PLAYERS OF TH-CELL DIFFERENTIATION AND IMMUNE DYSREGULATION
- focus on GIMAP family genes

Mirkka Heinonen
“You look at science (or at least talk of it) as some sort of demoralising invention of man, something apart from real life, and which must be cautiously guarded and kept separate from everyday existence. But science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment.”

-Rosalind Franklin
ABSTRACT

Mirkka Heinonen
Players of Th-cell differentiation and immune dysregulation – focus on GIMAP family genes
Faculty of Biomedicine, Department of Medical Microbiology and Immunology, University of Turku
Turku Centre for Biotechnology, University of Turku and Åbo Akademi University
Turku Doctoral Programme of Molecular Medicine (TuDMM)

The balance of T helper (Th) cell differentiation is the fundamental process that ensures that the immune system functions correctly and effectively. The differentiation is a fine tuned event, the outcome of which is driven by activation of the T-cell in response to recognition of the specific antigen presented. The co-stimulatory signals from the surrounding cytokine milieu help to determine the outcome. An impairment in the differentiation processes may lead to an imbalance in immune responses and lead to immune-mediated pathologies. An over-representation of Th1 type cytokine producing cells leads to tissue-specific inflammation and autoimmunity, and excessive Th2 response is causative for atopy, asthma and allergy. The major factors of Th-cell differentiation and in the related disease mechanisms have been extensively studied, but the fine tuning of these processes by the other factors cannot be discarded.

In the work presented in this thesis, the association of T-cell receptor costimulatory molecules CTLA4 and ICOS with autoimmune diabetes were studied. The underlying aspect of the study was to explore the polymorphism in these genes with the different disease rates observed in two geographically close populations. The main focus of this thesis was set on a GTPase of the immunity associated protein (GIMAP) family of small GTPases. GIMAP genes and proteins are differentially regulated during human Th-cell differentiation and have been linked to immune-mediated disorders. GIMAP4 is believed to contribute to the immunological balance via its role in T-cell survival. To elucidate the function of GIMAP4 and GIMAP5 and their role in human immunity, a study combining genetic association in different immunological diseases and complementing functional analyses was conducted. The study revealed interesting connections with the high susceptibility risk genes. In addition, the role of GIMAP4 during Th1-cell differentiation was investigated. A novel function of GIMAP4 in relation to cytokine secretion was discovered. Further assessment of GIMAP4 and GIMAP5 effect for the transcriptomic profile of differentiating Th1-cells revealed new insights for GIMAP4 and GIMAP5 function.

Keywords: GIMAP4, GIMAP5, human Th1-cell differentiation, Type 1 Diabetes, Asthma and Allergic sensitization, IFN-γ, transcriptome, population association study
TIIVISTELMÄ

Mirkka Heinonen
Pelaajat Th-solujen erilaistumisessa ja immunihiiriöissä – keskiössä GIMAP perheen geenit
Biolääketieteen laitos, Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto
Turun Bioteknikan keskus, Turun yliopisto ja Åbo Akademi
Turun molekulaarisen lääketieteet tutkijaohjelma (TuDMM)

T-auttaja(Th)solujen erilaistuminen on avainasemassa immuunijärjestelmän tehokkaan
toiminnan kannalta. Erilaistumisprosessi on tarkasti säädelty. Prosessia ohjaavat antigeeniä
esittelevät solut, Th-soluja aktivoivat molekyylit sekä Th-solua ympäröivät sytokiinit.
Prosessin häiriintymisestä voi seurata Th-solujen alaluokkien epätasapaino ja immunologinen
epäjärjestys. Liiallinen Th1-solu vaste johtaa autoimmunisairauksiin ja liiallinen Th2-solu
vaste aiheuttaa kroonisia tulehduksia; atopiaa, astmaa ja allergiaa. Vaikka Th-solujen
erilaistumiseen vaikuttavia päätekijöitä ja mekanismeja on tutkittu paljon, immuunivasteen
hienosääteelyyn vaikuttavat tekijä ja mekanismit ovat vielä laajalti selvittämättä.

Tässä tutkimuksessa on selvitetty yhdessä T-solureseptorin kanssa Th-solu aktivaatioon
tarvittavien, CTLA4 ja ICOS, molekyylien yhteys tyyppi 1 diabetekseen kahdessa eri
maantieteellisesti läheisessä populatiotessa, joissa sairauden esiintyvyys on hyvin erilainen.
Tutkimuksen pääpaino on keskittynyt yhdessä GIMAP eli Immunitetin assosioitteeseen proteiinit -
perheen pienten GTPaasien toimintaan. GIMAP geenit ja proteiineja on yhdistetty
immuunivälitteisiin sairauksiin eläinmalleissa. GIMAP4:n on osoitettu vaikuttavan
Th-solujen solukuolemana eli apoptoosiin ja GIMAP5:n on osoitettu olevan yhteydessä rotan ja
hiiren autoimmunihäiriöihin. Väitöskirjatyössä GIMAP4:n ja GIMAP5:n roolia Th-solujen
erilaistumisessa ja immunitetin häiriötiloissa selvitettiin yhdistämällä populaatiogeneettinen
tutkimus toiminnallisen tutkimuksen kanssa. Selvitimme GIMAP4:n assosioituvan astmaan ja
allergiaan, sekä GIMAP5 assosioituvan astmaan ja allergiaan, sekä tyyppi 1 diabetekseen.
Tutkimme lisäksi kyseisten GIMAP geenien välästä yhteisvaikutusta muiden tunnettujen
tyyppi 1 diabetekseen vaikuttavien tekijöiden kanssa. Lisäksi, havaitsimme GIMAP4:n
vaikuttavan Th-solujen sytokiinin tuotteen varhaisen Th-solu erilaistumisen aikana sekä
löysimme uusia seikoja GIMAP4:n ja GIMAP5:n toiminnasta Th1-soluun transcriptomin
tasolla.

Avainsanat: GIMAP4, GIMAP5, ihmisen Th1 solu erilaistuminen, Tyyppi 1 Diabetes, Astma
and Allerginen herkistyminen, IFN-γ, transcriptomi, populaatio assosiaatio tutkimus
# Table of Contents

ABSTRACT ......................................................................................................................1

TIIVISTELMÄ ..................................................................................................................2

LIST OF ORIGINAL PUBLICATIOS ...............................................................................8

1 INTRODUCTION .........................................................................................................9

2 REVIEW OF THE LITERATURE ..............................................................................11

2.1 The immune system ............................................................................................ 11

2.2 T-cell development ............................................................................................. 12

2.3 CD4+ T helper (Th) cells .................................................................................... 13

   2.3.1 Th-cell activation ....................................................................................... 14

   2.3.2 Th-cell function and regulators of differentiation ....................................... 15

   2.3.3 Induction of Th1-cell differentiation .......................................................... 18

      2.3.3.1 TCR, co-stimulation with NFAT, AP1 and NFkB ...................... 20

      2.3.3.2 Cytokines and transcription factors ..................................... 21

   2.3.4 Th-cells and immune dysregulation ........................................................... 23

      2.3.4.1 Type 1 diabetes (T1D) ........................................................ 23

      2.3.4.2 CTLA4 and ICOS in type 1 diabetes ................................... 25

      2.3.4.3 Asthma ............................................................................... 26

      2.3.4.4 The smaller effectors in immune dysregulation ................... 27

2.4 GIMAP family of Small GTPases ....................................................................... 28

   2.4.1 Gene and protein structure ...................................................................... 29

   2.4.2 GIMAP function ..................................................................................... 30

   2.4.3 GIMAP4 ................................................................................................ 32

   2.4.4 GIMAP5 ................................................................................................ 33

3 AIMS OF THE STUDY .............................................................................................. 36

4 MATERIALS AND METHODS ................................................................................. 37

   4.1 Ethics consideration (I-III) ........................................................................... 37
4.2 Population genetic association studies (I, II) ....................................................... 37
   4.2.1 Subjects ..................................................................................................... 37
   4.2.2 Genotyping ............................................................................................. 38
   4.2.3 Statistical analyses .................................................................................... 38
4.3 Human primary CD4+ T-cell isolation (II, III) ...................................................... 39
4.4 Cell culture and transfections (II, III) ................................................................ 39
   4.4.1 Human primary CD4+ T-cell culture and nucleofection ......................... 39
   4.4.2 Hela-cell culture and transfections ......................................................... 40
4.5 Luciferase reporter constructs and measurement (II) ........................................... 40
4.6 Confocal microscopy (III) .................................................................................. 40
4.7 Flow cytometry (II, III) .................................................................................... 41
   4.7.1 Cell surface staining .............................................................................. 41
   4.7.2 Intracellular staining .............................................................................. 41
4.8 Extracellular cytokine profiling (III) .................................................................. 42
4.9 Western blotting (III) ..................................................................................... 42
4.10 Quantitative real-time RT-PCR (II, III) ........................................................... 42
4.11 Chromatin immunoprecipitation (Unpublished) ............................................... 43
4.12 Transcriptomics (III) ..................................................................................... 43
   4.12.1 Microarray ............................................................................................. 43
   4.12.2 RNA-sequencing (Unpublished) .............................................................. 44
5 RESULTS AND DISCUSSION .............................................................................. 47
5.1 The association of CTLA4, ICOS, GIMAP4 and GIMAP5 with immune-mediated diseases (I, II) ................................................................. 47
   5.1.1 CTLA4, but not ICOS, is association with T1D in Finnish and Estonian populations ......................................................................................... 47
   5.1.2 GIMAP5 associates with T1D, asthma and allergic sensitization ............. 48
5.1.3  GIMAP5 and INS interaction and the GIMAP5 3’ UTR genotypic variance.................................................................50

5.1.4  GIMAP4 association with asthma & allergic sensitization and IL2RA interaction ..........................................................52

5.2  The role of GIMAP4 and GIMAP5 in early human Th-cell differentiation (III) .................................................................54

5.2.1  GIMAP4 localizes into the intracellular trafficking compartment ..........54

5.2.2  GIMAP4 regulates the expression of ER localizing chaperone VMA21.....55

5.2.3  GIMAP4 is regulated by STAT4, but not essential for TCR activation ......55

5.2.4  The role of GIMAP4 in IFNγ signaling and Th1-cell differentiation ........58

5.2.5  The effects of GIMAP4- and GIMAP5-depletion on Th-cell transcriptome during early differentiation (Unpublished) ...............62

5.2.6  GIMAP4 and VMA21 revisited (Unpublished) ........................................64

6  LIMITATIONS OF THE STUDIES AND DISCUSSION OF THE IMPROVEMENTS..........................................................................66

7  CONCLUSIONS .................................................................................................................................................................68

ACKNOWLEDGEMENTS .........................................................................................................................................................70

REFERENCES ........................................................................................................................................................................72

ORIGINAL PUBLICATIONS ..................................................................................................................................................91
ABBREVIATIONS

API  Activator protein 1, transcription factor complex
APC  Antigen presenting cell
BBDP Bio-breeding diabetes prone rat
BCL  B-cell lymphoma 2
BrdU Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
BSA  Bovine serum albumin
cDNA Complementary deoxyribonucleic acid
CFSE Carboxyfluorescein succinimidyl ester
ChIP Chromatin Immunoprecipitation
CTLA4 Cytotoxic T-lymphocyte-associated protein 4
CXCR CXC chemokine receptor
DC  Dendritic cell
DMEM Dulbecco’s Modified Eagle’s Medium
DN  Double negative
DP  Double positive
ECL Electrochemiluminescence
EF1α Eukaryotic translation elongation factor 1 alpha
FACS Flow activated cell sorting
FBS Fetal bovine serum
FDR False discovery rate
FITC Fluorescein isothiocyanate
FOXP3 Forkhead box P3
FPDRNF Finnish pediatric diabetes registry nuclear family
GADD45 Growth arrest and DNA-damage-inducible 45
GATA3 GATA binding protein 3
GIMAP GTPase of the immune associated protein family
HLX H2.0-like homeobox
HRP Horseradish peroxidase
ICAM Intercellular adhesion molecule
ICOS Inducible T-cell co-stimulator
IFNγ Interferon gamma
Ig  Immunoglobulin
IL  Interleukin
IRF Interferon regulatory factor
IS  Immunological synapse
iTreg Inducible T regulatory
JAK Janus kinase
JNK c-Jun N-terminal kinase
kDa Kilo Dalton
LAT Linker for activation of T-cells
LFA1 Lymphocyte function-associated antigen 1
MAP Mycobacterium avium paratuberculosis
MAPK Mitogen activated protein kinase
MHC Major histocompatibility complex
NFAT Nuclear factor of activated T-cells
Abbreviations

NFκB  Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NOD  Non-obese diabetic
nt  Nucleotide
OX40  CD134, tumor necrosis factor receptor
OR  Odds ratio
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PKC  Protein kinase C
PLC  Phospholipase C
RNA  Ribonucleic acid
RNAi  RNA interference
ROR  RAR-related orphan receptor
rRNA  Ribosomal RNA
RT-qPCR  Real time quantitative PCR
RUNX  Runt-related transcription factor
siRNA  Small interfering RNA
SN  Single negative
SNP  Single nucleotide polymorphism
SP  Single positive
STAT  Signal transducer and activator of transcription
T1D  Type 1 diabetes
TBET  T-Cell-Specific T-Box Transcription Factor, Tbx21
TCR  T-cell receptor
TDT  Transmission disequilibrium test
Tfh  Follicular T helper
TGF  Transforming growth factor
TGN  Trans Golgi network
Th  T helper
Thp  Progenitor T helper
TIM  T-cell immunoglobulin and mucin domain containing
Treg  T regulatory
VMA21  Vacuolar ATPase assembly integral membrane protein 21
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by roman numerals (I-III). The thesis includes unpublished data.


* and # Authors have contributed equally to the article

The original publications have been reproduced with the permission of the copyright holders.
1 INTRODUCTION

Our health and survival are jeopardized by the continuous attack of foreign objects, pathogens, which range from bacteria to viruses to parasites. Beyond the unspecific barriers formed by skin and mucus, a more specific system is needed to fight the unwanted invaders – the immune system. This marvelous defense mechanism can be divided in two, the innate and adaptive immune systems, which, working together and apart, make sure that the invaders are efficiently cleared. Innate immunity is the first line of defense. The adaptive immunity, which can form memory, is more efficient in its response at pathogen re-encounter.

T-helper (Th)-cells are part of the adaptive immune system. The innate immunity cells serve as antigen presenting cells (APC) introducing the foreign molecules to Th-cells, thus stimulating the invader-depended response. The memory of the immune system is created by triggering the differentiation of Th-subtypes (Th1, Th2, Th17, Treg, Tfh). This process is highly controlled and conveyed by the co-stimulatory molecules and the cytokine milieu at the time of APC-Th-cell priming. On the basis of their original discovery and classification, the Th1-cells are in charge of clearing the system from intracellular pathogens, and Th2-cells take care of the extracellular invaders. The main functional mechanism for these cells, and the other Th-cell subtypes discovered later, is the secretion of hallmark cytokines. The cytokine profile of a given Th-cell type is the characteristic by which they are also distinguished from each other.

An immunological imbalance occurs if the differentiation or the mechanistic function of one or multiple subtypes is distorted. This may lead to various immune-mediated diseases, the phenotype of which depends on the subtype-specific response that is taking over. The relative proportion of the cell-type-specific cytokines drives the pathological immune response. Roughly, the pathologies can be divided to be caused by type 1 or type 2 immune response. The former occur when the Th1-type cytokine milieu is expressed excessively and results in autoimmunity and tissue destruction. The Th2-type responses result in chronic inflammation, atopy, asthma and allergic reactions. Importantly, the regulation of the immune system is also controlled by cytokines produced by the T-regulatory (Treg)-cells. The distorted immune regulation mostly requires both, an excess of one Th-cell subtype and a malfunction in its regulation.

Type 1 diabetes (T1D) is an autoimmune disorder, which has a great socioeconomical impact. Worldwide, the highest incidence is found in Finland. Genetic factors account for only 50% of the disease risk of which the human leucocyte antigen, *HLA*, gene region confers 50% and the rest is most likely influenced by environmental factors. Beyond *HLA*, most of the other genetic factors that are studied in detail, have been those with the highest conferring risk. *CTLA4*, which is one of the driving molecules in immune suppression, has been shown to have a high genetic effect on the risk for T1D. Additionally, genetic variance in other
genes important in Th-cell function, such as IL2Ra and PTPN22, have been shown to have a moderate risk effect in the development of T1D. The association of CTLA4, together with ICOS, in two geographically close populations with different disease rates was studied in this thesis. The results indicate that variance in CTLA4 genotype may reflect the different disease rate observed in these populations.

There are, however, a number of other susceptibility genes that have not been found by large scale genome wide association studies (GWAS), but are nevertheless, important players in the disease etiology. The small GTPase GIMAP family genes were recently recognized as factors in Th-cell function. The GIMAP family proteins are highly regulated during Th-cell development and differentiation, thus making them interesting targets of studies in immune regulation and dysfunction. The functions of the GIMAP protein family members have, as of yet, been poorly characterized. The majority of investigations have concentrated on the role for GIMAP5 in rodent lymphopenia and there are only a few human studies published to date. In the work presented in this thesis, the role of GIMAP4 and GIMAP5 in early human Th-cell differentiation was studied. Specifically, the role and function of GIMAP4 during early Th1-cell differentiation was investigated. The population genetic studies of asthma, allergy and type 1 diabetes were conducted to elucidate GIMAP4 and GIMAP5 role in immune dysregulation. Interesting genetic interactions between the GIMAPs and other Th-cell specific T1D susceptibility genes were found. Additionally, functional studies were conducted to strengthen the findings of genetic association. Overall, the results highlight the role of GIMAP proteins in Th-cell differentiation and in the pathways important for Th-cell function.
2 REVIEW OF THE LITERATURE

2.1 The immune system

The immune system is one of the most sophisticated systems in vertebrate biology. It has an amazing capability to attack an outsider pathogen in seconds, and it is adaptable. In the absence of correct regulation, it may attack one's own self and this can lead to harmful consequences. Immunity plays a central role in cancer, neurological diseases and metabolic syndromes, among others. Thus, the well-functioning immune system is the basis for a healthy organism and understanding of the immune regulation is essential in comprehending the balance of health and disease.

All the hematopoietic cells, lymphoid and myeloid, develop from the hematopoietic stem cells in the bone marrow. The differentiation is orchestrated by various and precise regulatory pathways. Of the myeloid lineage, macrophages are crucial for the innate immunity responses. Dendritic cells are important for the adaptive immune responses, as they serve often as antigen presenting cells (APC) for effector lymphocytes. Innate immunity also involves the humoral complement system and other cellular components, such as eosinophils, basophils, natural killer cells and mast cells. Upon pathogenic invasion, the innate immunity cells take the front line in the battle. Due to the lack of memory formation, the innate immune system cannot be educated through exposure to an invader. Thus, innate immunity reacts in a more or less similar way to a particular foreign agent. In vertebrates, another system has developed to act alongside and in close cooperation with the innate immunity. The lymphocyte driven adaptive immunity has memory and can modify and expedite its response according to the pathogen once it has been encountered for the first time. Importantly, the adaptive immunity is specific and has to distinguish the self from non-self. (Murphy et al. 2012)

The naming of the lymphocytes originates from their source, as B-cells develop in the bone marrow and T-cells develop in the thymus. Both of these cell types are able to recognize pathogen-specific antigens via specific cell surface receptors, resulting in an appropriate immune response to eliminate the menace. In addition to their cooperation within innate immunity, B- and T-cells form an elaborate alliance with each other. The basis of the immune response mediated by these cells involves the secretion of soluble antibodies in response to extracellular pathogens. These antibodies either neutralize the foreign antigens or activate the innate immunity response by binding to the pathogen. A high genetic variability of the B-cells, due to the somatic DNA recombination of the immunoglobulin genes, ensures a vast repertoire of different antibodies that recognize a large variety of antigens (Li et al. 2004). Once a B-cell recognizes an antigen “A” via interaction with the cell surface B-cell receptor, it becomes activated in the presence of co-stimulatory signals from the T-cells (Crotty 2011). The activation is followed by clonal expansion resulting in plasma cells, which secrete antibodies against the antigen “A”. In addition, the plasma cell expansion creates memory cells, which ensure fast and efficient defense against the pathogen when re-encountered. T-
cells can make the process more effective by steering the antibody class switch to be more suitable for a particular pathogen clearance. (Murphy et al. 2012). The T-cells induce this class switch of the Ig constant region from the first expressed IgM to IgG1, -2, -3 or -4, IgE or IgA, depending on the Th-cell subtype and the cytokine produced (Crotty 2011; Delves and Roitt 2000; Mond et al. 1995).

