Effects of Toxoplasma gondii Infection on NK Cells and ILC1s

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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
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Effects of *Toxoplasma gondii* Infection on NK Cells and ILC1s
by
Eugene Park

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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# Table of Contents

List of Figures ........................................................................................................ iv
List of Tables ........................................................................................................... vi
Acknowledgments ................................................................................................... vii
Abstract ................................................................................................................ ix

Chapter 1: Introduction .......................................................................................... 1
  1.1 Conventional Natural Killer Cells ................................................................. 1
  1.2 NK Cell Memory ............................................................................................. 3
  1.3 NK Cell Heterogeneity .................................................................................. 5
  1.4 Innate Lymphoid Cells ................................................................................... 6
  1.5 Comparison of NK Cells and Type I ILCs .................................................... 7
  1.6 NK Cells and ILC1s in *Toxoplasma gondii* Infection .................................. 8

Chapter 2: Characterization of NK Cells and ILC1s in *T. gondii* Infection ........ 9
  2.1 Protective Effects ........................................................................................... 9
  2.2 Phenotype of Splenic Cells ......................................................................... 11
  2.3 RNA Sequencing Analysis ........................................................................... 11
  2.4 Anatomical Distribution .............................................................................. 13
  2.5 Epigenomic Analysis .................................................................................... 15

Chapter 3: Development of *T. gondii*-Induced ILC1s ........................................ 44
  3.1 Role of Ongoing Infection ............................................................................ 44
  4.2 Effect of Tbet Deficiency ............................................................................. 45
  4.3 Requirement of Eomes ................................................................................. 47
  4.4 Cell-Extrinsic Factors ................................................................................. 48

Chapter 4: Discussion .............................................................................................. 69
  4.1 Overview ...................................................................................................... 69
  4.2 Significance .................................................................................................. 70
  4.3 Future Directions ......................................................................................... 73

Chapter 5: Methods ................................................................................................. 76
  5.1 *Toxoplasma gondii* Infection ...................................................................... 76
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2 Mice</td>
<td>77</td>
</tr>
<tr>
<td>5.3 Luciferase Imaging</td>
<td>77</td>
</tr>
<tr>
<td>5.4 Cell Isolation</td>
<td>77</td>
</tr>
<tr>
<td>5.5 Flow Cytometry and Cell Sorting</td>
<td>78</td>
</tr>
<tr>
<td>5.6 Stimulations and Intracellular Staining</td>
<td>79</td>
</tr>
<tr>
<td>5.7 Generation of Bone Marrow Chimeras</td>
<td>80</td>
</tr>
<tr>
<td>5.8 RNA Sequencing</td>
<td>80</td>
</tr>
<tr>
<td>5.9 ATAC Sequencing</td>
<td>81</td>
</tr>
<tr>
<td>5.10 Single-cell RNA Sequencing</td>
<td>82</td>
</tr>
<tr>
<td>5.11 Quantification and Statistical Analysis</td>
<td>83</td>
</tr>
<tr>
<td>References</td>
<td>84</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1: Effect of α-NK1.1 treatment on *T. gondii* parasite burden ........................................ 17
Figure 2: Effect of α-NK1.1 treatment on survival following *T. gondii* infection ......................... 18
Figure 3: Quantification of cNK cells and ILC1s after *T. gondii* infection ................................. 19
Figure 4: Quantification of cNK cells and ILC1s 4 months after *T. gondii* infection .................. 20
Figure 5: Expression of cNK cell maturation markers ............................................................. 21
Figure 6: Surface markers differentially expressed by cNK cells and ILC1s ................................. 22
Figure 7: Expression of Ly49 receptors ...................................................................................... 23
Figure 8: Cytokine production by cNK cells and ILC1s ............................................................ 24
Figure 9: Expression of Ly6C by cNK cells and ILC1s .............................................................. 25
Figure 10: Gating strategy to sort cNK cells and *T. gondii*-induced ILC1s ............................... 26
Figure 11: RNA-seq of cNK cells and ILC1s from *T. gondii*-infected mice ............................... 27
Figure 12: Gene Set Enrichment Analysis for signature ILC1 and NK cell genes ......................... 28
Figure 13: Heatmaps of differentially expressed genes .............................................................. 29
Figure 14: Gene Set Enrichment Analysis for signature genes of tissue-residency and circulation .................................................................................................................. 30
Figure 15: Expression of chemokine receptors and adhesion molecules ..................................... 31
Figure 16: *T. gondii*-induced ILC1s in additional organs ................................................................. 32
Figure 17: Organs that do not increase in *T. gondii*-induced ILC1s ........................................... 33
Figure 18: Ly6C expression by ILC1s in *T. gondii*-infected mice .................................................. 34
Figure 19: Homing of NK cells and ILC1s in lymphopenic recipients .......................................... 35
Figure 20: Epigenomic Differences in cNK cells and *T. gondii*-induced ILC1s ......................... 36
Figure 21: *Eomes* and *Tbx21* loci ......................................................................................... 37
Figure 22: Features of differential regulatory elements ................................................................. 38
Figure 23: Comparison of regulatory element accessibility and gene transcription ..................... 39
Figure 24: Effect of sulfadiazine treatment on development of *T. gondii*-induced ILC1s

Figure 25: Effect of *Cps1*-1 *T. gondii* on development of *T. gondii*-induced ILC1s

Figure 26: Effect of deficient bradyzoite formation on development of *T. gondii*-induced ILC1s

Figure 27: Effect of *T. gondii* infection on *T. gondii*-induced ILC1 development in BALB/c mice

Figure 28: Tbet expression and requirement in ILC1s

Figure 29: Eomes<sup>+</sup> CD49a<sup>+</sup> cells develop from wild-type and *Tbx21<sup>−/−</sup>* NK cells

Figure 30: Development of Eomes<sup>+</sup> CD49a<sup>+</sup> cells in the liver during *T. gondii* infection

Figure 31: Clusters identified by single-cell RNA-seq analysis

Figure 32: Gene expression by distinct clusters

Figure 33: Quantification of NK cell and ILC1 populations in the liver by flow cytometry

Figure 34: Expression of signature ILC1 and cNK cell genes by *T. gondii*-induced ILC1 subpopulations

Figure 35: Characterization *T. gondii*-induced ILC1 subpopulations

Figure 36: Lack of NK cells in *Ncr1<sup>Cre<sup> Eomes<sup>f/f</sup>* mice

Figure 37: Impaired development *T. gondii*-induced ILC1s in *Ncr1<sup>Cre<sup> Eomes<sup>f/f<sup>* mice

Figure 38: Cell intrinsic role of Eomes in development of *T. gondii*-induced ILC1s

Figure 39: Cell intrinsic role of STAT4 in development of *T. gondii*-induced ILC1s

Figure 40: Effect of IL-12 on NK1.1<sup>+</sup> NKp46<sup>+</sup> cells in vivo

Figure 41: Effect of IL-12 on sorted cNK cells and ILC1s

Figure 42: Effect of TGF-b signaling on development of *T. gondii*-induced ILC1s
List of Tables

Table 1: Signature ILC1 gene set .......................................................... 40
Table 2: Signature NK cell gene set ...................................................... 41
Table 3: Signature tissue-residency gene set ........................................ 42
Table 4: Signature circulating gene set .................................................. 43
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ABSTRACT OF THE DISSERTATION

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by

Eugene Park

Doctor of Philosophy in Biology and Biomedical Sciences

Immunology

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Professor Wayne Yokoyama, Chair

Natural killer cells and Type I innate lymphoid cells (ILCs) are subsets of ILCs. In C57BL/6 mice, they share expression of the surface markers NK1.1 and NKp46, and can produce the cytokine interferon-γ. These similarities led to the initial classification of natural killer cells and Type I ILCs together under the category of Group 1 ILCs. However, more recent studies found that natural killer cells and ILC1s develop from distinct progenitor cells and utilize transcription factor in distinct manners. Whereas ILC1s require Tbet for their development and are Eomes-independent, natural killer cells require Tbet only for terminal maturation and are Eomes-dependent. As such, these populations were reclassified as separate ILC subsets. In the context of *Toxoplasma gondii* infection, we identified a new population that blurs these strict delineations. Our data suggest that *T. gondii* induces the development of ILC1-like cells that primarily result from the downregulation of Eomes and the upregulation of Tbet. We further validated these findings with epigenomic profiling and single-cell RNA sequencing.
Chapter 1: Introduction

1.1 Conventional Natural Killer Cells

Conventional natural killer (cNK) cells are innate cells, relying on signals through germline-encoded receptors to discern healthy cells from infected or transformed cells. Although cNK cells develop from the common lymphoid progenitor (CLP), like B and T lymphocytes, they are unique in being effective without priming, possessing preformed granules containing perforin and granzymes. This allows cNK cells to mount swift responses early in infection, keeping pathogens at bay while adaptive immune cells activate and expand (Yokoyama, 2013).

Conventionally, cNK cells arise from CLPs in the bone marrow (Rosmaraki et al., 2001). This process relies on IL-15 (Kennedy et al., 2000), which induces expression of transcription factors that regulate cNK cell development. Among these transcription factors are Ets-1, Pu.1, and Id2, which are shared across multiple hematopoietic lineages. In contrast, Nfil3 uniquely specifies the cNK cell lineage, as revealed by Nfil3-deficient mice, which lack peripheral cNK cells but maintain normal numbers of other immune cell types (Gascoyne et al., 2009). In addition, Tbet deficiency results in arrested cNK cell maturation and reduction of peripheral cNK cells (Gordon et al., 2012; Townsend et al., 2004). Eomesodermin (Eomes), like Tbet, is a T-box transcription factor that drives cNK cell development, and the deletion of Eomes within the lymphoid lineage results in severely reduced cNK cell numbers and complete absence of mature cNK cells.
In addition to transcription factor expression, the canonical model of cNK cell development also corresponds to the expression of surface markers. Early in development, cNK cells gain expression of NK1.1 (in C57BL/6 mice) and various activating and inhibitory receptors (Kim et al., 2002). Terminally mature cNK cells express the α2 integrin (i.e. CD49b/DX5). Additionally, cNK cell maturation can be followed based on expression of CD27 and CD11b, where cNK cells begin as double-negative, and progress through CD27+ CD11b−, CD27+ CD11b+, and CD27− CD11b+ stages as they differentiate (Chiossone et al., 2009).

For many years the major focus of cNK cell studies has been on their roles during early immune processes. In Murine cytomegalovirus (MCMV) infection, cNK cells target infected cells through the interaction between the activating NK cell receptor Ly49H and the viral protein m157 expressed on the surface of infected cells (Arase et al., 2002; Brown et al., 2001; Smith et al., 2002). cNK cells can also lyse tumor cells due to their recognition of "missing self," wherein inhibitory receptors lose inhibitory signaling when their ligands are downregulated on target cells.

cNK cell activity is usually insufficient for complete control of viral infection or tumorigenesis, and collaboration with adaptive lymphocytes is often required. In MCMV infection, for instance, infection of Rag1−/− mice, in which cNK cells are intact but adaptive lymphocytes are absent, leads to recrudescence of infection due to mutations in MCMV that allow for evasion of cNK cell recognition (French et al., 2004). Thus, development of an adaptive immune response is necessary for identification of escape mutants and viral elimination, exemplifying the principle
that cNK cells are critical early in immune processes, while adaptive lymphocytes are often required during later responses. However, cNK cells may also contribute to late immune responses and though this possibility remains largely unexplored.

