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Review

Screening for new macrophage therapeutics

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Abstract

Myeloid derived macrophages play a key role in many human diseases, and their therapeutic modulation via pharmacological means is receiving considerable attention. Of particular interest is the fact that these cells are i) dynamic phenotypes well suited to therapeutic manipulation and ii) phagocytic, allowing them to be efficiently targeted with nanoformulations. However, it is important to consider that macrophages represent heterogeneous populations of subtypes with often competing biological behaviors and functions. In order to develop next generation therapeutics, it is therefore essential to screen for biological effects through a combination of *in vitro* and *in vivo* assays. Here, we review the state-of-the-art techniques, including both cell based screens and *in vivo* imaging tools that have been developed for assessment of macrophage phenotype. We conclude with a forward-looking perspective on the growing need for noninvasive macrophage assessment and laboratory assays to be put into clinical practice and the potential broader impact of myeloid-targeted therapeutics.

Key words: macrophage therapeutics, screening

Macrophage Types and Therapeutics

Phagocytic cells in the tumor microenvironment (TME) are typically myeloid derived cells recruited from the bone marrow [1]. Tumor-associated macrophages (TAMs) in particular can diversify into a spectrum of states, with either promoting or limiting tumor functions. At the time of writing, the field remains incompletely understood; however, a "parts list" of molecular cell types is emerging [2]. Traditionally, macrophages have been classified as either classically (M1) or alternatively (M2) activated in response to defined stimuli. These phenotypes are associated with anti- and pro-tumor activities, respectively [3]. *In vivo*, however, macrophages typically display complex phenotypes that extend well beyond canonical M1 and M2 conventions [2, 4–6]. Using single-cell RNA sequencing, new macrophage states have been uncovered [2, 7]. Most recently, the Pittet and Klein groups have identified 14 distinct TAM subtypes. In human cancers, the majority of these states are representative of the immunosuppressive M2 phenotype, with few anti-tumoral M1 phenotypes represented [2]. In addition, there was limited overlap in myeloid cell population structures between the blood and tumor; hence, profiling of circulating myeloid cells is poorly representative of the TME, motivating the local assessment of TAM phenotypes.

Numerous strategies for manipulation of macrophages have been proposed in the context of cancer immunotherapy. These methods include targeting of selective cell functions (e.g., enzymatic activity, recruitment), TAM depletion, and phenotypic re-polarization. All of these strategies are promising methods for combination with checkpoint immunotherapies [8], and activation of TAMs toward a functional phenotype better suited to preventing tumor growth has decently demonstrated excellent success [9, 10]. To date, there are relatively few clinically approved therapeutics that promote anti-tumorigenic polarizations. Various therapeutic

strategies have been proposed, including small molecule compounds, nanoformulations, biologics, and vaccines. From a biological perspective, a promising approach is to stimulate pattern recognition receptors (PRRs) that control innate immune pathways, such as toll-like receptor (TLR) and cytosolic nucleic acid sensing pathways [11–14]. Activation of these pathways triggers secretion of pro-inflammatory and anti-viral cytokines, indicative of anti-tumorigenic polarizations. Most activators of these pathways are large, complex compounds, such as CpG DNA, lipopeptides, cyclic dinucleotides, double-stranded RNA as well as their synthetic mimetic (poly(I:C)) that require nanoformulations and/or specialized delivery systems. A key exception are imidazoquinolines, which are small molecule TLR agonists. This group has shown that resiquimod, a TLR7/8 agonist, (R848) promotes M1 polarization, and its anti-cancer effects can be enhanced via cyclodextrin nanoparticle delivery [15]. Other small molecule agonists of TLRs and STING are beginning to emerge, and other strategies for macrophage activation have been explored, such as inhibition of CSF1R [16, 17]. Given the panoply of pharmacological agents, dosages and formulations, screening for biological effects is critical to narrowing down viable therapeutic approaches. In this review, we describe *in vitro*, *in vivo,* and translational approaches useful in the systematic investigation of new myeloid-targeted therapeutics with an emphasis on anti-tumorigenic macrophage activation.

Cell Based Screens

Robust screening assays will expedite the future discovery of candidate therapeutics. In search of ideal myeloid screens, several variables need to be considered. These include cell type (primary isolate vs. cell line), cell source (human vs. mouse) and assay type (gene expression assays, high-content screens, co-culture screens; **Tables 1-2**). A number of different assays have been described for screening macrophage polarization, with most using genetically engineered reporters [18–20], phenotypic screens [15, 21] or molecular secretion assays [22, 23]. Each method has certain advantages and disadvantages that need to be considered when embarking upon a screen. In the subsequent sections, we discuss considerations of cell sourcing and screening methodologies.

Table 1: Model cell lines for cell-based screens. Physiological relevance of the cell source, difficulty of cell handling, and utility in high-throughput screening (HTS) assays are qualitatively scored (negligible: (-), low (+) to high (+++)).

Name	Description	Origin	Source	Comment		
				Relevance	Difficulty	HTS
RAW264.7 or J774.A.1	Immortalized murine macrophage cell line, with or without reporter	Mouse	ATCC, InvivoGen	++	-	+++
THP-1 or U937	Immortalized monocyte cell line, with or without reporter	Human	ATCC, InvivoGen	+	-	+++
HEK293 Reporter Cell lines	Immortalized human embryonic kidney cells, overexpressing pattern recognition receptor	Human host, Overexpressing mouse or human pathway	InvivoGen	-	-	+++ Immune pathway analysis only
BJ or BJ5ta	Fibroblasts isolated from foreskin (BJ); immortalized with telomerase (BJ5ta)	Human	ATCC	-	-	+++ Immune pathway analysis only
BMDM	Primary isolate	Mouse (B6)	Bone marrow B6 mice	+++	+	++
PBMC	From blood	Human	Blood Banks	+++	++	++
TAM	Primary MF isolate from TME	Murine tumors		+++	+++	+

Table 2: Assays for macrophage activating drug activity.

Assay Types	Description	Advantages	Disadvantages
Immune Cell Reporter Lines	THP-1 or RAW264.7 reporter assays	Probe multiple pathways Fast, cheap, easy	Physiological relevance Bulk measurements
HEK293 Reporter Cell lines	Test ligand binding to PRR of interest	Pathway specific Fast, cheap easy	Not examining macrophages directly
ELISAs and/or qPCRs for immune cytokines	Treat immune cells with drugs and measure cytokine levels	Probe multiple pathways Applicable to any cell type	Costly Bulk measurement Antibody dependent (ELISA)
High-content screening	Treat immune cells with drugs and stain for markers of interest	Physiological relevance Applicable to any cell type Single cell analysis	Costly More difficult Antibody dependent
Single cell RNAseq	Analysis of drug effects on genome, at single cell level	Comprehensive analysis of drug effect on many cell types Can identify heterogeneous effects	Very costly Not suitable for high-throughput screening Relatively new technology

Model Systems for Screening

Human Immune Cells

Perhaps the most practical and physiologically relevant models for human disease are primary macrophages derived from peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) are typically isolated from a leukopak (using a Ficoll column), and CD14+ monocytes are then purified using either flow or magnetic sorting [24, 25]. Once CD14+ monocytes are isolated, macrophages can be obtained by treating the cells with recombinant human M-CSF for one week. These cells are then polarized into either an M1, M2, or other state using the appropriate stimuli [26]. The final cells should be adherent, elongated, and are compatible with a number of different assays that are further described below. While these macrophages are not immortalized and thus cannot proliferate, isolation from a single donor can yield millions of cells, allowing for evaluation of hundreds to even thousands of compounds in high-throughput plate formats [23].

