A Novel Population of Natural Killer Cells Plays a Critical Role in the Depletion of Splenic B2 B Cells During Experimental African Trypanosomiasis

Deborah Frenkel

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A NOVEL POPULATION OF NATURAL KILLER CELLS PLAYS A CRITICAL ROLE IN THE DEPLETION OF SPLENIC B2 B CELLS DURING EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

A Dissertation Presented
by
DEBORAH RUTH FRENKEL

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of
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THE DEPLETION OF SPLENIC B2 B CELLS DURING EXPERIMENTAL AFRICAN
TRYPANOSOMIASIS

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Samuel J. Black, Chair

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DEDICATION

I dedicate this dissertation to my family who have been by my side to support and encourage me throughout the entire process. You have offered your love, and your belief in me unconditionally and I have relied on you for internal strength and perseverance.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Samuel J. Black, for his guidance, his patience and unwavering optimism and exuberance. I express my gratitude to the members of my committee, Dr. Cynthia Baldwin for generously offering time, guidance and support, Dr. Robert Woodland, for his contributions to and thoughtful insights regarding the research and his willingness to travel.

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A very special thank you to all of the family and friends, who have supported me, guided me, pushed and pulled me and generally encouraged me to pursue and finish this step.
ABSTRACT

A NOVEL POPULATION OF NATURAL KILLER CELLS PLAYS A CRITICAL ROLE IN THE DEPLETION OF SPLENIC B2 B CELLS DURING EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

FEBRUARY 2015

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Directed by: Professor Samuel J. Black

Loss of humoral immune competence in *T. brucei*-infected mice is associated with the apoptotic depletion of splenic transitional, marginal zone and follicular B cells as well as a depletion of CD8$^+$ T cells. This occurs rapidly after infection and impairs responses to vaccine antigens in addition to responses to newly arising VSG antigenic variants, leading to uncontrolled parasite growth and death of the infected mice. Infection-induced B2 B cell and CD8$^+$ T cell loss requires the presence of a novel population of natural killer (NK) cells and is mediated by a perforin-dependent process consistent with perforin- and granzyme-mediated cytotoxicity. The absence of perforin in deficient mice prevents infection-induced loss of splenic B cells, enhances trypanosome-specific antibody responses, reduces infection-associated anemia and prolongs survival after infection thus enhancing trypanosomiasis-resistance.

Additionally, the parasite enzyme phospholipase C (PLC) is required for B2 B cell and Cd8$^+$ T cell depletion. Abrogation of infection induced immunopathology during infection with a PLC deficient parasite (PLC$^{-/-}$ *T. brucei*) is dependent on the presence of the antigen presenting molecule CD1d, as B2 B cell depletion and CD8$^+$ T cell depletion occur during infection of CD1d$^{-/-}$ mice with PLC$^{-/-}$ *T. brucei*. Natural killer cells acquire the same novel phenotype during infection with intact or PLC$^{-/-}$ *T. brucei*, however only during infection with the intact parasites are the NK cells shown to degranulate.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The parasites</td>
<td>2</td>
</tr>
<tr>
<td>VSG</td>
<td>4</td>
</tr>
<tr>
<td>Trypanosomiasis – the disease</td>
<td>5</td>
</tr>
<tr>
<td>Tsetse control</td>
<td>6</td>
</tr>
<tr>
<td>Chemotherapy/drug resistance</td>
<td>8</td>
</tr>
<tr>
<td>Vaccination</td>
<td>9</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>11</td>
</tr>
<tr>
<td>Trypanopsomiasis resistance and susceptibility</td>
<td>11</td>
</tr>
<tr>
<td>Focus of this thesis</td>
<td>13</td>
</tr>
<tr>
<td>II. NKP46&lt;sup&gt;Ve&lt;/sup&gt; NATURAL KILLER CELLS DELETE B2 B CELLS AND SUPPRESS HUMORAL IMMUNITY IN MURINE AFRICAN TRYPANOSOMIASIS</td>
<td>19</td>
</tr>
<tr>
<td>Abstract</td>
<td>19</td>
</tr>
<tr>
<td>Author summary</td>
<td>20</td>
</tr>
<tr>
<td>Introduction</td>
<td>21</td>
</tr>
<tr>
<td>Results</td>
<td>24</td>
</tr>
<tr>
<td>Retention of splenic B2 B cells and production of trypanosome antigen-specific igg in T. brucei-infected perf&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>24</td>
</tr>
<tr>
<td>T cells and NKT cells are not required for T. brucei infection-induced B cell deletion</td>
<td>26</td>
</tr>
<tr>
<td>NK cells are required for T. brucei infection-induced B cell deletion</td>
<td>26</td>
</tr>
</tbody>
</table>
NK cells in T. brucei infected mice have a novel phenotype .......... 27

Discussion ............................................................................................................ 29

Materials and methods ............................................................................................................ 34

Ethics statement ............................................................................................................ 34

Mice and trypanosomes ............................................................................................................ 35

Mouse weight and packed cell volume measurements ............................................................................................................ 35

Antibodies: Flow Cytometry ............................................................................................................ 35

Cell isolation and flow cytometric analysis ............................................................................................................ 36

Immunofluorescent staining of mouse spleen ............................................................................................................ 37

NK cell depletion ............................................................................................................ 38

Western blot analysis of trypanosome-polypeptide specific antibodies in mouse serum ............................................................................................................ 38

Enzyme linked immunosorbent assay (ELISA) of trypanosome-specific antibodies ............................................................................................................ 39

Statistics ............................................................................................................ 39

III. NK CELL AND PERFORIN DEPENDENT DEPLETION OF CD8 T CELLS IN TRYANOSOMA BRUCEI INFECTED MICE ............................................................................................................ 54

Abstract ............................................................................................................ 54

Introduction ............................................................................................................ 56

Results ............................................................................................................ 59

Experimental strategy ............................................................................................................ 59

T. brucei infection induces depletion of splenic CD8^+ T cells in C57BL/6 mice ............................................................................................................ 59

CD8^+ T cells are not deleted from the spleens of C57BL/6 mice depleted of NK1.1^+ cells ............................................................................................................ 60

CD8^+ T cells are depleted from the spleens T. brucei infected CD1d^−/− C57BL/6 mice ............................................................................................................ 61
CD8+ T cells are not depleted from the spleens of T. brucei infected perforin−/− C57BL/6 mice but are depleted from the spleens of T. brucei infected CD16−/− mice.

Expression of myd88 and trif is required for T. brucei infection induced depletion of splenic CD8+ T cells.

Trypanosome lysate injections do not induce depletion of splenic CD8+ T cells in C57BL/6 mice.

Discussion

Materials and methods

Ethics statement

Mice and trypanosomes

Cell isolation and flow cytometric analysis

Preparation of T. brucei lysate

Antibodies and detection reagents

NK cell depletion and FasL Blocking

Statistics

IV. TRYPANOSOMA BRUCEI PLC AND HOST CD1D JOINTLY REGULATE INFECTION INDUCED DEPLETION OF SPLENIC B2 B CELLS BY NK CELLS

Abstract

Introduction

Results

Infection of C57BL/6 mice with PLC−/− T. brucei does not cause depletion of splenic B2 B cells.

In the absence of host CD1d, virulence is restored to the PLC−/− T. brucei.

Neither T cells nor natural killer T cells are required for low virulence of PLC−/− T. brucei.
Co-infection with PLC<sup>−/−</sup> <i>T. brucei</i> does not reduce the virulence of <i>T. brucei</i> Antat1.1e in C57BL/6 mice ......................................................... 83
Depletion of NK1.1<sup>+</sup> cells prevents loss of splenic B2 B cells during PLC<sup>−/−</sup> infection of CD1d<sup>−/−</sup> mice ............................................................. 84
CD1d-dependent suppression of NK cell degranulation in mice infected with PLC<sup>−/−</sup> <i>T. brucei</i> ...................................................................................................... 84
Intravenous administration of <i>T. brucei</i> Antat1.1e lysate to C57BL/6 mice infected with PLC<sup>−/−</sup> <i>T. brucei</i> does not cause depletion of splenic B2 B cells or increase virulence ............................................................. 86

Discussion ............................................................................................................ 87

Materials and methods .......................................................................................... 92

   Ethics statement .................................................................................................. 92
   Mice and trypanosomes ...................................................................................... 92
   Cell isolation and flow cytometric analysis ....................................................... 92
   Preparation of <i>T. brucei</i> lysate ....................................................................... 93
   Antibodies and detection reagents ..................................................................... 94
   NK cell depletion and FasL Blocking ............................................................... 94
   Statistics ............................................................................................................ 94

V. CONCLUDING COMMENTS ............................................................................. 107

Future Directions .................................................................................................. 111

BIBLIOGRAPHY ..................................................................................................... 115
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>SCIENTIFIC CLASSIFICATION OF <em>T. BRUCEI</em></td>
<td>15</td>
</tr>
<tr>
<td>2.1</td>
<td>B CELL, NK CELL, NKT CELL DIFFERENIATION ANTIGENS</td>
<td>53</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>1.1</td>
<td>T. brucei in bloodstream</td>
<td>15</td>
</tr>
<tr>
<td>1.2</td>
<td>Tsetse fly before and after bloodmeal</td>
<td>16</td>
</tr>
<tr>
<td>1.3</td>
<td>Distribution of cattle and tsetse in Africa</td>
<td>16</td>
</tr>
<tr>
<td>1.3</td>
<td>Cases of HAT in DRC 1926-2011</td>
<td>17</td>
</tr>
<tr>
<td>1.5</td>
<td>Trypanosoma brucei life cycle</td>
<td>17</td>
</tr>
<tr>
<td>1.4</td>
<td>VSG homodimer</td>
<td>18</td>
</tr>
<tr>
<td>1.7</td>
<td>Tsetse trap and catch</td>
<td>18</td>
</tr>
<tr>
<td>2.1</td>
<td>Splenic B cells during infection of perforin deficient mice with T. brucei</td>
<td>41</td>
</tr>
<tr>
<td>2.2</td>
<td>Splenic B cells during infection of intact C57BL/6 mice with T. brucei</td>
<td>42</td>
</tr>
<tr>
<td>2.3</td>
<td>B220 and MOMA staining of infected mouse spleen</td>
<td>43</td>
</tr>
<tr>
<td>2.4</td>
<td>Detection of anti-trypanosome antigen antibodies in mouse spleen via ELISA and Western Blot analysis</td>
<td>44</td>
</tr>
<tr>
<td>2.5</td>
<td>Splenic B cells during infection of TCR deficient mice with T. brucei</td>
<td>45</td>
</tr>
<tr>
<td>2.6</td>
<td>Depletion of NK cells and splenic B cells during infection of NK cell depleted mice with T. brucei</td>
<td>46</td>
</tr>
<tr>
<td>2.7</td>
<td>NK cell expression of differentiation antigens during T. brucei infection</td>
<td>47</td>
</tr>
<tr>
<td>2.8</td>
<td>Splenic B cell expression of death receptors during T. brucei infection</td>
<td>48</td>
</tr>
<tr>
<td>2.9</td>
<td>Splenic B1 B cells during infection of C57BL/6 mice with T. brucei</td>
<td>49</td>
</tr>
<tr>
<td>2.10</td>
<td>Expression of MHC I on B cells during T. brucei infection</td>
<td>50</td>
</tr>
<tr>
<td>2.11</td>
<td>Splenic B cells during infection of β2 microglobulin deficient mice with T. brucei</td>
<td>51</td>
</tr>
<tr>
<td>2.12</td>
<td>Splenic B cells during infection of CD16 deficient mice with T. brucei</td>
<td>52</td>
</tr>
</tbody>
</table>
3.2 Splenic CD8+ T Cells During Infection of C57BL/6 Mice with T. Brucei

3.3 Splenic CD8+ T Cells During Infection of NK Cell Depleted C57BL/6 Mice with T. Brucei

3.4 Depletion of NK Cells in C57BL/6 Mice

3.5 Splenic CD8+ T Cells During Infection of CD1d Deficient Mice with T. Brucei

3.6 Splenic CD8+ T Cells During Infection of Perforin Deficient Mice with T. Brucei

3.7 Splenic CD8+ T Cells During Infection of CD16 Deficient Mice with T. Brucei

3.8 Splenic CD8+ T Cells During Infection of Myd88 Deficient Mice with T. Brucei

3.9 Splenic CD8+ T Cells in C57BL/6 Mice After Injection with T. Brucei Whole Cell Lysate

4.1 Splenic B Cells During Infection of C57BL/6 Mice with Intact or PLC Deficient T. Brucei

4.2 Splenic B Cells During Infection of CD1d Deficient Mice with Intact or PLC Deficient T. Brucei

4.3 Anemia During Infection of CD1d Deficient Mice with Intact or PLC Deficient T. Brucei

4.4 Splenic B Cells During Infection of TCR Deficient Mice with Intact or PLC Deficient T. Brucei

4.5 Splenic B Cells During Coinfection of C57BL/6 Mice with Intact and PLC Deficient T. Brucei

4.6 Splenic B Cells During Infection of CD1d Deficient Mice Depleted of NK Cells with Intact or PLC Deficient T. Brucei

4.7 NK Cell Expression of Activation Markers During PLC Deficient T. Brucei Infection

4.8 NK Cell Expression of Differentiation Antigens During PLC Deficient T. Brucei Infection

4.9 Expression of MHC I on B Cells and Receptors for MHC I on NK Cells During Infection of CD1d-/- or Intact C57BL/6 Mice with PLC Deficient T. Brucei
4.10  EXPRESSION OF CD1D ON B CELLS AND THE CD1D RECEPTOR 2B4 ON
INTACT AND CD1D DEFICIENT MICE DURING INFECTION WITH INTACT OR
PLC DEFICIENT T. BRUCEI..................................................................................................................105

4.11  SPLENIC B2 B CELLS IN C57BL/6 MICE AFTER INJECTION WITH T. BRUCEI
WHOLE CELL LYSATE DURING INFECTION WITH PLC DEFICIENT T. BRUCEI..................................................................................................................................................................................106
CHAPTER I

GENERAL INTRODUCTION

African Trypanosomiasis is a parasitic disease caused by protozoan parasites of the genus Trypanosoma (Fig. 1; for classification see Table 1.) which are transmitted to mammalian hosts by the bite of an infected tsetse fly (*Glossina* spp; Fig 2). African Trypanosomiasis is endemic to the tsetse habitat, which encompasses an area approaching 10 million km$^2$ and including 37 countries[1]. There are about 30 species and subspecies of tsetse which are divided into 3 main groups known as the Morsitan group, the Palpalis group, and the Fusca group. Tsetse species of the Morsitan group can be found in much of the savannah (grassy woodland) of Africa, whereas those of the Palpalis group are found in the mangrove swamps, the rain forest, the lake shores and the gallery forests along rivers, and those of the Fusca group are broken down into species which inhabit rather dry country, those that inhabit riverine valleys, and those that inhabit forested areas. Each species seems to be specialized to a particular climate condition [3,4,5]. Each species of *Glossina* has preferred host species on which it feeds but seems to be flexible if the presence of their preferred species is low or absent. Within the tsetse habitat there is a notable absence of cattle (Fig 3 Cattle and Tsetse Distribution) purportedly due to trypanosomiasis which is often fatal in trypanosomiasis-susceptible hosts including humans and livestock. There are several species of pathogenic African trypanosomes. Of these, two subspecies of *Trypansoma brucei* (*T. b gambiense* and *T. b. rhodesiense*) cause disease in humans (human African trypanosomiasis; HAT; respectively called west African HAT, and east African HAT) and a third sub species of *T. brucei* (*T. b. brucei*) together with *T. vivax* and *T. congolense* cause disease, called nagana, in livestock (animal African trypanosomiasis; AAT).
HAT epidemics have occurred periodically in Africa (Fig 4), one recently arising in the 1970s. This was controlled by a rigorous regime of diagnosis and treatment implemented by the World Health Organization and sustained today[2]. Although the number of cases of HAT is currently maintained at relatively low levels, the same cannot be said for AAT, which is more prevalent and widely distributed than HAT. There are no straightforward, broadly applicable and affordable (for African countries) ways of controlling AAT, which therefore imposes substantial constraints to food sufficiency and socio-economic development of sub-Saharan Africa[6]. As indicated in Fig 3, the presence of tsetse and AAT precludes cattle based agriculture from vast areas of fertile land in the humid and semi-humid zones of sub-Saharan Africa. Even where tsetse and trypanosome challenge is relatively low, i.e., on the fringes of the tsetse habitat where animal agriculture can be practiced only with support from chemotherapy and tsetse control, there is an estimated loss in cattle production alone at well over 1 billion U.S. dollars annually as a result of AAT.

The parasites

Trypanosoma brucei spp., has is the most extensively studied of the African trypanosomes. Trypanosomes of T. brucei spp. are spindle shaped and measure about 20µm in length and 2µm in diameter at their widest point (Fig 1, 5). Distinctive features of T. brucei include the large single mitochondria which spans the length of the cell, and the kinetoplast which is situated at the base of the flagellum and consists of circular (minicircles and maxicircles) mitochondrial DNA called kDNA (Figure 5) after which the Order Kinetoplastidae is named (Table 1 – classification). African trypanosomes including T. brucei have a single flagellum attached to the cell by an undulating membrane which allows motility. The flagellum originates within the flagellar pocket, an invagination of the parasite’s plasma membrane which serves as the endosomal gateway. Endocytosis, exocytosis and surface membrane turnover are all restricted to the trypanosome flagellar pocket [7]. T. brucei’s spindle shape and the unique way that the flagellum and
undulating membrane wraps around the parasite make it well adapted to motion in the crowded, viscous environment of its host blood plasma and interstitial fluids [8].

*T. brucei* are digenetic parasites having life cycle stages in both mammalian hosts and their *Glossina* spp., vectors (Figure 5)[9,10]. In the mammalian blood stream, the trypanomastigote transitions from a long slender, dividing form to a short stumpy non-dividing form which is adapted to infect the tsetse fly vector. Within the tsetse midgut, ingested bloodstream trypomastigotes transform into dividing procyclic forms which travel from the midgut to the proventriculus and further differentiate to the long epimastigote and then the short epimastigote. The parasite further migrates to the salivary gland where it differentiates into the non-dividing metacyclic (Figure 4). It is the metacyclic form which is deposited into the mammalian host with the saliva of the tsetse fly beginning the cycle again.

There are three sub-species of *T. brucei*, namely *T. b. gambiense* and *T. b. rhodesiense* which infect humans, and *T. b. brucei* which does not. The human infective *T. brucei* can survive in human serum during short term incubations, whereas *T. b. brucei* is rapidly lysed by human serum. This forms the basis of a simple diagnostic test. Lysis of *T. b. brucei* in human serum, which excludes the parasites from human infectivity, is mediated by 2 serum “trypanolytic factors” (TLF) called TLF1 and TLF2. Both contain haptoglobin related protein and ApoL1 which contribute to their activity [11]. Uptake of the ApoL1 by *T. b. brucei* is most efficient when in complex with human haptoglobin, which binds specifically to the parasites. Within the *T. b. brucei* endosomal system Apolipoprotein L-1 forms ionic pores that destabilize lysosomes resulting in trypanosome autolysis. Resistance of *T. b. rhodesiense* to ApoL1 is dependent on the expression of a serum resistance associated (SRA) gene which has been identified as a lysosomal protein [11,12] belonging to the VSG gene family [13]. Studies using transfection of SRA to *T. b. brucei* show that SRA binds ApoL1 preventing it being processed to form membrane destabilizing ionic pores thus conferring resistance to TLA [14]. *T. b. gambiense* is also resistant
to ApoL1 lytic activity although it does not express the SRA gene. Rather it uses several mechanisms to detoxify the ApoL1 [15].

The serum of baboons and other old world primates also has trypanolytic activity, which like that of human serum lyses *T. congolense* and *T. vivax* in addition to *T. b. brucei* [16,17,18,19]. Ongoing projects seeks to create transgenic cattle which express the human trypanosome lytic factor and baboon lytic factors and are thus engineered to resist infection with trypanosomes that cause Animal African Trypanosomiasis[20]. While this project is promising and has the potential to open the door to agriculture in Tsetse endemic regions by generating trypanosomiasis-resistant cattle, no transgenic resistant cattle have yet been reported. Moreover, the introduction of strains of livestock which express one or more human trypanolytic factors, possibly at sub-optimal levels, carries the risk of selecting for resistance to TLA in *T. b. brucei* and other species of African trypanosomes over time.

**VSG**

The primary adaptation of all African trypanosomes to life in the blood plasma is expression of a variable surface glycoprotein (VSG) coat. VSG is a glycophasphatidylinositol (GPI) anchored glycoprotein of about 65kD of which $10^7$ copies are packed on the surface of the protozoan parasite as homodimers (Fig. 6)[9].

Parasite phospholipase C (PLC) cleaves the GPI-anchor, releasing soluble VSG from the parasite surface. *In vitro* this has been induced with different stressors including hypo-osmotic shock, acidic stress and calcium flux. PLC is a virulence factor in *T. brucei* as PLC deficient *T. b. brucei* infected mice suffer less pathology and have significantly prolonged survival times as compared to animals infected with intact parasites[21,22]. The PLC$^{-/-}$ parasites retain their capacity to infect tsetse flies as well as mammals.
Parasites with an intact VSG coat poorly activate complement and hence are not subject to antibody-independent opsonization and phagocytosis, or antibody-independent complement mediated lysis\[23,24\]. In addition, antigenic variation of VSGs precludes rapid immune termination of infection. In this regard, the *T. brucei* genome has been shown to encode about 1000 VSG genes and pseudogenes, the diversity of which is expanded by formation of mosaic VSG genes\[2\]. VSG gene expression is tightly regulated so that only a single VSG gene is expressed at a time. However the expressed VSG gene is varied stochastically resulting in a seemingly unlimited parade of new antigenic variants\[2\].