1.2 NK Cell Memory

cNK cells were once thought to become activated in response to stimuli and return to a basal resting state after stimulation subsides. However, recent studies have shown that cNK cells can adopt long-lasting alterations that accompany functional changes. Whereas initial study of MCMV infection focused on the role of cNK cells during the first week of infection (Arase et al., 2002; Brown et al., 2001; Smith et al., 2002), more recently, studies have expounded upon these findings to unearth adaptations that cNK cells undertake even after MCMV clearance. Following clonal expansion and contraction of Ly49H+ cNK cells, MCMV-experienced Ly49H+ cNK cells persist, rapidly expand upon challenge, and confer better protection compared to naïve cNK cells (Sun et al., 2009a). IL-12 signaling during acute infection is thought to be crucial in endowing cNK cells with the capacity for memory (Sun et al., 2012).

Phenotypically, MCMV-induced memory cNK cells resemble naïve cNK cells. Examination of cNK cells at various time points post-infection by microarray showed that memory cNK cells have very few transcriptional changes, relative to the Ly49H+ effector cNK cells at 7 dpi. Indeed, when Ly49H+ cNK cells were adoptively transferred from wild-type mice into Ly49H-deficient recipients, challenged with MCMV, and sorted for microarray 27 dpi, CD49a, Ly6C, and CD11b were the only differentially expressed surface markers (Bezman et al., 2012). However, subtle transcriptional differences belied indelible imprints on the chromatin landscape. Analysis of memory cNK cells by the assay by the assay for accessibility for transposase-accessible
chromatin using sequencing (ATAC-seq) showed unique patterns of chromatin accessibility in memory cNK cells, some of which bear semblance to those of memory CD8\(^+\) T cells (Lau et al., 2018). Motif binding analysis also showed enrichment of distinct transcription factor binding sites in differentially accessible regions, and in particular, loss of NK-\(\kappa\)b binding motifs in memory cNK cells.

In humans, clonal expansion of cNK cell is thought to occur following Human cytomegalovirus (HCMV) infection, as HCMV seropositivity correlates with an increased frequency of NKG2C\(^+\) cNK cells (Guma et al., 2004; Guma et al., 2006). In these individuals, NKG2C\(^+\) cNK cells often have additional distinctions, such as lack of FcR\(\gamma\) and alterations in signaling molecules. Like MCMV-induced memory cNK cells in mice, these FcR\(\gamma\)– cNK cells (g–NK cells) have striking differences in chromatin accessibility and DNA methylation, which correspond to transcriptional differences (Lee et al., 2015; Schlums et al., 2015). Functionally, these memory-like cNK cells display enhanced expansion in the presence of HCMV infected cells and autologous plasma, compared to FcR\(\gamma\)\(^+\) NK cells, suggesting these cNK cells may confer protection in conjunction with antibodies.

An \textit{in vitro} corollary of memory NK cells shows that pre-activation of cNK cells with IL-12 and IL-18 results in the development of memory-like NK cells, which are long-lived and possess an enhanced ability to produce IFN\(\gamma\) even months later (Cooper et al., 2009). This finding has been mirrored in recent studies in humans that showed that pre-activation with cytokines resulted in enhanced tumoricidal capacity and therapeutic benefits in patients with acute myeloid leukemia.
Both MCMV-induced memory NK cells and cytokine-induced memory-like NK cells require IL-12 signaling to adapt long-lasting functional changes (Sun et al., 2012).

1.3 NK Cell Heterogeneity
Interestingly, examination of cNK cells under steady state conditions does not always predict behavior under stimulation. For instance, cNK cell developmental requirements change during MCMV. Whereas IL-15 signaling through IL-15 receptor is required for cNK cell development in steady state, it may be dispensable during MCMV infection as Ly49H$^+$ NK cells expand in Il15ra$^{-/-}$, Il15$^{-/-}$, and Rag2$^{-/-}$ I2rg$^{-/-}$ mice (Sun et al., 2009b). Moreover, while Nfil3 is normally required for development of cNK cells, Ly49H$^+$ NK cells expand in Nfil3-deficient mice during MCMV infection (Firth et al., 2013). Lastly, inducible deletion of Tbet and Eomes reveal differing roles of these T-box transcription factors as well. Whereas Eomes is required for NK cell development, its deletion only impairs expansion during acute MCMV infection, but does not affect naïve or memory NK cell maintenance. On the other hand, Tbet was found to be required for both NK cell expansion during MCMV infection, as well as memory NK cell maintenance at later time points (Madera et al., 2018).

Additional instances of NK cell heterogeneity have been uncovered under steady state conditions. The majority of NK cells circulate through the bloodstream and these circulating cNK cells were once considered the predominant NK cell population. However, parabiosis studies have shown there are additional tissue-restricted populations that differ in their phenotype and transcription factor dependence. Under steady state conditions, DX5 and CD49a expression is mutually exclusive and denotes circulating and tissue-resident NK cells, respectively (Peng et
al., 2013). Populations of tissue-resident NK cells exist in the salivary gland and uterus, where they outnumber cNK cells (Cortez et al., 2016). Interestingly, these populations are Nfil3-independent, unlike cNK cells (Cortez et al., 2014; Sojka et al., 2014).

### 1.4 Innate Lymphoid Cells

NK cells were once thought to be the predominant innate lymphocyte population. However, in the last decade, additional innate lymphocyte subsets that also develop independently of antigen receptor rearrangement and are present in Rag-deficient mice have been identified. These additional innate lymphocyte populations widely differ in development, phenotype, and distribution. Their discovery has resulted in reframing cNK cells within the broader context of innate lymphoid cells (ILCs).

Apart from cNK cells, ILCs display tissue-residency within mucosal sites and require signaling through the IL-7 receptor for development. Initially, ILCs were classified into three major categories based on their cytokine production and transcription factor dependence, which mirrored CD4+ T cell subsets (Spits et al., 2013). Group 1 ILCs encompassed NK cells and ILC1s, which both produce IFNγ. Group 2 were defined as cells that produce IL-5 and IL-13. Group 3 ILCs encompassed lymphoid tissue inducer (LTi) cells and ILC3s, which could produce IL-17 or IL-22. These distinct cytokine production profiles are thought to underlie distinct functionality of these cells, which occupy different tissues and are thought to be specialized for their own microenvironments.
1.5 Comparison of NK Cells and Type I ILCs

NK cells and ILC1s were once classified together as Group 1 ILCs because they express Tbet, produce IFNγ, and identifiable based on co-expression of NK1.1 and NKp46. However, they were recently reorganized as distinct subpopulations of ILCs, owing to recent observations that their developmental paths diverge downstream of the CLP (Vivier et al., 2018). NK cells develop from the NK Precursor (NKP) while other ILCs arise from the common helper ILC precursor which further differentiates into the ILC precursor (ILCp) (Constantinides et al., 2014; Klose et al., 2014; Rosmaraki et al., 2001). In contrast to NK cells, ILC1s are entirely Tbet-dependent and develop uninterrupted in the absence of Eomes (Daussy et al., 2014; Sojka et al., 2014).

In additional to developmental differences, NK cells and ILC1s are found at distinct anatomical sites. ILC1s are thought to be tissue-resident, as parabiosis studies show they are retained within organs such the liver and small intestine without recirculating (Gasteiger et al., 2015; Sojka et al., 2014). Like tissue-resident NK cells, ILC1s express CD49a. In addition, ILC1s can produce TNFα in addition to IFNγ, while NK cells only produce IFNγ.

The function of ILC1s remains unclear, but work thus far has focused on their contributions in clearing acute infection and limiting tumor formation, much like cNK cells. Like cNK cells, T cell receptor expressing ILC1-like cells can lyse tumor cells through the release of cytotoxic granules (Dadi et al., 2016). Moreover, in MCMV infection, liver ILC1s have the potential to limit early viral spread by producing IFNγ (Weizman et al., 2017). Furthermore, in Toxoplasma
*Toxoplasma gondii* infection, both cNK cells and ILC1s produce IFNγ to stimulate inflammation (Goldszmid et al., 2012; Klose et al., 2014).

**1.6 NK Cells and ILC1s in *Toxoplasma gondii* Infection**

*Toxoplasma gondii* is an intracellular parasite that infects a third of the global population (Montoya and Liesenfeld, 2004). Its definitive hosts are cats, which can transmit infectious *T. gondii* oocysts through their feces into the environment (Dubey and Frenkel, 1972). These oocysts are resistant to environmental conditions and can persist for long periods of time. Most warm-blooded animals are susceptible to infection and can become infected by consuming infected foods. After entering hosts via the digestive tract, *T. gondii* differentiates into rapidly replicating tachyzoites that disseminate systemically (Hunter and Sibley, 2012).

Upon recognition of the parasite, dendritic cells produce IL-12. This cytokine subsequently stimulates cNK cells to produce IFNγ and ILC1s to produce IFNγ and TNFα (Goldszmid et al., 2012; Klose et al., 2014). A strong adaptive response correlates with the clearance of acute infection, and mounting immune pressure eventually causes tachyzoites to differentiate into slowly replicating bradyzoites. The presence of bradyzoites, which are primarily encysted within cells of the central nervous system and skeletal muscle, marks the chronic phase of *T. gondii* infection (Yarovinsky, 2014).
Chapter 2: Characterization of NK Cells and ILC1s in T. gondii Infection

2.1 Protective Effects
Previous analysis of the impact of NK cells in the protection of C57BL/6 mice during infection with T. gondii relied on depletion of NK cells using α-asialoGM1 (Goldszmid et al., 2007), which depletes not only NK cells but also basophils (Nishikado et al., 2011). To eliminate NK1.1-expressing cells specifically, i.e. NK cells and ILC1s, we administered α-NK1.1 to C57BL/6 mice prior to i.p. (intraperitoneal) infection with 200 tachyzoites of the type II Prugniaud (Pru) strain of T. gondii. We used a luciferase-expressing strain, PRU-FLuc-GFP, which allowed for monitoring of parasites in vivo. We observed that infection resulted in increased parasite load in mice treated with α-NK1.1 at 6 dpi (Figure 1). Moreover, half of the α-NK1.1-treated mice died 10-15 dpi (Figure 2), consistent with previous reports.

2.2 Phenotype of Splenic Cells
The acute phase of Type II T. gondii infection in C57BL/6 mice subsides by 3-4 weeks post-infection. Around the same time, adaptive immune cells become activated and CD8+ T cells become major producers of IFNγ. Additionally, NK cells can be depleted 3 wk post-infection without causing increased mortality (data not shown). Regardless, we observed changes among NK1.1+ NKp46+ cells that were maintained even at later time points, suggesting ongoing activation or long-lasting alterations to NK cell homeostasis. We assessed the effect of T. gondii infection on NK1.1+ NKp46+ populations by following expression of Eomes and CD49a, as cNK cells express Eomes and not CD49a, whereas ILC1s do not express Eomes but do express
CD49a. In the spleen under steady state conditions, Eomes$^+$ CD49a$^-$ cNK cells comprise the majority of NK1.1$^+$ NKp46$^-$ cells, while 2-3% are Eomes$^-$ CD49a$^+$, which is the phenotype of ILC1s (Figure 3). Interestingly, over the course of infection, cNK cells decreased both as a proportion of NK1.1$^+$ NKp46$^-$ cells and in absolute number. By contrast, beginning at 21 dpi, there was an increase in Eomes$^-$ CD49a$^+$ ILC1s. Interestingly, which acute *T. gondii* infection unusually resolves within 3-4 weeks, we observed increased numbers of *T. gondii*-induced ILC1s even 4 months post-infection (Figure 4).

