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Stoichiometry of Signalling Complexes in Immune Cells: Regulation by the Numbers

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1. Introduction

Host protection against pathogens and tumor cells is mediated mainly via white blood cells or leukocytes. As an inappropriate immune response can result in damage to the host and/or failure in pathogen clearance, the activation and function of leukocytes are tightly regulated processes. Regulation of immune cells is carried out by complex networks of receptors and cellular mediators. While the progress of the signal cascade is necessary for leukocyte activation and the development of an immune response, improper signaling and cellular activation are associated with various pathologies. The study of such networks constitutes a cornerstone of immunological research and has great implications for the understanding of the immune system and for the development of immunotherapies for cancer, infectious diseases, as well as autoimmunity.

In this chapter, we will describe the importance of the stoichiometry of signalling complexes in the regulation of leukocyte activation and function. We will focus on techniques used to analyze the formation, composition, and stoichiometry of multiprotein complexes, and we will also review current information and implications of stoichiometry on immune-cell activation and regulation.

1.1 Leukocytes and their regulation

Protection against infectious diseases is mediated by the immune system, which includes both humoral and cellular responses that enable the protective function or resistance against pathogens (Viret & Janeway, 1999). While humoral immunity is mediated by secreted proteins, peptides and small molecules and participates in host protection, responses of immune cells are cardinal for most immunological functions.

Immune cells, or leukocytes (white blood cells) are divided into two main cell types based on their nuclear shape; these include mononuclear cells (including monocytes, macrophages, dendritic cells and lymphocytes) and polymorphonuclear cells, also termed "granulocytes" (including neutrophils, eosinophils, and basophils). In general, each of these

cell types plays a role in a different aspect of the immune response (Risso, 2000). Some of the leukocytes such as macrophages, neutrophils and dendritic cells, also termed "phagocytes", are responsible for the phagocytosis of pathogens. Others, as the granulocytes, secrete bactericidal agents, while one type of lymphocytes (B lymphocytes- described below) produce and secrete target-specific antibodies. The leukocytes are also responsible for the destruction of virus infected cells, as well as for the identification and eradication of cancerous cells (Doherty, 1996; Jager et al., 2001).

The leukocytes belong to two arms: either to the innate or to the adaptive immune system. Cells of the innate immune system constitute the first responders to pathogens and cancerous cells. Phagocytes are responsible for pathogen removal as they engulf, ingest and digest these invaders. Cells belonging to the mononuclear phagocyte family, such as macrophages and dendritic cells, process ingested particles, releasing peptide fragments and displaying them on their cell surface. The presentation of these peptide chains, or antigens, in the context of immune cell recognition, constitutes the corner stone of the activation of the adaptive arm of the immune system (Davis & Bjorkman, 1988). The cells that display foreign antigen complexes on their surfaces are termed “antigen presenting cells” (APCs) and include, among others, macrophages and dendritic cells.

The lymphocytes are divided into three cell types: Natural killer cells (NK), T lymphocytes (also termed T cells) and B lymphocytes (also termed B cells). While NK cells play a major role in the innate immune response, T and B cells play a major role in the adaptive immune response. In contrast to NK cells, which do not express receptors for specific antigens, T and B cells express a stochastically generated receptor, the T-cell antigen receptor (TCR) or the B-cell receptor (BCR), capable of interacting with a single specific antigen. The large repertoire of different lymphocyte clones expressing different receptors allows the recognition of virtually all antigens (Davis & Bjorkman, 1988). T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity (secretion of antibodies) (Davis & Bjorkman, 1988).

Antigen presentation is mediated via the Major Histocompatibility Complex molecules (MHC). Class I MHC is expressed by all nucleated cells while Class II MHC is expressed only by dedicated APCs mentioned above, and B lymphocytes.

Class I MHC molecules, in addition to their role in antigen presentation, act as NK inhibiting ligands. Disruption of MHC Class I expression, occurring in certain virus infected cells and in tumor cells, while facilitating escape from recognition by T cells, reduces NK inhibitory signaling, thereby enhancing cytotoxic NK activity (Chini & Leibson, 2001; Wu & Lanier, 2003).

In response to pathogens, a type of T cells, called T helper cells, produce cytokines that direct the immune response, while another type, called cytotoxic T cells, produce also cytotoxic granules, similarly to NK cells, which induce the death of pathogen infected cells (Chini & Leibson, 2001; Wu & Lanier, 2003).

Cytokines are proteins that act as messengers between cells. In the immune system, cytokines facilitate communication among immune cells and between immune cells and other host cells. Cytokines are responsible for inducing immune cell proliferation

and for enhancing, suppressing and terminating immune responses (Weber & Iacono, 1997).

Given their role in the coordination of the immune response, the development of long term immunity, and their role in the recognition and elimination of cancerous and virally infected cells, T lymphocytes are the subject of a vast amount of studies. Deciphering the processes governing T cell activation constitutes a focal point of immunological research (Smith-Garvin et al., 2009; Wucherpfennig et al., 2010).

T cell activation begins with the binding of the TCR complex to peptide bound MHC (Dembic et al., 1986; Saito & Germain, 1987). Along with this engagement, T cell specific co-receptors called either CD4 (present on T helper cells) or CD8 (present on T cytotoxic cells) recognize and bind to MHC class II or MHC class I, respectively (Viret & Janeway, 1999) (Fig. 1). During these binding processes, the TCR and co-receptor molecules undergo clustering, allowing the protein kinase, Lck, bound to the intercellular portion of either CD4 or CD8, to phosphorylate tyrosine sites on cytoplasmic proteins of the TCR complex (Rudolph et al., 2006). These tyrosine motifs belong to a family called ITAMs (immunoreceptor tyrosine based activation motifs) (Love & Hayes, 2010; Reth, 1989). Upon phosphorylation, these tyrosine residues on the TCR ζ chain associate with the SH2 (Src Homology 2) domains of the kinase ZAP-70 (zeta chain-associated protein of 70 kDa) (Chan et al., 1992). As ZAP-70 is recruited to the complex, it undergoes phosphorylation by Lck, and is thereby itself activated (Barber et al., 1989; Samelson et al., 1986; Samelson et al., 1990; Veillette et al., 1988). ZAP-70 then phosphorylates the downstream T cell signalling molecules, LAT (linker for the activation of T cells) (Zhang et al., 1998) and SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) (Bubeck Wardenburg et al., 1996; Samelson, 2002). Phosphorylated LAT complexes with SLP-76 via the adaptor proteins Gads and Grb2 and acts as a scaffold for the recruitment of additional signalling proteins, promoting downstream activation events (Liu et al., 1999; Sommers et al., 2004). PLC-γ (Phosphoinositide phospholipase C γ) recruited to phosphorylated SLP-76, catalyzes the breakdown of the membranal phospholipid Phosphatidylinositol 4,5-bisphosphate (PIP2) into the secondary messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) (Beach et al., 2007; Ebinu et al., 1998). DAG activates PKCθ (Protein kinase C θ), and through it, activates the cellular transcription factors NF-κB (nuclear factor κB) and AP-1 (activator protein 1) (Melowic et al., 2007; Smith-Garvin et al., 2009), while IP3 induces the opening of calcium channels, further facilitating T cell activation (Imboden & Stobo, 1985). Increased cellular calcium levels induce the release of the nuclear factor, NFAT (Nuclear factor of activated T-cells), from the calcium binding protein, Calcineurin (Hogan et al., 2003). These transcription factors promote the expression of proteins necessary for T cell activation and for its effector functions, including the production and secretion of cytokines governing immune responses (Smith-Garvin et al., 2009).

