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In vitro response of macrophage polarization to a keratin biomaterial

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ABSTRACT

Macrophage response to biomaterials is emerging as a major focus in tissue repair and wound healing. Macrophages are able to differentiate into two distinct states, eliciting divergent effects. The M1 phenotype is considered pro-inflammatory and up-regulates activity related to tissue destruction, whereas the M2 phenotype is considered anti-inflammatory and supports tissue remodeling. Both are necessary but a fine balance must be maintained as dysregulation of naïve macrophages to M1 or M2 polarization has been implicated in several disease and injury models, and has been suggested as a potential cause for poor outcomes. Keratin biomaterials have been shown using different animal models to promote regeneration in several tissues. A potential common mechanism may be the general capability for keratin biomaterials to elicit beneficial inflammatory responses during the early stages of regeneration. In the present study, a keratin biomaterial was utilized in vitro to examine its effects on polarization toward one of these two macrophage phenotypes, and thus its role in inflammation. Exposure of a monocytic cell line to keratin biomaterial substrates was shown to bias macrophages toward an M2 phenotype, while a collagen control surface produced both M1 and M2 macrophages. Furthermore, keratin treatment was similar to the M2 positive control and was similarly effective at down-regulating the M1 response. Keratin biomaterial influenced greater production of anti-inflammatory cytokines and decreased amounts of pro-inflammatory cytokines. The use of a keratin biomaterial in regenerative medicine may therefore provide additional benefit by regulating a positive remodeling response.

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1. Introduction

Biomaterials have many current and potential applications, including those in tissue engineering and regenerative medicine (TE/RM). Regardless of their intended use, all biomaterials elicit a reaction from their host, the foreign body response, which exerts a great influence over the degree of success or failure in TE/RM applications. Prominent participants in the response of the body to an implanted biomaterial are macrophages. Exposure to implanted materials generally causes macrophages to fuse into multinucleated giant cells, which ultimately leads to fibrous encapsulation and scar tissue formation around the implant [1,2]. Multinucleated giant cells are generally associated with chronic inflammation and, depending on the signals encountered within the environment, can arise from both ends of the macrophage phenotype continuum [2–4]. Within the context of TE/RM, it has been demonstrated that adherent macrophages on biomaterials (precursors to foreign body giant cells) revealed a profile that was neither M1- nor M2-polarized but somewhere in the middle [2]. While many strategies aim to avoid this process and the host immune response completely, macrophages have recently emerged in a different light as an important component of the innate immune system that can modulate and attenuate tissue remodeling following injury [5–8]. More recently it has been suggested that the key to tissue regeneration approaches may be the concept of regulating the balance between two distinctly different sub-types of macrophages.

The general utility of keratin biomaterials has been described by several investigators for applications such as drug delivery, tissue regeneration, hemostasis and wound healing [9–24]. Three general findings have been reported: excellent biocompatibility, cell adhesion and improved tissue healing. As early as 1982, scientists reported work on the general biocompatibility of wool-based keratin biomaterials [25]. This Japanese-language publication describes the preparation of both oxidized and reduced, solubilized keratins that were used to coat polyester meshes with a glutaraldehyde-crosslinked film of keratin biomaterial prior to implantation into the dorsal muscle of dogs and rabbits. After 2, 4 and 6 weeks, the implants were scored for degree of foreign body reaction by examining histological sections. The investigators found that the degree of foreign body reaction was low in all cases, with no

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apparent distinction between the different forms of keratin biomaterials used in the study. More recently, several authors have expanded on this initial finding of good biocompatibility in papers utilizing a variety of in vitro and in vivo model systems, sometimes by blending keratin with other biomaterials [13,15,26–32]. Cell adhesion to keratin substrates has also been demonstrated by several authors [33–37], and tissue healing (i.e. regeneration) studies have included skin, bone, nerve, cornea and heart, with consistent findings of improved tissue repair and little notable scar formation reported [12,13,16,17,23].

Based on our group's earlier experience in peripheral nerve regeneration [11,14,22,23], we undertook a pilot study to investigate the potential for a keratin biomaterial hydrogel to facilitate neuronal regeneration in the spinal cord [38]. Along with several observations demonstrating improved functional recovery, the data from this study suggested that downstream tissue damage normally seen due to the inflammatory cascade was mitigated by keratin biomaterial treatment. Interestingly, Kigerl et al. demonstrated that these secondary injury mechanisms in the spinal cord are dominated by a pro-inflammatory M1 macrophage phenotype, a response that overpowers the relatively smaller and transient anti-inflammatory M2 macrophage phenotype [39]. Limited staining of the spinal cord tissue from the aforementioned pilot study revealed a strong M2 presence and a notably smaller M1 population. Other studies have shown that a keratin-based implant such as a hydrogel quickly becomes infiltrated with resident cells, but that a classical foreign body reaction does not ensue, overall cell population decreases, and the relatively small, initial inflammatory response resolves itself quickly [30,31]. These observations suggest that keratin biomaterials may be influencing the cellular response to tissue injury, particularly inflammation. Based on this previous research, we postulated that keratin biomaterials may be capable of inducing macrophage polarization at sites of injury, and that this may represent a common mechanism that is partly responsible for the beneficial tissue regeneration reported by different investigators around the world, including our group.

