SELECTIVE ADSORPTION OF IGG TO DEPROTEINIZED BOVINE BONE MINERAL ENHANCES IL-10 EXPRESSION IN HUMAN PRIMARY MACROPHAGES

Ludovica Parisi[1,2,*], Massimiliano G. Bianchi[2,*], Beatrice Mozzoni[1,2,*], Benedetta Ghezzi[1,2], Martina Chiu[2], Federico Rivara[1,2], Andrea Toffoli[1,2], Ovidio Bussolati[2], Guido M. Macaluso[1,2]

[1] *Centro Universitario di Odontoiatria, Università degli Studi di Parma, Parma, IT*
[2] *Dipartimento di Medicina e Chirurgia, Università degli Studi di Parma, Parma, IT*

[*] Equally contribution of the Authors

Riassunto

L'osso bovino deproteinizzato (DBBM) è il sostituto osseo più comunemente utilizzato negli innesti ossei. Studi recenti hanno descritto la capacità del DBBM di influenzare il sistema immunitario dell'ospite inducendo i macrofagi a differenziare in senso antinfiammatorio (M2). Dopo aver confermato la capacità del DBBM di indurre il fenotipo macrofagico M2, l'obiettivo di questo studio è stato quello di identificarne un potenziale meccanismo regolatore. Abbiamo ipotizzato che la corona proteica che si forma attorno ai granuli di DBBM dopo il loro contatto con il sangue possa svolgere un ruolo importante nell'attivazione macrofagica. I risultati ottenuti con SDS-PAGE e Western Blot hanno evidenziato una selettività del DBBM per le immunoglobuline G (IgG). Pertanto, abbiamo analizzato la polarizzazione di macrofagi primari umani coltivati a contatto con il DBBM in presenza o in assenza di IgG. I risultati di espressione genica (RT-PCR) hanno rivelato un'attivazione macrofagica più marcata sul DBBM e indirizzata al fenotipo M2. Inoltre, l'induzione della citochina antinfiammatoria IL-10 è risultata significativamente più elevata di quella delle citochine pro-infiammatorie IL-8 (p=0.00116) e TNFα (p=0.0091). I risultati di questo studio indicano chiaramente che l'adsorbimento preferenziale di IgG al DBBM stimola i macrofagi a produrre IL-10 che, a sua volta, è plausibilmente implicata nella formazione di un microambiente favorevole all'osteogenesi.

Abstract

Deproteinized bovine bone mineral (DBBM) is the gold standard xenograft used in bone augmentation procedures. Recent findings have demonstrated that DBBM exerts and impact on host immune response by inducing macrophage polarization towards an alternative activation (M2). In this study, we confirmed the capacity of DBBM to induce the M2-phenotype and further identified a potential controlling mechanism. We hypothesized that the protein-corona formed around DBBM granules after their insertion plays an important role in the control of macrophage polarization. SDS-PAGE and Western Blot results identified a selectivity of DBBM for immunoglobulins-G (IgG). Therefore, we analyzed the polarization of primary human macrophages, derived from peripheral blood, on DBBM granules in the presence or in the absence of IgG. RT-PCR results demonstrated a higher activation of macrophages on DBBM and a tendency to shift their phenotype toward the M2 polarization. Furthermore, we detected a more marked induction of the anti-inflammatory interleukin IL-10 if compared to the pro-inflammatory IL-8 (p=0.0116) and TNFα (p=0.0091) expression. The results from this study indicate that the selective adsorption of IgG to DBBM preferentially stimulates the production of the anti-inflammatory IL-10 in human macrophages, which could be potentially involved in the creation of a microenvironment suitable for osteogenesis.

Introduction

Over the last decades, bone grafts have been widely used for bone augmentation procedures and the management of periodontal bone defects[1-3]. Among them,