Phosphorylated SLP-76 is also a key player in the activation of the cellular actin polymerization machinery, facilitating the reorganization of the cytoskeleton necessary for enhancing the T-cell/APC interface and enabling T cell effector functions (Jordan et al., 2006; Reicher & Barda-Saad, 2010). By recruiting the adaptor protein Nck (non-catalytic

region of tyrosine kinase adaptor protein) and VAV1, an activator of Rho family GTPases, SLP-76 mediates between TCR proximate activation events and actin polymerization and reorganization (Koretzky et al., 2006). Nck, in turn, recruits the actin nucleation factor, WASp (Wiskott–Aldrich Syndrome Protein) (Zeng et al., 2003). With the recruitment of WASp by Nck, and activation of actin nucleation and polymerization machinery by VAV1 activated GTPases, the cytoskeleton undergoes remodelling, enabling enhanced cell spreading and the reorientation of cellular polarity (Billadeau et al., 2007). In cytotoxic T cells, cellular polarity allows for the direction of cytotoxic granules at the intended target cell (Reicher & Barda-Saad, 2010; Smith-Garvin et al., 2009).

B cells share many points of similarity with T cells, in aspects of antigen-induced cellular activation, subsequent signalling complex formation, and cytoskeletal remodelling. In analogy to T cells, B cell activation is triggered by ligation of the BCR to its cognate antigen (Fig. 1). The BCR is comprised of a membrane-bound immunoglobulin (mIg) component with a short cytoplasmic domain that has no direct interaction with downstream signalling molecules. Therefore, the mIg associates with two additional immunoglobulin chains, Igα/β, which contain ITAMs (Pierce, 2009; Tolar et al., 2005). Antigen binding by the BCR initiates rapid phosphorylation of the ITAMs within the Igα/β chains by Src family kinases e.g. Lyn, and leads to the recruitment of intracellular signalling molecules and adapters, including Syk (Spleen tyrosine kinase), Blnk (B cell linker), VAV and PLCγ2. These molecules interact to form a multi protein signalling complex known as the signalosome (Batista et al., 2010). Similar to T cells, the membrane recruited signalling molecules induce calcium influx (mediated by PLCγ2) and the subsequent activation of transcription factors such as NF-κB. Eventually, antigen binding by the BCR leads to B-cell proliferation, differentiation, and antibody production and secretion. Actin cytoskeleton reorganization is pivotal for the activation and function of B-cells, as it facilitates BCR clustering and organization, and B cell spreading (Fleire et al., 2006; Treanor et al., 2010; Weber et al., 2008). In this context, signalling molecules such as VAV may also facilitate actin polymerization in B-cells (Weber et al., 2008), by functioning as activators of Rho GTPases. However, the exact molecular mechanisms underlying actin reorganization in B-cells are not completely understood, and are under extensive investigation.

Deciphering of the underlying mechanisms of lymphocyte activation is of great importance to the understanding of the direct activity of T cells in anti-tumoral and anti-viral immunity, as well as its function as a key regulator of immune responses and autoimmunity. The regulation and function of numerous cellular processes are dependent on protein-protein interactions. In the immune system, protein-protein interactions are the main mechanisms leading the regulation of lymphocyte activation, and indeed, rule the initiation and termination of the immune response by various cells of the immune system. Understanding the molecular mechanisms underlying these multi-protein complexes requires the characterization of their composition and stoichiometry.

Below, we describe major techniques for exploring protein-protein interactions regulating immune cell activation and function, with the focus on technologies used for determining the stoichiometric ratios of protein complexes.

