LIPID RAFTS AND THE REGULATION OF IL-2 RECEPTOR
SIGNALLING IN T CELLS: MODULATION OF IL-2R FUNCTION
BY GPI-AP AND GANGLIOSIDES

By

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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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Lipid Rafts and the Regulation of IL-2 Receptor Signalling in T Cells: Modulation of IL-2R Function by GPI-AP and Gangliosides.

Graduate Department of Immunology, University of Toronto.

Lipid rafts are plasma membrane microdomains characterised by a unique lipid environment enriched in gangliosides and cholesterol, leading to their insolubility in non-ionic detergents. Many receptors are constitutively or inducibly localised in rafts, which play a role in co-ordinating the induction of signalling pathways. Results presented in this thesis characterise a role for lipid rafts in regulating signalling through IL-2R. The study presented in Chapter 3 demonstrates that mAb-mediated immobilisation of components of lipid rafts, glycosyl-phosphatidyl-inositol anchored proteins (GPI-AP), inhibited anti-TcR-induced proliferation in T cells. Neither IL-2 production, nor the effector function of cytotoxic T lymphocytes was impaired in these circumstances. Rather, the responsiveness of T cells to IL-2 was inhibited, and immobilised mAb specific for GPI-AP blocked IL-2-mediated heterotrimerisation of IL-2Rα, β and γ chains. The phosphorylation of JAK1 and JAK3, indicative of their activation states, in response to IL-2 was correspondingly reduced. The results presented in Chapter 4 demonstrate that mAb specific for GPI-AP or ligand-mediated immobilisation of another component of lipid rafts, the GM1 ganglioside, inhibited the proliferation of T cells in response to exogenous IL-2. Investigation of the subcellular localisation of components of the IL-2R complex revealed that IL-2Rα is constitutively enriched in lipid rafts. Furthermore, the proportion of IL-2Rα in rafts was increased in the presence of immobilised anti-Thy-1, relative to controls. In contrast, IL-2Rβ and γ, as well as JAK1 and JAK3, are localised in detergent soluble membrane fractions, and their localisation is not altered in the presence of immobilised anti-Thy-1. IL-2-mediated heterotrimerisation of IL-2R chains occurs within soluble membrane fractions, as does activation of JAK1 and JAK3. These results suggest that signalling through IL-2R
takes place in detergent soluble membranes, and consistent with these observations, the
disruption of lipid raft integrity upon extraction of membrane cholesterol did not impair IL-2-induced signalling. Inhibition of IL-2R signalling by components of rafts appears to be
due to blocking the mobility and/or intermolecular interactions of IL-2Rα in lipid rafts with
molecules outside these microdomains. The sequestration of IL-2Rα in lipid rafts functions
to decrease its association with IL-2Rβ and γ chains and thus regulate IL-2-mediated
signalling.
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I am extremely grateful to the other members of the lab, Bernadine and Dominik, as well as Sheela and Philippe for their friendship, support and advice, both scientific and otherwise. Finally, I would like to thank my family and friends for their love and laughter.
CHAPTER 1: INTRODUCTION

Overview/Context

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GPI-AP mediate T cell activation
GPI-AP modulate T cell activation via TcR/CD3
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Expansion of GPI-AP cells in PNH
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>aplastic anaemia</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AICD</td>
<td>activation induced cell death</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BcR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>Cbp</td>
<td>Csk-binding protein</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CIS</td>
<td>cytokine inducible SH2 domain containing protein</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>cSMAC</td>
<td>central supra-molecular activation complex</td>
</tr>
<tr>
<td>CT</td>
<td>cholera toxin β subunit</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DIGs</td>
<td>detergent insoluble glycolipid-enriched membrane domains</td>
</tr>
<tr>
<td>DP</td>
<td>double positive (CD4^+CD8^+)</td>
</tr>
<tr>
<td>DRM</td>
<td>detergent resistant membranes</td>
</tr>
<tr>
<td>DSS</td>
<td>disuccinimidy suberate</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Etx</td>
<td>E. coli heat-labile enterotoxin</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FcεRI</td>
<td>high affinity receptor for IgE</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE inhibitory protein</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>Gads</td>
<td>Grb-2-related adaptor downstream of Shc</td>
</tr>
<tr>
<td>GaMIg-HRP</td>
<td>horseradish peroxidase-conjugated goat-anti-mouse IgG</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial-cell derived neurotrophic factor receptor-α</td>
</tr>
<tr>
<td>EGF</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GEMs</td>
<td>glycosphingolipid enriched membrane domains</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GFR</td>
<td>GDNF family receptors</td>
</tr>
<tr>
<td>GlcNAc-PI</td>
<td>N-acetyl-glucosaminyl-phosphatidylinositol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidyl-inositol</td>
</tr>
<tr>
<td>GPI-AP</td>
<td>glycosylphosphatidyl-inositol-anchored proteins</td>
</tr>
<tr>
<td>Grap</td>
<td>Grb-2 related adaptor protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine trisphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine trisphosphatase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEL</td>
<td>hen egg lysozyme</td>
</tr>
<tr>
<td>HSA</td>
<td>heat stable antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol-3,4,5-trisphosphate</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>
</tr>
<tr>
<td>Itk</td>
<td>inducible T cell kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus family kinases</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KIR</td>
<td>killer cell inhibitory receptor</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>LFA-1</td>
<td>leukocyte function antigen 1</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAL</td>
<td>myelin and lymphocyte protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>MCD</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PAG</td>
<td>phosphoprotein associated with GEMs</td>
</tr>
<tr>
<td>Pak</td>
<td>p21 associated kinase</td>
</tr>
<tr>
<td>PEP</td>
<td>PEST-domain phosphatase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI-3K</td>
<td>phosphatidylinositol-3-phosphate kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
</tbody>
</table>
PKC  protein kinase C
PLAP  placental alkaline phosphatase
PLCγ1  phospholipase C γ1
PNH  paroxysmal nocturnal hemoglobinuria
PRR  proline rich region
pSMAC  peripheral supra-molecular activation complex
PTPase  protein tyrosine phosphatase
PTK  protein tyrosine kinase
SAPK  stress activated protein kinase
SCID  severe combined immunodeficiency
SH2  Src homology domain 2
SH3  Src homology domain 3
SHIP  SH2-containing inositol phosphatase
SHP  SH2-containing tyrosine phosphatase
SLP-76  SH2 domain containing leukocyte protein of 76 kD
SMAC  supra-molecular activation complex
SOCS  suppressors of cytokine signalling
Sos  son-of-sevenless
SP  single positive (CD4+CD8+ or CD4+CD8−)
STAT  signal transducers and activators of transcription
TcR  T cell receptor
TGF-β  transforming growth factor β
TSA-1  thymic shared antigen 1
WASP  Wiscott-Aldrich Syndrome protein
ZAP-70  ζ-associated protein kinase 70kD
CHAPTER 1

INTRODUCTION
Overview/Context

T lymphocytes recognise antigen through the T cell receptor (TcR) composed of polymorphic α and β chains derived from gene rearrangement of the TcR loci. TcRαβ chains are associated with invariant CD3 molecules that mediate receptor signalling. The TcR/CD3 complex recognises antigen derived peptides presented in the context of major histocompatibility complex (MHC) molecules on antigen presenting cells (APC). CD8⁺ T cells recognise antigen in the context of MHC class I molecules, and can differentiate into cytotoxic T lymphocytes (CTL) that mediate cell lysis through the release of perforin and other enzymes. CD4⁺ T cells or helper T cells (Th) recognise antigen in the context of MHC class II, and promote the differentiation and effector functions of other cells, including CTL and B cells.

T cells develop within the thymus and are subject to positive and negative selection events which are based upon antigen receptor specificity. The end result of these processes ensures that mature T cells emigrating from the thymus are responsive to self-MHC in conjunction with foreign peptides, but lack auto-reactivity.

Signals resulting in the clonal expansion and differentiation of peripheral T cells are initiated upon the recognition of antigenic peptides presented by APC concomitantly with signals derived from co-stimulatory molecules. The most clearly defined T cell co-stimulatory molecules are the ligands for CD28, CD80 and CD86, the expression of which is upregulated on APC as a consequence of their interaction with microbes and/or by cytokines produced by cells comprising the innate immune system. Productive stimulation of T cells by antigenic peptides and co-stimulatory molecules results in the secretion of cytokines and the acquisition of cytokine responsiveness. Secretion of the major T cell growth factor,
interleukin-3 (IL-3) and subsequent signalling through the IL-2 receptor promote T cell expansion.

Homeostatic mechanisms serve to limit the magnitude and duration of immune responses. Activated T lymphocytes undergo passive cell death by apoptosis in the absence of the continued presence of antigen, co-stimulatory signals and cytokines. Further, T cell activation is tightly coupled to the induction of feedback mechanisms that limit clonal expansion and function through the induction of activation-induced cell death.

This thesis will discuss the current understanding of the function of glycosyl-phosphatidyl-inositol-anchored proteins (GPI-AP) in the regulation of T cell development, activation and the maintenance of homeostasis. GPI-AP exert signalling functions through their localisation in plasma membrane microdomains called lipid rafts, and the function of lipid rafts in signalling are presented. Lipid rafts are also critical for signalling through TcR/CD3, and TcR/CD3-induced signalling pathways are discussed with emphasis on the role of lipid rafts in co-ordinating these events. In addition, the function of the interleukin-2 receptor (IL-2R) is addressed, prior to the presentation of results demonstrating that lipid rafts play a role in the regulation of IL-2R signalling.

**GLYCOSYL-PHOSPHATIDYL-INOSITOL-ANCHORED PROTEINS (GPI-AP)**

A group of proteins are tethered to the outer leaflet of the plasma membrane via a complex glycolipid structure called a glycosyl-phosphatidyl-inositol (GPI) anchor. GPI-anchors have been highly conserved throughout evolution, and a common core structure composed of phosphatidyl-inositol-glucosamine-(mannose)₃-phosphoethanolamine is seen in GPI-AP in mammalian cells, yeast, and protozoa (2). A diagram of the structure of the conserved core of the GPI-anchor is shown in Figure 1-1.
The core GPI-anchor structure is composed of ethanolamine phospho-mannose, 3-glucosamine-inositol-phospholipid, and is conserved in all eukaryotes (1). Modifications of the core structure are found in most GPI-AP, and include extra phosphoethanolamine residues and extra sugars such as mannose, N-acetyl-galactosamine and sialic acid. The lipid portion of GPI-anchors can be composed of several alkyl and acyl species. In addition, some anchors contain an extra acyl group esterified to inositol, which confers resistance to cleavage by PI-PLC. The addition of the anchor to the protein moiety likely proceeds via a transamidation reaction, resulting in an amide linkage of the ethanolamine amino group with the C-terminal amino acid of the polypeptide chain. The cleavage sites of phosphatidyl-inositol specific phospholipases C (PI-PLC) and D (PI-PLD) are indicated.
GPI-anchor biosynthesis is complex, and as depicted schematically in Figure 1-2, at least 12 proteins are known to be involved in mammalian cells. The GPI molecule is synthesised by the sequential addition of carbohydrates to phosphatidyl-inositol on the cytoplasmic leaflet of the endoplasmic reticulum (ER) membrane. An intermediate is flipped across the ER membrane, and additional carbohydrate moieties are added on the lumenal face of the ER. Proteins destined to be GPI-anchored have a signal sequence directing translocation across the ER membrane (2). Further, a second signal sequence directs its cleavage and replacement by the mature GPI anchor via a transamidation reaction.

GPI-anchoring confers several properties upon protein moieties, including subcellular localisation in specialised membrane micro-domains, signalling capacities, changes in lateral mobility in the membrane, sorting to the apical surface of polarised cells, and susceptibility to cleavage by GPI-specific phospholipases C and D (1, 3). The current understanding of the function of GPI-AP expressed on T lymphocytes is discussed below. As a starting point, the expression patterns, structural characteristics and functions, where known, of the members of this class of proteins expressed on T lymphocytes are listed in Table I.

**GPI-AP in T cell development**

Early thymocytes lack expression of the CD4 and CD8 co-receptors (double negative, DN), progress to co-express CD4 and CD8 (double positive, DP) and subsequently downregulate one of the co-receptors to become either CD4+ or CD8+ single positive (SP) thymocytes. Negative selection, which results in the removal of cells from the system by apoptosis, occurs if thymocytes rearrange their TcR genes to encode an antigen receptor with a high affinity for self-peptide bound to MHC. Further, thymocytes that do not undergo
The pathway of GPI-anchor biosynthesis is schematically depicted, and the mammalian genes known to be involved are listed. Newly synthesised proteins bearing GPI-attachment signals are transferred to the GPI-anchor via a transamidation reaction (2). Subsequently, GPI-AP are transported to the cell surface by the secretory pathway. In some GPI-AP, inositol is deacylated.
Table I  
GPI-AP expressed on T lymphocytes (16)

<table>
<thead>
<tr>
<th>CD24 (HSA)</th>
<th>Expression pattern in hematopoietic cells</th>
<th>Structural characteristics / function</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells, granulocytes, monocytes, mouse thymocytes (except DP), RBC</td>
<td>putative role in growth and differentiation</td>
<td></td>
</tr>
</tbody>
</table>

| CD48 | T cells, B cells, macrophages, dendritic cells | 2 Ig superfamily domains  |

| CD52 | lymphocytes, monocytes | CD2 family, major ligand of mouse and rat CD2 |

<table>
<thead>
<tr>
<th>CD55 (DAF)</th>
<th>all hematopoietic cells</th>
<th>also expressed in transmembrane form in mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56 (NCAM)</td>
<td>human NK cells, some T cells</td>
<td>4 complement control protein domains complement regulation : inhibits autologous C3 convertase</td>
</tr>
</tbody>
</table>

| CD58 (LFA-3) | most hematopoietic cells  ġ half of B and T cells | also expressed in transmembrane form  |
| CD59 (MIRL) | leukocytes, RBC, platelets | 2 Ig superfamily domains  |
| CD73 | subsets of mature B and T cells | CD2 family, binds CD2  |
| CD87 (uPAR) | few resting T cells, activated T cells, monocytes, granulocytes, some NK cells | 1 Ly-6 domain complement regulation - binds C8 and C9, interferes with assembly/insertion of membrane attack complex  |
| CD90 (Thy-1) | human prothymocytes, mouse and rat thymocytes, mouse T cells | 1 Ig superfamily domain  |
| CDwl08 | T and B cells, myeloid and stromal cells | 3 Ly-6 domains binds urokinase plasminogen activator involved in leukocyte extravasation  |
| CD109 | activated T cells, platelets |  |
| gp97 (melano-transferrin) | T cell line | transferrin family (17)  |
| Ly-6A.2/E.1 (Sca-1, TAP) | hematopoietic stem cells, some resting and all activated T and B cells | 1 Ly-6 domain putative role in lymphocyte development and maturation  |
| Ly-6C | half of CD8 T cells, BM cells, monocytes, neutrophils | 1 Ly-6 domain  |
| PrPc | lymphocytes | cellular isoform of scrapie agent protein (18)  |
| Qa-2 | lymphoid, myeloid and stem cells (19) | non-classical MHC class I  |
| RT6 | rat peripheral T cells | 2 Ig superfamily domains  |
| ThB | mouse thymocytes, B cells | ADP-ribosyltransferase NAD-metabolising activity  |
| TSA-1/Sca-2 | immature thymocytes and precursors, B cells | 1 Ly-6 domain (17)  |
productive TcR rearrangement, or encode a receptor that does not interact with MHC with the appropriate affinity, fail to be positively selected, and are also removed from the system through death by apoptosis. Thus, a central mechanism underlying intrathymic T cell development involves selection events regulated by TcR-MHC/peptide interactions. However, a role for other cognate interactions in intrathymic T cell development and selection has also been established. For example, ligation of CD40L and CD30 appear to play critical roles in the developmental progression of thymocytes (20, 21).

GPI-AP have also been shown to perturb thymocyte development/selection, putatively by modulating antigen receptor-mediated signalling. A role for Thy-1 in negative selection was proposed upon the demonstration that mAb-mediated co-aggregation of Thy-1 and TcR results in thymocyte apoptosis (22-24). Moreover, antigen-mediated deletion of DP thymocytes in vitro was inhibited by anti-Thy-1 (25). Thymic shared antigen 1 (TSA-1) may also have a negative regulatory role in TcR-mediated signalling, as anti-TSA-1 blocks anti-CD3-induced, but not steroid or radiation-induced deletion of thymocytes (26). However, anti-TSA-1 does not affect positive selection of thymocytes (26). TSA-1 may also be involved in thymocyte development as TSA-1 specific mAb blocked DN to DP transition in fetal thymic organ culture (27). Importantly, the capacity to modulate thymocyte development may not be generalizeable to all GPI-AP, as mAb-mediated co-aggregation of ThB and TcR did not induce thymocyte apoptosis (24). The putative role of GPI-AP in thymocyte development was further analysed in GPI-AP transgenic and knockout mice, and is discussed below.

**GPI-AP mediate T cell activation**

Many, if not all, GPI-AP can induce the activation of mature T cells, and a listing of cellular responses induced by GPI-AP on T lymphocytes is provided in Table II. Increases in
the phosphotyrosyl content of intracellular substrates and in intracellular calcium concentrations are induced by mAbs specific for Thy-1, Qa-2, CD48, CD55, CD59, and CD73 (28-34). Furthermore, in the presence of accessory cells and/or co-stimulatory reagents such as PMA, T cell proliferation and cytokine secretion are observed following mAb-mediated aggregation of Thy-1, Ly-6A/E, Ly-6C, Qa-2, CD48, CD52, CD55, CD59 and CD73 (32, 35-41). Co-expression of the TcR/CD3 complex is critical for proliferation and IL-2 secretion, but not increases in intracellular calcium concentrations, in response to mAbs specific for Thy-1, Ly-6A/E, CD55, and CD73 (31, 42-44). Specifically, expression of the CD3ζ chain is required for T cell proliferation and cytokine production via GPI-AP (42, 43). Although there are some qualitative differences between signalling through some GPI-AP and TcR/CD3 (45), signalling through GPI-AP may result, at least in part, from an association with elements of the antigen receptor complex.

**GPI-AP modulate T cell activation via TcR/CD3**

It has been hypothesised that GPI-AP are critical for the activation of T cells through TcR/CD3. Reduction of Ly-6A/E expression following in vitro mutagenesis, or treatment of cells with antisense oligonucleotides, resulted in decreased antigen receptor responsiveness (58, 59), as a result of reduced surface TcR expression (60). Further, the earliest responses to TcR/CD3-induced signalling, including the tyrosine phosphorylation of CD3ζ and increases in intracellular calcium concentrations, were decreased in cell lines or human peripheral blood cells lacking expression of GPI-AP due to mutations in GPI-anchor biosynthesis (61, 62). However, the above results remain controversial, as other investigators detected comparable antigen-receptor-induced responses in human peripheral blood cells expressing or lacking GPI-AP (32, 63, 64). Moreover, removal of cell surface GPI-AP by PI-PLC does not affect responsiveness to anti-CD3 (65).
<table>
<thead>
<tr>
<th>GPI-AP</th>
<th>Cells</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD48</td>
<td>human T cell line</td>
<td>increased $[Ca^{2+}]_{i}$ (30)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>IL-2 secretion, proliferation with PMA (39)</td>
</tr>
<tr>
<td></td>
<td>murine T cell line</td>
<td>inhibition of anti-CD3ε-induced IL-2 secretion, proliferation, increased $[Ca^{2+}]_{i}$, and PI turnover (39, 46)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>synergizes with anti-CD3ε to induce proliferation (47)</td>
</tr>
<tr>
<td>CD52</td>
<td>human T cells</td>
<td>IL-2 secretion, proliferation with PMA (40)</td>
</tr>
<tr>
<td>CD55</td>
<td>human T cell line</td>
<td>tyrosine phosphorylation (31)</td>
</tr>
<tr>
<td></td>
<td>human T cells</td>
<td>IL-2 secretion, proliferation with PMA (48)</td>
</tr>
<tr>
<td>CD59</td>
<td>human T cell line</td>
<td>increased $[Ca^{2+}]_{i}$, PI turnover (32)</td>
</tr>
<tr>
<td></td>
<td>human T cells</td>
<td>IL-2 secretion, proliferation with PMA (32)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>inhibition of anti-CD3ε-induced proliferation (49)</td>
</tr>
<tr>
<td>CD73</td>
<td>human T cells</td>
<td>increased $[Ca^{2+}]_{i}$, tyrosine phosphorylation (33, 34)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>IL-2R expression, IL-2 secretion, proliferation with PMA (41, 44)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>synergizes with anti-CD3ε to induce proliferation (33)</td>
</tr>
<tr>
<td>CD90</td>
<td>murine T cell hybridoma</td>
<td>proliferation (35)</td>
</tr>
<tr>
<td>(Thy-1)</td>
<td>murine T cells</td>
<td>increased $[Ca^{2+}]_{i}$ (28, 51)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>proliferation with PMA (51)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>inhibition of anti-CD3ε-induced IL-2 secretion, proliferation (52, 53)</td>
</tr>
<tr>
<td></td>
<td>murine thymocytes</td>
<td>apoptosis (24)</td>
</tr>
<tr>
<td></td>
<td>murine T cell clone</td>
<td>synergizes with anti-CD3ε to induce increased $[Ca^{2+}]_{i}$, tyrosine phosphorylation, IP$_1$ formation and apoptosis (22)</td>
</tr>
<tr>
<td>Ly-6A/E</td>
<td>murine T cells, T cell hybridomas</td>
<td>IL-2 secretion, proliferation with PMA (36)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>inhibition of anti-CD3ε-induced IL-2 secretion (55)</td>
</tr>
<tr>
<td></td>
<td>murine thymoma</td>
<td>induction of NFkB, AP-1 and NF-AT transcription factors (45)</td>
</tr>
<tr>
<td></td>
<td>murine thymoma</td>
<td>inhibition of anti-CD3ε-induced NFkB, AP-1 and NF-AT transcription factors (45)</td>
</tr>
<tr>
<td>Ly-6C</td>
<td>murine thymoma</td>
<td>IL-2 secretion (37)</td>
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<tr>
<td></td>
<td>murine T cell clone</td>
<td>inhibition of anti-CD3ε-induced IL-2 secretion (37)</td>
</tr>
<tr>
<td></td>
<td>murine T cell clone</td>
<td>inhibition of antigen-induced cytolytic activity and IFN-γ secretion (54)</td>
</tr>
<tr>
<td>Qa-2</td>
<td>murine T cells</td>
<td>increased $[Ca^{2+}]_{i}$ (29)</td>
</tr>
<tr>
<td></td>
<td>murine T cells</td>
<td>IL-2R expression, IL-2 secretion, proliferation with PMA (29, 38, 59)</td>
</tr>
<tr>
<td>TSA-1 (Sca-2)</td>
<td>murine T cell lines</td>
<td>inhibition of anti-CD3ε-induced IL-2 secretion and CD3ε phosphorylation (56, 57)</td>
</tr>
<tr>
<td></td>
<td>murine T cell hybridoma</td>
<td>inhibition of anti-TCRB- or anti-CD3ε-induced thymocyte apoptosis (26)</td>
</tr>
<tr>
<td></td>
<td>in vivo in mice, murine T cell hybridoma</td>
<td></td>
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</tbody>
</table>
The demonstration that T cells lacking all GPI-AP due to the tissue specific disruption of a gene critical in GPI-anchor biosynthesis, can respond to antigen in vitro and in vivo (66), suggests that GPI-AP are not critical for the generation of signals through the antigen receptor complex on T lymphocytes. However, GPI-AP may function in the potentiation of early signals generated through the TcR. For example, soluble anti-CD48 enhanced TcR-induced tyrosine phosphorylation of intracellular substrates and cytokine secretion (67).

GPI-AP may also play an important role in the negative regulation of T cell responses generated via the TcR/CD3 complex (see Table II), and mAb specific for Thy-1, Ly-6A/E, Ly-6C, CD48 and TSA-1 can inhibit anti-TcR/CD3-induced T cell proliferation and cytokine secretion (37, 39, 53, 55, 68). Early responses generated through TcR/CD3, including the tyrosine phosphorylation of CD3ζ and other intracellular substrates (57), as well as increases in intracellular calcium concentrations (46), were inhibited by mAbs specific for GPI-AP.

Thus, GPI-AP can function in T cell activation, as well as in modifying cellular responses to TcR/CD3 ligation. Importantly, the distinct consequences of signalling through various GPI-AP on the physiology of T lymphocytes in vitro depends upon the protocol used to ligate these molecules. Aggregation of GPI-AP-specific mAb by secondary antibodies results in cellular activation. In contrast, soluble mAb specific for GPI-AP, in the presence of FcR-bearing accessory cells, inhibits T cell activation induced by anti-TcR/CD3, blocking the de novo transcription and translation of IL-2. The distinct effect of mAbs presented in these systems may be a result of their differential capacity to prevent the interaction of GPI-AP with ligands, to induce signalling events, or to impede the mobility of the molecules within the plasma membrane.
**GPI-AP transgenic and knockout mice**

While functions imparted by the GPI-anchor, including those involved in modulating signalling, may be redundant, the extracellular portion of many GPI-AP are important in regulating signalling by binding ligands, as well as in other cellular processes, such as adhesion. The function of individual GPI-AP can be assessed upon analyses of transgenic mice and of mice lacking given molecules through targeted disruption of the respective loci.

A role for Thy-1 in growth control was supported by the demonstration that the expression of Thy-1 in inappropriate tissues (B lymphocytes and kidney tubular epithelia) of transgenic mice leads to proliferative abnormalities (69, 70). Thy-1 is not required for T cell development, and the number and phenotype of thymocytes and mature T cells in Thy-1-deficient mice were normal (25, 71), although alterations in thymic membrane contacts (71) may reflect a putative role for Thy-1 in the binding of murine thymocytes to resident epithelial cells (72). Thy-1 was not required for either positive or negative selection (25). However, thymocytes from Thy-1−/− mice exhibited modest increases in responsiveness to stimulation through the antigen receptor, and the differentiation of thymocytes from the DP to the SP stage was decreased, perhaps due to increased TcR-signalling and negative selection (71). Thy-1 may be also involved in the regulation of peripheral T cell responses, as Thy-1-deficient mice displayed decreased contact and delayed type hypersensitivity responses *in vivo* (73). Furthermore, anti-CD3ε-induced tyrosine phosphorylation of intracellular substrates, calcium mobilisation and proliferation were decreased in Thy-1-deficient mice (73). Whether and how the developmental defects of Thy-1−/− T cells is related to the generation of mature peripheral T cells with decreased TcR-mediated signalling capacity remains to be elucidated.
CD48 is expressed on T and B lymphocytes, macrophages and dendritic cells. Mice deficient in CD48 exhibit normal lymphoid development, however TcR/CD3-induced proliferative responses were partially impaired (74).

Ly-6A.2, or its alternate allelic form Ly-6E.1, is expressed on pluripotent hematopoietic stem cells, thymocyte precursors and DN thymocytes. Ly-6A/E expression is downregulated on DP thymocytes and re-expressed on the majority of mature T cells. Ly-6A expression is not required for T cell development, but T cell activation was dysregulated in Ly-6A" mice (75). Relative to cells from normal littermates, Ly-6A" T cells exhibited a higher and prolonged rate of proliferation in response to stimulation through TcR/CD3 in vitro, using either mAbs or allogeneic stimulator cells. The increased amplitude and prolonged kinetics of proliferative responses observed in Ly-6A-deficient mice are consistent with a role for this molecule in downregulating T cell responses to antigen.

Although Ly-6A is not required for T cell development (75), transgenic overexpression of Ly-6A.2 abrogates thymocyte maturation, resulting in a profound (90%) reduction in thymus cellularity, affecting both DP and SP thymocytes (76). Although the block in development was found to occur at the stage of development at which Ly-6A expression is normally downregulated, the overexpression of Ly-6A rather than its dysregulated expression may be responsible for the block in maturation, as transgenic mice which expressed lower levels of Ly-6A were less profoundly affected.

