



Article

Immuno-Instructive 3D Tendon Biomimetic Scaffolds Functionalized with Amniotic Epithelial Stem Cell Secretome for Controlled Inflammation and Targeted Macrophage Polarization

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Abstract

Tendon healing is often hindered by unresolved inflammation and dysregulated immune responses, highlighting the need for innovative regenerative strategies. This study developed an immune-informed platform by functionalizing validated 3D tendon-mimetic poly(lactide-co-glycolide) (PLGA) scaffolds with immunomodulatory conditioned media (CM), referred to as CM_{INF} to emphasize its anti-inflammatory and immunomodulatory properties, derived from ovine amniotic epithelial stem cells (AECs), offering a potential cell-free therapeutic solution. Three functionalization methods were compared: physical adsorption, and hydrochloric acid (HCl) or sodium hydroxide (NaOH) pre-treatments. FT-IR spectroscopy and protein adsorption analyses identified NaOH as the most effective method, enhancing retention and release of Amphiregulin (AREG), an AEC key immunomodulatory protein. Kinetic studies revealed a sustained, controlled release of AREG over 7 days (d) from CM_{INF}-functionalized scaffolds (3D-CM_{INF}), preserving bioactivity. Functionally, 3D-CM_{INF} scaffolds significantly suppressed T-cell activation and peripheral blood mononuclear cell (PBMC) proliferation. The released CM from 3D-CM_{INF} (CM_R) exhibited time-dependent immunomodulatory effects: early T-cell inhibition (6–72 h) and delayed suppression of PBMC proliferation (48 h–7 d). Macrophage polarization analysis revealed a shift towards the pro-regenerative M2 phenotype, with increased expression of M2 over M1 markers in 3D-CM_{INF}-adherent cells. Flow cytometry confirmed a preferential induction of regulatory M2b macrophages alongside reductions in pro-inflammatory M1 and pro-fibrotic M2a subsets. These results demonstrate that 3D-CM_{INF} scaffolds can finely modulate immune responses, balancing inflammatory and reparative cues relevant to early tendon healing processes. This platform, integrating structural and immunomodulatory elements, presents a promising, cell-free, and translational immunoengineering strategy to control inflammation and support tendon repair.

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1. Introduction

Inflammation is a fundamental biological response to injury, initiating tissue repair by recruiting immune cells and activating stromal and resident cells [1]. In tendons, this response is particularly critical, as impaired healing is often associated with persistent inflammation and dysregulated immunomodulation, posing a significant clinical challenge [2–5]. Following injury, tendon healing progresses through three overlapping phases— inflammation, proliferation, and remodeling [6], with the early inflammatory stage playing a pivotal role in orchestrating tissue healing [7]. In particular, the inflammatory phase is pronounced during the first 3–7 days post-injury, during which immune cell recruitment, cytokine release, and macrophage polarization critically shape the downstream reparative and remodeling responses [7–9]. However, excessive or prolonged inflammation can lead to fibrosis, extracellular matrix (ECM) degradation, and compromised tissue functionality [6]. Given that the acute inflammatory phase of tendon healing is largely confined to the first week post-injury, biomaterial-based immunomodulatory strategies should be specifically designed to act within this critical temporal phase [10,11].

Macrophages are key regulators of the immune tissue response during tissue healing, including in tendons [9]. Classically activated M1 macrophages initiate the pro-inflammatory response necessary for debris clearance, whereas alternatively activated M2 macrophages promote ECM deposition and functional recovery [2]. Emerging evidence indicates that macrophage plasticity is essential for effective tissue repair, as timely transitions between inflammatory and reparative programs critically determine healing outcomes [12–14]. Within a dynamic and context-dependent continuum, macrophages can reversibly adopt mixed or intermediate phenotypes in response to microenvironmental cues [12–14]. Consequently, guiding macrophage polarization or modulating macrophage-associated functional states toward a pro-regenerative M2 phenotype has become a central goal in regenerative medicine and tendon tissue engineering (TE). In this context, the design of scaffolds capable of actively modulating the immune response has emerged as a core component of immunoengineering strategies [15–17]. Such scaffolds can influence macrophage polarization, favoring the anti-inflammatory M2 over the pro-inflammatory M1 phenotype, and modulating stem cell behavior, particularly enhancing their paracrine activity and regenerative efficacy [10,11,18–22].

Beyond their structural role, scaffolds can act as dynamic delivery systems for bioactive molecules or stem cell-derived products, further expanding their immunomodulatory and regenerative potential [23–25].

Within this framework, 3D tendon-mimetic Poly(lactide-co-glycolide) (PLGA) scaffolds have been shown to enhance key biological functions of amniotic epithelial stem cells (AECs), particularly their paracrine signaling, while also attenuating inflammation and immune responses [10,18].

AECs, derived from the innermost layer of the amniotic membrane, possess distinctive characteristics [26]. In addition to their intrinsic teno-differentiation potential *in vivo* [27–31], AECs act as secretory biofactories, releasing a complex secretome rich in cytokines, growth factors, and extracellular vesicles that modulate the inflammatory microenvironment [10,18,32–34] and enhance tendon regeneration [35–37]. The therapeutic potential of the AEC-derived secretome, particularly in the form of conditioned media (CM), is increasingly recognized as a viable cell-free alternative to stem cell-based therapies [33,34,37,38].

However, a major challenge in leveraging the AEC secretome is achieving effective delivery and retention of its bioactive components at the injury site. Scaffold functionalization with CM represents a promising strategy to overcome this limitation, enhancing the stability, bioavailability, and therapeutic efficacy of cell-free approaches [39–43]. The choice of functionalization method, whether through physical adsorption or chemical surface modification, affects the binding, release kinetics, and biological activity of the loaded molecules, directly influencing therapeutic outcomes [44–47].

Based on these premises, this research was designed to optimize the functionalization of previously developed 3D tendon-mimetic PLGA scaffolds with an immunomodulatory CM (CM_{INF}) derived from AECs [33], which has been previously validated and molecularly characterized at proteomic and lipidomic levels under defined experimental conditions. The present work, therefore, builds upon an already characterized secretome platform, aiming to integrate its anti-inflammatory and immunoregulatory properties into an immune-informed platform capable of modulating inflammation and immune cell behavior while promoting tendon regeneration.

To this end, three scaffold functionalization strategies were evaluated: (i) physical adsorption (PA) involving direct application of CM_{INF} onto 3D scaffolds [42,47] and scaffold pre-treatment with either (ii) HCl [45,48] or (iii) NaOH [45,49] before the CM_{INF} application. The most effective method was identified based on the efficiency of total protein and AREG adsorption.

Subsequently, the immunomodulatory properties of the functionalized scaffolds were assessed in vitro using peripheral blood mononuclear cells (PBMCs) and Jurkat T lymphocyte reporter cells through both direct assays, in which the immune cells were cultured in physical contact with the functionalized scaffolds, and indirect assays, in which immune cells were exposed to the conditioned media released from the CM_{INF}-functionalized scaffolds (CM_R). In the direct approach, a local immune response at the implantation site was mimicked, while in the indirect approach, the paracrine effects of secreted bioactive factors were simulated over a 7-day period. Finally, the impact on macrophage polarization was investigated, focusing on the pro-inflammatory M1 and pro-regenerative M2 phenotypes.

By integrating the structural guidance of biomimetic 3D PLGA scaffolds with the immunomodulatory activity of CM_{INF} via optimized functionalization, this study allowed the development of an innovative immunoengineering strategy of a functionalized scaffold-based, cell-free approach for tendon regeneration. The proposed platform not only attenuates inflammation and the immune response but also supports ECM remodeling that may ultimately enhance functional tendon recovery.

2. Results

2.1. Chemical Pretreatment Modulates Fiber Topography and Mechanical Performance of Electrospun PLGA Scaffolds

To understand the effect of chemical pretreatment on PLGA scaffolds, ultrastructural analysis was performed. According to Figure 1A, SEM analysis revealed that net untreated PLGA scaffolds exhibited highly aligned, continuous, and bead-free fibers with smooth morphology with an average fiber diameter of $1.20 \pm 0.10 \mu\text{m}$.

Following chemical pretreatment, significant differences in fiber diameter were observed. In particular, NaOH-pretreated PLGA scaffolds showed a marked increase in fiber diameter ($1.71 \pm 0.19 \mu\text{m}$) compared to net PLGA ($p < 0.0001$). Similarly, HCl-pretreated PLGA scaffolds exhibited an intermediate increase ($1.56 \pm 0.17 \mu\text{m}$) compared to net PLGA ($p < 0.0001$) but remained significantly lower than NaOH-pretreated scaffolds ($p < 0.0001$).

Morphologically, NaOH-pretreated fibers preserved a homogeneous and continuous structure without visible defects. In contrast, HCl-pretreated scaffolds, although

maintaining overall fiber integrity and alignment, showed slight surface irregularities and localized morphological alterations compared to net and NaOH-pretreated scaffolds, suggesting a mild impact of acid treatment on fiber surface topography (Figure 1A).

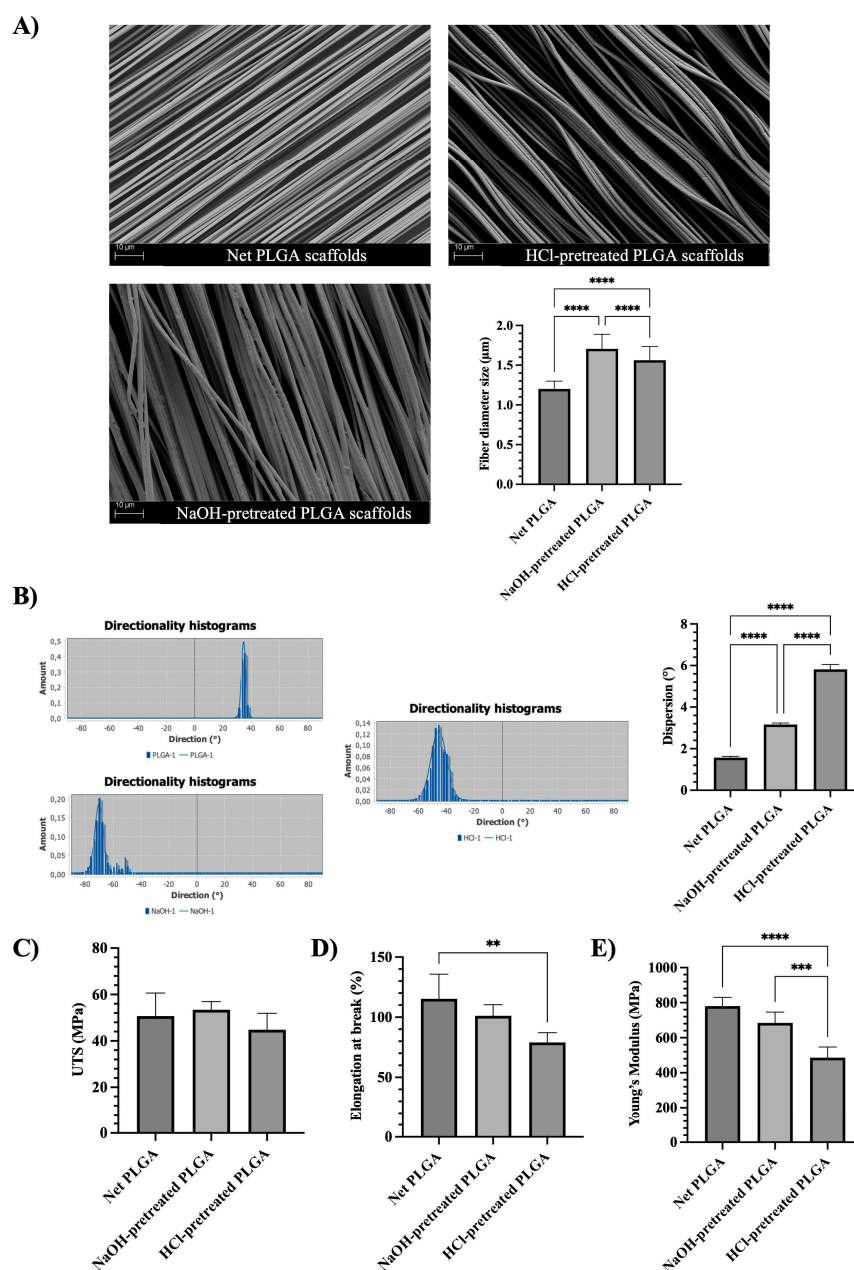


Figure 1. Morphological, orientational, and mechanical characterization of net and chemically pretreated PLGA scaffolds. **(A)** Representative SEM micrographs of net PLGA, NaOH-pretreated PLGA, and HCl-pretreated PLGA scaffolds and quantitative analysis of fiber diameter (scale bar = 10 µm). **(B)** Directionality histograms showing a Gaussian distribution of fiber orientation and corresponding dispersion values. **(C–E)** Mechanical properties evaluated by uniaxial tensile testing, including **(C)** ultimate tensile strength (UTS), **(D)** elongation at break, and **(E)** Young's modulus. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. **, ***, and **** Statistically significant difference between groups ($p < 0.01$, 0.001, and 0.0001, respectively).