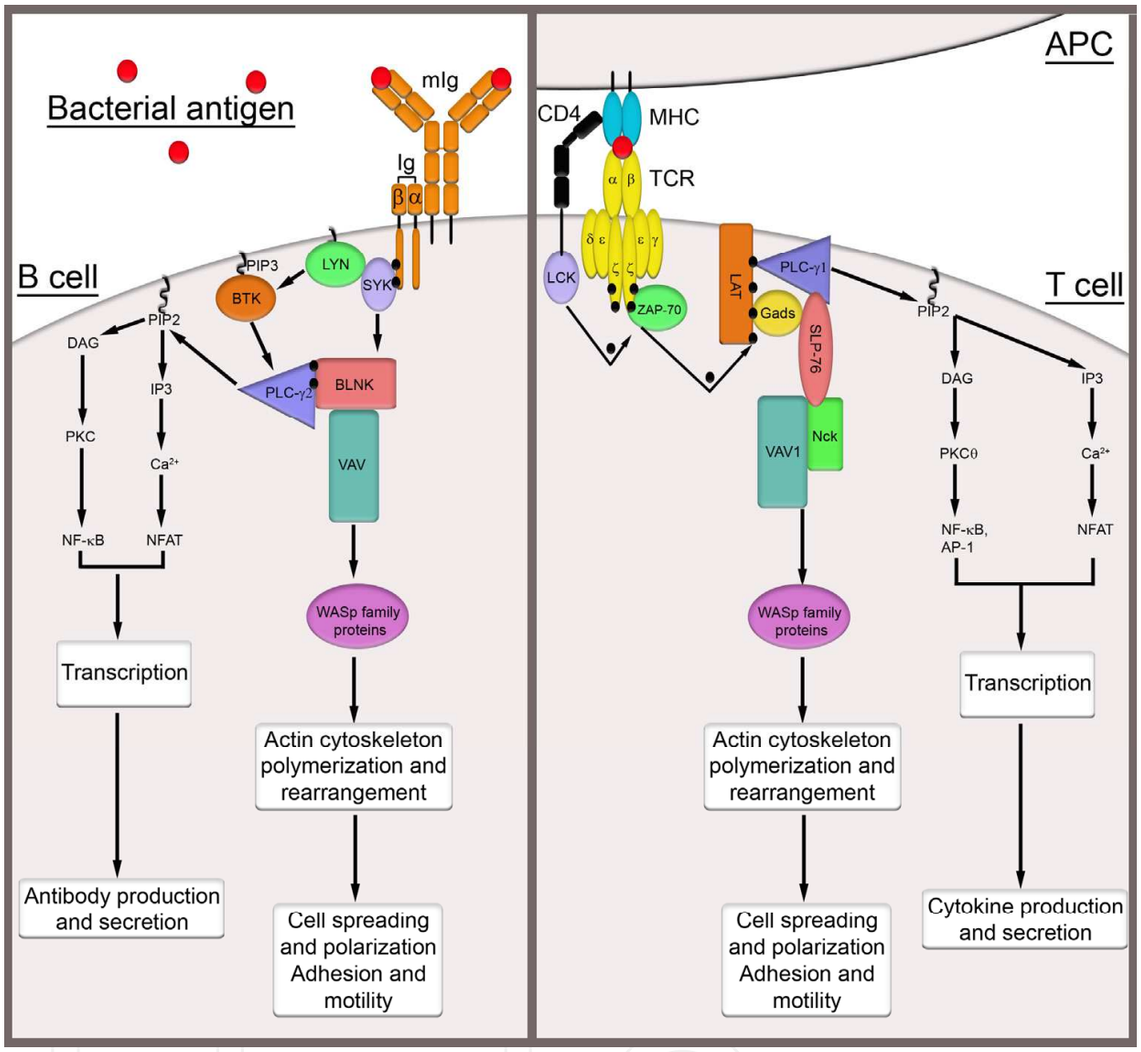

Fig. 1. B and T cell signalling cascades.

The above scheme describes key events downstream of TCR (right panel) or BCR (left panel) activation that are mentioned in the current review. The black spots represent phosphorylation sites.

2. Methods used in researching the stoichiometry of leukocyte regulatory complexes

As protein/protein interactions regulate and control a multitude of cellular functions, various methods for investigating protein-protein binding were developed. Here we review leading methods used for the investigation of inter protein binding and to determine the stoichiometry of these interactions.

2.1 Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance-based sensing makes use of the photonic excitation of electrons into surface plasmons - delocalized electrons that oscillate at the interface between a molecule adsorbed onto a metal film and a dielectric medium (Ritchie, 1957).

The interaction between photons, electrons in the metal film, and the adsorbent allow the investigation of inter-molecular interactions. Binding of molecules, such as proteins or small molecular ligands, to proteins immobilized to the metal film changes the interaction of the projected light photons and the electrons in the metal, causing a shift in the intensity of reflected light, and thus allows the investigation of protein-protein binding, as detailed below (McDonnell, 2001; Rich & Myszka, 2000).

Light is beamed at the metal through a prism. As light passes between two media differing in their refractive indices, some of the light can be reflected at the point of the inter-medium interface. Projection of light at a certain angle, called "critical angle" (itself dependent on the proportion of the two refractive indices), or at larger angles, results in the entirety of the light to be reflected, a phenomenon called total internal reflection. Even though photons are reflected at the interface between the two media, the reflected photons create an electric field reaching about one wavelength beyond the inter-medium boundary (Pattnaik, 2005). This field, called evanescent wave, allows photons reaching the metal surface at a certain angle to excite surface plasmons at the opposite side of the metal sheet (Fig. 2) (Pattnaik, 2005). Exciting photons are absorbed by the electrons of the metal film, leading to a detectable reduction in reflected light (Fig. 2).

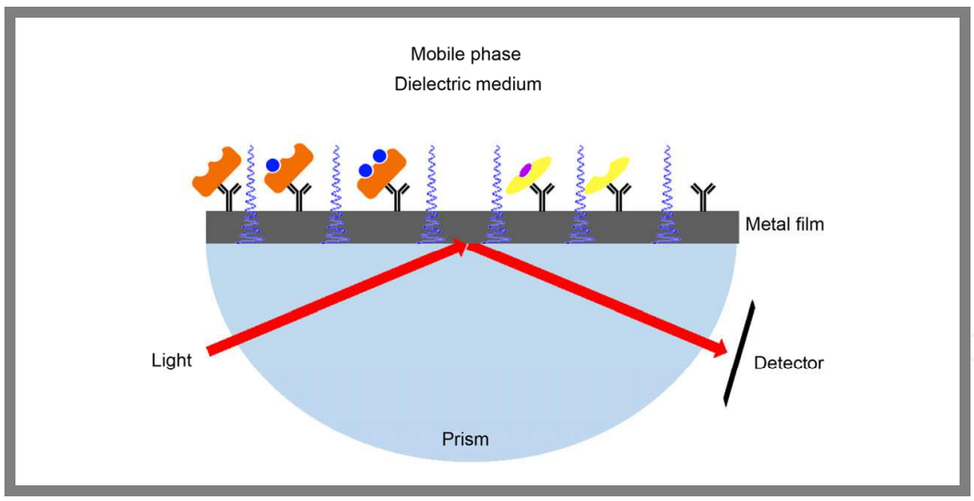

Fig. 2. Illustration of the principles underlying Surface Plasmon Resonance (SPR) technology.

Light reflected at the intermedium boundary can cause surface plasmon resonance on a metal film, reducing the intensity of the reflected light. The induction of surface plasmon is highly dependent on the angle of light incident, as the evanescent waves responsible for SPR propagate into the other side of the metal film, and the angle at which incident light can induce surface plasmon is dependent on the conditions proximate to the metal film. Proteins are adsorbed to the metal film either by direct immobilization or with the use of antibodies.

As proteins adsorbed to the metal film bind ligands (either other proteins or small molecules), the angle of maximal SPR changes, enabling the monitoring of the binding.

The angle at which photons are capable of exciting plasmon is dependent on the refractive indices of the metal and the adjacent. As proteins are adsorbed, the refractive index at the boundary changes, thus affecting the degree at which light can excite surface plasmons. By plotting the intensity of reflected light as a function of incidence angle and finding the angle at which light reflection is minimal, it is possible to find the angle of maximal surface plasmon induction. Comparison of the difference between angles of maximal surface plasmon induction before and after protein adsorption allows the calculation of the difference in refractive index of the medium, from which the mass of the protein adsorbate is easily calculable (due to the high identity between the reflective indices of all proteins) (Pattnaik, 2005).