2.2 T-cell development

T-cells are divided functionally into two categories, the major histocompatibility complex (MHC) class II-reactive CD4+ Th-cells and the MHC class I-reactive CD8+ T-cytotoxic (Tc) -cells. The main function for Tc-cells is to destroy infected cells by releasing cytotoxic proteins. The main function of Th-cells is to assist other immune cells by secreting cytokines. All T-cells differentiate from common precursor thymocytes in the thymus. The developmental process involves seven distinct maturation stages, based on the expression of specific cell surface molecules; double-negative 1-4 (DN1-4), two stages in double-positive (DP) and finally a single-positive (SP) stage. The different developmental steps take place in different thymic compartments (Figure 1).

During the DN stages the expression of CD44, which allows T-cells to migrate into the proliferation zone, is gradually shut down and CD25/IL2Rα is transiently expressed. The TCR gene rearrangements begin in the DN2 phase and the cells, which express β chain, loose CD25 and obtain the pre-α chain. The two chains form the pre-TCR, which together with the invariable CD3 chain enable cell proliferation (DN4) and the expression of the CD4 and CD8 co-receptors (DP). At the DP stage, the α chain genes rearrange and mature TCR expression gradually begins. Positive selection of the T-cells occurs during this stage. The mature TCR is tested for the ability to recognize the MHC molecules and those unable to do so undergo programmed cell death, apoptosis. The cells start to express only one of the co-receptors and become SP cells. The negative selection, in which the cells are tested for autoreactivity (reaction to self-antigens), starts at this stage. Most T-cells have the TCR dimer formed from α and β chains, and only a small portion of the circulating T-cells express γδ TCR. The γδ T-cells are most frequent in the mucosal epithelia and mediate both innate and adaptive immune responses. (Carpenter and Bosselut 2010; Murphy et al. 2012; Vantourout and Hayday 2013).
Figure 1. The Thymic development of T helper cells. T-cells develop in three stages, which include the four modes in the double-negative (DN) stage, two in double-positive (DP) stage and finally the single-positive (SP) stage. The pre-T-cell receptor (TCR) is expressed and tested for self-recognition in the DN stage. The CD8 and CD4 receptors are expressed in the DP stage, during which also the TCR matures. The SP stage is defined either by CD8 or CD4 expression.

2.3 CD4+ T helper (Th) cells

Th-cells are divided functionally into different subtypes including Th1, Th2, Th17, Treg (T-regulatory) and Tfh (follicular T-helper). The discovery of the different subtypes originates in the finding of different cytokine profiles of the Th1- and Th2-cells (Mosmann and Coffman 1989). Later, the knowledge of different Th-cell subtypes, defined by the expression of cytokines, chemokine receptors and transcription factors, has multiplied. A strict separation of one cell type from another has become complex due to the growing understanding of effector Th-cell plasticity (O'Shea and Paul 2010; Sundrud et al. 2003). Th-cells orchestrate and guide the cell mediated immunity, thus the cellular dysfunction of these cells mediates
immunopathologies (Chapter 2.3.4). Dysfunction of the immune system is often caused by the imbalance between the functional subtypes of Th-cells, due to abnormality in cell number and/or cell function. The understanding of the Th-cell differentiation process, from naive to effector cell, is therefore important in understanding the mechanisms of immune-mediated diseases.

2.3.1 Th-cell activation

Once fully developed, the naive Th-cells migrate to the periphery. Under normal conditions, i.e. the lack of infection, the Th-cell pool is maintained in a steady state by weak signals from the TCR complex and interleukin 7 (IL7) (Surh and Sprent 2008). Upon appropriate contact with the specific antigen presenting APC, the naive cells are activated, start to differentiate and proliferate. The activation requires four types of signals. The process starts when the APC attaches to the T-cell through interaction of non-specific adhesion molecules. The MHC class II bound antigen is presented to the TCR. If the binding is specific and strong enough, the immunological synapse (IS) forms at the site of contact. Besides their role in activation, the cytokines drive the epigenetic chromatin remodeling. The epigenetic changes induce or repress the expression of cell-type-specific genes (Inami et al. 2004; Kanno et al. 2012). The main co-stimulatory signals in Th-cell activation are mediated by two molecule families; the immunoglobulin superfamily (CD28, ICOS, CTLA4, PD-1 and TIM molecules) and the tumor necrosis factor (TNF) receptor superfamily (CD27, CD30, CD40, OX40/CD134) (Abdoli and Najafian 2014). The TNF family members OX40, the immunoglobulin family member TIM, Delta or Jagged binding Notch and LFA-1 binding ICAM, are important in defining cell fate after activation (Amsen et al. 2009; Salomon and Bluestone 1998; Amsen et al. 2007; Ito et al. 2005; Smits et al. 2002).

The co-stimulatory signals mediated by the CD28 family are most important for Th-cell activation. The cells are able to proliferate and differentiate once the IL2 and IL2R (IL2 receptor) expression has started as a result from CD28 priming (Zhou et al. 2002). Without proper interaction between the B7 molecules expressed on the APC and CD28 family molecules expressed on the Th-cell surface, the activated cell undergoes apoptosis or enters an anergic non-responsive state (Bour-Jordan et al. 2011). TCR activation coupled with co-stimulation activates the proximal signaling complex. CD4 recognizes the same MHC class II molecule as the TCR does. The TCR cytoplasmic immuno-receptor tyrosine-based activation motifs (ITAMs) of the CD3 dimer are phosphorylated by the Src non-receptor family kinase Lck. The tyrosine kinase ζ-chain associated protein (ZAP-70) is then recruited to the proximal signaling complex. Activated ZAP-70 phosphorylates the scaffold protein linker of activated T-cells (LAT). Adapter proteins called Gads assist LAT and ZAP-70 binding with activated SLP-76. The ZAP-SLP -complex activates phospholipase C gamma (PLC-γ) together with the membrane associated Ltk tyrosine kinase and CD28 induced co-stimulatory signals. The activated PLC-γ initiates the hydrolysis of phosphatidylinositol-4,5-Bisphosphate (PIP₂) and the release of 1,2-diacylglycerol (DAG) and inositol triphosphate (IP₃).
The secondary messengers, DAG and IP3, activate multiple signaling cascades, which lead to the elevation in intracellular calcium. After this, the following three steps take place: (1) Initiation of calcium regulated signaling pathways, which leads to the transcriptional regulation by the nuclear factor of activated T-cells (NFAT) family members. (2) Protein kinase C-theta (PKC-θ) induces the nuclear factor kappa light chain enhancer in B-cells (NFκB) family regulated transcription. (3) Initiation of the ras GTPase activated mitogen activated protein kinase (MAPK) pathway results in activator protein 1 (AP-1) regulated transcription. One of the main functions of NFAT, NFκB and AP-1 together is to stimulate the IL2 production, and promote T-cell differentiation and proliferation (Zhang and Nabel 1994). The transcriptional regulation led by these pathways is also an important part in defining the future lineage commitment (Aronica et al. 1999; Corn et al. 2005; Tripathi et al. 2012; Yoshida et al. 1998). (Murphy et al. 2012; Smith-Garvin et al. 2009).

2.3.2 Th-cell function and regulators of differentiation

The Th-cell lineage commitment process (Figure 2) is launched at the time of TCR activation and greatly influenced by the strength of the TCR stimulus. The strength affects the subsequent intensity of calcium signaling and thus, the line of differentiation that the cell will engage (Constant et al. 1995; Leitenberg and Bottomly 1999; Noble et al. 2000; Sloan-Lancaster et al. 1997). The specific pathogen induced, co-stimulatory molecules on the surface of the APC influence which additional signaling pathways are activated in the Th-cell (Kara et al. 2014). Differentiation inducing signaling is mediated through Th-cell transmembrane receptors, which include the co-stimulatory molecules, cytokine receptors and the cell migration enabling chemokine receptors (Geginat et al. 2003; Inami et al. 2004; Zhang et al. 2000). The developing subsets are able to regulate their own differentiation and that of the other subtypes (Yamane and Paul 2013). At the end, a given cell fate is determined by the combination of signals, both inductive and repressive. The initial signals activate a set of specific transcription factors, which drive the differentiation. Transcriptional activation also further reinforces the lineage-specific cytokine production, often creating a positive self-regulatory effect. (Kara et al. 2014; Murphy et al. 2012; Smeets et al. 2012)

The function of Th1-cells is to protect against intracellular bacteria and viruses, which are phagocytosed, but not killed by macrophages. Th1-cells secrete interferon gamma (IFNγ) in a targeted manner through the IS (Huse et al. 2006), which further stimulate and assists macrophages to destroy the invaders. The function of GIMAP proteins, particularly in Th1-cells, is in the focus of this thesis and thus Th1-cell differentiation is described in detail later (chapter 2.3.3.) The other Th-cell subtypes: Th2, Th17, Treg and Tfh are discussed briefly and the reader is referred to several recent extensive reviews for further information (Yamane and Paul 2013; Korn et al. 2009; Piccioni et al. 2014; Crotty 2011).

Review of the Literature
Figure 2. CD4⁺ T helper cell differentiation. The SP CD4⁺ progenitor Th-cell (Thp) begins to differentiate into distinct subtype upon antigen encounter. The surrounding cytokines and the stimulatory signals from the APC define the direction of differentiation. The different subtypes are classified by the key transcription factors driving the process as well as by the effector cytokines the cells secrete. The hallmark cytokines determine the nature of the resulting immune response. The arrows pointing from the subtypes (Treg, Th17, Th1, Th2 and Thfh) indicate the specific cytokines each cell type secretes. The arrows pointing to the subtypes indicate the cell differentiation inducing factors.
Th2-cell differentiation

Th2-cells fight against extracellular parasites by assisting in B-cell class switching to produce the IgE antibody isotype. They also activate cells primarily functioning in innate immune system, such as eosinophils and mast cells. IL4 is the Th2-cell effector cytokine, which is secreted both synaptically and in a multidirectional manner (Huse et al. 2006). One of the earliest signals initiating differentiation towards the Th2-cell lineage after TCR stimulus is the activation of the extracellular signal-regulated kinase (ERK) transcription factor (Tao et al. 1997; Dong et al. 2002). Additionally, coupling of two co-stimulatory molecules, ICOS and CD28, promotes Th2-cell differentiation (Coyle et al. 2000). NFAT and NFκB can promote both Th1- and Th2-cell differentiation depending on other factors, such as the chromatin state (Agarwal et al. 2000; Kiani et al. 2001; Lee et al. 2004). The main effector cytokines secreted by Th2-cells are IL4, IL5 and IL13. The differentiation of Th2-cells is strongly regulated by IL4. It induces the expression of the two key transcription factors, GATA binding protein 3 (GATA3) and signal transducer and activator of transcription 6 (STAT6). The secretion of Th2-cell cytokines is orchestrated by signal transduction via STAT6. (Horiuchi et al. 2011; Kaplan et al. 1996). GATA3 also induces Th2-cell differentiation by suppressing Th1-cell signal transducers (Zhu et al. 2006). GATA3 can be activated independently from IL4 stimulation and it also positively regulates its own expression, thus maintaining the differentiation process (Ouyang et al. 2000). In addition to STAT6, STAT5 has a necessary role in Th2-cell differentiation via IL2 signaling and it is independent from IL4 and GATA3 (Zhu et al. 2003). Other important factors in Th2-cell differentiation include IL6, Insulin regulatory factor 4 (IRF4) and NFATc2, which activate the IL4 promoter, induce GATA3 and inhibit Th1-cell differentiation. (Luckheeram et al. 2012; Murphy et al. 2012)

Th17-cell differentiation

Th17-cells mediate host immunity against extracellular bacteria and fungi. Their main function is to stimulate neutrophil migration to the inflammatory site, by inducing epithelial-cell chemokine secretion. Th17-cells secrete IL17A, IL17F and IL21. Their differentiation is driven by transforming growth factor beta (TGFβ), a cytokine that inhibits Th1-/Th2-cell polarization together with IL6 and IL23 (Annunziato et al. 2007). TGFβ upregulates the expression of IL21 cytokine and IL23 receptor. IL21 is important for Th17-cell amplification and the role of IL23 is to maintain the Th17-cell population (Korn et al. 2007; Langrish et al. 2005). These cytokines also induce the expression of retinoic acid receptor related orphan receptor gamma T (RORγT) via STAT3 mediated signaling (Stockinger and Veldhoen 2007). Other factors influencing Th17-cell differentiation are RORα, which acts together with RORγT and Runt-related transcription factor 1 (RUNX1). RORα can be either an inducer or inhibitor, depending on its interacting partners. Additionally, the transcription factors AHR, Batf and IRF4 have an effect on Th17-cell function. (Yang et al. 2008, Ciofani et al. 2012).

Treg-cell differentiation

Treg-cells inhibit antigen specific T-cell responses and protect against autoimmunity (Sakaguchi et al. 2006) and thus control the adaptive immune responses. They express
CTLA4 and IL2RA and secrete IL10 and TGFβ. Transcription factor STAT5 mediates the induction of the Th17-cell hallmark transcription factor forkhead box 3 (FOXP3), which acts as a transcription repressor (Arvey et al. 2014; Sakaguchi et al. 2008). Treg-cells can develop in thymus (nTreg) or in periphery from other subsets (iTreg). The iTregs have similar characteristics to those of a Th-cell progenitor, which allows the functional suppression of other Th-cell subtypes (Lehtimaki and Lahesmaa 2013).

**Tfh-cell differentiation**

Tfh-cells support the proliferation and expansion of B-cells and express high levels of CXC chemokine receptor 5 (CXCR5) chemokine receptor, ICOS and other receptors that are required for interaction with the B-cell (Breitfeld et al. 2000; Liu et al. 2014). The secreted cytokines defining Tfh-cells are IL4, IL10 and IL21, the latter of which is also a Tfh differentiation inducing cytokine. The master regulatory transcription factor, promoting Tfh-cell differentiation and suppressing the Th1-, Th2- and Th17-cell regulatory factors, is achaete-scute family BHLH transcription factor 2 (ASCL2) (Liu et al. 2014). Also STAT3 and BCL6 have an important part in Tfh-cell differentiation (Crotty 2011). (Luckheeram et al. 2012; Murphy et al. 2012).

**2.3.3 Induction of Th1-cell differentiation**

The hallmark cytokines IFNγ and IL12, and the transcription factors TBET (also Tbx21), STAT4 and STAT1 are the major factors driving the epigenetic and transcriptional changes in the course of Th1-cell differentiation (Figure 3) (Luckheeram et al. 2012). The differentiation process is initiated at the time of Th-APC priming by the pathways immediately induced at the TCR activation. In addition, IL18 and IL27 cytokine signaling and transcriptional regulation by RUNX play their own part in collaboration with the hallmark factors. Together they optimize an efficient Th1-cell driven immune response. IFNγ and IL12, together with TBET and STAT4 work in parallel and the pathways induced by them are intertwined resulting in fully activated Th1-cells (Thieu et al. 2008). There are some known differences between human and mouse in the mechanisms of differentiation of Th1-cells, reviewed in Rautajoki et al. (2008) and, Tuomela and Lahesmaa (2013). Here, however, we concentrate on the known facts of human Th1-cell differentiation.
Figure 3. Th1-cell differentiation. Differentiation is initiated by priming of the TCR by an antigen presented by MHC class II molecules on antigen presenting cells. Th1-cell differentiation is favored by a strong TCR stimulus, which leads to high intracellular calcium concentrations. The arrows indicate the following relationships: LFA-1 and Notch signaling regulates Th1-cell differentiation by promoting TBET expression. The co-stimulatory molecules (CD28, CD4) together with the TCR proximal signaling complex induce the Ca^{2+}/NFAT, Ras-MAPK and NFκB signaling pathways. These regulate transcription and chromatin remodeling in unison. NFAT primarily induces IFNγ. MAPK induces TBET and IFNγ. NFκB induces IL12Rβ2, TBET and CXCR3. The extracellular cytokine milieu regulates Th1-cell differentiation mostly by signals from IL18R (regulates expression of IFNγ together with IL12R, and expression of IL12Rβ2), IL12R (STAT4 and STAT1 targets), IL27R (STAT1 targets) and most importantly, IFNγR (STAT1 targets). IL18 mediates its effect also via NFκB. At the end, the STATs and TBET together with the NFAT, MAPK and NFκB induce the transcription of other Th1-specific genes and regulate also their own transcription.
2.3.3.1 TCR, co-stimulation with NFAT, AP1 and NFκB

The first stimulus initiating Th-cell differentiation is the strength of the TCR-antigen binding. A strong stimulus induces Th1-cell differentiation by sustained calcium influx from the extracellular space and from the ER to the cytoplasm (Constant and Bottomly 1997). Strong stimulus promotes Th1-cell lineage differentiation with the co-stimulatory signals from ICAM binding LFA-1 (Ruedl et al. 2000). LFA-1 can also drive Th1-cell differentiation if there is a weak IL12 stimulus (Smits et al. 2002). The importance of the co-stimulatory CD28/B7 coupling has been shown to be more important in the development of Th2-cells under weak TCR stimulus (Kallinich et al. 2005; Nurieva et al. 2003; Smeets et al. 2012). However, the strength of the TCR stimulus seems to be the primary determinant (Tao et al. 1997). In addition to LFA-1/ICAM, also CD27/CD70 has been shown to boost effector cell survival and Th1-cell differentiation by promoting expression of TBET and IL12Rβ2 (Smits et al. 2002; van Oosterwijk et al. 2007). TCR stimulus coupled only with CD3, i.e. without CD28, results in Th1-like signaling and transcriptional pattern, mainly due to differences in calcium signaling (Smeets et al. 2012). The Th1-cell differentiation promoting, CD3-coupled activation involves both Lck and PKC-θ. Their activation promotes the fast increase in intracellular calcium and calcineurin, which increases the NFATc1 nuclear translocation (Gallo et al. 2006; Noble et al. 2000; Porter and Clipstone 2002; Brogdon et al. 2002).

The sustained calcium influx also requires the activation of Tec family non-receptor kinases (Readinger et al. 2009). One of the family members, Th1-cell specific TXK has been shown to induce IFNγ transcription and possibly bind to the promoters of other Th1-cell inducing genes (Rajagopal et al. 1999; Takeba et al. 2002). PKC induces transcription factor NFκB family mediated regulation of differentiation via IFNγ, TBET and STAT4 expression (Aronica et al. 1999; McCracken et al. 2007; Sun et al. 2000). Other important factors in Th1-cell differentiation, especially when considering autoimmunity and allergic response, are the TIM family receptors. TIM3 is specifically a negative regulator of Th1 response (Khademi et al. 2004; Meyers et al. 2005). The role of the Notch pathway in Th1-cell differentiation is not clear cut. Delta-like ligand on the APC surface has been shown to bind the Th-cell surface Notch and to promote Th1-cell differentiation via TBET expression. It also suppresses Th2-cell differentiation by downregulating IL4 signaling (Amsen et al. 2009; Minter et al. 2005; Yu et al. 2007).

The TCR proximal signaling events and the co-stimulatory signals are coupled with Ca²⁺/NFAT signaling or with activated NFκB and AP-1, or both (Macian 2005). NFAT1, 2 and 4 are translocated to the nucleus as a result of activation and subsequent calcium signaling. NFAT1 favors Th1-cell differentiation by binding to the IFNγ promoter and regulating its transcription (Kiani et al. 2001; Lee et al. 2004). NFAT does not convey its effect on its own, but by interacting with other transcription factors, i.e. AP-1, c-Maf, IRF4, EGR1 and GATA3. Interaction with STATs and TBET leads to NFAT driven modifications of the chromatin state (Agarwal and Rao 1998; Hogan et al. 2003; Savignac et al. 2007). The MAPK pathway promotes both Th1-cell and Th2-cell differentiation, but the decision is
determined by the set of proteins which are activated along the pathway. TCR activation leads to JNK2 activation in Th1-cells and induces IFNγ and IL12Rβ2 during early differentiation (Yang et al. 1998). Additionally, p38 MAPK positively regulates IFNγ production (Dong et al. 2002; Rincon et al. 1998). The MAPK pathway is also involved in stress signaling promoted Th-cell differentiation. After TCR stimulation, the expression of GADD45 family proteins induce JNK and p38 MAPK pathways, and increase IFNγ production in fully committed effector cells (Lu et al. 2001). Additionally, IL18 and IL12 induce GADD45β, which favors IFNγ production (Yang et al. 2001). A small Ras related GTPase Rac2, activator of NFκB, JNK and p38 MAPK, is expressed in Th1-cells and promotes IFNγ expression (Li et al. 2000). The calcium-induced pathways and the Ras-MAPK pathways cross over in cooperation between NFAT and AP-1. The AP-1 complex is composed of either Fos-Jun or Jun-Jun dimers, the first of which promotes Th1-cell differentiation (Cippitelli et al. 1995; Jorritsma et al. 2003; Penix et al. 1996; Rauscher et al. 1988). In synergy, AP1, ATF2, ATF3 and JUN upregulate IFNγ (Filén et al. 2010; Jones and Chen 2006; Samten et al. 2008). NFκB joins the group and together these transcription factors regulate Th-cell differentiation in general (Zhu et al. 2010). NFκB signaling is also activated through IL18 receptor signaling (Corn et al. 2003; Kiani et al. 2001; Matsumoto et al. 1997; Sica et al. 1997). NFAT and NFκB induce TBET expression together, NFAT by activating the chromatin remodeling and NFκB by binding to the promoter, (McCracken et al. 2007; Placek et al. 2009).

