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TRANSCRIPTIONAL PROFILING OF ORGAN-SPECIFIC AUTOIMMUNITY IN HUMAN

by

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TURUN YLIOPISTO
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To my family

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ABSTRACT

Our understanding of the pathogenesis of organ-specific autoinflammation has been restricted by limited access to the target organs. Peripheral blood, however, as a preferred transportation route for immune cells, provides a window to assess the entire immune system throughout the body. Transcriptional profiling with RNA stabilizing blood collection tubes reflects *in vivo* expression profiles at the time the blood is drawn, allowing detection of the disease activity in different samples or within the same sample over time.

The main objective of this Ph.D. study was to apply gene-expression microarrays in the characterization of peripheral blood transcriptional profiles in patients with autoimmune diseases. To achieve this goal a custom cDNA microarray targeted for gene-expression profiling of human immune system was designed and produced. Sample collection and preparation was then optimized to allow gene-expression profiling from whole-blood samples. To overcome challenges resulting from minute amounts of sample material, RNA amplification was successfully applied to study pregnancy related immunosuppression in patients with multiple sclerosis (MS). Furthermore, similar sample preparation was applied to characterize longitudinal genome-wide expression profiles in children with type 1 diabetes (T1D) associated autoantibodies and eventually clinical T1D.

Blood transcriptome analyses, using both the ImmunoChip cDNA microarray with targeted probe selection and genome-wide Affymetrix U133 Plus 2.0 oligonucleotide array, enabled monitoring of autoimmune activity. Novel disease related genes and general autoimmune signatures were identified. Notably, down-regulation of the HLA class Ib molecules in peripheral blood was associated with disease activity in both MS and T1D. Taken together, these studies demonstrate the potential of peripheral blood transcriptional profiling in biomedical research and diagnostics. Imbalances in peripheral blood transcriptional activity may reveal dynamic changes that are relevant for the disease but might be completely missed in conventional cross-sectional studies.

Keywords: Autoimmunity, gene-expression, DNA microarray, type 1 diabetes (T1D), multiple sclerosis (MS)

Tuomas Nikula

GEENIEN ILMENTYMINEN IHMISEN KUDOS-SPESIFISISSÄ AUTOIMMUUNISAIRAUKSISSA

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TIIVISTELMÄ

Kohdekudosten hankala saatavuus on rajoittanut kudos-spesifisten autoimmuunisairauksien tutkimusta. Immuunijärjestelmää voidaan kuitenkin tarkastella myös potilaan verestä, joka toimii immuunijärjestelmän solujen tärkeimpänä kuljetusreittinä. Käyttämällä erityisesti RNA-molekyylien säilyttämiseksi tarkoitettuja näytteenottoputkia, voidaan tarkastella geenien ilmentymistä elimistössä näytteenottohetkellä ja siten seurata immuunijärjestelmän aktiivisuutta.

Tämän väitöskirjatyön tavoitteena oli tarkastella DNA-mikrosirujen avulla geenien ilmentymistä potilaiden veressä immuunijärjestelmän aktiivisuuden muuttuessa. Tätä tarkoitusta varten suunniteltiin ja valmistettiin keskeiset immuunijärjestelmän geenit sisältävä cDNA-mikrosiru, jota käytettiin raskauden aikaansaaman immuunivasteen heikkenemisen tarkasteluun MS-potilailla. Tutkimusta varten optimoitiin verinäytteiden keruu- ja RNA-eristysmenetelmät, ja koska verinäytteiden RNA-määrät olivat pieniä, eristetty RNA monistettiin ennen analysointia DNA-mikrosiruilla. Samaa näytteenkäsittelymenetelmää käytettiin myös kerättäessä näytesarjoja lapsista, joilla oli jo havaittu tyypin 1 diabetekseen yhdistettyjä autovasta-aineita. Näytesarjat lapsista, jotka myöhemmin sairastuivat tyypin 1 diabetekseen, analysoitiin kaupallisella koko genomin kattavalla sirulla.

Tutkimuksissa löydettiin aikaisemmin autoimmuunijärjestelmään yhdistettyjen geenien lisäksi uusia löydöksiä sekä itse suunniteltua ja valmistettua ImmunoChip cDNA-mikrosirua että koko genomin kattavaa Affymetrix U133 Plus 2.0 oligonukleotidisirua käytettäessä. Erityisen merkillepantavaa oli luokan 1b HLA geenien hiljeneminen sekä MS-taudin että tyypin 1 diabeteksen aktiivisuuden lisääntyessä. Väitöskirjatyön tutkimukset osoittivat, että immuunijärjestelmän aktiivisuutta voidaan seurata potilaiden verinäytteissä ilmenevien geenien kautta, ja veren soluissa ilmenevien geenien tarkastelua voidaan hyödyntää biolääketieteen tutkimuksessa ja diagnostiikassa. Lisäksi, geenien ilmentymisen seuraaminen saman potilaan peräkkäisissä näytteissä voi paljastaa toiminnallisia muutoksia, jotka perinteisessä poikkileikkaustutkimuksessa saattaisivat jäädä kokonaan huomioimatta.

Avainsanat: Autoimmuniteetti, geenien ilmentyminen, DNA mikrosiru, tyypin 1 diabetes (T1D), multippeli skleroosi (MS)

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ABBREVIATIONS

AICD	Activation induced cell death
CAGE	Cap analysis of gene-expression
CNS	Central nervous system
CTLA	Cytotoxic T-lymphocyte antigen
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent activated cell sorter
FDR	False discovery rate
FISH	Fluorescent in situ hybridization
FOXP3	Transcription factor forkhead box P3
FPR	False positive rate
HLA	Human leukocyte antigen system
IFIH1	Interferon induced with helicase C domain 1
iTreg	Induced T regulatory cell
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
nTreg	Naturally occurring T regulatory cell
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
Probe	Defined nucleotide sequence immobilized onto the microarray surface
PTPN22	Protein tyrosine phosphatase non-receptor type 22
Q-RT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAGE	Serial analysis of gene-expression
T1D	Type 1 diabetes
Target	The sample nucleotide sequence of interest that is hybridized on the microarray probes
TCR	T cell receptor
T_{FH}	Follicular T helper cell
TGF-β	Transforming growth factor beta
Th	T helper cell
Treg	T regulatory cell (either induced (i)Treg or naturally occuring (n)Treg)

LIST OF ORIGINAL PUBLICATIONS

I Nikula, T., West, A., Katajamaa, M., Lönnberg, T., Sara, R., Aittokallio, T., Nevalainen, O. S. and Lahesmaa, R. (2005) A Human ImmunoChip cDNA microarray provides a comprehensive tool to study immune response. *J.Immunol.Methods* 303, 122-134.

II Nikula T, Mykkänen J, Simell O, Lahesmaa R. Genome-wide comparison of two RNA stabilizing reagents for transcriptional profiling of peripheral blood. *Manuscript.*

III Airas L, Nikula T, Huang YH, Lahesmaa R, Wiendl H. (2007) Postpartum-activation of multiple sclerosis is associated with down-regulation of tolerogenic HLA-G. *J.Neuroimmunol.* 187, 205-211.

IV Elo LL*, Mykkänen J*, Nikula T, Järvenpää H, Simell S, Aittokallio T, Hyöty H, Ilonen J, Veijola R, Simell T, Knip M, Simell O, Lahesmaa R. (2010) Early suppression of immune response pathways characterizes children with prediabetes in genome-wide gene-expression profiling. *J.Autoimmun.* 35, 70-76.

**Authors have an equal contribution to this article*

This thesis includes also unpublished data.

1. INTRODUCTION

Autoimmunity is a state where the adaptive immune system attacks the cells and tissues of its own host due to a failure to recognize them. Traditionally autoimmune diseases are classified into two groups: Systemic autoimmune diseases where autoantibodies are targeted to antigens that are not tissue-specific, and organ-specific autoimmune diseases affecting specific organ or tissue. Type 1 diabetes (T1D) and multiple sclerosis (MS) are classical examples of organ-specific autoimmune diseases where inflammation involves a T cell response. Although genetic factors are pivotal, environmental factors are known to modulate autoimmunity prior to clinical disease state, which is often preceded by relatively long symptom-free period. Thus, studies that reveal the mechanisms related to autoimmune activity may lead to new treatments potentially delaying or even preventing clinical autoimmunity.

Transcriptional profiling with DNA microarrays provides a comprehensive tool to assess expression levels of thousands of genes simultaneously. In addition, modern bioinformatics is able to reveal pathways and co-regulated molecular networks among the differentially expressed transcripts. Gene-expression microarrays are established as a fundamental tool in genomic research and experimental medicine. As blood is a preferred transportation route for cells of the immune system, and thus reflects imbalances in the immune system of an individual, peripheral blood is the most practical specimen to profile gene-expression of the human immune system in clinical setting. This is particularly important for organ-specific autoimmune diseases such as T1D and MS, where access to the target organ is limited. However, while transciptional profiling of peripheral blood can monitor the secondary response of the inflammation, detection of on-site triggering events and development of the disease in the target tissue is limited.

Although animal models of different autoimmune diseases have proven very useful, there are inherent differences between the expression of T cells in human and murine models. The study of the transcriptional profiles reflected in the peripheral whole blood of patients with an autoimmue disease, therefore, have a great potential in the identification of immunoregulatory pathways explaining the loss of tolerance in disease prone individuals. The tendency with most published studies is that they have focused on a single aspect of health or treatment, such as treated vs. non-treated or diseased vs. healthy individuals. The main objective of this study was to characterize temporal peripheral blood transcriptional changes related to increased disease activity in organ specific autoimmune diseases. In order to achieve this aim, a custom cDNA microarray, tailored for targeted profiling of human immune system as well as collection and handling of peripheral blood samples for transcriptional profiling, were optimized.

2. REVIEW OF THE LITERATURE

2.1 Transcriptional profiling with microarrays

2.1.1 Transcriptional profiling

By definition, transcriptional profiling is a method to collect and analyze gene-expression levels in different samples (e.g. normal vs. diseased), or within the same sample over time. Microarrays were first introduced for 20 years ago and transcriptional profiling has been one of the key applications since then (Fodor et al. 1991a, Fodor et al. 1993, Schena et al. 1995, Lockhart et al. 1996). As one of the first microarray applications, transcriptional profiling has often been considered as synonymous to microarrays. However, during the last decade several other microarray applications have also emerged. Microarrays are used for genotyping and comparative genomic hybridization as well as to study alternative splicing and exon usage (Hoheisel 2006, Shearer et al. 2007, Thorland et al. 2007, Bemmo et al. 2008, Johnson et al. 2003, Lee and Roy 2004).

Although gene-expression microarrays have established a steady position as a fundamental tool in genomic research and experimental medicine, there are other technologies for transcriptional profiling that differ in sample throughput and the number of transcripts studied (Table 1). Whereas most of the other methods are limited by the number of gene products included in the analysis, or are more laborious and demanding as compared to microarrays, array based technologies provide fast high throughput analysis with a scalable range from targeted arrays to whole genome transcriptional profiling.

Table 1. Different technologies available for transcriptional profiling and/or validation of the resulting gene sets.

	Sample throughput	The scope of targeted transcripts
SAGE	Low	All
2nd generation and single-molecule RNA sequencing	Medium	All
Microarrays	Medium	High
Multiplex PCR	High	Medium
Bead hybridization assays	High	Medium
In situ expression profiling	Low	Low

Taq-based sequencing methods serial analysis of gene-expression (SAGE) and its variation, cap analysis of gene-expression (CAGE), convert mRNA into double-stranded cDNA carrying a biotin label (Velculescu et al. 1995, Shiraki et al. 2003, Kodzius et al. 2006). Then the cDNA is digested and fragments captured using the biotin labels are ligated to restriction site adapters. Cleavage with restriction enzyme produces SAGE/CAGE tags corresponding to each transcript in the original sample, and the tags are then cloned into standard plasmid vectors for sequencing. The

advantage of CAGE, as compared to SAGE, is the capability to identify promoter sequences in parallel with gene-expression monitoring. Like gene-expression microarrays, Taq-based sequencing methods are capable for producing information on large numbers of transcripts in a single sample analysis, but are more laborious and demanding to perform and cannot compete in sample throughput or costs.

Massively parallel signature sequencing (MPSS) was developed to multiply the sequencing throughput of SAGE (Brenner et al. 2000), and the recent development in next generation sequencing (NGS) techniques has introduced novel methods that overcome the disadvantages of Tag-based sequencing (Mortazavi et al. 2008, Cloonan et al. 2008, Wang et al. 2009). As the NGS technologies provided by several commercial manufacturers are capable in more detailed analysis of RNA expression, those are already replacing some of the microarray-based gene expression studies (Metzker 2010). The main benefits of NGS include an ability to detect sequence variants and to identify and quantify transcripts without a prior knowledge (Wang et al. 2009). The continuing progress in sequencing technologies has already made single molecule sequencing possible without a preceding amplification step (Ozsolak et al. 2009, Thompson and Milos 2011). The full potential of these methods has not yet been revealed, but it is reasonable to expect that RNA sequencing based diagnostics will be seen in near future (Ozsolak and Milos 2011).

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique available for mRNA detection. Quantitative real time RT-PCR methods (Gibson et al. 1996, Heid et al. 1996) are widely used to validate microarray results but also utilized for targeted studies focusing on subsets of interesting genes. Typically, these methods first convert mRNA into cDNA with reverse transcription and the cDNA is then used as a template in gene specific PCR. Several manufacturers have instruments for multiplex PCR assays to measure different transcripts in a single PCR reaction, using differently labeled probes.

Bead-assisted techniques, Luminex's xMAP technology (Dunbar 2006) and transcript analysis with the aid of affinity capture TRAC (Rautio et al. 2008), utilize polystyrene or magnetic beads to capture transcripts in liquid phase. In both techniques, probe hybridization to RNA is possible in cell lysates without a need for RNA cleanup or converting RNA into cDNA. Quantification of the hybridized RNA-probe complexes takes place with the Luminex instrument or capillary electrophoresis for TRAC. Both techniques can rapidly detect limited sets of mRNA's of interest.

Recently, development in confocal microscopy, fluorescent dyes and probe design has made multiplexed FISH (fluorescent *in situ* hybridization) an alternative to profile gene-expression of few targets at a time (Kosman et al. 2004). The benefit of FISH is that it gives information on cellular location and temporal changes in expression during the cell cycle or other cellular processes. Although the number of transcripts studied in parallel is still very limited, RNA FISH could provide important knowledge in the validation of array results.

2.1.2 Gene-expression microarrays

2.1.2.1 Array supports

Regardless of the probe chemistry used, microarrays need some type of solid support. Early arrays were manufactured on porous membranes, typically nitrocellulose or charged nylon membranes (Duggan et al. 1999), but the need to increase the probe density on the arrays rapidly lead to use of nonporous supports, typically glass (Cheung et al. 1999, Fodor et al. 1991b). Plastic supports, such as aminated polypropylene can also be used (Matson et al. 1995). Nonporous supports minimize the hybridization volume and enhance hybridization kinetics. Glass has also several other advantages as a supporter: it is durable, the glass surface is easy to activate and it has low self-fluorescence resulting in a relatively low background signal. The size of glass supports varies from the standard 1 x 3 inch microscope slides to miniaturized custom formats. However, especially for *in situ* synthesized oligonucleotides, supports other than planar glass are used (Matson et al. 1995, Guimil et al. 2003, Baum et al. 2003, Walt 2000, Steemers et al. 2000, Ferguson et al. 2000, Ferguson et al. 1996).

To print microarrays, nano- or picoliter quantities of the pre-synthesized probes (~500 – 5000 base long cDNA or synthesized oligonucleotides) are dispensed on the surface of the solid support using an array printer with ink jet (Lemmo et al. 1998, Stimpson et al. 1998) or pin technology (Schena et al. 1995). To increase the physical attachment of the probes, the surface can be pretreated. Coating of the arrays with a positively charged layer, e.g. polylysine, facilitates the physical attachment of the negatively charged probes via electrostatic interactions (Schena et al. 1995). Activation of the array support with reactive groups such as amines or epoxides increases the covalent attachment of amino-modified presynthesized probes. In addition to coating the surface with a single silane compound (e.g. amine-, epoxy- or aldehyde-silanes), binding efficiency can be further increased with a combination of tertiary amine- and epoxy-silanes (Chiu et al. 2003). For amine-derivatized surfaces, an exposure to ultraviolet radiation (254 nm) links the photochemically reactive thymine residues of DNA molecules covalently to amine groups on the surface (Cheung et al. 1999). As an alternative to modifications of the array support, probes can be covalently cross-linked in solution to an active silyl moiety before array printing, which allows rapid and efficient immobilization of these silanized nucleic acids directly on unmodified glass surface (Kumar et al. 2000, Cai et al. 2002).

Instead of applying a printer to dispense probes in desired locations on a solid support, fiber-optic gene arrays can be assembled randomly from pre-synthesized probes that have been covalently attached to silica microspheres (beads) (Ferguson et al. 2000, Ferguson et al. 1996, Steinberg et al. 2004). Beads coupled with array probes are randomly assembled in nano-wells that are constructed on a solid surface (Pantano and Walt 1996), and combinatorial decoding is used to detect random positions for each probe (Epstein et al. 2003).

