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Regulators, Processes, and Intricacies of the Innate Immune System with a Focus on the NEMO Complex

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SUMMARY Insight into immune system regulation is critical to understanding the homeostasis of the immune system, diagnosis of various autoimmune diseases, and cancer prevention measures in the body. The immune system is incredibly complex, from the organ to tissue to cell to molecular level. The innate immune system is contained within each somatic cell and can react to pathogen and damage-associated molecular patterns via pattern recognition receptors. These receptors initiate innate immune pathways that lead to inflammation. Understanding the mechanisms and regulators of the innate immune system and methods by which researchers map innate pathways provides a broader and more holistic context for the immune system's role in many diseases and disorders. To that end, this paper aims to record and analyze multiple proteins and mechanisms of the innate immune system, the role of the leucine rich repeat structure in the innate immune system, structural/functional elements of proteins that contribute to immune regulation of the NEMO (NF-kappa-B essential modulator), standard methodologies for testing signal pathways, and limitations/possible new methods for immune testing.

INTRODUCTION

The history of research into the innate immune system and the knowledge already gained is massive. Given that the immune system is relatively significant for medical analysis, a broad and rich understanding of immune processes is required for diagnosis and overall health. However, while our knowledge of the adaptive immune system is rich, our understanding of the intricacies of the innate immune system contains many holes that have the potential to be filled using testing methods propagating in the field.

Additionally, much is left to be desired when it comes to treatment methods for humanity's worst ailments, especially with cancer. Cancer is extremely difficult to target and treat. Mainstream strategies such as chemo- and radiotherapy, along with new therapies such as oncolytic viral therapy, present a credible threat to the general health of a sick patient, which is not ideal [1].

To add, understanding the regulation and minutia of the innate immune system's action would allow researchers to better understand adjacent systems and mitigate threats through autoimmune disorders or regenerative medicine. Perhaps understanding how the body reacts to viruses or bacteria at a molecular level could present new solutions to questions of aging and degradation [2].

In general, understanding the action of the immune system and the methods by which researchers investigate elements of immune cascades is crucial to developing a toolkit to prevent further harm and push biological research ever closer to the frontier.

In that spirit, this paper's objective is to summarize the functions and features of the innate immune system, discuss the regulation of the NEMO (NF-kappa-B essential modulator)

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complex, analyze the role of the LRR domain, comment on current testing methods, and discuss how modulation of the innate immune system can aid in medical research.

FUNCTIONS AND FEATURES OF THE INNATE IMMUNE SYSTEM

The innate immune system is the most significant part of the immune system to analyze, as each cell is primed with such a system, and it has the potential to call the entire immune response into action. In addition, it is often described as the first line of defense for the body's immune response. The first step of the innate immune response is the recognition of molecular patterns via pattern recognition receptors (PRRs). Molecular patterns are molecules present in multiple pathogens or other sources. Thus, PRRs suited to binding various molecular patterns allow for accurate and efficient innate immune system activation [3].

There are two types of molecular patterns: pathogen-associated molecular patterns (PAMPs) and damage/danger-associated molecular patterns (DAMPs). PAMPs recognize molecules generally associated with pathogens. These structures tend to be highly conserved, meaning they have remained essentially unchanged throughout the pathogen's evolutionary history. A PAMP could be flagellin or bacterial liposaccharides (LPS) [3]. DAMPs recognize structures that are released due to necrosis of bodily cells. A DAMP could be a free radical species or mtDNA [3]. Thus, by categorizing different molecular patterns in these groups, the immune system can more readily face any threat with more precise targeting. Pattern recognition receptors (PRRs) vary significantly in their method of reception and function. There are various types of PRRs suited to specific PAMPs or DAMPs. Upon recognition of PAMPs or DAMPs, PRRs initiate the innate immune cascade.

Innate immune cascades are categorized into various pathways that inevitably end with the production of cytokines through IRF phosphorylation or activation of NF-kB. IRF is a protein known as interferon-regulatory factor, while NF-kB is known as nuclear factor kappa B. Both are transcription factors, proteins that regulate target gene transcription. NF-kB and IRF are specifically responsible for initiating the production of cytokines, among other immune-related proteins. Cytokines are signaling protein and hormone messengers that act as ligands to illicit adaptive and innate immune responses inside and outside surrounding cells. Some cytokines include tumor necrosis factors (TNFs), interleukins (ILs), and interferons. Both of these methods are stimulated by PRR transduction [3].

