Characterizing the Dynamics of Macrophage Polarization and Signaling

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ABSTRACT:

Apart from their primary functions in innate immunity and phagocytosis, macrophages are critical regulators of inflammation through their ability to adopt polarization states, a spectrum of phenotypes in which macrophages change their morphology and produce large amounts of pro/anti-inflammatory cytokines and biomolecules. Dysregulated macrophage polarization has been implicated in numerous chronic inflammatory diseases. However, as a result of the tightly-regulated nature of macrophage polarization, it is difficult to design immunomodulatory strategies for modulating macrophage polarization without a robust understanding of the temporal dynamics involved. As a result, there is a need for a deeper understanding of polarization dynamics that can be used in conjunction with computational modeling to develop better immunomodulatory strategies. In this thesis, RAW 264.7 murine macrophages are subject to a number of different stimulation strategies in order to temporally characterize how these cells translate inputs (such as pro-inflammatory cytokines and biomolecules) into a key marker of pro-inflammatory polarization output (iNOS expression over time). Specifically, I measured iNOS expression primarily through immunocytochemistry performed in either 96-well microwell plates or in PDMS microfluidic devices for three-dimensional (3D) culture experiments. Performing polarization experiments in microfluidic devices revealed that 3D culture environments exhibit differences in polarization dynamics. In particular, I found that the M1 response to lipopolysaccharide (LPS) stimulation is always transient regardless of whether macrophages are re-stimulated with fresh LPS-containing medium. Subsequently, I investigated re-stimulating with an M1 cytokine that activates different pathways than LPS to upregulate pro-inflammatory genes, referred to in this thesis as an orthogonal stimulus. Interestingly, further re-stimulation with an orthogonal stimulus, which

upregulates iNOS through a separate pathway, such as interferon-γ, can extend M1 polarization and reach greater levels of iNOS expression than any single stimulus alone, in an order and dose-dependent fashion. I also showed that culturing macrophages in a three-dimensional environment within microfluidic devices delays their response to LPS but exhibits the same critical maximum of iNOS expression, suggesting a potential role for the extracellular matrix in regulating polarization dynamics. This thesis contributes novel findings to our understanding of macrophage polarization and proposes new avenues of investigation into the dynamics of other important cytokines in both 2D and 3D culture environments. These results may inform the development of novel models for understanding the temporal dynamics of macrophage polarization and direct further work into modulating polarization states for the treatment of inflammatory diseases.

INTRODUCTION:

Macrophages are a subset of white blood cells which serve as one of the first lines of defense in the immune system. Their primary function is to combat infection by engulfing pathogens through a process known as phagocytosis[1]. Over the course of the host's response, macrophages polarize to different states, where they promote or resolve inflammation by secreting signaling molecules, i.e. cytokines. Macrophages first polarize towards a pro-inflammatory (M1) state, marked by increased inducible nitric oxide synthase (iNOS) expression. M1 polarized macrophages induce inflammation in the local tissue environment and are important for anti-microbial responses during infections. Then, to resolve inflammation,

macrophages enter an M2 state, marked by Arginase-1, in which they upregulate proteins involved in tissue repair and remodeling[2].

Regulation of macrophage polarization is a promising area of research for diseases characterized by chronic inflammation, such as Alzheimer's disease[3] and diabetes[4]. M2 macrophages are also known to play a role in cancer biology in the form of tumor-associated macrophages, which locally attenuate immune response within the tumor microenvironment[5]. The translational relevance of modulating macrophage polarization to a diverse range of common and serious health issues suggests that it may be an attractive target for therapy.

However, the tightly-regulated and temporal nature of macrophage polarization poses challenges for developing methods to modulate their phenotype in a controlled-manner and without interfering with other important functions of the immune system. While the cytokines and biomolecules responsible for macrophage polarization have been well characterized, the dynamics of how these stimuli contribute to promoting chronic inflammation has been mostly unexplored.

This thesis investigates how inputs to macrophage polarization, such as cytokines and biomolecules, give rise to outputs in the form of polarization outputs. I have characterized macrophage polarization responses across a diverse range of stimulation strategies, as well as illuminating new differences in polarization dynamics across two-dimensional and three-dimensional culture platforms. Likewise, my work has demonstrated that macrophage polarization exhibits hysteretic dynamics, in which prior states of macrophage polarization affect the future dynamics of polarization. This has substantial implications for modeling macrophage polarization trajectories because it demonstrates that models for macrophage polarization must

take prior states of the system in consideration to fully recapitulate the dynamics.