Oligonucleotide based arrays can also be synthesized *in situ* using physical barriers, surface tension, microdispensing methods, or photochemical reactivity to define the locations on the support to form a microarray. Light-directed synthesis exploiting photosensitive protection groups in combination with photolithographic masks (Fodor et al. 1991a, Pease et al. 1994) or digital light processing with micro mirrors (Guimil et al. 2003, Baum et al. 2003, Singh-Gasson et al. 1999) have been successful in commercial array platforms. Light-directed synthesis can create very dense arrays for oligonucleotides up to 25 bases in length, with yields of >95% demonstrated (Pease et al. 1994). In addition, methods using physical barriers (Maskos and Southern 1992a, Maskos and Southern 1992b, Southern et al. 1992) or surface tension (Butler et al. 2001) can effectively localize chemical reactions in the desired locations on the array support. In addition, microdispension with repetitive cycles of deposition, reaction and washing can synthesize *in situ* oligonucleotide arrays (Matson et al. 1995).

2.1.2.2 Sample RNA

Preparation of intact high-quality total RNA or poly(A)$^+$ mRNA is critical to obtain reproducible results in gene-expression analysis. For a successful assay, RNA must be free of contaminants, especially RNAses, as degradation of the RNA leads to truncated labeling products. Most of the total RNA consists of other types of RNA (e.g. tRNA and rRNA) and only 1-3% is mRNA (Nygaard and Hovig 2006). Thus, oligo(dT) priming specific for poly(A)$^+$ mRNA is typically used for first strand synthesis from total RNA. If mRNA is isolated, random priming can also be employed. However, the array platform chosen for the study may also affect the selection of the reverse transcription primer. If microarray probes are based on 3' region of the mRNA, oligo(dT) priming biased towards the same region will yield more probe specific targets. For long cDNA probes and oligonucleotide probes designed for splice variants or open reading frames, random priming is recommended as it eliminates the interfering 3' bias (Clinical and Laboratory Standards Institute 2006).

Due to the limited amount of starting material in many gene-expression studies, direct incorporation of the labels during the *in vitro* cDNA synthesis is not an option. With amplification of the sample RNA or the signal, the microarray experiment can be performed with less starting material. Several technologies such as indirect aminoallyl amplification, 3DNA dendrimers, tyramide signal amplification, rolling circle amplification reaction and quantum dot or gold nanoparticles have been introduced to increase the signal intensity per transcript (Ladner et al. 2001, Karsten et al. 2002, Stears et al. 2000, Vora et al. 2008). However, whereas none of the signal amplification methods increases the amount of hybridized targets and do not allow repeated experiments or validation studies to be made, amplification of the target mRNA usually yields enough amplified (a)RNA for multiple hybridization or validation experiments. At the same time amplification further purifies the sample, which results very high quality aRNA, and even partially degraded RNA can yield amplification products for reliable measurement of relative expression levels (Schoor et al. 2003).

Although multistep amplification procedures are laborious to perform, and hybridization specificity and relative transcript abundance may be slightly distorted because of amplification (Barker et al. 2005, Nygaard et al. 2003, Puskas et al. 2002), the benefits of the amplification over non-amplified targets are substantial. Not only has amplification of mRNA proven to be both repeatable and reproducible (Nygaard et al. 2003, Wang et al. 2000, Baugh et al. 2001, Wilson et al. 2004), but aRNA is more sensitive than non-amplified mRNA, detecting significantly more transcripts (Nygaard et al. 2003, Puskas et al. 2002). Furthermore, other molecular methods have confirmed that the transcripts that were not detected in non-amplified material are actually expressed in the cells and not amplification artifacts or unspecific binding (Hu et al. 2002). The increased sensitivity arises as the relative amount of the labeled aRNA targets is 3-10 higher than the corresponding mRNA among the total RNA targets, and it appears that low copy number transcripts have greatest amplifying effect (Nygaard and Hovig 2006).

2.1.2.3 Experimental designs

As only one sample is hybridized on each single-color microarray there is little need to consider the experimental design. A variety of different designs are available for two-color microarrays to reduce variation caused by dye or labeling bias, and to maximize the power of the experiment with minimum number of hybridization (Kerr and Churchill 2001, Knapen et al. 2009). The objective of the experiment defines possible hybridization designs for two-color microarrays. In general, different options fall into three main categories: direct comparison, reference design and loop design. In direct comparisons treated and non-treated samples are hybridized against each other on the same array, following different sets of technical or biological replicates. In the replicate hybridizations, samples are often labeled with opposite colors (dye-swap) to reduce labeling bias. In the reference design, large quantities of pooled RNA from a single source is used in each hybridization to allow simple but reliable comparisons between different arrays hybridized over time (Novoradovskaya et al. 2004). Here the dye-swaps (a replicate hybridization with opposite dyes) are not necessary if the comparison between the samples of interest is based on the expression ratios relative to the reference sample (Dobbin and Simon 2002, Simon and Dobbin 2003, Dobbin et al. 2003). The loop designs leave out the common reference pool and are able to collect twice as many sample replicates with the same amount of arrays and utilize dye-swaps to reduce labeling bias (Kerr and Churchill 2001). Loop designs are tolerable to hybridization failures as sample pairs are connected via multiple paths. Prior knowledge on the number of samples in the experiment and possible replications is required to create the loop design. Biological studies, that are complex by nature, favor different models of loop designs, whereas in medical and biomedical studies direct comparison and reference design are applied more often (Knapen et al. 2009).

For two-color microarrays, reference design has clear advantages in a clinical setting. Reference designs have worked well in class discovery applications (Knapen et al. 2009) and as long as the reference material is available, new samples can be added

to the experiment and independent experiments compared (Churchill 2002). Thus, reference design fits well for the longitudinal studies where samples are collected over extended periods, however, the quality and availability of the reference sample greatly affects the success of the study. The reference does not need to represent a biologically relevant sample or treatment, but it should be chosen so that it has expression profile similar to the sample material (Simon and Dobbin 2003, Simon et al. 2002). If the reference sample does not contain target material for some of the array probes, they will not provide reliable measurements (Sterrenburg et al. 2002). The manufacture of large quantities of pooled RNA from cell lines has been demonstrated to be a reproducible option, allowing reliable comparisons of gene-expression data within and between different laboratories (Novoradovskaya et al. 2004).

Regardless of the microarray platform, single or two-color, the experimental design must include an adequate number of biological replicates to ensure statistical power. Whereas technical replication looks only at measurement variability, biological replicates measuring both technical and biological variability simultaneously have become a necessity (Churchill 2002, Allison et al. 2006). However, the use of technical replication only is justified in quality control studies, or when the costs to obtain more biological replicates greatly exceeds the cost of technical replication (Allison et al. 2006). When setting up the array experiments, it is essential to know the adequate number of biological replicates that gives the required statistical power for the study (Churchill 2002, Allison et al. 2006). Different methods are available to address the number of replicates to detect differential expression between sample groups at a single (Yang et al. 2003) or sequential time points (Yang et al. 2003, Gadbury et al. 2004, Pawitan et al. 2005, Page et al. 2006), or sample sizes for accurate classification (Dobbin and Simon 2005, Garge et al. 2005).

2.1.2.4 Data analysis

The analysis of microarray data is a three-tier process. The first tier contains the image analysis and preprocessing of the data, which includes extraction of the raw intensities of hybridized targets, normalization of the data, and detection of expression levels. The second tier contains data filtering and identification of differentially expressed genes, whereas high-level data analysis such as unsupervised/supervised learning algorithms, diagnostic classification and pathway level analysis of gene-expression comprise the last tier. On tiers two and three, virtually all microarray data can be treated similarly despite of the used array. The array platform may affect the format of the data extracted in the image analysis, but the principles remain much the same. The first tier on the contrary, is highly dependent on the array platform used for transcriptional profiling.

Although careful experimental design decreases the biological and technical variation in the microarray experiment, removal of possible measurement errors is required with all array platforms. Signal extraction from the scanned microarray image is the first step of this low-level microarray analysis. Generally, features

(labeled targets hybridized to the probes on the array) are recognized and identified according to given probe coordinates, and segmented by classification of pixels as part of either foreground or background. Each array probe is assigned a specific numerical intensity value with software or array type dependent quality measures. Quality measures typically include pixel-statistics for each extracted feature, to determine if a particular signal is reliable for further analysis. The quality statistics assigned to the probe signals can be used to exclude bad data points before normalization, which further improves the quality of the array data (Beissbarth et al. 2000, Aittokallio et al. 2003). Many different methods, often data type dependent, are available to normalize signal intensities between arrays (Allison et al. 2006, Aittokallio et al. 2003, Quackenbush 2002). It is very difficult, if not impossible, to define an ideal normalization method for any array platform, but for Affymetrix arrays (www.affymetrix.com) robust multi-array average (RMA) is generally recommended (Bolstad et al. 2003, Choe et al. 2005). For other platforms, normalization of probe signals is typically done using locally weighted linear regression (lowess) (Yang et al. 2002) or other non-linear fitting models, such as cubic splines (qspline) (Workman et al. 2002).

There is a variety of techniques available that can be used to identify differentially expressed probes among the normalized array data. Many of the algorithms do not perform efficiently with thousands of genes; therefore probes behaving badly within the data set (according to preprocessing quality control), not showing any signal at all or showing no variation across the sample set, can be removed. The most simple and, thus very popular, fold change approach is a reasonable choice to measure effect size, but it does not assign any confidence level to the fold change and should not be used without statistical tests (Allison et al. 2006, Hsiao et al. 2004). Gene-specific variance estimates are likely to be imprecise as only a few data points are usually available for each gene. To overcome this, variance shrinkage methods that take benefit of the whole array data set using a weighted combination of data from the specific probe and the full set of probes, have been successfully applied (Tusher et al. 2001, Baldi and Long 2001, Cui et al. 2005). Biologists tend to accept relatively many false positive and false negative results as long as all the biologically relevant changes are recorded. To estimate the amount of false positive and false negative findings in the data, false discovery rate (FDR) and mixture model methods (MMM) approaches have been developed to surrogate traditional statistics (Allison et al. 2002, Benjamini and Hochberg 1995, Storey 2003). Of these, MMMs tend to be more powerful as they group results according to differential expression and take this into consideration when assigning reliability values (Allison et al. 2006).

Prior knowledge on the transcripts can be used to assess biological relevance of the gene-expression data. Several software packages that use publicly available geneontology (GO) annotations (Ashburner et al. 2000) or predefined gene sets (Ashburner et al. 2000, Biocarta Pathway Collection , Kanehisa 1997, Kanehisa et al. 2002) exist to aid interpretation of the long transcript lists by categorizing these into functionally related groups. Permutation analyses that shuffle and recalculate the data multiple times to gain statistical confidence, have been shown to be very effective in detection of differential expression of predefined gene sets

(Subramanian et al. 2005, Mootha et al. 2003, Tomfohr et al. 2005). The results can also be visualized graphically as pathways and networks within multiple different software packages (Shannon et al. 2003, Nikitin et al. 2003, Ingenuity® Systems).

In addition to discovery of differential expression, networks and pathways function as a *priori* knowledge in classification of the samples (Rapaport et al. 2007). Generally, microarray data can be categorized by array probes and/or hybridized samples into pre-existing groups (supervised) or based on similarity of expression without a *priori* assumptions (unsupervised). With the use of unsupervised algorithms for microarray data analysis, the aim is to place objects (array probes and/or samples) into clusters where objects are more similar than in neighboring clusters (Quackenbush 2002). To do this, the desired number of clusters, a distance measure and similarity metrics need to be specified; no pre-assumptions are needed and resulting of predetermined number of clusters is guaranteed. Practically no information or guidelines exist for parameter selection, which unfortunately tends to yield biologically irrelevant clusters that often are even irreproducible due to the limited amount of data in most of the microarray experiments (Allison et al. 2006, Garge et al. 2005). Thus, although unsupervised clustering may work well in some cases, it should be used carefully. To avoid formation of irrelevant clusters, semi-supervised methods use available functional gene information (Boratyn et al. 2006) or clinical sample data (Bair and Tibshirani 2004) to evaluate the biological rationality of the clusters and select optimal clustering algorithms (Datta and Datta 2006). As standard unsupervised algorithms do not provide any information to indicate whether the resulting clusters arise from random sampling variation rather than reflect real differences in the population, it is important to apply re-sampling of subsets from the original data to assess reproducibility of the method (Garge et al. 2005, Datta and Datta 2003, Bryan 2004).

Contrary to unsupervised algorithms, supervised methods use the knowledge of which probes and samples should cluster together (Aittokallio et al. 2003). Supervised methods are typically used in classification, where the algorithm is first teached with a training data set to build a class predictor, which then classifies the test samples. The quality of the training data highly determines the success in the actual classification process. Supervised algorithms choose the best classifier for the available data, and the use of a small training data set relative to the classification parameters, increases the risk of overfitting, where algorithm performs perfectly well with the training data but poorly with the test samples. With the small sample sizes that are typical for microarray experiments, overfitting is quite possible and the simplest methods are likely to perform most efficiently. Although different classifiers perform best with different data sets, linear discriminant analyses (LDA) based methods generally classify better than more sophisticated methods (Lee et al. 2005). Furthermore, in a comparison study between two LDA-based nearest centeroid classifiers, rather simplified “classification to nearest centeroids” (ClaNC) (Dabney 2006, Dabney 2005) seemed to outperform more complex “prediction analysis for microarrays” (PAM) (Tibshirani et al. 2002). To avoid selection bias in estimation of classification accuracy, one should determine the adequate amount of test samples

(Dobbin and Simon 2005) and cross-validate the classifier with fully independent test data (Ambroise and McLachlan 2002).

2.1.3 Validity and reliability of transcriptional profiling

Different microarray platforms have shown to be complementary rather than competing (Lee et al. 2003, Yuen et al. 2002, MAQC Consortium et al. 2006), however, alternating sample and data processing protocols complicate individual study designs and may act as a source of bias in meta-analyses over different microarray experiments. The importance of standardization of storage and analysis of microarray data were understood early on and the Microarray Gene-expression Data society (MGED; www.mged.org) was established already in 1999 to facilitate sharing of functional genomics array data (Ball and Brazma 2006). A document outlining the minimum information about a microarray experiment (MIAME) was drafted to give a guidance on publishing microarray data in a usable manner to support conclusions presented in peer-reviewed journals (Ball et al. 2002, Brazma et al. 2001). Unfortunately, MIAME guidelines are still today improperly followed, though several MIAME compliant public databases do exist. Although major MIAME violations were not noticed, a recent study re-analyzing published datasets detected several different types of incompleteness in reported study details and repeatability of published microarray studies was very limited (Ioannidis et al. 2009). In spite of the poor reproducibility with "unknown" data, different microarray platforms and different laboratories do yield consistent results within a controlled experiment setup (MAQC Consortium et al. 2006). This outlines the importance of pre-analytical sample processing and technical performance of the microarray lab in order to retrieve valid transcriptional profiles from the data analysis.

The MicroArray Quality Control (MAQC) consortium demonstrated high intra- and inter-platform agreement between resulting gene lists (MAQC Consortium et al. 2006, Guo et al. 2006, Canales et al. 2006, Patterson et al. 2006, Shippy et al. 2006, Tong et al. 2006) and reliable identification of different transcriptional profiles between sample groups (Irizarry et al. 2005, Strauss 2006). The second phase of the MAQC project, focusing on predictive models based on gene-expression data, recently reported that different one- and two-color array platforms resulted in similarly performing signature genes and classifiers, and the classifiers generated with one platform can accurately predict samples profiled with another platform (Shi et al. 2010a, Oberthuer et al. 2010, Fan et al. 2010). Furthermore, classification algorithms were demonstrated to choose phenotype-related genes, supporting broader application in predictive medicine (Shi et al. 2010b). Still to date only few of several thousand gene-expression microarray studies have emerged into clinical practice (Ioannidis 2007, Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group 2009, Kim et al. 2010) but the recent work by MAQC consortium indicates that in standardized and controlled use gene-expression microarrays are valid for clinical diagnostics.

