

Showcase on Research

Towards a Systems Biology View of Macrophage and Osteoclast Function

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Note: Figures for this article are reproduced in colour on the inside back cover of this issue.

Introduction

The Mononuclear Phagocyte System (MPS) is defined as a population of cells derived from progenitor cells in the bone marrow, which differentiate to form blood monocytes, circulate in the blood and then enter tissues to become resident tissue macrophages. Mononuclear phagocytes, as defined originally, were found to share many characteristics that had been studied in detail for many years. These common features include the following: morphology and ultrastructural features observed by light and electron microscopy; expression of certain enzymes that could be detected by histochemical staining (notably non-specific esterase, lysosomal hydrolases and ecto-enzymes); non-specific uptake of particles such as latex or colloidal carbon and specific endocytic receptors especially for the Fc portion of immunoglobulin and for complement-coated particles.

Mononuclear phagocytes are the classical two-edged sword in host defence and homeostasis. On the one hand, they are central to innate immunity, recognising and engulfing potential pathogens and removing damaged tissue and dying cells in normal development and wounds. Their capacity for antigen processing and presentation, and their production of regulatory cytokines, are not only essential to development of an acquired immune response but also direct the nature of that response. Mononuclear phagocytes are also major effector cells once an acquired response has occurred. On the other hand, their destructive capacity, if unleashed inappropriately, underlies tissue destruction in inflammatory and infectious diseases, atherosclerosis and malignancy. Interference with the recruitment and function of macrophages represents a major focus for development of new anti-inflammatory therapies. The two faces of mononuclear

phagocytes and their many functions reflect a complex gene expression profile and a remarkable plasticity that are the central foci of research in our group. Several aspects of our research program are outlined below. The group is a major node of the newly-awarded CRC for Chronic Inflammatory Diseases, and also interacts with the ARC Special Research Centre for Functional and Applied Genomics in the Institute for Molecular Bioscience at the University of Queensland.

Comprehensive gene expression profiling

Key staff: Christine Wells, Timothy Ravasi, Ian Ross

In a collaborative program involving our laboratory, the RIKEN Genome Sciences Center in Yokohama, Japan, the Institute for Systems Biology in Seattle, and the ARC Special Research Centre, we have assembled a very large set of mouse macrophage-expressed genes for comprehensive gene expression profiling. The set of genes expressed in macrophage populations is influenced by many different variables, including differentiation state and tissue location, exposure to an enormous diversity of growth factors, lymphokines and microbial products and endocytic targets. Our own studies have also revealed that the genotype of the mice also profoundly alters the gene expression profile under any particular condition, probably reflecting the divergence in innate immunity between individuals (see **Fig. 1**). When one combines array information from many different conditions, it is possible to use computational methods to identify sets of genes that always track together (these are called "clusters"). Very tight regulatory clusters tend to contain genes that act in the same pathway, or contribute to the same overall biological response. Amongst the thousands of macrophage-expressed genes, the majority are still annotated as hypothetical protein, with no structural clues. In many cases, clues as to function (and level of interest) come from

recognition of the company a gene keeps.

Gene expression profiling is also being used to ask specific questions about mechanisms of cell signalling and the role of specific gene products in functional regulation of macrophages. For example, in a collaboration with Prof. Brandon Wainwright, we are using array profiling to identify the set of genes in macrophages, epithelial cells and intact lung that underlies the hyper-inflammatory state of the common genetic disease cystic fibrosis.

Transcription control in the macrophage lineage

Key staff: Roy Himes

A macrophage displays its characteristic properties because it expresses a particular combination of genes that is distinct even from related cell types such as granulocytes. We have worked for a number of years on delineating the characteristics of macrophage-specific promoters that direct transcription solely in the macrophage lineage. One gene of special interest, *c-fms*, encodes the receptor for macrophage colony-stimulating factor (CSF-1), which is the major growth factor for cells of the mononuclear phagocyte lineage. In the course of this project, we have identified the minimal elements, the proximal promoter and a highly-conserved intronic enhancer, required to direct expression of transgene in a manner specific to the macrophage lineage. Using this information, we have produced mice which express green fluorescent protein (GFP) in all the cells of mononuclear phagocyte lineage (**Fig. 2**). Our efforts to identify the transcriptional regulators that bind these key elements proceed on a number of tracks. Major collaborations involve Dr. Connie Bonifer in Leeds, UK, and Dr. Mike Ostrowski in Columbus, Ohio. Macrophage-specific promoters are unconventional, having no TATA-box or other CG-rich sequences. Instead they utilise purine-rich elements that bind PU.1 and other members of the Ets transcription factor family to delineate