A significant liability with primary cells is donor-donor variability, so any drug candidate discovered in a primary cell based screen needs to be verified across multiple donors. Along these lines, primary cells are not necessarily robust. Cells from certain donors may not be very responsive even to strong positive controls, making evaluation of compounds difficult. Lastly, an additional challenge with primary human cells is the lack of translation into a cell line. Any initial finding in a primary human cell type may not necessarily reproduce in a similar cell line or mouse model, complicating further studies of the drug's mechanism and efficacy.

Stem cell derived macrophages are an emergent tool for the study of human macrophage behavior. These cells may be produced by deriving monocytes from human embryonic stem cells (hESC) or human induced pluripotent stem cells (hiPSC) with subsequent M-CSF treatment to differentiate macrophages [27]. These models allow for the development of proliferative cell lines and high cell numbers unavailable in patient-derived primary cells. Resultant cells are susceptible to standard polarization procedures. hiPSC-derived monocyte cell lines are compatible with a number of cell based assays, including the development of co-culture systems, discussed below [28].

A key advantage with stem cell derived macrophages is the ability to create genetically modified macrophages lines. Macrophages are very difficult to manipulate genetically. Stem cells, on the other hand, are relatively susceptible to genetic modifications via viral transfection. As such, one may genetically manipulate progenitor stem cells and subsequently create a macrophage cell line with engineered traits. The ability to develop genetically engineered macrophages perpetuates the study of macrophage migration in the TME, such as by fluorescent labeling [29], or examination of therapeutic treatments including induced cytokine expression [30]. Despite these advantages, deriving primary macrophages from stem cells are still less common than obtaining cells from blood donors. This is partly due to the fact that this method is more complex and costly, requiring specialized media as well as other reagents. Advances in the stem cell biology field will likely increase the prevalence of this model system in the future.

In addition to primary cells, a number of immortalized human cell lines exist which are readily accessible. One common option is the THP-1 cell line. These cells are CD14+ "monocyte-like" cells derived from an AML patient. THP-1 cells are maintained in suspension culture, but can be differentiated into "macrophage-like" adherent cells through treatment with phorbol 12-myristate 13-acetate (PMA) or M-CSF [16, 31, 32]. THP-1 cells can also be skewed towards different polarizations, using protocols similar to those used for primary cells [31]. While THP-1 cells lack physiological relevance compared to primary cells, they are easier to handle, can be grown to large volumes, and are more robust. THP-1 cells express several innate immune pathways responsible for promoting anti-tumorigenic polarizations, such as TLR2/4/8, STING, and RIG-I. Thus, screening in a THP-1 cell line allows simultaneous probing of multiple pathways relevant to macrophage polarization. Currently, THP-1 cells are especially convenient screening platforms because several reporter cell lines that secrete luciferase and/or alkaline phosphatase enzymes are now commercially available (InvivoGen). These cell lines are compatible with high-throughput assays, reducing the need for time consuming and costly ELISAs and/or qPCRs in a primary screen. Additionally, several genetic KO cell lines are now commercially available, allowing further dissection of relevant molecular pathways.

There are a number of less common human cell lines that may be useful for screening. These include, for example, U937 cells which are another immune cell line isolated from a histiocytic lymphoma. While similar to THP-1 cells, they are less common and used to study the behavior and differentiation of monocytes. U937 cells differentiate in response to soluble stimuli, adopting the morphology and characteristics of mature macrophages [33]. Non-immune cell lines may also have utility in

screening. While they cannot be made into macrophages to study polarization directly, some express the aforementioned innate immune pathways relevant in macrophage polarization. BJ-1 fibroblasts are one such example [34].

Murine Immune Cells

While human cell sources have a higher degree of clinical relevance, murine cells have the advantage of providing a closed-loop system for experimentation where screening hits may be further evaluated using *in vivo* mouse models of disease. There are several potential sources of murine macrophages, including splenic, peritoneal, and bone-marrow derived macrophages (BMDMs). BMDMs are among the most common. In this method, cells from the bone marrow of femurs and tibias are isolated, and differentiated using M-CSF [35–37]. As with human cells, polarization can be tuned with the addition of specific growth factors. Use of primary murine cells confers some unique advantages over human cells. A primary advantage is the ability to isolate cells from genetically engineered mice, including from cytokine reporter mice (e.g., IL-12 or IFNγ reporters, discussed later) such that the genetically engineered marker (e.g. fluorescent protein) can be used directly for an assay readout, forgoing antibody based assays. Furthermore, primary murine cells exhibit little donor variability as compared to primary human cells, and a number of knock-out models exist from which derived cells are a valuable tool for pathway validation.

In the context of cancer immunotherapy, a more physiological relevant model is tumor-associated macrophages (TAMs). Implantation of various tumor lines, such as MC38, into immunocompetent mice causes robust macrophage infiltration. These TAMs can be accessed by flow sorting macrophages (e.g. CD68+ or F4/80+) from resected tumors. TAMs can be seeded directly onto high-throughput plates and treated as in a regular screen. In contrast to BMDMs though, yields for TAMs are much lower.

For long term culture, it is possible to immortalize BMDMs by infecting them with a retrovirus [38]. However, there are also immortalized macrophage cell lines, such as RAW264.7, which was derived from a tumor-bearing BALB/c mouse. These cell lines remain a very commonly used model to study macrophage polarization [39]. They are an adherent cell line, which can also be polarized towards M1 or M2 phenotypes with various growth factors. Like THP-1 cells, they express several innate immune pathways relevant in macrophage polarizations, allowing for examination of multiple pathways. Convenient reporter lines as well as genetic KOs are now commercially available from InvivoGen. J774.A.1 cells are another macrophage line derived from a BALB/c mouse. Like RAW264.7 cells, J774 cells also express several inflammatory pathways and are responsive to various PRR agonists. An engineered line with enzymatic reporters is available from InvivoGen, though genetic KOs are not readily available.

Non-immune Cell Types for Pathway Specific Analysis

Common non-immune cell lines, such as HEK293 and HeLa, have also been used in screening. These cell lines have either low or no expression of immune pathways, thereby requiring receptors, enzymes, and reporters to be overexpressed. Commercial vendors offer various sets of HEK293 reporter cell lines, expressing different pattern recognition receptors, such as STING, TLR2, and TLR8 (InvivoGen). While the host cell line is human, receptors for either human or mouse can be incorporated. Due to the tendency for some of these cells lines to lose expression with passage, care should be taken to follow manufacturer's protocols for positive selection, assay at early passage, and use of positive controls. When performed correctly, these assays efficiently screen for compounds that directly activate a specific receptor of interest.