2.3.3.2 Cytokines and transcription factors

IL12, IL18 and STAT4
IL12 is mainly produced by dendritic cells and macrophages, and was the first Th1-cell defining cytokine discovered (Hsieh et al. 1993; Watford et al. 2004). IL12 promotes both innate and adaptive immune responses (Chan et al. 1991; Frucht et al. 2001; Valiante et al. 1992; Yoshimoto et al. 1997, 2000). The naive Th-cells lack the expression of the IL12 receptor subunit IL2Rβ2, and are thus unresponsive to IL12 signaling (Szabo et al. 1997). TCR signaling triggers low expression of IL2Rβ2 by activating the BAF chromatin remodeling complex. The expression is then further enhanced by IFNγ induced TBET signaling, and endogenously expressed IL12. (Afkarian et al. 2002; Chang et al. 1999; Letimier et al. 2007). Additionally, together with TBET, IL12 also maintains IFNγ production (Letimier et al. 2007), thus creating a self-reinforcing regulatory loop. Once fully upregulated, IL12R mediates signal through JAK-STAT activation. IL12 binding to the receptor causes Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) activation and STAT4 phosphorylation (Watford et al. 2004). Once STAT4 is activated, IL12 also autoregulates its own responsiveness by further inducing IL12Rβ2 expression via STAT4. IL12 induces the regulation of other Th1 lineage-specific genes, like IL18 receptor 1 (IL18R) (Lawless et al. 2000; Letimier et al. 2007; Nishikomori et al. 2002; Thieu et al. 2008; Wei et al. 2010), which are not expressed in naive CD4+ T-cells (Nakahira et al. 2001). Together, IL12 and IL18 cooperate to enhance IFNγ production, IL12 being the determining factor (Micalele et al. 1996; Takeda et al. 1998; Yoshimoto et al. 2000). IL12 and STAT4 regulate interferon
regulatory factor (IRF) transcription factors (mainly IRF1 and IRF2), which promote IFNγ by binding to the IL4 promoter. They repress IL4 signaling and Th2-cell differentiation. (Coccia et al. 1999; Elser et al. 2002). At the later stage of differentiation, IL12 and STAT4 also induce TBET transcription (Schulz et al. 2009). Although STAT4 is considered more of an administrator than inducer of Th1-cell differentiation, it is an important mediator of Th1-cell function. For one, this is shown by STAT4-deficient patients having reduced Th1-cell response (Chang et al. 2009). TCR and STAT4 are the most important factors for IL12 signaling in human, but in mouse also IFNγ signaling is important for the induction of IL12Rβ2 expression (Afkarian et al. 2002; Athie-Morales et al. 2004).

**IFN-γ, IL27, TBET and STAT1**

IFNγ is mostly secreted by the Th1-cells and also to a great extent also by NK-cells. It is also secreted to some extent by naive T-cells, B-cells, macrophages and dendritic cells. IFNγ is the primary inducer of Th1-cell differentiation through STAT1. Like IL12, it has an important role in both innate and adaptive immunity and its main function is to help eliminate the invaders engulfed by the macrophages. (Szabo et al. 2003; Szabo et al. 2002). STAT1 translocates to the nucleus once it is phosphorylated and homodimerized. This is triggered by the binding of IFNγ to the IFNγ receptor leading to the JAK1 and JAK2 kinase activation. In the nucleus STAT1 induces the transcription of IFNγ target genes, the most important for Th1-cell differentiation being TBET and IL12Rβ2. By doing this, it also creates a positive self-regulatory loop. (Afkarian et al. 2002; Greenlund et al. 1994; Lawless et al. 2000; Letimier et al. 2007; Lighvani et al. 2001; Ramana et al. 2002; Szabo et al. 1997, 2000). In human, STAT1 phosphorylation is also induced independently of IFNγ by IL12 induced TBET upregulation (Ylikoski et al. 2005). IL27, which is related to IL12 and IL18, signals through STAT1 promoting IFNγ and IL12Rβ2. In synergy with IL12, IL27 supports Th1-cell differentiation (Hibbert et al. 2003; Pflanz et al. 2002; Takeda et al. 2003). The receptor for IL27, IL27R, is expressed only transiently during early differentiation and it is downregulated by TCR activation (Owaki et al. 2005; Pflanz et al. 2002). Also, another interferon family cytokine, a type I interferon IFNα, has been shown to promote human Th1-cell differentiation by inducing phosphorylation of STAT4 and ATF3, and expression of IL12Rβ2 (Filén et al. 2010; Letimier et al. 2007; Rogge et al. 1998).

TBET, the Th1-cell master regulator, works in close collaboration with IFNγ and STAT1, as it is induced by these factors, and TBET induces them ((Lighvani et al. 2001; Mullen et al. 2001; Szabo et al. 2002)). In addition, TBET has been shown to support IL12Rβ2 expression and the IL12/STAT4 signaling pathway (Afkarian et al. 2002). TBET binds to the promoter region of IFNγ and IL2Rβ2, and its own promoter. In this way, TBET drives the epigenetic chromatin changes enabling Th1-cell differentiation In a similar way, TBET also suppresses the expression of other Th-cell subtype-specific genes. (Hwang et al. 2005; Kanhere et al. 2012; Oestreich et al. 2011; Zhu et al. 2012). TBET regulates Th1-cell effector functions and immune response by binding to the CXCR3 promoter. CXCR3 is a Th1-cell-specific chemokine receptor, which enables Th1-cell migration to the site of inflammation (Lord et al. 2005; Thieu et al. 2008; Zhu et al. 2012). However, once IL2Rβ2 expression has been
established, it seems that TBET is redundant for the maintenance of the IFNγ expression (Usui et al. 2003). Eomesodermin, another member of the T-box family, is also a TBET independent regulator of IFNγ (Suto et al. 2006). The function of TBET as a regulator is closely connected to its interacting partners, such as RUNX3 and H2.0-like homeobox protein (HLX). The transcription of both of these co-factors is regulated by TBET (Djuretic et al. 2007; Mullen et al. 2002; Thieu et al. 2008). The enhancement in IFNγ expression and the repression of IL4 by TBET is gained in collaboration with RUNX3 (Djuretic et al. 2007; Naoe et al. 2007) and HLX plays its part in boosting IFNγ expression (Mullen et al. 2002). During an active immune response, TBET suppresses IFNγ together with BCL-6. This suppression is important in avoiding IFNγ overproduction and possible development of immunopathologies. (Oestreich et al. 2011).

### 2.3.4 Th-cells and immune dysregulation

The immune-mediated pathologies can be roughly divided in two categories: (1) autoimmunity, when the immune system attacks self-tissue, and (2) atopy or hypersensitivity, in which the immune system becomes mistakenly activated against an allergen, a non-infectious agent. Over 150 different autoimmune disorders have been reported by the American Autoimmune Related Diseases Association (AARDA, www.aarda.org). Traditionally, the tissue damage in autoimmunity is mediated through excessive Th1-cell response and chronic inflammation. The atopic responses are mediated through excessive Th2-cell response. However, the role of Th17- and Treg-cells combined with Th-cell plasticity have been in the center of investigations during the past years. The effect of different Th-cell subtypes is mediated through the cytokines produced, which recruit other adaptive and innate immune cells. Thus, the mechanistic background for the immunopathologies lies in the cytokine milieu produced by the cells, not necessarily in the composition of distinct cell subsets. This is partly explained by the Th-cell plasticity. Figure 2 summarizes the functional roles of the Th-cell subsets and their hallmark cytokines in health and disease. Here, the Th-cell role in autoimmune diabetes (type 1 diabetes, T1D) and asthma are discussed in more detail.

#### 2.3.4.1 Type 1 diabetes (T1D)

T1D occurs when the body can no longer produce insulin due to the destruction of the pancreatic β-cells. This proinflammatory autoimmune reaction is caused by one’s own immune system, which has escaped the regulation of immunetolerance. The autoimmune reaction usually starts at childhood, but adulthood onset can occur. These late onset cases may be mistakenly diagnosed as type 2 diabetes (Palmer et al. 2005; Thunander et al. 2008). The prevalence of T1D between different countries and populations is highly variable, Finland leading the statistics with an incidence of approximately 6 out of 10 000 cases yearly (Patterson et al. 2009). In neighboring country, Estonia, the incidence is notably only one third of this. Interestingly, the risk for genetic susceptibility, conveyed by the human leucocyte antigen, HLA genes, has become more rare in the general population. However, the
prevalence of the disease keeps climbing (Steck et al. 2011). This indicates an involvement of strong factors other than the \textit{HLA} region. Additionally, although the concordance for monozygotic twins is as high as 50\%, causative triggers other than the genetic factors are implied (Imkampe and Gulliford 2011; Steck and Rewers 2011).

The \textit{HLA} gene region, which codes for the antigen presenting and self-recognizing MCH class I and II molecules, has by far the most substantial odds ratio (OR), ranging from 0.02 to more than 11, depending on the \textit{HLA} haplotype (Erlich et al. 2008). OR indicates the odd of T1D occurring with a given haplotype or genotype in comparison to T1D occurring in the absence of the given haplotype or genotype. Other words, OR indicates the likelihood of T1D. The polymorphisms within the insulin coding gene region, \textit{INS}, has the second largest influence, with an OR of over 2. The mechanistic explanation for the \textit{INS} conferred risk lies in the variable number of tandem repeats (VNTR) in the \textit{INS} promoter region. The short alleles are shown to be predisposing over the longer ones. This is most likely due to the insulin levels in the thymus during negative selection. The shorter alleles associate with lower levels of thymic insulin, thus allowing the escape of insulin reactive T-cells (Meigs et al. 2005; Pociot et al. 2010; Pugliese 2005). The next most influential genes, \textit{IL2RA} and \textit{PTPN22}, have ORs of just over 1.5. The rest of the other dozen susceptibility loci carry an OR of 1.1—1.3. Interestingly, the majority of the most influential loci, such as \textit{CTLA4}, code for genes important in immune regulation and response (Concannon et al. 2009; Pociot et al. 2010). \textit{IL2RA} is essential in IL2 signaling and thereby important in Treg-cell function. In addition, \textit{IL2RA} promotes effector T-cell activation and Th17-cell differentiation when the IL2 signal is weak (Malek and Castro 2010). Treg-cells also express high levels of \textit{CTLA4}, which is essential in repression of excessive immune responses (Josefowicz et al. 2012). Most of the knowledge about the mechanisms how immune system dysregulation leads to T1D has come from non-obese diabetic (NOD) mouse studies. The NOD mouse shares characteristics of the disease with humans, and some of the susceptibility genes are the same, such as \textit{IL2}, \textit{CTLA4} and \textit{INS} (Ridgway et al. 2008).

CD4+ T-cells are the main focus of this review of the literature, however, it has be noted that also CD8+ T-cell-mediated reactions are required for the induction of T1D. For that, also dendritic cells need to be activated to cross-present exogenous antigen by MHC class I molecule, which normally presents endogenous peptides. This occurs through dendritic cell interaction with activated CD4+ T-cells. Nevertheless, the CD4+ T-cells seem to have the major role, since the disease incidence decreases upon their depletion in the NOD mouse. This has been found to be caused by the CD4+ T-cell effect through macrophages. The NOD mice, in which CD4+ T-cells are specific for islet antigens and show extensive T-cell infiltration in the pancreas, do not develop T1D with full penetrance. If the Treg-cell compartment is disturbed, the penetrance rapidly rises to 100\%. (Wallberg and Cooke 2013). The CD4+ T-cells of human T1D patients produce high levels of IFN\gamma and TNF\alpha together with a high Th1/Treg-cell ratio, whereas the cells from healthy controls produce Treg-cell cytokine IL10 (Chujo et al. 2013; Du et al. 2013). A role for Th17-cells in T1D has also been indicated in human. IL17 has been found to strengthen the apoptotic effect of Th1-cell
cytokines on human pancreatic β-cells in vitro (Arif et al. 2011). Additionally, 50% of children with a recent T1D diagnosis showed an increase in Th17-cell production and their pancreatic draining lymph node was infiltrated with excessive Th17-cells, accompanied by a lack of Treg-cell suppression (Ferraro et al. 2011; Honkanen et al. 2010). Also, in the NOD mice, the level of functional memory FOXP3+ Treg-cells is decreased as some of these cells are turned into IFNγ and TNFα producers (Du et al. 2013; McClymont et al. 2011; Zhou et al. 2009). Th17-cells, through the secretion of IL23, have been shown to be more potent in inducing disease compared to Th1-cells (Langrish et al. 2005). The Th17-cell differentiation is inhibited by IL2 signal through STAT5, which is indispensable for Treg-cell function (Laurence et al. 2007; Veldhoen et al. 2006). Treg-cells inhibit inflammation and contribute to their own induction via the secretion of IL10 and TGFβ. In addition to the cytokine signaling, the induction of Treg-cells is promoted by low CD28 and high CTLA4 expression (Josefowicz et al. 2012).

The role of dysregulated IL2 signaling in T1D is seen first and foremost at the genetic level. A number of different genetic polymorphisms, which alter gene expression and are associated with T1D, have been identified in IL2 related genes i.e. IL2, IL2RA and IL2RB. The molecular mechanism behind the role of IL2 in T1D involves both the lower expression of IL2Rα and the higher amounts of soluble IL2Rα. Each of which results in changes in the IL2Rβ2/CD25 surface expression and changes in the response to IL2 (Cerosaletti et al. 2013; Dendrou et al. 2009; Maier et al. 2009). This is again tied with the Treg-cell function, as one of the mechanisms for the Treg-mediated suppression of immune response is through IL2 signaling (Wallberg and Cooke 2013). NOD mice have reduced Treg-cell pool resulting from low IL2 production. In the Treg-cells and memory Th-cells of human T1D subjects, an increase in soluble IL2Rα and decreased response to IL2 has also been detected. These differences lead to a low phosphorylation rate of the Th17-cell regulator STAT5. (Bradfield et al. 2011; Orban et al. 2010). The decreased IL2 response results from impaired IL2-mediated FOXP3 expression, impaired Treg-cell survival and increase in Th17-cell differentiation (Curotto de Lafaille and Lafaille 2009; Thien et al. 2004). The role of Th1-, Th17- and Treg-cells in T1D remains controversial. It has been suggested that the Th17-cells orchestrate tissue inflammation by inducing Th1-cell recruiting proinflammatory cytokines and chemokines. The Treg-cells accumulating in the site of tissue damage are not efficient enough to override the vast amount of pro-inflammatory cytokines and the disease progression. (Dardalhon et al. 2008).

2.3.4.2 CTLA4 and ICOS in type 1 diabetes

CTLA4 and ICOS are molecules of interest considering autoimmune diseases. They share close chromosomal location and structural similarities with CD28 (Harper et al. 1991; Hutlof et al. 1999), but have an opposing effect on T-cell response. CD28/ICOS co-signaling results in stimulation of T-cell activity, whereas competitive CTLA4 binding to the B7 or competitive accumulation in IS leads to repression of CD28 signaling and T-cell response (Coyle et al. 2000; Krummel and Allison 1995; Rudd et al. 2009; Simpson et al. 2010;
Yokosuka et al. (2010). CTLA4 expression is induced by TCR activation in both CD4+ T-cells and CD8+ T-cells (Sharpe and Freeman, 2002). The downregulation of the immune response by CTLA4 is important in maintaining the immunological balance. The critical feature for Treg-cell suppressive ability is the phosphorylation of Tyr201 in CTLA4 intracellular domain (Stumpf et al. 2014). As mentioned in the previous section, CTLA4 is one of the human T1D high risk susceptibility genes. CTLA4-deficient mice have excessive proliferation of lymphocytes and die within 3 weeks of age (Khattri et al. 1999). By inhibiting CTLA4, an aggressive T-cell infiltration of the islets and T1D can be manifested in mice, even without T-cell activation (Luhder et al. 1998). A naturally occurring CTLA4 isoform, li-CTLA4 has been shown to affect TCR signaling, T-cell proliferation and cytokine production (Vijayakrishnan et al. 2005). Additionally, it has been shown that altered expression of this isoform is most likely the primary cause of CTLA4 association with T1D (Ueda et al. 2003; Stumpf et al. 2013).

ICOS is another important controller of Th-cell effector function. It is induced upon T-cell activation and has a role in T-cell differentiation and effector function (Greenwald et al. 2005). Whereas CTLA4 is important in Treg-cell function, ICOS has been found to be important in Th-cell function (Crotty 2011). The ICOS gene region, like the CTLA4 region, has been shown to associate with several autoimmune disorders, including T1D. The ICOS gene region has a high number of variance, but most of the polymorphisms are silent and do not change the sequence of the ICOS protein. Several attempts in showing ICOS gene association with T1D have not been able to pinpoint the causative loci. (Scandizzi et al. 2011). However, ICOS expression is found to be significantly lower in children diagnosed with T1D (Luczynski et al. 2009).

2.3.4.3 Asthma

Asthma is a respiratory disease involving a chronic airway inflammation that is caused by the infiltration of immune cells to respiratory system. Asthma is triggered by the allergens from the surrounding environment, and also by exercise, drugs or infections in the respiratory track. The prevalence of asthma is on the rise and approximately 400 million people worldwide are affected, most of which reside in the western countries (Braman 2006; Masoli et al. 2004). The airway inflammation in asthma involves Th-cells secreting Th2-cell type cytokines (IL4, IL5, IL9, IL13), but also eosinophils, neutrophils and mast cells (Nakajima and Takatsu 2007). Asthma and allergic reactions are seen as Th2-mediated responses, although the role of factors other than Th2-cell-type cytokines has been shown to be of importance. Mice lacking TBET resemble phenotypically human asthmatic patients with low TBET expression (Finotto et al. 2002). TBET polymorphisms have also been associated with some asthmatic phenotypes in human (Akahoshi et al. 2005; Raby et al. 2006). However, no association with high IgE-levels and TBET variants have been found. The expression of GATA3 is shown to be higher in asthmatics than in healthy controls (Robinson et al. 1992).
In addition to IL4 and IL5, IL13 has been shown to be important in asthma in both mouse and human (Cousins et al. 2002; Locksley 2009). The IL13 mRNA and protein expression is increased in asthmatic patients (Berry et al. 2004; Humbert et al. 1997). IL13 shares a role with IL4 in B-cell IgE switching, and in activating monocytes and macrophages (Wills-Karp 2004). IL13 induces the production of IL9, which enhances Th2-cell-type cytokine production and has been shown to be increased in asthmatic patients (Shimbara et al. 2000; Steenwinckel et al. 2007). Another cytokine produced by Th2-cells is IL6, which boosts Th2-cell differentiation, memory Th2-cell proliferation and inhibits Treg-cell function (Pasare and Medzhitov 2003). Inhibition of soluble IL6 receptor in mouse reduces airway inflammation, whereas inhibition of membrane-bound IL6 receptor increases the number of Treg-cells (Doganci et al. 2005). In addition, the inflamed tissue secretes cytokines such as GATA3 expression favoring IL25, IL33, IL7 related thymic stromal lymphopoietin (TSLP) and osteopontin, which further enhance the Th2-cell-driven asthma (Liu 2007; Saenz et al. 2008, 2010; Wang et al. 2007; Xanthou et al. 2007).

IL25 signaling could account for the Th2-cell-like cytokine profile in asthma (Hurst et al. 2002; Neill et al. 2010; Saenz et al. 2010). It is more likely that the interplay between the non-Th2-cells and Th2-cells, and innate and adaptive immune response, act in unison upon possible abnormalities in the epithelial tissue. Also, Th17-cell specific mRNA is elevated in asthmatic patients, but it is not clear whether it is from Th17-cells or from other cell types that have gained the ability to secrete IL17 (McKinley et al. 2008; Schnyder-Candrian et al. 2006; Wakashin et al. 2008). Mouse Th17-cells have been shown to express IL13, which also downregulated IL17 expression. In human, a Th17-cell clone that produces IL4 was also discovered, but the role for Th17-cells in asthma remains enigmatic. Additionally, the function of Treg-cells in asthma has been under investigation. There is evidence, that under atopic allergic disease, the suppression of Th2-cell response by Treg-cells is deficient and the peripheral FOXP3 expression is reduced. (Robinson 2010).

