Quantitative Proteomics Reveals GIMAP Family Proteins 1 and 4 to Be Differentially Regulated during Human T Helper Cell Differentiation*[S]

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T helper (Th) cells differentiate into functionally distinct effector cell subsets of which Th1 and Th2 cells are best characterized. Besides T cell receptor signaling, IL-12-induced STAT4 and T-bet- and IL-4-induced STAT6 and GATA3 signaling pathways are the major players regulating the Th1 and Th2 differentiation process, respectively. However, there are likely to be other yet unknown factors or pathways involved. In this study we used quantitative proteomics exploiting cleavable ICAT labeling and LC-MS/MS to identify IL-4-regulated proteins from the microsomal fractions of $CD4^+$ cells extracted from umbilical cord blood. We were able to identify 557 proteins of which 304 were also quantified. This study resulted in the identification of the down-regulation of small GTPases GIMAP1 and GIMAP4 by IL-4 during Th2 differentiation. We also showed that both *GIMAP1* and *GIMAP4* genes are up-regulated by IL-12 and other Th1 differentiation-inducing cytokines in cells induced to differentiate toward Th1 lineage and down-regulated by IL-4 in cells induced to Th2. Our results indicate that the GIMAP (GTPase of the immunity-associated protein) family of proteins is differentially regulated during Th cell differentiation. ***Molecular & Cellular Proteomics 8: 32–44, 2009.***

T helper (Th)[1] lymphocytes play a pivotal role in the regulation of the immune system, orchestrating the immune response of other leukocytes against microbes and toxins. Their importance is clearly illustrated with the collapse of the immune system that is observed following the destruction of Th cells in the development and progression of AIDS. Th cells produced in the thymus differentiate from their naïve state to specialized cell populations with specific functions and cytokine secretion profiles. The direction of Th differentiation is determined by the antigen encountered, its concentration, the cytokine environment, the presence of costimulatory molecules, and the epigenetic status of the cells (1–3). Following the original description of Th1 and Th2 subsets in the eighties (4, 5), the more recent characterization of new subsets, like $FOXP3^+$ T regulatory (Tr) cells, interleukin (IL)-10-producing Tr1 cells, transforming growth factor-β-secreting follicular Th cells, and IL-17A-producing Th17 cells, has revealed a more complex picture (for a recent review, see Reinhardt *et al.* (6)).

IL-4 is the key cytokine promoting Th2 cell differentiation and a major regulator of IL-4 production by the differentiated Th2 cell, although it powerfully inhibits Th1 differentiation and IFN-γ production (7–9). IL-4 also participates in the humoral immune response, for example, by acting as a mitogen for B cells and by enhancing their IgE production (10, 11). T cell receptor (TCR)-activated Th2 cells, basophils, mast cells, natural killer T cells, γ/δ T cells, and eosinophils have been reported to produce IL-4 (10). When IL-4 binds to IL-4 receptor α subunit (IL-4Rα), this in turn binds to the common γ chain (12, 13). The formation of this functional receptor-ligand complex for IL-4 signaling leads to binding and phosphorylation of the kinases JAK1 and JAK3 at the IL-4Rα and the common γ chain, respectively (10, 14–17). These kinases induce phosphorylation of IL-4Rα that leads to binding, phosphorylation, and dimerization of STAT6. The phosphorylated STAT6 dimer translocates to the nucleus, binds to $TTCN_4GAA$-containing DNA sequences, and activates IL-4-responsive genes (10, 11, 18, 19). Th2 response plays a major role in defense against extracellular parasites, particularly against gastrointestinal helminth infections. However, abnormal Th2 response and activation of Th2 cells can lead to pathophysiological conditions such as allergy and asthma (20, 21).

The characterization of the molecular mechanisms leading to Th cell differentiation is important for a better understand-

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[1] The abbreviations used are: Th, T helper; GIMAP, GTPase of the immunity-associated protein; STAT, signal transducer and activator of transcription; TCR, T cell receptor; cICAT, cleavable ICAT; IL, interleukin; IFN, interferon; FPR, false positive rate; PHA, phytohemagglutinin; SCX, strong cation exchange; shRNA, short hairpin RNA; siRNA, small interfering RNA; Thp, precursor T helper; FAM, carboxyfluorescein; TAMRA, carboxytetramethylrhodamine.

ing of T helper cell-mediated diseases. Transcriptomics studies have resulted in the identification of genes differentially regulated during Th cell activation and differentiation (22–32), and proteomics studies have provided complementary information about the processes (33–39).

The development of the technologies for stable isotope labeling of proteins/peptides and their application in conjunction with high throughput LC-MS/MS methodology have provided the means to rapidly screen for changes in protein expression (40–43). Although for the most part the analyses of these data are automated, it is important to address the reliability of peptide/protein identifications. The automated interpretation of data from the MS/MS spectra of peptides is generally achieved through the comparison of the observed spectra with the theoretical spectra generated from a sequence database. With the reversal or randomization of these databases, a decoy database may be created, and by a likewise comparison the false positive rates (FPRs) of inferred identification may be estimated, and the criteria for data acceptance can be chosen (44–46). Although searches against decoy databases are now routinely used to provide estimates of false positive rates of identification, consensus identification by different data analysis algorithms can also be used as an additional measure of validation that can also improve the scope of data analysis. The combination of the approaches of consensus identification and FPR evaluations provide an extra measure of certainty and complimentary information for both identification and quantification.

In the present study our aim was to characterize the proteome of the microsomal fraction extracted from naïve Th lymphocytes and, in particular, to identify proteins whose expression would be regulated in response to IL-4-induced Th2 differentiation. We used stable isotope labeling with cleavable ICAT (cICAT) and LC-MS/MS (47–49). To increase the number of protein identifications and at the same time improve their confidence, we performed duplicated data analysis with the database searching algorithms ProICAT/ProID and SEQUEST/XPRESS (Fig. 1). Using this proteomics approach differential expression of a number of IL4-regulated proteins was observed. Of these the expression of STAT1, MXA, and GIMAP4 was corroborated with Western blotting. GIMAP1 and GIMAP4 proteins were selected from the data set for further studies based on their biological and immunological properties.

The different members of the GIMAP (GTPase of the immunity-associated protein) (also termed immune-associated nucleotide-binding proteins (IANs); the nomenclature used here follows that used by Krücken *et al.* (50)) family have been associated in thymocyte development (51), T cell apoptosis (52, 53), antiapoptotic effects (54), T cell survival (55, 56), autoimmunity (57, 58), and leukemia (59), all suggesting that the GIMAP family is important for the immune system. However, there are no previous reports describing the regulation of the GIMAP family members during Th1 or Th2 differentiation. Here we demonstrate that *GIMAP1* and *GIMAP4* genes are up-regulated by IL-12 and down-regulated by IL-4 during human Th1 and Th2 differentiation. The proteomics application and Western blotting further confirm the similar regulation at the protein level.

EXPERIMENTAL PROCEDURES

Fig. 1 summarizes both the sample preparation and nano-LC-MS/MS analysis protocols and the data analysis pipeline.

Cell Culture—Mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences) gradient centrifugation either from cord blood samples, which were collected from healthy neonates at Turku University Hospital, or from buffy coats from healthy blood donors (Red Cross Finland Blood Service, Helsinki, Finland). $CD4^+$ T cells were enriched from mononuclear cells by anti-CD4 magnetic beads (Dynal Biotech, Oslo, Norway). $CD4^+$ T cells were cultured in Yssel's medium (60) supplemented with 1% AB serum (Red Cross Finland Blood Service). Cells were activated with plate-bound anti-CD3 (2.5 μg/ml for coating) and 500 ng/ml soluble anti-CD28 (both from Immunotech, Marseille, France) or with 100 ng/ml PHA (Murex Diagnostics, Chatillon, France) and irradiated CD32/B7-transfected mouse L fibroblasts. 2.5 ng/ml IL-12 (R&D Systems, Minneapolis, MN) and 10 ng/ml IL-4 (R&D Systems) were used to induce Th1 and Th2 differentiation, respectively. No cytokines were used for the neutral Th0 condition. To support proliferation of the cells, 17 ng/ml IL-2 (R&D Systems) was added on the 2nd culture day to 6–7-day cultures. Instead of IL-12, 100 units/ml highly purified human leukocyte IFN-α (Finnish Red Cross) or 25 ng/ml IL-18 (Medical & Biological Laboratories, Nagoya, Japan) were also used to induce Th1 differentiation. Where indicated, 3 μg/ml neutralizing anti-IFN-γ (clone 25718, R&D Systems) was used to block the endocrine production.