The purpose of the current study was to investigate the role of macrophage response in keratin's capacity as a regenerative biomaterial. We hypothesized that keratin can contribute to macrophage polarization, and ultimately tissue regeneration, by favoring the growth- and regeneration-promoting M2 phenotype. To examine this, an in vitro culture system employing a human monocytic cell line was used to determine the relative ratio of M1 and M2 macrophage phenotypes that arise at different time points following growth on a keratin biomaterial substrate, as well as cytokines secreted by these cells, compared to cells grown on tissue culture plastic (TCP) and collagen substrates.

2. Materials and methods

2.1. Preparation of keratin biomaterial and coatings

The keratin biomaterial was extracted and prepared as previously described [11,31,38]. Briefly, a 2% peracetic acid solution was used to oxidize human hair fibers. Following washing with deionized (DI) water to remove residual oxidant from the hair fibers, tris(hydroxymethyl)-aminomethane (Tris) base and DI water was used to extract the soluble keratin proteins. The solution was then dialyzed against DI water, neutralized to pH 7.4 with NaOH, lyophilized and ground into a powder. The keratin powder was sterilized via exposure to a 25 kGy dose of γ-irradiation and aseptically reconstituted in phosphate-buffered saline (PBS). Keratin and type-I rat tail collagen ($\geqslant$90% purity; BD Biosciences) were diluted to a final concentration of 200 μg ml^{-1} and 1 ml of these respective solutions was added to the wells of glass chamber slides (Nunc, Thermo Fisher Scientific) and incubated for 24 h at 37 °C to form coatings. After incubation, excess solution was removed and the coated surface rinsed with PBS prior to cell seeding.

2.2. Human macrophage cell culture

The THP-1 human monocytic cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.05 mM 2-mercaptoethanol (Sigma). To generate adherent THP-1-derived macrophages (TDM), 1×10^6 cells were added to wells in an untreated TCP six-well plate (Becton Dickinson) and treated with 5 ng ml^{-1} of phorbol myristate acetate (PMA; Sigma) dissolved in media for 48 h at 37 °C, 5% CO_2. Macrophage phenotype (CD14$^+$) was confirmed using flow cytometry (data not shown), and for the purposes of this study, will be considered as having an M0 phenotype. TDMs were then washed with PBS, detached using 0.25% trypsin/0.1% EDTA (HyClone), pelleted and resuspended in complete media. 1×10^6 TDMs were then plated and reattached on corresponding substrates of the glass chamber slides (Table 1). For control treatments, TDMs were induced to a polarized phenotype by culturing cells with either lipopolysaccharide (LPS, 100 ng ml^{-1}; Sigma) and human recombinant (hr) interferon gamma (IFNγ, 20 ng ml^{-1}; Sigma) to produce M1 macrophages, or hr interleukin 4 (IL-4, 20 ng ml^{-1}; Sigma) to produce M2 macrophages in glass chamber slides [40]. Media, including that of the control treatments that contained cytokines, were changed every 3 days. TDM M0 macrophages were produced by incubating to their respective time points in the presence of complete media only (Table 1, no coating treatment group).

2.3. Immunocytochemistry

All stains were performed at room temperature (RT), manually, using an optimized double-immunofluorescence technique. Briefly, macrophages cultured in glass chamber slides were washed with PBS, fixed in 4% paraformaldehyde for 20 min at RT and washed with a buffer containing 0.1% bovine serum albumin (BSA) in 1× PBS. After blocking non-specific staining for 45 min (10% BSA), the first primary antibody was added (CD86 for M1 specificity, 10 μg ml^{-1}; R&D Systems) and incubated for 1 h at RT. After washing, the secondary antibody was added (NL-557, 1:200; R&D Systems) and incubated in the dark for 1 h. After rinsing with the wash buffer, the second primary antibody was added to the wells (CD206 for M2 specificity, 15 μg ml^{-1}; R&D Systems) and incubated for 1 h. Cells were incubated with the final secondary antibody (NL-493, 1:50; R&D Systems) for an additional hour, washed and the gasket removed from the slide. Slides were mounted with ProLong® Gold Antifade (Life Technologies) mounting media and visualized using a Zeiss LSM510 inverted confocal microscope.

2.4. Macrophage quantitative analysis

Quantitative analysis of CD86+(M1), CD206+(M2), CD86+/CD206+(M1/M2; co-expressing phenotype) and CD86−/CD206−(M0) cells for each treatment group at each time point

Table 1
Time points and treatment culture conditions for TDMs. *n* = 6 was analyzed for each condition at each time point.