Mating Ly-6A-transgenic mice with mice lacking MHC class I and class II molecules led to the striking observation that CD4" T cells are generated in these animals (77). Thus, Ly-6A overexpression resulted in an MHC-independent signal sufficient to result in the maturation of CD4" T cells, which functioned normally with respect to anti-CD3e-induced proliferation and activation-induced expression of CD40L. The interaction of Ly-6A with a putative ligand in the thymus may mimic TcR signalling, possibly through the utilisation of
components of the TcR/CD3 complex for signalling. The development of CD4\(^+\), but not CD8\(^-\) T cells in Ly6A-transgenic mice is likely due to progression along the default developmental pathway, consistent with the demonstrated requirement for additional signals for development along the CD8\(^-\) T cell lineage (78).

Heat stable antigen (HSA) or CD24 is expressed on early thymocyte precursors and DN thymocytes. Its expression decreases upon maturation such that DP thymocytes express low levels of HSA and SP thymocytes are HSA\(^-\). Transgenic overexpression of HSA in thymocytes and T cells led to a pronounced defect in thymocyte maturation, characterised by a 10-20 fold decrease in the number of DP and SP thymocytes, but without change in the number of DN thymocytes (79). In addition, decreased expression of TcR and CD3 was detected on many DP thymocytes in HSA-transgenic mice. This phenotype is similar to that observed in mice expressing a transgenic TcR specific for self antigen, which results in the negative selection of the majority of thymocytes (80).

These results demonstrate that Thy-1, CD48 and Ly-6A/E are dispensable for T cell development. In striking contrast, transgenic overexpression of two GPI-AP, Ly-6A and HSA, led to a profound block in T cell maturation. In the absence of GPI-AP, TcR signalling proceeds and thymocyte development is relatively unaffected. However, when present at increased levels on the cell surface, aberrant signalling through GPI-AP, presumably as a result of interaction with ligands in the thymus, may synergise with TcR/CD3-mediated signalling to modulate the intensity or duration of the signal and result in a signal of sufficient intensity to mimic negative selection and ensuing apoptosis. In the absence of ligand for TcR, signalling through a GPI-AP may be of an intensity consistent with positive selection resulting in further thymocyte development, as was observed in Ly-6A-transgenic, MHC knockout animals (77). An exception to the consequences of forced expression of GPI-AP on thymocyte development may provide mechanistic insight into the requirements
for signalling through GPI-AP. Transgenic expression of CD73 on murine thymocytes does not affect T cell development (81), possibly because CD73 is an ecto-5'-nucleotidase, which may not signal upon binding to its soluble substrates AMP and dAMP.

The developmental block observed in Ly-6A and HSA-transgenic mice was at the stage at which their expression is normally downregulated (76, 79). In this context, it is of interest to note that the expression of many GPI-AP postulated to have thymic ligands is subject to developmental regulation. The expression of Ly-6A/E and HSA on thymocytes is downregulated upon maturation of thymocytes from the DN to DP stage, while Thy-1 and TSA-1 are downregulated upon maturation of DP thymocytes to the SP stage. The downregulation of these GPI-AP may be required to correctly temper antigen receptor signalling in aid of achieving the appropriate physiological outcome. Further insights into the function of GPI-AP as a class of molecules is derived from analysis of cells lacking expression of all GPI-AP due to defects in GPI anchor biosynthesis.

**Paroxysmal nocturnal hemoglobinuria**

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease in humans in which a proportion of hematopoietic cells lack of expression of GPI-AP [reviewed in (82)]. PNH is characterised by pancytopenia, venous thrombosis and intravascular hemolysis due to complement-mediated lysis of red blood cells as a result of the absence of two GPI-anchored complement-regulatory molecules, CD55 and CD59. Increased susceptibility to venous thrombosis may be due to the lack of expression of the GPI-anchored receptor for a plasminogen activator (83). Following diagnosis, the median survival time of patients with PNH is 10 to 15 years, although roughly one quarter of patients survive for more than 25 years, with approximately half undergoing spontaneous remission.
In all reported cases of PNH, the molecular defect resulting in the inability to express GPI-AP is a somatic mutation in the \textit{PIG-A} gene in a hematopoietic stem cell (HSC). \textit{PIG-A}, which encodes a protein involved in the first step of GPI anchor biosynthesis, is localised on the X chromosome. Somatic cells are therefore haploid with respect to \textit{PIG-A} as a result of X chromosome inactivation, and a single mutation in the expressed copy of \textit{PIG-A} results in a deficiency in GPI anchor biosynthesis. Mutations in the \textit{PIG-A} gene in PNH patients include small deletions, insertions and nonsense mutations, resulting in a lack or deficiency in enzymatic activity or correct localisation to the endoplasmic reticulum (82).

The proportion of blood cells lacking GPI-AP in PNH patients varies widely, with reports of between 0.5 to >95% affected erythrocytes and granulocytes (84). Almost all PNH patients have GPI-deficient T, B and NK cells, albeit at a lower proportion than granulocytes or red blood cells (84-87). The range of affected T cells varies between 0.2 to 52.7% of T cells. Abnormalities in the numbers of lymphocyte subsets are common in PNH, with low numbers of B cells and NK cells detected in 95% of patients, and mild reduction in the numbers of CD4$^+$ T cells in 53% of patients (84). These abnormalities are similar to those observed in patients with aplastic anaemia (AA), and may reflect a general defect in hematopoiesis associated with PNH.

In PNH patients, a higher frequency of CD45RA$^+$ (naïve) T cells were GPI$^-$ than were CD45RO$^+$ (memory) T cells (63, 85). One possible explanation is that T cells lacking GPI-AP cannot respond to antigen with transition to the memory phenotype. However, CD45RA$^+$ cells isolated from PNH patients acquired a CD45RO$^+$ memory phenotype when stimulated with PHA \textit{in vitro} (85). Thus, an alternative and favoured explanation is that affected T cells in PNH represent a more recently generated population of cells which have not yet encountered antigen.
Expansion of GPI⁻ cells in PNH

In PNH, one or several HSC clones bearing a somatic mutation in PIG-A expands such that it accounts for a significant proportion of hematopoiesis. The expansion of GPI⁻ cells may be due to an intrinsic growth or survival advantage imparted by the lack of expression of GPI-AP. Alternatively, external factors may exert a selection pressure on hematopoiesis to which GPI⁻ cells may be less susceptible, and thus would expand relative to their GPI⁺ counterparts. The selective pressure allowing for the expansion of GPI⁻ cells may be an environment of bone marrow failure, and PNH is characterised by pancytopenia. Moreover, there is a close relationship between PNH and AA, with roughly half of patients with AA developing PNH. As bone marrow failure in AA is immune mediated, it was hypothesised that the environment enabling the relative expansion of GPI⁻ cells in PNH was an autoimmune condition leading to generalised bone marrow failure, while selectively sparing cells lacking GPI-AP. Potential causes for the preferential survival of GPI⁻ cells are that one or several GPI-AP may be the autoantigen(s) driving the immune reaction, or adhesion or co-stimulatory molecule(s) contributing to immune-mediated clearance of normal cells.

Evidence of bone marrow failure or suppression in PNH is supported by decreased numbers and clonogenic potential of hematopoietic precursor cells in PNH patients (88). Moreover, GPI⁻ and GPI⁺ cells from individual patients displayed similar colony forming potential, suggesting an underlying proliferative defect common to both normal and GPI⁻ cells (88). Nonetheless, precursors lacking expression of GPI-AP may have an advantage in hematopoiesis as the proportion of mature GPI⁻ cells observed in peripheral blood or in vitro cultures was greater than the proportion of precursor cells with this phenotype (88, 89).

Analysis of peripheral blood cells of normal healthy individuals revealed a low frequency of cells lacking expression of GPI-AP due to a mutation of the PIG-A gene (90).
Moreover, one control exhibited a mutation identical to one found in a PNH patient. Thus, a mutation in \( PIG-A \), even one associated with disease, is not sufficient to result in PNH, and cannot therefore be solely responsible for the disease condition. While it cannot be ruled out that a subsequent mutation in \( PIG-A \) mutant cells leads to their expansion in PNH patients, a more likely explanation is that cells lacking GPI-AP will expand only in the context of selection pressure which confers a selective advantage.

Further evidence supporting a model whereby \( PIG-A \) mutant cells constitute the majority of a hematopoietic lineage following an immune-mediated selective pressure was provided by \textit{in vivo} treatment of humans with an antibody specific for a GPI-AP. Patients with rheumatoid arthritis or B cell lymphoma were treated with an antibody specific for CD52, a GPI-anchored glycoprotein expressed on lymphocytes. Following treatment, a proportion of these patients displayed CD52$^-$ lymphocytes, which also lacked expression of other GPI-AP (64, 91). These findings are consistent with the presence of mutant cells in patients prior to treatment. The absolute numbers of cells lacking GPI-AP did not increase over the course of observation and the proportion of GPI$^+$ lymphocytes decreased upon re-emergence of normal lymphocytes post-treatment, again suggesting that the lack of GPI-AP does not confer a growth advantage.

In contrast, several reports provide evidence for an intrinsic proliferative and/or survival advantage in cells lacking GPI-AP. In 11 out of 15 PNH patients, following \textit{in vitro} stimulation of T cells, the proportion of GPI$^+$ cells relative to GPI$^-$ cells increased over their proportion prior to stimulation (85). Further, stem cells from PNH patients, but not controls, engrafted sub-lethally irradiated SCID mice in the absence of human cytokines (92). Moreover, despite the chimerism between GPI$^+$ and GPI$^-$ cells from PNH patients, all engrafted human cells lacked expression of GPI-AP. As the immune system in SCID mice is
compromised and was further impaired by sub-lethal irradiation, it is unlikely that GPI\textsuperscript{-} cells displayed preferential engraftment as a result of escape from an immune-mediated clearance.

A survival advantage in GPI\textsuperscript{-} cells is supported by the demonstration that granulocytes from PNH patients were resistant to apoptosis following serum starvation, relative to cells from controls (93). Furthermore, when GPI\textsuperscript{-} and GPI\textsuperscript{+} cells from PNH patients were fractionated, the GPI\textsuperscript{-} population exhibited a decreased rate of cell death. Moreover, upon restoration of GPI-AP expression in a mutant lymphoblastoid cell lines, the induction of apoptosis following serum starvation, or DNA damage by ionising radiation was increased relative to the GPI\textsuperscript{+} variants. An anti-apoptotic effect of a mutation in PIG-A could account for clonal dominance, as well as functioning as a pre-disposing factor in neoplastic transformation. Indeed, PNH can be considered a pre-leukemic condition, as the development of acute leukemia is a complication of PNH with a risk factor 85 times over that in the general population (82).

However, it remains controversial whether cells lacking expression of GPI-AP have a proliferation and/or survival advantage. Although several studies confirmed that cells from PNH patients were less susceptible than control cells to spontaneous, serum starvation- or anti-Fas-induced apoptosis, they found no close correlation between apoptosis resistance and the dominance of the PNH phenotype (94, 95). Moreover, relative resistance to apoptosis was also observed in GPI\textsuperscript{+} cells from patients with AA or myelodysplastic syndrome (94). These authors suggested that the resistant phenotype is independent of PIG-A, and may result from the bone marrow damage common to these diseases. Further, and in direct contradiction to previously reported results (93), GPI\textsuperscript{+} and GPI\textsuperscript{-} variants of same JY5 lymphoblastoid cell line used in the latter studies exhibited similar rates of apoptosis following serum starvation, anti-Fas treatment or ionising radiation (95).
**PIG-A-deficient mice**

The role of GPI-AP in the growth and differentiation of hematopoietic cells was further investigated upon the generation of mice chimeric for *PIG-A*-deficient cells (96-100). GPI-AP are essential for mouse development, and *PIG-A*-deficient cells could not contribute to the development of heart, lung, kidney, brain and liver (98). In contrast, these proteins are not essential for hematopoiesis, and GPI-AP red blood cells, T cells and B cells were generated in the chimeric mice. Further, mice lacking expression of GPI-AP exclusively on T lymphocytes were generated by directing expression of the Cre recombinase to thymocytes of mice carrying *lox-P* sites flanking the *PIG-A* gene (66). Analysis of these mice confirmed that GPI-AP are not essential for T cell development, or for antigen receptor-mediated responses *in vivo* and *in vitro*.

The proportion of GPI-AP hematopoietic cells in chimeric mice remained constant or declined with time (96, 97, 99, 100), demonstrating that *PIG-A*-deficiency was insufficient to result in clonal dominance of GPI-AP cells. Further, expression of the disrupted *PIG-A* allele was not over-represented in hematopoietic cells from *PIG-A*-/- chimeric mice, as would be the case if GPI-AP cells displayed a growth advantage (98). However, the proportion of T cells lacking GPI-AP was 3 to 10-fold higher than other hematopoietic lineages (96, 97, 99, 100). The increased representation with respect to other lineages may reflect a proliferative or survival advantage of GPI-AP T cells relative to GPI-AP T cells. Alternatively, T cell differentiation may be less affected by the absence of GPI-AP, relative to other hematopoietic lineages.

The mechanism underlying the clonal expansion of GPI-AP cells in PNH patients remains unclear since analyses of *PIG-A*-deficient animals demonstrate that the lack of expression of GPI-AP is insufficient for clonal dominance of the affected cells. However, GPI-AP may function in the regulation of proliferation and survival, and their absence may
lead to a proliferative and/or survival advantage following stimulation. While the proportion of GPI- hematopoietic cells, including lymphocytes, did not increase over time in mice chimeric for \( PIG^{-}\) null cells (96, 97, 99, 100), these mice were likely immunologically naive, housed in specific pathogen free conditions with minimal exposure to antigens inducing T cell responses. Insight into a putative role for GPI-AP in the regulation of T cell responses could be provided by the repeated immunisation of such chimeric mice. Assuming the presence of antigen reactive cells amongst both GPI\(^{+}\) and GPI\(^{-}\) T cells, cells from each pool would become activated and expand in response to immunisation. The combined or individual consequences of proliferative and/or survival advantages of GPI\(^{+}\) cells following activation may lead to their accumulation over time.

**Signalling through GPI-AP: association with transmembrane proteins**

The mechanism through which GPI-AP transduce signals is not understood, as they are tethered to the outer leaflet of the plasma membrane and lack transmembrane and cytoplasmic domains. One possibility is that signalling events are transmitted via a transmembrane protein associated with GPI-AP. Several GPI-anchored receptors function in this manner, including the GDNF (glial-cell derived neurotrophic factor receptor-\( \alpha \)) family receptors (GFRs). Upon ligand binding, signalling through GDNF and neurturin receptor-\( \alpha \) occurs via association of GFRs with and subsequent activation of the Ret transmembrane receptor protein tyrosine kinase (PTK) (101-104). Similarly, ligand binding by GPI-anchored ciliary neurotrophic factor receptor-\( \alpha \) results in signalling via its association with gp130, a transmembrane protein which results in JAK kinase activation (105). Furthermore, in myeloid cells, GPI-anchored CD14, Fc\( \gamma \)RIIIB and CD87 associate with the \( \beta 2 \) integrin CR3, and CD87 also interacts with \( \beta 1 \) integrins (106).
In T lymphocytes, a transmembrane protein, p100, was shown to co-immunoprecipitate with Thy-1 and ThB specific mAbs (107). Expression of p100 seemed to be required for signalling through Thy-1, as a sub-population of primary CD4+ T cells lacking p100 could not be activated upon aggregation of Thy-1 (108). In addition, myelin and lymphocyte protein (MAL), a proteolipid, was co-precipitated with GPI-AP and Src family PTK in T cells, and co-localised with these molecules in the plasma membrane (109). A 43kDa transmembrane protein associated with CD55 was detected in B cells and T cells, and mAb specific for this protein induced a similar pattern of tyrosine phosphorylated intracellular proteins as did CD55-specific mAbs (110).

These integral transmembrane proteins may link GPI-AP with the cytosolic signalling machinery, however, they cannot account for the signalling capacity of all GPI-AP. Expression of p100 is restricted to T cells (107), and although it is implicated in Thy-1-mediated signalling, Thy-1 can activate basophilic cell lines or transfected B cells in the absence of p100. Further, MAL is not expressed on all T cells (109), and the 43kDa CD55-associated protein was not associated with CD59, another GPI-AP (110). As described below, signalling through the majority of GPI-AP may result from direct effects on the activity of signalling molecules co-localised with GPI-AP in specialised membrane microdomains referred to as lipid rafts.

**Lipid rafts: signalling platforms in the plasma membrane**

It is remarkable that GPI-AP, while structurally diverse, are capable of mediating similar signalling functions. This supports the contention that the GPI-anchor imparts signalling competency upon protein moieties, which may result from the anchor-dependent localisation to specialised sub-domains of the plasma membrane postulated to function as platforms for signalling and membrane trafficking (111). In addition to GPI-AP, these
domains are enriched in cholesterol and glycosphingolipids (gangliosides), as well as signalling molecules such as Src family PTK, the adaptor protein LAT, heterotrimeric and small G-proteins and phosphoinositides (112-116). These membrane areas, referred to as lipid rafts, are characterised by a relative insolubility in non-ionic detergents, and thus have also been called detergent-resistant membranes (DRMs), detergent-insoluble glycolipid-enriched membrane domains (DIGs), and glycosphingolipid-enriched membrane domains (GEMs) (117).

Lipid rafts are most commonly isolated on the basis of their detergent insolubility and low buoyant density, using discontinuous sucrose gradient centrifugation of non-ionic detergent lysates (111). In addition, immunofluorescence microscopy of rafts aggregated to form membrane patches has revealed co-clustering of lipid raft components and their segregation away from non-raft proteins (118), and has been used to identify weak, detergent-sensitive associations of proteins with lipid rafts (119). Lipid rafts have also been visualised by immuno-electron microscopy (120). Importantly, lipid rafts have been detected in living cells using chemical cross-linking to identify raft protein complexes (121). Further, studies using fluorescence resonance energy transfer (FRET) have demonstrated the proximity of raft components, confirming the presence of lipid rafts in living cells and leading to the estimation that lipid rafts are less than 70 nm in diameter (122, 123). In addition, analyses using fluorescence recovery after photobleaching (FRAP) and single particle tracking revealed that GPI-AP are trapped in membrane microdomains (124-126).

Caveolae, a subset of lipid rafts, are flask-shaped membrane invaginations also thought to function in membrane trafficking, endocytosis and signalling (127). These two plasma membrane domains share common features, including detergent insolubility, low density, and enrichment in cholesterol. Caveolae can fuse with lipid rafts under certain experimental conditions, for example. GPI-AP and glycosphingolipids are sequestered into
caveolae following antibody-mediated cross-linking (128, 129). However, these membrane
domains can be separated experimentally (130, 131), and moreover, show differing protein
composition and morphology. In addition, lipid rafts are present in cells, such as
lymphocytes, which do not express caveolin, a cholesterol-binding integral membrane
protein critical for caveolae formation (132, 133).

The formation and composition of lipid rafts appears to result from interactions of
lipid groups. Glycosphingolipids and cholesterol in the exoplasmic leaflet of the membrane
are thought to organise into ‘liquid-ordered domains’ of decreased membrane fluidity, which
separate from glycerophospholipids and most transmembrane proteins in the plasma
membrane (111). The long saturated acyl chains of glycosphingolipids impart a high degree
of order which is further stabilised by intercalating cholesterol molecules. The acyl and alkyl
chains of the GPI anchor target GPI-AP to lipid rafts (134). Indeed, lipid raft localisation is
predicated by the presence of long saturated fatty acids, and was not observed for a GPI-AP
containing acyl and alkyl chains with 14, instead of 16 or 18, carbon molecules (135).

The lipid environment of the cytoplasmic leaflet of lipid rafts remains to be fully
characterised, although it is likely enriched in cholesterol and phospholipids with saturated
fatty acids. Modification of intracellular proteins with saturated fatty acids can result in their
targeting to the membrane microdomains. The localisation of Src family PTK Lck and Fyn
to lipid rafts is dependent upon both N-terminal amide-linked myristoylation on glycine and
thioester-linked palmitoylation of two cysteine residues (136). Src, which is myristoylated
but not palmitoylated, does not co-localise with GPI-AP in these microdomains (136).
Similarly, the myristoylation and palmitoylation of Gα subunits results in their localisation to
lipid rafts (137). In addition, the localisation of LAT to lipid rafts requires dual
palmitoylation of the transmembrane protein on two juxtamembrane cysteine residues (114).
Although palmitoylation is not sufficient for targeting to lipid rafts and proteins in detergent
soluble membranes contain palmitate (138), myristoylation and palmitoylation or dual palmitoylation appear to constitute raft localisation signals and are sufficient to target exogenous proteins to the membrane microdomains (139-141). Importantly, hydrophobic interactions mediated by lipid groups are not sufficient to impart raft localisation, as lipid modification of proteins with unsaturated prenyl groups, which are branched and bulky structures not predicted to fit well into an ordered environment, appears to result in their specific exclusion from lipid rafts (138).

Some transmembrane proteins are enriched in lipid rafts, although the majority appear to be excluded from these membrane areas. The lipid raft targeting of several transmembrane proteins, including LAT and CD8, is dependent on their palmitoylation (114, 142), while integrins may localise to lipid rafts as a result of lectin-like interactions with carbohydrates on GPI-AP (106, 143). The basis of the raft localisation of other transmembrane proteins remains unclear, but requires critical residues within the transmembrane domain and particularly hydrophobic residues in contact with the exoplasmic leaflet of the lipid bilayer (144). In addition, multiple transmembrane receptors, including TcR/CD3, FceRI and BcR, are inducibly associated with lipid rafts upon ligand binding. The mechanism underlying this association has not yet been characterised, but likely relates to an increased affinity of the oligomerised proteins for lipid rafts (117).

Targeting to lipid rafts, and specifically, co-localisation with Src family PTK, may underlie the remarkable capacity of all GPI-AP to induce Src family PTK activation and result in similar patterns of tyrosine phosphorylation of intracellular substrates and subsequent T cell signalling. Src family PTK were first implicated in signalling through GPI-AP as these were co-precipitated in non-ionic detergents (112). Moreover, the association of GPI-AP with Src family PTK predicated their capacity to induce tyrosine phosphorylation and cellular growth (119, 145). In addition, the signalling capacity of
purified CD59, which incorporates into the plasma membrane, was dependent on its incorporation into lipid rafts and association with Src family PTK (146). Importantly, lipid rafts have also been shown to be critical for those GPI-AP in which transmembrane proteins are important for signalling. Following binding of GDNF, GPI-linked GFRα recruits the Ret receptor PTK to lipid rafts, and the lipid raft localisation of Ret is required for optimal signalling (147).

The critical nature of lipid rafts in signalling via GPI-AP was further supported by studies involving changes in the lipid environment in lipid rafts. CD59 and CD48-induced increases in intracellular calcium concentrations were inhibited by decreasing the cellular cholesterol content, and thus the cholesterol content in lipid rafts (148). Furthermore, GPI-AP-mediated signalling was inhibited by treatment of T cells with polyunsaturated fatty acids, which resulted in decreased protein palmitoylation (149) and the displacement of Lck and Fyn from lipid rafts (150).

Consistent with the notion that GPI-AP function in signalling as a result of their localisation in lipid rafts, gangliosides enriched in lipid rafts can mediate comparable signalling functions. Gangliosides are glycosphingolipids composed of a hydrophobic ceramide moiety anchored in the lipid bilayer and hydrophilic carbohydrate and sialic acid moieties which extend from the outer leaflet of the plasma membrane and can be ligated using mAbs (151). In brain tissue, the GD3 ganglioside co-localises with the Src family PTK Lyn in lipid rafts, and mAb-mediated ligation of GD3 results in Lyn activation (152). GD3 specific mAb also induce proliferation in T cells (153-155). Similarly, in mast cells and basophilic leukemia cells, the GD1b ganglioside associates with and activates Lyn (156), resulting in the tyrosine phosphorylation of multiple intracellular proteins (157). The GM1 ganglioside can be ligated using the β subunit of cholera toxin (CT) and E. coli heat-labile enterotoxin (ETx) (158), which modulate both T and B cell responses in vitro and in vivo
In T cells, GM1 ligation by CT results in increases in intracellular calcium concentrations, the induction of tyrosine kinase activity, IL-2 production and proliferation (160-162). In addition, and similar to the effects of mAb specific for GPI-AP, CT and ETx can inhibit antigen receptor-induced T cell proliferation (163, 164), as well as induce T cell apoptosis (164-166).

Signalling may be effected by the long fatty acid chains of GPI-AP and glycosphingolipids which penetrate the cytoplasmic leaflet of lipid rafts and may interact directly with the acyl chains of Src family PTK and other signalling molecules. Another possibility is that signalling through GPI-AP and glycosphingolipids may be mediated through an associated transmembrane protein. As described above, putative linking molecules include p100, which is associated with Thy-1 and ThB (108), and MAL, a proteolipid localised in lipid rafts (109). Alternatively, a change in the distribution, conformation or localised micro-environment of Src family PTK caused by aggregation of GPI-AP or glycosphingolipids may result in their activation. Aggregation of GPI-AP in the exoplasmic leaflet of lipid rafts may cause a redistribution of the cytoplasmic leaflet and/or cause the aggregation of several microdomains, resulting in the auto/trans-phosphorylation and activation of Src family PTK, and the initiation of signalling cascades. A diagram depicting signalling through GPI-AP in lipid rafts is shown in Figure 1-3. Indeed, ligation of GPI-AP or gangliosides has been shown to result in the formation of membrane patches due to the actin-dependent aggregation of individual rafts (118, 119). Further, co-capping of Src family PTK with ligated GPI-AP or the GM1 glycosphingolipid suggests that redistribution of the outer leaflet of lipid rafts does cause a concomitant redistribution of inner membrane components (118, 167). Aggregated rafts may amplify signalling as a result of increased interactions between adaptor proteins, enzymes and substrates, and may also exclude negative regulatory molecules, such as the protein tyrosine phosphatase (PTPase) CD45.
The function of lipid rafts in co-ordinating signalling processes is discussed in greater detail below in the context of signalling through TcR/CD3.

**Role of the GPI-anchor in signalling**

Signalling via different GPI-AP induces qualitatively similar responses despite their unrelated protein moieties, suggesting that the GPI-anchor is critical. As discussed above, GPI-anchoring may impart signalling competency upon protein moieties as a result of anchor-dependent targeting to lipid rafts. Consistent with a critical role for the GPI-anchor, proliferation does not ensue following aggregation of transmembrane forms of Ly-6A/E, Qa-2 and CD55 (38, 145, 170). Importantly, the transmembrane form of CD55 was no longer associated with Lck and Fyn (145), presumably as a result of its inability to localise to lipid rafts. Importantly, a transmembrane form of placental alkaline phosphatase did not localise to lipid rafts (171). In contrast to Ly-6A/E, Qa-2 and CD55, a transmembrane form of CD73 was able to induce IL-2 secretion (44). In addition, both GPI-anchored and transmembrane forms of CD58 functioned in activation of a human B cell line and induction of cytokine secretion (172). However, the localisation of transmembrane CD73 and CD58 were not determined and it remains possible that these transmembrane molecules were targeted to lipid rafts despite the absence of a GPI-anchor, perhaps as a result of lateral interactions of the protein moieties with lipid raft resident molecules. Indeed, several transmembrane proteins are localised in lipid rafts (173), and moreover, at least one transmembrane protein, CD44, can activate T cells due to this localisation (174). Notably, the same pattern of tyrosine phosphorylated proteins associated with both transmembrane and GPI-anchored forms of CD58 (172). Alternatively, some GPI-AP may activate both anchor/raft-dependent and -independent signalling pathways, and anchor-independent signalling may be sufficient to result in cytokine secretion.
Figure 1-3  Signalling through GPI-AP in lipid rafts

A. Lipid rafts are schematically represented as plasma membrane areas enriched in cholesterol and glycosphingolipids and organised into a 'liquid-ordered domain' which separates from glycerophospholipids and most transmembrane proteins [adapted from (3)]. Lipid modification of proteins with saturated acyl groups is a targeting signal for lipid rafts, which are therefore enriched in GPI-AP and Src family PTK, as well as other acylated signalling proteins.