In parallel with directing the technology closer to clinical use, MAQC studies have pointed out directions for future work. Methods to effectively combine microarray results with complementary studies and technologies or existing clinical databases and decision-making tools, are not available. Although many studies suggest clinical utility and diagnostic use of gene-expression profiling, several challenges to translate gene-expression studies into genomic medicine do exist (Table 2), and clinical utility should always be evaluated in prospective clinical trials with broad heterogeneous populations. (Kim et al. 2010, Tillinghast 2010)

Table 2. Translation of gene-expression studies into genomic medicine - Challenges and recommendations. (Kim et al. 2010)

Biological	Dynamic nature of gene-expression	Synthesis and degradation rates of different transcripts affect the measured steady-state levels and stability of some transcripts may vary between different tissues.
	Sampling	The choice of sampling method and variations in sampling locations may affect gene-expression results by affecting the proportions of different biological fractions in the sample.
	RNA quality	Stabilization of clinical samples during transportation and storage is critical to preserve intact expression profiles.
	Gene-expression arrays are just a part of the picture	Protein modifications such as glycosylation, methylation, acetylation and phosphorylation are involved in many biologically important changes that are not visible in RNA level.
Technical	Microarray platforms	Although different platforms are able to produce comparable results under standardized protocols, extra caution is needed when comparing results between different studies or across different platforms.
	Binding efficiency	Ensuring similar binding properties for all the probes on array is challenging, thus assessment of relative changes in transcription levels is more meaningful than absolute probe signals measured.
	Sources of technical variation	Proper planning and sound experimental design, RNA quality control and appropriate data processing protocols can minimize technical bias. For prospective studies, uniformity of experimental conduct is a necessity.
Statistical and Bioinformatical	Microarray study design	Sample characteristics and possible selection bias determine the validity of the gene-expression study. Data algorithms cannot compensate fundamental errors in study design or inadequate amount of samples.
	Normalization and transformation	Normalization to remove non-biological variation and transformation stabilizing variance are essential, but different normalization and transformation methods may yield different outcomes.
	Analytical methods	The study designs define appropriate methods for inference testing and classification.
	Multiple testing	Multiple testing correction is needed to minimize the false-discovery rate.
	Result validation	An independent test data set or cross-validation is required for all predictors and classifiers.
	Gene regulatory networks	Due to exponential complexity of network inference, several alternative equally probable networks can be constructed. Integration of different data sources can help to identify disease-related genes.
Informatic	Different nomenclatures	Use of different gene nomenclatures makes comparison between different gene-expression studies complicated.
	Different probes for the same gene	Different microarray platforms have different probes against the same transcript. Even if the actual mRNA level is similar, differences in probe design may lead to differences in measured expression levels.
	Common quality standards, data format and data repositories	Procedural guidelines for microarray assays and data analyses are described by the MAQC project. The MIAME guideline provides a template to report an adequate description of the microarray experiment. Assay data shall be stored in public MIAME compliant databases.

2.1.4 *Ex vivo* gene-expression studies on human peripheral blood

Although the effect of the minor cell types is masked by the dominant ones when profiling gene-expression in bulk tissue (Szaniszlo et al. 2004), peripheral blood has proven to be the most practical specimen to profile gene-expression of the human immune system in a clinical setting. As blood is the preferred transportation route for immune cells, transcriptional activity in peripheral blood reflects possible imbalances in the immune system of the patient. Particularly with organ-specific autoimmune diseases, such as multiple sclerosis (MS) and type 1 diabetes (T1D), where access to the target organs is restricted (Kaizer et al. 2007, Achiron et al. 2007, Singh et al. 2007), peripheral blood provides a window to monitor activity of the entire immune system throughout the body. Blood transcriptome analyses with microarrays are able to reveal disease related molecular pathways, genes and susceptibility loci, general autoimmune signatures shared between different autoimmune diseases, and expression signatures that allow monitoring of disease activity and response treatment. (Chaussabel et al. 2010, Pascual et al. 2010).

The expression profiles of PBMCs are highly sensitive to *ex vivo* incubation and sample handling prior the RNA extraction (Baechler et al. 2004). To overcome this, methods with the inclusion of RNA stabilizing reagents in the collection tube have been established (Rainen et al. 2002, Shou et al. 2005). These tubes immediately stabilize the RNA at the time the blood is drawn and allow profiling of the true physiological state without unexpected changes in transcriptional profiles. This is particularly important in clinical multicenter studies, where RNA isolation after blood collection is not possible and samples are shipped to a central laboratory for further processing (Matheson et al. 2008). Although these tubes contain reagents that prevent RNA degradation, extended incubation at room temperature before RNA extraction can reduce the quality of RNA (Shou et al. 2005, Kagedal et al. 2005). Freezing the collection tubes after the blood is drawn and RNA stabilized, yields high quality RNA for expression studies and allows extended sample collection and storage periods (Shou et al. 2005, Thach et al. 2003, Wang et al. 2004, Feezor et al. 2004, Vartanian et al. 2009). This also reduces technical assay variation, as simultaneous batch processing is possible.

Variability in the content of peripheral blood makes it a complicated source material for transcriptional studies. Although the peripheral blood transcription profiles of healthy adults are generally constant, variation has been detected in relation to gender, age, time of day that the blood was drawn, and different cellular composition of the blood sample (Whitney et al. 2003). Both the absolute numbers and relative proportions of blood lymphocyte subpopulations show developmental and age related variation (Hannet et al. 1992, Comans-Bitter et al. 1997, Hulstaert et al. 1994), and variation is also detected between gestational trimesters (Brain 2006). Thus, selection of matched healthy controls is important in order to avoid bias in transcriptional profiles measured from patient samples. Another important source of variation for peripheral blood transcriptional profiles is the high abundance of globin mRNA, which can mask other targets present in whole blood RNA, resulting in decreased signal detection and increased variability between sample replicates.

Hemoglobin levels of peripheral blood also vary over time, between individuals, and by gender (Brain 2006). To bypass the globin-effect, methods for reduction of globin transcripts have been developed (Vartanian et al. 2009, Liu et al. 2006, Wright et al. 2008). However, globin reduction protocols may reduce total RNA quality by depleting other transcripts. As an alternative to globin reduction, an amplification method less sensitive to globin transcripts can be used (Vartanian et al. 2009).

2.2 Organ-specific autoimmune diseases

2.2.1 Autoimmunity

Autoimmunity is a state where the adaptive immune system attacks its host tissues. Some form of autoimmune disease affects about 3-5% of the population and at least 80 different autoimmune diseases have been characterized (Waldner 2009, Jacobson et al. 1997). However, not all self-directed inflammation is autoimmune. It is likely that there is a variety of immune-mediated diseases with different combinations of autoimmune-autoinflammatory mechanisms, while only rare monogenic autoimmune diseases would be purely autoimmune (McGonagle and McDermott 2006). This together with environmental and genetic factors explains the huge variation in clinical outcomes of different patients and populations with immune-mediated diseases.

Traditionally, the scope of the inflammation divides autoimmune diseases into two main classes: organ-specific and systemic. Several organs of the human body have their own organ-specific autoimmune diseases, where expression of specific antigens in those particular organs directs the tissue damage. In systemic autoimmunity, antigens to which immune response is directed to are expressed throughout the body. In both organ-specific and systemic autoimmune diseases, the organ damage is mediated by T cells and/or antibodies. Autoimmune diseases where tissue damage is directed by antibodies can be further classified to diseases mediated by immune-complex (e.g. rheumatoid arthritis, RA and systemic lupus erythematosus, SLE), cell-surface or matrix antigens (autoimmune hemolytic anemia) or cell-surface receptors (Grave's disease) (Janeway et al. 2001). T cell mediated autoimmunity, where organs express autoantibodies that serve as markers of the antigen-specific T cell response, is involved diseases such as type 1 diabetes (T1D) (Knip and Siljander 2008) and multiple sclerosis (MS) (McFarland and Martin 2007). It is also possible that immunopathogenesis mediated by both T cells and autoantibodies operate in parallel, which is the case with rheumatoid arthritis (RA) (Kotzin et al. 2000). Nonetheless, $CD4^+$ T cells are always involved in autoimmunity.

2.2.1.1 T lymphocytes in autoimmunity

$CD4^+$ T helper (Th) cells represent the central population in immune protection. They are capable of producing cytokines and chemokines that regulate the immune defense reactions; they induce macrophages, recruit other cells of the immune

system to sites of the inflammation, and help B cells to produce antibodies. Classically, Th cells were divided into two subtypes, Th1 and Th2, based on production of specific signature cytokines interferon gamma (IFNγ) and interleukin 4 (IL-4), respectively (Mosmann et al. 1986). Th1 cells have been regarded to be critical for protection against intracellular pathogens and development of organ-specific autoimmunity; whereas Th2 cells provide protection against extracellular pathogens and are coupled with responses for allergic inflammation and asthma (Mosmann and Coffman 1989, Romagnani 1996, Romagnani 1994). The balance between Th1 and Th2 cells was long thought to be the main contribution to the outcome of immune response, since overexpression of either cell population leads to progression of inflammatory or autoimmune type response (Romagnani 1994). There is indeed a fine cross-regulation mechanism keeping this balance, however, not only between Th1 and Th2 cells but controlling several different Th subsets characterized by unique expression products and functions. Namely, these are Th1, Th2, Th17, follicular T helper (T_{FH}) cells, induced T regulatory (iTreg) cells and Th9 cells, and there may well be more subtypes to be found. Specific cytokines and transcription factors in each different lineage act as positive feedback signals, but cross-inhibition of other lineages is also actively involved (Zhu and Paul 2008).

So far very little is known about the recently described T_{FH} and Th9 cells. T_{FH} cells induce humoral immune response by promoting B cell differentiation. Differentiation of T_{FH} cells is induced by IL-6 and IL-21. Though IL-6 induces also Th17 cells and IL-21 could amplify Th17 differentiation through a positive feedback loop (Zhou et al. 2007, Korn et al. 2007a), production of IL-17 is repressed by transcription factor B-cell lymphoma 6 (BCL-6) that drives the T_{FH} differentiation (Nurieva et al. 2008, Bauquet et al. 2009, Chtanova et al. 2004, Yu et al. 2009). It is likely that despite of their functional distance, T_{FH} cells might share parts of early differentiation with Th17 cells (Bauquet et al. 2009, Debes and Reiner 2009). A Th9 specific transcription factor has not yet been identified but Th9 cells are differentiated in the presence of IL-4 and transforming growth factor beta (TGF-β), which inhibits Th2 cytokines by suppression of GATA binding protein 3 (GATA3) and promotes IL-9 and IL-10 production instead (Veldhoen et al. 2008, Soroosh and Doherty 2009). Although Th9 cells can be induced from Th2 cells and have been linked to allergic diseases, the capability of IL-9 to promote Th17 cells via induction of IL-23R and IL-9 production by Tregs, Th1 and Th17 cells indicates that Th9 cells might influence pathogenesis of autoimmune diseases (Li and Rostami 2009).

In addition to Th1 and Th2 cells, Th17 cells constitute a third effector T helper cell population with distinct cytokine expression and effector functions from Th1 and Th2 cells (Harrington et al. 2005, Park et al. 2005). The highly pathogenic nature of Th17 cells and their capability to induce autoimmune inflammation was demonstrated already at the time Th17 cells were first observed (Langrish et al. 2005). The clearance of pathogens that cannot be effectively handled by Th1 and Th2 cells seems to be the primary function of Th17 cells, but in addition to that, Th17 cells can induce tissue inflammation and are associated with autoimmune pathogenesis. When innate IL-17 response mediated by other than Th17 cells is not sufficient to clear pathogens, the presence of TGF-β and IL-6 in site of inflammation

provide Th17 favoring conditions and Th17 T cells act as a secondary response. Autoimmunity would arise only if also this secondary response fails to clear the pathogens, thus leading to chronic inflammation (Stockinger and Veldhoen 2007, Stockinger et al. 2007, Dardalhon et al. 2008). Several animal models have emphasized the importance of Th17 cells in the pathogenesis of organ-specific autoimmune diseases, but direct proof for pathogenic role of human Th17 cells is not available (Oukka 2008). However, the importance of Th17 cells in several inflammatory diseases has been suggested and the expression of IL-17 itself is associated with several human autoimmune diseases (Dardalhon et al. 2008, Matusevicius et al. 1999, Kolls and Linden 2004, Onishi and Gaffen 2010, Korn et al. 2009). On the other hand, IL-17 is not specific to Th17 cells but is produced by several other cells types (O'Connor et al. 2010). While the specific role of Th17 cells in human autoimmune pathogenesis remains to be clarified, it is notable that Th1 cells are always present at the sites of Th17-mediated inflammation. The way in which these two T cell populations interact or their specific roles have not been established either, but it has been shown that Th17 cells and Th1 cells have similar counterregulation as there is between Th1 and Th2 (Damsker et al. 2010). At the same time Th17 and Th1 cells show plasticity, this allows conversion from Th17 to Th1 cells, thus allowing modulation of the disease pathogenesis (Damsker et al. 2010, Lee et al. 2009, Zhou et al. 2009). It is proposed that in induction of organ-specific autoimmunity Th17 cells would constitute the initial wave of effector T cells in the site of inflammation and recruit further waves of effector T cells, namely Th1 cells, by inducing pro-inflammatory cytokines IL-6, IL-1 and TNFα and chemokines that recruit Th1 cells on site of inflammation (Dardalhon et al. 2008, Oukka 2008). Production of enormous amounts of pro-inflammatory cytokines, such like IL-6, appears to protect Th17 cells from the suppressive function of Treg cells (Korn et al. 2007b), while Treg-mediated suppression is efficient for Th1 cells *in vivo* (Li et al. 2007). As the Th1 effector population seems to be more easily controlled by Treg cells, it is suggested that immunopathogenesis of organ-specific autoimmunity would be driven by sequential changes in effector T helper cell compositions (Dardalhon et al. 2008).

Treg cells were first characterized by their expression of CD25 (Sakaguchi et al. 1995). Later transcription factor forkhead box P3 (FOXP3) was assigned as a master regulator of Treg differentiation, and expression of FOXP3 as a specific marker for Treg cells (Hori et al. 2003, Fontenot et al. 2003). In parallel with iTreg cells differentiating from naïve $CD4^+$ T cell in the periphery, naturally occurring regulatory T cells (nTreg) with similar phenotype and function are distinguished already in thymus (Zhu and Paul 2008, Sakaguchi 2004). Phenotypically and functionally iTreg cells resemble nTreg cells and distinguishing either function or relative importance of these two Treg populations has been difficult in both humans and animal models (Zhu and Paul 2008, Waldmann et al. 2006). However, the nTreg and iTreg populations seem to differ by their epigenetic control and stability of FOXP3 expression. Whereas nTreg cells have naturally stable expression of FOXP3, acquired Treg cell phenotype of iTreg is instable and antigen restimulation in the absence of TGF-β leads to loss of FOXP3 expression, which causes a loss of suppressor function as well (Huehn et al. 2009). Treg populations without FOXP3 expression, Th3 and TR1

regulatory T cell subtypes, have also been reported, but most likely these represent just certain circumstantial states of $CD4^+$ T cells rather than being own distinct cell lineages (Zhu and Paul 2008, Shevach 2006). Synergistic action of TCR signaling, co-stimulatory molecules and cytokine signals are needed for effective FOXP3 transcription, but the precise mechanisms how these affect molecular level expression of FOXP3 have not yet been solved. Shorter duration and weaker strength of TCR stimulation favors T regulatory cell development for both nTreg and iTreg subtypes, both of which require different specific co-stimulatory signals (Huehn et al. 2009).

Treg cells have a major role in maintaining immunological self-tolerance and immune homeostasis and although reduced prevalence of circulating Treg cells does not reliably reflect the inflammation, genetic anomalies and environmental factors that shift the balance towards self-reactive conventional T cells may induce autoimmunity (Wing and Sakaguchi 2010). It has been shown that in order to mediate their suppressive function to the target tissue, Treg cells have similar organ-specific homing properties as conventional T cells and as compared to peripheral blood, levels of Treg cells are typically higher at the site of inflammation (Siewert et al. 2007, Dejaco et al. 2006). However, the suppressive function of Treg cells is clearly not sufficient to prevent autoimmune diseases. The activation threshold of autoreactive T cells has reported to be lower in patients with autoimmune disease as compared to healthy individuals, also, decreased FOXP3 levels and functional defects of peripheral blood $CD4^+CD25^{high}$ Treg cells have been reported in patients with several autoimmune diseases, including T1D and MS, (Costantino et al. 2008b). Although genome wide association studies have not revealed linkage between FOXP3 and autoimmune diseases, CTLA-4, PTPN22, IL-2 and CD25, which are controlled by FOXP3 have been associated with autoimmune susceptibility (Wing and Sakaguchi 2010, Costantino et al. 2008b).

In comparison to $CD4^+$ T cells, which have been studied more in context of autoimmunity, not much is known on the role of $CD8^+$ T cells. Similarly to $CD4^+$ T cells, $CD8^+$ T cells may also exist with autoreactive or suppressive functions. The natural function of $CD8^+$ T cells is to protect against viral infections and tumors by destruction of target cells via cell-mediated cytotoxicity directed to class I peptide-MHC complexes. As virtually all cells in the human body express class I MHC molecules, autoimmune disease might be initiated during the clearance of viral or tumorigenic cells, when self-antigens released from $CD8^+$ T cell lysis are presented back to $CD4^+$ or $CD8^+$ T cells (Fujinami et al. 2006). Animal models for organ-specific autoimmune diseases suggest that $CD8^+$ T cells participate in initiation, progression and regulation of autoimmune diseases (Walter and Santamaria 2005). Elevated levels of class I MHC molecules and significant amount of $CD8^+$ T cells have been detected in pancreatic islets of T1D patients and MS lesions (Knip and Siljander 2008, Zozulya and Wiendl 2008, Itoh et al. 1993). Autoreactive $CD8^+$ T cells use Fas ligand (FasL) or perforin/granzyme-induced apoptosis to mediate the tissue damage. Recently, it was shown that in mice this tissue damage releases self-antigens that cause deletion of naïve autoreactive $CD8^+$ T cells; thus autoreactive $CD8^+$ T cell response is self-limiting and not capable to sustain chronic autoimmunity in healthy

mice, but additional sustaining signal would be needed (Parish et al. 2009). Autoreactive $CD4^+$ T cells in turn can be self-sustaining (Vanderlugt and Miller 2002), and it is likely that both $CD4^+$ and $CD8^+$ cells are required for chronic autoinflammation both in mice and human. Suppressive capabilities of several different types of adaptive inducible $CD8^+$ T cells have been described in human, but it is not clear whether these are derived from a single source or represent different cell populations (Suzuki et al. 2008). Naturally occurring suppressive $CD8^+CD25^+Foxp3^+$ T cells, similar to $CD4^+$ Treg cells, have also been identified (Cosmi et al. 2003). Recently, a potential of these cells to inhibit the induction of autoimmune response non-cytotoxicly, via direct cell-cell contact, was demonstrated with samples derived from MS patients during exacerbation and remission (Correale and Villa 2010). Earlier, an impaired suppressive function of $CD8^+$ Treg cells has been suspected in MS patients. In patients vaccinated with $CD4^+$ T cells, the majority of Tregs appeared to be $CD8^+$ T cells (Zozulya and Wiendl 2008). Induction of $CD8^+CD25^+Foxp3^+$ T cells was also detected in human PBMCs during continuous antigen stimulation, and in patients with T1D, who clinically responded to anti-CD3 mAb given to induce immunological tolerance (Bisikirska et al. 2005, Mahic et al. 2008). Still, the understanding of the roles and importance of suppressive $CD8^+$ T cells in human autoimmunity is very limited.