**Trypanosomiasis – The disease**

The earliest sign of infection in HAT and AAT is the appearance of a chancre, which is an indurated ulceration that arises at the site of infection between 5 to 15 days after tsetse bite. This is followed by a haemolymphatic stage in which the parasite escapes from the site of tsetse feeding and saliva deposition and invades the blood and tissues inducing immunopathology which results in lymphadenopathy, splenomegaly, hepatomegaly, cachexia and anemia\[10,20\]. During this stage the patient develops recurring fevers that coincide with waves of antigenically distinct parasites in the blood, and this is accompanied by enlargement of lymph nodes, joint pain, and facial edema. The infected hosts cannot clear infections with HAT and AAT parasite because these organisms are exemplars of antigenic variation as discussed above, stochastically changing variable surface glycoprotein (VSG) coats at low frequency which ensures a practically unlimited parade of new antigenic variants which evade on-going immune responses and maintain chronic infection.

In the case of patients with HAT, the haemolymphatic stage occurs one to three weeks after the initial tsetse bite\[25\]. It lasts for only a few weeks in the case of infections with *T. b. rhodesiense* which are typically acute and characterized by high parasite loads in the blood.
stream, and for several months in the case of infections with *T. gambiense* which is typically characterized by low levels of parasitemia and prolonged chronic or even asymptomatic infection. Over time human infective trypanosomes invade the central nervous system in many individuals causing the second stage of the disease, the neurological stage, which manifests as loss of coordination, dizziness, headaches, confusion, dementia, disruptions of circadian rhythm, after which “sleeping sickness” is named, and eventual coma and death[25].

Animal African Trypanosomiasis, also called Nagana, is caused by a range of Trypanosome species including *T. b. brucei, T. b. gambiense, T. vivax*, and *T. congolense*. “Nagana is a wasting disease and manifests in initial waves of high parasitemia and fever. Infected cattle become “progressively thinner, their coat becomes rough and unkempt and the skin is drawn tightly over the ribs and pelvis lacking the looseness of healthy animals. There may be discharge from the eyes, visible swellings of the superficial lymph nodes and the tail bush may fall out”[26], all symptoms of systemic and peripheral inflammation. These infected animals are unsuitable for meat, dairy, or draft [26,27,28]. The pathologic effects of trypanosomiasis on livestock not only reduces the availability of meat and dairy, but critically denies the use of cattle and horses for inedible fodder conversion to fertilizer, and for traction and transport. Agricultural communities are then limited to what can be tilled by hand which leaves them vulnerable to food shortages, starvation, famine. FAO (1994) estimated that for the whole of Africa, overall agricultural losses attributable to trypanosomiasis would total more than US$ 4 billion annually, a figure independently corroborated in 1999, when it was estimated that agricultural benefits accruing to tsetse elimination could reach US$ 4.5 billion per year[29].

**Tsetse Control**
The tsetse fly is a relative slow breeder with a female giving birth to just one offspring at a time and up to 8 during its lifespan[30]. Some scientists believe that this low rate of reproduction facilitates tsetse control and hence trypanosomiasis control at least in restricted regions as a means of reducing infection incidence [31,32,33]. Tsetse Chemoattractants have been identified and a variety of traps developed that can be used to deplete tsetse from agricultural areas if widely distributed and rigorously maintained (Fig. 7).

However, this approach is limited by lack of resources and of central organization.[3,34]. When traps and targets are effective it is a challenge to maintain local interest in maintaining the trap as the idea that tsetse are a threat wanes with lowered incidence of infection of humans and livestock leading to potential reemergence[35]. Tsetse can also be controlled by spraying of insecticides, and ambitious programs have been proposed to clear large areas of infested land in this manner. However, insecticide spraying is expensive and would have to deplete tsetse from the entirety of Africa to have a lasting effect because the tabanids can re-infest cleared areas[6,32,33]. Currently local insecticide application, in the form of underbelly spraying is used to protect individual animals but is a practice reserved for valuable animals and used by relatively wealthy farmers. Widespread release of sterile male tsetse, which compete with fertile males for mates, has also been explored as a general control measure, but is very expensive and at best a temporary measure[33,36]. Overall, the large number of tsetse species, their wide distribution, reduced application of control measures during times of political unrest and low funding, reduces the efficacy of tsetse control (along with all efforts to control disease instance) as a means of controlling trypanosomiasis [37]. Although large scale efficacy of tsetse control is debatable, many still believe this to be an integral limb of the multifaceted effort to control Trypanosomiasis and efforts to control the vector and disease continue. As an example, case numbers in the Democratic Republic of Congo are shown in Figure 4.
Chemotherapy / Drug Resistance

The drugs used to treat Trypanosomiasis were introduced before 1950 with very few innovations in drug development since[38]. For over 50 years there have been just a small number of primary drugs on the market to control Animal African Trypanosomiasis (AAT). These drugs are Homidium salts in the form of Homidium-chloride (Novidium) or Homidium-bromide (Ethidium), Diminazine acerurate (Berenil), Isometamidium chloride (Samorin, Trypanidium), and more recently Quinapyramine sulphate, which has been used to combat trypanosomiasis in camels[38,39]. Treatment of individual infected animals is still the most common method of control used by farmers with an estimated 30,000 thousand therapeutic doses being purchased per year, which is estimated to be only one tenth the number of doses needed given the number of animals at risk of infection.

Use of chemotherapy is moderately effective in a few scenarios [39], for example, in the case of ranchers moving cattle temporarily into tsetse habitat in order to take seasonal advantage of grazing land and available water or while driving cattle through tsetse infested areas. Chemotherapy is also a helpful tool for minimizing losses in the event of a sporadic outbreak of Trypanosomiasis in a region which does not experience much trypanosomiasis[40] or, when used in combination with traps or screens, chemotherapy can make cattle raising feasible in areas of low tsetse prevalence.

However, in tsetse endemic areas it is not feasible to control AAT with chemotherapy[39]. Treatment of an animal or herd does not preclude reinfection meaning that in endemic areas animals would require regular treatment with trypanocidal drugs. The cost of repeated treatments is prohibitive to most farmers. Moreover, when trypanocidal drugs are used over time, drug resistance begins to emerge and has been documented in several countries [41,42,43]. In addition, administration of trypanocidal drugs to livestock is typically carried out
without veterinary oversight and under-dosing is common in order to save money and leads to uncured animals and raised risk of emergence of drug resistant Trypanosome species.

Similar issues prevail in the treatment of Human African Trypanosomiasis (HAT) in that there are limited options for chemotherapy, most of which are antiquated and resistance has begun to emerge. HAT must be treated differently depending on whether the disease has progressed to late meningo-encephalitic stage in which the parasites have invaded the central nervous system (CNS). Early or haemo-lymphatic stage HAT is treated with one of 3 drugs, pentamidine, suramin, and berenil which, because of its effectiveness is widely used by physicians despite it being licensed only for veterinary use. Late stage disease is more difficult to treat as drugs must cross the blood-brain barrier. Melarsoprol has been the mainstay for treating late stage trypanosomiasis but has a high rate of toxicity which manifests in arsenical-induced encephalopathy followed by pulmonary edema and death. More recently, eflornithine has entered the market for treating late stage HAT. Some of the issues clinics are confronted with are the extended and complex nature of the treatment regime for both early and late stage disease[25]. Treatment is carried out via intravenous or intramuscular injection over many days to weeks. For instance eflornithine, which has lower incidence of side effects than melarsoprol, requires four daily injections for 7 to 14 days. This poses both financial as well as practical challenges in treating HAT. As with treatment of AAT, HAT is running up against treatment failures which are likely the result of drug resistance. With a limited number of drugs available for the treatment of HAT, all of which have been on the market for up to and over 50 years, emergence of drug resistance would lead to a resurgence of HAT.

**Vaccination**
No efficacious vaccine has been developed against any of the African trypanosomes and it is a widely held view that vaccines capable of inducing sterile immunity cannot be developed against these parasites because of antigenic variation of the VSG coat. This view is supported by studies showing that the VSG coat masks conserved plasma membrane antigens with the exception of receptors for macromolecular nutrients, which do not serve as vaccine antigens. As mentioned above, the surface of a trypanosome is covered with a 10 million copies of a variant surface glycoprotein (VSG) arranged in a contiguous array of homodimers. Expression of surface VSG is under constant low level stochastic change. The volume and arrangement of VSG homodimers on the cell surface does not allow for interaction with antibody molecules with underlying invariant parasite surface molecules. Inoculation with and subsequent antibody production against receptors for macromolecule nutrient uptake which protrude out from the VSG coat in the flagellar pocket have proven ineffective at offering protection[44]. Antibodies against these receptors are outcompeted by nutrient ligand and, once bound by ligand, receptors are internalized and therefore antibodies fail to opsonize the parasite for phagocytosis or compliment mediated lysis. Because of the vast potential for variation in surface glycoprotein and lack of protection offered from antibodies against flagellar pocke exposed receptors it is widely believed that it is unlikely that effective vaccine against T.brucei will be developed.

AAT and HAT have proven difficult to control with chemotherapy alone and even combined with vector control measures success is limited and quickly reversed when the efforts of aid agencies are interrupted for any length of time[38,39,45]. Currently utilized chemotherapeutics are old, treatment regimens and toxicity are major obstacles, drug resistance is an emerging problem in both HAT and AAT, and an effective vaccine has proven elusive. We are left with a vacuum in the place of effective treatment and control of a disease which reigns over a large region of the African continent. Deeper insight into the immunological interface between parasite and host has the potential to lead the field to new targets and tools leading to
effective disease control and prevention. Though there is little hope for a vaccine that can prevent infection with African trypanosomes, there remains hope that anti-pathology vaccines can be developed, particularly a vaccine that prevents infection-associated depression of humoral immunity and improves VSG-specific primary and secondary immune responses at all stages of infection thus allowing infected hosts to better control trypanosome parasitemia and eventually self-cure.

**Immunosuppression**

During infection there is general disruption of lymphoid tissue structure; trypanosusceptible species develop splenomegaly, hepatomegaly, hypergammaglobulinemia, anemia and weight loss [46,47,48,49,50]. Paradoxically, hypergammaglobulinemia is accompanied by progressive immunodeficiency as parasite specific IgM and IgG responses decline along with the decline of a recall response to vaccine antigen as infection progresses [51]. Although immunosuppression is a common characteristic of trypanosomiasis in all susceptible host species, this is not the case with trypanosomiasis resistant host species [52,53]. Understanding the mechanisms behind immune suppression, in particular loss of the humoral immune response may pave the way to developing effective vaccine strategies for control of disease and disease pathology associated with African Trypanosomiasis. If the humoral response can be spared or restored during infection there is hope for increased resistance to trypanosomiasis, i.e., sustained control of parasitemia, reduced anemia and prolonged survival.

**Trypanosomiasis resistance and susceptibility**

Some animals e.g., Cape buffalo and Eland, which co-evolved with tsetse and African trypanosomes, rapidly suppress levels of parasites to a minimal (cryptic) level and show no signs
of disease[52,53]. Similarly some breeds of cattle, including the West Africa N’dama are less affected by AAT than others and can be maintained under levels of tsetse and trypanosome challenge that rapidly lead to death of other breeds [54]. Analyses of disease resistance mechanisms in these animals have so far proven unrewarding. Long term control of trypanosome parasitemia in Cape buffalo is associated with the very efficient development of IgG antibody responses against newly arising trypanosome variable surface protein (VSG) types, but not with the development of antibodies against common protective antigens[52,53]. In addition, to suppressing parasitemia to a cryptic level, infected Cape buffalo do not develop anemia or other signs of disease. Similarly, infected N’dama develop lower levels of parasitemia and less severe anemia than similarly infected trypanosomiasis-susceptible Boran cattle, and mount stronger primary and recall IgG responses against trypanosome antigens that Borans [55,56]. Strains of mice have also been shown to differ in their capacity to control infections with *T. brucei* and *T. congoense* with the more resistant strains developing lower levels of parasitemia, surviving for a longer period and mounting stronger VSG specific antibody responses than the more trypanosomiasis-susceptible mice[57,58,59,60].

Immune responses of infected mice to African trypanosomes are not restricted to production of VSG-specific and other antibodies. T cells are also induced to respond and their responses have been shown to contribute to control of infection [61,62]. Both mouse and human response to trypanosome infection include production of interferon γ (INF-γ), tumor necrosis factor alpha (TNF-α), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), and transforming growth factor beta (TGF-β)[61,63,64],[61,63,64]. Interferon gamma is thought to contribute to control of trypanosomes in tissues by stimulating macrophages to produce H₂O₂ and NO which are potent trypanocides, and to contribute to control of trypanosomes in the blood by stimulating IgG1 responses. However, the cytokine also has strong pro-inflammatory activity and sustained production is debilitating for the host. Thus longer survival is associated with an early
TH1-like response with production of INF-γ which transitions to a TH2 like response later in infection characterized by increased expression of the counter-inflammatory cytokine IL-10, whereas sustained production of INF-γ leads to early death\[21,65,66\].

The mechanism through which \textit{T. brucei} regulates Th1 and Th2 responses is not resolved. However, deletion of the \textit{T. brucei} gene encoding GPI-PLC reduces its virulence\[22\], facilitates in the host a switch from a pro-inflammatory TH1 to an anti-inflammatory TH2 T cell response during infection\[57,67\], and reduces the degree of immunosuppression and pathology induced by the parasites\[21\]. It is therefore possible that soluble VSG, which is released from \textit{T. brucei} upon cleavage of GPI by GPI-PLC, or remnant GPI play a role in stimulating the potent inflammatory response to the parasites. This is consistent with \textit{in vitro} studies showing that.

Responses of inflammatory macrophages and TNF-producing/iNOS-expressing dendritic cells are induced by trypanosome components, particularly the GPI-PLC-cleavage fragments of the membrane form of VSG\[61,68\]. Inflammatory macrophages and TNF-producing/iNOS-expressing dendritic cells (tipDC) are implicated in mechanisms of \textit{T. brucei}-induced immunosuppression and immunopathology\[69,70,71,72,73,74\], although it is unclear how they down regulate VSG specific IgG antibody responses, and indeed humoral immunity in general, which is a key feature of trypanosomiasis-susceptibility in infected cattle as well as in infected mice.

\textbf{Focus of this thesis}

We have recently shown in collaboration Stefan Magez that loss of humoral immune competence in \textit{T. brucei}-infected mice is associated with the apoptotic depletion of splenic transitional, marginal zone and follicular B cells\[75\]. This occurs rapidly after infection and impairs responses to vaccine antigens in addition to responses to newly arising VSG antigenic
variants, leading to uncontrolled parasite growth and death of the infected mice[76]. B cell loss has also been shown to occur in mice infected with the S. American trypanosome T. cruzi, where it is associated with a Fas-L dependent fratricidal reaction and with production of prostaglandins[77,78,79]. However, this does not appear to be the mechanism of B cell depletion in murine African trypanosomiasis, which occurs to a similar degree in lpr, i.e., Faslo mice[75], in mice administered antibodies against Fas-ligand and in mice treated with indomethacin to prevent cyclooxygenase activity and production of prostaglandins. In addition T. brucei–induced B cell loss does not require host production of TNFα showing that it is independent of TNF dependent death pathways[75].

In this thesis I have attempted to elucidate the mechanism of T. brucei-infection induced B cell apoptosis using mice lacking various aspects of the immune system as a result of gene deletion or antibody administration. In addition, I have examined the role of T. brucei GPI-PLC in this process and whether cells in addition to splenic B cells are affected. I consider that understanding the mechanism of T. brucei infection-induced B cell deletion in the infected mice will contribute to a fundamental understanding of infection-associated immunopathology, will stimulate similar investigations in livestock species and has the potential to lead to a greater understanding of the mechanism of trypanosomiasis susceptibility in general.
### Table 1.1 Scientific classification of *T. brucei*

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<td>Species</td>
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Figure 1.1. Gimsa stain of *T. brucei* in bloodstream of mammalian host. - Parasitology Images: An Auto-tutorial Thomas P. Buckelew, Ph.D.
Figure 1.2 Photos of Glossina (a) before and (b) after blood meal used with permission from Dr. Ray Wilson.

Figure 1.3 Distribution of Tsetse. Grey shading denotes tsetse endemic areas.
Figure 1.4 Number of cased of HAT in the Democratic Republic of Congo by year[2].

Figure 1.5 Trypanosoma brucei life cycle and anatomy.
Fig 1.6 Graphic representation of GPI anchored VSG homodimer

Figure 1.7 Tsetse trap and April 1931 catch
CHAPTER II

NKP46\textsuperscript{\textsuperscript{VE}} NATURAL KILLER CELLS DELETE B2 B CELLS AND SUPPRESS HUMORAL IMMUNITY IN MURINE AFRICAN TRYPANOSOMIASIS

Abstract

Infection with \textit{Trypanosoma brucei} results in recurring waves of parasitemia and often fatal disease in humans and livestock. Clearance of the highly antigenically variable extracellular parasites from the bloodstream is mediated by antibodies specific for their variable surface glycoproteins (VSG). However, these responses, particularly VSG specific IgG responses, are suppressed in trypanosomiasis-susceptible hosts. In previous studies, using the \textit{T. brucei} AnTat 1.1E C57BL/6 mouse model, we have shown that suppression of VSG-specific antibody responses results from severe depletion of splenic transitional, marginal zone and follicular B cells. We now show that this process is mediated by a novel subpopulation of natural killer (NK) cells as follows: \textit{T. brucei} infection-induced B cell deletion was similar in intact and T cell deficient mice excluding involvement of T cells and NKT cells, but did not occur in mice depleted of NK1.1\textsuperscript{+} lymphocytes by mAb PK136 indicating the involvement of NK cells. These NK cells did not express the NKp46 activating receptor or CD49b the integrin \(\alpha_2\) subunit of the collagen receptor, distinguishing them from mature NK cells and tumor-destructive NK cells. Although the NK cells expressed both high levels of the death ligand FasL and the cytotoxic granule marker CD107a, B cell deletion was primarily perforin-dependent and hence a result of cell mediated cytotoxicity. Perforin\textsuperscript{-/-} mice retained splenic B2 B cells and B cell follicles throughout infection and developed high titer IgG responses against
many trypanosome polypeptides unlike similarly infected intact mice. In addition, they had a lower level of parasitemia, did not develop anemia and had prolonged survival compared to similarly-infected intact mice. We conclude that a population of NKp46^+ NK cells in *T. brucei* infected mice kills trypanosome antigen-specific and other B2 B cells and inactivation of their cytotoxic function improves control of parasitemia and decreases pathology thus increasing trypanosomiasis-resistance.

**Author Summary**

African trypanosomiasis caused by *Trypanosoma brucei* species is fatal in humans and trypanosomiasis-susceptible animals and cannot be combated by vaccination because of extensive parasite antigenic variation. Effective trypanosome control and clearance from the bloodstream involves the action of antibodies specific for the parasite’s highly diverse variable surface glycoprotein antigens. However, experimental *T. brucei* infection in mice depletes splenic B cells which results in a loss of protective anti-trypanosome antibodies. Here, we show that infection-induced B cell loss requires the presence of a novel population of natural killer (NK) cells and is mediated by a perforin-dependent process consistent with perforin- and granzyme-mediated cytotoxicity. The absence of perforin in deficient mice prevents infection-induced loss of splenic B cells, enhances trypanosome-specific antibody responses, reduces infection-associated anemia and prolongs survival after infection thus enhancing trypanosomiasis-resistance.
Introduction

Trypanosoma brucei spp., are highly antigenically variable protozoan parasites that inhabit the blood plasma and tissue fluids of their mammal hosts. The tsetse-transmitted parasites are endemic throughout the tsetse habitat which encompasses a land mass of approximately ten million square kilometers of the humid and semi-humid regions of sub-Saharan Africa. Two subspecies of T. brucei, namely T. b. gambiense and T. b. rhodesiense cause Human African Trypanosomiasis (HAT;); and a third subspecies, T. b. brucei, together with T. congolense and T. vivax, which belong to the same suborder but different subgenera to T. brucei, cause Animal African Trypanosomiasis (AAT). HAT and AAT are characterized by recurring wave of parasitemia in which new antigenic variants evade prior and ongoing immune responses and boost immunopathology often killing the host.

HAT threatens 60 million people and is controlled by a rigorous and sustained regime of diagnosis and treatment. AAT excludes cattle from vast tracts of the tsetse habitat. Where ranching of trypanosomiasis-susceptible livestock is possible, it is supported by chemotherapy and anti-vector strategies with an estimated AAT-associated production loss of about four billion US dollars per year [80]. Research on sustainable control of AAT has focused both on prevention of infection and alleviation of disease in infected animals. Approaches include development of a broad-acting anti-infection vaccine, which has so far proven unsuccessful [81], prophylactic anti-parasite transgenesis which is ongoing [82], targeted inhibition of parasite enzymes that cause pathology, which has yet to be achieved [83], and prevention of trypanosomiasis-
associated immunosuppression, which we show here using a mouse model of AAT increases host resistance to trypanosomiasis.

