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Proteomic Profiling of Mouse Helper T Cell Differentiation

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Helper T cell differentiation is a key process in the regulation of adaptive immune responses. Here, mouse Th1 and Th2 cells are profiled using high-throughput proteomics to increase the understanding of the molecular biology of Th differentiation to support the design of prophylactic and therapeutic intervention strategies for (infectious) diseases. Protein profiling of Th1/Th2 differentiated cells results in the quantification of almost 6000 proteins of which 41 are differentially expressed at FDR < 0.1, and 19 at the FDR < 0.05 level, respectively. Differential protein expression analysis identifies a number of the expected canonical Th differentiation markers, and gene set analysis using the REACTOME database and a hypergeometric test (FDR < 0.05) confirms that helper T cell pathways are the top sets that are differentially expressed. Additionally, by network analysis, many differentially expressed proteins are associated with the Th1 and Th2 pathways. Data are available via PRIDE database with identifier PXD004532.

The adaptive immune response is of key importance in protecting a host from pathogens. Helper T (Th) cells play a pivotal role in regulating this response. The role of $CD4^+$ Th cells is to coordinate the activity of other immune cells by the production and release of cytokines, including the induction of class switching in B cells, activation and proliferation of cytotoxic T-cells, and, for instance, activation of macrophages. Th cells have the ability to differentiate into several distinct subtypes, of which Th1 and Th2 are best understood. By releasing the inflammatory cytokine IFN-γ, Th1 cells support cellular effector mechanisms that counter intracellular pathogens. Th2 cells promote humoral responses that are antibody-based responses mostly directed against extracellular pathogens, but also to allergens and toxins.[1] Inducing the appropriate Th driven immune phenotype is crucial as the inappropriate or 'wrong' phenotypic response may be ineffective and may even lead to severe immunopathology.[2] Understanding of the molecular mechanisms of Th differentiation will contribute to the development of better vaccines to combat infectious diseases, as well as to improved therapies against autoimmune and other immune-mediated diseases.[1–5]

In the past few decades, high-throughput technology has been used to discern the precise molecular details of helper T cell differentiation. These studies have been performed in both human as well as mouse cells; Th1 and Th2 cells have been discovered in mouse models, and mice have since remained an important model system for Th cell differentiation. A range of studies have investigated the transcriptomes of Th cells for both human and mouse Th,[6–9] using both in vitro and in vivo approaches. Early attempts in profiling the proteome of human Th1 and Th2 cells revealed very few proteins being differentially regulated between Th1 and Th2.[10] Moulder et al. focused their investigation on the nuclear fraction of Th2 cells unravelling the involvement of novel proteins like SATAB1.[11] However, very few studies have been performed in mouse Th cells. A recent study investigated the differentiation of T cells to Th17 and iTreg subsets[12] providing a valuable resource for further characterization of these cell types. In this study, we set out to perform comprehensive proteome profiling of mouse Th1 and Th2 cells.

In order to access the differences in protein expression in primary mouse Th1 and Th2 cells, we designed the following experiment: $CD4^+$ cells were isolated from the spleens of 32 C57Bl/6 mice and ex vivo stimulated to differentiate into Th1, Th2, or Th0 (neutral) cells. The spleens were divided into four groups and were homogenized using 100 µm Cell Strainers (Falcon) in OpTimizer CTS medium (Gibco A10221-01) as four separate pools to obtain four biological replicates for every treatment. This step was necessary to obtain enough material and helps to remove inter-individual variability. Every sample consisted of 6M cells, cultured in 96-well plates at a density of 250 000 cells per well. Red blood cells were removed using RBC lysis buffer (Roche Diagnostics GmbH 11814389001). $CD4^+$ T cells were isolated using a MACS $CD4^+$ Cell Isolation Kit II (mouse, Miltenyi Biotech 130-095-248). Cells were activated using Dynabeads Mouse

