

Single cell biochemistry to visualize antigen presentation and drug resistance

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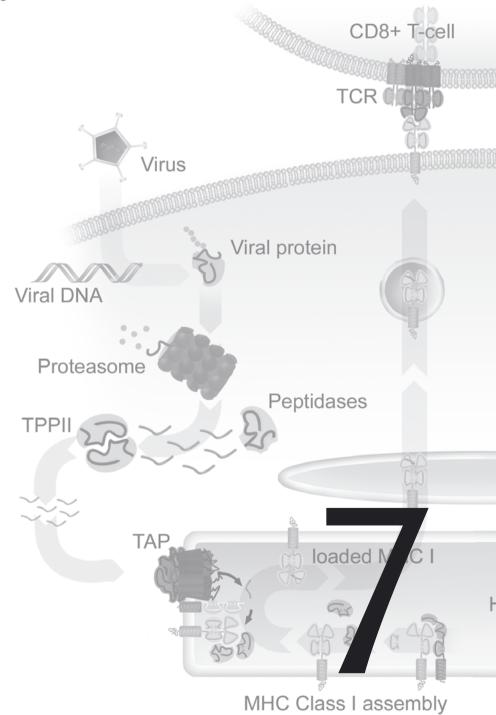
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Summary and Discussion

Antigen Presentation



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Antigen presentation forms the foundation on which the adaptive immune response is build. In humans, three different proteins facilitate the cell surface presentation of antigens to cells of the immune system, each part of a separate antigen presentation pathway containing distinct steps of antigen processing and loading. Whereas the CD1 molecule presents lipids from both endogenous and exogenous origin, the MHC Class I and MHC Class II molecules mainly present peptides (reviewed in Chapter 1). The MHC class II pathway is operational in professional antigen presenting cells only, and endocytosed material forms the source of antigens presented by this pathway. In contrast, the MHC class I pathway is functional in all nucleated cells, and continuously samples the intracellular peptide pool derived from degraded old and incorrectly synthesised new proteins (reviewed in Chapter 2). In the first work described here, we studied this process of peptide sampling that has evolved as a sidestep from protein degradation.

The majority of peptides is generated by the proteasome, a large multi-subunit complex that destroys proteins tagged for degradation. They will ultimately be recycled into single amino acids by the cellular peptidases for reuse in newly synthesised proteins. However, some peptides manage to escape degradation by transport into the lumen of the endoplasmic reticulum by the Transporter associated with Antigen Processing (TAP), and binding to MHC class I molecules. This balance between peptide degradation and presen-

tation is crucial for a proper MHC class I mediated immune response. Surprisingly, it also formed a territory that thus far had been largely unexplored.

In Chapter 3 we have visualised the fate and dynamics of intracellular peptides in living cells. Using fluorescently labeled

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peptides, we show that peptides are distributed over two different but interconnected compartments, the cytoplasm and the nucleus, and that they diffuse rapidly through and between these compartments. The TAP transporter is excluded from the nuclear face of the nuclear envelope, consequently nuclear peptides have to leave the nucleus in order to be sampled for antigen presentation. If they do, these peptides encounter the cytosolically localised peptidases. The nucleus thus forms a save harbour for peptides, especially because we show that peptides can interact with chromatin, preventing diffusion back into the cytosol. The potential function of this nuclear accumulation remains elusive to date. Finally, we have determined the halflife of peptides by microinjection of chemically synthesised peptides containing both a fluorescent group and a quencher moiety.

Antigen Presentation

Cleavage by peptidases will separate quencher from fluorophore, and unquenched fluorescence can be detected. We show that the cytosolic peptidases degrade all injected peptides within seconds, demonstrating a huge cellular capacity to recycle proteins targeted for destruction back to single amino acids. As a result, peptide degradation is far more efficient than translocation, and most peptides will be lost for antigen presentation by MHC class I molecules.

One important conclusion from these findings is that in order to be presented to the immune system, a peptide has to be either relatively resistant to peptidase activity, and thus very stable, or it has to be produced in large quantities. Examples of both situations were indeed found in more recent work from our group. Varying the sequence of injected peptides showed only minor differences in half-life between peptides of different composition (Reits et al., Immunity 20, 2004), although there were some notable exceptions. N-terminal dibasic peptides form poor substrates for cellular peptidases, and hence show a prolonged half-life. Accordingly, these peptides were found over-represented in the pool of peptides presented by MHC class I molecules at the cell surface (Herberts et al., JI 176, 2006). The other scenario in which a peptide is likely to be presented is when the original protein is produced in large numbers. In line with the short half-life we observed, it has been calculated that on average only one out of a hundred to ten thousand peptides makes it to the cell surface (Villanueva et al., Immunity 1, 1994; Yewdell, TiCB 11, 2001). Proteins that are only moderately expressed will therefore not be seen by the immune system, the cell can simply not allow the accumulation of peptides at the risk of aggregation. Only when changes in protein expression raise the number of generated peptides above this threshold, like in the event of a viral infection or oncogenic transformation, enough peptides escape the highly efficient peptide degradation machinery to alert the monitoring CD8+ T cells.