Time points	Treatment conditions
24 h, 3 days, 7 days, 14 days	Keratin coating Collagen coating No coating LPS/IFNγ IL-4

was conducted by selecting nine random areas per slide at 20× magnification and capturing digital images. The number of each phenotype, or co-expressing phenotype, present in the selected field was manually counted based on the positive staining of the cell. The mean ($n = 9$) for each cell phenotype was calculated for each sample. The per cent total expression was determined for each phenotype by dividing the number of positively staining cells by the total cell count (as determined by the corresponding digital interference contrast (DIC) images). Six independent replicates of each treatment condition were analyzed for each experimental group at each time point (i.e. six separate chamber slides).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Culture media supernatants were collected immediately prior to immunostaining and centrifuged for 10 min at 1000*g* to remove any cell debris or particulates. Supernatants were aliquoted and stored at −80 °C until analysis. To measure cytokine production, a multi-analyte ELISArray kit (Qiagen) was used to simultaneously detect levels of multiple cytokines. The manufacturer's directions and standard ELISA techniques were followed. Briefly, using a 96-well microplate coated with a panel of 12 capture antibodies, assay buffer was added to each well followed by experimental and control samples to their corresponding wells; samples were then incubated for 2 h at RT. Wells were washed, the detection antibody solution added and incubated for an additional 1 h at RT. After another wash, avidin-horseradish peroxidase was incubated for 30 min in the dark. Finally, a development solution was added for 15 min in the dark, followed by a stop solution. Absorbance levels were measured at 450 nm using a Spectramax M5 microplate reader (Molecular Devices) with a 570 nm correction wavelength.

2.6. Statistical analysis

For macrophage phenotype quantitation, a two-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used to determine significant differences due to time or culture conditions. For cytokine secretion data, negative control (i.e. uncoated TCP) values from ELISAs were subtracted from the corrected absorbance readings. Relative absorbance was calculated by normalizing data to the IL-8 absorbance signal (consistent across all time points and treatment groups). First, secretion levels were compared for significant differences due to time within a treatment group using a two-way ANOVA with a Bonferroni's post hoc test, then differences between treatment groups across time were compared for significance for each cytokine using the same method. All analyses were completed using Prism v. 5.0 (GraphPad Software, Inc.) and data presented as mean ± standard error of the mean (SEM) with $P < 0.05$ being considered statistically significant.

3. Results

3.1. Macrophage phenotypic response to a keratin biomaterial

Representative confocal fluorescent images of the double-stained treatment groups at their corresponding time points