Various PRRs are essential to this paper, including Mitochondrial antiviral signaling proteins (MAVs), Toll-like receptors, Nod-like receptors, and RIG-1-like receptors. These exist in various parts of the cell, though notably in the cytoplasm and the cell membrane [3].

Toll-like receptors, or TLRs, are a family of leucine-rich repeat-containing (LRR) proteins containing a Toll-like receptor domain, transmembrane, and LRR-containing domains [4], [3], [5]. A protein domain is a section of a protein's folding defined by the structure and location of the region. In this case, the TLR domain indicates Toll-like proteins, while the LRR-containing domain indicates a similar structure within other proteins. The transmembrane domain is defined not only by the structure but also by the region of the cell in which this domain exists. All protein domains, primarily the LRR domain, are defined by their similarity with other proteins, an example of molecular evolution. This is partly supported by the ubiquitousness of LRR-containing proteins in many animals, including plants, microorganisms, and most especially jawless fish [6], [7], [8]. The molecular evolution of the innate immune system, containing proteins in the LRR superfamily, indicates the importance, age, and adaptations of the innate immune system and its ingrained role in the history of physiology. LRR stands for leucine-rich repeats, a protein motif containing a repeating set of leucines separated by a variable number of other amino acids, usually in the form of LxxLxxLxLxxN [9], [5], [10], [11]. A protein motif is an unfolded amino acid sequence shared among many proteins, also displaying molecular evolution. Both domains and motifs are helpful in categorizing proteins into families. Proteins within a family or superfamily tend to share similar functions, allowing researchers to predict functions in newly discovered proteins more accurately [7]. In this case, the LRR-containing domain displays a curved solenoid structure [4], [5], [6], [7]. The curved structure is created by a concave, highly conserved motif and a convex, variable motif, creating a binding region for any number of ligands (Figure 1).

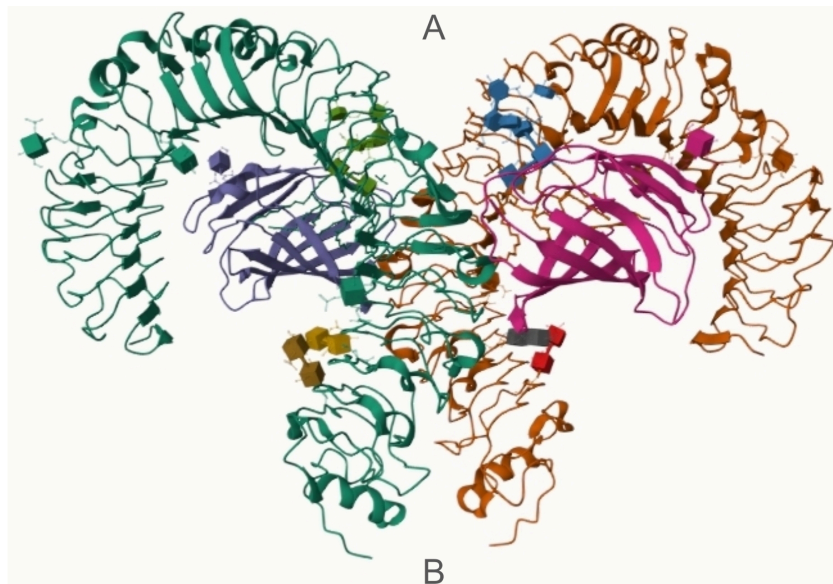

FIG. 1 Image depicting the curved solenoid structure from Interpro Adapted from [4]. The figure displays a dimerized TLR4 in complex with *E. coli* liposaccharide. The liposaccharide ligand binds under the solenoid framework of the LRR domain (A). The solenoid framework allows the TLR domain (B) to form, allowing TLR adaptors such as MyD88, TRAM, etc, to bind.

