In vitro macrophage activation: A technique for screening anti-inflammatory, immunomodulatory and anticancer activity of phytomolecules

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Macrophage activation plays a significant role in homeostasis of organisms. Various internal and external stress factors may affect their function, leading to adverse effects on the body. *In vitro* macrophage activation techniques provide us with a window to understand the mechanisms of inflammation and response of macrophages to the modulating interventions. Apart from infectious diseases, inflammation is also the major culprit in pathogenesis of many noncommunicable diseases such as arthritis, obesity, metabolic syndrome, diabetes, cancer, cardiovascular disease etc. *In vitro* macrophage activation allows us to study the role of polarized macrophages in the process of pathogenesis. This emerging technique leads to newer diagnostics, understanding pathophysiological mechanism/s, drug development and management of chronic inflammatory diseases. We, at MRC-KHS, use this technique for screening of medicinal plant-derived phytomolecules for their anti-inflammatory, immunomodulatory and anticancer activities. This review briefly outlines the different experimental models of *in vitro* macrophage activation and their applications for understanding the pathophysiological mechanisms of underlying chronic inflammation and screening of therapeutic activity of plant-based phytomolecules.

Keywords: Chemokines, Cytokines, Herbal, Homeostasis, Inflammation, Tumor associated macrophages (TAMs)

Introduction

The monocyte derived macrophages are flexible immune cell types, exhibiting a wide spectrum of phenotypes. These cells originate from circulating peripheral blood mononuclear cells, which develop from hematopoetic stem cells in bone marrow and sequentially give rise to monocytes. They circulate and migrate to various tissues and get differentiated into tissue specific macrophages, which reside in the resting phase unless stimulated. They rapidly respond to any endogenous or exogenous stimuli which could arise from stress, tissue damage, infection or homeostatic processes by executing phagocytosis and clearance of infectious microorganisms, tissue debris and apoptotic cells. They also play a vital role in wound repair of tissues. As a link between innate and adaptive immunity activated macrophages secrete small protein molecules known as cytokines. These molecules initiate the signaling cascade of immune system and exert the acute-phase response. These cytokines can influence the function of other cytokines in additive, antagonistic or synergistic manner1,3.

Macrophages are a vital component of the immune system and are characterized by functional and morphological plasticity4. Recently, some biotechniques have been evaluated to assess their role in various pathophysiological states like inflammation, diabetes, metabolic syndrome, atherosclerosis, stroke, myocardial infarction and cancer5. The balance between M1 and M2 types appears to decide the course of a disease e.g. Repair or fibrosis in inflammation in any organ, regression or progression of tumours with or without treatment. The detailed description of their functional and morphological attributes and variations in each disease state is beyond the scope of this article; however, considering the recent burst of new literature, we have tried to briefly summarize the various techniques of evaluation of macrophage activation to M1 or M2 types for potential investigational and therapeutic approaches in health and diseases, including cancer6,7.

Molecular mechanism of macrophage activation

Macrophage activation refers to the changes in morphology, metabolism and function of resting macrophages in response to any stimulation8. Depending upon the surrounding microenvironment macrophages display polarized phenotype and
functions. There are 2 major phenotypes of macrophages, M1 and M2. T helper cells are responsible for the polarization of macrophages into Classical (M1) or Alternative (M2) phenotype. Interleukin-2 (IL-2), gamma-Interferon-gamma (IFN-γ), and Tumor Necrosis Factor alpha (TNF-α) induce classically activated macrophage phenotype M1. They release proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, IL-23). They also express high levels of MHC-I, complementary factors and Class II antigens for phagocytosis. These pro-inflammatory cells promote arginine metabolism into Nitric oxide and Citrulline by secreting inducible nitric oxide synthase (iNOS).

Conversely, Interleukin-4 (IL-4) and interleukin-13 (IL-13) induce alternatively activated macrophage phenotype, M2. These anti-inflammatory M2 cells express IL-10, mannose receptor (MR) and scavenger receptor (SR). Their major functions are immune modulation, tissue remodeling, parasite clearance, and tumor progression.

