Module 1

PRISON BREAK

The natural egress is a tightly programmed event in *Toxoplasma gondii*

Soldati-Favre Lab
Faculty of Medecine – University of Geneva (Switzerland)
Dominique Soldati-Favre (dominique.soldati-favre@unige.ch)
Gaelle Lentini (gaelle.lentini@unige.ch)
Hugo Bisio (hugo.bisio@unige.ch)
Adi Mukherjee (budhaditya.Mukherjee@unige.ch)

I. INTRODUCTION TO *TOXOPLASMA GONDII*

Toxoplasma gondii (*T. gondii*) belongs to the phylum of Apicomplexa. The Apicomplexa phylum grouped diverse unicellular eukaryotes that are all obligate intracellular parasites. *T. gondii* is one of the most ubiquitous parasite of this phylum as it commonly infects many warm-blooded animals and is able to replicate in a various number of host cells.

Its life cycle is complex and can be divide in two parts (**Figure 1**). The first one occurs in the definitive host, the Felidae where the parasite multiplies sexually, forming oocyst that are then released in the feces. By ingestion of oocyst the intermediate hosts (warm-blooded animal) get infected constituting the second part of the life cycle. Oocysts will release sporozoites that differentiate into tachyzoites and invade tissue where they fastly multiply. A proportion of tachyzoites differentiate into bradyzoites, the latent form of the parasite mainly found in brain, liver and muscle. Bradyzoite are trapped inside a cyst and can persist as it for years or decades constituting a *T. gondii* reservoir. The life cycle starts over when cysts are ingested by Felidae (by predation for example) releasing bradyzoites that will convert in male or female gametocytes and form a new zygote that will mature in oocyst.

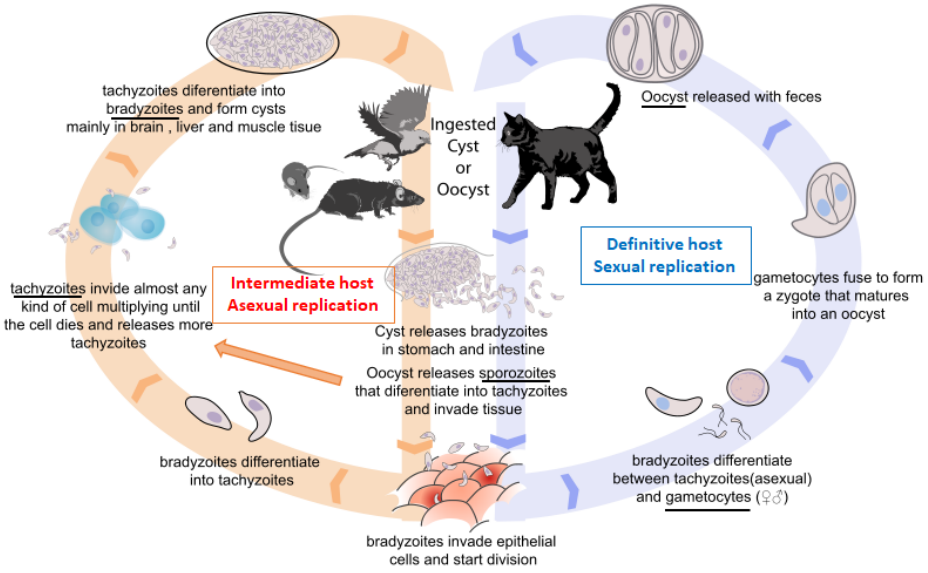


Figure 1. Life cycle of *Toxoplasma gondii* (wikipedia).

In the intermediate hosts, *T. gondii* is only able to divide in an asexual manner called endodyogeny. During endodyogeny, the two daughter cells are formed inside the mother cell. Following the emergence of the two daughter cells, the mother cells will degenerate, and the daughter cells will be ready to enter a new division cycle (around 8 hours for the full division cycle) (**Figure 2**). Parasites can be easily maintained *in vitro* during their asexual cycle whereas no system is currently available to study the sexual cycle in Felidae otherwise that in *vivo*.

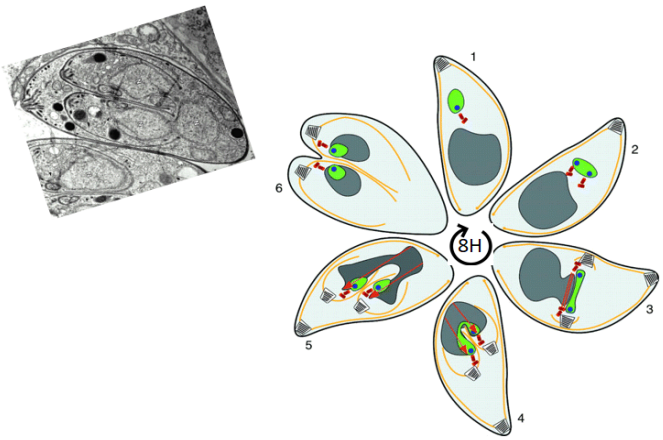
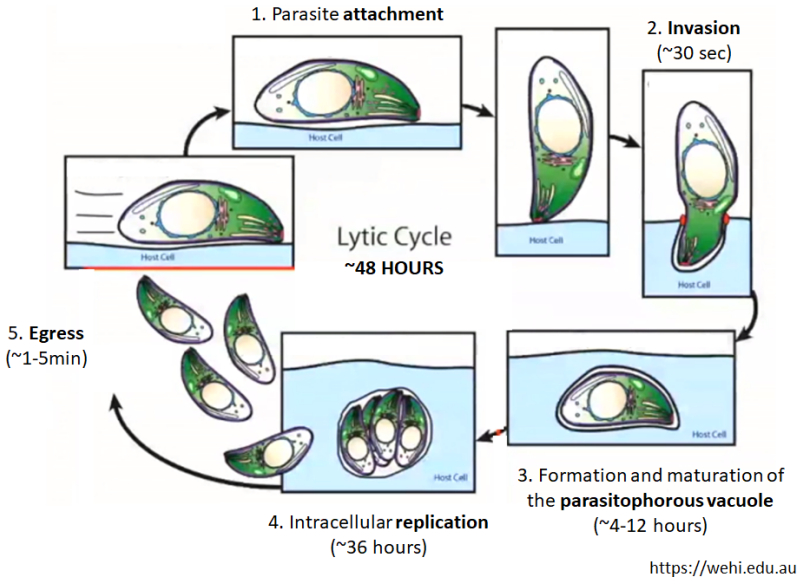


Figure 2. Cell division cycle of *T. gondii*. *T.gondii* divides by endodyogeny in which 2 daughter cells are built inside a mother cell (cf steps 3 to 6 and electron microscopy). Striepen *et al.* 2000

Studies in vitro of the asexual cycle have been incredibly useful to dissect the cellular and molecular events that composed the lytic cycle of *T. gondii*. As an obligate intracellular parasite, the lytic cycle of *T. gondii* is define as the set of events that occurred from the recognition of the host cell by the parasite to the lysis of the host cell due to intracellular parasite replication (**Figure 3**). For *T. gondii*, the lytic cycle takes approximately **48 hours**.