Immunosuppression is a universal but poorly understood feature of African trypanosomiasis in susceptible hosts [84,85]. Studies in vitro [86] and in vivo [87] implicate nitric oxide (NO) produced by macrophages and dendritic cells in impaired proliferative responses of leukocytes in mouse models of trypanosomiasis consistent with a role in infection-induced immunosuppression. However, studies in cattle with AAT suggest that NO is not responsible for reduced leukocyte proliferation [88]. Similarly, studies in T. brucei-infected mice show that prostaglandin E2 production parallels development of immunosuppression [89]. However, ablation of its production by indomethacin treatment did not prevent the development of immunosuppression in the mice [75] and did not restore mitogen-induced proliferative responses of T cells from cattle with AAT [90]. B cell clonal exhaustion has also been proposed as the cause of immunosuppression [49], consistent with the development of lymphopenia in cattle and sheep with AAT [91,92,93] and with ablation of bone marrow lymphopoiesis and deletion of splenic transitional, marginal zone (MZ) cells and follicular (Fo) B cells in mice infected with T. brucei [75,94]. The loss of these B cells, which are from the B2 lineage [95,96], severely compromises trypanosome specific antibody production [76], and occurs after infection with any of several different isolates of T. brucei (Black Laboratory, unpublished data) as well as with a T. congolense isolate [97]. Splenic B cell deletion also occurs in mice infected with T. vivax [98] although this has not been specifically typed to the B2 lineage.
In contrast to splenic B2 B cells, which are depleted from the spleens of *T. brucei* infected mice, the number of IgM^+^CD5^low^CD11b^+^ splenic B cells increases significantly (supplementary Figure 1). The origin of these B cells has not been established but their phenotype suggests they are related to B1a-derived B cells (IgM^+^CD5^+^CD11b^+^) [99] and B1b B cells (IgM^+^CD5^-^CD11b^+^) [100]. Splenic B1 B cells also increase in number in infected trypanosomiasis-susceptible cattle [101] which, in common with *T. brucei*-infected mice, mount poor trypanosome antigen-specific IgG responses indicating poor stimulation of T cell dependent antibody responses [85]. Although B cell dynamics have not been studied in patients with *T. b. rhodesiense* HAT, which is an acute disease, analysis of peripheral blood B cells has been performed in patients with *T. b. gambiense* HAT, which is a chronic disease. In these patients there is a significant increase in T cell-independent B cells and a significant decrease in T cell-dependent B cells as a percentage of total blood lymphocytes [102] accompanied by a significant, although non-threatening, decrease in anti-measles antibody in serum, relative to age, gender and habitat matched uninfected individuals. The infection-induced mechanism(s) that cause the deletion of splenic B2 B cells in trypanosome infected mice, and those that prevent the development of trypanosome-specific IgG antibodies in infected cattle and cause the proportional decrease in T cell dependent relative to T cell independent B cells in the peripheral blood of *T. b. gambiense* infected people have not been identified.

Here we used gene knock-out mouse strains and antibody-mediated deletion of putative effector cells to show that B2 B cells are deleted from the spleens of infected mice by T cell-independent but natural killer (NK) cell-dependent processes. Importantly, we show that splenic B2 B cells are not deleted in *T. brucei*-infected mice that lack the
gene encoding perforin (perf<sup>-/-</sup>) which therefore have a greatly increased capacity to mount trypanosome antigen specific antibody responses, especially those of the IgG class, compared to similarly-infected intact mice. In addition the infected perf<sup>-/-</sup> mice controlled parasitemia, maintained their body weight and blood packed cell volume and survived for up to 90 days after infection, whereas similarly infected intact mice lost the capacity to control parasitemia, developed severe anemia and died by 30 to 35 days after infection. The studies identify NK cell mediated cytotoxicity as the mechanism of trypanosome infection-induced B2 B cell deletion in infected mice and show that abrogation of this process increases the resistance of the infected mice to AAT.

**Results**

**Retention of splenic B2 B cells and production of trypanosome antigen-specific IgG in T. brucei-infected perf-/-mice**

Perforin is a pore forming protein found in cytotoxic granules of cytotoxic T cells, NKT cells and NK cells. The protein works together with granzymes, which are serine proteases found in the same granules, to kill cells on which they are deposited [103]. To determine the contribution of perforin to T. brucei infection induced B cell killing, intact and perf<sup>-/-</sup> mice were infected with T. brucei Antat 1.1E and the percentage and number of transitional, MZ and Fo B cells as determined by staining with antibodies listed in Table 1 determined at intervals thereafter. Although mice of both strains developed similar levels of first wave parasitemia (Fig.1A vs Fig 2A), perf<sup>-/-</sup> mice, for the most part, maintained splenic transitional type 1, type 2, and type 3 B cell populations (Fig. 1B-D, H, I) as well as splenic MZ and Fo B cell populations , (Fig. 1E, F, J, K) at,
or greater than, pre-infection levels throughout infection whereas these cell populations were depleted from the spleens of infected intact mice (Fig 2B-F, H-K). Our results indicate that *T. brucei*-induced splenic B cell depletion is perforin-dependent and thus is most likely caused by cytotoxic cells, the only cells to express perforin.

Not only were splenic transitional, MZ and Fo B cell populations maintained in the spleens of perf$^{-/-}$ mice infected with *T. brucei* AnTat 1.1E, the mice did not develop anemia evidenced by maintained blood packed cell volume (Fig 1G). In contrast, blood packed cell volume declined significantly in infected intact mice (Fig 2G).

B cell follicles, identified in cross sections of spleen as a large cluster of B220$^+$ B cells with a rim of MOMA$^+$ marginal zone macrophages, were numerous and similar in distribution in uninfected C57BL/6 mice and perf$^{-/-}$ C57BL/6 mice (Fig 3A, B). However, the follicles were greatly reduced in distribution (not shown) and size in the spleens of C57BL/6 mice 10 days post infection (dpi) and those remaining had substantially decreased presence of MOMA$^+$ cells (Fig 3C). B cell follicles were completely absent from the spleens of C57BL/6 mice at 30 dpi (Fig 3E), but remained prevalent in the spleens of perf$^{-/-}$ C57BL/6 mice at 10 and 30 dpi (not shown). However, despite retaining large numbers of B220$^+$ cells B cells, follicles in infected perf$^{-/-}$ mice also showed decreased presence and staining intensity of MOMA$^+$ cells (Fig 3D,F) which requires further investigation. Importantly, *T. brucei* infected perf$^{-/-}$ mice developed and sustained robust trypanosome antigen specific IgM and IgG responses while those of similarly infected intact mice were of lower titer and shorter duration (Fig. 4A,C). Moreover infected intact mice generated IgM and IgG antibodies against only a few trypanosome
polypeptides by day 30 after infection, whereas infected perf$^+$ mice generated antibodies against a large number of trypanosome polypeptides (Fig. 4B,D).

**T cells and NKT cells are not required for *T. brucei* infection-induced B cell deletion**

CD8$^+$ cytotoxic T cells kill target cells via the perforin/granzyme pathway, as do NKT cells and NK cells [103,104,105,106]. Mice that lack the genes encoding the T cell receptor beta and delta chains (TCR$^{-}$) and consequently $\alpha\beta$ and $\gamma\delta$ T cells as well as Natural Killer T (NKT) cells were used to determine the impact of these cell types in *T. brucei* infection-induced B cell deletion. The numbers of transitional, MZ and Fo B cells were significantly decreased in TCR$^{-}$ mice infected for 10 days with *T. brucei* AnTat 1.1E compared to naïve TCR$^{-}$ mice (Fig. 5A-E) and the extent of splenic B cell deletion was similar to that in intact mice infected with the parasites (Fig 2B-F, H-K). Therefore, T cells are neither directly nor indirectly responsible for perforin-dependent deletion of splenic B cells in the infected mice.

**NK cells are required for *T. brucei* infection-induced B cell deletion**

NK cells (CD3$^-$ NK1.1$^+$) can be eliminated from mice through treatment with monoclonal antibody against the NK1.1 differentiation antigen, which also depletes NKT cells (CD3$^+$ NK1.1$^+$) [107,108,109,110]. The intraperitoneal administration of NK1.1 specific monoclonal IgG2a antibody to C57BL/6 mice resulted in almost complete loss of NK1.1$^+$ spleen cells within 4 days (not shown) and NK1.1$^+$ cell depletion was maintained for 10 days by subsequent NK1.1-specific antibody administration on days 4, and 7 after the initial treatment (Data for day 10 anti-NK1.1 shown in Fig. 6A top panel). In contrast,
administration of an irrelevant IgG2a monoclonal antibody to intact C57BL/6 mice under the same regime did not affect splenic NK1.1+ cells (Fig 6A, bottom panel). Neither the anti-NK1.1 nor the irrelevant IgG2a monoclonal antibody affected the number of transitional, MZ or Fo B cells in the spleen (not shown). However, depletion of NK1.1+ cells by antibody administration to T. brucei AnTat 1.1E-infected mice on days -1, 3 and 7 after infection prevented, with the exception of transitional type 2 B cells, depletion of splenic B cells, whereas similar administration of control antibody did not prevent B cell depletion (Fig 6B-F). In 4 repeat experiments, treatment with the anti-NK1.1 mAb always prevented infection-induced elimination of T3, MZ and FoB cells, and in 2 of these studies also prevented infection-induced elimination of T2 B cells (data not shown). These data show that NK1.1+ cells are required for T. brucei infection-induced depletion of splenic T3, MZ and Fo B cells and taken together with results obtained in perforin-/- mice (Fig 1) and TCR^-/- mice (Fig 5) indicate that NK cells are responsible for the perforin-dependent, T cell-independent loss of splenic B cells from the infected mice.

**NK cells in T. brucei infected mice have a novel phenotype**

Most splenic NK cells in naïve mice expressed the natural cytotoxicity receptor NKp46 (Fig 7A) and the integrin alpha subunit CD49b (Fig 7B). They also expressed little to no FasL (Fig 7C) or TRAIL (Fig 7D), which are ligands for the death receptors Fas and DR5 respectively, and expressed little or no lysosomal-associated membrane protein 1 (LAMP-1 also called CD107a) on their surface (Fig. 7E) as expected for resting
cells. Most splenic NK cells at 7 days after infection, which is the time of remission of the first parasitemic wave (Fig 2A), had the same phenotype as those present in naïve mice (data not shown), however by 10 days post infection, by which time viable splenic B cells are predominantly deleted, expression of NKp46 and CD49b by splenic NK cells was greatly decreased in comparison to NK cells in naïve and day 7 infected mice (Fig 7A,B), and that of FasL, and CD107a was greatly increased (Fig 7C,E), while expression of TRAIL was only slightly increased (Fig 7D).

CD107a facilitates delivery of perforin to the cytotoxic granules of NK cells [111,112,113,114] and is placed on the surface of the cells upon their degranulation which occurs during target-cell killing. Thus, the increased expression of CD107a on the surface of infection-activated NK cells (Fig. 7E), together with the perforin-dependence of B cell deletion from the spleens of infected mice (Fig 1) support the view that infection-activated NK cells delete B cells by perforin and granzyme-dependent cytotoxicity. Never-the-less, because infection-activated NK cells also express high levels of FasL (Fig 7C) and slightly increased levels of TRAIL (Fig 7D), it is also possible that perforin- and granzyme-dependent killing is amplified by death receptor signaling. To address this possibility, the expression of Fas and DR5 (respectively death receptors for FasL and TRAIL) was determined on membrane impermeable (7AAD⁻; healthy) versus membrane permeable (7AAD⁺; dieing/dead) IgM⁺ B cells in mice that had been infected 10 days earlier with T. brucei AnTat 1.1E. The study showed that Fas expression was similar on IgM⁺ cells of naïve and infected mice irrespective of whether or not they took up 7AAD (Figs 8A,B). In addition, although DR5 expression was
slightly increased on 7AAD\(^+\) compared to 7AAD\(^-\) IgM\(^+\) cells of naïve mice (Fig 8D and 8C), this difference was not observed in 7AAD\(^+\) and 7AAD\(^-\) IgM\(^+\) cells of infected mice. Thus cells with increased Fas and DR5 death receptor expression did not selectively accumulate in the 7AAD\(^+\) (dead and dying) B cell population at 10 days after infection suggesting against a contribution of death receptor signaling to B cell death at this stage of infection.

**Discussion**

Infection of C57BL/6 mice with *T. brucei* results in severe deletion of splenic transitional, marginal zone and follicular B cells [75], but an increase in the number of CD5\(^{low}\)CD11b\(^+\)IgM\(^+\), putatively B1 B cells, in the spleen (Fig S1). Splenic elevations of B1 B cells suggest that these B cells are not as sensitive to NK mediated deletion as B2 B cells, or that their proliferation exceeds their depletion. Depletion of the splenic B2 B cells is accompanied by loss of humoral immune responses against newly arising trypanosome variant antigenic types [76] showing that the B2 B cells have a critical role in mediating this response. We now show that infection-induced deletion of splenic B2 B cells and suppression of the trypanosome-specific antibody response does not occur in mice lacking the gene encoding the pore forming protein perforin, implicating cell-mediated cytotoxicity in the deletion process. Importantly, in the absence of perforin, infected mice were able to suppress parasite levels in the blood, to maintain their body weight and blood packed cell volume, and to survive for much longer than similarly infected intact mice, which jointly are characteristics of trypanosomiasis resistance [53,55,115]. In addition, the *T. brucei* infected perforin gene-deleted animals retained B
cell follicles and developed high titer IgM and IgG responses against a wide range of trypanosome polypeptides in contrast to infected intact mice, which responded to a restricted range of trypanosome polypeptides only and which lost this limited response and B cell follicles rapidly after infection. An ability to mount high titer and sustained IgG responses against trypanosome antigens including VSG is also a characteristic of trypanosomiasis resistant animals [53,85,116]. Taken together, these data show that abrogation of perforin-dependent cytotoxicity prevents \( T. brucei \)-induced deletion of B2 B cells and greatly increases the resistance of the infected mice to trypanosomiasis. This may not be solely linked to improved antibody-mediated control of trypanosome parasitemia because B cells also produce regulatory cytokines [117,118,119] which might affect the development of life threatening inflammatory responses in the infected animals.

Two complementary lines of evidence suggest that NK cells are responsible for the perforin-dependent deletion of splenic B cells in the \( T. brucei \)-infected mice. Firstly, transitional and mature B cell populations were similarly depleted in the spleens of intact mice and mice lacking genes encoding both the beta and delta chains of the T cell antigen specific receptor and thus lacking all T cells and NK T cells. Secondly, the splenic B2 B cells were retained in infected mice from which NK1.1\(^+\) cells were removed by repeated administration of PK136 monoclonal antibody [107,120], whereas the B cells were deleted from infected mice that were administered a control antibody of the same Ig class.

Although NK cells kill virus-infected or transformed cells and do not typically delete healthy self-cells, there are instances where this boundary is broached. Thus NK cells have been shown to kill specific CD4\(^+\) T cells in mice with acute systemic LCMV
infection with resultant suppression of adaptive immunity [121,122]. They have also been shown to exacerbate pathology in mice exposed to high dose influenza virus infection although the mechanism is not resolved [123]. In addition, NK cells have been shown to regulate auto- and allo-reactive lymphocytes [124,125], by pathways that include deletion of activated T cells [126,127] and in which perforin-dependent cytolysis is implicated [128,129]. In addition, there are several reports that antibody responses are suppressed by NK cells [130,131,132] consistent with a role for NK cells in down-regulation of B cell responses per se, and with their more profound role in deletion of splenic B2 B cell and generalized immunosuppression of humoral immunity documented here.

Paradoxically, whereas putative B2 B cell destructive NK cells were found to accumulate in the spleen of infected mice within a few days of clearing first wave parasitemia, splenic NK cell activity against tumor cells decreases precipitously during this period [133]. This raises the possibility that B2 B cell and tumor cell destructive NK cells belong to distinct subpopulations. In this regard, the activating receptor NKp46 has been shown to play an important role in tumor cell killing by NK cells [134,135,136] and loss of this activity in T. brucei infected mice [133] correlates, as shown here, with loss of splenic NK cells bearing NKp46 and their replacement by, or differentiation to, NK cells that lack this receptor. The putative B cell destructive NK cells also lack expression of the integrin α2 subunit CD49b of the collagen receptor [137] which distinguishes them from mature NK cells, but have increased expression of the death ligand FasL in common with activated mature NK cells [138]. In addition, they also have greatly increased expression of the cytotoxic granule marker CD107a, which is placed on the cell surface.
when cytotoxic granule fuse to the plasma membrane during degranulation and hence is a functional marker for cell-mediated cytotoxicity [139].

Activated NKp46− NK cells also arise in the lungs of mice infected with a high dose of influenza virus and exacerbate pathology leading to weight loss and death [123]. Potent activation of self-destructive NKp46− NK cells by influenza virus- and *T. brucei*-infection is unexpected because NKp46 is an activating receptor and a substantial decrease in its expression would be expected to decrease rather than elevate NK cell activity. However, a loss of function mutation in the gene encoding NKp46 has been shown to result in NKp46− NK cells that are hyper-responsive to various stimuli [140]. These hyper-responsive NK cells enhance the innate control of cytomegalovirus infected cells but decrease activation of virus-specific CD4+ and CD8+ T cells. However, it is unclear whether the decreased adaptive immune response results from T cell deletion or another mechanism.

There is little or no information on the mechanism of induction of the B cell-destructive NKp46− NK cells that arise in *T. brucei* infected mice, or mice infected with other pathogens. They might arise from infection activated mature NK cells as a result of ligand induced modulation of the NKp46 receptor or another stimulus, e.g., NKp46 down regulation has been reported to result from L-arginine starvation of cultured NK cells [141]. Alternatively, the NKp46− NK cells might arise as a separate NK subpopulation during normal development as is suggested by the presence of both NKp46+ and NKp46− NK cells in the peripheral blood of healthy pigs [142] and in the intestinal mucosa of healthy humans [143]. We anticipate that their origin in mice infected with *T. brucei* can
be resolved by adoptive transfer of tagged NKp46⁺ and NKp46⁻ NK and subsequent fate analysis.

Identity of the NK cell receptor and ligand interactions that result in B cell-deletion is also unresolved. NK cell killing is regulated by a balance of receptor and ligand interactions that are either activating or inhibitory to the NK cell [144]. The NK cells are known to kill target cells that have decreased MHC I on their plasma membrane, designated “missing self”. However, in the case of the *T. brucei*-infected mice, MHCI expression is actually increased on IgM⁺ cells (Fig S2). We therefore conclude that “missing self”, at least in the context of class I MHC molecules, is not involved in making these B cells into targets for NK cells. Furthermore, *T. brucei*-induced B cell killing is similar in infected intact mice and in infected mice lacking the gene encoding β2 microglobulin (Fig S3) which is required for transport of MHC class I molecules to the cell surface, showing that elevated MHCI is not a requirement for B cell killing, and indeed that NK cell licensing which occurs during development in the presence of MHCI [145] is not required for infection-associated B cell destruction. NK cells also express an FcγIIIA receptor (CD16) and can kill cells with bound immune or auto-immune IgG by antibody and cell mediated cytolysis (ADCC). However, although *T. brucei* infected mice generate auto-reactive antibodies [146,147], anti-lymphocyte antibodies have not been reported and ADCC is not required for splenic B cell deletion, which is similar in infected intact mice and infected mice lacking the gene encoding CD16 (Fig S4). It is anticipated that a comprehensive analysis of receptors expressed on the NKp46⁺ and NKp46⁻ NK cells as well as ligands expressed on splenic B1 and B2 B cells in *T. brucei*-
infected mice will provide insights into other candidate receptors and target ligands that mediate killing of the B2 B cells by the NKp46⁻ NK cells.

To conclude, we have shown that *T. brucei* infection of mice induces NK cells which deplete splenic B2 B cells resulting in loss of humoral immune competence. Phenotypic analysis of the putative B cell destructive NK cells show that they lack NKp46 expression in common with pro-pathology NKp46⁻ NK cells that arise in mice heavily infected with influenza virus [123]. NK cells arising in *T. brucei*-infected mice require perforin to delete splenic B cells and facilitate the development of cachexia, anemia, and ultimately death, central features of trypanosomiasis susceptibility that are ameliorated or delayed in infected perf⁻/⁻ mice compared to intact mice. Further investigation is needed to identify the cytokines that induce and activate the B cell destructive NKp46⁻ NK cells in the infected mice, the trypanosome components that elicit their production, the receptor:ligand interactions that mediate B cell killing, and the contribution, if any, of infection-induced NK cells to trypanosomiasis susceptibility of other host species.

**Materials and Methods**

*Ethics Statement:* The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Guidelines for the Use of Laboratory Animals in Research, Teaching and Testing of the International Council for Laboratory Animal Science. All animal studies were approved by the Institutional Animal Care and Use
Committee, University of Massachusetts, Amherst, MA01003 USA, as documented in protocol #s 2010-0028, 2013-0049 and 2013-0050.

**Mice and Trypanosomes:** Male C57BL/6 (Taconic, Germantown, NY), C57BL/6-\textit{prf1}\textsuperscript{tm1sdz/1} (perforin\textsuperscript{−/−}), B6.129P2-\textit{Tcrb}\textsuperscript{tm1mom}\textit{Tcrd}\textsuperscript{tm1mom/J} (TCR\textsuperscript{−/−}), B6.129P2-\textit{Fcgr3}\textsuperscript{tm1Sjv/J} (CD16\textsuperscript{−/−}) and B6.129P2-\textit{B2m}\textsuperscript{tm1Unc} (β2microglobulin\textsuperscript{−/−}) mice (Jackson Laboratory, Bar Harbor, ME), (7-9 week old) were housed under barrier conditions. Mice were infected by intraperitoneal (i.p.) injection of 5000 exponentially growing pleomorphic \textit{Trypanosoma brucei} Antat 1.1E or sham infected by i.p., injection of Dulbecco’s Phosphate Buffered Saline (DPBS; GIBCO, Life Technologies). The parasites were derived from EATRO 1125 stock [148] and grown in immunocompromised C57BL/6 donors (600r from a 127 Cesium source 24 hours prior to i.p. injection of cryopreserved parasites). Parasitemia was assessed in tail blood by dilution in DPBS and counting using a hemocytometer.

**Mouse Weight and Packed cell volume measurements:** Weight was assessed using an Ohaus\textsuperscript{TM} brand digital scale. Packed cell volume was calculated as the percent of packed red blood cells to serum volume after spinning blood in heparinized capillary tubes in a hematocrit centrifuge (ADAMS MHCTII; BD, San Diego) for 8 minutes.

