The role of circadian rhythm in the immune response to *Trichuris muris*

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The role of circadian rhythm in the immune response to *Trichuris muris*

Circadian rhythms have been implicated in severity and outcome of infection and disease. Commonly, LPS and bacterial infection have been used to identify the mechanisms behind the difference in immune responses depending on the time of day of the challenge. In this thesis, the colon dwelling nematode parasite *Trichuris muris*, which elicits a Th2 immune response in resistant mice, was used to identify if circadian rhythms influence infection outcome 3 weeks post infection.

C57BL/6 mice infected with 200 eggs of *T. muris* at ZT0 (7am, lights on) expelled the parasite more efficiently than mice infected at ZT12 (7pm, lights off), which expelled with a delay of several days compared to ZT0 infected mice. Analysis of cell infiltration into the colon during the first days of infection suggested that there was no visible difference in the local immune response. There also were no differences in macrophage and dendritic cell numbers in colon tissue of naïve mice at ZT0 or ZT12.

Further experiments examined immunomodulation of the immune response to *T. muris* by pushing the immune response towards a Th1, by low dose infection, or a Th2 response, by vaccination with excretory/secretory antigen. In both cases any circadian influence was overwritten. Mice infected at ZT0 or ZT12 with only 40 eggs of *T. muris* were equally susceptible to infection and mice infected at ZT3 10 days after vaccination at ZT0 or ZT12 were equally resistant to infection.

Mice food restricted to mid-light phase and infected at ZT0 were not significantly delayed in their worm expulsion or polarised more towards a Th1 immune response compared to ZT0 infected mice fed during the dark phase. Therefore it is unlikely that feeding behaviour during the first days of infection is able to polarise towards a Th1 response and lead to delayed worm expulsion.

Transgenic mice were used to dissect the mechanism underlying the delay in worm expulsion in ZT12 infected mice. mPer2::luc mice were used to confirm rhythmic Per2 expression in colon tissue and dendritic cells. Infection of mPer2::luc mice at ZT0 or ZT12 with *T. muris* showed similar worm expulsion, antibody and cytokine production when infected at ZT0 or ZT12. Bmal1 flox LysM cre mice, which lack rhythmic clock gene expression in macrophages and granulocytes, produced a stronger Th2 antibody response in a primary infection at ZT3 than wild-type littermate controls. Newly generated mPer2::lucBmal1 flox CD11c cre mice showed the no difference in worm burden and parasite specific antibody production between ZT0 and ZT12 infected mice. Only IL-10 and IL-6 levels were significantly lower in ZT12 infected mPer2::lucBmal1 flox CD11c cre mice compared to ZT12 infected wild-type littermates. Confirmation of removal of exon 8 of the *Bmal1* gene was not achieved; therefore it is not clear if circadian rhythm in dendritic cells has any impact on the immune response to *T. muris* or if the mPer2::lucBmal1 flox CD11c cre mice and littermate controls both contain circadian rhythm in dendritic cells and therefore cannot be used to identify the role of the dendritic cell clock in the time of day differences in infection outcome.

This thesis shows that time of day of the infection impacts on the outcome of infection with *Trichuris muris*. 
Declaration

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And finally my friends who proof-read parts of my thesis and found all those bits that did not make sense.

And finally thanks to everyone else, who has not been named, but has not been forgotten. Without your friendship and support I would not have made it this far.
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AHTC</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>BMAL1</td>
<td>Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow dendritic cells</td>
</tr>
<tr>
<td>BrDU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>Carb/bicarb</td>
<td>carbonate/bicarbonate</td>
</tr>
<tr>
<td>CBA</td>
<td>cytokine bead array</td>
</tr>
<tr>
<td>CCL/CCR</td>
<td>CCL chemokine ligand/ CCR chemokine receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Circadian Locomotor Output Cycles Kaput</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre (causes recombination) recombinase</td>
</tr>
<tr>
<td>CRY</td>
<td>Cryptochrome</td>
</tr>
<tr>
<td>CT</td>
<td>circadian time</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DBP</td>
<td>D site of albumin promoter (albumin D-box) binding protein</td>
</tr>
<tr>
<td>DEPC-H2O</td>
<td>Diethylpyrocarbonate water</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E/S</td>
<td>excretory/secretory</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FEO</td>
<td>food entrainable oscillator</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Flox</td>
<td>Flanked by loxP sites</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intra peritoneal</td>
</tr>
<tr>
<td>IFA</td>
<td>incomplete Freund’s adjuvant</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LoxP</td>
<td>Locus of X-over P1</td>
</tr>
<tr>
<td>LPL</td>
<td>lamina propria leukocyte</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
</tbody>
</table>
LysM  Lysozyme M
MHC  major histocompatibility complex
MgCl₂  Magnesium Chloride
MLN  mesenteric lymph nodes
NaOH  Sodium hydroxide
NfkB  nuclear factor kappa-light-chain-enhancer of activated B cells
NK T cell  Natural killer T cell
OVA  ovalbumin
PAS staining  Periodic acid Schiff staining
PBS  phosphate-buffered saline
PBS-T  phosphate-buffered saline-tween 20
PCR  Polymerase chain reaction
PE  Phycoerythrin
PER  Period
PLN  peripheral lymph node
PMT  photomultiplier tube
qPCR  quantitative real-time polymerase chain reaction
R receptor
RNA  Ribonucleic acid
ROR  Retinoic acid-related orphan nuclear receptor
RORE  ROR response element
RPMI  Roswell Park Memorial Institute medium
RT  room temperature
RT-PCR  Real-time polymerase chain reaction
SA-POD  streptavidin peroxidase
SCID  Severe Combined Immunodeficient
SCN  suprachiasmatic nucleus
SEM  Standard error of the mean
TBE buffer  Tris/Borate/EDTA buffer
Th  T helper
TMB  3,3′,5,5′-Tetramethylbenzidine
TNF  tumour necrosis factor
TLR  Toll-like receptor
Tris-HCl  tris(hydroxymethyl)aminomethane
TSLP  Thymic stromal lymphopoietin
UV  Ultraviolet
VIP  vasoactive intestinal peptide
WHO  world health organisation
WT  wild-type
ZT  Zeitgeber time
Introduction
1. Circadian rhythm

Circadian clocks are a pervasive feature of the biology of all living organisms. The biological timing mechanism reflects external time cues, allowing the organism to adapt to its surroundings and maximise the ability to survive. The internal clock follows a near 24 hour pace (circa—about, dies—day). Circadian rhythms orchestrate body function appropriately depending on the time of day, for example by restricting activity to times where the animal is less likely to be discovered by predators or by anticipating food for efficient digestion (Panda & Hogenesch, 2004).

Without a functioning clock, the appropriate synchronism for physiology is impaired, and circadian rhythm disturbances lead to long term complications, for example metabolic disorders and inflammation. Clock disruption can result from aging, but also as a consequence of changes in the daily sleep wake cycle (such as jetlag or shift-work). It is now well established that circadian rhythms are cell-autonomous processes and are a dominant feature of the biology of both, single-celled and complex multi-cellular organisms.

1.1. Hierarchy of clocks in advanced organisms

Fig. 1 Schematic diagram of entrainment of peripheral oscillators. Light is taken up by the retina, which synchronises the SCN. The SCN entrains peripheral tissues via the autonomous nervous system and the adrenocorticotropic hormone (ACTH) axis. Corticoids oscillate as well and entrain peripheral tissue, as well as feed back to the brain. Organs associated with the digestive system also can be entrained by food, while activity and body temperature can entrain all peripheral organs.
Circadian rhythm is regulated by a central clock in the suprachiasmatic nucleus (SCN) in the brain, which in turn regulates the oscillation of the peripheral clocks (Zheng & Sehgal, 2008) throughout the body. The organisation of the entrainment pathways can be seen in Figure 1, which shows how peripheral tissues can be entrained via the autonomous nervous system, adrenocorticotropin hormone (ACTH) axis and corticoids. Details how the SCN and peripheral oscillators are regulated are described below. Within each peripheral organ, individual cells containing a molecular clock are entrained to follow the same rhythm in one tissue.

1.1.1. Suprachiasmatic Nucleus (SCN)

The main circadian oscillator, or “master clock” is now known to be neural (Moore & Lenn, 1972; Stephan & Zucker, 1972) and serves a major role by synchronising the activity of multiple peripheral oscillators throughout the body, thus maintaining overall circadian synchrony (Aton & Herzog, 2005).

The central clock is located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus. It is assumed that the SCN consists of several autonomous oscillating cells, which are linked to form the main clock (Aton & Herzog, 2005; Harmar et al., 2002). In mammals, the SCN sets the period and the phase of the circadian rhythm throughout the body. The SCN was discovered to be the site of the main circadian clock in 1972 independently by the laboratories of Robert Moore and the laboratory of Irving Zucker and Friedrich Stephan (Moore & Lenn, 1972; Stephan & Zucker, 1972). Later it was shown that transplanting SCN tissue from rats into SCN-lesioned animals could restore the circadian rhythm (Ralph et al., 1990), which showed that the SCN is the site of the central clock.

SCN cells can communicate via vasoactive intestinal peptide (VIP) signalling, as gaps between cells are small enough for VIP molecules secreted from one cell to be recognised by VPAC2 receptors on neighbouring cells (Aton & Herzog, 2005). Due to this signalling, clock genes are expressed and more VIP is secreted. The cells are thereby synchronised to one rhythm.

1.1.1.1. Entrainment of the SCN

The light-dark cycle entrains the SCN every day acting as a ‘Zeitgeber’ (time-giver) leading to 24h rhythmicity. Melanopsin, a vitamin A-based opsin photopigment, found in intrinsically photosensitive retinal ganglion cells, was shown to entrain the SCN (Provencio et al., 2000; 1998). Rods and cones, which lack melanopsin, have also been shown to signal to the SCN (Sollars et al., 2003). Mouse models which lacked rods and cones in their retina are still entrained by light, suggesting that melanopsin is the main photopigment required for entrainment by light (Freedman et al., 1999; Lucas et al., 1999; Rea et al., 2005).

1.1.2. Peripheral clocks are entrained by output from the SCN clock

Peripheral tissues also show independent circadian clock rhythm, which is assumed to be synchronised by the SCN (Reppert & Weaver 2002; Schibler et al., 2002). Tissues with a peripheral clock include: liver, kidney, lung pituitary, brain regions other than the SCN and
kidney (Miller et al., 2007; Yoo et al., 2004). The interaction between the local clocks in the peripheral tissue and the SCN ensures oscillation throughout the body, even though the exact mechanism of synchronisation is still unclear (Dibner et al., 2010; Stratmann & Schibler, 2006). The SCN is supposed to synchronise the peripheral tissue via neuronal and humoral pathways (Dibner et al., 2010; Stratmann & Schibler, 2006). The adrenal glands are synchronised by the SCN via the paraventricular nucleus (PVN) through the sympathetic nervous system (Berthoud, 2002; Ruiter & Kalsbeek, 2006), while the liver can be synchronised by the SCN through the feeding centre (Bartness et al., 2001). These results show that circadian rhythms can be found in most peripheral tissues.

Through SCN-lesion studies it has been established that the clock in the peripheral tissues continues to function in the absence of a central clock. Rhythmic mPer2 expression could be measured in peripheral tissues, such as cornea and lung of mPer2::luc mice (Yoo et al., 2004), independent of SCN entrainment. Rhythms in tissue and cells can be observed for days ex-vivo. mPer2 rhythms could be seen in SCN, liver and lung tissue for up to 20 days under photomultiplier tubes (Yoo et al., 2004). More recently, Keller et al., (2009) demonstrated robust circadian rhythms from a range of immune tissues and cells, including spleen and macrophages. Individual cells which make up a peripheral oscillator process - autonomous clocks allowing them to maintain rhythmicity in isolation. Cell-to-cell communication between these individual oscillators is essential to facilitate synchronisation between cells and thus maintain overall rhythmicity.

1.1.2.1. Non-photic entrainment

It appears that the peripheral clock can be maintained in absence of the SCN and maybe be entrained by other means, such as regular feeding times (Damiola, 2000; Preitner et al., 2002; Stokkan et al., 2001).

Food restriction can entrain mice and other animals to anticipate food. This anticipation can change the sleep-wake cycle and behaviour, as well as clock gene expression in peripheral organs, such as the colon and liver, but cannot influence the SCN (Damiola, 2000). Therefore restricted feeding is commonly used to de-couple peripheral oscillators from the SCN and alter circadian gene expression in peripheral tissues (Hussain & Pan 2009). A shift of colon clock-genes can be observed in the first 48h of food restriction (Hoogerwerf, et al. 2007; Hoogerwerf 2010), which shows how quickly the system adapts to changes in food availability.

Another potential entraining cue to cells in peripheral tissue is a change in body temperature. This is clearly demonstrated in vitro, where a change of only one degree in temperature can cause re-synchronisation of oscillation in fibroblast cells (Brown et al., 2002; Buhr et al., 2010), therefore changes in temperature also have to be taken into consideration when analysing changes in circadian rhythm.
1.2. Circadian timing in experimental settings

Time is described as ‘Zeitgeber time’ (ZT) when animals are maintained in a light dark (LD) cycle, with the light acting as an entraining signal (zeitgeber). In the absence of this light cue, under constant conditions, complete darkness (D/D) or complete light (L/L), time is described as ‘circadian time’ (CT). Animals placed in constant conditions have to rely on their intrinsic clock to ensure rhythmic activity, where CT0 corresponds to subjective dawn and CT12 to subjective dusk.

1.3. The mammalian molecular clock

Circadian rhythms are generated within cells through the rhythmic transcription and translation of a set of core clock genes (Fig. 1). Genetic screening in different animals helped to understand the molecular components of the circadian clock, which differ in unicellular and multi-cellular organisms (reviewed in Bell-Pedersen et al., 2005). Table 1 lists the known components of the mammalian molecular clock.

Table 1: Known molecules involved in the molecular clock

<table>
<thead>
<tr>
<th>Genes in mammals</th>
<th>Deletion of single genes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1, (2)</td>
<td>Arrhythmic (rhythmic)</td>
<td>Bunger et al., 2000; Shi et al., 2010</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Short period</td>
<td>Debruyne et al., 2006</td>
</tr>
<tr>
<td>Period (Per) 1, 2 and 3, (4)</td>
<td>Short period</td>
<td>Zheng et al., 2001</td>
</tr>
<tr>
<td>Cryptochrome (Cry) 1 and 2</td>
<td>Cry1&lt;sup&gt;−/−&lt;/sup&gt;:short period</td>
<td>Horst et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Cry2&lt;sup&gt;−/−&lt;/sup&gt;: long period</td>
<td></td>
</tr>
<tr>
<td>CKIα, δ, ε</td>
<td>CKIδ&lt;sup&gt;−/−&lt;/sup&gt;: Lethal in mammals</td>
<td>Etchegaray et al., 2009</td>
</tr>
<tr>
<td></td>
<td>CKI&lt;sup&gt;−/−&lt;/sup&gt;: long</td>
<td>Meng et al., 2008a</td>
</tr>
<tr>
<td>Rev-ERBα and β</td>
<td>REV-erba&lt;sup&gt;−/−&lt;/sup&gt;: Short period</td>
<td>Preitner et al., 2002</td>
</tr>
<tr>
<td>ROR α, β, γ</td>
<td>staggerer (α) short period</td>
<td>Baggs et al., 2009; Liu et al., 2008</td>
</tr>
<tr>
<td>Fbxl3</td>
<td>Long period</td>
<td>Shi et al., 2013</td>
</tr>
<tr>
<td>NPAS 2</td>
<td>Short period</td>
<td>Dudley et al., 2003</td>
</tr>
<tr>
<td>Glycogen synthase kinase 3 (gsk3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-element binding protein (DBP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4BP4</td>
<td></td>
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</tr>
</tbody>
</table>

So far these molecules have been identified to be involved in the different stages of the circadian clock. The genes involved in the main feedback loop are highlighted in grey. Bmal1 and CLOCK are the main proteins of the circadian clock. Period, Cytochromes and CKI are involved in the first feedback loop, while Rev-ERBs and RORs are found in the secondary feedback loop. The other molecules can be found as well at different stages of the circadian clock. Single gene deletion changes behaviour and period in most of animals.
Many clock genes have multiple homologues, such as *Per* and *Cry*, while others have paralogs. *Npas2* is a functional paralog for the *clock* gene and *Bmal2*, which can only be found in peripheral tissue, but not the SCN, is the paralog for *Bmal1* (Looby & Loudon, 2005). Duplication of gene suggests that there is redundancy in many elements of the molecular clock; *mCry1*\(^-/-\) and *mCry2*\(^-/-\) show almost normal clock function, while a double knockout of *Cry1* and *Cry2* disrupts the molecular clock (Horst et al., 1999). Generally one can say that all the core clock genes are important, but the presence of homologues and paralogs shows that many core clock genes are not essential and redundancy ensures that circadian rhythm is not disrupted by disruption of individual clock genes (Debruyne et al., 2006). Some clock genes, such as the casein kinases, are not only regulators of clock function and therefore disruption does impact not only on circadian rhythm but also other signalling pathways.

### 1.3.1. Concept of rhythmic negative feedback as a regulation

This main oscillator is regulated via two negative feedback loops (Fig. 1). The feedback loops consist of transcriptional-translational and but also post-transcriptional regulatory mechanisms (Allada et al., 2001; Preitner et al., 2002; Reppert & Weaver, 2001).

![Figure 2: The mammalian molecular clock](image)

**Figure 2: The mammalian molecular clock**

The molecular clock consists of two interlocked feedback loops. The CLOCK-BMAL1 dimer binds to E-Boxes in gene promoters, which up-regulates the transcription of genes such as *Crys*, *Per2*, *Rora* and *Rev-erba*. Gene-products CRYs and PERs heterodimerise and form a complex with CK1\(\varepsilon\\delta\), which inhibits BMAL1/CLOCK activity, which in turn stops *Cry/Per* transcription, forming the first negative feedback loop. In the second feedback loop *Rev-ERBa* and *RORa* influence transcription of *Bmal1*. RORa up-regulate *Bmal1* expression, while Rev-ERBa blocks *Bmal1* transcription.

The core components of the molecular clock are CLOCK and BMAL1. When these proteins heterodimerise, it initiates the transcription of target genes containing E-boxes with a cis-
regulatory enhancer element, for example Periods 1, 2, and 3 or Cryptochromes 1, 2 and 3 (Kwon et al., 2011). CRY and PER proteins can be found accumulating in the cytoplasm forming a heterocomplex, before they move into the nucleus. This process takes several hours. Inside the nucleus, the PER-CRY complex represses the activity of the CLOCK-BMAL1 heterodimer (Lee et al., 2001; Meng et al., 2008b; Preitner et al., 2002; Reppert & Weaver, 2002), which in turn inhibits transcription of Crys and Pers (Gallego & Virshup, 2007). Proteasomal degradation breaks down the inhibitory complex. Casein-kinase1ε and δ (CKIε and δ) cause this degradation process (Lee et al., 2001; Meng et al., 2008a).

The second feedback loop regulates Bmal1 expression and it consists of RORα and Rev-ERBα (Gallego & Virshup, 2007). RORα and Rev-erba transcription is also controlled by the CLOCK-BMAL1 heterodimer. Rev-ERBα and RORα in turn access the Bmal1 promoter as the ROR response elements (RORE) are open structures (Onishi et al., 2008). RORα is the activating and Rev-ERBα the repressing regulator (Akashi & Takumi, 2005; Guillaumond et al., 2005) and both positive and negative regulation results in cyclic Bmal1 expression (Kwon et al., 2011).

1.3.2. Pace setting

Posttranslational processes also lead to a time delay in the feedback loop, thereby creating the 24h cycle (Kalsbeek et al., 2011; Lee et al., 2001). It was assumed that the delay leading to a 24h day was due to the mRNA to protein conversion. It has been shown however, that this process only gives a 2-3h delay, which would not be enough to form a 24h rhythm (Kalsbeek et al., 2011; Kwon et al., 2011), which indicates other factors have to play a role in lengthening the period to 24 hours. So far, several post translational modification steps, as proteins mediating acetylation, phosphorylation, SUMOylation, and ubiquitination have been identified which regulate period (Kwon et al., 2011; Lee et al., 2001). Casein kinase enzymes CK1 phosphorylate PER proteins, which increases the time before the proteins are degraded and the cycle is re-started by about 8 hours. The next circadian cycle is started with the de-repression of CLOCK-Bmal1 activity, which initiates the transcription of Cry and Per genes (Meng et al., 2008b).

1.2. Rhythmic outputs

The circadian clock regulates many rhythmic outputs, influencing gene expression as well as hormone secretion and behaviour (Panda & Hogenesch, 2004). Individual clock gene components, such as casein kinases, RevERBα and Bmal1, also have non-clock functions, where they regulate transcription of genes involved in metabolism and DNA repair (reviewed in Feng and Lazar 2012). In mammals, examples of circadian rhythms are sleep-wake cycles, energy homeostasis, blood pressure, body temperature, liver metabolism and renal activity (reviewed in Preitner et al., 2002). The following are examples of rhythmic output.
1.4.1. Sleep-wake cycle

One of the aspects of behaviour affected by circadian rhythm is the sleep-wake cycle. Sleep-wake cycles are part of the normal 24h rhythm and both aspects of the cycle are under circadian control. The structure and timing of sleep are regulated by homeostatic and circadian systems (Borbély & Achermann, 1999; Pace-Schott & Hobson, 2002), which are entrained by the SCN. Melatonin levels are associated with induction of sleep (Liu et al., 1997), while a rise of glucocorticoids is linked to wakefulness.

The circadian variations in sleep are eliminated when the SCN is lesioned, which causes fragmented sleep, distributed over the 24h period (Edgar et al., 1993; Mistlberger, 2005; Trachsel et al., 1992). It is therefore possible, that the SCN not only regulates time of sleep and wake cycles but also regulates sleep stages (Rosenwasser 2009). Removing the SCN can also increase the sleep time in animals such as squirrel monkeys (Edgar et al., 1993) and some mouse strains (Easton et al., 2004). Regular sleep has been found to influence the immune responses. Loss of sleep increases susceptibility to infection and disease (Everson, 1993; Mohren et al., 2002). Patients with regular sleeping patterns after vaccination against Hepatitis A had to higher antibody titres compared to patients who were sleep deprived following vaccination (Lange, 2003).

So far it is still unclear, if sleeping rejuvenates the immune system, but it has been shown that sleep deprivation impairs defence mechanisms, bacterial infection were able to spread though the blood in sleep deprived, but not control rats (Everson, 1993), which suggests regular sleeping patterns are beneficial to health.

1.4.2. Metabolism

The circadian clock is now known to be involved in the regulation of many metabolic processes. Disruption of metabolic circadian rhythms is often seen in shift workers and commonly results in the development of pathologies including obesity (Debruyne, 2008; Gangwisch et al., 2005; Rudic et al., 2004; Turek et al., 2005) and diabetes (Oster et al., 1988; Velasco et al., 1988). This is replicated in the laboratory and studies show that mutations in the different clock genes often lead to metabolic phenotypes.

The same mutation of the *Clock* gene, *Clock*Δ19, leads to different metabolic phenotypes depending on the strain of mouse used for the experiments, but in all cases metabolic pathways were affected, resulting in altered glucogenesis, metabolic syndrome and obesity in mice on a C57BL/6J background, insulin sensitivity and glucose intolerance in mice on a BALB/c/CBA background and mice on a JcI:ICR background had lower levels of fatty acids in serum and no obese phenotype (reviewed in Froy, 2011). It is commonly known that different inbred strains develop a variety of inflammatory and metabolic conditions depending on the genetic background (Mills et al. 2000, Nishikawa et al. 2007, Stanya et al. 2013). C57BL/6 mice for example have a trend towards Th1 bias when challenged, while BALB/c or DAB/2 mice produce
strong Th2 responses (Mills et al. 2000). C57BL/6 mice also appear to be more prone to obesity and metabolic disease compared to BALB/cA mice, which require a high fat diet to develop symptoms. Furthermore, sex and age of the animals also impact on the development of the disease (Nishikawa et al. 2007). Stanya et al. (2013) recently showed that IL-13 signalling via Stat3 is critical in the control of hepatic glucose production, which explains the difference in strains of mice is dependent on the bias of their immune response (Mills et al. 2000).

Knock out of the Bmal1 gene has been linked to reduced adipogenesis (Shimba et al., 2005) and mice lacking Per2 expression have a disrupted feeding rhythm, which, on a high fat diet leads to obesity (Yang et al., 2009). These transgenic mice lacking a functional core clock show metabolic phenotypes, therefore metabolism appears to be closely linked to clock gene expression, but different core clock mutations result in a variety of metabolic conditions, which is most likely dependent on the genetic background of the mouse strain (reviewed in Sellers et al. 2012). Mice on a high fat diet, which were allowed to feed ad lib., developed obesity, hyperinsulinemia and inflammation, while feeding regularly throughout 24h. When these mice were placed on restricted feeding, while consuming the same amount of calories, these conditions were not observed (Hatori et al. 2012). This is a further example of the close connection between circadian rhythm and metabolism. Genetic background has also been shown to be important for timekeeping in mice kept in total darkness, as free running periods vary between strains (Schwartz and Zimmerman 1990). Manipulation of the diurnal behaviour, by changing light-dark cycles, did result in different amounts of wheel-running and sleep patterns between strains of mice (Oliverio and Malorni, 1979). Therefore one can conclude that strain backgrounds play an important role in circadian rhythm, immune responses and metabolism.

Despite several examples of rhythmic fluctuation in metabolism, it is not completely clear how the circadian clock controls metabolic events. Rhythmic expression of nuclear hormone receptors has been found in many metabolic tissues, such as liver, skeletal muscle, white and brown adipose tissues (Yang et al., 2006). More than half of the 45 nuclear hormone receptors found in the mouse are expressed rhythmically, such as Rev-ERBs, RORs, which are also linked to core clock gene expression, but also peroxisome-proliferator-activated-receptors (PPARs), Sirtuin 1, AMP protein activated kinase and PPARγ co-activator-1α (reviewed in Froy, 2011; Yang et al., 2006). RORα plays part in the regulation of lipogenesis and lipid storage (Lau et al., 2004) and PPARα is also involved in lipid, as well as in glucose metabolism. Transcription of PPARα is mediated directly by core clock genes, therefore lipid and glucose metabolism are directly regulated by the circadian rhythm (Inoue et al., 2012; Oishi et al., 2005). Rev-ERBα is pro-adipogenic transcription factor which is linked to differentiation of adipose tissue (Chawla & Lazar, 1993). Rev-ERBα−/− mice have a metabolic phenotype, including disrupted lipid metabolism and decreased bile acid accumulation (Le Martelot et al., 2009), indicating that the metabolic disruption seen in mice with disrupted central clocks could be a consequence of the clock gene’s direct action on metabolic pathways.
1.4.2.1. Circadian rhythm in the intestine

The intestine is regulated by its own peripheral clock, similar to liver, lung and other peripheral tissue (Lee et al. 2001; Hoogerwerf 2010). Food intake is an important part in the regulation of gut rhythmicity (Hoogerwerf et al., 2007; Konturek et al., 2011; Sládek et al., 2007). Rhythmicity can still be observed in the gut even if the vagus nerve of an animal is resected, disrupting signalling from the brain to the intestine, which could affect clock gene expression in peripheral tissues (Hoogerwerf, et al., 2007). Furthermore, food entrainment can maintain rhythm in peripheral tissues in SCN-lesioned animals (Marchant & Mistlberger, 1997; Stephan et al., 1979). Nonetheless, circadian rhythms in the gut can still be observed during food deprivation and housing in D/D conditions (Hoogerwerf et al., 2007; Hoogerwerf 2010), which suggest that the SCN can regulate circadian rhythm in the colon, but in its absence feeding habits can entrain clock gene expression in the intestine.

In the colon of the rat, rhythmic gene expression of Per1 and 2, Cry1, Bmal1, Rev-ERBα and NHE3 was observed in colon tissue sampled every 4 hours and analysed using RT-PCR. The genes were expressed in synchrony with the peripheral clock in the liver, but delayed by about 8 hours compared to the main clock of the SCN (Sladek et al., 2007). The expression of many clock genes e.g. clock and per2 in the human colonocytes was shown by RT-PCR (Pardini et al., 2005), which could play a role in the time-dependent varying efficiency of colorectal cancer therapy (Fu & Lee, 2003; Mormont & Levi, 2003). Clock genes in various tissues of the mouse intestine have also been shown by Hoogerwerf et al. (2007, 2008). RT-PCR of colon and stomach tissue collected every 4 hours revealed rhythmic expression of Per1, Bmal1, Per2, Per3 as well as Cry1 and Cry2. These results highlight the presence of circadian rhythm in the intestine.

In general it is assumed that circadian rhythms in the intestine influences gut motility, absorption rates, enzyme activity, cell proliferation and migration (Merle et al., 2000; Scheving 2000; Froy and Chapnik 2007; Hoogerwerf 2010; Hussain & Pan, 2009; Konturek et al., 2011). Secretion of gastric acid for example is increased at night, whereas motility of the colon is higher in the morning (Hoogerwerf et al., 2007). In Per1/Per2-/- mice, stool output lost rhythmicity, as in wild-type mice most stool is past during the dark cycle (Hoogerwerf, 2010a/b). The same result was found for colonic pressure activity, indicating that many intestinal functions are under circadian control. Absorption of macronutrients from the small intestine occurred in a circadian manner, Pan and Hussain (2009) showed that mice absorbed higher levels of glucose, lipids and peptides during the night than during the day, which most likely coincides with feeding habits. Taking these results together, circadian rhythm has major involvement in the function of the intestine.

Further research indicates that circadian rhythm also regulates innate immunity within the small intestine. In mice, mRNA of intestinal defensins (cryptidins) can be found cycling, with their peak at the end of the dark cycle (Froy & Chapnik 2007) and mRNA transcripts of most toll-like
receptors (TLRs) have been shown to follow circadian rhythm in mouse jejunum and Paneth-enriched crypt base cells (Froy et al. 2005; Froy & Chapnik 2007). Mice mainly feed in the beginning of the dark cycle (Hoogerwerf 2010), therefore it was expected that the level of TLRs in the gut was highest in the second half of the night and early morning, as it takes several hours for the food to reach the intestine (Froy & Chapnik 2007). During an infection the immune system is up-regulated, but the basal level of TLR and defensin expression is regulated by the circadian rhythm, with levels higher when infection is more likely.

Commensal microorganisms have been shown to signal through TLRs to regulate local clock gene expression in the small intestine, as most TLRs are expressed in a rhythmic manner. Treatment with antibiotics therefore disrupts the clock machinery in the small intestine (Mukherji et al., 2013). These findings lead to the suggestion that time keeping in the intestine is not only dependent on food, but also on the microbiota found within the gastrointestinal tract.

1.4.3. Melatonin and glucocorticoid rhythms

Melatonin and glucocorticoids are two hormones which are secreted in circadian fashion and are linked to circadian output in peripheral tissues.

Melatonin is a hormonal regulator of peripheral circadian rhythm (Konturek et al., 2011) produced predominantly in the pineal gland (Barriga et al., 2001). Not all strains of mice are able to produce melatonin; C57BL/6 mice for example have no melatonin expression. In mice which do produce melatonin, expression peaks around midnight, while under stressed conditions this peak is dampened (Barriga et al., 2001). The rise in melatonin levels has been linked to Th1 immune responses and can activate T cells, monocytes and NK cells, which enhances inflammatory cytokine production late at night (Esquifino & Cardinale, 1999; Maestroni et al., 1986). Rhythmic melatonin secretion has been linked to influence gut motility (Delagrange et al., 2003; Merle et al., 2000) and has been used to improve IBS symptoms (Lu et al., 2005) by reducing pro-inflammatory cytokine levels (Konturek et al., 2007). These studies show that melatonin is an important hormone influencing health in a circadian manner.

Glucocorticoids are a family of steroid hormones which are secreted predominantly from the adrenal glands. Rhythmic glucocorticoid secretion is important for entraining peripheral tissues in general, as it acts on transcriptional pathways in diverse contexts, such as metabolism and inflammation (Biddie et al., 2012; Kalsbeek et al., 2012). Corticosterone in mice or cortisol in humans peaks before the onset of activity (Kalsbeek et al., 2012; Malisch et al., 2008), but a pulsatile pattern has been identified, where a pulse of glucocorticoids is released every hour (Biddie et al., 2012). Under stress conditions the rhythm appears to be lost and levels are up-regulated (Barriga et al., 2001), as glucocorticoids boost energy levels available (Kalsbeek et al., 2012). Glucocorticoids are strong immunosuppressants and are commonly used to treat inflammatory conditions (Biddie et al., 2012). A reduction in cortisol secretion, which was observed in humans with abnormal sleep cycles (Vgontzas et al., 1999), could lead to elevated
Inflammation, infection and circadian rhythm

The immune system has evolved to combat invading viruses, bacteria and parasites effectively. In order to deal with these pathogens effectively, the immune system is linked to the internal rhythm which synchronised bodily functions. Immune parameters which are up-regulated during the day in diurnal mammals would be expected to be up-regulated 12 hours later in nocturnal animals, as they are optimised to encounter pathogens during activity or rest (Płytycz & Seljelid, 1997).

Humans are the only species to voluntarily disrupt natural circadian rhythm, for example through shift work and jet lag, which is linked now to many diseases (Harrington, 2010). Chronic jet lag experiments in mice have showed increased rates in cancer development (Filipski et al., 2004) and earlier mortality rates (Davidson et al., 2006).

Circadian rhythm has been implicated in many examples of inflammation, such as arthritis and asthma, myocardial infarction and stroke (Cutolo et al., 2003; Gupta & Shetty, 2005; Muller et al., 1985; Panzer et al., 2003; Smolensky et al., 2007). In the intestine, chronodisruption can cause or exacerbate existing conditions, such as inflammatory bowel disease, irritable bowel syndrome, ulcer formation, gastrointestinal reflux disease and even cancer (Hoogerwerf, 2009; Konturek et al., 2007), which is likely due to the involvement of circadian rhythm in most intestinal functions. Therefore maintaining circadian rhythm within the intestine appears important to keep a healthy digestive system.

The lung is circadian rhythmic, with the clock contributing to diurnal variations in pulmonary physiology. In nocturnal asthma one can see how the circadian rhythm influences the asthmatic reaction of the lung (Jarjour, 1999; Lebowitz et al., 1997; Martin, 1999), as symptoms get worse during the night, which is associated with increased infiltration of eosinophils and higher levels of IL-5 in serum as well as airway constriction (Panzer et al., 2003). However, environmental factors, such as the presence of house dust mites in bedding, cannot be ruled out as a factor for worsening in asthma symptoms at night. Clara cells, the main epithelial cell in the lung, also appear to follow a circadian rhythm (Gibbs et al., 2009). When Clara cells are reduced in the lung, the clock protein Per2 expression is lowered. In the lung, Per2 is supposed to be involved in the secretion of a hypophase layer of bronchoalveolar fluid, local stem cell function within the bronchial epithelium, modulation of pulmonary immune response and the metabolism of
xenobiotics (Elizur et al., 2007; Singh & Katyal, 2000), showing direct involvement of clock gene expression in healthy lung function.

Patients with rheumatoid arthritis often suffer from stiffness and exacerbated symptoms in the morning (Harkness et al., 1982). Worsening of symptoms could occur due to diurnal rhythm in pro-inflammatory cytokine expression, such as TNFα and IFNγ (Petrovsky et al., 1998). Melatonin levels are increased in many rheumatoid patients, while the cortisol production is impaired (Cutolo et al., 2003; Sulli et al., 2002). Hashimoto et al., (2010) showed that in rheumatoid patents rhythmic expression of PER protein and Bmal1 and dbp mRNA are disrupted in several tissues. Cry1^+/Cry2^- mice, lacking circadian rhythm show increased joint swelling and inflammation compared to wild-type controls. Administration of anti-TNFα reduced these symptoms, by reducing pro-inflammatory cytokine levels. CRY is thought to repress TNFα production and thus this is why blocking TNF improves inflammation in the CRY double knockouts. These studies show clearly an involvement of circadian rhythm in the severity of symptoms in rheumatoid arthritis. Nonetheless environmental factors have to be taken into account when analysing the symptoms disease, as long periods of immobility could contribute to the exacerbation of symptoms in arthritis in the morning.

Shift work has been identified as a risk factor for breast cancer (Schernhammer et al., 2006), as well as colorectal, endometrial and prostate cancer (Kubo et al., 2006; Schernhammer et al., 2003; Viswanathan et al., 2007), as cancer has been shown to be more prevalent in shift workers (Straif et al., 2007). This is likely due to de-synchronisation of the circadian clock through a disrupted sleep-wake cycle, which in turn leads to alterations in clock regulated processes, such as hormone production and cell repair. Breast cancer patients often show non-cycling levels of cortisol, with a reduced peak in the morning, but elevated levels in the afternoon (Abercrombie et al., 2004) as well as supressed melatonin levels (Schernhammer & Hankinson, 2005; Schernhammer et al., 2008). Skin cancer development has been linked to circadian rhythm as the repair mechanism dealing with damage caused by UV is subject to circadian control and more effective in the afternoon evening in a mouse model (Gaddameedhi et al., 2011). Exposure to UV rays when repair activity was low led to quicker skin cancer development as when repair activity was high. Circadian rhythms could therefore play a role in many different cancers.

The time of a pathogen entering the host can be important in the outcome of infection. Early studies showed that mice were more susceptible to lethal doses of endotoxin lipopolysaccharide (LPS) administered i.p. during the day, rather than night (Halberg et al., 1960; Liu et al., 2006). Infection with Salmonella enterica serovar Typhimurium showed a time of infection dependent difference in severity of colonization and inflammation, which was absent in CLOCK^- mice. The number of bacterial colonies isolated from the gut of infected wild-type mice was higher in mice infected in the morning compared to the evening, as were pro-inflammatory cytokine levels (Bellet et al., 2013). In a sepsis induction model, the time of gut perforation also changed the severity of disease. The model depends on the expression of TLR-9 (Plitas et al., 2008;
Yasuda et al., 2008), which has been shown to be expressed in a diurnal fashion. TLR-9 expression was higher at ZT19, at which time perforation of the colon led to higher mortality, higher pro-inflammatory cytokines and more bacterial infiltration into the peritoneum compared to mice where sepsis was induced at ZT7 (Silver et al., 2012a). Infection with live pathogens show that the immune system anticipates infection and time of infection influences how successful the host responds to the pathogen.

