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For the three of you,

in honour of truth, hope and love

TABLE OF CONTENTS

Abstract

Discrimination between foreign and potentially harmful antigens, and the body's own tissue is one of the most crucial first steps that lays at the basis of a proper immune response. Immunoreceptors are cell membrane embedded molecules that aid immune cells in identifying and interacting with its environment. Because of their key importance, they are therefore a frequent subject of research in immunology. It is becoming increasingly clear that the organization of immunoreceptors in space and time on the plasma membrane directly impacts on the way they function. Over the past two decades, novel microscopy techniques and biophysical tools have been developed to directly visualize molecular events in immune cells with unprecedented spatial and temporal resolution. These technical advances have led to the emergence of a new and active field of research: Nano-immunology. Biophysical tools and super-resolution imaging have been exploited in this thesis to unravel the spatiotemporal behaviour of different immunoreceptors, with a particular emphasis on the tyrosine kinase immunoreceptor MerTK. These studies have contributed to further our understanding of immune cell biology at the molecular level.

In **Part I** of this thesis, I will discuss several advanced imaging techniques and microfabrication approaches that I used throughout my doctoral studies. For each technique, the fundamental principles as well as the quantitative analysis associated to them will be explained. Their specific advantages in the field of nano-immunology will be highlighted. In addition, an example of how each technique has been exploited to answer a specific biological question in the field will be given. All of the examples presented in part I correspond to publications that I co-authored during my PhD research.

In **Part II**, I will address the subcellular organization of the immunoreceptor MerTK in human dendritic cells (DCs). By exploiting STED nanoscopy, we discovered that MerTK organizes in nanoclusters on the plasma membrane of tolerogenic DCs, where MerTK is highly expressed. Moreover, we will show that this membrane receptor is also found at very high levels in the nucleus of DCs. To place this in the context of immunity, we established a direct correlation between DC differentiation and the amount of nuclear MerTK. We enquired the route by which MerTK translocates to the nucleus, and dissected some of the main molecular factors involved in promoting this trafficking. In a first attempt to identify its nuclear function, we additionally mapped the spatial relationship between MerTK and chromatin with nanometre accuracy using super-resolution STORM imaging, in single intact DCs nuclei at different stages of their differentiation. We will finally place our findings in a broader perspective and suggest future lines of investigation that may further unravel the molecular mechanism of action of MerTK in particular, and the functional role of membrane receptors in the nucleus in general.

General introduction

Discriminating non-self, threatening antigens from the body's own, self-material is a vital first step in an adequate immune reaction[1]. This process normally occurs by means of frequent interactions between immune cells and other cells in our body, but also by interactions of immune cells with their surrounding environment, and/or interactions of immune cells with each other. Normally, these interactions start at the level of the cell membrane and involve a myriad of multiple receptors and their respective ligands working in a concerted dynamical fashion together with other molecular cell players. Optical techniques, and in particular fluorescence microscopy, are very important tools in studying these interactions due to a combination of chemical specificity coming from fluorescent labelling, and their non-invasive character compatible with live-cell imaging. Classical wide-field and confocal microscopy has greatly advanced our understanding in life sciences at the micrometre scale allowing for example the visualization of cellular organelles or bacteria. However, information at the nanoscale, including the visualization of individual protein complexes at the cell membrane or the structure of chromatin inside the nucleus, is lacking behind. In the case of fluorescence microscopy within the visible range, the lateral resolution limit is around 250 nm, which is set by the diffraction limit of light[2]. Especially in the crowed environment of the cell membrane, with transmembrane proteins that are typically 1-10 nm in size and are expressed at very high density levels of 1000-2000 proteins/μm^2 [3], the resolution limit hampers full understanding of the molecular landscape of the cell membrane. A similar crowded environment is also found in the nucleus of the cell, and in general, in every single subcellular compartment.

Over the past two decades, novel microscopy techniques and biophysical tools have been developed and exploited to directly visualize molecular events in immune cells with unprecedented spatial and temporal resolution. Important characteristics to investigate in order to further understand the behaviour of membrane receptors at the molecular level are: *a)* their localization at the micrometric level and organization at the nanometric scale, *b)* their dynamic behaviour and, *c)* their positioning relative to and interaction with other proteins. In **Part I** of this thesis, I will focus on two newly emerged imaging techniques that are able to break the diffraction limit of light: Stimulated Emission Depletion (STED) nanoscopy and Stochastic Optical Reconstruction Microscopy (STORM) super-resolution imaging. Both techniques allow for the direct visualization of how biomolecules are organized at the nanoscale with unprecedented spatial resolution.