**Antibodies:** \textit{Flow Cytometry} – Avidin-APC-Cy7 (San Diego), anti-DR5-biotin (clone MD5-1), anti-FasL-PE (clone MFL3), anti-Fas-PE-Cy7 (clone 15A7), anti-H2kb-PE
(clone eBM2a), anti-NKp46-PE (clone 29A1.4), anti-IgM-PE (clone II/41), anti-NK1.1-APC (clone pk136), anti-TRAIL-biotin (clone N2B2), anti-CD11b-APC-Cy7 (clone M1/70), anti-CD23-APC-Cy7 (clone B3B4), anti-CD23-FITC (clone B3B4), anti-CD45R (B220)-FITC (clone RA3-6B2), anti-CD93–APC (clone 1D4B) purchased from eBioscience (San Diego, CA); anti-CD5-APC (clone 53-7.3) anti-CD21-APC (clone 7G6), anti-CD49b FITC (clone DX5) purchased from BD Biosciences (San Diego, CA); 7-amino-actinomycin D (7AAD) purchased from EMD Chemicals (San Diego, CA).

IFA - Anti-MOMA-1(biotin) AbCam (Cambridge MA); Avidin-FITC, BD Pharmingen (San Diego, Ca); anti-human/mouse CD45 (B220) eBiosciences (SanDiego, Ca).

Cell isolation and flow cytometric analysis: Mice were killed by lethal CO\textsubscript{2} inhalation, spleens excised and mechanically dissociated in cold FACS Buffer (1.0% fetal bovine serum, FBS, [Atlanta Biologicals] in DPBS). Cell pellets prepared by centrifugation (500 g, 10 min, 4°C) were suspended in 10ml of cold ammonium chloride lysis buffer (ACK; 0.15M NH\textsubscript{4}Cl, 1.0 mM KHCO\textsubscript{3}, 0.1mM Na\textsubscript{2}-EDTA) and incubated for 4 minutes on ice to lyse red blood cells. Remaining leukocytes were pelleted as above, washed twice in DPBS and suspended in FACs buffer. Aliquots (100 µl) containing 10^6 cells were added to the wells of a 96 well V bottom plate, stained with combinations of specific monoclonal antibodies against B cell, T cell and NK cell differentiation antigens (listed in Table 1) and analyzed by multicolor flow cytometry as described by us [75]. Briefly, cells were incubated with Fc block (anti-CD16/CD32 Fc III/II, eBioscience, San Diego, CA; 1:1000 dilution) for 20 minutes at 4°C, pelleted, resuspended in 100 µl aliquots of
biotin- or fluorochrome-conjugated primary antibodies (listed above) for 30 minutes at 4°C, washed twice in cold FACs buffer, resuspended in 100 µl aliquots of FACs buffer with or without streptavidin (SA) conjugated fluorochromes and incubated for an additional 30 minutes at 4°C. Samples were washed twice and resuspended in 300 µl FACs buffer with 1 µg of 7AAD, a fluorescent DNA intercalating agent that binds to DNA in membrane permeable (dead or dying) cells, (EMD Chemicals, San Diego, CA). Analyses were performed using a flow cytometer (LSRII BD Biosciences, San Jose, CA) and data processed using FLOWJO software (Tree Star Inc., Ashland, OR) to determine the percentages of 7AAD− and where stated also 7AAD+ cells in fluorochrome-tagged subsets. The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number of viable leukocytes in the donor spleen.

*Immunofluorescence staining of the mouse spleen:* Spleens from uninfected and infected C57BL/6 and Perf−/− mice were embedded in OCT (Sakura Finetek, Torrance, CA), frozen in dry ice, stored frozen at -80°C and incubated at -20°C for 30 minutes before cryostat sectioning. Frozen sections (10) µm were cut and adhered to Poly-Prep slides (Sigma-Aldrich, St. Louis, MO), air dried and fixed in ice cold acetone for 10 minutes. Slides were blocked with 5% bovine serum albumin (BSA) and Fc blocker CD16/CD32 (eBioscience) for 30 minutes, then stained with biotin conjugated antibody MOMA-1 at room temperature for one hour. Sections were washed and treated for one hour at room temperature with Avidin FITC and antibody B220. Stained sections were washed, air dried at room temperature and mounted with ProLong® Gold antifade reagent (Life
Technologies) and covered with glass coverslips (Corning). All the slides were read on a Zeiss MOT200° inverted microscope with a Zeiss apotome at 20x magnification.

NK cell depletion [108,110]: Mice were injected intraperitoneally with 500 ug anti-NK1.1 IgG2a monoclonal antibody purified from hybridoma (PK136, ATCC Manassas, Virginia) culture supernatant by Protein G chromatography, or with an irrelevant control IgG2a antibody (M9144, Sigma-Aldrich). Treatments were repeated on days 4 and 7 after the first injection of anti-NK1.1 and splenic B cell, T cell and NK cell populations assessed on days 3, 7 and 10 using antibody staining and multicolor flow cytometry as described above.

Western Blot analysis of trypanosome-polypeptide specific antibodies in mouse serum: Parasite lysate was prepared by suspending \(10^8\) DEAE52 purified \[149\] washed \textit{T. brucei} AnTat 1.1E in 5 ml lysis buffer (DPBS, containing 0.4%NP40 and protease inhibitor cocktail [Complete mini tablets, Roche, Indianapolis, IN]). Insoluble material was removed by centrifugation at 1000g for 5 mins. An aliquot (600ug protein content) of trypanosome lysate was fractionated by reducing SDS-PAGE on a 10% polyacrylamide gel in a single gel-spanning slot and transferred to a polyvinylidene difluoride membrane using a mini trans-blot transfer apparatus (Bio-Rad) according to the manufacturer’s protocols. The membrane was blocked with 5\% nonfat milk in (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5\% Tween 20; TBST) for 60 min, washed once with TBST, inserted in a slot blot apparatus (Mini Protean Multi Screen II, Biorad) and serum, prepared from blood of control and trypanosome-infected mice (1:200 dilution, 600 µl/slot), was added.
to individual slots and incubated at 4 °C for 2 h. Membranes were washed three times (10 ml TBST, room temperature, 10 min) and incubated with horseradish peroxidase-conjugated anti-mouse IgM, or IgG antibodies (ABCAM 0.6 µg antibody/ml TBST) for 1 hour, then washed 3 times with TBST before developing with chemiluminescence substrate (ThermoPierce).

**Enzyme linked immunosorbent assay (ELISA) of trypanosome-specific antibodies:** Wells in a 96 well plate (NUNC) were coated with 0.1 µg T. brucei Antat1.1E NP40 extract (prepared as above) in 100 µl DPBS containing 0.05% NP40, washed with ELISA wash buffer (PBS + 1% bovine serum albumin [BSA] + 0.05% TWEEN20) and non-specific protein binding sites were blocked by incubation with 200 µl aliquots of PBS + 1% BSA. Aliquots (100 µl) of control and post-infection mouse serum diluted in DPBS were added to each well, incubated for 2 hours at room temperature and wells were washed 3 times with ELISA wash buffer to remove unbound serum immunoglobulins. Bound antibodies were revealed by addition of biotinylated anti-mouse IgM or anti-mouse IgG (BD biosciences) to each well and bound second step antibody was quantified after washing by incubation with avidin-conjugated Horse radish peroxidase (BD biosciences). Captured antibody was visualized with Ultra TMB ELISA (Thermo scientific) and absorbance readings were made at 450nm, using a 96 well plate spectrophotometer.

**Statistics:** Cell population and other data obtained from infected animals and from non-infected controls were subjected to two-tailed T tests with significant differences reported
as follows: \( p \leq 0.05 \), (**) \( p \leq 0.01 \), (***) \( p \leq 0.001 \). Differences among multiple groups were analyzed using ANOVA and means were compared using Tukey’s honest significant difference (HSD) test when \( p \leq 0.05 \) (GraphPad Prism v.4.0, GraphPad Software Inc. San Diego, CA).

Fig. 3 Distribution of B220\(^+\) B cells and MOMA\(^+\) macrophages in spleens of intact and perforin/-mice infected with *T. brucei*. Thin sections (10 \( \mu \)m) of OCT embedded frozen mouse spleens, were air dried, fixed in acetone, rehydrated and stained with anti-B220 (green) to detect B cells and anti-MOMA (red) to detect marginal metallophilic macrophages. For images presented in this figure, the slides were stained and read on the same day on a Zeiss MOT200\(^+\) inverted microscope with a Zeiss apotome at 20x magnification. Scale bars, 50 micrometers. A– Uninfected C57BL/6 mice; B – uninfected perf/-C57BL/6 mice; C,E C57BL/6 mice infected for 10 and 30 days with *T. brucei* AnTat 1.1E; D,F Perf/-C57BL/6 mice infected for 10 and 30 days with *T. brucei* AnTat 1.1E. Results are representative of 5 mice studied in each group.
Fig 2.1 Splenic B cells and parameters of infection in perf² mice infected with T. brucei AnTat 1.1E. Splenic B cells in naïve C57BL/6 or C57BL/6 mice infected with T. brucei AnTat 1.1E were stained for surface markers used to define transitional and mature B2 B cells as described in Table 1 and analyzed using flow cytometry. Results are presented as total numbers of: (A) T. brucei/ml blood; (B-D) Transitional B cells (E) Marginal Zone B cells (F) Follicular B cells/spleen. Data are presented as mean and standard deviation, of 3 mice per group. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one-way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals. (G) Body weight in grams and % blood packed cell volume (PCV) measured as the ratio of blood cell pellet to plasma. Results are representative of 3 identical experiments.
Fig 2.2 Splenic B cells and parameters of infection in C57BL/6 mice infected with T. brucei AnTat 1.1E. Splenic B cells in naïve C57BL/6 or C57BL/6 mice infected with T. brucei AnTat 1.1E were stained for surface markers used to define transitional and mature B2 B cells as described in Table 1 and analyzed using flow cytometry. Results are presented as total numbers of: (A) T. brucei/ml blood throughout infection; (B-D) Transitional B cells/spleen; (E) Marginal Zone B cells/spleen; (F) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 or more mice per group. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals. (G) Body weight in grams and blood packed cell volume (PCV) measured as the ratio of blood cells to plasma. Results are representative of 3 identical experiments.
Fig. 2.3 Distribution of B220⁺ B cells and MOMA⁺ macrophages in spleens of intact and perforin-/- mice infected with *T. brucei*. Thin sections (10 µm) of OCT embedded frozen mouse spleens were air dried, fixed in acetone, rehydrated and stained with anti-B220 (green) to detect B cells and anti-MOMA (red) to detect marginal metallophilic macrophages. For images presented in this figure, the slides were stained and read on the same day on a Zeiss MOT200™ inverted microscope with a Zeiss apotome at 20x magnification. Scale bars, 50 micrometers. 

A– Uninfected C57BL/6 mice; B – uninfected perf-/- C57BL/6 mice; C,E C57BL/6 mice infected for 10 and 30 days with *T. brucei* AnTat 1.1E; D,F Perf-/- C57BL/6 mice infected for 10 and 30 days with *T. brucei* AnTat 1.1E. Results are representative of 5 mice studied in each group.
Fig. 2.4 Detection of trypanosome antigen specific antibodies by ELISA and Western blotting. Trypanosome lysate was coated on wells of an ELISA plate and used to detect trypanosome antigen specific antibodies of the IgM (A) and IgG (C) classes in dilutions of serum from groups of uninfected (naïve) mice and mice that had been infected for 10, 20 or 30 days with T. brucei AnTat 1.1E (n=6/group). Trypanosome lysate was subjected to SDS-PAGE, transferred to membrane and stained with pooled serum from a group of uninfected mice (n=6), or mice infected with T. brucei AnTat 1.1E for 10, 20 or 30 days as indicated (n=6/group) to detect polypeptide-specific antibodies of the IgM (B) and IgG (D) classes. Results are representative of results obtained in individual mice of each group in 2 identical experiments.
Fig 2.5 Splenic B cell populations in naïve and *T. brucei* AnTat 1.1e-infected TCR/− C57BL/6 mice. Splenic B cells in naïve TCR/− mice and TCR/− mice that had been infected with *T. brucei* AnTat 1.1 for 10 days were stained for surface markers used to define transitional and mature B2 B cells as described in Table 1 and analyzed using flow cytometry. Results are presented as total numbers of: (A-C) Transitional B cells/spleen; (D) Marginal Zone B cells/spleen; (E) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 mice per group. Significance (**p<0.01; ***p<0.001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals. Results are representative of 3 identical experiments.
Fig 2.6 Efficacy of NK1.1 cell deletion and impact on splenic B cell survival in mice infected with T. brucei Antat 1.1E. (A) NK1.1+ CD3+ (NKT) and NK1.1+ CD3- (NK) cells in spleens of C57BL/6 mice administered 500ug pk136 anti-NK1.1 monoclonal antibody (upper panel) or an irrelevant IgG2a monoclonal antibody (lower panel) i.p., on days 0, 3, and 7 and collected on day 10. (B-F) Splenic B cells in naive or T. brucei Antat 1.1E infected C57BL/6 mice treated with control or anti-NK1.1 mAbs for 10 days as outlined in above were stained for surface markers used to define developing, transitional and mature B cells as described in Table 1 and analyzed using flow cytometry. Results are presented as total numbers of: (B-D) Transitional B cells/spleen; (E) Marginal Zone B cells/spleen; (F) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 mice per group. Significance (*<0.05) was determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals. Results are representative of 4 identical experiments, with the exception that in 2 of these studies, transitional type 2 B cells were significantly reduced in the spleens of anti-NK1.1 treated infected mice compared to those of naive mice.
Splenocytes from naïve C57BL/6 mice and mice infected 10 days earlier with *T. brucei* Antat1.1E were stained with mAb specific for NK cell differentiation antigens, and CD3− NK1.1+ cells expressing (A) Nkp46, (B) CD49b, (C) FasL, (D) TRAIL, and (E) CD107a identified by multicolor flow cytometry and FACS analysis. Blue lines present results obtained with cells from uninfected mice and red lines present results obtained with cells from infected animals. Results are representative of 3 identical experiments.
Fig 2.8 Expression of death receptors FAS and DR5 on viable (7AAD⁻) and membrane compromised (7AAD⁺) IgM⁺ spleen cells during infection with *T. brucei* Antat1.1E. Splenocytes from C57BL/6 mice at day 10 post infection with *T. brucei* Antat1.1E were stained with the vital dye 7AAD and mAbs specific for Fas, DR5 and IgM and analyzed by flow cytometry. (A) Fas expression on 7AAD⁻ IgM⁺ cells; (B) Fas expression on 7AAD⁺ IgM⁺ cells; (C) DR5 expression on 7AAD⁻ IgM⁺ cells; (D) DR5 expression on 7AAD⁺ IgM⁺ cells. Blue lines present results obtained with cells from naïve animals, red lines present results obtained with cells from infected animals. Results are representative of 3 identical experiments.
**Fig 2.9 Splenic IgM⁺, B-1 cells expand within infected mouse spleens.** IgM positive splenocytes from naïve C57BL/6 mice and mice infected 10 or 20 days earlier with *T. brucei* Antat1.1E were further subdivided based on their expression of CD11b and CD5. (A) Representative flow cytometry plots showing the gates used to identify CD11b⁺ and CD5⁺ IgM⁺ splenocytes. (B) Data are presented as the mean number and standard deviation of IgM⁺, CD5lo, CD11b⁺ splenocytes and (C) IgM⁺, CD5⁺, CD11b⁻ splenocytes from 3 mice per group. Significance (*p<0.05) is determined using a one way ANOVA and Tukey’s multiple comparison test comparing uninfected controls to infected mice. Results are representative of 2 identical experiments.
Fig 2.10 Expression of class I MHC on IgM⁺ cells on naïve and *T. brucei* Antat1.1E-infected C57BL/6 mice. IgM positive splenocytes from naïve C57BL/6 mice and mice infected 10 days earlier with *T. brucei* Antat1.1E were stained with monoclonal antibody specific for MHCI and analyzed by flow cytometry. Blue lines represent cells from naïve animals, red lines represent cells from infected animals. Results are representative of 3 identical experiments.
Fig 2.11 Splenic B cell populations in naïve and *T. brucei* AnTat 1.1e-infected β2 microglobulin−/− C57BL/6 mice. Splenic B cells in naïve B6.129P2-B2m<sup>−/−</sup> and B6.129P2-B<sub>2</sub>m<sub>−/−</sub> tm1Unc mice infected with *T. brucei* AnTat 1.1 for 10 days were stained for surface markers commonly used to define transitional and mature B cells as described in Table 1 and analyzed by flow cytometry. Results are presented as total numbers of: (A-C) Transitional B cells/spleen; (D) Marginal Zone B cells/spleen; (E) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 mice per group. Significance (*p<0.05; **p<0.01) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals.
Fig. 2.12 Splenic B cell populations in naïve and *T. brucei* AnTat 1.1e-infected CD16<sup>+</sup> C57BL/6 mice. Splenic B cells in naïve CD16<sup>+</sup> mice and CD16<sup>−</sup> mice infected with *T. brucei* AnTat 1.1e for 10 days were stained for surface markers commonly used to define transitional and mature B cells as described in Table 1 and analyzed using flow cytometry. Results are presented as total numbers of: (A-C) Transitional B cells; (D) Marginal Zone B cells; (E) Follicular B cells. Data are presented as individual values, mean and standard deviation, of 3 or more mice per group. Significance (*p*<0.05) is determined using one way ANOVA and Tukey's HSD test comparing uninfected controls to infected individuals.
Table 1  B cell, NK cell and NKT cell differentiation antigens defined by specific monoclonal antibodies

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Differentiation Antigen Expression</th>
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<tr>
<td>Type 1 Transitional B cell</td>
<td>B220⁺, AA4.1⁺, CD23⁻, IgM⁺</td>
</tr>
<tr>
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<td>B220⁺, AA4.1⁺, CD23⁺, IgM³⁺</td>
</tr>
<tr>
<td>Type 3 Transitional B cell</td>
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<td>IgM⁺, AA4.1⁻, CD23⁻⁻⁻, CD21⁺</td>
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<tr>
<td>B1-a B Cell</td>
<td>IgM⁺, AA4.1⁻, CD5⁺, CD11b⁻</td>
</tr>
<tr>
<td>B1-a derived B cell</td>
<td>IgM⁺, AA4.1⁻, CD5⁺, CD11b⁺</td>
</tr>
<tr>
<td>B1-b B cell</td>
<td>IgM⁺, AA4.1⁻, CD5⁻, CD11b⁺</td>
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<td>CD3⁻, NK1.1⁺</td>
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<td>Natural Killer T cell</td>
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CHAPTER III

NK CELL AND PERFORIN DEPENDENT DEPLETION OF CD8 T CELLS IN

TRYPANOSOMA BRUCEI INFECTED MICE

Abstract

Trypanosoma brucei is a highly antigenically variable flagellated protozoan that causes fatal
disease in people and livestock. Infected trypanosomiasis susceptible hosts develop severe
immunodepression which compromises their capacity to control the on-going trypanosome
infection and increases their susceptibility to secondary infections. Using a C57BL/6 mouse
model of human and animal African trypanosomiasis we have shown in chapter 1 that loss of
humoral immune competence results from depletion of splenic B lymphocytes by a novel NKp46
population of natural killer (NK) cells and requires expression of the gene encoding the pore
forming protein perforin but not that encoding the low affinity Fcγ receptor, CD16, consistent
with cell mediated, but not antibody dependent, cytotoxicity. We show here using the same
T. brucei infection model, gene knock strains of mice, and multicolor flow cytometry
that T. brucei infection induced NK cells also deplete splenic CD8 T cells with similar kinetics to
B2 B cell depletion and with a similar requirement for perforin gene expression and lack of
requirement for CD16 gene expression. CD4 T cells were less affected than CD8 T cells. Unlike
depletion of B2 B cells, NK cell dependent depletion of CD8 T cells required host expression of
genes encoding MyD88 and TRIF which are adaptor proteins for Toll-like receptor signaling,
suggesting that this pathogen molecular pattern recognition system is specifically involved in the
CD8 T cell recognition rather than NK cell activation. Repeated injection of naïve mice with T.
brucei AnTat 1.1E lysate did not elicit CD8 T cell or B cell destructive NK cells. These results
are discussed in the context of the regulation of adaptive and innate immune responses during acute infections.
Introduction

*Trypanosoma brucei* is a highly antigenically variable protozoan parasite that resides in the blood plasma, tissue fluid and cerebrospinal fluid of its mammalian hosts. It is transmitted by the bite of the tsetse fly (spp. Glossina), and is endemic in the tsetse habitat which encompasses 10 million square kilometers of the humid and semi-humid regions of Africa. *T. brucei* contributes to animal African trypanosomiasis (AAT) and causes human African trypanosomiasis (HAT), diseases that constrain food production and socio economic development of sub-Saharan Africa.

As an adaptation to life in the host extracellular fluids each *T. brucei* is coated with an almost contiguous monolayer of a single species of variable surface glycoprotein (VSG) which is encoded by a single VSG gene. This is selected from a large repertoire of genomic VSG genes and pseudogenes and varied stochastically and infrequently resulting in the generation of antigenic variants [150]. Some of the antigenic variants that are generated do not react with, and hence evade clearance by, VSG-specific antibodies that are induced by the dominant VSG types in the trypanosome population. The surviving antigenic variants expand to restore the parasite burden upon antibody mediated elimination of the dominant VSG types, a process that results in recurring waves of parasitemia.

Mammal hosts vary in their ability to control infections with *T. brucei* and other African trypanosomes. Some hosts, e.g., Cape buffalo, which were selected by co-evolution with African trypanosomes and tsetse vectors, carry the parasite with few or no signs of disease [53], while hosts that were not selected to resist the disease e.g., humans, livestock and mice, develop severe inflammation, cachexia, anemia, and immune system dysfunctions which result in life threatening immunopathology and increased susceptibility to secondary infections [151]. Analyses in murine models of AAT and HAT show that life threatening inflammation is associated with a polarized TH1 T cell response [73,152] and an inadequate regulatory IL-10 response [153] which results in the early and sustained production of pro-inflammatory cytokines, IFNγ and TNFα.
supports TH1 T cell polarization, which consequently is self-sustaining in the absence of regulatory cytokines. TH1 T cell polarization is additionally facilitated by production of IFNγ by CD8+ T cells in response to trypanosome cytoskeleton associated T lymphocyte triggering factor [154] and by infection-induced deletion of splenic B2 B cells including marginal zone B cells [75,155] which produce IL-10 [156].

