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Surface characteristics on commercial dental implants differentially activate macrophages in vitro and in vivo

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Abstract

Objectives: Biomaterial implantation provokes an inflammatory response that controls integrative fate. M2 macrophages regulate the response to implants by resolving the inflammatory phase and recruiting progenitor cells to aid healing. We have previously shown that modified titanium (Ti) disks directly induce M2 macrophage polarization. The aim of this study was to examine macrophage response to commercially available Ti or Ti alloy implants with comparable roughness and varying hydrophilicity.

Material and Methods: Eleven commercially available Ti (A-F) or Ti alloy (G-K) dental implants were examined in this study. Surface topography, chemistry, and hydrophilicity were characterized for each implant. To compare the immune response in vitro, human monocyte-derived macrophages were seeded on implants and secreted pro- and anti-inflammatory proteins measured. To evaluate the inflammatory response in vivo, mice were subcutaneously instrumented with clinical implants, and implant adherent macrophage populations were characterized by flow cytometry.

Results: Macrophages on hydrophobic Implant C produced the highest level of pro-inflammatory proteins in vitro. In contrast, hydrophilic Implant E produced the second-highest pro-inflammatory response. Implants F and K, both hydrophilics, produced the highest anti-inflammatory protein secretions. Likewise, pro-inflammatory CD80hi macrophages predominated in vivo on implants C and E, and M2 CD206 + macrophages predominated on implants F and K.

Conclusions: These findings show that hydrophilicity alone is insufficient to predict the anti-inflammatory effect on macrophage polarization and that other properties—surface composition or topography—determine immune modulation. This in vivo model may be a useful screening method to compare the immunomodulatory response to clinical implants of disparate geometry or size.

KEYWORDS

hydrophilicity, inflammation, macrophages, surface chemistry

1 | INTRODUCTION

Dental implants, one of the most common bone-dwelling biomaterials, are a highly reliable treatment for patients with missing one or more teeth. While early implant materials were chosen for their biological inertness, newer dental implants are now designed for bioactivity, inducing formation of bone by promoting differentiation of mesenchymal stem cells (MSCs) into osteoblasts (Lotz et al., 2017; Olivares-Navarrete et al., 2010). Such bioactive responses are driven not only by the proper selection of materials, such as titanium (Ti) or titanium alloys (Ti+), but also by modification of implant surfaces, such as increased surface roughness or changes in surface chemistry and energy (Donos, Hamlet, et al., 2011; Hamlet et al., 2012; Meirelles et al., 2008). These modifications are produced by methods such as sandblasting, acid etching, surface coating, plasma etching, anodization, and by varying the environmental conditions used during any of these procedures (Fu et al., 2017; Guehennec et al., 2007; Lopez-Lopez et al., 2012; Sul et al., 2002; Velasco-Ortega et al., 2019; Wennerberg et al., 2018). Ultimately, these modified surfaces have been found clinically to increase the rate of healing and success of dental implants (Bruyn et al., 2017; R et al., 2016). However, due to the wide range of materials and processing methods used to produce these surface qualities, small, but not insignificant, differences may arise between resulting surfaces, despite their common topographical features or hydrophilicity.

The final desired outcome of all bone-implanted biomaterial is the de novo bone formation connecting the implant to the host bone, termed osseointegration, and the majority of the efforts to develop and test new dental implant modifications rely on tests using osteoblasts or MSCs to predict the implant outcome (Bressel et al., 2017; Fu et al., 2009; Jiang et al., 2015; Lotz et al., 2017; Zanicotti et al., 2018). While these types of tests are important to understand the behavior of bone-forming cells in response to biomaterial modifications, they ignore the contribution of the other cellular and biological processes that occur in the peri-implant environment in the 14–21 days before bone formation begins that also affect osseointegration and ultimately dictate the fate of the implanted biomaterial (Biguetti et al., 2018; Vlahović et al., 2017). Interestingly, a vast number of reports have shown that increased surface roughness induced differentiation of MSCs and osteoblastic cells (Boyan et al., 2016b; Jung et al., 2018). These reports support the clinical outcomes where high success of osseointegration (~95%) is obtained implants with roughened surfaces, independently of implant composition, topography, or wettability (Annunziata & Guida, 2015; Bruyn et al., 2017). However, the speed of bone formation and implant loading has been shown to be affected by chemical changes in the surface oxide layer on dental implants (Romero-Ruiz et al., 2019; Sartoretto et al., 2015). In particular, modifications increasing the wettability or hydrophilicity have been shown to decrease healing times and faster bone formation in animal and clinical studies (Lang et al., 2011; Pinotti et al., 2018; Trisi et al., 2017).