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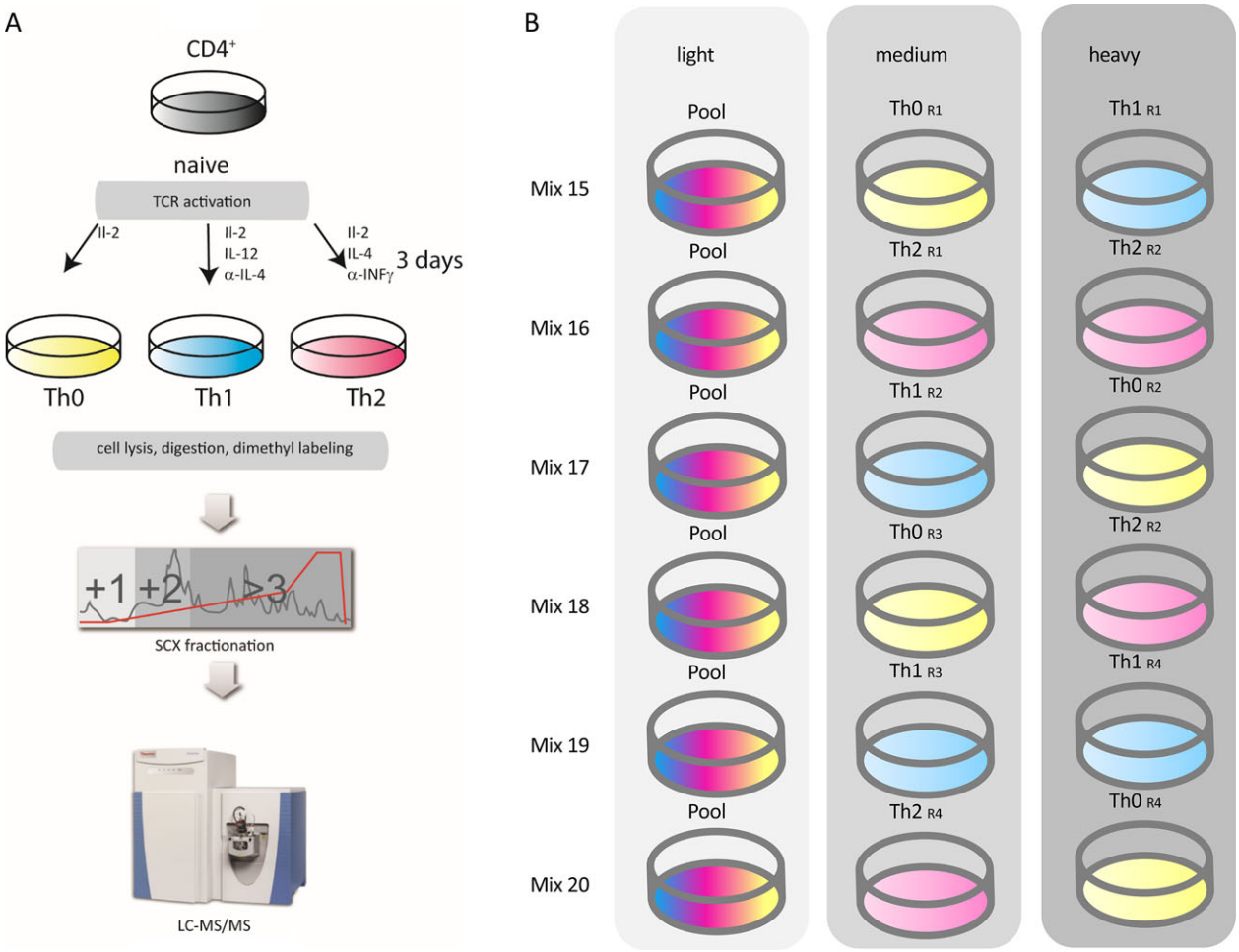

Figure 1. A) Setup of the experiment. Cells were isolated from mouse spleens, homogenized, activated, and processed to isolate proteins. The protein samples were digested, labeled, and mixed, and the resulting peptide mixtures were fractionated by strong-cation exchange (SCX) chromatography, and eventually measured using RP-LC–MS/MS. B) Mixing scheme for the three-plex dimethyl labeling. R1–R4 indicate biological replicate experiments.

