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Immunogenicity assay of peripheral blood mononuclear cells cultures in response to bovine bone xenograft scaffold

Pralita Kusumawardhini[1*], David Buntoro Kamadjaja[2], Coen Pramono Danudiningrat[3], Ni Putu Mira Sumarta[4], Aries Muharram[5]

[1]Clinical Medicine Magister Study Programme, Faculty of Medicine, Universitas Airlangga, Surabaya 60132, Indonesia
[2-5]Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya 60132, Indonesia

Abstract

Chronic inflammation is a major factor contributing to the failure of bone grafting treatments, particularly by impeding the integration of commonly used bone substitutes such as bovine bone xenografts. This research is conducted to better understand the immunogenic response to these biomaterials. This study examined the expression of key immune cell markers including CD14 (monocytes), CD3 (T-lymphocytes), and CD19 (B-lymphocytes) in Peripheral Blood Mononuclear Cells (PBMCs) exposed to different bone graft preparations: deproteinized bovine bone mineral (DBBM), Freeze-Dried Bovine Bone (FDBB), and decellularized FDBB (dc-FDBB). Expression levels were evaluated on days 1, 3, and 7 using flow cytometry, and results were analyzed descriptively and statistically for normality, homogeneity, and group comparisons. Notably, by day 7, the PBMC+dc-FDBB group showed the highest expression of both CD14 and CD3, indicating prominent monocyte and T-lymphocyte involvement, while the PBMC+FDBB group exhibited the highest CD19 expression, reflecting B-lymphocyte activity. Statistical analysis demonstrated significant differences in immune cell marker expression between all groups at each observation point. These findings suggest that the choice and processing of xenograft material can distinctly influence the immune cell response, underlining the importance of biomaterial selection in bone grafting procedures.

Keywords: CD14, CD3, CD19, Bovine bone scaffold, PBMC

Introduction

Critical-sized bone defects, which arise from trauma, congenital anomalies, or tumor resection, are challenging to treat because they lack the intrinsic ability to heal due to insufficient osteogenesis and angiogenesis.[1,2] Autologous bone grafts are considered the gold standard because of their osteoinductive and osteoconductive properties and excellent histocompatibility. However, donor site morbidity, limited tissue availability, and prolonged surgical time restrict their use.[3,27,4] These limitations have driven the search for alternative biomaterials, with bovine-derived xenografts emerging as promising substitutes due to their structural similarity to human bone and wide availability. In Muslim-majority regions such as Indonesia, bovine xenografts are particularly preferred over porcine sources for cultural and religious reasons.[5–7]

Despite their clinical success, bovine xenografts are not entirely free from immunological concerns. Residual proteins or cellular components can trigger innate and adaptive immune responses, leading to macrophage activation, T and B cell stimulation, and the release of pro-inflammatory cytokines, which may compromise graft integration.[8–11] To address this, various processing techniques such as deproteinization (DBBM), freeze-drying (FDBB), and decellularization of FDBB (dc-FDBB) have been developed to reduce antigenicity while attempting to preserve osteoconductive and osteoinductive properties.[12–16] However, it remains unclear which preparation offers the lowest immunogenicity without compromising biological performance.

Peripheral blood mononuclear cells (PBMCs) provide a valuable in vitro model to assess immunogenicity, as they encompass monocytes, T cells, and B cells, which play key roles in inflammation and graft tolerance.[17–19] Flow cytometry analysis of CD14 (monocytes), CD3 (T cells), and CD19 (B cells) expression can help characterize immune responses to different scaffold types.[20]

This study aimed to compare the immunogenicity of DBBM, FDBB, and dc-FDBB scaffolds by evaluating CD14, CD3, and CD19 expression in human PBMC cultures over 1, 3, and 7 days. We hypothesized that dc-FDBB would elicit the lowest immune response, indicating superior biocompatibility. The findings

Correspondence: Pralita Kusumawardhini, Clinical Medicine Magister Study Programme, Faculty of Medicine, Universitas Airlangga, Surabaya 60132, Indonesia, **e-mail:** pralita.kusumawardhini-2022@fk.unair.ac.id, **Received:** October 27, 2025 **Accepted:** December 29, 2025

provide insights into selecting xenograft scaffolds with minimal immunogenicity to improve clinical outcomes in bone regeneration.

Materials and Methods

This study used an in vitro laboratory experimental design with a post-test only control group. The expression of CD14, CD3, and CD19 was assessed on days 1, 3, and 7 across four groups: (1) PBMC only (control), (2) PBMC + DBBM, (3) PBMC + FDBB, and (4) PBMC + dc-FDBB. Each group contained four samples, exceeding the minimum recommended sample size of three replicates.