LITERATURE REVIEW:

Macrophages were first identified by Russian zoologist Élie Metchnikoff in 1882 due to their unique ability to perform phagocytosis[1]. However, over the past century, macrophages are now understood to display a diverse range of functional niches and phenotypes that far extend beyond their role as phagocytes. In 1962, macrophage activation was first described in a paper[6] that observed how peritoneal macrophage antibacterial activity rapidly increased in response to listeria infection, allowing the cells to acquire resistance to the bacteria. It was not until 1992 that alternatively activated macrophages (AAM, or later, M2) were described in IL-4 stimulated murine macrophages, which displayed decreased secretion of pro-inflammatory cytokines and increased mannose receptor activity[7]. By 2001, the modern terminology for polarization states had entered the literature, with M1 and M2 replacing classical and alternative activation, respectively[8].

Macrophages also play a crucial role in promoting the survival and proliferation of cancerous tumors. Tumor-associated macrophages (TAMs) are found in many solid tumors, and their density often correlates with disease severity and survival rates. These macrophages exhibit a chronic M2-like phenotype and secrete anti-inflammatory cytokines that attenuate healthy immune response in the tumor microenvironment[9]. Additionally, recent work has shown that TAMs are involved in the breakdown of the extracellular matrix (ECM) via uptake and degradation of collagen via cathepsins in lysosomes. This breakdown of the healthy ECM potentially promotes metastasis and growth, as cancerous tumors must break down healthy tissue in order to expand[10].

There have been many recent developments in refining the understanding of macrophage polarization from a binary dichotomy into a spectrum of different phenotypes. For instance, macrophage polarization dynamics are now known to differ between macrophage lineages. Macrophage lineages are often described by their cellular origin, such as monocyte-derived macrophages which arise from circulating monocytes within the bloodstream, or as tissue-resident macrophages which arise from embryonic tissue[1]. Microglia have been found to exhibit more resistance to adopting an M2 phenotype than peripheral macrophages, which may help explain why many diseases of the CNS are characterized by chronic neuroinflammation. Additionally, recent work has shown that microglia exhibit different gene expression profiles in polarization than peripheral macrophages[11]. Different macrophage lineages/sub-populations are also known to show different levels of transcriptional and post-transcriptional regulation of polarization-associated genes[12]. Furthermore, through high-throughput sequencing platforms and systems biology techniques, macrophage polarization has been reassessed by some authors as a spectrum of phenotypes with complex characterization[13].

Macrophage polarization is believed to play a key role in the pathophysiology of many diseases. Microglia, the tissue-resident macrophages of the central nervous system (CNS), are chronically-activated in Alzheimer's disease, and the characteristic plaques of amyloid beta are known to synergistically induce M1 polarization in microglia in conjunction with pro-inflammatory cytokines[14]. Additionally, mouse models of AD with the Nos2 gene knocked-out (which encodes iNOS, a canonical M1 marker protein) show significantly less cognitive decline[14]. Traumatic brain injury (TBI) is also known to involve chronic microglial activation, and PET scan experiments have shown that microglial activation after an injury can persist for

over 17 years[15]. TBI is thought to be a risk factor for AD, and microglial activation may help to explain the interconnection between these two diseases.

Although there is extensive ongoing work into understanding the complete stratification of microglial activation states, there is a need for further investigation into time-domain computational modeling of macrophage polarization. These models can be employed to develop immunomodulatory strategies that target macrophage phenotypes without systemically disrupting immune response or inducing a pathological polarization response. My work has furnished a dataset suitable for training computational models that will model macrophage polarization as a control system. By doing so, it may be possible to predict future macrophage polarization given a cytokine stimulation strategy, and determine what inputs are needed to shift polarization to a new setpoint. This form of modeling based on *in vitro* polarization responses has not been previously described in the literature.