Screening Assays

The general pipeline of a screening project is described in **Figure 1.** In any *in vitro* screen, one tests anywhere from tens to thousands of compounds and evaluates their ability to promote a macrophage phenotype of interest. The initial screen is referred to as a primary screen. After conducting a primary screen, hits are then further characterized by studying their effects in additional, secondary screening assays. These assays are distinct from the primary assays and allow one to profile the compounds' mechanisms in more detail. Doing so can aid in prioritizing which compounds have the most therapeutic promise and may shed light on the mechanisms of drug action. Many assays can be used for both primary and secondary screening assays. Some assays may be more appropriate for early phases of a project, while others may be better suited for the later phases. We describe these assays below, and discuss considerations to take into account when designing a screen.

Bulk Gene Expression Assays - Genetic Reporters

In bulk gene expression assays, one treats cells with a set of compounds and subsequently evaluates mRNA or protein expression changes, typically on the time scale of 24-48 hours. There are several ways bulk gene expression assays can be implemented in the

context of macrophage polarization. Activation of innate immune signaling pathways typically triggers M1 polarization, so most screens assay for pathway activation as a surrogate for general polarization response. Screening for activators of these pathways can most easily be done by using commercial reporter cell lines, which use either luciferase or fluorescent reporters as a readout. These assays are typically very robust. They have high Z-factor (Z') and strictly standardized mean difference (SSMD) scores, which are metrics used in high-throughput screening to measure how well separated positive and negative control groups are. Greater Z-factor or SSMD scores indicate that an assay is more reliable, as hits can be more easily discriminated in a screen. For gene expression assays related to macrophage polarization, an easy option is to take advantage of the commercially available THP-1 and RAW264.7 lines with luciferase reporters. Other published screens have used cell lines with engineered reporters in conceptually identical assays. The discovery of G10, a human-specific STING pathway activator, was made in a screen using fibroblasts with luciferase under control of an interferon stimulated response element (ISRE) [20]. Additionally, the HEK-TLR reporter lines were originally developed as a tool for discrimination of imidazoquinoline agonism of human and murine TLR7/8 [40], and have more recently been used to validate CU-T12-9 as an agonist of the TLR1/2 heterodimer [41].

Bulk Gene Expression Assays - ELISA and qPCR

When primary cells without genetic reporters are investigated, ELISA or qPCR become frequently used read-out methods. Such assays are conceptually identical to those mentioned above, but with the exception that measurements must be made on endogenous mRNA and/or protein. Sandwich ELISAs are excellent for measurements of various cytokines. Compared to qPCR, ELISAs are generally easier, faster, less noisy, and provide a direct read of the amount of protein produced. However, ELISAs are antibody dependent and are not ideal for measurements of intracellular proteins. Moreover, certain cytokines can bind tightly to their extracellular receptors or are difficult to detect in culture media, complicating analysis. qPCRs on the other hand are very sensitive, requiring fewer cells are per assay condition. Species dependence of suitable polarization markers has been established [26].

Similar to ELISAs, Luminex assays are an additional option [22]. Conceptually similar to sandwich ELISAs, these assays allow for simultaneous measurement of up to 50 cytokines in a single sample through conjugation of the capture antibodies to color-coded polystyrene beads, while the detection antibody is conjugated to phycoerythrin (via biotin-streptavidin). Using a specialized laser-scanning/flow device, one can measure all levels of all 50 targets using the color-code of the beads along with the PE intensity. This assay type is also high-throughput compatible. Disadvantages are cost and the need for specialized detection equipment. While it also provides a wealth of multi-dimensional data, it is arguably not necessary in a primary screen and more suited to later stages of investigation.

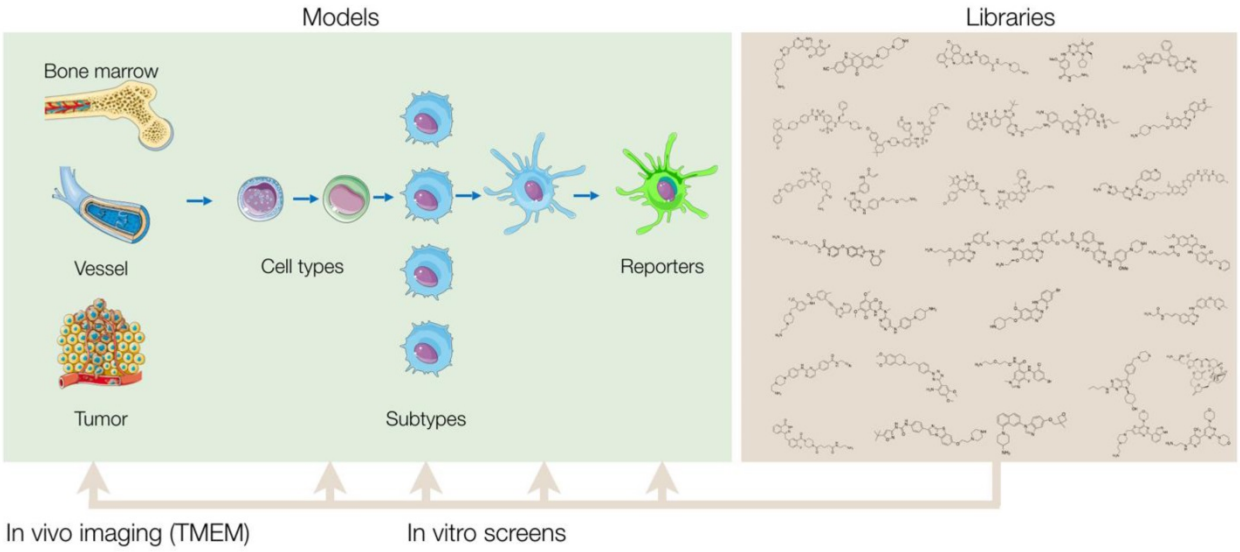

Figure 1. Overview of screening approach. Small molecule libraries can be screened in a variety of primary *in vitro* assays that vary based on cell source, subtype, and assay readout. After validation of the results, hits from the primary screen are then tested in additional secondary assays to further characterize the compounds' biological effects. By comprehensively profiling the biological effects of the initial hits, one can then prioritize and narrow down the final list of compounds to be tested *in vivo*. Ultimately, a single or select set of compounds can be administered into mice and other *in vivo* model systems, and a combination of intravital microscopy and various other methods can be used to quantify the therapeutic effect of the compounds in myeloid cells of the tumor microenvironment.

Analysis of Macrophage Phenotype by Single Cell RNA-seq (scRNA-seq)

Single cell RNA-seq (scRNA-seq) has emerged as an incredibly powerful tool for analysis of cell types. Its use is particularly prevalent in immunology, where it has been instrumental in refining immune cell types and classifications beyond what has currently been possible via immunohistochemistry and flow cytometry. In cancer immunotherapy, scRNA-seq has been applied heavily to the study of anti-tumor T cells [42, 43], but a thorough characterization of myeloid cells, both in the blood and in the tumor microenvironment, is also emerging [2, 14, 44]. As referenced above, our group has shown, with scRNA-seq, that M2 macrophages express varying levels of Arg1, challenging the conventional view that all M2 macrophages are Arg1+ [7]. Moreover, we also showed that anti-PD-1 therapy causes depletion of Arg1+ TAMs, thereby suggesting that Arg1 inhibitors would have little utility in combination with anti-PD-1 therapy.