In the SPR based inter-protein interaction assays, a protein is first immobilized on one side of the metal film, via binding to different bait molecules attached to the metal film such as streptavidin or carboxymethyl groups. During the experiment, changes in the intensity of reflected light, occurring due to the excitation of surface plasmon, are detected with a charge-coupled device (CCD). As ligand proteins in the mobile phase bind to the immobilized proteins, the refractive index at the inter-medium boundary changes, affecting light reflection, from which various aspects of the protein-protein binding can be deciphered. By measuring protein adsorption and dissociation from the immobilized proteins bound to the metal film, the equilibrium constant (Kd), the association and dissociation constants (ka and kd, respectively) and the stoichiometry governing the protein complex formation can be calculated (McDonnell, 2001; Pattnaik, 2005).

Biacore is the oldest and most commonly commercially available SPR system.

2.2 Isothermal Titration Calorimetry (ITC)

Inter-protein interactions results in the formation and termination of non-covalent bonds such as van der Waals interactions, hydrogen bonds and hydrophobic interactions. Isothermal titration calorimetry takes advantage of the thermodynamic outcomes of such reactions to measure molecular interactions. By recording heat changes due to binding enthalpy, isothermal titration calorimetry allows the monitoring of molecular interactions. Measurement of the heat change occurring during the reaction allows for the determination of the thermodynamic variables of the molecular interaction: changes in enthalpy, entropy and free energy (ΔH, ΔS and ΔG, respectively), binding constants (Ka), heat capacity (Δ Cp) and the reaction stoichiometry (n) (Ladbury, 2007).

The ITC apparatus is composed of two identical cells, or chambers, housed in an isothermal jacket. One chamber contains a solution of one of the components participating in the reaction, while the second chamber is filled with buffer (or water) and serves as a control (Fig. 3). The jacket is cooled, thus requiring energy investment to maintain the temperature of the chambers. Temperature detectors (thermopile/thermocouple circuits) allow heaters to keep the temperature of each chamber fixed (Ladbury, 2007; Liang, 2008; Roselin et al., 2009).

At the beginning of the experiment, the two chambers are at the same temperature. The reacting component is injected into the sample chamber, resulting in enthalpic change; in the

case of an exothermic reaction, less heat per time will be needed to maintain the sample chamber at a temperature equal to that of the reference chamber, while an endothermic reaction will require more heat to maintain the equilibrium between the two chambers. As titrant aliquots are injected into the sample chamber, changes in power required to maintain the sample chamber at the same temperature of the reference chamber are recorded (Fig. 3). In addition to heat changes due to the reaction between the two macro-molecules, dilution of the reactant adds its own enthalpy. In order to compensate for dilution heat, the reactants are independently added to the buffer, with heat changes recorded and subtracted from raw ITC data.

Using data from multiple titrations, a titration plot is drawn, with the relation between energy released or used during the reaction plotted against the reactants' molar ratio. From the curve, the reaction enthalpy, binding constant and stoichiometry are calculated in a single experiment, using non-linear regression. Using these variables, the other thermodynamic constants can be calculated with the use of basic thermodynamic relations (Ladbury, 2007).

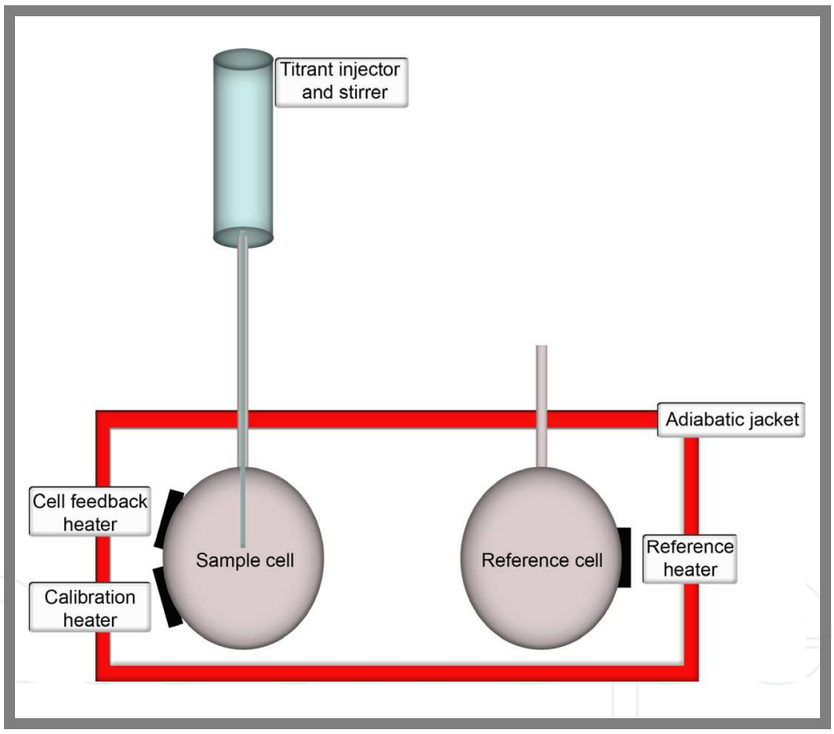

Fig. 3. Illustration of the principles of Isothermal Titration Calorimetry (ITC).

The sample cell is filled with one of the reactants, and the injector is filled with the other. The reference cell is filled either with distilled water or buffer. Continuous power is applied to the reference heater, while the cell feedback heater keeps the temperature of the sample cell equal to that of the reference cell. As titrant is injected into the sample cell, heat is either taken up or evolves. This causes the power required by the cell feedback heater to keep the temperature of the cells equal to either increase or decrease, respectively. These changes are monitored to produce ITC data.

While both ITC and SPR are powerful methods for the real-time in-vitro analysis of protein-protein interactions, ITC is preferable to SPR in the study of stoichiometry ratios of units of protein complexes, as it offers greater resolution and does not require prior immobilization of one of the proteins (Jecklin et al., 2009).

2.3 Analytical Ultracentrifugation (AUC)

Making use of the analysis of the sedimentation of macromolecules under centrifugal forces, analytical ultracentrifugation allows the observation of the shape, size, and stoichiometry of complexes, as well as association constants, molecular mass and intermolecular interactions. Analytical Ultracentrifugation was first developed by Nobel Prize laureate Theodor Svedberg in 1925, but costly instrumentation and arduous data acquisition and processing limited its usage. With the development of computerized data management and new types of detectors, the increased usability and versatility of AUC occurred starting in the 1990s (Cole et al., 2008).

Analytical Ultracentrifugation aims to differentiate and characterize macromolecules based on their behaviour under acceleration. Prior to centrifugation, the distribution of the macromolecules in the solution is dependent on inter molecular forces and on entropy, with the effect of gravity being negligible. During centrifugation, molecules in the solution are redistributed, as centrifugal forces compete with diffusion (Fig. 4).

