Visualization of Immune Cells during *Toxoplasma gondii* Infection

Dissertation

zur Erlangung des akademischen Grades
des Doktors der Naturwissenschaft (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie
der Freien Universität Berlin

vorgelegt von

Seong-Ji Han

geboren in Berlin

Januar 2012

1. Gutachterin: **Prof. Dr. Ellen Robey**

University of California, Berkeley
Department of Molecular & Cell Biology
142 Life Sciences Addition
Berkeley, CA 94720
USA

2. Gutachter: **Prof. Dr. Burghardt Wittig**

Freie Universität Berlin
Molecular Biology and Bioinformatics
Arnimallee 22
14195 Berlin
Germany

Disputation am: **25. September. 2012**

사랑하는 어머님과 아버님께
감사드리는 마음으로

Mit diesen Worten möchte ich mich bei meinen lieben
Goethianern bedanken, die mich seit nun mehr als 23 Jahren
in meinem Leben begleitet und unterstützt haben.

A special thanks to my mentor Ellen Robey
who always supported me through my graduate research
and had a large influence in my personal and career
development. Further, I would like to thank all Robey lab
members, especially Heather Melichar, Janine Coombes,
Ena Ladi and Shiao Chan.
The Robey lab is the best that could happen to me.

Auch geht mein Dank an meine Geschwister, die
mich stets bestärkt und an mich geglaubt haben.

진심으로 감사합니다

Vielen, vielen Dank

Thanks so much

1. Introduction

The body's biological defense system is called the immune system (immunis latin = free, pure, clean), which overcomes attacks from different invading microorganisms. The immune system is a complex composition of different, specialized cells, tissues and organs that work together through a series of regulated steps to protect individuals against microorganisms such as bacteria, viruses, fungi and parasites. Unchecked, these organisms can lead to more severe diseases. To find therapeutics or vaccinations against these diseases it is necessary to understand the immune response against these organisms.

This thesis aims to understand the immune response to the obligate intracellular parasite *Toxoplasma gondii* (*T. gondii*), an important human pathogen infecting approximately one third of the world's population, with limited drugs to treat chronically infected individuals. One advantage of using *T. gondii* as a model parasite for laboratory study is that it is easily amenable to genetic manipulation, including the introduction of fluorescence proteins. There is a significant amount of information about the immune response to *T. gondii*, but much of this research has been done either *in vitro* or using black box mouse studies. We are interested in studying the interaction between immune cells and infected cells in a physiological environment. Therefore, we are using two-photon laser-scanning microscopy (TPLSM) to study the behavior of immune cells during *T. gondii* infection. Specifically we studied the interaction between an innate cell population, neutrophils, and an adaptive immune cell population, $CD8^+$ T cells, with infected cells during infection to generate unique information about dynamic cell-cell interactions within a natural environment. This method allows us to fill in gaps in our knowledge of the immune response and to build a complete picture of the interplay of immune cells during *T. gondii* infection.

The following chapter gives a broad picture of our current understanding of the host immune response to *T. gondii* as it relates to the role and importance of neutrophils, $CD8^+$ T cells and infected antigen-presenting cells during infection and highlights holes in our knowledge about how these cells interact at a cellular level.

1.1 *Toxoplasma gondii*

Toxoplasma gondii, a member of the phylum Apicomplexa, is an obligate intracellular protozoan parasite that can infect most warm-blooded animals. Within the host, the parasite can invade and replicate in almost all nucleated cell types. The life cycle of *T. gondii* is divided in two distinct components: the asexual and the sexual components. While the sexual phase takes place in the intestine of its definitive host, the feline, the asexual component occurs in its intermediate hosts (1).

In intermediate hosts, including humans and mice, the parasite exists in two interconvertable forms: a fast replicating tachyzoite, and the slowly replicating bradyzoite. Infection of an intermediate host is initiated by oral ingestion of raw or undercooked meat containing bradyzoite filled cysts. As the cysts pass through the digestive tract, the cyst wall is disrupted and the bradyzoites are released. Free bradyzoites then infect the epithelium of the small intestine and convert to tachyzoites. Rapid intracellular replication leads to host cell lysis and subsequent infection of the neighboring cells (1). The parasite infects circulating cells and can use them as a "Trojan horse" to gain access to protective tissues such as the brain, where entry of immune cells is restricted (2, 3).

Tachyzoite infection activates the immune system, which typically eliminates the majority of parasites. Under normal conditions, the immune system is able to control parasite infection. However, some tachyzoites escape the immune system, convert back to bradyzoites and persist as cysts in brain and muscular tissue to establish a life-long chronic infection. In healthy individuals, the infection is usually asymptomatic, but reactivated infection in immunocompromised patients can lead to toxoplasmosis, and acute infection during pregnancy can cause damage to the developing fetus (1).

Most *T. gondii* strains used in laboratories have been isolated from human and animals with toxoplasmic encephalitis. They are organized into three clonal lines: Type I, II and III strains. Infection with even low doses of the hyper-virulent Type I strain causes overwhelming parasite growth, systemic overproduction of pro-inflammatory cytokines, and death in C57BL/6 mice. Type II and III strain parasites cause nonlethal infection and lead to a chronic latent infection of brain and muscular tissue (4).

Table1: Characteristic strain differences in *Toxoplasma gondii*

Strain	Virulence	Cysts formation	Acute phase (Tachyzoites)	Chronic phase (Tachyzoites and Bradyzoites)
Typ I (eg.: RH)	+++	-	+	-
Type II (eg.: Pru)	++	+	+	+
Type III (eg.: CEP)	+	+	+	+

1.2 Immunity to *Toxoplasma gondii*

T. gondii is a major food-borne pathogen in humans and mice. Our current view of the immune defense to toxoplasmosis derives largely from infection models in the mouse. The experimental model of *T. gondii* infection in the mouse has made a significant contribution to our understanding of the cellular immune response.

T. gondii is a pathogen which induces a robust type I immune response. The control of acute and chronic infection depends highly on the pro-inflammatory cytokine, interleukin-12 (IL12) (Figure 1 illustrates the initiation of the acute immune response following *T. gondii* infection) (5). Innate immune cells such as dendritic cells (DCs), macrophages, neutrophils and inflammatory monocytes (IMs) are capable of producing IL12 in a myeloid differentiation factor 88 (MyD88) -dependent manner during *T. gondii* infection. IL12 is the major cytokine triggering synthesis of IFNγ by NK and T cells (5-7). Resistance to *T. gondii* is highly dependent on IFNγ and therefore on IL12. Mice deficient in either IL12 or IFNγ are extremely susceptible to infection (8). Together, the IFNγ and IL12 pathways activate effector mechanisms in a variety of cell types, leading to control of *T. gondii* infection (5, 9-11).

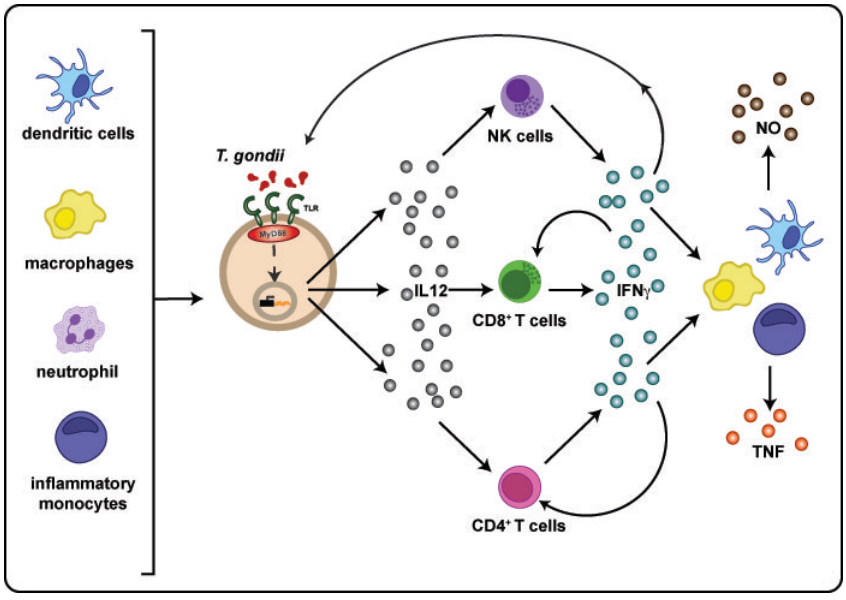


Figure 1. Initiation of the acute immune response to *Toxoplasma gondii*

1.3 Pathogen recognition during the innate immune response: TLR/MyD88

The innate immune response is the first line of defense against infectious diseases. The defense against invading microbes depends on the recognition of non-self pathogen-associated molecular patterns (PAMPs) through germline-encoded pattern receptor molecules (PRR) mostly expressed on innate immune cells. One class of PRRs are the Toll-like-receptors (TLRs). Activation of these receptors leads to recruitment of effector cells and production of pro-inflammatory cytokines, which then modulate innate and adaptive immune responses (12).

