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A Mendelian Disease Of Autoimmunity Reveals Gimap5 As A Novel Member Of The Ragulator Complex

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Abstract

The incidence of autoimmune diseases, many of which lack effective treatments, is rapidly increasing in the developed world. Mendelian diseases allow the study of autoimmunity in humans, enabling new insights into the underlying pathology. In this study, I have identified a patient cohort suffering from a novel recessive Mendelian disease of immune dysregulation characterized by severe lymphopenia, splenomegaly, thrombocytopenia and liver failure. Whole exome sequencing revealed mutations in GIMAP5, a small GTPase primarily expressed in T, NK and endothelial cells. The missense mutations in these patients destabilize the protein in vitro and lead to a near complete loss of protein in patient cells. Animal models lacking GIMAP5 develop a disease remarkably similar to that observed in the human patients; however, the molecular role of this gene in the immune system remains obscure. To address this, I defined the interactome of GIMAP5 via immunoprecipitation and high-throughput mass spectrometry. This revealed a robust interaction with all seven members of the Ragulator complex which I went on to confirm via endogenous co-immunoprecipitation and proximity ligation assays. This complex has recently been described as a key regulator of mTORC1, Erk signaling and lysosome positioning. In order to study the functional relevance of this interaction I utilized an in vitro CRISPR mediated approach to knockout Gimap5 in murine primary T cells. I observed a very rapid and robust induction of apoptosis accompanied by significant increases in ceramide levels following the loss of GIMAP5 which is consistent with the lymphopenic phenotype. Future studies will relate the increased ceramide to the Ragulator complex and induction of apoptosis in GIMAP5-deficient T cells and leverage these findings to develop novel treatments for GIMAP5 deficiency and other autoimmune diseases.

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ABSTRACT

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The incidence of autoimmune diseases, many of which lack effective treatments, is rapidly increasing in the developed world. Mendelian diseases allow the study of autoimmunity in humans, enabling new insights into the underlying pathology. In this study, I have identified a patient cohort suffering from a novel recessive Mendelian disease of immune dysregulation characterized by severe lymphopenia, splenomegaly, thrombocytopenia and liver failure. Whole exome sequencing revealed mutations in GIMAP5, a small GTPase primarily expressed in T, NK and endothelial cells. The missense mutations in these patients destabilize the protein *in vitro* and lead to a near complete loss of protein in patient cells. Animal models lacking GIMAP5 develop a disease remarkably similar to that observed in the human patients; however, the molecular role of this gene in the immune system remains obscure. To address this, I defined the interactome of GIMAP5 via immunoprecipitation and high-throughput mass spectrometry. This revealed a robust interaction with all seven members of the Ragulator complex which I went on to confirm via endogenous co-immunoprecipitation and proximity ligation assays. This complex has recently been described as a key regulator of mTORC1, Erk signaling and lysosome positioning. In order to study the functional relevance of this interaction I utilized an *in vitro* CRISPR mediated approach to knockout

Gimap5 in murine primary T cells. I observed a very rapid and robust induction of apoptosis accompanied by significant increases in ceramide levels following the loss of GIMAP5 which is consistent with the lymphopenic phenotype. Future studies will relate the increased ceramide to the Ragulator complex and induction of apoptosis in GIMAP5-deficient T cells and leverage these findings to develop novel treatments for GIMAP5 deficiency and other autoimmune diseases.

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CHAPTER I - Introduction

I. Summary

Lymphocytes are critical effectors of the immune system whose dysregulation can lead to immunodeficiency and/or autoimmunity. Proper regulation of lymphocyte number in addition to function is key for an appropriate immune response. Thus, the rates of generation of new lymphocytes, proliferation of existing ones as well as death of older ones tightly controlled. The generation of new lymphocytes generally occurs at a steady rate, and in the case of T cells, steadily decays with age. On the other hand, proliferation and death are regulated by a complex system of competition for soluble cytokines, exposure self-antigens and pro-apoptotic surface receptors. Genetic lesions affecting any of these processes lead to immunodeficiency and/or immune dysregulation due to abnormalities of lymphocyte numbers. Many autoimmune diseases have high heritability, indicating a strong genetic component. Harnessing genetics to study autoimmunity and lymphocyte regulation has the potential to both improve our understanding of the etiology of autoimmune disease and also develop novel, more effective therapeutics targeting previously poorly understood pathways.

II. Human Genetics and Whole Genome Sequencing

A) The history of DNA sequencing techniques and human genetic variation

DNA sequencing became possible in the 1970s due in part to pioneering work by Frederick Sanger (Sanger, Nicklen and Coulson, 1977). His method exploited the sequential addition of chain-terminating dideoxynucleotides to a DNA synthesis reaction to generate DNA fragments with single nucleotide resolution, allowing the derivation of

the DNA sequence. While labor-intensive and expensive, 'Sanger Sequencing' remained the principal method used to sequence DNA for four decades and was used to sequence the human genome. The initial draft of the sequence of the human genome was published in 2001 with roughly 90% coverage and at cost of approximately 3 billion USD (Petersen *et al.*, 2017). This sequence is actually a haploid mosaic of several different human donors and thus does not always represent the most common alleles and remains incomplete. However, attempts are made to represent relatively common alleles that are present in multiple different populations. While automation and increased use of computers drastically drove down the cost of Sanger sequencing, a major paradigm shift occurred in the mid 2000s as massively parallel 'Next-generation' sequencing was developed. This method hybridizes many fragments of the DNA sequence of interest to a glass slide, followed by a DNA polymerization reaction that emits light when a specific dideoxy-base is incorporated. A camera can then interpret the sequence of DNA from the sequences of light emission following each cycle of base addition on the glass slide and thus generate many short sequences, or "reads" of DNA from the fragments (Alekseyev *et al.*, 2018). These techniques have slashed both the turnaround time and cost of sequencing a human exome or genome by several orders of magnitude. As of this writing sequencing a human exome/genome costs in the range of hundreds of dollars per sample when done for research purposes (Petersen *et al.*, 2017). These developments have combined to usher in a golden age in human genetics and a surge in research studying the human genome.

Following the completion of the human genome project, the focus has shifted to translating these developments in basic science into clinical benefits. The contribution of genetic variation to the development of human disease is a spectrum. One end is composed of Mendelian, or monogenic diseases which are caused by rare mutations which are highly deleterious with high penetrance. The opposite end of the spectrum is composed of polygenic, or complex diseases, which are typically conceptualized as being caused by a combination of many low impact genetic variants in combination with environmental influences (Kaiser, 2012). Broadly speaking, human genetic variation can be broken down into Single Nucleotide Variants (SNVs), small Insertions or Deletions (indels) or larger-scale structural variation. The latter exists on a large spectrum, all the way up to aneuploidies involving duplications or losses of entire chromosomes. They also include inversions of large sequences as well as duplication/deletion of large stretches of DNA termed Copy Number Variations (CNVs). These structural variations are best interrogated by array-based Comparative Genome Hybridization (array-CGH) (Feuk *et al.*, 2006). Meanwhile, SNVs and small indels are best interrogated by WES/WGS.

B) GWAS and the heritability of human disease

Twin studies have long given an estimate of the heritability of a variety of diseases (Tenesa and Haley, 2013). Human disease runs the spectrum from low to high heritability, with presumably the remaining incidence occurring due to environmental influences. Many diseases of the immune system such as Systemic Lupus Erythematosus (SLE) and Type one diabetes (T1D) exhibit very high heritability – both greater than 60%

(Guerra, Vyse and Cunninghame Graham, 2012; Leslie, 2018). Initial hypotheses argued that alleles with large damaging effect sizes would be strongly selected against and thus extremely rare in the population and not contributing significantly to the total heritability of these diseases. Thus, research into the genetic contributions to human disease focused largely on the mapping of relatively common Single Nucleotide Variants (SNVs) with small effect sizes. This was called the Common Disease-Common Variant (CD-CV) model and the underlying hypothesis was that cumulatively, the combination of many such alleles would predispose individuals to disease when exposed to the appropriate environmental conditions (Reich and Lander, 2001). The hope was that identification of these alleles would lead to novel molecular pathways involved in the development and pathogenesis of these diseases that could then be targeted therapeutically. This led to the generation of the Hapmap project and a proliferation of Genome Wide Association Studies (GWAS)(Gibbs *et al.*, 2003).

GWAS rely on genetic recombination during meiosis. This process allows variants on one chromosome to 'cross over' to the other, and thus potentially be inherited independently of other variants present on the parent chromosome. This process typically occurs at hotspots throughout the genome, resulting in stretches of DNA or 'haplotypes' which are frequently inherited together (Evangelou, 2018). Various groups were then able to develop panels of SNVs spread across these haplotypes, genotype hundreds to thousands of individuals either carrying or lacking the phenotype of interest, and associate various haplotypes with given traits.

Some initial GWAS generated very promising results – in particular, a study investigating Macular Degeneration (MD) using a relatively small number of participants yielded polymorphisms accounting for a very large fraction of the heritability (Klein *et al.*, 2005). However, the majority of the studies to follow only revealed relatively small fractions of the heritability for many complex diseases such as (Maher, 2008; Manolio *et al.*, 2009). Boyle *et al.* have proposed an alternative framework which they call the 'omnigenic' model (Boyle, Li and Pritchard, 2017). This pushes the CD-CV model even beyond GWAS and hypothesizes that all aspects of cell biology are so intertwined that complex, polygenic diseases being caused by a small number of genetic variants is naïve. Rather, a large number of low-effect SNVs mostly in the non-coding regions of the genome predispose individuals to common diseases and the effect sizes are so small that even the largest GWAS had insufficient power to detect them.

C) *Monogenic Mendelian diseases and the contribution of rare variants to human disease*

More recently, several groups have proposed that a larger fraction than previously estimated of the heritability of common diseases could in fact be caused by rare, highly deleterious alleles (Lupski *et al.*, 2011; Casanova, 2015). For example, many patients suffering from Autism Spectrum Disorders (ASD) have been found to carry rare, (defined as having a MAF of less than 1%) *de novo*, deleterious genetic variants (Geschwind and State, 2015). The number of *de novo* variants varies as a function of the father's age, however, on average one can expect 1-2 *de novo* coding mutations per individual (Kong *et al.*, 2012). Recent estimates propose that 20-40% of ASD cases may be caused by *de*

novo SNVs or structural rearrangements (Iossifov *et al.*, 2014). The contribution of rare genetic variants to other complex diseases remains unclear, however, recent studies have estimated that each individual carries hundreds of deleterious loss of function SNVs in their genome (Macarthur *et al.*, 2012).

The massive drop in sequencing costs has led to the realistic use of Whole Exome and, more recently, Whole Genome Sequencing (WES/WGS) as a diagnostic tool for Mendelian disease. WES uses a hybridization approach in order to exclusively sequence the coding regions of the genome (1-2% of the total sequence), while WGS also captures the non-coding regions by sequencing the entire genomic DNA. It is estimated that 85% of Mendelian disease-causing mutations are in the coding regions of genes, thus WES offers a significant cost reduction relative to WGS while still capturing most of the relevant information (Rabbani *et al.*, 2012; Seleman *et al.*, 2017). However, the hybridization approach does result in relatively poor sequence quality for some fraction of genes, an estimated 15% of disease-causing variants will be missed and other drawbacks to WES likely means that over time if sequencing costs continue to fall WGS will eventually be favored. One key observation of large scale exome or genome sequencing is that the number of variants is much larger than was previously thought.

The number of Mendelian diseases identified has vastly increased with the advent of WES/WGS analysis: as of 2015, 1000 new genes had been associated with Mendelian traits through WES (Chong *et al.*, 2015). Specifically in the immune system, over 350 inborn errors of immunity have been described and recognized (Cunningham-Rundles *et al.*, 2017). In some cases, these diseases can exhibit extensive incomplete penetrance

whereby related family members may also carry the pathogenic SNV but not exhibit any clinical manifestations (Bleesing, Nagaraj and Zhang, 2004; Avery *et al.*, 2014; Schubert *et al.*, 2014). In addition to increasing our understanding of the genetic mechanisms underlying complex diseases the study of Mendelian diseases has other translational advantages to offer. Firstly, by sequencing large cohorts of patients one can essentially replicate high-throughput forward genetic screening that was previously only possible in model organisms. The vast majority of potential therapeutics fail in the transition from animal models to the human and screening for important therapeutic targets directly in humans can bridge the divide (Mak, Evaniew and Ghert, 2014). Secondly, Mendelian diseases represent a more homogenous cohort of patients for the testing of novel therapeutics. This can greatly facilitate testing the efficacy of new therapeutics before offering them to the broader cohort of patients suffering from autoimmune disease. Lastly, the promise of personalized medicine will be most easily realized initially in monogenic diseases as the impacts of these alleles are the most straightforward. It is clear that variations in both complex disease manifestations and responses to therapies are caused in part by genetic.

Miller syndrome was the first Mendelian disease whose underlying genetic cause was determined by Whole Exome Sequencing (WES) in 2010 (Ng *et al.*, 2010). In the intervening years, WES and more recently Whole Genome Sequencing (WGS) have seen remarkable success in both diagnostic and discovery roles. Early studies reported a variable success rate of 20-50% in providing a diagnosis to patients, with this variation very likely to be a function of the initial patient cohort selected for the study (Dixon-

Salazar *et al.*, 2012; Need *et al.*, 2012; Yang *et al.*, 2013; Zhu *et al.*, 2015). As data accumulates over time the diagnostic success rate of WES/WGS is likely to increase greatly and it will undoubtedly become a standard tool in the physician's repertoire.

III. Lymphocyte development, homeostasis and autoimmunity

A) Autoimmune disease and the contribution of lymphocytes

The concept of autoimmunity stretches back to the idea of 'horror autotoxicus' in which the immune system resists attacking self and this resistance occasionally fails as proposed by Paul Ehrlich. However, it was not until the middle of the 20th century that the concept was broadly accepted (Arthur M. Silverstein, 2001). Autoimmune disease is defined as the loss of self-tolerance whereby an immune response is triggered against self, or host tissues. The incidence of autoimmune diseases such as SLE and T1D has markedly increased in developed countries in the modern era and are now collectively a leading cause of death among young and middle-aged women (Walsh and Rau, 2000; Lerner, Jeremias and Matthias, 2016). Furthermore, therapies for many diseases are ineffective and often cause significant side effects. New approaches to understand and treat autoimmune diseases are urgently needed. Harnessing recently developed genomic technologies to discover novel pathways and drug targets has led to breakthroughs in knowledge of genetic diseases of the immune system (Milner and Holland, 2013; Cunningham-Rundles *et al.*, 2017; Comrie and Lenardo, 2018).

Decades of research in model organisms and more recently in human patients have demonstrated a critical role for the regulation and homeostasis of lymphocytes in

avoiding the development of autoimmune disease (Fisher *et al.*, 1995; Avery *et al.*, 2014; Lo *et al.*, 2015). The immune system is staggeringly complex with a multitude of cell types in constant equilibrium, and disruption frequently leads to both susceptibility to specific pathogens as well as dysregulation and consequently autoimmunity. Lymphocytes are critical effectors of the immune system as evidenced by individuals with severe defects in lymphocyte development, termed Severe Combined Immunodeficiency (SCID), invariably face early mortality in the absence of bone marrow transplants (Linte *et al.*, 2015). Similarly, selective defects in either $CD4^+$, $CD8^+$ or B cell development/function lead to severe disease with well characterized groups of infections (Agammaglobulinemia *et al.*, 2000; Zhang *et al.*, 2009; Matthews *et al.*, 2014; Cirillo *et al.*, 2015). As one of many examples, loss of function mutations in MAGT1 result in a drastic loss of NKG2D surface expression on $CD8^+$ T cells leading to a selective susceptibility to Epstein-Barr Virus (EBV) infection and consequently lymphomas (Li *et al.*, 2011; Chaigne-Delalande *et al.*, 2013; Matthews *et al.*, 2014) Interestingly, many of these defects actually simultaneously result in both autoimmunity and immunodeficiency. For instance, loss of the AIRE gene causes Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED), which is characterized by autoimmune mediated hypoparathyroidism, adrenal insufficiency, susceptibility to Candida infections (Bosticardo, Peterson and Villa, 2016; Ferre *et al.*, 2016).

B) Lymphocyte development and homeostasis

Conventional αβ T cells originate in the thymus and are subject to stringent selective processes based on their T cell receptor specificity. Hematopoietic Stem Cells

(HSCs) first differentiate to Common Lymphoid Progenitors (CLPs) which migrate to the thymus and further differentiate to $CD4^{-}CD8^{-}$ Double Negative (DN) T cells. At this point, the DN T cells rearrange the Trb locus in a Rag1/2 dependent manner and successful signaling through the pre-T Cell Receptor (TCR) leads to a burst of proliferation and progression to the $CD4^{+}CD8^{+}$ Double Positive (DP) stage (Shah and Zuniga-Pflucker, 2014). Cells that were unable to productively rearrange the Trb locus either die by neglect or are shunted towards a different developmental pathway. At this point, the DP T cells rearrange the Tra locus and again test the nascent TCR against self-ligands. Current models posit that cells with excess affinity for self will either die via negative selection or become regulatory T cells, whereas if the T cell is unable to recognize self-MHC at all they die by neglect (Krueger, Ziętara and Łyszkiewicz, 2017). Finally, the mature naïve T cells emigrate from the thymus to the periphery. Defects in any step of the process described above lead to severe immunodeficiency and at times autoimmunity.

One critical aspect of lymphocyte biology that is closely regulated is lymphocyte number. Many studies in both animal and human models have shown that depletion or chronic low numbers of lymphocytes predispose or outright induces autoimmunity with the pancreas and thyroid being particularly susceptible (Allen and Braverman, 1996; Wong and Roth, 2007; Surh and Sprent, 2008; Onoe *et al.*, 2010; Sauer *et al.*, 2012; Jones *et al.*, 2013). For example, depletion of lymphocytes via the monoclonal antibody alemtuzumab leads to the development of autoimmunity in one in three patients (Jones *et al.*, 2013). In the case of T lymphocytes, thymic output greatly decreases with age and

thus homeostatic proliferation in the periphery is required to maintain a complete cohort of T cells (Hale *et al.*, 2006). Defects in both thymopoiesis and peripheral survival have been shown to cause lymphopenia associated with autoimmunity. This process is critically dependent on self-peptide/MHC complexes weakly interacting with the TCR as well as signaling by γ-chain cytokines (Surh and Sprent, 2008). Similar to B lymphocytes, conditional knockout of the TCR or proximal TCR signaling molecules in mature T lymphocytes leads to a drastic decrease in T cell half-life (Labrecque *et al.*, 2001; Polic *et al.*, 2001; Seddon and Zamoyska, 2002). Furthermore, this TCR/self-MHC interaction appears to be antigen specific as transferring transgenic T cells with different specificities won't compete with each other, while transferring large numbers of a single transgenic T cell clone reduces their lifespan (Hataye *et al.*, 2006). This data supports a model with constant competition amongst T cells in the periphery for weakly-interacting self-antigens. It has been proposed that this is an additional mechanism to ensure a diverse polyclonal repertoire in the periphery even after thymic selection has occurred.

Soluble cytokines are the other major mechanism by which the organism regulates the size of the peripheral lymphocyte repertoire. For T lymphocytes the common γ-chain family of cytokines are recognized as particularly critical. This is supported by the fact that mutations in the common γ chain receptor, the receptor subunit specific for IL-7 or downstream signaling molecules lead to SCID (Lin and Leonard, 2018). IL-2, 7, 15 and others all share a the common γ chain, IL-2 and IL-15 share a common β chain coupled with a unique receptor chain specific for each cytokine (Waickman, Park and Park, 2016). Signaling by IL-7 is required for naïve T cell survival

and development, IL-15 is thought to be required primarily by memory T cells and IL-2 is thought to support the expansion of activated T cells as well as regulatory T cells (Surh and Sprent, 2008). Indeed, blocking IL-7 signaling reduces survival of naïve T cells whereas overexpression of IL-7 leads to an increase in the size of the naïve T cell pool and autoimmunity (Tan *et al.*, 2001; Williams *et al.*, 2016) However, much of the data regarding IL-7 activity and function is indirect as measurement of endogenous levels of IL-7 *in vivo* is difficult. It is thought that endogenous IL-7 is captured by the extracellular matrix of Secondary Lymphoid Organs (SLO), increasing the local concentration as has been described for other cytokines (Wrenshall and Platt, 1999). Indeed, naïve T cells from mice lacking SLOs have drastically decreased half-lives, although this result is complicated by the fact that these T cells may also have decreased interaction with endogenous TCR ligands (Ruddle and Akirav, 2009). Furthermore, the genetic program of naïve T cells ensures that homing receptors for SLOs are constantly expressed. This simultaneously ensures their exposure to IL-7/self-antigens and allows them to monitor local Antigen Presenting Cells (APCs) for their cognate antigens.

Signaling through the common γ-chain family of cytokines occurs via the JAK/Stat pathway. In particular, IL-7 signaling has been shown to regulate the intrinsic apoptosis pathway because Bcl-2 overexpression can reverse many of the defects in IL-7 deficient mice (Akashi *et al.*, 1997).

C) Lymphocyte survival, apoptosis and necroptosis

Multiple other pathways are critical for controlling the survival/apoptosis of lymphocytes both at rest and during the course of an immune response. Classically,

apoptosis has been broken down into the intrinsic, extrinsic and perforin/granzyme pathways (Elmore, 2007). Multiple initiating stimuli such as genotoxic or mitochondrial stress and death receptor signaling can stimulate the intrinsic and extrinsic apoptosis pathways respectively. The intrinsic pathway is controlled by the balance of pro and anti-apoptotic factors on the surface of the mitochondria (Galluzzi *et al.*, 2018).

More recently, necrosis and the related programmed form of cell death termed necroptosis have been described. These two forms of cell death are much more inflammatory than apoptosis. Necroptosis is known to be dependent on the kinases RIPK1/3 downstream of signaling through TNF family receptors (Grootjans, Vanden Berghe and Vandenabeele, 2017). The kinase activity of both enzymes is thought to be important, as well as the dimerization of RIPK1 which is both necessary and sufficient to initiate the process (Christofferson and Yuan, 2010). Necroptosis is entirely independent of the caspases which are critical for the process of apoptosis. MLKL is a pseudokinase downstream of RIPK1/3 and is thought to be the sole and critical effector of necroptosis via the formation of pores in the membrane (Grootjans, Vanden Berghe and Vandenabeele, 2017). More recently, CAMK2D has also been described as a substrate for RIPK3 which can induce a pronounced influx of ions leading to cellular swelling and death (Zhang *et al.*, 2016). Apoptosis and to a lesser extent necroptosis have been well documented as being critical for the regulation of the lymphocyte compartment (Fisher *et al.*, 1995; Ch'en *et al.*, 2011).

Sphingolipid metabolism is a metabolic pathway that has long been linked to the induction of cell death by various ceramide species. Ceramides are sphingolipids

consisting of a sphingoid base conjugated to an acyl chain of varying lengths via an amide bond. Ceramides are both precursors for more complex lipid species such as glycerosphingolipids/sphingomyelins as well as potent signaling molecules in their own right. Ceramide levels are maintained via both *de novo* synthesis and degradation of glycerosphingolipids and sphingomyelins. *De novo* synthesis of ceramides occurs through a series of enzymatic reactions primarily in the ER, with further modification to more complex lipid species occurring in the ER and Golgi. The final step of ceramide synthesis is catalyzed by one of six Ceramide Synthase (CerS) enzymes (CerS1-6). Each enzyme has a specificity for producing ceramides with different acyl chain lengths as well as different tissue distributions. The length of the acyl chain has been shown to have profound effects on the biological function. Depending on the cell line being studied and the stimulus used to induce apoptosis, different acyl chain lengths can be either protective or pro-apoptotic in different contexts (Grösch, Schiffmann and Geisslinger, 2012). Overall, the underlying biology is complex and remains incompletely understood. Conversely, ceramide production via recycling of more complex glycerosphingolipids occurs in the lysosome. Acid Sphingomyelinase (aSMase) cleaves sphingomyelin into ceramide and choline. Humans carrying mutations in various enzymes involved in the catabolism of ceramide and sphingomyelins suffer from what are termed Lipid Storage Disorders (LSDs) which affect multiple organ systems and are frequently characterized by large cellular lipid deposits visible via conventional light microscopy.

Multiple lines of evidence have pointed towards ceramide levels influencing cell survival. Firstly, induction of cell death via extracellular receptors such as TNFR and Fas

or DNA damage can lead to increased ceramide levels (Taha, Mullen and Obeid, 2006). Specifically, the CerS and SMase enzymes appear to be key regulatory points within the pathway that can be influenced by diverse upstream mechanisms such as p53/ATM, caspases, Bcl-2 family members, pro-apoptotic surface receptors and various signaling kinases. Both the activity and expression of these enzymes have been observed to increase during certain forms of cell death (Mullen and Obeid, 2012; Kuzmenko and Klimentyeva, 2016). Secondly, addition of exogenous ceramides to cells can induce apoptosis in multiple *in vitro* models. Lastly and most convincingly, inhibition of the CerS enzymes via Fumonisin B1 (FB1) can also block cell death induced by genotoxic agents such as daunorubicin (Bose *et al.*, 1995).

D) Lymphopenia and lymphopenia induced proliferation

In a lymphopenic host the remaining T cell pool will undergo Lymphopenia Induced Proliferation (LIP) in order to attempt to replenish the full complement of peripheral T cells. LIP was first recognized following experiments showing that the transfer of splenocytes from a healthy animal into an athymic nude rat eventually generated a full complement of T cells (Bell *et al.*, 1987). Multiple models of lymphopenia have different effects on the remaining T cells and likely reflect a complex system of replenishment with many interacting variables. LIP is thought to occur by two different mechanisms termed fast and slow LIP. Fast LIP is thought to be driven by T cell clones specific for antigens from the gut microbiota. It occurs even in the absence of IL-7 but is blocked when cells are transferred to irradiated germ-free mice (Kieper *et al.*, 2005; Min *et al.*, 2005). As expected from the nomenclature, the proliferation driven by fast LIP

is orders of magnitude higher than slow LIP. Slow LIP is driven by the increased availability of self-peptide/MHC and IL-7 in lymphopenic hosts and can be blocked by preventing IL-7 signaling (Min *et al.*, 2005). Microarray analysis of T cells undergoing LIP in a lymphopenic host upregulate a similar set of genes (at a smaller magnitude) to T cells responding to cognate antigen, consistent with a persistent low-level tonic signal as opposed to a brief high-affinity binding (Goldrath *et al.*, 2004). The relevance of these two mechanisms is likely dependent on the context. For example, acute lymphopenia induced by chemotherapeutic agents or irradiation in the context of cancer treatment could much more resemble the RAG transfer animal model (and thus fast LIP), although this is complicated by the age and thus the rate of thymic output in the patient. On the other hand, chronic lymphopenia caused by genetic conditions or infections could more closely resemble models of Omenn syndrome, and thus slow LIP (Khiong *et al.*, 2007; Marrella *et al.*, 2007). While LIP does induce T cells with a phenotype associated with memory cells, these cells have not mounted a true response to cognate antigen (Murali-Krishna and Ahmed, 2000).

IV. GTPase Immune Associated Protein (GIMAP) Family

A) Introduction

The "GTPase of immunity-associated proteins" (GIMAPs; previously "immune-associated nucleotide-binding" proteins: IANs) are proteins containing an AIG type GTP binding domain, highly conserved among vertebrates and higher plants and apparently descended via duplication from a primordial gene (Nitta and Takahama, 2007). In humans, there are 7 translated GIMAP genes and one pseudogene (GIMAP3) linked on

chromosome 7q36.1. All the GIMAPs are highly expressed in thymus, spleen and lymph node tissues, while all except for GIMAP3 and GIMAP9 (neither of which are expressed in humans) are also highly expressed in the lung (Nitta *et al.*, 2006). On the other hand, human GIMAP2 appears to have no direct homologue in *Mus musculus* (Krücken *et al.*, 2004).

While relatively little work has been done on any GIMAP in the human system, multiple genetic association studies have linked this locus to human autoimmune conditions. Different studies have linked polymorphisms in GIMAPs to Behçet's disease, asthma, T1D and SLE (Lee, Horie, Graham R Wallace, *et al.*, 2013; Lee, Horie, Graham R. Wallace, *et al.*, 2013; M. T. Heinonen *et al.*, 2015). This data suggests that GIMAPs play a similar role in regulation of the immune system and lymphocyte homeostasis in humans as well as the mouse model. After decades of study the *in vivo* impacts of GIMAP deficiency have been well described in multiple models but the molecular function of this family of proteins is still very poorly understood.

Guanosine Triphosphate hydrolases (GTPases) are a large class of regulatory switches for internal cellular functions (Gilman, 1987; Wittinghofer and Vetter, 2011). The classical paradigm for small GTPases utilizes the conformational change induced by the binding/hydrolysis of GTP to modulate the interacting partners and enable effector functions. The GTP bound form of the enzyme is typically 'on' or able to perform its biological function while the GDP bound form is 'off.' The transition from GDP to GTP bound forms is catalyzed by a class of proteins called GTPase Exchange Factors (GEFs)

while the hydrolysis of GTP is induced by GTPase Activating Proteins (GAPs) and the regulation of these two classes of proteins is critical for appropriate signaling.

Broadly speaking, GIMAPs are thought to be distinct in their regulation from the classical small GTPases described above and more similar to the less studied GTPase families such as the septins and paraseptins. Septins are distinct in that GTP binding induces the formation of large heterogeneous oligomers. The septins are peculiar relative to the small GTPases in two other ways as well. Firstly, at least in some model organisms, it is unclear whether GTPase activity is even required for proper function at baseline (Zent and Wittinghofer, 2014; Abbey *et al.*, 2016). The ability to bind GTP seems to be sufficien, or, in some cases, is completely dispensable. Secondly, in humans, septin proteins appear to be highly redundant with knockout of all the septin genes being necessary in T lymphocytes to reveal a selective cytokinesis defect (Mujal *et al.*, 2015).

Paraseptins include protein channels such as the Translocon on Outer Chloroplast membrane (Toc) and the Signal Recognition Particle Receptor (SRPR) and along with the septin family are termed G proteins Activated by nucleotide-dependent Dimerization (GADs). These proteins are thought to have a relatively lower affinity for GTP relative to conventional small GTPases, potentially foregoing the need for GEFs and GAPs to regulate their activity. Rather, homo or heterodimerization itself regulates the GTP hydrolysis cycle with both members of the complex providing residues required for GTP hydrolysis (Gasper *et al.*, 2009). GTP binding prompts dimerization, which is thought to be the active complex capable of carrying out a biological function. One subgroup of

GADs is the "GTPase of immunity-associated proteins" (GIMAPs) that is primarily expressed in lymphocytes (Krücken *et al.*, 2004).

B) GIMAP1

Endogenous murine Gimap1 has been shown to localize to the Golgi in the cell line C1498, although endogenous localization in primary cells has not been tested (Vivian W Y Wong *et al.*, 2010). GIMAP1 is expressed at all stages of T and B cell development (Saunders, Louise M C Webb, *et al.*, 2010). Ablation of a floxed Gimap1 allele with either a CD2- or CD79a-restricted Cre in mice leads to severe peripheral lymphopenia of T and B cells (Saunders, Louise M C Webb, *et al.*, 2010; Webb *et al.*, 2016). Interestingly, in both cases the numbers of thymocytes and immature/developing B cells are not significantly different from controls, with the exception of the $CD8^+$ single positive ($CD8^+$ SP) stage which are roughly 2-3 fold reduced. (Saunders, Louise M C Webb, *et al.*, 2010; Webb *et al.*, 2016). The lymphocytes which did survive in the periphery express Gimap1 and thus represent rare clones which failed to delete the gene completely, suggesting that expression of this gene is critical for survival in the periphery. While overall numbers are normal, survival of $CD4^+$SP thymocytes is deficient *in vitro* despite IL-7 signaling being intact. Overexpression of Bcl-2 is unable to rescue survival of GIMAP1 deficient B cells in the periphery, suggesting that GIMAP1 does not affect B cell survival via the classical intrinsic apoptosis pathways as has been suggested for other GIMAPs (Chen *et al.*, 2011a; Webb *et al.*, 2016). Consistent with such a drastic decrease in survival in the absence of GIMAP1, acute ablation of GIMAP1 via administration of tamoxifen to ERT2-Cre GIMAP1f/f mice led to a severe reduction

in B cell responses, germinal center formation, plasma cell generation and B cell memory recall responses to secondary challenges (Webb *et al.*, 2016).

C) GIMAP2

GIMAP2 is absent from the mouse genome, suggesting that it arose later in evolution (Krücken *et al.*, 2004). Overexpressed, GFP-tagged GIMAP2 has been shown to localize to the surface of lipid droplets, presumably via insertion of its C-terminal hydrophobic segments into the surface phospholipid monolayer (Schwefel *et al.*, 2010). While knockout mice have not been generated and little is known about the role of GIMAP2 in lymphocytes, the crystal structure of a construct lacking the C-terminal hydrophobic sequences ($GIMAP2^{1-260}$) was solved and extensive biochemical characterization has been done. $GIMAP2^{1-260}$ binds GTP and GDP with a K_d of 40 and 630 nM respectively, which is at the low end of normal compared to classical small GTPases which typically bind guanine nucleotides in the picomolar to low nanomolar range (Bos, Rehmann and Wittinghofer, 2007). Interestingly, *in vitro* purified GIMAP2 both self-oligomerizes and heterodimerizes with GIMAP7 in a GTP-dependent fashion (Schwefel *et al.*, 2010, 2013). The truncated construct does not show significant intrinsic GTPase activity *in vitro*, suggesting that some aspect of the C-terminal domain, insertion into a membrane *in vivo*, or a GTPase Activating Protein (GAPs) is required. Finally, mixtures of GIMAP2 and GIMAP7 showed that GIMAP2 possessed GAP activity for GIMAP7 *in vitro* in a GTP-dependent manner. Interestingly, GIMAP2 mutants unable to hydrolyze/bind GTP are less efficient at catalyzing GTP hydrolysis in GIMAP7 (Schwefel *et al.*, 2013). The fact that GIMAP2 is absent from mice raises interesting

questions about how GIMAP7 may be regulated in different organisms and suggests that other GAPs must be involved. However, as virtually nothing is known about the physiological roles of either GIMAP2 or GIMAP7 *in vivo*, it is difficult to evaluate the relevance of this GAP activity or the GTPase activity of these proteins more generally.

D) GIMAP3

Relatively little is known about GIMAP3, most likely due to its being a pseudogene in humans (Krücken *et al.*, 2004). One study claimed that GIMAP5-deficient mice have a very mild phenotype that was greatly exacerbated in GIMAP3/GIMAP5 double knockouts, suggesting that these genes were synergistic (Yano *et al.*, 2014). However, two other independently generated GIMAP5-deficient mouse lines showed severe lymphopenia even in the presence of GIMAP3, suggesting that GIMAP5 has a critical, non-redundant role in lymphocyte homeostasis and survival (Ryan D. Schulteis *et al.*, 2008; Aksoylar *et al.*, 2012). This discrepancy could be due to genetic background or environmental factors specific to different animal facilities. Furthermore, the absence of GIMAP3 in humans argues against it synergizing with GIMAP5. Another genome-wide linkage study between BALB/c and CAST/Ei mouse strains suggested that GIMAP3 is responsible for differences in mitochondrial DNA segregation in hematopoietic tissues (Jokinen *et al.*, 2010). However, this argument was weakened by the fact that ectopic expression of GIMAP3 had no effect on this phenotype and no loss-of-function model was explored.

