Revista de Osteoporosis y Metabolismo Mineral

La activación del sistema purinérgico modula la formación de células gigantes a cuerpo extraño en presencia de diferentes aleaciones metálicas de uso clínico

The activation of the purinergic system modulates the formation of foreign body giant cells in the presence of different metal alloys for clinical use

10.20960/RevOsteoporosMetabMiner.00058

11/20/2024

Original 00058

The activation of the purinergic system modulates the formation of foreign body giant cells in the presence of different metal alloys for clinical use

La activación del sistema purinérgico modula la formación de células gigantes a cuerpo extraño en presencia de diferentes aleaciones metálicas de uso clínico

John Jairo Aguilera-Correa[1,2], Miguel Marco-Bonilla[3], Estíbaliz Torrecilla-Sábada[1], Ana Conde[2,4], María Ángeles Arenas[2,4], Juan José de Damborenea[2,4], Raquel Largo[3], Gabriel Herrero-Beaumont[3], Jaime Esteban[1,2], Aránzazu Mediero[3]

[1]Clinical Microbiology Department. IIS-Fundación Jiménez Díaz. Universidad Autónoma de Madrid. Madrid, Spain. [2]CIBERINFEC - Consorcio Centro de Investigación Biomédica en Red (CIBER) de Enfermedades Infecciosas. Madrid, Spain. [3]Bone and Joint Research Unit. IIS-Fundación Jiménez Díaz. Universidad Autónoma de Madrid. Madrid, Spain. [4]Department of Surface Engineering, Corrosion, and Durability. Centro Nacional de Investigaciones Metalúrgicas - CENIM. CSIC. Madrid, Spain

Received: 08/08/2024
Accepted: 14/11/2024
Correspondence: Aránzazu Mediero. Bone and Joint Research Unit. IIS-Fundación Jiménez Díaz. Universidad Autónoma de Madrid. Avda. Reyes Católicos, 2. 28040 Madrid, Spain
e-mail: aranzazu.mediero@quironsalud.es

Authors´ contributions: AM designed the experiments, performed experiments, analyzed and interpreted the results, and wrote and

revised the manuscript. JJA-C and ET were responsible of bacterial preparation and plate coating, interpreted the results, and wrote and revised the manuscript. MM-B. performed HPLC analyses, interpreted the results, and wrote and revised the manuscript. AC, MA, JJD supplied and prepared the samples of both alloysfor the present study and revised and edited the manuscript, RL, GH-B and JE interpreted the results, and revised and edited the manuscript.

Data availability statement: the original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

Conflict of interest: the authors declare no conflict of interest.

Artificial intelligence: the authors declare not to have used artificial intelligence (AI) or any AI-assisted technologies in the elaboration of the article.

ABSTRACT

Objective: two of the major complications after arthroplasty are prosthetic joint infection, mainly due to *staphylococcus,* and the experience of a foreign body reaction by macrophages and foreign body giant cells (FBGCs) regardless of the infection. Our aim is to study the role of purinergic receptors with fusogenic function (P2X7, adenosine A1 and A2A receptors) in the formation of FBGCs induced by *S. aureus* and their possible differential modulation in the presence of Ti–6Al–4V and Cr-Co-Mo alloys.

Methods: RAW264.7 cells were differentiated to FBGCs with 20 ng/ml of IL-4 in the presence of adhered unviable *S. aureus,* metal alloys and/or CGS21680/ZM241385 1 µM. Cell supernatant was collected for nucleotide analysis by HPLC as well as cytokine expression, and cells were lysed for RNA expression.

Results: the presence of *S. aureus* induces an increase in FBGCs formation in a concentration-dependent manner, and phalloidin staining demonstrated that both Ti–6Al–4V and Cr-Co-Mo alloys reduce FBGCs formation. The expression of adenosine A1 and A2A receptors were increased after 5 days of differentiation in the presence of *S. aureus*, and this expression was enhanced with metal alloys. HPLC analyses showed an increase in adenosine in the presence of Ti–6Al–4V and Cr-Co-Mo alloys while ATP was not modified in any of the conditions. The presence of metal alloys induced an increased in IL1β, IL-6 and RANTES.

Conclusions: the increase in the levels of adenosine and the adenosine A2A receptor induced by the presence of Ti–6Al–4V and Co-Cr-Mo alloys would be responsible for the inhibition of cell fusion and the subsequent reduction of FBGCs induced by *S. aureus*.

Keywords: Adenosine. A2A receptor. FBGCs. Metal alloys. Prosthetic joint infection.

RESUMEN

Introducción: dos de las principales complicaciones tras la artroplastia son la infección de la prótesis articular, principalmente por estafilococos, y la experiencia de una reacción a cuerpo extraño por parte de macrófagos y células gigantes de cuerpo extraño (FBGC) independientemente de la infección. Nuestro objetivo es estudiar el papel de los receptores purinérgicos con función fusogénica (P2X7, receptores de adenosina A1 y A2A) en la formación de FBGC inducidas por *S. aureus* y la presencia de aleaciones de Ti–6Al–4V y Cr-Co-Mo.

Métodos: se diferenciaron células RAW264.7 a FBGC con IL-4 20 ng/ml en presencia de *S. aureus* adherido inviable, aleaciones metálicas y/o CGS21680/ZM241385 1 µM. El análisis de nucleótidos mediante HPLC y la expresión de citoquinas se realizaron en sobrenadante celular. Las células se lisaron para estudios de ARN.

Resultados: *S. aureus* indujo un aumento en la formación de FBGC de manera concentración dependiente, y la presencia de Ti–6Al–4V y Cr-Co-Mo redujeron la formación de FBGC. La expresión de los receptores de adenosina A1 y A2A es clave en la formación de FBGC, y el análisis por HPLC mostró un aumento de adenosina en presencia de Ti–6Al–4V y Cr-Co-Mo. La presencia de aleaciones metálicas indujo un aumento de IL1β, IL-6 y RANTES.
Conclusiones: el aumento de los niveles de adenosina y del receptor A2A inducido por la presencia de Ti–6Al–4V y Co-Cr-Mo sería responsable de la inhibición de la fusión celular y la posterior reducción de FBGC inducida por *S. aureus.*

Palabras calve: Adenosina. Receptor A2A. FBGC. Aleaciones metálicas. Infección de prótesis articulares.

INTRODUCTION

Arthroplasty has improved the quality of life of millions of patients and is highly effective, although implant-related complications can arise (1). Prosthetic joint infection (PJI) is one of the most important complications with a high morbidity, mortality, and costs associated. The incidence varies from country to country, between 0.5 and 2 % (2). Staphylococci are the most common etiological agents associated with PJI, with *S. aureus* (30-40 %) and *S. epidermidis* being two of the main microorganisms involved (3). PJI results from the ability of microorganisms to develop a biofilm, a conglomerate of bacterialcells of at least one species that adheres to a surface or interface with complex sociomicrobiological interactions among them (4).

