A Role for Glycosylphosphatidylinositol Anchored Proteins in T Lymphocyte Homeostasis

by

Alessandra Ferzoco

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Immunology
University of Toronto

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A ROLE FOR GLYCOSYLPHOSPHATIDYLINOSITOL ANCHORED PROTEINS IN T LYMPHOCYTE HOMEOSTASIS

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2017

ABSTRACT

The glycosylphosphatidylinositol (GPI) anchorage of proteins to the outer leaflet of the plasma membrane imparts unique properties and controversial functionality. While all GPI-anchored proteins (GPI-AP) contain a conserved GPI core structure, they represent a large class of functionally diverse proteins with known capacities for intracellular signalling, protein trafficking, and localization within membrane microdomains, termed lipid rafts (LR). Many studies have implicated GPI-AP as regulators of T cell receptor (TCR)-mediated cellular activation, however, as they are not transmembrane molecules, mechanisms underpinning their signalling capacity remain elusive.

We have isolated a mutant clonal variant devoid of GPI-AP. This T cell clone produced 10-100-fold more TCR-induced interleukin (IL)-2 than the GPI-AP+ parent clone. As GPI-AP reside exclusively within LR, we hypothesized that within this signalling scaffold, they may function as essential regulators of TCR/CD3-mediated “on” and “off” signals.
Model systems utilizing GPI-AP* and GPI-AP− variants of the antigen specific, IL-2-dependent T cell clone, and primary GPI-AP− T cells were generated towards directly testing this hypothesis. In the clonal system, GPI-AP deficiency was shown to impart a prolonged TCR/CD3-induced clonal expansion, which correlated with both enhanced IL-2 messenger RNA (mRNA) and protein production. The re-expression of GPI-AP abrogated the prolonged growth/survival phenotype, consistent with a GPI-AP-dependent attenuation of TCR/CD3 signalling. The phenotype was recapitulated in primary GPI-AP− CD4+ T cells. Hypersensitive TCR/CD3 signalling in the primary model correlated with an enhanced basal kinase activity of the Src-family protein tyrosine kinase Fyn, essential for TCR/CD3 induced IL-2 production.

The role of the GPI-anchor, as opposed to that of one or more GPI-anchored proteins in supporting this phenotype was assessed using GPI-anchor sufficient, GPI-AP deficient primary T cells. Preliminary results demonstrated that the TCR/CD3-induced IL-2 phenotype of GPI−/GPI-AP− closely resembles that of GPI-AP*, wild-type T cells. While a mixed genetic background in these GPI−/GPI-AP− T cells precludes formal proof of this conclusion, results thus far attribute a potentially novel role for GPI in regulating homeostatic T cell physiology. The biological significance of the results may provide insight into the mechanism underpinning the human disease paroxysmal nocturnal hemoglobinuria, the maintenance of cellular homeostasis and proliferative disease states 

writ large.
ACKNOWLEDGEMENTS

The completion of this project would not have been possible without the assistance and encouragement of many individuals in numerous ways.

First and foremost, I would like to acknowledge my supervisor, Dr. Michael Julius, who has been a source of guidance, knowledge, and encouragement since my days as a summer student within his laboratory. My experiences with him opened my eyes to the world of academic science for the first time and gave me the inspiration to pursue a PhD. His mentorship has moulded my mind, taught me how to think critically and approach the daily challenges of basic research, all while celebrating in the joys of scientific discovery. Through him, I have come to appreciate what it truly means to be a scientist. It has been an honour and privilege to work alongside him for all of these years.

I would also like to acknowledge my committee members, Dr. Katherine Siminovitch and Dr. Juan Carlos Zúñiga-Pflücker for their continuous support. I am incredibly grateful for their expertise, understanding, thoughtful discussions, and thorough critiques. Their insight and cooperation have helped shape this project into what it is today.

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also grateful to her for sharing in my frustrations and providing encouragement at each hurdle this project has faced.

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ABBREVIATIONS:

AA  aplastic anemia
Ab  antibody
AICD  activation induced cell death
ALL  acute lymphatic leukemia
AML  acute myelogenous leukemia
AP-1  activator protein-1
APC  antigen presenting cell
Av  streptavidin
BLIMP1  B lymphocyte-induced maturation protein 1
Ca^{2+}  calcium
Cas9  CRISPR associated protein 9
Cbp  Csk-binding protein
CD  circular dichroism
CD28RE  CD28 response element
CFSE  carboxyfluorescein succinimidyl ester
CLL  chronic lymphatic leukemia
ConA  Concanavalin A
Csk  C-terminal Src kinase
cSMAC  central supramolecular activation cluster
CRISPR  clustered regularly interspaced short palindromic repeats
CTLA-4  cytotoxic T lymphocyte antigen-4
DAF  decay accelerating factor
ddPCR  digital droplet polymerase chain reaction
DL1  Delta-like-1
DN  double negative
DNA  deoxyribonucleic acid
DP  double positive
ELISA  enzyme-linked immunosorbent assay
EMMA  European mouse mutant archive
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERES</td>
<td>ER exit site</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>EtN-P</td>
<td>ethanolamine phosphate</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence acquired cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment, crystallizable</td>
</tr>
<tr>
<td>FOXP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>FTOC</td>
<td>fetal thymic organ culture</td>
</tr>
<tr>
<td>Fyb</td>
<td>Fyn-T-binding protein</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA-binding protein 3</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GlcN</td>
<td>glucosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<tr>
<td>GlcNAc-PI</td>
<td>N-acetylglcosaminyl-PI</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<tr>
<td>GPI-AP</td>
<td>glycosylphosphatidylinositol-anchored proteins</td>
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<td>GPI-GnT</td>
<td>glycosylphosphatidylinositol-N-acetylglcosaminyl-transferase</td>
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<tr>
<td>gRNA</td>
<td>guide ribonucleic acid</td>
</tr>
<tr>
<td>^3H-TdR</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>HSPCs</td>
<td>hematopoietic stem/progenitor cells</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>Iso Ctl</td>
<td>isotype control</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
<td>ITSM</td>
<td>immunoreceptor tyrosine-based switch motif</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus family tyrosine kinase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KIR</td>
<td>killer immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LR</td>
<td>lipid raft</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>mESC</td>
<td>mouse embryonic stem cell</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mIL-2</td>
<td>mouse interleukin-2</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NKG2D</td>
<td>natural killer group 2, member D</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T</td>
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<td>Nnt</td>
<td>nicotinamide nucleotide transhydrogenase</td>
</tr>
<tr>
<td>OCT-1</td>
<td>octamer transcription factor-1</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAG</td>
<td>phosphoprotein associated with glycosphingolipid-enriched membrane domains</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed death-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>programmed death ligand-1</td>
</tr>
<tr>
<td>PD-L2</td>
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<tr>
<td>PEP</td>
<td>PEST-enriched protein tyrosine phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEST</td>
<td>Peptide sequence enriched in proline (P), glutamic acid (E), serine (S), and threonine (T)</td>
</tr>
<tr>
<td>PGAP</td>
<td>Post-GPI-attachment to proteins</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
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<td>PIG</td>
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<td>Protein kinase A</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<td>Phospholipase C gamma-1</td>
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<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<td>PNH</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
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<td>PTK</td>
<td>Protein tyrosine kinase</td>
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<td>PTPase</td>
<td>Protein tyrosine phosphatase</td>
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<td>PTP-PEST</td>
<td>PTPase with PEST-sequences</td>
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<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
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<td>RORγt</td>
<td>Retinoic-acid receptor-related orphan receptor γt</td>
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<td>rIL-2</td>
<td>Recombinant interleukin-2</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SLP-76</td>
<td>SLAP-associated protein 76</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>T cell receptor</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
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\text{TSA-1} \quad \text{thymic shared antigen 1}

\text{WT} \quad \text{wild-type}
CHAPTER 1: INTRODUCTION

Overview of Glycosylphosphatidylinositol Anchored Proteins:

Glycosylphosphatidylinositol (GPI) is a glycolipid structure that is covalently attached post-translationally to the C-terminus of many proteins, within many different species. Despite the broad nature of this modification and the diversity of GPI-anchored proteins (GPI-AP), all characterized GPI anchors have been found to contain a highly conserved core sequence consisting of an identical ethanolamine-phosphate-6Manα1-2Manα1-6Manα1-4GlcNα1-6myoinositol-phospholipid backbone, where Man is mannose and GlcN is glucosamine [1]. A diagram of the conserved GPI core is illustrated in Figure 1-1. This glycolipid modification occurs on the luminal side of the plasma membrane and functions in anchoring the attached protein to the outer leaflet of the cell membrane. Specifically, GPI-AP are localized within detergent-insoluble membrane microdomains termed, lipid rafts (LR), enriched in glycosphingolipids, cholesterol, and other lipidated proteins [2, 3]. GPI-AP constitute approximately 0.5% of all cellular proteins in eukaryotes [4]. Approximately 250 different types of GPI-AP have been identified to date, expressed on nearly all types of cells and tissues examined [5]. While the conserved core backbone is a common to all GPI-AP, they represent a large class of functionally diverse proteins.
The conserved core backbone structure of a GPI-anchor is comprised of ethanolamine-phosphate-Manal-2Manal-6Manal-4GlcNal-6myo-inositol. Spanning the exoplasmic leaflet of the plasma membrane lies one phospholipid, whose complex head-group is associated with a phosphodiester-linked inositol, which is coupled onto a glucosamine linked to a linear chain of three sequential mannose sugars, the last of which is bound to a phosphoethanolamine. Finally, an amide bond fosters the attachment of the C-terminus of a protein moiety with the phosphoethanolamine amino group. The lipidic portion of the anchor can be composed of either diacyl or alkyl-acyl chains, or a ceramide, depending on the protein or species. Modifications of the glycan core are common and include the addition of phosphoethanolamine residues, mannose, galactose, sialic acid, or other sugars. The addition of palmitic acid onto the inositol ring confers resistance to cleavage by the phosphatidylinositol specific phosphatase, PI-PLC. The phosphatases PI-PLC and PI-PLD can cleave the GPI anchor at the indicated cleavage sites.

Figure 1-1: Structure of a representative GPI-anchor.
The Biosynthesis of GPI-AP:

Twenty-two known genes are involved in the biosynthesis of GPI-AP (Figure 1-2) [1]. The biosynthetic pathway for mature GPI-AP is initiated in the endoplasmic reticulum (ER) with free phosphatidylinositol (PI), from which GPI is synthesized [1]. On the cytosolic side of the ER, free PI is coupled onto N-acetylglucosamine (GlcNAc) by GPI-N-acetylglucosaminyl-transferase (GPI-GnT), a transferase complex consisting of six PIG (phosphatidylinositol glycan) genes products; PIG-A, -C, -H, -P, and -Q [6-9]. The PIG-L gene is also involved at this step as it functions to deacetylate GlcNAc, producing GlcN-PI [10]. With aid from a potential flippase, it is thought that GlcN-PI has the capacity to flip from the cytosolic to luminal side of the ER, where further modifications can take place [11, 12]. Modifications to the composition of the GPI lipid chains have been implicated at this point. Specifically, while most PI lipid chains are diacylated, modifications at this stage replace an acyl chain for an alkyl chain, allowing the majority of PI moieties within surface GPI-AP to consist of alkyl-acyl lipid chains [13].

On the luminal side of the ER, PIG-W then transfers an acyl chain from acyl-CoA onto the 2-position of the GlcN-PI inositol group to form GlcN-(acyl)PI [12]. PIG-M/PIG-X, PIG-V, and PIG-B then perform the sequential addition of three mannose groups, followed by the coupling of one ethanolamine phosphate (EtN-P) onto the now mature GPI anchor, and two EtN-Ps as side-chains linked onto mannose units by the PIG-N, PIG-O/PIG-F, and PIG-G/PIG-F genes [14-21]. All additional modifications to the GPI anchor, which can vary among species, are performed during these GPI biosynthesis
Figure 1-2: The mammalian biosynthetic pathway of GPI-AP.
Of the genes involved in this pathway, those involved in the synthesis of GPI are named the PIG (phosphatidylinositol glycan) family of genes, while genes termed PGAP (post-GPI-attachment to proteins) are involved in the structural remodelling of GPI-AP, acting later in the pathway. Modified from [1].
stages, prior to the protein attachment. Lastly, a GPI-transamidase complex, consisting of PIG-K, the catalytic subunit, PIG-S, PIG-T, PIG-U, and GAA1, recognizes and removes the GPI-attachment signal peptide from a protein, allowing the transfer of the mature anchor onto the protein and the formation of a putative GPI-AP [22-26]. The catalytic site of PIG-K assists in the linkage by creating transient intermediates with the precursor protein GPI-attachment signal peptide [24, 27]. The EtN-P associated with the third mannose group also assists in the linkage through the formation of an amide bond bridging the GPI anchor with the processed carboxy terminus of the precursor protein [28].

Once the GPI is transferred onto the precursor protein, additional modifications including structural GPI remodelling take place within the ER or the Golgi. The first remodelling step occurring prior to exiting the ER is a Post-GPI-Attachment to Proteins (PGAP)1-catalyzed inositol-deacylation event [29]. The second remodelling step, which also takes place within the ER, involves PGAP-5-catalyzed removal of EtN-P from the second mannose group [30]. It has been suggested that this modification is critical to effectively sort GPI-APs to the ER exit site (ERES). To assist in efficient sorting into ERES, p24 family members have been implicated as potential cargo receptors, binding to GPI-AP and mediating their transport out of the ER [31, 32]. Budding of the GPI-AP from the ER initiates their recruitment into COPII vesicles that utilize Sec24C/D isoforms in their transportation to the Golgi [33]. Once localized to the Golgi, further structural remodelling events can ensue, particularly fatty acid remodelling for GPI-AP transport to the cellular surface.
While the lipid chains of GPI and GPI-AP localized within the ER are predominantly unsaturated, most GPI-AP expressed on the cell surface have replaced an unsaturated fatty acid chain with a saturated chain. This fatty acid remodelling reaction occurs in the Golgi lumen and requires PGAP3 for the removal of the unsaturated lipid chain and the addition of a saturated chain, typically stearoyl, by PGAP2 [34, 35]. Interestingly, this fatty acid remodelling coincides with the accumulation of GPI-AP into LR, likely due to their two straight saturated fatty acid chains [35]. Fatty acid remodelling and the concentration of GPI-AP into LR is posited to affect the biological function of GPI-AP, including trafficking, signal transduction, and spatial distribution [2].

Should a step in the biosynthetic pathway be defective and incomplete generation of mature GPIs arise, the formation and expression of GPI-AP on the cell surface fails. In this case, the unbound precursor proteins may be retained by chaperone proteins and targeted for ER-associated degradation [36]. Alternatively, if the precursor protein has already coupled with the GPI-transamidase to form a transient intermediate, hydrolysis and secretion of a soluble form of the protein can occur. Thus, a defective step in the biosynthetic pathway impacts the generation of all GPI-AP, and consequently results in a complete deficiency in GPI-AP surface expression.
Tethering to Lipid Rafts:

GPI-linkage imparts a stable association of proteins within the lipid bilayer. As mentioned above, these molecules are concentrated into specialized membrane microdomains, termed LR. The small and dynamic raft structures, as well as GPI-AP, are characterized by their insolubility in the nonionic detergent Triton X-100 at 4°C [2]. This enriched partitioning of glycolipids, sphingolipids, cholesterol, and other lipidated proteins is thought to serve as a highly ordered platform critical for numerous cellular functions [37-39]. As a result of protein lipid modifications, the composition of these platforms is dynamic [40]. The GPI anchor itself has been suggested to potentially serve as an apical targeting domain, initiating the association of a protein within LR microdomains [2, 41]. Some of the molecules known to concentrate into LR include signalling proteins, such as protein tyrosine kinases (PTKs) p59<sup>fyn</sup> (Fyn), and transiently p56<sup>ck</sup> (Lck). These proteins along with other cytosolic PTKs, including Hck and Fgr, have been shown to co-immunoprecipitate with GPI-APs and thus supported the hypothesis that GPI-AP-mediated signalling may involve tyrosine phosphorylation [42, 43].

The conserved GPI moiety has been shown to function in the transduction of signals across the membrane of mammalian cells. Intracellular signalling via GPI-AP may occur through protein-protein interactions linking the protein ectodomain of GPI-AP to transmembrane proteins with cytosolic signalling components [44]. Signalling may be mediated through these interactions with signal transduction partners such as integrins, PTKs, or heterotrimeric GTP-binding proteins [45, 46]. Alternative mechanisms include
a lectin-like interaction of these transmembrane proteins with the GPI-moiety itself, or an indirect lipidic interaction of the GPI-AP with signalling molecules within LR [47].

Regardless of the mechanistic means, the localization of GPI-AP within LR may be of central importance to GPI-AP functionality, as LR segregation via cholesterol and sphingolipids is often mandatory for GPI-AP physiology. In this regard, cholesterol and sphingolipid depletion can impair the function, sorting, and signalling capacity of some GPI-AP [48].

_Diverse Roles of GPI-AP:_

While hundreds of different GPI-AP, expressed in eukaryotes, from protozoa to mammals, share a common core backbone sequence, they are functionally diverse, playing variety of distinct and critical roles in various cell functions. Within mammals, functions of GPI-AP include but are not limited to contributions to transmembrane signalling, intracellular targeting, cellular adhesion, and most noteworthy, embryonic development; as GPI-AP deficiency results in embryonic lethality in mice [49-51].

In regards to the role(s) of GPI-AP in T lymphocyte physiology, some GPI-AP have been implicated in T cell development/selection due to their capacity to modulate antigen receptor-mediated signalling. Briefly, T cell development involves three major consecutive stages named based on the expression of the CD4 and CD8 co-receptors: the CD4⁺CD8⁻ double negative (DN) stage of early thymocytes, which progresses to the CD4⁺CD8⁻ double positive (DP) stage and finally maturation into the CD4⁺CD8⁻ or CD4⁻
CD8+ single positive stage [52]. DP thymocytes bearing the expression of a successfully rearranged T cell receptor (TCR) that is capable of recognizing self major histocompatibility complex (MHC)-peptide complexes with intermediate affinity are positively selected within the thymus [53]. A second critical developmental checkpoint is the negative selection and apoptotic removal of thymocytes expressing a high affinity TCR for self antigens-bound MHC molecules on antigen presenting cells (APCs) [53]. The GPI-AP Thy-1 (CD90) has been identified as a potential contributor to negative selection as thymocyte apoptosis was induced following monoclonal antibody (mAb)-mediated coaggregation of Thy-1 with TCR [54-56]. Furthermore, anti-Thy-1 was also found to inhibit the antigen-induced depletion of DP thymocytes in vitro [57]. The GPI-AP thymic-shared antigen 1 (TSA-1) has also been suggested to play a role in thymocyte development as anti-TSA-1 was found to block the transition from DN to DP in fetal thymic organ culture (FTOC) [58].

Many studies implicate GPI-AP as regulators of TCR mediated cellular activation. Antibodies to GPI-AP have been shown to either directly induce T cell proliferation and IL-2 production or completely inhibit anti-TCR/CD3 signalling [59-61]. Examples of mAbs capable of upregulating both the phosphotyrosyl content of intracellular substrates and intracellular calcium concentrations include anti-Thy-1, -Qa-2, -CD48, -CD55, -CD59, and CD73 [62-65]. Antibody mediated aggregation of these molecules as well as anti-Ly-6A/E, -Ly-6C, and -CD52 is also sufficient for the initiation of T cell proliferation and cytokine secretion, in the presence of accessory cells or co-stimulatory reagents [66-68]. Interestingly, in order to generate these GPI-AP-mediated responses,
the CD3ζ chain must be expressed on the T cell surface [69, 70]. While these responses may suggest a role for GPI-AP in the potentiation of early signals through the TCR complex, it must be noted that GPI-AP deficiency imparted by a tissue specific disruption of GPI-AP biosynthesis does not obviate the ability of a T cell to respond to antigen [71]. Additionally, while aggregation of some GPI-AP correlates with T cell activation, antibody mediated ligation of others has been implicated in the attenuation of T cell responses. Specifically, antibodies to Thy-1, Ly-6A/E, Ly-6C, CD48, and TSA-1 have been shown to inhibit: CD3ζ tyrosine phosphorylation; increases in intracellular calcium concentrations; T cell proliferation; and cytokine secretion [72-76]. Thus GPI-AP can modulate TCR signalling both positively and negatively, and their capacity to do so may be predicated by their localization to LR.

The Role of the GPI Anchor:

While numerous studies have identified roles for many GPI-AP, the significance of the GPI-linkage itself remains controversial. The highly conserved nature of the GPI core structure along with the complicated nature of GPI protein anchorage compared to simple lipid or more stable transmembrane protein linkages suggests that the GPI anchor may provide a functional role besides protein linkage. A potential caveat to this hypothesis is that some GPI-AP have been shown to function similarly when the GPI anchor is replaced with a transmembrane version. In this regard, the GPI-AP DAF can continue protecting against complement-induced cytotoxicity, and the GPI-AP CD73 can still mediate T lymphocyte activation [77, 78]. Additionally, some GPI-AP including CD58 are expressed on the cell membrane in either a transmembrane or a GPI-anchored
form with no notable functional differences [79]. Conversely, for some GPI-AP such as Ly-6A/E, functionality is abolished with the loss of the GPI anchor [80]. To investigate the impact of the GPI anchor, Su et al. constructed and expressed a transmembrane form of the Ly6E antigen and demonstrated that in the transmembrane form, Ly6E lost the capacity to initiate T cell activation through antibody crosslinking [80]. This finding suggests that the expression of an intact GPI anchor is essential for signalling via Ly6A/E on T cells.

In addition to bestowing subcellular localization into LR and signalling capacities as described above, GPI-linkage confers changes in lateral mobility, sorting to the apical surface of polarized cells, and the susceptibility to the phosphatidylinositol specific phosphatase PI-PLC and PI-PLD [81]. An additional attribute of GPI anchorage that has been noted among GPI-AP is a slower protein turnover rate, allowing a prolonged residence on the plasma membrane compared to transmembrane proteins [82-84]. Further, GPI-linkage affects the conformation of its associated protein. For example, if the GPI linkage on the GPI-AP Thy-1 is cleaved by PI-PLC, binding of the Thy-1-specific antibody OX7 is prevented [85]. Furthermore, using circular dichroism (CD) as a tool to estimate protein secondary structure, the CD spectra of Thy-1 was found to differ between GPI-anchored and soluble forms [85]. Additional findings suggest an influence of GPI-linkage on protein conformation by causing the protein to interact with the cell membrane. Computer modeling has predicted that the protein moiety of Thy-1 rests directly on the cell membrane, with the anchor occupying a carbohydrate-binding site of the protein domain [86]. Proteins such as Thy-1 and DAF not only sit on the cell
membrane, but also have the capacity to diffuse freely and move rapidly in response to stimulation [87]. These attributes related to GPI-linkage may underpin mechanisms facilitating cellular communication and signalling.

The mitogenicity of mAbs to different GPI-AP whose protein moieties are otherwise unrelated, suggests that the GPI anchor is critical to the engagement of shared signalling pathways. Their capacity to modulate TCR/CD3 signalling in specific fashions may relate to the specificity of their protein moieties that interact, in most cases, with yet unknown ligands to control the balance between cellular proliferation, survival and death [88].

Paroxysmal Nocturnal Hemoglobinuria:

The role of GPI-AP in regulating cellular homeostasis has human disease analogues, specifically paroxysmal nocturnal hemoglobinuria (PNH), and possibly related neoplasias [89]. PNH is defined as a non-inherited mono- or oligo-clonal disorder almost always arising from one or more somatic mutations in the 16-kb-long human PIGA gene on Xp22.1 in an early hematopoietic cell [90]. Typically, complete GPI-AP deficiency constitutes embryonic lethality at an early stage of development [49-51], however as only clonal hematopoietic cells are affected; mutations arising in PNH are compatible with life. The prevalence of the disease (10^6 to 10^5) is approximately equal in both males and females, as the PIGA somatic mutation occurs post-X-chromosome inactivation [91]. As described above, PIGA encodes a critical catalytic component of the GPI-GnT, which initiates GPI-AP biosynthesis by transferring N-acetylglucosamine from
UDP-N-acetylglucosamine to phosphatidylinositol [92]. All affected progeny cells from the mutated self-renewing, hematopoietic stem cell are incapable of GPI-AP biosynthesis and have the potential to preferentially expand in the bone marrow [90]. As all GPI cells are derived from the single somatic stem cell mutation, PNH patients present as somatic cell mosaics; the co-existence of two discrete GPI-AP deficient and GPI-AP sufficient populations of somatic cells [91].

The basis for the preferential expansion of hematopoietic stem cell (HSC) clones bearing the somatic PIGA mutation is not well characterized. While the PIGA mutation is required for PNH onset, PIGA mutations have also been detected in healthy people (~1 in 50,000 granulocytes), indicative of additional contributors to the disease [93, 94]. The clinical disease outcomes only become relevant upon expansion of the PNH clone. Additionally, a potential mechanistic hypothesis must take into consideration the pathophysiologic correlation between PNH and acquired aplastic anemia (AA), a T cell-mediated autoimmune bone marrow failure disorder characterized by the depletion of hematopoietic stem cells [95].

Two theories have been forwarded: the expansion of GPI cells due to an intrinsic growth/survival advantage imparted by the lack of GPI-AP expression, or an autoimmune selection mechanism whereby GPI-AP deficiency confers protection against a natural killer cell (NK) or CD8+ cytotoxic T cell-mediated attack targeting the bone marrow [96-100]. The majority of evidence points to the latter, suggesting that GPI itself may be the target of autoreactive cells in PNH. With roughly half of the patients with immune-
mediated AA developing PNH, it supported the hypothesis that the GPI cells in these patients were selectively spared during the autoimmune destruction of bone marrow hematopoietic stem/progenitor cells (HSPCs) [101]. Investigation into this potential mechanism has identified a greater proportion of circulating T cells expressing the activating forms of killer immunoglobulin-like receptor (KIR) [100, 102], and an increase in expression of CD25, CD54, and human leukocyte antigen (HLA)-DR on PNH T cells [103]. Additionally, GPI-AP deficient K562 cells were found to be less susceptible to NK cell killing than their GPI-AP sufficient counterparts, potentially due to the absence of the stress-inducible GPI-AP, ULBP1 and ULBP2 [104]. The ULBP1/2 proteins are ligands for the natural killer group 2, member D (NKG2D) receptor, which is expressed on both NK cells and CD8+ cytotoxic T cells. In the case for GPI-AP deficient cells, if ligand-receptor engagement is ablated, NKG2D would fail to promote cell death and the PNH cells may profit from a ULBP1/2-deficient survival advantage and be spared from immune attack.

CD1d-restricted, GPI-specific T cells have also been proposed as a mediator of immune killing in PNH patients [105]. This may be a contributing factor to PNH bone marrow failure and pathogenesis as GPI has been identified in the presentation groove of CD1d, a molecule expressed on HSPCs. Recently, a study provided direct evidence that both endogenous and exogenous GPI have the capacity to activate CD1d-restricted, GPI-specific autoreactive T cells, with these cells and their effectors being significantly enhanced in PNH patients [105]. These results are consistent with the targeting of GPI-
AP positive HSPCs contributing to bone marrow failure in PNH, while providing the linkage to AA diagnosis.

While controversial, an intrinsic proliferative and/or survival advantage in GPI cells has also been described in these patients. Various reports have documented an enhanced proliferative capacity of isolated GPI cells stimulated in vitro, more efficient engraftment into sub-lethally irradiated severe combined immunodeficiency (SCID) mice, and a greater resistance to serum starvation- or anti-CD95-induced apoptosis [106-108]. This potential anti-apoptotic effect of a PIGA mutation may predicate both the clonal dominance of the GPI cells, and the PNH predisposition to neoplastic transformation. Indeed, with 5-15% of PNH patients developing hematologic malignancies, the GPI-AP deficiency does impart a heightened risk of leukemic development relative to the general population [109]. While the majority of such blood dyscrasias are of myeloid origin, most notably, acute myelogenous leukemia (AML), incidences of non-myeloid dysplasias including acute lymphatic leukemia (ALL) and chronic lymphatic leukemia (CLL) have been reported as well [109, 110]. Again, this predisposition is reflective of a GPI-AP deficient clonal dominance and survival advantage in PNH patients.

