Discussion

Summary & Discussion
Nederlandse Samenvatting

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Major histocompatibility class II molecules (MHC class II) are one of the key regulators of adaptive immunity because of their specific expression by professional antigen presenting cells (APC). They present peptides derived from endocytosed material to T helper lymphocytes. Consequently, MHC class II is fundamental in orchestrating both cellular and humoral immune responses. A genetic association of certain MHC class II alleles with autoimmunity has long been established. The molecular mechanisms underlying this association are only poorly understood. An in depth understanding of the antigen presentation pathway by MHC class II is essential for the improvement of current therapies. General aspects of MHC class II antigen presentation and ways to manipulate it have been widely discussed in the Introduction Chapter and Chapter 3. Here, the tools to arrive at a systems understanding of MHC class II antigen presentation will be discussed. What are the advantages and disadvantages of a genome-wide screen? And how can a multi-dimensional, data-integrating approach increase the understanding of the systems biology of MHC class II?

In 2006, the Nobel Prize in Medicine was awarded to Andrew Fire and Craig Mello for their work on RNAinterference (RNAi) in C. elegans [1]. RNAi occurs in many eukaryotic organisms and is thought to be an antiviral mechanism triggered by double stranded (ds)RNA, which leads to sequence-specific mRNA degradation. Endogenously encoded micro (mi)RNA molecules target the 3' untranslated region (UTR) of mRNA and are involved in gene regulation. Nowadays, RNAi is applied in mammalian cells as means to silence the expression of genes of interest. As early as 2004, first efforts had been undertaken to perform genome-wide RNAi screens [2-4]. And this was just the beginning. Ever since its discovery, RNAi has been applied many a times to study signal transduction, cancer biology and host-pathogen interactions, to just name a few of the biological processes addressed (reviewed in [5]). The setup of these screens including the analysis methodology, however, varied greatly.

Different species of RNA can be used. Short hairpin RNA (shRNA) is often delivered by viral transduction, which has the advantage of stable integration in the genome and expression of selection markers. Libraries of shRNA are often provided in pools of several thousand different sequences, which makes it a more economic approach. Another very popular format is small interfering RNA (siRNA). These chemically synthesized 19-27 nucleotide long sequences can be easily introduced into cells by lipid-based transfection methods. Libraries of siRNA are often designed in an arrayed format with every well of a 96- or 384-well plate containing siRNAs targeting a single gene only. This naturally leads to a larger format; hence, automated liquid handling and high throughput analysis equipment are required. An siRNA species called enzymatically generated siRNA (esiRNA) can be easily produced by the researcher himself. For an overview of advantages and disadvantages of the various setups see [6].

Depending on the RNA format, the analysis assay might have to be performed in a high throughput manner. Robustness is a necessity. Various normalization procedures have been described [7, 8]. The cutoff levels are arbitrarily chosen and in turn influence the rate of false-positive and -negative candidates. Validation steps need to be applied to obtain a list of more reliable candidates. Again these have to be carefully chosen. Various reviews are available, which highlight tips, tricks and pitfalls for RNAi screening [6, 9, 10]. The take-home message is; clever design is crucial. By choosing proper controls one can get an idea of the 'screening window' (the maximum changes expected for a certain read-out), which will ultimately determine the robustness of the entire screen. A common normalization method is the so-called z-score [11], which is mainly dependent on the standard deviation of the control samples. The primary screen is often followed by one or several follow-up screens aiming at candidate validation. Furthermore, these screens may provide additional information about the candidate genes, which may become useful when plotting interaction networks. While other groups have established screening regimes suitable for the scientific questions they addressed [12-15], we have come up with a unique multi-dimensional approach that integrated various data sets to arrive at a more complete understanding of a crucial cellular process – MHC class II antigen presentation (described in Chapter 1 and 2 of this thesis).