The major steps of the lytic cycle are the following:

1. Parasite Attachment to the host cell
2. Invasion of the host cell
3. Formation and maturation of the parasitophorous vacuole
4. Intracellular replication
5. Egress (lyse of the host cell)
6. Parasite gliding motility to move to a neighbor cell



Interestingly, all the steps of the lytic cycle are dependent of parasite-secreted proteins. T. gondii possesses 3 types of secretory organelles. The micronemes, the rhoptry and the dense granules. Micronemes and rhoptry are apical organelle that are secreted in a temporal fashion whereas dense granules are uniformly distributed in the cell body and are secreted in a constitutive manner. A boost of microneme secretion occurs just after the first contact between the parasite and its host cell, followed directly by rhoptry secretion. This sequential secretion of microneme and rhoptry is crucial for parasite penetration into the host cell. While the parasite enters, it derives host cell plasma membrane to form a parasitophorous vacuole that will reside in the cytoplasm of the infected cell and protect the intracellular parasites from host attack. Dense granules are secreted during all the intracellular life of *T. gondii* and participates to the establishment of the parasitophorous vacuole, its maturation and its enlargement.

1. Parasite Attachment

The first contact between *T. gondii* and its host cell occurs via interaction between parasite surface glycoproteins and mammalian cell surface proteoglycans. This first interaction triggers a boost of microneme proteins (MICs) among which several adhesins will be associated with the parasite plasma membrane following secretion. These adhesins will establish a strong interaction between the parasite and its host cell.

2. Invasion

Once the parasite is strongly attached to the host cell, the rhoptry proteins (ROPs) will be secreted. On the contrary to the MICs, ROPs are directly injected in the cytoplasm of the host cell by an unknown mechanism. Following secretion, some of the rhoptry protein will remain associated with the cytoplasmic face of the host plasma membrane. These proteins will form a complex called the moving junction (red dots on step 2, Figure 3) that is crucial for the entry of the parasite in the host cell and for the formation of the parasitophorous vacuole in which the parasite resides during its intracellular life. Other ROPs will be targeted to the host cytoplasm, the host organelles or nucleus to modulate the host response to infection.

3. Formation and maturation of the parasitophorous vacuole (PV)

The PV is form during the invasion process and derives from the host plasma membrane. The PV is an important element of the infection process because it plays the role of an interface between the host cytoplasm and the parasite surface. The PV is modified by parasite proteins mainly dense granule proteins and will protect the parasite from the host immune response. The PV membrane is also the place where the parasite will engulf host nutrients for its own growth. To do so, host organelles such as the mitochondria, endoplasmic reticulum, Golgi and endo-lysosomes are recruited at the parasitophorous vacuole membrane.

4. Replication

Once well established in the parasitophorous vacuole, a single *T. gondii* parasite will replicate by endodyogeny - two daughter cells are formed inside a mother cell. As the daughter cells emerge from the mother, this last one degenerate and the two daughter cells are ready to start a new cycle. The approximative duration of one division cycle is 8 hours. However, inside a cell, the parasite will undergo

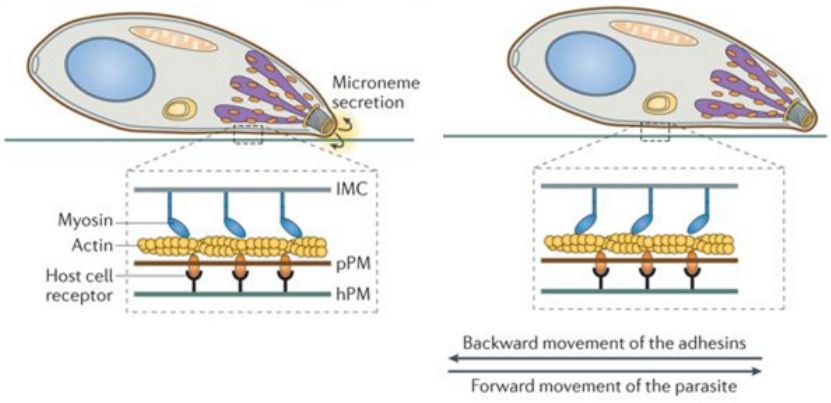
more or less 6 cycles of division – reaching the number of 64 parasites / cell before actively getting out by lysing the PV and the host cell.

5. Egress

In our culture model that consist in fibroblast cells infected by *T. gondii*, intracellular parasites naturally egress from their host cell roughly 48 hours post-infection. Egress is the result of a signaling cascade that start with external stimuli and lead to microneme secretion. Among the secreted MICs, some proteins such as proteases will play a role in the rupture of the parasitophorous vacuoles and other MICs will initiate parasite motility thought to mechanically help the egress of parasites from the host. Interestingly, egress do not happen always at 48 hours post-infection. For example, parasites undergo a higher stress in macrophages due to their oxidative nature and parasite egress occurs earlier. The inconsistency of egress time account for a regulated sensing mechanism turning on active egress signaling cascade rather than mechanical constraints triggering host cell rupture.

6. Gliding motility

Gliding motility is an energy-dependent mechanism by which the parasite moves without deforming its cell body. To do so, transmembrane secreted MIC (orange) exposed at the surface of the parasite interact with host cell receptors on their extracellular side and with the complex motor-actin on the parasite cytoplasmic site. MyosinA (MyoA – blue) forms a motor complex that will displace the MICs-actin filament complex along the plasma membrane of the parasite generating a movement of adhesins going from the apical to the basal end of the parasite. Gliding motility will be used by the parasite to efficiently egress from the host cell and to reach a new host cell to infect.