The most prominent symptom in PNH patients is anemia resulting from the chronic intravascular destruction of red blood cells (RBC). This hemolysis occurs due to the absence of the cell surface GPI-linked complement regulators; CD59, which prevents terminal compliment C9-mediated lytic pore formation by the membrane attack complex (MAC), and DAF (CD55), which blocks the formation of the C3 convertase [111]. The
absence of these two molecules thereby promotes RBC hypersensitivity to complement [112, 113].

While a mutation of any gene in the GPI-AP biosynthetic pathway could potentially result in PNH, the frequency of a causal PIGA mutation is significantly higher, as it is the only X-linked enzyme [114]. However, there is a report of a PNH patient bearing inactivating mutations in both alleles of PIGT on chromosome 20 [115]. Specifically, a heterozygous germline splice site mutation in PIGT followed by a PIGT somatic deletion in granulocytes permitted the block in GPI-AP biosynthesis. As classical PIGA-mediated PNH is initiated through a deficiency in GPI anchor biosynthesis, this PIGT-targeted PNH patient provides evidence that PNH can occur in the presence of the glycolipid moiety.

Piga Deficient Mice:

Much effort has been devoted to create a mouse model to elucidate the genetic basis of PNH. The generation of a mouse model also sought to characterize the functional roles of GPI-AP within development and on specific tissues. Initial attempts were focused on the creation of a chimeric mouse using embryonic stem cells (ESC) with a homologous recombination-induced disruption of the Piga (murine homologue of PIGA) gene [49]. High chimerism (>50%) with respect to the surface expression of GPI-AP equated with significant lethality in the Piga-deficient mice, with only low chimeric mice surviving post birth. This initial study proffered two fundamental revelations; firstly, that the expression of GPI, GPI-AP, or both is crucial for mouse development, and secondly,
that the clonal dominance seen in PNH patients requires more than the *PIGA* mutation alone. This second conclusion derives from the finding that in all but one mouse, GPI deficient erythrocytes remained constant in number and failed to clonally dominate. Interestingly, one chimeric mouse did show a clonal expansion of erythrocytes, T cells and B cells, yet the event triggering the expansion remains unknown. A second study using *Piga*-deficient ESC recapitulated the finding that the gene disruption alone cannot confer a proliferative advantage on the PNH clone [50]. Furthermore, this finding showed *in vitro* and *in vivo* that hematopoiesis with erythroid and myeloid differentiation ensues even in the absence of GPI-AP.

Studies to follow the initial stem cell disruption of *Piga* graduated to the use of Cre-\textit{loxP} recombinase systems for the specific deletion of GPI-AP in targeted tissues, thus creating conditional knock-out mice. A crossbreed of mice with \textit{loxp} sites flanking exon 2 of the *Piga* gene with mice expressing the EIIa-promoter specific \textit{cre} recombinase transgene, was established to target GPI-AP expression in early embryogenesis [116]. This objective is possible since the EIIa promoter functions in the temporal restriction of Cre expression to early embryonic life, with no subsequent *Piga* gene recombination at later developmental stages. GPI-AP were shown to contribute to the development of heart, lung, kidney, brain and liver as mosaic analyses found the predominant expression of the wild-type *Piga* gene in these tissues. Recapitulating the previous findings of unaltered erythroid and myeloid differentiation, this study showed a balanced number of PIGA sufficient and PIGA deficient cells within the thymus, spleen and RBC population.
As these mosaic mice resemble a PNH patient with the coexistence of circulating blood cells both bearing the expression and lacking the expression of GPI-AP, Bessler et al. utilized this animal model for a second study on the hematopoietic impact of Piga inactivation [117]. Similar to the PNH phenotype, Piga-deficient RBCs derived from these mice displayed an enhanced sensitivity to complement-mediated lysis and reduced half-life. However, in contrast to PNH, where GPI-AP deficient lymphocytes exist but are a minority population relative to the greater number of affected granulocytes and RBC, lymphocytes were the majority population of Piga-deficient cells in the mosaic mice. In this regard, T lymphocytes were the largest population affected, with Piga-deficient CD8⁺ T cells being the only cell type to expand in number throughout the 1-year investigation. Moreover, like the predecessor studies, the lack of clonal expansion of all Piga-deficient cells supports the hypothesis of additional factors responsible for the initiation of clonal expansion and pathogenic onset in PNH.

Further investigation of Piga-deficient lymphocytes merited the creation of a conditional mouse model with GPI-AP deficient T cells. By crossing mice expressing the cre recombinase transgene under the regulation of the T-lymphocyte-specific p56lk proximal promoter with mice bearing loxP sites within the Piga gene, Takahama et al. successfully disrupted the Piga gene exclusively on mouse T cells [71]. This study documented the ability of peripheral T cells lacking the expression of GPI-AP to be functionally competent and capable of undergoing TCR-induced activation both in vitro and in vivo. Expanding on this study, Takeda’s group generated a second conditional mouse model of GPI-AP deficiency exclusively in cells of hematopoietic origin. They did
so by targeting *Piga* with Cre recombinase under the *hCMV* promoter to affect most cell types at the preimplantation stage, followed by fetal liver cell transplantation from affected female mice into irradiated recipients [118]. While the authors also documented the GPI-AP negative T cell component to exceed that of the GPI-AP sufficient population within the blood, this study characterized an enhanced proportion of both CD4+ and CD8+ GPI-AP deficient T cells in the spleen and lymph nodes. Thymic analysis indicated a high percentage of GPI-AP negative cells even at the double negative, early T cell developmental stage. These findings demonstrate a role for GPI-AP in early T cell development at which point deficiency confers dominance over cells expressing GPI-AP.

*The Role of Lipid Rafts in TCR Mediated Cell Activation:*

Perhaps the most fundamental role of GPI-linkage is to achieve the stable association of proteins within the specialized, dynamic LR structures. By means of protein lipid modifications, these dynamic platforms permit the lateral segregation of proteins within the plasma membrane, with the capacity to either recruit molecules in, enable egress, or prevent their entrance [45]. It is the GPI-AP localization within the LR, the subsequent association with signalling elements, and the functional commonalities seen independent of protein structure that suggests that the anchorage within LR imparts signalling competency upon the protein moiety [81]. Particularly upon the surface of a T cell, these LR are crucial for both the activation and attenuation of T cell responses. The importance of LR in the initiation of T cell activation is validated by the findings that cholesterol depletion and palmitoylation-deficiency inhibited T cell activation, while
cholera-toxin-mediated copatching experiments partially induced T cell activation [40]. However, the role GPI-AP play in this regard remains uncharacterized.

The dynamic nature of LR is thought to be a central component to the current model of T cell activation, whereby multiple nanoassemblies coalesce to form larger, stabilized platforms for membrane signalling and protein trafficking (Figure 1-3). The LR structures within resting cells are ~50 nm in diameter and are composed of only a small number of proteins [45, 119, 120]. Ligand binding and TCR crosslinking triggers the clustering and stabilization into structures ranging in size from hundreds of nm to µm in diameter [45, 121, 122]. Upon binding of the TCR with its MHC-bound cognate peptide on APCs, a cascade of signalling events triggers the formation of an immunological synapse between the contacting cells [123]. The central part of the immunological synapse is referred to as the central supramolecular activation cluster (cSMAC) and is surrounded by highly concentrated adhesion molecules and signalling components [40]. The LR hypothesis suggests that it is the condensation and stabilization of LR that forms the foundational basis of the growing synapse and recruits the signalling elements required for T cell activation.

Localization of the TCR and the Src family kinases within this lipid structure is of central importance [124-126]. Following TCR engagement and activation upon TCR microclusters, rapid actin-mediated translocation into the cSMAC ensues [127, 128]. This process assists in enhancing the kinetics and affinity of the TCR-pMHC interaction, as well as in aiding the specific recognition of 10-100 cognate pMHC among the $10^4-10^6$
Figure 1-3: The clustering of lipid rafts for the formation of the immunological synapse.
A simplified depiction of the progression from resting cells with numerous small raft fractions to their aggregation and the formation of the immunological synapse following TCR engagement. Ligand binding induces the translocation of the TCR into the raft fractions and the initiation of a signalling cascade, represented by *. Modified from [129].
self-peptide-MHC interactions occurring simultaneously [40, 130]. TCR aggregation results in the dephosphorylation of Y505 on the Src family tyrosine kinase Lck by the protein tyrosine phosphatase (PTPase) CD45 outside of LR, which opens its structural conformation, enhances enzymatic activity, and permits auto/trans-phosphorylation of Y394 within the activation loop of its catalytic domain [131]. Further regulation of Lck is thought to depend on its association with the cytoplasmic tail of the CD4 co-receptor outside of LR [132, 133]. In this regard, CD4 may serve as a “gate keeper” sequestering 75-95% of associated Lck outside of LR in non-activated CD4+ T cells. As illustrated in Figure 1-4, within 10 seconds post TCR/CD4 coaggregation outside of LR, some Lck, within TCR-CD4 complexes translocates into LR at the T cell-APC interface where the juxtaposition of Lck-CD4 with TCR/CD3 functions in the ongoing phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) on the CD3 chains [126]. Additionally, the translocation of Lck into LR induces the activation of LR-resident Src family tyrosine kinase Fyn, whereby the activating pY417 levels of Fyn increase five-fold and pY394 levels of Lck increase seven-fold within LR after 10 seconds of stimulation [126]. Importantly, in resting CD4+ T cells 75-95% of Lck resides outside of LR while 95% of Fyn is LR resident, thus the LR serves as a partition separating the signalling molecules and regulating cellular activation [126]. These activation events ensue seconds post-TCR engagement, ahead of establishment of a stabilized immunological synapse, underscoring the importance of LR in initiating the full sequelae of TCR-induced signalling events.
Figure 1-4: The involvement of LR in T cell activation.
This schematic illustration of T cell receptor depicts the role of LR in partitioning signalling elements either outside or in LR. In a resting T cell, the majority of Lck is excluded from LR, associated to the CD4 co-receptor, and maintained in its inactive, closed conformation. Similarly, Fyn, which resides exclusively in LR, is also maintained in its inactive, closed conformation due to suppressive phosphorylation by LR-resident Csk. Upon engagement of the TCR with peptide/MHCII, TCR/CD3 aggregates are formed and a signalling cascade dependent on the re-organization of LR is initiated. T cell activation allows for the dephosphorylation of Csk and results its dissociation from PAG, its displacement from LR, and the loss of LR Lck/Fyn suppression. The aggregation of TCR with CD4 also results in the CD45-mediated dephosphorylation of Y505 on Lck, which functions to open its structural conformation. CD4-associated kinase active Lck, can then phosphorylate the ITAM motifs of the CD3 chains and on the TCRζ homodimer, which leads to the recruitment of additional ZAP-70 molecules. A portion of the TCR complex is then translocated into LR, juxtaposing Lck with Fyn, and allowing its physical interaction and activation to occur. This activation cascade ultimately culminates in the de novo production of IL-2.
LR-resident Lck also supports the recruitment and activation of the Tyr kinase TCRζ chain-associated protein ZAP-70 [134]. ZAP-70 activation commences the phosphorylation of the transmembrane LR-resident linker for activation of T cells (LAT), initiating further downstream signalling by recruiting additional signalling molecules including phospholipase C gamma-1 (PLCγ1) and the adaptor molecules Grb2 and Gads [135, 136]. The localization of LAT within LR has been shown to be crucial for its functionality as mutants incapable of LR association fail to support downstream signalling post-TCR engagement [136, 137].

The downstream events include Ca²⁺ influx, actin polymerization, Ras activation, and transcriptional events culminating in cellular proliferation, survival, and production of the T cell growth factor interleukin-2 (IL-2) [138-140]. The production of IL-2 is largely dependent on the Lck-mediated activation of Fyn within LR; TCR aggregation in the absence of Fyn does not induce IL-2 [141]. Fyn activation sequentially activates and phosphorylates the kinases Pyk2, the Fyn-T-binding protein (Fyb), SLAP-associated protein 76 (SLP-76), and the Src kinase associated phosphoprotein 55 (SKAP55) [142-144]. Cumulatively, these molecules regulate integrin clustering, adhesion, calcium-induced ion channels and activate the mitogen-activated protein kinase (MAPK) signalling pathway, which ultimately culminates in the synthesis of IL-2. Moreover, this cascade of events further supports the stabilization and growth of the immunological synapse.
LR have also been shown to play a role in the negative regulation of Lck and thus dampening TCR-mediated cellular activation. As discussed, tyrosine phosphorylation mediates multiple components of the TCR-induced activation cascade; however, these activating phosphorylation events are reversible. One molecule imperative in this regulation is the protein tyrosine kinase C-terminal Src kinase (Csk), which negatively regulates both TCR-induced tyrosine phosphorylation and IL-2 protein production [145]. In resting T cells, Csk is concentrated in LR by means of its association with the pY317 in the LR transmembrane adapter protein, Csk-binding protein (Cbp) or phosphoprotein associated with glycosphingolipid-enriched membrane domains (PAG) [146, 147]. It is within LR that Csk maintains the suppressive phosphorylation of Y505 in Lck and Y528 in Fyn during resting conditions [148, 149]. However, upon TCR engagement, the dephosphorylation of Csk and its subsequent dissociation from Cbp/PAG results in its displacement from LR and the loss of Lck/Fyn suppression [146, 150]. The exclusion of Csk from LR enables the activation of Lck and thereby regulates the initiation of the tyrosine phosphorylation cascade. Lck/Fyn mediated rephosphorylation of Y317 on Cbp/PAG enables relocalization of Csk to LR and the downregulation of TCR responses after 2-5 minutes, and is therefore essential to homeostatic mechanisms [151].

TCR-induced activation of protein kinase A (PKA) type I within LR also promotes regulation and dampens responses by phosphorylating and activating Csk [152]. Additional inhibitory molecules that have been implicated in this homeostatic network include the PTPases CD45, as discussed, PEST-enriched protein tyrosine phosphatase
(PEP), PTPase with PEST-sequences (PTP-PEST), and Src homology 2-domain-containing protein tyrosine phosphatase 1 (SHP1), among numerous others [153-155]. Ultimately, these molecules share functionality in common; their temporal partitioning into LR is fundamental to the role of these structures in homeostatic regulation of TCR signalling. Two examples follow.

The inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) is essential for the downregulation of TCR responses. Surface expression of CTLA-4 is rapidly induced upon TCR-mediated cellular activation and peaks 2 to 3 days post activation [156, 157]. CTLA-4 is a CD28 homologue, its physiological ligands are B7 family members and CTLA-4-B7 interaction counteracts CD28-mediated co-stimulation with an opposing inhibitory signal [158]. The opposing signal serves to impede cell cycle progression, block IL-2 gene transcription, and ultimately inhibit T cell proliferation [157, 159]. Mice lacking the expression of CTLA-4 exhibit a severe lymphoproliferative disease and eventually succumb to death after 3-4 weeks [160]. Importantly, the function of this inhibitory receptor has shown to depend on its translocation into LR, where it colocalizes and associates with the TCR complex in activated T cells [161]. CTLA-4 has been shown to directly interact with the phosphorylated form of the TCRζ chains. This finding suggests that CTLA-4 may function by regulating the association and retention of TCR within LR [162].

A second inhibitory molecule that has been identified to require LR localization for its functionality is programmed death-1 (PD-1). Similar to CTLA-4, PD-1 is also
upregulated upon TCR-mediated cellular activation and counteracts CD28 co-stimulation by binding to B7 family members (Figure 1-5) [163, 164]. In the case of PD-1, binding is specific for the B7 family members programmed death ligand-1 (PD-L1), expressed on both leukocytes and nonhaematopoietic cells, and programmed death ligand-2 (PD-L2), expressed exclusively on dendritic cells and monocytes [165, 166]. CD28-mediated T cell activation involves the recruitment and activation of phosphatidylinositol-3 kinase (PI3K), the accumulation of 3-phosphorylated lipids and their binding and modulation of Akt [167, 168]. Both CTLA-4 and PD-1 have the capacity to augment CD3/CD28-mediated induction of glucose metabolism and Akt activity. However, these inhibitory molecules are not functionally redundant, as CTLA-4 and PD-1 suppress the PI3K/Akt through distinct mechanisms [169].

Neither CTLA-4 nor PD-1 molecules contain enzymatic activity and are instead dependent on motifs in their cytoplasmic domains for the recruitment of inhibitory phosphatases including SHP-1 and SHP-2 [170-173]. In contrast to CTLA-4, PD-1 contains both an immunotyrosine-based inhibitory motif (ITIM) and an immunoreceptor-tyrosine based switch motif (ITSM); with its signalling capacity dependent on factors binding to the latter [170, 171]. Through these cytoplasmic motifs, it has been suggested that CTLA-4 activates the phosphatase PP2A for the direct inhibition of Akt, whereas PD-1 augments more membrane-proximal events by targeting P13K for Akt suppression [169]. Furthermore, high concentrations of PD-L1 followed by PD-1 activation have been shown to induce the translocation of PD-1 and its associated SHP-1 and SHP-2 into the immunological synapse [171, 174]. This redistribution into LR/the immunological
synapse has been proposed to assist in the dephosphorylation of colocalized TCR signalling intermediates [171, 175]. Collectively, these findings support a central role for LR in partitioning inhibitory receptors such as PD-1 and CTLA-4 and thus, regulating their functions in modulating TCR signalling.
Figure 1-5: The role of PD-1 in the downregulation of T cell activation.
Programmed death 1 (PD-1) is an inhibitory receptor that regulates the adaptive immune response by counteracting CD28 costimulation. Shortly following T cell activation, PD-1 upregulation and binding to its B7 family ligands, PD-L1 or PD-L2, initiates a negative signal cascade to downregulate T cell activation and cytokine production. PD-1 engagement on the cell surface leads to phosphorylation of PD-1 cytoplasmic tyrosines and increases SHP-2 association with the ITSM of PD-1. Recruitment of SHP-2 dephosphorylates signalling through the PI3K pathway and downstream signals through Akt. PD-1 ultimately decreases the induction of cytokines, such as IFN-γ and IL-2, and cell survival proteins such as Bcl-xL.
**The Diverse Roles of Interleukin-2:**

Interleukin 2 (IL-2) plays a fundamental role in the maintenance of T cell homeostasis. The main producers of IL-2 are activated peripheral CD4^+ T cells [176], and to a lesser extent, CD8^+ T cells [177], natural killer (NK) cells [178], natural killer T (NKT) cells, activated dendritic cells [179], and mast cells [180]. T cell activation and subsequent growth is contingent upon the parallel expression and function of both the α/β-TCR and the IL-2 receptor (IL-2R). Signalling events downstream of TCR engagement ultimately culminate in transcriptional induction of the IL-2 gene, with ensuing secretion of IL-2, as well as the upregulated expression of the high-affinity IL-2R [181]. In turn, signalling through the high-affinity IL-2R mediates a plethora of immune enhancing functions, including a role in supporting cellular proliferation and survival [182-184], as well as the differentiation of naïve T cells into either antigen-specific effector T helper 1 (Th1) [185, 186] or T helper 2 (Th2) [187, 188], and memory cells.

IL-2-secreting CD4^+ T cells are known to be multipotent, as they have the capacity to differentiate into several lineages upon antigen exposure. In response to IL-12, via a STAT4 driven pathway, differentiation into Th1 effector cells can take place. Contemporaneously, this pathway also drives the upregulation of the Th1 signature transcription factor, T-bet, and the expression of IL-12Rβ, the proliferation and survival of the Th1 cells, and the production of IFNγ through a STAT5-dependent pathway [186, 189, 190]. Differentiation into this Th1 effector cell type contributes to host defense against intracellular pathogens and viruses, but can also mitigate inflammatory pathogenic diseases as a result of their upregulation of IFNγ [185].
Stimulation by IL-2 is also required to drive the generation of Th2 cells. IL-2-mediated promotion to this CD4⁺ lineage involves high-level expression of the transcription factor GATA3, as well as the induced production of IL-4, IL-5, and IL-13 [191]. Cellular responsiveness to IL-4 is achieved via an IL-2 and STAT5-dependent mechanism, as IL-2 functions to promote chromatin accessibility at both the \( I_{l4} \) locus, and the \( I_{l13} \) locus [187]. This IL-2 and STAT5-dependent mechanism, along with TCR engagement, also functions in the upregulation of IL-4Rα and IL-4 expression, further promoting the differentiation into Th2 cells [188]. The generation of Th2 cells is critical in mediating allergic inflammatory responses and regulating humoral responses to extracellular parasites [191].

In contrast to its roles in cellular survival, IL-2 has also been implicated in activation induced cell death (AICD), specifically for the clonal contraction of mature T cells [192]. Previous exposure to high concentrations of IL-2 can trigger apoptosis post antigen receptor stimulation in both CD4⁺ and CD8⁺ T cells. This functionality is in part mediated by the transcription factor B lymphocyte-induced maturation protein 1 (BLIMP1), which is activated in response to IL-2, and in turn silences the IL-2 gene [193]. A negative feedback mechanism is initiated whereby the prolonged exposure to antigen promotes the upregulation of BLIMP1. As the capacity to secrete IL-2 is progressively reduced, the T cells become terminally differentiated or exhausted. The presence of IL-2 coupled with prolonged antigen stimulation can also regulate homeostasis and the elimination of potentially harmful autoreactive T cells by inducing
the expression of death receptor FAS (CD95) and its ligand, FasL (CD95L) [194]. These molecules are imperative in limiting the expansion phase of an immune response via AICD. Thus, IL-2 is essential for T cell homeostasis as it functions as not only a potent growth factor but also predisposes cells for apoptosis.

The roles of IL-2 have been further diversified with the findings of its participation in anti-inflammatory responses and its role in the production and function of regulatory T (T_{Reg}) cells [182]. The latter predicates the maintenance of self-tolerance [195-197], as deficiencies in IL-2, or components of the IL-2R (CD25 and CD122), result in the spontaneous development of systemic autoimmunity, an accumulation of autoreactive T cells, and a lack of T_{Reg} cells in mice [198]. Unlike activated effector CD4+ T cells, T_{Reg} cells are unable to synthesize significant amounts of IL-2 either in vitro or in vivo, and are thus dependent on the IL-2 produced by neighbouring cells. These IL-2 signals can upregulate both CD25 expression and the suppressive capacity of T_{Reg} cells by sustaining high expression levels of its signature transcription factor forkhead box P3 (FoxP3) [199, 200]. In addition to suppressing autoimmunity, these IL-2 dependent cells have been implicated in the control of tumour immunity, microbial infection, feto-maternal tolerance, allergy, and the maintenance of transplantation tolerance [201].

In addition to supporting FoxP3+ T_{Reg} cells, IL-2 signals are essential for the reciprocal balance and generation of pathogenic T_H17 cells [183, 202]. In contrast to the
IL-2 dependent T\textsubscript{Reg} cells, which decline in numbers in the absence of IL-2 signals, the numbers of IL-17 producing T\textsubscript{H}17 cells increase, particularly in the presence of transforming growth factor beta (TGF-β) and IL-6 following TCR stimulation [195, 202]. This increased generation of T\textsubscript{H}17 cells has been attributed to an enhanced susceptibility to autoimmune disease and inflammatory disorders [195]. IL-2 is a central component regulating this homeostatic balance as the presence of IL-2 functions to lower the expression of the IL-6 receptor β chain on the surface of activated CD4\textsuperscript{+} T cells [203]. This downregulation dampens the IL-6-mediated STAT3 activation, which is essential for the development of retinoic acid receptor-related orphan receptor γt (ROR\textgamma t) expressing T\textsubscript{H}17 cells. Furthermore, STAT5 can be activated by IL-2 and subsequently compete with STAT3 for the binding site on the \textit{Il17} locus [204]. Therefore, the influence of IL-2 on the generation of T\textsubscript{H}17 cells dictates the balance between the transcription factors STAT3 and STAT5.

\textit{Regulation of IL-2 Gene Expression:}

The \textit{Il2} locus is maintained in a transcriptionally silent state within quiescent naïve CD4\textsuperscript{+} T cells, and its transcriptional induction is initiated upon TCR stimulation, mediated by multiple transcription factors, and further stabilized by costimulatory signals. Extending approximately 300 base pairs upstream of the transcriptional start site of the \textit{IL}-2 gene lies a highly conserved proximal promoter/enhancer region containing binding sites for several transcription factors. The regulatory proteins involved include the transcription factors, the nuclear factor of activated T cells (NFAT) family proteins [205],
activator protein 1 (AP-1, FOS/JUN family dimers), nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), and the octamer transcription factor (OCT-1) [206] as well as the architectural protein HMGI [207]. Each of these regulatory elements must come into play to achieve maximal induction of IL2 gene expression.

With the exception of OCT-1, the binding of these transcription factors is induced upon TCR signalling. The binding of NFAT is dependent on the induced AP-1, as it functions to upregulate active NF-κB p65/rel and trigger the calcineurin-mediated dephosphorylation of NFAT, thereby allowing its translocation into the nucleus and increasing its affinity for DNA [182]. T cell activation contributes to the elevated levels of intracellular calcium [Ca\(^{2+}\)], which supports the activation of calcineurin and its activation and dephosphorylation of NFAT via Ca\(^{2+}\)-dependent binding of calmodulin to the calcineurin phosphatase. Ultimately, it is the cooperative binding and activation of these transcription factors that promotes \(IL-2\) gene transcription.

\(IL-2\) transcription is significantly enhanced in response to CD28 costimulation via the composite NF-κB-AP-1 CD28 response element (CD28RE) situated between -164 and -152 base pairs upstream of the transcriptional start site [208]. The effect of CD28 costimulation positively impacts the stability of \(IL-2\) mRNA [209]. In addition, binding of CD28RE to the \(IL-2\) gene contributes towards a five-fold increase of \(IL-2\) enhancer activity [208], and augments expression of the endogenous gene at least one hundred-fold [210, 211]. Ligation of CD28 promotes the activation of PI3K along with its target protein Akt [212, 213]. It also results in the degradation of IκB, thereby increasing the
nuclear concentration of NF-κB dimers along with the transcriptional activation of NF-κB reporters. Taken together, CD28 costimulation plays a fundamental role in the regulation of IL-2 post T cell activation as it functions in the stabilization of IL-2 mRNA.

*The IL-2 Receptor:*

Three distinct membrane subunits, the IL-2-specific, LR-resident, alpha chain (IL-2Rα or CD25), the beta chain (IL-2Rβ or CD122) [214-216], and the common cytokine receptor gamma chain (IL-2Rγ or CD132) [217-219], together comprise the high-affinity trimeric receptor complex for IL-2 [220]. The IL-2Rα chain is undetectable on resting T cells and is upregulated upon T cell activation [221, 222]. This subunit is capable of binding to IL-2 with low-affinity (K_d = 10^{-8} M), and unlike the β and γ chains, its short cytoplasmic tail does not participate in the recruitment of cytoplasmic signal transduction molecules. While the expression of the IL-2Rβ chain can also be induced in response to TCR engagement [223], the IL-2Rγ chain is constitutively expressed on lymphoid cells [224]. The IL-2Rβ chain alone binds poorly with low affinity to IL-2 (K_d = 1 µM), but in combination with the IL-2Rγ chain, the dimeric IL-2 receptor complex is formed and the affinity for IL-2 is increased (K_d = 10^{-9} M). Although the subunit pairing improves affinity, the dimeric intermediate-affinity receptor can only be responsive to IL-2 if expressed at high levels. This dimeric receptor is expressed at low levels on naïve CD8^+ T cells and memory CD4^+ T cells, and at higher levels on memory CD8^+ T cells and NK cells. Formation of the high-affinity IL-2R (K_d = 10^{-11} M) [225] is induced upon TCR engagement [226], when the upregulated IL-2Rα initiates the first binding of IL-2 within
LR and translocates outside of LR for the heterotrimerization with the IL-2Rβ and IL-2Rγ chains (Figure 1-6) [227]. It is there that this quaternary complex induces IL-2 signalling by means of the cytoplasmic tails of the IL-2Rβ and IL-2Rγ chains.

**IL-2 Receptor Signalling:**

It is the signalling through the IL-2R, which promotes clonal expansion and differentiation of antigen-specific peripheral T cells [181]. The IL-2-induced trimerization of the IL-2R subunits enables the juxtaposition of Janus family tyrosine kinases, JAK1 and JAK3, constitutively associated with IL-2Rβ and IL-2Rγc, respectively, and their subsequent trans-phosphorylation and activation [228, 229]. Activated JAK1/3 allows for the association of the adapter Shc with the phosphorylated tyrosine residues on IL-2Rβ [230], and provides docking sites for Src homology 2 domain signal transducer and activator of transcription (STAT) proteins STAT5a, STAT5b, and STAT3, the latter to a lesser extent [198]. Phosphorylation of the associated STAT proteins leads to their dimerization and activation, followed by their translocation into the nucleus where they function to regulate gene transcription [230, 231]. In addition to JAK/STAT activation, Shc associates with Grb2 and provides a platform to induce the recruitment and activation of PI3K and Ras/mitogen-activated protein kinase (MAPK) pathways [232]. These signal transduction cascades mediate T cell clonal expansion and survival, the latter primarily through the activation of the downstream serine/threonine protein kinase B/Akt [182, 233]. Activation of this effector kinase can then function in diverse cellular processes including cytokine synthesis and the regulation of glucose metabolism and glycolysis, ultimately promoting cell survival.
Figure 1-6: A schematic illustration of the LR-mediated spatial regulation of the heterotrimeric IL-2 receptor complex in IL-2 signalling.