The expansion of ILC1-like cells may be due to *T. gondii*-induced expansion of preexisting ILC1s. However, it is also possible that infection induces a novel ILC1-like population, or that the ILC1-like cells are heterogeneous and comprise multiple subpopulations. We further compared the cNK cells and ILC1s in uninfected and mice infected 35 d earlier to determine if differences existed between the ILC1s in uninfected and infected mice. In terms of the maturation markers CD27 and CD11b, the cNK cells in both uninfected and infected mice were distributed across CD27$^+$ CD11b$^-$, CD27$^+$ CD11b$^+$, and CD27$^-$ CD11b$^+$ stages, while ILC1s were predominantly CD27$^+$ CD11b$^-$, as previously reported (Chiossone et al., 2009; Sojka et al., 2014). Surprisingly, the ILC1s in uninfected mice were predominantly CD27$^-$ CD11b$^+$, with a smaller frequency of CD27$^+$ CD11b$^-$ cells (Figure 5).

Flow cytometric analysis also showed that ILC1s from the spleens of infected mice expressed Neuropilin-1 and KLRG1 at higher levels than the other populations, and DNAM-1 and NKG2A more highly than cNK cells (Figure 6). We next assessed expression of Ly49 receptors. We
found that similar to the ILC1s in uninfected mice, the ILC1s from infected mice expressed most canonical NK receptors at lower frequencies than cNK cells (Figure 7). Moreover, there were minor alterations of Ly49 receptor expression between the cNK cells in uninfected and infected mice, suggesting that *T. gondii* infection may alter the antigen recognition properties of NK cells and perhaps cause expansion of certain subpopulations.

To further compare these populations, we assessed their ability to produce cytokines. Following stimulation with IL-12, IL-12 + IL-18, or PMA + Ionomycin, we found that all populations were able to produce IFNγ (Figure 8A). Interestingly, higher frequencies of cNK cells from infected mice produced IFNγ following stimulation. We also assessed TNFα production following PMA + Ionomycin stimulation, which is a hallmark of ILC1s. Whereas ILC1s in uninfected mice produced TNFα, the ILC1s in infected mice did not (Figure 8B).

### 2.3 RNA Sequencing Analysis

To understand how *T. gondii* infection shapes NK1.1+ NKp46+ populations in greater depth, we performed RNA-sequencing (RNA-seq) of the predominant NK1.1+ NKp46+ populations in the uninfected and infected spleen: cNK cells from uninfected mice and cNK cells and *T. gondii*-induced ILC1s from infected mice. We found that Ly6C is not expressed by most ILC1s that are already present in the uninfected spleen, but highly expressed by ILC1s following infection (Figure 9). For RNA-seq analysis, we identified cNK cells as CD49a− Ly6C− cells, which expressed Eomes. Meanwhile, we identified *T. gondii*-induced ILC1s as CD49a+ Ly6C+ cells, which accurately identified Eomes− cells (Figure 10). Principal component analysis and hierarchical clustering showed a higher degree of similarity between cNK cells from uninfected
and infected mice, relative to the *T. gondii*-induced ILC1s, which displayed more variability (Figure 11A-B). As expected, *Ly6c1, Ly6c2, Itga1,* and *Eomes* were differentially expressed (Figure 11C).

The small number of splenic CD49a⁺ ILC1s in uninfected mice precluded the possibility of RNA-seq of this specific population. Instead, we performed gene set enrichment analysis (GSEA) with previously published lists of signature ILC1 genes to determine if *T. gondii*-induced ILC1s possessed core ILC1 traits (Robinette et al., 2015). An ILC1 signature was indeed enriched in *T. gondii*-induced ILC1s relative to cNK cells from infected mice (Figure 12A), with 17 of 53 signature ILC1 genes expressed at higher levels, including *Itga1* and *Cxcr6* (Table 1). Consistently, *T. gondii*-induced ILC1s also expressed 14 of 35 signature cNK cell genes at lower levels compared to cNK cells, including *Eomes* (Figure 12B, Table 2). However, *T. gondii*-induced ILC1s expressed lower levels of the signature ILC1 gene *Tmem154* and higher levels of the signature cNK cell genes *Gpx8, Klrg1,* and *Cdc20b.* Thus, *T. gondii*-induced ILC1s were enriched for the ILC1 signature, but also expressed cNK cell genes, incompletely recapitulating all aspects of either cell type. Overall, the data suggest that *T. gondii*-induced ILC1s possess some characteristics of both cNK cells and ILC1s, but also display unique features.

*T. gondii*-induced ILC1s differed from both cNK cells and steady state ILC1s in their expression of surface markers, transcription factors, secreted factors, adhesion molecules, chemokine receptors, signaling molecules, and NK cell receptors (Figure 13), which the transcriptional profile verifying our flow cytometric analysis above (Figures 6-7). In addition, we observed
striking differences in the expression patterns of transcription factors such as increased expression of *Foxn3*, *Rbpj*, and *Tbx21* and decreased expression of *NfkB1*, *Tox*, and *Batf3* in *T. gondii*-induced ILC1s relative to cNK cells. Cytokine receptor expression also differed, with *T. gondii*-induced ILC1s expressing higher levels of *Tgfbr2*, *Il2ra*, and *Il7ra*. Together these findings suggested the *T. gondii*-induced ILC1s may have unique developmental requirements, as compared to cNK cells.

### 2.4 Anatomical Distribution

In addition to expressing the tissue-residency marker CD49a, *T. gondii*-induced ILC1s expressed high levels of *Zfp683* (Figure 13), a transcription factor that programs tissue-residency (Mackay et al., 2016). GSEA revealed enrichment of tissue-residency genes (Milner et al., 2017) among genes expressed more highly by *T. gondii*-induced ILC1s (Figure 14A, Table 3). However, genes comprising a circulatory signature were not lost (Figure 14B, Table 4). Moreover, RNA-seq showed that *T. gondii*-induced ILC1s differentially expressed chemokine receptors and adhesion molecules compared to cNK cells and ILC1s from uninfected mice (Figure 13). Flow cytometric analysis verified that ILC1s from infected mice expressed CX3CR1, CCR8, CXCR6, and PECAM-1 and did not express CD62L (Figure 15). These features suggested that *T. gondii*-induced ILC1s may exhibit distinct patterns of distribution or migration as compared to cNK cells and steady state ILC1s.

In contrast to ILC1s in uninfected mice, ILC1s were increased in the blood of infected mice, indicating that these cells circulate (Figure 16). Consistently, ILC1s were also increased in the spleen and lung. However, they were unaltered or decreased in the mesenteric lymph node, brain,
peritoneum, bone marrow, uterus, and salivary gland (Figure 17). In the liver, the frequency of ILC1s did not increase (Figure 16), but this was possibly confounded by a sizeable ILC1 population already present at steady state (Peng et al., 2013; Sojka et al., 2014). To assess this in further depth, we examined expression of Ly6C, which is not expressed in the liver under state conditions but is expressed by ILC1s following *T. gondii* infection. Indeed we found that Ly6C was expressed by approximately half of the ILC1s from livers of infected mice, as well as by ILC1s in the blood and lungs of infected mice (Figure 18). Accordingly, the number of Ly6C⁺ ILC1s was increased relative to uninfected mice, while the number of Ly6C⁻ ILC1s was comparable, which suggested that some of the ILC1s that are present during steady state remain unchanged by *T. gondii* infection.

To compare homing of NK cells and ILC1s cells from infected mice, we isolated Eomes-GFP⁺ CD49a⁻ and Eomes-GFP⁻ CD49a⁺ cells from congenically distinct infected Eomes-GFP mice for adoptive transfer into naïve *Rag2²/² Il2rg²/²* mice (Figure 19). When we assessed the recipient mice 24 days later, we detected both populations in the spleen and liver (Figure 19). Although cNK cells upregulated CD49a upon transfer into *Rag2²/² Il2rg²/²* mice, as previously reported (Gao et al., 2017), the Eomes expression generally remained the same, suggesting that *T. gondii*-induced ILC1s may persist independent of infection. Interestingly, while the percentage of transferred ILC1s was higher than transferred cNK cells in the spleen, this percentage was even more skewed in the liver, where ILC1s comprised nearly all transferred cells, suggesting their homing pattern may be altered, even though they circulate in the blood. Nonetheless, overall our data indicated that *T. gondii* induced a population of circulating ILC1s.
2.5 Epigenomic Analysis

Chromatin accessibility patterns differ in cNK cells and ILC1s (Shih et al., 2016), and infection can induce long-lasting epigenetic changes in cNK cells (Lau et al., 2018). To determine if *T. gondii*-induced ILC1s possess a stable chromatin landscape that is distinct from that of cNK cells, we performed ATAC-seq (Buenrostro et al., 2015), comparing splenic cNK cells from uninfected mice and cNK cells and *T. gondii*-induced ILC1s from infected mice. As in the RNA-seq analysis, the accessibility pattern of *T. gondii*-induced ILC1s was distinct from that of the cNK cell populations in naïve and infected mice, which more closely resembled each other (Figures 20A-B). The majority of differentially accessible regulatory elements (REs) were within introns or intergenic regions (Figure 20C), consistent with categorization as putative enhancers. These findings suggested that *T. gondii*-induced ILC1s are a discrete and stable population. To determine if ILC1 epigenetic features accounted for differences, we referred to published ATAC-seq data of liver cNK cells and ILC1s (Shih et al., 2016). Indeed, the *Eomes* locus was less accessible in *T. gondii*-induced ILC1s than cNK cells, and resembled the *Eomes* locus of ILC1s in uninfected mice (Figure 21A). However, *T. gondii*-induced ILC1s displayed increased accessibility of an NK cell-specific enhancer in the *Tbx21* locus, which was inaccessible in ILC1s (Figure 21B).

We organized differentially accessible REs into a non-redundant peak set containing 6 clusters (Figure 22A). Clusters 1, 3, and 5 contained REs that were uniquely more accessible in uninfected cNK cells, cNK cells from infected mice, and *T. gondii*-induced ILC1s, respectively. Clusters 2, 4, and 6 contained REs that were shared between two groups. Cluster 2 encompassed REs that were more accessible in both cNK cell groups relative to *T. gondii*-induced ILC1s, and
accounted for the largest fraction of peaks, showing that cNK cells share many epigenetic features regardless of infection. Somewhat surprisingly, Cluster 4, which contained REs that were more accessible in both cNK cells and *T. gondii*-induced ILC1s from infected mice relative to uninfected cNK cells, was the second largest cluster, suggesting that infection induces epigenetic changes, some of which are common to both populations.

We also sought to identify additional differences between cNK cells and *T. gondii*-induced ILC1s following infection by directly comparing the REs in Clusters 3 and 5. Analysis of transcription factor binding motifs revealed motifs that were significantly enriched in each population, such as NF-κB in Cluster 3, whose motif was present in 13.2% of REs, and Klf4 in Cluster 5, whose motif was present in 25.8% of REs (Figure 22B). Enriched motifs were found within a differentially accessible REs in the *Tbx21* locus shown above (Figure 21B). We also identified the specific loci that contained the most differentially accessible REs (Figure 23A). Generally, the presence of more accessible REs correlated with greater gene expression. Notably, many of the epigenetic features shared by memory NK cells and memory CD8⁺ T cells (Lau et al., 2018) were among the loci that gained accessibility in *T. gondii*-induced ILC1s as compared to cNK cells (Figure 23B).
Figure 1: Effect of α-NK1.1 treatment on T. gondii parasite burden

(A) Representative images of luciferase imaging of undepleted mice and α-NK1.1-treated mice lying supine, at indicated time points after i.p. infection with 200 Pru.Luc T. gondii parasites.