2.1. Intrinsic circadian rhythm can be found in individual components of the immune system

Various studies have found rhythmic clock gene expression in immune cells. Silver et al., (2012b) established diurnal rhythms in macrophages, dendritic cells and B cells enriched from spleens using RT-PCR. Oscillation in macrophages has also been shown by Keller et al. (2009), in vivo as well as ex vivo. Macrophage did not only show molecular expression of clock genes (Keller et al., 2009) but also functional changes in phagocytosis activity, which was greater during the night (Barriga et al., 2001). Natural killer cells enriched from rats also showed rhythmic clock gene expression, as well as a circadian rhythm in cytolytic activity, which is highest in during the night (Arjona et al., 2004; Arjona & Sarkar, 2005, 2006). Bollinger et al., (2009; 2010; 2011) showed that CD4+ T cells possess an intrinsic clock machinery, which results in rhythmic proliferation and cytokine production.

Most immune cells not only show intrinsic circadian rhythm, but their numbers show diurnal differences in circulation (Abo et al., 1981; Kawate et al., 1981). In humans, naive T cell levels are lowest midday, while CD8+ T cells peak about this time (Dimitrov et al., 2009). Cortisol rhythms have been linked to the naive T cell rhythms in a negative correlation and peak during the night, while cortisol peaks in the morning (Dimitrov et al., 2009). Sensitivity to cortisol is related to the rhythmic expression of CXCR4 and its ligand CXCL12, which regulates T cell migration to the bone marrow in response to increases in cortisol in serum (Mendez-Ferrer et al., 2008)

Cytokine levels are also expressed in a circadian manner, and have been linked to melatonin and glucocorticoid rhythms (Petrovsky & Harrison, 1997). In Cry1/Cry2−/− mice, which lack a rhythmic core clock, pro-inflammatory cytokine levels of TNFα and IL-6 are up-regulated through increased signalling via NfκB (Narasimamurthy et al., 2012).

These examples show that multiple components of the immune system are under circadian control, either by intrinsic molecular clocks or the presence or absence of hormonal regulators. Most likely a combination of both factors, hormones and cell intrinsic rhythms, influences a time of day difference in immune responses (Fortier et al., 2011).
2.2. The circadian clock can influence the immune system; in turn the immune system can also regulate the clock

The ability of the immune system to influence clock gene expression has been shown by negative regulation of core clock proteins by TNFα (Bozek et al., 2009), which is up-regulated in a general immune response. IFNα and γ have both been shown to impact on clock gene expression. IFNγ administration blunts the amplitude of PER1 expression (Kwak et al., 2008) while IFNα affects the expression of Per1, 2 and Cry 1 mRNA as well as the levels of CLOCK and BMAL1 proteins (Koyanagi & Ohdo, 2002). Low doses of LPS up-regulate pro-inflammatory cytokines which induces a phase delay in locomotor activity when LPS is administered late in the evening, which was comparable to light pulse (Marpegán et al., 2005). LPS is likely regulating the circadian system by signalling via TLR-4, as TLR4−/− mice do not show a phase delay compared to wild-type controls (Paladino et al., 2010). These results lead to the suggestion that cytokines are able to suppress clock gene expression, which might occur especially in chronic inflammatory diseases.

Due to the circadian rhythms in several immune functions, a variation in susceptibility to infection can be linked to time dependent variations in the immune system. This can influence clinical parameters in diagnosis as well as pharmacological therapy (Haus & Smolensky, 1999).

2.3. Circadian drug therapy

Chronotherapy is a newly emerging field of pharmacology, trying to administer remedies at the time of day when the effect is highest while the side effects lowest. For cancer treatment, chronotherapy has shown promise, as in some patients slowing of cancer growth and reduced treatment toxicity could be observed (Lévi et al., 1997; 1990; Schmiegelow et al., 1997). Chronotherapy is also being developed for asthma patients. Administering one dose of synthetic glucocorticoids at night was able to reduce nocturnal exacerbation of the condition and constriction of the airway (Burioka et al., 2010), which reduced the glucocorticoids dose over the day. The same schedule has been proposed to alleviate symptoms in rheumatoid arthritis, as a small dose of cortisone acetate late in the evening is able to suppress the diurnal increase seen in the early morning in pro-inflammatory cytokines as TNFα, IFNγ, IL-1 and IL-12 (Arvidson et al., 1997; Petrovsky et al., 1998).

Administration of IFNα, which is used to treat viral infections and tumour growth, has severe disruptive effects on locomotor activity, body temperatures and even clock gene mRNA expressions. Injection of the drug at ZT12 led to blunting of clock gene expression, locomotor activity, which was not seen at ZT0 (Ohdo et al., 2001). These results show that chronotherapy should be evaluated for many different conditions, as side effects could be more severe at certain times of the day.

Vaccination following the principles of chronotherapy has been evaluated again and again since 1967. So far, no clear pattern has emerged for vaccination to be more successful in the morning.
or the afternoon, but for several vaccines a diurnal effect could be observed (Feigin et al., 1967; Langlois et al., 1995; Phillips et al., 2008; Pollmann & Pollmann 1988), suggesting a diurnal effect has to be taken into account in vaccination.

As inflammation and circadian rhythm are closely intertwined, an infection model that allowed development of inflammation in the colon was needed to establish the influence of circadian rhythm on infection.

3. Experimental parasite model *Trichuris muris*

Worldwide 2 billion people, about 24% of the world’s population are infected with intestinal helminths, predominantly the roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale*, which can be transmitted via contaminated soil and water due to poor sanitation (World Health Organisation, 2013). Infection rates are especially high in the developing world and often multiple parasites can be found in one host. Despite being rarely fatal, intestinal helminths cause a high level of morbidity. Infection is especially common in children and is a cause of malnutrition, stunted growth and reduced education (Bethony et al., 2006; World Health Organisation, 2013). Helminth infection can also be found in livestock, which reduces growth of animals and overall productivity (Artis, 2006).

Antihelminthic drug treatment is successful for short term, however re-infection rates are high and drug resistance is emerging, therefore new treatment ideas have to be developed. Successful vaccines for helminths in livestock have been developed so far, but a vaccine for humans is still out of reach (Maizels et al., 1999).

*Trichuris muris*, a whipworm naturally occurring in mice, is used to model human infection with the whipworm *Trichuris trichiura*, contributing to the better understanding of the immune response to parasitic infection (Roach et al., 1988). *T. muris* is a nematode dwelling in the caecum and large intestine of its host. The parasite causes a mild to moderate infection in the intestine, leading to only slight changes in animal behaviour, without obvious malaise. The disruption caused by the presence of the worm in the colon of infected mice appears to be similar to inflammatory bowel disease and *T. muris* infection is an ideal model for this disease (Levison et al., 2010). Contrary to these findings, administration of *Trichuris suis* eggs, the related parasite causing trichuriasis in pigs, reduces IBD symptoms in some patients (Summers, et al., 2005; Summers, 2003). This method is still questioned and chronic infection could be a possible outcome of medication with *T. suis* (Kradin et al., 2006). Immune responses against parasite are supposed to be regulatory in nature, affecting the bacterial flora likely to cause IBD (DuPont & DuPont, 2011).
3.1. *T. muris* Life cycle

The lifecycle of *T. muris* starts with the ingestion of embryonated eggs, which contain the infective first stage (L1) larvae. The eggs travel to the caecum, where the larvae hatches and invades epithelial cells in the crypts of Lieberkuhn (Panesar, 1981). This can occur as early as 90 minutes after indigestion, depending on the time it takes for the egg to reach the right environment.

![Diagram of T. muris lifecycle]

*T. muris* eggs, containing infective L1 worms, are ingested by mice

Eggs pass in feces, embryonate approx. in 2 months

- d28-34 L4 molt to adult worm
- Adult worm mature and mate. After d35 female worms release eggs

L1 hatches in caecum and invades epithelial cells in crypts

- L1 migrate through epithelial cells.
- d9-11 molt to L2

L1 hatches in caecum and invades epithelial cells in crypts

- d21: L2 molt to L3. Posterior end of worm emerges into the gut lumen

**Fig: 3: Life cycle of *T. muris* parasites.** Embryonated eggs hatch when they reach the caecum and invade epithelial cells. Through a series of molts they reach maturity and mate around day 35. Females then release eggs into the gut lumen (Adapted from deSchoolmeester 2002).

Hatching only occurs in the presence of bacteria with bacterial type 1 fimbrae on the surface, as for example *Escherichia coli*, which indicate that the egg has reached the parasites niche of the intestine. The fimbrae allow the bacteria to adhere to the egg (Hayes et al., 2010). The presence of these bacteria is mainly found in the caecum, which is most likely the reason which the main worm burden can be found in this part of the colon (Klementowicz et al., 2012). Antibiotics can reduce the number of worms, most likely by inhibiting hatching (Hayes et al., 2010). The L1 larvae migrate through epithelial cells, creating a syncitial tunnel. The epithelial cells merge together, forming a multi-nuclear space for the worm (Tilney et al., 2005). As illustrated in figure 2, the larvae go through four molts before reaching maturity, at day 28-35.
The first moult (L1 to L2) occurs between day 9 and day 11 post infection. The second moult occurs around day 21, becoming L3 larvae and the posterior end of the worm emerges into the gut lumen. L3 larvae then moult to become L4 larvae between day 24-28 post-infection and finally the worms reach maturity between day 28 to 34 (Fahmy, 1954). By this time, the parasite is up to 1.5cm long. Mating occurs between posterior ends of the parasite in the gut lumen and female worms start to release eggs. The eggs are passed in feces and embryonate in soil for approximately 2 months (Beer, 1976).

3.2. T. muris strains

In the lab today, three different T. muris strains are available, the E, the S and the E-J strain. The E-strain was cultured in Edinburgh, the E-J strain developed from the Edinburgh strain sub-cultured in Japan for a long time and the S strain was cultured in Sombredo. The three strains vary in the response they provoke in the host, as expulsion by day 32 in C67BL/6 mice is only evident in strain E and E-J. The S strain generally is not expelled, which is associated with failure to produce an adequate IL-4 response, which can be seen after infection with the E and the E-J strain (D’Elia et al., 2009; Koyama & Ito, 1996). Excretory/secretory protein isolated from the S-strain stimulates higher levels of IL-6 and IL-10 compared to the E and the E-J strain (D’Elia & Else, 2009), which create a regulatory, rather than Th2 cytokine environment. In this project, only the E strain was used.

3.3. Excretory/secretory antigen

Throughout their development, T. muris secretes complex antigen into the lumen of the colon, called excretory/secretory protein (E/S) (Jenkins & Wakelin, 1983). This antigen is capable of inducing immune responses and is frequently used to re-stimulate cells ex vivo. Wakelin and Selby, (1973) used somatic worm antigen from whole, but also posterior and anterior worm tissue. Homogenates of anterior tissue given 30 days prior to infection generated up to 92% protection against T. muris infection. In 1983, E/S was first generated by Jenkins and Wakelin, by placing whole worm into media for several hours and collecting the antigen released into the surrounding. E/S is assumed to be originating in the stichosome, the anus and the reproductive tract (Jenkins & Wakelin, 1983). Subcutaneous administration of 100μg E/S antigen in Freunds’ incomplete adjuvant promotes immunity to T. muris infection, but also with oral administration some immunity can be achieved (Jenkins & Wakelin, 1983). The ease of generating E/S in comparison to homogenised antigens led to the use of E/S antigen in subsequent studies. The different larval stages produce antigen with different antigenic properties (Preston et al., 1986), but most research, including this thesis, uses E/S released from adult worms, as eggs can be obtained at the same time.

3.4. Spectrum of T. muris infection in mice

The ability to expel T. muris parasites depends on the genetic background of the laboratory strain used, as well as on gender. Susceptible mice form an inadequate immune response to an
Infection with *T. muris*, while resistant mice are able to expel the parasite before it reaches maturity. Different inbred mouse strains vary in their response to the infection. Most inbred strains have been found to be resistant (Wakelin, 1975). The major histocompatibility complex (MHC) gene H-2 haplotype has been found to be important for the immune response to *T. muris*. Congenic strains sharing the background genes, BALB or B.10, have different H-2 haplotypes. The H-2^b^ haplotype, found in BALB/k and B10.BR mice makes these mouse strains more susceptible to infection in comparison to other haplotypes, H-2^b^ and H-2^a^, found in resistant strains. H-2^d^ can be found in resistant as well as susceptible mice, suggesting that this is not the only gene determining the ability to expel the worm burden (Else & Wakelin 1988). H-2 recombinant strains of mice were analysed to examine the genes within the H-2 complex affect resistance. Analysis of the genetic variations in these mice revealed resistance (q, b) and susceptibility (k, d) alleles in the mouse I-A region. At the end of the D end of the H-2 complex, especially the q or d allele further enhanced resistance. To analyse the importance of non H-genes, antibody production after *T. muris* infection in mice on different backgrounds with the H-2^a^ haplotype was examined. All mice were able to expel their parasitic burden, but did so with varying kinetics, showing that non-H-2 genes also influence the immune response (Else et al., 1990). The MHC complex influences the complex immune response to *T. muris*, but until now it remains unclear how all the different components of a protective response against the parasite are regulated.

Gender can also play a part in whether a mouse will be susceptible or resistant. Male BALB/c IL-4^−/−^ mice are susceptible to *T. muris* infection and unable to expel their worm burden, while female mice are as resistant as wild-type mice, due to higher levels of IL-13 produced by restimulated mesenteric lymph node cells (Bancroft et al., 2000). TNFαR^−/−^ mice show the same gender bias as BALB/c mice (Hayes et al., 2007a). Research by Hepworth et al., (2010) indicates that the susceptibility in male mice is due to the male sex steroid dihydrotestosterone, which leads to reduced ability of dendritic cells to activate T cells. The female related hormone 17-β-estradiol on the other hand appears to enhance the generation of Th2 responses in vitro.

The dose of *T. muris* eggs also influences the outcome of infection. Wakelin (1973) established that high dose infections with 400 eggs were readily expelled, compared to infections with 10 eggs, which led to chronicity in outbred mice. Trickle infection with low doses of eggs led a build-up in worm numbers, but eventually to expulsion of the worm burden (Bancroft et al., 1994; Wakelin & Selby, 1973). Resistant mice remain able to expel their worm burden in subsequent infections following a high dose of infective eggs, but often develop chronicity when given a primary low dose infection. Susceptible mice will not be able to expel a second infective dose and remain susceptible (Bancroft et al., 2001).

Resistance and susceptibility is dependent on the immune response formed, AKR mice for example are susceptible and mount a Th1 response, while BALB/c mice are resistant and mount a Th2 response. C57BL/6 mice are intermediate as they mount both a Th1 and a Th2 response, but more about T cell responses will be discussed below.
3.5. T cell mediated immunity is required for the expulsion of *T. muris*

Adoptive transfer studies of cells and serum in 1973 by Selby and Wakelin established that cell mediated immunity was required for worm expulsion, even though serum transfer also led to a decrease in worm numbers, but not as consistently as with cell transfer. Adoptive transfer experiments examining which cell type transfers immunity showed that enriched T cells but not B cells from *T. muris* infected mice transferred immunity to the parasite into naive mice (Lee *et al.*, 1983). To establish if CD4$^+$ T cells and CD8$^+$ T cells were required to mediate expulsion, mice were given monoclonal antibodies to neutralise CD4$^+$ or CD8$^+$ cells, which established that the absence of CD8$^+$ T cells did not influence protective immunity and showed that the presence of CD4$^+$ T cells is required for worm expulsion (Koyama *et al.*, 1995). Adoptive transfer of CD4$^+$ T cells to SCID mice, which are deficient for T and B cells, enabled these mice to mount a protective immune response (Else & Grencis, 1996), demonstrating not only the importance of T cells but that B cells are not required for protection. CD4$^+$ T cells have been shown to be more effective during the larval stages of *T. muris*, as adoptive transfer of CD4$^+$ T cells to mice with an established infection with adult worms did not induce expulsion. Gut homing receptors β7 and αE integrins as well as the gut homing ligand MadCAM-1 ensure the T cell responses occur locally and inhibition of these ligands using monoclonal antibodies inhibits CD4$^+$ T cell mediated expulsion (Betts & Else, 1999; Svensson *et al.*, 2010).

Table 2 summarises the cytokines involved in the immune response to *T. muris*, either by supporting protective immune responses or by developing the environment for chronic infection.

**Table 2: cytokines associated susceptibility and resistance in *T. muris* infection.**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Resistance</th>
<th>Susceptibility</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>✓</td>
<td></td>
<td>Bancroft <em>et al.</em> 1998; Else <em>et al.</em>, 1994; Else &amp; Grencis, 1996</td>
</tr>
<tr>
<td>IL-5</td>
<td>✓</td>
<td></td>
<td>Else &amp; Grencis, 1991</td>
</tr>
<tr>
<td>IL-13</td>
<td>✓</td>
<td></td>
<td>Bancroft <em>et al.</em>, 1998; Else &amp; Finkelman, 1998; Else &amp; Grencis, 1991</td>
</tr>
<tr>
<td>IL-9</td>
<td>✓</td>
<td></td>
<td>Faulkner <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>TNFα</td>
<td>✓</td>
<td>✓</td>
<td>Artis <em>et al.</em>, 1999b; Hayes <em>et al.</em>, 2007a,b</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>✓</td>
<td></td>
<td>Cliffe &amp; Grencis, 2004; Else &amp; Finkelman, 1998</td>
</tr>
<tr>
<td>IL-12</td>
<td>✓</td>
<td></td>
<td>Bancroft <em>et al.</em>, 1997; Helmby <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>IL-18</td>
<td>✓</td>
<td></td>
<td>Helmby <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>IL-25</td>
<td>✓</td>
<td></td>
<td>Saenz <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>IL-10</td>
<td>✓</td>
<td></td>
<td>Schopf <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>IL-33</td>
<td>✓</td>
<td></td>
<td>Humphreys <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>IL-6</td>
<td>✓</td>
<td></td>
<td>Else &amp; Finkelman, 1998</td>
</tr>
</tbody>
</table>
To ensure *T. muris* is expelled in mice, a strong Th2 response has to be mounted, consisting of antibody IgG1 and cytokines IL-4, IL-13, IL5 and IL-9. In the presence of a Th1 response, comprising of high antibody IgG2a/c and cytokines IFNy, IL-12 and IL-18, a chronic worm burden is established (Else & Grencis 1991).

3.5.1. Cytokines associated with resistance

IL-4 has been described as the most important cytokine in resistance to *T. muris* infection. The administration of IL-4 monoclonal antibodies blocks IL-4 function and leads to a chronic infection. In the absence of IL-4, a Th1 response is established, featuring increased levels of IFNy and Th1 associated antibody IgG2a (Else *et al.*, 1994). Susceptible AKR mice, treated with IL-4 complex, which consist of IL-4 and a neutralising anti-IL-4 monoclonal antibody and was intended to increase IL-4 effects, early in infection (day 8-11), display worm expulsion. Administration later during the infection reduces worm numbers (Else *et al.*, 1994).

The absence of IL-4 or IL-13 turns resistant animals susceptible. IL-4−/− mice were bred on a C57BL/6 background develop chronic *T. muris* infections. In the absence of IL-4 other Th2 associated cytokines such as IL-5 and IL-13 are secreted in decreased levels by mesenteric lymph node cells when re-stimulated with E/S, while levels of IFNy are increased (Bancroft *et al.*, 1998).

IL-13 is closely linked to IL-4 and important for the generation of the immune response to *T. muris*. IL-13−/− mice, bred on a resistant background are unable to expel the parasite, even though they are able to produce Th2 response, as IL-4, IL-5, IL-9 and TH2 associated IgG1 antibody response could be measured (Bancroft, *et al.* 1998). Treating female IL-4−/− mice on a BALB/c background, which are able to expel an infection, with soluble IL-13 receptor protein to block IL-13 prohibits expulsion, while IL-13 administration to male IL-4−/− mice, which are susceptible to infection, enables them to expel the parasite infection (Bancroft *et al.*, 2000).

Production of IL-5 has been linked to protective immune responses to *T. muris* and controls eosinophilia (Else & Grencis, 1991; Else *et al.*, 1992a). Ablation of IL-5 by treatment with an anti-IL-5 antibody did result in elimination of eosinophilia, but resistant mice were still able to expel their worm burden. Therefore, levels of IL-5 and resulting eosinophilia are markers of an on-going Th2 immune response, but likely not essential for protective immunity to *T. muris*.

IL-9 is produced by CD4+ T cells early in the responses to *T. muris* infection in resistant mice, even before an IL-4 response is formed, but not at any time point in susceptible mice (Else *et al.*, 1992b; 1993; Faulkner *et al.*, 1998). Faulkner *et al.* (1998) showed that adoptive transfer of IL-9 producing T-cells increases mast cell infiltration, MCP-1 levels and overall faster worm expulsion in susceptible mice. Using IL-9 transgenic mice, which overexpress IL-9, the importance of IL-9 was further evaluated. IL-9 transgenic mice expel their worm burden quicker than most resistant strains (Faulkner *et al.*, 1998). Vaccinating mice with an IL-9-OVA complex neutralised IL-9 by inducing the production of auto-antibodies, which turns resistant mouse
strains susceptible (Richard et al., 2000). IL-9 also is important in muscle hyper-contraction, which is described below in more detail (Khan et al., 2003).

IL-10 is another cytokine linked to resistance in T. muris infection. Deficiency in IL-10 or both IL-10 and IL-4 leads to chronic T. muris infection, rapid weight loss and mortality after 25 days of infection (Schopf et al., 2002). Mice deficient for IL-10 and IL-12 on the other hand were resistant to infection, as they did not mount an IFNγ response, but secreted high levels of Th2 cytokines (Schopf et al., 2002). These experiments indicate that IL-10 is important for host survival during T. muris infection.

TNFα has also been shown to support cytokine function and enhance the dominant T cell lineage (Artis et al., 1999b; Hayes et al., 2007a,b). Male mice deficient in TNFα become susceptible to T. muris infection, while female mice retain resistance (Hayes et al., 2007a). TNFα is generally not associated with a Th2 response, but in the context of T. muris seems to aid resistance (Artis et al., 1999b). On the other hand, TNFα can also increase Th1 responses in susceptible animals. Recombinant TNFα treatment can increase Th1 or Th2 immune responses, depending on the immune response mounted against T. muris by the mouse strain in question. Blocking TNFα on the other hand does not diminish resistant immune responses (Hayes et al., 2007a). High levels of TNFα have generally been associated with delayed expulsion in C57BL/6 mice, as the production of a Th1 response is increased further than the Th2 response when treated with TNFα (Hayes et al., 2007a).

3.5.2. Cytokines associated with susceptibility

Susceptibility to T. muris infection is linked to high levels of IFNγ, IL-12 and IL-18 (Cliffe & Grencis, 2004).

IFNγ is the dominant Th1 cytokine driving chronic parasite infection (Else & Grencis, 1991; Else et al., 1992a; 1994; Bancroft et al., 1998). Comparing IFNγ levels produced by re-stimulated mesenteric lymph node cells isolated from susceptible B10.BR mice with IFNγ levels measured in resistant BALB/k mice, showed lower levels of IFNγ in resistant mice, which correlated to lower worm burden (Else et al., 1992a; Else et al., 1993). Depletion of IFNγ in susceptible mice changed the immune response generated from a Th1 response to a Th2 response, which was evident in the reduction of Th1 associated antibody IgG2a/c and higher levels of Th2 associated antibody IgG1 and the ability to expel the parasite (Else et al., 1994).

IL-12 is commonly produced by monocytes, macrophages and dendritic cells and linked to Th1 responses, as it stimulates IFNγ production (D’Andrea et al., 1992). Treatment of resistant mice with recombinant IL-12 resulted in the development of chronic infection, with elevated worm burden as late as day 35 and high levels of IgG2a, but reduced mastocytosis and IgE levels (Bancroft et al., 1997). The effect recombinant IL-12 can be negated by adding anti-IFNγ monoclonal antibody, which resulted in worm expulsion comparable to control mice, indicating
that IL-12 is dependent on IFNγ (Bancroft et al., 1997). Mice deficient in IL-12 p40 are able to expel high dose infections as well as low dose infections (Bancroft et al., 2001; Helmby et al., 2001), showing that IL-12 is important for the development of a chronic infection.

IL-18 has also been associated with susceptibility to *T. muris* infection. IL-18 stimulates NK cells, T cells, B cells and macrophages to secrete IFNγ especially in conjunction with IL-12 (Okamura et al., 1995; Ahn et al., 1997; Robinson et al., 1997; Kohno et al., 1997). IL-18 mRNA levels in the large intestine are up-regulated during *T. muris* infection. In susceptible mice these levels are higher than in resistant mice and peak between day 8-12 post infection (Helmby et al., 2001). IL-18−/− mice are resistant to *T. muris* infection and expel even a low dose infection by day 35. Resistant C57BL/6 mice treated with recombinant IL-18 are susceptible to infection, with reduced levels of IL-4 and IL-13 (Helmby et al., 2001). These results suggest that IL-18 inhibits IL-4 and IL-13 rather than stimulating IFNγ. This process is also IFNγ independent, as recombinant IL-18 is able to reduce IL-13 production in mesenteric lymph node cultures taken from IFNγ−/− mice (Helmby et al., 2001).

### 3.6. Effector mechanisms in *T. muris* infection

Even though CD4+ T cells have been identified as the main effector cell type required for expelling *T. muris*, several other effector mechanisms are involved in the efficient clearance of the parasite infection.

#### 3.6.1. Dendritic cells

In order for adaptive immunity to take effect, naïve CD4+ T cells need to be activated in the mesenteric lymph nodes by antigen presenting cells. Dendritic cells can activate and direct naïve T cells to become Th1 or Th2 type T cells (Cruickshank et al., 2009). During parasite infection dendritic cells are recruited to the gut by epithelial cells and migrate then to the mesenteric lymph nodes to present antigens to T cells (Cruickshank et al., 2009). Several different DC subsets can be found in the mucosal tissue of the gut.

The exact role in of dendritic cells in *T. muris* infection is still controversial. Cruickshank et al. (2009) established that resistant mice expressed more cell surface markers e.g. CD80/86, MHCII and CCR7 on dendritic cells, while their epithelial cells express higher levels of chemokines such as CCL2, CCL3, CCL5, CCL20 and TSLP. The resistance/susceptibility in BALB/c and AKR mice respectively, has been shown to depend on dendritic cell migration within the first days of the infection (Cruickshank et al., 2009). A combination of CD11c and CD103 normally indicates gut homing dendritic cells, nonetheless CD103−/− mice do not show impaired protective immune responses but rather heightened Th2 associated IgG1 levels when infected with *T. muris* and were able to expel the parasite (Mullaly et al., 2011).

In B10.BR mice, dendritic cell expansion in the mesenteric lymph node occurs at the same time as expulsion of *T. muris*, when a protective Th2 immune response has formed (Koyama, 2005).
Koyama et al. (2008) identified dendritic cells in as important for IL-10 production in mesenteric lymph nodes. This cytokine has been shown to inhibit Th1 responses, which allows Th2 polarisation and IL-10 also plays a role in worm expulsion in the context of T. muris infection (Moore et al., 2001; Schopf et al., 2002). These results suggest another function for dendritic cells in T. muris infection as cytokine producing cell.

Dendritic cells have shown to be very important in the context helminth of Schistosoma mansoni infection (Phythian-Adams et al., 2010), which, together with the differences in DC recruitment observed by Cruickshank et al. (2009) suggest that dendritic cells play a role in T. muris infection.

3.6.2. Basophils

Basophils have also been linked to the emergence of a Th2 response to T. muris. It has been suggested that basophils aid dendritic cells in Th2 differentiation by producing IL-4 and other Th2 associated cytokines or even directly act as antigen presenting cells (Perrigoue et al., 2009; Sokol et al., 2009; 2008; Wynn, 2009). Perriogue et al. (2009) showed that MHC<sup>CD11c</sup> mice, which only have MHC class II expression on CD11c<sup>+</sup> cells, did not produce Th2 cytokines as a response to an infection with T. muris, and therefore CD11c<sup>+</sup> cells might not be required for Th2 cytokine development. Depletion of FcεRI<sup>+</sup> cells, including basophils, using MAR-1 monoclonal antibody on the other hand impaired Th2 cytokine responses and led to incomplete expulsion of T. muris. These experiments lead to the assumption that basophils are required for Th2 responses in infections with T. muris and basophils would be a candidate for this role (Perrigoue et al., 2009).

In the context of helminth Nippostrongyles brasiliensis and Schistosoma mansoni infection, depletion of basophils does not affect clearance of the parasite (Kim et al., 2010; Phythian-Adams et al., 2010). Despite the evidence for an involvement of basophils in protective immunity to T. muris the exact role for basophils remains unclear.

3.6.3. Macrophages

Macrophages are a diverse population of cells and generally have been described in two activation stages, classical activation (M1) and alternative activation (M2) (Dalton et al., 1993). CCL2 deficient mice are unable to expel their T. muris worm burden and show reduced Th2 responses in mesenteric lymph nodes and lack macrophage numbers in the colon (deSchoolmeester et al., 2003). Furthermore Little et al., (2005) established that macrophage numbers are significantly up-regulated in colon tissue during expulsion in resistant mice, while the increase seen in susceptible mice is only small. These studies lead to the suggestion that there is a role for macrophages in T. muris infection, which will need further investigation.
3.6.4. Eosinophils

Mice resistant to *T. muris* show elevated numbers of eosinophils in the colon and MLN during infection and in resistant mice eosinophils have been described as IL-4 producing cells (Dixon *et al*., 2006; Svensson *et al*., 2011). These cells are recruited to the colon by IL-5 in synergy with chemokine CCL11 (Dixon *et al*., 2006; Else & Finkelman, 1998). The use of IL-5 neutralising antibodies reduces the number of eosinophils in the colon by approximately 75% (Betts & Else, 1999) and CCL11−/− mice also have delayed recruitment of eosinophils to the intestine (Dixon *et al*., 2006). In IL-5/CCL11−/− mice no eosinophils can be found. There is no visible effect on worm expulsion or Th2 responses in mice with reduced or depleted eosinophil numbers and high levels of parasite specific IgG1 could be measured (Betts & Else, 1999; Dixon *et al*., 2006), which suggests that eosinophils do not have a significant role in the production a protective Th2 response against *T. muris* infection.

3.6.5. Innate Lymphoid cells

Innate lymphoid cells are newly described innate cell populations, mainly driven by IL-25 and IL-33 production, and presumed to secrete Th2 cytokines early in helminth infection (Neill & McKenzie, 2011). All of these cells are lineage negative and have to be compared further to distinguish real populations, as some markers as IL-25R and IL-33R can be found overlapping between them.

One of these new cell populations are multi-potent progenitor type 2 cells (MPP<sup>type2</sup>), which have been linked to protective immune responses in *T. muris* infection (Saenz *et al*., 2010). MPP<sup>type2</sup> cells can differentiate into mast cells, basophils and macrophages if cultured with stem cell factor and IL-3 and can be found in all compartments of the gut associated lymphoid tissue. Administration of IL-25 leads to expansion in the MLN, Payer’s patches and caecal patches, but not in the spleen and bone marrow. IL25−/− mice are susceptible to *T. muris* infection, with reduced Th2 cytokine production but the adoptive transfer of MPP<sup>type2</sup> cells into IL-25−/− mice turns them resistant to infection, able to reduce their worm burden by day 20 and produce high levels of IL-4, IL5 and IL13 as well as Th2 associated antibody IgG1 (Saenz *et al*., 2010). Other innate lymphoid cells are nuocytes (Neill & McKenzie, 2011), natural helper cells (Moro *et al*., 2010) and innate type 2 helper cells (Price *et al*., 2010), which have been described to provide Th2 cytokines in the protective immune response to *Nippostrongylus brasiliensis* and will need investigating in the context of *T. muris* infection.

3.6.6. NK T cells

NK T cells are producer of high levels of IL-4 (Yoshimoto & Paul, 1994), which implicated a role in the development of a protective immune response to *T. muris* infection. Koyama *et al.* (2002) established that the source of this cytokine during *T. muris* infection is independent of NK T cells. Depletion of NK1.1+ cells in B10.DR mice, a normally resistant mouse, with neutralising
antibodies did not affect Th2 cytokine levels or parasite specific antibody production and therefore did not impair the ability to expel the worm burden in these experiments (Koyama, 2002). This experiment revealed no involvement of NK T cells in the ability to expel T. muris.

3.6.7. Mast cells

Increases in mast cells are found in mice with T. muris infection, although the accumulation of mast cells is not required for the expulsion of the parasite, as accumulation of mast cells at the site of infection does not correlate to worm expulsion (Lee & Wakelin, 1982). Subsequent experiments by Betts and Else (1999) indicated that worm expulsion is not disrupted when mast cells are depleted by blocking stem cell factor receptor c-kit and therefore are deemed not necessary for the generation of a successful immune response again T. muris, although mast cells might be important in the immune response to other parasites as Trichinella spiralis (Faulkner et al., 1997). Naturally mast cell-deficient (WBB6F1-W/W') mice on the other hand showed delayed T. muris worm expulsion, compared to wild-type controls (Koyama & Ito, 2000). Taken together these experiments show that the role of mast cells is minor in the protective immune response to T. muris.

3.6.8. B cells and antibody production

T. muris infection is accompanied by an antibody response, Th2 and Th1 associated antibodies IgG1 and IgG2a/c can be measured in serum post infection. More resistant animals produce higher IgG1 levels, while IgG2c is elevated in susceptible animals (Koyama et al., 1999). In resistant BALB/c mice, elevated levels of B cell marker B220 can be found in mesenteric lymph node cells, as well as IgG and IgA producing cells, at day 14 during expulsion (Koyama et al., 1999).

B cells and parasite specific antibodies have been associated with protective immunity against T. muris infection (Blackwell & Else, 2001; Koyama et al., 1995) but the importance of B cells and antibody production for protective immune responses remains controversial. Several studies suggest that antibody and B cell presence is required for optimal parasite expulsion, while others claim that there is no important role for B cells and antibodies in protective responses to T. muris.

B cell deficient mice (µMT mice) are highly susceptible to T. muris infection, produce high levels of IFNγ and only naïve levels of Th2 cytokines. Adoptive transfer of B cells, isolated from infected, resistant C57BL/6 mice or administration of Th2 associated parasite specific IgG1 from resistant NIH mice turned µMT mice resistant to infection (Blackwell & Else, 2001).

Serum transferred from resistant mice infected with T. muris to naïve mice at the time of infection resulted in decreased worm numbers and therefore partial protection against T. muris infection in 5 out of 9 recipient mice (Selby & Wakelin, 1973), while antibody transfer alone appears to be insufficient for protection against the parasite (Else et al., 1990). B cells alone
are not able to elicit expulsion, as B cell transfer into SCID mice, which are lacking B and T cells, does not render these mice resistant, while transfer of CD4^+ T cells alone was successful in inducing expulsion (Else & Grencis, 1996; Lee et al., 1983).

These experiments suggest that B cells and antibodies do play a role in resistance to *T. muris* but only in the presence of T cells.

### 3.6.9. Goblet cells and mucin production

Goblet cells can be found in the crypts of colon and caecum, where they produce mucins, forming the major component of the mucus layer lining the large intestine (Klementowicz et al., 2012). In the context of *T. muris* infection, resistant as well as susceptible animals show goblet cell hyperplasia (Artis et al., 2004), although the type of mucins produced varies and the mucus layer is thinner and less charged in susceptible mice (Hasnain et al., 2010, 2011a, b). Mucus production by goblet cells has been associated with the presence of Th2 cytokines IL-9 and IL-13 (Kondo et al., 2002; Taube et al., 2002; Whittaker et al., 2002). One of the mucins associated with worm expulsion is Muc2, which is secreted by goblet cells but expression is not up-regulated in susceptible mice (Hasnain et al., 2010). *T. muris* secretes serine proteases, which make the mucus barrier more porous by depolymerising Muc2. Resistant mice have higher levels of serine protease inhibitors preventing the loss of Muc2 compared to susceptible mice (Hasnain et al., 2012). Another mucin found in resistant mice is Muc5ac. Without this mucin present, strong Th2 responses were not sufficient to expel the parasite (Hasnain et al., 2011b). The thick mucin defence layer under the mucus layer lining the intestinal wall is thinner in susceptible mice compared to mice resistant to *T. muris* infection (Hasnain et al., 2011a, b).

### 3.6.10. Epithelial cells

*T. muris* established a close relationship with the epithelial cells of the large intestine, as it burrows tunnels into the epithelial lining (Tilney et al., 2005). Epithelial turnover is also described as the “epithelial escalator”, where cells are moved from the proliferating zone at the bottom of the crypt to the shedding zone at the top of the crypt. This way, the parasite burrowed into the epithelial cells is moved from the bottom of the crypt towards the lumen of the intestine, where it is shed (Cliffe et al., 2005). Epithelial turnover can be measured using bromodeoxyuridine (BrdU) incorporation and is accelerated in resistant animals around the time of expulsion. In susceptible mice, epithelial turnover speed is delayed compared to resistant mice and the epithelial turnover is not enough to displace larger worms and shedding of epithelial cells into the lumen is less than in resistant mice (Cliffe et al., 2005; Klementowicz et al., 2012).

The Th2 cytokine IL-13 and chemokine CXCL10 control the speed of epithelial cell turnover. IL-13^+^ mice have a similar epithelial turnover rate as susceptible AKR mice. CXCL10 expression was measured on day 21 in susceptible mice, when levels of IFNγ were high and found to reduce epithelial cell turnover. Neutralisation of CXCL10 in susceptible AKR mice and SCID
mice led to increased epithelial turnover and decreased worm burden. Artis et al. (1999a) described crypt hyperplasia, which is caused by hyperproliferation of epithelial cells, to be under IFNγ control in chronic infection, as depletion of IFNγ prevents crypt hyperplasia in mice susceptible to *T. muris* infection (Artis et al., 1999a; Cliffe et al., 2007).

### 3.7. Mechanisms leading to expulsion

*T. muris* expulsion depends on many different factors. T cells are the main effector cell, orchestrating the immune system to mount an inflammatory response against the parasite. The immune system nonetheless does not directly kill the parasite, but creates a hostile environment which removes the worm from the synovial tunnel and expels it with the natural bowl movement. Mucin production and epithelial cell turnover, the main mechanisms leading to expulsion have been described in more detail above. A schematic of how T cell activation is presumed to leads to expulsion can be seen in figure 4.

**Figure 4: Schematic diagram of T cell activation leading to *T. muris* expulsion.** Naïve dendritic cells home to the colon where they sample antigen which they present to naïve T cells in the draining lymph nodes. Naïve T cells become Th1 or Th2 cells, depending on the cytokine environment and co-stimulatory factors in the lymph node. Activated lymph nodes home back to the colon, where they secrete cytokines. Cytokine secretion recruits immune cells to the site of infection, upregulates of mucus production, muscle contractility and epithelial turn over, resulting in the expulsion of the parasite from the epithelial lining.
3.8. Intrinsic circadian rhythm within *T. muris*

So far no molecular clock has been established within the *T. muris* genome. Given that the helminth *C. elegans* contains a functioning circadian clock (Temmerman et al. 2011); it is likely that a molecular clock can be identified for *T. muris*. Nonetheless, the relevance of a functioning clock for the life cycle of *T. muris* in the intestinal niche is low. Most likely circadian rhythm within the parasite would entrain to the gastrointestinal environment and the feeding habit of its host. Parasitic eggs are kept in in the dark at 4°C and hatching is dependent on the presence of bacteria, rather than an intrinsic rhythm. For this project, any circadian rhythm within the parasite is presumed to be entrained by the host and therefore unlikely having an effect on the priming of the immune response.