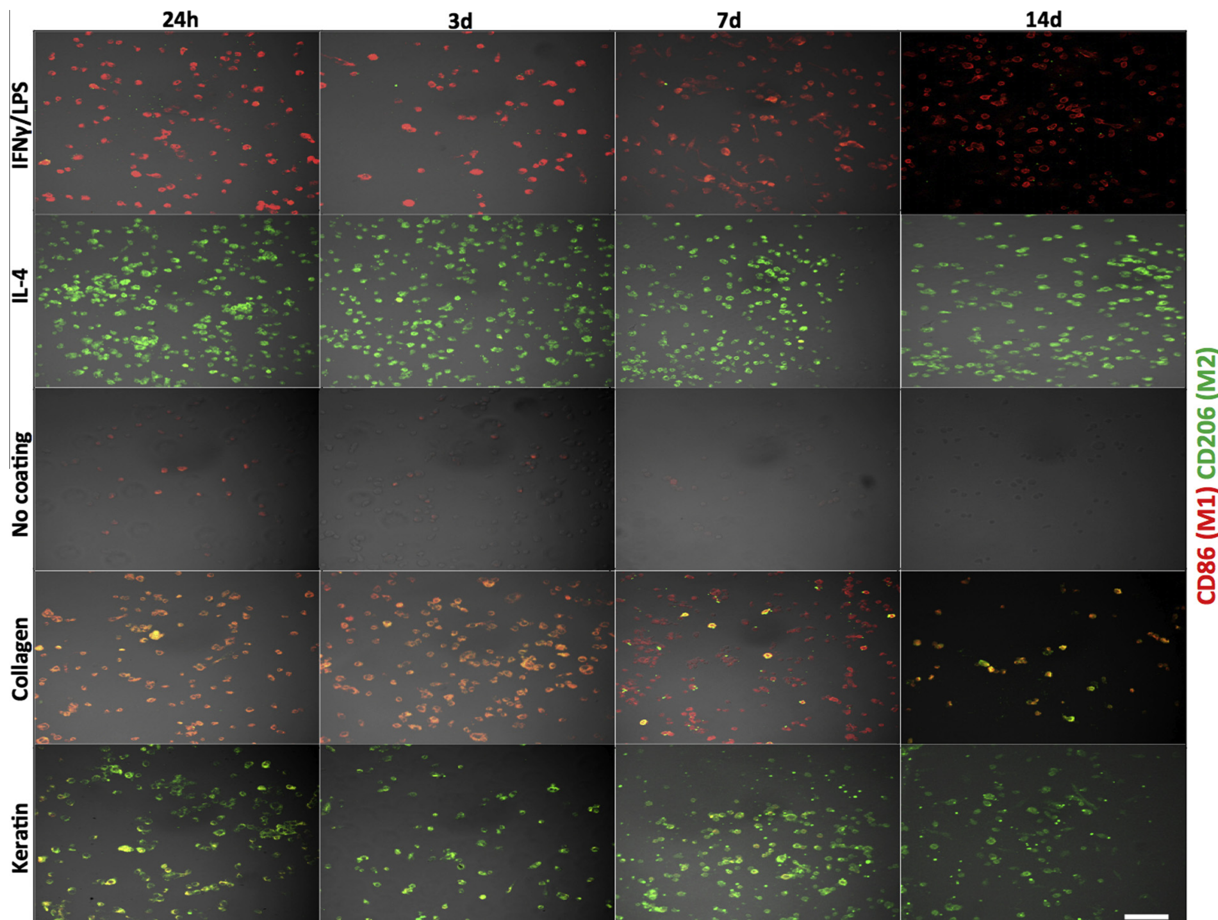

Fig. 1. Representative confocal fluorescent images of treatment conditions and time points. M1 phenotype is indicated in red, M2 phenotype is indicated in green, mixed M1/M2 phenotype is shown as yellow, and M0 phenotype are cells without staining. Keratin-exposed TDMs exhibited a predominantly M2 phenotype across all time points, with a peak M1/M2 co-staining observed at 24 h. Scale bar = 100 μm.

demonstrates that M1 and M2 control treatments are effective at maintaining high levels of their respective phenotype across all time points (Fig. 1). These images also suggest that keratin treatment increases CD206+ staining (M2 macrophages), especially after 24 h, whereas collagen treatment shows cells simultaneously expressing both M1 and M2 antigens. Furthermore, cells cultured with no coating did not express high amounts of either CD86 or CD206 (M1 or M2 markers, respectively; Fig. 1).

Quantification of the cell counts from the confocal images indicates TDMs can be pushed to an M1 phenotype, with LPS/IFNγ treatment producing the greatest amounts of CD86+ staining cells (Fig. 2A). Similarly, the cells can be differentiated to an M2 phenotype with IL-4 treatment, where the highest levels of CD206+ staining cells among treatment groups was observed across all time points (Fig. 2B). Both control treatments produced minimal M0 and co-staining M1/M2 phenotypes. TDMs cultured on TCP (i.e. no coating) display significantly more unlabeled M0 macrophages compared to M1, M2 and M1/M2 macrophages at 3, 7 and 14 days (Fig. 2D), suggesting these cells remain unactivated in a more naïve phenotypic state. These untreated cells produced higher levels of the M1 phenotype at 24 h, but there was no statistical difference compared to the M0 phenotype. Moreover, this observation disappeared by day 3. Also, despite higher levels of M1-staining cells at 24 h, there was no statistical difference between M1 and M0 percentage expression. Collagen coatings demonstrated significantly more concomitant M1/M2-staining cells compared to M1 and M2 phenotypes at 3, 7 and 14 days, as well as significantly greater numbers of M1 than M2 macrophages across all time points (Fig. 2C). When cultured on a keratin biomaterial coating, TDM phenotype displays an obvious shift toward a predominantly M2 phenotype, where there is a significantly higher population of M2-staining cells at all time points compared to M1, M0 and M1/M2 phenotypes (Fig. 2E). When comparing the keratin-induced cell populations to the collagen-induced populations, keratin-treated TDMs look more similar to the IL-4 (M2)-induced control populations. Moreover, there is no significant difference between M2 populations from keratin and M2 control treatments, except for the 24 h time point (Fig. 3A). Keratin also produces a significantly greater population of M2-staining cells compared to untreated cells, collagen coating and M1 control treatment (Fig. 3A). In contrast, collagen consistently produced significantly more M1 macrophages compared to keratin across all time points, and there was once again no statistical difference between M2 control treatment and keratin (Fig. 3B).

3.2. Cytokine profiles following keratin exposure

Cytokine production by cells in M1 and M2 control groups demonstrate what would be typically considered pro- and anti-inflammatory profiles, respectively (Fig. 4A, B). Generally speaking, M1 control macrophages showed higher levels of overall cytokine secretion compared to M2 controls, in particular IL-1β, IL-6, IFNγ and tissue necrosis factor alpha (TNF-α), which all reached statistical significance compared to their 24 h values. The M2 control treatment is known to produce cells that secrete higher levels of IL-4 and IL-10, both common anti-inflammatory cytokines, and indeed IL-10 was secreted in significantly higher amounts at 7 and 14 days compared to 24 h and 3 days. There was a significant