Immediately after implantation with and without a perioperatory PJI, the implant surface is coated with plasma proteins that direct cellular adhesion and activation. Last step in the prosthesis implantation process is a foreign body reaction, that occurs when the inflammatory and wound healing responses havetaken place and is composed of macrophages and foreign body giant cells (FBGCs) (9). These FBGCs

are present on the surface of the implants throughout the life of the implant and produce stress cracks and oxidative damage (7). These macrophages and FBGCs secrete cytokines that in the early stages will modulate neutrophils and lymphocytes recruitment and activation (8). IL-4 and IL-13, both secreted by CD4+ T cells, are key cytokines for the formation of FGBCs involved in material degradation and implant rejection (9). In each of the four phases for cell fusion, similar and different mechanisms for the formation of FBGCs or osteoclasts (multinucleated bone cells with the function of resorbing bone) have been described (10). At the end of this process, adherent macrophages and FBGCs inducebiomaterialdegradation with ensuing clinical device failure (5). Therefore, it is under exploration if different materials respond differently to FBGCs adherence and how this biomaterial surface can be modulatedto alter the presence and activity of adherent macrophages and FBGCs (11).

The purinergic signalling is also activated in cell fusion, especially in inflammation. Pharmacologically, two families of purinergic receptors are known: P1 receptors selective for adenosine, and P2 receptors selective for purine and pyrimidine nucleotides and dinucleotides (11,12). Adenosine is generated extra- and intra-cellularly by the hydrolysis of adenine nucleotides and performs its physiological functions through the activation of G-protein coupled transmembrane receptors (A1, A2A, A2B and A3), whose tissue distribution, pharmacological profile and effects are different (12). Purine and pyrimidine nucleotides activate two types of P2 receptors: P2X receptors —ionotropic, ion-linked channels— and P2Y receptors —metabotropic, G protein-coupled (11). It is well-known the role of several P1 and P2 receptors in bone cells. The adenosine A1 receptor is critical and essential for differentiation and function of osteoclasts (13), while the adenosine A2A receptor induces an inhibition ofosteoclast formation and function both *in vitro* (18) and in the osteolysis model (16). The adenosine A2B receptor is involved in osteoblast differentiation and function, with a more ambiguous role

for the A2A receptor (16,17). Activation of adenosine A2A receptor both directly (by specific agonist) or indirectly (by drugs that increase extracellular adenosine levels by blocking its transport) promotes bone formation at levels similar to BMP-2 (bone morphogenic protein 2) (17). Among P2 receptors, P2X7 is especially relevant as its expression is positively regulated by pro-inflammatory cytokines (18). The P2X7 receptor is activated and is involved in cell fusion, where it intervenes in the late phases of membrane fusion and establishes union bridges between the cytoplasm of adjacent cells (19).

In Orthopaedic Surgery and Traumatology, the most used medical alloys are Ti–6Al–4V and Co-Cr-Mo. Ti–6Al–4V alloy has favourable biocompatibility, mechanical properties and corrosion resistance (20). Co-Cr-Mo alloy shows also high biocompatibility levels, with exceptional properties including abrasion, cracking-corrosion, pitting and wearresistance, as well as malleability anda high fatigue resistance and ductility (21).

Surprinsingly, nothing is known about the effect that the presence of bacteria, such as *S. aureus*, may or may not have during the formation process of these FBGCs. Therefore, we aim to investigate if variations in the metal alloys of the implants would modify the recruitment of macrophages to the implant area and, therefore, the formation of FBGCs since they would modulate the cell fusion process. More specifically, we believe that a differential activation of the purinergic system may be involved, and especially the P2X7 receptor and adenosine A1 and A2A receptors, as they are implicated in the fusion of macrophages to osteoclasts.

METHODS

Materials

An 18-mm diameter bar of ELI grade Ti–6Al–4V manufactured to ASTM F136-02, was supplied by Surgical Co., SAU (Valencia, Spain). The rod was cut into 2 mm thick disc specimens and ground through

successive grades of SiC paper up to 2000 grade. To achieve a smooth surface with a final controlled roughness, chemical-mechanical polishing was then carried out in a commercial colloidal silica suspension (OP-S Suspension. 0.25 μm from Struers, Copenhagen, Denmark) with hydrogen peroxide with a volume ratio of 9:1.

A rodof Co-Cr-Mo alloy with a diameter of 19 mm was supplied by Carpenter Technology Corp. (Philadelphia, PA, USA), which was melted and tested in accordance with ASTM F1537-11. The rod was cut into 2 mm thick discs. One side of each disc was ground on silicon carbide paper and polished to a 3 μm diamond finish. The chemical composition of both alloys has been described elsewhere (22).

Cell lines

Inmortal murine macrophage line RAW264.7 (ATCC, LGC Standards S.L.U., Barcelona, Spain) were used. Cell culture was maintained in alpha MEM with 10 % FBS and 1 % penicillin/streptomycin and maintained in a humidified chamber at 37 °C, in 95 % air and 5 % CO_2.

The *S. aureus* strain chosen was a smallcolony variant strain (Sa35) isolated from a total hip PJI of a 79 years-old woman identified in the Clinical Microbiology Department at the Hospital Universitario Fundación Jiménez Díaz, Madrid (Spain). Several colonies of Sa35 grown on chocolate-blood agar were inoculated in an 8-mL tube of brain-heart infusion (BHI, BD, New Jersey, United States) at 37 °C for 24 h, then centrifuged at 3500 rpm at 22 °C for 10 min. The supernatant was removed, and the pellet was washed three times with sterile 0.9 % NaCl saline solution (SS) (B. Braun, Melsungen, Germany) and then resuspended and diluted in SS to get a bacterial solution (approximately, $1.64 \pm 0.22 \cdot 10^9$ CFU/mL). The bacterial solution was autoclaved at 121 °C for 20 min. Both types of alloy coupons were incubated in 5 mL of this 1:100-bacterial solution in a sterile nontreated six-well plate (Thermo Fisher Scientific, Waltham,

MA, USA) at 37 °C for 24 h to allow bacterial adhesion. Afterwards, the supernatant was discarded, and the metallic samples were used in the next described experiment.

Foreign body giant cells (FBGCs) formation

To determine the optimal conditions for infection by *S. aureus* in the RAW264.7 cells, proliferation (alamar blue, Bio-Rad, Madrid, Spain) has been carried out on the cells at 72 hours, in three different culture medias: a) alpha MEM with 10 % FBS without penicillin/streptomicin; b) RPMI with 5 % FBS without penicillin/streptomycin; and c) RPMI with 50 % bovine serum albumin (BSA) ($n = 5$).

Ten thousand RAW264.7 cells were differentiated with 20 ng/ml of IL-4 in the presence of adhered unviable*S. aureus* ($1.61 \pm 0.22 \cdot 10^7$, 10^6 and 10^5 CFU/ml) for 5 days for giant cell formation. In some of the wells the co-culture will be carried out in the presence of the material of the two aforementioned alloys: Ti–6Al–4V and Co-Cr-Mo, and other well will be incubated with selective adenosine A2A agonist (CGS21680) and antagonist (ZM241385) 1 µM each. Cells were stained using the quick panoptic stain (PanReac Applichem, ITW Reagents, Barcelona, Spain) a non-vital, differential stain based on the May Grünwald-Giemsa staining. To check FBGCs formation in the metal alloys, phalloidin staining was performed when RAW264.7 cells were differentiated with 20 ng/ml of IL-4 in the presence of adhered unviable *S. aureus* ($1.61 \pm 0.22 \cdot 10^7$ CFU/ml) for 5 days. Briefly, cells were fixed with 4 % PFA, blocked in PBS BSA 1 % and 0.1 % Triton X-100 for 30 minutes and stained Alexa Fluor 555-phalloidin (Invitrogen, Fisher Scientific, Madrid, Spain) for 30 minutes.