2.3.4.4 The smaller effectors in immune dysregulation

The current view is that asthma and allergic reactions are more profoundly Th2-cell-driven than autoimmunity is Th1-cell-driven. As more is known about the Th-cell plasticity and about the genetic factors beyond the major susceptibility loci the knowledge about the mechanisms increases in both instances. For example, beyond the genes shown to be associated with autoimmunity, the function of the corresponding proteins has to be taken into consideration when resolving the question of variance in disease susceptibility between different populations. Although through multiple GWAS studies the major variants have been found, the role of minor factors has yet to be studied. In regard to genetic association, Thirunavukkarasu et al. (2014) state that in order to understand new biological findings, the molecular connections between the genes in question need to be solved and genes conferring small effects cannot be over looked. They also point out that biological associations may not be found by the genetic studies, as they are hidden under the complexity of gene interactions.
These gene interactions may not be directly connected via protein-protein or gene-gene interactions, but can pathologically affect the development of a disease by acting in the same mechanistic network. (Thirunavukkarasu et al. 2014). CTLA4 has been shown in many studies to associate with T1D and this is most likely due to its function in Treg-cells. ICOS is found to be important for Th-cell function and to have a protective effect over autoimmune reactions. The first study in this thesis investigated CTLA4 and ICOS association with T1D, but did not further consider their role in immunity or in the disease. The main focus of this PhD thesis is in GIMAP4 and GIMAP5 function, as they were more extensively investigated in the second and in the third study. GIMAP family of small GTPases make rather new group of molecules to be recognized in immune mediated diseases. There is no evidence of GIMAP association with T1D or asthma in GWAS studies. However, their functional role in immunity strongly suggests that they have a role in health and disease.

2.4 GIMAP family of Small GTPases

The first GIMAP (GTPase of the Immunity Associated Protein) family GTPase, originally named AIG1, was initially discovered in the early 90s in Arabidopsis thaliana (Reuber and Ausubel 1996) and was functionally assigned to defense mechanisms. The mouse (imap38, mIAN-1, mIAN4) and rat (r-IAN5, rIAN4) homologues were described shortly afterwards (Hornum et al. 2002; Krucken et al. 1997; MacMurray et al. 2002; Poirier et al. 1999). The first human GIMAP gene, himap1/hian1 was reported in 2002 (Cambot et al. 2002; Stamm et al. 2002a). The nomenclature was set in accordance with the human GIMAP-nomenclature (Wain et al. 2002), with exception of A. thaliana, which follows the IAN-nomenclature (Liu et al. 2008). Interestingly, unicellular organisms and insects seem to lack homologous genes for GIMAPs (Liu et al. 2008). The structural and functional annotations of GIMAPs in vertebrates indicate the potential ability to hydrolyze GTP (Poirier et al. 1999) and a strong connection with the immune system. The following discussion of genetic, structural and functional aspects will be restricted to the current knowledge of GIMAP family members in vertebrates, specifically concentrating on GIMAP4 and GIMAP5.

Early studies with mouse and rat Gimap genes discovered their tight regulation during thymic T-cell development. Rat Gimaps 1, 6, 8 and 9 were expressed most highly in the DP thymocyte developmental stage, and 3, 5 and 7 were highest in T-cells. Additionally, the expression of all other rat Gimaps, except Gimap4, were elevated when switching from DN stage to DP stage. Rat Gimaps 1 and 6 were also highly expressed in B-cells. In mouse, Gimap4 is highly expressed only in SP and DN4 stages, and regulated by TCR and IL7-mediated signaling during thymic development. Additionally, mGimap1 protein expression is critically required throughout T-cell development. (Dion et al. 2005; Duthie et al. 2007; Poirier et al. 1999; Saunders et al. 2010; Schnell et al. 2006).
2.4.1 Gene and protein structure

There are eight human GIMAP genes (Figure 4), of which seven are functional and one is a pseudogene. They form a cluster in chromosome 7q36.1 extending across approximately 300kb (Krucken et al. 2004). The clustering of the GIMAP genes is also characteristic for the other vertebrates studied (Liu et al. 2008; Nitta et al. 2006). In human, the molecular weight of the transcribed proteins varies between 24.1-74.9 kDa (Dion et al. 2005), with GIMAP8 being the largest and one of the three GIMAP6 isoforms the smallest. Multiple isoforms are transcribed for each of the genes, and at least one isoform expands over two GIMAP genes, GIMAP1 and GIMAP5. The highest expression of the GIMAP genes has been found in the cells of the immune system, but other cell types also express these genes to some extent (Hornum et al. 2002; Krucken et al. 2004; Saunders et al. 2010). Thus, the functional studies so far have concentrated on their function as putative GTPases in the T-cell survival and homeostasis.

All GIMAPs have a GTPase domain, with GIMAP8 making the exception of harboring three domains. The GTPase domain consists of five motifs G1-G5, and is found within the AIG1 domain, the latter named after the original finding in *A.thaliana*. In addition, the AIG1 domain has a characteristic conserved box (Krucken et al. 2004). The human GIMAPs have also been shown to have coiled-coil domains, which indicate putative protein-protein interactions, transmembrane domains and hydrophobic sequences (Krucken et al. 2004; Schwefel et al. 2010; Wong et al. 2010). Localization studies have indicated that GIMAPs are localized in the cytoplasm, ER and Golgi (Krucken et al. 2004; Nitta et al. 2006; Schnell et al. 2006; Stamm et al. 2002a, 2002b). Additionally, GIMAP2 and GIMAP7 have been shown to colocalize with lipid droplets (Schwefel and Daumke 2011; Schwefel et al. 2013) and GIMAP5 to lysosomes (Wong et al. 2010).

GIMAP GTPases are structurally distinctive from the other small GTPases, such as the RAS-superfamily GTPases and the heterodimeric G proteins (Poirier et al. 1999). The mechanisms of their functions have remained poorly characterized. Recently, Schwefel et al. (2010 and 2011) showed by studying GIMAP2 structure, that GIMAPs belong to septin GTPases, with similarities to those of Toc and Dynamin GTPases. As the family is structurally highly conserved, they speculated that the whole family would fall into this structural category. Septins have a wide range of functions in the cell, but most notably they are important in cell division, cell compartmentalization and acting as scaffolding proteins (Mostowy and Cossart 2012).
The GIMAP family consists of eight functional genes. The GIMAP4 and GIMAP5 are highlighted in red, as they are in the focus of the work presented in this thesis. The arrows indicate the direction of transcription and the relative size of the transcript. The molecular mass for each primarily transcribed protein from left to right are 8: 74.9 kDa, 7: 34.5 kDa, 4: 37.5 kDa, 6: 35.4 kDa, 2: 45.5 kDa, 1: 34.4 kDa, 5: 39.5 kDa.

2.4.2 GIMAP function

Initially it was thought that GIMAP4 was the only family member to have intrinsic GTPase activity (Cambot et al. 2002). Later, Schwefel et al. (2013) also found that GIMAP2 and GIMAP7 can act as a heterodimer, in which GIMAP7 stimulates GIMAP2 GTPase activity. Additionally, they showed that as a septin-type GTPase, their GTP hydrolyzing activity is triggered by dimerization. In general, the family members are likely to form pairs in which the one partner is tightly localized in cellular organelle and binds GTP and, the other family member triggers the GTP hydrolysis. Corresponding results were also found in mouse studies by Yano et al. (2014), which concentrated on the cooperative function of Gimap3 and Gimap5 in T-cell survival. The study revealed that a lack of Gimap3 did not have an effect on the T-cell number, whereas the double knock-down of Gimap5 and Gimap3 resulted in a greater defect than a single Gimap5 knock-down. It was concluded that there is cooperation among the Gimap proteins in regulating the T-cell pool. Gimap3 and Gimap5 are also both involved in mitochondrial function, regulating mitochondrial DNA segregation and mitochondrial calcium buffering, respectively. Whether Gimap3 co-localizes with Gimap5 in lysosomes is unclear. (Jokinen et al. 2010, 2011; Wong et al. 2010).

There are some controversial findings regarding the role of GIMAPs in immune cell function. As a one example, mouse Gimap1 was shown to be highly expressed in the splenic macrophages during parasitic infection (Plasmodium chabaudi) and less so in B- or T-cells (Krucken et al. 1997). Later studies, however, revealed no such upregulation (Saunders et al. 2009, 2010). Mouse Gimap8 has been shown to be highly expressed in the thymus, whereas the human counterpart is highly expressed in the spleen (Krucken et al. 1997, 2004, 2005). GIMAP5 has been reported to be apoptotic in human (Sandal et al. 2003), but rat studies have indicated both anti- and pro-apoptotic function (Dalberg et al. 2007; Pandarpurkar et al. 2003). Additionally, regarding their function in T-cell survival, it has been shown that GIMAP4 and GIMAP5 have an opposing effect (Chen et al. 2011; Schnell et al. 2006).
According to Schwefel et al. (2013), the septin-like structure, and thus the septin-like function, might explain this phenomenon. They speculate that GTP binding GIMAP5 forms an apoptotic scaffold, whereas the interplay with GIMAP4 may induce its GTPase activity and interrupt the scaffold formation (Schwefel et al. 2013).

GIMAP proteins could also have other functions in immunity than those involved in apoptosis. Pascall et al. (2013) found GIMAP6 to regulate and bind cytosolic, non-membrane bound GABARAPL2 (also GATE-16), which is a mammalian homologue for the yeast autophagy-related protein Atg8. Mutation at the suspected GAPARAPL2 binding sequence in GIMAP6 had no effect on the binding, whereas C-terminally truncated GIMAP6 could not bind GABARAPL2 and mouse Gimap6 had only a weak binding. The C-terminal part of the GIMAPs is less conserved between species and is naturally truncated in mouse and rat in comparison to human. This may explain the differences in mouse and human findings. The GIMAP6-GAPARAPL2 complex is translocated into autophagosomes as a result of cell starvation, most likely to selectively regulate GIMAP6 degradation. (Pascall et al. 2013). The functional significance of this interaction in immune response remains to be discovered. The observation is corroborated by a study in corals (Acropora millepora), in which the GIMAP family members 1, 2, and 3 were found to be upregulated upon muramyl dipeptide (MDP) introduction, and speculated to take part in the phagolysosomal processing (Weiss et al. 2013).

GIMAP proteins are thought primarily to have a role in cell survival, and some new discoveries are thus interpreted on the basis of that predisposition. Thirunavukkarasu et al. (2014) found GIMAP6 to be highly downregulated in cattle exposed to Mycobacterium avium paratuberculosis (MAP), a causative of a chronic granulomatous intestinal disease affecting ruminants called Johne's disease. The concluding explanation regarding the role of GIMAP6 in MAP was linked through the role of GIMAP5 in calcium dependent apoptotic processes. A likely link between GIMAP6 downregulation in MAP infection is through autophagy. GIMAP6 is recruited to autophagosomes (Pascall et al. 2013), autophagy is induced by cholesterol depletion (Cheng et al. 2006) and MAP favors cholesterol (Keown et al. 2012). Another example of an apoptosis prone interpretation is involved with the finding of the differential regulation of GIMAP6 and GIMAP8 in non-small cell lung cancer. Shiao et al. (2008) discovered that the expression of all of the GIMAP genes was downregulated in the tumor tissue, but most notably GIMAP6 and GIMAP8. In the surrounding tissue, GIMAP8 was upregulated in comparison to healthy lung tissue (Shiao et al. 2008). These observations support the tissue-specificity of GIMAP protein function. GIMAP1 and GIMAP4 might have a function in skeletal muscle in human and in pig (Liaubet et al. 2011; Ran et al. 2014; Raymond et al. 2010), and GIMAP1, interestingly, in higher human consciousness (Ravnik-Glavac et al. 2012). These findings demonstrate that the function of GIMAP extends beyond the immune system, and these aspects should be taken into consideration in the future interpretations regarding their function.
2.4.3 GIMAP4

GIMAP4 has several unique features in comparison to the other GIMAP proteins, which makes it an interesting target of immunological studies. Firstly, as previously mentioned, it has intrinsic GTPase activity, as shown by Cambot et al. (2002). Secondly, mGimap4 has a functional calmodulin binding IQ-domain and six phosphorylation sites phosphorylated via PKC signaling (Schnell et al. 2006). PKC and Calmodulin are known to be involved in calcium signaling, which is crucial in many cellular processes, including TCR signaling and T-cell activation (Quintana et al. 2007; Sugiura et al. 2001). Four of the phosphorylation sites in mouse Gimap4 are also found in the human orthologue. Mouse Gimap4 is rapidly, but transiently, phosphorylated upon PMA/ionomysin stimulus to the TCR (Schnell et al. 2006), thus indicating an important role for GIMAP4 in lymphocyte signaling. GIMAP4 has been found to localize in the cytosol, ER and Golgi (Krucken et al. 2004; Nitta et al. 2006; Schnell et al. 2006) and most recently in the microsomal membrane fraction of the human CD4+ Th-cells (Filén et al. 2009).

GIMAP4 protein expression is tightly regulated after TCR activation. Schnell et al. (2006) observed a cycle of phosphorylation, dephosphorylation and degradation of mouse Gimap4 immediately after activation. The GIMAP4 mRNA was shown to be stable while the protein amount decreased slowly after activation, thus indicating post-transcriptional regulation to be the primary regulatory mechanism. However, the human GIMAP4 mRNA and protein expression seem to follow a different pattern (Filén et al. 2009). GIMAP4 protein expression is high in naive human CD4+ Th-cells. Upon TCR activation the protein expression is rapidly downregulated, but increases at least for 7 days under IL12 stimulus. IL4/STAT6 stimulus sustains the low expression over the same period. The mRNA expression is upregulated upon TCR activation and IL12 stimulus.

Functionally, GIMAP4 is mostly connected with peripheral T-cell survival and is found to accelerate mouse T-cell death, but not to initiate apoptosis (Schnell et al. 2006; Carter et al. 2007). GIMAP4 is not necessary for the development of T-cells, although its expression is briefly upregulated during thymic development (Nitta et al. 2006; Schnell et al. 2006), and when expressed ectopically it can increase the apoptosis of immature mouse thymocytes (Nitta et al. 2006). The proapoptotic role for GIMAP4 was further verified by its association with the BCL-2 family member BAX (Hacker et al. 2006; Nitta et al. 2006). Another molecule shown to bind with GIMAP4 is amyloid precursor protein, APP (Olah et al. 2011), which associates strongly with Altzheimer’s disease (AD). There are recent indications that innate immunity also plays a role in the pathogenesis of AD (Golde et al. 2013). Moreover, GIMAP4 has been shown to be downregulated in the cultured human myotubes in comparison to human muscle biopsy samples (Raymond et al. 2010). These findings indicate that according to the level of expression, GIMAP4 has a primary role in the immune cell
functions and in processes involving immune-cell regulation, but function in other tissues cannot be overlooked.

Recently there has also been an indication of GIMAP4 playing a role in human immune cell-mediated diseases. GIMAP4 was found to be associated with Behçet's disease in Japanese and Korean populations (Lee et al. 2013). However, similar studies from a European population did not show any disease associations with GIMAP4 (Ortiz-Fernandez et al. 2014). Additionally, GIMAP4 has been shown to be downregulated upon the downregulation of plant homeodomain finger protein 11, PHF11 (Rahman et al. 2010). This is interesting when considering the function of PHF11 in asthma and dermatitis (Jang et al. 2005; Zhang et al. 2003). Rahman et al. (2010) show in their study that the overexpression of PHF11 increased the binding of NFκB in the IFNγ promoter, and its downregulation affected T-cell viability, NFκB nuclear transport, IFNγ and IL2 expression in addition to GIMAP4 transcription.

2.4.4 GIMAP5

GIMAP5 is known to be expressed in most human tissues (Sandal et al. 2003; Zenz et al. 2004). Recent studies have shown GIMAP5 to be important in calcium signaling, to have a role in mitochondrial function, and to have other roles that extend beyond the immune system. Like GIMAP4, GIMAP5 is upregulated upon TCR stimulation (Dalberg et al. 2007) and binds with most of the BCL-2 family members (Nitta et al. 2006), but not BCL-2 itself (Keita et al. 2007). Previous localization studies were mostly performed by detecting exogenously expressed GIMAP5, and reported it to be found in the mitochondria, ER, centrosome and plasma membrane (Daheron et al. 2001; Nitta et al. 2006; Pandarpurkar et al. 2003; Sandal et al. 2003; Stamm et al. 2002a; Zenz et al. 2004). However, a recent result obtained by detecting endogenous GIMAP5 indicated its localization in lysosomes (Wong et al. 2010). This is also supported by the finding by Aksoylar et al. (2012) in Gimap5-deficient mice, which have a weakened CD8+ T- and NK-cell survival. These cells are abundant with cytotoxic granules, and the low survival rate might be caused by the malfunctioning of the lysosomal compartment. GIMAP5 has two transcripts (Krucken et al. 2004, 2005), one of which also includes the neighboring GIMAP1 (Krucken et al. 2004; Prakash et al. 2010; Stamm et al. 2002a). However, the localization studies by Wong et al. (2010) indicated that GIMAP1 localizes in the Golgi rather than colocalizing with GIMAP5 in lysosomes. This suggests, that the translation is separate for these two proteins.

GIMAP5 is the most studied family member to date. This is mostly due to the diabetes-prone BioBreeding (BB-DP) rat, which has a premature stop codon inducing frameshift mutation in the Gimap5 gene. This mutation results in lymphopenia with a human T1D-like condition and is missing in the diabetes-resistant BioBreeding (BB-DR) rat (Hornum et al. 2002; MacMurray et al. 2002; Michalkiewicz et al. 2004; Rabinovitch 1994; Rutledge et al. 2009). The molecular basis for the lymphopenia in these rats includes the apoptosis of old and new, peripheral and recently developed, T-cells (Ramanathan and Poussier 2001). Multiple groups
Review of the Literature

have reported, that the BB-DP rats have an altered Treg-/Th17-cell ratio, in favor of the Th17-cells, and it has been shown that the Treg-cells of the BB-DP rat do not sufficiently suppress the Th17-cell function (Hillebrands et al. 2006, 2011; Poussier et al. 2005; van den Brandt et al. 2010; Visser et al. 2009). This, in particular, indicates that rGimap5 function may also be important for Treg-cell suppressor function.

By combining the genetic background of the BB-DP rat with that of a WOKW rat, which has a metabolic syndrome, Bahr et al. (2011) created a WOKW.4BB strain that has the Gimap5 mutation together with the MHC RT1 (u) haplotype, both needed for T1D occurrence in the BB-DP rat. The rats developed lymphopenia, but not hyperglycemia. This strongly indicated that Gimap5 mutation is causative in BB-DP rat lymphopenia. Moreover, when comparing the rGimap family gene expression in the spleen and mesenteric lymph node (MLN) between two congenic lines, diabetes-resistant DR.+/+ rat and diabetes-resistant, rGimap5 mutated DR.lyp/lyp rat, all the Gimap genes were downregulated. However, only half of the rGimap genes (1,4, 5 and 9) were downregulated in the thymus (Rutledge et al. 2009). Moralejo et al. (2011) further clarified these results in their study, in which the expression of Gimap family members was detected in sorted T- and B-cells. They stated that the Gimap5 frameshift mutation is the primary defect in the peripheral cells and concluded that the impaired expression of the entire Gimap gene family is due to the lack of Gimap5 protein.

