III. INVASION

Characterization of moving-junction proteins involved in *T. gondii* invasion
Invasion in Apicomplexa parasites

Cryptosporidium parvum
Toxoplasma gondii tachyzoite
Plasmodium falciparum sporozoite or merozoite
Theileria sporozoite or merozoite

1. Actin polymerization
2. Feeder organelle
3. Motility driven invasion

Invasion by zippering

M-J Gubbels, M.T. Duraisingh, 2012
The moving junction (MJ)

The moving junction of Apicomplexa

*Toxoplasma gondii*  
*Plasmodium knowlesi*

Aikawa et al., J cell Biol 1978

JF Dubremetz
Time-lapse video-microscopy of a tachyzoite invading a human fibroblast
Sequential events of *Plasmodium* Merozoite invasion in a red blood cell

Aikawa et al., J cell Biol 1978
PV maturation and host cell hijacking

Gliding motility and attachment to host cell

Moving junction
The MJ acts a molecular sieve during invasion
Mordue et al., J Exp Med 1999
Molecular sieve... a protection against lysosomal degradation

Transferrin receptor

Vacuolar proton pump

Manose 6 Phosphate Receptor

LAMP1

All the endocytic markers are excluded from the PVM 30 min. post-invasion

Mordue et al., Exp Parasitol 1999
What is the composition of the MJ?
An antibody (Mab4H1) that recognizes the MJ
The MJ protein is a rhoptry neck protein, RON4

Localisation dans le col des rhoptries dans des parasites intracellulaires

Morphological description of the MJ

Molecular characterization of the MJ


Lebrun et al. Cell Microbiol 2005
Molecular characterization of the MJ complex

Macromolecular complex

Ron8
Ron5
Ron2
Ron4

Rhoptry neck

Intracellular parasite: Rhoptry neck

Ron4
Ron2
Ron5
Ron8
merge

Invading parasite: MJ

Ron2
Ron4

Ron5
Ron8
merge
Complex AMA1/RON2/4/5/8

- RON8
- RON5
- RON2
- RON4

**Microneme**

**Rhoptry neck**

**Intracellular parasite: microneme**

**Invading parasite: MJ**

**IP αRON4**
Lebrun et al. Cell Microbiol 2005

**IP αAMA1**
Alexander et al. PloS Pathogen 2005

Image of protein complex and images of parasites with labels and references.
Conservation of the MJ complex in *Plasmodium*

**Complex AMA1/RON2/4/5**

![Image showing protein bands for PfRON2, PfRON4, PfRON5, PfAMA1, and PfAMA1_83](image)

- **Rhoptry neck**
- **Microneme**

**AMA1 and RONs:**

Collaboration between MIC and RONs to establish the MJ

- **AMA1**
- **RON4**

- **IP αRON4**
  - Alexander et al., Eukaryot Cell, 2006
  - Collins et al. PloS Pathogen 2009

- **Invading merozoite into red blood cell:**
  - AMA1 and RON4 at the Moving junction

- **Riglar et al. Cell Host and Microb 2011**
Conservation of the MJ components in Apicomplexa

<table>
<thead>
<tr>
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<th><em>P. falciparum</em></th>
<th><em>T. gondii</em></th>
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<th><em>E. tenella</em></th>
<th><em>Theileria annulata</em></th>
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<td>✓</td>
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Adapted from S. Besteiro *et al.* *Cell Microbiol* 2011

http://tolweb.org/Apicomplexa (Jan Sapleta)
What is the organization of the MJ?
Export of the RONs complex in the host cell

The RONs are associated with the cytosolic face of the host cell membrane

Lamarque et al. (2011) PLoS Pathogens
Besteiro et al. (2009) PLoS Pathogens
The RONs are associated with the **cytosolic** face of the host cell membrane

RON2 is inserted in the host plasma membrane

Lamarque *et al.* (2011) *PLoS Pathogens*
Tyler *et al.* (2011) *PLoS Pathogens*
RON2 binds AMA1

RON2 exposes a short domain that binds to AMA1

Lamarque et al. (2011) PLoS Pathogens
Tyler et al. (2011) PLoS Pathogens
Besteiro et al. (2009) PLoS Pathogens
Molecular organization of the MJ complex

Direct interaction between AMA1 and RON2
Direct interaction between AMA1 and RON2

The αRON2-2 antibody fails to co-immunoprecipitate TgAMA1 and does not label the MJ

→ The binding region of TgRON2 to TgAMA1 corresponds to the RON2-2 region
AMA1-RON2 interaction is conserved in *Plasmodium* and *Eimeria*

*Plasmodium falciparum* AMA1

<table>
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<th>Phase</th>
<th>F8.12.19</th>
<th>α-GST</th>
<th>Merge</th>
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**PfAMA1** Anti-GST