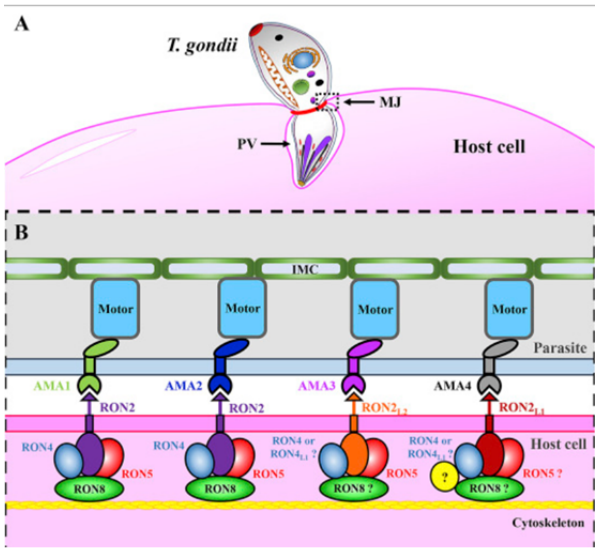


Frénal et al. 2017

Figure 4. Gliding motility

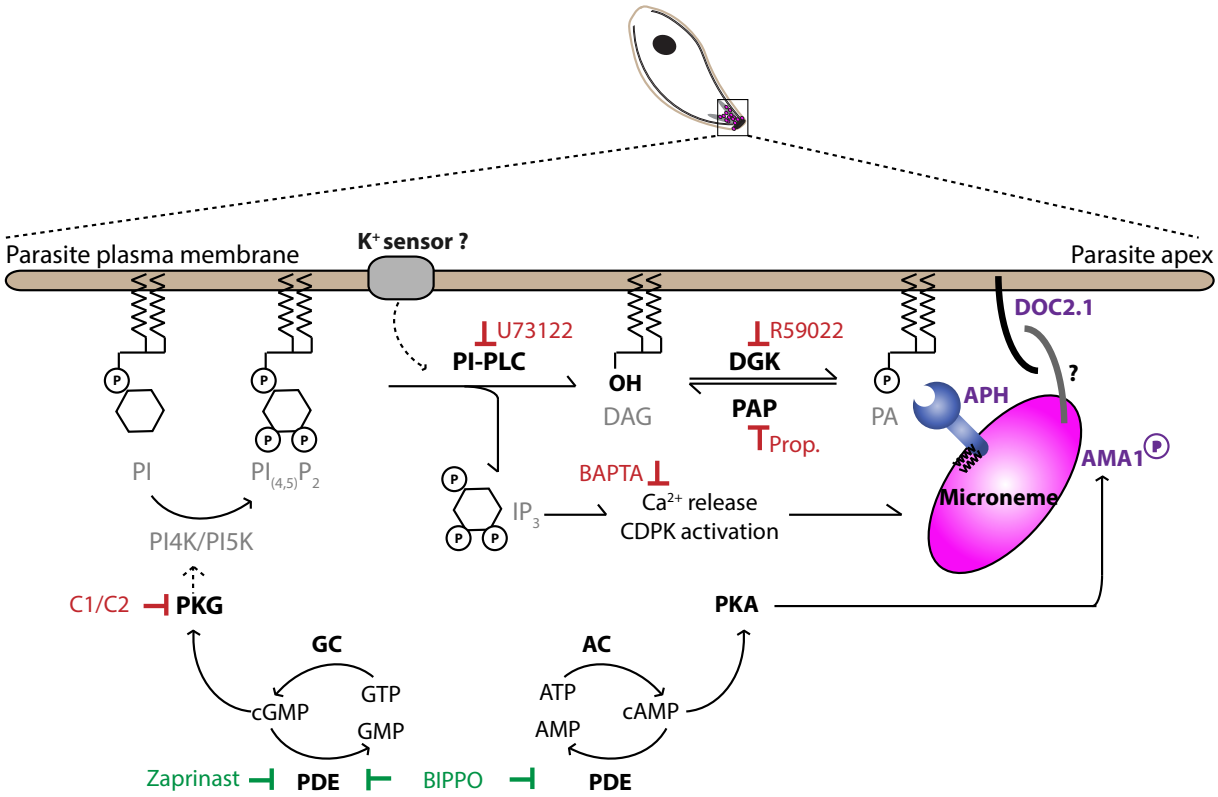
A variant of gliding motility is used during invasion where the microneme proteins AMA1-4 do not interact with host receptors but with ROPs proteins. The ROPs proteins are injected in the host cytoplasm during rhoptry secretion and remain associated with cytoplasmic face of the host plasma membrane where they serve as a receptor for the microneme proteins AMA1-4 that is anchored to the parasite plasma membrane. By this mechanism, the parasite establishes a tight molecular link

between its own plasma membrane and the host plasma membrane (called the moving junction) and uses this anchor to actively propel itself inside the host cell.



Liu et al. 2017

A signaling cascade leads to microneme secretion



Cyclic nucleotides cAMP and cGMP are important second messengers with diverse roles in eukaryotic cells. cGMP is produced by a unique guanylate cyclase encoded in *T. gondii* genome and degraded by phosphodiesterases (PDE), of which there are 18 putative genes in *T. gondii*. cGMP mediated activation of PKG triggers a signaling cascade that involves a phosphoinositide phospholipase C (PI-PLC) and leads to a rise in intracellular calcium and the production of intracellular phosphatidic acid (PA), two key mediators of microneme exocytosis. PA, produced through the reversible reaction of diacylglycerol kinase 1 (DGK1) and phosphatidic acid phosphatases (PAPs/lipins) is sensed by TgAPH, an acylated pleckstrin homology domain containing protein at the surface of the micronemes, which allows membrane fusion in a SNARE-dependent manner. Several signals are known to induce microneme secretion: change in K+ level, pH or seroalbumin concentration (**why do you think the parasite is sensing these signals?**). cAMP regulates microneme secretion via PKA, which senses cAMP produced by adenylate cyclases repressing exocytosis. In addition, several inhibitors and inducers have been shown to act in different enzyme in this pathway and are highlighted in the figure.