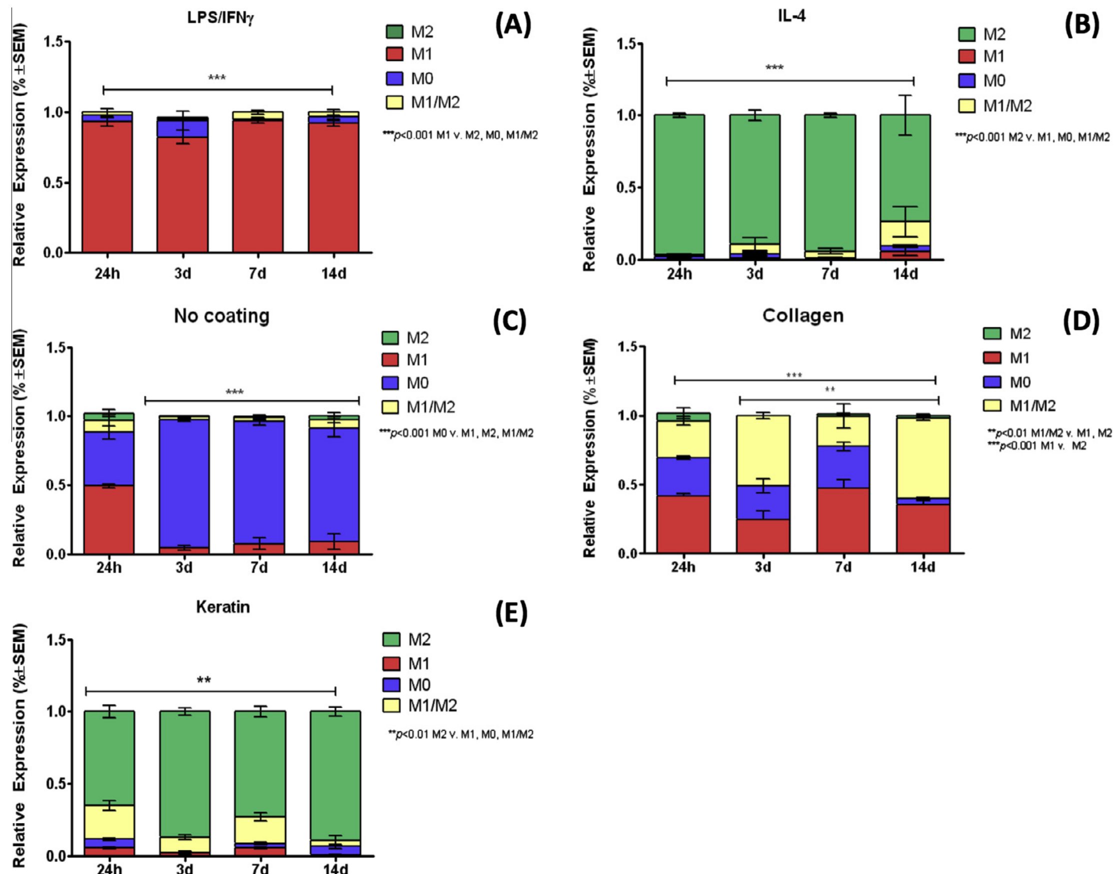

Fig. 2. Percent expression of each macrophage phenotype. M1 and M2 control treatments polarize TDMs (A, B). Collagen coatings express mostly M1 and co-staining M1/M2 cells (C). Except for 24 h, non-treated TDMs do not express M1 or M2 cell surface markers (D). TDMs seeded on keratin coatings express significantly more M2 macrophage phenotype (E).

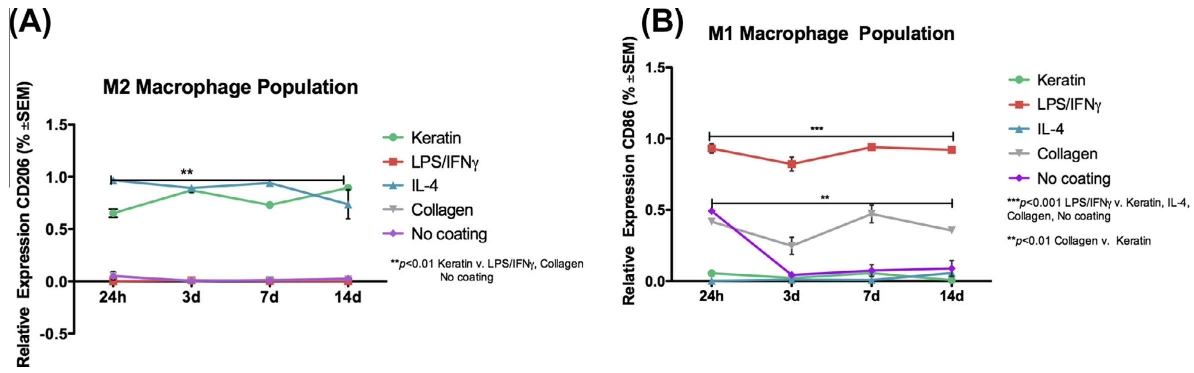

Fig. 3. CD86 and CD206 expression over time. Keratin polarizes macrophages to an M2 phenotype significantly better than collagen or no coating (A). Collagen coatings promote an M1 macrophage phenotype significantly more than keratin (B).