The depletion of B cells in T. brucei infected mice is mediated by natural killer (NK) cells and is dependent on expression of the pore forming molecule perforin consistent with cell mediated cytotoxicity. Thus deletion of NK cells from the infected mice by administration of NK1.1 specific antibody prevents infection induced depletion of transitional and mature splenic B2 B cells, and the B cells are not deleted from the spleens of infected perforin−/− C57BL/6 mice. Not only do the T. brucei infected perforin−/− mice mount high titer IgG antibody responses against many trypanosome antigens unlike similarly infected intact mice, they maintain their body weight and blood packed cell volume showing that they do not develop cachexia and anemia. Taken together these observations suggest that infection induced NK cell cytotoxicity has broad immunopathologic consequences and hence that NK cells may affect leukocytes in addition to B2 B cells.

It has been established that NK cells that accumulate in the spleen of mice undergoing B cell apoptosis have an activated immature phenotype. They do not express the integrin α2 chain CD49b of the collagen receptor, or the NK cell activating receptor NKp46, both of which are characteristically expressed by mature NK cells. They do however, have high expression of the death ligand FasL in common with activated mature NK cells as well as high surface expression of the cytotoxic granule marker CD107a indicating degranulation [157] which is associated with cell mediated cytotoxicity.
It has been recently shown that NKp46\(^*\), CD107a\(^+\) cells predominate in the lungs of mice acutely infected with influenza virus and that depletion of the NK cells significantly reduces mortality and reduces lung tissue damage[123]. In addition, during acute infections of mice with lymphocytic choriomeningitis virus, NK cells mediate mortality and lung pathology via a cytolytic depletion of antigen specific CD4\(^+\) T lymphocytes and downstream inactivation of antigen specific CD8\(^+\) T cells [121,145]. The observation that B cell destructive NK cells arising in *T. brucei* infected mice have a similar differentiation antigen phenotype to the T cell destructive NK cells that arise in mice with acute virus infections raises the possibility that the cells might also have functional similarity, i.e., that *T. brucei* infection-induced NK cells might kill T cells as well as B cells. We investigate this possibility here using mice in which NK1.1\(^+\) cell presence and function is modulated through treatment with anti-NK1.1 specific monoclonal antibody and through knock out of genes encoding CD1d, an MHC-1 like molecule required for natural killer T (NKT) cell development [158], perforin which is required for cell mediated cytotoxicity [159], and CD16 which is required for antibody dependent cell mediated cytotoxicity [160]. In addition we investigate the possible role of signaling thorough Toll like receptors in deletion of B cells and T cell by *T. brucei*-infection induced NK cells.

Toll like receptors (TLRs) recognize specific molecular patterns of microbial pathogens referred to as pathogen associated molecular patterns (PAMPS). TLRs are located on the plasma membrane (TLRs 1, 2, 4-6, 11), where they recognize components of foreign microorganisms, or intracellularly (TLRs 7, 8, 9, 3), where they recognize Viral nucleic acids [161]. Signaling through Toll like receptors induce inflammatory responses including the production of type 1 interferons and plays an important role in innate immunity to bacterial, fungal, and viral pathogens as well as playing important role in homeostasis and tolerance in the liver, and in systemic autoimmunity[162,163,164]. Natural killer cells possess TLRs and are stimulated to produce INF\(\gamma\) upon ligation of TLRs [165]. TLRs signal through the adaptor proteins MyD88 or,
in the case of TLR3, through the TIR-domain-containing adapter-inducing interferon-β (TRIF)[165,166,167]. The majority of TLRs require MyD88 for signaling (TLR1, TLR2, TLR5, TLR6, TLR11 on the cell surface, and TLR7, TLR8, TLR9, TLR13 intracellularly). Whereas the intracellular TLR3 does not require MyD88, but requires TRIF. Furthermore, TLR4, which localizes at both the plasma membrane and in endosomes, can signal through TRIF in the absence of MyD88[168]. Here we show that Both MyD88 and TRIF are required for depletion of CD8⁺ T cells during *T. brucei* infection.

**Results**

**Experimental Strategy**

All studies reported below were done on individual strains of mice and were repeated at least 3 times for each strain. Infections, in each case were with 5000 exponentially growing *T. brucei* Antat 1.1E which were grown from a cryostabilate for 4 days in immunocompromised (600 r) C57BL/6 mice prior to injection into the experimental mice. Control mice were uninfected age and gender matched and were received on the same day as the mice receiving *T. brucei* infections. In all cases spleen cell numbers and types from infected mice were compared statistically to those of matched uninfected mice of the same strain which are considered to represent day 0 of infection. The studies therefore report the impact of *T. brucei* AnTat1.1E on splenic T cells in each strain of mice. Comparative infections were not performed between strains of mice although results are discussed in the context of strain differences. The impact of infections on the splenic B cell content of the same mice are reported previously in this thesis.

**T. brucei infection induces depletion of splenic CD8⁺ T cells in C57BL/6 mice**
Depletion of splenic B2 B cells occurs after remission of the first parasitemic wave in C57BL/6 mice which occurs between 7 and 8 days after infection with 5000 exponentially growing *T. brucei* AnTat 1.1E and is almost complete by 10 days post infection [75] (and described in the previous chapter). A comparison of results presented in Figure 1 panels A and B versus panels C and D show that the proportion of CD4+ T cells (CD3+, CD4+, CD8-) remains similar in a representative infected and uninfected mouse, whereas proportion of CD8+ T cells (CD3+, CD4-, CD8+) decreases in the infected mouse compared to the uninfected mouse. Results presented in Figure 1 also show that numbers of splenic T cells (CD3+) and CD8+ T cells significantly decreased on days 10, 20 and 30 after infection of C57BL/6 mice with *T. brucei* AnTat 1.1E (Fig 1 Fig 1A-D, E,G). In contrast, the number of splenic CD4+ T cells did not decrease significantly on days 10 and 30 after infection (Fig. 1F), although it was decreased significantly at 20 days after infection (Fig. 1G). As with splenic B cells (previous chapter), there was no significant difference in the numbers of splenic T cells (CD3+), CD4+ T cells and CD8+ T cells in mice that had been uninfected 7 days earlier and those infected 7 days earlier with *T. brucei* AnTat 1.1E (data not shown).

**CD8+ T cells are not deleted from the spleens of C57BL/6 mice depleted of NK1.1+ cells**

Removal of NK and NKT cells by administration of anti-NK1.1 monoclonal antibody to C57BL/6 mice on days -1, 3 and 7 post infection with 5000 *T. brucei* AnTat1.1E prevents the depletion of splenic B2 B cells (previous chapter). Results presented in Figure 2 show that T cells (CD3+), and CD8+ T cells are not depleted from the spleens of NK1.1+ antibody treated mice that had been infected for 10 days with *T. brucei* AnTat1.1E. Thus individual antibody treated uninfected and *T. brucei* infected mice had similar proportional representation of CD4+ and CD8+ T cells (Fig 2, panels A-D), and similar numbers of total T cells, CD4+ T cells and CD8+ T cells. The anti-NK1.1 protocol depletes both NK cells (CD3- NK1.1+) and natural killer T (NKT; CD3+ NK1.1+) cells (Fig 3).
CD8+ T cells are depleted from the spleens T. brucei infected CD1d-/- C57BL/6 mice

Mice lacking the gene encoding CD1d were used to determine the contribution of NKT cells to the T. brucei infection induced depletion of CD8+ T cells. CD1d is specialized to present glycolipid antigens to NKT cells and in its absence NKT cells do not develop, whereas development of other T cells and NK cells is not prevented [169,170]. Despite the absence of NKT cells, CD8+ T cells are depleted proportionally and numerically from the spleens of CD1d-/- C57BL/6 mice infected 10 days earlier with 5000 T. brucei AnTat 1.1E (Fig 4). There was no significant difference in the total number of T cells CD3+ or CD4+ T cells in the day 10 infected mice compared to uninfected mice. These data suggest a critical role for NK cells but not NKT cells in Trypanosome induced depletion of CD3+ and CD8+ T cells.

CD8+ T cells are not depleted from the spleens of T. brucei infected perforin-/- C57BL/6 mice but are depleted from the spleens of T. brucei infected CD16+/- mice.

We have previously shown that B2 B cells are depleted from the spleens of intact C57BL/6 mice, but not from the spleens of perforin+/- C57BL/6 mice infected with 5000 T. brucei Antat 1.1E (previous chapter). This also proved to be the case with splenic CD8 T cells. There was neither a proportional nor a numerical decrease in T cells (CD3+), CD4+ T cells, or CD8+ T cells from the spleens of the infected perforin+/- mice as compared to uninfected perforin+/- mice up to 60 days after infection with 5000 T. brucei Antat 1.1E. Results for 10 days post infection are presented in Figure 5.

NK cell mediated cytotoxicity is regulated by the interaction of an array of inhibitory and activating receptors expressed on the surface of the NK cells with ligands on the target cell [144],
and is facilitated by the interaction of target cell bound antibody with the activating receptor CD16 on NK cells [160], referred to as antibody dependent cell mediated cytotoxicity (ADCC). CD16 is the low affinity receptor for the Fc portion of some IgGs. Results presented in Fig 6 show that CD8+ T cells are proportionally and numerically decreased from the spleens of CD16−/− C57BL/6 mice 10 days after infection with 5000 T. brucei AnTat 1.1E. Although the mean numbers of T cells (CD3+) and CD4+T cells were reduced relative to those in uninfected mice these reductions were not statistically significant.

**Expression of MyD88 and TRIF is required for T. brucei infection induced depletion of splenic CD8+ T cells**

Mice which are lacking the gene encoding the adaptor protein MyD88 or TRIF, and thus cannot signal through TLRs (excepting TLR3 in the case of MyD88−/−, and only TLR3 in the case of TRIF) were infected with T. brucei. At day 10 post infection both MyD88−/− and TRIF−/− mice had maintained numbers of total CD3+, Cd4+ and CD8+ T cells to levels similar to those in uninfected MyD88−/− or TRIF−/− mice. Though both MyD88−/− and TRIF−/− mice have low numbers of T cells as compared to intact C57BL/6 mice, these data suggest that TLR signaling is important for loss of CD8+ T cells during T. brucei infection.

**Trypanosome lysate injections do not induce depletion of Splenic CD8+ T cells in C57BL/6 mice**

Freeze thaw lysate of T. brucei was prepared in the presence of protease inhibitors. C57BL/6 mice were administered lysate from 10⁸ T. brucei via 3 retro-orbital injections on consecutive days. Numbers of CD3+, CD4+ and CD8+ T. cells were unchanged in mice having received T.brucei lysate as compared to animals that did not receive injections of lysate. These data
suggest that soluble portions of *T. brucei* in the blood plasma are not sufficient to cause a depletion of T cells in C57BL/6 mice.

**Discussion**

We have previously established that during *T. brucei* infection NKp46- NK cells are responsible for depletion of splenic B2 B cells and loss of humoral immune competence. B cell deletion occurs after remission of the first parasitemia wave, results from perforin-dependent NK cell mediated cytotoxicity, and does not involve ADCC (previous chapter). We show here that *T. brucei* infection induced NK cells also deplete splenic CD8+ T cells and do so with similar kinetics to the depletion of B2 B cells. Thus at the peak of first wave parasitemia i.e., by 7 days after infection with 5000 exponentially growing *T. brucei* AnTat 1.1E, the number and class composition of splenic T cells is similar to that in uninfected mice, whereas by 3 days later the number of CD8+ cells in the spleen is decreased by about 90% compared to uninfected mice. As with infection-induced depletion of B2 B cells, the depletion of CD8 T cells is mediated by NK1.1+ cells and does not occur in mice that lack the gene encoding perforin. Furthermore, depletion of the CD8 T cells does not require NKT cells, which we show here using mice that do not express the gene encoding CD1d which is required for NKT cell development [158]. In addition, similar to depletion of splenic B cells, the depletion of splenic CD8 T cells occurs in mice that lack the gene encoding CD16 and which therefore do not support FcγR-dependent ADCC.

The activation of NK cells that subvert lymphocyte responses and adaptive immunity is not restricted to *T. brucei* infection. Virus specific CD8+ cells are decreased during acute mouse cytomegalovirus (MCMV) infection by NK cell- [140] and perforin- [121,145] dependent cell mediated cytotoxicity. In addition, both virus and *T. brucei* infection-induced NKp46- NK cells contribute to host pathology. Thus inactivation of the cytotoxic mechanism in *T. brucei*-infected perforin−/− mice sustains splenic B2 B cells (previous chapter) and CD8 T cells (shown here),
prevents weight loss and the development of anemia and greatly extends survival compared to similarly infected intact mice (previous chapter), whereas depletion of NK cells (predominantly NKp46 NK cells) from mice acutely infected with influenza virus abrogates lung tissue pathology and results in lower mortality rates[123]. NKp46 is an NK cell activating receptor, which reacts with viral hemagglutinins [171] and with unknown ligands expressed by tumor cells, antigen presenting cells, and pancreatic beta cells [172]. However, despite its designation as an activating receptor, NKp46 also has a critical role in preventing over-activation of NK cells. Thus, NKp46 NK cells that arise in mice bearing the natural cytotoxicity triggering receptor 1 mutation noe which prevents placement of NKp46 on the cell surface but not its synthesis, are hyper-responsive to a variety of stimuli. They are protective during MCMV infection but at the expense of adaptive immunity which they decrease by deleting virus responsive T cells [140]. Depletion of self-reactive lymphocytes by NKp46 NK cells may also be an important feature of self-tolerance, and lack of expression of NKp46 is a feature of NKs in tolerance organs i.e., organs in which antigen presentation results in tolerance instead of an adaptive immune response, such as intestine, uterus, and liver [140,148]. Little is known regarding the induction, activation and specificity of NKp46 NK cells in infected mice and in tolerogenic organs.

The correlation between development of lymphocyte destructive NK cells, and the depletion of splenic CD8 T cells and B2 B cells and clearance of first wave parasitemia in T. brucei infected mice is consistent with NK cell activation by products (antibodies, cytokines, chemokines etc.,) of responding leukocytes, or by components of trypanosome that are released during immune clearance, or combinations of these. In this regard, depletion of the splenic B2 B cells and CD8 T cells, is associated with loss of expression of NKp46 and CD49, and gain of expression of FasL and CD107a, i.e., loss of some mature NK cell differentiation antigens and acquisition of markers of activation and degranulation. It has been recently shown that repeated stimulation of human NK cells with antibody and antigen complexes that bind to CD16, which is the low affinity Fcγ
receptor, causes down regulation of NKp46 expression [173]. However, while immune complexes are certainly present in blood plasma and tissue fluids upon antibody mediated clearance of trypanosomes, loss of B2 B cells occurs in infected CD16⁺ mice to a similar degree to that in infected intact mice (previous chapter) and hence does not require binding of immune complexes to CD16. In addition, activation of NK cells to kill B cells occurs to a similar degree in intact mice and mice lacking both T cells and NKT cells, and their activation to deplete CD8 T cells occurs to a similar degree in intact mice and mice lacking NKT cells, excluding a requirement for T cell and NKT cell derived immune modulators for induction of lymphocyte destructive NK cells. NK cells express Toll-Like receptors (TLRs) that recognize pathogen associated molecular patterns [165] as do accessory cells that are important in NK cell activation. However, while TLRs are unlikely to play a role in activation of B2 B cell destructive NK cells in T. brucei infected mice because depletion of splenic B2 B cell populations is similar in intact mice and in mice lacking the genes encoding MyD88 or TRIF, which are adaptor protein required for TLR signaling, mice lacking MyD88 or TRIF maintain T cells during infection to similar levels seen in uninfected MyD88 or TRIF knockout mice suggesting a potential role for TLR signaling in NK cell dependant depletion of CD8⁺ T cells.

Finally, we have not observed loss of NKp46 expression by NK cells in mice injected intravenously, daily for 3 days, with the freeze thaw lysate of 10⁸ T. brucei AnTat 1.1E prepared either in the presence or absence of protease inhibitors, and this regime did not result in the depletion of splenic B2 B cells or CD8 T cells suggesting against direct or indirect activation of the NK cells by water soluble trypanosome components in blood plasma. However, we have observed that injection of naïve C57BL/6 mice with day 10 post infection serum from mice infected with T. brucei AnTat 1.1E, but not with normal mouse serum, induces a substantial portion (40-50%) of splenic NK cells to lose expression of NKp46 and CD49b and to express the activation marker CD69, but not FasL or CD107a suggesting partial activation (Hilliard,
unpublished). Further study in this system might therefore provide insights into the developmental pathway and activation of the NKp46 NK cells.

Not only is the mechanism of T. brucei infection induced NK cell activation unresolved, so is the mechanism of target cell specificity. The T. brucei infection induced NK cells deplete splenic B2 B cells but not B1 B cells (previous chapter) and, as shown here, also deplete CD8 T cells but not, or to a much lesser extent CD4 T cells. NK cells comprise several subpopulations that differ in ontogeny and differentiation antigen expression but share regulation by inhibitory and activating signals received through an array of receptors that express either inhibitory or activating motifs [174]. Hence it is reasonable to hypothesize that activated CD8 T cells and B2 B cells lack, or express a lower amount of, an inhibitory ligand for NKp46 NK cell cytotoxicity. The best studied negative regulator of NK cells is MHC class I which signals mouse NK cells through the Ly49 family of receptors [175], however the level of expression of MHC I is increased by three fold on B2 B cells (previous chapter) and on CD8 T cells (not shown) of C57BL/6 mice infected with T. brucei Antat 1.1E, as it is on the B1 B cells and CD4 T cells (not shown), excluding this regulatory axis. MHC I is not the sole ligand for NK cell inhibitory receptors. 2B4 is another important inhibitory NK cell receptor, for which the ligand (CD48), is expressed on hematopoietic cells. Altered expression of CD48 on target cells or of 2B4 on NK cells results in a lack of inhibitory signal to the NK cells and killing[176]. In the future, it will be informative to assess the expression of known NK cell activating and inhibitory receptors as well as expression of their cognate ligands on the activated B cells and T cells of T. brucei infected mice.

**Materials and Methods**

*Ethics Statement:* The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and
Guidelines for the Use of Laboratory Animals in Research, Teaching and Testing of the International Council for Laboratory Animal Science. All animal studies were approved by the Institutional Animal Care and Use Committee, University of Massachusetts, Amherst, MA01003 USA, as documented in protocol #s 2010-0028, 2013-0049 and 2013-0050.

Mice and Trypanosomes: Male C57BL/6 (Taconic, Germantown, NY), C57BL/6-prf1^tm1sdz^-/-, C57BL/6-prf1^tm1sdz^-/-, B6.129P2-Fcgr3^tm1Sjv^-/-, B6(C)-Cd1d^tm1.2Aben^-/- (Jackson Laboratory, Bar Harbor, ME) add MyD88^-/- and TRIF^-/- (generously provided by Katherine Fitzgerald, University of Massachusetts Medical School), (7-9 week old) were infected by intraperitoneal (i.p.) injection of 5000 exponentially growing pleomorphic Trypanosoma brucei Antat 1.1E (derived from EATRO 1125 stock, described in[148]) in blood collected from immunocompromised C57BL/6 donors (600r from a 127 Cesium source, 24 hours prior to i.p. injection of cryopreserved parasites) and diluted in Dulbecco’s Phosphate Buffered Saline (DPBS; GIBCO,Life Technologies), or uninfected by i.p., injection of DPBS.  Parasitemia was assessed in blood collected from the tail vein during infection by dilution (1:100) in DPBS and counting using a hemocytometer. All mice were housed under barrier conditions.

Cell isolation and flow cytometric analysis: Spleens were dissected from mice that were killed by lethal CO2 inhalation and mechanically dissociated in cold FACS Buffer (1.0% fetal bovine serum, FBS, [Atlanta Biologicals] in DPBS). Supernatant was collected, cell pellets prepared by centrifugation (500 g, 10 min, 4°C) and red blood cells were lysed by re-suspending each spleen cell pellet in 10ml of cold ammonium chloride lysis buffer (ACK; 0.15M NH4Cl, 1.0 mM KHCO3, 0.1mM Na2-EDTA). Incubations in ACK buffer were for 4 minutes on ice after which cells were pelleted as above, washed twice in FACs buffer, re-suspended in FACs buffer and 100 µl aliquots containing 10^6 cells were added to the wells of a V bottom plate and stained with combinations of specific monoclonal antibodies against B cell, T cell and NK cell differentiation antigens (listed in Fig S1) to identify these leukocyte populations and subpopulations by
multicolor flow cytometry as described [75]. Briefly, cells were incubated with a 1:1000 final concentration Fc block (CD16/CD32 Fc III/II, eBioscience, San Diego, CA) for 20 minutes at 4°C, pelleted, resuspended with biotin- or fluorochrome-conjugated primary antibodies (listed below) for 30 minutes at 4°C, washed twice in cold FACs buffer, resuspended in 100 µl buffer with or without streptavidin (SA) conjugated fluorochromes, which detects cell bound biotinylated antibodies, and incubated for an additional 30 minutes at 4°C. Samples were washed twice and resuspended in 300 µl FACs buffer with 1 µg of 7-amino-actinomycin D (7AAD), a fluorescent DNA dye that binds to membrane permeable (dead or dying) cells, (EMD Chemicals, San Diego, CA). Analyses were performed using a flow cytometer (LSRII BD Biosciences, San Jose, CA) and data processed using FLOWJO software (Tree Star Inc., Ashland, OR) to determine the percentages of 7AAD−ve and where stated also 7AAD+ve cells in fluorochrome-tagged subsets. The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue. Differences among multiple groups were analyzed by ANOVA and means were compared using Tukey’s HSD test when p ≤ 0.05 (GraphPad Prism v.4.0, GraphPad Software Inc. San Diego, CA).