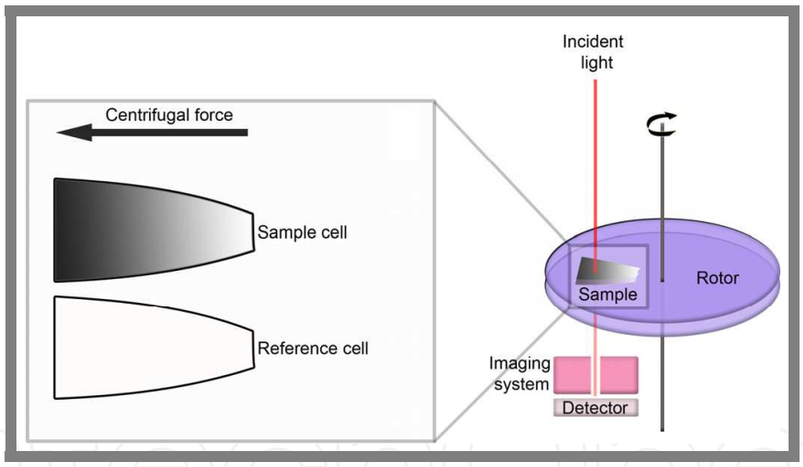

Fig. 4. Illustration of the principle technology of Analytical Ultracentrifugation (AUC).

Under centrifugation, protein complexes are redistributed according to their mass and hydromantic properties. In sedimentation velocity experiments, an optical detector is used to measure the radial concentration of proteins during sedimentation. In sedimentation equilibrium, the sedimentation gradient is monitored not during its formation but at the end of the process, when the centrifugal force is at equilibrium with diffusion. Sample cell readings are compared to those of a reference cell.

Concentration distribution under centrifugation is described by the Lamm equation:

$$\frac{\partial c}{\partial t} = D\left[\frac{\partial^2 c}{\partial r^2} + \frac{1}{r}\frac{\partial c}{\partial r}\right] - s\omega^2\left[r\frac{\partial c}{\partial r} + 2c\right] \quad (1)$$

The solution concentration at time t and position r is represented by c. D is the diffusion coefficient; s represents the sedimentation coefficient; and ω is the angular speed of the rotor.

In sedimentation equilibrium experiments, the equilibrium state of molecule distribution under acceleration is monitored. The sedimentation equilibrium is the state where sedimentation due to centrifugation (simulating gravity) equals diffusion-driven transport. By examining the rate at which the solution reaches the new equilibrium it is possible to observe intermolecular interactions and define the shape, the size, and the stoichiometry of the complexes. On the other hand, in sedimentation equilibrium experiments, the concentration distribution at equilibrium is examined, allowing the determination of molecular mass, association constants and complex stoichiometry. In sedimentation equilibrium experiments, the hydrodynamic properties of the investigated molecules do not influence the results, which are dependent only on thermodynamic properties, simplifying data analysis (Balbo et al., 2005; Schuck, 2010b; Zhao et al., 2011).

However, the advantages of sedimentation velocity measurements, namely, shorter run time (hours instead of days), higher precision, wider versatility and the collection of multiple data points, combined with the robust data processing offered by modern analysis programs, make it the most commonly used AUC technique.

During centrifugation, molecules are radially redistributed. Measurements of radial concentration distribution, also called “scans”, are acquired at various time intervals (minutes in sedimentation velocity experiment, hours in sedimentation equilibrium experiments) (Demeler, 2010). Detection of proteins in analytical ultracentrifugation experiments is performed via optical detectors. Absorbance detection is the most commonly used method, making use of the strong excitation peaks of proteins (and nucleic acids, when relevant) in the UV range. It does require the use of non-absorbing buffer, preventing its use in cases where the sedimentation experiment is performed in the presence of UV absorbing additives, such as nucleotides or certain reducing agents, such as dithiothreitol.

Interference optics uses differences in refractive indices between the centrifuged sample and a reference. In addition to being unaffected by UV absorption of medium components, interference optics offers greater precision than absorbance-based detection, and has a higher dynamic range, a characteristic advantageous for the measurement of highly concentrated solutions. Data density is also higher than that gained from absorbance optics. A disadvantage of interference optics is that all the components dissolved in the medium affect its interference pattern; thus, the reference buffer components must match in content and concentration to the buffer used for the sample. Use of interference optics also requires the use of measurement cell windows that do not affect the refraction pattern. The sample should also be devoid of components with absorbance at the wavelength of the laser used in this system (commonly 675nm). Another option is the use of fluorescence detection; this method allows greater sensitivity and selectivity compared to absorbance optics, but requires the prior labelling of the proteins. This requirement negates a major advantage of AUC, namely, the ability to use untagged proteins (Cole et al., 2008; Demeler, 2010).

Data analysis is performed using dedicated programs such as SEDANAL, SEDFIT/SEDPHAT, BPFIT and ULTRASCAN. In sedimentation velocity experiments, the analytical programs are commonly used to interpret the data by computationally fitting it to

the Lamm equation, an approach called discrete Lamm equation modelling (DLEM) (Brown et al., 2009; Schuck, 2009, 2010a).

2.4 Analytical Native Antibody-based Mobility-Shift (NAMOS) assay

Gel electrophoresis is a common process utilized in multiple protein research methods. Unlike the widely used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and other gel electrophoresis assays preformed under denaturing conditions, blue native polyacrylamide gel electrophoresis (BN-PAGE) allows the separation of proteins according to their sizes in a native, non-denaturing environment (Schagger & von Jagow, 1991; Schamel & Reth, 2000; Swamy et al., 2006). The negatively charged Coomassie blue reagent binds nonspecifically to all proteins, bestowing its negative charge uniformly, thus allowing the proteins to migrate through the polyacrylamide gel during electrophoresis. Unlike electrophoresis performed in the presence of denaturing agents, the BN-PAGE assay allows the conservation of multi-protein complexes (Schagger & von Jagow, 1991; Schamel & Reth, 2000; Swamy et al., 2006). The native antibody-based mobility-shift (NAMOS) assay, developed and used to investigate the stoichiometry of the TCR complex, uses antibodies specific to various components of the TCR protein complex (Swamy et al., 2006). Samples of the complex are incubated in the presence of various antibodies at different concentrations prior to electrophoresis. Antibody binding to the complex increases its overall mass and size, thereby slowing down its passage through the gel. Furthermore, antibody binding to the complex is dependent on the availability of sites recognized by the antibody (epitopes) and the complex/antibody ratio. In the absence of antibodies, all the protein complex molecules migrate in the gel according to the original size of the complex, forming a single band. As antibody binding slows down the movement of the protein complex, bound complexes will form additional bands, corresponding to the increased size of the complex that includes bound antibody. As complexes are treated with increasing concentration of antibodies, the band corresponding to the unbound complex begins to disappear while the band or bands corresponding to bound complexes increase in intensity. If two antibodies are able to bind to the complex, as antibody concentration is increased, the band corresponding to the binding of one antibody begins to disappear as well, with the band corresponding to the protein bound to two antibodies increasing in strength. Additional binding sites will result in bands corresponding to higher molecular weight. It is thus possible to learn from the resulting band pattern the number of complex sites recognizable by the antibodies, and thereby deduce complex stoichiometry (Swamy et al., 2006).