deproteinized bovine bone mineral (DBBM), which is basically hydroxyapatite obtained with the removal of any organic components from bone of bovine origin, is the gold standard xenograft. Even though DBBM lacks any intrinsic osteoinductive potential, large preclinical studies have confirmed its osteogenic and osteoconductive capacities, which prominently enhance the speed and the quality of new bone formation[4, 5]. Interestingly, previous studies have also convincingly demonstrated the capacity of DBBM to induce the formation of large multinucleated giant cells (MNGCs) *in vivo*[6, 7]. This evidence, together with the proof that cells from the monocytic lineage are among the first to come in contact with biomaterials[8, 9], suggests a potential important role of the host innate immunity in bone regeneration.
Macrophages are phagocytic cells involved in innate immunity that circulate in blood or reside in specialized tissues contributing in the maintenance of local homeostasis[10]. They are typically characterized by their plasticity in response to the exposure to different milieu signals, which leads to the switch of their metabolic and functional properties into a killing/inhibitory capacity (M1) or into a heal/promoting setting (M2)[11, 12]. Therefore, it is convincible that the role of these cells in bone tissue is highly influenced by the implantation of a foreign biomaterial, such as DBBM[13]. In this regard, *in vitro* studies on murine models have convincingly shown that osteoblasts differentiation on DBBM is not a direct consequence of the material contact[14] but, rather, an effect of macrophage polarization towards an M2 phenotype[15]. However, the mechanism that controls macrophage phenotype upon interaction with DBBM has not been investigated yet.
During the surgical procedure DBBM is often used in combination with patient's own blood. It follows that plasma proteins are quickly adsorbed on material surface shortly after its insertion, and the formation of this so-called protein-corona is the first and decisive step to define cell-DBBM interaction[16]. Protein adsorption to biomaterials is a complex process, and when DBBM is exposed to blood plasma, certain molecules are preferentially deposited from the bulk. The most abundant protein species present in the bulk solution are adsorbed firstly. Subsequently, molecules endowed with greater affinity for the substrate may induce the detaching of the previously adsorbed and less affine ones[17, 18]. Therefore, the characterization of blood plasma protein adsorption to DBBM is the first step to understand the mechanisms behind its clinical performance.
Immunoglobulins are among the most abundant proteins in blood (15-20mg/ml) and are capable to elicit a variety of cellular responses through their Fc portion. Immunoglobulins-G (IgG) represent the most abundant antibody class in blood (~12mg/ml) and are capable of binding various members of the Fcγ receptor family (FcγRs), which are expressed on several immune cells[19, 20]. Human macrophages express FcγRI and, interestingly, IgG4 binding to this receptor has been described to trigger the M2-like phenotype[21].
Considering these premises, the aim of this work was to study the adsorption pattern of blood plasma protein to DBBM and, therefore, to determine potential mechanisms triggered by IgG in inducing the activation of macrophages towards M2-like phenotype.

Materials and Methods

This study has been conducted using BioOss® granules kindly provided by Geistlich Pharma AG (Wolhusen, CH) and primary macrophages isolated from whole human blood according to the approval by the local ethic committee (#3182/2018).

Protein adsorption studies

The adsorption pattern of blood plasma proteins to DBBM has been investigated by soaking DBBM granules (range size 0.25-1mm) in 2% human serum. The amount of protein adsorption has been monitored through Bradford assay, while the composition of

the protein-corona has been investigated through SDS-PAGE and Western Blot (WB) analysis.

Bradford assay – Seventy-mg of DBBM were weighed and soaked for 1h at room temperature (RT) on an orbital shaker with 500µl of PBS (Thermo Fisher, Waltham, MA – USA) supplemented with 2% of IgG-depleted human serum (IHPLA-SER-GF, Innovative Research, Novi, IT) in the presence or in the absence of a 2%-diluted physiological concentration (0.3mg/ml) of human IgG (I4506, Sigma-Aldrich, Saint-Louis, MI – USA). Ten-µl aliquots of supernatants were collected after 5, 15, 30, 45 and 60min and used for protein quantitation. Bradford assay (BIO-RAD Protein Assay, BIO-RAD, Hercules, CA – USA) was performed mixing serum aliquots with 200µl of Bradford Working Solution. Specimens were incubated at 37°C for 2min and their absorbance was assessed at 620nm with a Multiskan FC plate reader (Thermo Fisher).

SDS-PAGE – Seventy-mg of DBBM were weighed and soaked for 1h with 2% human serum in the presence or in the absence of IgG as described above. For total proteins recovering, samples were incubated with 100µl of Sample Buffer 1X (Tris-HCl 62.5mM pH 6.8, SDS 1,5%w/v, DTT 100mM and traces of Bromophenol Blue), cooled at -20°C overnight, sonicated for 10min and boiled at 95°C for 5min. Equal volumes of samples were thus loaded on a 12% polyacrylamide gel (Sigma-Aldrich) and separated at 110V for 1h and 30min. To reveal proteins, gel was exposed to the Silver Stain solution (Silver Stain solution kit, BIO-RAD) and images were acquired by Personal Densitometer SI (Molecular Dynamics, GE Healthcare, Little Chalfont, UK).