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the region of transcription initiation. Through a combination of conventional protein identification via *in vitro* binding assays, gene expression profiling and genetics studies, we have identified a series of candidate regulators that interact with PU.1, such as the leukaemia-associated AML1 transcription factor complex, and the four members of the microphthalmia transcription factor family. Transcriptional regulators are not commonly considered as drug targets, but if they could be targeted effectively, they clearly have the potential to modulate a wide range of macrophage functions in a strictly lineage-restricted manner.

Differentiation of osteoclasts

Key staff: Ian Cassady, Barbara Fletcher

Osteoclasts are the major cell type able to decalcify bone (Fig. 3). They derive from a progenitor shared with other mononuclear phagocytes, and share the major growth factor, CSF-1. Although the major interest in osteoclasts has previously been based upon their role in calcium homeostasis, and possible regulation in osteoporosis, there is rapidly emerging recognition that osteoclast-like cells arise in many inflammatory lesions. Of particular relevance is their association with bone destruction in arthritis, and their appearance in atherosclerotic lesions in which calcification of the vessel wall is clinically important. Our interest in osteoclasts started with detailed studies of the expression, structure and function of the osteoclast marker gene, tartrate-resistant acid phosphatase (TRAP), which is also expressed in some mature macrophage populations. Our group cloned the mouse TRAP gene, produced a high resolution crystal structure of the pig protein (1.7Å) and generated a transgenic mouse that over-expresses TRAP. These studies have provided a framework for validation of TRAP as a drug target in osteoporosis and inflammatory bone destruction. In parallel, we have carried out detailed analysis of the TRAP promoter, as a model for understanding transcription control during osteoclast-macrophage divergence. Osteoclasts use a distinct proximal promoter and distal enhancer for transcription control. Amongst the transcription factors that interact with TRAP, the microphthalmia transcription factor (MITF) interacts with the macrophage-specific PU.1 factor. In

collaboration with Mike Ostrowski at Ohio State University, we are searching for other targets of MITF expressed in osteoclasts, and protein-protein interaction partners that could contribute to the function of MITF. As in the macrophage systems, further identification of candidate regulators, and osteoclast-expressed genes that might be alternative drug targets, involves us in detailed gene expression profiling using cDNA clone sets derived specifically from purified osteoclasts. These studies have been expedited by the recent discovery of cell line systems in which phenotypic transformation towards an osteoclast-like phenotype can be induced.

Differentiation of osteoclasts in our cell culture systems and *in vivo* is influenced by the steroid hormone vitamin D₃, which acts through the VDR, a member of the nuclear hormone receptor superfamily. Many effects of vitamin D₃ are indirect, through actions on osteoblasts. Another class of nuclear hormone receptors strongly implicated in the control of osteoclastogenesis is the peroxisome proliferation activator receptors (PPARs), particularly the PPAR- γ and PPAR- δ isoforms, which are expressed in osteoclasts. The role of PPAR ligands in osteoclastogenesis will be examined by expression profiling of cells treated with ligands and by the generation of novel PPAR- δ knockout mice. The possibility exists for the development of a completely new approach to the regulation of osteoclast activity in acute and chronic osteoclast pathologies.

Response of macrophages to CpG DNA and LPS

Key staff: Kate Stacey, Matthew Sweet

The development of acquired (T and B cell-mediated) immunity to pathogens depends on the ability of the components of the innate immune system such as macrophages to recognise characteristic microbial products. Bacterial cell wall products such as lipopolysaccharide (LPS), lipoarabinomannan and peptidoglycan and characteristic sugar residues of yeast and fungi activate macrophages to become cytotoxic to the pathogen and to secrete a range of cytokines which recruit and activate other immune cells. In recent years it has become apparent that microbial DNA is another product capable of stimulating

the immune system in this manner, and we provided the first evidence that bacterial DNA directly activates macrophages. The ability of the host to discriminate foreign from self DNA relies on the presence of unmethylated CpG motifs in bacterial but not mammalian DNA. CpG DNA *per se* provides an immunological adjuvant in naked DNA vaccines and this activity is retained in stable phosphorothioate-stabilised single-stranded oligonucleotides, which may have many applications as immunological adjuvants.

