



Membrane Organization and Physical Regulation of Lymphocyte Antigen Receptors: A Biophysicist's Perspective

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Received: 2 May 2019 / Accepted: 18 July 2019 / Published online: 27 July 2019
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Abstract

Receptors at the membrane of immune cells are the central players of innate and adaptative immunity, providing effective defence mechanisms against pathogens or cancer cells. Their function is intimately linked to their position at and within the membrane which provides accessibility, mobility as well as membrane proximal cytoskeleton anchoring, all of these elements playing important roles in the final function and links to cellular actions. Understanding how immune cells integrate the specific signals received at their membrane to take a decision remains an immense challenge and a very active field of fundamental and applied research. Recent progress in imaging and micromanipulation techniques have led to an unprecedented refinement in the description of molecular structures and supramolecular assemblies at the immune cell membrane, and provided a glimpse into their dynamics and regulation by force. Several key elements have been scrutinized such as the roles of relative sizes of molecules, lateral organisation, motion in the membrane of the receptors, but also physical cues such as forces, mediated by cellular substrates of different rigidities or applied by the cell itself, in conjunction with its partner cell. We review here these recent discoveries associated with a description of the biophysical methods used. While a conclusive picture integrating all of these components is still lacking, mainly due to the implication of diverse and different mechanisms and spatio-temporal scales involved, the amount of quantitative data available opens the way for physical modelling and numerical simulations and new avenues for experimental research.

Keywords Immunobiophysics · Lymphocyte · Cell adhesion mechanisms · Receptors · Mechanobiology

Abbreviations

AFM	Atomic force microscopy
APC	Antigen presenting cell
BCR	B cell receptor
BFP	Bio-membrane force probe
CAR	Chimeric antigen receptor
CDR	Complementary determining regions
FcR	Constant fragment receptor
FCS	Fluorescence correlation microscopy
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
ICAM	Inter-cellular adhesion molecule
Ig	Immunoglobulin
ITAM	Immuno-receptor tyrosine-based activation motifs

ITIM	Immuno-receptor tyrosine-based inhibition motifs
LFA-1	Lymphocyte function-associated antigen 1
LFC	Laminar flow chamber
NK	Natural killer
pMHC	peptide Major histocompatibility complex
RICM	Reflection interference contrast microscopy
SIM	Structured illumination microscopy
SMLM	Single-molecule localisation microscopy
TCR	T-cell receptor
TFM	Traction force microscopy
TIRF	Total internal reflection fluorescence

Introduction

Receptors in Immunity

Receptors at the membrane of immune cells are the main specific molecular actors of innate and adaptative immunity, providing the defence of our organism against pathogens or

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cancer cells. Their structure, localisation, interactions and collective organisation in the membrane determine their functional behaviour. In a nutshell, these receptors are in charge of recognising a given class or a specific species of pathogenic antigens in order for a cellular immune response to start, which will include the destruction of the pathogen. Specificity is crucial for avoiding autoimmunity i.e. an immune response against self-antigens. Additionally, sensitivity and speed in the recognition are required because a pathogen should be detected as early as possible among an immense variety of non-agonist molecules (Krogsgaard and Davis 2005). Despite the number of genetic and biochemical data gathered on these aspects, how this is achieved remains to be fully understood, in particular by linking the different scales in space and time. In this context, complementary experimental approaches and concepts should be considered. Thus, integrating biochemical, structural and biophysical cues has become central to decipher immune cell function (Malissen and Bongrand 2015; Hivroz and Saitakis 2016).

Receptors Self-Organisation and Mechanical Regulation

The study of mechano-biological processes has exploded in the recent years, reaching also the field of immunology, thanks to progresses in imaging and micro-manipulations of molecules and cells, allowing mechanical measurements and perturbations of individual cell components and living cells. Indeed, immunological cells and receptors present various original features making them ideal model systems for mechano-biological approaches. Immune cells are fundamentally circulating cells which are not strictly part of a tissue, justifying *per se* *in vitro* studies of individual cells. They are soft and easily deformable due to rapid cytoskeleton reorganisations (Burkhardt et al. 2008). They are often motile with labile adhesion and signalling platform due to rapid reorganisation of the receptors at and below the membrane (Dustin and Groves 2012). How these mechanical and chemical aspects are integrated to achieve immune cell function is still largely unknown. At the molecular level, antigenic receptors have the capability of recognising a huge diversity of molecules due to combinatorial variations in recognising motifs. Understanding how receptors, together with co-receptors and adapter/signalling molecules, self-organise to induce specific recognition and controlled immune response remains a big challenge (He and Bongrand 2012). For example, controlled multivalency as for antibodies is an important feature of immune receptors which still lacks a precise definition and a quantitative understanding. Finally, adaptative immunity is based on the production of new receptor variants by an accelerated evolutionary process; it provides thus a rich system to explore the role of mechanical dimension in molecular evolution.

Outline

In this review, we will focus on how biophysical parameters, in particular forces, are playing a role in the organisation and function of immune receptors in the cellular membrane. A first important aspect to emphasise is that recognition often occurs at the interface between two cells, the immune cell and the antigen-presenting cell, thus imposing important geometrical and physical constraints to the processes. Molecules are confined in crowded, 2D environment and subjected to lateral or tangential forces with respect to the membrane they are embedded in. For example, receptors of B and T lymphocytes recognise their antigen when presented by other cells. Cytotoxic T cells are scanning the surface of encountered cells, looking for information about their state, by palpating the surface and circulating around the target cell. Here, we will detail the limitations of traditional biochemistry-derived techniques in this context and present recently developed biophysical techniques aimed at reproducing better the physiological situation, as well as extracting new important parameters, such as resistance and sensitivity to force or mechanical characteristics of cells, largely ignored until recently.

Structure and Function of Antigen Receptors

Different Types of Receptors (Fig. 1)

An important diversity of immune receptors are present at the surface of the various immune cell types and play a central role in their identification, communication and function. We will focus mainly on the receptors present at the surface of lymphoid cells like B and T lymphocytes, as well as Fc Receptors of natural killer (NK) cells. These cells have the capacity to form immune synapses, a close and highly structured contact with partner cells, the term coined by analogy with the neuronal synapse (Grakoui et al. 1999). B cell receptors (BCRs) are in charge of recognising extracellular antigens and are precursor of antibodies. T cell receptors (TCRs) are in charge of recognising intracellular antigens, in the form of short peptide fragments (~ 10 amino acids) carried by MHC (Major Histocompatibility Complex) present at the surface of nucleated cells (Murphy and Weaver 2016). Two subtypes exist, one is present on nucleated cells (MHCI), the other only on professional antigen-presenting cells (APCs) such as dendritic cells and macrophages (MHCII). Natural killer (NK) cells possess a variety of surface receptors which can be targeted for cancer immunotherapy (Chiossone et al. 2018). Among them, Fc receptors, present also on

