Vascularization for Bone Tissue Engineering through Modulation of Macrophage Behavior

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Statement of Purpose: Angiogenesis is critical for normal bone repair and for the success of bone tissue engineering strategies. The natural inflammatory response is a crucial determinant of the success or failure of an implant. Immediately following injury, macrophages infiltrate the wound and differentiate into a pro-inflammatory phenotype (M1), and at later stages of healing they exhibit a pro-healing phenotype (M2). Biomaterials that cause a predominance of M1 macrophage behavior are chronically rejected, while those that promote the M2 phenotype become integrated and vascularized (Badylak SF. Tis Eng A 2008; 14:1835-1842). The goal of this study was to understand the role of macrophage behavior in angiogenesis and to control this process using biomaterial strategies.

Methods: Primary human monocytes, derived from peripheral blood using sequential density gradient centrifugation, were differentiated into macrophages through the addition of monocyte colony stimulating factor (MCSF) over 5 days. Macrophages were polarized to M1, M2A, and M2C phenotypes by incubation for 48hrs with lipopolysaccharide and interferon-γ (IFNγ), interleukin-4 (IL4) and IL13, or IL10, respectively. To probe the role of macrophage phenotype in angiogenesis, human umbilical vein endothelial cells (HUVECs) were co-cultured with polarized macrophages or unactivated control macrophages (M0) or in their conditioned media, and analyzed in terms of transwell migration over 5hrs, tube formation on fibrin gel and Matrigel, and gene expression after 24hrs. Then, macrophage-polarizing cytokines were conjugated to decellularized bone scaffolds with the goal of specifically directing macrophage phenotype. Biotinylated IFNγ, IL4, or IL10 were conjugated to biotinylated scaffolds using the biotin-avidin system. Unactivated macrophages were seeded on these scaffolds and then analyzed in terms of gene expression of markers of the M1 or M2 phenotypes after for 2 days. Data is presented as mean ±SEM and statistical significance was determined by ANOVA.

Results: M1 macrophages recruited HUVECs more than M0 and M2C macrophages after 5hrs across transwell inserts, relative to cytokine controls ($p<0.05$, n=4) (Figure 1a). However, tube formation of HUVECs on Matrigel, as measured by total tubes, average number of tubes, and total length of tubes using ImageJ, was lower in media conditioned by M1 macrophages compared to M0 and M2A macrophages (Figure 1b). In addition, HUVECs organized into network-like structures on fibrin when conditioned media from M1 and M2A macrophages was applied sequentially over 7 days (data not shown). Co-culture with M1 and M2C macrophages caused HUVECs to significantly upregulate *PECAM* and *VCAM1*, respectively. Interestingly, co-culture with HUVECs caused polarized macrophages to decrease expression of M1 markers *CCR7* and *CD80*, with no effect on M2 markers *CD206*, *CD163*, and *CCL18* (data not shown).

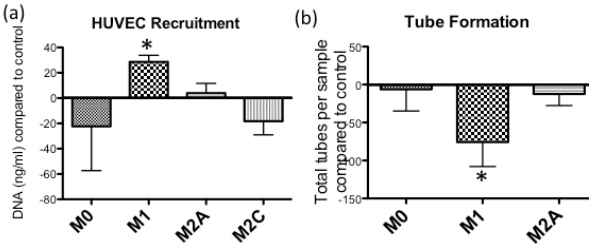

Figure 1. (a) Recruitment of HUVECs by macrophages over 5hrs and (b) tube formation by HUVECs on Matrigel over 16hrs in macrophage-conditioned media.

The polarizing cytokines IFNγ, IL4, and IL10 were conjugated to bone scaffolds to direct macrophages to the M1, M2A, or M2C phenotypes, respectively. Immobilized IFNγ increased expression of the pro-inflammatory cytokine and M1 marker tumor necrosis factor-α (TNFα) compared to scaffolds without cytokines ($p<0.05$, n=5) (Figure 2). Likewise, IL4 caused upregulation of the M2 markers CD206 and CCL18 ($p<0.05$, n=5). The conjugation of IL10 did not cause significant changes in any of the genes assessed.

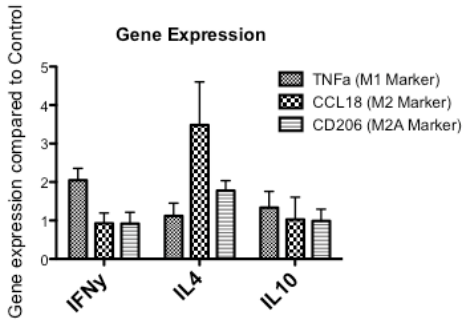

Figure 2. Gene expression, normalized to negative controls, of macrophages seeded on bone scaffolds with immobilized cytokines IFNγ, IL4, and IL10 (n=5).

Conclusions: These data suggest that M1 macrophages are important for recruiting endothelial cells at early stages of wound healing. However, they inhibit organization into networks, a necessary step in angiogenesis. The precise role of M2 macrophages in angiogenesis has yet to be defined, as most of the information regarding their angiogenic role is derived from the cancer literature. Current inhibition studies are aimed at evaluating the mechanisms of crosstalk between polarized macrophages and HUVECs, to shed light on a possible role of endothelial cells in mediating the M1-to-M2 transition. Importantly, the conjugation of polarizing cytokines to scaffolds resulted in specific control over macrophage phenotype, which may allow the in vivo assessment of macrophage behavior in healing and vascularization.