OCN in osteoblasts following macrophage co-culture in media following were also determined using a two-way ANOVA and subsequent post-hoc analysis with Bonferroni correction for multiple comparisons (GraphPad Prism 7, La Jolla, CA). Descriptive statistics of the osteogenic gene expression data is also provided as supporting information (Expression_stats.xlsx).

RESULTS

Macrophage Phenotype Response to Titanium Surface Modification.

The M1 phenotype of the rat macrophages was confirmed by the demonstration of significantly increased secretion of IL-1β (Supplementary Figure 1a) after 48 hrs culture of the bone marrow derived rat macrophages with LPS. Similarly, the M2 phenotype was confirmed by demonstrating significantly increased secretion of IL10 following 48 hrs incubation of the bone marrow derived rat macrophages with IL-4 (Supplementary Figure 1b).

M1 Macrophages: A titanium surface-induced effect on both iNOS and ARG1 gene expression in M1 macrophages was observed over the 7 days of culture. iNOS gene expression was significantly higher in M1 cells cultured on the SLA surface at all observed

time points compared with that from M1 cells cultured on the modSLA surface (Figure 1a). Conversely, significantly higher levels of ARG1 gene expression were observed in M1 cells cultured on the modSLA surface compared to culture on the SLA surface (Figure 1b).

Following immunostaining, on the SLA surface (Figure 2a-c), the observed predominantly red fluorescence signal from PE-CD11c labelled M1 cells persisted over the 7 days of culture, whereas on the modSLA surface (Figure 2d-f), there was an obvious change in observed fluorescence towards a predominantly green FITC-CD163 signal over the 7 days of culture. Merged and component immunofluorescent images are provided as supplementary data (Supplementary figures 2&3).

To account for potential differences in macrophage attachment to the two titanium surfaces, quantification of the normalised PE-CD11c and FITC-CD163 signal intensities i.e. per macrophage (as described in the statistical analyses) was performed which confirmed these observations. In cells cultured on the SLA surface, compared to the mean signal/cell after 1 day of attachment, by 7 days the mean PE-CD11c signal/cell increased 51% whereas the FITC-CD163 signal/cell only increased marginally by 25% (Figure 2g). On the modSLA surface however, PE-CD11c expression/cell in M1 macrophages decreased by 48% over 7 days of culture whereas as early as day 4 of culture, there was a 249% increase in mean CD163 expression/cell by the M1 macrophages which was maintained at 203% after 7 days of culture (Figure 2h).

M2 Macrophages: Again, titanium surface effects on both INOS and ARG1 gene expression in M2 cells were observed over the 7 days of culture. M2 cell culture on the SLA surface was associated with significantly higher levels of INOS expression compared with that from M2 cells cultured on the modSLA surface (Figure 3a). M2 cell culture on the modSLA surface was also associated with significantly higher levels of ARG1 expression compared to that from cells cultured on the SLA surface (Figure 3b).

Following immunostaining (Figure 4a-c), after 1 day of culture on the SLA surface, the observed fluorescence signal was predominantly green, as would be expected from FITC-CD163 labelled M2 macrophages. However by day 7 of culture, the observable fluorescence signal was a mixture of both red PE-CD11c and green FITC-CD163 signals. By comparison, culture of the M2 macrophages on the modSLA surface (Figure 4d-f) showed an initial green FITC-CD163 fluorescence signal which persisted over the 7 days of culture. Merged and component immunofluorescent images are provided as supplementary data (Supplementary figures 4&5).

Similar quantification as above of the normalised signal intensities/cell by the M2 macrophages cultured on the SLA surface showed that by day 7, CD11c expression increased 115% while FITC-CD163 expression/cell remained relatively unchanged (mean 15% increase) reflecting the changes observed visually (Figure 4g). In contrast, on the modSLA surface over the same period of time, mean CD11c expression/cell decreased by 83% after 7 days of culture while the mean FITC-CD163 expression/cell remained at ~60% of that at Day1 (Figure 4h).

Macrophage Cytokine Response to Titanium Surface Modification.

The macrophage phenotype changes demonstrated by the gene expression and surface marker assays also resulted in significant changes to the levels of secreted cytokines examined. High levels of the inflammatory cytokines IL1α and IL1β characteristic of M1 macrophages, generally decreased over the 7 days of culture. However there was a significant titanium surface driven effect whereby significantly lower levels of these cytokines were released by M1 cells cultured on the modSLA surface compared to the same cells cultured on the SLA surface (Figure 5a,b). A similar pattern of secretion of MIP1α was also seen in both M1 and M2 cells (Figure 5c). MCP1 levels on the contrary increased with time of culture in both M1 and M2 macrophages, however the titanium surface effect was still apparent with levels significantly lower in both M1 and M2 cells cultured on the modSLA surface compared to those cultured on the SLA surface by Day 7 (Figure 5d).