The NAMOS assay allows the investigation of the stoichiometry of complexes in a relatively simple manner, requires only small amounts of protein complexes (femtograms to nanograms), and does not require their prior purification. On the other hand, the NAMOS assay is highly dependent on the ability of the antibodies to reach and bind to all relevant binding sites, as well as on the properties of the antibodies used. Some antibodies form aggregates, creating an unintelligible “ladder” pattern instead of bands, and are thus unusable for this assay. Bivalent antibodies may bind two complex molecules at the same time, creating an additional band interfering with correct stoichiometry analysis. This problem may be mitigated with the use of monovalent antigen binding fragments (Fab fragments) derived from the whole antibodies. Epitopes of the target can also be sterically inaccessible, thereby causing an underestimation of the complex stoichiometry. If the

inaccessibility is caused by the proximity of the epitopes, with one antibody sterically interfering with the binding of an antibody to the adjacent epitope, use of Fabs may also negate this problem, as they are considerably smaller than full sized antibodies (Swamy et al., 2006).

Based on an understanding of the most common analytical techniques, we will now review major studies that contributed to the understanding of the stoichiometry of regulatory protein complexes in immune cells.

3. Current understanding of stoichiometry of the complexes involved in immune cell regulation

With the development and application of new technologies allowing the investigation of protein-protein contacts, studies conducted to understand the key mechanisms of immune cell regulation have shed light on the stoichiometric ratios in fundamental immune regulatory complexes.

The stoichiometry of the BCR was initially investigated by quantifying radioactivity of protein complexes incorporating 35S-labeled methionine. As the number of methionine residues in each of the BCR proteins is known, it was possible to calculate the molar ratios between different subunits of the receptor complex. A 1:1 stoichiometry was found between the immunoglobulin and the Igα/β heterodimer required for signal transduction (Swamy et al., 2006).

This finding was later confirmed in a study utilizing quantitative fluorescence resonance energy transfer (FRET) analysis to characterize the distances between the different chains of the BCR, its conformation during cell rest and during activation, and the clustering of multiple BCRs during antigen binding. In that experiment, FRET between BCR complex proteins genetically tagged with the fluorescent protein, monomeric YFP (mYFP), and Cy3-labeled antibodies specific to either the immunoglobulin or to the Igβ chain, indicated a 1:1 stoichiometry between the immunoglobulin and the Igα/β heterodimer (Tolar et al., 2005).

Knowledge of the stoichiometric ratio between the Ig-α/β heterodimer and the immunoglobulin was later used in the development of the NAMOS assay, validating its use in the investigation of the TCR complex stoichiometry (Swamy et al., 2007).

In the past, stoichiometry of the TCR complex was determined using classical methods, making use of 35S-labeled methionine incorporation into the proteins, followed by the purification of the radiolabeled protein complexes, as described above for the BCR. The subunits were detected by phosphoimaging and quantified by densitometry. The results indicated that a TCRα-TCRβ heterodimer binds a single CD3δ-CD3ε heterodimer, a single CD3γ-CD3ε heterodimer and a CD3ζ-CD3ζ homodimer. Thus, the overall stoichiometry of the TCR complex was found to be 1:1:1:1:2:2 (for αTCR, βTCR, CD3γ, CD3δ, CD3ε, and CD3ζ, respectively) (Rudolph et al., 2006; Wucherpfennig et al., 2010) These findings corroborated the stoichiometry proposed in a previous study (Blumberg et al., 1990), and are supported by other results, as well (Call et al., 2004). However, other works suggested that a second αβ heterodimer was incorporated into the complex, suggesting an $(\alpha\beta)_2\gamma\varepsilon\delta\varepsilon\zeta\zeta$ stoichiometry (Exley et al., 1995; Fernandez-Miguel et al., 1999; San Jose et al., 1998). Finally, an $\alpha\beta\ (\gamma\varepsilon)_2\delta\varepsilon\zeta\zeta$ configuration was also suggested (Rubin et al., 2002).

A later study utilized the NAMOS technique to study the stoichiometry of the TCR-CD3 complex (Swamy et al., 2007). The assay was first validated using the BCR complex, whose stoichiometry (two heavy chains, two light chains and a single Ig-α/β heterodimer) is well characterized (Reth et al., 2000; Schamel, 2001; Schamel & Reth, 2000). Purified BCRs were incubated with increasing concentrations of antibodies specific to various subunits of the complex. The protein complexes incubated with antibodies were then subjected to electrophoresis. Antibody binding to protein complexes slows their movement through the electrophoresis gel. With increasing concentration of antibodies specific to the Ig-α/β heterodimer, the band corresponding to BCR not bound to antibodies began to disappear, and a single band at a higher molecular weight appeared. However, increasing the concentration of antibodies specific to the heavy chain caused two bands to appear along with the disappearance of the original band, with the first band corresponding to the binding of one heavy chain specific antibody appearing at lower antibody concentration, and a second one, corresponding to the binding of two antibodies specific to the BCR heavy chain appearing at higher antibody concentrations, along with the disappearance of the band corresponding to the binding of a singular antibody. Use of antibodies specific to the BCR light chain yielded the same pattern. From this, a 2:2:1 heavy chain, light chain, Ig-α/β heterodimer was suggested, in accordance with previous findings (Reth et al., 2000; Schamel, 2001; Schamel & Reth, 2000; Tolar et al., 2005).