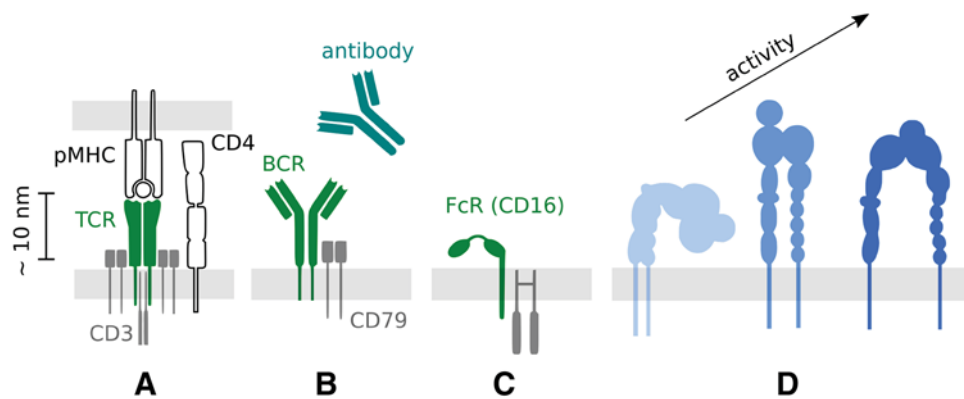


Fig. 1 Different types of receptors on immune cells. **a** Antigen receptor complex specific of T cell (or TCR-CD3 complex) shown with the TCR (in green, extension of its external domain ~10 nm) bound to its natural ligand, the peptide Major Histocompatibility Complex (pMHC), displayed by an antigen-presenting cell. A co-receptor CD4 or CD8 is also shown. **b** Antigen receptor complex specific of B cell (or BCR) and one antibody, the soluble form of the BCR (dimension ~15 nm). **c** Fc Receptor present on the surface of NK cells and

macrophages, binding the Fc domain of antibodies (here example of the low affinity FcγRIII receptor aka CD16). **d** Integrins, the main adhesion receptor of lymphocytes, represented in different conformations, corresponding to different states of activation (Nordenfelt et al. 2016). The membrane(s) is depicted as a shaded rectangle(s). Associated proteins, exhibiting intracellular domains responsible for signal transduction, are shown in grey (Color figure online)

many immune cell types, are in charge of binding the constant fragment of the antibodies; this mechanism permit for innate immune cells to exploit the unique specificity of antigen receptors characteristics of adaptative immune cells via antibodies. While NK cells may be considered as lymphocytes of the innate immunity, their recently discovered ability to retain immunological memory (Adams et al. 2016; Boudreau and Hsu 2018), confer them an additional trait of adaptative immunity (Vivier et al. 2011). Adhesion receptors, some of them specific of immune cells, are part of the large families of integrins, selectins and immunoglobulins. Some other important immune receptors, which bind soluble ligands, will not be considered in this review and include: cytokine and chemokine receptors (some G-protein coupled receptors) and Toll-like receptors at the surface of innate immune cells.

Structure and Generation of Antigen Receptors

Antigen receptors for T and B cells are formed by sequences of immunoglobulin (Ig) domains and possess a distal hyper-variable domain (or Complementary Determining Regions, CDR), capable of recognising virtually any antigen. Each B or T lymphocyte clone displays a single type of receptor exhibiting a particular specificity for a given antigen, according to the clonal theory. The wide diversity in the specificity is obtained by an accelerated molecular evolution process alternating genetic modifications (gene rearrangements or point mutation) and receptor selection against the antigen (Murphy and Weaver 2016). Importantly, the selection occurs in specialised organs (e.g. thymus) and takes place at the interface between two cells.

The B cell receptor displays two identical Fab fragments recognising the antigen, providing higher binding stability. Mature B cells secrete antibodies (Abs), derived from their BCR, which are composed of a variable fragment (Fv) identical to the BCR, and a constant fragment (Fc) (Murphy and Weaver 2016). The Fv is responsible for the antigen recognition, and the Fc determines the functional property of the Ab. Thus, Fc receptors of NK cells or macrophages bind the Fc fragment of antibodies and are formed by two Ig domains. While Fc receptors are not antigen receptor per se, their capability to bind Fc fragment confer them indirectly an antigenic-like specificity. The relative role of the specificity will depend on the relative affinity on the Fc receptor side and on the antigen side. For low affinity Fc receptors, antibody may likely cover the antigenic target first, while the situation may be reverse for higher affinity Fc receptors.

The T cell receptor is a hetero-dimer very similar to a Fab fragment of immunoglobulin and directly anchored in the membrane. It possesses a very short cytoplasmic domain which is not implicated in transferring the signal to the inside of the cell. TCR is found associated with the CD3 complex which provides different cytoplasmic domains in charge of signalling. Associated with antigen receptors are co-receptors CD4 (or CD8) on T-cell surface, recognising directly MHC class II (class I, respectively) (Murphy and Weaver 2016).

Molecular Recognition and Binding Kinetics

The central function of antigen receptors is to recognise their antigen. This recognition occurs by the fitting of the encountering protein surfaces at the atomic scale. It generates a

reversible and non-covalent interaction involving a variety of physical interactions, including electrostatic, hydrogen, Van der Waals and hydrophobic forces (Murphy and Weaver 2016). They determine the specificity and the affinity of the interaction.

For this reason, the measurement of binding properties in solution has long been an important asset for the biophysical characterisation and understanding of antigen receptors. As previously noticed, while relevant for the action of soluble antibodies performing inhibition of pathogens, the affinity in solution only poorly reflects molecules' behaviour at the surface of cells as well as between cells (Malissen and Bongrand 2015). The possible motions, active or passive (undulations) of the cell surface/membrane induce a non-equilibrium situation where kinetic constants become the most relevant ones to evaluate. Thus, rather than equilibrium affinity, on-rate and off-rate were measured to predict receptor recognition properties (Aleksic et al. 2010). At the surface of cells, multivalency effects, for example due to divalent antibodies, change the effective concentration or binding constant. This effect is contained in the concept of avidity (Mammen et al. 1998; Fasting et al. 2012). The same occurs between cellular surfaces for the bridging function of antibodies as well as for the common functioning situation of monovalents BCR and TCR (Huppa et al. 2010; Huang et al. 2010). This may reflect the action of rebinding effects and forces at the interface of cells which are known to modify kinetics rates (Bell 1978; Seifert 2002). As a consequence, the kinetic "constants" should be rather considered as complex functions of distances between membranes, i.e. ligand and receptors, molecular crowding and accessibility for the formation of the bonds, magnitude and duration of applied forces, for their ruptures (Bongrand 1999; Fenz et al. 2017; Huang et al. 2019).