Levels of the anti-inflammatory cytokine IL10 were unchanged over time in M1 cells regardless of the culture surface. However IL10 levels increased significantly with culture of M2 cells on the modSLA surface compared to the SLA surface (Figure 5e). No significant titanium surface effects for either MIP2, TNF or RANTES secretion were observed (Figure 5 f,g,h).

Osteoblast response to macrophage phenotype.

In rat osteoblasts, significantly higher levels of ALP, COL1A1, BMP2, OCN and RUNX2 gene expression were found at all three time-points when the culture media was supplemented with media from M2 macrophages (compared to M1 macrophages) cultured on either the SLA or modSLA surfaces (Figure 6). Few differences were noted for BSP expression. Moreover, for each surface, significantly higher levels of ALP, COL1A1, BMP2, OCN and RUNX2 gene expression were again demonstrated at most time-points when the culture media was supplemented with media from the M1 or M2 macrophages that were cultured on the modSLA surface (Figure 6).

Osteoblast Signalling Pathways.

Differential expression of osteoblast genes associated with the TGFβ/BMP pathway as a result of co-culture with SLA and modSLA surface-stimulated macrophages was seen from day 1 (Table 1). At this earliest time-point, the expression of 10 osteoblast genes associated with the TGFβ / BMP pathways including BMPR2, SMAD3, TGFβ2 and TGIF1 were significantly ($p<0.05$) up-regulated following co-culture with modSLA induced macrophages compared to their expression following co-culture with SLA induced macrophages. Three genes were also down regulated (COL1A2, ENG, LTBP1) at this time point. COL1A2 is broadly associated with extracellular matrix formation, ENG, a component of the TGFβR complex and latent TGFβ binding protein 1 (LTBP1), both have a role in TGFβ activation and secretion.

By day 3 of osteoblast-macrophage co-culture, few differences in gene expression were observed apart from BMPER (almost 4-fold up-regulation) which is known to be associated with BMP (particularly 2 & 4) regulation. After 7 days of co-culture, 9 genes were significantly up-regulated. Interestingly by this time, genes also associated with bone formation e.g. BMP3 (2.5 fold), BMP5 (2.7 fold), GSC (3.3 fold), INHA (3.7 fold) and AMHR2, a SMAD transcriptional regulator (3.7 fold), were significantly up-regulated by the modSLA surface-induced macrophage secretome along with four further genes regulating TGFβ i.e. LEFTY1, TGFBR2, TGFBR3 and TSC22D1.

DISCUSSION

The interplay between bone formation and the immune system (Takayanagi 2007; Lorenzo & Choi 2005) allows a strong argument to be made that biomaterial-directed modulation of the immune response may well represent a potential therapeutic strategy in bone engineering applications. Biomaterial features such as topography, particle size, porosity, and ion release, are all biomaterial-specific factors able to influence the immune response. Hydrophobic materials for example have been shown to enhance monocyte adhesion (Hezi-Yamit et al. 2009) whereas hydrophilic or neutral surfaces can inhibit macrophage adhesion but enhance the release of pro-inflammatory cytokines and chemokines (Jones et al. 2007, Hamlet et al. 2012; Alfarsi et al. 2014; Hotchkiss et al. 2016; Hotchkiss et al. 2017). More recently, a hydrophilic titanium surface with nanotopography (modSLA) has been shown to modulate the early inflammatory response *in vivo* promoting polarization of macrophages to an M2-like phenotype, even in diabetic conditions where an exaggerated pro-inflammatory environment is a distinguishing feature (Lee et al. 2017). Titanium surface nanotopography

has also been shown to regulate both macrophage cell shape (McWhorter et al. 2013), the release of cytokines (Luu et al. 2015) and restrict cytoskeletal remodelling-associated signalling by macrophages leading to reduced cell to cell fusion, potentially moderating the foreign body reaction (Padmanabhan et al 2016).

Differences in titanium surface topography and or chemistry have been shown to affect the amounts and configurations of serum protein adsorbed onto the titanium surface that influence the immune response of macrophages, either through the stimulation of Toll-like receptors to release pro-inflammatory cytokines (TNF-α, IL-6) via NF-kB during macrophage activation, or through other intracellular signalling pathways (Trinchieri & Sher 2007). The surface characteristics of the titanium discs used in this study have been well-described in the literature. At the micron level, blasting and etching produces a severely pitted surface and subsequent storage in saline not only results in a hydrophilic surface (modSLA), but also the spontaneous formation of nanoscale sized surface structures (Wennerberg et al. 2013). These features have been shown by our group (Hamlet et al. 2012; Alfarsi et al. 2014) and others (Hotchkiss et al. 2016; Hotchkiss et al. 2017; Lü et al 2015; Lu & Webster 2015) to affect both the attachment of monocytes and macrophages, as well as the gene expression and secretion of pro-inflammatory cytokines.