Western blot – After SDS-PAGE analysis, separated proteins were blotted on a PVDF membrane (Immobilon-P, Darmstadt, D) at 100V for 1h. Non-specific sites were blocked for 1h at RT in Tris-buffered saline (TBS, TrisHCl 50mM ph 7.5 and NaCl 150mM) containing 10% of blocking reagent (Roche S.p.A., Segrate, IT). Membrane was then incubated overnight with an anti-human HRP-conjugated IgG antibody (abcam, Cambridge, UK) diluted 1:10000 (Sigma-Aldrich). Immunoreactivity was visualized with enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP, Sigma-Aldrich).

Samples preparation

To simplify the model and to better understand the effects of IgG on macrophage polarization, an albumin solution at serum concentration supplemented or not with human IgG was used for DBBM coating.

DBBM coating – Human albumin solution (Alburex®, CSL Behring, Bern, CH) was diluted at 2% in PBS (0.8mg/ml) and supplemented or not with IgG (Privigen®, CSL Behring) at a final 2%-diluted physiological concentration (0.3mg/ml). The 2 different solutions were then used to coat 70mg of DBBM granules or the bottom of 48-well tissue culture plates, which were used as controls, for 1h at RT on an orbital shaker. A WB analysis (see above) was then performed to confirm albumin and IgG adsorption on granules. Albumin detection was performed using an anti-albumin (Cell Signalling) primary antibody diluted 1:800 in 0.1% Tween20 in TBS supplemented with 5%BSA and revealed with an HRP-conjugated secondary antibody diluted 1:10000 (Cell Signalling).

In vitro assays

The polarization of human macrophages obtained after selection and differentiation of human monocytes isolated from whole blood plasma has been studied through RT-PCR.

Blood samples – Ten 5ml specimens of whole human plasma were collected. Eligible volunteers were identified from the members of the research team of the School of Dental Medicine of the University of Parma, Italy. The presence of hematological, immunological or diabetic diseases was an exclusion criterium. All the subjects gave written informed consent (#3182/2018), and full medical history was recorded. Blood samples were

collected in the morning and immediately transferred to the laboratory for monocyte isolation that was completed within 8h.

Macrophage isolation and culture – Ficoll-Paque Plus solution (Sigma-Aldrich) was used to separate peripheral blood mononuclear cells (PBMCs), followed by two rinses in PBS for better removal of platelets. After having determined the cell number, PBMCs were resuspended in RPMI-1640 (EuroClone, Pero, IT) supplemented with 5% of human serum (Innovative Research), L-Glutammine 2mM (Thermo Fisher) and 1% PenStrep (Thermo Fisher) and plated on a 6-well plate. Thirty minutes after seeding non-adhering cells were gently removed and the culturing media was further supplemented with M-CSF 50ng/ml (BioTechne, Minneapolis, MN – USA) for macrophage differentiation. After 7 days, macrophages were recovered and seeded on DBBM at a final concentration of 300000cells/sample in complete RPMI-1640 supplemented with 2% human albumin (CLS Behring), L-Glutammine 2mM and 1% PenStrep.

RT-PCR analysis – Total RNA from human macrophages was extracted 48h after seeding by TRIzol (Thermo Fisher) and further purified using the GeneJET RNA purification kit (Thermo Fisher). Five hundred-mg of RNA were used as a template for cDNA synthesis using a RevertAid RT Reverse Transcription kit (Thermo Fisher). The expression of cluster differentiation related genes CD68 (M0), CD86 (M1) and CD36 (M2), as well as the expression of the inflammatory-related interleukin-8 (IL-8) and tumor necrosis factor alpha (TNFα), and of the anti-inflammatory IL-10 was detected by RT-PCR. The specific primer sets are outlined in **Table 1**. RT-PCR was performed in a total volume of 20µl with the Power UP SYBR Green Master Mix (Thermo Fisher) and the target gene expressions were evaluated with a StepOne Plus Real-Time PCR System (Applied Byosistem, Foster City, CA – USA). Data analysis was made according to the relative standard curve method. Expression data were reported as the ratio between each investigated mRNA and RPL15 mRNA.