Nitta *et al.* also investigated the impact of gain/loss-of-function of GIMAP3 in Fetal Thymus Organ Cultures (FTOCs). Here, the authors showed no effect of GIMAP3

overexpression but a significant reduction in $CD4^+$SP cells following knockdown with shRNA-expressing retroviruses (Nitta *et al.*, 2006). Furthermore, in a 293T overexpression system, the authors showed an interaction with Bcl-2, Bcl-xl, Bax, Bak, Bad and BimEL, suggesting GIMAP3's involvement in the intrinsic apoptosis pathway.

E) GIMAP4 (mIAN1)

Overexpressed GIMAP4 in HeLa cells is spread throughout the cytosol (Schnell *et al.*, 2006). Staining for GIMAP4 in primary stimulated T cells is more punctate, although it does not seem to significantly co-localize with vesicular markers such as Rab27, LAMP1 or EEA-1 (Mirkka T. Heinonen *et al.*, 2015). It is unclear whether the difference in localization between the HeLa and primary T cells is a function of the cell type or the activation status of the T cells. Interestingly, while unstimulated T cells from the knockout mice show slightly higher cell death following serum starvation, both lymphocyte development and numbers in the periphery are identical to controls (Schnell *et al.*, 2006). Consistent with this, authors showed an interaction with Bax in a 293T overexpression system (Nitta *et al.*, 2006). Depletion of GIMAP4 via electroporation of siRNAs in primary human $CD4^+$ T cells showed no difference in viability or proliferation but a mild decrease in Interferon-gamma (IFNγ) secretion (Mirkka T. Heinonen *et al.*, 2015). Given the normal T cell development and absence of peripheral lymphopenia in GIMAP4-deficient mice, GIMAP4 could possibly be redundant in the mouse model, the phenotype could be masked by the genetic background or the gene may be involved in some currently unknown pathway distinct from the other GIMAPs. While the crystal structure has not been solved, Cambot *et al*. purified GST-GIMAP4 and showed that it

can bind both GTP and GDP with relatively low affinities (Kd of 0.47 and 6 μM respectively) (Cambot *et al.*, 2002).

F) GIMAP5

GIMAP5 is the best studied protein in the GIMAP family. It was first recognized as a critical regulator of lymphocyte survival and homeostasis in the Biobreeding rat model of T1D as well as Lymphocytic Thyroiditis (LT) (BioBreeding rat – Diabetes Prone abbreviated as BBDP). This model spontaneously develops antibodies specific for multiple pancreatic proteins and mirrors many aspects of the human disease (Allen and Braverman, 1996). Two groups independently mapped the gene responsible for the pronounced lymphopenia in the BBDP rat strain to a small locus on chromosome 4 and discovered a homozygous frameshift mutation in GIMAP5 (Hornum, Rmer and Markholst, 2002; MacMurray *et al.*, 2002). While other loci were also required for the development of T1D, wild-type GIMAP5 could rescue the phenotype demonstrating that it was necessary but not sufficient. Work using T cells from the BBDP rat showed defective calcium flux, Endoplasmic Reticulum (ER) and mitochondrial function. However, these studies were likely significantly affected by the lymphopenic environment and did not validate these phenotypes with bone marrow chimeras or *in vitro* knockout strategies (Pandarpurkar *et al.*, 2003; Keita *et al.*, 2007; Ilangumaran *et al.*, 2009; Pino *et al.*, 2009; Chen *et al.*, 2013). Another group suggested that GIMAP5 regulated NF-kB activity and showed that this was maintained in bone marrow chimeras, controlling for the lymphopenic environment (Kupfer *et al.*, 2007). Overall, while the lymphopenic/diabetic phenotype *in vivo* is very reproducible, a clear *in vitro* phenotype

that can either be rescued by wild type GIMAP5 or induced in wild type cells via loss-of-function approaches is lacking. Furthermore, there is controversy as to the function of GIMAP5 likely due to many groups observing phenotypes secondary to LIP. Interestingly, crossing the loss-of-function GIMAP5 allele from the BioBreeding to the PVG background did not lead to DM but rather an eosinophilic colitis suggesting that genetic background has a significant impact on the GIMAP5 deficiency phenotype (Cousins *et al.*, 2006).

The subcellular localization of GIMAP5 has been controversial in the literature, with initial overexpression studies of GFP-fused GIMAP5 co-localizing with markers of the ER, Golgi, centrosome, nuclear membrane, Multi-Vesicular Body (MVB) and mitochondria (Sandal *et al.*, 2003; Nitta *et al.*, 2006; Dalberg, Markholst and Hornum, 2007; Pino *et al.*, 2009). More recent studies using a monoclonal antibody measuring endogenous GIMAP5 in Jurkats or overexpression systems using lower amounts of plasmid showed significant colocalization with LAMP1 (Vivian W Y Wong *et al.*, 2010; Serrano *et al.*, 2017). These more recent studies have cast doubt on previous work suggesting that GIMAP5 regulates the intrinsic apoptosis pathway via direct interaction with Bcl-2 family members at the mitochondrion.

Multiple studies have shown GIMAP5 to be primarily expressed in lymphocytes (Dahéron *et al.*, 2001; Stamm *et al.*, 2002; Vivian W Y Wong *et al.*, 2010). Northern blots have also shown expression in non-lymphoid human tissues such as liver and lungs but it is unclear whether this is due to resident lymphocytes or some other cell type

(Krücken *et al.*, 2004). To date, the crystal structure, affinity for GTP/GDP, potential GEFs/GAPs and *in vitro* GTP hydrolysis rates remain unknown.

Three groups independently generated GIMAP5-deficient mice – two groups generating complete knockouts via conventional gene targeting, while a third group reported an N-ethyl-N-nitrosourea(ENU)-induced missense mutation (p.G38C mutation, referred to hereafter as GIMAP5$^{sph/sph}$) that severely destabilizes the protein and likely results in a complete loss-of-function (Ryan D. Schulteis *et al.*, 2008; Barnes *et al.*, 2010a; Yano *et al.*, 2014). While two groups reported a phenotype very similar to GIMAP1-deficient mice with peripheral lymphopenia but relatively normal thymopoiesis, the third reported that GIMAP3 also had to be knocked out in order to observe lymphopenia (Yano *et al.*, 2014). The reason for this discrepancy is unclear but could be related to facility specific environmental factors or minor genetic differences in the parental mouse strains used to generate the knockouts. In the two congruent studies, GIMAP5-deficient mice die between 12 and 20 weeks of age and suffer from profound progressive lymphopenia, anemia, liver failure, extramedullary hematopoiesis and colitis (Ryan D Schulteis *et al.*, 2008a; Barnes *et al.*, 2010a). There is virtually a complete absence of iNKT cells, CD8$^+$ T cells and a significant reduction in CD4$^+$ T cells, NK cells, granulocytes and B cells. Absolute numbers of myeloid lineages are largely unaffected although experiments testing their function have not been reported to date. Naïve T cells are drastically decreased, likely due to LIP, and while the absolute number of FoxP3$^+$ regulatory T cells is decreased the relative fraction of the CD4$^+$ T cell pool is normal (Barnes *et al.*, 2010a; Aksoylar *et al.*, 2012). Furthermore, TCR/B Cell Receptor

(BCR)-dependent proliferation of lymphocytes is severely deficient. This could be secondary to the lymphopenic environment, although the authors later showed that inhibition of GSK3β by lithium chloride or 6-bromoindirubin-3'-oxime (BIO) could rescue proliferation (Patterson *et al.*, 2018). This phenotype may be secondary to lymphopenia because the severity of the proliferative defect increases with age along with the lymphopenia, possibly due to or exacerbated by decreased thymic output and a progressive transition of the T cells from a naïve to an exhausted memory state (Aksoylar *et al.*, 2012). Survival of GIMAP5-deficient mice can be rescued by antibiotic treatment, depletion of $CD4^+$ T cells or intravenous transfer of wild type splenocytes/$FoxP3^+$ regulatory T cells (Barnes *et al.*, 2010a). One GIMAP5-deficient human patient has been recently described in the literature (Patterson *et al.*, 2018). The patients phenotype is consistent with that observed in the mouse, including lymphopenia, splenomegaly and a susceptibility to infections. Proliferation of the patients peripheral T cells was defective relative to one control and could be rescued with lithium chloride treatment. Barnes et al. showed that on a C57BL/6J background $GIMAP5^{sph/sph}$ mice develop severe colitis (Barnes *et al.*, 2010a). This gut pathology could be consistent with fast LIP leading to gut inflammation in the lymphopenic environment of the $GIMAP5^{sph/sph}$ mouse.

Many different hypotheses have been put forward with regard to the mechanism behind the phenotype observed *in vivo* in GIMAP5-deficient mice. Chen et al. proposed that GIMAP5 is required for Hematopoietic Stem Cell (HSC) survival due to direct interactions between GIMAP5 and HSC70, Mcl-1 and Bcl-xl (Chen *et al.*, 2011a). Defective HSC function is supported by competitive engraftment models wherein wild

type cells dominate the bone marrow. These findings can account for the anemia and B cell defects, but considering that thymopoiesis is largely normal in GIMAP5 deficiency it is unlikely that the peripheral T cell lymphopenia is due to HSC defects. Furthermore, this model ignores the finding that GIMAP5 is largely absent from the mitochondria and primarily localizes to the surface of lysosomes and other vesicular compartments (although it is worth noting this study was in T cell lines and not HSCs), making direct interactions with members of the intrinsic apoptosis pathway unlikely (Vivian W Y Wong *et al.*, 2010). A different group has proposed that GIMAP5 controls FOXO1 levels. FOXO1 is known to regulate the transcription of genes critical for naïve T cell survival in the periphery through an unclear mechanism (Kerdiles *et al.*, 2009; Aksoylar *et al.*, 2012). This same group also showed that GIMAP5 is a negative regulator of GSK3β and that both the *in vitro* and *in vivo* T cell defects of GIMAP5 deficiency can be rescued either by inhibitors or genetic ablation of GSK3β activity (Patterson *et al.*, 2018). Increased GSK3β activity in the absence of GIMAP5 was shown to lead to increased DNA damage and consequently apoptosis. This finding would be consistent with another group that showed overexpression of GIMAP5 in Jurkat cells could protect against okadaic acid and γ-radiation induced apoptosis, suggesting that GIMAP5 protects against apoptosis. Chen *et al.* proposed that GIMAP5 is both a negative regulator of Akt and also required for appropriate IL-7 signaling, although neither of these publications accounted for the lymphopenia with either a rescue or acute depletion of GIMAP5 in wild type cells (Chen *et al.*, 2015, 2016)

Multiple groups have published conflicting results regarding overexpression or knockdown of GIMAP5 *in vitro*. One study showed an increase in apoptosis in Jurkat and primary human T cells following transient overexpression of GFP-tagged GIMAP5, but no effect of RNAi knocking down GIMAP5 (Dalberg, Markholst and Hornum, 2007). However, this publication did not show the efficiency of the knockdown and the overexpressed tagged form of GFP-GIMAP5 localized throughout the cytosol rather than in vesicular puncta, raising the possibility of artifactual results. Another group showed increased apoptosis following siRNA knockdown of GIMAP5 in Jurkat cells, although again validation of the knockdown at the protein level was lacking (Pandarpurkar *et al.*, 2003). Another group showed an anti-apoptotic function for GIMAP5 in T-ALL lines (Chadwick *et al.*, 2010). Despite decades of research and dozens of publications, multiple studies have reached different conclusions regarding the function of GIMAP5 and a clear consensus on its molecular function has yet to emerge.

G) GIMAP6

Conditional GIMAP6 knockout mice have a milder but similar phenotype to the GIMAP1/GIMAP5-deficient lines with B and T cell lymphopenia but no liver disease or colitis reported. It is worth noting that the mice were generated using a CD2 specific Cre, restricting the absence of GIMAP6 to B and T lymphocytes which could be responsible for some of these differences (Pascall *et al.*, 2013, 2018). Interestingly, lymphocytes from these GIMAP6-deficient mice were shown to have defects in autophagy. At baseline in the absence of bafilomycin treatment GIMAP6-deficient mice have an accumulation of LC3-II, suggesting that there may be a defect in autophagosome processing or

degradation. There is no published data concerning autophagy in the context of any other GIMAP deficiency which would be interesting given the similarity in phenotypes between mice deficient for different GIMAP family members.

GFP tagged GIMAP6 overexpression studies in 293T cells have shown localization dispersed throughout the cytoplasm, although upon starvation it was recruited to autophagosomes. In a separate study the same group demonstrated that GIMAP6 interacts with GABARAPL2, an Atg8 homologue, in overexpression systems (Pascall *et al.*, 2013). Contrary to what is observed with most G proteins which interact with effectors through the switch regions in the GTPase domain, the interaction between GIMAP6 and GABARAPL2 was mapped to the C-terminal domain of GIMAP6. The authors also showed that endogenous GIMAP6 was recruited to autophagic vesicles following starvation in both Jurkat and Human Vascular Endothelial (HUVEC) cells (Pascall *et al.*, 2013).

Another group showed that knockdown of GIMAP6 in Jurkat cells via siRNA led to a mild increase in apoptosis at baseline as well as greater sensitivity to apoptotic stimuli such as FasL, okadaic acid and hydrogen peroxide. Furthermore, knockdown led to increased activation kinetics and an increase in GIMAP6 levels in an NF-κB dependent manner (Ho and Tsai, 2017).

H) GIMAP7

From a functional point of view, GIMAP7 has been very poorly studied but the crystal structure has been solved. GIMAP7 has been shown to localize to the surface of lipid droplets, similarly to GIMAP2. Indeed, these two proteins have been shown to

heterodimerize *in vitro*, and furthermore GIMAP2 is thought to act as a GAP for GIMAP7 (Schwefel *et al.*, 2013).

I) GIMAP8

GIMAP8 is unique in the GIMAP family and GTPases in general in that it carries three GTP binding and hydrolysis domains. When a C-terminally His tagged GIMAP8 was overexpressed in Chinese Hamster Ovary (CHO)-K1 cells it localized broadly throughout the cytoplasm, ER, Golgi and mitochondria (Krücken *et al.*, 2005). This strategy may be prone to artifacts as the membrane targeting domains of GIMAP proteins are typically C-terminally located and thus most studies have used N-terminal tags.

While structurally GIMAP8 is unique it is unclear whether or not it has a non-redundant role in the immune system. Webb *et al.* generated GIMAP8-deficient mice and saw no major differences in the peripheral T and B cell compartments (Webb *et al.*, 2014). There were minor increases in apoptosis following irradiation or dexamethasone treatment in T cells from GIMAP8-deficient mice, supporting an anti-apoptotic role for this gene. However, T dependent immune responses were normal again leaving it unclear how this gene functions in the murine immune system (Webb *et al.*, 2014).

J) Summary

While a role in the intrinsic apoptosis pathway is attractive due to the lymphopenia phenotype of multiple GIMAP deficient mice and various groups have shown interactions with proteins regulating the intrinsic apoptosis pathway in 293T overexpression systems, the evidence for this hypothesis is weak. Specifically, GIMAPs are broadly distributed throughout the cell and none of them have been shown to localize

to the mitochondria in primary cells. As a result, it is difficult to imagine how these proteins are interacting with members of the intrinsic apoptosis pathway *in vivo.* Thus, these results may be artifacts of overexpression systems or co-immunoprecipitation approaches which mix proteins from different cellular compartments during cell lysis.

While multiple GIMAP knockout mice share similar phenotypes (GIMAP1, GIMAP5 and to a lesser extent GIMAP6), the subcellular localization of these proteins differ from one another. This raises the possibility that at least some members of this family of proteins have non-redundant roles in a common pathway. Furthermore, the restricted expression pattern prompts me to speculate that this pathway may be specific to lymphocytes or at least play some unique role in lymphocytes distinct from other tissues. However, to date most proposed explanations for possible biochemical roles of GIMAPs have only been explored in cells isolated from GIMAP1/GIMAP5-deficient hosts which have significant systemic immune perturbations, raising a caveat to conclusions based on these findings. Very little work has shown either a rescue of the proposed phenotype or an *in vitro* phenocopy via genetic loss-of-function approaches. The closest to meeting this standard is a study from Patterson *et al.* showing that both the *in vitro* and *in vivo* phenotypes can be rescued via inhibition of GSK3β (Patterson *et al.*, 2018). However, the precise molecular mechanism of GIMAP5 in this case as well as the downstream function of GSK3β in activated T cells remains unclear. Overall, after decades of study the *in vivo* impacts of GIMAP deficiency have been well described in multiple models but the molecular function of this family of proteins is still very poorly understood.

V. The Influence of Cellular Metabolism on the T cell Response

A) Cell culture media and metabolism

The metabolic status of lymphocytes has recently been recognized as critical for both quiescence/homeostasis and also maintaining an appropriate number of cells in the periphery. Recent publications have highlighted the influence of lipids, glucose, and amino acid metabolism on both the magnitude and characteristics of the T cell response (Pearce *et al.*, 2009; van der Windt and Pearce, 2012; Sinclair *et al.*, 2013; O'Sullivan *et al.*, 2014; Hosios *et al.*, 2016; Buck *et al.*, 2017; Jacobs *et al.*, 2018). This realization has influenced and enhanced my understanding of T cell activation in both *in vivo* and *in vitro* model systems.

Formulations of common cell culture media such as RPMI-1640 were developed in the middle of the 20th century and have undergone remarkably little change. The compositions of these media were designed to optimize growth of early cancerous cell lines such as the immortalized murine fibroblast line 'Strain L' (Yao and Asayama, 2017). Despite this renewed focus on the effects of metabolic needs on T cell activation and proliferation very little effort to date has been made to develop culture conditions that more closely resemble the *in vivo* milieu that which T lymphocytes experience. More recently, development of medias such as x-Vivo 15 allow for the culture of T lymphocytes in serum-free conditions which can be critical for clinical applications (Medvec *et al.*, 2018). However, one approach that has been less explored in the literature

is a culture media designed to mimic the *in vivo* environment rather than optimize the growth of tumor cell lines.

Recently, several groups have added various additional supplements to RPMI with the aim of either improving growth in cell culture or better modeling the *in vivo* environment (Favaro *et al.*, 2012; Birsoy *et al.*, 2014; Schug *et al.*, 2015; Pan *et al.*, 2016). Most comprehensive was a study from Cantor *et al.* in which the authors measured levels of various metabolites in human plasma and formulated Human Plasma-Like Media (HPLM) containing a cocktail of small metabolites that are absent from RPMI and other standard culture media. They may be present in non-physiological levels in Fetal Bovine Serum (FBS) which is the most widely used medium supplement (Cantor *et al.*, 2017). In addition to this, the levels of amino acids, glucose and various ions were also calibrated to physiological values for humans in HPLM.

B) Lymphocyte activation and metabolic demands

Quiescent, unstimulated T cells have drastically different metabolic programs relative to activated T cell blasts. Similarly, naïve and memory T cells also have different metabolic requirements. Quiescent T cells are mainly in interphase/G1 and only undergo a very slow homeostatic proliferation to maintain total cell numbers. Broadly speaking, these cells have a catabolic metabolism whereby they mostly degrade nutrients completely via oxidative phosphorylation in order to obtain the maximal amount of energy. On the other hand, stimulated T cells preferentially use anaerobic glycolysis to promote anabolism and accumulate the biomass and macromolecules required for growth and cell division (Heiden *et al.*, 2009). Massive changes occur in the cell to accommodate

this: for example, the main glucose transporter of the cell GLUT1 is highly upregulated following TCR stimulation (Jacobs *et al.*, 2018). Furthermore, the same study showed that overexpression of GLUT1 was sufficient to skew the T cell pool away from a naïve phenotype and towards a $CD44^{hi}$ effector phenotype. In *in vitro* assays of memory/effector T cell skewing, increased glycolysis is inversely correlated with memory formation while inhibition skews the cells towards a memory fate (Sukumar *et al.*, 2013). Memory T lymphocytes also have a greater requirement for glycerol and fatty acid metabolism as the upregulation of glycerol transporters is necessary for memory T cell formation (Cui *et al.*, 2015).

C) mTORC1 signaling in T lymphocytes

Multiple sensors of the metabolic status of the cell have an influence on T cell activation, proliferation and the requisite switch to a glycolytic metabolism. One of the most studied sensors is the mechanistic Target Of Rapamycin Complex 1 (mTORC1), a critical nexus for sensing multiple metabolic and signaling inputs and inducing proliferation, cell growth, global protein translation and T cell differentiation. This complex consists of the mTOR kinase, Raptor, MLST8, PRAS40 and DEPTOR proteins. The mTORC1 signaling pathway couples growth and proliferation to nutrient availability and can be dysregulated in a plethora of diseases (Altomare and Gitto, 2015). The mTORC1 is regulated by two sets of small GTPases upstream – the Rag GTPases and Rheb (Groenewoud and Zwartkruis, 2013; Bar-Peled and Sabatini, 2014). These in turn are thought to be governed by nutrient availability and growth factor signaling

respectively. Downstream, the mTOR kinase phosphorylates a variety of targets involved in protein translation, cell metabolism and proliferation.

The mTORC1 has been shown to play a critical role in the regulation and homeostasis of T lymphocytes. It has been recognized that TCR stimulation activates pathways stimulating protein translation even before the mTOR kinase had been discovered (Calvo, Bierer and Vik, 1992). Genetic ablation of one of the main negative regulators of mTORC1 activity, Tsc1, has no major effect on thymocyte development (although thymocytes are larger consistent with increased mTORC1 activity) but does lead to a loss of naïve cells and decrease in lymphocyte numbers in the periphery (Wu *et al.*, 2011). T lymphocytes from these mice also exhibit increased proliferation *in vivo* although it is unclear whether this is due to LIP or a primary effect of the ablation of Tsc1. Curiously, T lymphocytes from these mice also exhibit increased apoptosis although the mechanism behind this remains unclear. A separate study with similar findings showed that the defect could be partially rescued by overexpression of a Bcl-2 transgene suggesting that the intrinsic apoptosis pathway may be affected (Yang *et al.*, 2011). Puzzlingly, human patients carrying germline autosomal dominant negative mutations in TSC1/2 do not suffer from lymphopenia and their T cells activate and proliferate normally with no evidence of increased mTORC1 activity (Pilipow *et al.*, 2014). There is some evidence that cell survival may be affected in these cells similarly to the mice. Conversely, at least in B cells, loss of Raptor (and consequently mTORC1 signaling) also led to defective survival of these cells (Iwata *et al.*, 2016). It is worth noting that a complete loss of function of mTORC1 leads to effects on many other

pathways such as an increase in mTORC2 signaling which could compensate for some functions (Xie and Proud, 2013). This could explain the finding that in some contexts both loss and gain of function mutations in the mTORC1 pathway lead to defective survival. It could also be that appropriate metabolic regulation is required for cell survival and perturbations in either direction reduce cell fitness.

mTORC1 activity also plays a major role in the response to cognate antigen as well as memory formation following a successful immune response. Araki *et al.* demonstrated that rapamycin treatment during acute infection with LCMV has no major impact on the magnitude of the initial response (although this finding was dose dependent) but generated a much larger pool of memory T cells (Araki *et al.*, 2009). They also confirmed these findings via genetic approaches as well. Previous studies have shown that mTORC1 and glycolysis are required for development of effector functions during the T cell response, which may demonstrate a potential drawback of this strategy to augment memory responses (Chang *et al.*, 2013; Millet *et al.*, 2017). GAPDH is sensitive to glycolytic flux and can directly bind regulatory elements in the 3' Untranslated Region (UTR) of IFNγ and TNFα, releasing translational inhibition when glycolytic flux is high. These findings likely account for some previous findings suggesting that mTORC1 inhibition affects T cell effector function. mTORC1 signaling regulates T helper differentiation in addition to memory formation and the induction of effector functions. Genetic depletion of Raptor and Rictor, unique components of mTORC1 and mTORC2 respectively, had inverse effects on Th1, Th17 and Th2 differentiation. Ablation of Raptor and consequently mTORC1 signaling prevented the

generation of Th1 and Th17 cells, whereas loss of Rictor and mTORC2 signaling blocked the formation of Th2s (Delgoffe *et al.*, 2011).

Upon TCR stimulation, secondary growth signals such as CD28 ligation or IL-2 promote the metabolic switch to glycolysis via activation of multiple signaling pathways. The Phosphoinositide 3-kinase (PI3K)/Akt and LKB1/AMPK signaling axes have been shown to be particularly important in the TCR mediated metabolic switch (van der Windt and Pearce, 2012). Activation of PI3K leads to the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2). PIP3 recruits both Akt and its activator, PDK1, to the plasma membrane via PIP3 binding Pleckstrin Homology (PH) domains. Furthermore, it induces a conformational change in Akt exposing the critical threonine 308 residue for phosphorylation by PDK1 (Mora *et al.*, 2004). Activated Akt can then go on to phosphorylate the Tuberous Sclerosis Complex proteins 1/2 (TSC1/2) which are critical negative regulators of mTORC1. The TSC complex acts as a GAP for the small GTPase Rheb on the surface of the lysosome. In the absence of TSC GAP activity Rheb can become GTP loaded and mediate the phosphorylation of mTOR in mTORC1 which is required for its kinase activity.

In parallel, a second signaling axis is required for appropriate activation of mTORC1. It has long been known that amino acid availability can impact mTOR activity, however, only recently have the molecular underpinnings been elucidated. Initially it was thought that this was mediated by the TSC1/2/Rheb axis as well; however, the discovery that $Tsc2^{-/-}$ Murine Embryonic Fibroblasts (MEFs) are still sensitive to

amino acid starvation pointed towards an alternative pathway (Smith *et al.*, 2005). A combination of genetic and molecular functional approaches led to the discovery of the Rag GTPases, Ragulator complex and lysosomal V-ATPase as the molecular components of this alternative signaling pathway (Kim *et al.*, 2008; Sancak *et al.*, 2008). The Ragulator complex consists of 5 scaffolding proteins: Late endosomal/lysosomal Adaptor, MAPK and mTOR activator 1 (LAMTOR1) through LAMTOR5. Some of these proteins and the general mechanism of regulation are conserved all the way back to yeast. The crystal structure of these in complex demonstrates a hydrophobic tail of LAMTOR1 anchoring the complex to the lysosomal membrane (Yonehara *et al.*, 2017). LAMTOR1, 2 and 3 have also previously been recognized as scaffolds for Mek/Erk in the MAPK signaling pathway indicating that the Ragulator complex has other functions beyond its role in the mTORC1 signaling pathway (Nada *et al.*, 2009). The Ragulator complex serves as a scaffold for a heterodimer of another set of small GTPases – RagA or RagB heterodimerized with either RagC or RagD. A series of elegant studies demonstrated that the GTP/GDP loading state of this heterodimer mediated the recruitment of mTORC1 to the surface of the lysosome. The active heterodimer has RagA/RagB in the GTP bound state and RagC/RagD in the GDP bound state (Bar-peled *et al.*, 2012). The Ragulator complex works in conjunction with the lysosomal V-ATPase as a GEF for RagA/RagB to stimulate a switch to the active form of the complex. This complex can then interact with Raptor, recruiting mTORC1 to the surface of the lysosome to phosphorylate downstream substrates such as p70-S6K, 4EBP1 and Ulk1.

Upstream of the Ragulator complex are the sestrin and GATOR complexes which are able to directly sense amino acid levels in the cell. GATOR1 and GATOR2 form a complex and GATOR1 is able to interact with the Rag GTPases. Loss of function studies were initially confusing as ablation of GATOR1 led to constitutively active mTORC1 and insensitivity to amino acid starvation whereas loss of GATOR2 led to deficient mTORC1 signaling. Epistasis experiments later demonstrated that GATOR2 acts upstream as a negative regulator of GATOR1, which in turn acts as GAP for RagA/B (Bar-peled *et al.*, 2013). Sestrin2 acts as a further regulator of this pathway upstream of GATOR2. Sestrin2 binds to GATOR2 and acts as a negative regulator of mTORC1; however, in the presence of sufficient levels of leucine it dissociates from GATOR2 thus allowing for the inhibition of GATOR1. Sestrin2 directly binds leucine and presumably acts as a sensor for levels for this specific amino acid in the cell (Wolfson *et al.*, 2015).

D) Autophagy in lymphocyte metabolism

Another critical metabolic pathway controlling lymphocyte metabolism is macroautophagy (hereafter referred to as autophagy). Autophagy is an ancient and highly conserved intracellular pathway for degrading macromolecules, organelles and even invading pathogens in lysosomal compartments. Autophagy has been shown to be critical for lymphocyte fitness. Multiple studies have shown that TCR stimulation drastically increases levels of autophagic flux (Li *et al.*, 2006; Hubbard *et al.*, 2010). In other cell types, inhibition of mTORC1 has long been recognized to induce autophagy. Current models posit that the mTORC1 senses starvation conditions and the absence of mTORC1 signaling induces autophagy. The molecular mechanism has recently been described with

AMPK and mTORC1 directly phosphorylating Ulk1 with opposing effects (Egan *et al.*, 2011). T cell specific ablation of autophagy genes Atg5, Atg7 and Rab7 led to relatively minor effects on thymopoiesis but major defects in peripheral T cell fitness and proliferation following stimulation (Pua *et al.*, 2007; Roy *et al.*, 2013; Lin *et al.*, 2014). Curiously, both autophagic flux and mTORC1 signaling are greatly increased during TCR stimulation despite the fact that these two pathways are typically inversely regulated. Furthermore, rapamycin treatment of activated T lymphocytes has little effect on autophagic flux contrary to many other cell lines that have been used to study autophagy (Botbol, Patel and Macian, 2015). While the molecular underpinnings of these effects remain unclear, there is some evidence that Jnk signaling may drive autophagy initiation in recently activated T lymphocytes (Li *et al.*, 2006).

The phenotypes of Rab7 deficient T lymphocytes and GIMAP1/GIMAP5-deficient T cells is remarkably similar (Ryan D Schulteis *et al.*, 2008a; Saunders, Louise M C Webb, *et al.*, 2010; Roy *et al.*, 2013). Both have largely regular thymopoiesis with peripheral lymphopenia, increased apoptosis of peripheral T lymphocytes, a more drastic defect in $CD8^+$ T cells and defective T cell proliferation *in vitro*. Several studies have demonstrated abnormalities in mitophagy (the envelopment of mitochondria in autophagosomes and their subsequent degradation) and consequently mitochondrial numbers. This in turn leads to altered levels of genes involved in the intrinsic apoptosis pathway as well as increased levels of reactive oxygen species T lymphocytes with genetic defects in autophagy (Pua *et al.*, 2009; Watanabe *et al.*, 2014).

The classical pathway involves the formation of double membraned autophagosomes containing the cargo in question and then subsequent delivery to lysosomes for degradation (Mizushima, Yoshimori and Ohsumi, 2011). This process is initiated by a complex of UNC-51-like Kinases 1 and 2 (Ulk1 and Ulk2) activating another complex of PI3KC3, Vps34, Vps15, Atg14 and Beclin1 which can then initiate the formation of the autophagosome membrane via lipidation of LC3 with phosphatidylethanolamine to LC3-II (Liang *et al.*, 1999; Eisuke Itakura,*† Chieko Kishi,* Kinji Inoue, 2010). A cascade of other Atg proteins then mediate the elongation of this membrane and trafficking of the mature autophagosome to the lysosome for processing and degradation. A family of small GTPases, the Rab proteins, are also critical for appropriate trafficking of the autophagosomes (Ao, Zou and Wu, 2014). Measurement of autophagic activity in the cell is typically expressed as a flux – baseline levels of LC3-II are measured and compared to cells treated with bafilomycin to inhibit LC3-II degradation. Overall, while these studies establish that autophagy is required for proper T cell function and survival, the mechanism of cell death and biochemical role of autophagy in T cell activation remain enigmatic.

CHAPTER 2: A Novel Mendelian Disease of Autoimmunity Caused by Mutations in GIMAP5

Authors: Michael Leney-Greene, Silvia Vilarinho, Yu Zhang, Alex George, Aiman Faruqi, Mike Leipold, Joy Edward-Hicks, Aaron Morawski, Ahmet Ozen, Helen C. Su, Michael J. Lenardo. Michael Leney-Greene designed research, conducted experiments, performed analysis and wrote this section. Alex George and Aiman Faruqi performed SPADE analysis of cytof data. Aaron Morawski tested GIMAP5 expression in monocytes and B cells. Mike Leipold performed cytof assay. Silvia Vilarinho, Yu Zhang and Michael Leney-Greene performed analysis of WES data. Joy Edward-Hicks conducted lipidomics analysis in Gimap5-deficient murine T cells. Helen C. Su and Michael J. Lenardo helped design experiments and write the paper.

I. Summary

Whole exome sequencing offers the promise of diagnosis to many patients as well as an avenue for discovering novel genes involved in the regulation of the immune system and allowing their study in the human model. Here, I describe for the first time a novel autosomal recessive Mendelian disease of autoimmunity caused by mutations in the small GTPase GIMAP5. Characterization of a cohort of patients carrying mutations in GIMAP5 allowed me to describe for the first time the broad clinical and immunological impacts of the loss of this gene in humans. Virtually every patient suffered from lymphopenia, thrombocytopenia, mild anemia, bronchiectasis, varying degrees of liver disease, hepatosplenomegaly and lymphadenopathy. Extensive immunological phenotyping and functional studies in patient T cells revealed major disruptions in the balance between naïve and memory T cells, although, surprisingly, no *in vitro* evidence of defective survival or proliferation in contrast to the murine model. I then undertook high-throughput RNA-Seq in an siRNA knockdown model as well as BioID2 labeling studies to define the interactome. The former revealed surprisingly little impact of GIMAP5 knockdown on the transcriptome while the latter identified three main complexes in close proximity to GIMAP5 in the cell: the Ragulator complex, the T cell receptor complex and various complexes involved in cellular trafficking. I went on to confirm interactions between GIMAP5 and the Ragulator complex via two independent methods. These data define for the first time the human presentation of GIMAP5 deficiency as well as multiple new interacting partners.

II. Results

Clinical phenotype of a cohort of patients suffering from a novel Mendelian disease of autoimmunity

We identified 9 affected individuals from 4 pedigrees suffering from a similar autoimmune disease (Figure 2.1A). Clinical problems common to the patients were fatigue related to anemia, bleeding, splenomegaly, lymphadenopathy and compromised breathing due to bronchiectasis which was presumably secondary to recurrent respiratory infections. Clinical testing revealed severe lymphopenia and thrombocytopenia that were consistent and in some cases progressive over time (Figure 2.1B, Table 1). Nearly every measurement of the GIMAP5-deficient patients showed values lower than the 5^{th} percentile (bottom dotted line). CT scans of the lungs of P1.4 and others revealed ground glass opacities and bronchial wall thickening typical of bronchiectasis (Figure 2.1C – white arrows). This clinical finding could represent either a congenital role for GIMAP5 in airway formation or, more likely occurred secondarily to recurrent lung infections. At the time of the scan the patient was also suffering from an acute fungal infection in his lungs (Figure 2.1C – red arrows). Three affected individuals from pedigree 1 are deceased - two of infections and liver failure and one of hypoxemic respiratory failure due to pulmonary embolus. A bone marrow biopsy was performed on one patient (P1.5) and revealed findings suggestive of myelodysplasia with increased, atypical megakaryocytes, increased erythroid progenitors, granulocytic hypoplasia and normal fractions of lymphocyte lineages. Liver biopsies were taken from P1.4 who had elevated levels of circulating liver enzymes as well as abnormal CT scan findings. A biopsy

showed a mild lymphocytic infiltrate (black arrow) as well as increased $CD34^{+}$ staining in the endothelial cells consistent with a diagnosis of Nodular Regenerative Hyperplasia (NRH) (Figure 2.1D). Detailed clinical information for each of the patients is available in Table 1.