Macrophages are critical mediators of inflammation and important for the integration of biomaterials such as titanium into recipient tissues. These macrophages are a heterogeneous cell population, each with different surface markers and functions (Mosser & Edwards 2008). Their phenotype can be broadly defined by their functional properties and pattern of gene expression (Mills et al. 2000). As such, the classical 'M1' phenotype is characterized by the expression of high levels of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, promotion of a Th1 response, and strong microbicidal and tumoricidal activity. In contrast, 'M2' macrophages characterized by low levels of pro-inflammatory cytokines and high expression of anti-inflammatory cytokines, play a major role in promoting growth and regeneration. M2 macrophage phenotype has been further sub-classified as either M2a, M2b, M2c or M2d based on the type of stimulation received and their subsequent expression of surface molecules and cytokines reflecting functional and molecular phenotypes (Mantovani et al. 2004, Röszer et al. 2015). In the present study, further classification of the M2 macrophage phenotype is difficult due to the relatively small number of cytokines assessed and the large number of cytokines such as IL10 that are co-expressed among these subgroups.

No clear consensus exists however as to which macrophage phenotype is the most beneficial for osteogenesis. For example, classical M1 macrophages are well known to secrete pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β that induce osteoclastogenesis and bone resorption. Conversely however, recent studies have shown macrophage production of oncostatin-M, a pro-anabolic molecule by M1 but not M2 macrophages, induces osteogenesis in MSCs (Guihard et al. 2012). Alternatively, activated M2 macrophages on the other hand are closely associated with later stage tissue repair resulting in either fibrosis via TNFα, TGF-β1 and TGF-β3 release that isolates an inflammatory reaction centre from normal bone tissue, or the formation of new bone through the secretion of cytokines such as BMP2 (Champagne et al. 2002). However, an excessive switch to the M2 phenotype, results in scar tissue delaying wound healing (Brown & Badylak 2013).

In a healing wound site, whether the transition from a predominantly M1 to M2 macrophage population is the result of the arrival of new already polarised macrophages or the result of a 'reprogramming' of existing macrophages to adopt a new phenotype, is currently unclear. Evidence suggests macrophage phenotypes most likely span a continuum from the classical inflammatory 'M1' to the anti-inflammatory or pro-regenerative 'M2'phenotype (Lü et al. 2015) and whose functions are highly heterogeneous and dependent on micro-environmental cues from within the injury niche. This view is consistent with the concept that cells within different tissues have diverse responses in inflammation.

Greater understanding of the specific functions of various macrophage phenotypes in tissues is difficult due to the complexity of signal integration *in vivo* and the lack of a defined set of distinguishing markers for each class of macrophage. Indeed, a limitation of this study was the number of macrophage phenotype markers i.e. for M1: Cd11c and INOS and for M2: CD163 and ARG1 used to characterize cellular behaviour following exposure to the titanium surfaces. As macrophages may display a broad spectrum of phenotypes, including hybrid phenotypes, depending on their current signalling environment (Wolf et al. 2014), a wider panel of surface markers would be required in order to fully characterize their behaviour. Despite this, the subsequent *in vitro* findings of the present study showed that both titanium surfaces were able to modulate the phenotype over time. The SLA surface promoted a change in the degree of M2 macrophage surface marker expression to that more representative of an M1 phenotype as demonstrated by increasing the CD11c fluorescence signal over seven days of culture. In contrast, the modSLA surface promoted changes more representative of an M2 macrophage phenotype as shown by a concurrent increase in CD163 signal expression in what were originally M1 phenotype macrophages. Furthermore, these titanium surface-driven

changes in macrophage phenotype were also supported by similar changes in the gene expression of other well recognised M1 (iNOS) and M2 (ARG1) phenotype markers (Ma et al. 2014).

The change in macrophage phenotype also resulted in the secretion of distinct cytokine profiles that were able to modulate osteogenic gene expression in osteoblasts in a paracrine fashion. While this is in agreement with a recent direct co-culture study which also showed macrophage plasticity and subsequent phenotypic changes can influence osteogenic gene expression (Loi et al. 2016), the design of the present study meant it was not possible to differentiate whether the M2 phenotype promoted osteogenic gene expression, or whether osteogenic gene expression was being downregulated by M1 macrophages. This study showed for the first time however, that the upregulation of osteogenic gene expression in osteoblasts following co-culture with M2 phenotype macrophages was even further enhanced if the macrophages were cultured on the modSLA surface. This suggested temporal modulation or transition in macrophage phenotype from inflammatory to pro-osteogenic provides a plausible biological mechanism for the faster rate of osteogenesis and osseointegration seen clinically with the modSLA surface (Lang et al. 2011). As gene expression does not necessarily reflect the proteins actually released, caution in the interpretation of this data is required.