Preparation of T. brucei lysate: Parasite lysate was prepared by suspending 10⁸ DEAE52 purified [149] washed T. brucei AnTat 1.1E in 5 ml lysis buffer (DPBS, containing 0.4%NP40 and protease inhibitor cocktail [Complete mini tablets, Roche, Indianapolis, IN]). Insoluble material was removed by centrifugation at 1000g for 5 mins.

Antibodies and detection reagents: The following antibodies were added to 100 µl aliquots of 10⁶ Fc-blocked (anti-CD16/CD32 clone 93) leukocytes: anti-CD3-FITC (clone 145-2C11), anti-CD8-PE (clone 53-6.7), anti-CD4–APC-Cy7 (clone GK1.5 ), anti-IgM-PE (clone II/41), anti-CD23-PE-Cy7 (clone B3B4), anti-CD45R (B220)-FITC (clone RA3-6B2), anti-CD93–APC (clone AA4.1) purchased from eBioscience (San Diego, CA); anti-CD21-APC (clone 7G6) purchased
from BD Biosciences (San Diego, CA); 7-amino-actinomycin D (7AAD), (EMD Chemicals, San Diego, CA)

NK cell depletion [177,178] and FasL Blocking: Mice were injected intraperitoneally with 500 ug anti-NK1.1 IgG2a monoclonal antibody purified from hybridoma (PK136, ATCC Manassas, Virginia) culture supernatant by Protein G chromatography, or with an irrelevant control IgG2a antibody (M9144, Sigma-Aldrich). Treatments were repeated on days 4 and 7 after the first injection of anti-NK1.1 and depletion of splenic NK1.1^+ cells was assessed on days 3, 7 and 10 using flow cytometry as described above. Blocking of Fas-FasL signaling was performed as previously described[179]; anti-FasL MFL4 antibody (Ab) [180] was injected intraperitoneally (500 µg per mouse).

Statistics: Results comparing data obtained from infected animals and from non-infected controls were subjected to two-tailed T tests with significant differences reported as follows: p≤0.05, (**), p≤0.01, (***) p≤0.001. Differences among multiple groups were analyzed using ANOVA and means were compared using Tukey’s honest significant difference (HSD) test when p≤0.05 (GraphPad Prism v.4.0, GraphPad Software Inc. San Diego, CA).
Fig 3.1 Splenic CD8+ T cells are depleted in C57BL/6 mice infected with T. brucei AnTat 1.1E. Mice were infected with 5000 T. brucei AnTat 1.1E. Spleens were taken at days 10, 20 and 30 post infection and T cells (CD3, CD4 and CD8) were analyzed using FACS. Uninfected mice were used as controls at each time point. A,B - Detection of splenic CD4 and CD8 T cells in a representative naïve C57BL/6 mouse. C,D - Detection of splenic CD4 and CD8 T cells in a representative C57BL/6 mouse 10 days after infection. E-G - Mean number of cells/spleen+/− 1 standard deviation of: (E) CD3+ (F) CD4+ (G) CD8+ T cells (n=3). Day 0 values are the mean of all sham infected mice. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected to infected mice.
Fig 3.2 Splenic T cells are maintained in the absence of natural killer cells in C57/BL6 mice infected with T. brucei AnTat 1.1E. Mice were infected with T. brucei AnTat 1.1E. They were treated with anti-NK1.1 mAb on days -1, 4, 7 post infection. Spleens were taken at day 10 post infection and T cells (CD3, CD4 and CD8) were analyzed using FACS. Uninfected mice and uninfected mice treated with anti-NK1.1 mAb were used as controls. A,B - Detection of splenic CD4 and CD8 T cells in a representative naïve C57BL/6 mouse treated with anti-NK1.1. C,D - Detection of splenic CD4 and CD8 T cells in a representative anti-NK1.1 treated C57BL/6 mouse 10 days after infection. E-F - Total numbers of: (E) CD3+ (F) CD4+ (G) CD8+T cells/spleen of control untreated mice (-0), control NK1.1 specific antibody treated mice (+0) and T. brucei infected NK1.1 specific antibody treated mice (+10). Data are presented as mean number of cells/spleen +/- 1 standard deviation (n=3). Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals.
Fig 2.3 Depletion of NK1.1+ cells: Spleens were collected from C57BL/6 mice injected i.p. 4 days earlier with 500µg of an irrelevant IgG2a mAb (panel A), or 500µg pk136 (anti-NK1.1) mAb (panel B). CD3+ and NK1.1+ cells were detected by FACS.
Fig 3.4 Splenic CD8+ T cells are depleted in CD1d deficient C57BL/6 mice infected with *T. brucei* AnTat 1.1E. CD1d/-/- C57BL/6 mice were infected with 5000 *T. brucei* AnTat 1.1E on day 0. Spleens were taken at days 10, post infection and T cells (CD3, CD4 and CD8) were analyzed using FACS. Uninfected mice were used as controls.

A, B - Detection of splenic CD4 and CD8 T cells in a representative naïve C57BL/6 mouse.

C, D - Detection of splenic CD4 and CD8 T cells in a representative C57BL/6 mouse 10 days after infection.

E-G - Mean number of cells/spleen +/- 1 standard deviation of: (E) CD3+, (F) CD4+, (G) CD8+T cells (n=3). Day 0 values are the mean of sham infected mice. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected to infected mice.
Fig 3.5 Splenic CD8+ T cells are maintained in perforin−/− mice infected with *T. brucei* AnTat 1.1E. Perforin−/- C57BL/6 mice were infected with 5000 *T. brucei* AnTat 1.1E. Spleens were taken at days 10 post infection and T cells (CD3, CD4 and CD8) were analyzed using FACS. Uninfected mice were used as controls. A,B - Detection of splenic CD4 and CD8 T cells in a representative naïve C57BL/6 mouse. C,D - Detection of splenic CD4 and CD8 T cells in a representative C57BL/6 mouse 10 days after infection. E-G - Mean number of cells/spleen +/- 1 standard deviation of: (E) CD3+ (F) CD4+ (G) CD8+ T cells (n=3). Day 0 values are the mean of uninfected mice. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected to infected mice.
Fig 3.6 Splenic CD8+ T cells are depleted in CD16<sup>−</sup> mice infected with <i>T. brucei</i> AnTat 1.1E. CD16<sup>−</sup>-C57BL/6 mice were infected with 5000 <i>T. brucei</i> AnTat 1.1E on day 0. Spleens were taken at days 10, post infection and T cells (CD3, CD4 and CD8) were analyzed using FACS. Uninfected mice were used as controls. 

**A,B** - Detection of splenic CD4 and CD8 T cells in a representative naïve C57BL/6 mouse. 

**C,D** - Detection of splenic CD4 and CD8 T cells in a representative C57BL/6 mouse 10 days after infection. 

**E-G** - Mean number of cells/spleen ± 1 standard deviation of: (E) CD3<sup>+</sup> (F) CD4<sup>+</sup> (G) CD8<sup>+</sup>T cells (n=3). Day 0 values are the mean of uninfected mice. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected to infected mice.
Figure 3.7 T cells are not depleted from the spleens of T. brucei AnTat1.1E infected MyD88-/- or TRIF-/- C57BL/6 mice. Spleens were taken from naïve mice and mice infected 10 days earlier with 5000 T. brucei AnTat 1.1E and T cells (CD3, CD4 and CD8) were analyzed using FACS. Data are presented as mean number of CD3+, CD4+ or CD8+ T cells/spleen +/- 1 standard deviation (n=3) of MyD88-/- mice (panels A-C) and TRIF-/- mice (panels D-E). Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey's HSD test comparing uninfected controls to infected individuals.
Figure 3.8 T cells are not depleted from the spleens of C57BL/6 mice injected with lysate from T. brucei AnTat1.1E. Spleens were taken from mice injected with PBS and mice injected 4 times with whole cell lysate of 1x10^8 T. brucei AnTat 1.1E (prepared in the presence of protease inhibitors) and T cell populations analyzed using multicolour flow cytometry. Data are presented as mean number of CD3+, CD4+ or CD8+ T cells/spleen± 1 standard deviation (n=3). Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected injected controls to lysate injected individuals.
Abstract

We have previously shown that depletion of splenic B2 B cells in *T. brucei* infected mice, which suppresses trypanosome specific antibody responses, requires perforin dependent NK cell-mediated cytotoxicity. Here we show that B2 B cell depletion is regulated by both *T. brucei* variable surface glycoprotein glycosylphosphatidylinositol specific phospholipase C (PLC) and host CD1d. Thus splenic B2 B cells were not depleted from C57BL/6 mice infected with PLC<sup>−/−</sup> *T. brucei* and these mice controlled parasitemia and survived for 125 days post infection (dpi), whereas splenic B2 B cells were depleted by a NK1.1<sup>+</sup> cell- and perforin-dependent process in CD1d<sup>−/−</sup> C57BL/6 mice infected with the same PLC<sup>−/−</sup> *T. brucei*, and the mice died with fulminating parasitemia by 35 dpi. Lack of splenic B2 B cell depletion in PLC<sup>−/−</sup> *T. brucei* infected intact C57BL/6 mice was not due to a protective response because the B cells were rapidly depleted from these mice following super-infection 3 to 15 days later with a cloned intact *T. brucei* from the same isolate. Retention of B2 B cells in intact mice infected with PLC<sup>−/−</sup> *T. brucei* and their loss from CD1d<sup>−/−</sup> mice infected with the same parasites was associated with differential NK cell cytotoxic granule degranulation evidenced by differential expression of CD107a. The degranulation marker was highly expressed by NK cells from infected CD1d<sup>−/−</sup> but not intact mice, suggesting the absence of signals that cause NK cell degranulation. Phenotypic analyses of splenic NK cells from PLC<sup>−/−</sup> *T. brucei* infected intact and CD1d<sup>−/−</sup> mice showed these were similar (NKp46<sup>−</sup>, CD49b<sup>−</sup>, Ly49 C,F,I, Ly49 H, FasL<sup>+</sup>, 80% 2B4<sup>+</sup>, and CD160<sup>lo</sup>) as were splenic B2 B cells from both strains of infected mice (MHC class I<sup>hi</sup>, Fas<sup>+</sup>, CD48<sup>+</sup> and CD60<sup>+</sup>) suggesting
that differences in expression of these receptors and ligands are not responsible for differential
NK cell degranulation and depletion of B2 B cells. However, while the level of CD1d expression
was similar on splenic B2 B cells of PLC$^+/ -$ T. brucei infected and naive intact mice, it was
strikingly increased on NK cells from the infected intact mice, raising the possibility that negative
signaling through CD1d by an unknown T. brucei PLC-sensitive ligand prevents degranulation
and cytotoxic depletion of B2 B cells in these mice.

**Introduction**

*Trypanosoma brucei* expresses on its surface about 10 million copies of a single species of
variable surface glycoprotein (VSG) encoded by a single VSG gene. These VSGs, which form an
almost contiguous monolayer, assemble as non-covalent homodimers with each VSG attached to
the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor [181]. The VSG coat
contributes to the survival of the flagellated extracellular protozoan by masking plasma
membrane components that activate the alternative complement pathway[24]. In addition, GPI
anchoring of VSGs on the outer lipid leaflet of the plasma membrane allows the homodimers to
independently rotate and diffuse in the plane of the membrane, and their packing on the
trypanosome surface results in only a subset of N terminal VSG epitopes being accessible to
antibodies[182]. As a result of these properties and of trypanosome motility, the complexes of
VSG and antibody that form on the surface of intact trypanosomes are small and are moved
directionally [183] by hydrodynamic force [184] into the flagellar pocket, where they are
endocytosed and the antibody degraded in endosomes [185,186] thus reducing the efficacy of
antibody mediated trypanosome clearance. Eventually, an adequate concentration of VSG
specific antibodies is achieved in host plasma to cause trypanosomes to be cleared by
complement dependent phagocytosis in the liver and other organs [187,188]. However, VSG
antigenic variants [189] remain and expand the parasitic burden until a new antibody response is
generated. This process recurs, resulting in waves of parasitemia, each arising from new variant antigenic types.

Trypanosome infections can eventually resolve through self-cure in trypanosomiasis-resistant hosts e.g., African wildlife species[52,190,191], possibly because immune responses of these hosts reduce the parasite burden below that required to sustain antigenic variation which has been shown to occur at rates of $1.9 \times 10^{-6}$ to $6.9 \times 10^{-3}$ switches/cell/generation depending on the parasite variant antigenic type examined [192]. In contrast, trypanosome infections do not typically resolve in trypanosomiasis-susceptible mammals, perhaps as a result of immunosuppression which severely compromises IgG antibody responses against VSGs or other trypanosome antigens in infected people and livestock [84,85] and which rapidly inhibits trypanosome specific IgM and IgG antibody responses in infected mice [75,76]. Mechanisms of *T. brucei* induced immunosuppression of antibody responses are unresolved in people and cattle, but partially resolved in mice.

Suppressed humoral immunity in *T. brucei* infected mice results from infection-induced depletion of splenic B cells of the B2 lineage [75] as a result of natural killer (NK) cell mediated cytotoxicity [193]. Inactivation of this process through perforin gene deletion results in the development of high titer IgM and IgG antibody responses against VSG and many other trypanosome antigens, sustained control of trypanosome parasitemia, sustained blood packed cell volume and body weight and prolonged survival after infection, all of which are characteristics that are shared by infected trypanosomiasis resistant animals. The findings suggest that diverse indicators of pathology in infected trypanosomiasis susceptible hosts are linked to trypanosomiasis-induced immunosuppression.

Inflammatory macrophages and TNF-producing/iNOS-expressing dendritic cells (tipDC) are implicated in mechanisms of *T. brucei*-induced immunopathology[69,70,71,72,73,74] and studies
in animal models show that responses of these cells are induced by trypanosome components, particularly the PLC-cleavage fragments of the membrane form of VSG [61,68]. The contention that PLC cleavage products play a major role in trypanosomiasis-associated immunopathology is supported by findings that removing PLC coding sequence from the *T. brucei* genome decreases parasite virulence in mice infected with pleomorphic *T. brucei* [22]. It facilitates in the host a switch from a pro-inflammatory TH1 to an anti-inflammatory TH2 T cell response during infection [57,67], and reduces the degree of immunosuppression and pathology induced by the parasites [21] resulting in sustained humoral immune competence and control of parasitemia [21]. Thus intact mice infected with PLC<sup>−/−</sup> *T. brucei* have a similar absence of immunopathology to perforin<sup>−/−</sup> mice infected with intact *T. brucei* consistent with the possibility that PLC is required to elicit the B cell destructive NK cell response.

PLC indirectly enhances transferrin acquisition by the parasites[194] and has a minor role in excision of the VSG coat during parasite differentiation to tsetse fly gut forms[195]. However, these functions of the enzyme are not essential for viability or infectivity and it is generally agreed that the PLC serves mainly as a virulence factor in mammals. This suggests that PLC was selected through evolution to enhance survival of *T. brucei* in its mammal hosts. Consequently elucidating the PLC-dependent virulence mechanism is a critical step towards developing interventions that will negate its effect on the immune system in trypanosomiasis-susceptible hosts. Here we address the possibility that *T. brucei* PLC plays a key role in infection-induced deletion of B2 B cells by NK cells. In support of this possibility our findings show that infection of intact C57BL/6 mice with PLC<sup>−/−</sup> *T. brucei* does not cause the deletion of splenic B cells. However, the same parasites delete splenic B 2 cells from CD1d<sup>−/−</sup> C57BL/6 mice with comparable efficacy to that of intact *T. brucei*. Additional studies reported here suggest against induction of an anti-pathology response by the PLC<sup>−/−</sup> *T. brucei* in intact mice and implicate
antagonism of NK cell degranulation by negative signaling as the key mechanism in determining B2 B cell survival.

**Results**

**Infection of C57BL/6 mice with PLC<sup>−/−</sup> T. brucei does not cause depletion of splenic B2 B cells**

In order to assess the role of T. brucei PLC in infection induced loss of splenic B2 B cells we compared splenic B cell dynamics in C57BL/6 mice infected with 5000 exponentially growing T. brucei AnTat 1.1E [196] and 5000 T. brucei EATRO 1125 which are engineered to lack a functional gene encoding PLC (PLC<sup>−/−</sup>) [197]. T. brucei Antat 1.1E was cloned from EATRO 1125 after several passages in rats and mice [196]. As shown previously (see chapter 1) C57BL/6 mice infected with T. brucei Antat 1.1E lose splenic B2 B cells and humoral immune competence by 10 days after infection [75] and die between 30 and 40 days post infection with fulminating parasitemia [76,182,198]. In contrast C57BL/6 mice infected with PLC<sup>−/−</sup> T. brucei survived for 100 -125 days post infection (Fig. 1A) and numbers of splenic transitional type 1, 2 and 3 B cells, marginal zone B cells, and follicular B cells were not reduced during infection with the PLC<sup>−/−</sup> parasite at 10 dpi (Fig. 1B-F), and for at least 90 dpi (data not shown). Thus T. brucei PLC is required for infection-induced depletion of splenic B2B cells in intact C57BL/6 mice.

**In the absence of host CD1d, virulence is restored to the PLC<sup>−/−</sup> T. brucei**

CD1d is a MHC1 like molecule that has been shown to present glycolipid antigen, including T. brucei VSG-GPI, to the T cell receptors of NKT cells [199]. Administration of GPI to mice prior to infection with T. brucei provides a degree of protection against infection-induced pathology [200] and is host CD1d-dependent[201], raising the possibility that loading of CD1d by VSG-GPI
in mice infected with PLC\(^+\) \textit{T. brucei} might stimulate an anti-pathology response. In order to discern whether CD1d plays a role in preventing depletion of splenic B2 B cells by PLC\(^-\) \textit{T. brucei}, CD1d\(^-\) C57BL/6 mice were infected with the PLC\(^+\) parasites or with \textit{T. brucei} Antat 1.1E. Results presented in Fig 2 show that infection of the CD1d\(^+\) mice with either intact or PLC\(^+\) \textit{T. brucei} caused depletion of splenic B2 B cells. In addition, the infected CD1d\(^-\) mice developed anemia evidenced by a decrease in blood packed cell volume similar to that observed in mice infected with intact \textit{T. brucei}(Fig3) and died between 30 and 40 dpi irrespective of infection with intact or PLC\(-/-\) \textit{T. brucei} (data not shown). Thus low virulence of PLC\(^+\) \textit{T. brucei} requires host CD1d expression.

**Neither T cells nor Natural killer T cells are required for low virulence of PLC\(^+\) \textit{T. brucei}**

To determine whether NKT cells or other T cells play a role in maintaining the low virulence of PLC\(^+\) \textit{T. brucei} in intact C57BL/6 mice, C57BL/6 mice lacking the genes for both the beta and delta chains of the T cell receptor and hence lacking all T cells and NKT cells (TCR\(^-/-\) C57BL/6) were infected. Splenic B2 B cells were not depleted from the spleens of the PLC\(^+\) \textit{T. brucei} infected TCR\(^-/-\) mice by 10 dpi (Fig4 A - E) or at later stages of infection and the infected mice survived for > 60 dpi (data not shown) at which time the study was terminated. These data show that neither NKT nor other T cells are required to maintain the low virulence of PLC\(^+\) \textit{T. brucei}.

**Co-infection with PLC\(^+\) \textit{T. brucei} does not reduce the virulence of \textit{T. brucei} Antat 1.1E in C57BL/6 mice**

Despite the lack of requirement for NKT and other T cells in suppressing the virulence of PLC\(^+\) \textit{T. brucei} in intact C57BL/6 mice, it remains possible that PLC\(^+\) parasites stimulate a CD1d-dependent anti-pathology response. In order to assess this possibility intact mice were infected with PLC\(^+\) \textit{T. brucei} at the same time as, or 15 days prior to, infection with \textit{T. brucei} Antat 1.1E. At day 10 post infection with \textit{T. brucei} Antat 1.1E, spleen cells were harvested and analyzed for
the portion and numbers of B2 B cells. Transitional, marginal zone and follicular B cells were significantly reduced in the co-infected mice as compared to that in naïve mice (day -15 co-infection data shown in Fig 5) and the levels of depletion were similar to that obtained in C57BL/6 mice infected with *T. brucei* AnTat 1.1E alone (see Fig 1). Furthermore, co-infected mice died within 30 to 40 days after infection with *T. brucei* AnTat 1.1E (data not shown) showing that the co-infection with PLC<sup>−/−</sup> parasites did not reduce the virulence of *T. brucei* Antat1.1E in C57BL/6 mice.

**Depletion of NK1.1<sup>+</sup> cells prevents loss of splenic B2 B cells during PLC<sup>−/−</sup> infection of CD1d<sup>−/−</sup> mice**

*T. brucei* AnTat1.1E infection-induced B cell depletion is dependent upon the presence of NK cells and is abrogated through depletion of these cells during infection. To assess whether NK cells are also required for depletion of B2 B cells during PLC<sup>−/−</sup> *T. brucei* infection of CD1d<sup>−/−</sup> mice, NK1.1<sup>+</sup> cells were depleted from the mice by administration of anti-NK1.1 mAb on days -1, 3, and 7 after infection. Control infected Cd1d<sup>−/−</sup> mice were administered an irrelevant IgG2a mAb as an isotype control. Splenic B2 B cells were depleted from infected CD1d<sup>−/−</sup> mice administered the irrelevant mAb and retained in mice treated with anti-NK1.1 mAb (Fig 6). Thus PLC<sup>−/−</sup> *T. brucei* infection induced depletion of B2 B cell from the spleens of CD1d<sup>−/−</sup> mice requires NK1.1<sup>+</sup> cells similar to the depletion of B2 B cells from the spleens of intact mice by *T. brucei* AnTat 1.1E.

