Research Article

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Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions

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Background & Aims: Kupffer cells (KCs), the resident tissue macrophages of the liver, play a crucial role in the clearance of pathogens and other particulate materials that reach the systemic circulation. Recent studies have identified KCs as a yolk sac-derived resident macrophage population that is replenished independently of monocytes in the steady state. Although it is now established that following local tissue injury, bone marrow derived monocytes may infiltrate the tissue and differentiate into macrophages, the extent to which newly differentiated macrophages functionally resemble the KCs they have replaced has not been extensively studied.

Methods: We studied the two populations of KCs using intravital microscopy, morphometric analysis and gene expression profiling. An ion homeostasis gene signature, including genes associated with scavenger receptor function and extracellular matrix deposition, allowed discrimination between these two KC sub-types.

Results: Bone marrow derived "KCs" accumulating as a result of genotoxic injury, resemble but are not identical to their yolk sac counterparts. Reflecting the differential expression of scavenger receptors, yolk sac-derived KCs were more effective at accumulating acetylated low density lipoprotein, whereas surprisingly, they were poorer than bone marrow-derived KCs when assessed for uptake of a range of bacterial pathogens. The two KC populations were almost indistinguishable in regard to i) response to lipopolysaccharide challenge, ii) phagocytosis of effete red blood cells and iii) their ability to contain infection and direct granuloma formation against *Leishmania donovani*, a KC-tropic intracellular parasite.

Conclusions: Bone marrow-derived KCs differentiate locally to resemble yolk sac-derived KC in most but not all respects, with implications for models of infectious diseases, liver injury and bone marrow transplantation. In addition, the gene signature we describe adds to the tools available for distinguishing KC subpopulations based on their ontology.

Lay summary: Liver macrophages play a major role in the control of infections in the liver and in the pathology associated with chronic liver diseases. It was recently shown that liver macrophages can have two different origins, however, the extent to which these populations are functionally distinct remains to be fully addressed. Our study demonstrates that whilst liver macrophages share many features in common, regardless of their origin, some subtle differences in function exist.

Data repository: Gene expression data are available from the European Bioinformatics Institute ArrayExpress data repository (accession number E-MTAB-4954).

Introduction

Kupffer cells (KCs), the resident tissue macrophages of the liver have a crucial role in both the pathogenesis and the resolution of various liver diseases and inflammatory states including alcohol-induced liver injury [1], non-alcoholic fatty liver disease associated with obesity [2], ischemia reperfusion injury [3], immune tolerance to organ transplantation [3] and infectious disease [4].

Resident tissue macrophages, including KCs, were historically considered a hematopoietic population, with replenishment of the tissue reservoir from monocyte-derived precursors in the steady state. This view has now been challenged with the majority of tissue macrophages shown to develop independently of haematopoietic stem cells, being seeded in the tissues prior to birth from a population of yolk sac (YS) derived macrophages [5,6]. These cells show some level of radiation resistance [7] and are independent of replenishment by monocytes in the steady state [8,9]. Parallel studies that identified the transcription factors MafB and c-Maf as the factors that control the self-renewal of

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differentiated macrophages [10], and the observation that tissue macrophages are capable of self-renewal in models of acute inflammation [11] and under T-helper cell 2 conditions in the presence of interleukin (IL)-4 [12], have confirmed that mature tissue macrophages are capable of proliferation and self-renewal. Finally, recent landmark studies have demonstrated that macrophage identity is unique to each macrophage population and is plastic, with phenotype conferred by microenvironment rather than cellular origin [13–15]. Together, the above studies represent a paradigm shift in the field of tissue macrophage biology.

The studies described above all examined the origin of tissue macrophages only in the steady state. However, there is clear evidence that infection or tissue injury, and the associated inflammatory response, promotes the recruitment of myeloid cells, mostly monocytes into peripheral tissues. It is unclear when, and if, the newly infiltrated monocytes undergo differentiation into macrophages *in situ*. In fact, some authors have described the infiltrating cells as tissue macrophages [16], sometimes as early as 24 h post infiltration [17] without defining any phenotypic or functional changes in the cells. Although once infiltrated, monocytes begin differentiating into cells that are similar to the macrophages in the tissue that they reside in [18], the function of these bone marrow (BM)-derived monocytes once they are present in the tissue has not been fully investigated. Given that KCs are implicated in the pathogenesis and the resolution of a number of liver diseases [2–4,19–21] and their phagocytic capacity makes them an easy target for particle based therapeutics [22], a greater understanding of KC biology and heterogeneity will facilitate the development of targeted liver therapeutics. Understanding whether distinct functions can be attributed to KCs of different origin will also be important for the design of new anti-infective strategies.