Table 1: Clinical phenotype of GIMAP5-deficient patients

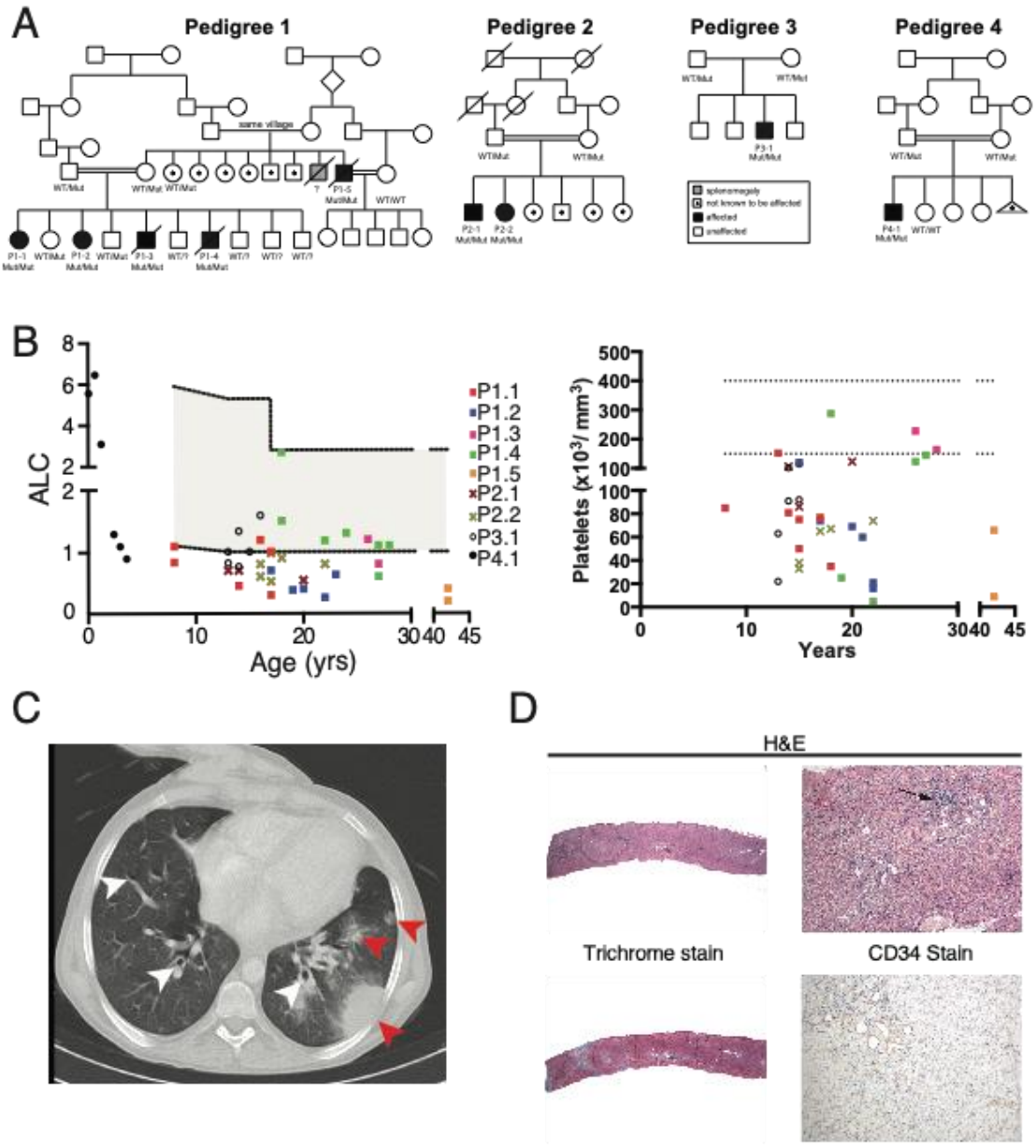
	P1-1	P1-2	P2-1	P2-2	P2-3	P2-4	P2-5	P3-1	P4.1
Age at Presentation (yo)	13	11	17	na	15	2	35	8	4
Current age (yo)	22	20	29	27	Died at 22	Died at 17	Died at 44	16	6
Clinical Presentation	Ecchymosis	Hemoptysis	Abdominal and back pain	Recognized during screening of family members	Fatigue, epistaxis	Recurrent Infections	Fatigue	Psoriasis	
Ethnicity	Turkish		Turkish					European	Turkish
Genotype, *GIMAP5*	p.I47T/I47T		p.L223F/L223F					p.L204P/ p.N221S	p.P109L
IMMUNODEFICIENCY & AUTOIMMUNITY									
Coombs Test	Negative	Negative	Negative	NA		Negative	Negative	Negative	Negative
Auto-antibodies	No	No	No	NA	No		No		Negative
Blood Cell counts									
Hemoglobin (NR: 12-17.5)	**12.3**	13.6	**9.8**	**11.1**	**5.3**	**9**	**7.5**	**10.2**	**10.5**
WBCs (NR: 4500-11000)	**3600**	**3500**	NA	NA	NA	NA	NA	NA	2960
Neutrophils (NR: 1900-8000)	2200	2500	2100	3100	**110**	**300**	**700**	**230**	**1950**
Lymphocytes (NR:900-5200)	**800**	**540**	1100	**800**	**380**	**830**	**300**	**790**	**720**
Platelets (NR: 130-400 x10e3/µL)	**74**	**123**	**25**	164	**69**	**35**	**9**	**44**	**81**
Lymphocyte subsets (%)									
CD3+	NA	NA	69% (55-83)	76% (55-83)	62% (55-83)	67% (52-78)	75% (55-83)	81% (56-84)	68%
CD3+CD4+	NA	NA	39% (28-57)	37% (28-57)	**13%** (28-57)	**23%** (25-48)	48% (28-57)	48% (31-52)	45%
CD3+CD8+	NA	NA	28% (10-39)	30% (10-39)	**48%** (10-39)	**44%** (9-35)	28% (10-39)	30% (18-35)	18%
CD19+	NA	NA	19% (6-19)	9% (6-19)	20% (6-19)	22% (8-24)	7% (6-19)	9% (6-23)	26%
CD3-CD16+/CD56+	NA	NA	**5%** (7-31)	7%(7-31)	6% (7-31)	7% (6-27)	**5%** (7-31)	5% (3-22)	6%
Immunoglobulins									
IgG (NR: 700-1600mg/dL)	1520	1700 (↑)	1610 (913-1894)	3360 (913-1894)	1340 (913-1894)	**360** (764-2134)	1720 (913-1894)	**380** (588-1573)****	**928** (700-1600)
IgM (NR: 40-230mg/dL)	59.5	90.4	215 (88-322)	156 (88-322)	178 (88-322)	118 (69-387)	246 (88-322)	**874** (57-237)	**10.5** (40-230)
IgA (NR: 70-400mg/dL)	190	310	246 (135-378)	596 (135-378)	176 (135-378)	**39** (70-303)	260 (135-378)	**18** (46-287)	**63.5** (70-400)
Thoracic CT Findings	Not done	Chronic fibrotic changes in the lingula	Bronchiectasis	Bronchiectasis	Bronchiectasis	Left lung consolidation	Bronchiectasis	Ground glass nodular pulm. infiltrates	Normal

Numbers in boldface represent values outside the normal range. Lowest values for each patient are given; NR: Normal Range. **** This value is post Rituxan treatment.

Prepared by M.L.G., A.O. and S.V.

Figure 2.1

Clinical phenotype of a cohort of patients suffering from a novel Mendelian disease of autoimmunity



A) Four pedigrees suffering from GIMAP5 deficiency are shown.
B) Plots showing ALC and platelet counts in GIMAP5-deficient patients over time. (continued on next page).

C) Chest CT scans with evidence of bronchiectasis (white arrows) and acute infection (red arrows).

Figure 2.1A,C,D were prepared by M.L.G. and S.V., 2.1B by M.L.G. and A.O.,

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WES reveals autosomal recessive mutations in GIMAP5

Given the inheritance patterns, I hypothesized that affected individuals in my cohort suffered from an autosomal recessive Mendelian disease. Genomic DNA was isolated from both affected and unaffected individuals in each pedigree for WES. In my initial bioinformatics analysis I considered protein-altering variants with minor allele frequency <0.1% in dbSNP, NHLBI, 1000 Genomes, Exome Aggregation Consortium (ExAC) and Yale exome databases. This approach revealed recessive mutations in GIMAP5 that were predicted to be highly deleterious to protein function in every affected individual, but these alleles were either heterozygous or absent in over 20 unaffected relatives who were sequenced. Three of the four pedigrees (Pedigrees 1, 2 and 4) had multiple generations of consanguinity and thus carried homozygous mutations while the fourth had no reported consanguinity and carried compound heterozygous mutations. Affected individuals from pedigree 1 were homozygous for a mutation encoding a p.L223F (c.667C>T) while P3.1 carried compound heterozygous mutations causing p.L204P (c.610T>C) and p.N221S (c.662A>G) (Figures 2.2A). Affected individuals in pedigree 2 carried a homozygous mutation causing a I47T (c.140T>C) substitution and the proband in pedigree 4 harbored a homozygous mutation encoding p.P109L (c.326C>G) (Figures 2.2A, B).

The I47T and L223F mutations were unique and had never before been seen according to multiple genomics databases (Figure 2.2B). The P109L and N221S mutations were extremely rare, with allele frequencies of 3.98×10^{-6} and 1.19×10^{-5} respectively. While the L204P mutation was more common (0.2% of the population), it was paired with the rarer N221S. Another study published a patient carrying homozygous L204P alleles with similar clinical features to the patient cohort described here, although whether patients homozygous for the L204P have complete penetrance remains to be seen (Patterson *et al.*, 2018). I confirmed the status of all of these mutations in every affected individual (barring P1.2 who died before I could acquire a sample) via Sanger Sequencing (Figure 2.2B).

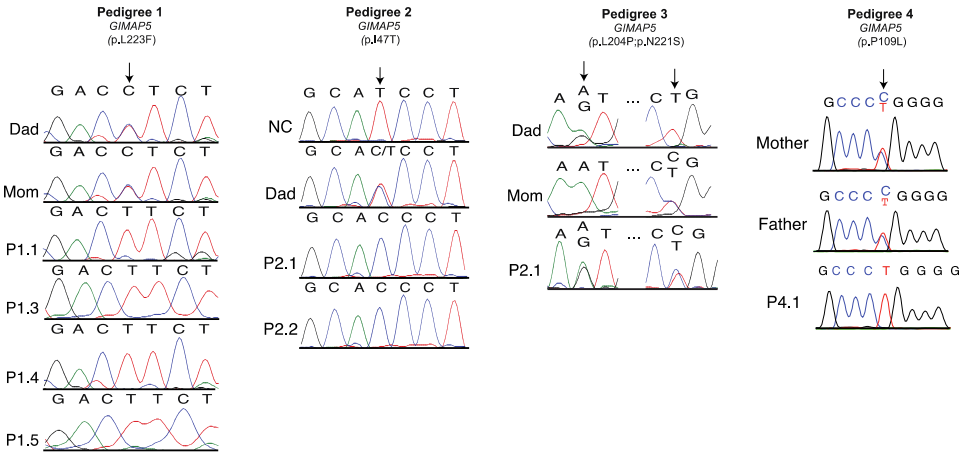
Figure 2.2

Whole exome sequencing reveals autosomal recessive mutations in GIMAP5

A

Kindred #	Chr: position (hg19)	Amino acid change	Polyphen-2 (Prediction)	NHLBI	1000G	Turkish DB	gnomAD (overall)	gnomAD (highest frequency)
1	7:150439367	I47T[#]	1.0 (D)	0.00	0	0	0	0
2	7:150439894	L223F[#]	0.999 (D)	0	0	0	0	0
2	7:150439889	N221S[D]	0.996 (D)	0	0	0	1.220e-05	6.498e-05 (South Asian)
3	7:150439838	L204P[D]	0.946 (D)	0.0036	0.001	0.00168	0.002103	0.003024 (European, Non-Finish)
4	7:150439553	P109L[#]	1.0 (D)	0	0	0	4.067e-06	8.979e-06 (European, Non-Finish)

B



A) Chart listing the GIMAP5 mutations and allele frequencies carried by affected individuals in the pedigrees in (A)
B) Sanger sequencing confirmation of the mutations carried by each surviving affected individual.

Figure 2.2A prepared by M.L.G. and S.V., Figure 2.2B by M.L.G.

Parametric linkage analysis using the WES data from the parents was also performed using affected individuals and two unaffected siblings in pedigree 1. Pedigree

3 yielded a LOD score of 1.8, pedigree 4 yielded a LOD score of 1.2 and pedigree 1 yielded a LOD score of at least 6.2, providing a highly significant combined LOD score of 9.2 (data not shown). Overall, the genetics data alone provide a strong argument that mutations in GIMAP5 are responsible for the phenotype of the patients and furthermore that the disease is 100% penetrant with a modest variation in expressivity.

GIMAP5 residue conservation and expression pattern in human tissues

The amino acid residues of GIMAP5 that were altered in the patient cohort are normally highly conserved in primates, more distantly related mammals and also other GIMAP family members. I47 and P109 are absolutely conserved, likely due to their close proximity to the active site of the GTPase domain (Figure 2.3A). The other mutated residues were generally conserved across different members of the human GIMAP family (Figure 2.3A). More distantly related organisms such as fruit flies and zebrafish did not have direct homologues to GIMAP5. Nevertheless, the strong conservation of these residues and the extremely low minor allele frequency of the alleles observed in the patient cohort suggest that the mutated residues are normally critical for GIMAP5 function.

Some mutations (I47T, P109L, L204P) were present in the GTPase domain and close to the active site of the enzyme, while others were very distal and localized in a α-helix outside the GTPase domain (N221S, L223F) (Figure 2.3A). This led me to speculate that the GTPase dynamics or subcellular localization of the protein would be affected. Surprisingly, all mutations had profoundly destabilizing effects on GIMAP5 with T cells from the patients showing drastically reduced levels of GIMAP5 protein

(Figure 2.3B). Furthermore, given the homology of the different GIMAP family members and possibility that they act in the same pathway I hypothesized they may be upregulated in the absence of GIMAP5 in an attempt to compensate. However, in three patients from three different pedigrees, I did not observe significant or reproducible differences in the levels of any other GIMAP family member (Figure 2.3B).

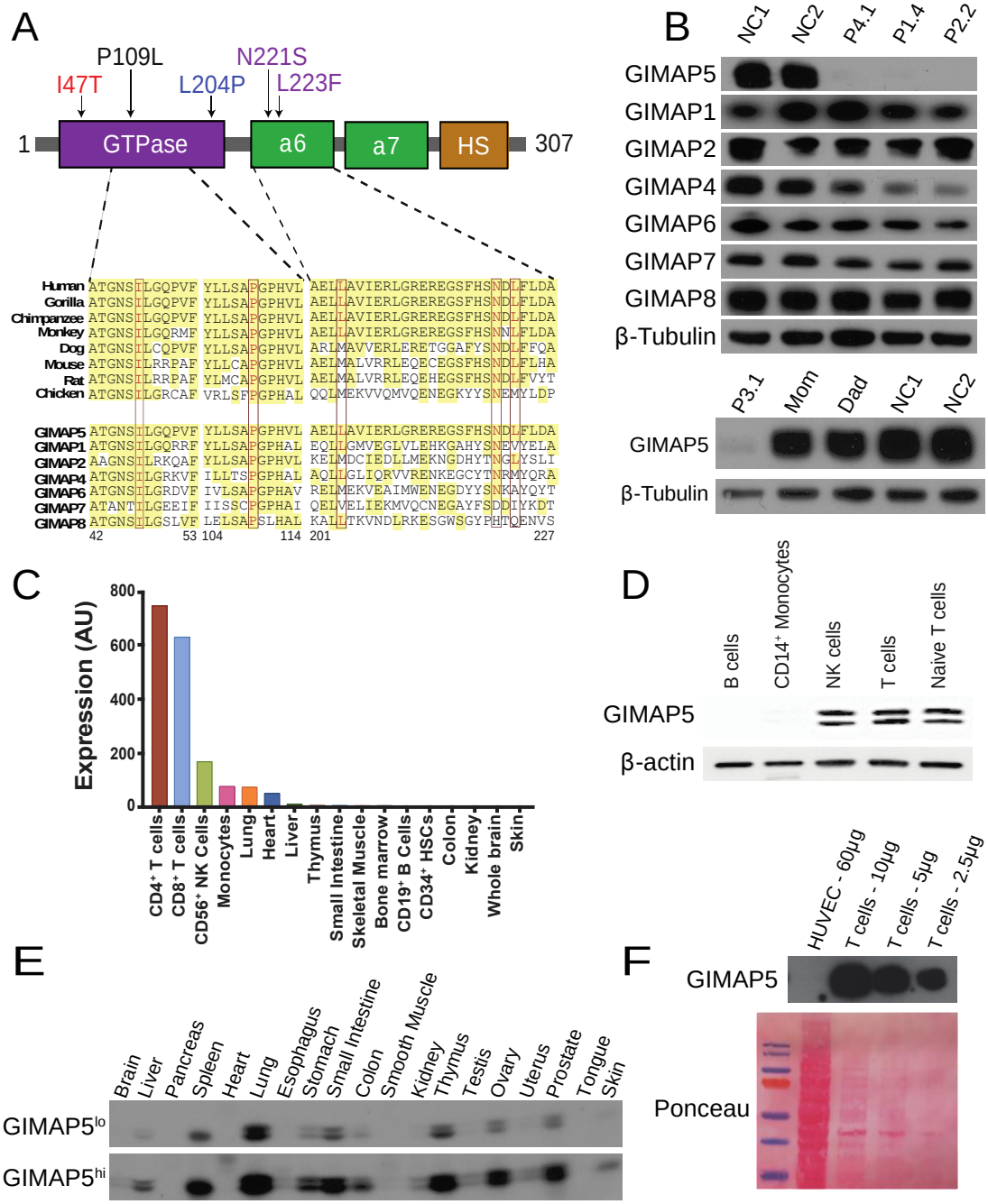
As most studies of GIMAP5 have been performed in mice, I investigated the expression pattern of GIMAP5 in human cells. Using a publicly available database of microarray data from 78 different human tissues, significant GIMAP5 expression was observed in $CD4^+$ and $CD8^+$ T cells (Figure 2.3C). Less substantial expression was also observed in NK cells, monocytes, lungs and heart tissue although in the latter two cases it is unclear if resident immune cells were responsible or if other cell types in these tissues actually express GIMAP5. Interestingly, virtually no expression was observed in $CD19^+$ B cells which is in contrast to what is observed in mice and puzzling given the B cell lymphopenia of the patients (Vivian W.Y. Wong *et al.*, 2010). I confirmed this result by isolating B cells, monocytes, NK cells and T cells and running western blots for GIMAP5 (Figure 2.3D). As expected, robust bands were observed in T and NK cells, very low expression in monocytes and no detectable GIMAP5 in B cells. I also ran a western blot with lysates from a wide variety of other human tissues. Interestingly, I saw significant GIMAP5 expression in a wide variety of tissues such as the liver, lungs, stomach, intestines, ovary and prostate (Figure 2.3E). Unfortunately, similar to the microarray data, it is impossible to determine whether this staining is due to resident lymphocytes or, for example, hepatocytes or alveolar cells. Apparent expression of GIMAP5 in the liver and

lung is particularly provocative due to the liver disease and bronchiectasis observed in multiple GIMAP5-deficient patients. I hypothesized that detection in these tissues may be due to GIMAP5 expression in endothelial cells which has been reported in some online databases (Schaum *et al.*, 2018). To test this I blotted protein lysates from Human Umbilical Vein Endothelial Cells (HUVECs) for GIMAP5. No GIMAP5 expression was detected relative to the titrated lysate from primary human cycling T cell blasts, despite loading 24 times as much total protein in the HUVEC lane (Figure 2.3F). Thus, if GIMAP5 protein is truly expressed in endothelial cells it must be at extremely low levels or is only induced under specific conditions.

Also of interest was my observation that human GIMAP5 migrates as a prominent doublet between 30 and 35 kilodaltons (Figures 2.3B, D, E). I observed that both bands were decreased upon electroporation of siRNA or Cas9-RNP complexes specific for GIMAP5 and were resistant to treatment with phosphatases, Endoglycosidase H and PNGase F (Figure 2.8C, Figure 3.1D, data not shown). This was also observed in Rat, but not murine, Gimap5 (Vivian W Y Wong *et al.*, 2010). Furthermore, overexpression of human or mouse GIMAP5 results in a single band suggesting a genetic origin rather than a post-translational modification, although there are only two known exons with coding sequence which would rule out alternative splicing mechanisms. The precise nature of these two bands remains unclear.

Figure 2.3

Tissue distribution and expression of GIMAP5 in GIMAP5 deficiency



A) Sequence alignments indicating the location and conservation of each of the GIMAP5 mutations carried by patients identified in Figure 2.1. Top alignments show conservation between species while bottom shows conservation amongst the GIMAP family members expressed in humans.
B) Western blots measuring GIMAP family protein levels in patients representing each of the pedigrees described in figure 2.1.
C) Ranked order plot of microarray data showing GIMAP5 expression in various human tissues. Shown are the 17 highest expressing tissues from a larger dataset of 72 tissues (Su *et al.*, 2004).
D) Western blot validating GIMAP5 expression in indicated human cell types.
E) Western blot probing for GIMAP5 expression in a wide variety of human tissues.
F) Western blot measuring GIMAP5 expression in HUVECs.

Figure 2.3A prepared by M.L.G. and S.V., Figure 2.3B, C, E and F by M.L.G and 2.3D by A.M.

Data in B, D and F are representative of 2-3 independent repeats.

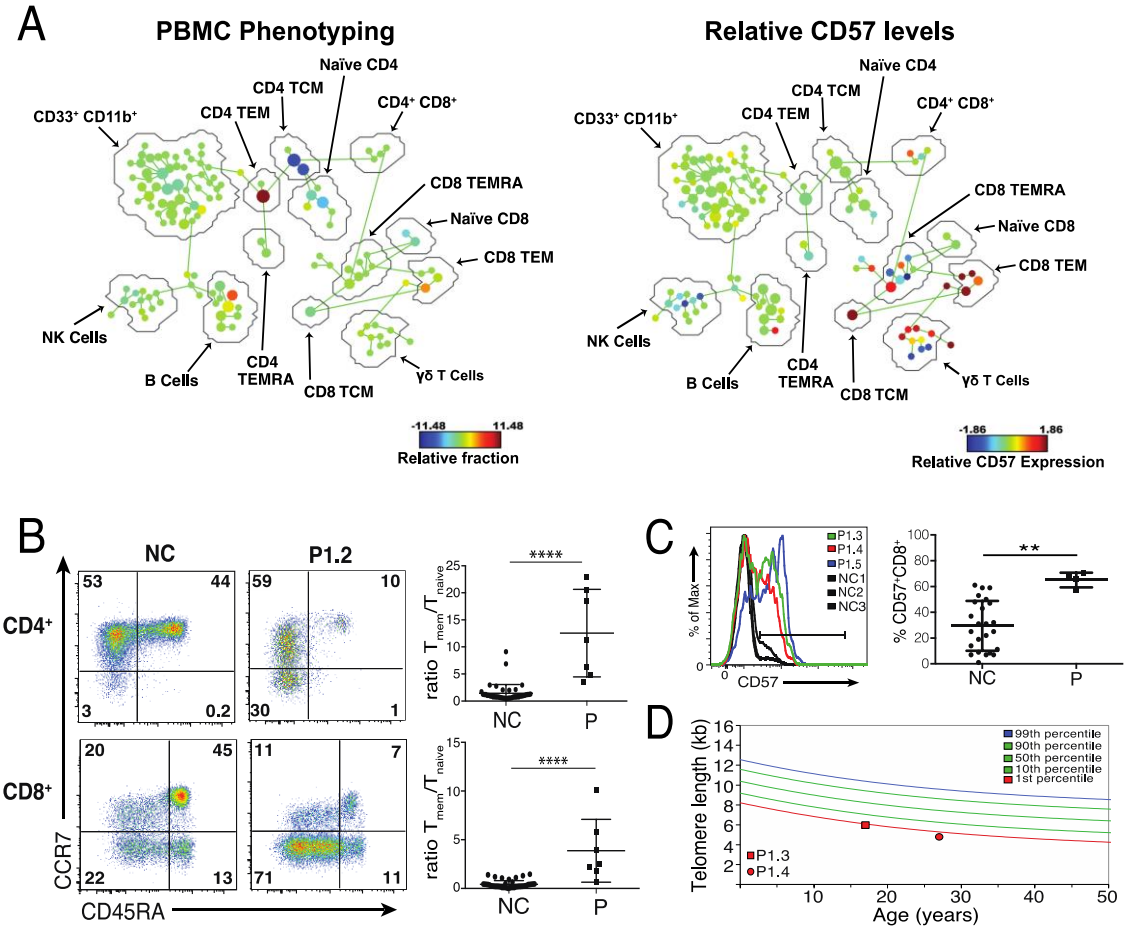
Immune cell phenotyping of patients suffering from GIMAP5 deficiency

Deep phenotyping of the patient immune cell populations from isolated Peripheral Blood Mononuclear Cells (PBMCs) was carried out using Cytometry by Time-Of-Flight (CyTOF) with the goal of gaining some insight into the etiology of the disease caused by GIMAP5 (Leipold and Maecker, 2015). A panel of 38 different surface markers was analyzed via the SPADE software to obtain a global view of the immune dysregulation (Qiu *et al.*, 2011). While major differences in the surface phenotype of αβ T cells were observed, most other immune cell populations were largely unaffected (Figure 2.4A, left panel). Little to no effect was observed on NK cells, γδ T cells, monocytes or dendritic cell populations. The patients showed a decrease in naïve and central memory T cell subsets and a corresponding increase in effector memory subsets (Figure 2.4A, left panel). Another major difference was a pronounced increase of CD57 expression on

virtually all CD8$^+$ and γδ T cell subsets (Figure 2.4A, right panel). CD57 is a marker of replicative senescence and T cell dysfunction, making this finding consistent with LIP.

Figure 2.4

Immune cell phenotyping of GIMAP5-deficient patients



A) CyTOF mass cytometry data of 38 phenotyping markers collected on peripheral blood mononuclear cells (PBMCs) from affected individuals or healthy controls for which higher order data was analyzed and visualized via SPADE. The left panel shows a phenotyping tree with subsets overrepresented in the patient in red and subsets underrepresented in blue. The right panel shows the same tree with coloration showing increased/decreased CD57 expression in the patient.

B) Measurement of naïve T cell fractions (CCR7$^+$CD45RA$^+$) via conventional flow cytometry in healthy donor or GIMAP5-deficient PBMCs. (continued on next page)

C) Measurement of CD57 expression on T cells isolated from human GIMAP5-deficient patients or healthy donors via flow cytometry.
D) Measurement of telomere length in GIMAP5-deficient patients via flow-FISH.

Figure 2.4A prepared by M.L.G., A.F., A.G., M.L., Figure 2.4B,C,D prepared by M.L.G.

Individual points plotted in (B) and (C) represent the mean of individual patients and pooled normal controls averaged across 1-5 independent measurements. Statistical significance was calculated by Student's t-test **p<0.01, ****p<0.0001.

I confirmed the drastic loss of naïve cells and corresponding increase in senescent effector memory cells in both $CD4^+$ and $CD8^+$ T cell populations in these patients via conventional flow cytometric analysis. More specifically, I observed a marked decrease in $CD45RA^+CCR7^+$ naïve phenotype $CD4^+$ and $CD8^+$ T cells with a corresponding increase in $CD45RA^-CCR7^-$ effector memory T cells with little change in the $CD45RA^+CCR7^-$ central memory T cells (Figure 2.4B). Consistent with the CyTOF data I observed a marked expansion of $CD57^+$ T cells in the patients suggesting their cells had undergone multiple rounds of proliferation and became replicatively senescent (Figure 2.4C). Finally, telomere lengths of T cells from two patients were within the 1st percentile for their age consistent with the idea of replicative senescence (Figure 2.4D). Overall, this phenotype is consistent with LIP caused by the chronic lymphopenia observed in the absence of functional GIMAP5. The depletion of naïve T cells and small remainder of senescent T cells can contribute to the immunodeficiency and autoimmunity observed in these patients, particularly considering the very limited expression pattern of GIMAP5 in humans.

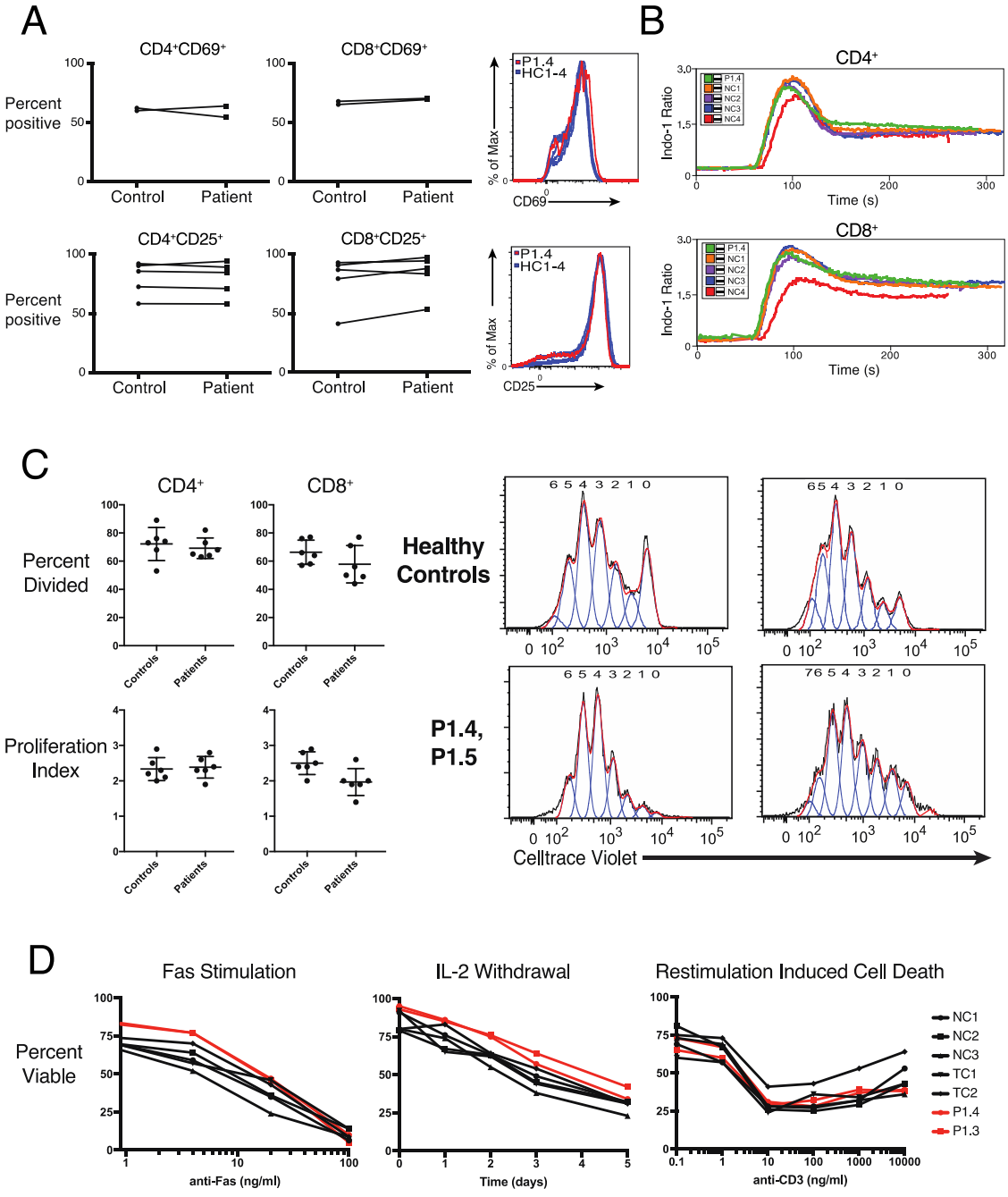
T cells from GIMAP5-deficient patients activate and proliferate normally

I next performed a battery of functional tests on GIMAP5-deficient patient T cells. Murine T cells deficient for Gimap5 show profound defects in survival, activation (calcium flux) and proliferation leading me to hypothesize that these phenotypes would be present in patient cells as well (Ryan D Schulteis *et al.*, 2008a; Ilangumaran *et al.*, 2009; Barnes *et al.*, 2010a). I have been able to confirm the survival and proliferation phenotypes in T cells isolated from GIMAP5$^{sph/sph}$ mice. It is worth pointing out that to date no *in vitro* rescue or acute knockdown/depletion strategy for GIMAP5 has shown that this is a primary cell intrinsic phenotype and not secondary to the lymphopenia or other abnormalities in the GIMAP5$^{sph/sph}$ mice, with the exception of one study showing lithium chloride treatment rescued T cell proliferation (Patterson *et al.*, 2018). Many different experiments looking at activation of T cells from GIMAP5-deficient patients showed no defects in CD69 or CD25 expression following 24 or 72 hours of activation respectively (Figure 2.5A). I next expanded GIMAP5-deficient T cell blasts in IL-2 and acutely restimulated them to measure calcium flux. I observed no defects in calcium flux in these cells relative to controls, contrary to previous studies in rodent models of GIMAP5 deficiency (Figure 2.5B). Even more puzzlingly, acute activation of T cells isolated from GIMAP5-deficient patients revealed no apparent defect in either survival or proliferation in GIMAP5-deficient T cells from multiple patients (Figure 2.5C). While I did observe variability from individual to individual, there was no significant difference in GIMAP5-deficient T cells relative to healthy controls. Finally, I also exposed expanded GIMAP5 deficient human T cell blasts to a variety of apoptotic stimuli such as anti-Fas crosslinking antibody, Restimulation Induced Cell Death (RICD) and cytokine

withdrawal. RICD refers to the phenomenon whereby activated T cells restimulated through the T cell receptor will undergo cell death. None of these treatments showed any increased susceptibility of patient T cells to apoptosis (Figure 2.5D). This result is also contrary to the model put forth by multiple groups claiming that GIMAP5 regulates the intrinsic survival pathway of T lymphocytes via direct interactions with Bcl-2 family members (Chen *et al.*, 2011a).

Figure 2.5

T cells from GIMAP5-deficient patients activate and proliferate normally



A) Paired plots and flow cytometry histograms showing expression of early activation markers CD25 (day 3) and CD69 (day 1) following stimulation of T cells from GIMAP5-deficient patients or healthy donors.
B) Calcium flux plots in expanded T cell blasts from either GIMAP5-deficient patient T cells or healthy donors.
C) Plots showing either the percent divided or proliferation index (left panel) or sample plots of celltrace violet dilution (right panel) in stimulated T cells from either GIMAP5-deficient patient cells or healthy donors.
D) Plots showing viability of T cell blasts from either GIMAP5-deficient patients or healthy donors. Cells were treated with either anti-Fas crosslinking antibody (left panel), cytokine withdrawal (center panel) or TCR restimulation (right panel).

Figure 2.5A, B, C, D prepared by M.L.G.

Defective survival and proliferation of murine GIMAP5$^{sph/sph}$ T cells can be rescued via overexpression of GIMAP5

To date, nearly all studies into the phenotype of GIMAP5 deficiency have used genetic *in vivo* knockout rodent models with loss of GIMAP5 either in all tissues or specifically in CD2 expressing tissues (Ryan D Schulteis *et al.*, 2008b; Barnes *et al.*, 2010a; Aksoylar *et al.*, 2012; Patterson *et al.*, 2018). However, none of the phenotypes described (defective survival, calcium flux, autophagy or hyperactive mTORC1 activity) could be recapitulated in GIMAP5-deficient patient cells or GIMAP5 knockout Jurkat lines. This strongly suggested to me that these phenotypes could be T cell extrinsic and caused by the inflammatory/lymphopenic environment of the Sphinx mouse. However, it was puzzling that most of my expression data in humans pointed to GIMAP5 and the GIMAP family more broadly being mostly lymphoid restricted.