MATERIALS & METHODS:

All cell culture experiments performed in this thesis were conducted using the RAW 264.7 cell line, an adherent macrophage line which recapitulates polarization responses observed in primary cells. Using the RAW 264.7 (ATCC TIB-71™) macrophage cell line cultured from passages P5 to P10, we explored the dynamics of M1 polarization in response to temporal stimulation strategies using lipopolysaccharide (LPS, Sigma-Aldrich L2880), interferon-γ (IFN-γ, R&D Systems 485-MI), and interleukin 4 (IL-4, PeproTech 214-14). LPS and IFN-γ were used to induce an M1 macrophage phenotype, as measured by increased expression of iNOS. Protein expression was measured using immunocytochemistry and Western blotting.

For immunocytochemistry experiments, cells were cultured in 96-well microwell plates until 70% confluence, and subsequently stimulated with LPS, IFN-γ, or IL-4 at given concentrations. Cells were fixed in 4% PFA, solubilized in 0.1% Triton-X, and stained with α-iNOS (Cell Signaling Technology, Cat No. 13120, 1:400) and α-Arg1 (BD Bioscience, Cat No. 610709, 1:400). iNOS expression was quantified by dividing total iNOS fluorescence by total cell count.

For Western blotting experiments, cells were cultured in 6-well microwell plates and lysed in RIPA buffer (Boston BioProducts, BP-115), phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich P7626-1G), and Mini-Complete Tablets (Sigma-Aldrich 11836153001). Membranes were probed for alpha-tubulin, iNOS, and Arginase-1. Membranes were imaged on an Odyssey CLx machine and analyzed in ImageStudio. These results were used to validate key findings in immunocytochemistry.

To elucidate differences in polarization responses between 2D and 3D culture environments, a microfluidic platform developed by Dr. Levi B. Wood was employed to perform polarization experiments. Microfluidic devices were fabricated with polydimethylsiloxane (PDMS) on silicon microstructures and bound to glass coverslips. Microfluidic devices were treated with poly-D-lysine and filled with a 5 million cell per milliliter RAW 264.7 cell-suspension in Matrigel using a previously established protocol[16].

RESULTS:

The primary objective of this work was to produce an *in vitro* dataset to enable the computational modeling of macrophage polarization, with the goal of producing novel immunomodulatory strategies for chronic inflammatory diseases. These experiments investigated macrophage polarization responses to lipopolysaccharide (LPS), interferon-γ (IFN-γ), and interleukin-4, as measured by iNOS protein expression. While measuring iNOS does not capture the full spectrum of polarization responses, it is unique as a functional effector of polarization that is rapidly induced after stimulation. Because we have previously shown iNOS expression to peak after 24 hours post-stimulation, we have chosen a time sampling interval of 24 hours to measure M1 polarization after LPS stimulation[17].

The results of re-stimulating RAW 264.7 macrophages with 1μg/mL LPS-conditioned medium by media replacement indicate that re-stimulation alone is insufficient for sustaining macrophage polarization beyond 24 hours. On the contrary, re-stimulation appeared to accelerate the decline in iNOS expression after 24 hours, with re-stimulated macrophages exhibiting lower iNOS expression at 48 hours than macrophages stimulated continuously for 48 hours without media replacement, as measured by immunocytochemistry (Figure 1). These effects were observed as soon as six hours after re-stimulation with LPS-conditioned media.

