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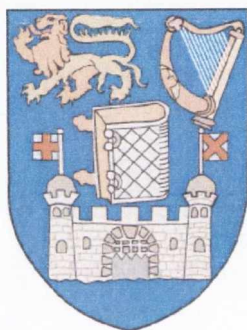
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**THE QUEUINE-TRANSFER RNA PATHWAY
AS A NOVEL DRUG MECHANISM TO TREAT
MULTIPLE SCLEROSIS**

SREEJA VARGHESE

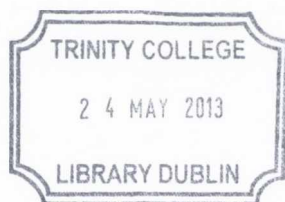


**A THESIS SUBMITTED TO
TRINITY COLLEGE DUBLIN
FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY**

**RNA BIOLOGY GROUP
SUPERVISOR: DR. VINCENT P. KELLY**

**SCHOOL OF BIOCHEMISTRY AND IMMUNOLOGY
TRINITY COLLEGE DUBLIN**

2012



Thesis 10077

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Abstract

Queuine is a pyrrolopyrimidine molecule that is irreversibly incorporated into the anticodon loop of transfer RNA (tRNA) for the amino acids Asparagine, Aspartate, Tyrosine and Histidine. Only bacteria synthesize queuine, yet almost all eukaryotic species harvest queuine from food or bacteria in the gut and insert it into the same tRNA by means of the Queuine tRNA ribosyltransferase (QTRT) complex, comprising the proteins TGT and QTRTD1 and referred to here as the queuine tRNA pathway. This study shows that the queuine analogue 6-thioguanine (6TG) can influence immune function when incorporated into tRNA *via* the queuine-tRNA pathway and furthermore, that this is an effective strategy to ameliorate the physical, immunological and neurological disabilities observed in the animal model of multiple sclerosis known as experimental autoimmune encephalomyelitis (EAE). The tRNA of somatic cells is generally fully modified with queuine, whereas hyperproliferative cells (including cancer cells) are deficient. Previously, it was shown that incorporation of 6TG into the anticodon loop of tRNA can limit HL-60 cancer cell proliferation and promote differentiation. 6TG is a known anti-cancer agent that conveys its anti-metabolic effect *via* incorporation into newly synthesized DNA, causing genotoxicity and growth arrest. This effect is dependent on the HPRT enzyme. Therefore, to remove this primary drug route and to avoid DNA incorporation studies were conducted on *hprt* deficient primary cells and animals. 6TG acting through the queuine-tRNA pathway resulted in a dose-dependent growth inhibition of T-cell proliferation in splenocyte cultures. Competition experiments between queuine and 6TG demonstrated that queuine can dose-dependently outcompete the anti-proliferative effect of 6TG, verifying the efficacy of the queuine-tRNA therapeutic strategy. Genetic evidence is also presented showing that the anti-proliferative effect of 6TG is lost when repeated on a double *hprt::tgt* null background, proving that the HPRT-independent effect of 6TG is conveyed exclusively through the queuine tRNA pathway. Administration of 6TG post-onset of paralysis in EAE diseased animals was able to halt and reverse disease progression. The animals regained motor ability to perform physical tasks, suggesting successful repair of the damaged myelin. Tissue analysis showed a marked reduction in the T_H1 and T_H17 immune response in both the peripheral and central nervous system. Immunohistochemical analysis of the brain also revealed a lack of EAE-induced T cell population and microglial activation in the striatum, hippocampus, brain stem and cerebellum. Importantly, the effects of 6TG administration were abolished in *hprt::tgt* double knockout animals—suggesting that the therapeutic effects *in vivo* are conveyed exclusively through the queuine tRNA pathway. In conclusion, this project provides a preliminary validation of the queuine tRNA pathway as a novel therapeutic strategy for multiple sclerosis and other T cell mediated auto-immune, inflammatory disorders.

Abbreviations

6MP	6-Mercaptopurine
6TG	6-Thioguanine
ALL	Acute lymphoblastic leukaemia
APC	Antigen presenting cell
Asn	Asparagine
Asp	Aspartic acid
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3
BBB	Blood brain barrier
B cell	Hematopoietic stem cells that matured in the bone-marrow
Ca ²⁺	Calcium ions
CCR6	CC-chemokine receptor 6
Cdc25	Cell division cycle protein 25
CFSE	Carboxyfluorescein Succinimidyl Ester
CHK 1/2	Cell cycle checkpoint proteins
CLA	Cutaneous lymphocyte antigen
CNS	Central nervous system
CNT	<u>con</u> centrative <u>n</u> ucleoside <u>t</u> ransporters
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
CXCR3	CXC motif containing chemokine receptor 3
CXCR5	C-X-C motif bearing Chemokine receptor-5
DC	Dendritic cell
DD	Daily dose
DN	Double negative
DNA	Deoxyribonucleic acid
Dok1/2	Downstream of kinase 1/2
DP	Double Positive
EAE	Experimental autoimmune encephalomyelitis
EB/AO	Ethidium Bromide/Acridine Orange

EGF	Endothelial growth factor
ENT	<u>E</u> quilibrative <u>n</u> ucleoside <u>r</u> eceptors
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
GA	Glatiramer Acetate
GMP	Guanosine monophosphate
hCNT	Human family of CNT
His	Histidine
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
IBD	Inflammatory Bowel disease
ICAM 1	Intracellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL 2	Interleukin 2
IL 2R	Interleukin 2 receptor
IMP	Inosine monophosphate
ITAMS	Immunoreceptor tyrosine-based activation motifs
ITIMS	Immunoreceptor tyrosine-based inhibitory motifs
KGF	Keratinocyte growth factors
LAT	Linker for the activation of T cells
LDH	Lactate dehydrogenase enzyme
LFA1	Leukocyte function-associated 1 antigen
LND	Lesh-Nyhan disease
MAP	Mitogen-activated protein
MBP	Myelin basic protein
mENT	Murine family of ENT
me-TGMP	Methylated-TGMP
MHC I/II	Major histocompatibility complex I/ II
MMR	Mismatch repair

MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MS	Multiple Sclerosis
MTOC	Microtubule organising centre
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Programmed cell death-1
PDGF	platelet-derived growth factor
PDLA 1/2	Programmed cell death ligand 1/2
PLCγ1	Phospholipase Cγ1
PLP	Proteolipid protein
PTK	Protein tyrosine kinase
QTRT	Queuine tRNA ribosyltransferase enzyme
QTRTD1	Queuine tRNA-guanine transglycosylase domain containing 1
Qv0,1,2	QTRTD1 spliced variants 0, 1 and 2
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SAS	Subarachnoid space
SD	Single dose
SH2	Src homology 2
SHP1	SH2 domain containing protein tyrosine phosphatase 1
SLE	systemic lupus erythematosus
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SP	Single Positive
T cell	Hematopoietic stem cells that matured in the thymus
TCR	T cell receptor
TGDP	Thioguanosine diphosphate
TGMP	Thioguanosine monophosphate
TGT	tRNA-guanine transglycosylase

TGTP	Thioguanosine triphosphate
TNF	Tumor necrosis factor
TNFR	TNF-receptor
TPMT	Thiopurine methyltransferase
T _{reg}	T regulatory cell
tRNA	Transfer RNA
Tyr	Tyrosine

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Chapter 1: Introduction

1.1 Transfer RNA, tRNA: structure and modifications

1.1.1 Nucleic acids and RNA

Johann Friedrich Miescher's attempt to understand the chemical composition of leukocytes in 1868 brought to light the existence of a component found in the nuclei called 'nuclein' (Dahm *et al.*, 2008). The preliminary extraction studies of nuclein showed the presence of organic molecules such as carbon, hydrogen, oxygen and oddly sulphur—probably due to some protein contamination in the extract (Miescher 1871). This misled most scientists into believing that nuclein was just a subset of proteins (Dahm *et al.*, 2008). Strongly convinced otherwise, Miescher confirmed in his later studies with more pure nuclein extractions, that nuclein lacked sulphur and moreover contained high amounts of phosphorous suggesting that nuclein was non-proteinaceous and a distinct cellular component in its own right (Miescher, 1874). Soon, various scientists became interested in this novel component. One such Nobel Laureate, Albrecht Kossel reported that nucleic acids (originally nuclein) were composed of purine and pyrimidine bases (collectively called nucleic acids), one sugar (ribose) and phosphoric acid (Portugal and Cohen, 1977)—making deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Kossel took his studies further to show that nucleic acids combined with chromosomal structural proteins (now commonly known as histones) to form chromatin in the nucleus thereby confirming Miescher's original findings (Olby, 1994; Portugal and Cohen, 1977). In an era where scientists believed that twenty different amino acids were sufficient to carry the complex genomic information, the discovery of the nuclein molecule stirred controversy.

In the 1950's, the research focus of many scientists moved to nucleic acids and in a combined effort they investigated the potential of nuclein to act as the carrier for genetic information. Two studies were crucial in validating this hypothesis. Firstly, a functional study by Al Hershey, Marta and co-workers showed that infection of bacteria with the DNA of T2 bacteriophage, but not viral protein, lead to propagation of the virus (Hershey and Chase 1952). Secondly, in the subsequent year (1953) a structural study by Watson and Crick was published elucidating the double-helical

nature of the DNA molecule (Watson and Crick 1953). They showed that the two anti-parallel DNA strands are held together by two major forces: base-stacking interactions and hydrogen bonding between purine (Guanine, G and Adenine, A) and pyrimidine bases (Cytosine, C and Thymidine)—also known as Watson-Crick base-pairing. Although these interactions provide DNA with stability they must also be periodically broken to allow the strands to unwind for replication and transcription (Dahm *et al.*, 2008). Now that Watson-Crick had posited the central dogma, proteins were re-defined as synthesised information, however it remained unclear as to ‘how’ the information was decoded from DNA into protein. The conceptual leap came from a study by Torbjorn Caspersson, Jean Brachet and Jack Schults in which they reported that another tetranucleic molecule composed of ribonucleic acid (RNA), served to link nuclein to protein synthesis (Caspersson, 1950). This marked the start of the next stage in nucleic acid research—the process of decrypting the DNA code, often referred to as ‘cracking’ the genetic code (Davies 2002).

RNA, like DNA, is a polymeric chain of nucleic acids consisting of four major canonical bases and several minor bases, which are chemically transformed derivatives of the latter (Helm 2006). Three of the four major bases are the same as in DNA: A, G and C. The fourth RNA base is Uracil; where it substitutes for T. This is believed to occur due to two main reasons: Firstly, cytosine can be degraded into uracil, which would threaten DNA integrity and therefore the presence of thymidine, allows the DNA enzymes to detect uracil as a degraded C base and signal repair processes. Secondly, uracil requires less energy to synthesise than thymidine, thereby making it better-suited for the high turnover rate experienced by RNA molecules. RNA is a very dynamic cellular component and this is reflected in the different moieties of RNA present in the cell, each with a specific function. Hubert Chartrenne was the first to illustrate the function of one of these RNA moieties by showing it can act as a messenger transcript for protein synthesis, hence it being termed messenger RNA or mRNA (Chartrenne, 1965). Within the next decade additional RNA species in protein translation were discovered: ribosomal RNA (rRNA) and transfer RNA (tRNA).

1.1.2 tRNA structure

Originally identified as ‘small’ RNAs, there are a total of 45 different tRNA molecules in higher eukaryotes. Their primary role in the cell is to act as an isotype specific carrier molecule for an amino acid during protein translation. They successfully complete the task only upon recognition of their complementary sequence on mRNA in the ribosomal complex and transferring the amino acid onto the growing polypeptide chain.

The first tRNA to be sequenced was the yeast tRNA molecule for Alanine, tRNA^{Ala}, a work for which Dr. Robert Holley was awarded the Nobel prize in 1965. His further studies reported that all tRNA molecules were 73-94 ribonucleotides in length. The tRNA molecules are primarily synthesised as precursor tRNA, pre-tRNA by RNA polymerase III. This transcript is then subjected to extensive trimming and alterations: briefly, the 5' leader sequence is cleaved by the ribonucleoprotein complex, RNase P followed by removal of the 3' trailer sequences by exo- and endonucleases. Finally, a nucleotidyltransferase enzyme catalyses the base-by-base synthesis of the CCA sequence found on the acceptor arm of all tRNA; note that in bacterial species the CCA terminal sequence is genetically encoded. The resulting product is a single stranded linear transcript where certain ribonucleotides follow Watson-Crick pairing: A=U and G≡C as shown in **Figure 1.1a**. The base-paired regions form ‘stem’ like structures followed by ‘loops’ that contain un-paired bases. This stem-loop secondary structure as shown in **Figure 1.1b**, resembles a ‘cloverleaf’ and is strengthened by base-stacking interactions and intramolecular hydrogen bonding.

A tRNA molecule composes four distinct structural regions: the D-arm, the amino acid acceptor arm, the T-arm and the anticodon arm. The D-arm, made of the D-stem and loop contains the modified uracil base, dihydrouridine—from which it derives its name. The amino acid acceptor arm of tRNA, as the name suggests, accepts an amino acid at the 3'terminal OH group, a reaction catalysed by the aminoacyl tRNA synthase enzymes. The T-arm is a 5 base-pair stem-loop containing thymidine, pseudouridine and cytidine residues in tandem, often written as TΨC. The function of this arm is to facilitate recognition of the tRNA by the ribosome during protein synthesis. Finally, the 7 bp loop at the base of the tRNA molecule is defined

by the three decoding nucleotides at positions 34, 35 and 36, collectively called the anticodon. This anticodon sequence base pairs with its complementary codon found on the incoming mRNA in the ribosome. The correct codon:anticodon base pairing allows the transfer of the tRNA bound amino acid onto the growing polypeptide chain during protein translation. The first position of the anticodon (position 34) is called the 'wobble' base. Its name is derived from its non-Watson base interaction displayed during codon recognition allowing the tRNA codon to recognise and pair with more than one specific codon sequence on the mRNA. There are four known wobble base-pairings: Guanine:Uracil (G:U), Inosine-Uracil/Adenine/Cytosine (I:U; I:A; I:C). This phenomenon was postulated by Francis Crick to explain why there are fewer tRNAs, 45 for a total of 64 codons. Although each tRNA is specific for a certain amino acid, the genetic code can use different codons to encode for the same amino acid. Therefore, the wobble-base helps accommodate this flexibility necessary for the anticodon to recognise the different codons.

Later X-ray crystallography analysis of tRNA secondary structure showed that tRNA molecules can fold into a higher order L-shaped configuration (Voet and Voet, 1995) as shown in **Figure 1.1c**. In addition to base stacking interactions and intramolecular hydrogen bonding, there are additional hydrogen bonds made between residues in different regions of the folded tRNA molecule. The D loop is also known to play a role in stabilizing this tertiary structure. Moreover, other structural features such as magnesium ions and modified nucleobases, discussed further below, also enhance structural integrity.

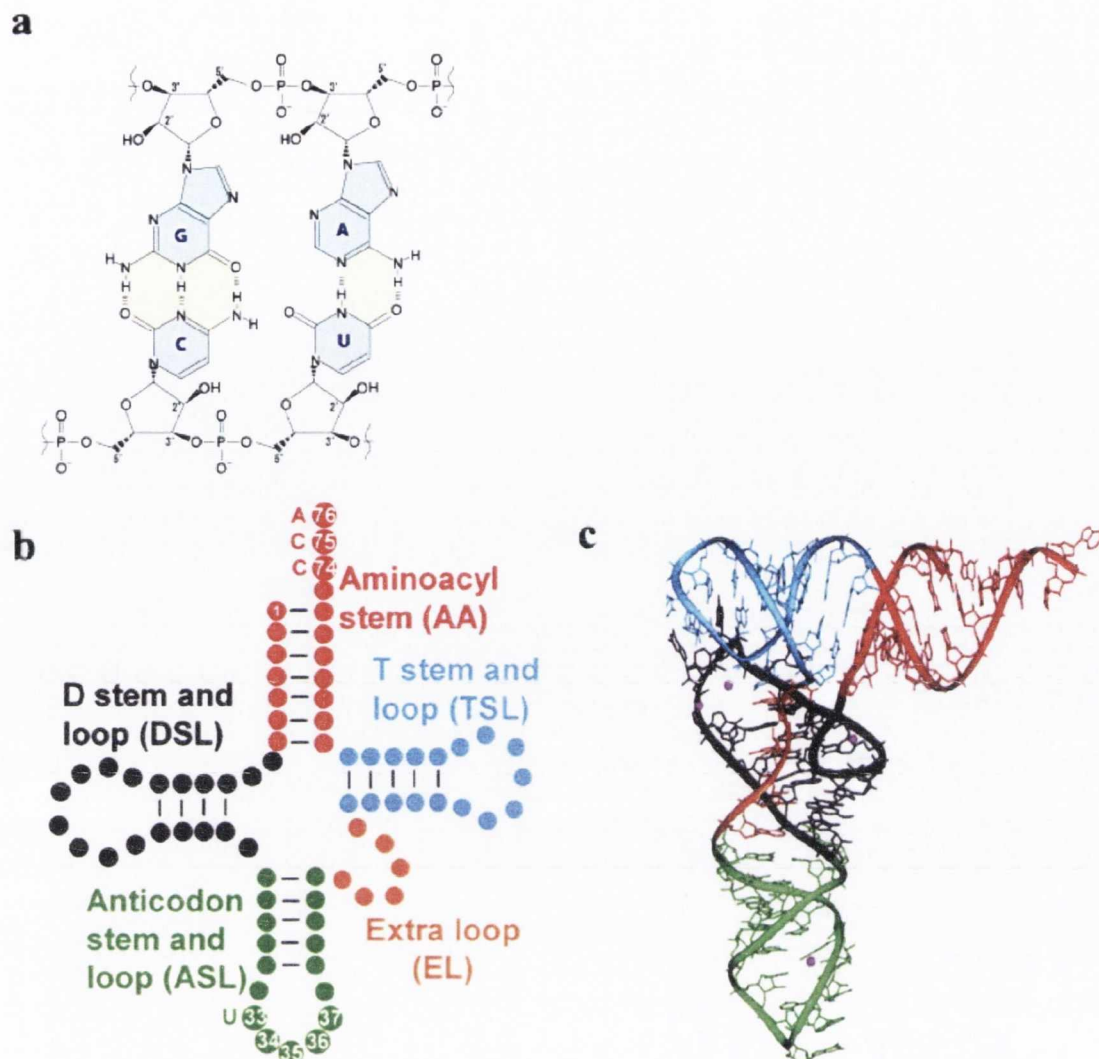


Figure 1.1: tRNA structure

Canonical RNA bases adhere to the rules of Watson-Crick base pairing where Adenine forms a double hydrogen bond with Uracil ($A=U$), and Guanine forms a triple hydrogen bond with Cytosine ($G\equiv C$) (**a**). These bonding forces give rise to ‘stems’ (regions of paired bases) and ‘loops’ (regions of unpaired bases) that direct the folding of the single stranded tRNA molecule into its familiar cloverleaf secondary structure (**b**) consisting of four regions: a D loop (**black**), an aminoacyl acceptor arm (**red**), a T loop (**blue**) and an anticodon loop (**green**). A high-order L-shaped tertiary structure (**c**) is elaborated when the tRNA stem-loop regions fold and are stabilized by other forces such as intramolecular hydrogen bonds, base-stacking forces, base modifications and magnesium ions (illustrated as **purple** dots). Adapted from (Agris, 2004).

1.1.3 tRNA modification

tRNA, like other nucleic acid polymers, contains many modified bases. Originally identified as non-canonical structures, Hotchkiss was the first to discover a methylated cytosine base, deoxy 5-methylcytosine (dm^5C , Wyatt, 1950) in DNA. Soon to follow was the discovery of a similar non-canonical base in RNA hydrolysate that was identified a few years later as 5-ribosyluracil (Cohn and Volkin, 1952). Since then, numerous studies have reported on the existence, pattern and function of these minor bases. To date, over a 100 different naturally occurring chemical modifications have been found to occur on up to 10% of the total number of ribonucleosides in any particular tRNA molecule (Iwata-Reuyl, 2003). In fact it has been postulated that the 2'-hydroxyl group on the ribose of each purine and pyrimidine moiety can be a potential modification site.

Modifications occur as part of the tRNA maturation process. Each modification is carried out by a specific enzyme(s) at a post-transcriptional level. Modifications can range from simple to complex—simple modifications such as methylations, thiolations, isomerisations require mostly just a one step mechanism to chemically alter the genetically encoded ribonucleoside. Complex modifications on the other hand can involve a multi-step enzymatic pathway resulting in hypermodification such as queuine annotated as (*Q) in **Figure 1.2a**, wybutosine and isopentyladenosine. The exact role of many modifications has yet to be determined but it is hypothesised that most modifications contribute towards shielding the tRNA from enzymatic degradation (Engelke *et al.*, 2006) in an otherwise high-turnover family of molecules that is RNA (Alexandrov *et al.* 2006). Studies comparing the properties of unmodified tRNA against fully modified tRNA have reported that the lack of modification affects thermal stability and molecular dynamics of the tRNA molecule rather than its structural folding (Helm, 2006). Pseudouridine (Ψ) is one such common post-transcriptional tRNA modification. The pseudouridine synthase enzymes catalyse the conversion of uridine ribonucleotides at specific sites on the tRNA molecule. This process is preserved across all three kingdoms: archaea, eubacteria and eukarya, and its deletion has been reported to affect tRNA stability, cell growth and protein synthesis (Mengel-Jorgensen *et al.*, 2002).

The function of a modification is related to the site where it occurs. In tRNA, studies have identified two major areas where a cluster of ribonucleosides may be subjected to modifications or even hyper modifications: the core of the L-shaped tertiary structure and the anticodon loop (Grosjean *et al.* 1997). A vast majority of the modifications (methyations, isomerisations) that occur within the L-shaped core, within the D and T loops contribute mainly to structural stability. Disruptions of such modifications have been reported to affect tRNA folding, for example within mammalian systems, absence of the methyl modification at position A9-N¹ (the nitrogen atom on Adenine base at position 9) causes the human mitochondrial tRNA to adopt a non-cloverleaf structure, disabling its optimal recognition by the translation apparatus (Helm *et al.*, 2004). In stark contrast, the same modifications occurring in the anticodon loop are involved in aminoacylation, codon recognition, frameshifting abilities and more (Hopper *et al.* 2003). For example, studies with *S. cerevisiae*, reported that the presence of a carboxymethylaminomethylation at the wobble position (34) of the nuclear encoded tRNA^{Lys}_{CUU} (tRK1) allows this tRNA to decode the AAA and AAG codons under stress conditions (Kamenski *et al.*, 2007), thereby demonstrating how a single modification can alter translational flexibility. Furthermore, studies on the Human Immunodeficiency virus-1 (HIV-1) that uses the human tRNA₃^{Lys} as its primer for reverse transcription (RT) upon infection reported that the virus was unable to form the initiation complex in the absence of modified tRNA. Upon using different recombinant modified tRNA₃^{Lys} in *E.coli*, the study demonstrated the crucial role of a thiolated-Uracil at the wobble position in the viral RT process (Tinse *et al.*, 2000; Helm, 2006). Similarly, other modifications have been reported to affect/alter base-pairing capacity, initiation of translation or even maintenance of the reading frame (Bjork and Durand, 1999; Persson, 1993).

Each modification is carried out by specific enzymes that are coded in the genome. In bacteria alone, there are up to 50 different tRNA modifying enzymes (Buck *et al.*, 1983). Similarly, eukaryotes also devote a significant amount of their genetic code towards the production of these modifications that occur at a post-transcriptional level mainly as a nuclear event. But there are a few exceptions where the modification occurs in the cytoplasm after the tRNA has exited the nuclear compartment- queuine modification.

1.2 Queuine: a post-transcriptional tRNA modification

1.2.1 Introduction to queuosine

Queuine or Q-base (7-(((4,5-cis-dihydroxy-2-cyclopenten-1-yl) amino) methyl)-7 deazaguanosine) is a hypermodified guanine base. Its secondary amine group attached to a cyclopentenyl diol ring structure classifies it as being structurally unique from other nucleosides as shown in **Figure 1.2b**. The nucleoside of queuine is referred to as queuosine (Q) and it is found in four forms: free Q-base, free nucleotide, free nucleoside and nucleoside incorporated into tRNA. Queuine in tRNA is found inserted in the wobble position (34) of the anticodon loop of four cognate tRNA isotypes for Aspartate (Asp), Asparagine (Asn), Histidine (His) and Tyrosine (Tyr) (Kuchino *et al.*, 1976). All the aforementioned tRNA molecules contain a GUN sequence (where N stands for any ribonucleoside) at the anticodon loop position 34-37 in their primary transcript, collectively being referred to as the family of GUN tRNA, tRNA_{GUN} or the queuosine family of RNAs, Q-tRNAs.

The insertion of queuine into tRNA is an irreversible post-transcriptional event. It occurs on the wobble position (34) of the nascent tRNA_{GUN} transcript that genetically encodes a guanine base. In a base-for-base exchange reaction, known as transglycosylation, the guanine base is excised and exchanged for the hypermodified queuine base. Queuine is found in all bacterial and eukaryotic tRNAs, except plant leaf cells (Kersten *et al.*, 1988), archaea (Gregson *et al.*, 1980) and *S. cerevisiae* (Walden *et al.*, 1982)—although other yeast species examined shows the existence of the TGT and QTRTD1 protein (Boland *et al.*, 2009).

Queuine is synthesised *de novo* exclusively by bacterial organisms. The **upper panel of Figure 1.3** gives a schematic representation of the multi-step pathway in bacteria, beginning with the conversion of nucleotide triphosphate GTP into preQ₀ and then into preQ₁. The subsequent tRNA insertion step is carried out by the tRNA guanine transglycosylase (TGT) enzyme which utilises either guanine, preQ₀ or preQ₁ as a substrate for incorporation into the wobble position of the anticodon, preQ₁ being its preferred substrate.

Eukaryotes on the other hand are incapable of queuosine biosynthesis. They must salvage free queuosine nucleoside and queuine/Q-base from the gut flora or food and from tRNA turnover reactions within the cell (Dirheimer *et al.*, 1995) as

represented in **Figure 1.3, lower panel**. This was demonstrated in the work by Gündüz and co-workers, where they cultured Vero cells, a monkey kidney cell line, in queuine depleted conditions and then re-administrated radiolabelled queuine, [^3H]dihydroqueuine, to track the eukaryotic queuine salvage mechanism (Gündüz and Katze, 1982). The HPLC analysis consistently reported a single peak corresponding to the size of dihydroqueuine suggesting lack of any degraded products. However, further studies involving cell extracts from the queuine salvage deficient mouse fibroblast L-M cells, reported the presence of Q-monophosphate—an intermediate base capable of acting as a substrate for salvage activity when added back to Vero cells. By HPLC, this intermediate was identified as queuosine 5'-phosphate (Gündüz and Katze, 1982).

The salvaged queuine base is inserted into the same site as in bacterial tRNA by a eukaryotic homologue of the bacterial TGT enzyme and its partner protein QTRTD1, as explained in further detail below. Therefore in both kingdoms, preQ₁ (eubacteria) and queuine (eukaryotes) is inserted by a TGT homologue into the tRNA anticodon wobble position. In archaea, the archaeal TGT homologue inserts a 7-deazaguanine analogue called archeosine into position 15 (D loop) of the tRNA. The insertion of queuine into tRNA does not require the cleavage of the phosphodiester backbone, nor does it require any energy input (for example ATP hydrolysis). However the N-C glycosidic bond of queuine is more stable than typical purines (Reyniers *et al.*, 1981). Also, once incorporated into the tRNA_{GUN} wobble position, queuine can be subjected to further modifications such as the addition of mannose (ManQ) or galactose (GalQ), as represented in **Figure 1.2b**. These sugars are covalently appended to the hydroxyl group at the 2' position of the cyclopentenylidol ring. To date, only two of the four Q-tRNA species, tRNA^{Asp} and tRNA^{Tyr}, are known to be modified by sugars. The physiological purpose of these secondary modifications is currently unknown (Gündüz and Katze 1984).

1.2.2 Queuine insertion enzymes

The initial efforts to understand the enzymes responsible for this transglycosylase/queuine insertion activity came from studies in bacteria, principally *E.coli* and *Z. mobilis* (Iwata-Reuyl *et al.*, 2003). The crystal structure of the *Z. mobilis* TGT was the first to be elucidated. The structure revealed that the protein adopted a TIM (triosphosphate isomerase enzyme) barrel structure, providing a basis to annotate the α -helices and β -strands (Romier *et al.*, 1996). It was found that tertiary structural elements along with the active site nucleophile, Asp 280 were highly conserved across species (Stengl *et al.*, 2007). Mutational and kinetic studies of the recombinant *E.coli* protein, informed by the *Z. mobilis* crystal structure, revealed the catalytic mechanism by which TGT recognises and binds tRNA (Boland *et al.*, 2009; Romier *et al.*, 1996). This enzymatic reaction was found to conform to a ping-pong reaction mechanism (Garcia *et al.*, 2005) where the tRNA is bound first followed by cleavage of the guanine from the wobble position. The active site of the transglycosylase then binds queuine before its insertion into the TGT-tRNA intermediate resulting in the insertion of queuine into tRNA at position 34 (Bjork and Durand, 1999).

Despite knowing the identity of the bacterial enzyme, it was only recently that the eukaryotic Q-tRNA insertase enzymes were discovered through work carried out in our laboratory (Boland *et al.*, 2009). Our group showed that the activity of eukaryotic TGT is absolutely dependent on forming a heterodimeric complex with the mitochondrial localised protein queuine tRNA-guanine transglycosylase domain containing 1 (QTRTD1) (**Figure 1.3 lower panel**). Together TGT and QTRTD1 are known as the queuine tRNA ribosyltransferase enzyme (QTRT). These results were later confirmed by Chen and co-workers where they co-purified the co-expressed human QTRTD1 (hQTRTD1) with the hTGT form. Their further structural analysis, using mass spectrometry and size exclusion chromatography, re-confirmed our results reporting that the two proteins do indeed exist as a heterodimer (Chen *et al.*, 2010).

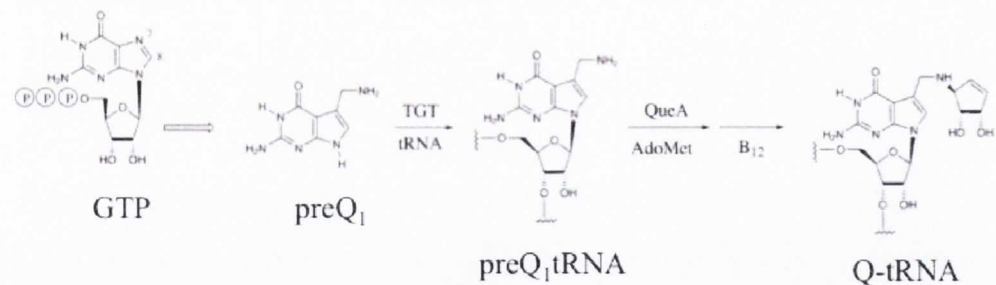
The study by our group also showed that the QTRTD1 protein exists as three spliced variants (Qv0, Qv1 and Qv2) out of which Qv1 is the predominant form expressed in adult tissue (Boland *et al.*, 2009). TGT however only exists as one spliced variant. The expression of TGT and Qv1 was analysed by Northern blot and both proteins were found to have identical expression patterns. Moreover, confocal and immuoblot analysis showed that while Qv1 is stably associated with the mitochondrial outer membrane, the TGT partner protein is only weakly associated —

suggesting the queuine tRNA ribosyltransferase reaction occurs in the cytoplasm (juxtaposed to the mitochondrial membrane). The mitochondrial localization of the proteins may also help explain the modification of mitochondrial tRNA with queuine; although there is still a large amount of mechanistic analysis to be done. The possibility that the queuine insertase reaction occurs at the outer mitochondrial membrane is partially vindicated by earlier *Xenopus* oocyte microinjection experiments. In these studies, cytoplasmic microinjection of yeast tRNA_{Asp} resulted in queuine modification whereas injection of yeast tRNA_{Tyr} into the nucleus failed to generate modified product (Haumont *et al.*, 1987).

The studies by our group also demonstrated that the association of both proteins, TGT and Qv1 was requisite for any transglycosylase activity to occur where the TGT supposedly performs the transglycosylation step by associating with the tRNA and the modified base, whereas Qv1, as reported by Chen and co-workers, could be a TGT partner protein which may have a role in queuine salvage during tRNA turnover (Boland *et al.*, 2009; Chen *et al.*, 2010).

Conservation of the queuine tRNA insertion enzymes across the vast majority of species make it possible to speculate on their evolutionary origin. The Qv1 protein sequence diverges significantly from bacterial enzymes indicating that it is perhaps the older of the two and possibly carried into the eukaryotic genome following fusion with the pro-mitochondrial bacterium. On the other hand, the similarity between the TGT proteins found in all three kingdoms suggests the eukaryotic enzyme was acquired later during evolution, being acquired by lateral transfer from the pro-mitochondria (Anantharaman *et al.*, 2002; Boland *et al.*, 2009). The above, compounded with the fact that insertion of the bacterial base queuine is a terminal modification present extensively in most eukaryotes (Nishimura *et al.*, 1983), raises the question as to its physiological role in the cell. Studies by Kersten and co-workers have investigated into this very aspect of queuine and linked it to various physiological effects such as differentiation, development, hypoxic stress management, tyrosine biosynthesis and protein synthesis. Although a definitive biochemical function for queuine has yet to be verified, the above physiological effects have been clearly linked to queuine modification, or its lack thereof (Kersten *et al.*, 1990).

Bacteria



Eukaryotes

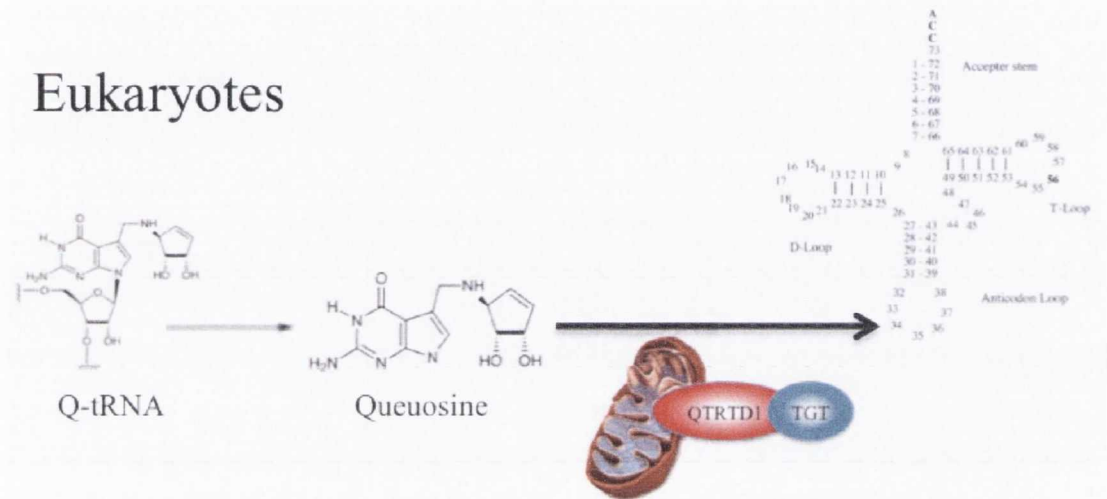


Figure 1.3: Queuine biosynthesis in bacteria and insertion into eukaryotic tRNA
Queuine biosynthesis in bacteria (**upper panel**) begins with the conversion of GTP to preQ₁, a bacterial TGT substrate, which is inserted into the wobble position of tRNA harboring a GUN anticodon sequence (Q-tRNA). A series of enzymatic steps then acts to convert the inserted preQ₁ into queuine on the tRNA molecule. To allow the salvage of queuine by eukaryotic species from the gut flora or diet, it is presumed that tRNA bound queuine is released either as a free base or nucleoside residue which is then taken up by eukaryotic cells. The eukaryotic QTRT enzymes (TGT and QTRTD1), which are mitochondria bound, insert queuine into the wobble position of GUN tRNA (**lower panel**) similar to bacterial species. In both bacteria and eukaryotes, queuine modification is an irreversible event. Adapted from (Iwata-Reuyl *et al.*, 2003.)

1.2.3 The physiological role of queuine

In the 1980's, a series of *in vivo* studies by Walter Farkas and his co-workers attempted to investigate the physiological role of the queuosine modification in eukaryotes by placing eight week old germ-free (axenic) mice on a specialized queuine-free diet. In short term studies (after 4 weeks), which equates to nine tRNA half-lives, the levels of queuine modified tRNA^{His} and tRNA^{Asn} were found to decrease by 70% however, the other Q-tRNA isoacceptors remained fully modified. It took a full year for the animals to become fully depleted in Q-tRNAs (Farkas, Jacobson *et al.*, 1981). Due to lack of any pathological manifestations in these animals, the study could merely conclude that in the absence of queuine from diet, the robust eukaryotic salvage pathway is able to maintain sufficient levels of queuine (Reyniers *et al.*, 1981). It was not until 1997 that a second study by Farkas's group revealed a pathological effect of withdrawal of both queuine and tyrosine from the diet of germ free mice. Within eighteen days, each animal in this experimental cohort presented with behavioural symptoms ranging from squinting, stiffness, staggered movement, seizures and eventually death—a pathological effect that could be reversed by re-supplementing the diet with either tyrosine or queuine. This study was re-visited by our group using TGT genetrap mice (a functionally disrupted gene mutant) showing that Q-tRNA deficient conditions impaired tyrosine biosynthesis as a result of the accumulation of the oxidised form of the phenylalanine hydroxylase co-factor, tetrahydrobiopterin. Furthermore, this study also explored how queuine, through tetrahydrobiopterin depletion, could influence the synthesis of biogenic amines (dopamine, adrenaline, serotonin), all of which depend on tetrahydrobiopterin for their synthesis. Behavioural abnormalities were observed in female mice, the animals displaying lower anxiety and higher explorative activity (Rakovict *et al.*, 2011). A further metabolic change commonly observed in the absence of queuine is an increase in lactate dehydrogenase (Reisser *et al.*, 1994). Conversely, studies by Langutt and Vinayak reported that upon administering queuine to transformed HeLa cells, the activity of the lactate dehydrogenase enzyme (LDH) is decreased (Reisser *et al.*, 1994; Pathak *et al.*, 2005).

Other studies in bacteria and eukaryotic cell lines focused on the effect this anticodon specific queuine modification could have on tRNA aminoacylation and the interaction between the anticodon and the degenerate codons on mRNA (Meier *et al.*, 1985). Work by Noguchi and co-workers using *tgt* gene deletion mutants in *E.coli*

showed how the lack of queuine modification negatively affected cell viability in competitive growth experiments (Noguchi *et al.*, 1982). Interestingly, other mutational studies in the *tgt* homologue in *Shigella flexneri* called *vacC* (virulence associated chromosomal locus), resulted in loss of pathogenicity by 50-60%. Neither of the above studies found any changes in the aminoacylation ability of the Q-tRNA or the levels of mRNA, instead the studies highlighted how the lack of queuine increased read-through of the non-sense codon by almost two fold (Noguchi *et al.*, 1982; Durand *et al.*, 1994). Similar studies by Singhal and his co workers in L-M cell line reported the presence of the queuine modification increased the affinity of the synthetase enzyme for its substrate from a $V_{\max}/K_m$ of 5 units in unmodified tRNA to a ratio of 12 units in queuine modified tRNA (Singhal and Vakharia 1983). Suggesting queuine modification has an effect on translational speed and reading frame selection (Noguchi *et al.*, 1982; Kersten 1982).

From a more biological point of view, the importance of queuine peaked the interests of scientists when variability in the levels of this modification was reported in studies during stages of development and tumour malignancies. Studies by Kersten and co-workers reported that despite sufficient levels of queuine in the amniotic fluid of humans, the placental tissue was devoid of queuine modification whereas embryonic tissue was found to be only partially modified. Furthermore, regenerating rat liver tissue only showed partial extent of modification, suggesting the importance of queuine as a differentiation marker especially as all the fully differentiated/mature cells were fully modified with queuine (Okada *et al.*, 1978; Kersten *et al.*, 1990). This phenomenon gained more attention when the work by Okada's group reported that malignant transformed cells were deficient in queuine modified tRNAs (Okada *et al.* 1980). Emmerich extended this study using human leukaemia and lymphoma cells showing that the levels of queuine modified Q-tRNA decreases accruing to the stage of malignancy suggesting Q-tRNA levels could be used as a tool for malignancy grading (Emmerich *et al.*, 1995; Langgut, Reisser *et al.*, 1990; Iwata-Reuyl, 2003). Another study by Huang reported the link between unmodified Q-tRNA and the stages of tissue differentiation in lung cancer patients (Huang *et al.*, 1992), represented in **Figure 1.4a**. This theory was re-confirmed by Baranowski who similarly found that hypomodified Q-tRNA is indicative of increased malignancy suggesting it is the result of the increasingly poor differentiation status of cancer cells (Baranowski *et al.*, 1994). Huang then re-analysed lung cancer patients post-surgery

and found that levels of unmodified Q-tRNA could even be extrapolated to assess patient survival (Huang, Wu *et al.*, 1992), represented in **Figure 1.4b**.

Despite the extent of published literature, none of the studies provided a valid explanation as to why the Q-tRNAs demonstrated this characteristic loss in queuine modification in developing tissues and tumours. Studies by Kersten investigated whether this outcome was due to loss of TGT activity or catalytic activity in these tissue or insufficient substrate availability or even higher tRNA turnover rate. All the above studies gave inconclusive data to pinpoint the cause of this phenomenon. However the studies did point out a difference in the levels of queuine uptake.