Implantation of bone-dwelling biomaterials generates an immune response predominated by innate immune cells such as neutrophils and macrophages, which arrive in response to release of damage-associated molecular patterns from the injury to the implant site and to the material itself (Trindade et al., 2018). Our group has shown that macrophages are particularly important to this response, ultimately driving the conclusion of the inflammatory phase and recruiting mesenchymal stem cells (MSCs) to begin the reparative phase or recruiting other inflammatory cells to delay the healing response (Hotchkiss et al., 2016, 2018). Moreover, we have shown that macrophage ablation results in persistent pro-inflammatory phase, dramatically decreasing MSC recruitment to the implantation site (Hotchkiss et al., 2018). Macrophages are also implicated in undesirable implant outcomes, spurring chronic inflammation and fibrotic responses. To mediate a desirable transition from inflammation to healing, macrophage populations at an injury or implantation site shift from a pro-inflammatory (M1) phenotype into an anti-inflammatory (M2) phenotype. We and others have demonstrated that macrophage activation in response to biomaterial surface modifications in vitro is predictive of macrophage activation, inflammation resolution, and MSC recruitment in vivo (Donos et al., 2011; Hamlet et al., 2019; Hotchkiss et al., 2016; Hotchkiss et al., 2017; Hotchkiss et al., 2018; Wang et al., 2019a).

Inflammatory cells are affected by implant composition, design, and surface characteristics, but it has been difficult to predict the outcome of current implants due to the lack of access to clinical samples suited for in vitro studies. Furthermore, it is unclear whether implants with moderate to rough topographies or hydrophilicity modulate immune cell phenotype in the same way. The objective of this study was to elucidate the immunomodulatory effects of a battery of titanium and titanium alloy implants with surface modifications in vitro and in vivo in their commercially available form.

2 | MATERIALS AND METHODS

2.1 | Clinical implants

Eleven commercially available dental fixtures were compared in this study (Table 1). Of these, six were titanium [Implant A (Osseotite®, Biomet 3i™), Implant B (OsseoSpeed™, Astra-Tech™), Implant C (TiUnite™, Nobel-Biocare®), Implant D (Ti SLA®, Institut Straumann AG®), Implant E (CM Alvim, Neodent®), and Implant *F* (Ti SLActive®, Institut Straumann AG®) and 5 were titanium alloy [Implant G (MDI, 3M ESPE™ [titanium-aluminum-vanadium]), Implant H (Laser-Lok 3.0, BioHorizons® [titanium-aluminum-vanadium]), Implant I (Roxolid SLA®, Institut Straumann AG® [titanium-zirconium]), Implant J (Facility, Neodent® [titanium–aluminum–vanadium]), and Implant K (Roxolid SLActive®, Institut Straumann AG® [titanium-zirconium]). Of these implants, E, F, J, and K were reported to be hydrophilic.

2.2 | Material characterization

Surface topography was visualized using scanning electron microscopy (*SEM*, Zeiss Auriga, Carl Zeiss, Jena, Germany) using 5kV and a

2mm working distance. Differences were assessed at 500x magnification. Surface roughness was quantified using confocal microscopy (μsurf explorer, NanoFocus AG, Germany) using a 20x objective with a total measurement area of 798 μm × 798 μm. The arithmetical mean height of the scale limited surface (Sa) was calculated using a moving average Gaussian filter with a cut-off wavelength of 30 μm. In the case of very steep slopes due to the macro design of the implants, the measurement area was reduced while keeping the shorter side of the rectangular analysis area always larger than 10 times the cut-off wavelength. Mean surface roughness was measured at six different areas of three separate implants from each group.

Surface hydrophilicity was evaluated by sessile drop contact angle goniometry using a Rame-Hart goniometer 250 (Model 100-25a, Rame-Hart Instrument Co). 0.5 μl of deionized water was dropped on each implant, and the contact angle was measured. A contact angle of 0° was considered hydrophilic. Contact angle measurements were performed on the most cervical part of the implant avoiding the thread area. Contact angle was measured in three separated implants.

Oxide layer composition was determined by X-ray photoelectron spectroscopy (XPS). XPS analysis was performed using a PhI5000 VersaProbe spectrometer (ULVAC-PHI, Inc.) equipped with a 180° spherical capacitor energy analyzer and a multi-channel detection system with 16 channels. Spectra were acquired at a base pressure of 1 10–7 Pa using a focused scanning monochromatic Al-Ka source (1,486.6 eV) with a spot size of 200 μm. The instrument was run in the FAT analyzer mode. Pass energy used for survey scans was 187.85 eV and 46.95 eV for detail spectra. Data were analyzed using the program CasaXPS. The signals were integrated following Shirley background subtraction. Sensitivity factors were calculated using published ionization cross-sections, (Scofield, 1976) corrected for attenuation, transmission function of the instrument and source to analyzer angle. Three separated areas from each implant were used for analysis.

2.3 | Cell culture

Human macrophages were derived from purchased human peripheral blood monocytes (STEMCELL Technologies, Vancouver, BC, Canada) and cultured in RPMI 1,640 (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 50U/mL penicillin-50 μg/ml streptomycin (Thermo Fisher Scientific), and 50 ng/ml macrophage colony-stimulating factor (M-CSF, BioLegend, San Diego, CA) to generate macrophages. Fresh media supplemented with M-CSF was added after four days. After seven days of exposure to M-CSF, macrophages were passaged with Accutase (Innovative Cell Technologies) for experiments.