Next we switch to the other major antigen presentation route, the MHC class II pathway (reviewed in Chapter 4). We again studied the process of peptide sampling as a sidestep from protein degradation, but now of proteins taken up by the cell from the extracellular environment, and degraded by proteases in the endocytic pathway. MHC class II molecules including HLA-DR are targeted to a late endocytic structure named MIIC. These compartments have a peculiar architecture, consisting of a limiting membrane and

multiple internal membranes. In the MIIC, HLA-DR interacts with HLA-DM, a specialised chaperone that stabilises HLA-DR during peptide exchange, and is critical for successful peptide loading. In Chapter 5 we followed this process in living cells by generating a cellline containing HLA-DR3/CFP, HLA-DM/YFP and the invariant chain. We visualised HLA-DR/DM interactions in MIICs by Fluorescence Resonance Energy Transfer (FRET). In contrast to findings from *in vitro* experiments, these interactions were not pH sensitive, again stressing the importance to assay biological processes in their normal cellular context.

Interestingly, using high resolution FRET microscopy we could show that HLA-DR/DM interactions occurred only in the internal structures and not at the limiting membrane of the MIIC. The MIIC can thus no longer be considered a homogeneous envi-

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ronment for molecular interactions, but instead contains different subdomains that selectively support the formation and/or sorting of molecular complexes. What makes up this newly discovered MHC class II loading microenvironment is a logical next question. One candidate protein family is of particular interest. The tetraspanins CD63 and CD82 have been found to interact with HLA-DR and HLA-DM and are almost exclusively present on the internal membranes of the MIIC. Moreover, a role for tetraspanins in support of peptide loading has been suggested in previous literature reports. If indeed these proteins play a role in the formation of a subvesicular microdomain is the subject or our current studies.

Proteins alone do not tell the whole story however. The lipid composition of the different subvesicular membranes almost certainly plays a crucial role as well. By manipulating the lipid phosphorylation machinery we could prevent the formation of internal structures, and hence disrupt HLA-DR/DM interaction. Likewise, in a cellular model of infection using Salmonella, phagosomes formed a limiting membrane surrounding internalized bacteria without internal structures. HLA-DR and HLA-DM did not interact

in Salmonella-induced vacuoles, and HLA-DR was not loaded with antigens as long as the bacterium was alive. The absence of HLA-DR and HLA-DM interactions at the limiting membrane prevents local loading of MHC class II molecules in phagosomes, which may allow these bacteria to successfully evade the immune system. Whether Salmonella actively prevents the formation of internal vesicles as part of its large arsenal of host manipulating tricks, or that it is merely a consequence of its size remains to be studied. Likewise, it is still a question to what extent Salmonella can fully suppress MHC class II mediated presentation of its antigens. We observed normal HLA-DR/DM interaction in other vesicles within the same cell, and shed material that ends up in these MIIC is likely to be presented.

In Chapter 6 we encounter another example of the fine balance between an effective immune response and immune escape, again with Salmonella in the spotlight, but this time in B cells. We demonstrate that B cells, which in general show poor phagocytic behaviour, after triggering of their B cell receptor (BCR) can rapidly and efficiently take up large particles, including complete Salmonella bacteria. We show that B cells that recognize and take up Salmonella are indeed present in blood, of naive and memory B cell type. Importantly, although the B cell initiates uptake of the bacterium through binding to its specific BCR, entry into the cell is actively facilitated by the bacterium. Once inside, Salmonella is not able to replicate, but neither can the B cell kill this hitchhiker in dormant state. The B cell is not inert however. It reacts as a true professional Antigen Presenting Cell (APC) and samples antigens from the bacterium, which it subsequently presents to surrounding CD4⁺ T cells. Moreover, the massive cross linking of its BCR and the uptake of Salmonella activate the B cell to such an extent that it starts to

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produce and excrete IL-6, and Salmonella specific antibodies. This autocrine behaviour is further stimulated by feedback from activated autologous T cells, which thus live up to their role as true helper cells. Still, as long as the bacterium stays inside the B cell it evades the immune system. Perhaps this is the reason why the majority of the B cells actively excretes Salmonella back into the environment. Alternatively, excretion of the bug could simply be a consequence of B cell activation, one that comes at a potentially high cost because the expelled bacterium is still alive and capable of infecting other cells. We directly show that B cells form a niche in which Salmonella can temporarily hide and later escape to infect a different type of host cell in which it does replicate, and which allows Salmonella to establish a distant infection.

Although the contribution of B cells in Salmonella infection biology has seen increased interest, historically most studies have primarily looked at the role of macrophages and neutrophils. In the light of our results it is worth to further study the role of B cells, especially when it comes to spreading of Salmonella from the site of primary infection. Salmonella preferentially localises to spleen and lymph nodes, sites that it could very well reach through B cells mediated transfer. That this temporary evasion of the immune system is sufficient to protect the bacterium during the course of infection seems unlikely. The B cell becomes activated and it samples enough antigens to start a humoral immune response. Still, it might give Salmonella a head start that proves hard to counteract.