B. Signalling through GPI-AP, depicted here using mAbs, may result in a re-distribution of the protein in the exoplasmic leaflet of the plasma membrane, causing a concomitant re-distribution and subsequent activation of Src family PTK in the cytoplasmic leaflet. Signalling cascades initiated by activated Src family PTK result in the functional and signalling sequelae observed following stimulation of T cells with mAbs specific for GPI-AP. The re-distribution of GPI-AP may occur within one lipid raft, or may involve the aggregation of GPI-AP in several lipid rafts, depicted in C as occurring following the addition of secondary mAbs.
The observation that all GPI-AP can mediate T cell activation begged the question of whether any protein expressed on the cell surface by a GPI-anchor could transduce activation signals. When MHC class I extracellular domains were expressed in GPI-anchored form in transgenic mice, mAb-mediated aggregation of the fusion protein resulted in T cell activation (38). However, and in contrast, GPI-anchored CD4 molecules were not functional in this context (175). Furthermore, GPI-anchoring of complement receptor-1 and tissue inhibitor of metalloproteinase did not result in their association with Src family PTK (176), but the localisation of these recombinant proteins to lipid rafts was not determined.

Taken together, accumulated evidence is consistent with the following conclusions: GPI-anchoring, which results in localisation to lipid rafts, confers signalling capacity upon proteins. Some proteins, whether normally GPI-anchored or transmembrane proteins, may be targeted to lipid rafts in the absence of a GPI-anchor. Conversely, some proteins, even when expressed in GPI-anchored form, may be excluded from lipid rafts as a result of interactions mediated by their extracellular domains, and are therefore not competent for signalling.

As is the case for T cell activation by GPI-AP, it is striking that structurally unrelated proteins have similar capacities to inhibit TcR/CD3-induced responses when attached to cell membranes by a GPI-anchor, suggesting that the GPI-anchor is important for the inhibitory effects of GPI-AP. However, transmembrane forms of Ly-6A/E and TSA-1 mediate inhibition of TcR/CD3-induced cytokine secretion and cell growth (37, 57). As the plasma membrane localisation of transmembrane forms of Ly-6A/E and TSA-1 was not assessed, these molecules may function comparably to their GPI-anchored wild type counterparts as a result of targeting to lipid rafts by their extracellular domains. Alternatively, the proteinaceous portions of those molecules which function in both GPI-anchored and transmembrane forms may be involved in T cell signalling, independently and in addition to
functions imparted by the GPI anchor. In this regard, the same transmembrane Ly6E molecule, a fusion protein with the transmembrane and cytoplasmic domains of MHC class I D^b, was capable of mediating inhibitory, but not activating effects (37, 170), suggesting that if T cell activation by Ly6A/E is dependent on the GPI-anchor or localisation to lipid rafts, inhibition of T cell activation may be GPI-anchor/raft independent. However, different cells were used by these two groups, and it remains possible that differences in the plasma membrane and lipid raft composition in these cells resulted in differential localisation of transmembrane Ly-6E to lipid rafts, and thus differential functionality.

TcR/CD3

Aggregation of lipid rafts and formation of the immunological synapse

Recent studies have demonstrated that in addition to GPI-AP-mediated signalling, lipid rafts are involved in signalling through TcR/CD3. A proportion of CD3ζ (~10%) is constitutively associated with lipid rafts. Stimulation of cells through TcR/CD3, accomplished with either anti-CD3ε or antigen-MHC complexes presented by APC, results in a further recruitment of CD3ζ to lipid rafts (169, 177). Alternatively, TcR/CD3 may have a weak, detergent-sensitive constitutive association with lipid rafts which is stabilised and rendered detergent resistant following receptor ligation (119). The association is potentially due to multiple co-operative weak interactions of oligomerised TcR, and does not require the induction of tyrosine phosphorylation (119). TcR/CD3 recruitment/stabilisation in lipid rafts results in the activation of resident Src family PTK and the initiation of multiple signalling pathways. Studies using mutant thymocytes have revealed a critical role for the TcRα chain connecting peptide domain and the extracellular and/or transmembrane domains of CD3ζ in the ligand-induced formation of a TcR/CD3 signalling complex in lipid rafts (178, 179).
In contrast to the inducible localisation/stabilisation of TcR in lipid rafts, the pre-TcR in thymocytes, composed of pTα and TcRB, is constitutively associated with lipid rafts, possibly as a result of palmitoylation of pTα on one or both of two juxtamembrane cysteine residues (180). The constitutive localisation in lipid rafts may underlie the putative ligand-independence of pre-TcR signalling in thymocyte development (181).

Stimulation of cells through TcR/CD3 results in a redistribution of lipid rafts to the TcR contact site, which is enhanced by CD28 (182). The aggregation of lipid rafts may promote TcR-induced signalling by concentrating raft-associated signalling molecules. In addition, phosphorylation may be promoted by the exclusion of the PTPase CD45 (168, 169).

The T cell:APC contact site is further organised into a highly structured junction called the immunological synapse, which has been visualised using real-time microscopic imaging (183). The immunological synapse forms over several stages, beginning with integrin-mediated adhesion of the T cell to the APC. TcR then engages peptide-MHC complexes and is transported to the centre of the contact area. The mature immunological synapse is characterised by the formation of a central assembly of TcR/CD3, together with adhesion and signalling molecules, including CD28, Lck, Fyn, and PKCθ, referred to as the central supramolecular activation cluster or cSMAC (184). The cSMAC is surrounded by a peripheral SMAC (pSMAC) ring of integrin family adhesion molecules such as LFA-1, and cytoskeletal proteins such as talin (184).

The formation of the immunological synapse is proposed to allow TcR signalling to overcome several barriers (185). The transport of engaged TcRs to the central area of the contact site serves to concentrate rare peptide-MHC complexes and may allow for a threshold of signalling to be achieved. The low affinity of the TcR:MHC interaction may be overcome by the concentration of these molecules in the cSMAC. Further, topological consideration of the T cell surface suggests that large, abundant glycoproteins such as CD43
and CD45 may pose a steric barrier to the TcR:MHC interaction, estimated to be approximately 15 nm in size. TcR signalling may require sustained and close apposition of cell surfaces, which occurs in the context of the formation of the immunological synapse. The size of the CD2:CD48 interaction, as well as the interaction of other molecules such as CD4 and CD28 with their ligands, may facilitate positioning of the T cell and APC membranes at an optimal distance for TcR:MHC interactions (186). As predicted by this model, larger forms of CD48 inhibited T cell activation (187). The close apposition of the T cell:APC membranes may be further supported by the exclusion of large molecules such as CD43 and CD45 from the contact areas (188, 189).

Lipid rafts aggregated at the T cell:APC contact may facilitate and stabilise the formation of the immunological synapse in several ways (185). Lipid rafts may represent areas of decreased steric hindrance as a result of their enrichment in gangliosides and small GPI-AP, and thus may favour TcR:MHC interactions and contribute to the optimal packing of proteins at the contact site. Furthermore, the increased membrane rigidity in lipid rafts may stabilise the immunological synapse. Lipid rafts may also contribute to the spatial organisation of key signalling molecules in SMACs, concentrating PTK and accessory molecules while excluding phosphatases such as CD45. Further, lipid rafts may direct the actin cytoskeleton-mediated reorganisation of cell surface proteins required for the formation of the immunological synapse (186). Indeed, lipid rafts have been proposed to be sites of actin nucleation regulated by tyrosine phosphorylation, phosphoinositides, WASP and the Arp2/3 complex (190).
Lipid rafts and the regulation of Src family PTK

Activation of the Src family PTK Lck and Fyn are required for signalling through TcR/CD3, as well as through GPI-AP. The activity of Src family PTK is tightly regulated, and the localisation of Lck and Fyn in lipid rafts may play an important role in their regulation. Phosphorylation of a C-terminal tyrosine residue (tyrosine 505 in Lck and tyrosine 531 in Fyn) negatively regulates the tyrosine kinase activity, while auto- or trans-phosphorylation of a tyrosine residue within the activation loop of the kinase domain is required for kinase activity. The structural basis for the kinase inactivation is an intramolecular interaction between the SH2 domain of the PTK and the C-terminal phosphotyrosine, which maintains the PTK in an inactive ‘closed’ configuration (191-193). The ‘closed’ configuration is further supported by an intramolecular association between the SH3 domain and a proline rich region between the SH2 and kinase domains (191). The structure of the Src family PTK Fyn and Lck, as well as aspects of their regulation, are depicted in Figure 1-4. Interactions of Src family PTK with ligands that bind the SH2 or SH3 domains interfere with the intramolecular interactions and promote a catalytically active ‘open’ configuration (194). For example, binding of the SH3 domain with HIV Nef protein or binding of Lck to tyrosine phosphorylated CD3ζ or ZAP-70 increases the kinase activity (195-198).

The C-terminal tyrosine residue is phosphorylated by the cytoplasmic C-terminal Src kinase Csk (199-201). Csk may be regulated by SH2 domain-mediated binding to a molecule localised in lipid rafts, called the Csk-binding protein (Cbp) or phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) (202, 203). In resting cells, Cbp is phosphorylated and binds Csk, resulting in the recruitment of Cbp to lipid rafts in proximity to Lck and Fyn. In addition, the SH3 domain of Csk mediates its association
The structure and regulation of the Src family PTK Lck and Fyn

A. Src family PTK contain a C-terminal negative regulatory tyrosine residue, a kinase domain with a positive regulatory autophosphorylation site, SH2 and SH3 domains and a unique region. The unique region of Lck and Fyn contains sites that are modified by the addition of myristate and palmitate.

B. Activated Src family kinases are characterised by phosphorylation of the positive regulatory tyrosine residue. Phosphorylation of Cbp by activated Src family PTK leads to the recruitment of Csk and PEP, which phosphorylate the negative regulatory tyrosine and dephosphorylate the positive regulatory tyrosine, respectively. The Src family PTK is then maintained in an inactive "closed" configuration due to intramolecular interactions between the SH2 domain and the C-terminal phosphotyrosine, and between the SH3 domain and the linker region. CD45 is also critical for the regulation of Src family PTK, and can dephosphorylate both regulatory tyrosines. See text for details.
with the PTPase PEP, which can negatively regulate the activity of Src family PTK by dephosphorylating the positive regulatory tyrosine residue (204-206). Upon stimulation through TcR/CD3, Cbp is dephosphorylated (202, 203). The resulting release of Csk may lead to the segregation of cytosolic Csk from Src family PTK in lipid rafts, supporting their activation. Src family PTK may subsequently contribute to a negative feedback loop to limit their own activity as they can phosphorylate Cbp.

The CD45 transmembrane PTPase plays a critical, albeit incompletely understood role in the regulation of Src family PTK [reviewed in (207-209)]. CD45 can dephosphorylate the negative regulatory tyrosine of Src family PTK, thus priming the kinases for activation. However, CD45 can also dephosphorylate the positive regulatory tyrosine residues, and the hyperphosphorylation of Src family PTK observed in CD45−/− cells corresponds to phosphorylation at both these sites. Src family PTK can be enzymatically active in the presence of phosphorylated C-terminal tyrosine residue. Thus, although several reports demonstrate diminished TcR/CD3 signalling in the absence of CD45, CD45−/− thymocytes display increased Lck activity (210). Indeed, multiple studies have shown increased activity of Src family PTK in CD45-deficient cells (209).

The activity of CD45 may be regulated by its localisation in the plasma membrane relative to its substrates. Several studies have shown that CD45 is excluded from lipid rafts (119, 168, 169), which may render Src family PTK in these domains inaccessible to CD45. In addition, the exclusion of CD45 from lipid rafts may contribute to its exclusion from the T cell:APC contact site, thereby preventing the dephosphorylation of activated Src family PTK. In two reports, Lck in lipid rafts was shown to be hyperphosphorylated and displayed low kinase activity relative to non-lipid raft associated Lck (168, 211). In contrast, in one of these studies, an increase in the activity of Fyn in lipid rafts relative to Fyn in soluble membranes was observed, and others reports have shown increased increased Src PTK
activity in lipid rafts (212, 213). Thus, although the localisation of Src family PTK to lipid rafts and the exclusion of CD45 from these domains likely plays an important role in the regulation of kinase activity, a complete understanding of this regulation requires further investigation.

The plasma membrane compartmentalisation of Fyn and Lck may be subject to regulation. While both of these Src family PTK are enriched in lipid rafts, the extent of the enrichment varies, with greater than 90% of Fyn but only 10-40% of Lck detected in detergent insoluble microdomains (unpublished observations from our laboratory). The palmitoylation of both Fyn and Lck is critical for their localisation to lipid rafts (214, 215). However, palmitate is added to proteins post-translationally through a labile thioester linkage and proteins can be depalmitoylated in a regulated manner. For example, G protein α subunits are depalmitoylated upon stimulation (216). The palmitoylation of Src family PTK also appears to be dynamic, as palmitate groups on Lck have a half-life of less than 6 hours while the half-life of the protein is 20 to 30 hours (217). Differential palmitoylation of Fyn and Lck could present a mechanism for their differing enrichments in lipid rafts.

Lck associates with the CD4 and CD8α, and its kinase activity and membrane localisation may be regulated by these co-receptors. Both CD4 and CD8β can be palmitoylated, and a proportion of CD4 and CD8αβ heterodimers thus localise to lipid rafts (142, 173, 218). Importantly, the association of CD8 with Lck, as well as the activation of Lck following CD8 binding to MHC class I, involved mainly Lck in lipid rafts (142). Further, the localisation of Lck may be dynamically regulated in an activation dependent manner, as an increased amount of Lck in lipid rafts was observed upon MHC- or mAb-mediated ligation of the TcR/CD3 complex (142).
TcR/CD3 signalling: co-ordination of signalling pathways by lipid rafts

A model of T cell activation has emerged in which lipid rafts play a critical role in coordinating the interaction of many signalling molecules (3). Multiple signalling pathways amongst those induced following stimulation of T cells through TcR/CD3 are schematically depicted in Figure 1-5 and are discussed below, with emphasis on the role of lipid rafts in regulating these processes. Antigen-receptor engagement results in the recruitment/stabilisation of CD3ζ in lipid rafts and the activation of resident Src family PTK, which phosphorylate tyrosine residues within the ITAM motifs of CD3 chains. ZAP-70 is tyrosine phosphorylated and activated following its recruitment to lipid rafts through an SH2 domain-mediated association with phosphorylated CD3ζ. ZAP-70 then phosphorylates LAT co-localised in lipid rafts, enabling the recruitment of many signalling molecules, including phospholipase Cγ1 (PLCγ1), Grb-2, Grap, Gads, SLP-76, Vav, Cbl, and the p85 subunit of PI-3K (219), thus coupling the antigen receptor to multiple signalling pathways, schematically represented in Figure 1-5, culminating in the activation of transcription factors required for the de novo transcription and translation of genes including interleukin-2 (IL-2), and cellular differentiation and proliferation.

Once recruited to lipid rafts, PLCγ1 is positioned to be phosphorylated and activated by the Tec family kinases Itk and Rlk. Itk associates with lipid rafts, at least in part through the binding of its PH domain to inositol trisphosphate generated by PI-3K (220), while Rlk contains a cysteine string motif resulting in its constitutive palmitoylation and association with Fyn (221). Following signalling through TcR and CD28, Itk and Rlk are activated through trans-phosphorylation by Src PTK, and phosphorylate and activate PLCγ1 (222). Activated PLCγ1 cleaves phosphotidylinositol-bisphosphate (PIP2) co-localised in lipid rafts (223) generating the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG), which in turn lead to increased intracellular calcium concentrations and activation of
Signalling pathways induced through TcR/CD3

Signalling through TcR/CD3 results in the activation of multiple signalling pathways, which converge to activate transcription factors required for the transcription of genes, including the T cell growth factor IL-2. See text for details.
the serine/threonine protein kinase C (PKC). Importantly, the increased local concentration of PIP₂ in rafts may be required for signalling, and PIP₂ dilution into bulk plasma membrane upon disruption of rafts results in inhibition of phosphoinositide turnover (224).

Increased intracellular calcium concentrations lead to the activation of calcineurin, a serine/threonine phosphatase, which dephosphorylates cytoplasmic NFAT (NFATc) resulting in its nuclear translocation and transcriptional activation (225). DAG can result in the activation of the small guanosine trisphosphate (GTP)-binding protein Ras by PKC-mediated suppression of Ras-GAP, a Ras-GTPase activating protein (226). DAG can also bind RasGRP, a Ras guanine nucleotide exchange factor (GEF) which catalyses the exchange of GDP for GTP, thereby activating Ras (227, 228). Activation of Ras results in the activation of the Erk1 and Erk2 MAP kinases through serial activation of the MAPK kinase kinase Raf and the MAPK kinases Mek-1/2 (229).

In addition to DAG, multiple adaptor proteins are implicated in the regulation of Ras in T cells. Tyrosine phosphorylated LAT binds to both Grb2 and the Grb2-like accessory protein Grap, which constitutively associate with Sos through SH3-proline rich region (PRR) interactions (230, 231). Ras is constitutively found in lipid rafts (169, 232), and recruitment of Sos, a Ras GEF, results in Ras activation. The Shc adaptor protein, recruited to the signalling complex in lipid rafts (169) by SH2 domain-mediated binding to phosphorylated CD3ζ and subsequently tyrosine phosphorylated, may also mediate the recruitment of Grb2-Sos (233). Importantly, modification of Shc with sequences encoding its acylation and constitutive localisation in lipid rafts resulted in constitutive Shc phosphorylation and MAPK activation (234).

Activation of the Ras-ERK pathway is critical for positive selection of thymocytes (235). Thymocytes deficient in CD3δ or bearing a mutation in the TcRα chain connecting peptide domain are defective in ERK activation, and fail to undergo efficient positive
selection (178, 179). In these cells, activated Lck, as well as phosphorylated CD3ζ, ZAP-70 and LAT are not detected in lipid rafts, again underscoring the critical role of rafts in coordinating the signalling events leading to activation of the Ras-ERK pathway.

Phosphorylated LAT and Shc bind Gads, a Grb2-related adaptor protein which is constitutively associated with the SLP-76 adaptor protein (236), resulting in the recruitment of the latter molecules to lipid rafts (237). SLP-76 is phosphorylated on tyrosine residues and binds to the SH2 domains of several proteins, including Itk, Vav and Nck (236, 238). Both LAT and SLP-76 are required for TcR/CD3-induced activation of PLCγ1 and Ras (239, 240), possibly due to the recruitment of Itk to the LAT-Gads-SLP-76 complex, thus positioning this kinase to phosphorylate and activate PLCγ1. Targeting SLP-76 to lipid rafts rescues TcR-induced activation of PLCγ1 and Ras in the absence of LAT, demonstrating that a critical role of LAT is to mediate recruitment of SLP-76 to lipid rafts (237).

Phosphatidylinositol-3-phosphate kinase (PI-3K) is activated following stimulation through both TcR/CD3 and CD28, and plays a role in multiple signalling pathways involved in cell survival, trafficking, actin rearrangement and cell growth (241). PI3K phosphorylates phosphoinositides at the 3' position, creating binding sites for PH domain-mediated recruitment of signalling molecules to the plasma membrane. The p110 catalytic subunit of PI-3K is regulated by the p85 subunit, which contains two SH2 domains and an SH3 domain and can therefore mediate protein-protein interactions with molecules such as CD28, LAT, Grb2 or the adaptor protein TRIM (231, 242, 243), bringing PI-3K to the membrane in proximity to its substrates. Importantly, PI-3K is recruited to lipid rafts following TcR/CD3 stimulation (169), and moreover, at least one of its substrates, PIP2, is enriched in lipid rafts (223). Thus, the generation of 3'-phosphoinositides and the recruitment of signalling molecules containing PH domains, including Tec family kinases as described above, may preferentially occur in lipid rafts. Another important effector of PI-3K is the serine/threonine
kinase PKB, which is positioned to be phosphorylated and activated by the combined action of the PDK1 and PDK2 kinases following the PH-domain dependent recruitment of these kinases to the membrane (244). PKB regulates cell survival (245), in part by phosphorylating the pro-apoptotic protein Bad, which results in its dissociation from the anti-apoptotic molecule Bcl-Xl.

Vav, a GEF for the Rho/Rac/Cdc42 family of GTPases, is regulated by subcellular localisation and tyrosine phosphorylation. Vav is recruited to lipid rafts upon TeR/CD3-mediated stimulation (169) through binding to phosphorylated SLP-76, as well as PH domain-mediated binding of phosphoinositides generated by PI3K (246). Vav is then tyrosine phosphorylated by Src or ZAP-70 family PTK, and optimal phosphorylation of Vav requires co-stimulation through CD28 (247). Vav is critical for actin polymerisation and T cell polarisation towards APC (248, 249), through a pathway involving Cdc42 and the Cdc42-associated Wiscott-Aldrich syndrome protein (WASP) (250, 251). Phosphorylated SLP-76 may co-ordinate interactions between Vav, WASP and the serine/threonine p21 associated kinase (PAK) via the adaptor protein Nck (252), facilitating the activation of WASP and PAK by Rho/Rac/Cdc42 GTPases and resulting in cytoskeletal re-organisation. As CD28 is required for optimal Vav activation, this may be the mechanistic basis for the requirement for CD28 in effective aggregation of lipid rafts (182), a process dependent on actin reorganisation (167, 253).

Vav-induced activation of Rho/Rac/Cdc42 GTPases also results in the activation of PKCθ and its translocation to the central area of the immunological synapse (254), as well as activation of the SAPK/JNK pathway through MKK1 and MKK4/Sek1 (255). PKCθ is implicated in the CD28-dependent activation of IKK, which phosphorylates IκBα and IκBβ resulting in their degradation and the release of NF-κB transcription factors into the nucleus (256-258). Lipid rafts may co-ordinate this pathway, as stimulation of cells with anti-CD3
and anti-CD28 resulted in the recruitment of PKCθ and activated IKK to lipid rafts (259).
Vav also mediates the Rac-dependent activation of phosphatidylinositol 4-phosphate 5 kinase (PIP5K), resulting in the localised accumulation of the PLCγ1 substrate PIP2 (260) which may be important for sustained calcium fluxes.

Signalling sequelae downstream of TcR/CD3 result in the activation of transcription factors required for the expression of genes required for T cell activation, differentiation and proliferation (261). Important regulators include NFAT, which is induced downstream of increases in intracellular calcium concentrations and calcineurin activation. In addition, ERKs phosphorylate the Elk-1 transcription factor which then participates in initiating the transcription of c-Fos, while SAPK/JNK phosphorylates c-Jun to increase its transcriptional activity. c-Fos associates with Jun to form AP-1 dimers, an important transcription factor that co-operates with NF-AT, NF-κB and Oct proteins in initiating the transcription of genes in activated T cells, including the major T cell growth factor IL-2. IL-2 secretion and subsequent signalling through the IL-2 receptor promote T cell proliferation and survival, and will be discussed below.

**TcR/CD3-induced signalling: negative regulation**

TcR/CD3-induced signalling pathways are transient, and are regulated through endocytosis and downregulation of surface levels of activated receptors, the induction of negative regulatory pathways and the recruitment of phosphatases. In addition, proliferation and effector functions are subject to negative regulation by inhibitory cytokines and through the induction of cell cycle arrest and Fas-mediated activation-induced cell death.

Pathways involved in the negative regulation of TcR/CD3-induced signalling are depicted in Figure 1-6. TcR/CD3-induced signalling results in the activation of tyrosine kinases of the Src, Syk/ZAP-70 and Tec families, however the levels of tyrosine
phosphorylation of intracellular substrates are also regulated by the action of protein tyrosine phosphatases (PTPase). CD45 is critical for T cell activation (207), and in combination with Csk and PEP, regulates the activity of Src family PTK. CD45 and PEP may have additional targets in activated T cells, and both may negatively regulate TcR/CD3-induced signalling by dephosphorylating CD3ζ and ZAP-70 (204, 262, 263). SHP-1 and SHP-2 are cytoplasmic SH2 domain-containing PTPases which are activated upon binding to phosphotyrosine residues. SHP-1 downregulates TcR/CD3-mediated signalling, and has been shown to interact with ZAP-70, Vav, PI-3K and Grb-2, and to dephosphorylate ZAP-70 and Src family PTK (264).

MAPK activated through TcR can also be inactivated through dephosphorylation by several tyrosine specific, serine/threonine specific or dual-specificity phosphatases (265). In addition, dephosphorylation of inositol phospholipids is important in the regulation of the many signalling pathways which are dependent on these molecules (266). The SH2 domain-containing inositol phosphatases SHIP1 and SHIP2 hydrolyse the 5'-phosphate of IP3, thus countering the effects of PI-3K activation. The activity of SHIP1 and SHIP2 may be regulated by their recruitment to the proximity of their substrates. Indeed, SHIP1 is phosphorylated following TcR/CD3-induced signalling and can bind CD3ζ and Shc. Thus, SHIP1, inducibly recruited to lipid rafts following BcR-mediated stimulation of B cells (267), is also predicted to be recruited to lipid rafts in T cells, where both tyrosine phosphorylated CD3ζ and Shc, as well as SHIP’s substrate IP3, are localised. PTEN, another inositol phosphatase and a tumour suppressor protein, may function constitutively to maintain low basal levels of IP3 (266).

CTLA-4 is a critical negative regulatory molecule in T cells (268), and CTLA-4-deficient mice exhibit massive lymphoproliferation and autoimmunity (269, 270). Induction of CTLA-4 expression competes with CD28 for binding to CD80 and CD86, thus inhibiting
Signalling through TcR/CD3 is dampened by tyrosine and inositol phosphatases. Further, Cbl functions as a ubiquitin ligase and as an adaptor protein to negatively regulate signalling. See text for details.
the T cell response. Furthermore, CTLA-4 binds to the tyrosine phosphatase SHP-2 (271), which may inhibit T cell activation by dephosphorylating CD3ζ and Shc. Notably, CTLA-4 inhibits the activation of Erk2 and SAPK/JNK (272).

Cbl family members. Cbl-c. Cbl-b and Cbl-3, are also critical negative regulators of TcR/CD3-induced signalling (273). Cbl family members are ubiquitin ligases which direct the ubiquitination, and subsequent internalisation, lysosomal sorting and proteosome-mediated degradation of receptor PTK (274, 275). The ubiquitin ligase activity of Cbl is dependent upon its tyrosine phosphorylation following its recruitment to activated receptors (274). Cbl may function in a similar manner to direct the ubiquitination of molecules involved in TcR/CD3-induced signalling, as it is also recruited to the signalling complex by phosphotyrosine dependent binding to ZAP-70 and becomes tyrosine phosphorylated. Indeed, mutants of Cbl which lack ubiquitin ligase activity do not downregulate TcR/CD3-induced signalling (275-277). Further, stimulation through TcR has been shown to induce the tyrosine phosphorylation-dependent ubiquitination of CD3ζ (278, 279). Cbl also enhances the degradation of Fyn (280). and Cbl-induced down-regulation of TcR is supported by the increased expression of TcR in thymocytes from c-Cbl-deficient mice (281, 282).

Mice lacking Cbl-b display CD28-independent T cell proliferation and cytokine production, and are prone to autoimmunity (283, 284). The signalling defect in Cbl-b−/− mice is dysregulated activation of Vav, as the tyrosine phosphorylation of Vav and activation of its GEF activity do not require co-stimulation through CD28 (283, 284). Furthermore, actin-dependent aggregation of lipid rafts to the TcR contact site is CD28-independent in Cbl-b−/− cells (285). Thus, Cbl-b functions to negatively regulate the activation of Vav and downstream activation of Rho/Rac/Cdc42 GTPases which control reorganisation of the actin cytoskeleton and raft polarisation. As levels of Vav are unchanged in Cbl-b deficient cells
(283, 284). The target of Cbl responsible for regulating raft aggregation remains unknown, however it likely involves an upstream regulator of the Vav-Rho/Rac/Cdc42 pathway. The development of autoimmunity in Cbl-b-deficient mice highlights the importance of regulated lipid raft aggregation in T cell responses.

In addition to mediating ubiquitination, Cbl can function as an adaptor protein to negatively regulate TcR/CD3-induced signalling in several ways (273). Cbl binds ZAP-70 and decreases its kinase activity. Cbl constitutively associates with Grb2, competitively excluding binding of Sos which may decrease Grb2-mediated Ras activation. Furthermore, following its phosphorylation by Fyn, Cbl associates with the adaptor protein CrkL and C3G, a GEF which activates the small G protein Rap-1. The activation of Rap-1 inhibits MAPK activation by Ras through the sequestration of Raf, and has been implicated in the induction of T cell anergy (286).