The Arg1 example above highlights just one example of how significant time and effort could be placed on studying a suboptimal biomarker. Hence, characterization of myeloid cell by scRNA-seq could be an important step prior to initiating a screen, in order to establish a relevant biomarker. As an assay to be used directly in an *in vitro* screen, however, scRNA-seq has limited utility due to its low throughput and very high cost. To profile drug activity, its use is more warranted at the later stages of a screening project, in which the effect of one drug needs to be characterized comprehensively across a population of cell types.

High-Content Screening (HCS)

High-content screening refers to assays in which several phenotypes can be captured at single cell resolution. It most often refers to screens using high-throughput microscopy as a readout; though, other assays such as flow cytometry and CyTOF can also be employed in high-throughput format [45, 46]. In high-content screening, cells of interest are again treated with the compound library and subsequently imaged using one of the above methods. While ELISA/qPCR based assays are often easier and have higher Z' scores than HCS, high-content screening is a more effective approach that can better capture the intricacies of macrophage phenotypes in various model systems.

HCS is most commonly done in standard 96, 384, or 1536 well formats which lend themselves to high-throughput robotic automation. Adherent cells are ideal, though it is also possible to screen in suspension cells using biocompatible adhesive reagents, such as CellTak [47]. The most common approach is to fix and immunostain the cells for various markers with fluorescently labeled antibodies and/or dyes. Typically, up to four markers can be simultaneously imaged without concern of spectral overlap. However, various methods are being pursued with the goal of developing higher multiplexed assays in which several more phenotypes can be measured. In cyclic imaging for example [48, 49], one stains cells with a cocktail of markers, signal is bleached or washed away, and cells re-stained with a separate cocktail of markers. These methods allows for detection of as many as 30 markers at once. Other approaches involve barcoding antibodies with DNA, using the sequence information to identify levels of each marker [50, 51].

Various phenotypes can be imaged in macrophages to search for agents that induce M1 polarization. Several previous studies have used levels of arginase 1 (Arg1) as a distinguishing marker of polarization, with Arg1 levels decreasing upon polarization from M2 to M1. However, recent work has shown that there is significant heterogeneity in Arg1 levels across M2 macrophages [7]. Alternatively, the production of nitric oxide synthase 2 (iNOS, NOS2) expression, as wells as levels of various cytokines such as IL-12 or CXCL10, is more robust in M1 macrophages [24, 52]. Staining for these markers upon polarization to M1 is likely a better approach for a direct M1/M2 comparison (**Figure 2**). These proteins are good targets to use for HCS, and validated antibodies are commercially available. Antibody staining is also possible in murine assays; though, it may be simpler to use primary bone marrow isolates from cytokine reporter mice where possible.

In addition to protein markers, other features pertaining to general cellular states may also help discriminate between macrophage states. Cell morphology is a particularly powerful tool to assess functionally relevant phenotypes [21]. For example, M2 murine macrophages are typically elongated, with high length to width ratios, while M1 murine macrophages are round (**Figure 2**) [15, 53]. In Rodell et. al., features pertaining to cellular shape were used to identify the TLR agonist resiquimod (R848) as an inducer of M1 polarization. Overall, an advantage with high-content microscopy is that general features of morphology can be simultaneously extracted while also staining for protein levels. Additionally, use of common dyes to label cellular components can allow for extraction of hundreds of parameters [54]. This information can then be analyzed in parallel with protein level measurements in an integrated manner [55]. Methods from machine learning will likely prove

useful in analyzing high-content data, clustering macrophage subtypes, and scoring small molecules in screening assays (**Figure 2**) [56]. Going forward, it will be interesting to evaluate whether other general cellular phenotypes (e.g., mitochondrial morphology) correlate with macrophage polarization. When using broader phenotype indicators, however, one must be careful as these cellular phenotypes are controlled by several regulators and pathways. In the case of morphological phenotypes for example, drugs that disrupt the cytoskeleton may score as false hits.

Co-culture and Trans-well Screening

Tumor-associated macrophages are just one component of the TME. In addition to other immune cell types (neutrophils, dendritic cells, granulocytes, lymphocytes), stromal and tumor cells can also influence macrophage phenotypes. As such, drugs perturbing these other components of the TME can indirectly affect macrophage activity. For example, cancer cells can release various factors (i.e., danger associated molecular patterns, DAMPs) into the local environment during immunogenic cell death (ICD) [57]. These DAMPs directly activate myeloid cells through innate signaling pathways [14].

Given the complex interplay between cancer, stromal and immune cells, there is occasionally a need to develop co-culture screening assays in which immune cells are cultured with other cell types. Cells can be plated together, if contact interactions are necessary. In this case, use of fluorescent reporters and/or morphology can be used to distinguish cell types with a high-content imaging assay. Alternatively, if contact interactions are not necessary, one may use trans-well plates, in which one cell type is plated into the well, while the other(s) are on a semi-permeable insert, allowing for media exchange between the cell types.