Fiber orientation analysis using the Directionality plugin generated Gaussian distribution curves for all groups (Figure 1B). net PLGA scaffolds exhibited the sharpest Gaussian curve, indicating highly oriented fibers with minimal angular variability. In contrast,

both NaOH- and HCl-pretreated scaffolds showed broader Gaussian distributions, reflecting increased variability in fiber orientation. Among them, HCl-pretreated scaffolds displayed the broadest angle distribution curve. Quantitative assessment of fiber dispersion, calculated as the standard deviation of the Gaussian distribution (Figure 1C), confirmed these observations. Net PLGA scaffolds exhibited the lowest dispersion ($1.57 \pm 0.06^\circ$). NaOH pretreatment significantly increased dispersion to $3.16 \pm 0.07^\circ$ ($p < 0.0001$ vs. Net), while HCl pretreatment resulted in the highest dispersion values ($5.81 \pm 0.24^\circ$, $p < 0.0001$ vs. both net and NaOH).

Despite the increased angular variability observed after chemical treatments, all groups maintained a Gaussian distribution profile, indicating preservation of overall fiber alignment.

Moreover, mechanical characterization was performed to evaluate the impact of chemical pretreatments on tensile properties (Figure 1C–E). Regarding ultimate tensile strength (UTS, Figure 1C), no statistically significant differences were observed among groups ($p > 0.05$). Net PLGA scaffolds showed a UTS of 50.70 ± 9.94 MPa, NaOH-pretreated scaffolds 53.40 ± 3.58 MPa, and HCl-pretreated scaffolds 44.80 ± 7.11 MPa. Elongation at break analysis (Figure 1D) revealed that NaOH pretreatment did not significantly alter ductility compared to net PLGA ($101.00 \pm 9.30\%$ vs. $115.40 \pm 20.41\%$, $p > 0.05$). In contrast, HCl-pretreated scaffolds showed a significant reduction in elongation at break ($79.05 \pm 8.13\%$) compared to net PLGA ($p < 0.01$), indicating increased brittleness following acid treatment. Young's modulus analysis (Figure 1E) demonstrated that net PLGA scaffolds exhibited the highest stiffness (780.20 ± 50.10 MPa). NaOH-pretreated scaffolds showed a non-significant reduction (684.40 ± 61.80 MPa, $p > 0.05$ vs. net), whereas HCl-pretreated scaffolds presented a significant decrease in Young's modulus (486.20 ± 61.10 MPa, $p < 0.0001$ vs. net; $p < 0.001$ vs. NaOH).

Overall, NaOH pretreatment increased fiber diameter and angular dispersion without significantly compromising mechanical performance, whereas HCl pretreatment induced greater fiber disorganization and significantly reduced mechanical stiffness and elongation at break.

2.2. Chemical Characterization of Liquid Media and 3D Functionalized Scaffolds

2.2.1. Analysis of Media

A preliminary FT-IR spectroscopic analysis was performed on all liquid media (SF, SF+AREG, and CM_{INF}) to establish their baseline chemical signature before scaffold functionalization (Table 1 and Figure 2A).

This characterization was essential to later identify which molecular components from these liquids were successfully transferred to the scaffold surfaces during functionalization. The spectroscopic analysis revealed that all three liquid formulations shared similar chemical profiles with three characteristic bands at 701, 1643, and 3389 cm^{-1} , corresponding to out-of-plane H-C-H bending, amide I (N-H) stretching, and O-H stretching vibrations, respectively (Figure 1A and Table 1). These signature bands represent key functional groups that contribute to the bioactive properties of these media.

Table 1. FT-IR spectral analysis of liquid samples.

Samples	Wavenumber (cm^{-1})	Functional Group Assignment
SF	701	Out-of-plane H-C-H bending
SF+AREG	1643	Amide I (N-H) stretching
CM _{INF}	3389	O-H stretching

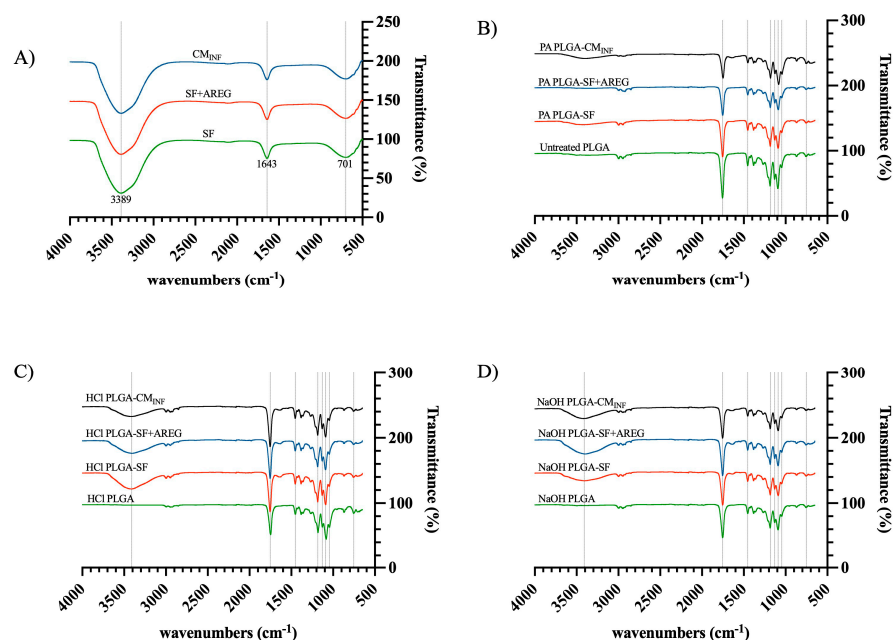


Figure 2. Chemical characterization of 3D scaffolds before and after functionalization. Infrared spectra for (A) liquid (SF, SF+AREG, and CM_{INF}), (B) PA, (C) HCl, and (D) NaOH-treated PLGA scaffolds, functionalized or not with SF+AREG and CM_{INF}, and reported in the 650 and 4000 cm⁻¹.

Notably, while CM_{INF} is derived from SF through cellular conditioning (where cells release bioactive molecules into the SF) and SF+AREG contains supplemented AREG, the fundamental spectral features remained consistent across all three media. This spectral consistency indicated that the cellular conditioning process and the addition of AREG did not substantially alter the primary chemical structure of the SF-based media, confirming the chemical stability of these formulations prior to scaffold functionalization.

2.2.2. Characterization of PLGA Scaffolds and Effects of Surface Modifications

The chemical structure of untreated 3D PLGA scaffolds was first confirmed via FT-IR spectroscopy (Figure 2 and Table 2), which revealed characteristic spectral features consistent with their chemical structure, including peaks associated with ester carbonyl groups (1754 cm⁻¹), ether linkages (1085, 1091 cm⁻¹), and various C-H stretching vibrations (2945 and 2996 cm⁻¹), consistent with the typical PLGA signature [27,29].

To assess the chemical impact of different functionalization techniques (PA, HCl, and NaOH pre-treatments) with SF, SF+AREG, and CM_{INF} on the scaffold's surface, distinctive spectral modifications were studied, each reflecting specific chemical interactions at the scaffold surface and possible biomolecule adsorption (Figure 2 and Table 2).

Table 2. FT-IR spectral analysis of PLGA scaffolds after different treatments.

Treatment Method	Liquid Type	Characteristic Peaks at	Functional Groups Affected
Untreated PLGA	None	1044 cm ⁻¹	C-CH ₃ symmetric stretching
		1085, 1091 cm ⁻¹	C-O-C ether stretching
		1754 cm ⁻¹	C=O ester stretching
		2945, 2996 cm ⁻¹	C-H, C-H ₃ , C-H ₂ stretching
		1424, 1454 cm ⁻¹	C=O ester bending
Treatment Method	Liquid Type	Key Spectral Changes	Functional Groups Affected
PA	SF	↑ Transmittance (~8%) at 1089, 1130, 1183, 1754 cm ⁻¹	C-O-C, C-O, C=O groups
		↑ Transmittance (>8%) at 1089, 1130, 1183, 1754 cm ⁻¹	C-O-C, C-O, C=O groups
PA	SF + AREG	Additional ↑ (~7%) at 1454 cm ⁻¹	CH ₂ groups
		Peak shift: 2049 → 2036 cm ⁻¹	Structural modification

PA	CM _{INF}	↑ Transmittance (>8%) at 1089, 1130, 1183, 1754 cm ⁻¹	C-O-C, C-O, C=O groups
		Additional ↑ (~7%) at 1454 cm ⁻¹	CH ₂ groups
		Peak shift: 2049 → 2036 cm ⁻¹	Structural modification
HCl Pre-treatment	None	↓ Transmittance at 754, 1044 cm ⁻¹	Increased absorption
		↑ Transmittance at 1181, 1453, 1747 cm ⁻¹	Decreased absorption
		Peak shifts: 1091 → 1083 cm ⁻¹ , 1754 → 1747 cm ⁻¹	Structural modification
HCl Pre-treatment	SF	↓ Transmittance (12%) at 1046 cm ⁻¹	C-O groups
		↑ Transmittance at 1454, 1754 cm ⁻¹	CH ₂ , C=O groups
		New peak at 3418 cm ⁻¹	O-H stretching
		Peak shifts: 1083 → 1089 cm ⁻¹ , 1747 → 1754 cm ⁻¹	Partial reversion to original
HCl Pre-treatment	SF+AREG	↓ Transmittance (12%) at 1046 cm ⁻¹	C-O groups
		↑ Transmittance at 1454, 1754 cm ⁻¹	CH ₂ , C=O groups
		New peak at 3418 cm ⁻¹	O-H stretching
		Peak shifts: 1083 → 1089 cm ⁻¹ , 1747 → 1754 cm ⁻¹	Partial reversion to original
HCl Pre-treatment	CM _{INF}	↓ Transmittance (12%) at 1046 cm ⁻¹	C-O groups
		↑ Transmittance at 1454, 1754 cm ⁻¹	CH ₂ , C=O groups
		New peak at 3418 cm ⁻¹	O-H stretching
		Peak shifts: 1083 → 1089 cm ⁻¹ , 1747 → 1754 cm ⁻¹	Partial reversion to original
NaOH Pre-treatment	None	↑ Transmittance at 1047 cm ⁻¹ (5% vs. PA, 17% vs. HCl)	C-O groups
		↑ Transmittance at 1090 cm ⁻¹ (14% vs. both)	C-O-C groups
		↑ Transmittance at 1130 cm ⁻¹ (12% vs. PA, ↓ 7% vs. HCl)	C-O stretching
		↑ Transmittance at 1184 cm ⁻¹ (14% vs. PA, 7% vs. HCl)	Ester bonds
		↑ Transmittance at 1454 cm ⁻¹ (6% vs. PA, no change vs. HCl)	CH ₂ groups
		↑ Transmittance at 1753 cm ⁻¹ (17% vs. PA, ↓ 4% vs. HCl)	C=O groups
		Peak shift: 2049 → 2036 cm ⁻¹	Structural modification
NaOH Pre-treatment	SF	Minimal changes	
		Peak shift: 2036 → 2049 cm ⁻¹	Reversion to untreated state
		New peak at 3414 cm ⁻¹	O-H stretching
NaOH Pre-treatment	SF+AREG	Minimal changes	
		Peak shift: 2036 → 2049 cm ⁻¹	Reversion to untreated state
		New peak at 3395 cm ⁻¹	O-H stretching
NaOH Pre-treatment	CM _{INF}	↓ Transmittance at 1090 cm ⁻¹ (7%)	C-O-C groups
		↓ Transmittance at 1130 cm ⁻¹ (5%)	C-O stretching
		↓ Transmittance at 1754 cm ⁻¹ (9%)	C=O groups
		Peak shift: 2036 → 2049 cm ⁻¹	Reversion to untreated state
		New peak at 3400 cm ⁻¹	O-H stretching

↑ indicates an increase in transmittance (decrease in absorption) and ↓ indicates a decrease in transmittance (increase in absorption).

- PA resulted in a general decrease in transmittance across multiple wavenumbers, with more pronounced effects observed after functionalization with SF+AREG and CM_{INF} compared to SF alone. These changes, along with the characteristic peak shift from 2049 to 2036 cm⁻¹, indicate varying degrees of surface adsorption of biomolecular components, although without significant changes to core polymer features.
- HCl pre-treatment induced more substantial modifications to the PLGA structure, as evidenced by notable transmittance changes and peak shifts, particularly at wavenumbers associated with ester and ether functionalities (1083 and 1747 cm⁻¹), indicating chemical alterations to the PLGA surface. When these acid-treated scaffolds were functionalized, a partial reversion toward the spectral profile of untreated PLGA was observed, along with the appearance of a new O-H stretching band at 3418 cm⁻¹, suggesting successful incorporation of the bioactive molecules.
- NaOH pre-treatment caused the most notable alterations in the FT-IR spectra and thus in the PLGA surface chemistry, with substantial changes in transmittance values across the spectrum compared to both untreated (PA) and HCl-treated scaffolds.

These changes reflect the alkaline hydrolysis of the polyester backbone, creating more reactive surface moieties. The subsequent functionalization with SF or SF+AREG resulted in minimal additional spectral changes, while CM_{INF} functionalization produced more pronounced modifications, particularly at wavenumbers associated with C-O-C (1090 cm⁻¹), C-O (1130 cm⁻¹), and C=O (1754 cm⁻¹) vibrations. These spectral changes, coupled with the appearance of a new O-H stretching band at 3400 cm⁻¹, suggest a stronger molecular interaction between CM_{INF} and the alkaline-treated scaffold surface compared to the other bioactive molecules.