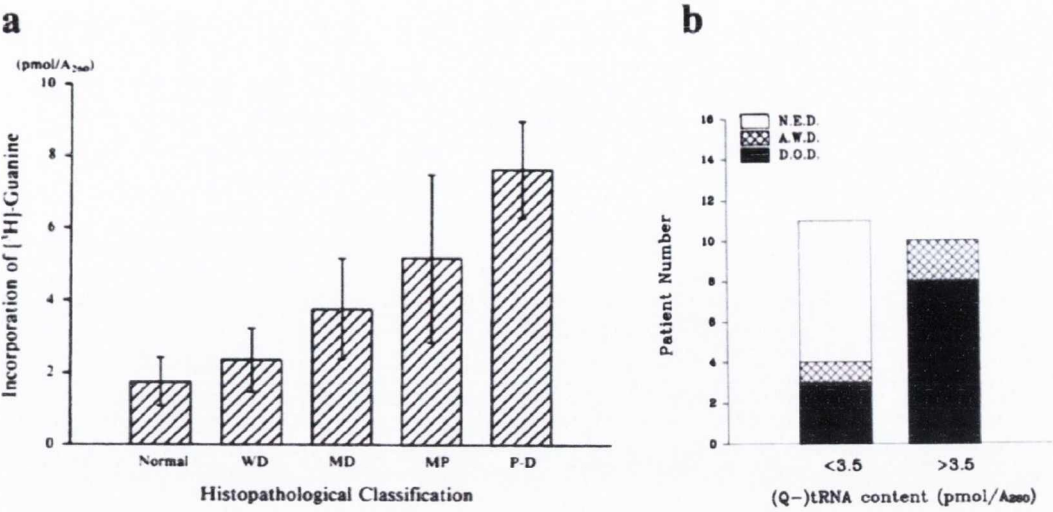


Figure 1.4: The effect of queuine deficient tRNA on malignancy and patient survival

Studies investigating the role of queuine in malignant cancers like Lung cancer (**a**) show that the level of ³H-guanine incorporated into queuine deficient Q-tRNAs is inversely related to the stages of differentiation- represented here in terms of Normal (<2pmol ³H-G), well-differentiated (WD; >2pmol ³H-G), moderately differentiated (MD; <4pmol ³H-G), moderately to poorly differentiated (MP; 5pmol ³H-G) and finally poorly differentiated (PD; <8pmol ³H-G) lung cancer patient tissue. Upon analysing queuine content in lung cancer patients four years post-surgical intervention, a direct correlation was observed in terms of level of queuine modified Q-tRNA present and patient survival (**b**). Out of 21 patients analysed, 11 had higher than 3.5 pmol Q-tRNAs, with 7/11 alive (N.E.D), 1/11 alive with relapse (A.W.D) and 3/11 dead (D.O.D), whereas in the remaining 10 patients with lower than 3.5 pmol Q-tRNAs, only 2/10 were alive with relapse (A.W.D) and the remaining 8/10 were dead (D.O.D.). N.E.D, No evidence of disease; A.W.D, alive with disease; D.O.D, died of disease. Adapted from (Huang *et al.*, 1992).

1.2.4 Queuine uptake

Eukarya are incapable of synthesising queuine *de novo*, but the studies of Walter Farkas showed that animals can extract queuine from ingested modified tRNA to replenish their own unmodified tRNAs with queuine (Farkas, 1980; Reyniers *et al.*, 1981). Mammalian cell lines grown in culture obtain queuine from animal serum, principally foetal calf serum (FBS) (Katze, 1978). The above therefore strongly suggest a robust mechanism exists in eukarya to accumulate dietary queuine across the cell membrane—a step prerequisite for the queuine insertion into Q-tRNA. Notwithstanding this, the Q-tRNAs isolated from neoplastically transformed cells consistently show a characteristic loss in queuine modification in their Q-tRNAs and instead their wobble position on the anticodon contains the genetically encoded guanine nucleoside (Katze *et al.*, 1983; Nishimura, 1983). Studies by Shindo-Okada and co-workers showed that levels of TGT were equivalent in both normal and transformed cells (Shindo-Okada *et al.*, 1980) leaving open the question as to whether queuine uptake was affected in these cells containing hypomodified Q-tRNAs.

Queuine uptake is indispensable for its cellular role. Studies by Elliot and co-workers have demonstrated that its uptake is sensitive to phorbol esters such as Phorbol-12, 13-didecanoate (PDD), a tumour promoting agent (TPA) (Elliot *et al.*, 1985; Elliot *et al.*, 1986). Upon treating human foreskin fibroblast cultures with PDD, intracellular levels of a radioactively labelled queuine analogue, dihydroqueuine decreased leading to loss of queosine content of Q-tRNA along with increased cell culture density. Concurrent addition of exogenous queuine effectively inhibited PDD-induced culture density and also restored levels of queuine modified Q-tRNA back to normal levels. (Elliot *et al.*, 1984). Their data also concluded that due to the structural dissimilarity between PDD and queuine, the observed effect must have occurred via an uptake independent mechanism- perhaps a signalling dependent pathway. This finding was re-visited when a later study commented on the effect of protein kinase C (PKC) on the uptake of queuine. PKC was found to have a modulatory effect on the TGT activity. So treatment of fibroblasts with PKC activators stimulated queuine uptake whereas chronic treatment with phorbol esters – inhibitors of PKC, reduced queuine uptake along with its insertion into tRNA leading to lower amount of queuine modified Q-tRNAs. However, the effect on queuine uptake can only be referred to as

a depressive modulation since uptake levels were only brought down to base-line (Morris *et al.*, 1995).

With no significant changes in cellular TGT levels along with at least basal levels of cellular queuine concentration present, the reason behind the undermodified state of Q-tRNAs in transformed cells remains to be elucidated. However, the above observations also re-enforce the fact that the lack of queuine insertion is a substrate availability-independent phenomenon and more so a transformed-cell specific feature. Without delay, this deduction beckoned for studies to investigate the viability of other substrates/queuine analogues to be exploited by the TGT enzymes present.

1.3 6-Thioguanine, a queuine analogue

1.3.1 Thiopurines and QTRT

Although queuine is known to be the main substrate for the QTRT enzymes, Farkas and Jacobson tested a number of 7-deazaguanine derivatives reporting the ability of other purine analogues to act as inhibitors or substrates in the exchange reaction (Farkas *et al.*, 1984). Their study also suggested that while incorporation of queuine, dihydroqueuine, 7-aminomethyl-7-deazaguanine, or 7-deazaguanine was irreversible, the substitution of C-7 for N-7 prevents the reversible incorporation of queuine into tRNA- for example in guanine, structure represented in **Figure 1.5a**. There are a group of such purine/guanine analogues that all contain an N-7 instead of a C-7 called thiopurines. Their name is derived from their chemical structure containing a purine base where the oxygen at C6 is substituted by a thiol group (SH) in 6-Thioguanine (6TG, in **Figure 1.4b**) and 6-Mercaptopurine (6MP, in **Figure 1.4c**) or by a 6-(1-methyl-4-nitro-5-imidazolythio) purine group in Azathioprine (Aza, in **Figure 1.4d**) (Elion, 1993). These compounds, like other purine analogues, have similar properties to that of a guanine base, however the substituted thiol group at position C6 makes it more chemically reactive than the usual nucleic bases (Karran, 2006).

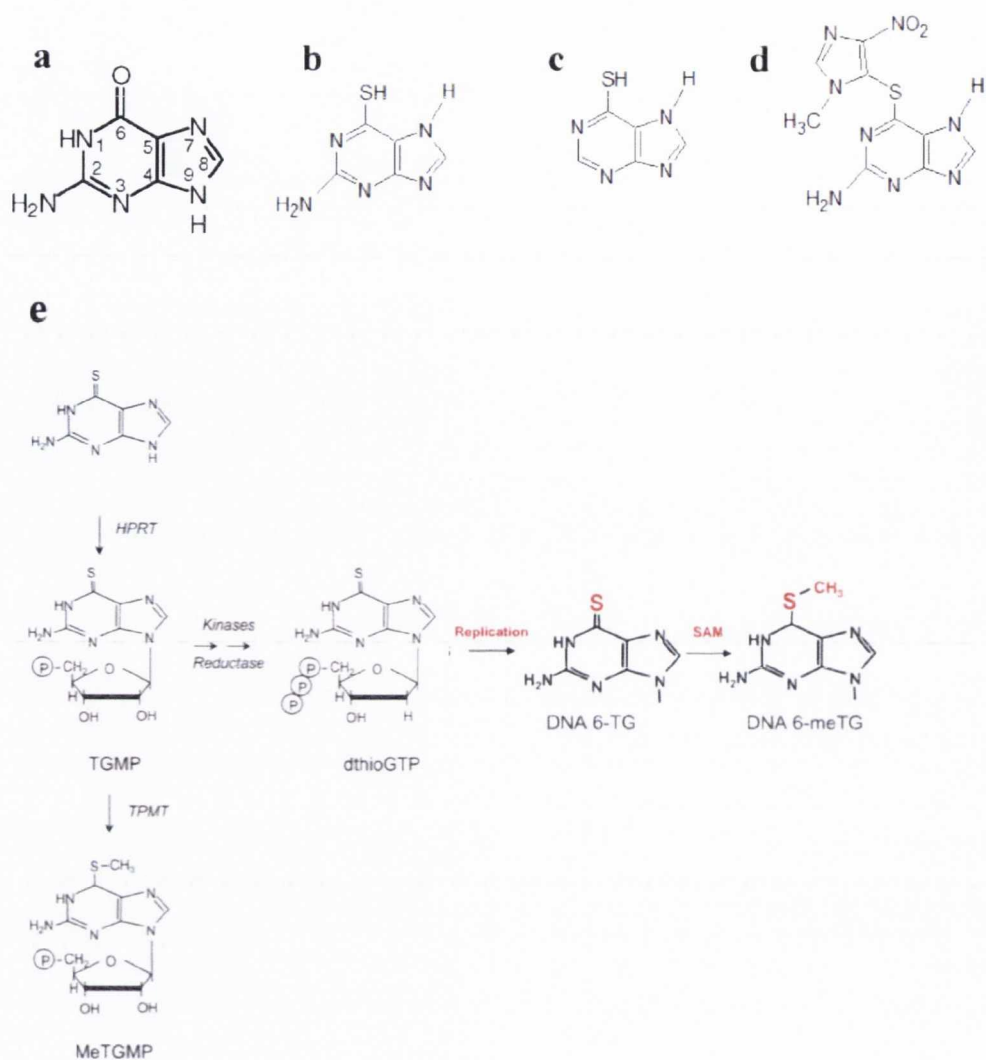


Figure 1.5: Structure and metabolism of thiopurine drugs.

The chemical structure of a canonical guanine base is shown (a), to allow comparison with a number of thiopurine analogues commonly used in human therapeutics. Whereas guanine has an oxygen molecule at the C6 position of the purine ring, a thiol (SH) is found in 6-Mercaptopurine (6MP) (b) and 6-Thioguanine (6TG) (c) or is substituted by a 1- methyl-4-nitro-5-imidazolythio group in the case of Azathioprine (Aza) (d). In the cell, Aza is as pro-drug to 6-MP. So, 6MP and 6TG are the active metabolites. 6TG is a substrate for the purine salvage enzyme HPRT (e) which converts it into thioguanine monophosphate (TGMP) and through a series of phosphorylation and reduction step this is converted to 2'-Deoxy-6-thioguanosine 5'-triphosphate (dthioGTP) and incorporated into replicating DNA (denoted in the figure as DNA 6-TG). Further modification of DNA 6-TG by methyl transfer to the C6 thiol-group from S-adenosylmethionine (SAM) generates the highly genotoxic residue DNA 6-meTG. In a competing catabolic reaction, TGMP is also a substrate for TPMT resulting in the formation of MeTGMP (an effective inhibitor of *de novo* purine biosynthesis). Adapted from (Karran 2006; Karran and Attard, 2008).

1.3.2 6TG uptake and metabolism

Discovered back in the 1950's by Gertrude B. Elion as a purine analogue, the effectiveness of 6TG in dampening allogenic immune-rejection in organ transplants made it the first ever immunosuppressive agent to be developed—work for which Gertrude B. Elion and George Hitchings were awarded the Nobel Prize in Medicine in 1988 (Elion 1993). Ever since, medical interest in the immunosuppressive properties of these analogues was conveyed by their ability to exploit the uptake and metabolism pathways of the canonical purine bases.

The purine and pyrimidines uptake mechanism exploited by their analogues consists of two families of nucleotide/nucleoside transporters classified according to whether they passively uptake purine molecules, the equilibrative nucleoside receptors (ENT), or rely on a Na⁺-dependent uptake mechanism, the concentrative nucleoside transporters (CNT). The CNT family of transporters consists of 3 members in humans (hCNT 1, 2 and 3) and 4 members of the ENT family in humans and mice (hENT 1, 2, 3 and 4 and mENT 1, 2, 3 and 4) (Acimovic and Coe 2002; Fotoohi, *et al.*, 2006; Loffler *et al.*, 2007). Along with the uptake of purine and pyrimidine nucleosides (adenosine, guanosine, inosine, uridine, thymidine and cytidine), these transporters also provide a channel for related drug analogues such as azidothymidine (used in HIV-1 therapy) and ribavirin (a Hepatitis-C drug) to gain entry into the cellular space (Loffler *et al.*, 2007).

Similarly, the uptake of thiopurines like 6TG also occurs via the ENT and CNT transporters. Although the expression of these receptors varies across cell populations, the level of hCNT2 and all four members of hENT1, 2, 3 and 4 family are expressed at high levels in T lymphocytes—a key feature especially as their immunosuppressive role is pre-dominantly exploited in hyper-lymphoproliferative disorders such as Psoriasis, Leukaemias, Colitis and many more (Loffler *et al.*, 2007; Karran, 2006).

In the early years after its discovery however, the exact mechanism of 6TG mechanism and metabolism was unknown, inviting various scientists to investigate into its anti-metabolic properties. Preliminary studies by LePage and Jones on the malignant, poorly-differentiated mouse breast and connective tissue carcinoma-Ehrlich ascites and Sarcoma 180 tumour respectively, reported that administration of 6TG had its maximum anti-tumour effects in rapidly proliferating cells where the

radiolabelled 6TG was retained completely in DNA. Moreover although these cells were still viable, their inability to either grow or divide hinted strongly that the tumour-growth inhibitory effects were mediated by the robust substitution of the natural base guanine by 6TG in the synthesis of nucleic acids; both DNA and RNA (LePage, 1961; LePage, 1963; Sartorelli and LePage, 1958). Later studies on primary human cells, Chinese hamster ovary cell lines and even *in vivo* in mice (Christie *et al.*, 1984; Petit *et al.*, 2008; Aubrecht *et al.*, 1997) confirmed these preliminary findings along with providing the exact mechanism of 6TG metabolism in the cell — its incorporation into replicating DNA via the action of the HPRT (hypoxanthine-guanine phosphoribosyl transferase 1) enzyme (Murphy *et al.*, 1999), a schematic for which is represented in **Figure 1.5e**.

The HPRT enzyme is the first step in the purine salvage pathway, converting 6TG into thioguanosine monophosphate (TGMP) (Aubrecht *et al.*, 1997). Aza (converted into 6MP) and 6MP itself are also metabolised by HPRT into TGMP, however it is via a different enzymatic steps (Karran, 2006; Karran and Attard, 2008). At this point, thiopurine methyltransferase (TPMT) can inactivate the molecule by methylating TGMP into methylated-TGMP (me-TGMP). Methylation prevents the molecules from being further metabolised. However, usually TGMP functions as a substrate for the inosine-5'-monophosphate dehydrogenase enzyme that converts TGMP into thioguanosine diphosphate (TGDP) (al-Deen *et al.*, 1992). This product is further metabolised into thioguanosine tri-phosphate (TGTP) by Guanosine monophosphate synthase (GMP) (al-Deen *et al.*, 1992) yielding thioguanosine nucleoside (6TGN) (Cara *et al.*, 2004). 6TGN is then readily incorporated into synthesising nucleic acid polymers (Murphy *et al.*, 1999). The erroneously incorporated 6TGN is not a substrate for pre-replicative excision repair thereby allowing 6TG to be retained in replicating DNA (DNA 6TG). It has been discussed that the incorporation of 6TG alone is insufficient to convey its entire anti-metabolic effects. The thiol containing group is very reactive and as a result, is a substrate for *in situ* methylation (to DNA 6-meTG) by non-enzymatic agents such as S-adenosylmethionine (SAM) (Karran, 2006). The final product interferes with DNA processes such as transcription and replication.

Similar to most drugs, the benefits of thiopurines also comes with limitations of use. Its basic mechanism of misincorporation into replicating DNA conveys its therapeutic effects in the short-term by mainly being toxic to rapidly proliferating

cells. However long-term exposure to these analogues leads to: gain of resistance to the drug and off-target mutations in potentially any replicating cell. Such accumulation of aberrations/insults to the genetic material has been extensively reported to be a leading cause for the development of either leukaemia in patients treated with 6TG in chemotherapy, or brain tumours in patients treated for childhood ALL (acute lymphoblastic leukaemia) and also skin cancer in patients treated for graft versus host disease. It is still noteworthy that despite these drastic effects, the use of thiopurines is still prevalent as an immunosuppressive agent, however mainly in combination with other therapies allowing low doses to be given (Karran, 2006).

1.3.3 6TG and tRNA

Although extensive clinical and scientific research focused on 6TG incorporation into DNA to explain its immunosuppressive effects, early studies on bacteria and murine tumours, were aware of the lesser but significant incorporation of 6TG into RNA. Early studies on *in vitro* transformed cell lines or injected tumours described the ability of ^{14}C -radiolabelled 6TG to be incorporated into DNA and/or RNA in terms of ‘sensitivity’ of the cells to 6TG—where cells sensitive to 6TG were found to retain cytotoxic 6TGNs incorporated in their DNA whereas cells resistant to the analogue were reported to contain 6TG incorporated into intact RNA (Sartorelli and LePage, 1958; LePage and Jones, 1961; LePage, 1963) (**Figure 1.6a**).

Trewyn’s group carried out one of the preliminary studies to suggest that the anti-proliferative effects of 6TG in the HPRT-deficient leukaemia cell line, HL-60 was conveyed by its incorporation into the queuine deficient wobble position of tRNA—suggesting an HPRT-independent, alternative pathway for 6TG mediated anti-proliferative effects. He confirmed this further by administering exogenous queuine and showing the ability of these 6TG cells to reverse the anti-proliferative effects of 6TG (**Figure 1.6b**). Additionally, these results also confirmed the hypothesis that 6TG was a substrate and not an inhibitor of the queuine-tRNA pathway (Kretz *et al.*, 1985; Morgan *et al.*, 1994) (**Figure 1.6c**).

It is noteworthy the origin of the terms ‘sensitive’ and ‘resistant’ to describe the cell’s susceptibility to the analogue could be explained by the status of its HPRT enzyme which enables the incorporation of 6TGN into replicating DNA more robustly when compared to its incorporation into and effect on RNA, a less acute effect. This is

also demonstrated by the fact that it is only at very high concentrations of 6TG treatment that incorporation of 6TG into RNA is observed. This could explain why any RNA-mediated effect was overlooked and instead the HPRT mediated DNA pathway has been studied in great detail for thiopurine therapeutics in treatment of cancers, autoimmune and inflammatory disorders.

a EFFECT OF 6-THIOGUANINE ON GUANINE-
8-¹⁴C INCORPORATION INTO
NUCLEIC ACID GUANINE

THIOGUANINE DOSE (mg/kg)	PER CENT INHIBITION	
	Sensitive	Resistant
0.5	40	
2.5	49	
5.0	58	27
10.0	61	33
15.0	78	52

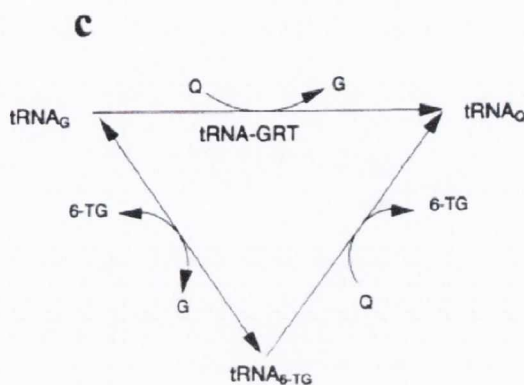
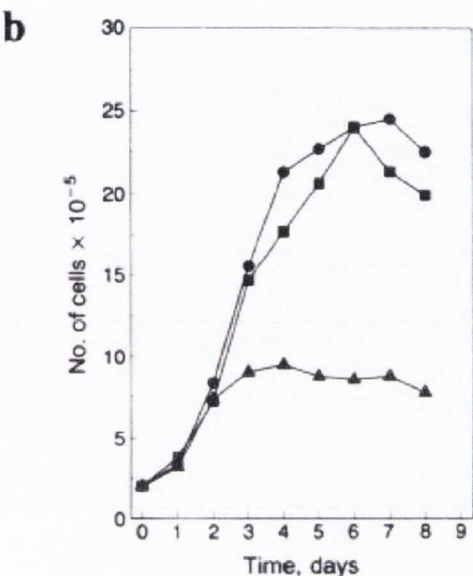


Figure 1.6: 6TG can be incorporated into queuine deficient tRNA

Studies in 1958 reported that injections of 6TG into Ehrlich ascites tumour-bearing mice inhibited the incorporation of ¹⁴C-8-guanine into nucleic acids at increasing doses (a). The 6TG ‘sensitive’ cells show 40% inhibition at the lowest injected 6TG dose of 0.5 mg/kg, going up to 78% inhibition at the highest injected dose of 15 mg/kg. The 6TG ‘resistant’ cells do not show any inhibition at the injected doses of 0.5 and 2.0 mg/kg, instead they start showing inhibition of 27% starting at 5mg/kg rising to a maximum of 52% at 15 mg/kg (Sartorelli and LePage, 1958). In 1991, researchers at Ohio State University using 6TG resistant HL-60 cells, correctly re-defined as being HPRT-deficient (b), showed that normal cell viability (solid circle) was significantly decreased by 6TG administration (solid triangles) and this effect could be reversed by co-administration of queuine (solid squares). The role of 6TG in tRNA was presented by an accompanying schematic (c) where the genetically encoded guanine at position 34 of GUN tRNA (tRNA_G) could be either directly replaced by queuine, yielding the irreversible product tRNA_Q, or by 6TG to give tRNA_{6TG} which can be displaced by queuine to generate tRNA_Q. Adapted from (French *et al.*, 1991).

1.4 Introduction to Multiple Sclerosis: an autoimmune disorder

In 1868, the French neurologist Jean Martin Charcot noted multiple demarcated inflammatory lesions or ‘sclerotic plaques’ in the brain and spinal cord of patients who had been suffering from neuronal dysfunction. His original description of these lesions as *sclérose en plaques disséminées* is now recognized as the very first account of Multiple Sclerosis (Charcot, 1868; Charcot, 1877; Lassmann, 1999).

Multiple Sclerosis (MS) is a progressive neurological disorder of the brain and spinal cord affecting over 2.5 million adults worldwide. The affected age group is between the ages of 20 and 40 years with a susceptibility index found to be three times higher in women than in men (Costantino *et al.*, 2008; Hohlfeld, 2009). Gross examination of the CNS tissue from MS patients reveals the presence of multiple inflammatory lesions with a predilection to the optic nerves and the neuron rich white matter, as described previously by Charcot (Hafler, 2004). These lesions represent a massive heterogeneous infiltrate of activated immune cells including T and B-lymphocytes, macrophages, microglia and soluble factors such as cytokines, chemokines and antibodies (Bruck, 2005). Together, this ‘cocktail’ of immune components elicits an attack against the protective myelin lipid layer of the neurons, causing demyelination (Hafler, 2004; Bruck, 2005). Damage to the neuronal axons leads to deficits in sensation and neurocognitive function that manifests in a variety of motor dysfunctions ranging from fatigue, muscle weakness, impaired vision to tremors and paralysis. While some patients experience these symptoms in its chronic form, i.e. deteriorating over time from onset (primary progressive MS), up to 80% of the patients experience deterioration in so-called clinical ‘episodes’ or relapse-remitting pattern (RRMS) of the illness with intermittent periods of recovery (secondary progressive MS). However the massive inter-individual heterogeneity compounded by the unpredictable pattern of the clinical episodes, has lead to many misdiagnosed or undiagnosed cases of MS (Hohlfeld, 2009). An example of this is highlighted is MS cases historically recorded by physicians—as early as in the year 1300—or by patient themselves, as in the case of Augustus d’Este (Maeder, 1979; Murray, 2005). Due to the lack of knowledge about such an illness, along with an absence of diagnostic tools, the disease was simply classified as a form of ‘mental illness’. Even today, despite sophisticated technology such as magnetic resonance

imaging (MRI), many patient cases are never diagnosed clinically (Bruck, 2005). It is noteworthy that although it is a relief to some patients to have their disability recognized as a neurological condition, as opposed to a mental one, being an MS patient does not bring much comfort given that it is a life-long debilitating disease with no cure and moreover an unknown pathogenesis.

Etiology of Multiple Sclerosis: The etiology of MS is unknown. However many groups over the past few decades have focused heavily on the genetic, environmental and microbial factors contributing to the disease (Herndon, 2000).

Epidemiological twin-based studies have reported that the risk of genetically identical individuals developing MS was 2.5 fold higher than that of Type 1 Diabetes, and that there was a 20-40 fold increased risk in first-degree relatives when compared to unrelated individuals (Ebers, 1998; Ebers and Dyment, 1998; Costantino *et al.*, 2008). Genetic studies have identified variations in the human leukocyte antigen class II gene (HLA, human equivalent of the major histocompatibility complex, MHC) HLA-DRB* as a contributory risk factor to developing MS, but its etiological pattern is not always consistent (Oksenberg *et al.*, 2005; McFarland and Martin, 2007). Therefore, despite a number of likely genetic associations, no single gene alone is known to be responsible for the disease—reflecting the polygenic nature of MS as originally postulated by Pratt (Pratt *et al.*, 1951; Hayes, 2008). This prompted other groups to investigate the potential involvement of environmental factors as well. Two such factors that have been found prominent in MS patients are deficiencies in Vitamin D and the exposure to past viral infections (Acheson *et al* 1960; Hayes 2000).

The hormone Vitamin D (1,25-dihydroxyvitamin D₃) is peculiar in that its synthesis requires sunlight (Hayes, 2000). Moreover, Vitamin D specific receptors are expressed on the surface of activated T and B cells and shown to alter T cell proliferation either directly by affecting IL-2 production or indirectly by affecting antigen presentation by B cells. Viral infections on the other hand, such as Epstein Barr Virus (EBV), can cause activation and expansion of auto-reactive T cells either through molecular mimicry of CNS self-peptides or by leading to increased amounts of oligoclonal immunoglobulins in the CSF (Hayes, 2000; Hayes and Acheson, 2008). This theory, based on the inter-play between the two factors, has gained much interest in MS research, especially since individuals classified as ‘genetically susceptible’ and who suffer from recurrent infections during the darker winter months are those that

most often present with MS. However this theory also fails to apply to all MS cases. Moreover, up to 14 different viruses have been isolated from MS patient brains and none have shown an etiological pattern (Herndon, 2000). This suggests therefore that, similar to genetic association studies, no single environmental agent can be singled out as an initiator of the disease.

The identification of a range of contributory factors has led to MS being defined as a syndrome rather than a single disorder (Coyle *et al.*, 2011). The influence of more than one genetic or environmental factor on the course of the illness is proof that the disease is much more complex than previously thought (Hafler, 2004). It is however noteworthy that irrespective of the trigger, the course of the illness is characterized by an autoimmune inflammation in the CNS—as previously described by Charcot (Charcot 1877), mediated by myelin-reactive T cells. This was confirmed by Kabat in 1948 who reported that in the CNS, an otherwise immune-privileged site, MS lesions were found with elevated levels of the immune secretory product, oligoclonal immunoglobulin in the CSF (Kabat *et al.*, 1948; Kabat *et al.*, 1950). From this, stemmed the now generally accepted hypothesis that MS pathology is secondary to an autoimmune reaction that has been initiated against self-antigens; the prevalence of which is influenced by genetic susceptibility factors (Costantino *et al.*, 2008).

This is why perhaps, despite the unknown etiology of MS, scientists continue to study the animal model experimentally induced autoimmune encephalomyelitis (EAE) since it resembles the inflammatory encephalomyelitis that occurs post-infection/post-immune activation.

1.4.1 EAE as a model for MS

Louis Pasteur's attempts to develop a vaccine against Rabies in 1884, brought to light the potency of injected CNS tissue at causing neurological complications. Repeated injection of dried rabies infected rabbit spinal cord emulsions into recipient patients provided immunity against Rabies, however it also brought about sporadic cases of paralysis (Stuart *et al.*, 1928). These cases were histologically distinct from rabies, characterized by lymphoid infiltrates and demyelination—suggesting a distinct neuropathological condition (Baxter, 2007). At first, these complications were attributed to an incomplete inactivation of the injected virus. But around 1909 Thomas Rivers, a virologist at that time, saw a parallel between this study and other reported complications of viral diseases affecting the CNS such as smallpox, measles and vaccinia (Stuart and Krikorian, 1930). He hypothesized that this was an immune reaction triggered by the repeated injection of CNS tissue. To test his theory, he carried out a landmark study demonstrating that immunization of naïve primates with myelin from the CNS of normal (non-diseased) rabbits caused limb weakness, wobbly gait, and CNS demyelination, followed by periods of recovery—establishing the first ever EAE model in animals (River *et al.*, 1933; River and Schwentker *et al.*, 1935).

The only setback in River's EAE protocol was the cumbersome immunization regime requiring up to 85 injections over a period of a year (Baxter, 2007). Subsequent studies by Kabat therefore developed a technique to fast-track the immunization by combining the brain extracts from genetically homologous monkeys with heat-killed *Mycobacterium tuberculosis* (MTB) in paraffin oil—an adjuvant strategy described by Freund (Kabat, 1946; Morgan 1947). This concoction induced the disease in one single injection, making it a very practical protocol. Without delay, this technique gained interest and various groups employed it to successfully and reproducibly induce EAE in a wide range of animals including guinea pigs, goats, mice, rats, sheep and even chicken (Baxter, 2007).

Until the late 1980's EAE was defined as an 'allergic' immune response. A landmark study by Ben-Nun in 1981, demonstrated that the adoptive transfer of MBP-specific T cells, not antigens, into naïve mice was sufficient to trigger the same immune reaction (Ben-Nun *et al.*, 1981). The auto-aggressive nature of the selected T cells suggested that the immune reaction was 'autoimmune' rather than 'allergic' (Schluesener and Wekerle, 1985; Gold *et al.*, 2006). This work afforded EAE

researchers two fundamental guiding principles, (i) the autoimmune reaction is mediated by auto-aggressive T cell clones, (ii) the auto-aggressivity was mediated against CNS myelin peptides—two features consistently observed in human MS samples. Taking this a step further, studies revealed that the auto-reactivity of the T cell clones is not restricted to a single/specific myelin antigen suggesting that a breach in self-tolerance precipitates an autoimmune attack against a variety of CNS antigens *via* ‘epitope spreading’ (Lehmann *et al.*, 1992). This is reflected in the wide range of CNS antigens capable of inducing EAE, ranging from chemically different myelin peptides such as Myelin Basic Protein (MBP), Myelin oligodendrocyte glycoprotein (MOG), Proteolipoprotein (PLP) to non-myelin protein S-100B. MBP, PLP and protein S-100 induce a chronic or relapse-remitting course of disease lacking demyelination, whereas MOG together with myelin auto-antibodies induced a chronic, demyelinating yet monophasic EAE (Sriram and Steiner, 2005).

These studies revealed that despite the MS-like pathology obtained from these models, the pattern of disease progression relies on the CNS antigen used for immunization; hence the argument on the validity of EAE as a model of MS. One of the most obvious discrepancies was the immune-stimulatory bolus, containing strong immune adjuvants, required to sustain a brut-force inflammatory reaction in EAE. It is in itself a contradiction to the spontaneously occurring nature of MS in humans (Gold *et al.*, 2006). In more recent studies, transgenic animal models have been developed to ‘spontaneously’ succumb to EAE, however they depend on loss of gene expression to override the intrinsic regulatory mechanisms of self-tolerance (Waldner *et al.*, 2000; Bettelli *et al.*, 2003; Zehntner *et al.*, 2003; Gold *et al.*, 2006). Furthermore, the use of in-bred and genetically homogeneous strains for EAE studies fails to represent the genetic heterogeneity found in MS.

Notwithstanding these shortcomings, given that MS is a complex syndrome and EAE is simply an experimental system for research, it is naïve to believe that any single model of EAE would suffice at representing MS. Today, the different EAE models have therefore been categorized according to the aspect of MS they best represent: MOG₃₅₋₅₅ induces T-cell and Macrophage mediated chronic EAE in C57BL/6 (Mendel *et al.*, 1995), and in CNTF (ciliary neurotrophic factor)-deficient mice (Linker *et al.*, 2002), oligodendrocyte damage can also be observed. Meanwhile, MOG₁₋₁₂₅ produces a more T-cell and antibody mediated chronic EAE in Dark Agouti (DA) rats (T’Hart *et al.*, 2004), while in marmosets it causes axonal injury and

cortical demyelination (Pomeroy *et al.*, 2005).

EAE is therefore considered as one of the first and best-studied animal models for a human autoimmune disorder. Each model contributes towards understanding the role of one of more underlying components in MS (Gold *et al.*, 2006). And in that matter, two of the principal aspects defining the usefulness of EAE are i) in understanding the role of the immune system in CNS inflammation and ii) to act as an *in vivo* model system for the development and validation of potential MS therapies.

1.5 Aims of the project

The purpose of this study is to investigate the anti-proliferative capacity of 6TG through the queuine pathway and the ability of this pathway to inhibit lymphoproliferation *in vitro* and *in vivo*, an illustrated model to represent the same is shown in **Figure 1.7**. The objectives of the study are as follows:

1. Establish the experimental techniques necessary to examine the growth inhibitory effects of 6TG *via* the queuine tRNA pathway by means of transgenic animals and primary T cell assays *in vitro*.
2. Examine the efficacy of the queuine-tRNA pathway in treating experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis.
3. Characterise the physiological and immunological effect on mice of 6TG working through the queuine-tRNA pathway.

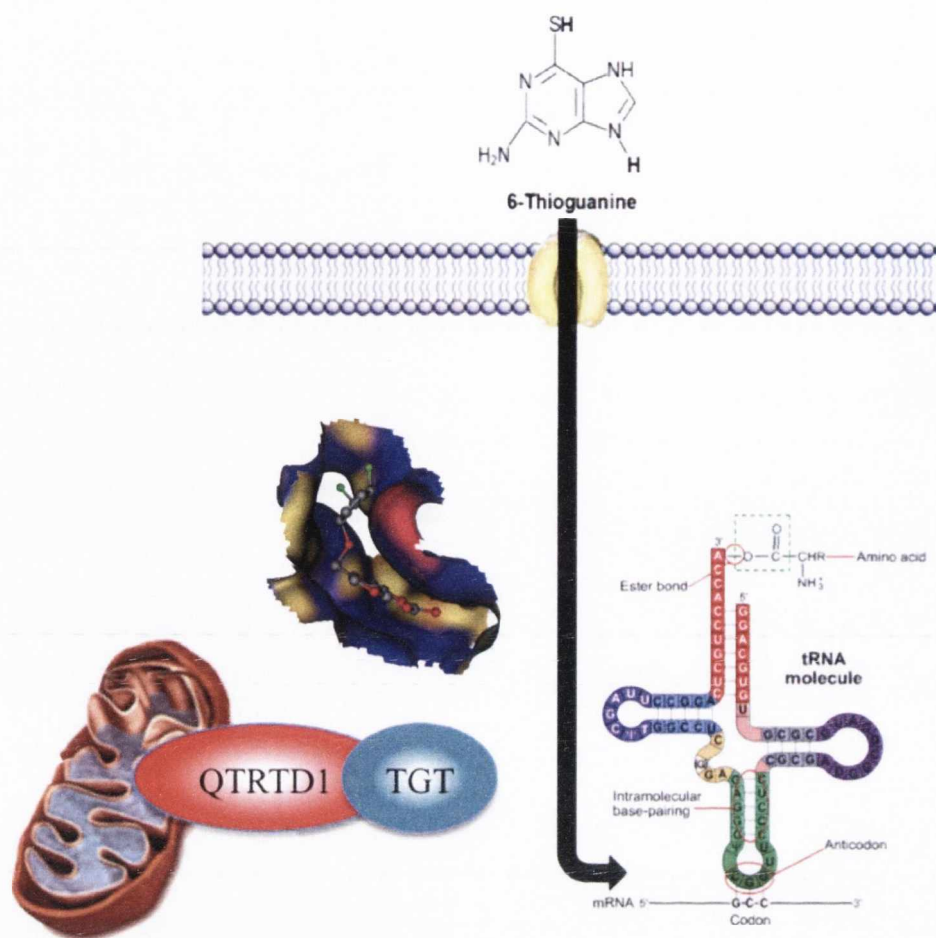


Figure 1.7: Exploring the therapeutic potential of the queuine-tRNA pathway
 The aim of this study was to examine if the queuine-tRNA pathway could be exploited as a therapeutic strategy for the treatment of lymphoproliferative disorders. 6TG, a queuine analogue, was specifically directed through the queuine-tRNA pathway (TGT and QTRTD1) by means of genetically modified primary T cells and transgenic mouse lines, thereby avoiding the likely genotoxic effects of DNA incorporation.

Chapter 2: Materials and Methods

All plastic ware was bought from Corning (UK) and BD Falcon (UK). The samples were centrifuged in the Eppendorf 5415R bench top centrifuge purchased from Eppendorf (Ireland).

2.1 Genotyping using genomic DNA and cDNA

2.1.1 Isolation of genomic DNA from mouse ear punches

Whole genomic DNA was isolated from ear punches taken from 21 day old mice. Tissue samples were transferred to a 1.5 ml tube and digested immediately or stored at -20 °C. To each ear punch 504 µl of digestion buffer, which was composed of 450 µl of 1 X SSC (20 mM Tris-HCl, pH 7.5, 15 mM trisodium citrate, 150 mM NaCl and 1 mM EDTA), 50 µl of 10 % SDS and 4 µl of Proteinase K was added and the tube incubated at 55 °C, with gentle agitation, overnight. The next day 500 µl of a 50:50 mixture of phenol:chloroform was added and the tubes mixed by inversion for 5 minutes. The tube was centrifuged at 16,000 x g for 5 minutes and upper aqueous layer removed and transferred to a new 1.5 ml tube. Then 500 µl of isopropanol was added to the tube and the DNA precipitated by inversion of the tube until the DNA fibres became visible. The DNA was then pelleted by centrifugation at 5,900 x g for 30 seconds and the pellet washed with 70% ethanol and resuspended in 100 µl of water. The tube, with the lid open, was incubated at 65 °C to allow any excess ethanol to evaporate. The genomic DNA was stored at -20 °C.

2.1.2 Isolation of total RNA from tail cut samples

Mouse tail cut samples were placed in eppendorfs containing 500 µl of RNAlater solution (Ambion, applied Biosystems, UK) and placed at 4°C overnight. The following day, the QIAgen RNeasy kit (QIAgen, UK) followed according to the manufacturer's protocol for total cell RNA isolation. The protocol for 'Purification of total RNA from animal tissue using spin technology' was followed according to the manufacturer's instructions. The additional 'Optional On-column DNase digestion with RNase-Free DNase set' protocol steps were also followed to ensure removal of any residual DNA material from samples. The final RNA was eluted in 50 µl of RNase-free water and stored at -70°C till further use.

2.1.3 Reverse Transcription (using random primers)

A 2 µl volume of each sample was applied to the NanoDrop1000 (Thermo Scientific, USA) to determine RNA concentration. The reading was obtained in (ng/µl). The following RTPCR kit (Applied Biosystems, UK) protocol was used: a total of 250 ng of RNA was added to 1 µl of 200 ng/µl Random primers and 4 µl Takara dNTP mix (2.5 mM of each nucleotide). RNase free water was added to bring the total volume to 13 µl. The tubes were then placed on a heating block at 65°C for 5 minutes. The tubes were then immediately placed on ice for 2 minutes and given a quick spin to collect any condensation. To each sample, 4 µl 5 x First strand buffer, 1 µl 0.1M DTT, 1 µl SuperInasin and 1 µl Superscript III RT was added. The total sample volume of 20 µl was mixed and incubated at 25°C for 5 minutes. Following this, the samples were placed on the heating block at 50°C for an hour. The samples were then incubated for 15 minutes at 70°C to inactivate the reverse transcriptase enzyme. 1 µl of RNase A (QIAGEN, Ireland) was added to each sample which were then incubated at 37°C for 10 minutes to remove any remaining RNA from the sample. To purify the cDNA, each sample was run through the QIAGEN Nucleotide removal kit (QIAGEN, UK). The protocol was followed according to manufacturer's instructions. The final cDNA was eluted in 30 µl of RNase free water and stored at -20°C till further use.

2.1.4 Reverse Transcription (using gene specific primers)

A 2 µl volume of each sample was applied to the NanoDrop1000 to determine RNA concentration. The reading was obtained in (ng/µl). The following RTPCR protocol was used: To 1 µl of total RNA, 2 pmol/µl of gene specific primers, 4 µl Takara dNTP mix (2.5 mM of each nucleotide) were added. The total volume was brought up to 13 µl by adding RNase free water. The mixture was incubated on a heat block at 65°C for 5 minutes and then immediately placed on ice for 2 mins. The samples were briefly centrifuged to collect any condensation. A volume of 1 µl of RNase inhibitor (Suprase In) along with 4 µl of 5x First Strand Buffer, 1 µl 0.1M DTT and 2 µl of Superscript III RT. The reaction was gently mixed and placed on heat block at 55°C for an hour. The enzyme was then inactivated by heating at 70°C for 15 minutes. Any residual RNA was removed by adding 1 µl RNase A. The cDNA was purified using the QIAquick PCR purification kit according to the manufacturer's guide. The cDNA was eluted in 32 µl RNase free water.