Preparation of bone scaffolds

DBBM

DBBM was prepared from 20 µm cancellous bovine bone granules subjected to chemical cleaning with 3% hydrogen peroxide, rinsing, sintering at 1000°C, oven-drying to less than 10% moisture, and sterilization by gamma irradiation. A 2% suspension was created by homogenizing 0.25 g of DBBM in 10 mL Roswell Park Memorial Institute (RPMI) medium.

FDBB

FDBB, derived from 20 µm bovine femoral bone granules, underwent similar cleaning procedures, freezing at -80°C, freeze-drying, and gamma sterilization, followed by preparation of a 2% suspension as above.

Dc-FDBB

dc-FDBB was obtained from 5×5×5 mm bovine femur blocks, cleaned, frozen, freeze-dried, then decellularized using sodium lauryl ether sulfate (SLES), and sterilized. A 2% suspension was prepared by mixing 0.25 g scaffold with 10 mL RPMI medium.

PBMC isolation and culture

PBMCs were isolated at the Central Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya. Blood samples collected in heparin- or EDTA-coated tubes were diluted 1:1 with Phosphate-Buffered Saline (PBS) and layered onto 3 mL Histopaque-1077, followed by centrifugation at 400×g for 30 minutes. The PBMC layer was aspirated, washed with PBS, and red blood cells were lysed with 1X RBC lysis buffer. Cells were washed again with PBS, and viability was confirmed via trypan blue exclusion. PBMCs were counted using a hemocytometer and resuspended in RPMI medium. Cells were plated in 24-well plates at 5×10^5 cells/500 µL per well, with six wells per group containing 2 mL of PBMC suspension at 1×10^6 cells/mL. Treatment groups received 1 mL of the respective 2% scaffold suspension and were incubated at 37°C in 5% CO_2.

Flow cytometry immunophenotyping: On days 1, 3, and 7, two wells from each group were harvested. Cells were detached using trypsin, washed with PBS, centrifuged at 1500 rpm for 5 minutes, and resuspended in 250 µL Fluorescence-Activated Cell Sorting (FACS) buffer with 2% fetal calf serum (FCS). Cells were stained at 4°C for 30 minutes with monoclonal antibodies: FITC-conjugated anti-human CD3 (T cells), PE-conjugated anti-human CD19 (B cells), and FITC-conjugated anti-human CD14 (monocytes). After staining, cells were fixed with 150 µL CellFix and stored at 4°C. Prior to analysis, 230 µL FACS Flow buffer with 2% FCS was added. Flow cytometry was performed using a FACSort instrument and CellQuest software (BD Biosciences). Expression levels of CD14, CD3, and CD19 were analyzed relative to isotype controls. Each condition was analyzed in duplicate for technical validation.

Statistical analysis: Data were analyzed using SPSS version 26. The normality of data distribution for each group and time point was assessed using the Shapiro-Wilk test, with p-values greater than 0.05 indicating normal distribution. Homogeneity of variance was evaluated using Levene's test, where p-values greater than 0.05 indicate equal variances across groups. For datasets meeting the criteria of normality and homogeneity, One-Way Analysis Of Variance (ANOVA) was performed to compare marker expression levels among groups at each time point. Post hoc multiple comparisons were conducted using Tukey's HSD test to identify specific intergroup differences. For data violating assumptions of normality or homogeneity, the non-parametric Kruskal-Wallis test was applied. Statistical significance for all tests was set at $p < 0.05$. Analyses were performed separately for CD14, CD3, and CD19 expression data at days 1, 3, and 7, using four biological replicates per group. Plant molecular pharming (PMP) has afforded the production of

pharmaceuticals such as vaccines in plants.[21] Transgenic plants allow the production of recombinant proteins that are essential for disease prevention, diagnosis, and treatment. The choice of a host plant species during the production of vaccines is very important.

The expression levels of CD14, CD3, and CD19 analyzed in this study were measured using flow cytometry and based on four groups: PBMC, PBMC+DBBM, PBMC+FDBB, and PBMC+dc-FDBB. Each group was evaluated at three different time points: day 1, day 3, and day 7.