Whenever an RNAi screen is set up one has to be aware of its strengths as well as its limitations. In our approach we ensured robustness using a melanoma cell line, which possesses a complete and functional MHC class II antigen presentation machinery [16]. A cell line facilitates reproducible transfection efficiency over long periods of time. There is virtually no limit in resources as opposed to primary cells (which would show donor to donor variation, thus affecting the statistical power of the assay). Two monoclonal antibodies were used to detect MHC class II on the cell surface in two different states, hence distinguishing target genes

involved in the regulation of overall surface levels and/or peptide loading. We have integrated these primary results with information on the intracellular distribution of MHC class II gathered in a secondary microscopy-based assay. Microscopy in combination with automated image analysis opens new venues to study complex cellular processes, such as intracellular trafficking [17], which are of great importance in MHC class II antigen presentation. It will certainly be further exploited in the near future, as high-resolution (semi)-automated microscopes and the corresponding image software undergo constant improvement.

MHC class II is selectively expressed on a subset of immune cells. Most of the genes discovered in our screen are affecting MHC class II expression at the cell surface when silenced. Therefore, they should contain those candidates that control tissue-specific MHC class II gene expression. The networks controlling these factors are unknown. We used our RNAi screen results to address this issue and unravel general cell biological terms defining tissue-selective expression of MHC class II. Therefore, high-throughput quantitative PCR was performed to investigate effects of gene silencing on the expression of the MHC class II locus. From this screen we were able to deduct a complex feedback regulated network of signaling molecules and transcription factors, which in turn regulate the levels of CIITA – the master regulator of MHC class II transcription. Further bioinformatics investigation revealed general cell biological cues such as signaling and chromatin modifications as modifiers of tissue-selective expression of MHC class II. Using RNAi to unravel transcriptional networks is being acknowledged as a powerful tool to uncover regulatory interactions that modulate transcription factor activity [18].

In general, the introduction of bias of any kind was prevented as much as possible by always investigating the list of candidates as a whole. We have subjected them to multiple assays, which we finally integrated to obtain networks of similarity in phenotype. To ensure that we are not studying artifacts due to the use of a melanoma cell line, we have additionally determined the expression levels of all genes in a panel of human primary immune cells. Only those candidates expressed in immune cells were considered for further studies.

Nevertheless, false-positive or -negative candidates can never be entirely excluded, without losing too many 'real hits'. Any screening setup has limitations which need to be taken into account. One major issue is the variability of knockdown efficiency achieved by different siRNA pools. The transfection method has been optimized beforehand targeting a few representative genes, but it will never be optimal for all. Often lethality caused by silencing particular genes (such as PLK1) can be observed and this has to be corrected for. Usually an arbitrary cut-off is chosen, and conditions of too low cell viability are excluded from the analysis. Despite the fact that the melanoma cell line MelJuSo has been shown to be properly presenting peptides bound to MHC class II [16] artifacts due to these cells' origin can never be entirely excluded. Hence, a thorough confirmation of selected candidates in primary APC has to be executed.

Dendritic cells (DC) have first been described in the 1970s [19], a discovery which has recently been awarded the Nobel Prize in Medicine for Ralph Steinman. DC are the main professional APC in our body. Starting in the early 1990s, culture methods have been described to obtain DC from bone marrow precursors or peripheral blood monocytes. Again these findings were pioneered by Ralph Steinman together with Kayo Inaba [20, 21].

We have used our integrated data sets (cell surface as well as intracellular MHC class II localization, expression in immune cells, and effects on MHC class II locus transcription) to select candidates that we think are involved in orchestrating the redistribution of MHC class II from intracellular late endosomal structures to the plasma membrane during DC activation (maturation). Establishing a lentiviral transduction method for delivery of shRNA constructs into monocytes that are then differentiated into immature DC enabled us to study the function of the selected candidates in human primary immune cells (described in Chapter 4 of this thesis). One striking finding was that the small GTPase ARL14 (sometimes referred to as ARF7) seems to be a negative regulator of outward transport of MHC class II. When silenced in immature DC, the majority of MHC class II molecules are redistributed to the plasma membrane leaving late endosome void of MHC class II. While those ARL14-silenced cells remain immature in terms of other activation markers they strikingly resemble a mature DC in regards to their MHC class II localization (see Chapter 1 and 2 of this thesis). This is a unique observation. By silencing one gene we have uncoupled two processes that were hitherto coupled: export of MHC class II and maturation of DC.