As a well-defined model of a Th2 response, *T. muris* will be useful model to identify the involvement of circadian rhythm in the establishment of Th2 immune responses in the large intestine. Even though it is known that T cells are the main effector cells, the focus of this thesis lies with the establishment of the immune response against *T. muris* and therefore focuses also on dendritic cells, which are thought to activate T cells and macrophages, as they produce large quantities of cytokines. Both cell types have been shown to contain an oscillating molecular clock, which could impact on the immune response developed depending on the time of day the challenge is administered.
Aims and objectives

A circadian influence on the immune system is traditionally observed in autoimmune diseases and acute immune responses (Bollinger et al. 2010; Silver et al. 2012a/b; Keller et al. 2009; Gibbs et al. 2009; Cutolo et al. 2003). Using an inflammation model which takes weeks to be resolved, I question whether circadian rhythm can impact on the early events which prime for the outcome of a long-term infection. In the setting of C57/BL6 mice, a high dose of T. muris worms is expelled over the time course of 21 to 35 days. It is well known that the intestinal niche these parasites dwell in is under strong circadian rhythms but nobody has examined the impact of these rhythms on parasitic helminth infections before.

In general laboratory studies, experimental mice are infected with T. muris throughout the working day, without a defined, specific time of day, as the infection is usually carried until day 21 and thus time of day of infection has been assumed unimportant. In my thesis I challenge this assumption and examine if infections delivered 12h apart show any differences in the quality of the immune response mounted to infection and/or outcome/resolution of infection.

The following questions were addressed within this thesis

- Does the time of day of infection influence the outcome of an infection with T. muris?
- Do gut tissue and/or cells of the immune system follow a circadian rhythm?
- Can the immune response to infection be manipulated to identify the mechanisms influencing worm expulsion which are under circadian control?

In Chapter 3 I explore the effect of T. muris infection administered at ZT0 and ZT12 over a time course infection. I describe the antibody and cellular immune response to infection at day 21 post infection and day 35 post infection; with further time points between day 21 and day 35 also included to more precisely capture any differences in worm expulsion due to the time of day of infection.

In order to understand the mechanisms underlying time of day dependency on resistance to worm infection, the infection model/immune response to infection was manipulated in various ways: by reducing the dose of parasite eggs given by oral gavage, by subcutaneous vaccination of mice with parasite derived antigen, by food restriction (Chapter 4) and the use of transgenic mice (Chapter 5). Reducing the dose of eggs polarises the host immune response towards a Th1 response, which supports a chronic infection. Vaccination on the other hand pushes the immune system more towards a Th2 response. Utilizing food restriction, I examine the impact of food and circadian rhythm within the colon and surrounding tissue on the expulsion of the worm burden. Restricting feeding alters the local clock in the intestine, but not the main oscillator in the brain.

Taking advantage of the per2::luc transgenic mouse line, which expression of the clock gene Per2 is linked to the expression of the fluorescent luciferase marker, colon tissue be examined,
which indicates if local tissue is under circadian rhythm. Further, the circadian rhythm in dendritic cells will be examined, with dendritic cells being one of the key cellular candidates underlying rhythmic responses in the colon.

Transgenic mice with clock gene expression targeted in specific cell types allow the key contributing cell types underlying the influence of time of day on infection to be narrowed down, thus allowing for a more targeted approach to the question of which cells are involved in the circadian impact on the immune response to *T. muris* infection. Specifically, I generate and use transgenic mice where CD11c+ dendritic cells lack core clock gene Bmal1 expression and ask whether the absence of rhythmic Bmal1 expression in dendritic cells alters the time of day difference in infection outcome and/or immune responses to *T. muris* infection.
Material and methods
1. Mice
Male C57 BL/6 mice, age 6-8 weeks, were bought from Harlan, UK. Male Bmal1<sup>flox</sup>LysM<sup>cre</sup> mice (Clausen et al. (1999), Storch et al. (2007)), mPer2::luc mice (Yoo et al. (2004) and mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> mice (Yoo et al. (2004), Storch et al. (2007), Travis et al. (2007)) were bred locally. All experimental procedures were within the guidelines of the animals (Scientific procedures) Act, 1986. All mice were maintained at a temperature of 20-22°C in a 12h light, 12h dark lightening schedule, in sterile, individually ventilated cages with food and water ad lib unless otherwise stated.

1.1. Food restriction
C57BL/6 mice were restricted to ad lib food for selected periods of time. Two food regimens were used: (i) mice were allowed food for 12h during the light or the dark phase, (ii) mice were allowed food for 6h mid-light or mid-dark phase. Mice were kept on the restricted diet for 12 days before cull or infection. Infected mice were kept on 6h feeding for further 7 days before returning to ad lib feeding.

1.2. Genotyping
Genotyping was carried out using ear punches prior to infection and using tail-snips to confirm the genotype after sacrificing the animals. Tissue was stored until use in individual Eppendorf tubes at -20°C. Genomic DNA was extracted from tissue using QIAamp DNA Mini Kit (Quiagen, Manchester, UK).

After the genomic DNA was extracted, it was amplified using PCR (GSTorm PCR machine, GSTorm, Somerton, UK). Primers were ordered from Eurofins (Ebersberg, Germany).

To establish if Bmal1 is transcribed in Bmal1<sup>flox</sup>LysM<sup>cre</sup> mice or mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> PCR was carried out to amplify bmal gene exon 8. The primer sequences are: forward primer 5’-gggccacagtcagattgaaa-3’ and reverse primer 5’-gctgaacagccatccttagc-3’.

0.7μl High fidelity Taq, 2 μl dNTP (25mM, 1:10, Bioline Reagents Ltd; London, UK), 16.75μl, 2.5 μl buffer, 1μl reverse and 1 μl forward primer as well as 1 μl cDNA were used per reaction. Samples were heated to 94°C for 2 minutes and then cycled 35 times; 15 sec 94°C, 60 sec 55°C, 60 sec 72°C. In the final heating step the samples were held at 72 °C for 7 minutes before keeping at 4°C. The product for exon 8 is 137 bp long.

To genotype for the presence of floxed sites around the Bmal1 gene, the following sequences were used: Bmal1 forward 5’-actggaagtaactttatcaaactg-3’, Bmal1 reverse 5’-ctgaccaacttgctaacaatta-3’. Per reaction 15.675μl H₂O, 2 μl dNTP (25mM, 1:10, Bioline Reagents Ltd; London, UK), 0.5μL Primer 1 and primer 2, 0.125μL Go TAQ (Promega, Southampton, UK) and 1μL genomic DNA were mixed. The samples were heated to 94°C for 2 minutes, and then cycled 35 times, 30 sec 94°C, 60 sec 61.6°C, 60 sec 72°C, after which it is heated to 75°C for 5 minutes, before storing at 4°C.

To genotype for the presence of the luciferase fusion protein with Per2, the following sequences were used: common 5’-ctgtgtttactgcgagagt-3’, WT reverse 5’-ggttcaggtgattagaaac-3’ and
mutant reverse 5’-taaaaccggaggtagatgaga-3’. Per reaction 9.527µL H2O, 5µL buffer, 2 µL MgCl2, 2.208µL, dNTP (1:10, 2.5 mM, Bioline Reagents Ltd; London, UK), 1.38µL Primer 1, 2 and 3, 0.125µL Go TAQ (Promega, Southampton, UK) and 2µL genomic DNA were added. The reaction is heated to 94°C for 2 minutes, and then cycled 30 times, 30 sec 94°C, 30 sec 58°C, 60 sec 72°C, after which it is heated to 75°C for 5 minutes, before storing at 4°C.

To genotype for the presence of CD11ccre or floxed sites around the vβ8integrin gene, the following primers were used: CD11c-cre Forward 5’-acttggcagctgttccaag-3’, CD11c-cre Reverse 5’-gcgaacatcttcaggttctg-3’, vβ8integrinfloxed Forward 5’-gagatgcaagagtttacc-3’ and vβ8integrinfloxed Reverse 5’-cactttagtgctaatgag-3’. Per reaction 15µL H2O, 2.5µL buffer, 1.5µL MgCl2, 0.75µL dNTP (25mM, Bioline Reagents Ltd; London, UK), 1µL Primer 1 and primer 2, 0.25µL BioTaq DNA Polymerase (Bioline, Reagents Ltd; London, UK) were added to 3µL genomic DNA. Samples were heated to 95°C and then cycled 34 times, 30 sec 95°C, 30 sec 56°C, 30 sec 72°C, before holding for 5 minutes at 72°C, then storing at 4°C. the product for CD11c was about 300bp long.

1.5g agarose was dissolved in 100 ml 0.5% TBE solution by heating in the microwave for 3 min. After cooling down to touch, 1µl Ethidium bromide (10mg/ml, Sigma Aldrich Company Ltd, Dorset, UK) or 3µl SaveView (NBS Biologicals, Huntington, UK) was added to the liquid gel. The gel was then poured and left to set. 5µl loading dye was added to each sample before loading 15µl sample onto the gel. To determine the size of the band, Hyperladder IV (Bioline Reagents Ltd; London, UK) was run on the gel as well. The PCR products were run on the 1.5% agarose gel for 1 hour at 70V. After 1h, the gel was examined under UV light to identify the PCR products.

2. Trichuris muris

2.1. Maintenance of the Trichuris muris life cycle

For all experiments, the Edinburgh strain (E) was used. To maintain the parasite, SCID mice were infected with about 200 Trichuris muris eggs via oral gavage. Worms reach adulthood at day 32 post infection with egg production from females peaking at 40-45 days post infection, when mice were sacrificed. The colon and large intestine were collected, cut open longitudinally, washed first in PBS, then in PBS with Penicillin (500U/ml) and Streptomycin (500µg/ml,Sigma Aldrich Company Ltd, Dorset, UK) before being stored in pre-warmed RPMI 1640 media (Sigma Aldrich Company Ltd, Dorset, UK) containing Penicillin (500U/ml) and Streptomycin (500µg /ml).The worms were pulled alive from the gut tissue using fine forceps and kept in 6 well plates (Costar, Corning Corporation, NY, USA) in 10ml pre-warmed RPMI 1640 containing Penicillin (500U/ml) and Streptomycin (500µg /ml). Plates were incubated at 37°C in a humid environment for 4 hours after which time the 4h supernatants were removed and exchanged with fresh RPMI 1640 containing Penicillin (500U/ml) and Streptomycin (500 µg/ml). The worms were incubated overnight under the same conditions as before and supernatants collected.
The supernatants were spun down for 10 minutes at 720g to pellet shed eggs, the supernatant removed for E/S preparation (2.2) and eggs re-suspended in ultra-pure distilled water. The egg suspension was collected into a tissue culture flask (Costar, Corning Corporation, NY, USA) through a 100μm nylon cell strainer (BD Biosciences, Oxford, UK). The flasks were wrapped in foil, so the eggs could embryonate for 6 weeks in the dark at room temperature before storing them at 4°C until needed.

2.2. E/S antigen preparation

4h and overnight supernatants were concentrated by repeatedly centrifuging for 30 minutes, at 4°C at 2000g using Centriprep tubes (Millipore Corporation, MA, USA) until a volume of about 5ml was reached. The remaining 5ml were dialysed in three bottles of 5L PBS for 2h at 4°C. The concentration of the protein was measured using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK). Using a 0.22μm millex filter (Millipore Corporation, MA, USA) the 4h E/S was sterile filtered for cell culture. Overnight E/S was used for parasite-specific antibody ELISAs as the target protein. Both types of E/S were stored until use at -80°C.

2.3. Egg infectivity testing

SCID mice were infected with approximately 200 embryonated eggs to assess the infectivity of each batch of eggs. After 42 days the mice were sacrificed and the worm burden counted. The number of worms recovered per number of administered eggs resulted in the percentage of infectivity. The final infectivity was calculated from the mean of three infectivity-counts.

2.4. Infection

For a high dose infection, up to 200 eggs were given per oral gavage to each mouse. Stock eggs were spun to a pellet, 10min at 720g, the supernatant discarded and the eggs were suspended in 15ml fresh dH$_2$O. The egg suspension was mixed using a magnetic stirrer and 50μl aliquots were counted to allow the total number of eggs in 200μl to be calculated. The volume was adjusted to 200 eggs in 200μl, taking infectivity into account. For a low dose infection the number of eggs to be administered (30-40) was counted into individual Eppendorf tubes in a volume of 200μl.

3. Blood collection

Blood was collected by opening the lung cavity under the ribs and removing the left section of ribs. The heart was snipped and the blood sucked out of the lung cavity using a plastic pipette. Blood was left to clot, then spun at 15700g for 10 minutes. Clear serum was collected into Eppendorf tubes and stored at -20°C.

4. MLN collection and re-stimulation

Mesenteric lymph nodes (MLN) were removed prior to removal of the gut, to prevent contamination and stored in RPMI 1640 (Sigma Aldrich Company Ltd; Dorset, UK) containing
10% FCS (Sera Laboratories International, West Sussex, UK) 1% L-glutamine and 1% penicillin/streptomycin (both Sigma Aldrich Company Ltd; Dorset, UK) (complete medium) on ice until use. Under sterile laminar-flow conditions, a cell suspension was made by pushing individual MLNs through a 100μm cell strainer (BD Biosciences, Oxford, UK) into a sterile petri dish in a volume of 5ml complete medium. The cells suspension was collected, cells were spun down at 1500g for 5 min, re-suspended in 5ml medium before counting using a CasyCounter (CASYS® 1, Schärfe Systems, Germany). 1x10^6 cells in 200μl were re-stimulated with 4h E/S antigen (50μg/ml). Cells were cultured for 48h in a 96 well plate with E/S at 37°C (5% CO₂) in a humid incubator. After incubation cell cultures were spun down to remove cells and the supernatants collected and stored at -20°C until use.

5. Worm burden assessment
Infectivity of the administered T. muris eggs was assessed at the end of the experiment as previously described (Else et al., 1990). Frozen caecum and colon were defrosted in a petri dish containing water and cut open longitudinally. After shaking the content into the petri dish the tissue was removed to petri dish with clean water. Using forceps, the gut mucosa was scraped off to remove epithelia and worms from the gut tissue. Adult worms could be removed by pulling out of the tissue with fine forceps. All worms were counted under a binocular microscope. The gut contents were also checked for T. muris worms.

6. Gut tissue collection
For worm counts, the entire colon and caecum were removed and stored at -20°C until use. Tissue for wax, frozen sections and RNA extraction were collected prior to freezing. 5mm tissue each was taken from the proximal colon, after removal of the caecum. Tissue for wax processing was put into a wax processing cassette and stored overnight in 10% Formaldehyde fixative (400ml dH₂O, 50ml PBS, 50ml Formaldehyde (Fischer Scientific UK Ltd, Loughborough, UK), then transferred to 70% ethanol until processing. Frozen sections were embedded into OCT (Raymond A Lamb, Thermo Fischer Scientific, Runcorn, UK) and frozen on dry ice, then stored at -80°C until cut using a Microm HM560 cryostat, collected onto gelatine-coated glass slides, and stored at -80°C until staining. Sections for RNA extraction were put into TriSure (Bioline, London, UK) and frozen on dry ice, then stored at -80°C until use.

6.1. Wax Tissue processing
Proximal colon tissue, fixed in 10% formaldehyde overnight and stored in 70% ethanol until use, was processed using a Microtom STP 120 Tissue Processor (Microm international, Germany) through 70% ethanol for 15 minutes, 90% ethanol for 30 minutes, 95% ethanol for 30 minutes, twice 100% ethanol for 60 minutes, 100% ethanol for 20 minutes xylene for 15 minutes, twice xylene for 30 minutes (all at 40°C) and twice in fibrowax pastillated wax (BDH Lab Supplies, UK) for 1h at 60°C. Using a Microm EC-350 embedding system (Microm International,
Germany) the processed tissue was mounted in wax blocks and cut into 5µm serial sections using a Microm HM325 microtome (Microm International, Germany). Sections were mounted on glass slides and dried.

6.2. Histological analyses on wax embedded tissue

6.2.1. Haematoxylin and eosin staining

Wax sections were dewaxed in Citroclear (HD supplies, UK) twice for 10 minutes each and rehydrated in decreasing concentrations of alcohol: 100%, 90%, 70% and 50% and distilled water. Harris’s haematoxylin solution (Sigma Aldrich Company Ltd; Dorset, UK), filtered prior to use, was used to stain the sections for 5 minutes. Sections were differentiated for 10 seconds in acid alcohol (1%HCl in 70% ethanol). After the sections were washed in running tap water for 5 minutes, they were stained with Eosin (Sigma Aldrich Company Ltd; Dorset, UK) for 30 seconds. Following another wash step, the sections were dehydrated through increasing ethanol concentrations, cleared in Citroclear and mounted using DPX mounting medium (Raymond A. Lamb Ltd, UK).

6.2.2. Goblet cell staining

Alcian blue/PAS staining to visualise acid and neutral mucopolysaccharides was used to stain for Goblet cells in wax sections, which appear blue/magenta/purple. Wax sections were dewaxed and rehydrated, as described above. First sections were stained for 5 minutes in 1% alcian blue with 3% acetic acid (pH 2.5, Sigma Aldrich Company Ltd, Dorset, UK), then after washing in distilled water, treated for 5 minutes with 1% periodic acid. The sections were then washed first in distilled water, then 5 minutes in tap water and rinsed again in distilled water before staining for 15 minutes with Schiff’s reagent (Phillip Harris Education, UK). The previous wash step was repeated, the slides stained for 1 minute with Mayer’s haematoxylin (Sigma Aldrich Company Ltd, Dorset, UK) and blued in tap water. After dehydrating the slides to 100% ethanol they were cleared in Citroclear and mounted in DPX mounting medium (Raymond A. Lamb Ltd, UK). Slides were made anonymous and goblet cells per 20 crypts counted in two sections per mouse.

6.2.3. Histological measures

Tissue sections stained with H&E or PAS staining were used to measure the average crypt length and muscle wall thickness of the proximal colon. The muscle wall thickness was measured between the serosal edge of the colon wall and the inner wall prior to the lamina propria. For measurements of crypt length and muscle wall, Wright Cell Imaging Facility Image J was used.
6.3. Immunohistochemistry staining for CD45, CD11c, CD11b and F4/80 on frozen tissue

CD45 is a leukocyte antigen found on all cells of hematopoietic origin. Staining for CD45 therefore allows an estimate of the inflammatory cellular infiltrate in the proximal colon. CD11c is used to identify dendritic cells, CD11b and F4/80 for macrophages.

Frozen sections on slides were dried under a fan and the tissue circled with a grease pen. When dry, the slides were fixed in 4% Paraformaldehyde (4g Paraformaldehyde (Sigma Aldrich Company Ltd, Dorset, UK)/100mL dH2O, heated to 65°C, then NaOH added until the liquid is clear and stored refrigerated) on ice for 10min and washed twice for 3min in PBS. 100ml glucose oxidase type II-S: from Aspergillus Niger (1.5U/ml, Sigma-Aldrich Company Ltd, Dorset, UK) was added per 100ml of preheated glucose PBS (1.8mg/ml D-glucose, Sigma-Aldrich Company Ltd, Dorset, UK) to quench endogenous peroxidase activity. Slides were immersed in glucose PBS containing glucose oxidase and incubated for 20min at 37°C. The wash step was repeated as above. The slides were blocked with 7% rat serum (Sigma-Aldrich Company Ltd, Dorset, UK) in PBS for 1h. After washing, endogenous avidin and biotin sites were blocked using a commercial kit (Avidin/Biotin Blocking kit, Invitrogen, Life Technologies, Paisley, UK) according to the manufactures instructions. After washing the slides, they were incubated with the primary antibody for 1hour. Biotin-conjugated anti-mouse CD45 (clone 30-F11), CD11c (clone N418) antibodies (eBioscience, Hatfield, UK) were used at 5µg/ml, CD11b (clone M1/70, BD Pharmigen, BD Biosciences, Oxford, UK) and F4/80 (clone Cl-A3-1, AbD Serotec) were used at 2.5µg/ml in PBS. The slides were washed prior to the use of a Vectorstain Elite avidin-biotin peroxidise complex kit (Vectastain ABC kit, Vector Laboratories Inc. Peterborough, UK), according to the manufacturer's instructions. Following another wash step, a Peroxidase Substrate DAB kit (Vector Laboratories Inc. Peterborough, UK) was used to stain the sections according to the manufacturer's instructions. The colour development was watched under the microscope. When sufficient colour had developed, slides were washed in running tap water and counter stained with HaemQS (Vector Laboratories Inc. Peterborough, UK) for 1 minute. The slides were then blued in tap water and Aquatex aequus mounting agent (MERCK KgaA, Darmstadt, Germany) was used to mount the slides. Images of stained tissue were obtained using a SPOT Insight QE camera and SPOT software (Diagnostic Instruments Inc., MI, USA). Image-Pro Plus software (MediaCybernetics, Version 6.3.0.512) was used to quantify CD45 staining and calculate the percentage area of positively stained gut tissue, as described by Humphreys & Grencis (2002).

6.4. Immunofluorescence for dendritic cell subsets on frozen tissue

After air-drying the slides, the tissue sections were circled with a grease pen and fixed in 4% Paraformaldehyde for 5 min on ice. The slides were then washed in PBS with 0.05%BSA for 5 minutes. To block unspecific binding 200µl tyramide blocking kit (TSA™ Fluorescein Tyramide Reagent Pack, Perkin Elmer, Waltham, Massachusetts, USA) was used for 30 min. Endogenous avidin and biotin sites were blocked using a commercial kit (Avidin/Biotin Blocking kit, Vector Laboratories Inc.) according to the manufactures instructions. All antibodies were
diluted in 0.1M Tris-HCl (pH7.5) (TNB) unless stated otherwise. After washing the slides in wash buffer, purified rat anti-mouse CD103 antibody (1:100, clone M290, BD Pharmigen, Oxford, UK) was added for 1h. Following the wash steps, the slides were kept in the dark. The secondary antibody goat-anti-rat IgG Cy5 (1:100, Invitrogen, Life Technologies Ltd; Paisley, UK) was added for 60 minutes, followed by a wash step. Biotinylated anti-mouse CD11c (1:500, clone N418, eBioscience, Hatfield, UK), and anti-mouse cytokeratin-FITC (1:100, clone C-11, Sigma Aldrich, Dorset, UK), were added together in one tube of TNB, and then added to the slides, leaving them for 60 min. After washing the slides, Streptavidin Horseradish peroxidase (1:1000, Invitrogen, Life Technologies Ltd; Paisley, UK ) was added for 30 min. Tyramide cy3 detection antibody (1:100 in amplification buffer, Perkin Elmer, Waltham, Massachusetts, USA) was added after further washing and left for 5 min, followed by a final wash step. The slides were mounted with a pro-long, anti-fade agent containing DAPI (Vector Laboratories Inc.).

7. ELISA
7.1. IgG2c/IgG1 Antibody ELISA
96 well, flat-bottomed plates were coated with 50µl/well ON E/S (0.5µg/ml) in 0.05M carbonate/bicarbonate buffer (pH 9.6, 1.59g/L Na₂CO₃, 2.93g/L NaHCO₃) and stored at 4°C in the dark overnight. After washing the plates 3 times with PBS containing 0.05% Tween 20 (PBS-T, Sigma Aldrich Company Ltd, Dorset, UK) using a plate washer (SKAN Washer 400, Molecular Devices, CA, USA), plates were blocked with 3% bovine serum albumin (BSA, PAA Laboratories Ltd, Yeovil Somerset, UK) in PBS-T (100µl/well) at 37°C for 30 minutes. 8 serial dilutions (1/20-1/2500) of each serum in PBS were added to the plate (50µl/well) after washing and incubated for 1h at room temperature. The antibodies (Biotin-anti mlG1 (1:500) and Biotin-anti mlG2a/c (1:1000), both BD Biosciences, Oxford, UK) were added after repeated washing and stored at RT for 45 min. Following washing, Streptavidin-POD conjugate (1:1000, Roche Diagnostics Limited, West Sussex, UK) was added at 75µl/well and incubated for 45 min at RT. After another wash step, TMB substrate kit (BD Biosciences, Oxford, UK) was used according to the manufacturer's instructions and left to develop in the dark. The reaction was stopped using 2NH₂SO₄, when sufficient colour had developed. The plates were read by a MRX II microplate reader (DynexTechnologies, VA, USA) at 405nm, with reference of 490nm.

7.2. Cytokine ELISA
Cytokine ELISAs were mainly used when cytokine concentrations fall above CBA sensitivity. ELISA plates were coated with 50µl capture antibody/well in carb/bicarb buffer (pH 9.6) and incubated overnight at 4°C. Antibodies against mouse IFNγ, IL-4, 5, 6, 9, 10 and TNF-α were sourced from BD Pharmigen (Oxford, UK), while IL-12, 13 and IL-17 were sourced from R&D systems (R&D Systems Europe Ltd; Abingdon, UK). Capture antibodies were used as followed:
2μg/ml anti-IFNγ, 2.5μg/ml anti-IL-4, 2μg/ml anti-IL-5, 2μg/ml anti-IL-6, 2μg/ml anti-IL-9, 2μg/ml anti-IL-10, 6μg/ml anti-IL-12p70, 6μg/ml anti-IL13 and 6μg/ml anti-IL17A. After washing in PBS-Tween (0.5mL Tween 20/L PBS), the plates were blocked with 150µl 10% FCS in PBS/well and incubated at 37°C. Standards were serial diluted in complete RPMI using the following top standards: 100ng/ml IFNγ, 2000pg/ml IL-4, 500 U/ml IL-5, 10ng/ml IL-6, 1000 U/ml IL-9, 10ng/ml IL-10, 10ng/ml IL-12p70, 10ng/ml IL13 and 10ng/ml IL17A. 50µl standard and sample were added to the ELISA plate and incubated for 1h at 37°C. After washing the plates, 50µl biotinylated detection antibody at the following concentration, 1μg/ml anti-IFNγ, 1μg/ml anti-IL-4, 2μg/ml anti-IL-5, 1μg/ml anti-IL-6, 1μg/ml anti-IL-9, 2μg/ml anti-IL-10, 0.5μg/ml anti-IL-12p70, 0.8μg/ml anti-IL13 and 0.8μg/ml anti-IL17A; in PBS-T containing 1%BSA/well were added to the plate and incubated for 45min at RT. The plates were washed again and 75µl SA-POD (1/1000 in PBS-T, Roche Diagnostics Limited, West Sussex, UK)/well were added, incubated 1h at RT. Following washing, TMB substrate kit (BD Biosciences, Oxford, UK) was used according to manufacturer’s instructions and incubated until sufficient colour developed, then 50µl stop solution (2N H₂SO₄) was added. The plates were read at 450nm, with correction at 570nm using a MRX II micro plate reader (DynexTechnologies, VA, USA).

7.3. Corticosterone ELISA
Corticosterone in serum was measured using the Corticosterone EA Kit (Enzo Life Sciences, Exeter, UK) according to manufacturer’s instructions. Corticosterone standards were prepared using the 100µL 200,000pg/mL Corticosterone standard solution in 900µL. This top standard was further diluted by serial dilution, resulting in standards of 20,000, 4,000, 800, 160 and 32pg/µL. Wash buffer was prepared using deionized water. Using the included plate layout, samples were allocated to wells. All samples were used in doublets. 150µL of Assay Buffer 15 were added to the wells labelled NSB and 100 µL to the Bo (0pg/µL Standard) wells. For each standard 100µL were added to the designated wells. 50µL of Blue Conjugate were added to each well, except the Total Activity and blank wells, followed by 50µL yellow Antibody to each well apart from Blank, Total Activity and NSB wells. After sealing the plate, it was incubated on a plate shaker. After 2h incubation, the plate was emptied, then washed 3 times using 400µL wash solution in each well. Following the final wash step, the plate was emptied before adding 5 µL of Blue Conjugate to the Total Activity well and 200µL of the pNpp Substrate solution to all wells. The plate was incubated at RT for 1h, before adding 50µL Stop Solution to each well. The plate was read using a plate reader (MRX II micro plate reader (DynexTechnologies, VA, USA) at 405nm with correction at 570nm. The concentration of corticosterone was then calculated from the optical density results. First the average net Optical Density (OD) was calculated for each standard and sample by subtracting the average bound OD from the average NSB OD. Next the percentage of bound was calculated by dividing the average net OD by the net Bo OD before multiplying by 100. Using Exel, the percentage bound of the standards was plotted against the log10 of the known
concentration of corticosterone in each standard. From the standard curve the log10 values for each sample could be read. This log10 values were then inversed and the dilution factor removed. The concentration in pg/mL was converted to ng/mL.

8. Cytokine bead array (CBA) analysis
CBA analysis was used to identify the following cytokines: IFNγ, IL-10, IL-13, IL-17a, IL-4, IL-5, IL-6, TNFα, IL-12/23 and IL-9 in MLN supernatants using BD Cytometric Bead Array (CBA) Mouse/Rat soluble protein flex set system (BD Bioscience, Oxford, UK). All standard spheres were pooled in 4ml of assay diluent in a 15 ml falcon tube (BD Biosciences, Oxford, UK) to prepare the flex set standards. The standards were left to equilibrate for 15 minutes; serial dilutions from 1:2 to 1:256 were prepared. Protein Flex Set Capture Beads and PE Detection reagents were diluted 1:50 with Capture Bead Diluent or Detection Reagent Diluent. Capture beads and detection reagent were added to each sample so that 0.2μl of each analyte was mixed with each sample. Plates were pre-wet with wash buffer before 10μl of each standard/sample and 10μl mixed capture beads were added to each well and mixed. After incubating at RT for 1h, mixed PE detection reagents (10μl/well) were added, mixed again, and then incubated for another hour at RT. The plates were drained, 150ml wash buffer added to each well and to re-suspend the beads, mixed for 5 min before analysis by Flow cytometry (LSR II, BD Biosciences, Oxford, UK). For analysis, FCAP Array v1.0.1 software (BD Cytometric Bead Array) was used.

9. Colonic lamina propria isolation and Flow cytometry staining
Lymphocytes were isolated from the lamina propria of caecum and colon, which were removed from mice, opened longitudinally, cleaned of faeces and fat and soaked in ice cold PBS. Tissue was then washed in 2% FCS HBSS (Sigma Aldrich Company Ltd; Dorset, UK ), cut into 0.5cm segments and kept on ice in 50ml Falcon tubes with 10ml 2% FCS HBSS. After vigorous shaking, supernatants were removed and 10ml 2mM EDTA/HBSS (Sigma Aldrich Company Ltd; Dorset, UK) was added to the tubes. These were then incubated in a shaker for 15 minutes at 37°C. The tissue was washed in warmed HBSS and incubated for 30 minutes again in 10ml 2mM EDTA/HBSS. Following another wash step in warmed HBSS, the tissue was digested by shaking for approximately 45 minutes at 37°C in an enzyme cocktail containing 1.25mg/ml collagenase D (Roche, West Sussex, UK), 0.85mg/ml collagenase V (Sigma-Aldrich Company Ltd; Dorset, UK), 1mg/ml dispase (Gibco, Life Technologies Ltd; Paisley, UK) and 30µg/ml DNase (Roche, West Sussex, UK) dissolved in complete RPMI media until all tissue was digested. The suspension was passed through 40µm cell strainers and re-suspended in complete RPMI. The single cell suspension was stored on ice until use.
2x10⁶ cells were added to FACS tubes and washed in FACS Buffer (PBS with Ca²⁺ and Mg²⁺ plus 4% FCS, Sigma Aldrich Company Ltd; Dorset, UK). FC receptors are blocked using anti CD16/CD32 antibody (1:100, BD Biosciences, Oxford, UK) for 20 minutes on ice. FC Block was washed off and cells were stained with PE-anti mouse CD103 (clone M290, 1:200, BD
Pharmigen, BD Biosciences, Oxford, UK), Alexaflor 700- anti mouse CD45 (clone 30-F11, 1:200, BD Biosciences, Oxford, UK) FITC- anti mouse CD11b (clone M1/70 1:200), PE-Cy7-anti mouse CD11c (clone N418 1:200), Pacific Blue-anti mouse IA-IE (clone M5/114.15.2, 1:400), APC-anti mouse F4/80 (clone BM8,1:200, all eBioscience, Hatfield, UK) and Biotin- anti mouse Dec205 (NLDC-145,1:200 Biolegend, Cambridge, UK) and left for 20-30 minutes on ice. After washing in FACS Buffer, SAv-QDot 605 (Invitrogen, Life Technologies Ltd; Paisley, UK) was added to the FACS tubes containing Biotin-anti mouse Dec205 and incubated for further 15 minutes. Following another wash cycle, 7AAD (10µL, BD Biosciences, Oxford, UK) was added to 200µl FACS Buffer remaining in each tube and about 1x10^6 cells were acquired using LSR II Flow cytometer (BD Biosciences, Oxford, UK).

10. RNA-to cDNA Conversion for RT-PCR

10.1. RNA extraction

Gut tissue in TriSure contained in Lysing Matrix D 2ml tubes (MP Biomedicals LLC.; Ohio, USA) had been kept at -80°C after autopsy and were defrosted before homogenising for 1 minute at 6m/s using a FastPrep-24 machine (MP Biomedicals LLC.Ohio, USA) until completely homogenised. The fluid was transferred to a RNA-free Eppendorf tube and centrifuged at 4°C at 12000g for 10 minutes. The supernatant was transferred to a fresh Eppendorf tube and incubated at RT for 5 minutes. 0.2ml Chloroform (Sigma-Aldrich Company Limited, Dorset, Uk) per 1ml TriSure (Bioline Reagents Ltd., London, UK) was added to the supernatant and shaken vigorously for 15 seconds, then centrifuged at 4°C at 12000g for 15 minutes. The aqueous phase was transferred into an Eppendorf tube containing 0.5ml isopryl alcohol (Sigma-Aldrich Company Limited, Dorset, UK) per ml of TriSure used and incubated for 10 minutes at RT after which the samples were centrifuged at 4°C at 12000g for 10 minutes. The supernatant was removed and 1ml 75% ethanol (Sigma-Aldrich Company Limited, Dorset, UK), per ml of TriSure used, was added, mixed and spun down for 5 min at 4°C at 7500g. Having repeated this step, the supernatant was removed fully and the samples were air-dried at 40°C for 20 minutes. The dried pellet was re-suspended in 20µl DEPC H2O (Ambion Ltd-The RNA Company, UK), then incubated for 10 minutes at 55-60°C.

The RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK), diluted to 500ng/ml, and then stored at -80°C.

10.2. cDNA creation

To ensure DNAses were removed from the extracted RNA, the samples were treated for DNAses by adding 3µl DEPC H2O, 1µl RQ1 DNase 10x Reaction Buffer, 2µl RQ1 RNase-free DNase (both Promega, Southampton, UK) to 4µl RNA. The samples were incubated in a Peltier Thermal Cycler (DNA Engine DYAD, MJ Research, Global Medical Instrumentation Inc; MN, USA) PCR machine for 30 min at 37°C. After adding 1µl RQ1 DNase Stop Solution (Promega, Southampton, UK), the samples were incubated for further 10min at 70°C. For reverse transcription 12µl DEPC H2O and 2µl Oligo (dT) 15 Primer
(Promega, Southampton, UK) were added to 11μl DNase treated RNA and heated to 70°C for 5 minutes.

A mastermix of 8μl 5x Reaction Buffer, 4μl dNTP, 2μl RNase inhibitor and 1μl Bioscript (Taq-polymerase) (all Bioline, Reagents Ltd; London, UK) was added to each sample and heated in the thermal cycler for 1 hour at 42°C, 10 minutes at 70°C and finally cooled to 4°C.

10.3. RT PCR for clock gene expression

To measure clock gene expression in cDNA created from RNA extracted from gut tissue the FAST BLUE qPCR MasterMix Plus dTTP kit ( Eurogentec, Seraing, Belgium) was used. 10μL 2x reaction buffer master mix was mixed with 7.8 μl H2O, 0.4 μl Primer 1, Primer 2 and Probe as well as 1 μl cDNA for the either Bmal1, Per2 or DBP, as well one mix containing a β-actin Probe (the housekeeping gene) to normalise the gene-expression. The sequences for βActin probes and primers are the following: Probe: 5’-tgccacaggattccatacacaagaag-3’; Sense primer 5’-aggtcatcttggctcagca-3’ and Antisense primer 5’-caacctgatggaattgtagtt-3’. The sequences for Bmal1 probes and primers are the following: Probe: 5’-tgacccatcttgcaacagca-3’; Sense primer 5’-ccaagaaggtgaatgtcagcacaa-3’ and Antisense primer 5’-gtcattcttgatttccttgggt-3’. The sequences for mPer2 probes and primers are the following: Probe 5’-aactgctcactacgacacctcgt-3’, Sense primer 5’-gctctgacactcagatgcagc-3’ and Antisense primer 5’-tttggtgctgctttggcaggtt-3’. Samples were added in duplicate to low profile 96 well plates (Applied biosystems, Life Technologies Ltd; Paisley, UK) Plates were read using RT-PCR (Applied Biosystems StepOnePlus Realtime PCR system; Life Technologies Ltd; Paisley, UK), before RQ values were calculated in Exel (Microsoft).

11. Sorting of bone marrow dendritic cells

11.1. Bone marrow derived dendritic cells

Femur and tibia bones were cleaned off tissue and collected in complete RPMI (RPMI 1640 (Sigma Aldrich Company Ltd; Dorset, UK) containing 10% FCS (Sera Laboratories International, West Sussex, UK) 1% L-glutamine and 1% penicillin/streptomycin (both Sigma Aldrich Company Ltd; Dorset, UK). The ends of the bone were cut off and using a small needle (0.5x16mm) the bone marrow was flushed out of the bone. With a bigger needle (0.8x16mm) the medium containing the bone marrow was collected. Cells were spun down at 1500g for 5 minutes at 4°C, the supernatant decanted, the pellet re-suspended and the cells counted using a Casy*1 cell counter (Schärfe System, Germany). 2x10⁶ cells/ml were plated into a culture flask (Corning Limited - Life Sciences) with complete RPMI and 0.5ml GMCSF (40ng/ml, PeproTech, London, UK).