The NAMOS assay was then used to explore the stoichiometry of the TCR-CD3 complex. Use of antibodies specific for either TCRα or TCRβ each resulted in the appearance of a single band, indicating a single TCRα and TCRβ subunit in the complex. Use of antibodies specific to CD3ζ chain yielded the appearance of two bands, indicating the presence of two CD3ζ chains in the complex (Swamy et al., 2007). It should be noted that even at high concentrations of CD3ζ specific antibodies, the band corresponding to a complex bound to a single antibody did not disappear completely, possibly due to the small size of the CD3ζ subunit, resulting in steric interference with the simultaneous binding of two antibodies at once to the spatially proximate CD3ζ monomers. Next, antibodies specific to CD3ε were used. The band pattern included, aside from the original band of complexes not bound to antibodies, the appearance of three bands, interpreted as the TCR-CD3 complex bound to either one antibody, two antibodies, or the binding of one antibody to two TCR-CD3 complexes. In order to avoid this complicated pattern, monovalent CD3ε binding antibody fragments were used, yielding a band pattern consistent with the presence of two CD3ε in the complex. Complexes incubated with antibodies specific for CD3γ yielded an electrophoresis pattern consistent with the binding of a single antibody per complex. Finally, use of CD3δ specific antibodies did not result in an intelligible band pattern, as these antibodies tend to form aggregates, resulting in a smear. Therefore, it was impossible to directly observe CD3δ stoichiometry in the TCR-CD3 complex from these results. However, given that the presence of two CD3ε and one CD3γ, and that previous studies showed that CD3ε forms heterodimers with CD3γ and with CD3δ (Alarcon et al., 2003) an overall stoichiometry of the TCR-CD3 complex as αβγεδεζζ was suggested (Swamy et al., 2008; 2007).

A later study used electron microscopy to characterize the structure of the TCR-CD3 complex. It revealed that the actual three dimensional structure of the complex is larger than expected given a composition of αβγεδεζζ. Due to this discrepancy, the authors suggested

the possible existence of a second αβ heterodimer in the complex, sterically inaccessible to antibodies, which would explain its lack of detection with the use of the NAMOS assay and in previous studies. This study therefore contradicts the model of αβγεδεζζ stoichiometry, reviving the suggestion of $(\alpha\beta)_2\gamma\varepsilon\delta\varepsilon\zeta\zeta$ complex stoichiometry (Arechaga et al., 2010). Further research is therefore necessary to clarify the issue of the stoichiometry of the TCR-CD3 complex.

At the initiation of the T cell activation, recognition of the peptide bound MHC by the TCR is accompanied by binding of the appropriate co-receptor (CD4/8) to the MHC protein, bringing together the co-receptor bound protein kinase, Lck, with its target ITAMs in the TCR complex, and allowing ZAP-70 to be recruited to the TCR complex. The stoichiometric ratios between the clustered TCR molecules and the Lck-bound co-receptors, also governed by the dynamics of lipid rafts, remain the subject of further studies.

ZAP-70, with its recruitment to the TCR complex, phosphorylates LAT, thereby enabling its recruitment to cellular membrane and allowing the recruitment of additional signalling proteins to the activation site. LAT constitutes a major adaptor protein, forming a complex of multiple proteins taking part in T cell activation.

Grb2 is an adaptor protein that recruits SOS1 to LAT, thereby allowing SOS1 to perform as a guanine exchange factor, activating GTPases of the Ras family (Koretzky, 1997; Samelson, 2002; Zhu et al., 2003). Alternatively, Grb2 molecules bind Cbl, an E3 ubiquitin-protein ligase capable of chemically attaching ubiquitin monomers to proteins involved in the cellular activation process, modifying their functionality and regulating their degradation (Samelson, 2002). Binding of these two proteins is facilitated by the Grb2 SH3 domain, capable of recognizing their proline rich domains. SPR was used for determining the binding affinity of the signalling adaptor molecule, Grb2, to the epidermal growth factor receptor (EGFR) and to SOS1 and the stoichiometry of the complex. Grb2 uses the same binding motifs for interacting with either EGFR or, in T cells, with LAT (Lowenstein et al., 1992; Weber et al., 1998). Therefore, these findings are also relevant for the Grb2-SOS1 binding-mediated regulation of Ras activity during the activation of T cells. Using the BIAcore SPR system, the binding of Grb2 to the epidermal growth factor receptor was determined to be of 1:1 stoichiometry, while the binding stoichiometry of Grb2 to SOS1 was 2:1 (respectively) (Lemmon et al., 1994). A later study utilized SV ultracentrifugation to measure the binding of Grb2 to SOS1 and to LAT. It was found that two Grb2 molecules are able to bind to a single SH3 ligand (either SOS1 or Cbl). ITC was used to examine Grb2 binding with either the N terminal domain or the C terminal domains of SOS1. It was determined that the binding stoichiometry Grb2 to either protein fragment was 1:1, while binding of Grb2 to SOS1 proteins containing both proline rich Grb2 binding domains was found to be 2:1 (Houtman et al., 2006). These results were in disagreement with those of a previous study that described a 1:1 stoichiometry between Grb2 and SOS1 (Chook et al., 1996); the cause of this discrepancy may be due to the usage of gel filtration analysis and of SE ultracentrifugation, which are of relatively lower resolution compared to the SV ultracentrifugation method; the techniques used may be less suitable to differentiate between 2:1 and 1:1 Grb2:SOS1 sedimenting species, along with the use of the full length SOS1 in the older study (Houtman et al., 2006). Stoichiometric analysis of the binding of Grb2-SOS1 complexes to LAT molecules was performed with the use of SV ultracentrifugation. While a 1:2:1 LAT–Grb2–SOS1 complex was observed, detection of

peaks signifying of sedimentation of larger complexes indicates that Grb2-SOS1 complexes can bind additional LAT molecules, facilitating the clustering and oligomerization of LAT. The presence of these rapidly sedimenting species is indeed highly dependent on the concentration of LAT, Grb2 and SOS1, indicating that they are formed by clustering of LAT in the presence of Grb2-SOS1 complexes. This mechanism serves to oligomerize LAT molecules subsequent to TCR activation. LAT clustering serves as a mechanism enhancing T cell activation, and may play a critical role in T cell activation under weakly stimulating conditions, occurring *in-vivo*. Indeed, transfection of Jurkat E6.1 T cell line with truncated SOS1 proteins, containing only the C-terminal proline-rich, Grb2- binding domain, reduced T cell activation in comparison to mock transfected cells and to cells transfected with the SOS1 containing both proline rich Grb2 binding domains. This effect was more pronounced with the use of low concentration of anti-CD3 activating antibody (Houtman et al., 2006). The function of Grb2-SOS complexes in LAT clustering was later verified with the use of ITC technology (Houtman et al., 2007).

To analyze the interaction between SLP-76, a scaffold protein and a key player in T cell activation, and Gads, an adaptor protein responsible for the recruitment of SLP-76 upon cellular activation events downstream to TCR engagement, ITC was used. To this end, an 18mer oligopeptide of SLP-76, and the Gads C-SH3 domain to which it binds were titrated. Calorimetric analysis showed that the stoichiometry of this inter-protein interaction responsible for T cell activation is 1:1 (Seet et al., 2007).