We then set out to unravel the mechanism underlying the MHC class II transport regulation by ARL14. Through a series of proteomic assays we have established various interaction partners, one of which is a so far unknown protein, now named ARF7 effector protein (ARF7EP). We propose that ARL14 is recruited to late endosomes by its guanine exchange factor PSD4, which binds to specific lipids on late

endosomes through its pleckstrin homology domain. ARL14 is linked to the actin cytoskeleton via ARF7EP and the motor myosin 1E. This complex seems to orchestrate the positioning of MHC class II-positive late endosomal vesicles along the cytoskeleton (see Chapter 1 and 2 of this thesis).
Other members of the ARF-like family of GTPases have recently been found to be implicated in lysosomal trafficking [22]. Lysosome-associated ARL8B was shown to regulate endosome to lysosome traffic of various cargos. It directly recruits, VPS41, a subunit of the HOPS (*ho*motypic fusion and *pro*tein sorting) complex. The HOPS complex has been well described in yeast, where it is involved in trafficking to the vacuole, an organelle similar to the lysosome [23]. The described function of ARL8B does not seem to be mediated by any of the known effectors of late endosome (LE) to lysosome trafficking. These are the small GTPase RAB7, RILP (RAB7 interacting lysosomal protein) [24], the cholesterol sensor ORP1L and the ER-resident protein VAP [25]. Lately, this machinery has been linked to the HOPS complex too. RILP seems to act as a bridge between two LE decorated with the RAB7/RILP/HOPS complex [Rik van der Kant, personal communication]. While the GTPase ARL14 is involved in positioning LE along the actin cytoskeleton, the GTPases ARL8B, RAB7 and possibly others seem to control late endosome and lysosome vesicle tethering and fusion.

RNAi screening will become a more and more affordable and feasible tool for the understanding of molecular processes in immune cells as the development of high-throughput techniques and automated analysis software progresses [26]. Studying the MHC class II pathway on a genome-wide scale opens up many opportunities of manipulation (see Chapter 3 in this thesis). Understanding antigen presentation in more detail will ultimately lead to new strategies of inhibiting unwanted immune responses (e.g. in autoimmune diseases) as well as boosting immunity (e.g. in cancer or vaccine development).
Yet, defining novel factors using the new technologies described in this thesis (Chapters 1, 2 and 4) should be considered as a basis for a deeper understanding of biology, which still requires much and hard work. The findings presented here may be further supported by the large data sets that appear in the public domain. We believe that an integration of wet-lab and computer-based biology will guide future cell biology and immunology to a deep understanding of important biological processes and to new ways to manipulate these in disease. Manipulating MHC class II may be of relevance for auto-immune diseases, transplantation as well as vaccination strategies and I hope that my work may provide a basis for novel insights and improvement.

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Nederlandse samenvatting

Major Histocompatibility Complex klasse II moleculen (MHC klasse II) zijn belangrijke regulatoren van de adaptieve immuun respons. Ze komen specifiek tot expressie in professioneel antigen presenterende cellen (APC) waar ze peptiden, afkomstig van opgenomen materiaal, presenteren aan T helper lymphocyten. MHC klasse II is fundamenteel voor het dirigeren van zowel de cellulaire als de humorale immuun respons.

