Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines

Peter J. Murray,[1,*] Judith E. Allen,[2] Subhra K. Biswas,[3] Edward A. Fisher,[4] Derek W. Gilroy,[5] Sergij Goerdt,[6] Siamon Gordon,[7] John A. Hamilton,[8] Lionel B. Ivashkiv,[9] Toby Lawrence,[10] Massimo Locati,[11] Alberto Mantovani,[11] Fernando O. Martinez,[12] Jean-Louis Mege,[13] David M. Mosser,[14] Gioacchino Natoli,[15] Jeroen P. Saeij,[16] Joachim L. Schultze,[17] Kari Ann Shirey,[18] Antonio Sica,[19,20] Jill Suttles,[21] Irina Udalova,[22] Jo A. van Ginderachter,[23,24] Stefanie N. Vogel,[18] and Thomas A. Wynn[25]

[1]Departments of Infectious Diseases and Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
[2]Centre for Immunity, Infection, and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JR, UK
[3]Singapore Immunology Network, A*STAR, 8A Biomedical Grove, Immunos Building, Level 4, Singapore138648, Singapore
[4]Center for the Prevention of Cardiovascular Disease, New York University School of Medicine, Smilow 7, 522 First Avenue, New York, NY, USA
[5]Division of Medicine, Rayne Institute, University College London, 5 University Street, London WC1 6JJ, UK
[6]Department Dermatology, University Medical Center Mannheim, University of Heidelberg, 68167 Mannheim, Germany
[7]Sir William Dunn School of Pathology, University of Oxford, Headington, Oxford, OX1 3RE, UK
[8]Department of Medicine, University of Melbourne and Royal Melbourne Hospital, Parkville, VIC 3050, Australia
[9]Hospital for Special Surgery and Weill Medical College, Cornell University, 535 East 70th Street, New York, NY 10021, USA
[10]Centre d'Immunologie de Marseille-Luminy, 13009 Marseille, France
[11]University of Milan School of Medicine, Istituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano, Milan, Italy
[12]Botnar Research Centre, Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences, University of Oxford, Headington, Oxford OX3 7LD, UK
[13]Infectious Diseases, Aix Marseille University, 27 Boulevard Jean Moulin, 13285 Marseille, France
[14]Department of Cell Biology, University of Maryland, College Park, MD 20742, USA
[15]Department of Experimental Oncology, European Institute of Oncology, Via Adamello 16, 20146 Milan, Italy
[16]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[17]Genomics & Immunoregulation, LIMES-Institute, University of Bonn, 32115 Bonn, Germany
[18]Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[19]Istituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano, Milan, Italy
[20]Department of Pharmaceutical Sciences, Università degli Studi del Piemonte Orientale "Amedeo Avogadro," Via Bovio 6, 28100 Novara, Italy
[21]Microbiology & Immunology, University of Louisville School of Medicine, 319 Abraham Flexner Way, Louisville, KY 40292, USA
[22]Kennedy Institute of Rheumatology, University of Oxford, Headington, Oxford, OX3 7FY, UK
[23]Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium
[24]Laboratory of Myeloid Cell Immunology, VIB, Pleinlaan 2, 1050 Brussels, Belgium
[25]Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
*Correspondence: peter.murray@stjude.org
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Description of macrophage activation is currently contentious and confusing. Like the biblical Tower of Babel, macrophage activation encompasses a panoply of descriptors used in different ways. The lack of consensus on how to define macrophage activation in experiments in vitro and in vivo impedes progress in multiple ways, including the fact that many researchers still consider there to be only two types of activated macrophages, often termed M1 and M2. Here, we describe a set of standards encompassing three principles—the source of macrophages, definition of the activators, and a consensus collection of markers to describe macrophage activation—with the goal of unifying experimental standards for diverse experimental scenarios. Collectively, we propose a common framework for macrophage-activation nomenclature.