After 3 days of culturing at 37°C, 10ml medium with 0.5ml GMCSF were added per plate. Medium was replaced after 6 days. The medium was collected, spun down and cells were re-suspended in fresh medium with GMCSF, and then returned to the cell culture flask. Cells were harvested between day 7-10. This method was adapted from Lutz et al. (1999).
11.2. Sorting of bone marrow derived dendritic cells
Bone marrow dendritic cells were harvested into 50ml falcon tubes. The cells were then counted using Casy*1 cell counter (Schärfe System, Germany). After spinning down (1500g, 5 minutes), cells were re-suspended in 2ml FACS buffer (PBS with Ca\(^{2+}\) and Mg\(^{2+}\) plus 4% FCS, Sigma Aldrich Company Ltd; Dorset, UK). FC receptors were blocked using anti CD16/CD32 antibody (1:100, BD Biosciences, Oxford, UK) for 20 minutes on ice. After washing, cells were stained with CD11c-PE (1:100, clone N418, eBioscience, Hatfield, UK) for 30 minutes on ice. Following washing, cells were adjusted to 5-10x10\(^6\) cells/ml in 25mM HEPES buffered DMEM, containing 1% penicillin/streptomycin (both Sigma Aldrich Company Ltd; Dorset, UK) in a 15ml Falcon tube. To ensure a single cell solution, the cells are passed through a 50 micron cup type Filcon (BD Biosciences (Oxford, UK). The cells were sorted for presence of CD11c using the FACS sorting core-facility on site. Positive and negative fractions were collected in 15 ml Falcon tubes containing 1.5ml complete media.
Sorted cells were lysed using TriSure and cDNA was created using methods described above to genotype for Bmal1 exon 8.

12. Photomultiplier tubes (PMT) use
Tissue and cells was synchronised by Dexamethasone (100nM) or serum shock, by replacing of media. Synchronisation was carried out 1 hour prior to placing tissue or cells on the PMT (H6240 MOD1; Hamamatsu Photonics, Shizuoka, Japan). Glass covers for the 35mm PMT dishes were sterilised by spraying with 70% ethanol and drying under U.V. light. For tissue samples, membrane inserts were used (Millipore (UK) Ltd; Watford, UK). After synchronisation, the media was removed and cells were washed with 1% PBS to remove synchronisation compound and remove phenol red from growth media. Instead filter-sterilised recording media (1000mg/l glucose-low glucose DMEM, 4g/l Glucose powder, 4.7ml/l Sodium Bicarbonate, 10ml/ml HEPES (all Sigma Aldrich Company Ltd; Dorset, UK), 2.5ml/l Pen Strep (Gibco Invitrogen, Life Technologies Ltd; Paisley. UK), 5% FBS, 100µl luciferin/l (0.1M, Promega,Southampton, UK)) (Yamazaki & Takahashi, 2005) were added, 2ml for cells and 1 ml for tissue. Coverslips were sealed onto the 35mm dish using grease (Dow Corning Corporation, Midland, USA) before putting on the PMT. Period of oscillation is calculated using a rhythm-analyzing program (RAP) program (Okamoto et al., 2005).

13. Vaccination
The vaccine was made by adding 2ml 2mg/ml O/N E/S drop wise to 2ml Freund’s adjuvant (Sigma Aldrich Company Ltd, Dorset, UK) and emulsifying until the emulsion was thick and white. The emulsion was left in the fridge overnight, to thicken up further. A 0.8x16mm needle was used to inject 100µl (100µg E/S) subcutaneously on the left or right side of the abdomen of the mouse. Mice were vaccinated at ZT0 or ZT12.
14. Statistics
Where statistics are quoted, Mann-Whitney U test was used for non-parametric data comparing two groups. Kruskal-Wallis test, with Dunn’s multiple comparison post hoc was used to compare three or more groups. A p-value of <0.05 was classed as significant. Statistical analysis was carried out using GraphPad Prism for windows, version 5.
Chapter 3:
Circadian influence on the immune response to the gut dwelling parasite *Trichuris muris*
Introduction:

The importance of circadian rhythm in inflammation

Various infectious settings show that circadian rhythms influence the outcome of infection and inflammation (Cutolo et al., 2003; Gibbs et al., 2009). In chronic inflammatory diseases, such as rheumatoid arthritis, symptoms are more severe in the morning is due to a circadian production of pro-inflammatory cytokines (Cutolo et al., 2003; Hashiramoto et al., 2010) whereas asthma commonly worsens at night time or the early morning (Burioka et al., 2010; Smolensky et al., 2007). Also, in acute inflammation, the time of insult can result in a different outcome of inflammation (Gibbs et al., 2011; Marpegán, et al., 2005; Marpegán et al., 2009; Silver, et al., 2012).

Communication between the main circadian oscillator in the brain and the immune system: corticosterone - a candidate mediator

Corticosterone is a steroid hormone secreted by the adrenal glands. It is expressed in a circadian manner, peaking just before the active phase in mammals (Malisch et al., 2008), which in mice is at dusk. As corticosterone levels cycle throughout the day, the presence of high levels of corticosterone could influence priming of the immune response at the time of infection. Corticosterone levels maintain importance in chronic infection. For example, rheumatoid arthritis patients still show rhythmic, but reduced, levels of corticosterone, and therefore are unable to dampen the inflammatory response. Thus symptoms worsen during the natural nadir of corticosterone secretion (Cutolo et al., 2003; Cutolo & Straub, 2008).

Definition of circadian rhythm in immune cells is through ex vivo studies

Several cellular components of the immune system have an intrinsic circadian rhythm. Through primarily quantitative PCR analysis for clock gene expression, Keller et al. (2009), Kurepa et al. (1992), Silver, et al. (2012b) and others (Arjona & Sarkar, 2008; Fortier et al., 2011; Hayashi et al., 2007; Suzuki et al., 1997) showed that macrophages, NK cells, dendritic cells, T cells and B cells follow an intrinsic rhythm ex vivo. Even red blood cells, which do not contain a nucleus, follow a circadian rhythm in culture (Ebisawa et al., 2010). The intrinsic rhythm in these immune cells is likely the underlying reason for circadian effects on inflammation. Cytokine secretion has been found to be under circadian control and this too may contribute to the changes in symptoms in arthritis and asthma according to time of day (Cutolo & Straub, 2008). However, our understanding of the precise ways in which circadian rhythms influence components of the immune system remains incomplete.

Exploring circadian rhythm in vivo - the Trichuris muris mouse model

As most research on intrinsic circadian rhythm in immune cells is conducted in vitro or ex vivo, in vivo models are needed to ensure that the findings can be translated to a whole organism. To this end, intestinal nematode parasite Trichuris muris in the mouse represents a physiological
relevant infection model. The impact of an infection with *T. muris* on the host circadian rhythm has not been examined. In addition, the effect of circadian rhythms on the host’s ability to expel *T. muris* is also unclear. The only external circadian cue able to reach these gut dwelling parasites is the feeding behaviour of the host. Whole colon tissue exhibits circadian rhythms (Froy & Chapnik, 2007; Hoogerwerf *et al*., 2007; Hussain & Pan, 2009). This peripheral tissue is regulated by the suprachiasmatic nucleus (SCN) in the brain under normal circumstances, and by feeding rhythms under restricted conditions, when food is only available for defined times during the day or only in restricted quantities. This leads to the assumption that immune cells within colonic tissue are under circadian control. Cell numbers in colon tissue could therefore oscillate throughout the day in anticipation of insult. Furthermore, the ability of antigen presenting cells to present antigen or secrete cytokines could also be gated by circadian rhythm. Hayashi *et al.* (2007) showed a functional rhythm in the phagocytosis of peritoneal macrophages. In a similar manner, the recognition of *T. muris* antigen by host dendritic cells or epithelial cells, which in turn condition dendritic cells, could be under circadian control and prime the immune response differently depending on the time of day of infection.

Rhythm in immune responses conserves energy and leads to effective responses when the body anticipates the insult. In nature, *T. muris* infects the host via the faecal-oral route when infective eggs, which can be found in the soil, are ingested, most likely during feeding. Mice are nocturnal and therefore the body should be anticipating infection with parasites such as *T. muris* during their active feeding time at night.

*T. muris* is a gut dwelling nematode which can develop into an acute or chronic infection depending on the dose of infective eggs given or the genetic background of the experimental mouse strain used. C57BL/6 mice are resistant to high dose infections of about 200 eggs and above, and start expulsion of the parasite around day 21, when the cytokine response peaks. At about the same time, the larvae moult from the L3 to the L4 stage. Typically in C57BL/6 mice 1 in 8 mice retain the worm burden and become chronically infected. Successful expulsion is associated with a strong Th2 dominated immune response, while susceptibility is due to a stronger Th1 response (Else & Grencis, 1991). Why some mice of the same genetic background respond differently than others is unclear, but it is thought to be a parasite induced effect.

**Early events in *T. muris* infection could be under circadian control**

CD4+ T cells have been shown to control the innate effector mechanisms needed for expulsion of *T. muris*. Activation of T cells in the lymph node relies on antigen presenting cells. Silver *et al.*, (2012b) established by quantitative PCR that antigen presenting cells (macrophages, dendritic cells, B cells) enriched from spleens of mice housed in 12h light, 12h dark settings expressed clock genes and generated a functional clock output in a circadian manner. Keller *et al.* (2009) previously found circadian expression of clock genes in peritoneal macrophages harvested under constant dark conditions (CT), also using quantitative PCR. Rhythmic
responses have also been defined in T cells (Fortier et al., 2011). Thus T cells extracted from lymph nodes and costimulated with anti-CD3 or anti-CD28 proliferated better when extracted at CT6 than CT14. Further mice, housed in light/dark conditions, immunized with dendritic cells loaded with OVA-peptide at ZT6 or ZT18 show significantly stronger T cell proliferative responses when immunization was given at ZT6 compared to ZT18.

In summary, circadian rhythms influence a variety of immune responses. The biology of immune cells which respond to infection with the gut dwelling nematode parasite *Trichuris muris* in the gut are known to have biological rhythm. Therefore the aim of this chapter is to establish whether the time of day of infection has an impact on the ability of the immune system to mount a protective immune response to infection and therefore influence resistance to the parasite up to 3 weeks post infection.

The ability of the host to expel the parasite can be established by quantifying worms in colonic tissue. In addition, parasite specific antibody responses and mesenteric lymph node cytokine production are used as indicators of the predominant type of T helper cell response (Th1 or Th2), to identify the underlying T cell polarization. The extent of damage caused by the inflammatory response to the parasite infection can be measured by quantifying crypt length, muscle wall thickness and cellular infiltrates in histological sections in proximal colon tissue.

**Aims**

- To establish whether infection of mice with a high dose *T. muris* infection at ZT0 or ZT12 impacts on the immune response which subsequently develops.
- To investigate if there are underlying differences in naïve mice prior to infection at ZT0 versus ZT12.
- To characterise the local immune response to infection over the first 3 days post infection in order to identify early differences between ZT0 and ZT12 infected mice.
- To quantify the level of corticosterone found systemically in sera at the time of autopsy.
Results:

3.1. Infection of mice at ZT0 or ZT12 resulted in a different outcome of infection and altered T helper cell response

To establish if long term persisting worm infection is affected by circadian rhythm in the immune system, C57BL/6 mice were infected with *Trichuris muris* by oral gavage at 7 am in the morning (ZT0) or 7 pm at night (ZT12). These times were chosen as they coincide with on the offset and onset of mouse activity respectively. Following two preliminary experiments, mice were infected and culled on several days. After an error at day 25/28 in the initial experiment, which led to small numbers in one of the groups, day 28 was repeated as a single time point. Day 21, 25 and 28 were repeated in 2 further repeats. Experiments 2-4 were culled at time of infection, while in the first experiments mice were culled at one time point. All time points accessed over several experiments are shown below in Figure 1.

![Figure 1: The schematic of the time course of ZT0 and ZT12 infected mice.](image)

Over several experiments, different time points were assessed. Mice were infected at ZT0 or ZT12. On day 13, worm establishment was measured. On the following time points, inflammation and expulsion of the parasite was analysed.

3.1.1. Mice infected with *T. muris* at ZT0 or ZT12 showed no difference in the number of worms establishing as determined at day 13 post infection

![Figure 2: Worm burden of ZT0 and ZT12 infected C57BL/6 mice with 200 *T. muris* eggs at day 13 post infection.](image)

Mice were sacrificed at day 13 and worm burdens accessed by counting worms found in the colon and caecum. The symbols represent individual mice and histograms show mean +/- SEM. The data was combined from 2 experiments.
The ability of the *T. muris* infection to establish was assessed at day 13 (Fig. 2) by counting the larvae in the caecum and colon. Equal numbers of *T. muris* worms established at this time point in ZT0 and ZT12 infected mice, with an average of about 80 worms. Even though 200 infective eggs were administered, not all of the eggs produced larvae that established in the gut. In addition, day 13 larvae are still very small at this stage and difficult to count. This explains the difference between the number of worms counted at day 13 (Fig. 2) and the higher worm burden found at day 21 (Fig. 3A).

3.1.2. Worm expulsion was significantly delayed in ZT12 infected mice at day 21 and day 28 post infection

![Figure 3: Worm burden of ZT0 and ZT12 infected mice over time. C57BL/6 mice were infected with 200 *T. muris* eggs by oral gavage at ZT0 (7am) and ZT12 (7pm). Mice were sacrificed at several time points and worm burden was accessed by counting worms found in the colon and caecum SEM at day 21 (A), 28 (B) and 35 (C). The symbols represent individual mice and histograms show mean +/- Statistics: Mann-Whitney U-test. *P < 0.05, **P< 0.01, d21 n=22 (pooled from 3 experiments), d28 n=28/36 (pooled from 4 experiments), d35 n=12 (Data from 2 preliminary experiments).

By day 21, C57BL/6 mice start to expel a high dose of *T. muris* parasites under normal laboratory settings. *T. muris* moults from an L2 larval stage to L3 stage at this point. In mice infected at ZT0 significantly fewer worms were recovered than in mice infected at ZT12 (Fig. 3A). By day 28 post infection mice infected at ZT0 still had significantly lower worm burdens than mice infected at ZT12 (Fig. 3B). At day 35 post infection almost all mice infected at ZT0 had expelled their worm burden (Fig. 3C). There was no significant difference between ZT0 and ZT12 infected mice, but 4 out of 12 ZT12 infected mice (Fig. 3C) retained a high worm burden.

3.1.3. Parasite specific Th2 antibody IgG1 was significantly increased at day 21 in ZT0 infected mice, while parasite specific Th1 antibody IgG2c was significantly increased at day 28 in ZT12 infected mice

Infection of mice with *T. muris* results in the production of an antigen specific antibody response. Even though there is controversy as to whether antibodies are involved in *T. muris* expulsion, Th1 and Th2 type antibody production (IgG2c and IgG1 respectively) can at least be used as an indicator for the overall skewing of the immune response. The stronger the Th2 response is compared to the Th1 response, the more likely the worm burden will be expelled.
Parasite specific antibody is cumulative and can even be measured post expulsion. Therefore it is an ideal parameter to monitor in order to indicate the type of T cell response to *T. muris* infection. If circadian rhythm impacts on immune responses leading to delayed worm expulsion, antibody production can indicate if the immune response is skewed towards an inappropriate Th1 response.  

![Figure 4: Parasite specific IgG1 (A and B) and IgG2c (C and D) antibody production at day 21 (A and C) and day 28 (B and D). Mice were infected with 200 *T. muris* eggs and sacrificed at day 21 and day 28 post infection. The serum was serially diluted (1/20-1/2560) and screened against parasite ES antigen at 0.5μg/ml; the data shown is dilution 1/320 only, as it falls within the linear range of the titration curve. The symbols represent individual mice, histograms show mean +/- SEM. Statistics: Mann-Whitney U-test. *p < 0.05, **p< 0.01, d21 n=6, d28 n=10. Data shows one of four experiments.](image-url)

Optical densities of Th2 associated antibody IgG1 and Th1 associated antibody IgG2c were measured in serum via serial dilution ELISA. As there is no standard for *T. muris* specific antibodies, the dilution 1:320 was selected for comparing groups, based on ODs falling on the linear part of the titration curve. Levels of IgG1, a Th2-associated antibody isotype, were significantly higher in mice infected at ZT0 than in mice infected at ZT12 (Fig. 4A). By day 28, IgG1 levels were similar at both times of infection (Fig. 4B). Levels of IgG2c, a Th1-associated antibody isotype, had a tendency to be higher at day 21 in ZT12 infected mice compared to ZT0 infected mice although ODs are low overall (Fig. 4C). By day 28 the differences between ZT0 and ZT12 reached significance with significantly higher levels of IgG2c in ZT12 infected mice (Fig. 4D). High levels of IgG2c, the Th1-associated antibody, are indicative of retaining worm burdens, while high levels of IgG1, the Th2-associated antibodies coincide with quick expulsion. These variations in the level of parasite specific antibody indicate that mice infected at ZT0 are more efficient at mounting a Th2 response, while mice infected at ZT12 tend to produce a stronger Th1 response. Indeed the IgG1 response seen in mice infected at ZT12, even at day 28, never reaches the high ODs seen at day 21 in ZT0 infected mice.
3.1.4. Localised cytokine responses were skewed towards Th2 in ZT0 infected mice and skewed towards Th1 in ZT12 infected mice

Figure 5: Mesenteric lymph node cell (MLN) cytokine profiles at day 21 post infection. Mice were infected with 200 T. muris eggs. 5x10⁶ MLN cells were isolated from mice infected at ZT0 and ZT12 at day 21 and cultured with T. muris E/S (50µg/ml) for 48h. The supernatants were analysed using cytometric bead array (CBA). The data for Th2 associated cytokines (A-C), anti-inflammatory cytokine IL-10 (D), Th1 associated cytokines (F) and proinflammatory cytokines (E and G) are shown as histograms expressing mean +/- SEM as well as symbols representing individual mice. Statistics: Mann-Whitney U-test, *p < 0.05, **p< 0.01. n=8. Data shown one experiment of 4.
Antibodies are not the only measurement indicating the dominant type of T helper cell response found in mice infected at ZT0 or ZT12. Local cytokine production can also show which immune response is dominant in the mesenteric lymph node that drain the site of infection.

Mesenteric lymph node cells cultured with T. muris E/S antigen were analysed for the localised cytokine response in mice infected at ZT0 or ZT12 and culled at day 21. Levels of Th2 cytokines, IL-4 and IL-5 (Fig. 5B and C), were similar in mice infected at ZT0 or ZT12, while IL-13 levels were significantly higher in ZT0 infected mice (Fig. 5A). Anti-inflammatory cytokine IL-10 levels (Fig. 5D) were similar in ZT0 and ZT12 infected mice. Pro-inflammatory cytokines and Th1 cytokines IL-6, IFNγ and TNFα were detected at significantly higher levels in mice infected at ZT12 (Fig. 5E-G). These results follow the same line as worm burden and also antibody responses, indicating stronger Th1 responses are produced when mice are infected at ZT12 rather than ZT0.

3.1.5. No significant differences could be observed in muscle wall thickness, crypt length and goblet cell hyperplasia between ZT0 and ZT12 infected mice

![Figure 6: Gut pathology measurements at day 28 post infection with 200 T. muris eggs.](image)

T. muris larvae burrow through synovial tunnels in the epithelial lining of the colon tissue. This, as well as the immune response mounted to fight the infection, causes damage to the lining of
the intestine. These pathological changes in the large intestine were quantified by PAS-staining wax embedded colon tissue sections and measuring muscle wall thickness, crypt length and the number of goblet cells. No differences could be observed between mice infected at ZT0 and ZT12 for any of these parameters (Fig. 6). These results suggest that the even though ZT12 infected mice show a delay in forming an appropriate immune response and therefore present with a delayed worm expulsion, tissue damage at time of expulsion is equal between ZT0 and ZT12 infected mice.

3.1.6. CD45⁺, F4/80⁺, CD11b⁺ and CD11c⁺ cell infiltration did not differ between mice infected at ZT0 or ZT12

![Figure 7: Immunohistochemistry (staining for CD45⁺, F4/80⁺, CD11b⁺ and CD11c⁺) on proximal colon tissue recovered from mice 28 days post an infection delivered at ZT0 or ZT12. Mice were infected with 200 T. muris eggs and sacrificed at day 28 post infection. A) CD45 positive cells B) F4/80 positive cells C) CD11c positive cells D) CD11b positive cells. The symbols represent individual mice,](image-url)
In addition to pathological measurements, the cellular infiltrate into inflamed tissue can be measured, indicating whether circadian rhythm causes the lymphocytes, macrophages or dendritic cells to infiltrate the infected tissue differently depending on the time of infection.

Frozen proximal colonic tissue was stained for cell surface markers representing lymphocyte infiltrate (CD45, Fig. 7A), Macrophages (F4/80 and CD11b, Fig. 7B and D), eosinophils (F4/80 Fig. 7B) and dendritic cells (CD11b and CD11c, Fig. 7D and C). Staining for these markers showed no significant differences in cell numbers or percentage of cells stained for any of these cell types (Fig. 6). This indicates that cell numbers are not affected by circadian rhythm, but this does not mean that functional aspects are not under circadian control.

3.2. Culling naïve mice at ZT0 and ZT12 shows differences in crypt length but not in muscle wall thickness, goblet cell hyperplasia or in F4/80+ and CD11c+ cell numbers in colon tissue

Analysing naïve mice will investigate if the dichotomy in the T helper cell response observed 3 weeks post infection is determined by factors regulated by underlying circadian differences in some T cell conditioning factor/cell type in naïve animals which varies by time of day. Therefore mice were culled at ZT0 and ZT12 respectively prior to infection.

The tissue of naïve animals can be analysed in the same way as in inflammation. Pathology measurements on PAS-stained proximal colon tissue showed no differences in muscle wall thickness or goblet cell numbers between ZT0 and ZT12 naïve mice, but a significant difference in crypt length was identified, with ZT0 naïve mice having shorter crypts than ZT12 naïve mice (Fig. 8A-C). The difference between crypt lengths was surprising, as it was not expected that epithelial cell proliferation would be higher at night. Also no differences in crypt lengths were observed during infection at day 28 (Fig. 6) or day 1 (Fig. 11).

Quantification of immune cells in colon tissue could reveal if immune cell numbers vary in a circadian manner and therefore influence the immune response at the time of infection. DAB-staining on frozen proximal colon tissue for F4/80 revealed no differences in macrophages and eosinophils between ZT0 and ZT12 naïve mice (Fig. 8D). CD11c staining, representing dendritic cells, also revealed no differences between ZT0 and ZT12 naïve proximal colon tissue (Fig. 8E). These results indicate that the number of cells found in proximal colon tissue does not change
in a circadian manner. This does not preclude immune cells from having a circadian rhythm in response to infection.

**Figure 8: Changes in gut morphology in untreated mice according to time of day.** Naïve mice were sacrificed at ZT0 or ZT12. Pathology measurements were analysed on wax embedded proximal colon tissue. A) Muscle wall thickness B) Crypt length C) Goblet cell numbers. Immunohistochemistry was carried out on frozen proximal tissue sections (D and E). D) F4/80 positive cell numbers E) CD11c positive cell numbers. The symbols represent individual mice. Histograms show mean +/- SEM. F) Representative photographs of PAS-stained naïve proximal colon. Goblet cells stain pink-purple. G) Representative photographs of immunohistochemistry using anti-F4/80 monoclonal antibody on frozen proximal colon H) Representative photographs of immunohistochemistry using anti-CD11c monoclonal antibody on frozen proximal colon. F4/80 and CD11c positive cells are stained brown. Photographs were taken at 400x magnification. Scale bar represents 100μm. Statistics: Mann-Whitney U-test, PAS-staining n=5 mice per group, Immunohistochemistry staining n=5 mice per group.
3.3. Early events post ZT0 or ZT12 infection showed no difference in cell infiltrate and tissue responses in mice culled at day 1-3

Assuming that circadian rhythm impacted on the priming of the immune response to the worm infection, tissue from mice infected at ZT0 or ZT12 early post infection was analysed. The kinetics of early dendritic cell response to *T. muris* worms has been shown to significantly correlate with the ability to expel a high dose infection (Cruickshank et al., 2009). Therefore colon tissue was examined at day 1 of infection. Worm hatching cannot be measured as early as day 1, but it can be assumed that the eggs hatch within a few hours, therefore the worms should have hatched and successfully established in the caecum and colon after 24 hours.

3.3.1. F4/80+ and CD11c+ cell infiltrate on frozen sections showed no significant differences between ZT0 and ZT12 infected mice at day 1

In response to the infection, immune cells, such as dendritic cells and macrophages are activated and drive further innate and adaptive immune responses. Measuring macrophage and dendritic cell numbers in gut tissue at day 1 post infection could reveal if responses to *T. muris* infection are enhanced at ZT0 compared to ZT12 due to a faster cellular infiltration.

Immunohistochemistry on OCT embedded frozen tissue stained for macrophage marker F4/80 (Fig. 9A and C) and dendritic cell marker CD11c (Fig. 9B and D) showed no significant differences in F4/80 positive cells or CD11c positive cells between mice infected at ZT0 and ZT12.
Figure 9: Immunohistochemistry on frozen proximal colon tissue taken at day 1 post infection and stained for F4/80 positive cells (macrophages) and CD11c (dendritic cells). Mice were infected with 200 T. muris eggs and sacrificed at day 1 post infection. Proximal colon was stained for A) F4/80 positive cells and B) CD11c positive cells. The symbols represent individual mice; histograms show mean +/- SEM. Representative photographs of F4/80 (C) and CD11c (D) immunohistochemistry staining using anti-F4/80 or CD11c monoclonal antibody. Positive cells stain brown. Photographs were taken at 400x magnification. Scale bar represents 100μm. Statistics: Mann-Whitney U-test, n=10 mice per group.
Figure 10: Fluorescent staining for CD11c and CD103 positive cells on frozen proximal colon tissue from day 1 post infection. Mice were infected with 200 T. muris eggs and sacrificed at day 1 post infection. Tissue was stained for A) CD11c positive cells B) CD103 positive cells C) CD11c and CD103 double positive cells. The symbols represent individual mice, histograms show mean +/- SEM. D) Representative photographs of CD11c and CD103 double staining. CD11c positive cells stained red, CD103 positive cells stain blue. Double positive cells would appear purple. Green staining shows epithelial cells. Photographs were taken at 400x magnification. Scale bar represents 100μm. Statistics: Mann-Whitney U-test, n=5 mice per group.

As immunohistochemistry did not show differences in cell infiltration using DAB staining, immunofluorescent staining was used. In agreement with the single staining analysis, there was no significant difference between ZT0 and ZT12 infected mice for CD11c and CD103 single positive cells (Fig. 10A and B), or CD11c/CD103 double positive cells in frozen proximal colon tissue (Fig. 10C). These results showed that at day 1 post infection rhythmic influxes of macrophages and dendritic cells are not visible.
3.3.2. No differences in muscle wall thickness, crypt length or goblet cell hyperplasia were visible at day 1 in mice infected at ZT0 or ZT12.

![Pathology measurements on PAS-stained proximal colon tissue at day 1 post infection.](image)

Muscle wall thickness, crypt length and goblet cell numbers were measured on PAS-stained wax embedded sections of day 1 ZT0 or ZT12 infected mice. At day 1, no significant differences could be observed between muscle wall thicknesses, crypt length or goblet cell numbers (Fig. 11A-C) in mice infected at ZT0 and ZT2 culled at day 1.

3.3.3. Flow cytometry analysis of lamina propria leukocytes harvested at ZT0 or ZT12 from uninfected mice, as well as on day 1-3 from mice infected at ZT0 or ZT12, showed no differences in macrophage and dendritic cell numbers.

To more precisely quantify leukocytes in gut tissue, lamina propria lymphocytes were isolated from gut tissue on days 0, 1, 2 and 3 post infection. The isolated cells were stained with fluorescent dye for Flow cytometry to identify cell surface markers on infiltrating lymphocytes, macrophages and dendritic cells. The gating strategy to identify these cells can be seen in Figure 12.
Cells were selected on the forward/side scatter plot to exclude debris (Fig. 12A). In the next step, doublet cells were excluded by plotting forward scatter height against forward scatter area (Fig. 12B). Single cells were then analysed for viability (7AAD) and the expression of CD45 (Fig. 12C). The cells negative for 7AAD and positive for CD45 showed all leukocytes in the lamina propria. Live leukocytes were analysed for the expression of CD11c and MHCII, classical dendritic cell markers (Fig. 12D). As these can also be found on some macrophages, the double positive cells were further analysed for the dendritic cell markers CD103 and Dec205 (Fig. 12E). To exclude that these cells were macrophages, they were analysed for the expression of F4/80 and CD11b as well (Fig. 12F). Live leukocytes were also analysed for the expression of F4/80 and MHCII (Fig. 12G). The double positive cells were expected to be macrophages and examined for the expression of CD11b and CD11c (Fig. 12I). F4/80 negative MHCII positive cells could be dendritic cells and were analysed for the expression of CD11c (Fig. 12H).

Roughly 8% of live leukocytes were double positive for CD11c and MHCII (Fig. 13A). This percentage did not significantly differ between ZT0 and ZT12 infected mice and also did not increase from naïve levels up to day 3. About 50% of these cells also stained double positive for
CD11c and Dec205 (Fig. 13B). Again, this percentage did not increase significantly over the time course or differ significantly between mice infected at ZT0 or ZT12. Six per cent of CD11c*MCHII+ cells were single positive for CD103, while 30% were single positive for Dec205 (Fig. 13B).

Between 35 and 45% of CD11c ‘MCHII’ cells were double positive for F4/80 and CD11b (Fig. 13C). These percentages did not increase significantly from naïve levels up to day 3 or differ significantly between mice infected at ZT0 or ZT12. There is a non-significant increase of F4/80 single positive cells over time, which increased from about 1% to 4%. On the other hand, a non-significant decrease in CD11c ‘MCHII’ cells single positive for CD11b from about 27% to 20%

Figure 13: Flow cytometry analysis showing the relative percentage of CD11c MHCII double positive cells found in the lamina propria of C57BL/6 mice infected with 200 T. muris eggs at ZT0 or ZT12 culled at day 0 to day 3. Live leukocytes were selected and analysed for expression of CD11c and MHCII as seen in Figure 11 D (A). These cells were selected and analysed for CD103 and Dec205 expression as seen in Figure 11 E (B). Cells expressing CD11c and MHCII are considered to be dendritic cells, but these markers can also be found on macrophages. Dec205 is a further dendritic cell marker. CD103 is a dendritic cell marker for gut homing. CD11c MHCII double positive cells were also analysed for F4/80 CD11b expression in Figure 11 F (C), which are macrophage markers.
was observed (Fig. 13C). These non-significant changes could indicate a slow change over time, which has not reached significance by day 3.

About 8% of live leukocytes were F4/80"MHCII" (Fig. 14A). Of these, over 80% of cells stained CD11b and CD11c double positive (Fig.14B). The remaining cells stained CD11b positive; therefore it can be assumed that these cells were macrophages.

Live leukocytes were to 50-60% MHCII single positive (Fig. 15A). Only about 2% of these cells were single positive for CD11c (Fig.15B). Roughly 6% were double positive for CD11c and Dec205 (Fig. 15B). Dec205 single positive cells made up 60% of these MHCII single positive cells (Fig. 15B). Therefore only a small percentage of MHCII" cells are dendritic cells. Further analysis of LPLs using other cell surface markers should reveal the cell types making up this cohort.

Figure 14: Flow cytometry analysis showing the percentage of F4/80 MHCII double positive cells found in the lamina propria of C57BL/6 mice infected with 200 T. muris eggs at ZT0 or ZT12 culled at day 0 to day 3. Live leukocytes were selected and analysed for expression of F4/80 and MHCII as seen in Figure 11 G (A). These cells were selected and analysed for CD11b and CD11c expression as seen in Figure 11 I (B). Cells expressing F4/80, MHCII and CD11b are considered to be macrophages. CD11c is also often expressed on macrophages.

Overall there were no significant differences in the dendritic cell/macrophage cell populations found in the lamina propria, either over time or between ZT0 and ZT12. This leads to the assumption that cell numbers do not change markedly in the first three days from naive levels. These results further suggest a functional rather than a numerical circadian variation between mice infected at ZT0 or ZT12.
Figure 15: Flow cytometry analysis showing the percentage of MHCII positive cells found in the lamina propria of C57BL/6 mice infected with 200 T. muris eggs at ZT0 or ZT12 culled at day 0 to day 3. Live leukocytes were selected and analysed for expression of F4/80 and MHCII as seen in Figure 11 G (A). MHCII single positive cells were selected and analysed for CD11c and Dec205 expression as seen in Figure 11 H (B). Cells expressing MHCII and CD11c are considered to be dendritic cells. Dec205 is a further DC marker.

3.4. Mice infected at ZT12 or ZT0 showed no reversed phenotype

As mice were infected at ZT0 and ZT12 but autopsied at a single time of day in the initial experiments, it is possible that delayed worm expulsion in ZT12 mice could reflect later time of infection. Therefore C57BL/6 mice were infected at ZT12 (7pm) and ZT0 (7am) to ensure differences observed in worm burden, IgG antibody production and cytokine production were not due to a 12h delay, i.e. the order of infection was changed, infecting ZT12 mice first, then infecting ZT0.

3.4.1. No significant differences could be observed when mice were infected at ZT12, then ZT0

Worm burdens counted in caecum and colon at day 21, were not significantly different between ZT12 and ZT0 infected mice (Fig. 16A). Even though mice infected at ZT0 had been exposed to the infection for about 12h less, only 3/10 mice still harbour > 20 worms. This is in contrast to mice infected at ZT12; with 6/10 mice still harbouring worms. Despite the worm burden not being significantly lower in ZT0 infected mice compared to ZT12 infected mice, the worm expulsion phenotype (lower number of worms if mice infected at ZT0) is not reversed when the order of infection time is reversed.
Optical densities of Th2 associated antibody IgG1 and Th1 associated antibody IgG2c were measured in serum via serial dilution ELISA. The dilution 1:320 was selected for comparing groups, based on ODs falling on the linear part of the titration curve. Parasite specific antibodies measured in serum revealed that the IgG1 and IgG2c levels were not significantly different between the two groups, ZT12 and ZT0 infected mice (Fig. 16B/C). The lack of any significant differences in antibody levels shows that there is no stronger Th2 response in ZT0 infected mice and no stronger Th1 response in ZT12 infected mice as previously seen. The fact that the Th1/Th2 polarization is the same in ZT12 or ZT0 infected mice explains why the worm expulsion data is not significantly different.

3.4.2. ZT0 infected mice had only significantly elevated IL-13 cytokine levels, other cytokine levels were similar in ZT0 and ZT12 infected mice

Cytokines were measured in supernatants of T. muris E/S (50μg/ml) re-stimulated mesenteric lymph nodes cells using cytokine bead array. Levels of Th2 cytokines IL-13 and IL-4 were not significantly higher ZT0 infected mice (Fig. 17A and B), while IL-5 was significantly elevated in ZT0 infected mice (Fig.17C) compared to ZT12 infected mice. Anti-inflammatory cytokine IL-10 (Fig. 17D), Th1 and pro-inflammatory cytokines IL-6, IFNγ and TNFα (Fig. 17E-G) were not significantly different between ZT0 and ZT12 infected mice. Thus only IL-5 levels were higher in ZT0 infected mice compared to ZT12 infected mice. Both ZT0 and ZT12 infected mice produced pro-inflammatory and Th1 cytokines with no significant differences between groups.
Figure 17: Mesenteric lymph node cell cytokine profiles at day 21 post infection at ZT12 or ZT0. Mice were infected with 200 *T. muris* eggs at ZT12 or ZT0 and sacrificed at day 21. MLN cells were stimulated with *T. muris* E/S (50μg/ml) antigen for 48h. The supernatants were analysed using cytometric bead array (CBA). Data for Th2 associated cytokines (A-C), anti-inflammatory cytokine IL-10 (D), Th1 associated cytokines (F) and proinflammatory cytokines (E and G) are shown as histograms expressing mean +/- SEM as well as symbols representing individual mice. Statistics: Mann-Whitney U-test, *p < 0.05. n=8 mice per group.

3.4.3. Pathological measurements and CD45<sup>+</sup> and CD11c<sup>+</sup> cell infiltrate did not differ between ZT0 and ZT12 infected mice

Pathological measurements were made from PAS-stained wax embedded proximal colon sections. No differences can be seen between ZT12 and ZT0 infected mice for muscle wall
thickness, crypt length and goblet cell numbers (Fig. 1A-C), as previously seen in ZT0 and ZT12 infected mice (Fig. 6).

Figure 18: Quantitative and qualitative analysis of pathology of proximal colon tissue from mice infected at ZT12 and ZT0. Mice were infected with 200 T. muris eggs and sacrificed at day 21. Pathological measurements were analysed on wax embedded proximal colon tissue for A) Muscle wall thickness B) Crypt length C) Goblet cell numbers. Immunohistochemistry of frozen proximal colon tissue was stained for D) CD45 positive cells and E) CD11c positive cells. Each dot represents the mean for one individual mouse with 20 crypts counted. Histograms show the mean for 10 mice +/- SEM. Representative photographs of goblet cells using PAS staining on wax embedded proximal colon tissue (F), CD45 positive cells in immunohistochemistry staining on frozen proximal colon tissue (G) and CD11c positive cells in immunohistochemistry staining on frozen proximal colon tissue (H). Photographs were taken at 400x magnification. Scale bars represent 100μm. Goblet cells appear pink-purple. CD45 or CD11c staining show positive cells stained with anti-CD45 monoclonal antibody or CD11c monoclonal antibody in brown. Goblet cells and CD11c positive cells were counted directly under the microscope, for analysis of CD45 staining Image-Pro Plus software was used. Statistics: Mann-Whitney U-test, n=10 mice per group.
Infiltrating lymphocytes were analysed in frozen proximal colon sections. No differences were observed for leukocytes (CD45 positive staining) between ZT12 and ZT0 infected mice (Fig. 18D). CD11c positive staining, the marker for dendritic cells numbers, was also equal between ZT12 and ZT0 infected mice (Fig. 18E). The results were thus equivalent to when mice were infected first at ZT0, and then ZT12.

3.5. The levels of corticosterone were reduced by day 3 post infection in ZT12 infected mice then remained low throughout infection

Corticosterone levels influence the immune response and are generally associated with suppression of inflammation. Corticosterone levels were measured in sera of mice sacrificed day 0-3 post infection as well as on day 21 and day 28 (Fig. 18). Mice were culled at ZT0 and ZT12 respective to the time of infection. The concentration was higher in ZT12 infected and culled mice, which was expected as corticosterone levels peak before onset of activity (Malisch et al., 2008). By day 3 however levels of corticosterone in ZT12 sacrificed animals reduced and stayed low throughout infection (Fig. 19). In ZT0 sacrificed mice, levels of corticosterone remained low throughout the time course. No significant difference could be observed between time points of mice infected and culled at ZT0. Groups of mice infected at culled at ZT12 also did not show significant differences over the time course. A comparison between ZT0 and ZT12 was not possible, as serum was only collected from mice sacrificed at ZT0 or ZT12 depending time of infection and therefore it is unclear if the rhythm in corticosterone is dampened during the course of infection.