(B) Total photon flux at indicated time points after infection (n=5; mean±SEM; unpaired t-test, *p ≤ 0.05).
Figure 2: Effect of α-NK1.1 treatment on survival following T. gondii infection

Percentage of mice surviving at indicated time points after i.p. infection with 200 T. gondii tachyzoites Pru strain (n=10; Mantel-Cox test; *p ≤ 0.01).
Figure 3: Quantification of cNK cells and ILC1s after T. gondii infection

(A) Expression of Eomes and CD49a by CD3− CD19− NK1.1+/NKp46+ cells from spleens of uninfected mice and mice at indicated time points after i.p. infection with 200 tachyzoites of T. gondii Pru strain.

(B) Frequency and absolute number of CD3− CD19− NK1.1+/NKp46+ Eomes+ CD49a− and CD3− CD19− NK1.1+/NKp46+ Eomes− CD49a+ cells from spleens of uninfected mice and mice at indicated time points after infection (n=5; mean±SEM; one-way ANOVA with Bonferroni correction, *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001).
Figure 4: Quantification of cNK cells and ILC1s 4 months after T. gondii infection

(A) Expression of Eomes and CD49a by CD3− CD19− NK1.1+ NKp46+ cells from spleens of uninfected mice and mice 4 mo after i.p. infection with 200 tachyzoites of T. gondii Pru strain.

(B) Frequency (of CD3− CD19− NK1.1+ NKp46+ cells) and absolute number of cNK (Eomes+ CD49a−) and ILC1s (Eomes− CD49a+) from spleens of uninfected mice and mice 4 mo post-infection (n=5; mean±SEM; one-way ANOVA with Bonferroni correction, *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001).
Expression of CD27 and CD11b by cNK cells (CD3\(^{-}\) CD19\(^{-}\) NK1.1\(^{+}\) NKp46\(^{+}\) Eomes\(^{+}\) CD49a\(^{-}\)) and ILC1s (CD3\(^{-}\) CD19\(^{-}\) NK1.1\(^{+}\) NKp46\(^{+}\) Eomes\(^{-}\) CD49a\(^{+}\)) from spleens of uninfected mice and mice infected 35 d earlier by i.p. injection of 200 tachyzoites of \textit{T. gondii} Pru strain (n=3-5, mean+SEM; one-way ANOVA with Bonferroni correction, *\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\), ****\(p \leq 0.0001\)).
Figure 6: Surface markers differentially expressed by cNK cells and ILC1s

Expression of Neuropilin-1, KLRG1, DNAM-1, and NKG2A by cNK cells (CD3− CD19− NK1.1+ NKp46+ Eomes+ CD49a−) and ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) from spleens of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain.
Figure 7: Expression of Ly49 receptors

Expression of indicated Ly49 receptor by cNK cells (CD3\(^-\) CD19\(^-\) NK1.1\(^+\) NKp46\(^+\) Eomes\(^+\) CD49a\(^-\)) and ILC1s (CD3\(^-\) CD19\(^-\) NK1.1\(^+\) NKp46\(^+\) Eomes\(^-\) CD49a\(^+\)) from spleens of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain.
Figure 8: Cytokine production by cNK cells and ILC1s

(A) Intracellular IFNγ after 4 hr of culture in media alone (unstim), or media supplemented with IL-12, IL-12 + IL-18, or PMA + Ionomycin (P+I) in cNK cells (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁺ CD49a⁺) and ILC1s (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁻ CD49a⁺) from spleens of uninfected and mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain.

(B) Intracellular TNFα in cNK cells and ILC1s after 4 hr of culture in media alone (unstim), or media supplemented with P+I (n=3; mean+SEM; one-way ANOVA with Bonferroni’s multiple comparisons test, ns not significant, ****p ≤ 0.0001).
Figure 9: Expression of Ly6C by cNK cells and ILC1s

(A) Expression of Ly6C by cNK cells (CD3<sup>-</sup> CD19<sup>-</sup> NK1.1<sup>+</sup> NKp46<sup>+</sup> Eomes<sup>+</sup> CD49a<sup>-</sup>) and ILC1s (CD3<sup>-</sup> CD19<sup>-</sup> NK1.1<sup>+</sup> NKp46<sup>+</sup> Eomes<sup>-</sup> CD49a<sup>+</sup>) from the spleens of uninfected mice and mice at indicated time points after infection with *T. gondii* Pru strain.

(B) Frequency of cNK cells and ILC1s expressing Ly6C at indicated time points post-infection (n=5; mean±SEM; one-way ANOVA with Bonferroni correction, **p ≤ 0.01, ****p ≤ 0.0001).
Figure 10: Gating strategy to sort cNK cells and T. gondii-induced ILC1s

(A) Sorting strategy to isolate splenic cNK cells from uninfected mice and cNK cells and T. gondii-induced ILC1s from mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain (gated on CD3^− CD19^− NK1.1^+ NKp46^+).

(B) Expression of Eomes in each sorted population.
**Figure 11: RNA-seq of cNK cells and ILC1s from T. gondii-infected mice**

(A) Principal component analysis using top 2000 most variable genes comparing splenic cNK cells from uninfected mice (n=3), and cNK cells and T. gondii-induced ILC1s from mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain (n=5). Points represent biological replicates.

(B) Unsupervised hierarchical clustering of samples in (A).

(C) Volcano plots showing Log2(Fold Change) (FC) differences versus -Log10(False Discovery Rate) (FDR) for indicated comparisons. Genes exhibiting Log2FC > 1 and False Discovery Rate (FDR) < 0.1 are colored.
Figure 12: Gene Set Enrichment Analysis for signature ILC1 and NK cell genes

GSEA of signature (A) ILC1 and (B) NK cell genes (Robinette et al., 2015), using genes ranked by DESeq2 Wald statistic of *T. gondii-*induced ILC1s relative to cNK cells from mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain.
Figure 13: Heatmaps of differentially expressed genes

Heatmaps showing centered DESeq2 variance-stabilized expression values of manually selected genes exhibiting Log$_2$FC > 1 and FDR < 0.1 by cNK from uninfected mice and cNK cells and *T. gondii*-induced ILC1s from mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain.
Figure 14: Gene Set Enrichment Analysis for signature genes of tissue-residency and circulation

GSEA of signature (A) tissue-residency and (B) circulation genes (Milner et al., 2017), using genes ranked by DESeq2 Wald statistic of *T. gondii*-induced ILC1s relative to cNK cells from mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain.
Expression of CX₃CR1, CCR8, CXCR6, PECAM-1, and CD62L by cNK cells (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁺ CD49a⁻) and ILC1s (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁻ CD49a⁺) from spleens of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain (n=3-5, mean±SEM; one-way ANOVA with Bonferroni correction, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).
Figure 16: *T. gondii*-induced ILC1s in additional organs

(A) Expression of Eomes and CD49a by CD3<sup>-</sup> CD19<sup>-</sup> NK1.1<sup>+</sup> NKp46<sup>+</sup> cells from the blood, lung, and liver of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain.

(B) Frequency of ILC1s (Eomes<sup>-</sup> CD49a<sup>+</sup>) among CD3<sup>-</sup> CD19<sup>-</sup> NK1.1<sup>+</sup> NKp46<sup>+</sup> cells in indicated organs from uninfected and infected mice (n=3-5, mean±SEM; one-way ANOVA with Bonferroni correction, ns not significant, ***p ≤ 0.001, ****p ≤ 0.0001).

(C) Absolute number of ILC1s (CD3<sup>-</sup> CD19<sup>-</sup> NK1.1<sup>+</sup> NKp46<sup>+</sup> Eomes<sup>-</sup> CD49a<sup>+</sup>) from the blood, lung, and liver (n=3-5, mean±SEM; unpaired t-test, ns not significant, *p ≤ 0.05).
Figure 17: Organs that do not increase in T. gondii-induced ILC1s

(A) Expression of Eomes and CD49a by CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ cells from indicated organs of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain.

(B) Absolute number of ILC1s (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁻ CD49a⁺) from indicated organs of uninfected mice and infected mice (n=3-5, mean±SEM; unpaired t-test, ns not significant, *p ≤ 0.05).
Figure 18: Ly6C expression by ILC1s in *T. gondii*-infected mice

(A) Expression of Ly6C by ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) from the liver of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain.

(B) Frequency of liver ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) expressing Ly6C, and absolute number of Ly6C+ ILC1s and Ly6C− ILC1s in the liver (n=3-5, mean±SEM; unpaired t-test, *ns* not significant, *p* ≤ 0.05, ***p* ≤ 0.001).
Figure 19: Homing of NK cells and ILC1s in lymphopenic recipients

(A) Overview of competitive transfer experiment. NK cells and ILC1s were sorted from day 35-infected CD45.2 and CD45.1 x CD45.2 Eomes-GFP reporter mice, respectively. Cells were combined in a 1:1 ratio and intravenously injected into Rag2\(^{-/-}\) Il2rg\(^{-/-}\) mice. Twenty-four days later, the recipient spleen and liver were assessed for transferred cells.

(B) Expression of CD45.1 and CD45.2 by CD3\(^{-}\) CD19\(^{-}\) NK1.1\(^{+}\) NKp46\(^{+}\) cells from the spleen and liver of Rag2\(^{-/-}\) Il2rg\(^{-/-}\) mice, and frequency of transferred Eomes-GFP\(^{+}\) CD49a\(^{-}\) cells (CD45.2\(^{+}\)) and Eomes\(^{-}\) CD49a\(^{+}\) cells (CD45.1\(^{+}\) CD45.2\(^{+}\)), 24 days after transfer (n=5, mean+SEM; two-way ANOVA, ***p ≤ 0.001).

(C) Expression of CD49a and Eomes-GFP by transferred NK cells and transferred Eomes-GFP\(^{-}\) CD49a\(^{+}\) cells and frequency of Eomes\(^{-}\) CD49a\(^{+}\) cells within each population, 24 days post-transfer (n=5, mean+SEM; unpaired t-test, **p ≤ 0.01, ****p ≤ 0.0001).
Figure 20: Epigenomic Differences in cNK cells and T. gondii-induced ILC1s

(A) Unsupervised principal component analysis comparing splenic cNK cells from uninfected mice, and cNK cells and T. gondii-induced ILC1s from mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain (n=3). Points represent biological replicates.

(B) Unsupervised hierarchical clustering of samples in (A).

(C) Quantification of REs with log2FC>1 and FDR<0.05 within indicated genomic regions in pairwise comparison of cNK cells from infected mice versus cNK cells from uninfected mice (left), T. gondii-induced ILC1s and cNK cells from infected mice (middle), and T. gondii-induced ILC1s and cNK cells from uninfected mice (right).
**Figure 21: Eomes and Tbx21 loci**

UCSC genome browser tracks showing ATAC-seq peaks in cNK cells from uninfected mice, and cNK cells and *T. gondii*-induced ILC1s from mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain, and cNK cells and ILC1s from the liver of uninfected mice (Shih et al., 2016) at the (A) *Eomes* and (B) *Tbx21* genomic loci. Differentially accessible regions between cNK cells and *T. gondii*-induced ILC1s from infected mice are highlighted in blue. Peach arrow denotes a previously identified NK cell-specific enhancer (Shih et al., 2016).
Figure 22: Features of differential regulatory elements

(A) Heatmap of REs that are differentially accessible in cNK cells and *T. gondii*-induced ILC1s, centered on peaks and showing 3 kb upstream and downstream of peak center.