Gene	Forward (5'-end)	Reverse (3'-end)
CD68	GGAAATGCCACGGTTCATCCA	TGGGGTTCAGTACAGAGATGC
CD86	ACAAAAAGCCACAGGAATG	CAGGGAATGAAACAGACAAGC
CD36	CTGTCATTGGTGCTGTCCTG	GCGTCCTGGGTTACATTTTC
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
IL-10	TCAAGGCGCATGTGAACTCC	GATGTCAAACTCACTCATGGCT
TNFa	ATGAGCACTGAAAGCATGATCC	GAGGGCTGATTAGAGAGAGGTC

Table 1: Primer sequence used for RT-PCR

Statistical analysis

Data were analyzed using Prism8 (GraphPad, La Jolla, CA – USA) and are reported as the mean ± SD of three repeated experiments performed in multiple replicates. Differences between groups were evaluated with either t-test or one-way ANOVA statistical test with Tukey multiple comparison post-hoc test and considered significant when $p<0.05$.

Results

DBBM protein-corona formation

A Bradford assay was exploited to study the time-course of blood plasma protein adsorption to DBBM. Protein adsorption followed a hyperbolic trend (R^2=0.9672) showing a massive adsorption within 15min without any marked difference between IgG-depleted and IgG-supplemented sera (**Fig.1a**).
When the protein-corona composition was qualitatively analyzed by SDS-PAGE and WB, the patterning of DBBM adsorbed proteins was similar to that of complete human serum (**Fig.1b** lane3 vs. lane5). However, the intensity of revealed bands showed that some proteins were better adsorbed than others. Interestingly, the band around 70kDa, which is putatively that of serum albumin, was only moderately adsorbed on DBBM, while the one around 50kDa (putatively containing IgG heavy chains) and the one around 23.5kDa (putatively containing of IgG light chains) were more evident in the DBBM sample (lane5). Since electrophoresis data can only be considered semi-quantitative, to further characterize protein adsorption to DBBM, we performed an immunoblot. IgG heavy chains were massively present in the pool of DBBM-adsorbed proteins (**Fig.1c**). Noteworthy, DBBM showed a greater affinity for the IgG heavy chain, while no adsorption of other Ig isoforms, such as IgA or IgM was detectable as in the complete serum counterpart. Additionally, although roughly equal amounts of proteins were adsorbed to DBBM (**Fig.1a**), no evident pattern change, besides IgG-containing bands, was visible when IgG-depleted medium was used (**Fig.1b**).

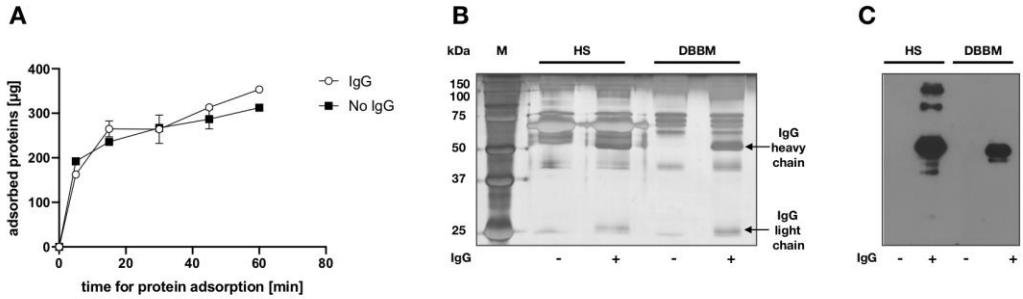

Figure 1: *Protein adsorption study*. (**A**) Time course of complete or IgG-depleted 2% human serum proteins to DBBM. (**B**) SDS-PAGE of complete or IgG-depleted 2% human serum proteins adsorbed for 1h to DBBM. (**C**) Western Blot analysis of IgG adsorbed to DBBM.

The affinity of DBBM for IgG was further confirmed when DBBM or controls were incubated with serum or albumin solution in the presence or in the absence of IgG. While DBBM evidently adsorbed IgG (**Fig.2b**), no IgG adsorption was detected for controls (bottom of culture wells). Moreover, controls showed a remarkable affinity for albumin, which, on the contrary, was less evidently adsorbed on DBBM (**Fig.2a**).