Overview

Activation of macrophages has emerged as a key area of immunology, tissue homeostasis, disease pathogenesis, and resolving and nonresolving inflammation (Biswas and Mantovani, 2010; Gordon and Martinez, 2010; Lawrence and Natoli, 2011; Mantovani et al., 2008; Mantovani et al., 2005; Martinez et al., 2008; Murray and Wynn, 2011b; Nathan and Ding, 2010; Wynn et al., 2013). Over the last several years, diverse terms have been applied to macrophage activation and "polarization," where a stimulus such as a cytokine or toll-like receptor (TLR) agonist produces distinct patterns of gene and protein expression. Here, we use the term "activation" to mean the perturbation of macrophages with exogenous agents in the same vein as many use "polarization." We also note the ability of macrophages to change their activation states in response to growth factors (e.g., CSF-1 and GM-CSF) and external cues, such as cytokines, microbes, microbial products, and other modulators, including nucleotide derivatives, antibody-Fc receptor stimulation, glucocorticoids, infection, phagocytosis, and potentially any other entity capable of being recognized by macrophages. Because macrophage activation is involved in the outcome of many diseases, including metabolic diseases, allergic disorders (such as

airway hyperreactivity), autoimmune diseases, cancer, and bacterial, parasitic, fungal, and viral infections, we need to establish a common language for describing the properties of the macrophages under investigation.

Background to the Problem

We note widespread use of at least four definitions of macrophage activation, including terms such as M1 and M2, alternative and classical activation, "regulatory" macrophages, and subdivisions originating from the parent terms. The origins of these terms originated in the early 1990s when differential effects of interleukin-4 (IL-4) in comparison to those of interferon γ (IFN-γ) and/or lipopolysaccharide (LPS) on macrophage gene expression were described (Martinez and Gordon, 2014; Stein et al., 1992). Compared to IFN-γ, IL-4 was described to induce "alternative activation." It should be noted that the term "classical" activation, which originally referred to macrophages stimulated with IFN-γ, is now interchangeably used with IFN-γ and TLR stimulation (Martinez and Gordon, 2014). The second definition came several years later when Mills proposed the M1-M2 terminology (Mills et al., 2000). Mills's idea originated from the differential metabolism of arginine between macrophages from C57BL/6 and macrophages from Balb/c mice, an effect he correlated with differences between T helper 1 (Th1) and Th2 cell responses in the same strains. Mills and colleagues went further and proposed that the M1-M2 dichotomy was an intrinsic property of macrophages associated with transitions from inflammation to healing, would occur in the absence of an adaptive immune response, and arose early in evolution (Mills, 2012). Several lines of evidence suggest that this theory requires rethinking. First, C57BL/6 mice bear a deletion in the promoter of *Slc7a2*, encoding the key arginine transporter in macrophages, causing large differences in arginine utilization between C57BL/6 and BALB/c mice. This genetic difference between the strains was not known at the time that Mills's hypothesis was published and was therefore not taken into account (Sans-Fons et al., 2013). Second, although Mills's notion on "innate" shifts in macrophage activation might be true, most immunologists are concerned with immunity in the presence of lymphocytes, which profoundly affect the activation state of macrophages through cytokine secretion. Third, no molecular definition has yet accounted for an "innate" M1-to-M2 transition, although new information from epigenetics and metabolism (see below) might provide a means of dissecting intrinsic macrophage activation states.

The third set of nomenclature (M2a, M2b, etc.) expanded the M1-M2 definitions to account for different activation scenarios and was balanced by the idea that activation exists on a spectrum and cannot be easily binned into defined groups (Biswas and Mantovani, 2010; Edwards et al., 2006; Mantovani et al., 2005; Martinez and Gordon, 2014; Stout et al., 2005; Stout and Suttles, 2004). The fourth definition refers to macrophages grown in GM-CSF-1 as M1 and macrophages grown in CSF-1 as M2 (Joshi et al., 2014). Notably, significant differences have been documented in the transcriptomes of macrophage populations primarily generated with the use of CSF-1 or GM-CSF, with and without exogenous perturbation (Fleetwood et al., 2009), but there is no compelling evidence to assign CSF-1- or GM-CSF-derived macrophages as M1 or M2.