T-Activator CD3/CD28 (Gibco, 11452D) and were cultured in OpTimizer medium supplemented with 10 ng mL^{-1} murine IL-2 (PeproTech Inc.; catalog number 212-12, A2313) in 96-well plates (250 000 cells per well). $CD4^+$ cells were skewed toward Th1 by adding in 25 ng mL^{-1} IL-12 (PeproTech Inc.; catalog number 210–12, G2111) and 1 µg mL^{-1} αIL-4 antibody (FGP anti-mouse IL4, clone 11B11, dat# 16-7041-85) to the culture medium. $CD4^+$ cells were skewed toward Th2 by adding 10 ng mL^{-1} IL-4 (PeproTech Inc.; catalog number 214-14, F2613) and 1 µg mL^{-1} αIFN-γ antibody (FGP antimouse IFN-γ, clone XMG1.2, catalog number 16-7311-81) to the culture medium. No skewing cytokines were added to the "neutral" stimulated Th0 cells (**Figure 1**).

The differentiation into Th1 and Th2 phenotypes can already be detected within 1 day post-activation at the mRNA level.[6,8] For proteome analysis, we allowed the cells for full phenotype development for 3 days. In order to evaluate successful differentiation at the mRNA level, we collected RNA at day 3 and measured the expression of Th cell marker genes *Tbx21* (Tbet), *Ifng* (IFN-γ), *Gata3*, and *Il4*. Cells were harvested and immediately collected in TRIzol, and processed as described before.[6] Briefly, total RNA was isolated, purified, and transcribed into cDNA. Expression of the key transcription factor and cytokine marker genes was verified using qRT-PCR.[6] qRT-PCR values were normalized against β-actin mRNA expression levels using the $-\Delta\Delta$Ct method as done in ref. 13. As expected, Tbet and IFN-γ are upregulated in Th1 cells, but not in Th2 cells; conversely, Gata3 and IL-4 are upregulated in Th2 and downregulated in Th1 (Figure S1, Supporting Information). This shows that the differentiation of cells was successful and that we are profiling "bona fide" Th1 and Th2 cell cultures.

Protein profiling was performed as described before[14,15] and detailed methods are provided in the Supporting Information. Briefly, from a third part of each sample, we created a pool that was labeled with a "light" stable isotope dimethyl label and used in each run mix as a common reference. Two individual replicate samples were chemically labeled with either "medium" or "heavy" stable isotope dimethyl labels and subsequently combined with a "light"-labeled pool sample aliquot resulting in six mixtures for each of the six runs. By swapping the medium and heavy label between biological replicates, labeling efficiency bias was avoided (Figure 1B). Labeling efficiency was >95% (data not shown). Analysis of prefractionated peptide mixtures by