In the present study, it was shown that the macrophage secretome in response to the modSLA surface resulted in the differential expression of genes associated with the TGFß/BMP signalling pathway in co-cultured osteoblasts that were not in direct contact with the macrophages. This secretome was able to influence osteoblast TGFß/BMP signalling related gene expression as early as day 1. However, by day 7, the expression of genes associated with osteoblast differentiation were clearly shown to be upregulated by the modSLA surface-induced macrophage secretome. Furthermore, recent genomic and proteomic analysis also showed subtle differences in the pro-osteogenic properties of the two titanium surfaces used in this study (Calciolari et al 2018). Hydrophilic surfaces were able to enhance the timing of activation of several signalling pathways such as mitogen-activated protein kinase, phosphoinositide-3 kinase-AKT, Wnt, Notch, transforming growth factor-β, directly or indirectly involved in bone formation (Calciolari et al 2018).

The concept of a surface dependent macrophage secretome is further supported by real-time monitoring of macrophages on titanium surfaces which showed a nanoscale-rough surface could restrict cellular cytoskeleton movement and inhibit pro-inflammatory cytokine secretion (Lee et al 2011). We have previously demonstrated similar morphological changes

in macrophages following culture on the SLA and modSLA titanium surfaces *in vitro* when compared to macrophage culture on polished titanium (Alfarsi et al 2014) supporting the hypothesis that the downstream changes in osteoblast gene expression may indeed be driven by surface-induced changes in morphology and the subsequent cytokine secretome.

Monocytic cell lines such as the THP-1 cells used in this study are frequently used to model macrophage function since primary tissue macrophages cannot be readily expanded *ex vivo*. Unfortunately, compared to primary macrophages, their differentiation state has meant that inferences drawn from such experiments may not always accurately predict the behaviour of differentiated tissue macrophages. In the current study however, the THP-1 cells are activated with PMA then rested in culture for two days prior to use. This protocol has been shown to produce a macrophage phenotype that most closely resembles that of human monocyte derived macrophages (Daigneault et al. 2010), which are the most widely used model for studying macrophage-osteoblast interactions.

CONCLUSIONS

Using defined M1 and M2 polarized macrophages, this study showed *in vitro* that titanium-adherent macrophages can modulate their phenotype in response to biomaterial surface cues resulting in the secretion of distinct cytokine profiles that are able to stimulate osteogenic gene expression in osteoblasts in a paracrine fashion via up-regulation of the TGFß/BMP signalling pathway. These results suggest that immunomodulation of the macrophage phenotype by titanium surface topography could modulate osteogenesis *in vivo,* ultimately resulting in enhanced early osseointegration of titanium implants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Gene Symbol	Day 1	Day 3	Day 7
AMHR2			3.72
BMPER		-1.45	
BMPR2	1.51		
BMP3			2.51
BMP5			2.68
CDC25A	2.32		
COL1A2	-2.40		
COL3A1	1.56		
ENG	-1.99		
FST	1.55		
GSC			3.3
ID1	1.81		
IL6	2.32		
INHA			3.69
LEFTY1			2.44
LTBP1	-1.90		
SMAD3	2.00		
STAT1	1.93		
TGFB2	1.77		
TGFBR2			1.49
TGFBR3			2.62
TGIF1	1.35		
TSC22D1			1.55

Table 1: TGFß/BMP signalling pathway gene expression in human osteoblasts after one, three and seven days culture was assessed following co-culture with human macrophages seeded onto the SLA and modSLA titanium discs. Only statistically significant ($p<0.05$) fold change differences in osteoblast gene expression (modSLA c.f. SLA) are shown. Sign (-) indicates down regulation.

Figure Legends

Figure 1: Fold change relative iNOS and ARG1 gene expression by M1 macrophages following culture on the SLA and modSLA titanium surfaces over 7 days *in vitro* compared to cells cultured on tissue culture plastic (n=3). **(a)** Significant (*$p<0.0005$) increases in INOS expression were apparent in M1 macrophages cultured on the SLA surface when compared to M1 macrophages cultured on the modSLA surface. **(b)** Conversely, significant (*$p<0.0005$) increases in ARG1 expression were apparent in M1 macrophages cultured on the modSLA surface compared to M1 macrophages cultured on the SLA surface.

Figure 2: (a - f) Immunofluorescence images of M1 macrophages cultured *in vitro* for 1, 4 and 7 days on the SLA (a-c) and modSLA (d-f) titanium discs. Red phycoerythrin-CD11c and green fluorescein-isothiocyanate-CD163 labelled antibodies were used to differentiate M1 and M2 phenotype respectively. Cells were counterstained with DAPI to visualize cell nuclei. (**g & h**) To confirm the observed changes in red CD11c and green CD163 florescence in M1 macrophages over time, the quantitative signal intensity (normalized per macrophage) is shown as a percentage of the signal seen at day 1 (line at 100%).