2.3. NaOH Pretreatment Maximizes Functionalization Efficiency

The FE of CM_{INF}- and AREG-functionalized 3D PLGA scaffolds was quantified through both total protein adsorption analysis and specific molecule detection, respectively, to comprehensively characterize the adsorption process (Figure 3). This dual approach allowed us to evaluate not only the overall molecular binding capacity of the scaffolds but also their ability to retain AREG.

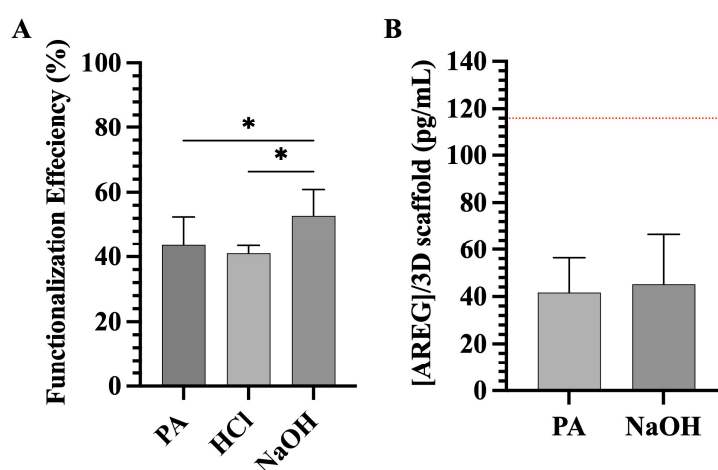


Figure 3. Histograms representing (A) the FE (%) of CM_{INF} total proteins absorbed on the 3D scaffolds subjected to three different functionalization methods: PA, HCl, and NaOH pretreatments; and (B) AREG quantification in CM_{INF} (red line) or adsorbed on the 3D scaffold functionalized with PA or NaOH pre-activation. * Statistically significant between groups for $p < 0.05$.

Total protein adsorption was quantified by using the Bradford assay, which demonstrated differential efficiency across three treatment methodologies. As shown in Figure 3A, the results indicate that both PA and HCl pre-treatment exhibited comparable functionalization efficiency (43.65 ± 8.85% vs. 42.02 ± 2.46%, respectively; $p > 0.05$; Figure 3A), suggesting that acid-based surface modification did not enhance biomolecule binding relative to PA scaffolds. In contrast, NaOH-pretreated scaffolds showed a significantly higher functionalization efficiency (52.75 ± 8.06%) compared to PA and HCl-pretreated scaffolds ($p < 0.05$; Figure 3A), confirming FTIR analysis. As expected, on the 3D sample, no protein adsorption was detected.

Based on the FE results, HCl was excluded from further analysis, and PA was used as a comparison for NaOH pre-treatment. To better determine whether NaOH pre-treatment positively modulated the FE compared to PA, the adsorption of the recombinant AREG molecule on the 3D scaffold was evaluated. For this purpose, the concentration of AREG in the native CM_{INF} was quantified at 116.35 ± 6.60 pg/mL (red line; Figure 3B). The concentration of 120 pg/mL was therefore used to functionalize 3D scaffolds with PA and pretreated with NaOH, which were then subjected to a comparative analysis of the adsorbed AREG molecule (Figure 3B). The results showed that AREG adsorption levels for

PA were 41.76 ± 14.68 pg/mL, and for NaOH, they were 45.24 ± 21.37 pg/mL, corresponding to FE of 35.34% and 38.79%. Although no statistically significant differences were observed between methodologies ($p > 0.05$; Figure 3B), a trend toward enhanced AREG-specific adsorption was noted in NaOH.

Taken together, the combined structural, mechanical, chemical, and protein adsorption data supported the selection of NaOH pre-treatment as the most suitable activation strategy under the tested conditions for subsequent biological evaluations.

2.4. Kinetic Insights Reveal Faster Initial but Sustained AREG Release from CM_{INF}-Functionalized Scaffolds

The release kinetics of AREG were evaluated over 7 days (d) from NaOH-pretreated 3D PLGA scaffolds functionalized either with the complete secretome (3D+CM_{INF}) or with recombinant AREG (3D+AREG) (Figure 4).

In detail, 3D+AREG exhibited a faster initial release compared to those functionalized with CM_{INF} (3D+CM_{INF}). At 6 h, approximately 52% of AREG was released from 3D+AREG, versus 40% from 3D+CM_{INF} ($p < 0.01$; Figure 4). This release profile continued to be significantly different also at 24 h, reaching a percentage of AREG release of around 60% compared to 50% ($p < 0.05$ vs. 3D+CM_{INF}; Figure 4).

In the mid phase (between 48 and 72 h; Figure 4), the release profiles of both groups achieved a comparable release level of around 70% and 75% at 48 h and 72 h, respectively ($p > 0.05$). Interestingly, at 168 h (7 d) (Figure 4), the release profiles became different, with a faster release for 3D scaffolds functionalized with CM_{INF} compared to 3D+AREG (99% vs. 84%, $p < 0.001$).

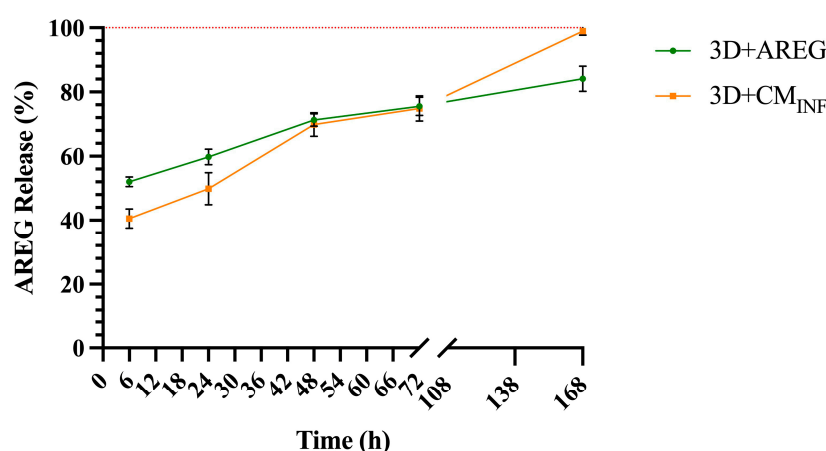


Figure 4. Kinetic release profile of AREG from 3D+CM_{INF} and 3D+AREG at different time points. The red line represents the maximum concentration of AREG adsorbed on the surface of 3D scaffolds previously exposed to NaOH pre-treatment.

2.5. Distinct Inhibitory Profiles of PBMC Proliferation and NFAT Activation in Jurkat T Cells by CM_{INF}-Functionalized 3D Scaffolds and Their Released Media

The immunomodulatory potential of 3D scaffolds functionalized with CM_{INF} was evaluated by analyzing their direct effects and the sustained activity of their derivative released CM (CM_R) on immune cells over time. Overall, these analyses showed a distinct inhibitory effect of 3D scaffolds functionalized with CM_{INF} and their CM_R on PBMC proliferation and NFAT activation in Jurkat T cells, with differing temporal patterns and sensitivities between the two cell types (Figure 5). Moreover, the CM_R from 3D scaffolds functionalized with CM_{INF} further modulated these inhibitory effects, albeit with different dynamics for PBMC proliferation and NFAT activation.

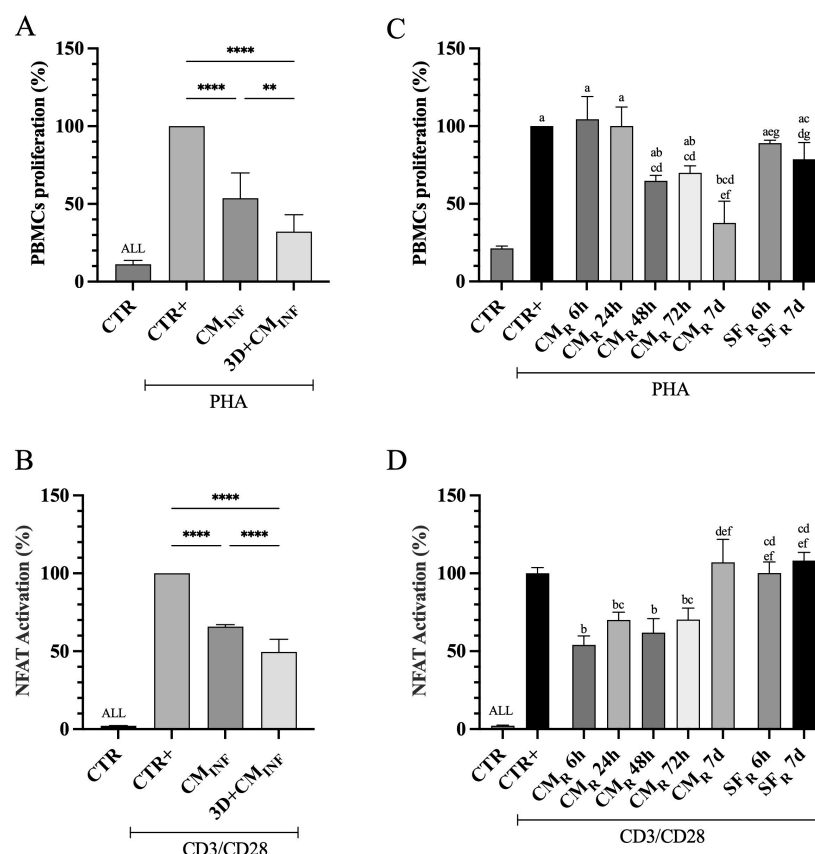


Figure 5. Immunomodulatory profile of CM_{INF}, CM_{INF}-functionalized 3D scaffolds, and released media (CM_R) on activated PBMCs and Jurkat cells after 48 h of culture. Histograms representing PBMC proliferation (%) and NFAT activation in response to CM_{INF} and 3D+CM_{INF} (A,B) and the CM_R obtained from 3D+CM_{INF} collected at different time points (C,D). ^a Statistically significant vs. (p < 0.05). ^b Statistically significant vs. CTR+ (p < 0.05). ^c Statistically significant vs. CM_R 6 h (p < 0.05). ^d Statistically significant vs. CM_R 24 h (p < 0.05). ^e Statistically significant vs. CM_R 48 h (p < 0.05). ^f Statistically significant vs. CM_R 72 h (p < 0.05). ^g Statistically significant vs. CM_R 7d (p < 0.05). ** and **** Statistically significant differences between groups (p < 0.01 and 0.0001, respectively).

2.5.1. Direct Exposure of Immune Cells to CM_{INF}-Functionalized 3D Scaffolds over Time

In PBMC cultures, the mitogenic stimulus induced by PHA (CTR+) significantly increased proliferation compared to untreated cells (vs. CTR, $p < 0.0001$; Figure 5A), confirming robust activation. Direct exposure to CM_{INF} alone significantly reduced PBMC proliferation compared to CTR+ ($p < 0.001$; Figure 5A), indicating the immunosuppressive effect of CM_{INF}-derived factors. Interestingly, PBMCs directly co-cultured with 3D scaffolds functionalized with CM_{INF} (3D+CM_{INF}) further enhanced this inhibition ($p < 0.01$ compared to both CM_{INF} alone and CTR+ groups; Figure 5A).

A similar inhibitory profile was observed for Jurkat T cells, measured through the analysis of NFAT activation, stimulated with CD3/CD28. The CTR+ group displayed the highest NFAT activation ($p < 0.0001$ compared to unstimulated cells; Figure 5B). Similar to PBMCs, Jurkat T cells cultured directly with CM_{INF} exhibited significant inhibition of NFAT activation compared to the CTR+ ($p < 0.0001$; Figure 5B), reflecting the suppressive effects of CM-derived factors again. The direct Jurkat co-culture with 3D scaffolds functionalized with CM_{INF} (3D+CM_{INF}) demonstrated an even greater inhibitory effect ($p < 0.01$ compared to the CTR+ and CM_{INF} groups; Figure 5B), highlighting the scaffolds' enhanced immunomodulatory potential compared to soluble factors alone. Importantly, 3D scaffolds alone (3D) did not show any inhibitory effect on either immune cells, indicating that

the enhanced immunosuppressive activity observed in 3D+CM_{INF} conditions was attributable to secretome functionalization rather than scaffold topology per se.

2.5.2. Time-Dependent Effects of Released Media (CM_R) Derived from CM_{INF}-Functionalized 3D Scaffolds

The CM_R derived from CM_{INF}-functionalized 3D scaffolds, collected at 6 h, 24 h, 48 h, 72 h, and 7 d, was then examined on immune cells—PBMCs and Jurkat T lymphocytes—showing inhibitory effects in distinctly time-dependent patterns (Figure 5C,D).

In detail, in PBMCs, CM_R collected at early time points (6 and 24 h) did not significantly inhibit proliferation ($p > 0.05$ vs. CTR+) (Figure 5C), while CM_R collected at 48 h induced a significant reduction ($p < 0.05$), with the strongest inhibition observed at 7 d ($p < 0.01$ vs. CTR+ and SF controls). Notably, at this time point, in response to CM_R, PBMC proliferation was comparable to unstimulated cells ($p > 0.05$ vs. CTR; Figure 5C). The SF_R had no significant effect on PBMC proliferation at any time point, confirming the specificity of the CM_{INF} inhibition driven by CM-derived factors ($p > 0.05$ vs. CTR+; Figure 5C).