In studies with the Gimap5-deficient sphinx mouse, Barnes et al. (2010) found lymphopenia, weight loss and intestinal inflammation. The Gimap5-deficient T- and B-cells develop normally, but their proliferation upon antigen stimulus is affected. Additionally, it was discovered that the morbidity of these mice was caused by the immune response to microbial flora. Later it was shown, in concordance with the studies in DP rats (Hillebrands et al. 2011), that the cells are polarized to the Th17-cell lineage. Also, the suppressive ability and the number of Treg-cells is diminished leading to colitis in these mice. Moreover, they found that the changes in the T-cell pool are linked to the loss of FOXO family transcription factor expression (Aksoylar et al. 2012). To date, only a few human GIMAP5 studies have been conducted. However, these studies have discovered that a common polyadenylation polymorphism, causing a defective polyadenylation signal resulting in non-terminated GIMAP5 mRNA, is associated with Systemic Lupus Erythematosus, SLE (Hellquist et al. 2007) and an increased risk to develop Islet Antigen-2, IA-2 autoantibodies (Shin et al. 2007). In addition to GIMAP4, GIMAP5 is also downregulated by PHF11 downregulation (Rahman et al. 2010), indicating that is might play some role in the pathogenesis of asthma. GIMAP5 has also been shown to be upregulated by Notch signaling in T-cell acute lymphoblastic leukemia, and these cancer cells are protected from apoptosis (Chadwick et al. 2010). Interestingly, a study by Rojewska et al. (2014) found rat Gimap5 to be upregulated during neuropathic pain. How this is related to Gimap5 function remains to be resolved, but is an interesting aspect as GIMAP4 has been associated with neurological disease as well. Additionally, GIMAP5 has been shown to have an importance in mouse hematopoietic stem cell development (Chen et al. 2011, 2013), indicating that there is more to this protein than its importance in immunity.
The mechanisms underlying the role of GIMAP5 in T-cell survival are not fully understood. The reduced number of live cells has been implicated to result from an impaired TCR induced Ca\(^{2+}\) response (Ilangumaran et al. 2009). Additionally, the T-cells isolated from BB-DP rat have a weak mitochondrial membrane potential (Keita et al. 2007; Pandarpurkar et al. 2003). Recently, Chen et al. (2013) revisited this phenomenon and concluded that a functional GIMAP5 is needed for mitochondrial Ca\(^{2+}\) accumulation. They showed that the accumulative process upon capacitive entry requires a microtubule network. The importance of GIMAP5 in calcium signaling extends beyond the immune cells. A study by Wang et al. (2014) investigated adipose tissue and the role of CDGSH iron–sulfur cluster-containing protein 2, CISD2 in Wolfram syndrome. This is a rare genetic disease that often has diabetes mellitus as its first manifestation. They discovered that CISD2 interacts with GIMAP5 and affects the mitochondrial Ca\(^{2+}\) uptake. The two proteins are expressed similarly during adipocyte differentiation, which is impaired in the absence of GIMAP5.
3 AIMS OF THE STUDY

The overall goal of the work presented in this thesis was to study factors with a known role in Th-cell function and differentiation, which may have a role in immune dysregulation. CTLA4 and ICOS were studied for their association with type 1 diabetes in two geographically close, but genetically distinct populations, Finnish and Estonians. The main studies, focusing on GIMAP4 and GIMAP5, aimed to evaluate their role in human Th1-cells and in immune mediated diseases, type 1 diabetes in Finnish population and asthma and allergic sensitization in Swedish population.

The specific aims of the PhD thesis project included:

1. To study the association of CTLA4 and ICOS with T1D in Finnish and Estonian populations and assess whether either of these molecules could explain the differences in disease rates between the study populations. (I)
2. To study the association of GIMAP4 and GIMAP5 with T1D and asthma and allergic sensitization in Finnish and Swedish populations, respectively. (II)
3. To characterize the role of GIMAP4 (III) and GIMAP5 during early human Th1-cell differentiation by using molecular genetics and transcriptomic approaches.
Materials and Methods

The sequences for all the used PCR probes, primers and siRNA oligos are listed in Table 1A and for all the antibodies used are listed in Table 1B.

4.1 Ethics consideration (I-III)

All the patients in the association studies (Original publication I, II) were diagnosed according to the World Health Organization (WHO) criteria. In both of the association studies, the sample collection was approved by the Ethics Committees of the participating universities and university hospitals, and all participants gave informed consent for the study samples collected. The use of human blood from unknown donors (Original publications II and III) was permitted by the Finnish Ethics Committee.

4.2 Population genetic association studies (I, II)

4.2.1 Subjects

Two populations of different ethnic origin were used in the study of ICOS and CTLA4 association with T1D. The Estonian population consisted of 170 patients and 230 controls, and the Finnish population of 404 patients and 725 controls. The mean age at blood collection was 29.5 ± 17.9 years for the Estonian patients and 9.8 ± 3.4 years for the Finnish patients. The full data set used in the GIMAP-T1D association analysis included 1732 Finnish Pediatric Diabetes Registry T1D nuclear families (FDPRNF). These families consisted of trios, two parents and one affected child. The data set was analyzed in two series, of which the first included 956 families and the second included 776 families. The samples were collected within the framework of the Finnish Pediatric Diabetes Register and Biobank in Finnish pediatric units. All the subjects were diagnosed under the age of 15 years according to the WHO criteria.

The study population in GIMAP-Asthma and allergic sensitization included the samples from the Swedish BAMSE (Children, Allergy, Milieu, Stockholm, Epidemiology) cohort. BAMSE is an unselected, population-based Swedish birth cohort (Kull et al. 2010) consisting in total of 4089 newborn children from four municipalities in Stockholm county. A questionnaire regarding health, symptoms and environmental exposures was conducted at the ages of 1, 2, 4, 8 and 16 years. The response rates were 96%, 94%, 92%, and 84%, respectively. The blood samples were taken at 4 and/or 8 years of age and DNA was available from 2033 subjects collected at 8 years of age (Acevedo et al. 2013). The asthma cases (n=440) were selected based on criteria of asthma diagnosis at any follow-up from 1 to 16 years and non-asthmatic, non-allergic persons were enrolled as controls (n=839). Subjects with diagnosed inhalant or food allergic sensitization with IgE 0.35 kU/l from 1-16 years were considered as
allergic sensitization cases (n=1046) and non-allergic children were enrolled as controls (n=979).

4.2.2 Genotyping

The CTLA4 and ICOS gene polymorphisms studied in the Finnish population were analyzed with Taqman SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). A polymerase chain reaction–restriction fragment length polymorphism assay was used for the Estonian population CTLA +49 and CT60 genotyping. All of the investigated polymorphisms, except the CTBC217_1 in Estonian patients (p =0.006) and CTIC159 in the Finnish controls (p=0.002), were in Hardy-Weinberg equilibrium (HWE, p=0.05).

The genotyping for the FDPRNF and BAMSE cohort samples was done with MALDI-TOF mass spectrometry (matrix-assisted laser desorption/ionization-time of flight; Sequenom GmbH, John Hopkins Court San Diego, CA, USA) at the Genome Center of Eastern Finland, University of Eastern Finland, Kuopio and in the Mutation Analysis Core Facility (MAF) at Karolinska Institutet, respectively. The genotyping had a success rate of 98%. Primers for multiplex PCR and extension reactions were designed by the SpectroDesigner software (Sequenom GmbH, San Diego, CA, USA) and the reactions were performed according to manufacturer’s standard protocols. For the FPDRNF, a number of random samples were re-typed as a genotyping quality control. The healthy parents were analyzed for HWE and Mendelian inconsistencies. No deviation from HWE (p>0.001) was found and Mendelian errors were sorted out from the analysis. For the BAMSE cohort, each assay was validated with 24 unrelated Caucasians, 3 CEPH (CEU) DNA samples and 14 trios from the CEU population.

4.2.3 Statistical analyses

The R program version 2.3.1 was used to statistically analyze the CTLA4 and ICOS genotype results in the Finnish and Estonian populations. The Haplotype construction was done with SHEsis software (Shi and He 2005). The Hardy-Weinberg equilibrium was analyzed with the Genepop (Raymond and Rousset 1995). The sequences at the GIMAP4 and GIMAP5 5’ loci (±400 bp) associated with the diseases studied were examined. The predictions were determined with HOMER (Heinz et al. 2010) motif track, which includes known motif positions for the human genome, based on HOMER-motifs. The detection threshold specifying if any given sequence is a match, is optimized by the HOMER software.

For the heterogeneity analysis, the FPDRNF families were divided into subgroups of South-West (SW) Finland and North-East (NE) Finland (based on the place of birth). The SNPs tagging the GIMAP4 and GIMAP5 gene region, with r² thresholds of 0.8 (FPDRNF) and 1.0 (BAMSE cohort), were selected by using the Tagger (de Bakker et al. 2005) software tool in Haploview (Barrett et al. 2005). PLINK v1.07 software (Purcell et al. 2007) was used for the Transmission/disequilibrium and interaction (--epistasis, pseudo controls) tests including the
adjustment for multiple testing in the FPDRNF and allelic case-control test (--assoc) in the BAMSE cohort. Genotype counts for the pseudo controls (Table S4, II) were deduced by using the UNPHASED 3.0.13 software (Dudbridge 2008). The interaction test and the asthma and allergic sensitization association test p-values were adjusted for multiple testing with R package multtest (Pollard et al. 2011). The multiple testing adjustment (Benjamini and Hochberg 1995) was done according to Benjamini & Hochberg false discovery rate (FDR) correction (alpha=0.05).

The p-value of less than 0.05 (p<0.05) was considered to be statistically significant in all the analyses across the studies.

4.3 Human primary CD4+ T-cell isolation (II, III)

Mononuclear cells were first isolated from umbilical cord blood samples by Ficoll-Paque (GE Healthcare, Little Chalfont, UK) gradient centrifugation collected from healthy neonates at Turku University Hospital. Secondly, anti-CD4 conjugated magnetic beads (Dynal Biotech, Thermo Fisher, Waltham, MA, USA) were used to enrich the CD4+ Th-cells from the mononuclear cell pool. All cultures consisted of a pool of CD4+ T-cells isolated from at least three donors.

4.4 Cell culture and transfections (II, III)

4.4.1 Human primary CD4+ T-cell culture and nucleofection

The isolated CD4+ T-cells were either nucleofected and incubated at a density of 1.5-2x10^6 cells per mL in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml Penicillin/100 µg/ml Streptomycin and 2 mM L-glutamine at 37°C for 43 h before activation, or activated immediately. The cells were nucleofected with non-targeting siScramble or a pool of two siGIMAP4, siGIMAP5 or siSTAT4 gene specific RNAi oligos at a density of 1.5 ng/4x10^6 cells per transfection. The cell density for all nucleofections was 4x10^6 cells per 100 µl Opti-MEM I (Invitrogen, Thermo Fisher, Waltham, MA, USA) using the Nucleofector Device (program U-14, Amaxa, Lonza, Cologne, Germany). The nucleofected cells were then harvested and activated similarly to the cells activated immediately after isolation. The cell activation was induced by 2.5 µg/mL plate-bound anti-CD3 and with 500 ng/mL soluble anti-CD28 (Immunotech, Vaudreuil-Dorion, Quebec, Canada) at a density of 1.7-2 x 10^6 cells/mL in Yssel’s medium supplemented with 1% AB serum (Red Cross Finland Blood Service, Helsinki, Finland). To induce Th1-cell differentiation, 2.5 ng/mL of IL12 (R&D Systems, Minneapolis, MN, USA) was added to the culture medium at the time of activation. On the second day of culture, 17 ng/mL of IL2 (R&D Systems, Minneapolis, MN, USA) was added. In the IFNγ rescue experiment, the cells were supplemented with 100U of extracellular IFNγ (ab119140, Abcam, Cambridge, England, UK).
4.4.2 **Hela-cell culture and transfections**

ATCC HeLa-cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Thermo Fisher, Waltham, MA, USA), which was supplemented with 50 µg/mL of penicillin and streptomycin, 2 mM L-glutamine and 10% FBS. The cells were co-transfected with the 3’UTR-con structs R1, R2 and an internal control, pGL3 construct using Lipofectamine 2000 (Invitrogen, Thermo Fisher, Waltham, MA, USA). For immunostainings, cells were cultured on glass cover slips (0.5 x 10^6 cell/mL). The luciferase expression was measured in cells from three biological and technical replicates harvested 48 h after transfection, according to the manufacturer’s instructions by Dual Luciferase Reporter Assay System (Promega, Fitchburg, WI, USA).

4.5 **Luciferase reporter constructs and measurement (II)**

Previous publications by Pitarque et al. (2004) and Wang et al. (2006) were used as methodological basis for building the reporter construct. The human GIMAP5 3’ UTR sequence specific PCR primers were designed according to the (NCBI Reference Sequence: NM_018384.4). A 580 bp region was cloned to replace SV40 polyA signal downstream of the luciferase gene in pGL3-promoter firefly luciferase expression plasmid (Promega, Madison, WI, USA). The primer sequence for amplification featured XbaI and BamHI restriction sites, which enable the replacement. Two different constructs were generated by incorporating the GIMAP5 3’UTR SNP (rs2286899) variable genotype in the amplifying reverse primer, TT R1-construct and CC R2-construct. Genomic DNA was isolated from whole blood of a healthy donor by Qiagen FlexiGene DNA Kit (Qiagen, Valencia, CA, USA). The genotype and sequence of the constructs were verified by sequencing.

4.6 **Confocal microscopy (III)**

The non-activated CD4+ T-cells, anti-CD3/anti-CD28 activated and IL12 treated CD4+ T-cells cultured for 4 days and HeLa-cells were washed with PBS, fixed with 4% PFA (paraformaldehyde) and permeabilized with 0.2% saponin. The cells were probed with primary antibodies against GIMAP4, β-tubulin, EEA1, Rab27A, syntaxin-6 and LAMP1-Cy3, and then with the secondary antibodies. Vectashield HardSet medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used for mounting of the washed cells. Confocal imaging was performed with a Zeiss LSM 510 laser-scanning confocal microscope and ZEN lite software (Zeiss, Oberkochen, Germany) was utilized for the linear fluorescence profile analysis. The quantification of co-localized proteins was performed with the BioImageXD program (Kankaanpää 2012). All the stained cells were randomly selected from multiple technical replicates for imaging.
4.7 Flow cytometry (II,III)

The cord blood driven CD4+ T-cells used in all flow cytometric measurements were nucleofected with siGimap4 specific or with non-targeting siScramble siRNA oligos and activated with anti-CD3/anti-CD28, with IL12 (Th1 induction) or without (Th0). The culture time and time point for the detection varied. The active caspase 3 was measured also from non-activated cells. For each individual experiment, 0.1–0.5x10^6 cells were used and at minimum 10,000 cell counts were measured per sample using FACSCalibur Flow Cytometer and analyzed with cellQuest Pro (BD Biosciences, San Jose, CA, USA). The significance of difference between the values in GIMAP4 depleted cells against the control cells was calculated from three biological replicates by using a two-tailed t-test.

4.7.1 Cell surface staining

After harvesting, the cells were washed with PBS and the staining antibodies were introduced into the staining buffer. For the surface staining of IL2RA, half of the IL12 supplemented (Th1) and non-supplemented (Th0) cells were supplemented with IL2 at 48 h after activation and other half was left as a non-IL2 controls. All the cells were harvested at 72 h and incubated with specific and isotype control antibodies. The surface expression of IL12Rβ2 was measured from Th0 and Th1 induced cells at 24 h, 48 h and 72 h by incubating with a specific antibody or with no antibodies added, and then with the secondary antibody. The expression of CXCR3 was detected at 24 h and 72 h after initial activation in Th1 induced cells and CD69 was measured at 6 h and 24 h from activated Th0 cells as described.

4.7.2 Intracellular staining

After harvesting or surface staining, cells were washed with 0.5%BSA/PBS (w/v). The cells were fixed with 4% PFA and 0.01% NaN_3 was used for permeabilization. GIMAP4 was measured from the same cells already stained for IL2RA, to assess the status of knock-down. The expression of IFNγ was measured from Th1 induced cells at 24 h, 48 h and 96 h. Brefeldin A was added five hours prior harvesting to halt the protein export and to concentrate the IFNγ produced. The harvested cells were treated as in the intracellular GIMAP4 staining, incubated with corresponding antibodies and isotype control. The cell viability with FITC BrdU Flow kit (BD Pharmingen, San Jose, CA, USA) and apoptosis marker Active Caspase-3 (#550914, BD Pharmingen San Jose, CA, USA) detections were done according to the manufacturer’s instructions. The BrdU was added to the cells at the time of activation and the IL12 (Th1) induced cells were harvested at 48 h and 72 h. The Active caspase 3 was measured firstly from cell samples collected before activation. Half of the activated cells were supplemented with Brefeldin A to induce apoptosis 4 h after activation. The Active caspase 3 was measured secondly from these apoptosis induced and non-induced cell samples harvested at 24 h after activation.
4.8 Extracellular cytokine profiling (III)

Before detecting the secreted cytokines, the GIMAP4 knock-down was verified by western blotting at 24 h after activation. The cytokines were measured daily until the day five from a 200-400µl aliquot of cell culture medium. The samples were stored in -70°C until analysis and the concentrations were measured according to the manufacturer’s instructions with MILLIPEX™ MAP -kit (MPXHCYTO-60K, Bio-Rad, Hercules, CA, USA). The preliminary measurements for TNF-β (lymphotoxin), IL13 and IL10 were done from one biological replicate, but for IFNγ three biological replicates were measured. The detection by Luminex 100 IS 2.2 (Luminex, Bio-Rad, Hercules, CA, USA) was performed at the Plexlaboratory, Medicity, University of Turku. The significance of differences was calculated by using two-tailed t-test.

4.9 Western blotting (III)

The harvested cell samples were first lysed with Triton-X-100 lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5% Triton-X-100, 5% glycerol, 1% SDS, 1mM Na3VO4, 10mM NaF), which was supplemented with 1x Complete Mini protease inhibitor (Roche # 11-836-170-001) and 1x PhosphoStop (Roche # 04-906-837-001). The lysate was incubated in +97 ºC for 7 minutes and sonicated for 5 minutes with 0.5 min pulses. The protein concentration was measured utilizing chemiluminescence and loaded equally to 10-12% SDS-PAGE gel in 1x SDS-sample buffer. The separated proteins were transferred to a nitrocellulose membrane (Hybond enhanced chemiluminescence [ECL]; GE Healthcare Niagara Inc., Mississauga, ON, Canada) and detected by corresponding antibodies. The protein bands were visualized with ECL (GE Healthcare Niagara Inc., Mississauga, ON, Canada). The protein quantitation against GAPDH or β-actin was done with MCID 5+ image analyzer (GE Healthcare Niagara Inc., Mississauga, ON, Canada), which was integrated with a Leica DMRB fluorescence microscope (Leica, Wetzlar, Germany) and a light table or with ImageJ software (http://rsb.info.nih.gov/ij 20.3.2013). The significance of the differences was calculated by using two-tailed t-test.

4.10 Quantitative real-time RT-PCR (II, III)

The total RNA was isolated with RNeasy minikit (Qiagen, CA, USA), the samples were treated with DNase I (Invitrogen, CA, USA) and cDNA synthesis was done with SuperScript II (Invitrogen, CA, USA). The qRT-PCR analyses were performed with ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA). Duplicate samples from three biological replicates were run in two technical replicates. Relative expression levels were calculated from EF1α normalized Ct values (ΔCt). The significance of the differences were calculated from the ΔCt values using two-tailed paired Student’s t-test.

The HeLa-cells were treated with Actinomyosin D (ActD 1 µg/ml, Sigma Aldrich, MO, USA) 24 h after reporter plasmid transfection to suppress transcriptional activity.
samples were collected at the time of ActD addition (0 h), and 2 h, 4 h, 6 h, 8 h, and 24 h after the treatment for the mRNA stability experiments. The luciferase (luc) mRNA expression was detected by RT-qPCR. The luc mRNA and IFNγ expression was normalized to a housekeeping gene EF1a.

4.11 Chromatin immunoprecipitation (Unpublished)

The cells in this experiment were treated differently to ensure the IL12 responsiveness and to follow the protocol in Good et al. (2009). The cells were activated and supplemented with IL2 at 48 h after activation. The cells were cultured for 68 h and supplemented with IL12 at this point. The optimal sample collection time point for phosphorylated STAT4 expression was verified from total cell lysate and nuclear extracts with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Thermo Fisher, Waltham, MA, USA) from samples collected at 0 h, 10 min, 1 h, 2 h, 4 h, 6 h and 24 h after addition of IL12. The optimal collection time was found to be at 1.5 h after the initial IL12 addition. The following ChIP protocol was executed as instructed according to the ChIP kit (Agarose ChIP Kit, Pierce, Thermo Fisher, Waltham, MA, USA) with the STAT4 specific antibody (SC485 (C-20), Santa Cruz Biotechnology, Dallas, TX, USA). The GIMAP4 promoter amplifying primers (550 bp upstream the transcription start site) were used to detect the bound STAT4 in the GIMAP4 promoter region. The GIMAP4 specific PCR amplicon intensity was measured with MCID 5+ image analyzer (GE Healthcare Niagara Inc., Mississauga, ON, Canada).