The diversity of terminology and inconsistent use of markers to describe macrophage activation impedes research in several ways. First, researchers entering the field encounter confusion about which terms to use and which markers are representative of their experimental or human-based system; many researchers might erroneously consider there to be only "two types of macrophages." Second, established researchers in the field have yet to agree on nomenclature or standards for describing activation. Third, grant and manuscript writers and their reviewers, funding and regulatory agencies, and journal editors can be exasperated at the breadth of terminology in use. Fourth, the lack of experimental standards impedes studies where comparisons are required (e.g., microarray and proteomic data sets). Fifth, deployment of therapeutic macrophage modulators requires that standards be translatable across disciplines so that pharmaceutical and regulatory bodies can draw meaningful comparisons in terms of diagnostic or efficacy metrics. A sixth and final issue is the diversity in macrophage activation across species (discussed briefly below).

To address the obstacles and pitfalls in describing macrophage activation and in achieving experimental standards, a small group of macrophage biologists met informally at the International Congress of Immunology in Milan in August 2013. We discussed the issues surrounding terminology and set out to provide an initial set of nomenclature and experimental guidelines. A draft letter was then circulated to a broader group of researchers active in this area. In this perspective, we do not attempt to capture everyone who has published on macrophage activation and polarization; rather, we aim to attain consensus about the problems within the field and to propose solutions. As such, discussion and revision will be essential for refining the properties and mechanisms of macrophage polarization.

Recommendations

A Reproducible Experimental Standard

We concluded that a starting point was to frame a nomenclature system within a reproducible in vitro experimental standard. CSF-1-cultured macrophages from murine bone marrow and human peripheral-blood monocytes remain the predominant in vitro systems used for generating macrophages and therefore will be used as references (Figure 1A). Other commonly used macrophage sources are peritoneal macrophages (resident or elicited) from mice and GM-CSF-cultured macrophages from murine bone marrow (Figure 1A), and each of these can be perturbed to generate activated populations of macrophages with gene-expression profiles overlapping those of CSF-1-generated cells. On this basis, the culture conditions for generating the two paradigmatic in vitro M1 and M2 populations are straightforward, i.e., postdifferentiation stimulation with IFN-γ or IL-4. IL-4 and IFN-γ often exert clear-cut antagonistic effects on macrophage polarization mediated by STAT6 and STAT1, respectively. Furthermore, IL-4 and IFN-γ induce defined and comprehensively investigated macrophage subpopulations (Lawrence and Natoli, 2011; Mills, 2012; Rutschman et al., 2001; Taub and Cox, 1995; Wynn et al., 2013).

Recommendation for Minimal Reporting Standards

Incomplete descriptions of how macrophages are isolated, stimulated, and analyzed are contrary to the value of replication and reproducibility across laboratories. To this end, macrophages