In contrast, CM_R showed an early inhibitory profile on NFAT activation (Figure 5D). Already, CM_R collected at 6 h significantly suppressed NFAT activation compared to CTR+ ($p < 0.01$), and this suppression persisted with CM_R collected at 24, 48, and 72 h ($p < 0.01$ vs. CTR+; Figure 5D). However, with CM_R collected on day 7, NFAT activity returned to control levels ($p > 0.05$ vs. CTR+), indicating a loss of inhibitory effect over time (Figure 5D). Media from SF_R had no significant impact on NFAT activation, confirming the specificity of CM_{INF}-derived factors ($p > 0.05$).

2.6. Immunomodulatory Effects of CM_{INF}-Functionalized 3D Scaffolds on Macrophage Phenotypic Profiles

PBMC-derived adherent cells on the 3D scaffolds functionalized with CM_{INF} or SF were characterized by using immunofluorescence (Figure 6), assessing two key macrophage markers: CD86, indicative of pro-inflammatory M1 macrophages; and CD206, associated with anti-inflammatory M2 macrophages.

The results revealed that 3D scaffolds functionalized with CM_{INF} promoted monocyte adherence to the scaffold and modulated macrophage-associated polarization. Specifically, 3D+CM_{INF} increased the expression of both M1 (CD86⁺) and M2 (CD206⁺) markers, indicating modulation of macrophage-associated phenotypic markers. However, a predominance of CD206 expression was observed, suggesting a relative enrichment of CD206⁺ cells within the adherent population (Figure 6A–C). In contrast, as shown in Figure 6A–C, scaffolds conditioned with SF media induced lower expression of both CD86 and CD206 markers compared to CM_{INF}-functionalized 3D scaffolds. In particular, the percentages of CD86 ($31.10\% \pm 7.37$) and CD206 ($71.65\% \pm 1.79$) positive cells were significantly higher in scaffolds functionalized with CM_{INF} compared to SF scaffolds ($p < 0.05$; 8.17 ± 1.70 for CD86 and $46.99\% \pm 2.37$ for CD206; Figure 6B,C). Nonetheless, even in the absence of CM_{INF}, a higher proportion of CD206⁺ cells relative to CD86⁺ cells was still observed, suggesting a topology-driven enrichment of M2-associated marker expression. However, the addition of CM_{INF}-derived bioactive factors significantly enhanced this effect, resulting in a higher relative proportion of CD206⁺ cells compared to CD86⁺ cells.

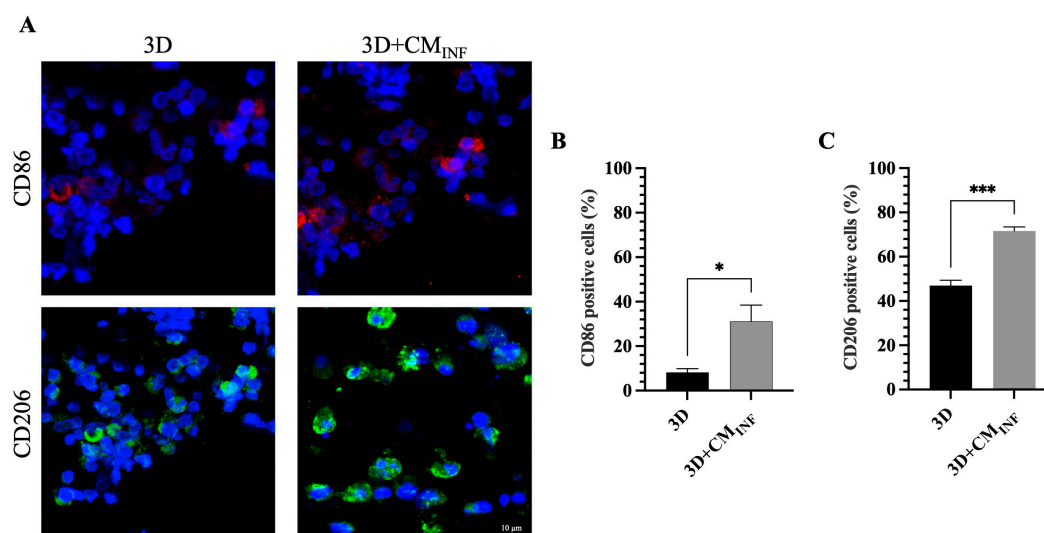


Figure 6. Effect of functionalization of 3D scaffolds with CM_{INF} on macrophage polarization. (A) Representative immunofluorescence images of CD86 (M1 pro-inflammatory marker, red fluorescence), and CD206 (M2 anti-inflammatory marker, green fluorescence) expressed within PBMCs adhered on 3D scaffolds exposed to SF (3D) and functionalized with CM_{INF} (3D+CM_{INF}). DAPI (blue fluorescence) counterstained nuclei. Histograms representing the percentages of (B) CD86 and (C) CD206 positive cells of PBMCs within the different tested groups quantitatively. * and *** denote statistically significant differences between groups for $p < 0.05$ and $p < 0.001$, respectively. For all images, the scale bar = 10 μm.

2.7. CM_{INF}-Functionalized Scaffolds Are Associated with Enrichment of M2b Subsets and Reduced M1 Pro-Inflammatory Macrophages

The immunomodulatory potential of CM_{INF} and CM_{INF}-functionalized 3D scaffolds was evaluated under inflammatory conditions using PHA-activated PBMCs (CTR+), preserving native cellular heterogeneity. All experimental groups were initially stimulated with PHA to mimic a pathological inflammatory microenvironment, resembling in vivo immune activation and establishing a relevant baseline activation state.

Following immune activation, the phenotypic impact of CM_{INF} and CM_{INF}-functionalized 3D scaffolds on immune cells was investigated by flow cytometry, focusing on surface markers associated with immune activation and macrophage-related responses.

Flow cytometry analysis showed a general rightward shift in fluorescence intensity for all experimental conditions compared to the negative control (CTR−), indicating increased marker expression at the population level (Figure 7). This trend was observed for CD14, CD80, and CD163, the analyzed markers, with comparable distribution profiles among CTR, CM_{INF}, and 3D+SF and 3D+CM_{INF} conditions. For CD86 and CD206, a slightly more pronounced rightward shift was observed in cells exposed to 3D+CM_{INF} compared to the other treatments, suggesting a stronger modulation of these markers under this condition (Figure 7). In addition, the 3D+CM_{INF} condition consistently showed the highest event counts at peak fluorescence intensity relative to the other treatments, indicating a higher proportion of cells expressing elevated levels of the analyzed markers (Figure 7). Despite these shifts, fluorescence distributions remained predominantly unimodal, supporting a homogeneous population response without the emergence of distinct subpopulations.

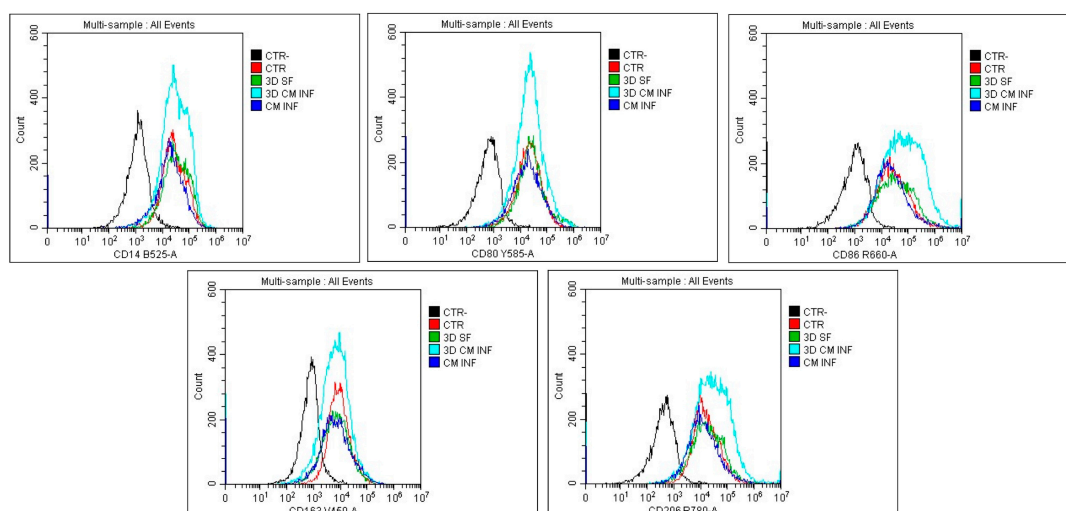


Figure 7. Flow cytometry analysis of immune cell surface marker expression under inflammatory conditions. Representative overlaid histograms show the fluorescence intensity distribution of CD14, CD80, CD86, CD163, and CD206 in PHA-activated PBMCs cultured under different experimental conditions: negative control (CTR−), activated control (CTR), CM_{INF}, 3D+SF, and 3D+CM_{INF}. The x-axis represents fluorescence intensity (log scale), and the y-axis indicates event count. Histograms illustrate population-wide shifts in marker expression across treatments, with distributions remaining predominantly unimodal.

Moreover, macrophage-associated markers were analyzed in total, scaffold-adherent, and supernatant fractions to evaluate 3D scaffold/CM_{INF}-specific effects across experimental conditions (Figures 8–10).

In the total cell population, no significant differences were detected for CD14, CD80, and CD163 MFI across all treatment groups ($p > 0.05$; Figure 7). In contrast, CD86 MFI was significantly increased in both LPS and 3D+CM_{INF} conditions compared to CTR ($p < 0.05$, Figure 8A), with 3D+CM_{INF} also showing significantly higher CD86 expression than IL-4, CM_{INF}, and 3D ($p < 0.05$, Figure 8A). Notably, CD206 MFI was significantly increased among treatments, with IL-4, CM_{INF}, and 3D+CM_{INF} vs. CTR ($p < 0.05$, Figure 7). These data confirm the results observed in the IF analysis (Figure 6).

In the scaffold-adherent fraction, MFI levels of all analyzed markers were comparable between 3D and 3D+CM_{INF} groups, except for CD86 and CD206 ($p > 0.05$, Figure 8B). Particularly, markers CD86 and CD206 showed significantly higher MFI within 3D+CM_{INF} compared to 3D ($p < 0.05$; Figure 8B), confirming the results obtained with IF (Figure 5). In contrast, in the supernatant fraction, CD163, CD86, and CD206 expression was significantly higher in 3D+CM_{INF} compared to 3D ($p < 0.05$, Figure 8C).

To further characterize how these marker expression changes translated into distinct macrophage phenotypes, a combinatorial flow cytometry analysis was performed using paired surface marker expression as follows: M1 (CD80⁺CD86⁺), M2a (CD163⁺CD206⁺), M2b (CD86⁺CD80[−]), and M2c (CD206⁺CD163[−]). Macrophage subsets were operationally defined based on established combinations of the selected surface markers, acknowledging that these phenotypes represent context-dependent functional states rather than fixed lineages. Bivariate dot plots were used to visualize marker co-expression and population distribution across total, scaffold-adherent, and supernatant fractions (Figure 9), while histogram-based analyses were used to compare the relative abundance of the corresponding macrophage subpopulations across experimental conditions (Figure 10).