Fig. 4. Cytokine profiles of treatment conditions. M1 and M2 control treatments produce more pro- and anti-inflammatory cytokines, respectively (A, B). TDMs cultured under normal conditions do not produce significant amounts of most cytokines (D), while those cultured on a collagen coating produce higher levels of pro-inflammatory cytokines (C). TDMs exposed to keratin produce a cytokine profile closer to that of the M2 control (E).

increase in IL-1α, which is sometimes considered pro-inflammatory, at 7 days as well, but this cytokine has also been shown to play a major role in beneficial tissue remodeling and wound healing.

Collagen coatings demonstrate a cytokine profile that appears dissimilar to either M1 or M2 controls, but the inflammatory cytokines IL-1β and TNF-α are significantly higher compared to 24 h and 3 days (Fig. 4C), though they did not reach levels observed within the M1 control group. This trend seems to correspond to the immunofluorescent staining results where the majority of cells were positive for the CD86 M1 antigen as well as co-staining CD86+/CD206+ for both M1 and M2 antigens. TDMs cultured with no coating, with the exception of IL-1α at 7 days, do not produce significant levels of either pro- or anti-inflammatory cytokines compared to the other time points (Fig. 4D). There were elevated levels of IL-6, IL-10, IFNγ and TNF-α, but those did not reach statistical significance. Generally, this corresponds to the immunofluorescent staining of these cells, noted as having scarce M1, M2 and M1/M2 staining, suggesting the majority of these cells remain in an undifferentiated, M0 phenotypic state.

Cytokines produced in the presence of keratin appear consistent with an M2 phenotype, where there is a significant shift toward increasing levels of IL-10 with time, as well as low levels of IL-1β and IL-6 with time (Fig. 4E). This is more consistent in the M2 control cytokine profile (Fig. 4B), and although there is a significant increase in TNF-α, there is no statistical difference between keratin and the M2 control. The only group to produce significantly higher levels of TNF-α was the M1 control group.

Keratin and collagen elicit different effects on TDM cytokine production, particularly when looking more closely at several cytokines that showed more profound responses (Fig. 5). The highest levels of IL-1β were produced by the M1 control group across all time points (Fig. 5A). However, at 7 days collagen treatment produced significantly more IL-1β compared to keratin and there was no statistical difference between keratin and the M2 control group. Likewise, the M1 control group produced the greatest amounts of IL-6 and there was no statistical difference between collagen and the M1 control group at 24 h and 3 days (Fig. 5B). Collagen treatment also produced significantly more IL-6 compared to keratin at 7 days. Additionally, keratin treatment was responsible for producing significantly higher levels of IL-10 at 7 and 14 days compared to collagen, and there was, again, no statistical difference between the M2 control and keratin (Fig. 5C).

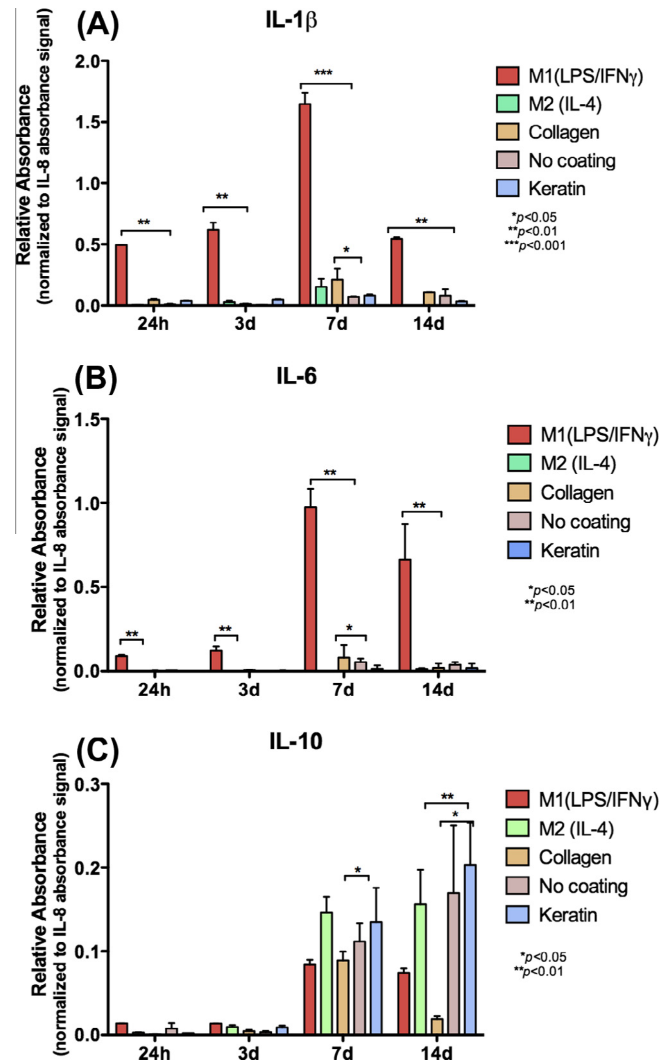

Fig. 5. Individual cytokine production by treatment groups. TDMs produce significantly more IL-1β when cultured on collagen coatings at 7 and 14 days (A) and significantly more IL-6 at 7 days compared to keratin coatings (B). Keratin coatings promote significantly greater production of IL-10 than collagen at 7 and 14 days (C).