4.12 Transcriptomics (III, unpublished)

4.12.1 Microarray

The samples for microarray studies were harvested at two time points, first before activation and secondly, 2 h after activation and Th1 induction. GIMAP4 downregulation was verified by western blotting. The samples were treated and analyzed at the Finnish Microarray and Sequencing Centre, Turku, Finland. RNeasy minikit (Qiagen, Valencia, CA, USA) was used for the total RNA isolation. The sample preparation for the GeneChip oligonucleotide hybridizations was done according to Affymetrix’s instructions. In total of 250 ng RNA was used as a starting material with GeneChip 3’ IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized into HG-U219 arrays using a GeneTitan® (Affymetrix Santa Clara, CA, USA). The normalization of the raw data was done by using robust multi-array average algorithm implemented in the Bioconductor suite. The genefilter package in R, was used for the removal of duplicate and non-annotated probe sets, and the probe sets with the highest inter quartile range were retained. By using the standard EM algorithm implemented in mix tools package in R, present and absent calls for probe sets were generated by fitting the chip-wide expression data to a two-component Gaussian distribution. A probe set was defined to be present if it has an expression value higher than the threshold where the two components of the Gaussian distribution densities meet. Differential expression analysis was done using
unpaired, moderated t-test. A differentially expressed gene was defined with Benjamini-Hochberg adjusted p-value (<0.05) and log fold change (<-1 or >1).

4.12.2 RNA-sequencing

The isolated cells were divided in three and nucleofected with, siGimap4, siGimap5 and non-targeting control siRNA oligos. The activated (Th0) and, activated and IL12 supplemented (Th1) cell samples for each siRNA targeted condition were collected at 24 h, 48 h and 72 h from three biological replicates. The downregulation of both GIMAP4 and GIMAP5 was verified by quantitative PCR. The RNA isolation, cDNA synthesis, library preparation and sequencing, and initial quality control steps in between, were performed in the Finnish Microarray and Sequencing Centre, Turku, Finland. Sequence reads were mapped using Tophat (version 2.0.8) (Trapnell et al. 2009) and Bowtie (version 2.1.0) (Langmead et al. 2009) with default parameters to the GRCh37 human reference genome and Ensemble human transcriptome (release 70). Expression levels for genes were estimated using Python package HTSeq (Anders, Pyl, Huber 2015) with parameters "--type=exon --idattr=gene_id --stranded=no". Differential expression calling between the two conditions was done using Bioconductor package edgeR (Robinson et al. 2010). For each comparison (Thp:siGIMAP vs. Thp:SCR, Th1:siGIMAP vs. Th1:SCR, Th0:siGIMAP vs. Th0:SCR, Th1:SCR vs. Th0:SCR and Th1:siGIMAP vs. Th0:siGIMAP), only the genes with RPKM value more than 1 in at least at one time point per culture condition measured were included in differential expression analysis. Identification of transcription factor binding motifs was done with HOMER software (Heinz et al. 2010). Binding motifs were searched using two different regions around TSS of differentially expressed genes; [-1000,100] and [-500,300]. The differentially expressed genes were annotated and analyzed for known functional pathways with the Ingenuity Pathway Analysis Software (IPA, Ingenuity Systems, QIAGEN Redwood City, www.ingenuity.com).
### Table 1A. Sequences of PCR probes, PCR primers and siRNA oligos.

<table>
<thead>
<tr>
<th>OLIGOS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RNAi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>target</td>
<td>sequence</td>
<td></td>
</tr>
<tr>
<td>siScramble</td>
<td>5’-gcgcgctttgtaggattcg-3’, Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>siGIMAP4</td>
<td>GIMAP4HSS124340, Invitrogen</td>
<td></td>
</tr>
<tr>
<td>siGIMAP5</td>
<td>GIMAP5HS124411, Invitrogen</td>
<td></td>
</tr>
<tr>
<td>siSTAT4</td>
<td>5’-gguaacaguguaccaac-3’, Sigma-Aldrich</td>
<td></td>
</tr>
</tbody>
</table>

| PCR |   |   |
| **GIMAP5 3’ UTR reporter construct** | Forward primer | 5’-catctagatctggaccctggagca-3’, Oligomer |
|   | Reverse primer R1 | 5’-tgggatccaaattttatattttgaatg-3’, Oligomer |
|   | Reverse primer R2 | 5’-tgggatccaaatttttattttgaatg-3’, Oligomer |

| **STAT4 ChIP: GIMAP4 primers** | Forward primer F1 | 5’-tttcctacttcagctgtacc-3’, Oligomer |
|   | Forward primer F2 | 5’-ccggtaagacccgtagtac-3’, Oligomer |
|   | Reverse primer | 5’-gtcaccagctcaggta-3’, Oligomer |

| RT-PCR |   |   |
| **Luciferase/mRNA stability** | primer 1 | 5’-atccggagagccagaccaag-3’, Oligomer |
|   | primer 2 | 5’-cggtgaacctccgcttcgatac-3’, Oligomer |
|   | probe | #70, Roche |

|   |   |   |
| **EF1a** | primer 1 | 5’-ctgaacacacecagggcacaac-3’, Oligomer |
|   | primer 2 | 5’-gcgtggtggcaatcata-3’, Oligomer |
|   | probe | #87, Roche |

| GIMAP4 |   |   |
| primer 1 | 5’-tgccgctactgcagtgatc-3’, Oligomer |
| primer 2 | 5’-tggaacgcaacccagcagca-3’, Oligomer |
| probe | 5’-FAM-acaaggcaacaggcgctgagca-TAMRA-3’, Oligomer |

| GIMAP5 |   |   |
| primer 1 | 5’-accttcctgagaggagaccag-3’, Oligomer |
| primer 2 | 5’-ggaatcttcctgaccttc-3’, Oligomer |
| probe | #87, Roche |

| IFNγ |   |   |
| primer 1 | 5’-gtcgaacagcaggaatcaca-3’, Oligomer |
| primer 2 | 5’-ctggaacagcagtaaagacct-3’, Oligomer |
| probe | 5’-FAM-tggtgccgagctgagca-TAMRA-3’, Oligomer |
### Table 1B. Antibodies

<table>
<thead>
<tr>
<th><strong>ANTIBODIES</strong></th>
<th><strong>Western blot</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>primary antibodies:</strong></td>
<td><strong>provider</strong></td>
</tr>
<tr>
<td>rabbit anti-GIMAP4-antiserum</td>
<td>a gift from Dr. Cambot</td>
</tr>
<tr>
<td>rabbit anti-STAT4, sc485 C-20</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>rabbit anti-pSTAT4, sc-16317-R Ser 721-R</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>mouse anti-IkBα, L35A5</td>
<td>Cell Signaling Technology Inc.</td>
</tr>
<tr>
<td>mouse anti-GAPDH</td>
<td>Hytest Ltd.</td>
</tr>
<tr>
<td>mouse anti-β-ACTIN</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>rabbit anti-pSTAT1, Tyr 701-R</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>rabbit anti-STAT1</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>rabbit anti-VMA21</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

**HPR conjugated secondary antibodies:**

<table>
<thead>
<tr>
<th><strong>anti-rabbit IgG</strong></th>
<th><strong>anti-mouse-IgG</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Signaling Technology Inc.</td>
<td>Santa Cruz Biotechnology</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>FACS</strong></th>
<th><strong>BD Biosciences, San Jose, CA, USA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>rat anti-human IL2RA/CD25-FITC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>isotype control: rat IgG1-FITC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>rat anti-human IL12Rβ2/CD212 , #550722</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>secondary: rat anti-human IgG2a-FITC, #554016</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>mouse anti-human CXCR3-APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>isotype control: mouse IgG1k-APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD69-FITC antibody, #347823</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>isotype control: #MG101</td>
<td>Caltag</td>
</tr>
<tr>
<td>rabbit anti-HIMAP4-antiserum</td>
<td>Dr. Cambot</td>
</tr>
<tr>
<td>secondary: anti-rabbit-Alexa649</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>mouse anti-human-IFN-γ-FITC, #IFG601</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>isotype control: #MG101</td>
<td>Caltag</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Immunostainings</strong></th>
<th><strong>BD Biosciences, San Jose, CA, USA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit anti-HIMAP4-serum</td>
<td>a gift from Dr. Cambot</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>EEA1</td>
<td>Abcam</td>
</tr>
<tr>
<td>rab27A</td>
<td>Abcam</td>
</tr>
<tr>
<td>Syntaxin6</td>
<td>Abcam</td>
</tr>
<tr>
<td>Lamp1-Cy3</td>
<td>Abcam</td>
</tr>
<tr>
<td>F-actin staining, Alexa Fluor® 546 phalloidin</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Secondary: Alexa Fluor® 488 or 546</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>

BD Biosciences, San Jose, CA, USA
Abcam, Cambridge, England, UK
Caltag, Buckingham, UK
Dr. Cambot, INTS, Paris, France
Cell Signaling Technology Inc., Danvers, MA, USA
Hytest Ltd., Turku, Finland
Life Technologies, Thermo Fisher, Waltham, MA, USA
Santa Cruz Biotechnology, Dallas, TX, USA
5 RESULTS AND DISCUSSION

5.1 The association of CTLA4, ICOS, GIMAP4 and GIMAP5 with immune-mediated diseases (I, II)

5.1.1 CTLA4, but not ICOS, is association with T1D in Finnish and Estonian populations

CTLA4 is a well characterized T-cell surface receptor, which has been associated with multiple autoimmune diseases (Donner et al. 1997a, 1997b; Haller et al. 2004; Marron et al. 2000). Due to its inhibitory role in T-cell responses and its role in Treg-cell biology, CTLA4 may play an important role in the development of autoimmunity (reviewed in Romo et al. 2013). The CTLA4 gene region harbors two genes, CD28 and ICOS, which code for TCR costimulatory molecules and are expressed by T-cells. CD28 and ICOS are structurally similar to CTLA4, but functionally opposing. The mechanism of T-cell response inhibition by CTLA4 is based on shutting down the CD28-induced signaling through binding to the CD28 ligands at the APC surface (Qureshi et al. 2011). Previous studies have mapped this gene region as one of the T1D susceptibility regions. The genetic polymorphisms in CTLA4 have been suggested to be promising effector candidates in autoimmune disorders by Ueda et al. (2003).

In our study we investigated the association between autoimmune diabetes and polymorphisms in the CTLA4-ICOS 2q33 chromosomal gene region. The study was conducted in two geographically and linguistically related populations, Finnish (404 cases and 725 unaffected controls) and Estonians (170 cases, 230 unaffected controls), which have a considerable difference in the disease incidence. It is noteworthy that Finland has a higher standard of living together with a higher disease rate, when compared to Estonia. In both of the populations studied, we observed higher frequencies of the CTLA4 +49 GG, CT60 GG and CTBC217_1 TT in the T1D patients, in comparison to the corresponding age and gender matched healthy controls (Table 1, I). Additionally, the frequencies of CTLA4 +49 G and CTBC217_1 T risk-alleles were higher in the Finnish population. The data from both populations was combined (the T1D cases in both populations vs. the matched controls in both populations) in order to assess the impact of causative alleles to the risk of T1D (Table 3, I). In the Finnish patients, the CTLA4 +49/CT60/CTBC217_1 GGT haplotype was found to be associated with the T1D risk, whereas in Estonian patients the CTLA4 +49/CT60/CTBC217_1 GGG haplotype was enriched. In the combined sample set, the CTLA4 +49/CT60/CTBC217_1 GGT haplotype was again enriched among patients and the CTLA4 +49/CT60/CTBC217_1 AAC and AGC haplotypes in healthy controls. The analysis of allelic, genotypic and haplotypic frequencies of ICOS markers revealed no differences in either of the populations (Tables 2 and 4, I), but in the combined sample set, ICOS
CTIC154_1 T was observed with a lower frequency in patients in comparison to the healthy controls.

We were thus able to show a T1D association with the known CTLA4 +49 risk marker in both of the populations studied. Additionally, we confirmed the association with the putative risk CTLA4 markers, CTBC217_1 and CT60, which were first reported by Ueda et al. (2003). We did not find any strong evidence of association with ICOS polymorphisms, which was also in line with the previous finding by Ueda et al. (2003). However, when combining the two populations, an indication of an association with a marker residing in ICOS 3’ UTR was found. Further studies are still needed regarding the possible effects of the CTIC154_1 genotype on ICOS mRNA stability. The changes in the stability of the mRNA may affect T-cell function by altering ICOS protein expression. Moreover, the CTLA4 CTBC217_1 marker resides at the 3’ UTR of the gene and is also close to the ICOS promoter. The genotypic variance in the CTLA4 CTBC217_1 could therefore modulate the CTLA4 mRNA stability and gene expression, and/or affect the function of ICOS promoter region.

The aspect of differential disease incidence between the study populations was also considered. It was known that the age of T1D onset is higher in Estonians (15-16 years) than in the Finnish population (early childhood). We observed that the allele frequencies of the associating risk markers and haplotypes were higher in the Finnish than in Estonian population. The frequency of CTLA4 +49 G risk allele in the Finnish cases was 54%, whereas in Estonians, it was 46%. Additionally, the frequency of CTLA4 CTBC217_1 risk-allele T in the Finnish population was 50%, against the frequency of 47% in Estonians. Considering the corresponding controls, the genotype distributions were significantly different between the Estonian and Finnish populations (p=0.047). Our results could offer one explanation for the difference in the disease rate. The genotypic variance in the CTLA4 region in the Finnish population may result in altered regulation and function of CTLA4. This would further lead to altered T-cell function resulting in higher disease prevalence.

5.1.2 **GIMAP5 associates with T1D, asthma and allergic sensitization**

The importance of T-cells in the development of autoimmunity and immune-mediated diseases is well established. Several GWAS studies have identified disease susceptibility loci, some of which are also shared between the different immune-mediated diseases. However, there are also other factors that affect the function of the cells involved in the disease development or maintaining the disease phenotype. The GIMAP family of genes and proteins are such candidates, although the knowledge of their function is incomplete. Interestingly, all seven functional human GIMAP family members are differentially expressed in the course of Th-cell differentiation (Filén et al. 2009; Filén unpublished), suggesting that they have a functional role in the differentiation process. Some of the family members have been connected with immune-mediated diseases in both human and mouse studies. GIMAP5 polymorphisms, in particular, have been associated with SLE and diabetes in the BB-DP rat is caused by a mutation in the Gimap5 gene (Hellquist et al. 2007; Hornum et al. 2002;
In the work presented in this thesis, the GIMAP5 gene region was studied in Finnish T1D trio families of the FPDRNF dataset (1732 families in total) and in Swedish asthma and allergic sensitization BAMSE birth cohort (440 asthma cases, of which 300 are also allergy cases; 979 allergy cases of which 140 are also asthma cases and 839 healthy, non-asthmatic, non-allergic controls). When the whole population was considered we did not find any statistically significant association of GIMAP5 with T1D in the Finnish FPDRNF family-based sample set. However, the geographical stratification of the data enabled the discovery of a significant GIMAP5 rs6965571 association with T1D in South-Western (SW) population (Tables 1 and 2, II). The basis for this stratification is well justified. The genetic structure of Finnish population is exceptionally heterogenic between the South-West and the North-East. The geographical division is one such method that enables the detection of association in the Finnish population, especially when considering disease markers with low odds ratio (Salmela et al. 2008; Zondervan and Cardon 2004). The power of association detection can be modified by the differences between SNP allele frequencies, the functional polymorphisms, interaction between genes, and polymorphism interplay with effect size (Hunter 2005; Moonesinghe et al. 2008). The association study for the same markers in the Swedish BAMSE cohort revealed an association with the same GIMAP5 rs6965571 marker as in the Finnish T1D sample set (Tables 1 and 3, II). Most interestingly, the marker has an opposing genotypic effect in these two theoretically opposing diseases. The GIMAP5 genotype, which is protective in T1D, was a risk in asthma and allergic sensitization and vice versa. Although, the association with T1D did not survive the correction for multiple hypothesis testing, it is likely that it may modulate the disease via the function of GIMAP5 in T-cell immunity. Interestingly, it has been proposed earlier that genotypic variance in the genes associated with both asthma and autoimmunity may also result in variability in the immunopathogenesis in question (Li et al. 2012). Thus, these types of genetic variants may also act as modulators of disease susceptibility. Additionally, these types of variants may affect the direction to which, the immune system distorts in the individuals at risk, i.e. to autoimmunity or chronic infection.

The marker associated with asthma and allergic sensitization and T1D is located at the 5’ end of the GIMAP5 gene, and thus it may affect the regulation of the gene expression. The variation in the genomic sequence may alter the binding properties of transcription factors or transcription factor complexes. We did not discover any known transcription factor binding sites in the immediate vicinity of disease associated marker loci, but the closest ones are interesting in terms to T-cell function and T1D. Expanding 7-14 nucleotides upstream from GIMAP5 rs6965571 is a predicted Forkhead box protein O1 (FOXO1) binding site, and 38-47 nucleotides further downstream is a predicted NFAT binding site. NFAT transcription factors are known to be important in immune response and are regulated by calcium signaling. GIMAP5 has been shown to play a functional part in calcium signaling as it affects the
mitochondrial ability to buffer calcium (Chen et al. 2013). Moreover, TCR activation results in fast calcium release in the cell. Thus, a potential mechanism by which GIMAP5 rs6965571 is associated with immune dysregulation could be explained by a change in the ability of NFAT to regulate GIMAP5. This would lead to changes in GIMAP5 expression and in the mitochondrial buffering of the calcium and in the calcium induced signaling pathways, which regulate Th-cell function. On the other hand, FOXO1 is also an interesting factor when considering the association of GIMAP5 with T1D. In addition to its role in insulin signaling and glucose metabolism FOXO1 has been shown to promote Th-cell responses and Th2-cell function (Stentz and Kitabchi 2003; Viardot et al. 2007). Moreover, in CD4+ T-cells of the Gimap5-deficient mouse, the immunological tolerance is lost in association with the loss of the Foxo transcription factor (Aksoylar et al. 2012). Thus, the abnormal expression of GIMAP5, which is observed in response to altered regulation by FOXO1, could lead to an imbalance of the Th1- and Th2-cells. This could further promote either the diabetic condition or the inflammatory response.

5.1.3 GIMAP5 and INS interaction and the GIMAP5 3’ UTR genotypic variance

Once an association was established between GIMAP5 and T1D, it became of interest to determine whether GIMAP5 might affect T1D by interacting with other well established T1D genes. The gene-gene interaction was calculated for CTLA4, PTPN22, INS and IL2RA risk genotypes. The INS risk marker rs689 showed indication of an interaction with GIMAP5 rs2286899 (Table 4, II). The interaction results show that the INS risk allele A was detected more often with the GIMAP5 rs2286899 (T/C) T allele than expected, when taking the weak risk effect of GIMAP5 into consideration. In other words, the patients with diagnosed T1D more often have the INS risk allele with GIMAP5 rs2286899 T rather than C, making the C allele possibly protective against the INS conferred risk. This is an interesting observation, particularly when regarding the association GIMAP5 with T1D and the associating loci being close to insulin regulator, FOXO1 binding site. Additionally, in an earlier study on GIMAP5 polymorphisms in SLE (Hellquist et al. 2007), the same marker locus was indicated as a splice site. We investigated the genotypic effect of GIMAP5 rs2286899 variance on mRNA stability and protein expression by a reporter construct transfected to HeLa cells.

The Hela-cells were transfected with one of the three luciferase reporter constructs; control-construct, TT-construct and CC-construct (Figure 3A, II). The amount of luciferase produced was measured 48 h after transfection. The mRNA was measured from samples taken over a time course after global transcription halting actinomycin D treatment. There were no major differences in the rate of mRNA degradation, but the amount of luciferase measured was significantly lower in the cells transfected with the CC-construct than with the TT-construct (Figure 3B, II). Thus, the genotypic variance at the GIMAP5 3’ UTR rs2286899 affects the protein amount produced. As the effect is not seen in the mRNA degradation rate, it is most likely caused by the difference in the amount of mRNA transcribed. Considering the INS-GIMAP5 interaction and the effect on protein expression together, the diabetic patients have the GIMAP5 high protein producing genotype more often together with the genetic INS risk
factor. Taking this the other way around, there are less diabetics with the INS risk genotype and GIMAP5 low protein producing genotype. To conclude from this data, the low production of GIMAP5 could possibly be a protective mechanism against the INS conferred risk. The most popular hypothesis for the mechanism of how the INS rs689 genotype affects susceptibility is based on the amount of insulin produced in the thymus (Vafiadis et al. 2001). The INS risk genotype might result in low insulin production in the thymus, which leads to impaired negative selection and to the production of insulin self-reactive T-cells. In this situation, if the low GIMAP5 production alters the thymic T-cell development, the global self-reactivity may be neutralized to some extent. Another possible hypothesis is that the GIMAP5-low insulin autoreactive T-cells have a lower activation and differentiation capacity, and thus the self-reactivity to the pancreatic β-cells is reduced.