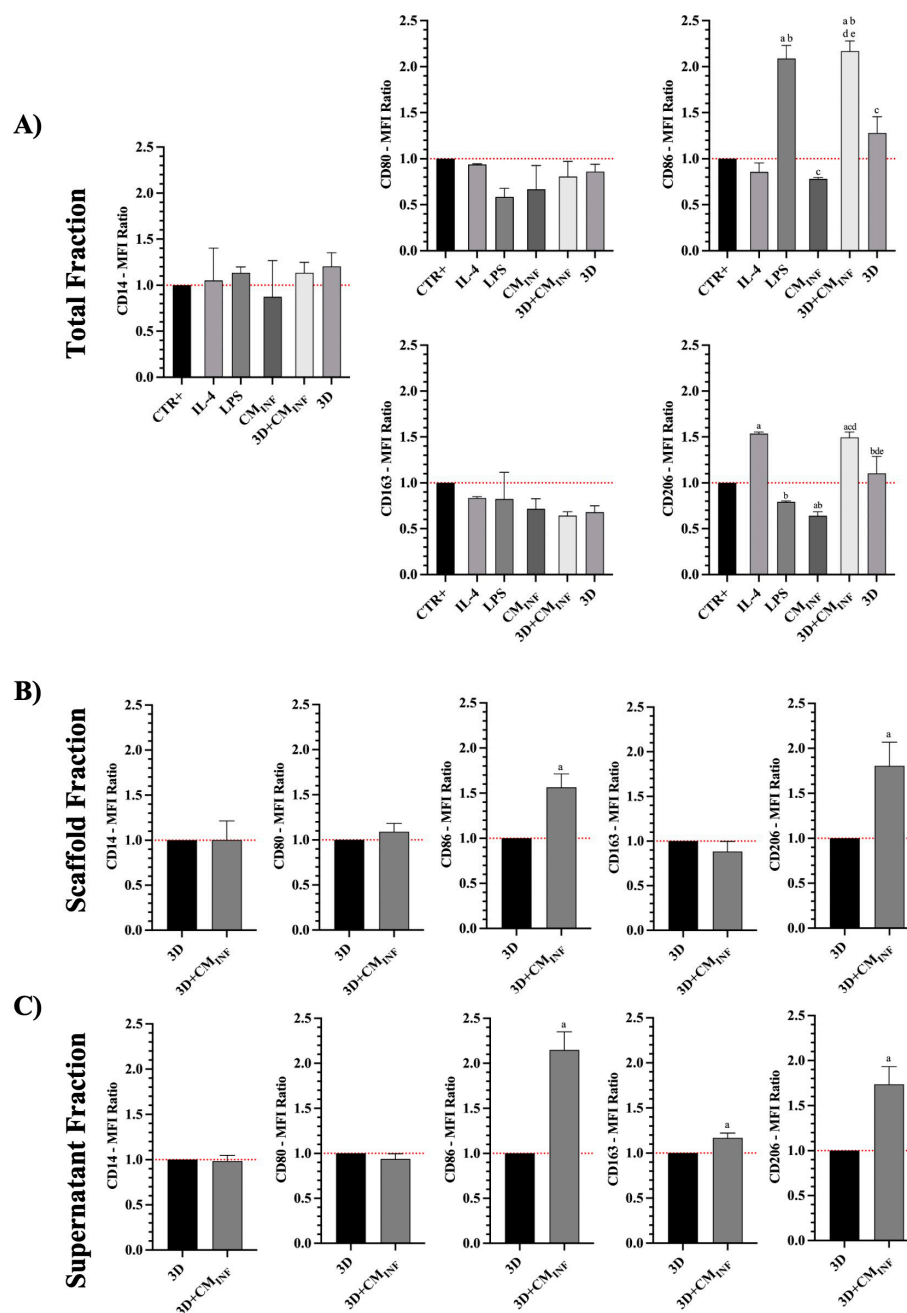


Figure 8. Immunomodulatory effect of CM_{INF} and CM_{INF}-functionalized 3D scaffolds on activated PBMCs. Representative histograms of the MFI of CD14, CD80, CD86, CD163, and CD206 within PHA-activated PBMCs subjected to different treatments: (A) total, (B) scaffolds, and (C) supernatant fractions. Data were normalized to the CTR (panel (A)) and 3D (panels (B,C)) conditions, respectively, and are presented as mean $\pm$ S.D.; the red line indicates the corresponding reference condition in each panel. Statistically significant values between the different studied groups were obtained for at least $p < 0.05$. For the total fraction: ^a vs. CTR, ^b vs. IL-4, ^c vs. LPS, ^d vs. CM_{INF}, and ^e vs. 3D+CM_{INF}. For the supernatant fraction: ^a vs. 3D+CM_{INF}.

In Figure 9, representative bivariate flow cytometry dot plots illustrate the co-expression of macrophage-associated surface markers in PHA-activated PBMCs across the total, scaffold-adherent, and supernatant fractions under the different experimental conditions. In the total fraction, dot plots are shown for CTR, IL-4, LPS, CM_{INF}, 3D+SF, and 3D+CM_{INF}, providing an overview of macrophage phenotypic distribution in the whole cell population. In the scaffold-adherent and supernatant fractions, dot plots are reported for 3D+SF

and 3D+CM_{INF} conditions, allowing visualization of the phenotypic distribution of cells directly interacting with the scaffold or present in the culture medium. Quadrant gating based on paired marker expression enables qualitative comparison of macrophage-associated phenotypes across fractions and treatments.

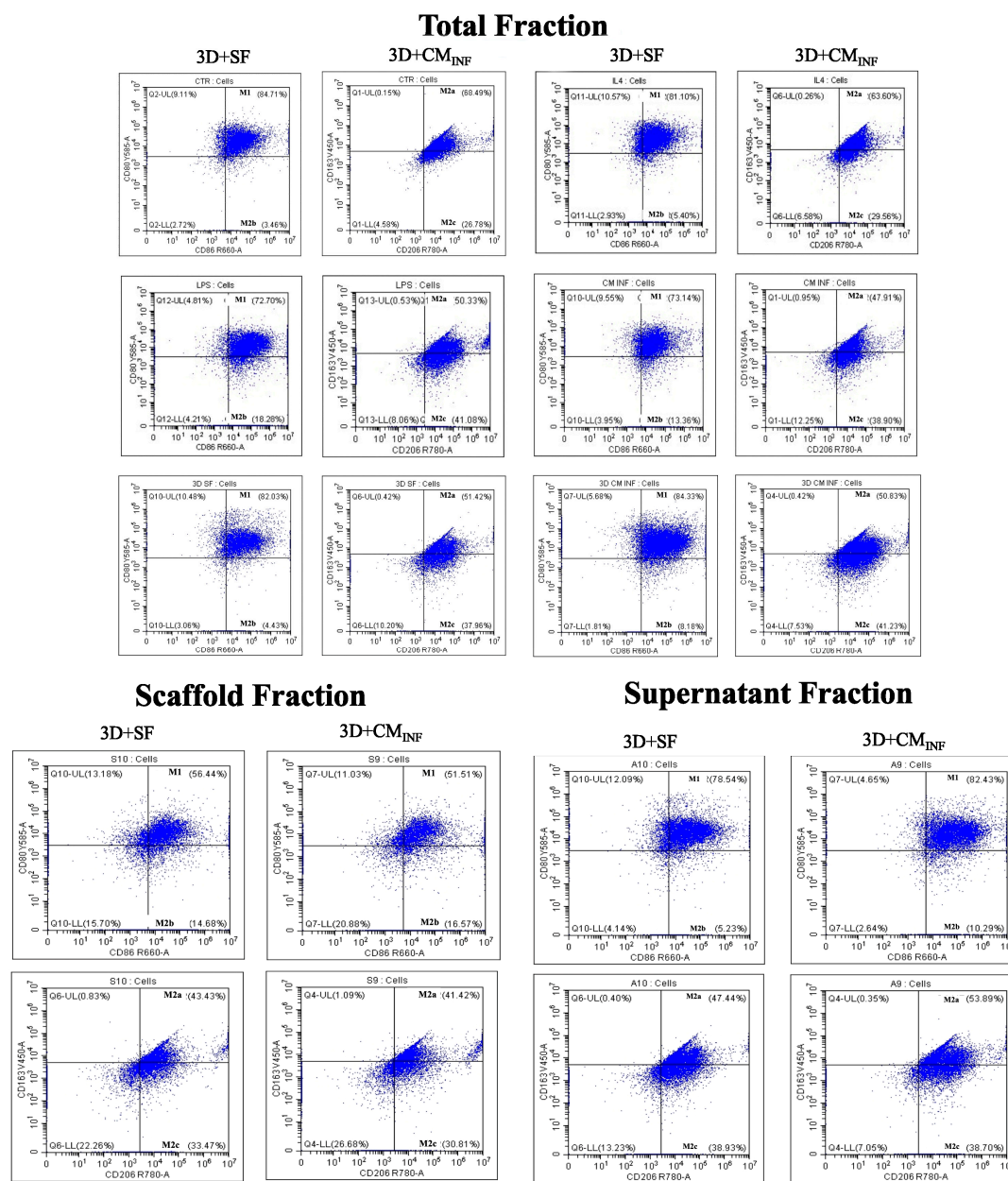


Figure 9. Bivariate flow cytometry dot plot analysis of macrophage-associated surface marker co-expression in PHA-activated PBMCs. Representative dot plots show the co-expression of CD80/CD86 and CD163/CD206 used to define macrophage phenotypic subsets within the total, scaffold-adherent, and supernatant fractions. In the total fraction, PBMCs subjected to CTR, IL-4, LPS, CM_{INF}, 3D+SF, and 3D+CM_{INF} are shown. In the scaffold-adherent and supernatant fractions, dot plots are reported for 3D+SF and 3D+CM_{INF} conditions. Quadrant gates indicate the distribution of cells according to paired marker expression, providing a qualitative overview of macrophage phenotypic distribution across experimental conditions.

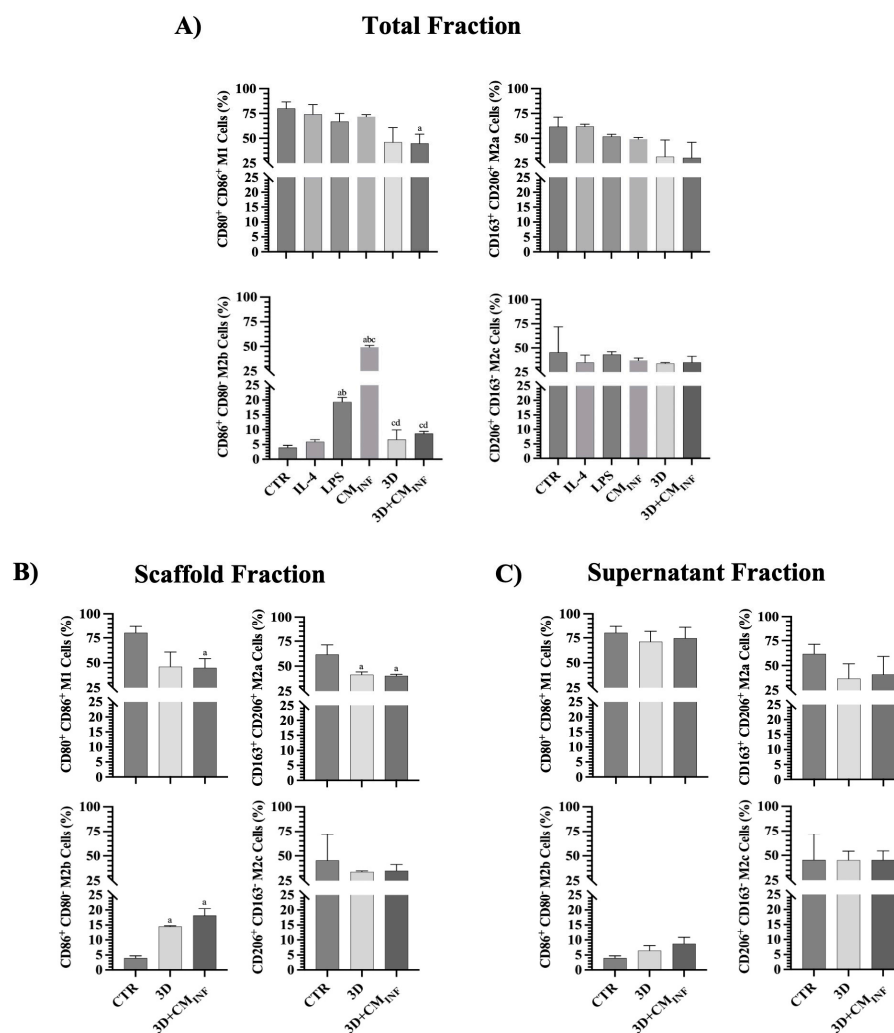


Figure 10. Phenotypical analysis of macrophage subpopulation within PHA-activated PBMCs exposed to CM_{INF} and 3D+CM_{INF}. Representative histograms of the macrophagic subpopulations identified within the PHA-activated PBMCs subjected to different treatments combining different markers: M1 (CD80⁺CD86⁺), M2a (CD163⁺CD206⁺), M2b (CD86⁺CD80⁺), and M2c (CD206⁺CD163⁺) within (A) total, (B) scaffolds, and (C) supernatant fractions. Data are presented as mean $\pm$ S.D. Statistically significant values between the different studied groups were obtained for at least $p < 0.05$. For total fraction: ^a vs. CTR, ^b vs. IL-4, ^c vs. LPS, and ^d vs. CM_{INF}.

Based on these distribution patterns, macrophage subpopulations were quantitatively compared across experimental conditions according to their defined marker combinations (Figure 10). In the total fraction, the pro-inflammatory M1 subset (CD80⁺CD86⁺) was significantly reduced in 3D+CM_{INF}-treated cultures compared to CTR ($p < 0.05$, Figure 10A). However, while M2a (CD163⁺CD206⁺) and M2c (CD206⁺CD163⁺) subpopulations remained largely unchanged across conditions, the M2b subset (CD86⁺CD80⁺) was notably expanded in LPS, CM_{INF}, 3D, and 3D+CM_{INF} groups compared to CTR and IL-4 ($p < 0.05$, Figure 10A). Soluble CM_{INF} induced the strongest M2b expansion overall ($p < 0.05$, Figure 10A), but interestingly, both 3D and 3D+CM_{INF} led to lower M2b (CD86⁺CD80⁺) levels than soluble CM_{INF}, suggesting that scaffold-functionalization modulates the availability or spatial presentation of CM_{INF}, and that scaffold-mediated presentation spatially constrains and modulates the magnitude of M2b expansion compared to soluble exposure, resulting in localized immune instruction rather than system-wide skewing. Interestingly, analysis of macrophages that adhered to scaffold surfaces (scaffold fraction; Figure 10B) revealed that both 3D and 3D+CM_{INF} were associated with significant changes in macrophage

subpopulation distribution compared to CTR conditions. The pro-inflammatory M1 subset (CD80⁺CD86⁺) was significantly reduced in 3D+CM_{INF}-treated cultures compared to CTR ($p < 0.05$, Figure 10B). No significant differences in M1 expression were observed between 3D and 3D+CM_{INF} ($p > 0.05$, Figure 10B). Importantly, both 3D scaffold conditions significantly decreased the M2a (CD163⁺CD206⁺) subset compared to CTR, indicating that this modulation was dependent on the scaffold itself. Conversely, M2b (CD86⁺CD80⁻) subsets were significantly elevated in both 3D scaffold-treated cultures compared to CTR, with more pronounced expression in the 3D+CM_{INF}-treated culture ($p < 0.05$, Figure 10B). The M2c (CD206⁺CD163⁻) subset remained unchanged across all treatments ($p > 0.05$, Figure 10B). Regarding the supernatant fraction (Figure 10C), no significant differences were observed among experimental conditions for any of the macrophage subsets examined ($p > 0.05$, Figure 10C).