Our ongoing studies address many aspects of the response of macrophages to bacterial DNA. Mice deficient for the toll-like receptor, TLR9 do not respond to CpG DNA and it has been postulated that TLR9 directly recognises CpG DNA. The most abundant intracellular protein that recognises single stranded DNA is replication protein A (RPA) and we have found that RPA has a selective affinity for immunostimulatory CpG sequences versus non-activating sequences. We are now investigating whether TLR9 and RPA interact to form a recognition complex for CpG DNA. We have also performed a rigorous analysis to identify why self DNA does not activate immune cells. The lack of activity of self DNA appears to result from a combination of CpG suppression, methylation of CpG motifs that are present, and the presence of inhibitory motifs within self DNA that suppress responses to activating sequences. Finally, many of our studies focus on the comparison of LPS and CpG DNA action. Although LPS and CpG DNA have many overlapping actions on macrophages, *in vivo* administration of LPS but not CpG DNA is highly toxic. We are defining the differences between LPS and CpG DNA that may account for the differential toxicities of these two stimuli. One approach has been to assess global gene expression profiles upon LPS and CpG DNA stimulation using cDNA micro-arrays. Our analysis has identified genes that are both LPS- and CpG DNA-specific in macrophages. In addition, we have studied the effects of priming cytokines on macrophage responses to LPS and CpG DNA. This study demonstrated that CSF-1, a cytokine that is constitutively present *in vivo*, enhances the LPS-induced inflammatory response but suppresses the same response to CpG DNA. Thus CSF-1 may contribute to the differential toxicities of LPS and CpG DNA *in vivo*.



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Structural genomics

Key staff: Pawel Listwan, Richard Kidd, Nathan Cowieson, Bostjan Kobe, Jenny Martin

Our ability to predict the folding patterns and possible functions of proteins without a known functional/structural motif is still very limited. Structural genomics is essentially a systematic attempt to gain more information about the diversity of possible protein folds. As a relatively small group compared to the massive international endeavours, we have decided to focus our efforts on sets of proteins where we can utilise the availability of protein and expression plasmids for other purposes. In simple terms, we establish genes of interest based upon their expression profile in macrophages and osteoclasts. With access to the resources of the ARC Special Research Centre, we have established a cloning/expression pipeline in which macrophage/osteoclast specific genes, or those that are induced in an informative manner are selected. Many candidates fail at each of the steps in the pipeline; for example, the protein might not be expressed, it might be insoluble, it may not crystallise, and so on. In parallel, we use epitope-tagged expression plasmids to investigate the intracellular location of the gene product and the effect of over-expression on macrophage function, and we can use the expressed protein to produce antibodies and to identify protein interaction partners. The identification of protein interaction networks has been greatly enhanced by the introduction of Surface-Enhanced Laser-Desorption-Ionisation (SELDI) technology to the laboratory, allowing us to capture interaction partners on protein chips, and “walk through” sets of interacting proteins.

Overview

We have entered a new era in bioscience research, with complete mouse and human genomes, and transcriptomes becoming available. The global aim of our laboratory is to understand macrophages and osteoclasts as a System. We wish to know all of the mRNAs and all of the proteins that these cells can make, and to be in a position to predict how they will change, and how cell function will change, in response to external perturbations. It is no longer a pie in the sky.

Further information about the laboratory, a summary of publications, and resources related to macrophage biology, can be found at on our web site at - www.imb.uq.edu.au/groups/hume

