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# Pleiotropic role for monocyte C-fms protein in response to vascular injury: Potential therapeutic target

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ABSTRACT

*Objectives:* We examined the role of C-fms+ cells in response to vascular injury with a focus on the temporal and spatial platelet interactions, monocyte survival and proliferation within the evolving neointimal lesion and monocyte proliferation within the circulation and specified monocyte reservoir sites. Finally, we investigated the therapeutic effect of C-fms kinase inhibition (CFKI) on neointimal hyperplasia post vessel injury.

*Methods and results:* We utilized murine carotid-wire injury, a transgenic C-fms reporting mouse model, confocal microscopy, shear-flow studies, specific C-fms signalling inhibition to determine the activation, mobilization and recruitment of C-fms+ monocytes in the context of early and late vessel remodelling. C-fms+ cells were recruited as early as 4 h and accumulated over time in the neointima following injury. Monocyte interaction with platelet thrombus under flow and in vivo, in addition to monocyte mobilisation into the circulation post-injury was impaired by CFKI administration. Sustained inhibition of C-fms over 1–2 weeks abrogated the neointimal response but preserved re-endothelialisation post-injury.

*Conclusion:* These data establish C-fms as a key regulator of the vascular response to injury and a potentially attractive therapeutic target in disease states where neointimal hyperplasia, monocyte activation and pathologic remodelling are prominent and endothelial homeostasis is desirable.



## 1. Introduction

Neointimal hyperplasia following vascular injury generates luminal restriction, downstream ischemia and, if severe, vessel occlusion [1] leading to restenosis in approximately 30% of interventional angioplasty procedures [2]. The cellular components of this response include platelets, cells of the mononuclear phagocyte system (MPS) and smooth muscle cells (SMC). Over the past two decades targeting of the platelet and SMC proliferative responses to vascular injury has resulted in improved clinical outcomes after percutaneous coronary intervention (PCI) with a reduction in late restenosis [3]. However, no clinical therapy currently exists that specifically targets the MPS response to vascular injury. The MPS, consisting of monocytes, macrophages and dendritic cells accomplishes several tasks post injury including clearance of dead cells and cellular debris by phagocytosis and activation of specific immunological defence through antigen-presentation mechanisms [4]. Resident macrophages also facilitate a rapid response to wound healing and angiogenesis [5,6]. Given the scavenger function of the MPS during the acute inflammatory response, ablation of monocytes or inhibition of monocyte recruitment appears to result in reduced neointima formation [7,8]. However, despite these data, key regulators of the MPS contribution to neointimal hyperplasia in the context of vascular injury remain poorly understood.

The maturation and development of monocytes are dependent on signalling resultant from binding of macrophage colony-stimulating factor (M-CSF) to its receptor C-fms [9,10]. Mice deficient for either the C-fms receptor or M-CSF have been shown to possess substantially reduced numbers of blood monocytes [11,12]. The expression of C-fms is lineage-specific to myeloid cells and is largely restricted to monocytes, macrophages and their bone marrow (BM) precursors [13]. Although the role of C-fms in neointimal hyperplasia is still unknown, there is indirect evidence that its ligand M-CSF is increased after balloon angioplasty [14], accelerates neointima formation after exogenous administration in a femoral wire-injury model [15] and rises rapidly in the serum of human subjects after PCI [16].

We show here that C-fms-expressing cells participate in vascular injury from as early as 4 h to as late as several weeks following vessel perturbation. C-fms regulates multiple aspects

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of the MPS response to injury including monocyte recruitment, monocyte–platelet adhesion, monocyte invasion, survival and proliferation. Inhibition of C-fms kinase signalling in the first two weeks post injury abrogates neointimal hyperplasia. These data suggest that targeted inhibition of C-fms could prove beneficial in human vascular disease states associated with neointimal hyperplasia.

## 2. Materials and methods

### 2.1. Mice, carotid wire-injury and CFKI administration

C57BL6/J mice were obtained from Harlan Laboratory, UK. All procedures were approved by University College Cork Ethics Committee in addition to being licensed by the Department of Health and Children in Ireland. Mafia mice were obtained from Jackson Laboratory with MTA from Ariad Pharmaceuticals Inc. (Cambridge, MA). Mice weighing 23–25 g were anesthetised by i.p. administration of ketamine (90 mg/kg) and xylazine (10 mg/kg) and the left common carotid artery at its bifurcation was exposed by a standard microsurgical procedure, before injury by five endoluminal passages of an angioplasty guide wire (0.25 mm diameter, Brivant Ltd., Galway, Ireland). CFKI ($C_{20}H_{22}N_4O_3$), was synthesised by the UCC Chemistry department, based on a previous study [17]. The synthesised sample, confirmed by $^1$H NMR studies, was identical to the commercial sample of CFKI GW2580 (EMD Biosciences). CFKI (in 0.5% hydroxy propyl methylcellulose and 0.1% Tween 80) was administered at 75 mg/kg per dose by oral gavage using soft-tip flexible plastic feeding tubes (Instech Solomon, US).