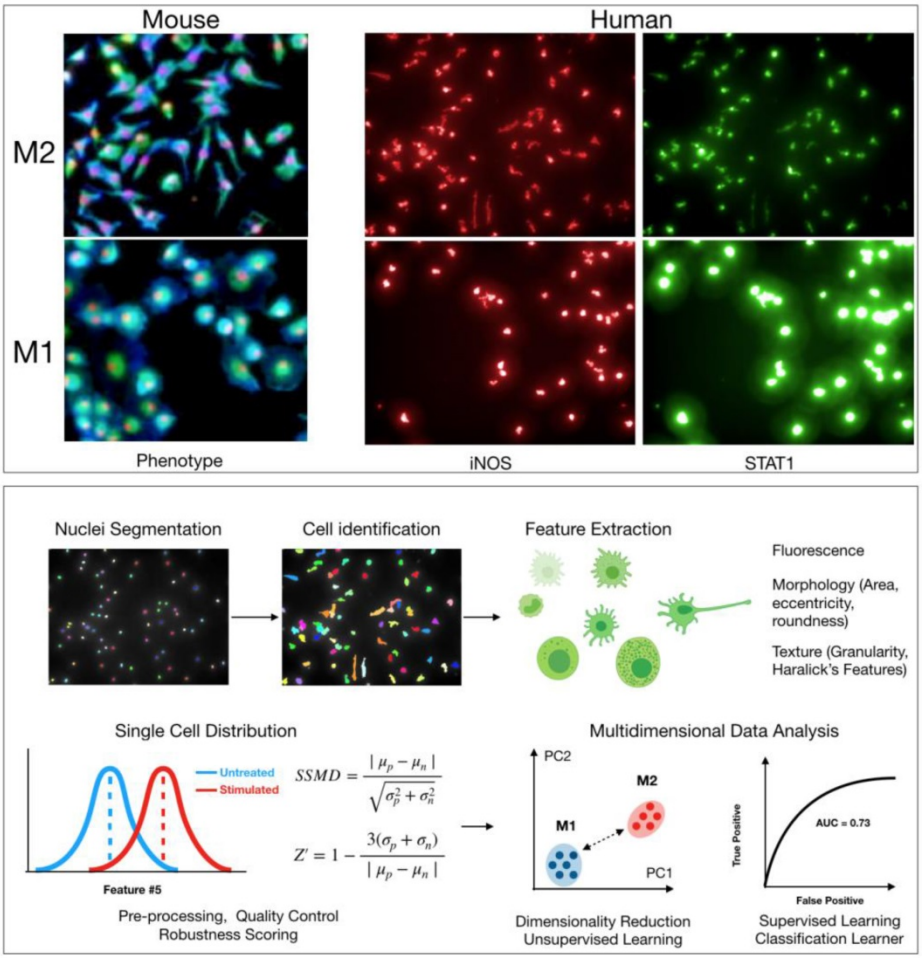

Figure 2. Cell based screen. High-content screening allows for quantification of hundreds of features pertaining to morphology, texture, and levels of protein markers in any cell type. *Top*: Example images of M2 and M1 macrophages from either mouse (bone marrow derived macrophages, red: nuclei, blue: wheat germ agglutinin, green: actin) or human (macrophages derived from peripheral blood monocytes). Cellular morphology can be used to robustly discriminate macrophage phenotype in both mouse and human. Levels of protein markers (iNOS, STAT1) can also distinguish M1 and M2 phenotypes in human cells. *Bottom*: Sample computational workflow in high-content screening. In HCS, nuclei are often first segmented using either a DAPI or Hoechst stain, and then the surrounding cellular area is identified using a membrane and/or protein level stain. All quantified features can be examined at single cell resolution. Features can be analyzed individually or in an integrated, multidimensional fashion. For the latter, it is increasingly common to use methods from machine learning to robustly discriminate phenotypes. Methods include principal component analysis, k-means clustering, Gaussian mixture models, classification learners, etc. Assay robustness can be quantified using either SSMD or Z-factors.

Co-culture assays are particularly useful in rapidly profiling how cytotoxic chemotherapeutics affect macrophage polarization. It has long been known that cytotoxic chemotherapeutics and radiation therapy can induce inflammatory response in patients. One reported mechanism is that cytotoxic drugs induce ICD, causing the release of the protein HMGB1 and subsequent TLR4 pathway activation [58]. Another emerging mechanism linked to several therapeutics is micronuclei formation. Micronuclei are fragmented pieces of DNA that arise in the cytosol [59, 60]. Cytotoxic drugs, such as DNA-damaging agents, can cause micronuclei formation. These micronuclei then trigger the cGAS-STING pathway, expressed in various cancer types, resulting in pro-inflammatory cytokine secretion and macrophage polarization.

Macrophages also influence the therapeutic response of other immune cells to drugs. Microscopy based assays have been developed to quantitate the activity of CD8+ T cells, including how TAMs repress their cytotoxicity [61, 62], as well as how macrophages contribute to checkpoint blockade resistance through disadvantageous antibody uptake [37]. It would be straightforward to develop high-content screening assays to identify therapeutics that perturb these processes. Given that it is now possible to image most types of immunocytes [63], numerous other co-culture screening assays can be developed to further identify macrophage immunomodulators.

Advanced Models

A number of more complex culture systems have been developed to more accurately mimic aspects of the *in vitro* environment. These include, for example, multicellular 3D culture platforms (e.g., spheroids, printed scaffolds) and microfluidic organs on a chip representing tumor-lymph node immune trafficking [64, 65]. Some of these model systems, such as spheroids, are also compatible with all of the high-throughput assays mentioned above. These systems capture some aspects of the native environment, such as 3D architecture or select cell-cell interactions; however, they still often cannot fully recapitulate complex *in vivo* systems.

In Vivo Imaging

One limitation of screening assays described above is the lack of a fully functional immune system. Intravital imaging approaches have thus emerged as a powerful method to study therapeutics in the presence of a fully intact immune environment, physiological forces, and a panoply of cells — all of which cannot be fully captured by model culture setups. Over the past decades, there has developed a veritable toolbox of methods for assessing the inflammatory state *in vivo*. These include methods to quantitate immune cell populations and phenotype through *in vivo* imaging. Successful execution of experiments requires good pairing of available animal models, imaging methods, and imaging probes to examine drug pharmacokinetics (PK) and pharmacodynamic (PD) outcomes. This section will review these important components of the intravital imaging toolbox, highlighting pertinent examples which examine myeloid cell activation or complex *in vivo* immune response in the tumor immune microenvironment and beyond.

Animal Models

A number of animal models are well suited to imaging at different scales, summarized in **Figure 3**. Zebrafish embryos are unique in that they are mostly transparent and can be readily imaged. Zebrafish have therefore been employed as a model to predict macrophage-associated biodistribution of nanoparticle therapeutics in the tumor environment [66], identify pH-dependent probes as indicators of macrophage activation [67] and to study macrophage polarization with fluorescent reporter systems [68] (**Table 3**). While there is broad conservation of macrophage functions and immune signaling across species, drug discovery applications may be limited by known differences in the regulatory pathways between zebrafish and higher level vertebrates [69–71], as well as limited tumor models.

Mice are the most commonly studied species. As a model system, they are relatively low-cost, easy to breed, and make an excellent platform for study due to the depth of prior characterization and availability of advanced tools such as transgenic and humanized mice. Indeed, a wide variety of genetically engineered variants are available, including knockout and reporter systems useful in drug screening. To aide in the identification of existing murine models, a number of resources are available such as the International Mouse Strain Resource (IMSR, http://www.findmice.org/) and Jackson Lab's Mouse Genome Informatics (MGI, http://www.informatics.jax.org/) database, amongst others. In addition, emergent tools such as CRISPR/Cas9 enable the rapid development of new mouse strains for study [72]. Mice, whether wild-type or genetically engineered, are well suited to an array of imaging techniques, including both whole-body imaging and intravital microscopy to examine behavior at the single cell level. As discussed in subsequent sections, these capabilities are essential for PK/PD studies of therapeutic candidates.