Preparation of Microsomal Fraction—Microsomal fractions were prepared as described previously (35). Briefly cells were lysed in hypotonic buffer with a tightly fitting Dounce homogenizer. The nuclear fraction was removed from the cell lysate by centrifugation. The microsomal fraction was then pelleted from the postnuclear supernatant by centrifugation.

cICAT Labeling—The ICAT labeling protocol was performed according to the previously described methods (47, 61) and the protocol provided with the cICAT reagents (Applied Biosystems, Foster City, CA). Proteins from the microsomal fraction were dissolved in denaturing buffer (6 M urea, 0.05% SDS, 5 mM EDTA, and 50 mM Tris, pH 8.5) and reduced for 30 min with 1 mM tris(2-carboxyethyl)phosphine. Equal amounts of microsomal fraction from IL-4-treated and non-treated cells were labeled with heavy and light cleavable ICAT reagent (2 mM per label), respectively. After 2 h of labeling 20 mM DTT was added to stop the reaction. Then the ICAT-labeled samples (light and heavy) were mixed, diluted, and digested with trypsin (1:100 (w/w)) (sequencing grade modified trypsin, Promega, Madison, WI) overnight at 37 °C.

Fractionation of the Peptides—The resulting peptide mixtures were fractionated by a strong cation exchange (SCX), avidin affinity, and reversed-phase chromatography. A BioCAD chromatography work station (PerSeptive Biosystems, Freiburg, Germany) was used with a 4.6 × 200 mm-Polysulfoethyl A column (Poly LC Inc., Columbia, MD) for SCX chromatography. Peptides were eluted from the SCX column with a linear gradient from 0 to 60% phase B in 30 min while collecting 1-min fractions. The A and B phases both consisted of 5 mM KH_2PO_4, 25% acetonitrile, at pH 3, with the B phase containing 0.6 M KCl. The cICAT-labeled peptides were then isolated from the SCX fractions by avidin affinity separation (Applied Biosystems) as described in the protocol. "Flow-through" fractions representing non-labeled peptides were retained for MS analysis.

Nano-LC/ESI-MS/MS Analysis—Avidin chromatography fractions, including the ICAT-labeled peptides, were first dried in a vacuum centrifuge, and then cleaving reagents were added (Applied Biosystems) to remove the biotin tag from the ICAT label. After cleavage, the peptides were dried again and suspended to 0.1% HCOOH prior to LC-MS/MS analysis. Flow-through fractions from avidin chromatography were also dried and suspended to 0.1% HCOOH. An LC-MS/MS system consisting of a nano-LC system (Famos, Switchos-II, and Ultimate, LC Packings, Amsterdam, Netherlands) coupled to a QSTAR® Pulsar ESI-hybrid quadrupole time-of-flight instrument (Applied Biosystems/MDS Sciex) was used. On-line sample concentration and desalting followed by reversed-phase separation were performed with a 0.3 × 5-mm PepMap C_{18} μ-precolumn (LC Packings) and a 75-μm × 200-mm Magic C_{18} (5 μm, 120 Å, Michrom BioResources) analytical column (prepared in house), respectively. A separation gradient was developed from 5 to 40% phase B over 120 min and then from 40 to 95% phase B over 10 min. The flow rate was 200 nl/min. The phase compositions were as follows: phase A, 5% ACN, 0.1% HCOOH; phase B, 95% ACN, 0.1% HCOOH. The mass spectrometer was set to perform survey scans of 1 s followed by two 3-s MS/MS scans of the two most intense peaks from the survey scan. The program AnalystQS (Applied Biosystems) was used for data acquisition and instrument control.

Data Processing—Two pairs of identification and quantification algorithms were used primarily to increase the confidence of the protein identifications and quantitative information related to protein expression. The data analysis steps are schematically presented in Fig. 1 and described in details in the following paragraphs.

ProICAT and ProID—Data were analyzed using the Applied Biosystems Analyst scripts, ProICAT (version 1.0, service pack 2) for the ICAT fractions and ProID for the non-labeled peptide fractions. The data were searched against the Swiss-Prot human-specific database (Swiss-Prot Release 44.5 containing 11,461 human sequence entries) using a precursor and product ion mass tolerances of 0.3 and 0.2 Da, respectively, while specifying trypsin digestion and one missed cleavage. Labeling with the cICAT reagent was set as a fixed modification of cysteines in the ProICAT searches. A confidence value ≥90 was used to filter the data. ProICAT detects peptide pairs labeled with light and heavy ICAT reagent and calculates their ratio from the relative areas of the precursor ion chromatograms extracted from MS scans. Default parameters of ProICAT were used for the quantification of peptides and proteins.

SEQUEST, XPRESS, and the Transproteomic Pipeline—SEQUEST (ThermoFinnigan) and XPRESS (ThermoFinnigan) were used for comparative and complementary data analysis as described in Fig. 1. The QSTAR data files were converted to mzXML format and then to the SEQUEST-compatible *.dta format with the Sashimi transproteomic pipeline tools mzStar and mzXML2other, respectively (SourceForge, Inc.) as described previously (62). SEQUEST searches were performed against the Swiss-Prot human-specific database (as above) with the fixed cICAT modification of the cysteine residue specified as 227.14 Da and an associated differential mass of 9.0 Da; the precursor ion tolerance was set to 0.3 Da. The searches were made for tryptic peptides, and the results were filtered such that only assignments with a maximum of one missed cleavage were included. Peptides were filtered using Xcorr values suggested by the reversed database searches. The XPRESS calculations were made using the default mass tolerance of 1.0, specifying cysteine labeling, co-elution of the peaks, and the mass differences between the ICAT pairs as described in the SEQUEST parameters. With XPRESS, ICAT ratios are calculated from the relative areas of the extracted ion chromatograms of the precursor ions and their isotopically distinct equivalents. Finally SEQUEST- and XPRESS-processed data were filtered and combined using the Sashimi tools Interact (61) and ProteinProphet (63).

Reversed Database Searches to Determine the False Positive Rates for ProICAT and ProID and to Determine the SEQUEST Xcorr Values—The Sashimi tool subsetdb was used to create a reversed form of the human-specific Swiss-Prot database. Using the relationship described by Peng *et al.* (45) that estimates the false positive rate from the number of protein identifications found with reversed (n_{rev}) and normal (n_{norm}) database searches (*i.e.* % false = $2(n_{rev}/(n_{rev} + n_{norm}))$), the ProID/ProICAT false positive rates were calculated, and the SEQUEST data inclusion parameters were selected. The filtering criteria subsequently applied for SEQUEST results were such that the false positive rate per single peptide was ~7% for both 2+ and 3+ ions. Using the ΔCN value of 0.1 with Xcorr values of 2.38 and 3.08 gave an estimated false positive rate of 1.3% for proteins identified with at least two peptides.

Statistical Methods and Data Management—The reports from ProteinProphet and ProICAT were exported into Microsoft Excel format, and the protein measurements were normalized such that the median ICAT ratio was unity for both sets of results (XPRESS and ProICAT). Kensington (InforSense Ltd., London, UK) was used for data comparisons between the sets of search results and statistical processing.

Western Blot Analysis—The microsomal fraction and cell lysate samples were dissolved in SDS-PAGE loading buffer and boiled for 5 min prior to loading to the SDS-PAGE gel. After electrophoresis, the proteins were transferred from the gel to nitrocellulose membrane (Hybond ECL, Amersham Biosciences). The membranes were stained with P-STAT6 (Cell Signaling Technology Inc.), STAT1 (Cell Signaling Technology Inc.), MXA (a gift from Dr. Julkunen), STAT6 (BD Biosciences), GIMAP4 (a gift from Dr. Cambot (64)), and β-actin (Sigma-Aldrich) antibodies/antiserum. Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology Inc.) and anti-mouse-IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

Quantitative Real Time RT-PCR—Total RNA was isolated from the cells using the RNeasy minikit (Qiagen, Valencia, CA). RNA was treated with DNase I (Invitrogen) and used as a template for cDNA synthesis by SuperScript II (Invitrogen). For detection of *GIMAP4* mRNA expression the following primers and probes were used: 5′-FAM-acaaggcaacaggcgctgagca-TAMRA-3′, 5′-tgaccgctactgtgcgttaaa-3′, and 5′-tggatcaggcccagcaa-3′ for simultaneous detection of both the long and short isoforms and 5′-FAM-ttcctgctccggttttacccactaacac-TAMRA-3′, 5′-gagagggcattcagtgctcc-3′, 5′-caggggccagttatgggc-3′, and 5′-gcagtgccagaatgaaacactt-3′ for the detection of the differentially spliced isoforms. *GIMAP1* mRNA was detected using Universal ProbeLibrary probe 24 (Roche Applied Science) together with primers 5′-cgtggacactccggacat-3′ and 5′-tcctcacagccaggatctgt-3′. Housekeeping gene *EEF1A* was used for normalization and detected using primers 5′-ctgaaccatccaggccaaat-3′ and 5′-gccgtgtggcaatccaat-3′ and probe 5′-FAM-agcgccggctatgcccctg-TAMRA-3′. Quantitative real time RT-PCR analyses were performed with an ABI Prism 7700 Sequence Detector or ABI 7900HT Fast Real-Time PCR system (Applied Biosystems) as described earlier (65). Duplicate samples were run at least twice, and samples from at least three cultures were used. The obtained Ct values (the PCR cycle during which the signal exceeded the set threshold value) were normalized to housekeeping gene *EEF1A*, thus obtaining ΔCt values for each sample. ΔΔCt values for sample "*n*" were calculated by $\Delta\Delta Ct^n = \Delta Ct^{Thp} - \Delta Ct^n$. -Fold differences were obtained by $FD = sign(\Delta\Delta Ct) \cdot 2^{|\Delta\Delta Ct|}$. The significance of the gene expression differences were calculated from ΔΔCt values using Student's *t* test.