2.1.5 Polymerase Chain Reaction (PCR)

The GoTaq (Promega, USA) reaction was performed according to the manufacturer’s instructions. To a PCR tube, 1 µl of cDNA/DNA sample was added along with 5 µl of 5 x Green GoTaq Flexi Buffer, 2 µl MgCl₂ 2.5 mM solution, 4 µl Takara dNTP 2.5 mM mix, 1 µl Forward and Reverse primer- each at 10 (pmol/µl) (Eurofins MWG Operons, Germany), 0.25 µl GoTaq DNA polymerase (5 units/ µl) and 10.75 µl MilliQ water. The reaction samples were placed in the Biometra T professional PCR bock (Analytic Jena, Germany). The PCR primer sequences are shown in **Table 2.1** and the conditions used are shown in **Table 2.2** below:

Primer name	Sequence
18s For	5’ – CGT CTG CCC TAT CAA CTT TC – 3’
18s Rev	5’ – TGC CTT CCT TGG ATG TGG – 3’
HPRT For	5’ – GTT ATT GGT GGA GAT GAT CTC TCA AC – 3’
HPRT Rev	5’ – AGT CTG GCC TGT ATC CAA CAC TTC G – 3’
TGT For	5’ – GAG GCC GGT GTG TGG ATT CGA TCT G – 3’
TGT Rev	5’ – CAG AGC ATT CTG GAT CTC CAC CG – 3’
GT β geo For	5’ – GAG GCC GGT GTG TGG ATT CGA TCT G – 3’
GT β geo For	5’ – TCT AGC CTC GAG GTC GAC GGT ATC G – 3’

Table 2.1: PCR primer sequences for 18S, HPRT, TGT and GT β geo amplification.

18s		35 cycles
Temperature	Time	
98	2 minutes	
98	10 seconds	
56	30 seconds	
72	30 seconds	
72	8 minutes	
4	∞	

HPRT		35 cycles
Temperature	Time	
98	2 minutes	
98	10 seconds	
60	30 seconds	
72	30 seconds	
72	8 minutes	
4	∞	

TGT		35 cycles
Temperature	Time	
98	5 minutes	
98	10 seconds	
68	30 seconds	
72	2 minutes	
72	8 minutes	
4	∞	

GT (β geo)		35 cycles
Temperature	Time	
98	5 minutes	
98	10 seconds	
68	30 seconds	
72	1 minutes	
72	8 minutes	
4	∞	

Table 2.2: PCR conditions for 18S, HPRT, TGT and GT β geo amplification.

2.1.6 DNA agarose gel

A 1.5% (w/v) agaorse gel was made in 1x TAE Buffer (0.04 M Tris, 1.1% (v/v0 acetic acid, 1 mM EDTA, pH 8.5, made up with MilliQ water). Ethidium Bromide added at a concentration of 20 μ g/ml. The gel was allowed to set for 20 mins. The gel was placed in an electrophoresis tank (BioRad, Ireland) and submerged in 1 x TAE buffer. The samples were loaded alongside DNA Wide Range marker (Sigma, Ireland). The gel was run at 20 milliamps for approximately an hour.

2.2 Quantitative PCR analysis

2.2.1 Isolation of Macrophages, B and T lymphocytes using the cell sorter

Spleens isolated from C57BL/6 mice were mashed through a 40 μ M cell strainer. The splenocyte cell suspension was diluted in sterile PBSA (500 mls PBS supplemented with 0.1% FBS) to a total volume of 50 mls. A 10 μ l aliquot was taken to determine the cell count using EBAO. The cell suspension was centrifuged at 300 x g for 5

minutes. The supernatant was discarded and the pellet was re-suspended in 2 mls pre-warmed Lysis Buffer. The mixture was incubated at 37°C for 10 minutes. The mixture was adjusted to 50 mls with PBSA. The cell suspension was centrifuged at 300 x g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 2 mls PBSA. To the cell suspension, a cocktail of desired cell surface marker antibodies (all purchased from BD Pharmingen, UK) was added: PE anti-mouse CD3 (T lymphocytes); CD19- FITC anti-mouse (B lymphocytes); Pe-Cy5 anti-mouse F4/80 (macrophages) and F_c block antibody (to prevent non-specific binding of the antibodies). The suspension was mixed and incubated at room temperature in the dark for 20 minutes. The total suspension volume was adjusted to 50 mls with PBSA. The cells were pelleted at 300 x g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 500 µl of fresh PBSA. The suspension was filtered using a 30 µm Partec filter (Cell Trics, UK) into a fresh FACS tube. The filter was washed with 500 µl of fresh PBSA to bring the total cell suspension to 1 ml. The cells were then sorted into the various populations using the Dako MoFlo cell sorter, Denmark) with the kind assistance of FACS technician, Mr. Barry Moran.

2.2.2 Isolation of total cell RNA from organs and cell sorted populations

All tissues were isolated from male C57BL/6 mice. Whole lungs, liver, spleen and kidney were briefly rinsed in fresh PBS to wash off any excess blood. The washed organs were dabbed on tissue before being cut into small pieces and placed in a 6-well plate with 2 mls RNAlater solution (Ambion, applied Biosystems, UK) overnight at 4°C. Mouse tail cut samples were placed in eppendorfs containing 500 µl of RNAlater solution and placed at 4°C overnight. The following day, total cell RNA was isolated from the organ tissues in RNAlater and the cell sorted Macrophages, B and T lymphocytes from spleen, using the QIAgen RNeasy kit (QIAgen, UK) according to the manufacturer's protocol for total cell RNA isolation.

2.2.3 Quantitative Polymerase Chain Reaction (QTPCR)

Each sample was prepared using the reagents provided in the SYBR green PCR kit (Stratagene, UK) according to the manufacturer's instructions: 5 µl 10 x SYBR PCR buffer, 6 µl 25 mM MgCl₂, 4µl dNTP (2.5 mM), 0.25 µl AmpliTaq Gold (5 U/µl), 0.5 µl AmpErase UNG (1U/µl), 10 µl primer (1 pmol/µl), 10 ng Template for each reaction sample. The samples were set-up in a 96 well reaction plate (MicroAmp Fast

Optical, applied systems, UK) with the 7500 Fast Real-Time PCR system (Applied Systems, UK). The QTPCR primer sequences are shown in **Table 2.3** and the conditions used are shown in **Table 2.4** below:

Primer name	Sequence
18s For	5' – CGT CTG CCC TAT CAA CTT TC – 3'
18s Rev	5' – TGC CTT CCT TGG ATG TGG – 3'
TGT For	5' – CCC TGA GTG TCC CTG TCC – 3'
TGT Rev	5' – CGG TTA GGT GGT GTA GTG C – 3'
QTRTD1 For	5' –TCG TTA ATG GCT GTC GTC – 3'
QTRTD1 For	5' –TAG GGC TGA GAG TGT GAG – 3'

Table 2.3: QTPCR program set-up for QTPCR of 18S, TGT and QTRTD1.

Temperature	Time	
95	10 minutes	} 50 cycles
95	15 seconds	
52	30 seconds	
72	1 minutes	
72	8 minutes	
4	∞	

Table 2.4: QTPCR conditions for QTPCR of 18S, TGT and QTRTD1.

2.2.4 Non-denaturing polyacrylamide gel

A 10 % non-denaturing acrylamide gel was made using 3.75 mls 40 % acrylamide/Bis 19:1 (Sigma, Ireland) and 1.5 mls 10 x TBE buffer (containing a final concentration of 900 mM Tris-Base, 900 mM Boric Acid, 20 mM EDTA) adjusted to a final volume of 15 mls with MiliQ water. To this solution, 25 µl of TEMED (Sigma, Ireland) and 25 µl of 25 % Ammonium Persulphate (APS- Sigma, Irelad) were added. The final solution was poured into the PAGE apparatus (BioRad, Ireland) with 0.75-1.5 mm spacers and comb, and allowed to set for about 30 minutes. The set gel was then placed in a gel tank and filled to the top with 1 x TBE. A 10 µl volume of each sample

was pipetted into the wells alongside with 8 µl of the Hyperladder V marker (Bioline, UK). The gel was run at 20 milliAmps for 40 minutes. The gel was stained with about 4 µl of Ethidium Bromide (Sigma, Ireland) (diluted in ultrapure water) and photographed under UV.

2.3 HPLC based HPRT activity assay

2.3.1 Murine facial vein bleed sample

Two drops of blood were collected from the facial vein bleed of 12 weeks (or older) C57BL/6 mice into an eppendorf containing ice cold PBS to prevent coagulation. The erythrocytes were separated from blood plasma by pelleting them down at 10 minutes for 200 x g. The pellet was re-suspended and washed twice in PBS and the final pellet was resuspended in 9 volumes of ultrapure water. The samples were stored in -70 freezer with 10% glycerol (Sigma, Ireland).

2.3.2 Determining protein concentration

Bradford assay was used to determine protein concentration. A 10 µl volume of the protein sample and 200 µl of 1 x Bradford Reagent (BioRad, Ireland) (diluted in ultrapure water) were aliquoted together in a 96 well plate and the absorbance was read on the Spectra max 340PC machine (Molecular devices, Cambridge Scientific, UK). Bovine serum albumin (BSA- Sigma, Ireland) was used for protein concentration standards which ranged between 50-500 µg/µl.

2.3.3 A radioactive HPLC technique to determine HPRT activity in mouse blood

To a total volume of 200 µl reaction mixture containing 55 mM Tris-HCl (pH 7.4), 5.5 mM MgCl₂, 1 mM 5-Phospho-D-Ribose 1-diphosphate pentasodium salt (PPRP- Sigma, Ireland) and 10 µM ³H-Hypoxanthine (Amersham, UK), 250 µg of sample blood protein was added. The reaction sample was incubated at 37°C for 4 hours and quenched by adding 40 µl of 1.5 M HClO₄. Denatured proteins were pelleted and discarded at 375 x g for 3 minutes. The supernatant was transferred into a fresh eppendorf. A volume of 22 µl of 3.5 M K₂CO₃ was added to neutralise the supernatant to pH 7.0. The cloudy precipitate was discarded by centrifuging at 375 x g for 5 minutes. The supernatant was applied to a 0.45 µm spin filter (Millipore,

Ireland). The samples were centrifuged at $3.3 \times g$ for 3 minutes. The flow-through was applied to a $0.22 \mu\text{m}$ spin filter (Millipore, Ireland) and centrifuged at $3.3 \times g$ for 3 minutes. The clear flow-through was applied to a Zorbax 300SB-C18 column (Agilent, Ireland) on the High Pressure Liquid Chromatography (HPLC- Shimadzu, Japan). The solvent delivery system consisted of a DGU 14A on-line degasser coupled to a LC-10AT quaternary pump. Column temperature was maintained using a CT0-10A column oven and on-line solvent preheater. Samples were injected with a SIL-10AD autoinjector maintained at ambient temperature. An RF-1-AXL scanning fluorescence detector was used. The eluant A was 0.1 M potassium phosphate containing 6 mM tetrabutylammonium phosphate, pH 5.5. Eluant B was HPLC grade Methanol (Sigma, Ireland). The gradient was set as follows: Isocratic phase at 7% Eluant B for 3 minutes, up to 30% Eluant B holding for 3 minutes and then back to 7% Eluant B for 6 minutes to restore the initial conditions. The total run time was 12 minutes and flow rate was 1 ml/min. The procedure was performed at room temperature and the absorbance was monitored at 260 and 280nm using an on-line photodiode array. All fractions were collected in vials containing 4 mls of scintillation fluid (Biosciences, UK). The vials were placed in the Tri-Carb 21000 TR (Packard, Canberra, UK) scintillation counter to quantify the radioactivity of the sample. The counts were recorded in dpm over 3 minutes.

2.4 Cell culture

2.4.1 Animals

Specific pathogen-free C57BL/6 wild type mice were purchased from Harlan[®], HPRT transgenic female mice generated from ES cell line HPRT: E.2.4.2.8 were purchased from Jackson Laboratories[®] and TGT transgenic animals generated from the ES: FHCRC-GT-S12-11A1 using ROSAFARY, were all housed and maintained under the regulations and guidelines of the Irish Department of Health and Children and with the approval of Trinity College Dublin BioResources Ethics Committee.

2.4.2 Primary murine splenocyte culture

Whole spleens isolated adult mice were extruded through a $40\mu\text{m}$ nylon mesh (Millipore, Ireland) using a 3 ml syringe plunger. The cells were washed in a total volume of 50 mls of sterile PBS (Sigma, Ireland) and centrifuged at $300 \times g$ for 5

minutes to discarded supernatant. The cell pellet was re-suspended in 20 mls PBS to determine total cell count.

2.4.3 Determining total live cell count using Ethidium Bromide/Acridine Orange (EB/AO)

Total cell density was calculated by staining a 10 µl aliquot of the total cell suspension with EBAO (Sigma, Ireland) in a 1:1 ratio. A 10 µl aliquot of this mixture was applied onto a disposable haemocytometer (Hycor medical, Stratagene, UK) and counted under a fluorescence microscope (Olympus BX51, Japan). EBAO is a combination of two individually used intercalating agents: ethidium bromide (EB) and acridine orange (AO) which stains nucleic acid content. In apoptotic cells the membrane is permeable to both EB and AO, which then bind DNA and give orange colour fluorescence. In non-apoptotic cells however, only the cell-permeable dye AO is able to penetrate the cell and stain the DNA content green. Therefore, their combination can distinguish between green/viable cells and orange/apoptotic cells. The total live cell count per ml was calculated using the following formula:

$$\text{Cell Number/ml (x10}^6\text{)} = \text{Live cell count} \times \text{Dilution factor} \times \text{Total volume of suspension}$$

2.4.4 Labelling cells with Carboxyfluorescein Succinimidyl Ester (CFSE)

From a stock concentration of 100 µM CFSE dye (Molecular Probes, UK) in PBS, a 100 µl aliquot was added to the 20 ml splenocyte suspension. Other titrated concentrations of CFSE were obtained by using aliquots of 10 - 75 µl to obtain final CFSE labeling concentration of 0.1 – 0.75 µM. The cells were incubated for labeling with CFSE for 15 minutes at 37°C. The reaction was quenched by adding 10% Horse serum (Sigma, Ireland) followed by incubation on ice for 10 minutes. The cells were centrifuged at 300 x g for 5 minutes and the supernatant discarded. The cell pellet was then washed with 20 mls of XVivo 15 medium (Biowhittaker, LONZA, UK). Finally, the pellet was re-suspended in XVivo Medium and seeded at a density of 1-1.5 x10⁶ cells per ml in a 24 well-plates. The plates were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.4.5 T lymphocyte specific stimulations

Cell culture 24 well plates were coated with 2 µg/ml of anti-CD3ε (BD Biosciences, UK) in warm PBS for 2 hours at 37°C. Prior to plating, the unbound anti-CD3ε antibodies in the wells were aspirated off. Post-plating, the cells were also stimulated with 5 ng/ml of soluble Interleukin-2 (IL-2) (RnD systems, UK) every 48 hours starting on Day 0.

2.4.6 B lymphocyte specific stimulations

B cell proliferation was induced by stimulating whole splenocyte cultures with 1 µg/ml of LPS (Sigma, Ireland) and 0.5 µg/ml of soluble αCD40 (BD Pharmingen, Ireland) per well on day 0.

2.4.7 Drug treatment with 6-Thioguanine

All 6TG (Sigma, Ireland) dilutions were made in XVivo 15 medium and stored at 4°C. The dilutions were pre-warmed at 37°C before cell-culture treatment.

2.4.8 Queuine co-administration

A 10 mM stock of chemically synthesized queuine (a generous gift from Dr. Susumu Nishimura, Japan) was prepared in ultrapure H₂O (Millipore, Ireland) and stored at 4°C. Dilutions of a range of queuine concentrations were made in pre-warmed XVivo 15 medium for administration to cells in culture.

2.4.9 Cell-culture pictures

Pictures of the cell-culture plates were taken using a camera (3.2 Megapixel Sony Ericsson, Ireland) just prior to harvest.

2.4.10 Antibody labelled cell analysis by Florescence associated cell sorter (FACS)

Cells were harvested on Day 5 post-plating (unless stated otherwise) for analysis by Cyan florescence associated cell sorter (Cyan FACS- Denmark). Sample from each well was de-clumped and collected into a fresh FACS tube using a transfer pipette. A 1 ml volume of FACS buffer (1 x PBS, 2 % FBS, 0.2 % Sodium Azide) was added to each tube and the mixture was gently vortexed. The cells were pelleted at 200 x g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in any

residual buffer. An aliquot of 1 μ l of Pe-Cy7 CD3 ϵ antibody (BD Pharmigen, UK) was added to all samples (except negative control) and incubated in the dark at room temperature for 20 minutes. The incubated reaction was quenched by adding 2 mls of FACS buffer and gently vortexing the tubes. The cells were pelleted at 200 x g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in 300 μ l FACS buffer. A final concentration of 1 μ g/ml of Propidium Iodide (Sigma, UK) diluted in XVivo, was added to the samples and they were incubated in the dark at room temperature for 1 hour. The samples were then directly analysed on the FACS.

2.5 EAE immunization

2.5.1 Animals

Specific pathogen-free animals that screened positive for HPRT transgenic female mice generated from ES cell line HPRT: E.2.4.2.8 purchased from Jackson Laboratories[®], TGT transgenic animals generated from the ES: FHCRC-GT-S12-11A1 using ROSAFARY and the *tgt::hpert* double knockouts generated from the single knockout strains were all housed and maintained under the regulations and guidelines of the Irish Department of Health and Children and with the approval of Trinity College Dublin BioResources Ethics Committee.

2.5.2 MOG-EAE Immunization

Chronic, monophasic EAE was induced in 8-10 week old female mice by subcutaneous (s.c.) injection of a 200 μ l emulsion containing 150 μ g 35-55 MOG peptide in Complete Freund's Adjuvant (CFA) (containing 5mg/ml of heat-inactivated *Mycobacterium tuberculosis*). On the same day, mice were administered 500 ng Pertussis Toxin intra-peritoneally (i.p.) and again 48 hours later on day 2.

2.5.3 EAE score

Day 2 post-immunization, disease severity was recorded every 24 hours in each animal according to the standard EAE scoring system shown in **Table 2.5** below:

EAE Score	Clinical symptoms
0	Normal
1	Limp tail
2	Wobbly gait
3	Hind limb weakness
4	Hind limb paralysis
5	Tetraparalysis/Moribund/Dead

Table 2.5: EAE Scoring of clinical symptoms.

2.5.4 Animals weights

The total body weight of each animal was recorded on a digital balance every 24 hour interval starting on Day0, day of immunization.

2.5.5 Motor tests: *Horizontal bar test:* A 26 cm long metal wire/bar, 0.2 cm in diameter was supported by two 19.5cm high solid metal columns at either end. Each mouse was held by its tail, allowed to grab the metallic wire with its front paws at the central point and rapidly released along with the start of the stopwatch. A time was assigned to each mouse depending on whether and when the mouse fell, held on for 60 seconds or reached either supporting column- also called the ‘safety platforms’. The maximum time allotted was 60 seconds per mouse. Padding was provided to cushion any fall.

2.5.6 6TG administration regime

One tablet of the commercially available *Lanvis*[®], containing 40 mgs of active 6TG, was dissolved in 15 mls of 7.5 mM sterile filtered NaOH in PBS, to obtain a stock concentration of 2.66 mg/ml of active 6TG. From this stock, an injection volume of about 100 µl (to obtain a final concentraion ≈ 0.0147 mg per gram of body weight ≈ 15 mg/Kg). This would bring the final *in vivo* concentration of about 1.31 mM) was administered intra-peritoneally. The injection regime consisted of 6 consecutive doses (each 24 hours apart) from day 9-14 post-immunization, followed by 3 alternative doses (each 48 hours apart) on days 16,18 and 20.

2.5.7 Video

All animal videos were taken using a Nikon Camera[®].

2.6 Immunological analysis of EAE animals

2.6.1 Organ isolation

Mice were anaesthetized with 40 µl pentobarbitol sodium (Euthanol) injected intraperitoneally. Upon loss of all reflexes, the mice were dissected and lung cavity exposed for easy access to the heart. The animals were then perfused intracardially with 20 mls of ice-cold PBS. Whole peripheral (spleen, inguinal lymph nodes and cervical lymph nodes) and central nervous organs (brain) were isolated into 5 ml cold aliquots of Hanks Balanced Salt Solution supplemented with 3% FCS (cHBSS).

2.6.2 Mononuclear cell (MNC) isolation from whole brain

Whole brain was extruded through a 70 µm cell strainer (Nunc), washed through with cHBSS, centrifuged at 170 x g for 10 minutes at 18°C. The pellet was enzymatically digested in 1mg/ml collagenase D and 10 µg/ml DNase I for 1 hour at 37°C. The digestion reaction was quenched and resuspended in 9 mls of cHBSS, this was underlayered with 5 mls 1.122 g/ml Percoll, overlaid with 9 ml 1.072 g/ml Percoll; 9ml 1.030 g/ml Percoll and 9ml of 1x PBS. Percoll gradients were centrifuged at 1250 x g for 45 minutes at 18°C. The Mononuclear cells (MNCs) were carefully removed from the 1.088:1.072 and 1.072:1.030 g/ml interfaces and washed twice with cHBSS.

2.6.3 *In vitro* re-stimulation and FACS analysis

Whole spleen, lymph nodes (cervical and inguinal) were extruded through a 70 µm cell strainer to obtain a single cell suspension. All samples, including the MNCs were re-stimulated for six hours at 37°C in the 5 % CO₂ cell culture incubator with 10 ng/ml PMA and 1 µg/ml ionomycin. Cytokine secretion was blocked with 5 µg/ml Brefeldin A. Cells were washed and incubated with 1 µg/ml Fcγ Block for 15 minutes to eliminate any non-specific binding. The cells were now stained with cell surface markers: anti CD3 (FITC), anti-CD4 (PeCy7), anti-CD8, anti-F4/80 (PeCy5), anti-Ly-6G (PeCy7), anti-δγ TCR (APC) and anti-CD11b (PE). These cells were then fixed

and permeabilized (Fix and Perm Cell Permeabilization Kit; Catlag) and stained intracellularly for IL-17a (PE) and IFN γ (PerCP-Cy5.5). Flow cytometric analysis was performed using CyAn ADP flow cytometer that was calibrated using BD compensation beads (BD Biosciences). Results were analysed and facs plots were obtained using Summit (DakoCytomation) or FlowJo (Stanford University) software.

2.6.4 Antigen re-call assay

Antigen and mitogen induced T cell proliferation was assessed by monitoring [3 H]-Thymidine incorporation into newly synthesized DNA. Single cell suspensions of spleen, lymph nodes (inguinal and cervical) were washed in cRPMI at 1200 rpm for 5 minutes and counted using EBOA. Cells were seeded at a final density of 1×10^6 /ml to U-bottomed 96 well plates containing medium alone (negative control), or 100 μ l of 2 μ g/ml, 10 μ g/ml or 50 μ g/ml MOG, 10 ng/ml PMA and 1 μ g/ml α CD3 (positive control). At 72 hours, the plates were centrifuged at 1000 RPM for 3 minutes to allow the cells to settle down. The supernatants were carefully removed and the cells were pulsed with 0.5 μ Ci of [3 H]-Thymidine per well (Amersham, GE Healthcare Life Sciences) in fresh warm cRPMI overnight at 37°C. After 12-16 hours, the cells were harvested onto glass fibre mats using an automated cell Harvester. The mats were left out to dry, sealed in plastic bags with 5mls scintillation fluid (BetaScint, Wallac) and fixed onto a plate reader for the micro- β -counter (Wallac). Each sample was assayed in triplicate and the results were expressed as mean counts per minute (CPM).

2.7 Histological analysis

2.7.1 Fresh-frozen brain tissue

Mice were anaesthetized with 40 μ l pentobarbitol sodium (Euthanol) injected intraperitoneally. Upon loss of all reflexes, the mice were dissected to expose the lung cavity and provide easy access to the heart. The animals were perfused intracardially with 20 mls of ice-cold Heparanized-PBS. After full clearing of lungs (pink to white colour) and liver tissues (dark red to light brown colour), the whole brain was carefully isolated, quickly dabbed on tissue, briefly coated in some OCT (TissueTek, UK) and then dropped into a small aluminium foil pod containing OCT. The pod was

lowered into a beaker containing about 5 mls of isopentane, and placed in suspension over liquid nitrogen vapour in a canister. After about 10 minutes, the pods were taken out, the extra aluminium at the open end is sealed and the labelled pods were stored in the -20 freezer until further use.

2.7.2 APS coated-slides

Glass slides (25 x 75x 1 mm SuperFrost, VWR, Ireland) were washed in 10% Decon (AGB Scientific, UK) for an hour and then washed under running cold tap water for 20 minutes. After 2 x 15 minute distilled water washes, the slides were left in an oven at 60°C overnight. The following day, the slides were coated for 15 seconds in 2% APS (3-Aminopropyltriethoxy-silane) (Sigma, Ireland) in methanol. Excess APS was rinsed off with 2 x 15 minute washed in 100% methanol followed by 3x 15 minute washes in distilled water. The slides were dried off in the 37°C oven overnight. Finally the slides were put back into the box till further use.

2.7.3 Cryostat

Each aluminium pod containing the frozen brain was quickly transferred from the freezer to the cryostat to prevent any thawing during handling. The cryostat was constantly maintained around -18°C during use. Using forceps, the OCT-embedded tissue block was exposed and fixed onto the 'chuck' (sample holder) using fresh OCT. The chuck was set into place and 10 µm thickness sections were cut on the cryostat (LEICA, UK). Upto four slices were picked up onto each APS-coated slides. The labelled slides were allowed to air dry overnight and the next day they were all transferred to a labelled box to store in the -20 freezer until immunoassaying.

2.7.4 Immunohistochemical analysis

To stain for FA11-CD68 (late endosomal marker, microglia) and KT3-CD3 (T cell surface marker), slides were oven dried at 37°C for 30 minutes, rehydrated in 4% cold ethanol for 10 minutes and quenched with 1% hydrogen peroxidase in absolute methanol. Excess methanol was quickly washed off in milli Q water prior to 2 x 10 minute washes in PBS. Each slide was then blocked with 40 µl of 10% normal rabbit serum for 45 minutes. The serum was tapped off on tissue and 30 µl of Rat Anti-mouse 1° antibody (AB Serotec, UK) as applied for 90 minutes. Excess antibody was

washed off with 3 x 10 minutes PBS-Tween washes followed by 2 x 10 PBS washes. The Rabbit Anti-rat 2° antibody (BD Pharmingen) was made up in 10% BSA (Sigma, Ireland) at 1:40 dilution for FA11-CD68 and 1:200 dilution for KT3-CD3. A total volume of 30 µl of the 2° antibody was applied for 45 minutes and then washed off with 3 x 10 minute PBS washes. Labelling was visualised using the avidin-biotin-peroxidase complex method (ABC, Thermo Scientific) method using 0.015% vol/vol hydrogen peroxide as substrate and diaminobenzidine as a chromagen (Sigma, Ireland). The slides were visualised under LEICA Light microscope and upon obtaining adequate signal:noise ratio, the reaction was quenched in PBS and counterstained with Haemotoxylin (Sigma, Ireland). The slides were washed, dewaxed in Histoclear I and II (ABC Scientific) and coverslips were mounted with DPX mountant (Sigma, Ireland). The mounted slides were left to dry in the fume hood overnight. The next day, any excess mountant solution was gently scaped off. All pictures were taken using the LEICA software programme Cell-1 (Leica, UK).

2.8 Statistical analysis

Statistical analyses were performed using the computer based mathematical package GraphPad Prism software. Statistical differences in mean values between experimental groups were determined by student's *t* test. P-values of 0.05 or less were considered significant.

Chapter 3: Results

Foundation work: Establishing the experimental system

3.1 Introduction

To investigate the potential of the queuine tRNA pathway for novel drug development, tailored experimental systems had to be established. This chapter focuses firstly on the generation of the transgenic animals (their breeding and screening) and secondly on the development of an *in vitro* T cell proliferation assay under queuine free conditions. These will be used in subsequent chapters to examine the efficacy of the queuine tRNA pathway in controlling lymphoproliferation both *in vitro* and *in vivo*.

3.1.1 Mouse transgenesis

Many early gene manipulation studies aimed at understanding cellular function were conducted in either non-eukaryotic organisms or transformed cell lines. The unsatisfactory ability of either system to mimic the physiological environment of a eukaryotic organism, caused much speculation as to the relevance of these to the human situation. Better models to study the same had to wait until the 1980's when scientists started to gain a handle on animal transgenics; the deliberate manipulation of an organism's genetic material through introduction or deletion of specific DNA sequences (Boverhof *et al.*, 2011). Early transgenic studies in mice exploited retroviral infection of either pre- or pro-implantation embryos (Rubenstein *et al.*, 1986) or the microinjection of DNA into embryos at the pronuclear stage leading to a random integration of exogenous DNA into the genome (Gordon *et al.*, 1980). These techniques were heavily reliant on the efficiency of transfection and were limited by the ability of the modified embryonic cells to colonise the germline. These limitations spurred the exploration of more powerful technologies to craft the genome in a predictable and permanent fashion.

One such technology, that continues to underpin transgenesis even today, was the discovery of pluripotent embryonic stem (ES) cells by Martin J. Evans (Evans and Kaufman, 1981). Kemler's group combined Evan's technique with transgenesis and demonstrated the ability of cultured ES cells to colonise somatic cells and enter the

germline lineage, after being genetically altered *in vitro* (Gossler *et al.*, 1986). These studies revolutionised the field of transgenics by providing a method to screen and select cell colonies carrying a predetermined genetic alteration (Hooper *et al.*, 1987). This work helped re-define the use of ES cells as ‘vehicles for transgenesis’ (Gossler *et al.*, 1986). Martin J. Evans in collaboration with Mario Capecchi and Oliver Smithies used this ES cell technique to perform a gene specific knockout in mouse ES cells *in vitro* (Thomas and Capecchi, 1987)—a work for which they were awarded the Nobel prize in Medicine in 2007. The discovery of this new transgenic tool revolutionised the field of biological and biomedical studies by expanding the wealth of information that could be derived from the whole organism, ranging from fundamental studies on cellular and organ function to a mechanistic evaluation of drug action in pre-clinical studies (Rosenberg and Bortner, 1999; Boverhof *et al.*, 2011).

3.1.2 *hprt* deficient mice

Having mastered the technique of deriving mice from ES cells in culture, subsequent efforts were directed towards identifying rapid and efficient ways to manipulate the genome. Capecchi’s *in vitro* work exploited homologous recombination as a means to modify DNA in ES cells. An antibiotic resistance cassette (conferring neomycin resistance) and a coding segment of the *hprt* gene were microinjected into ES cells. Homologous recombination between the experimentally applied DNA and its cognate chromosomal sequence resulted in the loss of HPRT enzymatic activity which was selected by screening for 6TG resistant colonies (Thomas and Capecchi, 1987). The same year, Hooper and co-workers published another method to obtain a knockout in the HPRT enzyme. They generated *hprt* deficient ES cell colonies by culturing the cells in 6TG containing medium. Over time, cells that spontaneously gained resistance to 6TG were found to carry a 55Kbp deletion in the *hprt* locus spanning the promoter region and the first two coding exons. They then transferred these 6TG resistant ES cells into a recipient mouse, generating mice that were genetically deficient in *hprt*—giving rise to the first ever and arguably, best studied *hprt* deficient mouse. It is this genetic strain, annotated as *Hprt1*^{b-m3} that was used for the studies in this thesis (Hooper *et al.*, 1987).

The *hprt* gene is carried on the X chromosome, necessitating that males are either wild type or knockout since they possess only one copy of the X chromosome. Females, on the other hand, can also be heterozygous as a result of having two copies of the X chromosome. Due to lack of any overt phenotype, the HPRT status of *Hprt1*^{b-m3} mice necessitates the screening of offspring by biochemical or genetic means. Methods that have been used in the past include PCR analysis of the HPRT gene, flow cytometric analysis of peripheral blood cells for cell cycle arrest after 6TG treatment (Kolman *et al.*, 2002) and the culture of tail cells in 6TG supplemented medium (Nohmi *et al.*, 1996). With the exception of PCR analysis, the techniques described would prove cumbersome if used to analyse an expanding cohort of animals, and a more robust and quantitative method to determine HPRT status was sought. Based on a technique by Jacomelli and colleagues (Jacomelli, Micheli *et al.* 2002), this chapter describes the design and optimisation of a new HPLC based quantitative assay to measure HPRT enzymatic activity from minute amounts of peripheral blood.

Loss of HPRT has the ability to bestow resistance to toxic guanine analogues such as 6TG by preventing their incorporation into DNA. This has been comprehensively examined in toxicological studies. HPRT deficient animals have a 23 fold higher resistance to 6TG than wild-type counterparts (Aubrecht *et al.*, 1997). Importantly, and of particular relevance to this thesis, disruption of HPRT activity does not completely abolish the growth inhibitory effects of 6TG. Administration of high concentrations of 6TG to *hprt* deficient HL-60 cells was shown to have growth inhibitory effects. Upon further investigation, 6TG was found to be incorporated into queueine deficient tRNA at the wobble position—suggesting the existence of an alternative pathway for 6TG mediated growth inhibitory effects (French *et al.*, 1991).

3.1.3 *tgt* deficient mice

Queueine modification of the wobble position of GUN tRNA is an irreversible event (Kersten *et al.*, 1988). Therefore, the characteristic loss of queueine in hyperproliferative cancer cells garnered much interest from researchers in the queueine field. Previous *in vitro* studies had identified 6TG as a substrate of the TGT enzymes (French *et al.*, 1991) and furthermore, that its presence has a negative effect on the proliferation of promyelocytic HL-60 cells—a phenomenon that could be reversed by

re-administration of queuine back to the culture medium. Other studies using radiolabelled dihydro-queuine showed that administration of 6TG to queuine deprived Chinese hamster embryo cells lead to its incorporation into the wobble anticodon position of queuine deficient tRNA; inferred by the lack of radiolabelled queuine from isolated tRNAs (Muralidhar *et al.*, 1988). However, the above effects have only been studied in cells or *in vitro*.

Since 6TG can directly substitute for queuine (although its incorporation is reversible) an additional consequence of treatment would be the loss of queuine in tRNA. Non-bacterial organisms rely exclusively on the uptake of the bacterially derived queuine to modify their Q-tRNA. Therefore, depletion of this component from the organism's diet has been used to examine the effect of queuosine deficiency. The short generational times of both *Drosophila melanogaster* and *Dictyostelium discoideum* allows rapid depletion of queuine (Ott *et al.*, 1982, Jacobson *et al.*, 1981). Surprisingly, although earlier *in vitro* studies on cells in culture linked queuine to development, proliferation and other metabolic changes (Langgut *et al.*, 1993), neither the fly nor slime mould displayed any adverse effects when depleted of queuine.

A major limitation to achieving queuine deficiency in higher eukaryotes is the fact that many sources of queuine exist i.e. the gut flora, diet and cellular turnover/salvage reactions (Gündüz and Katze, 1984). In a landmark study by Marks and Farkas they succeeded in depleting mice of queuine by maintaining the animals under germfree (axenic) conditions and feeding them a chemically defined liquid diet. Although this eliminated the possibility of queuine being absorbed from the diet or gut flora, the authors reported that the already modified Q-tRNAs was highly resilient to queuine depletion. As a result, it took a year for the all the Q-tRNAs to be fully depleted of queuine modified tRNA (Marks and Farkas, 1980). Curiously, and in agreement with the earlier studies on fly and slime mould, no obvious physiological effects were observed in these mice. However, later studies, in which the animals were depleted in both queuine and tyrosine resulted in drastic behavioural and pathological changes such as: squinting, lethargy, stiff limbs, laboured beathing and eventual death (Farkas, 1981).

Recent work in our lab (mentioned previously) discovered and characterised the eukaryotic enzymes responsible for queuine modification. However, due to technical limitations, an exact replication of Marks and Faras's work was not a feasible option. Therefore the other option was gene disruption. Studies involving the

use of disrupting mutations of the queuine pathway have only been looked at in bacterial systems. Induced *E.coli* mutant strains (JE7736) or those naturally harbouring disruptions to the queuine modification pathway (B105), have consistently been reported to perform poorly in mixed competitive growth experiments against wild type strains (Noguchi *et al.*, 1982; Dineshkumar *et al.*, 2002). With the identification of the eukaryotic genes encoding the queuine transglycosylase machinery, disrupting these genes became possible and a gene-trap TGT knockout mouse line was successfully generated and validated by other members of our laboratory (Rakovict *et al.*, 2011; Boland *et al.*, 2009).

3.2 Results

3.2.1 Developing and validating a quantitative screen for HPRT activity in mice

The medical relevance of the purine salvage pathway to Lesch-Nyhan disease in humans—where patients have an intractable urge to chew off their lips and fingers if not restrained—has led to the development of *in vitro* assays to screen for the enzyme activities. The HPRT enzyme catalyses the first step of the purine salvage pathway. In the case of Hypoxanthine (Hyp), HPRT appends a D-ribose-5-phosphate moiety on to the nitrogen atom at position 9 of the purine ring to generate inosine-5'-monophosphate (IMP). The structural difference between the two (Hyp and IMP) allows for their chromatographic separation by HPLC. To verify the feasibility of this separation technique, Hyp and IMP were purchased from a commercial supplier and applied to a C18 reverse-phase HPLC column. The traces in **Figure 3.1** represent the elution peaks obtained for each compound singularly, Hyp (**Figure 3.1a**) and IMP (**Figure 3.1b**) and in combination Hyp plus IMP (**Figure 3.1c**). The traces show that Hyp is eluted in the fraction at four minutes while IMP is eluted in the fraction at eight minutes. When the two compounds were applied together on the HPLC, the elution times were consistent with controls (**Figure 3.1c**).

In preparation for future studies commercially available *hprt* deficient heterozygous female mice ($Hprt1^{+/+} Hprt1^{b-m3}$) were purchased from Jackson Laboratories[®] and crossed with a wild-type male ($Hprt1^{+/+} / Y$), as shown in the schematic **Figure 3.2a**. Previous studies have resorted mainly to the use PCR to determine HPRT status at the gene level. However, lack of published primer sequences (Hooper *et al.*, 1987) or of a gene map describing the deleted region, made it difficult to screen for *hprt* without first mapping the allele. Therefore, after several failed attempts, the HPRT status was screened for at the messenger RNA level. The RTPCR products from total cellular RNA samples from tails 1-5 are shown in **Figure 3.2b**. Each sample was amplified for HPRT message using primers that produce a 198 bp amplicon as well as for the 18S ribosomal subunit (used as a control) to yield a 124 bp amplicon. All tail samples 1-5 show a bright band for the 18S ribosomal subunit at 198 bp, however only tail samples 2-4 show a bright band for HPRT (at 124 bp). This demonstrates that tail samples 2-4 contain mRNA transcript for the HPRT gene. Tail 1 shows no band for HPRT suggesting it is HPRT deficient whereas Tail 5 shows a

faint band for HPRT at 124 bp suggesting that it is likely to be heterozygous. Also, a faint band could suggest cross contamination between samples. Therefore, screening *via* RTPCR gave a qualitative result for HPRT gene expression (at the RNA level). However, this method was not capable of distinguishing between heterozygous and wild-type female mice—where only one allele carries the mutation.

To cross-check the accuracy of the RTPCR results and in the hope of developing a better assay, commercially available radiolabelled hypoxanthine was purchased to serve as a substrate for HPRT. 8-[³H] Hypoxanthine contains a tritium atom (³H) at the 8th position which conveniently remains unaffected by the activity of the HPRT enzyme. Furthermore, upon conversion of Hyp to IMP, this group at position 8 will be retained in the reaction product compound IMP allowing an observable difference between the fractions that contain the radiolabelled entity. To assay the HPRT activity *in vitro*, 8-[³H] Hypoxanthine was incubated with just 50 µg of serum protein. The reaction was then quenched, neutralised and filtered prior to its application to a C18 HPLC column. The column was washed with phosphate buffer and the compounds were eluted in methanol. The fractions were collected every minute and the radioactivity was quantified by scintillation counting. The quantitative measure for radioactivity was then plotted as a trace, shown in **Figure 3.2c**. This graph shows the readout for two samples shown in two different colours (blue and red). The sample in blue shows a peak at the four minute fraction, representing the elution of radiolabelled Hyp (Hyp, **Figure 3.2c**). The unique peak at for Hyp suggests a lack of conversion to IMP, caused by an absence of HPRT enzymatic activity. However, the trace for the second sample in blue shows two visible peaks: a small peak at four minutes followed by a large peak at eight minutes. This trace indicates that though some of the radiolabelled hypoxanthine remains unconverted, however most of the compound has been converted to IMP by an active HPRT enzyme (IMP, **Figure 3.2c**). Therefore, it can be deduced that the sample in blue represents the HPRT knockout animal while the sample in red represents an HPRT wild type animal. This graph validates the newly designed HPLC technique.

3.2.2 TGT and Qv1 are expressed in abundant levels in adult murine tissues

The eukaryotic queuine tRNA ribosyltransferase enzyme consists of TGT and QTRTD1. While the TGT protein is a product of a single transcript, QTRTD1 exists as three different spliced forms: Qv0 (551 bp), Qv1 (757 bp) and Qv2 (1.6 kb)

(Boland *et al.*, 2009), shown in **Figure 3.3a**. Therefore, gene specific primers were designed to amplify the full-length transcript of TGT and all three spliced forms of QTRTD1. The RTPCR was performed on total cellular RNA from adult murine kidney, liver, lung and spleen. Results show a distinct band at 757 bp, indicating the presence of only the Qv1 variant in adult mice, shown in **Figure 3.3b**.

Using the gene specific primers, a QTPCR assay was set up to determine the relative mRNA expression levels of TGT and Qv1 in liver, lung, kidney, spleen and immune B and T cell populations that were isolated from spleen by FACS sorting. Primers that were specific for the ribosomal 18S subunit were used as control. The primer specificity for all three products was checked by gel electrophoresis, shown in **Figure 3.3c**. The distinct bands at 179 bp (Qv1), 98 bp (TGT) and 124 bp (18S) correspond to the expected product size and indicate a high level of specificity during the amplification process. The corresponding QTPCR amplification plot, shown in **Figure 3.3d** shows three sets of curves, which correspond to amplification runs for the 18S (left hand side of plot), TGT (middle of plot) and Qv1 (right hand side of the plot). Each set of amplicons (18S, TGT or Qv1) consists of triplicate curves corresponding to the seven samples assayed: liver, lung, kidney, spleen and FACS sorted immune B and T cell populations from spleen. A qualitative observation can be made by visual inspection of the amplification curves for each of the three transcripts. The most abundant message would be expected to reach saturation levels first, as seen for 18S transcript at cycle 27. The mRNA expression for TGT and Qv1 is not as high as that for 18S as their amplification curves reach saturation only by cycle 30 and cycle 45, respectively. The plot shows that all transcripts are expressed in the tissues and cells analysed.