Figure 18: Corticosterone levels measured in serum of mice sacrificed at various time points after a 200 egg T. muris infection delivered at ZT0 or ZT12. Sera collected at days 0, 1, 2, 3, 21 and 28 were analysed for levels of corticosterone. Corticosterone was measured using the Corticosterone EA Kit (Enzo Life Sciences, Exeter, UK). Statistics: Bonferroni ANOVAs, Dunn post hoc *p < 0.05, **p < 0.01, ***p< 0.001. d0-3 n = 3 mice per group, d21 n = 7 mice per group, d28 ZT0 n = 7 mice per group, d28 ZT12 n = 6 mice per group.
Discussion

For optimal worm expulsion a Th2 response is required, however in a C57BL/6 mouse, which expels *T. muris* relatively slowly, a mixed Th2/Th1 response develops. Worm expulsion is evident if a strong enough Th2 response is achieved (Else & Grenchis 1991).

The time of day of infection appears to impacts on the host’s ability to eventually expel the parasite, evident at 3 weeks post infection

Delayed worm expulsion correlated with a higher Th1 response and lower Th2 response in those mice infected at ZT12, reflected in the parasite specific antibody IgG1 and Th2 associated cytokines IL-4 and IL-13 and Th1 associated cytokine IFNγ. The infection at ZT12 did not render all mice fully susceptible, as a majority of the mice still expelled their worm burden by day 35. ZT0 infected mice produced a stronger Th2 response and most mice cleared their worm burden by day 28. Typically 1 in 8 C57BL/6 mice develop a chronic infection under normal lab conditions (Else, 1988). This ratio appeared to rise to 1 in 3 when mice were infected at ZT12 rather than ZT0.

The delay in worm expulsion seen in mice infected at ZT12 is not likely due to the 12h delay in infection, which occurs when mice are infected 12 hours apart (ZT0 to ZT12) but sacrificed at one time point. Thus when groups of C57BL/6 mice are infected 12 hours apart, but with the first infection at ZT12 rather than ZT0, mice infected at ZT12 did not show reduced worm burdens compared to mice infected at ZT0, despite being infected for 12 hours longer. Nonetheless mice infected at ZT12 also did not show a delay in worm expulsion. Furthermore Th2 cytokines expressed by MLN re-stimulated cells recovered from mice infected at ZT0 or ZT12 were not significantly different; reversing the order of infection did not enhance Th2 responses in ZT12 infected mice. As results were inconclusive as to whether that the delay in worm expulsion seen in mice infected at ZT12 simply reflects a 12 hour longer exposure to worms, late experiments involved sacrificing mice at ZT0 and ZT12 thus keeping worm exposure equal.

A positive correlation between IgG2c antibody levels and worm burden has previously been described (Else *et al.*, 1993) and these levels of IgG2c are indicative of persisting worms. The polarization of the ZT0 infected mice response towards Th2 is evidenced by low worm burdens, high IgG1 levels and high Th2 cytokine levels. ZT12 infected mice are polarized towards Th1 responses, with higher worm burdens, higher IgG2c and high Th1 cytokine levels. Th2 type antibody levels increase later during infection; by day 28 IgG1 levels rise, which might show a change from Th1 towards Th2, leading to the mice being able to expel their worm burden after 4, rather than 3 weeks. IL-4 and IL-13 cytokine levels also increase by day 28.
High levels of Th2 associated cytokines are linked to expulsion of *T. muris*

Th2 type cytokines IL-4 and IL-13, as well as IL-5 are associated with the expulsion of *T. muris* worm burden. Deficiencies in IL-4 and IL-13 have been found to lead to susceptibility to *T. muris* infection (Bancroft *et al.*, 2000; 1998; Else *et al.*, 1994). Else *et al.* (1994) showed that blocking IL-4 receptors in resistant BALB/K mice renders them susceptible to *T. muris* infection, while the administration of IL-4 to susceptible AKR mice enables them to expel the parasite. Bancroft *et al.* (1998) described the immune response to *T. muris* in IL-13−/− and IL-4−/− mice. Both strains were susceptible to infection, compared to C57BL/6 mice, clearly stating the importance for IL-13 and IL-4 in *T. muris* infection. In ZT0 infected mice, IL-13 was significantly higher than that seen in ZT12 mice, while IL-4 and IL-5 were not different in ZT0 and ZT12 infected C57BL/6 mice. Thus, IL13 levels support the faster worm expulsion in ZT0 infected mice, with IL-13 only found at low levels in ZT12 infected mice.

IL-10 is produced by many cell types including Th1, Th2, B cells, Treg cells, macrophages, dendritic cells and eosinophils (O’Garra & Vieira, 2007). IL-10 is generally associated with resistance to *T. muris*, as infected IL10−/− mice show higher susceptibility and mortality with increased IFNγ levels (Schopf *et al.*, 2002). In the case of ZT0 and ZT12 infected mice, IL-10 levels were not significantly different. It is likely that the levels of IL-13 and IL-4 are most important in expulsion of *T. muris* than the presence of IL-10.

TNFα has been linked to resistance against *T. muris*, even though generally classed as a pro-inflammatory cytokine (Artis *et al.*, 1999b; Hayes *et al.*, 2007b). Artis *et al.* (1999b) and Hayes *et al.* (2007b) blocked TNFα in C57BL/6 mice, which led to delayed worm expulsion. MLN cell supernatants from mice infected at ZT12 contained significantly higher levels of TNFα compared to those from mice infected at ZT0, perhaps reflecting the on-going inflammatory response in mice where worms were persisting.

High levels of Th1 associated cytokines are linked to susceptibility to *T. muris* infection

IFNγ is found in high levels in susceptible mice (Else *et al.*, 1994; 1992b; Else & Grencis, 1991). In 1992, Else *et al.* showed that in mesenteric lymph node cultures of susceptible B10.BR and AKR mice higher levels of IFNγ could be measured than in cultures of mesenteric lymph nodes taken from resistant BALB/K mice. Depleted IFNγ in susceptible AkR and B10.BR mice, showed a reduction in IgG2c, the Th1 associated antibody, and led to expulsion of the *T. muris* (Else *et al.*, 1994), showing that IFNγ is important for the development of a chronic infection and the presence of IgG2c is an indicator of Th1 responses. In ZT12 infected mice, IFNγ levels are significantly higher than in ZT0 infected mice. The high levels of IFNγ found in ZT12 mice are another indicator for a shift in T cell response towards Th1 instead of Th2 when mice are infected at ZT12 rather than ZT0.
Levels of Th2 associated cytokines were low, while Th1 associated and pro-inflammatory cytokines dominated in ZT12 infected mice, which suggests that ZT12 infected mice are more polarized towards Th1 responses than ZT0 infected mice.

**Intestinal pathology did not differ between mice infected at ZT0 and ZT12**

Quantification of gut pathology in mice infected at ZT0 and ZT12 revealed no difference, despite enlarged crypt length and goblet cell hyperplasia being known signs of a *T. muris* infection and secretion of mucins aids in expulsion (Artis *et al.*, 1999a; Artis *et al.*, 2002; D’Elia *et al.*, 2009b; Hasnain *et al.*, 2010, 2011a,b). Goblet cell hyperplasia nonetheless is seen in susceptible as well as resistant mice (Artis *et al.*, 2004), which might explain why there was no quantitative difference in goblet cell hyperplasia between ZT0 and ZT12 infected mice.

The composition of the mucus barrier varies between resistant and susceptible animals. Muc2 secretion by goblet cells correlates with worm expulsion and is up-regulated in resistant mice. Secretion of Muc5ac, another critical mucin, is only found in resistant mice (Hasnain *et al.*, 2010). The thickness of the glycocalyx, the mucin defence layer found under the mucus layer is thicker in resistant mice compared to susceptible mice during *T. muris* infection, as *Muc4, Muc13* and *Muc17* were up-regulated in resistant mice, leading to more glycoproteins in the mucus barrier (Hasnain *et al.*, 2011a,b). These glycoproteins were diminished in chronic infections (Hasnain *et al.*, 2011a,b). Mucins in the mucus barrier of chronic infected mice also appear less charged than in resistant mice (Hasnain *et al.*, 2011a, b). It has been established that *T. muris* secretes Serine proteases, which are able to depolymerise Muc2, leading to a more porous mucus barrier. In resistant mice, serine protease inhibitors are up-regulated, preventing the loss of Muc2, which is not seen in susceptible mice (Hasnain *et al.*, 2012).

As sheer numbers of goblet cells are the same in ZT0 and ZT12 infected mice, further research could look into the expression of Muc5ac or Muc2 by these cells, to evaluate if the composition of mucus secreted changes depending on the time of infection as for example, deficiency in Muc2 leads to delayed worm expulsion (Hasnain *et al.*, 2010). Mucus composition depends on the presence of Th1 or Th2 milieu. Th2 cytokines have been described to control goblet cell differentiation and mucin production (Kondo *et al.*, 2002; Taube *et al.*, 2002; Whittaker *et al.*, 2002). As more Th2 type cytokines are produced later during infection when ZT12 infected mice start to expel, changes in goblet cell mucin production could aid expulsion later on.

Muscle wall thickness is often used as a measure of pathology. The muscle wall thickness was very variable, especially depending on the amount of stool found in the section. Overall there appeared to be no difference in muscle wall thickness, which is assumed to be under IL-9 regulation (Khan *et al.*, 2003). IL-9 levels (not shown) were not different between ZT0 and ZT12 infected mice, and in most experiments were too low to detect. IL-9 is associated with expulsion of *T. muris* and overproduction of IL-9 in IL-9 transgenic mice leads to quick expulsion of the
parasite (Faulkner et al., 1998), but in the context of these experiments the levels of IL-9 were below the detectable threshold.

Epithelial cell turnover is increased as a response to *T. muris* infection. Rapid epithelial cell turnover, up-regulated post infection, can reduce worm burden, by shedding the parasite into the lumen. This forces the parasite to burrow further along the epithelial cell layer in order to maintain a status quo. Cliffe *et al.* (2005) established that epithelial cell turnover is under IL-13 control, which is significantly up-regulated in infected mice. In the context of ZT0 or ZT12 infected mice, proliferation was not analysed. BrDU, which is incorporated into dividing cells, could be used in future experiments to analyse if the higher level of IL-13 in ZT0 infected mice leads to a higher rate of epithelial cell turnover.

In naïve mice, pathology analysis revealed significantly longer crypts in mice culled at ZT12 compared to mice culled at ZT0. Crypts elongate when stem cells at the bottom of the crypt increase proliferation due to the presence of IFNγ, but the epithelial cell turnover, shedding old cells into the lumen to push the worms out of the epithelial lining does not increase (Artis *et al.*; 1999a; Cliffe *et al.*, 2005). The difference in crypt length of naïve mice was very surprising, as no IFNγ levels are expected in naïve mice and long crypts are an indicator of crypt hyperplasia, which is associated with helminth infection and not with steady-state colon. Furthermore, during infection, even as early as day 1 post infection, there was no visible difference in crypt length between ZT0 and ZT12 infected mice.

**Cell infiltration into the large intestine is similar in ZT0 and ZT12 infected C57BL/6 mice**

At day 21 and day 28, numbers of infiltrating lymphocytes did not show a significant difference between ZT0 and ZT12 infected mice. Macrophages and dendritic cell were also found in similar numbers.

To ensure there is no initial variation in cell number at the time of infection, naïve C57BL/6 mice were analysed. Cruickshank *et al.* (2009) showed that resistant mice had higher numbers of dendritic cells present in the intestine of resistant BALB/c mice compared to susceptible AKR mice under resting steady state conditions. At naïve levels, there is no difference in antigen presenting cell numbers between the ZT0 and ZT12 infected C57BL/6 mice. Analysing macrophage and dendritic cell numbers in colon tissue by immunohistochemistry and also flow cytometry did not show any differences in quantity. This does not exclude a functional difference in macrophages and dendritic cells in naïve mice sacrificed at ZT0 or ZT12.

**Expulsion of *T. muris* relies on T cell polarisation early in infection**

As the cellular events which underlie T cell polarization occur at the time of T cell priming it is likely that the important rhythmic factors driving the time of day differences in immune responses occur after infection, even though equal numbers of worms establish in mice infected at ZT0 compared to ZT12.
Early events post infection were measured in mice infected at ZT0 and ZT12 and culled on day 1, 2 or day 3 post infection. No differences in infiltrating leukocytes, macrophages or dendritic cells were identified by Flow cytometry. Therefore one can conclude that circadian variation in cell numbers present in the gut at time of infection is not the reason for delayed expulsion. A circadian rhythm inside these immune cells, regulating chemokine and cytokine production is nonetheless a possibility. Measuring intracellular cytokine production of Th2 and Th1 cytokines via flow cytometry might be a possible way to determine if the function of cells found in tissue is under circadian control.

One possibility to explain the delay in worm expulsion is the time it takes to mount the adaptive immune response and activate T cells. Rhythmic expression of clock genes has been found in antigen presenting cells, T cells and even some types of epithelial cells (Arjona & Sarkar, 2008; Fortier et al., 2011; Hayashi et al., 2007; Silver et al., 2012b; Suzuki et al., 1997) all of which could impact on the immune response during T. muris infection.

As circadian rhythm could be found in many cells involved in the priming of the immune response to T. muris, several candidates for the mechanism causing delayed worm expulsion in ZT12 infected mice could be suggested. Dendritic cells present antigen in the lymph node to activate T cells. Epithelial cells secrete cytokines and chemokines causing lymphocyte infiltration to the gut and circadian expression of these could lead to a delayed recruitment. T cells on the other hand are effector cells required for T. muris expulsion. Circadian rhythm in these cells could regulate cytokine production and activation, for example by the diurnal expression of co-stimulatory receptors.

**Dendritic cells prime T cell responses**

The speed of dendritic cell recruitment towards the gut epithelial cells has been implicated in resistance to T. muris (Cruickshank et al., 2009). Resistant mouse strains showed faster recruitment of dendritic cells towards the colon epithelial cells and higher levels of chemokine CCL5 and CCL20 production, measured by RT-PCR, increased post infection in the colon of resistant, but not in susceptible animals.

In susceptible BALB/c mice infected with a low dose of T. muris, dendritic cell numbers in colon tissue did not increase post infection, compared to the increase in cell infiltration found in BALB/c mice infected with a high dose of T. muris eggs, leading to the assumption that dendritic cell homing to the gut is mediated by the immune response rather than the strain of mouse (Cruickshank et al., 2009). Maturation of dendritic cells was nonetheless seen in susceptible, as well as resistant mice.

Given that dendritic cell recruitment to the gut epithelial cell layer is delayed in susceptible compared to resistant mice, it is likely that dendritic cell rhythm is involved in the delayed immune response in mice infected with a high dose of T. muris eggs at ZT12 compared to ZT0.
Epithelial chemokine production could be under circadian control

Thymic stromal lymphopietin (TSLP) is an IL-7 like cytokine, which is mainly produced by intestinal epithelial cells (Massacand et al., 2009; Taylor et al., 2009). TSLP expression increased in resistant and susceptible BALB/c mice at day 1 post infection, but reduced again by day 7 in susceptible BALB/c mice, while remaining high resistant BALB/c. In susceptible AKR mice however, TSLP levels decreased from day 1 post infection (Cruickshank et al., 2009).

For a strong Th2 response against T. muris infection, TSLP-TSLPR interactions are essential. TSLP does not directly affect Th2 cell differentiation but inhibits IL-12p40 production by epithelial cells and dendritic cells, leading to enhanced Th2 responses (Massacand et al., 2009; Taylor et al., 2009). A transgenic mouse model lacking TSLPR is susceptible to T. muris infection. Massacand et al. (2009) found higher levels of IL-12p40 in TSLPR−/− mice, which could be the reason for susceptibility to the parasite. According to Taylor et al. (2009) not only elevated pro-inflammatory cytokines, but also defective expression of Th2 cytokines lead to susceptibility. Neutralisation of IFNγ in TSLPR−/− mice restored Th2 cytokine levels and thereby worm expulsion, which leads to the conclusion that TSLP might not be essential for Th2 cytokine production in the intestine in the absence of IFNγ (Taylor et al., 2009).

TSLP expression is regulated by NFκB, which is under circadian control (Hayashi et al., 2007; Taylor et al., 2009). Hayashi et al. (2007) showed that knockdown of Bmal1 in RAW264.7 cells showed a reduction in NFκB activity, which suggested that clock genes regulate NFκB activity. NFκB is essential for helminth infection, as it regulates cytokine expression promoting Th1 and Th2 responses (Artis et al., 2002), therefore clock-gene controlled NFκB signalling could be a possible mechanism pushing ZT12 infected mice towards a Th1 immune response.

Another chemokine associated with T. muris expulsion is CC chemokine ligand 2 (CCL2), which recruits activated T cells, NK T cells, basophils and monocytes/macrophages. Mice resistant to T. muris secrete almost 5-fold higher levels of CCL2 after infection than susceptible mice, while mice deficient in CCL2 are unable to clear their worm load. Susceptible mice produce high levels of CCL3 instead, while resistant mice only showed a slight increase in the chemokine (deSchoolmeester et al., 2003). Chemokine expression by epithelial cells at ZT0 and ZT12 might therefore be worth evaluating in the future.

Circadian rhythm has been identified in T cells

Peripheral CD4+ T cell numbers vary according to the time of day. In humans, total numbers of CD4+ and CD8+ T cell numbers are lowest at 2pm in the afternoon, and peak early in the morning when analysed by Flow cytometry, while fully differentiated CD8+ effector T cells peak during the day time (Dimitrov et al., 2009). Suzuki et al. (1997) described the peak of CD8+ T cells in the daytime, while CD4+ T cells and T cells in general peaked during the night. Rhythmic clock gene expression was measured by Bollinger et al. (2011) in enriched ex vivo human T cells over 24 hours. After stimulation in vitro, T cells expressed IL-2, IL-4 and IFNγ in a rhythmic
An intrinsic rhythm in isolated T cells (Bollinger et al., 2011), suggests that T cells are not dependent on external time cues, for example from corticosterone, but able to generate their own circadian rhythm.

In mice, CD4+ T cells and CD8+ T cells extracted from lymph nodes proliferate faster in culture with anti-CD3, when lymph nodes were harvested late in the evening, compared to the morning. CD4+ and CD8+ T cell numbers in lymph nodes appeared to be stable over the course of the day, as opposed to the circadian variation observed in blood (Fortier et al., 2011). Vaccinating mice with OVA-loaded dendritic cells at ZT6 or ZT18 resulted in a higher fold expansion in ZT6 vaccinated mice compared to ZT18 vaccinated mice, which suggests that the circadian response of T cells is not depending on circadian rhythm in antigen presenting cells, but intrinsic to T cells (Fortier et al., 2011). The ability of T cells to respond differently to antigen presenting cells depending on the time of day could be the cause for Th2 polarisation at ZT0 and Th1 polarization at ZT12. Fortier et al. (2011) analysed key molecules downstream of the T cell receptor, establishing a circadian expression of the cytosolic tyrosine kinase ZAP70, which could be under circadian T cell clock control.

It is unclear if T cells themselves contain the rhythm that causes this difference in response to antigens, or if the circadian element lies in the antigen presenting cell or the gut epithelial cell secretion which influences DC biology.

Taken together, circadian rhythm in dendritic cells, but also T cells or epithelial cells could be the mechanism causing delayed worm expulsion in C57BL/6 mice infected at ZT12 compared ZT0.

**Corticosterone levels have been shown to influence several parasitic infection models**

A possible circadian regulator influencing immune cells is corticosterone. Corticosterone expression in mice and cortisol expressions in humans is rhythmic over the day (Born et al., 1997; Kalsbeek et al., 2012; Malisch et al., 2008). Cortisol rhythms negatively correlate with naïve, memory and effector memory CD4+ and CD8+ T cell numbers peaking during the night, while effector CD8+ T cells numbers peak during day time and positively correlate to ephedrine levels (Dimitrov et al., 2009). Dimitrov et al. (2009) showed that the up-regulation of CXCR4 in naïve and central memory CD4+ and CD8+ T cells is important for cortisol-dependent control of circadian rhythm, which enhances redistribution of T cells to the bone marrow in the early morning. Th2 cell responses are associated with high levels of cortisol (Dimitrov et al., 2009). The impact of cortisol on dendritic cells or epithelial cells is still unclear.

Nonetheless corticosterone can affect the immune response to parasite infection. Corticosterone is a steroid with anti-inflammatory properties, which in mice with chronically elevated levels of corticosterone, reduces immune responses. This has been observed in the context of infection with the helminth *Nippostrongylus brasiliensis* (Malisch et al., 2009). As with most helminths, a strong Th2 response is required for the expulsion of this parasite. The
presence of corticosterone reduced Nippostrongylus brasiliensis expulsion rates, inhibited cytokine responses and blocked T cell maturation (McEwen et al., 1997; Sapolsky et al., 2000), with a positive correlation between parasite infection and corticosterone levels (Malisch et al. 2009, 2008, 2007; Faulkner et al. 1998). Faulkner et al. (1998) used corticosterone administration to ensure T. muris parasite survival, as high levels of corticosterone suppress Th2 responses. Mice infected at ZT12 had higher levels of corticosterone at the point of infection and mounted a stronger Th1 response, leading to the speculation that the level of corticosterone at the point of infection could be part of a mechanism by which the CD4+ T cells polarize towards a Th1 or a Th2 response according to time of day of infection, as high levels of corticosterone at the point of infection coincide with delayed worm expulsion. As it is assumed that corticosterone levels oscillate, elevated corticosterone levels could only have an impact in the first hours of infection.

Corticosterone levels in ZT12 infected mice were measured at ZT12 and decreased over the infection with T. muris, leading to the hypothesis that a reduction in corticosterone later during the infection could enable the generation of a Th2 response and allow the host to expel the worm burden, although with delayed kinetics. Corticosterone levels in ZT0 infected mice, sacrificed at ZT0 were low throughout the time of infection, but the corticosterone level of ZT0 infected mice was not evaluated at ZT12 at this time. Measuring the corticosterone levels of ZT12 infected mice at ZT0 and of ZT0 infected mice at ZT12 would allow analysis of the corticosterone rhythm and whether it is dampened over the course of infection.

The interaction of other parasites with their host’s circadian rhythm has been explored. The causative agent of sleeping sickness, Trypanosoma brucei brucei appears to be able to influence the clock function of the host and disrupt endocrine signals, which cause some of the symptoms associated with the disease (Lundkvist et al., 2002; 2010; Grassi-Zucconi et al., 1995; Brandenberger et al., 1996), and thereby ensure its survival. Malaria on the other hand has been shown to adapt to the host’s circadian behaviour, being able to synchronise its own biology to the host in order to survive (Hawking, 1970; O'Donnell et al., 2011; Reece et al., 2009). Melatonin levels in serum might be used by the parasite to gauge circadian rhythm of the host (Hotta et al., 2000). Both protozoan parasites have adapted to their niche inside the mammalian host by adapting to their hosts circadian changes. Helminth parasites are less likely directly affected by the host’s circadian rhythm, as they reside inside the intestinal tract and not within the circulation. Overall, corticosterone cannot be discounted as an influence on worm expulsion.

The data gathered in this chapter suggests that circadian rhythm does have an impact on the immune response to T. muris infection and leads to a delay in worm expulsion in ZT12 infected mice. Nonetheless, the ZT12/ZT0 experiment was inconclusive and large animal numbers were needed for the study. Further research has to be carried out to understand the mechanisms underlying the difference in polarization of T cells in ZT0 and ZT12 infected mice.
Summary

- ZT12 infected mice have exhibited delayed expulsion of *T. muris* and a Th1 dominated immune response.

- ZT0 infected mice make a strong Th2 response and expel the parasite faster than mice infected at ZT12.

- Measures of pathology and infiltrating cells in colon tissue showed no differences between mice infected at ZT0 or ZT12 on day 21 and day 28.

- Measures of pathology and infiltrating cells in colon tissue showed no difference in naïve mice at ZT0 or ZT12 and on day 1-3 of ZT0 or ZT12 infected mice.

- Corticosterone levels measured at ZT12 are reduced in ZT12 infected mice over time of infection, while corticosterone levels measured at ZT0 in ZT0 infected mice remained low throughout the course of infection.
Chapter 4:
Immune response manipulation: Low dose infection, vaccination and food restriction
Introduction
The quality of immune response to *T. muris* can be manipulated using low dose infection and vaccination. Low dose infection in C57BL/6 mice pushes the immune system towards a Th1 dominated response, whilst vaccination will induce a more Th2 dominated immune response. This chapter uses low dose infection and vaccination in order to explore the mechanisms underlying the delayed worm expulsion observed in ZT12 infected mice in chapter 3.

Circadian rhythm is generally described to be re-set daily by light via the retina. The light signal entrains the SCN and this in turns entrains peripheral tissues. Nonetheless light is not the only factor entraining peripheral tissues. Food is a strong entrainment factor in the digestive system and liver. When restricting food availability, the digestive system is de-coupled from the central circadian oscillator in the SCN and mice display strong food anticipation (Damiola 2000; Le Minh et al., 2001; Takasu et al., 2012). The rhythmic expression of clock genes in colon tissue is therefore subject to feeding habits and thus the rhythm can be reversed by food restriction to the light or dark phase. This allows examination of the importance of the clock gene expression in intestinal tissue during infection with *T. muris* and how early circadian rhythm influences priming of the immune response, as mice will be only food restricted during the first 7 days of infection.

**Low dose infection with Trichuris muris of C57BL/6 leads to strong Th1 responses**

One of the advantages of working with C57BL/6 mice is the dose dependent expulsion phenotype when infecting with *T. muris*. The resistant phenotype was explored in chapter 3, where C57BL/6 mice were infected with a high dose of 200 *T. muris* eggs. High dose infected C57BL/6 mice produce a mixed Th1/Th2 response, where a strong Th2 response is required for worm expulsion.

A susceptible phenotype is established in C57BL/6 mice when mice are infected with a low dose of about 30-40 *T. muris* eggs (Bancroft et al., 2001; deSchoolmeester & Else, 2002). When given a low dose, C57BL/6 mice initiate a strong Th1 response, which prohibits worm expulsion. No Th2 response is found in low dose infection. By day 42 *T. muris* are present and are able to reproduce. By this point, the infection has become chronic and will only be removed using anti-helminthic drugs.

Circadian rhythm impacts on the immune response to a high dose of *T. muris* (chapter 3); where ZT12 infection polarizes towards a more Th1-like immune response, while ZT0 infection polarizes towards Th2 immune responses. In low dose infections, the immune response is Th1 dominated. By infecting mice with a low dose infection at ZT0 or ZT12, it is possible to establish if Th1 responses can be driven towards a Th2 response when the infection is given at ZT0 and/or a ZT12 low dose infection leads to a more robust Th1 response. Worm numbers were quantified at day 42, when worms reach adulthood. Gut pathology was assessed and MLN
cytokine responses analysed to establish if there is a circadian element influencing the immune response to a low dose *T. muris* infection.

**Vaccination with *Trichuris muris* excretory/secretory antigen primes strong Th2 responses**

Vaccines against helminth parasites, which have been developed so far for livestock, are mostly based on antibody-reactivity (Maizels *et al.*, 1999). The importance of B cells and antibodies in helminth infection varies between parasites; therefore no single vaccine approach will work for all species of helminths. The role of antibodies in the immune response to *T. muris* infection is still controversial; therefore any vaccine should be designed to promote T cell as well as B cell memory.

T cells are the main effector cell in *T. muris* infection. Else and Grencis (1996) showed that adoptive transfer of CD4+ T cells alone enables mice to expel their worm burden. Nonetheless, absence of B cells led the T cell response to be skewed towards Th1 rather than Th2 and worm burdens were significantly higher at day 35 (Blackwell & Else, 2001). Although no vaccine against any of the human helminths exists, the development of a commercially available *Trichuris trichuria* vaccine would address the rising problems of drug resistant parasite populations and high reinfection rates post drug treatment.

Petrovsky & Harrison (1997) suggested that time of day of vaccination could play a role in efficacy, due to diurnal rhythms in cortisol and melatonin levels, influencing Th1/Th2 type cytokine production. As early as 1967, it has been shown that some vaccines do show diurnal differences (Feigin *et al.*, 1967; Langlois *et al.*, 1995; Phillips *et al.*, 2008; Pollmann & Pollmann 1988). For example, vaccination with attenuated Venezuelan equine encephalomyelitis vaccine showed elevated antibody titres earlier when vaccinated at 8 am rather than 8 pm (Feigin *et al.*, 1967) and vaccination against Influenza A/Philippines strain proved most effective when given between 11 am and 1 pm (Langlois *et al.*, 1995). Taken together, vaccination efficacy against *T. muris* could be dependent on the time of administration.

The administration of *T. muris* excretory/secretory (E/S) antigen in incomplete Freund's adjuvant, enables susceptible mouse strains to expel the worm burden given (Dixon *et al.*, 2010) by priming a Th2 immune response and thereby allowing mouse strains susceptible to a primary infection to expel their worm burden.

Vaccine efficacy is dose-dependent and100μg O/N E/S has been shown to effectively protect mice against *T. muris* infection (Dixon *et al.*, 2010). C57BL/6 mice are susceptible to a low dose infection, but can be made resistant if vaccinated with E/S antigen. In the context of a high dose infection, vaccination accelerates the expulsion rate when infective eggs are administered 10 days post vaccination. Vaccinated C57BL/6 mice infected with a high dose of *T. muris* eggs expel their worm burden rapidly by day 14, compared to sham vaccinated mice where worm expulsion occurred around day 21.
In this study, C57BL/6 mice were vaccinated with 100μg O/N E/S in Freund's incomplete adjuvant or PBS in Freund's incomplete adjuvant at ZT0 or ZT12. At day 10 post vaccination, proliferation of - and cytokine production by peripheral lymph node cells were measured in a very small group of animals to establish if vaccination was successful in priming a Th2 response. Other groups of vaccinated mice were infected with a high dose of *T. muris* eggs at ZT3. At day 14 post infection, worm burden, antibody production, cytokine production as well as gut pathology measurements and immunohistochemistry on colon tissue were analysed.

**Food restriction changes clock gene expression and could impact on immune responses to *T. muris***

Despite the equal establishment of worm burden in ZT0 and ZT12 infected mice, shown in Chapter 3, food could play a role in the delayed expulsion of the worm burden as colon functions are diurnal and can be influenced by restricted feeding and changes in clock gene expression (Hoogerwerf 2010; Malloy *et al.*, 2012; Soták *et al.*, 2011).

To eliminate food anticipation at the site of infection as a factor in circadian entrainment of the immune response to a high dose of *T. muris*, C57BL/6 mice were food entrained to mid-light and mid-dark phase for 2 weeks before infection at ZT0 and a subsequent week of food entraining during the first seven days of infection. This reversed normal feeding rhythm in mice food restricted to mid-light phase. Before infection, clock gene expression in colon tissue was measured using RT-PCR. The level of corticosterone in serum was quantified at the day of infection as well to establish if the corticosterone rhythm was reversed. This establishes if the colon clock is reversed at the time of infection. Food restricted mice were infected at ZT0, when mice should be able to expel the worm burden quickly, unless the food restriction influences the ability of the immune system to respond to the high dose *T. muris* infection. Intestinal clock genes take about 2 days to align again with the SCN when returned to ad lib. conditions after food reversal (Mistlberger 2011).

At day 21 post infection with a high dose of *T. muris* eggs, worm burden, antibody production, cytokine production as well as pathology measurements and immunohistochemistry on colon tissue were analysed.

**Aims:**

- To establish if circadian rhythm influences the immune response to a low dose infection.
- To evaluate if vaccination with 100μg O/N E/S at ZT0 or ZT12 results in better protection against a high dose *T. muris* infection given at ZT3.
- To investigate the role of food in *T. muris* infection, food availability was restricted to mid-light or mid-dark prior and during the first week of ZT0 *T. muris* high dose infection.
Results:

4.1. Circadian rhythm does not impact on the outcome of a low dose infection of C57BL/6 mice or the establishment of a Th1 response

To establish if the delayed expulsion and stronger Th1 immune response in C57/BL6 mice infected at ZT12 was only visible when mice were infected with a high dose, C57/BL6 mice were infected with a low dose of 30-40 eggs. Mice were sacrificed at day 42, when worms should be at the adult stage.

The worm burden at day 42 was equal between mice infected at ZT0 and ZT12 (Fig. 1A). Pathological analyses of muscle cell wall thickness, crypt length and goblet cell numbers showed no significant difference between mice infected at ZT0 and ZT12 (Fig. 1B and C). Cytokine expression, measured in the supernatant of re-stimulated mesenteric lymph node cells using ELISA, showed no significant differences for Th2 cytokines IL-13, IL-4 and IL5, anti-inflammatory cytokine IL-10 or the Th1 cytokine IFNγ and pro-inflammatory cytokine TNFα (Fig. 1E). There appears to be no difference in infection outcome or the quality of the immune response between mice infected at ZT0 and ZT12 when infected with a low dose.

The results indicate that a low-dose infection, which in C57BL/6 mice results in a Th1 response and no expulsion, is not influenced by the time of day of infection at ZT0 or ZT12.
Figure 1: Analysis of immune response to infection of mice given a low dose infection of 30 eggs at ZT0 or ZT12. Mice were sacrificed at day 42, when worms were mature. A) Worm burdens were counted in the caecum and colon. Pathology measures (B-D) were taken from wax embedded, PAS stained proximal colon tissue B) Muscle wall thickness C) Crypt length D) Goblet cell numbers. E) Cytokine profile. 5x10^6 MLN cells/ml, isolated from mice sacrificed at ZT0 and ZT12, were stimulated with 50µg/ml of E/S and harvested after 48h. The supernatant was analysed using Cytokine Elisa. Th2 associated cytokines are IL-4, IL-5 and IL-13 and Th1 associated cytokine is IFN-γ and proinflammatory cytokine IL-6. The symbols represent individual mice; histograms show mean +/-SEM. F) Representative photographs of mucosal colon tissue using PAS staining. Phototgraphs were taken at 400x magnification, scale bars represent 100µm. Statistics: Mann-Whitney U-test, n=6.
4.2. Vaccination

Vaccinating mice with excretory/secretory antigen from *T. muris* can help prevent chronic infection in susceptible mice and enhance expulsion in resistant mice (Dixon *et al.*, 2010; Robinson *et al.*, 1995; Wakelin & Selby, 1973). To establish if the time of vaccination could influence the immune response when challenged, mice were vaccinated at ZT0 or ZT12 with 100μg O/N E/S in incomplete Freund’s adjuvant, a dose which has been shown by Dixon *et al.* (2010) to provide protection against *T. muris* infection. The time points for vaccination were chosen to keep in line with other infection experiments. Control mice were vaccinated at ZT0 or ZT12 with PBS in incomplete Freund’s adjuvant. At day 10 post vaccination, uninfected mice were sacrificed at ZT0 and ZT12 to establish the proliferation in the local peripheral lymph nodes (PLN) due to the vaccination as well as the ability of peripheral lymph node cells to promote an antibody response when stimulated with *T. muris* antigen.

Two further groups of 6 C57BL/6 mice, one vaccinated at ZT0, one at ZT12, were infected at day 10 post vaccination at ZT3 with 200 eggs of *T. muris* and sacrificed at day 14 post infection to establish if the vaccination was successful at both time points and impaired the progress of the infection to the same extent. Mice infected at ZT3 have previously been shown to have a resistant phenotype, therefore this time point was suitable to establish if expulsion is accelerated post vaccination. The experimental time table can be seen in Figure 2. Sham vaccinated mice were not expected to expel the worm burden by day 14. Worm burden, antibody production and cytokine responses were assessed, as well as pathology and infiltrating cells measured within gut tissue.

![Experimental time table](image_url)

**Figure 2. Schematic of the vaccine experiments.** Mice were vaccinated at ZT0 or ZT12. After 10 days one group was removed from the study to establish whether the vaccination was successful. A second cohort was infected at ZT3, which were culled after 14 days.
4.2.1. Vaccination of C57BL/6 mice with 100μg O/N E/S led to the priming of a Th2 response at day 10 post vaccination

At day 10 post vaccination, uninfected mice vaccinated at ZT0 had no significantly higher cell number in peripheral lymph nodes compared to mice vaccinated at ZT12 (Fig. 3A), there also was no difference in cell number compared to sham vaccination at ZT0 and ZT12. Antibody production was measured in serum samples of ZT0 and ZT12 sham vaccinated and vaccinated mice using serial dilution. Dilution 320 was selected as it fell on the linear part of the dilution curve. Mice vaccinated at ZT0 or ZT12 mounted a strong Th2 associated IgG1 antibody response, while only a small antibody response was seen in sham vaccinated mice (Fig. 3B). Vaccinated mice also produced some Th1 associated IgG2c, but levels were almost as low as in sham vaccinated animals (Fig. 3C). These results indicate that the vaccination was successful, independent of the time of vaccination. Levels of IgGs were not measured previously as early as day 14 and even though vaccinated mice expelled the worm burden, antibody levels were lower this early in the time course compared to ZT0 and ZT12 infected mice in chapter 3.

Ten days following vaccination, cytokine levels were measured in supernatants of re-stimulated peripheral lymph node cell cultures, to establish if the vaccine was able to prime the immune response against T. muris antigen. No differences in cytokine production could be observed between ZT0 and ZT12 vaccinated and sham vaccinated mice (Fig.4A, B, D-G). As the groups of mice were very small, any differences in cytokine levels in ZT0 vaccinated mice did not reach significance. Only IL-13 levels were significantly upregulated in vaccinated ZT0 mice from sham vaccinated mice at ZT0 (Fig.4C). It is unclear, why sham vaccinated mice produced pro-
inflammatory IL-6 and TNFα as well as IFNγ cytokines at all (Fig.E-G), possibly it was a reaction to the injection, rather than the vaccine. The levels were not different from vaccinated mice.

Figure 4: Uninfected C57BL/6 mice vaccinated at ZT0 with *T. muris* antigen produced similar levels of cytokines in both mice vaccinated at ZT12 with *T. muris* antigen and mice given PBS at ZT0 or ZT12 measured at day 10 post vaccination. 5x10⁶ MLN cells/ml, isolated from mice sacrificed at ZT0, were stimulated with 50µg/ml of E/S and harvested after 48h. The supernatant was analysed using cytometric bead array CBA. Th2 associated cytokines are IL-4 (A), IL-5 (B) and IL-13 (C), anti-inflammatory cytokine IL-10 (D), the Th1 associated cytokine is IFNγ (E) and proinflammatory cytokine IL-6 (F) and TNFα (G). The symbols represent individual mice; histograms show mean +/- SEM. Statistics: Kruskal-Wallis test, Dunn post hoc, n=3 mice per group.
4.2.2. Vaccinated mice were able to expel their worm burden by day 14 post infection, while sham vaccinated mice retained their worm burden, however there was no difference between mice receiving the vaccine at ZT0 versus ZT12

Worm burden assessment at day 14 post infection showed the vaccination was successful, as most worms were expelled in both vaccinated groups, while sham vaccinated mice still harboured worms (Fig. 5A). There was no difference between ZT0 and ZT12 vaccinated mice. By day 14 no worm expulsion was expected to be seen in sham vaccinated mice, as previously seen in ZT0 and ZT12 infected mice (Chapter 3, Fig. 2). No difference in PLN-cell numbers was observed between sham and vaccinated mice both at ZT0 or ZT12 (Fig. 5B). The same was found in MLN cell numbers, there was no difference observed between groups (Fig. 5C), as only 1 mouse in the ZT0 sham vaccinated group reached very high cell numbers.