Lipid raft-mediated T cell activation is known to culminate in the *de novo* production of the major T cell growth factor, interleukin 2 (IL-2), and signalling through the IL-2 receptor (IL-2R). IL-2 is thought to first bind the LR-resident IL-2Rα chain, which induces its translocation outside of LR and the trimerization of IL-2Rα, IL-2Rβ and IL-2Rγc chains to form the high affinity IL-2R complex. The trimerization and activation of the IL-2R complex enables the juxtaposition of the JAK1 and JAK3 kinases constitutively associated with IL-2Rβ and IL-2Rγc, respectively, and their subsequent trans-phosphorylation and activation. Activated JAK1/3 provide docking sites for SH2-domain signal transducer and activator of transcription (STAT) proteins [STAT 3 and 5], their dimerization and activation followed by their translocation into the nucleus where they function to regulate gene transcription. In addition to JAK/STAT activation, IL-2R signalling induces the recruitment and activation of both phosphatidylinositol 3 kinase (PI3K) and phospholipase C gamma-1 (PLCγ1). Ultimately, these signal transduction cascades mediate T cell clonal expansion and survival, the latter primarily through the activation of the downstream serine/threonine protein kinase B/Akt. Activation of this effector kinase can then function in diverse cellular processes including cytokine synthesis and the regulation of glucose metabolism and glycolysis, ultimately promoting cell survival.
CHAPTER 2: MATERIALS & METHODS

Cell Culture:

Clone 2.10 is an IL-2-dependent, CD4^+, Vβ4-expressing murine T cell clone, specific for ovalbumin (OVA) peptide residues 143-157, within the context of I-A^b restriction. Clone 2.10 was cultured in serum-free Iscove’s Modified Dulbecco’s Media (IMDM) base supplemented with 3.1ng/ml (0.5%) rIL-2 and 0.1% L-α-Phosphatidylcholine (soybean lecithin) (Sigma-Aldrich) as previously described [132]. The GPI-AP deficient (GPI^−) 2.10 variant was isolated from the total 2.10 population based on the loss of CD90 expression. The GPI-AP deficiency of sorted cells remained stable in culture. Flow cytometric analysis confirmed the loss of additional GPI-AP, including CD48 and Sca-1 in the GPI 2.10 variant. The purity of the GPI^- 2.10 variant was maintained by occasional sorting for CD90 positivity.

Reconstituted Expression of Pigp:

To reconstitute the expression of GPI-AP in GPI^- 2.10 cells, PIG-P was reintroduced via the green fluorescent protein (GFP)-expressing, bicistronic pMIEV retroviral vector. 293 fibroblast cells were transfected with either the empty pMIEV vector (MIEV-0) or the pMIEV vector containing cDNA encoding PIG-P (MIEV-PIG-P) using Lipofectamine Plus (Life Technologies). Supernatant was harvested from the infected 293 cells after 48 hours, pelleted to remove any contaminating cells, and transferred with the addition of polybrene (1 μg/ml) onto adherent GP+E packaging cells. The expression of GFP was determined flow cytometrically as a measurement of efficient
GP+E infection and GFP⁺ cells were positively sorted for the establishment of infected GP+E cell lines for future experiments. Supernatant containing the infectious virions was harvested from the infected GP+E cells, pelleted to remove any contaminating cells, and loaded onto RetroNectin®-coated plates (Takara) at 200 μl/cm². The RetroNectin®-coated plates were incubated with the supernatant for 4-6 hours at 37°C. Following incubation, the supernatant was discarded and plates were gently washed twice with IMDM base. 1.5x10⁵ GPI-AP deficient cells of clone 2.10 cells were seeded per (RetroNectin®)-coated plate in a volume of 2 ml of media and cultured for three days at 37°C, 5% CO₂. Following the required incubation, both non-adherent and adherent cells were collected, centrifuged, washed with IMDM base, and resuspended in phosphate buffered saline (Wisent Inc.) plus 3% fetal calf serum (Sigma Aldrich) (PBS+3%FCS) for staining with the GPI-AP anti-CD90 (Thy-1). Cells cultured on MIEV-0-coated plates remained CD90 negative and were sorted for GFP positivity. GPI⁺ 2.10 cells cultured on MIEV-Pig-P plates rescued the expression of GPI-AP and were sorted for GFP⁺ CD90⁺.

**In Vitro Differentiation of Embryonic Stem Cells into T Cells:**

Cell lines and reagents used for the generation of embryonic stem cell (ESC)-derived T cells were provided by Dr. Juan Carlos Zúñiga-Pflücker and established in vitrō as previously described [234, 235]. Briefly, GPI⁺ and GPI⁻, Ly5.2⁺ ESCs were thawed and expanded on 3000 rads gamma-irradiated mouse embryonic fibroblasts and then transferred onto OP9-GFP cells for 8 days of co-culture at 37°C, 5% CO₂. The cells were then harvested and co-cultured with the Delta-like-1 ligand (OP9-DL)-expressing OP9 stromal cell line for an additional 8 days. On day 16, CD25⁺ DN2/3 cells were
isolated by FACS and the proportion of cells derived from GPI⁺ and GPI⁻ ES cells was determined. Following CD25⁺ selection, 1×10⁵ sorted GPI⁺ cells and 5×10⁵ sorted GPI⁻ cells were intravenously injected into RAG2⁻/γc⁻ mice by tail vein. Spleens were harvested after 40 days of reconstitution and the proportion of CD4⁺ and CD8⁺ T cells was assessed by flow cytometry.

*Primary T cells from LckCre/Piga<sup>fl</sup>ox mice:*

Mice bearing loxP sites flanking exon 6 of the Piga gene were acquired from Dr. Taroh Kinoshita and bred with mice transgenic for the Cre recombinase driven by the T cell-specific Lck proximal promoter (Jackson Laboratories) [236] for the generation of LckCre/Piga<sup>fl</sup>ox mice [71]. F2 progeny generated primary T lymphocyte-specific GPI-AP deficiency, and are termed GPI⁻ mice. Mice lacking Cre expression, yet positive for loxP (Piga<sup>fl</sup>ox mice) maintained the expression of GPI-AP and were used as littermate control mice, termed wild-type (WT). All mice were kept in specific pathogen-free conditions and all experiments were approved by Sunnybrook Research Institute Animal Care Committee, following guidelines imposed by the Canadian Council on Animal Care. Primary CD4⁺ or CD8⁺ T cells were purified from the spleens of WT and GPI⁻ mice at 6-10 weeks old using the EasySep™ CD4⁺ or CD8⁺ T cell isolation kit and magnet (STEMCELL Technologies). For the selection of GPI⁻ primary T cells, the selection cocktail was supplemented with biotinylated anti-CD90, due to leakiness of the Cre system. Post negative selection, the T cell preparation was consistently found to be >95% CD4/8⁺TCRαβ⁺CD90⁺ for WT cells and >95% CD4/8⁺TCRαβ⁻CD90⁻ for GPI⁻ cells when assessed by flow cytometry.
**Antibodies and Reagents:**

The following antibodies used in this study include mAbs coupled or not to fluorophores or biotin. The mAbs specific for Thy-1/CD90 (clone 30H12), CD48 (clone OX78), IL-2Rα/CD25 (clone 7D4), TCRβ (clone H57-597), CD4 (clone GK1.5), and CD8 (clone 53-5.8), CD3ε (clone 2C11), IL-2 (S4B6) and anti-IL-2 isotype control (rat IgG2) were affinity-purified from their respective hybridoma cultures at the Sunnybrook Research Institute Antibody Facility. Antibodies purchased from BD Pharmingen include fluorophore-labeled mAbs specific for Sca-1/Ly-6A/E (clone D7), PD-1 (clone J43), PD-L1 (clone MIH5), IL-2Rβ/CD122 (clone TM-Beta 1), and IL-2Rγ/CD132 (clone TUGm2). A fluorophore-labeled mAb specific for IgM (clone 1B4B1) was purchased from eBiosciences.

PD-L1-Fc was purchased from R&D Systems and its isotype control, ChromPure Human IgG, whole molecule was purchased from Jackson ImmunoResearch. Functional grade anti-CD28 was purchased from eBiosciences. Both PMA and Ionomycin were purchased from Sigma-Aldrich. Rabbit polyclonal anti-Lck and anti-Fyn antibodies used for immunoprecipitation and Western blotting have been previously described [125]. Brij-58, the cOmplete™ Mini Protease Inhibitor Cocktail Tablets, and cholera toxin B-HRP (CT-HRP) were purchased from Sigma-Aldrich. Streptavidin and enolase were purchased from Sigma-Aldrich, and [γ32-P]dATP was purchased from Perkin Elmer.
**Flow Cytometry:**

Flow cytometric analysis was performed following labelling of 1x10^5 cells in a solution of 100 μl of PBS+3%FCS with the indicated fluorochrome-labelled antibodies at concentrations recommended by the manufacturer/in-house facility. After staining for 10 minutes at 4°C, the cells were washed twice using PBS+3%FCS, and resuspended in PBS+3%FCS with the addition of propidium iodide (PI) as a viability marker. All flow cytometric analyses were performed on a FACS Calibur flow cytometer (BD Biosciences) and data files were analyzed with FlowJo (Tree Star). Viable cells were gated using forward and side scatter and PI positive cells were excluded from the analysis. All flow cytometry and cell sorting was performed at the Sunnybrook Research Institute Flow Cytometry Facility.

**Proliferation Assays:**

For antigen-induced proliferation, 2.5x10^4 2.10 clonal T cells were cultured with 5 μg/ml OVA^{143-157} peptide (Canpeptide), and 5x10^5 irradiated splenocytes isolated from 6-10 week old C57BL/6 mice (Jackson Laboratories) in 96-well (Corning Costar) plates. For mAb-mediated proliferation, 2x10^4 2.10 clonal T cells, or 4x10^4 primary GPI^+ and GPI T cells were cultured in wells of 96-well (Corning Costar) plates pre-coated overnight at 4°C with anti-TCRCβ at 9 μg/ml or anti-CD3ε at 3 μg/ml. Where described, the addition of soluble anti-CD28 was provided at 3 μg/ml into cultures of 4x10^4 primary WT and GPI CD4^+ T cells on 9 μg/ml plate-bound anti-TCRCβ. When required, triplicate cultures were pulsed with 1 μCi ^3^H-TdR, harvested 6 hours later on Unifilter
plates, and thymidine uptake was assessed on the TopCount NXT™ Microplate Scintillation and Luminescence Counter (Packard).

**IL-2 ELISA:**

Supernatant from stimulated 96-well plate cultures was harvested ahead of thymidine pulsing and frozen at each sequential time point. The level of IL-2 was determined using a mouse IL-2-specific ELISA kit (eBioscience) according to the manufacturer’s protocol. Absorbances at 450 and 570 nm were measured using the BioTek Eon spectrophotometer with the BioTek Gen5 software and IL-2 concentrations determined.

**Quantification of IL-2 mRNA:**

GPI+ and GPI- clonal and primary T cell variants were stimulated for the indicated time points on 9 µg/ml anti-TCRβ-coated or 3 µg/ml anti-CD3ε-coated 96 well plates, respectively. For PMA/Ionomycin stimulation, WT and GPI CD4+ T cells were cultured with PMA at 10 ng/ml and ionomycin at 500 ng/ml. At each time point, cells were harvested and lysed in Tri Reagent® (Sigma Aldrich) according to the manufacturer’s protocol. The RNA was extracted, quantified and assessed for purity using a NanoDrop spectrophotometer (Thermo Scientific), and used to prepare cDNA using reagents and protocol from Life Technologies. The cDNA was diluted based on 5 ng/µl of input RNA ahead of ddPCR reaction.
**gDNA Isolation:**

For the isolation of genomic DNA (gDNA), 1x10⁵ cells from each of the *Piga*, *Pigk*, and *Pigp* CRISPR/Cas9-edited clonal variants were harvested and pelleted by centrifugation at 1500 rpm for 10 minutes. The cell pellets were lysed in 200 μl of 10 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 10 mM NaCl, 0.5% N-lauroylsarcosine, supplemented with 1 mg/ml of fresh proteinase K overnight at 55°C. DNA was precipitated by addition of 400 μl of 75 mM NaCl in ethanol that had been precooled at -80°C. Following a 2 hour incubation at -80°C, tubes were centrifuged at 14,000 rpm for 10 minutes. The pellets were then washed twice with 70% ethanol and air dried at room temperature for 30-45 minutes. The DNA was dissolved in 100 μl of TNE buffer and the DNA concentration was measured ahead of ddPCR analysis.

**Digital Droplet PCR:**

QX200 droplet digital PCR (ddPCR) system (Bio-Rad) was used to quantify IL-2 mRNA in GPI⁺ and GPI⁻ clonal and primary T cell variants (primer based ddPCR). The QX200 ddPCR system was also used to determine the zygosity of *Piga, Pigk*, and *Pigp* CRISPR-edited clonal variants (probe based ddPCR). For IL-2 detection using primer based ddPCR the forward primer 5’-AGCAGCTGTTGATGGACCTA-3’, and reverse primer 5’-CGCAGAGGTCCAAGTTCAT-3’ (Invitrogen) sequences were used. This primer based ddPCR was performed in a 20 μl volume containing 10 ng of genomic DNA template in RNase/DNase-free water, 100 nM each of forward and reverse IL-2 primers, and 10 μl of 2X QX200 EvaGreen ddPCR supermix (Bio-Rad). The probe based
ddPCR for CRISPR analysis used a forward and reverse primer flanking two fluorescently conjugated probes (HEX and FAM) (Integrated DNA Technologies) for each of the *Piga*, *Pigk*, and *Pigp* targets. These sequences are defined in Table 2-1. The probe sequences included Locked Nucleic Acid (LNA) for increased sensitivity in detecting DNA base-pair mismatches, and the modified nucleotides are denoted as a + in Table 2-1. The probe based ddPCR was performed in a 20 µl volume containing 10 ng of genomic DNA in RNase/DNase-free water, 900 nM of the forward and reverse target primers, 250 nM FAM probe, 900 nM of the forward and reverse reference primers, 250 nM HEX probe, and 10 µl of 2X ddPCR supermix for probes (Bio-Rad). For both primer based and probe based ddPCRs, the assay mixtures were loaded into a disposable droplet generator cartridge (Bio-Rad), followed by the addition of 70 µl of droplet generation oil (Bio-Rad) into each of the eight oil wells. The cartridge was then placed inside the QX200 droplet generator (Bio-Rad). When droplet generation was completed, the droplets were transferred to a 96-well PCR plate (Eppendorf) using a multichannel pipette. The plate was heat-sealed with foil and placed in C1000 Touch Thermal Cycler (Bio-Rad). For the primer based ddPCR, thermal cycling conditions were as follows: 95°C for 5 minutes, then 44 cycles of 95°C for 30 seconds and 60°C for 1 minute, and 4°C for 5 minutes, 90°C for 5 minutes, and a 4°C indefinite hold. For the probe based ddPCR, thermal cycling conditions were as follows: 95°C for 10 minutes, then 44 cycles of 94°C for 30 seconds and 60°C for 1 minute, followed by 98°C for 10 minutes, and a 4°C indefinite hold. EvaGreen fluorescent signal, labelling the IL-2 DNA sequence in each droplet in the primer based ddPCR, and HEX and FAM fluorescent signals in each droplet of the probe based ddPCR were counted by QX200 digital droplet reader and
analyzed by QuantaSoft analysis software ver.1.7.4.0917 (Bio-Rad). All ddPCR analyses were performed at the Sunnybrook Research Institute Genomics Facility.

**CFSE Assay:**

WT and GPI− CD4+ splenic T cells were prepared at a concentration of 2x10⁷ cells/ml in PBS+3%FCS. Cells were incubated with 5 μM of CFSE (Sigma-Aldrich) for 5 minutes at room temperature and then washed 3 times with 10 times the volume of PBS+3%FCS. 4x10⁴ cells were then taken for confirmation of CFSE labelling at Day 0. The balance of cells was seeded at 1x10⁵ cells per well in plates pre-coated with 1 μg/ml of plate-bound anti-TCRCβ. The kinetics of CFSE dilution was measured flow cytometrically at days 1, 2, 4, 6, and 8. Dead cells were excluded based on forward/side scatter analysis and inclusion of PI.

**PD-1/PD-L1-Fc Inhibition Assays:**

GPI+ and GPI− clonal variants were cultured at 2x10⁴ cells/well on 96 well plates pre-coated with 3 μg/ml of anti-CD3ε alone, or with a titration of PD-L1-Fc or its isotype control. For primary cell experiments, CD4+ WT and GPI T cells were cultured at 4x10⁴ cells/well on 96 well plates pre-coated with 3 μg/ml of anti-CD3ε alone, or with a titration of PD-L1-Fc or its isotype control. At each assessed time point, cultures were pulsed with 1 μCi ³H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed. The effect of PD-L1-Fc was determined through the calculation and
plotting of ‘percent control’, a ratio of the PD-L1-Fc + anti-CD3ε co-stimulated cultures to the control stimulation of anti-CD3ε alone.

*Inhibition of IL-2 in Culture Supernatants:*

GPI− clonal variants were cultured at 2x10⁴ cells/well on 96 well plates coated with 3 µg/ml of anti-CD3ε alone, or co-coated with 3 µg/ml of anti-CD3ε and 9 µg/ml of PD-L1-Fc or its isotype control. For primary cell experiments, CD4+ GPI− T cells were cultured at 4x10⁴ cells/well on 96 well plates coated with 3 µg/ml of anti-CD3ε alone, or with 9 µg/ml of PD-L1-Fc or its isotype control. Control stimulation represents anti-CD3ε alone. Levels of IL-2 in the supernatant were neutralized using an anti-IL-2 mAb, S4B6, to normalize levels present in the GPI-AP deficient stimulated cultures with those from its GPI-AP sufficient analogue. S4B6 or its isotype control, rat IgG2a, was added at 1 µg/ml to all plated cell cultures containing PD-L1-Fc or its isotype control. Clonal cultures were incubated at 37°C for 1 day and primary cell cultures were incubated for 2 days and 4 days, at which time they were pulsed with 1 µCi ³H-TdR, and thymidine uptake was assessed as discussed above. Percent control was calculated as discussed above.

*Lipid Raft Analysis:*

Lipid rafts isolation was performed as previously described [125]. 1x10⁶ purified primary CD4+ T cells were lysed for 1 hour on ice in 250 µl of TKM buffer containing 50 mM Tris (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 0.5% Brij 58 detergent, and
cOmplete™ Mini Protease Inhibitor Cocktail. After lysis, the lysate was thoroughly mixed with 250 µl of 80% sucrose in TKM buffer. Very delicately, the 500 µl lysate solution was overlaid with 4.3 ml of a 36% sucrose solution and topped with 200 µl of a 5% sucrose solution. The tubes were subjected to equilibrium density gradient ultracentrifugation within an Optima™ L-100 XP Ultracentrifuge (Beckman Coulter) for 16 hours at 50,000 rpm, at 4°C.

Following the centrifugation, 500 µl were removed from the samples for consecutive fractions 1 to 10. 20 µl of each of the 10 sucrose gradient fractions was diluted with 80 µl of PBS-T, and loaded onto a nitrocellulose membrane (Whatman) using a dot-blot apparatus. The membrane was then blocked in a 5% milk TBS-T solution for 1 hour. The LR-resident ganglioside, GM1, was detected by incubation with cholera-toxin-subunit B (CTB)-HRP (Sigma-Aldrich) at a 1:3,000 dilution in 5% milk TBS-T. After a 1 hour long incubation, the membrane was washed 5 times with TBS-T and then developed with ECL solution (GE Healthcare). Rafts were deemed of high quality only if GM1 was found to localize in the earliest membrane fractions.

To determine the subcellular partitioning of Lck and Fyn protein, 30 µl of fractions 1, 2 and 3 representing LR fractions, and 30 µl of fractions 8, 9 and 10 representing soluble fractions were mixed with 10 µl of 4x Laemmlili loading buffer, boiled for 10 minutes, spun, and loaded onto a 9% SDS-PAGE, followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer). The membranes were then blocked in 5% milk-TBS-T for 1 hour at room temperature, and probed with either
anti-Lck or anti-Fyn at a 1:3,000 solution with 5% milk-TBS-T for 1 hour at room temperature. After washing with TBS-T, membranes were incubated with goat-anti-rabbit-HRP (BioRad) at a 1:3,000 solution with 5% milk-TBS-T for 1 hour at room temperature, washed, and developed with ECL solution (GE Healthcare).

**Immune Complex Fyn Kinase Assay:**

Primary CD4+ WT and GPI-1 T cells were prepared in PBS+1% bovine serum albumin (BSA) at 2x10^6 cells/ml and coated with biotinylated anti-TCRβ (B6H57) at 1 µg/ml and biotinylated CD4 (B GK1.5) at 0.3 µg/ml for 30 minutes at 4°C. Following incubation, cells were washed twice with ice cold PBS+1% BSA and 1x10^6 cells were aliquoted per sample and resuspended in 16 µl of ice cold PBS+. All samples were kept on ice until stimulation. Activation was induced by streptavidin-mediated TCR/CD4 aggregation as follows: each sample was pre-warmed at 37°C for 1 minute, followed by addition of streptavidin at 50 µg/ml and incubation at 37°C for the indicated time. Reactions were stopped by the addition of 480 µl of Fyn Lysis Buffer (20 mM Tris pH 8.0, 150 mM NaCl, 200 µg/ml Na3VO4, 0.9% NP-40) containing EDTA-free cOmplete™ Mini Protease Inhibitor Cocktail and lysed for 30 minutes on ice. Post nuclear centrifugation (15 minutes at 14,000 rpm, 4°C), supernatant was transferred to fresh tubes for Fyn immunoprecipitation. Samples were then immunoprecipitated with anti-Fyn covalently coupled to Sepharose 4B beads for 5 hours at 4°C.

Fyn immunoprecipitates were used for immune complex kinase assays as previously described [125]. Immunoprecipitates were washed four times in Fyn lysis
buffer and then twice in kinase buffer containing 20 mM Tris (pH 7.2), 10 mM MgCl₂, and 10 mM MnCl₂. Each immunoprecipitated sample was then incubated with 10 μl of Fyn kinase buffer supplemented with 0.5 μg of freshly acid-denatured enolase, 1 μM cold ATP, and 10 μCi of [γ-³²P]dATP for 30 minutes at 37°C. Following incubation, 10 μl of 4x Laemmli loading buffer was added, and immunoprecipitates were boiled for 5 minutes, centrifuged, and resolved on 9% SDS-PAGE. Proteins were transferred to a PVDF membrane and immunoblotted for Fyn. Kinase activity was detected by exposing the membranes to X-ray film for 1–3 days at -80°C. Once the enolase signal was determined by densitometric analysis, the membrane was blotted for Fyn. Fyn kinase activity was determined by normalizing the enolase signal to the quantification of total Fyn content.

_Densitometric Analysis:_

Densitometric analysis was performed using GS-800 densitometer and Quantity One software from Bio-Rad Laboratories on non-saturated signals.

_CRISPR/Cas9-Targeted Gene Disruption:_

Guide RNA (gRNA) sequences for CRISPR/Cas9-targeted gene disruption of _Piga, Pigk, and Pigp_ designed using the online MIT CRISPR Design software (crispr.mit.edu) are detailed in Table 5-1. Following two washes in PBS⁺⁻, 5x10⁵ GPI⁺ 2.10 clonal cells were electroporated using the Neon Transfection System (ThermoFisher) at 1,700 volts for 20 ms with one pulse. Each electroporation was performed in a total volume of 10 μl of Buffer R for adherent cells (ThermoFisher),
consisting of 10 µg of the GFP-expressing Cas9 vector (pCas9-EF1a-GFP) (OriGene) containing the required gRNA sequences. Electroporated cells were dispensed into 24 well plates containing 1 ml of pre-warmed IMDM Base media in the absence of antibiotics, and supplemented with 0.5% rIL-2. After 18 hours, wells were examined for GFP expression by fluorescent microscopy and transferred into T25 flasks for expansion. Once expanded, cells were sorted for GFP positivity, expanded, sorted for GPI-AP deficiency, expanded, and then single cell deposited into 96 well plates based on GPI-AP deficiency. Following clonal expansion, heterozygous and homozygous mutations were determined by dual probe based ddPCR as described above and in Table 2-1. All heterozygous and homozygous mutants were stimulated on 3 µg/ml of plate-bound anti-CD3ε for 24 hours. Supernatant from these cultures was harvested and IL-2 protein was quantified by IL-2 ELISA and 3H-TdR uptake was measured as described above.
Table 2-1: The primer sequences created for probe based ddPCR analysis of zygosity in CRISPR-edited clonal variants.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Piga</strong> (1&lt;sup&gt;st&lt;/sup&gt; attempt)</td>
<td>Forward</td>
<td>CTCTTTCACTGGTGCCTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTCCCACTGGTGGGTAGAAA</td>
</tr>
<tr>
<td></td>
<td>WT Probe</td>
<td>HEX - CT+TT+C+CG+A+CG+AT</td>
</tr>
<tr>
<td></td>
<td>Probe #2</td>
<td>FAM - CT+GCA+C+C+CA+TAA</td>
</tr>
<tr>
<td><strong>Piga</strong> (2&lt;sup&gt;nd&lt;/sup&gt; attempt)</td>
<td>Forward</td>
<td>TTTCTTAACACCTCCCTCACTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATGGATGACAAACACAGAACC</td>
</tr>
<tr>
<td></td>
<td>WT Probe</td>
<td>Hex - TG+G+CC+A+TC+GT+GG</td>
</tr>
<tr>
<td></td>
<td>Probe #2</td>
<td>FAM - TG+C+CA+GT+T+GTG</td>
</tr>
<tr>
<td><strong>Pigk</strong></td>
<td>Forward</td>
<td>CCTGCTTCCTCACTCTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCGGTCCTCAACAGACAC</td>
</tr>
<tr>
<td></td>
<td>WT Probe</td>
<td>HEX - CC+GC+C+T+TG+GC</td>
</tr>
<tr>
<td></td>
<td>Probe #2</td>
<td>FAM - CA+CA+TC+GA+G+G+TA</td>
</tr>
<tr>
<td><strong>Pigp</strong></td>
<td>Forward</td>
<td>GTTCCCTTTTCACAGATTGTCTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCAGTACTTACTGAAAGCCAAAT</td>
</tr>
<tr>
<td></td>
<td>WT Probe</td>
<td>HEX - AG+C+GAT+TT+AT+G+GC</td>
</tr>
<tr>
<td></td>
<td>Probe #2</td>
<td>FAM - CA+GG+GAA+GA+T+GG</td>
</tr>
</tbody>
</table>
Assessment of Cas9 vector and Cas9 protein effect:

To address the impact of the Cas9 vector on the 2.10 clonal variant, 5x10^5 GPI^+ 2.10 cells were electroporated in 10 µl of Buffer R (ThermoFisher) containing 10 µg of either an empty vector or a vector encoding the Cas9 DNA (pCas9-EF1a-GFP) (OriGene). Electroporation was performed using the Neon Transfection System (ThermoFisher) at 1,700 volts for 20 ms with one pulse per sample. For controls, non-electroporated GPI^+ cells were similarly treated in Buffer R, and non-treated GPI^+ and GPI 2.10 cells were also included. To formally prove the impact of the Cas9 protein on the 2.10 clonal variant, 5x10^5 GPI^+ 2.10 cells were electroporated in 10 µl of Buffer R (ThermoFisher) alone, or supplemented with 1 µg of purified Cas9 protein (Cedarlane). Non-treated GPI^+ and GPI 2.10 cells were included as a control. Following expansion of both Cas9 vector and Cas9 protein electroporated populations, cells were cultured onto 3 µg/ml of plate-bound anti-CD3ε and induced IL-2 production was measured from the supernatant of 24 hour stimulated cultures by IL-2 ELISA (eBiosciences).