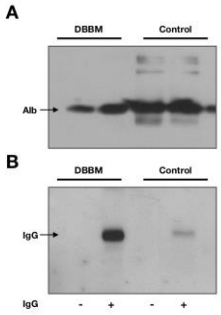

Figure 2: *Protein adsorption study*. (**A**) Western Blot analysis of albumin adsorbed to DBBM. (**B**) Western Blot analysis of IgG adsorbed to DBBM.

The effect of protein-corona on macrophage activation and differentiation

The study of macrophages activation was performed on DBBM and on controls previously exposed to a solution of albumin and IgG or of albumin at physiological concentrations.
The expression of the CD68 was markedly enhanced when IgG were adsorbed on DBBM (**Fig.3a** – Control vs. DBBM + IgG p=0.0016; Control + IgG vs. DBBM + IgG p=0.0014; DBBM vs. DBBM + IgG p=0.0029). Furthermore, when the fold-change IgG vs. No IgG was considered, a higher expression of the macrophage marker dependent on the presence of IgG was evident for DBBM if compared to the control (p=0.0130). Noteworthy, the expression of the pro-inflammatory marker CD86 was greatly diminished on DBBM both in the presence or in the absence of IgG, showing statistically significant differences with the control pre-adsorbed with IgG (**Fig.3b** – Control + IgG vs. DBBM p=0.0254; Control + IgG vs. DBBM + IgG p=0.0259), while no significant differences were assessed on DBBM in the presence or in the absence of IgG (p>0.9999). Consistently, the analysis of the fold-change showed lack of induction on DBBM even though the difference with control was not significant (p=0.0764). On the contrary, the analysis of the fold-change for the expression of the scavenger receptor CD36 showed a positive effect of DBBM against the control even though without significant differences (p=0.1445). The levels of CD36 expression were indeed comparable among all the samples and statistically significant differences were detected only between DBBM and controls in the absence of IgG (**Fig.3c** – Control vs. DBBM p=0.0434).

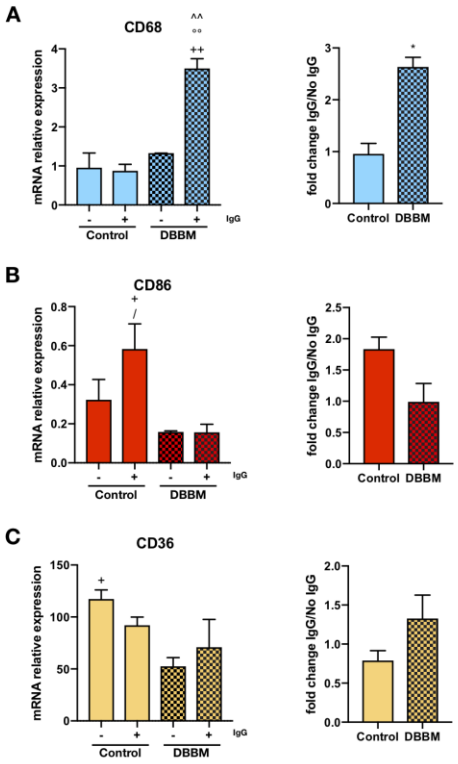

Figure 3: *CD expression analysis*. (**A**) Relative mRNA expression and IgG/No IgG fold change of CD68. (**B**) Relative mRNA expression and IgG/No IgG fold change of CD86. (**C**) Relative mRNA expression and IgG/No IgG fold change of CD36. ^=p<0.05 vs. Control + IgG; °=p<0.05 vs. Control + IgG; +=p<0.05 vs. DBBM; /=p<0.05 vs. DBBM + IgG; *=p<0.05 Control vs. DBBM.

The effect of protein-corona on cytokines expression

The influence of adsorbed proteins on macrophage polarization has been further investigated through the analysis of the expressed cytokines. Particularly, the transcription levels of IL-8, IL-10 and TNFα have been taken into consideration.