4. Discussion

The plasticity of macrophage phenotypes is diverse and dependent upon factors within the local environment [41,42]. M1 macrophages are considered "classically activated", pro-inflammatory macrophages that secrete cytotoxic compounds such as reactive oxygen and nitric oxygen intermediates, as well as inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α. M2 macrophages are defined as "alternatively activated" macrophages that are immunomodulatory and promote wound healing and angiogenesis, and produce anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 [43–45]. The underlying mechanisms remain poorly understood, but in vitro experiments have demonstrated that M1 and M2 phenotypes may be induced with various cytokines and microbial products (e.g. LPS and IFNγ, and IL-4, respectively) [6,39,46,40]. However, once polarized, the M1 or M2 phenotype is not fixed and studies have shown that M1 and M2 macrophages exposed to the opposing phenotype's induction signals can be differentiated to express the other's characteristic genes and cytokines [41,47]. This dynamic and highly regulated plasticity is believed to be a protective mechanism that allows a host response that is pathogen-appropriate, but also one that is able to resolve quickly and restore tissue homeostasis following injury or infection [6,48]. The dysregulation of macrophage polarization and failure to return M1 and M2 phenotypes to a normal balance is known to play a crucial role in chronic inflammation associated with injuries and disease states [6,39,48]. As such, some injury models such as skeletal muscle remodeling demonstrate an initial M1-dominant response, which serves to clear debris and secrete cytokines and chemokines that attract muscle progenitor and satellite cells [49–52]. The initial pro-inflammatory phase is followed by a change to an M2 phenotype that serves to resolve the inflammatory response, as well as promote the differentiation of recruited satellite and progenitor cells [53–55]. In the case of an aberrant state of macrophage polarization, such as muscular dystrophy, there exists a constant, mixed M1/M2 cell population with no shift to a resolving M2 phenotype, resulting in failure of progenitor cells to differentiate [55–57]. Another important example of dysregulation of the macrophage response related to tissue injury is in the central nervous system following a spinal cord injury (SCI). It has been shown that post-SCI, a predominantly M1 macrophage phenotype persists at the lesion site, and despite a small number of M2 macrophages present at early

time points, there is no observed M1 to M2 phenotypic shift as seen in normal tissue repair [39].

The use of biomaterials to modulate the inflammatory response is not a new concept, and as the field of TE/RM grows, it is becoming increasingly important to understand how biomaterials interact with the immune system. Macrophages are a crucial mediator in these processes and often facilitate scaffold degradation and, thus, remodeling of tissue constructs [5,7,8,58–60]. The role of M1 and M2 macrophages in regenerative tissue processes can be substantial. One study showed that neurons cultured in conditioned media from M2 macrophages displayed increased viability as well as greater neurite extension, while those cultured with M1-conditioned media showed decreased survival and neurite length [39]. Similarly, results have been reported wherein M1 macrophages caused lysis of muscle cells and M2 macrophages supported satellite cell proliferation and muscle regeneration [55,56]. This, along with data indicating that IL-10 and other M2-specific cytokines specifically down-regulated the pro-inflammatory response [43], suggests that M2 polarization promotes constructive tissue remodeling by abrogating the M1 response. However, abnormal polarization of M1 and M2 cell populations toward either extreme may have negative effects [39,56,61].

Thus, it is essential in the design of biomaterials to consider properties that maintain this delicate balance and support tissue remodeling and functional recovery. Some properties of implantable scaffolds and polymers have been shown to elicit primarily an M1 macrophage response. Specifically, those constructed using cross-linking agents [5,8], those containing cellular components [7], biomaterials derived from synthetic sources [40], and those of small or nonexistent fiber and pore size [46] have been shown to promote an M1 phenotype. This can correlate to a poor outcome of tissue remodeling and deposition of dense connective tissue and scarring, while an M2 macrophage response results in constructive remodeling [5,7,8,62]. An interesting observation in this study is the largely M1 response to collagen, particularly since previous studies using collagen-based extracellular matrix (ECM) scaffolds have demonstrated constructive tissue remodeling. Perhaps the use of rat tail collagen, an extracted and presumably damaged and denatured form of the protein, in the present study is one reason for these different outcomes.

Considering the robust M2 response elicited by keratin biomaterials in this study, as well as a tunable biodegradation profile that would allow bioresorption of a keratin scaffold to occur at a controlled rate, it is believed that keratin may offer an improved alternative to other biomaterial-mediated macrophage polarization strategies. The immunostaining and cytokine profiling data demonstrate that the M2 phenotypic character of the positive control and keratin-treated cells are statistically comparable. This suggests that keratin biomaterial is as good as the positive control at supporting an M2-dominated response, but it is also important to note that keratin appears to down-regulate differentiation toward an M1 phenotype. This response is more dramatic than an increase in M2 phenotype alone, and is essential in the role of macrophages in wound healing and tissue repair. It is also important to point out that in this study TDMs were exposed only to a thin coating of keratin biomaterial at the onset of seeding and were not re-seeded or otherwise exposed to additional doses of keratin. Therefore, the significant effects noted in the present study were elicited by a modest interaction with the keratin biomaterial and more profound effects may be seen with other cell treatment modalities.