**CD1d-dependent suppression of NK cell degranulation in mice infected with PLC<sup>−/−</sup> T. brucei**

During infection of intact mice with intact *T. brucei*, NK cells acquire a novel activation phenotype expressing characteristics of both immature NK cells (NK1.1<sup>+</sup>, CD3<sup>−</sup>, NKp46<sup>−</sup>, CD49b<sup>−</sup>) and activated degranulated mature NK cells (FasL<sup>+</sup>, TRAIL<sup>+</sup>, CD1d<sup>hi</sup>, CD107a<sup>+</sup>). During infection of C57BL/6 mice with PLC<sup>−/−</sup> *T. brucei* the NK cells also acquire a novel activation
phenotype as shown in Fig 7. The NK cells in C57BL/6 mice infected with PLC\(^{-}\) T. brucei do not express NKp46 distinguishing them from almost all NK cells in uninfected C57BL/6 mice (Fig 7A). They also express the death receptor ligand FasL, the MHCI like molecule C1d as well as low levels of TRAIL as do the NK cells in mice infected with T. brucei AnTat 1.1E (Fig 7B-D) and a high portion of the cells lose expression of CD49b although not as high as that seen in mice infected with T. brucei AnTat1.1E (Fig 7E). Unstimulated mature NK cells in uninfected C57BL/6 mice express NKp46 and CD49b (Fig 7A, E) but not FasL (Fig 7B). Importantly, most of the NK1.1\(^{+}\) CD3\(^{-}\) cells (NK cells) in intact C57BL/6 mice infected with PLC\(^{-}\) T. brucei do not acquire surface expression of CD107a, which distinguishes them from NK cells in C57BL/6 mice infected with T. brucei AnTaT 1.1E (Fig 7F). CD107a, also called LAMP1, is a cytotoxic granule marker and appears on the surface membrane of NK cells only upon degranulation. It is therefore a functional marker for cell mediated cytotoxicity, and its absence from the surface of splenic NK cells from PLC\(^{-}\) T. brucei infected C57BL/6 mice is consistent with lack of splenic B2 B cell depletion from these mice.

Several differences were observed in NK cells of naïve and PLC\(^{-}\) T. brucei infected CD1d\(^{-}\) C57BL/6 mice compared to naïve and similarly infected C57BL/6 mice. Firstly, most NK cells in naïve CD1d\(^{-}\) mice lack the NKp46 activating receptor (Fig 8A). Secondly, almost all NK cells in CD1d\(^{-}\) mice that are infected with PLC\(^{-}\) T. brucei lose expression of CD49b (Fig 8B), acquire expression of FasL (Fig 8C) and have strong surface expression of CD107a (Fig 8D) thus recapitulating the phenotype of B cell destructive NK cells in intact mice infected with T. brucei AnTat 1.1E. MHCI is upregulated on B2 B cells during PLC\(^{-}\) infection of both intact and CD1d\(^{-}\) mice (Fig 9A). Whereas the expression of the cognate inhibitory and activating receptors for MHCI, Ly49C, F, I (inhibitory) and H (activating), are downregulated on NK cells during infection of both CD1d\(^{-}\) (Fig 9A-C) and intact (Fig 9D-F) C57BL/6 mice with either intact or PLC\(^{-}\) T. brucei.
Surface expression of CD160, the natural killer cell receptor for CD1d, remains unchanged during infection of intact or CD1d−/− mice with either intact or PLC−/− T. brucei (representative data from intact C57BL/6 shown in Fig 10A) as does expression of CD1d on B cells in the intact mice (see Fig 8). The molecule CD244 (2B4) is expressed on NK cells and is thought to be an inhibitory receptor for NK cells while it is thought to act as a stimulatory ligand to lymphocytes that express CD48 [202]. 2B4 expression on NK cells is unchanged during intact or PLC−/− T. brucei infection of C57BL/6 mice (Fig 10B), except in the absence of host CD1d, in which case 2B4 expression is upregulated (Fig 10) and CD48 expression on B cells remains unchanged during intact or PLC−/− infection of either intact or CD1d−/− mice (representative data from C57BL/6 shown in Fig 10D).

**Intravenous administration of T. brucei Antat 1.1E lysate to C57BL/6 mice infected with PLC−/− T. brucei does not cause depletion of splenic B2 B cells or increase virulence**

PLC cleaves the GPI anchor of GPI-VSG resulting in the release of water soluble glycosylinositolphosphatase-VSG dimers (sVSG) and retention of T. brucei membrane bound dimyristyl glycerol. Both the dimyristyl glycerol and phosphoglycan moieties of VSG-GPI stimulate macrophages. In combination they induce production of TNFα [203] and the phosphoglycan moiety alone stimulates INFγ activated macrophages to produce TNFα [203]. Since sVSG cannot be generated by PLC−/− T. brucei it is possible that this parasite component, or its phosphoglycan moiety, alone or in combination with T. brucei membrane associated dimyristyl glycerol, causes the infection-induced depletion of splenic B2 B cells. To determine whether sVSG in combination with T. brucei membrane fragments contributes to splenic B2 B cell depletion, mice were infected with PLC−/− T. brucei or sham infected and repeatedly administered intravenous injections of 10^8 freeze-thaw lysed T. brucei AnTat 1.1E prepared in the presence of DNAase and protease inhibitors. The extracts contain sVSG and little or no
membrane form VSG determined by SDS-PAGE in 7% gels (data not shown). The parasite lysates were injected on days 6, 7, and 8 post infection which coincides with clearance of peak parasitemia in the PLC<sup>-/-</sup> T. brucei infected mice (see Fig 1A), or to uninfected animals, and splenic B2 B cell numbers were determined 2 days after the last injection. Administration of T. brucei AnTat 1.1E freeze thaw lysate did not cause depletion of splenic B2 B cells from either the uninfected mice, or from mice infected with the PLC<sup>-/-</sup> parasites (Fig. 11). Thus, T. brucei membrane fragments and sVSG in the lysate do not elicit B cell destructive NK cells in naïve mice, and in mice infected with the PLC<sup>-/-</sup> T. brucei, at least when administered by the intravenous route at the concentrations used.

Discussion

The studies reported above show that PLC<sup>-/-</sup> T. brucei are relatively avirulent in intact C576BL/6 mice, but of similar virulence to intact T. brucei in CD1d<sup>-/-</sup> C57BL/6 mice. This difference in virulence is at least in part a result of differential depletion of splenic B2 B cells in the two strains of infected mice. Thus intact C57BL/6 mice chronically infected with PLC<sup>-/-</sup> T. brucei maintain splenic B2 B cells at pre-infection levels, do not experience a decline in blood packed cell volume and survive for 125 dpi. Whereas CD1d<sup>-/-</sup> C57BL/6 mice that are infected with the same parasites, and intact mice infected with intact T. brucei, rapidly lose splenic B2 B cells, have a severe decline in blood packed cell volume and die by 35 dpi. These findings both clarify and complicate our understanding of how T. brucei PLC functions as a virulence factor.

T. brucei VSG specific PLC is capable of cleaving VSG-GPI resulting in the release of water soluble VSG (sVSG) bearing C terminal residual glycosylinositolphosphate (GIP) and leaving dimyristyl glycerol attached to the outer leaflet of the trypanosome plasma membrane. This occurs in parasites with ruptured membranes, but does not occur in healthy trypanosomes
The VSG-GPI substituents activate cells of the innate immune system and regulate macrophage and dendritic cell cytokine expression profiles and antigen presenting function by interaction with Type A scavenger receptors on the macrophage and dendritic cell membrane [206]. This stimulates an initial pro-inflammatory activation profile [203,207,208]. Target cell responses are modified by prior or subsequent interactions of IFNγ receptors with IFNγ, and pathogen pattern recognition receptors with CpG and other materials released by dead or dying trypanosomes [206,209,210]. The timing of these complex interactions may regulate the balance of TH1 and Th2 T cell responses by controlling the magnitude of response and functional commitment of the antigen presenting cells, and in so doing affect trypanosome virulence [208]. In this regard, control of infection is optimized by an initial Th1 T cell response, which promotes production of trypanocidal reactive oxygen and nitrogen intermediates by tissue macrophages, and which is subsequently balanced by an anti-inflammatory Th2 T cell response, which prevents lethal systemic inflammation. While these observations are consistent with the possibility that virulence of *T. brucei* is linked to production of GIP-sVSG and uncontrolled inflammation at high parasitemia, the studies reported here show that release of GIP-sVSG by PLC is not a requirement for infection-induced immunopathology and high parasite virulence in CD1d<sup>−</sup> mice thus indicating a GIP-sVSG independent virulence mechanism.

Our earlier studies show that high trypanosome virulence in chronically infected mice manifests as a rapid depletion of splenic B2 B cells [75], loss of humoral immune competence as well as an infection induced decline in blood packed cell volume and early death all of which are linked to infection-induced NK cell mediated cytotoxicity. NK cell mediated cytotoxicity is also likely to play a central role in the high virulence of PLC<sup>−</sup> *T. brucei* in CD1d<sup>−</sup> mice because antibody-mediated depletion of NK1.1 cells, from the infected mice resulted in maintenance of splenic B2 B cells. The similar nature of pathology arising in *T. brucei* infected C57BL/6 mice and PLC<sup>−</sup> *T. brucei* infected CD1d<sup>−</sup> mice, namely depletion of B2 B cells and decrease in blood packed cell
volume, as well as the requirement for a NK1.1+ cell strongly suggest that the same effector mechanism operates in both infection models.

How might the presence of CD1d prevent the induction or effector function of the NK cells? One possibility is that the PLC−/− *T. brucei* stimulate an anti-pathology response in intact mice but not in CD1d−/− mice, however, two lines of evidence suggest against this explanation. First, despite studies showing that B cell help is provided by NKT cells that are stimulated by VSG-GPI loaded on CD1d[118,199] ( ), NKT cells, indeed all T cells, appear to play no role in limiting virulence of PLC−/− *T. brucei* determined by infection of TCR−/− mice. Splenic B2 B cells were not depleted from intact or TCR−/− mice infected with the PLC−/− parasites and survival times of both strains of mice were similar and in excess of 100 dpi. Second, intact C57BL/6 mice that were infected with PLC−/− *T. brucei* EATRO 1125 up to 15 days before superinfection with *T. brucei* AnTat 1.1E, showed no protection against the latter parasites which were derived from the parent *T. brucei* EATRO 1125 isolate. Depletion of splenic B2 B cells from the co-infected mice occurred with the same kinetics to singly infected mice and both groups of mice died by 35 dpi.

It is also possible that NK cells acquire cytotoxic function in CD1d−/− but not intact mice infected with PLC−/− *T. brucei*. This possibility was investigated by comparative analysis of cell surface differentiation antigens expressed on the NK cells in both infection models. There was a good deal of similarity in the surface differentiation antigen profiles of NK cells from intact and CD1d−/− C57BL/6 mice infected with the PLC−/− *T. brucei*, and two striking differences. In both cases most of the NK cells in the infected mice were CD3−, NK1.1+. NKp46−, CD49b−, FASL+, TRAIL+, Ly49 C,F,I,H−, and 2B4+, thus differing from typical mature NK cells in uninfected mice which express NKp46, CD49b, and Ly49C,F,I, in addition to NK1.1 and do not express FasL. These infection-induced changes in NK cell differentiation antigen expression are similar to those arising on NK cells in intact mice infected with intact *T. brucei*. Two possibly important
distinctions were observed between the NK cells of intact and CD1d<sup>+</sup> mice before and after infection with PLC<sup>+</sup> T. brucei. First, splenic NK cells in naïve CD1d<sup>+</sup> mice did not express NKp46 unlike those in intact C57BL/6 mice suggesting that CD1d might play a role in normal NK cell development and sub-population commitment. To our knowledge this has not been reported previously. Second, NK cells in CD1d<sup>+</sup> mice infected with PLC<sup>+</sup> trypanosomes acquired strong expression of CD107a, which is expressed on cytotoxic granules and placed on the plasma membrane during NK cell degranulation, whereas intact mice infected with the PLC<sup>+</sup> trypanosomes did not acquire expression of CD107a. Thus, infection activated NK cells in intact C57BL/6 mice infected with PLC<sup>+</sup> T. brucei are not induced to degranulate consistent with retention of the putative target B2 B cells in the spleens of infected mice, whereas those in infected CD1d<sup>+</sup> mice are induced to degranulate consistent with splenic B cell depletion in these mice. Finally, NK cells in infected C57BL/6 mice have upregulated expression of CD1d on their cell surface, which is not possible in CD1d<sup>+</sup> mice.

NK cell killing is regulated by a balance of receptor and ligand interactions that are either activating or inhibitory to the NK cell. The NK cells are known to kill target cells that have decreased MHC I on their plasma membrane, designated “missing self” [211]. However, in the case of the T. brucei-infected mice, MHC I expression is actually increased on IgM<sup>+</sup> cells and, as shown here, is increased to a similar extent on IgM<sup>+</sup> cells in both intact and CD1d<sup>+</sup> mice infected with PLC<sup>+</sup> T. brucei. These findings show that “missing self”, at least in the context of class I MHC molecules, is not involved in making the B cells of the infected CD1d<sup>+</sup> mice into targets for NK cell. This conclusion is supported by analysis of Ly49 receptor family expression on NK cells of the infected mice. Ly49 family members are Type II C-type lectin-like NK cell receptors that bind MHC class I, delivering an activating signal in the case of Ly49H and Ly49D and inhibitory signals in the case of other Ly49 receptors that are expressed by the C57BL/6 NK cells,
namely Ly49A,C/I,F,G [212]. The Ly49 receptor profiles of NK cells show a decreased expression of both inhibitory Ly19C, F, I and activating Ly49H.

Mouse NK cells express an Ig-like receptor CD160 that recognizes both classical and non-classical MHC class I molecules, the later including CD1d [213]. Importantly, CD1d1 signaling to IL-2 activated CD1d1-binding mouse NK cells has been shown to negatively regulate their cytotoxic activity [214,215], raising the possibility that signaling though CD160 might also down regulate the cytotoxic activity of NK cells in intact mice infected with PLC<sup>-/-</sup> T. brucei accounting for low parasite virulence. Furthermore, the absence of CD1d-dependent CD160 signaling in NK cells of CD1d<sup>-/-</sup> mice might account for high NK cytotoxic activity and high virulence of PLC<sup>-/-</sup> parasites in this strain. However, this attractive hypothesis is unlikely to be valid because of the unchanged expression of CD1d on B cells and unchanged expression of CD160 on NK cells during infections.

Increased expression of surface CD1d on NK cells occurs during infection of intact mice with both PLC<sup>-/-</sup> T. brucei and intact T. brucei raising the possibility that NK cell degranulation on B2 B cells is inhibited by negative signaling through NK expressed CD1d which is somehow circumvented by T. brucei PLC. Direct CD1d signaling has not been reported for NK cells and, disappointingly, the absence of PLC in infecting T. brucei could not be compensated by repeated intravenous injection of freeze thaw lysed intact T. brucei containing membrane associated PLC, as well as GPI substituents generated by PLC. Nevertheless we consider that testing this hypothesis is a reasonable next step in resolving how host CD1d and T. brucei PLC jointly control depletion of B2 B cells in infected mice, and thus the efficacy of trypanosome specific antibody responses. In addition, discovery based approaches including gene expression, plasma membrane proteome and cell phosphoproteome analyses of NK and B2 B cells from intact and
CD1d−/− mice before, and after, infection with intact and PLC−/− T. brucei, might provide insights into signaling pathways that regulate cytotoxic granule degranulation and B2 B cell depletion.

**Materials and Methods**

*Ethics Statement:* The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Guidelines for the Use of Laboratory Animals in Research, Teaching and Testing of the International Council for Laboratory Animal Science. All animal studies were approved by the Institutional Animal Care and Use Committee, University of Massachusetts, Amherst, MA01003 USA, as documented in protocol #s 2010-0028, 2013-0049 and 2013-0050.

*Mice and Trypanosomes:* Male C57BL/6 (Taconic, Germantown, NY), C57BL/6-prf1tm1sdz/J (perforin−/−), B6.129P2-Fcgr3tm1Sjv/J (CD16−/−), B6(C)-Cd1d1tm1.2Aben/J (CD1d−/−)(Jackson Laboratory, Bar Harbor, ME) add MyD88−/− and TRIF−/− (generously provided by Katherine Fitzgerald, University of Massachusetts Medical School), (7-9 week old) were infected by intraperitoneal (i.p.) injection of 5000 exponentially growing pleomorphic Trypanosoma brucei Antat 1.1E or PLC−/−(both derived from EATRO 1125 stock, described in[148]) in blood collected from immunocompromised C57BL/6 donors (600r from a 127 Cesium source, 24 hours prior to i.p. injection of cryopreserved parasites) and diluted in Dulbecco’s Phosphate Buffered Saline (DPBS; GIBCO,Life Technologies), or uninfected by i.p. injection of DPBS. Parasitemia was assessed in blood collected from the tail vein during infection by dilution (1:100) in DPBS and counting using a hemocytometer. All mice were housed under barrier conditions.

*Cell isolation and flow cytometric analysis:* Spleens were dissected from mice that were killed by lethal CO2 inhalation and mechanically dissociated in cold FACS Buffer (1.0% fetal bovine serum, FBS, [Atlanta Biologicals] in DPBS). Supernatant was collected, cell pellets prepared by centrifugation (500 g, 10 min, 4°C) and red blood cells were lysed by re-suspending each spleen
cell pellet in 10ml of cold ammonium chloride lysis buffer (ACK; 0.15M NH$_4$Cl, 1.0 mM KHCO$_3$, 0.1mM Na$_2$-EDTA). Incubations in ACK buffer were for 4 minutes on ice after which cells were pelleted as above, washed twice in FACs buffer, re-suspended in FACs buffer and 100 µl aliquots containing 10$^6$ cells were added to the wells of a V bottom plate and stained with combinations of specific monoclonal antibodies against B cell, T cell and NK cell differentiation antigens (listed in Fig S1) to identify these leukocyte populations and subpopulations by multicolor flow cytometry as described [75]. Briefly, cells were incubated with a 1:1000 final concentration Fc block (CD16/CD32 Fc III/II, eBioscience, San Diego, CA) for 20 minutes at 4°C, pelleted, resuspended with biotin- or fluorochrome-conjugated primary antibodies (listed below) for 30 minutes at 4°C, washed twice in cold FACs buffer, resuspended in 100 µl buffer with or without streptavidin (SA) conjugated fluorochromes, which detects cell bound biotinylated antibodies, and incubated for an additional 30 minutes at 4°C. Samples were washed twice and resuspended in 300 µl FACs buffer with 1 µg of 7-amino-actinomycin D (7AAD), a fluorescent DNA dye that binds to membrane permeable (dead or dying) cells, (EMD Chemicals, San Diego, CA). Analyses were performed using a flow cytometer (LSRII BD Biosciences, San Jose, CA) and data processed using FLOWJO software (Tree Star Inc., Ashland, OR) to determine the percentages of 7AAD$^-$ and where stated also 7AAD$^+$ cells in fluorochrome-tagged subsets. The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue. Differences among multiple groups were analyzed by ANOVA and means were compared using Tukey’s HSD test when p$\leq$0.05 (GraphPad Prism v.4.0, GraphPad Software Inc. San Diego, CA).

*Preparation of T. brucei lysate:* Parasite lysate was prepared by suspending 10$^8$ DEAE52 purified [149] washed T. brucei AnTat 1.1E in 5 ml lysis buffer (DPBS, containing 0.4%NP40 and protease inhibitor cocktail [Complete mini tablets, Roche, Indianapolis, IN]). Insoluble material was removed by centrifugation at 1000g for 5 mins.
**Antibodies and detection reagents:** Avidin-APC-Cy7 (San Diego), anti-CD1d-biotin (clone 1B1), anti-CD48-biotin (clone HM4810), anti-CD244-APC (clone eBio244f4), anti-CD160-PE (clone eBioCNX46-3), anti-DR5-biotin (clone MD5-1), anti-FasL-PE (clone MFL3), anti-Fas-PE-Cy7 (clone 15A7), anti-H2kb-PE (clone eBM2a), anti-Ly49C/I/F/H-FitC (clone 14B11), anti-Ly49H-biotin (clone 3D10), anti-NKp46-PE (clone 29A1.4), anti-IgM-PE (clone III/41), anti-NK1.1-APC (clone pk136), anti-TRAIL-biotin (clone N2B2), anti-CD23-APC-Cy7 (clone B3B4), anti-CD23-FITC (clone B3B4), anti-CD45R (B220)-FITC (clone RA3-6B2), anti-CD93–APC (clone AA4.1), anti-CD107a (clone 1D4B) purchased from eBioscience (San Diego, CA); anti-CD21-APC (clone 7G6), anti-CD49b FITC (clone DX5) purchased from BD Biosciences (San Diego, CA); 7-amino-actinomycin D (7AAD) purchased from EMD Chemicals (San Diego, CA).

**IFA -** Anti-MOMA-1(biotin) AbCam (Cambridge MA); Avidin-FITC, BD Pharmingen (San Diego, Ca); anti-human/mouse CD45 (B220) eBiosciences (San Diego, Ca).

**NK cell depletion [177,178] and FasL Blocking:** Mice were injected intraperitoneally with 500 µg anti-NK1.1 IgG2a monoclonal antibody purified from hybridoma (PK136, ATCC Manassas, Virginia) culture supernatant by Protein G chromatography, or with an irrelevant control IgG2a antibody (M9144, Sigma-Aldrich). Treatments were repeated on days 4 and 7 after the first injection of anti-NK1.1 and depletion of splenic NK1.1+ cells was assessed on days 3, 7 and 10 using flow cytometry as described above. Blocking of Fas-FasL signaling was performed as previously described[179]; anti-FasL MFL4 antibody (Ab) [180] was injected intraperitoneally (500 µg per mouse).