To test this, I undertook two separate strategies. I first established a retroviral expression system to rescue the defects present in the sphinx mice. I cloned either GIMAP5^{WT}, GIMAP5^{L223F} (analogous to a mutation present in human patients),

GIMAP5^{A240X} (mutant lacking C-terminal hydrophobic domains), GIMAP5^{S41N} (unable to bind nucleotide) or GIMAP5^{R122D} (constitutively active mutant, unable to hydrolyze GTP (Schwefel *et al.*, 2013)) into the pMSGV1 vector with a C-terminal T2A-GFP sequence to identify successfully transduced cells. I also generated a 'GIMAP1-GIMAP5 switch' construct which swapped the C-terminal hydrophobic domains of GIMAP5 with that of GIMAP1. This should target this construct to the Golgi complex rather than the lysosome. I then generated retrovirus, tittered it in NIH-3T3 cells which lack endogenous GIMAP5 and verified expression of the various alleles (Figure 2.6A). Despite similar transduction efficiencies, the p.L223F, p.S41N and G1-5 switch alleles had significantly less protein, while the p.A240X allele had virtually none. This would suggest that inability to bind nucleotide or localize to the lysosome lead to degradation of GIMAP5. The constitutively active p.R122D protein was expressed at a similar level to the GIMAP5^{WT} condition.

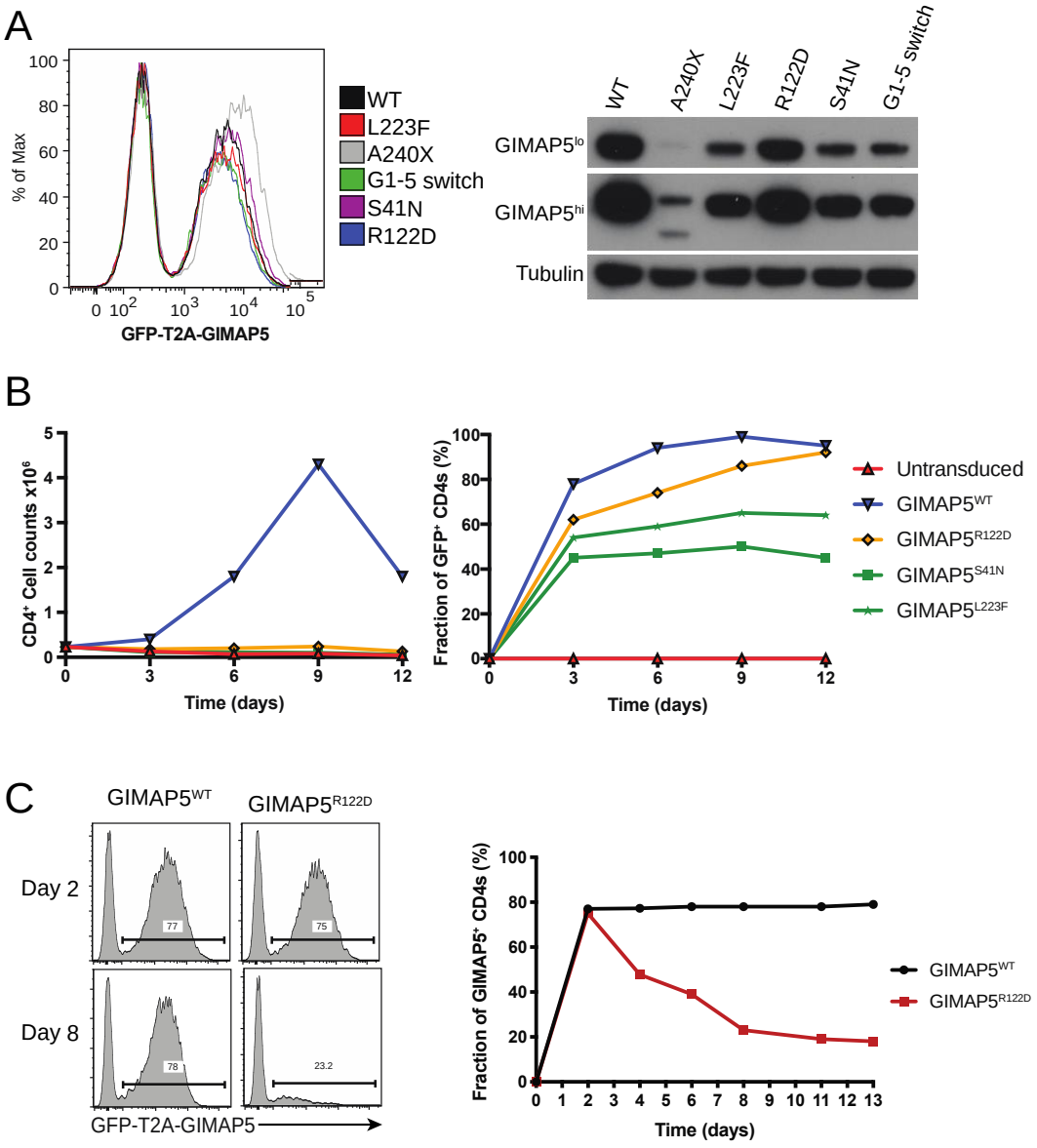
I next activated enriched CD4^{+} T cells from Sphinx mice and transduced with the indicated retroviruses at 24 hours post-activation. As expected, untransduced T cells from Sphinx mice were unable to expand significantly. However, I achieved impressive expansion of the culture when transducing with the GIMAP5^{WT} retrovirus, but none of the other retroviruses (Figure 2.6B). However, all of the constructs were able to infect the T cells as evidenced by 50-80% of the cells becoming GFP^{+} by day 3 post activation (Figure 2.6B). While the WT was slightly higher already at day 3 post activation, I hypothesize that this difference is likely due to these cells already having a survival advantage over their untransduced counterparts. As further evidence that expression of

the GIMAP5^{WT} protein is rescuing the survival of these cells, the culture transduced with this retrovirus rapidly becomes nearly 100% GFP^{+} while presumably the untransduced GFP^{-} cells die or are unable to proliferate. On the other hand, cultures transduced with the S41N or L223F constructs undergo little change between day 3 and day 12, suggesting that these cells have no survival advantage. Interestingly, the R122D^{+} cells are gradually selected for in the culture over time, but do not accumulate and rescue the cell numbers (Figure 2.6B). Overall, these data provide very robust evidence that the survival defect of GIMAP5-deficient murine cells is T cell intrinsic and furthermore capable of being rescued with expression of GIMAP5. Furthermore, it also provides the first evidence to my knowledge that regulation of the GTP-hydrolysis of GIMAP5 is required for proper functioning of the protein.

We also transduced T cells from GIMAP5^{WT} littermates with the same viruses. Interestingly, while I observed no difference in cell counts or in the fraction of GFP-T2A-GIMAP5^{+} cells between GIMAP5^{WT} and most of the other alleles, I did observe a reproducible decrease in fitness in host cells transduced with GIMAP5^{R122D}. While there were identical fractions of cells expressing GIMAP5 at 48 hours post-transduction, the fraction of GFP^{+} cells in the GIMAP5^{R122D} cultures rapidly decreased by nearly half between 48 and 96 hours following exposure to the retrovirus (Fig. 2.6C). This highlights that proper regulation of GTP hydrolysis is required for GIMAP5 function and also that GIMAP5^{R122D} is likely constitutively active and dominant over the endogenous wild type GIMAP5.

Figure 2.6

Defective survival and proliferation of murine Gimap5 $^{sph/sph}$ T cells can be rescued via overexpression of Gimap5



A) Flow cytometry histograms (left panel) and western blots (right) measuring GFP-T2A-GIMAP5 expression in NIH-3T3 cells transduced with the indicated retroviruses.

B) Cell counts (left panel) and fraction of GFP-T2A-GIMAP5^{+} cells in stimulated GIMAP5$^{sph/sph}$ T cells transduced with the same retroviruses as in (A).

C) Sample flow plots and fraction of GFP-T2A-GIMAP5^{+} cells in stimulated GIMAP5WT/WT T cells transduced with the same retroviruses as in (A).

Figure 2.6A, B, C prepared by M.L.G.

Data shown is representative of 2 (A) or 3-4 independent experiments (B,C).

Murine T cell GIMAP5 CRISPR-RNP model recapitulates the phenotype of GIMAP5$^{sph/sph}$ T cells and reveals a link between GIMAP5 and ceramide metabolism

While the rescue experiment did convince me that decreased survival of murine T cells lacking GIMAP5 is a cell intrinsic effect, I also wished to generate an acute loss-of-function model *in vitro* both to cement these findings and provide a convenient model for further functional experiments. To date, no group has published an acute loss of function model to study the role of GIMAP5 and thus previous studies have all been done on T cells generated in a lymphopenic environment. I turned to the recently developed CRISPR-RNP model in order to acutely and permanently eliminate GIMAP5 (Seki and Rutz, 2018). I generated gRNAs *in vitro* by mixing custom CRISPR RNAs (crRNA) with trans-activating crRNAs (tracrRNA) complexed to a fluorescent tag. These gRNAs specific for either GIMAP5 or Thy1 were then bound to purified Cas9 enzyme *in vitro* and electroporated into activated and cycling WT murine T cells.

We observed a remarkably high electroporation and knockout efficiency. Over 90% of the T cells were positive for the tracrRNA fluophore and virtually all of these cells lost surface expression of Thy1.1 within 48 hours (Figure 2.7A). Cells electroporated with the GIMAP5 crRNAs were equivalently positive for the gRNA fluophore but as expected did not lose Thy1.1 expression. I also observed the gRNA

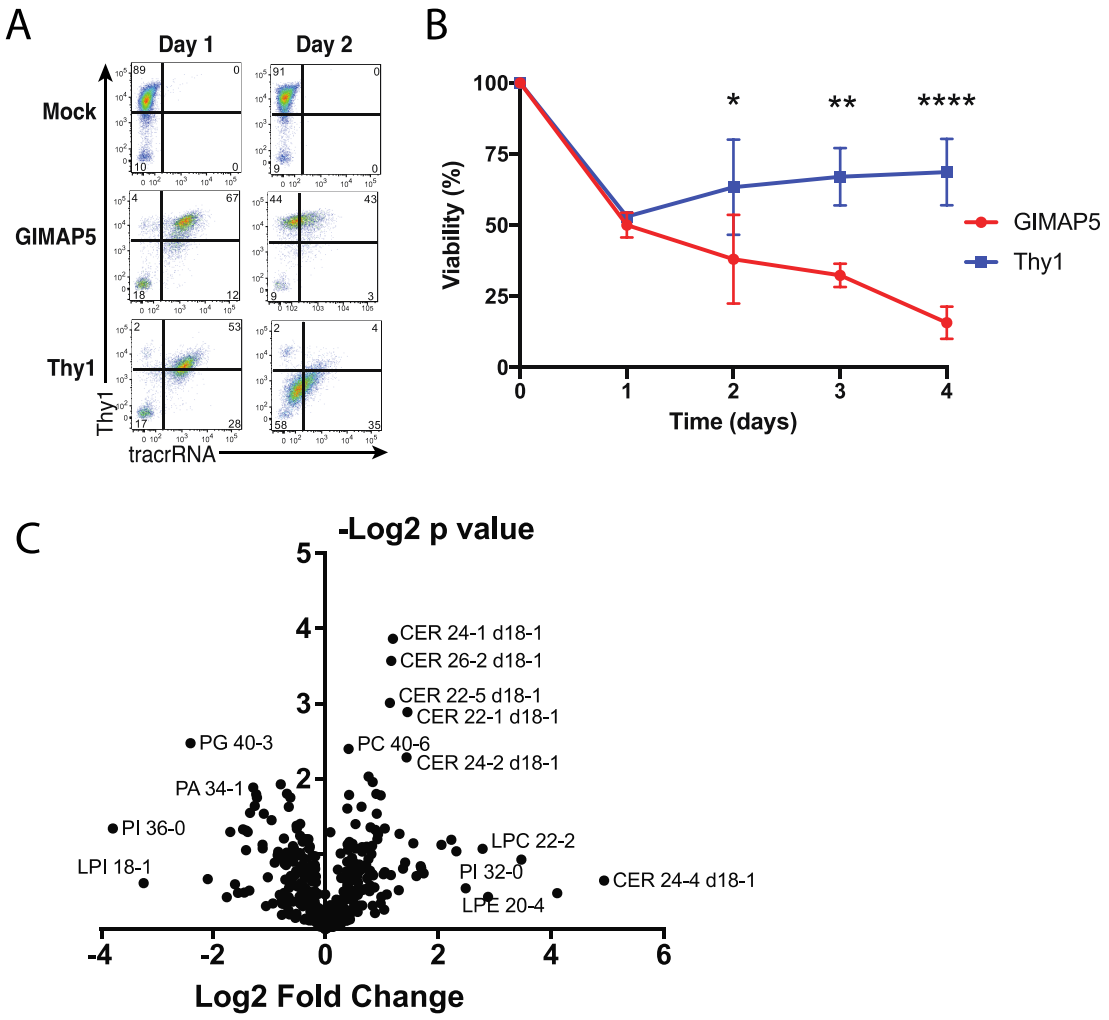
fluophore being rapidly diluted as the cells were actively cycling. Western blotting confirmed a loss of GIMAP5 relative to the Thy1.1 control cultures.

Following electroporation, I tracked viability in the two cultures daily. In every experiment the T cells electroporated with GIMAP5 crRNAs rapidly began dying between 48 and 72 hours post electroporation while the Thy1.1 cells recovered and proliferated (Figure 2.7B). This directly and acutely recapitulated the phenotype of the Sphinx T cells in a well-controlled manner, both verifying the finding that this phenomenon is directly caused by GIMAP5 in a T cell intrinsic manner and providing me with a cleaner model for untangling the biological processes affected by GIMAP5.

In order to begin to understand the biochemical role of GIMAP5 I extracted either lipids or cytoplasmic metabolites for high-throughput analysis via mass spectrometry. I ran five biological replicates comparing cells electroporated with gRNAs specific for GIMAP5 or Thy1 as a negative control. In the lipidomics analysis I observed a significant overrepresentations of multiple ceramide species with long fatty acid chains (Figure 2.7C). Ceramides have long been known to be pro-apoptotic as well as generated during cell death (Bose *et al.*, 1995). Thus, this could potentially represent the biological mechanism by which loss of GIMAP5 leads to cell death.

Figure 2.7

Acute depletion of Gimap5 in murine T cells leads to cell death and accumulation of long-chain ceramides



A) Flow cytometry plots showing CRISPR-RNP electroporation efficiency (tracrRNA) and Thy1.1 expression over time.

B) Viability of stimulated murine T cells over time following electroporation of CRISPR-RNPs specific for GIMAP5. Cells were stimulated for 7-10 days prior to electroporation/day 0. (continued on next page)

C) Plots comparing the Log2 Fold Change (GIMAP5$^{-/-}$/control) versus the p value of various metabolites in GIMAP5/Thy1 CRISPR-RNP electroporated murine T cells, with the most significant/affected species highlighted.

Figure 2.7A, B prepared by M.L.G., Figure 2.7C prepared by M.L.G. and J.E.H.
(continued on next page)

Data shown in (A-B) are representative of over 7 experiments with *p<0.05, **p<0.01, ****p<0.0001. Data in C represent the averages of samples collected from 5 distinct electroporations and extractions.

III. Discussion

These results represent the first description of a substantial cohort of patients suffering from GIMAP5 deficiency. Overall, the clinical and immunological phenotype were similar to that previously described in rodent models (Ryan D Schulteis *et al.*, 2008b; Barnes *et al.*, 2010b). Surprisingly, the functional manifestations in patient T cells were different from what has previously been described in the literature. Indeed, I was unable to discern any consistent defect in GIMAP5-deficient patient T cells in contrast to previous murine studies or a previous study with a single patient carrying mutations in GIMAP5 (Barnes *et al.*, 2010a; Aksoylar *et al.*, 2012; Patterson *et al.*, 2018). Thus, I sought to cast a wider net by defining the transcriptome of human T cells depleted of GIMAP5 via siRNA knockdown. These data again were negative, showing virtually no differences between T cells depleted for GIMAP5 or electroporated with a non-specific siRNA. Previous data has argued for a role for GIMAP5 in regulating FOXO family transcription factors, leading me to expect transcriptional differences (Aksoylar *et al.*, 2012). Overall, these data point towards a major difference in GIMAP5 function between mouse and man in spite of very similar phenotypes at the organism level. This underscores the value of studying the human model as the majority of GIMAP5 research has focused on the T cell survival and proliferation defect, while its absence in the human

model would argue that this is not the cause of the disease or our experimental conditions do not reflect the *in vivo* conditions in humans.

There are significant technical hurdles that could compromise the conclusions mentioned above. Specifically, the GIMAP5-deficient patients and the siRNA knockdown model have residual GIMAP5 protein. The former suffers from missense mutations rather than premature truncations, while the latter is limited both in terms of efficiency (we have measured a ~3-4 fold reduction in protein levels at peak knockdown) as well as duration. The knockdown efficiency shown in Figure 2.8C is at time zero of the RNA-Seq experiment, and thus it is likely that after 12-24 hours of TCR stimulation significant amounts of new GIMAP5 protein were produced. Previous data has shown in the murine model that T cell activation both *in vitro* and *in vivo* is required to induce the survival defect in GIMAP5-deficient cells (Patterson *et al.*, 2018). Thus, it is possible that in the siRNA knockdown model either the residual GIMAP5 protein is sufficient or following stimulation protein levels have already recovered.

Technical considerations aside, there are multiple possible explanations for the mouse-human difference in GIMAP5-deficient T cell phenotypes. Firstly, and what I believe is least likely, it is possible that another GIMAP family member is able to compensate for the loss of GIMAP5. In particular GIMAP2 is absent in mice, although it is possible that another GIMAP family member diverged sufficiently between mice and humans to acquire new functions (Krücken *et al.*, 2004). In order for this to be true GIMAP2 or another family member would have to be able to compensate for the loss of GIMAP5 *in vitro* but not *in vivo* as the GIMAP5-deficient humans are still severely

lymphopenic. Furthermore, I did not observe upregulation of GIMAP2 or any other GIMAP family member in T cells isolated from my GIMAP5-deficient patients. A second possibility is that the survival and proliferation phenotype previously reported in GIMAP5-deficient murine T cells are either T cell extrinsic or caused by the lymphopenic/inflammatory environment. The former at least is very unlikely as depletion of $CD4^+$ T cells in $GIMAP5^{sph/sph}$ mice rescues survival of the animals (Barnes *et al.*, 2010a; Patterson *et al.*, 2018). The latter is more difficult to rule out and would require investigation of the survival/proliferation phenotype either in a mixed bone marrow chimera or by acutely depleting GIMAP5 *ex vivo* from murine T cells as I have done above in human cells. To date, neither of these experiments have been done to formally demonstrate that the survival phenotype is T cell intrinsic and directly caused by a loss of GIMAP5. A third possibility is that GIMAP5 deficiency causes the same defect in both mice and humans, while the specific metabolic alterations lead to much more pronounced survival defects in the murine cells *in vitro.* In this model, the defect in the human GIMAP5-deficient cells is extremely subtle/not captured by the data I present in the previous section or perhaps only present *in vivo*.

GIMAPs are members of the paraseptin family of GTPases. The regulation and function of GTPase activity in these proteins is much less understood than conventional small GTPases whose activity is regulated by GEFs and GAPs. Rather, their GTPase activity is thought to be regulated by homo/heterodimerization (Ghosh *et al.*, 2006; Sirajuddin *et al.*, 2009). Within the GIMAP family only GIMAP2 and GIMAP7 have been shown to have GTPase activity, with GIMAP7 thought to stimulate the GTPase

activity of GIMAP2 (Schwefel *et al.*, 2013). These authors also generated variants which were either unable to hydrolyze GTP and thus constitutively active or unable to bind nucleotide. I generated analogous mutations in GIMAP5 and observed that the analogous mutant that was unable to bind nucleotide was extremely unstable relative to wild type protein, however the constitutively active form was expressed normally. However, neither was able to significantly rescue Sphinx cells from cell death. This provides the first evidence that both binding of nucleotide and appropriate regulation of GTP hydrolysis are required for GIMAP5 function. However, I did not directly demonstrate that the R122D mutant was unable to hydrolyze GTP due to the lack of an assay for endogenous GTPase activity. In support of this being a constitutively active mutant I did observe that overexpression of GIMAP5^{R122D} was toxic to wild type T cells. Overexpression of the same construct in NIH-3T3 cells had no effect, showing that the protein was broadly cytotoxic.

To conclude, I have comprehensively described the functional and clinical characteristics of GIMAP5 deficiency in humans. These studies underscored the importance of studying humans as well as I observed significant differences in the impact of loss of GIMAP5 on human T cells. However, major questions regarding the basic phenotype of loss of GIMAP5 in human and murine T cells remain. Further studies using acute loss-of-function models of GIMAP5 to differentiate between the possibilities outlined above are required to further shed light on the biochemical function of GIMAP5.

CHAPTER 3 – Functional studies of GIMAP5 deficiency

Authors: Michael Leney-Greene, Ann Park, Xijin Xu, Juan Ravell, Lixin Zheng Helen C. Su and Michael J. Lenardo. Michael Leney-Greene designed research, conducted experiments, performed analysis and wrote this section. Ann Park conducted endogenous Co-Immunoprecipitations. Lixin Zheng, Michael Leney-Greene and Xijin Xu performed BioID2 experiments. Juan Ravell and Michael Leney-Greene performed PLA experiments. Helen C. Su and Michael J. Lenardo helped design experiments and write the paper.

I. Summary

The biochemical role of GIMAP5 remains unclear despite decades of study and multiple conflicting mechanisms having been proposed. Furthermore, virtually all of these studies have been conducted using T cells isolated from Gimap5-deficient mice which could yield many conclusions based on phenotypes secondary to the LIP and the inflammatory *in vivo* environment. To address these issues, I undertook high-throughput RNA-Seq in an siRNA knockdown model as well as BioID2 labeling studies to define the interactome. The former revealed surprisingly little impact of GIMAP5 knockdown on the transcriptome while the latter identified three main complexes in close proximity to GIMAP5 in the cell: the Ragulator complex, the T cell receptor complex and various complexes involved in cellular trafficking. I went on to confirm interactions between GIMAP5 and the Ragulator complex via two independent methods. These new interacting partners suggested that GIMAP5 may play a role in MAPK/mTORC1 signaling from the surface of the lysosome.

In this section I performed further functional studies using siRNA/CRISPR strategies to acutely deplete GIMAP5 in murine and human T cells and test possible functional implications of the interaction with the Ragulator complex. While I was able to show clinical benefit in both mice and humans treated with mTORC1 inhibitors, multiple models of GIMAP5 deficiency showed no direct effect of acute GIMAP5 depletion on mTORC1 signaling or proliferation. Thus, the benefit conferred on the GIMAP5-deficient patients was most likely due to secondary immunosuppressive effects. I also describe for the first time defects in autophagy in murine T cells deficient for GIMAP5,

although I again was unable to reproduce this in human cells suggesting that this was secondary to the *in vivo* environment of the Sphinx mouse or a mouse-human difference. Lastly, as GIMAP5 is a lysosomal protein and the Ragulator has previously been reported to regulate lysosomal dynamics, we tested lysosome number in GIMAP5-deficient human T cells (Pu *et al.*, 2015; Pu, Keren-Kaplan and Bonifacino, 2017a). Overall, I conclude that there are clear mouse-human differences in the *in vitro* manifestation of the underlying phenotype in T cells, and furthermore, that in human cells GIMAP5 most likely does not regulate mTORC1. Thus, the functional importance of the interaction with the Ragulator complex remains unclear.

II. Results

BioID2 reveals three major clusters of proteins in close proximity to GIMAP5 in human T cells

As previous work on GIMAP5 has focused primarily on the *in vivo* phenotype of GIMAP5-deficient mice and the molecular role of this gene remains unclear, I sought to investigate which pathways GIMAP5 may be involved in via a series of high-throughput approaches. In particular, knowledge of the GIMAP5 interactome and identification of true interacting partners could shed light upon its biochemical role within the cell. BioID2 is a recently developed method which involves overexpressing a fusion protein of the bacterial biotin ligase BirA to the gene of interest (bait) (Kim *et al.*, 2016). The bait fusion protein is able to biotinylate prey proteins within a 10-25nm radius depending on the size of the linker of the fusion protein. Biotinylated proximal partners can then be captured via streptavidin and identified by mass spectrometry (Figure 3.1A).

With this goal in mind constructs containing GIMAP5 N-terminally tagged with the BirA enzyme or the BirA enzyme alone were cloned and used to generate stably expressing Jurkat cell lines. A Jurkat cell line whose endogenous GIMAP5 locus had been disrupted via CRISPR in order to ensure that 100% of the GIMAP5 expressed in these cells was tagged was used. The full results of the enriched genes are shown in Table 2, with the enrichment being calculated as the ratio of intensities for a given protein in cell lines expressing BirA-GIMAP5 versus BirA alone. The list of enriched genes was also plotted comparing overall intensity, enrichment in the GIMAP5 cells compared to negative controls, and the enrichment p-value added as a heatmap (Figure 3.1A). Some of the most significantly enriched proteins, including GIMAP5, members of the Ragulator complex (LAMTOR1-5) and several CD3 subunits are labeled.

Table 2: Most significantly enriched proximal proteins by BioID2

Gene names	mean log2 ratio	Peptides	Unique peptides	-Log10 T-test p-value	Protein names
AF1Q;MLLT11	5.35	4	4	1.72	Protein AF1q
TMEM237	4.38	11	11	2.00	Transmembrane protein 237
TMEM138	4.12	3	3	4.81	Transmembrane protein 138
CD3E	3.69	8	8	2.75	T-cell surface glycoprotein CD3 epsilon chain
CD3D	3.55	7	7	1.55	T-cell surface glycoprotein CD3 delta chain
LAMTOR5	3.43	3	3	2.62	Ragulator complex protein LAMTOR5
IGF2R	3.35	94	2	1.74	Cation-independent mannose-6-phosphate receptor
GIMAP5	3.31	24	20	2.44	GTPase IMAP family member 5
TPD52L2	3.01	13	13	2.54	Tumor protein D54
LAMTOR3	2.96	4	4	2.54	Ragulator complex protein LAMTOR3
SNAPIN	2.94	3	3	1.08	SNARE-associated protein Snapin
STIM1	2.79	26	26	2.41	Stromal interaction molecule 1
LOH12CR1	2.72	6	6	0.99	Loss of heterozygosity 12 chromosomal region 1 protein
RRAGC	2.67	9	4	2.80	Ras-related GTP-binding protein C
LNPEP	2.66	30	30	3.37	Leucyl-cystinyl aminopeptidase
ITGAL	2.59	31	31	1.26	Integrin alpha-L
ITGB1	2.54	13	13	1.75	Integrin beta-1
CD2	2.53	6	6	2.14	T-cell surface antigen CD2
LAMTOR4	2.53	2	2	1.54	LAMTOR4
TMPO	2.50	20	8	2.05	Thymopoietin
TPD52	2.44	9	2	4.45	Tumor protein D52
LAMTOR2	2.30	4	4	1.42	Ragulator complex protein LAMTOR2
TFRC	2.27	25	1	1.79	Transferrin receptor protein 1
SYBL1;VAMP7	2.21	6	6	1.42	Vesicle-associated membrane protein 7
LAMTOR1	2.19	6	6	2.11	Ragulator complex protein LAMTOR1
SNAP23	2.18	4	4	1.39	Synaptosomal-associated protein 23
YKT6	2.18	7	7	1.11	Synaptobrevin homolog YKT6
CD4	2.18	7	7	1.64	T-cell surface glycoprotein CD4
BLOC1S2	2.10	4	4	1.64	Biogenesis of lysosome-related organelles complex 1 subunit 2

In order to better visualize the data, I used the list of significantly enriched proteins to generate a protein-protein interaction network using STRING (Figure 3.1B)

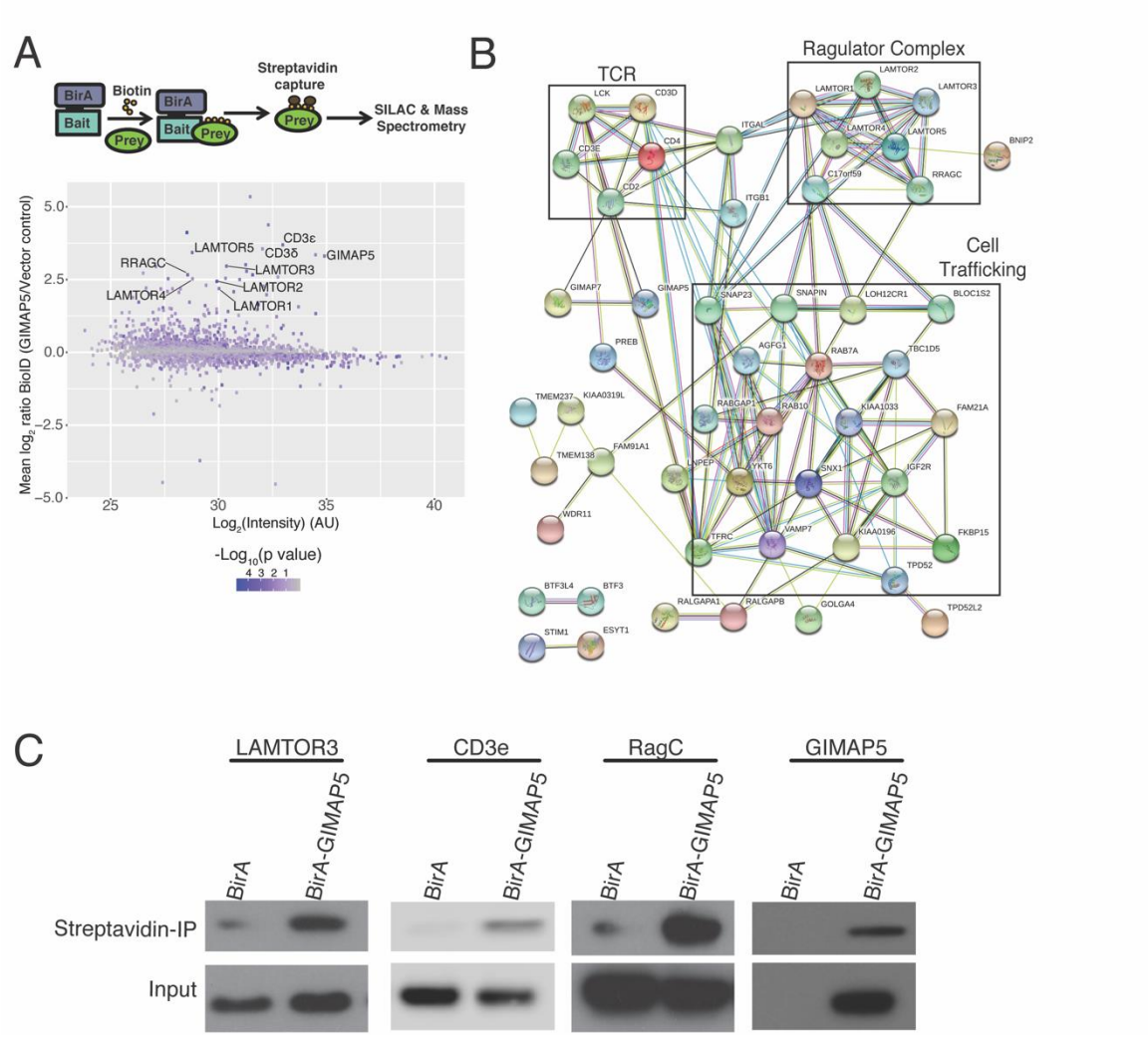
(Szklarczyk *et al.*, 2015). Strikingly, across three separate experiments I observed very high enrichment scores for every single member of the Ragulator complex as well as the Rag GTPases. While the precise member of the Ragulator complex that is directly binding to GIMAP5 (if any) remains unclear, this is nevertheless strong evidence that GIMAP5 is in close proximity to the complex. Interestingly, another cluster of candidate interacting proteins were members of the TCR complex. This may represent TCR proteins that have been internalized from the immune synapse and brought to the lysosome for degradation or may reflect GIMAP5 trafficking to be proximal to the cell surface. It is reminiscent of a complex on the surface of the lysosome recently described by Phelan *et al.* which comprises of the BCR, Ragulator complex and MyD88 (Phelan *et al.*, 2018a). The exact nature of this interaction is puzzling however considering that the biotin ligase end of the GIMAP5-BirA fusion protein should be cytosolic, while many of the biotinylated proteins identified only have small tails on the cytoplasmic side of the membrane.

To further confirm my results, I also carried out low-throughput verification by western blot of some candidate interacting genes. I did not label with SILAC media but otherwise followed the same lysis and biotin-streptavidin capture protocol. I confirmed that the BirA-GIMAP5 fusion protein was able to preferentially biotinylate LAMTOR3, RagC and TCRε relative to the BirA enzyme alone (Figure 3.1C). There was significant background signal from the enzyme alone, presumably from spurious non-specific labeling as it is likely that the enzyme disperses throughout the cytosol in the absence of any targeting sequence. It is also likely that the SILAC/mass-spectrometry approach is

more sensitive than the western blot and better able to demonstrate the enhanced biotinylation with the GIMAP5 construct. Overall these findings represent strong evidence that GIMAP5 is interacting with members of the Ragulator complex and may hint at a larger supercomplex including the TCR as well as various endocytic trafficking components.

Figure 3.1

BioID2 reveals three major clusters of proteins in close proximity to GIMAP5 in human T cells



A) Heatmap plotting the relative enrichment of a given protein in Jurkat cells expressing BioID2-GIMAP5 fusion protein relative to Jurkats expressing BioID2

alone, versus the Log2(intensity) of the mass spectrometry signal. The heatmap signal represents the associated p value.

B) STRING diagram showing a putative interactome of BioID2-GIMAP5 subdivided into three broad clusters.
C) Western blots for lysates of Jurkat cells expressing either BioID2 alone or BioID2-GIMAP5 followed by streptavidin capture. Blots for the indicated proteins were carried out in either the streptavidin capture or input.

Figure 3.1A prepared by L.Z. and X.X., Figure 3.1B by M.L.G. and L.Z., Figure 3.1C by M.L.G.

