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The adult murine heart has a sparse, phagocytically active macrophage population that expands through monocyte recruitment and adopts an 'M2' phenotype in response to Th2 immunologic challenge

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ABSTRACT

Tissue resident macrophages have vital homeostatic roles in many tissues but their roles are less well defined in the heart. The present study aimed to identify the density, polarisation status and distribution of macrophages in the healthy murine heart and to investigate their ability to respond to immune challenge. Histological analysis of hearts from CSF-1 receptor (*csf1-GFP*; MacGreen) and CX_3CR1 (*Cx3cr1*$^{GFP/+}$) reporter mice revealed a sparse population of GFP positive macrophages that were evenly distributed throughout the left and right ventricular free walls and septum. F4/80+CD11b+ cardiac macrophages, sorted from myocardial homogenates, were able to phagocytose fluorescent beads *in vitro* and expressed markers typical of both 'M1' (IL-1β, TNF and CCR2) and 'M2' activation (Ym1, Arg 1, RELMα and IL-10), suggesting no specific polarisation in healthy myocardium. Exposure to Th2 challenge by infection of mice with helminth parasites *Schistosoma mansoni*, or *Heligmosomoides polygyrus*, resulted in an increase in cardiac macrophage density, adoption of a stellate morphology and increased expression of Ym1, RELMα and CD206 (mannose receptor), indicative of 'M2' polarisation. This was dependent on recruitment of Ly6ChighCCR2+ monocytes and was accompanied by an increase in collagen content.

In conclusion, in the healthy heart resident macrophages are relatively sparse and have a phagocytic role. Following Th2 challenge this population expands due to monocyte recruitment and adopts an 'M2' phenotype associated with increased tissue fibrosis.

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Introduction

Well characterised resident macrophage (Mϕ) populations exist in the liver (as Kupffer cells), in the brain (microglia), in the lung (alveolar Mϕ), as well as the peritoneal cavity and bone marrow. In the heart there is plentiful evidence for robust monocyte recruitment after surgically induced myocardial injury (Frangogiannis 2006; Nahrendorf et al. 2007; McSweeney et al. 2010). However, until recently (Pinto et al. 2012; Epelman et al. 2014a), there had been relatively little focus on the regulation, activation status and function of the resident cardiac Mϕ population. This is important, as resident cells in the heart are likely to play a key role in determining the immediate response to 'sterile' injury or immunologic challenge (Epelman et al. 2014b).

It is clear that resident Mϕ populations are heterogeneous in terms of origin, activation status and function, due in part to age

Abbreviations: Mϕ, macrophages; cMϕ, cardiac macrophages; lMϕ, liver macrophages; pMϕ, peritoneal macrophages; IL, interleukin; TNF, tumour necrosis factor; Th2, T helper 2; CSF-1R, macrophage colony stimulating factor 1 receptor; CCR2, C-Cchemokine receptor type 2; RV, right ventricle; LV, left ventricle.

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and gender but also to stimuli present in their local environment (Mosser and Edwards 2008; Davies et al. 2013). Recent studies have suggested that resident cardiac Mϕ (cMϕ) are derived in part from the embryonic yolk sac and liver (Epelman et al. 2014a) but are also replenished from the circulating monocyte population (Molawi et al. 2014). While some studies suggest that they are an abundant population (Pinto et al. 2012; Heidt et al. 2014), others report that they are relatively sparse in the healthy myocardium (Frangogiannis et al. 2002; Macri et al. 2012; Yan et al. 2013). The reasons for these discrepancies are unknown but differences in detection methods e.g. specificity of antibodies in immunohistochemistry and flow cytometry, or use of reporter mice, may have contributed to these varying outcomes.

Resident Mϕ perform varying roles, including maintenance of tissue homeostasis by scavenging and phagocytosing of apoptotic cells arising during normal cell turnover (Lech et al. 2012; Davies et al. 2013). They also act as sentinels of damage and infection, performing immune effector functions. For this reason, Mϕ are highly plastic and signals that they encounter within tissue can alter their activation state, number and function (Mosser and Edwards 2008; Mylonas et al. 2009). Thus both sterile injury and bacterial infection can stimulate them to adopt a 'classical' or 'M1' phenotype that instigates further inflammatory cell recruitment through the production of pro-inflammatory mediators, such as IL-1β and TNF (Gordon and Taylor 2005; Mosser and Edwards 2008). In contrast, phagocytosis of apoptotic cells (Voll et al. 1997; McDonald et al. 1999) or exposure to Th2 type cytokines, such as IL-4, IL-13 and IL-10 can lead to adoption of an 'alternatively-activated' or 'M2' Mϕ phenotype. These 'M2' Mϕ tend to and typically express markers such as Ym1 and RELMα (Nair et al. 2005), suppress inflammation (Mosser and Edwards 2008; Gordon and Martinez 2010) and promote tissue repair, but also fibrosis (Mantovani et al. 2013; Hofmann et al. 2014). The abundant resident cardiac population described by Pinto et al. (2012) was polarised towards an alternatively activated or 'M2' phenotype and also stellate shaped but the underlying mechanism was not investigated. Th2 conditions, such as those arising during helminth infection, promote alternative macrophage activation (Gordon and Martinez 2010; Allen and Wynn 2011), and can also cause expansion of the tissue Mϕ population by resident cell proliferation, rather by recruitment of monocytes from the circulation (Jenkins et al. 2011). The response of the heart to this challenge has not however been investigated.