Signalling by Antigen Receptors

Upon antigen binding, immune cells should respond appropriately via intracellular signalling toward gene regulation and cytoskeleton reorganisation. BCR, TCR and FcR have membrane domains, but no identified signalling cytoplasmic domains. However, these receptors are part of protein complexes, with different components harnessing specific functions. Signalling occurs via membrane-bound proteins which are non-covalently associated with protein possessing the extracellular receptor domain the receptor. The former possess an intracellular domain with one or several ITAM domains (immuno-receptor tyrosine-based activation motifs) (Fig. 1).

For the TCR, these are the CD3 complex and zeta chains. For the BCR, the Ig alpha and Ig beta. For the FcR, they are connected to ITAM and ITIM domains (Getahun and Cambier 2015).

The precise mechanisms of phosphorylation upon antigen binding has been extensively studied in T-cells (Van der Merwe and Dushek 2011). Further signalling occurs upon phosphorylation by the recruitment of tyrosine kinases and adaptor proteins, which then target classical signalling pathways such as G-proteins and second messenger such as calcium and phosphoinositides. An important aspect of signalling involves cytoskeleton remodelling, which has been also shown to control BCR diffusion and signalling (Trenor et al. 2010). Co-stimulation through CD28 can also be involved in the TCR case. Inhibitory signal through receptors with phosphatases (CD45 for T-cells) or ITIM domains in the case of NK cells are also playing a role. Integrins (LFA-1 and VLA-4), in addition to their adhesive role, appear to increase the BCR and TCR sensitivity to antigen (Springer and Dustin 2012). Other important co-receptors and signalling proteins, which precise function will not be detailed here, include CD19/CD21/CD81 which amplify the BCR signalling and the inhibitory receptors CTLA-4 and PD-1 that suppress T-cell activation. The subtle balance of activator and inhibitory signal leading to cell activability and subsequent activation are only beginning to be understood (Vivier 2004; Chiossone et al. 2018).

Open Questions

We will, in the next sections, summarise some data and concepts that have been proposed over the last decades to answer the following questions, which are at the core of the most recent research on immune cell membrane receptors structure and function in the frame of the immune response:

- (i.) How are molecules organised at the membrane, before and after recognition?
- (ii.) What are the relevant physical parameters at molecular scale to describe the physiology of recognition?
- (iii.) How is the message transmitted through the membrane and beyond in the cytosol and integrated at cellular scale?

These elements are central for building integrative models which could unify the different time and space scales of observed data into the physiological, cellular responses of the defence system of the body.

Engineer, Measure, Perturb

Different experimental approaches have been applied, adapted and even developed to study immune receptors organisation and force regulation in their cellular, membrane context for the most of them. We have divided the recent

methodological progresses into three categories: (i) simplify or mimic the system using tools of genetic engineering, soft matter, or material sciences (ii) observe molecular organisation within the membranes with higher time and space resolution using new optical microscopy modalities (iii) apply controlled/localised perturbations on molecules and cells using physical and mechanical instruments. These strategies are represented on Fig. 2. Some strategies may overlap partly or be combined synergistically. An overall goal consists in obtaining better quantification and degree of control of these systems, for example by measuring receptor diffusion and velocity, co-localisation, interaction forces, kinetic constants, cellular visco-elastic properties.

Reductionist and Bio-Mimetic Engineering Approaches

Immune cells present the great advantage, for in vitro studies, of acting as isolated cells, even if their state and environment plays a crucial role in their behaviour. Various cell lineages as well as primary cells isolated from blood or various organs can be studied in vitro. Additionally, immune cells generally act by direct one-to-one contact with other

immune cells or infected cells, generically called antigen-presenting cells (APC). Some early progresses have been done in the description of immune receptors in action by replacing the APC by a glass substrate functionalised with ligands, such as antibodies or recombinant proteins, as well as supported-lipid bilayers, serving as surrogate APC (examples include Grakoui et al. 1999 for T-Cell, Natkanski et al. 2013 for B-Cell, Bertolet and Liu 2016 for NK-Cell). This permits a direct visualisation of receptor organisation at the cell–glass interface using fluorescence and high-resolution microscopy (Fig. 2a). This situation has been particularly exploited recently for super-resolution microscopy (see next section). Additionally, this strategy allows a precise control of the nature, average orientation and amount of ligands presented on the artificial surface to decipher the role of chemical stimulations. There has been also a constant effort to control more and more precisely the physical environment of the ligands in order to mimic one or more properties of cellular membranes and cellular material (Fig. 2a); this approach is often guided by the idea of mimicking as closely as possible the observed properties of APCs (Hivroz and Saitakis 2016). Metal barriers have been used to confine mobile ligands bound to a supported bilayer (Mossman et al.

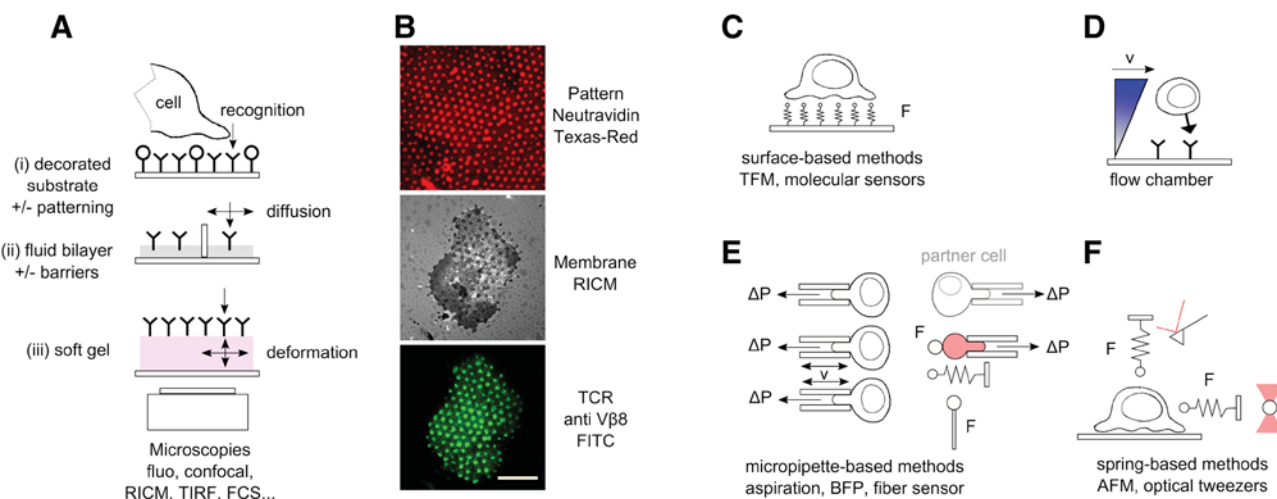


Fig. 2 Various surface and force-based methods to study antigen-receptor organisation and binding. **a** The antigen-presenting cell is replaced by an artificial substrate with well-defined physico-chemical features. The nature, number and position of the molecules presented, as well as their physical environment, can be controlled. This configuration is compatible with various high-resolution optical microscopy modalities, including RICM, TIRF, FCS and SMLM. **b** Examples of microscopy images of substrate with sub-micron pattern of anti-CD3 imaged in epifluorescence (top), lymphocyte membrane imaged in RICM (middle) and fluorescently labelled TCR imaged in TIRF (bottom). Bar = 10 μm . **c** Surface and molecular force sensors. In a situation analogous to (**a**), the “surrogate cell substrate” can be used to measure the forces exerted by the cell either by monitoring substrate elastic deformation via embedded beads or using force-sensitive FRET-based molecular probes. **d** Laminar flow chamber.