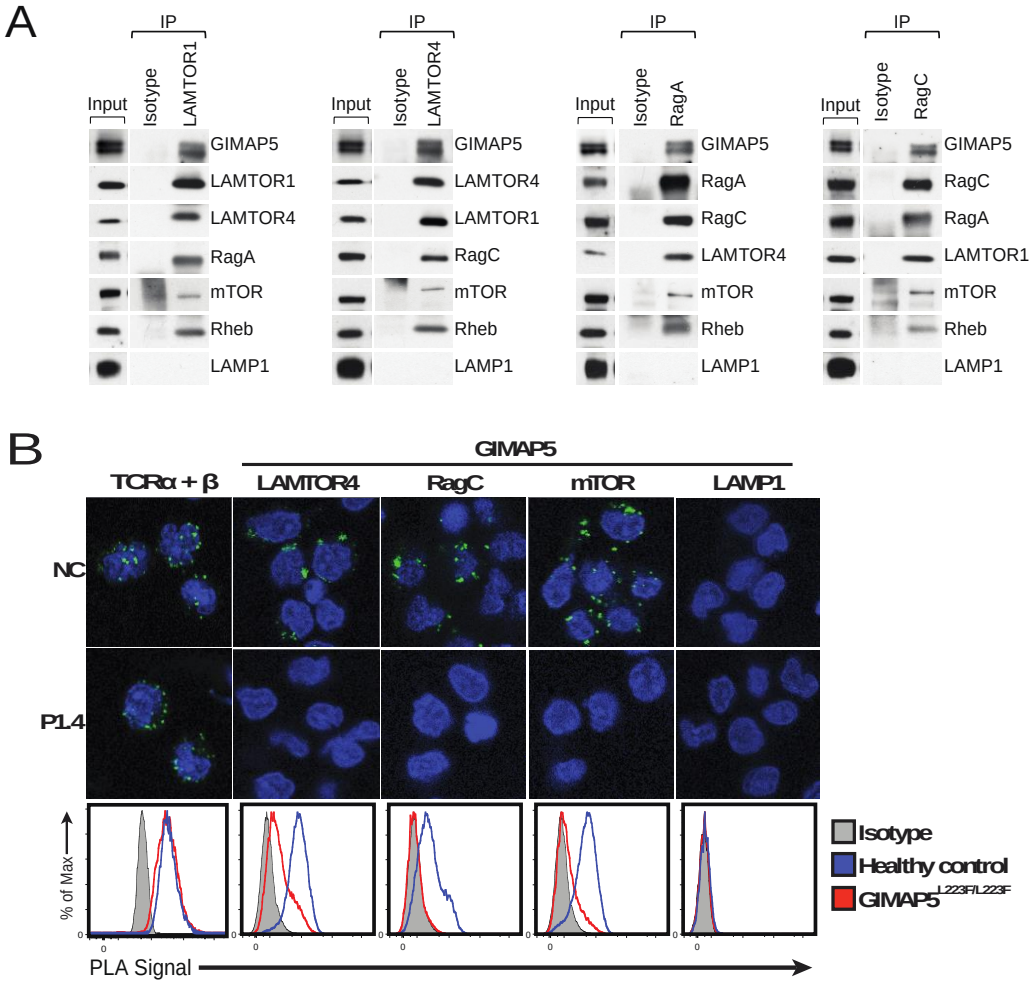
Data in (A-B) represent three pooled experiments. Data in (C) are representative of three independent experiments.

PLA and conventional Co-IPs confirm that GIMAP5 interacts with the Ragulator complex

I sought to confirm the data generated by the high-throughput BioID2 approach via conventional co-IPs. With this in mind, virtually all the different members of the Ragulator complex immunoprecipitated from human T cell lysates and blotted for GIMAP5. In line with the data from the high throughput approach, I observed robust association of GIMAP5 with LAMTOR1, LAMTOR4, RagA and RagC (Figure 3.2A). As expected from the literature, all of these proteins immunoprecipitated as a unit confirming that they act as a large complex in T lymphocytes similarly to findings from other cell types. Both bands from the GIMAP5 doublet interacted with the Ragulator complex equivalently. In addition to the isotype rabbit IgG used as a negative control, LAMP1 was also blotted for to be certain my immunoprecipitation strategy was not isolating the entire lysosome. There was no observed immunoprecipitation of LAMP1 across multiple repeats (Figure 3.2A).

Figure 3.2

PLA and conventional co-immunoprecipitations confirm that GIMAP5 interacts with the Ragulator complex



A) Western blots of Co-immunoprecipitations for the indicated proteins from T cell blasts of healthy donors.
B) PLA in T cell blasts from either healthy donors or a GIMAP5-deficient patient.

Figure 3.2A prepared by A.P., Figure 3.3B prepared by M.L.G. and J.R.

Data shown in (A-B) are representative of three independent experiments.

As further validation that GIMAP5 is truly interacting with the Ragulator complex, I utilized Proximity Ligation Assay (PLA) using complexed antibodies specific for GIMAP5, members of the Ragulator/mTORC1, or LAMP1 as a negative control on the surface of the lysosome (Figure 3.2B). This technique allowed me to rule out spurious interactions caused by dissolving all the cell membranes and mixing proteins that normally are not adjacent to each other as occurs during conventional co-immunoprecipitations. I used both flow cytometry and confocal microscopy to confirm my results, showing robust PLA signals in T cell blasts from both normal controls and GIMAP5-deficient patients when using antibodies specific for TCRα and TCRβ as a positive control. On the other hand, as a negative control, using PLA antibodies for GIMAP5 and LAMP1 (which was absent from my BioID2 and conventional co-immunoprecipitation data) showed no positive signal in T cells from healthy donors or a GIMAP5-deficient patient (Figure 3.2C). On the other hand, I observed robust signals between GIMAP5 and either LAMTOR4, RagC and mTOR. This confirms that GIMAP5 is in very close proximity to these proteins *in vivo*, on the order of 10nm or closer.

siRNA mediated knockdown of GIMAP5 in human T lymphocytes has little effect on the transcriptome

While the BioID2 and FLAG-immunoprecipitation studies were meant to define the interactome of GIMAP5, I sought to complement these with RNA-Seq studies following GIMAP5 depletion in order to discover pathways regulated by this protein. I hypothesized that loss of GIMAP5 would generate a unique transcriptional signature following disruption of the metabolism of the cell.

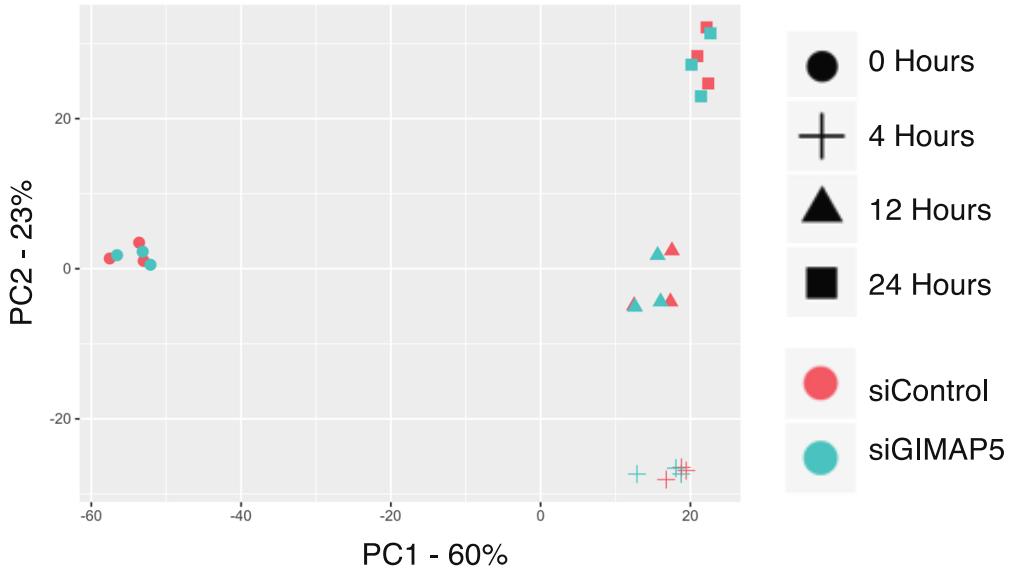
I isolated T cells from three healthy donors and electroporated them with siRNAs specific for GIMAP5. Following depletion of the protein (Figure 3.3C), cells were stimulated for 0, 4, 12 and 24 hours with anti-CD2/3/28 magnetic beads and RNA was isolated for sequencing. I then analyzed the data via Principal Component Analysis (PCA) and plotted the two largest principal components, accounting for 60% and 23% of the variability respectively (Figure 3.3A). PC1 seemed to account for most of the variability caused by stimulation, with the unstimulated samples separating from the 4, 12 and 24 hour samples along the x-axis. PC2 accounted for the variation caused during the TCR stimulation time course, with the 4, 12 and 24 hour samples clustering separately primarily on the y-axis. Contrary to my hypothesis, I observed no clear transcriptional signature caused by depletion of GIMAP5 in primary human T cells at any time point or condition. These samples clustered remarkably close to each other given that each was isolated from a different individual.

I plotted the most significantly affected genes in the GIMAP5 knockdown condition relative to the siCtrl (Figure 3.3B). A number of genes were highly significantly changed, although the magnitude was small with most showing less than a twofold change in expression. I chose the most reproducible and highest differentially regulated gene, WD Repeat domain 12 (WDR12), to verify the results. I saw roughly a twofold reduction in WDR12 levels following depletion of GIMAP5 from T cells of three independent donors which was consistent with my high-throughput RNA-Seq data (Figure 3.3D).

Figure 3.3

siRNA mediated depletion of GIMAP5 in human T lymphocytes has little effect on the transcriptome

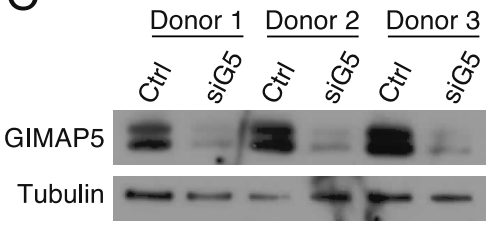
A



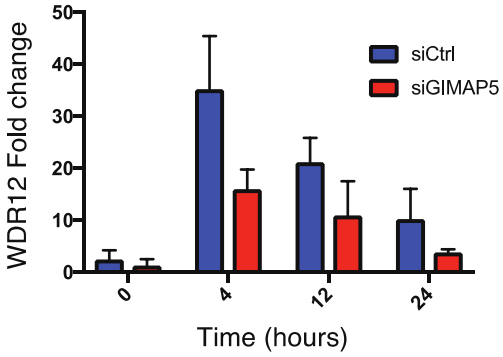
B

0hrs	log2 Fold Change	Adjusted p value
SUMO2	-0.69	1.78E-08
RPS27	-0.68	3.77E-08
ACTR3	-0.45	3.77E-08
ARL6IP1	-0.54	4.01E-08
LEPROTL1	-0.70	3.95E-07
NUCKS1	-0.75	6.54E-07
HSPA9	-0.62	5.30E-06
ATP13A3	-0.52	9.43E-06
4hrs		
ACTR3	-0.67	9.95E-21
HSPA9	-0.62	7.44E-06
WDR12	-0.79	1.15E-05
12hrs		
ACTR3	-0.60	5.54E-17
WDR12	-1.08	1.57E-12
HSPA9	-0.68	4.86E-08
24hrs		
WDR12	-1.09	4.25E-12
ATP13A3	-0.56	5.58E-07
KRAS	-0.76	1.53E-06

C



D



A) Principle component analysis of RNA-Sequencing data from healthy donor T depleted of GIMAP5 via siRNAs. Cells were stimulated for the indicated times.
B) Table indicating the most differentially regulated genes at each timepoint as well as the associated adjusted p value.
C) Western blots validating the knockdown efficiency from the same batches of cells used for analysis in (A-B).
D) Validation of the most differentially regulated gene identified in each condition of (C) via qPCR using samples independent from (A-C)

Figure 3.3A,B prepared by M.L.G. and Y.Z., Figure 3.3C, D prepared by M.L.G.

Data plotted in A-C represent three independent experiments. Data plotted in D are the means of three independent experiments.

Autophagy is dysregulated in GIMAP5-deficient murine but not human T cells

Multiple lines of evidence led me to suspect that GIMAP5 may play a role in autophagy and metabolism in T lymphocytes. Firstly, the phenotype of GIMAP5-deficient mice is very similar to the phenotype of T cells in which critical autophagy genes have been conditionally deleted (Pua *et al.*, 2007; Roy *et al.*, 2013). Both suffer from lymphopenia which is more pronounced in the $CD8^+$ T cell fraction, a decreased fraction of naïve T cells and exhibit defective T cell proliferation in response to antigen (Roy *et al.*, 2013). Secondly, both interaction studies (BioID2 and overexpression) I carried out suggested that GIMAP5 interacted with proteins involved in regulating autophagy, such as multiple Rab trafficking proteins as well as the Ragulator complex. Thus, I sought to evaluate the autophagic flux in GIMAP5-deficient mice and humans.

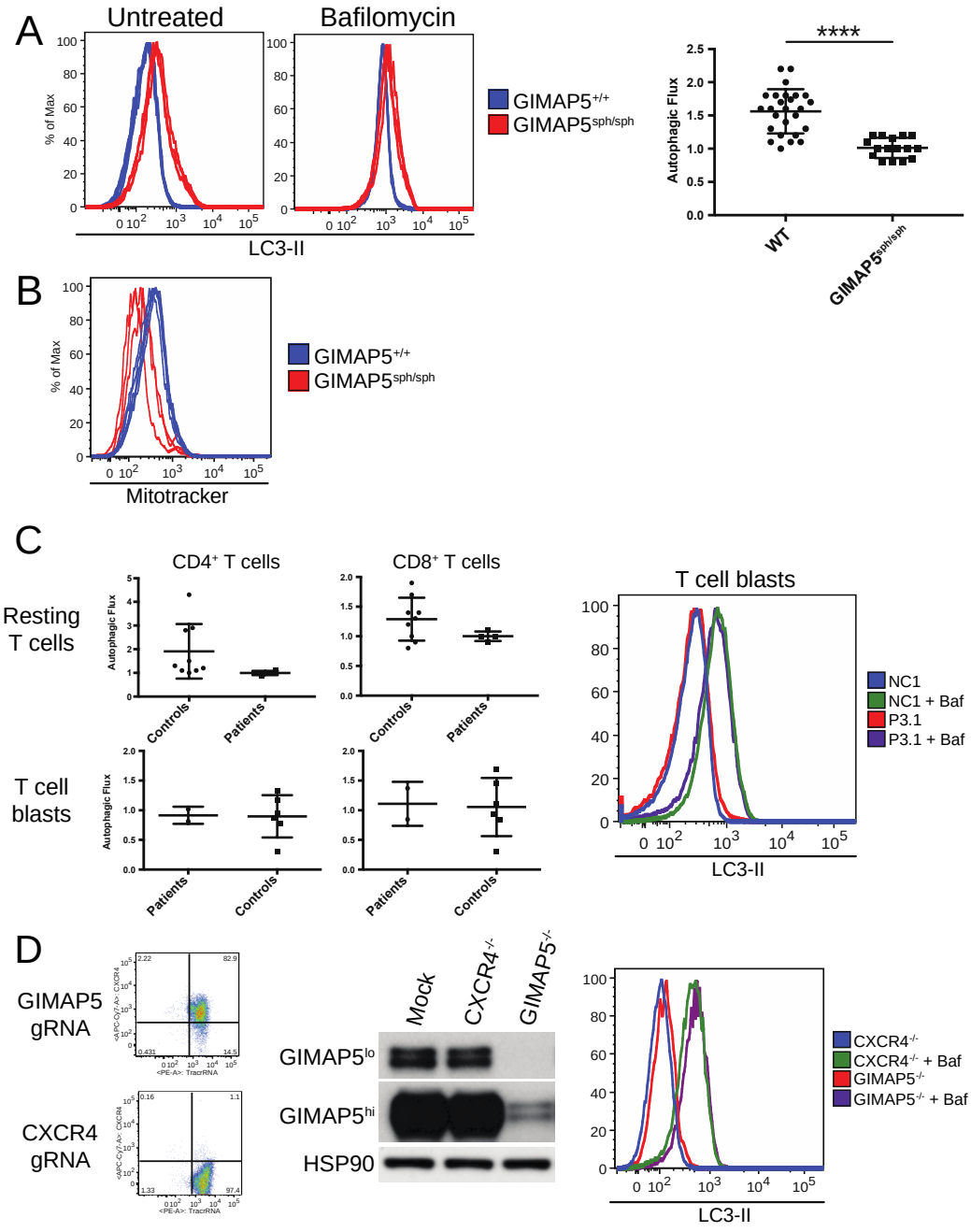
When analyzing T cells directly *ex vivo* from $GIMAP5^{sph/sph}$ mice, I consistently observed a highly significant increase in levels of LC3-II staining by flow cytometry (Figure 3.4A). Even in the absence of bafilomycin the $GIMAP5^{sph/sph}$ cells had high

levels of staining for LC3-II. These findings could be due to either a block in the trafficking/degradation of autophagosomes or increased activity through this pathway. In order to differentiate between the two, I treated the cells with bafilomycin to prevent acidification of the lysosomes and artificially block degradation of the autophagosomes, allowing LC3-II to accumulate. While I still observed increased LC3-II staining in the GIMAP5-deficient cells with bafilomycin treatment, the calculated autophagic flux in the GIMAP5$^{sph/sph}$ T cells was significantly lower, pointing towards a block in the pathway rather than an increase in the rate of autophagy initiation (Figure 3.4A right panel).

This led me to hypothesize that GIMAP5 may be involved in the fusion or trafficking of autophagosomes to the lysosome in unstimulated T cells. Unfortunately, the proliferation defect is so severe in T cells isolated from GIMAP5$^{sph/sph}$ mice that I was unable to evaluate autophagic flux in cycling Gimap5-deficient murine T cell blasts, and, to date I have not investigated this phenomenon in acute depletion models of murine Gimap5. In mouse models with T cells deficient for Atg5, Atg7 or Rab7 it has been shown that mitochondria accumulate due to defective mitophagy (Roy *et al.*, 2013). However, it is worth noting that the Rab7 knockouts had decreased mitotracker staining and increased TOMM20 staining relative to controls, potentially due to the autophagosome membrane being impermeable to the mitotracker dye. T cells from Sphinx mice more closely resembled the Rab7$^{-/-}$ mice than the Atg5$^{-/-}$ cells, again supporting a role for GIMAP5 in autophagosome trafficking and/or fusion with the lysosome rather than autophagosome initiation (Figure 3.4B).

Figure 3.4

Autophagy is dysregulated in Gimap5-deficient murine, but not human, T cells



A) Murine CD4^{+} T cells isolated from littermate controls or GIMAP5$^{sph/sph}$ murine lymph nodes and spleen were stained for LC3-II directly *ex vivo*, and autophagic flux was calculated as described in the methods section.

B) Cells as in (A) were stained and analyzed for Mitotracker.
C) Autophagic flux was measured in resting or activated human T cells from healthy controls or GIMAP5 patients.
D) GIMAP5 was knocked out of human T cells isolated from healthy donors and autophagic flux was measured.

Figure 3.4A, B, C, D prepared by M.L.G.

Data shown in (A) represents pooled data from three individual experiments with each point representing a mouse, ****$p<0.0001$ by Student's t test. Data in (C) represents pooled data from 2-4 experiments with each point representing an individual. Data in (B) and (C) are representative of 2-3 experiments.

I sought to test the same phenomenon in GIMAP5-deficient patient T cells to verify that this function was conserved in human cells deficient for GIMAP5 as well. To my surprise, I observed only a very mild defect in autophagy in unstimulated T cells isolated from GIMAP5-deficient patients which did not reach statistical significance (Figure 3.4C – top panel). At baseline in resting human T cells, the flux through the autophagy pathway was minimal. I reasoned that increasing flux through the system via TCR stimulation may reveal any defects in the system, especially if the loss of GIMAP5 leads to defective autophagosome trafficking or fusion with the lysosome. However, the flux from cycling patient T cell blasts was identical to that in normal controls (Figure 3.4C – bottom panel).

We hypothesized that this discrepancy between the human patients and mice deficient for Gimap5 may be due to residual GIMAP5 protein as all of the patients carry missense mutations. While Gimap5$^{sph/sph}$ mice also carry a missense mutation in Gimap5, it has been shown that protein levels are extremely low and they strongly phenocopy Gimap5 null mice (Barnes *et al.*, 2010a). Furthermore, the human patients have very few

naïve cells and are not age/sex matched to my normal controls leading me to believe that some of these factors may be preventing the detection of an autophagy defect in the patient cells. To address these possibilities, I isolated T lymphocytes from healthy human donors and electroporated the cells with Cas9/gRNA Ribonucleoproteins (RNPs) specific for GIMAP5 or CXCR4 as a negative control. This approach presumably would induce frameshift mutations at the GIMAP5 locus and yield human T cells truly devoid of GIMAP5 protein. I was able to track cells which acquired the complex via tracrRNA labeled with the Atto™ 550 dye and typically observed an electroporation rate of greater than 90% of the viable cells. Impressively, as soon as 24 hours post-electroporation I observed >95% of the tracrRNA^{+} cells electroporated with Cas9 RNPs specific for CXCR4 expressed virtually no protein on their surface (Figure 3.4D, left panel). As the GIMAP5 Cas9 RNPs gave similar electroporation efficiencies, I was confident that I was generating true GIMAP5 knockout cells. In order to ensure that all GIMAP5 protein was eliminated and also increase the autophagic flux, I stimulated the electroporated cells and cycled them in IL-2 for several days before measuring GIMAP5 levels via western blot. I observed a massive reduction in GIMAP5 levels, with the residual protein likely being present due to cells that were able to successfully repair the GIMAP5 locus or failed to be electroporated (Figure 3.4D, middle panel). However, even in these cells completely lacking GIMAP5, I did not observe significant defects in levels of LC3-II either with or without bafilomycin (Figure 3.4D right panel). The two most likely explanations for these data are that the defect in autophagy observed in the GIMAP5$^{sph/sph}$ murine T cells is a secondary effect caused by the LIP/inflammatory environment or there is a major mouse-

human difference in the biochemical role of GIMAP5. Furthermore, in order for the latter possibility to be true, this would have to mean that the clinical phenotype (which is very similar in mice and humans) is unrelated to the defect in autophagy and thus diminishes its importance.

Inhibition of mTORC1 provides clinical benefit in both murine and human GIMAP5 deficiency

The GIMAP5-deficient Sphinx mice share many characteristics with mice carrying alleles leading to hyperactive mTORC1 in addition to mice with defects in autophagy. For example, mice deficient for TSC1 (a negative regulator of mTORC1) also suffer from peripheral lymphopenia with normal thymopoiesis, increased apoptosis, defective proliferation of T lymphocytes and a loss of peripheral naïve T cells (Yang *et al.*, 2011). Similarly, human patients with mutations in the PI3K subunit p110δ have a clinical phenotype that significantly overlaps with the GIMAP5-deficient patients (Lucas *et al.*, 2014). Furthermore, previous work has shown that T cells isolated from GIMAP5$^{sph/sph}$ mice had increased mTORC1 activity at baseline as measured by the phosphorylation levels of pS6 at serines 235 and 236 (Chen *et al.*, 2015). This led me to hypothesize that GIMAP5 may be a T cell specific negative regulator of mTORC1 via interactions with the Ragulator complex.

We confirmed previous findings regarding increased levels of pS6 as well as increased cell size in peripheral T cells from GIMAP5$^{sph/sph}$ mice via flow (Figure 3.5A). Before testing this hypothesis in human cells, I sought to test whether inhibition of mTORC1 via rapamycin treatment could yield clinical benefits in the murine model in

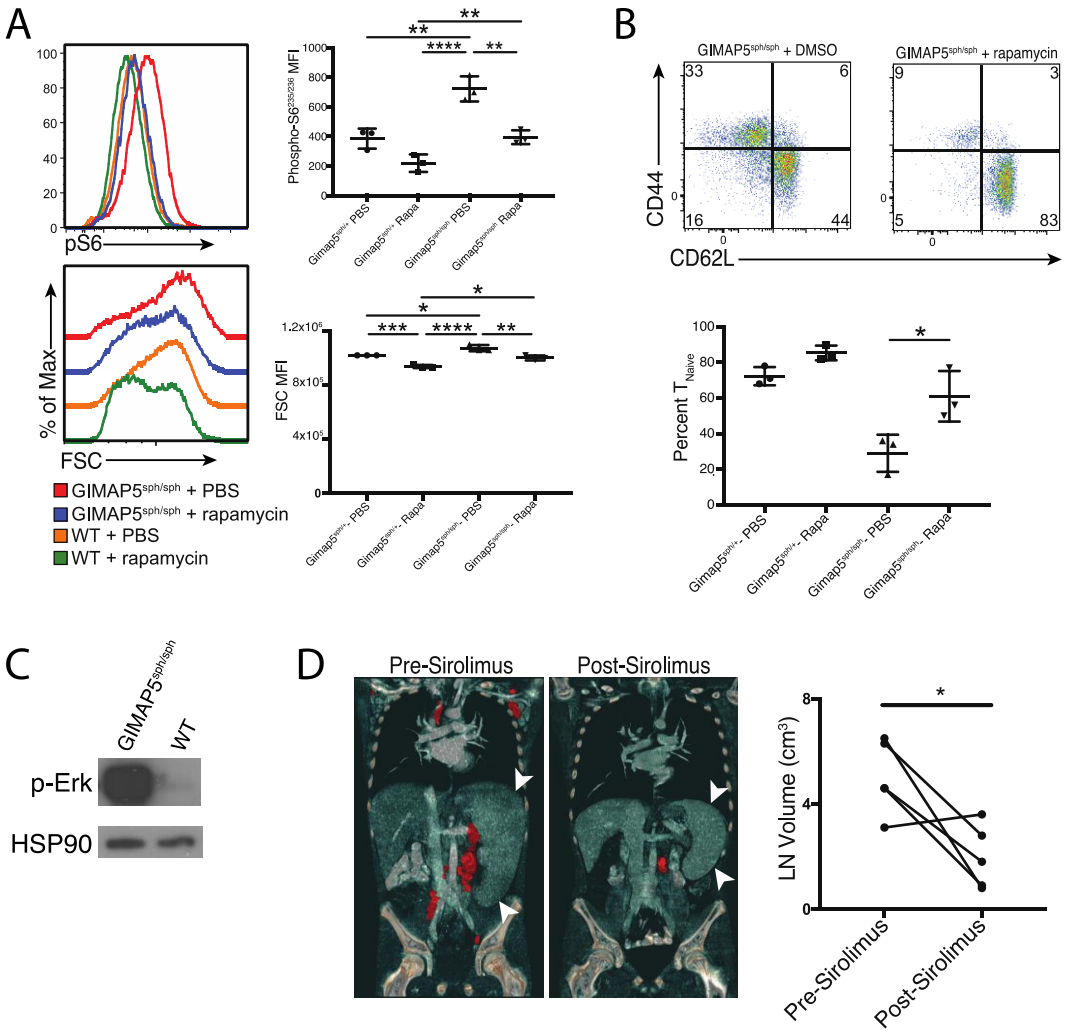
order to potentially translate these findings to the clinic. Thus, I injected mice daily with rapamycin intraperitoneally before measuring mTORC1 and physiological readouts. As expected, I was able to show that *in vivo* treatment of Gimap5$^{sph/sph}$ mice with rapamycin could significantly reduce both pS6 and cell size (forward scatter) to levels comparable to wild type controls (Figure 3.5A). The loss of naïve T lymphocytes is a major clinical finding in both the GIMAP5-deficient patients and Gimap5$^{sph/sph}$ mice which I suspect contributes to the pathology of the disease. As differentiation to effector phenotypes has been shown to be driven by mTORC1 during the immune response (Araki *et al.*, 2009) and inhibition of mTORC1 provides significant clinical benefit in other autoimmune diseases (Bruce, Rane and Schuh, 2014; Karen L. Bride, Tiffaney Vincent, Kim Smith-Whitley, Michele P. Lambert, 2, 3 Jack J. Bleesing, Alix E. Seif, Catherine S. Manno, James Casper, Stephan A. Grupp, 2015; Dimitrova *et al.*, 2017) I hypothesized that the loss of naïve T lymphocytes may be ameliorated via rapamycin treatment. I observed that *in vivo* treatment with rapamycin significantly increased the fraction of naïve T cells present in both the spleen and the blood (Figure 3.5B, data not shown) consistent with this phenotype being driven by hyperactive mTORC1.

The Ragulator complex is also known to be an anchor for and regulator of both Mek and Erk (Colaço and Jäättelä, 2017). I also investigated other signaling pathways in cells isolated from GIMAP5-deficient mice and observed an enormous increase in activity in the Erk pathway (Figure 3.5C). This demonstrates that the hyperactive signaling present in the Sphinx mice is not restricted to the mTORC1 pathway and is likely more generalized. This could complicate my attempts to pin down the precise signaling pathway being

affected and I expect clearer data from an *in vitro* acute loss-of-function model using CRISPR or siRNAs.

Figure 3.5

Inhibition of mTORC1 provides clinical benefit in both murine and human GIMAP5 deficiency



A) GIMAP5$^{sph/sph}$ mice or littermate controls were treated with rapamycin as described in the methods section and then readouts of mTORC1 activity (size, pS6$^{235/236}$) were measured in T cells via flow cytometry.

B) Mice were treated with rapamycin as in (A) and then the fraction of naïve cells in the spleen was measured.

C) Levels of phospho-Erk (Thr-202/Tyr-204) were measured in protein extracts isolated from either GIMAP5$^{sph/sph}$ mice or littermate controls.
D) Abdominal CT scans of a GIMAP5-deficient patient pre/post sirolimus treatment for 2 years. Three dimensional reconstruction was used to measure the volume of 5 lymph nodes. (continued on next page)

Figure 3.5A, B, C prepared by M.L.G and K.R.

Data shown in A-B is representative of three experiments, with significance calculated by ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data in C is representative of 3 separate experiments, while statistical significance was done via Student's t test in D.

Nevertheless, due to the improvements observed in my Gimap5$^{sph/sph}$ mice following rapamycin treatment I hypothesized that GIMAP5-deficient human patients might similarly benefit. P3.1 was treated with sirolimus (an inhibitor of mTORC1) beginning in November of 2013 and continuing up to the present and I observed a remarkable reduction in spleen/lymph node size (Figure 3.5D). In this same time period his severe psoriasis was similarly resolved. Taken together, these data suggest that mTORC1 inhibitors may be a valuable clinical intervention in treating human patients deficient for GIMAP5. The data also supports a model that GIMAP5 is a T cell specific negative regulator of mTORC1.

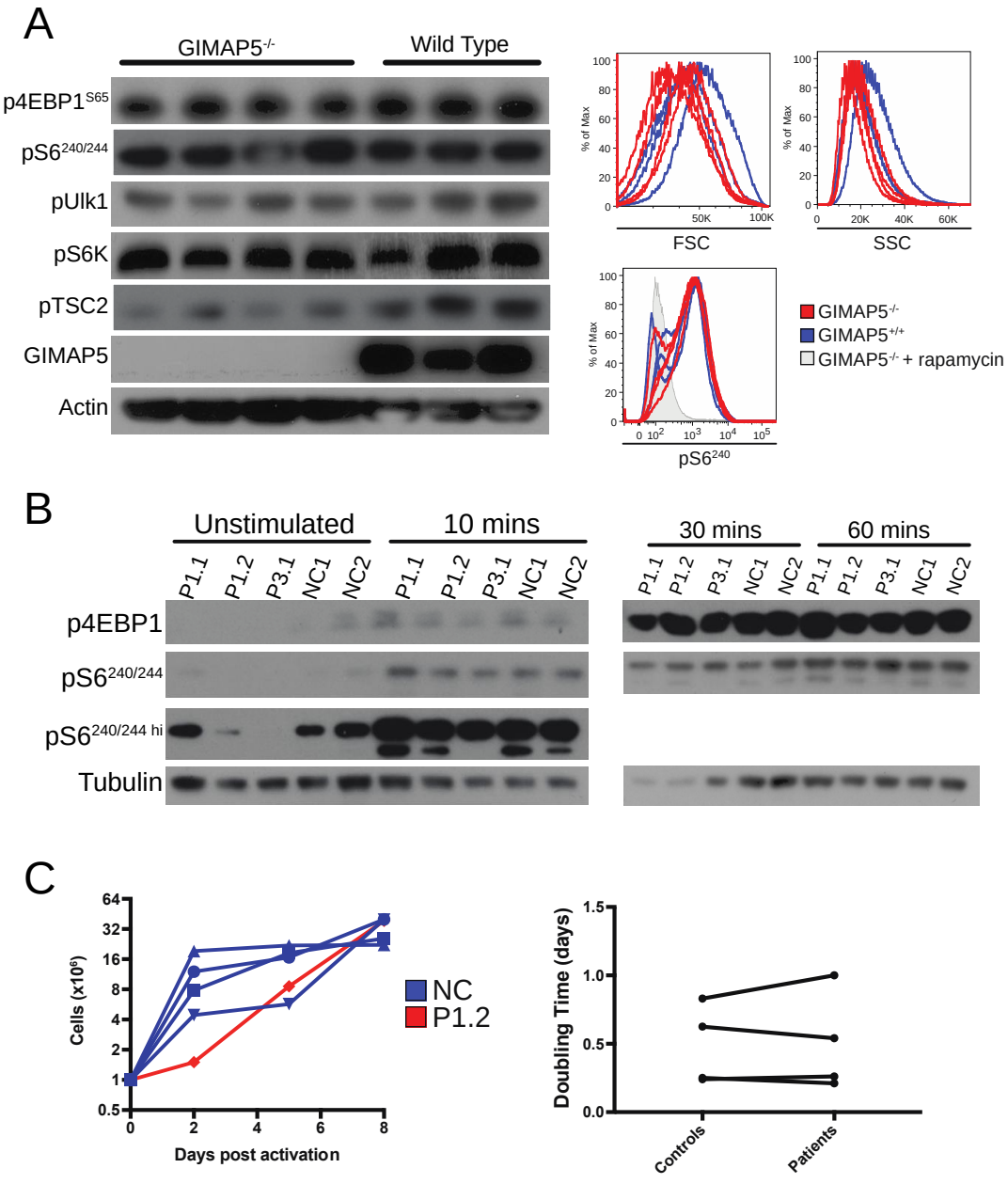
mTORC1 activity is normal in various GIMAP5-deficient human models

Previous work has shown that silencing of GIMAP5 via siRNA in Jurkat cells reduced the viability of these cells and increased their susceptibility to apoptosis, again reminiscent of mice with hyperactive mTORC1 signaling (Pandarpurkar *et al.*, 2003; Yang *et al.*, 2011). I hypothesized that this increase in death may be due to a dysregulation of cellular metabolism caused by increased mTORC1 activity in the

absence of GIMAP5. In order to test this hypothesis, I transiently transfected Jurkat cells with plasmids expressing both the Cas9 enzyme and guide RNAs (gRNAs) specific for the GIMAP5 locus. I then single cell-cloned Jurkat cell lines deficient for GIMAP5. I used 5 knockout and 3 control lines to test a multitude of mTORC1 downstream targets (p4EBP1, pS6, pUlk1, pS6K) as well as upstream regulators (pTSC2) as negative controls under basal culture conditions. While I confirmed that these cells were completely lacking GIMAP5, I did not observe any significant difference in any readout downstream of mTORC1 in more than five different experiments (Figure 3.6A). While I did observe significant differences from one clone to the next (even in the control lines) I have since repeated this experiment in non-clonal populations of Jurkats completely deficient for GIMAP5 and again obtained negative results (data not shown). mTORC1 activity has previously been shown to increase cell size. However, when cell size was measured via flow cytometry I observed the four Jurkat clones deficient for GIMAP5 trended towards being smaller than the three wild type lines (Figure 3.6A – right panel). I also repeated the measurement of levels of $pS6^{240}$ in the Jurkat lines via flow cytometry to confirm the results of my western blots. I confirmed that this site was exquisitely dependent on mTORC1 activity as rapamycin treatment completely abrogated the signal (grey trace). However, again I observed no significant difference in mTORC1 activity at baseline (Figure 3.6A). However, in most of the readouts tested there was significant variability from clone to clone suggesting that I may have obtained more reliable data from bulk populations of cells.

Figure 3.6

mTORC1 activity is normal in various GIMAP5-deficient human models



A) GIMAP5-deficient or wild type single cell Jurkat clones were generated via CRISPR and then a variety of mTORC1 readouts were measured via western blot (left panel). Cell size and $pS6^{240}$ were also measured via flow cytometry.
B) T cell blasts from GIMAP5-deficient human patients were expanded in IL-2, rested, restimulated with TCR crosslinking for the indicated time and then mTORC1 activity was measured.
C) T cells from bulk PBMCs were stimulated and cell counts over time were measured. The average doubling time of the culture beginning on day 2 was calculated.