Results: CD14 expression: The expression of CD14 varied significantly among the groups and over time (Figure 1). On day 1, the highest CD14 expression was observed in the PBMC control group (81.73 ± 2.70), with significantly lower expression in PBMC+dc-FDBB (50.26 ± 4.15) and PBMC+FDBB (70.18 ± 6.43) groups ($p < 0.01$). By day 3, CD14 levels remained highest in the control group (78.71 ± 1.55), while scaffold-treated groups had comparable expression levels around 70%. On day 7, expression in controls dropped sharply (10.61 ± 1.66), whereas PBMC+dc-FDBB maintained a significantly higher level (63.16 ± 9.33) compared to PBMC+FDBB (40.81 ± 1.47) and PBMC+DBBM (35.71 ± 1.55) ($p < 0.001$) (Table 1). Statistical tests confirmed significant differences among groups at every time point ($p = 0.000$). Post hoc analysis revealed that PBMC+dc-FDBB often induced CD14 expression significantly different from other scaffolds and controls, indicating prolonged monocyte activation.

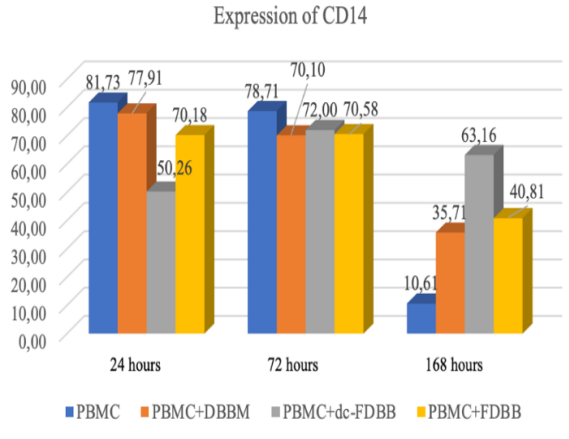

Figure 1. Mean of CD4 expression based on observation time

Table 1. Flow cytometry analysis of CD14 expression in PBMCs with bovine bone scaffolds (DBBM, FDBB, dc-FDBB) and control at days 1, 3, and 7

Time	Group	Mean	SD
Day 1	PBMC	81.73	2.70
	PBMC+DBBM	77.91	1.29
	PBMC+dc-FDBB	50.26	4.15
	PBMC+FDBB	70.18	6.43
Day 3	PBMC	78.71	1.55
	PBMC+DBBM	70.10	2.02
	PBMC+dc-FDBB	72.00	2.97
	PBMC+FDBB	70.85	3.22
Day 7	PBMC	10.61	1.66
	PBMC+DBBM	35.71	1.55
	PBMC+dc-FDBB	63.16	9.33
	PBMC+FDBB	40.81	1.47

CD3 expression: CD3 expression exhibited an increasing trend over time across all groups (Figure 2). On day 1, expression levels were similar among groups, with PBMC+FDBB showing the highest mean (74.31 ± 0.83). Day 3 showed a significant increase in CD3 expression for all groups, with PBMC controls reaching the highest level (84.63 ± 0.93). By day 7, PBMC+dc-FDBB displayed the highest CD3 expression (92.83 ± 1.18), surpassing both controls and other scaffolds (Table 2). Normality and variance homogeneity tests justified one-way ANOVA for days 1 and 3, with significant differences found ($p = 0.005$ and $p = 0.000$, respectively). Due to non-normal distribution on day 7, the Kruskal-Wallis test was used and confirmed significant group differences ($p = 0.005$). Post hoc analyses identified significant differences primarily involving the PBMC+dc-FDBB group, suggesting this scaffold enhances T cell activation.

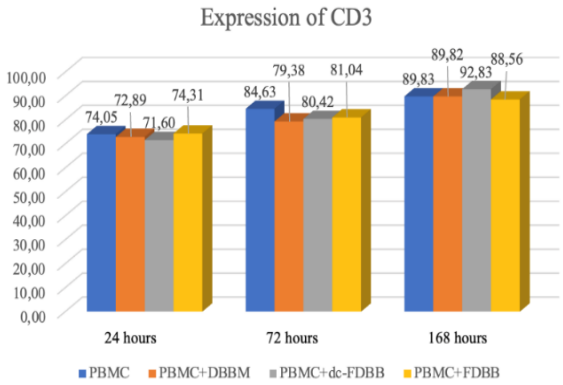

Figure 2. Mean of CD3 expression based on observation time

Table 2. Flow cytometry analysis of CD3 expression in PBMCs with bovine bone scaffolds (DBBM, FDBB, dc-FDBB) and control at days 1, 3, and 7

Time	Group	Mean	SD
Day 1	PBMC	74.05	0.64
	PBMC+DBBM	72.89	0.38
	PBMC+dc-FDBB	71.60	1.12
	PBMC+FDBB	74.31	0.83
Day 3	PBMC	84.63	0.93
	PBMC+DBBM	79.38	0.27
	PBMC+dc-FDBB	80.42	0.54
	PBMC+FDBB	81.04	0.84
Day 7	PBMC	89.83	0.25
	PBMC+DBBM	89.82	0.13
	PBMC+dc-FDBB	92.83	1.18
	PBMC+FDBB	88.56	0.38