![Graph A: Worm burden comparison](image1.png)

![Graph B: PLN cell numbers comparison](image2.png)

![Graph C: MLN cell numbers comparison](image3.png)

![Graph D: IgG1 comparison](image4.png)

![Graph E: IgG2c comparison](image5.png)

Figure 5: Immune responses at day 14 post infection in C57BL/6 mice vaccinated at ZT0 or ZT12 with *T. muris* antigen or PBS as control, and infected at ZT3 on day 10 post vaccination. Mice were sacrificed at d14 post infection. A) Worm burden were quantified by counting worms found in caecum and colon. B and C) PLN and MLN cell numbers, measured using CasyTon®. D and E) parasite specific IgG1 and IgG2c production measured in serum, screened against parasite E/S antigen at 0.5μg/ml. The serum was serially diluted; the data shown is dilution 160 only, as it falls within the linear range. Data is presented as histograms expressing mean +/- SEM as well as symbols representing individual mice. Statistics: Kruskal-Wallis test, Dunn post hoc n=6.

By day 14 post infection, IgG1 levels were similarly high in both groups of vaccinated mice, but sham vaccinated mice stayed at very low levels (Fig. 5D). IgG2c levels were low in all four groups (Fig. 5E). There was no difference in antibody production between ZT0 and ZT12...
vaccinated mice, which reflects the equal expulsion of the worm burden in vaccinated mice. Overall vaccination with 100μg O/N *T. muris* antigen induced a strong Th2 response, which appeared to override any impact of circadian rhythm.

4.3.3. Cytokine levels at day 14 post infection in C57BL/6 mice showed no significant differences between ZT0 and ZT12 vaccinated mice

![Cytokine responses at day 14 infection in C57BL/6 mice vaccinated at ZT0 or ZT12 with *T. muris* antigen or PBS as control, infected at ZT3 on day 10 post vaccination. 5x10⁶ MLN cells/ml, isolated from mice sacrificed at ZT0, were stimulated with 50μg/ml of E/S and harvested after 48h. The supernatant was analysed using cytometric bead array CBA. Th2 associated cytokines are IL-4 (A), IL-5 (B) and IL-13 (C), anti-inflammatory cytokine IL-10 (D), the Th1 associated cytokine is IFN-γ (E) and proinflammatory cytokine IL-6 (F) and TNFα (G). The symbols represent individual mice; histograms show mean +/- SEM. Statistics: Kruskal-Wallis test, Dunn post hoc, n=3 mice per group.](image-url)
Cytokine profiles show that by day 14, when both groups of vaccinated mice had expelled most of their worm burden, levels of Th2 associated cytokines (IL-4, IL-5 and IL-13) were equally elevated. Levels of anti-inflammatory cytokine IL-10 were also similar in ZT0 and ZT12 vaccinated mice (Fig. 6A-D). Sham vaccinated mice made no quantifiable Th2 cytokine response by day 14.

Pro-inflammatory cytokine TNFα and Th1 associated cytokine IFNγ showed similar levels in both sham vaccinated and vaccinated mice (Fig. 6E and G). Pro-inflammatory cytokine TNFα levels were significantly elevated from sham vaccinated to vaccinated mice when the inoculation was given at ZT0, but showed equal levels in both vaccinated groups (Fig. 6E). These similar cytokine levels in vaccinated mice align with the worm expulsion and IgG1 levels found in vaccinated mice, indicating a strong Th2 response in both E/S vaccinated groups. Cytokine levels were further spread in this experiment at day 14, with some mice reaching higher levels than seen in Chapter 3, in ZT0 and ZT12 infected mice at day 21.

4.2.4. Muscle wall thickness, crypt length and goblet cell hyperplasia showed no significant differences between sham vaccinated and vaccinated mice

Figure 7: Pathological measurements on day 14 post infection following a 200 egg *T. muris* infection at ZT3 in mice vaccinated at ZT0 or ZT12 10 days prior to infection. A) Muscle wall thickness B) Crypt length C) Goblet cell numbers. Wax embedded tissue sections were stained with PAS staining and ImageJ software was used to measure muscle wall thickness and crypt length. The symbols represent individual mice and histograms show mean +/- SEM. D) Representative photographs of PAS staining. Goblet cells stain pink/purple. Photographs were taken at 400x magnification. Scale bars represent 100μm. Statistics: Kruskal-Wallis test, Dunn post hoc. n= 6 mice per group.

Pathological measurements on PAS stained wax embedded proximal colon tissue showed no difference in inflammation between groups. Muscle wall thickness (Fig. 7A) and crypt length
(Fig. 7B), as well as goblet cell numbers (Fig. 7C), were not significantly raised in vaccinated mice compared to sham vaccinated mice. Furthermore there were no differences between ZT0 and ZT12 vaccinated or ZT0 and ZT12 sham vaccinated mice.

4.2.5. No differences were measured in CD11c⁺, B220⁺ and CD45⁺ cell infiltrate between sham vaccinated and vaccinated mice.

Figure 8: Immunohistochemistry on proximal colon tissue stained for CD11c, B220 and CD45 at day 14 post infection following a 200 egg *T. muris* infection at ZT3 in mice vaccinated at ZT0 or ZT12 10 days prior to infection. A) CD11c positive cells representing dendritic cells B) B220 positive...
cells, representing B cells C) CD45 positive cells, representing infiltrating lymphocytes. The symbols represent individual mice, histogram shows mean +/- SEM. D-F) Representative photographs for CD11c staining (D), B220 staining (E), and CD45 staining (F) in the colonic mucosa of ZT3 infected animals 10 days post vaccination. Immunohistochemistry using CD45⁺, B220⁺ or CD11c⁺ monoclonal antibodies stains CD45⁺, F4/80⁺ or CD11c⁺ cells brown. F4/80 and CD11c positive cells were counted directly, for analysis of CD45 staining Image-Pro Plus software was used. Photographs were taken at 400x magnification. Statistics: Kruskal-Wallis test, Dunn post hoc, n=6 mice per group.

Cell numbers quantified by immunohistochemistry on frozen proximal colon tissue showed no differences for CD11c positive cells (Fig. 8A), representing dendritic cells, or B220 positive cells (Fig. 8B) representing B cells. There was no difference in total leukocyte infiltrate in both groups of vaccinated mice (Fig. 8C) as well as sham vaccinated mice, shown by CD45 staining.

Overall the results show that vaccination with 100μg O/N T. muris antigen led to early expulsion of a high dose of T. muris compared to sham vaccinated mice, independently of the time of vaccination.

4.3. Mice food restricted to feeding during the day or during the night

Food restriction to the light phase reverses the expression of clock genes in the gastrointestinal tract and liver. As many functions of the intestine are under circadian control (Hoogerwerf 2010; Malloy et al., 2012; Soták et al., 2011), one can assume that clock gene expression has an impact on the immune response in the colon tissue. By reversing the natural feeding time into the light phase, clock gene expression in the colon should be reversed as well, identifying if the difference in response to T. muris infection at ZT0 or ZT12 seen in chapter 3 is dependent on the timing of peripheral clocks in gut cells in the initial establishment of the immune response.

![Figure 9. Schematic of the food reversal experiment. Mice were started on food reversal for 2 weeks prior to infection ZT0. Half of the mice were fed during the daytime, while control mice were fed during the dark phase. Mice were continued for further 7 days on reversed feeding regimen before being returned to ad lib. feeding for the final 2 weeks of infection.](image)

4.3.1. Food restriction to 12 or 6 hours mid-light or mid-dark phase did reverse clock gene expression in colon tissue and corticosterone levels

Initially, C57BL/6 mice were given access to food for 12 hours during day or night over 12 days. Tissue was collected at autopsy and analysed by RT-PCR for the rhythmic expression of clock genes Bmal1 and Per2. As seen in Figure 10A and B, 12h food restriction to the light phase for
12 days is sufficient to reverse clock gene \textit{Bmal1} rhythmic expression in colon, but \textit{Per2} expression was not completely reversed.

Repeating this experiment, the time of food availability was reduced to 6h mid-light or mid-dark phase to further enhance the reversal of the clock before infection. \textit{Bmal1} clock gene expression appears reversed in mice restricted to 6h feeding, but this did not reach significance. RT-PCR for the clock gene \textit{Per2} in mice restricted to 6h showed no reversal of clock gene expression in the colon of mice forced to feed during the light phase (Fig. 10C and D).

\textbf{Figure 10:} RT-PCR RQ values of clock gene \textit{Per2} (A+C) and \textit{Bmal1} (B+D) expression in food restricted mice for 12h in light or dark phase (A+B) or restricted to 6h mid-light or mid-dark phase (C+D). RNA was extracted from proximal colon tissue and converted to cDNA. FAST BLUE qPCR MasterMix Plus dTTP kit (Eurogentec, Seraing, Belgium) was used for RT-PCR. RQ values were calculated for \textit{Bmal1} and \textit{Per2} RNA expression, normalising to night fed ZT0 culled animals. 12h restriction: ZT0 cull, am fed n= 6 mice per group, other groups n=7. 6h restriction: day fed n=6, night fed, ZT0 cull n=5, ZT12 cull n=4.
Corticosterone measurements in serum of mice restricted to 6h feeding mid-light or mid-dark phase showed that mice feeding at night showed a normal corticosterone rhythm, with high levels of corticosterone in mice culled at ZT0 and low levels of corticosterone in mice culled at ZT12 while mice forced to feed during the day show a similar concentration of corticosterone when culled at ZT0 or ZT12 (Fig. 11).

Figure 11: Corticosterone levels measured in serum of mice sacrificed at ZT0 or ZT12 after 12 days of food restriction to either 6h mid-light phase (day fed) or mid-dark phase (night fed). Sera collected after 14 days of food restriction at ZT0 or ZT12 for both groups of restriction were analysed for levels of corticosterone using the Corticosterone EA Kit (Enzo Life Sciences, Exeter, UK). Statistics: Bonferroni ANOVAs, Dunn post hoc ***p< 0.001. n=3 mice per group.

4.3.2. Food reversal prior to and during the first week of infection led to higher Th1 associated antibody production in day fed mice, resembling ZT12 infected C57BL/6 mice in previous experiments, which are more susceptible to infection

C57BL/6 mice infected at ZT0 during food restriction for 6h to either mid-light or mid-dark phase were returned to ad lib feeding 7 days post infection and sacrificed at day 21 post infection. Worm burdens were assessed by counting worms found in colon and caecum. The worm burdens were not significantly different (Fig.12A). Parasite specific Th2 type antibody IgG1 and Th1 type antibody IgG2c were measured by ELISA. The serum was serially diluted and from the linear proportion of the titration curve the dilution 1: 320 was selected as representative of antibody production. There was no difference in Th2 antibody production, as expected from experiments in Chapter3, but Th1 antibody IG2c levels were significantly higher in mice forced to feed during the day (Fig.12B and C). This higher production in IgG2c was seen by day 28 in C57BL/6 mice infected at ZT12 in previous experiments (Chapter 3).
4.3.3. Food reversal prior to and during the first week of infection did not result in higher Th2 cytokine levels in re-stimulated mesenteric lymph nodes of mice fed mid-dark phase.
Cytokine responses showed no clear reversal when mice were forced to feed mid-light or mid-dark phase. Th2 associated cytokine IL-13 (Fig. 13A) and anti-inflammatory cytokine IL-10 (Fig. 13C) were similar between day and night fed mice. Levels of IL-5 were significantly higher in day fed mice (Fig. 13B). For pro-inflammatory cytokines IL-6 (Fig. 13D) and TNFα (Fig. 13F) as well as Th1 cytokine IFNγ (Fig. 13E), levels were similar in both day and night fed mice. Cytokine levels were similarly high in Fig. 5 to those in ZT0/ZT12 infected mice, despite the lack in significant differences (Chapter 3, Fig. 5).

4.3.4. Muscle wall thickness, crypt length and goblet cell hyperplasia as well as CD11c+, F4/80+ and CD45+ cell infiltrate at the site of infection did not show any differences between mid-light and mid-dark fed mice

Pathological measurements on wax embedded colon tissue stained with PAS stain for goblet cells showed no differences in muscle wall thickness, crypt length or goblet cell numbers (Fig. 14). Cell infiltrate into the colon tissue showed no differences in mice forced to feed mid-light or mid-dark. There was no difference visible in frozen sections stained for the cell surface marker CD11c by immunohistochemistry (Fig. 15A), indicating that there is no difference in the number of dendritic cells present in the tissue on day 21 post infection. There also was no variation in F4/80 staining (Fig. 15B), representing mainly macrophages, but also eosinophils. The overall
lymphocyte infiltrate, measured by CD45 staining showed no differences between mice fed during the light phase or the dark phase (Fig. 15C).

Figure 15: Immunohistochemistry on proximal colon tissue stained for CD11c, F4/80 and CD45 on day 21 post infection at ZT0 during 6h food restriction to mid-light or mid-dark phase. A) CD11c positive cells B) F4/80 positive cells C) CD45 positive cells. The symbols represent individual mice, histogram shows mean +/- SEM. D-F) Representative photographs for CD11c staining (D), F4/80 staining (E), and CD45 staining (F) in the colonic mucosa of ZT0 infected animals during 6h food restriction to mid-light or mid-dark phase. Immunohistochemistry using CD45⁺, F4/80⁺ or CD11c⁺ monoclonal antibodies stains CD45⁺, F4/80⁺ or CD11c⁺ cells brown. F4/80 and CD11c positive cells were counted directly, for analysis of CD45 staining Image-Pro Plus software was used. Photographs were taken at 400x magnification. Scale bar represents 100μm. Statistics: Mann-Whitney U-test; n=13 mice per group.
Discussion:

In C57BL/6 mice, infection with high dose of *T. muris* induces a mixed Th1/Th2 response. A strong Th2 response is needed to expel the worm burden, while a strong Th1 response leads to chronic infection. The feeding status at the time of infection could also have an impact on the priming of the immune response, especially as under natural conditions *T. muris* infection would occur during feeding.

This chapter uses a Th1 dominated infection regime, a Th2 dominated vaccination model and food restriction to the mid-light phase during the first days of infection to examine whether the diurnal difference in the quality of immune responses to *T. muris* infection and resulting difference in expulsion kinetics can be manipulated by altering specific parameters.

**Low dose infections induce a strong Th1 response in mice whether they are infected at ZT0 or ZT12, resulting in chronic worm burdens**

C57BL/6 mice given a low dose of *T. muris* eggs (30-40) mount a Th1 response, leading to a chronic infection, allowing parasite mating and egg release (Bancroft *et al.*, 1994; 2001). Administering a low dose of infective eggs at ZT0 or ZT12 did not impact on the outcome of infection. Both groups of mice harboured similar amounts of worms by day 42.

The delay in worm expulsion seen in C57BL/6 mice given a high dose infection at ZT12 (Chapter 3) could be due to a weak Th2 response in ZT12 or weak Th1 response at ZT0 infected mice. The results from the low dose infection study suggest that the difference in speed of expulsion most likely depends on the strength of the Th2 response mounted as low dose infected mice could not be tipped towards resistance by early morning infection (Bancroft *et al.*, 1994; 2001). Thus Th1 dominated responses appear unaltered by time of day of infection.

**Cytokine responses in mice infected with a low dose infection at ZT0 and ZT12 were equal for Th2, Th1 and pro-inflammatory cytokines measured**

In high dose infected mice (Chapter 3), levels of Th2 cytokines were higher in ZT0 infected mice, which correlated with worm expulsion. As low dose worms were retained to the same degree, it is not surprising to see that Th2 as well as Th1 cytokine levels were similar independent of time of infection.

Low dose infection of BALB/c and BALB/k mice also leads to a chronic infection. Cytokine levels of IL-4, IL-5 and IL-9 were lower than in high dose infections, while IFNγ was highly up-regulated (Bancroft *et al.*, 1994; deSchoolmeester & Else, 2002). The Th2 cytokine levels of low dose infection at ZT0 and ZT12 in C57BL/6 mice were comparably low, showing that low dose infected mice produced a Th1, but no Th2 response.
Vaccination at ZT0 or ZT12 results in strong priming of a Th2 response and enhanced expulsion when vaccinated mice are infected at ZT3 with a high dose of *T. muris* eggs

Early vaccination studies used adult male homogenate and stichosome extract, before settling on E/S antigen routinely used in vaccination (Jenkins & Wakelin, 1983; Selby & Wakelin, 1973; Wakelin & Selby, 1973). The route of administering the vaccine is important for the protection against *T. muris* infection. Subcutaneous vaccination with homogenate adult worm antigen in Freund’s complete adjuvant leads to over 95% reduction in worm burdens in quick responding BALB/c and slow responding C57BL/6, but only 41% reduction in non-responding B10.BR mice. Oral vaccination with homogenate adult worm antigen mixed with cholera toxin on the other hand induced 97% reduction in BALB/c mice, but only 59% in C57BL/10 mice and no protection in non-responder B10.BR (Robinson et al., 1995). These results show that both routes of vaccination can induce Th2 responses in quick and in slow responders, but only subcutaneous vaccination led to a reduction in the non-responder strain. As C57BL/6 mice are a slow responder strain, subcutaneous vaccination is more likely to induce a strong Th2 response, enabling the mice to expel a high dose *T. muris* infection faster than the normal 3 weeks they require for expulsion. Dixon et al. (2010) described the immune response in peripheral lymph nodes after subcutaneous vaccination of susceptible AKR mice. Mice were vaccinated with either E/S or PBS in Freund’s incomplete adjuvant, injected in one abdominal site. Vaccination with E/S led to proliferation of cells and production of parasite specific IgG1 antibodies and Th2 cytokines in the peripheral lymph nodes, but not mesenteric lymph nodes, as seen in primary infections.

Diurnal variations in vaccination where observed by Silver et al. (2012a). Mice vaccinated with OVA and TLR-9 ligand CpG ODN as an adjuvant at either ZT7 or ZT19, which corresponded to lowest and highest TLR-9 expression in macrophages, showed that the adjuvant maximises TLR-9 signalling and thus leads to higher cytokine responses in ZT19 vaccinates mice. 4 weeks post vaccination lymphocytes of ZT19 vaccinated mice showed increased proliferation and production of IFNγ compared to mice vaccinated at ZT7.

**No differences can be seen at day 10 post vaccination between mice vaccinated at ZT0 or ZT12**

Vaccination with a 100µg/100µL E/S emulsified in incomplete Freund’s adjuvant introduces a very strong Th2 response post ZT3 infection, independent of the time of vaccination. When vaccination was successful, peripheral lymph node cells expand (Dixon et al., 2010) but there was no significant difference between PLN numbers in ZT0 vaccinated mice compared to ZT12 vaccinated mice 10 days post vaccination. Parasite specific antibody levels also were similarly high in ZT0 and ZT12 vaccinated mice, compared to sham vaccinated mice. For the vaccination against *T. muris* to be successful, a high parasite specific antibody titre is not sufficient, as IgG1 antibody transfer to susceptible mice results only in partial protection (Blackwell & Else, 2001).
The cytokine results obtained at day 10 post vaccination are difficult to interpret, as the group number was only 3 and for some cytokines the values are more scattered than for others. No significant difference could be observed for sham and vaccinated mice for IL-4, IL5, IL-10, IFNγ and IL-6. I-13 levels were significantly up-regulated from sham to vaccinated mice inoculated at ZT0, but no difference could be seen between ZT0 and ZT12 vaccinated mice. In sham vaccinated mice, no increase in any cytokines was expected (Dixon et al., 2010), and despite the rapid expulsion, cytokine levels in vaccinated mice might have been too low to detect significant increases.

Small group sizes could have been a problem, as the diurnal effect seen in chapter 3 required bigger groups sizes to be significant. The small n number used to establish if vaccination was successful in priming the immune response was too small to predict a diurnal effect in vaccination efficacy.

**Vaccinated mice infected at ZT3 were able to expel their worm burden successfully by day 14 independent of the time of vaccination**

Once vaccinated, mice were infected with a high dose *T. muris* eggs at ZT3 and culled at day 14. The worm burden showed that mice vaccinated with E/S at ZT0 and ZT12 were equally capable of expelling the worm burden, compared to sham vaccinated mice, which still harboured high numbers of worms. Cell numbers in peripheral and mesenteric lymph nodes were the same in ZT0 and ZT12 sham and ZT0 and ZT12 E/S vaccinated groups; therefore the priming with antigen in the vaccination did not lead to increased lymphocyte accumulation in the lymph node up to day 14.

**Both E/S vaccinated groups of mice produced high levels of Th2 associated parasite specific antibody IgG1**

Parasite specific antibodies measured in serum of vaccinated and sham vaccinated mice showed a clear increase in Th2 associated antibody IgG1 in both ZT0 and ZT12 vaccinated mice, while sham vaccinated mice still had near naïve levels. Levels of Th1 associated antibody IgG2c on the other hand were barely up-regulated in any of the groups. Only one mouse vaccinated at ZT12 with E/S appeared to make higher levels of IgG2c. Very high levels of IgG1 and low levels of IgG2c in E/S vaccinated mice indicate clearly that the vaccination primed the immune response towards a strong Th2 response.

Th2 associated cytokine levels in infected mice did not increase significantly from low levels, while sham vaccinated mice still remained at low levels. Pro-inflammatory cytokines TNFα and IL-6 as well as Th1 associated cytokine IFNγ could be measured in ZT0 and ZT12 sham and E/S vaccinated mice. Only one ZT12 sham vaccinated mouse produced high levels of these cytokines, while the other mice in this group only secreted the same level of cytokines as ZT0 sham vaccinated mice. TNFα and IL-6 are up-regulated in in E/S vaccinated compared to sham vaccinated mice. Overall one can conclude that vaccination with E/S does induce higher
cytokine levels compared to sham vaccinated mice, but overall they were still low at day 14, compared to cytokine levels previously measured at day 21 in chapter 3.

Intestinal muscle wall thickness, crypt length and goblet cell hyperplasia was found to be the same in sham vaccinated and E/S vaccinated mice. Infiltrating CD11c+ and B220+ cells also did not vary in numbers between groups. The non-significant reduction in CD45+ cells in both vaccinated groups can be attributed to expulsion of worms and return to steady state (Little et al., 2005) while the inflammation is still active in sham vaccinated mice. No difference had been previously observed in chapter 3, therefore these results were as expected.

**Vaccination with E/S at both ZT0 and ZT12 both induces a strong Th2 response when infecting mice at ZT3, which overwrites circadian rhythm at time of vaccination**

Infection at ZT3 for mice vaccinated at ZT0 and ZT12 did not show any differences in worm expulsion but very strong Th2 responses in both groups, supporting the theory that the vaccine dose of 100μg E/S was stronger than the circadian rhythm, which was overwritten. This indicates the immune response is able to influence circadian rhythm as well.

Dixon (2007) showed that only 100μg E/S/100μL in incomplete Freund’s adjuvant (IFA) results in sterile immunity. Vaccination of susceptible mice with 50μg E/S/100μL IFA and 10μg E/S/100μL IFA still reduced worm burden, 50μg/100μL to a significant degree and 10μg/100μL to a non-significant degree. These concentrations of vaccine are less successful in forming a protective immune response, but might be more susceptible to circadian influences than 100μg E/S/100μL IFA used in the experiments in this chapter.

Another possibility why no diurnal difference was seen is the infection time. Mice infected at ZT0 mounted strong Th2 responses as seen in chapter 3, so mice infected at ZT3 would still be expected to expel their worm burden quickly. The combination of weak Th2 priming in ZT12 infected mice with a strong Th2 induction due to an infection in the morning could be the reason why quick expulsion was observed in both the ZT0 and ZT12 vaccinated groups of mice, which might not have been the case if mice had been infected at a later time point closer to ZT12.

**Vaccination studies in humans revealed diurnal differences in priming of the immune response**

Only a few studies so far have examined a diurnal effect in vaccination. Pollmann & Pollmann (1988) vaccinated patients at 7.30-9 am or at 1-3 pm with a hepatitis B vaccine and measured higher antibody titres in patients vaccinated around noon compared to morning vaccinations. The hepatitis B vaccine consisted of three injections, administered over 6 months and the antibody levels were measured 1-2 months following the final injection. Administration of hepatitis A vaccine at 10 am-noon did lead to a higher antibody titre than vaccination at 4-6pm only in male patients, but not in females (Phillips et al., 2008). The same gender difference was seen in the antibody titre levels following vaccination against influenza, administered at 8-11 am
or 1- 4 pm (Phillips et al., 2008). In both studies described by Philips et al. (2008), antibody titres were measured 1 month post vaccination. Live, attenuated Venezuelan equine encephalomyelitis virus vaccine was administered at 8 am or 8 pm and leukocyte numbers were measured daily at the 8 am and 8 pm for 10 days post vaccination. Leukocyte numbers peaked by day 2 post vaccination in 8 am vaccinated patients, while leukocyte numbers in 8 pm vaccinated patients did not peak until day 6 (Feigin et al., 1967). Langlois et al. (1995) evaluated 5 influenza vaccines, each with a different antigen, A/Chile/83 (H1N1), A/Philippines/82 (H3N2), B/USSR/83, A/Mississippi (H23N2) and B/Ann Arbour. Only in one out of 5 vaccines tested a diurnal effect was observed after the first injection. Most of the time, antibody levels increased about 5 fold and showed no diurnal effect, while the 12 fold increase of antibody response to the influenza virus antigen A/Philippines was accompanied by a diurnal effect. Following the second injection with the vaccine a year later, no diurnal effect was visible for any of the antigens tested. More often only diurnal side effects were recorded at the site of injection. Here first time immunized patients were more likely to suffer redness, hardness and soreness if vaccinated mid-day (Langlois et al., 1995; Pollmann and Pollmann 1988).

Taken together, all these vaccination models suggest that there is a possibility for diurnal variation post vaccination in the degree of priming of the immune response. It remains unclear however, how this affects protection post infection.

**Mice food-restricted to mid-day and infected at ZT0 did not show a significant delayed expulsion phenotype seen in ZT12 infected mice in previous experiments**

Food restriction is a common tool to decouple peripheral circadian tissues from the SCN (Damiola 2000). The time peripheral organs take to reset to changed feeding conditions is individual, but after 1 week of altered feeding regimen, liver, kidney, heart and pancreas reset the expression of clock genes to the feeding schedule. Reversing the feeding habit of rodents clearly changes internal rhythm of the colon (Hoogerwerf et al., 2007; Schibler et al., 2003; Sládek et al., 2007). Mice infected at ZT0 and restricted to feeding during the day, and not the night as they would naturally, were expected to have a similar delayed expulsion phenotype seen in mice infected at ZT12 (chapter 3), due to the assumption that the immune system anticipating insult depends on feeding entrainment but not presence or absence of food. Food anticipatory behaviour ensures that mice are active during food restriction to the light-phase, which is normally the resting phase in nocturnal animals. Anticipatory feeding behaviour is accompanied by intestinal motility and corticosterone secretion (Mieda & Sakurai, 2011), which is therefore both coupled to food availability.

**The food entrainment was only partly successful in reversing the clock gene expression in the colon after 12 days**

Mice are nocturnal, therefore feeding would occur primarily during the night. Under those circumstances, Bmal1 expression in colon tissue is up at ZT0 and low at ZT12 (Hoogerwerf et
Data presented in this chapter shows that mice restricted to 12h food availability during light or dark phase as well as mice restricted to 6h food availability mid-dark or mid-light phase were able to fully reverse Bmal1 gene expression, with high Bmal1 expression at ZT12 and low expression at ZT0. Per2 expression is anti-phase to the expression of Bmal1, therefore under normal feeding conditions Per2 expression is high at ZT12 and low at ZT0 (Hoogerwerf et al., 2007). In mice with 12h food availability during the light phase, the level of Per2 gene expression was the same in at ZT0 and ZT12 and therefore had not reversed. In mice with 6h food availability mid-light phase, the expression of Per2 was reversed compared to mice fed for 12h during the dark phase, but the level of Per2 expression in mice restricted to 6h mid-dark phase was lower in mice culled at ZT12 than in mice restricted to 12h feeding during the dark phase. Corticosterone levels in mice restricted to feeding 6h mid-light or mid-dark phase were not reversed in mice fed during the light phase. According to Le Minh et al., (2001) food restriction to day time results in bimodal corticosterone oscillations, peaking about ZT0 and ZT12. One of these peaks is dependent on the SCN, while the other is food-induced. The bimodal rhythm of corticosterone could explain why no difference in corticosterone could be observed in the experiment where mice are restricted to 6h mid-light phase.

**Mice fed mid-light phase have significantly higher levels of Th1 associated parasite specific antibody production, similar to ZT12 infected C57BL/6 mice in previous experiments in chapter 3**

C57BL/6 mice restricted to food availability for 6 hours mid-light or mid-dark phase for 12 days were infected at ZT0 with a high dose of eggs and kept on food restriction for further 7 days, as the emphasis of the experiment lay on the initial establishment of the immune response. No significant difference could be observed in worm burdens at day 21. Levels of parasite specific IgG1 were equal in both groups of mice, but IgG2c was significantly higher in mice fed during the light phase than in mice fed during the dark phase.

In various food restriction studies, it has become obvious, that food restriction impacts on intestinal functions, which could influence immune responses to *T. muris* infection. Wild-type mice showed circadian clock gene expression in the intestine and can be food entrained when food availability is restricted to 2h mid-light phase. Behaviour of clock mutant mice can also be entrained by food restriction, but clock gene expression in the small intestine is disrupted and cannot be entrained. Rhythmic clock gene expression is linked to the diurnal rhythm in lipid, macronutrients and monosaccharide absorption and in clock mutant mice this rhythm in absorption is lost (Pan & Hussain, 2009). These experiments showed that clock expression is important for the rhythmic functions of the gut. Surgical ablation of the SCN does disrupt circadian rhythm of feeding and digestion, which can be rescued when placed in a restricted feeding regimen at least temporarily (Malloy et al., 2012). These studies show that the peripheral clock in the intestine is not independent of the SCN in ad lib. conditions, but can be used to overwrite the main clock in the brain by restricting the access to food.
Measuring intestinal defensins mRNA over 24h, every 3h using RT-PCR showed circadian rhythm in the expression of innate immune components (Froy & Chapnik, 2007). Expression of Toll-like receptors in the intestine has also been shown to be under diurnal regulation (Froy & Chapnik, 2007; Mukherji et al., 2013; Silver et al., 2012a). Increased severity of TLR-9 dependent sepsis correlated to increased TLR-9 expression at ZT19 compared to ZT7 induction of sepsis by puncturing the caecum (Silver et al., 2012a). Froy and Chapnik (2007) showed rhythmic expression of TLR-4, TLR-2, TLR-3 and TLR-5 by RT-PCR, but did not see rhythmic expression of TLR-9. Expression of these receptors is highest when food is anticipated to reach the small intestine. As mice feed during the night, TLRs are up-regulated towards the morning. If food restriction is able to reverse rhythmic expression of these pathogen recognition receptors, it would be expected that the immune response to infection is altered as well. The response to LPS via TLR-4 is also gated by circadian rhythm. Administration of LPS at the beginning of the active phase leads to elevated levels of IL-6, IL-12 CCL2, CCL5 and CXCL1 compared to mice given LPS at the beginning of the resting phase. In Bmal1^{flo^{+}}LysM^{cre} and RevEBBa^{-/-} mice, this rhythm is abolished. In the absence of microbiota signalling via TLRs and other pattern recognition receptors was lost, which impaired expression of many genes involved in innate and adaptive immunity. The clock regulating the Toll-like receptors is therefore likely regulated by the microorganisms present in the intestine (Mukherji et al., 2013). Helmy and Grencis (2003) have established that Toll-like receptors signalling through MyD88, which includes TLR-2, TLR-4 and TLR-9, are not likely required to mount a protective immune response in a T. muris infection, therefore a circadian rhythm in these Toll-like receptors should not influence the immune response to T. muris.

Cytokine levels in mice fed mid-light phase were similar to ZT0 infected and not ZT12 infected C57BL/6 mice seen in previous experiments

Levels of Th2 associated cytokine IL-5 were significantly higher in mice forced to feed during the light phase compared to mice fed during the dark phase. There was no difference for Th2 associated cytokines IL-13 and IL4 in day or night fed mice. In ZT0 and ZT12 infected mice (chapter 3), Th2 associated cytokines were found at higher levels in ZT0 infected mice compared to ZT12 infected mice. Pro-inflammatory cytokines IL-6 and TNFα and Th1 associated cytokine IFNγ were expected to be higher in mice fed during the light phase and infected at ZT0 to resemble ZT12 infected mice seen in previous experiments. There was a broad spread in the IL-6, TNFα and IFNγ responses measured; the response of both groups of mice was not very uniform. The cytokine results therefore suggest that mice fed mid-light phase do not resemble ZT12 infected mice seen in chapter 3.

Intestinal muscle wall thickness, crypt length and goblet cell hyperplasia did not show a difference in severity of pathology between mice fed mid-light or mid-dark phase. This did resemble mice infected at ZT0 or ZT12 (chapter 3), where the parameters also did not show any differences between groups. Numbers of CD11c^{+}, F4/80^{+} and CD45^{+} cells infiltrating colon tissue were also the same in mice fed mid-dark or mid-light phase. Numbers of infiltrating cells
were not different in ZT0 and ZT12 infected mice in previous experiments, therefore these results are not surprising.

**Restricting food availability to mid-light during the first week of a ZT0 infection did not prove enough to significantly delay worm expulsion**

The parasite specific antibody production of mice fed mid-light phase during infection does resemble mice infected at ZT12 at day 28 in chapter 3, but the cytokine response resembles more ZT0 infected mice in previous experiments. It is possible that the clock gene reversal at the time of infection was not successful and therefore the phenotype did not establish as hypothesised. Another possibility is that the length of food reversal was too short during infection and when the colon clock reversed back to normal conditions with *ad lib.* food availability, the immune response to the infection was altered. Results were therefore inconclusive, as to the extent by which rhythmic clock gene in the colon result in the delayed worm expulsion seen in chapter 3.

Corticosterone administration during the second week of infection can prevent expulsion (Else *et al.*, 1992b), suggesting that important priming of the immune response to the infection with *T. muris* occurs during the second week of infection, where mice in this experiment were returned to *ad lib.* conditions. Administration of recombinant IL-13 to boost the immune response in IL-KO mice was carried out during the second week of infection (Bancroft *et al.*, 2000) resulted in worm clearance, while treatment between day 0 and day 14 with recombinant IL-12 led to chronic infection (Bancroft *et al.*, 1997). All of these experiments suggest that the immunomodulation has to take place not during the first week of infection, but also during the second week of infection to take effect.

Compared to chapter 3, no diurnal effect could be seen in low dose or vaccine experiments. Manipulating the immune response towards Th1 or Th2 did overwrite any circadian influence, leading to the suggestion that the mechanism leading to delayed worm expulsion in ZT12 infected mice can easily be overwritten by the immune response, if the stimulus is strong enough. The colon clock cannot be discounted as of yet as a player in the delayed worm expulsion in mice infected at ZT12, orchestrating the immune response to the parasite, even though the experiment in this chapter did not show a clear result.

**Summary:**

- Mice infected with a low dose infection mount a chronic infection independent of time of infection.
- Vaccination at either ZT0 or ZT12 leads to accelerated expulsion in mice infected with a high dose *T. muris* eggs at ZT3 compared to mice sham vaccinated at ZT0 or ZT12.
- Mice food restricted to mid-light phase prior and during the first week of a ZT0 infection do not resemble ZT12 infected mice.

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Chapter 5:
Infection of transgenic mice with *Trichuris muris*
Introduction

Transgenic mice are widely used tools to model disease and understand the influence of individual genes on mechanisms within the body. Due to the broad application for these animals, a variety of different transgenic mice are available for research. Global knockout mice describe mice with deleted genes in all tissues. These mice do not discriminate between different functions a gene can have in various compartments of the body and can sometimes lead to severely impaired animals or even embryonic lethality (Matthaei, 2007).

Conditional knockout mice show genetic alterations only in specific tissues or cells expressing a target gene or under inducible conditions, like the administration of tamoxifen (Metzger & Chambon 2001) or doxycycline (Guo et al., 2005). These mice are most frequently created using the Cre-loxP system or the Flp-FRT system (Kwan 2002). Conditional transgenic mice enable research into the function of individual genes in distinct cell or tissue types by restricting the mutation to that cell/tissue.

Reporter mice on the other hand generally have a fluorescent tag, as GFP, which can be excited with a laser or a bioluminescent tag, which leads to co-expression of a light signal and a target gene, inserted into their genome. Several luminescent reporter lines have been created for the study of circadian rhythms, using the firefly Luc gene (Yamazaki & Takahashi 2005).

Three different strains of transgenic mice were used in this thesis, mPer2::luc reporter mice, Bmal1<sup>lox</sup>LysM<sup>cre</sup> mice and mPer2::lucBmal1<sup>lox</sup>CD11c<sup>cre</sup> mice. Due to the close link between the molecular clock and the immune system, mice with a global disrupted clock often show disrupted immune responses as well. Clock<sup>-/-</sup> mice have reduced immune responses when cells were cultured with an immune stimulant (Sprenger et al. 2012). Cry1Cry2<sup>-/-</sup> mice on the other hand show upregulated levels of proinflammatory cytokines (Narasimamurthy et al. 2012). These changes in immune responses might skew the immune response to the parasite. Furthermore dendritic cells, as antigen presenting cells, and possibly macrophages, as cytokine producing cells, could play a role in the immune response to T. muris, therefore examining these cell-specific knock out animals is more specific in answering the question about the mechanism behind the delayed worm expulsion seen in chapter 3.

**mPer2::luc mice are used to measure mPer2 gene expression in cells or tissue**

mPer2::luc mice were generated by fusing a firefly luciferase gene to the terminal exon of the mPer2 locus, leading to a mPer2::luc fusion protein. Using luciferin substrate in the media with tissue or cells, the transcription of the mPer2 protein and luciferase is accompanied by a light signal, which can be detected using photomultiplier tubes (Yoo et al., 2004). Mice homozygous for the Per2::luc fusion gene follow the same circadian patterns as wild-type mice, which lead to the conclusion that normal clock function was not disrupted (Yoo et al., 2004). Therefore these mice can be used as real-time reporters for the expression of the Period2 expression and measure the circadian oscillation in peripheral tissue and ex vivo/in vitro cells.