Nucleotide concentration determination via high-performance liquid chromatography (HPLC)

Ten thousand RAW264.7 cells were differentiated with 20 ng/ml of IL-4 in the presence of adhered unviable *S. aureus* ($1.61 \pm 0.22 \cdot$

10^7CFU/ml) alone or in the presence of Ti–6Al–4V and Co-Cr-Mo for 3 or 5 days and supernatant were collected (n = 8 each). Samples were treated with EHNA and dipyridamole 1 µM each to avoid adenosine degradation/cellular uptaken. Protein denaturation and HPLC analysis were performed as described previously by Vivero-Lopez et al. (23). Briefly, a heat shock step was performed for 2 min at 98 °C, and samples were centrifuged at 13 000 g for 10 min at 4 °C. Supernatants were collected and stored at -80 °C until use. Inosine, adenosine, AMP, ADP and ATP concentrations were determined via high-performance liquid chromatography (HPLC) using a liquid chromatography with a reversed phase column (Agilent 1100 Series Liquid Chromatography) and a UV detector set at 254 nm. The buffer (0.1 mol/l KH_2PO_4 pH 7.5, 18 % acetonitrile) was run at 1.5 ml/min for 20 min. Compounds were identified and quantified by their retention times and peak areas of known standards, calibrated via spectrophotometry. The results are expressed as the mean ± standard error of the mean (SEM). All results were corrected according to heat shock lost and calculated as a percentage of basal cells.

Cytokine and chemokine array

Ten thousand RAW264.7 cells were differentiated with 20 ng/ml of IL-4 in the presence of adhered unviable *S. aureus* ($1.61 \pm 0.22 \cdot 10^7$ CFU/ml) alone or in the presence of Ti–6Al–4V and Co-Cr-Mo alloys for 5 days and supernatant were collected (n = 5-6 each). Cytokines were measured using the Mouse Cytokine Array Q1 (QAM-CYT-1-4, RayBiotech, CliniSciences, Madrid, Spain) which, simultaneously, detects 20 cytokines. The cytokines concentration was measured by fluorescence using GenePix 4000B in accordance with manufacturer´s recommendations.

RNA isolation and RT-PCR

Total RNA was isolated from cultures using TRIzol reagent according to the manufacturer's protocol. RNA (1 µg) was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (2.5 U/µL), with RNase Inhibitor 1 U/µL, Random Hexamers 2.5 U/µL, $MgCl_2$ 5 mM, PCR buffer II 1× and dNTPs 1 mM (Applied Biosystems, Foster City, CA, USA). Relative analysis of gene expression was performed via real-time RT-PCR on a Step One Plus with Power UP SYBR Green MasterMix (Applied Biosystems). The primers used are listed in table I. Gene expression levels were calculated with the ΔΔCt method.

Statistical analyses

Statistical significance among groups was determined using one-way ANOVA and Bonferroni post-hoc test. All statistics were calculated using GraphPad® software (GraphPad, San Diego, CA, USA).

RESULTS

The presence of adhered unviable *S. aureus* induces an increase in the formation of FBGCs that is abrogated in the presence of metal alloys

To understand the role of *S. aureus* in the formation of FGBCs, we first tested the most suitable media for the growth of RAW264.7 cells and *S. aureus*. As we can see in figure 1A, RAW264.7 cells proliferated adequately when they were seeded in αMEM medium with 10 % FBS and without antibiotics, while RPMI media with 5 % FBS but without antibiotics and RPMI with 50 % BSA, were not adequate for RAW264.7 cells proliferation. Next, we tested the number of RAW264.7 cells and the concentration and IL-4 necessary for the correct differentiation of FBGCs. In figure 1B we observe that the best formation of FBGCswas achieved with 10,000 RAW264.7 cells and 20 ng/ml of IL-4. Finally, we

tested if the presence of *S. aureus* modified FBGCs formation. We observed that the presence of *S. aureus* induced an increase in the formation of FBGCs in a concentration-dependent manner (54 ± 6 % increase for $1.61 \pm 0.22 \cdot 10^7$ CFU/ml, $p < 0.0001$).

Therefore, we decided to carry out all the experiments in αMEM with 10 % FBS and without antibiotics, 10,000 RAW264.7 cells, 20 ng/ml of IL-4 and $1.61 \pm 0.22 \cdot 10^7$ CFU/ml.

When RAW264.7 cells and *S. aureus* where co-cultured in the presence of IL-4 and the metal alloys, immunofluorescence staining with phalloidin demonstrated that both Ti–6Al–4V and Co-Cr-Mo alloys reduced the formation of FBGCs in the presence of $1.61 \pm 0.22 \cdot 10^7$ CFU/ml *S. aureus* (Fig. 1D).

S. aureus **induces the secretion of adenosine into the extracellular medium and the metal alloys Ti–6Al–4V and Co-Cr-Mo increase it**

The HPLC study of extracellular nucleotide concentrations reflected a significant increase in inosine in the presence of *S. aureus* at 3 and 5 days of differentiation with IL-4 20 ng/ml ($p < 0.0001$, $n = 8$), which were reduced significantly when the co-culture was done in the presence of Ti–6Al–4V alloy at 3 and 5 days of differentiation and by the Co-Cr-Mo alloy at 5 days ($p < 0.0001$, $n = 8$) (Fig. 2A). Regarding the extracellular concentrations of adenosine, we observed that no significant changes occurred in the presence of IL-4 20 ng/ml alone nor in the presence of *S. aureus* neither at 3 or 5 days of differentiation, but both Ti–6Al–4V and Co-Cr-Mo alloys induced a significant increase in extracellular adenosine levels when compared to both the basal and *S. aureus* at both time points ($p < 0.0001$ vs *S. aureus*, $n = 8$) (Fig. 2B). The extracellular levels of AMP increased significantly on day 5 of differentiationin all cases ($p < 0.001$, $p < 0.0001$, $n = 8$) without significant differences between groups

(Fig. 2C). No significant changes in either the extracellular concentrations of ADP or ATP were found (p = ns, n = 8) (Fig. 2D-E).

Adenosine A1 and A2A receptors and P2X7 receptors expression is modulated by *S. aureus* and the presence of metal alloys

Having the increase in extracellular adenosine levels, we next exert to comprehend whetherthe purinergic receptors implicated in fusogenic functions (adenosine A1 and A2A receptors and P2X7 receptors) areinvolved in the formation of FBGCs in the presence *of S. aureus* and the metal alloys. The expression of mRNA for adenosine A1 receptor in RAW264.7 cells increased at 3 and 5 days in the presence of 20 ng/ml IL-4 and was enhanced at day 5 of differentiation in the presence of *S. aureus* although this increase was not significant (p = ns, n = 3-4) (Fig. 3A). Ti–6Al–4V alloy was able to increase A1 receptor mRNA expression, been significant this increase at day 5 of differentiation (11120.6 ± 6630 fold change *S. aureus* + Ti–6Al–4V vs. 11.7 ± 8.073 fold change *S. aureus*, $p < 0.05$, n = 3-4) (Fig. 3A). Similar trend was observed for Cr-Co-Mo alloy, although in this case, the increased expression was not significant (p = ns, n = 3-4) (Fig. 3A).