SIT, a transmembrane adaptor protein, can negatively regulate T cell activation. SIT may function in part through recruitment of the phosphatase SHP-2 by its immunoreceptor tyrosine-based inhibitory motif (ITIM), although the inhibitory effects of SIT were independent of the ITIM (287). SLAP-130, an adaptor protein which binds to multiple signalling molecules including CD3ζ, ZAP-70, SLP-76, Vav and Cbl (288, 289), may also function to negatively regulate TcR/CD3 signalling (288). The mechanism by which these molecules regulate TcR/CD3-induced signalling requires further characterisation.

TcR/CD3-induced signalling: critical role of lipid rafts

The critical role of lipid rafts in TcR signalling was demonstrated by the inhibitory effects seen upon the disruption of lipid raft integrity, achieved using methyl-β-cyclodextrin (MCD) which results in the extraction of cholesterol by forming inclusion complexes within a hydrophobic cyclodextrin cavity (290), or nystatin and filipin, polyene antifungal agents
which complex cholesterol into multimeric globular deposits in the membrane (291, 292). MCD, filipin or nystatin inhibited anti-CD3ε-induced increases in intracellular calcium concentrations and tyrosine phosphorylation of CD3ζ and PLCγ1 (169). MCD also resulted in decreased pre-TcR-mediated signalling in thymocytes (180). CD3 responsiveness of T cells was also decreased upon addition of exogenous GM1 ganglioside (169), which may perturb raft stability and results in the internalisation of raft components.

Studies with acylation defective mutants of Lck, Fyn and LAT revealed that, in addition to membrane targeting, palmitoylation of these proteins was required for their correct sub-cellular localisation in lipid rafts, which in turn predicated their function in TcR/CD3 signalling (114, 214, 215). Re-targeting of mutated LAT to rafts using the acylation signals from Lck restored TcR/CD3 responsiveness, demonstrating that while the extracellular and transmembrane domains of LAT are dispensable, lipid raft localisation of this molecule is critical for signalling (293). Inhibition of acylation using the fatty acid analogs 2-hydroxymyristate or 2-bromopalmitate resulted in decreased levels of wild-type Fyn in lipid rafts, and impaired TcR/CD3-mediated signalling (149, 215). In addition, treatment of T cells with polyunsaturated fatty acids, resulting in the displacement of Lck and Fyn from lipid rafts as a consequence of decreased palmitoylation (149), correlated with diminished T cell responsiveness to signals induced through both GPI-AP and TcR/CD3 (150). These results provide a potential mechanistic basis for the immunosuppressive effect of orally administered polyunsaturated fatty acids in use clinically (294).

Modulation of TcR/CD3-induced signalling through effects on raft aggregation

GPI-AP may affect TcR-induced signalling by modulating the association of TcR/CD3 with lipid rafts. In support of this hypothesis, it has been demonstrated that co-engagement of TcR and CD48 was co-stimulatory for T cell activation, and led to an increase
in CD3ζ phosphorylation, and importantly, an increase in the induced localisation of CD3ζ to lipid rafts (67). This study went on to demonstrate that raft disruption by MCD blocked T cell activation, as well as the enhancement of T cell activation observed upon co-engagement of CD48. Further, other GPI-AP, including Thy-1 and CD59, as well as the GM1 ganglioside, potentiated TcR/CD3-induced signalling, notably enhancing the phosphorylation of LAT in lipid rafts (182, 295, 296). Similarly, mAb-mediated co-ligation of CD3 with CD2, CD5, CD9 and CD44, non-GPI-linked proteins present in lipid rafts, also potentiated the association of TcR/CD3 with lipid rafts and enhanced signalling (297). Thus, molecules in lipid rafts may modulate TcR/CD3 signalling by affecting the recruitment of lipid rafts to TcR/CD3 at the T cell-APC interface. This passive enhancement of raft aggregation is co-stimulatory, as is actin-driven enhancement of raft aggregation by CD28 (182, 298). Under different constraints, the ability of mAbs specific for GPI-AP to inhibit T cell activation through TcR/CD3 may likewise be due to effects on the recruitment of TcR/CD3 to lipid rafts. Thus, mAbs specific for GPI-AP may result in the functional sequestration of critical signalling molecules localised in lipid rafts away from TcR/CD3, as well as affecting the organisation of TcR, co-receptors and accessory molecules into specific membrane-protein aggregates. Alternatively, mAbs specific for GPI-AP may affect the higher order aggregation of lipid rafts which may be required for signalling.

In this context, it is interesting to speculate that the requirement for CD3ζ expression in the activation of T cells through GPI-AP (42, 43) is because CD3ζ is a critical substrate of GPI-AP-activated Src PTK, and that signalling pathways downstream of GPI-AP are similar to pathways involved in TcR/CD3 signalling. In support of this model, CD3ζ phosphorylation was observed following stimulation of T cells with mAbs specific for TSA-1 or CD55 (31, 68), and mAb-mediated cross-linking of CD59 led to the CD3ζ dependent activation of ZAP-70 (295). Furthermore, signalling effected by lipid raft resident
gangliosides may function in the same manner, and CD3 was detected in lipid rafts aggregated by cross-linking of both CD59 and GM1 (119).

**Lipid rafts: regulation of signalling via multiple receptors**

Lipid rafts function to co-ordinate signalling via other immunoreceptors in a manner similar to that outlined for TcR-mediated signal transduction. In B cells, stimulation through BcR results in the recruitment/stabilisation of BcR. Igα and Igβ to lipid rafts (267, 299-302), which are constitutively enriched in the Src family PTK Lyn. Activation of Lyn and subsequent tyrosine phosphorylation events lead to the recruitment and activation of many signalling molecules in lipid rafts, including Syk. PLCγ2, the Tec family kinase Btk, the adaptor protein BLNK, PI-3K, Vav and Shc (300, 301). Interestingly, as was observed for pre-TcR (180), pre-BcR is constitutively enriched in rafts (300), which may likewise underlie its capacity to signal in the absence of an extracellular signal. Lipid rafts are also implicated in BcR-mediated targeting of antigen for processing in the MHC class II peptide-loading compartment (299). Furthermore, MHC class II molecules on APC, including B cells, are enriched in lipid rafts (303). The increased local density of peptide-MHC complexes in lipid rafts may facilitate antigen presentation to T cells at low antigen concentrations (304).

FceRI is recruited/stabilised in lipid rafts following aggregation of FceRI by IgE:antigen complexes. Signalling is then initiated by the lipid raft resident-Src family PTK Lyn, which phosphorylates FceRI β and γ chains resulting in the recruitment of Syk and PLCγ1 to lipid rafts and subsequent downstream signalling events (305, 306). Lipid rafts are critical for signalling through BcR and FceRI, and signalling is inhibited upon disruption of raft integrity using MCD or filipin (300, 301, 307), or the cholesterol lowering agent lovastatin (308). Lipid rafts are also implicated in signalling though CD44, a transmembrane hyaluronan in T cells (174), CD20 in B lymphocytes (309), and FcαR in myeloid cells (310).
Lipid rafts may have a general role in signalling, acting as a repository of signalling molecules which can be activated by membrane receptor systems which are inducibly recruited to these domains. Furthermore, lipid rafts may function in the polarisation of immunoreceptors at the site of activation, as has been postulated for the site of contact of a T cell with an APC. Incubation of NK cells with sensitive targets also resulted in the aggregation of lipid rafts to the site of contact with the target cell (311). The aggregation of rafts was dependent on signalling, and was inhibited in the presence of pharmacological inhibitors of Src and Syk family PTK. Furthermore, and more physiologically relevant, the interaction of a killer inhibitory receptor (KIR) with its ligand MHC class I which results in the SHP-1 dependent inhibition of cytotoxicity, also inhibited raft reorganisation. Conversely, dispersion of rafts by MCD blocks not only raft aggregation at the site of contact with the target cell but also cytotoxicity. Thus, lipid rafts function to co-ordinate initial signalling events through multiple immunoreceptors, as well as stabilising the higher order organisation and polarisation of receptor systems at the site of activation.

**INTERLEUKIN 2 RECEPTOR (IL-2R)**

Stimulation of T cells through TcR/CD3 results in the activation of multiple signaling pathways as described above, which lead to the secretion of IL-2 as well as cytokine responsiveness (312). IL-2 then promotes cell survival and cell cycle progression by binding to its receptor. The high affinity receptor for IL-2 ($K_d = 10^{-11}$ M) is composed of the IL-2Rα chain, which functions solely in IL-2 binding, and IL-2Rβ and IL-2Rγ, which contribute to IL-2 binding and mediate signal transduction (313). Both mouse and human IL-2Rα alone exhibit relatively low IL-2 binding affinity ($K_d = 10^{-9}$ M) but cannot signal. Whereas human IL-2Rβ and γ chains display an intermediate binding affinity ($K_d = 10^{-9}$ M) and can function
in signalling, the murine IL-2Rβ and γ chains are incapable of binding IL-2 and thus, IL-2 dependent signalling requires expression of all three receptor chains in mouse cells (314, 315). IL-2Rβ and γ chains are members of the cytokine receptor superfamily. IL-2Rβ is also a component of the receptor for IL-15. The IL-2Rγ chain is shared by the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15, and is thus also referred to as the common γ chain (γc).

**IL-2R signalling**

Antigenic stimulation of T cells is required for the responsiveness of cells to IL-2, and results in the expression of the high affinity IL-2Rαβγ complex through the induction of IL-2Rα expression and the upregulation of IL-2Rβ expression. IL-2Rαβγ chains appear to form a pre-existing complex on the surface of T cells, which is brought into closer proximity by ligand binding (316), resulting in the aggregation of IL-2Rβ and γ chains required for signaling (317, 318). The Janus family kinases JAK1 and JAK3 are constitutively associated with IL-2Rβ and γ chains, respectively (319, 320) through interactions with box 1/box2 regions in the receptor chains (321, 322). In addition, JAK3 can contact IL-2Rβ (320, 323). Ligand-induced IL-2R aggregation results in the further recruitment of JAK3, and leads to the juxtaposition of JAK1 and JAK3 and their phosphorylation and activation. The activation of JAK kinases, which is essential for IL-2 induced proliferation, results in the phosphorylation of tyrosine residues on IL-2Rβ and γ chains, as well as on other signalling molecules. While the tyrosine residues on IL-2Rγ chains are dispensable for IL-2R signalling (324, 325), phosphorylation of IL-2Rβ is required for the recruitment and activation of multiple signalling molecules. Signalling pathways induced through IL-2R are schematically depicted in Figure 1-7.
Phosphorylated tyrosine residues 392 and 510 of IL-2Rβ lead to the SH2 domain mediated-recruitment of cytosolic signal transducers and activators of transcription (STAT) proteins STAT5a and STAT5b (326, 327). JAK-mediated phosphorylation of STATs leads to their dimerisation by SH2 domain-phosphotyrosine interactions, and their translocation to the nucleus where STATs can bind to conserved promoter sequences to regulate gene transcription. STAT3 is also tyrosine phosphorylated and activated in response to IL-2 (326, 328). STATs induce gene transcription via their C-terminal transactivation domain, and contribute to the expression of genes induced in response to IL-2, including IL-2Rα.

Partial impairment of IL-2-induced proliferation was observed in T cells lacking STAT5a, STAT5b or STAT3, due to a defect in IL-2 induced IL-2Rα expression (329-331). However, IL-2 induced proliferation was ablated in T cells lacking both STAT5a and STAT5b (332), demonstrating that although the highly related STAT5a and STAT5b are partially redundant, activation of at least one of the STAT5 proteins is critical for IL-2R-mediated proliferation. Consistent with an essential role for STAT5 in cell cycle progression, stimulation with IL-2 did not result in the induction of cyclins A, D2, D3 and E, as well as the cyclin dependent kinase (Cdk) Cdk6, in STAT5a/STAT5b−/− T cells (332). Further, the transactivation domain of STAT5 was shown to be required for IL-2-induced proliferation and expression of the c-Myc proto-oncogene, which is required for entry into the S phase of the cell cycle (333).

Signaling through the IL-2R also induces the recruitment of the p85 subunit of PI-3K to phosphotyrosine residue 392 in IL-2Rβ and the activation of PI-3K activity (334-336). JAK1 also interacts with p85, and contributes to the recruitment and activation of PI-3K (337). PI-3K is implicated in IL-2-mediated proliferation and survival through its downstream effectors PKB and p70 S6 kinase (338, 339). PKB activation is sufficient to prevent apoptosis of IL-2 dependent cell lines upon withdrawal of IL-2, and induces the
IL-2 induces the heterotrimerisation of the IL-2Rα, β and γ chains, resulting in the activation of the JAK1 and JAK3 kinases associated with the IL-2Rβ and γ chains, respectively. Subsequent phosphorylation of IL-2Rβ enables the recruitment and activation of signalling molecules including STAT5a, STAT5b, STAT3, Shc, PI-3K and Lck. See text for details.
expression of c-Myc and the anti-apoptotic protein Bcl-2 (338). PI-3K is also implicated in the IL-2-induced expression of Bcl-XL, although PI-3K independent mechanisms of regulating Bcl-XL expression are also induced by IL-2 (340). The role of PI-3K in regulating cell survival has also been examined in resting human T cells, which do not express IL-2Rα or JAK3. IL-2 signalling through the intermediate affinity IL-2Rβγ in these cells does not result in JAK kinase activation or proliferation, but rather promotes cell survival through the activation of Lck (discussed below) and PI-3K, and the induction of Bcl-2 expression (341, 342). IL-2-induced Bcl-2 expression in activated T cells has also been found to be JAK-independent (343, 344). The Rho GTPase may function downstream of PI-3K in regulating Bcl-2 levels, as dominant negative mutants of Rho inhibited IL-2-induced Bcl-2 expression (345). In addition to effects on cell survival, PI-3K activation stimulates cell cycle progression through G1 into S phase by downregulating levels of the cyclin/Cdk inhibitor p27kip1, and by inducing the activation of the E2F transcription factor, which in turn regulates cyclins and other genes important for cell cycle progression (346). Further, PI-3K-induced p70 S6 kinase activation regulates translation initiation and protein synthesis.

The tyrosine phosphorylation of IL-2Rβ on Y338 results in the phosphotyrosine binding domain-mediated recruitment of Shc and its subsequent phosphorylation (327, 347). Shc associates with Grb2 and couples IL-2R to the activation of the Ras/MAPK pathway. Optimal IL-2-induced proliferation requires both activation of STAT5 and Shc-mediated activation of Ras/MAPK (327). IL-2 also induces the tyrosine phosphorylation of the PTPase SHP-2 and the scaffolding protein Gab2, and results in the formation of a multi-molecular complex containing SHP-2, Gab2, Grb2 and the p85 PI-3K subunit (348-351). SHP-2 and Gab2 can support the IL-2 mediated activation of STAT5 and the Ras/MAPK pathway, and a catalytically inactive SHP-2 mutant inhibits IL-2-induced STAT5 and MAPK activation.
Lck associates with the acidic region of IL-2Rβ and is activated upon stimulation of T cells with IL-2 (352, 353). Lck can phosphorylate tyrosine residues within the IL-2Rβ chain (354), and may also support IL-2-mediated activation of PI-3K (355). IL-2Rβ also interacts with Syk (356). While the activation of Lck is independent of JAKs, IL-2 induces the JAK3-dependent activation of Syk, as well as the Pyk2 PTK (344, 357). Although dominant negative mutants of Pyk2 can inhibit IL-2-induced proliferation (357), the biological roles of Syk and Pyk-2 activation in IL-2R signalling remain unclear.

**Regulation of IL-2R signalling**

The responsiveness of T cells to IL-2 can be modulated by other cytokines. IL-4 suppresses IL-2-induced JAK1 and JAK3 activation, putatively by sequestering the IL-2Rγ chain into the IL-4 signalling complex (358, 359). The immunosuppressive cytokine TGF-β inhibits IL-2-induced activation of JAK1 but not JAK3 (360), at a stage following receptor binding (361). IL-2 induced JAK/STAT activation and T cell proliferation can also be inhibited by macrophage derived products, including nitric oxide and prostaglandin E₂ (362, 363).

Signalling through cytokine receptors is rapid but transient and is regulated by several pathways, including endocytosis and degradation of the receptor complex, the dephosphorylation of activated signalling components and the induction of molecules which suppress signalling [reviewed in (364, 365)]. Negative regulatory pathways involved in limiting IL-2R signalling are shown in Figure 1-8. Deregulation of IL-2R signalling has been implicated in the oncogenic transformation of T cells, and constitutive activation of the JAK/STAT pathways has been observed in T leukemia and lymphoma cells (366-369).

Ligand binding induces rapid internalisation of the IL-2/IL-2R complex which serves to limit the duration of IL-2 signalling. A mutant of IL-2 that results in decreased receptor
internalisation shows increased bioactivity (370). IL-2R internalisation is dependent on the induction of tyrosine kinase activity (371) and is not mediated by clathrin coated pits (372). Upon internalisation, IL-2Rα is recycled to the plasma membrane (373). In contrast, IL-2, IL-2Rβ and IL-2Rγ are routed to late endocytic compartments for proteosomal degradation (373, 374).

STAT3 dependent DNA binding and transcriptional activation is inhibited upon cytokine-induced binding to protein inhibitor of activated STAT (PIAS) 3 (375). IL-2R signalling is also regulated by several PTPases. Upon stimulation with IL-2, SHP-1 binds to the IL-2R and dephosphorylates IL-2Rβ, JAK1 and JAK3 (376). SHP-1 overexpression inhibits JAK1 and JAK3 phosphorylation, and the loss of SHP-1 in transformed T cells correlates with constitutive JAK/STAT signalling. Furthermore, T cells in SHP-1 deficient mice have increased proliferative responses to IL-2 (377). Despite evidence that the SHP-2 PTPase positively regulates IL-2R signalling, SHP-2 was shown to dephosphorylate STAT5 proteins in vitro suggesting a role in negative regulation (378). SHP-2 is also constitutively associated with JAK1 and JAK3 (348), but the outcome of this interaction is unclear.

The CD45 PTPase was recently shown to bind and dephosphorylate the JAK kinases JAK1, JAK2 and Tyk2, resulting in inhibition of kinase activity (379). CD45-deficient cells were hyper-responsive to stimulation with many cytokines including IL-3, IL-4, interferon α and erythropoietin. Although a negative regulatory role for CD45 in IL-2R signalling has not yet been demonstrated, it appears likely given the ability of CD45 to dephosphorylate JAK1, and the hyper-phosphorylation of JAK3 observed upon stimulation of CD45<sup>−/−</sup> B cells with IL-4 (379).

Suppressor of cytokine signalling (SOCS) family proteins are involved in the negative regulation of JAK/STAT signalling [reviewed in (364, 380, 381)]. SOCS proteins, characterised by a central SH2 domain and a C-terminal SOCS box, are induced in response
to cytokine stimulation, at least in part due to STAT transcription factors, and thus form a negative feedback loop to limit cytokine-induced signalling. Several SOCS proteins regulate signalling through IL-2R. The expression of CIS1 (cytokine inducible SH2 domain containing protein) is regulated by STAT5, and is induced in response to IL-2. CIS1 is thought to inhibit signalling by binding to phosphotyrosine residues in activated cytokine receptors and competing for STAT5 binding sites. CIS1 has been shown to associate with IL-2Rβ, and to inhibit the transcriptional activation of STAT5 (382). Mice transgenically overexpressing CIS1 have defects in IL-2-induced IL-2Rα expression and T cell proliferation, due to inhibited cytokine-induced activation of STAT5 (383).

The SH2 domain of SOCS1 binds to a critical tyrosine within the activation loop of activated JAK kinases resulting in the inhibition of kinase activity, possibly by preventing access of substrates and/or ATP (381). The SOCS1 promoter contains a binding site for STAT3, and its expression is induced in response to IL-2. SOCS1 overexpression inhibits IL-2-induced STAT5 phosphorylation and transcriptional activity (384), and SOCS1−/− mice are hyper-responsive to IL-2 (385).

SOC3 is also induced in response to IL-2 and suppresses IL-2-induced phosphorylation of STAT5 and proliferation through its interaction with JAK1 and inhibition of JAK1 phosphorylation (386). SOCS3 also binds activated IL-2Rβ, and optimal inhibition of JAK1 may occur when SOCS3 is bound to the receptor.

SOCS proteins may also regulate cytokine signalling by targeting proteins for degradation. The SOCS box in SOCS proteins binds to the elongin BC complex which associates with cullin-2, a putative ubiquitin ligase (387, 388). This interaction may target the ubiquitination and subsequent proteosomal degradation of SOCS proteins and the activated signaling molecules to which they bind. In support of this model, CIS1 can be
Signalling through the IL-2R is negatively regulated by PIAS3 and the SHP-1 phosphatase, and may also involve SHP-2 and CD45. In addition, stimulation with IL-2 induces the expression of the SOCS family proteins CIS, SOCS1 and SOCS3, which then suppress IL-2R signalling. See text for details.
ubiquitinated (389), and SOCS1 can target Vav for ubiquitination and degradation (390). Furthermore, IL-2-induced STAT5 activation is prolonged in the presence of proteosome inhibitors (389, 390).

**Genetic deficiencies in IL-2R and components of IL-2R signalling**

Analysis of mice deficient in IL-2, IL-2Rα and IL-2Rβ revealed normal T and B cell development in young animals, demonstrating that IL-2 signalling is not essential for lymphoid development (392, 393). However, with age, these mice develop splenomegaly, lymphadenopathy and autoimmune disease characterised by inflammatory bowel disease, auto-antibody production and hemolytic anemia. Further, a patient lacking IL-2Rα expression was found to have similar characteristics, with splenomegaly, lymphadenopathy and multi-organ lymphocytic infiltration (394).

Mutations leading to the inactivation or lack of expression of IL-2Rγ or JAK3 in humans leads to X-linked or autosomal severe combined immunodeficiency, respectively (392). These patients lack peripheral T and NK cells, and have normal numbers of B cells which are not functional. These observations reflect the critical developmental role of cytokines other than IL-2 which share usage of IL-2Rγ; signalling through the IL-7R is essential for T cell development in humans. Mice deficient in IL-2Rγ or JAK3 have decreased thymic cellularity, but the remaining T cells have normal development. Thus, IL-7R contributes to but is not essential for T cell development in mice. In contrast, B cell development is arrested at an early stage in IL-2Rγ or JAK3−/− mice, reflecting an essential role for IL-7R in B cell development in mice.

In mice lacking IL-2, IL-2R components, or JAK3, peripheral T cells accumulate with age and have an activated phenotype, as assessed by a higher rate of *in vivo* proliferation, the expression of cell surface markers and cell morphology (392).
accumulation of activated cells in these animals is antigen driven, and is not observed in germ free mice (395) or in T cells expressing high levels of transgenic TcR which do not encounter antigen (396-398). Surprisingly, T cells isolated from these mice do not proliferate or secrete IL-2 in response to stimulation through TcR/CD3 in vitro, however this finding has been attributed to in vivo hyper-activation (398).

IL-2 was first characterised as a T cell growth and survival factor, however the phenotype of mice lacking IL-2 or IL-2R chains demonstrate that IL-2R signalling is essential for the homeostatic regulation of T cell activation and the maintenance of peripheral tolerance. Following T cell activation by antigen, two major mechanisms support the programmed cell death of activated T cells. Clearance of antigen results in the cessation of TcR stimulation and IL-2 synthesis, and activated T cells undergo apoptosis as a result of cytokine deprivation. In addition, activation and cell cycle progression renders T cells susceptible to Fas-mediated activation-induced cell death (AICD). AICD is essential for the elimination of autoreactive T cells and the maintenance of tolerance. Other cytokines can compensate for IL-2 with respect to growth and survival, however IL-2 plays a unique and critical role in promoting AICD.

IL-2 potentiates the susceptibility of activated T cells to Fas-mediated AICD (399) through several mechanisms. Activation of STAT5 proteins by IL-2 synergises with signalling through TcR/CD3 to induce expression of Fas ligand (FasL) (400-402). Thus, T cells lacking both STAT5a and STAT5b also accumulate activated T cells (332). Further, IL-2 induces the downregulation of FLIP (FLICE inhibitory protein), an inhibitor of Fas-mediated caspase activation (400). The defect in T cell homeostasis and the development of autoimmunity in the absence of IL-2R signalling is due to the failure of T cells to undergo AICD, and is similar to the phenotype of mice defective in Fas or FasL expression (403). Consistent with the critical role of IL-2 in promoting AICD, impaired superantigen-mediated
deletion of peripheral T cells was observed in IL-2°, IL-2Rα° and IL-2Rγ° mice (397, 404, 405). Further, impaired activation-induced FasL expression was observed in IL-2Rγ° or STAT5α/STAT5b° T cells (313, 397), and IL-2° and IL-2Rα° T cells showed insensitivity to apoptosis in response to ligation of Fas (404, 406).

TcR-induced feedback inhibition of ongoing immune responses is further linked with IL-2R signalling. Re-stimulation of activated, cytokine dependent T cells through TcR/CD3 blocks IL-2-induced activation of JAK1, JAK3, STAT5 and PKB, through a MAPK dependent pathway (407). IL-2-induced expression of cell cycle regulatory proteins, cell cycle progression and proliferation were also inhibited by TcR-mediated signalling in activated T cells (407, 408). TcR-mediated inhibition of IL-2R signalling may occur in the presence of persistent or large amounts of antigen, and may result in apoptosis by mimicking cytokine withdrawal or by inducing the growth arrest postulated to be a prerequisite for Fas-mediated AICD.

**THESIS OUTLINE AND PREVIEW**

Accumulating evidence suggests that lipid rafts play a critical role in regulating signalling through many receptors. The studies described in Chapters 3 and 4 were initiated prior to the current understanding of how lipid rafts function in signal transduction. However, it was known that multiple GPI-AP with unrelated protein moieties functioned comparably to mediate both T cell activation and, under different constraints, to inhibit T cell responsiveness to stimulation through TcR/CD3. These studies were aimed at investigating whether and how GPI-AP mediated a common effect on TcR/CD3-induced T cell activation. Immobilised mAb specific for GPI-AP were used to enable the assessment of biological outcomes of T cell activation, such as proliferation and cytokine secretion, as well as
biochemical outcomes, including the tyrosine phosphorylation of intracellular substrates, under identical conditions. Although this system was set up to investigate the effects of GPI-AP on TcR/CD3-induced signalling, the use of immobilised mAb specific for GPI-AP instead uncovered a role for lipid rafts in IL-2R signalling.

In Chapter 3, results are presented demonstrating that immobilised mAb specific for GPI-AP inhibit TcR/CD3-induced T cell proliferation. TcR/CD3-mediated signalling appears unperturbed, and IL-2 secretion and cytolytic activity are not impaired. Rather, proliferation induced by endogenously secreted IL-2 is inhibited as immobilised mAb specific for GPI-AP block IL-2-induced heterotrimerisation of the IL-2R and activation of the JAK1 and JAK3 kinases.

In Chapter 4, T cell proliferation in response to exogenous IL-2 is shown to be inhibited by immobilised mAb specific for GPI-AP, as well as by ligand-mediated immobilisation of another component of lipid rafts, the GM1 ganglioside. Although IL-2Rα was constitutively localised in lipid rafts, the heterotrimeric IL-2Rαβγ signalling complex was not detected in membrane microdomains. Thus, IL-2Rα may dissociate from lipid rafts to interact with IL-2Rβ and γ in soluble membranes and initiate signalling. The proportion of IL-2Rα in lipid rafts was increased in the presence of immobilised mAb specific for GPI-AP, suggesting that components of lipid rafts may inhibit IL-2R signalling by blocking the mobility and/or intermolecular associations of IL-2Rα in lipid rafts.

These results characterise a role for lipid rafts in the regulation of IL-2R signalling. The implications of these findings, as well as questions remaining to be resolved and future directions for these studies are discussed in Chapter 5.
CHAPTER 2

MATERIALS AND METHODS
Primary T cells, clones and transfections

Primary CD8^+ T cells were purified from the lymph nodes of 6 - 10 week old C57Bl/6 mice purchased from Jackson Laboratories. Lymph node cell suspensions were incubated with rat anti-mouse CD4, and passed over columns (Cytovax Labs) pre-treated with goat anti-rat IgG to remove CD4^+ T cells and goat anti-mouse IgG to remove B cells. The CD8^+ T cell preparations were consistently found to be >95% CD8^+TcRαβ when assessed by flow cytometry.