Downstream of almost all TLRs as well as the IL1 receptor (IL1R) superfamily is the adaptor protein, MyD88. It plays a central role in activating the nuclear-factor-κB (NFκb) and mitogen-activated protein kinase (MAPK) signaling pathways, which subsequently lead to induction of pro-inflammatory cytokines such as IL-12 and tumor necrosis factor-α (TNFα). The importance of MyD88 in resistance to viral, bacterial and protozoan infection is extensively documented. Mice lacking MyD88 are highly susceptible to *T. gondii* infection whereas mice lacking IL1 or lL18 show normal resistance to *T. gondii* infection suggesting that the absence of MyD88 reflects a specific defect in TLR signaling (13).

It is still unclear which TLRs are involved in *T. gondii* recognition *in vivo*. *In vitro* studies using Chinese hamster ovary (CHO) cells demonstrated TLR2 and TLR4 dependent synthesis of TNFα using glycosylphosphatidylinositol (GPI) -anchored proteins extracted from tachyzoites (14). In addition, it has been demonstrated that TLR9 is required for an effective Th1 inflammatory response after *T. gondii* oral infection. Wild-type (WT), but not TLR9 knockout (KO) mice, develop Th1-dependent acute, lethal ileitis (15). Further, TLR11, which is expressed in mice but not in humans, has been identified to recognize Toxoplasma profilin and is able to induce IL12 in DCs (16). However, survival experiments with TLR11, TLR4, and TLR4/2 TLR9 KO mice showed no increase in susceptibility compared to WT mice (14-16). TLR2 KO mice showed increased susceptibility only under high dose infection (17). None of the single TLR KO mice phenocopied the MyD88 KO phenotype during *T. gondii* infection. Together, it seems that optimal resistance to *T. gondii* infection depends on multiple TLRs.

1.4 Dendritic Cells

The activation and maturation of DCs in response to infection plays a key role in initiating the innate and adaptive immune responses. DC maturation and activation is defined by the up-regulation of cell-surface major histocompatibility complex (MHC) and co-stimulatory molecules (18). In response to local infection, antigen loaded DCs migrate from the site of infection to the spleen and draining lymph nodes and present antigen to naive T cells. During *T. gondii* infection, the production of IL12 by DCs biases the $CD4^+$ T cell response to a parasitic-specific Th1 immune response (19).

Many attempts have been made to prove that DCs are the major source of IL12 after *T. gondii* infection (20, 21). For example, depletion experiments using CD11c-diphtheria toxin (DT) transgenic mice or selective depletion of MyD88 in CD11c expressing cells using the CD11c-Cre mice exhibit a decrease in IL12 production and increased susceptibility to *T. gondii* infection (22, 23). However, CD11c is expressed on several DC subsets and is also expressed on some macrophage subsets. More recent studies using Batf3-deficient mice, a transcription factor selectively expressed in $CD8\alpha^+$ conventional DCs (cDC), suggests that *in vivo*, the $CD8\alpha^+$ cDC subset is the primary source of IL12 production after infection with *T. gondii* (21).

1.5 Macrophages

Macrophages, together with DCs, provide the first line of cell-mediated defense in response to infection. Besides the production of pro-inflammatory cytokines such IL12, the major functions of macrophages are to detect, through several different PRRs on their cell surface, and eliminate, through phagocytosis, pathogen. To limit the initial dissemination and growth of pathogen, macrophages turn on their microbicidal effector mechanism such as phagolysosomal degradation and production of reactive oxygen intermediates (ROI) and nitric oxide (NO) (24, 25). Like DCs, macrophages are professional antigen presenting cells, and together with DCs, they trigger the adoptive immune system, including T cell activation.

Activated macrophages play a critical role in the host response to *T. gondii* infection by producing ROI and NO. Among the important molecules needed for macrophage activation, IFNγ is produced by NK and T cells whereas TNFα is produced by the macrophages themselves in response to PAMPS (24). These two signals lead to upregulation of inducible nitric oxide synthase (iNOS) and p47 GTPases (26).

Separately, activated macrophages are able to eliminate parasites that have invaded the cell. Invading parasites build a protective parasitophorous vacuole (PV) where the parasite undergoes several rounds of replication and then lyses the PV resulting in destruction of the host cell. The elimination of invaded parasites inside the PV is thought to be mediated by the process of autophagy (27). Interaction of CD40/CD40L can trigger the IFNγ-independent process of autophagy (28, 29). Autophagosomes surround the PV and initiate lysosomal degradation of the parasite. An IFNγ-dependant mechanism for elimination of parasites is the activation of p47 GTPases. IFNγ induced activation the GTPases causes GTPase migration to the PV where they disrupt it and release the parasites from the protective vacuole. The mechanism of this process is still unclear (26, 30).

To prevent immune-mediated pathology and survival after *T. gondii* infection, the production of pro-inflammatory cytokines has to be in balance with anti-inflammatory cytokines such as IL10 (5, 25). Another important function of macrophages, together with DCs and Th2 T cells during the mid-to-late acute phase, is the production of IL10. IL10 inactivates the microbicidal activity of DCs, T cells, NK cells and macrophages themselves. It inhibits antigen processing and presentation, as well as pro-inflammatory cytokine and chemokine production (5).

1.6 $CD169^+$ macrophages

Microorganisms arrive in the lymph node through the lymphatic vessels, which are located directly under the capsule. Here, specialized macrophages capture pathogens/antigen (31-33). Some $CD169^+$ macrophages in the lymph node reside in this area and are called subcapsular sinus (SCS) macrophages. Very little is known about the biological features or development of $CD169^+$ SCS macrophages. However, researchers are starting to

investigate the immunological function of these cells. In general, $CD169^+$ SCS macrophages are poor phagocytic cells, but they are very efficient in capturing small quantities of particulate antigen. This is in contrast to medullary macrophages, which are known to capture large quantities of antigen and are better phagocytic cells (34).

To study the function of SCS macrophages, two-photon imaging was used (31-33, 35-37). The ability of SCS macrophages to capture antigen from the lymphatics and present it for recognition by follicular B cells was demonstrated (32). SCS macrophages are also able to present immune complexes through non-cognate recognition by follicular B cells using complement receptors 1 and 2 (38). In addition, it has been shown that $CD169^+$ macrophages are able to activate $CD8^+$ T cells through cross-presentation of tumor antigens, while DCs were not essential for cross-presentation (35). Further, the activation of invariant natural killer T cells (iNKT) by presentation of lipid antigen by $CD169^+$ macrophages was shown (36). These studies demonstrate the important role of $CD169^+$ macrophages to capture and present antigen to different cell types for activation.

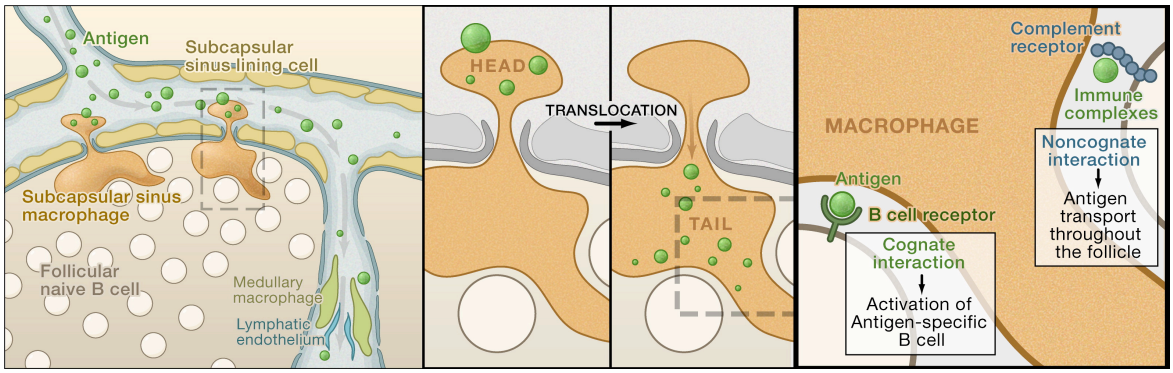


Figure 2. Graphic representation of SCS macrophages capturing and presenting antigen to B cells (Figure is published in Martinez-Pomares et al, 2007)

Depletion experiments have shown that SCS macrophages serve as a barrier to the peripheral nerves and therefore the central nervous system. In mice in which SCS macrophages were depleted, mouse vesicular stomatitis virus (VSV) was able to access the CNS, and susceptibility to the virus was increased. This increased susceptibility was also attributed to decreased pDC recruitment to the subcapsular region and decreased type 1 interferon (IFN-1) production. Therefore, SCS macrophages were responsible for the recruitment of pDC to the subcapsular sinus region, and they were identified as an IFN-1 producing cell type after VSV infection (37).

1.7 Neutrophils

Another IL12 producing cell type is neutrophils (39-41). However, the protective function of neutrophils during *T. gondii* infection is still controversial. Neutrophils are bone marrow derived immune cells, which, under steady-state conditions, are short lived in the bloodstream. During infection, neutrophils are rapidly recruited to the site of infection in response to a variety of chemo-attractants, where they phagocytose and release anti-microbal components to kill the pathogen (42). Another important function of neutrophils is the release of pro-inflammatory chemokines and cytokines to attract other immune cells to the site of infection (43).