A similar tendency was observed for adenosine A2A receptor in RAW264.7 cells. A2A receptor mRNA expression increased at 3 and 5 days in the presence of 20 ng/ml IL-4, and was enhanced at day 5 of differentiation in the presence of *S. aureus* although this increase was not significant (p = ns, n = 3-4) (Fig. 3B). Ti–6Al–4V alloy was able to increase A2A receptor mRNA expression, but this increase was not significant (p = ns, n = 3-4), with Co-Cr-Mo alloyable to significantly increase A2A receptor mRNA expression at day 5 of differentiation (15114 ± 6082-fold change *S. aureus* + Co-Cr-Mo *vs*. 8.17 ± 2.95-fold change *S. aureus*, $p < 0.05$, n = 3-4) (Fig. 3B).

The expression of the P2X7 ATP receptor does not increase significantly in the presence of IL-4 20 ng/ml, both at 3 and5 days of differentiation. However, it does increase in the presence of *S. aureus,* with this increase being furtherenhanced by both metal alloys. Nevertheless, this increase was not found to be statically significant (p = ns, n = 3-4) (Fig. 3C).

Adenosine A2A receptor activation inhibits *S. aureus*-induced FBGC formation

Incubation of RAW264.7 cells with the adenosine A2A receptor agonist CGS21680 1 µM, inhibited FBGCs formation induced by IL-4 20 ng/ml (reduction of 37.89 ± 1.97 % compared to 100 % FBGCs in IL-4, $p < 0.0001$, $n = 6$), being reversed in the presence of the A2A receptor antagonist ZM241385 1 µM (Fig. 4). In the presence of *S. aureus*, there was an increased in FBGCs formation (increase of 70.67 ± 2.33 % compared to 100 % FBGCs in IL-4, $p < 0.0001$, $n = 6$) as described above that was significantly decrease in the presence of CGS21680 1 µM (decrease of 43.17 ± 3.7 % compared with IL-4 and *S. aureus*, $p < 0.0001$, $n = 6$) which is also reversed in the presence of ZM241385 1 µM (Fig. 4).

***S. aureus* and metal alloys slightly change the cytokines prolife secretion of RAW264.7 cells**

Next, we tend to identify changes in cytokine secretion due to presence of *S. aureus* and metal alloys. For this purpose, we performed a cytokine array that measures 20 cytokines and chemokines (Table II). When we analyzed the expression of pro-inflammatory cytokines, we found that the presence of *S. aureus* only increased the expression of IL-6 and TNFα when we compared both with basal conditions and control conditions in the presence of IL-4 20 ng/ml (137.2 ± 23.12 pg/ml *S. aureus vs*. 2.28 ± 1.23 pg/ml IL-4 for IL-6; and 781.1 ± 289.9 pg/ml *S. aureus* vs 7.051 ± 3.287 pg/ml IL-4

for TNFα, $p < 0.0001$ and $p < 0.05$ respectively, $n = 5$-6) (Table II). When RAW264.7 cells were differentiated in the presence of IL-4 20 ng/ml and the Co-Cr-Mo alloy, no differences in pro-inflammatory cytokines were observed when compared to control conditions in the presence of IL-4 20 ng/ml (Table II). However, when RAW264.7 cells were differentiated in the presence of *S. aureus* and the Co-Cr-Mo alloy, we observed a significant increase in IL-1α, IL-1β and IL-6 expression when compared to all conditions (336.6 ± 139.5 pg/ml *S. aureus* + Co-Cr-Mo *vs.* 22.98 ± 10.5 pg/ml *S. aureus* for IL-1α; 114.5 ± 23.21pg/ml *S. aureus* + Co-Cr-Mo vs. 2.636 ± 1.895 pg/ml *S. aureus* for IL-1β; and 925.9 ± 283.621 pg/ml *S. aureus* + Co-Cr-Mo *vs.* 137.2 ± 23.12 pg/ml *S. aureus* for IL-6, $p < 0.05$, $p < 0.0001$ and $p < 0.0001$ respectively, $n = 5$-6) (Table II). In a similar manner, when RAW264.7 cells were differentiated in the presence of IL-4 20 ng/ml and the Ti–6Al–4V alloy, no differences in pro-inflammatory cytokines were observed when compared to control conditions in the presence of IL-4 20 ng/ml (Table II). Moreover, when RAW264.7 cells were differentiated in the presence of *S. aureus* and the Ti–6Al–4V alloy, we observed a significant increase in IL-1β and IL-6 expression when compared to all conditions (209.5 ± 52,88 pg/ml *S. aureus* + Ti–6Al–4V vs 2.636 ± 1.895 pg/ml *S. aureus* for IL-1β; and 3366 ± 818.3 pg/ml *S. aureus* + Ti–6Al–4V *vs.* 137.2 ± 23.12 pg/ml *S. aureus* for IL-6, $p < 0.0001$ and $p < 0.0001$ respectively, $n = 5$-6) (Table II).

No changes were observed among conditions in any anti-inflammatory cytokine (Table II).

RANTES, a classical chemotactic cytokine also known as CCL5, was increased in the presence of *S. aureus* (528.3 ± 66.47 pg/ml *S. aureus* vs 199.8 ± 37.12 pg/ml IL-4, $p < 0.001$, $n = 5$-6) and was enhanced when co-culture was performed in the presence of metal alloys (609.6 ± 81.64 pg/ml *S. aureus* + Co-Cr-Mo vs 528.3 ± 66.47 pg/ml *S. aureus* and 994.4 ± 185,4 pg/ml *S. aureus* + Ti–6Al–4V vs

528,3 ± 66,47 pg/ml *S. aureus*, $p < 0.001$ and $p < 0.0001$ respectively, $n = 5$-6) (Table II).

Finally, we found that the presence of IL-4 20 ng/ml induced an increased expression of VEGF (360.8 ± 44.25 pg/ml IL4 vs 82.94 ± 46.62 pg/ml basal, $p < 0.0001$, $n = 5$-6) that was not significantly reduced in the presence of *S. aureus* but it was significantly increased in the presence of both IL-4 and the Co-Cr-Mo alloy (669.3 ± 144.5 pg/ml Co-Cr-Mo vs 82.94 ± 46,62 pg/ml basal, $p < 0.0001$, $n = 5$-6) (Table II).

DISCUSSION

In this manuscript we have demonstrated that the presence of Ti–6Al–4V and Co-Cr-Mo alloys required purinergic system activation in macrophages in order to inhibit FBGCs formation induced by adhered unviable *S. aureus*.

As we can observe in our data, adhered unviable *S. aureus* is able to form FBGCs although no infectious activity. Although the use of non-viable *S. aureus* represents a limitation of our study, it is worth noting that during *S. aureus* biofilm formation bacterial lysis plays a key role (24), so this study may reveal what role these non-viable cells play in the development of FBGCs. *S. aureus* can attach to a biotic surfaces through electrostatic and hydrophobic interactions under static conditions, even if it is not viable (25). These adhered unviable staphylococci which are not forming a biofilm will be detected by the Toll-like receptors and will induce the phagocytic cell activation (26). Macrophage fusion to form a FBGC occurs in response to tissue injury and the presence of any biomaterial (7), whose physical features (e.g., substrate stiffness, topography, and surface chemistry) will determine such response. Interestingly, this foreign body response is also regulated by Toll-like receptors (27). Our results bring to light

that unviable *S. aureus* can induce the formation of FBGCs, and it is plausible that the presence of Ti–6Al–4V and Co-Cr-Mo metallic would be the necessary for the inhibition of that formation of FBGCs even in the presence of unviable *S. aureus*.