2.2.1.2 T cell repertoire and recognition of an antigen

The major histocompatibility complex (MHC), called human leukocyte antigen system (HLA) in humans, controls the antigen specificity of the autoimmune response. The T cell receptor (TCR) recognizes foreign peptides that class I or class II MHC/HLA molecules present. T cell repertoire of an individual, responsible for detecting foreign peptides in the periphery, is one of the key factors in development of autoimmune diseases. Based on mathematical modeling, for immune system to gain functional protection against all possible foreign epitopes, a single T cell should recognize 1×10^6 structurally similar peptides (Mason 1998). In reality, the number of recognized epitopes is likely to be far less. To overcome this challenge, T cells in the periphery have a significant level of cross reactivity (Kersh and Allen 1996b, Kersh and Allen 1996a, Anderson et al. 2000). As a result, some self-reactivity is likely to occur with most TCRs, and autoreactive T cells exist among the peripheral T cell repertoire of healthy individuals. Because of this necessity for cross reactivity, negative selection in thymus cannot be fully effective but the immune system needs to control autoreactive T cells in the periphery via different tolerance mechanisms, such as anergy or apoptosis, phenotype skewing and regulatory T cells (Walker and Abbas 2002). Alterations in negative regulation of TCR signaling and T cell signaling associated with survival have been linked to failed protection against autoimmunity (Oak and Fruman 2007, Huang and Gu 2008).

T cell cross reactivity is dependent on the sensitivity of a T cell to a peptide, phenomena known as avidity, which is determined by the binding strength of peptide to MHC and affinity of TCR to the peptide-MHC complex (Margulies 2001). As a result, different TCRs can bind the same peptide-MHC complex with different

affinity and a single T cell will respond differently to different peptides. Avidity also has a key role in central tolerance during positive and negative selection of T cells in thymus, where recognition of self-peptide-MHC with low avidity is required from $CD4^+CD8^+$ thymocytes but those with too high affinity TCR are directed to undergo apoptosis (Hogquist et al. 2005). Recent advances in structural studies on TCRs and TCR-peptide-MHC complexes (TCR-pMHC) have shed light on antigen recognition and biased use of TCR. The determined structures of autoimmune TCR-pMHC complexes indicate that autoimmune TCR recognition would be different from recognition of non-self-peptides (Ely et al. 2008). Structural studies have also shown that self-MCH reactivity is avoided by reshaping the TCR repertoire towards a viral determinant (Gras et al. 2009). As the number of reported TCR-pMHC structures continues to grow, it remains to be seen if autoimmune TCR recognition is truly divergent from cognate and allogeneic interactions.

For most of the autoimmune diseases in human, genetic susceptibility is strongly associated with MHC class II alleles, but some associations with particular MHC class I alleles are found as well (Janeway et al. 2001). Consequently, class II HLA alleles are the best characterized of genes that control antigen specificity in autoimmunity. Different alleles have been associated with different diseases, indicating antigen-specific nature of MHC class II effects. There are multiple parallel mechanisms, how autoimmunity is influenced by class II alleles. Predisposing to autoimmune disease may occur by increasing positive and/or decreasing negative selection of autoreactive T cells, or by inhibition of the selection of regulatory $CD4^+$ T cells in thymus. On the contrary, some alleles have shown to be protective and may inhibit autoimmunity by deleting autoreactive cells (Marrack et al. 2001). In addition to simply determining the susceptibility to a certain autoimmune disease by an ability to present autoantigenic peptides to autoreactive T cells, the HLA genotype may alter the susceptibility to autoimmunity by participating in shaping of individual's T cell repertoire by promoting the positive selection of autoantigen-specific thymocytes (Anderton and Wraith 2002).

2.2.1.3 Autoreactive T cells and peripheral tolerance

Stimulation of a T cell through TCR alone does not lead to activation of the T cell response. Instead, T cells become anergic and are unable to proliferate in response to restimulation. A co-stimulation signal, typically mediated by antigen-presenting cell, is needed to induce productive T cells. CD28, which is constitutively expressed on naïve $CD4^+$ and $CD8^+$ T cells, is a unique co-stimulatory molecule controlling the activation of both naïve and memory T cells (Shahinian et al. 1993, Sharpe and Freeman 2002). In addition to CD28 and its counterpart CD80, there are several different context dependent receptor-ligand interactions that amplify or modify the TCR signal (Goronzy and Weyand 2008), along with co-inhibitory pathways (Sharpe et al. 2007, Fife and Bluestone 2008, Murphy et al. 2006). The co-inhibitory signals that T cells can sense have been indicated to be as equally important for immune regulation as the signals that amplify or modify the immune response (Leibson 2004). Co-stimulation, or –inhibition, are not antigen specific and thus not specific

for autoimmune responses. On the other hand, all immune responses are context dependent since various receptor-ligand interactions are highly dependent on the environment. Co-stimulation does not necessarily happen spatially or temporally in parallel with TCR-pMHC complex formation but in both cases, it acts as a central decision point in immune response (Goronzy and Weyand 2008).

In addition to anergy, self-reactive T cells may be restrained via co-stimulation signals leading to apoptosis or skewing of the T cell phenotype, or via T cell ignorance. The state of ignorance results if the T cell never meets a self-antigen or T cell response is not triggered due to low avidity (Walker and Abbas 2002). Sometimes, although an autoreactive T cell is fully activated, tolerance is maintained by changing of the T cell phenotype. This phenotype skewing is induced by the cytokine environment and especially Th2 type cytokines have been reported to down-regulate autoimmunity in animal models (Young et al. 2000, Bradley et al. 1999). It is thought that phenotypic skewing can modulate the immune response by determining where self-reactive T cells migrate and which accessory cells they encounter (Walker and Abbas 2002).

The most effective way to remove a particular autoreactive T cell clone from the periphery is programmed cell death, apoptosis. Fas (CD95 or Apo-1) -mediated activation induced cell death (AICD) has been considered as a major regulator of clonal deletion of autoreactive T cells (Walker and Abbas 2002, Watanabe-Fukunaga et al. 1992). Fas-receptor signaling has no functional significance in the regulation of T cell responses triggered by non-self antigens but is limited to self-reactive T cells (Stranges et al. 2007). In addition, the absence of the Fas signal in activated T cells leads to systemic autoimmunity, probably because of extended survival of autoreactive T cells (Stranges et al. 2007). Based on observations from $Bim^{-/-}$ mice that cannot undergo apoptosis, it also appears that apoptotic clearance of autoreactive T cells is not indispensable but can be substituted by anergy and suppressor function of Treg cells (Barron et al. 2008). This result by Barron et al. agrees with the earlier indication that peripheral deletion is not complete and AICD would act merely to decrease the frequency of self-reactive T cells to a level where anergy can be effectively induced in the rest of the cells (Walker and Abbas 2002).

Whereas apoptosis and anergy provide two separate mechanisms for tolerance (Barron et al. 2008), Treg cells and apoptosis are coupled. IL-10, a cytokine secreted by Treg cells, has been reported to promote AICD (Georgescu et al. 1997). *In vivo* suppression by Treg cells is mediated by induced apoptosis of activated $CD4^+$ T cells (Pandiyan et al. 2007) and it has been demonstrated that Treg cells selectively induce the apoptosis of autoreactive T cells *in vivo* (Chang et al. 2009). However, no differences were detected in Fas expression between autoreactive and other T cells (Chang et al. 2009). Active death-receptor mechanisms were not detected either. In view of the latter it seems likely that cytokine deprivation-induced death would be a key component of Treg cell mediated suppression, by which Treg cells initially decrease effector $CD4^+$ cell population by apoptosis to a level where apoptosis-independent control of smaller T cell population is possible by immunosuppressive cytokines (Pandiyan et al. 2007).

Interestingly, naturally occurring $CD4^+CD25^+$ T regulatory cells (nTreg), which provide an important suppression mechanism for peripheral tolerance and have a central role in development of autoimmunity (Sakaguchi 2004, Dejaco et al. 2006, Piccirillo and Shevach 2004, Torgerson 2006), have a distinct TCR repertoire as compared to conventional T cells (Pacholczyk and Kern 2008). Conventional T cells and nTreg cells express different sets of TCRs directed for non-self and self-antigens, respectively. The suppressor function of the nTreg cell is dependent on TCR activation by self-antigen, and recognition with high affinity is required for nTreg lineage commitment (Pacholczyk and Kern 2008). In addition, nTreg cells are able to recognize foreign antigens with the same rate as conventional T cells, but with lower affinity (Pacholczyk et al. 2007). After activation, Treg cells suppress other T cells in a TCR-non-specific manner (Thornton and Shevach 2000).

2.2.1.4 Predisposing factors

According to animal models of autoimmune diseases, it is difficult to break tolerance in individuals without predisposition (Christen and von Herrath 2005). Development of an autoimmune disease is always a result from an unfortunate combination of the individual's genetic susceptibility and exposure to environmental factors promoting the disease. Genetic predisposition can almost entirely explain the generation of autoantibodies but cannot cause the autoimmune attack without environmental co-factors modulating the initiation of the autoimmunity. Prior to clinically overt autoimmune disease, both genetic and environmental factors influence the overall reactivity of the immune system, antigen recognition and the response of the target tissue (Marrack et al. 2001). Infections can induce strong inflammatory responses in various organs of susceptible individuals, which may initiate or modulate autoimmunity via multiple pathways.

Despite the well-characterized predisposing roles of HLA-genes, these account for only a half of the autoimmune disease risk (Pociot and McDermott 2002, von Herrath 2001). In addition, several other genes may alter the response in the target tissue and either promote or protect from autoimmunity by different mechanisms affecting the maintenance of tolerance (Griffith et al. 1995, Vafiadis et al. 1997, Mahoney and Rosen 2005, Viorritto et al. 2007). Gene functions after the onset of the autoimmunity can further modify the course of the disease. In addition to HLA-genes, different patients are predisposed by different genes and a hierarchy of a few major and several minor predisposing genes is likely to exist (von Herrath 2001). Indeed, the expansion of genome wide association studies (GWAS) during the past four years has revealed that predisposing and susceptibility genes are widely shared between different autoimmune diseases (Zhernakova et al. 2009, Invernizzi and Gershwin 2009, Xavier and Rioux 2008). Rather than being disease-specific, the group of common autoimmune susceptibility genes is likely to regulate the overall reactivity of the immune system and define the predisposition of an individual to develop an autoimmune disease. The observation of these common autoimmune susceptibility genes indicates pathway level commonalities in autoimmune disease pathogenesis. The development of a particular autoimmune disease is determined

by T cell recognition of antigen(s) (Anderton and Wraith 2002, Ohashi 2002) and the ability of the target tissue or organ to modulate autoimmune attack (Marrack et al. 2001).

Although the majority of patients with autoimmune disease do not have a family history of the disease, accumulation of different autoimmune diseases in the same family is relatively common and people with one autoimmune disorder have an increased risk to develop another one (Sloka 2002, Anaya et al. 2006). The relationship between genetic and environmental contribution in autoimmune disease pathogenesis has been elucidated in twin studies. The concordance of autoantibodies between monozygotic twins is nearly 95%, but disease penetrance only ~50% (Christen and von Herrath 2005). The disease concordance rate of identical twins varies between different autoimmune diseases and variable concordance rates are reported even within a disease (Brooks et al. 2010). Environmental factors could explain these differences and both disease promoting and protective effects of microbial and viral infections have been reported (Christen and von Herrath 2005, Chervonsky 2010). This bidirectional effect of inflammation is in congruence with the observation that in developed countries, where environments are getting more and more hygienic, the lack of antigen stimulation may have contributed to increased levels of allergic and autoimmune diseases (Bach 2001, Bach 2002). This is also supported by population-level studies describing the inverse relationships between the frequency of T1D and enterovirus infections (Viskari et al. 2004) or allergic asthma and parasites (van den Biggelaar et al. 2004), and low risk of allergic sensitization after exposure to viral infections (Seiskari et al. 2007).

Recently, epigenetic changes have been demonstrated to mediate environmental influences to autoimmune disease pathogenesis (Brooks et al. 2010, Hewagama and Richardson 2009), but overall understanding of the mechanisms how the environment modulates autoimmune pathogenesis is still rather poor. However, it seems that infectious events are more likely to promote development of autoimmunity in individuals with increased genetic risk towards autoimmunity, but are unlikely to induce autoimmunity in non-prone individuals. Furthermore, some of the genes associated with autoimmune diseases are related to innate immunity (Zhernakova et al. 2009), supporting the dual role of the immune system described in the continuum model of immunological diseases: the nature of immunological diseases varies from purely autoimmune to purely autoinflammatory through variable combinations of autoimmune-autoinflammatory mechanisms (McGonagle and McDermott 2006).

2.2.2 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), causing demyelination, axonal loss and brain atrophy (McFarlin and McFarland 1982a, McFarlin and McFarland 1982b). MS usually develops between the age of 20 and 40 years and it is the most common cause of paralysis in young adults

(Waldner 2009). In Northern Europe and North America 60-200/100,000 individuals suffer from MS (Sospedra and Martin 2005). MS is divided into two major forms: More frequent relapsing-remitting (RR)-MS, which covers 85-90% of all cases being two times more prevalent in women as compared to men, and primary progressive (PP)-MS where patients show steady progression after insidious disease onset (Sospedra and Martin 2005). Patients with RR-MS usually later develop secondary progressive (SP)-MS (Sospedra and Martin 2005). The onset of MS results from a combination of environmental factors with genetic susceptibility. Familiar aggregation, related to shared genetic material has been reported and several different genes and loci have been implicated in addition to the HLA locus (Dyment et al. 2004). Mechanisms that lead to myelin sheath and axonal damages are still poorly understood, but it is generally accepted that formation of acute inflammation lesions, characterized by blood-brain barrier (BBB) breakdown, initiate the MS disease (McFarland and Martin 2007).

2.2.2.1 Pregnancy and Multiple Sclerosis

During the third trimester of pregnancy, the risk of relapses in MS patients decreases significantly, but during the first four months postpartum it increases three-fold (Confavreux et al. 1998). The phenomena is explained by pregnancy-related alterations in maternal immune system that occur in order to protect the semiallogeneic fetus against an attack by the mothers immune system (Chaouat et al. 2004), but the underlying pathobiological mechanisms that modify the disease activity are not fully understood. The shift of the polarization of the immune response towards Th2 type immunity during the pregnancy and expansion of Treg cells are included among putative explanations (Sanchez-Ramon et al. 2005, Wegmann et al. 1993, Polanczyk et al. 2005). Pregnancy hormones can control MS-activity by a direct effect on the function of the immune cells (Sicotte et al. 2002). Pregnancy-related immunoregulatory factors include serum proteins and tolerance-promoting signaling molecules such as sHLA-G, CD200, Fas-ligand, alphafetoprotein and indoleamine 2,3-dioxygenase (Clark 2005, Irony-Tur-Sinai et al. 2006, Bebo and Dveksler 2005). Interestingly, peripheral blood frequencies of neither the pro-inflammatory Th17 subset nor Treg cells differed between MS patients and controls around and during the pregnancy (Neuteboom et al. 2010), thus suggesting that Th17 and Treg cells are not involved in MS disease course alteration during pregnancy.

2.2.2.2 MS disease pathogenesis

Two major biological reasons have complicated the search for proof of T cell mediated autoimmune responses in MS patients. Firstly, antigen-presenting cell presents antigens to $CD8^+$ T cells as MHC class I products and to $CD4^+$ T cells as MHC class II products. Transfer studies are thus excluded as a final proof of autoimmune reactivity because MHC identity would be required between T cell donor and recipient. In addition, the search for target autoantigens among processed peptides

is complicated. Second reason is that in contrast to many other tissues, possibilities for experimental investigation of CNS lesions are very limited. (Wekerle 2008)

It is unclear which antigen(s) initiate T cell activation in MS. However, most patients show reactivity to several myelin antigens (Ota et al. 1990). This agrees with an "epitope spreading" detected in an animal model of MS, experimental autoimmune encephalomyelitis (EAE), where mice injected with a particular peptide antigen become activated against other epitopes as well (Costantino et al. 2008a). In humans, the antigen specificity is very similar between patients with MS and healthy controls, and autoreactive T cells against myelin antigens can be isolated from peripheral blood of both MS and control group (Sospedra and Martin 2005, Costantino et al. 2008a). Molecular mimicry by viral infections has been proposed as an initiator of autoimmune pathogenesis, but a causal link between infection and MS has not been detected (Costantino et al. 2008a). In comparison to controls, MS patients show a significant TCR degeneracy that allows myelin-antigen recognition due to unconventional TCR binding pattern (Zhang et al. 2008, Hahn et al. 2005).