2.4 | Cell seeding

Human macrophages (200,000 cells/cm^2) were plated on implants as previously described (Hotchkiss et al., 2019). Briefly, macrophages were plated onto implant surfaces in 30 μl droplets spaced out over the implant surface to increase cell distribution and ensure direct interaction with the surface. Macrophages were incubated for 2 hr at 37°C to facilitate cell attachment before the implants were placed into custom culture vials containing 2 ml of full media to fully submerge the implant. Macrophages were cultured for 48 hr at 37°C, 5% CO_2, and 100% humidity.

2.5 | Protein Analysis

Secreted pro-inflammatory (IL-1B, IL-6, IL-12(p40), IL-17A, IL-23, TNF-a, CXCL-10) and anti-inflammatory (IL-1RA, IL-4, IL-10, CCL-17, Arginase) cytokines and chemokines were measured in the conditioned media using a custom human multiplex-ELISA panel LEGENDplex (BioLegend). Secreted protein levels were normalized to DNA content of implant adherent cells using a QuantiFluor® dsDNA System (Promega).

2.6 | In vivo implantation

Clinical implants ($n = 6$) were placed on 10–12 week old C57Bl/6 mice, one implant per animal, under the approval of the Virginia Commonwealth University Institutional Animal Care and Use Committee (Protocol: AD10001108). All experiments were carried out in accordance with approved procedures and reported according to ARRIVE guidelines. Mice were anesthetized by inhalation of 5% isoflurane gas in O_2. Legs were prepared by shaving and cleaned with isopropanol and chlorhexidine. While under anesthesia, an incision was made above the right knee, the right aspect of the inguinal fat pad overlaying the proximal quadriceps retracted, and the implant placed in the newly created space. The surgical site was closed using wound clips, and animals were administered 1mg/kg buprenorphine SR LAB subcutaneously to relieve post-operative pain. Animals were monitored for initial ambulation and then daily following the procedure. Mice were single housed, subjected to 12h light–dark cycle, with ad libitum access to food and water. On post-operative day 3, animals were euthanized by CO_2 asphyxiation and implants were retrieved.

2.7 | Flow cytometry

Characterization of macrophage populations on implants in vivo was performed by flow cytometric analysis. Following harvest, implants were immersed in Accutase (Innovative Cell Technologies) for 10 min at 37°C, followed by pulse vortexing at low speed to lift cells. The single cell suspension was then quenched by addition of cold staining buffer (1% bovine serum albumin in PBS, sterile filtered). Cells were then pelleted by centrifugation at 300*g*, washed with cold staining buffer, and incubated with anti-CD16/32 (BioLegend) for 30 min at room temperature to block Fc receptors. Next, cells were stained with antibody cocktails for M1

(CD45-Brilliant Violet 785, CD11b-Pacific Blue, CD68-Alexafluor 488, CD86-PE) or M2 (CD45-Brilliant Violet 785, CD11b-Pacific Blue, CD68-Alexafluor 488, CD206-APC) macrophage markers (BioLegend).

2.8 | Statistical analysis

In vitro experiments were conducted with a sample size of 6 for each implant. In vivo experiments were conducted with 6 animals per group, with pooling of cells from each group for flow cytometry. Prism GraphPad V7 software was used for statistical analysis. Data were first subjected to Shapiro–Wilk normality test. Results from this test indicate that the data were normally distributed. A one-factor, equal analysis of variance (ANOVA) was used to test the null hypothesis that group means were equal at a significance level of $\alpha = 0.05$, with post-hoc TUKEY-HSD for multiple comparisons. All experiments were repeated at least twice to confirm results.

3 | RESULTS

3.1 | Physical and chemical surface properties

Surfaces appeared visibly rough to varying degrees under *SEM* (Figure 1a). Despite having similar Sa values, the topographies were qualitatively different; for example, implants J and K had Sa values of 0.88 ± 0.13μm and 0.80 ± 0.08μm, respectively, but had visibly different topography (Figure 1b, Table 2). Contact angle demonstrated the hydrophilicity of implants E, F, J, and K (0°) and revealed varying degrees of hydrophilicity among the non-wettable implants (Table 2). Implant H had a contact angle of 62°, while the rest ranged from 93° to 108° (Figure 1b). XPS showed clear differences in oxide layer content, with surface carbon comparably low in hydrophilic implants (~20at %) and higher in the hydrophobic implants (>25at %).