Fibrotic encapsulation emerged as a crucial tissue response to foreign objects inadvertently becoming lodged in the body. The immune capacity to successfully block access to the rest of the body by fibrotic encapsulation is pivotal since these foreign items pose numerous hazards, including the potential to spread infection (28). If fibrotic encapsulation were to take place on the *S. aureus*-infected implant, the foci of infection would have access to fewer nutrients from the periprosthetic tissues. Hence, the inhibition of the development of FBGCs by those metallic alloys may inhibit the fibrous encapsulation of these staphylococci-covered alloys (29) and would favour the maintenance of the staphylococcal infection which would not have any fibrotic capsule impairing the arrival of nutrients into their immediate environment.

HPLC analysis showed an increase in adenosine extracellular levels induced by metal alloys that was not exert neither by IL-4 alone nor in combination with adhered unviable *S. aureus*. This adenosine increased correlates with the expression of adenosine receptors showed. Although both metal alloys increased in a similar manner adenosine levels, and both have the same trend in adenosine A1 and A2A receptors mRNA increased, Ti–6Al–4V only significantly increased mRNA levels for adenosine A1 receptor meanwhile Co-Cr-Mo only significantly induced adenosine A2A receptor. None of the conditions induce P2X7 changes, in correlation with no modifications of ATP levels. Having in consideration that both FBGCs and osteoclast share some mechanism during their formation process (10), it is plausible that adenosine receptors have a similar role in both FBGCS and osteoclast formation. It has been extensively demonstrated that adenosine A2A receptor inhibits osteoclast formation (14,15). The

increasein this receptor together with the risein adenosine levels induced by metal alloys, indicate that similar role might be award to this receptor in FGBCs formation, primary in the presence of Co-Cr-Mo alloy.

On the other hand, it has been demonstrated that adenosine A1 receptors participate in fusion of human peripheral blood monocytes into giant cellsalthough ithas not been fully established (30). It is known that adenosine A1 receptor induces osteoclast differentiation (13), and blockade or deletion of the receptor suppresses osteoclast differentiation *in vitro* and *in vivo* (31). It has been also demonstrated that adenosine A_1 receptor on both primary murine and human osteoclast precursors are constitutively active, with selective antagonists working as an inverse agonists at A1 receptor (32). This might be the reason why changes in adenosine A1 receptor mRNA expression in IL-4 and *S. aureus* alone groups when compared with basal conditions are not significant.

The increase in A1 receptor in the presence of metal alloys need to be address better, but this increase might be a compensatory mechanism induced by *S. aureus* as it has been observed that A1 receptor stimulation is essential for formation of giant cells *in vitro* in the syncytia in giant cell arteritis, a characteristic where inflammation is crucial (33). Our data indicate that the presence of metal alloys induces a significantly increase in IL-1β and IL-6, two key pro-inflammatory cytokines. This increase may be involved in the rise in adenosine A1 receptor. However, the inflammatory role of adenosine A1 receptor is controversial. As it has been observed in some sceneries, A1 receptor can induce anti-inflammatory effects, including renal ischemia reperfusion, lungs (where absence of adenosine A1 receptor induce leukocyte migration and increased cytokines), and sepsis where A_1KO mice exhibit a higher degree of renal dysfunction and higher release of pro-inflammatory cytokines (34). These data indicate that the presence of endogenous A1 receptor is necessary to

protect against exacerbation of the disease and organ dysfunction. It is plausible that as occurs in sepsis, the imbalance in inflammation induced by *S. aureus* promotes the activation of A1 receptor by metal alloys in our system to counteract the hyperinflammatory scenario, been in this situation not only adenosine A2A receptor the only anti-inflammatory receptor, but also the A1 receptor. Therefore, more data arerequired to understand why metal alloys induced A1 receptor expression in this system.

Finally, one of the limitations of this study was the impossibility of using live *S. aureus* bacteria in our assays. Although different bacterial infection assays were performed (data not shown), the results obtained were erratic and the amount of FBGCs remaining adhered after infection was too small to perform the assays performed in this study. It would be convenient to carry out experiments with viable bacteria, been the appropriate moment of infection, co-culture methods (adherence or non-adherence), etc. would have to be validated.

In conclusion, the increased in the levels of adenosine would be responsible for the inhibition of cell fusion and the subsequent reduction of FBGCs induced by *S. aureus.* been also relevant the activation of adenosine A2A receptor induced by the presence of Co-Cr-Mo alloys.

ACKNOWLEDGMENTS

This work was supported by grants from Becas FEIOMM INVESTIGACIÓN 2021 and from Instituto de Salud Carlos III through the “Miguel Servet” program (CP15/00053, CPII20/00017), co-funded by Fondo Europeo de Desarrollo Regional (FEDER), and a research grant from the Spanish Instituto de Salud Carlos III (PI19/00744, PI22/00347).

REFERENCES

1. Trampuz A, Zimmerli W. Prosthetic joint infections: update in diagnosis and treatment. Swiss Medical Weekly 2005;135(1718):243-51. DOI: 10.4414/smw.2005.10934
2. Ayoade F, Li DD, Mabrouk A, Todd JR. Periprosthetic Joint Infection. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024. Available from: http://www.ncbi.nlm.nih.gov/books/NBK448131/
3. Benito N, Mur I, Ribera A, Soriano A, Rodríguez-Pardo D, Sorlí L, et al. The Different Microbial Etiology of Prosthetic Joint Infections according to Route of Acquisition and Time after Prosthesis Implantation, Including the Role of Multidrug-Resistant Organisms. J Clin Med 2019;8(5):673. DOI: 10.3390/jcm8050673
4. Høiby N, Ciofu O, Johansen HK, Song Z jun, Moser C, Jensen PØ, et al. The clinical impact of bacterial biofilms. Int J Oral Sci 2011;3(2):55-65. DOI: 10.4248/IJOS11026
5. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20(2):86-100. DOI: 10.1016/j.smim.2007.11.004
6. Gibon E, Takakubo Y, Zwingenberger S, Gallo J, Takagi M, Goodman SB. Friend or foe? Inflammation and the foreign body response to orthopedic biomaterials. J Biomed Mater Res A 2024;112(8):1172-87. DOI: 10.1002/jbm.a.37599
7. Sheikh Z, Brooks PJ, Barzilay O, Fine N, Glogauer M. Macrophages, Foreign Body Giant Cells and Their Response to Implantable Biomaterials. Materials (Basel) 2015;8(9):5671-701. DOI: 10.3390/ma8095269
8. Brodbeck WG, Nakayama Y, Matsuda T, Colton E, Ziats NP, Anderson JM. Biomaterial surface chemistry dictates asherent monocyte/macrophage cytokine expression in vitro. Cytokine 2002;18(6):311-9. DOI: 10.1006/cyto.2002.1048
9. Fang J, Liu R, Chen S, Liu Q, Cai H, Lin Y, et al. Tuning the immune reaction to manipulate the cell-mediated degradation of a collagen

barrier membrane. Acta Biomaterialia 2020;109:95-108. DOI: 10.1016/j.actbio.2020.03.038