(B) *De novo* transcription factor binding motifs found enriched in Clusters 3 and 5 from (A), and *p*-values for enrichment compared to background regions.
Figure 23: Comparison of regulatory element accessibility and gene transcription

For each gene listed, Log2FC of gene expression from RNA-seq is indicated by position of bottom point. Each differentially accessible RE is depicted as a point, with Log2FC of peak size in *T. gondii*-induced ILC1s, relative to cNK cells indicated by fill color of each point.

(A) Loci containing the most differentially accessible REs.

(B) Loci shared by MCMV-induced memory CD8+ T cells and NK cells (Lau et al., 2018).
Table 1: Signature ILC1 gene set

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Table 4: Signature circulating gene set

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3.1 Role of Ongoing Infection
We postulated that the expansion of ILC1s following *T. gondii* infection was either a response to ongoing parasite replication or a permanent change that persisted even after infection subsided. Bradyzoites develop following acute infection with the Pru strain of *T. gondii* and parasite reactivation could potentially also account for changes to NK1.1\(^+\)NKp46\(^+\) cells during chronic infection. To test whether ongoing parasite replication played a role, we infected mice with *T. gondii* Pru strain, then suppressed growth of tachyzoites with sulfadiazine at various time points post-infection (Eyles and Coleman, 1955). Interestingly, infection induced expansion of ILC1s even in sulfadiazine-treated mice when treatment began 7 or 10 days post-infection, with cell numbers comparable to those of untreated mice (Figure 24). ILC1 expansion also occurred following repeated administration of the Cps1-1 *T. gondii* strain (Figure 25), which does not replicate in vivo and therefore does not persist in a chronic form (Fox and Bzik, 2002). Moreover, the number of ILC1s was comparable after infection with Δgra4 parasites on a Pru background, which are defective in cyst formation (Fox et al., 2011; Jones et al., 2017), and the WT parental strain, PruΔku80 (Figure 26). BALB/c mice, which have fewer cysts during chronic *T. gondii* infection (Suzuki et al., 1993), also had increased numbers of ILC1s after infection with Pru *T. gondii* (Figure 27). Thus, *T. gondii*-induced expansion of ILC1s persists independently of ongoing parasite replication. To investigate factors that affect the development of these cells, we further examined the impact of infection with the Pru strain of *T. gondii*.
4.2 Effect of Tbet Deficiency

Tbet is required for ILC1 development (Daussy et al., 2014; Sojka et al., 2014). Moreover, *T. gondii*-induced ILC1s express higher levels of Tbet (Figure 28A), and display increased accessibility of the *Tbx21* locus (Figure 21B). We therefore hypothesized that Tbet may be required for ILC1 expansion after infection. Inasmuch as *Tbx21*/* mice succumb to *T. gondii* infection (Harms Pritchard et al., 2015), we studied WT:*Tbx21*/* mixed bone marrow chimeras. In both uninfected and infected chimeras, cNK cells were present, though they were biased towards WT origin (Figure 28B), consistent with a requirement for Tbet for complete cNK cell maturation (Gordon et al., 2012). However, no *T. gondii*-induced ILC1s were found of *Tbx21*/* origin in infected WT:*Tbx21*/* chimeras, like ILC1s in naïve chimeras. Thus, *T. gondii*-induced ILC1s are Tbet-dependent.

![Image](image_url)

Surprisingly, we found that infection increased *Tbx21*/* Eomes" CD49a" cells in the livers, but not the spleens, of WT:*Tbx21*/* chimeras, which were also present in counterpart WT cells at lower frequencies (Figure 29). Moreover, Eomes" CD49a" cells emerged over the course of infection in the WT liver (Figure 30) and spleen (Figure 3). This suggested that cNK cells upregulate CD49a and subsequently lose Eomes expression. If so, *T. gondii*-induced ILC1s may be cNK cell-derived despite their semblance to ILC1s.

To explore this possibility, we performed single-cell RNA-seq on sorted WT (CD45.1") or *Tbx21*/* (CD45.2") NK1.1" NKp46" cells from the livers of uninfected and infected WT:*Tbx21*/* chimeras. Using t-distributed stochastic neighbor embedding (t-SNE) analysis, we grouped the
cells into 17 clusters, C1-C17 (Figure 31A). In the uninfected WT sample, C1 was the largest
cluster, whereas C1 was diminished in the infected WT and Tbx21−/− samples (Figure 31B-C). C1
cells expressed Eomes and Itgam but did not express Cd27 or Itga1 (Figure 32). These findings
are consistent with C1 being mature cNK cells (Figure 31D), which are indeed the predominant
NK1.1+ NKp46+ cells present in the steady state liver (Figure 33). C3 was the second largest
cluster in the uninfected WT sample, and C3 cells did not express Eomes or Ly6c2, expressed
Itga1, Cd27, and Cxcr6, and were absent in Tbx21−/− samples, indicating that C3 comprised
ILC1s.

C5 and C6 were present in the infected WT sample, but not the uninfected WT sample. These
cells co-expressed Eomes and Itga1, suggesting that these clusters include the Eomes+ CD49a+
cells that arose in the liver after infection (Figures 31-32). These clusters were especially
prominent in the infected Tbx21−/− sample, confirming that the Eomes+ CD49a+ cells that
accumulate among Tbx21−/− NK cells resemble cells that typically arise in the WT liver following
infection. While our current data do not allow definitive association of the Eomes+ CD49a+ cells
to ILC1s, the single-cell RNA-seq analysis revealed additional subsets of interest.

Two closely related clusters, C10 and C11, were unique to the infected WT sample (Figure 31).
Cells in C10 and C11 expressed Itga1, Ly6c2, and Klrk1, and did not express Eomes (Figure 32).
This specificity of infection, Tbet-dependence, and gene expression identified both clusters as T.
gondii-induced ILC1s (Figure 31D). Interestingly, the presence of two clusters indicated
heterogeneity within the T. gondii-induced ILC1s population. The t-SNE analysis placed C10
closer to mature cNK cells (C1) and C11 closer to ILC1s (C3). Comparison of C10 and C11 revealed that C10 expressed signature NK cell genes at higher levels (Robinette et al., 2015), whereas C11 expressed higher levels of signature ILC1 genes (Figure 34). Interestingly, C10 also highly expressed Cx3cr1 whereas C11 did not (Figure 32). Indeed, flow cytometric analysis confirmed that *T. gondii*-induced ILC1s in the infected liver contained a CX3CR1+ CXCR6− population that expressed higher levels of Zeb2, mirroring the C10 phenotype, and also a CX3CR1− CXCR6+ population that mirrors C11 (Figure 35).

4.3 Requirement of Eomes
Single-cell RNA-seq analysis revealed heterogeneity of NK1.1+ NKp46+ cells, including within *T. gondii*-induced ILC1s, and raised the possibility that these cells were derived, at least in part, from cNK cells. To assess if Eomes downregulation by cNK cells contributed to the ILC1 population following infection, we examined Ncr1Cre Eomes−/− (Eomes cKO) mice, in which NK1.1+ NKp46+ cells were reduced due to the Eomes-dependence of cNK cells (Figure 36), but numbers of CD49a+ ILC1s were normal at steady state (Figure 37A) (Gordon et al., 2012; Pikovskaya et al., 2016). Following *T. gondii* infection, the numbers of splenic CD49a+ and CD49a− Ly6C+ cells were significantly reduced in Eomes cKO compared to Eomes−/− controls (Figure 37A), showing that expansion of these cells requires Eomes. However, while the overall number of CD49a+ cells did not change between uninfected and infected Eomes cKO mice, the number of CD49a+ Ly6C+ cells increased slightly, and a greater frequency of the CD49a+ cells expressed Ly6C and KLRG1 (Figure 37), showing that ILC1s can also give rise to *T. gondii*-induced ILC1s. However, approximately three times as many CD49a+ Ly6C+ were derived from Eomes-dependent cells, i.e., cNK cells. These data are consistent with the single-cell RNA-seq
data, showing that *T. gondii* infection results in two closely related populations of *T. gondii*-induced ILC1s.

To confirm a cell-intrinsic requirement for Eomes during *T. gondii*-induced ILC1s expansion, we studied NK1.1^+ NKp46^+ cells in the spleens of mixed bone marrow chimeras reconstituted with WT cells and either Eomes^{f/f} or Eomes cKO cells. In uninfected chimeras, the contribution of Eomes^{f/f} and Eomes cKO cells to CD49a^+ cells was comparable, consistent with these cells being Eomes-independent ILC1s (Figure 38). This chimerism did not change upon infection of WT:Eomes^{f/f} chimeras. However, infection of WT:Eomes cKO chimeras resulted in a significant reduction of CD49a^+ cells of Eomes cKO origin. Thus, *T. gondii* infection resulted in Eomes^-CD49a^+ cells that were dependent on a cell-intrinsic effect of Eomes in NKp46-expressing cells, i.e., cNK cells; therefore, most *T. gondii*-induced ILC1s are ex-cNK cells.

### 4.4 Cell-Extrinsic Factors

We postulated that STAT4 may play a role in the expansion of *T. gondii*-induced ILC1s because STAT4 and IL-12 signaling is critical for NK cell and ILC1 activation during *T. gondii* infection (Cai et al., 2000; Hunter et al., 1994; Klose et al., 2014). To test the relevance of this signaling on the generation of *T. gondii*-induced ILC1s *in vivo*, we infected WT:Stat4^-/- mixed bone marrow chimeras. Indeed, STAT4-deficient Eomes^-CD49a^+ cells were diminished, but not absent, while chimerism of cNK cells and ILC1s remained equal between uninfected and infected chimeric mice (Figure 39), suggesting that *T. gondii*-induced ILC1s are dependent on STAT4 for development. However, *T. gondii*-induced ILC1s expansion was not observed in cNK cells upon culture of splenocytes from uninfected mice (Figure 40). Moreover, culture of
purified cNK cells in IL-12 did not result in Eomes\(^{-}\) NK cells, although a small decrease in Eomes expression was detected (Figure 41). On the other hand, \textit{in vitro} culture of splenocytes from infected mice resulted in an increased frequency of Eomes\(^{-}\) CD49a\(^{+}\) cells following culture in IL-2 and IL-12, compared to culture in IL-2 alone (Figure 40), suggesting that IL-12 may contribute to expansion of ILC1s. Taken together, these results suggest that STAT4 and IL-12 contribute to \textit{T. gondii}-induced ILC1s, but that other factors play additional roles.