The expression of IL-8 was promoted on DBBM and further enhanced by the presence of IgG (**Fig.4a** – Control vs. DBBM p=0.0041; Control vs. DBBM + IgG p=0.0006; Control + IgG vs. DBBM p=0.0289; Control + IgG vs. DBBM + IgG p=0.0017; DBBM vs. DBBM + IgG p=0.0177). However, when the fold-change IgG vs. No IgG was considered, the production of IL-8 was more markedly enhanced by the antibodies on controls than on DBBM (p=0.0046), suggesting that the stimulatory effect of IL-8 production detected in macrophages exposed to DBBM is somewhat blunted by IgG. At variance with IL-8 expression, DBBM did not influence the expression of IL-10 (**Fig.4b** – Control vs. DBBM p=0.2934; Control + IgG vs. DBBM + IgG p=0.1815), which was instead markedly influenced by the presence of IgG (**Fig.4b** – Control vs. Control + IgG p=0.0178; Control + IgG vs. DBBM p=0.0248; DBBM vs. DBBM + IgG p=0.0055). The analysis of the fold-change indicated that IgG effect was significantly higher with DBBM than under control conditions (p=0.0121). Surprisingly, the expression of TNFα was comparable among all the groups and the fold-change did not reveal any significant difference (**Fig.4c** – p=0.1335).

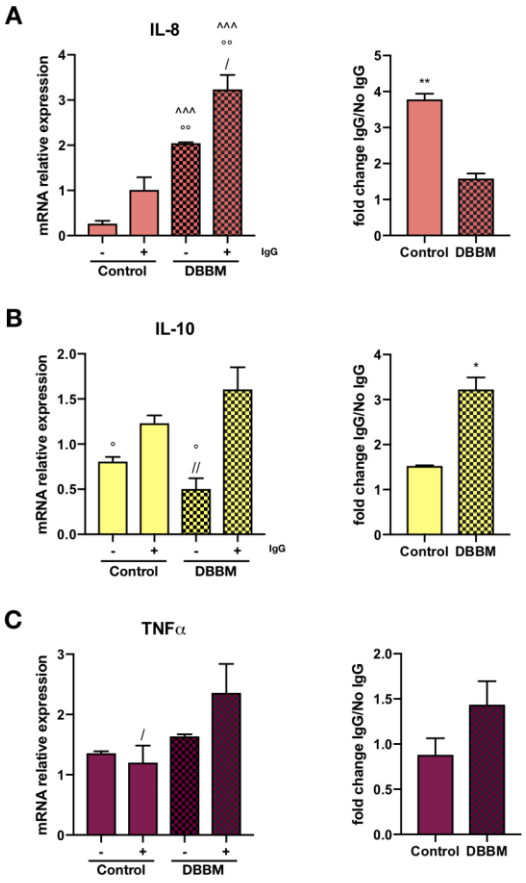

Figure 4: *Cytokine expression analysis*. (**A**) Relative mRNA expression and IgG/No IgG fold change of IL-8. (**B**) Relative mRNA expression and IgG/No IgG fold change of IL-10. (**C**) Relative mRNA expression and IgG/No IgG fold change of TNFα. ^=p<0.05 vs. Control + IgG; °=p<0.05 vs. Control + IgG; +=p<0.05 vs. DBBM; /=p<0.05 vs. DBBM + IgG; *=p<0.05 Control vs. DBBM.

The direct comparison of the fold-changes in cytokine expression induced by IgG in macrophages seeded on DBBM (**Fig.5**), highlighted a much higher induction of IL-10 compared to both IL-8 ($p=0.0116$) and TNFα ($p=0.0091$).

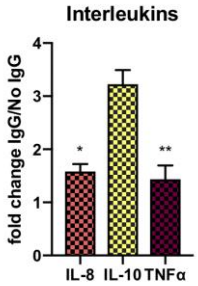

Figure 5: *Cytokine expression analysis*. **Comparison of** IgG/No IgG fold change of IL-8, IL-10 and TNFα. *=$p<0.05$.

Discussion

Macrophages represent the driving force that coordinate the chronic inflammation at biomaterials interface after insertion[22]. Once activated, these cells interact with the foreign material showing most often features of a classically activated pro-inflammatory phenotype (M1). However, macrophages exhibit a functional plasticity that allows a more or less complete switch of their metabolism and functional properties towards an alternatively activated anti-inflammatory and pro-resolving phenotype (M2)[12]. A debate on the mechanisms regulating macrophages commitment is still open and, together with the complexity of intermediate or mixed phenotypes, still renders difficult to understand the precise role played by macrophages during specific regenerative processes, such as the new-bone formation that occurs after augmentation procedures.