3.2 | Inflammatory cytokine production

Pro-inflammatory cytokines and chemokines (IL-1B, IL-6, IL-12(p40), IL-17A, IL-23, TNF-a, CXCL-10) were produced in the greatest quantities by human monocyte-derived macrophages cultured on implants C, E, and G and were produced in the lowest quantities on implants F and K. Likewise, macrophages on implants F and K produced the highest levels of anti-inflammatory (IL-1RA, IL-4, IL-10, CCL-17, Arginase) markers. Interestingly, while pro-inflammatory protein production varied greatly between implants, anti-inflammatory protein production was more similar between implants, outside of F and K. While implants E, F, J, and K all have hydrophilic surfaces, they did not have a comparable effect on inflammatory polarization relative to non-hydrophilic implants. These findings suggest that implants F and K not only serve as a less pro-inflammatory signal but also induce an M2 phenotype in human macrophages, and that this effect is not due to the surface properties of hydrophilicity or roughness alone.

3.3 | Inflammatory polarization in vivo

To compare the effect of the implants in vivo, mice underwent implantation in the right side of the inguinal fat pad. This location was chosen because the clinical implants were too large to fix into bone in a murine model. This region was chosen because, like bone, it is connective tissue, and it was large enough to encase the entire implant. The inguinal fat pad is also not superficial or in close proximity to any internal organs, which might put the animal at risk for organ injury or dehiscence of the surgical site, as some of the implants have rough, sharp threads. Flow cytometric analysis of cells adhered to the surface of these implants after 3 days demonstrated that pro-inflammatory macrophages were significantly more predominant on implants B, C, E, and J, as evidenced by higher proportions of CD45+/CD11B+/CD68+/CD86hi cells. Conversely, implants D, F, G, and H had the lowest proportions of these pro-inflammatory macrophages. However, similar to the protein data, implants F and K had

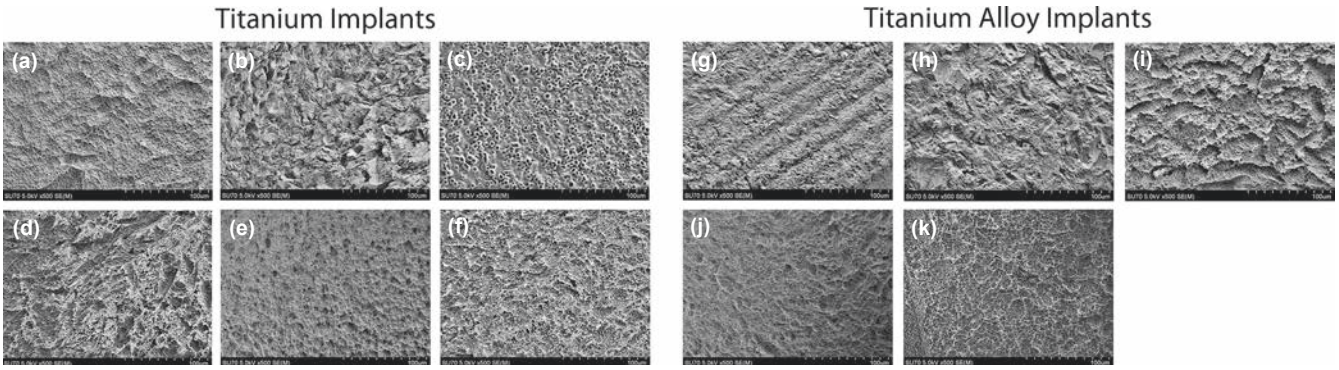

FIGURE 1 Comparison in implant surface characteristics. Qualitative *SEM* analysis at 500x from the different titanium and titanium alloy implants

TABLE 1 Descriptive information of the clinical implants used in this study

Code	Name	Manufacturer	Size	Diameter	Material	Surface Type	Lot
A	Osseotite®	Biomet 3i™	11.5 mm	4 mm	Titanium		2,015,101,457
B	OsseoSpeed™	Astra-Tech™	11 mm	4 mm	Titanium		181,886
C	TiUnite™	Nobel-Biocare®	11.5 mm	4.3 mm	Titanium		13,024,816
D	SLA®	Institut Straumann AG®	12 mm	4.1 mm	Titanium		LT534
E	Alvim CM Acqua	Neodent®	10 mm	3.5 mm	Titanium	Hydrophilic	122,094
F	SLActive®	Institut Straumann AG®	12 mm	4.1 mm	Titanium	Hydrophilic	GJ449
G	MDI	3M ESPE™	10 mm	2.4 mm	Ti alloy		4,417
H	Laser-Lok 3.0	BioHorizons®	10.5 mm	3.0 mm	Ti alloy		1,703,097
I	Roxolid SLA®	Institut Straumann AG®	10 mm	2.9 mm	Ti alloy		NX506
J	Facility Acqua	Neodent®	10 mm	2.9 mm	Ti alloy	Hydrophilic	116,912
K	Roxolid SLActive®	Institut Straumann AG®	10 mm	2.9 mm	Ti alloy	Hydrophilic	PH599

TABLE 2 Assessment of surface roughness by laser confocal microscopy and wettability by contact angle presented in mean ± standard deviation