Since signaling through TGF-\(\beta\) receptor 2 downregulates Eomes \textit{in vitro} and in tumors (Cortez et al., 2016; Gao et al., 2017), we evaluated \textit{Ncr1}\textsuperscript{iCre Tgfbr2}\textsuperscript{f/f} mice. After infection, we found decreased numbers of ILC1s compared to \textit{Tgfbr2}\textsuperscript{f/f} mice (Figure 42), consistent with TGF-\(\beta\) signaling contributing to \textit{T. gondii}-induced ILC1 development. However, there were also decreased numbers of Eomes\(^{+}\) CD49a\(^{-}\) NK cells in infected \textit{Ncr1}\textsuperscript{iCre Tgfbr2}\textsuperscript{f/f} mice, indicating that TGF-\(\beta\) also controls cNK cells more broadly following infection. Thus, TGF-\(\beta\) may act directly on cNK cells to downregulate Eomes or impact global NK cell homeostasis.
Expression of Eomes and CD49a by CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ cells from spleens of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain, treated with sulfadiazine (SDZ) beginning at indicated dpi, and maintained on sulfadiazine until 35 dpi, and frequency and absolute number of ILC1s (n=5-11; mean±SEM; one-way ANOVA with Bonferroni correction, ns not significant, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

*Figure 24: Effect of sulfadiazine treatment on development of *T. gondii*-induced ILC1s*
Figure 25: Effect of Cps1-1 T. gondii on development of T. gondii-induced ILC1s

(A) Mice were injected with the attenuated Cps1-1 mutant on the type I RH background as shown.

(B) Expression of Eomes and CD49a by CD3− CD19− NK1.1+ NKp46+ cells from the spleens of uninfected mice and mice administered Cps1-1 T. gondii as in (A), and frequency and absolute number of ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) 42 d after initial injection (n=5; mean±SEM; unpaired t-test, ***p ≤ 0.001, ****p ≤ 0.0001).
Expression of Eomes and CD49a by CD3− CD19− NK1.1+ NKp46+ cells from spleens of mice 35 dpi with 200 tachyzoites of PruΔku80 (WT) T. gondii or PruΔku80Δgra4 (Δgra4) T. gondii, and frequency and absolute number of ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) (n=8-11; mean±SEM; unpaired t-test, ns not significant).
Figure 27: Effect of *T. gondii* infection on *T. gondii*-induced ILC1 development in BALB/c mice

Expression of Eomes and CD49a by CD3−CD19−NKp46+ cells from spleens uninfected BALB/c mice and BALB/c mice infected 35 d earlier with 200 tachyzoites of *T. gondii* Pru strain, and frequency and absolute number of ILC1s (CD3−CD19−NK1.1+.NKp46+Eomes−CD49a+) (n=3; mean±SEM; unpaired t-test, *p ≤ 0.05, **p ≤ 0.01).
Figure 28: Tbet expression and requirement in ILC1s

(A) Expression of Tbet by cNK cells (CD3\(^-\) CD19\(^-\) NK1.1\(^+\) NKp46\(^+\) Eomes\(^+\) CD49a\(^-\)) and ILC1s (CD3\(^-\) CD19\(^-\) NK1.1\(^+\) NKp46\(^+\) Eomes\(^-\) CD49a\(^+\)) from spleens of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of \(T.\ gondii\) Pru strain (\(n=5\), mean+SEM; one-way ANOVA with Bonferroni correction, ****\(p \leq 0.0001\)).

(B) Chimerism of cNK cells (CD3\(^-\) CD19\(^-\) NK1.1\(^+\) NKp46\(^+\) Eomes\(^+\) CD49a\(^-\)) and ILC1s (CD3\(^-\) CD19\(^-\) NK1.1\(^+\) NKp46\(^+\) Eomes\(^-\) CD49a\(^+\)) from spleens of uninfected and infected WT: \(Tbx21^{-/-}\) bone marrow chimeras (\(n=4-5\), mean+SEM; one-way ANOVA with Bonferroni correction, ****\(p \leq 0.0001\)).
Figure 29: Eomes$^+$ CD49a$^+$ cells develop from wild-type and Tbx21$^-$ NK cells

(A) Expression of Eomes and CD49a by CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$ cells that are CD45.1-derived (WT) or CD45.2-derived (Tbx21$^-$), from spleens and livers of uninfected and infected WT: Tbx21$^-$ bone marrow chimeras 35 dpi with 200 tachyzoites of T. gondii Pru strain.

(B) Frequency of Eomes$^+$ CD49a$^+$ cells (of CD3 CD19 NK1.1$^+$ NKp46$^+$) in the spleen and liver (n=4-7, mean±SEM; one-way ANOVA with Bonferroni correction, ns not significant, *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$).
Figure 30: Development of Eomes+ CD49a+ cells in the liver during T. gondii infection

(A) Expression of Eomes and CD49a by CD3− CD19− NK1.1+ NKp46+ cells from livers of mice at indicated time points post-infection with 200 tachyzoites of T. gondii Pru strain.

(B) Frequency and absolute number of CD3− CD19− NK1.1+ NKp46+ Eomes+ CD49a+ cells from livers of uninfected mice and mice 35 dpi (n=5; mean+SEM; unpaired t-test, ***p ≤ 0.001, ****p ≤ 0.0001).
Figure 31: Clusters identified by single-cell RNA-seq analysis

(A) Biaxial t-SNE analysis of cells pooled across all samples. Points represent individual cells. Colors denote C1-C17.

(B) t-SNE plots showing the distribution of cells across C1-C17, by sample.

(C) Fraction of cells in C1-C17, within each sample.

(D) t-SNE plot labeled with putative cluster identities.
Figure 32: Gene expression by distinct clusters

(A) Heatmap of average expression of manually selected genes that are characteristic of C1-C17.

(B) Expression of indicated gene by cells in each sample.
Figure 33: Quantification of cNK cell and ILC1 populations in the liver by flow cytometry

(A) Expression of CD27 and CD11b by cNK cells (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁺ CD49⁻ ) from livers of uninfected mice and mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain.

(B) Fraction of CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ cells that belong to each subpopulation in uninfected and infected mice.
Figure 34: Expression of signature ILC1 and cNK cell genes by T. gondii-induced ILC1 subpopulations

Heatmap of average expression of signature ILC1 and NK cell genes (Robinette et al., 2015), by cells in Clusters 1, 3, 10, and 11.
Figure 35: Characterization T. gondii-induced ILC1 subpopulations

(A) Expression of CX3CR1 and CXCR6 by cNK cells (CD3− CD19− NK1.1+ NKp46+ Eomes+ CD49a−), and Ly6C− and Ly6C+ ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) from livers of mice infected 35 d earlier with 200 tachyzoites of T. gondii Pru strain.

(B) Expression of Zeb2 by CXCR6+ Ly6C+ ILC1s and CX3CR1+ Ly6C+ ILC1s from the liver of Zeb2-GFP mice 35 dpi (n=3; mean+SEM; paired t-test, *p ≤ 0.05).
Figure 36: Lack of cNK cells in Ncr1<sup>Cre</sup> Eomes<sup>ff</sup> mice

Expression of NK1.1 and NKp46 by CD3<sup>-</sup> CD19<sup>-</sup> cells from spleens of Eomes<sup>ff</sup> and Eomes cKO mice that were uninfected or were infected 35 dpi with 200 tachyzoites of <i>T. gondii</i> Pru strain (n=6-9, mean±SEM; one-way ANOVA with Bonferroni correction, ns not significant, ****p ≤ 0.0001).
Figure 37: Impaired development T. gondii-induced ILC1s in Ncr1\textsuperscript{icre} Eomes\textsuperscript{ff} mice

(A) Expression of Ly6C and CD49a by CD3\textsuperscript{−} CD19\textsuperscript{−} NK1.1\textsuperscript{+} NKp46\textsuperscript{+} cells from spleens of uninfected and d 35-infected Eomes\textsuperscript{ff} and Eomes cKO mice, and absolute number of ILC1s (CD3\textsuperscript{−} CD19\textsuperscript{−} NK1.1\textsuperscript{+} NKp46\textsuperscript{+} CD49a\textsuperscript{+}) and Ly6C\textsuperscript{+} ILC1s from spleens of uninfected and d 35-infected Eomes\textsuperscript{ff} and Eomes cKO mice (n=6-9, mean+SEM; one-way ANOVA with Bonferroni correction, ns not significant, *p \leq 0.05, ****p \leq 0.0001).

(B) Expression of Ly6C and KLRG1 by CD49a\textsuperscript{+} cells from the spleens of uninfected and d 35-infected Eomes\textsuperscript{ff} and Eomes cKO mice, and frequency of KLRG1\textsuperscript{+} and Ly6C\textsuperscript{+} cells (n=6-9, mean+SEM; one-way ANOVA with Bonferroni correction, ns not significant, ****p \leq 0.0001).
Figure 38: Cell intrinsic role of Eomes in development of T. gondii-induced ILC1s

Chimerism of ILC1s (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ CD49a⁺) from spleens of uninfected and 35 d infected WT:Eomes⁰/⁻ and WT:Eomes cKO bone marrow chimeras (n=4-5, mean±SEM; one-way ANOVA with Bonferroni comparison, ns not significant, ****p ≤ 0.0001).
Figure 39: Cell intrinsic role of STAT4 in development of T. gondii-induced ILC1s

Chimerism of cNK cells (CD3− CD19− NK1.1+ NKp46+ Eomes+ CD49a−) and ILC1s (CD3− CD19− NK1.1+ NKp46+ Eomes− CD49a+) from spleens of uninfected and 35 d infected WT:Stat4−/− bone marrow chimeras (n=4-5, mean+SEM; one-way ANOVA with Bonferroni comparison, ns not significant, ****p ≤ 0.0001).
Figure 40: Effect of IL-12 on NK1.1+ NKp46+ cells in vivo

Expression of Eomes and CD49a by CD3− CD19− NK1.1+ NKp46+ cells from the spleens of uninfected mice and mice infected 35 d earlier with *T. gondii* Pru strain after splenocytes were cultured for 36 h in 300 IU/mL IL-2, or 300 U/mL IL-2 + 20 ng/mL IL-12, and frequency of ILC1s (n=3-12, mean±SEM; one-way ANOVA with Bonferroni correction, ns *not significant, ****p ≤ 0.0001*).
Figure 41: Effect of IL-12 on sorted cNK cells and ILC1s

Expression of Eomes and CD49a by sorted cNK cells from uninfected mice and cNK cells and T. gondii-induced ILC1s from mice infected 35 d earlier with T. gondii Pru strain, after 36 h of culture in IL-2, or IL-2 + IL-12, and frequency of Eomes− CD49a+ NK cells and gMFI of Eomes (n=3-9, mean±SEM; one-way ANOVA with Bonferroni correction, ns not significant, **p ≤ 0.01, ****p ≤ 0.0001).
Figure 42: Effect of TGF-b signaling on development of T. gondii-induced ILC1s

Expression of Eomes and CD49a by CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ cells from spleens of Tgfr2⁺/⁻ and Tgfr2 cKO mice that were uninfected or infected 35 d earlier with T. gondii Pru strain, and frequency and absolute number of cNK cells (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁺ CD49a⁻) and ILC1s (CD3⁻ CD19⁻ NK1.1⁺ NKp46⁺ Eomes⁻ CD49a⁺) (n=7-11, mean+SEM; unpaired t-test, ns not significant, **p ≤ 0.01).
Chapter 4: Discussion

4.1 Overview

Our analysis initially centered on the comparison of *T. gondii*-induced ILC1s with both cNK cells and ILC1s. RNA-seq showed that *T. gondii*-induced ILC1s were enriched for an ILC1 gene signature and displayed fewer core cNK cells traits, relative to cNK cells. However, although the ILC1 gene set was designed to be representative of all ILC1 populations (Robinette et al., 2015), some signature ILC1 genes were expressed at lower levels in *T. gondii*-induced ILC1s. Moreover, *T. gondii*-induced ILC1s uniquely expressed some markers, including Ly6C, KLRG1, CD11b, Neuropilin-1, and CX3CR1, compared to both cNK cells and ILC1s.

