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Smooth Muscle Cell to Macrophage Differentiation in Atherosclerosis - Remaining Limitations and Questions

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Abstract
Macrophages contribute decisively to the initiation and progression of atherosclerosis. Although most studies conclude that plaque macrophages derive from circulating monocytes, there is growing evidence that vascular-resident smooth muscle cells (SMCs) may also differentiate into macrophages and contribute to the growing pool of foam cells in the atherosclerotic plaque. Understanding of SMC-to-macrophage differentiation has been clouded by inadequate fate-mapping studies and potentially inaccurate staining of SMC- or macrophage-specific markers. A new study published in *Nature Medicine* by Shankman et al., used a novel fate-mapping technique to label SMCs early in atherosclerosis and definitively assess their cellular fate throughout the progression of disease. The authors conclude that SMCs make up a striking number of cells in the atherosclerotic plaque, but lose several SMC-specific markers masking their inclusion in previous studies. Although this research illustrates the plasticity of SMCs and the importance of SMC retention in ameliorating atherosclerosis, it, unfortunately, does not confirm that SMCs differentiate into functional macrophages nor provide proof that SMC-to-macrophage differentiation is important for the progression of atherosclerosis.

Keywords: smooth muscle cells, macrophages, KLF4, atherosclerosis

Introduction

Atherosclerosis is a chronic inflammatory disease caused by the buildup of lipid plaques in arterial walls, and is the leading cause death and disability worldwide. Macrophages contribute decisively to the initiation and progression of atherosclerosis (Guo et al., 2003). Macrophages traffic to sites of lipid deposition in the artery intima and phagocytose cholesterol and lipid deposits. While macrophage uptake of oxidized lipoproteins may initially prevent damage to artery walls, once they become overwhelmed, lipid-engorged macrophages, or foam cells, take on permanent and inflammatory positions in the arteries contributing to the buildup and rupture of atherosclerotic plaques. Our understanding of how macrophages are recruited and proliferate within the plaques has significantly improved over the last decade (Guo et al., 2003; Guo et al., 2005; Swirski et al., 2006; Tacke et al., 2007; Potteaux et al., 2011; Dutta et al., 2012; Robbins et al., 2012; Robbins et al., 2013), but recent studies suggest our scope of investigation into macrophage dynamics and ontogeny has been too narrow.

Until recently, lesional macrophages were thought to develop exclusively from circulating myeloid-derived monocytes recruited to sites of lipid deposition. In 2013 it was also demonstrated that once in the lesion, macrophages have the potential to proliferate *in situ* and no longer rely on monocyte recruitment (Robbins et al., 2013). Nonetheless, circulating monocytes are still the initial precursors of proliferating macrophages. Several studies have challenged this monocyte-to-macrophage dogma in the atheroma, however, suggesting macrophage accumulation may result from local arterial smooth muscle cell (SMC) differentiation in addition to myeloid cell recruitment (Gomez and Owens, 2012).

Firm support for the SMC-to-macrophage hypothesis has been lacking due to potentially inaccurate SMC- or macrophage-specific markers in immunolabeling and fate-mapping studies (Feil et al., 2014). Previous reports have characterized CD68+Mac2+ cells as macrophages and α-actin+ cells as SMC (Papaspyridonos et al., 2008; Lovren et al., 2012), but these markers may include several additional cell types or misclassify SMCs as macrophages or visa versa. This is because multiple reports now show that in the appropriate environment, macrophages and SMCs can drastically alter their phenotypic markers (Gomez and Owens, 2012; Lavin et al., 2014). In human coronary artery atherosclerotic plaques, for

example, 10% of α-actin+ cells (thought to be SMCs) were myeloid derived (Caplice et al., 2003). Similarly, cholesterol loading of SMCs downregulated SMC-associated markers and upregulated macrophage-associated markers, including CD68 and Mac2 (Rong et al., 2003). These studies do not indicate, however, whether SMCs and macrophages can completely different into one another. Nonetheless they illustrate that staining for SMCs or macrophages alone may be unreliable in assessing the origin or role of a particular cell type within the atherosclerotic lesion.

Identifying the ontogeny of lesional macrophages is important because efforts to reduce their accumulation in atherosclerotic plaques will likely fail if we do not completely understand their origins. For example, current pharmacological efforts to reduce monocyte recruitment to the plaque (Rader and Daugherty, 2008; Tabas and Glass, 2013) may have no long-term benefits if macrophages can derive from SMCs. A recent study in *Nature Medicine* attempts to clearly define how many macrophages derive from SMCs and whether these cells can function as macrophages (Shankman et al., 2015).