Knockdown of STAT6 with siRNA Oligonucleotide/shRNAs—Human buffy coat $CD4^+$ T cells or neonatal cord blood $CD4^+$ T cells were transfected with 10 μg of plasmid DNA or 1.5 μg of oligonucleotide siRNAs, respectively, at a cell density of $4 \cdot 10^6$ cells/100 μl of Opti-MEM I (Invitrogen) using the Nucleofector Device (program U-14) (Amaxa, Cologne, Germany) as described earlier (66). The shRNAs

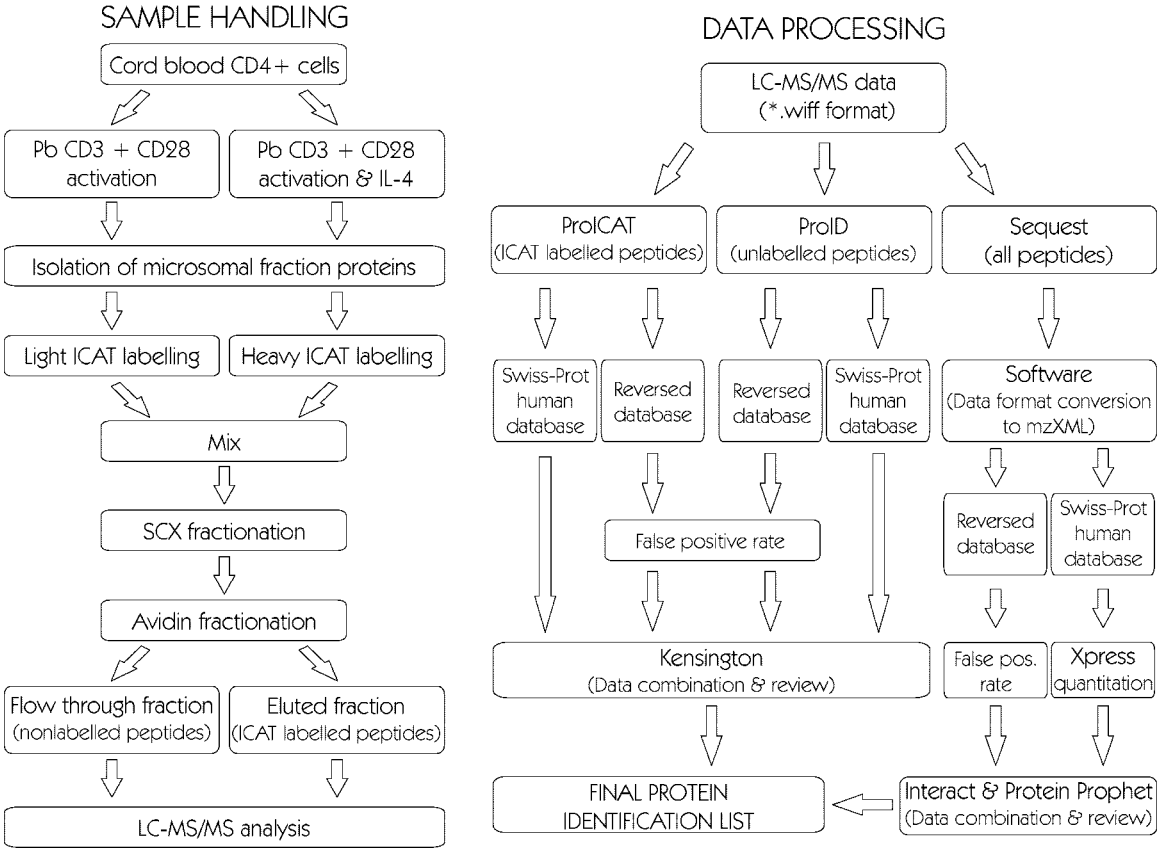

FIG. 1. **Sample preparation and data handling summary.** *Pb*, plate-bound; *pos.*, positive.

(DNA Technology, Aarhus, Denmark) were cloned into the XhoI/BglII-cleaved pSuper-H-2K^k vector (66). The sequences of the siRNA oligos and the targets of the shRNAs were: Scramble, 5′-gcgcgcuuuguaggauucguu-3′; and *STAT6*, 5′-caguuccgccacuugccaautt-3′, 5′-aagcaggaagaacucaaguuutt-3′, or 5′-gaaucagucaacguguugucatt-3′ (siRNA oligonucleotides from Sigma/Proligo, Evry Cedex, France). After nucleofection the cells were allowed to rest for 24 h in RPMI 1640 medium supplemented with 10% FBS, penicillin + streptomycin, and 2 mM L-glutamine. After resting the cells were harvested and induced to differentiate along the Th2 lineage as described above. If nucleofected with plasmid DNA, dead cell removal and enrichment of transfected cells were performed before plating as described earlier (66).

RESULTS

Characterization of the Microsomal Fraction Proteome of Human Th Lymphocytes Stimulated with IL-4—In the present study we used a proteomics-based isotope-coded affinity technology to characterize the expression differences in the proteome of the microsomal fraction of cord blood CD4$^+$ cells stimulated with Th2-promoting cytokine IL-4. The peptide mixtures labeled with cICAT reagents were fractionated by SCX and avidin affinity chromatography, and the resulting fractions were analyzed by LC-MS/MS (Fig. 1). Duplicate data analysis was applied to achieve confident protein identifications as comprehensively as possible (Fig. 1). Using the results of reversed database searches the data inclusion criteria were chosen, and the FPR was estimated; these were $<$0.1, $<$1.4, and $<$8.8% for ProICAT, SEQUEST, and ProID, respectively. With these criteria ProICAT/ProID and SEQUEST/XPRESS resulted in 2636 and 2007 peptide identifications, inferring the detection of 474 and 432 proteins, each with two or more unique peptides, respectively. Of these identifications, 349 proteins were common to both algorithms, whereas an additional 125 proteins were identified by ProICAT/ProID alone, and 83 were identified by SEQUEST/XPRESS alone. Supplemental Table 1 summarizes all the protein identifications. Table I (A) summarizes the number of peptide and protein identifications with different software algorithms and the false positive rates.

Relative Quantitation of the Protein Abundances in the Microsomal Fraction of Naïve Th Lymphocytes—Microsomal fraction proteins, extracted from IL-4-treated and non-treated cells, were labeled with heavy and light cICAT reagents, respectively (IL-4, heavy; non-treated, light). Using the programs ProICAT and XPRESS a total of 304 proteins were quantified of which 136 proteins were quantified by both algorithms, and 21 proteins and 147 were quantified by ProICAT and XPRESS only, respectively. Fig. 2*A* shows the correlation of the protein quantifications analyzed by the two different algorithms. The corresponding Pearson correlation value was 0.77. Statistics of quantitative information is summarized in Table I (B). A major difference in the numbers of

TABLE I
Protein identifications and quantifications

	ProICAT[a]	ProID[a]	Sequest/ Xpress[b]
A. Protein identifications[c]			
Proteins[c]		**474**	**432**
False positive rate[c] (%)	<0.1	8.8	1.4
Peptides	431	2205	2007
Matching proteins[c]		**349**	
All proteins[c]		**557**	
B. Protein identifications with quantitative information[c]			
Proteins[c]	**157**		**283**
Matching proteins[c]		**136**	
All proteins[c]		**304**	
1.4-fold up-regulated proteins		**3** (21)[d]	
1.4-fold down-regulated proteins		**5** (25)[d]	

[a] Filtered with confidence value > 90.
[b] Filtered with ΔCN = 0.1, Xcorr = 1.80, 2.38, and 3.08 for 1+,2+, and 3+, respectively.
[c] Refers to proteins identified by at least 2 different peptide sequences.
[d] Bold number represents the matching up- and down-regulated proteins, whereas the number in the parentheses indicates all the up- and down-regulated proteins (matching proteins and single algorithm observations).

protein ICAT ratios presented by the two programs arises because ProICAT only calculates ratios for peptides where both the light and heavy forms are identified, whereas XPRESS calculates extracted ion chromatograms and ratios for both forms by default.