An hydrodynamic flow is convecting cells or microspheres functionalised with receptors above a surface coated with antigens; the kinetics of on/off binding are obtained by recording parallelly the particle arrests duration and detachment by fast video-microscopy (Robert et al. 2011; Limozin et al. 2019). **e** Micropipettes-based methods. A cell is aspirated in a pipette to measure cell membrane or cortical tension. An APC cell can be put into contact and eventually pulled away (Sung et al. 1986). Alternatively, another pipette holding an aspirated red blood cell serving as a spring is used to present a functionalised bead for measuring receptor-ligand interaction (Bio-membrane force probe; Evans et al. 2005; Kinoshita et al. 2010). The second pipette can be replaced by a sensor fibre (Sawicka et al. 2017). **f** Spring-based methods used to apply and measure localised forces. Atomic force microscopy uses a soft cantilever while optical tweezers uses a highly focused infrared light beam trapping a bead

2005) mimicking corralling effects of the APC's cytoskeleton (Comrie et al. 2015); ligands have been presented on islands of different spacing and size varying from micron (Doh and Irvine 2006), to sub-micron (Pi et al. 2013, 2015) and nanometer (Delcassian et al. 2013) scales. This helps to assess the role of different length scales on receptor behaviour and to reproduce various spatial organisations of receptors on the APC (Fig. 2b). The effect of ligand mobility was explored by comparing ligands on fluid lipid bilayers and the same ligands immobilised on glass, maintaining also a precise control and reduction of non-specific interactions (Dillard et al. 2014). The effect underlying substrate stiffness was studied in a wide range using different types of elastomers (Wahl et al. 2019). Patterning and substrate stiffness can also be controlled at the same time (Alameddine et al. 2017). Overall, these approaches have provided a wealth of detailed information on the immune synapse, and recent developments may help in diagnosis (Cretel et al. 2011) and therapeutics design (Mensali et al. 2019) as well as bridging molecular and cell scales (Liu et al. 2016a). However, the cross-talk between effector cells and APCs, as well as the interactions in a 3D environment remain difficult to address with these strategies. Besides, the growing knowledge about various immune cell activation states, as well as tissue specificity, sets some limits on an exclusive *in vitro* approach.

A different and complementary approach consists in modifying selectively or reconstructing parts of the immune cell receptor machinery. Genetic engineering was used to express the immune receptor and certain co-receptors in a non-immune cell vehicle to decipher the minimal ingredients necessary for immune response (James and Vale 2012). Alternatively, a selection of these receptors could be associated with the membrane of Giant Uni-lamellar Vesicles, a common strategy to produce model cells (Limozin et al. 2003; Puech et al. 2004; Fenz and Sengupta 2012; Hui and Vale 2014; Carbone et al. 2017). Finally, the receptor itself can be replaced by engineered variants to modify the degree of recognition between receptor and antigen and assess the functional consequences on immune cell activation. This strategy was applied using complementary DNA strands of known binding kinetics to replace the TCR (Taylor et al. 2017). A similar approach where the T-cell receptor is replaced by an antibody fragment (chimeric antigen receptor or CAR) possesses a high potential for cell immunotherapy in cancer (Roybal et al. 2016), and in growing academic and industrial fields of research.

Increase Spatio-Temporal Resolution of Observables

The tremendous progress of fluorescence imaging, associated with genetically encoded fluorescent proteins, have reached the field of immune receptors in the recent years. The lymphocyte/surrogate APC configuration (Fig. 2a)

permits high-resolution total internal reflection (TIRF) microscopy used to study antigen receptors organisation in fixed samples at the scale of ca. 20 nm by single-molecule localisation microscopy (SMLM) (Lillemeier et al. 2010; Shelby et al. 2013; Baumgart and Schütz 2015; Paeon et al. 2016; Jung et al. 2016). TIRF was also used for dynamic studies of the fluorescently labelled membrane of primary T cells (Brodovitch et al. 2013, 2015). Increased resolution was obtained by structured illumination microscopy (SIM) to decipher dynamic actin structure at the synapse (Murugesan et al. 2016). Light sheet microscopy (LSM) was used to monitor cell dynamics in 3D at 0.1 μm spatial resolution and second time resolution (Chen et al. 2014; Cai et al. 2017). Alternatively, reflection interference contrast microscopy allows to follow membrane conformation of live cells at a surface without labelling (Limozin and Sengupta 2009; Dillard et al. 2014; Benard et al. 2018; González et al. 2019). The increased spatio-temporal resolution describing membrane and cytoskeleton resolution gives important insights on the structural base and environment of immune receptors, together with their impact on cell morphology and motion.

Fluorescence is now widely used to measure spatio-temporal parameters of antigen receptors. Labelling strategies vary from genetically encoded fluorescent probes to variants of fluorescently labelled antibodies. Diffusion of a population of receptors is measured by fluorescence recovery after photobleaching FRAP (Dushek et al. 2008). Diffusion of individual receptors is measured by fluorescence correlation microscopy FCS (Gakamsky et al. 2005; Guia et al. 2011; Chouaki-Benmansour et al. 2018), which measure transit time of a fluorophore in a focal spot or single-particle tracking SPT (Manz et al. 2011). Nanometer scale co-localisation is measured by forster resonance energy transfer FRET between a donor and an acceptor fluorophores. Innovative protein or peptide constructions can use FRET to report various signals. Thus, phosphorylation levels of certain molecules, downstream of TCR, can be measured using specific reporters (Cadra et al. 2015). Similarly, calcium fluxes, that are a common robust marker of the interpretation of a membrane signal for immune cells, can be measured using innovative reporter molecules, either chemical ones or genetically encoded ones (Palmer and Tsien 2006; Salles et al. 2013). Polyethylene glycol, DNA or peptide linkers with known elastic properties can serve as molecular force probes emitting a FRET signal dependant on the molecular extension (Ma et al. 2016). Thus, forces exerted by single receptors can be imaged at the cell-surface interface and quantified in the 10 pN range (Fig. 2c).