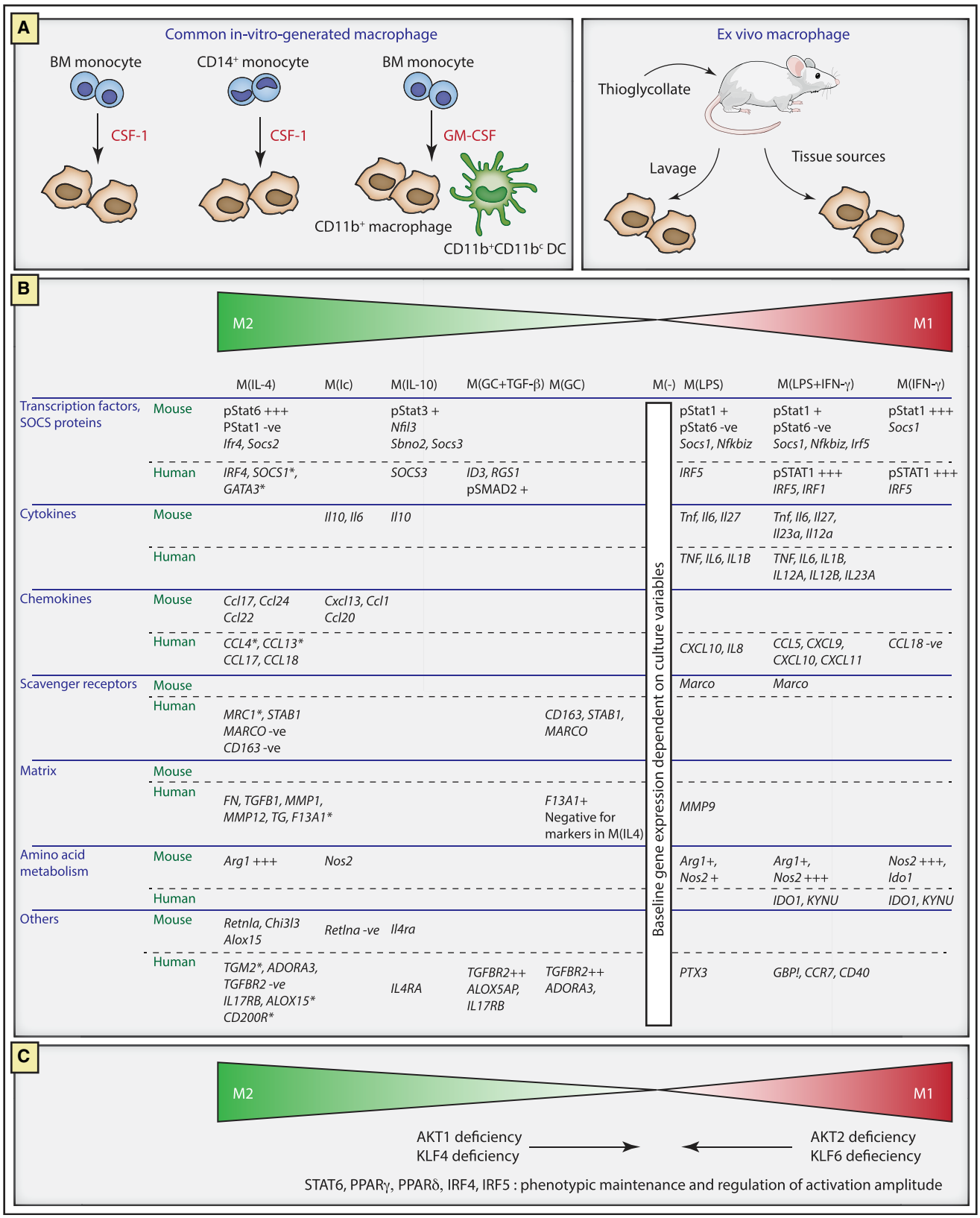

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Table 1. Reporting Standards for In Vitro Experiments

Parameter	Notes
Mouse strain	how the bone marrow is isolated and processed
Starting cell number, media, and supplements	media (DMEM versus RPMI) have substantial effects of growth rate, development, and activation status
Tissue-culture conditions	different types of plastic affect macrophage growth and activation; tissue-culture conditions should be documented for reproducibility
Time of culture	the precise conditions used and whether cytokines and/or media are supplemented during the culture period
Source and concentration of differentiation cytokines	the source and concentration of CSF-1
Macrophage yield	the yield relative to the starting number should be recorded
Activation conditions	variables include whether macrophages are rested prior to activation and how, whether CSF-1 is present in the activation cultures, the source and concentrations of the activating agents, and the time to assay
Processing and analysis	how the cells are processed and what marker readouts are used

isolated from in vitro and in vivo systems require, at a minimum, reporting standards encapsulated in Table 1. With these standards as a guide, in vitro experiments from different laboratories can be directly compared. Finally, we favor the use of purified endotoxin-free recombinant CSF-1 rather than L-cell-conditioned medium as the source of CSF-1 to generate bone-marrow-derived macrophages because the latter is not readily defined and can vary from batch to batch. For example, L-cell-conditioned medium contains variable amounts of type I interferons that could cause confounding effects in subsequent activation experiments (Warren and Vogel, 1985).