IL-4-induced Differences in the Protein Expression of the Microsomal Fraction of the CB CD4$^+$ Cells—Proteins that showed at least 1.4-fold difference in expression between the IL-4-treated and non-treated sample were considered as potential IL-4-regulated proteins. Table II lists all these 46 proteins. Eight of the proteins were quantified with both ProICAT and SEQUEST/XPRESS, whereas ProICAT detected independently 13 proteins, and SEQUEST/XPRESS detected independently 25 proteins. Fig. 2*B* presents the correlation of the protein quantifications between the two algorithms. The eight proteins detected by both algorithms (A and D in both Table II and Fig. 2*B*) were considered more reliable hits than the proteins detected by only a single algorithm (B, C, and E in both Table II and Fig. 2*B*). However, in view of the differences in the identification algorithms and the FRP estimates, we felt that it was important to also take into account the single algorithm hits: a particularly interesting example was the finding of the change observed in the expression of GIMAP4 protein. In our study four different proteins, namely STAT1 (P42224, STA1 in Table II), MXA (P20591, MX1),

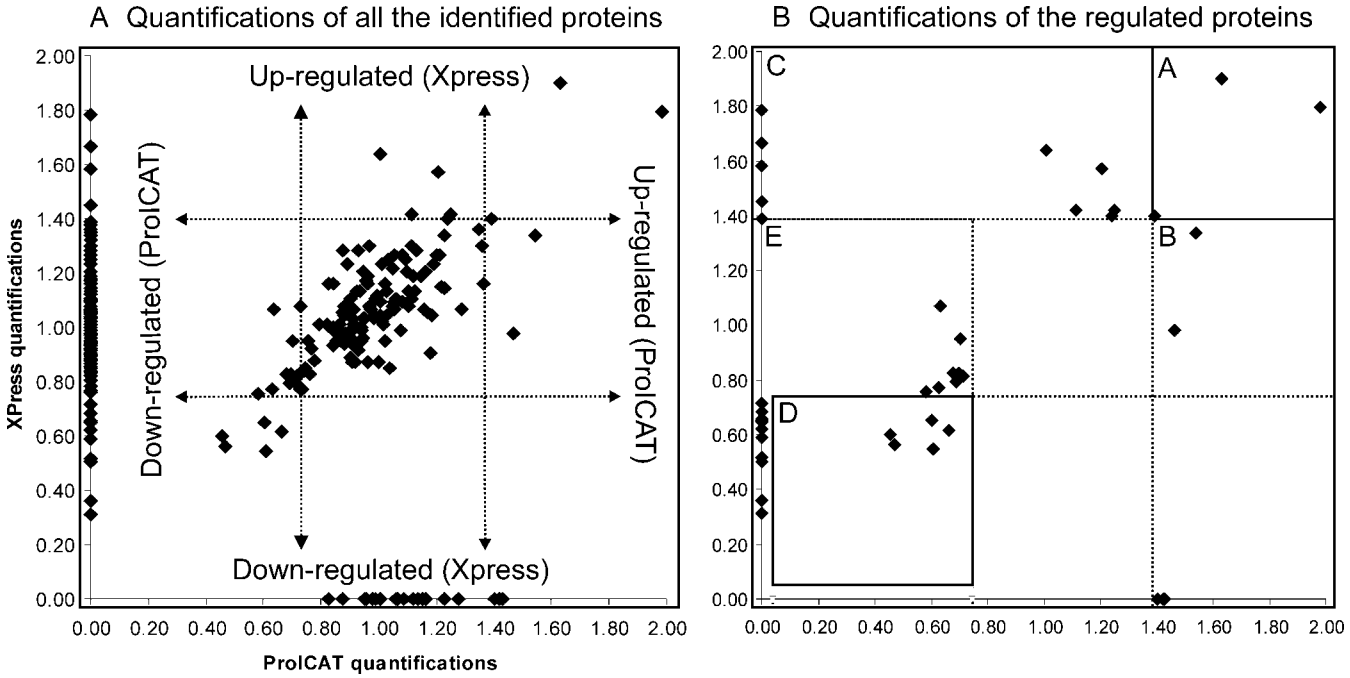

FIG. 2. **Correlation of the protein quantifications between ProICAT and XPRESS.** The *horizontal axis* presents ProICAT quantifications, and XPRESS quantifications are on the *vertical axis*. Proteins, which were quantified only by another of the algorithms, are presented on the *axis*. All the proteins are present in *A*, and proteins showing >1.4-fold expression difference are in *B*. The correlation was determined also by Pearson's test resulting in a value of 0.77. *A*, proteins detected by both algorithms and up-regulated; *B*, proteins detected by ProICAT and up-regulated; *C*, proteins detected by SEQUEST/XPRESS and up-regulated; *D*, proteins detected by both algorithms and down-regulated; *E*, proteins detected by ProICAT or SEQUEST/XPRESS and down-regulated.