**Statistics:** Results comparing data obtained from infected animals and from non-infected controls were subjected to two-tailed T tests with significant differences reported as follows: \( p \leq 0.05 \), (**) \( p \leq 0.01 \), (+++) \( p \leq 0.001 \). Differences among multiple groups were analyzed using ANOVA and
means were compared using Tukey’s honest significant difference (HSD) test when p≤0.05 (GraphPad Prism v.4.0, GraphPad Software Inc. San Diego, CA).
Figure 4.1 PLC is required for *T. brucei* induced loss of Splenic B2B cells: C57BL/6 mice were infected with 5000 *T. brucei* AnTat 1.1E or PLC-/- *T. brucei* on day 0. Spleens were taken at 10 days post infection and B Cell populations were analyzed by FACS. (A) survival of infected mice, and total number of Splenic B2 B cells (B,C,D) transitional type 1,2,3, (E) marginal zone and (F) follicular B cells of mice infected with intact *T. brucei* (WT) or PLC-/- *T. brucei*. Day 0 values are the mean of all uninfected mice. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected to infected mice.
Figure 4.2 B2 B cells are deleted during PLC<sup>−/−</sup> infection of CD1<sup>d<sub>−/−</sub></sup> mice. CD1<sup>d<sub>−/−</sub></sup> mice were infected with 5000 T. brucei AnTat 1.1E (WT) or PLC<sup>−/−</sup> T. brucei on day 0. Spleens were taken at 10 and 30 days post infection and B cell populations were analyzed by FACS. Results are presented as total numbers of: (A-C) Transitional (types 1, 2, and 3 respectively) B cells/spleen; (D) Marginal Zone B cells/spleen; (E) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 mice per group. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals.
Figure 3 CD1d−/− mice develop anemia during PLC−/− infection. Packed (red blood) cell standard deviation, of 3 mice per group. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected (naive) controls to infected individuals.
Figure 4.4 B2 B cells are not deleted during PLC⁻¹ infection of TCR⁻¹ mice

TCR⁻¹ mice were infected with 5000 PLC⁻¹ T. brucei on day 0. Spleens were taken at 10 and 30 days post infection and B Cell populations were analyzed by FACS. Results are presented as total numbers of: (A-C) Transitional (type 1, 2 and 3 respectively) B cells/spleen; (D) Marginal Zone B cells/spleen; (E) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 mice per group. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey's HSD test comparing uninfected controls to infected individuals.
Figure 4.5 PLC<sup>−/−</sup> T. brucei does not induce protective response against B2 B cell depletion during infection with intact T. brucei C57BL/6 mice were infected with 5000 PLC<sup>−/−</sup> T. brucei on day 0 and with 5000 intact T. brucei on day 15. Spleens were taken at 10 days post infection and B Cell populations were analyzed by FACS. Results are presented as total numbers of: (A-C) Transitional (type 1, 2 and 3 respectively) B cells/spleen; (D) Marginal Zone B cells/spleen; (E) Follicular B cells/spleen. Data are presented as individual values, mean and standard deviation, of 3 mice per group. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals.
**Fig 4.6** Splenic B2 B cells are maintained in the absence of natural killer cells in CD1d\(^+\) mice infected with PLC\(^+/\) T. brucei. CD1d\(^+\) mice were infected with PLC\(^+/\) T. on day 0. They were treated with anti-NK1.1 mAb on days -1, 3, 7 post infection. Spleens were taken at day 10 post infection and B2 B cells were analyzed using FACS. Total numbers of: (A-C) transitional (type 1, 2 and 3 respectively) (D) marginal zone (E) Follicular B cells/spleen of uninfected treated mice (naive) and PLC\(^+/\) T. brucei infected NK1.1 specific antibody treated mice (Day 10 + NK1.1). Data are presented as mean number of cells/spleen +/- 1 standard deviation (n=3). Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing uninfected controls to infected individuals.
**Fig 4.7 T. brucei infection-induced changes in NK cell differentiation antigen expression.** Splenocytes from naïve C57BL/6 mice and mice infected 10 days earlier with *T. brucei* Antat1.1E or PLC-/- *T. brucei* were stained with mAb specific for NK cell differentiation antigens, and CD3- NK1.1+ cells expressing (A) NKp46, (B) FasL, (C) CD1d (D) TRAIL, (E) CD49b and (F) CD107a were identified by FACS analysis. Blue lines present results obtained with cells from uninfected animals and red lines present results obtained with cells from *T. brucei* Antat1.1E (WT) infected animals and green lines present results obtained from PLC+ *T. brucei* (PLC+) infected animals. Results are representative of 3 identical experiments.
Fig 4.8 *T. brucei* infection-induced changes in NK cell differentiation antigen expression. Splenocytes from naïve (blue lines) CD1d\(^{-}\) mice and mice infected 10 days earlier with *T. brucei* Antat1.1E (red lines) or PLC\(^{-}\) *T. brucei* (green lines) were stained with mAb specific for NK cell differentiation antigens, and CD3\(^{-}\)NK1.1\(^{+}\) cells expressing (A) NKp46, (B) CD49b, (C) FasL, (D) CD107a were identified by FACS analysis.
Fig 4.9 *T. brucei* infection-induced changes in Class I MHC, LY49 antigen expression. Splenocytes from naïve (blue lines) CD1d<sup>+</sup> (A-C) or C57BL/6 (D-E) mice and mice infected 10 days earlier with *T. brucei* Antat1.1E (red lines) or PLC<sup>−/−</sup> *T. brucei* (green lines) were stained with mAb specific for MHC class I or NK cell differentiation antigens, IgM<sup>+</sup> cells expressing (A) MHC-I, or CD3<sup>−</sup> NK1.1<sup>+</sup> cells expressing (B, D) LY49F/E/C/H, (C, E) LY49H were identified by FACS analysis.
Fig 4.10  Expression of 2B4, CD160 on NK cells and CD48 on B cells during infection with intact or PLC−/− T. brucei of intact or CD1d−/− C57BL/6 mice. Splenocytes from naïve (blue lines) C57BL/6 (labeled C57) (A-B, D) or CD1d−/− (labeled CD1d−/−)(C) mice and mice infected with T. brucei Antat1.1E (red lines) or PLC−/− T. brucei (green lines) were stained with mAb specific for cell differentiation antigens, NK cells expressing (A) CD160, or (B-C)2B4 and B cells expressing (D) CD48 were identified by FACS analysis.
Figure 4.11 Splenic B2 B cells are not depleted from PLC⁻/⁻ infected C57BL/6 mice injected with lysate from T. brucei AnTat1.1E. Naïve or PLC⁻/⁻ infected C57Bl/6 mice injected 3 times (on days 6, 7 and 8 post infection) with whole cell lysate of 1x10⁸ *T. brucei* AnTat 1.1E (prepared in the presence of protease inhibitors) and T cell populations analyzed using multicolour flow cytometry. Data are presented as mean number of (A-C) transitional type 1, 2 and 3 respectively, (D) marginal zone and (E) follicular B cells/spleen+/−1 standard deviation. Significance (*<0.05, **<0.001, ***<0.0001) is determined using one way ANOVA and Tukey’s HSD test comparing Sham injected controls to lysate injected individuals.
Studies from the Magez and Black laboratories, on which I was a co-author (see appendix), had shown that *Trypanosoma brucei* infection induced immunosuppression in the C57BL/6 model of African Trypanosomiasis results from the depletion of developing and mature B2 B cells. My studies were aimed at elucidating the mechanism of B2 B cell depletion, and in the process of answering this question I showed that splenic CD8+ T cells were also depleted (chapter 2). To gain insights into possible effector mechanisms I first performed *T. brucei* infections in strains of C57BL/6 mice lacking genes encoding components of apoptotic cell death pathways (TNF receptor), cytotoxic cell effector molecules (C3, perforin), receptor molecules (TCR, MyD88, TRIF) and antigen presenting molecules (CD1d, beta-2 microglobulin). It was hoped that one or other gene deletion would prevent infection-induced deletion of the B cells, although it was realized that redundant pathways might preclude this.

In fact, as shown in Chapter 1 of this thesis, only one of the knock out models used gave an indication of the mechanism responsible for cell depletion in the infected mice. I discovered that perforin gene expression is required for depletion of both splenic B2 B cells as well as splenic CD8+ T cells (chapter 2). Infection of TCR deficient mice and CD1d deficient mice ruled out an essential role for T cells and natural killer T cells in B2 B cell depletion. Finally, treatment with monoclonal antibody that kills NK cells as well as NKT cells revealed that infection induced deletion of B2 B cells in C57BL/6 mice requires these cells. Thus, NK cell mediated cytotoxicity is most likely responsible for splenic B and CD8 T cell depletion in the *T. brucei* infected mice. This proved to have substantial biological consequence, because mice that were unable to mount
cell mediated cytotoxic responses, i.e., in perforin−/− mice, had a superior capacity to control infection with *T. brucei*. They survived for at least twice as long as similarly infected intact mice, retained splenic B2 B cells and CD8 T cells, generated high titer IgG1 and IgG2a antibody responses against trypanosome antigens, which infected intact mice were unable to do, and control parasitemia effectively up to the time studies were terminated because the infected mice developed gut hemorrhages of unknown etiology.

My studies also showed that natural killer cells in *T. brucei* infected mice take on a novel phenotype which shares qualities with both activated natural killer cells and with immature natural killer cells. The activating receptor NKp46 and the cell adhesion molecule CD49b (integrin alpha 2 subunit) are both markers of mature natural killer cells; both are lacking on natural killer cells by day 10 post infection with *T. brucei* when B2 B cells and CD8+ T cells are deleted. Yet Fas ligand is upregulated suggesting an activated state. The non-classical MHC protein CD1d is also upregulated on natural killer cells during infection while it remains unchanged on both B and T cells, while 2B4, the receptor for the B cell blast antigen CD48 is similarly expressed on naïve and activated NK cells and there is little change in expression of its ligand on naïve and infection activated B cells. Importantly, these natural killer cells are acting effector cells as indicated by the presence of the cytotoxic granule marker CD107a on their surface membrane.

Using parasites which lack the only known Trypanosome virulence factor, GPI-PLC, I observed in Chapter 3 that infection induced loss of splenic B2 B cells and CD8+ T cells in C57BL/6 mice requires expression by the *T. brucei* of VSG-GPI specific PLC (GPI-PLC). Paradoxically, I also observed that GPI-PLC−/− *T. brucei* were highly virulent in CD1d−/− C57BL/6 mice, indicating that virulence of *T. brucei* is not determined by GPI-PLC alone, but is also regulated by host CD1d. Interestingly, during infection of intact C57BL/6 mice with GPI-PLC−/− *T. brucei*, i.e., a combination in which the infection is relatively avirulent, NK cells were found to acquire most
but not all of the infection-induced phenotypic markers seen in mice infected with virulent intact
*Trypanosoma brucei*. In both cases the NK cells lacked expression of NKp46 and CD49b, and expressed high
levels of FasL and CD1d. However, whereas NK cells in mice infected with intact *T. brucei* has
a high level of surface CD107a indicative of cytotoxic granule degranulation, the NK cells in
intact mice infected with GPI-PLC⁻*T. brucei* did not have surface CD107a and thus had not
degranulated⁻.

What is activating NK cells to degranulate and kill splenic B2 B cells and CD8⁺ cells during
infection with intact *T. brucei* but not during infection with GPI-PLC⁺*T. brucei*? GPI-PLC
removes the lipid moiety of the *T. brucei* VSG-GPI anchor by hydrolyzing the phosphodiester
bond of phosphatidylinositol to form a free 1, 2-diacylglycerol and glycopeptide-bound inositol
cyclic-1, 2-phosphate. In the absence of GPI-PLC trypanosomes are unable to cleave the GPI-
anchored VSG. This presumably leaves the intact GPI available for processing by cells that
phagocytose antibody and complement coated trypanosomes and ultimately presentation of the
glycan-lipid moiety of GPI on the MHC class 1 like molecule CD1d. Because GPI-PLC⁻*T.
brucei* are avirulent in intact C57BL/6 mice but virulent in CD1d⁺ C57BL/6 mice it is reasonable
to hypothesize that CD1d-loaded with the lipoglycan from VSG-GPI provides a negative signal to
responding NK cells preventing these cells from degranulation. If so, infection induced NK cells
would be expected to express CD160 which is the receptor for CD1d, while B2 B cells and CD8⁺
T cells in the infected mice would be expected to express CD1d and to take up and process VSG-
GPI. While these predictions were not exhaustively studied, initial studies make it rather unlikely
that the hypothesis is correct.

I found that expression of CD1d on B2 B cells and CD8 T cells did not change in C57BL/6 mice
infected with intact *T. brucei*. Rather, it is the NK cells that upregulate CD1d expression during
infection and this occurs whether the mice are infected with intact or GPI-PLC⁻*T. brucei*. In
addition, expression of CD160, which is the receptor for CD1d did not increase on the NK cells
of infected mice. How then could CD1d on NK cells negatively regulate their degranulation in mice infected with GPI-PLC\(^{-/-}\) \textit{T. brucei}, while having little or no effect on their degranulation in mice infected with intact \textit{T. brucei}? Given that NKT cells respond to lipoglycan presented on CD1d, it could be argued that these cells somehow contribute to regulating NK cell degranulation. However, because intact \textit{T. brucei} and GPI-PLC\(^{-/-}\) \textit{T. brucei} retain their virulence profiles in intact mice and mice lacking all T cells, this possibility is excluded. This line of reasoning leads me to propose that CD1d on the \textit{T. brucei} infection induced NK cells serves directly as a signaling molecule that regulates degranulation and is inhibited by VSG-GPI. How this would work is unclear at this time. However, there is precedent that CD1d can serve as a signaling molecule, at least on intestinal epithelial cells where cross-linking of CD1d causes the production of the anti-inflammatory cytokine, IL-10, which is protective against inflammatory bowel disease. [216,217].

In Chapter 2 we show that toll like receptors potentially play an important role in the deletion of CD8\(^{+}\) T cells by NK cells in mice infected with intact \textit{T. brucei} (though not so in the case of B2 B cell deletion). I found that \textit{T. brucei} infected MyD88\(^{-/-}\) and TRIF\(^{-/-}\) mice do not have diminished splenic CD8\(^{+}\) T cell numbers. However, both uninfected MyD88\(^{-/-}\) and TRIF\(^{-/-}\) mice had very low splenic T cell numbers (less than half the numbers of total T cells, about a fifth the number of CD4\(^{+}\), and less than half the numbers of CD8\(^{+}\)) which increased during infection nearly to numbers seen in uninfected intact C57BL/6 mice. The low numbers of T cells in resting MyD88\(^{-/-}\) and TRIF\(^{-/-}\) mice is problematic when trying to draw conclusions on the data showing an increased number of CD8\(^{+}\) T cells during infection of these animals, but the fact that CD8\(^{+}\) cells are able to proliferate without deletion by natural killer cells does suggest a role for toll like receptors in \textit{T. brucei} induced CD8\(^{+}\) cell depletion. The role of MyD88 during infection is complex; while on the one hand MyD88 plays an important role in controlling \textit{T. brucei} parasitemia[218], on the other hand MyD88 has been shown to facilitate T cell and \textit{T. brucei}
infiltration across the blood brain barrier[218] and progression to meningoencephalitic stage of infection, thus contributing to virulence. It is unclear whether MyD88 regulates expression of an NK ligand on CD8\(^+\) T cells or a receptor for a CD8+ T cell ligand on the NK cells.

To summarize, we have shown that B2 B cell deletion during infection of C57BL/6 mice requires and most likely is mediated by a novel population of natural killer cells through a perforin dependent mechanism as is depletion of CD8 T cells. NK cell dependent killing of B2 B cells and CD8 T cells during *T. brucei* infection is dependent on factors both within the host as well as the parasite. Trypanosome GPI-PLC plays a critical role in parasite virulence, host ability to control parasitemia, survival and in the induction of natural killer cells which target splenic B2 B cells and CD8 T cells during infection. The presence of host CD1d molecule is also critical for low virulence phenotype of infection with PLC deficient parasites. In addition, MyD88 expression by the host is necessary for infection induced depletion of CD8 T cells but not B2 B cells. Thus, in answering the question of how B2 B cells are depleted in *T. brucei* infected mice, I have opened many new questions, notably: (i) how are the novel NK cells induced, (ii) do they, as seems likely from my studies, directly kill B2 B cells and CD8 T cells, (iii) if so what receptor and ligand interactions are involved and what are the roles of *T. brucei* GPI-PLC and host CD1d in regulating NK cell cytotoxic activity and (iv) is this apparently novel method of immunosuppression restricted to infected mice or a feature of acute trypanosomiasis and other diseases in other host species.

**Future Directions**

It will be important to know whether natural killer cells which arise during infection originate in the spleen or emigrate from elsewhere. Currently, it is unknown whether resident natural killer cells are stimulated by infection to differentiate into the NK1.1\(^+\), CD3\(^-\), NKp46\(^-\), CD49\(^-\), CD1d\(^+\)
natural killer cells we observe, whether these cells differentiate from a small population of progenitors which is resident in the spleen or elsewhere, or whether this population migrates into the spleen from another organ such as the liver, lung, intestine or bone marrow.

Currently studies are underway to determine whether natural killer cells taken from an intact mouse will be induced to differentiate into the B2 B cell killing cells (NK1.1<sup>+</sup>, CD3<sup>-</sup>, NKp46<sup>-</sup>, CD49<sup>-</sup>, CD1d<sup>+</sup>, CD107a<sup>+</sup>) in perforin knockout mice, and if they will indeed be induced to kill in the context of infection within perforin deficient animals. If the putative (*T. brucei* induced) B cell (and CD8<sup>+</sup> T cell) killer NK cells are resident splenic natural killer cells we would expect perforin<sup>+</sup> cells to differentiate and B2 B cells (and CD8<sup>+</sup> T cells) to be deleted in the *T. brucei* infected perforin<sup>-/-</sup> animal. If the donor natural killer cells do not differentiate into the novel infection-induced NK cells, this would suggest the possibility that the differentiated cells we see during infection are emigrating to the spleen from another tissue or are arising from a resident precursor cell.

Secondly, it will be beneficial to know what signaling pathway(s), are involved in both the differentiation and activation to effector function (killing). Given my data showing that under condition of a PLC<sup>-/-</sup> infection natural killer cells are differentiated into the activated, immature phenotype described above, but are not killing, the logical conclusion is that there are two mechanisms involved, one mechanism which induces natural killer cell differentiation, and a second which is the trigger for cytotoxic effector function. We have preliminary data which suggest that soluble factors in the serum of infected mice are enough to induce the appearance of *T. brucei* induced natural killer cell phenotype but not killing of B cells or CD8<sup>+</sup> T cells (unpublished results R. Hillard and S. J. Black). Also, it would be beneficial to these studies to show natural killer cell killing through cytotoxic assay as well as visualization of synapse with putative target cells. This poses a challenge as it has been established that natural killer cells from *T. brucei* infected mice do not kill yac-1 cells ex vivo and we have preliminary data that they also
do not kill resting splenic B2 B cells. Studies are on-going to determine if they kill myeloma B2 B cells, LPS activated B cells, and B cells from intact C57BL/6 mice infected with GPI-PLC$^{+/-}$ $T. brucei$. It would be helpful if we can identify a myeloma cell target as this could be genetically manipulated to investigate the relevance of expressed genes to killing, which might facilitate ligand identification.

It will also be important to know what receptors are expressed by the novel NK cells that arise in $T. brucei$ infected mice. So far we have approached this question by hypothesis and test, but a more discovery-based approach is warranted. Insights into relevant receptors, i.e., receptors that mediate the recognition of B2 B cells and CD8 T cells might be gained by characterization of the infection-induced NK cell plasma membrane proteome, and proteins associated with lipid rafts which are known to harbor several NK receptors including 2B4. It will also be important to identify and compare splenic NK cell plasma membrane antigens and associated proteins that are phosphorylated on serine/threonine and tyrosine residues in intact C57BL/6 mice infected with intact $T. brucei$ compared to GPI-PLC$^{+/-}$ $T. brucei$, and in intact mice infected with GPI-PLC$^{+/-}$ $T. brucei$ compared to CD1d$^{+/-}$ mice infected with the same parasites. These latter comparisons are expected to inform on signaling proteins that regulate cytotoxic granule degranulation.

It will be important to show presence (or absence) of intact GPI presentation during infection with PLC$^{+/-}$ $T. brucei$ infection versus antigen presentation during infection with intact $T. brucei$. It is known that endogenous GPI is a natural ligand for CD1d[219] and that CD1d is capable of binding Trypanosome GPI[220]. We will design experiments to assess GPI and VSG antigen presentation during infection. $T. brucei$ bloodstream form are unique in GPI formation in that they use a fatty acid remodeling in which GPI intermediates are replaced with myristic acid(C14). This can be exploited because trypanosomes grown in the presence of synthetically labeled C14 will then have the label intercalated into their GPI. This would allow us to assess whether GPI from PLC$^{+/-}$ $T. brucei$ is processed by NK cells and is then associated with CD1d on
NK cells. To compare presentation of mVSG and sVSG from PLC−/− parasites versus from intact *T. brucei* metabolic labeling of carbohydrate moieties would also be necessary.

Finally, Trypanosomiasis is socially and economically important as a disease which affects humans and cattle. Infection in human and trypanosusceptible cattle are also characterized by profound immune suppression [133,221,222]. Infected Zebu cattle have low antibody titer to vaccine antigens, and *T. congolense* infected cattle have depressed production of antibody[223] and 117 human subjects infected with Human African Trypanosomiasis have been described as having a lowered antibody titer to vaccine antigen after suffering trypanosomiasis [102], though the authors assumed that this reduced titer would still be protective. It would be important to assess whether susceptible cattle suffer the same loss of splenic B2 B cells and CD8+ T cells and importantly, whether natural killer cells differentiate (or arise) which have a similar phenotype to the natural killer cells in mice and whether natural killer cells play a role in pathology in cattle.

There is little known about the role of natural killer cells outside of the context of cancer and viral infection [224] and up until this point there has been little attention into the role of natural killer cells during trypanosome infections[225]. Future studies into the role of natural killer cells in the pathogenicity of trypanosomiasis could lead to a better understanding of not only the diseases caused by African Trypanosomes, but potential could lead to insights into other protozoan infections, and immune regulation.
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