Generation of Pigu deficient mice:

Heterozygous mice of strain C57BL/6NTac-PigU<tm1c(EUCOMM)Hmgug>/lcsOrl (Tm1c) were acquired from the European Mouse Mutant Archive (EMMA). To generate the T cell specific, conditional Pigu deficient animal, the Tm1c mouse was crossed with C57BL/6J mice expressing the Lck driven-Cre recombinase (Jackson Laboratories). The progeny of this crossing (Tm1d B6N/J) was then intercrossed to generate mice with conditional GPI-AP deficiency exclusively on T
cells. The generation of the conditional *Pigu* deficient mouse strain is illustrated in Figure 5-4. All mice were kept and bred within specific pathogen-free conditions and all experiments were approved by Sunnybrook Research Institute Animal Care Committee, following guidelines imposed by the Canadian Council on Animal Care.

*Genotyping:*

Tail samples were digested with RedExtract-N-Amp Tissue (Sigma) according to manual’s instruction. Genotyping was performed by PCR using the primers and conditions listed in Table 2-2.

*Statistical Analysis:*

P values among experimental groups were determined by the unpaired Student’s t-test. Error bars were calculated based on triplicate cultures from one experiment. Results from each described experiment have been reproduced a minimum of 2 times, to a maximum of 22 times, depending on the experiment.
Table 2-2: The list of primer sequences and PCR cycle conditions used for genotyping

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sequence</th>
<th>Cycle Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Piga</strong></td>
<td>P1: ACCTCCAAAGACTGAGCTGTG</td>
<td>93°C 3 min</td>
</tr>
<tr>
<td></td>
<td>P2: CCTGCTTAGTCTCCACGT</td>
<td>(93°C 1 min, 62.5°C 1 min, 72°C 2 min) x 28 cycles, 72°C 7 min 4°C indefinitely</td>
</tr>
<tr>
<td></td>
<td>P3: TGTGGGTTCAGTTCATTTCAGA</td>
<td></td>
</tr>
<tr>
<td><strong>Pigu</strong></td>
<td>Ef: CTGTTGGAGGTTGCAAGAAGGTAGAC</td>
<td>95°C 4 min</td>
</tr>
<tr>
<td></td>
<td>Er: GAGAAATAGCGTGCAACTAAGAAGTG</td>
<td>(94°C 30 s., 62°C 30 s., 72°C 1 min) x 34 cycles</td>
</tr>
<tr>
<td></td>
<td>Kr: CCAACAGCTTCCCCACACGG</td>
<td>72°C 7 min, 20°C 5 min, 4°C indefinitely</td>
</tr>
<tr>
<td></td>
<td>L3f: GATACCAAACGCTGAACCTCTTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L3r: GCATGTGGAGAAGCCGCACCTCAACAATG</td>
<td></td>
</tr>
<tr>
<td><strong>cre</strong></td>
<td>CATCGCTCGACACGGTTTAGT</td>
<td>95°C 4 min,</td>
</tr>
<tr>
<td></td>
<td>CGATGCAACGAGTGAG</td>
<td>(95°C 30 s., 61°C 30 s., 72°C 45 s.) x 35 cycles, 72°C 7 min, 4°C indefinitely</td>
</tr>
</tbody>
</table>
CHAPTER 3: CLONAL T CELL MODEL SYSTEM

RESULTS

Chapter Hypothesis: As GPI-AP reside exclusively within LR, they may function as essential regulators of TCR/CD3 mediated “on” and “off” signals within this signalling scaffold.

Identification of Pigg, a novel gene involved in the initial step of GPI-anchor biosynthesis:

Towards addressing the role of GPI-AP in T cell homeostasis, we have developed a model system utilizing an IL-2-dependent, CD4⁺, I-Aᵇ restricted, OVA¹⁴³⁻¹⁵⁷ specific, murine T cell clone, termed 2.10. The clonal line was found to be a heterogeneous mixture of cells either expressing GPI-AP, or presenting a complete deficiency in all assessed GPI-AP (Figure 3-1). The population of GPI-AP deficient (GPI) cells was isolated by fluorescence-activated cell sorting (FACS), and in collaboration with Dr. Taro Kinoshita, at the National Cardiovascular Centre Research Institute in Japan, we sought to characterize the lesion in GPI-AP biosynthesis. As a mutation in the initial steps of GPI-AP biosynthesis can render a cell devoid of GPI-AP, the activities of the early GPI-anchor biosynthesis enzymes were measured. The 2.10 GPI- cells were found impaired in their ability to transfer N-acetylglucosamine (GlcNAc) to phosphatidylinositol (PI), and were thus defective in the synthesis of N-
acetylglucosaminyl-PI (GlcNAc-PI), the first intermediate in GPI-AP biosynthesis. This transfer of GlcNAc is required for the production of N-acetylglucosaminyl-PI (GlcNAc-PI) and is catalyzed by GPI-N-acetylglucosaminyl-transferase (GPI-GnT). The deficiency in the clone 2.10 correlated with an autosomal mutation in a novel gene, phosphatidylinositol glycan class P, which was termed Pigp. Once cloned, Pigp was determined to constitute the fifth component of GPI-GnT, predicking GPI-GnT activity, with direct binding to Pigq and Piga [8].

Following the introduction of Pigp into the GFP-expressing, bicistronic, retroviral expression vector, MIEV, the GPI 2.10 cells were transduced with either MIEV-PIG-P, or the empty vector (MIEV-0). The transduced populations were sorted based on normalized levels of GFP expression, and the expression levels of GPI-AP, CD48, CD90, and Sca1, at the cell membrane were determined flow cytometrically. As illustrated in Figure 3-1, while infection of the GPI 2.10 cells with the empty vector, MIEV-0, did not impact GPI-AP deficiency, the cells expressing the MIEV-PIG-P vector regained the expression of all three assessed GPI-AP, CD48, CD90, and Sca1. Thus, the stable ectopic reconstitution of Pigp in the GPI clonal variant was found to successfully rescue GPI-AP expression.
Figure 3-1: Ectopic expression of Pigp rescues GPI-AP expression in GPI transduced clonal variants

The IL-2-dependent CD4, I-A\(^{b}\) restricted, OVA\(^{143-157}\) specific T cell clone [2.10] is a heterogeneous mixture of cells expressing or deficient in GPI-AP. These two populations of 2.10 are termed here as the Parent populations. GPI variants of 2.10 infected with either an empty vector (MIEV-0) or MIEV-PIG-P, are termed Transduced variants. All populations were stained with mAb specific for the GPI-AP: CD90, CD48, and Sca-1. Viable cells were gated using forward and side scatter and PI positive cells were excluded from the analysis. While the GPI\(^{+}\) Parent population (upper white histograms) expresses all three of the assessed GPI-AP, there is a complete deficiency in the GPI Parent population (upper grey histograms). Ectopic expression of Pigp rescues CD90, CD48, and Sca-1 expression in the MIEV-PIG-P Transduced variant (lower white histograms), while MIEV-0 Transduced variant (lower grey histograms) remain GPI-AP deficient.
First insight into the regulation of T cell activation by GPI-AP: an intrinsic growth/survival phenotype:

The capacity to stimulate the GPI⁺ and GPI⁻ 2.10 clonal variants, as well as the transduced GPI⁻ clonal variants (MIEV-0 and MIEV-PIG-P), was measured in response to antigen, OVA\textsuperscript{143-157} in the context of I-A\textsuperscript{b} expressing, gamma-irradiated antigen-presenting cells (APC). Tritiated thymidine uptake was assessed as a measure of activation-induced proliferation over time. While both the GPI⁺ and GPI⁻ 2.10 clonal variants exhibited a comparable initial proliferative response to antigen \textit{in vitro}, GPI-AP expression was found to impart a growth limitation on clonal expansion, with GPI⁻ variants exhibiting prolonged proliferation and survival. Following three days of stimulation, the measured thymidine uptake in the GPI⁺ 2.10 cells began to decline steadily over time. Conversely, the GPI⁻ 2.10 cells showed no such waning of proliferative responses; thymidine uptake in GPI⁻ 2.10 cells remained elevated, and at a plateau at each time point assessed. As illustrated in Figure 3-2A, by day 8 of antigen-induced stimulation, the level of thymidine uptake in the GPI-AP deficient cells was 9.6-fold greater than that in the GPI-AP sufficient variants. Thus, these findings suggest a novel growth/survival advantage phenotype imparted by the absence of GPI-AP.

The causal role of GPI-AP underpinning this phenotype was formally proven by ectopically re-expressing \textit{Pigp} in the GPI⁻ variant, which rescued both the expression of GPI-AP and the antigen-induced proliferation phenotype of the GPI⁺ variant. Specifically, the re-introduction of \textit{Pigp} circumvented the enhanced survival and proliferation seen in
Figure 3-2: GPI-AP deficiency in clonal variants confers an intrinsic growth/survival advantage.

Parental 2.10 GPI+ (black squares) and GPI- variants (white squares), and transduced GPI-MIEV-0 (white triangles), and GPI-MIEV-PIG-P (black triangles) 2.10 T cells were cultured at 2.5x10^4 cells per well with 5 μg/ml OVA^143-157 peptide and 5x10^5 irradiated splenocytes in A., at 2x10^4 cells per well in wells pre-coated with mAb H57 (anti-TCRβ) at 9 μg/ml in B., or at 2x10^4 cells per well in wells pre-coated with mAb 2C11 (anti-CD3ε) at 3 μg/ml in C. At each of the indicated time points, triplicate cultures were pulsed with 1 μCi ^3H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed.
antigen-stimulated GPI 2.10 cells. Figure 3-2A illustrates the waning antigen-induced thymidine update of the MIEV-PIG-P transduced variant, in comparison to the MIEV-0 transduced variant, which exhibited thymidine uptake at plateau levels at each time point assessed. The response of the MIEV-0 transduced variant precisely replicated that of GPI 2.10 cells, indicating that transduction with the MIEV vector plays no role in the proliferative phenotype. In contrast, the response of the MIEV-PIG-P transduced variant is similar to that of the GPI+ 2.10 cells, as the cells do experience an attenuation of antigen-induced proliferation and survival; however, the waning response is significantly accelerated. This may be attributed to the introduction of Pigp gene rescuing a nearly 10-fold greater level of GPI-AP expression (Figure 3-1). Ultimately, these results formally establish the correlation between the absence of GPI-AP and a prolonged and enhanced TCR-induced growth advantage.

As the novel GPI-AP deficient phenotype was observed post-stimulation with OVA/APC, the role of APC in mediating and/or contributing to this phenotype cannot be ruled out. To address this question, GPI+, GPI 2.10 clones, and MIEV-0 and MIEV-PIG-P infected GPI 2.10 clones were stimulated using plate-bound anti-TCRβ mAb. The response of clone 2.10 to anti-TCRβ is possible as it is CD4 deficient [132]. Specifically, MHC class II restricted CD4+ T cell clones [132] and primary CD4+ T cells, as will be seen in the next Chapter, are not responsive to anti-TCRβ mAb in the absence of co-aggregation with CD4/Lck [132]. In the absence of CD4, Lck is not sequestered in clone 2.10 and is free floating on the inner leaflet of the plasma membrane and available to interact with aggregates of TCR/CD3 induced by the plate-bound anti-TCRβ [132,
As illustrated (Figure 3-2B), the response to anti-TCRβ paralleled that observed with antigen. Once again, TCR-induced thymidine uptake declined over time in both the GPI and the MIEV-PIG-P clonal variants, while the GPI-AP deficient cells exhibited an extended growth/survival advantage (Figure 3-2B). Furthermore, as illustrated in Figure 3-2C, the GPI-AP deficient phenotype is recapitulated through stimulation with anti-CD3ε. Therefore, the mechanisms supporting these differential responses are intrinsic to the cell, and directly related to the expression of GPI-AP. This novel phenotype provided the first insight into the regulation of T cell activation by GPI-AP.
**Differential TCR-mediated induction of IL-2 in GPI clonal variants:**

IL-2 is a major T cell growth factor for the clonal expansion of antigen-activated T cells, and the maintenance of homeostatic processes. As the 2.10 clonal variants are IL-2 dependent, their sustained growth can be maintained through either the addition of exogenous IL-2, or the TCR-induced production of endogenous IL-2. In the absence of IL-2, cell death of the 2.10 clone ensues through the intrinsic apoptotic pathway. Thus, the first investigations into mechanisms correlating with the differential proliferation kinetics of the GPI+ and GPI 2.10 clonal variants focused on IL-2 biochemistry.

Two potential mechanisms to account for the differential kinetics of growth exhibited by GPI+ and GPI variants were posited. One possibility was that the waning antigen- and mAb-induced proliferative responses of the GPI+ 2.10 clonal variants reflected an inability of the IL-2 receptor (IL-2R) to bind and utilize the IL-2. Alternatively, if the IL-2R was indeed comparably functional over time, we then questioned whether the presence or absence of GPI-AP could impact the kinetics and magnitude of IL-2 message and protein production in response to stimulation. The first possibility was addressed by adding a fixed amount of exogenous recombinant IL-2 (rIL-2) to antigen- or anti-TCRβ-stimulated cultures once a day, from day 3 onward, and thymidine update was assessed 48 hours post addition, as a measure of *de novo* DNA synthesis. As illustrated in Figure 3-3, exogenous IL-2 rescued the waning responses of stimulated GPI+ variants, both the MIEV-*Pig-P* transduced and the GPI+ 2.10 parental line. Therefore, the GPI+ variants continue to express functional IL-2R over time.
Figure 3-3: Exogenous IL-2 rescues the response of GPI\(^+\) variants.
A. Replicate cultures of GPI-MIEV-0 (white triangles), and GPI-MIEV-PIG\(^+\)P 2.10 (black triangles) (2.5x10\(^4\)) were established with OVA\(^{143-157}/IA\(^b\) APC stimulation at each of the indicated time points. B. Replicate cultures of GPI 2.10 (white squares), and GPI\(^+\) 2.10 (black squares) (2x10\(^4\)) were stimulated on plate-bound anti-TCR\(\beta\) at each of the indicated time points. In both A. & B. cells were pulsed with 1 \(\mu\)Ci \(^3\)H-TdR, and harvested as in 3-2. Exogenous rIL-2 (3 ng/ml) was added at days 3 to 6 in A. and days 3 to 5 in B., and pulsed and harvested 48 hours later. The rescued responses are represented as white circles for GPI-MIEV-0 and GPI\(^+\) variants, and black circles for GPI-MIEV-PIG\(^+\)P 2.10 and GPI\(^+\) variants.
To further investigate IL-2R functionality, the consumption and utilization of a fixed amount of IL-2 was assessed. Specifically, a constant number of GPI+ and GPI− variants were cultured with 3 ng/ml of rIL-2 and the concentration of rIL-2 remaining in the supernatant at 24 and 48 hours was determined. Following one day of culture, both GPI+ and GPI− cells had consumed approximately 90% of the rIL-2, and by day 2 about 99% of the rIL-2 had been consumed (Figure 3-4A). These results support the conclusion that the absence of GPI-AP does not impart differential IL-2R expression or IL-2R mediated IL-2 uptake.

To address the issue of IL-2 utilization, a constant amount of IL-2 was added to varying numbers of GPI+ and GPI− variants and ³H-thymidine uptake was assessed at 48 hours. GPI+ and GPI− cells responded and proliferated comparably to the 3 ng/ml of rIL-2, over the full range of cell numbers cultured, with the amount of ³H-thymidine uptake increasing linearly with cell number (Figure 3-4B). Therefore, the expression/absence of GPI-AP plays a role with neither the consumption nor utilization of IL-2. Taken together, these results again provide confirmation of a functional IL-2R in both the GPI+ and GPI− 2.10 variants.

As neither the capacity to consume nor utilize IL-2 accounted for the differential growth/survival of GPI− clonal variants, we addressed the potential role of differential TCR induced IL-2 production. GPI-AP sufficient and deficient variants of clone 2.10 were stimulated with 9 μg/ml of plate-bound anti-TCRβ mAb and the induction of IL-2 mRNA over time was quantified using digital droplet PCR (ddPCR).
Figure 3-4: Comparable consumption and utilization of IL-2 in GPI+ & GPI- variants.

A. A constant number of GPI+ and GPI- cells (2x10⁴) were added to cultures containing 3 ng/ml of IL-2. At days 1 and 2 supernatants were collected and the concentration of contained IL-2 assessed using a mIL-2 specific ELISA. B. 3 ng/ml of IL-2 was added to cultures of varying numbers of GPI+ and GPI- cells and ³H-thymidine uptake assessed at 48 hours.
To determine the fold induction of IL-2 mRNA, the levels found at all time points were normalized to basal levels assessed from cultures of non-stimulated GPI+ and GPI- clonal variants. As illustrated in Figure 3-5A, while the kinetics of IL-2 mRNA induction in GPI+ and GPI- clonal variants was comparable, the fold induction of IL-2 mRNA was materially higher in the GPI+ stimulated cells at each time point assayed. Specifically, in both the GPI+ and GPI 2.10 cultures, the peak of IL-2 mRNA induction was at day one of stimulation, and was 3.4 fold greater in GPI-AP deficient variants. The level of IL-2 mRNA in GPI+ variants declined in parallel with their proliferative responses, as it did for the GPI- variants, but in contrast to levels observed over time in the GPI+ variants, levels of IL-2 mRNA remained close to 100-fold higher in GPI- variants. Specifically, at day 6, the final time point assessed, the fold induction of IL-2 mRNA over background was 0.03x and 2.84x in the GPI+ and GPI 2.10 cells, respectively. These results support the conclusion that the material increase and prolonged IL-2 mRNA produced in GPI+ variants, and the ensuing increase in available IL-2, underpin the extended growth/survival kinetics observed in GPI+ T cell clonal variants.

To formally demonstrate the role of GPI-AP in the regulation of TCR induced IL-2 mRNA, the above experiment was repeated with GPI+ MIEV-0 and the GPI-AP-rescued MIEV-PIG-P clonal transduced cells. As illustrated in Figure 3-5B, the levels of induced IL-2 mRNA in the GPI+ and GPI- transduced cells replicated the trends observed with the original GPI+ and GPI- variants. Likewise, the peak of IL-2 mRNA induction was on day 1 for both the MIEV-0 and the MIEV-PIG-P transduced cells, with the GPI-AP
Figure 3-5: Enhanced IL-2 production imparted by GPI-AP deficiency.
A-B. GPI+, GPI-, MIEV-PIG-P and MIEV-0 clonal variants were stimulated with plate-bound anti-TCR Cβ for days 1-6, or clonal variants were harvested directly from rIL-2 culture for day 1. RNA was extracted and cDNA was prepared for ddPCR. IL-2 message levels out of IL-2 were set to 1, and the fold induction in 9 µg/ml anti-TCR Cβ stimulated cultures was assessed at the indicated time points. C-D. The supernatants from the stimulated cultures were collected at the indicated time points and IL-2 content was assessed by mIL-2 specific ELISA. Black symbols are representative of GPI-AP deficient variants; white symbols are representative of GPI-AP sufficient variants. E. The IL-2 concentrations quantified in C & D are represented as ratios comparing the differential amount of IL-2 in GPI-AP sufficient parental or transduced cultures compared to their GPI-AP deficient counterparts.
deficient transduced variant producing 3.4x the IL-2 mRNA in comparison to that produced by the GPI-AP sufficient counterpart (Figure 3-5B).

To determine whether the enhanced and prolonged IL-2 mRNA production correlates with enhanced amounts of secreted IL-2 protein, IL-2 concentrations in supernatants of anti-TCRβ stimulated GPI and GPI+ clonal variants were assessed. As illustrated in Figure 3-5C, the amount of IL-2 in supernatants correlated well with levels of IL-2 mRNA observed in the same cells. While levels of IL-2 in supernatants declined over time in all cultures, there was significantly more IL-2 in supernatants of stimulated GPI-AP deficient variants. Specifically, 493 pg/ml compared to 2830 pg/ml of IL-2 was detectable at day 1 in cultures of GPI+ and GPI-2.10 variants, respectively. This 5.7-fold higher level of IL-2 detected in supernatants of GPI variants at day 1 increased to 64.3-fold by day 4. Similarly, in the transduced cultures, the rescue of GPI-AP expression through the introduction of the Pigp gene, markedly decreased the amount of IL-2 detected in the supernatant (Figure 3-5D). At all time points there was significantly more IL-2 in supernatants of the MIEV-0 cells compared to the MIEV-PIG-P stimulated cells, ranging from 4.6x to 44.8x higher levels. While the level of IL-2 detected in the supernatant is greatest at day one of stimulation in the MIEV-PIG-P stimulated cultures, like the GPI+ 2.10 clonal variant, the levels progressively decline over time, dropping to 19 pg/ml at day 6, a level 23.4x lower than that of its GPI-AP deficient counterpart. Given that uptake and utilization of IL-2 is comparable in GPI+ and GPI- clonal variants, these results support the conclusion that GPI-AP expression regulates IL-2 production.
Assessing the role of GPI-AP in the attenuation of T cell activation:

As the growth/survival advantage of GPI variants is due, at least in part, to their enhanced capacity to produce and maintain the production of IL-2, we posited that the quantity and prolonged production of IL-2 by GPI variants reflects dysregulated signalling through TCR/CD3. To investigate a potential role for GPI-AP in the attenuation of TCR signalling, the expression of known negative regulators of TCR signalling was assessed. The ITIM-containing inhibitory receptor, PD-1, and its agonistic ligand, PD-L1 were expressed at comparable basal levels on both the GPI-AP sufficient and deficient clones (Figure 3-6), and expression of both were comparably upregulated post anti-TCR\(\beta\) stimulation (Figure 3-6). PD-1 was of particular interest as it has been shown to attenuate TCR-mediated clonal expansion and induce cell death upon its translocation into the immunological synapse [174], where its colocalization with TCR recruits SHP2 to induce the dephosphorylation of TCR proximal signalling events [238]. As the expression of neither PD-1 nor its ligand is impacted by the absence of GPI-AP, we next sought to determine whether the prolonged growth/survival phenotype was consistent with a differential PD-1/PD-L1 functionality.

As GPI-AP are known to reside exclusively within LR, we hypothesized that the differential composition of LR in the GPI-AP deficient variants may prohibit the PD-1/PD-L1-mediated attenuation of TCR/CD3 signalling. This was assessed by stimulating GPI\(^+\) and GPI variants with anti-CD3\(\epsilon\) in the presence of increasing concentrations of PD-L1-Fc, a recombinant human B7-H1/PD-L1 Fc chimera that has been shown to inhibit anti-CD3 induced IL-2 secretion in human T lymphocytes [239]. The effect of
Figure 3-6: Anti-TCRβ-induced upregulation of PD-1 and PD-L1 on 2.10 clonal variants.

GPI+ and GPI− variants of clone 2.10 were cultured in either 0.5% (3 ng/ml) of recombinant mIL-2 (red line) or stimulated with 9 µg/ml of plate-bound anti-TCRβ (blue line). After 20 hours, cells were harvested and the expression of PD-1 and PD-L1 were assessed flow cytometrically. Negative control (green line) represents unstained cells. The data is representative of five experiments.
PD-L1-Fc was determined through the calculation and plotting of ‘% control responses’, specifically the ratio of responses obtained in the PD-L1-Fc/anti-CD3ε co-stimulated cultures to the control stimulation of anti-CD3ε in the presence of an isotype control for PD-L1-Fc. Increasing concentrations of PD-L1-Fc was found to suppress the clonal expansion of the GPI⁺ clonal variants, exclusively, as assessed by ³H-TdR (Figure 3-7A). Strikingly, in the presence of 9 µg/ml of PD-L1-Fc, the response of the GPI⁺ clonal variant was suppressed to 2.5% of the control response, while the response of the GPI⁻ clonal variant was actually enhanced to 155% of the control response.

To formally prove the role of GPI-AP expression in mediating the differential susceptibility of GPI⁺ and GPI⁻ variants to PD-1 mediated inhibition, the above experiment was replicated using the MIEV-0 and MIEV-PIG⁻P clonal transduced variants. Similar to the parental GPI⁺ and GPI⁻ variants, the MIEV-0 and MIEV-PIG⁻P transduced cells expressed basal levels of PD-1 and PD-L1, which were both upregulated after anti-TCRβ stimulation. Reconstitution of the GPI⁺ clonal variant with Pigp restores the expression of GPI-AP, and the MIEV-PIG⁻P transduced variant is as susceptible to PD-L1-Fc-mediated attenuation of TCR signalling and clonal expansion as the parental GPI⁺ clonal variant (Figure 3-7B). Specifically, as for the parental GPI⁺ variant, anti-CD3ε mediated growth of the MIEV-PIG⁻P variant was suppressed to 1% of control responses in the presence of 9 µg/ml of PD-L1-Fc, while the response of the MIEV-0 variants was unimpeded, or as for the parent GPI variant, the response was enhanced (Figure 3-7B).
Figure 3-7: Differential sensitivity of GPI clonal variants to PD-L1-Fc.
A. Triplicate cultures of 2x10^4 cells were stimulated on plate-bound anti-CD3ε alone, or with a titration of PD-L1-Fc or its isotype control. At day 2, cultures were pulsed with 1 µCi ^3H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed. Thymidine uptake counts per million with anti-CD3ε alone was considered the control (100%) and % Control was determined by comparing the counts per million of the PD-L1-Fc and isotype control stimulated cultures to that of the anti-CD3ε alone control. B. MIEV-0 and MIEV-PIG-P transduced cells were stimulated with plate-bound anti-CD3ε alone or with a titration of PD-L1-Fc. As in A., thymidine uptake and % Control was determined at day 1.
A mechanism underpinning PD-1-PD-L1 mediated attenuation of TCR signalling and proliferation is through inhibition of IL-2 production; and of note, provision of exogenous IL-2 overcomes PD-1-PD-L1 mediated effects [239]. Given the profoundly enhanced and prolonged production of IL-2 observed in GPI clonal variants we hypothesized that this distinct physiology underpinned their differential sensitivity to PD-L1 mediated attenuation of TCR signalling and proliferation.

To test this hypothesis we assessed IL-2 concentrations in the supernatants of PD-L1-Fc/anti-CD3ε stimulated cultures of GPI+, GPI, MIEV-0, and MIEV-PIG-P. In parallel to the PD-L1-Fc-mediated inhibition of proliferative responses in the GPI+ cell variants, there was a decline in detectable IL-2 in the supernatant with increasing concentrations of PD-L1-Fc (Figure 3-8A). Specifically, IL-2 present in supernatants of anti-CD3ε stimulated cultures of GPI+ clonal variants were reduced 100-fold at the highest concentration of PD-L1, from 550 pg/ml for the GPI+ parental variant and 1500 pg/ml in the MIEV-PIG-P clonal variant, in control cultures down to 10 pg/ml and 5 pg/ml in the presence of the 9 µg/ml of PD-L1-Fc in cultures of the GPI+ parent and MIEV-PIG-P variants, respectively (Figure 3-8A). Thus, the presence of GPI-AP renders these variants susceptible to PD-1-mediated negative regulation of proliferative responses by inhibiting IL-2 production, as expected.

Insight was derived when assessing IL-2 levels in supernatants of cultures of GPI parent and GPI+ MIEV-0 transduced cells stimulated with anti-CD3ε in the presence of increasing concentrations of PD-L1-Fc. Specifically, the efficacy of PD-L1-Fc
Figure 3-8: Differential sensitivity to PD-L1 is directly related to level of IL-2 production.

A. The supernatants from cultures stimulated on plate-bound anti-CD3ε alone, or with a titration of PD-L1-Fc, were collected at day 1 and IL-2 content was assessed by mIL-2 specific ELISA. B. The GPI parental 2.10 cells were stimulated on plates co-coated with 3 µg/ml of anti-CD3ε and either 9 µg/ml of PD-L1-Fc or its isotype control (Iso Ctl). Cultures were supplemented with 1 µg/ml mAb S4B6, such that levels of IL-2 present culture supernatants approximated those generated by GPI+ parental variant, or 1 µg/ml of S4B6 isotype control. Cultures were pulsed with 3H-TdR at 24 hours. Sensitivity to PD-L1-Fc- (grey bars) or its isotype control- (white bars) mediated inhibition in the presence of S4B6 (left bars) or its isotype control (right bars) is presented as % Control. % Control was determined by comparing the counts per million of the PD-L1-Fc and Isotype Control stimulated cultures to that of the anti-CD3ε alone control.
mediated inhibition of IL-2 production in these GPI variants was comparable to that observed in GPI\(^+\) variants, ranging from 50 to 100-fold in GPI parent and MIEV-0 variants, respectively (Figure 3-8A). This result proves that indeed PD-1-PD-L1 interaction was comparably functional in GPI\(^+\) and GPI clonal variants, and also reveals the basis for the differential susceptibility of GPI\(^+\) and GPI clonal variants to PD-L1-Fc mediated inhibition of proliferation as assessed by \(^3\)H-TdR uptake.