In order to quantitatively gauge the results, the value obtained for the TGT and QTRTD1 amplification was calculated relative to the value of 18S, which is considered to be a suitable control even across different tissue types. The resulting figure was then plotted against that of spleen, which was normalised to 1 as shown in **Figure 3.3e** for Qv1 levels and **Figure 3.3f** for TGT levels. From the two graphs, it can be deduced that both TGT and Qv1 are present at equal or greater levels in B and T cells when compared to the levels found in other cells. Also, Qv1 expression appears to be relatively higher in T and B cells than that of solid organs.

3.2.3 Characterization of *tgt* deficient mice

The *tgt* deficient mice were generated by using the gene-trap technique. The ES cell line FHCRC-GT-S12-11A1 contains an integrated copy of the ROSAFARY (Reverse Orientation Splice Aceptor For ARRaY) vector in the *Qtrtl* gene encoding the TGT subunit. Using nested PCR, the cassette was found to be inserted in exon 3 of the *Qtrtl* gene on chromosome 9. To obtain *Qtrtl*^{Gt/Gt} homozygous mice, a heterozygous *Qtrtl*^{Gt/+} pair were intercrossed as shown in the breeding schematic in **Figure 3.4a**. At each generation, genomic DNA isolated from ear punches from mice of unknown genotype was amplified by PCR to determine the *Qtrtl* status, as shown in **Figure 3.4b**. Samples that gave a unique band at 450 bp represent wild-type *Qtrtl*^{+/+} animals. Samples giving two bands at 751 bp (for Gt) and 450 bp (TGT) represent heterozygous *Qtrtl*^{Gt/+} animals carrying the genetrapp cassette in only one of the alleles. Finally, samples showing a unique band at 750 bp represent *Qtrtl*^{Gt/Gt} animals, homozygous for the *Qtrtl* gene. Other members of our group who worked on these mice have reported that the *tgt* deficiency did not influence fertility, survival/viability or sex bias (Rakovict *et al.*, 2011).

3.2.4 Characterisation of *hprt:tgt* double knockout mice

Having validated screening protocols for the *hprt* and *tgt* status of mice, the female *Hprt*^{b-m3} was mated to a *Qtrtl*^{+/Gt} male mouse, as shown in the breeding schematic in **Figure 3.5**. From each animal, serum proteins from blood and DNA from ear punches were extracted to assay for HPRT *in vitro* and *tgt* status by PCR, respectively. Note the offspring comprised of only one eighth of pups born in agreement with Mendelian principles. The animals that screened positively deficient for *hprt* and *tgt* did not manifest any infertility, viability issues or gender bias.

3.2.5 Optimisation of serum-free growth conditions for splenocyte cultures

Published studies exploring the role of queuine modification, have been conducted on either non-eukaryotic systems or in eukaryotic transformed cell-lines. In this study, being the first to investigate the queuine tRNA pathway in a primary cell system, it was decided to focus on primary T cells due to their ability to mimic the rapidly proliferating feature of cell-line systems without the complications of a transformed phenotype/genotype that could alter the cell's physiological response.

Traditionally, T cell *in vitro* proliferation assays use RPMI medium supplemented with 10% FBS. However this was unsuitable for our investigations due to the presence of queuine, estimated to occur at concentrations of ~300 nM in bovine serum (Elliott *et al.*, 1984). Therefore, it was necessary to develop new serum-free cell culture conditions for the maintenance of primary splenocytes *in vitro*. T cell activation may be mimicked by coating the cell culture plate with CD3 ϵ antibody, which binds to the TCR and transmits the primary activation signal. This is then followed by co stimulatory signals by non T cell populations, such as APCs (Kang *et al.*, 1992). Unfortunately, non T cell populations require serum to sustain their survival. Therefore, there was a need for alternative growth factors to be added to the serum-free medium such as IL-2 and for the efficiency of the overall protocol to be compared with that of previously established protocols (Kang *et al.*, 1992).

The lymphocyte growth factor IL-2 is well established as a potent enhancer of T cell responses *in vitro* (Antony *et al.*, 2006). Hence, whole splenocyte cultures were grown in XVivo serum-free medium supplemented with IL-2 (XVivo/IL-2) and plated on CD3 ϵ coated cell culture plates. T cell proliferation was compared to cells plated in RPMI supplemented with 10% FBS and IL-2, shown in **Figure 3.6**. The FACS plots show that in absence of IL-2, RPMI supplemented with 10% FBS has a greater ability to promote T cell proliferation compared to Xvivo. However, when both media are supplemented with IL-2, the proliferation observed in XVivo serum free medium is equivalent to that observed in RPMI supplemented with FBS. The proliferation plots from this study indicate that XVivo medium supplemented with IL-2 (XVivo/IL-2) is efficient at promoting T cell survival and proliferation.

Techniques to quantify T cell proliferation include the direct counting of the cells under a light microscope, quantification over time of DNA incorporation of [3 H]-thymidine and bromouridine and the counting of specific cell populations by FACS after ligation of a fluorescently tagged antibody to an extracellular or intracellular molecule. More recently the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) has been employed as a means to quantify cell proliferation. CFSE is a cellular membrane dye that is equally divided between the daughter cells at every population doubling (Fulcher and Wong, 1999). When observed on the FACS machine, cells that have passed through successive replicative cycles show decreasing dye intensity and can be separated into distinct populations that have replicated once, twice, three times and so on (Fulcher and Wong, 1999). Hence, identifying the

optimal CFSE concentration to stain the cells was crucial to ensure that the proliferative populations could be visualised because at low concentrations the dye would dissipate very quickly and at very high concentrations it could mask certain populations and/or be toxic to cells. CFSE concentrations ranging from 0.1 μM to 1 μM were used to stain splenocyte cultures and the CD3^+ T cell population was analysed by FACS, shown in **Figure 3.7a**. The proliferation plots show that CFSE dye at 1 μM concentration is optimal for cell staining because the proliferative populations were clearly visible while cell viability remained unaffected with values in the range of 99% survival, shown in **Figure 3.7b**.

The final parameter to be optimised was the harvest point. This was an important aspect of the study as it determines the amount of proliferation observed upon harvest and has an influence on the intensity of CFSE dye remaining for FACS analysis. Whole splenocyte cultures were stained with 1 μM CFSE and plated in XVivo/IL-2 medium and harvested at three different time points: Day 3, 5 or 7 **Figure 3.8a**. The proliferation plots show that on Day 3 the cells did not have adequate time to proliferate sufficiently. The CFSE dye for this plot is concentrated near the right side of the FACS plot and the proliferation recorded was 69.61%. By Day 7, the majority of CFSE stained cells were found to shift to left side of the FACS plot indicating an increased number of proliferating cells with a low fluorescent intensity of CFSE dye. Though the proliferation recorded was high at 96.46%, the significant shift in the CFSE stain intensity indicates that the staining capacity of the dye is nearing the lower limits of detection. The proliferation on Day 5 however conveniently fell between the values obtained on Day 3 and 7. The CFSE intensity is evenly spread out between the high intensity and low intensity end of the side-scatter spectrum while clearly retaining the proliferating populations. The proliferation recorded on Day 5 was of the range 91.47%, and though not as high as that recorded on Day 7, it provided a proliferation profile that is more informative and robust.

In conclusion, the above assays provide an optimised method to observe primary T cell proliferation in splenocyte cultures grown under serum free conditions. The conditions are summarised thus, i) freshly isolated splenocytes are pre-stained with a 1 μM concentration of CFSE dye, ii) the cells are plated in XVivo/IL-2 medium on $\text{CD3}\epsilon$ coated cell culture plates and iii) the cells are harvested on Day 5 for FACS analysis.

3.3 Discussion

In line with the competitive growth experiments in bacteria, *in vitro* studies by Helga Kersten's group highlighted the effect of queuine on the proliferation of mammalian cells. Kersten's group used chemically synthesized queuine and reported that addition of queuine *in vitro* encouraged proliferation of the human cervical carcinoma derived transformed cell line, HeLa S3 cells and primary non-transformed cells (Langgut *et al.*, 1990; Langgut *et al.*, 1993). However, later extensive studies by the same group showed that other transformed cell lines showed opposite effects, i.e. while in some cell lines queuine promoted growth, in others it inhibited growth—a phenomenon dependent on the type of oncogenes expressed and the cellular metabolic state according to the authors (Langgut *et al.*, 1994).

T cells, a primary cell with proliferative capacity comparable to transformed cells *in vitro* are arguably among the best studied primary cells in culture. A number of protocols exist for the *in vitro* proliferation of primary T cells and have variously used Roswell Park Memorial Institute (RPMI) RPMI medium or XVivo medium supplemented with either 10% FBS or human serum. Importantly, this system also efficiently sustains the survival of non-T cell populations, through ill-defined serum factors, and these cells in turn provide full activatory signals to the T cells which are indispensable for their survival *in vitro* (Kang *et al.*, 1992). The objectives of my project necessitated the use of medium that does not contain the dietary molecule queuine and since queuine is found in bovine and human sera, along with numerous other undefined components (Carlens *et al.*, 2000), it was not possible to use previously established protocols for T cell culture. Methods for the proliferation of T cells under serum free conditions were therefore developed and optimised. The protocol that was eventually employed required the optimisation of three key parameters, i) the choice of growth medium and supplements required to sustain/activate T cell expansion, ii) the choice of a proliferative assay to quantify cell expansion and iii) the identification of the most appropriate time point at which to harvest cells, so that the effect of 6TG on T cell proliferation could be evaluated.

In addition to plating conditions, which are crucial for the survival of primary T cells *in vitro*, it is necessary to ensure full activation of the T cells, allowing their clonal expansion. In conditions that are depleted of serum—which, as explained earlier do not support the survival of numerous non-T cell populations—it is crucial to provide the T cells with alternative survival/activation signals. Previous studies showed that a key signal for T cell responses *in vitro* is provided by the lymphocyte growth factor IL-2 (Kang *et al.*, 1992).

CFSE is known to spontaneously and irreversibly bind to the cellular membrane and with each population doubling the dye is equally divided between the daughter cells (Fulcher and Wong, 1999). Since the CFSE dye responds to high-energy excitation (blue light at 450-500 nm) and emits light in the FITC channel (green spectrum, lower wavelength of 500-550 nm) its fluorescence can be exploited for cell quantification and proliferation by using FACS. The dye intensity is an indication of the number of proliferative cycles that the cells have gone through (Fulcher and Wong, 1999).

Having optimised a novel *in vitro* primary T cell system that creates queueine free conditions, the transgenic mouse strains that were generated served as a source of the primary T cells to conduct our studies. It is noteworthy that deficiencies in the HPRT enzyme are known to occur in humans, resulting in Lesh-Nyhan disease (LND), an inborn error of metabolism. As the gene encoding the HPRT enzyme is found on the X-chromosome, LND is an X-linked disorder, meaning that the disease occurs far more frequently in males due to the presence of only one X chromosome. LND is characterised by hyperuricemia, mental retardation and a compulsive self-injurious behaviour such as chewing of the fingers and lips, sometimes resulting in the complete loss of appendages if the patient is not restrained. Mouse models for HPRT deficiency have been developed by means of chemical mutagenesis and more recently through targeted gene mutation. Unlike HPRT deficient humans, studies have shown that HPRT knockout mice do not display any of the abnormal behavioural characteristics of LND, due to the operation of an alternative purine salvage capability (Bertelli *et al.*, 2006).

TGT deficient mice were generated in an attempt to reproduce the study on germ-free mice by Marks and Farkas. The TGT deficient mice did not display any abnormal activity under normal diet and non-germ-free conditions. The Q-tRNA status of the animals was assessed by liquid chromatography-mass spectrometry

(LCMS) of bulk tRNA from the liver of six-week old $Qtrt1^{+/+}$ (wild type), $Qtrt1^{Gt/+}$ (heterozygous) and $Qtrt1^{Gt/Gt}$ (homozygous) mice. As expected, the wild type animals contained Q-tRNA whereas none was detected in the homozygous mice. The heterozygous animals showed an interesting phenomenon, although the six-week animals showed undetectable levels of Q-tRNA, at sixteen weeks, appreciable amounts of Q-tRNA were detected (Rakovict *et al.*, 2011). The homozygous remained deficient in any Q-tRNA. This was an important aspect to be considered especially for the double knockout mice generated. These mice generated purely for this study are the very first of their kind. The lack of any recent studies looking at this pathway in the primary system never called for the set-up of such a mouse strain.

Similarly, the mice deficient for both enzyme activities, *tgt:hpert* double deficient mice, did not display any loss of fertility, viability or sex bias. Moreover, no abnormal behavioral manifestation was observed, in line with the study of Marks and Farkas. Although it took almost a year to successfully breed and obtain the mice of the desired double deficiency genotype, the animals of this strain were fully devoid of any HPRT or TGT activity even when housed in non-germ-free conditions and fed a normal diet as for the other housed animals; a much easier and reliable system than placing axenic animals on a special diet. The focus of this chapter has been purely on the experimental setup required to conduct *in vitro* and *in vivo* studies on the queuine tRNA pathway. It is noteworthy however that the systems established in this chapter are novel and have been designed with the unique interest of studying the queuine tRNA pathway in the simplest system possible to avoid the use of any inhibitors or any special housing/dietary conditions for the animals, all while being able to obtain a reliable physiological cell or organism response—a goal that shall unfold in the subsequent chapters.

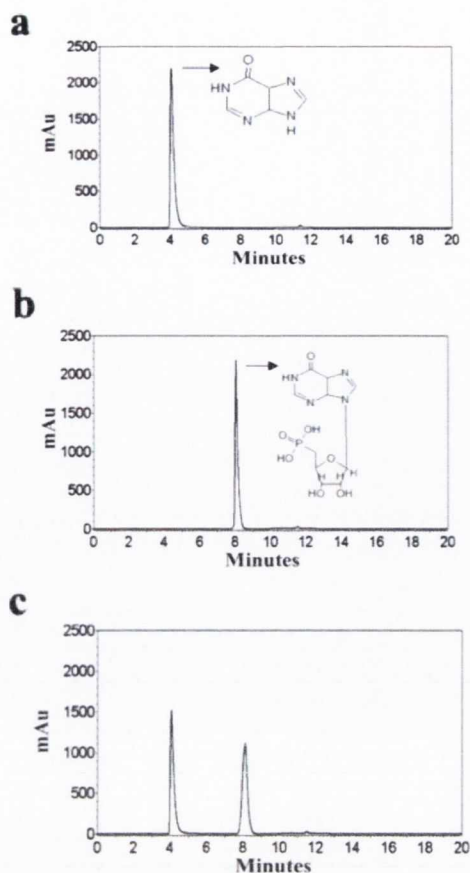


Figure 3.1: Separation of hypoxanthine and inosine 5' monophosphate by HPLC

(a) HPLC trace for 0.1 mM unlabelled hypoxanthine base

(b) 0.1 mM unlabelled inosine-5'-monophosphate and both unlabelled hypoxanthine and

(c) Inosine-5' monophosphate. The samples were filtered and applied to a C18 HPLC column with a run time of 20 minutes. The peaks were visualised by photodiode array at a wavelength of 251 nm.

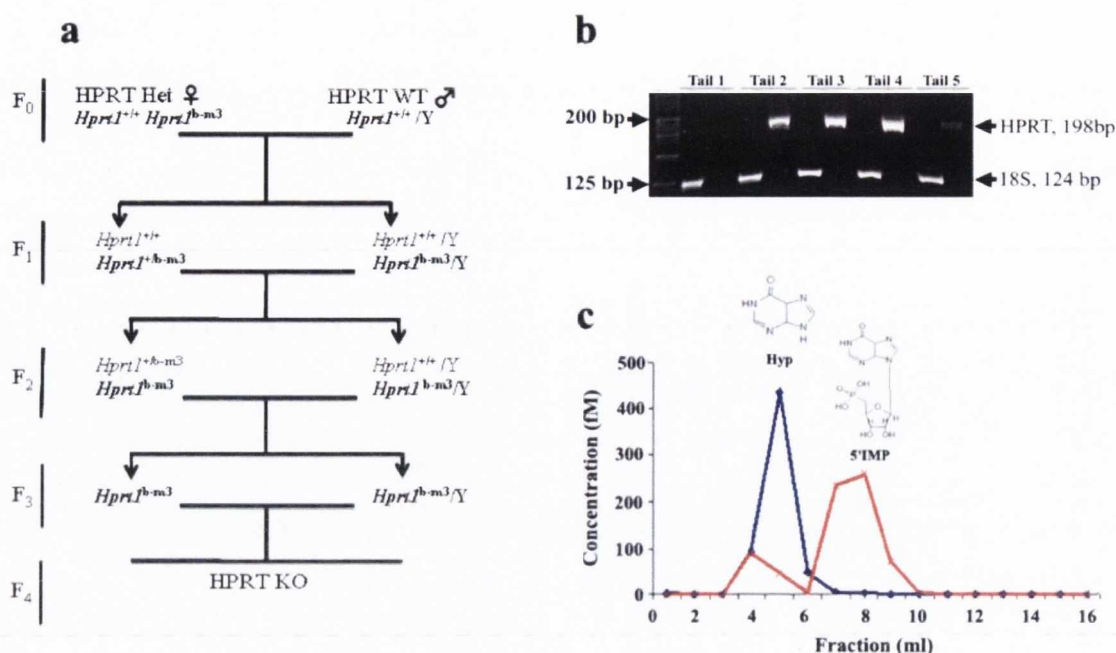


Figure 3.2: Breeding scheme and screening strategy for *Hprt1*^{b-m3} mice

(a) The *Hprt1*^{b-m3} allele is carried on the x-chromosome. Homozygous male and female mice were derived according to the scheme shown. Heterozygous (*Hprt1*^{+/b-m3}) HPRT females were mated to wild-type males to yield F₁ offspring according to the ratio 2:1:1 wild-type:heterozygous:knockout (W:H:K). Interbreeding of the siblings, heterozygous females with knockout males (carrying the single *Hprt1*^{b-m3} allele on the x-chromosome) generated W:H:K mice at a ratio of 1:1:2 in the F₂ generation. A final cross between knockout male and female mice yielded an F₃ generation of homozygous breeding pairs, which were interbred to generate sufficient numbers of knockout animals for experiments.

(b) Detection of the HPRT and Ribosomal 18S transcripts by RT-PCR amplification. Amplicons were resolved on a non-denaturing polyacrylamide gel alongside Hyperladder V DNA marker™ and visualised by ethidium bromide staining. The expected product sizes are indicated on the right hand side of the figure.

(c) HPLC based radioactive assay to determine HPRT activity *in vitro*. *In vitro* reactions using an aliquot of blood and radiolabelled hypoxanthine were filtered by spin columns and separated by HPLC. The radioactive counts (cpm) from each sample were converted to concentration of product (fM). The peak in fraction 5 comprises radiolabelled Hypoxanthine (Hyp), while the peak in fraction 8 represents the elution of radiolabelled Inosine-5'-Monophosphate (5'IMP).

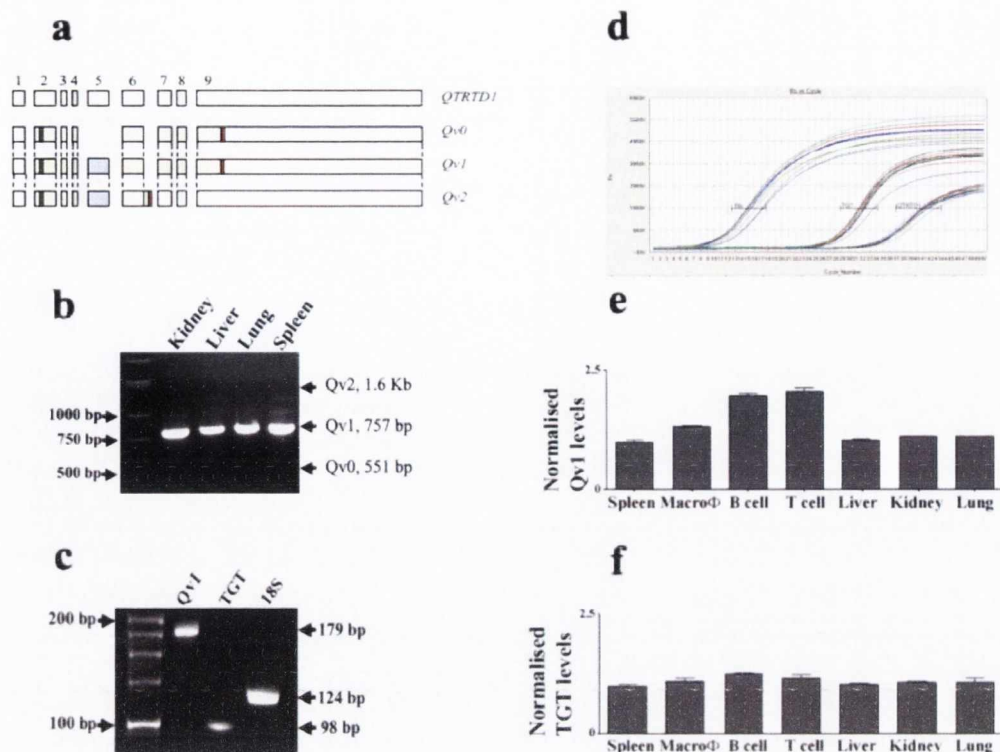


Figure 3.3: Qv1 and TGT expression in murine tissues and immune cells

(a) The genomic structure of the mouse *QTRTD1* allele. Alternative splicing of the *QTRTD1* gene leads to three mRNA transcripts which are illustrated underneath. The boxes, which are broadly proportional to their actual sequence length, are coloured to denote protein coding regions (yellow), the 5' and 3' untranslated regions (white), the start codon (green) and stop codon (red). Exon 5 (blue) is skipped in the Qv0 transcript whereas Exon 6 of Qv2 has a cryptic splice site leading to read-through into the downstream intron. The transcripts for Qv0 (2845 bases), Qv1 (3083 bases) and Qv2 (3935 bases) generate protein products of 345, 416 and 271 amino acids, respectively.

(b) Gene-specific RT-PCR amplification to detect *QTRTD1* splice variants. Total RNA was isolated from the tissues shown and amplified by RT-PCR. Products were resolved on an agarose gel alongside Wide-Range DNA markers™ and visualised using ethidium bromide staining. The size of the expected amplicon for each *QTRTD1* variant is shown on the right-hand side.

(c) Validation of the QRT-PCR amplification strategy of Qv1 and TGT. The amplicons for Qv1, TGT and 18S were resolved on a non-denaturing polyacrylamide gel alongside Hyperladder V marker™ and visualised using ethidium bromide staining. The expected product sizes are shown on right-hand side.

(d) Relative expression levels of Qv1 and TGT in mouse tissue and immune cells. Total RNA was extracted from mouse tissue (spleen, liver, kidney and lung) and immune cells (macrophages, B cells and T cells) and analysed by QT-PCR for the expression of (e) Qv1 and (f) TGT. Each sample was normalised to its respective ribosomal 18S control. The average value for sample triplicates was then plotted with respect to the expression level of spleen sample (=1) with standard deviation error bars.

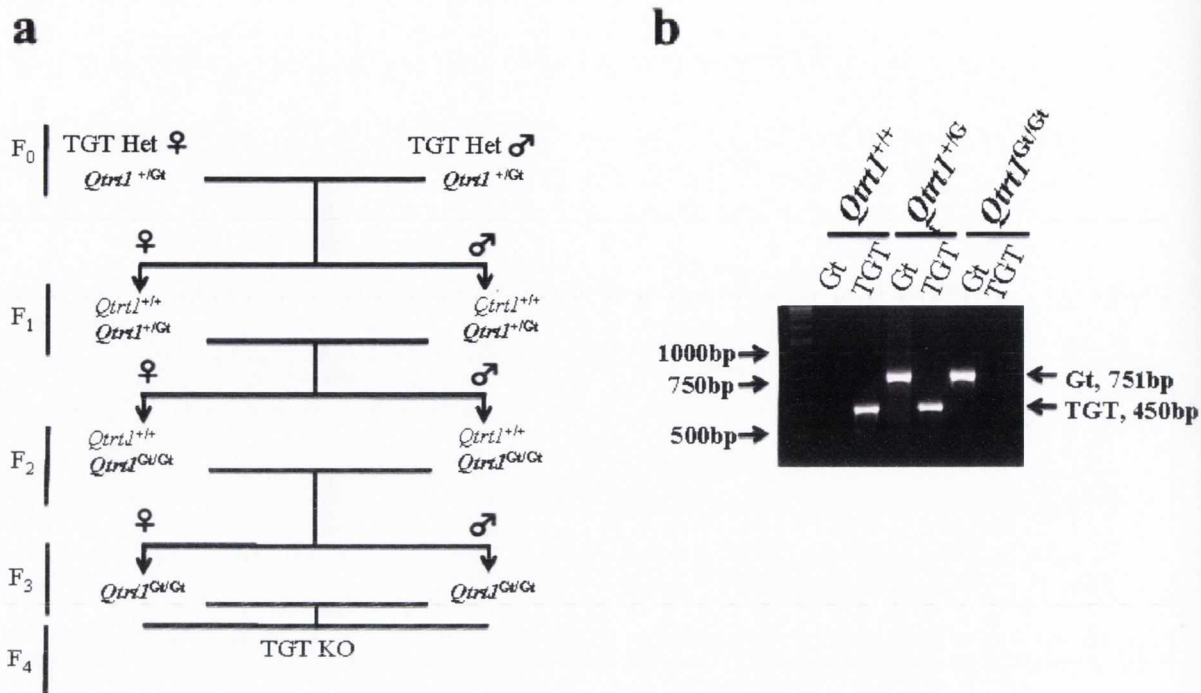


Figure 3.4: Breeding scheme and screening strategy for *Qtrt1*^{Gt/Gt} mice

(a) Breeding schematic to generate *Qtrt1*^{Gt/Gt} homozygous mice. Genetrap heterozygous mice were crossed to yield a 1:2:1 ratio of wild-type:heterozygous:homozygous F₁ offspring. Heterozygous mice were subsequently mated to yield mice at a ratio equivalent to the F₁ offspring. These F₂ animals acted as breeding stock for the production of F₃ and F₄ knockout mice used in experimental studies.

(b) PCR genotyping of TGT genetrap animals. Genomic DNA was isolated from ear punches of 21 day old mice and the *Qtrt1* gene and genetrap insert detected by PCR. Reaction products were resolved on an agarose gel alongside Widerange DNA markers™ and visualised with ethidium bromide staining. The expected product sizes are shown on the right hand side.

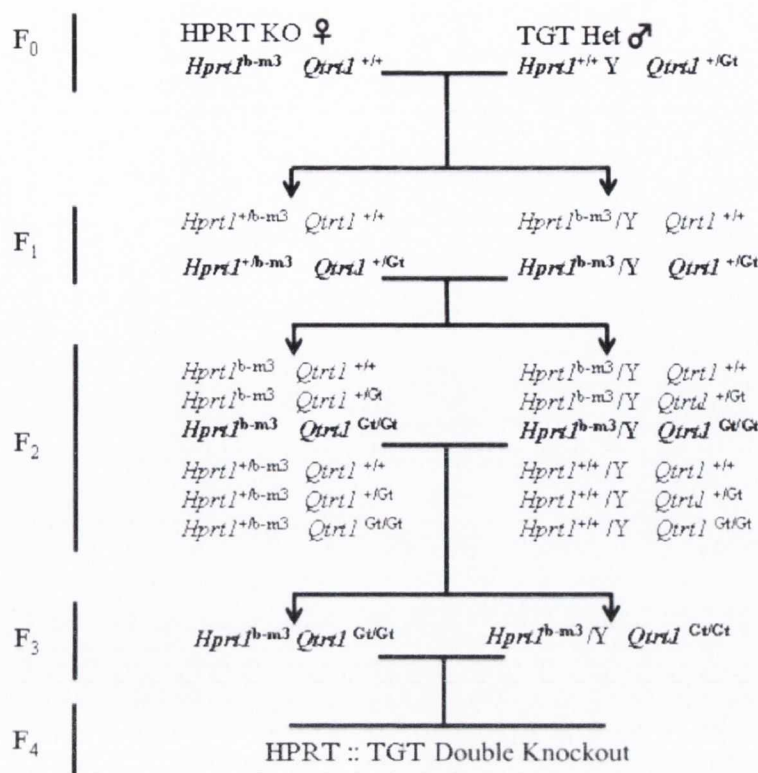


Figure 3.5: Breeding scheme to obtain *Hprt1*^{b-m3}::*Qtrt1*^{Gt/Gt} double knockout mice

A breeding pair of female *Hprt1*^{b-m3} mice were mated to *Qtrt1*^{+/Gt} male animals to establish a cohort of F₁ females that were heterozygous for both the *Hprt1*^{b-m3} and *Qtrt1*^{Gt} allele and male mice that are homozygous for the *Hprt1*^{b-m3} allele (due to the location of *Hprt1* on the x-chromosome) but heterozygous for the *Qtrt1*^{Gt} allele; shown in bold type. These animals were subsequently mated to establish double knockout male and female animals for the *Hprt1* and *Qtrt1* genes. Note these offspring comprised only one eighth of pups born in agreement with Mendelian principles. The mating pair between HPRT and TGT double knockouts, also shown in bold type, created double knockout offspring of the F₃ and F₄ generation, which were used for experimental studies.

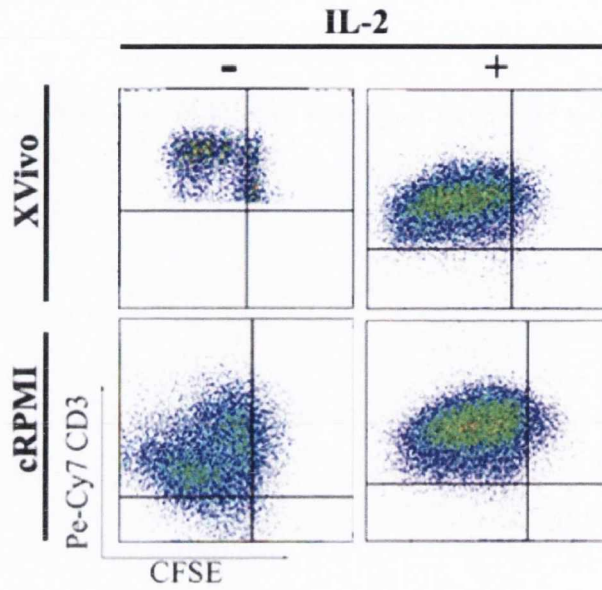


Figure 3.6: Effect of growth medium and IL-2 stimulation on T cell proliferation
 CFSE labelled wild-type spleenocytes were grown on plates coated with stimulatory anti-CD3 ϵ antibody. T cell proliferation was expanded in either XVivo serum-free medium (XVivo) or complete RPMI medium supplemented with 10% Foetal Bovine Serum (cRPMI). To evaluate the effect of IL-2 on proliferation, cultures were either left untreated (-) or treated with soluble IL-2 every 48 hours (+). The cells were harvested on Day 5 and labelled with Pe-Cy7 CD3 antibody for FACS analysis. The FACS plots shown are representative of the different samples assayed.

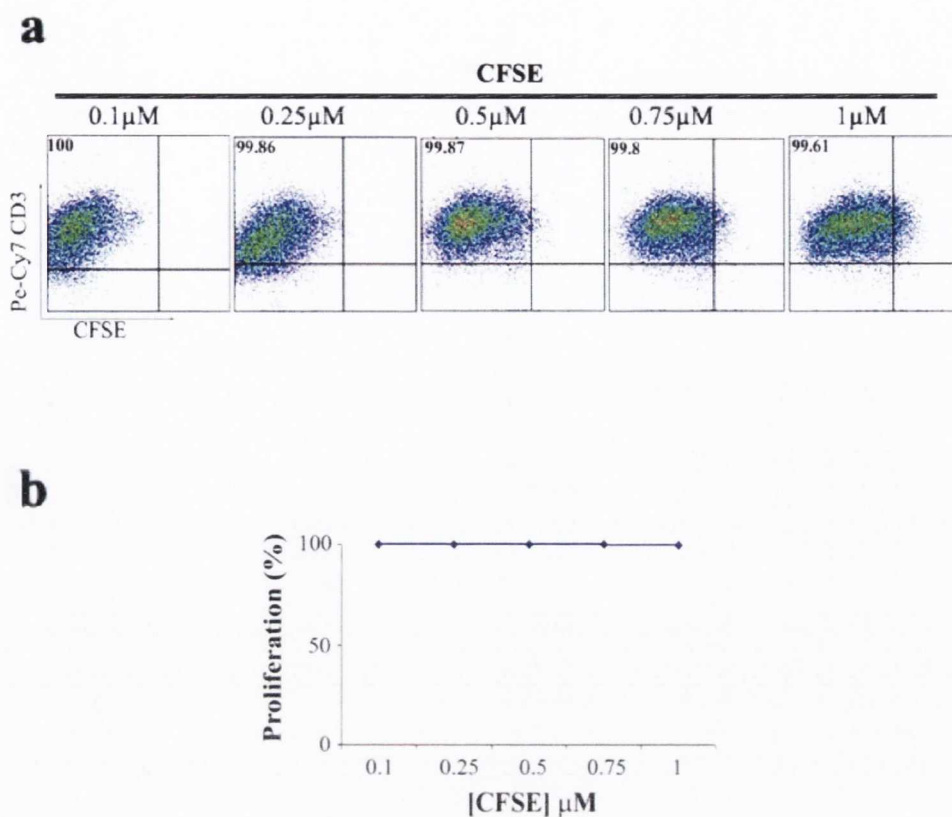


Figure 3.7: Evaluation of varying CFSE concentration on T cell proliferation

(a) Whole wild-type spleenocytes were labelled with a range of CFSE concentrations: 0.1 μM to 1 μM prior to plating on anti-CD3 ϵ antibody coated dishes in Xvivo/IL-2 medium. The cells were harvested on Day 5 and labelled with Pe-Cy7 CD3 antibody for FACS analysis.

(b) The percentage proliferation recorded for each CFSE concentration relative to CFSE control sample, which was left unstimulated with IL-2, is represented in the graph.

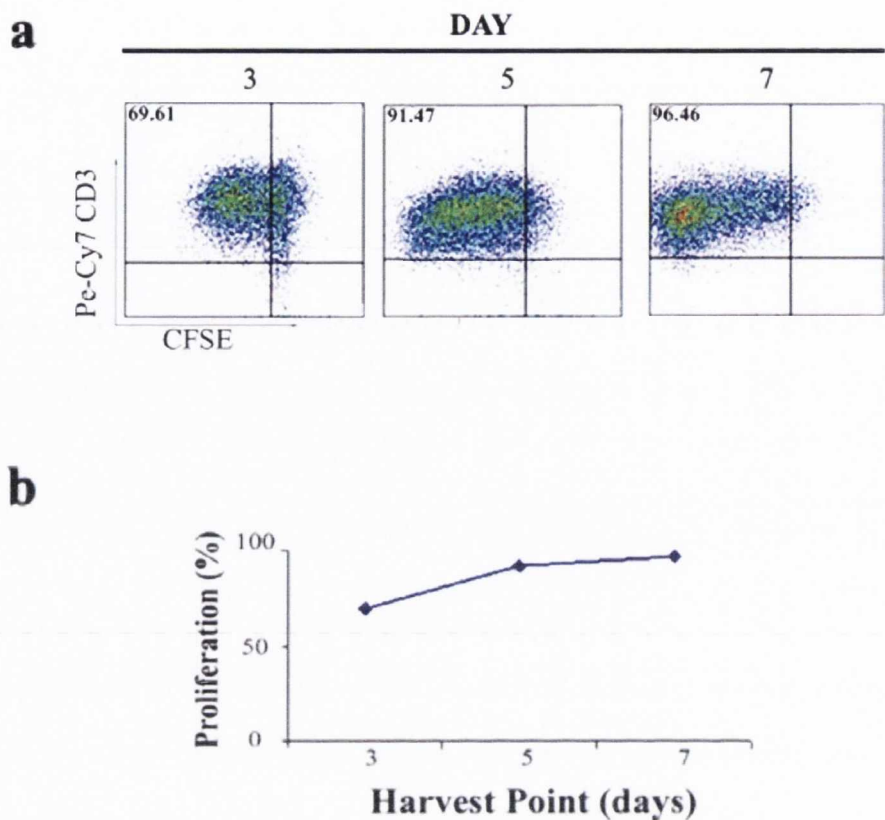


Figure 3.8: Evaluating the capacity of CFSE to quantitate T cell proliferation

(a) CFSE labelled wild-type splenocytes were plated on anti-CD3 ϵ coated dishes in Xvivo/IL-2 medium. Cells were harvested on day 3, 5 and 7 and labelled with Pe-Cy7 CD3 antibody for FACS analysis.

(b) The percentage proliferation recorded at day 3, 5 and 7 is represented as a graph.

Chapter 4: Results

6-Thioguanine selectively acting through the queuine tRNA pathway inhibits T cell proliferation *ex vivo*

4.1 Introduction

The discovery and synthesis of the thiopurines 6TG, 6MP and Aza, was the work of Getrude B. Elion and George Hitchings, over six decades ago (Elion and Hitchings, 1955). The approval of these drugs by the FDA (Food and Drug Administration, US) as anti-cancer and immunosuppressive agents, aroused intense scientific interest in their mechanism of action (Elion 1989; Karran and Attard, 2008). All three thiopurines are considered as pro-drugs that are metabolized into the active metabolite that is 6TG nucleotide. Based on the structural similarity of 6TG to the canonical purine base guanine, it was initially hypothesized that the analogue would interfere with many cellular metabolic processes. In a preliminary attempt to investigate the same, Sartorelli injected Elrich tumour carrying mice with 6TG. Using incorporation of radiolabelled ^{14}C -guanine as a quantitative marker, he reported that the decrease in ^{14}C -guanine isolated from nucleic acids was the principal effect of 6TG administration (Sartorelli and LePage, 1958). This indirect correlation was then re-visited by LePage, who used radiolabelled ^{14}C -6TG and isolated radiolabelled 6TG nucleotide monophosphate derivatives (6TGMP) from cellular extracts and nucleotide derivatives (6TGN) from nucleic acids (LePage, 1960). These studies suggested that in the cell, 6TG was transformed into various anti-metabolite derivatives amongst which incorporation of 6TG nucleotides (6TGN) into DNA was an inevitable event—a step responsible for the therapeutic effects of the thiopurines (Karran and Attard 2008).

6TG is readily taken-up and accumulated in the cell. Like other thiopurines, 6TG is metabolized in the cell by the purine salvage enzyme HPRT enzyme, which is the initial step towards its conversion into 6-thiodeoxyGTP (6-thiodGTP) (Footohi *et al.*, 2006). Being a dGTP analogue, 6-thiodGTP acts as a non-inhibitory substrate for the replicative DNA polymerases with a reported K_m value similar to that of unmodified dGTP (Karran and Attard, 2008). Studies on the content of 6TG in the DNA of patients given the drug suggests that during DNA replication, between 0.01

and 0.1 % of the DNA guanines (amounting to 10^5 - 10^6 residues per cell) can be substituted by 6TGN (Swann, 1996). It is noteworthy that 6TG cytotoxicity is consistently linked to the presence of 6TGNs in DNA, and therefore it was assumed that DNA incorporation alone was responsible for the genetic aberrations leading to cytotoxicity. This however is untrue because misincorporated 6TG is not a substrate for the pre-replicative excision repair machinery and therefore the accumulated 6TG is retained in DNA as part of the template strand and consequently is insufficient to convey toxicity (Karran, 2006; Tidd and Paterson, 1974). Moreover, *in vitro* primer extension studies have shown that 6-thiodGTP in DNA does not present a major obstacle for DNA polymerases during the next round of replication (Ling *et al.*, 1992). These studies therefore suggest a more complex mechanism for 6TG mediated toxicity which relies on other *in vivo* mechanisms.

4.1.1 Anti-metabolic mechanism of 6TG

Similar to other thiopurines, 6TG is associated with the property of ‘delayed toxicity’; that is, the toxic effects of the drug manifest at specific stages of the cell cycle (Karran and Attard, 2008). This was earlier assumed to be due to the need for a cell to passage through at least one S phase before incorporating the analogue into its DNA. Later studies claimed however that this could not fully explain the biochemical mechanism (Tidd and Paterson, 1974). In fact, the cytotoxicity is not observed until the following/second round of replication (Tan *et al.*, 2003). This very unusual phenomenon, requiring passage through a replicative phase, is shared by the carcinogenic base modification *O*⁶-methylguanine (*O*⁶-meG) (Roberts *et al.*, 1971). The *O*⁶-meG lesion is an erroneous *in vivo* product of methylating agents which append an alkyl/methyl group onto the oxygen molecule of guanine. Curiously, resistance to these methylating agents was also found to provide resistance to 6TG suggesting a similar mode of toxicity. To investigate this, Bignami and co-workers treated Chinese hamster ovary cells with an SN1-methylating agent called *N*-methyl-*N*-nitrosourea (MNU). This treatment led to the loss of *in vivo* alkylating/methylating agents in the cell, thereby providing methylation tolerance. When these cells were administered 6TG in culture, the methylation resistant clones incorporated 1.6 fold more of 6TG into DNA. This simultaneous resistance to methylating agents as well as thiopurines strongly suggested the importance of methylation of 6TG in DNA (Aquilina *et al.*, 1990; Karran and Attard, 2008). Successive *in vitro* studies reported

that the thiol group at position C6 of the non-canonical 6TGN was highly reactive and found to be readily alkylated. Thus it was postulated the sulphur was the site of methylation by *S*-adenosylmethionine (SAM)—known to act as a weak *in vivo* methylating agent. *In vitro* experiments showed that methylation of 6TGNs in DNA was 38 times higher than that of N7 guanine (Swann, 1996) i.e. the methylation of 6TG by SAM occurs *in situ* once incorporated into DNA. The methylation event gives rise to the formation of 6-meTG, which during the next round of replication is part of the DNA template strand (Swann, 1996). The DNA helicase enzyme unwinds the DNA helix to allow for the replicative DNA polymerases to synthesize the complementary daughter strand. However, the 6TG/6-meTG on the template strand fails to base pair correctly or forms a base-pair with thymine (6TG/6-meTG:T) which resembles a replication error/lesion similar to that of *O*⁶-meG containing DNA base pair. This provokes processing by the DNA mismatch repair enzymes to the error site (Karran and Marinus, 1982).