Figure 3.6A, B, C prepared by M.L.G.

Data shown in (A) and (B) are representative of 4-6 experiments. Data in (C) shows 4 experiments, each consisting of 1-2 GIMAP5-deficient patients and at least 3 normal controls.

We reasoned that Jurkat cells may be a poor model to study mTORC1 activation with due to the fact that this cell line has constitutive signaling through this pathway. Thus, I next evaluated a large selection of downstream mTORC1 targets in T cell blasts expanded from GIMAP5-deficient patient T cells. Cells were rested in serum free media for two hours to decrease the basal level of mTORC1 signaling and then restimulated via soluble anti-CD3 and cross-linking F(ab')2 fragment. Again, in a number of different experiments, I observed no difference in a number of different mTORC1 readouts at multiple timepoints (Figure 3.6B).

One main downstream biological function of mTORC1 signaling is cell proliferation and thus I predicted that patient cells would behave differently from controls. Furthermore, murine models of GIMAP5 deficiency show drastically decreased proliferation relative to controls (Barnes *et al.*, 2010a). To test this hypothesis, I activated T cells from GIMAP5-deficient patients *in vitro*, added IL-2 after three days and measured cell counts over time from this point forwards. Although I observed significant

variability from experiment to experiment likely related to the way the blood was handled during shipment and processing, once the GIMAP5-deficient patient T cells had been activated they were able to proliferate at the same rate as control cells (Figure 3.6C). I consistently observed an initial decrease in cell counts early in the activation cycle, however this was most likely an artifact caused by using PBMCs from lymphopenic individuals as starting material. While they were normalized for total cell counts, the lymphopenic patients had fewer T cells initially thus leading to the initial decrease. These data are in stark contrast to what has been published regarding murine T cells deficient for GIMAP5 which have striking proliferation defects. It is however consistent with the data shown in Figure 2.5 where I observed no major defects in Celltrace Violet dilution in GIMAP5-deficient patient cells acutely following stimulation.

Acute depletion of GIMAP5 in human T cells via siRNA has no impact on mTORC1 activity or proliferation

Increased mTORC1 activity has been observed in the peripheral T cells of GIMAP5$^{sph/sph}$ mice and there is a similarity between the clinical phenotype of GIMAP5-deficient patients and other diseases caused by enhanced mTORC1 activity. Moreover, I have observed improvement in disease course following treatment of both GIMAP5$^{sph/sph}$ mice and humans with mTORC1 inhibitors. Therefore, I hypothesized that GIMAP5 may be a negative regulator of mTORC1. This hypothesis was also supported by the robust interaction between GIMAP5 and members of the Ragulator complex which is known to be required for the recruitment of mTORC1 to the surface of the lysosome (Sancak *et al.*, 2008). However, many of these findings could have been secondary findings due to

lymphopenia or other disruptions of T cell homeostasis *in vivo* in GIMAP5-deficient mice/humans. Furthermore, my initial experiments using cells from the human patients and Jurkat cells deficient for GIMAP5 failed to reveal a consistent difference in mTORC1 signaling. This led me to develop *in vitro* methods to deplete GIMAP5 and study mTORC1 signaling in an internally controlled setting.

We electroporated either unstimulated primary human T cells or activated T cell blasts with siRNAs specific for either GIMAP5 or scrambled controls. It would appear the half-life of GIMAP5 is relatively long as significant depletion of GIMAP5 in human T cell blasts was not observed until 72 hours post electroporation and was most consistent at 96 hours post electroporation (Figure 3.7A). However, even after depletion of GIMAP5 in cycling T cell blasts no difference in levels of phospho-S6 (pS6) were observed. It is worth noting that even at the peak of GIMAP5 depletion residual levels of GIMAP5 remained which could complicate my analysis.

As mTORC1 is a critical regulator of proliferation and cell size, and furthermore, GIMAP5$^{sph/sph}$ murine T cells exhibit a drastic block in proliferation, I hypothesized that if these defects were not secondary to lymphopenia or other systemic factors siRNA knockdown of GIMAP5 should affect these cellular processes. To test this I electroporated GIMAP5 siRNA into unstimulated human T cells, waited 96 hours then stimulated them and measured proliferation, upregulation of cell cycle genes via qPCR and cell size. Cell cycle genes CDC25A and MCM10 were both upregulated 40-200 fold following T cell stimulation, however no significant difference was observed between T cells electroporated with scrambled siRNA or siRNA specific for GIMAP5 (Figure 3.7B,

top panel). Similarly, at three days post stimulation no defect was observed in T cell proliferation as measured by Celltrace violet dilution (Figure 3.7B, middle panel). It is worth pointing out that the transient knockdown was likely only efficient at the time of stimulation and GIMAP5 was likely re-expressed at some point during proliferation. Previous studies have shown that increased mTORC1 activity leads to an increase in cell size (Montagne *et al.*, 1999). Thus, I measured cell size by flow cytometry following siRNA depletion of GIMAP5 both before and after TCR stimulation. While stimulation greatly increased cell size as expected, no difference in cell size was observed in either condition in the absence of GIMAP5 (Figure 3.7B bottom panel).

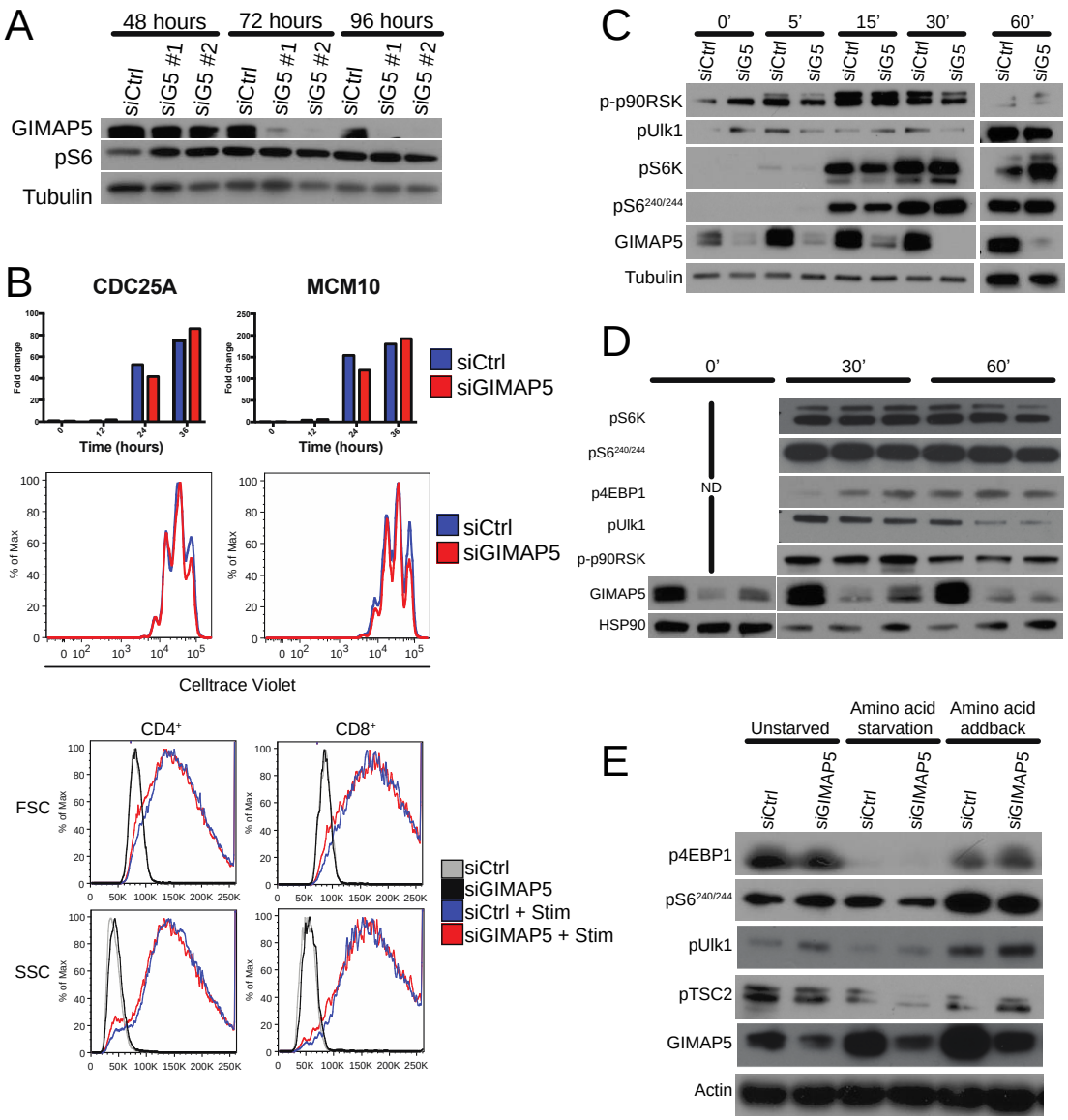
While I did not observe any broad effects on cell function following GIMAP5 knockdown, I reasoned that there may be differences in acute mTORC1 signaling following TCR stimulation. Thus, I depleted GIMAP5 in either cycling T cell blasts (Figure 3.7C – top panel) or unstimulated T cells (Figure 3.7C – bottom panel) from healthy human controls. I then stimulated the cells with soluble CD3 crosslinked with F(ab')2 fragments for the indicated time periods. While depletion of GIMAP5 was robust, I observed no difference in a number of direct and indirect mTORC1 targets (Ulk1, S6K, S6, 4EBP1) at multiple timepoints (Figure 3.7D). Finally, I hypothesized that GIMAP5 may be involved in the regulation of mTORC1 by amino acid sensing rather than TCR stimulation as it interacts with the Ragulator complex. As the Ragulator complex mediates the recruitment of mTORC1 to the surface of the lysosome in the presence of amino acids, I starved cycling GIMAP5 depleted T cell blasts of amino acids for 60 minutes and then returned them to cRPMI for 30 minutes. This strategy has

previously revealed defects in mTORC1 signaling in other cell types lacking components of the Ragulator complex (Bar-peled *et al.*, 2012). While I did observe a decrease in mTORC1 readouts following starvation and recovery after incubation with cRPMI, again there was no difference in mTORC1 signaling in the absence of GIMAP5.

Overall, I failed to replicate any phenotypes observed in the murine model of GIMAP5 deficiency with my siRNA/Cas9-RNP GIMAP5 depletion strategies, and more broadly, in GIMAP5-deficient patient cells or GIMAP5-deficient Jurkat lines (see Figure 3.6). These results could be due to a number of technical or biological causes. Primarily, both the siRNA knockdown model and GIMAP5-deficient patient cells have residual GIMAP5, while Jurkat cells have constitutive activity through the mTORC1 signaling pathway. It could also be that prolonged depletion of GIMAP5 is required to see some effect or some other factor is present *in vivo* that is lacking *in vitro*.

Figure 3.7

Acute depletion of GIMAP5 in human T cells via siRNA has no impact on mTORC1 activity or proliferation



A) GIMAP5 was measured in unstimulated human T cells electroporated with the indicated siRNAs at the indicated time points.
B) Measurements of genes required for cell cycle (top panel), proliferation (middle panel) or cell size (bottom panel) are shown from human T cells depleted of GIMAP5 via siRNA.
C) Western blots of various mTORC1 readouts following TCR stimulation of activated T cell blasts electroporated with either scrambled or GIMAP5 specific siRNA.
D) Western blots of various mTORC1 readouts following TCR stimulation of previously unstimulated human T cells electroporated with either scrambled or GIMAP5 specific siRNA.
E) Western blots of various mTORC1 readouts following amino acid starvation and addback to T cells electroporated with either scrambled or GIMAP5 specific siRNA.

Figure 3.7A, B, C, D, E prepared by M.L.G.

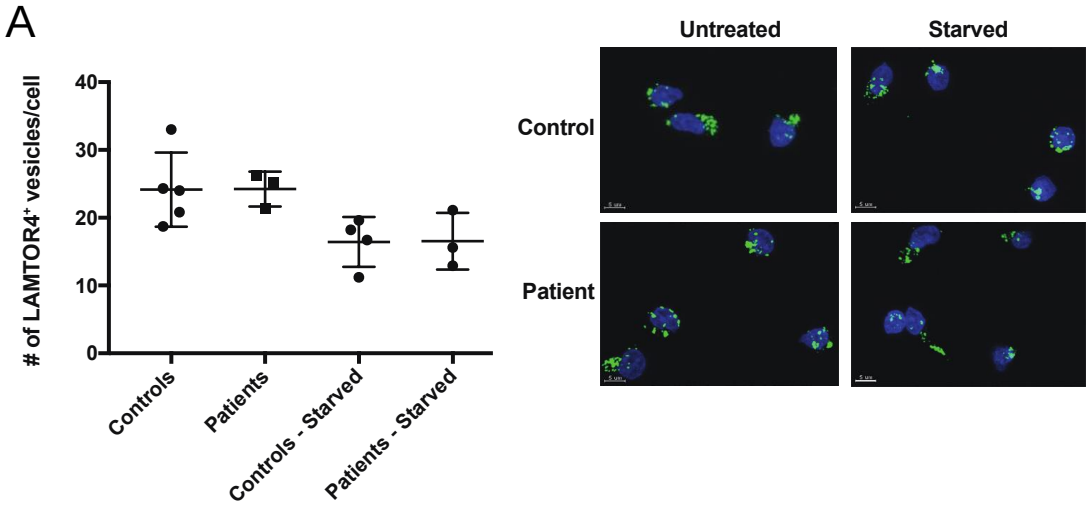
Data shown is representative of 2 (A), 3 (B, D) or 6 (C) experiments.

Lysosome number is normal in GIMAP5-deficient human T cells

The potential role of GIMAP5 in regulating lysosome number and function has not previously been tested. This may have been overlooked due to previous studies claiming that GIMAP5 localizes to the mitochondria or ER (Dalberg, Markholst and Hornum, 2007; Keita *et al.*, 2007). Furthermore, I have shown that GIMAP5 interacts with the Ragulator complex on the surface of the lysosome/late endosome. This complex has previously been shown to regulate lysosome numbers and dynamics in other cell types (Colaço and Jäättelä, 2017; Pu, Keren-Kaplan and Bonifacino, 2017b).

Figure 3.8

Lysosome number is normal in GIMAP5-deficient human T cells



A) Quantitation of lysosome number via confocal microscopy in either untreated or amino acid starved GIMAP5-deficient patient cells or healthy donors (left panel). Sample microscopy images are shown in the right panel.

Figure 3.8A prepared by M.L.G.

Data is representative of two individual experiments using 3 GIMAP5-deficient patients and 4-5 healthy donors each.

Thus, I sought to quantify lysosomes in proliferating T cells from patients. I stained patient T cell blasts for LAMTOR4 to detect lysosomes as well as DAPI to delineate individual cells and then imaged them via confocal microscopy. I also included cells which had been starved of amino acids which is known to induce autophagy and inhibit mTORC1 (Rabanal-Ruiz, Otten and Korolchuk, 2017). I observed no differences between cells isolated from GIMAP5-deficient patients relative to controls despite testing three individuals representing two independent pedigrees and five unrelated healthy

controls (Figure 3.8A). The variability in average lysosome number per cell was relatively low given sex, age and genetic background were not controlled in this assay and suggests that this is a strictly regulated parameter in T lymphocytes. However, it would appear that GIMAP5 does not regulate lysosome number in human T cells.

III. Discussion

I have undertaken extensive interaction and transcriptomic studies of GIMAP5. The RNA-Seq data analyzing the impact of GIMAP5 knockdown by siRNA in human T cells was not particularly fruitful, with the vast majority of differences between the samples being explained by T cell activation/proliferation. Comparison of human cells depleted of GIMAP5 versus those electroporated with control siRNAs showed virtually no differences, indicating that either technical issues (i.e. residual protein, culture conditions) were responsible or GIMAP5 does not affect the transcriptome in human T cells.

Regarding the interaction studies, most previous publications have described direct interactions between GIMAP5 and Bcl-2 family members (Chen *et al.*, 2011b). More recently studies of endogenous GIMAP5 have demonstrated a strict lysosomal localization suggesting that the previous interaction data may have been an artifact of overexpression systems (Vivian W.Y. Wong *et al.*, 2010). Encouragingly, the majority of the protein complexes identified by the BioID2 study were either known to be lysosomally localized (the Ragulator complex) or known to traffic to/from lysosomes (TCR complex, endosomal trafficking members). Furthermore, this association was

confirmed via two independent techniques including endogenous co-immunoprecipitations that GIMAP5.

While I am confident that I have truly identified GIMAP5 associated proteins in this study, a major open question is what the functional relevance of these interactions is. The Ragulator complex is known to regulate the activity of mTORC1 in response to the presence of amino acids as well as acting as a scaffold for MAPK signaling (Colaço and Jäättelä, 2017). Strikingly, previous work has shown increased Akt and mTORC1 activity in GIMAP5$^{sph/sph}$ T cells, suggesting that GIMAP5 may act as a negative regulator of this complex. The association of the TCR complex is also very reminiscent of a recent study describing a supercomplex of the BCR and Ragulator complexes on the surface of the lysosome in a B cell lymphoma cell line (Phelan *et al.*, 2018a). However, if the absence of GIMAP5 had any major impact on mTORC1 or TCR signaling I would have anticipated an impact on T cell activation or proliferation in GIMAP5-deficient patient cells or the siRNA knockdown model.

Based on the BioID2 interaction data and previous data showing that Sphinx T cells had increased mTORC1 activity (which I have also replicated) I hypothesized that GIMAP5 was a negative regulator of the mTORC1 complex by influencing the Ragulator complex. However, my data from multiple different model systems (Jurkat knockout lines, siRNA knockdown in unstimulated/blasting T cells, GIMAP5-deficient patient cells) clearly show no difference in mTORC1 activity in response to a variety of stimuli (TCR stimulation, amino acid starvation/addback). Thus, I am forced to conclude that the clinical benefit I observed with rapamycin treatment was most likely not due to the fact

that GIMAP5 directly regulated mTORC1 and rather that inhibition of mTORC1 non-specifically inhibited lymphoproliferation and autoimmunity. Furthermore, it would also argue that the Ragulator complex may have roles beyond tethering mTORC1/Erk to the surface of the lysosome which may be influenced by GIMAP5. Alternatively, it is unclear from my data whether GIMAP5 is upstream or downstream of the Ragulator complex. It is also possible that the activity of GIMAP5 is influenced by the Ragulator complex rather than the inverse. This model would posit that the Ragulator complex may act as a GAP or a GEF for GIMAP5 GTPase activity and would regulate some novel pathway downstream of the Ragulator complex independently of mTORC1.

CHAPTER 4: Human physiologic media reveals extracellular calcium concentrations as critical for T cell activation and effector function

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Michael Leney-Greene designed research, conducted experiments, performed analysis and wrote this section. Jason Cantor, Helen C. Su and Michael J. Lenardo helped design experiments and write the paper.

I. Summary

Lymphocytes are critical effectors of the immune system, playing major roles in the susceptibility to infectious and autoimmune disease. In order to effectively treat these diseases a better understanding of the *in vivo* function and conditions experienced by immune cells is required. Many assays require the use of *in vitro* culture conditions which are optimized for growth and thus are vastly different from the *in vivo* milieu. We hypothesized that media modeled after the *in vivo* environment would better support human T lymphocyte activation and survival *in vitro.* In this study, I showed that physiologic media supports a much more robust response to antigen stimulation in primary human T lymphocytes relative to RPMI. I demonstrated that this activation defect is due to RPMI being profoundly hypocalcemic relative to the *in vivo* milieu and show that addition of calcium chloride to RPMI can improve human T lymphocyte activation. I propose that commonly used media formulations be modified to contain physiological levels of ionic calcium to better model the *in vivo* environment.

II. Results

HPLM is superior to RPMI in supporting naive human T cell activation

Lymphocytes *in vivo* are activated in a rich milieu containing high levels of amino acids, lipids as well as various small organic metabolites. In contrast, typical *in vitro* culture methods using RPMI only contain a skeleton of essential amino acids, vitamins, glucose and salts all at non-physiological levels. Initially, I expected that HPLM may reveal a defect in human GIMAP5-deficient T cells although ultimately this wasn't the case. However, independently of GIMAP5, I hypothesized that either small organic

metabolites present in plasma but absent in RPMI or components common to both but at different concentrations may influence lymphocyte activation. In order to test this I took advantage of a synthetic media developed by Cantor *et al.* which contains physiological levels of 40 different metabolites that are absent from RPMI (Cantor *et al.*, 2017)(Table 3). I supplemented both RPMI and HPLM with dialyzed rather than complete FBS in order to better control the levels of metabolites as well as ions in both preparations while still maintaining protein growth factors required for cell survival.

Table 3: Detailed composition of RPMI, HPLM-Min and HPLM

	Concentration (µM)		
	RPMI	HPLM-Min	HPLM
Glucose	11111	5000	5000
Proteinogenic amino acids			
Alanine	0	430	430
Arginine	1149	110	110
Asparagine	378	50	50
Aspartate	150	20	20
Cysteine	0	40	40
Cystine	208	100	100
Glutamate	136	80	80
Glutamine	2055	550	550
Glycine	133	300	300
Histidine	97	110	110
Hydroxyproline	153	0	0
Isoleucine	382	70	70
Leucine	382	160	160
Lysine	219	200	200
Methionine	101	30	30
Phenylalanine	91	80	80
Proline	174	200	200
Serine	286	150	150
Threonine	168	140	140
Tryptophan	25	60	60
Tyrosine	111	80	80
Valine	171	220	220

	Concentration (µM)		
	RPMI	HPLM-Min	HPLM
Ions			
Na^+	138525	132271	132271
K+	5333	4142	4142
Ca2+	424	2390	2390
Mg2+	407	830	830
NH4+	0	40	40
Cl-	108781	116196	116196
HCO3-	23809	24000	24000
PO43-	5634	966	966
SO42-	407	350	350
NO3-	848	80	80

	Concentration (µM)		
	RPMI	HPLM-Min	HPLM
Additional polar metabolites			
2-hydroxybutyrate			50
3-hydroxybutyrate			50
4-hydroxyproline			20
Acetate			40
Acetone			60
Acetylglycine			90
Alpha-aminobutyrate			20
Betaine			70
Carnitine			40
Citrate			130
Citrulline			40
Creatine			40
Creatinine			75
Formate			50
Fructose			40
Galactose			60
Glutathione			25
Glycerol			120
Hypoxanthine			10
Lactate			1600
Malonate			10
Ornithine			70
Pyruvate			50
Succinate			20
Taurine			90
Urea			5000
Uric acid			350

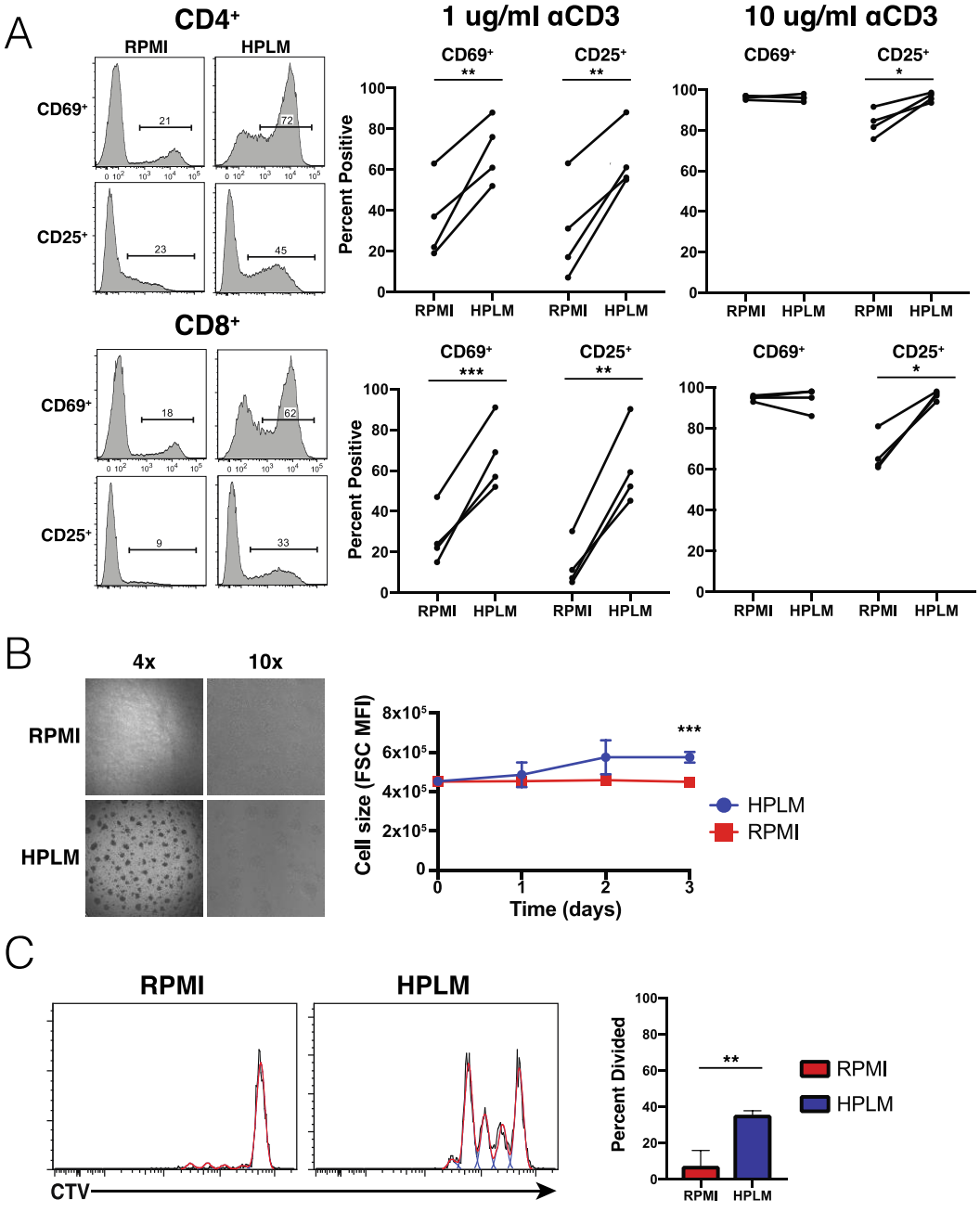
I purified naïve T cells from healthy human donors and stimulated them in either RPMI or HPLM using plate-bound anti-CD3/CD28 antibodies. I observed a striking increase in levels of activation markers CD25 and CD69 in cells from five different healthy donors cultured in HPLM compared to RPMI in both $CD4^+$ and $CD8^+$ naïve human T cells (Figure 4.1A). Higher concentrations of anti-CD3/CD28 antibodies used to

stimulate the cells could lead to nearly maximal response rates in both RPMI and HPLM (i.e. >90% positive for both CD25 and CD69), though still with slightly higher activation rates in HPLM. This demonstrates that cells stimulated in RPMI could be maximally activated, while activation in HPLM lowers the stimulus threshold. The improved activation of naïve T cells in HPLM was immediately apparent when studying the culture via microscopy which revealed large clusters of activated T cells in the HPLM cultures that were absent from cells cultured in RPMI (Figure 4.1B). This was accompanied by a significant increase in cell size as measured by flow cytometry. Finally, with the lower dose of TCR crosslinking antibody stimulation, I also observed robust proliferation of cells in HPLM that was slower in cells stimulated in RPMI as measured by CTV dilution (Fig. 4.1C).

Overall, my data suggests that some component of HPLM strongly augments naïve human T cell activation and proliferation compared to conventional culture media. I hypothesize that this is due to HPLM better modeling the *in vivo* milieu and providing a superior environment for T cell activation.

Figure 4.1

Human physologic media is superior to RPMI in supporting human T cell activation



A) Measurement of T cell activation markers CD25 and CD69 on purified naïve human T cells following stimulation with either 1 or 10μg of plate-bound anti-CD3/CD28 in HPLM or RPMI.
B) Bright-field microscopy images of naïve T cells stimulated with 1μg of plate-bound anti-CD3/CD28 in either HPLM or RPMI.
C) CTV dilution plots of naïve T cells stimulated in either HPLM or RPMI.
D) Data shown in (A-C) represent 3-4 pooled experiments with different donors, with B-C plotting the mean and standard deviation.

Figure 4.1A,B,C prepared by M.L.G.

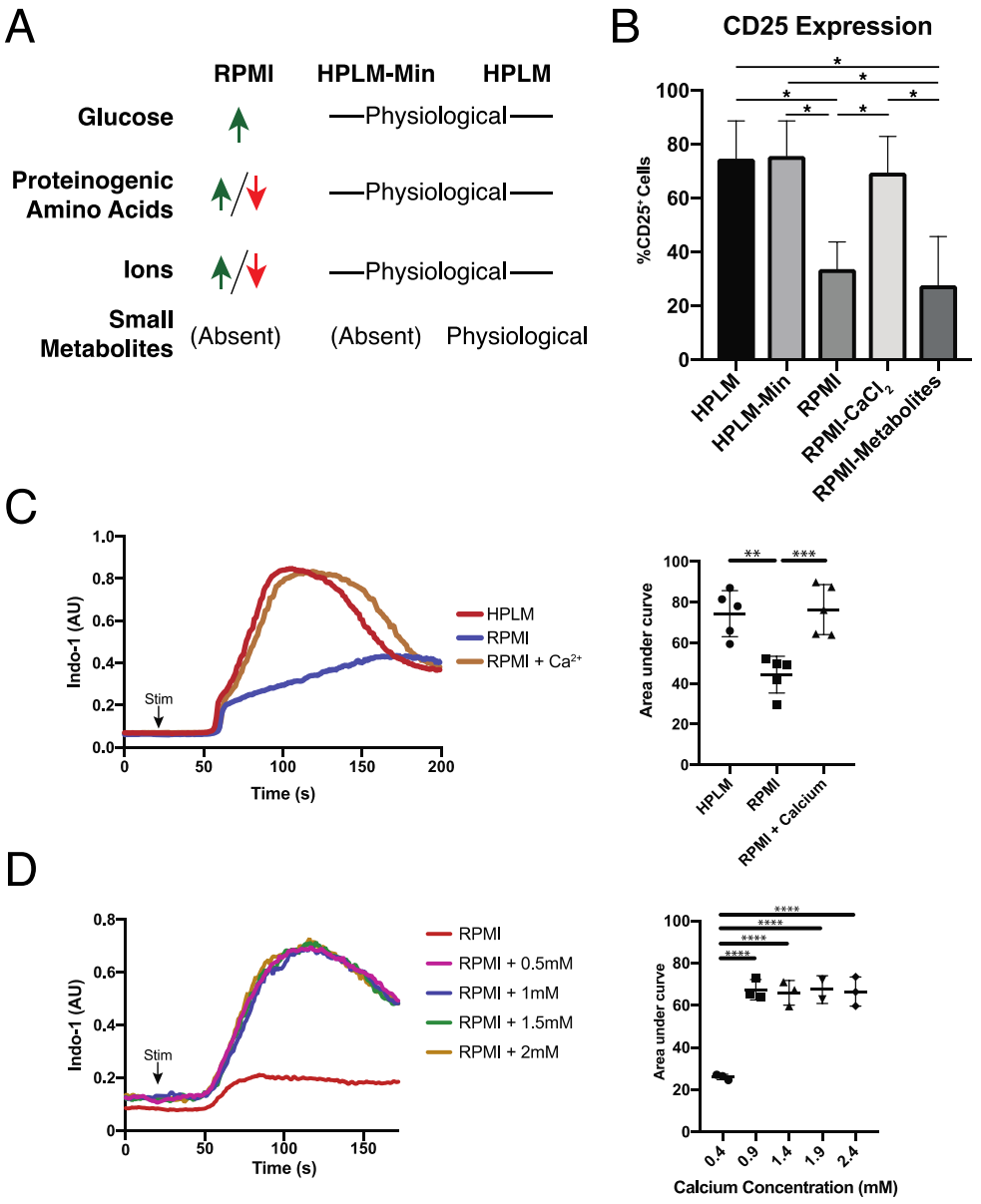
Statistical significance was calculated via paired t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The increased calcium concentration in HPLM augments T cell activation

I initially determined that HPLM greatly improved naïve human T cell activation compared to RPMI. My next goal was to determine which component of HPLM was responsible in order to both identify supplements that could improve T cell activation *in vitro* and potentially discover new metabolic pathways that influenced T cell biology. To do this, I compared the activation of naïve human T cells in either HPLM, HPLM-Min (which lacks the 40 small metabolites added to HPLM but maintains the same concentration of amino acids, glucose and ions) and RPMI. An overview of the composition of these three media is given in Figure 4.2A while the complete formula is given in table 3. I also included a condition which added all of the HPLM small metabolites back to RPMI (RPMI-Metabolites). I then repeated the activation assays done in Figure 4.1 using these different conditions.

Figure 4.2

RPMI is hypocalcemic relative to human plasma



A) Diagram showing the relative composition of HPLM, HPLM-Min and RPMI. Exact composition of each media is given in Supplemental Table 1.

B) Measurement of activation marker CD25 on $CD4^+$ T cells comparing RPMI and minimal-HPLM supplemented with various metabolite components unique to HPLM.

C) Calcium flux following primary stimulation of isolated human $CD8^+$ T lymphocytes in either RPMI or HPLM. (continued on following page)

D) Calcium flux following primary stimulation of isolated human $CD8^+$ T lymphocytes in RPMI supplemented with the indicated concentrations of calcium chloride.

Figure 4.2A, B, C, D prepared by M.L.G.

Data shown in (B-D) represent 3-5 pooled experiments with different donors, with B-D plotting the mean and standard deviation. Statistical significance was calculated via one way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To my surprise, I observed equivalent activation in the HPLM and HPLM-Min conditions, and significantly decreased activation in the RPMI and RPMI-Metabolites conditions (Figure 4.2B). These data suggest that the small metabolites in HPLM are dispensable for early T cell activation and rather that the phenotype I observed was due to differences in amino acid or ion concentrations in HPLM versus RPMI. One striking difference was the largely increased level of calcium in HPLM relative to RPMI, which is markedly hypocalcemic relative to the *in vivo* milieu (Table 3). Oddly, basal RPMI only contains 0.432 mM of calcium which is severely hypocalcemic relative to typical physiological levels of 2-2.5 mM (Goldstein, 1990). HPLM likely represents a hypercalcemic condition as it contains 2.4mM of ionic calcium. While this is within the physiological range of total serum calcium levels, it does not account for the fact that 50% of serum calcium is typically bound to albumin and thus potentially not bioavailable for diffusion across membranes. As such, I included a condition of RPMI supplemented with 2mM of calcium chloride to normalize it relative to HPLM (Figure 4.2B). The addition of calcium completely rescued the early activation defect in RPMI relative to HPLM.

In order to further verify my findings that HPLM led to superior T cell activation due to calcium levels I tested calcium flux following T Cell Receptor (TCR) stimulation in either RPMI, calcium supplemented RPMI or HPLM. Most calcium flux protocols are carried out in HBSS or Ringer's solution; however, some other studies have shown decreased calcium flux in RPMI (Prakriya *et al.*, 2006; Gwack *et al.*, 2008; Bertin *et al.*, 2014). I observed a striking decrease in calcium flux following activation in RPMI supplemented with either dFBS or cFBS relative to either HPLM or RPMI supplemented to equivalent calcium levels (Figure 4.2C). These data further support my hypothesis that the defective activation shown in Figure 4.1 was due to a defect in calcium flux during activation.