TABLE II
All proteins identified with two peptides

ALL PROTEINS ID'd WITH 2 PEPTIDES																				
Protein Information			ProID		ProICAT							SEQUEST				XPRESS				Protein Prophet
SP_ number[1]	entry_name[2]	protein_name	non_ cys_ pep[3]	max_ conf	cys_ peps[4]	max_ conf	total_ peps		seq_ cov (%)	H(IL4)/ L(Act)[5]	SD[5]	entry_name[2]	peps	all_ forms	calcs	L(Act)/ H(IL4)[5]	SD[5]	1/ xpress[6]	seq_ cov (%)[7]
A) IL-4 UP-REGULATED (Pro ICAT and XPRESS)																				
P32322	PROC_HUMAN	Pyrroline-5-carboxylate reductase	2	98	1	99	3	49	15.4	1.98	0.23	PROC_HUMAN	4	7	4	0.56	0.11	1.79	27.6
Q14103	ROD_HUMAN	Heterogeneous nuclear ribonucleoprotein D0	3	98	2	90	5	52	14.6	1.63	0.03	ROD_HUMAN	3	3	1	0.53		1.90	18.9
P12004	PCNA_HUMAN	Proliferating cell nuclear antigen	2	98	1	99	3	47	18.0	1.39		PCNA_HUMAN	2	2	1	0.72		1.40	18.0
B) IL-4 UP-REGULATED (Pro ICAT)																				
P49458	SR09_HUMAN	Signal recognition particle 9 kDa protein	1	92	1	90	2	22	25.6	1.54	0.02	SR09_HUMAN	3	8	6	0.75	0.18	1.34	38.8
P50213	IDHA_HUMAN	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	1	98	2	99	3	46	12.6	1.46		IDHA_HUMAN	6	16	14	1.02	0.15	0.98	21.0
P38117	ETFB_HUMAN	Electron transfer flavoprotein beta-subunit	4	98	1	90	5	67	26.3	1.43		ETFB_HUMAN	4	8					49.4
Q14764	MVP_HUMAN	Major vault protein	2	98	1	90	3	43	4.8	1.40		MVP_HUMAN							9.4
Q15424	SFB1_HUMAN	Scaffold attachment factor B	1	94	1	90	2	31	3.4	1.42	0.26	SFB1_HUMAN							3.4
C) IL-4 UP-REGULATED (XPRESS)																				
P05141	ADT2_HUMAN	ADP,ATP carrier protein, fibroblast isoform	4	98			4	44	14.8			ADT2_HUMAN	4	8	1	0.05		19.02	23.5
P54886	P5CS_HUMAN	Delta 1-pyrroline-5-carboxylate synthetase	5	98			5	67	8.4			P5CS_HUMAN	9	10	3	0.60	0.30	1.67	14.3
P62906	R10A_HUMAN	60S ribosomal protein L10a	2	98			2	27	12.4			R10A_HUMAN	4	8	2	0.63	0.05	1.58	31.5
Q14697	GA2A_HUMAN	Neutral alpha-glucosidase AB precursor	6	98	1	90	7	93	9.9	1.01		GA2A_HUMAN	4	4	2	0.61	0.22	1.64	11.8
Q02338	BDH_HUMAN	D-beta-hydroxybutyrate dehydrogenase, mitochondrial precursor	1	98	1	99	2	37	10.8	1.21	0.24	BDH_HUMAN	3	4	2	0.64	0.05	1.57	25.1
P51991	ROA3_HUMAN	Heterogeneous nuclear ribonucleoprotein A3	2	98	1	90	3	47	12.4	1.25		ROA3_HUMAN	4	13	8	0.70	0.03	1.42	16.1
Q8NBS9	TXN5_HUMAN	Thioredoxin domain containing protein 5 precursor	1	98	1	90	2	30	6.9	1.11		TXN5_HUMAN	4	6	5	0.70	0.06	1.42	28.5
Q16718	NUFM_HUMAN	NADH-ubiquinone oxidoreductase 13 kDa-B subunit						0				NUFM_HUMAN	2	5	2	0.72	0.01	1.39	22.6
Q13586	STM1_HUMAN	Stromal interaction molecule 1 precursor						0				STM1_HUMAN	2	2	1	0.69		1.45	12.7
Q9P2J5	SYLC_HUMAN	Leucyl-tRNA synthetase, cytoplasmic						0				SYLC_HUMAN	2	2	1	0.56		1.79	4.3
P08865	RSP4_HUMAN	40S ribosomal protein SA						0				RSP4_HUMAN	2	2	1	0.56		1.79	6.5
P53634	CATC_HUMAN	Dipeptidyl-peptidase I precursor						0				CATC_HUMAN	2	2	1	0.32		3.13	7.3
P13010	KU86_HUMAN	ATP-dependent DNA helicase II, 80 kDa subunit	8	98	1	90	9	123	16.8	1.24		KU86_HUMAN	12	18	6	0.72	0.18	1.40	23.9
D) IL-4 DOWN-REGULATED (Pro ICAT and XPRESS)																				
P42224	STA1_HUMAN[8]	Signal transducer and activator of transcription 1-alpha	7	98	1	90	8	108	14.4	0.66		STA1_HUMAN	8	12	1	1.62	0.27	0.62	21.6
Q8WWP7	IMP1_HUMAN[8]	Immunity-associated protein 1	1	98	1	99	2	37	12.1	0.61	0.00	IMP1_HUMAN	4	8	7	1.83	0.24	0.55	20.3
Q9Y3Z3	SAD1_HUMAN	SAM domain and HD domain-containing protein 1	6	98	1	99	7	91	14.5	0.60		SAD1_HUMAN	9	12	4	1.54	0.15	0.65	19.6
P20700	LAM1_HUMAN	Lamin B1	6	98	2	99	8	98	16.7	0.47	0.01	LAM1_HUMAN	9	13	4	1.78	0.16	0.56	19.5
P20591	MX1_HUMAN[8]	Interferon-regulated resistance GTP-binding protein MxA	6	98	2	99	8	131	19.8	0.46	0.04	MX1_HUMAN	8	14	6	1.67	0.23	0.60	23.1
E) IL-4 DOWN-REGULATED (Pro ICAT)																				
P13804	ETFA_HUMAN	Electron transfer flavoprotein alpha-subunit, mitochondrial precursor	6	98	2	90	8	121	36.3	0.63	0.14	ETFA_HUMAN	10	20	13	0.94	0.21	1.07	44.4
P07919	UCRH_HUMAN	Ubiquinol-cytochrome C reductase complex 11 kDa protein, mitochondrial precursor			3	90	3	34	37.4	0.70	0.08	UCRH_HUMAN	3	13	11	1.05	0.05	0.95	46.2
P35579	MYH9_HUMAN	Myosin heavy chain, nonmuscle type A	49	98	6	99	55	784	40.0	0.70	0.15	MYH9_HUMAN	72	145	47	1.21	0.20	0.83	47.1
P45880	POR2_HUMAN	Voltage-dependent anion-selective channel protein 2	5	98	3	90	8	114	38.8	0.68	0.07	POR2_HUMAN	8	10	4	1.21	0.08	0.83	38.9
P30405	PPIF_HUMAN	Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor	1	98	1	90	2	52	25.1	0.71		PPIF_HUMAN	3	4	3	1.23	0.14	0.81	29.5
P08575	CD45_HUMAN	Leukocyte common antigen precursor	12	98	6	99	18	249	19.1	0.69	0.04	CD45_HUMAN	24	59	41	1.26	0.14	0.79	28.4
Q8TDB6	BBAP_HUMAN	B-lymphoma- and BAL-associated protein	1	98	1	99	2	28	3.8	0.63		BBAP_HUMAN	4	5	4	1.29	0.09	0.77	8.9
Q9Y5M8	SRPB_HUMAN	Signal recognition particle receptor beta subunit	1	98	1	90	2	28	10.3	0.58		SRPB_HUMAN	3	5	3	1.33	0.14	0.75	25.1
E) IL-4 DOWN-REGULATED (XPRESS)																				
P27635	RL10_HUMAN	60S ribosomal protein L10	2	98			2	21	9.8			RL10_HUMAN	4	5	1	1.40		0.71	26.1
O75367	H2AY_HUMAN	Core histone macro-H2A.1	2	98			2	40	10.8			H2AY_HUMAN	4	5	1	1.52		0.66	24.0
P63104	143Z_HUMAN	14-3-3 protein zeta	3	98			3	52	21.2			143Z_HUMAN	6	10	2	1.94	0.63	0.52	44.1
P48643	TCPE_HUMAN	T-complex protein 1, epsilon subunit	3	98			3	53	9.8			TCPE_HUMAN	3	3	1	1.99		0.50	9.1
P22626	ROA2_HUMAN	Heterogeneous nuclear ribonucleoproteins A2	7	98			7	98	27.8			ROA2_HUMAN	6	15	1	3.19		0.31	31.7
Q99829	CNE1_HUMAN	Copine I						0				CNE1_HUMAN	2	2	1	2.77		0.36	9.9
P09326	CD48_HUMAN	B-lymphocyte activation marker BLAST-1 precursor						0				CD48_HUMAN	2	6	3	1.7	0.12	0.59	15.6
P07996	TSP1_HUMAN	Thrombospondin 1 precursor						0				TSP1_HUMAN	2	5	5	1.61	0.20	0.62	9.1
P22234	PUR6_HUMAN	Multifunctional protein ADE2						0				PUR6_HUMAN	2	2	2	1.54	1.00	0.65	10.8
P59665	DEF1_HUMAN	Neutrophil defensin 1 precursor						0				DEF1_HUMAN	2	3	3	1.54	0.04	0.65	16.8
Q9NUV9	IMP4_HUMAN[8]	Immunity-associated protein 4						0				IMP4_HUMAN	2	4	4	1.53	0.24	0.65	13.1
Q14258	Z147_HUMAN	Zinc finger protein 147						0				Z147_HUMAN	2	4	4	1.46	0.20	0.68	8.6

[1] Swiss-Prot (SP) accession number.
[2] Entry name of the accession number in Swiss-Prot Release 44.5.
[3] Peptides identified by ProID (not used for quantification).
[4] Peptides identified by ProICAT (used for quantification).
[5] Average ICAT ratio and its S.D. are calculated from all the constituent peptides (*e.g.* peptides identified from different fractions and peptides with different charge states).
[6] Corresponds to ProICAT quantification.
[7] Sequence coverage of SEQUEST-identified proteins was determined from the whole data set by ProteinProphet.
[8] Database entry names correspond to protein names STAT1, GIMAP1, MXA, and GIMAP4.

GIMAP1 (Q8WWP7, IMP1), and GIMAP4 (Q9NUV9, IMP4), were selected for further investigation based on the data and their immunological and biological properties. As the first stage of validation the cellular expression levels of STAT1, MXA, and GIMAP4 and their abundances in the microsomal fraction were verified with Western blot analysis (Fig. 3). The presence of IL-4 clearly decreased the expression of STAT1 and MXA and their amounts in the microsomal fraction in four different biological samples pooled from different individuals, whereas GIMAP4 showed a significant decrease in three of the four samples. Fig. 3 shows Western blot results from one of the samples. As an example of the quantitative MS data, a peptide of the GIMAP1 protein is presented in Fig. 4. At the time of this study there were no antibodies available for GIMAP1, and, thus, validation of the finding by Western blotting was not made.

Expression of GIMAP Family Members 1 and 4 in Th1 and Th2 Cells—The expression of *GIMAP1* and *GIMAP4* mRNA was studied in human cord blood $CD4^+$ cells that were stimulated using PHA and irradiated CD32-B7-transfected fibroblasts and induced to differentiate to either Th1 or Th2 lineage for 7 days or stimulated to proliferate by PHA and feeder cells (Th0) (Fig. 5*A*). Quantitative real-time RT-PCR analysis was performed on samples taken at different time points from four individuals, and statistical significance was determined by Student's *t* test. The results showed that IL-12 and IL-4 signaling differentially regulated both *GIMAP1* and *GIMAP4* genes during Th1 and Th2 differentiation. Furthermore, the genes showed remarkably similar expression profiles. Western blot analysis demonstrated the differential expression of GIMAP4 also on the protein level in peripheral blood $CD4^+$ cells induced to differentiate to the Th1 or Th2 direction (Fig. 5*B*).

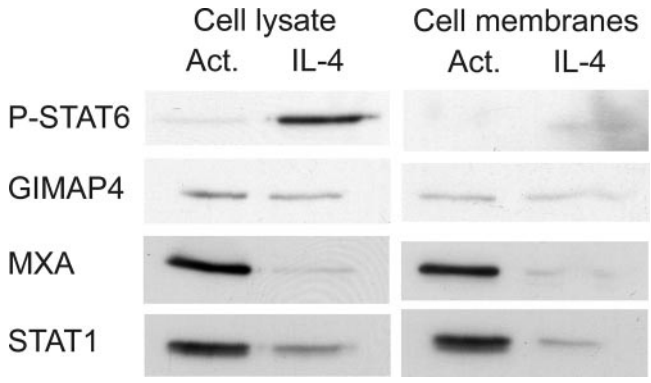

FIG. 3. **Western blot analysis of STAT1, MXA, and GIMAP4 confirmed the down-regulation by IL-4.** Human cord blood $CD4^+$ cells were activated by anti-CD3/anti-CD28 (*Act.*) and induced to differentiate toward Th2 cells with activation + IL-4. STAT1, MXA, and GIMAP4 were all down-regulated by IL-4.