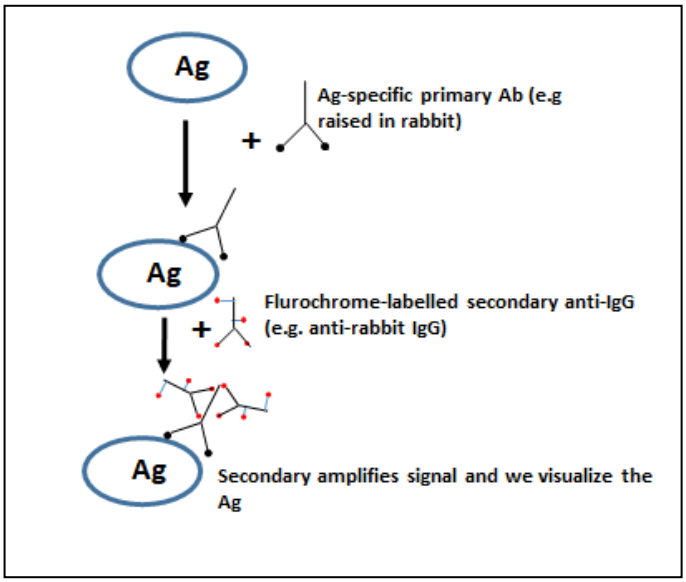
Materials and Methodology
Monday 23/07/18
Group A
Indirect Immunofluorescence assay (IFA) for DGK2-3ty localization

What is IFA and how can we do it?

This is the technique primarily used for the localization of protein of interest (antigen, Ag) in which binding of unlabeled primary antibody (Ab) to Ag is detected using a fluorochrome labelled secondary Ab raised against the immunoglobulin from the animal species in which the primary Ab is produced.

You can also 'Tag' (peptide sequence genetically grafted onto the protein) your protein of interest with commonly used protein tags like ty, His-tag, Strep tag and use primary Ab against these 'Tag'

Scheme



Reagents:

- 4% paraformaldehyde (PFA) or PFA + 0.005 % Glutaraldehyde (PFA/GA) in PBS, depending on the antibodies used
- 1X PBS/0.1M glycine
- 1X PBS/0.2% (w/v) Triton X-100
- 2%BSA/1X PBS/0.2% (w/v) Triton X-100
- Primary antibodies (in this case we are using mouse ty Ab to stain DGK2 and rabbit GAP45 Ab to stain the the parasite outline)
- Secondary antibodies (in this case we are using fluorescence anti-mouse Ab and anti-rabbit Ab)
- DAPI (to stain the DNA)
- Fluoromount G

Protocol:

Remove the medium from an infected HFF-IFA (24well plate with HFF monolayers on coverslips) and fix with PFA or PFA/GA for 10 min (250µl of fixative per well is sufficient).

1. Quench the reaction by adding 1X PBS/0.1M glycine, incubate for 5 min
2. Permeabilize with 1X PBS/0.2% Triton X-100, 20 min on the shaker

3. Block with 2%BSA/1X PBS/0.2% Triton X-100, 20 min on shaker
4. Incubate with primary antibodies in 2%BSA/1X PBS/0.2%Triton (dilution depends on the antibody used) for 1 h (250 µl/well)
5. Wash 3 times with 1X PBS (5-10 min each wash)
6. Incubate with secondary antibodies (1:3000 dilution; 250µl/well), 45 min.
   The plate should be kept in the dark from now on!
7. Wash 3 times with 1X PBS (5-10 min each wash)
8. Stain cells with DAPI for 5-10 min.
9. Wash with 1X PBS
10. Carefully mount coverslips on the slide with cell layer facing down using a drop of mounting solution (Fluoromount G)

Keep slides in the dark at RT in order to let them dry. Then check them using a fluorescence microscope. For long-term storage, keep them at 4°C, in the dark.

Group B

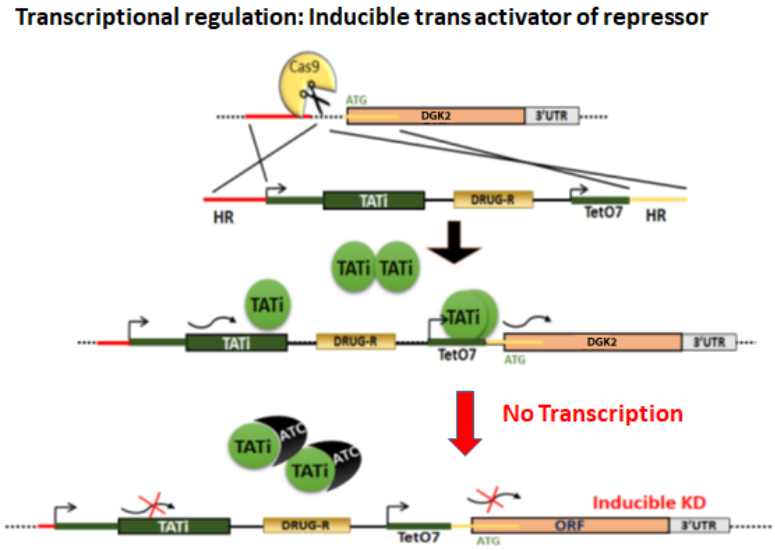
Overall phenotype of inducible knock down of DGK2

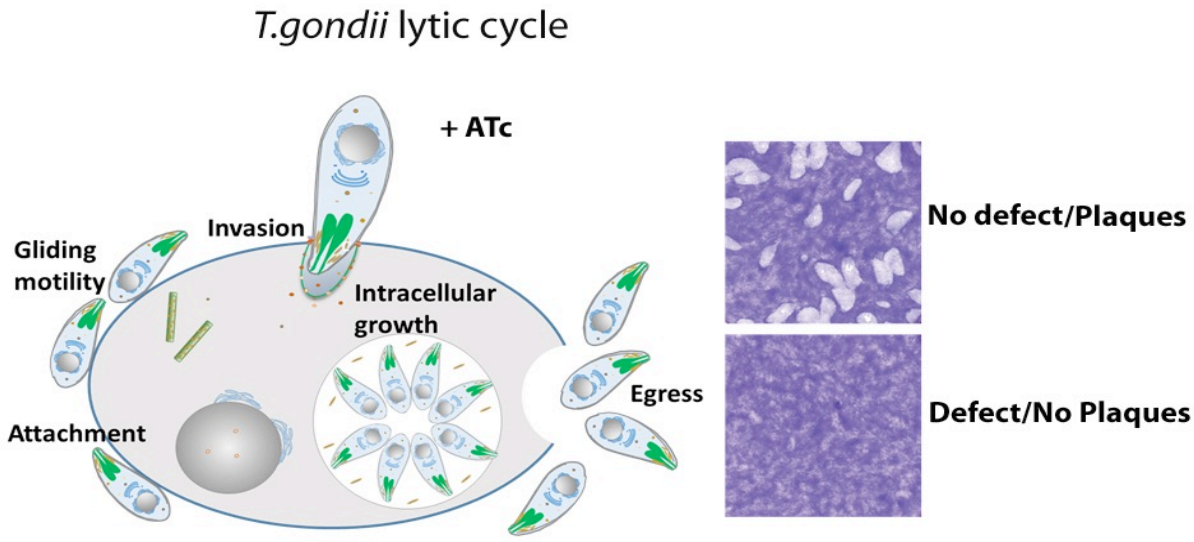
What is inducible knock down?