Lamarque et al. PlosPathogen 2011

*Eimeria tenella* AMA1

Unpublished **EtAMA1** Anti-GST

GST-RON2

GST

GST-PfRON2

Gb

Mb

Mb

III

Out

In

tail

AMA1

ectodomain
Co-evolution of AMA1 and RON2 partners within the Apicomplexa phylum

ELISA
Coating AMA1 from:

- **Plasmodium falciparum**
- **Toxoplasma gondii**
- **Plasmodium vivax**

**GST-RON2 from** *Plasmodium falciparum***

**GST-RON2 from** *Toxoplasma gondii***

**Conclusion:** No inter-species or inter-genus interaction between *Toxoplasma* and *Plasmodium* sp

Lamarque et al., PlosPathogen 2011
What is the function of the AMA1-RON2 interaction
Targeting of AMA1-RON2 interaction

TgRON2

N-term intracellular domain

TM

C-term ectodomain

Toxoplasma

DIVQHMEDIGGAPPVSCVTNEI--LGVTCPQAIKATT

P. falciparum

DITQQAKDIGAGPVASCFTTRMSPPQQQICLNSVVNTALS

37 AA synthetic peptide

Invasion Assay in fibroblasts

Red blood cell Invasion Assay

Toxoplasma RON2sp (μM)

Tonkin et al. Science, (2011)

P. falciparum RON2sp (μM)

Structural analysis of the AMA1-RON2 interaction
Co-structures AMA1-RON2sp from *Toxoplasma* and *Plasmodium*

*Plasmodium vivax*: Pizarro et al., Science 2005

*Plasmodium falciparum*: Bai et al, PNAS, 2005

*Toxoplasma gondii*: Crawford et al, JBC 2010
Toxoplasma
AMA1-\textit{TgRON2sp}

\begin{center}
\includegraphics[width=0.4\textwidth]{toxoplasmamodel.png}
\end{center}

\begin{itemize}
\item N-terminal helix
\item Cystine loop
\end{itemize}

\textit{Tonkin et al. Science} 2011

\begin{center}
\includegraphics[width=0.4\textwidth]{toxoplasmamodel.png}
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\end{itemize}

\textit{Vuillez-LeNormand et al. PloSPathgen} 2012

\begin{center}
\includegraphics[width=0.4\textwidth]{p.falciparum.png}
\end{center}

\begin{itemize}
\item N-terminal helix
\item Cystine loop
\end{itemize}

\textit{Vuillez-LeNormand et al. PloSPathgen} 2012
• DII loop is associated to the base of the hydrophobic cleft of AMA1
• A pair of Trp anchors the DII loop to the hydrophobic groove

Crawford et JBC 2000
Conformational change of the DII loop upon RON2 binding
Exposition of complementary surfaces

apoTgAMA1

Displaced DII loop

Ordered DII loop

TgAMA1-TgRON2sp

Exposed basic patch

Complementary acidic patch

TgRON2sp
Mapping of the residues critical for AMA1-RON2 interaction

Extensive mutagenesis analysis in *Toxoplasma* and *Plasmodium*

AMA1 side

RON2 side

**Surface Plasmon Resonance**

**Invasion inhibition assay**

DIC  *TgAMA1*  *TgRON2*

BHK21 –binding assay
The cystine loop is crucial to maintain the interaction
The cystine loop also governs the genus/species specificity

This illustrates at the structural level the distinct co-évolution of the MJ complex in Apicomplexa phylum.
From fundamental research to vaccination strategy with the AMA1-RON2 complex
Vaccination with AMA1-RON2 complex

Srinivasan et al. Nat Comm 2014
The efficacy is linked to qualitative differences in the antibody specificity.

Increased proportion of antibodies anti-AMA1 targeting the loops Dle et Dlf that surround the hydrophobic groove of AMA1.
Functional analysis of AMA1-RONs proteins by knock-out strategy
Wild-type

Host cell

- AMA1
- RON2

RON2 mutant affected in AMA1 binding

- AMA1
- RON2 IL/AA

Costructure AMA1/RON2

IL/AA
Generation of a conditional mutant: Knock-Down RON2

- ATc → RON2 expression
+ ATc → No RON2

promRON2 → TATi → 7 x tetO operator → RON2

DIC

Δku80

- ATc

KD-RON2

+ ATc

RON2 expression

αRON2

αROP1

kDa

Δku80
Δku80 + ATc
KD-RON2
KD-RON2 + ATc

170
130
100
KD-RON2 parasites exhibit a strong invasion phenotype.
A RON2 mutant affected in its capacity to interact with AMA1