Whereas most NK1.1+ NKp46+ cells that bear the Eomes−CD49a+ phenotype are tissue-resident ILC1s in uninfected mice, we detected *T. gondii*-induced ILC1s cells in the blood of infected mice, where they comprised approximately 20% of NK1.1+ NKp46+ cells. However, *T. gondii*-induced ILC1s were enriched for a tissue-residency gene signature and expressed higher levels of CD49a, CXCR6, and PECAM-1 and lower levels of CD62L, which are traits of ILC1s that are thought to confer tissue-residency. However, in a competitive adoptive transfer setting, we observed that ILC1s outcompeted cNK cells in homing to the liver. Thus, *T. gondii*-induced ILC1s circulate freely throughout the vasculature, unlike ordinary ILC1s, but also home differently than cNK cells.

*T. gondii*-induced ILC1s persist even after acute infection resolves, strongly suggesting a permanently changed population. This was confirmed by epigenomic analyses, with ATAC-seq
revealing sweeping chromatin accessibility differences between *T. gondii*-induced ILC1s and cNK cells. We utilized publicly available ATAC-seq data of cNK cells and ILC1s to determine if REs characteristic of ILC1s accounted for the differences we observed. Surprisingly, we found many instances of REs that were uniquely more accessible in *T. gondii*-induced ILC1s. In summary, *T. gondii*-induced ILC1s are distinct from cNK cells and ILC1s in their gene expression, circulatory patterns, and epigenetic profile.

Single-cell RNA-seq analysis revealed that *T. gondii*-induced ILC1s are heterogeneous, with activated ILC1s and ex-cNK cells giving rise to two closely related subpopulations that can be distinguished from the cNK cells and ILC1s in naïve mice. In contrast to the preexisting notion that ILC1s are Eomes-independent, Eomes downregulation results in ex-cNK, which make up the majority of *T. gondii*-induced ILC1s.

### 4.2 Significance

Distinguishing between cNK cells and ILC1s has been challenging since ILC1s were first discovered. Initially, liver ILC1s were considered a subset of tissue-resident NK cells. Then, cNK cells and ILC1s were categorized together as Group 1 ILCs. Subsequently, the identification of a discrete progenitor cell that gives rise to ILC1s and not cNK cells led to the current ILC classification system, wherein cNK cells and ILC1s are considered distinct ILC subsets. Our findings diverge from the current understanding of these cells by definitively showing that cNK cell conversion into ILC1s occurs in *T. gondii* infection. Our studies indicate that the current system of ILC classification, based on their phenotype and development under steady state conditions, may not apply following inflammation. Rather, plasticity may give rise to populations that resemble one another at first glance, but actually represent the convergence of
multiple developmental paths to form interrelated populations. It is possible that the potential for plasticity accounts for the confusion surrounding these cell populations.

The only prior observations of Eomes downregulation in cNK cells were made within the tumor microenvironment (Gao et al., 2017; Gill et al., 2012) and in vitro (Cortez et al., 2016). Akin to the intermediate ILC1s (intILC1s) described within tumors (Gao et al., 2017), we found *T. gondii* infection induced Eomes$^+$ CD49a$^+$ NK cells, which are potential intermediates between NK cells and ex-cNK cells. However, our findings differ in several significant ways from intratumoral intILC1s. The tumor studies indicated that Eomes$^-$ NK cells were restricted to tumors and emphasized the importance of the microenvironment (Gao et al., 2017; Gill et al., 2012). These considerations led to the interpretation that within tumors, Eomes downregulation transiently silences NK cells to hamper immunosurveillance (Silver and Humbles, 2017), since intratumoral Eomes$^-$ NK cells express high levels of *Tigit* and display hypofunctionality. By contrast, *T. gondii*-induced ILC1s are disseminated throughout the circulation, retain the ability to produce IFN$\gamma$, do not produce TNF$\alpha$, and are maintained in the absence of ongoing stimulus. These findings suggest a permanent transformation rather than a transient response to inflammation, a notion that is further bolstered by the observations that *T. gondii*-induced ILC1s possess a unique gene expression profile that includes increased expression of many transcription factors and widespread chromatin remodeling that encompasses many uniquely accessible REs. Furthermore, we observed reduced numbers of both cNK cells and ILC1s in *Tgfbr2* cKO mice following infection, suggesting that TGF-$\beta$ may have broad effects in *T. gondii* infection, unlike
the aforementioned studies that noted a selective impairment in development of Eomes− NK cells from NK cells.

Several factors may account for the reported differences between tumor-induced and *T. gondii*-induced Eomes downregulation. First, the reliance on DX5 and CD49a expression to identify NK cells and ILC1s may not always faithfully reflect Eomes expression, as is the case during *T. gondii* infection. Second, though there is clear evidence supporting conversion from NK cells to intILC1s, the relationship between intILC1s and ILC1s is more tenuous, owing to the inability to distinguish between preexisting ILC1s and *de novo* converted ILC1s. Indeed, this may explain discrepancies that currently exist within the field, such as the conflicting report that ILC1-like cells confer protection against certain tumors (Dadi et al., 2016).

The persistence of *T. gondii*-induced ILC1s after clearance of infection is reminiscent of classical immune memory and suggests that *T. gondii*-induced ILC1s play a role beyond what we have established for NK cells and ILC1s in *T. gondii* infection thus far. In *T. gondii* infection, IL-12 is critical for NK cell activation (Gazzinelli et al., 1993) and our data suggest that it can have long-lasting effects. IL-12 signals through STAT4, which can bind the Tbx21 locus and enhance Tbet expression (Madera et al., 2018). Although STAT4 signaling is thought to be important for NK cell activation and proliferation during *T. gondii* infection (Cai et al., 2000), we found cNK cells remained intact upon *Stat4* deletion, while ILC1s were reduced. This parallels the requirements for development of memory and memory-like NK cells induced by MCMV and cytokine stimulation (Cooper et al., 2009; Romee et al., 2012; Sun et al., 2012), where IL-12 in the acute
setting has durable effects. Future work may reveal a mechanism by which IL-12 signaling during acute infection confers long-lasting changes in ILC populations.

MCMV-induced memory NK cells and *T. gondii*-induced ILC1s share Ly6C expression, IL-12 dependence, and many epigenetic features (Bezman et al., 2012; Lau et al., 2018). Both cell types display alterations in their receptor repertoire, although *T. gondii*-induced ILC1s primarily express the inhibitory receptor NKG2A while MCMV-induced memory NK cells are Ly49H+ (Sun et al., 2009a). The epigenetic differences between *T. gondii*-induced ILC1s and NK cells also mirror differences found in human NK cells that do not express FcRγ (γ−NK cells), which display global differences in epigenetic programming and DNA methylation as compared to FcRγ-expressing NK cells (Lee et al., 2015; Schlums et al., 2015). Interestingly, γ−NK cells are associated with serological evidence of prior infection with human cytomegalovirus (Guma et al., 2004; Guma et al., 2006), suggesting that they are induced by infection. Like γ−NK cells, *T. gondii*-induced ILC1s alter their expression of signaling adaptors, lose accessibility at the *Zbtb16* locus and decrease PLZF expression, and change their receptor repertoire.

### 4.3 Future Directions
The rewiring of NK cells and ILC1s that occurs during *T. gondii* infection may represent an actual physiological response, as *T. gondii* is a natural mouse pathogen. Moreover, mice are thought to play important roles as secondary hosts in the *T. gondii* life cycle as they are prey for cats, which are the definitive hosts. Understanding the immune response to *T. gondii* infection is critical, as *T. gondii* infects humans and can have deleterious impact on immunocompromised individuals. Yet much remains unknown about *T. gondii* infection, especially during chronic
infection. The possibility that *T. gondii*-induced ILC1s contribute to the immune response in hitherto unknown ways raises the intriguing prospect of novel therapeutic approaches to treat *T. gondii* infection. Thus, our main focus for future studies involves identifying the role of *T. gondii*-induced ILC1s.

One possible function of *T. gondii*-induced ILC1s is to circulate and survey for ongoing infection. A similar observation was recently main in the ILC2 field. Although ILCs are canonically tissue-resident (Gasteiger et al., 2015), ILC2s were recently shown to circulate in response to activation by cytokines or infection (Huang et al., 2018). These inflammatory ILC2s can be distinguished from tissue-resident ILC2s based on their increased expression of KLRG1 and S1P receptors, features that are also characteristic of *T. gondii*-induced ILC1s. Likewise, *T. gondii*-induced ILC1s comprise subpopulations that differ in their expression of CXCR6 and CX3CR1. CXCR6 retains ILC1s within hepatic sinusoids, from whence they can mobilize in response to contact hypersensitivity and maintain recall responses (Paust et al., 2010; Peng et al., 2013; Zhang et al., 2016). By contrast, CX3CR1 regulates NK cell recruitment into the circulation (Ponzetta et al., 2013; Sciume et al., 2011). Future studies may illuminate that the different ILC1 subpopulations have specialized roles, based on their circulatory capacity.

Interestingly, the heterogeneity of *T. gondii*-induced ILC1s mirrors heterogeneity found among CD8+ T cells following activation. Lymphocytic choriomeningitis virus (LCMV) infection causes CD8+ T cells to differentiate into diverse subpopulations, and several of these processes are guided by the balance between Eomes and Tbet. For instance, Eomes and Tbet interact in a
nonredundant and cooperative manner during acute LCMV infection to regulate effector CD8$^+$ T cell responses (Pearce et al., 2003). Furthermore, Eomes and Tbet are thought to differentially affect virus-specific CD8$^+$ T cells during chronic LCMV infection (Paley et al., 2012). The balance of Tbet and Eomes may represent a paradigm that underlies the differentiation processes of not only T cells, but rather multiple cell types, including in NK cells during *T. gondii* infection.

Tbet gradients also dictate CD8$^+$ T cell differentiation into either short-lived effector cells or memory precursor cells during acute LCMV infection (Joshi et al., 2007). This process is inflammation-dependent, with an important role for IL-12. Moreover, in this setting, Tbet is thought to recruit Zeb2 to enhance expression of CX3CR1 and KLRG1 (Dominguez et al., 2015; Omilusik et al., 2015). This interaction has already been described in NK cell development (van Helden et al., 2015). Taken together, our findings parallel observations made in other fields and raise the possibility that common transcriptional networks may operate to give rise to ex-cNK cells. Further examination of the role of these transcription factors in *T. gondii*-induced ILC1s may shed light on host-pathogen interactions at play throughout the natural course of this infection.
Chapter 5: Methods

5.1 Toxoplasma gondii Infection

PRU-FLuc-GFP Type II strain of T. gondii was a generous gift from John C. Boothroyd, and used in all infections unless otherwise specified. PruΔku80ΔhxΔgra4::HX/mCherry and the wildtype control strain PruΔku80Δhx were previously described (Jones et al., 2017). The attenuated Cps1-1 mutant on the type I RH background was described previously (Fox and Bzik, 2002).

All T. gondii strains used in this study were maintained in human foreskin fibroblast (HFF) monolayers grown in D-10 medium (Dulbecco’s modified Eagle medium, 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES pH 7.5, 20 µg/mL gentamicin), maintained at 37 °C with 5% CO₂. The Cps1-1 mutant was grown as described above but supplemented with 200 µM uracil (Sigma). Mature parasites were lysed from host cells by vigorous pipetting and egressed parasites were filtered through 3 µm polycarbonate membranes and resuspended in HHE medium (Hanks’ balanced salt solution, 10 mM HEPES, 0.1 mM EGTA). Cell cultures were determined to be mycoplasma-negative using the e-Myco plus kit (Intron Biotechnology).