Sinds lange tijd is bekend dat er een genetisch verband bestaat tussen bepaalde MHC klasse II allelen en bepaalde auto-immuunziekten, maar tot nu toe is er nog geen goede verklaring voor het moleculaire mechanisme achter dit verband. Meer kennis over MHC klasse II antigeen presentatie is essentieel om de huidige behandelmethodes voor auto-immuunziekten te verbeteren.

Wij hebben een unieke methode ontwikkeld waarin verschillende datasets met elkaar zijn geïntegreerd om een compleet beeld te krijgen van dit belangrijke cellulaire proces, MHC klasse II antigeen presentatie (zie hoofdstuk 1 en 2 van dit proefschrift).

Bij het opzetten van een RNAi screen is het belangrijk de voordelen, maar ook de nadelen van de techniek te kennen. In onze aanpak hebben we voor robuustheid gezorgd door gebruik te maken van een melanoma cel lijn, welke een functionele MHC klasse II antigeen presentatie route bezit. Door gebruik te maken van twee monoclonale antilichamen konden we onderscheid maken tussen twee verschillende vormen van MHC klasse II aan de cel oppervlakte. Dit gaf ons de mogelijkheid genen te identificeren die betrokken zijn bij de regulatie van ofwel de totale MHC klasse II levels ofwel de peptide belading van MHC klasse II aan het cel oppervlak. Deze gegevens hebben we geïntegreerd met gegevens uit een tweede screen, in welke we gekeken hebben naar de lokalisatie van intracellulaire MHC klasse II moleculen met behulp van confocale microscopie. Daarnaast hebben we ook op grote schaal door middel van kwantitatieve PCR gekeken naar het effect van gen onderdrukken op de expressie niveaus van de MHC klasse II locus. Met behulp van de data die deze screen opleverde, konden we een complex CIITA regulerend netwerk tekenen, bestaande uit signaalmoleculen en transcriptiefactoren, met vele feedbackloops. CIITA is de belangrijkste regulator van MHC klasse II transcriptie.

Door geen voorselectie te maken, maar telkens de gehele lijst met kandidaat genen te gebruiken voor follow-up experimenten, hebben we uiteindelijk een unbiased dataset verkregen. De kandidaat genen zijn onderworpen aan verschillende testen/screens. Door de verschillende resultaten te integreren hebben we deze kandidaat genen kunnen clusteren naar gelijkende effecten op de MHC klasse II antigeen presentatie route.

Deze geïntegreerde dataset is gebruikt om kandidaat genen te selecteren die naar onze mening betrokken zijn bij het dirigeren van MHC klasse II moleculen van intracellulaire laat endosomale membranen naar het plasma membraan tijdens dentritische cel (DC) activatie (maturatie). Voor een aantal van deze genen hebben we de betrokkenheid bij dit proces kunnen bevestigen. Eén van deze genen, ARL14 hebben we in meer detail bestudeerd. Met behulp van proteomics hebben we interactiepartners van ARL14 geïdentificeerd. Dit ARL14 complex lijkt belangrijk te zijn voor de lokalisatie van MHC klasse II positieve laat endosomale vesicles langs het cytoskelet (zie hoofdstuk 1 en 2 van dit proefschrift). Het beter begrijpen van de systeembiologie van MHC klasse II presentatie is slechts één manier om nieuwe targets te vinden voor de behandeling van bijvoorbeeld auto-immuunziekten. De afgelopen tien jaar zijn verschillende methodes beschreven waarop de presentatie van antigeen door MHC klasse II gemoduleerd kan worden. Deze zijn uitgelicht in hoofdstuk 3 van dit proefschrift.

DCs hebben een grote bijdrage in immuun regulatie en antigeen presentatie. Daarom komt er bij het bestuderen van MHC klasse II moleculen altijd een moment waarop DCs een rol gaan spelen. In hoofdstuk 4 van dit proefschrift worden methoden beschreven die de onderzoeker in staat stellen de MHC klasse II route te bestuderen in dit professionele antigeen presenterende cel type.