Current practical limitations come from the existing trade-off between spatial and temporal resolution, inherent to super-resolution strategies. It is particularly significant for immune cells which exhibit fast and large concomitant

reorganisations in their shape and protein distribution at their membrane.

Mechanical Perturbation

A range of techniques coming from single-molecule physics and material sciences has been now adapted to study individual immune receptors or bonds, as well as their behaviour in the cellular context. Mechanical perturbations are applied through an external force field or a mechanical actuator. Forces are measured from the deformation of a spring constituted by a cantilever, a deformable cell, an optical beam or a flexible molecule. Measurements and stimulations can be performed on isolated purified proteins (acellular context) or directly on cells. We review here the main methods applied to immune receptors and cells. Biological results are discussed in the last chapter.

The laminar flow chamber (LFC; Fig. 2d) uses an hydrodynamic flow to convect cells or antigen functionalised microspheres above a surface coated with purified receptors. The method allows direct measurements of the lifetime of receptor-antigen complexes as well as a precise control of their association (Robert et al. 2011; Limozin et al. 2016). With the use of microspheres, the force range applied on individual bonds is 5–50 pN. Recent application showed that the unique ability of LFC for simultaneous measurements of association and dissociation can be instrumental to assess the kinetic properties of single TCR-pMHC bonds (Limozin et al. 2019).

The bio-membrane force probe (BFP; Fig. 2e) is based on micropipette aspiration of the cell of study and of a red blood cell serving as a ultra-soft spring and carrying an antigen-decorated bead. This was used to bind and stimulate a lymphocyte via its receptors and monitor calcium flow and suggest for the first time that TCR-pMHC exhibit catch bond behaviour (Liu et al. 2014). The force range is from 0.1 to 10 pN in the lateral direction, normal to the bead or cell surface. Micropipettes and micro-needles can also be used to control force and time in the interaction between one lymphocyte and an antigen-covered bead (Sawicka et al. 2017). The method can easily be coupled with fluorescence measurement to assess cell response, e.g. by following the burst of calcium flux (Liu et al. 2014). It was also extensively used for phagocytosis studies, where an antibody-coated bead or a parasite is presented to a neutrophil or a macrophage, taking advantage of the lateral view of the bead-cell contact (Heinrich 2015).

Atomic force microscopy (AFM; Fig. 2f), which uses a micro-cantilever with a nanometric tip to contact or bind a sample, was used to pull on antigen receptors at the cell surface (Puech et al. 2011). The accessible force range is from 10 pN to several nN in the vertical direction. The method can be coupled to fluorescence imaging to read a cellular

response for example via calcium signals (Cazaux et al. 2016; Hu and Butte 2016) and can even be extended to study the interaction between a T cell and an APC (Hoffmann et al. 2011). While useful to perform localized time-controlled stimulations by contact, the technique can also serve for measuring mechanical response of the cell and its evolution upon a stimulation (Franz and Puech 2008).

Optical tweezers (OT; Fig. 2f), which use a strongly focus infrared beam creating an optical trap to capture a microsphere, were used to stimulate the cell surface and applying force in a controlled direction (Kim et al. 2009; Feng et al. 2017). This unique feature was used to demonstrate that the TCR responds differently to forces applied in directions lateral (shearing) or normal (pulling) to the membrane (Feng et al. 2017). The force range is typically 1–50 pN in horizontal direction, and about $3 \times$ less in the vertical direction. The technique was also applied on purified proteins to measure receptor-antigen rupture under force (Das et al. 2015). The force curve obtained upon membrane tether pulling can be analysed to probe membrane tension (Masters et al. 2013) or the receptor link to the cytoskeleton, as already achieved with the BFP (Evans et al. 2005).

From Molecular to Cellular Scales

Antigen Receptor Conformations and Cis Interactions

Receptor diversity encoded in the CDR is responsible for its specificity and affinity for the antigen (Murphy and Weaver 2016; Pecht 2018). Since antigen receptors are deprived of cytoplasmic domains, a long-standing question was to understand how antigen binding is triggering downstream intracellular signal, i.e. passing through the membrane. One hypothesis is that antigen binding may lead to conformational changes in the receptor protein or its direct environment affecting its organisation and reactivity. Nuclear Magnetic Resonance studies have revealed that the cytoplasmic tails of CD3 change conformation upon antigen binding, by detaching from the membrane and providing an access for their phosphorylation sites ITAM (Xu et al. 2008). Numerical simulations have suggested recently that a conformational change of the TCR alpha-transmembrane domain changes its orientation during binding, which leads cytoplasmic tails of the CD3 to reorient for phosphorylation (Brazin et al. 2018). As detailed later, force exerted on the molecule upon binding maybe a driving parameter to trigger a conformational change of the subunits of the TCR (Kim et al. 2009; Feng et al. 2017).

Since TCR cannot be efficiently activated by soluble monomeric pMHCs, it has been proposed early that receptor triggering may occur through multimerisation of the receptors

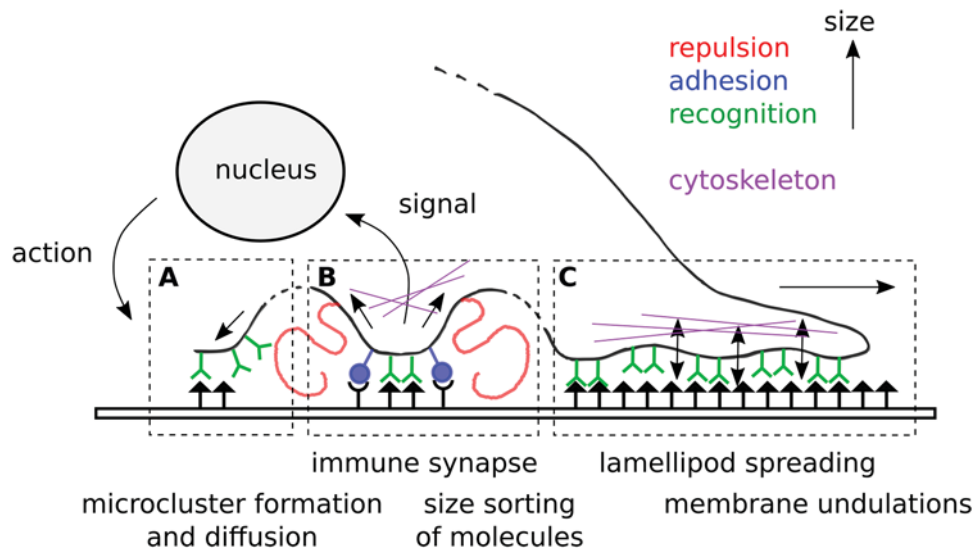


Fig. 3 Schematic overview of receptor organisation and dynamics at the lymphocyte cell membrane at different stages of recognition and/or under various experimental conditions. The lymphocyte interacts with an artificial surface acting as a surrogate APC. Receptors are shown in different subcellular contexts, which can be representative of different steps in lymphocyte physiology. Receptors of increasing size are shown: antigen receptors for molecular recognition (green); adhesion receptors such as integrins for adhesion (blue); proteins of the glycocalyx like CD45 for repulsion (red). Membrane and receptors may interact with the actin cytoskeleton represented in purple.