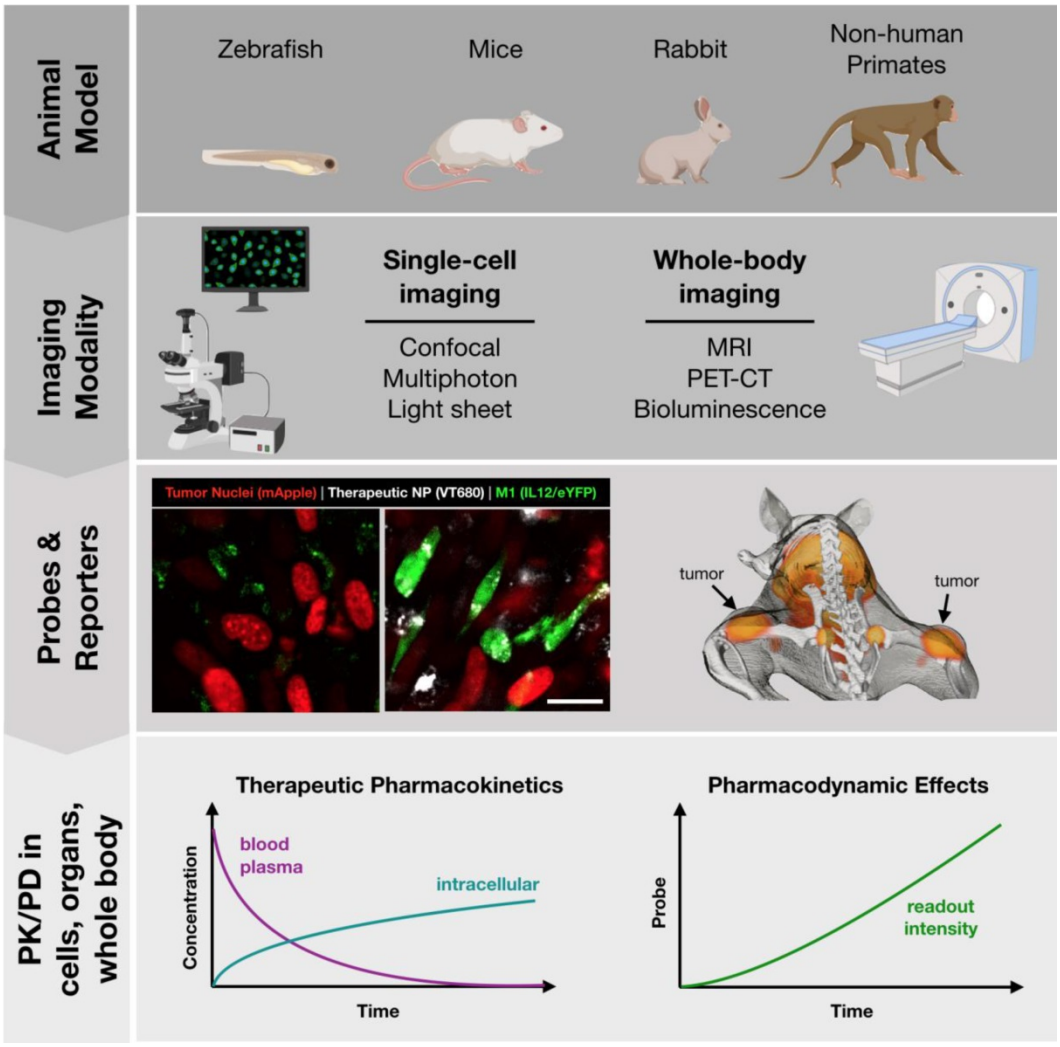

Figure 3. Toolbox for development of successful *in vivo* imaging studies. Development of *in vivo* imaging studies requires the selection and appropriate pairing of animal models, modes of imaging, and best readouts from appropriate imaging probes. Small animals (zebrafish, mice) are best suited for single-cell imaging approaches, such as cytokine reporters (imaged at left, Rodell unpublished data). Larger animal models (mice, rats, rabbits, primates) are best suited for whole-body imaging. Macrophage-targeted probes can indicate sites of active inflammation and dynamic response to therapy can be tracked longitudinally, such as by PET-CT probes (imaged at right; adapted with permission from [93], copyright 2018 American Chemical Society). These models allow for direct observation of therapeutic biodistribution to specific cell types (i.e., pharmacokinetics) as well as indicators of phenotypic changes such as cytokine production indicated by a eYFP reporter (i.e., pharmacodynamics).

Table 3: Common reporter systems for cell lineage and polarization.

Species	Reporter	Description & Notes	References
Zebra Fish	c-fms:mCherry	c-fms is also known as csf-1 receptor (CSF1R). Useful as a lineage reporter for macrophages.	[118]
	mpeg1:eGFP, mCherry	mpeg1 is macrophage expressed gene 1, a lineage reporter selective for macrophages vs neutrophils.	[119]
	mpeg1:mCherry, tnfa:eGFP	Double transgenic reporter for mpeg1 and tnfa (tumor necrosis factor alpha,), useful for identification of M1 activated macrophages.	[68]
Mouse		***Macrophage Lineage Reporter*: used to identify cell type, such as in PK studies.**	
	CSF1R	Available as MacGreen, MacBlue.	[120, 121]
	$CX3CR1^{+/GFP}$	Chemokine receptor also expressed by other immune cell types, including DC, NK, and T cells.	[122]
	$CCR2^{+/RFP}$	Monocyte/macrophage associated chemokine receptor useful in examination of monocyte recruitment. Also expressed in NK, T cells.	[123]
	$MerTK^{+/GFP}$	Receptor tyrosine kinase with excellent macrophage selectivity.	[124]
	Dye-conjugated dextran, particles, & antibodies	Administered before imaging to allow for macrophage uptake.	[63, 81]
		***Polarization Reporter*: used to identify cell phenotype, such as in PD studies.**	
	IL-12-eYFP	M1-associated. p40-IRES-eYFP reporter, also indicates activated DCs distinguishable by higher expression levels.	[125]
	IFNγ-eYFP	M1-associated. Also referred to as GREAT mice. Also expressed in NK, T cells.	[126]
	Arg1-eYFP	M2-associated. Also referred to as YARG mice.	[127]
	IL-10-GFP	M2-associated. Numerous analogous reporters developed. Also expressed in DCs, Treg.	[128]

As a complement to murine models, a number of larger animal models have been developed which are relevant for study of macrophage behavior. These include rabbit, pig, and primate models of atherosclerosis which are an excellent model for human disease [73–76], complementing the Apoe$^{-/-}$ murine model [77]. In the context of immuno-oncology, relevant large animal models include ovine lung cancer models for the study of alveolar macrophages and TAMs [78]. Additionally, the oncopig is uniquely suited for the study of cancer immunotherapeutics in the presence of comorbid immune-related diseases, such as obesity and non-alcoholic steatohepatitis [79]. Finally, non-human primates are a valuable model for general inflammatory response [80] and for the examination of macrophage-targeted imaging probes [74]. For image-based examination, large animal models are best suited for whole-body imaging approaches.

Imaging Modalities

There are a number of methods for imaging macrophage distribution and functional phenotype. Broadly, these can be classified into methods for single-cell and whole-body imaging (**Figure 3**). Methods of whole-body imaging include magnetic resonance imaging (MRI), nuclear imaging (PET-CT), and optical imaging (i.e., fluorescence mediated tomography, bioluminescence). Benefits of these methods include the potential for longitudinal tracking of response (over the course of hours to days), moderate throughput, and imaging deep within the body. These properties are particularly useful, as macrophage abundance is itself an important biomarker in diseases such as cancer, cardiovascular disease, and soft tissue injury. Over the years, a number of nanoparticle-based methods have been developed to track macrophages based on magnetic resonance imaging [81, 82]; though, PET probes provide improved imaging sensitivity, such as for identification of metastatic sites.

In some instances, whole-body imaging can also provide information on the amplitude of immune activation. Many of these systems rely on targeting of receptors associated with polarizations, such as the folate (M1) and mannose receptor (M2) [83–85]. More recently, Gambhir and colleagues have introduced a reporter system for optical imaging of macrophage polarization *in vivo*, where a luciferase reporter was placed under control of the Arg1 promoter, indicative of M2-like polarization. Adoptive transfer of these engineered macrophages into mice resulted in their migration to tumor sites as well as sites of soft tissue injury, identifiable by bioluminescence imaging [86]. To enable moderate throughout screening for polarization *in vivo*, there are a number of optical probes commercially available. These include protease-activated near-IR fluorescence sensors (e.g., PerkinElmer's ProSense 680, a cathepsin activated fluorescent probe) that can be used to identify enzymatic activity associated with local inflammation [80, 87, 88].