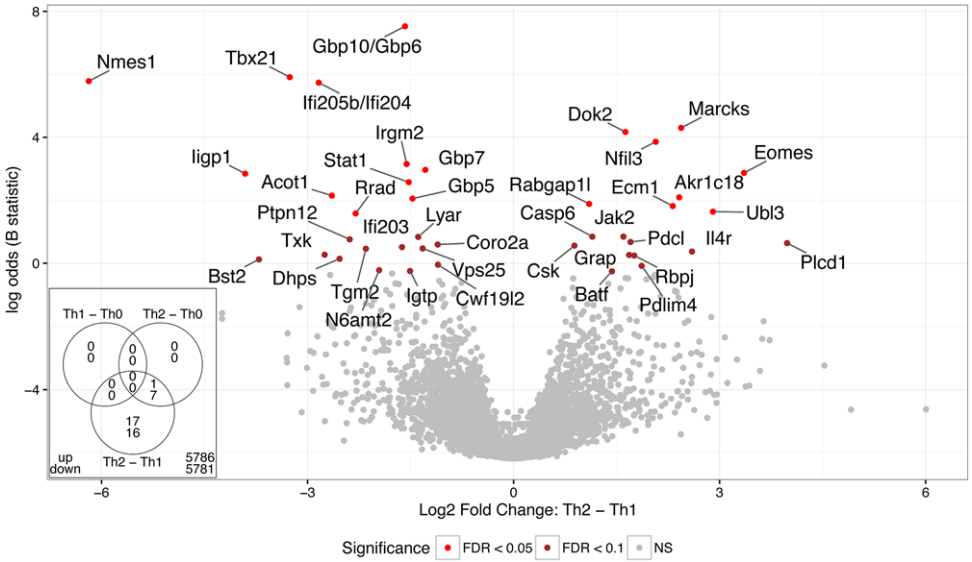

Figure 2. Differential protein expression for the Th2 versus Th1 contrast, with average $\log_2$-fold change plotted on the *x*-axis and log odds value (B-statistic) plotted on the *y*-axis. Significantly changing protein expression values are shown in light red (FDR < 0.1) and dark red (FDR < 0.05). Venn diagrams for all contrasts are given as inset.

RP-LC–MS/MS resulted in the identification of about 6000 proteins in the individual mixes (Figure S2A, Supporting Information) and 7410 proteins in all run mixes combined. We could quantify over 90% (6601) of the identified proteins. The identified proteins cover all cellular compartments (Figure S2B, Supporting Information) ensuring a representative view on the proteome of these cells.

In order to identify proteins that are differentially expressed between the Th1, Th2, and/or neutral phenotypes, we performed differential protein expression analysis using limma (version 3.20.8, Bioconductor Biobase 2.24.0, R 3.1.3) on nonimputed protein data. The mouse/spleen group from which the cells were derived was used as a co-variate to control for replicate associated effects, for details see refs. 16 and 17. The intensity data was processed using custom R scripts: after $\log_2$-transformation, the sample medians were shifted onto a single median value (Figure S3, Supporting Information). To determine protein intensity values, the peptide intensities were rolled up using median polish (R 3.1.3, base package stats, function medpolish).We compared all groups to one another, and we observed that the Th2–Th1 comparison yields 41 differentially expressed proteins at FDR < 0.1 (**Figure 2**, inset), and 19 at the FDR < 0.05 level (Figure 2, red dots). No proteins were found to change expression in the Th1–Th0 contrast. All of the differentially expressed proteins identified in the Th2–Th0 contrast (eight for FDR < 0.1, Figure 2, inset) are also identified in the Th2–Th1 comparison. We show the latter contrast using a volcano plot that depicts $\log_2$-fold change versus significance of differential expression (log odds, Figure 2). There are relatively few significant changes in the proteome between the different cell phenotypes. Pearson correlation analysis of all samples revealed weak to moderate correlation (0.4–0.8) of the individual samples with each other (Figure S4, Supporting Information). This might be related to the small fraction of differentially regulated proteins that are associated with cell differentiation. We performed over-representation analysis on the significant proteins of the Th2–Th1 contrast (FDR < 0.1) using REACTOME.[18] When applying a FDR < 0.05 threshold to the results of the gene set analysis, we found four pathways in cytokine signaling: IL4 and IL13 signaling, IFN signaling, and IL-6 signaling (Table S1, Supporting Information). These are pathways we expect to be differentially expressed as they are the direct result of cytokine signaling in T cells.