Figure 3: Fold change relative iNOS and ARG1 gene expression by M2 macrophages following culture on the SLA and modSLA titanium surfaces over 7 days *in vitro* compared to cells cultured on tissue culture plastic (n=3). **(a)** Significant (*$p<0.0001$) increases in INOS expression were apparent in M2 macrophages cultured on the SLA surface when compared to M2 macrophages cultured on the modSLA surface. **(b)** Conversely, significant (*$p<0.0001$) increases in ARG1 expression were apparent in M2 macrophages cultured on the modSLA surface compared to M2 macrophages cultured on the SLA surface.

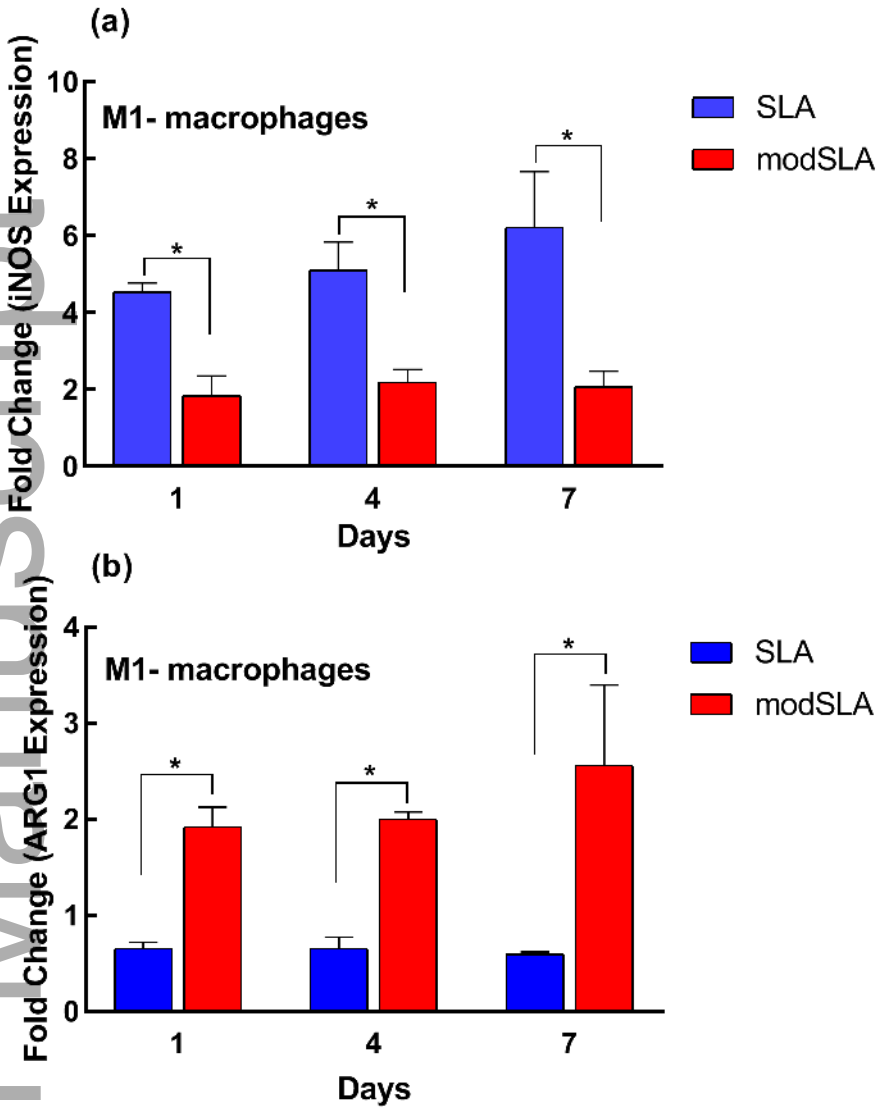
Figure 4: (a - f) Immunofluorescence images of M2 macrophages cultured *in vitro* for 1, 4 and 7 days on the SLA (a-c) and modSLA (d-f) titanium discs. Red phycoerythrin-CD11c

and green fluorescein-isothiocyanate-CD163 labelled antibodies were used to differentiate M1 and M2 phenotype respectively. Cells were counterstained with DAPI to visualize cell nuclei.

(g & h) To confirm the observed changes in red CD11c and green CD163 florescence in M1 macrophages over time, the quantitative signal intensity (normalized per macrophage) is shown as a percentage of the signal seen at day 1 (line at 100%).

Figure 5: Cytokine (IL-1α, IL-1β, TNFα, MCP1, IL10, MIP1α, MIP2 and RANTES) levels secreted into the culture media following macrophage incubation on the titanium surfaces for 1, 4 and 7 days were determined by multiplexed ELISA (n=3). (*) $p<0.005$ M1 macrophages on SLA cf. M1 macrophages on modSLA, (^) $p<0.005$ M2 macrophages on SLA cf. M2 macrophages on modSLA.