Our own immunohistochemical studies of Mϕ recruitment following myocardial injury (McSweeney et al. 2010) support suggestions that the resident population of the healthy heart is relatively sparse rather than abundant. The present study was designed to clarify the status of the resident cMϕ population more accurately in terms of density, localisation, function and polarisation status using 2 different Mϕ reporter mouse lines, CSF-1 receptor (*csf1-GFP*; MacGreen; Sasmono et al. 2003) and CX_3CR1 (*Cx3cr1*$^{GFP/+}$; Jung et al. 2000) reporter mice, as well as flow cytometry and *in vitro* functional analysis. The study also sought to determine the response of cMϕ to Th2 type immunological challenge (MacDonald et al. 2002) following infection with 2 separate helminth parasites *Schistosoma mansoni* (*S. mansoni*) or *Heligmosomoides polygyrus* (*H. polygyrus*).

Materials and methods

Mice

Mice (8–12 weeks old) were bred and maintained in conventional barrier unit facilities at the University of Edinburgh. These units are regularly tested in accordance with the Felasa 2014 recommendations, which involves testing for various infectious agents, including parasites. Experimental mice were age and sex matched, and C57BL/6 unless otherwise stated.

Ethics statement

All animal work was compliant with IACUC guidelines, conducted in accordance with the UK Government Animals (Scientific Procedures) Act 1986 and was approved by the University of Edinburgh Ethical Review Committee.

Transgenic mice and helminth infection

Naïve hearts from MacGreen mice, in which cells expressing the c-*fms* gene (CSF-1 receptor; i.e. Mϕ) are positive for enhanced green fluorescent protein (EGFP) (Sasmono et al. 2003), were kindly donated by Prof David Hume's lab (Roslin Institute, University of Edinburgh). In addition, hearts were collected from *Schistosoma mansoni* (*S. mansoni*) infected *Cx3cr1*$^{GFP/+}$ mice. In these *Cx3cr1*$^{GFP/+}$ mice one allele of the CX_3CR1 gene has been replaced by the gene encoding GFP (Jung et al. 2000). Hearts were also obtained from *Heligmosomoides polygyrus* (*H. polygyrus*) infected *Cx3cr1*$^{GFP/+}$ mice and from mice lacking CCR2 (CCR2KO; Boring et al. 1997). The infections were carried out according to published protocols. Briefly, *Cx3cr1*$^{GFP/+}$ mice were infected either percutaneously with ∼80 *S. mansoni* cercariae and hearts recovered 8 weeks later (Phythian-Adams et al. 2010), or with 200 *H. polygyrus* L3 by oral gavage before recovery of hearts 28 days later (Hewitson et al. 2011). These time points were chosen because they reflect the chronic infection stage, with egg production by adults, for these two distinct parasites.

Immunohistochemistry (IHC)

Frozen sections from MacGreen hearts (fixed in 4% paraformaldehyde and snap frozen), were air-dried for 20 min before mounting and coverslipping with Fluoromount aqueous mounting medium (Sigma). GFP positive Mϕ were visualised using the Zeiss Axioskop 2mot+ or Confocal LSM710.

As *Cx3cr1*$^{GFP/+}$ heart sections were fixed in 10% formalin following collection, dampening the GFP signal, GFP was detected in these hearts using a specific antibody (rabbit anti-GFP; 1:1000; AbCam Ab290), rather than direct detection of the GFP signal. Expression of GFP and of the 'M2' markers Ym-1 (rabbit anti-Ym1; 1/100; StemCell Technologies) and RELMα (rabbit anti-RELMα; 0.25 μg/mL; Peprotech) was assessed in heart sections by indirect immunoperoxidase staining.

Briefly, the paraffin embedded tissue sections were deparaffinised and rehydrated. After high temperature antigen unmasking (citrate buffer), endogenous peroxidase was quenched with aqueous 2–3% H_2O_2 (Sigma–Aldrich, UK) for 15–20 min. For GFP staining, slides were then incubated for 30 min in 2.5% Horse Serum (IMPRESS kit, Vector Labs), followed by incubation with primary antibody for 1 h at room temperature. For Ym1 and RELMα, sections were blocked in goat serum buffer (10% goat serum in phosphate buffered saline; PBS), then incubated overnight at 4 °C with primary antibodies. The secondary antibodies were goat anti-rabbit biotin for RELMα and Ym1 (1 mg/ml, Dako Cytomation, Denmark) and anti-rabbit IMPRESS reagent for GFP (IMPRESS kit, Vector Labs). Peroxidase-labelled ABC reagent for Ym1 and RELMα and DAB substrate (Vector Laboratories, UK) for all three were then added for signal visualisation. Finally, the sections were counterstained with haematoxylin, dehydrated through ethanol and xylene and mounted with DPX mountant (Sigma).

To carry out picro-sirius red (PSR) staining for collagen, sections were deparaffinised and rehydrated as above, before treatment in haematoxylin for 8 min. After washing, these were then stained

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