Control over Macrophage Behavior to Promote Scaffold Vascularization for Bone Regeneration

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Statement of Purpose: Given that bone has the capacity to heal small defects perfectly, without scarring (McKibbin, B. J Bone Joint Surg Br 1978; 60B:150), we hypothesize that scaffolds that exploit the natural healing response will achieve greater levels of bone regeneration than traditional scaffolds. 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) (Arnold, L. J Exp Med 2007; 204:1057-1069). We have previously shown that M1 macrophages promote angiogenesis and M2 macrophages promote vessel maturation (manuscript under review). Therefore, scaffolds that promote the M1 phenotype followed by the M2 phenotype of infiltrating macrophages should result in enhanced angiogenesis and healing. In this study, scaffolds were designed to cause sequential activation of the M1 and M2 phenotypes through quick release (~1 day) of the M1-activating cytokine interferon-gamma (IFNy) followed by sustained presence of the M2-activating cytokine interleukin-4 (IL4).

Methods: Biotinylated IL4 (Peprotech, biotinylated with EZLink from Pierce) was immobilized on decellularized bone scaffolds via avidin by reaction for one hour at room temperature (500ng IL4/scaffold). Scaffolds were washed four times in an excess of phosphate buffered saline. Then, scaffolds were immersed in a solution of IFNy (Peprotech, 325ng/scaffold) to allow passive adsorption for one hour at room temperature. Scaffolds with either immobilized IL4 or adsorbed IFNy, or neither, served as controls. 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) for 5 days and then seeded onto the scaffolds (1 million/scaffold). The media was collected after 3 and 6 days of culture and analyzed via enzyme linked immunosorbent assays for content of the M1 marker tumor necrosis-alpha (TNFa, Peprotech), the M2 marker CCL18 (R&D Systems), the angiogenic factor vascular endothelial growth factor (VEGF, Peprotech), and the blood vessel-stabilizing factor platelet-derived growth factor (PDGF-BB, Peprotech). The amounts of IL4 and IFNy released into the media from non-cell-seeded scaffolds were also determined by ELISA (Peprotech). Data is presented as mean ±SEM (n=5) and statistical significance for the effect of scaffold treatment was determined by one-way ANOVA with Tukey's post-hoc analysis.

Results: The passive adsorption of IFNy to bone scaffolds caused slight increases in macrophage secretion of the M1 marker TNFa and the angiogenic factor VEGF to values that were significantly higher than scaffolds with immobilized IL4 ($p<0.05$) (**Figure 1**). These values were not significantly higher than the negative control, which is probably due to low levels of IFNy adsorption to the scaffolds (detected levels were less than 1ng/scaffold). The immobilization of IL4 to the scaffolds caused significant increases in release of the M2 marker CCL18 at both 3 and 6 days ($p<0.001$). Secretion of the maturation-promoting growth factor PDGF was higher for scaffolds with immobilized IL4 than those with adsorbed IFNy. After 3 days, in which ~2ng IL4 was released from the scaffolds, the scaffolds released less than 50 pg/ml IL4 over 11 days (data not shown). Considering the strong interactions between biotin and avidin combined with the continued effects of IL4 on the macrophages at 6 days, it is likely that the IL4 remained immobilized on the scaffolds. Interestingly, the combination of adsorbed IFNy and immobilized IL4 did not cause any changes in cytokine secretion, perhaps reflecting conflicting signals of IFNy and IL4 on the macrophages.

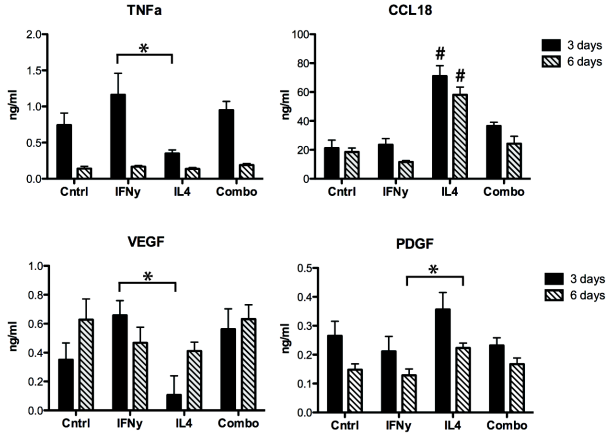

Figure 1. Factors secreted by macrophages seeded on scaffolds with adsorbed IFNy (IFNy), immobilized IL4 (IL4), or a combination (Combo), compared to the control (Cntrl). *=$p<0.05$; #=$p<0.001$ compared to other groups at the same time point.

Conclusions: These results indicate that scaffolds can be designed to control macrophage behavior. Macrophages are crucial in the healing process, and healing and angiogenesis are drastically reduced when they are depleted (Kubota, Y. J Exp Med 2009; 206:2089-1102). Scaffolds that promote the natural sequence of M1 and M2 macrophage polarization would cause host macrophages to release the myriad of growth factors involved in healing at the correct doses and temporal profiles, which is impossible to achieve using drug delivery techniques. As examples of the numerous cytokines that macrophages release, an M1 marker, an M2 marker, and two of the most important growth factors in angiogenesis (VEGF and PDGF) were analyzed. Although immobilized IL4 caused sustained M2 polarization, passive adsorption of IFNy was insufficient to cause significant M1 polarization. Future studies will investigate the use of a coating to release higher levels of IFNy before exposing IL4 for delayed M2 activation.