Clone 2.10 is an IL-2 dependent, Vβ4-expressing, CD4^- T cell clone specific for ovalbumin derived peptide comprised of residues 143-157 in the context of I-A^b (409). Forced expression of IL-2Rβ in clone 2.10 was achieved by electroporating 10^7 2.10 cells with 10 μg of the eukaryotic expression vector BCMGSneo (410), or with this vector containing cDNA encoding mouse IL-2Rβ (411). Following selection in 1 mg/ml G418, cells were sorted for high expression of IL-2Rβ on a FACStar Plus (Becton-Dickinson). Levels of IL-2Rβ expression remained stable when cells were propagated in the presence of G418.

2.10 clonal variants expressing or lacking GPI-AP were isolated by sorting Thy-1^- and Thy-1^- cells on a FACStar Plus, and the phenotype of sorted cells remained stable in culture. The expression of GPI-AP in GPl^- 2.10 was restored upon infection of cells with the MIEV retroviral vector (412) containing cDNA encoding PIG-P (8). The cDNA encoding PIG-P was cloned into MIEV 5' to an encephalomyocarditis virus internal ribosome entry site followed by cDNA encoding enhanced green fluorescent protein (EGFP). Thus, infected cells express a polycistronic mRNA resulting in the expression of PIG-P and EGFP, and can be isolated by sorting GFP^+ cells. The Phoenix-Eco packaging cell line (413) was transfected with 0.5 μg of empty MIEV or PIG-P-MIEV using Effectene (Quiagen). Transfected Phoenix-Eco cells transiently produced infectious virions, and supernatants from
these cells were harvested after 48 hours and used to infect GP+E packaging cells (414).
GP+E lines stably producing infectious virions were established by sorting GFP⁺ cells using
a FACStar Plus. Sorted GP+E cells were co-cultured for 48 hours with GPI⁻ 2.10 in the
presence of 8 µg/ml polybrene (Alrich), a polycationic substance which increases the
efficiency of infection by decreasing repulsion between cells and virions. Finally, GFP⁺
retrovirally-infected 2.10 cells were isolated by sorting using a FACStar Plus.

The CTLL-2 T cell line was obtained from American Type Culture Collection
( ATCC).

Antibodies and flow cytometry

Antibodies used in these studies include mAbs specific for Thy-1 [30H12 (415),
M5/49 (ATCC) and 5-3.2.1 (BD BioSciences )], Ly6A/E [D7, (416)], CD45 [M189 (417)],
CD5 [53-7.3 (415)], CD4 [GK1.5, rat IgG2b mAb isotype control (418), and HI29, rat IgG2a
isotype control (419), IL-2 [S4B6.34.1 (420)] and IL2Ra [PC61 (ATCC) and 7D4 (421)].
These mAbs were purified from hybridoma supernatants on Sepharose 4B beads conjugated
with mouse anti-rat Igκ. MARK-1 (422). MAbs specific for TcRCβ, H57-597 (423) and
CD48, 5-8A10 (424) were purified on Protein A-Sepharose. Normal hamster IgG (Jackson
Immunoresearch Laboratories) was used as an isotype control for anti-CD48. Unless
otherwise indicated, anti-Thy-1 refers to the 30H12 mAb.

Flow cytometric analysis was performed following labelling of 3 x 10⁵ cells in 100 µl
of PBS containing 5% FCS with the indicated antibodies for 10 minutes on ice, followed by
3 washes in PBS/FCS. IL2-R expression was analysed using 10 µg/ml of mAb specific for
IL-2Ra (PC61) followed by FITC-mouse anti-rat IgG (FITC-MaR Ig, Jackson
Immunoresearch Laboratories); phycoerythrin-labeled mAb specific for IL-2Rβ [PE-TM81
(Pharmingen)]; and mAb specific for IL-2Rγ [4G3 (425)] followed by FITC-MaR Ig.
Expression of GPI-AP was determined by labelling of cells with anti-Thy-1 (30H12) or anti-Ly6A/E (D7) followed by FITC-mouse anti-rat κ, or FITC-conjugated anti-CD48 (5-8A10). Prior to analysis on a FACScalibur (BD BioSciences), cells were resuspended in 1 μg/ml 7-amino-actinomycin-D (7AAD: Sigma). All plots shown exclude dead cells based on forward versus side scattering profiles as well as positive staining with 7AAD.

Cellular DNA content was determined by Vindelov's method (426). Cells were cultured as indicated, then harvested, pelleted and resuspended by drop-wise addition of Vindelov's solution [3.4 mM TrisHCl pH 7.6, 10 mM NaCl, 0.1% v/v NP-40, 50 μg/ml propidium iodide (Sigma) and 20 μg/ml Rnase A (Boehringer Mannheim)]. The proportion of cells with sub-diploid DNA content was assessed by flow cytometry on a FACScalibur (Becton-Dickinson), using doublet discrimination in the FL2 channel.

Proliferation assays

Purified cholera toxin β subunit (CT; Sigma) and all antibodies used in these studies were diluted to 10 μg/ml in Hanks basic salt solution (HBSS) without calcium or magnesium chloride. with the exception of anti-TcR, which was used at 1 μg/ml unless otherwise indicated. 50 μl of diluted solution was added to wells of 96 well cluster flat bottom tissue culture plates, and plates were incubated for 1 hour at 37°C. Following 2 washes with HBSS, 5 x10⁴ primary T cells, or 2 x 10⁴ 2.10 T cells or CTLL-2 cells, were added in 200 μl of serum free culture medium (409). In assays for IL-2-induced proliferation, cells were pelleted onto the coated plates by centrifugation and cultured for 1 hour at 37°C prior to the addition of cytokine. TcR-induced proliferation was assessed after 40 hours of culture and IL-2-induced proliferation was assessed after 20 hours of culture by pulsing each well with 1 μCi of ³H-thymidine for a further 6 hours. ³H-thymidine uptake was assessed by liquid scintillation spectroscopy using a Topcount Microplate scintillation counter (Camberra
Packard). In Figure 4-1, primary CD8+ T cells were stimulated in 24 well plates coated with anti-TcR. After 20 hours of culture, the activated cells were harvested, and viable cells were isolated on a Lympholyte M gradient (Cedarlane) and cultured as described above.

The bioassay for IL-2 content described in Figure 3-2 was performed by adding 5 x 10^3 CTLL-2 cells to the dilution of culture supernatant indicated. Cultures were brought to a final volume of 200 μl of serum free culture medium, and cultured for 48 hours prior to assessing ^3H-thymidine uptake as described above. Supernatants from the X630 hybridoma transfected with cDNA encoding IL-2 (427) were used as a source of cytokine. The activity of the IL-2-containing X630 hybridoma supernatant was quantitated by bioassay using CTLL-2. One unit was defined as resulting in half-maximal proliferation of 5 x 10^3 CTLL-2 cells cultured for 48 hours in 200 μl of medium and diluted supernatant.

**CTL assays**

Lymph node cells were obtained from mice transgenic for a Vα2/Vβ8 TcR specific for a lymphocytic choriomeningitis virus (LCMV) glycoprotein derived peptide, residues 33 - 41 (gp33), in the context of H-2D^b (428). 5 x 10^5 cells were stimulated in vitro for 2 days in the presence of 10^-7 M gp33 and 4 x 10^5 irradiated syngeneic splenocytes in 24 well cluster tissue culture plates in serum free medium. Viable cells were isolated on Lympholyte-M gradients and cultured in 25 U/ml IL-2 for 5 days, after which cells were harvested and restimulated with gp33 for 3 days, as described above. Viable cells isolated at this point were used in subsequent assays. Cells were pre-incubated for 1 hour at 37°C in flat bottom 96 well cluster tissue culture plates that had been coated with either anti-CD48 or normal hamster IgG. Following this pre-incubation, 10^5 51Cr (NEN/DuPont, Boston, Massachusetts)-labelled MC57G fibroblasts were added as target cells. Prior to their addition, the MC57G cells were pulsed with antigen by incubating 10^6 cells in 100 μl of 10^-7
M gp33 for 1 hour at 37°C, followed by 2 washes in medium. 

$^{51}$Cr release into the supernatant after a six hour culture period at 37°C was quantitated using LumaPlates and a Topcount Microplate counter. The percentage of specific lysis was calculated as (experimental release - spontaneous release)/(total release - spontaneous release) x 100. The growth inhibitory effects of anti-Thy-1 and anti-CD48 on these cells was confirmed by performing proliferation assays in parallel.

Northern blot analysis

2.10 T cells were untreated, or cultured for 20 hours in the presence of co-immobilised anti-TcRβ and either anti-CD48 or normal hamster IgG. Total RNA was extracted using TRIzol (Gibco-BRL) as per manufacturer's instructions. Briefly, cells were lysed in TRIzol and RNA was extracted by phenol-chloroform, precipitated in 50% isopropanol, and washed in 75% ethanol. The ratio of optical densities of the RNA samples at 260 nm and 280 nm was consistently >1.8. 15 μg of each RNA sample was electrophoresed on a 1.2% agarose gel containing 3% formaldehyde, 0.02 M MOPS, 8 mM sodium acetate and 1 mM EDTA. RNA bands were transferred to a GeneScreen nylon membrane (Dupont) and cross-linked with UV light. The blots were pre-hybridised overnight at 42°C in 25 ml of 6X SSC, 50% formamide, 0.5% SDS, 10% dextran sulfate, 5X Denhardt's solution and 100 μg/ml herring sperm DNA, and then hybridised for 8 hours with 25 x 10^6 cpm of the indicated probe. Probes were prepared by radiolabelling the 1.3 kb EcoRI insert of pSI mIL-2Ra; the EcoRI/NotI inserts of pSI mIL-2Rβ (1.8 kb); or pSI mIL-2Rγ (1.2 kb), using a commercial kit (Pharmacia). Labelled probes were separated from excess $^{32}$P-dCTP (Dupont) by chromatography on Sephadex G-50 columns (Pharmacia). After hybridisation, membranes were washed twice with 2X SSC for 10 minutes at room temperature, and then with 5X SSC, 1% SDS at 60°C. Results were visualised by
autoradiography, and quantitative analysis was performed using a PhosphorImager (Molecular Dynamics). Blots were stripped by washing with 0.1X SSC and 1% SDS at 80°C for 1 hour, and hybridisation was carried out as above with 32P-labelled L32 ribosomal protein cDNA, which provided a loading control to which signals for the IL-2R chains were normalised (429).

Isolation of lipid rafts

Lipid rafts were isolated by discontinuous sucrose density gradient ultracentrifugation. 10 - 30 x 10⁶ cells were lysed at 20 x 10⁶ cells/ml in TKM buffer (50 mM Tris pH 7.4, 25 mM KCl, 5 mM MgCl₂ and 1 mM EDTA) containing 0.5% w/v Brij58 and protease inhibitors leupeptin (2.5 μg/ml), aprotinin (2.5 μg/ml) and Pefabloc (2 mM), all from Boehringer Mannheim. The lysates were incubated on ice for 30 minutes, mixed with an equal volume of 80% w/v sucrose in TKM, and overlaid with 5.5 mls of 36% sucrose in TKM followed by 2.5 mls of 5% sucrose in TKM. The sucrose density gradients were subjected to ultracentrifugation at 250,000 x g for 16-18 hours in an SW41 rotor (BD BioSciences), and 1 ml fractions were collected from the top. The protein or glycolipid content in each fraction was assessed by blotting, as described below.

Immunoprecipitations and immunoblotting

For the determination of the phosphotyrosine content of JAK1 and JAK3 in Figure 3-6, cells were lysed in TX-100 buffer containing: 50 mM Hepes, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM Na₂VO₄, 1 mM PMSF or Pefabloc and 2.5 μg/ml each of aprotinin and leupeptin, adjusted to pH 7.5. JAK1 and JAK3 were immunoprecipitated from the post-nuclear fraction of lysates containing 3 x 10⁶ and 1 x 10⁶ cell equivalents, respectively, using 5 μl of specific antisera (UBI) collected with Protein A-
Sepharose beads (Pharmacia). Proteins were resolved by SDS-PAGE, and transferred to nitrocellulose. All immunoblotting was done in 5% milk, 10 mM Tris HCl, 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween-20, adjusted to pH 7.5. Immunoblotting was performed using the phosphotyrosine specific mAb 4G10 (430), followed by horseradish-peroxidase (HRP) conjugated goat-anti-mouse IgG (GaMlg-HRP, Sigma), and revealed by enhanced chemiluminescence (Amhersham). Membranes were stripped by incubating for 15 minutes at room temperature in buffer containing 150 mM NaCl, 10mM Tris-HCl, adjusted to pH 2.3, and levels of JAK1 and JAK3 were revealed by immunoblotting using anti-JAK1 (Transduction Laboratories) followed by GaMlg-HRP, or JAK3 specific antisera (UBI) followed by protein-A-HRP (ICN).

Immunoprecipitation of IL-2R chains in Figures 3-5 and 3-7 was performed following lysis of cells in NP-40 buffer containing: 0.5% NP-40, 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM Na2VO4, 1 mM PMSF or Pefabloc and 2.5 µg/ml each aprotinin and leupeptin, adjusted to pH 7.4. IL-2Rβ and γ chains were immuno-precipitated from the post-nuclear fraction of lysates containing 1.5 x 10⁷ cell equivalents using 0.5 ml 5H4 culture supernatant (411) or 20 µg 3E12 (425) respectively, bound to Protein-A Sepharose beads which had been pre-coated with rabbit anti-rat Ig (Jackson Immunoresearch). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotting was performed as indicated. Polyclonal anti-IL-2Rβ and anti-IL-2Rγ chains (Santa Cruz Laboratories) were used, followed by Protein A-HRP.

To determine the levels of PKB phosphorylation, 2.10 cells were cultured for 1 hour in plates pre-coated with anti-Thy-1 or isotype control and stimulated for the indicated times with 400 U/ml IL-2. Cells were lysed in 1X boiling SDS sample buffer (4X contains 25% glycerol, 5% SDS, 250 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol and bromophenol blue). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and
immunoblotting was performed using PKB and phosphorylated serine 473-PKB specific antibodies (New England Biolabs) followed by Protein-A-HRP.

The protein or glycolipid content of fractions isolated from sucrose density gradients was determined by immunoblotting. 5 μl of each fraction was diluted in TKM/Brij58 and transferred to nitrocellulose by dot-blotting. GM1 was detected by probing the membrane with HRP-conjugated cholera toxin β subunit (Sigma). The localisation of Fyn, Thy-1 and CD45 were assessed following subjecting of 15 μl of each fraction to SDS-PAGE, and transfer to nitrocellulose. Immunoblotting was performed using the Thy-1 specific mAb 30H12 and the CD45 specific mAb M189, followed by rabbit anti-rat IgG-HRP (Sigma), and anti-Fyn serum (provided by Dr. A. Veillette) followed by protein-A-HRP (ICN).

The membrane localisation of IL-2R chains (Figure 4-6) was assessed in CTLL-2 cells which were either starved for 16 hours in 1.25 U/ml IL-2 (unstimulated), or following stimulation with 400 U/ml IL-2. Cells were pelleted, lysed in TKM/Brij58, and lipid rafts were isolated. To assess the effect of immobilised mAb specific for Thy-1 on the localisation of IL-2R chains (Figure 4-10), lipid rafts were isolated from CTLL-2 cells cultured for 16 hours with 15 U/ml IL-2 in flasks coated with 10 μg/ml anti-Thy-1 or an isotype-matched control mAb. As a positive control for immunoblotting, immunoprecipitation of IL-2R chains was performed following lysis of 2.10 cells (40 x 10^6 cells/ml) in NP-40 buffer. IL-2Rα, β and γ chains were immunoprecipitated from post-nuclear fractions of lysates containing 10^7 cell equivalents using 7.5 μg 7D4, 0.5 ml 5H4 culture supernatant (411) or 20 μg 3E12 (425), respectively, bound to Protein-A Sepharose beads which had been pre-coated with rabbit anti-rat Ig. Immunoprecipitates or 150 μl of the fractions from the sucrose gradients were resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was performed using polyclonal rabbit anti-IL-2Rα, β and anti-IL-2Rγ (Santa Cruz Laboratories), revealed by Protein-A-HRP.
To determine the membrane localisation of JAK1 and JAK3 (Figure 4-7), equal amounts of pooled lipid raft and soluble fractions, as assessed by blotting for GM1, were diluted four-fold in TX-100 buffer. Immunoprecipitations were performed using anti-JAK1 (Transduction Laboratories) and JAK3 specific antisera, and collected using Protein-A or Protein-G Sepharose beads (Pharmacia), respectively. Proteins were resolved on SDS-PAGE gels run in parallel, and Western blot analysis was performed using anti-phosphotyrosine or anti-JAK1 followed by GaMIg-HRP, or JAK3 specific antisera followed by protein-A-HRP.

To determine the effect of disrupting lipid rafts on IL-2R-mediated signalling, 10⁷ cells/ml were incubated with 10mM methyl-β-cyclodextrin (MCD, Aldrich Chemicals) for 20 minutes at 37°C. Where indicated, 400 U/ml IL-2 was added and cells were incubated for a further 10 minutes. Cells were pelleted, lysed in TX-100 buffer, and immunoprecipitation and immunoblot analysis of JAK1 and JAK3 from post-nuclear lysates was performed as described for Figure 4-7. To assess the effect of MCD on TcR-induced signalling, 5 x 10⁶ MCD-treated 2.10 T cells were pelleted and incubated at 10⁷ cells/ml with 2.5 μg/ml biotin-labelled anti-TcRCβ and 10 mM MCD for 45 minutes on ice. Cells were pelleted, resuspended to 4 x 10⁶ cells/ml, and warmed to 37°C prior to stimulation with 20 μg/ml streptavidin for 30 seconds. Cells were lysed in TX-100 buffer and PLCγ1 was immunoprecipitated from post-nuclear lysates using a mixture of mouse mAbs (UBI). Western blot analysis was performed on gels run in parallel using 4G10 and anti-PLCγ1 revealed by GaMIg-HRP.

**1²⁵-I-IL-2 binding assays**

Ligand-receptor binding studies were adapted from a previous description (431). In Figure 3-7, neo-IL-2Rβ-2.10 cells were stimulated overnight at 25 x 10⁶ cells/flask in 175 cm² tissue culture flasks pre-coated with 25 mls of anti-TcRCβ and either anti-CD4 or
normal hamster IgG. All subsequent incubations were performed using neo-IL-2Rβ-2.10 cells at $2.5 \times 10^6$ cells/ml (Figure 3-7) or CTLL-2 cells at $1 \times 10^6$ cells/ml (Figure 4-8). Cells were washed twice in PBS with 0.1% Na azide and incubated for 1 minute in 10 mM Na citrate. 150 mM NaCl adjusted to pH 4.0 to dissociate IL-2 bound to IL-2R. Cells were then washed twice in PBS with 3% FCS and 0.1% Na azide prior to the addition of $5 \times 10^{10}$ M $^{125}$I-labelled IL-2 (NEN). After a 30 minute incubation on ice, cells were washed twice in PBS with 0.1% Na azide, and incubated for 10 minutes in 2 mM of the crosslinker disuccinimidyl suberate (DSS: Pierce). Unreacted DSS was quenched by the addition of 5 mM ammonium acetate for 1 minute, after which cells were washed twice in PBS/0.1% sodium azide.

In Figure 3-7, Neo-IL-2Rβ-2.10 cells were lysed in NP-40 buffer. IL-2Rα was immunoprecipitated from post-nuclear fractions of lysates containing $5 \times 10^6$ cell equivalents using mAb 7D4 (421), and IL-2Rβ and γ chains were immunoprecipitated from post-nuclear fractions of lysates containing $5 \times 10^7$ cell equivalents with 5H4 and 3E12, respectively. Immune complexes were recovered using protein-A Sepharose pre-coated with rabbit anti-rat Ig, and proteins were resolved by SDS-PAGE. In Figure 4-8, $3 \times 10^7$ CTLL-2 cells were lysed in TKM/Brij58. Discontinuous sucrose density gradient centrifugation was performed, and 150 µl from fractions corresponding to lipid rafts and soluble membranes, as assessed by blotting for GM1, were resolved by SDS-PAGE. All gels were fixed in 40% methanol and 10% acetic acid, dried and autoradiographed at -70°C.
The results presented in this chapter were generated by Mina Marmor, and have been published: Mina D. Marmor, Martin F. Bachmann, Pamela S. Ohashi, Thomas R. Malek and Michael Julius. Immobilization of GPI-anchored proteins inhibits T cell growth but not function. International Immunology (1999) 11:1381-1393.
Introduction

While not affecting the specificity of T cell activation, an increasing number of membrane molecules have been shown to alter signals induced through the T cell antigen receptor. The modifications mediated by accessory activation molecules can be extreme, resulting in cell growth, anergy, or death. As discussed in Chapter 1, antibodies specific for GPI-AP have been shown to function in this context. Specifically, Thy-1, Ly6A/E, CD48 and TSA-1 specific mAbs can inhibit anti-TcR/CD3 induced T cell activation (39, 53, 57, 432). GPI-AP have also been implicated in T cell activation. Proliferation and cytokine secretion follow direct mAb mediated aggregation of Thy-1, Ly6A/E, Qa-2 and CD48 on murine lymphocytes (35, 36, 39, 433). Further, aggregation of CD59, CD55 and CD73 on human lymphocytes has been shown to induce cellular growth (32, 41, 48). The distinct consequences of signalling through various GPI-AP on the physiology of T lymphocytes in vitro appears to depend on the protocol used to ligate the molecules. Specifically, the addition of soluble mAbs, compared with their deliberate aggregation, support fundamentally distinct consequences on T cell physiology. While the central role of GPI-AP in development is supported by evidence that targeted disruption of an enzyme central to the biosynthesis of the anchor moiety results in embryonic death (96), the role of GPI-AP in cellular activation and growth control in physiological circumstances remains unknown.

Signalling through different GPI-AP induces qualitatively similar responses, consistent with the involvement of shared signalling pathways. Furthermore, the mitogenicity of mAbs to different GPI-AP, whose protein moieties are otherwise unrelated, suggests that the GPI anchor is critical. Consistent with this notion is the demonstration that antibody-mediated aggregation of transmembrane forms of Ly6A/E, CD55 or Qa-2, does not result in cellular growth (38, 145, 170). As discussed in Chapter 1, one consequence that
GPI-linkage imparts upon membrane molecules is their localisation to detergent-insoluble sub-domains of the plasma membrane called lipid rafts (111). Lipid rafts are enriched in cholesterol and gangliosides, as well as Src family PTK and heterotrimeric and small G proteins (111). Src PTK have been implicated in signalling through GPI-AP as these are co-precipitated in non-ionic detergents (112), and as mAb mediated aggregation of GPI-AP leads to a rapid increase in the tyrosine phosphorylation of intracellular substrates (434). Furthermore, the association of GPI-AP with Src family PTK predicates the capacity of mAbs specific for GPI-AP to induce cellular growth (145).

While of interest due to their capacity to activate T cells and to fundamentally alter signals generated through TcR/CD3, the conceptual difficulty associated with analyses of signal transduction through GPI-AP stems from the fact that the majority of their physiological ligands remain uncharacterised. Putative ligands for GPI-AP may be expressed on neighbouring cells, on the same cell, or in the extracellular milieu. Furthermore, the physiological role of certain GPI-AP with known ligands remain unclear. For example, although CD48 has been shown to bind CD2 (435), the outcome of this interaction in vivo is equivocal.

The question addressed in the present study is whether a common effector function can be attributed to any protein tethered to the plasma membrane through a GPI-anchor. The results demonstrate that mAb-mediated immobilisation of multiple GPI-AP on both primary CD8\(^+\) T cells, as well as on an IL-2 dependent, antigen specific T cell clone, virtually ablated cellular growth induced through the T cell antigen receptor complex. However, in these same circumstances, neither IL-2 production and secretion, nor cell mediated cytotoxic effector function was impaired. The inability to utilise IL-2 to support cell growth demonstrated a lesion in IL-2/IL-2R signalling. Flow cytometric analysis revealed the absence of the IL-2R\(\beta\) chain, however, forced expression of this chain did not revert the anti-
GPI induced lesion in cell growth. Further analyses revealed that when GPI-AP are immobilised, IL-2 failed to induce the recruitment of IL-2Rα, β, and γ chains, and the generation of the heterotrimeric IL-2R complex was inhibited. Consistent with this defect is that while mAb specific for GPI-AP did not affect the stoichiometry of IL-2Rβ and IL-2Rγ associated JAK1 and JAK3, respectively, the latter did not become activated. These results highlight a novel mechanism through which T cells could mediate their effector functions in the absence of clonal expansion, and potential physiological counterparts reflecting these circumstances are discussed in terms of cellular homeostasis and function.

Results

Immobilisation of GPI-AP ablates T cell growth but not effector functions

To determine whether immobilisation of GPI-AP alters the consequences of signals emanating from the TcR/CD3 complex, we first assessed effects on primary T cells. CD8⁺ lymph node T cells were cultured on plates which had been pre-coated with mAb specific for TcR-Cβ in combination with mAb specific for Thy-1 (30H12), or an isotype control. As illustrated in Figure 3-1A, anti-TcR induced DNA synthesis was profoundly inhibited in the presence of anti-Thy-1, over a broad range of anti-TcR concentrations used to coat the culture wells. Further, while the coating concentration of the Thy-1 specific mAb used in this experiment was 10 μg/ml, titration of the coating concentrations for this mAb revealed that it mediated its effects from 100 to 3 μg/ml (data not shown). Anti-TcR induced DNA synthesis was unaffected by the isotype control (Figure 3-1A), or mAbs specific for other membrane molecules including CD45 and MHC Class I (data not shown). Further, the effects observed were not specific to mAb 30H12, as two other mAbs specific for Thy-1 (M5/49 and 5-3.2.1) mediated identical effects (data not shown). Importantly,
immobilisation of these mAbs was required, as the addition of soluble mAb to plates coated with anti-TcR had no effect on the induction of T cell growth.

In aid of the further biochemical analysis of the signalling lesions in this system, we next determined whether the above observations could be extended to T cell clones. We have utilised the antigen specific, IL-2 dependent T cell clone, 2.10 (409). As illustrated in Figure 3-1B, Thy-1 specific mAb inhibited anti-TcR induced DNA synthesis of 2.10. Furthermore, mAbs specific for CD48 and Ly6A/E mediate the same effects as anti-Thy-1 in this assay system, on both 2.10 (Figure 3-1C) and primary CD8+ T cells (data not shown). We have also assessed the effect of mAbs specific for Qa-2 (436), and observed less profound inhibition, which in turn correlated with lower levels of membrane expression as assessed by flow cytometry (data not shown). Thus, the phenomenon appears to be generalisable to GPI-AP.

Culture of primary T cells or clone 2.10 in plates coated with immobilised mAb specific for Thy-1 resulted in profound morphological changes. Within 1 hour of culture at 37°C, 2.10 cells appeared flattened, with irregularities in cell shape and spindle-like projections. In contrast, cells proliferating in response to co-immobilised anti-TcR and control mAbs have spherical morphology and grow in clusters (Figure 3-1D). The photomicrographs presented in Figure 1D are at the same final magnification, and thus emphasise the marked difference in the apparent size of the cells in the presence of immobilised isotype control and Thy-1 specific mAbs. These alterations were observed in conjunction with all of the mAbs specific for GPI-AP assayed, but not with mAbs specific for the transmembrane proteins CD8 or MHC class I. The changes in cell morphology are dependent upon the immobilisation of mAb specific for the GPI-AP, and are not dependent
Figure 3-1  Antibodies specific for GPI-AP inhibit anti-TcRCβ-induced T cell proliferation

Primary CD8⁺ T lymphocytes from lymph nodes (A) or the 2.10 T cell clone (B) were cultured in wells that had been coated with the indicated concentrations of anti-TcRCβ and 10 µg/ml of mAb specific for Thy-1 (circles) or a mAb isotype control (squares). After 40 hours, each culture was pulsed with 1 µCi of ³H-thymidine for 6 hours, harvested, and thymidine uptake assessed by liquid scintillation spectroscopy. (C) The proliferation of 2.10 cells in response to the optimal concentration of anti-TcRCβ (1 µg/ml), in the presence of Thy-1, Ly6A/E and CD48 specific mAb, or their isotype-matched control Abs. was assessed as above. (D) Photographs (at X 100 magnification) of 2.10 cells cultured for 20 hours in the presence of immobilised anti-TcRCβ and anti-Thy-1 (left) or isotype matched control mAb (right).
on the presence of co-immobilised anti-TcR (data not shown). Thus, the GPI-AP-mediated alterations in cell morphology are likely induced independently of signals generated upon TcR aggregation.