Implant	Sa	CA
A	0.67 ± 0.07 μm	102 ± 5.1°
B	1.41 ± 0.02 μm	108 ± 3.5°
C	1.35 ± 0.24 μm	98 ± 2.3°
D	1.27 ± 0.05 μm	96 ± 3.3°
E	1.29 ± 0.09 μm	0°
F	1.22 ± 0.07 μm	0°
G	0.35 ± 0.02 μm	93 ± 1.9°
H	1.13 ± 0.12 μm	62 ± 2.7°
I	0.88 ± 0.11 μm	103 ± 4.3°
J	0.88 ± 0.13 μm	0°
K	0.80 ± 0.08 μm	0°

higher proportions of M2 CD45+/CD11B+/CD68+/CD206 + macrophages. Again, no specific effect of hydrophilicity was observed.

4 | DISCUSSION

Dental implants are one of the most successful biomaterials in clinical use. Typically, the success of these implants is measured by integration of the implant to the adjacent bone without formation of fibrous tissue over a period of time usually measured in years. While the direct contact between the implant and the adjacent bone at 1, 5, or 10 years is the measurement of success, osseointegration can take from weeks to months and is affected dramatically by the inflammatory process. Here, we show the in vitro macrophage inflammatory response to different commercial clinical implants of varying material composition, surface roughness, chemistry, and wettability. Furthermore, we performed in vivo implantation in a mouse model to elucidate the macrophage inflammatory response. The results of this study highlight that the initial inflammatory cellular response differs between most of the clinical implants analyzed. We found that changes in surface roughness, chemistry, and wettability play a significant role in the activation of macrophages and production of inflammatory microenvironment. Additionally, we found that the cellular response observed in our in vitro experiments is similar to the in vivo response to implanted biomaterials, suggesting this method can be used to rapidly screen inflammatory response to dental implants using the clinical materials instead of surrogate implants designed for murine models.

Implant placement generates an inflammatory response characterized by the infiltration of innate immune cells. These inflammatory cells infiltrate the injury site and interact both with the damaged tissue and the implant (Hotchkiss et al., 2018). Macrophages are one of the primary innate effector cells that determine whether the inflammatory response will be resolved or prolonged as chronic inflammatory response, depending on the molecular cues from the injury site and the interaction of cells with the biomaterial (Hotchkiss et al., 2018; Wang, Zhang, et al., 2019). Traditionally macrophages have been classified by the inflammatory microenvironment that they produce. Macrophages with a pro-inflammatory phenotype secrete pro-inflammatory cytokines and chemokines (e.g., IL-1β, IL6, IL-12, IL17A, TNFα). This phenotype propagates the inflammatory response and causes additional inflammatory cells to be recruited to the injury site (Ogle et al., 2016; Shapouri-Moghaddam et al., 2018). M2 macrophages produce cytokines associated with inflammation resolution (e.g., IL-4 and IL-10) and are able to recruit progenitor and stem cells to the site of injury for new tissue formation (Kim & Nair, 2019; Zhang et al., 2018).

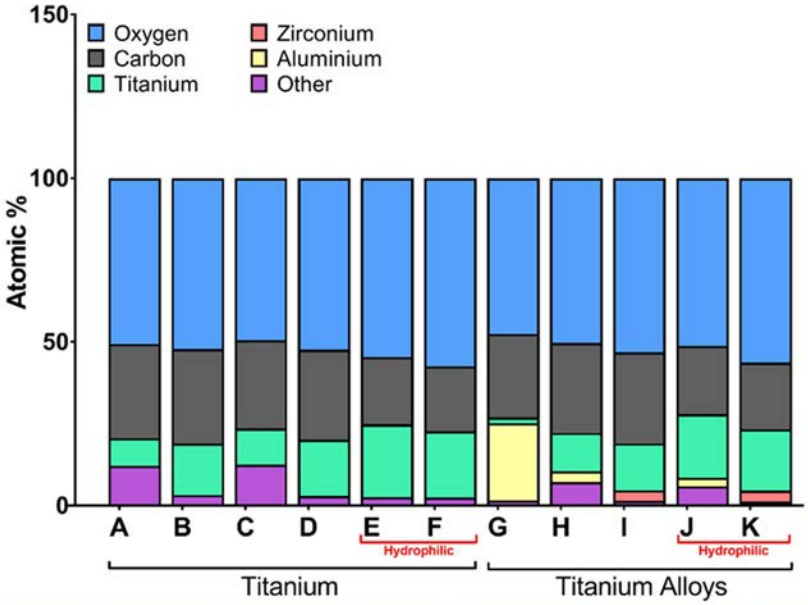

FIGURE 2 XPS analysis of titanium and titanium alloy implants showing atomic percentages of oxygen, carbon, titanium, zirconium, and aluminum on implant surface layer