**LPS stimulated macrophage activation**

Macrophages identify and act in response to the pathogen associated molecular patterns (PAMPs) through the cell membrane and cytoplasm receptors. Lipopolysaccharide (LPS) is a component of the outer membrane of gram negative bacteria. It binds to membrane receptor CD 14 with LPS binding protein (LBP)\(^\text{10}\). LPS is transferred inside the cell through antigen 96 (MD-2) and Toll-like receptor 4 (TLR-4). TLR4 after binding MyD88 and Receptor-interacting serine-threonine kinase 2 (RIPIK2), initiates NF-kB-activating pathway\(^\text{11}\). Activated NF-kB starts transcription of many proinflammatory cytokine genes such as TNF-α, IL-1β, IL-6, and IL-8\(^\text{12}\). Fig. 1 illustrates the mechanism of LPS stimulated macrophage activation. Measurement of these cytokines released in culture medium gives a clue about the degree of inflammation.

**Ex vivo models for macrophage activation**

Several models have been developed to understand the mechanism of macrophage activation and evaluate their patho-physiological role in various diseases. These models are briefly described below:

**PBMC derived macrophage activation**

Peripheral blood mononuclear cells (PBMC) primarily contain monocytes and lymphocytes. After
density gradient centrifugation the buffy coat is collected and seeded in culture vessels. Non adherent cells (lymphocytes) are removed after overnight incubation and the remaining cells (monocytes) are treated with activating agents such as LPS, IFNγ. These cells can be further used for: (a) Phagocytosis studies for engulfment of bacteria, yeast, etc.; (b) expression markers like CD64, CXCL10, CD23, CCL22 etc.; (c) Nitri Oxide (NO) production by spectrophotometry; and (d) cytokine and chemokine release in the culture supernatant such as IL1β, IL-6, TGFβ, TNFα, CX3CL1 CXCL 9, etc.13.

Primary murine macrophage activation

Primary murine macrophages can be collected from the peritoneal cavity and the bone marrow. An inflammatory response is created using thioglycollate broth or Bio-Gel polyacrylamide beads injection into the peritoneal cavity. These macrophages are harvested and seeded into tissue culture vessels. After 7 days these cells are labeled with specific antibody to recognize the cell surface markers as above and further sorted and identified by flow cytometer14.

Alveolar macrophage activation

Alveolar macrophages are present in the alveoli and alveolar ducts. These macrophages are isolated from broncho-alviolar lavage fluid. These cells are activated by IFNγ and used for phagocytosis, cytokine release, and chronic inflammatory studies. RAW 264.7 and U937 are developed by viral transformation to study a variety of studies, surface receptors etc. RAW 264.7 and U937 are primary murine macrophage cell lines and are primary used for studying behaviour of macrophages release, and chronic inflammatory studies activated by IFNγ and used for phagocytosis, cytokine release, and chronic inflammatory studies. RAW 264.7 and U937 are developed by viral transformation to study a variety of studies, surface receptors etc.

Macrophage cell lines

Many macrophage like cell lines have been developed by viral transformation to study a variety of functions such as cell mediated cytotoxicity, metabolic studies, surface receptors etc. RAW 264.7 and U937 cell lines are primarily used for studying behaviour of monocytes and macrophages. They are activated by LPS or PPD (purified protein derivative from Mycobacteria). These cells express macrophage like morphology, growth characteristics and cytochemistry in vitro16.

Macrophage co-culture with other cells

Macrophage co-culture with other cells e.g. Fibroblasts, adipocytes, etc., is used as a physiological model for that particular cell or tissue type. The functional phenotype of the macrophage depends upon the surrounding microenvironment. This model is employed to understand the cell-cell interactions which mimic the in vivo conditions17,18.

Biomarkers for evaluation of inflammation

Monocytes and activated macrophage subpopulations differ in terms of gene expression, receptor expression, cytokine and chemokine production and effector function. Classically alternatively activated macrophages also express different markers, depending upon the surrounding environment. in vitro macrophage activation technique utilizes these markers, specific to M1 or M2 phenotype, to evaluate the degree of inflammation. These are functional or phenotype markers. Some genomic variations of these cells like M2a, M2b, M2c and M2d are also reported (Table 1)9,19,20.