Define the Activator

In general, given that diverse mediators have been used alone or in various combinations for the generation of polarized macrophage populations, we propose that researchers describe stimulation scenarios and adopt a nomenclature linked to the activation standards, i.e., M(IL-4), M(Ig), M(IL-10), M(GC), M(IFN-γ), M(LPS), and so forth (Figure 1B). Such a system avoids the complexity of M2a, M2b, etc., where one laboratory might experimentally define activation differently than another, and allows new activation conditions to be compared and contrasted with these core examples. Figure 1 also depicts the concept of a "spectrum" of activation to denote activation "states" commonly observed (Mosser and Edwards, 2008; Stout et al., 2005; Stout and Suttles, 2004; Xue et al., 2014). The employment of the spectrum concept is useful where ambiguity exists or when researchers are operating outside the in vitro CSF-1 schema described above. In summary, we note that standards need to be simple for adoption but at the same time not cause sudden conceptual shifts. Therefore, researchers should consider harnessing the terminology and markers for CSF-1-grown macrophages activated under defined conditions as a reference standard (Xue et al., 2014). Where ambiguity exists—for example, in a macrophage population isolated from an in vivo system—researchers should emphasize the marker combinations used and state the closest relative(s) along the spectrum shown in Figure 1 (discussed below).

Terms to Avoid

We propose that the term "regulatory" macrophages should be avoided because all macrophages are regulatory in some capacity. The use of macrophages derived from mice with specific targeted mutations that prevent development of an M(IL-4) profile (e.g., through the use of IL-4Rα- or STAT6-deficient macrophages) is recommended to confirm a specific phenotype. Some researchers often ascribe the subset terminology M1 and M2 to GM-CSF- and CSF-1-generated macrophages, respectively; such terminology should be abandoned. When CSF-1 or GM-CSF is used for generating activated macrophage populations, it should be clearly indicated. A further complication is that GM-CSF cultures contain substantial numbers of $CD11c^+$ cells with distinct antigen-presenting activities that need to be accounted for in gene profiling or functional analyses.

Markers of Activation

CD4 defines $CD4^+$ Th cells. Within $CD4^+$ cells, Foxp3 defines regulatory T cells. These are just two examples of markers defining cell lineages. By contrast, macrophage activation is

Figure 1. Framework for Describing Activated Macrophages

(A) Examples of widely used macrophage preparations. CSF-1-grown mouse adherent macrophages from bone marrow (BM) or $CD14^+$ monocytes are used as the exemplars for marker evaluation and standardized activation conditions. Macrophages can also be generated with GM-CSF, where a $CD11c^+$ dendritic cell (DC) population is also present depending on the culture conditions. In mice, thioglycollate injection followed by peritoneal lavages is used for generating macrophage populations with differing yields and properties, whereas many organ systems in mice and humans are sources of tissue-infiltrating macrophages.

(B) Marker systems for activated macrophages. Shown are functional subdivisions according to stimulation of mouse CSF-1 macrophages or human monocyte-derived CSF-1 macrophages with the existing M1-M2 spectrum concept (Martinez and Gordon, 2014; Mosser and Edwards, 2008; Stout and Suttles, 2004). Stimulation conditions are IL-4, immune complexes (Ic), IL-10, glucocorticoids (GC) with transforming growth factor β (TGF-β), glucocorticoids alone, LPS, LPS and IFN-γ, and IFN-γ alone. Marker data were drawn from a wide range of published and unpublished data from the authors' laboratories and represent a starting consensus (Edwards et al., 2006; Fleetwood et al., 2009; Gratchev et al., 2008; Gundra et al., 2014; Krausgruber et al., 2011; Lang et al., 2002; Shirey et al., 2008; Shirey et al., 2014; Shirey et al., 2010; Xue et al., 2014). An asterisk indicates corroboration of human IL-4 genes by deep sequencing (K.A.S. and S.N.V., data not shown).