Study

In an article published on June 21, 2015 in *Nature Medicine* the authors Shankman et al. use a rigorous fate-mapping approach to permanently label SMCs early in life (6-8 weeks) and evaluate their fate in transgenic mice prone to atherosclerosis (*ApoE*$^{-/-}$) (Shankman et al., 2015). SMCs were labeled using the *Myh11*-CreER ROSA floxed STOP eYFP *ApoE*$^{-/-}$ mice and tamoxifen injection. Following tamoxifen injection all Myh11+ cells were permanently labeled with eYFP. Because Myh11 appears to be the best marker of SMCs during steady state (Gomez et al., 2013), this technique allowed researchers to label >95% of SMCs within the arteries and track their fate during atherosclerosis.

The first finding from these studies was that an unappreciated number of cells in the atherosclerotic lesion were derived from SMCs. The authors report that 82% of eYFP+ cells were negative for another key SMC marker, α-actin, indicating previous reports using α-actin to label SMCs in the lesion missed nearly 80% of the cells. The authors go on to show that a proportion of eYFP+ cells were also positive for multiple macrophage markers including Mac2, F4/80, and CD11b. Using immuno-transmission electron microscopy the authors show that a subset of these cells were lipid-laden and actively engulfing neighboring cells - functions attributed to macrophages.

Further support for a SMC origin to macrophages within the atherosclerotic lesion was provided through epigenetic labeling studies. The authors previously reported detection of a novel and permanent epigenetic modification in SMCs (Gomez et al., 2013). By performing an *in situ* hybridization assay for the histone H3K4diMe in the Myh11 promoter (PLA+) the authors identified any cell that was, at one point, a smooth muscle cell. Using this technique they further confirmed macrophage markers on PLA+ cells in mouse and human lesions, suggesting plaque macrophages in humans can also derive from SMCs.

A key transcription factor involved in SMC phenotypic alterations is KLF4 (Cordes et al., 2009; Salmon et al., 2012; Turner et al., 2013). KLF4 is a negative regulator and downregulates its target genes by binding to their promoter. In the 2015 Shankman et al. paper, the authors report that the binding of KLF4 to promoter regions of several SMC genes was markedly unregulated in *ApoE*$^{-/-}$ mice fed a high fat diet. This suppression of SMC genes, presumably, allowed for the upregulation of genes associated with macrophages or mesenchymal stem cells. In the absence of SMC-specific KFL4, atherosclerotic lesions were reduced. The authors suggest the attenuated atherosclerosis my have resulted from reduced differentiation of SMCs into macrophages. Indeed, their *in vitro* studies show reduced Mac2 expression and reduced phagocytosis in cholesterol-loaded SMCs that lacked KLF4.

Limitations

This study attempts to conclusively show that macrophages within the atherosclerotic plaque can and do derive from SMCs. Although the authors used novel transgenic approaches to elegantly label and fate-map SMCs during atherosclerosis, they did not provide concrete evidence that the SMC-derived cells functioned as macrophages, nor did they establish the

importance of SMC-to-macrophage differentiation in atherosclerosis.

A primary concern in these studies was that the proportion of lesional macrophages deriving from SMCs was never shown. Although the authors claim nearly 30% of lesional macrophages derived from SMCs, this was not quantitatively or qualitatively demonstrated in any figure. There is high contention in the atherosclerotic field as to the origins of lesional macrophages (Tabas et al., 2015) and thus showing these data was critical to the message of the manuscript. The authors could have clearly shown the contribution of SMC-to-macrophage differentiation in the flow cytometric studies of Figure 1. Moreover, an important point never addressed in the manuscript was whether the defined "SMC-derived macrophages" were CD45 positive or negative. This is important because most studies investigating macrophages in the atherosclerotic lesion exclude CD45- (non BM-derived) cells from flow cytometric analysis (Lessner et al., 2002; Swirski et al., 2006; Robbins et al., 2013). Thus, studies using flow cytometry to quantify lesional macrophages may not even include SMC-derived macrophages in their analysis. This would be a huge revelation for the field and could be clearly shown by examining the proportion of macrophages that were eYFP+CD45+, eYFP+CD45-, and eYFP-CD45+.

The second major limitation with this study was the lack of definitive evidence that SMC-derived macrophages function as macrophages. The authors were likely aware of this limitation because they classified SMC-derived cells as "macrophage-like." The authors show one electron micrograph image of a lipid-laden eYFP+ cell engaged in phagocytosis, but state that this cell type was rare. A more relevant study would have been to isolate eYFP+ (SMC-derived) and eYFP- (myeloid-derived) macrophages and determine their phenotypic and functional differences *ex vivo*. Can these cells equally produce inflammatory mediators and respond to oxidized lipoproteins or TLR agonists? Is the phagocytosis rate and quality similar? In the end, it does not matter if SMCs take on macrophage-specific markers if they do not take on the detrimental functions of foam cells in the lesion.