Specifically, the amount of IL-2 detected in culture supernatants of GPI cultures stimulated with anti-CD3\(\varepsilon\) and 9 \(\mu\)g/ml of PD-L1-Fc, while reduced 30-80-fold relative to controls, was on average 10-50-fold higher compared to levels observed in supernatants of GPI\(^+\) parent and MIEV-\(PIG-P\) transduced cells, respectively (Figure 3-8A). Thus, while the fold reduction of IL-2 detected is comparable in the GPI-AP sufficient and deficient variants, the overall enhanced amount of IL-2 present in culture supernatants of GPI-AP deficient clonal variants is adequate for the maintenance of continued clonal survival and proliferation.

The prediction follows that reduction of available IL-2 in culture supernatants of stimulated GPI\(^+\) clonal variants should render them susceptible to PD-L1-Fc mediated inhibition of survival and clonal expansion. This was directly tested by assessing the impact of 9 \(\mu\)g/ml of PD-L1-Fc on anti-CD3\(\varepsilon\) stimulated GPI\(^+\) clonal variants in the presence of the IL-2 neutralizing mAb, S4B6, such that available IL-2 in the supernatants of the stimulated GPI\(^+\) variants approximated that observed in the supernatants of the GPI\(^+\) variants stimulated in the same way. Specifically, the addition of 1 \(\mu\)g/ml of S4B6
to the anti-CD3ε-stimulated GPI² cultures was sufficient to reduce contained IL-2 to levels comparable to those within the GPI⁺ cultures, while not significantly decreasing ³H-TdR levels as well. Thus, 1 µg/ml of S4B6 or its isotype control, rat IgG2a, was added to cultures and sensitivity to 9 µg/ml of PD-L1-Fc or its isotype control was assessed.

As illustrated in Figure 3-8B, stimulation of GPI² variants with anti-CD3ε in the presence of 9 µg/ml of PD-L1-Fc and 1 µg/ml of S4B6 resulted in roughly 70% inhibition of ³H-TdR uptake compared with roughly 5% inhibition mediated by the isotype control for S4B6. Again, the inhibition observed is specifically mediated by PD-L1-Fc as there is minimal to no effect of S4B6 on the isotype control for PD-L1-Fc. Thus, the enhanced amount of IL-2 present in culture supernatants of GPI⁻ variants is necessary and sufficient for the evasion of PD-1-mediated downregulation of proliferative responses.
CHAPTER 4: PRIMARY T CELL MODEL SYSTEMS

RESULTS

Chapter Hypothesis: The growth/survival advantage attributed to GPI-AP deficiency in the clonal T cell model system will be recapitulated in GPI primary T cells, confirming physiological relevance.

GPI-AP in T lymphocyte development:

While attaining some clarity with respect to the role of GPI-AP in regulating TCR/CD3 signalling and cellular homeostasis using T cell clones, a key objective is to assess the cell biology and biochemistry of TCR signalling in GPI-AP deficient primary T cells, to ensure physiological relevance. Toward this end, and because deficiency in GPI-AP constitutes embryonic lethality [49-51], we sought to capitalize on key advances made that enable the development of mouse T cells in vitro [240]. While falling short of providing sufficient cells to assess the biology of GPI T cells, this system did provide some new information in regards to a role of GPI-AP in T cell development.

Utilizing Notch signalling to facilitate T cell differentiation, a cell culture system capable of differentiating mouse embryonic stem cells (mESC) into T-lineage cells was employed (Figure 4-1). Briefly, GPI\(^+\) or GPI\(^-\) mESC were expanded on gamma-irradiated mouse embryonic fibroblasts, and then transferred onto a GFP-expressing OP9 cell line.
Figure 4-1: GPI-AP deficient primary T cells show a developmental advantage.

GPI\(^\text{+}\) and GPI\(^{-}\), Ly5.2\(^{+}\) embryonic stem cells (ES) were thawed and expanded on gamma-irradiated mouse embryonic fibroblasts and then transferred onto OP9-GFP cells to begin hematopoiesis. At day 8, the cells were co-cultured with the OP9 cell line expressing Delta-like-1 ligand (OP9-DL) for an additional 8 days. CD25\(^{+}\) cells, representing DN2 and DN3 subsets of immature thymocytes, were isolated by FACS and the proportion of cells derived from GPI\(^{+}\) and GPI\(^{-}\) ES cells was determined. A titration of sorted CD25\(^{+}\) cells were intravenously injected into RAG2\(^{-}/^{-}\)/γc\(^{-}/^{-}\) mice by tail vein and the kinetics of reconstitution was assessed.
to initialize the mesoderm to hematopoietic differentiation conditions. At day 8 of co-culture, the cells were transferred onto OP9 cells expressing the Notch receptor ligand Delta-like-1 (OP9-DL1), to induce T cell development with the exclusion of other lineages. The co-culture on OP9-DL1, continued for an additional 8 days, after which CD25+ cells, representing DN2 and DN3 T-lineage cells, were isolated by fluorescence activated cell sorting. It was at this early stage of T lymphocyte development that a striking phenotype was noted. Sorting for CD25+ pro-T cells revealed that GPI-AP deficiency conferred roughly a 10-fold greater growth/survival advantage of GPI DN2/3 in vitro (Figure 4-1). While certain GPI-AP have been implicated in T cell development, this was the first time that a complete GPI-AP deficiency was attributed to a quantified developmental advantage of DN2/3 pro-T cells.

Buoyed by the selective advantage imparted by GPI-AP deficiency at early stages of T cell development, we sorted CD25+ DN2 and DN3 cells to reconstitute RAG-2+/γc− mice; enabling the final steps of intrathymic development to occur in vivo. The availability of RAG-2+/γc− mice proffered the opportunity to exclude the need to seed DN2/3 cells into fetal thymic organ cultures (FTOC) and implant the FTOC into RAG-2−/− recipient mice for continued T-lineage development in order to avoid the potential involvement of residual NK cells impeding engraftment [241].

A titration of GPI+ or GPI− DN2/3 cells were injected into RAG-2+/γc− mice and the level of successful engraftment was assessed at day 40 (Figure 4-2). The spleens of recipient mice were analyzed for the presence of TCR+ cells as an indication that the injected pro-T
cells had reached the thymus, completed development into mature T cells, and had subsequently migrated into the peripheral organs. Regrettably, the efficacy of engraftment precluded using this approach to create sufficient numbers of GPI-AP deficient T cells. Specifically, even at the highest dose of injected CD25^+ DN2/3 cells (0.5x10^6), the level of engraftment was minimal with only 3.3% of isolated splenocytes expressing TCRαβ (Figure 4-2). Further, the addition of IL-7/anti-IL-7 complexes, shown to enhance engraftment [242, 243], had no effect on the efficacy of engraftment in our hands (data not shown).

Rather than continuing this approach and adopting the use of FTOC implantation as described above, we chose to capitalize on the availability of a Cre-loxP system to enable the creation of conditional knockout animal for the production of GPI-AP deficient primary T cells.
Figure 4-2: Insufficient engraftment of OP9/DL1-derived GPI primary T cells following injection into RAG2−/−γc−/− mice.

GPI+ and GPI- embryonic stem cells (ES) were co-cultured with OP9 stromal cells for 8 days, and then transferred onto OP9-DL1 expressing stromal cells for 8 days. On day 16, CD25+ cells, representing DN2 and DN3 subsets of immature pro-T cells, were isolated by FACS. 1x10^5 sorted GPI+ cells and 5x10^5 sorted GPI- cells were directly injected into RAG2−/−γc−/− recipients. Spleens were harvested after 40 days of reconstitution and proportion of CD4+ and CD8+ T cells were determined on a FACS Calibur. Successful engraftment was only seen in the mice with 5x10^5 sorted GPI- cells, as shown. Of all isolated splenocytes, only 3.3% were TCR+. 
Conditional GPI-AP deficient mouse model:

We capitalized on an existing conditional knockout of GPI-AP [71]. The genetics of this mouse strain and the breeding scheme employed to generate GPI-AP deficient T cells is illustrated in Figure 4-3. Mice bearing loxP sites flanking exon 6 of Piga, an X-linked gene essential for the initial steps in GPI anchor biosynthesis, were mated with mice transgenic for the Cre recombinase driven by the T cell-specific Lck proximal promoter. This lck-cre recombinase transgene is preferentially expressed in the thymus at the double negative stage [244, 245]; supporting a thymus-specific disruption of Piga, and thus a T cell-specific GPI-AP deficiency.

Flow cytometric analysis of peripheral blood lymphocytes and eventually splenocytes provided confirmation of conditional GPI-AP deficiency in T cells from these animals. As illustrated in Figure 4-4, targeting of the Piga deletion was specific to T cells, as B cells maintain the expression of GPI-AP. T cells from pups that were Cre and positive for loxP were GPI-AP positive and were used as littermate controls, and termed wild-type (WT) in all of the following experiments. Importantly, both the Piga\textsuperscript{lox} mice and the LckCre/Piga\textsuperscript{lox} mice generated in these crosses were phenotypically normal and contained comparable numbers of total splenocytes and lymph node cells, as well as comparable proportions of contained T cells of both lineages. Hence, in contrast to using the ESC/OP9-DL1 system, GPI-AP deficient T cells derived from these conditional knockout mice provided sufficient primary T cells to extend the analyses of the homeostatic role of GPI-AP.
Figure 4-3: Generation of a conditional Piga deficient mouse.
Mice expressing loxP sites (red triangles) flanking exon 6 of the Piga gene were crossed with mice transgenic for the Cre recombinase driven by the T cell-specific Lck proximal promoter (orange rectangle) to generate a thymus specific disruption of Piga. F1 progeny were intercrossed and the resulting F2 progeny were phenotypically normal, with the successful generation of primary T lymphocyte-specific GPI-AP deficiency. Hemizygosity in male mice is represented with parentheses.
Figure 4-4: Lineage specific disruption of the Piga gene.
Spleens were harvested from both WT mice and GPI mice. A. For the assessment of GPI-AP deficient T cells, spleens were processed and FACS analysis on total splenocytes was performed after staining for TCR and a representative GPI-AP, CD48. B. To confirm that the LckCre expression was restricting the Piga deletion exclusively in T cells, total splenocytes were stained for IgM and a representative GPI-AP, CD48.
The targeted *Piga* is an X-linked gene and T cell specific GPI-AP deficiency was observed in both male and female mice. The mosaicism of GPI\(^+\) and GPI\(^-\) T cells in both male and female mice is likely attributed to the variation of penetrance in the Cre-\textit{loxp} system. To maximize the purity of GPI CD4\(^+\) or CD8\(^+\) T cells from the splenocytes of these animals, anti-CD90 antibody was incorporated in the T cell purification process to remove residual GPI\(^+\) cells. T cells used in the experiments described below were isolated from both male and female donors. Purity of GPI\(^-\) T cells through the negative selection process is illustrated in Figure 4-5, where an \textit{LckCre/Piga}\textit{floox} mouse (grey histogram) expressed 13.9\% contaminating CD48\(^+\) TCR\(^+\) splenocytes, which were removed in the purified post-CD4\(^+\) negative selection sample. The effective removal of >98\% of GPI\(^+\) contaminating cells through the addition of biotinylated-anti-CD90 is shown with the absence of CD90\(^+\) and Sca1\(^+\) expression, as representative GPI-AP. Conversely, the \textit{Piga}\textit{floox} control mice (open histograms) were 100\% positive for the expression of the representative GPI-AP - CD48, CD90, and Sca1. Importantly, both the \textit{Piga}\textit{floox} control mice and the \textit{LckCre/Piga}\textit{floox} mouse express similar levels of TCR\(^+\), CD4\(^+\) and CD8\(^+\) expression, post-selection (Figure 4-5).
Figure 4-5: Selection of CD4\(^+\) and CD8\(^+\) T cells from \(Lck\text{Cre}/Piga^{\text{flox}}\) mice and \(Piga^{\text{flox}}\) mice. Flow cytometric analysis of TCR\(^+\) pre-selection and post-selection CD4\(^+\) and CD8\(^+\) T cells isolated from spleens of \(Lck\text{Cre}/Piga^{\text{flox}}\) mice. Grey histogram represents analyses from GPI-AP deficient \(Lck\text{Cre}/Piga^{\text{flox}}\) mice and open histograms represent analyses from WT GPI-AP expressing \(Piga^{\text{flox}}\) control mice.
**Hyper TCR/CD3 responsiveness and prolonged growth kinetics of GPI primary CD4+ T cells:**

We first assessed the growth/survival phenotype of GPI-AP deficient CD4+ and CD8+ splenic T cells. CD4+ GPI T cells from LckCre/Piga\textsuperscript{floox} mice and CD4+ GPI+ T cells from Piga\textsuperscript{floox} mice (WT) were stimulated using plate-bound anti-TCR\(\beta\) or anti-CD3\(\varepsilon\) and activation/clonal expansion was measured by \(^3\)H-TdR uptake, as per the clonal model.

As mentioned in the Chapter 3, and described in detail by the Julius laboratory [132] the activation of CD4+ T cells is typically predicated by co-aggregation of the CD4 coreceptor, which can be bypassed by direct stimulation through CD3 [237]. The mechanism underpinning the uncoupling of TCR and CD3 signalling is thought to involve the sequestration of the majority of cellular Lck by CD4 [132, 133, 246, 247]. This phenotype is exhibited by the WT GPI' CD4+ T cells as illustrated in Figures 4-6A and 4-6C. However, and in contrast to WT GPI' CD4+ T cells, GPI' CD4+ T cells are responsive to anti-TCR\(\beta\), as illustrated in Figure 4-6A. Hence, the role of CD4 in sequestering Lck is not sufficient to inhibit anti-TCR\(\beta\) signalling. Over a time course of 2-10 days, GPI-AP-deficient cells responded with 8-36-fold, 10-26-fold, and 6-38-fold higher \(^3\)H-TdR uptake than their GPI-AP sufficient counterparts at 1, 3, and 9 \(\mu\)g/ml anti-TCR\(\beta\), respectively.

These results are extended to stimulation with anti-CD3\(\varepsilon\). While the differential activation of WT and the GPI CD4+ T cells are not as pronounced with anti-CD3\(\varepsilon\) as observed with anti-TCR\(\beta\) stimulation, the GPI prolonged and enhanced growth kinetics
Figure 4-6: Enhanced anti-TCR/CD3 responsiveness and prolonged growth kinetics of GPI primary CD4+ T cells.
A. WT and GPI CD4+ T cells were isolated from total splenocytes and cultured in wells pre-coated overnight at 4°C with anti-TCRβ at 1, 3, and 9 μg/ml. At each indicated time point, triplicate cultures were pulsed with 1 μCi ³H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed. B. WT and GPI CD8+ T cells were isolated from total splenocytes, stimulated with plate-bound anti-TCRβ, and harvested as described in A. C. & D. WT and GPI CD4+ (C.) or CD8+ (D.) T cells were isolated from total splenocytes and stimulated with 1, 3, or 9 μg/ml of plate-bound anti-CD3ε at each of the indicated time points. Thymidine uptake was assessed as described in A.
is recapitulated (Figures 4-6A and 4-6C). Throughout the time points and concentrations of anti-CD3ε stimulation, a 2-8-fold greater response of the GPI- CD4+ T cells is observed. Thus, in response to either anti-TCRβ or anti-CD3ε mediated activation, GPI primary CD4+ T cells recapitulate the prolonged growth/survival advantage observed in the GPI clones.

Interestingly, this phenotype was exclusive to the primary CD4+ T cells, as GPI-AP deficiency on CD8+ T cells did not confer growth/survival compared to WT CD8+ T cells. Also of note, and expected [248], unlike WT CD4+ T cells, WT CD8+ T cells responded robustly to anti-TCRβ. Further, as illustrated in Figure 4-6B, the kinetics of induction and maintenance of 3H-TdR uptake was comparable in the WT and GPI CD8+ T cells over the doses of anti-TCRβ stimulation tested. Both WT and GPI variants were ever more responsive to increasing concentrations of plate-bound anti-TCRβ. Similarly, when the activation of WT and GPI CD8+ T cells was induced via plate-bound anti-CD3ε, there was no apparent growth advantage related to GPI-AP deficiency (Figure 4-6D). The kinetics of stimulation with the lowest concentration of anti-CD3ε, 1 µg/ml, did show a marginally greater amount of 3H-TdR uptake in the GPI compared to the WT CD8+ T cells. However, at both 3 µg/ml and 9 µg/ml of plate-bound anti-CD3ε-induced activation, it was the WT CD8+ T cells that responded with higher levels of 3H-TdR uptake (Figure 4-6D). Elucidation of mechanism(s) underpinning the differential effect of GPI-AP expression on TCR/CD3 signalling in the two T cell lineages remain to be elucidated; and while their characterization may contribute to our understanding of the
role GPI-AP in T cell homeostasis, further assessment of TCR signalling in CD8$^+$ GPI$^+/-$ T cells is beyond the scope of this thesis.

Also unique to the primary GPI CD4$^+$ T cells is their hypersensitive response to both anti-TCR$\beta$ and anti-CD3$\varepsilon$. First the differential responsiveness of GPI$^+$ and GPI$^-$ CD4$^+$ T cells to anti-TCR$\beta$ mediated activation was assessed at the single cell level. This was demonstrated by pre-labelling GPI$^+$ and GPI$^-$ CD4$^+$ T cells with carboxyfluorescein succinimidy ester (CFSE) ahead of in vitro stimulation with 1 $\mu$g/ml of plate-bound anti-TCR$\beta$. As illustrated in Figure 4-7, GPI$^+$ CD4$^+$ T cells responded more robustly than their GPI$^+$ counterparts over the 8 day time course assayed. At each time point, cells were harvested and the dilution of CFSE was determined flow cytometrically as an assessment of cell division. While an undiluted level of CFSE stayed constant within the vast majority of WT CD4$^+$ T cells over the first 96 hours of stimulation, progressive halving of CFSE levels within GPI$^+$ CD4$^+$ daughter cells was evident within the first 48 hours with >80% of cells having divided at least once by 96 hours. These results demonstrate the differential efficacy of responsiveness of GPI CD4$^+$ T cells compared to the WT CD4$^+$ T cells to anti-TCR$\beta$, and are convergent with their enhanced $^3$H-TdR uptake observed in the same circumstances (Figures 4-6A and 4-6C). Further, they suggest that the TCR/CD3 complex on GPI CD4$^+$ T cells is in a hypersensitive state of activation.

The differential sensitivity of GPI$^+$ and GPI$^+$ CD4$^+$ T cells to activation was further assessed by measuring $^3$H-TdR uptake induced by a broad range of concentrations
Figure 4-7: Hypersensitive anti-TCR responsiveness of GPI CD4+ T cells.
WT and GPI CD4+ T cells were pre-labelled with CFSE and cultured onto wells pre-coated with 1 µg/ml of plate-bound anti-TCRβ. Cells were harvested and cell division was tracked flow cytometrically based on CFSE dilution at each of the indicated time points. Dead cells were excluded based on forward/side scatter analysis and inclusion of PI.
of plate-bound stimulating anti-TCRβ or anti-CD3ε mAb. As illustrated in Figure 4-8, 5x and 6x less anti-TCRβ or anti-CD3ε, respectively, was required to achieve 1000 cpm in the GPI CD4+ T cells than in the GPI+ CD4+ control population. Taken together, these results support the conclusion that the expression of GPI-AP impedes TCR/CD3-mediated activation of DNA synthesis.
Figure 4-8: Hypersensitivity of GPI-CD4+ T cells to anti-TCR Cβ and anti-CD3ε.
A. & B. WT and CD4+ T cells (4x10⁴) were cultured in wells pre-coated overnight at 4°C with a titration of anti-TCR Cβ or anti-CD3ε in A. and B., respectively. Forty-eight hours later, triplicate cultures were pulsed with 1 µCi ³H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed.
Enhanced kinetics and amplitude of TCR/CD3 induced responses of primary GPI CD4+ T cells correlates with supra-physiological production of IL-2:

As the prolonged growth/survival advantage of GPI-AP deficient cells was found to correlate with enhanced IL-2 production in the clonal variants, we sought to assess whether the phenotype would be recapitulated in the primary T cell model. Towards this end, the supernatants of anti-TCRβ and anti-CD3ε stimulated cultures of WT and GPI-AP deficient CD4+ T cells were harvested over time and contained IL-2 was assessed by ELISA. As illustrated in Figure 4-9A, supernatants of GPI-AP deficient T cells stimulated with anti-TCRβ contained 100-fold more IL-2 over the time course assessed in comparison to their GPI+ counterparts. Similarly, stimulation with anti-CD3ε resulted in roughly a 50-fold differential in IL-2 detected in supernatants of GPI-AP deficient T cells (Figure 4-9B). Thus, as for results observed in the clonal T cell system, these results are consistent with enhanced TCR/CD3 induction of IL-2 in the absence of GPI-AP.

To support this conclusion we assessed the kinetics and the fold-induction of IL-2 mRNA. WT and GPI-AP deficient CD4+ T cells were stimulated with plate-bound anti-CD3ε and harvested at each of the indicated time points. Following mRNA isolation and cDNA preparation, we assessed IL-2 mRNA content using ddPCR analysis. In addition to the daily time points at which amounts of IL-2 were measured in culture supernatants, early time points of 2 hours, 6 hours and 1 day post-stimulation were included for assessment of the kinetics of IL-2 mRNA. Baseline levels of IL-2 mRNA were established using non-stimulated ex-vivo cells. This baseline level of IL-2 mRNA was set to “1” in each of
Figure 4-9: Enhanced IL-2 production in primary GPI CD4+ T cells.
A. WT and GPI CD4+ T cells were cultured in wells pre-coated with either 3 µg/ml or 9 µg/ml of anti-TCR Cβ for the indicated periods of time. Supernatant was harvested and IL-2 production was measured using a mouse IL-2 specific ELISA. B. WT and GPI specific ELISA. B. WT and GPI CD4+ T cells were cultured as in A. but on plates pre-coated with 3 µg/ml of anti-CD3ε. Culture supernatants were harvested and IL-2 content was assessed by ELISA. C. The IL-2 concentrations quantified in B. are represented as ratios comparing differential IL-2 in the GPI cultures to WT cultures.
the WT and GPI samples, and fold-induction was calculated at each of the indicated time points, post stimulation. As observed with the clonal variants, induction of IL-2 mRNA was significantly enhanced in the GPI-AP deficient primary CD4+ T cells (Figure 4-10A). The results demonstrate that TCR/CD3-mediated enhanced IL-2 mRNA induction occurs at the earliest time points assessed and persists over the extended time course of the assay. The enhancement in GPI CD4+ T cells ranged from 3- to 5.5-fold compared to levels observed in GPI+CD4+ control T cells. Further, the results demonstrate that the kinetics of induction in GPI CD4+ and GPI+CD4+ T cells are comparable; with the exception of the 6 hour time point, the time course of early induction and second wave of induction presumably mediated by IL-2 produced [249, 250] are comparable.

IL-2 detected in culture supernatants in the above kinetics experiment was assessed in parallel. As illustrated in Figure 4-10B, 3-fold, 3.6-fold, and 8.5-fold more IL-2 was detected in supernatants of anti-CD3ε stimulated GPI CD4+ T cells at 2 hours, 6 hours and 1 day, respectively, in comparison to their WT controls. The higher levels of IL-2 detected in supernatants of GPI CD4+ T cells increased over the time course assayed. In sum, these results validate that the GPI growth/survival advantage can be attributed to their enhanced and prolonged levels of de novo IL-2 production.

To formally prove that the enhanced levels of de novo mRNA induction observed in GPI CD4+ T cells was a result of differential TCR/CD3 signalling, we directly compared the induction of IL-2 specific mRNA in response to anti-CD3ε and phorbol myristate acetate (PMA) in combination with the calcium ionophore Ionomycin (Iono).
Figure 4-10: Confirmation of enhanced IL-2 message and protein production imparted by GPI-AP deficiency.

A. WT and GPI CD4+ T cells were stimulated with plate-bound anti-CD3ε and harvested at each of the indicated time points. mIL-2 message was detected by ddPCR analysis. Fold induction at each time point was calculated relative to the amount of IL-2 message detected in non-stimulated cultures. B. Supernatant of stimulated cultures in A. was harvested and IL-2 protein was measured by mouse IL-2 ELISA.
The latter stimulation should bypass any differential TCR/CD3 signalling in GPI⁺ and GPI⁻ T cells as PMA/Iono will directly activate Protein Kinase C (PKC) [251-253]. The addition of ionomycin functions in enhancing the activation of calcium dependent PKC, as well as trigger the activation of NFAT via the mobilization of intracellular calcium [253-256]. As illustrated in Figure 4-11, the enhanced anti-CD3ε-mediated induction of IL-2 specific mRNA observed in GPI⁻CD4⁺ T cells is not observed subsequent to PMA/Iono stimulation of these cells. While the level of IL-2 specific mRNA induction mediated by PMA/Iono was roughly 10-fold higher in both GPI⁺ and GPI⁻ T cells, the key observation is that the differences in fold-induction between these two cell types was virtually obviated when stimulation bypassed the TCR/CD3 complex (Figure 4-11). The normalization of levels of IL-2 specific mRNA induced by PMA/Iono support the conclusion that GPI-AP are in some fashion regulating TCR/CD3 signalling, IL-2 production, or both.
Stimulation with PMA/ionomycin minimizes the differences in induced IL-2 message within GPI-AP sufficient and deficient CD4+ T cells.

WT and GPI-AP CD4+ T cells were cultured on either plate-bound anti-CD3ε, or with the addition of PMA and ionomycin. At 2, 6, and 24 hours of stimulation, cells were harvested and IL-2 message was assessed by ddPCR analysis. Data represent the fold induction relative to the amount of IL-2 detected in non-stimulated cells. Values above each set of data points represent fold differences between the WT and GPI-AP CD4+ T cells.
Soluble anti-CD28 does not normalize IL-2 production in WT and GPI CD4+ T cell responses to anti-TCRβ:

The involvement of the co-stimulatory immunoglobulin supergene family molecule, CD28, in optimal T cell activation has been well established [257, 258]. Engagement of the T cell specific CD28 molecule with members of the B7 family expressed on professional APCs provides the costimulatory signals that amplify proliferation and survival of the activated T cell. In an in vitro system of T cell activation, cross-linking of the TCR/CD3 complex with the addition of anti-CD28 increases IL-2 transcription and stabilizes resulting mRNA [210, 259]. In addition to promoting increased cytokine expression, CD3/28 costimulation amplifies glucose metabolism, provides a resistance to apoptosis, and ultimately allows for the expansion of activated T cells [210, 260-262].

We sought to assess whether the inclusion of CD28/CD3 co-stimulation would normalize the striking differential responses observed upon stimulation of GPI CD4+ and GPI+ CD4+ T cells with anti-TCRβ. Toward this end, WT and GPI CD4+ T cells were stimulated with 9 µg/ml of plate-bound anti-TCRβ, with or without the addition of 3 µg/ml soluble anti-CD28. After 4 days of stimulation, the cultures were pulsed with ³H-TdR and uptake was read as a measure of DNA synthesis and cell proliferation. As reported above, there was a highly significant difference in ³H-TdR uptake between WT and GPI cells stimulated with anti-TCRβ alone; and the addition of anti-CD28 failed to normalize these differences (Figure 4-12A). Of note, the provision of a positive control
Figure 4-12: The addition of soluble anti-CD28 is unable to normalize the differential thymidine uptake and IL-2 production observed in primary WT and GPI CD4+ T cells.

A. Triplicate cultures of 4x10^4 WT and GPI CD4^+ T cells were cultured on 9 µg/ml plate-bound anti-TCRβ alone, or with the addition of 3 µg/ml anti-CD28. At day 4, cultures were pulsed with 1 µCi ^3H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed. B. Supernatant of stimulated cultures in A. was harvested and IL-2 protein was measured by mouse IL-2 ELISA. ns: not significant
for the addition of anti-CD28, is that its co-stimulation with anti-TCRβ significantly increased levels of \(^{3}\text{H}-\text{TdR}\) uptake in CD4\(^+\) GPI\(^+\) T cells (Figure 4-12A).