A number of studies have reported a large influx of neutrophils after *T. gondii* infection. However these responses have been observed after non-physiologic, high dose intraperitoneal (ip) infection with a highly virulent *T. gondii* strain (RH), in which the host is unable to control the infection (40). More recently, oral inoculation, with an avirulent *T. gondii* strain, did not show a large influx of neutrophils to the site of infection (44). In addition, neutrophils have been proposed to play a protective role in response to *T. gondii*. Depletion experiments using Gr1 (clone RB6-8C5) antibody suggested a critical and protective role of neutrophils during *T. gondii* infection (45, 46). However, Gr1 is also expressed in high levels on IMs (47). Therefore, depletion with RB6-8C5 antibody led to a depletion of neutrophils and IMs. In contrast, deletion experiments using an antibody specific to neutrophils, Ly6G (1A8), demonstrated no such increase in susceptibility to infection (48).

Multiple studies using different antibodies and different strains of knockout mice have yielded a vast array of contradictory data (40, 44, 45, 48-50). Thus far, it appears that neutrophils play an important role in host defense during T. gondii infection. However, the role of neutrophils in *T. gondii* infection remains controversial and their contribution to control infection remains poorly defined. This highlights a need for additional research into neutrophil interactions with infected cells and other immune cell subsets, which will be a major focus of this thesis.

1.8 Inflammatory monocytes

Murine monocytes are divided, by cell surface expression of lineage markers, into two major subsets of monocytes: the $CX3CR1^{hi}CCR2^{-}Ly6C^{-}$ (referred to as $Ly6C^{low}$ monocytes) and $CX3CR1^{lo}CCR2^{+}Ly6C^{hi}$ (referred as $Ly6C^{hi}$ monocytes). Both subsets are derived from the bone marrow. After *Listeria* infection, $Ly6C^{hi}$ monocytes migrate to the spleen and differentiate into so-called TNF/iNOS (Tip) producing DCs, where they play an important role to control the infection (51, 52). While these cells express low levels of CD11c and produce high levels of TNFα and iNOS in a *Listeria* infection model, after *T. gondii* oral infection, $Ly6C^{hi}$ monocytes in the gut do not express CD11c and are referred to in the literature as IMs (44).

The recruitment of TipDCs/IMs from the bone marrow into the bloodstream depends on the concentration of CCL2, the ligand of CCR2, in the blood (51). Therefore, to demonstrate the relevance of IMs after *T. gondii* oral infection, survival and transfer experiments were performed in CCR2 and CCL2 KO mice. *T. gondii* infected CCL2 and CCR2 KO mice showed increased parasite burden and susceptibility when compared to WT mice. The fact that the levels of IL12 and IFNγ in these mice are unchanged suggests that a deficiency in IMs results in lack of control of parasite replication rather than an indirect alteration of cytokine production (44).

1.9 Natural Killer cells

NK cells are a major source of IFNγ in the very early phase of the immune response to *T. gondii* infection (53). IL12 initiates NK cell killing of infected cells. Factors stimulating NK cell proliferation during infection are only beginning to be characterized. However, it has been shown that NK cells constitutively express IL18 receptor α to bind IL18 produced by macrophages and DCs. IL18 then stimulates the production of IFNγ. But, IL18 alone is not sufficient to drive NK cell proliferation. IL18 cooperatively acts with IL15 to stimulate the proliferation of NK cells to enhance IL12 stimulus of NK cells to produce IFNγ (54).

NK cells develop in the bone morrow and circulate in the blood. After infection, NK cells migrate to the lymph node and then to the site of infection where they release IFNγ to stimulate activated macrophages increasing cell-surface MHC class II expression. NK cells express a variety of chemokine receptors. In particular, however, *T. gondii* infected CCR5 KO mice had decreased numbers of NK cells, suggesting an important role for CCR5 in NK cell trafficking to the infection site in response to *T. gondii* infection (55).

Production of IFNγ by NK cells is stimulated by IL12, which is produced by different innate immune cell subsets. But, it has been shown that direct interactions between DCs and NK cells enhances production of IFNγ by NK cells as well as increasing IL12 production by DCs. The interaction between NK cells and DCs is mediated through the NKG2D receptor expressed on NK cells and the ligand expressed on DCs. While NKG2D ligands are not generally expressed on normal cells, they are up-regulated in transformed, stressed or infected cells (56).

1.10 T cell immune response

As described above, during the early immune response to *T. gondii*, IFNγ is induced in a T cell-independent manner, in particular though the up-regulation of IL12 by NK cells. This IFNγ, which is present before T cell recruitment, limits the replication and promotes the killing of the parasite through activation of microbicidal macrophages (7). Another important feature of IFNγ is to direct synthesis of chemoattractants such as macrophage induced gene (MIG) or IFNγ inducible protein-10 (IP10) to recruit T cells and initiate the development of T helper (Th) precursor cells (57).

In this early stage of infection, T cells are recruited by different chemoattractants to the site of infection. The cytokine milieu, especially IFNγ, promotes the differentiation toward Th cells. Through the recognition of antigen, presented by antigen presenting cells (APCs), by Th precursor cells, the differentiation and proliferation to Th1 cells is initiated, and cytokines such as IL2 and additional IFNγ are produced (58). Further, the release of IL2 in the system triggers the activation and proliferation of antigen-specific $CD8^+$ effector T cells. These antigen-specific $CD8^+$ effector T cells have cytotoxic activity, which leads to the killing of infected cells and more IFNγ production. Altogether, the activation of T cells creates a feedback loop that induces the production of more IFNγ (59).

The importance and protective function of $CD8^+$ T cells in the acute and chronic stage of infection has been demonstrated through adoptive transfer experiments. Mice challenged with the highly virulent type 1 parasite strain prior to adoptive transfer of $CD8^+$ T cells from infected or immunized mice demonstrate the highly protective function of $CD8^+$ T cells (60). Depletion experiments during the chronic phase of infection showed increased mortality and demonstrate the importance of $CD8^+$ T cells in long-term resistance to *T. gondii* infection (60). In contrast, *T. gondii* infected CD4 KO mice did not show changes in mortality (61). To protect the host against *T. gondii* infection, $CD8^+$ T cells need to efficiently produce IFNγ and differentiate to antigen specific effector $CD8^+$ T cells with cytotoxic activity. Experiments with infected β_2-microglobulin (β_2m) KO mice that lack $CD8^+$ T cells leads to compensation for the lack of IFNγ production by $CD8^+$ T cells in

this system with an increased number of NK cells (62). Although β_2m KO mice survive the acute stage of infection, they succumb during the chronic stage of infection. In order for $CD8^+$ T cells to differentiate into effector cells, the presence of IL2 produced by $CD4^+$ T cells is required. Depletion of $CD4^+$ T cells leads to a failure in the generation of $CD8^+$ T cell activity and antigen driven $CD8^+$ T cell proliferation (63). In *T. gondii* infected MHC class II (Aβ) KO mice, where the $CD4^+$ population is missing, $CD8^+$ T cells differentiate into effector cells and produce IFNγ. The differentiation to $CD8^+$ effector T cells in the absence of $CD4^+$ T cells in this model can be explained by the fact that $CD4^+$ $NK1.1^+$ T cells provide IL2 for the development of $CD8^+$ effector T cells. $CD4^+$ $NK1.1^+$ T cells develop in the thymus through a MHC class II-independent pathway, therefore $CD4^+$ $NK1.1^+$ T cells are present in Aβ KO mice and can provide $CD8^+$ T cells with IL2 (64). However, as in the β_2m KO mice, Aβ KO mice survive the acute phase of infection, but not the chronic stage of infection.

The mode of antigen recognition by $CD8^+$ T cells during *T. gondii* infection is unclear; which cells are presenting antigen to $CD8^+$ T cells and their mechanisms of antigen presentation is still not fully known. Antigen needs to be efficiently presented to $CD8^+$ T cells by MHC I molecules. In general, for antigen presentation by MHC class I molecules, the antigen needs to be in the cytoplasm for proteasomal processing. The peptides are transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP), where it is associated with the MHC class I heavy chain and β2-microglobulin. Exocytosis to the cell surface allows presentation of the antigen-peptide to $CD8^+$ T cells (65, 66).

MHC class II molecules acquire peptide that is generated by proteolytic degradation within the phagolysosome in endosomal compartments. Therefore, proteins of these peptides were endocytosed/phacytosed from the extracellular environment. The peptide containing phagolysosome fuses with endosomes, the peptide associates with the MHC class II moleces that are inside of the endosome, and the complex is then transported to the cell surface for presentation to $CD4^+$ T cells (66).

Another mechanism of antigen presentation that may occur during *T. gondii* infection is cross-presentation of exogenous antigens by MHC class I molecules. However, the

existing data are very controversial and the exact mechanism of this process is still poorly understood (18, 67-69). One possible model is the transport of antigen through the parasite protective PV into the cytosol. The PV functions as a molecular filter, which allows the diffusion of small molecules (<1300 Da) (70). These molecules can then be presented using the conventional MHC presentation pathways. Another possibility is a cross-priming event, where one cell takes up antigen and presents it to another cell (71) (72).