The role of the DNA MMR is to correct any replication errors committed by DNA polymerases. The presence of an incorrect base pair or supernumery bases that escape proof-reading can engage the MutS α (made of MSH2 and MSH6 proteins) of the MMR complex **Figure 4.1**. MutS α then recruits the MutL α (heterodimer of MLH1 and PMS2) subunit of MMR which binds to MutS α . Together, they recruit exonucleases and DNA polymerases to excise the mismatched section of the synthesized DNA strand and re-synthesize it with the accurate base pair (Stojic *et al.*, 2004). But the lack of appropriate base pairing causes the replication to enter an erroneous loop, creating nicks in the replicating strand and thereby delaying the exit from S phase. This leads to growth arrest or DNA single strand damage (Yan and Marko, 2003). Repetitive formation of DNA single-strand breaks causes damage that is sensed by the Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 (ATR) proteins. Activated ATM/ATR proteins phosphorylate the cell cycle checkpoint proteins CHK1 and CHK2 leading to their activation (Yamamoto *et al.*, 2006) and phosphorylation of the cell division cycle phosphatase Cdc25. Phosphorylation renders Cdc25 inactive causing it to be sequestered in the cytoplasm by the 14-3-3 proteins. Sequestration of Cdc25 prevents the activation of downstream cyclinB/Cdk1 complex. As a result, the cell cycle is unable to progress past the G2 phase (Yamamoto *et al.*, 2006). ATM/ATR activation also activates p53-dependent signaling, which ensures the cell is maintained in a G2 arrested state, by upregulating

the 14-3-3 proteins (to further sequester Cdk1 in the cytoplasm) (Yamamoto *et al.*, 2006). The cell cycle therefore is maintained in G2 arrest until either the damage is completely repaired, or the cell experiences damage induced cell death (Karran, 2006).

In intact cells, low levels of DNA 6TG are tolerated as they are neither toxic nor highly mutagenic. However, a more extensive substitution is associated with eventual chromosomal damage and cell death and is entirely dependant on the active purine salvage pathway (Warren *et al.*, 1995; Cuffari *et al.*, 2004). The HPRT enzyme is indispensable in this case, for the bioactivation of 6TG. As cells only possess a single active copy of the X-linked *Hprt1* gene that encodes for the HPRT enzyme, at high concentration of 6TG, the cells gain resistance by a single mutation in the gene. A widely used technique to develop drug resistant cells (Karran and Attard, 2008). In the absence of this HPRT enzyme, the cell is unable to metabolise 6TG and as a result, 6TGNs are not found incorporated into replicating DNA. It is noteworthy that cell division, which of course entails DNA replication, is crucial for 6TG incorporation into DNA. A study conducted on non-dividing liver cells *in vitro* showed that none of the administered thiopurines were incorporated into DNA in the absence of replication (Berman *et al.*, 1985).

Interestingly however, previous studies have also indirectly reported that incorporation into DNA is not the sole mechanism by which 6TG can convey its toxic effects. These studies, albeit focusing on the incorporation of 6TG in DNA, have repeatedly reported detectable amounts of analogue found incorporated into RNA as well (LePage, 1961; Sartorelli and LePage, 1958). This should be of no surprise as RNA, like DNA, is also a nucleic acid macromolecule and thereby a potential site for the incorporation of the purine analogue. Notwithstanding this observation, successive studies have concentrated on the effects of 6TG on DNA to explain the therapeutic mechanism of thiopurine toxicity.

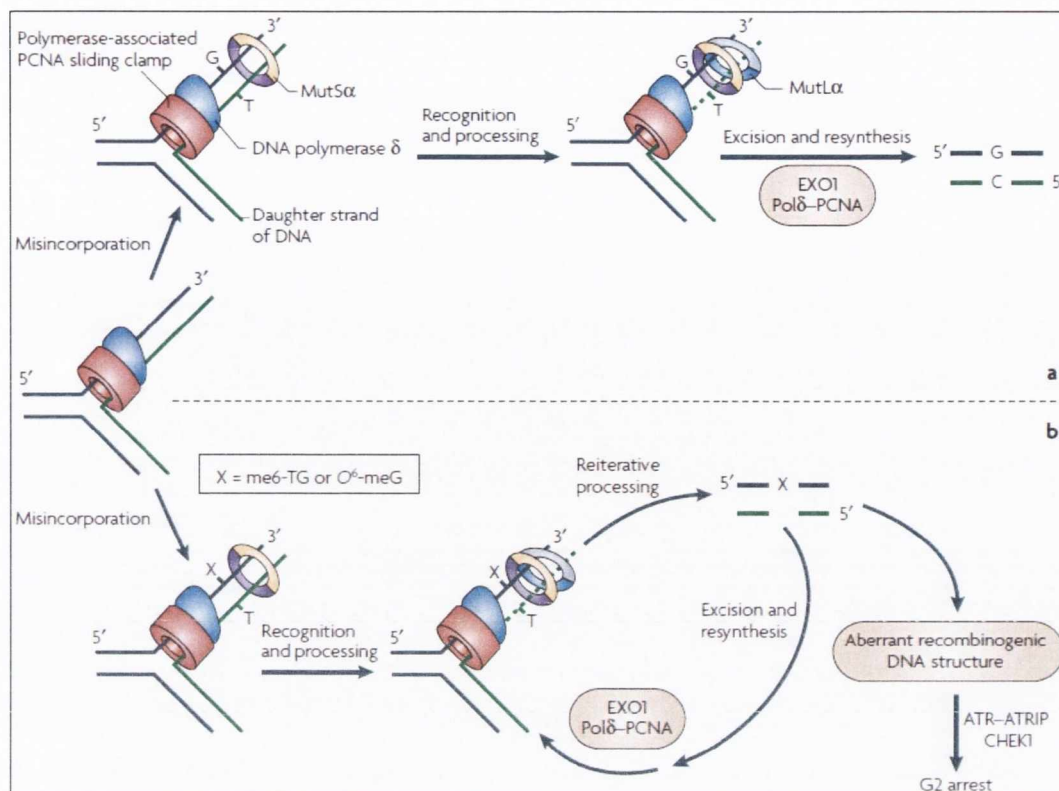


Figure 4.1: Mechanism of DNA MMR

The role of the DNA MMR is to correct any replication errors. Erroneous incorporation of a non-complementary base (a) by DNA polymerase δ and its associated PCNA (polymerase-associated proliferating cell nuclear antigen) sliding clamp triggers recruitment of the mismatch recognition MSH2-MSH6 heterodimer called MutS α . Successful recognition recruits another MMR factor MLH1-PMS2 heterodimer called MutL α . The endonuclease activity of MutL α introduces nicks in the mismatch region to allow for EXO1 to dock onto the 5' end of the misincorporated nucleotide. The 5'-3' exonuclease activity of EXO1 excises the section of the daughter strand that carries the mismatch. This missing stretch of DNA is then resynthesized by the DNA polymerase δ -PCNA complex. The polishing finish is provided by DNA ligase which seals the remaining nick in the strands- restoring the complementary base pairing between the template and daughter DNA strands. Misincorporation of a base analogue X (me-6TG or O⁶-meG) (b) in the template strand also recruits the MMR surveillance for a similar corrective mechanism, however the process enters a futile cycle due to the inability of the methylated analogue to form a correctly formed complementary base pair with a canonical base. This leads to the formation of DNA lesions, thus triggering activation of the ATR and ATRIP DNA-damage signaling pathway and subsequent G2 cell cycle arrest- a stage where the cells are stuck at until the repair is successful. If not, the cell goes into arrest leading to eventual cell death (Adapted from Karran and Attard, 2008).

4.1.2 6TG in tRNA

The preliminary reports of 6TG incorporation into RNA came from studies by LePage and co-workers where they isolated the administered analogue from RNA of the so-called '6TG-resistant' cells—which were transformed cells deficient in HPRT (Sartorelli and LePage, 1958; LePage and Jones, 1961; LePage, 1963). Mandel then furthered this concept using radioactive 6TG (6TG-³⁵S), recovering the intact compound from total RNA fractions; comprising all species of RNA (Kwan *et al.*, 1973). Around the same time another study on mouse lymphoma LS178Y cells also reported isolation of 6TG from purine ribonucleotides (Tidd and Paterson, 1974). Trewyn, based on these findings was the first to apply the concept to growth studies in culture. Using the promyelocytic HPRT-deficient leukaemia cell line HL-60, his studies showed that despite lack of incorporation into DNA, the cells were still sensitive to the anti-proliferative effects of the analogue presumably through its effects of RNA (Kretz *et al.*, 1987). The biochemical basis for this effect was only postulated later showing that the HPRT-independent cytotoxic effects conveyed by the analogue were due to its incorporation into tRNA, more specifically at the queuine deficient position 34 (French *et al.*, 1991). These findings strongly suggested an alternative mechanism for 6TG mediated growth inhibition which was independent of its incorporation into DNA as well as independent of its metabolism/bio-activation by HPRT- therefore a distinct alternative pathway in its own right.

Incorporation into the queuine deficient position 34 of tRNA implied that the QTRT enzymes carried out the reaction. Studies by Farkas and Jacobson reported that among other purine analogue that act as inhibitors and substrates, 6TG was characterized as a non-inhibitory substrate of the QTRT enzymes (Farkas *et al.*, 1984) — suggesting the possibility of other analogues to be incorporated into this queuine deficient site and perhaps alter tRNA function. Preliminary studies in bacterial systems have suggested that the base analogue is capable of impeding RNA synthesis even more so than DNA synthesis. Also, the formation/synthesis of certain proteins was altered (Mandel *et al.*, 1965). A study by Gray and Rachmeler took this further and they reported that incorporation of 6TG in tRNA had an effect on the tRNA molecule's amino acid acceptor function (Gray and Rachmeler, 1967). In 1973, Mandel's group published a study conducted in Mouse sarcoma 180 ascite injected tumour cells where they found that incorporation of 6TG into tRNA had an affect on

protein synthesis and activity (Kwan *et al.*, 1973). Although these studies weren't really conclusive, they did however correlate with similar conclusions derived from studies on lack of queuine modification which caused increased read-through of the UAG codons (Bienz *et al.*, 1981; Frey *et al.*, 1989) and in other cases, altered codon choice (Bjork *et al.*, 1999; Meier *et al.*, 1985).

Another interesting feature of 6TG in tRNA was the fact that unlike its irreversible incorporation into a cell's genetic material, in tRNA, it is completely reversible, unlike the natural substrate queuine. In substrate competitive growth experiments, Trewyn's group showed that while administration of 6TG to proliferating HL-60 cells mediated growth inhibitory effects through incorporation into tRNA, re-supplementation of the culture medium with queuine was able to reverse 6TG incorporation and restore the proliferative capacity of cells in culture (Kretz *et al.*, 1985; French *et al.*, 1991). It is noteworthy that queuine has been consistently reported to be characteristically absent from rapidly proliferating cells, such as cancer cells. Therefore, 6TG would be expected to convey growth inhibitory effects only in queuine deficient cells.

In this chapter therefore, for the first time, we explore the growth inhibitory effects of 6TG in an *in vitro* primary, non-transformed T cell system.

4.2 Results

4.2.1 Antiproliferative effects of 6-Thioguanine following incorporation into DNA

6TG is a prominent therapeutic agent used in the treatment of various cancers, as an immuno-suppressive drug, in organ-transplantation and as a treatment for inflammatory and autoimmune disease. The anti-proliferative and cytotoxic effects of 6TG are principally mediated by its incorporation into replicating DNA (Murphy *et al.*, 1999). A key step in its mechanism is the conversion of 6TG into a thioguanosine nucleotide by the HPRT enzyme (Cara *et al.*, 2004). 6TG is structurally similar to the guanosine nucleotide and can be incorporated into newly synthesised DNA and RNA (Murphy *et al.*, 1999). Interference with DNA metabolic processes contributes to the antimetabolic activity of the drug.

Previous *in vitro* and *in vivo* analyses have shown the importance of HPRT for 6TG mediated cytotoxicity (Aubrecht *et al.*, 1997) and as part of our investigation, we wished to study similar effects by treating primary murine splenocytes under our newly validated cell culture conditions. Therefore, whole murine splenocytes were isolated and plated according to the optimised protocol. Cells were treated with increasing concentrations of 6TG ranging from 0.1 nM to 1.5 mM. The effect of increasing 6TG concentration on sample proliferation is demonstrated by the titration curve showing the proliferation of each sample by FACS analysis using CFSE intensity as a marker of replication **Figure 4.2a**. The graph shows the sensitivity of proliferating splenocytes to increasing 6TG concentration at ED₀, ED₂₅, ED₅₀ and ED₁₀₀. The titration curve gives an ED₅₀ value of approximately 0.06 mM as shown in **Figure 4.2b**. The highlighted area of the graph indicates the *in vivo* drug range recorded in the plasma of patients who were administered 6TG during clinical studies (Murphy *et al.*, 1999). This shows that the antiproliferative effects of 6TG under the new protocol conditions are in line with previously published findings and validate the use of this protocol for the analysis of 6TG's anti-proliferative capacity.

4.2.2 The anti-proliferative effect of 6TG in HPRT null cells

6TG incorporation into nascent DNA and RNA polymer *via* the action of HPRT has gained large medical interest due to the ability to arrest proliferation (Aubrecht *et al.*, 1997). However, studies by Trewyn and colleagues showed that 6TG treatment was able to mediate an anti-proliferative effect even in HPRT deficient HL-60 leukaemia cells, and further that the anti proliferative effect was due to the incorporation of 6TG into RNA, and more specifically into tRNA (Elliott *et al.*, 1984). This suggested an important role for tRNA as an alternative anti-proliferative mechanism of 6TG. However, caution must be observed when translating results found in cell lines, particularly drug effects, to animal models and most importantly animal models of disease. Therefore, we examined the anti-proliferative effect of 6TG on primary T cells from HPRT knockout mice.

Using our previously validated protocol for the anti-proliferative effect of 6TG on T cells, we examined the effect of 6TG on splenocyte cultures from HPRT knockout mice. The radioactive HPLC assay (described in Chapter 3) was used to distinguish wild-type from HPRT knockout siblings. The isolated splenocytes were labelled with CFSE and plated onto anti-CD3 ϵ coated plates in XVivo/IL-2 medium. At 24 hours post-plating, the cells were treated with a range of 6TG concentrations between 0.1 nM and 1.5 mM, while the control sample (labelled Cntrl) was left untreated. The cells were left a further 4 days until harvesting. The proliferation of the harvested cells was evaluated from the decreasing intensity of CFSE dye fluorescence **Figure 4.3a**. The FACS plots show the sensitivity of splenocyte cultures to increasing concentration of 6TG treatment at ED₀, ED₂₅, ED₅₀ and ED₁₀₀. A titration curve was obtained for the same giving an ED₅₀ value of 0.65 mM as shown in **Figure 4.3b**. The area highlighted in grey on the leftmost side of the graph corresponds to the *in vivo* concentration of 6TG found in the blood patients administered 6TG during treatment. This concentration window corresponds to the highlighted area on the 6TG titration graph for HPRT wild type model in **Figure 4.2b**. The shift of the concentration window to the far left axis in **Figure 4.3b** is due to the higher concentrations of 6TG needed to exert anti-proliferative effects in HPRT knockout cells.

4.2.3 Queuine incorporation into tRNA provides a protective effect against 6TG-mediated anti-proliferation.

6TG treatment has been long known for its anti-proliferative effects due to its incorporation into replicating DNA (Aubrecht *et al.*, 1997). However LePage and Jones have shown that 6TG incorporation into DNA is usually accompanied by its incorporation into the RNA as well (Lepage and Jones, 1961; Lepage, 1963). Mandel treated mouse sarcoma 180 ascites cells with radioactive 6TG (6TG-³⁵S), radioactivity and recovered it as an intact compound from RNA (Kwan *et al.*, 1973). These findings are in line with the results discussed thus far showing that in primary murine T cells, 6TG treatment has an anti-proliferative effect independent of the HPRT-pathway (**Figure 4.3**), and hence independent of DNA incorporation. However, there has been no concrete proof in a non-transformed primary system to date to conclude that this occurs *via* incorporation into tRNA. Previously published studies that suggested tRNA incorporation of 6TG could account for the alternative anti-proliferative pathway were only conducted in cell lines and tumor lines, not in primary murine T cells. Therefore, the hypothesis addressed by my project, that 6TG can inhibit growth *via* incorporation into tRNA, was yet to be verified.

There are no currently used techniques to verify the incorporation of 6TG into tRNA. In established protocols, commercially available radio-labelled 6TG was used. However, this is no longer available, unless custom made. The previously validated cell culture protocol however served as an efficient tool to study the effect of queuine administration on the anti-proliferative effects of 6TG treatment. The irreversible incorporation of queuine into the tRNA anticodon loop would prevent 6TG at that same site. This should therefore lead to protection against 6TG-mediated inhibition of proliferation. Murine splenocytes were isolated from HPRT knockout mice for cell culture. The samples were then divided into different treatment groups **Figure 4.4a**: Samples left untreated with queuine or 6TG were labelled as (-) and those treated for both individually were labelled as (+). Co-treated samples were administered 0.65 mM 6TG, on day 1 post-plating and 1 μ M queuine was administered as part of two regimes: Single dose (SD, on day 0) and Daily dose (DD, Between day 0 – 5). On its own, queuine administration does not enhance the proliferation of the control samples (96.25 %) when given either as a SD (96.37 %) or as a DD (96.54 %). Treatment of HPRT KO T cells with the ED₅₀ 6TG (0.65 mM) gives an expected 50% inhibition of proliferation (~47.75%). The co-treatment of HPRT deficient

splenocytes with 6TG and queuine shows an interesting trend; when given as a SD, queuine gives a marginal enhancement of proliferation of around 49.67% whereas when administered as a daily dose, cell viability and proliferation is enhanced up to 75.90%, which is more than a 50 % increase in cell viability. The same is represented as a graph in **Figure 4.4b** where the 'Ctl' represents the untreated controls, SD and DD similar to that explained in **Figure 4.4a**. The light grey bars represent the samples treated for queuine alone (no 6TG administered) whereas the dark grey bars represent the co-treated samples. The results here would suggest that queuine can directly counteract the anti-proliferative drug effects of 6TG in the HPRT knockout model.

To analyse this concept further, the same protocol was followed where the samples were treated with increasing concentrations of queuine, **Figure 4.5**. This was to verify whether the effect observed was concentration dependent. Whole HPRT knockout murine splenocytes were isolated and cultured according to the previously discussed and validated cell culture protocol. The samples were either subjected to a SD, **Figure 4.5a** or DD, **Figure 4.5b** treatment regime with a range of queuine concentrations: 0 μ M, 0.1 μ M, 1 μ M, and 10 μ M. Control samples were left untreated ('No 6TG' samples, **Figure 4.5 a** and **b**). In the absence of 6TG, queuine alone, whether given as a SD or DD, does not enhance T cell proliferation, which is consistently in the range of ~92%. Upon queuine administration to the 6TG treated samples, a similar trend can be observed as above for **Figure 4.4**. In the samples administered ED_{50} 6TG, proliferation does not exceed 53.4 % when co-administered 0 μ M, 0.1 μ M, or 1 μ M queuine as a SD. However, at the high dose of 10 μ M, queuine enhanced proliferation up to 77.9 %. This could suggest that there is competition between queuine and 6TG for uptake into the cell (in addition to competition for insertion into tRNA) since, at a concentration of 10 μ M queuine is vastly in excess of physiological levels (~300 nM) and the lower concentrations would be expected to easily outcompete 6TG for tRNA insertion.

In the samples that were co-administered the ED_{50} of 6TG and queuine as a DD, a gradual enhancement of proliferation can be observed across increasing concentrations. A DD of 0.1 μ M queuine enhances proliferation to 71.34 % which is further enhanced in the samples administered 1 μ M queuine up to 71.34 %. The administration of the highest dose of 10 μ M queuine, pushes the proliferation to 84.68%, which is close to the proliferation seen in control, untreated samples. A graphical representation of the proliferation data is shown **Figure 4.5 b** where the SD

samples are the solid square symbol and the DD are the open circle symbol. Both SD and DD lines show a gradual increase in cell viability, with almost full restoration of proliferative capacity when administered 10 μ M queuine as a SD or DD. This result is an extended study of the effect observed in **Figure 4.4**. The repeated pattern observed suggests that the effect of queuine administration observed in 6TG treated T cells is of a mechanistic nature.

4.2.4 T cell Selectivity

The flexibility provided by the ability to clonally expand primary T cells *in vitro* has greatly benefited studies such as this to investigate the properties of certain drugs and pathways (Kang *et al.*, 1992). Moreover, using T cells as an experimental system also proves beneficial when the intention is to further study immunological models. Many disorders are caused, mediated or propagated by pathogenic, hyperproliferative or auto-reactive T cells (Rose and Mackay, 2006; Davidson and Diamond, 2001). This is also reflected in the fact that many therapeutic drugs have been developed to counteract the proliferation of T-cells. Therefore, with all the studies conducted on T cells, it makes it one of the best studied primary, non-transformed cell types. Notwithstanding this, one should be cognisant of the equally important role of other immune cells in mediating immune disorders; Macrophages, dendritic cells, natural killer cells and B cells to mention but a few. Moreover, even though these other cell systems may not be as well studied as T cells, protocols have been validated by various groups to promote their isolation and *in vitro* primary culture (Sugita *et al.*, 2010).

In this study, while using whole splenocyte cultures to specifically induce T cell clonal expansion, it was also possible to assay for the effect of 6TG treatment on B cells. The choice of B cells was for two main reasons. Firstly, 6TG affects proliferation and therefore, it was preferable to use an immune cell type that displayed similar proliferative capacity to that of T cells. Secondly, the established protocol for the *in vitro* culture of B cells similarly involves the use of whole splenocyte cultures to induce proliferation.

Whole splenocyte cultures from wild type and HPRT deficient animals were isolated and labelled with CFSE and plated onto anti-CD3 ϵ coated wells in XVivo/IL-2 medium for T cells and in uncoated wells for B cells. The B cell sample wells were stimulated with LPS and soluble α CD40 on Day 0 itself. At 24 hours post-plating the

cells were treated with ED₀, ED₅₀ and ED₁₀₀ 6TG for wild type and HPRT knockout separately. The cells were left a further 4 days until harvesting. On day 5, the cells were harvested for FACS analysis. A histogram of CFSE dye intensity in the proliferating cells is shown for wild type T and B cells **Figure 4.6a** and for HPRT knockout T and B cells **Figure 4.6b**. In the FACS plots, the brown trace histogram represents the unstimulated control that had harvest at the same day to represent proliferation independent of the provided stimulation, while the green trace represents the proliferation recorded in the samples that were specifically stimulated with the respective antibodies/cytokines.

In splenocyte cultures from wild type mice **Figure 4.6a**, administration of ED₀, ED₅₀ and ED₁₀₀ of 6TG gave T cell proliferation values of ~90%, ~50 % and ~10%, respectively. At ED₀ of 6TG (i.e no drug), B cells give a maximum proliferative capacity of 90%, which was similar to that of T cells. However, at the ED₅₀ and ED₁₀₀ of 6TG the concentrations were too toxic for the B cells to tolerate and therefore only gave rise to very low proliferative percentages of around 20% and 10%, respectively.

In the HPRT knockout samples **Figure 4.6b**, the administration of ED₀, ED₅₀ and ED₁₀₀ of 6TG to HPRT knockout T cells again gave expected proliferative values of ~90%, ~50% and ~10%, respectively. When identical concentrations were administered to B cells, apart from a slight decrease in proliferative capacity from ED₀ to ED₅₀ and ED₁₀₀, they seemed to be resistant to 6TG. Even at doses where the T cells fail to sustain viability, 6TG did not seem to impede the proliferative capacity of the B cells.

4.2.5 The effect of 6TG on the proliferation of HPRT::TGT null T-cells

Previously published studies examined the incorporation of 6TG into tRNA as an additional mechanism for 6TG toxicity. The work of Ronald Trewyn's group, using HL60 cells, provided proof that 6TG can be incorporated at the same position in the tRNA as queuine (position 34) and showed the 6TG incorporation could be reversed by queuine administration to the cells (Elliott *et al.*, 1984). However, none of the studies have employed a system where both enzymes could be inhibited to observe if there was yet other mechanism by which 6TG could be metabolized. Perhaps it wasn't detrimental to the previous studies as the aim was to elucidate the growth inhibitory effects of 6TG which would appear to be mainly through incorporation into DNA or

tRNA. The complete restoration of cell viability and proliferative capacity upon adding back queuine to the 6TG treated cells was reason enough to perhaps conclude that there were no other significant pathways by which 6TG could convey its growth inhibitory effects. It is noteworthy that the above studies were conducted in a transformed cell-line HL-60 where the deficiency in HPRT was acquired by positive selection of the resistant clones from cultures grown in 6TG supplemented medium. Although these clones are deficient in HPRT, it is certain that a myriad of other mutations have been induced by 6TG treatment. Therefore, in our work we decided to establish a double knockout strain of mouse. As explained in Chapter 3, these double knockout mice are a novel transgenic strain established primarily for the purpose of this study. Using the PCR-based TGT screening and the HPLC based HPRT screening, the animals deficient in both were selected for to investigate the anti-proliferative effects of 6TG, if any, in the absence of both growth-inhibitory mechanisms.

The isolated splenocytes were labelled with CFSE and plated onto anti-CD3 ϵ coated plates in XVivo/IL-2 medium. At 24 hours post-plating the cells were treated with a range of 6TG concentrations between 2 mM and 18 mM, while the control sample (labelled Cntrl) was left untreated. The cells were left a further 4 days until harvesting. The proliferation of the harvested cells was evaluated from the decreasing intensity of CFSE dye fluorescence in **Figure 4.7a**. The FACS plots show the sensitivity of splenocyte cultures to increasing concentration of 6TG treatment at 0, 2, 10 and 18 mM. Due to lack of any observable dose-dependent response, a titration curve could not be obtained **Figure 4.7b**. The area highlighted in grey on the leftmost side of the graph corresponds to the *in vivo* concentration of 6TG found in the blood of patients administered 6TG during treatment. This concentration window corresponds to the highlighted area on the 6TG titration graph for HPRT wild type model in **Figure 4.2b**. The shift of the concentration window to the far left axis in **Figure 4.7b** reflects the high concentrations that were assayed. In fact, concentrations of up to 18 mM 6TG—180 times that of drug concentrations reported in patients (Murphy *et al.*, 1999)—were completely ineffective at inhibiting proliferation. The lack of any growth inhibition at these high concentrations strongly indicates that the HPRT and the queuine-tRNA pathway are the only two mechanisms through which 6TG exerts its growth inhibitory effect on proliferating T cells *in vitro*.

4.3 Discussion

The presence of queuine, or more specifically the lack thereof, has attracted intermittent interest in the cancer field over the past 20 years. While some studies have suggested changes in queuine modification as a tool for malignancy grading (Emmerich *et al.*, 1990; Reisser *et al.*, 1990; Iwata-Reuyl *et al.*, 2003) others studies have proposed it may function as a biomarker to predict patient survival (Huang *et al.*, 1992). Ronald Trewyn was the first to investigate and postulate that this characteristic deficiency may be a potential therapeutic target (Kretz *et al.*, 1985; Morgan *et al.*, 1994). Farkas and co-workers reported the ability of the thiopurine 6TG to act as a non-inhibitory substrate for the QTRT enzymes (Farkas *et al.*, 1984). Trewyn applied this concept to his growth studies on the transformed leukaemia cell line HL-60. Although a landmark study in its own right, one of the set-backs was the use of a transformed *in vitro* system. Given that the study was to speculate the potential of a new therapeutic avenue, use of a cell line was sub-optimal for any subsequent translation into higher eukaryotes. Moreover, previous studies on the effect of queuine on cell proliferation have reported that a cell's physiological response is much dependent on the oncogenes involved in the cell's transformed phenotype (Langgut *et al.*, 1993; Langgut *et al.*, 1994). Different cell lines have given contradicting data on queuine's ability to enhance proliferation. Therefore the use of knockout animals here has been doubly beneficial. It has provided a means to obtain a cleaner/non-transformed system to study queuine related effects and the results validate the drug-related findings of Trewyn in a primary cell model—allowing its potential translation into eukaryotic systems.

In this study we demonstrate that 6TG can convey growth inhibitory effects on proliferating wild type T cells at an ED₅₀ value that lies within the *in vivo* drug concentration found in patients administered the drug. A similar dose dependent response is found even in the HPRT deficient animals, at a high concentration range—about 10 times higher. This suggests that while there is an alternative HPRT-independent mechanism for 6TG, the DNA incorporation pathway dominates any observable therapeutic effect. To determine whether 6TG incorporation was occurring at the queuine deficient position of the tRNA anticodon loop, we re-administered queuine to the culture medium and observed a dose dependant reversal of 6TG cytotoxicity. Although the result is only correlative, it would suggest that the anti-

proliferative effects observed in the HPRT deficient mice occurs through specific incorporation of 6TG into the queuine deficient tRNA at position 34; although other unknown substrates for the queuine-tRNA ribosyltransferase complex may exist. It is noteworthy that even at the highest concentration of queuine used to counteract 6TG's antiproliferative effect, it is still sixty-five times lower than the dose of 6TG administered. The results are in line with Trewyn's original findings and have now been validated in a primary non-transformed eukaryotic system.

This ability of queuine to restore cell viability in the presence of 6TG to almost control levels in HPRT deficient assays would suggest that tRNA is the only HPRT-independent pathway for 6TG toxicity. It would be interesting to mention here that studies carried out by Neurath's group reported that thiopurines could induce apoptosis in human T cells (Tiede *et al.*, 2003). The induction of apoptosis required the costimulation of T cells by CD28 which caused blockade of Rac-1 activation through binding of 6TGTP instead of GTP. In our study however we showed that in absence of HPRT and TGT 6TG was unable to mediate any growth inhibitory effects in proliferating T cells, casting doubt on the significance of the Rac-1 mechanism. In other words, even if this Rac-1 GTP pathway is valid, it is not enough to convey any significant anti-metabolic effects vis-à-vis DNA or tRNA incorporation.

The use of 6TG has proved to be very informative for the study of the queuine tRNA pathway. Firstly, the incorporation of 6TG into tRNA despite the lack of the purine salvage enzyme HPRT, suggests that the queuine-tRNA pathway is fully distinct from the HPRT pathway. Secondly, 6TG is a non-inhibitory substrate for the QTRT enzyme complex and its incorporation into queuine deficient tRNA leads to growth inhibition of proliferating cells. Thirdly, 6TG incorporation and its anti-proliferative effects are reversible by queuine, the preferred QTRT substrate, whose incorporation is irreversible. Although the exact therapeutic mechanism of 6TG in tRNA has yet to be elucidated, it can be hypothesized from previous literature, that it may effect translational capacity of the Q-tRNAs and even perhaps the activity of the enzymes synthesized (Kwan *et al.*, 1973). Unlike in DNA, where the incorporation of thiopurine is irreversible and eventually toxic, incorporation into tRNA provides a reversible and non-retainable inhibition of growth. Also, incorporation into DNA tampers with the genetic blueprint with the concomitant risk of gene mutation. This feature is made all the more dangerous by the fact that 6TG is bio-available to every cell. In the short-term there may not be a huge cost to pay as the major effects are

observed on the targeted rapidly proliferating cells, however in the long-term 6TG has the potential to cause either cell death, cell cycle arrest, toxicity or malignancy (Zackheim *et al.*, 1994). Studies on thiopurine treated leukemic and psoriatic patients have reported hepatotoxicity, bone marrow toxicity (De Bruyne *et al.*, 2006) and even skin cancer. This complication is completely avoided by exploiting the queuine-tRNA pathway since i) the DNA code remains unaffected by drug treatment and ii) queuine deficiency only occurs in rapidly proliferating cells.

Another interesting feature highlighted in our study is some degree of selectivity for T cells over B cells by targeting the queuine-tRNA pathway. Both immune cell populations are hyper-proliferative and known to be mediators of various immune disorders. In some diseases like MS, the two have been shown to converse at different progressive stages of the disease (Goverman, 2009). Although the data presented here does not provide any information as to the mechanism behind this selectivity, based on previously published data, its possible that the metabolism of the T cell is being affected through the inhibition of protein synthesis (Fox *et al.*., 2005). T cells are subject to a marked metabolic switch upon receipt of instructions to proliferate, from a cell type that favours oxidative phosphorylation to a state of aerobic glycolysis, which is needed to sustain increased proliferation and biosynthesis through the provision of carbon cytoskeleton (MacDonald *et al.*, 1979; Miller *et al.*, 1994; Fox *et al.*, 2005; Marko *et al.*, 2010). Other immunosuppressive drugs, such as Rapamycin, are known to inhibit T cell activation though impeding energy metabolism and have been shown to decrease the number of pro-inflammatory T cell types but selectivity increase the number of regulatory (CD4⁺25⁺) T cells (Battaglia *et al.*, 2005).

It would be of great interest to understand the exact mechanism whereby 6TG affects proliferation through the queuine-tRNA. However, from the point of view of validating this novel pathway as an effective therapeutic strategy, subsequent work on this project focused on the queuine tRNA pathway in an *in vivo* animal disease model EAE.

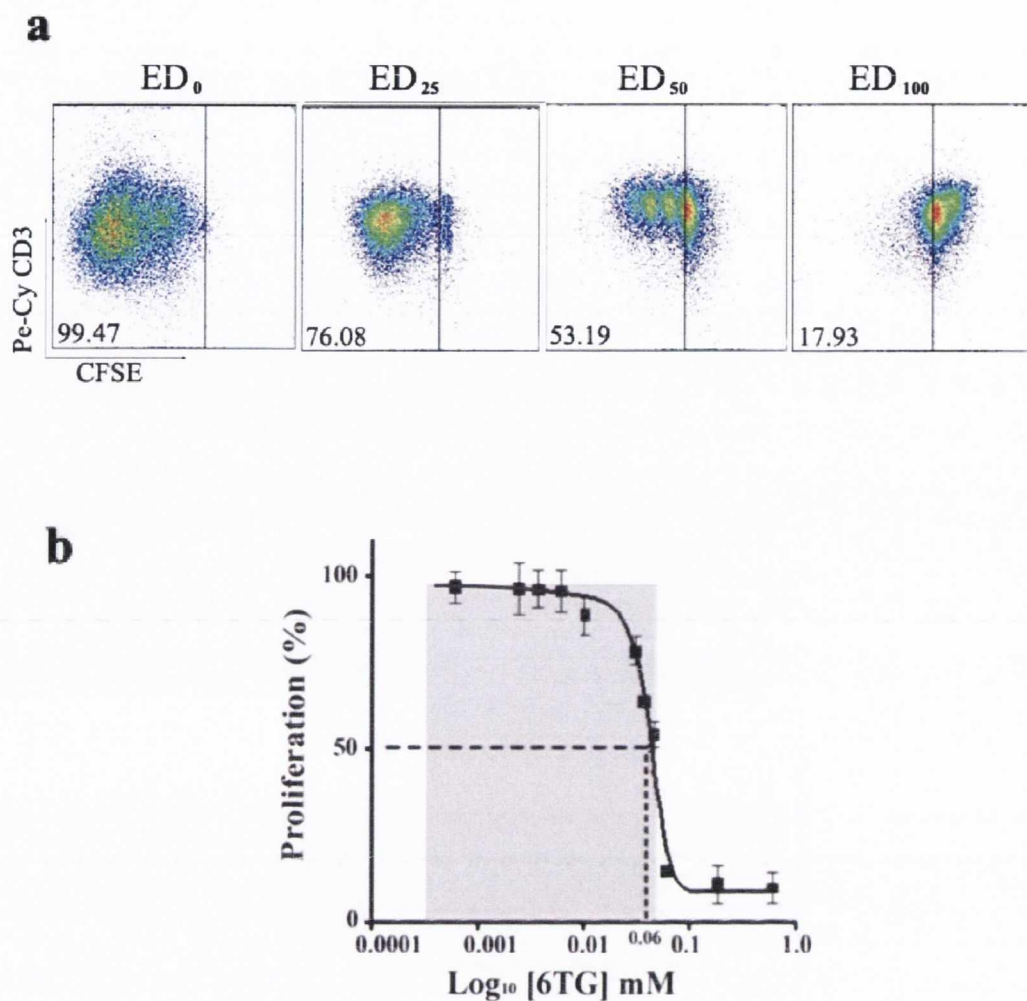


Figure 4.2: Inhibitory effect of 6TG on the proliferation of wild-type T cells in culture
(a) Whole wild-type splenocytes were isolated, labelled with $1\mu\text{M}$ CFSE and plated in anti-CD3 ϵ coated dishes in Xvivo/IL-2 medium. On day 1 post-plating, cells received increasing concentrations of 6TG. On day 5, cells were harvested, labelled with Pe-Cy7 CD3 and analysed by FACS. Representative FACS plots show the dilution of CFSE fluorescence when proliferating T cells were treated with ED₀, ED₂₅, ED₅₀ and ED₁₀₀ of 6TG.
(b) Titration curve showing the average percentage proliferation from triplicates of T-cells treated with increasing concentration of 6TG. The ED₅₀ value—the concentration of 6TG that inhibits cell proliferation by fifty percent—of wild-type T cells corresponds to a concentration of 0.06 mM. The shaded area of the graph indicates the concentration of 6TG found in the serum of psoriatic patients who had been treated with the drug (Murphy *et al.*, 1999).

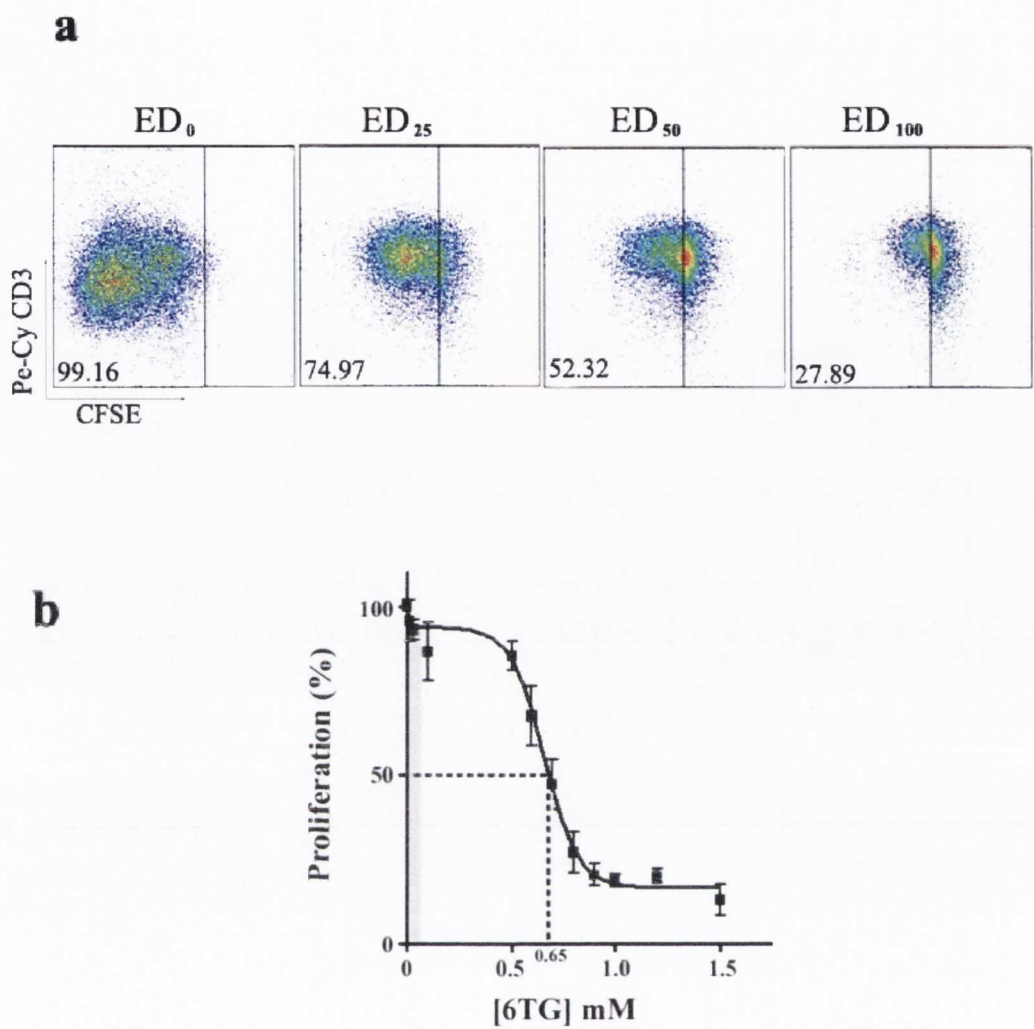


Figure 4.3: Inhibitory effect of 6TG on the proliferation of *Hprt1^{b-m3}* T cells in culture
(a) Whole *Hprt1^{b-m3}* splenocytes were isolated, labelled with 1μM CFSE and plated on anti-CD3ε coated dishes in Xvivo/IL-2 medium. On day 1 post-plating, cells recieved increasing concentrations of 6TG. On day 5, cells were harvested, labelled with Pe-Cy7 CD3 and analysed by FACS. Representative FACS plots show the dilution of CFSE fluorescence when proliferating T cells were treated with ED₀, ED₂₅, ED₅₀ and ED₁₀₀ of 6TG.
(b) Titration curve showing the average percentage proliferation from triplicates of T-cells treated with increasing concentration of 6TG. The ED₅₀ value—the concentration of 6TG that inhibits cell proliferation by fifty percent—of *Hprt1^{b-m3}* T cells corresponds to a concentration of 0.65 mM. The shaded area of the graph indicates the concentration of 6TG found in the serum of psoriatic patients who had been treated with the drug (Murphy *et al.*, 1999).