This raised the possibility that calcium levels *in vivo* could influence T cell activation, and that hypocalcemic/hypercalcemic patients could be more prone to immunodeficiency or autoimmunity, respectively. Furthermore, this provided strong evidence that RPMI was a poor approximation of the *in vivo* condition and rather represented a severely hypocalcemic environment. To test these ideas, I titrated calcium levels in basal (FBS free) RPMI in 0.5 mM increments up to a maximum of 2.2 mM and then measured calcium flux following TCR stimulation. I observed that basal RPMI gave a poor calcium flux that could be improved with every concentration of calcium from hypocalcemic (0.7 mM) through the physiological range and into the hypercalcemic range (2.2mM). The concentrations tested yielded virtually identical magnitude and kinetics of calcium fluxes (Figure 4.2D). This suggests that at physiological concentrations of extracellular calcium the rate of increase of intracellular calcium is

already at maximal and extracellular calcium concentrations likely do not affect the sensitivity of T cells to activation *in vivo*. However, this finding again highlights that the levels of calcium in RPMI are too low for full T cell activation when using dialyzed serum. When using complete serum, this problem is compensated by calcium derived from the serum. My data shows that only increasing the external calcium levels by 0.5mM would overcome the deficit in RPMI.

Effector cytokine production by $CD8^+$ T cells is superior in HPLM

I hypothesized that HPLM would be an improvement over RPMI in modelling the physiological environment that T lymphocytes encounter during an *in vivo* immune response. Thus, while my initial experiments comparing RPMI and HPLM underlined the hypocalcemic nature of RPMI, I sought to unravel the impact of small serum metabolites on T cell activation and acquisition of effector functions *in vitro*. Thus, I designed a series of experiments comparing T cells cultured in HPLM to HPLM-Min (See Table 3 for comparison). This would control for levels of amino acids, vitamins and small ions while only the HPLM condition would contain the small metabolites. I also included cells initially activated in RPMI and then cultured in IL-2 containing media for a further 14-21 days prior to restimulation and measurement of cytokine production.

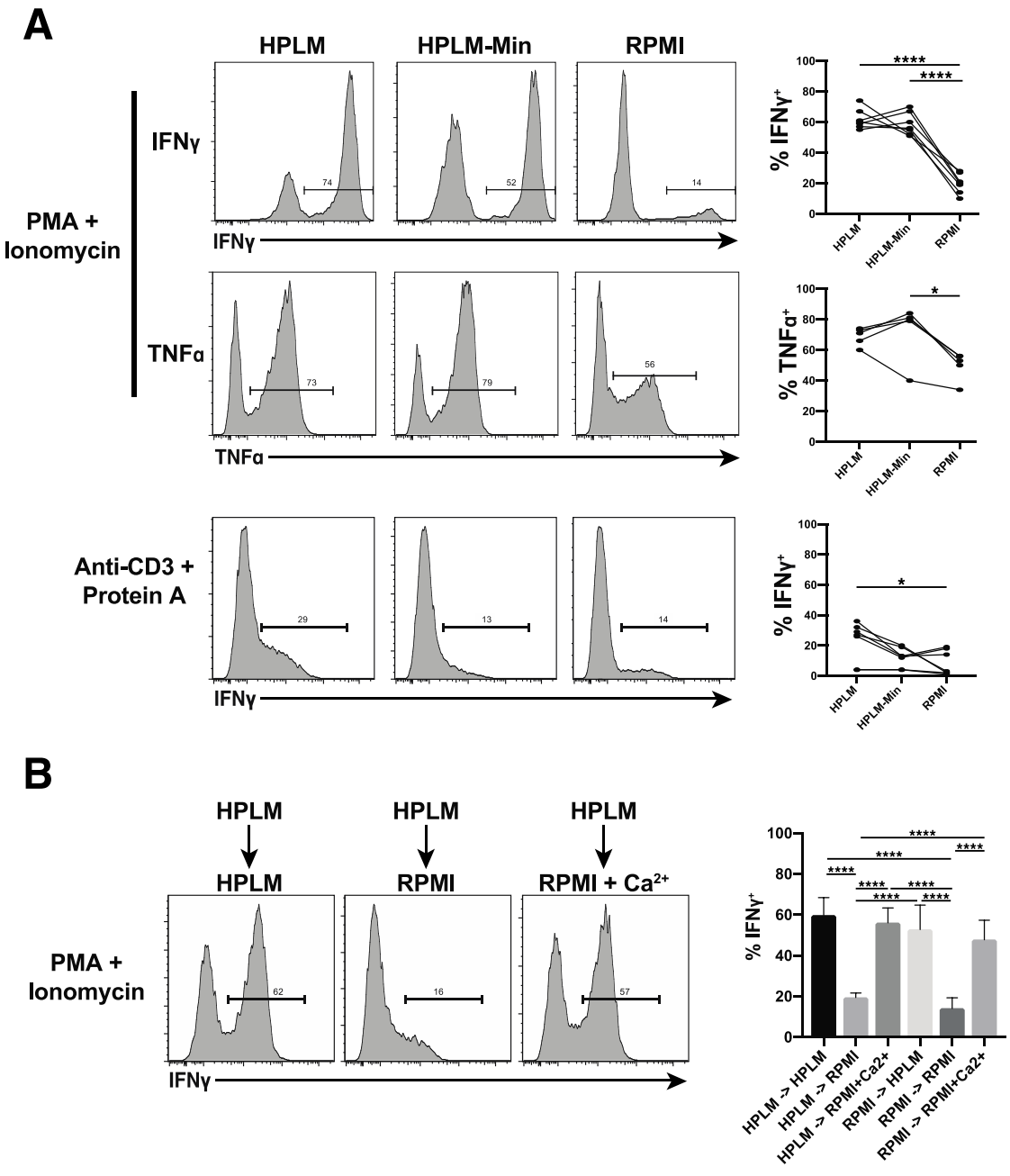
I measured TNFα and IFNγ production following restimulation of T cell blasts with PMA and ionomycin. I did not observe any significant differences in cytokine production between cells cultured in HPLM or HPLM-Min, indicating that the additional metabolites added to HPLM do not majorly influence cytokine production under these conditions (Figure 4.3A). However, as expected, I did observe a large decrease in both

TNFα and IFNγ production in T cells cultured in RPMI following restimulation. This difference was consistent in cells restimulated with either PMA and ionomycin or anti-CD3 antibodies crosslinked with protein A. This pattern was very reminiscent of the result I observed with T cell activation, with HPLM and HPLM-Min being equivalent and RPMI performing substantially worse. Again, the most likely interpretation is that the small metabolites uniquely present in HPLM do not affect effector cytokine secretion, while the different with RPMI is due to concentrations of either amino acids, glucose or ions (most likely calcium).

This data did not differentiate between whether this difference in cytokine production was due to the composition of the media during the initial stimulation, the subsequent expansion phase or acutely during the restimulation phase. To test these possibilities, I activated and expanded the cells in the media as in Figure 4.3A and then immediately prior to restimulation moved them to fresh media. Thus, I observed robust cytokine production in cells that were activated in HPLM or RPMI and then transferred to HPLM or RPMI supplemented with 2 mM Ca^{2+}. However, I observed equally poor cytokine production in cells that were activated in HPLM or RPMI and transferred to RPMI with no calcium supplement. Thus, I conclude that under these conditions the concentration of calcium is critical acutely during restimulation, but dispensable during the initial activation and expansion phases. This is consistent with previous findings demonstrating that in murine T cells the concentration of calcium can influence cytokine production (Zimmermann, Radbruch and Chang, 2015).

Figure 4.3

HPLM supports more robust cytokine production in human CD8⁺ T cells



A) Levels of cytokine production in primary human T lymphocytes following restimulation after being expanded in the indicated media.
B) Two week old human T cell blasts that were activated and cultured in the indicated media were transferred to fresh media of the indicated type. Shown are levels of cytokine production following restimulation.

Figure 4.3A, B prepared by M.L.G.

Data shown represent 4-6 donors tested across three different experiments and each point representing a distinct donor. Statistical significance was calculated via one way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

CD19 CAR-T cell construct transduction efficiency is similar in HPLM and other medias

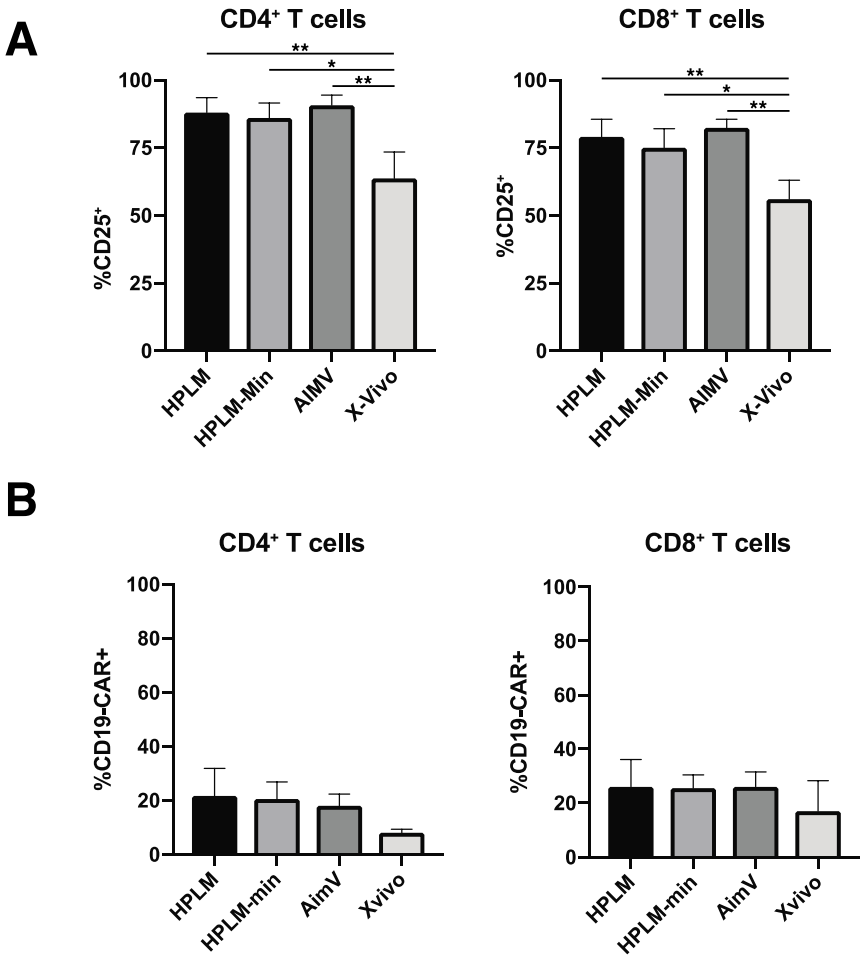
A large variety of recently developed clinical protocols require the *in vitro* culture and expansion of large numbers human T cells (Newick *et al.*, 2018). One of the major correlates of the effectiveness of these treatments is the metabolic and differentiation state of the T cell. For example, in Chimeric Antigen Receptor (CAR)-T cell therapy patients with positive responses to therapy have been shown to have T cells skewed towards a memory rather than effector phenotype (Fraietta *et al.*, 2018).

Due to the improved activation of human T cells cultivated in HPLM relative to RPMI I hypothesized that I may observe an improvement in transduction with lentiviruses expressing CAR-T cell receptors. I sought to compare HPLM to strategies typically used in clinical protocols which use two other synthetic medias: a mixture of AIM V and RPMI (referred to as AIM V here) and X-VIVO 15. The latter has the added advantage of being serum free, while HPLM/HPLM-min are supplemented with dialyzed FBS and AIM V is supplemented with 5% human serum. I observed equivalent activation in HPLM, HPLM-Min and AIM V media when stimulated with plate-bound anti-CD3/CD28 antibodies as measured by CD25 (Figure 4.4A). However, I did observe a

significant reduction in T cell activation in X-VIVO 15 media relative to any of the other conditions.

Figure 4.4

T cell transduction in HPLM is comparable or superior to commonly used media



A) Levels of CD25 expression 24 hours after stimulation in primary naïve human T cells cultured in the indicated media.
B) Fraction of primary human T cells positive for the CD19-CAR transgene 48 hours after transduction in the indicated media.

Figure 4.4A, B prepared by M.L.G.

Data shown represents 3 separate experiments of 2 donors with the mean and standard deviation shown. Statistical significance was calculated via one way ANOVA, *$p<0.05$, **$p<0.01$.

Transduction of T cells with lentiviruses typically depends strongly on how robustly they are activated. Based on the data in Figure 4.4A I anticipated that HPLM, HPLM-Min and AIM V media would have equivalent transduction efficiencies while X-VIVO 15 would be lower. The results essentially tracked the activation data perfectly with X-VIVO 15 media supporting a lower rate of transduction relative to HPLM and AIM V (Figure 4.2B). Thus, HPLM performs as well or better than commonly used culture media for the generation of CAR-expressing T cells.

III. Discussion

The development of cell culture techniques in the mid 20th century heralded an enormous shift in life sciences research by offering much more rapid and reproducible assay systems. However, these techniques have changed little in the intervening period as my knowledge has grown and in particular media formulations developed in the 1950s are still universally used today. Here I adapted a recent effort to modernize the field via the development of a cell culture media modeled closely after the *in vivo* environment to the study of primary human lymphocytes. I anticipated that this approach would both potentially improve cell culture techniques for lymphocytes and also reveal insights into the metabolism of T cell activation.

Calcium has long been known to be required for lymphocyte activation, with more recent studies demonstrating the molecular machinery involved (Zhang *et al.*, 2005;

Prakriya *et al.*, 2006). In fact, the molecular mechanisms underpinning this phenomenon were accomplished in part through the identification and study of a novel Mendelian PID (Feske *et al.*, 2006). However, despite this fact, RPMI has been the primary culture media used to study lymphocyte function for decades despite the fact that it contains subphysiological levels of calcium. While some groups have observed increased *in vitro* secretion of effector cytokines in IMDM (which has a a concentration of 1.5mM) or calcium supplemented RPMI relative to basal RPMI in murine T cells *ex vivo* (Zimmermann, Radbruch and Chang, 2015), I am the first to show such a drastic impact on activation, proliferation and effector cytokine secretion in primary human T cells. It is also likely that the use of FBS as a common supplement to cell culture augments the calcium concentration of RPMI considerably, as I measured my current stock of FBS at 3.9 mM – raising the concentration to roughly 800 mM when added at a concentration of 10%. Based on my calcium flux data, this would appear to be sufficient to achieve maximal calcium flux in my assay (Figure 4.2D). However, this still corresponds to a strongly lymphopenic environment and it is quite possible that the cross-linking anti-CD3 antibody used in the calcium flux masks an underlying defect that would be present in response to an *in vivo* antigen response. Furthermore, the calcium flux assay only measures calcium changes for the first several minutes of a response, whereas *in vivo* the process is much more dynamic and spread out over the course of hours/days.

Looking beyond calcium, a comparison of great interest to me was the HPLM versus HPLM-min conditions. As these two media shared the same concentrations of amino acids, glucose and ions they allowed a direct measurement of the impact of the plasma

metabolites on T cell activation and proliferation. I hypothesized that human T cells would behave differently in a media designed to more closely mimic the *in vivo* environment. Curiously, comparing these two media revealed no differences in CD25/CD69 expression, proliferation or cytokine production after stimulation. It is nevertheless possible that there are underlying metabolic differences between the cells grown in HPLM compared to HPLM-Min; however as they seem to have no functional impact in this model system it seems unlikely that they are important regulators of T cell biology. The same caveat applies to these experiments whereby the stimulus used to active the T cells is very strong and artificial relative to a true antigen response and could be masking a role for these serum metabolites.

Broadly speaking, my data underscores the artificiality of commonly used cell culture conditions for T lymphocytes and identifies one component in particular which can easily and rapidly be remedied by researchers in the field. This approach highlights the value of studying human physiologic media and also further work to attempt to improve the physiological relevance of *in vitro* techniques.

CHAPTER 5 – Discussion

I. Summary

Despite decades of study the function of the GIMAP family remains enigmatic. GIMAP5 is the most extensively studied family member. Previous work has demonstrated a drastic decrease in peripheral murine lymphocyte numbers following loss of GIMAP5 protein. Various hypotheses including defective calcium flux, dysregulation of Bcl-2 family members and aberrant GSK3β signaling have been proposed. Furthermore, the biochemical study of this protein including the role (if any) of GTP hydrolysis, interacting partners and specific metabolic/signaling processes influenced by GIMAP5 remains nearly completely unexplored. Finally, the role of any of these proteins in human biology remains nearly completely unstudied.

In this study I have described the first large scale human cohort of patients suffering from a disease caused by loss-of-function mutations in GIMAP5. This recessive Mendelian disease has 100% penetrance across four pedigrees and nine affected individuals. Broadly speaking, the clinical features of the GIMAP5-deficient human patients were remarkably similar to the phenotype of two independent mouse strains carrying similar mutations (Ryan D Schulteis *et al.*, 2008a; Barnes *et al.*, 2010a). These patients suffered from lymphopenia, thrombocytopenia, lymphadenopathy/splenomegaly, NRH and bronchiectasis in addition to other pedigree specific autoimmune conditions. I also developed the first *in vitro* loss-of-function CRISPR/siRNA models to acutely deplete GIMAP5 in both murine and human T cells. I leveraged these models to reveal a striking difference in T cell phenotype between mouse and man, as well as a previously

unknown link between GIMAP5 and ceramide biology. I also established a retroviral transduction model to rescue T cells from the sphinx mouse and used this to demonstrate that regulation of GIMAP5 GTPase activity was required for proper function in addition to definitively showing that the L223F patient mutation is non-functional. Lastly, I also undertook an extensive characterization of the GIMAP5 interactome and showed three main clusters of GIMAP5 interacting proteins. One cluster (the Ragulator complex) was validated via two independent approaches.

Taken together, the body of work in this thesis sheds light on some of the major mysteries surrounding GIMAP5 and the GIMAP family more broadly but also poses new questions. Primarily, what is the explanation for the significant difference between the mouse and human phenotypes in T cells *in vitro* given that their clinical features are so similar? Furthermore, how does GIMAP5 regulate ceramide levels, and what is the function of its interaction with the Ragulator complex?

II. Clinical and functional phenotyping of GIMAP5-deficient patients

My findings in the human model both extend previous results published in the murine model as well as shed light on new phenotypes that had not been described. GIMAP5-deficient human patients share many cardinal clinical features across the entire cohort as well as with GIMAP5-deficient mice on a C57bl/6 background. B/T/NK cell lymphopenia, mild anemia, thrombocytopenia and liver dysfunction were common to all patients as well as the two murine models. However, in the human patients I also observed some inter-pedigree variation likely caused by different genetic backgrounds as

well as the harsher environment humans experience relative to laboratory. Furthermore, I also observed an additional set of phenotypes in the human patients not present in the murine model on a C57bl/6 background. For example, nearly all of the patients suffered from recurrent lung infections and consequently bronchiectasis which was not apparent in the SPF conditions experienced by the mice. In addition, studying the human model revealed varied pedigree specific autoimmune conditions such as psoriasis (P3-1), uveitis (P2-1), and autoantibody production (2-3, 2-4, 3-1). This raises the possibility that loss of GIMAP5 predisposes to autoimmunity more generally and that other environmental and genetic factors can contribute to the manifestation of these lower penetrance phenotypes.

We also analyzed some phenotypes shared with the murine model in more depth in the human model than was previously done. Schulteis *et al.* show that in a knockout murine model of GIMAP5 broad liver dysfunction occurs with histology showing large areas of necrosis (Ryan D Schulteis *et al.*, 2008c). I observed elevated liver enzymes in the serum of the patients as well as one patient experiencing acute liver failure due to nodular regenerative hyperplasia. Furthermore, some patients initially presented to gastroenterologists rather than immunologists suggesting that the liver disease may be more debilitating than the immunological issues in some cases. Histological findings were very typical of NRH, with mild lymphocytic infiltrates and aberrant expression of CD34 on the vasculature. Thus, GIMAP5 deficiency could represent the first monogenic knockout murine model of NRH, as previous models relied on overexpression of both IL-6 and the IL-6R on hepatocytes (Maione *et al.*, 1998). Interestingly, Schulteis *et al*. claim that crossing GIMAP5 to *Rag2*$^{-/-}$ deficient mice had no effect on the liver phenotype,

suggesting that GIMAP5 plays a critical role in other cell types beyond lymphocytes (Ryan D Schulteis *et al.*, 2008a). This finding could be of great interest to the NRH field as the study of GIMAP5 could reveal specific cell types and pathways involved in the pathology of NRH which to date has remained very obscure. Conversely, this finding could also point towards IL-6 playing a role in the liver pathology of GIMAP5-deficient patients. This would suggest that therapies targeting the IL-6 axis may be more effective at controlling liver disease in GIMAP5 patients than therapies aimed at controlling autoreactive lymphocytes.

Unfortunately, my current study offers little understanding as to the etiology of all clinical findings in the patient cohort. I hypothesize that many of the autoimmune symptoms observed in these patients could be secondary to the lymphopenia, although further work is required to test this.

We also carried out much more extensive profiling of PBMCs on these patients than has previously been done in the murine model. Previous studies have shown a decrease in naïve T cells (via CD44/CD62L expression). I observe a similar phenomenon using roughly analogous human markers CD45RA and CCR7. I also carried out deep phenotyping of patient PBMCs using an extensive CyTOF panel with 38 different markers. These data reinforced my hypothesis that the major defects observed in GIMAP5 deficiency were due to the lymphoid compartment as these patients had no major phenotypic differences in any of the myeloid compartments. However, I did observe perturbations consistent with extensive LIP – namely, a loss of naïve T cells, an expansion of effector memory subsets and a drastic increase in the fraction of

$CD57^{+}CD8^{+}$ T cells. Again, consistent with LIP I also observed extremely short telomeres in patient T cells suggesting that significant homeostatic proliferation had occurred after egress from the thymus. Curiously, the CyTOF phenotyping also revealed differences in the B cell compartment of GIMAP5-deficient patients. This was particularly surprising given my finding that human B cells do not express GIMAP5, in contrast to murine B cells. This demonstrates that the mature B cells are being influenced by the inflammatory/pro-autoimmunity environment generated by loss of GIMAP5 in other cell types, or GIMAP5 is required at some earlier point in B cell development and the rate of naïve B cell production is impaired. In terms of B cell function, nearly the entire patient cohort shared B cell lymphopenia although the consequences of this were much more varied with some individuals exhibiting hypergammaglobulinemia, others hypogammaglobulinemia as well as some testing positively for autoantibodies. Overall, my phenotyping data is strongly indicative of a disease characterized by normal myeloid compartments but severe lymphopenia.

We hypothesized that this lymphopenia was the prime perturbation to the immune system induced by loss of GIMAP5 and was central to my attempts to study the gene. My current data does not allow me to differentiate between peripheral lymphopenia caused by defective thymic output, trafficking defects resulting in sequestration in peripheral lymphoid organs or a peripheral survival defect. Previous studies have published conflicting claims with regards to T cell development in the murine model of GIMAP5 deficiency. One group has claimed that while the fraction of each thymic subpopulation is unaffected, HSC function and survival in the bone marrow is impaired (Ryan D Schulteis

et al., 2008a; Chen *et al.*, 2011a). Another has claimed that thymic involution is accelerated in the Sphinx mouse with the total number of thymus cells decreasing more rapidly relative to controls (Chen *et al.*, 2016). Moreover, it is clear that there is an *ex vivo* survival defect of T cells isolated from GIMAP5-deficient mice which would support a model with defective T cell survival in the periphery. More experiments designed to differentiate between these possibilities are required to answer this question.

With regards to the human model even less data is available as I have been unable to study thymic tissue. However, in contrast to the murine model, I have shown that survival of GIMAP5-deficient T lymphocytes *in vitro* is normal. This would cast doubt on the hypothesis that the lymphopenia is caused by defective peripheral survival, although it is also possible that there is some element *in vivo* which I cannot replicate in my *in vitro* culture system. While the lymphadenopathy observed in the GIMAP5-deficient patients could indicate a trafficking disorder, this finding is discordant with the murine model which have smaller lymph nodes with fewer cells. Measurement of naïve T cell production by testing for T cell Receptor Excision Circles (TRECs) in the peripheral T cells of humans and mice deficient for GIMAP5 would be extremely helpful in differentiating between these possibilities.

III. GIMAP5 expression, structure and genetics

GIMAP5 has a GTPase domain with homology to other small GTPases. In addition to this it also contains a C-terminal hydrophobic sequence which is thought to be required for insertion into the lysosomal membrane. Unfortunately my attempts to test this by mutating this sequence were stymied by the fact that deletion of this domain

dramatically destabilized the protein. It is unclear whether this was due to an intrinsic destabilization of the protein or stability of the protein is dependent on localization and/or insertion into a membrane. Two of the alleles observed in the patient cohort (p.L223F and p.N221S) were located in this domain. Thus, these mutations could lead to a loss of protein due to destabilization or defective function of this C-terminal domain.

All affected individuals in my cohort carried either homozygous or compound heterozygous mutations in GIMAP5. Individuals carrying these alleles in the heterozygous state did not report any clinical complications, although it is possible that my cohort is too small as the GIMAP locus has been linked to various autoimmune conditions (Lee, Horie, Graham R Wallace, *et al.*, 2013). As GIMAP5 is a GTPase, I was surprised to see that many of the patient mutations were localized to sections of the protein distal from the active site of the enzyme. Specifically, The P109L, N221S and L223F alleles were all located either in the C-terminal hydrophobic regions of the protein or in the GTPase domain but very distal from the active site. The I47T and L204P alleles affected residues closer to the active site. However, this puzzle was resolved when I measured levels of GIMAP5 in patient T cells via western and observed a dramatic decrease in protein. Further work from my collaborators demonstrated that these alleles intrinsically destabilized the protein even when synthesized and purified from bacteria. Thus, it appears that the human patient mutations lead to a near complete loss-of-function primarily due to destabilization of the protein rather than affecting rates of GTP hydrolysis.

Also of interest is the fact that some of these alleles are relatively common in the general population. The Turkish patients in the cohort carried the I47T, P109L and L223F alleles which had not previously been detected as of this writing. These could represent alleles that are common in Turkey but underrepresented in available databases. On the other hand, the Minor Allele Frequency (MAF) of the L204P mutation is on the order of 0.1%, suggesting that there are a relatively high number of unions between individuals heterozygous for this mutation. Indeed, another group has recently published a study describing a single patient homozygous for the L204P mutation with clinical features resembling my cohort (Patterson *et al.*, 2018).

We also investigated the expression patterns of GIMAP5. Initially, I investigated publicly available microarray data from a large variety of human tissues (Su *et al.*, 2004). This dataset showed significant expression in $CD4^+$ and $CD8^+$ T cells, some expression in NK cells and monocytes and virtually no other tissues. I confirmed these findings at the protein level via western blot from primary human tissues as well. However, I suspected there were functionally significant non-lymphoid tissues which required GIMAP5 due to the clinical phenotypes of GIMAP5-deficient patients. In particular, the bronchiectasis and liver disease could both be due to GIMAP5 playing a role in non-lymphoid tissues. The latter is also supported by the finding that $GIMAP5^{-/-}Rag2^{-/-}$ mice still suffer from liver disease. Thus, I also investigated GIMAP5 expression at the protein level from a large variety of human tissues and saw robust expression in tissues with large populations of T and NK cells such as the spleen and thymus. However, I also observed high levels of GIMAP5 in other tissues such as lungs, ovary and prostate. It is unclear at this time

whether this signal can be completely explained by resident lymphoid populations or not. However, this finding prompted me to investigate publicly available single cell RNA-Seq databases for GIMAP5 expression patterns, and observed robust GIMAP5 mRNA expression in endothelial cells. I think it is likely that the liver phenotype in both my GIMAP5-deficient mice and humans is in part or entirely caused by loss of GIMAP5 in endothelial cells, although future studies in this area are required.

IV. GIMAP5 interactome data

The most critical lacunae in our knowledge of the GIMAP family is which biochemical cellular processes it regulates. I sought to address this via the elucidation of the GIMAP5 interactome. Previous work using overexpression systems in 293T or B cell leukemia cell lines (which do not naturally express endogenous GIMAP5 and likely many other lymphoid restricted binding partners) has posited that GIMAP5 directly interacts with and regulates Bcl-2 family members on the surface of the mitochondria. This finding was attractive due to the lymphopenia exhibited by GIMAP5-deficient murine T cells as well as the decreased *in vitro* survival of GIMAP5-deficient murine lymphocytes (Barnes *et al.*, 2010a). However, more recent work by (Vivian W.Y. Wong *et al.*, 2010) as well as our group studying endogenous GIMAP5 in primary cells rather than overexpression systems provides strong evidence that GIMAP5 is restricted to the lysosome and is virtually absent from mitochondria where Bcl-2 family members typically reside. This would suggest that the previous findings could be an artifact due to the overexpression and encouraged me to explore other approaches to identify interacting partners.

Our BioID2 interactome represents the first robust dataset describing the interactome of GIMAP5 in a T cell line. Interestingly, I did not observe any of the Bcl-2 family members that have previously been described to interact with GIMAP5. I also did not observe GSK3β, any genes related to calcium flux or FOXO family proteins all of which are biochemical functions previously attributed to GIMAP5 (Ilangumaran *et al.*, 2009; Aksoylar *et al.*, 2012; Patterson *et al.*, 2018). However, I did observe a very robust signal for the entire Ragulator complex (LAMTOR1-5, RagC, other Rag proteins were present but not statistically significant) as well as multiple components of the TCR signaling complex (CD2, CD3*ε*, CD3*δ*, CD4 and Lck). These findings do tie into one previous publication suggesting that GIMAP5$^{sph/sph}$ T cells have increased Akt and mTORC1 signaling (Chen *et al.*, 2015). The TCR complex cluster, however, was completely unexpected despite being among the most enriched proteins in the BioID2-GIMAP5 condition. Specifically, I was initially unsure as to how a lysosomal protein could be interacting with the TCR complex. However, a fascinating study recently published describes an analogous situation in immortalized B cell lines. The authors describe a novel complex of the BCR, MYD88 and the Ragulator complex on the surface of the lysosome (Phelan *et al.*, 2018b). Furthermore, this complex forms specifically in B cell leukemia lines whose growth is sensitive to inhibitors of the BCR pathway. In the case of GIMAP5, its role in the TCR/Ragulator complex remains unclear.

Lastly, I also observed a large cluster of proteins (Rab10, SNAP23, VAMP7, Syntaxin1, etc.) which I broadly labeled as genes involved in cellular trafficking. From this data alone it is unclear whether these are simply the genes involved in trafficking

GIMAP5 to the lysosome/late endosome or whether GIMAP5 has a direct functional role in their regulation. One possible model implied by these datasets is that GIMAP5 regulates the internalization and recycling/degradation of the TCR complex from the cell surface.

Despite its strengths the BioID2 system still relies on overexpression of the bait protein and could be prone to artifacts. This was addressed this via endogenous co-immunoprecipitations as well as PLA. The latter has the added bonus of showing that the two proteins are temporally and spatially in contact in a cell and thus avoids artifacts caused by lysing the cell and disrupting membranes and spatial localization of protein complexes. Furthermore, these data greatly strengthen my confidence that GIMAP5 is truly interacting with the Ragulator complex and more broadly suggests that my BioID2 dataset is robust.

One drawback of these approaches has been defining the precise protein(s) in direct contact with GIMAP5. For example, it is extremely unlikely that GIMAP5 makes direct contact with each protein of the regulator complex. Unfortunately, my attempts to determine this were hindered by non-specific binding of GIMAP5 overexpressed in 293Ts as well as the fact that endogenous LAMTOR proteins form an obligate pentamer, making it difficult to simultaneously overexpress the entire complex (data not shown). Future approaches using Förster Resonance Energy Transfer (FRET) may be more precise than the BioID2 technique and allow the determination of the direct binding partner. Alternatively, crystallization of the Ragulator/GIMAP5 complex or purification

of large quantities of each individual member in a bacterial expression system may enable clarification of this point in the future.

V. The functional role of GIMAP5 in lymphocytes

Due to my robust and reproducible data demonstrating that GIMAP5 is a T cell specific member of the Ragulator complex, I invested a significant amount of effort investigating downstream cellular processes. The most detailed studies to date have shown the Ragulator complex to be required for mTORC1 recruitment to the lysosome when sufficient amino acids are present, allowing for consequent activation by the TSC1/2 complex (Bar-Peled and Sabatini, 2014). However, older studies have also described these proteins as being scaffolds for Mek/Erk as well as being involved in lysosomal trafficking to and from the perinuclear space (Pu *et al.*, 2015; Pu, Keren-Kaplan and Bonifacino, 2017b). I and others have shown that T cells isolated from GIMAP5$^{sph/sph}$ mice have increased cell size and mTORC1 signaling activity relative to controls (Chen *et al.*, 2015). This led me to hypothesize that GIMAP5 is a negative regulator of mTORC1, most likely via its interaction with the Ragulator complex. My collaborators and I also demonstrated that treatment of both mice and humans deficient for GIMAP5 with mTORC1 inhibitors could lead to clinical benefit lending support to this hypothesis. However, after extensively investigating multiple different models of GIMAP5 deficiency in human cells (Jurkat CRISPR knockouts, GIMAP5-deficient patient cells, GIMAP5 siRNA/CRISPR-RNP depletion strategies) I observed no difference in a large number of mTORC1 readouts either at baseline, following TCR stimulation, or amino acid starvation/addback. I measured proliferation, cell size,

phosphorylation of targets such as S6, 4EBP1, Ulk1 and S6K. While the single-cell Jurkat clones and individual patients/healthy donors showed some variability, more controlled models such as the GIMAP5 siRNA knockdowns or CRISPR-RNPs showed virtually no differences between cells lacking GIMAP5 and appropriate controls. Overall, these data are very strong evidence GIMAP5 is not regulator of mTORC1 activity. While it is possible that there are specific components of the *in vivo* milieu that are required for this phenotype to become evident, I would argue it is much more likely that the increased mTORC1 activity observed in the GIMAP5$^{sph/sph}$ mice is due to a broader ongoing autoimmune reaction which can be alleviated with rapamycin treatment. Furthermore, despite very similar clinical phenotypes, T lymphocytes isolated from human patients do not exhibit increased mTORC1 activity directly *ex vivo*. I also observed a broad increase p-Erk in lymph node cells from Sphinx mice suggesting that other pathways besides mTORC1 are affected. I would propose instead that loss of GIMAP5 leads to cell death independently of mTORC1, creating a lymphopenic environment where the remaining T cells become activated by increased availability of self-peptide and cytokines. This indirect effect can then be inhibited by rapamycin leading to abatement of some symptoms in GIMAP5 deficiency. As mentioned previously, crossing GIMAP5-deficient mice to Rag2$^{-/-}$ mice did not improve the liver phenotype. While the human patient who was treated with rapamycin did experience a significant reduction in lymphadenopathy as well as psoriasis following treatment, he continued to have elevated liver enzymes in his blood suggesting that this is occurring via a separate mechanism.