We also assessed the amount of IL-2 present in culture supernatants in the above experiment. As illustrated in Figure 4-12B, provision of anti-CD28 significantly increased levels of IL-2 detected in supernatants of both GPI-AP sufficient and deficient cells; consistent with its role in enhancing IL-2 transcription and stabilizing resulting mRNA. In sum, these results demonstrate that neither of the aforementioned functions of CD28 are dependent on GPI-AP expression, nor is CD28 directly involved in the mechanism(s) supporting the enhanced and prolonged IL-2 production observed in GPI CD4\(^+\) T cells.
Involvement of the IL-2 Receptor:

As described, the interaction of IL-2 with its heterotrimeric receptor, IL-2R, promotes both clonal expansion and the development of effector function [182-184, 206]. Of the three chains that comprise the high affinity IL2-R, it is CD25 (IL-2Rα) which functions to increase the IL-2R affinity for IL-2 by 10-100-fold, while the CD122 (IL-2Rβ) and CD132 (IL-2Rγ) mediate downstream signal transduction [263-265]. As we have characterized enhanced and prolonged IL-2 production as a key biochemical basis for the growth/survival advantage of GPI CD4+ T cells, we sought to explore the kinetics of IL-2R chain expression.

While the expression of all three chains of the IL-2R is upregulated upon TCR/CD3 engagement, the fold-upregulation of CD25 is the most profound [222, 266]. Ahead of assessing the kinetics of IL-2R chain expression post stimulation, basal levels of CD25, CD122, and CD132 were determined. While no differences in the basal levels CD122 and CD132 were observed in non-stimulated WT and GPI CD4+ T cells (Figure 4-13C), it is of note that a significant difference in the proportion of CD25+ cells was observed (Figure 4-13A&B). Over an average of 4 experiments, 3.3% and 7.4% of ex vivo WT CD4+ and GPI CD4+ T cells T cells were CD25+, respectively. This increase may reflect overall higher levels and prolonged IL-2 production in the system driving a skewed differentiation of T_{Reg} cells in the GPI-AP deficient mice. Of note in this regard is that the frequency of CD4+CD25+FoxP3+ T cells in the spleens of GPI animals is nearly 1.5x that observed in WT controls (Figure 4-14)
Figure 4-13: Differential CD25 expression on non-stimulated ex vivo CD4⁺ T cells.
A. The proportion of CD25 positive cells in total purified, non-stimulated, viable CD4⁺ T cells from the spleens of WT and GPI⁻ mice determined flow cytometrically. Figure depicts one representative experiment. B. The percentage of CD25 positive cells from total non-stimulated CD4⁺ T cells from the spleens of WT and GPI⁻ mice, averaged over 4 experiments. *** Significance represents P ≤ 0.001. C. The basal level expression of CD122 and CD132 in non-stimulated purified CD4⁺ T cells from WT (red line) and GPI⁻ mice (blue line).
Figure 4-14: The quantification of CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells in spleen from WT and GPI⁻ mice.

CD4⁺ T cells were purified from spleens of WT and GPI⁻ mice and the proportion of CD4⁺CD25⁺FoxP3⁺ T_{Reg} cells was determined flow cytometrically. The gated value represents the percentage of FoxP3⁺ cells in the population of gated CD25⁺ T cells, post CD4⁺ T selection. One representative experiment is depicted above.
Having established basal levels of expression of each IL-2R chain, we assessed the kinetics of expression after stimulation with plate-bound anti-CD3ε (Figure 4–15). The IL-2Rγ chain, CD132, was the most highly expressed of the three chains at 2 hours of stimulation. Its expression waned by 24 hours, and there were no significant differences in the levels of expression detected between GPI⁺ and GPI⁻ CD4⁺ T cells. No differences in the expression profile of the IL-2Rβ chain, CD122, were noted at any point during the kinetics. In contrast, from the 6 hour time point post stimulation and onward, there was a marked upregulation of the IL-2Rα chain, CD25, which was significantly greater in GPI⁻CD4⁺ T cells (Figure 4-15). In sum, these results support the following sequelae. CD25 expression is known to be the limiting chain in establishing the high affinity hetero-trimeric IL-2R [225, 267]; further, IL-2-IL-2R interaction is known to increase IL-2 production [268]; and the ensuing IL-2R signalling cascade upregulates the expression of CD25 by promoting the binding of signal transducer and activator of transcription 5 (STAT5) to the Il2ra gene locus [176, 198]. Thus, the enhanced production of IL-2 imparted by GPI-AP deficiency may potentiate this increased expression of CD25 and further drive the IL-2R positive signalling loop.
Figure 4.15: Upregulated expression of IL-2 receptor chains on anti-CD3ε stimulated WT and GPI CD4⁺ T cells.

CD4⁺ T cells were stimulated on 3 µg/ml of plate-bound anti-CD3ε. Cells were harvested at each of the indicated time points and expression levels of CD122, CD132, and CD25, comprising the chains of the IL-2R were measured flow cytometrically. Mean Fluorescence Intensities represent averages over 2-4 experiments.

* Significance represents P ≤ 0.05.
Differential resistance of GPI⁺ and GPI primary CD4⁺ T cells to PD-L1-Fc inhibition of DNA synthesis:

As for the clonal system described in Chapter 3, the results presented support the conclusion that the mechanism underpinning the enhanced growth/survival phenotype of CD4⁺ GPI⁻ primary T cells is due, at least in part, to their enhanced capacity to produce and maintain production of IL-2. We next assessed whether, as for the GPI⁻ clonal T cells, this would impart differential susceptibility to PD-1 mediated attenuation of TCR/CD3 signalling.

We first assessed the expression of both PD-1 and its ligand, PD-L1, on both WT and GPI⁻ CD4⁺ primary T cells. As illustrated in Figure 4-16, in contrast to easily detectable basal levels of PD-1 expressed on clonal variants, the frequency of PD-1⁺ cells in unstimulated primary T cell populations is low, revealing only 4% of the population. In contrast, basal levels of PD-L1 expression appear constitutive and are expressed at comparable levels on ex vivo unstimulated WT and GPI⁻ CD4⁺ primary T cells (Figure 4-16). Stimulation of these cells using plate-bound anti-CD3ε resulted in comparably upregulated expression of both PD-1 and PD-L1, independent of GPI-AP expression (Figure 4-16).

To determine differential functionality, cells were cultured on flat-bottom plates co-coated with anti-CD3ε and a titration of PD-L1-Fc, and DNA synthesis was assessed by measuring ³H-TdR uptake. Results are plotted as ‘percent control’; a comparison of
**Figure 4-16:** The upregulated expression level of PD-1 and PD-L1 on anti-CD3ε stimulated WT and GPI CD4+ T cells.
CD4+ T cells were isolated from spleens of WT and GPI mice and expression of PD-1 and PD-L1 was assessed directly *ex vivo*. Cells were also stimulated on 3 µg/ml of plate-bound anti-CD3ε for 1-2 days, harvested, and the upregulated expression of PD-1 and PD-L1 was assessed flow cytometrically. Red line represents isotype control and blue line represents either PD-1 or PD-L1 staining. Data represents one experiment of two.
the PD-L1-Fc/anti-CD3ε co-stimulated cultures to the control stimulation of anti-CD3ε in the presence of an isotype control for PD-L1-Fc. As was demonstrated for GPI+ clonal T cell variants, PD-L1-Fc was found to suppress ³H-TdR uptake, in a dose dependent fashion, only in GPI+ primary T cells (Figure 4-17). Nine µg/ml of PD-L1-Fc inhibited 85% of ³H-TdR uptake in WT CD4+ primary T cells at day 2 and 98% at day 4, while the isotype control for PD-L1-Fc failed to mediate significant inhibition. In striking contrast, and exactly comparable to results obtained with the GPI clonal T variants, GPI-CD4+ primary T cells are insensitive to inhibition with PD-L1-Fc. Indeed, as illustrated in Figure 4-17, the GPI response to PD-L1-Fc is 143% and 303% of control responses at days 2 and 4, respectively. Thus, the GPI resistance to PD-L1-Fc mediated attenuation seen in the clonal model is mirrored in the GPI CD4+ primary T cells.

As described in the context of the clonal T cell variants, the differential capacity of PD-L1-Fc to attenuate TCR mediated ³H-TdR uptake was inversely proportional to the amount of IL-2 in supernatants of stimulated cells. We set out to establish whether precisely the same mechanism underpins the resistance of CD4+ GPI primary T cells. Firstly, the relative amounts of IL-2 detectable in supernatants of cultures of GPI+ and GPI primary CD4+ T cells stimulated with anti-CD3ε in the presence of PD-L1-Fc or its isotype control were assessed at days 2 and 4 (Figure 4-18A). Control culture supernatants of GPI T cells contained 19-fold and 16-fold more IL-2 than did supernatants of GPI+ T cells at days 2 and 4, respectively. PD-L1-Fc mediated a 10-fold reduction in the amount of detectable IL-2 at day 2 and a 10-100-fold reduction at day 4, independent of GPI-AP expression. However, and critically, while the IL-2 was
Figure 4-17: A GPI resistance to PD-L1-Fc-mediated attenuation of proliferative responses.
Triplicate cultures of 4x10⁴ WT and GPI CD4⁺ T cells were stimulated on 3 µg/ml of plate-bound anti-CD3ε alone, or with a titration of PD-L1-Fc or its isotype control (Iso). At day 2 and day 4, cultures were pulsed with 1 µCi ³H-TdR, harvested 6 hours later on Unifilter plates and thymidine uptake was assessed. Thymidine uptake counts per million with anti-CD3ε alone was considered the control (100%) and % Control was determined by comparing the counts per million of the PD-L1-Fc and isotype control stimulated cultures to that of the anti-CD3ε alone control. Data represents one experiment of three.
Figure 4-18: Differential sensitivity to PD-L1 is directly related to levels of IL-2 production.

A. Supernatant was collected from cultures in Figure 4-17 at days 2 and 4, and IL-2 content was assessed by mIL-2 specific ELISA. B. CD4+ GPI- T cells were cultured at 4x10^4 cells/well on 96 well plates coated with 3 µg/ml of anti-CD3ε alone, or with 9 µg/ml of PD-L1-Fc or its isotype control (Iso Ctl). Levels of IL-2 in the supernatant were neutralized using an anti-IL-2 mAb, S4B6, such that levels present in the GPI- stimulated cultures were made comparable to levels in their WT counterparts. The GPI sensitivity to PD-L1-Fc (grey bars) or its isotype control (white bars) was assessed in terms of % Control, as described in Figure 4-17. The experiment was repeated with the isotype control for S4B6 in the place of S4B6. A. and B. were both repeated three times.
substantially reduced in supernatants of the GPI T cells, the absolute amounts of IL-2 remained 18-fold and 1861-fold higher at days 2 and 4, respectively, than detected in supernatants of GPI+ T cells (Figure 4-18A). As such, these results predict that a similar mechanism might underpin the resistance to PD-1 mediated attenuation of TCR/CD3 signalling in GPI CD4+ primary T cells as that characterized in GPI T cell clonal variants.

This was formally addressed and demonstrated by the identical approach used for GPI clonal variants. Specifically, the prediction that neutralization of IL-2 in supernatants of GPI CD4+ primary T cells to levels detected in supernatants of GPI+ CD4+ T cells would render them susceptible to PD-L1-Fc mediated attenuation of 3H-TdR uptake was tested. As for the clonal system, the anti-IL-2 monoclonal antibody, S4B6, was utilized to neutralize the IL-2 in the supernatant of GPI cultures to levels observed in GPI+ cultures. The capacity of this neutralizing concentration of S4B6 on the sensitivity of GPI CD4+ T cells to either 9 µg/ml of PD-L1-Fc or its isotype control was then assessed. As illustrated in Figure 4-18B, S4B6 neutralization of IL-2 in culture supernatants of GPI CD4+ T cells rendered them as susceptible to PD-L1-Fc-mediated, attenuation of clonal expansion, as assessed by 3H-TdR uptake, as that observed for GPI+ CD4+ T cells. Specifically, these cells responded at 62% and 14% of control responses at day 2 and 4, respectively. As illustrated in Figure 4-18B, this attenuation was predicated by the presence of both S4B6 and PD-L1-Fc as no inhibition was observed in the presence of either of their respective isotype controls. These results support the conclusion that enhanced and prolonged production of IL-2 is a concordant mechanism
supporting sustained proliferation and survival in the presence of PD-1 engagement in GPI T cell clonal variants and GPI CD4+ primary T cells.

The role of Fyn in hypersensitive TCR/CD3 signalling and enhanced IL-2 production in GPI CD4+ primary T cells:

Distinct and interdependent roles for the Src-family protein tyrosine kinases, Lck and Fyn, have been characterized as central components to the most proximal signals emanating from the TCR/CD3 complex [125, 269]. Specifically, Lck mediated Fyn activation predicates TCR/CD3 induced IL-2 production, and ultimately supports T cell expansion in vivo [141]. Briefly, Lck and Fyn reside in distinct subcellular locations in primary resting CD4+ T cells, with >90% of CD4-associated Lck residing outside of LR, while >95% of Fyn is LR-resident [126, 133]. Upon TCR/CD4 engagement, Lck is activated and functions as a mobile signalling element, translocating into LR, where it physically associates with and is critical for Fyn activation [124-126, 141].

As Fyn is constitutively associated with LR, the distinct composition of LR in GPI CD4+ T cells may directly or indirectly affect either or both of the cellular localization or physiology of Fyn, and as such contribute to the hypersensitive TCR/CD3 signalling and enhanced IL-2 production observed. This was investigated by assessing the spatial localization of Lck and Fyn in GPI+ and GPI CD4+ T cells. LR and soluble fractions were prepared from nonstimulated GPI+ CD4+ and GPI CD4+ primary T cells by equilibrium density centrifugation. LR and soluble fractions were harvested from these gradients and
the distribution of contained GM1 ganglioside, Lck, and Fyn were assessed by immunoblotting. The first question was whether the absence of GPI-AP affected the existence and quality of LR. As illustrated in Figure 4-19A, the purity of LR derived from both cell types was comparable. Specifically, cholera toxin binds to the GM1-ganglioside that localizes exclusively to LR. As is evident in Figure 4-19A, the distribution of GM1 is unaltered in the absence of GPI-AP. The same is true for Fyn distribution. Specifically, the absence of GPI-AP did not significantly alter the proportion of Fyn localizing to LR (Figure 4-19A). In contrast, significantly more Lck localizes to LR in resting GPI-CD4+ primary T cells (Figure 4-19A). The mean proportions of Fyn and Lck localizing to LR and soluble fractions of 4 independent experiments were quantified densitometrically (Figure 4-19B).

As discussed above, TCR/CD3/CD4 engagement induces the translocation of a portion of cellular Lck into LR [126]. The increased proportion of LR-resident Lck in GPI-CD4+ primary T cells may reflect increased tonic TCR/CD3 signalling in vivo [270, 271] and may contribute to the observed hypersensitive TCR/CD3 signalling phenotype and enhanced IL-2 production. If this were the case, the prediction follows that the physiology of Fyn should be altered.

To investigate this hypothesis, we assessed the basal kinase activity of Fyn in GPI- and GPI+ CD4+ primary, unstimulated T cells as well as the kinetics of TCR/CD4 mediated activation of Fyn in both cell types. Fyn activation was assessed using immune
Figure 4-19: A greater localization of Lck in LR fractions of GPI-AP deficient cells.  
A. A representative Western blot of the distribution of Lck, Fyn, and GM1 in the lipid raft and soluble sucrose-gradient fractions of non-stimulated WT and GPI-AP CD4+ T cells.  
B. Densitometric analysis of Lck and Fyn localization in lipid raft and soluble fractions averaged from 4 experiments.
complex kinase assays; and as 90% of cellular Fyn is LR-resident in both cell types, equilibrium density centrifugation was not done, rather Fyn was immunoprecipitated from total cell lysates. Specifically, CD4+ T cells were isolated from GPI+ and GPI- mice and co-coated with biotinylated anti-TCR (B57) and biotinylated anti-CD4 (BGK1.5). Coaggregation of TCR and CD4 was induced with streptavidin (Av). Fyn was immunoprecipitated from lysates and immune complex kinase assays using enolase as the exogenous substrate were conducted. Levels of phosho-enolase (pY Enolase) were quantified and normalized for levels of contained Fyn to determine the specific relative kinase activity of Fyn at each time point. Thus, specific kinase activity was expressed as a ratio between pY Enolase and total Fyn.

As illustrated in Figure 4-20A, the induction of Fyn kinase activity in GPI+ CD4+ T cells proceeded over time with TCR-CD4 co-aggregation, peaking at 270 seconds with a 5.9x increase in pY Enolase and waning at 810 seconds, consistent with previously described kinetics [126]. In striking contrast, Fyn activation barely proceeded over background in GPI- CD4+ T cells, throughout the time course assayed, with a 1.7x increase in pY enolase at 810 seconds (Figure 4-20A). The basis of this difference is the basal kinase activity of Fyn in GPI+ T cells. While Fyn distribution was unaffected in the absence of GPI-AP (Figure 4-19), the average basal kinase activity of Fyn in GPI+ CD4+ T cells was 3.7-fold higher than that observed in GPI+ CD4+ T cells (Figure 4-20B). The mechanism(s) underpinning the inability of TCR-CD4 co-aggregation on GPI+ CD4+ T cells to increase Fyn kinase activity remains to be elucidated. However, at this juncture, we can conclude that the marked difference in the basal levels of Fyn kinase activity in
GPI CD4+ primary T cells is consistent with the increased proportion of LR-resident Lck, and in turn the hypersensitivity of TCR/CD3 signalling and enhanced induction of IL-2 mRNA and protein. Importantly, while the basal Fyn activity is enhanced in GPI CD4+ primary T cells, IL-2 production is not induced in the absence of TCR/CD3 activation. Therefore, in addition to the increased basal activity of Fyn, other mechanisms must be contributing to the regulation of IL-2 induction.
Figure 4-20: Enhanced basal level Fyn kinase activity in GPI-AP deficient CD4+ T cells.

A. WT and GPI CD4+ T cells were co-coated with biotinylated anti-TCRβ (H57) and biotinylated CD4 (GK1.5). Activation was induced at each of the indicated time points by streptavidin (Av)-mediated aggregation. Fyn was immunoprecipitated and the kinase activity of Fyn was assessed by in vitro immune complex kinase assays, performed using enolase as an exogenous substrate. Once the enolase signal was determined by densitometric analysis, the membrane was blotted for Fyn. Fyn kinase activity was determined by normalizing the enolase signal to the quantification of total Fyn content.

B. Densitometric analysis of normalized Fyn kinase activity in non-stimulated WT and GPI CD4+ T cell Fyn immunoprecipitates averaged over 4 experiments.
CHAPTER 5: MECHANISTIC INSIGHT THROUGH THE
GENERATION OF GPI-AP\(^{-}\) GPI\(^{+}\) T CELLS:

RESULTS:

Chapter Hypothesis: Mutations in the initial transferase complex may result in
dysregulated PI metabolism in GPI anchor deficient cells, and may implicate the anchor
rather than a particular protein moiety in the GPI clonal and primary growth/survival
phenotypes.

All comparative analyses characterizing differences in T cell physiology between
GPI-AP\(^{-}\) and GPI-AP\(^{+}\) cells have utilized GPI T cells devoid of not only GPI-AP, but
also the GPI anchor (GPI). The GPI-AP deficiencies in the clonal system and the primary
T cell system are mediated by either a natural mutation in Pigp expression in the clonal T
cell model system, or the deliberate conditional deletion of Piga in the primary T cell
model system. Both Pigp and Piga encode transferases that are two of a six member
transferase complex, which in aggregate predicate the synthesis of the first intermediate
in the biosynthetic pathway of a GPI anchor [7]. At this juncture, we cannot conclude
whether one or more GPI-AP, or the GPI anchor itself contribute to the mechanism(s)
regulating the enhanced TCR/CD3 induced and prolonged IL-2 production observed in
GPI-AP deficient cells in either clonal or primary T cell model systems.
To address this question, we sought to create T cells lacking the expression of GPI-linked protein moieties, while maintaining the expression of the GPI anchor at the plasma membrane. The strategy involved targeting the 5 member GPI-transamidase complex, as illustrated in Chapter 1 Figure 2, which recognizes and removes the GPI-attachment signal peptide from a protein, enabling the transfer of a mature anchor onto the protein and the formation of a GPI-AP [7]. The catalytic subunit of the transamidase complex, PIG-K, assists in the linkage by creating transient intermediates with the precursor protein GPI-attachment signal peptide. Through the utilization of genomic engineering to selectively target and disrupt the Pigk gene, its absence should prevent the GPI transamidase complex from transferring fully assembled GPI units onto proteins. As has been described in an increasing number of systems, specific genome editing can be achieved utilizing the RNA-guided microbial clustered regularly interspaced short palindromic repeat (CRISPR) Cas9 nuclease system [272-274]. The CRISPR-Cas9 system recognizes a 20 nucleotide targeting sequence within its guide RNA (gRNA) which pairs with its DNA target, immediately 3’ of a requisite 5’ protospacer adjacent motif (PAM), most commonly NGG [273-275]. This recognition mediates the creation of double stranded breaks and can promote efficient editing of the target genomic locus by non-homologous end joining [274, 276]. We therefore employed the CRISPR/Cas9 system to introduce a frameshift and/or an early stop codon via short indel mutations at the Pigk locus in GPI-AP⁺ clone 2.10. This approach should yield GPI-AP⁺, GPI⁺ T cells.
In addition to targeting \textit{Pigk}, we chose to target two members of the initial transferase complex (\textit{Piga} and \textit{Pigp}) as controls for the use of CRISPR-Cas9 technology. Specifically, to ensure that deletion of either \textit{Piga} or \textit{Pigp} yielded clonal T cell variants, devoid of both GPI-AP and GPI, which recapitulated the enhanced and prolonged IL-2 production observed in the primary and clonal T cell models, respectively. The online MIT CRISPR Design software (crispr.mit.edu) was used to design the gRNA sequences with the greatest score for locus specificity, while minimizing off-target specificity (Table 5-1).

GPI$^+$ 2.10 clonal variants were electroporated with a GFP-expressing vector containing the required gRNA sequences for CRISPR-mediated targeted disruption of the \textit{Piga}, \textit{Pigp}, and \textit{Pigk} genes. Following the positive selection and clonal expansion of GFP$^+$ cells at 24 hours to ensure expression of the vector, GPI-AP deficient cells were isolated by FACS and cloned by FACS-mediated single-cell deposition into 96 well plates. Upon clonal expansion, a portion of cells from wells containing expanding clones was harvested and the specific loci assessed for successful editing using a dual probe method of ddPCR analysis (Table 2-1, Figure 5-1). This ddPCR assessment of the locus enabled determination of zygosity within each edited clone, for each of the loci targeted (Figure 5-1). As illustrated in Table 5-2, in total, 494 clones were screened post expansion from the single cell deposition. Of those treated with \textit{Pigk} gRNA, 142 clones were generated, 7 of which were successfully edited, with 1 heterozygous and 6 homozygous mutants detected. \textit{Pigp} targeted CRISPR editing generated 45 clones, with only 16 containing mutations, all of which were heterozygous. \textit{Piga} targeted CRISPR
editing generated 307 clones of which 177 contained an edited \textit{Piga} locus; 139 of these clones were heterozygous and 38 were homozygous mutants. In sum, the ddPCR analysis identified successful CRISPR targeting in 200 of the 494 GFP$^+$ clones isolated, from which GPI-AP negative cells were cloned.
Table 5-1: The CRISPR/Cas9-directed gRNA sequences used to target the *Piga*, *Pigk*, and *Pigg* genes.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Exon</th>
<th>Sequence</th>
<th>Score</th>
<th>Off-Targets (in genes)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Piga</em></td>
<td>2</td>
<td>TGCAGGTCCTATCGTGGA</td>
<td>96%</td>
<td>47</td>
</tr>
<tr>
<td>(1st attempt)</td>
<td></td>
<td></td>
<td></td>
<td>(6 in genes)</td>
</tr>
<tr>
<td><em>Piga</em></td>
<td>5</td>
<td>AGCGTTCTGATGGCCATG</td>
<td>86%</td>
<td>73</td>
</tr>
<tr>
<td>(2nd attempt)</td>
<td></td>
<td></td>
<td></td>
<td>(12 in genes)</td>
</tr>
<tr>
<td><em>Pigk</em></td>
<td>1</td>
<td>ACAAGAGCCAGCGGCGCC</td>
<td>81%</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(26 in genes)</td>
</tr>
<tr>
<td><em>Pigg</em></td>
<td>1st common exon</td>
<td>TTGCCAGAAAGAGCGGTTA</td>
<td>31%</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4 in genes)</td>
</tr>
</tbody>
</table>
Figure 5-1: Analysis of CRISPR/Cas9-mediated locus editing via a dual probe method of ddPCR.

Pro WT represents the fluorescent probe specific for the targeted gene of interest cleavable by gRNA-directed CRISPR/Cas9 endonuclease. Pro 2 represents a fluorescent probe capable of binding upstream of the targeted sequence. Arrows in each representation signify the area amplified by PCR. A. In a WT, non-edited locus, Pro WT and Pro 2 are able to bind to their specific DNA sequences on both alleles. Similar to flow cytometric analysis, the dual probe binding can be seen as a population of double positive fluorescence. B. In a heterozygous clone, where CRISPR/Cas9 gene deletion was successful only on one allele, two fluorescing populations will be present. Both the Pro WT and Pro 2 will bind and fluoresce on the non-edited allele. Pro WT will fail to bind to its specific target sequence if the CRISPR editing was successful. Thus, on the edited allele, Pro 2 will bind and fluoresce, yet Pro WT will fail to fluoresce. C. In a homozygous clone, successful editing of the target sequence will occur on both alleles. In this case, a single population of only Pro 2 fluorescence will be observed as Pro WT will fail to bind and fluoresce.
Table 5-2: Quantification of CRISPR/Cas9-induced homozygously and heterozygously mutated *Pigk*, *Pigp*, and *Piga* clones.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th># Clones Generated</th>
<th># Homozygous Clones</th>
<th># Heterozygous Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pigk</em></td>
<td>142</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td><em>Pigp</em></td>
<td>45</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td><em>Piga</em></td>
<td>307</td>
<td>38</td>
<td>139</td>
</tr>
</tbody>
</table>
We next assessed the impact of either heterozygous or homozygous mutants of each of the 3 loci targeted on TCR/CD3 induced growth, as assessed by \(^3\)H-TdR uptake, and IL-2 production, as assessed by ELISA, at 24 hours post stimulation with plate-bound anti-CD3\(\varepsilon\). \(^3\)H-TdR uptake by those clones that survived 24 hours post stimulation is illustrated in Figure 5-2A. As illustrated, the majority of edited clones did not respond to anti-CD3\(\varepsilon\) stimulation (Figure 5-2A, open circles). Of those surviving \(Piga, Pigk, and Pigp\) edited clones that did respond, levels of \(^3\)H-TdR uptake were significantly lower than those observed in the non-edited GPI\(^+\) or GPI\(^-\) control variants. Further, and strikingly, levels of IL-2 in supernatants of clones that responded to plate-bound anti-CD3\(\varepsilon\) with at least 1000 cpm were roughly 100-300-fold lower than those observed in supernatants of the non-edited GPI\(^+\) and GPI\(^-\) control variants (Figure 5-2B). These unexpected results are discordant with those obtained with natural GPI-AP deficient clones (Figure 5-2B – and Chapter 3) and primary T cells (Chapter 4), and motivated our re-assessment of the use of the CRISPR/Cas9 system to create GPI-AP\(^-\), GPI\(^+\) T cell variants. Specifically, we next assessed the effect of the Cas9 endonuclease on TCR/CD3 induced IL-2 production in this T cell clonal system.

Toward this end, GPI\(^+\) cells were electroporated with either an empty vector or a vector encoding the Cas9 DNA. As illustrated in Figure 5-3A, while electroporation alone, specifically with a vector not encoding Cas9 DNA (V no Cas9) had no impact on levels of anti-CD3\(\varepsilon\) induced IL-2 detected in culture supernatants, electroporation with a Cas9 encoding vector (V + Cas9) reduced levels of IL-2 detected in culture supernatants by 7.4-fold (Figure 5-3A). The impact of Cas9 alone was verified through the
Successful CRISPR/Cas9-mediated editing of the *Pigk*, *Pigp*, and *Piga* locus was determined via a dual probe method of ddPCR. **A.** All identified heterozygously and homozygously mutated clones were plated on 3 μg/ml of anti-CD3ε and thymidine uptake was assessed after 24 hours. Non-edited GPI⁺ and GPI⁻ parental 2.10 variants were also stimulated for comparison. CRISPR-edited clones generating thymidine uptake values of less than 1000 cpm were deemed unable to respond to stimulation (*open circles*). **B.** Supernatant was harvested from all clonal variants generating thymidine uptake values above the proliferation threshold of 1000 cpm (*red circles*). The amount of IL-2 in the supernatant was determined by mIL-2 ELISA.