Implant	Oxygen	Carbon	Titanium	Zirconium	Aluminium	Other
A	50.7	28.7	8.4	0	0	12.2
B	52.3	28.8	15.7	0	0	3.2
C	49.5	26.9	11.1	0	0	12.5
D	52.4	27.5	17.2	0	0	2.9
E	54.6	20.6	22.3	0	0	2.5
F	57.5	19.8	20.3	0	0	2.4
G	47.6	25.5	1.8	0	23.5	1.6
H	50.4	27.3	11.8	0	3.3	7.2
I	53.2	27.8	14.4	3.1	0	1.5
J	51.3	20.8	19.4	0	2.6	5.9
K	56.3	20.4	18.8	3.3	0	1.2

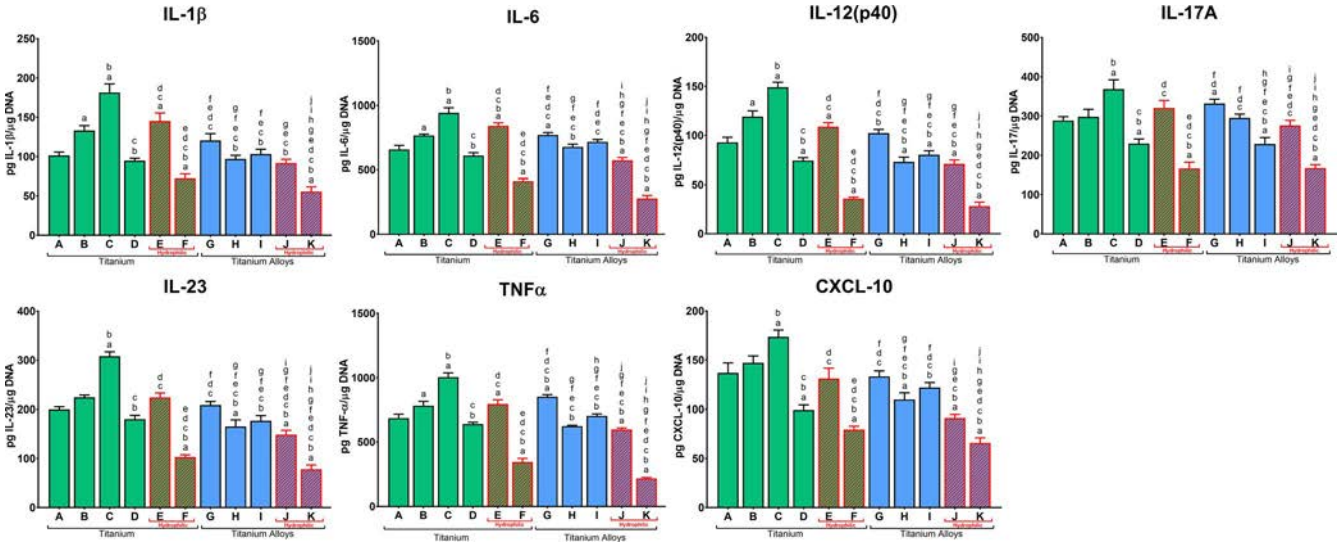

FIGURE 3 Pro-inflammatory protein secretion from primary macrophages cultured on implants for 48 hr. $p < .05$: a versus. implant A, b versus. implant B, c versus. implant C, d versus. implant D, e versus. implant E, f versus. implant F, g versus. implant G, h versus. implant H, i versus. implant I, j versus. implant J