Here, we have used an irradiation bone marrow chimera model to enforce loss of YS-derived KCs and repopulation of the KC niche from BM-derived precursors. Using intravital microscopy to characterise the morphological and dynamic properties of YS- and BM-derived KCs *in situ*, and microarray analysis to examine gene expression profile, we show that after 6 weeks of differentiation in the liver, BM-derived "KCs" closely resemble but are not fully identical to the YS-derived KCs they have replaced. Whilst uptake of acetylated low density lipoprotein (Ac-LDL) was more prominent in YS-derived macrophages and the converse was true for bacterial uptake, for most of the functional studies we performed, these populations were functionally similar. This was particularly notable in their capacity to exert early control of and direct granuloma formation in response to infection with the KC-tropic intracellular protozoan parasite *Leishmania donovani*. These findings demonstrate that in the context of enforced liver inflammation, BM derived monocytes transition into KCs, which are as capable of protecting the host from infectious challenge as their YS-derived counterparts.

Materials and methods

Mice and infection

C57BL6 or B6.CD45.1 mice were obtained from Charles River (UK) or the Australian Resource Centre (WA). mT/mG [1], lysMcre [2] and B6.MacGreen [3] mice have been previously described. Mice were bred and housed under specific pathogen-free conditions and used at 6–12 weeks of age. The Ethiopian strain of *Leishmania donovani* (LV9) and tandem Tomato fluorescent protein expressing LV9 (tdTom.LV9) [4] were maintained by serial passage in *Rag1*$^{-/-}$ mice. Amastigotes were isolated from infected spleens [5], and mice were infected with 3×10^7 *L. donovani* amastigotes intravenously (i.v.) via the tail vein in 200 µl of RPMI 1640 (GIBCO, UK). For the generation of chimeras, mice were placed on acidified water for at least 2 days prior to irradiation. Donor mice were irradiated with 1100 rads on a split-dose regimen (550 rads per dose, 24 h apart) and were then reconstituted with 2–5 $\times$ 10^6 donor bone marrow cells via tail vein injection. Reconstituted mice were treated with oral antibiotics (Baytril) for 4 weeks post-reconstitution.

Liver enzyme analysis

Heparinized blood was immediately centrifuged for 10 min at 300 g. Plasma was stored at −80 °C until analysis. Alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were determined using a Beckman Unicell DxC800 analyzer in a single batch.

Confocal microscopy

Confocal microscopy was performed on 20 µm frozen sections. For tissue containing tdTom expressing parasites, tissues were fixed in 4% paraformaldehyde (PFA) for two h before overnight incubation in 30% sucrose and embedding in OCT medium (Sakura). Antibodies were conjugated to Alexa488 or Alexa647 (eBioscience, UK). Slides were blinded before imaging on a Zeiss LSM510 axioplan microscope (Carl Zeiss Microimaging). Data were rendered and analysed using Volocity software (Improvision).

Ethics statement

All experiments were approved by the University of York Animal Welfare and Ethical Review Body and performed under UK Home Office license ('Immunity and Immunopathology of Leishmaniasis' Ref # PPL 60/3708) or approved by the Queensland Institute of Medical Research Berghofer (QIMRB) animal ethics committee Ref #P2076 (A1412-614).

Intravital imaging

Mice were anaethetised and surgery performed similar to previously described [23] except that anaethesia was maintained by inhalation of 4% isofluorane (Abbott laboratories, UK). Images were acquired on an inverted LSM 780 multiphoton microscope (Carl Zeiss Microimaging), maintained at 36 °C by a blacked-out environmental chamber (Solent Scientific, UK). Images were acquired with a 40x 1.1 water immersion objective and fluorescence excitation provided by a Chameleon XR Ti:sapphire laser (Coherent) tuned to 870 nm.

Whole genome array

RNA was isolated from purified KC and amplified via Agilent low-input Quick Amp labelling kit (Agilent Technologies). Amplified RNA was then assayed with Agilent SurePrint G3 mouse GE 8 $\times$ 60 k microarray chips that were scanned with an Agilent C Scanner with Surescan High Resolution Technology (Agilent Technologies). The data were normalized using the percentile shift method to the 75th percentile. Comparison of the gene expression data between liver resident and BM-derived KCs was performed using the Benjamini and Hochberg false discovery rate (FDR) correction [24]. This analysis was performed with GeneSpring software (version 9; Agilent) as a standard 5% FDR, with the variances assessed by the software for each *t* test performed. A 2-fold expression criterion was then applied to each gene list. Gene ontology analysis was performed using the GeneSpring (Agilent) and Ingenuity pathway systems analysis software packages (Ingenuity Systems). Gene expression data is available from European Bioinformatics Institute ArrayExpress (accession number E-MTAB-4954).

Further methodology may be found in the Supplementary materials and methods.

Results

Radiation-induced liver injury causes loss of a proportion of liver resident KCs and their replenishment from the bone marrow

To study KCs, we used (LysM-Cre x mT/mG)$_{F1}$ mice. To confirm that the majority of KCs expressed Cre recombinase and were