FINAL REPORT

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Introduction:

Although the immune system, both the innate and adaptive components, have long been known to be important and necessary for tissue healing following injury, the neutralization of infectious agents, and the host response to foreign materials such as surgical meshes and orthopedic implants, its role in normal tissue/organ development and tissue regeneration has only recently been recognized [1-5]. In fact, it is now understood that immune cells such as neutrophils, macrophages, and lymphocytes possess robust plasticity with respect to phenotype. For example, macrophages typically show a marked pro-inflammatory (M1-like) phenotype when presented with certain antigens (for example, synthetic foreign materials or bacteria), but then transition to pro-healing, anti-inflammatory and constructive phenotype when subsequently influenced by alternative signaling molecules.

In brief, a "normal" response to injury involves an initial pro-inflammatory cell response that must then switch to a pro-healing phenotype lest there be continuous, non-healing inflammation and tissue destruction. The phenotype of cells such as macrophages can be determined, at least in part, by the expression of certain markers that are detected by immunolabeling. A 2012 paper further showed that macrophage phenotype during the early response (i.e., 7-14 days) to an implanted foreign material is predictive of the downstream outcome [6]. An early M1-like response was associated with chronic inflammation and fibrosis; whereas an early M2-like response was associated with minimal fibrosis and constructive and functional tissue remodeling.

With the above concepts in mind, it is now possible to conduct in vitro and/or in vivo studies that evaluate the macrophage response to a biomaterial. The present report includes results of a highly reproducible in vitro assay that characterizes the response of primary murine bone marrow-derived macrophages to ZFuze™ and Xiphos PEEK™.

Objective:

The objective of this study is to compare the gene and protein expression changes in macrophages exposed in vitro to ZFuze and Xiphos PEEK products, as well as cytokine controls.

Materials and Methods:

Isolation and Culture of Bone Marrow Derived Macrophages: Bone marrow were isolated from the femurs and tibias of C57bl/6 mice and subsequently cultured in complete growth media, including Dulbecco's modified Eagles medium, 10% fetal bovine serum (FBS), 10% L929 supernatant, 0.1% beta-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM nonessential amino acids, and

10 mM HEPES buffer, for 7 days with complete media changes every 48 h until mature bone marrow-derived macrophages are obtained.

$2*10^6$ macrophages were then plated onto Tissue Culture Plastic, Z-FUZE, or Xiphos PEEK, using 1 cm inner-diameter steel rings to confine the cells to the surface of the tiles. At the first media change the rings were removed.

Macrophage Activation: Mature macrophages were exposed to the following treatments for 24h: complete media (M0 control), 20 ng/ml IFN-γ and 100 ng/mL lipopolysaccharide LPS (M1 control), 20 ng/mL interleukin IL-4 (M2 control). For the cytokine challenge study, cells were exposed for 6h to 20 ng/ml IFN-γ and 100 ng/mL LPS, washed and then placed in 10% FBS 1% P/S DMEM for 24h. After the incubation period at 37°C, cells were washed with sterile PBS and fixed for 30 minutes with 2% paraformaldehyde (PFA) for immunolabeling, or harvested with TRIzol lysis reagent for RNA assessment, respectively.

Macrophage Immunolabeling: Fixed cells were washed with 1X PBS followed by incubation in a blocking solution composed of PBS, 0.1% Triton-X, 0.1% Tween-20, 4% goat serum, and 2% bovine serum albumin for 1 h at room temperature. The blocking buffer was then be removed and the cells will be incubated in a solution of one of the following primary antibodies: anti-F4/80 at 1:100 dilution as a pan-macrophage marker, anti-inducible nitric oxide synthase (iNOS) at 1:100 dilution as an M1-like marker, and anti-Fizz1 and anti-Arginase1 at 1:200 dilution, each as M2-like markers. The cells were incubated in primary antibody at 4°C for 16 h, after which the primary antibody was removed, and the cells washed with PBS. A solution of fluorophore-conjugated secondary antibody wasadded to the appropriate well for 1 h at room temperature in blocking solution. The antibody wasl then be removed, the cells washed with PBS and the nuclei counterstained with DAPI. Cytokine-activated macrophages was used to establish standardized exposure times (positive control), and held constant throughout groups thereafter.