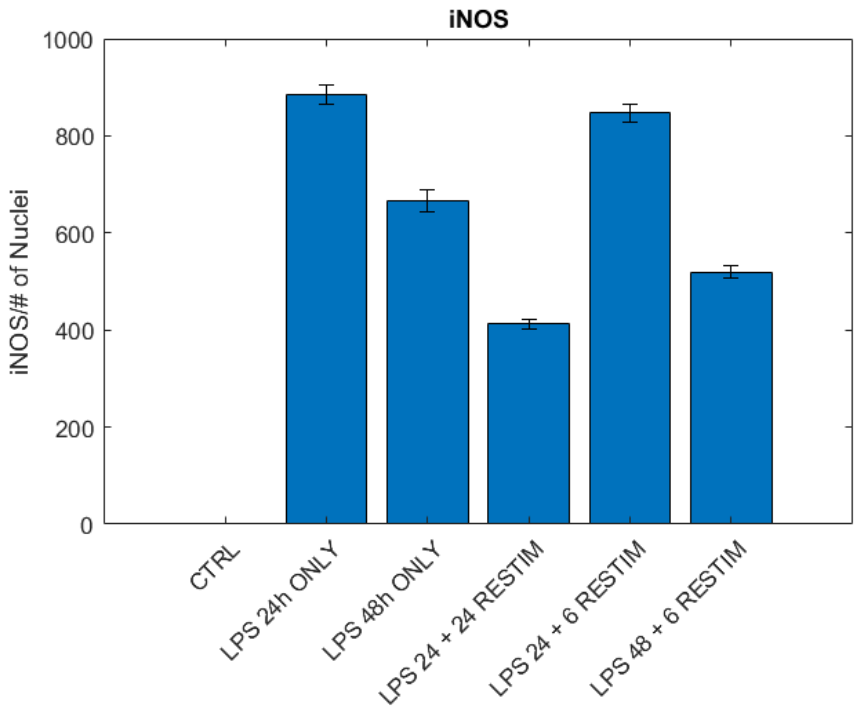

Figure 1. M1 polarization as measured by immunocytochemical staining for iNOS expression. Re-stimulation was performed using media replacement (n=3, mean±SEM).

To determine whether re-stimulation with an orthogonal stimulus, such as interferon-γ, could sustain or increase iNOS expression beyond 24 hours, RAW 264.7s were cultured for 24 hours in 1μg/mL LPS-conditioned media and re-stimulated with 100ng/mL IFN-γ conditioned media for an additional 24 hours. In contrast to the previous experiment, re-stimulation with an orthogonal stimulus resulted in markedly increased iNOS expression, as demonstrated in representative immunocytochemistry images (Figure 2).

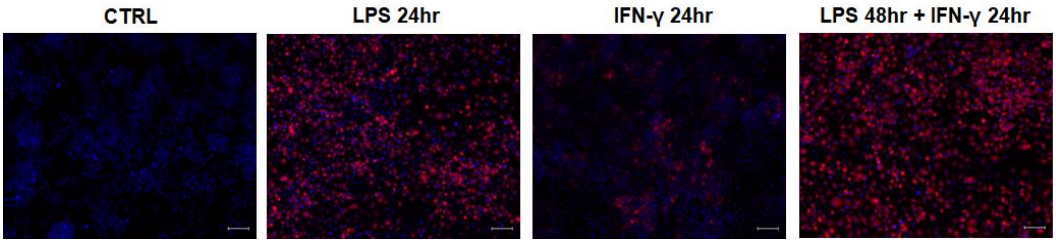

Figure 2. Representative immunocytochemistry images demonstrate that re-stimulation of LPS-treated macrophages with interferon gamma results in increased M1 polarization (n=3, representative images shown).

While orthogonal stimulus successfully induced greater iNOS expression beyond 24 hours, it was not known whether this effect persisted at a broader range of concentrations for LPS and IFN-γ. Subsequently, a range of concentrations for LPS (10ng/mL to 1μg/mL) and IFN-γ (1ng/mL to 100ng/mL) were investigated, wherein RAW 264.7s were treated with either LPS or IFN-γ for 24 hours and subsequently re-stimulated with the opposite condition for an additional 24 hours. This experiment was performed to verify that the increase in iNOS expression observed in Figure 2 persisted at lower concentrations of LPS and IFN-γ. For each combination of conditions, iNOS expression was measured at the 48 hour endpoint by immunocytochemistry (Figure 3). When RAW 264.7s were first treated with LPS and re-stimulated with IFN-γ, they exhibited further increases in iNOS expression which were dose-dependent. However, RAW 264.7s treated first with IFN-γ and re-stimulated with LPS exhibited a plateau of iNOS expression even at the lowest concentrations of both stimuli.