**Figure 5-2: Assessment of thymidine uptake and IL-2 levels in CRISPR/Cas9-edited clones.**
Figure 5-3: The impact of CRISPR/Cas9 on TCR-induced IL-2 production in the clone 2.10.

A. GPI⁺ 2.10 cells were electroporated (V) with either an empty vector or a vector encoding the Cas9 DNA. Similarly treated non-electroporated GPI⁺ cells were included as a control, as were non-treated GPI⁺ and GPI⁻ 2.10 cells. Following expansion of electroporated populations, cells were cultured onto plate-bound anti-CD3ε and induced IL-2 production was measured from the supernatant of 24 hour stimulated cultures by mIL-2 ELISA. B. GPI⁺ 2.10 cells were electroporated either with or without 1 μg of purified Cas9 protein. Non-treated GPI⁺ and GPI⁻ 2.10 cells were included as a control. All populations were cultured onto plate-bound anti-CD3ε and induced IL-2 production was measured from the supernatant of 24 hour stimulated cultures by mIL-2 ELISA.
introduction of purified Cas9 protein. As illustrated in Figure 5-3B, voltage alone, in the absence of the Cas9 protein, showed no significant effect on anti-CD3ε induced IL-2 detected in the culture supernatants. In striking contrast, electroporation at the same voltage but with the inclusion of 1 µg of Cas9 protein results in a 10-fold reduction in the level of IL-2 detected in the supernatant of stimulated T cells. Thus, and in contrast to expectation, Cas9, in the absence of gRNA, has a profound effect on the physiology of clone 2.10, which in turn precluded its utilization in generating GPI-AP+ GPI-AP−T cells for our purposes. Specifically, while technically successful in effecting the desired genome editing, the use of Cas9 rendered TCR/CD3 signalling and IL-2 production in recipient cells profoundly compromised.

*Generation of a GPI-AP deficient, GPI anchor sufficient mouse model:*

We sought a mouse model that would enable addressing the differential role of GPI and GPI-AP in supporting the enhanced and prolonged IL-2 production in GPI-AP and GPI T cells as observed in the Pigp and Piga mutant clonal and primary T cells, respectively. The objective was to target one of the 5 members of the transamidase complex within the GPI-anchor biosynthetic pathway, and to do so in a conditional manner to avoid embryonic lethality observed for PIGA deficiency [49-51].

We next contemplated which of the 5-member transamidase complex would best lend itself to the genetic manipulations required in support of its conditional expression. We discovered that floxed-*Pigu* embryos were available, and could enable the generation
of a conditional *Pigu* knock-out. As for *Pigk*, *Pigu* is also one of the 5 obligatory transferases the expression of which predicates the biosynthesis of mature GPI-AP, and its absence should prevent the GPI transamidase complex from transferring fully assembled GPI units onto proteins [26]. As acquisition of these embryos would accelerate achieving our goal, we contracted the European Mouse Mutant Archive (EMMA) Repository to ship us heterozygous mice containing the floxed exon 2 of *Pigu*, referred to as Tm1c. This strain was housed and bred in the specific pathogen free (SPF) environment at the Sunnybrook Research Institute for preparation of the conditional *Pigu* deficient strain.

To achieve T cell specific *Pigu* deficiency, which would not constitute embryonic lethality, a strategic breeding program analogous to that employed for the generation and maintenance of the *Piga* conditional mutants was implemented (Figure 5-4). It is important to note that unlike *Piga*, *Pigu*, is autosomal, and therefore the breeding scheme to generate *Pigu*− required modification as described below. The Tm1c mice were bred with mice transgenic for Cre recombinase under the control of the proximal Lck promoter, termed *Lck-Cre*. The Lck proximal promoter is preferentially expressed in the thymus [277, 278]; when mature single positive TCRαβ+ thymocytes of both lineages emigrate to the periphery, the control of Lck expression preferentially switches to the Lck-distal promoter [279, 280]. The differential expression of the proximal and distal Lck promoters in the thymus and periphery respectively, are 10:1-fold and 1:10-fold, respectively. This promoter switch, while ensuring the peripheral T cells are PIGU deficient, also safeguards against unwanted Cre-expression in peripheral T cells of these animals.
The proposed breeding plan has been initiated and the first [Tm1c x Lck-Cre]F₁ progeny have been genotyped and phenotyped. As both parents are heterozygous for the genes of interest, the pups are a blend of homozygous WT and heterozygous for mutant Pigu allele. As mentioned above, as the Pigu locus is autosomal the generation of PIGU deficient animals requires interbreeding to yield F2 animals.
Figure 5-4: The generation of a conditional Pigu deficient mouse.
To generate a conditional Pigu deficient animal, the Tm1c mouse was crossed with mice expressing the Lck driven-Cre recombinase (Lck-Cre). The Lck proximal promoter is represented as an orange rectangle in this schematic. This crossing should generate mice with Cre mediated recombination at the loxP sites (red triangles) flanking exon 2 of Pigu, producing the F1 progeny LckCre/Pigu\textsuperscript{lox} \textsuperscript{Tm1d}. As Pigu is autosomal, the creation of conditional GPI-AP deficiency exclusively on mouse T cells requires the intercrossing of F1 Tm1d mice to yield F2 LckCre/Pigu\textsuperscript{lox} mice bearing the disruption of Pigu on both alleles.
Preliminary findings with the GPI-AP deficient, anchor sufficient T cells:

As illustrated in Figure 5-5, the conditional Pigu deficient mice achieved through our strategic breeding program will elucidate whether the absence of one or more GPI-AP, or the GPI anchor itself, predicates the enhanced and prolonged TCR-induced IL-2 production of GPI-AP deficient, GPI anchor deficient T cells.

There are two material issues related to moving the assessment of Pigu<sup>-</sup> mice forward. The first, as described above, is generation of PIGU deficient animals through interbreeding the Pigu<sup>-/-</sup>, cre<sup>+</sup> F<sub>1</sub> animals. The second is derived from observations we have made in regards to the impact of the mixed genetic background of the PIGU deficient animals. The latter will be explained in detail below.

First, to the long awaited characterization of TCR/CD3 induced IL-2 production in PIGU deficient, GPI-AP, GPI<sup>+</sup> T cells. We have currently assessed anti-CD3ε mediated IL-2 production from the T cells derived from two animals in two independent experiments. GPI CD4<sup>+</sup> T cells were isolated from splenocytes deriving from Pigu<sup>-/-</sup> mice, and GPI<sup>+</sup> CD4<sup>+</sup> T cells were isolated from Lck-Cre deficient, WT littermates. Ahead of experimentation, we sought to confirm GPI-AP deficiency in the Pigu<sup>-/-</sup> T cells by both PCR and flow cytometric analysis. As illustrated in Figure 5-6B, PCR analysis confirmed the deletion of the floxed exon 2 exclusively in DNA derived from Pigu<sup>-/-</sup> mouse T cells, with the verified presence of the cre transgene. Total splenocytes isolated from Pigu<sup>-/-</sup> were stained for the GPI-AP, CD48, and either TCR as a marker for T cells, or IgM to identify B cells (Figure 5-6C). The flow cytometric analysis corroborated the PCR
Figure 5-5: Elucidating the role of the GPI anchor in TCR/CD3 signalling and IL-2 production.

WT, Piga\textsuperscript{+/-}, and Pigu\textsuperscript{+/-} CD4\textsuperscript{+} T cells were stimulated for 24 hours on plate-bound anti-CD3\textsubscript{\varepsilon}. Piga\textsuperscript{+/-} CD4\textsuperscript{+} T cells, lacking both GPI-AP and the GPI anchor, produce significantly enhanced and prolonged IL-2 compared to their WT counterparts. The response of the Pigu\textsuperscript{+/-} CD4\textsuperscript{+} T cells to stimulation remains in question. If levels of IL-2 protein are low and comparable to WT cultures, it would indicate that an enhanced IL-2 phenotype is dependent on anchor deficiency. Further directions will be focused on lipidomic analysis to elucidate how the anchor regulates TCR/CD3 signalling and IL-2 production. If levels of IL-2 protein are high and comparable to Piga\textsuperscript{+/-} cultures, efforts will be placed in determining which GPI-AP regulates the growth/survival phenotype of anchorless cells.
Figure 5-6: T cell-specific disruption of the *Pigu* gene.
A. Schematic representation of *Pigu* exon 2 showing the *loxP* sites (red triangles) and the positions of primers used for PCR analysis. B. Tail, B cell or GPI T cell-derived DNA was used in PCR to assess the presence or the deletion of exon 2 with 2 different couples of primers. The presence of the *cre* transgene was also verified. C. To confirm that the *Lck-Cre* expression was restricting the *Pigu* deletion exclusively in T cells, total splenocytes were stained for either TCR or IgM, along with a representative GPI-AP, CD48. D. CD4+ T cells were negatively selected from total splenocytes of *Pigu<sup>−/−</sup>* Tm1d *Lck-Cre*<sup>+</sup> mice or their WT control littermates. T cell purity was assessed post-selection by flow cytometry.
findings, indicating the successful induction of Cre-mediated recombination at the loxP sites flanking *Pigu*, and the creation of conditional T cell-specific GPI-AP deficiency. Furthermore, the negative selection procedure utilized resulted in >95% pure populations of GPI-AP\(^{-/-}\), TCR\(^{+}\), CD4\(^{+}\) T cells for use in stimulation prior and IL-2 assessment (Figure 5-6D).

To afford a direct comparison of the impact of the GPI-anchor on the enhanced and prolonged TCR/CD3-induced IL-2 production observed in *Piga\(^{+/-}\)* GPI-AP deficient, GPI anchor deficient T cells; T cells from *Pigu* and *Piga* deficient mice, along with those derived from their WT littermate controls were assayed in parallel. The results described in Figure 5-7 provide the first evidence for a role of the GPI-anchor in regulating TCR/CD3 signalling.

WT and GPI CD4\(^{+}\) T cells derived from both *Pigu* mice and *Piga* mice and their WT littermate controls were stimulated on plate-bound anti-CD3\(\varepsilon\) and culture supernatant was harvested at days 2, 4, 6, and 8 for IL-2 quantification by ELISA. For ease of direct comparison, the data are presented as a ratio of the IL-2 concentrations produced by the two different GPI T cells divided by that derived from their cognate GPI\(^{+}\) controls. As illustrated in Figure 5-7 this ratio for the *Piga\(^{+/-}\)* versus cognate WT T cells increased from 3.4 to 23 over the 8 day assessment period, as expected and highlighted for these GPI CD4\(^{+}\) T cells in Chapter 4. In striking contrast, this ratio for the *Pigu\(^{+/-}\)* versus cognate WT T cells ranged from 2 - 2.5 over the 8 day assessment period. While the ratio is not 1, these results are consistent with GPI-AP GPI\(^{+}\) T cells behaving more similarly to their
Figure 5-7: Reduced IL-2 differential in GPI Pigu deficient versus WT CD4+ primary T cells.

GPI-AP deficient, GPI anchor sufficient CD4+ splenic T cells (PIGU GPI) were isolated from Pigu<sup>−/−</sup> cre<sup>+</sup> mice and stimulated on 3 μg/ml of plate-bound anti-CD3ε; in comparison with their Pigu-expressing WT counterparts. GPI-AP deficient, GPI anchor deficient CD4<sup>+</sup> T cells from LckCre/Piga<sub>flax</sub> mice and their WT Piga<sub>flax</sub> littermate controls were also stimulated on 3 μg/ml of plate-bound anti-CD3ε. At each indicated time point, culture supernatants were collected and IL-2 was quantified by ELISA. The fold difference in the amount of IL-2 detected in GPI cultures compared to WT cultures was calculated and represented as a ratio of differential IL-2 concentration (GPI/WT) for the comparison of IL-2 production by each cell type. At day 8, the peak IL-2 concentrations were as follows: 62.1 pg/ml for PIGA WT, 1421.8 pg/ml for PIGA GPI, 114.1 pg/ml for PIGU WT, and 286.3 pg/ml for PIGU GPI. WT and GPI PIGU IL-2 concentrations varied within 10% over the 8 day time course, as did those from WT PIGA cells; in contrast to increasing IL-2 levels observed in supernatants from GPI PIGA cells.
cognate WT control T cells. As such, the results underscore a potential and fundamental role of the GPI-anchor in regulating TCR/CD3 induced IL-2 production and T cell homeostasis. As will be further discussed in Chapter 6, this reproducible finding targets a role for inositol and associated lipid metabolism underpinning the IL-2 phenotype observed in GPI-AP, GPI T cells.

A material observation limits formal proof of the above conclusion at this juncture. As described in the Materials & Methods, the heterozygous Pigu+/- embryos obtained from the EMMA Repository are on a C57BL/6N (B6N) genetic background. Further, introduction of lck-cre was achieved by breeding the Pigu+/- B6N mice with C57BL/6J (B6J) carrying the lck-cre transgene, resulting in Pigu+/-, lck-cre+ mice on a B6N/J genetic background. Further still, generation of Pigu+/-, lck-cre+ mice required interbreeding these B6N/J F1 mice, resulting in unpredictable genetic mixtures of the B6J and B6N backgrounds. Through extensive whole-genome comparison of C57BL/6N and C57BL/6J mice, Simons et al. recently identified multiple genetic variations existing between the substrains. Specifically, 34 coding SNPs (single nucleotide polymorphisms), 2 coding small indel (insertion or deletion) mutations, 146 noncoding SNPs, 54 noncoding small indels, and 43 structural variants were identified, including the B6J nicotinamide nucleotide transhydrogenase (Nnt) mutation known to correlate with mitochondrial redox imbalances and contribute towards moderately impaired glucose clearance rates [281, 282]. Ultimately, a total of 51 genes were identified as expressing either sequence or structural variants, with the potential to affect gene function [281]. While a proportion of the genetic variants may have little or no phenotypic consequence,
there are some notable phenotypic differences in metabolism, including glucose tolerance, as well as in eye development and inflammatory responses [281, 283]. While upon inspection none of the differing loci appear to encode products that would interfere with T cell physiology and IL-2 production, we chose a simple approach to assess potential consequences of a mixed B6N/J genetic background in $Pig\gamma^+$ animals on T cell production of IL-2. Specifically, we created a $Pig\alpha^{+\alpha_\gamma}$, $lck-cre^+$ B6N/J F$_1$ mouse.

The $Pig\alpha^{+\alpha_\gamma}$ $LckCre/Pig\alpha^{\text{floxed}}$ mice on the B6J genetic background were bred with WT B6N mice to create GPI$^+$ and GPI$^-$ B6N/J F$_1$ mice. $Pig\alpha$ is X-linked, hence crossing B6J $LckCre/Pig\alpha^{\text{floxed}}$ female mice with B6N males would provide B6N/J male pups, 50% of which would be $Pig\alpha^-$. WT and GPI$^+$ B6J CD4$^+$ T cells as well as WT and GPI$^-$ B6N/J CD4$^+$ T cells were isolated. As illustrated in Figure 5-8A, the purity of these populations post-selection was comparable. Each of these populations was then stimulated on 3 $\mu$g/ml of plate-bound anti-CD3$\varepsilon$ and culture supernatant was harvested daily for the comparison of differential IL-2 production over a four day period. As for the results presented in Figure 5-7, data presented in Figure 5-8B are presented as a ratio of the IL-2 concentrations produced by the different GPI$^-$ T cells divided by that derived from their cognate GPI$^+$ controls. As illustrated in Figure 5-8B, the $Pig\alpha$ mutation on a B6J showed the material differential production of IL-2 by GPI$^-$ versus GPI$^+$ controls, peaking at 15-fold at day 4. By contrast, on a B6N/J F$_1$ background, while the $Pig\alpha$ mutation still revealed a significant differential production of IL-2 relative to its GPI$^+$ control, it peaked at only ~4-fold; roughly a 4-fold reduction in differential in comparison to that observed by T cells on a B6N background.
Figure 5-8: The influence of genetic background on detectable IL-2 levels.
_LckCre/Piga<sup>lox</sup>._ mice on the B6J genetic background were bred with B6N mice to create GPI<sup>+</sup> and GPI<sup>-</sup> primary T cells on the mixed B6N/J background. GPI<sup>+</sup> and GPI<sup>-</sup> CD4<sup>+</sup> T cells of both the B6J and B6N/J backgrounds were isolated. **A.** Purity of GPI<sup>+</sup> and GPI<sup>-</sup> CD4<sup>+</sup> B6J and B6N/J T cells.

**B.** These four T cell populations were stimulated on 3 µg/ml of plate-bound anti-CD3ε. At each indicated time point, culture supernatants were collected and IL-2 was quantified by ELISA. The fold difference in the amount of IL-2 detected in GPI<sup>-</sup> cultures compared to WT cultures was calculated and represented as a ratio of differential IL-2 concentration (GPI/WT) for the comparison of IL-2 production by each cell type. At day 4, the peak IL-2 concentrations were as follows: 726 pg/ml for B6J WT, 10922 pg/ml for B6J GPI<sup>-</sup>, 1201 pg/ml for B6N/J WT, and 4337 pg/ml for B6N/J GPI<sup>-</sup>.
We cannot assume that the impact on the observed differential IL-2 production supported by the \textit{Piga} mutation on the B6N/J background will also impact the \textit{Pigu} mutation. If it were to have a similar impact it would challenge the conclusions drawn from results presented in Figure 5-7. Specifically, the IL-2 ratios obtained on the mixed genetic background would have to be adjusted upwards. The extent of this increase, if any, cannot be predicted with any certainty. The resolution will have to come experimentally, and backcrossing the \textit{Pigu} mutation onto a B6J background is underway. We are currently breeding the third generation backcross. To ensure that we have accomplished a sufficient number of backcrosses we are also initiating the re-creation of the B6J \textit{Piga}^+/, \textit{Ick-cre}^+ and associated IL-2 phenotype by backcrossing the \textit{Piga}^-/, \textit{Ick-cre}^+ B6N/J F\textsubscript{1} mouse to B6J. The \textit{Pigu}^-/ mice will be retested at that number of backcrosses at which the \textit{Piga} backcrossing regime generates pups containing GPI CD4^+ T cells that recapitulate the IL-2 phenotype observed on the B6J background. We will await the 5\textsuperscript{th} backcross progeny, containing roughly 97% B6J background, for the first reassessment of the \textit{Pigu}^-/ genotype. A definitive resolve of the role of the GPI-anchor on TCR/CD3 induced IL-2 production and T cell homeostasis awaits these results. Strategies for future directions with the conditional \textit{Pigu}^-/ animal model will be discussed in Chapter 6.
CHAPTER 6: DISCUSSION

Defining a role for GPI-AP in T cell homeostasis:

Approximately 10-20% of all eukaryotic membrane proteins are tethered to the outer leaflet of the plasma membrane by a complex, conserved glycolipid structure [28]. The GPI anchorage imparts functional attributes upon membrane molecules, including localization to lipid rafts, lateral mobility, and importantly, the capacity to transduce intracellular signalling. In regards to their signalling role in T lymphocytes, GPI-AP have been implicated in diverse processes; most notably, T cell development, and the modulation of TCR responsiveness, both positively and negatively. As GPI-AP are neither transmembrane molecules nor do they exhibit intrinsic enzymatic activity, the manner in which they mediate intracellular signalling remains to be elucidated. It has been proposed that it is their localization within LR that predicates their signalling capacity [2, 3, 42]. The LR residence is thought to permit their association with potential signal transduction partners, such as co-localized protein tyrosine kinases [42]. GPI-AP have been characterized as regulators of T lymphocyte physiology. The first, and striking evidence that the GPI-anchor (GPI) itself, common to all GPI-AP, may play a seminal role in GPI-AP signalling is presented herein. However the balance of signalling capacity is shared between the protein moiety of the GPI-AP and the GPI, the results presented in this thesis define a novel role for GPI and/or GPI-AP in T cell antigen receptor signalling, regulation of IL-2 production, and T cell homeostasis.
First insights into the growth/survival advantage imparted by GPI-AP deficiency in the clonal model:

The first insights into a role of GPI-AP in the regulation of T lymphocyte homeostasis followed the characterization of GPI-AP deficient variants of the T cell clone 2.10. GPI-AP deficiency in clone 2.10 was proven to be due to the impaired expression of the novel autosomal gene termed *Pigp*, that encodes a transferase, the expression of which predicates the earliest step of GPI-anchor biosynthesis [8]. Availability of these clonal variants therefore enabled an assessment of both GPI and GPI-AP on T cell physiology, providing a significant opportunity to study these molecules, as GPI-AP deficiency in nature constitutes embryonic lethality [49-51].

The results provided herein have revealed a novel growth/survival advantage with ensuing TCR engagement in GPI-AP deficient T cells; specifically, prolonged proliferation, which directly correlated with a material increase in both transcription and translation of IL-2. Importantly, the kinetics of anti-TCR induced proliferation of GPI-AP sufficient cells could be extended by addition of exogenous IL2 and no differences in either consumption or utilization of IL-2 was observed in GPI-AP sufficient and deficient clonal variants. Taken together, these results support the conclusion that the differential kinetics observed in GPI-AP deficient clonal variants was indeed due to differential IL-2 production rather than differential function of the IL-2R.
Ectopic expression of wild-type Pigp in GPI-AP deficient (GPI−) clonal variants rescued the expression of GPI-AP and reverted the growth/survival phenotype of these transduced cells to that comparable to the GPI-AP sufficient (GPI+) 2.10 clonal variant. Further, the underpinning mechanistic basis for this reversion correlated with the loss of the extended kinetics and amplitude of IL-2 message and protein produced in these transduced cells. These correlates support the conclusion that GPI-AP play a central role in the attenuation of TCR mediated cell activation and growth.

**Identifying a developmental advantage of GPI DN2/3 pro-T cells:**

The first step toward elucidating the mechanisms underpinning GPI-AP regulation of TCR signalling was to validate the physiological relevance of these observations in primary T cells. As GPI-AP deficiency constitutes embryonic lethality, our first attempt was to create ESC-derived primary GPI pro-T cells *in vitro*. GPI ESCs were differentiated into CD25+ DN2/3 cells, followed by their injection into RAG2<sup>−/−</sup>γ<sup>−/−</sup> recipients for the final steps of intrathymic development and differentiation into functionally mature GPI primary T cells. The technique failed to support sufficient thymus engraftment and the ensuing peripheralization of GPI-AP deficient T cells. The failure to support sufficient engraftment can be attributed to two main challenges: the limiting numbers of DN2/3 cells available for transfer; and the efficiency with which transferred cells homed to the thymus. Notwithstanding, the experiment did reveal novel insight into the GPI growth/survival phenotype.
At the early DN2/3 stage of T cell development \textit{in vitro}, GPI-AP deficiency conferred a 10-fold greater growth/survival advantage. Previous studies have suggested a regulatory role for various GPI-AP in thymocyte maturation; most notably, in their ability to perturb thymocyte development/selection. An inhibitory role has been proposed for Thy-1 (CD90) as mAb mediated coaggregation with TCR was found to enhance thymocyte apoptosis [54, 56, 284]. An inhibitory role may also apply for the GPI-AP lymphostromal molecule, TSA-1, as mAb mediated blockage reduced total thymocyte yield and inhibited DN to DP differentiation in fetal thymic organ culture [58]. A third study implicating a GPI-AP in the regulation of hematopoiesis found a tight control of Sca-1 expression during thymocyte differentiation. Specifically, Sca-1 expression is extinguished early in thymocyte development, and transgenic over-expression was found to arrest differentiation at the CD3^+CD4^+CD8^-CD44^-CD25^- stage where Sca-1 expression is normally terminated [285]. Each of these studies are consistent with and indeed predict that GPI-AP deficiency will result in the observed perturbations in intrathymic pro/pre-T cell development, which we now extend to include a developmental advantage of GPI DN2/3 pro-T cells.

\textit{Extending the GPI growth/survival advantage to primary \textit{CD4}^+ GPI T cells:}

As the \textit{in vitro} system for T cell development proved inefficient, we capitalized on the availability of a Cre-\textit{loxP} conditional targeting system to enable the creation of primary GPI-AP deficient T cells [71]. The generation of this \textit{LckCre/Piga^{lox}} mouse involved the targeted deletion of the \textit{Piga} gene, which encodes an essential transferase required for the initial step of GPI-anchor biosynthesis. The Cre recombinase was driven
by the Lck proximal promoter to support a thymus-specific disruption of Piga, and ensure a T cell-specific GPI-AP deficiency. Takahama et al. first examined the responsiveness of total splenocytes or enriched splenic T cells from this animal model to the mitogen ConA, CD3 specific mAb, PMA/Ionomycin, and Staphylococcal enterotoxin B [71]. While these studies resolved that GPI-AP deficiency did not perturb T cell activation either in vitro or in vivo, no significant differences between GPI− and GPI+ samples were observed. Most notably, in the context of the present studies, neither enhanced proliferation nor IL-2 production in GPI− splenic T cells was observed in response to ConA stimulation. The same group published a second analysis demonstrating a significant enhancement of ConA induced 3H-TdR uptake using total splenocytes from the LckCre/Piga^{flx} mice [286]. While the conflicting results were not addressed in the second study, the discrepancies may relate to the different genetic backgrounds of the mice used in the studies, as described herein. T cell-specific GPI-AP deficiency was achieved using mice of the B6C3F1 genetic background in the Takahama et al. study, in contrast to the C57BL6-129v genetic background used in the second study by Hazenbos et al. Furthermore, it is also possible that the discrepancies may be attributed to different assays and indicators used as readouts. Importantly, Hazenbos et al. extended the GPI growth/survival advantage in the second study to LckCre/Piga^{flx} CD4+ LN T cells, which displayed a strongly enhanced proliferative response to alloantigen expressed on bm12 cells [286] compared to Piga^{flx} littermate controls. These later studies are clear harbingers to the results presented herein, using LckCre/Piga^{flx} mice on the C57BL/6 genetic background.
We have demonstrated that primary GPI CD4+ T cells from the \textit{LckCre/Piga}^{\text{lox}} animals recapitulated the GPI growth/survival phenotype observed in the 2.10 clonal T cell model. As described above, conflicting results from our study compared to the two former studies, may potentially relate to differences in the genetic background of all three mouse model systems. A key distinction that may also underpin the differences we have observed is our use of purified splenic CD4+ T cells and differential stimuli in the \textit{LckCre/Piga}^{\text{lox}} studies presented herein. The earlier studies assessed the stimulatory capacity of total splenocytes or enriched splenic T cells, which could have masked the differences observed in our analyses with purified CD4+ GPI T cells. Hazenbos \textit{et al.} also demonstrated that isolation and stimulation of a CD4+ GPI OVA specific T cell clone from the \textit{LckCre/Piga}^{\text{lox}} mouse exhibited an enhanced response to ConA stimulation [286]. However, in response to 48 hours of stimulation with its OVA-specific protein Ag, they concluded no differential proliferation between the GPI clone and its \textit{PIGA}-restored GPI+ counterpart. As described, we too have assessed the responsiveness of OVA-specific clonal T cells. In response to OVA stimulation, GPI deficiency in the 2.10 clonal variants confers an enhanced and prolonged proliferative capacity. A notable difference between our study and the former lies in the kinetics of stimulation: at only 48 hours of stimulation the differences in $^3$H-TdR uptake is negligible, and only over time is the GPI growth/survival advantage observed in the GPI 2.10 clones.

As with the clonal T cell system, the growth/survival advantage of GPI primary T cells was attributed to the material increase in TCR-mediated induction of IL-2 mRNA and protein. In fact, the differential production of IL-2 in these primary GPI versus GPI+
T cells was even more exaggerated than that observed in the clonal T cell model, and may correlate with the hypersensitive responses seen exclusively in GPI CD4+ T cells.