TLRs exist in endosomes within the cytoplasm or as transmembrane proteins. As previously described, ligands bind under the solenoid framework that characterizes the LRR domain. Many different processes can occur to activate the TLR system from the inception of ligand binding, all resulting in the dimerization of TLRs. Dimerization is the polymerization of 2 different things into 1. In TLR1/2, TLR2/6, the TLR10 homodimer, and the TLR3 homodimer, a section of the targeted ligand binds to the concave pocket of the LRR for each TLR subunit so that the entire ligand binds both TLRs. This brings the TLRs closer together, causing dimerization along the planar surface of the transmembrane domain [6], [8]. In TLR9 and other TLRs, however, the binding of a ligand institutes a conformational change in the transmembrane domain that brings the homodimer together. Indeed, especially for TLR9, single-stranded DNA with the CpG motif can only bind as a ligand to TLR9 when the Z-Loop between the LRR14 and 15 residues is cleaved, another conformational change [12]. For TLR4, receptor signaling is appended by an MD-2 (Myeloid Differentiation factor 2) mediator that binds to the liposaccharide, changing its morphology to meet with the LRR pocket on TLR4, allowing for dimerization [6]. A conformational change, or post-translational modification, is a change to a protein that happens after protein folding and after the protein enters its natural conformation (most efficient folded structure). By dimerizing, the internal Toll-like receptor domain is activated. This dimerization allows proteins to bind to the Toll-like receptor domain, starting the cascade.

Adaptor proteins containing a Toll-like domain include MyD88 (Myeloid differentiation primary response 88), TIRAP (TIR domain containing adaptor protein), TRIF (TIR-domain-containing adapter-inducing interferon-β), SARM (Sterile alpha and TIR motif containing), and TRAM (Translocating chain-associated membrane protein) [3]. However, it has been shown that TLRs tend to primarily utilize MyD88, likely due to its role in the production of the myddosome [13], [8]. The myddosome is a structure formed from the binding of the death domains on MyD88 and IRAK4/1/2 (interleukin-1 receptor-associated kinase). These proteins combine to recruit TRAF6 (TNF receptor associated factor 6) extremely efficiently, making it an ideal adaptor for the TLR family. The other adaptors are used for more specific signaling, as shown in TRIF's role in interferon signaling [8], [13], [14]. TRAF6 is polyubiquitinated via various E2 ubiquitination enzymes. Ubiquitination is a process of post-translational modification where small proteins known as ubiquitins bind to lysine acids in a folded protein. Ubiquitination is done using E1,2, and 3 ligases. There are 3 E1 ligases, ~20 E2 ligases, and many E3 ligases. A ubiquitin will be bound to an E1 ligase, transferring to an E2 ligase, which then transfers to an E3 ligase to be bound to the target protein. Ubiquitination is used for protein degradation via the proteasome (an important regulation step in the innate immune system) or post-translational modification. This builds up to the recruitment of the NEMO complex.

The K63 polyubiquitin chain added to TRAF6 allows for the recruitment of NEMO via its ubiquitin-binding domain. Additionally, TRAF6 recruits TAB2/3 (TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein 2/3), which activates TAK1 (Transforming growth factor-β activated kinase 1) [3], [15]. TAK1 phosphorylates the subunits IKKa and IKKb (Inhibitory-κB Kinase alpha and beta), which bind to the IKK binding sites on NEMO. NEMO is also polyubiquitinated, though the purpose of this is speculatory [15]. Current theories suggest that it either allows TAK1 to be closer to IKK subunits, which increases efficiency, or provides for the oligomerization of NEMO complexes, as more NEMOs bind to the chains on other NEMOs [15]. Oligomerization is the polymerization of "some" amount, precisely more than one. NEMO contains two linker domains, each binding to NEMO binding domains on IKKa and IKKb [15]. Both IKKa and IKKb contain helix-loop-helix domains, allowing for allosteric modulation by regulators [15]. The IKK complex thus phosphorylates IkBa (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha), an NF-kB regulator [15], [3]. This phosphorylation is recognized by B-TrCp (Beta-transducin repeats-containing proteins) ubiquitin ligase [15], which ubiquitinates IkBa and destroys it via proteasomal degradation. Thus, the free NF-kBs can upregulate cytokines, regulators, and cytosolic sensors such as AIM2.