RNA Isolation: RNA was isolated from 8×10^6 cells using the RNeasy Mini Kit according to the manufacturer's instructions. Isolated RNA concentration was subsequently determined using a NanoDrop spectrophotometer. Reverse transcription of 500 ng of RNA to cDNA was performed via a high-capacity reverse transcriptase kit according to the manufacturer's instructions. SYBR Green gene expression assays was used to determine the relative expression levels of: gapdh, inos, tnf-α, arg1, fizz1, il1β, il6, and gapdh. All results were analyzed by the ΔΔCt method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize the results. Fold change was compared to media control macrophages (M0) as the baseline.

Quantification and Statistical Analysis: Exposure times for each antibody was determined using cytokine controls and then held constant throughout. Images were then quantified using CellProfiler. Imaging was performed by taking a representative image of each treatment group, and then averaging the number of green positive nuclei per treatment group across all three replicated and compared using a one-way ANOVA. Significant differences were then subjected to a Tukey's HSD.

Results:

Immunolabeling:

Legend:

0: expression comparable to M0	+: increased expression compared to M0	**0/+:** slightly higher expression than M0

First Biological Replicate

	F4/80	iNOS	Arg1	Fizz1
M0	+	0	0	0
M1	+	+	0	0
M2	+	0	+	+
ZFuze	+	+	0	+
PEEK	+	+	0	0/+
M1 → M0	+	+	0	0
M1 → ZFuze	+	+	0	+
M1 → PEEK	+	+	0	0/+