Viability of clone 2.10 is strictly dependent on IL-2. The presence of this cytokine, provided exogenously or produced endogenously following antigen receptor stimulation, results in cell growth. Thus, it was striking to observe that the cells were not dying in conditions in which DNA synthesis was ablated by mAbs specific for GPI-AP. We therefore assessed whether anti-TcR induced IL-2 production was perturbed in these circumstances. As illustrated in Figure 3-2B, supernatant from growth inhibited cultures contained more IL-2 than those from cultures containing isotype control mAb, consistent with the fact that it was not being utilised in support of proliferation. Proliferation of the indicator cell line was shown to be mediated by IL-2, as thymidine uptake was inhibited by anti-IL-2 (Figure 3-2B), but not by mAb specific for IL-4 or IL-5 (data not shown). These results are identical to those obtained with primary T cells (Figure 3-2A). Again, immobilisation of these mAbs was required in aid of generating this phenotype. Thus, while immobilisation of GPI-AP, with concomitant activation through the antigen receptor complex, ablated cell growth, cytokine-mediated effector functions remained intact.

To assess the effects of this treatment on cell mediated effector function, we determined whether mAb specific for GPI-AP perturbed CTL activity. We utilised lymph node T cells from mice transgenic for a lymphocytic choriomeningitis virus (LCMV) specific TcR (428). Lymph node cells from these transgenic animals were stimulated with gp33 peptide (residues 33-41) from LCMV glycoprotein in vitro, and CTL activity assessed in the presence of anti-CD48 or isotype control. As illustrated in Figure 3-3A, the CTL activities were indistinguishable in these two culture conditions, over the indicated titration of effector:target ratios. To ensure that the effects of immobilising GPI-AP on these CTL
Figure 3-2  Anti-TcR-induced IL-2 secretion is not inhibited by mAbs specific for GPI-AP

Supernatants from primary CD8\(^+\) T cells (A) or clone 2.10 (B) cultured for 20 hours with anti-TcR and anti-Thy-1 (circles) or an isotype control (squares) were diluted as indicated, and IL-2 content was assessed by culture with the IL-2 dependent cell line CTLL-2. Uptake of \(^3\)H-thymidine was assessed after 40 hours of culture. Closed symbols indicate pre-incubation of the respective supernatants with anti-IL-2 prior to the addition of CTLL-2.
paralleled those observed in primary CD8\(^+\) T cells and clone 2.10, we determined the affect of anti-CD48 and anti-Thy-1 on proliferation assays set up in parallel with the CTL assays. These effector cells were derived from antigen stimulated cultures, and IL-2-induced proliferation was observed in the absence of further TcR stimulation. This growth was inhibited in the presence of either anti-CD48 or anti-Thy-1 (Figure 3-3B). Furthermore, anti-TcR induced proliferation was also inhibited by mAbs specific for GPI-AP (Figure 3-3C). Thus, as for T cell effector functions supported by cytokines, a cell mediated T cell function was not perturbed by the GPI-mediated growth inhibition.

**Immobilisation of GPI-AP inhibits IL-2R\(\beta\) expression**

Notwithstanding the production of IL-2 by T cells stimulated with co-immobilised mAbs specific for TcR and GPI-AP, the T cells exhibit a growth inhibited phenotype. This suggests a lesion in IL-2R signalling. To determine the presence of the IL-2R chains necessary for IL-2 mediated T cell growth, we assessed the levels of membrane expression of IL-2R components. As illustrated in Figure 3-4A, the level of IL-2R\(\alpha\) was in fact higher in the presence of anti-CD48 than in the presence of isotype control. Further, the level of IL-2R\(\gamma\) was indistinguishable in the two culture conditions. In marked contrast, membrane expression of IL-2R\(\beta\) was virtually undetectable in cultures containing anti-CD48.

Steady state levels of mRNA for each of the three chains were assessed by Northern blot analysis (Figure 3-4B). Total RNA from unstimulated cells or cells cultured with anti-TcR and co-immobilised anti-CD48 or an isotype control was isolated, electrophoresed on an agarose gel and transferred to a nylon membrane. Hybridisation with \(^{32}\)P-labelled probes specific for murine IL-2R\(\alpha\), \(\beta\) and \(\gamma\) revealed the characteristic pattern of bands. An increase in the level of IL-2R\(\alpha\) mRNA and comparable amounts of IL-2R\(\beta\) and IL-2R\(\gamma\) mRNA
Figure 3-3  The cytotoxic T cell response is not affected by anti-CD48

(A) In vitro activated lymph nodes cells from TcR transgenic mice specific for LCMV glycoprotein derived peptide gp33 (428) were incubated for 1 hour in plates coated with anti-CD48 (circles) or normal hamster IgG (squares). $^{51}$Chromium labeled target cells (MC57G fibroblasts) incubated with gp33 (open symbols) or not (closed symbols) were then added, and chromium release into the supernatant was assessed 6 hours later. The growth inhibitory effect of anti-CD48 and anti-Thy-1 on these cells was confirmed by assessing $^3$H-thymidine uptake, after 40 hours of culture, in response to 25 U/ml mIL-2 (B) or co-immobilised anti-TcR (C).
2.10 cells were untreated, or cultured for 20 hours in the presence of immobilised anti-TcRβ and co-immobilised anti-CD48 or normal hamster IgG. (A) Cells were analyzed by flow cytometry for the presence of IL2R α, β and γ chains. Open histograms represent staining with secondary antibodies alone, while shaded histograms represent specific staining. Numbers in the upper right corner represent the fold increase in mean fluorescence intensity over background.

(B) Northern blot analysis of total RNA was performed with 32P-labeled murine IL-2R α, β and γ cDNA (425). The bottom panel shows northern blot analysis of the membranes stripped and re-probed with 32P-labeled L32 ribosomal protein cDNA as a loading control (429). Sizes of RNA standards are indicated.
were detected by Northern blot analysis in cells cultured in the presence of anti-CD48 relative to isotype control (Figure 3-4B). Thus, the profound decrease in membrane expression of IL-2Rβ is not accounted for by comparable changes in steady state levels of mRNA, which in turns suggests regulation at the level of translation, internalisation, or indeed the capacity to detect the protein at the membrane of cells treated with immobilised mAb specific for GPI-AP.

**Forced expression of exogenous IL-2Rβ does not revert GPI-mediated growth inhibition**

If the growth inhibited phenotype mediated by immobilisation of GPI-AP was secondary to the lack of membrane expression of IL-2Rβ, the prediction follows that forced expression of exogenous IL-2Rβ should rescue growth. Towards testing this hypothesis, clone 2.10 was transfected with a construct encoding IL-2Rβ. Cells were selected in G418, and populations expressing increased levels of IL-2Rβ were isolated using a FACStar Plus. The resulting population (neo-IL-2Rβ 2.10) expressed 3-4-fold higher levels of IL-2Rβ compared to those expressed by either clone 2.10, or variants of 2.10 which had been transfected with the "empty" vector (neo-2.10), and selected in G418 (Figure 3-5A, upper panel). Further, the exogenous IL-2Rβ was functional, as over-expressing cells displayed increased survival in limiting concentrations of IL-2, relative to the parental 2.10 or neo-2.10 lines (data not shown). The question follows whether forced expression of this IL-2R chain reverts the phenotype mediated by immobilising GPI-AP.

Flow cytometric analysis revealed that, as was seen in untransfected clone 2.10, membrane expression of IL-2Rβ on neo-IL-2Rβ-2.10 was decreased in anti-TcR-stimulated cells in the presence of anti-CD48, relative to controls (Figure 3-5A). Nonetheless, the expression of IL-2Rβ in these circumstances was comparable to its expression on untransfected cells in control cultures (Figure 3-5A, compare upper left and middle lower
Figure 3-5  Forced expression of IL-2Rβ does not rescue anti-TcR-induced proliferation in the presence of mAbs specific for GPI-AP

A

2.10  neo IL-2Rβ 2.10  neo 2.10

isotype ctrl  

anti-CD48

B

C

2.10 cells were transfected with a vector encoding the neoR gene, or neoR as well as the IL-2Rβ gene. (A) The expression of IL-2Rβ on G418 resistant cells was assessed by flow cytometry as in Figure 4A. Numbers in the upper right corner represent the fold increase in mean fluorescence intensity over background. (B) 3H-Thymidine uptake by untransfected 2.10 (circles), neo-IL-2Rβ 2.10 (triangles) and neo-2.10 (squares) in response to co-immobilised anti-TcRβ and either anti-CD48 (closed symbols) or normal hamster IgG (open symbols) was assessed as in Figure 1. (C) IL-2Rβ was immuno-precipitated from the indicated cell lines following culture for 20 hours in the presence of immobilized anti-TcRβ and co-immobilized anti-CD48 or normal hamster IgG. Proteins were resolved by SDS-PAGE, material transferred to nitrocellulose, and immunoblotting was performed using polyclonal rabbit anti-IL-2Rβ.
panels). Notwithstanding the maintenance of levels of IL2Rβ expression comparable to those observed in cells which respond to IL-2 with growth, cells stimulated with co-immobilised anti-TcR and anti-CD48 exhibited a growth inhibited phenotype (Figure 3-5B). Thus, the lack of membrane IL-2Rβ expression per se does not account for the observed lack of IL-2 mediated growth.

In order to determine whether IL-2Rβ was still present in the cell in circumstances where it cannot be detected on the cell membrane by immunofluorescence, IL-2Rβ was immunoprecipitated from cells cultured with anti-TcR and anti-CD48 or normal hamster IgG. As illustrated in Figure 3-5C, IL-2Rβ was revealed in immunoprecipitates of neo-2.10 and neo-IL-2Rβ-2.10 cells cultured in the presence of CD48-specific mAb, despite undetectable or decreased levels of cell surface expression.

**Im mobilisation of GPI-AP correlates with the lack of Janus kinase activation**

The lesion in IL-2R signalling in this system was further analysed by assessing the status of the Janus kinases, JAK1 and JAK3, which are required for IL-2 induced cellular growth (437). Towards this end cellular JAK1 and JAK3 were immunoprecipitated from lysates of clone 2.10 which had been cultured in the presence of co-immobilised anti-TcR and either anti-CD48 or an isotype control. Immunoprecipitates were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with phosphotyrosine specific mAb. As illustrated in Figure 3-6, the phosphotyrosyl content of JAK1 and JAK3, indicative of their activation states (437), was profoundly reduced when cells were cultured in the presence of anti-CD48 relative to isotype control. To ensure that IL-2 was not limiting in these cultures, despite the observed increase in levels of JAK tyrosine phosphorylation in control cultures, some cultures were supplemented with 1000 U/ml of exogenous IL-2. As illustrated in Figure 3-6, even this concentration of IL-2 did not rescue the increase in
Figure 3-6  Antibodies specific for GPI-AP inhibit IL-2-induced JAK activation

2.10 cells were untreated, or cultured for 20 hours in the presence of co-immobilised anti-TcR Cβ and either anti-CD48 or normal hamster IgG. JAK1 and JAK3 were immunoprecipitated from cell lysates using specific antisera, and immunoblotting was performed using the phosphotyrosine specific mAb (4G10). Lanes 4 and 5 are lysates from cells that had been pulsed with 1000 units/ml of recombinant IL-2 (427) for 15 minutes prior to the end of the 20 hour culture period. Membranes were stripped, and levels of JAK1 and JAK3 were revealed by immunoblotting.
JAK1/3 phosphotyrosyl content observed in the presence of isotype control mAb. To ensure that the observed difference in JAK1 and JAK3 phosphotyrosyl content were not due to underloading in samples from cells cultured in the presence of immobilised anti-CD48, immunoblots were stripped and reprobed with either JAK1 or JAK3 specific antisera.

Identical results were obtained using primary CD8+ T cells. and variants of clone 2.10 overexpressing IL-2Rβ (not shown). These results suggest that the IL-2R signalling lesion is membrane proximal in that coupling to the first second messenger generating system known to be operative in IL-2 mediated signal transduction is perturbed. While demonstrating a reduction in the activation of cellular JAK kinases, these results leave open the possibility that the stoichiometry of JAK1 and JAK3 association with IL-2Rβ and IL-2Rγ chains, respectively, is altered as a consequence of immobilising GPI-AP. The absence or reduction of constitutively associated JAK kinases with IL-2R could account for the observed phenotype.

**Im mobilisation of GPI-AP inhibits IL-2-induced formation of the heterotrim eric IL-2R complex**

To establish whether the defect in IL-2R mediated JAK kinase activation could be due to the disruption of the association with their respective IL-2R chains, IL-2Rβ and γ chains were immunoprecipitated from lysates of neo-IL2Rβ-2.10 cells stimulated with co-immobilised anti-TcR and either CD48-specific mAb or an isotype control mAb. Immunoprecipitates were resolved by SDS-PAGE, transferred to membranes and immunoblotted with JAK1 or JAK3 specific antisera. As illustrated in Figure 3-7A, the stoichiometry of neither the JAK1/IL-2Rβ, nor the JAK3/IL-2Rγ association appeared altered in cells from anti-CD48 containing cultures compared to cells from cultures containing the isotype control mAb. To ensure equivalent loading, these immunoblots were stripped and re-
probed with IL-2Rβ and γ specific antisera (Figure 3-7A). This result obviates the possibility that the absence of Janus kinase activation in this system is due to their lack of constitutive association with their respective IL-2R chains. Thus, the basal composition of the IL-2R chains in this context appeared poised to respond to IL-2, yet did not.

The activation of JAK1 and JAK3 is mediated by IL-2 induced heterotrimerisation of the IL-2R chains. Ligand mediated aggregation of these chains results in the juxtaposition of associated kinases, leading to their transphosphorylation and activation (437). Since we have demonstrated that GPI-mediated growth inhibition is not due to the disrupted association of these kinases with their respective IL-2R chains, it is plausible that the impaired activation of JAK kinases reflects the inability of IL-2 to recruit the IL-2R chains, resulting in defective heterotrimerisation and impaired IL-2-induced signalling.

This question was addressed by taking advantage of the capacity of the anti-IL-2Rα mAb 7D4 to efficiently immunoprecipitate IL-2 cross-linked to either IL-2Rα, IL-2Rβ or IL-2Rγ as a stable multi-subunit complex. The variant of clone 2.10 in which IL-2Rβ was over-expressed was used to ensure the presence of sufficient levels of membrane receptor. Thus, neo-IL-2Rβ-2.10 cells derived from cultures containing co-immobilised anti-TcR and either anti-Thy-1 or isotype control mAb were incubated with 125-I-iodine-labelled IL-2. Cell bound IL-2 was chemically cross-linked to membrane proteins using disuccinimidyl suberate, followed by lysis and the immunoprecipitation of IL-2R chains from the post-nuclear fraction of lysates. In cells stimulated with anti-TcR and an isotype control, immunoprecipitation of IL-2Rα revealed three bands. The mobility of these bands corresponded to that expected for IL-2 complexed with each of IL-2Rα, γ and β (Figure 3-7B). Immunoprecipitation with anti-IL-2Rβ and γ confirmed the identity of the two upper bands (Figure 3-7B). In marked contrast, immunoprecipitation from lysates of anti-Thy-1-growth inhibited cells with anti-IL-2Rα revealed a dominant band migrating with an apparent molecular weight of 70 kDa, and
Figure 3-7  Anti-Thy-1 inhibits IL-2-induced receptor heterotrimerisation

Neo-IL-2Rβ cells were cultured for 20 hours in the presence of co-immobilised anti-TcRβ and either anti-CD48 or normal hamster IgG. (A) IL-2R β or γ chains were immuno-precipitated, proteins resolved by SDS-PAGE, material transferred to nitrocellulose, and immunoblotting was performed as indicated. (B) Cells were incubated with $^{125}$I-labelled interleukin-2. After two washes, bound IL-2 was cross-linked to cell-surface proteins. Following lysis, IL-2Rα, β and γ were immunoprecipitated, and proteins resolved by SDS-PAGE. Gels were fixed, dried, and visualised by autoradiography.
thus corresponding to IL-2 complexed with IL-2Rα. The bands consistent with IL-2 complexes of IL-2Rβ and γ chains were not observed in anti-IL-2Rα precipitates from lysates of these cells. As illustrated in Figure 3-7B, and consistent with a profound reduction in the generation of the heterotrimeric IL-2R complex in the presence of immobilised anti-Thy-1, direct precipitation with mAbs specific for IL-2Rβ and γ did not reveal bands consistent with IL-2 complexed with either of these IL-2R chains.

Discussion

A GPI anchor imparts functional attributes upon membrane molecules, including localisation to lipid rafts and increased lateral mobility in the membrane. Furthermore, as many GPI-AP have been implicated in T cell signalling, and moreover, have been shown to mediate similar effects, the GPI anchor may impart signalling functions. The aims of this study were to address whether GPI-AP mediate a common function in regulating T cell growth, and to characterise the mechanism through which this regulation occurs. The approach taken was to determine the effects of immobilised mAbs specific for a panel of GPI-AP on T cells stimulated with co-immobilised anti-TcR. The effect of immobilised mAbs may be to impede the mobility of GPI-AP in the membrane, to prevent their interaction with ligands, or to induce signalling events.

We demonstrated that in both primary CD8+ T cells and T cell clones, anti-TcR-induced proliferation was profoundly inhibited in the presence of co-immobilised mAb specific for the GPI-AP Thy-1, CD48 and Ly6A/E. Other transmembrane molecules expressed on the surface of these T lymphocytes, including CD45 and MHC Class I, did not alter proliferative responses. Thus, inhibitory effects on T cell proliferation are generalisable to GPI-AP, and may reflect the perturbation of common signalling pathways. Src family
PTK, associated with GPI-AP in glycosphingolipid membrane microdomains and implicated in signalling through these proteins, may be critical in growth-inhibition. Importantly, there is a precedent for Src PTK in inhibition of growth, as the anti-proliferative effects of interferon-α in T cells have been shown to require Lck (438).

Immunobilised mAbs specific for GPI-AP resulted in rapid and profound morphological changes in T cells. In contrast to the spherical shape and growth in clusters which was observed in control cultures, mAb-mediated ligation of GPI-AP resulted in cell flattening and irregularities in cell shape, such as the generation of spindle-like projections. These morphological changes may reflect re-organisation of the actin cytoskeleton, and may also involve activation of Src family PTK, which have been implicated in cytoskeletal changes (106). Consistent with this hypothesis, similar morphological changes have been observed in response to immobilised anti-CD45 (439), and are blocked by inhibitors of actin polymerisation, as well as by a PTK inhibitor. Moreover, the phosphoproteins induced by immobilised anti-CD45 are co-immunoprecipitated with anti-Lck (439). Further, the GPI-AP uPAR and Src PTK have been co-precipitated with the β2 integrin (440), and other GPI-AP undergo lateral associations with integrins (441), presenting another mechanism through which signalling and cytoskeletal organisation can be effected by proteins tethered to the membrane through a GPI-anchor.

A hallmark of the growth inhibited phenotype described herein is that T cell effector functions are retained. Thus, despite the growth inhibition induced by co-immobilised mAbs for TcR and GPI-AP, both primary cells as well as the IL-2 dependent 2.10 T cell clone, remained viable. This was particularly striking for the latter, as its viability is strictly IL-2 dependent. Assessment of the amount of IL-2 in culture supernatants of growth inhibited T cells revealed the presence of more IL-2 than in control cultures, consistent with the lack of utilisation of this cytokine in support of proliferation. Further, cell mediated cytolysis
proceeded unimpaired in circumstances of growth inhibition. Consistent with these observations is that while Ly6A-deficient cells from knockout mice exhibit dysregulated anti-TcR-induced proliferative responses, cytolytic functions were not perturbed (75).

It need be emphasised that the GPI-mediated state of T cell physiology reported here required the immobilisation of mAbs specific for the various GPI-AP. Other methods of ligating these molecules result in a distinct T cell phenotype. Corresponding with previous reports (39, 53, 55), when T cell activation was achieved through the addition of soluble anti-TcR, the addition of soluble mAb specific for GPI-AP inhibited both anti-TcR induced proliferation and IL-2 secretion (data not shown). In this context, it has been demonstrated that inhibition of T cell cytokine secretion and cell growth by soluble mAbs specific for Ly6A/E and Tsa-1 (Sca-2) was independent of the GPI anchor, as transmembrane forms of these molecules mediated the same effects as wild type molecules (37, 57). However, we cannot infer that the GPI anchor is dispensable in mediating the effects described in this study, and it remains to be determined whether transmembrane forms of Ly6A/E or Tsa-1 can mediate the growth inhibited phenotype supported by their GPI-linked counterparts. Furthermore, it is plausible that the proteinaceous portions of Ly6A/E and Tsa-1 are involved in the inhibition of T cell activation, independently and in addition to functions imparted by the GPI-anchor. Importantly, and in contrast to the dispensability of the GPI-anchor in processes supporting the inhibition of cytokine secretion and cellular growth, the activation of T cells following aggregation of GPI-AP appears to be dependent upon the GPI-anchor, as aggregation of transmembrane forms of Ly6A/E, CD55 or Qa-2 does not result in cell growth (38, 145, 170). The demonstration in the present study that all the GPI-AP tested to date mediated the same phenotype strongly suggests that the GPI-anchor is critical.

Towards elucidating the basis for GPI-mediated growth inhibition, notwithstanding the presence of IL-2, we assessed the levels of membrane expression of IL-2R components.
Immobilised mAb specific for GPI-AP resulted in perturbations in the expression of the heterotrimeric IL-2 receptor. While IL-2Rα and IL-2Rγ expression was increased or unchanged, membrane expression of IL-2Rβ was virtually undetectable in cultures containing anti-CD48. This result was not reflected in steady state mRNA levels, as Northern blot analysis revealed only a modest decrease in IL-2Rβ mRNA. IL-2Rβ expression may be regulated at the level of translation, or by receptor internalisation. Alternatively, the ability to detect IL-2Rβ at the cell surface by flow cytometry may be impaired as a result of conformational changes or membrane alterations in cells treated with immobilised mAb specific for GPI-AP. It is of note in this context that despite undetectable levels of expression of membrane IL-2Rβ by immunofluorescence, the protein was readily detectable in lysates of growth inhibited cells. However, the lack of membrane IL-2Rβ expression per se did not account for the observed lack of IL-2 mediated growth. Forced expression of exogenous IL-2Rβ did not rescue anti-TcR induced proliferation, despite levels of expression in the presence of GPI-specific mAb comparable to untransfected cells in control cultures. Thus, membrane expression of all three of the IL-2R components in circumstances of growth inhibition is consistent with disruption of IL-2R signalling.

The protein tyrosine kinases JAK1 and JAK3 are constitutively associated with the IL-2Rβ and γ chains, respectively, and are critical in IL-2R signal transduction. We demonstrate that these interactions are not disrupted in the presence of mAb specific for GPI-AP. Nonetheless, IL-2 failed to induce increases in the phosphotyrosyl content of these associated kinases, which is indicative of their activation. This result suggests that the uncoupling of IL-2 mediated JAK kinase activation may be due to a block in ligand mediated receptor heterotrimerisation. A defect in the aggregation of the receptor chains would result in the failure to juxtapose JAK1 and JAK3, and in turn, their impaired transphosphorylation and activation. IL-2 binding assays were performed using clonal variants overexpressing IL-
2Rβ to ensure the ability to immunoprecipitate IL-2R chains. IL-2 mediated receptor aggregation was indeed perturbed in circumstances of growth inhibition. Immunoprecipitation of IL-2Rα revealed three bands corresponding to IL-2 cross-linked to IL-2Rα, γ and β. In contrast, immunoprecipitation of IL-2Rα from cells stimulated in the presence of anti-Thy-1 revealed a predominant band corresponding to IL-2 cross-linked to IL-2Rα. Furthermore, bands visualised following the immunoprecipitation of IL-2Rβ and γ were decreased in these circumstances. Thus, and possibly reflected in the profound changes in cell morphology induced by these culture conditions, IL-2 induced redistribution of membrane IL-2R chains was inhibited, and subsequent growth responses to IL-2 proportionately reduced. The impaired generation of the high affinity heterotrimeric IL-2R complex in these circumstances suggests that the mobility of some of the chains may be restricted by the treatment.

These results characterise a novel mechanism through which T cells could mediate their effector functions in the absence of clonal expansion. There are data consistent with a physiological role for GPI-AP in the maintenance of cellular homeostasis and function. Specifically, insight is derived from analyses of patients suffering from paroxysmal nocturnal hemoglobinuria (PNH), an acquired hematopoietic stem cell disorder characterised by a lack of GPI-AP due to a mutation in the Pig-A gene, which is required for anchor biosynthesis (4). As several complement regulatory molecules are GPI-anchored, PNH is characterised by complement-mediated hemolysis. In PNH patients, a stem cell clone with a somatic mutation in Pig-A expands such that a large proportion of hematopoietic cells harbour this mutation. This suggests that the lack of expression of GPI-AP imparts a growth advantage (442). Consistent with this notion, following in vitro stimulation of peripheral blood cells from patients with PNH, the proportion of GPI-deficient T lymphocytes increased relative to GPI+ T cells from the same patient (85).
Studies using targeted gene knockout mice also support a role for GPI-AP in regulating T cell growth in vivo. Mice lacking GPI expression on T lymphocytes were generated as a result of a T cell-specific disruption of the Pig-A gene (66), and have demonstrated that GPI-AP are not critical in the initiation of T cell activation, as had been previously suggested (58, 61, 443). These mice do not have defects in T cell development and peripheral responses to superantigen. However, the role of GPI-AP in growth control was not assessed by extensive characterisation of T cell responses. Thymocytes from Thy-1-deficient mice show modest increases in responsiveness to stimulation through the antigen receptor, and the differentiation of thymocytes from the double positive to the single positive stage is decreased, perhaps due to increased TcR-signaling and negative selection (71). Further, T cells lacking Ly6-A expression display prolonged proliferation following stimulation through the antigen receptor complex (75). These results suggest that in the absence of any given GPI-AP, subtle defects in growth control occur, however, signalling mediated by the remaining GPI-AP may compensate to maintain growth regulation. In vitro, the outcome of anti-TcR induced activation is profoundly altered using immobilised antibodies specific for a given GPI-AP, however in vivo, all GPI-AP expressed on the surface of a cell may contribute to the regulation of T cell growth. In support of this, mAb specific for Thy-1 and CD48 inhibit the proliferation of CD8+ lymph node T cells from Ly6-deficient animals (data not shown), demonstrating that other GPI-AP can modify anti-TcR induced proliferation of Ly6A-deficient cells. The extent to which a GPI-AP contributes to the regulation of cell growth may depend upon its level of expression. MAbs specific for Qa-2 mediated less profound inhibition of anti-TcR-induced proliferation than Thy-1, CD48 or Ly6A/E, which in turn correlated with lower levels of membrane expression as assessed by flow cytometry.
A plausible physiological paradigm consistent with the observations presented in this study characterises a role for GPI-AP in minimising clonal expansion. Effector T cells specifically localising to sites of inflammation could be confronted with an array of previously described and/or as yet uncharacterised ligands for GPI-AP expressed on the surrounding inflamed tissues, which in turn could effect the circumstances which we have created in vitro. The consequences would ensure that the peripheral pool of T cells is not unduly diluted, while effector function towards clearance of the insult would proceed unimpaired.
The results presented in this chapter were generated by Mina Marmor and have been accepted for publication: Mina D. Marmor and Michael Julius. A role for lipid rafts in regulating IL-2 receptor signalling. Manuscript in press. Blood (2001).
Introduction

As discussed in Chapter 1, lipid rafts are plasma membrane microdomains postulated to function in signalling (3, 111, 117). Lipid rafts are enriched in gangliosides (glycosphingolipids) and cholesterol which form 'liquid-ordered domains' of decreased membrane fluidity. The long saturated acyl chains of gangliosides impart a high degree of order which is further stabilised by intercalating cholesterol molecules, leading to the insolubility of lipid rafts in non-ionic detergents. Lipid rafts are commonly isolated on the basis of their detergent insolubility and low buoyant density using discontinuous sucrose gradient ultracentrifugation of non-ionic detergent lysates. Importantly, lipid rafts are not an artefact of detergent extraction and have been detected in living cells using chemical cross-linking and fluorescence resonance energy transfer (121, 122).