### 2.2. Carotid tissue section, immunohistochemistry and immunofluorescent microscopy

At designated time points after wire injury of the carotid artery, mice were euthanized by i.p. injection of urethane. Perfusion was done with 3 mL of ice-cold buffer (10 U/mL heparin in PBS) followed by 3 mL of ice-cold 4% paraformaldehyde (PFA) through the left ventricle of the still beating heart. Fixed carotid artery was dissected out and cleaned of surrounding fat and debris before embedding in OCT for cryosectioning. For intima/media (I/M) ratio analysis tissue was cut at 5 μm thickness through the entire length of the injured carotid and haematoxylin & eosin (H&E) stained. I/M ratios were calculated from digitally photographed H&E stained sections using Nikon NIS-Elements Br imaging software.

For confocal microscopy (Nikon C1 plus) the following antibodies were used; anti-SMA (IA4) and anti-von Willebrand Factor (Dako), anti-C-fms (clone ab61137) from Abcam, anti-CD42b (Clone Xia.G5) from Emfret, anti-F4/80 (Clone BM8) from eBioscience and anti-Ly-6G (Clone 1A8) from BD Pharmingen. Appropriate isotypes were used as background control. DAPI nuclear stain and secondary antibodies – goat anti-mouse IgG and goat anti-rabbit IgG conjugated to Alexa Fluor 488 or 546 – were from Molecular Probes, Invitrogen.

En face microscopy sections of carotid arteries were prepared by cutting the carotids longitudinally after perfusion with PBS followed by PFA. The carotids were then placed in Cytofix/Cytoperm solution (BD Pharmingen) + 0.5% Chicago Sky Blue (Sigma) to quench and shift the green auto-fluorescence from the elastic laminae to red. The carotids were washed and mounted flat with Prolong media (Invitrogen) onto a glass slide/cover slip.

### 2.3. Flow cytometry

Blood was collected from facial vein with EDTA as anti-coagulant and red blood cells were lysed using Pharm Lyse buffer (BD Pharmingen). Cells, in PBS 3% FBS, were stained on ice with anti-C-fms (CD115) APC or PE (eBioscience). BM cells were harvested from the tibia and stained directly without RBC lysis. Splenic cells were obtained from PBS perfused spleens and passed through 70 μm strainer to achieve single cell suspension.

### 2.4. Cell culture

BM-derived monocytes (BMM) were generated by incubating BM cells (BMC) harvested from the tibias and femurs of adult C57BL/6J up to 1 week in X-Vivo 15 media (BioWhittaker), 5% FCS and 50 ng/mL rm-MCSF (Peprotech).

### 2.5. In vivo cell proliferation

100 μL of 10 mg/mL BrdU was injected intraperitoneally per day of the experiment. Cells were harvested from desired organs and stained for BrdU incorporation using BrdU Flow Kit (BD Pharmingen) as per the manufacturer's instructions.

### 2.6. Myeloid progenitor-colony forming unit (M-CFU) assay

20,000 BM cells harvested from the tibia of each mouse were mixed with methylcellulose-based media (M3534-MethoCult, StemCell) supporting the growth of CFU-granulocytes/monocytes and cultured for 1 week. The number of colonies were then enumerated by the investigator and subsequently verified by an independent observer in a blinded fashion.

### 2.7. In vitro PBMC dynamic adhesion assay

A 40 mL volume of whole human blood from healthy volunteers was drawn into a syringe containing sodium citrate anticoagulant (0.38%). Half of the blood was labelled using mepacrine (10 μM, Sigma) and from the remainder, mononuclear cells were isolated using density gradient centrifugation (1000 × *g*, 30 min, 4 °C without brake) with Biocoll (Biochrom, Germany). Monocyte enrichment of the PBMC fraction was achieved through attachment to cell culture flasks. Separate aliquots of attached cells were simultaneously labelled with CellTracker Orange, CTO (Invitrogen) and treated with vehicle, 1 μM or 10 μM CFKI. The cells were washed three times with sterile PBS and detached with versene. The cells were added to 10 mL assay volumes of mepacrine-labelled whole blood at a final concentration of $2 \times 10^5$/mL prior to the commencement of flow, with vehicle or CFKI accordingly. Perfusion slides (μ-slide VI, ibidi, Munich, Germany) were coated with fibrillar Type I Collagen (100 μg/mL, Helena Labs). Labelled blood was perfused over collagen at arterial shear (15 dyn/cm$^2$) and images were taken at the indicated timepoints using confocal microscopy (Nikon T2000E). Cell attachment was quantified using NIS Elements BR software (Version 3.0, Nikon, Japan).

### 2.8. Statistical analysis

Data represent mean ± SEM and were analyzed by 1-way analysis of variance, or Kruskal–Wallis test followed by Dunn's Multiple Comparison test where appropriate, using Prism4 software (GraphPad, La Jolla, California). A $p$-value <0.05 was considered significant.

## 3. Results

### 3.1. C-fms+ cell mobilisation and homing to the vessel wall after vascular injury

After injury, carotid arteries showed immediate platelet-rich thrombus formation in the early phase (4 h–1 day), followed by