In addition, while unclear, NEMO acts within the TLR3/4 IFN pathway [3]. TLR3/4 binds with TRIF in this pathway to activate the TBK1 (TANK-binding kinase 1)/IKKi complex, which acts as a kinase complex similar to the IKK complex. Therefore, NEMO regulators may act on IKKi in the same way they act on the NEMO complex. Additionally, TRIF is reported to interact with TRAF6. TBK1/IKKi phosphorylates IRF3/7, allowing it to upregulate the production of type 1 interferon.

NEMO may also exist in the MAVS Viral RNA pathway. Upon sensing viral RNA, RIG-1 (Retinoic acid-inducible gene 1) activates MAVS [3]. MAVS provides a foundation for recruiting TBK1/IKKi and the IKK complex, producing type 1 interferon and other cytokines/factors produced via NF-kB promotion. MAVS acts as a base for kinase activation by recruiting TRAF6 and other TRAF proteins, all responsible for supporting the K36 polyubiquitin chains that bind NEMO [3].

It is no surprise that TLRs would be present in immune cells along with normal somatic cells. For example, TLR3 and 4 exist within endosomes created through phagocytosis, a prominent function of macrophages and dendritic cells [3]. Additionally, NF-kB is one of many transcription factors responsible for the production of major histocompatibility complex, or MHC complex, subunits, which bind to antigens in destroyed bacteria or viruses present in the endosomes of macrophages and dendritic cells and present them on the surface of those cells [16]. Thus, helper-T cells, which are called to the scene by interferons (also being released by TLR signaling), recognize that these antigens exist via CD4 receptors on their surface binding to MHC-II units on the immune cell's surface. This allows helper T cells to initiate crosstalk between each other and other immune cells by producing IL-6 and interferons, providing more support against the infection. B cells and cytotoxic T cells are recruited to fight infection. Cytotoxic T cells utilize pyroptotic factors such as granzyme and perforin to lyse infected cells. These cells are recognized by the production and presentation of MHC-I complexes on their surfaces. B-cells attempt to find antibodies with variable regions that match antigenic epitopes. Once determined, the B-cell will differentiate into memory and plasma varieties. Plasma B cells produce large amounts of antibodies, which agglutinate pathogens by binding two or more pathogens together via their shared epitopes. Thus, macrophages can engulf and digest these pathogens, ending an infection.

However, as mentioned before, cytotoxic T cells are most relevant to mitigating viral infection and are most prevalently used to curb the infection of normal somatic cells. Thus, surface TLRs play a much broader and more critical role. In this scenario, NLRs and adjacent cytosolic sensors are most important.

In addition to being endosomal, TLR4 can be transmembrane and binds with LPS, a common protein found in gram-negative bacterial cell walls. TLR4 binds with MyD88, leading to the cascade, which inevitably ends with the upregulation of cytokines and cytosolic sensors in the NLR and ALR families. Specific cytokines include pro-IL-18 and pro-IL-1B [3].

Marked LPS is used frequently to determine the purpose and placement of specific immune components. LPS testing is specifically relevant to the scope of this paper, as it is commonly used in papers involving NLRC5 and LRRC14, both being immune regulators [11], [9]. This is discussed more specifically in the coming sections.

NLR and AIM-2-like receptor (ALR) PRRs are cytosolic sensors vital to forming inflammasomes. An inflammasome is a protein complex made of an NLR or ALR base, ASC, and caspase-1 (Figure 2). Its function is to cleave proteins, specifically pro-ILs and pro-gasdermin. Many proteins in the human body exist as zymogens, or precursors to the actual protein. The "pro" or zymogen group must be removed to form the active protein. When a protein like AIM2, an ALR, senses dsDNA in the cytoplasm, its conformation changes, allowing ASC proteins to bind to the pyrin sites. Apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) or ASC binds to the pyrin sites on the AIM-2. The CARD domain of procaspase-1 then binds to the CARD of ASC, forming active caspase-1. These caspases cleave pro-gasdermin and pro-IL-18/1B, forming active components. Gasdermin opens pores in the cell membrane, lysing the cell. The released interleukin and DAMPs from cell death all contribute to recruiting the adaptive immune system and triggering PRRs for innate response in surrounding cells [3], [17].