Homozygous mPer2::luc mice were used as reference animals for work with photomultiplier tubes, to establish if colon tissue and bone marrow derived dendritic cells follow
a circadian rhythm. In order to compare results from *T. muris* infection in wild-type mice, it was essential to establish the immune response of mPer2::luc mice to *Trichuris muris* infection. mPer2::luc mice were bred on a C57BL/6 mouse background (Yoo et al., 2004), therefore the immune response should be the same, or at least similar to conventional C57BL/6 mice. Slightly different genetic backgrounds of transgenic mice can underlie variations in their immune response, especially in the context of *T. muris*, which makes it difficult to distinguish if the immune response is influenced by the genetic manipulation or the strain of mouse used for breeding the transgenic mouse line. mPer2::luc mice can only be used successfully as reference animals to C57BL/6 mice if the immune responses described in chapter 3 are also seen in mPer2::Luc mice. Thus, to compare the immune responses of mPer2::luc mice with C57BL/6 mice, mPer2::luc mice were infected at ZT0 and ZT12 and culled at day 21, when worm burden, antibody production, cytokine production as well as pathology measurements and immunohistochemistry of colon tissue were analysed.

**Bmal1**<sup>flo</sup>**LysM<sup>cre</sup>** mice lack circadian rhythm in macrophages

* Bmal1 is one of the core clock genes of the circadian feedback loop. When expression of Bmal1 is disrupted, other circadian clock genes also cease to oscillate (Bunger et al., 2000; Gibbs et al., 2012; McDearmon et al., 2006).

In Bmal1<sup>flo</sup>LysM<sup>cre</sup> mice, Bmal1 gene expression is disrupted in cells expressing LysM, which is mainly found expressed in macrophages, but also monocytes (Clausen et al., 1999; Faust et al., 2000).

Using the CreLoxP system from the bacteriophage P1, LoxP sites were inserted around the exon 8 of the *Bmal1* gene (the gene of interest), now described as a 'floxed gene' as it is flanked by LoxP sites, while recombinase enzyme Cre expression is under the control of the LysM promoter (Matthaei 2007; Metzger & Chambon, 2001). When the Cre recombinase is expressed, the 'floxed' sites are recombined and the targeted exon is removed, leaving the rest of the gene intact but unable to function, as the result is as null protein. Cells which do not express Cre recombinase expression are unaffected (Bunger et al., 2000; Kwan, 2002; Matthaei, 2007; Smedley et al., 2011).

With exon 8 of the *Bmal1* gene removed, mice show no circadian rhythm in macrophages. Even though macrophages have not been shown to be significant for worm expulsion in *T. muris* infection, they are antigen presenting cells and secrete a variety of cytokines (Bowcutt et al., 2011), therefore a lack of rhythm in these cells could still impact on the immune response to *T. muris*. In resistant mice, large quantities of macrophages can be found at the time of expulsion (Little et al., 2005).

A primary infection experiment was carried out in Bmal1<sup>flo</sup>LysM<sup>cre</sup> mice. ZT3 infected Bmal1<sup>flo</sup>LysM<sup>cre</sup> mice and littermate controls were analysed at day 21 for worm burden, antibody production, cytokine production as well as pathology measurements and immunohistochemistry or colon tissue.
mPer2::LucBmal1\textsuperscript{flo}\textsuperscript{CD11c\cre} mice lack circadian rhythm in dendritic cells

To establish if the circadian rhythm displayed by dendritic cells causes the day-night difference in the immune response to \textit{T. muris} infection, a new mouse line had to be created that did not have a functional circadian oscillator in dendritic cells. Classical characterization of dendritic cells describes them as highly positive for the cell surface marker CD11c (Denning \textit{et al.}, 2011; Hashimoto \textit{et al.}, 2011). The CD11c marker can also be found at low levels on other cells, as for example some subsets of macrophages and activated T, B and NK cells (Hashimoto \textit{et al.}, 2011), but is still widely used to identify dendritic cells and has been used before as a target to deplete dendritic cells (Phythian-Adams \textit{et al.}, 2010). Cruickshank \textit{et al.}, (2009) showed that the speed of dendritic cell recruitment to the colon is crucial in determining resistance to infection. BALB/c mice, which expel their parasite burden within 2 weeks and mount a strong Th2 response, have very quick dendritic cell recruitment. AKR mice on the other hand develop chronic infections, mount a Th1 immune response and show very slow dendritic cell recruitment to the gut.

Dendritic cells sample antigens in the colon and activate T cells upon their return to the lymph node. Circadian rhythm in dendritic cells could influence the activation of T cells towards Th2 rather than Th1 and therefore modulate the immune response, leading to quicker expulsion in mice infected at ZT0 rather than ZT12.

In house V\textbeta8\textsuperscript{flo}\textsuperscript{CD11c\cre} mice were bred through a number of generations with in-house mPer2::LucBmal1\textsuperscript{flo} mice, breeding out the V\textbeta8\textsuperscript{flo} gene, to create the new transgenic mouse line. The resulting triple transgenic mPer2::LucBmal1\textsuperscript{flo}\textsuperscript{CD11c\cre} mouse line was infected at ZT0 or ZT12 with \textit{T. muris} infection alongside wild-type littermates and analysed at day 21 for worm burden, antibody production, cytokine production as well as pathology measurements and immunohistochemistry of colon tissue.

Aims:

- Analyse the immune response of mPer2::Luc mice to \textit{T. muris} infection to ensure the genetic background does not influence the responses to the parasite and is still comparable to C57BL/6 mice.
- Establish if there is a phenotype in Bmal1\textsuperscript{flo}\textsuperscript{LysM\cre} mice, which lack circadian rhythm in macrophages, when infected with \textit{T. muris} alongside littermate controls.
- Characterise the importance of dendritic cell rhythmicity in mPer2::LucBmal1\textsuperscript{flo}\textsuperscript{CD11c\cre} mice, which lack a rhythm in CD11c positive cells, in the context of \textit{T. muris} infection.
Results:

5.1. mPer2::luc mice

mPer2::luc mice are generally used as reporter mice to establish the circadian expression of the Period 2 gene. Using media containing luciferin substrate, luminescence can be measured which correlates with the expression of the Period 2 gene. Photomultiplier tube (PMT) devices are routinely used to measure the luminescence in tissue or cell cultures.

5.1.1. mPer2::luc mice tissue shows circadian rhythm in the intestine

Circadian expression of clock genes has been shown in a variety of peripheral tissue such as liver, lung and kidney (Yoo et al., 2004). Using RT-PCR, Hoogerwerf et al., (2007) showed rhythmic expression of clock genes in the murine intestinal tract, including colon tissue. To examine the circadian expression of Per2 in colon tissue of mPer2::luc mice, proximal colon tissue slices were placed under the PMT. The average period was measured to be slightly over 24 hours after tissue was synchronised using dexamethasone (Gibbs et al., 2009), which has been shown to be a strong phase-shifting agent (Balsalobre, 2000) (Fig. 1). This shows that colon is also a peripheral tissue under circadian control.

![Oscillation of small rings of proximal colon tissue placed under photomultiplier tubes after synchronisation with dexamethasone](image)

**Figure 1:** Oscillation of small rings of proximal colon tissue placed under photomultiplier tubes after synchronisation with dexamethasone. Oscillation was visible for several days under the photomultiplier tube and normalised. The average period, analysed between 20-70 hours of recording, was 24.87 hours. Erratic measurement approx. 40h due to opening of the incubator.
5.1.2. Bone marrow derived dendritic cells generated from mPer2::luc mice displayed circadian rhythm

After establishing that whole tissue in the colon was under circadian control, the next step was to show that dendritic cells themselves are rhythmic. Arjona & Sarkar (2006); Keller et al., (2009) and Silver et al., (2012b) showed that many innate cells, including macrophages, dendritic cells and NK cells expressed a circadian rhythm. B cells were also shown to be rhythmic. Dendritic cell rhythms have previously been measured using RT-PCR for clock gene expression (Silver et al., 2012b). Culturing bone marrow dendritic cells from mPer2::luc mice allowed Per2 expression to be measured directly and could be tracked over time. Two cultures of BMDCs were synchronised using dexamethasone, while others were synchronised only via serum change. Both groups showed circadian rhythm over 3-5 days, but surprisingly synchronisation did not reset both groups to the same point in the expression of Per2. As expected, dexamethasone induced stronger circadian expression of Per2, but synchronisation with serum change induced rhythm in BMDCs as well. This indicates that dendritic cells were under circadian control and could play a role in circadian priming of T cells.

![Figure 2: Bone marrow derived dendritic cells show rhythmic mPer2 expression.](image)

1x10^6 Bone-marrow derived dendritic cells were synchronised using dexamethasone (BMDC+Dex) or by serum change alone (BMDC serum change). Higher amplitude oscillation was triggered by adding dexamethasone. Under both conditions cells oscillated for at least 3 days.
5.1.3. Infection of mPer2::luc mice at ZT0 or ZT12 with a high dose of *Trichuris muris* showed no difference in immune response

To ensure the immune response in mPer2::luc mice to *Trichuris muris* infection resembled that of C57BL/6 mice, mPer2::luc mice were infected with a high dose of *T. muris* eggs at ZT0 or ZT12 and culled on day 21 post infection.

5.1.3.1. Worm burden, antibody responses and cytokine levels did not show a significant difference between mPer2::luc mice infected at ZT0 or ZT12

Figure 3: Analysis of immune response to infection of mPer2::luc mice at day 21 post infection at ZT0 or ZT12 with a high dose of 200 *T. muris* eggs. A) Worm burden were quantified by counting worms found in caecum and colon. B and C) parasite specific IgG1 and IgG2c production measured in serum, screened against parasite ES antigen at 0.5μg/ml. The serum was serially diluted; the data shown is dilution 160 only, as it falls within the linear range. D-I) MLN cells were isolated from mice infected at ZT0 and ZT12 and cultured with *T. muris* E/S (50μg/ml) for 48h. The supernatants were analysed using cytometric bead array (CBA). Th2 associated cytokines (D-F), Th1 associated cytokines (G) and pro-inflammatory cytokines (H, I) are shown. The data is presented as histograms expressing mean +/- SEM.
as well as symbols representing individual mice. Statistics: A) Mann-Whitney U test, B-N) Kruskal-Wallis test, Dunn post hoc *p < 0.05. Naïve n=3, infected n=5.

The worm burden was not significantly higher in ZT12 infected mPer2::luc mice compared to mice infected at ZT0; only 1 mouse in the ZT0 infected mPer2::luc mouse group retained a high worm burden at day 21 (Fig.3A). Worm burdens were significantly lower in ZT0 compared to ZT12 infected mice in chapter 3. Antibody levels for IgG1 were elevated from naïve levels, but no significant differences were seen between mice infected at ZT0 versus ZT12 (Fig. 3B). In ZT0 infected mice IgG1 levels were higher in Chapter 3, while ZT12 levels were similar to Figure 3b). The difference between IgG1 levels was not significant, while in chapter 3, levels of IgG1 were higher in ZT0 infected C57BL/6 mice. No differences could further be observed between IgG2c levels in naïve, ZT0 and ZT12 infected mice (Fig. 3C), which was similar to day 21 in ZT0 and ZT12 infected C57BL/6 mice in Chapter 3.

Cytokine production for Th2 associated cytokines IL-4, IL5 and IL-13 (Fig. 3D-F) as well as Th1 associated IFNγ (Fig. 3G) and pro-inflammatory cytokine IL-6 (Fig.3I) showed no significant difference, while pro-inflammatory cytokine TNFα (Fig. 3H) was expressed at significantly higher levels in ZT12 infected mice, which was also seen in C57BL6 mice in chapter 3. C57BL/6 mice furthermore showed significant differences for IL-13 in ZT0 compared to ZT12 infected mice, while ZT12 infected mice had higher significantly higher levels of IFNγ and IL-6.

Thus, C57BL/6 mice in chapter 3 do not resemble mPer2::luc mice in their immune response to *T. muris*.

### 5.1.3.2. Muscle wall thickness, crypt length, goblet cell hyperplasia or CD11c+ and CD45+ cells infiltrating colon tissue did not show any significant differences between ZT0 and ZT12 infected mPer2::luc mice

When analysing the pathology (crypt length, muscle wall thickness, goblet cell numbers) of colon tissue, no significant differences could be seen between ZT0 and ZT12 infected mice and no differences were visible when comparing to naïve mice (Fig. 4A-C). Immunohistochemistry on frozen colon tissue revealed a significantly increased influx of CD45+ cells in ZT12 infected mice (Fig. 4E), while no difference was seen in CD11c+ cells (Fig. 4D). The reduced leukocyte infiltrate at ZT0 versus ZT12 may reflect the more complete worm expulsion and return to resting state. There was no difference visible in pathology and cell infiltration in chapter 3, therefore these results are similar.

Despite the time of day infection experiment in mPer2::luc mice failing to give significant differences in worm burden between groups infected ZT0 versus ZT12, with the group size of 5 mice, breeding mice which lack circadian rhythm in dendritic cells used the mice on a mPer2::luc background.
Figure 4: Pathology and immunohistochemistry analysis of mPer2::Luc mice at day 21 post infection at ZT0 or ZT12 with a high dose of 200 T. muris eggs. A-C) pathological measurements: A) muscle wall thickness B) crypt length C) goblet cell numbers. D) Representative photographs of PAS staining. Goblet cells stain purple/blue. E and F) Immunohistochemistry on frozen gut tissue: E) CD11c positive staining, F) CD45 positive cells. The symbols represent individual mice, histograms show mean +/- SEM. G+H) Representative photographs for CD11c staining (G) and CD45 staining (H). Immunohistochemistry using CD45+ or CD11c+ monoclonal antibodies stains CD45+ or CD11c+ cells.
brown. CD11c positive cells were counted directly, for analysis of CD45 staining Image-Pro Plus software was used. Photographs were taken at 400x magnification. Statistics: Kruskal-Wallis test, Dunn post hoc *=p<0.05. Naive n=3, infected n=5.

5.2. Infection of Bmal1\textsuperscript{flox}LysM\textsuperscript{cre} mice and littermates with a high dose of Trichuris muris

Macrophages have been shown to be non-essential for T. muris expulsion. Nonetheless macrophages are antigen presenting cells and produce many cytokines implicated in resistance to T. muris infection. Even though no differences could be observed in numbers between ZT0 and ZT12 infected mice at early events as well as the end point of the infection time course, macrophages could play a vital role in providing cytokines in the establishment of the inflammatory response in a rhythmic manner. Keller \textit{et al.} (2009) and others showed that macrophages contain a functional molecular clock and in LPS challenge models these mice showed time of day dependent differences to the stimulus (Gibbs \textit{et al.} 2012). Therefore Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice and wild-type littermates were infected to establish whether Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice, which lack a circadian rhythm in macrophages, responded differently than their wild-type littermates to infection with T. muris.

Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice and littermate controls were infected at ZT3 to establish if there was a difference in immune response to a high dose infection with T. muris. ZT3 in the animal unit corresponds with 10am, which should in quick expulsion in littermate controls. Mice were sacrificed at day 21 and worm burden, antibody production and cytokine responses were assessed, as well as gut pathology and leukocyte infiltrate.

5.2.1. Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice expel their worm burden and have similar levels of MLN cytokines as their littermate controls, but produce higher levels of parasite specific antibodies

Most Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice and littermate controls expelled their worm burden by day 21 (Fig. 5A). The level of parasite specific Th2 associated antibody IgG1 was significantly higher in Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice compared to their wild-type littermates (Fig. 5B). No difference was seen in parasite specific Th1 associated antibody IgG2c production in Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice compared to their wild-type control (Fig 5C). There were no significant differences in levels of Th2 cytokines IL-4 and IL-5 (Fig. 5D and E), Th1 cytokine IFN\(\gamma\) (Fig. 5F) or pro-inflammatory cytokine IL-6 (Fig. 5G) measured using ELISA. Worm burdens were as low as in ZT0 infected C57BL/6 mice seen Chapter 3, Fig. 3 on day 21. IgG1 levels on day 21 were as high as in Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice, while littermates were lower in this experiment. Levels of IgG2c were similar in Bmal1\textsuperscript{flox}LysM\textsuperscript{cre+} mice and littermate controls as ZT0 and ZT12 infected mice on day 21 in Chapter 3, Fig. 4b.
Fig 5: Immune response at day 21 of Bmal1<sup>flox</sup>LysM<sup>cre+</sup> mice and littermates to high dose <i>T. muris</i> infection at ZT3. A) Worm burden quantified by counting worms found in caecum and colon. B and C) IgG1 and IgG2c production measured in serum. The serum was serially diluted; the data shown is dilution 320 only, as it falls within the linear range. D-G) MLN cells were isolated from mice and cultured with <i>T. muris</i> E/S for 48h. The supernatants were analysed using Cytokine Elisa. Th2 associated cytokines (D-E), Th1 associated cytokines (F) and pro-inflammatory cytokines (G) are shown. The symbols represent individual mice, histograms show mean +/- SEM. Statistics: Mann-Whitney U-Test, *=p<0.05, **=p<0.01. + = Bmal1<sup>flox</sup>LysM<sup>cre+</sup> n=4, - = littermates n=5.

5.2.2. No differences in muscle wall thickness, crypt length, goblet cell hyperplasia or F4/80<sup>+</sup>, CD11b<sup>+</sup>, CD11c<sup>+</sup> and CD45<sup>+</sup> cells infiltrating the lamina propria were visible between Bmal1<sup>flox</sup>LysM<sup>cre+</sup> mice and control littermates

When analysing pathology of the colon tissue, no significant differences could be seen between Bmal1<sup>flox</sup>LysM<sup>cre+</sup> mice and littermate controls (Fig. 6A-C). Immunohistochemistry on frozen colon tissue revealed no difference in the influx of F4/80<sup>+</sup> cells, representing mainly macrophages, and eosinophils; CD11b<sup>+</sup> cells, another marker for macrophages; CD11c<sup>+</sup> cells, representing dendritic cells as well as CD45<sup>+</sup> cells, a marker for infiltrating leukocytes (Fig. 6E-H). Overall there appears to be no difference in cell infiltration or pathology between Bmal1<sup>flox</sup>LysM<sup>cre+</sup> mice and littermate controls.
Figure 6: Pathology analysis and immunohistochemistry at day 21 of Bmal1^flox^LysM^cre^+ and littermate control gut tissue. A-C) pathological measurements: A) muscle wall thickness B) crypt length C) goblet cell numbers D) Representative photographs of PAS staining. Goblet cells stain purple/blue. E-H) Immunohistochemistry on frozen gut tissue. E) F4/80 positive cells F) CD11b positive cells G) CD11c positive cells H) CD45 positive cells. The symbols represent individual mice, histograms show mean +/- SEM. I-L) Representative photographs for F4/80 staining (I), CD11b staining (J), CD11c staining (K) and CD45 staining (L). Immunohistochemistry using CD45^+^, F4/80^+^, CD11c^+^ or CD11b^+^ monoclonal antibodies stains CD45^+^, F4/80^+^, CD11c^+^ and CD11b^+^ cells brown. F4/80, CD11c and CD11b positive cells were counted directly, for analysis of CD45 staining Image-Pro Plus software was used. Photographs were taken at 400x magnification. Scale bars represent 100μm. Statistics: Mann-Whitney U-test, Bmal1^flox^LysM^cre^+ : Cre+ n=4, littermates : Cre- n=5.
5.3. Mice with clockless dendritic cells: mPer2::lucBmal1^{flox}CD11c^{cre}mice

Dendritic cells have been shown to follow a circadian rhythm (5.1.2., Fig. 2) and antigen presenting cells could be one circadian player influencing the T cell activation, leading to delayed worm expulsion in ZT12 high dose *T. muris* infected C57BL/6 mice. To establish if dendritic cells are a key player, mice were bred which lacked a functional circadian clock in dendritic cells. If circadian rhythm of dendritic cells is important in the time of day of infection effects, mice with dendritic cells which lack the core clock gene Bmal1 should lose any time of day effect on infection outcome.

5.3.1. Breeding of mPer2lucBmal1^{flox}Cd11c^{cre}mice

To establish if dendritic cell rhythms cause the difference in immune priming for long-term worm expulsion, mice lacking circadian clock function in dendritic cells were bred. Dendritic cells have classically been discriminated by the expression of the integrin CD11c on their cell surface (Denning *et al.* 2011, Hashimoto *et al.* 2011). Therefore CD11c was selected as the Cre recombinase promoter region. vβ8integrin^{flox}CD11c^{cre} mice and mPer2::lucBmal1^{flox} mice were used to breed the first generation of mice.

![Breeding strategy for mPer2::lucBmal1^{flox}Cd11c^{cre} mice](image)

*Figure 7: Breeding strategy for mPer2::lucBmal1^{flox}Cd11c^{cre} mice.* Mice were bred from vβ8integrin^{flox}Cd11c^{cre} mice and mPer2::lucBmal1^{flox} mice over 3 generations.
F1 offspring were crossed back onto mPer2::lucBmal1\textsuperscript{flox} mice. In the F2 generation, mice expressing floxed vβ8integrin sites were excluded from further breeding. Mice expressing CD11c\textsuperscript{cre} were crossed with mice homozygous for mPer2::luc and Bmal1\textsuperscript{flox}, but there were also breeding pairs heterozygous for mPer2::luc or Bmal1\textsuperscript{flox} to increase chances for the right genotype combination. Only offspring homozygous for mPer2::luc and Bmal1\textsuperscript{flox} were used for experiments. The breeding strategy can be seen in Figure 7.

5.3.2. Infection of mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls at ZT0 or ZT12

mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls were infected with 200 eggs of \textit{T. muris} at either ZT0 or ZT12. The mice were sacrificed at day 21, which in previous experiments had shown a significant difference in worm expulsion between C57BL/6 mice infected at ZT0 or ZT12 (chapter 3). A schematic of the experiment can be seen in Figure 8.

<table>
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<tr>
<th>ZT0 infection</th>
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<td>Cre+</td>
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Figure 8. Schematic of ZT0 and ZT12 infection of mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and Cre- littermate controls. Mice were infected at either ZT0 or ZT12, then culled at day 21 to establish whether removal of circadian rhythm in CD11c+ cells affected delayed worm expulsion observed in C57BL/6 mice (Chapter 3).

5.3.2.1. Infection of mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls at ZT0 or ZT12 revealed no delayed worm expulsion in ZT12 infected mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls compared to ZT0 infected mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls

Worm burden in mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls were assessed at day 21. Figure 9A shows no difference between ZT0 and ZT12 infected littermate controls, as well as between ZT0 and ZT12 infected mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice. The Th1 and Th2 associated antibodies IgG2c and IgG1 respectively were measured to analyse the skewing of the immune response. Levels of Th2 associated antibody IgG1 were significantly higher in wild-type ZT0 infected littermates compared to ZT12 infected wild-type littermates (Fig. 9B). This difference cannot be found in mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice infected at ZT0, compared to those infected at ZT12 (Fig. 9B). IgG2c antibody levels did not vary between any of the groups, with no difference seen between ZT0 and ZT12 infected mice nor between mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} mice and littermate controls (Fig. 9C). A significant difference was seen in C57BL/6 mice (chapter 3) between ZT0 and ZT12 infected mice in worm expulsion and IgG1 antibody production. The results of mPer2::lucBmal1\textsuperscript{flox}Cd11c\textsuperscript{cre} do not resemble mice in chapter 3, while littermate controls are only similar in their antibody response but do not show the delay in worm expulsion in ZT12 mice expected from previous experiments in C57BL/6 mice.
Figure 9: Analysis of immune response at day 21 post infection of mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> mice and littermates infected with 200 eggs of *T. muris* at ZT0 or ZT12. A) Worm burden. Worms were counted from the colon and caecum of all mice sacrificed. B + C) IgG1 and IgG2c response. The serum was serially diluted (1/20-1/2560) and screened against parasite ES antigen at 0.5μg/ml; the data shown is dilution 1/160 only, as it falls within the linear range of the titration curve. The symbols represent individual mice, histograms show mean +/- SEM. Statistics: Kruskal-Wallis test, Dunn post hoc *=p<0.05, **=p<0.01.

WT ZT0 and ZT12 n=9, Cre+ ZT0 and ZT12 n=6.

5.3.2.2. Th1 and Th2 cytokine levels did not show any significant differences

Cytokines were measured in the supernatant of mesenteric lymph node cells re-stimulated with E/S for 48h. Th2 associated cytokine IL-5 (Fig. 10A) and IL-13(Fig. 10B) showed no difference between mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> mice littermates, independent of time of day infection. In C57BL/6 mice (Chapter 3, Fig.5), IL-13 levels were significantly elevated in ZT0 infected mice, which was not seen in littermate controls in this experiment. IL-5 levels were not different in chapter 3, which also was seen in mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> and littermate controls.

Levels of the anti-inflammatory cytokine IL-10 were significantly lower in ZT12 infected mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> mice compared to their ZT12 infected littermates (Fig. 10C), but no difference was seen in ZT0 and ZT12 littermate controls. In C57BL/6 mice, no difference could be measured between ZT0 and ZT12 infected mice (chapter 3).
Figure 10: Measurement of cytokines at day 21 in mPer2::lucBma1floxCD11ccre mice and littermates infected at ZT0 or ZT12 with a high dose of 200 \textit{T. muris} eggs. 5\times10^6 MLN cells/ml, isolated from mice sacrificed at ZT0 or ZT12, were stimulated with 50\mu g/ml of E/S and harvested after 48h. The supernatant was analysed using cytometric bead array CBA. Th2 associated cytokines are IL-5 (A) and IL-13 (B), anti-inflammatory cytokine IL-10 (C), the Th1 associated cytokine is IFN-\gamma (E) and pro-inflammatory cytokine IL-6 (D) and TNF\alpha (F). The symbols represent individual mice; histograms show mean +/- SEM. Statistics: Mann-Whitney U-test, WT ZT0 n=9, WT ZT12 n=9, Cre+ZT0 n= 6, Cre+ ZT12 n=6.
The pro-inflammatory cytokine IL-6 was also significantly lower in ZT12 infected mPer2::lucBmal1^{flox}Cd11c^{cre} mice compared to littermate controls (Fig. 10D). IL-6 levels were significantly higher in ZT12 infected C57BL/6 mice compared to ZT0 infected C57BL/6 mice (Chapter 3, Fig. 4), but there was no significant difference in IL-6 levels in ZT0 and ZT12 infected Cre negative littermate controls. There is no significant difference in IFNγ levels (Fig. 10E) and TNFα (Fig. 10F) between ZT0 and ZT12 infected mPer2::lucBmal1^{flox}Cd11c^{cre} mice and littermate controls, which had been observed in C57BL/6 mice in chapter 3.

5.3.2.3. Muscle wall thickness, crypt length and goblet cell hyperplasia measured on PAS-stained wax embedded sections showed a significantly thinner muscle wall thickness in ZT0 infected mPer2::lucBmal1^{flox}Cd11c^{cre} mice compared to ZT0 infected littermate controls. T. muris damages the colon tissue by burrowing through the epithelial lining forming synovial tunnels. To assess the damage caused by infection, muscle wall thickness, crypt length and goblet cell numbers were quantified on PAS-stained wax embedded proximal colon tissue. Muscle wall thickness was lower in ZT0 infected mPer2::lucBmal1^{flox}Cd11c^{cre} mice compared to littermate controls, while no difference was visible between ZT12 infected mPer2::lucBmal1^{flox}Cd11c^{cre} mice and littermate controls (11A). No difference could be measured between ZT0 and ZT12 infected littermate controls for muscle wall thickness, which resembles the results in C57BL/6 mice infected in chapter 3. There was no difference visible between mPer2::lucBmal1^{flox}Cd11c^{cre} mice and littermate controls in terms of crypt length or goblet cell numbers, independent of time of infection (Fig. 11B and C), which also had not been seen in C57BL/6 mice in chapter 3.
Figure 11: Gut pathology measurements at day 21 post a 200 egg *T. muris* infection of mPer2::lucBmal1<sup>flox</sup>CD11c<sup>cre</sup> mice and littermates at ZT0 or ZT12. A) Colonic muscle wall thickness B) Colonic crypt length C) Colonic goblet cell numbers. The symbols represent individual mice and histograms show mean +/- SEM. D-G) Representative photographs of PAS staining. Wax embedded tissue sections were stained with PAS staining and ImageJ software was used to measure muscle wall thickness and crypt length. Goblet cells stain pink/purple. Photographs were taken at 400x magnification. Kruskal-Wallis test, Dunn post hoc *=p<0.05, *=p<0.01 WT ZT0 n=9, WT ZT12 n=9, Cre+ZT0 n= 6, Cre+ ZT12 n=6.

5.3.2.4. The number of CD45<sup>+</sup>, CD11c<sup>+</sup> and F4/80<sup>+</sup> cells did not differ according to time of day infection or between mPer2::lucBmal1<sup>flox</sup>Cd11c<sup>cre</sup> mice and Cre negative littermate controls

Immunohistochemistry was used to quantify the cellular infiltrate into the colon tissue at day 21 post infection. These analyses will indicate whether circadian rhythm influences lymphocyte, macrophage or dendritic cell infiltration into the infected tissue according to time of infection and/or the presence or absence of a circadian rhythm in dendritic cells.
Figure 12: CD45⁺, CD11c⁺ and F4/80⁺ cells in proximal colon tissue recovered from mPer2::lucBmal¹²/CD11c⁻/⁻/mice and littermates at day 21 post infection with 200 T. muris eggs, delivered at ZT0 or ZT12. A) CD45 positive cells B) CD11c positive cells C) F4/80 positive cells. The symbols represent individual mice, histogram shows mean +/- SEM. Representative photographs for CD45 staining (D), CD11c staining (E) and F4/80 (F) staining in the colonic mucosa of ZT0 and ZT12 infected animals. Immunohistochemistry using CD45⁺, F4/80⁺ or CD11c⁺ monoclonal antibodies stains CD45⁺, F4/80⁺ or CD11c⁺ cells brown. F4/80 and CD11c positive cells were counted directly, for analysis of CD45 staining Image-Pro Plus software was used. Photographs were taken at 400x magnification. WT ZT0 n=9, WT ZT12 n=9, Cre⁺ ZT0 n=6, Cre⁺ ZT12 n=6. For Immunohistochemistry CD11c Cre⁺ ZT12 n=5.
Cell surface marker CD45 (Fig. 12A) represents lymphocyte infiltrate, cell surface marker CD11c can mostly be found in dendritic cells, but also some macrophages (Fig. 12B) and cell surface marker F4/80 represents eosinophils and macrophages (Fig. 12C). No significant differences were observed between wild-type littermates infected at ZT0 or ZT12 as previously observed in C57BL/6 mice in chapter 3 for CD45⁺ and F480⁺ infiltrating cells. Also no significant differences were observed in CD45⁺, CD11c⁺ and F4/80⁺ cell numbers between ZT0 and ZT12 infected mPer2::lucBmal1floxCd11ccre mice (Fig. 12B). The lack of difference in infiltrating cell numbers was also observed in C57BL/6 mice in chapter 3. These results lead to the conclusion that cell numbers are not affected by circadian rhythm.

5.3.3. Confirmation of Bmal1 rhythm removal

The infection of mPer2::lucBmal1floxCd11ccre mice did not lead to the expected loss of the time of day effect in the immune response to an infection with T. muris. Before dendritic cells can be dismissed as the gating factor, it is important to establish whether the cre-LoxP system successfully removed exon 8 from the Bmal1 gene, thereby disrupting clock gene expression in CD11c positive cells. Initial attempts to show a loss of oscillation using PMTs was not successful due to a limited viability after cell sorting using FACS.

Bone-marrow derived dendritic cells grown from mPer2::lucBmal1floxCd11ccre mice were sorted using FACS for CD11c expression. cDNA of pure CD11c positive sorted cells and non-sorted cells was used to perform PCR for Bmal1 exon 8, which should be missing in CD11ccre positive cells.

The 137bp Bmal1 exon 8 band can be found in CD11c positive as well as non-sorted cells of both CD11ccre positive and CD11ccre negative littermate control mice (Fig. 12). Therefore Bmal1 exon 8 has not been cut in CD11c positive cells from mPer2::lucBmal1floxCd11ccre mice.

![Figure 13: PCR for the presence of Bmal1 exon 8 in CD11c+ sorted and non-sorted bone marrow dendritic cells from a mPer2::lucBmal1floxCd11ccre mouse and a littermate control. Bmal1 exon 8 is 137 bp long and can be found in all four samples (highlighted in the blue box). 300bp and 500bp bands were not expected. PCR for GapDH, as quantity control, is shown on the right. Cre⁺ = Per2::lucBmal1floxCd11ccre mice, Cre⁻ = Cre⁻ littermate controls, CD11c⁺ = CD11c positive sorted cells, ns = non-sorted cells.](image)

To ensure that the positive sorted cells did contain CD11ccre positive cells, PCR for CD11ccre was performed on the sorted cells. A band for CD11ccre was found in CD11c positive sorted and non-sorted dendritic cells from mPer2::lucBmal1floxCd11ccre mice (Fig. 13). There were also CD11ccre positive cells in the non-sorted fraction of the littermate controls. It is unclear why there is a bigger band (approx. 600bp) found in the CD11c⁺ fraction of the littermate control (Fig. 13).
This band does not correspond to CD11c\textsuperscript{cre}. The result of this PCR suggests that possibly both mice were CD11c\textsuperscript{cre} positive or that some of the samples got contaminated during RNA-to-cDNA conversion.

Further investigation will be needed to establish if the mPer2::\textit{LucB\textsubscript{mal1}flo}\textsuperscript{CD11c\textsuperscript{cre}} mice have clockless dendritic cells or not given that these data are based upon a sample size of one and there is some ambiguity over the detection of Cre recombinase.

**Figure 14:** PCR for the presence of CD11c\textsuperscript{cre} in CD11c\textsuperscript{+} sorted and non-sorted bone marrow dendritic cells from a mPer2::\textit{LucB\textsubscript{mal1}flo}\textsuperscript{CD11c\textsuperscript{cre}} mouse and a littermate. The band for CD11c\textsuperscript{cre} can be seen in the blue box. A faint band is visible for CD11c positive sorted BMDCs from the mPer2::\textit{LucB\textsubscript{mal1}flo}\textsuperscript{CD11c\textsuperscript{cre}} mouse. The approximately 600bp band in the littermate control CD11c\textsuperscript{+} cells is unexpected. Both non-sorted populations of the mPer2::\textit{LucB\textsubscript{mal1}flo}\textsuperscript{CD11c\textsuperscript{ cre}} mouse and the littermate control showed the presence of CD11c\textsuperscript{cre}.
Discussion:

Transgenic mice with disrupted core clockwork have been used to identify the mechanisms and contributing cells to time of day effects in the literature. Nakamura et al. (2011) used mice lacking functional mPer2 expression to identify a time of day dependent difference in cutaneous anaphylactic reaction. Wild-type but not mPer2−/− mice, when they were treated subcutaneously with TNP IgE before being challenged with CNP-BSA intravenously, showed a time of day dependent response. This time of day response was not seen in aged or adrenalectomised mice. These results indicate that this phenotype is dependent on rhythmic mPer2 expression and its impact on corticosterone secretion. Analysing a time of day dependent difference in the colonization of Salmonella enterica serovar typhimurium in the colon, Bellet et al. (2013) compared wild-type mice to CLOCK−/− mice, which lack circadian rhythm. Wild-type mice harboured more colonies of the bacterium when infected at 10 am than mice infected at 10 pm, compared to clock mutant mice, which had similar numbers of S. typhimurium independent of the time of infection, indicating that a functional clock is required for the time of day effect.

The nuclear hormone receptor Rev-ERBα has been shown to be the gating factor in time of day inflammatory response to LPS, as the administration of LPS to Rev-ERBα−/− mice did not show a time of day effect, compared to wild-type mice, where CT12 injected mice exhibited a stronger immune response than mice injected at CT0 (Gibbs et al. 2009).

Also mice with the clockwork disrupted in targeted cells have enabled the discovery that the circadian rhythm of individual cells is important for time of day dependent effects. Peritoneal macrophages, isolated from Bmal1fl/flLysMcre mice, lack functional circadian rhythm and therefore exhibit no diurnal inflammatory response to LPS administration, compared to macrophages isolated from wild-type mice (Gibbs et al. 2009).

In this chapter, immunity to high dose T. muris infection is explored in several clock transgenic mouse lines to identify possible mechanisms and contributing cell types behind the delay in worm expulsion seen in ZT12 infected mice compared to ZT0 infected mice. The T. muris model takes three to four weeks to resolve the infection and will answer whether circadian rhythm at the time of infection influences the outcome of a long-term infection.

Using mPer2::luc mice, rhythmic clock gene expression was visualised in colon tissue and bone marrow derived dendritic cells

Rhythmic expression of clock genes in the mouse intestine has previously been described using RT-PCR, microarray analysis and western blotting (Froy & Chapnik, 2007; Hoogerwerf et al., 2008; Pan & Hussain, 2009). mPer2::luc mice co-express a firefly luciferase gene with the Per2 gene and are generally used to visualise circadian rhythm of mPer2 expression by adding luciferin substrate to the media with cells and tissues when using photomultiplier tubes (PMT) (Yoo et al., 2004). Colon tissue of naïve mice did show rhythmic mPer2 expression for about 3 days after synchronisation with 200 nM dexamethasone for 1 hour (Gibbs et al. 2009), thus
confirming a peripheral circadian clock in the colon tissue of mice. This allows the speculation that local circadian rhythm at the site of infection could play a role in the immune response to \textit{T. muris} infection.

Rhythmic clock gene expression has been described in many immune cells, among them dendritic cells. Silver \textit{et al.}, (2012b) used RT-PCR to confirm clock gene expression in CD11c positive dendritic cells enriched from spleen over 24h, with samples taken every 4 hours. Bone marrow derived dendritic cells cultured from mPer2::luc mice also display circadian expression of \textit{mPer2} when synchronised with 200nM dexamethasone for 1 hour (Gibbs \textit{et al.} 2009), but also using serum shock (50% serum, 1 hour, Gibbs \textit{et al.} 2012). Cells in culture retained their rhythmicity for about 3 days, confirming rhythmic clock gene expression in dendritic cells. Dendritic cells present \textit{T. muris} antigen to T cells and thereby prime the immune system to respond to the infection. Diurnal variations in dendritic cell function could therefore impact on the immune response against \textit{T. muris}, which was investigated using the new transgenic mPer2::luc\textsuperscript{Bmal1\textsuperscript{floxed}CD11c\textsuperscript{cre}} mouse line.