3. Discussion

The present study investigated the immunomodulatory potential of a validated 3D tendon biomimetic PLGA scaffold [10,18] functionalized with CM_{INF} derived from AECs, known for their potent immunomodulatory potential [32,33,36]. The aim was to develop a cell-free, immune-informed biomaterial platform capable of modulating the immune response to support tendon regeneration. The development of such immunomodulatory scaffolds represents a key innovation in tendon immunoengineering, where precise control of inflammation is essential for effective and functional tissue repair [50]. Electrospun scaffolds offer a versatile foundation for immunoengineering, enabling functionalization with bioactive molecules that mimic the native tissue environment and influence immune cell behavior [11,46,51].

While many approaches have incorporated isolated factors or growth factors to stimulate healing, these strategies are inherently limited by the narrow molecular spectrum they deliver, often insufficient for the complex orchestration of tenogenesis and inflammation resolution [52–57]. In contrast, stem cell-derived secretomes such as CM_{INF} from AECs provide a broad and physiologically relevant mixture of cytokines, growth factors, and extracellular vesicles, offering more comprehensive immunomodulatory capabilities [10,18,32–34].

Before functionalization, the structural and biomechanical features of the electrospun 3D constructs were carefully evaluated, as anisotropic fiber organization and tensile competence are essential requirements for tendon-mimetic scaffolds. SEM analysis confirmed a defect-free, highly aligned fibrous architecture, and orientation histograms displayed narrow Gaussian distributions indicative of preserved anisotropy. Although chemical pretreatments slightly broadened fiber angle distributions, particularly following HCl exposure, the overall aligned architecture was preserved. Such structural preservation is critical, as fiber alignment directly influences cell guidance, mechanotransduction, and ECM deposition in tendon TE.

Mechanical testing further demonstrated that NaOH pretreatment preserved ultimate tensile strength and Young's modulus within the same mechanical range as untreated PLGA scaffolds, whereas HCl pretreatment was associated with reduced stiffness and elongation capacity. Mechanical strength is a critical parameter for implantable biomaterials, as scaffolds should mimic the mechanical behavior of the target tissue to provide adequate support during the remodeling phase. In the present study, NaOH-treated scaffolds maintained tensile properties compatible with tendon-oriented applications, preserving both anisotropic architecture and biomechanical competence [58]. This observation is consistent with a previous report on hydrolyzed PLGA nanofibrous membranes, in which alkaline treatment enhanced surface reactivity without significantly altering Young's modulus compared to pristine membranes [58]. Although

absolute modulus values differ due to variations in scaffold architecture and fabrication parameters, both studies converge on the key principle that controlled alkaline hydrolysis can improve surface reactivity while preserving bulk mechanical integrity.

Following structural and mechanical validation, surface hydrolysis induced by NaOH introduced carboxylate groups ($-\text{COO}^-$), as confirmed by FT-IR spectroscopy, thereby increasing scaffold hydrophilicity and molecular reactivity [59,60]. These alterations are consistent with previous studies showing that alkaline treatment improves wettability and promotes protein immobilization by introducing carboxyl functionalities on polyester-based biomaterials [45,49,61–63]. The spectral shifts observed after CM_{INF} functionalization on NaOH-pretreated scaffolds further support the establishment of stable scaffold–biomolecule interactions compared to PA and HCl pretreatment.

Collectively, these findings indicate that NaOH-mediated surface activation enhances protein adsorption through increased hydrophilicity, electrostatic attraction via carboxyl groups, and improved availability of non-covalent binding sites [64–66], while maintaining the structural anisotropy and biomechanical properties required for tendon-oriented applications. The integration of preserved architecture, mechanical stability, and improved adsorption capacity, therefore, provided the rationale for selecting NaOH pretreatment for subsequent biological investigations.

Release studies were subsequently conducted on NaOH-pretreated scaffolds, which demonstrated superior protein adsorption efficiency and more stable scaffold–biomolecule interactions compared to PA and HCl pretreatment based on Bradford quantification and FT-IR analysis. On this basis, NaOH activation was selected as the most suitable functionalization strategy for further kinetic evaluation. Within this context, release kinetics were investigated by comparing CM_{INF} -loaded scaffolds with recombinant AREG-loaded scaffolds, both applied onto NaOH-pretreated constructs. The aim of this analysis was not to compare release behavior across all pretreatment strategies but rather to determine whether the selected activation method could provide sustained and biologically relevant delivery of secretome components relative to a single recombinant factor.

Release experiments were performed under controlled agitation conditions to promote homogeneous diffusion and minimize boundary-layer effects, thereby providing a dynamic *in vitro* framework for assessing protein retention and release within a consistent surface activation context. A biphasic release profile was observed for CM_{INF} -loaded scaffolds, characterized by an initial release phase followed by a more gradual and sustained delivery over a 7-day period of AREG, a representative immunoregulatory protein within CM_{INF} , known for its roles in tissue repair and M2 macrophage modulation [33,67]. Compared to recombinant AREG alone, CM_{INF} exhibited a slower and more consistent release pattern, likely reflecting the complex composition of the secretome, in which soluble factors and extracellular components may contribute to retention within the scaffold matrix [68–70]. Although the *in vitro* setup cannot fully replicate the complex fluid turnover of the *in vivo* tendon environment, the use of agitation provides a conservative model of protein exchange rather than a purely static incubation condition.

Importantly, the 7-day release window aligns with the duration of the early inflammatory phase in tendon healing, during which immune-mediated events critically regulate macrophage polarization and shape downstream reparative vs. regenerative trajectories [7,71]. Within this framework, the release profile of CM_{INF} -functionalized 3D scaffolds should be interpreted as immune-phase targeting rather than as an attempt to span the entire regenerative cascade [7,25,70,72,73]. By focusing on the acute inflammatory window, the platform is designed to intervene during the biologically decisive phase of immune modulation, when macrophage programming exerts maximal influence on subsequent healing outcomes.

Functionally, both soluble CM_{INF} and CM_{INF}-functionalized scaffolds exhibited marked immunosuppressive effects, significantly reducing PBMC proliferation and T-cell NFAT activation. Notably, this activity was preserved despite the use of frozen CM_{INF}, indicating that the bioactive cargo of the CM remains functionally stable under the applied storage conditions. This observation is consistent with previous molecular characterizations demonstrating that frozen AEC-derived CM retains extracellular vesicle-associated lipid mediators involved in lipid-mediated signaling and ER/mitochondria-related pathways, supporting the structural and functional preservation of the secretome after freezing [33,34]. Importantly, the AREG concentrations detected in CM_{INF} were within the range previously reported to inhibit PBMC activation. In the study by Prencipe et al. [33], AREG was demonstrated to be the key downstream effector of the COX-2/PGE₂/EP₄ axis and essential for the immunosuppressive properties of AEC-derived CM: AREG neutralization abolished PBMC suppression, while exogenous AREG addition restored it. In the present work, AREG was employed as a quantifiable surrogate marker to monitor scaffold functionalization and release behavior, rather than as the sole functional effector of CM_{INF}. While AREG contributes to the immunomodulatory activity of AEC-derived CM [33], the differential immune kinetics observed in this study suggest a multi-component mode of action likely involving both soluble mediators and vesicle-associated cargo previously characterized in AEC-derived secretomes [33,34]. Taken together, the results indicate that scaffold-mediated delivery preserves the functional immunomodulatory properties of the complex secretome over time, without implying that AREG alone accounts for the observed biological effects. Indeed, these results align with existing evidence showing that biomaterial-mediated delivery can potentiate the therapeutic efficacy of immunomodulatory factors [15,74,75].

Temporal profiling of the released CM_R collected from 3D+CM_{INF} revealed differential effects on immune cell subsets. Notably, PBMC proliferation was inhibited starting at 48 h, with maximal suppression at 7 d, whereas NFAT activation in T cells was significantly reduced at 6 h up to 72 h but declined over time. The differential temporal sensitivity between PBMCs and T cells suggests cell-type-specific responses to distinct components/molecules within CM_{INF} [10,32,34] with varying half-lives, receptor affinities, and cell-specific targets. Similar patterns of differential immune cell responsiveness have been observed with mesenchymal stem cell secretomes, suggesting a common feature of stem cell-derived therapeutic products [76–79]. Such temporally distinct immunomodulation may benefit tendon healing by dampening acute inflammation early (via T-cell suppression) while facilitating more regulated resolution in later stages (via PBMC inhibition) [80–82].

Most significantly, CM_{INF}-functionalized scaffolds were associated with significant changes in macrophage-associated marker expression patterns within PHA-activated PBMC cultures. It should be noted that macrophage polarization states are highly plastic and context-dependent, and the subsets described herein are operationally defined based on established surface-marker combinations rather than representing fixed or terminal phenotypes [12–14]. The 3D architecture and fiber topography of the scaffold alone allowed an enrichment of M2 marker expression profiles, likely through biophysical cues that guide monocyte adhesion and fate. This result was further modulated by co-culturing PBMCs on functionalized CM_{INF} scaffolds, highlighting a synergistic immunomodulatory environment. Analysis of macrophage markers (CD86, CD206, CD163) revealed a distinct shift toward anti-inflammatory, pro-regenerative phenotypes within scaffold-adherent populations, suggesting that secretome-loaded scaffolds modulate the localized immune microenvironment at the biomaterial interface, an effect that could be leveraged in vivo to enhance regenerative outcomes [6,7,83].

Importantly, CM_{INF}-functionalized scaffolds were associated with an increased representation of the M2b (CD86⁺CD80⁻) regulatory subset, known for its role in maintaining immune balance while supporting tissue repair. This subset has been described as exhibiting context-dependent regulatory properties, often characterized by IL-10 production alongside moderated pro-inflammatory cytokine expression (e.g., IL-12) [84–87]. In the present study, M2b identification is based on surface-marker combinations and does not directly imply a defined cytokine secretion profile. The induction of an M2b regulatory phenotype by 3D-CM_{INF} scaffolds suggests a shift toward an immune-modulatory microenvironment potentially favorable for tissue repair. While M2b macrophages have been associated with regulatory and anti-fibrotic functions in certain contexts, our data are limited to phenotypic marker expression and do not directly establish the cytokine secretion profile or functional regenerative outcome. Conversely, CM_{INF} functionalization showed reduced proportions of M2a (CD163⁺CD206⁺) macrophages, a subset commonly associated with pro-fibrotic signaling and excessive ECM deposition through TGF- β and IGF production [9,84,88]. Although these observations are derived from an in vitro immune model, they support the hypothesis that selective modulation of macrophage phenotypic distribution by 3D+CM_{INF} may contribute to an immunological context potentially less permissive to fibrotic remodeling. The spatial confinement of these effects, mainly within the scaffold-adherent macrophage population, highlights the importance of local delivery and cellular interaction. Non-adherent immune cells showed minimal phenotypic changes, suggesting that direct cell-scaffold contact is key to effective phenotypic modulation. This compartmentalized immune modulation represents a novel approach to fine-tuning regenerative environments.

The differential effects observed between total and scaffold-adherent fractions further emphasize the importance of spatial presentation and localized delivery, as CM_{INF} functionalization appeared to modulate macrophage-associated marker expression patterns specifically within the scaffold microenvironment. The scaffold-mediated enrichment of M2b subsets relative to M2a profiles within the adherent fraction suggests spatially confined modulation of macrophage-associated phenotypic distribution rather than systemic amplification of immune responses. This suggests that the scaffold does not amplify immunomodulatory potency per se but rather refines the intensity and localization of the immune response through controlled release dynamics. In this context, the observed redistribution may contribute to a localized regulatory environment compatible with tissue repair. A balanced immune environment is crucial to support tissue regeneration by avoiding both excessive inflammation and premature resolution that might impair proper tissue remodeling [83,89].

These results highlight the therapeutic rationale of the developed immune-informed, cell-free scaffolds (3D+CM_{INF}) capable of selectively modulating the inflammatory response rather than inducing broad immune suppression, a feature that is critical to preventing fibrotic repair and supporting functional tendon regeneration [9,37,83]. The present in vitro study represents a mechanistic preclinical validation step, not proof of regenerative efficacy in vivo, aimed at defining immune-modulatory mechanisms prior to animal testing. By combining structural support with the controlled delivery of stem cell-derived paracrine factors [90,91], the platform enables a multi-targeted immunomodulatory strategy that extends beyond single-factor approaches [45] while avoiding the regulatory and manufacturing constraints associated with cell-based therapies. In this context, the simplicity, scalability, and GMP compatibility of the NaOH-based functionalization method further support the translational relevance of the proposed system. Based on our data, scaffold topology alone partially influences macrophage phenotypes; however, the addition of the secretome significantly modulates

and spatially refines this effect, promoting a more favorable and controlled immunomodulatory profile. Therefore, the observed immunomodulation is best interpreted as a synergistic interaction between physical (topological) and biochemical (secretome-mediated) cues, rather than the dominance of one over the other.

It is important to note that the release profile of CM_{INF} from 3D scaffolds was designed to target the early inflammatory phase of tendon healing, rather than to span the entire regenerative process. In tendons, immune-mediated events occurring within the first week post-injury critically influence macrophage polarization and downstream reparative trajectories [7,9]. Accordingly, the observed 7-day release window was selected to align with this biologically relevant timeframe, during which immunomodulation is expected to exert maximal impact on subsequent healing outcomes. Within this framework, the present work provides a mechanistic and functional validation of immune modulation at the biomaterial-immune cell interface, establishing a necessary foundation for subsequent *in vivo* investigations.