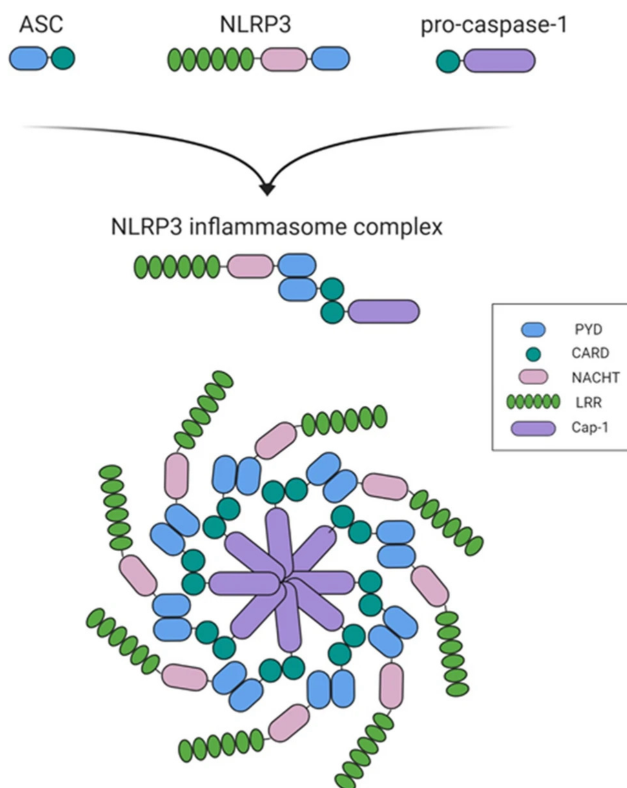

FIG. 2 Diagram of Inflammasome structural components. Taken from [27]. The image above displays the structural components of each of the inflammasome proteins. NLRP3's LRR domain curls into a solenoid when activated. The pyrin domains (PYD) of NLRP3 and ASC also bind together, recruiting ASC. In general, this results in large amounts of ASC recruiting, as ASCs can bind to the pyrins of other ASCs. The CARD domain recruits procaspase, activating it to cleave pro-gasdermin and pro-IL1b/16.

REGULATION OF THE NEMO/IKK COMPLEX

Considering each of these processes relies on NEMO and the general IKK complex, regulation of such a complex is necessary for homeostasis of the innate immune system.

One such regulator is NLRC5. Like its other NLR counterparts, NLRC5 contains a CARD domain. However, it also includes a large LRR region and a central NOD domain, which separate it from inflammasome subunits [9]. Due to the relative size of these regions, it has been shown that NLRC5 likely competes with NEMO for binding of IKKa. The LRR domain on NLRC5 binds to the kinase domain of IKKa, blocking it from being able to phosphorylate IkB for degradation. Additionally, the size of NLRC5 blocks the C-terminus binding domain for NEMO on IKKa, competing with NEMO for activation of IKK subunits [9], [18].

Additionally, observations of upregulation of NLRC5 in MyD88-deficient and control cells have shown that NLRC5 is likely produced by NF-kB, suggesting that NLRC5 acts as a negative regulator of NEMO. This is because NLRC5, a repressor, must exist to inhibit NF-

kB. With LRRC14, the regulator binds to the HLH region of IKKb, blocking IKKb from being able to bind with NEMO. The HLH region is a domain that acts as a kinase modulator, similar to an allosteric site. This changes the conformation of IKKb [11].

STANDARD RESEARCH METHODS

In testing NLRC5 and LRRC14, liposaccharide (LPS) is often used to start the innate immune cascade. LPS is an agonist of TLR4, triggering the NEMO complex formation downstream. An agonist is a molecule that binds to a receptor to produce some response. Then, various stains and fluorescent protein markers are used to identify cell structures and point out the production of a target protein.