Inflammatory cytokines

Cytokines are low molecular weight soluble protein molecules for intercellular signalling. They are effectors of macrophage function and transmit the inflammatory signal to other cells. They can be divided into two major groups on the basis of the secretory cell type: pro-inflammatory and anti-inflammatory. Cytokines released by classically and alternatively activated macrophages are listed in Table 1. In vitro secretion of cytokines is measured by ELISA in media supernatant21.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>M1</th>
<th>M2a</th>
<th>M2b</th>
<th>M2c</th>
<th>M2d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulatory factors</td>
<td>IFN-γ and LPS or TNF-α</td>
<td>IL-4 and IL-13</td>
<td>TLR/IL-1R ligands</td>
<td>IL-10</td>
<td>IL-6, MCF</td>
</tr>
<tr>
<td>Cytokines secreted</td>
<td>TNF-α, IL-1β, IL-6, IL-12, IL-23</td>
<td>IL-10, TGF-β, IL-1Ra</td>
<td>TGF-β, IL-10</td>
<td>TGF-β, IL-10, IL-12</td>
<td></td>
</tr>
<tr>
<td>Chemokines and receptors</td>
<td>CCL5, CXCL9, CXCL10, CXCL11, CXCL16, CX3CL1</td>
<td>CCL17, CCL18, CCL22, CCL24</td>
<td>CCL1</td>
<td>CCL13, CCL16, CCL5, CXCL10, CXCL16</td>
<td></td>
</tr>
<tr>
<td>iNOS expression</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cells Surface markers</td>
<td>CD80, CD86, TLR2, TLR4, IL1R1</td>
<td>CD-163, CD-206, IL1RII, Decoy R TGM2</td>
<td>CD86</td>
<td>CD163</td>
<td>TLR1, TLR8</td>
</tr>
</tbody>
</table>

Adapted from Hao et al. 20129 and Duluc et al. 200720.
Chemokines
Chemokines are small chemotactic protein molecules from cytokine family. Their major role is, trafficking immune cells through a concentration gradient by activation of G protein coupled receptor. These cells are accumulated at the site of chemokine production and exert their function. Chemokine function is essentially regulated by cytokines. Macrophage polarization intensely modulates the profile of chemokine production.

Nitric oxide
It is generated in activated macrophages by Inducible Nitric oxide synthase (iNOS) enzyme. Expression of this enzyme is regulated by cytokines and LPS. Once generated NO inhibits the activity of iron containing enzymes due to its high affinity to iron molecule. Nitric oxide can be measured in macrophage culture employing various spectrophotometric techniques. It requires extra care and accurate assays as the half life of NO is very short (few milliseconds). Measurement of NO concentration in cell culture supernatant, involves quantitation of Nitrate and nitrite, catalyzed by nitrate reductase enzyme.

Tumor associated macrophages (TAMs)
Macrophages, present in the vicinity of tumor cells are known as tumor associated macrophages. They play an important role in manipulating the tumor environment. At the primary stage of cancer, macrophages express proinflammatory phenotype (M1) and show tumoricidal activity, whereas at late stage of cancer, they express anti inflammatory phenotype (M2) which provides favourable microenvironment to the tumor progression. They release immunosuppressive mediators and Migration Stimulation Factor (MSF) to promote the tumor growth. Presence of TAMs in high numbers is associated with advanced stages of cancer. This makes them a key prognostic marker for cancer progression as well as target for therapeutic strategies.

Cell Surface Markers
Macrophage subsets are also defined by expression of cell surface markers along with their functions. Cell surface markers are proteins specifically expressed in a particular cell type. In case of macrophages, these proteins are pathogen receptors, cytokine receptors, chemokine receptors, adhesion molecules or costimulators.

As seen in Table 1 these markers singly cannot define the subset of macrophage. There is always an overlapping of the markers in macrophage subsets. Therefore, combination of these markers should be utilized for characterization and identification of polarized macrophages.

Applications of in vitro macrophage activation
Screening of Anti-inflammatory drugs
Inflammation is a physiological response to internal or external stimuli for protection. If this response is not regulated, it results in chronic inflammation. A wide variety of medicinal plants contain active phytochemicals which are believed to suppress inflammatory cascade at multiple levels. Veres has reviewed the anti-inflammatory activity of polyphenols which are metabolites of all traditional medicines and most natural products. They exert their anti-inflammatory activity through the modulation of MAPK, Akt and NF-kB pathways, by inhibition of NF-kB phosphorylation, thus transcriptional inactivation of proinflammatory cytokines and chemokines.