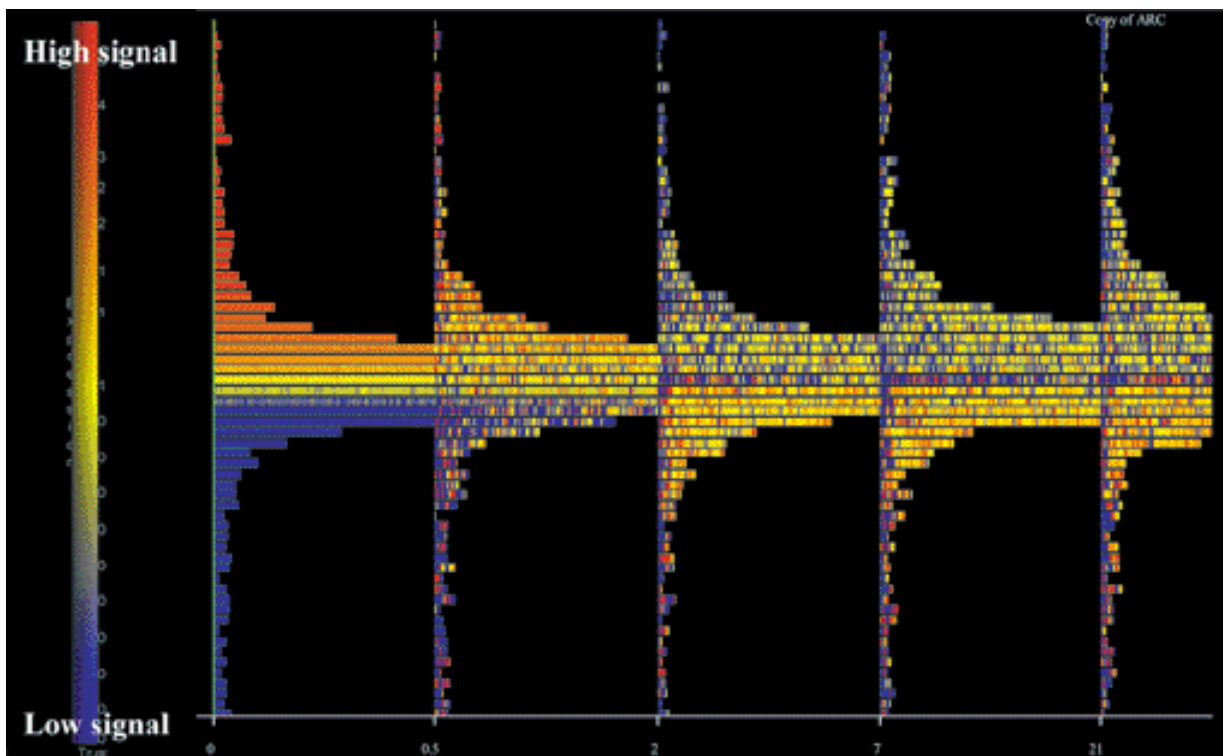


Fig. 1. Gene expression profiling.

Distribution of 20,000 genes across five time points (0, 0.5, 2, 7, 21 hr) after exposure of mouse bone marrow-derived macrophages to lipopolysaccharide (LPS). Each gene is coloured according to the signal intensity measured at time 0. Red indicates the gene was highly expressed at time zero, yellow moderately expressed and blue indicates the gene was not expressed at time 0. Most of the blue elements are induced across the time course, while most of the red elements are repressed. Very few elements remain static, suggesting that global gene induction occurs in parallel with repression and degradation of existing mRNAs.

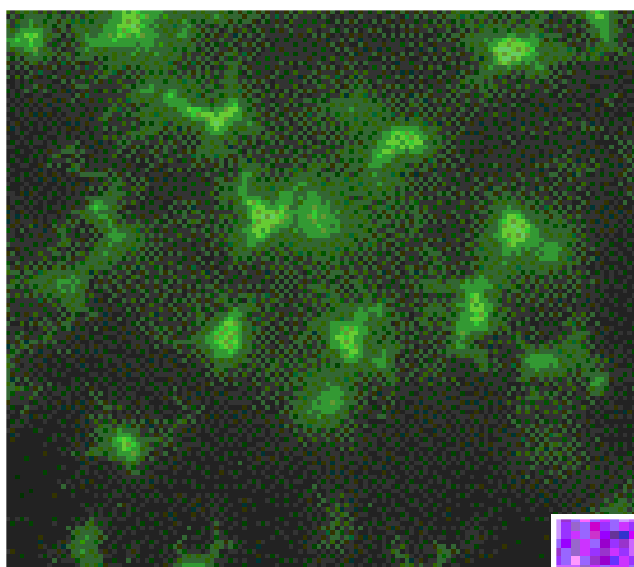
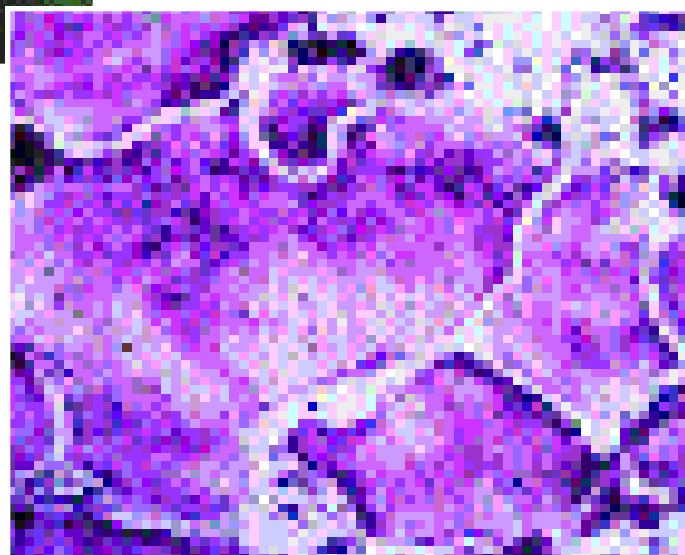