Table 3. Flow cytometry analysis of CD19 expression in PBMCs with bovine bone scaffolds (DBBM, FDBB, dc-FDBB) and control at days 1, 3, and 7

Time	Group	Mean	SD
Day 1	PBMC	11.40	0.36
	PBMC+DBBM	13.44	0.52
	PBMC+dc-FDBB	6.81	0.28
	PBMC+FDBB	8.81	2.68
Day 3	PBMC	4.25	0.25
	PBMC+DBBM	4.74	0.33
	PBMC+dc-FDBB	1.56	0.28
	PBMC+FDBB	5.34	0.71
Day 7	PBMC	4.27	1.66
	PBMC+DBBM	4.34	0.27
	PBMC+dc-FDBB	1.79	0.51
	PBMC+FDBB	5.32	0.20

CD19 expression

CD19 expression varied both with scaffold and over time (Figure 3). On day 1, PBMC+DBBM exhibited the highest expression (13.44 ± 0.52), whereas PBMC+dc-FDBB showed consistently the lowest across all time points. Expression levels decreased on day 3 for all groups, though PBMC+FDBB maintained relatively higher values. By day 7, the pattern remained, with PBMC+FDBB expressing the highest level (5.32 ± 0.20) (Table 3). Statistical analysis indicated significant differences in CD19 expression among groups at all-time points ($p < 0.005$). Notably, PBMC+dc-FDBB's CD19 expression was significantly lower than other groups on day 3 ($p < 0.001$), suggesting suppressed B cell activation associated with this scaffold.

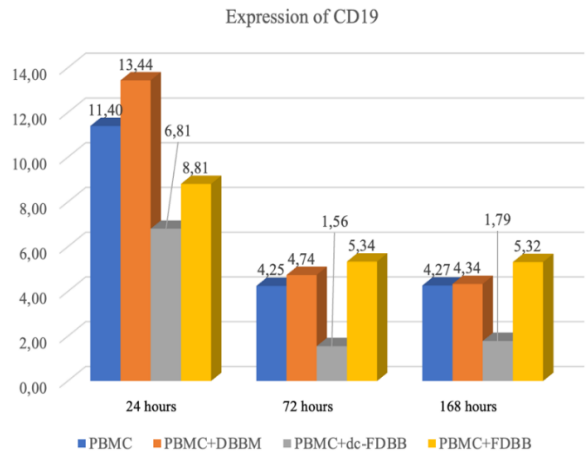

Figure 3. Mean of CD19 expression based on observation time

Overall, the dc-FDBB scaffold showed a distinctive immunogenic profile characterized by sustained monocyte (CD14) and T cell (CD3) activation, but reduced B cell (CD19) expression compared to DBBM and FDBB. These patterns suggest that scaffold processing influences immune cell responses, which may impact graft integration and success.

Understanding the immunological functions of the specific immune cell subsets observed in this study which are monocytes, T lymphocytes, and B lymphocytes is essential for interpreting the immune dynamics in response to bovine bone xenograft scaffolds. Monocytes, key components of the innate immune system, function primarily as phagocytes that recognize and eliminate foreign agents. They also differentiate into macrophages with distinct roles: pro-inflammatory M1 or anti-inflammatory M2, depending on microenvironmental cues. T lymphocytes, central to the adaptive immune response, recognize antigens through T cell receptors (TCRs) and destroy target cells, but require activation by Antigen-Presenting Cells (APCs) and co-stimulatory signals. Meanwhile, B lymphocytes mediate humoral immunity by producing antibodies and establishing immunological memory.

The selection of days 1, 3, and 7 as observation time points was based on the natural phases of the immune response. Day 1 represents the immediate innate immune activation following scaffold exposure, marked by monocyte activity and pro-inflammatory signaling. Day 3 corresponds to the transitional phase, during which the innate response

begins to subside, and the adaptive immune system becomes activated. By day 7, adaptive immunity is expected to be more fully established, indicated by specific activation of T and B lymphocytes in response to scaffold-derived antigens.

Our findings demonstrate distinct patterns in CD14, CD3, and CD19 expression across treatment groups and time points, reflecting the dynamic progression of both innate and adaptive immune responses to bovine bone scaffolds processed via deproteinization, freeze-drying, and decellularization.