One of the most surprising results from this study has been the striking difference in the *in vitro* phenotypes of murine versus human T cells deficient for GIMAP5. This is most clearly illustrated by the fact that in my experiments knocking GIMAP5 out via CRISPR-RNP electroporation in human T cells has no effect on survival or proliferation following stimulation whereas the vast majority of murine T cells will die within 96 hours in the absence of GIMAP5. T cells isolated from GIMAP5-deficient patients or generated via GIMAP5 siRNA may still retain sufficient protein for residual activity, however, the CRISPR model should result in frameshift mutations leading to a complete loss in protein providing very strong evidence that GIMAP5 is not required for survival in human T cells. GIMAP3 is a pseudogene in humans, while GIMAP2 does not exist in the mouse suggesting that perhaps another GIMAP family member can compensate for the loss of GIMAP5 in human T cells. However, this seems very unlikely as firstly I have shown that T cells from GIMAP5-deficient patients do not increase expression of other GIMAP family members. Secondly, the fact that GIMAP5-deficient patients still suffer from similar clinical features to the mice would argue that another gene cannot compensate for GIMAP5 when it comes to *in vivo* phenotype. I hypothesize there are two likely explanations for this finding. Firstly, the artificial *in vitro* environment used to culture my human T cells could lack some critical factor that is present *in vivo* that would alter the metabolism of the cells and lead to cell death. Similarly, my *in vitro* assays typically use antibodies to cross-link CD3 and CD28 with high efficiency rather than the much weaker self-peptide/TCR contacts and IL-7/IL-15 *in vivo*. Alternatively, it is possible that some/most of the clinical features of GIMAP5 deficiency are T cell

independent, or at least not caused by decreased survival. Studies in the murine model would argue against this possibility as it has been shown that multiple manipulations of T cells in GIMAP5$^{sph/sph}$ mice can rescue survival of the animal, implying an intrinsic defect (Barnes *et al.*, 2010a; Aksoylar *et al.*, 2012; Patterson *et al.*, 2018). Regardless, this example has highlighted the importance of studying the human model as these findings would have been completely overlooked if the field had been restricted to animal models.

VI. Rapamycin as a treatment for GIMAP5 deficiency

GTPases are typically thought of as 'molecular switches' commonly thought to be involved in signal transduction. Previous work which I have confirmed and extended has shown increased mTORC1 signaling in T lymphocytes isolated from Sphinx mice (Chen *et al.*, 2015). Furthermore, multiple critical regulatory points of the mTORC1 pathway have already been shown to regulate mTORC1 activity through modulation of GTPase activity. Firstly, activation of the PI3K signaling pathway leads to activation of the TSC1/2 complex, which in turn acts as a GEF for the small GTPase Rheb (Huang and Manning, 2008). Rheb can then potentiate the activity of the mTOR kinase (Armijo *et al.*, 2016). Secondly, recent work has described heterodimers of the Rag GTPases recruiting mTORC1 to the surface of the lysosome (which is thought to be required for its activity) in response to the availability of amino acids (Bar-peled *et al.*, 2012; Efeyan *et al.*, 2013; Bar-Peled and Sabatini, 2014). Thus, considering these previous results I hypothesized that GIMAP5 provided a novel layer of mTORC1 regulation specific to the extreme metabolic demands of lymphocyte activation. This was further supported by its

localization to the surface of the lysosome and clinical phenotype which has significant overlap with other Mendelian diseases and animal models leading to hyperactive mTORC1. For example, T cell specific ablation of TSC1 in mice led to a very similar phenotype to GIMAP5-deficient mice (Yang *et al.*, 2011; Zhang *et al.*, 2012). In the human model, disorders of hyperactive PI3K and consequently increased mTORC1 also share many features with GIMAP5 deficiency (Lucas *et al.*, 2014).

Consistent with my hypothesis, I observed significant clinical improvement in one patient as well as the Sphinx murine model of GIMAP5 deficiency. The lymphadenopathy and splenomegaly were both drastically reduced and the psoriasis was also largely resolved. However, serum liver enzymes remained elevated suggesting that this aspect of the disease was caused by a different mechanism. Unfortunately, I did not receive samples prior to commencement of the treatment which precluded an analysis of the impact of rapamycin on the immune cell subpopulations. In the murine Sphinx model, treatment with rapamycin was observed to reduce mTORC1 signaling and cell size as expected, but also prevented the loss of naïve T cells and development of effector memory T cell populations which I suspect are the root cause of many of the clinical symptoms. Thus, the treatment appeared effective in at least one patient for certain aspects of the diseaseas well as in the mouse model.

However, virtually all of my mechanistic data exploiting multiple models and readouts showed no impact of the loss of GIMAP5 on mTORC1 signaling. Rapamycin is also a general immunosuppressant and thus it is likely that the clinical benefit I observed was due to an indirect effect of inhibiting mTORC1 signaling rather than a direct

targeting of the pathway affected by GIMAP5 deficiency. Determination of the molecular pathways regulated by GIMAP5 remains key to developing more effective therapies that may address all of the clinical features of the patients. My data demonstrating increased levels of ceramides in murine T cells acutely depleted of GIMAP5 provides a new candidate pathway to target. Unfortunately while there are inhibitors of multiple enzymes in this pathway and none of these are approved for clinical use and appear to be quite toxic (Colvin, Cooley and Beaver, 1993). Furthermore, as of now my data remains *in vitro* in nature and requires more work both *in vitro* and in *in vivo* murine models to test potential efficacy. Overall, rapamycin or potentially bone marrow and/or liver transplantation likely remain the best treatment options at this point.

VII. Proposing a new model of GIMAP5 function

As discussed above, many models of GIMAP5 function have been put forward over the years. While I have been able to directly confirm many previous results from the literature in the GIMAP5$^{sph/sph}$ model, none of them were consistent in the human model or *in vitro* models of acute GIMAP5 depletion.

Curiously, GIMAP1 and (to a lesser extent) GIMAP6 deficient mice have very similar phenotypes to GIMAP5$^{sph/sph}$ or knockout mice (Saunders, Louise M.C. Webb, *et al.*, 2010; Pascall *et al.*, 2013, 2018; Webb *et al.*, 2016). The other GIMAP family members are less clear as human GIMAP2 does not have a direct murine homologue, GIMAP7 knockout mice have not been generated, and GIMAP8 deficient mice have virtually no phenotype (Krücken *et al.*, 2004; Webb *et al.*, 2014). Despite having similar phenotypes at the organismal level, the subcellular localizations of these proteins are very

different. GIMAP1 localizes to the Golgi, GIMAP2 and 7 are thought to heterodimerize on the surface of lipid droplets, GIMAP5 to the surface of the lysosome and GIMAP6 is thought to be cytosolic but recruited to autophagosomes when autophagy is induced (Nitta and Takahama, 2007; Vivian W.Y. Wong *et al.*, 2010).

The similarity in phenotypes combined with the different localizations of the GIMAP family proteins suggests that these are all essential, non-redundant genes at different steps in the same pathway. Thus, I propose a new model of GIMAP family function in regulating lipid metabolism and/or transport. Experimentally, this is supported by my high-throughput experiments showing dramatic alterations in lipid metabolism in GIMAP5-deficient murine lymphocytes. Furthermore, each GIMAP family member localizes to an organelle important for lipid metabolism. For example, the heterodimer of GIMAP2/7 localizes to the surface of lipid droplet presumably via the hydrophobic C-terminal domain of GIMAP2. Lipid droplets serve as cellular reservoirs of lipids and cholesterol. Thus, GIMAP2/7 could regulate mobilization/storage of lipids from lipid droplets. Most lipids are synthesized in the ER and then transported through the Golgi to their destinations throughout the cell. I speculate that GIMAP1 may be involved in this process. Further experiments to test this model requires metabolic profiling of lymphocytes deficient in GIMAP1, 2, GIMAP6 and GIMAP7 as well as the generation of mice deficient for GIMAP7.

One other question raised by this model is why lymphocytes would require specific machinery for lipid metabolism that is absent from other cell types. Multiple genetic Lysosomal Storage Diseases (LSDs) that disrupt lipid metabolism have been

identified and studied, however, these individuals have not been reported to suffer from immunological abnormalities. One method of diagnosis involves detecting lipid bodies in lymphocytes isolated from PBMCs directly via light microscopy (Kanzaki, 2011). These lipid bodies are not evident in mice or patients lacking GIMAP5, indicating a metabolic defect of a different nature. I hypothesize that the extraordinary metabolic demands of T cell activation and rapid cell division in the face of pathogen attack requires additional levels of regulation, some of which could be provided by members of the GIMAP family.

VIII. HPLM as a novel media for the culture of human T lymphocytes

In vitro cell culture techniques have greatly accelerated biological research and provide much more easily accessible and high throughput methods. However, despite this most media used for cell culture protocols has remained virtually unchanged for decades. Improvements to cell culture media or the development of media more closely modeling the *in vivo* environment would be easy to adopt by many research groups and have a large benefit to the field at large.

Cantor *et al.* have recently developed a media designed to more closely mimic human plasma, termed HPLM. However, to date only one study on HPLM has been published and no attempt was made to test the function and growth rate of lymphocytes in HPLM. I hypothesized that HPLM would better support the growth of human T cells in cell culture. My data did in fact show much greater activation of naïve human T cells in HPLM relative to RPMI. I showed this was due to the physiological levels of calcium in HPLM, while RPMI is severely hypocalcemic relative to the *in vivo* environment. Of

note, these experiments were conducted in HPLM/RPMI supplemented with dialyzed FBS in order to preserve the composition of each media while still adding the required growth factors. The dialyzed FBS should be stripped of all ions and small organic molecules. I measured the calcium concentration in complete FBS to be nearly 4 mM, thus under common culture conditions with a supplement of 10% FBS the amount of calcium in RPMI is roughly doubled. Based on my calcium flux data, it would appear that while this concentration would still be severely hypocalcemic (~0.8mM) at least under those conditions functionally that appears to be sufficient. It remains unclear if culturing cells in 0.8mM rather than physiological concentrations of calcium affects other aspects of T cell biology or not.

Other groups have observed similar results in different conditions. For example, Zimmermann *et al.* in 2015 first induced colitis in mice via T cell transfer (Zimmermann, Radbruch and Chang, 2015). They then isolated lamina propria T cells and restimulated them acutely *in vitro* in either IMDM, RPMI or RPMI supplemented with 1mM $CaCl_2$. Under these conditions, they observed slightly decreased secretion of effector cytokines IL-10, IFNγ, IL-17, TNFα and IL-22. I observed a much more robust difference between my RPMI and HPLM/RPMI + Ca^{2+} conditions. This is likely due to the fact that I activated and expanded my cells prior to restimulation, ensuring that the vast majority were strongly activated and primed to secrete cytokines following restimulation as opposed to measuring this directly *ex vivo*. I also extend these results by showing that under the hypocalcemic conditions of RPMI both the initial calcium flux into the cell following activation is deficient as well as early activation events including CD25 and

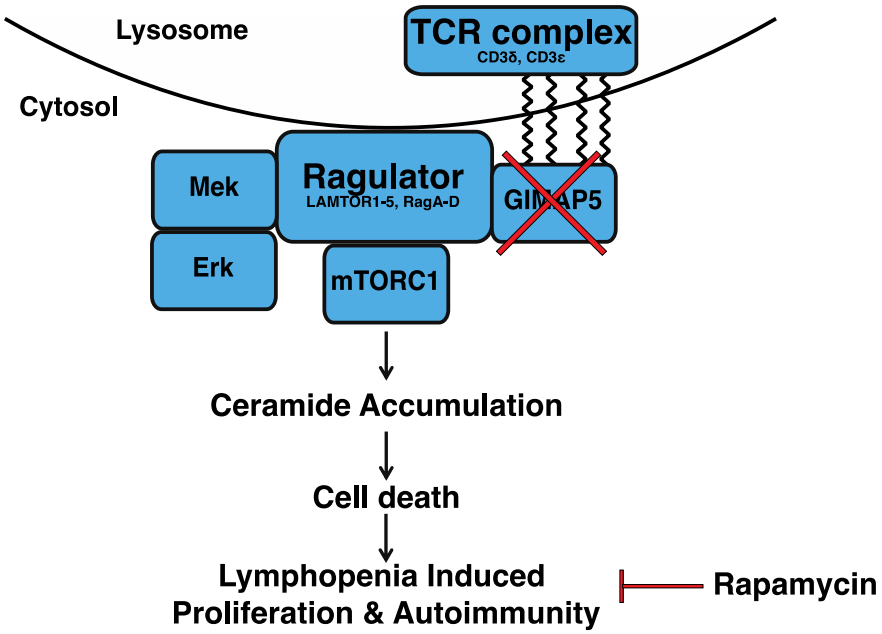
CD69. Other groups have also observed decreased calcium flux in basal RPMI relative to other media (Prakriya *et al.*, 2006; Gwack *et al.*, 2008; Bertin *et al.*, 2014).

While my work does not provide convincing evidence that the small metabolites present in HPLM have a significant impact on T cell function or biology, I nevertheless did show improved activation in HPLM due to higher levels of calcium relative to RPMI. Either the use of HPLM or the addition of supplemental calcium to RPMI has the potential to improve the efficiency and physiological relevance of *in vitro* T cell assays.

IX. Conclusion

These findings have led me to propose a new model of GIMAP5 function: that GIMAP5 is a novel, T cell specific member of the Ragulator complex on the surface of the lysosome (Figure 5.1). Based on my interactomics data it also appears to either form a larger supercomplex consisting of various members of the TCR complex, the Ragulator complex as well as endosomal trafficking proteins or is able to sequentially associate with these groups of proteins. From this point the loss of GIMAP5 leads to a dysregulation of ceramide biology, resulting in an excess accumulation of ceramides and cell death.

Figure 5.1
A new model of GIMAP5 function



A) Figure showing a model of GIMAP5 activity incorporating the novel interactions between GIMAP5 and the TCR/Ragulator complexes. The absence of GIMAP5 leads to ceramide accumulation, cell death and consequently lymphopenia induced proliferation and autoimmunity which can be relieved by Rapamycin treatment.

There are still major conceptual questions that remain with regards to the biology of GIMAP5. Firstly, while I both define the interactome and cellular pathways that GIMAP5 regulates in murine T cells, a molecular mechanism connecting the Ragulator complex/TCR/GIMAP5 and ceramide biology is lacking. Further studies into the link between the Ragulator/TCR complex and ceramide biology, perhaps by lipidomics studies of Ragulator-deficient T cells, are required. The second major unanswered question is the nature of the mouse-human difference in terms of the *in vitro* phenotype

of GIMAP5-deficient T lymphocytes. Whether the human cells required specific conditions or stimuli in order to exhibit defective survival, T cells are not the cell type causing the *in vivo* condition or some other possibility remains to be seen.

Materials and Methods

Immunoblotting

Protein lysates were isolated from cell pellets via a 20 minute incubation in lysis buffer (1% NP-40, 150 mM sodium chloride, 50mM Tris-HCl, pH 7.4 plus Roche c0mplete™ protease inhibitor cocktail and then clarified via centrifugation at 14,000 to 16,000 x g at 4°C for 20 minutes. Total protein levels were measured by BCA assay (Pierce) prior to reduction with 5% beta-mercaptoethanol and SDS Loading Solution (Quality Biological) at 95°C for 15 minutes. Equal amounts of protein were electrophoresed on either 4-12% Bis-Tris or 10% Bis-Tris gels (NuPAGE, Life Technologies) and wet-transferred to nitrocellulose membranes. In the case of human total tissue western blots, equivalent amounts of protein from 19 different human tissues were run on a gel (Zyagen) and then processed in a similar fashion. These were then blocked in 5% non-fat milk in 0.05% TBST and probed with primary antibodies to GIMAP1, GIMAP4, GIMAP5, GIMAP6 and GIMAP7 (generous gifts from Dr. Geoffrey Butcher); GIMAP2 and GIMAP8 (produced by GenScript); IkBα (662402, Biolegend), β-Actin (ab20272)(purchased from Abcam); LAMTOR1 (C11orf59, #8975), LAMTOR4 (C7orf59, #13140), RagA (#4357), RagC (#3360), mammalian Target Of Rapamycin (mTOR, #2983), Rheb (#13879), LAMP1 (#15665), Raptor (#2280), phospho-4E Binding Protein 1^{S65} (p4EBP1, #9451), phospho-S6 ribosomal $protein^{S240/244}$ (pS6, #5364), phospho-p70 S6 kinaseThr389 (pS6K, 9234) (all purchased from Cell Signaling Technology). Binding of primary antibodies was then measured via HRP-tagged antibodies specific for the relevant species

and visualized by chemiluminescent substrate (Luminata Classico/Forte western HRP substrate or Thermo Fisher SuperSignal West Femto Maximum Sensitivity Substrate).

Co-Immunoprecipitations

$3x10^7$ cycling primary human T cell blasts were incubated with cRPMI containing 0.5 Units/ml (U/ml) of IL-2 overnight and restimulated with HIT3α (BioLegend, 10 ug/ml) and 10 μg/ml $F(ab')_2$ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch) or 100 U/ml of IL-2 for the indicated timecourses. Alternatively, T cell blasts were starved of amino acids via incubation in amino acid free RPMI (US Biological, R9010-01) containing dialyzed FBS (Thermo Fisher, A3382001) for one hour. They were then restimulated for the indicated times with complete RPMI (cRPMI). ACell lysates were precleared with unconjugated Dynabeads Protein G (Thermo Fisher Scientific) for 30 minutes with rotation at 4°C. Primary antibodies were then added to the precleared lysates and incubated with rotation overnight at 4°C. Dynabeads Protein G were added and incubated for an additional 1 hour at 4°C. Immunoprecipitates were washed four times with lysis buffer. Immunoprecipitated proteins were eluted from the dynabeads via 5 minutes at 95°C in SDS sample loading buffer resolved by electrophoresis and analyzed by immunoblotting.

***In Vivo* Rapamycin treatments**

Rapamycin (LC labs) was dissolved in DMSO to generate a concentrated stock solution. This was then further diluted in PBS for *in vivo* use. 7-9 week old age and sex matched mice were injected intraperitoneally with 2mg/kg of rapamycin daily for two weeks prior to isolation of tissues for further experimentation.

Cytof

This assay was performed with the Human Immune Monitoring Center at Stanford University as previously described (Leipold and Maecker, 2015). Frozen PBMCs from patients or healthy controls were thawed, washed twice, resuspended in CyFACS buffer (PBS supplemented with 2% BSA, 2 mM EDTA, and 0.1% sodium azide), and viable cells were counted by Vicell. $1.5x10^6$ viable cells were stained for 60 minutes on ice with 50µl of the antibody-polymer conjugate cocktail described in Table S4. Sources for antibodies are listed in Table S4 while the polymer and metal isotopes were purchased from DVS Sciences. Following staining, the cells were washed twice in FACS buffer before resuspension in 100µl PBS buffer containing 2 µg/ml Live-Dead (DOTA-maleimide (Macrocyclics) containing natural-abundance indium). The cells were washed twice with PBS before fixation with 100µl of 2% PFA in PBS and placed at 4°C overnight. The next day, the cells were pelleted and washed by resuspension in fresh PBS. The cells were washed twice in MilliQ water before resuspension in 700µl of MilliQ water before injection into the CyTOF (DVS Sciences). Data analysis was performed using FlowJo v9.3 (CyTOF settings) by gating on intact cells based on the iridium isotopes from the intercalator, then on singlets by Ir-191 vs cell length, then on live cells (Indium-LiveDead minus population). Further analysis on these populations was then carried out using SPADE v. 3.9 using the following definitions:

T cells: $CD3^+$ $CD19^-$ $CD33^-$
$CD4^+$ T cells: $CD4^+$ $CD8^-$ $TCR\gamma\delta^-$
$CD8^+$ T cells: $CD8^+$ $CD4^-$ $TCR\gamma\delta^-$
$TCR\gamma\delta$ T cells: $CD3^+$ $TCR\gamma\delta^+$
Naïve T cells: $CD45RA^+$ $CCR7^+$
TEMs: $CD45RA^-$ $CCR7^-$

TCMs: $CD45RA^{-}$ $CCR7^{+}$
TEMRAs: $CD45RA^{+}$ $CCR7^{-}$
NK cells: $CD56^{+}$ HLA-DR- $CD3^{-}$ $CD19^{-}$ $CD33^{-}$
B cells: $CD19^{+}$ $CD20^{+}$ $CD3^{-}$ $CD33^{-}$

Flow Cytometry

For human experiments, patient or control PBMCs were washed once in PBS and stained with 50μl of Zombie Aqua™ (Biolegend) viability dye for 20 minutes on ice. After washing once with FACS buffer, cells were stained in 50μl of FACS buffer containing diluted antibodies for 30 minutes on ice. Cells were then washed three times in FACS buffer and fixed before acquisition on either a LSR II or LSRFortessa (BD Biosciences). Murine cells isolated from either spleen or lymph nodes were stained and analyzed in a similar manner. Data was analyzed using FlowJo v. 9.9.5.

Telomere analysis

Frozen PBMCs from patient P1.3 and P1.4 were shipped to Repeat Diagnostics for measurement of telomere length via Flow-FISH. A fluorescently labeled nucleic acid probe was hybridized with the TTAGGG repeats in telomeres. Signal strength was then plotted relative to telomere lengths for healthy control subjects in order to calculate the percentile for each patient.

Confocal/STED microscopy

Cycling T lymphocytes were either treated with Mitotracker were incubated for 30 min at 37oC with 6.25 nM of Mitotracker Red CMX-ROS in complete RPMI with 100 U/mL IL-2. For confocal imaging, cells were plated onto poly-lysine coated coverslips for five minutes before addition of an equal volume of 8% paraformaldehyde for 30 min at room

temperature. After washing the coverslips twice with PBS, the samples were permeabilized for 15 min using 0.1% Triton X-100, washed, and then blocked with 5% BSA for at least 45 minutes. Samples were then incubated with primary antibodies diluted in PBS with 0.05% Tween-20 and 5% goat serum for two hours. After washing twice, the samples were then treated with secondary antibodies and Hoescht 33342 for 30 min at room temperature in the dark. Following two washes with PBS, then coverslips were mounted with Prolong Gold onto slides and left to dry overnight. Confocal images were acquired on a Leica SP5 X-WLL microscope and for STED, images were acquired using a Leica SP8 STED microscope. Analysis was performed using Imaris software.

Proximity Ligation Assay

Primary human T cell blasts were washed once in PBS and then fixed in 4% paraformaldehyde for 30 minutes at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature, washed twice with PBS and resuspended in 1ml of Duolink blocking solution for 1 hour. Cells were washed twice with PBS and resuspended in 50µl of the specified primary antibody solutions overnight with shaking at 4°C. The following morning, cells were washed twice in Duolink Buffer A, then incubated in secondary antibody for 1 hour at 37°C. Samples were sequentially washed twice, incubated with ligase for 30 minutes at 37°C, washed twice and incubated with Duolink In Situ Detection Reagents Green (Sigma Aldrich) at 37°C for 2 hours. Cells were then washed twice and either analyzed by flow cytometry or mounted on glass slides in Prolong Antifade with DAPI (Life technologies, P36971) for acquisition with a Leica SP5 X-WLL microscope.

Cell Culture & T cell expansion

In the case of patient cells, freshly isolated or frozen PBMCs were stimulated at $5x10^6$/mL in cRPMI with 2 ug/mL of PHA-L (lectin from red kidney bean (*Phaseolus vulgaris*), Sigma-Aldrich L2769), in the presence of 1 ug/mL anti-CD28 (Biolegend) and 1 ng/mL recombinant IL7 (Biolegend, Cat) for 48 hours. The cells were washed twice with cRPMI and resuspended in media at 1 x 10^6/mL with 100 IU/mL Recombinant Human IL2 (rhIL2), and cultured for up to 3 weeks with fresh rhIL2 and medium supplemented every 2 days. HUVECs were cultured using L200 growth medium supplemented with low serum growth supplement (Thermo Fisher).

Exome and Whole Genome Sequencing Analysis.

Genomic DNA was isolated from PBMCs of proband, parents and healthy relatives from each pedigree. Exome sequencing were generated using SureSelect Human All Exon 50Mb Kit (Agilent Technologies) coupled with Illumina HiSeq sequencing system (Illumina). Whole Genome Sequencing (WGS) were generated based on Standard Coverage Human WGS from Broad Institutes. For individual samples, WES produced ~50-100X sequence coverage for targeted regions and WGS produced 60X coverage for proband and 30X coverage for family members. DNA sequence data was aligned to the reference human genome (build 19) using Burrows-Wheeler Aligner (BWA) with default parameters and variants were called using the Genome Analysis ToolKit (GATK) (Li and Durbin, 2009). Variants were then annotated by functional impacts on encoded proteins and prioritized based on potential disease causing genetic model. Variants with minor allele frequency < 0.1% in the dbSNP (version 137), 1000 Genomes (1,094 subjects of

various ethnicities; May 2011 data release), Exome Sequencing Project (ESP, 4,300 European and 2,203 African-American subjects; last accessed August 2016), ExAC databases (61,000 subjects of various ethnicities; March 2016 data release) or Yale internal database (2,500 European subjects) were filtered. Autosomal-recessive inheritance was investigated and genes with rare homozygous or compound heterozygous variants were prioritized.

Sanger Sequencing of Genomic DNA

Direct bidirectional Sanger sequencing of *GIMAP5* mutation, p.I47T, from genomic DNA of two affected patients (P1-1 and P1-2) and their parents was performed following PCR amplification using forward primer: 5'-AAGATAACTTGTCTGCAACACCA-3', and reverse primer: 5'-GTAGCAGTCCCCGATGTTCT-3'. Nomenclature of the *GIMAP5* variants is based on NCBI reference sequence NM_080916.2.

Orthologues and Other Human GIMAPs

Full-length orthologues of GIMAP5 protein sequences from several species and related human GIMAP-family members (GIMAP1, 2, 4, 6, 7 and 8) were obtained from GenBank. Protein sequences were aligned using the Clustal Omega algorithm. For microarray data measuring GIMAP5 expression in human tissue, data was exported from (Su *et al.*, 2004).

T cell activation and proliferation assays

Primary human T cells from either GIMAP5-deficient patients or healthy donors were isolated from PBMCs. Following any manipulations (such as siRNA/CRISPR-RNP mediated depletion – see other sections) cells were then labeled with CellTrace Violet

(CTV) (Thermo Fischer Scientific). Briefly, a 5mM stock of CTV in DMSO was diluted 1000 fold in PBS to generate a 5μM working stock. T cells were then resuspended in the working stock and incubated for 20 minutes at 37°C, then washed once in RPMI. Cells were then stimulated with either CD2/3/28 magnetic beads (Miltenyi) or a dose response of plate-bound CD3/CD28 for 3-5 days. Cells were then stained for various surface markers as described elsewhere and CTV dilution was analyzed via flow cytometry.

To measure early T cell activation events, unlabeled primary human T cells/total PBMCs were activated either by plate-bound CD3/28, CD2/3/28 activation beads or soluble CD3/CD28. Cells were analyzed by flow cytometry either 24 hours (CD69 expression) or 72 hours (CD25 expression) following stimulation.

BioID2

pCI-BioID2-GIMAP5 was constructed by InFusion cloning technology according to the manufactural instructions (Clontech). A synthesized gBlock DNA that composed of a Kozak sequence, the BirA biotin ligase (BioID2), a 9x poly-glycine linker, and human GIMAP5 coding sequences into the pCI-neo backbone (Promega). The empty vector only contains the biotin ligase (BioID2) coding sequence and the 9x polyG linker. GIMAP5-deficient Jurkat cells were transfected with the two linearized BioID2 via BTX. The transfected cells were transferred to RPMI and stable transfectants were selected by limiting dilution against 800 ug/mL of G418 for over three weeks.

Jurkat clones were cultured in stable isotope labeling amino acid culture (SILAC) RPMI-1640 media (ThermoFisher). The low, medium, and heavy isotope supplementation and preparation were detailed previously (Phelan *et al.*, 2018b), with low molecular weight

isotope medium used for culturing a Jurkat clone expressing BioID2 alone and separately with medium and heavy isotopes for BioID2-GIMAP5. The SILAC cultures were expanded for 2 weeks before 50 uM D-biotin (Invitrogen) was added 20 hours prior to harvesting a total of 100 million cells from each culture. The cells were harvested, washed once with PBS and then all cultures were pooled into one tube and washed twice more with PBS. Cell pellets were lysed with 30 uL/million of lysis buffer (50 mM Tris.Cl pH7.5, 145 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.05% SDS, 10% glycerol, 1 x EDTA-free protease inhibitor cocktail (Roche)), at 4 °C for 30 minutes. The lysate was isolated by 10,000 x g centrifuging 10 minutes at 4 °C. Biotinylated proteins in the remaining lysate were isolated by via a two hour incubation with 400 uL of lysis-buffer-washed Dynabeads MyOne Streptavidin C1 (Invitrogen 65002) at 4ºC. After washes, the beads were resuspended in LDS sample buffer plus reducing agent (NuPAGE), and boiled for 5 min before PAGE electrophoresis separation and cutting of the gel into 20 slices, which were processed as previously described (Oellerich *et al.*, 2019). After tryptic digestion samples were separated by a UltiMate 3000 RSLCnano HPLC system coupled to a Q Exactive HF mass spectrometer. Data analysis was done using the MaxQuant software.

siRNA mediated depletion of GIMAP5

T cells were isolated from PBMCs of healthy donors and either stimulated and expanded in IL-2 for 6-10 days or processed immediately. 10-30x10^6 cells were used per condition. Cells were centrifuged, then resuspended in 9ul of P3 electroporation buffer (Lonza) plus 1ul of 20μM siRNA specific for GIMAP5 or a scrambled negative control. Cells were

then electroporated with the EO-115 program using the Lonza 96 well shuttle system. 160μl of warm RPMI (plus 100U/ml of IL-2 for the T cell blasts) was immediately added and the cells were then transferred to 6ml of RPMI (plus 100U/ml IL-2 for T cell blasts). Significant depletion of GIMAP5 was not detected until at least 72 hours post electroporation.

CRISPR-RNP mediated depletion of GIMAP5

CRISPR-RNP mediated knockout of both primary human and murine T cells were done as previously described (Seki and Rutz, 2018). Briefly, in the human model, T cells were isolated from PBMCs of healthy donors and immediately used while in the murine model T cells were activated with ConA or plate-bound anti-CD3/CD28 plus IL-2 for 6-9 days prior to use. 10-30x10^6 cells were used per cuvette with the volumes scaled accordingly. 2-3 pre-designed crRNAs (IDT) per gene were conjugated to ATTO550 labeled tracrRNA at a 1:1 ratio to form gRNAs. The resulting gRNAs were added to purified Cas9 enzyme (QB3 Macrolab – Berkeley) at a 3:1 molar ratio and conjugated at room temperature for 10 minutes, with 180pmol of Cas9 enzyme used per 10 million cells. Cells were centrifuged and resuspended in 20μl of P2 (human) or P4 (murine) electroporation buffer (Lonza) per 10 million cells. 15ul of gRNA-Cas9 conjugates were then added to 20μl of cells and incubated for 2 minutes before electroporating using the amaxa 4D nucleofector and the EH100 program (human) or the CM137 program (murine). 1ml of warm RPMI was immediately added to the cells and then they were transferred to 6ml of RPMI (plus 100U/ml of IL-2 for the pre-activated murine cells). In the human cells significant depletion of GIMAP5 was observed after 48-72 hours, while

in the pre-activated murine T cells significant depletion could already be observed at 24 hours with the peak being around 48 hours post-electroporation.

RNA-Seq

Primary human T cells were purified from PBMCs of healthy human donors using a magnetic human pan T cell isolation kit (Miltenyi) and GIMAP5 was depleted as described in the siRNA knockdown section. After 72 hours, the unstimulated T cells were stimulated with CD2/3/28 beads (Miltenyi) for 0, 4, 12 or 24 hours before RNA was isolated via TRIzol (Invitrogen) with some aliquots of cells from the unstimulated timepoint being used to validate knockdown efficiency. RNA-Seq and data analysis was then performed by Merck via proprietary internal pipeline.

qPCR

RNA was isolated from T cells previously electroporated with either siRNAs specific for GIMAP5 or negative controls using standard TRIzol (Invitrogen) extraction procedures. cDNA was then generated using the Firststrand cDNA synthesis kit (Roche). All RT-PCR was performed using a 7500 Real Time PCR System (ABI). The expression of genes of interest were first normalized to GAPDH expression levels and then expressed using the $2^{-\Delta\Delta Ct}$ method.

Apoptosis Assays

PBMCs from either GIMAP5-deficient patients or healthy donors were stimulated with anti-CD2/3/28 beads as described above and expanded in 100U/ml of IL-2 for 15-20 days. Apoptosis was then induced in cycling T cells via a variety of methods. Firstly, cells were treated with a dose response of 1-100 ng/ml of anti-Fas crosslinking antibody

(clone CH11) or 0.1-10,000 ng/ml anti-CD3 (Hit3α) for 24 hours. Alternatively, cells were washed twice and transferred to RPMI supplemented with FBS but devoid of IL-2 and viability was tracked over 5 days. In both assays the fraction of viable cells was defined as AnnexinV$^-$LiveDead$^-$.

Autophagy Assays

Autophagic flux was measured in cycling T cells from either patients deficient for GIMAP5 or T cells from healthy donors rendered deficient for GIMAP5 via CRISPR-RNP electroporation. Cells were split and either treated with 50nm of Bafilomycin A1 (Sigma) or incubated in RPMI. Cells were then stained for surface proteins. Intracellular LC3-II levels were measured according to the Flowcellect kit instructions (EMD Millipore). The autophagic flux was defined via the Mean Fluorescence Intensity (MFI) of LC3-II staining via the following formula: $(MFI_{Baf} - MFI_{basal})/MFI_{basal}$ as previously described (Klionsky *et al.*, 2008).

Plasmids and retroviral transduction

Various GIMAP5 cDNAs were cloned into the MSGV1 retroviral plasmid vector (Genscript). These plasmids were transfected via Turbofect (Thermo Fisher Scientific) into Platinum-E cells (Cell Biolabs, Inc.). Supernatants were collected for viral transductions on day 2, sterile filtered and stored at 4°C.

Murine CD4$^+$ T cells from GIMAP5$^{sph/sph}$ mice were enriched from lymph nodes using a MojoSort CD4$^+$ T cell isolation kit (Biolegend). Cells were then stimulated at a concentration of $2x10^6$ cells/ml with 3μg/ml of Concanavalin A in RPMI for 24 hours. Cells were washed, resuspended in viral supernatant plus 5μg/ml of Polybrene plus

100U/ml of IL-2 and spun at 2000rpm for 60-120 minutes at 35°C. 24 hours later an equivalent amount of RPMI containing 100U/ml of IL-2 was added to the culture to dilute the viral supernatant twofold. 24 hours following this, the viral supernatant was washed out completely and the transduced cells were resuspended in RPMI containing 100U/ml of IL-2. Viability and the fraction of transduced cells were then monitored every three days for the following two weeks.

mTORC1 Activation assays

Following siRNA mediated depletion of GIMAP5 in either unstimulated or pre-activated human T cells (see siRNA knockdown section) cells were (re)stimulated through the TCR. T cells were (re)activated with 10μg/ml of HIT3α and 10μg/ml of $F(ab')_2$ fragment (Jackson Imunoresearch) for the indicated timepoints. Protein lysates were generated and analyzed for various downstream mTORC1 targets as described in the immunoblotting section.

Alternatively, T cells depleted of GIMAP5 via siRNA were incubated in amino acid free RPMI plus 10% dialyzed FBS for two hours. Samples were taken prior to amino acid starvation or at the end of two hours, while a final batch of cells were resuspended in amino acid replete RPMI containing 10% FBS for another hour. Protein lysates were then generated and analyzed for levels of various phosphorylated proteins downstream of mTORC1 as described in the immunoblotting section.

Statistical Analyses

Data were analyzed using Graphpad Prism 7.0. Depending on experimental design statistical significance was tested via either two-tailed unpaired or paired Student's t test.

P-values ≤ 0.05 were considered significant (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001), p > 0.05 non-significant (n.s.). Flow cytometry data was analyzed via Flowjo 9.9.5 (Treestar). No statistical methods were used to pre-determine sample size. Details on the test used can be found in the respective figure legends.

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