With LRRC14, Wu, Yang et al. (2016) used green fluorescent protein to track the production of LRRC14 and used DAPI (4',6-diamidino-2-phenylindole) to stain the nuclei of the cultured cell. The cell line used to test various TLR agonists (agonists for TL4, TLR1/2, TLR7/8) and their effect on LRRC14 production was a human leukemia cell line known as THP-1. This was achieved using real-time PCR. Real-time PCR, or qPCR, is a polymerase chain reaction method used to identify the quantity of a target sequence produced at any given time. This is facilitated using the normal PCR process. However, instead of taking the data at the end of amplification, fluorescent dyes track increased cDNA production over time. For gene expression purposes, often the increase of the target gene is proportionally related to the increase of an expected gene over time, showing relative transcription ratios and providing a dataset that characterizes the quantity of the gene transcribed. Thus, the quantity of genetic material expressed after certain stimuli can be observed, which is the case in this study.

THP-1 cells work as viable models for human cells and contain the requisite PRRs on their surface. The paper also measured LRRC14 levels in various mouse tissues, with heavy expression in immune structures such as the spleen, lungs, blood, and thymus.

To identify where in the cell LRRC14 diffuses, HeLa cells were stained with the dyes to indicate the movement and intensity of LRRC14. Before stimulation by an agonist, LRRC14 passively diffuses into the cytoplasm. However, after stimulation with TNFa (tumor necrosis factor-alpha), a cytokine used throughout the experiment, LRRC14 is present in large quantities. The researchers suggest that the activation of the NF-kB pathway redistributes LRRC14.

The next step in the identification process is using a luciferase reporter assay. Every gene contains a regulatory region, a promoter, upstream of the genetic sequence. This allows regulators to bind, controlling the rate and amount of the gene being transcribed. Usually, the entire promoter-target sequence ends with a terminator sequence. With luciferase reporting, a recombinant structure is created such that the target gene, which is the gene that NF-kB upregulates, is upstream of a luciferase sequence. The termination point is built to exist after the luciferase, causing the target gene and the luciferase to be controlled by the same regulatory factors. Thus, the amount of target gene is measurable by luciferase. Luciferase is a protein that exists in bioluminescent organisms, namely fireflies. It catalyzes the breakdown of luciferin, emitting light as a result. In the assay, a substrate containing luciferin initiates the bioluminescent process, and a light meter measures the amount of light emitted from a sample. This way, the target gene's rate/quantity change can be calculated [19].

Using various cytokines and agonists (TNFa, IL-1B, and LPS), as LRRC14 increased, Nf-kB-luc reporter decreased, suggesting that LRRC14 regulates the activation rate of NF-kB. Additionally, small interfering RNA (siRNA) was used to interfere with the production of LRRC14 from the recombinant structure to check whether it regulated Nf-kB reporter concentration and not some other protein. Indeed, stimulation with LPS in cells with LRRC14 siRNA interference increased the production of cytokines as the byproduct of the NF-kB pathway.

The researchers then needed to identify where in the cascade LRRC14 acts. As previously established, many innate pathways converge on the NEMO complex. The investigators reasoned that the upregulation of signaling proteins in the pathway would identify the effect of LRRC14 expression on activation in a measurable way. Using overexpression of MyD88, TRIF, and their downstream counterparts in a step-by-step fashion revealed that LRRC14 inhibited all action except for p65-mediated activation of NF-kB (IkBa regulation).

Immunoprecipitation results from tagged IKKb confirmed that LRRC14 must function before p65, most likely interfering with the NEMO complex to block IkBa phosphorylation.

Coimmunoprecipitation is a lab process by which a target protein, LRRC14, is bound to an antibody. Anything in complex with LRRC14, including any of the proteins it interacts with, will also be bound in complex with the antibody. Any proteins unrelated to LRRC14 are washed away, allowing researchers to isolate what LRRC14 interacts with.

To identify which part of the NEMO complex LRRC14 interacts with, researchers isolate IKKa, IKKb, and NEMO separately and conduct coimmunoprecipitation and immunoblotting to each of these molecules. It was revealed that IKKa and IKKb interacted with LRRC14. To determine where on each protein, investigators created various versions of IKKa and IKKb containing mutations that removed each binding region individually, thus identifying that the HLH domain of IKKb was interacting with LRRC14. To test whether this affected IKKb-NEMO interaction, investigators measured the rate of this interaction at various increasing levels of LRRC14, thus indicating that LRRC14 blocked NEMO complex formation by binding to the HLH domain of IKKb, as mentioned in the Immune Regulation section.