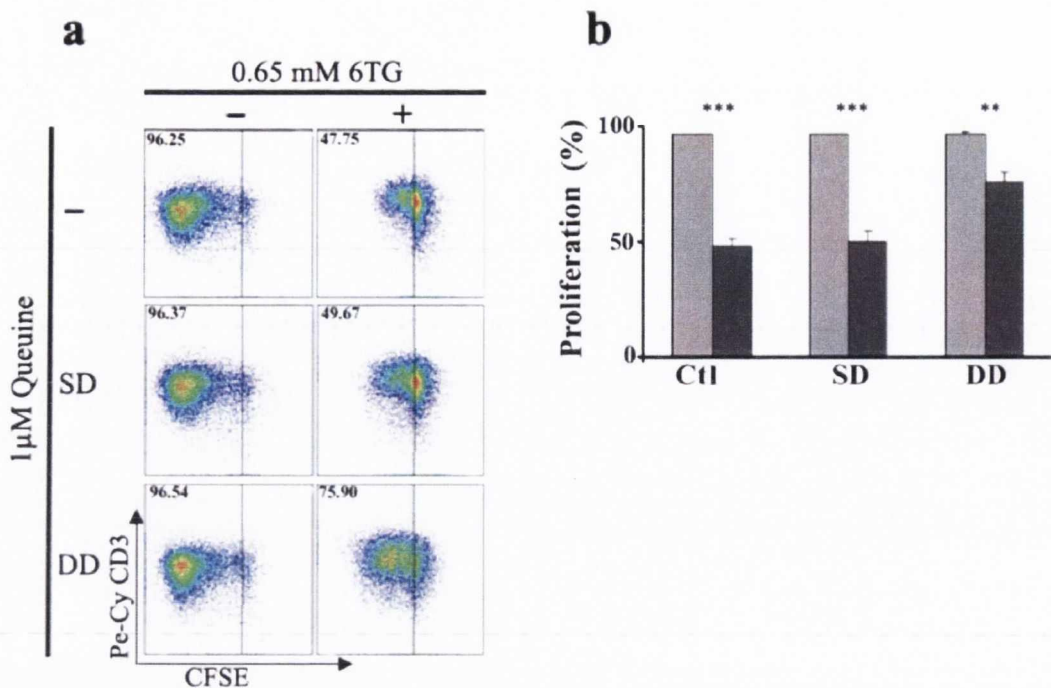


Figure 4.4: Queuine can reverse the anti-proliferative effects of 6TG in *Hprt1^{b-m3}* T cells
(a) Representative FACS plots for *Hprt1^{b-m3}* spleenocytes cultures administered a single dose (SD) or daily dose (DD) of 1 μM queuine with (+) or without (-) co-administration of 0.65 mM 6TG. Control samples were either left untreated with queuine (-) or 6TG (-) or received each chemical individually.

(b) Percentage proliferation of *Hprt1^{b-m3}* T-cells treated with 6TG and queuine. The light grey bars represent the control samples, that remained untreated or were given a SD or DD of queuine, whereas the dark grey bars represent samples that were administered the ED₅₀ concentration of 6TG (0.65 mM) and had been left untreated or received queuine as a SD or DD. **, $P < 0.004$ and ***, $P < 0.0001$.

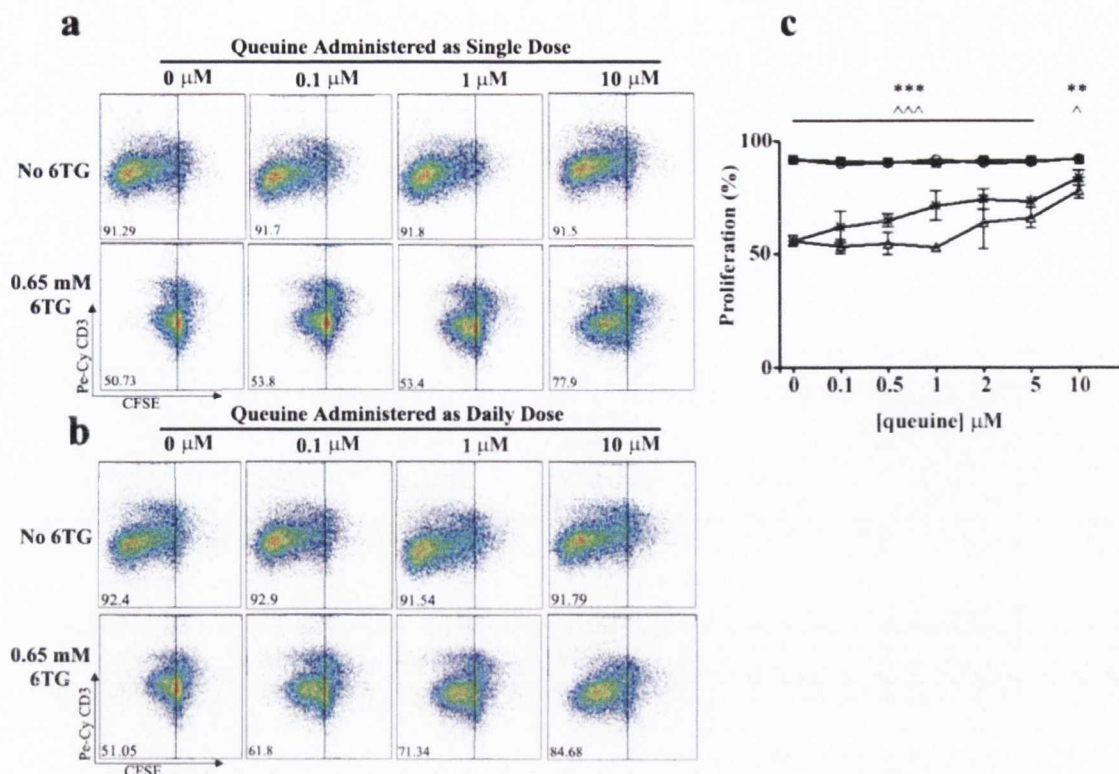


Figure 4.5: Queuine administration can counteract the anti-lymphoproliferative effect of 6TG

Representative FACS plots for *Hprt1^{b-m3}* spleenocytes cultures that were administered queuine as a Single Dose, SD (a) or Daily Dose, DD (b) at the concentrations shown, and either with (+) or without (-) 6TG at the ED₅₀ concentration. Control samples were either left untreated with queuine (-) or 6TG (-) or treated with each chemical individually.

(c) Percentage proliferation of *Hprt1^{b-m3}* T-cells treated with 6TG and increasing concentrations of queuine. Control samples that were treated only with queuine as a SD (■) or DD (○) show normal proliferation. Samples co-administered 6TG at the ED₅₀ concentration on day 0 and queuine as a SD on day 0 (Δ) or as a DD from days 0 to day 5 show a reversal of the anti-proliferative effects of 6TG. Each data point shows the mean of triplicate stimulations $\pm$ standard deviation. Statistical significance for SD (*) and DD (^) were as follows: ***, $P < 0.0001$; ^^^, $P < 0.0001$; **, $P < 0.0029$ and ^, $P < 0.0128$.

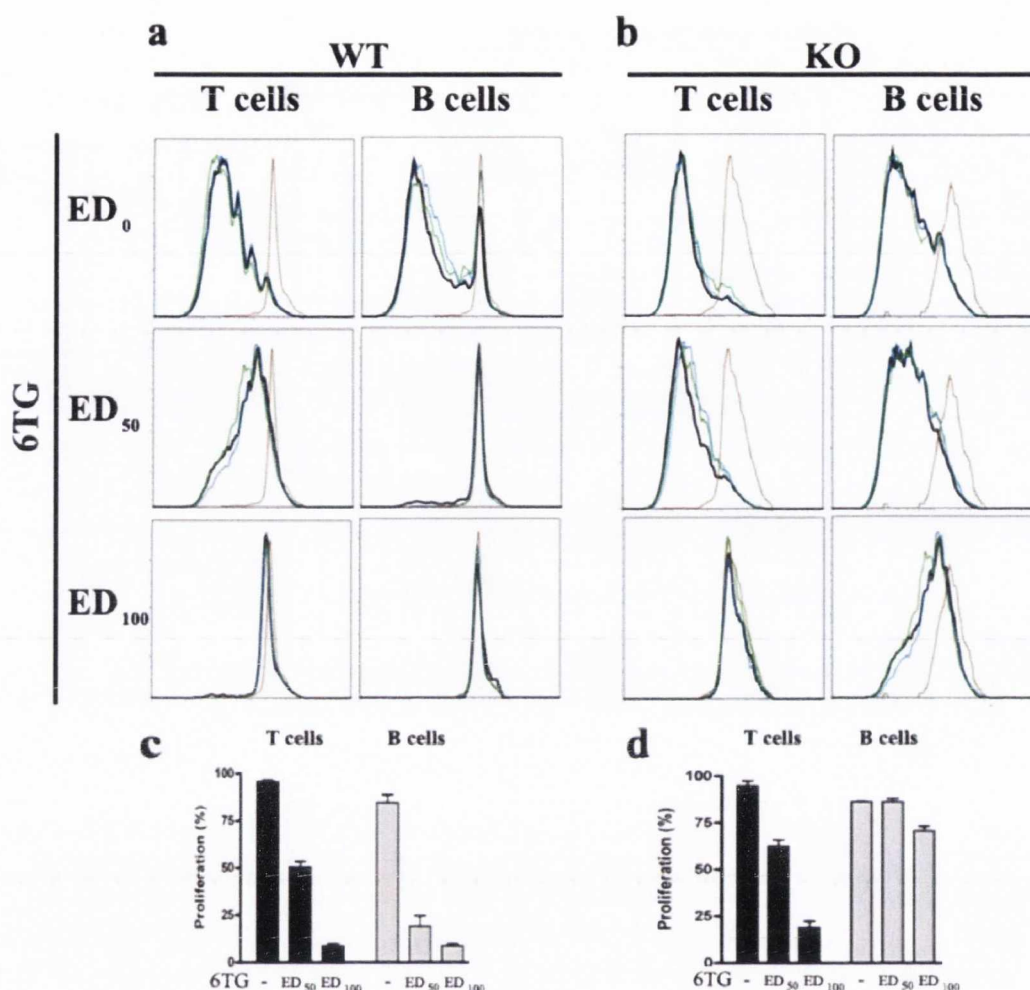


Figure 4.6: Sensitivity of wild-type and *Hprt1^{b-m3}* T and B cells to the anti-proliferative effects of 6TG

Representative FACS plots show the dilution of CFSE fluorescence of proliferated T cells (Pe-Cy7 CD3 labelled) and proliferated B cells (AlexaFluor647-CD45R labelled) from spleenocyte cultures generated from wild-type (**a**) and *Hprt1^{b-m3}* or KO (**b**) female animals. Stimulations for the expansion of T cells (plate bound anti-CD3 ϵ in XVivo/IL2 medium) and B cells (with LPS and soluble α CD40) were carried out on day 0. On day 1 post-plating cells were administered ED₀, ED₅₀ (0.06 mM, 6TG for wild-type cells and 0.65 mM 6TG for *Hprt1^{b-m3}* cells) and ED₁₀₀ (1.5 mM 6TG). The brown trace represents the lack of proliferation in the control unstimulated sample, while the green traces represent triplicates at the corresponding concentration.

Percentage proliferation of T and B cells in 6TG treated spleenocyte cultures from wild-type (**c**) and *Hprt1^{b-m3}* (**d**) animals.

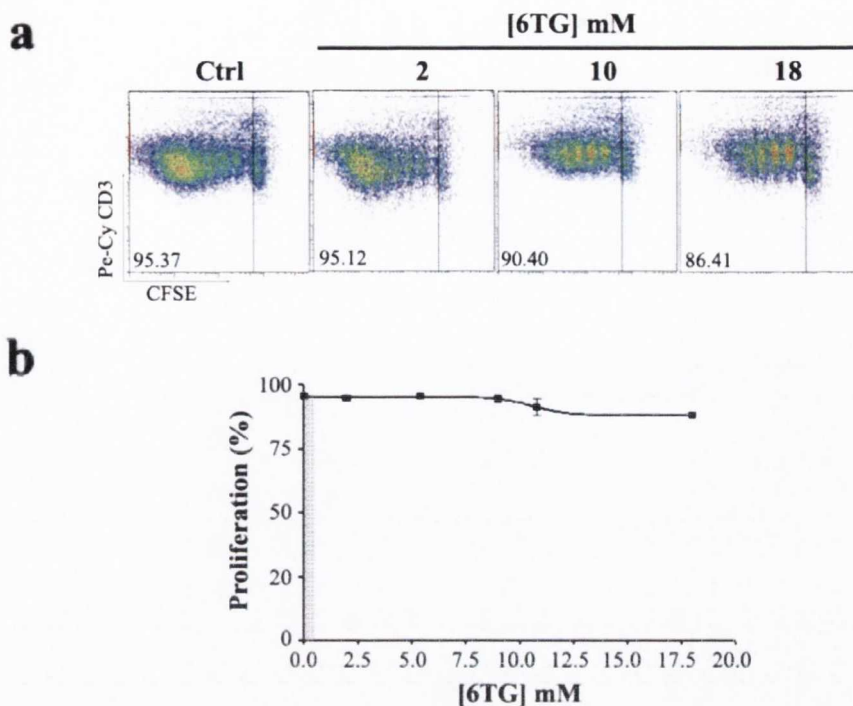


Figure 4.7: Growth inhibitory effect of 6TG on proliferating *Hprt1^{b-m3}::Qtrt1^{Gt/Gt}* T cells
(a) Whole spleenocytes were isolated from *Hprt1^{b-m3}::Qtrt1^{Gt/Gt}* animals and labelled with 1 μ M CFSE before being plated on anti-CD3 ϵ coated dishes in Xvivo/IL-2 medium. On day 1 post-plating cells individual wells were administered increasing concentrations of 6TG. On day 5 cells were harvested, labelled with Pe-Cy7 CD3 and analysed by FACS. Representative FACS plots show the dilution of CFSE fluorescence of proliferated T cells which had been treated with 0, 2, 10 and 18 mM 6TG.
(b) Titration curve showing the average percentage proliferation from triplicates of T-cells treated with increasing concentration of 6TG. 6TG treatment of *Hprt1^{b-m3}::Qtrt1^{Gt/Gt}* cells did not have any appreciable effect on T cell proliferation. The shaded area of the graph indicates the concentration of 6TG measured in the serum of psoriatic patients treated with the drug (Murphy *et al.*, 1999).

Chapter 5: Results

Therapeutic validation of the queuine-tRNA pathway in EAE

5.1 Introduction: Role for T cells in MS Pathology

Based on EAE models, MS was regarded as a T cell mediated disorder, more specifically an antigen-specific CD4⁺ T cell disorder—consistent with the genetic risk associated with HLA class II / MHC class II polymorphism. This was further supported by the ability of adoptively transferred CD4⁺ T cells to induce EAE and the *in vitro* studies reporting an expansion of antigen-specific CD4⁺ T cells to exogenous antigens (McFarland and Martin, 2007). However, later EAE studies in mice showed that disease progression was not restricted to just one T cell subset, since CD8⁺ T cells were also found to be equally encephalitogenic *in vivo* and *in vitro* (Huseby *et al.*, 2001; Sun *et al.*, 2001; Gold *et al.*, 2006). Immunohistochemical analyses demonstrated that while CD4⁺ T cells were detected mainly around perivascular cuffs, CD8⁺ T cells were found to be more prevalent in the centre or borders of the inflammatory lesions (Neumann *et al.*, 2002; Hemmer *et al.*, 2006, Gold *et al.*, 2006).

Today, after decades' of accumulated knowledge on EAE and MS pathology, the list of immune components involved in MS pathogenesis has grown extensively. However, it is still widely held that the T cell population is the principle instigator and mediator of the disease (Epps, 2005). This role is attributed to them for their unique ability to clonally expand upon activation, infiltrate into the CNS where they secrete soluble factors and recruit other immune components to sustain the inflammatory response in the CNS, as illustrated in **Figure 5.1**.

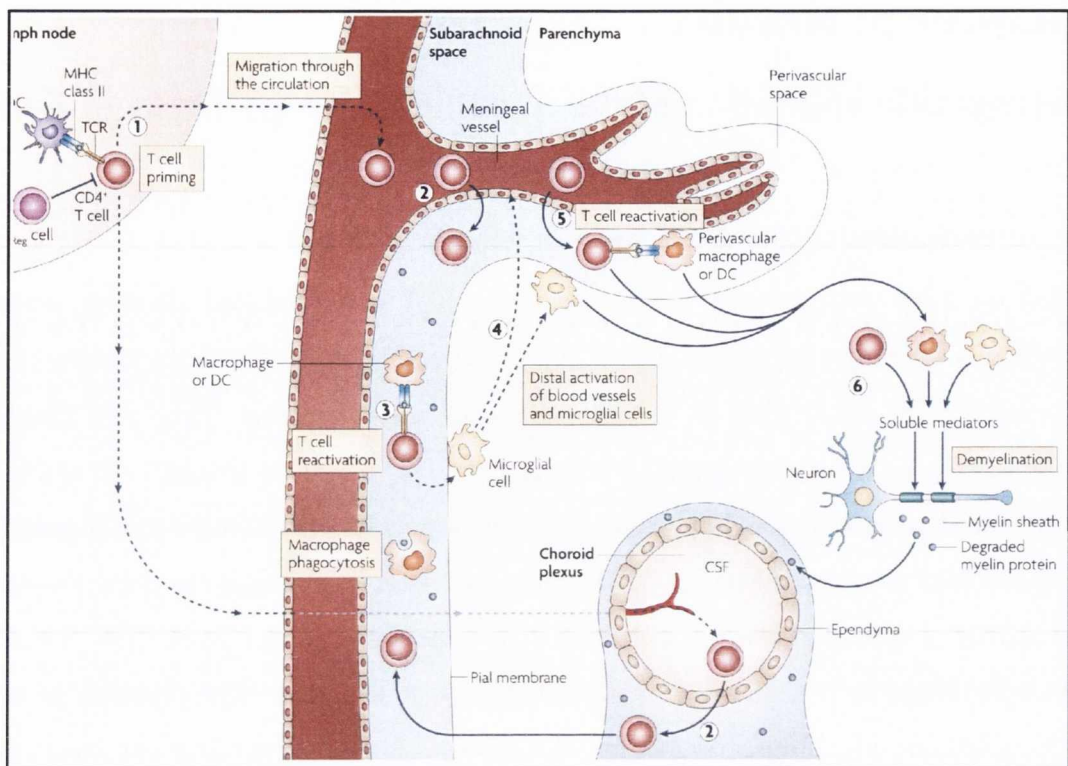


Figure 5.1: Multiple Sclerosis, a T cell pathology.

CD4⁺ T cells activated in the periphery against either the myelin-epitopes presented on peripheral DCs or soluble myelin antigens that are drained from the CNS to lymph nodes to be phagocytosed by local APCs (1). The activated CD4⁺ T cells now express adhesion molecules allowing them entry into the CNS via the subarachnoid space (SAS) by crossing the blood–cerebrospinal fluid (CSF) barrier either in the choroid plexus or the meningeal venules (2); In the CNS, the T cells are re-activated by MHC class II-expressing macrophages and DCs expressing myelin epitopes (3). The now reactivated T cells activate local microglia leading to eventual activation of distal microglial cells and blood vessels (4). The activated T cells adhere to and cross the activated blood–brain barrier, enter the perivascular space and are reactivated by perivascular macrophages and DCs (5). T cells enter the parenchyma and, together with activated macrophages and microglial cells, secrete soluble mediators that trigger demyelination (6). Adapted from (Goverman, 2009).

Peripherally activated T cells in the MS brain: The CNS consists of the brain, spinal cord and neuronal network that are separated from the rest of the body by a physiological barrier that demarcates the peripheral blood vessels from the brain parenchyma. This barrier, commonly termed the blood brain barrier (BBB), is formed by specialized tight junctions between the endothelial cells of the blood vessel, surrounded by a basement membrane which is further protected with a membrane consisting of astrocyte feet and microglia (Goverman, 2009). The physiological role of this membrane is to limit the migration and infiltration of peripheral immune cells into the CNS—partially accounting for the limited immunological activity of the brain (Wenkel *et al.*, 2000; Rezai-Zadeh *et al.*, 2009). Studies have shown that upon injection of neurotoxins or xenogenic tumour implants into the CNS parenchyma, the trigger for a typical immune response is either absent or delayed (Perry, Bell *et al.* 1995). This delayed reaction to immune challenge helps prevent an increase in pressure in the inelastic dural lining from inflammatory swelling—a condition that could lead to tissue damage. In addition, due to the restricted regenerative capacity of the brain, the barrier ensures the selective or modified immune reactivity of the CNS (Goverman, 2009).

The brain sustains its immune-privileged state by modulating the expression of surface adhesions according to the immune response required. During inflammation, expression of these adhesion molecules is upregulated to recruit immune cells to distinct regions of the CNS. In the absence of any inflammation, the endothelial cells do not express any adhesions molecules on their surface, thus depriving immune cells from any means to gain entry into the CNS. However, memory T cells are known to frequently traffic in and out of the CNS for the purpose of immune surveillance. It has been proposed that these cells enter the CNS *via* the subarachnoid space (SAS)—the region between the arachnoid and pial membrane (Ransohoff *et al.*, 2003). Entry *via* the SAS is facilitated by the constitutive expression of selectins and adhesion molecules on memory T cells.

Unlike memory or activated T cells, naïve T cells do not express molecules to bind selectins and therefore cannot enter the CNS, even *via* the SAS. This route of entry has been reported to be important for T cell infiltration into the brain in EAE (Kivisakk *et al.*, 2009). This means that in EAE, the myelin-reactive T cells must

initially be activated in the periphery before infiltrating into the CNS. Activation of a self-reactive T cell requires the self-antigen to be present in the periphery where the naïve auto-reactive T cells circulate. Studies have shown that transgenic mice expressing TCR specific for proteolipid protein (PLP) or myelin basic protein (MBP) develop spontaneous EAE (Furtado *et al.*, 2008). This suggests that PLP and MBP, products of the oligodendrocytes (that reside uniquely in the CNS), are constitutively present in the periphery and can trigger activation of potential auto-reactive T_H1 (T helper 1, pro-inflammatory) CD4⁺ T cells (Goverman, 2009). These peripherally activated T cells, upregulate surface adhesion molecules thereby allowing entry into the CNS.

Studies on the expression of chemokine receptor-6 (CCR6) by a subset of pathogenic T cells has reported its ability to facilitate T cell entry into the CNS *via* ligation to its chemokine ligand-20 (CCL20), which is constitutively expressed by the epithelial cells of the choroid plexus—in line with the initial studies showing that T cells first cross the CSF-barrier to initiate EAE (Reboldi *et al.*, 2009). This is also consistent with the reports that inflammation in EAE is first detected in the SAS, the primary site for CD4⁺ T cell infiltration (Lassmann and Wisniewski, 1978; Goverman, 2009). Once within the CNS, the activated T cells bind to their cognate ligands presented to them on MHC II APCs such as macrophages and microglia, the most predominant macrophage types found in the perivascular space. The reactivated T cells are triggered to proliferate and form large T cell aggregates. Along with the proliferative expansion of T cells in the SAS, the secretion of tumor necrosis factor (TNF) induces activation of the perivascular endothelial cells which then upregulate adhesion molecules to further enhance CD4⁺ T and other immune cell recruitment and infiltration (Goverman, 2009). EAE studies also revealed the presence of other T_H1 cytokines in the CNS and CSF such as interferon- γ (IFN γ) and IL-2, the expression of which has been reported to correlate with increased clinical activity in MS patients (Gutcher and Becher, 2007). Secretion of IFN γ is known to upregulate MHC II expression in the CNS triggering production of chemokines to attract and activate macrophages and monocytes, thus amplifying the pro-inflammatory milieu of the CNS (Segal and Shevach, 1996).

The above scenario was the generally accepted paradigm until studies in IL-12 deficient animals showed these mice succumbed to even more severe EAE, despite

the absence of this T_H1 differentiating factor (Steinman, 2007). From this work, the identification of a distinct T-cell subset arose referred to as T_H17 cells, which are characterized by production of IL-17 (A and F) and IL-22. The ability of this pathogenic subpopulation to promote the production of pro-inflammatory cytokines such as IL-6, granulocyte/macrophage colony-stimulating factors (GM-CSF), matrix metalloproteinases and CXC chemokines, has strongly suggested its potent role in mediating CNS inflammation (Korn, 2009; Goverman, 2009). Therefore, it comes as no surprise that recent studies have suggested and reported that T cells expressing IL-17 and IFN γ could be crucial in EAE pathogenesis (Axtell *et al.*, 2006; Ivanov *et al.*, 2006; Goverman, 2009).

Similar to naïve CD4⁺ T cells, naïve CD8⁺ T cell are also unable to gain entry into the CNS (Goverman, 2009). Their peripheral activation is required therefore, but the mechanism remains unclear. It has been suggested that activation of naïve CD8⁺ T cells could be accomplished by peripheral APC's that express antigen by MHC class I. However this hypothesis does not stand very strong as MHC I only presents antigen proteins that are synthesized within the cell and in this case, the only myelin-synthesizing cell is the oligodendrocyte (ODc), which is solely resident in the CNS and therefore unable to present myelin to the naïve CD8⁺ T cell in the periphery. But as bone-derived cells are known to synthesize and present myelin peptides on MHC I molecules, perhaps there are other APC's that can present the same in the periphery (Perchellet *et al.*, 2008). Alternatively, either the myelin peptides are cross-presented by dendritic cells (DCs), macrophages or possibly the endothelial cells present degraded myelin products on their surface on MHC I molecules. The exact mechanism is yet to be fully characterized.

Once in the CNS, CD8⁺ T cells must be re-activated. In non-inflamed CNS, expression of MHC I is limited to cells that do not synthesize myelin and hence theoretically are incapable of interacting with myelin-specific CD8⁺ T cells (Galea *et al.*, 2007). Perhaps, this may be overcome by some cross-presentation facilitated by DCs and macrophages, but again this mechanism is unclear. However under an inflammatory environment, all CNS-resident cells are known to upregulate MHC I along with much higher incidence of cross-presentation, providing many venues for CD8⁺ T cell reactivation (Na *et al.*, 2008). Similar to CD4⁺ T cells, re-activation of CD8⁺ T cells leads to massive proliferation. Studies in MS samples have reported that

CD8⁺ T cells compose a significant percentage of the inflammatory cell pool (Babbe *et al.*, 2000; Neumann *et al.*, 2002; Hemmer *et al.*, 2006). The pathogenicity of CD8⁺ T cells is conveyed by their cytotoxic ability that results in lysis upon cell-contact. Studies on early stage MS have shown that axonal injury correlates with the number of CD8⁺ T cells in the CNS, where the cytotoxic granules are polarized towards the oligodendrocytes and axons in the MS lesion. In EAE also, CD8⁺ T cells are more prevalent in the centre or borders of lesions that mediate CNS pathology (Gold *et al.*, 2006). Further damage is promoted by the secretion of pro-inflammatory cytokines such as IFN γ , TNF, and IL-4 among others. Immunohistochemical analysis has shown that up to 70% of the T cell infiltrate are IL-17 expressing cells, equally distributed between CD4⁺ and CD8⁺ T cells. Although both populations have been implicated in MS their mechanism of damage induction is different. Variation in the CD4⁺ and CD8⁺ T cell populations in human MS patients could therefore account for the heterogeneity seen in tissue damage and clinical progression of the illness (Friese *et al.*, 2008; Goverman, 2009).

Continuing the focus on T cells, another subset that has recently emerged as being distinctly pathogenic in EAE is the $\gamma\delta$ T cell population (Blink and Miller, 2009). They are so named from the distinct expression of $\gamma\delta$ chains on their surface instead of the commonly found $\alpha\beta$ chains that are indicative of CD4⁺/CD8⁺ T cells. $\gamma\delta$ T cells constitute 1-5% of the total blood lymphocyte population and are commonly found in the skin and mucosal tissues where they comprise up to 50% of the T cells. $\gamma\delta$ T cells have been linked to pathogenesis and have been isolated from the serum of patients with autoimmune disorders including diabetes, arthritis and MS. Similarly, the role for $\gamma\delta$ T cells in disease pathogenesis in EAE has been studied and validated. However, their exact function is yet to be elucidated because while this subset has been shown to concentrate in EAE and MS lesions contributing to inflammation, their depletion has also been reported to exacerbate the clinical severity of the disease by impairing clinical remission (Ponomarev and Dittel, 2005). The importance given to this subset in EAE also goes hand-in-hand with the discovery of the new IL-17 producing pathogenic cells. Adoptive transfer experiments have reported that up to 60% of the IL-17 producing cells were $\gamma\delta$ T cells. Moreover, when this experiment was conducted in the *IL17*^{-/-} mice, EAE severity was found to ameliorate (Lees *et al.*, 2008). Therefore, although further experiments are required to clarify their exact role

in disease, the current focus given to this subset cannot be ignored.

Regulatory T cells in the MS brain: Studies on the spontaneous T cell transgenic EAE model, found that Forkhead Box P3 (FOXP3) expressing T cells, known as Tregs, could prevent EAE progression by suppressing the activation of myelin-specific CD4⁺ T cells (Lafaille *et al.*, 1994). The immunosuppressive property of this cell type is characterized by secretion of the anti-inflammatory cytokines IL-10 and TGFβ (Cabbage *et al.*, 2007). It was hypothesized that in the absence of Tregs, any immunogenic stimuli would overcome regulation, causing a potential breach in tolerance (Goverman, 2009). This idea was soon questioned as the suppressive abilities of Tregs were only found to be effective in the CNS when the local levels of pro-inflammatory IL-6 and TNF were low. In MS patient samples, the function of peripheral Tregs was found to be impaired, but they were undetected in areas of lesions activity (O'Connor and Anderton, 2008). Therefore, their exact role in EAE/MS is still under scrutiny.

B cells in the MS brain: Based on the early studies by Kabat, which reported elevated levels of immunoglobulin (Ig) in the CSF of MS patients (Kabat *et al.*, 1948; Kabat *et al.*, 1950), and the requirement for co-administration of auto-antibodies in the MOG EAE protocol, a potential role for antibody producing cells was suspected for the pathogenesis of MS (Genain *et al.*, 1996). This has been supported further by B-cell depletion studies in MS patients which provided substantial benefit to patients (Hauser *et al.*, 2008). The role of B cells in MS ranges from antigen-presentation to auto-reactive T cells to the production of anti-myelin/axon/neuronal antibodies (McLaughlin and Wucherpfennig, *et al.*, 2008). They share similar proliferative, infiltration and cytokine production ability with T cells during an immune response. Immunoglobulin gene rearrangements differ between B cells isolated from CSF and periphery of patients suggesting that the B cells are required to mature in the CNS by responding to specific antigens (Colombo *et al.*, 2000). Although most antigen-specific antibodies are yet to be characterized, recent analysis of MS patients revealed specificity of B cells for neurofascin—a myelin protein found in the myelin-axon interfaces, called nodes of Ranvier. When these neurofascin antigens were subjected to EAE experiments, it was found to exacerbate the clinical severity of EAE by

specifically causing axonal injury (Mathey *et al.*, 2007; Goverman, 2009). However, in contrast to their pathogenic role, B cells have also been recently shown to mediate an immunoregulatory response by interacting with Treg cells and secreting IL-10—an anti-inflammatory cytokine, important during recovery (Mann *et al.*, 2007; Fillatreau *et al.*, 2002). It is noteworthy that the IL-10 producing B cells, sometimes referred to as Breg cells, vary with respect to disease progression especially between EAE studies and samples from MS patients (Duddy *et al.*, 2007; Goverman, 2009). So, although it is suggested B cells participate in disease progression, as opposed to disease initiation, their mechanism needs to be further investigated.

CNS-resident cells in the MS brain: The heterogeneity of MS clinical activity observed between patients has lead to an immunopathologic classification based on lesion pattern. Pattern I plaques are characterized by T-cell and macrophage associated myelin damage, whereas Pattern II lesions are caused by antibody and complement components at the site of myelin degradation. Pattern III plaques are caused by oligodendrocyte dystrophy, apoptosis and brain hypoxia while Pattern IV is characterized by degeneration of oligodendrocytes adjacent to demyelinated plaques (Herndon, 2000). It is noteworthy that regardless of the phenotype/pattern, all MS lesions present with inflammation-induced damage involving lymphocytes, activated macrophage and microglia (Lassmann, 1999).

The ability of the different EAE models to recapitulate certain key aspects of each MS pattern has been very beneficial to studying pathology. Berger studied the effect of the number of T cells adoptively transferred on the severity of the lesions. He reported that rather than correlating with the number of activated T cells, clinical severity correlated with the absolute number of activated macrophages in the parenchyma instead (Berger *et al.*, 1997; Lassmann, 1999; Gold, 2006). This is reflected in MS Pattern I EAE model immunized with MOG₃₅₋₅₅ where activation of macrophages is found pronounced in the CNS. Macrophages can either infiltrate from the periphery into the CNS upon activation, or can be resident APCs in the CNS, the microglia. Activation of these cells causes them to cluster at the lesion site, known as microgliosis. This is a key marker of neuroinflammation as they cause tremendous damage to the surrounding tissue. It has already been discussed that post-immune stimulation in the CNS the resident immune cells ‘take-over’ and sustain the

inflammation caused by infiltrating immune cells (Berger *et al.*, 1997). They do so by secreting large numbers of cytokines—such as TNF α , IL-1 β , IL-2, 4, 6 and 18—leading to functional blockade and structural damage to axons. These cytokines affect the function of other non-immune glial cells such as astrocytes and oligodendrocytes in the CNS. While astrocytes are known to provide neurotrophins that promote neuronal growth, oligodendrocytes are specialized myelin producing cells that coil around the axonal bodies or neurons providing insulation for transmission of action potential (Ponomarev *et al.*, 2005).

Activation of microglia is not always associated with damage induction, as these cells also promote repair through the secretion of growth factors; for example, BDGF (brain derived growth factor) and EGF (epidermal growth factor). Moreover, even after activation, microglia are known to switch from T_H1 to a T_H2 (anti-inflammatory) response in the presence of cytokines like IL-10 and TGF β , discussed above (Allen *et al.*, 2001; Ponomarev *et al.*, 2005; McFarland and Martin, 2007).

Even with only a few components of the EAE/MS pathology covered above, one gets a sense of the complex inter-play of immune and non-immune cells in this disease. Similar to other autoimmune diseases, MS can be seen as an imbalance of the immune response caused by a breach in tolerance and regulation.

5.1.1 Current MS therapies

Currently, there are no cures for MS. The range of MS drugs encompasses antigen-specific and antigen non-specific molecules that aim to ameliorate patient disability. They do so by a number of means including preventing acute attacks, controlling/reducing relapses, slowing disease progression or promoting repair.

The drugs either target immune cell activation, proliferation, cytokine production or even migration to lymphoid organs and the CNS. The effectiveness of the majority of these drugs was tested in EAE models prior to gaining FDA approval. Although some of the EAE validated drugs have shown different effects *in vivo* or have even had to be retracted from the market, EAE still provides an unparalleled and cost-effective *in vivo* platform to further our knowledge on drug action and efficacy.

The Queuine t-RNA pathway in MS: In this project, the queuine tRNA pathway has been shown to convey T cell anti-proliferative activity *in vitro*. The current chapter builds on this earlier work to investigate the therapeutic capacity of this pathway *in vivo* using the MOG induced chronic EAE model.

5.2 Results

5.2.1 6TG *via* the queuine-RNA pathway ameliorates clinical severity in EAE

EAE, as an animal model for MS, provides an unparalleled *in vivo* system to study the potential therapeutic benefit of new drug molecules (Farooqi *et al.*, 2010). Despite the criticisms leveled at the reliability of this model, at present it is the only cost-effective system available for preliminary/pre-clinical observations. Moreover, even post FDA approval it is common practice for drug studies to include work on EAE models to further elucidate the mechanism of the drug.

Both EAE models—chronic monophasic and RREAE—are actively induced and are by nature contradictory to the spontaneously occurring human disease. Despite this shortcoming, if one is interested in the post-induction phase of EAE, as we are, then the model provides extrapolatable resemblance to the clinical episodes in MS. That is why in studies involving currently approved MS therapies like GA, Interferons, Fingolimod, Natalizumab, the effect of the drug post-disease onset is more valuable than as part of a preventive regime, especially when one considered that, in patients the disease is only diagnosed after onset and at times only confirmed once clinical disability has been established. Therefore, a therapy with palliative/therapeutic capacity, as opposed to prophylactic activity, is needed. In line with the above, the ability of the queuine tRNA pathway to convey therapeutic amelioration was assessed by administering 6TG to Hprt knockout (KO) female mice after the onset of clinical disability.

Clinical severity score: After an intensive period of coordinated breeding of knockout animals, a total of 32, 8-10 week old age-matched HPRT knockout female mice were equally divided into four experimental groups of eight mice each: Naïve group (solid black circles, ●) was left untreated, 6TG group (solid black squares, ■) was treated with 6TG alone, EAE group (solid black inverted triangles, ▼) was immunized with EAE alone and EAE+6TG animals (empty red circles, ○) were immunized for EAE and administered 6TG as per the indicated regime (inverted red triangles ▼), as shown in **Figure 5.2a**.

As per the standard protocol, EAE was induced by subcutaneous immunization with MOG in CFA and by inter-peritoneal (i.p) injection of pertussis toxin on day 0 followed by a booster i.p injection of pertussis toxin on day 2. This induces a monophasic chronic disease with a clearly visible clinical onset by day 6-7 as shown in **Figure 5.2a**. The start of disease is defined by a limp tail-tip, an early indication of paralysis. Progressively, this paralysis ascends towards the base of the tail (score of 0-1.5) before affecting the hind limbs (score of 1.75-3.5) and subsequently the forelimbs (score of 3.75-4.75) until the animal reaches a moribund state (score of 5; requiring that the animal be sacrificed).

The clinical severity for the EAE group correlated with weakness in both hind limbs and an average clinical score of 2 by day 9. The disability further progressed to the forelimbs around day 13 where the average clinical score reached a peak of 3.75. This also marked the entry into the monophasic stage of the disease where the physical disability was sustained at an average clinical score of 3.25-3.5 until the end of the experiment on day 21.

The animals in the EAE+6TG group, similar to the EAE group, showed a similar disease onset attaining an average clinical score of 2 by day 9, as shown in **Figure 5.2a**. Over the next 13 days, these animals received a total of 9 6TG injections consisting of 6 daily injections followed by 3 injections on alternate days, as denoted in **Figure 5.2a**, by the ▼ symbol. Following 6TG administration, animals in the EAE+6TG group showed a much different trend to those in the EAE group. On day 10, which was 24 hours after the first 6TG injection, the clinical score of the animals did not deteriorate further. In fact, within 24 hours the clinical severity started to decline and over the next seven days the paralysis continued to abate with an approximate decrease in clinical score of 0.25 every 48 hours. Three days before the end of the experiment the overall score continued to decrease but at a slower rate. On day 21, the average score recorded for the EAE+6TG animals was 0-0.25, with a total of 5 out of 8 animals showing complete lack of clinical disability. The individual disease progression for the eight animals in the EAE+6TG group is illustrated in **Figure 5.2b**. Here, out of the eight animals, two sub-groups of four animals are discernable. One sub-group show a clinical score of 2-3 (High-EAE+6TG) as shown in **Figure 5.2b**, **dark blue lines**, while the second subgroup presented with a clinical score of 1.5-2 (Low-EAE+6TG), as shown in **Figure 5.2b**, **light blue lines**. Out of the four animals in the High-EAE+6TG sub-group, one animal showed a clinical score of

0 by day 18, whereas the others showed a decreased clinical score of 0.25 for two animals by day 20-21 and a score of 1.25 for the fourth animal by day 18, as shown in **Figure 5.2b, dark blue lines**. All four animals in the Low-EAE+6TG sub-group show complete recovery from clinical disability, having a clinical score of 0 on days 12, 13, 16 and 19 dpi for the individual animals, as shown in **Figure 5.2b, light blue lines**. In the control experimental groups, Naïve (un-immunized and untreated) and 6TG (un-immunized), no clinical signs of were visible throughout the EAE experiment, as expected. Importantly, the pattern described above for the clinical score for each group, correlated with the physical agility and animal weights, as described further below.

Physical disability test: During the course of the disease there is a gradual loss of physical strength and motor function. The clinical score, although universally accepted as a means to record disease progression, may be considered slightly biased due to its semi-quantitative nature. Therefore, for a more unbiased approach, as well as to restore further confidence in the clinical scheme, each animal was examined using the Horizontal Bar Test. On three consecutive days prior to immunization, all animals were habituated to the apparatus, to ensure uniformity in task completion between groups. Following EAE immunization, each animal was subjected to this physical test every 72 hours.

The average time taken for animals from each group to arrive at the second platform of the apparatus is graphed in **Figure 5.3a**. On days 0-3, the average time taken by all animals was around 4-6 seconds. Between days 6-9, the EAE (▼) and EAE+6TG (prior to any 6TG administration; ○) animals showed a slight increase in the average time taken to perform the same task; 7-9 seconds. The animals in the Naïve control (●) and 6TG control (■) groups continued to take approximately 4-6 seconds to complete the task. Over the next 6 days, animals in the EAE group began to fall off the bar as soon as their front paws were placed on the beam, correlating with the loss of motor function seen earlier. This loss in grip and subsequent falling is shown as a drastic decrease in the ‘time on bar’, dropping to a value of 0 seconds by day 15, without any subsequent recovery. The animals in the EAE+6TG group however (from day 9) showed a gradual amelioration of their physical/motor functions. By day 15, the animals take 5-6 seconds to complete the task similar to control Naïve and 6TG groups. The animals in the control groups do not show variation in their ability to perform the task during the course of the experiment.

Body weight: Similar to the clinical manifestation of many acute illnesses, in EAE there can be a loss in body weight up to 15%. Any loss in body weight exceeding 18 % is considered beyond experimentally expected variation and the animals are sacrificed to prevent any further distress.

The graph in **Figure 5.3b** shows the change in average body weight for animals in their respective groups during the experimental time line. Over the 21 day study, the animals in the Naïve and 6TG control groups showed a gradual increase in average body weight from 17.5g at the start of the experiment to around 20.25g by the end of the experiment. By contrast, the diseased animals in the EAE group showed a gradual loss of total of total body weight from an initial 17.5g to an average mass of 15.25g by the end of the experiment. It is worth stressing that at the onset of clinical disability, food and water were provided daily in small petri-dishes placed on the floor of the cage to ensure animals had access to food and water. Therefore the loss of body weight is linked to illness and not to starvation.

The EAE+6TG animals were approximately 18g at the start of the experiment and followed a similar trend to that of the EAE diseased animals up to day 12. Subsequently however, they started to gain body weight similar to the Naïve control animals. By day 21, they had a weight of 20-20.25g, correlating with the lack of disease progression.