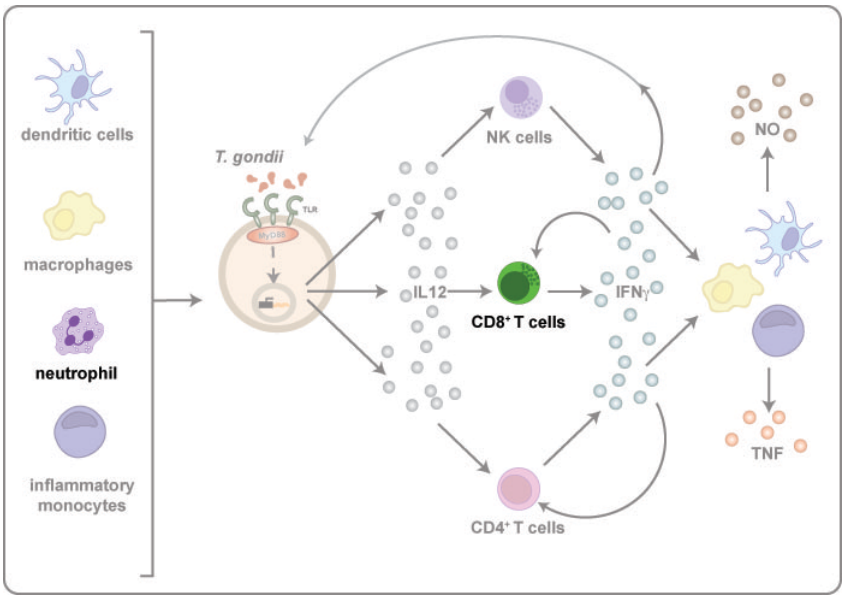
The cell types and the relative contribution of these antigen presentation mechanisms within the natural environment during infection are important questions that remain. One of the goals of this thesis is to fill in gaps in our knowledge of antigen recognition by T cells during T. gondii infection. We explore which cells are possible APCs and determine if these cells are infected with parasite or not.

1.11 Role of the immune system in the pathological progression during the acute phase of *T. gondii* infection

Despite the important role of the immune system in protecting the host from *T. gondii* infection, the immune response itself can cause tissue pathology. During the acute phase of infection after oral inoculation, the susceptible mouse strain C57BL/6, develops severe intestinal pathology characterized by erosion of the intestinal villi and severe necrosis of mucosal tissue. The pathology associated with the acute phase of infection may be mediated by the overproduction of cytokines. Experiments with *T. gondii* infected athymic nude and euthymic B6 mice showed that mucosal pathology is a result of activated T cells rather than parasite burden. Further experiments using $CD4^+$, $CD8^+$ and IL10 KO mice showed that within the T cell populations, IFNγ production by $CD4^+$ T cells mediated necrosis of the small intestine (73, 74).

2. Aims

One powerful method to study the dynamic behavior of immune cells during infection is microscopy. Microscopy can provide information about when, where and how pathogens and host cells interact within physiologically relevant tissue during infection. Widefield epifluorescence microscopy and confocal microscopy have been used for dynamic *in situ* imaging, but both methods are limited in their ability to penetrate into the tissue, restricting analysis to the surface area. An alternative method is two-photon laser-scanning microscopy (TPLSM). Similar to confocal microscopy, TPLSM uses a laser to excite fluorescently labeled cells, but it uniquely allows imaging greater than 200 microns into the tissue with minimal photo-damage. Using TPLSM, we are able to explore three-dimensional time-lapse imaging of intact living tissue where we attain information about cell-cell interactions and cell motility in a physiological environment. In the past, TPLSM has been primarily used to study the dynamics of the immune response using model antigens, but the dynamics of the immune response to pathogens remained underexplored (75, 76). In this thesis, TPLSM was used to understand the immune response to the obligate intracellular parasite, *Toxoplasma gondii*.



Aim 1: As described in the introduction, neutrophils play an important role during *T. gondii* infection. Although neutrophils may not be necessary for the protective immune response against *T. gondii*, they bolster and support the immune cells as they respond

early and are quickly recruited to sites of infection. Early in infection, they participate in IL12 production, which is crucial for protection against *T. gondii* infection. Further, they are a part of the immune response that is responsible for killing parasite invaded cells to limit parasite dissemination throughout the host body. Another very important function of neutrophils is the release of chemoattractants to initiate the recruitment of other immune cells to the site of infection.

Despite the fact that neutrophils play an important role after infection, very little known is about the behavior of this cell type in the lymph node. Therefore, the first aim of this thesis is to study the behavior of neutrophils after *T. gondii* infection. Using TPLSM, we addressed the following questions: Are there unique characteristics of neutrophil migration to sites of infection in the lymph nodes? How do neutrophils interact with infected cells and what are the consequences of such interactions in a physiological environment? Does the recruitment of neutrophils to the site of infection affect the local architecture of the lymph node?

Aim 2: While two-photon imaging has been extensively used to study the behavior of naïve T cells, especially the interaction of T cells with DCs during T cell priming using model antigens, the behavior of T cells during recall responses after pathogen infection is still underexplored. The second aim of this thesis is to examine the dynamic behavior of memory $CD8^+$ T cells in an intact lymph node in respect to their important protective role during *T. gondii* infection. We aimed to compare the localization and migration patterns of of naïve and memory $CD8^+$ T cells during *T. gondii* infection, as well as characterize the naïve and memory T cell interactions with infected cells and determine the consequences of such interactions.

Aim 3:

Toxoplasma gondii is able to persist, in the form of cysts, in the brain of the host and to establish a life-long chronic infection. To keep the infection under control and to prevent the reactivation of infection, the presence of an active immune response in the brain is necessary. As illustrated in the introduction, $CD8^+$ T cells play a key role during the chronic stage of infection and protect the host through the production of pro-inflammatory cytokines. However, how $CD8^+$ T cells behave in chronically infected brain and if and with which APCs they interact with has not yet been explored. The final

part of this thesis is to examine the behavior of effector $CD8^+$ T cells in the brain during the chronic stage of infection. We further characterized the T cell response to *T. gondii* in the brain as well as identified important APC subsets, and we addressed whether $CD8^+$ T cells in the brain interact with cells containing intact cysts or respond to isolated parasites.

Dynamics of Neutrophil Migration in Lymph Nodes during Infection

Chtanova T*, Schaeffer M*, **Han SJ***, van Dooren GG, Nollmann M, Herzmark P, Chan SW, Satija H, Camfield K, Aaron H, Striepen B, Robey EA.
Immunity. 2008 Sep 19;29(3):487-96. **(* joined first authors)**

MANUSCRIPT I

The original article is available online at:
http://dx.doi.org/10.1016/j.immuni.2008.07.012

Experimental contribution

Out of 6 figures, 3 supplementary figures and 14 supplementary movies, I performed the experiments presented in Figure 1, Figure 5A-5D and Figure 6. In addition, I was involved in the setup of the experiments to generate the supplementary movies.

Dynamics of T cell, Antigen-Presenting Cell, and Pathogen Interaction during Recall Response in the Lymph Node

Chtanova T, **Han SJ**, Schaeffer M, van Dooren GG, Herzmark P, Striepen B, Robey EA. Immunity. 2009 Aug 21;31(2):342-55.

MANUSCRIPT II

The original article is available online at:
http://dx.doi.org/10.1016/j.immuni.2009.06.023

Experimental contribution

Out of 6 figures, 6 supplementary figures and 11 supplementary movies, I performed the experiments presented in Figure 1, Figure 4B-4D, Figure 5, Figure 6B and supplementary Figure S2 and part of supplementary Figure S3. In addition, I was involved in the setup of the experiments to generate the supplementary movies.

Dynamic Imaging of T cell-Parasite Interaction in the Brain Chronically Infected with *Toxoplasma gondii*

Schaeffer M, **Han SJ**, Chtanova T, van Dooren GG, Herzmark P, Chen Y, Roysam B, Striepen B, Robey EA.
J Immunol. 2009 May 15;182(10):6379-93.

MANUSCRIPT III

The original article is available online at:
http://dx.doi.org/10.4049/jimmunol.0804307

Experimental contribution

Out of 9 figures and 11 supplementary movies, I performed the experiments presented in Figure 1E, Figure 4A and 4C, Figure 7 and Figure 8A.

6. Discussion

In this thesis, the dynamic behavior of neutrophils and $CD8^+$ T cells during *Toxoplasma gondii* infection was determined. To supplement the known function of neutrophils and $CD8^+$ T cells, TPLSM was used to explore the behavior of these cells in three-dimensional intact living tissues, in response to natural antigen.

To study immune cell migration and interactions with infected cells within a physiological environment, we took advantage of the highly synchronized earflap model, where we inject large numbers of the fluorescently labeled *T. gondii* parasites into the earflap and examine the behavior of immune cells in the draining lymph node shortly after infection by two-photon and confocal microscopy. We, and others, have found that after subcutaneous injection of *T. gondii* or other particulate antigen, the parasite or antigen drains through the lymphatics to the draining LNs and gets captured predominantly by $CD169^+$ subcapsular macrophages (31-33, 77). $CD169^+$ macrophages are distinct from other macrophages particularly in their location in the LN. Very little is known about the function of these macrophages, but as mentioned in the introduction, $CD169^+$ macrophages are able to release cytokines such as type 1 interferon (IFN-1) and are responsible for recruiting other immune cells such as pDCs to the infection site (37), possibly by release of chemoattactants. Our results agree with previous results; we observed rapid recruitment of neutrophils and antigen independent relocalization of memory and naïve T cells to the infection site, strongly suggesting that recruitment of these immune cell subsets is directed by the production of different chemoattractants by highly infected $CD169^+$ macrophages after *T. gondii* infection.