Th1 cells initiate the acute lesions in MS disease, however, the idea of MS as a Th1 cell mediated autoimmune disease has been revised since the identification of interleukin 17 producing Th17 cells. Several studies have shown that human leukocyte antigen (HLA) class II genes have a major influence on the genetic risk of MS, which is in agreement with the involvement of a $CD4^+$ T cell mediated process (McFarland and Martin 2007, Dyment et al. 2004). As evidence of disease association with Th17 cells, elevated levels of IL-17 mRNA have been detected in blood and cerebrospinal fluid mononuclear cells (Matusevicius et al. 1999) and brain tissue from patients with multiple sclerosis (Lock et al. 2002). Furthermore, expansion of the Th17 cell population in patients with MS is reflected by dendritic cells producing increased amounts of IL-23 (Vaknin-Dembinsky et al. 2006), which is required for T cells commitment to Th17 lineage (Harrington et al. 2005, Park et al. 2005).

$CD8^+$ T cells are also involved in inflammatory lesions of MS patients (Traugott et al. 1983), and it has been speculated that after MS lesion is initiated with $CD4^+$ T cells, $CD8^+$ T cells follow, mediating the amplification and damage (McFarland and Martin 2007). This is supported by the finding of $CD8^+$ T cells in the perivascular regions, whereas $CD4^+$ T cells are usually present only at the edge of the lesion. Moreover, it is also reported that more myelin recognizing $CD8^+$ T cells are found from patients with MS than in healthy controls (Crawford et al. 2004). Clonal expansion of $CD8^+$ T cells has also been detected in the lesions from brain tissue of MS patients (Babbe et al. 2000).

Both $CD4^+$ and $CD8^+$ T cells have significant roles in regulation over MS disease. There is somewhat contradictory information if levels of $CD4^+CD25^{high}$ regulatory T cells are altered between patients with MS and healthy controls. However, it is clear that suppressor function of $CD4^+CD25^{high}$ Treg cells is impaired in patients with MS, but the mechanism of the functional defect remains still unknown (Costantino et al. 2008a). $CD8^+$ Treg cells have been identified to balance the immune response against CNS in MS patient by recognizing and lysing myelin-reactive $CD4^+$ T cells (Correale

and Villa 2008). In addition, a distinct thymus-derived Treg subset expressing HLA-G has been identified in MS patients (Feger et al. 2007) and these HLA-G^+ Treg cells have normal functionality (Zozulya and Wiendl 2008).

2.2.3 Type 1 Diabetes

Type 1 diabetes (T1D) is another example of an organ-specific T cell mediated autoimmune disease in human. In T1D, insulin producing β-cells in the islets of Langerhans of the pancreas are selectively destroyed by autoreactive T cells, leading to clinical disease after only 10-20% of the β-cells remain active (Knip 1997). Throughout the world, the yearly increase in T1D incidence is 2.5-3.0% and continues to grow steadily, especially in developed countries, being highest in Finland where 64.2 cases were detected per 100 000 individuals in 2005 (Harjutsalo et al. 2008). Like in most autoimmune diseases, the onset T1D is a combined effect of genetic susceptibility and environmental factors and is preceded by long symptom-free period (Figure 1). The importance of environmental triggering is highlighted by a concordance rate of only about 50% between identical twins (Todd 2010) and huge differences in incidence rates between different Caucasian populations (Knip et al. 2005). Numerous environmental factors may contribute to T1D already during pregnancy and early childhood. Early influence is supported by recent findings that children that develop T1D show distinct metabolic profiles already after birth (Oresic et al. 2008) and the gut microbiome of children that develop T1D is less diverse and stable as compared to healthy children (Giongo et al. 2010).

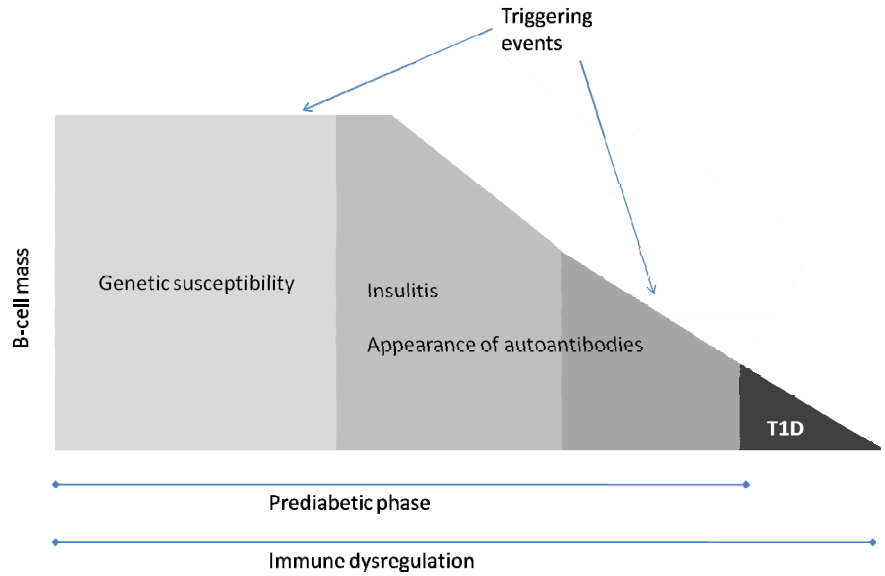

Figure 1. Progression of T1D. (Devendra et al. 2004)

2.2.3.1 T1D susceptibility and environmental modulation

Clinically overt diabetes is preceded by the appearance of autoantibodies against islet cells (ICA), insulin (IAA), protein tyrosine phosphatase-related IA-2 protein (IA-2A), glutamic acid decarboxylase (GADA) and recently identified cation efflux transporter ZnT8 (Wenzlau et al. 2007). The appearance of more than one type of these autoantibodies vastly increases the disease risk; for first-degree relative of a patient with T1D the risk increases from 20 to 90%, when they become positive for second autoantibody (Bluestone et al. 2010). Autoantibodies have a great predictive value for disease progression and recent studies have demonstrated that in combination with HLA genotype this is also true for the population without familial association (Knip et al. 2010, Siljander et al. 2009). HLA class II molecules are the ones to recognize T1D associated autoantibodies in the thymus and periphery, and certain alleles in DR and DQ loci are associated with increased disease risk. Though not considered in risk models, some HLA class I molecules have also been associated with T1D (Ziegler and Nepom 2010) and both class II and class I -specific antigens are present in blood and pancreatic islets of T1D patients (Bluestone et al. 2010). In addition to a strong genetic contribution by human leukocyte antigen (HLA) genes, more than 50 non-HLA regions contribute to the risk towards T1D (Pociot et al. 2010, Smink et al. 2005). However, while genome-wide association studies have rapidly increased the number of T1D candidate genes, they have not revealed that much about T1D pathogenesis. In a recent large genome-wide association study of copy number variations (CNV) and common human diseases, only the HLA loci were associated with T1D but several artifacts that could lead to false-positive associations were identified (Wellcome Trust Case Control Consortium et al. 2010). The non-HLA susceptibility is largely related to T cell mediated immunity, but nonexistence of direct pathways suggests that initiation and development of T1D are associated with chronic dysregulation of the immune system (Concannon et al. 2009, Barrett et al. 2009).

Whereas genetic susceptibility of the HLA region has an undisputable role in the decision whether autoimmunity activates in the first place, proteins coded by other susceptibility genes are implicated in progression of T1D. Polymorphism of the variable number of tandem repeats (VNTR) region in the promoter of the insulin gene (INS) is associated with low insulin expression in the thymus, which may hamper the negative selection of insulin-specific autoreactive T cells (Vafiadis et al. 1997, Bennett et al. 1995, Pugliese et al. 1997). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is suggested to maintain peripheral tolerance by down-regulating T cell activation (Scalapino and Daikh 2008) and polymorphism in the CTLA-4 region is associated with T1D (Ueda et al. 2003, Atabani et al. 2005). Intracellular lymphoid protein tyrosine phosphatase non-receptor type 22 (PTPN22) inhibits T cell receptor signaling and the C1858T SNP in PTPN22 is associated with T1D and several other autoimmune diseases (Vang et al. 2008, Bottini et al. 2004). This gain of function mutation, with higher catalytic activity, reduces IL-2 secretion and intracellular calcium release, impairs activation of $CD4^+$ memory T cells and decreases expression of CD25 and IL-10 (Bottini et al. 2004, Zoledziewska et al. 2008, Vang et al. 2005, Rieck et al. 2007). IFIH1 (interferon induced with helicase C domain 1), also known as

MDA5 (melanoma differentiation-associated protein 5), is a mediator of the innate immune response and associated with T1D as a predisposing gene (Smyth et al. 2006). Recently, the importance of IFIH1 in T1D pathogenesis was highlighted when certain rare variants of IFIH1, with reduced IFIH1 expression in peripheral blood lymphocytes and monocytes, were shown to be protective against T1D (Nejentsev et al. 2009, Downes et al. 2010).

As not everyone with high genetic susceptibility proceeds to T1D, environmental factors triggering the disease onset must be involved. As a consequence of the increased T1D rates observed in developed countries, studies have focused in lifestyle-related differences, such as infections and dietary factors. Different viruses have been investigated as potential T1D inducers, but enteroviruses have remained as primary candidates since 1969 (Oikarinen et al. 2011, Gamble et al. 1969, TEDDY Study Group 2008). Enteroviruses are able to damage pancreatic β-cells *in vitro* (Roivainen et al. 2000) and *in vivo* (Ylipaasto et al. 2004), and seasonal linkage between enteroviral infections and T1D has been demonstrated (Oikarinen et al. 2011, Salminen et al. 2004, Kimpimäki et al. 2001). Although several protective or causative nutritional agents have been reported in the context of T1D during the past three decades with no definite associations, reduced exposure to microbes via food, together with other dietary changes in industrialized countries may have contributed to increased levels of T1D (Virtanen and Knip 2003). However, it is likely that exposure to complex dietary proteins during early childhood increases the risk for T1D, as it was observed that, for children with a high-risk HLA genotype and a first-degree relative with T1D, dietary intervention with highly hydrolyzed milk formula during infancy was able to decrease the risk of T1D predictive autoantibodies (Knip et al. 2010).

2.2.3.2 Molecular mechanisms of β-cell damage in human

The initiation of T1D takes place in pancreatic lymph nodes, where antigen-presenting cells present T1D autoantigens to naïve HLA class II reactive T cells (Knip and Siljander 2008). These autoreactive $CD4^+$ T cells then invade the pancreatic islets and cause further damage that leads to release of self-antigens and epitope spreading (Bluestone et al. 2010). Though class II molecules seem to be responsible for initiation of the disease, class I antigens and class I antigen specific $CD8^+$ T cells are present in the peripheral blood and pancreatic islets of T1D patients (Bluestone et al. 2010, Willcox et al. 2009). As neither $CD4^+$ nor $CD8^+$ T cells can effectively transfer T1D in NOD mice, it is possible that different mechanisms of T cell mediated β-cell death works in parallel: Cytotoxic $CD8^+$ T cells can kill β-cells in direct contact while cytokines inducing FAS death receptor can be secreted by both $CD4^+$ and $CD8^+$ T cells (Lehuen et al. 2010). In addition to well-characterized adaptive immunity, innate immunity and inflammatory mediators contribute in the early stages of T1D pathogenesis. Cells of the innate immune system that are found in pancreatic infiltrates and lymph nodes are capable of modulating the immune response of islet antigen specific T cells and may contribute to β-cell death directly (Lehuen et al. 2010, Eizirik et al. 2009). Furthermore, inflammatory responses induced by viral

infections are known to accelerate T1D pathogenesis and recently a novel interferon regulatory factor 7 (IRF7) driven inflammatory network enriched with viral response genes was implicated in regulation of T1D pathogenesis (Heinig et al. 2010). One of the genes implicated in this network is viral RNA recognizing IFIH1, shown to be protective to T1D (Nejentsev et al. 2009, Downes et al. 2010).

According to the classical Th1/Th2 paradigm T1D is a Th1 mediated autoimmune disease, but the capability of highly pathogenic Th17 cells to induce autoimmune inflammation and their importance in animal models of organ-specific autoimmunity suggests a pathogenic role for Th17 cells in human as well. Recently, two different groups have reported increased levels of IL-17 in naïve and memory $CD4^+$ T cells (Honkanen et al. 2010, Marwaha et al. 2010) and $CD8^+$ T cells (Marwaha et al. 2010) isolated from peripheral blood of children with newly onset T1D. Furthermore, Honkanen et al. show that Th17 immunity stays up-regulated after longer duration of the disease and that IL-17 is able to promote human islet cell damage in vitro (Honkanen et al. 2010). Both studies also report elevated expression levels of the Treg marker gene FOXP3. However, instead of naïve ($CD45RA^+CD25^{int}FOXP3^{low}$) or memory Tregs ($CD45RA^-CD25^{high}FOXP3^{high}$), the elevated FOXP3 levels result from an increase in the population of non-suppressive $CD45RA^-CD25^{int}FOXP3^{low}$ cells secreting IL-17. These $IL\text{-}17^+FOXP3^+$ T helper cells may be an intermediate lineage between plastic Th17 and Treg lineages, and local cytokine environment defines suppressive/proinflammatory role for $IL\text{-}17^+FOXP3^+$ Th cells (Marwaha et al. 2010). Furthermore, monocytes derived from T1D patients secrete proinflammatory cytokines driving differentiation and expansion of Th17 cells, thus supporting general Th17 response in T1D (Bradshaw et al. 2009).

Although regulatory failure is acknowledged as a root cause for T1D autoimmunity, the lack of proper definition for human regulatory T cells has resulted in contradictory information on the number, proportion and functionality of Treg cells in patients with T1D. In addition to the unreliability of Treg markers, human studies are somewhat limited to peripheral lymphocytes, which may be of little relevance and does not reflect Treg levels at the site of the autoimmune inflammation. Nevertheless, Treg cells are critical for autoimmune pathogenesis. Complete loss of Treg cells, caused by mutations switching of Treg marker gene FOXP3, leads to a multi-organ autoimmune disease IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) (Bennett et al. 2001). In T1D patients, both naturally occurring Treg cells and inducible $CD4^+FOXP3^+$ precursor cells are present before and after the disease onset (Ziegler and Nepom 2010). Some of the induced Treg cells in T1D patients are islet antigen specific and indistinguishable from similar cells from non-T1D individuals (Long et al. 2009). Instead of loss in Treg functionality, pathogenic T cells may escape from regulatory mechanisms by unresponsiveness of autologous effector T cells in T1D. Whereas effector T cells from healthy controls can be equally suppressed by autologous control Treg cells and T1D Treg cells, neither T1D nor control Tregs are able to normally suppress T effector cells from T1D patients (Schneider et al. 2008). Effector T cells from T1D patients are resistant to both naturally occurring and induced Treg cells, which may partly explain variable results regarding functionality of Treg in T1D patients.

3. AIMS OF THE STUDY

The goal of this PhD study was first to design and optimize a custom tailored cDNA microarray for gene-expression profiling of the immune system, and validate its performance. Furthermore, to allow gene-expression profiling from the minute amounts of total RNA resulting from whole peripheral blood samples, suitable sample collection and preparation protocols were optimized. The main objective was to apply gene-expression microarrays to characterization of transcriptional profiles in peripheral blood of patients with autoimmune diseases.

The specific aims of this Ph.D. thesis were:

- To design cDNA microarray for targeted study of signal transduction and molecular networks related to immune-mediated diseases and validate the array with a case study of T helper cell differentiation.

- To set up total RNA stabilization and mRNA amplification methods to be used in clinical studies.

- To apply gene-expression microarrays to study pregnancy related immunosuppression in patients with multiple sclerosis.

- To characterize type 1 diabetes associated gene-expression profiles during the disease progression in at-risk children who developed autoantibodies and clinical T1D.

4. MATERIALS AND METHODS

4.1 Samples and specimen

4.1.1 In vitro polarization and activation of T helper cells

Mononuclear cells were first isolated from neonatal cord blood using Ficoll Paque Isolation (Amersham Pharmacia Biotech, Uppsala, Sweden) and $CD4^+$ lymphocytes were recovered with magnetic beads (Dynal Biotech, Oslo, Norway). The $CD4^+$ cells were stimulated in the presence of 100 ng/ml PHA (Murex Diagnostics, Chatillon, France) and irradiated (60 Gy) with CD32-B7 transfected fibroblasts. The final densities of the cell cultures were 1 x 10^6 $CD4^+$ cells and 0.5 x 10^6 feeder cells in 1 ml, cells were grown in Yssel's medium (Irvine Scientific, Santa Ana, CA) supplemented with 1% AB-serum (Red Cross, Helsinki, Finland).