Characterization of the stoichiometric ratios between LAT and SLP-76 and PLCγ was also performed. Using ITC, these proteins were found to exhibit a 1:1 binding ratio (Houtman et al., 2004). The interactions of SLP-76 with its binding partners VAV1 and Nck was studied by our group. Study of these interactions and their stoichiometry was preformed with the use of SV ultracentrifugation and ITC technologies (Barda-Saad et al., 2010)

First, ITC was used to measure the affinity, specificity and stoichiometry of the binding of Nck and VAV to SLP-76. To that end, short (17mers) phosphopeptides bearing the sequences of phosphorylated SLP-76 binding domains were prepared. Our results indicated that VAV1 and Nck both bind at the pY113 and pY128 sites. We then used longer peptides (49mers), containing both pY113 and pY128, both pY113 and pY145, both pY128 and pY145, or pY113, pY128 and pY145. Binding stoichiometry of VAV1 to the pY113-pY128 doubly phosphorylated peptide was surprisingly 1:1, while VAV1 was able to bind to both the pY113 and the pY128 containing peptides. Although Nck binding to the pY113-pY145 or to pY113-pY145 peptides was of a 1:1 stoichiometry, Nck engaged in low affinity binding to pY145, in contrast to VAV1. Surprisingly, binding of either Nck or VAV1 to the pY113-pY128-pY145 triply phosphorylated peptide was of 2:1 stoichiometry.

In order to further study the SLP76-Nck-VAV1 complex, we utilized ultracentrifugation. We performed SV analytical ultracentrifugation, using full sized Nck protein, the SH3–SH2–SH3 domains of VAV1 (which are responsible for its inter-protein interactions), and SLP-76 peptides containing different combinations of binding sites. The VAV1 truncated protein was tagged with a site specific label (VAV-FAM), and SLP-76 peptides were labelled with rhodamine TAMRA. We examined the formation of complexes of different SLP-76 derived peptides, Nck and truncated VAV1, by monitoring the effects of different combinations of SLP-76 derived peptides, in the presence of either Nck, VAV1, or both, on the sedimentation

velocity. The AUC results corroborated the results of the ITC experiments, suggesting that the stoichiometry of the complex SLP76-Nck-VAV1 was 1:2:2, respectively (Barda-Saad et al., 2010).

The activity of WASp, an actin nucleation-promoting factor, is required for reorganization of the actin cytoskeleton and therefore is crucial for T cell activity (Jordan et al., 2006; Reicher & Barda-Saad, 2010). WASp is recruited to the site of TCR activation by Nck, and is activated by VAV1. WASp, by activating the Arp2/3 complex, promotes the branching of actin filaments and the development of cytoskeletal networks (Machesky et al., 1999; Rohatgi et al., 1999). The C-terminal domain of WASp, called VCA, binds to the Arp2/3 complex. The VCA domain is divided into three regions, with the C and A regions contributing most to the association energy of the Arp2/3 complex (Marchand et al., 2001), driving a conformational change in Arp2/3 and facilitating its activity (Chereau et al., 2005; Dayel & Mullins, 2004; Rodal et al., 2005); the V region binds an actin monomer, delivering it to the Arp2/3 complex upon Arp2/3-WASp binding (Dayel & Mullins, 2004; Machesky & Insall, 1998; Marchand et al., 2001; Rohatgi et al., 1999). The increased activity of dimerized VCA domains suggests that more than one WASp molecule is able to bind to the Arp2/3 complex (Padrick et al., 2008; Padrick et al., 2011). To study the binding stoichiometry of Arp2/3 and WASp, the WASp VCA domain was tagged with Alexafluor-488 (Padrick et al., 2011). Its binding stoichiometry with Arp2/3 was then investigated using SV AUC. Experiments were performed in the presence of excess VCA molecules. The co-sedimentation pattern of Arp2/3 and the tagged WASp VCA domain indicated a 2:1 VCA:Arp2/3 binding stoichiometry. While Arp2/3 was shown to bind two VCA molecules simultaneously, it is possible that it can accommodate only one VCA molecule bound to an actin monomer at the same time. To address this issue, VCA bound to actin was used. Analysis of SV ultracentrifugation results indicated that two VCA-actin molecules were able to simultaneously bind to the Arp2/3 complex. Mass spectroscopic analysis of these species yielded an apparent mass of 333 kDa, consistent with the molecular mass predicated for one Arp2/3, two VCA and two actin molecules (Padrick et al., 2011). Together with the increased activity of VCA dimers (Padrick et al., 2008), these findings suggest that VCA:Arp2/3 complexes of 2:1 stoichiometry are likely to be the main mechanism for WASp activation of Arp2/3. WASp proteins are bound to scaffolding proteins, limiting their orientation. Since simultaneous binding of two WASp molecules may bias the orientation of the Arp2/3 complex, this new insight suggests that the 2:1 binding stoichiometry may limit the directions in which Arp2/3 may promote actin filament branching. This mechanism may guide the creation of actin networks towards the cellular membrane, facilitating cell spreading required for its immune functions (Padrick et al., 2011).

4. Conclusion

The study of the stoichiometry of the protein complexes governing immune-cell activity constitutes an important element in understanding the processes controlling the immune system. Methods used for the investigation of the stoichiometric ratios between these proteins yield new insights regarding the mechanism and the function of immune signalling complexes. As illustrated, the binding stoichiometry of Grb2 and SOS1 to LAT serves as a mechanism of LAT oligomerization responsible for a lower cellular activation threshold, thus facilitating T-cell activation under physiological conditions (Houtman et al., 2006). The

study of the stoichiometry of WASp binding to the actin nucleating factor Arp2/3 suggests a mechanism that is responsible for the directionality of actin-filament branching, guiding actin polymerization, and thus assisting in the cell spreading necessary for the function of immune cell (Padrick et al., 2011).

While the new, innovative technologies and techniques have greatly assisted in the ongoing effort to decipher the mechanisms controlling the activation of immune cells, especially those regulating T-cell activity, much remains to be discovered in the field of interactions and stoichiometric ratios between immune regulatory proteins.

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Stoichiometry and Research - The Importance of Quantity in Biomedicine

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The aim of this book is to provide an overview of the importance of stoichiometry in the biomedical field. It proposes a collection of selected research articles and reviews which provide up-to-date information related to stoichiometry at various levels. The first section deals with host-guest chemistry, focusing on selected calixarenes, cyclodextrins and crown ethers derivatives. In the second and third sections the book presents some issues concerning stoichiometry of metal complexes and lipids and polymers architecture. The fourth section aims to clarify the role of stoichiometry in the determination of protein interactions, while in the fifth section some selected experimental techniques applied to specific systems are introduced. The last section of the book is an attempt at showing some interesting connections between biomedicine and the environment, introducing the concept of biological stoichiometry. On this basis, the present volume would definitely be an ideal source of scientific information to researchers and scientists involved in biomedicine, biochemistry and other areas involving stoichiometry evaluation.

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