To further investigate functional relationships of the proteins that are differentially expressed, we constructed protein–protein interaction networks using our results (Figure 2) and the REACTOME FI PlugIn in Cytoscape v3.2. The REACTOME Functional Interaction network is a highly reliable, manually curated pathway-based protein functional interaction network. When all differentially expressed proteins are used to create a network, we could connect nine out of the 41 most significant proteins (**Figure 3**A). The transcriptional regulator Stat1 that activates transcription of the Th1 cell master regulator T-bet (TBX21)[19] is positioned in the center of this network. The antiviral protein Bst2 is also a direct interactor of the IFN-γ/Stat1 pathway (Figure 3A) and was found in an early proteomics study as being more abundant on the surface of Th1 cells.[20] If we add six non-observed proteins to extend the Th1 network, many other observed Th1-related proteins become connected (Figure 3B). The tyrosine kinase TXK may potentiate IFN-γ signaling.[21] The tyrosine phosphatase PTPN12 is known as positive regulator of secondary T cell activation, but also prevents anergy and induction of autoimmunity.[22] For the other proteins in the network, a connection to the Th1 phenotype is less obvious. Furthermore, we can link 14 out of the 18 proteins that show higher abundance in Th2 into another network by adding only seven linker proteins (Figure 3C). The cytokine IL-4 drives Th2 differentiation. After recognition by its receptor a signaling cascade involving Jak2 and Stat6 is activated, leading to the expression of the master regulator GATA3. We found the IL-4 receptor to be upregulated in Th2 cells as well as Jak2 that transmits the signal from the receptor