10. Pereira M, Petretto E, Gordon S, Bassett JHD, Williams GR, Behmoaras JV. Common signalling pathways in macrophage and osteoclast multinucleation. J Cell Sci 2018;131(11):jcs216267. DOI: 10.1242/jcs.216267
11. Jacobson KA, Delicado EG, Gachet C, Kennedy C, von Kügelgen I, Li B, et al. Update of P2Y receptor pharmacology: IUPHAR Review 27. Br J Pharmacol 2020;177(11):2413-33. DOI: 10.1111/bph.15005
12. Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine Receptors—An Update. Pharmacol Rev 2011;63(1):1-34. DOI: 10.1124/pr.110.003285
13. He W, Cronstein BN. Adenosine A1 receptor regulates osteoclast formation by altering TRAF6/TAK1 signaling. Purinergic Signal 2012;8(2):327-37. DOI: 10.1007/s11302-012-9292-9
14. Mediero A, Perez-Aso M, Cronstein BN. Activation of adenosine A2A receptor reduces osteoclast formation via PKA- and ERK1/2-mediated suppression of NFκB nuclear translocation. Br J Pharmacol 2013;169(6):1372-88. DOI: 10.1111/bph.12227
15. Mediero A, Kara FM, Wilder T, Cronstein BN. Adenosine A2A Receptor Ligation Inhibits Osteoclast Formation. Am J Pathol 2012;180(2):775-86. DOI: 10.1016/j.ajpath.2011.10.017
16. Mediero A, Frenkel SR, Wilder T, He W, Mazumder A, Cronstein BN. Adenosine A2A Receptor Activation Prevents Wear Particle-Induced Osteolysis. Sci Transl Med 2012;4(135):135ra65. DOI: 10.1126/scitranslmed.3003393
17. Mediero A, Wilder T, Perez-Aso M, Cronstein BN. Direct or indirect stimulation of adenosine A2A receptors enhances bone regeneration as well as bone morphogenetic protein-2. FASEB J 2015;29(4):1577-90. DOI: 10.1096/fj.14-265066

18. Miller CM, Boulter NR, Fuller SJ, Zakrzewski AM, Lees MP, Saunders BM, et al. The Role of the P2X7 Receptor in Infectious Diseases. PLoS Pathog 2011;7(11):e1002212. DOI: 10.1371/journal.ppat.1002212
19. Falzoni S, Chiozzi P, Ferrari D, Buell G, Di Virgilio F. P2X7 Receptor and Polykarion Formation. Mol Biol Cell 2000;11(9):3169-76. DOI: 10.1091/mbc.11.9.3169
20. Long M, Rack HJ. Titanium alloys in total joint replacement—a materials science perspective. Biomaterials 1998;19(18):1621-39. DOI: 10.1016/S0142-9612(97)00146-4
21. Sánchez-De Jesús F, Bolarín-Miró AM, Torres-Villaseñor G, Cortés-Escobedo CA, Betancourt-Cantera JA. Mechanical alloying of biocompatible Co-28Cr-6Mo alloy. J Mater Sci: Mater Med 2010;21(7):2021-6. DOI: 10.1007/s10856-010-4066-9
22. Martín-García M, Aguilera-Correa JJ, Arenas MÁ, García-Diego IM, Conde A, de Damborenea JJ, et al. Differences in In Vitro Bacterial Adherence between Ti6Al4V and CoCrMo Alloys. Materials (Basel) 2023;16(4):1505. DOI: 10.3390/ma16041505
23. Vivero-Lopez M, Pereira-da-Mota AF, Carracedo G, Huete-Toral F, Parga A, Otero A, et al. Phosphorylcholine-Based Contact Lenses for Sustained Release of Resveratrol: Design, Antioxidant and Antimicrobial Performances, and In Vivo Behavior. ACS Appl Mater Interfaces 2022;14(50):55431-46. DOI: 10.1021/acsami.2c18217
24. Sadykov MR, Bayles KW. The control of death and lysis in staphylococcal biofilms: a coordination of physiological signals. Curr Opin Microbiol 2012;15(2):211-5. DOI: 10.1016/j.mib.2011.12.010
25. Moormeier DE, Bayles KW. Staphylococcus aureus Biofilm: A Complex Developmental Organism. Mol Microbiol 2017;104(3):365-76. DOI: 10.1111/mmi.13634
26. Hanke ML, Heim CE, Angle A, Sanderson SD, Kielian T. Targeting macrophage activation for the prevention and treatment of S.

aureus biofilm infections. J Immunol 2013;190(5):2159-68. DOI: 10.4049/jimmunol.1202348

27. Garrigues GE, Cho DR, Rubash HE, Goldring SR, Herndon JH, Shanbhag AS. Gene expression clustering using self-organizing maps: analysis of the macrophage response to particulate biomaterials. Biomaterials 2005;26(16):2933-45. DOI: 10.1016/j.biomaterials.2004.06.034
28. Welch NG, Winkler DA, Thissen H. Antifibrotic strategies for medical devices. Adv Drug Deliv Rev 2020;167:109-20. DOI: 10.1016/j.addr.2020.06.008
29. Anderson J, Cramer S. Chapter 2 - Perspectives on the Inflammatory, Healing, and Foreign Body Responses to Biomaterials and Medical Devices. In: Badylak SF, editor. Host Response to Biomaterials [Internet]. Oxford: Academic Press; 2015. *p*. 13-36. Available from: https://www.sciencedirect.com/science/article/pii/B9780128001967000025. DOI: 10.1016/B978-0-12-800196-7.00002-5
30. Haskó G, Pacher P. Regulation of Macrophage Function by Adenosine. Arterioscler Thromb Vasc Biol 2012;32(4):865-9. DOI: 10.1161/ATVBAHA.111.226852
31. Kara FM, Doty SB, Boskey A, Goldring S, Zaidi M, Fredholm BB, et al. Adenosine A1 Receptors (A1R) Regulate Bone Resorption II Adenosine A1R Blockade or Deletion Increases Bone Density and Prevents Ovariectomy-Induced Bone Loss. Arthritis Rheum 2010;62(2):534-41. DOI: 10.1002/art.27219
32. He W, Wilder T, Cronstein BN. Rolofylline, an adenosine A1 receptor antagonist, inhibits osteoclast differentiation as an inverse agonist. Br J Pharmacol 2013;170(6):1167-76. DOI: 10.1111/bph.12342
33. Merrill JT, Shen C, Schreibman D, Coffey D, Zakharenko O, Fisher R, et al. Adenosine A1 receptor promotion of multinucleated giant cell formation by human monocytes. A mechanism for

methotrexate-induced nodulosis in rheumatoid arthritis. Arthritis & Rheumatism 1997;40(7):1308-15.