(Fig. 3a). Antigen binding favours the formation of clusters of TCR, identified early by TIRF microscopy (Varma et al. 2006). It was proposed that these clusters serve as signalling platforms (Dustin and Groves 2012). Progresses in super-resolution imaging have evidenced cluster of smaller and smaller size, down to nanometer sizes, questioning the realm of micron-size clusters initially observed (Pageon et al. 2016; Liu et al. 2016a). Additionally, the resting state of the membrane is still the subject of contradictory studies, reporting either preformed clusters (Crites et al. 2014) or their absence (Rossboth et al. 2018). Vale and colleagues reconstituted in vitro a collection of essential signalling proteins on a lipid bilayers and observed protein phase separation following initial instants of molecular recognition at the membrane surfaces (Su et al. 2016).

By analogy with the TCR, the mechanism of triggering of the BCR was also thought to be based on receptor dimerisation, suggested by the early observation that the BCR is not activated by soluble monovalent ligands. This hypothesis was challenged by a quantitative bi-fluorescence complementation assay, suggesting that the resting state of BCR on the cell membrane was already oligomeric (Yang and Reth 2010), and that BCR activation was dependent on its immunoglobulin class (Übelhart et al. 2015). BCR oligomers were also shown to be open by treatment with different monovalent stimuli (Volkman et al. 2016). As in the case

Receptor triggering induces signalling to the nucleus which feedbacks to the membrane and cytoskeleton. a Micro-cluster formation and diffusion. b Formation of small scale or large scale immunological synapse with size sorting of molecules, potentially serving as a platform for either signalling efficiently, which in turns may affect membrane structure and dynamics, among other, though triggering specific cellular programs in the frame of the TCR, or as a recycling/extracting platform for e.g. antigens in the frame of the BCR. c Lamellipodial extension during cell spreading or migration, which may include membrane undulation and actin retrograde flow (Color figure online)

of the TCR, there is no current consensus on the pre-existence of clusters prior to antigen binding (Liu et al. 2016a). Additionally, how force may influence this organisation upon antigen binding is still unknown.

For Fc Receptors, the classical hypothesis for their triggering relies on cross-linking, like assembly or concentration mediated by the binding of opsonised targets densely covered by Ig (Murphy and Weaver 2016). However, mobile receptors can also trigger FcRI receptors on mast cells (Carroll-Portillo et al. 2010). To our knowledge, no consensus exists on the resting distribution of these receptors.

Membrane Regulation of Antigen Receptor Organisation

After being long under-estimated, due to a dominant view of the involvement of protein receptors, the lipid membrane composition, domain organisation and its mechanical state are now more and more considered as a regulator of antigen receptor organisation and triggering.

T-cell activation is regulated by various lipid membrane components including phospholipids, cholesterol and phosphoinositides (Wu et al. 2016). Lipid nature and organisation in rafts could typically modulate antigen receptors conformation, mobility and interactions (Wu et al. 2016). Early studies with raft markers have suggested the involvement of

cholesterol-rich micro-domains associated with TCR clustering (Gaus et al. 2005). The acidic phospholipids of the membrane help sequestering the CD3 cytoplasmic tails, preventing phosphorylation prior to antigen binding (Xu et al. 2008). The role of phosphoinositide de-phosphorylation in this process was supported by the measurement of augmented TCR mobility by FCS (Chouaki-Benmansour et al. 2018). Lipids and derivatives are also key messengers of cell activation, e.g. PIP2, PIP3, DAG for T-cell activation pathways (Murphy and Weaver 2016).

For FcRI on mast cells, an increase in receptor clustering was observed upon by SMLM calcium stimulation (Shelby et al. 2013). Membrane cholesterol content was found to play a role in this change. More generally, lipid composition induced phase separation in the plasma membrane could be a driving force for receptor reorganisation and triggering, as shown for the BCR again using SMLM (Stone et al. 2017).

Membrane tension is primarily involved in phagocytosis, the process by which certain immune cells engulf their targets, by regulating membrane trafficking and biochemical signalling (Masters et al. 2013). Increased tension modifies small GTP-ase action and repartition as well as cytoskeletal organisation, which in turn regulates receptor signalling in macrophages (Masters et al. 2013). Similar mechanisms are probably also involved in signalling by the TCR and BCR (Rossy et al. 2018). For example membrane tension may affect Ca^{2+} influx (Cazaux et al. 2016). Recently, the professional mechanotransducer membrane channel Piezo1 was found to regulate T-cell activation, supporting the role of membrane tension in TCR triggering (Liu et al. 2018).

Trans Interactions and Force Regulation

Interactions of antigen receptor with their ligand occurs in many instances at the interface between two cells. The proximity and the interactions between the two cell surfaces influences deeply reaction kinetics, which in turn determine antigen recognition. This has lead to use of the concept of 2D reactions (Zarnitsyna and Zhu 2012; Malissen and Bongrand 2015). Compared to reactions in solution (3D), increased off-rate and increased on-rate were measured between TCR and pMHC for T cells interacting with an artificial surface, resulting in an increased 2D apparent affinity (Huppa et al. 2010). The accelerated off-rate was observed in particular for agonist peptides (Huang et al. 2010). Later studies showed that cooperative binding of TCR-pMHC and CD28 co-receptor was achieved globally upon antigen recognition by increased on-rate (Pielak et al. 2017). Furthermore, simultaneous monitoring of T-cell activation and counting of each binding and unbinding events by single-particle tracking reveal long tails distribution in dwell times involved in cellular activation (Lin et al. 2019). This shows that reactions at the interface are modified in a complex

manner at the cell–cell interface, and that a small number of outlier events could be responsible for cell scale response.

In this context, it became crucial to measure individual TCR-pMHC bond kinetics under controlled force. The first study in an acellular context, using LFC on purified proteins, revealed that an applied force was usually increasing off-rate, and that, for a series of peptide of variable T-cell activation potency, a better correlation between off-rate and cytokine production was obtained for an applied force around 40 pN (Robert et al. 2012). Experiments with BFP showed that T-cell activation, as monitored by intracellular calcium fluxes, was correlated with a catch bond behaviour of the TCR-pMHC bond with a peak in lifetime for around 10 pN (Liu et al. 2014). Since, one single group observed a catch bond behaviour in an acellular system (Das et al. 2015), whether the catch bond behaviour observed in cellular context (Liu et al. 2014; Sibener et al. 2018) is solely encoded in the TCR-pMHC bond is questionable. A recent study suggests that the intracellular coupling of CD8 with TCR-pMHC is determinant to observe the catch bond behaviour (Hong et al. 2018). Indeed, new cell-free measurements (i.e. without implication of co-receptors) of association and dissociation showed that no catch-bond behaviour of single TCR-pMHC interactions for a set of five activating peptides could be evidenced (Limozin et al. 2019).