Fig2. Tissue-specific expression of GFP

Live action image of a transgenic mouse ear, in which the enhanced green fluorescent protein (EGFP) is expressed off the CSF-1 receptor (c-fms) promoter. The labelled cells are epidermal macrophages or Langerhans cells, which have a function in control of epidermal renewal as well as activation of the immune system.

Fig3. Expression of TRAP in cultured osteoclasts.

An image of massive multinucleated osteoclasts grown in cell culture. The cells have been stained for expression of the marker tartrate-resistant acid phosphatase (TRAP), a core interest of our group. The cells are not counterstained. A very large single cell fills most of the field. TRAP activity is concentrated in the vicinity of clusters of nuclei, which are correspondingly highlighted by exclusion of reaction product.



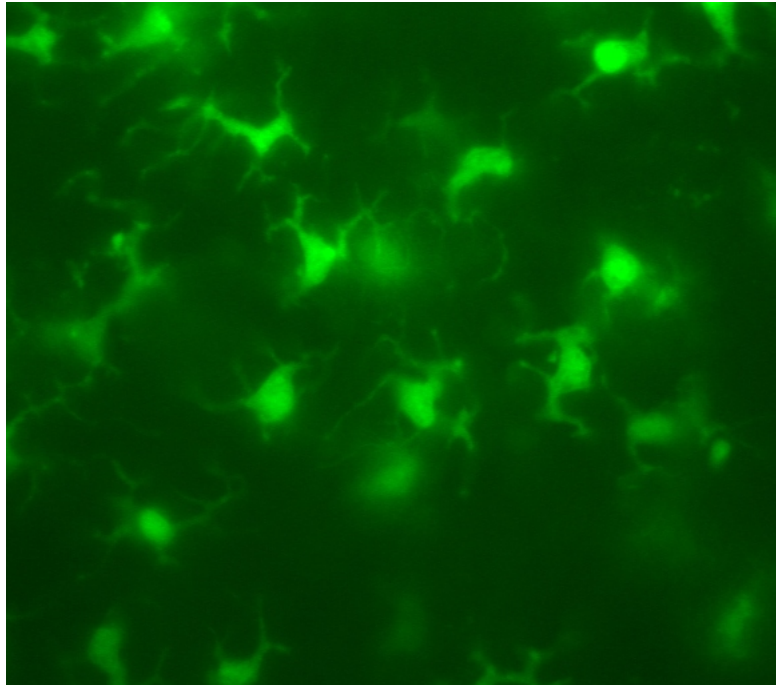


Fig. 2

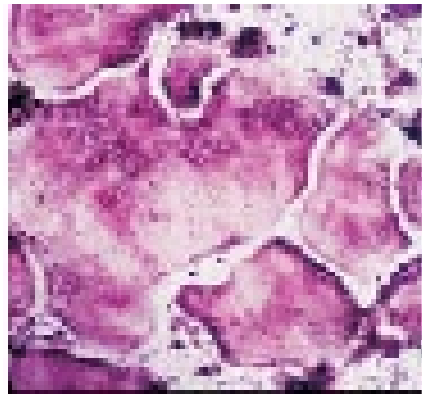


Fig. 3