It has been previously established that if an implanted biomaterial is able to support the normal M1-to-M2 shift, there will be greater tissue remodeling and more beneficial downstream effects that avoid the fibrotic, scar tissue response [5,6,8,62]. In previous studies, a keratin biomaterial has been shown to tolerate cellular and vascular infiltration, evade foreign-body giant cell formation, chronic inflammation and graft rejection, as well as elicit a minimal fibrous capsule response [25,28,30,31]. Keratin has also demonstrated versatility in regeneration and tissue repair. In a bone defect model, a keratin scaffold loaded with bone morphogenetic protein 2 supported greater tissue remodeling and regeneration [63]; a keratin biomaterial hydrogel has been shown to significantly improve nerve repair in tibial and sciatic nerve defect models [11,14,22,23,64]; a keratin hydrogel injected into an infarcted mouse heart was able to improve cardiac function [17]; and keratin biomaterial was shown to prevent wound enlargement and promote faster wound closure in both thermal and chemical burns [24]. In our own pilot study using the SCI hemisection model in rats, better functional outcomes were observed [38], as well as a stronger presence of M2 macrophages at the lesion site (unpublished data). Taken together, these data suggest one of the potential mechanisms, and possibly a common mechanism, by which keratin biomaterials may promote tissue repair.

While no mechanistic investigation was undertaken here, previous results using keratin biomaterials may suggest a role for integrin signaling through ligand-like activity of the keratin biomaterial itself. Human hair keratins contain the peptide-binding motif leucine–aspartic acid–valine, a cell adhesion sequence recognized by the $\alpha4\beta1$ integrin [65]. Prior studies have shown that blockage of the $\beta1$ integrin subunit results in decreased cell adhesion to keratin [66,67]. Most leukocytes, including macrophages, express the $\alpha4\beta1$ integrin, suggesting a potential ligand–receptor relationship between keratin and macrophage behavior. However, it has also been shown that cells are capable of interacting with keratin substrates even when they are known to lack the $\alpha4\beta1$ integrin [68], suggesting a different cell–matrix relationship such as recognition of intact surface glycans. Furthermore, functional blockage of the $\beta3$ integrin subunit has been shown to prevent cell adhesion to keratin [66]. The $\beta3$ subunit has been implicated in impaired and aberrant macrophage migration and subsequent polarization [61]. Additional investigation is needed to confirm such potential interactions and a more focused study to elucidate the mechanisms at play.

This study utilized a simplified in vitro culture model system and as such may not be directly comparable to in vivo situations. In particular, M1 and M2 phenotypes were identified based on a limited number of cell surface markers. Although these markers are known to be highly expressed and indicative of their respective polarized phenotypes [39,69], it would be advantageous in future studies to expand upon this characterization. Moreover, this study focused on the major role of macrophages in the host response to biomaterials. There are undoubtedly more cellular and related molecular players found in the in vivo system that would come to bear in a TE/RM animal model. The present data is also based on an immortalized cell line whose behavior has been analyzed and confirmed in numerous studies [70], but there is still a potential uncertainty as to whether the observed effects can correlate with primary cells and explain the tissue regeneration responses seen with keratin biomaterial in other animal studies. Additional experiments employing primary cells and, importantly, animal models that specifically delineate the role of macrophages in tissue regeneration and the influence of keratin biomaterials are needed before any definitive conclusions can be made. However, and despite these potential limitations, the present study results may have profound implications for the use of keratin biomaterials in the future and the existence of a common mechanism not seen with other biomaterials.

5. Conclusion

In certain cases, it appears that the requirements for beneficial macrophage polarization in response to biomaterial scaffolds

include a highly porous structure, lack of modifications (e.g. cross-linking agents, cellular components), and the ability to degrade at a rate that maintains function but avoids a long-term presence that would impede tissue growth. Based on such conditions, and because its efficacy in several injury models showed it to be better than current treatment standards, keratin biomaterials may represent a unique prospect for modulation of macrophage phenotype in TE/RM applications. Even though there are no known mammalian keratinases, keratin is still subject to proteolytic degradation, though its degradation rate is typically slower than that of other ECM proteins. These characteristics represent strong justification for further investigation of keratin biomaterial systems, with the data generated in this study providing intriguing evidence of a potential common mechanism for keratin's activity in other TE/RM research.

Conflict of interest statement

Dr. Mark Van Dyke holds stock and is an officer of KeraNetics, LLC, who provided partial funding for this research. Wake Forest University Health Sciences has a potential financial interest in KeraNetics through licensing agreements.

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Appendix A. Figures with essential colour discrimination

Certain figure in this article, particularly Figs. 1–5, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.04.003.

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