While whole-body imaging is adept toward cell tracking and gross magnitude of immune response, single-cell imaging is uniquely able to observe cell interactions with each other and their environment that control bulk outcomes. For example, recent work by Uderhardt, et al. has shown that tissue resident macrophages rapidly envelop microlesions, blocking neutrophil activation and swarming. This short-term activation of macrophage cloaking behavior is crucial toward preventing neutrophil-associated inflammation, maintaining tissue homeostasis [89]. Macrophages may also be activated in response to other insults, such as in the case of biomaterial implants. Recent examination of the foreign body response by nonlinear intravital microscopy has revealed M1 macrophage accumulation on the material surface that leads to development of multinuclear giant cells, implicated in release of VEGF, vascularization, and continued progression of fibrous encapsulation [90].

These examples highlight the ability of intravital microscopy to reveal single cell behaviors that drive tissue-level response. Similarly, there is a growing understanding that response of individual cells to pharmacological modulation contributes to overall response in cancer treatment [91]. Examination of drug distribution (PK) and drug activity (PD) on individual cell populations in the TME therefore requires the use of intravital microscopy, and the application of these methods toward immunology has been recently reviewed [63, 91]. Single-cell imaging *in vivo* relies upon optical imaging methods that include confocal fluorescence, multiphoton, and harmonic generation microscopy. While these methods uniquely allow for the direct visualization of drug location and cell phenotypic markers with excellent spatiotemporal resolution, limiting aspects include the timeframe of investigation possible (on the order of hours), high cost, and a high degree of technical ability needed for the experimental setup.

Imaging Probes and Reporter Systems

Among the simplest methods of imaging macrophage location is their uptake of nanoparticles as imaging agents [81]. A number of MRI and PET tracers have been described, and some of these are in clinical use or progressing towards clinical trials [92, 93]. In contrast, assessment of macrophage

phenotypes *in vivo* necessitates the use of imaging probes or reporter systems as a readout of therapeutic response. These include aforementioned probes for discrimination of phenotype based on relative expression of surface receptors as well as transporter proteins (TSPO, Slc18b1/SLC1881) [94, 95]. A number of fluorescent probes have also been designed as indirect probes of macrophage activity, including pH indicating probes for ROS activity (e.g., PhagoGreen) [67]. Another important indirect measure of macrophage activation is activity of secreted enzymes, including matrix metalloproteinases (MMPs) and cathepsins. Due to their inherent lytic activity, these enzymes lend themselves to the development of FRET probes. These classes of smart fluorescent probes have been thoroughly reviewed elsewhere [96].

The final method for assessing macrophage lineage and phenotype is the use of endogenous fluorescent reporter systems. Indeed, genetically engineered reporter mice are an invaluable tool in immunology and are an exceedingly good method for intravital imaging, compatible with an array of established tumor models. The reporters are engineered to express fluorescent reporter proteins alongside either cell lineage markers (e.g., CSF1R, MerTK) or cytokines well associated with polarization states (e.g., IL-12, Arg1). Myeloid phenotype reporters may be used to identify macrophages *in vivo* so that biodistribution of labeled drugs may be studied at the single cell level [91]. On the other hand, polarization reporter systems can be used to directly observe changes in cell state following treatment. While reporter systems and methods of imaging macrophages by nanoparticle labeling have been more extensively reviewed elsewhere [63, 81, 97], a summary of pertinent models is provided in **Table 3**. A notable concern with reporter systems is somewhat limited specificity. For example, IL-10 is commonly associated with M2-like polarization. However, the cytokine is also highly expressed in T cell subsets, indicative of Th2-type response. Caution should therefore be taken to utilize a secondary phenotype indicator (e.g., fluorescently labeled ferumoxytol or dextran) if cytokine production is to be linked exclusively to macrophages.

Pharmacokinetic (PK) and Pharmacodynamic (PD) Studies

These many imaging tools come together in the execution of PK/PD studies of promising therapeutic candidates, from which imaging datasets can yield quantifiable parameters to extract relevant PK and PD data including i) systemic half-life, ii) biodistribution to different organs, iii) cellular and subcellular distribution within the target tissue, and iv) magnitude of phenotype change. In order to examine all of these properties, a combination of independent imaging studies is often required. For initial studies of therapeutic biodistribution and efficacy, mouse models are the most appropriate as they lend themselves to both whole-body and single cell imaging approaches as well as follow-up studies on therapeutic efficacy.

In a typical experiment, drug candidates, such as those identified in cell based screens, can be examined either directly or following nanoformulation. Nanoformulation is a highly effective means of targeting therapeutics to myeloid cells, due in large part to their propensity to uptake a wide variety of materials on the nanoscale. Considerations for nanoparticle size, charge, and other properties to exploit this feature have been recently reviewed [98]. For imaging purposes, labeling of the therapeutic drug or nanoparticle is typically required. For biodistribution at the whole-body level, development of radiotracers is common, such as through drug synthesis incorporating suitable isotopes for PET imaging [99], or by chelation of these isotopes with the nanoparticle [74, 93, 100, 101]. The radiometric quantification of the therapeutic can then be monitored following administration by serial blood draw (to determine half-life) and by harvesting of tissues of interest (to determine organ biodistribution). Similar analysis can be accomplished by the development of fluorescently labeled drug derivatives as a companion imaging agent [102], by fluorescently labeling the nanoparticle [81], or by use of companion particles for imaging [103]. For fluorescent tracers, similar quantification of therapeutic biodistribution proceeds by time-lapse microscopy of the vasculature (to determine half-life) and by image analysis of resected tissues (to determine organ biodistribution). Chief concerns for imaging agents are attenuation of fluorescence with depth as well as the potential for fluorescent drug conjugates to have altered subcellular distribution compared to the base compound. These issues may be addressed by use of long wavelength emitting fluorophores and screening of fluorophore conjugated libraries *in vitro*, respectively.

The TME is a complex environment, composed of a variety of host cell types. To examine drug distribution within the tumor and resulting drug effects on macrophage activation (**Figure 4**), intravital microscopy techniques are most appropriate. Techniques and equipment for intravital imaging of immune cell behavior have recently been expertly reviewed [63]. For PK/PD studies, imaging may be performed on exteriorized organs, or by placement of optical imaging windows for visualization within

skin, lung, brain, or other tissues [104, 105]. Typically, tumors are implanted in these tissues by injection of suspended cells and allowed to grow until established and vascularized tumors are formed prior to treatment.

Experiments should be thoroughly planned before their execution to identify the necessary components to be visualized. Most confocal setups typically have up to four color channels, allowing visualization of labeled therapeutics (drugs, nanoparticles), structural components (extracellular matrix, vasculature), cell populations (tumor, stromal, or immune cells), and/or a probe or reporter system for the pharmacodynamic readout. Through pairing of appropriate labels, it is possible to discriminate between tissue and cell compartments which can be processed by quantitative image analysis to yield the desired PK/PD readouts. By establishing a delivery route (i.e., intravenous catheter) or injecting immediately preceding imaging, early distribution of the therapeutic through the vasculature and eventual vascular clearance can be assessed. Moreover, uptake by phagocytic cells in the TME can be quantified, as can the dynamic polarization response of these cells. Notably, one should understand that there may be a temporal lag in polarization responses (e.g., fluorescent reporter protein synthesis), as neither phenotypic changes or reporter protein synthesis are immediate processes, and may therefore require several hours to reach quantifiable levels.