It is the technique commonly applied to study the function of essential genes (genes that cannot be knock out completely) due to the essential role they play in the life cycle of the parasites.

Applied at the transcriptional (mRNA) level or at post-transcriptional (protein) level.

For DGK2, at the transcription level to generate iKD DGK2 as illustrated by the scheme.





Evaluation technique by Plaque Assays

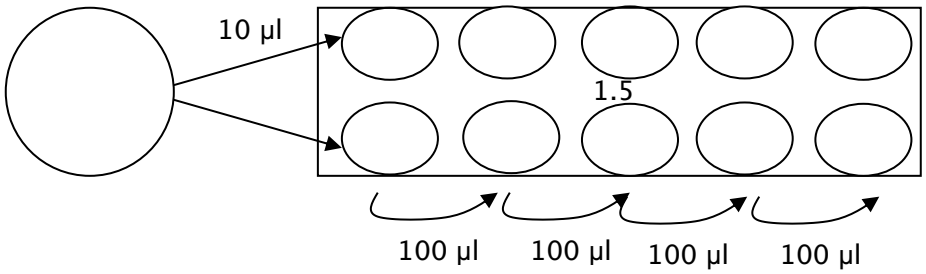
Reagents:

- Crystal violet
- 4% Paraformaldehyde (PFA)

This assay will reveal if the parasites are able or not to be propagated in culture. It will give you a general idea of the fitness but you will not know which step of the lytic cycle is affected. Here only the size of the plaques is informative, not their number. Set up duplicate dishes or wells for each condition.

Protocol:

1. Prepare the HFF plate to receive the parasites: add to each well fresh medium and ± drug (if necessary).
2. Transfer 10 µl of parasites from a totally lysed dish into the first well of an IFA plate and pass 100 µl to the neighboring well. Continue the dilution process up to the end of the line.



3. Incubate for 7-8 days without disturbing the wells (don't bump them, move them or slam incubator doors).
4. After incubation for 7-8 days, aspirate the medium, rinse the infected monolayer with PBS, fix for 10 minute with 4% paraformaldehyde, and stain with crystal violet for 15 min. The solution provided is already diluted. (1/5 dilution of crystal violet in water, filter the solution with 0.22 µm filters).
5. Aspirate the crystal violet solution, rinse twice with water and let it dry.

Group C

Observation of GC localization by using HA and GAP45 Ab

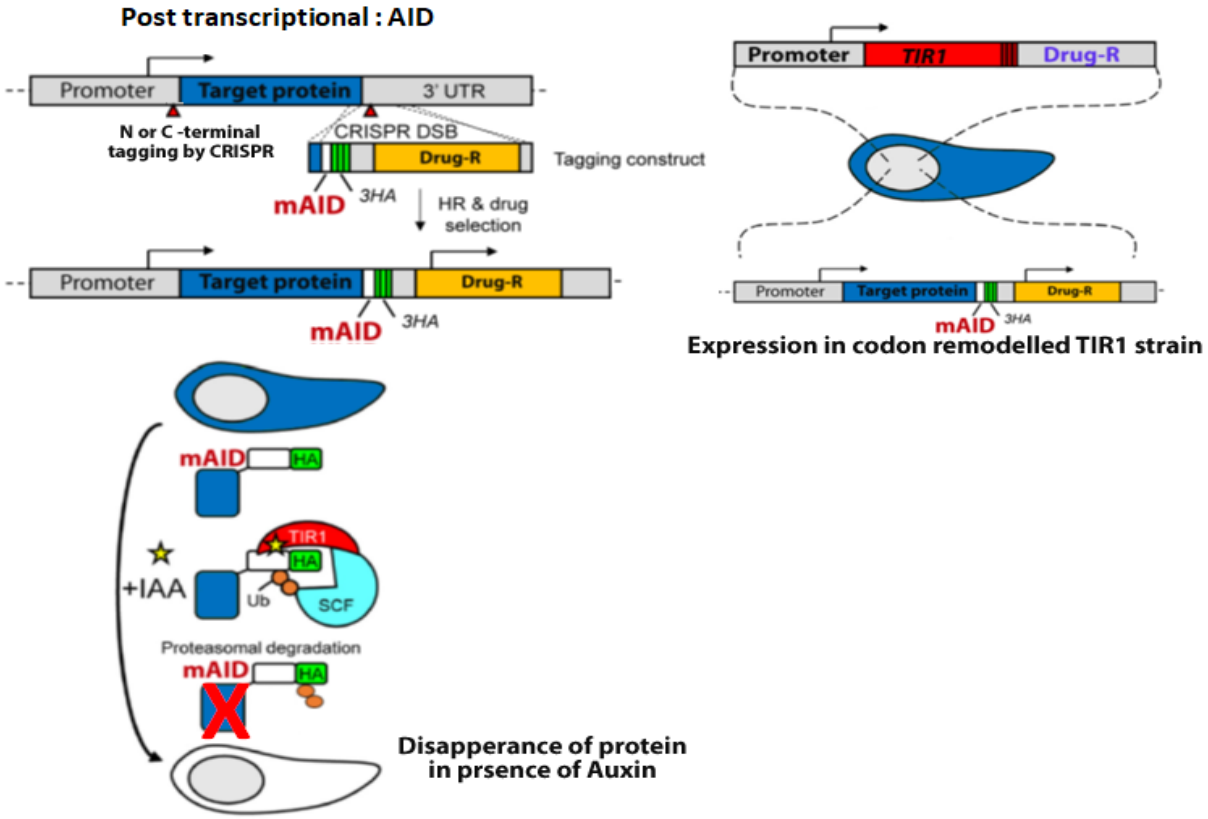
Protocol same as before except we use anti-HA Ab to detect GC

Group D

Overall phenotype of iKD GC by Plaque assay

Protocol same as before.