The BCR-antigen bond may also be submitted to force during antigen recognition and endocytosis by the B lymphocyte (Natkanski et al. 2013; Wan et al. 2015; Pierobon and Lennon-Duménil 2017; Spillane and Tolar 2018). Therefore, it is relevant to study the force dependence of this bond to account for the physiological context of the BCR as well as in situation where the antibody bridges cellular structures between antigen and Fc receptor. Early AFM study revealed a good correlation between antibody unbinding force and their thermal dissociation rate (Schwesinger et al. 2000). BCR and classical antibodies are also good models to define more strictly and study quantitatively the concept of avidity of divalent or multivalent receptors (Lo Schiavo et al. 2012). While antigen–antibody bonds would generally exhibit a slip bond behaviour, a catch bond characteristic was recently evidenced for a single domain antibody–Fc Receptor bond using the LFC (González et al. 2019).

Size-Based Lateral Organization of Receptors

When two cellular surfaces form a close contact like in the immunological synapse, the efficient binding between receptors and ligand on each side is highly dependant on the relative size of the molecules which form the bonds. The archetypal immunological synapse is traditionally described as concentric rings of molecules establishing a hierarchical topography between the T cell and the APC (Fig. 3b). It is formed after several minutes of adhesive

contact with a surrogate APC and extends on about 10 μm diameter. The central adhesion patch contains TCR-pMHC bond, surrounded by an adhesive ring of integrins LFA-1-ICAM bonds, and an external ring of CD45 and other glycoproteins (Yokosuka and Saito 2010). It was shown that elongating the ectodomains of pMHC was reducing T-cell activation, suggesting that a close apposition of membranes is necessary for TCR triggering (Choudhuri et al. 2005). A possible mechanism involves the exclusion from the contact zone of the long glycoprotein CD45 by steric repulsion (Fig. 3b), which was recently reproduced in a bio-mimetic system (Carbone et al. 2017). CD45 cytoplasmic tail exhibit phosphatase domains which exclusion may favour kinase action in a kinetic segregation model for TCR triggering (Van der Merwe and Dushek 2011). Recently, it was shown that TCR could be dispensable for T-cell activation in case of sufficient CD45 local exclusion (Chang et al. 2016). However, additional mechanisms involving coupling of long glycoproteins like CD43 to the cytoskeleton via adapter protein moesin, may contribute to alter the topography of the immune synapse (Delon et al. 2001). The supramolecular organisation of the synapse was also proposed to be adapted to the migration phase displayed by lymphocytes by a symmetry breaking leading to a kinapse (Dustin 2008).

Examination of earlier contacts between T cell and a surrogate APC revealed the existence of micro adhesion-rings, reproducing the supramolecular organisation of the synapse at a subcellular scale (Hashimoto-Tane et al. 2016). The dynamic interplay of inter-cellular bond formation and rupture with the topographical constraints and lateral phase separation remains to be understood in the cell context (Schmid et al. 2016). Moreover, the exact role of the complex structure of the mature IS remains to be established. For B cells, similarly organised synapse are the zones where antigen extraction is performed from opposing surfaces, being artificial or cellular (Spillane and Tolar 2018). The relative size of activator and inhibitory receptors was shown to regulate the balance responsible for NK cell activation (Köhler et al. 2010). Using a reconstituted model of antibody opsonised target, Fletcher et al. recently showed that phagocytosis was strongly reduced for antigens larger than 10 nm, and that this was due to the lack of exclusion of CD45 from the cell-target contact zone (Bakalar et al. 2018).

Cytoskeleton-Receptor Interaction and Force Generation

The importance of actin cytoskeleton in T lymphocyte activation was recognised early by use of pharmacological perturbations (Burkhardt et al. 2008). The TCR micro-clusters at the immune synapse display a centripetal motion linked to actin cytoskeleton tread-milling, as observed on surrogate APC with TIRF microscopy (Varma et al. 2006). Substrates

composed of metallic barrier partitioning a functionalised supported bilayer were used to hinder TCR motion by blocking their ligand, suggesting that a frictional coupling between cytoskeleton and receptor was operating (Mossman et al. 2005). Using structured illumination microscopy, TCR micro-clusters were shown to be propelled by actomyosin arcs within the immunological synapse (Murugesan et al. 2016). Sorting mechanisms leading to the segregation of antigen receptors and adhesive molecules observed in the synapse may also rely partly on corralling or even differential binding to the cytoskeleton (Hartman et al. 2009). The interaction of integrin LFA-1 with the actin cytoskeleton and upon binding to its immobilised ligand ICAM on the dendritic cell helps to modulate integrin activation by a conformational change and adhesion regulation (Comrie et al. 2015). In turn, regulation of the integrin-actin link by adapter proteins may strongly influence the actin retrograde flow, providing a feedback to antigen receptor signalling (Jankowska et al. 2018). The comparative use of substrates exhibiting immobilised or mobile anti-CD3 in combination with cytoskeletal drugs evidenced the instrumental role of actin-receptor friction in T-cell spreading (Dillard et al. 2014). The same driving force produced by the actin retrograde flow was also proposed, in combination with ligand-receptor force-dependent unbinding, to explain the optimal substrate stiffness observed for TCR-mediated spreading (Wahl et al. 2019) (Fig. 3c). The interaction of actin and TCR was also proposed to be responsible for TCR triggering by inducing conformational change upon ligand binding (Feng et al. 2017). More generally, actin dynamics may be a generic mechanism for T cells to adapt the time and length scales of their activation (Colin-York et al. 2019). TCR actin interaction probably occurs non-specifically by corralling effects but the existence of a direct molecular link is still lacking experimental evidence. Additionally, cross-talk between TCR and LFA-1 regulates adhesion as well as potentially the interaction of both receptors with the cytoskeleton (Benard et al. 2018).

BFP studies had first shown force generation by the T lymphocyte upon receptor triggering by an anti-CD3-coated bead, with an initial pushing followed by pulling (Husson et al. 2011). More recently, using AFM, force was shown to require TCR engagement and to precede calcium flux, a common marker of T-cell activation (Hu and Butte 2016). DNA-based tension sensors were used to measure pulling forces exerted by single TCR, in the 10–20 pN range (Liu et al. 2016b). Consistently with BFP studies discussed above (Liu et al. 2014), it suggests a possible cellular mechanism used to enhance antigen discrimination by force application.