The above data reports the therapeutic effect of exploiting the Queuine tRNA pathway on the physical disability and body weight in chronic EAE. The various parameters examined—clinical score, weight and motor function—are universally accepted as a means to document EAE progression prior to sample harvest and analysis. However, these assessment techniques are inadequate to prove therapeutic changes in either the immune system or CNS. To investigate this, peripheral and CNS organs were isolated upon termination of the experiment for biological analysis.

5.2.2 6TG via the queuine-RNA pathway reduces T_H1 and T_H17 T cells in peripheral/secondary lymphoid organs

Following sensitization of mice with MOG, the subcutaneously injected bolus on the animals back provides a site for immune stimulation. The MOG antigen initiates an immune response in which immune cells are recruited and activated. It is noteworthy that in the actively induced EAE models, the injected concoction elicits a peripheral immune response against a CNS peptide (MOG) requiring the self, MOG-activated

immune cells to infiltrate into the CNS to complete their immune task.

Peripheral immune stimulation entails activation and proliferation of T cells against the injected MOG prior to an even greater re-activation once inside the CNS. The spleen, cervical and inguinal draining lymph nodes (ILN, CLN) are some of the secondary lymphoid organs where the post-thymic differentiated naïve T cells rest prior to involvement in an immune response. These organs were harvested therefore and analyzed for their total immune cell number and their antigen specific responses.

Whole spleen, ILN and CLN cells were labeled with FACS antibodies to quantify the various T-cell populations (CD4, 8 and $\gamma\delta$) in the periphery. Overall, the same pattern was observed in each lymphoid organ within the same experimental group i.e. all controls gave similar data relative to diseased and treated animals. The Naïve and 6TG control groups showed basal T-cell levels, with slightly elevated levels in the 6TG samples. This difference, when statistically analyzed was found not to be significant (ns). The animals in the EAE group showed a drastic elevation in cell number, suggesting an on-going immune reaction and the proliferation of T cells. In the EAE+6TG group, despite EAE induction, drug administration caused a dramatic decrease in the total cell numbers. The decrease brings the average number of cells down to similar values to the 6TG control group and subsequently similar to that of the Naïve group. The consistent pattern is seen in each cell type analyzed from Spleen (**Figure 5.4**), ILN (**Figure 5.5**) and CLN (**Figure 5.6**). This is indicative of the systemic effect conveyed by exploiting the queuine tRNA pathway.

5.2.3 6TG *via* the queuine-RNA pathway dampens MOG-specific T cell responses in the periphery

Although a significant reduction was observed in all the immune-cell populations analyzed, a variety of mechanisms could be postulated to explain the effect. Therefore, it seemed reasonable to look at the MOG-specific encephalogenic T cell response and the mitogen (α CD3/PMA)-specific control proliferative response of the T cell population.

For this, single cell suspensions were prepared from the spleen and lymph nodes (inguinal and cervical) and plated in either medium alone (negative control), or increasing concentration of MOG at 2 μ g/ml, 10 μ g/ml or 50 μ g/ml, and finally α CD3/PMA as a positive control, as shown in **Figure 5.7**. Proliferation was

determined by scintillation counting in terms of radioactive counts per sample. The data recorded shows that in all the peripheral lymphoid organs analysed, both Naïve and 6TG control groups do not respond to MOG antigen, whereas the CD3/PMA mitogenic stimulus gave a robust proliferation of about $2.0\text{--}3.5 \times 10^5$ counts, as would be expected. By contrast, in the EAE animals, MOG induced a dramatic dose-dependent T cell proliferative response. Spleen gave the most robust response at 50 $\mu\text{g/ml}$ MOG, yielding $\sim 3.5 \times 10^4$ counts, while the proliferative stimulus with CD3/PMA was yet higher still at $\sim 3.0 \times 10^5$ counts. Remarkably, when splenocytes from the EAE+6TG groups was subjected to the same stimulations, they respond weakly to even the highest concentration of MOG, $\sim 0.5 \times 10^4$ counts, whereas the response to the mitogenic CD3/PMA stimulation was still robust, at $\sim 2.8 \times 10^5$ counts, as shown in **Figure 5.7**. The same pattern was observed in the ILN where the response to MOG in the EAE samples was $\sim 1.9 \times 10^4$ counts whereas in the EAE+6TG group that fell to an average of $\sim 0.8 \times 10^4$ counts. The samples from CLN do not show significant MOG-specific proliferation even in the diseased samples. In all groups however the mitogenic stimulated cells give a robust proliferation.

5.2.4 6TG via the queueine-RNA pathway reduces immune cell number in diseased brain

The peripheral immune response has a major impact on the inflammatory status of the CNS in EAE. One would expect that prior to CNS infiltration a suppression of the ongoing peripheral activation would dampen the immune response and as a result alter/reduce CNS inflammatory activity. However in this experiment, the animals were allowed to reach a clinical score of 2 before drug treatment; a situation where CNS inflammation and damage must already be in progress. Importantly, the queueine tRNA pathway proved effective in ameliorating the clinical and physical disability of the animals. At the physiological level, this should correlate with a reduction in inflammatory activity in the CNS. To investigate this, whole brain from each group was harvested and subjected to Percoll gradient separation to allow the isolation of mononuclear cells (MNCs). The MNCs were then labeled with FACS antibodies to look at total T cell number (CD3) and various T-cell sub-populations (CD4, CD8 and $\gamma\delta$) in the CNS and the intracellular levels of the cytokines IL-17 and IFN γ .

FACS analysis: The CNS has been long considered an immune privileged organ where the physiological barrier ensures minimal exchange with the periphery. In Naïve and 6TG control samples, our FACS analysis demonstrate a lack of immune cell trafficking in and out of the CNS, as represented by the very low total levels of CD3, CD4 and $\gamma\delta$ T cells, **Figure 5.8**. There does seem to be a significant amount of CD8 cells, however most of them are negative for intracellular IL-17 and IFN γ , suggesting that they are non-pathogenic memory or naïve T cells.

The EAE diseased brain samples show an increase in the average total cell count by almost 2-3 fold whereas in the EAE+6TG group, the average total count is almost 50% lower. The same pattern is observed amongst the T-cell sub-populations that are positive for intracellular IL-17 and IFN γ . All sub-populations of T cells can produce either cytokine, as shown in **Figure 5.8**.

Apart from T cells, other immune cells such as macrophage and neutrophils are known to gain entry into the CNS during inflammation. By exploiting cell surface markers such as CD11b, Ly6G and F4/80, the levels of mature/activated macrophages, neutrophils and microglia can be assessed, as shown in **Figure 5.9**. Exactly the same trend was observed as that described for the T cell populations. The Naïve and 6TG control groups showed basal cell numbers that escalate dramatically in the EAE group. In the EAE+6TG group, despite the continued immune challenge, the cell numbers are almost equivalent to basal levels.

5.2.5 The queuine-RNA pathway ameliorates CNS pathology by affecting microglia and T cell population in EAE

To visually observe the physiological effect of reduced CNS inflammation, whole brains were extracted from PBS perfused animals, embedded in OCT and frozen immediately over liquid nitrogen vapour. The brains were sliced at 10 μ m thickness and stained for CD3 (T cell marker) and CD68 (macrophage and lysosomal marker in activated microglia). Three distinct regions of the brain were sectioned and stained: Striatum, Hippocampus and Brain stem/cerebellum. These markers have been shown to be prominent in lesions of the CNS. The overall lesion/inflammatory activity observed across these three brain regions for the EAE and EAE+6TG animals are summarized in **Table 5.1**.

A total of four brains from the EAE and EAE+6TG groups were analyzed. Each of the four animals within a group were labeled 1-4 along with their clinical disease score at the end of the experiment; shown in parenthesis. This is followed by the brain activity assessment across the three regions where '-' signifies lack of any visible activity, '+' represents visible yet low activity, '++' represents medium level activity, corresponding to immune cell infiltration and some parenchymal activity, and '+++' represents a significant amount of lesions with ventricular and parenchymal activity.

Amongst the animals with EAE there is consistent activity in the hippocampus and brain stem regions whereas only one of the four animals (number 3) showed any activity in the striatum. By contrast, in the EAE+6TG group, the disease activity observed is neither uniform between the regions nor between animals. Animals 1 and 3 from the EAE+6TG group do not show any disease activity in any region of the brain. Animals 2 and 4 show low to medium activity in the hippocampus and brain stem. This correlates with the immunohistochemical staining for each animal explained below, **Table 5.1**.

Immunohistochemistry: Staining for CD68 and CD3 in the striatum is shown in **Figure 5.10**. Naïve and 6TG control animals do not show any positive staining for CD68 or CD3. The diseased animals of the EAE group however show a very active lesion that stains prominently for CD68 and CD3. A higher magnification (x40) is provided to illustrate clearly the recruitment and infiltration of the stained cells. In the EAE+6TG group, none of the animals showed any activity in the striatum.

In the hippocampal region, three areas were examined i) the ventricles under the hippocampus (Lesion area 1), ii) the fimbria (Lesion area 2) and iii) the parenchymal area under the hippocampus (Lesion area 3). Across all regions, neither the Naïve, nor the 6TG control animals show any immune activity. By contrast, in the EAE group, a pronounced signal for CD68 was apparent in each of the hippocampal regions. Staining for CD3 however, proved not to be uniform across the lesions. It is likely that in lesion areas 1 and 2, the CD3 stain correlates with the recruitment and infiltration of T cells to the site of macrophage, and microglial activation whereas, in lesion area 3, the lack of any co-staining of CD3 with CD68 may be due to the activation of the parenchymal microglia, as opposed to the recruitment of macrophage.

Immunohistochemical analysis of the brain stem is shown in **Figure 5.12**. Two main areas have been highlighted—the base and the side. Again, for the animals in the Naïve and 6TG control groups, no activity is visible for either stain. For the EAE group however, CD68 shows staining around the base of the brain stem, in the meninges as well as some activity in the stem parenchyma. The corresponding CD3 stain is concentrated more around the meninges, with fainter staining visible within the parenchyma. Images taken from the same region of the EAE+6TG animals shows no discernable parenchymal activity with very faint meningeal activity. It is noteworthy that in the immunohistochemical slices, slight curling of the slice at the edges also leads to darker staining.

At the side of the brain stem, there is a very active and intense lesion area in one of the EAE animals with a significant staining for CD68 in the meninges as well as into the brain stem parenchyma. The corresponding CD3 stain also shows clusters of CD3⁺ cells in the parenchymal area of the lesion. In the EAE+6TG animals however no significant activity was visible in the parenchyma of any animal. There was some CD68 positive staining around the meninges which could suggest a certain degree of recruitment of peripheral and CNS Macrophages, **Figure 5.12**.

In the cerebellum sections, again neither the Naïve nor the 6TG control animals show any CNS activity, **Figure 5.13**. The EAE animals showed many lesions that stain positive for both CD68 and CD3 markers. Most of these lesions, in panels labeled lesion area 1 and 2, can be described as clusters of infiltrating and activated macrophages, microglia (CD68) and infiltrating activated T cells (corresponding CD3 stain). Lesion area 3 shows a different activity pattern where there are many scattered positively stained cells for CD68 along with CD3 stained cells as well for T cells. Although different from the cluster formation, this lesion is also representative of the CNS activity during EAE involving the CD68 and CD3 expressing immune cells. True to the pattern observed in the EAE+6TG animals so far, in the cerebellum, hardly any significant lesion formation, cell recruitment, infiltration or activation was visible as areas failed to stain positive for CD3 or CD68, **Figure 5.13**.

5.2.6 The therapeutic effect of 6TG is abolished in animals deficient for the queuine tRNA pathway

To ensure that the above effects observed are purely and directly a result of exploiting the queuine tRNA pathway, EAE was induced in the double knockout animals and the effect of 6TG administration was studied.

As explained in Chapter 3, the double knockout animals were generated by crossing transgenic knockout lines for the genes encoding HPRT and TGT. After an intensive round of breeding and screening, pure double knockout 8-10 week old female mice were set-up for the experiment. A total of six animals per experimental group: Naïve, 6TG, EAE and EAE+6TG. The Naïve and 6TG control groups show no sign of clinical disease as expected. When immunized with MOG (**Figure 5.14a** ▽ symbol), the EAE group developed MS symptoms in a similar pattern to that seen in the previous experiments (**Figure 5.14a** ▼ symbol). When the animals in the EAE+6TG group are administered 6TG (**Figure 5.14a** ▼ symbol) according to the exact same regime as that explained for Hprt knockout animals (please see **Figure 5.2**), no therapeutic effect for 6TG could be observed (**Figure 5.14a** ◇ symbol). As such, the EAE and EAE+6TG groups follow the same pattern of disease deterioration during the course of the experiment, proving that the therapeutic effects observed in the *in vivo* model uniquely occur through the queuine tRNA pathway. The schematic illustrated in **Figure 5.14b** represents the pathway for a potential queuine tRNA specific drug molecule.

5.3 Discussion

The absence of a cure for MS has driven many investigators to develop potential drug molecules using not only human MS samples but also the animal model of MS, EAE. The wide range of MS therapies in use today can be broadly classified as being antigen specific or non-antigen specific.

The antigen specific therapies such as cytokines, integrin and receptor antagonists were mainly developed against the EAE model or were initially created to treat other autoimmune disorders, then later applied to MS. This strategy of applying autoimmune therapies across different diseases has not always proved successful. For example, anti-TNF α treatment of inflammatory autoimmune disorders like RA and Crohn's disease was a phenomenal success and suggested that it may be a potential new treatment for MS (O'Shea *et al.*, 2002; Feldmann and Steinman, 2005). However, when used as an MS therapy, the long-term suppression by this potent cytokine, created a dangerously immunosuppressive environment, resulting in an unexpected increase in opportunistic infections such as Tuberculosis, Pneumonia and even drug-induced autoimmune disorders (Feldmann and Steinman 2005). Nonetheless, despite the downfall, it did emphasize the role of other cytokines such as type-1 beta interferons: IFN β -1a (Avonex, Rebif) and IFN β -1b (Betaseron, Betaferon). Although these were not initially validated in EAE, they were one of the first immunomodulatory drugs to be approved for MS treatment due to their ability to inhibit leukocyte proliferation, migration and antigen presentation, as well as promoting the secretion of anti-inflammatory cytokines (Heremans and Biliau, 2004; Friese *et al.*, 2006). Type-1 interferons are known to reduce relapse rate by 30% in patients suffering from RRMS. However, these drugs are effective in less than half of MS patients and due to uncomfortable side effects, such as inflammation at the site of injection and continuous flu-like symptoms, up to 20% of patients fail to maintain their treatment (Clegg and Bryant, 2001).

Other immunomodulatory therapies have focused on immune cell migration, identifying new therapeutic targets such as selectins, integrins and chemokines. From studies in EAE, a crucial role for the integrin molecule $\alpha_4\beta_1$ was discovered in lymphocyte homing and an antibody was developed which was capable of blocking its function (Tysabri) (Miller *et al.*, 2003; Balague *et al.*, 2009). Short-term blockade reduced MS lesions, however blocking $\alpha_4\beta_1$ is not specific in inhibiting immune cell

migration into the brain, instead interfering with lymphocyte homing in general (Feldmann and Steinman, 2005). As a result, rare, opportunistic infections like multifocal encephalopathy were found to occur (Steinman, 2004). Recently, Fingolimod (FTY720) has emerged as a promising therapeutic agent. It acts on sphingosine-1-phosphate (S1P) receptors and sequesters lymphocytes into lymph nodes without directly inhibiting their effector function. Results from Phase II & III clinical trials demonstrate that Fingolimod is highly effective in RRMS (Mandala *et al.*, 2002). At the moment, it is the very first orally available disease-modifying agent to be approved by the US FDA for the treatment of relapsing forms of multiple sclerosis (MS). However, studies have reported skin cancer, hemorrhaging focal encephalitis and inflammation/bleeding of the brain in patients treated with Fingolimod. Therefore, the long-term effects are yet to be fully documented.

Alongside the antigen-specific immunotherapies, many non-antigen specific therapies were also developed and approved. One success story is that of Glatiramer Acetate (GA) (Copolymer-1, Copaxone). It is a non-interferon, non-steroidal drug developed by Teva pharmaceuticals (Friese *et al.*, 2006). Its random amino acid composition (glutamate, tyrosine, alanine and lysine) had been shown to mimic the myelin basic protein (MBP) and is thought to act as a decoy to divert any myelin directed immune response (Feldmann and Steinman 2005). The mode of action of GA has not been fully characterized, it is known to effectively inhibit the presentation of CNS antigens on MHC II molecules (Gilgun-Sherki *et al.*, 2003) and also cause activation of GA-specific T regulatory cells, which secrete anti-inflammatory cytokines (Teitelbaum *et al.*, 2004). GA is an approved drug for the treatment of RRMS and had shown remarkable efficiency with regards to slowing disease progression and relapse rate all while keeping within a high safety drug profile, making it a very effective drug (Arnon and Aharoni, 2004; Feldmann and Steinman, 2005). However other similar molecules such as altered peptide ligand (APL) consisting of a mutated MBP-derived peptide was found to exacerbate disease progression (Ruiz *et al.*, 2001).

In the quest to develop a cure for MS, novel MS-specific therapeutic agents have provided a means to better understand the disease itself—although in most cases, the long-term side effects are yet to be elucidated. Current therapeutic strategies have focused on inhibiting T lymphocyte activation, signaling, migration or proliferation. Therefore, not surprisingly, the use of older, better understood drugs—originally developed to mediate immune proliferation in other diseases—were applied to MS. These drugs work principally through the non-antigen specific route and have the advantage that their long-term side-effects are better understood.

Immunosuppressive drugs in MS therapy

Broadly termed immunosuppressive agents, these non-selective chemical molecules, which include Methotrexate, Metoxantrone Cyclophosphamide, Cyclosporine, Azathioprine, etc (Clegg and Bryant, 2001; Fernandez *et al.*, 2004) were discovered and approved as anti-cancer agents. Although bioavailable to all cells, the anti-metabolites target cells with high metabolic activity and in particular hyper-proliferative cell populations. For this feature, these molecules have also been exploited as anti-inflammatory agents in combinatorial therapy for MS.

Methotrexate (MTX, an anti-folate), Metoxantrone (Type II topoisomerase inhibitor) and Cyclophosphamide (alkylating agent, nitrogen mustard) all impede DNA synthesis and repair during cell growth and division (Wood, 1997; Fernandez *et al.*, 2004; Clegg and Bryant, 2001). Cyclosporin, is another immunosuppressive agent that selectively targets T lymphocytes, by inhibiting the cytosolic protein immunophilin which concomitantly impedes the transcription of IL-2. As a result, T cell activation and subsequent clonal expansion is inhibited (Wood, 1997).

Azathioprine is a purine analogue and pro-drug of 6-mercaptopurine (6MP). It is metabolized into the nucleotide intermediate thioguanosine monophosphate (TGMP) by a three-step enzymatic pathway, the rate-limiting step for which depends on HPRT. TGMP is then converted to its triphosphate form prior to incorporation into RNA and DNA—the principle mechanism through which thiopurine drugs exert their cytotoxic activity (Karran and Attard, 2008).

The ability of the above molecules to slow or inhibit proliferation has been proven to be efficacious in treating different types of cancers and autoimmune disorders (RA, Psoriasis, SLE). And therefore, when MS was understood in terms of immune inflammation against oneself, these molecules were administered to suppress the hyperproliferative cell populations in MS patients. As expected, the immunosuppressive environment dampened immune cell proliferation, inflammation and disease progression. More importantly, the therapeutic effect was observed in RRMS as well as in the slow (secondary progressive) and chronic (primary progressive) forms of SP/PPMS (Wood, 1997). Most of these drugs are not FDA approved, however the myelosuppressive agent Azathioprine has been approved for MS therapy since 1963 in the UK (Clegg and Bryant, 2001).

6TG is also a thiopurine but is not a downstream product of Aza or 6MP. The structure of 6TG allows the HPRT enzyme to convert it directly into TGMP for its action as a RNA/DNA anti-metabolite (Karran and Attard, 2008). Unlike Aza or 6MP, 6TG can also act as an analogue of the hypermodified guanine base, queuine and hence be a substrate for the QTRT enzymes in the queuine tRNA pathway. This specific property was exploited in this chapter to investigate the potential efficacy of this pathway for treating MS.

The results demonstrated that exploiting the queuine tRNA pathway was a potent means to ameliorate the clinical severity of the disease. Even as a therapeutic/palliative dosing regime post-onset of paralysis, the amelioration of the clinical score corresponded with restoration of the animal's physical/motor ability caused by the autoimmune inflammation—suggesting that therapeutically, this pathway was able to halt disease progression and even reverse the damage caused to a considerable extent.

From our data, one cannot categorically rule out that the effect observed is a result of non-selective systemic immunosuppression, but certain key points in the data set indicate otherwise. Firstly, the analysis of the total cell numbers in the periphery and CNS show a marked decreased in total proliferation of cells, but never lower than the basal level of cell numbers recorded in the Naïve and 6TG control groups. Moreover, this inhibition of proliferation appeared to be targeted against the self-antigen (MOG)-specific cells as the proliferative response against mitogens (CD3/PMA) was still intact in all groups. These are important factors as this shows

that the pathway is not conveying its effect via creating an immunosuppressive environment, unlike the case of Aza and other anti-cancer drugs. Importantly, the lack of systemic immunosuppression ensures the organism would not become susceptible to other infection agents such as those caused by Natalizumab and Fingolimod.

Another important aspect of this pathway is that during the course of the experiment, dosing of 6TG did not cause any loss of appetite or any visible changes in food or water consumption. In a study conducted by Aubrecht and colleagues, administration of 6TG to HPRT knockout mice did not affect the gut lining in the animals, which correlates with an intact appetite and lack of excessive weight loss during the dosing regime (Aubrecht *et al.*, 1997). Most immunosuppressive agents are known to causing intensive nausea, vomiting (Aza, Metotrexate) and restricted appetite which do not appear to be the case as far as the animal studies are concerned (Clegg and Bryant, 2001). Therefore, it would seem that the exploiting this pathway, halts inflammation and promotes a tolerogenic environment resulting in damage repair and subsequent amelioration.

One could argue that given the significant amounts of antigen and non-antigen specific therapies already available for MS, how could this pathway provide a more attractive market. From the preliminary data shown in this project, the benefit of this pathway in the field of MS therapeutics could be explained as being manifold. Firstly, its specificity for the target cell population would be naturally selected on the basis of the queueine deficient pool of tRNA molecules. Thus despite its off-target bioavailability to other cells, the pool of queueine-modified tRNA would prevent any drug incorporation and therefore activity. Such a natural selection process would help overcome the incidence of fatal infections and secondary tumors caused by antigen-specific Natalizumab (antagonizing even non-pathogenic cell expressing the integrin) or even immunosuppressive agents that alter normal cell DNA as well. Also, the double knockout experiments are a proof that even in the target cell, the queueine-deficient tRNA would be the only site to mediate any drug-related effect.

Secondly, the target site is in the RNA, which eliminates any irreversible damage caused to the genomic DNA. In the long-term therefore, exploiting this pathway would prevent the generation of drug-resistant cell clones or even malignant tumors. So, unlike in the case of Thiopurines, Metotrexate, Cyclophosphamide and others, the cells' DNA would not be tampered with or altered. This also highlights the ability of this pathway to be exploited long-term or at frequent intervals—a

phenomenon not possible with DNA interfering non-specific molecules.

Furthermore, although this project is a preliminary validation of a novel pathway using 6TG, one can associate certain known properties of 6TG to the properties of the potential queueine tRNA-specific drug molecule. 6TG, like other thiopurines are orally administrated in tablet form—a highly probable feature of a potential drug for the queueine tRNA pathway.

Finally, a very important factor for MS patients is the feasibility of a therapy. In a cost effectiveness study on MS drugs conducted in 2001, the annual cost incurred to a patient undergoing immunomodulatory therapies such as IFN treatment was around £10,000-20,000 in the UK, and around £10,000 for Glatiramer Acetate. For the non-antigen therapies, the costs were much lower starting at around £50-300 for Metatrexate, Cyclophosphamide and Aza, going upto £6,000 for Cladribine (Clegg and Bryant, 2001). So, one can predict that a potential Queueine tRNA pathway compound could also be an orally administered, highly cost-effective therapeutic regime. Also, due to its unique mode of action, it can be administered as part of a combinatorial-therapeutic regime without causing any lethal cross-talk between other drug pathways.

The above features therefore provide an attractive drug profile so far although much validation work still remains. It is still worthwhile noting that the non-antigen specific action of this pathway provides a potent means to apply this to treat other types of inflammatory and autoimmune disorders such as RA, Psoriasis, SLE, Crohn's disease, etc. This novel pathway therefore leads down a whole new field of immunotherapeutic agents that can be applied clinically to help improve patient welfare if not cure their symptoms completely.

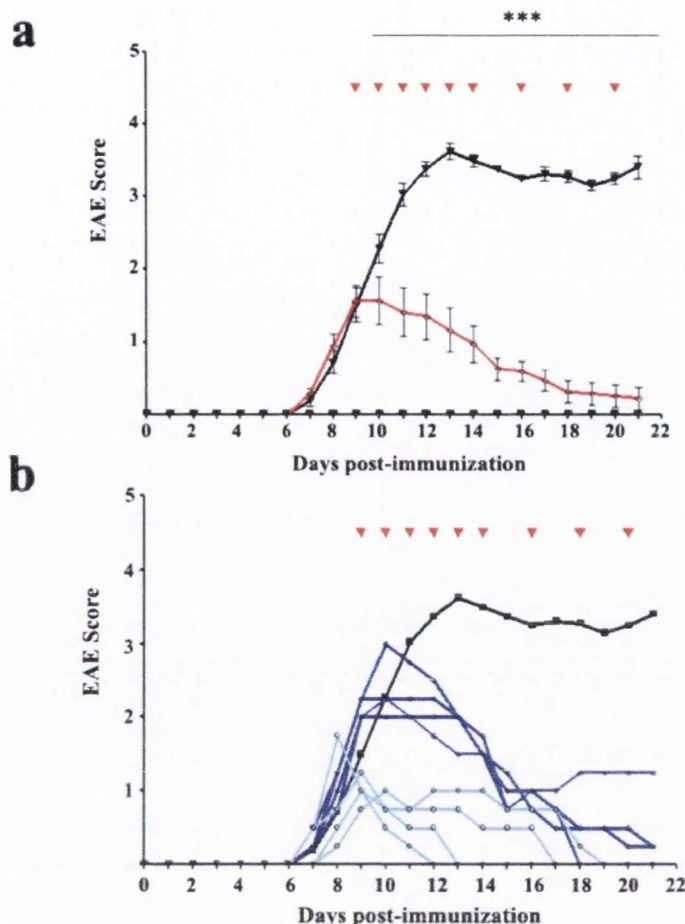


Figure 5.2: 6TG via the queuine-tRNA pathway halts and reverses clinical progression of chronic EAE in *Hprt1^{b-m3}* mice.

(a) Average clinical EAE score in 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice. A total of four groups of eight mice each were set up: Naïve (●), 6TG (■), EAE (▼) and EAE + 6TG (○). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. Starting on day 9, 6TG was administered intra-peritoneally according to the indicated regime (▼). The Naïve animals were neither immunized nor treated and 6TG animals were left un-immunized. Every 24 hours, each animal was allocated a clinical score: Grade 0, normal; grade 1, limp tail; grade 2, wobbly gait; grade 3, hind limb weakness; grade 4, hind limb paralysis; and grade 5, tetraparalysis/death. The average clinical score values are plotted SEM (standard error of mean) error bars. ***, $P < 0.0001$.

Please find CD attached to end of thesis, containing a video of animals taken on day 21; to be viewed in QuickTime player®.

(b) Individual clinical EAE score for each animal in the EAE+6TG experimental group. On day 9 out of a total of eight animals in the EAE+6TG group, 4 mice showed a disease score of 2.0-3.0 (dark blue line) whereas the other 4 mice scored between 1.0-1.75 (light blue lines). By day 21, a total of 5 out of 8 mice from this EAE+6TG group no longer showed any clinical symptoms of EAE and the remaining 3 showed low/minimal signs.

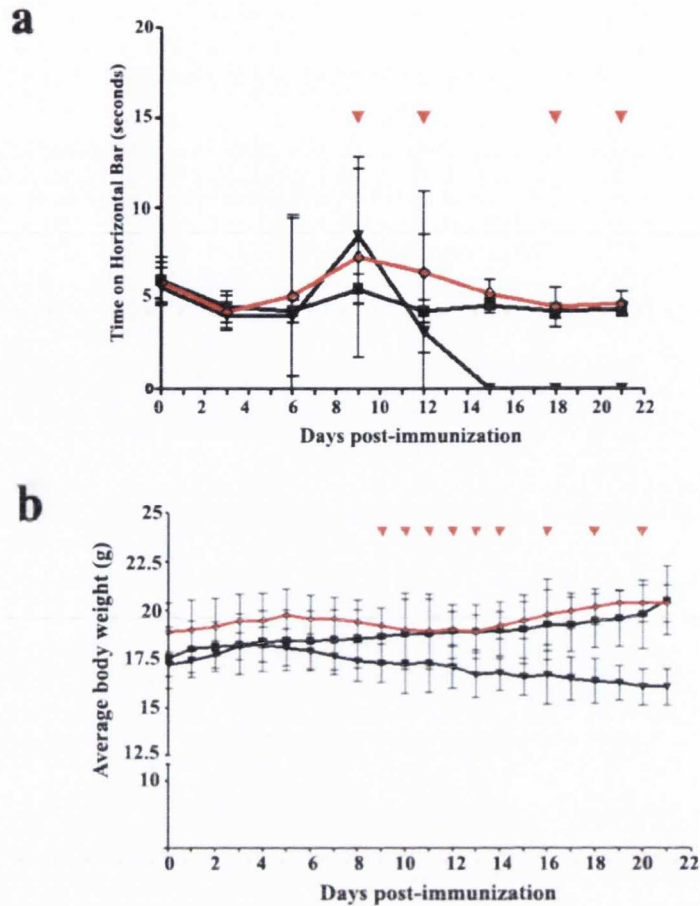


Figure 5.3: 6TG via the queuine-tRNA pathway prevents EAE-induced physical disability and weight loss in *Hprt1^{b-m3}* mice.

(a) Average time on the horizontal bar test for C57BL/6 *Hprt1^{b-m3}* female mice. A total of four groups of eight mice each were set up: Naïve (●), 6TG (■), EAE (▼) and EAE + 6TG (○). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. Starting on day 9, 6TG was administered intra-peritoneally once a day according to the indicated regime (▼). The Naïve animals were neither immunized nor treated and 6TG animals were left un-immunized. Each animal was subjected to the horizontal bar physical test every 72 hours starting on day 0, till the end of the experiment on day 21. The 6TG and EAE+6TG groups received 6TG injections (▼) on indicated days. The average time on bar for each group is plotted along with the respective SEM values.

(b) Average body weight of C57BL/6 *Hprt1^{b-m3}* female mice. Each animal was weighed every 48 hours starting on day 0, till the end of the experiment on day 21. The 6TG and EAE+6TG groups received 6TG injections (▼) on indicated days. The average body weight for each group was graphed along with the respective SEM values.

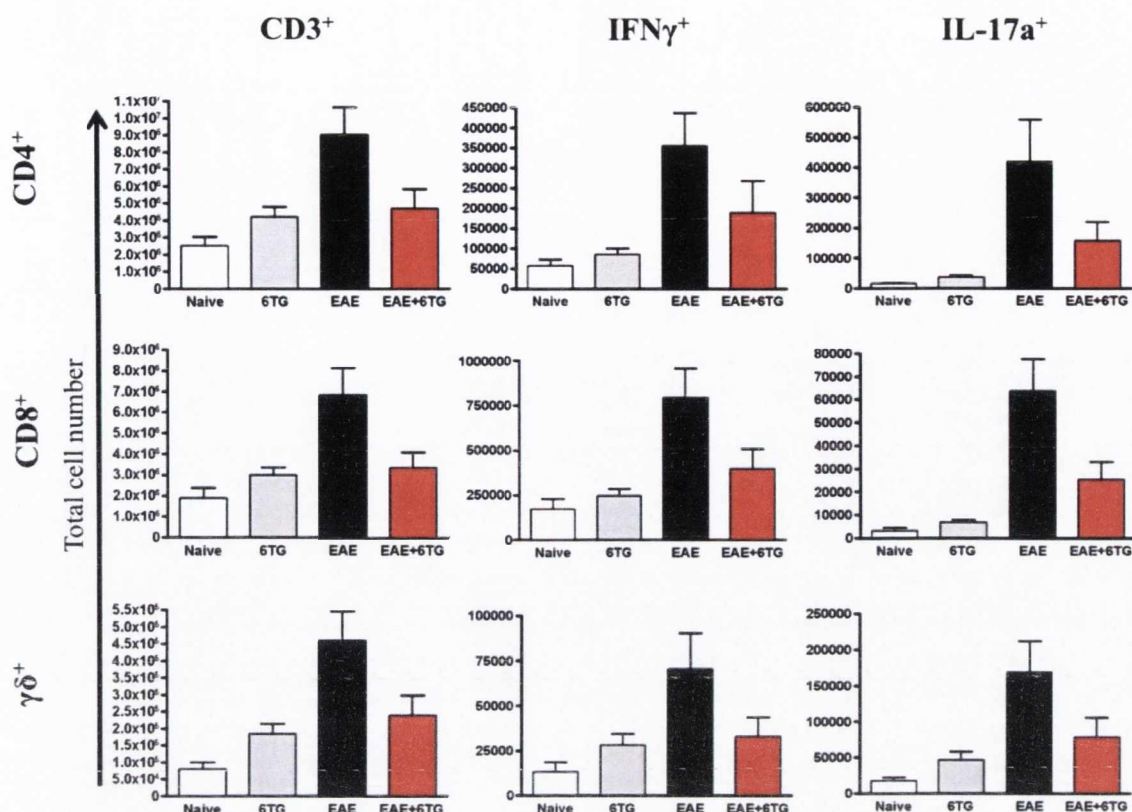


Figure 5.4: 6TG via the queuine-tRNA pathway reduces EAE-induced T_H1 and T_H17 cells in spleen in *Hprt1^{b-m3}* mice.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole spleen was isolated from the PBS-perfused animals on day 21, single cell suspensions were re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 µg/ml) and treated with Brefeldin A for a total of 6 hours. The cells were then counted, labelled for CD3, CD3⁺4⁺, CD3⁺8⁺ and CD3⁺γδ⁺ and intracellular IL-17a and IFNγ for each sub-population and analysed by FACS. The percentage of cells obtained was then calculated with respect to their absolute numbers and plotted as a bar graph. Each bar, represents the average of triplicate values with its respective SEM values.

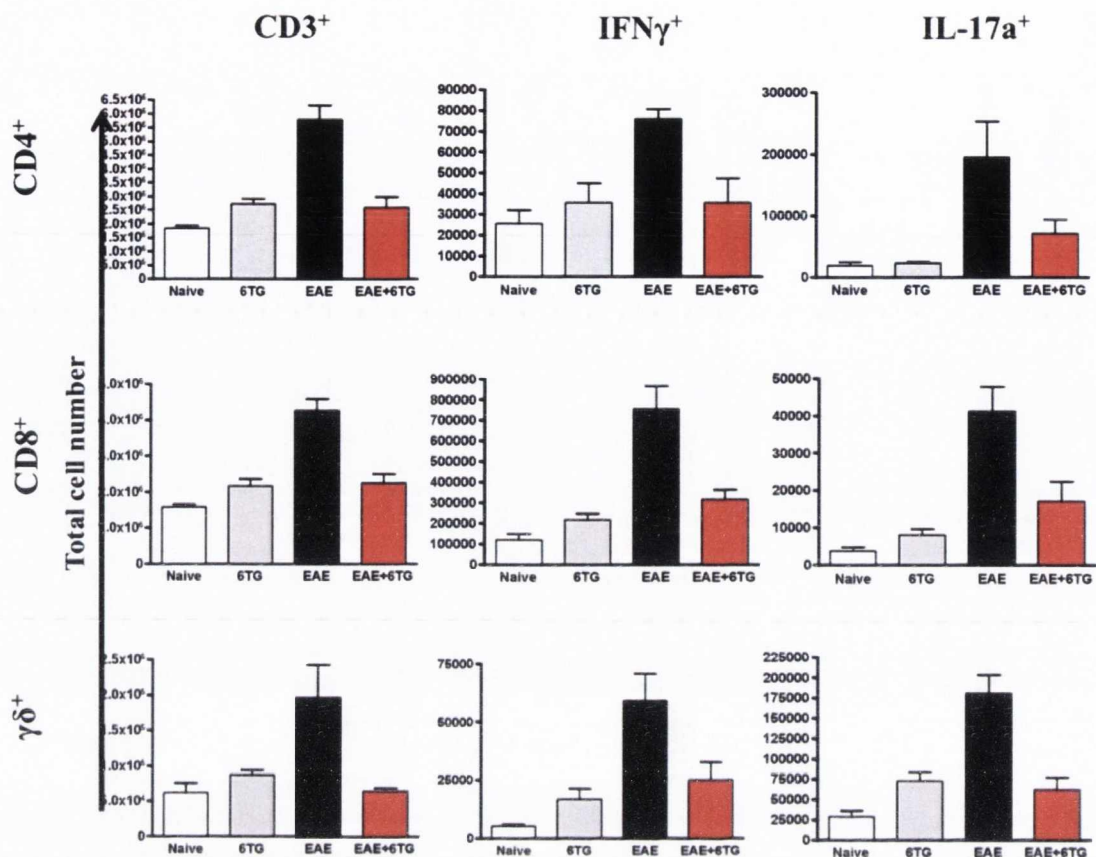


Figure 5.5: 6TG via the queuine-tRNA pathway reduces EAE-induced T_H1 and T_H17 cells in inguinal lymph nodes in *Hprt1^{b-m3}* mice.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole inguinal lymph nodes were isolated from the PBS-perfused animals on day 21, single cell suspensions were re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 μ g/ml) and treated with Brefeldin A for a total of 6 hours. The cells were then counted, labelled for CD3, CD3⁺4⁺, CD3⁺8⁺ and CD3⁺ $\gamma\delta^+$ and intracellular IL-17a and IFN γ for each sub-population and analysed by FACS. The percentage of cells obtained was then calculated with respect to their absolute numbers and plotted as a bar graph. Each bar, represents the average of triplicate values with its respective SEM values.

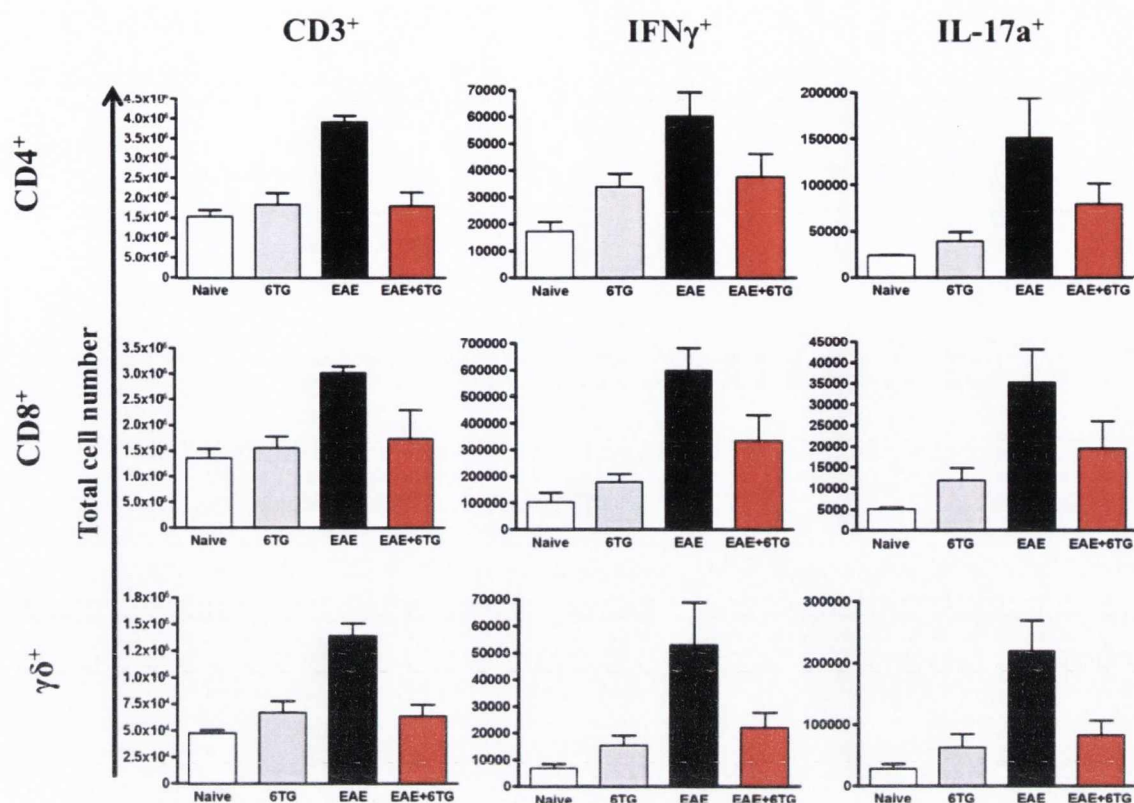


Figure 5.6: 6TG via the queuine-tRNA pathway reduces EAE-induced T_H1 and T_H17 cells in cervical lymph nodes in *Hprt1^{b-m3}* mice.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole cervical lymph nodes were isolated from the PBS-perfused animals on day 21, single cell suspensions were re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 µg/ml) and treated with Brefeldin A for a total of 6 hours. The cells were then counted, labelled for CD3, CD3⁺4⁺, CD3⁺8⁺ and CD3⁺γδ⁺ and intracellular IL-17a and IFNγ for each sub-population and analysed by FACS. The percentage of cells obtained was then calculated with respect to their absolute numbers and plotted as a bar graph. Each bar, represents the average of triplicate values with its respective SEM value.