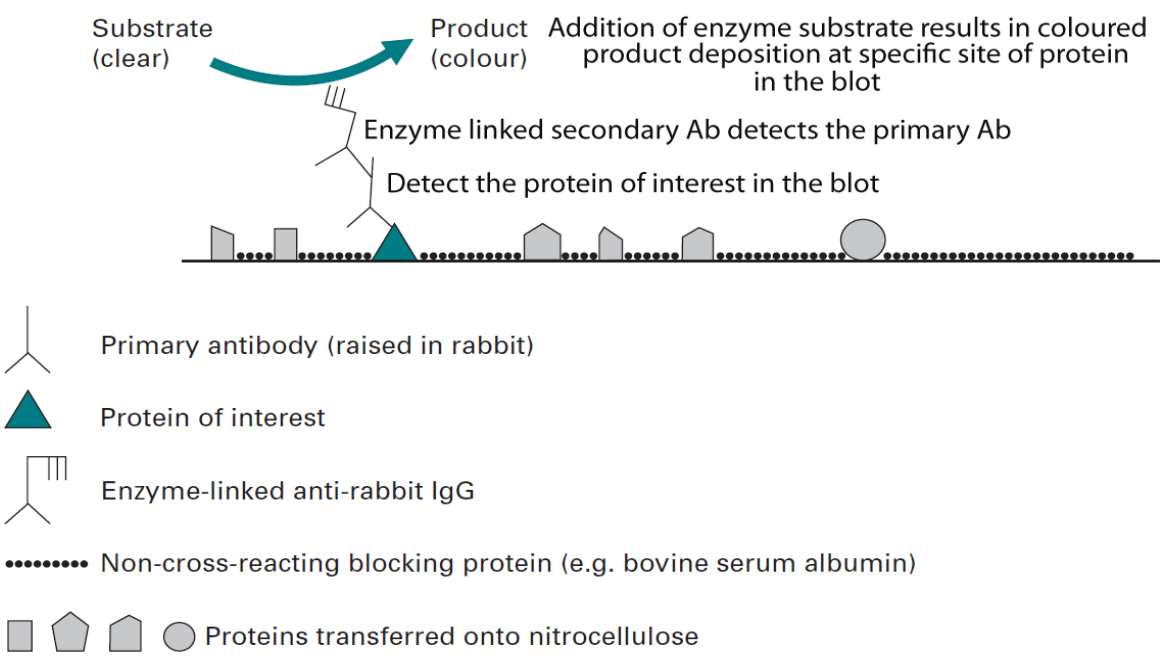
For GC post translation regulation is applied using AID Degron as described in the scheme



Tuesday 24/07/18
Group A
Regulation of DGK2 at protein level by western blot

What is western blot and what is its use?

The analytical technique combines SDS-PAGE, which achieve fractionation of a protein mixture during the electrophoresis process. It is possible to make use of this fractionation to examine further individual separated proteins. The first step is to transfer or blot the pattern of separated proteins from the gel onto a sheet of nitrocellulose paper. The method is known as protein blotting, or western blotting. Transfer of the proteins from the gel to nitrocellulose is achieved by a technique known as electroblotting. In this method, a sandwich of gel and nitrocellulose is compressed in a cassette and immersed in buffer between two parallel electrodes. A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and into the nitrocellulose sheet. The nitrocellulose with its transferred protein is referred to as a blot. Once transferred onto nitrocellulose, the separated proteins can be examined further. This involves probing the blot, usually using an antibody to detect a specific proteins.



SDS-PAGE gel running, western blotting

Preparation of parasite protein lysate:

A fully lysed well from a 6 well plate produces enough parasites ($2x10^7$) to run several gels

1. Harvest the freshly lysed parasites and centrifuge at 1000g, 10 min
2. Wash the parasite pellet in 1X PBS
3. Resuspend the parasite pellet in 100µl 1X PBS
4. Add 80 µl 2xSDS loading buffer and 20 µl of DTT (1 M), boil at 95°C for 10 min
5. The lysate can be used directly or stored at -20°C

SDS-PAGE gels:

1. Use the precast gels
2. Run each gel at constant 35 mA per gel (approx. 45 minutes running)

Western Blotting with ECL detection kit

Transfer the proteins from the gel onto a nitrocellulose membrane with a semi-dry transfer machine

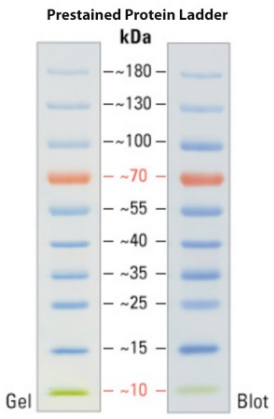
Reagents:

- 1xPBS/0.05% Tween20
- Milk powder
- 5% non-fat milk powder in 1X PBS/0.05% Tween20
- Primary antibodies DGK2 and actin (as loading control)
- Secondary antibodies, HRP conjugated
- ECL plus kit

Protocol:

1. Blot the membrane with 5% milk/PBS/Tween solution 45 min, at room temperature (RT) or overnight at 4°C
2. Incubate the membrane with the appropriate dilution of the primary antibody in 5% milk/PBS/Tween for 1 h, RT or overnight at 4°C
3. Wash the membrane 3 times in 1xPBS/0.05% Tween, 10 min each, RT
4. Incubate the membrane with secondary antibodies (1:3000 dilution) in 5% milk/PBS/Tween for 1 h, RT
5. Wash the membrane 4 times in 1xPBS/0.05% Tween, 10 min each, RT
6. Briefly dry the membrane, then incubate it with the ECL plus detection kit
7. Expose to capture the chemiluminescent signal

Note: For reprobing, the membrane can be stripped by incubating for 1 h, RT with stripping solution (PBS/2%SDS and 7µl/ml of β-Mercaptoethanol). Wash extensively with PBS and PBS/0.05% Tween and re-block membrane with 5% milk before incubation with antibodies.