Figure 6: Osteoblast RUNX2, BMP2, BSP, COL1, OCN and ALP gene expression following 72hrs culture in macrophage conditioned media. The conditioned media was collected from both M1 and M2 culture on SLA and modSLA surfaces. (#$p<0.05$, *$p<0.01$, ^$p<0.005$, n=3).



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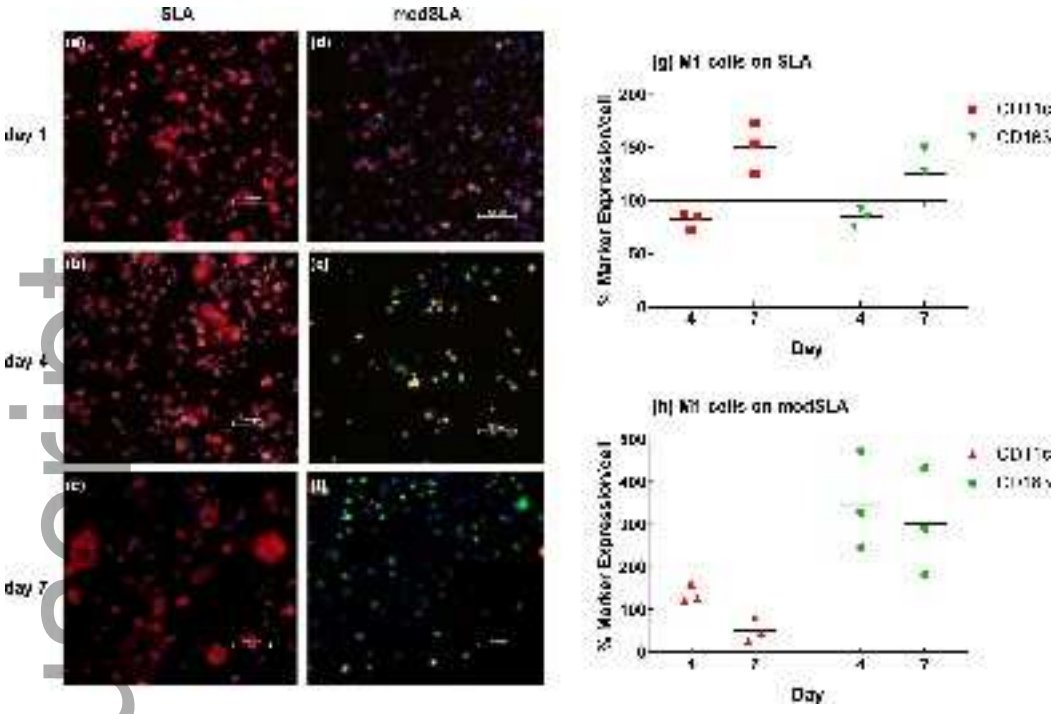

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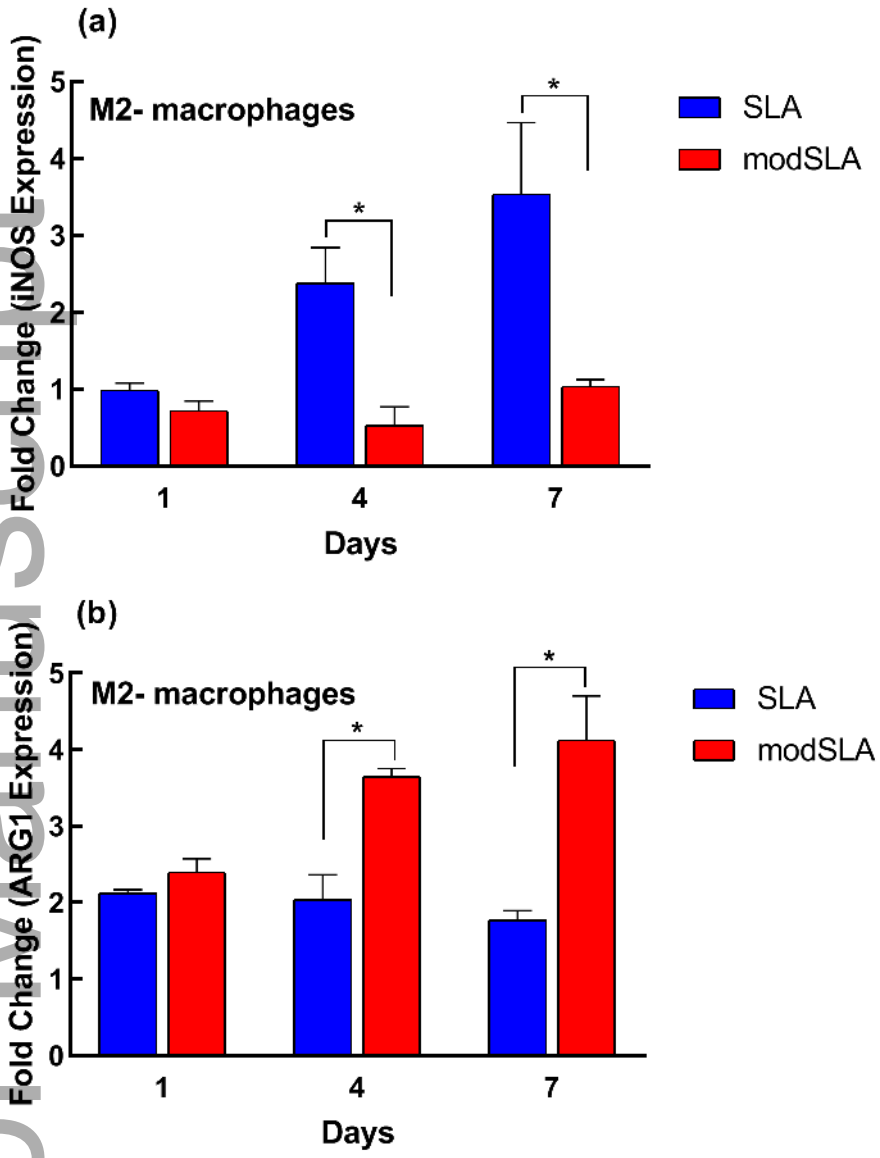