Recent findings have shown that DBBM, a typical bone xenograft commonly used in dentistry, promotes macrophage polarization toward an M2-pro-resolving phenotype, which further contributes to the creation of a microenvironment suitable for osteoblast maturation and new-bone deposition[15]. Nevertheless, the mechanisms that drive M2-polarization on DBBM remains to be characterized. In this study, we have analyzed the role of plasma protein adsorption at the interface of DBBM on macrophage activation and polarization.

When serum proteins were adsorbed on DBBM, a massive contribution of IgG in forming the protein-corona was evident (**Fig.1b-c**). Since, as a consequence of the Vroman effect[18], albumin is the first molecule to be adsorbed on surfaces, we can presume that DBBM possesses a remarkable affinity for IgG. Therefore, we investigated if IgG could influence the response of macrophages when cultured on DBBM. The analysis of the CDs (cluster of differentiation) revealed that the expression of CD68, a general marker for macrophages activation, was promoted on DBBM when IgG were adsorbed (**Fig.3a**). Furthermore, the expression of CD86, which is expressed at low levels in monocytes and up-regulated on macrophages surface under inflammatory conditions[23], was stimulated by IgG adsorbed to culture plastic but not to DBBM (**Fig.3b**). Even though no significant differences were detected, the expression of the CD36, a scavenger receptor that is an important player in collagen-guided platelet adhesion[24], exhibited a positive trend in macrophages seeded on DBBM as an effect of IgG adsorption (**Fig.3c**). According to Shi et al., our findings confirm that DBBM elicited effects on the behavior of macrophages by shifting their phenotype toward the M2 extreme and suggest that IgG adsorption can influence this effect[15].

Additional information has been obtained from the analysis of cytokine expression, which clearly identified a potential anti-inflammatory role of IgG when adsorbed on DBBM. Consistently with the literature, the expression of the gene for IL-8, which is a potent pro-inflammatory mediator, was promoted on DBBM and further enhanced by the presence of IgG both on DBBM and on controls[25]. However, the effect of IgG was much smaller on macrophages seeded on DBBM and, interestingly, DBBM-dependent induction was laved in the presence of IgG, compared with samples treated with albumin alone, suggesting that IL-8 increase should be attributed to DBBM. Conversely, a 2-folds induction of IL-10 gene (**Fig.4a**), which is mainly expressed and secreted by immunocompetent cells to limit and control the inflammatory response, was observed when IgG were adsorbed on DBBM (**Fig.4b**). IL-10 is a potent anti-inflammatory cytokine that inhibits macrophage activation by suppressing the production of inflammatory cytokines[26]. Consistently with this, the presence of adsorbed IgG seems to recover the expression levels of TNFα (**Fig.4c**), but this data should be confirmed with further analysis.
It should be stressed that our data have been obtained with primary human macrophages. To the best of our knowledge, previous efforts that aimed to study macrophage polarization on biomaterials *in vitro* were mostly conducted using murine cells or cell lines of human or murine origin. Since murine and human macrophage models are known to possesses high discrepancies in their M1/M2 profiles, such as different capability of inducing NO synthase (NOS) activity, as well as of expressing in cytokines or chemokines receptors[27, 28], the use of primary macrophages isolated from whole blood plasma confers reliability to our results.
In conclusion, these data are consistent with the hypothesis that, while contact with DBBM promotes the acquisition of functional competence by blood-derived macrophages, preferential IgG adsorption to DBBM, due to a marked affinity of these proteins for the material, may promote anti-inflammatory and pro-repair responses of human macrophages through IL-10 expression. If expression data will be confirmed with determinations of secreted cytokines, we can furthermore speculate that macrophage-derived IL-10 could be involved in bone regeneration on two different levels. First, IL-10 is known to activate osteoblasts by stimulating the secretion of osteoprotegerin (OPG) and inhibiting that of the receptor activator of nuclear factor kappa-B ligand (RANKL), needed for osteoclast differentiation[29]. On the other hand, IL-10 is an inhibitor of the TNFα production[26]. High levels of TNFα enhance the secretion of the IL-6, a potent effector of the osteoclastogenesis, and further induces osteoclastogenesis by direct stimulation of macrophages when exposed to RANKL[30, 31]. IL-10 could be involved in inhibiting this pathway. Further studies investigating the effects of macrophages conditioned medium on osteoblasts and osteoclasts activity are needed to validate these mechanisms.

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Corresponding Author:
Dr. Ludovica Parisi
E-mail: ludovica.paris@gmail.com