Recent examples have highlighted the ability of these methods to unveil a novel understanding of drug PK/PD, where single cell behaviors are critical to overall therapeutic outcomes. These include the development of fluorescent prodrugs capable of selectively targeting and depleting M1 macrophages for rescue of a pro-regenerative response following injury [106]. Macrophage depletion has similarly been examined in the TME, observing effects of anti-CSF1R blocking antibody therapy. In this instance, imaging revealed depletion of both TAM and M-DC phenotypes; however, therapeutic efficacy was modest [107]. A contrasting approach to TAM depletion is TAM activation. We have recently demonstrated the development of a supramolecular nanocarrier [15, 108], which is rapidly uptaken by TAMs and acts as a vehicle for small molecule drug delivery through guest-host association [109]. When paired with a potent TLR7/8 agonist (R848) for stimulation of TAMs, M1-like activation was indicated in IL-12 reporter models, resulting in therapeutic efficacy.

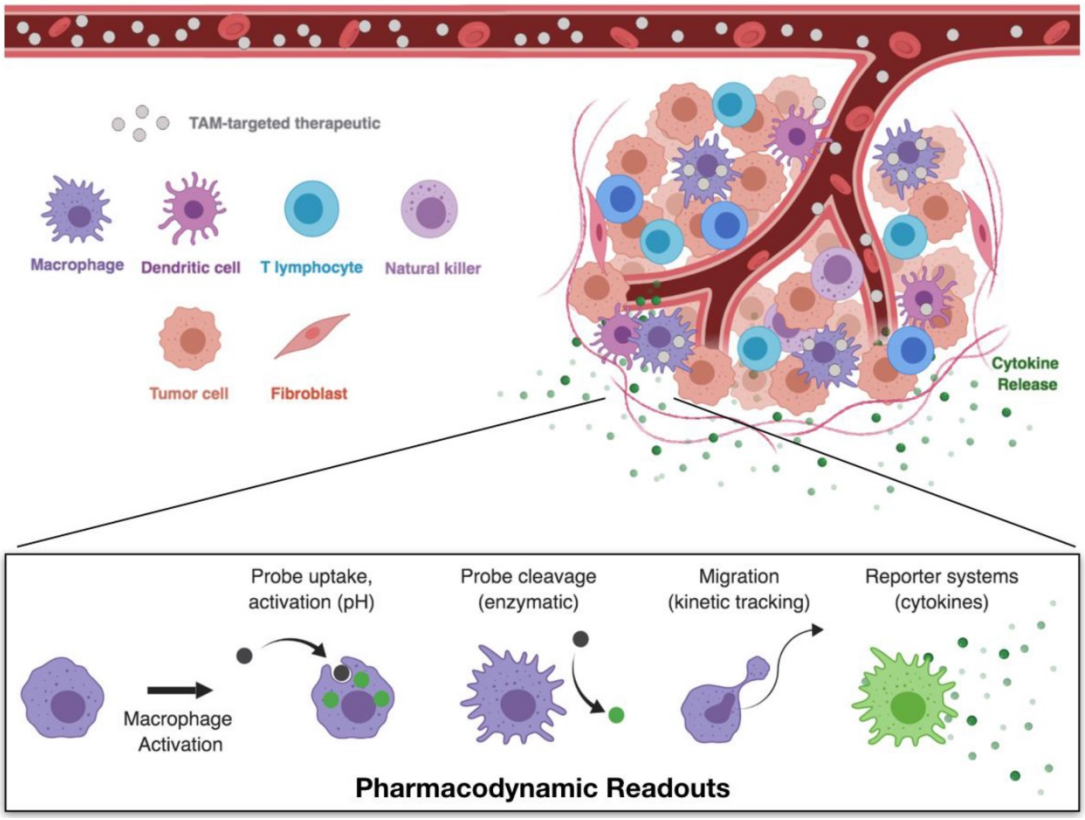

Figure 4. Pharmacokinetic and pharmacodynamic studies through single-cell imaging. Intravital microscopy allows for the simultaneous imaging of therapeutic biodistribution and macrophage phenotype. Following the administration of fluorescently labeled TAM therapeutics, compartment fluorescence may be monitored in the vasculature, interstitial space, or in specific cell types. These models therefore allow for direct observation of pharmacokinetics — the therapeutic biodistribution to specific tissues and cell types. Pairing of these techniques with appropriate model readouts for cell phenotype enable simultaneous monitoring of the pharmacodynamic effects — the direct observation of cell functions which are correlated with cell phenotype.

Additional studies have shown that macrophages are also an important mechanism of therapeutic action and resistance. For example, macrophage-associated drug uptake has been observed in the case of bisphosphonate, revealing TAM uptake as a driver of therapeutic efficacy [110]. TAMs may also act as a therapeutic reservoir for continued, local release of anticancer therapeutics [111]. Conversely, pharmacokinetic studies of anti-PD-1 have revealed an important resistance mechanism, whereby TAMs remove anti-PD-1 from the target T cells through Fcγ receptors and thereby undermine treatment [37]. Efficacy of checkpoint therapies may therefore be improved through engineering of antibodies that avoid these resistance mechanisms. In sum, these studies highlight the ability of PK/PD studies to provide a clear picture of drug actions, and specifically how macrophages may influence therapeutic outcomes.

Future Needs

New macrophage therapeutics will invariably emerge over the next few years given the central role of these cells in many human diseases and the strong rationale for developing them. From a translational perspective, there are two key questions: i) what clinical readouts are available to quantitate the efficacy of these new drugs and ii) what are some of the opportunities for other class specific cellular modulators?

Pharmacokinetic and pharmacodynamic readouts are essential in clinical trials [112]. With respect to macrophage therapeutics, there exist at least three possible biomarkers: i) macrophage imaging using MRI (ferumoxytol) or PET (Macrin); ii) biopsies and fine needle aspirates (FNA); and iii) analysis of secreted proteins or extracellular vesicles in the peripheral blood. The most clinically advanced of these methods is macrophage MR imaging with super paramagnetic nanoparticles [81, 103, 113], which can be performed repeatedly in the same patient. More recently, PET based imaging agents with high affinity for macrophages have been developed [74, 93, 114]. It is expected that some of these agents will enter clinical trials in 2020. Tumor biopsy by image guidance is currently a routinely performed method to sample cells. More recently, FNA methods have been developed that use smaller needles, have reduced morbidity, are better tolerated and allow immune cell profiling such as by cyclic imaging [48]. Finally, it is well known that host immune cells shed extracellular vesicles into circulation; research is underway to identify these vesicles in peripheral blood [115]. These methods could indeed provide a much needed window into the TME composition, as peripherally circulating immune cells have been show to poorly reflect the TME.

While we have mostly focused on macrophages in this topical review, a number of other myeloid derived immune cells are receiving increasing attention as therapeutic targets. Among these are myeloid derived suppressor cells (MDSC) [116], tumor-associated neutrophils (TAN) [1], dendritic cells (DC) [117] and granulocytes. It is hoped that some of the above described technology could be further adapted to testing emerging therapeutics for these immune cell populations as well. These approaches are important, both as monotherapies and in combination with standard of care checkpoint blockade therapies where improvement in clinical response rates would be hugely impactful.

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Competing Interests

The authors have declared that no competing interest exists.

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