In vitro macrophage activation assay has been used for accessing anti-inflammatory activity of various compounds by inhibiting cytokine release. It may provide essential information about inflammation in many diseases. Exposure of therapeutic compounds to the macrophages, reduces the release of proinflammatory cytokines in response to LPS. This model is employed to study in vitro anti-inflammatory activity of natural compounds such as alkaloids, polyphenols, and other extracts. They suppress the activity of COX and iNOS and decrease the production of ROS/RNS. Biological modulation and reduction of LPS-inducible inflammatory factors are considered to be an effective strategy for inflammatory diseases.

Polyphenols present in the Red wine are reported to have antioxidant and anti-inflammatory activity which reduce the risk of cardiovascular diseases. Bognar et al. has accessed the anti-inflammatory activity of Malvidin, a red wine polyphenol in RAW 264.7 macrophages by LPS stimulation. In LPS treated cells, Malvidin reduced phosphorylation of NF-kB p65 on Ser536 which is responsible to enhance its transcriptional activity but did not affect the expression of the protein. It also prevented nuclear translocation and DNA binding of NF-kB in LPS stimulated cells. Malvidin suppressed LPS-induced ROS production, MAPK and PARP activation in RAW 264.7 macrophages.
In vitro anti-inflammatory activity of 2-methoxyestradiol (2-MEO), a 17-estradiol metabolite was demonstrated by Shand et al.29. Antiproliferative and antiangiogenic activity of 2-MEO is well characterized in vitro and in vivo30. It is also reported for the prevention of arthritis and osteoporosis in postmenopausal women as an alternative for hormone replacement therapy31. Anti-inflammatory activity of 2-MEO was seen in LPS activated human blood monocytes and mouse macrophage cell lines. It suppressed production of PGE2 and IL-6 in vitro as well as in vivo. Whereas there was no effect on TNF-α and nitrite production in vitro.

Molecular mechanism responsible for anti-inflammatory activity of Ethanolic extract of Myagropsis myagroides (EMM) in LPS activated RAW 264.7 macrophages was investigated by Joung et al.32. Cells preincubated with extract were stimulated by LPS and concentrations of NO, PGE2, IL-1β, IL-6, and TNF-α were measured in cultured media. EMM significantly reduced production of NO and inflammatory cytokines in a dose dependent manner. It also inhibited phosphorylation of MAPKs and Akt thus preventing translocation of NF-kB in nucleus in LPS stimulated cells. Similarly, Calendula officinalis reduced levels of TNF-α in LPS stimulated macrophage culture supernatant as well as other proinflammatory cytokines i.e IL-1β, IL-6, IFN-γ and CRP in serum of treated mice. It also inhibited expression of Cox-2 in treated mice33.

Adipocyte-macrophage co-culture system was utilized for the assessment of anti-inflammatory activity of a flavonoid known as nobiletin, present in citrus fruit peels. It inhibited NO, TNF-α and MCP-1 secretion in a dose dependent manner and also blocked many transcription factors expressed by adipocytes in the interaction with macrophages. Thus, it breaks the paracrine loop between macrophages and adipocytes and inhibits inflammatory changes34.

In vitro anti-inflammatory activity of various medicinal plants extracts has been studied by our group using human PBMC culture. Raut et al.35 have studied effect of aqueous, hydroalcoholic and alcoholic extracts of Commiphora wightii on monocyte derived macrophage culture model. They observed a reduction in TNF-α and IL-1β levels in media supernatant. Commiphora wightii is commonly used as an anti-inflammatory and anti arthritis drug in Ayurvedic clinical practice.

We have also investigated anti-inflammatory activity of phytomolecule, curcumin and compared it with CurcuWIN™ (curcumin soluble in water)36. Water soluble curcumin significantly reduced TNF-α release in macrophage cells activated by LPS. Similarly, we have assessed anti-inflammatory activity of Curcuma longa and Tinospora cordifolia extracts individually and synergistically using the same method. It was observed that the extracts significantly reduced IL-6 and TNF-α secretion in macrophages36.