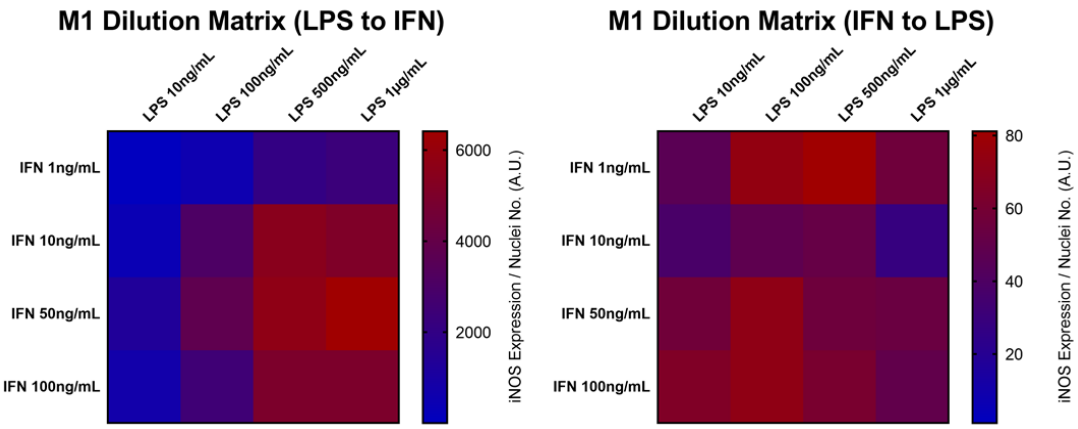

Figure 3. iNOS expression after re-stimulation is dependent on the order of stimulation, as measured by immunocytochemistry (n=3, mean).

It is known that there are differences in cell responses in three-dimensional culture environments, but it was not known whether the dynamics of macrophage polarization are significantly altered in 3D. To investigate whether dynamics observed in prior two-dimensional cell culture experiments exhibited the same patterns in iNOS expression in a three-dimensional environment, the LPS re-stimulation experiment described in Figure 1 was replicated using RAW 264.7s cultured within PDMS microfluidic devices. Microfluidic devices were seeded with a suspension of 5 million RAW 264.7s per milliliter in Matrigel and treated with LPS-conditioned media for the indicated timepoints (Figure 4). Re-stimulation with LPS-conditioned media after 24 hours resulted in an accelerated decline in iNOS expression as observed in Figure 1. However, unlike RAW 264.7s treated in microwell plates, peak iNOS expression in microfluidic devices was not reached until 48 hours of stimulation, indicating a delayed response.

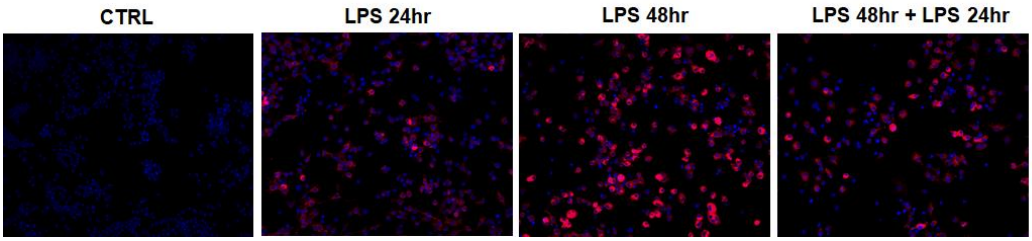

Figure 4. iNOS expression trajectory after LPS stimulation is extended in a microfluidic, three-dimensional culture platform (n=3, representative images shown)

DISCUSSION:

I first sought to establish whether a stimulation scheme involving one pro-inflammatory stimulus, such as LPS, could sustain M1 polarization at a constant level for an extended period of time. Using various M1 stimulation strategies that incorporated re-stimulation (media replacement with fresh 1μg/mL LPS in cell-culture medium) strategies at a variety of different time points, I used immunocytochemistry to measure iNOS expression and quantified the degree of M1 polarization (Figure 1). Surprisingly, no single strategy maintained constant M1 polarization, and media replacement appeared to accelerate the decline of M1 polarization. This was hypothesized to result from a loss of paracrine signaling, as M1 macrophages are known to secrete pro-inflammatory cytokines such as TNF-α, which in turn promote greater M1 polarization. Other inhibitory processes may also explain this effect, such as downregulation of the TLR4 receptor and loss of sensitivity to LPS, which must be explored in future work.

I next characterized whether re-stimulation with an orthogonal M1 stimulus, such as interferon-γ, is capable of sustaining M1 polarization and reaching a greater space of iNOS expression than one single stimulus alone. Interferon-γ is referred to as an orthogonal stimulus in this text as it activates genes required for M1 polarization via the JAK/STAT signaling pathway, while LPS drives TLR4 signaling. Because these pathways are largely separate, this was

hypothesized to increase the degree of M1 polarization in combination. I conditioned RAW 264.7 macrophages with LPS and then re-stimulated with IFN-γ, which resulted in greater iNOS expression than any group that received LPS alone (Figure 2). This suggests that orthogonal stimulus can induce greater M1 polarization than any single stimulus alone. One possible explanation for this result is that negative feedback and inhibition processes that sufficiently limit response to LPS alone are overcome by orthogonal stimulation.