(C) Using genetics to aid in macrophage-activation studies. Mutations in *Akt1* and *Klf4* cause a "switch" to M(LPS)- and M(IFN-γ)-associated gene expression, whereas mutations in *Akt2* and *Klf6* show the reverse phenotype. Mutations in *Stat6*, *Ppard*, *Pparg*, and *Irf4* and IRF5 depletion are involved in the maintenance and/or amplitude of activation.

associated with substantial shifts in gene expression (hundreds of genes) depending on the specific stimuli, but none define a sublineage or activation state of macrophages. To the researcher outside the macrophage sphere, marker use probably appears confusing because immunologists are accustomed to tight marker-lineage association. An example of problematic marker use is expression of Arginase-1 (Arg1) as a "marker" for M2 or M(IL-4) spectrum macrophages, which has led to interpretive problems because Arg1 is also induced in M1 spectrum macrophages, expressed in some resident macrophage populations, and highly induced in mycobacteria-infected macrophages, further emphasizing the need for criteria encompassing multiple markers (El Kasmi et al., 2008). Accordingly, we favor an approach using combinations of markers (or a lack of marker expression) to ascribe activation outcomes as outlined in Figure 1B. Clearly, there is significant scope to expand upon marker assignment such as transcription factor and cell-surface marker combinations within the standardized experimental framework proposed here, and this should serve as a starting cartography for the field.

Translation to In Vivo Experiments

When isolating macrophages from tissue and analyzing their activation state, each laboratory will confront a familiar problem: what do we call them? What if there are different populations present? Our recommendation is to acquire sufficient evidence to place a given population within the framework shown in Figure 1. It seems unlikely that a particular in vivo scenario will fall exactly within the groups in Figure 1. However, as more macrophage populations are dissected ex vivo, more information will accumulate to help us understand the general and specific nature of in vivo macrophage activation.

Ex Vivo Characterization of Macrophage Activation

Each laboratory has individualized macrophage isolation procedures. Because of the breadth of conditions used, we favor describing in detail how macrophages are isolated, which tissue and pathological or homeostatic condition they are from, and which marker combinations are used for ascertaining macrophage activation. All authors stress the need for rapid isolation techniques to preserve the underlying phenotype quickly and without additional ex vivo culture. Advances in technologies for in situ gene expression within individual tissues and cells will most likely advance the understanding of spatial macrophage activation. Regardless of the technology employed, combinations of markers need to be applied to the populations being analyzed, and a full description of the isolation techniques needs to be provided. For example, the Immgen Consortium has a mandate for isolation and sorting conditions for immune cells, and we favor their degree of descriptive rigor for ex vivo macrophages (Gautier et al., 2012). Another complication from ex vivo analysis of macrophage activation is plasticity across different disease stages. For example, in obesity research, macrophages residing in adipose tissue are thought to become more proinflammatory as fat accumulates and thus fall toward the M1 end of the activation spectrum (Wynn et al., 2013). In atherosclerosis, resolution of lesions is associated with the reverse: macrophage populations on the M1 spectrum convert to the M2 part of the spectrum without evidence of local STAT6 activation by IL-4 or IL-13 (Moore et al., 2013). One solution to the problem of describing macrophage activation in scenarios in vivo is to begin with an explicit description of the populations under investigation and how they were isolated (as Immgen defines, for example). Markers can then be used to reflect the perturbations they have encountered. For example, $Arg1^{hi}Retnla^{hi}pSTAT6^{+}pSTAT1^{-}$ could be used to enhance the description of a specific lung macrophage population isolated from a Th2-cell-type-driven disease and thus be reasonably related to the M(IL-4) cells (Figure 1B). Reporting the time points of ex vivo macrophage isolation and analysis are therefore mandatory in the description of tissue- and disease-associated macrophage populations.