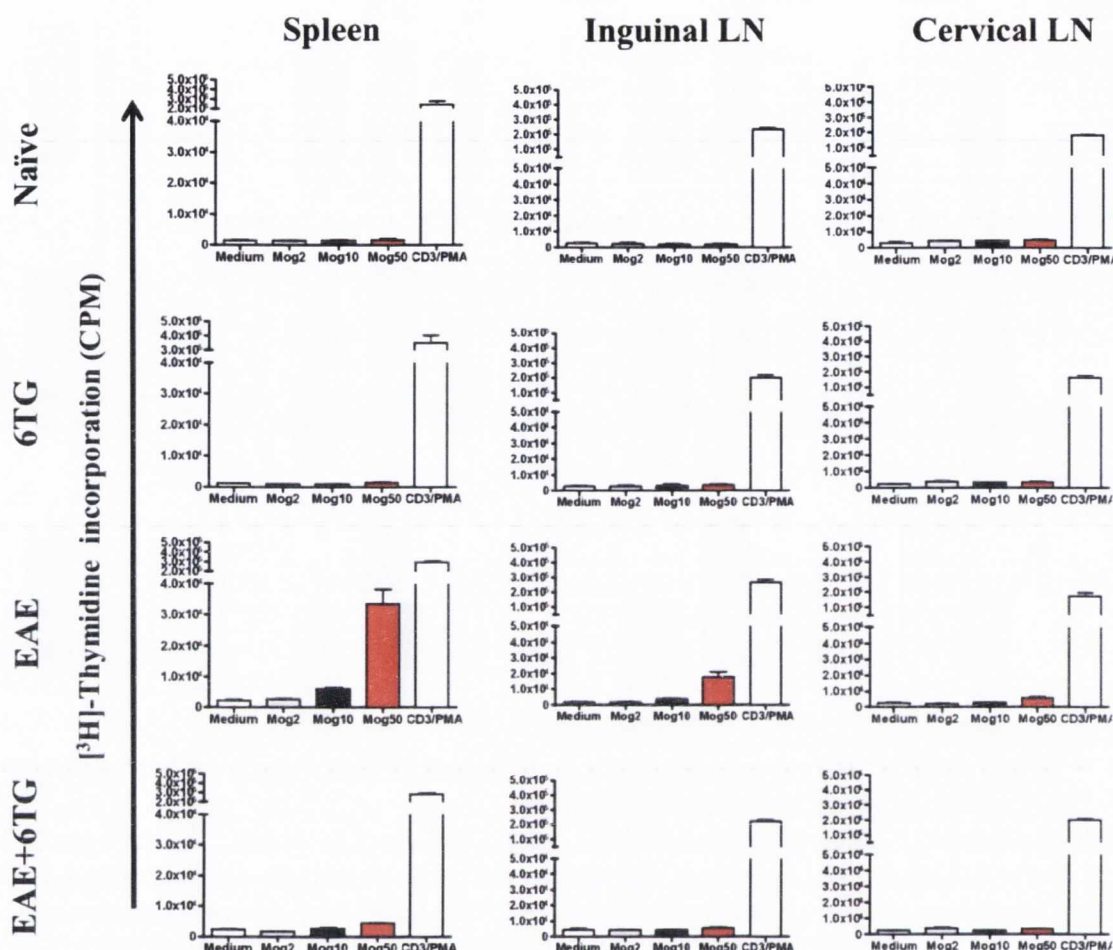


Figure 5.7: 6TG via the queuine-tRNA pathway reduces peripheral MOG-specific T cell response in the spleen, inguinal and cervical lymph nodes in *Hprt1^{b-m3}* mice.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole spleen, inguinal and cervical lymph nodes were isolated from the PBS-perfused animals on day 21. Single cell suspensions were plated at 1 x10⁶ per well containing either medium alone (negative control), or stimulated with either 2 µg/ml, 10 µg/ml or 50 µg/ml of antigen MOG, or 10 ng/ml PMA and 1 µg/ml αCD3 (positive control). At 72 hours, the supernatants were removed and the cells were pulsed with 0.5 µCi of $[^3\text{H}]$ -Thymidine per well overnight at 37°C. The cells were harvested onto glass fibre mats, air-dried, sealed in plastic bags with 5mls of scintillation fluid and read using the micro-β-counter. The average counts per minute for each sample in triplicate was plotted as a bar graph with its respective SEM values.

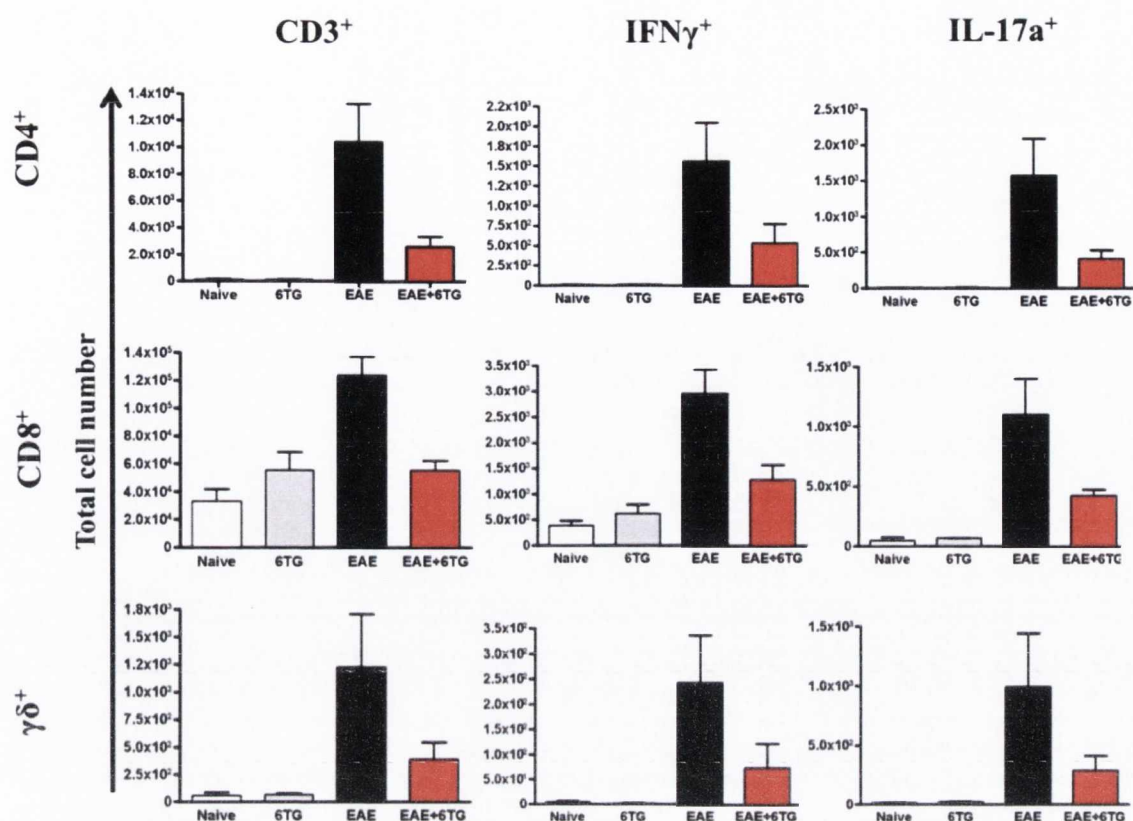


Figure 5.8: 6TG via the queuine-tRNA pathway reduces EAE-induced T_H1 and T_H17 cells in brain in *Hprt1^{b-m3}* mice.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole brain was isolated from PBS-perfused animals on day 21. The MNCs were isolated using Percoll and re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 μ g/ml) and treated with Brefeldin A for a total of 6 hours. The cells were then counted, labelled for CD3, CD3⁺4⁺, CD3⁺8⁺ and CD3⁺ $\gamma\delta^+$ and intracellular IL-17a and IFN γ and analysed by FACS. The obtained percentage of cells was then calculated with respect to their absolute numbers and plotted as a bar graph. Each bar, represents the average of triplicate values with its respective SEM values.

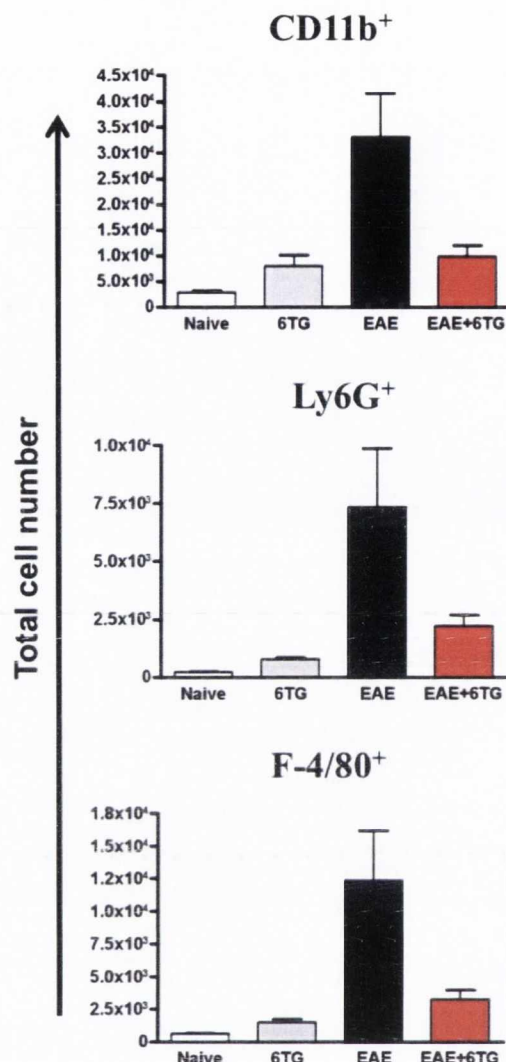


Figure 5.9: 6TG via the queuine-tRNA pathway reduces EAE-induced total activated macrophages and neutrophils in *Hprt1^{b-m3}* mice brain.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole brain was isolated from PBS-perfused animals on day 21. The MNCs were isolated using Percoll and re-stimulated *in vitro* with PMA (10 ng/ml) and ionomycin (1 µg/ml) and treated with Brefeldin A for a total of 6 hours. The cells were then counted, labelled for CD11b⁺, Ly6G⁺ and F4/80⁺ and analysed by FACS. The obtained percentage of cells was then calculated with respect to their absolute numbers and plotted as a bar graph. Each bar, represents the average of triplicate values with its respective SEM values.

EAE Brain	Striatum	Hippocampus	Brain Stem
1 (3.75)	-	+	+
2 (2.75)	-	+++	++
3 (3.75)	+++	++	++
4 (4.25)	-	++	+++
EAE + 6TG Brain	Striatum	Hippocampus	Brain Stem
1 (0)	-	-	-
2 (0)	-	+	+
3 (0)	-	-	-
4 (0.25)	-	+	++

Table 5.1: Overall EAE lesion activity observed in diseased and treated *Hprt1^{b-m3}* mice.

Immunohistochemical analysis for T cells (CD3) and activated microglia (CD68) was performed on fresh-frozen brain section from three distinct regions of the brains: Striatum, Hippocampus and Brain stem (including cerebellum). The overall lesion activity/staining intensity observed in sections from EAE and EAE+6TG animals has been summarised in this table above. For each of the four animals per group, the EAE clinical score on day 21 is indicated in brackets and the lesion activity is indicated qualitatively where: '-' represents lack of any visible activity, '+' represents visible yet low activity, '+ +' represents medium level activity- infiltration and some parenchymal activity, and '+ + +' represents significant amount of lesions with ventricular and parenchymal activity.

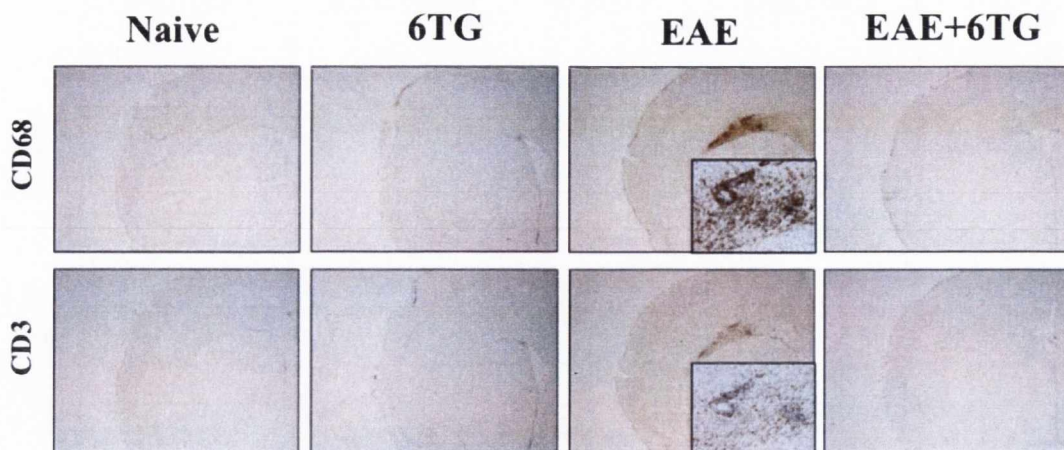


Figure 5.10: 6TG via the queuine-tRNA pathway reduces microglial activation and T cell population in striatal EAE lesions

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole brain was isolated from heparanized-PBS animals on day 21. The brains were fresh-frozen in OCT embedding medium over liquid nitrogen vapour. Sections of 10 micron thickness were cut on the cryostat onto APS-coated glass slides, stained for activated microglia (CD68) and T cells (CD3) by the ABC method and counter stained with Haemotoxylin. The ABC is conjugated to the HRP enzyme which uses the chromagen DAB as a substrate to produce a brown precipitate- a signal for positive staining. Haemotoxylin stains for cellular nuclei. The representative images were taken at x2.5 magnification and the inset for lesion in the EAE slices at x40 magnification.

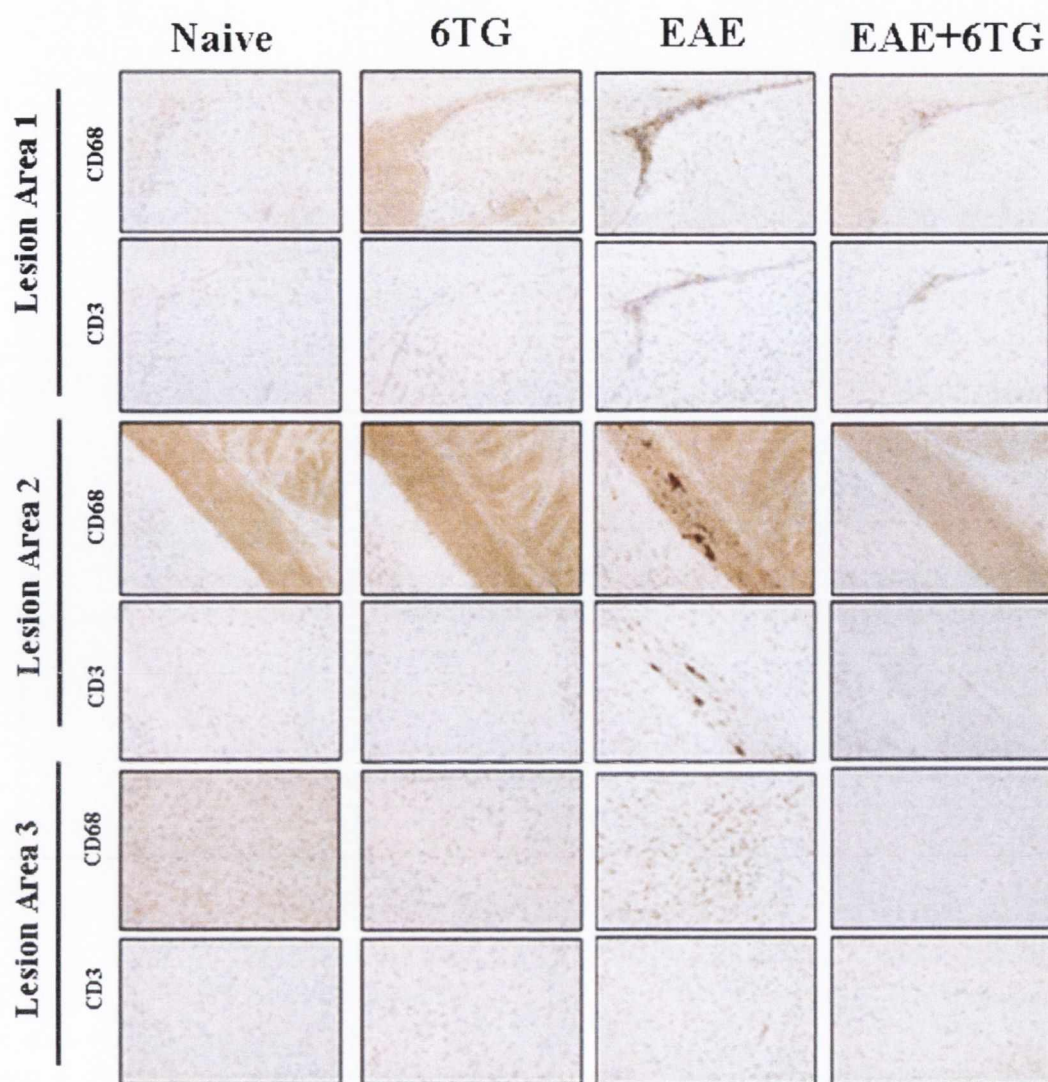


Figure 5.11: 6TG via the queuine-tRNA pathway reduces microglial activation and T cell population in hippocampal EAE lesions.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole brain was isolated from heparanized-PBS animals on day 21. The brains were fresh-frozen in OCT embedding medium over liquid nitrogen vapour. Sections of 10 micron thickness were cut on the cryostat onto APS-coated glass slides, stained for activated microglia (CD68) and T cells (CD3) by ABC method and counter stained with Haemotoxylin. Three regions of the hippocampus that were found to contain lesion activity are hippocampal ventricle (Lesion Area 1), fimbriae (Lesion Area 2) and the parenchyma (Lesion Area 3). The representative images were taken at x20 magnification.

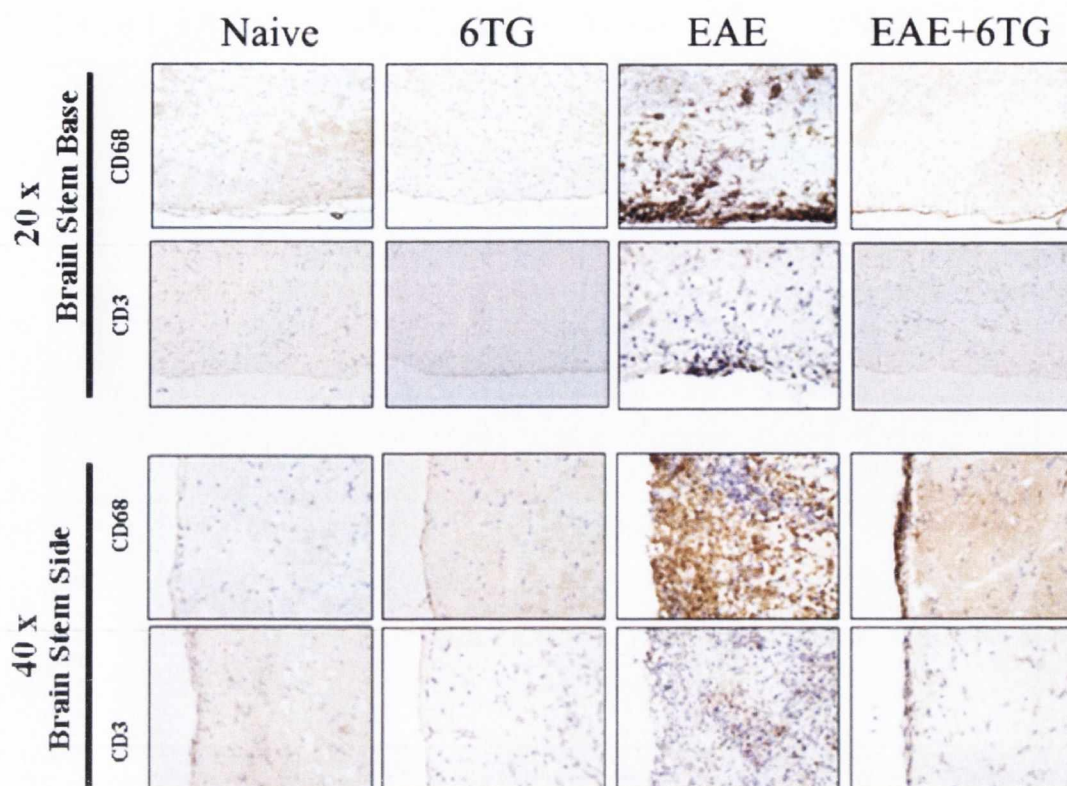


Figure 5.12: 6TG via the queueine-tRNA pathway reduces microglial activation and T cell population in brain stem EAE lesions.

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole brain was isolated from heparanized-PBS animals on day 21. The brains were fresh-frozen in OCT embedding medium over liquid nitrogen vapour. Sections of 10 micron thickness were cut on the cryostat onto APS-coated glass slides, stained for activated microglia (CD68) and T cells (CD3) by ABC method and counter stained with Haemotoxylin. The representative images for Brain Stem Base were taken at x20 magnification and those for Brain Stem Side were taken at x40 magnification.

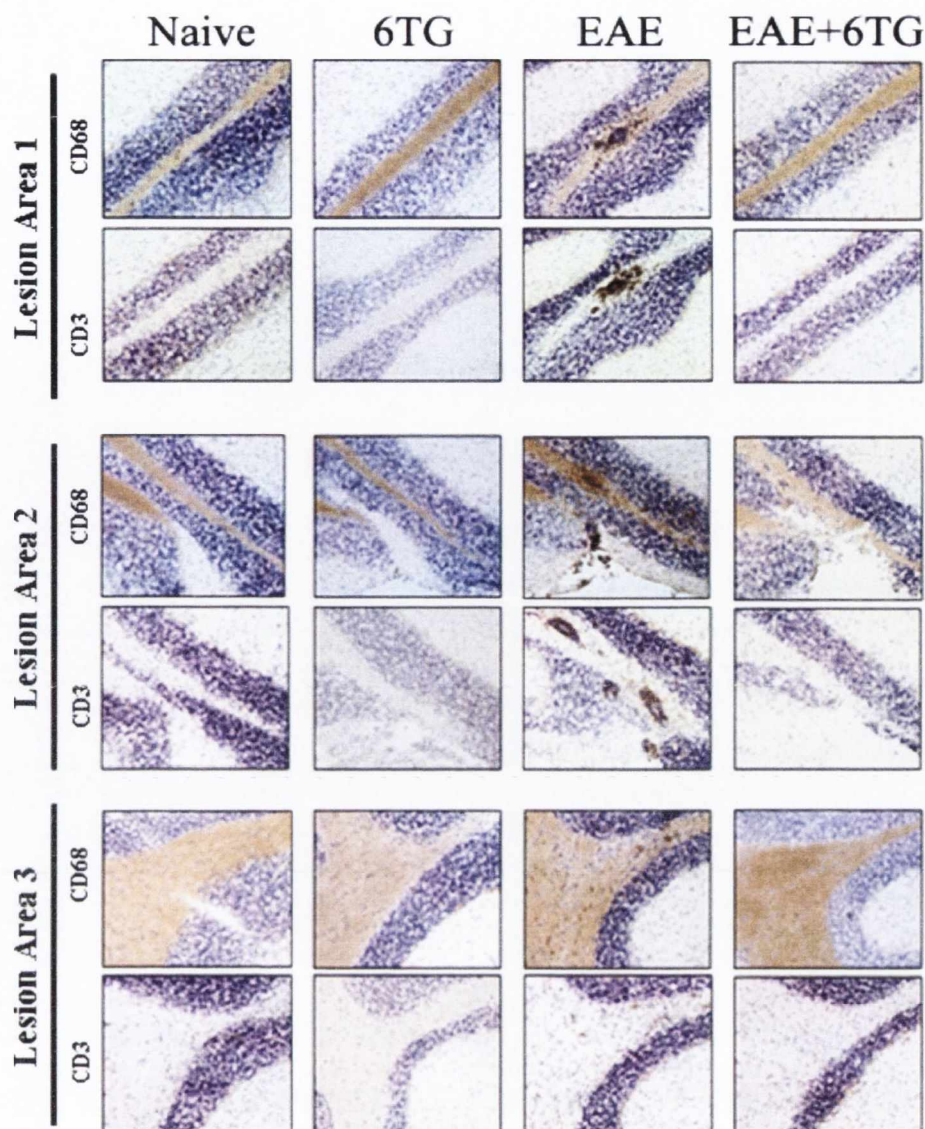


Figure 5.13: 6TG via the queuine-tRNA pathway reduces microglial activation and T cell number in the cerebellum of EAE diseased animals

Four groups of four 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice each were set-up as follows: Naïve (un-immunized, untreated), 6TG (treatment only), EAE (immunization only) and EAE + 6TG (immunization + treatment). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. 6TG was administered intra-peritoneally according to the regime (indicated previously). Whole brain was isolated from heparanized-PBS animals on day 21. The brains were fresh-frozen in OCT embedding medium over liquid nitrogen vapour. Sections of 10 micron thickness were cut on the cryostat onto APS-coated glass slides, stained for activated microglia (CD68) and T cells (CD3) by ABC method and counter stained with Haemotoxylin. The representative images for all three Lesion Areas 1, 2 and 3 were taken at x20 magnification.

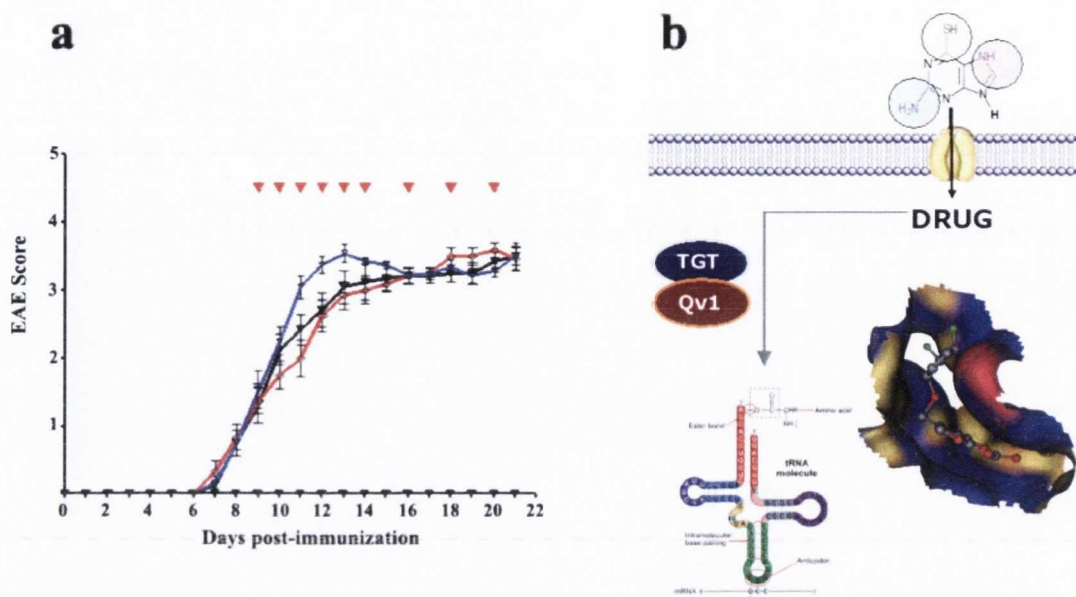


Figure 5.14: Therapeutic effect of queuine tRNA pathway abolished in *Hprt1^{b-m3}::Qtrt1^{G/Gt}*
Average clinical EAE score in 8-10 week old C57BL/6 *Hprt1^{b-m3}* female mice (**a**). A total of four groups of eight mice each were set up: Naïve (●), 6TG (■), EAE (▼) and EAE + 6TG (◆). Chronic EAE was induced by sub-cutaneous immunization with MOG in complete CFA and intra-peritoneal injection of Pertussis Toxin on day 0 and day 2. Starting on day 9, 6TG was administered once a day intra-peritoneally according to the indicated regime (▼). The Naïve animals were neither immunized nor treated and 6TG animals were left un-immunized. Every 24 hours, each animal was allocated a clinical score: Grade 0, normal; grade 1, limp tail; grade 2, wobbly gait; grade 3, hind limb weakness; grade 4, hind limb paralysis; and grade 5, tetraparalysis/death. The average clinical score values are plotted SEM error bars. The EAE disease severity score recorded for the *Hprt1^{b-m3}* (▼) animals in **Figure 5.2** has been superimposed on this graph.

The schematic illustrates how a potential drug can be incorporated into tRNA via the queuine tRNA pathway (**b**). The shaded regions on the 6TG chemical structure represent positions that can be altered during drug design. A potential drug will not be a substrate for the HPRT enzyme and instead be specific for the queuine tRNA pathway. Upon uptake, it will act as a natural substrate for the QTRT enzymes (TGT-Qv1 complex) that will catalyse its insertion into the queuine deficient wobble position 34 in the tRNA anticodon. The incorporation of the drug will alter cell growth and proliferation.

Chapter 6: Discussion

6.1 General discussion

Queuine is exclusively synthesized by bacteria yet is found irreversibly inserted into the QtRNA anticodon of both bacteria and eukaryotes. It is not surprising therefore that much of the research effort on this molecule has focused on understanding the physiological importance of this wobble position modification. Preliminary studies on *E.coli* and eukaryotic cell lines *in vitro* have made various assertions of queuine's role in development, cellular growth rate, differentiation and metabolism (Noguchi *et al.*, 1982; Dineshkumar *et al.*, 2002; Langgut *et al.*, 1990; Langgut *et al.*, 1993). Studies in higher eukaryotes were complicated by the tenacious retention of queuine by modified tRNA. In animal studies by Walter Farkas' group over a year was required to successfully deplete all QtRNA isoacceptors of queuine—leading to the surprising discovery that queuine deficiency does not cause adverse effects in animals (Marks and Farkas, 1980). Similarly, studies in fly (*Drosophila melanogaster*) and slug (*Dictyostelium discoideum*) showed a complete lack of physiological changes caused by a lack of queuine (Ott *et al.*, 1982, Jacobson *et al.*, 1981; Marks and Farkas, 1980). In this project, mice were made deficient in queuine by a transgenic manipulation of the *tgt* gene. Although an improvement on Farkas's protocol, the queuine deficient animals still did not show any phenotypic/behavioural manifestations. Moreover even at a physiological level, the lack of queuine had no impact on the animal's immune response in EAE. Therefore, for a modification that has been conserved ubiquitously across the prokaryotic and eukaryotic kingdoms, its physiological importance is still quite puzzling (Okada *et al.* 1979).

The eukaryotic QTRT enzyme complex, responsible for the insertion of queuine, was recently identified and characterised in our lab (Boland *et al.*, 2009). Immunoprecipitation and confocal studies found that both proteins, TGT and Qv1, interact *in vivo*. In this report, all adult mouse tissues co-express TGT and Qv1 at equal or greater levels in B and T cells—confirming their co-expression in immune cell types. It is postulated that TGT is principally found in the cytosol where it docks to its mitochondrial bound partner protein Qv1. This is in line with the studies in *Xenopus laevis* oocytes where yeast tRNA^{Asp} was modified with queuine only when

injected to the cytosol, and not the nucleus (Carbon *et al.*, 1982). Interestingly, in the opossum and rat (Morl *et al.*, 1995; Randerath *et al.*, 1984), mitochondrial tRNA was also found modified with queuine, implying the need for a specific export/import shuttle to allow the mitochondrial tRNAs to be modified cytosolically. The very fact that both cytosolic and mitochondrial tRNA is required to be modified with queuine suggests its importance for tRNA function.

The anticodon region, especially the phosphodiester bond of the wobble position between residues 33 and 34, is known to display high flexibility—a feature highly dependent on the nucleotide sequence and modifications found at this site (Gautheret and Cedergren, 1993). When compared to the unmodified QtRNAs (those containing the genetically encoded guanine instead), queuosine modification has been shown to alter read-through of UAG stop codons, increase the translation rate of HIV, HTLV-1 (Human T-cell Leukaemia Virus, type-1) genes (Hatfield *et al.*, 1989), as well as increase the K_m of charging tRNA^{tyr} by around 75% and decrease the K_m for tRNA^{asp} isoacceptor by around 50% (Singhal and Vakharia, 1983). Furthermore, the bias displayed by tRNA^{his} for codon sequence CAC over CAU, is eliminate by queuine modified tRNA (Smith and Hatfield, 1986). These features described above have been suggested to occur as a result of the multi-functional group attached to the base structure of queuine. The functional groups alter the hydrogen bonds donor/acceptor profile that contributes majorly towards stabilizing the tRNA anticodon loop (Morris *et al.*, 1999)—a feature that is compromised upon incorporation of an analogue at the site.

Although queuine is the preferred substrate, 6TG has also been listed as a non-inhibitory substrate of the QTRT enzymes (Kretz *et al.*, 1985; Morgan *et al.*, 1994). In this project, administration of 6TG to HPRT-deficient proliferating T cells mediates a dose-dependent growth inhibitory effect. The lack of the HPRT enzyme suggests that 6TG is conveying this effect *via* an alternative pathway. The proliferation assay from double knockout T cells (deficient in *hprt* and *tgt*) proves that the queuine tRNA pathway is the only alternative pathway responsible for the anti-proliferative effects. Unlike queuine, 6TG lacks a bulky multi-functional group and instead possesses a highly-reactive thiol group (SH) at the purine C6 position. Either the presence of this sulphur group and/or the lack of the multi-functional group could be the cause for the antagonistic effects of 6TG conveyed upon incorporation into the queuine-deficient

wobble position. Using the promyelocytic HPRT-deficient leukaemia cell line HL-60, Trewyn was the first to report that 6TG could impede cell proliferation *via* its reversible incorporation into the queueine deficient tRNA position 34 (Kretz *et al.*, 1987; French *et al.*, 1991). In this project, the same effect is observed. The growth-inhibitory effect conveyed by 6TG alone is abolished upon co-administrating queueine. Even at concentrations of queueine:6TG at a 1:65 ratio, queueine is able to irreversibly out compete any 6TG mediated growth-inhibition. In the animals, exploiting this queueine tRNA pathway *in vivo* proved to be of high therapeutic potential. Administration of 6TG to HPRT-deficient animals was able to halt and reverse clinical disease progression in EAE. At a behavioral and physical level, animals were able to regain their physical strength and motor abilities. At a pathological level, there was a marked reduction in the inflammatory T cell populations in the periphery and the CNS. The growth-inhibitory effects observed *in vitro* could explain this phenomenon, but only to a certain extent. *In vitro*, the effect of exploiting the queueine tRNA pathway was analyzed mainly on T cells. Although there seemed to be some selectivity for T cells, this does not discount potential physiological effects on other immune cells. Within MS, the role of non-hyper proliferative populations such as antigen presentation cells (Dendritic cells, microglia) has been shown to be an important feature of the disease (Ransohoff and Perry, 2009). In human MS samples, demyelinating lesions in the subpial areas are very prominent. Here, the inflammatory lesions consist of lymphocytes, monocytes and robust microglial activation (Peterson *et al.*, 2001; Ransohoff and Perry, 2009). Microglia are sensitive to the slightest changes in the CNS immune environment and can elicit diverse effector functions. Under conditions of minimal immune challenge, microglia are induced to become anti-inflammatory/neuroprotective, whereas in case of extensive injury they create a pro-inflammatory/neurodegenerative environment. They have therefore been implicated in sustaining CNS inflammation during disease (Streit, 2002). In our immunohistochemical analysis there was a lack of staining for the microglial marker in the EAE animals treated with 6TG. It would be interesting to determine whether this is caused by lack of microglial activation by T cells, or due to direct modulatory effects conveyed by the queueine tRNA pathway.

An important failing of most immune-therapies is toxicity. 6TG is known to be highly toxic to cells due to its ready incorporation into the genomic material (Karran and Attard, 2008). This aspect of toxicity would be avoided by drugs specific

to the queuine-tRNA pathway as they would not interfere with DNA. Also, from the data available, the lack of weight loss or appetite suppression caused by 6TG treatment would suggest that there has not been any significant toxicity caused. Moreover, during all physical tests and the video recorded, none of the animals show any overt behavioral changes. However, one cannot overlook the fact that a drug targeting the hyper proliferative cell populations could affect the bone marrow. Therefore, quantifying the level of platelet-clearance in animal blood by flow cytometry is a possible means to assess thrombocytopenia (Liang *et al.*, 2010). Also, assessing levels of AST (aspartate aminotransferase), ALT (alanine aminotransferase) and Bilirubin from animal blood are important to detect possible hepatotoxicity (Halevy *et al.*, 1994).

Nevertheless, the data from the double knockout studies show that the effect is being conveyed uniquely through the queuine tRNA pathway, which in itself is a novel drug mechanism yet to be exploited in the field of disease therapeutics. The currently available drug molecules for MS can only provide a means to alleviate disease symptoms in terms of slowing disease progression, reduced relapse intensity, etc. A cure for MS is yet to be discovered. The fact that by exploiting this pathway, the disease progression was found to not only halt, but also reverse, lends credence to the possibility that this therapeutic strategy is promoting repair. There was a marked reduction in total T cell number in the brain and a lack of inflammatory activity within the lesion, according to the immunohistochemical analysis. This would suggest that the pathway is promoting, if not inducing a tolerogenic environment. To check this further, the extent of re-myelination on nerve fibers can be assessed either by an immunochemical stain for myelin, or by MRI imaging (Gilgun-Sherki *et al.*, 2003; Frohman *et al.*, 2006). Other factors that contribute to an anti-inflammatory environment are regulatory cytokines such as IL-10, TGF- β and regulatory lymphocyte subsets, T-regs and B-regs (Constantino *et al.*, 2008; Gold *et al.*, 2006).

Finally, it is also worth mentioning that while working towards developing this new avenue for MS therapy further, one cannot overlook the potential of this novel field of therapeutics for other disorders. Autoimmune and inflammatory disorders such as RA, SLE and IBD, are all examples of immune malfunction that leads to a proliferative burst in self-reactive immune system. Such cases could also benefit from a non-invasive immuno-modulatory therapy such as drug molecules targeting this pathway.

6.2 Future work

The first aim towards pursuing this study further would be to design, synthesize and validate drug molecules that would work specifically through the queuine-tRNA pathway. Knowing the chemical properties of both queuine and 6TG, an ideal drug for this pathway would act as a naturally irreversible substrate for the queuine tRNA pathway (like queuine) while conveying antagonistic properties displayed by 6TG. That way, it would conform to the non-DNA interfering profile of the drug while being able to provide a sustainable effect—improving thus on the drug-safety profile. Moreover, like most thiopurines, drugs specific for this pathway have the potential to be administered orally providing an attractive treatment regime (Clegg and Bryant, 2001). The drug should have an immediate effect on disease progression upon administration. Also, the drug would not have many side effects-allowing frequent or long-term administration of it to patients. Also, given that it's a unique pathway, it would not interfere with any of the other existing therapies if administered in combination.

Having stated the above, future work would involve subjecting the candidate molecules to *in vitro* recombinant assays to test specificity for the QTRT and HPRT enzymes. The radioactive assay for QTRT enzymes has been optimized in the lab and is both quantitative and rapid (*unpublished data*). The HPRT assay is the HPLC-based radioactivity assay that has been described and validated as shown in Chapter 3. Candidate molecules that screen positive as substrates for the QTRT enzymes, will be applied to the earlier described *in vitro* T cell proliferation assays. The dose-dependent growth inhibition will be assessed. The successful drug candidates from this round of screening will then proceed to an *in vivo* EAE study in wild type animals. The study will be extensive in terms of dose administered, route of administration, therapy regimes (starting at disease onset or at late phase of disease) and finally the effect of the therapy post drug-withdrawal. During the experiment, along with the clinical score, weights and physical tests, serum samples will be collected at regular intervals to assess drug concentration and conduct toxicity studies. MRI will also need to be performed to assess disease progression and lesion activity in whole mouse brain. At the end of the experiment, tissue samples will be collected to assess physiological effects of drug in peripheral and CNS organs.

Work on actual human MS samples would also be of much benefit to assess the ability of drug effects seen in the animal model when applied to human samples. Many of the new immunotherapeutic strategies that have shown promise in animals models have failed when applied to clinical trials. For example application of anti-TNF (Sriram and Steiner, 2005) or development of a myelin peptide APL (Kappos *et al.*, 2002; Friese *et al.*, 2006) and the anti-CD40L (Dumont 2002; Friese *et al.*, 2006) all showed promise in EAE, but failed in MS. So, by assessing the effect of the drug on the proliferation and cytokine profile of human samples, a clearer picture could be gained on the efficacy and viability of the queuine tRNA pathway as a drug target. This could also be further applied to other human samples from patients, for example synovial samples from RA patients. Current techniques have been even based on simple ELISA based analysis of the key pro-inflammatory RA cytokines such as IL-1 β , IL-6, IL-8, TNF α and MMP3 (matrix metalloproteinase 3) (Rosengren *et al.*, 2003).

From a mechanistic point of view, it would be worth investigating the exact effect of a specific drug on tRNA function. Based on next generation sequencing technology used to sequence the different mRNAs expressed, Ribosome Profiling has the ability to quantify the amount of protein synthesis. This technique was originally described by Johnathan Weissman, and involves isolating up to 30 nucleotide long mRNA fragments that are protected by a bound ribosome during translation (ribosome footprint). These mRNA fragments are converted to a DNA library, then sequenced. This technique can provide a means to quantify the type and extent of translational activity occurring for a specific protein transcript (Ingolia *et al.*, 2009). Alternatively, a new non-radiochemical means to assess global protein translation in T cells and DCs is the SUnSET (surface sensing of translation) technology. This technique is based on using the antibiotic puromycin, an analogue of aminoacyl-tRNA. When applied to cells at low amounts, puromycin gets incorporated into nascent polypeptide chains that can be detected using monoclonal antibodies to puromycin by flow cytometry (Schmidt *et al.*, 2009).

Other structural studies could also be performed to learn more about the drug interactions with the tRNA molecules. The information from the crystal structure of the *Z. mobilis* TGT revealed the conserved nucleophile activity of residue Asp 280

(Romier *et al.*, 1996). Similarly, a crystal structure of the drug bound to the QTRT enzymes would demonstrate the effect that the functional groups are conveying during the catalytic reaction—data that could contribute to improving the design of drug molecules to the queuine tRNA pathway.

Chapter 7: References

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