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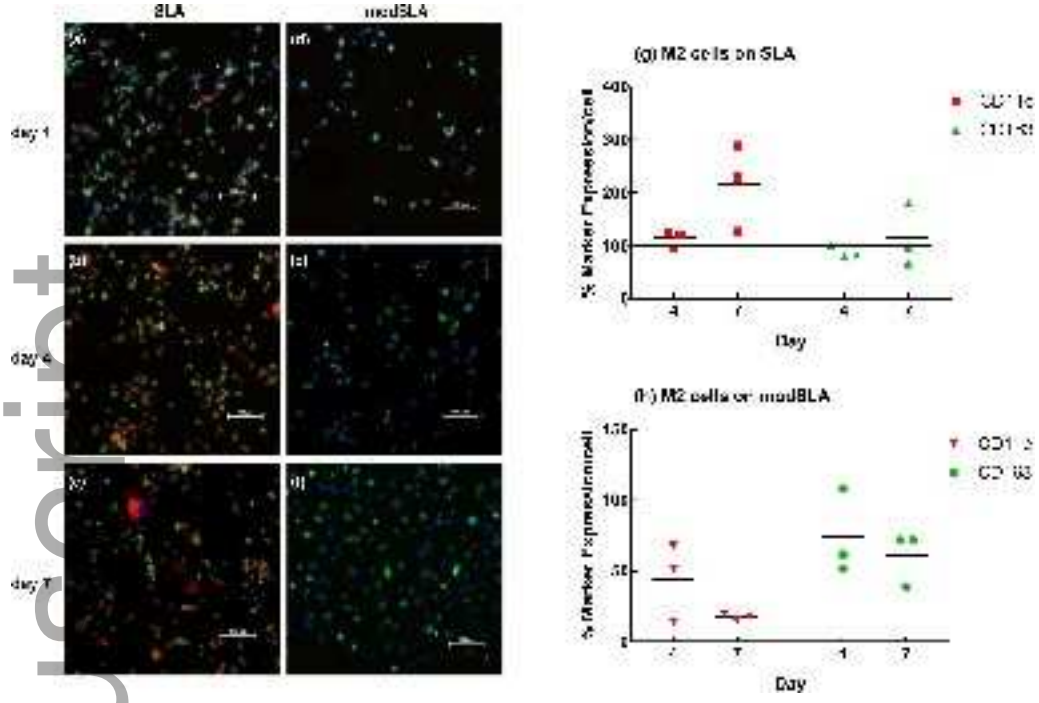