However, the prior experiment only measured M1 polarization in macrophages that were first treated with LPS and second with IFN-γ. It is possible that there are order-specific effects on M1 polarization when multiple cytokines or biomolecules are being used. Using four different concentrations of LPS and IFN-γ, I conditioned RAW 264.7 macrophages for 24 hours with either LPS or IFN-γ and then subsequently re-stimulated with the orthogonal stimulus for an additional 24 hours (Figure 3). In agreement with the previous experiment, combinations of stimuli resulted in far greater iNOS expression than a single stimulus alone. However, surprisingly there were order-specific effects, where IFN-γ followed by LPS appeared to reach a critical maximum at far lower concentrations. In contrast, LPS followed by IFN-γ followed a much more linear dose-dependent response. This suggests that models of macrophage polarization must account for information about prior stimuli before predicting responses to subsequent stimulation.

I also sought to show whether any differences exist between M1 polarization dynamics in a three-dimensional cell culture environment versus a two-dimensional culture. One of the major limitations of *in vitro* culture is that cells are suspended in a flat shape that fails to properly recapitulate the microenvironment of living tissue. Performing cell-culture experiments in a three-dimensional platform allows for studying cell responses in an environment which more

closely recapitulates the properties of native tissue and the extracellular matrix[18]. Previous investigators have demonstrated that the structural properties of an extracellular scaffold exert effects on macrophage polarization[19]. Thus, it was hypothesized that macrophages may exhibit altered dynamics when cultured in 3D or in the presence of an extracellular matrix. Using a PDMS microfluidic device with Matrigel as an extracellular protein scaffold, I conducted an experiment to investigate how LPS re-stimulation is altered in three-dimensional cultures (Figure 4). Using the same stimulation schemes as described in Figure 1, I observed a similar response to re-stimulation where iNOS expression decreased at a greater rate than no re-stimulation at all. However, macrophages cultured in a three-dimensional environment also exhibited a more extended response to LPS, showing increases in iNOS past 24 hours and up to 48 hours, which is considerably longer than responses observed in two-dimensional microwell plates. These results present a novel effect of the culture environment on the dynamics of macrophage polarization and suggest that M1 polarization dynamics observed *in vitro* may exhibit a more delayed response *in vivo* under the same conditions. Further work may investigate whether injected macrophage cell lines exhibit different dynamics in a native murine tissue environment.

CONCLUSION:

Macrophages are a crucial part of the immune system's front line of defense, and their polarization states make them a versatile effector over the course of inflammatory responses. However, dysregulated dynamics of macrophage polarization are implicated in a number of pathologies such as Alzheimer's disease and solid-tumor cancers. As a result, it is vital to understand the dynamics of how macrophages polarize in response to cytokines and biomolecules in order to correct for dysregulated polarization states in disease.

Through this work, I have shown that M1 polarization is a refractory and transient process that cannot be sustained through one type of stimulus alone. In the case of LPS, iNOS expression begins to rapidly decline after 24 hours and is accelerated by replacement of media with fresh LPS. However, re-stimulation with a secondary orthogonal stimulus, such as IFN-γ, allows for greater iNOS expression and extended M1 responses. This response is order specific, and switching the stimulus type used in the first and second round of treatment has dramatic effects on the M1 polarization observed. Furthermore, three-dimensional culture environments show intriguing differences in M1 polarization responses versus two-dimensional environments, which may have implications for the process of translating the results macrophage polarization responses into therapeutic interventions for live organisms. For instance, the delayed macrophage response to LPS in 3D culture indicates that successful inhibition of inflammation *in vivo* may require more prolonged delivery of inhibitory inputs (i.e. M2 cytokines, receptor inhibitors) than what is sufficient for experiments performed in 2D culture environments.

These results expand our understanding of how macrophages transduce signals from their environment into a rich spectrum of polarization responses with complicated dynamics. These results can form the basis for future models that may allow us to develop new immunomodulatory therapies for inflammatory diseases.

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