While cloning *GIMAP4* we found two splicing variants. The isoforms differ by 42 amino acids. The shorter isoform is the previously described transcript (*i.e.* NM_018326) (64), and the longer isoform has been described as a full-length expressed sequence tag in GenBank™ (*i.e.* CF594134). To further study the regulation of the expression of the differentially spliced *GIMAP4* isoforms in $CD4^+$ cells real time RT-PCR was performed with reaction conditions specific for either the short or the long isoform. Plate-bound anti-CD3 and soluble anti-CD28 antibodies were used for TCR stimulation, and polarizing cytokines IL-12 or IL-4 were added to the cell culture media. A portion of the cells was cultured in neutral conditions without any polarizing cytokines. Because $CD4^+$ T cells start to produce large amounts of IFN-γ after activation, neutralizing anti-IFN-γ was added to rule out the effects of the IFN-γ produced by the cells themselves. Both isoforms of *GIMAP4* were regulated during the early differentiation of both Th1 and Th2 cells (Fig. 6). Furthermore the observed regulation of the

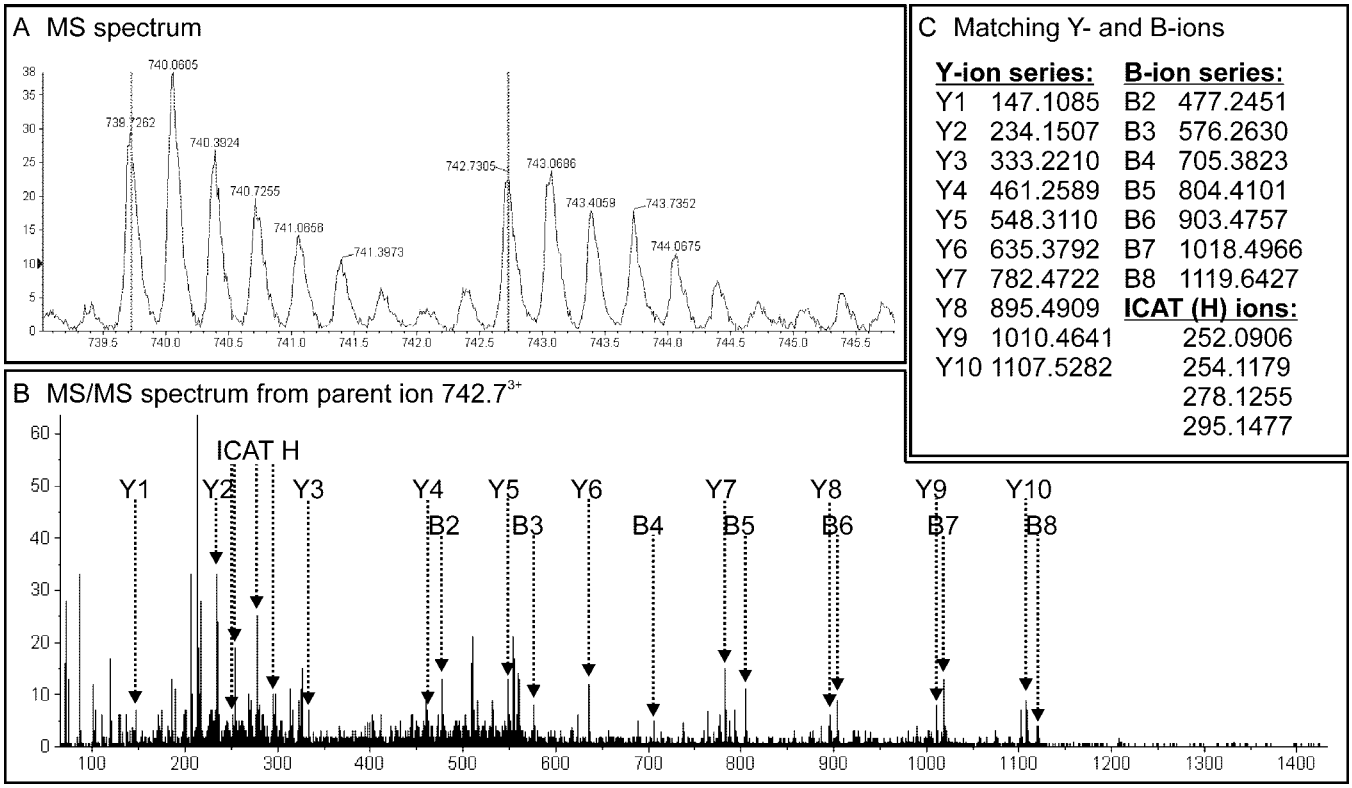

FIG. 4. **MS and MS/MS data from protein GIMAP1.** MS quantification of the peptide shows the down-regulation of GIMAP1 in the microsomal fraction of IL-4-treated cord blood $CD4^+$ cells (*A*). Peptide fragmentation by MS/MS is shown in *B*, and Y- and B-ions present in the fragmentation spectrum are shown in *C*.