Actin is also regulating B cell response to antigens by controlling BCR diffusion and by force generation in the immune synapse (Tolar 2017). Antigen discrimination by internalisation following BCR binding was found to be

actin dependent, and force dependent, as shown by the use of AFM (Natkanski et al. 2013). Using molecular force sensor, the BCR was later shown to exert a direct pulling force on the antigen (Spillane and Tolar 2017). As for T cells, force generation in their key functional events makes the B cell naturally sensitive to substrate stiffness (Zeng et al. 2015).

For Fc receptors, force induced mechanisms are commonly accepted in phagocytosis (Ostrowski et al. 2016). Integrins regulate Fc receptor aggregation by creating an adhesive physical barrier to their diffusion (Freeman et al. 2016). Interestingly, the actin cytoskeleton is also involved in this corralling effect via transmembrane glycoproteins interacting with the extracellular matrix and operating as cytoplasmic pickets (Freeman et al. 2018). F-actin network at the lytic synapse of T and NK cells was shown to be crucial in controlling lytic vesicle release by nanoscale actin dynamism (Carisey et al. 2018). By analogy to the cytotoxic synapse of T cells, force are also expected to occur at the NK cell synapse or during their formation (Matalon et al. 2018), as recently suggested by the use of force-dependent anti-CD16 nanobodies (González et al. 2019).

Membrane Ultra-Structure During Cell Exploration and Adhesion

The close link between receptor organisation and cytoskeletal dynamics evidenced in the immunological synapse is suggesting that this association maybe relevant in various cell membrane ultra-structures at different scales and at different steps of lymphocyte function (Fig. 3). The interplay of membrane dynamics and structure with TCR signalling was reviewed recently (He and Bongrand 2012; Pettmann et al. 2018). Brodovitch et al. have observed the change in filopodial dynamics upon specific binding (Brodovitch et al. 2013). A subsequent study using light sheet imaging, has described how filopodia explore efficiently their environment (Cai et al. 2017). One has to note that the mobilisation of the actin cytoskeleton upon TCR triggering was noticed early (Valitutti 1995). The situation is now seen as more complex, since (i) TCR clusters were shown to promote the nucleation of actin foci by the WASP pathway (Kumari et al. 2015) and (ii) conversely, cell protrusions were shown to organise antigen receptors: microvilli (Jung et al. 2016; Razvag et al. 2018), lamellipods (Varma et al. 2006; Ritter et al. 2015). One intriguing point, not yet clearly addressed, is whether the cytoskeleton only ‘corrals’ the TCR in membrane regions and related molecules (together with potential lipid raft effects) or if a ‘direct’ link of TCR to cytoskeleton exists which may explain e.g. its mechanosensitive properties, in particular solve the controversy about the TCR being able of behaving as a catch bond.

Modelling and Integrating Experimental Data

Progresses in molecular dynamics have permitted to study the structure of antigen receptors in interaction with their ligand, illustrating the effect TCR-pMHC docking geometry on activation (Adams et al. 2011). For the BCR, modelling of the hinge region was used to predict the valency effect of different antibody classes (Übelhart et al. 2015). More recently, it was suggested that activating peptides may rely on catch bonds, but it is still delicate to rely finely measured kinetic properties to observed or calculated structures (Sibener et al. 2018). Thus, recent data obtained with the laminar flow chamber suggesting the need of a minimal encounter time for TCR-pMHC association can not be linked yet to a detailed structural base (Limozin et al. 2019).

Attempts to integrate experimental data at a slightly larger scales, are exemplified by the variations proposed around the kinetic segregation model of TCR triggering (Van der Merwe and Dushek 2011), where receptors size and lateral organization are coupled at the immune synapse. In this context, Monte-Carlo simulations may help decipher quantitatively the respective roles of lateral crowding, binding protein affinity, and thermally driven membrane height fluctuations in membrane proteins segregation (Schmid et al. 2016), where cytoskeleton and activity are missing.

More recently, a clutch model integrating cytoskeleton generated forces in bond kinetics was proposed to explain the non-monotonous spreading response of T-cells on substrates of increasing rigidity (Wahl et al. 2019). At the cellular scale, phagocytosis of antibody-opsonised particles was modelled with finite elements simulations, emphasising the role of cytoskeleton-receptor bonds in driving the phagocytic cup progression (Herant et al. 2011).

Outlook

We have seen that, when considering some of the most important receptors that are embedded in the plasma membrane of immune cells, different scales in space and time are involved. From molecular recognition to the organisation of clusters of proteins which may serve as platforms for signalling at cellular scale, from very brief molecular contacts (msec-sec) to the integration of the read signal (up to several hours for the immune response to take place), the idea has been to dissect how recognition of a danger signal modifies cell’s program. Biochemical and biophysical parameters, such as chemistry and structure of the recognition pockets, geometry of the partners, mobility of the molecules in the plane of the membrane, but also the forces that may be acting on them due to cellular or membrane-cytoskeleton motion, have been identified and quantified. All in all, a large corpus of data exists,

and some discrepancies are still remaining. We identified two crucial points that are sometimes under-estimated, and where the membrane is a key player.

First, recording changes in membrane structure or protein organisation when the cell is activated by a given signal requires a very good knowledge of the “starting state” of the cell. Lipid rafts and protein clusters of smaller and smaller sizes are proposed to pre-exist, a baseline of phosphorylation state has also been identified for the underlying signalling hubs, but no clear view of what the membrane looks like in its resting state exists, providing this resting state may be well defined. This is the most difficult experiment ever: how to observe something that is made to change upon the most subtle and gentle perturbation? More and more evidence points toward the fact that we know very little about that, and the most used control situation may still bias the membrane structure (Ponjavic et al. 2018; Santos et al. 2018).

Second, the large jumps in scale that are at play in the process leading from protein interaction and membrane apposition between an immune cell and its partner to the development of a signature of an efficient recognition (such as phosphorylation, calcium fluxes, cytokine production, cell cycle modification,...) render very difficult the establishment of causal links between diverse phenomena. At each step, non-linear effects may be at work, in order to be more robust to noise, false recognition and false amplification. Moreover, trying to block some of the signalling pathways, or even modulating the organisation of proteins at the membrane may only reveal that other mechanisms may be compensating for the deleted, more direct way. Redundancy is a key concept in this complex world of membrane receptors in immune cells.

A trivial, but argued conclusion could be that a lot of experimental, but also conceptual work has still to be done to be able to propose an integrated view of at least the first min of protein recognition at cellular membrane in the context of the immune response. As briefly discussed above, in silico approaches, either at molecular scale or cellular scale, could help identifying new avenues for this field, based on existing multi-parametric data.

Acknowledgements We thank Pierre Dillard and Fuwei Pi for the microscopy images of Fig. 2b, Pierre Bongrand and Kheya Sengupta for scientific exchanges and critical reading of the manuscript, as well as all present and past members of Laboratoire Adhesion and Inflammation for numerous discussions.

Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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