The overall hypothesis for the interplay of GIMAP5 interaction with INS, the GIMAP5 association with T1D and possible regulation of GIMAP5 by FOXO1 is illustrated in Figure 5. FOXO1 binding to DNA is stimulated by insulin (Guo et al. 1999; Puig and Tjian 2005; Zhang et al. 2012). The INS risk genotype results in low insulin production, possibly disrupting negative selection and leading to the destruction of insulin producing β-cells. This results in defect in insulin signaling and impairs FOXO1 binding, thus promoting Th1-cell differentiation over Th2 differentiation. In the situation when GIMAP5 expression is downregulated by FOXO1, the loss of FOXO1 binding results in upregulation of GIMAP5. This may endorse the Th1-cell differentiation over Th2-cell differentiation and enhance the progression of T1D. In the situation when GIMAP5 expression is upregulated by FOXO1, the loss of FOXO1 results in GIMAP5 downregulation and endorsement of Th2 differentiation. If the disease progression has already started, it also might occur slower in case of GIMAP5 upregulation. On the other hand, in the case of low GIMAP5 production due to the rs2286899 CC genotype, the up- or downregulation by loss of FOXO1 is irrelevant. The 3’ UTR CC genotype effect decreases GIMAP5 protein expression and results in protection from the insulin conferred risk, regardless of FOXO1 regulation. The true mechanism by which GIMAP5 alters the progression to T1D and/or the insulin conferred risk and the FOXO1 regulation remains an interesting aspect of future studies.
Figure 5. Hypothetical model of the effect of GIMAP5 on T1D under FOXO1 regulation. Given a predisposing situation in which the genetic milieu is biased towards immune dysregulation and making the assumption that FOXO1 is a GIMAP5 repressor, the FOXO1 regulated GIMAP5 transcription can either promote Th2 differentiation and asthma or Th1-cell differentiation and T1D. Under the 3’UTR genotype CC, the GIMAP5 expression is low by default and thus protects from the effects of an INS conferred risk in T1D.

5.1.4 GIMAP4 association with asthma & allergic sensitization and IL2RA interaction

In previous studies, GIMAP4 has not been strongly associated with immune-mediated diseases. GIMAP4 has been indicated to be a susceptibility gene for Behcet’s disease in a Korean GWAS study, but this result was not reproducible in a recent European study (Lee et al. 2013; Ortiz-Fernandez et al. 2014). Like GIMAP5, GIMAP4 is found to be regulated by PHF11, which has an effect on allergic asthma, total IgE and eczema (Jang et al. 2005; Rahman et al. 2010; Zhang et al. 2003). We found GIMAP4 to be associated with asthma and allergic sensitization in the BAMSE cohort (Table 3, II). The association was stronger for allergic sensitization than for asthma, but this was most likely due to smaller sample size of the asthma cases. The asthma associating rs13222905 (A/G) is located at the 5’ end of the GIMAP4 gene. Thus, the in silico prediction of transcription binding sites was also applied in this case. Again, there were no putative binding sites at the exact site of the SNP in question. The nearest upstream binding site, expanding 22-29 nucleotides, was ZFX/ZNF711. MYB
and Eomes sites, expanding 20-27 nucleotides and 21-30 nucleotides, were the nearest downstream binding sites, respectively. Additionally, the binding site for STAT6, the master regulator of the Th2-cell and known GIMAP4 regulator, was predicted to be located at 161-172 nucleotides downstream. It is possible, that the dysregulation of GIMAP4 distorts the immune reaction towards a Th2-cell-like response and thus the association with asthma and allergy is detected. The predicted transcription binding sites around the associating loci suggest that GIMAP4 expression is regulated during Th-cell development. This is in line with the results from the earlier study by Schnell et al. (2006), in which they observed a high upregulation of GIMAP4 during the development of mouse T-cells. Thus, GIMAP4 may affect the development of the disease during T-cell development in the thymus, but the mechanism requires further investigations.

There was no significant GIMAP4 association with T1D detected. The gene-gene interaction analyses were nevertheless applied for GIMAP4 and the known T1D susceptibility genes CTLA4, PTPN22, INS and IL2RA. The results displayed a significant interaction between GIMAP4 and IL2RA. The IL2RA rs2104286 risk allele A was more often present in diabetic patients with a GIMAP4 rs9640279 T allele in comparison to those with the GIMAP4 rs9640279 G allele. This indicates that GIMAP4 G allele could possibly be protective from the IL2RA conferred risk. As IL2 signaling is crucial for the T-cell expansion and function, the effect of RNAi induced GIMAP4 downregulation to IL2RA expression during T-cell differentiation was examined. GIMAP4 was downregulated in human umbilical cord blood driven CD4+ Th-cells. The IL2RA expression was measured from cells under Th0 (activated) and Th1 (activated with IL12) cell culture conditions, with or without IL2 in the culture. In all of the six biological replicates, the IL2RA expression was approximately 10% downregulated in response to GIMAP4 downregulation (p-value in all conditions: <0.05, Figure 3. II). Additionally, the results indicated (not statistically significant), that under IL2 induction, GIMAP4 expression is upregulated on average 80% (Figure 6).
Figure 6. IL2 signaling promotes GIMAP4 expression. Samples treated with non-targeting Scramble siRNA show a difference in GIMAP4 expression, which is dependent on IL2 supplementation (IL2 +) in both activated (act. +) Th1 inducing (IL12 +) and activated Th0 (IL12 -) culture conditions. The figure illustrates the average GeoMean value at 72 h after activation from three biological replicates.

Although GIMAP4 does not have an impact on T1D, the interaction with \textit{IL2RA} indicated that it could still be a noteworthy modulator of the disease. \textit{IL2RA} conferred risk in T1D is suggested to be mechanistically based on its function in the IL2 signaling cascade in memory T-cells and most importantly, in Treg-cell function (Garg et al. 2012). The decrease in IL2RA expression upon GIMAP4-depletion is in line with the observed decrease in IL2 signaling in diabetes subjects compared to healthy controls reported by Garg et al. (2012). Here, the effect is detectable as a co-function with IL2RA and the IL2 pathway. Thus, the lack of association, but finding of gene-gene interaction, could be explained by the moderate nature of the effect in IL2 signaling. It is likely that in a situation where IL2RA plays a part in disease progression, GIMAP4 may strengthen the effect. This results in discovery of a genetic interaction between these genes in the T1D population data, but lack of direct genetic association.

5.2 The role of GIMAP4 and GIMAP5 in early human Th-cell differentiation (III)

5.2.1 GIMAP4 localizes into the intracellular trafficking compartment

Earlier studies in mouse indicated that GIMAP4 is localized in the cytoplasm, ER and Golgi (Krucken et al. 2004; Nitta et al. 2006; Schnell et al. 2006). Additionally, GIMAP4 has been found in the microsomal fraction of human CD4+ T-cells, indicating that it localizes in cell membranes (Filén et al. 2009). In this study (III), an earlier finding of GIMAP4 differential regulation under IL12 (Th1-cell) induction in comparison to IL4 (Th2-cell) induction during human Th-cell differentiation (Filén et al. 2009) was followed up. In theory, the cellular
loca
tization of a given protein indicates in which functional category it may belong. No previous localization data on human GIMAP4 was available, thus we investigated cellular localization of endogenous GIMAP4 in HeLa-cells by confocal imaging. HeLa-cells were initially used due to their better imaging properties over primary Th-cells. The localization was thereafter studied also in the human primary CD4+ T-cells. The hypothesis was that, as a microsomal fraction residing septin-related GTPase, the most likely localization of GIMAP4 would be in the membranes and microtubules.

In HeLa-cells, we found a co-localization or partial co-localization of GIMAP4 with actin filaments, β-tubulin and syntaxin6 (Figure 1a-d, III). This suggested that the functional role of GIMAP4 is connected with the trans-golgi network (TGN). The localization with the machinery involved in endocytosis was excluded, as GIMAP4 showed no co-localization or – distribution with the early endosomes or lysosomes. Similar results were recovered in human primary Th-cells. There was no co-localization with early- or functional endosomes. GIMAP4 co-localized in the same cellular compartment as lysosomes and more so with syntaxin6 (Figure 1e-f, III). In agreement, the results from Hela-cells and primary T-cells indicated that TGN and microfilaments are the most likely organelles to harbor GIMAP4. We hypothesized that GIMAP4 is likely to have a role in secretory processes. TGN has an important function in sorting the proteins that are transported to the cell membrane, in order to be secreted, or are transported to lysosomes. Moreover, microfilaments also have a crucial role in the secretory processes.

5.2.2 GIMAP4 regulates the expression of ER localizing chaperone VMA21

When RNAi GIMAP4-depleted cells were compared to the controls, VMA21 (Vacuolar ATPase assembly integral membrane protein) mRNA and protein were shown to be differentially regulated in the non-activated Thp cells (Figure 2, III). VMA21 has been mostly studied in yeast and it is known to localize in the ER and act as a chaperone required for V-ATPase proton pump assembly. Most human studies have concentrated on the role of VMA21 in X-linked myopathy with excessive autophagy (XMEA). One of the studies reported that VMA21 expression decreased in cultured human muscle fibers upon ER-stress (Nogalska et al. 2010). Additionally, GIMAP4 expression has been shown to be downregulated in cultured human muscle fibers when compared to muscle biopsy samples (Raymond et al. 2010). Adding these together, it would be of interest to investigate the roles of GIMAP4 and VMA21 in ER-stress, and also the role of ER-stress in the functions of GIMAP4 and VMA21.

5.2.3 GIMAP4 is regulated by STAT4, but not essential for TCR activation

Considering the role of GIMAP4 in the course of Th-cell differentiation, the first question arises from the GIMAP4 expression kinetics. Does the high GIMAP4 expression prior to TCR activation have a role in the activation process or the immediately following signaling pathways? Mouse Gimap4 is upregulated during T-cell development in the thymus (Schnell
et al. 2006). The human GIMAP4 protein is highly expressed in the Thp-cell state and the expression decreases upon TCR activation, after which it is either downregulated by STAT6/IL4 or upregulated by IL12 (Filén et al. 2009; Filén and Lahesmaa 2010). This led us to examine if GIMAP4 downregulation prior to TCR activation affects the TCR signaling machinery and cell activation. As IL12 induced STAT4 has been shown to regulate GIMAP4 in mouse cells (Good et al. 2009), we wanted to investigate whether GIMAP4 is under IL12/STAT4 regulation during human Th1-cell differentiation. STAT4 plays an important role in early Th1-cell polarization and in the maintenance of the differentiated lineage by regulating IFN\(\gamma\) and other Th1 lineage-specific genes (Lund et al. 2003, 2004; Ouyang et al. 1999).

For the assessment of the effect on TCR activation, the RNAi GIMAP4-depleted cells and their matching controls were collected before activation and 2 h after activation. The total RNA was isolated, hybridized and the changes in the transcriptomic profile between GIMAP4 downregulated and control samples were examined. There were no differentially expressed genes found between the siGimap4 treated samples and the non-targeting siRNA treated controls, which thus ruled out any essential role in the immediate TCR activation pathways. Additionally, the effect to the cell activation was verified by measuring CD69 expression under GIMAP4-depletion and controls at 6 h and 24 h after initial activation. The results were in line with the transcriptomic profile, as there were no differences between GIMAP4-depleted cells and the controls (Figure 7).

**Figure 7. Fluorescence intensity of CD69 expression under GIMAP4 downregulation.**

The activation marker CD69 expression was measured by flow cytometry at 6 h and 24 h after initial TCR activation in siGimap4 and siScramble treated CD4+ T-cells. The measurements were made with two biological replicates.
The ability of STAT4 to bind to the GIMAP4 promoter was addressed by chromatin immunoprecipitation (ChIP). The optimal conditions were first established by measuring STAT4 at multiple time points in total cell lysates and nuclear extracts in response to IL12 stimulation (Figure 8A). To ensure IL12 responsiveness of the cells this was done in a similar manner as that established in the study by Good et al. (2009). The time point with the highest STAT4 phosphorylation proved to be from one to two hours after IL12 supplementation. The samples for the following ChIP experiments were thus collected accordingly, 1.5 h after IL12 supplementation. The result in Figures 8B and 8C illustrate the relative intensity of STAT4 binding upstream of the GIMAP4 transcription start site, with and without IL12 induction, and a representative image of PCR agarose gel. In Figure 8C, the input sample shows the total amount of chromatin prior to the STAT4 specific immunoprecipitation and rabbit IgG is the control for unspecific binding. The ChIP results show that STAT4 binds to the GIMAP4 promoter region. In addition, the effect of STAT4 downregulation on GIMAP4 expression was detected at protein level (Figure 8D). GIMAP4 was found to be downregulated upon STAT4 downregulation. Thus, it is likely that the IL12 induced upregulation of GIMAP4 is regulated through STAT4.

An in silico screen for STAT4 binding sites in the GIMAP4 promoter region, however, did not reveal any known STAT4 binding sites (data not shown). We utilized HOMER software and searched 1000 nucleotides upstream and 500 nucleotides downstream of the GIMAP4 transcription start site. This, however, does not diminish the validity of the ChIP results as IL12/STAT4 may regulate GIMAP4 expression in an indirect manner or STAT4 may bind as a part of a regulatory complex. It has been noted that several of the STAT proteins can be a component of different DNA binding protein complexes, and that the STAT protein complexes can bind to several STAT-related DNA sequences with different affinities.
5.2.4 The role of GIMAP4 in IFNγ signaling and Th1-cell differentiation

One of the functions of STAT transcription factors during Th-cell differentiation is to regulate cytokine secretion. Considering this and the earlier findings of the GIMAP4 localization study, we wanted to further investigate the role of GIMAP4 in cytokine secretion. First, the effect of RNAi based GIMAP4-depletion on the cytokine secretion was addressed the measurement of multiple cytokines in both Th1- and Th2-cell types. The results showed that cytokine secretion was diminished under GIMAP4 downregulation.
(Supplemental Figure 1, III). The most affected cytokine was IFNγ, and this result was then corroborated in multiple replicates at 24 h and 72 h from activation and induction of Th1-cell differentiation (Figure 3a-b, III). Additionally, neither the intracellular concentration nor the mRNA levels of IFNγ were affected during the first three days of cell culture (Figure 3c-d, III). The results show that GIMAP4 is not necessary for the production of IFNγ, but it is specifically required for the secretion.

The main target for IFNγ signaling during Th1-cell differentiation is the STAT1 transcription factor, which is phosphorylated and activated via the IFNγ-receptor. To establish whether the low level of IFNγ induced by GIMAP4-depletion has an effect on Th1-differentiation, the IFNγ downstream pathway and the IFNγ-receptor were considered. The concentration of total STAT1 and phosphorylated STAT1 were measured under GIMAP4-depletion at 24 h after activation and induction of Th1-cell differentiation. The results revealed that GIMAP4-depletion induces a decrease in STAT1 and especially in phosphorylated STAT1 expression (Figure 4a, III). To verify whether this was due to the low IFNγ in the extracellular space, exogenous IFNγ was added to the cell culture (Figure 4b, III). Accordingly, the higher amount of IFNγ in the extracellular space rescued STAT1 and phosphorylated STAT1 expression despite GIMAP4-depletion. Additionally, this result revealed, that GIMAP4-depletion has no effect on the functionality of the IFNγ-receptor. As phosphorylated STAT1 is known to regulate the expression of the IL12Rβ2, we studied if the lower amount of phosphorylated STAT1, due to GIMAP4-depletion, affects the cell surface expression of IL12Rβ2. Indeed, lower IL12Rβ2 expression under GIMAP4-depletion was observed in comparison to control (Figure 4c, III). The expression of another Th1-specific, but not STAT1-regulated cell surface receptor, CXCR3, was also studied in the same setting (Figure 4c, III). The CXCR3 surface expression was found not to be affected by GIMAP4-depletion. Finally, the role of GIMAP4 in apoptosis and Th-cell survival was taken into consideration. The effects on cell cycle, proliferation and apoptosis were studied under GIMAP4 downregulation and found not to be affected during early differentiation (Figure 5, III). The role of GIMAP4 in human T-cell survival needs to be further studied since it has been shown that the Gimap4 knock-down mouse splenic T-cells are protected from apoptosis, but in our study design such an effect was not observed.

Overall, these results suggest the following model. In differentiating Th1-cells, GIMAP4 is upregulated upon TCR activation. It then accumulates in the ER-Golgi region and microtubules, in which it has a role in the processes involving cytokine secretion. The Th1-cell differentiation driving IFNγ is secreted in a directed manner through the IS and its secretion is assisted by GIMAP4. Upon GIMAP4-depletion, the system is disrupted leading to a dysfunction in differentiation, as illustrated in Figure 9. Interestingly, when CD4+ Th-cells, isolated from human cord blood, are cultured in Th1- and Th2-cell differentiation inducing conditions for two weeks, the level of GIMAP4 mRNA expression in Th2-cells increases close to the same level as in Th1-cells (Figure 10A). Additionally, GIMAP4 was detected from isolated human peripheral blood CD4+ Th-cells, that were sorted for CXCR3+ (Th1-cells) and CXCR3- (other Th-cells). The sorted cells were activated and cultured for 24
h and 48 h before protein expression detection (the cells were from the experiment described in Tahvanainen et al. 2013). There was no difference in GIMAP4 expression observed between the CXCR3+ and the CXCR3- cells (Figure 10B). The latter indicates that the difference in GIMAP4 expression is necessary only for the early differentiation stage. In the later stages of differentiation and in memory Th-cells GIMAP4 might have another function.
Figure 9. Graphical illustration of GIMAP4 function in Th1-cell differentiation. A) Under normal conditions GIMAP4 assists the intracellular transport of IFNγ carriers and IFNγ secretion. This further leads to the expression of phosphorylated STAT1 dimers and transcription of the downstream targets such IFNγ and IL12Rβ2, which enhance the Th1-cell differentiation. B) Under GIMAP4-depletion, IFNγ secretion is hindered and thus, the self-regulatory loop disrupted leading to low expression of the STAT1 target molecules and changes in Th1-cell differentiation.
Figure 10. GIMAP4 mRNA kinetics under Th1- and Th2-cell differentiation and protein expression in peripheral blood CXCR3+/- cells. A) The GIMAP4 mRNA was measured before activation (0) and at days 1, 2, 4, 5, 7, 8, 9, 12, and 14 after initial anti-CD3/anti-CD28 activation (act.). The cells in the culture were induced to differentiate either by IL4 (Th2) or IL12 (Th1), or differentiation was not induced (Th0). The cells were re-activated (act. 2) with anti-CD3 at day 7. B) The CD4+ cells isolated from human peripheral blood were divided by flow cytometric cell sorting to CXCR3 positive (Th1-cells) and CXCR3 negative (other Th-cell types) pools. The cells were then activated and cultured for 24 h and 48 h before protein measurements. The GIMAP4 expression and GAPDH loading control were detected by western blot in three individual replicates.

5.2.5 The effects of GIMAP4- and GIMAP5-depletion on Th-cell transcriptome during early differentiation (Unpublished)

To better understand the role of GIMAP4 and GIMAP5 in the course of Th-cell activation and differentiation, the effect of their downregulation on the transcriptome was addressed. GIMAP4 and GIMAP5 were downregulated by RNAi in non-activated and in early differentiating CD4+ Th-cells. The differentiation was initiated by anti-CD3/anti-CD28 activation only (Th0), or together with IL12 induction (Th1). The time points studied after the initiation of differentiation were 24 h, 48 h and 72 h. The study was repeated in three biological replicates. The knock-down of GIMAP4, GIMAP5 or no knock-down in the control was verified by RT-PCR and Western blot. The total RNA was isolated and sequenced. The reads per kilobase per million mapped reads (RPKM) values showed successful downregulation of both of the target genes. The RPKM values showed GIMAP4 to be IL12-induced and expressed in higher amounts than GIMAP5. The criteria used to designate the DE genes between the GIMAP-depleted samples and the controls was based on the cut off of |logFC|>1.5 and FDR<0.05. The DE genes were selected for further analyses if the cut off criteria was met in any of the time points measured.
The downregulation of GIMAP5 resulted in greater number of differentially expressed (DE) genes than downregulation of naturally more abundant GIMAP4. After TCR activation in GIMAP4-depleted cells, additional to GIMAP4, three genes were differentially expressed over the time course studied. These genes were IFIT3, TBX21 (TBET) and CXCR3. IFIT3 was also DE at 0 h. After IL12 induction, there were no genes DE across the whole time course studied. The corresponding genes in the GIMAP5-depleted cells were IL8, LPL and IFIT3. IL8 was also DE at 0 h. After Th1 induction, LPL was the only gene DE throughout the whole time course studied. A motif analysis was performed in order to find any enrichment for binding sites of certain transcription factors that could be responsible for regulating the DE genes observed. The areas chosen were 1000 nt upstream to 100 nt downstream and 500 nt upstream and 300 nt downstream from each transcription start site. GIMAP4-affected transcriptional changes seem to be under NFκB regulation before TCR activation, and 24 h after TCR activation they are under Ets1-distal regulation. For GIMAP5 after activation and IL12 induction, the regulation is under interferon-induced transcription factors and FOXA1. Additionally, NFκB is also represented in GIMAP5-depleted data.