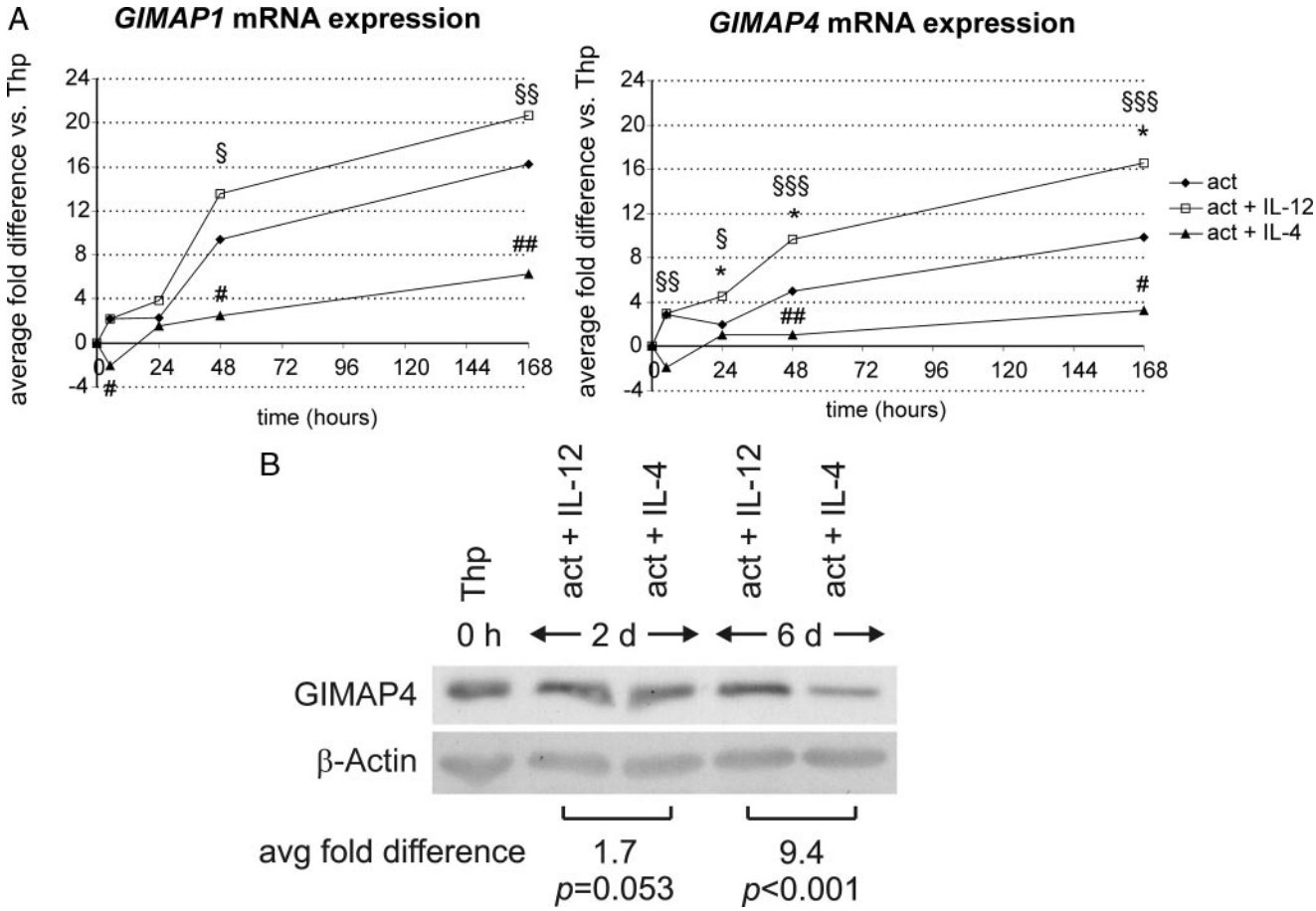

FIG. 5. ***GIMAP1* and *GIMAP4* are differentially regulated during Th1 and Th2 differentiation.** *A*, human cord blood $CD4^+$ T cells were activated with PHA and irradiated CD32/B7-transfected mouse L fibroblasts (*act*) and cultured in the presence of IL-12 or IL-4 to promote Th1 and Th2 differentiation, respectively, or in neutral Th0 conditions for 7 days. *GIMAP1* and *GIMAP4* mRNA expression was analyzed using quantitative real time RT-PCR. Each sample was analyzed in duplicate at least twice, and samples from at least three individuals were used for calculations. An *asterisk* (*) indicates a significant difference between Th1 and Th0, # indicates a significant difference between Th2 and Th0, and § indicates a significant difference between Th1 and Th2. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, human peripheral blood $CD4^+$ cells were induced to the Th1 or Th2 direction by anti-CD3/anti-CD28 activation and cytokines IL-12 and IL-4, respectively. GIMAP4 expression was detected by Western blotting 2 and 6 days after culture. *d*, days; *avg*, average.

GIMAP4 gene was not a secondary effect of the differential regulation of IFN-γ by IL-12 and IL-4. If that were the case, adding anti-IFN-γ to the cultures would have abolished the differential regulation of *GIMAP4* observed in response to IL-12 and IL-4. It is also notable that although both of the *GIMAP4* isoforms follow a similar pattern of gene expression, the up-regulation of the long isoform in response to the indicated cytokines is clearly more potent. However, the short isoform was expressed at a significantly higher level than the long isoform in Thp cells (~44-fold difference measured by real time RT-PCR, $p = 0.003$, data not shown).

We also studied the effects of other Th1-inducing cytokines, namely IFN-α and IL-18, in the regulation of GIMAP4 protein expression (Fig. 7, *A* and *B*). Again anti-IFN-γ was added to inhibit autocrine IFN-γ signaling. The results showed that GIMAP4 protein is slightly up-regulated also by IFN-α and IL-18. It is also notable that the differential regulation of GIMAP4 in cells induced to the Th1 or Th2 direction was not detected on the protein level until 48 h of culture, although on the mRNA level the differential regulation was highly significant already after 5 h of polarization.

IL-4-induced STAT6 Signaling Negatively Regulates GIMAP4 Expression—The finding that *GIMAP4* gene expression was significantly down-regulated by IL-4 (Fig. 5) led us to examine whether the expression was regulated by STAT6 signaling pathway. The expression of STAT6 was knocked down in $CD4^+$ cells using RNA interference, and cells were induced to differentiate along the Th2 lineage. Fig. 8 shows that the IL-4-induced repression of GIMAP4 expression was released when STAT6 signaling was disturbed by RNA interference. On the mRNA level the increase in *GIMAP4* expression induced by STAT6 knockdown was, on average, 2-fold after 48 h of culture (data not shown). Thus, STAT6 signaling negatively regulates the GIMAP4 expression.

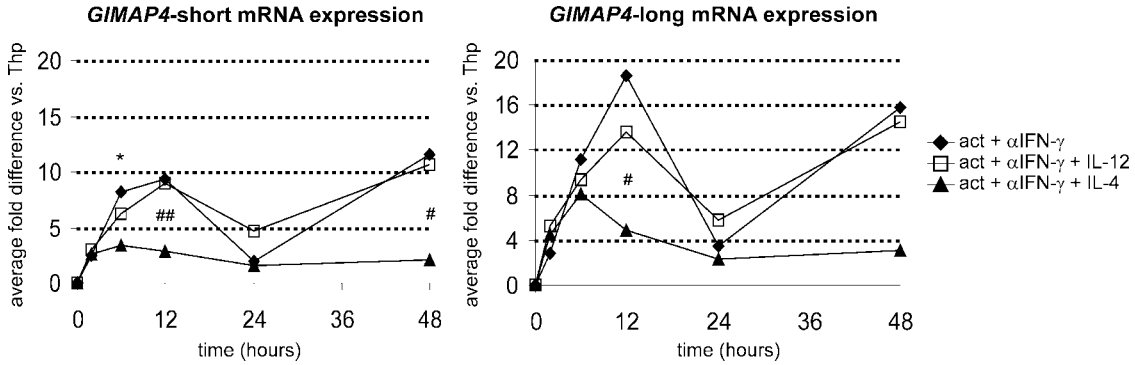

FIG. 6. **The regulation of differentially spliced variants of *GIMAP4* mRNA during early Th1 and Th2 differentiation follows similar kinetics of gene expression.** Human cord blood $CD4^+$ T cells were activated by anti-CD3/anti-CD28 (*act*) and induced to differentiate toward Th1 and Th2 lineages by IL-12 and IL-4, respectively, or activated only. Anti-IFN-γ was added to culture medium to block the autocrine IFN-γ signaling. Samples from three independent cultures were harvested and analyzed using quantitative real time RT-PCR. Differences in gene expression were calculated as -fold differences *versus* naïve Thp cells, and the significance of difference between samples was determined by Student's *t* test as in Fig. 5.

DISCUSSION

An LC-MS/MS shotgun proteomics approach was used in combination with cICAT reagents to elucidate qualitatively and quantitatively the microsomal fraction of the proteome of Th lymphocytes isolated from cord blood. Although the application of such methodology can create large volumes of informative data, the validation and interpretation process can be labor-intensive, time-consuming, and prone to error. To facilitate this task a number of powerful software algorithms have been developed (for a recent review, see Palagi *et al.* (67)). Comparisons of different software programs have shown that in addition to the overlapping protein identifications there are also unique protein identifications for individual programs (62, 68, 69). In this study a paired algorithm strategy was used to both speed up the data processing in an automated way and to analyze the data as comprehensively and reliably as possible. Protein identification and quantification with two different algorithms increase the confidence of the results obtained with both the algorithms. However, the proteins detected with a single algorithm might be as important in offering novel biological information.

Characterization of the microsomal fraction proteome of cord blood $CD4^+$ cells treated with Th2-polarizing IL-4 resulted in 557 protein identifications with at least two distinct peptides (Fig. 2). ICAT quantification was determined for 304 of the identified proteins (Table II). Proteins with a 1.4-fold difference were filtered as potential IL-4-regulated proteins. The -fold difference was set to 1.4-fold because we wanted to filter potential IL-4-regulated proteins as comprehensively as possible. Eight of the proteins were identified and quantified by both ProICAT and SEQUEST/XPRESS, whereas 13 of the proteins were detected by ProICAT alone, and 25 of them were detected only by SEQUEST/XPRESS. Differential regulation of STAT1, MXA, and GIMAP4, discovered by the ICAT and LC-MS/MS analysis, was validated with Western blotting.

GIMAP1 and GIMAP4 are members of the GIMAP family, a newly described family of putative small GTPases highly conserved among *Arabidopsis*, mouse, rat, and human (50). There are seven functional *GIMAP* genes and one pseudogene clustered in human chromosome 7, eight functional genes and one pseudogene in mouse chromosome 6, and seven functional genes in rat chromosome 4. The GIMAP family members all share an evolutionary highly conserved AIG1 domain, which includes the GTP binding motif. Initially it was shown that the expression of human GIMAP4 is for the most part restricted to spleen (64). In a more recent report a wider tissue distribution of GIMAP4 in non-lymphoid organs, especially of the reproductive system, was demonstrated, although the highest expression was detected in the cells of the immune system (50). GIMAP4 has been shown to localize mainly in cytoplasm (51, 52) but also in endoplasmic reticulum and Golgi (50). GIMAP1 is preferably expressed in spleen and lymph nodes, but the expression is not restricted to cells of the immune system (70). GIMAP1 has been shown to localize in endoplasmic reticulum (70). This study suggests membrane localization of both GIMAP1 and GIMAP4 because they were identified from the microsomal fractions of human $CD4^+$ T cells by mass spectrometric analysis.

In the context of T cell biology the studies on the expression and molecular function of the individual GIMAP members have mostly dealt with T cell development in the thymus and apoptosis and cell survival. The studies by Nitta *et al.* (51) showed differential regulation of mouse GIMAP proteins in the developing thymocytes and associated GIMAP4 with proapoptotic BAX and GIMAP3 and GIMAP5 with antiapoptotic proteins BCL-2 and BCL-XL. Studies on rat *Gimap* genes also showed differential regulation of the *Gimap* genes during thymocyte development (71).