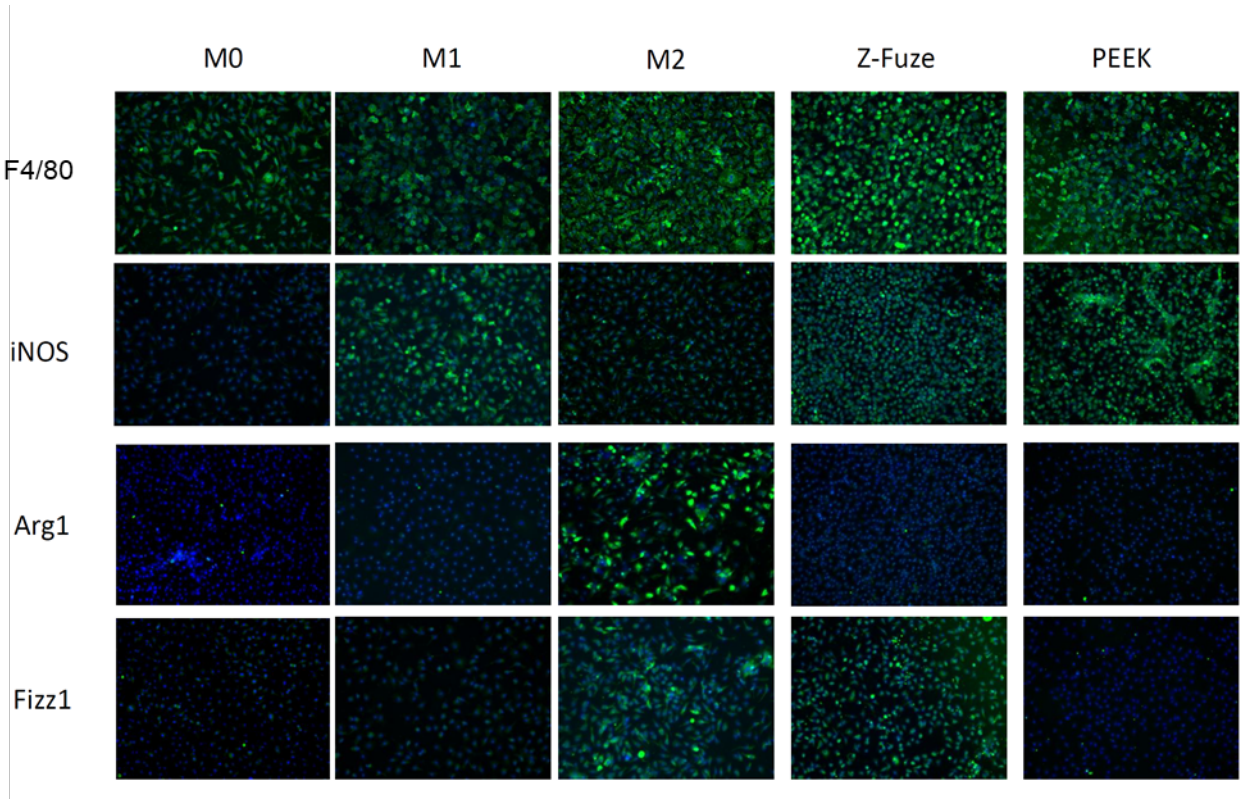
Second Biological Replicate

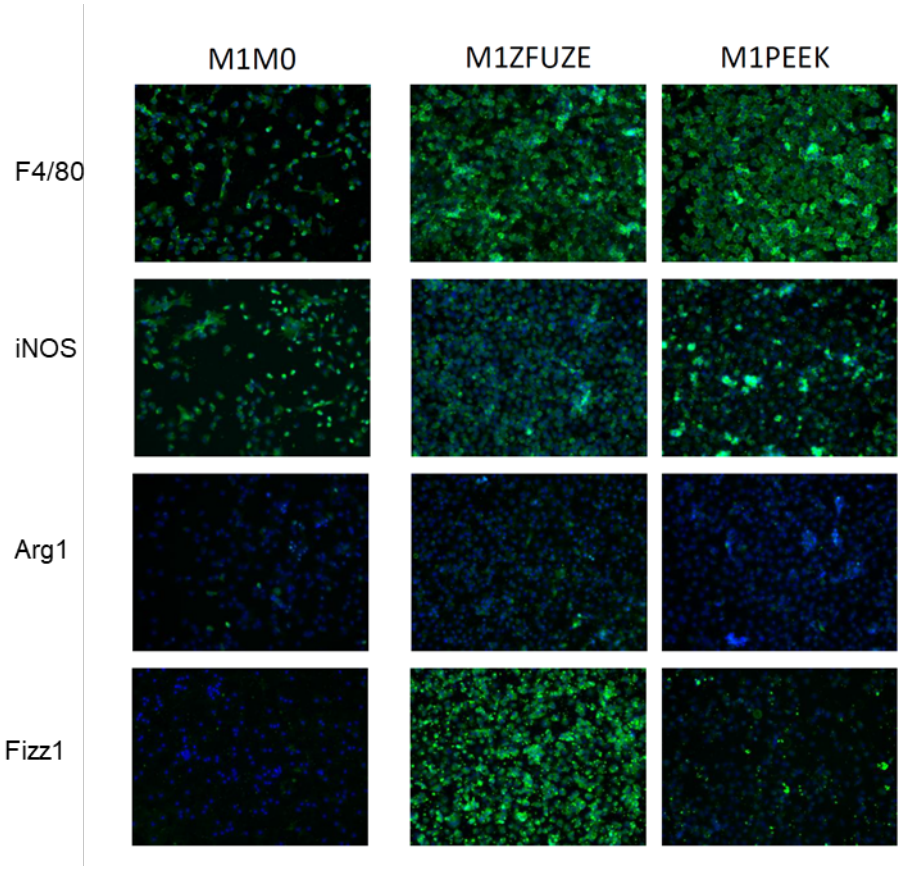
	F4/80	iNOS	Arg1	Fizz1
M0	+	0	0	0
M1	+	+	0	0
M2	+	0	+	+
ZFuze	+	+	0	+
PEEK	+	+	0	0
M1 → M0	+	+	0	0
M1 → ZFuze	+	+	0	+
M1 → PEEK	+	+	0	+

Third Biological Replicate

	F4/80	iNOS	Arg1	Fizz1
M0	+	0	0	0
M1	+	+	0	0
M2	+	0	+	+
ZFuze	+	+	0/+	+
PEEK	+	+	0	0
M1 → M0	+	+	0	0
M1 → ZFuze	+	+	0/+	+
M1 → PEEK	+	+	0	0

Representative Images:

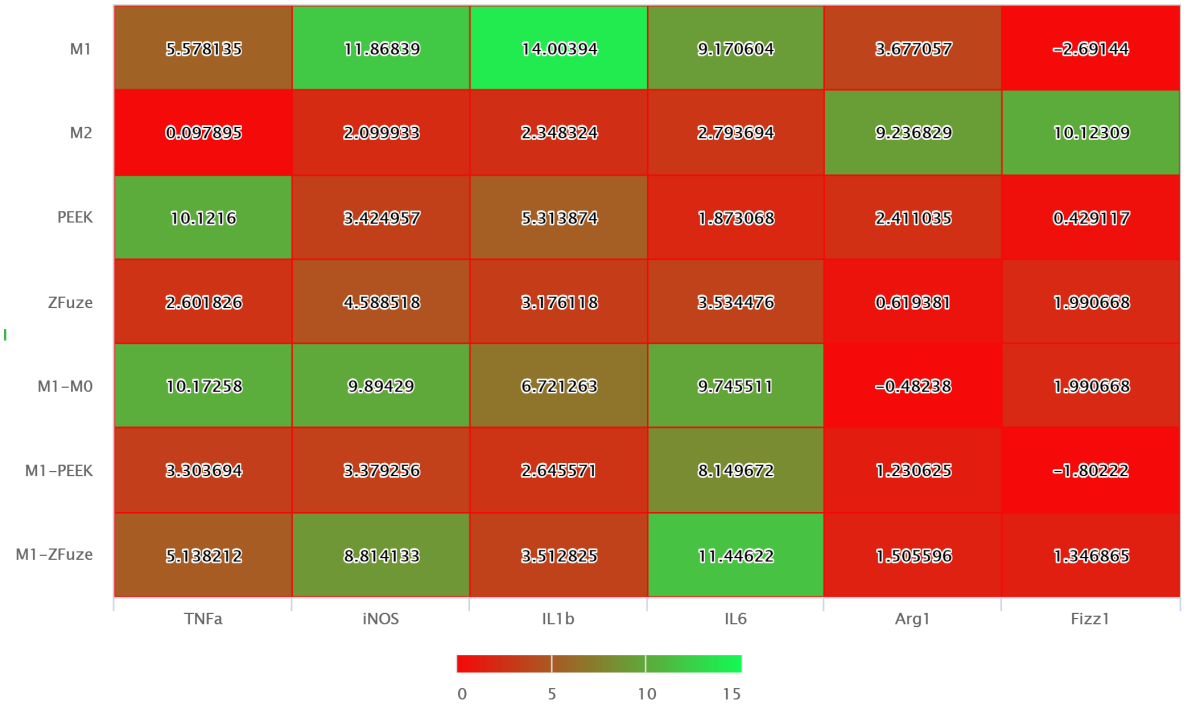

M0
M1
M2
Z-Fuze
PEEK
F4/80
iNOS
Arg1
Fizz1


M1M0
M1ZFUZE
M1PEEK
F4/80
iNOS
Arg1
Fizz1

Immunolabeling Summary:

- Z-Fuze: iNOS^{+}/Fizz1^{+} (Arginase activation is inconclusive).
- Xiphos PEEK: iNOS^{+}/Arg^{-}/Fizz1^{-}

qPCR:



- Heatmap of Log_2 fold expression changes relative to M0 control.
  - Values represent the average fold change in three biological replicates, as well as three technical replicates.
- Summary of Results:
  - qPCR results corroborate immunolabeling results showing that ZFuze^{TM} promotes greater Fizz1 expression than Xiphos PEEK^{TM} test articles. Both products increase the expression of iNOS compared to media controls.
  - ZFuze^{TM} test articles led to only low activation of TNF-α similar to IL-4 treated macrophages. However, Xiphos PEEK^{TM} test articles promoted a >2-fold increase in TNF-α.
  - IL-1 expression was greater in macrophages exposed to Xiphos PEEK^{TM} test articles than ZFuze^{TM}.
  - IL-6 expression was greater in macrophages exposed to ZFuze^{TM} test articles than Xiphos PEEK^{TM} treated cells.

Conclusion:

Results of the present study suggest that ZFuze^{TM}, but not Xiphos PEEK^{TM}, promotes activation of several genes associated with an M2-like macrophage phenotype. Results of protein expression data are corroborated by gene expression data. In particular, ZFuze^{TM} test articles promote increased expression of

Fizz1 and decreased expression of IL-1β; both of which correlate with a pro-repair M2-like macrophage phenotype.

References:

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