On day 1, CD14 expression which marking monocyte activation was dominant. CD14 is a well-established monocyte surface marker and key component of the innate immune response. Upon detection of foreign materials or tissue injury, monocytes rapidly migrate to the site of inflammation and participate in phagocytosis. They may subsequently differentiate into M1 macrophages that drive inflammation, or into M2 macrophages that promote resolution and tissue repair.[22,23] The high CD14 expression observed on day 1 suggests that, despite undergoing processing, the xenograft scaffolds still triggered early innate immune activation. The declining CD14 expression on days 3 and 7 indicates resolution of the acute inflammatory response and a possible shift toward immune adaptation or tolerance.

Interestingly, the dc-FDBB group exhibited the highest CD14 expression across all groups. Although decellularization is intended to reduce antigen content and immunogenicity, residual matrix molecules or DNA fragments may still be recognized by the immune system, prompting an inflammatory response. Moreover, residual processing agents such as Sodium Lauryl Ether Sulfate (SLES) may cause tissue irritation and further activate monocytes.[24]

CD3 expression, indicating T cell activation, gradually increased, and peaked on day 7. This aligns with the immunological principle that adaptive immunity requires antigen processing and presentation by APCs, leading to T cell activation over time. The highest CD3 expression was also observed in the dc-FDBB group, suggesting that, although decellularized, this scaffold still contained bioactive or irritating components capable of activating dendritic cells and subsequently T lymphocytes. This highlights that morphologically "clean" scaffolds may still pose immunological risks at the molecular level.

In contrast, CD19 expression, a marker of B cell activity, showed a different pattern. Unexpectedly, the highest CD19 levels were observed on day 1 in the FDBB group. While B cell activation typically occurs later in the adaptive response, early CD19 upregulation may result from residual antigenic proteins insufficiently removed during freeze-drying. Freeze-dried biomaterials are known to elicit stronger early inflammation, potentially explaining the heightened early B cell response.[25,26]

Notably, CD19 expression declined by day 7 across all groups, as shown by a significant overall difference identified by the Kruskal-Wallis test ($p = 0.003$). Although no pairwise comparisons were conducted, the PBMC+dc-FDBB group consistently showed the lowest CD19 levels descriptively, indicating reduced and stable B cell activity. This pattern reflects a lower humoral immune response and a potential shift toward immunological tolerance.[17,20]

Decellularized scaffolds offer a key advantage in minimizing immunogenicity by removing cellular and antigenic components while preserving the Extracellular Matrix (ECM). ECM components are not only less immunogenic but may also modulate immunity by promoting macrophage polarization toward the M2 phenotype, which secretes anti-inflammatory cytokines such as IL-10 and TGF-β that aid tissue regeneration. In contrast, deproteinized scaffolds tend to elicit a moderate immune response, while freeze-dried materials may provoke stronger inflammation due to higher cellular residue.

The elevated CD3 expression on day 7 in the dc-FDBB group suggests that this scaffold type supports a more regulated and targeted adaptive immune response, particularly T cell-mediated. Meanwhile, the concurrently low CD19 expression implies limited B cell involvement and a lack of prolonged humoral activation. The preserved ECM in decellularized scaffolds may selectively support T cell activation while suppressing unnecessary humoral responses.

Collectively, these findings indicate that bovine bone xenograft scaffolds, despite processing, retain some immunogenic potential. The dc-FDBB scaffold consistently induced higher CD14 and CD3

expression, suggesting engagement of both innate and adaptive immune responses. FDBB scaffolds, meanwhile, triggered early CD19 activation, likely due to residual antigens. Over time, CD19 expression declined significantly across groups (Kruskal-Wallis, $p < 0.05$), suggesting immune adaptation or tolerance. Specific intergroup differences were not assessed due to the absence of post hoc analysis. Among the scaffold types studied, the decellularized form appears to offer the best immunological profile for clinical application, as it promoted a mild inflammatory response with dominant T cell activation and minimal B cell stimulation.

Clinically, these results underscore the need for further strategies to reduce scaffold immunogenicity, including improved decellularization techniques, elimination of irritant residues, and co-culture with immune cells to evaluate immunomodulatory properties more comprehensively. This study contributes to a better understanding of immune responses to bone scaffolds and provides valuable insights for developing next-generation, immunocompatible biomaterials for bone regeneration.

Conclusion

This study found that bovine bone xenograft scaffolds influenced immune cell activation differently. dc-FDBB induced the highest CD14 and CD3 expression, suggesting strong monocyte and T cell activation, while CD19 expression peaked in FDBB on day 1 but declined by day 7. DBBM showed the lowest overall immune marker expression, indicating better biocompatibility. Freeze-dried and decellularized scaffolds triggered more controlled immune responses and may support tissue healing through M2 macrophage polarization.

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