IL-2-mediated signalling is contingent on the upregulated expression the IL-2Rα chain and formation of the high-affinity IL-2R [176, 266]. The signalling cascade ensuing from IL-2-IL-2R interaction is known to further induce both IL-2 production and upregulation of the IL-2Rα chain (CD25) by promoting the binding of signal transducer and activator of transcription 5 (STAT5) to the Il2ra gene locus [176, 198]. In this regard, the IL-2Rα chain, which is known to initiate binding of IL-2 within LR [227], was more highly expressed on GPI primary CD4+ T cells in comparison to their WT counterparts; and may facilitate binding of IL-2 and further drive the IL-2-IL2R positive signalling loop. Upon IL-2 binding, the IL-2Rα chain is known to translocate outside of LR enabling the heterotrimerization with IL-2Rβ and IL-2Rγ chains [227] and the initiation of IL-2R signalling via the cytoplasmic tails of the IL-2Rβ and IL-2Rγ chains. This finding was revealed to Marmor et al. when immobilized mAbs to GPI-AP sequestered IL-2Rα in LR and in turn inhibited IL-2-induced T cell proliferation. Thus, the absence of GPI-AP in our T cell model systems, which will directly impact the composition of LR, may do so in ways in which retention and/or translocation of IL-2Rα may be significantly altered. This in turn may enhance the formation of the high affinity IL-2R complex and IL-2 mediated signalling in CD4+ GPI cells, and contribute to the prolonged growth/survival phenotype observed.
Strikingly, the growth/survival advantage of GPI primary T cells was unique to the CD4 lineage. No differences in either sensitivity to TCR induced activation or the ensuing growth kinetics was observed in GPI CD8+ T cells. In this regard, activated primary CD4+ T cells are known to be the majority source of IL-2 in primary immune responses, and while primary CD8+ T cells do produce IL-2 they do so to a much lesser extent [183, 198]. This lineage specificity reveals the likely role of IL-2 production and function as underpinning the mechanism in support of the distinct cellular responses to TCR mediated activation in GPI+ and GPI T cells.

**CD28 costimulation:**

We further investigated the ancillary but fundamental function of CD28 in the regulation of TCR induced IL-2 production in GPI+ and GPI primary CD4+ T cells. Co-stimulation through CD28 during TCR engagement promotes IL-2 enhancer activity and stabilizes IL-2 mRNA [208, 209]. In addition, CD3/28 costimulation enhances cytokine expression, amplifies glucose metabolism, and supports increased resistance to apoptosis, thereby promoting the expansion of activated T cells. As the GPI growth/survival advantage was achieved using plate-bound TCRCβ or CD3ε specific mAbs in the absence of CD28 co-stimulation, we investigated whether addition of soluble CD28 specific mAb would impact the differential TCR induced IL-2 production in GPI+ and GPI CD4+ T cells. These assays revealed that the GPI growth/survival advantage is not attributable to a GPI-AP-dependent differential function of CD28.
Investigation into differential involvement of the inhibitory molecule, PD-1:

In both clonal and primary T cell models, the enhanced and prolonged production of IL-2 by GPI variants may reflect dysregulated TCR/CD3 signalling; specifically, an inhibitory role of GPI-AP. Reports of the hyper-responsiveness of Ly-6A deficient lymphocytes [287] and Thy-1 deficient thymocytes [288] to various stimuli support this hypothesis, as does the demonstration of Ly6A mediated downregulation of TCR induced IL-2 production [289]. Taken together, we hypothesized that GPI-AP may attenuate TCR induced cellular activation and homeostasis via an association with a known inhibitory molecule(s).

The role of PD-1 as a negative regulator of TCR signalling was of particular interest as it has been characterized extensively in its capacity to suppress cytokine production [239, 290]. PD-1 upregulation and binding to its B7 family ligands, PD-L1 or PD-L2, initiates the recruitment of SHP-2 for the dephosphorylation and inhibition of CD3/CD28-mediated PI3K and AKT activity [170, 171, 173]. This downregulation of T cell activation ultimately decreases the induction of cytokines, including IL-2. When differential PD-1 functionality was assessed in our model systems, again, what was first identified in the clonal model was mirrored with primary T cells. Both PD-1 and its ligand, PD-L1, were comparably upregulated in response to TCR/CD3 engagement on GPI-AP sufficient and deficient cells. Further, GPI-AP deficiency was found to impart a resistance to PD-L1-Fc mediated dampening of TCR/CD3 induced clonal expansion. The differential sensitivity to PD-L1 directly related to the significantly greater amount of IL-2 produced by the GPI primary and 2.10 T cells. Neutralizing IL-2 in cultures of GPI
cells to levels observed in their GPI\(^+\) counterparts, rendered the GPI cells as sensitive to PD-L1 mediated inhibition of both DNA synthesis and clonal expansion. These studies further underscore the central role of a dysregulated production of IL-2 underpinning the growth/survival advantage observed in GPI T cell variants.

*Involvement of the Src family protein tyrosine kinases, Lck and Fyn, in the GPI phenotype:*

TCR induced *de novo* synthesis of IL-2 is contingent on the activation and physical association of the Src family protein tyrosine kinases, Lck and Fyn. These kinases are fundamental in generating the most proximal signals emanating from the TCR/CD3 complex. TCR/CD4 coaggregation induces the activation of CD4-associated Lck, followed by its translocation into LR at the T cell-APC interface. Lck mediated phosphorylation of CD3 chains initiates outside of LR and continues upon the translocation of the TCR/CD3/CD4-Lck into LR. This results in the juxtaposition and physical association of activated Lck with LR-resident Fyn, and the ensuing activation of the latter [124]. Lck-mediated activation of Fyn is essential for TCR induced IL-2 production [141].

We then investigated whether dysregulated IL-2 production observed in GPI-AP deficient T cells may, at least in part, be attributable to either or both of the differential localization or function of Lck and Fyn. Consistent with a role for GPI-AP in the regulation of both spatial distribution and function of Lck and Fyn in primary CD4\(^+\) T
cells, we demonstrated that in resting, pre-activated CD4+ T cells there was a 150% increase in the proportion of LR-associated Lck, and remarkably, a 4-5-fold increase in the basal kinase activity of Fyn in GPI CD4+ T cells in comparison to their GPI+ counterparts. While the mechanism(s) through which the absence of GPI-AP underpins these observations remains to be elucidated, they would provide the GPI T cells with a “head start”. Specifically, the enhanced basal level of Fyn kinase activity could be more efficiently increased, at a faster rate subsequent to TCR engagement, enabling more efficient activation of Fyn-dependent kinase Pyk2, and phosphorylation of the Fyn-T-binding protein (Fyb), SLAP-associated protein 76 (SLP-76), and the Src kinase associated phosphoprotein 55 (SKAP55) [142-144]. Enhanced activity of these molecules may collectively support the cascade of signalling events promoting an overproduction of IL-2 synthesis in GPI-AP deficient cells.

Further and in this regard, the increased proportion of LR-resident Lck and the enhanced basal Fyn kinase activity may also underpin the hypersensitive TCR signalling phenotype attributed by GPI-AP deficiency in primary CD4+ T cells. A continuous low level interaction of self-peptide and MHC class II molecules in the periphery supports tonic signalling in resting naïve CD4+ T cells [270, 291]. Src family tyrosine kinases, most notably Lck and Fyn, support these tonic signals essential for naïve T cell survival by maintaining a basal level of activity and phosphorylation [292]. Taken together, these findings are consistent with increased TCR/CD3 tonic signalling in GPI T cells in vivo, which in turn underpins the observed spatial alteration of Lck and increased basal activity of Fyn.
Challenges with the CRISPR/Cas9 mediated genomic engineering strategy:

The GPI-AP deficiency in both the clonal model and the primary T cell model was achieved through either a natural or deliberate disruption of the earliest transferase step of GPI-AP biosynthesis, respectively. In the case of the clonal system, the GPI-AP deficiency was imparted by a natural mutation in Pigp expression, whereas the primary T cell model system involved the deliberate conditional deletion of Piga. Both Pigp and Piga are constituents of a six member transferase complex, which predicates the synthesis of the first intermediate in the biosynthetic pathway of a GPI anchor [8, 9]. Thus, in both models the GPI T cells were devoid of not only GPI-AP, but also the GPI anchor. It has been demonstrated that ligation of many different GPI-AP can induce comparable physiological responses, as described above. The functional commonalities seen upon perturbation of many different GPI-AP, suggest that the specific protein moiety of the GPI-AP may not be involved in mediating the physiological effect, rather the conserved GPI anchor may be critical. To determine whether the mechanism(s) underpinning the enhanced and prolonged TCR induced IL-2 production was due to the absence of one or more GPI-AP, or the GPI anchor itself, we sought to create GPI-AP deficient T cells maintaining the expression of the GPI anchor.

The first strategy was to use CRISPR/Cas9 mediated genomic engineering to edit and disrupt the Pigk gene. A mutation of this gene, which encodes one of a 5 member transamidase complex, would prevent the transfer of protein moiety to fully assembled GPI anchors [26]. Importantly, it is known that free GPI anchors (GPI) exist within
mammalian cells within the range of $10^5$ to $10^7$ molecules/cell, and can be expressed at the plasma membrane, the Golgi, and all major subcellular organelles following their synthesis in the ER [293, 294]. As critical controls for the use of the CRISPR/Cas9 system, the Piga and Pigp loci were targeted to ensure that the growth/survival advantage of GPI-AP deficient, GPI-deficient cells could be recapitulated using this technology. While the strategy was successful in targeting the loci of interest and creating hundreds of edited clones, none of the mutated variants were capable of inducing a robust response to TCR/CD3 stimulation. We further determined that the TCR non-responsiveness and failure to induce enhanced levels of IL-2 in these clonal variants related directly to the presence of Cas9, which even in the absence of gRNA had a profound effect on the physiology of clone 2.10.

Although RNA-guided genome editing by CRISPR/Cas9 nucleases is now a widely accepted technique, the system is also known to induce substantial genome-wide off-target mutations. The potential for these unexpected genomic instabilities is one of the major concerns of the technique and may be causal in the physiological effect of CRISPR/Cas9 edited 2.10 cells. Off-target mutations are most predominantly found at sequences that resemble the on-target site [295]. Fu et al. have also described these RNA-guided nucleases as being highly active, even in circumstances of imperfectly paired RNA-DNA interfaces [295]. As we are seeing the deleterious effect in the absence of gRNA, we can conclude that the Cas9 endonuclease is not well tolerated in our clonal model, which we underscore are IL-2 dependent cells that are constantly cycling [132]. In accordance with this, Duan et al. has suggested that off-targets may be cell type
dependent and related to various factors including the degree of DNA methylation and chromatin accessibility, and the integrity of double-stranded break repair pathways [295-297]. Much effort is now being focused on optimization of the CRISPR/Cas9 endonuclease system to curb the potential for genome-wide off-target recognition. The dampening of off-target effects may be achieved through optimization of the gRNA sequence, targeting an alternative PAM, or selecting the correct Cas9 amount, delivery system, or orthologue [297]. In this regard, Kleinstiver et al. have provided an alternative to the widely used Streptococcus pyogenes Cas9 (SpCas9), focusing on the high-fidelity variant SpCas9-HF1 [272]. While maintaining robust on-target activities in human cells, the SpCas9-HF1 was shown to reduce nearly all genome-wide off-target effects to undetectable levels, as determined by GUIDE-seq and targeted next-generation sequencing.

**Generation of a mouse model to achieve GPI-AP deficient, GPI anchor sufficient T cells:**

As both the Cas9 vector and purified Cas9 protein resulted in impaired TCR-mediated IL-2 induction, a new strategy was sought for the creation of GPI-AP deficient, GPI anchor sufficient T cells. We acquired existing floxed-*Pigu*-deficient mouse embryos from the European Mouse Mutant Archive (EMMA) Repository. As *Pigu* is one of the 5 obligatory transferases essential for the biosynthesis of mature GPI-AP, its absence should prevent the GPI transamidase complex from transferring protein moieties onto fully assembled GPI units [26]. Moreover, creating a conditional mouse model where the *Pigu*-deficiency was restricted to T lymphocytes would circumvent the embryonic lethality observed with complete GPI-AP deficiency. The T cell restriction was achieved
by breeding with mice transgenic for Cre recombinase under the control of the thymus-specific Lck proximal promoter, as was the case for the conditional Piga deficient mouse line. The initiation of this mouse model began with the thawing of heterozygous embryos at the EMMA Repository in France, followed by a rigorous mating scheme for the generation of the conditional Pigu deficient strain at Sunnybrook Research Institute. The goal of the conditional Pigu deficient mouse model is the conclusion of whether the mechanism underpinning the enhanced TCR/CD3 responses and the enhanced and prolonged TCR-induced IL-2 production in GPI-AP deficient, GPI anchor deficient T cells is due to the absence of one or more GPI-AP, or the GPI anchor itself.

We report on results from two independent experiments assessing the IL-2 production from Pigu deficient, GPI-AP⁺, GPI⁺ CD4⁺ T cells. The results are striking, in that they provide the first evidence that the GPI-anchor is contributing to the dysregulated TCR-induced IL-2 production observed in GPI-AP⁺, GPI⁺, CD4⁺ T cells. Specifically, the 10-100-fold increase in IL-2 production observed in the latter, was reduced to roughly 2-fold, relative to GPI-AP⁺ controls, in Pigu deficient CD4⁺ T cells. These results support a key role for GPI, rather than the absence of one of more GPI-AP in regulating TCR-induced IL-2 production.

While an exciting and novel finding, we must consider these results as preliminary, as the mixed genetic background of the Pigu⁻/⁻ mice could be impeding TCR mediated IL-2 production. Recall that while Piga⁻/⁻ deficient mice are on a C57BL/6J genetic background, the Pigu⁻/⁻ deficient mice are on a C57BL/6N (B6N) genetic
background. Further crossing with the *Lck-Cre* recombinase C57BL/6J (B6J) mice, results in conditional *Pigu*\(^{-}\) mice on a mixed C57BL/6J/N (B6N/J) background, additionally complicated by having to inter-cross these *Pigu*\(^{-}\) F\(_1\) animals.

We reasoned that the background could be an issue, so that ahead of obtaining the first results with *Pigu*\(^{-}\) mice we set out to assess the potential impact of a mixed B6J/N WT background on TCR-induced IL-2 production in *Piga*\(^{-}\) deficient animals. This was enabled as *Piga* is X-linked, hence crossing B6J *LckCre/Piga*\(^{\text{floxed}}\) female mice with B6/N males would provide B6N/J male pups, 50% of which would be *Piga*. The *Piga*, GPI\(^{-}\), CD4\(^+\) T cells from these animals showed a 3-4-fold reduction in differential IL-2 production observed in GPI\(^{-}\) controls.

While both C57BL/6N and C57BL/6J strains are now widely used, previous studies have identified numerous genomic and phenotypic differences between the two inbred lines, spanning multiple physiological, biochemical, and neurobehavioural systems [281, 283]. Through comparison of the C57BL/6N and C57BL/6J genomes, Simons *et al.* identified 34 coding SNPs, 2 coding small indel mutations, 146 noncoding SNPs, 54 noncoding small indels, and 43 structural variants, including the well-known B6J Nnt mutation. Ultimately, a total of 51 genes were identified as expressing either sequence or structural variants, with the potential to affect gene function [281]. Fontaine *et al.* stress that the use of mixed B6N/J mice, as we observed in our initial *pig u*\(^{-}\) experiment, introduces substantial variability among littermates and breeding pairs, with unknown proportions of B6N and B6J traits, and the creation of subcolonies within the
Hence, at this juncture, attributing a key role of GPI in modulating TCR-induced IL-2 production is premature. While the results derived from the $F_2$ Pigu$^{+/+}$ mice may indeed accurately reflect this conclusion, one cannot predict whether the collateral effect of the mixed genetic background will impact Piga and Pigu deficient T cells comparably. Resolution will require the ongoing backcross of the Pigu mutation onto a B6J background.

A physiological relevance:

Ultimately, all responses observed thus far, in both the clonal and primary T cell model systems, converge on an IL-2 mediated growth/survival advantage in cells lacking the expression of the GPI anchor and all associated GPI-AP. It is important to note the physiological relevance of this finding. Paroxysmal nocturnal hemoglobinuria (PNH) is a human disease that develops almost always as a result of a loss of function PIGA mutation in an early hematopoietic stem cell. All affected progeny cells derived from the single mutated somatic stem cell are incapable of GPI-AP biosynthesis, and thus are devoid of both GPI and GPI-AP, as in our model systems. PNH patients describe with somatic cell mosaicism; the co-existence of two discrete GPI-AP deficient and GPI-AP sufficient populations of somatic cells [91]. In relation to the clonal and primary GPI T cell growth/survival phenotype discussed above, the GPI progeny cells in PNH patients have the potential to preferentially expand in the bone marrow [90]. While the mechanism underpinning the clonal dominance is not well characterized, it is thought to
stem from an intrinsic proliferative and/or survival advantage of the GPI cells. The current theory for the expansion of GPI clones favours a cell-mediated autoimmune process, which specifically targets GPI\(^+\) hematopoietic stem cells, sparing and allowing the expansion of \textit{PIGA} mutated GPI\(^-\) cells [102]. Conversely, the studies supporting an intrinsic proliferative advantage in PNH have identified enhanced proliferation of \textit{in vitro} stimulated GPI cells relative to GPI\(^+\) cells, as well as enhanced capacity for PNH stem cell engraftment in sub-lethally irradiated SCID mice [106, 107]. Moreover, and possibly related to the growth/survival advantage of GPI-AP and GPI deficient cells in PNH patients, is the predisposition for neoplastic transformation [109, 110], with 5-15\% of PNH patients developing hematologic malignancies, significantly higher than in the general population. Furthermore, and as for the T cell models described herein, it is not known whether the underpinning mechanistic basis of growth/survival advantage of GPI-AP deficient cells in PNH is due to the absence of one or more protein moieties, or the anchor itself; hence insights derived from the \textit{Pigu\(^-\)} mice may shed light on the pathogenesis of PNH.

Of note, while attention has been placed on elucidating the role of GPI-anchor versus that of GPI-AP(s), an alternative mechanism related to the yet unknown contribution of the PIG genes themselves may predicate the GPI-AP deficient phenotype. In addition to serving as a prerequisite gene for the biosynthesis of GPI-AP, recent evidence has described further functionality for the \textit{PIGN} gene. Ihara \textit{et al.} have characterized a conserved and non-canonical function of \textit{PIGN} in the prevention of protein aggregates and undue stress in the ER of \textit{Caenorhabditis elegans} and mammalian
Interestingly, the regulation of protein quality was independent of the known PIGN phosphoethanolamine transferase activity. Additional roles of PIGN were extended by Teye et al. with the identification of its role in the regulation of mitotic integrity and genomic stability via its binding to the spindle assembly checkpoint protein, MAD1 [299]. Furthermore and in relation to the PNH PIGA predisposition for neoplastic transformation, Teye et al. identified a correlation with PIGN deficiency, genomic instability, and leukemic transformation/progression. Taking these non-canonical roles of PIGN into consideration, we cannot eliminate the possibility that either PIGA or PIGU may too have additional specific roles with the potential to impact IL-2 secretion/T cell homeostasis, independent of their roles in GPI-AP biosynthesis.

Future Directions:

Characterization of the role of the GPI-anchor versus that of GPI-AP(s) is fundamental to mechanistic insight. Two approaches will be taken contemporaneously. The first involves backcrossing the Pigu mutation onto a B6J background. In parallel, the B6N/J F1, LckCre/Piga- mice described above will also be backcrossed to B6J. The number of backcrosses required to rescue the full 10-100x differential in TCR-induced IL-2 production from Piga- CD4+ T cells will provide a good indication of the number required for the Pigu- mice. That said, each of these backcrosses will generate unpredictable genetic backgrounds, hence ten backcrosses yielding a 99.9% B6J background may be required. With potentially confounding genetic variables related to the B6N strain mitigated, the first experiments with the Pigu- mice will focus on TCR/CD3 signalling and IL-2 production: isolated CD4+ T cells and their cognate GPI-
control CD4+ T cells will be isolated from spleens of WT, Piga<sup>−/−</sup>, and Pigu<sup>−/−</sup> mice; stimulated for varying times on plate-bound anti-CD3ε and supernatants screened for differential production of IL-2 amongst the variants, as described in Chapter 4, for the GPI-AP deficient T cells derived from Piga<sup>−/−</sup> mice. These results will be confirmed by evaluating the kinetics of TCR-induced IL-2 mRNA.

As the described breeding will be time intensive, we will also revisit the genome editing approach using GPI<sup>+</sup> clone 2.10 in combination with the higher fidelity SpCas9-HF1. It would be of great utility to have this editing system working in the clonal system, specifically if results elucidate the underpinning role of one or more GPI-linked proteins and potential transmembrane partners in the regulation of TCR-induced IL-2 production.

In the best of circumstances, specifically, if either the GPI-associated protein(s), or the GPI anchor, underpins the IL-2 phenotype, then the results will resolve with clarity. As illustrated schematically in Figure 5-5, if the mechanism is mediated by GPI-AP, then the Pigu deficient T cells will behave as do Piga deficient T cells; if mediated by GPI, then the Pigu deficient T cells will behave as WT cells. Thus, if the levels and kinetics of IL-2 specific mRNA and levels of IL-2 detected in the culture supernatants of anti-CD3ε stimulated T cells from Pigu<sup>−/−</sup> and Piga<sup>−/−</sup> deficient animals are the same, and an order of magnitude higher than observed in cells and supernatants of stimulated cognate WT T cells, respectively, it would rule out the role of the GPI anchor, exclusively. This result would implicate the involvement of one or more GPI-linked proteins in the growth/survival advantage observed in Piga<sup>−/−</sup> and Pigp<sup>−/−</sup> deficient primary and clonal T
cells, respectively. Conversely, if levels and kinetics of IL-2 specific mRNA and levels of IL-2 detected in supernatants of cultures of anti-CD3ε stimulated Pigu<sup>−/−</sup> T cells are identical to those in supernatants of stimulated WT T cells, and an order of magnitude lower than those observed in cells and supernatants of Piga<sup>−/−</sup> stimulated T cells, respectively, it would directly implicate the anchor per se.

Should the latter be the case, a key control will be required to formally demonstrate that the GPI-anchor is expressed on the plasma membrane of Pigu deficient T cells. A novel technique for the labelling of cell surface GPI-AP and GPI on live cells has recently been described [300]. The strategy involves metabolic engineering of the GPI core structure by introducing a molecular handle in the form of an azide-modified precursor of GPI-AP biosynthesis, a partially acetylated inositol derivative. The acetylated inositol derivative is lipophilic and can easily be incorporated into the cell, where esterases remove the acetyl group allowing the integration of free inositol into the forming GPI anchor. Following incubation with the inositol derivative, Biotin-PEG₄-alkyne and CuSO₄ were introduced to initiate a Cu¹-catalyzed azide-alkyne cyclo bioorthogonal click reaction. Staining with streptavidin-APC allowed the visualization of GPI incorporation. We plan on following a similar protocol to ensure the expression of the GPI anchor on our GPI-AP deficient Pigu deficient T cells.

In collaboration Dr. Zhongwu Guo, we have acquired inositol intermediates required for the click chemistry [300]. PIGU deficient T cells will be pulsed with these inositol intermediates during the course of a 24 hour stimulation with plate-bound anti-
CD3ε. PIGA deficient T cells will be treated in the same way as control. The prediction follows that while both T cell populations will be GPI-AP deficient, PIGA deficient T cells will remain APC-streptavidin−, as they lack the capacity to generate GPI; while PIGU deficient T cells, having the capacity to create mature GPI, will be APC-streptavidin+. This formal proof will enable the direct assessment of the roles of GPI-AP versus GPI in supporting the IL-2 and IL-2 mediated sequelae observed in GPI-AP deficient T cells thus far.

Future directions towards conclusion of the mechanism(s) underpinning enhanced TCR signalling and enhanced and prolonged IL-2 production will be contingent on the results with Pigu deficient T cells. If the response to anti-CD3ε-induced stimulation is similar to that of the Piga−/− CD4+ T cells, efforts will be dedicated to elucidating which GPI-AP is responsible for the growth/survival advantage. This option would involve sequentially knocking out/down of each of 20-30 GPI-AP expressed on the plasma membrane of mature CD4+ T cells, either individually and/or in combination, and quantifying anti-CD3ε-induced IL-2 production of these variants. Given the labour intensity of executing this strategy in primary T cells, our focus on rescuing fidelity of gene ablation with the CRISPR/Cas9 system and use of clone 2.10 is critical. Once one or more GPI-AP are identified as essential to phenotype observed in Piga−/− T cells, the appropriate knock-out mouse would be prepared and results obtained in the clonal T cell system confirmed with primary T cells.
Conversely, should *Pigu*−/− CD4+ T cell responses to anti-CD3ε-induced stimulation recapitulate those of WT CD4+ T cells, efforts will focus on determining how the GPI anchor regulates IL-2 production. This direction would entail lipidomic analyses to quantify differences in lipid species and lipid signalling within LR of *ex vivo* and TCR/CD3-stimulated GPI-AP deficient, anchor-expressing (*Pigu*−/−) and anchorless (*Piga*−/−) CD4+ T cells.

Particular attention will be placed on phosphatidylinositol (PI) metabolism, as this lipid species is the initial substrate utilized for GPI anchor biosynthesis. Thus, in the absence of the transferase complex to utilize PI in anchor biosynthesis, steady state levels may be altered in *Piga*- and *Piga*-deficient T cells. While PI constitutes less than 15% of the total eukaryotic phospholipids, together with their phosphorylated products, termed phosphoinositides, they have a unique signalling capacity and regulatory role in cellular physiology [301]. The phosphoinositide species PtdIns(4,5)P2 and PtdIns(3,4,5)P3 have been shown to concentrate at the plasma membrane, possibly within LR structures [301]. Here, they may support high-affinity membrane-protein interactions, associating with small GTPases and effector molecules such as the protein kinase Akt [302]. The phosphoinositide species can then initiate the recruitment and activation of actin regulatory proteins and the regulation of ion channels, ultimately impacting cellular proliferation, migration, differentiation, and survival, among other metabolic effects [303-305]. Importantly, hydrolysis of the phosphoinositide species PtdIns(4,5)P2 occurs rapidly during T cell activation and is critical for the induction of IL-2 secretion and the regulation of T cell rigidity at the T cell/APC interface [306]. To investigate how the GPI
anchor regulates IL-2 production, we will assess differential levels of phosphatidylinositol (PI), monophosphorylated phosphatidylinositol (PIP), bisphosphorylated phosphatidylinositol (PIP2) such as PtdIns(4,5)P$_2$, and trisphosphorylated phosphatidylinositol (PIP3) such as PtdIns(3,4,5)P$_2$. As discussed, this lipidomic analysis will identify these species within LR of *ex vivo* and TCR/CD3-stimulated GPI-AP deficient, anchor-expressing (*Pigu*<sup>−/−</sup>) and anchorless (*Piga*<sup>−/−</sup>) CD4<sup>+</sup> T cells.

If anchor deficiency proves key to the growth survival advantage, our hypothesis follows that PI metabolism is fundamentally dysregulated in GPI anchor deficient T cells. The expectation is that these analyses will reveal differential phosphoinositide levels in anchor-deficient and anchor-sufficient variants and conclude our underlying mechanism(s).

*Final Thoughts:*

In sum, the results discussed in this thesis characterize a novel role for the GPI anchor and/or GPI-AP in T cell homeostasis. In both a clonal T cell model system, and in primary CD4<sup>+</sup> T cells, the absence of both GPI-AP and the GPI anchor constitutes a novel growth/survival phenotype. The IL-2-dependent prolonged proliferation and hypersensitive responses in the clonal and primary models, respectively, support the conclusion that GPI-AP and/or the GPI-anchor itself (GPI) play a seminal role in regulating T cell clonal contraction by augmenting early T cell activation. In this regard,
the GPI anchor or a specific GPI-AP may function as an inhibitory molecule or associate with an inhibitory molecule to dampen T cell responses. Further, and more generically, altered growth kinetics may contribute towards transformation events including lymphocytic leukemias. The growth/survival advantage of GPI-AP deficient cells in PNH patients, as described above, correlates with 5-15% of patients developing hematologic malignancies, including AML, ALL, and CLL.

Thus, our findings linking enhanced growth/survival with differential growth factor production may provide novel insight into a broader role for GPI-AP in the maintenance of cellular homeostasis and transformation. While the predisposition is reflective of a GPI-AP deficient clonal dominance and survival advantage in PNH patients, it extends beyond lymphocytes with the majority of blood dyscrasias originating from myeloid cells. As myeloid cells can also be sensitive to certain cytokines, perhaps they too may be impacted in an analogous fashion to our GPI-AP deficient T cells. Further investigation into the role for GPI-AP in the regulation of cellular homeostasis and transformation may provide insight into the mechanism underpinning the PNH phenotype, with the possible extension to other cell types and cytokines involved.
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