Nonetheless, some limitations must be acknowledged. The present study was conducted exclusively *in vitro*, and *in vivo* validation will be required to confirm scaffold integration, immune response, and regenerative efficacy in tendon injury models. Moreover, while macrophage polarization profiles provide valuable insight into the immunological environment shaped by the scaffold, this study does not directly assess fibroblast activation, extracellular matrix deposition, or tissue-level remodeling. Moreover, among the macrophage activation spectrum, these cells are highly plastic cells that can transition towards a broad array of functional states. For this reason, the *in vitro* macrophage subsets observed do not necessarily reflect stable *in vivo* phenotypes. Therefore, the proposed link between selective modulation of macrophage subsets and attenuation of fibrotic outcomes remains to be directly validated. Future studies will address these aspects through analyses of fibroblast activation markers (i.e., α -SMA expression), collagen expression (collagen I/III ratios) and organization, and angiogenic responses, together with *in vivo* validation in tendon injury models. In addition, further molecular characterization of CM_{INF}, including proteomic profiling and identification of critical quality attributes, will be required to support clinical-grade standardization [92,93] and deepen mechanistic understanding of the observed immunomodulatory effects. Additionally, elucidating the contributions of different secretome fractions (soluble factors vs. extracellular vesicles) to the observed effects would advance our understanding of the therapeutic mechanisms and inform future optimization strategies. Finally, while the current platform focuses on early immune modulation, future scaffold designs may explore sequential or multi-phase release strategies to address later proliferative and remodeling stages of tendon healing.

4. Materials and Methods

4.1. Ethical Statement

AECs were obtained from the amniotic membranes of Appenninica breed sheep, sourced as discarded reproductive tissues from animals processed for food consumption. Simultaneously, ovine peripheral blood mononuclear cells (PBMCs) were isolated from blood freshly collected after slaughter. As these samples were derived from waste tissues, no ethical approval was required for their use.

4.2. AECs Isolation and Culture

As previously described by Barboni et al. [31], amniotic membranes were collected from ovine fetuses at approximately 2–3 months of gestation (mid-gestation) to isolate AECs. The epithelial layer of the amniotic membrane was enzymatically digested using

0.25% Trypsin-EDTA (Sigma Chemical, St. Louis, MO, USA), and the enzymatic activity was blocked by adding 10% Fetal Bovine Serum (FBS). The resulting cell suspension was filtered through a 40 µm cell strainer, followed by centrifugation. AECs were then counted using the LUNA-II™ Automated Cell Counter (Logos Biosystems Inc., Dongan-gu Anyang-si, Republic of Korea) with trypan blue vital staining.

Before use, AECs were characterized by the absence of hematopoietic markers (CD14, CD58, CD31, and CD45), positivity for adhesion molecules (CD29, CD49f, and CD166) and stemness markers (TERT, SOX2, OCT4, and NANOG), low expression of MHC class I, and the absence of MHC class II (HLA-DR) antigens, in accordance with previously published reports [26,31,36,37]. Additionally, freshly isolated AECs were tested for the absence of tendon-related gene markers, including SCX, COL1, and TNMD [18,27,31,36,94].

Fresh AECs were seeded at a density of 3×10^3 cells/cm² on Petri dishes and cultured in α -Minimum Essential Medium Eagle (α -MEM) supplemented with 20% FBS, 1% ultraglutamine, 1% amphotericin, and 1% penicillin/streptomycin. Cells were incubated at 38.5 °C with 5% CO₂ until they reached 70–80% confluence before use for further experiments.

4.3. Production of Conditioned Media with Immunomodulatory Properties (CM_{INF})

The production of AECs' inflammatory conditioned media (CM_{INF}) was performed according to the previously published protocol [33]. Briefly, the cells at 70–80% confluence were incubated at 38.5 °C with 5% CO₂ in a serum-free medium (SF) for 4 h and then stimulated with lipopolysaccharides (LPS, 10 µg/mL) for 1 h. Afterwards, the cells were washed and maintained in SF conditions for an additional 24 h at 38.5 °C with 5% CO₂. At the end of the culture period, CM_{INF} was recovered and centrifuged at 2500 rpm for 10 min to eliminate any residual cellular debris. The collected CM_{INF} was aliquoted and stored at −80 °C. All subsequent functionalization procedures and biological assays were performed using thawed CM. This approach was adopted based on previous evidence demonstrating that freezing and thawing do not alter the overall biological activity and molecular integrity of AEC-derived CM [33,34]. Moreover, the CM_{INF} used in the present study was previously characterized at proteomic and lipidomic levels [34], where both soluble and extracellular vesicle fractions were extensively analyzed.

The CM_{INF} was tested for Amphiregulin (AREG) concentration, selected as a representative immunomodulatory molecule secreted by AECs [33,67], using the “Sheep Amphiregulin (AREG) ELISA kit” (#MBS044705; MyBiosource, Southern California, San Diego, CA, USA). The assay was performed according to the manufacturer's instructions. The AREG concentration recorded was then considered for functionalization procedures.

4.4. Fabrication of 3D PLGA Scaffolds

Poly (lactide-co-glycolide) (PLGA, PLG8523) scaffolds with highly aligned fibers were fabricated, according to previously optimized and validated protocols [10,27,28], through electrospinning using a commercial E-Spintronic electrospinning apparatus with a climate control machine. In detail, a PLGA 12% (%wt/wt) solution was prepared by dissolving the polymer in hexafluoroisopropanol under magnetic stirring overnight. The equipment setup for the electrospinning consisted of a 3 mL syringe connected to a polytetrafluoroethylene tube (PTFE) and a stainless-steel needle. The electrospinning process was carried out at about 22.5 °C and an air humidity of about 65% [10,27,28]. Other conditions applied were voltage = 33 kV and flow rate = 0.25 mL/h. Fibers were collected using baking paper on a rotating drum collector, with a rotational speed of 1000 rpm. The resulting PLGA fleeces were obtained by electrospinning 250 µL of PLGA solution.

To obtain the 3D scaffolds, the fabricated PLGA fibers were cut into rectangular pieces with a length of 1.5 cm and a width of 0.7 cm, and subsequently shaped into a three-dimensional form [10,18].

4.4.1. Imaging and Morphological Analysis of Untreated and Chemically Pretreated 3D Scaffolds

The ultrastructural morphology of the net (untreated) and chemically pretreated electrospun PLGA scaffolds was assessed by Field-Emission Scanning Electron Microscopy (FE-SEM; Supra 55 VP SEM, Carl Zeiss AG, Jena, Germany). Samples were sputter-coated with gold prior to imaging and observed at an accelerating voltage of 5 kV, according to previously published data [10,18,27,29]. Fiber morphology and diameter size were measured using ImageJ software (ImageJ 1.54i, NIH, USA). Approximately 100 randomly selected fibers per sample were measured from SEM micrographs ($n = 3$ independent scaffolds per group), and the average fiber diameter was calculated.

Fiber orientation was analyzed using the Directionality plugin in ImageJ ($n = 3$ per group). This tool divides images into square regions and computes Fourier power spectra to determine orientation parameters. The output generates Gaussian distributions of fiber angle alignment characterized by direction (center of the Gaussian) and dispersion (standard deviation of the Gaussian curve). Dispersion values were used as quantitative indicators of angular variability and fiber alignment.

4.4.2. Mechanical Properties Assessment of Untreated and Chemically Pretreated 3D PLGA Scaffolds

Mechanical properties of net PLGA and chemically pretreated PLGA scaffolds were evaluated by uniaxial tensile testing under dry conditions. Rectangular specimens (5 cm in length) were mounted onto a Texture Analyzer TA.XT2i (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell. The gauge length was set at 50 mm, and samples were secured between two clamps prior to testing. Tensile loading was applied at a constant crosshead speed of 1 mm/min until complete failure of the specimen.

Before mechanical testing, the thickness of electrospun fleeces and the diameter of rolled 3D scaffolds were measured using a digital micrometer in order to calculate the cross-sectional area required for stress determination. Mechanical parameters were derived from the stress–strain curves and included ultimate tensile strength (UTS, MPa), elongation at break (%), and Young's modulus (MPa). For each experimental group, five independent samples were analyzed ($n = 5$ for each experimental group).

4.5. Functionalization of 3D PLGA Scaffolds

Three different scaffold functionalization methods were employed to evaluate the most effective approach for maximizing the adsorption of bioactive factors from CM_{INF} onto the scaffold surface.

For this purpose, 3D scaffolds were treated according to previous works [45,47–49] with minor modifications, as follows:

1. Physical adsorption (PA) [47].
2. Acid pre-treatment: Scaffolds were pretreated with 2 M HCl for 24 h at 37 °C under agitation before functionalization [45,48].
3. Alkaline pre-treatment: Scaffolds were pretreated with 0.1 M NaOH for 20 min at 37 °C under agitation before functionalization [45,49].

Following HCl and NaOH pre-treatment, 3D scaffolds were washed with DPBS to neutralize their pH. The pH of the DPBS wash solution was assessed using pH indicator

strips to confirm neutralization. PA, as well as NaOH- and HCl-pretreated scaffolds, were incubated for 24 h at 37 °C under agitation as follows:

- Incubation with 1 mL of CM_{INF} (3D+CM_{INF}) produced as described in Section 4.3.
- Incubation with 1 mL of purified AREG recombinant protein (MBS963544, MyBiosource, Southern California, San Diego, CA, USA; 3D+AREG) prepared at the same concentration recorded in CM_{INF} as previously described in Section 4.3. AREG was selected as a representative potency marker present within CM_{INF} [33,34].

The incubation of 3D scaffolds with SF (3D) was used as an internal control for the experiments. All 3D scaffolds before and after functionalization were used for further investigations.

4.6. Chemical Characterization of Untreated and HCl- and NaOH-Treated 3D Scaffolds Before and After Functionalization

Fourier transform infrared spectroscopy (FT-IR) was used to characterize both the media (SF, SF+AREG, and CM_{INF}) and the 3D scaffolds before and after surface modifications. All spectra were acquired with a Nicolet Apex FIRT spectrometer (Thermo Fisher Scientific, S.p.A., Milan, Italy) at 4 cm⁻¹ resolution (4000–650 cm⁻¹; 64 accumulations).

Liquid samples (50 µL) were analyzed prior to scaffold functionalization to verify their chemical composition. For scaffold analysis, the following groups were examined (n = 3 per condition): untreated PLGA scaffolds, scaffolds subjected to different pre-treatments (HCl and NaOH), and pretreated scaffolds after functionalization with SF, SF+AREG, or CM_{INF}, together with those functionalized through the PA method. All scaffolds were dried at room temperature for 24 h before being placed directly on the ATR crystal for spectral acquisition.

Characteristic peaks were identified and assigned to specific functional groups to assess chemical modifications resulting from various treatments.

4.7. 3D Scaffolds Functionalization Efficiency

The functionalization efficiency (FE) of 3D scaffolds under all conditions was assessed by measuring the total protein content in the pre- and post-functionalization media (CM_{INF}) using the Bradford assay, according to the manufacturer's instructions (Quick Start™ Bradford 1× Dye Reagent, Bio-Rad Laboratories, Inc., Hercules, CA, USA) [95]. After 24 h of functionalization, scaffolds were washed three times with PBS to remove unbound molecules, then incubated for 5 min with 2% SDS in diH₂O supplemented with protease and phosphatase inhibitors to extract the immobilized molecules from the scaffold surfaces. The absorbance (Ab) was measured at 595 nm using a multi-mode microplate spectrophotometer (INNO-S).

The functionalization efficiency (FE, %) was calculated using the following formula [96]:

$$FE (\%) = \frac{Ab_{\text{pre-functionalization}} - Ab_{\text{post-functionalization}}}{Ab_{\text{pre-functionalization}}} \times 100$$

This approach allowed an indirect quantification of the amount of protein adsorbed onto the 3D scaffold surface.

Based on FE results, AREG quantification was performed on pretreated NaOH and PA scaffolds using a commercially available ELISA kit (Sheep Amphiregulin ELISA kit, MyBiosource, Cat#MBS044705) according to the manufacturer's instructions.

4.8. Quantification of the Release Profile of AREG from Functionalized NaOH and PA Scaffolds

To better understand the release profile of AREG from functionalized 3D scaffolds, AREG release kinetic studies were performed on the best functionalization strategies. To this end, 3D scaffolds pretreated with PA or NaOH were functionalized with CM_{INF} (3D+CM_{INF}) or recombinant AREG protein (3D+AREG) at an equivalent concentration to that measured in CM_{INF} as described in Section 4.3. Functionalized scaffolds were subsequently incubated in SF media under agitation conditions, and the corresponding released media (CM_R) were collected at multiple time points (6, 24, 48, 72 h, and 7 d) for AREG quantification using the same ELISA kit and biological activity assessment on immune cells (Figure 11A).