Assessment of Immunomodulation

Immunomodulation stands for stimulation or suppression of immune response in defense against pathogens or autoimmunity. The basic function of immunomodulator is balancing and stabilizing the immune system. Devasagayam & Sainis37 have earlier reviewed the mechanisms and studies based on immunomodulatory and antioxidant activity of Indian medicinal plants. Many of these studies are based on the macrophage activation technique. The mechanism of immunomodulation involves many aspects of immunity. These aspects can be studied to access in vitro immunomodulatory activity of compounds by macrophage activation.

In a study to improve phagocytotic activity using BioBran, a modified arabinoxylan present in rice bran, three in vitro macrophage activation models was utilized. Macrophages were activated by PMA and then incubated with BioBran and yeast. Percent phagocytic cells, cytokine and NO production were measured. Macrophages treated with BioBran showed enhanced spreading ability, increased production of cytokines and nitric oxide38.

The role of Myeloperoxidase in the in vitro release of TNF-α by the peritoneal macrophages, has been shown by Lefkowitz et al.39. It was suggested that possible mechanisms for the TNF release could be radical produced by MPO or binding of MPO to the mannosylfucosyl receptor. Guan et al.40 employed macrophage activation assay for measurement of nitric oxide production and Phagocytosis assay. They demonstrated that crude polysaccharide fraction stimulated macrophage proliferation, phagocytosis and nitric oxide production in a dose dependent manner. Orsi et al.41 evaluated immunomodulatory activity of Propolis. It induces dose dependent elevation in H2O2 release and an inhibition in NO generation by activated macrophages. Madaan et al.42 utilized dendritic cells from bone marrow and evaluated immunostimulatory activity of chyavanprash in vitro. Dendritic cells treated with chyavanprash solution
showed increased levels of IL-1β, IFN-γ and MIP-1α without any other stimulation as compared to controls. It also significantly enhanced phagocytic activity of treated macrophages as compared to non-treated macrophages\(^ {42}\).

A three dimensional culture model was utilized by Wang et al.\(^ {43}\) to understand the immune mechanism of Human Pappiloma Virus therapeutic vaccine. Dendritic cells generated from PBMC were cocultured with T cells in the 3D culture model. The culture treated with HPV16mE7 increased proliferation of T-cells and IFN-γ expression. In a study by Rao et al.\(^ {44}\), α-linolenic acid modulated the generation of reactive oxygen species in macrophages in vitro and in vivo. Rats were fed diet containing partially hydrogenated vegetable fat (PHVF) including elaidic acid and linseed oil which contains α-linolenic acid. After 10 weeks, the peritoneal macrophages of the PHVF fed rats generated higher amount of reactive oxygen, NO and peroxide species as as compared to the macrophages of rats fed with linseed oil along with PHVF.

**Cancer progression and targeted therapy**

One of the primary functions of macrophages is known to provide a defense mechanism against cancer cells. They need to be stimulated for tumor cytotoxicity either by LPS, MDP or cytokines IFN-γ and GM-CSF. TNF-α and NO release in response to the stimulus have tumoricidal activity\(^ {45}\). Macrophages present inside the tumor are classified as Tumor Associated Macrophages (TAMs). These cells display characteristics of M2 macrophages such as expression of anti-inflammatory molecules, (IL-10, TGF-β) and pro-angiogenic mediators (VEGF, COX-2-derived PGE2, and matrix metalloproteinase). They also overexpress M2-specific genes, macrophage galactose type C-type lectin-2, ARG, etc. Depending upon the tumor stage, TAMs also express M1 specific cytokines such as TNF-α, IL-6, IL-1β, IL-23p19, and CXCL8\(^ {46}\). TAMs are recruited at the malignant site by tumor derived chemotactic factor CCL2 and cytokines such as M-CSF, VEGF and PDGF\(^ {47}\).

Heusinkveld et al. have observed that cervical cancer tumour cells, which can produce PGE2 and IL-6, induce conversion of local macrophages to M2 type\(^ {48}\). This promotes tolerance to tumours and allows tumour growth. Interaction of the M2 macrophages with TH1 cell types the M2 types can be converted to M1 type indicating that immune system plays an important role in the growth and progression or regression of tumours. Recently, Sanchez-Reyes et al.\(^ {49}\) have shown that supernatant from cervical cancer cells can change M1 to M2 types and increased expression of TLRs (Toll-like receptors). This has been confirmed for HPV 16 associated cervical cancer in vivo mouse model by Lepique et al.\(^ {50}\) in studies utilizing the TAMs.