As alluded to earlier, we showed that neutrophils rapidly migrate through the lymphatics and blood vessels to the site of infection in the lymph node and form transient and persistent dynamic swarms presumably triggered by local chemokine production. This dynamic behavior seems to be initiated by the release of multiple chemoattractants, which are produced and released by neutrophils themselves as well as upon parasite egress from cells. Interestingly however, we found that removal of $CD169^+$ macrophages occurs as a consequence of neutrophil swarm formation. Both features of neutrophil

behavior, swarm formation and removal of CD169$^+$ macrophages, were also observed under a more physiologically relevant infection model-after oral infection in the MLN.

The consequences of CD169$^+$ macrophage removal by neutrophils remain unknown. It is clear that these macrophages are especially important when antigens/microorganisms are arriving through the lymphatics into the LN, and in this scenario, these cells contain most of the antigen/microorganisms. Flow cytometry data suggest a decrease in numbers of CD169$^+$ macrophages after infection, suggesting that CD169$^+$ macrophages were killed, removed or changed their phenotype rather than relocalized to another part of the LN. Swarm formation and removal of CD169$^+$ macrophages was seen primarily in areas where CD169$^+$ macrophages were heavily infected. Therefore, one explanation for removing the layer of infected CD169$^+$ macrophages could be to find a balance between the presence of these crucial macrophages to support the immune response or to kill infected host cells to prevent the spread of infection. Another explanation could be that the removal of CD169$^+$ macrophages has immunoregulatory functions. It is possible that the purpose of CD169$^+$ macrophage depletion is to regulate or subdue the production of chemoattractants and control the recruitment of different cells. *In vitro* experiments have shown that after LPS and CpG treatment, CD169$^+$ macrophages are able to produce different cytokines and chemoattractants such as RANTES and MIP-1α (35). It is still unclear what the impact of CD169$^+$ macrophage removal by neutrophils has on the production of cytokines and the downstream immune response.

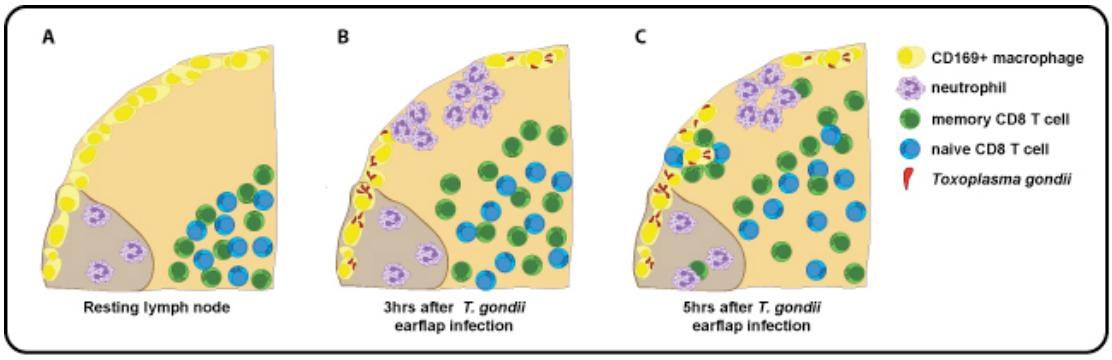


Figure 3. Time line of neutrophil and T cell recruitment after *T. gondii* earflap infection.
A. In a resting lymph node, naïve and memory T cells are evenly distributed in the T cell zone of the lymph node. B. Between 1 and 3 hrs post earflap infection, neutrophils migrate to the site of infection and remove CD169$^+$ macrophages through swarm formation. C. 5 hrs post infection, memory and naïve T cells relocalize to the subcapsular sinus and form clusters around infected CD169$^+$ macrophages.

Our data contribute to recently appreciated roles of CD169$^+$ macrophages to shape the immune response through the release of chemokines after infection, and also to present

antigen to $CD8^+$ T cells. To study antigen-specific interactions between $CD8^+$ T cells and infected $CD169^+$ macrophages, we took advantage of genetically engineered parasites expressing a red fluorescent protein (RFP) and the model antigen OVA as well as OVA-specific, OT1 TCR transgenic T cells. We found that, as characteristic of a memory response, during the very early stages of infection, memory T cells relocalized more rapidly to the site of infection and migrated significantly faster than naïve T cells. Further, we found antigen independent relocalization of memory and naïve T cells towards the foci of infection, suggesting a relocalization of T cells as a response to chemoattractants from the site of infection. Furthermore, we observed antigen dependent stable T cell cluster formation around infected $CD169^+$ macrophages, suggesting that infected $CD169^+$ macrophages are also able to present antigen efficiently to $CD8^+$ T cells. This is in agreement with previous data showing that $CD169^+$ macrophages act as APCs and present antigen to B cells (31, 38). The majority of stable T cell clusters were seen around $CD169^+$ macrophages and DCs. While a large part of T cell clusters surrounded infected $CD169^+$ macrophages, the DCs around which T cell clusters formed did not contain visible parasites, suggesting that the antigen presented by DCs was presented via cross-presentation. This is an intriguing finding since in other systems it has been shown that $CD169^+$ macrophages can cross-present tumor antigens whereas DCs cannot. Thus, the mechanisms of cross-presentation and the cell types presenting antigen in this manner may be specific to the type of antigen involved.

Clusters of T cells consisted of both memory and naïve T cells. Naïve and memory T cells within a cluster had similar behavior; both were able to form long-lasting contacts with infected cells. In addition, we observed T cell clusters break up, where T cells and parasites disperse rapidly. Surprisingly, in some of these cases, we observe invasion of T cells by the parasite.

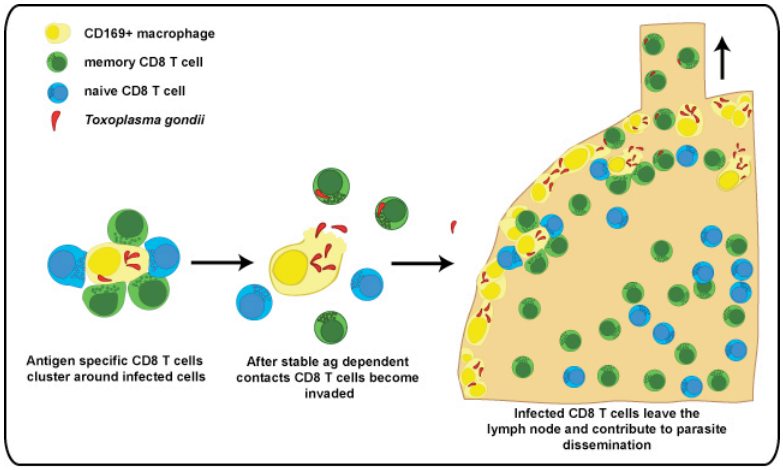


Figure 4. Infected T cells contribute to parasite dissemination.
Within the supcabsular sinus, T cells cluster around infected CD169$^+$ macrophages. During this process, clusters of T cells break up and the parasites invade T cells. Egress of infected T cells from the lymph node contributes to parasite dissemination.

One unexpected result reported in this thesis is the invasion of T cells by parasites after antigen-dependent cluster formation. The question of how parasites access various tissues is still poorly understood. Previous studies have suggested that *T. gondii* infected DCs and macrophages contribute to parasite dissemination throughout the host body (2, 3). Another cell type that could be used as a "Trojan horse" is T cells. We saw in three different experimental setups, that parasites invaded CD8$^+$ T cells during stable antigen-dependent contacts-after earflap infection in the cervical lymph node, after oral infection in the MLN and during the chronic phase of infection in the brain. Further, we saw that ~50% of infected cells in the MLN and blood after oral infection were T cells. There are several scenarios that could explain the high infection rate of T cells. One possibility could be cell composition of the MLN, where 40% of the cells in the MLN are T cells. Also, we observed invasion of T cells after tight contacts, such that T cells are positioned such that they are the first cellular host seen after parasite release from another infected cell. Further, previous data has shown that Fas/FasL interactions and perforin release trigger parasite egress from the host cell (78). This complements our observations that parasite invasion of CD8$^+$ T cells occurs predominantly during tight contacts with infected cells and may reflect directed killing of the infected target cell. In addition, to test the contribution of infected T cells to parasite dissemination, we blocked T cell egress from the LN and saw decreased parasite spread beyond the MLN. Altogether, our data provide evidence that infected T cells may provide a route for the parasite to disseminate throughout the host body.

$CD8^+$ T cells play a protective role during the chronic phase of *T. gondii* infection. Whether $CD8^+$ T cells protect the host through cell-cell mediated recognition or through production of cytokines such as IFNγ is still unknown. To study and visualize the interaction of specific T cells with APCs in the brain of chronically infected mice, we used two-photon and confocal microscopy.