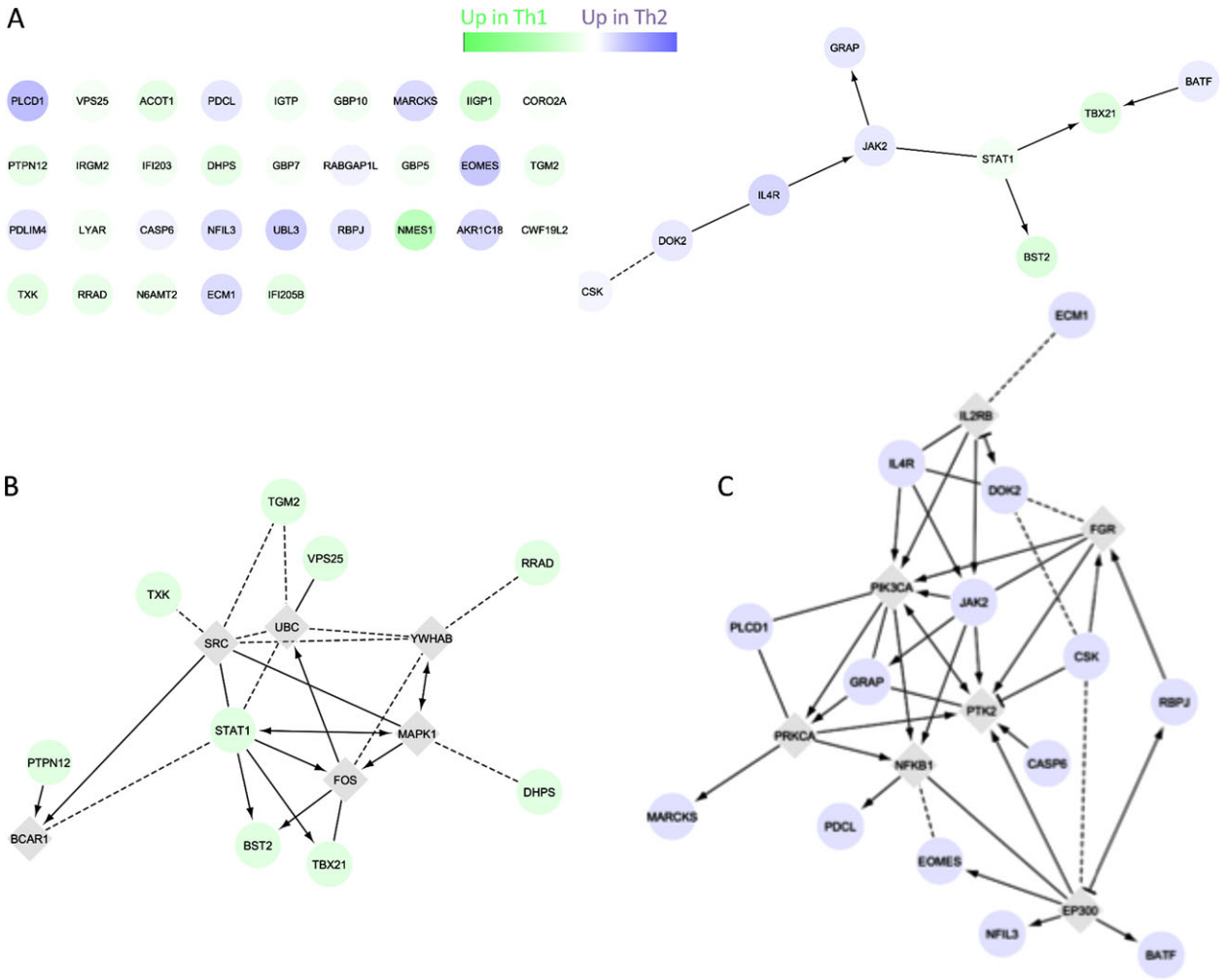

Figure 3. Network analysis of significantly (FDR < 0.1) changing proteins. A) Network of all significantly changing proteins that could be mapped. The color-coding indicates the fold change in the comparison Th1 versus Th2. The darker the color the stronger the fold change. B) Reactome network of Th1 specific proteins (green circles) supplemented with linkers (grey diamonds). C) Reactome network of Th2 specific proteins (purple circles) supplemented with linkers (grey diamonds). The connections between the proteins indicate their relationship to each other "→" for activating/catalyzing, "-|" for inhibition, "-" for functional interactions extracted from complexes or inputs, and "—" for predicted functional interactions.

to Stat6. However, IL4 can also activate PI3K signaling pathway via Janus kinases. Furthermore, this pathway activates the adaptor protein GRAP, a negative regulator of the Erk signaling.[23] With Dok2, we identified another adaptor protein that may negatively regulated TCR signaling.[24] Also tyrosine kinase CSK is a negative regulator of TCR signaling. It phosphorylates inhibitory residues on Src-family kinases.[25] In conclusion, many of the differentially expressed proteins have a well-documented role in Th1–Th2 differentiation (Table S2, Supporting Information); for others, no clear role has been described.

In this study, we have profiled neutral, Th1, and Th2 cells that were induced by skewing primary naive Th cells derived from mouse spleens. Our differential protein analysis shows that, as expected, cytokine signaling is one of the main molecular pathways that are activated. Transcription factor Tbet (TBX21), the Th1 master regulator, is significantly upregulated in Th1 compared to Th2 cells. Even though the Th2 signature transcription factor GATA3 does not emerge as significantly regulated at the protein level, we could detect differential protein expression of upstream members of the GATA3 induction pathway, such as IL4R and Jak2. They are known to activate the transcription factor Stat6, which drives GATA3 transcription. Furthermore, we could show differential expression of many genes at the protein level previously linked to Th skewing in mRNA profiling studies. For instance, we suggested that RBPJ is involved in the regulation of the Th2-like Th9 phenotype,[26] and that Batf is involved in Th2 skewing.[6] This indicates that we have indeed identified some of the major protein players in Th1/Th2 differentiation in our study. Despite the successful quantification of 6600 annotated proteins, only a small number of differentially expressed proteins linked to Th differentiation could be identified. This may be due to run-to-run variability in the assay. Consequently, the proteins that are detected differ from run to run, resulting in missing quantifications in the (replicate values of the) data set. Although these can be imputed, we recently showed that this leads to high levels of false positives and hence, is not recommended for high-throughput

protein analysis.[16] Future proteomics experiments will yield additional proteins involved in Th skewing, but the observation that only a small fraction of all (quantified) proteins are associated with Th differentiation is in line with our genome wide mRNA profiling experiments. These experiments also showed that only a relative small fraction (hundreds of genes) of all genes is differentially regulated in similar Th skewing conditions (6). The currently identified set of proteins associated with Th skewing appears "bona fide" hits, which is confirmed by the large overlap obtained with our earlier mRNA results.[6] The presently identified proteins will support future research on the biology of Th differentiation.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

helper T cell differentiation, LC-MS/MSexosomes, limma

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