Another limitation was that the KLF4-deficiency studies provided weak support for a role of SMC-derived macrophages in atherosclerosis. In the absence of KLF4, the percentage of α-actin+ cells (i.e. SMCs) in the lesion increased, though by the authors' own admission α-actin is not a good marker to denote SMCs. This rise in α-actin+ cells, however, only resulted in a minute ($p=0.07$) change in the proportion of macrophages. Thus, the improvement in atherosclerosis may not have resulted from a deficit in macrophages, but from an increase in the fibrous cap and SMC stability (Akhtar et al., 2013; Lopes et al., 2013).

Finally, the last main figure attempts to prove that the absence of KLF4 prevents the differentiation of cholesterol loaded SMCs into macrophages. This figure only shows one image of a KLF4-deficient cell, however, and in this cell Mac2 expression and bead uptake were still noted. Perhaps KLF4 is sufficient but not necessary to induce differentiation.

Conclusions

Overall the Shankman et al., *Nature Medicine* paper provides further support that, in the right conditions, SMCs have the capacity to upregulate markers typically associated with macrophages. This idea is not entirely new but required further support as previous fate-mapping and immunolabeling studies relied on markers that may be robustly downregulated on SMC during atherosclerosis. Furthermore, as a recent view-point paper suggests, if a mesenchymal-derived cell can differentiate into a myeloid-derived cell *in vivo*, this would change how we approach our interpretation of many diseases - cell origins would no longer be a given and immune cell culprits could come from a variety of tissue sources (Rosenfeld 2015). Unfortunately, this manuscript did not provide definitive evidence of SMC-to-macrophage differentiation in terms of functionality, nor did it provide a conclusion as to whether SMC-derived-macrophages are important in the pathogenesis of atherosclerosis.

The strongest conclusion appeared to be that 82% of SMC-derived cells do not maintain SMC-specific marker expression (i.e., α-actin) 18 weeks after tamoxifen. Staining of α-actin 1 week after tamoxifen would also have been informative here as it is unclear whether these cells ever expressed α-actin, or lost expression over time. Similarly, staining of Myh11 1 week and 18 weeks after

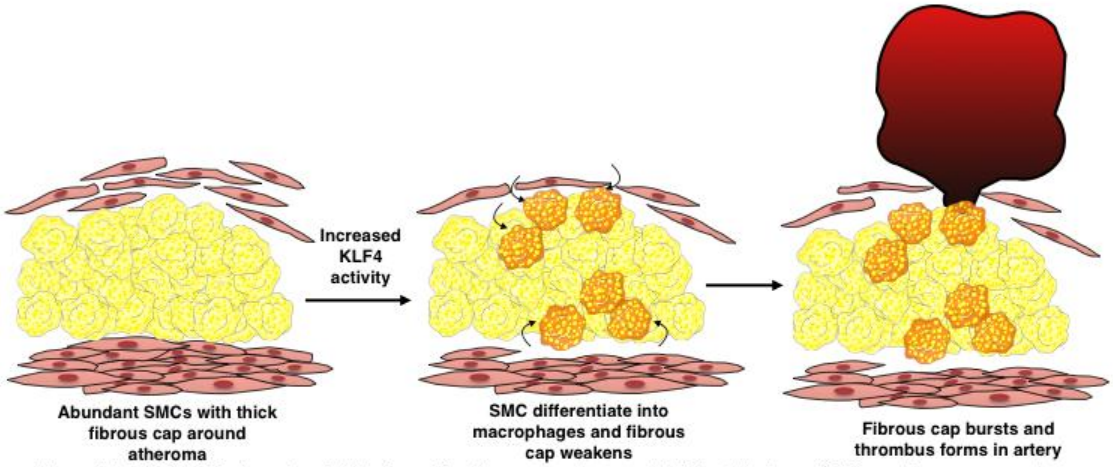

Figure 1. Loss of KLF4 allows for stabilization of the fibrous cap. Increased KLF4 activity lowers SMC-specific gene expression associated with the upregulation of macrophage-specific markers. Whereas the authors hypothesize that an increased number of macrophages results in a larger lesion and more severe atherosclerosis, the loss of SMCs may actually be more detrimental. Increased KLF4 activity results in reduced SMC function and loss of SMCs as they differentiate into macrophages. This loss of SMCs would weaken the fibrous cap resulting in more frequent plaque rupture, thrombus formation, and occurrence of heart attack and stroke - with no functional consequence associated with increased macrophage number per say. Figure created through *Servier Medical Art.*

tamoxifen would be helpful to understand whether these cells lose all SMC marker expression over time, or only a subset of markers.

Another convincing conclusion was that inactivation of KLF4 promotes the retention of SMC activity and allows for more stable fibrous caps. Whether this would ameliorate macrophage accumulation in the lesion is yet unclear, but thickening of the fibrous cap would reduce risk of plaque rupture and subsequent heart attacks and strokes (Figure 1).

Understanding how to block KLF4 on SMCs *in vivo* may be a promising avenue for reducing the detrimental sequelae of atherosclerosis (Yan et al., 2008), independent of macrophage origin, number, or function.

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