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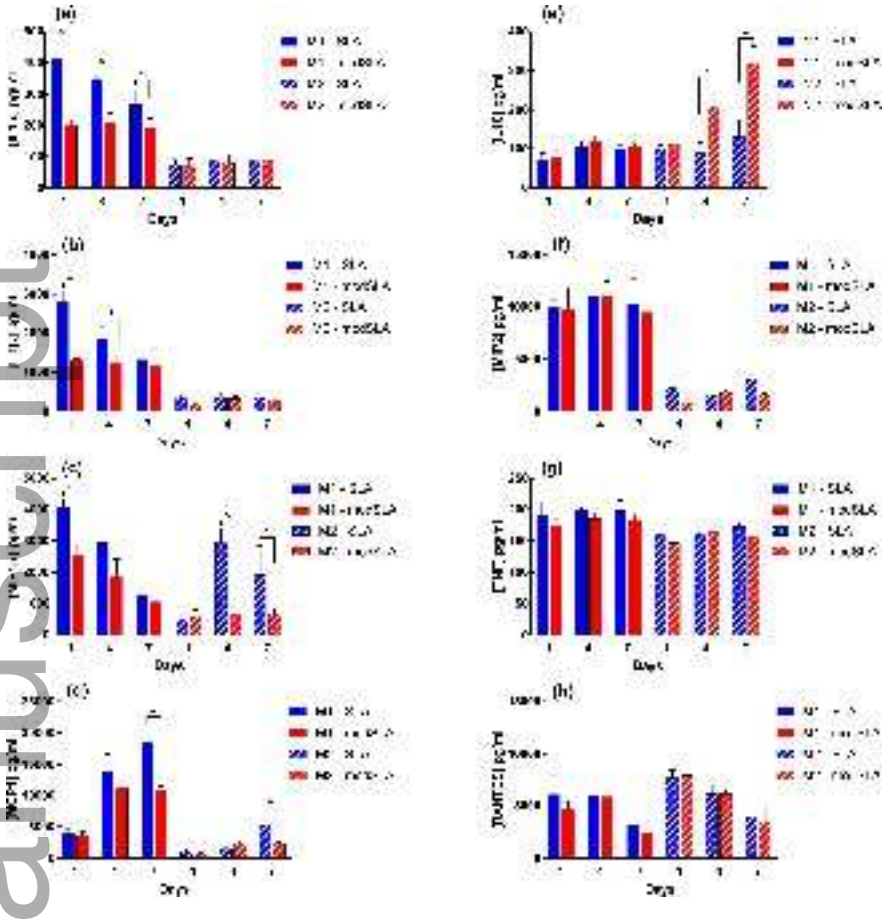

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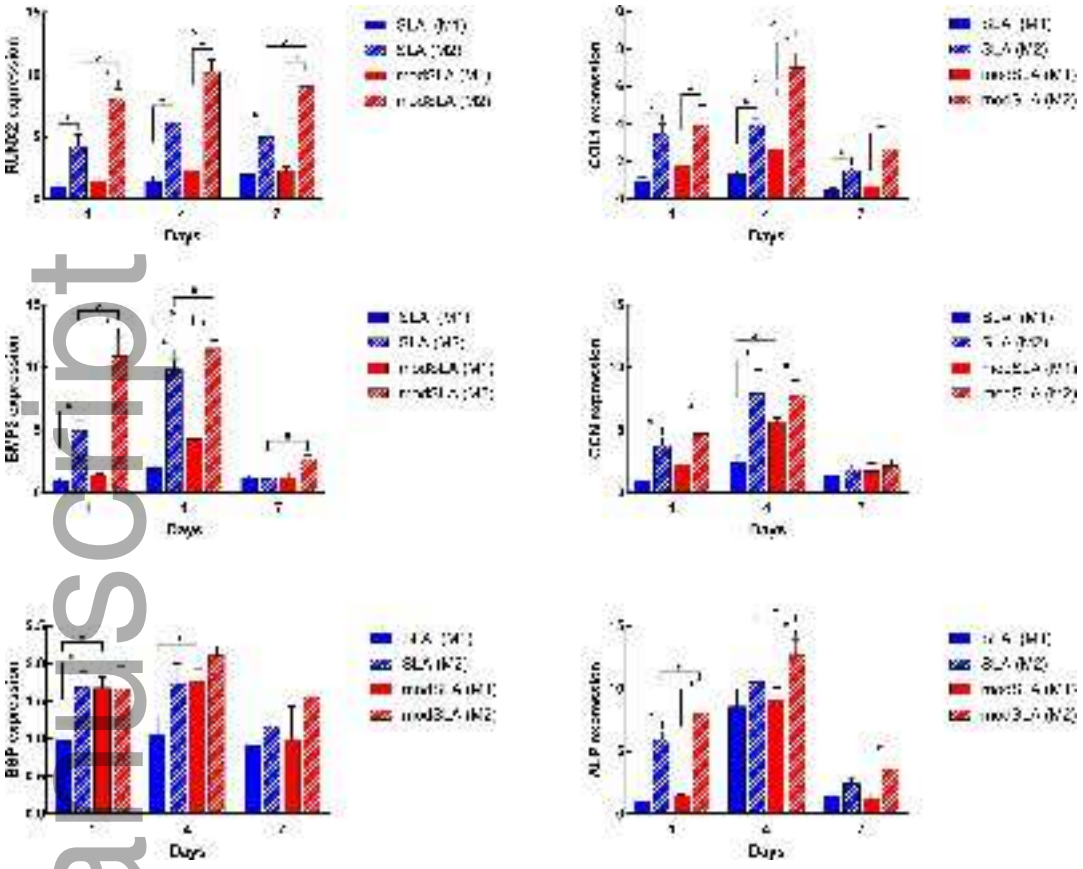

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