Observing the processes of protein testing is essential to understanding the broader functionality of these immune regulators. With the rigorous methodology of LRRC14 identification and function, it is evident that every aspect of the protein should be identified through testing. The tools by which these tests can occur are necessary to understand, and their relevance can be observed in many papers discussing any number of protein analyses. Researchers testing NLRC5 also underwent these test processes.

In general, the process can be synthesized then into six steps: identify which tissues contain the protein, determine where in the cell the protein diffuses, identify how the protein affects the cascade, identify where in the cascade the protein exists, and identify where/with what the protein interacts. Along the way, steps are taken to quantify these processes. Standard methods progress through real-time PCR, staining, luciferase reporter assays, and coimmunoprecipitation and immunoblotting until an answer is reached.

MODERN IMMUNE TESTING

Given the age of many of the previously discussed methodologies, there have been discoveries regarding the limitations of testing. One common consideration is the type of luciferase that would fit the particular application. For example, in [20], investigators tested luciferase reporter fluctuations in various practical applications, namely recombinant/molecular and complex fluid applications. Each standard luciferase reporter affects data differently; thus, investigators who choose the wrong luciferase may find the data collected unusable. By selecting the correct luciferase, researchers can highlight the data they need, which is one strength of luciferase reporting. In the case of [20], luciferases appeared to act more favorably than other reporter methods (fluorescent proteins) in the testing environment, being various complex body fluids, showing higher transcription and activation rates. There was also an apparent variance between the effectiveness of different luciferases for intracellular and complex fluid environments. Through the methods discussed in this paper, scientists can isolate favorable luciferases with a clear testing standard, making it a reliable tool for intracellular signal testing.

In papers such as [21], researchers commonly use a standard test known as Western blotting. Western blotting is used to detect, find the quantity, see the size, and compare protein concentrations in multiple samples [19]. In the realm of detection and quantification, it can be compared with luciferase reporting. However, the distinct difference is the usage: luciferase reporting affects the target gene. Both tests consider the protein concentration when analyzed after interacting with some agonist. With [21], the independent variable is LCZ696, an angiotensin II neprilysin inhibitor that affects NLRP3 action. Thus, western blotting is used for many different protein concentration and action tests, i.e., NF-kB concentration to test NLRP3 transcription, IL-1b, and other cleaved interleukins.

The western blotting process begins with running a gel of a protein sample [19], [22]. Proteins in the sample will spread based on weight and charge. However, the general charge is normalized to a negative due to the use of SDS buffer, with smaller, lighter, and less numerous concentrations moving faster toward the positive electrode through electrostatic force. Once a gel is prepared, the proper buffer acts as a medium to move the gel bands onto

a nitrocellulose membrane electrostatically. Once transferred, the filter paper is treated with antibodies designed to bind to a target protein. The antibodies are also conjugated with antibodies bound to a dye, which develops the color and identifies concentration and other data about the protein [19], [22].

Luciferase reporting and western blotting compare and contrast in many different functions and use cases, although western blotting is more cost-effective and less complicated than luciferase reporting. However, luciferase reporting may be more helpful in mapping specific inhibitor locations than generally characterizing a protein and its substrates/cofactors, as is the use case for western blotting. Despite this, western blotting may be analyzed to test for the same use cases as luciferase reporting in specific ways. Generally, both are viable and efficient ways to test signal transduction.

One criticism of immune testing and innate immune action in immunobiology papers is in vivo testing. Every mechanism mapped by labs worldwide is highly controlled by easily measurable testing parameters, such as using specific concentrations of agonists like LPS or cytokines. This makes for a potentially unrealistic analysis of how immune pathways work in vivo. We can see how particular inhibitors function by inducing model organisms with certain deficiencies or disorders, such as NLRP3 action in ulcerative colitis. However, there may not be certain diseases that target nearly every signaling protein in a pathway, making in vivo testing by this method impossible for proteins like TRAF6.