Loading of Gel
Lane 1-Molecular weight marker
Lane 2-Intracellular parasite + ATC 24h
Lane 3-Extracellular parasite
Lane 4-Extracellular parasite + ATc 6h
Lane 5-Extracellular parasite + ATc 24h

Group B
What is the issue in the lytic cycle with Indirect Immunofluorescence assay
Same as before but using GRA3 and GAP45 antibody

Group C
Regulation of GC protein level by western blot
Same as before but we use HA ab to detect GC, actin remain as loading control

Loading of Gel
Lane 1-Molecular weight marker
Lane 2-GC without Auxin (IAA)
Lane 3-GC + IAA 6h
Lane 4-GC+IAA 24h

Group D
What is the issue in the lytic cycle with Indirect Immunofluorescence assay
Same as before using GRA3 and GAP45 antibody

Wednesday 25/07/18
Group A
Completion of the western blot left in overnight primary Ab

Group B
What is the issue in the lytic cycle (continue)
Invasion assay
In *T. gondii*, invasion, like egress, is an active process. This process follows sequential steps: (i) microneme discharge and attachment of the parasite to the host cell, (ii) apical reorientation of the parasite, (iii) rhoptries secretion within the host cell, iv) formation of the moving junction and (v) translocation of the junction propelling the parasite forward inside the host cell.
The principle of the assay consists of letting the parasite invade for 30 min before fixation with PFA. Then, antibody incubation is performed without permeabilization, staining the parasite with

a surface marker. The absence of permeabilization will block the antibodies outside the host cell, leaving the intracellular parasites unlabelled. Samples will then be permeabilized and stained with a second marker to label the total population. The invasion rate can be then be easily calculated as a ratio of the parasites inside (single stained) to the total (single stained + double stained parasites).

Reagents:

- ATc
- PFA/GA
- 1% formaldehyde in PBS
- α-GAP45
- α-SAG1
- Secondary anti-rabbit
- Secondary anti-mouse
- PBS and Triton-X100 (TX100)
- BSA

Protocol:

A. Resuspend parasites from a 6 cm dish.
B. Dilute parasites 1:10 in pre-warmed medium (100 µl parasites + 1 ml medium)
C. Inoculate IFA plate with 100 µl of the diluted parasites and incubate for 30 min (± drug) at 37°C.
D. Fix the cells with PFA/GA. **Do not wash the cell** otherwise you can detach non-invaded parasites.
E. IFAs are then process as follow:

1. without triton!!
- blocking for 30 min with 2% BSA/PBS
- incubation for 20 min with α-SAG1 diluted in 2% BSA/PBS
- 3 washes of 5 min in PBS.
2. Cells were then fixed with 1% formaldehyde for 7 min and washed once with PBS.
3. without triton!!
- permeabilization for 20 min using 0.2% TX100/PBS
- incubation for 30 min with α-GAP45 diluted in 2% BSA/0.2% TX 100/PBS
- 3 washes with 0.2% Triton X-100/PBS
- incubation with secondary antibodies for 30 min.
- 3 washes with 0.2% Triton X-100/PBS
- DAPI staining and mounting

Intracellular growth/replication assay

T. gondii divides synchronously within a vacuole but asynchronously across the different vacuoles. Counting the number of parasites/vacuole across several vacuoles is thus an easy and efficient way to determine the replication rate of the parasites.

Reagents:

- ATc
- PFA/GA
- α-GAP45

- Secondary anti-rabbit
- PBS
- Triton-X100
- BSA

Protocol:

1. Transfer 10-15 µl of parasites from a fully lysed dish to wells of an IFA plate
2. Incubate for 24-30 hr, 37°C
3. Fix with PFA/GA.
4. Perform IFA using α-GAP45 Abs and secondary α-rabbit Abs and count the number of parasites/vacuole (only vacuoles with at least 2 parasites are counted). Count 100 vacuoles per coverslip.

The experiment should be done at least 3 times to have standard deviations.

Natural Egress

Parasites were allowed to infect for 5 mins and then washed.
Parasites were allowed to divide ~48-50 hr, at 37°C
Fixed with PFA/GA
Perform IFA using GRA3 and GAP45 Ab.

Group C

Completion of the western blot left in overnight primary Ab

Group D

What is the issue in the lytic cycle (continue)

Same as described before

Thursday 26/07/2018

Group A

Digging into the DGK2 phenotype

Comparison of iKD DGK2 (with or without ATc) phenotype with parental strain Ku80 by IFA

Induced Egress Assay

In *T. gondii*, egress is an active process relying on the parasites' ability to sense that their host cell is dying. Treatment with the BIPPO/PA leads to change in pH, which artificially induces parasite egress as early as two hours post-infection. During this process, the conoid protrudes, the micronemes discharge their content and the parasites become motile.

Reagents:

- BIPPO/PA
- DMEM w/o serum
- DMSO
- ATc
- PFA/GA
- α-GAP45
- α-GRA3
- Secondary anti-rabbit
- Secondary anti-mouse
- PBS and Triton-X100
- BSA

Protocol:

1. Resuspend parasites from a 6 cm dish.
2. Dilute parasites 1:10 in pre-warmed medium (100 µl parasites + 1 ml medium).
3. Inoculate IFA plate with 30-50 µl of the diluted parasites and incubate for 30 h at 37°C. Don't forget to plate for a control well.
4. Aspirate the medium and wash with DMEM **w/o serum**
5. Incubate the cells with DMEM **w/o serum** containing either BIPO (10 uM)/PA (50 nM) or DMSO, for 7-10 min, 37°C
6. Fix the cells with PFA/GA and perform IFA (as mentioned before) with α-GAP45 that stain the parasites and α-GRA3 Abs that stain the parasitophorous vacuole.

Group B

Comparison of iKD DGK2 (with or without ATc) phenotype with parental strain Ku80 by Western blot

Microneme Secretion assay

Protocol:

1. Resuspend parasites in IC buffer (Intracellular Buffer) to remove secreted protein and block further secretion.

Pellet

2. Remove supernatant. Resuspend in IC buffer and split sample into required number of tubes. For eg, uninduced, induced, etc. (Don't use too much IC as it makes it hard to efficiently pellet the sample)

   Pellet at 2000 rpm / 5mins
3. Remove supernatant and resuspend in egress buffer +/- stimulus

   BIPO (10 uM for 10 mins)

   PA (50 nM for 30 mins)
4. Pellet at **1000rpm/5min/4ºC**

   Transfer supernatant to new tube (ESA). Very important! If you don't do this, you get pellet in your supernatant and your results are hard to interpret

   Keep **Pellet** also.
5. Spin supernatant from the previous step at **2000rpm/5min/4ºC**

   Transfer sup to fresh tube, avoiding any pellet! Leave few L at the bottom to ensure you don't get any pellet in supernatant. This is your **final ESA**
6. Resuspend **Pellet** in PBS. Pellet at **2000rpm/5min/4ºC**. Remove PBS
7. Immediately add Sample buffer.