The lipid modification of proteins with saturated acyl groups can result in their localisation within lipid rafts. Thus, these microdomains are enriched in GPI-AP and many signalling molecules such as Src family protein tyrosine kinases, the adaptor protein LAT, heterotrimeric and small G-proteins and phosphoinositides (112-116, 134). In addition, several transmembrane receptors are inducibly recruited to or stabilised within lipid rafts, including TcR, BcR and FceRI (3). The subsequent activation of signalling molecules in lipid rafts may facilitate signalling through these immunoreceptors. Lipid rafts may act to segregate molecules in the plasma membrane and regulate signalling through the spatial co-ordination of intermolecular associations.

Stimulation of T cells through TcR results in the activation of multiple signalling pathways leading to IL-2 responsiveness and the secretion of IL-2, and resulting in autocrine cell growth (312). As discussed in Chapter 1, the high affinity receptor for IL-2 is composed of the IL-2Rα chain, which functions solely in IL-2 binding, and IL-2Rβ and γ chains, which
contribute to IL-2 binding and mediate signal transduction (313). IL-2-induced proliferation requires activation of the Janus family kinases JAK1 and JAK3, which are constitutively associated with IL-2β and γ chains respectively (319, 320). Ligand-induced IL-2R aggregation leads to the juxtaposition of JAK1 and JAK3, resulting in their phosphorylation and activation. The subsequent phosphorylation of tyrosine residues in receptor chains leads to the SH2-mediated recruitment of signal transducer and activator of transcription (STAT) proteins, STAT5a and STAT5b (313). JAK-mediated phosphorylation of STAT proteins leads to their dimerisation by SH2 domain-phosphotyrosine interactions, and their translocation to the nucleus where STAT proteins can bind to conserved promoter sequences to regulate gene transcription. Signalling through the IL-2R also induces the recruitment and activation of PI-3K (335, 336), which is implicated in IL-2-mediated proliferation and survival through its downstream effector PKB/Akt (338, 339). Further, the tyrosine phosphorylation of IL-2Rβ results in the recruitment of Shc and Grb2, and activation of the Ras/MAPK pathway. The non-receptor protein tyrosine kinases Lck, Syk and Pyk-2 also associate with IL-2Rβ, however the functional outcome of these interactions remain unclear (313).

Similar to many other receptors, the IL-2R is not randomly distributed in the lipid bilayer. IL-2Rαβγ chains appear to form a pre-existing complex on the surface of T cells, which is brought into closer proximity by ligand binding (316), resulting in the aggregation of IL-2Rβ and γ chains required for signalling (317, 318). In addition, IL-2Rα has been found in clusters on the cell surface, which appear to correspond to lipid rafts (444).

Results described in Chapter 3 demonstrate that mAb specific for GPI-AP inhibit T cell proliferation, but not IL-2 secretion, following stimulation through TcR/CD3. Further, in these circumstances, IL-2-induced heterotrimerization of the IL-2R complex is inhibited, with a corresponding decrease in IL-2-induced signalling. These results suggest that GPI-AP
inhibit the responsiveness of T cells to endogenously produced IL-2. In this study, we demonstrate that GPI-AP and another component of lipid rafts, the GM1 ganglioside, modify signalling through IL-2R in response to exogenous IL-2, and characterise the involvement of lipid rafts in IL-2R signalling.

Results

**Immobilised mAb specific for GPI-AP inhibit IL-2-induced proliferation**

As described in Chapter 3. in the presence of immobilised mAb specific for GPI-AP, anti-TcR-induced proliferation was inhibited, despite the production of IL-2 (445). In addition, IL-2-induced signalling was inhibited in these circumstances. These results were consistent with a signalling defect in the responsiveness of T cells to endogenously produced IL-2. To confirm that GPI-AP inhibited signalling through IL-2R, we determined the effect of mAb specific for GPI-AP on T cell proliferation in response to exogenous IL-2.

The IL-2 dependent 2.10 T cell clone (Figure 4-1A) or the CTLL-2 T cell line (Figure 4-1B) were cultured in wells coated with anti-Thy-1, or an isotype matched control mAb in the presence of the indicated concentrations of IL-2. IL-2 induced DNA synthesis was determined by assessing the uptake of $^3$H-thymidine after 20 hours of culture. Immobilised anti-Thy-1 inhibited the proliferation of both 2.10 and CTLL-2 in response to IL-2 at a range of concentrations. Analysis of the growth inhibitory effects of anti-Thy-1 was extended to primary T cells, which require stimulation through TcR to induce expression of the high affinity IL-2R and acquire responsiveness to IL-2. Thus, unstimulated CD8$^+$ lymph node T cells did not proliferate in response to exogenous IL-2 (Figure 4-1C). Primary CD8$^+$ T cells were stimulated for 20 hours with anti-TcR, harvested, and re-stimulated with exogenous IL-2. As seen in Figure 4-1C, exogenous IL-2 induced the proliferation of activated primary T
Figure 4-1  Anti-Thy-1 inhibits IL-2-induced T cell proliferation

The 2.10 T cell clone (A) or the CTLL-2 T cell line (B) were cultured in wells that had been pre-coated with mAb specific for Thy-1 (open circles) or a mAb isotype control (closed circles) and the indicated concentration of IL-2. (C) Resting or TcR-stimulated CD8⁺ lymph node T cells were cultured in wells pre-coated with anti-Thy-1 or an isotype control in the presence of 15 U/ml IL-2. Uptake of [³H]-thymidine was assessed after 20 hours of culture.
cells, which was inhibited by anti-Thy-1.

Anti-TcR-induced proliferation, but not IL-2 production, was inhibited by mAb specific for multiple GPI-AP, as described in Chapter 3. Therefore, we determined the effect of GPI-AP in addition to Thy-1 on T cell proliferation induced by exogenous IL-2. Stable GPI" and GPI' clonal variants of the 2.10 T cell line were established by FACs sorting of cells based on expression of Thy-1. The GPI-AP Thy-1, Ly6A/E and CD48 are expressed on GPI" but not GPI' 2.10 T cells, as determined by flow cytometric analysis (Figure 4-2A,B). IL-2-induced proliferation of GPI' 2.10 (Figure 4-2C) and CTLL-2 (data not shown) is inhibited by several mAb specific for Thy-1, as well as mAb specific for Ly6A/E and CD48. The proliferation of the GPI' clonal variant is unaffected by these mAbs, as predicted for cells that lack expression of these proteins, demonstrating that the inhibition of proliferation is not due to non-specific toxic effects of the mAb preparations. IL-2-induced proliferation is not affected by isotype controls, or by mAbs specific for the transmembrane proteins CD45 and CD5 (Figure 4-2C). In addition, IL-2-induced proliferation of GPI", but not GPI' 2.10 was inhibited by mAb specific for Qa-2 and CD73, albeit to a lower extent, which in turn correlated with a decreased level of surface expression of these GPI-AP as detected by flow cytometry (data not shown). Thus, immobilised mAb specific for all GPI-AP tested to date inhibit T cell proliferation in response to exogenous IL-2.

**GPI 2.10 is defective in GPI anchor biosynthesis due to a defect in PIG-P expression**

GPI' 2.10 lacked expression of all GPI-AP tested, including Thy-1, Ly6A/E and CD48 (Figure 4-2A,B), suggesting that this cell line is defective in GPI-anchor biosynthesis. As illustrated in Figure 1-2, the first step in GPI-anchor biosynthesis is the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol to generate N-acetyl-glucosaminyI-phosphatidylinositol (GlcNAc-PI) (446). In a collaboration with the
Figure 4-2  IL-2-induced proliferation is inhibited by mAb specific for the GPI-anchored proteins Thy-1, Ly-6A/E and CD48

The expression of Thy-1, Ly6A/E and CD48 on GPI\(^{-}\) (A) and GPI\(^{+}\) (B) variants of the 2.10 T cell clone was analyzed by flow cytometry. The first histogram represents staining with secondary antibodies alone. (C) GPI\(^{-}\) and GPI\(^{+}\) 2.10 T cells were cultured in wells that had been pre-coated with the indicated mAbs and 15 U/ml IL-2. Uptake of \(^{3}\)H-thymidine is represented as the percentage of the proliferative response to IL-2 in the absence of added mAbs.
laboratory of Taroh Kinoshita at Osaka University, GPI' 2.10 was determined to be defective in the generation of GlcNAc-PI. The generation of GlcNAc-PI is catalysed by an enzyme complex which was previously known to consist of at least four proteins, PIG-A, PIG-C, PIG-H or GPI-1 (7). Somatic cell fusion of GPI' 2.10 with mutant cells lacking expression of PIG-A, PIG-C, PIG-H or GPI-1 resulted in Thy-1 expression, demonstrating that GPI' 2.10 expressed each of these proteins, and suggesting that GPI' 2.10 is defective in a fifth gene required for the generation of GlcNAc-PI. A novel protein, PIG-P, was isolated on the basis of its interaction with PIG-A. Transfection of GPI' 2.10 with a vector encoding PIG-P results in the re-expression of all GPI-AP, demonstrating that GPI' 2.10 lack expression of PIG-P. Moreover, these results demonstrated that PIG-P is a requisite part of the enzymatic complex that catalyses the first step in GPI-anchor biosynthesis. These experiments were performed by Yoshiko Murakami and Reika Watanabe, and were published in The EMBO Journal, R. Watanabe, Y. Murakami, M. Marmor, N. Inoue, Y. Maeda, J. Hino, K. Kangawa, M. Julius and T. Kinoshita. Initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-P and is regulated by DPM2. 19;4402-4411.

Re-constitution of the expression of GPI-AP on GPI' 2.10 upon infection with a retroviral vector containing cDNA encoding PIG-P (Figure 4-3A) restores the capacity of mAb specific for GPI-AP to inhibit proliferative responses induced by IL-2 produced endogenously following stimulation with anti-TcR (Figure 4-3B).

**Immunoblot specific for GPI-AP result in the dissociation of IL-2-mediated survival and proliferation**

The 2.10 T cell clone is IL-2-dependent and undergoes apoptotic cell death upon cytokine withdrawal. Strikingly, although IL-2-mediated proliferation was inhibited by immobilised mAb specific for Thy-1, IL-2 still supports cell survival in these circumstances.
Figure 4-3  Re-expression of GPI-AP in GPI 2.10 restores inhibitory capacity of GPI-AP specific mAb

(A) Flow cytometry was used to assess the expression of CD48 on GPI' 2.10, and GPI' 2.10 infected with a retroviral vector (GPI' MIEV-2.10), or the vector encoding PIG-P (GPI' PIG-P-MIEV-2.10). The first histogram represents staining with secondary antibodies alone. (B) GPI' 2.10 and infectants were cultured in wells that had been pre-coated with anti-Thy-1 or an isotype control in the presence of 15 U/ml IL-2. \(^{3}\)H-Thymidine uptake was assessed after 20 hours of culture.
Figure 4-4A demonstrates that in the absence of IL-2, the majority of cells undergo apoptosis by 24 hours following cytokine withdrawal. In the presence of IL-2, cells cultured with anti-Thy-1 or an isotype control mAb remain viable, despite the growth inhibition mediated by anti-Thy-1 in cultures set up in parallel (Figure 4-4B). Similar results are observed using mAb specific for CD48 or Ly6A/E, and using CTLL-2 cells (data not shown). In addition, mAb specific for GPI-AP inhibit proliferation induced by IL-2 produced endogenously upon stimulation through TcR (Chapter 3, and Figure 4-5C), yet endogenous IL-2 supported cell survival, as 2.10 T cells underwent apoptosis upon mAb-mediated neutralisation of IL-2 (data not shown). The increase in the number of control cells undergoing apoptosis at the 48 hour time-point is consistent with the IL-2 being used and catabolised by the proliferating cells. In the presence of immobilised anti-Thy-1, IL-2 is not utilised in aid of proliferation and continues to support cell survival (Figure 4-4A).

The ability of IL-2 to support cell survival but not proliferation in the presence of immobilised mAb specific for GPI-AP may be a result of residual signalling through IL-2R. Alternatively, cell survival in the absence of proliferation may result from distinct signals induced through IL-2R that are differentially affected by GPI-AP. The activation of PI-3K, and its downstream effector PKB, have been implicated in IL-2-mediated survival (338, 339). However, in the presence of mAb specific for Thy-1, IL-2-induced PI3K activation, as determined by PKB phosphorylation levels, was decreased relative to controls (Figure 4-4C). This result, in addition to our previous finding that immobilised anti-Thy-1 inhibits IL-2-induced IL-2R heterotrimerisation (Chapter 3, Figure 3-7B), is consistent with an inhibition of all signalling pathways induced through IL-2R, and suggests that viability may be mediated by residual signalling.
Figure 4-4  Anti-Thy-1 inhibits IL-2-induced proliferation, but not cell survival

(A) GPI+ 2.10 T cells were cultured in the presence of immobilized anti-Thy-1 or a mAb isotype control, in the presence or absence of 15 U/ml IL-2. After 20 hours, the percentage of apoptotic cells in each condition was assessed by flow cytometric quantitation of cells with subdiploid DNA content. (B) ^3H-thymidine uptake in response to 15 U/ml IL-2 was assessed in cultures set up in parallel to (A). (C) IL-2-induced phosphorylation of PKB was assessed by immunoblotting in cells cultured in the presence of anti-Thy-1 or an isotype control.
Another component of lipid rafts, the GM1 ganglioside, inhibits IL-2-induced proliferation

The ability of all GPI-AP tested to date to inhibit IL-2R-induced proliferation, despite their unrelated protein moieties, suggests that a characteristic imparted by the GPI anchor is critical for this inhibition. GPI anchoring results in the localisation of proteins to lipid rafts, and we hypothesised that the localisation of GPI-AP in lipid rafts underlies their inhibitory capacity. Therefore, we determined the effect of immobilising an additional component of lipid rafts, the GM1 ganglioside, on IL-2-induced proliferation.

To establish that plasma membrane compartmentalisation into lipid rafts and soluble membranes occurs in cells expressing or lacking GPI-AP, lipid rafts were isolated from GPI⁺ and GPI⁻ 2.10 T cells lysed in buffer containing Brij58, a weak non-ionic detergent which preserves lipid rafts. Fractions were collected following discontinuous sucrose density gradient ultracentrifugation, and the localisation of proteins and glycolipids was assessed by immunoblotting. In both GPI⁺ and GPI⁻ clonal variants, the transmembrane tyrosine phosphatase CD45 is found almost exclusively in soluble membranes, as previously reported (119, 168), while the GM1 ganglioside, detected using the β subunit of cholera toxin, is found almost exclusively in lipid rafts (Figure 4-5A,B). In addition the majority of the Fyn PTK is found within lipid rafts, as is the GPI-anchored Thy-1. As predicted, Thy-1 cannot be detected in immunoblots of fractions isolated from GPI⁻ cells (Figure 4-5A,B).

Immobilisation of GM1 inhibited the proliferation of both GPI⁺ and GPI⁻ T cells in response to IL-2 produced endogenously upon stimulation with anti-TcR (Figure 4-5C), or provided exogenously (Figure 4-5D). Immobilised mAb specific for Thy-1 inhibited anti-TcR and IL-2-induced proliferation of GPI⁺ but not GPI⁻ cells. The effects of immobilising GM1 using cholera toxin appear identical to those of GPI-AP using mAbs, as IL-2-induced proliferation but not cell survival or TcR-induced production of IL-2 were inhibited (data not
Figure 4-5  Proliferative responses of T cells to anti-TCR and IL-2 are inhibited by immobilised CT

(A, B) Plasma membrane compartmentalisation into lipid rafts in the presence or absence of GPI-anchored proteins. Lysates of GPI- (A) or GPI+ (B) 2.10 T cells were subjected to discontinuous sucrose density gradient ultracentrifugation. Fractions were collected from the top of the gradient, and the distribution of CD45, Fyn, Thy-1 and GM1 was analyzed by blotting. The fractions corresponding to lipid rafts and soluble membranes are indicated. (C,D) Immobilised CT inhibits anti-TCR and IL-2-induced proliferation. GPI+ and GPI- 2.10 T cells were cultured in wells pre-coated with CT, anti-Thy-1 or an isotype control mAb and co-immobilised anti-TcR-Cβ (C) or in the presence of 15 U/ml IL-2 (D). 3H-thymidine uptake was assessed after 20 hours of culture.
These results are consistent with the ability of components of lipid rafts to modify IL-2R signalling in T cells.

**IL-2Rα is enriched in lipid rafts, however IL-2R signalling occurs in soluble membranes**

The ability of components of lipid rafts to inhibit IL-2-induced proliferation suggests that lipid rafts may play a role in the regulation of IL-2R signalling. As a first step in assessing the role of lipid rafts in IL-2R signalling, we determined the localisation of IL-2R chains. CTLL-2 are maintained in IL-2, therefore in order to minimise potential effects of IL-2 on the distribution of its receptor chains, CTLL-2 cells were cultured for 16 hours in very low concentrations of IL-2 (1.25 U/ml). In these circumstances, IL-2R signalling, as assessed by the tyrosine phosphorylation of JAK1 and JAK3, was not detectable (Figure 4-7). CTLL-2 were not further treated (unstimulated) or were stimulated for 10 minutes with 400 U/ml IL-2 prior to lysis, and membranes were fractionated by discontinuous sucrose density gradient ultracentrifugation. Immunoblot analysis of fractions from these gradients revealed that a large proportion of IL-2Rα was localised in lipid rafts (Figure 4-6). In contrast, IL-2Rβ and γ chains were not enriched in lipid rafts, and the vast majority of these receptor chains were detected in soluble membranes. No significant differences in the localisation of IL-2Rα, β or γ chains were detected upon stimulation of cells with IL-2.

The JAK1 and JAK3 kinases are constitutively associated with the IL-2Rβ and γ chains, respectively (319, 320), and their activation following stimulation by IL-2 is critical for signalling through the IL-2R (437). We therefore assessed the localisation of JAK1 and JAK3. The kinases were immunoprecipitated from pooled fractions of sucrose density gradients derived from CTLL-2 corresponding to lipid rafts and soluble membranes, as determined by blotting for GM1. Figure 4-7 demonstrates that JAK1 and JAK3 are found in soluble membranes. In addition, those JAK1 and JAK3 molecules involved in IL-2R
CTLL-2 cells were left untreated, or stimulated for 10 minutes with 400 U/ml IL-2 prior to lysis. The lysates were subjected to discontinuous sucrose density gradient ultracentrifugation, and fractions were collected and separated by SDS-PAGE. The localization of IL-2α, β or γ chains was analyzed by immunoblotting. The final lane in each blot consists of immunoprecipitations of IL-2α, β or γ chains as positive controls for immunoblotting.
Figure 4-7  JAK1 and JAK3 are localised in detergent soluble membranes

CTLL-2 cells were left untreated, or stimulated for 10 min with 400 U/ml IL-2 prior to lysis and sucrose density gradient ultracentrifugation. JAK1 and JAK3 were immuno-precipitated from pooled fractions of sucrose gradients corresponding to lipid rafts (R) and soluble membranes (S).

Immunoprecipitations were split in two, resolved by SDS-PAGE, and immunoblotting was performed using phosphotyrosine (PY). JAK1 or JAK3 specific antibodies.
signalling, assessed by IL-2-induced tyrosine phosphorylation, were found in the soluble membranes. JAK1 or JAK3, as well as IL-2Rβ and γ chains, were not detected in lipid rafts at multiple additional time points following stimulation with IL-2 (1, 3, 9 or 27 minutes, data not shown). Similar results were observed using 2.10 T cells (data not shown).

IL-2 binding to receptor chains can be assessed using labelled cytokine. CTLL-2 cells were incubated with $^{125}$I-labelled IL-2, which was subsequently chemically cross-linked to bound receptor chains. The fractions from sucrose density gradients which corresponded to lipid rafts and soluble membranes were determined by blotting for GM1 (Figure 4-8A). Proteins in these fractions were separated by SDS-PAGE, and proteins cross-linked to $^{125}$I-labelled IL-2 were visualised by autoradiography. $^{125}$I-labelled IL-2 is cross-linked to IL-2Rα, β and γ chains in soluble membranes (Figure 4-8B). This result is consistent with the presence of the signalling complex, composed of IL-2 and IL-2Rαβγ, in soluble membranes exclusively.

Further support for IL-2R signalling occurring within soluble membranes was derived by assessing the effect of disrupting the integrity of lipid rafts. This was achieved using methyl-β-cyclodextrin (MCD), which results in the extraction of cholesterol by forming inclusion complexes within a hydrophobic cyclodextrin cavity (290). As shown in Figure 4-9A and B, MCD did not inhibit IL-2 induced tyrosine phosphorylation of JAK1 and JAK3 in CTLL-2. As a positive control for the disruption of lipid rafts by MCD, we assessed the effects on a TcR-induced signalling pathway known to be dependent on lipid rafts (169). While IL-2-induced tyrosine phosphorylation of JAK1 in 2.10 T cells was unaffected by MCD (Figure 4-9C), TcR-induced tyrosine phosphorylation of PLCγ1 was virtually ablated (Figure 4-9D).

Thus, although IL-2Rα was enriched in lipid rafts, the signalling components of the IL-2R, the IL-2Rβ and γ chains, as well as JAK1 and JAK3 phosphorylated in response to IL-
Figure 4-8  The heterotrimeric receptor complex composed of IL-2 bound to IL-2Rαβγ is detected in detergent soluble membranes

(A) CTLL-2 cells were incubated with ¹²⁵I-labelled interleukin-2, and bound IL-2 was cross-linked to cell-surface proteins. Following lysis, sucrose density gradient ultracentrifugation was performed. The fractionation into lipid rafts and soluble membranes was determined by blotting for GM1. (B) Proteins in fractions corresponding to lipid rafts and soluble membranes were resolved by SDS-PAGE and proteins cross-linked to ¹²⁵I-labelled interleukin-2 were visualised by autoradiography. Bands corresponding to ¹²⁵I-IL-2 cross-linked to IL-2Rα, β and γ are indicated.
Figure 4-9 Disruption of lipid raft integrity does not affect IL-2-induced tyrosine phosphorylation of JAK1 and JAK3

(A,B) CTLL-2 cells were left untreated or incubated with 10 mM MCD prior to stimulation with 400 U/ml IL-2. JAK1 and JAK3 were immunoprecipitated from post-nuclear lysates. Immunoprecipitations were split in two, resolved by SDS-PAGE, and immunoblotting was performed using phosphotyrosine (PY), JAK1 or JAK3 specific antibodies. (C,D) 2.10 cells were left untreated, or incubated with 10 mM MCD. (C) Cells were stimulated with IL-2, and JAK1 immunoprecipitated from post-nuclear lysates was immunoblotted using anti-phosphotyrosine (PY) or anti-JAK1. (D) Cells were stimulated with anti-TCR, and PLCγ1 immunoprecipitated from post-nuclear lysates was immunoblotted using anti-phosphotyrosine (PY) or anti-PLCγ1.
2. were not found in lipid rafts. The active heterotrimeric receptor complex composed of IL-2 bound to IL-2Rαβγ was not detected in lipid rafts. Further, IL-2R-induced signalling is not affected by the disruption of lipid rafts. Taken together, these results support the conclusion that IL-2R signalling occurs in soluble membranes.

**Immobilised anti-Thy-1 results in an increased proportion of IL-2Rα in lipid rafts**

The ability of lipid raft components to block IL-2R signalling suggests that lipid rafts can regulate signalling through the IL-2R, despite evidence that signalling occurs in soluble membranes. Lipid rafts may regulate IL-2R signalling by segregating elements of the receptor complex in the plasma membrane. IL-2 may result in the dissociation of IL-2Rα from lipid rafts, and its interaction with IL-2Rβ and γ chains in soluble membranes to initiate signalling. The inhibition of IL-2R signalling observed upon immobilisation of GPI-AP or the GM1 ganglioside in lipid rafts may be due to impairment of the mobility of IL2Rα, thus preventing its dissociation from rafts. Further, IL-2Rα may be in a dynamic equilibrium between lipid rafts and soluble membranes, and immobilisation of lipid raft components may shift this equilibrium by 'trapping' IL-2Rα chains in lipid rafts. In either case, the prediction would follow that a greater proportion of IL-2Rα would be present in lipid rafts in these circumstances. We therefore assessed whether immobilisation of components of lipid rafts affected the distribution of IL-2Rα.

CTLL-2 cells were cultured for 16 hours in the presence of immobilised anti-Thy-1 or isotype control mAb in the presence of 15 U/ml IL-2, conditions in which IL-2-induced proliferation is inhibited by anti-Thy-1. Membranes from cell lysates were fractionated by discontinuous sucrose density gradient ultracentrifugation and subjected to immunoblot analysis. Figure 4-10A and B demonstrates that the proportion of IL-2Rα localised in lipid rafts was higher in cells that had been cultured in the presence of immobilised anti-Thy-1,
Figure 4-10  The proportion of IL-2Rα in lipid rafts is increased in cells cultured in the presence of immobilized anti-Thy-1

(A) CTLL-2 cells were cultured in the presence of immobilized anti-Thy-1 or an isotype matched control mAb prior to lysis and sucrose density gradient ultracentrifugation. The localization of IL-2Rα and IL-2Rβ chains was assessed by immunoblotting the fractions of the sucrose gradients corresponding to lipid rafts and soluble membranes. (B) The proportion of IL-2Rα and IL-2Rβ in lipid rafts and soluble membranes was quantified by scanning densitometry. The data shown are represented as the percentage of the total IL-2Rα or β localised in lipid rafts, and are the averages from three independent experiments.
relative to an isotype matched mAb (61.4 +/- 15.8% versus 24.6 +/- 6.0%). In contrast, IL-2Rβ was not enriched in lipid rafts in the presence or absence of anti-Thy-1, and the proportion of IL-2Rβ in lipid rafts was less than 5% in all experiments. Thus, in the presence of immobilised anti-Thy-1, IL-2Rα is further enriched in lipid rafts and is segregated from IL-2Rβ and γ localised in soluble membranes. This demonstration is consistent with our earlier report that IL-2-induced receptor heterotrimerisation was inhibited by anti-Thy-1 (445).

Discussion

The results presented demonstrate that immobilised mAb specific for GPI-AP inhibit IL-2-induced proliferation of primary T cells and two T cell lines, GPI* 2.10 and CTLL-2. Multiple GPI-AP, including Thy-1, Ly6A/E and CD48, can mediate this effect suggesting that the ability to affect IL-2R signalling can be generalised to GPI-AP, and may reflect a common feature of these proteins as a class of molecules. An important characteristic imparted by the GPI-anchor is localisation to lipid rafts. Lipid rafts are microdomains in the plasma membrane which have a unique lipid composition and are enriched in cholesterol and glycosphingolipids, including the ganglioside GM1 (117). Consistent with the notion that their localisation to lipid rafts underlies the capacity of GPI-AP to modify IL-2R signalling, immobilisation of GM1 using the β subunit of cholera toxin also inhibits IL-2-induced proliferation.

The ability of multiple components of lipid rafts to modify cellular responsiveness to IL-2 suggests that lipid rafts can regulate IL-2R signalling, notwithstanding the fact that IL-2R signalling does not appear to occur in these membrane microdomains. Although IL-2Rα was enriched in lipid rafts isolated by sucrose density gradient ultracentrifugation, the IL-2Rβ
and $\gamma$ chains responsible for signal transduction were found in detergent soluble membranes both before and after stimulation of cells with IL-2. In addition, the Janus family kinases phosphorylated in response to IL-2, JAK1 and JAK3, were not detected in lipid rafts.

Disruption of lipid rafts using MCD did not perturb IL-2R-induced signalling. Finally, in cells proliferating in response to IL-2, the active heterotrimeric receptor complex composed of IL-2R$\alpha\beta\gamma$ bound to $^{125}$I-labelled IL-2 was detected only in soluble membranes.