34. Gallos G, Ruyle TD, Emala CW, Lee HT. A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis. Am J Physiol Renal Physiol 2005;289(2):F369-76. DOI: 10.1152/ajprenal.00470.2004

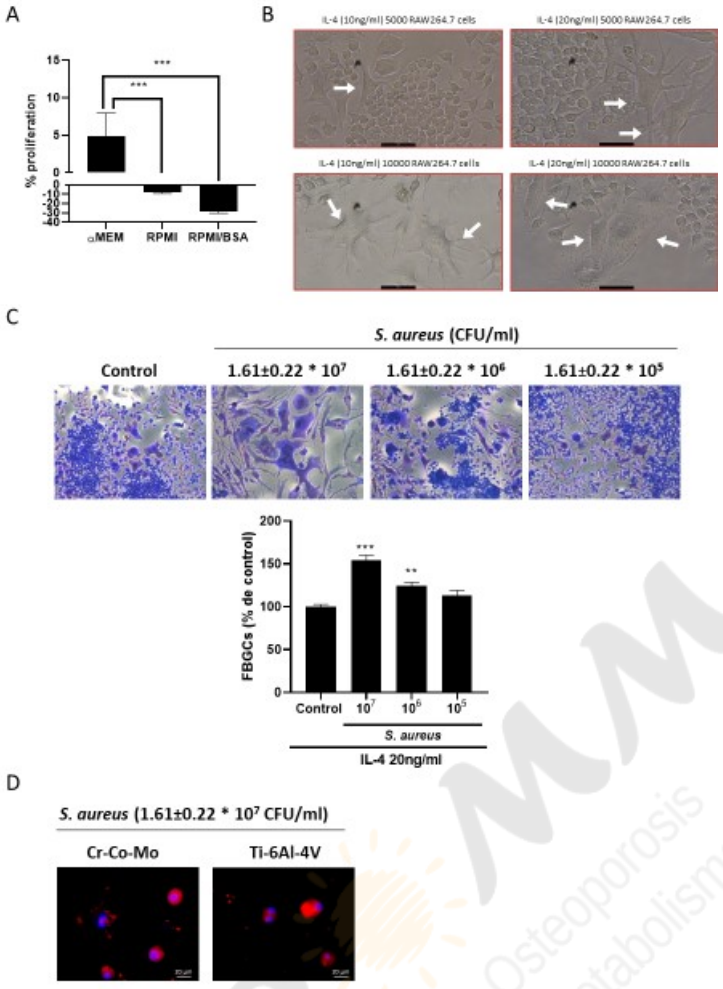

Fig. 1. Adherent unviable *S. aureus* induces formation of FBGCs and the presence of metal alloys inhibits it. A. Optimal culture media condition determination. B. Determination of Raw264.7 cell number and IL-4 concentration optimal for culture. White arrows indicate the presence of giant cells. C. Panoptic staining representative images and quantification for FBGCs formation with adherent unviable *S. aureus*. D. Representative images for FBGCs formation with adherent unviable *S. aureus* in the presence of IL-4 and Ti–6Al–4V and Co-Cr-Mo alloys. All images were taken at 40x magnification. Data are presented as the mean ± SEM. ***$p < 0.0001$ and **$p < 0.001$ vs control. One-way ANOVA and Bonferroni *post-hoc* test.

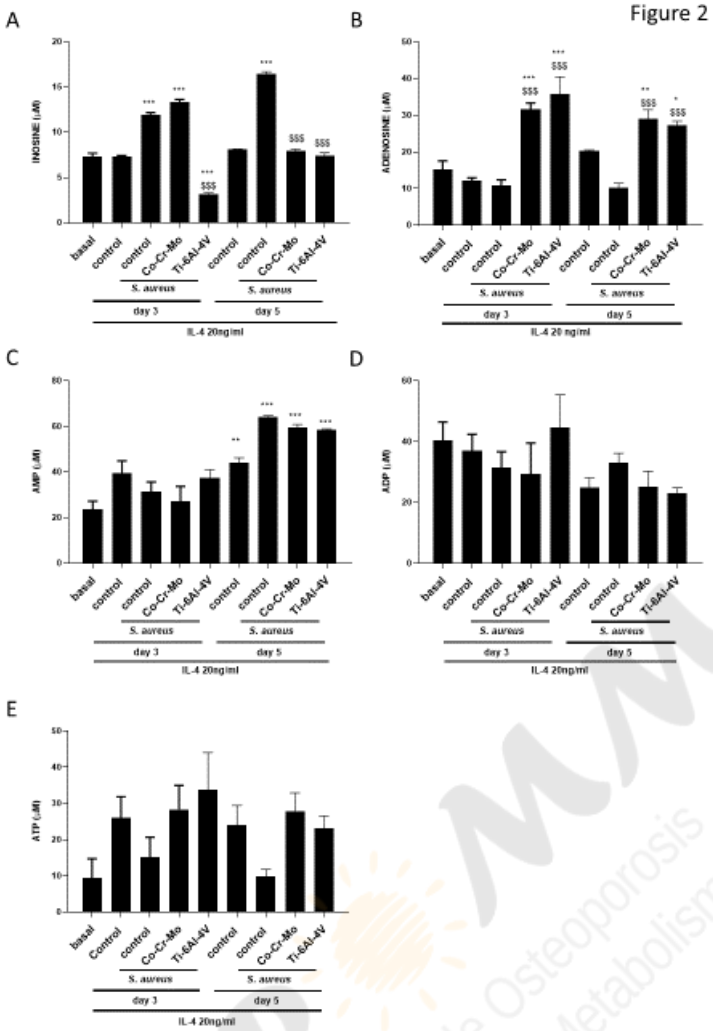

Fig. 2. Nucleotide analysis via HPLC at day 3 and 5 of differentiation in the presence ofadherent unviable *S. aureus* and metal alloys. A. Extracellular concentrations of inosine. B. Extracellular concentrations of adenosine. C. Extracellular concentrations of AMP. D. Extracellular concentrations of ADP. E. Extracellular concentrations of ATP. Data are presented as the mean ± SEM. ***$p < 0.0001$ vs basal; **$p < 0.001$ vs -basal; $$$$p < 0.0001$ vs *S. aureus*. One-way ANOVA and Bonferroni *post-hoc* test.

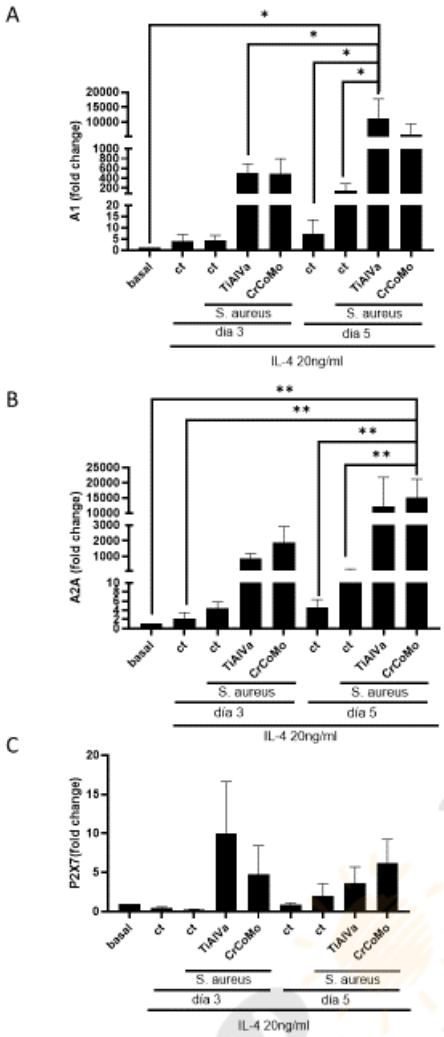

Fig. 3. Purinergic receptor mRNA expressionat day 3 and 5 of differentiation in the presence ofadherent unviable *S. aureus* and metal alloys. A. mRNA expression for adenosine A1 receptor in the presence of adherent unviable *S. aureus* and Ti–6Al–4V and Co-Cr-Mo alloys. B. mRNA expression for adenosine A2A receptor in the presence of adherent unviable *S. aureus* and Ti–6Al–4V and Co-Cr-Mo alloys. C. mRNA expression for P2X7 receptor in the presence of adherent unviable *S. aureus* and Ti–6Al–4V and Co-Cr-Mo alloys. Data are presented as the mean ± SEM. *$p < 0.05$. One-way ANOVA and Bonferroni *post-hoc* test.