Co-structure AMA1/RON2

AMA1 / RON2

ELISA

Recombinant protein (µg/ml)

OD 492 nm

GST-RON2

GST-RON2 IL/AA

GST
Complementation of KD-RON2 mutant

**IFA**

- cKD-RON2 WT
  - DIC
  - αRON2
  - αRON4
  - Merge

- cKD-RON2 IL/AA
  - DIC
  - αRON2
  - αRON4
  - Merge

**Western-Blot**

- Δku80
- KD-RON2
- cKD-RON2 WT
- cKD-RON2 IL/AA

- αRON2
  - 130 kDa

- αROP5
  - 70 kDa
AMA1-RON2 interaction is crucial for invasion

Invasion (%)

<table>
<thead>
<tr>
<th></th>
<th>Δku80</th>
<th>KD-RON2</th>
<th>cKD-RON2 WT</th>
<th>cKD-RON2 IL/AA</th>
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<tr>
<td>Invasion</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

Lamarque et al. Nat Comm 2014
KD-RON2 mutant displays a striking phenotype ...

iKO-AMA1
KD-RON2
KD-RON2 complemented RON2(IL/AA)

Accumulation of ROPs vesicles observed, with a dot of RON4, but no parasite in the vicinity

- Initial attachment
- Rhoptry secretion
- Initiation of MJ formation, but parasites detach

Failure to establish a firm and robust attachment via the formation of the MJ

Lamarque et al. Nat Comm 2014
Why and how some parasites invade in absence of AMA1?

How the MJ is formed in absence of AMA1?
Generation of a direct AMA1 knock-out line

Lamarque et al. Nat Comm 2014
An invasion pathway independent of AMA1 ...
The loss of AMA1 leads to a strong invasion phenotype... But...

KO-AMA1

promAMA1

KO-AMA1

Δku80

KO-AMA1

2 months

4 months

12 months

% of Invasion

22.3

12.8

10.1

0

20

40

60

80

100

120

0

20

40

60

80

100

120

0

20

40

60

80

100

120

% of Invasion

Δku80

KO-AMA1

Marker

MA1

3’UTR

2 months

4 months

12 months
Does RON2 have another micronemal partner?

In situ binding assay

permeabilization

Washes

IFAtαGST, αAMA1

% invasion in KO-AMA1

KO-AMA1

Δku80

promAMA1

Marker MA1 3’UTR

KO-AMA1

parasite

Host cell

PV

AMA1

GST-RON2

Phase

AMA1

GST-RON2

Phase

AMA1

GST-RON2

0 20 40 60 80 100 120

-TgRON2sp +TgRON2sp

% invasion in KO-AMA1

Does RON2 have another micronemal partner?
2 proteins homologues of AMA1 encoded in *T. gondii* genome

<table>
<thead>
<tr>
<th></th>
<th>AMA1</th>
<th>AMA2</th>
<th>AMA3</th>
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<tr>
<td>AMA1</td>
<td>100%</td>
<td>38%</td>
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<tr>
<td>AMA2</td>
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*Toxoplasma* life cycle

Environmental transmission
AMA2 is up-regulated in KO-AMA1

2 AMA1 homologs in *Toxoplasma* genome

Is AMA2 the new binding partner of RON2?
AMA2 interacts with RON2

Recombinant proteins AMA

ELISA

AMA1 + RON2
AMA2 + RON2

Absorbance 492 nm

Recombinant protein (µg/ml)

- AMA2 + GST-RON2-2
- AMA2 + GST
- AMA1 + GST-RON2-2
- AMA1 + GST
AMA2 interacts with RON2 at the MJ

Invading KO-AMA1 parasite

Phase RON4 AMA2-HA₃ Merge
KO-AMA1 relies on AMA2-RON2 interaction for invasion

25% reduction

60% reduction

Other players?
Plasticity of AMA-RONs pairs to ensure invasion
What is the role of the other RONs located at the cytoplasmic face of the host plasma membrane?
Yeast two hybrid screen reveals host interactants

- Y2H screen of TgRON4 against a human cDNA library
- Identification of 2 host proteins: ALIX and CIN85
- ALIX is an accessory member of the endosomal sorting complex (ESCRT). It participates in ESCRT-dependent membrane fission events (virus budding, cytokinesis, membrane repair...), while CIN85 is a direct partner of ALIX.

ALIX recruitment is RON4-dependent and important for parasite virulence.
Model of the host cell part of the MJ

- Toxoplasma gondii
- Parasite membrane
- Host cell membrane
- Cytoskeleton
- ALIX binding site (YP(x)_nL)
- CIN85 binding site (Px(P/A)xPR)
- TSG101 binding site (P(T/S)AP or PPPY)