We found that during chronic infection, antigen-specific $CD8^+$ T cells migrated to the brain and accumulated in regions with isolated parasites, whereas in areas with no visible parasites or intact cysts, the density of $CD8^+$ T cells was lower. Furthermore, $CD8^+$ T cells ignored cells with cysts, but they slowed down and transiently arrested close to areas with cells infected with individual parasites. These isolated parasites were often found in or near $CD11b^+$ aggregates, but cysts were never observed within these $CD11b^+$ aggregates. These $CD11b^+$ aggregates have very similar structures to granulomas seen in the liver after *Mycobacterium bovis* infection (79). In this system, granuloma seem to serve as a physical barrier to prevent bacterial dissemination during the chronic phase of infection. The function of $CD11b^+$ granuloma-like structures in chronically infected brain remains unclear; further investigation will help to determine the function of these granuloma-like structures.

Further, we found that $CD8^+$ T cells in the $CD11b^+$ aggregates migrate more slowly and arrested more frequently compared to T cells outside of the $CD11b^+$ aggregates. In the past, antigen-recognition, effector function, and priming of $CD8^+$ T cells are associated with arrest and cluster formation of $CD8^+$ T cells (75). Neither the arrest nor clustering of $CD8^+$ T cells was observed in chronically infected brains. However, very recent two-photon data showed that transient interactions between APCs and T cells could be linked to antigen recognition, during low levels of antigen (80, 81). In the chronic phase of *T. gondii* infection, most of the parasites are "hidden" in cysts and most *T. gondii* antigen is most likely coming from ruptured cysts. Therefore, a low level of antigen is present in chronically infected brains, which could be one of the reasons for such a different behavior of $CD8^+$ T cells in the brain compared to the $CD8^+$ T cells during the acute phase in LNs. Altogether, these behaviors suggest that $CD8^+$ T cells do not recognize cells containing cysts, $CD8^+$ T cells within granuloma structures recognize antigen.

Another notable behavior of $CD8^+$ T cells within the granuloma-like structures is that they did not interact with isolated parasites; they more often appeared to be evenly distributed throughout the entire aggregate. This behavior suggests detection of antigen on aggregates of APCs rather than presented from single cells, and it seems that antigen presentation may occur through cross-presentation rather than through the classical MHC class I antigen presentation pathway. Recent studies in other tissues showed evidence of *T. gondii* antigen being cross-presented to $CD8^+$ T cells (68, 69). Unfortunately, the exact pathway of cross-presentation is still unclear. Understanding the mode of cross-presentation during *T. gondii* infection may clarify the mechanisms of antigen-presentation within granulomas in a chronically infected brain. Together, these observations indicate the presence of ongoing antigen recognition in the brain during the chronic stage of infection.

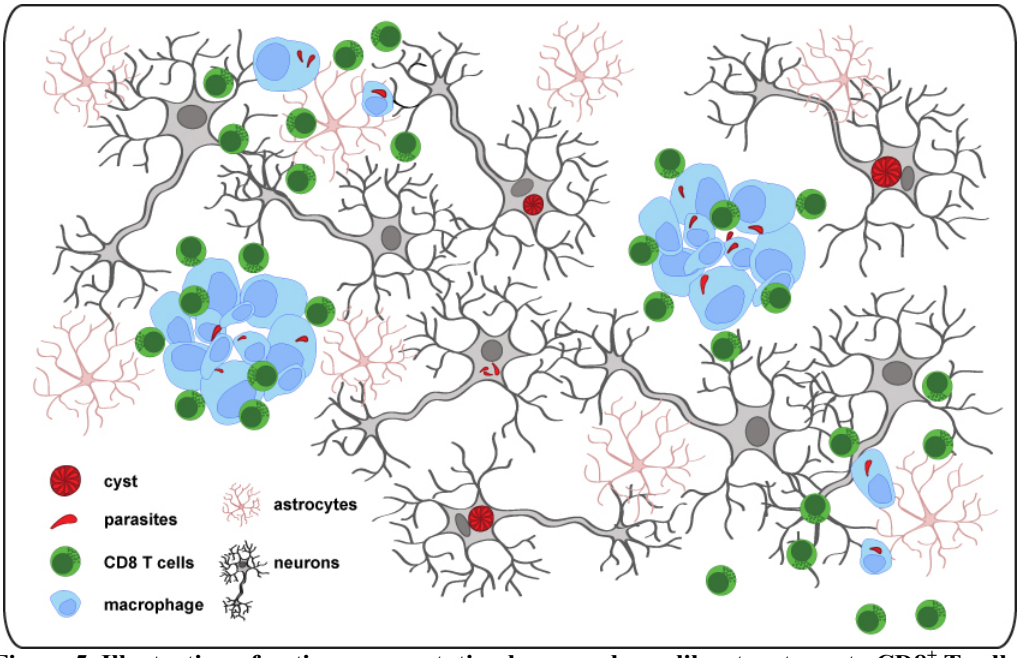


Figure 5. Illustration of antigen-presentation by granuloma-like structures to $CD8^+$ T cells.
$CD8^+$ T cells interact with granuloma-like structures and are present at a higher density in areas with single parasites but ignore cysts.

In summary, the current thesis starts to characterize the early immune response in lymph nodes using living parasites expressing the model antigen OVA. We found that $CD8^+$ T cells migrate in an antigen-independent manner to the subcapsular sinus of the lymph node, the site of infection, and interacts there in an antigen-dependent manner with infected $CD169^+$ macrophages. This T cell response may be modulated through another

observation of ours: the $CD169^+$ macrophage layer is diminished during neutrophil swarm formation. Further, we found that $CD8^+$ T cells form transient, antigen-dependent contacts with granuloma-like aggregates in the brain, but ignored cells with intact cysts. Our work provides information about how the immune cells recognize and interact with each other, which contributes to our understanding of the immune system and the mechanisms by which it protects us from pathogens.

7. Summary

Toxoplasma gondii is an intracellular protozoan parasite that infects most warm-blooded animals. While in healthy individuals the infection is asymptomatic, in immunocompromised individuals, the infection can lead to serious diseases. Like the human, the mouse is a natural host for the parasite, and the mouse immune response to *T. gondii* is similar in many respects to the human. Therefore, mouse models provide an excellent experimental model for understanding the process of the immune response to *T. gondii* infection. To understand how the mammalian immune system responds to *T. gondii* during the acute and chronic phase of infection, two-photon laser scanning microscopy was used to visualize and track the dynamics and interactions between parasites and immune cells in living tissues such as the lymph node and brain.

To study very early immune events after *T. gondii* infection, we chose a highly synchronized system where high numbers of the parasites were injected into the earflap. In this system, the parasites arrive within minutes of inoculation in the draining lymph nodes and get captured by subcapsular sinus macrophages. We found that at the very early stage of infection, neutrophils migrate to the lymph node and start to form, in cooperative action, transient and persistent swarms in highly infected areas. Further, we could correlate swarm formation with removal of $CD169^+$ macrophages at the subcapsular sinus, which may be a defense mechanism to prevent further spread of the parasite. Shortly after neutrophil recruitment, $CD8^+$ T cells migrate to foci of infection and form antigen-specific clusters with remaining infected $CD169^+$ macrophages at the subcapsular sinus. During these stable interactions, target cells are lysed, and parasites often invade T cells, contributing to parasite dissemination to other tissues.

The parasite *T. gondii* has developed strategies to escape the immune system and to hide in organs, such as the brain, where the entry of immune cells is restricted. These organs need to have special immune regulatory mechanisms to prevent the reactivation of inflammation. We found that during chronic phase of infection, $CD8^+$ T cells did not interact with intact cysts, but instead responded to granuloma-like structures in the brain that contained individual parasites or had isolated parasites nearby. $CD8^+$ T cells within the granuloma structures move slowly and arrested more frequently compared to

$CD8^+$ T cells outside of the granuloma structures, consistent with ongoing antigen recognition. But, within the granuloma structures, $CD8^+$ T cells did not slow or arrest near parasites. Rather, the $CD8^+$ T cells were distributed evenly in the entire granuloma structure. Here we hypothesize that those granuloma structures cross-present antigens to $CD8^+$ T cells.

Studies to characterize the behavior of additional immune cells will allow us to fill in the gaps of the immune response and allow us to build a complete picture of the orchestration of an immune response during infection. Our work provides information about how the immune cells recognize and interact with each other, which contributes to our understanding of the immune system and the mechanisms by which it protects us from pathogens.

8. Zusammenfassung

Toxoplasma gondii ist ein einzelliger, intrazellulärer Parasit, der die meisten warmblütigen Tiere infizieren kann. Während bei gesunden Individuen eine Infektion mit *T. gondii* meist asymptomatisch verläuft, kann eine Infektion bei immungeschwächten Individuen zu schweren Krankheiten führen. Das Mausmodell eignet sich ausgezeichnet als experimentelles Tiermodell, um die Immunantwort auf eine *T. gondii*-Infektion zu untersuchen und zu verstehen, da die Maus ebenso wie der Mensch ein natürlicher Wirt des Parasiten ist und zudem die Immunantwort der Maus in vielerlei Hinsicht der Immunantwort des Menschen ähnelt.

Um die Dynamik und die Interaktionen zwischen infizierten Zellen und Immunzellen in lebendem Gewebe, wie Lymphknoten und Gehirn, während einer Infektion zu visualisieren und zu verfolgen, wurde die Zwei-Photonen-Laserscanner-Mikroskopie verwendet.