It has been proposed that GIMAP genes form functional heterodimers and act together in addition to acting as homodimers (Schwefel et al. 2013). Upon removal of those DE genes, which were shared between the GIMAP4 and GIMAP5 data sets, unique effects on transcription could be observed. The gene sets were primarily analyzed by using Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com, IPA) tool. The overall scenario of the most significantly associated gene network was formed by averaging the values over all the time points studied. The biological functions mostly affected by GIMAP4-depletion under IL12 induction were generally involved in Th-cell-to-cell interaction, cell migration and cell survival. The most significant IPA networks described the following functions: cellular movement, hematological system development and function, immune cell trafficking and cell-to-cell signaling and interaction. Unsurprisingly, the top upstream regulator was IFNγ. The GIMAP5 unique DE genes were also analyzed as described above. For the GIMAP5 analyses the Th0 condition was chosen, as GIMAP5 is not under as strong IL12-regulation as GIMAP4. In general, the analysis revealed that the top biological functions affected by GIMAP5 downregulation were involved with immunological disorders, cell migration and, not surprisingly, calcium signaling. The top networks were antimicrobial response, inflammatory response, dermatological diseases and conditions. The top upstream regulators were type I interferons. In comparison to GIMAP5, GIMAP4 appears to more involved in cell-to-cell communication, whereas GIMAP5 has a more global impact in cellular function.

To assess the effects of GIMAP downregulation more specifically on Th1-cell differentiation, the DE genes with differential expression between the activated (Th0) and Th1-induced conditions during the course of normal differentiation were selected. This was done by comparing the Th0 and Th1 DE genes in the non-targeting siScramble treated sample set. The DE genes that were unique for Th1 condition were selected. A similar comparison was then made for the GIMAP4 or GIMAP5 targeted sample sets. Finally, the shared genes between
the Th1-normal condition and the Th1-target conditions (GIMSAP4 or GIMAP5) were removed from the target lists. This resulted in a list of genes that were differentially expressed during early Th1-cell differentiation due to GIMAP4 or GIMAP5 downregulation. The values for these unique DE genes in the sets were again averaged over the time course studied, and analyzed using the IPA tool.

The IPA biological functions that were enriched for GIMAP4 downregulation and included the largest number of molecules were concentrated around cellular movement, cell-to-cell interaction and inflammatory response. Interestingly, connective tissue, skeletal and muscular disorders were also highly ranked. For GIMAP5, the corresponding pathways were solely involved in Th-cell death and survival. These findings are in line with the earlier findings and implications of GIMAP4 and GIMAP5 function. However, these differences show that although some functional compensation is expected within this gene family, their regulation results in specific changes in the Th-cell transcriptome. In addition, a comparison analysis was performed with IPA. The comparison was made between the original DE genes from the normal control and the GIMAP4 and GIMAP5 targeting gene lists. The resulting canonical pathways that were most different between the GIMAP4 and GIMAP5 downregulated conditions were chemokine signaling and UVC-induced MAPK signaling. Finally, the clearest difference was in NFκB signaling, in which the effect of GIMAP4-depletion was inactivating and GIMAP5-depletion was activating.

5.2.6 GIMAP4 and VMA21 revisited (Unpublished)

The idea of the potential connection and functional interplay between GIMAP4 and VMA21 came from the transcriptomic study described in the original publication III. It has been shown that GIMAP4 interacts with APP (Olah et al. 2011) and has a role in IFNγ secretion (III). VMA21 has been shown to interact with IKBKE, which is an inhibitor of NFκB inhibitor IκBα (Ewing et al. 2007). Additionally, it was found in a preliminary experiment that IκBα was downregulated upon GIMAP4 downregulation, an hour after TCR activation (Figure 11A). The IPA tool was used to construct a network around GIMAP4, APP, VMA21, IKBKE, NFκB and IFNγ. The tool uses published information about molecular interactions and other associations. The network was built by using the list of genes found to be differentially expressed under GIMAP4-depletion, induced by siGimap4, in comparison to the normal situation induced by the non-targeting control (Figure 11B). The intensity of the node color indicates the average logFC value of the given gene over the four time points studied. The analysis resulted in a network in which all the components are connected to the observed effect of GIMAP4-depletion on the Th1-cell transcriptome. Although these results are preliminary, they strongly indicate that NFκB signaling should be studied in the future in order to find out the mechanism behind GIMAP4 and VMA21 interaction. Additionally, the results strengthen the findings concerning the effect of GIMAP4 on Th1-cell differentiation, through IFNγ, and on IL12Rβ2 expression.
Results and Discussion

Figure 11. GIMAP4 and VMA21 in IFNγ and NFκB pathways. A) A single experiment showing IκBα downregulation upon GIMAP4 downregulation after 1 h cell culture under TCR activation and IL12 induced Th1 differentiation. B) An IPA network constructed around GIMAP4, APP, VMA21, IKBKE, NFκB and IFNγ. The nodes display the average expression (red=upregulation, green=downregulation) of differentially expressed genes during early Th1-cell differentiation, resulting from GIMAP4-depletion in comparison to normal control condition (color intensity represents the average logFC value). The connections in the network are based on known information of the given genes and proteins. The large arrows are added later to indicate the findings within this PhD work. The dashed arrows indicate indirect interaction and the intact arrows indicate direct interaction. Connecting lines indicate known binding partners.
6 LIMITATIONS OF THE STUDIES AND DISCUSSION OF THE IMPROVEMENTS

The CTLA4 and ICOS case-control association study (I) conducted in Finnish and Estonian diabetic subjects corroborated the earlier results on autoimmune diseases published by Ueda et al. (2003). However, the population size in the study was rather small and thus the results are not fully conclusive. Additionally, an open question about the effects of mRNA stability and transcriptional control in the CTLA4-ICOS gene region remained to be addressed. It was speculated that CTLA4 could be a causative gene regarding the observed differences of disease rate between the two populations. This aspect should be further studied and validated in different populations. Somewhat similar considerations should be taken into consideration regarding the study of GIMAP4 and GIMAP5 association with T1D, asthma and allergic sensitization (II). The results for each of the disease condition were obtained from a single population although two populations were studied. The T1D results were from a Finnish population, and the asthma and allergic sensitization from a Swedish population. To make a conclusive assessment of the association of GIMAPs with T1D, asthma and allergic sensitization, these studies should be repeated in a separate cohorts/sample sets and in a different population. Although the T1D study was repeated in a Finnish population with a different sample set of Finnish diabetes families, the results were finally combined and thus equivalent to a single study. Further functional experiments were carried out in the study to strengthen the association results. However, the role of FOXO1 in GIMAP5 regulation, and the role of GIMAP4 in IL2 signaling remained open and are in need of further attention. Additionally, functional studies utilizing patient samples are needed for conclusion.

In the second (II) and third study (III), an electric transfer-mediated RNAi method was used. The efficiency of GIMAP4 and GIMAP5 downregulation varied between the individual experiments and was at its best 60%. In order to optimize the best possible knock-down and to gain better downstream effect, alternative methods for gene silencing should be considered in future experiments. These methods could include lentivirus-mediated RNAi gene silencing or the CRISP/Cas system, both of which have been shown to produce highly efficient downregulation. Possible disadvantages with these alternative methods may include immune reaction towards the virus, off-target effects and the need of thorough and time consuming optimization for the human primary Th-cell system. Another aspect to be considered regarding experimental settings in human primary Th-cells, is the genetic variation of the samples. The methods utilized and, the time and effort in obtaining reliable results was multiplied by the variance in the results. In the future, the use of T-cell lines for optimizing the study settings, and for obtaining the primary results should be considered.

The use of Hela-cells in imaging was criticized by the reviewers of the original publication III. The goal was to show the localization of GIMAP4 within the hypothesized functional cell compartments. Jurkat-cells could have been used, but the HeLa-cells were chosen for their good imaging properties. The results were corroborated thereafter with primary Th-cells. The
effect of GIMAP4 on the Th1-cell specific cytokine secretion was intriguing. However, the effect was also seen with Th2-cell specific cytokines, even though GIMAP4 is downregulated under IL4-mediated Th2-cell induction. To understand the role of GIMAP4 in Th-cell differentiation, aspects about its function in other Th-cell types remain to be addressed. These include the role of GIMAP4 in non-activated naive cells and how GIMAP4 is regulated. Also, there is a need for clarification of the cellular function of GIMAP in general. In the transcriptomic study, we found that GIMAP4 is likely involved in NFκB signaling cascades, but which factors regulate GIMAPs remains to be discovered. As GIMAPs are small GTPases, and not nuclear transcription factors, the results of the transcriptomic study reflect an effect in pathways resulting in change in transcription, not a direct effect on gene regulation. Also, the IPA tool used for the construction of the regulatory pathways relies on published knowledge and as such cannot be considered as fully confirming. To further study the mechanistic networks in which GIMAP4 and GIMAP5 might function, protein interactomic approaches and more advanced imaging methods could be utilized in the future.

The main focus of this thesis was to study the events involving GIMAPs during early Th-cell differentiation. The expression kinetics in the late phase of differentiation were not in the scope, although this aspect was briefly considered. More detailed studies concerning GIMAP function in memory cells would be of interest. Studies of all the Th-cell subsets should be conducted, and based on the results of the transcriptomic study and the association studies in immune-mediated diseases, the role of GIMAPs in Th17-cells would be an especially interesting topic. The role of GIMAPs in human Th-cell apoptosis and the co-function of GIMAP4 and GIMAP5 still remains of interest, and should be added to the future agenda.
7 CONCLUSIONS

The first study, which assessed the association of CTLA4 and ICOS with T1D in Finnish and Estonian populations, was rather confined in comparison to the second and third studies. However, on the basis of this study we can conclude that the earlier results of CTLA4 association with autoimmune disorders are reproducible. ICOS was only found to associate with T1D through analysis based on the combination of the samples, indicating that there is still a need for further studies regarding the role of ICOS in T1D. Novel information was gained in relation to the CTLA4 CTBC217_1 allele in T1D, whereby it was specifically shown to be more frequent in cases than controls. The difference in the T1D disease rate between the two populations was addressed in this work, and it was found that the CTLA4 risk alleles and haplotype were more common in the Finnish control subjects compared with the Estonians controls. Together, this indicates that genetic variance in the CTLA4 locus could account for the higher disease incidence observed in Finland. The question left to be addressed in the future was the functionality of the T1D associating loci. The genomic localization of ICOS CTIC154_1 indicates that this genotype could have an effect on mRNA stability. On the other hand, the genomic localization of CTLA4 CTBC217_1 could have a dual effect on either CTLA4 mRNA stability or ICOS promoter function.

GIMAP family genes and the proteins they code for are a relatively new group of small GTPases that have been of interest in immunology during the past two decades. The knowledge about GIMAP family members 4 and 5 to date is mostly based on mouse and rat studies on their role in T-cell survival, apoptosis and autoimmunity. During recent years, reports of the role of GIMAPs in intracellular transport processes, as well as their function in other cellular systems than the immune system have been published. The study presented in this thesis was focused on elucidating GIMAP5 and especially GIMAP4 function in human Th-cell differentiation, with the emphasis on Th1-cells. The aim in connecting functional and genetic approaches was to find holistic insights for the role of GIMAP4 and GIMAP5 in immune system and in immune regulation. RNA interference was exploited to investigate how these small GTPases influence T-cell activation and/or Th1-cell differentiation. The intracellular localization of GIMAP4 was first studied in order to gain insight of its function and this was followed by the finding of GIMAP4 role in cytokine secretion. In addition, samples from type 1 diabetes trio families and prospective asthma and allergic sensitization cohorts were used for genetic association studies and gene-gene interaction studies of GIMAP4 and GIMAP5. These studies were complemented by a transcriptomic study aiming in finding trace of the cellular pathways in which these GIMAPs play their part.

For GIMAP5, the most interesting finding was the association with T1D and in asthma and allergic sensitization with dual but opposing effects. In both cases the biological and functional mechanisms could be based on the possible FOXO1 regulation of GIMAP5, as FOXO1 is tightly connected with insulin signaling and also with Th2-cell function. It has to be noted, that no major conclusions can be made based on one population. It is possible that GIMAP5 has a minor but noteworthy effect on T1D pathology and role of GIMAP5 in
human T1D should be studied further in the future. Interestingly, the transcriptomic study revealed that GIMAP5-depletion has a greater effect on the transcriptome than GIMAP4, although the level of GIMAP5 expression is lower. It was also evident that the role of GIMAP5 during Th1-cell differentiation is more closely connected with the T-cell apoptosis, cell death and survival mechanisms than that of GIMAP4. This aspect is also noteworthy when considering GIMAP5 role in immune-mediated diseases. To what extent these two proteins compensate each other, or what is the possible role of GIMAP4-GIMAP5 heterodimer, remains to be discovered.

GIMAP4 was localized within cell compartment and cytoskeletal components, which have a known function in intracellular transport. The localization results were supported by the functional finding of GIMAP4 having a role in cytokine secretion. This was further linked with Th1-cell specific differentiation, as the most notable effect was on IFNγ signaling and IFNγ downstream pathways. A novel finding was that GIMAP4 regulated mRNA and protein expression of VMA21. As VMA21 localizes in the ER, in a similar cell compartment as GIMAP4, additional support was provided for the role of GIMAP4 in the processes and/or in the structures important for intracellular traffic. The results from association study indicated the involvement of GIMAP4 in asthma and allergic sensitization. Interestingly, although lacking association with T1D, the gene-gene interaction analyses revealed a connection between GIMAP4 and IL2 signaling. This is also relevant in the context of the association of GIMAP4 with asthma and allergic sensitization as IL2 has been shown to have a role in asthmatic inflammatory response. Finally, the transcriptomic study indicated that under IL12/STAT4-driven Th1 induction, the effects of GIMAP4-depletion on Th-cell transcriptome are minor. A lack of strong IL12 signal, however, strengthens the negative effect of GIMAP4 downregulation on Th-cell specific pathways during the first culture days of activated Th-cells. In addition, the observation that NFκB was one of the enriched transcription regulators under GIMAP4-depletion, suggests that GIMAP4 may be relevant for IL18 signaling as well. All the observed effects of GIMAP4-depletion, under differentiation, point towards Th1-cell specificity and that GIMAP4 is needed for Th1-cell related processes.

In conclusion, these studies have demonstrated an interesting snapshot of the involvement of GIMAP proteins in the human immune system and provide a basis for future investigations.

“DNA neither cares nor knows. DNA just is.
And we dance to its music.”

- Richard Dawkins
ACKNOWLEDGEMENTS

This PhD thesis was carried out at the Turku Centre for Biotechnology, University of Turku and Åbo Akademi University. The thesis was supervised by Adjunct Professor, PhD Tiina Henttinen and Professor, MD, PhD Riitta Lahesmaa. These two strong women guided my way through this scientific endeavour and by their individual views of science, they helped me to become the scientist I am. Tiina has been a remarkable teacher, mentor and a friend through these years and I cannot thank her enough. Tiina has taught me to be self confident with my results and to believe what I see, even others may not. Riitta has provided a vibrant, scientific work environment and all the necessary nick nacks for my accomplishments. Riitta has also taught me to appreciate differences in every aspect of science and life in general.

I warmly acknowledge the former Turku Doctoral Programme of Biomedical Sciences (TuBS), currently Turku Doctoral Programme of Molecular Medicine (TuDMM) for the possibilities in education, financial support for attendance in scientific conferences, social networking and extra scientific activities with other PhD students. I want to express my gratitude to my supervisory board members PhD Kati Elima and Professor Jorma Ilonen. I warmly thank Professors Harri Alenius and Olli Vainio for an excellent review of my thesis, good feedback and constructive criticism. PhD Robert Moulder reviewed the language of my thesis with uttermost precision and is highly acknowledged for that.

All the co-authors and collaborators, PhD Antti-Pekka Laine, MD, PhD, Robert Hermann, Msc. Kati Lipponen, MD, Riitta Veijola, Prof. Olli Simell, Prof. Mikael Knip, Prof. Harri Lähdesmäki, Msc. Kartiek Kanduri, Msc Sini Rautio in Finland, MD, PhD, Konstantinos Douroudis, Prof. Raivo Uibo, Prof. Kalle Kisand in Estonia and PhD Cilla Söderhäll, PhD Olena Gruzieva, Prof. Erik Melén, Prof. Göran Pershagen and Prof. Juha Kere in Sweden, have been crucial assets for me and for this accomplishment. I warmly thank you all. One, however, deserves extra special credit. I cannot thank enough Antti-Pekka Laine, who truly, but uncredited, supervised my masters thesis by guiding, sharing a room, discussing and simply tolerating my temperament. AP has been a friend, a colleague, an important individual behind my scientific accomplishments during my PhD journey.

The staff at the Turku Centre for Biotechnology and the Finnish Centre for Microarrays and Sequencing deserve a special thank you for their support in various aspects in science, IT and official dilemmas. I owe a huge gratitude to ATLAS group marvelous lab technicians Marjo Hakkarainen, Sarita Heinonen and Elina Pietilä for all the help and support in the lab.

All the rest ATLAS group members, past and present, who have shared their knowledge, ideas of life and science, I salute you: Kanchan Bala, Santosh Bhosale, Zhi Chen, Sanna Edelman, Bogata Fezazi, Sumedha Gattani Goel, Bhawna Gubta, Walteri Hosia, Saara Hämäläistö, Terhi Jokilehto, Päivi Junni, Kartiek Kanduri “the roommate”, Moin Khan, Lingjia Kong, Juha Korhonen, Anne Lahdenperä, Essi Laajala, Sari Lehtimäki, Riikka Lund,
Acknowledgements

The foundation of my well-being during the years of my PhD training has been created and supported by my friends and family, thus I owe the utter most gratitude for them. Äiti ja isä, te olette antaneet minulle kaiken mitä ikinä olen tarvinnut edetää menestyksekästä elämässäni. Te annitte minulle tiedonjanon, päämäärittäjäsi, henkisen ja taloudellisen tuen. Te olette antaneet minulle kaiken mitä ikinä olen tarvinnut edetää menestyksekästä elämässäni. Te annitte minulle tiedonjanon, päämäärittäjäsi, henkisen ja taloudellisen tuen. Minulla on aina ollut koti, jonka olisi ollut nauramaan, itkemään, lohduttautumaan ja lepäämään. Te opettelette, että maailma on jännä paikka, muurahaisi sinä maalata pilkkuja, miten hienoa on ratkaista arvoituksia ja ennen kaikkea, mikä elämässä on oikeasti tärkeää. Mikael, my other half, calm and collected. You have always supported my ambition and done your best in making my upsets disappear. You took care of our home and made sure that Axu is a happy puppy, when I had no time. You have given me strength and self confidence when my own mind was fighting back. You are the bedrock of my being, ever constant, ever present, ever loving, no matter what <3.

This work was supported by the Academy of Finland, the Sigrid Juselius foundation, the Maud Kuistila foundation, the Waldemar von Frenkells foundation, the Ida Montinis foundation, the Turku University Foundation, the Oskar Öflunds foundation and the former Turku Doctoral Programme of Biomedical Sciences (TuBS), current Turku Doctoral Programme of Molecular Medicine (TuDMM).

Turku, September 2015

Mirkka Heinonen
REFERENCES


References


Braman SS. 2006. The global burden of asthma. Chest 130(1 Suppl):4S-12S.


Josefowicz SZ, Lu LF, Rudensky AY. 2012. Regulatory T-cells: Mechanisms of


Nogalska A, D'Agostino C, Terracciano C, Engel WK, Askanas V. 2010. Impaired autophagy in sporadic inclusion-body myositis and in endoplasmic reticulum stress-provoked...


Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, et al. 2007. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81(3):559-75.


References


Zhang L and Nabel GJ. 1994. Positive and negative regulation of IL2 gene expression: Role of