Although these techniques are extremely powerful, they do not provide yet the required temporal resolution to visualize dynamic processes at the nanoscale. Therefore, to inquire

on the dynamic behaviour of proteins across the cell membrane, I will here describe the use of modern single molecule dynamic approaches, such as Single Particle Tracking (SPT) and Fluorescence Correlation Spectroscopy (FCS). These techniques provide quantitative information on the diffusion of proteins, a characteristic that lays at the basis of their spatial organization and ultimately the way they function. The cell is a very crowed place and biomolecules do not function individually. It is therefore very important to additionally study the interaction between different molecules, which can be done by scaling the techniques mentioned above up to imaging 2 or 3 molecules at the same time. Finally, I will discuss an innovative approach to study the direct interaction between proteins using microfabrication techniques. In this thesis, I will describe the working principle of all these techniques, providing examples of how they have furthered our understanding on different biological questions, with an accent on the immune system. Importantly, I will additionally discuss the advantages and drawbacks of these complementary techniques and their suitability depending on the biological process to be investigated. Finally, I will show an example of how we have exploited each tool, in combination with quantitative image analysis, to answer specific questions within the field of nano-immunology.

Unravelling the subcellular organization of the tyrosine kinase receptor MerTK in the context of human dendritic cells will be the main focus of **Part II** of this doctoral thesis. By combining biochemical tools and novel imaging techniques, we aimed to decipher the spatial distribution of this membrane receptor and its interaction with other key proteins in order to get further insight into its molecular functioning. Transmembrane receptor MerTK forms together with Tyro3 and Axl the small TAM family within the larger group of receptor tyrosine kinases (RTKs). This receptor in involved in a wide variety of cellular functions, including apoptosis, migration, transcription regulation[4–6] and immunotolerance[7–13]. MerTK is mainly expressed on macrophages and dendritic cells[14], where the receptor plays a role in the phagocytosis of apoptotic cells[8] and contributes to the downregulation of proinflammatory cytokine secretion[15]. Loss of function of the protein leads to inflammation and an increased susceptibility for auto-immune disorders[9,12]. Aberrant expression of MerTK has been found in many different tumour cells, where it increases cell survival, invasion and therapy resistance by activating several oncogenic signalling pathways [5,6].

MerTK is a membrane receptor with a single transmembrane domain, and a conserved intracellular kinase domain. The fully mature protein is heavily glycosylated, but partially glycosylated forms of a lower molecular weight are found as well[16]. A soluble form of MerTK also exists, which is the cleaved-off extracellular part of the receptor[17]. The receptor is activated by ligand-induced dimerization and subsequent auto-phosphorylation

of the kinase domain at several well defined residues[18]. This in turn leads to the phosphorylation of different signalling molecules which can stimulate a wide range of intracellular pathways, depending on cell type and environment. Well studied ligands that can activate MerTK at the cell membrane are ProS[19–21] and Gas6[22,23].

The broad involvement of MerTK in cancer as well as auto-immune disorders made this molecule an interesting clinical target. Many reports demonstrated the effectiveness and specificity of MerTK inhibition in tumour suppression[24–28]. In the context of immunity, a recent study in human dendritic cells (DCs) showed that MerTK is highly upregulated upon several days of tolerogenic treatment with Dexamethasone[13]. These so-called tolerogenic DCs suppress both T cell expansion and pro-inflammatory cytokine production by T cells[13] in a MerTK dependent manner. The same study also reported the presence of a large intracellular pool of MerTK in human DCs. However, its precise subcellular location as well as its function remained completely unknown. Several ongoing clinical trials focus on injecting tolerogenic DCs to battle varies auto-immune disorders, exploiting the immune tampering function of MerTK that is highly expressed on the membrane of these cells[29,30] The notion that MerTK related therapy is being explored in so many occasions makes it extremely apparent how important a more extensive understanding of the molecular mechanism of action of the receptor is.

In this thesis, we used biochemical tools as well as advanced optical imaging techniques, including super-resolution microscopy, to investigate the spatial organization of MerTK on immunogenic and tolerogenic human DCs. Our results show that the receptor is organized in small, well-defined nanoclusters on the plasma membrane. Interestingly, we identified that intracellular MerTK is mainly located in the nucleus, and super-resolution studies show that it is associated to euchromatin. We furthermore determined its route of internalization and identified ligand ProS and endocytic receptor LRP-1 as key factors in triggering nuclear translocation. Importantly, our work shows that the degree of nuclear localization strictly relates to DC maturation, uniquely indicating a physiological function for the nuclear translocation of a RTK in the context of immunity.

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