In dieser Arbeit wurde zur Untersuchung der Immunantwort nach einer Infektion mit *T. gondii* ein hochsynchronisiertes System genutzt, bei dem zunächst eine große Dosis von *T. gondii*-Parasiten in die Ohrmuschel von Mäusen injiziert wird. Anschließend gelangen die Parasiten von dort durch das lymphatische System innerhalb weniger Minuten nach der Injektion in die nahegelegenen Lymphknoten, wo sie von subkapsulären Makrophagen „eingefangen“ werden.
In dieser Arbeit wurde gezeigt, dass in der frühen Phase nach der Ohrmuschel-Infektion neutrophile Zellen zu den Lymphknoten und innerhalb dieser in die Regionen mit verstärkt infizierten Zellen wandern, bei denen es sich hauptsächlich um $CD169^+$-Makrophagen handelt. Dort bilden die neutrophilen Zellen transiente und persistente Schwärme. Des Weiteren belegt diese Arbeit, dass die Bildung der Schwärme von neutrophilen Zellen mit einem Rückgang von $CD169^+$-Makrophagen am subkapsulären Sinus korreliert. Bei diesem Vorgang könnte es sich um einen Mechanismus handeln, der die Dissemination der Parasiten verhindern soll. Kurz nach der Rekrutierung von neutrophilen Zellen wandern auch $CD8^+$-T-Zellen zum Infektionsherd und bilden im subkapsulären Sinus antigenspezifische Cluster mit den verbliebenen infizierten $CD169^+$-Makrophagen. Während dieser stabilen Interaktion kommt es zur Lysis der

infizierten Zellen, wobei die freigesetzten Parasiten anschließend in die nahegelegenen T-Zellen eindringen und sich somit in andere Gewebe ausbreiten.

Der Parasit *T. gondii* hat verschiedene Strategien entwickelt, um der Immunantwort zu entgehen und sich in Organen mit limitiertem Zugang für Immunzellen, wie z.B. dem Gehirn, zu verbergen. Diese Organe benötigen daher spezielle, immunregulatorische Mechanismen, um ein Wiederaufflammen der Entzündung zu verhindern. In der vorliegenden Arbeit konnte gezeigt werden, dass die $CD8^+$-T-Zellen im Gehirn während der chronischen Phase einer Infektion nicht mit Zellen interagieren, die mit intakten Zysten infiziert sind, sondern mit Granulom-ähnlichen Strukturen, die einzelne Parasiten beherbergen oder isolierte Parasiten in unmittelbarer Nachbarschaft aufweisen. Konsistent mit einer fortwährenden Antigen-Erkennung bewegen sich die $CD8^+$-T-Zellen innerhalb der Granulom-Strukturen langsamer und stoppen häufiger als $CD8^+$-T-Zellen außerhalb der Granulom-Strukturen. Allerdings erfolgt diese Verlangsamung bzw. das Anhalten der $CD8^+$-T-Zellen in den Granulom-Strukturen nicht im Zusammenspiel mit infizierten Zellen, sondern die $CD8^+$-T-Zellen verteilen sich vielmehr gleichmäßig in der gesamten Granulom-Struktur. Das führt zu der Vermutung, dass den $CD8^+$-T-Zellen die Antigene in diesen Granulom-Strukturen durch *cross-presentation* präsentiert werden.

Diese Arbeit liefert Informationen darüber, wie sich Immunzellen erkennen und miteinander interagieren, und trägt so zum Verständnis über das Immunsystem und seiner Mechanismen bei, mit denen es uns vor Krankheitserregern schützt. (Zudem bringt diese Arbeit neue Erkenntnisse über die Art und Weise, auf die der Parasit *T. gondii* der Immunantwort entgeht.) Allerdings muss insbesondere das Verhalten anderer an der Immunantwort beteiligter Immunzellen in weiteren Studien charakterisiert werden, um ein noch umfassenderes Bild der Immunantwort auf eine *T. gondii*-Infektion zu erhalten.

9. References

1. Black MW, Boothroyd JC. 2000. Lytic cycle of Toxoplasma gondii. *Microbiol Mol Biol Rev* 64: 607-23
2. Lambert H, Hitziger N, Dellacasa I, Svensson M, Barragan A. 2006. Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination. *Cell Microbiol* 8: 1611-23
3. Courret N, Darche S, Sonigo P, Milon G, Buzoni-Gatel D, Tardieux I. 2006. CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood* 107: 309-16
4. Blader IJ, Saeij JP. 2009. Communication between Toxoplasma gondii and its host: impact on parasite growth, development, immune evasion, and virulence. *APMIS* 117: 458-76
5. Aliberti J. 2005. Host persistence: exploitation of anti-inflammatory pathways by Toxoplasma gondii. *Nat Rev Immunol* 5: 162-70
6. Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, Medzhitov R, Sher A. 2002. Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *J Immunol* 168: 5997-6001
7. Gazzinelli RT, Hieny S, Wynn TA, Wolf S, Sher A. 1993. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc Natl Acad Sci U S A* 90: 6115-9
8. Yap G, Pesin M, Sher A. 2000. Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. *J Immunol* 165: 628-31
9. Trinchieri G, Gerosa F. 1996. Immunoregulation by interleukin-12. *J Leukoc Biol* 59: 505-11
10. Reis e Sousa C, Hieny S, Scharton-Kersten T, Jankovic D, Charest H, Germain RN, Sher A. 1997. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J Exp Med* 186: 1819-29
11. Gazzinelli RT, Hayashi S, Wysocka M, Carrera L, Kuhn R, Muller W, Roberge F, Trinchieri G, Sher A. 1994. Role of IL-12 in the initiation of cell mediated immunity by Toxoplasma gondii and its regulation by IL-10 and nitric oxide. *J Eukaryot Microbiol* 41: 9S
12. Kawai T, Akira S. 2005. Pathogen recognition with Toll-like receptors. *Curr Opin Immunol* 17: 338-44
13. Yarovinsky F. 2008. Toll-like receptors and their role in host resistance to Toxoplasma gondii. *Immunol Lett* 119: 17-21
14. Debierre-Grockiego F, Campos MA, Azzouz N, Schmidt J, Bieker U, Resende MG, Mansur DS, Weingart R, Schmidt RR, Golenbock DT, Gazzinelli RT, Schwarz RT. 2007. Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from Toxoplasma gondii. *J Immunol* 179: 1129-37
15. Minns LA, Menard LC, Foureau DM, Darche S, Ronet C, Mielcarz DW, Buzoni-Gatel D, Kasper LH. 2006. TLR9 is required for the gut-associated lymphoid

tissue response following oral infection of Toxoplasma gondii. *J Immunol* 176: 7589-97

16. Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S, Sher A. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308: 1626-9
17. Mun HS, Aosai F, Norose K, Chen M, Piao LX, Takeuchi O, Akira S, Ishikura H, Yano A. 2003. TLR2 as an essential molecule for protective immunity against Toxoplasma gondii infection. *Int Immunol* 15: 1081-7
18. Gubbels MJ, Striepen B, Shastri N, Turkoz M, Robey EA. 2005. Class I major histocompatibility complex presentation of antigens that escape from the parasitophorous vacuole of Toxoplasma gondii. *Infect Immun* 73: 703-11
19. Reis e Sousa C, Yap G, Schulz O, Rogers N, Schito M, Aliberti J, Hieny S, Sher A. 1999. Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology. *Immunity* 11: 637-47
20. Scott P, Hunter CA. 2002. Dendritic cells and immunity to leishmaniasis and toxoplasmosis. *Curr Opin Immunol* 14: 466-70
21. Mashayekhi M, Sandau MM, Dunay IR, Frickel EM, Khan A, Goldszmid RS, Sher A, Ploegh HL, Murphy TL, Sibley LD, Murphy KM. 2011. CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. *Immunity* 35: 249-59
22. Liu CH, Fan YT, Dias A, Esper L, Corn RA, Bafica A, Machado FS, Aliberti J. 2006. Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice. *J Immunol* 177: 31-5
23. Hou B, Benson A, Kuzmich L, DeFranco AL, Yarovinsky F. 2011. Critical coordination of innate immune defense against Toxoplasma gondii by dendritic cells responding via their Toll-like receptors. *Proc Natl Acad Sci U S A* 108: 278-83
24. Stafford JL, Neumann NF, Belosevic M. 2002. Macrophage-mediated innate host defense against protozoan parasites. *Crit Rev Microbiol* 28: 187-248
25. Mosser DM. 2003. The many faces of macrophage activation. *J Leukoc Biol* 73: 209-12
26. Taylor GA, Feng CG, Sher A. 2007. Control of IFN-gamma-mediated host resistance to intracellular pathogens by immunity-related GTPases (p47 GTPases). *Microbes Infect* 9: 1644-51
27. Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJ, Yap GS. 2006. Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed effector macrophages. *J Exp Med* 203: 2063-71
28. Yap GS, Ling Y, Zhao Y. 2007. Autophagic elimination of intracellular parasites: convergent induction by IFN-gamma and CD40 ligation? *Autophagy* 3: 163-5
29. Andrade RM, Wessendarp M, Gubbels MJ, Striepen B, Subauste CS. 2006. CD40 induces macrophage anti-Toxoplasma gondii activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. *J Clin Invest* 116: 2366-77
30. Martens S, Parvanova I, Zerrahn J, Griffiths G, Schell G, Reichmann G, Howard JC. 2005. Disruption of Toxoplasma gondii parasitophorous vacuoles by the mouse p47-resistance GTPases. *PLoS Pathog* 1: e24
31. Phan TG, Grigorova I, Okada T, Cyster JG. 2007. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol* 8: 992-1000

32. Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, Fink K, Henrickson SE, Shayakhmetov DM, Di Paolo NC, van Rooijen N, Mempel TR, Whelan SP, von Andrian UH. 2007. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* 450: 110-4
33. Carrasco YR, Batista FD. 2007. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* 27: 160-71
34. Gretz JE, Norbury CC, Anderson AO, Proudfoot AE, Shaw S. 2000. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med* 192: 1425-40
35. Asano K, Nabeyama A, Miyake Y, Qiu CH, Kurita A, Tomura M, Kanagawa O, Fujii S, Tanaka M. 2011. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity* 34: 85-95
36. Barral P, Polzella P, Bruckbauer A, van Rooijen N, Besra GS, Cerundolo V, Batista FD. 2010. CD169(+) macrophages present lipid antigens to mediate early activation of iNKT cells in lymph nodes. *Nat Immunol* 11: 303-12
37. Iannacone M, Moseman EA, Tonti E, Bosurgi L, Junt T, Henrickson SE, Whelan SP, Guidotti LG, von Andrian UH. 2010. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. *Nature* 465: 1079-83
38. Phan TG, Green JA, Gray EE, Xu Y, Cyster JG. 2009. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat Immunol* 10: 786-93
39. Bliss SK, Zhang Y, Denkers EY. 1999. Murine neutrophil stimulation by Toxoplasma gondii antigen drives high level production of IFN-gamma-independent IL-12. *J Immunol* 163: 2081-8
40. Bliss SK, Butcher BA, Denkers EY. 2000. Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. *J Immunol* 165: 4515-21
41. Sukhumavasi W, Egan CE, Denkers EY. 2007. Mouse neutrophils require JNK2 MAPK for Toxoplasma gondii-induced IL-12p40 and CCL2/MCP-1 release. *J Immunol* 179: 3570-7
42. Nathan C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 6: 173-82
43. Denkers EY, Butcher BA, Del Rio L, Bennouna S. 2004. Neutrophils, dendritic cells and Toxoplasma. *Int J Parasitol* 34: 411-21
44. Dunay IR, Damatta RA, Fux B, Presti R, Greco S, Colonna M, Sibley LD. 2008. Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen Toxoplasma gondii. *Immunity* 29: 306-17
45. Bliss SK, Gavrilescu LC, Alcaraz A, Denkers EY. 2001. Neutrophil depletion during Toxoplasma gondii infection leads to impaired immunity and lethal systemic pathology. *Infect Immun* 69: 4898-905
46. Sayles PC, Johnson LL. 1996. Exacerbation of toxoplasmosis in neutrophil-depleted mice. *Nat Immun* 15: 249-58
47. Serbina NV, Pamer EG. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 7: 311-7

48. Dunay IR, Fuchs A, Sibley LD. 2010. Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice. *Infect Immun* 78: 1564-70
49. Khan IA, Murphy PM, Casciotti L, Schwartzman JD, Collins J, Gao JL, Yeaman GR. 2001. Mice lacking the chemokine receptor CCR1 show increased susceptibility to Toxoplasma gondii infection. *J Immunol* 166: 1930-7
50. Del Rio L, Bennouna S, Salinas J, Denkers EY. 2001. CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during Toxoplasma gondii infection. *J Immunol* 167: 6503-9
51. Shi C, Pamer EG. 2011. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* 11: 762-74
52. Serbina NV, Jia T, Hohl TM, Pamer EG. 2008. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol* 26: 421-52
53. Hunter CA, Subauste CS, Van Cleave VH, Remington JS. 1994. Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infect Immun* 62: 2818-24
54. French AR, Holroyd EB, Yang L, Kim S, Yokoyama WM. 2006. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. *Cytokine* 35: 229-34
55. Khan IA, Thomas SY, Moretto MM, Lee FS, Islam SA, Combe C, Schwartzman JD, Luster AD. 2006. CCR5 is essential for NK cell trafficking and host survival following Toxoplasma gondii infection. *PLoS Pathog* 2: e49
56. Guan H, Moretto M, Bzik DJ, Gigley J, Khan IA. 2007. NK cells enhance dendritic cell response against parasite antigens via NKG2D pathway. *J Immunol* 179: 590-6
57. Gazzinelli RT, Amichay D, Sharton-Kersten T, Grunwald E, Farber JM, Sher A. 1996. Role of macrophage-derived cytokines in the induction and regulation of cell-mediated immunity to Toxoplasma gondii. *Curr Top Microbiol Immunol* 219: 127-39
58. Villegas EN, Lieberman LA, Carding SR, Hunter CA. 2002. Susceptibility of interleukin-2-deficient mice to Toxoplasma gondii is associated with a defect in the production of gamma interferon. *Infect Immun* 70: 4757-61
59. Gigley JP, Bhadra R, Khan IA. 2011. CD8 T Cells and Toxoplasma gondii: A New Paradigm. *J Parasitol Res* 2011: 243796
60. Parker SJ, Roberts CW, Alexander J. 1991. CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to Toxoplasma gondii in mice. *Clin Exp Immunol* 84: 207-12
61. Gazzinelli R, Xu Y, Hieny S, Cheever A, Sher A. 1992. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii. *J Immunol* 149: 175-80
62. Denkers EY, Gazzinelli RT, Martin D, Sher A. 1993. Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to Toxoplasma gondii in MHC class I-deficient mice. *J Exp Med* 178: 1465-72
63. Gazzinelli RT, Hakim FT, Hieny S, Shearer GM, Sher A. 1991. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated Toxoplasma gondii vaccine. *J Immunol* 146: 286-92
64. Denkers EY, Scharton-Kersten T, Barbieri S, Caspar P, Sher A. 1996. A role for CD4+ NK1.1+ T lymphocytes as major histocompatibility complex class II

independent helper cells in the generation of CD8+ effector function against intracellular infection. *J Exp Med* 184: 131-9

65. Shastri N, Cardinaud S, Schwab SR, Serwold T, Kunisawa J. 2005. All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunol Rev* 207: 31-41
66. Goldszmid RS, Sher A. 2010. Processing and presentation of antigens derived from intracellular protozoan parasites. *Curr Opin Immunol* 22: 118-23
67. Dzierszinski F, Pepper M, Stumhofer JS, LaRosa DF, Wilson EH, Turka LA, Halonen SK, Hunter CA, Roos DS. 2007. Presentation of Toxoplasma gondii antigens via the endogenous major histocompatibility complex class I pathway in nonprofessional and professional antigen-presenting cells. *Infect Immun* 75: 5200-9
68. Goldszmid RS, Coppens I, Lev A, Caspar P, Mellman I, Sher A. 2009. Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in Toxoplasma gondii-infected dendritic cells. *J Exp Med* 206: 399-410
69. John B, Harris TH, Tait ED, Wilson EH, Gregg B, Ng LG, Mrass P, Roos DS, Dzierszinski F, Weninger W, Hunter CA. 2009. Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii. *PLoS Pathog* 5: e1000505
70. Schwab JC, Beckers CJ, Joiner KA. 1994. The parasitophorous vacuole membrane surrounding intracellular Toxoplasma gondii functions as a molecular sieve. *Proc Natl Acad Sci U S A* 91: 509-13
71. Heath WR, Carbone FR. 2001. Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol* 19: 47-64
72. Yewdell JW, Reits E, Neefjes J. 2003. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat Rev Immunol* 3: 952-61
73. Araujo FG. 1992. Depletion of CD4+ T cells but not inhibition of the protective activity of IFN-gamma prevents cure of toxoplasmosis mediated by drug therapy in mice. *J Immunol* 149: 3003-7
74. Liesenfeld O, Kosek J, Remington JS, Suzuki Y. 1996. Association of CD4+ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with Toxoplasma gondii. *J Exp Med* 184: 597-607
75. Cahalan MD, Parker I. 2008. Choreography of cell motility and interaction dynamics imaged by two-photon microscopy in lymphoid organs. *Annu Rev Immunol* 26: 585-626
76. Coombes JL, Robey EA. 2010. Dynamic imaging of host-pathogen interactions in vivo. *Nat Rev Immunol* 10: 353-64
77. Szakal AK, Holmes KL, Tew JG. 1983. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells, including cells with dendritic morphology. *J Immunol* 131: 1714-27
78. Persson EK, Agnarson AM, Lambert H, Hitziger N, Yagita H, Chambers BJ, Barragan A, Grandien A. 2007. Death receptor ligation or exposure to perforin trigger rapid egress of the intracellular parasite Toxoplasma gondii. *J Immunol* 179: 8357-65
79. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. 2008. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity* 28: 271-84

80. Egen JG, Rothfuchs AG, Feng CG, Horwitz MA, Sher A, Germain RN. 2011. Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity* 34: 807-19
81. Filipe-Santos O, Pescher P, Breart B, Lippuner C, Aebischer T, Glaichenhaus N, Spath GF, Bousso P. 2009. A dynamic map of antigen recognition by CD4 T cells at the site of Leishmania major infection. *Cell Host Microbe* 6: 23-33

Page 41 to 107 include the original manuscripts.