8. Load ESA and pellet to run the gel and perform the western blot as described before with MIC2 and Catalase Ab.

Loading of the gel

Loading of Gel#1

Lane 1-Molecular weight marker

ESA

Lane 2-Ku80 (–ATc) +BIPO
Lane 3-Ku80 (+ATc) +BIPO
Lane 4-iKD DGK2 (-ATc) +BIPO
Lane 5-iKD DGK2 (+ATc) +BIPO
Lane 6-Ku80 (–ATc) +DMS0
Lane 7-Ku80 (+ATc) +DMSO
Lane 8-iKD DGK2 (-ATc) +DMSO
Lane 9-iKD DGK2 (+ATc) +DMSO
Lane 10-Moleculat weight marker
Lane 11, 12, 13, 14-pellet control

Loading of Gel#2

Same as gel#1 but we will use the PA stimulated sample instead of BIPO stimulated one.

Group C

Digging into GC phenotype

Comparison of iKD GC(with or without IAA) phenotype with Tir1 parental strain IFA

Induced Egress assay, same as before

Group D

Comparison of iKD GC (with or without IAA) phenotype with Tir1 parental by Western blot

Microneme secretion assay same as before.

Friday 27/07/2018

- All groups (A, B, C and D) analysis of results of IFA experiments from 26/07/2018
- And finishing the western blot left in primary ab overnight on Thursday.

DGK2 Team

	MONDAY 07/23	TUESDAY 07/24	WEDNESDAY 07/25	THURSDAY 07/26	FRIDAY 07/ 27
GROUP A	**DGK2 Localization** Observation of DGK2 localization. Strain DGK2-3ty (C-terminal tag) Indirect Immunofluorescence assay (IFA) 1. Ty and GAP45 Abs 2. Ty and GRA1 Abs	**Regulation of DGK2 protein level PART 1** Timing of regulation of DGK2 under Atc treatment Strain iKD DGK2 Western Blot 1. Migration 2. Transfert 3. Blocking 4. Primary antibody DGK2 (overnight)	**Regulation of DGK2 protein level PART 2** Timing of regulation of DGK2 under Atc treatment Strain iKD DGK2 Western Blot 5. Washes 6. Secondary antibody 7. Washes 8. Revelation **END OF THE AFTERNOON WITH MEHDI**	**Digging into the DGK2 phenotype PART 1** IFA Comparing parental strain (Ku80) and iKD DGK2 under Atc treatment	**Digging into the DGK2 phenotype PART 2** Analysis of the IFA (07/26) Comparing parental strain (Ku80) and iKD DGK2 under Atc treatment **Preparation of presentation MODULE 1**
GROUP B	**Overall phenotype of iKD DGK2** Evaluation of the ability to accomplish several lytic cycle of the iKD DGK2 parasites under Atc treatment Plaque assay	**What is the issue in the lytic cycle? PART 1** Immunofluorescence at different time post-infection to assess which step is impaired Strain iKD DGK2 under Atc treatment Immunofluorescence at different Indirect Immunofluorescence assay (IFA) Time post-infection : 12 - 24 - 48 - 60hpi GRA3 and GAP45 Abs	**What is the issue in the lytic cycle? PART 2** Immunofluorescence at different time post-infection to assess which step is impaired Visual evaluation of 1. Invasion 2. Intracellular growth 3. Natural Egress **END OF THE AFTERNOON WITH MEHDI**	**Digging into the DGK2 phenotype PART 1** Comparing parental strain (Ku80) and iKD DGK2 under Atc treatment Western Blot 1. Migration 2. Transfert 3. Blocking 4. Primary antibody MIC2 (overnight)	**Digging into the DGK2 phenotype PART 2** Comparing parental strain (Ku80) and iKD DGK2 under Atc treatment Western Blot 5. Washes 6. Secondary antibody anti-mouse HRP 7. Washes 8. Revelation **Preparation of presentation MODULE 1**

GC Team

	MONDAY 07/23	TUESDAY 07/24	WEDNESDAY 07/25	THURSDAY 07/26	FRIDAY 07/ 27
GROUP C	**GC Localization** Observation of GC localization. Strain mAID-HA-GC (N-terminal tag) Indirect Immunofluorescence assay (IFA) 1. HA and GAP45 Abs 2. HA and GRA3 Abs	**Regulation of GC protein level PART 1** Timing of regulation of GC under IAA treatment Strain mAID-HA-GC Western Blot 1. Migration 2. Transfert 3. Blocking 4. Primary antibody HA (overnight)	**Regulation of GC protein level PART 2** Timing of regulation of GC under IAA treatment Strain mAID-HA-GC Western Blot 5. Washes 6. Secondary antibody anti-rat HRP 7. Washes 8. Revelation **END OF THE AFTERNOON WITH MEHDI**	**Digging into the GC phenotype PART 1** IFA Comparing parental strain (Tir1) and mAID-HA-GC under IAA treatment	**Digging into the GC phenotype PART 2** Analysis of the IFA (07/26) Comparing parental strain (Tir1) and mAID-HA-GC under IAA treatment **Preparation of presentation MODULE 1**
GROUP D	**Overall phenotype of iKD GC** Evaluation of the ability to accomplish several lytic cycle of the mAID-HA GC parasites under IAA treatment Plaque assay	**What is the issue in the lytic cycle? PART 1** Immunofluorescence at different time post-infection to assess which step is impaired Strain mAID-HA-GC under IAA treatment Immunofluorescence at different Indirect Immunofluorescence assay (IFA) Time post-infection : 12 - 24 - 48 - 60hpi GRA3 and GAP45 Abs	**What is the issue in the lytic cycle? PART 2** Immunofluorescence at different time post-infection to assess which step is impaired Visual evaluation of 1. Invasion 2. Intracellular growth 3. Natural Egress **END OF THE AFTERNOON WITH MEHDI**	**Digging into the GC phenotype PART 1** Comparing parental strain (Tir1) and mAID-HA-GC under IAA treatment Western Blot 1. Migration 2. Transfert 3. Blocking 4. Primary antibody MIC2 (overnight)	**Digging into the GC phenotype PART 2** Comparing parental strain (Tir1) and mAID-HA-GC under IAA treatment Western Blot 5. Washes 6. Secondary antibody anti-mouse HRP 7. Washes 8. Revelation **Preparation of presentation MODULE 1**