These aspects of TAMs are well utilized for assessing in vitro anticancer activity of compounds by activation of macrophages. In vitro tumor promoting activity of monocyte derived macrophages was evaluated in two different culture conditions. Their morphologic variation, cytokine expression, phagocytic activity, surface receptor expression and effect of macrophage media supernatant on tumor cells were analyzed\(^ {47}\). Investigation of cytotoxic effect of activated macrophages using the MTT method has been done in tumor cell line\(^ {52}\). Antitumor activity of Squalene-treated Cell Wall Skeleton of Nocardia rubra was pursued by activation of macrophages by compound and culturing them with tumor cell lines FBL-3, and CEM\(^ {53}\). Baay et al.\(^ {54}\) have reviewed the potential targets and strategies for cancer therapy associated with TAMs. They have focussed on the secreted proteins which are responsible for interaction between tumor cell and macrophage as intervention targets.

TAMs, therefore, are recognized as prospective targets for therapy whereby their entry into tumours may be blocked or M2 types may be converted to M1 types for a favourable therapeutic outcome in cancer\(^ {55}\). A recent study has reported that non classical patrolling monocytes play a key role in controlling tumor growth and metastasis in the lungs. The orphan nuclear receptor Nr4a1 is abundantly expressed in these monocytes as compared to other myeloid cells and regulates their function. These cells recruit and activate Natural Killer (NK) cells to inhibit tumor growth. They also actively monitor the endothelial cells of vasculature and remove the dead cells and microparticals. These properties facilitate the resolution of inflammation and suppression of tumor growth and metastasis, which makes these cells a possible target for cancer therapy\(^ {56}\).

**Limitations of macrophage activation techniques**

All experimental techniques of macrophage activation in vitro or in vivo are likely to have limitations with respect to species differences and artificial experimental conditions.
Though current *in vitro* models are easy, cost effective and provide a direction for research, they may not give an accurate idea of the cell response *in vivo*. The cell receives multiple positive and negative feedbacks from several other cells *in vivo* and this microenvironment cannot be exactly replicated *in vitro*. Macrophage co-culture and 3 dimensional culture techniques solve this problem to some extent. Moreover, minor changes can also alter the behavior of monocytes and macrophages. For example PBMCs collected from healthy individuals, even from the same individual at different times, may not give similar response to the stimulation. In case of primary cell cultures genetic make of the animal or human subject may affect the cell response and behavior. Whereas, transformed macrophage cell lines are more stable but may not express the desired marker. Standard methods, reagents and careful handling are very crucial for consistent results in this technique.

Hence, sometimes the results may not be directly extrapolated to the varied clinical situations. However since clinical studies are difficult because of confounding factors and ethical considerations, experimental studies provide insights into mechanisms of action and can identify new drug candidates.

### Conclusion

*In vitro* Macrophage activation technique provides an opportunity to investigate various mechanisms of immune function ie inflammation, cytokine action, cell mediated cytotoxicity, immunomodulation and understanding of molecular pathways involved in the polarization and plasticity of macrophage activation. With emerging techniques we are able to study distinct macrophage populations, their biological roles and their interactions. Based on the *in vitro* results, similar strategies can be explored for clinical interventions and new therapeutic approaches to address various diseases.

Traditional medicines may work as preventive as well as therapeutic agents for chronic inflammatory diseases such as diabetes, arthritis, obesity, etc. Cancers such as cervical cancer which can be detected in precancerous phase and may revert to normal after treatment of infections and inflammation can be managed with anti-inflammatory and anticancer drugs without any side effects. Inhibition of tumor progression can be achieved by inhibiting macrophage conversion in TAMs or switching M2 phenotype to M1 or reducing TAM survival.

Examples of phytomolecules which have been studied for anti-inflammatory, immunomodulatory or anticancer activity include curcumin, malvidin, nobiletin, lycopene, quercetin and tyrosol, etc. However, more studies are required to understand the mechanisms by which macrophages regulate the diverse range of biological systems in homeostatic and disease associated conditions. Thorough understanding of macrophage function may widen the scope of potential clinical therapies for pathological challenges.

### References