T cell differentiation was primed with 2.5 ng/ml of IL-12 (R&D Systems, Minneapolis, MN) for Th1 cells and with 10 µg/ml anti-IL-12 (R&D Systems) and 10 ng/ml IL-4 (R&D Systems) for Th2 cells. To enhance proliferation of the lymphocytes, 40 U/ml of IL-2 (R&D Systems) was added to the cultures after 48 h. For longer time points, cultures were divided at days 3 and 10. At day 7 part of the cells were collected and the rest were re-stimulated and cultured for another 7 days as described above. At both 7 and 14 days, half of the collected cells were activated by plate-bound αCD3 (500 ng/well for coating) and 0.5 µg/ml soluble αCD28 (Immunotech, Marseille, France) for 6 h, whereas the other half was treated similarly without antibodies. Polarization efficiency was controlled by intracellular FACS staining of IFN-γ and IL-4.

Alternatively, $CD4^+$ cells were separated as described above, but one aliquot was reserved as untreated sample of naïve T cells that were cultured in "neutral conditions". The rest of the cells were cultured for 48 h after primary activation with plate-bound αCD3 (1000ng/µl for coating) and 500 ng/µl soluble αCD28 (Immunotech). Culture density was 0.5 – 2 x 10^6 cells/ml in Yssel's medium (Irvine Scientific) including 1% AB-serum (Finnish Red Cross). Polarization was performed in the presence and absence of TGF-β (R&D Systems) with 2.5 ng/ml IL-12 (R&D Systems) for Th1 cells or 10 ng/ml IL-4 (R&D Systems) for Th2 cells. Both CD3+CD28-activation and polarizing cytokines were given at the same time.

4.1.2 Pooled PBMC reference sample

To compare hybridization performed at different times, an RNA pool representing normal expression was collected and pooled from total RNA in PBMC from altogether 41 normal blood donors (Finnish Red Cross) to be used as a reference sample. PBMC were first isolated by Ficoll gradient centrifugation (Amersham Biosciences) and total RNA was isolated with Trizol (Gibco BRL®) and stored at -70 ˚C. Collected samples were then combined and purified with Qiagen's RNeasy Maxi kit

with on column DNAse treatment. After the purification step, the yield from different columns were pooled again, aliquoted for later use and stored at at -70 ˚C.

4.1.3 Human peripheral blood

Peripheral blood samples for microarray studies were collected with with the PAXgene™ Blood RNA System (Qiagen/BD) where RNA stabilization reagents in the collection tube ensure that transcription profiles reflect the physiological state at the time of the blood drawn. After the blood was drawn, PAXgene™ collection tubes were frozen at -70 ˚C for transportation and storage. The extraction of total RNA was done according manufacturer's instructions using specific materials and reagents provided with the kit. Before proceeding to gene-expression microarrays, the total RNA isolated was amplified with RiboAmp® RNA Amplification Kits (Arcturus) according to the manufacturer's instructions.

Alternatively, peripheral blood was drawn to TEMPUS™ Blood RNA Tube (Applied Biosystems) for the comparison of different RNA stabilizing collection methods for peripheral blood gene-expression studies.

4.1.4 Characterization of peripheral blood lymphocyte subpopulations

Simultest™ IMK-Lymphocyte Kit appended with $CD4^+/CD25^+$ marker (BD Biosciences) was used to characterize peripheral blood lymphocyte subpopulations in T1D prone and control children. Samples were collected in three to six month intervals during DIPP (Type 1 Diabetes Prediction and Prevention) study visits and processed same day according to the Simultest kit insert.

4.1.5 Samples collected from MS patients

Peripheral blood samples were collected from pregnant MS patients and pregnant healthy control persons at 10-12 gestational weeks (gw), at 26-28 gw and at 4-5 weeks postpartum (PP). In addition to samples drawn in the PAXgene™ Blood RNA System (Qiagen/BD) for peripheral blood gene-expression studies with microarrays, PBMC were collected with Ficoll-Hypaque™ PLUS (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation and frozen in DMSO for flow cytometry and as a dry pellet for RNA isolation at -135 ˚C. Serum samples were frozen at -40 ˚C.

4.1.6 Samples collected from children with T1D

Study subjects were recruited within the Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland (Kupila et al. 2001). Samples for peripheral blood gene-expression were collected with the PAXgene™ Blood RNA System (Qiagen/BD) in 3-6

month intervals. During the same visits to the DIPP clinic, the blood levels of T1D-associated autoantibodies (ICA, GADA, IAA and IA-2A) were measured.

4.1.7 Samples to study whole-blood RNA stabilization and mRNA amplification methods

To compare two methods available, peripheral blood from three healthy volunteers was collected simultaneously in TEMPUS™ or PAXgene™ collection tubes at two different times. After the blood was drawn, the samples were treated as recommended by manufacturer and stored at -70˚C. All samples were stored frozen 2-3 months before thawing and isolation of totRNA with ABI Prism™ Nucleic Acid PrepStation (TEMPUS™) or with specific spin columns (PAXgene™) according to manufacturer's instructions.

To address the long-term stability of PAXgene™ blood RNA samples as they are stored frozen at -70˚C, simultaneously drawn PAXgene™ samples were taken from two healthy volunteers. The first ones were directly isolated and the others after 6, 18 and 24 months. In addition, to asses stability of RNA in PAXgene™ tubes stored at room temperature, five tubes were simultaneously drawn from a single donor and processed on sequential days during a week.

4.2 Gene-expression microarrays

4.2.1 ImmunoChip cDNA microarray

All the cDNA microarrays used in this thesis work were specifically printed on glass slides at the Finnish DNA Microarray Centre, Turku Centre for Biotechnology. The sequences to be printed were picked from the Research Genetics' Human Sequence Verified 40K Library, or were cloned in-house. Some of the spots on these ImmunoChip(s) represent different cDNA clones from the same gene and some were empty spots that did not contain DNA. The printing configuration gave three technical replicates of each sequence placed on different parts of the slide. The first version of ImmunoChip (original publication I) included ~2000 sequences implicated in lymphocyte activation or differentiation including cytokines, chemokines and their receptors, transcription factors and genes involved in signaling, apoptosis and cell cycle regulation. An extended version of the ImmunChip contained an updated collection of ~5000 genes (used in original publication II).

All RNA samples, total RNA or amplified aRNA, were quality controlled by their A_{260}/A_{280} absorbance ratio detected using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis, or with Experion automated electrophoresis system (Bio-Rad Laboratories, Espoo, Finland). For each of the hybridizations, equal amounts of RNA from similarly treated test and reference sample (amplified or total RNA) were labeled directly during cDNA synthesis. The reference sample was always labeled with FluoroLink^{TM} Cy3-dUTP

(Amersham Pharmacia Biotech) and the sample of interest with Cy5-dUTP (Amersham Pharmacia Biotech). The labeled reference and the desired sample were mixed with hybridization reagents in one tube before hybridization.

Before hybridization printed slides were fixed with UV light (90 mJ) and pre-hybridized with Succinic Anhydride or bovine serum albumin (BSA). In the first case, 6 g of succinic anhydride was dissolved in 335 ml 1-methyl-2-pyrrolidinone and 15 ml borate (1 M, pH 8.0) was added immediately. The solution was poured into a slide-staining dish and microarray slides were plunged 5 times in the solution and mixed 15 – 20 min on orbital shaker at room temperature. Meanwhile, 750 ml sterile water was heated to boiling point and the slide rack was transferred to 95 ˚C water and plunged in hot water for 2 minutes. Lastly, the slide rack was plunged 5 times in 95% ethanol and slides were dried in a centrifuge. For BSA treatment, the slides were treated 30 min at 50 ˚C in buffer containing 5 x SSC, 0.1% SDS and 1% BSA, and washed first 3 min in 2 x SSC and then 3 min in 0.2 x SSC. Both SSC washes were done at room temperature.

The hybridization mixture containing labeled cDNA was placed on the array under a glass cover slip. Hybridizations were performed overnight at 65 ˚C in Corning hybridization chambers with humidity maintained by 3 x SSC. After hybridization, slides were dipped in 0.5 x SSC/0.1% SDS to remove the cover slip. Slides were then washed under agitation for 15 min in 0.5 x SSC/0.1% SDS, 15 min in 0.5 x SSC/0.01% SDS, 2 min in 0.06 x SSC and 1 min in 0.06 x SSC. All washing steps were performed at room temperature. After the last washing steps, the slides were dried one by one in microarray high-speed centrifuge (Telechem) or by centrifugation for 1 min/700 rpm.

4.2.2 Experimental design for cDNA microarrays

PBMC pool was used as a standard sample on microarrays (Churchill 2002). As a combination of expression profiles of 41 healthy individuals it was taken to represent normal expression, where signals provided by genes with high expression levels will be enhanced whereas genes with low expression level and random variation will disappear. Samples of interest were labeled with Cy5 and hybridized against Cy3 labeled reference RNA pooled from PBMCs and treated similarly as the test samples. Thus, all array-wise ratios give the expression rate against the same control, which significantly reduces variation caused by technical performance and simplifies the interpretation of the results.

4.2.3 Microarray quality control – RRPlot software

The quality of ImmunoChip hybridizations was assessed using RRPlot quality control tool. The latter was developed within this study for cDNA arrays printed in Finnish DNA microarray Centre, but can be applied to any array that contains two or more technical replicates and includes spots containing only print buffer without DNA. Many of the quality measures are based on the difference detected between

technical replicates, and “empty spots” are used to evaluate the quality of spotting and background intensity levels.

Instead of giving a binary decision concerning the array quality, the idea in RRPlot is to provide visualizations and measures to aid the user in determination of the array quality. Furthermore, removal of improper data points based on replicate statistics can also improve normalization (Aittokallio et al. 2003, Fan et al. 2004). RRPlot processes data in three steps: Filtering, normalization and visualization. First, it uses the signal measured from “empty spots” to set a cut-off value and filters the data by removing spots with intensity near or below “empty spots”. Second, the filtered data is normalized and different visualizations are drawn from the data. Visualization includes spot and background variation across the array, differences between technical replicates and intensities of the predefined empty spots. As a result, following options are available to handle the data measurements for each sequence: use the median of more than two good technical replicates; use the average of two remaining good technical replicates; use the only technical replicate accepted; or omit the sequence.

4.2.4 cDNA microarray analyses

Separate images for Cy3 and Cy5 dyes were acquired using ScanArray™ 5000 laser scanning microscope (Packard Biosciences). Images were then combined for data reduction in QuantArray® microarray analysis software from the same company. Gene transcript levels were determined from the fluorescent intensities of the scanned data image files. Systemic variation in measured intensity levels was removed using intensity dependent normalization with robust scatter plot smoothing algorithm (Yang et al. 2002).

InforSense Knowledge Discovery Environment (KDE) (InforSense, London, UK) was used to extract fold differences and statistical significance that were mainly used to detect differentially expressed genes. Filtering and normalization implemented in RRPlot (Yang et al. 2002, I) were recalculated from the raw data in KDE. The microarray data analysis web tool (MIDAW), built on R/Bioconductor packages (Romualdi et al. 2005, R Development Core Team 2008, Gentleman et al. 2004), was used for descriptive data analysis and feature extraction. In addition to KDE and MIDAW, Cluster 3.0 and TreeView software were used for clustering and visualization of the data (de Hoon et al. 2004, Saldanha 2004, Eisen et al. 1998).

4.2.5 Affymetrix GeneChip expression arrays

Affymetrix HG-U133A or U133 Plus 2.0 arrays were prepared according to the manufacturer’s small sample protocol, which includes an amplification step of total RNA. All total RNA were quality controlled with A_{260}/A_{280} absorbance ratio, determined with a NanoDrop ND-1000 detector (NanoDrop Technologies,

Wilmington, DE, USA) and agarose gel electrophoresis, or with Experion automated electrophoresis system (Bio-Rad Laboratories).

For T cell differentiation studies hybridized on Affymetrix HG-U133A arrays, data extraction was performed with Affymetrix GeneChip Microarray Suite, version 5 (MAS5). For the prospective sample series from diabetic children, robust multiarray average (RMA) procedure implemented in the Bioconductor affy package was used to process Affymetrix data (Gentleman et al. 2004, Irizarry et al. 2003).

4.2.6 Assessment of transcriptional profiles in longitudinal sample series

Differential expression in autoantibody positive (Ab^+) children or T1D-progressors as compared to healthy, persistently autoantibody negative (Ab^-) controls was mined using gene-level statistical analysis (Huang et al. 2001). Shortly, for each subject, expression intensity of probe set x at each time point was given a z-score, $z=(x-m)/s$, where m is the mean and s the standard deviation calculated for the probe x over all the time points in the sample series of Ab^- controls. Thus, z-score penalizes the probes that show high variation in control samples. A probe set was considered changed if $|z|>3$ at two or more time points in each of the Ab^+ and T1D-progressor sample.

K-means clustering with Euclidean distance was then applied to set of up- or down-regulated genes to investigate the overall behavior across the samples. The number of clusters was determined based on figure of metric (FOM) statistics (Yeung et al. 2001).

4.2.7 Computing inter-platform agreement

The inter-platform agreement was calculated between ImmunoChip and Q-RT-PCR, and between ImmunoChip and Affymetrix HG-U133A oligonucleotide microarray. With each platform, expression results were divided into three categories: Expressed in Th1, expressed in Th2, or equally expressed. For genes common to both platforms in a comparison, SAS system (v.8) was used to calculate unweighted Cohen's κ-statistics with one-sided *p*-values (Gwet 2002).

4.2.8 Network and pathway level analyses of gene-expression

Gene-expression results were viewed on a pathway level with Ingenuity Pathway analysis (Ingenuity® Systems, www.ingenuity.com). In addition, Gene Set Enrichment Analysis (GSEA) was used to map differential gene-expression to enriched pathways (Subramanian et al. 2005, Mootha et al. 2003). Final GSEA analysis was made based on gene rankings detected with Bioconductor RankProd package (Breitling et al. 2004).

4.2.9 Comparison of peripheral blood collection tubes with Illumina® expression arrays

Total RNA extracted from TEMPUS™ and PAXgene™ tubes and pooled total RNA from PBMC were first amplified for gene-expression studies with RiboAmp® OA 1 Round RNA Amplification Kit (Arcturus). Purified cDNA was then used as a template for *in vitro* transcription to synthesize labeled cRNA with Illumina® RNA Amplification Kit (Ambion®). Labeled cRNA was hybridized on Illumina Sentrix HumanRef-8 Expression BeadChips according to manufacturer's protocol. Cubic spline normalization method (Workman et al. 2002) was applied simultaneously with data extraction in Illumina Inc. BeadStudio (version 1.5.0.34). Normalized data was filtered first by detection values generated in BeadStudio and after that second filtering based on measured expression intensity was applied. Data sets from different sample types were separately filtered. Illumina probes with detection value ≥0.9 (detection = 1 - *p*-value) and measured intensity level ≥100 in at least one of the sample collection methods, were considered for further analysis.

4.3 Verification of differential gene-expression

A variety of different methods can be applied to verify differential gene-expression detected with microarrays. For each study the choice of verification method was based on available sample material and overall goals and experimental setup.

4.3.1 Array probe sequencing

All cDNA clones giving an interesting expression signal on the array were sequenced with Applied Biosystems ABI PRISM 3100 Genetic Analyzer using universal primers. Sequencing was performed from the same plates used for printing the arrays and sequencing results were then blasted against databases.

4.3.2 Q-RT-PCR

Quantitative real-time RT-PCR studies were performed using Applied Biosystems TaqMan gene-expression assays on ABIPrism 7700 Sequence Detection System. Housekeeping genes were used to normalize the RNA levels (Hamalainen et al. 2001b).

4.3.3 Enzyme-linked immunosorbent assay (ELISA)

Soluble HLA-G (sHLA-G) ELISA (Exbio, Prague, Czech Republic) was performed as described (Wiendl et al. 2005).

4.3.4 Flow cytometry

Monoclonal antibodies were used to detect HLA-G expression with flow cytometry. PBMC were washed with 1 x PBS supplemented with 0.1% BSA and 0.1% sodium azide, and incubated with monoclonal antibodies for 30 min at 4 ˚C. Cells were analyzed with FACS-Calibur™ using Cell Quest™ software (Becton Dickinson, Heidelberg, Germany). Monoclonal antibodies are listed in original publication III.

5. RESULTS AND DISCUSSION

5.1 ImmunoChip cDNA microarray

Our goal was to design and produce a targeted custom printed cDNA microarray for transcriptional profiling of immune-mediated diseases. To achieve this, we chose a comprehensive selection of genes belonging to the key signaling pathways and molecular networks implicated in human immune system. At the same time, ImmunoChip project was used to set up cDNA microarray manufacturing processes in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. The first version of the ImmunChip, including c.a. 2400 different transcripts, was used to validate the platform (I). For the final version of ImmunoChip that was later used, among many other things, to study postpartum activation in multiple sclerosis (II), the number of immune response genes on the array was expanded to around 5000. Due to the higher number of transcripts, changes to print configuration were required but no other changes were introduced. The original number of transcripts was adequate for reliable analysis of the cDNA array performance.

Although many commercial microarray platforms are available today and competition has lowered the price per sample, large genome wide microarray studies are still rather expensive to perform. However, the price structure between commercial and home-brew array platforms has balanced, or in some cases even turned the other way round, such that customized oligonucleotide probe selections are available from several manufacturers. Thus, oligonucleotide platforms have overtaken DNA probes, which require laborious and costly maintenance of clone libraries and are more error prone during manufacturing. Nevertheless, the ImmunoChip showed to be valuable tool to analyze immune-related gene-expression.