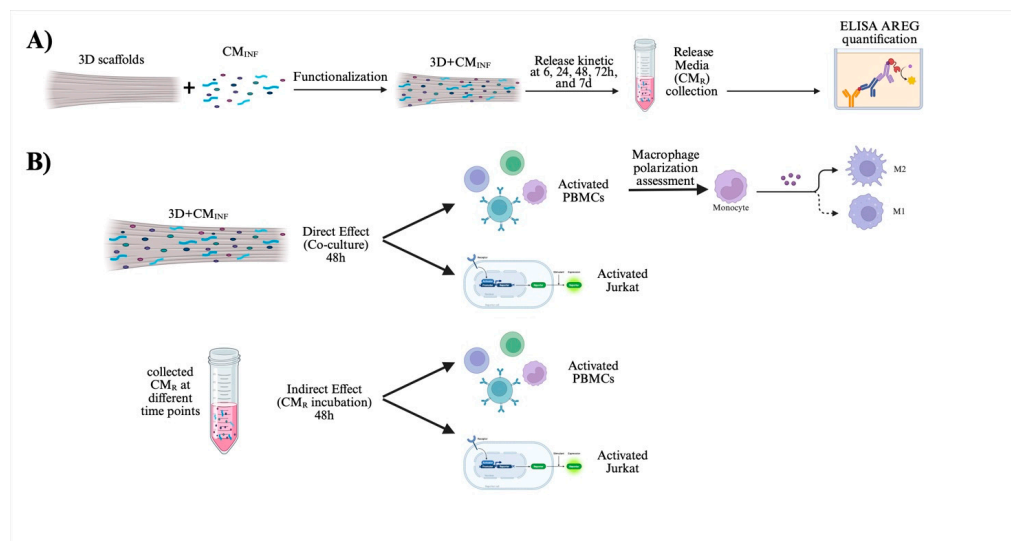


Figure 11. Experimental design. (A) Functionalization of 3D scaffolds with CM_{INF} (3D+CM_{INF}), production of released media (CM_R) at different time points (6, 24, 48, 72 h, and 7 d), and quantification of AREG from the collected CM_R. (B) Biological activity assessment of 3D+CM_{INF} (direct effect) and each CM_R collected at different time points: 6, 24, 48, 72 h, and 7 d (indirect effect) on activated PBMCs and Jurkat cells after 48 h of culture. Analysis of the 3D+CM_{INF} influence on macrophage polarization was performed on total, scaffold-adherent, and supernatant fractions.

4.9. Direct and Indirect Immunomodulatory Effects of 3D Functionalized Scaffolds on Immune Cells

The direct and indirect immunomodulatory effects of 3D scaffolds functionalized with CM_{INF} (3D+CM_{INF}) were evaluated on ovine PBMCs and Jurkat cells (Figure 11B). Direct effects were assessed by culturing immune cells in contact with 3D+CM_{INF} or with SF alone (3D), while indirect effects were tested using the media containing the released molecules from functionalized scaffolds (CM_R) (Figure 10). Activated ovine PBMCs and Jurkat cells cultured in the absence of scaffolds or CM_R were used as a positive control (CTR+), while non-activated immune cells were considered as the negative control (CTR). All experimental conditions, including CTR+ and CTR, were cultured for 48 h in SF media supplemented with 10% FBS, according to the experimental group.

Ovine PBMCs were isolated from 16 mL of peripheral blood by density gradient centrifugation using 12 mL of Ficoll-Paque™ PLUS (#GE17-1440-02; Cytiva, Marlborough, MA, USA), following the manufacturer's instructions. After isolation, PBMCs were cryopreserved in liquid nitrogen until use. PBMCs were seeded at a density of 3×10^5 cells and activated with 10 µg/mL of phytohemagglutinin (PHA; Sigma-Aldrich, Milan, Italy).

To investigate intracellular immune signaling related to cell proliferation, Jurkat-Lucia™ NFAT reporter cells (#jktl-nfat-cd28; InvivoGen, Toulouse, France) were used following previously established protocols [33,34] to assess NFAT-dependent T-cell

activation via an NFAT-inducible Lucia luciferase construct. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; #12440053; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated FBS (#10270-106; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 µg/mL penicillin/streptomycin (#DE17-602E; Lonza, Basel, Switzerland), 100 µg/mL Normocin™ (#ANT-NR-2; InvivoGen, Toulouse, France), 5 µg/mL Blasticidin (#ANT-BL-1; InvivoGen, Toulouse, France) and 100 µg/mL Zeocin® (#ANT-ZN-1; InvivoGen, Toulouse, France) at 37 °C under a 5% CO₂ atmosphere. Cells were passaged every 2 d and used after the third passage. Jurkat cells were seeded at a density of 3×10^5 cells per well in a 96-well high-binding plate (Corning #9018) pre-coated with anti-CD3 (2 µg/mL, #16-0037-81) and anti-CD28 (2 µg/mL, #16-0289-81) antibodies (Invitrogen, Thermo Fisher Scientific).

4.9.1. Direct Effects of CM_{INF}-Functionalized 3D Scaffolds (3D+CM_{INF})

For the evaluation of direct effects, activated PBMCs and Jurkat cells were cultured in contact with 3D+CM_{INF} scaffolds or non-functionalized 3D scaffolds (SF only) for 48 h [33,34]. At the end of the culture period, PBMC proliferation was quantified using the "CellTiter 96® AQueous One Solution Cell Proliferation Assay" (#G3582; Promega Corporation, Madison, WI, USA), following the manufacturer's instructions. Absorbance was measured at 490 nm using a multi-mode microplate spectrophotometer (INNO-S), and data were normalized to the CTR+ condition.

The percentage of PBMC proliferation was calculated using the following formula:

$$\text{PBMCs Proliferation (\%)} = \frac{\text{Ab}_{\text{sample}}}{\text{Ab}_{\text{CTR+}}} \times 100$$

NFAT activity in activated Jurkat cells was measured after 48 h [33,34] under one of the described conditions using the QUANTI-Luc™ Luciferase Detection Reagent (InvivoGen #REP-QLC4LG1). Luminescence was recorded with an EnSpire® Multimode Plate Reader (PerkinElmer), and the obtained results were normalized to the signal obtained from CD3/CD28-stimulated Jurkat cells.

4.9.2. Indirect Effects of the Released CM (CM_R) from 3D+CM_{INF}

To evaluate the indirect immunomodulatory effects on the considered immune cells, conditioned media released from 3D+CM_{INF} and CM_R were collected at 6 h, 24 h, 48 h, 72 h, and 7 d under agitation. The released CM from a non-functionalized 3D scaffold incubated with SF alone (SF_R) was used as a control. All collected CM_R were applied to activated PBMCs and Jurkat cells, and immunomodulatory effects were evaluated using the same proliferation and NFAT-activation assays as described above.

4.10. Effect of Functionalized 3D Scaffold on Macrophage Polarization

To evaluate the ability of functionalized 3D scaffolds to modulate macrophage subpopulations' phenotypic profile, ovine PBMCs were co-cultured for 48 h with both types of functionalized 3D scaffolds (3D+CM_{INF} and 3D) (Figure 11) and analyzed as in the following paragraphs.

4.10.1. Immunofluorescence

At the end of the PBMCs co-culture period, both types of scaffolds were washed three times with PBS (Cat# P3813; Sigma-Aldrich) and fixed with 4% paraformaldehyde (Cat# 11699404; VWR) for 15 min. Scaffolds were incubated in PBS containing 1% BSA for 1 h to block non-specific binding. Primary antibodies against CD86 (M1 marker; Serotec, Gibbstown, NJ) and CD206 (M2 marker; R&D System Cambridge, UK) (1:200 in PBS/BSA 1%) were then applied, and samples were incubated overnight at 4 °C. After washing with

PBS, samples were incubated with Alexa Fluor 488-conjugated anti-mouse (#AB150077; Molecular Probes, Göteborg, Sweden) and Cy3-conjugated anti-mouse (#AP124C; Merk Life Science S.r.l., Milan, Italy) antibodies (1:500 in PBS) to detect CD206 and CD86, respectively, for 1 h at room temperature (RT). After incubation, samples were washed in PBS (three times), followed by DAPI staining (1:2000 in PBS; #D9542; Sigma-Aldrich, St. Louis, MO, USA) to label cell nuclei. Negative controls lacking primary antibodies were included to verify staining specificity. All controls were negative.

Confocal fluorescence microscopy (Nikon A1r laser confocal microscope) was used to detect the fluorescent signal as follows: DAPI channel ($\lambda_{\text{excitation}} = 359 \text{ nm}$, $\lambda_{\text{emission}} = 461 \text{ nm}$), FITC channel ($\lambda_{\text{exc}} = 488 \text{ nm}$; $\lambda_{\text{em}} = 525 \text{ nm}$), and TRITC channel ($\lambda_{\text{exc}} = 544 \text{ nm}$; $\lambda_{\text{em}} = 570 \text{ nm}$).

The percentage of M1 and M2 macrophage subpopulations was quantified by analyzing at least 100 DAPI-positive nuclei per condition/the number of CD86⁺ (M1, red) and CD206⁺ (M2, green) cells.

4.10.2. Flow Cytometry

PBMCs were activated with PHA as described in Section 4.9. Subsequently, cells were exposed to IL-4 (20 ng/mL; #6507-IL; R&D Systems, Minneapolis, MN, USA) or LPS (100 ng/mL; #L2637; Sigma-Aldrich, St. Louis, MO, USA) as internal controls for M2 and M1 polarization, respectively, or cultured with CM_{INF}, 3D+CM_{INF}, or 3D. After 72 h of culture under different treatments, cells were washed three times with DPBS (#D8662; Sigma-Aldrich, St. Louis, MO, USA) and processed for flow cytometric analysis to characterize macrophage subpopulations. Total, scaffold-associated, and supernatant cell fractions were analyzed. For the analysis of the scaffold-associated cells, these were detached from the scaffold using Accutase™ (#07920, Innovative Cell Technologies, Inc., San Diego, CA, USA) for 10 min. Flow cytometry was performed using a CytoFLEX SRT system (Beckman Coulter, Brea, CA, USA), acquiring 10,000 events per sample. The following fluorochrome-conjugated antibodies were used for all samples: CD163 (5 μL /test; #333612, BioLegend, San Diego, CA, USA), CD206 (5 μL /test; #321124, BioLegend, San Diego, CA, USA), CD80 (10 μL /test; #MCA2436PE, Bio-Rad Laboratories, Italy), CD86 (10 μL /test; #MCA2437A647, Bio-Rad Laboratories, Italy), and CD14 (10 μL /test; #LS-C43762, LifeSpan Biosciences, Newark, CA, USA). Excitation was performed using 405 nm, 488 nm, 561 nm, and 638 nm lasers, and fluorescence was detected in the V450, B525, Y585, R660, and R780 channels using a standard logarithmic scale. Data were analyzed and visualized using CytExpert SRT software (version 1.2.10004; Beckman Coulter, Brea, CA, USA).

4.11. Statistical Analysis

For each experimental condition, a minimum of three biological replicates was used (AECs derived from independent fetuses and PBMCs obtained from individual animals). For scaffold characterization (including morphological, mechanical, physicochemical, functionalization, and release analyses), at least three independent scaffolds per experimental group were evaluated. Each experiment was conducted in three independent experimental runs, and every run was performed in technical replicate, resulting in a minimum of nine data points per group. Results are expressed as mean $\pm$ standard deviation (S.D.). Prior to statistical testing, data distribution was assessed using the D'Agostino–Pearson and Shapiro–Wilk normality tests, and variance homogeneity was assessed with Levene's test. When more than two groups were present and both assumptions were met, one-way ANOVA followed by Tukey's post hoc test was applied. For experiments involving only two groups, comparisons were performed using either an unpaired two-tailed Student's *t*-test or the Mann–Whitney U test, depending on whether

parametric assumptions were satisfied. When datasets did not meet the criteria for parametric analyses, even after data transformation, the Kruskal–Wallis test followed by Dunn’s correction was used. Statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant for at least $p < 0.05$.

5. Conclusions

This study demonstrates, in an in vitro immune model, the development of an immune-informed 3D PLGA tendon-mimetic scaffold functionalized with immunomodulatory conditioned media (CM_{INF}) derived from AECs. The resulting platform offers a potent, cell-free immunoengineering strategy capable of modulating T-cell activation, suppressing PBMC proliferation, and inducing measurable shifts in macrophage phenotypic distribution, including enrichment of the M2b-associated subset and reduction in pro-inflammatory markers. The data indicate a spatially confined modulation of macrophage polarization through scaffold-mediated presentation of bioactive factors.

The spatio-temporal immunomodulatory effects induced by the developed immune-inspired platform suggest a controlled immune-guidance mechanism within the tested in vitro setting. Overall, this immune-responsive biomaterial represents a promising immune-instructive biomaterial platform for tendon tissue engineering, advancing the field of regenerative immunotherapy through the integration of structural, biochemical, and immunological cues.

Further in vivo validation will be required to determine the extent to which these immune-modulatory effects translate into improved tendon healing outcomes.

Author Contributions: A.M., B.B., and V.R. designed the research; M.E.K. carried out the electrospinning; M.E.K. performed the functionalization of the 3D scaffolds and ELISA analysis; C.A. and M.E.K. performed FT-IR analyses and analyzed the data; G.P., M.E.K., and M.T. performed cell cultures and collected the CM_{INF}; G.P. conducted immunological and FACS analyses and analyzed the data with M.E.K.; M.E.K.; V.G.F. conducted IF analyses; O.D.G. acquired IF images through confocal microscope; P.B. supervised IF analyses; V.R. provided the resources; M.E.K., V.R., G.P., A.M., and wrote the original draft with the involvement of all the co-authors; M.E.K., A.M., G.P., P.B., B.B., and V.R. critically revised the draft. All authors validated the data, reviewed, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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