\textbf{mPer2::luc mice showed no significant difference in worm expulsion and antibody production between ZT0 and ZT12 infected mice}

For breeding of a new transgenic mouse line, using mPer2::luc mice gives an advantage as cells and tissue can be analysed directly under a PMT with luciferin in recording media, allowing confirmation of whether cells or tissue retain their oscillation in knockout models. mPer2::luc mice were generated on a C57BL/6 background (Yoo \textit{et al.}, 2004), therefore it is expected that the immune response to \textit{T. muris} infection closely resembles that seen in wild-type C57BL/6 mice. If this is not the case, any changes in the immune response to \textit{T. muris} infection could not only be accredited to the planned disruption of clock gene expression in dendritic cells, but also changes in genetic background. To ensure the immune response of mPer2::luc mice is similar to C57BL/6 mice; mPer2::luc mice were infected at ZT0 and ZT12 with a high dose of \textit{T. muris} eggs and culled at day 21.

mPer2::luc mice infected at ZT0 did not have a significantly lower worm burden than ZT12 infected mice. Parasite specific antibodies were very similar in both ZT0 and ZT12 infected mice, while IgG1 antibody production was significantly elevated in ZT0 infected C57BL/6 mice in chapter 3. The lack of significant differences between ZT0 and ZT12 groups could be due to small numbers of mice per group, as group numbers in chapter 3 were higher.

\textbf{Th2 associated cytokine production in mPer2::luc mice infected at ZT0 was not as high as expected compared with C57BL/6 mice}

Th2 associated cytokine levels measured were not similar to those measured in C57BL/6 mice. Levels of IL-4, IL-5 and IL-13 did not differ between groups. Th1 associated cytokine IFN\gamma levels were also not higher levels in ZT12 infected mice, but 1 out of 5 mice in the ZT0 infected group produced higher levels of IFN\gamma than in mice infected at ZT12. Pro-inflammatory cytokine IL-6
was not significantly different between groups, but levels of TNFα were significantly up-regulated in ZT12 infected mice.

**Pathological measurements were similar in ZT0 and ZT12 infected groups**

Muscle wall thickness, intestinal crypt length and goblet cell numbers, measured to establish severity of pathology, did not show any differences between ZT0 and ZT12 infected mice, but also no change compared to naïve mice. In ZT0 and ZT12 infected C57BL/6 mice no difference in pathology was visible and it can be summarised that the same can be said for mPer2::luc mice.

**The high number of CD45^+ cells found in ZT12 infected mPer2::luc mice was not expected when comparing to C57BL/6 mice**

There was a no significant difference in numbers of CD11c^+ cells in ZT0 and ZT12 infected mice, but significantly more CD45^+ cells were found in intestinal tissue from ZT12 infected mice compared to ZT0 infected mice. In ZT0 and ZT12 infected C57BL/6 mice in chapter 3, no differences in infiltrating cells were seen. The high number of CD45^+ cells in the tissue of ZT12 infected mice might have changed the ability to expel the worm burden, as there was no significant difference in worm burden.

These elevated levels of infiltrating cells could be an indicator for persisting worm infection in ZT12 infected mice, while the number of CD45^- and CD11c^- cells in the colon tissue of ZT0 infected mice already returned to pre-infection levels, as most of the worms were expelled. Overall, the difference seen in C57BL/6 mice was not observed in mPer2::luc mice. This could be due to smaller group numbers or an incomplete similarity in genetic background. Mice containing the mPer2::luc reporter were nonetheless used for subsequent breeding of the new transgenic mouse line.

**Bmal1^floX^LysMcre mice produced a significantly higher IgG1 antibody titre than wild-type littermate controls**

Bmal1^floX^LysMcre mice lack circadian rhythm in macrophages, some granulocytes and few dendritic cell subsets (Hume 2011). Macrophages have so far not been assigned a role in successful expulsion of a *T. muris* infection (Bowcutt et al., 2011; deSchoolmeester et al., 2009), despite it being established that larger numbers of macrophages are found in resistant mice at the point of expulsion (Little et al., 2005).

An initial infection at ZT3 of Bmal1^floX^LysMcre mice and Cre negative littermate controls with a high dose of 200 *T. muris* eggs suggested that a stronger Th2 immune response to the parasite was produced in mice lacking functional Bmal1 expression and therefore circadian rhythm in macrophages. Even though the worm burden was not significantly lower in Bmal1^floX^LysMcre mice compared to littermate controls, these mice produced a significantly higher Th2 associated parasite specific antibody IgG1 response than the littermates. Parasite specific antibody IgG2c
was similar in Bmal1^flox^LysM^cre+^ mice compared to wild-type littermates. These results suggest that a lack in circadian rhythm in macrophages changes the local environment when the immune response is primed skewed towards a Th2 response.

**Cytokine responses in Bmal1^flox^LysM^cre+^ mice were no different compared to their wild-type littermate controls**

Interestingly, the up-regulation in Th2 response suggested by the antibody production was not seen in Th2 associated cytokine production. Levels of IL-4 and IL-5 were both similar in Bmal1^flox^LysM^cre+^ mice and wild-type littermate controls. Th1 associated cytokine IFNγ and pro-inflammatory cytokine IL-6 were also produced in similar levels in Bmal1^flox^LysM^cre+^ mice and wild-type littermate controls.

The lack of circadian rhythm in macrophages appears to only up-regulate Th2 associated antibody production, but could not be seen in Th2 associated cytokine production at day 21. Parasite specific antibody is cumulative, whilst cytokines are assayed at a single time point, therefore the difference could be seen at a different time point. These results suggest that macrophages have an impact on B cells and subsequent antibody production. Macrophages in the intestine mainly produce IL-10 and TNFα (Bain & Mowat, 2011). Using ELISA, IL-10 and TNF-α could not be measured in serum of Bmal1^flox^LysM^cre+^ mice and littermate controls, as they were below the level of detection. Therefore it is likely that these cytokine levels could have been too low to influence the immune response to *T. muris*.

**Analysis of muscle wall thickness, crypt length and goblet cell hyperplasia**

Muscle wall thickness, crypt length and goblet cell hyperplasia were measured to quantify pathology of colon tissue. No significant difference could be measured for any pathological measurements. No differences were seen for F4/80^+^, CD11b^+^, CD11c^+^ or CD45^+^ cells infiltrating into colon tissue, showing that cells with macrophage markers F4/80 and CD11b were found in similar numbers in the gut at day 21 post a ZT3 infection. Future ZT0/ZT12 infections could possibly show a difference in macrophage recruitment in mice infected at ZT12 compared to those infected at ZT0.

**LysM is not an exclusive marker for macrophages**

LysM has been extensively used as a marker for macrophages but also is also found on granulocytes. Clausen (1999) established that LysM^cre^ mice crossed with a mouse line expressing a *LoxP* site resulted in about 80% removal of the target gene in bone marrow derived macrophages and even 95% in fully differentiated cells from the peritoneum. Only about 16% of CD11c positive cells were affected by the deletion, most likely CD11c positive macrophages rather than functional dendritic cells.
Despite the expression of LysM on granulocytes, the impact on neutrophils, eosinophils or basophils was not examined in this experiment, which focused on the impact on macrophages. So far only the impact on neutrophils has been described (Clarke & Gordon, 1998; Clausen et al. 1999), leaving it unclear whether eosinophils or basophils commonly express LysM and are therefore lacking clock gene expression in Bmal1^flox/LysM^cre mice.

Granulocytes have not been found to be critical in T. muris expulsion, even though some are able to aid the process (Betts & Else, 1999; Perrigoue et al., 2009). In particular basophils have been described as T cell inducing cells, possibly replacing dendritic cells in the context of T. muris infection (Perrigoue et al., 2009; Wynn, 2009). In general, LysM does not allow a distinction between myeloid cells and therefore the increase of IgG1 found in Bmal1^floxlLysM^cre could possibly also be attributed to granulocytes.

Overall the results of the initial infection of Bmal1^floxlLysM^cre+ mice suggests that the disruption of circadian rhythm in macrophages causes a shift towards Th2 associated antibody production compared to littermate controls in the context of a ZT3 infection.

**Crossing two mouse lines to create mPer2::lucBmal1^floxCd11c^cre mice resulted in viable offspring**

mPer2::lucBmal1^floxCd11c^cre mice were bred from mPer2::lucBmal1^floxF and vβ8integrin^floxCd11c^cre transgenic mouse lines, until only the desired combination of genes could be found via PCR genotyping. All mice expressed luciferase with mPer2 expression, which enabled tracking of mPer2 expression in cells and tissue.

**Infection of mPer2::lucBmal1^floxCd11c^cre mice and wild-type littermate controls showed no difference in worm burden or parasite specific antibody production**

ZT0 infected wild-type littermate controls did not have significantly lower worm burdens than ZT12 infected wild-type littermates, which had been seen in ZT0 infected C57BL/6 mice established in chapter 3. ZT0 and ZT12 mPer2::lucBmal1^floxCd11c^cre mice were also not significantly different. Levels of parasite specific Th2 associated antibody IgG1 were significantly higher in ZT0 infected wild-type littermate controls compared to ZT12 infected wild-type littermates. This significant elevation in the level of IgG1 was also seen in ZT0 infected C57BL6 mice in chapter 3. mPer2::lucBmal1^floxCd11c^cre mice were not significantly different in IgG1 levels in ZT0 infected mice compared to ZT12 infected mice. Levels of Th1 associated parasite specific antigen IgG2c were not different between ZT0 and ZT12 infected mice and not between mPer2::lucBmal1^floxCd11c^cre mice and wild-type littermate controls.

Taken together, the no difference was seen comparing worm burden, compared to the delay seen in ZT12 infected C57BL/6 mice in chapter 3. Only Antibody production in littermate controls mirrored results in chapter 3, while no significant difference could be found in mPer2::lucBmal1^floxCd11c^cre mice.
Levels of cytokines IL-10 and IL-6 were reduced in ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice compared to ZT12 infected wild-type littermate controls, while Th1 and Th2 associated antibody levels did not differ between mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice and littermate controls.

Th2 associated cytokines measured in the supernatant of re-stimulated mesenteric lymph node cells showed no differences between mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice and wild-type littermate controls. Levels of IL-5 and IL13 were similar in ZT0 and ZT12 infected of mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice and ZT0 and ZT12 infected littermate controls. The Th1 associated cytokine IFNγ, which had generally been found at higher levels in ZT12 infected C57BL/6 mice (chapter 3), was found at similar levels in mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice and littermate controls. Levels of anti-inflammatory cytokine IL-10 were significantly higher in ZT12 infected wild-type littermates compared to ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice. IL-10 levels were not significantly higher in ZT12 infected wild-type mice compared to ZT0 infected wild-type controls, which is the same observed in C57BL/6 mice in chapter 3. There was no difference in IL-10 measured in ZT0 and ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice. Levels of pro-inflammatory cytokine IL-6 were also significantly higher in ZT12 infected wild-type controls compared to ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice. IL-6 levels were not significantly higher in ZT12 infected wild-type mice compared to ZT0 infected wild-type controls, while it was significantly higher in ZT0 C57BL/6 mice in chapter 3. There was no difference between levels of IL-6 in ZT0 and ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice. The level of pro-inflammatory cytokine TNFα was similar in all groups of mice. In chapter 3, TNFα levels measured in ZT12 infected C57BL/6 mice were significantly higher than in ZT0 infected mice, while lymph node cells from wild-type controls in this chapter produced similar levels of TNFα in ZT0 and ZT12 infected mice.

Taken together, Th1 and Th2 cytokines are the same in ZT0 and ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice as in wild-type littermate controls. In C57BL/6 mice IL-13 levels were significantly higher in ZT0 compared to ZT12 infected mice. There was no differences observed in IFNγ between ZT0 and ZT12 infected mice in this experiment, while in chapter 3 ZT12 infected C57BL/6 mice had significantly elevated IFNγ levels compared to ZT0 infected mice.

It appears only anti- and pro-inflammatory cytokines IL-10 and IL-6 were affected by removing the expression core clock gene Bmal1 from dendritic cells, as there was a significant difference between ZT12 littermate controls and mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice. Perrigoue et al. (2009) suggest that CD11c\textsuperscript{+} cells are not sufficient to induce Th2 cytokine immune responses to *T. muris* and therefore a lack of CD11c\textsuperscript{+} cells would not impact on the worm burden. This could explain the lack of a phenotype in mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice. Basophils have been described by Perrigoue et al. (2009) and Sokol et al., (2009; 2008) as a stronger antigen presenting cell than dendritic cells in the context of helminth infection and allergic responses and are able to secrete Th2 promoting cytokines. There is no indication in the literature if
basophils express clock genes in a diurnal way and therefore no indication if basophils could be involved in the delayed expulsion phenotype seen in ZT12 infected C57BL/6 mice.

**Muscle wall thickness was significantly reduced in ZT0 infected mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice compared to wild-type littermate controls, while crypt length and goblet cell hyperplasia showed no difference between ZT0 and ZT12 infected mice**

Measurements of muscle wall thickness as an indicator of pathology has not showed any differences between C57BL/6 mice infected at ZT0 and ZT12 in previous chapters. There was nonetheless a significant reduction in muscle wall thickness in ZT0 infected mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice compared to littermate controls. This difference was not seen in ZT12 infected mice. Muscle wall thickness is a read-out for muscle hypercontractility, which is regulated by IL-9. Neutralization of IL-9 using IL-9-OVA complex vaccination reduced contractility of the colon in a subsequent *T. muris* infection and the worm burden was significantly higher in mice vaccinated with IL-9-OVA complex compared to mice vaccinated with OVA alone, indicating the importance of muscle contractility in *T. muris* expulsion (Khan et al. 2003). In the infection of mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice and littermate controls, levels of IL-9 were below the threshold of detection by cytometric bead array, leading to the assumption that any differences in IL-9 production should be too low to influence pathology.

Crypt length and goblet cell hyperplasia, two further indicators of severity of pathology showed no difference between ZT0 and ZT12 infected mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice and littermate controls, which was also seen in ZT0 and ZT12 infected C57BL/6 mice in chapter 3.

**Infiltrating CD45\(^{+}\), F4/80\(^{+}\) and CD11c\(^{+}\) cells were found in similar numbers in mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice and wild-type littermate controls**

The number of CD45\(^{+}\) lymphocytes infiltrating colon tissue was similar in mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice and wild-type littermate controls. F4/80\(^{+}\) cells were also found in similar quantities, while CD11c\(^{+}\) cell numbers were non-significantly lower in ZT0 infected wild-type controls than ZT0 infected mPer2::lucBmal1\(^{flox}\)CD11c\(^{cre}\) mice. Previous analysis of cells infiltrating colon tissue post infection at ZT0 and ZT12 in C57BL/6 mice showed no differences in cell numbers for CD45\(^{+}\), F4/80\(^{+}\) and CD11c\(^{+}\) cells. This leads to the conclusion that the lack of clock gene expression in CD11c\(^{+}\) cells does not influence lymphocyte homing to colon tissue in the context of this experiment. Dendritic cells recruit T cells to the colon and secrete cytokines, which could be gated by circadian rhythm and therefore affect the number of cells infiltrating colon tissue following infection.
Bone marrow derived dendritic cells cultured from mPer2::lucBmal1\textsuperscript{flo}\textsuperscript{x}CD11c\textsuperscript{cre} mice and wild-type littermate controls were analysed for the absence of Bmal1 exon 8 in CD11c positive cells

As there was no different phenotype in mPer2::lucBmal1\textsuperscript{flo}CD11c\textsuperscript{cre} mice compared to wild-type littersates in worm burdens, parasite specific antibody production and Th1/Th2 associated cytokines, it had to be evaluated if the circadian rhythm was indeed disrupted in dendritic cells of mPer2::lucBmal1\textsuperscript{flo}CD11c\textsuperscript{cre} mice. Exon 8 of the Bmal1 gene should have been removed when Cre is expressed under the CD11c promoter. Comparing PCR results for the presence of Bmal1 exon 8 in cDNA of CD11c positive cells and non-sorted bone marrow derived dendritic cells cultured from one mPer2::lucBmal1\textsuperscript{flo}CD11c\textsuperscript{cre} mouse and one littermate control showed that the 137bp band for the Bmal1 exon 8 gene was present in all four samples. Surprisingly, there were also 300bp and 500bp bands on the gel. It is unclear why these bands showed on the gel and lead to the speculation that the samples could have been contaminated during RNA extraction or cDNA conversion.

Subsequent genotyping of CD11c positive sorted and non-sorted bone-marrow derived dendritic cells cultured from the mPer2::lucBmal1\textsuperscript{flo}CD11c\textsuperscript{cre} mouse and littermate control for the presence of CD11c\textsuperscript{cre} showed that both non-sorted cell populations contained CD11c\textsuperscript{cre} positive cells, leading to the assumption that both mice analysed were indeed CD11c\textsuperscript{cre} positive. Cells sorted previously for the presence of CD11c showed a band for CD11c\textsuperscript{cre} in the mouse expected to be CD11c\textsuperscript{cre} positive, but in the mouse assumed previously to be CD11c\textsuperscript{cre} negative, a band appeared at 600bp rather than 300bp, the band expected in CD11c\textsuperscript{cre} positive samples.

Further analysis of mPer2::lucBmal1\textsuperscript{flo}CD11c\textsuperscript{cre} mice and littermate controls will be necessary to confirm if Bmal1 exon 8 was successfully removed from CD11c positive cells using genomic DNA rather than cDNA, as even the smallest amount of contaminating CD11c negative cells would lead to a false positive band on a gel. Primers for the ‘floxed’ product as well as the Δ product, the removed section of the gene, will be required. The ratio between the results of these two can be compared and will finally conclude whether Bmal1 exon 8 was successfully removed from CD11c\textsuperscript{cre} positive cells.

The Cre\textsuperscript{LoxP} system is able to remove the target gene in most cells expressing the promoter

Many mice expressing Cre recombinase have been created over the years and, depending on the site of the target gene, can vary in efficiency, especially in tissue or cell specific targets (Nagy 2000). Comparing CD11c\textsuperscript{cre} and LysM\textsuperscript{cre} reporter mouse lines in the literature, even though they do not always reach 100% removal of the target gene (Caton \textit{et al.}, 2007; Clausen \textit{et al.}, 1999) the Cre\textsuperscript{LoxP} system commonly leads to physiological changes. By using established mouse lines with functional CD11c\textsuperscript{cre} and Bmal1\textsuperscript{flo} to breed the new transgenic mouse line, efficient gene excision was expected.
If the circadian expression of Bmal1 was successfully disrupted, it is unlikely that circadian rhythm in dendritic cells is the mechanism resulting in the delayed worm expulsion in ZT12 infected mice, as there was no difference in ZT0 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice versus ZT0 infected littermate controls and no difference in ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice versus ZT12 infected littermate controls.

The lack of phenotype would also be explained if mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice have no disrupted circadian rhythm in CD11c\textsuperscript{+} dendritic cells and Bmal1 exon 8 is not removed. Cytokines IL-10 and IL-6 were expressed differently in ZT12 infected wild-type controls compared to ZT12 infected mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice, leading to the suggestion that there is a difference in the phenotype of the transgenic mouse compared to the wild-type littermate control, even if it is not as pronounced as hypothesised and dendritic cells are unlikely to be the mechanism causing the delay in worm expulsion.

Only cells expressing the cell surface marker CD11c should lack a circadian rhythm. This marker is traditionally associated with dendritic cells but can also be found on some macrophage subsets and T cells (Hume, 2011). CD11c\textsuperscript{cre} mice have been found however to not only disrupt dendritic cells, but also many macrophage subpopulations (Bar-On & Jung, 2010). Nonetheless, CD11c is commonly still used as a dendritic cell marker even though it has been shown by several studies (reviewed in Hume, 2011) that macrophages are depleted as well as dendritic cells using this cell surface marker.

**LysM and CD11c cell markers can be found on overlapping cell populations**

CD11c is found on macrophages and dendritic cells. LysM is also expressed by macrophages, dendritic cells and even granulocytes (Clausen et al., 1999). Comparing Bmal1\textsuperscript{fox}LysM\textsuperscript{cre} and mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice, it is interesting to see that Bmal1\textsuperscript{fox}LysM\textsuperscript{cre} mice have an up-regulated expression of IgG1 compared to their littermate controls while mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice produce the same amount of IgG1 in a ZT0 infection as their littermate controls. Bmal1\textsuperscript{fox}LysM\textsuperscript{cre} mice also appear to expel their worm burden more efficiently than their wild-type littersmates while the worm burden in mPer2::lucBmal1\textsuperscript{fox}CD11c\textsuperscript{cre} mice and littermate controls is similar. Therefore the cell types affected in the two transgenic lines must be different, even though LysM and CD11c can be found in overlapping populations.

Concluding, one can say that myeloid cells do play a role in the immune response to *Trichuris muris* but further investigation is needed to establish if these cells are the gating factor leading to delayed worm expulsion in ZT12 infected mice, as results presented in this chapter are still inconclusive.
Summary:

- mPer2::luc mice did not show a difference between ZT0 and ZT12 infected mice.
- Bmal1\textsuperscript{flox}LysM\textsuperscript{cre} mice produce a stronger Th2 antibody response than littermate controls in an initial infection at ZT3.
- mPer2::lucBmal1\textsuperscript{flox}CD11c\textsuperscript{cre} mice show no difference in worm burdens, Th1 or Th2 associated parasite specific antibody production and Th1/Th2 associated cytokine production, while only Th2 antibody production is significantly elevated in ZT0 compared to ZT12 infected wild-type littermate controls.
- mPer2::lucBmal1\textsuperscript{flox}CD11c\textsuperscript{cre} mice have lower IL-10 and lower IL-6 levels in ZT12 infected mice compared to littermate controls.
- It is unclear whether Bmal1 exon 8 is successfully removed in bone marrow derived dendritic cells cultured from mPer2::lucBmal1\textsuperscript{flox}CD11c\textsuperscript{cre} mice, therefore dendritic cells may still retain a functional clock.
Summary discussion
Circadian rhythms can be found throughout the mammalian body. Sleep-wake cycles, metabolism, hormone secretion and many other physiological processes are regulated in a 24 hour rhythm, which allows anticipating changes in the environment and adapting to external circumstances. Circadian rhythm of the immune response enables mammals to anticipate infection and mount the adequate response when encountering a pathogen.

Diurnal rhythms of many components of the immune response are one of the physiological rhythms found throughout the body. Lymphocyte numbers oscillate during the day in peripheral blood and the spleen (Abo et al., 1981; Kawate et al., 1981), and have been negatively correlated to the daily rhythms of cortisol in humans and corticosterone in mice (Cutolo et al., 2003). The distribution of leukocytes in tissues has also been linked to circadian rhythms, as adhesion molecules and chemokine expression is up-regulated in the active phase (Scheiermann et al., 2012). Therefore even something as simple as taking a blood sample at different times of the day might lead to varying interpretations of the results, as the number of leukocytes and cytokines measured could be dependent on the time of blood collection.

Not only do endocrine and hormonal signals orchestrate the oscillation of immune cells, many cells within the immune system follow their own intrinsic rhythm, such as macrophages, natural killer cells, T cells and dendritic cells (Arjona & Sarkar, 2005; Fortier et al., 2011; Keller et al., 2009; Logan & Sarkar, 2012; Silver et al., 2012b). Intrinsic circadian rhythms could lead to rhythms in function such as diurnal variations in phagocytic ability and cytotoxicity in NK cells, which peaks during the night in mice (Arjona & Sarkar, 2005; Logan & Sarkar, 2012) and T cell proliferation, whereby T cell cytokine production and responses to antigen are also under circadian control (Bollinger et al., 2011; Fortier et al., 2011). A diurnal rhythm occurs in humans, with Th1 immune cells and cytokines peaking during the night and Th2 immune responses and cells peaking in the morning (Cutolo et al., 2003).

Disruption of circadian rhythms, due to shift work or jet lag, has been implicated in many diseases (reviewed in Harrington, 2010) and many studies showed that disruption of the core clock result in up-regulated inflammatory responses (Hashiramoto et al., 2010; Narasimamurthy et al., 2012). Lack of sleep or disrupted sleep has been suggested to increase the danger of infection, as stress responses dampen the immune system (Barriga et al., 2001; Bollinger et al., 2009; Lange et al., 2006). Regular circadian rhythms could therefore important to enable the body to anticipate infection and mount appropriate inflammatory responses (Preston et al., 2009). Identifying the time of day the body is most able to defend against a pathogen could aid drug development and treatment regimen through reduction of drug doses and therefore possible side effects.

To summarise, the research indicates that there could a close interplay between circadian rhythms and the immune system; that there is scope for further investigation and such knowledge can aid the field of translational medicine.
Diurnal rhythms in LPS models

Several studies have shown that the time of day in infection can influence the outcome of infection or treatment. Early studies often involved administration of LPS, which is a strong immunogenic compound. Lethal doses, such as 20mg/kg, of LPS lead to higher mortality when administered during the light phase compared to the dark phase, but not if mice were housed in constant conditions (Halberg et al., 1960; Marpegan et al., 2009). Administration of low doses, such as 25μg/kg, of LPS resulted in a phase delay in locomotor activity when given at circadian time (CT) 15 compared to CT3 in mice housed in constant conditions (Marpegan et al., 2005). I.p. injection with LPS also showed a difference in the magnitude of the cytokine response depending on the time of injection when mice were housed in constant conditions. Levels of IL-6, IL-12 and TNFα were higher when LPS was given at CT12 compared to CT0, which was not seen in vehicle treated controls or Bmal1flo−/−LysMcre mice treated with LPS (Gibbs et al., 2012). The diurnal effect seen through LPS treatment depends on TLR-4, as no phase delay could be observed when mice were treated with low doses of LPS (Paladino et al., 2010). Even though these experiments show fundamental circadian responses to LPS administration in a time dependent manner, LPS is not an ideal representative of infection, as it solely signals through TLR-2 and TLR-4, while infection with a whole pathogen might interact with the host in a more diverse fashion and not all pathogens signal via TLR-4. TLR expression has been shown to be rhythmic (Froy & Chapnik, 2007; Mukherji et al., 2013), which would explain the diurnal response to LPS treatment.

Diurnal responses in in vivo models

More in vivo models emerge showing circadian timing in infection with bacteria. Infection with Salmonella enterica serovar typhimurium in the morning leads to higher infection rate and a stronger immune response, with higher cytokine production and more severe pathology, compared to later time points of infection. This could be due to rhythmic expression of antimicrobial peptides in the intestine (Bellet et al., 2013). A perforated bowel model for sepsis showed increased severity of colonisation in the peritoneum when the colon was punctured at ZT19 compared to ZT7. These results related to the diurnal expression of TLR-9 within the colon (Silver et al., 2012a).

Both experiments show that the use of whole pathogen results in strong time of day dependent infection outcomes. Nonetheless, bacteria induce a Th1 inflammatory response and the T. muris model shown in this thesis aimed to evaluate a Th2 inflammatory setting, suggesting that even after 3 weeks of infection, time of day of infection dependent responses can still be seen. ZT12 infected mice did show delayed worm expulsion and were more skewed towards Th1 responses than ZT0 infected mice, which were skewed towards Th2 responses. Future work could include keeping mice in constant conditions, to observe if the difference in immune response between ZT0 and ZT12 infected mice can also be found at CT0 and CT12, or if entrainment of the SCN by light is required.
C57BL/6 mice used for ZT0 and ZT12 infection produce a mixed Th1/Th2 response when infected with *T. muris*. The further mice are skewed towards Th2 and less towards Th1, the faster expulsion occurs. This balance might be influenced by circadian rhythm, as ZT0 and ZT12 mice both produce Th1 and Th2 responses, but while mice infected at ZT0 respond with a stronger Th2 response, ZT12 infected mice initially produce a strong Th1 response, which is later overtaken by a Th2 response, leading to delayed worm expulsion.

When the immune system is pushed towards either Th1 or Th2 responses, by either administering a low dose infection, which results only in a Th1 response and chronic *T. muris* infection, or by vaccination, which primes strong Th2 responses in ZT0 as well as ZT12 vaccinated mice, no diurnal differences can be observed. Diurnal effects seen in IL-6 cytokine production after induction of Th1 responses using LPS (Gibbs *et al.*, 2012) were not seen in the *T. muris* model. These results suggest that given the right stimulus, the immune response overwrites circadian rhythm and produces strong Th1 or Th2 responses required to fight the infection. As vaccination with 100 μg *T. muris* excretory/secretory antigen induced strong Th2 responses in mice whether they are vaccinated at ZT0 or ZT12, a dose that is less efficient at inducing sterile immunity could show there is a circadian element to vaccination with *T. muris* E/S. 50 μg has been shown by Dixon (2007) to induce a significant worm expulsion, and even as little as 10 μg can still reduce worm numbers, even though this did not reach statistical significance. Exploration of these concentrations may elucidate doses allowing the difference in immune priming between the time points of vaccination to become obvious. Future work should also investigate the mice vaccinated at ZT0 and ZT12 but infected at ZT12, when Th2 responses seemed less adequate for quick worm expulsion.

**Gating factor of delayed expulsion responses**

As many components of the immune response are under circadian control, experiments in this thesis aimed to identify which cell type could be the gating factor in the early establishment of inflammatory responses in the *T. muris* model. As antigen presenting cells are required to initiate an immune response to the pathogen and have been shown to possess intrinsic circadian rhythm (Keller *et al.*, 2009; Silver *et al.*, 2012b), macrophage and dendritic cell numbers were examined during the first days post infection, but no differences in cell infiltration could be observed. This did not exclude a functional difference, therefore transgenic knock-out mice were used to examine a lack of circadian rhythm in macrophages and dendritic cells. Bmal1<sup>lox/lox</sup>LysM<sup>cre</sup> mice, lacking circadian rhythm in macrophages showed a stronger Th2 antibody response in a primary morning infection compared to wild-type littermates. Future work could include a comparative study of ZT0 and ZT12 infected Bmal1<sup>lox/lox</sup>LysM<sup>cre</sup> mice and littermate controls, to establish if the difference seen in the primary infection also occurs in mice infected in the evening. mPer2::LucBmal1<sup>lox/lox</sup>CD11c<sup>cre</sup> mice, which should lack circadian rhythm in dendritic cells, showed the no significant difference between ZT0 and ZT12, as well as in wild-type littermate controls. It is still unclear whether rhythmic clock gene expression in these
mice was disrupted, therefore future work needs to investigate if exon 8 of the Bmal1 gene was successfully removed, disrupting Bmal1 expression or if CD11c positive cells still oscillate.

If it is established that dendritic cells are not influencing infection outcome depending on the time of day of infection, T cells and epithelial cells should be analysed as possible candidates. Breeding of a mouse line lacking circadian rhythm in T cells has already been carried out and it has been established that these mice lack rhythmic clock gene expression in T cells. These mice could be used in ZT0 and ZT12 infection experiments to establish the influence of intrinsic T cell rhythm on the immune response to T. muris infection. Breeding a mouse line lacking circadian rhythm in the epithelial lining of the gut could also prove beneficial using Bmal1\textsuperscript{fox} and Vili\textsuperscript{cre} mouse lines. This mouse line would not only examine the involvement of circadian rhythm within epithelial cells of the intestine, but also highlight the importance of chemokines produced by the epithelial lining in the recruitment of immune cells to the site of infection.

**Rhythmic activity in the intestine is beneficial for a healthy bowel**

The intestinal tract is designed to digest food and absorb nutrients, which depends on feeding rhythms. In shift work regular meal times are disrupted, which can change intestinal motility, cell signalling and regulation of inflammation and lead to constipation or diarrhoea and even diseases such as IBD, IBS, ulcers and cancer (reviewed in Malloy et al., 2012). Infection with T. muris causes similar inflammatory responses to IBD; therefore infection with this parasite is used to model the disease.

The presence of microbiota in the intestine is required for hatching of T. muris (Hayes et al., 2010), as well as time keeping in the intestine. Expression of clock genes, but also genes needed in the innate and adaptive immune response, is controlled by signalling of microbiota via TLRs, which appears to be a self-regulating system. In the absence of microorganisms in the intestine, rhythm appears to be lost in colon tissue (Mukherji et al., 2013).

It has been shown in the small intestine that immune components such as toll-like receptors and cryptidins are expressed in a circadian manner, which could influence the immune response mounted in the gut (Froy & Chapnik, 2007; Mukherji et al., 2013; Silver et al., 2012a). In mice, which normally feed during the night, TLRs are up-regulated in the morning, when food is reaching the intestine (Froy & Chapnik, 2007). Infection models with time of day dependent immune responses in the colon mentioned above (Bellet et al., 2013; Silver et al., 2012a) can be linked to rhythmic expression of immune components in the gut. Even though T. muris infection appears to be independent of TLRs which signal through MyD88, such as TLR-4, TLR-2 and TLR-9, (Helmby & Grencis, 2003) other pathogen recognition receptors could follow a similar circadian expression pattern. So far it has not been established how the host recognises the parasite and therefore it cannot be examined if the pathogen recognition receptor is expressed in a diurnal fashion.
In the absence of the SCN, restricted feeding can be used to entrain clock gene expression in peripheral tissues (Hoogerwerf et al., 2007; Sládek et al., 2007), thanks to a food entrainable oscillator which is semi-independent of the SCN (Hoogerwerf et al., 2007; Polidarová et al., 2011). Clock gene expression in different sections of the intestinal tract regulates periodic activity depending on the availability and anticipation of food, as do innate immune components such as TLRs and cryptidins. To establish if the clock gene expression in the colon in the first week of infection is able to influence the outcome of infection with T. muris, mice were food restricted to mid-light or mid-dark phase, which should reverse clock gene expression in colon tissue. This was nonetheless only observed partially in this thesis. No delayed worm expulsion could be seen in mice fed during the light phase and infected at ZT0 compared to mice fed during the dark phase. Therefore feeding status alone cannot be the gating factor in the delayed worm expulsion seen in ZT12 infected mice in chapter 3.

Corticosterone has been identified as a potential factor underlying time of day dependent effects in chronic infections such as asthma and arthritis. In T. muris, administration of corticosterone can ensure parasite survival (Else et al., 1992b), despite the link of corticosterone to Th2 responses in humans (Cutolo et al., 2003). Over the time course, in ZT12 infected mice, corticosterone levels dampened. Future work could investigate corticosterone rhythm and amplitude of corticosterone levels at ZT0 and ZT12 in both groups of mice, as well as the ability to expel the parasite in mice where corticosterone levels are kept constantly high or low to identify the magnitude of the role corticosterone plays in time of day dependent immune responses in T. muris infection.

Overall it has to be acknowledged that an inflammatory response in turn can also impact on circadian rhythm and overwrite rhythmic clock gene expression. The immune response and circadian rhythm are closely intertwined. Inflammatory responses for example result in increases of temperature at the site of infection and it has been shown that rising of temperature by few degrees can result in resynchronisation of cells in culture (Buhr et al. 2010). Further analysis of clock gene expression in colon tissue taken from infected animals could shed light on the influence the inflammatory responses has on the local circadian clock in the intestine.

**Chronotherapy suggests that time of day matters**

Chronotherapy is an emerging practice of adjusting the time of drug administration so that the benefits are maximised while side effects are reduced. Nocturnal asthma and rheumatoid arthritis patients both suffer from exacerbation of disease symptoms in the late night or early morning respectively, which can be countered by administration of glucocorticoids in the evening as it dampens the magnitude of pro-inflammatory cytokines (Petrovsky et al., 1998). This form of chronotherapy reduces the amount of drug needed over the course of the day (Burioka et al., 2010).
In cancer treatment and vaccination the time of drug administration can influence the outcome of the treatment in some patients. Chronotherapy in cancer treatment aims to modulate cell proliferation cycles. When patients are treated at the point when tumour cell proliferation is highest, while host cell proliferation is low, effects of anti-tumour drugs appear to be maximised, while side effects are low (Lévi et al., 1997; 1990; Mormont & Lévi, 2003; Schmiegelow et al., 1997). Anti-tumour treatment with IFNβ and anti-viral treatment with IFNα have been established to be less disruptive of body temperature and locomotor activity when administered in the morning than in the evening in mice (Ohdo et al., 2000; Takane et al., 2000). Mice vaccinated with OVA and TLR-9 ligand CpG ODN as an adjuvant at ZT19 had higher cytokine responses compared to mice vaccinated at ZT7. At ZT19 TLR-9 expression was highest, therefore immune responses were maximised due to the use of a TLR-9 ligand as adjuvant. Even 4 weeks after vaccination, ZT19 vaccinated mice showed increased lymphocyte proliferation and cytokine production compared to ZT7 vaccinated mice (Silver et al., 2012a). Human vaccination trials showed elevated antibody titres when vaccinated against Hepatitis B at noon compared to the morning (Pollmann & Pollmann, 1988) and increased numbers of leukocytes could be measured earlier in patients vaccinated with live, attenuated Venezuelan equine encephalomyelitis virus vaccine at 8 am compared to 8 pm (Feigin et al., 1967).

Together these results show that the time a drug is administered could impact on the outcome of disease. Parasites become more and more resistant to anti-helminthic drugs as re-infection rates are high. Development of new drugs and vaccines would greatly improve conditions in the developing world. Establishment of an optimal time to administer existing drugs could prolong the time existing drugs can be used.

Concluding words

Overall the experiments in this thesis conclude that circadian rhythm could play a role in the ability of the immune system to prime a strong Th2 response. C57BL/6 mice are classed as slow responders to T. muris infection and in this animal model, high dose infection at ZT0 are more effectively expelled than at high dose infections given at ZT12. Subsequent experiments were nonetheless often inconclusive to the extent of the day-night difference and significance was only reached in large group sizes. Therefore the importance of delayed worm expulsion has to be examined further.

Many early models looking at the circadian influence of inflammation used LPS. LPS is commonly used to induce Th1 inflammatory responses, in ex vivo/ in vitro cultures. In vivo LPS is not a very realistic model, as it is not representative of actual infection. Now more models are published using bacterial infection models (Bellet et al., 2013; Silver et al., 2012a), representing interaction between pathogen and immune responses better than LPS by itself. The T. muris model is useful to examine the balance between Th2 and Th1 immune responses, while bacterial infections result only in Th1 responses.
In laboratory settings, experimental design is often restricted by working hours and the time of infection or treatment is depending on the daily work load of the individual researcher and not fixed to one time of day. The *in vivo* studies showed that the time of infection impacts on the outcome of many infection models and the results presented in this thesis show the importance of regular schedules in mouse experiments. Time of treatment administration should also be under close scrutiny. Therefore, within a study, a schedule should be adhered to which ensures that results are not skewed by circadian rhythm. Studies examining asthma, arthritis or other conditions with circadian aspects should be monitored closely for time of day in experimental set up to ensure that results are physiologically relevant. For *T. muris*, infection occurs with ingestion of food and is therefore likely to occur during the night in nature and reach the colon in the morning, which is when immune responses are optimal for expulsion.
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