5.1.1 Technical quality of the ImmunoChip

The clones selected for ImmunoChip were picked from the Research Genetics' Human Sequence Verified 40 K Library, which showed 83% agreement between library annotation and our sequencing results from array print plates. This was fairly good number in comparison with other studies, for example, using Research Genetics' and IMAGE consortium (Integrated Molecular Analysis of Genomes and their Expression) cDNA clone libraries, which reported 62.2% (Halgren et al. 2001) and 79% (Taylor et al. 2001) concordance rates, respectively.

We developed the RRPlot quality control tool to assess hybridization quality. Pair wise overlay images of the three technical replicates printed on the same slide provided user-friendly visualization that allowed quick localization of potential problems (I, Figure 2). Though all the hybridizations of the validation experiments were of high quality and did not require any hybridizations to be excluded or repeated, removal of array spots of bad quality in the RRPlot improved the data

significantly (I, Figure 3). The importance of data filtering before normalization was emphasized by the behavior of the average replicate correlation in filtered and normalized data (Pearson p = 0.930), as compared to raw data (Pearson p = 0.895) and normalized data before filtering (Pearson p = 0.578). (I)

In addition to hybridization quality control, RRPlot is applicable for pre-hybridization quality control. Arrays printed in a salty buffer can be raw-scanned prior to the pre-hybridization washes to assess availability of array targets revealed by light scattering from the salt crystals. Thus, valuable samples will not be wasted on arrays lacking the transcript of interest.

5.1.2 Inter-platform agreement of the ImmunoChip

In order to evaluate the sensitivity of the ImmunoChip to detect differentially expressed transcripts, we performed a mutual validation between ImmunoChip, commercial oligonucleotide array and Q-RT-PCR with a case study of T cell differentiation (I). Considering the high influences of cell culture conditions and sample treatments have on gene-expression, direct comparisons between different platforms were limited to experiments done within the same lab under similar conditions. Time points and conditions for cell cultures were matched to previously published Q-RT-PCR results from our group (Chen et al. 2003, Chen et al. 2003, Lund et al. 2003a, Lund et al. 2003b, Hamalainen et al. 2000, Hamalainen et al. 2001a). Cohen's κ-statistics was used to assess similarity between ImmunoChip and previously reported Q-RT-PCR results (I, Table 1). In addition, at the 7 day time point total RNA of the untreated and activated Th1 and Th2 cells from two different individuals was hybridized to Affymetrix HG-U133A oligonucleotide arrays, similarity values of k=0.30 (p=0.0003) for polarized and k=0.43 (p<0.0001) for polarized and activated samples were observed (I, Figure 5).

Although there are differences in platform and sample preparation, the results obtained with ImmunoChip are in agreement with previous data (Chen et al. 2003, Lund et al. 2003b, Chtanova et al. 2001, Hamalainen et al. 2001a, Lund et al. 2003a, Rogge et al. 2000). In our validation study, the same samples hybridized on oligonucleotide arrays confirmed majority of the gene-expression changes detected with the ImmunoChip. These findings are further supported by studies reporting cDNA and oligonucleotide arrays as complementary rather than competing platforms, although comparison of the results from different platforms is not straightforward (Lee et al. 2003, Yuen et al. 2002, Järvinen et al. 2004, Petersen et al. 2005). Furthermore, general inter- and intraplatform reproducibility of gene-expression measurements was demonstrated by the MicroArray Quality Control (MAQC) project, though no cDNA array corresponding to ImmunoChip was included in the study (MAQC Consortium et al. 2006).

5.2 Transcriptional profiling of whole peripheral blood

The success of a microarray experiment is highly dependent on the quality of the sample RNA. Therefore, to ensure adequate amounts of high quality RNA were available for the transcriptional profiling of peripheral blood, we first optimized blood collection and sample RNA processing. We also examined the longitudinal variation in relative composition of peripheral blood lymphocyte subpopulations in children at risk of T1D and their genotypically matched controls.

5.2.1 *Ex vivo* stabilization and following mRNA amplification allow transcriptional profiling of peripheral blood samples

Two well-established methods, TEMPUS™ Blood RNA Tube (Applied Biosystems) and PAXgene™ Blood RNA System (PreAnalytix, Qiagen/BectonDickinson) are available for *ex vivo* transcriptional profiling and biomarker discovery from human peripheral blood. Both methods include RNA stabilizing reagents in the collection tube to ensure that the transcription profiles reflect the physiological state at the time the blood was drawn, thus providing a feasible and much needed means for gene-expression profiling and biomarker discovery from clinical sample material. Even though both manufacturers promise stable transcription profiles for collection tubes stored for days at room temperature, our preliminary finding with PAXgene tubes was that this leads to the degradation of RNA (Figure 2). Similar degradation of RNA in PAXgene tubes stored at room temperature was later reported (Kagedal et al. 2005). As our study designs did not allow immediate processing of collected peripheral blood samples, collection tubes were frozen and stored at -70˚C after the blood was drawn. We first performed a parallel comparison of the two methods available and demonstrated the long-term stability at -70˚C for PAXgene Blood RNA System, which was chosen for subsequent studies (II).

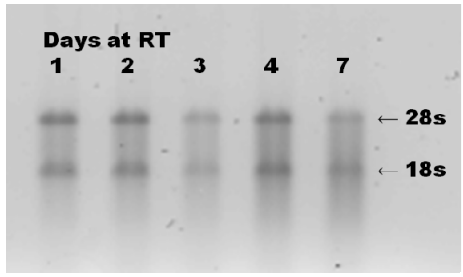

Figure 2. Poor intensity ratio of 28s and 18s RNA at day 7 indicates that RNA stabilization is incomplete and PAXgene tubes are not suitable for long-term storage at room temperature (RT). All tubes were collected at the same time from the same donor and processed after 1, 2, 3, 4 and 7 days of storage at RT.

Both the TEMPUS and PAXgene blood RNA systems yielded total RNA of high quality (A260/A280 > 1.93), but with low quantities, showing averages of ~2.5 µg for PAXgene and ~5.5 µg for TEMPUS tubes. Different blood volumes of 2.5 mL in PAXgene and 3 mL in TEMPUS cannot completely explain the difference in total RNA yield, since TEMPUS resulted in almost twice as much of total RNA/mL of blood than PAXgene. However, amplification of mRNA is required with both methods to ensure sufficient starting material for further microarray and/or validation experiments.

For effective labeling of minute amounts of total RNA for ImmunoChip cDNA array, we first tested signal amplification with a dendrimer based 3DNA technology (Genisphere Inc., Hatfield, PA, USA). This captures gene-expression signatures without RNA amplification, using only 1 – 5 µg of total RNA as a starting material. Even though dendrimer labeling was successful (data not shown), amplification of mRNA was chosen to preserve original total RNA for possible further needs, e.g. for processing the same sample to be analysed with different platforms. The 3-10 times higher relative amount of labeled aRNA targets when compared to the corresponding mRNA among the total RNA targets, has also been reported to increase the sensitivity of the assay (Nygaard and Hovig 2006).

The high relative abundance of globin mRNA has been reported to mask other transcripts in Affymetrix small sample protocol (Shou et al. 2005, Vartanian et al. 2009, Li et al. 2008), resulting in a signal decrease and increased replicate variability. We noticed that our chosen RiboAmp® mRNA amplification (Arcturus/Applied Biosystems) did not notably amplify globin mRNA (II, Figure 1). Therefore, although globin mRNA is over-represented in RNA isolated by both sample collection methods, additional globin depletion kits are not necessary with RiboAmp. Whereas Shou et al. claim reduced haemoglobin interference with TEMPUS tubes after a Affymetrix small sample protocol (Shou et al. 2005), our data suggests similar performance for both TEMPUS and PAXgene tubes after RiboAmp RNA amplification (Figure 3). We also detected that enzymatic depletion of globin transcript reduces the quality of total RNA and thus is not recommended (II, Figure 1).

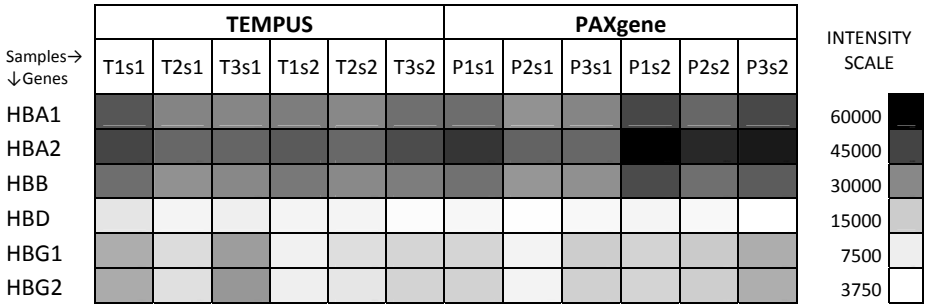

Figure 3. Samples collected using TEMPUS and PAXgene methods (columns) show similar expression of hemoglobin genes (rows) on Illumina Sentrix HumanRef-8 Expression BeadChips after RiboAmp OA 1 Round RNA Amplification. Numbers one and two after the tube ID (T=Tempus, P=PAXgene) indicate different individuals, whereas suffixes s1 and s2 indicate the order in which the samples were collected.

Altogether 5200 transcripts, about 20% of all the transcripts on Illumina Sentrix HumanRef-8 Expression BeadChip, were detected in RNA derived from TEMPUS, PAXgene or peripheral blood mononuclear cells (PBMC). Roughly 4000 transcripts were detected in each different sample type and 3000 of those transcripts were common to all three (II, Supplemental Figure 1). Although the comparison of differential expression between PBMC pool and peripheral blood collected with RNA stabilizing methods was not in our interest, the pooled reference was included in the study as a common reference sample in studies requiring comparative dual-label hybridization. In a large pooled sample, highly expressed transcripts will mask those with low abundance. For reliable measurement of relative expression in TEMPUS or PAXgene samples against the PBMC pool on the same array, it is important that the same transcripts are present with both methods. High overlap with PBMC also demonstrates that most of the signal detected with the stabilizing collection tubes arises from mononuclear cells.

Overall similarity of the three sample types was further assessed with principal component analysis (PCA) and Euclidean clustering. In addition, Pearson product moment correlation coefficients were calculated. The correlation within TEMPUS or PAXgene samples is remarkably good ($r \geq 0.94$) when samples are drawn at the same time. In PAXgene tubes collected to test stability of the RNA in frozen tubes after 6, 18 and 24 month storage periods (Samples P4 and P5 in Figure 4), intra-individual correlation was even higher ($r \geq 0.98$). The same phenomenon appeared when principal component analysis (PCA) and Euclidean clustering were applied on differentially expressed transcripts (Figure 4); microarray experiments are grouped according to sample types and the time of the sample collection.

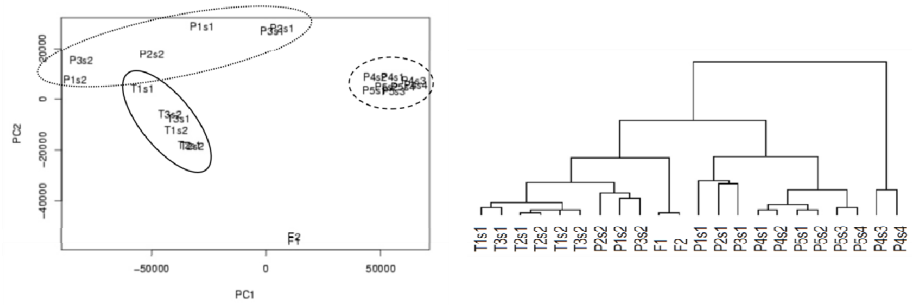

Figure 4. Principal component analysis (PCA) and clustering results of gene-expression measured from TEMPUS (T1-3) and PAXgene (P1-3) tubes and from peripheral blood mononuclear cells (F). Numbers after the sample ID indicate different individuals and suffixes s1-s4 the order in which the samples were collected. For PBMC samples F1 and F2 indicate technical replicates from the same pooled sample. **Left:** PCA shows closer distribution of TEMPUS samples (solid ellipse) as compared to PAXgene samples (dotted ellipse). The PAXgene results obtained after freezing (dashed ellipse) and PBMC samples (F1 and F2 at the bottom) form clear distinct clusters. **Right:** The euclidean cluster tree (complete linkage) shows TEMPUS samples clustering more closely with each other than PAXgene samples. The PAXgene samples taken to test stability in response to freezing (P4 and P5) are separated from the others. The PBMC samples (F1 and F2) form their own cluster closer to PAXgene than TEMPUS samples.

The studies reported in publications III and IV of this thesis give examples of clinical studies where sample collection is continuous and samples are collected from the same individual as a function of time. In both studies, collected samples had to be transported and stored for extended periods prior to the centralized processing of the samples of interest. As a benefit, this sample collection strategy allows better planning of the experiments. Large prospective studies can be carried out so that only a subset of all collected samples will be processed, rather than processing every single sample before knowing which ones to include based on the clinical data. Technical bias is also likely to reduce when the samples are processed in representative batches - or all in parallel. To overtake sample collection problems, we demonstrated that PAXgene™ tubes can be frozen to -70˚C for extended storage and transportation. Undoubtedly, the same applies for TEMPUS™ tubes, which have been tested and adapted for other studies (Shou et al. 2005, Matheson et al. 2008, Prezeau et al. 2006). Today both two methods are available with manual and automated extraction systems, but at the time the data was generated the TEMPUS™ system was only available with an automated ABI 6100 extraction instrument (Applied Biosystems). In our case studies, the flexibility of the manual spin-column RNA purification favored the selection of PAXgene™ Blood RNA kit.

In these measurements, we have observed that, hundreds of transcripts are differentially expressed solely as a result of collection in either the PAXgene™ or

TEMPUS™ tubes; therefore, usage of both tube types in the same experimental setup cannot be recommended. Taken together, we have here successfully demonstrated the long-term stability of RNA as frozen in PAXgene™ tubes and that PAXgene™ Blood RNA method combined with RiboAmp® mRNA amplification is suitable for biomarker discovery in clinical studies.

5.2.2 Relative proportions of lymphocyte sub-populations show little or no variation in longitudinal peripheral blood samples

Cellular composition has been shown to affect transcriptional profiles measured from peripheral blood RNA extracted with the PAXgene Blood RNA System (Qiagen/BD), and at the same time temporal variation was detected in inter-individual samples collected (Whitney et al. 2003). Thus, we wanted to see the level of intra-individual variation in different lymphocyte populations in pre-diabetic children and their matched control population. Three consecutive follow-up samples were analyzed from 30 pairs of children with diabetes associated autoantibodies and their controls (herein Ab^+ and Ab^-, respectively). There was little longitudinal variation in any of the measured lymphocyte subpopulations (Figure 5), indicating that changes in transcription profiles would reflect intracellular modifications rather than changes in relative proportions of peripheral blood lymphocyte populations. Statistical analysis with a T-test did not reveal any significant changes between the groups of Ab^+ and Ab^- children. Temporal variation in lymphocyte subpopulations was not detected in either group, although there was a slightly significant increase of activated T lymphocytes in Ab^+ children between the first and second samples. It seems that the proportion of the activated T lymphocytes keeps increasing in Ab^+ children, but is not anymore significant due to high inter-individual variation. The reported percentage increase of activation markers, including HLA-DR, from infancy to adulthood (Hulstaert et al. 1994) and similar trend seen in Ab^- children indicate that this is associated with maturation rather than being a consequence of immune activation in Ab^+ children.

Whitney et al. also reported a gender related cluster of differentially expressed autosomal transcripts in adults (Whitney et al. 2003) and gender bias is widely associated with autoimmunity (Zandman-Goddard et al. 2007). Thus, it might be worthwhile to study the gender effect on gene expression also in juvenile cohort. However, as we are here studying for difference in intra-individual variation between Ab^+ and Ab^- children within a longitudinal sample collection, gender effect is abrogated. Age was not considered in this analysis either, as with few exceptions the samples were collected between the ages of 1 and 5 years, and the relative sizes of the subpopulations fall into the expected range (Hannet et al. 1992). We did not detect such inter- or intra-individual variation over time, or differences between control and at risk T1D populations, that could be used to match samples for the microarray analysis.

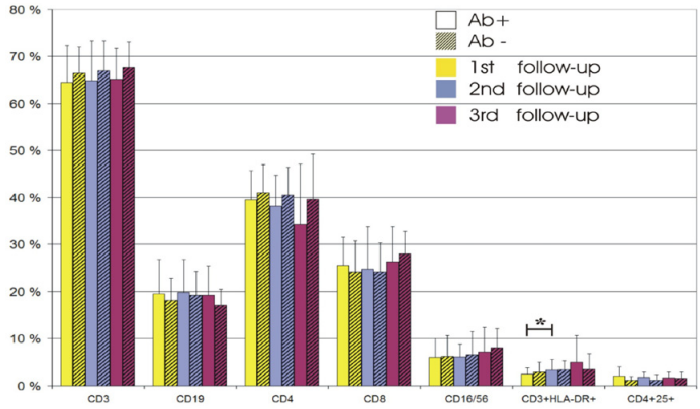

Figure 5. Average distribution of T lymphocytes (CD3), B lymphocytes (CD19), helper/induced lymphocytes (CD4), suppressor/cytotoxic lymphocytes (CD8), natural killer lymphocytes (CD15/56), activated T lymphocytes (HLA-DR) and regulatory type T cells (CD4/CD25) is similar between the groups of children with diabetes-associated autoantibodies (Ab^+) and controls (Ab^-). Within both groups, the relative proportions of the lymphocyte populations remain relatively constant over time. The only statistically significant difference (T-test, $p < 0.05$) resulted between first and second sample in activated T lymphocytes from Ab+ children (marked with an asterix).