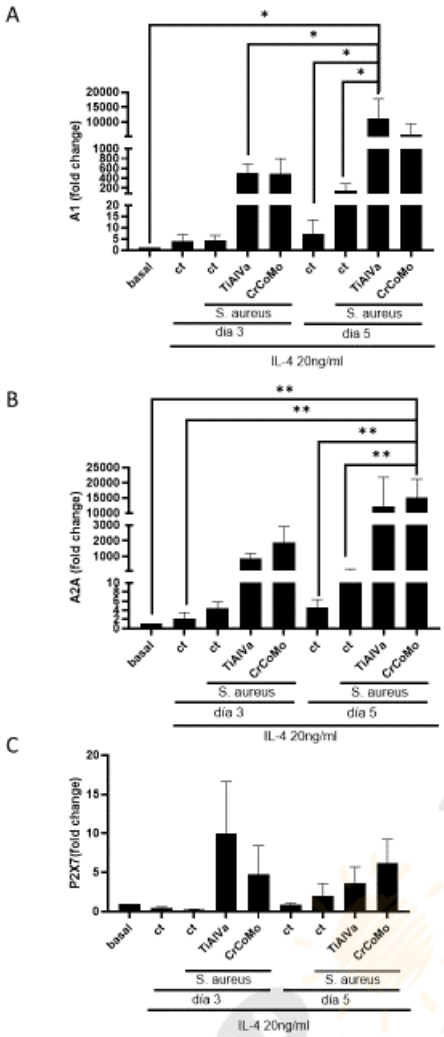

Fig. 4. The formation of FBGCs in the presence of *S. aureus* is decreased by the adenosine A2A receptor agonist CGS21680. Panoptic staining representative images and quantification for FBGCs formation. RAW264.7 cells treated with IL-4 20 ng/ml and adenosine A2A receptor agonist CGS21680 1 μM and antagonist ZM241385 1 μM alone or in the presence of adherent unviable *S. aureus*. All images were taken at 40x magnification. Data are presented as the mean ± SEM. ***$p < 0.0001$ vs control; $$$$p < 0.0001$ vs *S. aureus*. One-way ANOVA and Bonferroni *post-hoc* test.

Table I. List of primers used for RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
A2AR	TCGCCATCCGAATTCCACTC	TTTGTGCCCACAGATCTAGCC
A2BR	ATGGGCCAGATTAGGAGCAC	CTCCAAAAGGGGACCCAGTC
P2X7	GACAAACAAAGTCACCCGGAT	CGCTCACCAAAGCAAAGCTAAT
GAPDH	CTACACTGAGGACCAGGTTGTCT	GGTCTGGGATGGAAATTGTG

Table II. Inflammatory cytokines level measured using the Mouse Cytokine Array Q1

Cytokines (pg/ml)	**Basal**	**Control**	***S. aureus***	**Cr-Co-Mo**	***S. aureus* + CrCoMo**	**Ti–6Al–4V**	***S. aureus* + Ti-6Al4V**
Pro-inflammatory							
IFNg	12,33 ± 9,696	12,54 ± 2,968	23,58 ± 14,54	31,18 ± 16,48	6,729 ± 3,414	15,01 ± 3,93	63,93 ± 30,65
IL-1α	0 ± 0	0,1422 ± 0,08105	22,98 ± 10,5	5,4793,424	336,6 ± 139,5*	83,27 ± 59,56	581,82 ± 281,9
IL-1β	0 ± 0	0 ± 0	2,636 ± 1,895	15,11 ± 5,793	114,5 ± 23,21***	26,07 ± 10,79	209,5 ± 52,88***
IL-2	0 ± 0	0,1456 ± 0,101	0,4513 ± 0,289	0,3433 ± 0,210	0,2461 ± 0,181	0,1256 ± 0,080	5,51 ± 3,437

		8		3	3	59	
IL-3	0 ± 0	0 ± 0	0 ± 0	0,0008575 ± 0,0008575	0 ± 0	0,0008473 ± 0,0008473	0,06079 ± 0,06079
IL-6	0,08741 ± 0,08741	2,282 ± 1,226***	137,2 ± 23,12***	18,17 ± 8,755	925,9 ± 283,6***	62,05 ± 20,41	3366 ± 818,3***
IL-12	36,8 ± 27,04	22,1 ± 13,84	22,2 ± 22,2	53,17 ± 34,78	1,024 ± 0,9375	50,31 ± 34,34	57,24 ± 25,82
IL-17	18,81 ± 12,08	45,7 ± 21,8	49,75 ± 32,69	57,45 ± 32,05	36,84 ± 23,44	23,41 ± 15,74	44,41 ± 43,1
TNFα	28,1 ± 10,07	7,051 ± 3,287	781,1 ± 289,9*	27,71 ± 19,72	638,8 ± 258,3	206,1 ± 108,9	839 ± 282,4

Anti-inflammatory							
IL-4	0 ± 0	990,3 ± 417,9	839,1 ± 313,3	1243 ± 553,6	1043 ± 448	1055 ± 576,4	1053 ± 382,2
IL-5	12,08 ± 12,08	11,57 ± 6,139	6,548 ± 3,407	68,58 ± 45,6	4,693 ± 2,211	102,2 ± 64,11	106,2 ± 48,8
IL-9	32,74 ± 32,74	0 ± 0	72,46 ± 53,64	68,58 ± 68,58	2,842 ± 2,842	43,19 ± 41,89	294,2 ± 135,7
IL-10	9,099 ± 6,118	26,76 ± 17,81	26,94 ± 13,94	40,2 ± 18,28	21,57 ± 12,59	31,86 ± 10,41	205,7 ± 124,8
IL-13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0,04131 ± 0,04131	79,55 ± 78,01
Chemokines (pg/ml)							
KC	0 ± 0	0,2814 ± 0,225	0,05176 ± 0,05	1,039 ± 0,7841	0,2598 ± 0,259	0,01222 ± 0,01	49,18 ± 32,2

		9	176		8	222	
RANTES	184,2 ± 51,74	199,8 ± 37,12	528,3 ± 66,47**	312,7 ± 92,16	609,6 ± 81,64**	230,2 ± 109,4	994,4 ± 185,4***
Monocyte chemoattractant (pg/ml)							
MCP-1	19344 ± 10709	24237 ± 11396	70425 ± 37955	196673 ± 138323	101614 ± 55319	8901 ± 5512	57306 ± 37478
Differentiation and proliferation of macrophages (pg/ml)							
GM-CSF	0 ± 0	0,4957 ± 0,495	0,7988 ± 0,798	16,03 ± 11,65	0,4237 ± 0,423	0,3985 ± 0,398	33,38 ± 18,46

		7	8		7	5	
M-CSF	0 ± 0	0,03008 ± 0,02416	0 ± 0	0,01709 ± 0,0115	0 ± 0	0 ± 0	1,844 ± 1,839
Vasculogenesis and angiogenesis (pg/ml)							
VEGF	82,94 ± 46,62	360,8 ± 44,25***	24,77 ± 12,02	669,3 ± 144,5***	177,4 ± 32,91	299,6 ± 132,7	457,2 ± 172,1

Cytokines and chemokines in pg/ml. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.001$, and ^***$p < 0.0001$. One-way ANOVA and Bonferroni *post-hoc* test.