Mouse GIMAP4 has been linked to T cell apoptosis also in a study where splenic T cells from *Gimap4*-deficient knockout mice showed a delayed apoptosis phenotype in response to proapoptotic stimuli (serum starvation, γ-irradiation, etopo-

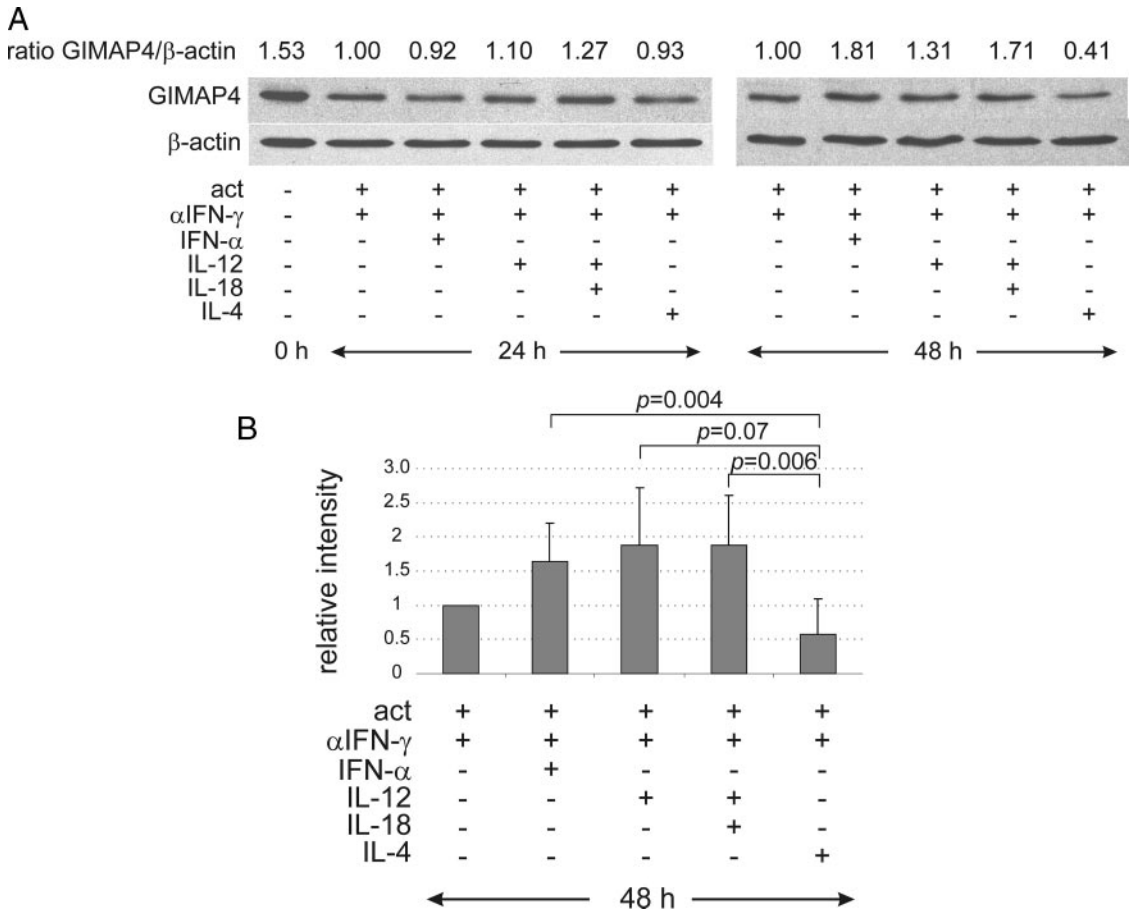

FIG. 7. **GIMAP4 is up-regulated by Th1-inducing cytokines IFN-α and IL-18.** Human umbilical cord blood CD4$^+$ cells were cultured under the indicated conditions for 24 and 48 h, and the level of GIMAP4 protein expression was analyzed by Western blotting. *A*, besides IL-12, other Th1-inducing cytokines, namely IFN-α and IL-18, induced up-regulation of GIMAP4 expression. Cytokine-induced regulation of GIMAP4 was detectable only after 48 h of culture. GIMAP4/β-actin ratios were calculated from the Western blot presented. *B*, relative quantitation of GIMAP4 protein level after 48 h of culture showing average values from four individual cultures. *Error bars*, S.D.; *p* values were obtained using Student's *t* test. *act*, activation.

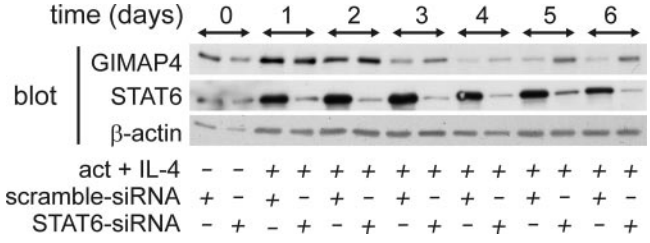

FIG. 8. **GIMAP4 gene expression is regulated by the STAT6 signaling pathway.** Human CD4$^+$ cells were transiently transfected with shRNA/siRNA targeting *STAT6* or with control (*scramble*) and induced to differentiate toward the Th2 lineage using anti-CD3/anti-CD28 activation (*act*) and IL-4. Western blotting showed an effective knock down of STAT6 by STAT6 siRNA and high dependence for GIMAP4 expression on STAT6. The figure is a representative of three individual experiments showing a similar pattern of expression.

side, and dexamethasone) compared with wild type cells (52). Similar results, although not as pronounced, were reported in a study describing a natural hypomorphic variant of rat *Gimap4* gene (53). However, GIMAP3, GIMAP5, and GIMAP8 have been shown to be antiapoptotic and important for T cell survival (51, 54, 56). On the other hand, overexpression of *GIMAP4* in a pre-B hematopoietic cell line, BaF3, had no effect on apoptosis (64). Thus, not only is the function of the GIMAP members different but the molecular function of the individual GIMAPs seems to be dependent on the cell type.

This is the first report where differential regulation of the expression of GIMAP1 and GIMAP4 proteins during Th1 and Th2 differentiation is shown. Their expression was found to be down-regulated in microsomal fractions of cells treated with IL-4. Our results also showed that both *GIMAP1* and *GIMAP4* gene expression was up-regulated by TCR signaling in cord blood CD4$^+$ T cells, further enhanced by IL-12, a Th1-inducing cytokine, and down-regulated by IL-4, a Th2-inducing cytokine. Our results are in keeping with the findings of Cambot *et al.* (64) and Schnell *et al.* (52) indicating that the amount of GIMAP4 protein was decreased in activated T cells. However, contrary to their results we found that activation of human cord blood CD4$^+$ T cells by

anti-CD3/anti-CD28 up-regulated the mRNA expression instead of stable gene expression. We also investigated the expression of two different splice variants of *GIMAP4* of which the long isoform has not been previously studied. Although it was notable that the up-regulation of the long isoform by activation and IL-12 was more potent than that of the short isoform, the short isoform was clearly more abundant during the 2-day culture.

In this study, we demonstrated that *GIMAP4* gene expression is negatively regulated by STAT6 signaling pathway during the early phases of Th2 differentiation by showing that IL-4-induced STAT6 signaling is necessary for effective repression of GIMAP4 expression. Studies verifying the binding of STAT6 to the regulatory areas of the *GIMAP* gene cluster are underway in our group. Notably a recent genome wide gene expression analysis performed in our group showed markedly similar gene expression kinetics for the whole *GIMAP* gene cluster in response to IL-12 or IL-4 stimuli during the early phases of Th1 and Th2 differentiation, respectively.[2] The molecular mechanisms responsible for this remain to be studied.

A functional role for GIMAP4 in Th1/Th2 differentiation is supported by the finding that the inbred Brown Norway rat, which carries a natural hypomorphic variant of *Gimap4* (53), is biased to Th2-type immune responses (72) and generates very high IgE responses (73). In addition, Brown Norway rats are susceptible to Th2-mediated autoimmunity (for a review, see Ref. 74). A role for GIMAP5 in the regulation of Th1/Th2 balance is suggested by a recent report describing PVG-*RT1^u*, *lyp*/*lyp* rats (the *lyp*, *i.e.* lymphopenia, gene encodes for GIMAP5), that develop spontaneous intestinal inflammation with Th2-related characteristics (75). Although there is growing interest in GIMAP family members, the cellular functions of these proteins remain ill defined. It will be interesting to determine in which signaling pathways these GTPases are participating and whether they share similar or different functions in the Th1/Th2 polarization process. Defining the possible role of GIMAP family members in the regulation of the Th cell differentiation process warrants investigation.

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S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

¶ Both authors made equal contributions to this work.

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[2] L. Elo, H. Järvenpää, S. Tuomela, H. Ahlfors, S. K. Raghav, B. Gupta, R. Lund, J. Tahvanainen, M. Oresic, O. Rasool, K. V. Rao, T. Aittokallio, and R. Lahesmaa, unpublished observations.

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