The function of lipids rafts in the co-ordination of signalling may be two-fold. Lipid rafts are enriched in multiple signalling molecules, including Src family PTK, heterotrimeric and small G proteins and phosphoinositides, and may act as a repository of signalling molecules (117). Multiple membrane receptors are inducibly recruited to or stabilised within these domains, including TcR, BcR and Fc$\varepsilon$RI, and the subsequent activation of critical signalling molecules in rafts may facilitate signalling (3). However, lipid rafts may also function to segregate signalling molecules in resting cells, maintaining low basal levels of signalling in the absence of stimulation. Thus, while disruption of lipid rafts resulted in decreased TcR/CD3-induced signalling (169), increased basal levels of tyrosine phosphorylation were observed upon treatment with the cholesterol extracting agent MCD (211). In addition, MCD resulted in activation of the Ras-ERK MAPK pathway (211, 447). Furthermore, there is evidence suggesting that the localisation of Src PTK in rafts may segregate these kinases from their substrates. Palmitoylation of Fyn, which results in its localisation to lipid rafts, prevented its ability to phosphorylate a non-lipid raft resident chimeric Ig$\alpha$ molecule (448). Src, which is not palmitoylated, as well as a non-palmitoylated mutant of Fyn, were able to phosphorylate Ig$\alpha$, suggesting that membrane compartmentalisation regulates protein-protein interactions.

Thus, lipid rafts may be involved in a spatial regulation of intermolecular associations in the plasma membrane. In the context of TcR or BcR signalling, lipid rafts result in a
segregation of enzymes (Src family PTK) and substrates, and signalling is initiated upon the regulated association of receptors with lipid rafts (169, 177, 299). In the context of IL-2R signalling, lipid rafts may mediate the segregation of receptor chains. Lipid raft components can affect the initiation of signalling in both receptor systems by interfering with the regulated assembly of molecules. Thus, the first indications that lipid rafts played a role in regulating TcR/CD3-induced signalling were the demonstrations that mAb specific for GPI-AP could inhibit or potentiate signalling through the antigen receptor [reviewed in (449)], possibly by modifying the inducible association of TcR/CD3 with lipid rafts. The demonstration herein that components of lipid rafts can modify IL-2 responsiveness likewise implicates lipid rafts in the regulation of IL-2R signalling. Furthermore, lipid rafts may be involved in regulating signalling through multiple cytokine receptors, as our preliminary evidence suggests that immobilisation of GPI-AP inhibits the responsiveness of T cells to IL-4 and IL-15 in addition to IL-2, of B cells to IL-7 and of mast cells to IL-3 (MDM and MJ, unpublished observations).

Although enriched in lipid rafts, IL-2Rα likely exists in a dynamic equilibrium between lipid microdomains and soluble membranes. Binding of IL-2 may result in a dissociation of IL-2Rα from lipid rafts, enabling its strengthened interaction with IL-2Rβ and γ chains in soluble membranes to initiate signalling. Alternatively, signalling may be mediated only by that proportion of IL-2Rα present in soluble membranes. We propose that the inhibition of IL-2-induced proliferation observed upon immobilisation of GPI-AP or GM1 in lipid rafts reflects effects on the mobility of IL-2Rα. These inhibitory effects may involve steric hindrance of the association of IL-2Rα with the β and γ chains, blocking of a ligand induced dissociation of IL-2Rα from lipid rafts and/or through a shift in the equilibrium such that IL-2Rα becomes ‘trapped’ in lipid rafts and is unable to initiate
signalling. In support of its altered mobility, the proportion of IL-2Rα in lipid rafts is 2.5 fold higher in cells cultured in the presence of immobilised anti-Thy-1, relative to controls.

Fluorescence resonance energy transfer analysis has revealed that IL-2Rα, β and γ chains exist in close proximity in resting T cell lymphoma lines, suggesting a pre-formed complex (316). Upon binding of IL-2, the chains are brought into closer proximity, consistent with strengthened interactions or movement of the chains in the plasma membrane. IL-2Rα may be localised at the periphery of lipid rafts, where it can maintain a loose association with IL-2Rβ and γ chains. Binding of IL-2 and the subsequent strengthening of the interactions among IL-2Rα, β and γ chains may result in the dissociation of IL-2Rα from lipid rafts and the initiation of signalling in soluble membranes. A similar segregation of receptor components in the lipid bilayer has been proposed for heterotrimeric G proteins. Many G protein α subunits are myristoylated and palmitoylated (450), which directs their localisation to detergent insoluble membrane microdomains (216, 451). In contrast, Gβγ subunits are prenylated and excluded from lipid microdomains (138). It has been postulated that the Gαβγ complex exists on the periphery of rafts allowing each subunit to reside in its preferred lipid environment (137). Moreover, the association of acylated Gα with prenylated Gβγ may regulate the localisation of Gα in rafts. The majority of Gα expressed in synthetic lipid bilayers localises to raft-like domains, however expression of Gβγ results in a decreased association of Gα with rafts (137). The localisation of IL-2Rα in lipid rafts may likewise be dynamically regulated by ligand binding and intermolecular interactions with IL-2Rβ and γ chains.

No significant differences in the membrane distribution of IL-2Rα were detected upon stimulation of cells with IL-2 for between 1 to 27 minutes (Figure 4-6 and data not shown). As the level of IL-2Rα chain expression on the cell surface exceeds those of IL-2Rβ and γ chains (452), only a fraction of available IL-2Rα will dissociate from lipid rafts and
participate in signaling in detergent soluble membranes. In these acute experiments, the change in the distribution of IL-2Rα is not detectable using Western blot analysis. In contrast, the difference in the proportion of IL-2Rα in lipid rafts in Figures 4-6 and the isotype control in Figure 4-10 may reflect ligand-induced changes in the localization of IL-2Rα upon prolonged exposure to IL-2. In Figure 4-6, lipid rafts were isolated from 'starved' CTLL-2, cultured for 16 hours in minimal concentrations of IL-2 compatible with sustaining survival. In these circumstances, the proportion of IL-2Rα in lipid rafts varied between 37 to 70% in 8 experiments. Results presented in Figure 4-10 are derived from rafts isolated from CTLL-2 in log phase response to IL-2, circumstances in which 24.6 +/- 6.0% of IL-2Rα was localised in rafts. This difference may be due to a shift in the equilibrium of IL-2Rα between rafts and soluble membranes as a consequence of prolonged stimulation with IL-2 and the resultant dissociation of IL-2Rα from rafts. Further, the IL-2R complex is internalised upon IL-2 binding. IL-2Rα is subsequently recycled to the plasma membrane (373), and it is unknown whether recycling IL-2Rα molecules localize to lipid rafts or soluble membranes. Thus, while the dynamic equilibrium of IL-2Rα localisation is altered in the presence of ligand, the time needed to observe a re-equilibration of IL-2Rα likely reflects constraints imposed by both receptor physiology (re-utilisation) and relative abundance of IL-2R chains.

These results confirm two recent reports demonstrating the localisation of IL-2Rα in lipid rafts (444, 453), and extends the analysis to IL-2Rβ and γ, as well as to ligand binding. Moreover, the results presented provide the first demonstration of a functional role for lipid rafts in the regulation of IL-2R signalling. Field et. al. demonstrated that in transfected CHO cells, IL-2Rα became associated with TX-100 insoluble domains upon cross-linking (453). In contrast, we have observed an IL-2 independent enrichment of IL-2Rα in lipid rafts. This apparent contradiction likely reflects the differing detergents used (0.5% Brij58 versus 0.05% TX-100) and the differing detergent:cell ratios, and highlights a limitation
imposed by the most common methodology used to examine lipid rafts constituents.
Specifically, weak associations of proteins with lipid rafts may be disrupted using detergents.
Field et. al. also observed that aggregation of FcεRI was required for its association with
TX-100 insoluble domains (305, 453). However, recent data suggest that the localisation of
FcεRI in lipid rafts is constitutive but weak, and rather is strengthened and rendered
detergent-resistant upon cross-linking. Constitutive localisation of FcεRI in lipid rafts was
observed upon cell lysis in very low concentrations of detergent (453). Furthermore, high
resolution immunogold labelling and electron microscopy revealed that monomeric FcεRI is
distributed in small clusters of less than 100 nm in unstimulated cells (120). These clusters
also contain the Src family PTK Lyn, and likely represent individual lipid rafts. Similarly,
the association of IL-2Rα with lipid rafts is likely constitutive but weak, and we have
observed that although it is maintained in the presence of Brij58, 0.5% TX-100 disrupts the
association of IL-2Rα, but not acylated Src PTK and GPI-AP with lipid rafts (data not
shown). Consistent with this interpretation, relative to other non-ionic detergents, Brij58 has
been shown to better preserve associations of proteins with lipid rafts (440). Indeed, using
immunogold labelling and electron microscopy, IL-2Rα was shown to localise in clusters in
cells cultured in the presence or absence of IL-2 (444). Clusters of IL-2Rα co-localised with
clusters of CD48, and moreover the cluster size was modulated by methyl-β-cyclodextrin,
suggesting they represented lipid rafts. The results presented herein demonstrate that only
IL-2Rα was enriched in lipid rafts isolated in the presence of Brij58. However, it remains a
possibility that IL-2Rβ and γ may associate with lipid rafts even more weakly than does IL-
2Rα, and that this association is sensitive to Brij58. Electron microscopic analysis of IL-
2Rα, β and γ chains prior to and post-stimulation with IL-2 should provide a definitive picture
of the membrane compartmentalisation of the IL-2R.
A recent report detected interferon receptors and JAK1 and JAK3 in caveolae isolated from mouse embryonic fibroblasts (454). Caveolae, flask-shaped membrane invaginations, are a subset of lipid rafts. Although these two plasma membrane domains share common features, including detergent insolubility, low density, and enrichment in cholesterol, they can be separated experimentally (130, 131), and moreover, show differing protein composition and morphology. In addition, lipid rafts are present in cells, such as lymphocytes, which do not express caveolin, a cholesterol-binding integral membrane protein essential for caveolae formation (132, 133). The localisation of JAK kinases to caveolae may not be relevant to their subcellular localisation in lymphocytes, as many molecules localise to caveolae as a result of a direct association with caveolin proteins. Indeed, examination of the sequences of all Janus family kinases reveals the presence of a caveolin binding motif, $\Phi X \Phi \text{XXXX}\Phi$, where $X$ represents any amino acid and $\Phi$ represents any of the aromatic amino acids tyrosine, phenylalanine or tryptophan (455). Furthermore, whereas in the present study lipid rafts were isolated in the presence of detergent, the localisation of JAKs in caveolae was assessed using a detergent free method to isolate caveolae (454). Differential results with regard to the localisation of some proteins have been observed using detergent and non-detergent fractionation of membranes (168, 212), thus further study is required to confirm the caveolar localisation of JAKs in fibroblasts.

These results demonstrate that components of lipid rafts inhibit IL-2R-induced proliferation in vitro, however a potential role for GPI-AP and/or lipid raft-associated gangliosides in regulating IL-2R-mediated signalling in vivo remains to be determined. Components of lipid rafts may be able to regulate IL-2 responsiveness, however T cell proliferative responses appear normal in the absence of GPI-AP in mice bearing a T cell specific disruption of a gene critical for GPI anchor biosynthesis (66). In contrast, T cells from mice lacking expression of all complex gangliosides, including GM1, due to targeted
deletion of the gene encoding the GM2/GD2 synthase, display decreased IL-2-induced signalling and proliferation (456). Whether the defects in IL-2R signalling in these cells relates to lipid rafts awaits further investigation.
CHAPTER 5

DISCUSSION
Effects of lipid raft components on T cell signalling

Although it has long been known that GPI-AP can function in signalling, the manner in which these molecules, anchored in the outer leaflet of the plasma membrane by a complex glycolipid structure, coupled to the intracellular signalling machinery has only begun to be elucidated in recent years. As described in Chapter 1, the signalling capacity of GPI-AP is now thought to be predicated upon their localisation in lipid rafts and their ability to activate Src family PTK co-localised in these plasma membrane microdomains. The capacity of GPI-AP and gangliosides to activate T cells or inhibit T cell activation through the TcR/CD3 complex can be re-examined in light of the current understanding of the critical role of lipid rafts in signalling. In circumstances where mAbs or ligands specific for GPI-AP and gangliosides result in T cell activation, the coalescence of individual lipid rafts into patches and the activation of signalling pathways in lipid rafts are observed (119). Aggregation of lipid rafts, using CT or mAbs specific for CD59 in the presence of secondary antibodies, results in the co-localisation of TcR and signalling molecules such as Lck and ZAP-70 in lipid raft patches (119). Cytokine secretion and proliferation are thought to ensue from the activation of similar signalling pathways in lipid rafts to those described for TcR/CD3-induced signalling in Chapter 1. In contrast, soluble mAb specific for GPI-AP inhibit signalling induced through TcR/CD3. It would be of interest to examine whether the inhibitory effect of soluble mAbs specific for GPI-AP is due to the inhibition of either the stabilisation of TcR/CD3 in lipid rafts or the aggregation of individual lipid rafts. These experiments could provide mechanistic insight into the requirements for lipid raft aggregation for productive T cell signalling leading to proliferation and the secretion of cytokines.

Multiple GPI-AP with unrelated protein moieties function comparably in T cell activation, as well as the inhibition of T cell activation. The studies described in Chapters 3
and 4 were initiated prior to the characterisation of the role of lipid rafts in signalling, and were aimed at determining whether and how GPI-AP mediated a common effect on T cell responsiveness to stimulation through the TcR/CD3 complex. These studies were performed using immobilised mAb specific for GPI-AP, which allowed for the assessment of biological outcomes of T cell activation, such as proliferation and cytokine secretion, as well as biochemical outcomes, such as the tyrosine phosphorylation of intracellular substrates, under identical conditions and without the complication of contaminating APC. As described in Chapters 3 and 4, antibody-mediated immobilisation of GPI-AP inhibited anti-TcR-induced proliferation in T cells. However, in contrast to the effect of soluble mAbs, ligation of GPI-AP using immobilised mAbs did not inhibit TcR/CD3-induced production of IL-2. In addition, the effector function of cytotoxic T lymphocytes was not impaired in the presence of mAb specific for GPI-AP. As cytotoxicity and IL-2 secretion require the induction of multiple signalling pathways, these results suggested that signalling through TcR/CD3 was unaffected by immobilised mAb specific for GPI-AP. Rather, immobilised mAb specific for GPI-AP inhibit proliferation by inhibiting signalling in response to IL-2 produced endogenously following stimulation through TcR/CD3. Further, proliferation in response to IL-2 provided exogenously was inhibited by mAbs specific for GPI-AP. Immobilisation of an additional component of lipid rafts, the GM1 ganglioside, mediated the same effects on IL-2R-induced signalling as do GPI-AP.

These results uncovered a novel mechanism through which GPI-AP and gangliosides can modulate T cell function, resulting in TcR/CD3-induced T cell effector functions in the absence of clonal expansion induced by IL-2. Importantly, T cell proliferation and the effector function of cytokine secretion have been uncoupled in vivo. In chimeric mice reconstituted with a high frequency of antigen-specific T cells, antigenic stimulation results in cytokine secretion with only minimal proliferation (457). A potential role for GPI-AP
and/or lipid raft-associated gangliosides in the regulation of IL-2R-mediated signalling in vivo remains to be determined. As previously mentioned, IL-2-induced signalling and proliferation is decreased in mice lacking expression of complex gangliosides (456), however the mechanism underlying the signalling defect has not yet been characterised. In contrast, T cell responses appear normal in mice lacking expression of GPI-AP (66), although signalling through the IL-2R was not stringently assessed. However, even if signalling through IL-2R is not impaired in the absence of GPI-AP, this class of molecules may be able to modulate IL-2 responsiveness when expressed.

The biological consequences induced by mAb-mediated ligation of GPI-AP depends on how the mAb is presented to the GPI-AP expressing cell. Thus, the biological outcome of cognate interactions between GPI-AP and their physiological ligands, which may be expressed on neighbouring cells, on the same cell, or in the extracellular milieu, may depend on the spatial distribution of the latter. Many of the physiological ligands of GPI-AP, including Thy-1 and Ly6A/E, remain uncharacterised, and even in circumstances in which cognate pairs have been characterised, such as CD48-CD2 (435), the outcome of this interaction in vivo remains equivocal. A clear understanding of the functions of GPI-AP requires the identification of their ligands, and further characterisation of the outcome of interactions with GPI-AP in physiological circumstances.

**Lipid raft components inhibit IL-2-induced receptor heterotrimerisation**

The ability of components of lipid rafts to inhibit IL-2-induced proliferation suggests that these membrane microdomains can regulate IL-2R signalling, therefore the nature of the signalling defect, as well as the role of lipid rafts in IL-2R signalling, were investigated. In the presence of immobilised mAb specific for GPI-AP, IL-2-mediated recruitment of IL-2Rα, β and γ chains, resulting in the formation of the high affinity heterotrimeric IL-2R, was
inhibited. The resulting phosphorylation of JAK1 and JAK3, indicative of their activation states, was correspondingly reduced.

Investigation of the subcellular localisation of components of the IL-2R revealed that IL-2Rα is constitutively enriched in lipid rafts. Furthermore, the proportion of IL-2Rα in lipid rafts was increased in the presence of immobilised anti-Thy-1, relative to controls. In contrast, IL-2Rβ and γ, as well as JAK1 and JAK3, are localised in detergent soluble membrane fractions, and their localisation is not altered in the presence of immobilised anti-Thy-1. IL-2-mediated heterotrimerisation of IL-2R chains occurs within soluble membrane fractions, as does activation of JAK1 and JAK3. These results suggest that signalling through IL-2R takes place in detergent soluble membranes, and consistent with these observations, the disruption of lipid raft integrity upon extraction of membrane cholesterol did not impair IL-2-induced signalling.

These results provide the first demonstration of a functional role for lipid rafts in regulating IL-2R signalling. While signalling through IL-2R occurs outside lipid rafts, the major ligand binding component of the receptor complex, IL-2Rα, is constitutively enriched in rafts. IL-2Rα may dissociate from lipid rafts upon ligand binding to interact with IL-2Rβ and γ chains in detergent soluble membranes to initiate signalling. Inhibition of IL-2R signalling by immobilisation of components of lipid rafts results in a further enrichment of IL-2Rα in lipid rafts, and thus appears to be due to blocking the mobility and/or intermolecular interactions of IL-2Rα with molecules outside microdomains. This sequestration of IL-2Rα in lipid rafts may impair its association with IL-2Rβ and γ chains and thus regulate IL-2-mediated signalling.

The ability of mAb or ligand-induced immobilisation of GM1 and all GPI-AP tested to date to inhibit IL-2R-signalling strongly suggests that it is their localisation in lipid rafts that is critical for this function. Further, GM1 and the GPI-AP utilised in these studies bear
distinct extracellular protein and carbohydrate moieties. Thus, it appears unlikely that modulation of IL-2R function could be mediated by interactions between IL-2Rα and the extracellular portions of GM1 and multiple GPI-AP. To determine the requirements for inhibition of IL-2R signalling, chimeric molecules could be constructed to encode transmembrane forms of GPI-AP, and conversely, GPI-anchored forms of transmembrane proteins. As the GPI anchor is critical for the constitutive localisation of GPI-AP in lipid rafts, assessing the effect of these chimeric proteins on IL-2-induced proliferation would determine whether localisation in lipid rafts is sufficient to enable inhibition of IL-2R signalling. Furthermore, if a transmembrane form of a GPI-AP could no longer inhibit IL-2R signalling, this would demonstrate that the extracellular portion of a given GPI-AP is not sufficient for the inhibitory capacity. Importantly, the localisation of these proteins would need to be assessed to ensure that GPI-anchoring is both sufficient and required for the localisation of the chimeric proteins in lipid rafts.

The capacity of components of lipid rafts to inhibit IL-2R signalling is consistent with a role for lipid rafts in mediating the spatial regulation of intermolecular associations in the plasma membrane. Lipid rafts may function as a repository of signalling molecules that can be activated following ligand-induced localisation of immunoreceptors with lipid rafts. In addition, rafts may function to segregate receptor components and/or signalling molecules, maintaining low basal levels of signalling in the absence of stimulation.

In this context, it is interesting to consider the function of caveolae in the regulation of signalling. Caveolae, as discussed in Chapters 1 and 4, are plasma membrane invaginations and vesicles which constitute a subset of lipid rafts detected in cells that express caveolin, notably adipocytes, endothelial cells and fibroblasts (458). Similar to lipid rafts, caveolae are enriched in many signalling molecules, including receptor PTK, Src family PTK and components of the Ras-MAPK pathway, and are thought to concentrate
signalling molecules in the plasma membrane and co-ordinate the regulated activation of signalling pathways. Caveolin appears to be critical for the formation of caveolae, through its oligomerisation and interaction with cholesterol and glycosphingolipids. In addition, caveolin contains a scaffolding domain which interacts with a caveolin binding motif found within the catalytic domain of many signalling molecules, including several serine/threonine and tyrosine kinases (459). The interaction with caveolin suppresses the catalytic activity of these kinases, and thus caveolin may function to maintain low basal levels of signalling in the absence of stimulation. Indeed, caveolin is postulated to be a tumour suppressor protein, and downregulation of caveolin is sufficient to result in constitutive Ras-MAPK activation, and drive anchorage independent growth and other hallmarks of cell transformation (459).

Although lacking caveolin, lipid rafts in cells such as lymphocytes may also serve to maintain low basal signalling through the sequestration of signalling molecules and the separation of receptor components in the plasma membrane. In addition, functional homologues of caveolin may be expressed in cells lacking caveolin. Several integral membrane proteins which localise in detergent insoluble microdomains have been identified, including the MAL proteolipid, flotillin-1 and flotillin-2, and are expressed in cells lacking caveolae, including T lymphocytes (109, 460, 461). Overexpression of MAL and flotillin-1 results in the formation of vesicular structures (461, 462), suggesting that these proteins may mediate similar functions as caveolin in the formation of detergent insoluble vesicular microdomains. However, whether these proteins can associate with and inhibit the activity of signalling molecules remains to be determined.
The nature of the localisation of IL-2R and immunoreceptors in lipid rafts

Recent data has demonstrated the importance of the compartmentalisation of proteins in the plasma membrane for the regulation of signalling. The understanding of this area has just begun, and many exciting and important questions remain to be answered.

Multiple immunoreceptors such as BcR, TcR and FcεRI are now thought to have a weak constitutive association with lipid rafts which is enhanced or stabilised, and rendered detergent resistant, upon ligand binding (119, 120). Signalling pathways are induced through the activation of molecules constitutively localised in lipid rafts, as well as upon the recruitment of additional signalling proteins to these membrane microdomains (3). The signals resulting in the targeting of intracellular proteins to lipid rafts have been characterised, and involve the modification of proteins with saturated fatty acids such as myristate and palmitate. Furthermore, several transmembrane proteins are targeted to lipid rafts as a result of palmitoylation. However, the molecular mechanism(s) underlying the inducible stabilisation of the association of immunoreceptors with lipid rafts upon ligand binding remains unclear. This change in membrane localisation is very rapid and can be detected with seconds of ligand binding (177, 267, 301). Moreover, this recruitment/stabilisation does not require signalling, and occurs in the presence of pharmacological inhibitors of tyrosine kinases (119, 302, 305). Multiple co-operative weak interactions of the oligomerised receptors, a ligand-induced conformational change in these receptors, post-translational modifications such as palmitoylation, or inducible associations with raft resident lipids or proteins may individually or together result in the stabilisation of transmembrane receptors in lipid rafts.

Interestingly, BcR did not localise into detergent insoluble domains following antigenic stimulation of tolerant B cells from transgenic mice co-expressing anti-hen egg lysozyme (HEL) and soluble HEL (302). The defect in subcellular partitioning of BcR
components may underlie the signalling abnormalities in tolerant cells (463, 464), and highlight the critical role of lipid rafts in signalling through immunoreceptors. Furthermore, this observation suggests that localisation to detergent insoluble membrane domains is subject to a level of regulation in addition to ligand binding.

Regulation of the partitioning of TcR and BcR into lipid rafts also appears to be subject to developmental regulation, and may result in the distinct developmental requirements and outcomes of antigen receptor ligation. A constitutive detergent-resistant localisation of pre-TcR and pre-BCR in lipid rafts was observed (180, 300), which may underlie their putative ligand-independent signalling. In mature peripheral T cells, a small proportion of CD3ζ (~10%) is constitutively associated with lipid rafts (169, 177), and it is interesting to hypothesise that this pool of CD3ζ may play a role in antigen receptor-mediated maintenance of naïve peripheral T cells. However, in mature T and B cells, antigenic stimulation is required for the detergent-resistant association of a large proportion of antigen receptors with lipid rafts, and results in cellular activation. In contrast, immature T and B cells are refractory to activation induced through the antigen receptor, and instead undergo apoptosis. Consistent with the hypothesis that association with lipid rafts is critical for the generation of the full complement of activation signals, antigen receptor cross-linking does not result in a detergent-resistant localisation to lipid rafts in an immature B cell line (465), or in lipid raft aggregation in immature DP thymocytes (253).

The structural requirements for the association of immunoreceptors with lipid rafts have begun to be investigated. Studies using thymocytes expressing mutant TcR/CD3 chains have revealed a role for the TcRα chain connecting peptide and the extracellular and/or transmembrane domains of CD3δ in mediating the induced association of the TcR/CD3 complex with lipid rafts (178, 179). FcεRI is composed of α, β and γ chains. Notably, the β and γ chains of FcεRI can be palmitoylated (466), which may be dynamically regulated and
may contribute to the partitioning of FcεRI into lipid rafts. However, neither the cytoplasmic tail of the FcεRI β and γ chains, nor the entire β chain, were required for association with lipid rafts (453). The transmembrane and/or cytoplasmic domain of the FcεRIα chain appears to be required for the association with lipid rafts, as chimeric molecules containing the extracellular domain of FcεRIα chain with the transmembrane and cytoplasmic domains of the type 1 IL-1R or the α4 integrin did not associate with lipid rafts (453).

The structural requirements and mechanisms responsible for the regulated association of immunoreceptors with lipid rafts may provide insight into the as yet unclear means through which non-palmitoylated transmembrane proteins associate with lipid rafts. This in turn may lead to an understanding of how IL-2Rα is constitutively enriched in lipid rafts. IL-2Rα has a short cytoplasmic domain of 13 amino acids (313), which does not contain any cysteine residues which could potentially be palmitoylated. As described above, the extracellular domain of FcεRIα is not sufficient for association with lipid rafts. However, a chimeric FcεRIα molecule containing the transmembrane and cytoplasmic domains of IL-2Rα could associate with lipid rafts, implicating these domains of IL-2Rα in mediating the association with membrane microdomains (453). Interestingly, IL-2Rα has been shown to interact with a plasma membrane phospholipid expressed on activated T cells (467). The phospholipid interacts with the IL-2 binding domain of IL-2Rα, but does not affect IL-2 binding. The interaction of IL-2Rα with a phospholipid may affect the membrane compartmentalisation of IL-2Rα. Further, a mAb which binds this phospholipid potentiated responses to IL-2, suggesting that its interaction with IL-2Rα may have biological effects on IL-2Rα function. A potential effect of phospholipid interactions on the localisation of IL-2Rα in lipid rafts or its association with IL-2Rβ or γ would be consistent with effects on signalling. In addition, exogenous gangliosides have been shown to inhibit IL-2-induced
proliferation by decreasing the binding of IL-2 to IL-2R, including binding to IL-2Rα (468, 469). However, this is due to the direct interaction of gangliosides with IL-2, not with IL-2R chains (468, 470).

Elucidation of the requirements for the association of IL-2Rα with lipid rafts will allow for the expression of mutant molecules which do not localise to microdomains. Alternatively, chimeric molecules composed of domains of IL-2Rα fused to non lipid-raft-associated proteins could be constructed. Mutant IL-2Rα or chimeric proteins which do not localise to lipid rafts but retain IL-2 binding and interactions with IL-2Rβ and γ would prove useful in determining the physiological effects of the localisation of IL-2Rα in lipid raft for IL-2R signalling. For example, a role for lipid rafts in regulating the interactions between IL-2Rα and IL-2Rβ and γ chains would be consistent with an increased sensitivity to IL-2 in cells expressing a non-lipid raft associated IL-2Rα. In addition, upon internalisation of the IL-2/IL-2Rαβγ complex following ligand binding, the IL-2Rα chain is differentially sorted through a recycling pathway, while IL-2, IL-2Rβ and IL-2Rγ chains are targeted for proteosomal degradation (373). Lipid rafts have been implicated in endocytic pathways and protein sorting (111), and whether the affinity of IL-2Rα for lipid rafts relates to its distinct intracellular fate could also be investigated.
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