One interesting method that could aid in this issue is complex fluid analysis, which allows for testing neutrophil concentrations, cytokine concentrations, etc. This was discussed in [20]. However, more testing with the results of specific signaling intermediates is still needed. Luciferase reporting in real-time could present a viable solution. For example, a rat model can be used to test the inhibitive qualities of LRRC14. Injecting the rat with LPS or infecting it with a common bacteria may initiate TLR signaling. Treat the rat model with LRRC14 and test upregulation rates of NF-kB by tying NF-kB to luciferase by placing it upstream, perhaps with a viral agent or embryonic genetic editing. Then, using data from controlled experiments, after a specific time, euthanize the rat and extract tissue to determine concentration. However, this method is speculatory.

Another potential method is using a fluorescent-protein signal monitoring system like Tango. While Tango is used to characterize GPCR activity, edits to the Tango system, as suggested by [23], display a modular receptor signaling monitor that uses a CRISPR effector to access endogenous genes as a way to showcase success rate. Theoretically, any effector could be placed, forming a versatile monitoring method that could be adapted to immune testing.

DISCUSSION AND FUTURE DIRECTIONS

The immune system is a vital body system that prevents infection and death from external pathogens and damage. It exists in every cell and can respond in every tissue with ease. Additionally, it has a "scorched earth" effect on the body, attempting to use every method possible to rid the body of disease, even at the expense of its integrity. The importance of immune regulators is evident: protecting the body from immune overaction. Each feedback loop and cascade requires regulation to function correctly and efficiently. Despite these regulators, the immune system still overacts in many ways. The existence of autoimmune disorders such as type-1 diabetes and vitiligo prove this. By understanding immune regulation, medical researchers and practitioners can find new ways to protect valuable cell structures and may find ways to pinpoint or modulate the immune system to attack more dangerous structures such as tumors. This study is limited to current information and the author's education level. However, the topics discussed in this paper can inform research into developing new identification methods or methods to attack cancers with less harm to the rest of the body, unlike the dangers associated with oncolytic viral therapy and chemotherapy/radiotherapy.

Regarding the function and components of the innate immune system, understanding the role of the NEMO complex is pivotal to understanding the various methods by which the innate immune system acts. Especially important to innate immune system analysis is comprehending TLR and PRR signaling mechanisms as well, as they lead to activation of the NEMO complex. In addition, understanding the processes of ubiquitination and proteasomal

degradation is vital to understanding the intricacies of immune regulation. Each part of the innate immune system falls on feedback loops guided by ubiquitination and diverse regulation by transcription factors. These facts are necessary to understand when looking at the action and broader scope of the immune system.

In addition, understanding the structural elements and functions of the leucine-rich domain is vital to finding similarities and inferring roles for various regulatory and transduction proteins in the immune system and broader contexts, such as decorin in the skeletomuscular system [24]. The LRR superfamily covers many proteins across the body and in other organisms, opening up questions regarding the evolutionary importance and age of the immune system relative to the history of life. Another possible future research question regarding the LRR domain is if and how various LRR proteins cross system pathways. Given the varied nature of LRR functions, it's plausible that a protein like LRRC14 has other purposes. This is upheld in papers such as [25].

The methodologies of papers in the niche of immune regulation through innate immune pathways reflect a more significant trend in biotechnological innovation. Some of the papers cited date back five or more years, yet the standards and tests in those papers track with newer ones adjacent to this topic [26], [27]. Researchers are finding reliable standards to work from as the frontier is pushed. These standards will likely increase efficiency and reproducibility, providing more validity and information on this topic.

Immune regulation has plenty of exciting prospects and uses in the near future. As mentioned above, cancer-targeting could focus more on specific immune promoters or regulators. However, another exciting use could be in organ transplantation. Organ transplants often fail because the transplanted organ's antigens and the host's antibodies are incompatible, causing tissue agglutination and organ rejection. In these procedures, specialists reduce the intensity and strength of the immune response, giving the body time to adapt to the new organ. If targeted regulatory factors are built from preexisting regulators, perhaps within innate and adaptive immune cells such as macrophages, T cells, and B cells, the immune system does not have to be subdued to accept an organ. Instead, regulators would block specific pathways regarding agglutination and rejection so that the body isn't entirely immunocompromised.

In general, topics regarding immune regulation and signal components have a promising future, but there are still significant gaps in our understanding, such as the nature of NLRP3 signaling and the specific role of LRR domains. However, many regulators and feedback loops have been mapped in the previous years, perhaps signifying new growth in our knowledge of the innate immune system.

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