Translation to Human Macrophages

How can we define and categorize activated human macrophages? This question continues to confound researchers in part because human macrophages are generally isolated from blood monocytes as opposed to bone marrow or tissues commonly used in murine studies. This distinction is particularly important with the new knowledge that many tissue-resident populations are not of bone marrow origin (Sieweke and Allen, 2013). Many of the markers used for murine macrophages have not translated to human macrophages. Plausible reasons for these discrepancies have been discussed (Murray and Wynn, 2011a), but it is worth emphasizing that no study has systematically compared the responses of blood-monocyte-derived macrophages from mice and humans in a side-by-side manner. We expect a range of interspecies variability on macrophage activation to reflect different evolutionary outcomes sculpted by different pathogens, diets, longevity, etc. Despite the variables involved, experimental rigor can be used in the search for information about human (and any other species) macrophage biology according to the principles and practices outlined here. Recently, systematic studies have begun to explore the conservation between macrophages from different species, including swine, where large numbers of different tissue macrophages can be isolated (Fairbairn et al., 2011; Martinez et al., 2013; Schroder et al., 2012; Xue et al., 2014). Therefore, researchers should describe how they generate their macrophages and subsequently stimulate them. When microarray, deep sequencing, and proteomic studies are combined to interrogate human macrophages, a consensus will emerge about which pathways of human macrophage activation are amenable to new drug discovery.

Genetics to Alter Activation States

Recent work has identified genetic modifications producing shifts in activation phenotype. For example, deletion of transcription factor IRF4 or KLF6 fails to make M(IL-4) macrophages, whereas PPARγ and PPARδ are required for the amplitude of the M(IL-4) state (Chawla, 2010; Date et al., 2014; Ivashkiv, 2013). Ablation of proteins involved in anabolic growth, such AKT2 and PTEN, enhances an activation state where gene expression is linked to M(IL-4) macrophages, whereas deletion of TSC1, an inhibitor of mTOR, causes the opposite effect (Arranz et al., 2012; Byles et al., 2013; Yue et al., 2014). Other mutations in the mTOR pathway have produced disparate results. However, using the principles described here for systematic investigation of mTOR pathway mutants will most likely resolve why rapamycin-treated macrophages and macrophages from Raptor, Rictor, and TSC1 mutants have diverse phenotypes (Ai et al., 2014; Byles et al., 2013; Festuccia et al., 2014; Weichhart et al., 2008). Some of

these mutants are summarized in Figure 1C. We contend that these and related mutants will be increasingly useful for defining activation states. Finally, it is important to recognize the effect of timing on altering the activation state. Several parameters can affect activation state across time; these include (1) removal of the stimulus, (2) enforcement of feedback and feed-forward signaling loops, including autocrine production of cytokines, and (3) epigenetic and/or developmental effects built into the life history of a macrophage (Ivashkiv, 2013; Lawrence and Natoli, 2011; Porta et al., 2009). This would go back to Mills's notion of an activated-to-healing transition.

Perspectives and Conclusions

Understanding macrophage behavior is a keystone of deciphering disease pathogenesis. It is straightforward to isolate and propagate macrophages, facilitating their links to disease. By contrast, nomenclature and standardization issues are stunting progress because a lingua franca has yet to be established and accepted. We hope our attempts are a starting point to resolve some of the immediate issues. We emphasize that our goal is to initiate dialog rather than act as arbiters of language and experiment. In doing so, we hope scientists new to macrophage biology, established researchers, pharmaceutical companies, and regulatory agencies can appreciate the history of our field and the need for a common framework open to frequent revision.

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