5.3 Gene-expression signatures characterizing activity of organ-specific autoimmune diseases

5.3.1 Tolerogenic HLA-G was associated to postpartum-activation of multiple sclerosis

In order to assess the determinants of the immune system that modulate pregnancy-related disease activity in MS, transcriptional profiles from three patients with relapsing-remitting MS were compared to those measured from matched pregnant controls. Samples collected during the third trimester of pregnancy and 4 – 5 weeks postpartum were analyzed with ImmunoChip cDNA microarray. Principal component analysis of the filtered and normalized array data indicated clear difference in postpartum gene regulation between patients and controls (III, Supplemental Figure 1). We therefore focused on those transcripts that behave differentially between patients and controls when postpartum samples are compared to the samples from the last pregnancy trimester.

Of the few transcripts with indisputably differential regulation in patients and controls, HLA-G was the most prominent one (III, Figure 1). The expression of HLA-G was verified in wider sample material using Q-RT-PCR for HLA-G mRNA levels in PBMC (III, Figure 2A-B), ELISA for soluble HLA-G (III, Figure 3) and flow cytometry to investigate frequency and relative changes of HLA-G expressing PBMC (III, Figure 2C-

F). All verification assays corroborated the detected decrease in HLA-G activity, when postpartum disease activity was considered in the analyses. In the stable group of MS patients, a decrease of HLA-G protein level was not detected (III, Figure 2D and 2F). This indicates that HLA-G is critically involved in the immunoregulatory mechanism that balances inflammatory disease activity in MS.

HLA-G is a non-classical class I MHC molecule that is capable of direct inhibition of virtually all immune-effector subset, thus providing a shield against inflammation (Carosella and Lemaoult 2011). In addition, HLA-G can influence the development of regulatory and suppressor T cells (Feger et al. 2007, LeMaoult et al. 2004). In a recent study assessing the frequencies of circulating Th17 and Treg cells in a corresponding sample collection setting to ours, neither Th17 nor Treg cells were involved in MS amelioration or disease course alteration (Neuteboom et al. 2010). On the other hand, HLA-G^+ Treg cells are shown to accumulate at the sites of MS inflammation (Wiendl et al. 2005) and their function is not impaired in MS patients (Zozulya and Wiendl 2008). Thus, the suggested decrease of HLA-G^+ $CD4^+$ and HLA-G^+ $CD8^+$ lymphocytes in patients with increased disease activity may indicate a loss in functionally relevant HLA-G^+ Treg cell population. Furthermore, the lower baseline level of HLA-G in MS patients, compared to controls, implicates the loss in tolerogenic function of HLA-G in regulation of disease activity.

In conclusion, we identified HLA-G as an immunoinhibitory molecule with an inverse correlation to activity of MS. The protective role of HLA-G in MS is further supported by decreased levels of soluble HLA-G in cerebrospinal fluid (CSF) of relapsing-remitting MS patients with high disease activity (Fainardi et al. 2008). It remains to be seen if peripheral levels of HLA-G could predict MS activity or probability of relapses. Although the regulation mechanisms of HLA-G are still unclear, it might also provide therapeutic strategies to target autoinflammation.

5.3.2 Early suppression of immune response pathways characterizes the prediabetic phase in T1D-prone children

Prospective peripheral blood samples from children who had developed T1D associated autoantibodies (Ab^+) and eventually clinical T1D were analyzed to reveal transcriptional profile of diabetes-associated autoimmunity. PAXgene blood RNA samples were collected to monitor the disease progression in altogether five children, from which two remained Ab^+ and three progressed to clinical T1D. For T1D progressors, age, gender, birthplace and HLA-genotype matched controls with no diabetes-associated autoantibodies were also monitored. Unsupervised principal component analysis (PCA) was applied to visualize the overall variation of genome-wide transcription profiles in altogether 60 samples. Transcriptional profiles of autoantibody positive (Ab^+) children and the T1D progressors are indistinguishable in the two-dimensional plot of the first two principal components (IV, Figure 1A), suggesting that the primary determinants of global gene-expression overlap between Ab^+ children and those who progress to clinical T1D. The segregating variation in gene-expression was indeed seen already in the first samples collected

soon after autoantibody appearance. Thus, samples collected already before seroconversion would better characterize the change in transcriptional profiles.

Closer investigation of differential longitudinal variation in the expression of individual transcripts revealed consistent changes in 520 probe sets representing 424 different genes, when Ab^+ children and T1D progressors were compared to the healthy Ab^- controls. Roughly, half of the probe sets were up- and the other half down-regulated. The functionality of the analysis setup was demonstrated by the detection of several genes with previous association to T1D, such as PTPN22/LYP, dexamethasone-induced transcript (DEXI), LPS-induced TN factor (LITAF) and nipsnap homolog 1 (NIPSNAP1) (Barrett et al. 2009, Bottini et al. 2004, Hermann et al. 2006). In addition, altogether 18 genes from the HLA region were detected. Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) and gene set enrichment analysis (GSEA) (Subramanian et al. 2005) were then applied to emphasize co-expression networks within the differentially expressed gene set and to detect dynamic activation/repression of molecular pathways during T1D progression, respectively.

IPA clustering was able to identify a highly connected network of down-regulated immune system genes (IV, Figure 2) with biological functions assigned to cellular growth and proliferation ($p = 10^{-7}$), hematological system development and function ($p = 10^{-7}$) and immunological disease ($p = 10^{-8}$). The repression of HLA region was prominent and not restricted to the Class II genes, but HLA Class I genes are also present. The association of this network to central immune system genes, such as T cell receptor (TCR), insulin, NF-kB and transporter associated with antigen processing (TAP), demonstrates that Ab^+ children who are progressing towards clinical T1D suffer from a massive down-regulation of genes contributing to critical functions of the immune system. General suppression of immune response pathways was further demonstrated in GSEA analysis performed separately on samples collected 0 – 6 months after seroconversion or 0 – 3 months preceding T1D diagnosis, where altogether 37 down-regulated and only 3 up-regulated pathways were detected (IV, Table 2). Of the down-regulated pathways, 13 are shared between early and late detection points. In addition to down-regulation of ribosomal pathways, indicating reduced activity of translational machinery, these shared pathways were related to functions that are likely to be relevant for T1D development, such as lymphocyte signaling and proliferation, antigen procession and presentation. Interestingly, in a meta-analysis with published GWAS data for T1D and six other common diseases, significantly overrepresented pathways for T1D show overlap with the pathways we detected (Torkamani et al. 2008).

The detected down-regulation in translational activity of the immune system is in contradiction with previous attempts to characterize T1D related transcriptional profiles in peripheral blood cells, where activation rather than suppression of immunological functions has been reported (Kaizer et al. 2007, Collins et al. 2006, Reynier et al. 2010). All these studies identified different gene sets suggesting pro-inflammatory response in T1D patients. This is not surprising regarding the differences in sample collection and processing. Only one of the studies used RNA a

stabilizing blood collection system, and different microarray platforms and data analysis methods were applied. In a recent review article Carey, Purohit and She argue in favor of the pro-inflammatory state in T1D patients and in particular criticize our study on the basis of the limited number of different subjects (Carey et al. 2010). However, they fail to notice that in our longitudinal follow-up the high number of biological replicates increases study power. In addition, our study setting is unique. Instead of measuring relative gene-expression differences between two different conditions, our results demonstrate consistent repression of immune system genes in each individual with ongoing T1D autoinflammation.

Down-regulation of genes implicated in the immune system has been previously detected in $CD4^+$ T cells from T1D patients (Orban et al. 2007, Jailwala et al. 2009). In general, our results are in line with previous studies that show anergic and impaired TCR signaling in patients with T1D (Salojin et al. 1998, Dosch et al. 1999). An attenuated lymphocyte activation capacity in T1D may result from the lack of free ribosomal proteins (Cooper and Braverman 1977, Kay et al. 1975), which is supported by detected down-regulation of ribosomal pathways. Several of the other pathways are linked with T1D also. Insulin is a major autoantigen in T1D (Nakayama et al. 2005) and down-regulated thymic expression of insulin has been associated with insulin gene polymorphism (Pugliese et al. 1997). Furthermore, low insulin expression level in the thymus may lead to the escape of insulin-reactive T-cells (Vafiadis et al. 1997). Down-regulation of insulin signaling may be a result from ongoing autoimmunity against insulin that first leads to decreased insulin expression, which is then reflected at a systemic level repression of insulin-regulated genes and pathways. Other down-regulated pathways with T1D associated genes include IL-2 receptor signaling (Yamanouchi et al. 2007) and CSK, a negative regulator of PTPN22 (Bottini et al. 2004, Vang et al. 2005). The detected down-regulation of IL-12 receptor β supports the function of IL-2 in T1D pathogenesis and the defect in IL-2 signaling may cause impairment of Treg cells (Jailwala et al. 2009, Kaye et al. 1986). Defects in the NF-kB and CD40 pathways have been reported in patients with T1D and both these pathways are important for the function of antigen presenting cells (Mollah et al. 2008). Our results also suggest prediabetic functional defects in $CD8^+$ T cells and B cells, which together have been proposed to promote T1D development (Marino and Grey 2008).

At the late phase of T1D, several pathways were linked to signaling via PI3K. PI3K is an upstream activator of serine/threonine kinase AKT, a key player in growth, proliferation, metabolism and survival (Manning and Cantley 2007). PI3K/AKT signaling also inactivates NFAT (Okkenhaug et al. 2007), which fits to our observed up-regulation of the NFAT pathway in late samples preceding clinical T1D. In lymphocytes, PI3K has a pivotal role in insulin receptor and antigen receptor signaling. Especially the catalytic p110δ isoform, encoded by PI3KCD that is down-regulated in our data, is important for T and B cell development and function (Okkenhaug et al. 2007, Bilancio et al. 2006, Okkenhaug et al. 2002). In type 2 diabetes PI3K/AKT signaling has been associated with beta-cell survival and insulin resistance (Elghazi and Bernal-Mizrachi 2009), but no studies with T1D have appeared yet.

5.3.3 Decreased transcriptional activity of MHC Class Ib molecules was associated with autoimmune activation in both MS and T1D

Autoimmune susceptibility alleles are found independently from HLA Class II (HLA-DR/DQ) locus from classical HLA class Ia (HLA-A and HLA-B) and non-classical HLA class Ib (HLA-E, HLA-F and HLA-G) (Eike et al. 2009, Hodgkinson et al. 2000, Nejentsev et al. 2007, Burfoot et al. 2008). Class Ib molecules HLA-E, HLA-F and HLA-G have all been implicated in Treg cell mediated immunity. HLA-F is a surface marker of activated lymphocytes and regardless of Treg cells, all B cells, NK cells, monocytes and T cells express HLA-F after activation (Lee et al. 2010); the authors suggest that this activation induced surface expression of HLA-F is a message of immune system activation to the Treg cells. On the other hand, we know that HLA-G can influence the development of regulatory and suppressor T cells (Feger et al. 2007, LeMaoult et al. 2004) and in MS the decrease of HLA-G^+ $CD4^+$ and HLA-G^+ $CD8^+$ lymphocytes was associated with increased post-partum disease activity, probably due to a loss in functionally relevant HLA-G^+ Treg cell population. Also HLA-E may induce autoinflammation via impaired regulatory mechanism: Defects in HLA-E–restricted $CD8^+$ Treg cells to discriminate self- and non-self peptides in the periphery are linked to development and control of human T1D (Jiang et al. 2010). Thus, it is intriguing to speculate that lost transcriptional activity of these MHC Class Ib molecules could impair the delicate regulatory balance maintained by Treg cells and push the immune system towards autoimmune disease.

The HLA gene transcripts repressed in our studies included the non-classical class I MHC molecules (class Ib) HLA-E, -F, and -G. All three of which were down-regulated in children progressing towards T1D. In MS, not only HLA-G but also HLA-F was down-regulated when samples collected during and after pregnancy in MS patients were compared to healthy controls. The transcriptional profile of HLA-F was very similar to that of HLA-G, but the expression ratios were not as remarkable and thus less attention was paid for HLA-F when the results were first analyzed. More recently, HLA-F has been associated with increased susceptibility to MS in a Tasmanian population (Burfoot et al. 2008). Despite the similarity in detected expression profiles, these three class Ib genes differ in the nature of their transcriptional regulation (Gobin and van den Elsen 2000).

Contrary to the decreased peripheral blood HLA expression in children progressing towards T1D, up-regulation of all three has been reported in human pancreas and purified islets in T1D already at day 5 after the disease onset (Planas et al. 2010). It is probable that this up-regulation is not reflected in peripheral blood at all, or it would require more samples to be collected at later time points. Previously, down-regulation of HLA Class II expression has been reported in peripheral monocytes of T1D patients and down-regulation of HLA has been suggested to protect from ongoing autoimmunity (Parkkonen et al. 1993). For HLA Class I, either down-regulated (Fu et al. 1993, Faustman et al. 1991) or normal (Hao et al. 1996), expression has been reported in peripheral blood lymphocytes in T1D. The level of soluble HLA-G was studied in the three children who progressed to T1D (IV), using samples collected in parallel with the PAXgene tubes for transcriptional profiling.

However, no correlation was seen with soluble HLA-G and T1D progression (unpublished data). The less dramatic change in physiological conditions may partly explain this lack of correlation. Also, whereas soluble HLA-G circulating in maternal blood is known to mediate immunosuppressive functions during pregnancy (Hunt 2006), in T1D related autoimmunity the soluble forms of HLA-G may not be as important as its capability to influence the development of regulatory and suppressor T cells (Feger et al. 2007, LeMaoult et al. 2004). T1D-related surface expression of HLA-G in different lymphocyte subpopulations remains to be characterized.

6. CONCLUSIONS

In this Ph.D. thesis, transcriptional profiling with microarrays was used to monitor differentially expressed gene transcripts in peripheral blood of individuals with an autoimmune disease. Although the methods to capture whole genome transcriptional profiles in peripheral blood are now well established and readily available, due to status of this methodology at the beginning of this study, the first goal of this thesis work was to develop and characterize a cDNA microarray customized for targeted study of signal transduction and molecular networks in immune-mediated diseases. Furthermore, clinical sample collection and processing were optimized to allow gene-expression profiling of peripheral blood samples resulting minute amounts of total RNA.

The ImmunoChip cDNA microarray manufactured at the Finnish DNA Microarray Centre proved to be a valuable tool for transcriptional profiling of immune-mediated diseases. The agreement between ImmunoChip, Q-RT-PCR and commercial oligonucleotide microarrays was shown in a case study of T cell differentiation and the ImmunoChip was then used to study pregnancy related alterations in gene-expression in patients with multiple sclerosis. In spite of the success with the ImmunoChip, the rapid expansion of microarray technologies and increased competition between commercial array platforms allowed transition to genome-wide transcriptional profiling during between the studies included in this Ph.D. thesis. However, the methodology itself is still valid for clinical disease monitoring by gene-expression profiling of peripheral blood.

Characterization of pregnancy-related disease activity in MS identified a HLA class Ib molecule HLA-G as an immunoinhibitory molecule with inverse correlation to disease activity. HLA-G is known to influence maternal-fetal tolerance in pregnancy, and to participate in control of the immune system by directly inhibiting virtually all immune-effector subset. All verification assays corroborated the decreased HLA-G activity in patients with increased postpartum disease activity. In the stable group of MS patients a decrease of HLA-G at the protein level was not detected, indicating that HLA-G is critically involved in the immunoregulatory mechanism that balances inflammatory disease activity in MS. Furthermore, using data from our two independent studies with MS and T1D, decreased peripheral blood transcriptional activity of HLA class Ib molecules HLA-E, HLA-F and HLA-G was associated with organ-specific autoimmunity. Recent studies have implicated all three, HLA-E, HLA-F and HLA-G, in development and function of Treg cells. Thus, further studies on the role of HLA Class Ib molecules in development of autoimmunity are suggested.

Investigation of transcriptional profiles of prospective whole-blood samples from children with T1D-associated autoantibodies and subsequent development of T1D revealed general suppression of immune response pathways, including previously known key components of the immune system and several novel T1D-associated pathways. However, the autoinflammation towards T1D was clearly visible already in the first analyzed samples after appearance of autoantibodies, and thus timing of

transcriptional changes with the initiation of autoimmune inflammation was not possible. Studies on samples collected before and after seroconversion might further and more precisely characterize the molecular mechanisms leading to T1D. Further studies should also address epigenetic control of gene expression, since epigenetic factors are potential mediators for gene – environment interaction and have recently been implicated in regulation of autoimmunity.

Taken together, these studies have demonstrated the potential of transcriptional profiling in biomedical research and diagnostics. Transcriptional profiling of patients at different stages of the disease is a functional approach to identify factors influential for the disease activity, and whole-blood samples drawn into RNA stabilizing collection tubes allow this disease monitoring without *ex vivo* manipulation altering the expression profiles. Moreover, longitudinal genome-wide data was shown to reveal dynamic changes that are relevant for the disease but might be completely missed in conventional cross-sectional studies or in genome-wide association studies.

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