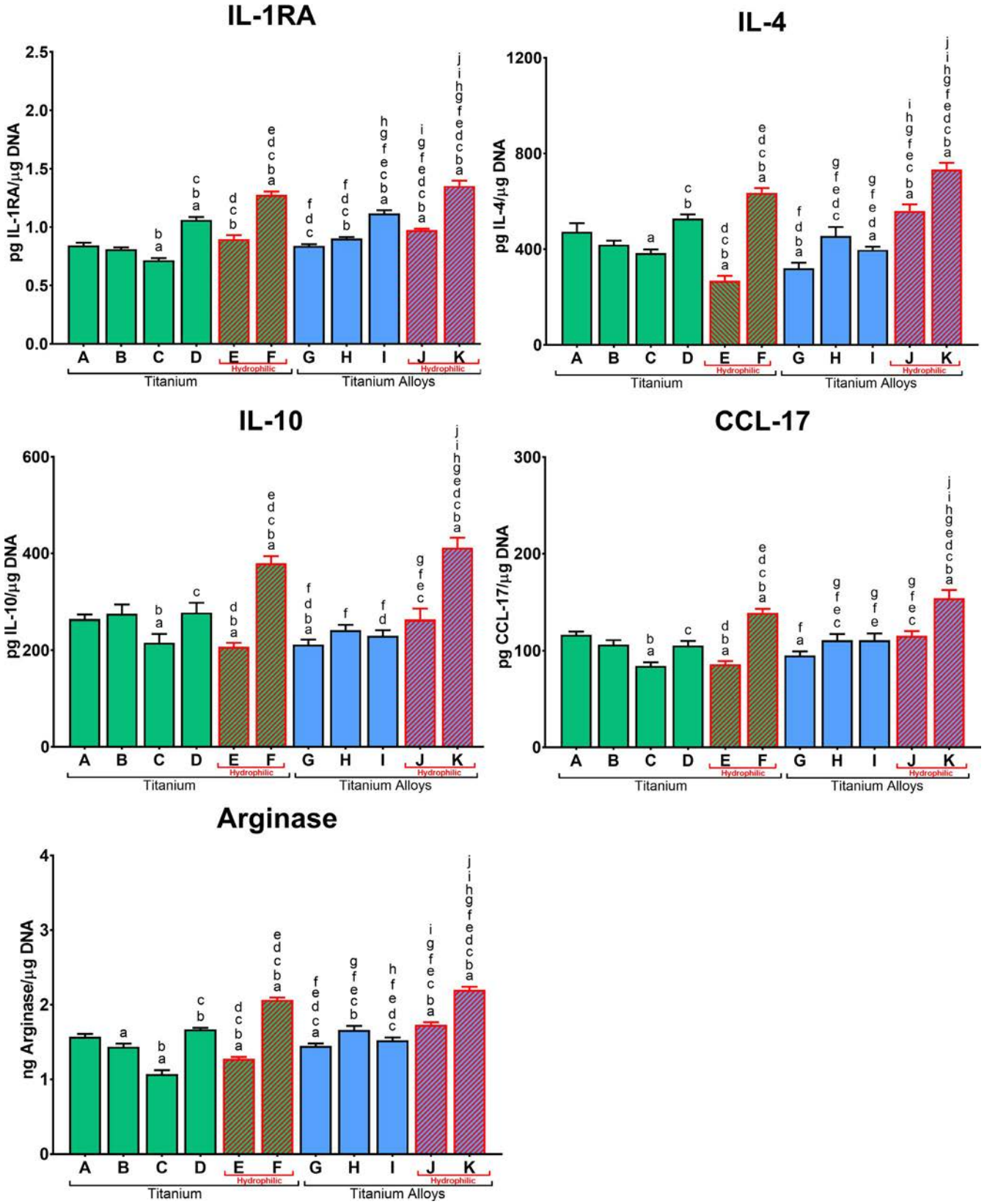

FIGURE 4 Anti-inflammatory protein secretion from primary macrophages cultured on implants for 48 hr. $p < .05$: a versus. implant A, b versus. implant B, c versus. implant C, d versus. implant D, e versus. implant E, f versus. implant F, g versus. implant G, h versus. implant H, i versus. implant I, j versus. implant J

There are currently a myriad of dental implants commercially available to clinicians who must decide which type of implant is the most appropriate for the therapeutic treatment based on the clinical parameters of the patient. Unfortunately, this selection is more complicated than expected since most clinical implants have some type of modification to the physicochemical implant surface properties (e.g., sand blasting, acid etching, anodization, surface coating, laser ablation, surface deposition) (Asensio and Rojo, 2019; Doornewaard et al., 2017; Wennerberg et al., 2018). These modifications increase the surface roughness, and in some cases also affect the wettability or hydrophilicity of the implants, parameters that have been shown to contribute in the osseointegration process (Rupp et al., 2018; Tallarico et al., 2019).

Historically, dental implant design has focused on surface modifications that increase osteoblastic differentiation and bone apposition (Boyan et al., 2016a; Mastrangelo et al., 2020). We have generated a rich body of research about specific parameters of dental implants that increase osteoblastic differentiation and bone to implant contact (Albrektsson & Wennerberg, 2019; Rupp et al., 2018). Increased surface roughness results in more bone formation, higher bone-to-implant contact, and increased osteoblastic differentiation of progenitor cells (Gittens et al., 2014). In our study, the 11 commercial implants analyzed had a wide range of average surface roughness (Sa = 0.67 to 1.41 μm), resulting from the physiochemical modifications used to produce the implant surface and from the bulk material used. While surface roughness has been shown to be a key factor in osteogenic differentiation, our results showed that macrophages are less sensitive to changes in surface roughness (Hotchkiss et al., 2016, 2019). Macrophages increased production of pro-inflammatory cytokines on implants with the lowest surface roughness analyzed (implant A [Sa 0.67 μm]) to a similar degree as macrophages grown on implants with the highest surface roughness (implant B [Sa 1.41 μm]).

We have previously reported that macrophages favor a pro-inflammatory phenotype when grown on hydrophobic titanium surfaces (smooth or rough) in comparison with hydrophilic surfaces in vitro (Hotchkiss et al., 2016, 2017) and in vivo (Abaricia et al., 2020; Hotchkiss et al., 2018). In the present study, we found that macrophages produced a more pro-inflammatory microenvironment on all hydrophobic implants and two hydrophilic implants. The only two implants with a marked decrease in pro-inflammatory and increase in anti-inflammatory markers were implants F and K. These results disproved the idea that any hydrophilic surface modifications will induce macrophages to produce a microenvironment with higher anti-inflammatory and lower pro-inflammatory cytokine production. Interestingly, the 4 hydrophilic implants analyzed, have similar surface roughness levels and oxygen, carbon, and titanium composition. One possible explanation is the limitation in surface characterization when using 3D structures instead of 2D, flat surfaces. It has been reported previously that implants F and K have nanoscale features on their surface. It is unknown if the other implants tested in this study have nanostructures on their surface as result of their surface modification, but we have previously demonstrated that increased wettability, and not nanostructures, increases M2 macrophage activation (Hotchkiss et al., 2016).

Most methods used to characterize these metallic materials were originally conceived to analyze flat 2D surfaces since it is the most accurate way to obtain information from the outermost atomic layers. However, these techniques have been used widely to characterize all types of biomaterials with or without complex surface structures (Kingshott et al., 2011; Rupp et al., 2014).

In vitro studies are extremely useful when comparing different biomaterials since variables can be isolated and parameters controlled. In this work, we analyzed the macrophage activation in vivo in response to clinical implants. Our results showed that there is not a single implant surface parameter that determines the M1 or M2 activation of macrophages, and our results suggest that a combination of surface roughness/topography, chemical composition, and wettability are responsible for macrophage activation toward a M1 or M2 phenotype. In our results, macrophage activation in vivo perfectly correlates to our in vitro pro-and anti-inflammatory cytokine release. We also observed that all implants activate macrophages

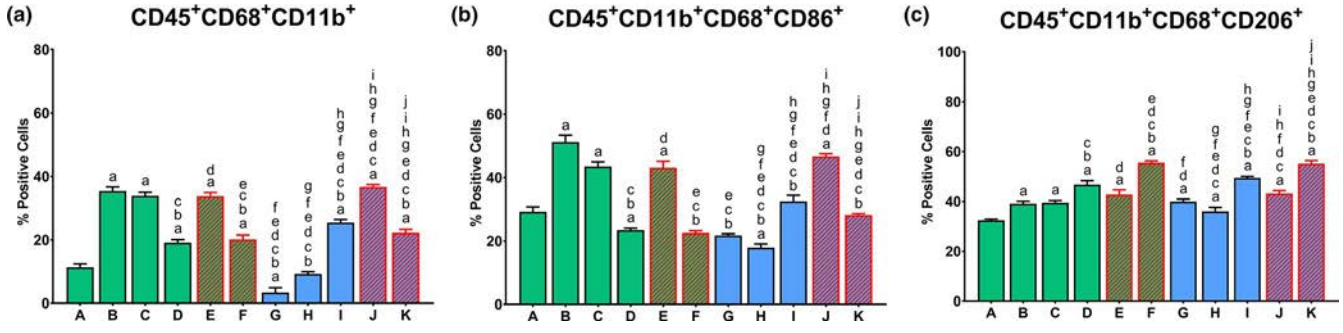

FIGURE 5 C57bl/6 mice were instrumented with titanium or titanium alloy implants. Implants were retrieved after 3 days post-implantation and immunophenotyping characterization from adherent cells was performed by flow cytometry to identify total macrophages (A, CD45 + CD68+CD11b+), M1 macrophages (B, CD45 + CD68+CD11b + CD80+), and M2 macrophages (C, CD45 + CD68+CD11b + CD206+). $p < .05$: $p < .05$: a versus. implant A, b versus. implant B, c versus. implant C, d versus. implant D, e versus. implant E, f versus. implant F, g versus. implant G, h versus. implant H, i versus. implant I, j versus. implant J

in vivo into both the phenotypes (M1 and M2), but the difference is the proportion of the two phenotypes. In this case, implants F and K were skewed to an M2 macrophage activation, and likewise had the highest anti-inflammatory cytokine production in our in vitro studies. One limitation of our in vivo study is that the implants were instrumented in soft tissue due to implant size instead of bone. We do not believe that macrophage activation and initial inflammatory response would be affected; however, it is possible that different tissue resident macrophages could be predetermine to activate to a more M2 phenotype as has been shown before (Burgess et al., 2019; SE et al., 2020).

5 | CONCLUSION

Our data suggest that while surface hydrophilicity, mean roughness, or chemical composition alter macrophage phenotype, any of these alone are insufficient to predict M1 or M2 modulation of macrophage phenotype. Moreover, hydrophilicity/wettability alone does not determine M2 macrophage phenotype. These parameters work in concert to direct macrophage activation and alter macrophage phenotype. This study demonstrates that placement of bone-dwelling implants in murine inguinal soft tissue produces similar early inflammatory outcomes in recruited macrophages as in vitro studies, suggesting this in vivo model may be a useful screening method to compare the immunomodulatory response to clinical implants of disparate geometry or size.

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AUTHOR CONTRIBUTION

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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