Mice were injected intraperitoneally (i.p.) with 200 PRU-FLuc-GFP, PruΔku80Δhx, or PruΔku80ΔhxΔgra4::HX/mCherry tachyzoites that were propagated in culture as described above. In indicated experiments, mice were treated with 0.5 g/L sulfadiazone in drinking water to suppress parasite growth. To generate non-persistent infections, 1x10⁵ Cps1-1 tachyzoites were injected 3 times, 2 wk apart.
5.2 Mice

*Eomes*<sup>f/f</sup>, *Tbx21<sup>−/−</sup>*<sup>−/−</sup>, and *Stat4<sup>−/−</sup>* were purchased from The Jackson Laboratory. C57BL/6, CD45.1<sup>+</sup>, and BALB/c mice were purchased from Charles River. *Ncr1<i>^{iCre}</i>* mice were a kind gift from Eric Vivier. *Zeb2-GFP* and *Ncr1<i>^{iCre} Tgfbr2<sup>f/f</sup></i>* mice were previously described (Cortez et al., 2016; Wu et al., 2016). *Ncr1<i>^{iCre}</i>* were bred to *Eomes<sup>f/f</sup>* mice to generate *Eomes cKO* mice and Cre-negative littermate controls. Age and sex-matched animals were used in all experiments. Mice were infected at 6-12 wk of age. All protocols were approved by the Institutional Animal Care and Uses Committee (Washington University School of Medicine, St. Louis, MO) under animal protocol number 20160002.

5.3 Luciferase Imaging

Mice were injected i.p. with 150 mg D-luciferin (Gold Biotechnology) per kg body weight, incubated for 10 min, then anesthetized with continuous isoflurane anesthesia at a flow rate of 1 L/min. Images were captured using an IVIS Spectrum In Vivo Imaging System (Perkin Elmer). Luminescence was quantitated using Living Image software (Perkin Elmer). NK1.1-depleted mice were injected i.p. with 100 µg of α-NK1.1, 3 d and 1 d prior to infection. Purified α-NK1.1 antibody was generated at the Rheumatic Diseases Core Center Protein Purification and Production Facility using the PK136 hybridoma (ATCC).

5.4 Cell Isolation

Spleens were mashed through a 70 µm cell strainer and treated with Tris-NH<sub>4</sub>Cl to lyse red blood cells. Livers were mashed through a 70 µm cell strainer, resuspended in isotonic 38.5% Percoll (Sigma- Aldrich), centrifugated at 325 x g for 20 min, and treated with Tris-NH<sub>4</sub>Cl. Peritoneal cells were isolated by lavage of the peritoneal cavity with PBS. Bone marrow was flushed from femurs and tibias, mashed through a 70 µm cell strainer, and treated with Tris-
NH₄Cl. Lymph nodes were mashed through a 70 μm cell strainer.Brains were mashed through a 70 μm cell strainer, resuspended in 38.5% isotonic Percoll, and centrifuged at 325 x g for 20 min. Lungs were perfused with PBS, minced, digested in RPMI-1640 containing 2% FBS, 1 mg/mL Collagenase Type IV (Sigma-Aldrich), and 0.2 mg/mL DNase (Sigma-Aldrich), mashed through a 70 μm cell strainer, resuspended in 38.5% isotonic Percoll, and centrifuged at 325 x g for 20 min. Uterus and salivary gland were minced, digested in RPMI-1640 containing 0.17 mg/mL Liberase TL (Sigma-Aldrich) and 0.1 mg/mL DNase (Roche), mashed through a 70 μm cell strainer, resuspended in 38.5% isotonic Percoll, and centrifuged at 325 x g for 20 min.

5.5 Flow Cytometry and Cell Sorting
Spleens were mashed through a 70 μm cell strainer and treated with Tris-NH₄Cl to lyse red blood cells. Livers were mashed through a 70 μm cell strainer, resuspended in isotonic 38.5% Percoll (Sigma- Aldrich), centrifuged at 325 x g for 20 min, and treated with Tris-NH₄Cl. Peritoneal cells were isolated by lavage of the peritoneal cavity with PBS. Bone marrow was flushed from femurs and tibias, mashed through a 70 μm cell strainer, and treated with Tris-NH₄Cl. Lymph nodes were mashed through a 70 μm cell strainer. Brains were mashed through a 70 μm cell strainer, resuspended in 38.5% isotonic Percoll, and centrifuged at 325 x g for 20 min. Lungs were perfused with PBS, minced, digested in RPMI-1640 containing 2% FBS, 1 mg/mL Collagenase Type IV (Sigma-Aldrich), and 0.2 mg/mL DNase (Sigma-Aldrich), mashed through a 70 μm cell strainer, resuspended in 38.5% isotonic Percoll, and centrifuged at 325 x g for 20 min. Uterus and salivary gland were minced, digested in RPMI-1640 containing 0.17 mg/mL Liberase TL (Sigma-Aldrich) and 0.1 mg/mL DNase (Roche), mashed through a 70 μm cell strainer, resuspended in 38.5% isotonic Percoll, and centrifuged at 325 x g for 20 min.
Cells were stained with Fixable Viability Dye and Fc receptors were blocked with 2.4G2 hybridoma (ATCC) culture supernatants prior to staining in PBS containing 2% FBS and 2.5 mM EDTA on ice. Data was acquired using a FACSCanto instrument (BD Biosciences) or FACSaria instrument (BD Biosciences) using FACSDiva software (BD Biosciences). Data was analyzed with Flowjo v10 (Treestar).

For cell sorting, splenocytes were enriched for NK1.1$^+$ NKp46$^+$ cells by negative selection. Splenocytes were incubated with 0.2 µg/mL biotin anti-mouse CD4, 0.2 µg/mL biotin anti-mouse CD8, and 1 µg/mL biotin anti-mouse CD19. One hundred µL of EasySep Mouse Streptavidin Rapidspheres (BD Biosciences) were added per 1x10^8 splenocytes. Cells were placed in an EasySep magnet (BD Biosciences) and enriched cells were poured off, and subjected to extracellular staining. Sorting was performed using a FACSaria instrument (BD Biosciences) using FACSDiva software (BD Biosciences). cNK cells were identified as live CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$ Ly6C$^-$ CD49a$^-$ and T. gondii-induced ILC1s were identified as live CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$ Ly6C$^+$ CD49a$^+$.

### 5.6 Stimulations and Intracellular Staining

Following extracellular staining, cells were fixed with FoxP3/Transcription Factor Staining Buffer Set for 30 min at room temperature and washed with 1x Permeabilization buffer (eBiosciences). Transcription factor antibodies were diluted in 1x FoxP3 permeabilization buffer and cells were incubated for 30 min at room temperature.
To assess Eomes downregulation after culture in IL-12, 5x10^6 splenocytes were cultured in R-10 medium (RPMI-1640 medium containing 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) with 300 IU/mL IL-2 with 20 ng/mL IL-12 (Pepro Tech). Unstimulated control wells contained only 300 IU/mL IL-2.

For detection of IFNγ production, 5x10^6 splenocytes were cultured for 5 h in R-10, with the addition of 20 ng/mL IL-12, 20 ng/mL IL-12 + 5 ng/mL IL-18, or 0.5 µg/mL PMA + 4 µg/mL Ionomycin. Brefeldin A was added after 1 h.

### 5.7 Generation of Bone Marrow Chimeras

Donor bone marrow was harvested by flushing femurs and tibias and mashing through a 70 µm cell strainer. Cells were mixed in a 1:1 ratio with CD45.1^+ bone marrow, and intravenously injected into irradiated mice. Recipient mice were lethally irradiated (900 rad) and reconstituted with a 1:1 ratio of CD45.1^+ bone marrow cells and CD45.2^+ Eomes cKO, Eomes^f/f, Tbx21^−/−, or Stat4^−/− bone marrow cells (Finotto et al., 2002; Kaplan et al., 1996; Zhu et al., 2010). Mice were maintained on sulfamethoxazole/trimethoprim oral suspension added to the drinking water for 2 wk after reconstitution and used for experiments 6-8 wk after reconstitution. To prevent chimeric mice from succumbing to acute *T. gondii* infection, bone marrow chimeras were treated with 0.5 g/L sulfadiazine in drinking water beginning 10 d post-infection and maintained on sulfadiazine until the end of the experiment.

### 5.8 RNA Sequencing

RNA was isolated from 5x10^4 sorted cells using Trizol. Libraries were prepared using the Clontech SMARTer Kit. Sequencing was performed using 1x50 single-end reads with a
HiSeq3000 instrument (Illumina). Reads were quantified using kallisto and differential expression was assessed using the DESeq2 package in R (Bray et al., 2016; Love et al., 2014). Using a log2FC cutoff of 1 and an FDR threshold of 0.1, we identified 657 differentially expressed (DE) genes between the cNK cells from uninfected and infected mice, 2288 DE genes between cNK cells from infected mice and *T. gondii*-induced ILC1s, and 1685 DE genes between cNK cells from uninfected mice and *T. gondii*-induced ILC1s. Variance-stabilized transform values were used for subsequent analysis. All plots were generated in R using the ggplot2, pheatmap, and hclust packages. GSEA was performed using the GSEA Preranked module in GenePattern (Mootha et al., 2003; Reich et al., 2006; Subramanian et al., 2005).

5.9 ATAC Sequencing
Samples were prepared as previously described (Buenrostro et al., 2015), then purified with MinElute spin columns (Qiagen). DNA fragments were amplified using Nextera index adapters as per manufacturer’s instructions (Illumina). Libraries were purified with AMPure XP beads (Beckman Coulter). Three libraries were pooled and sequenced with 2x50 paired end reads using a HiSeq3000 instrument (Illumina).

Sequences were aligned to the mm10 reference genome using Bowtie2 (Langmead and Salzberg, 2012). Reads with a quality score below 30 were removed with Samtools (Li et al., 2009) and duplicate reads were filtered with PicardTools (Broad Institute). Peaks were called using MACS2 (Zhang et al., 2008) with an FDR cutoff of 0.05. Narrowpeak files generated from MACS2 were converted to Bigwig files with deepTools (Ramirez et al., 2014), and visualized using the UCSC genome browser. Differential peaks were identified using Homer (Heinz et al.,
2010) and heatmaps of differentially accessible regions were generated with deepTools. Transcription factor binding motifs were identified *de novo* with Homer.

Across all samples, we identified 71,504 peaks. We further analyzed 9,640 discrete peaks that displayed two-fold changes between at least two groups. Between cNK cells from control and infected mice, there were 1,809 differential peaks (1,445 were larger in the cNK cells from infection and 364 were larger in cNK cells from uninfected mice), between cNK cells from infected mice and *T. gondii*-induced ILC1s, there were 6,710 differential peaks (4,572 were larger in the cNK cells and 2,138 were larger in *T. gondii*-induced ILC1s), and between cNK cells from uninfected mice and *T. gondii*-induced ILC1s, there were 5,938 differential peaks (3,065 were larger in the uninfected cNK cells and 2,973 were larger in *T. gondii*-induced ILC1s).

### 5.10 Single-cell RNA Sequencing

Sorted cells were subjected to droplet-based 3’ end massively parallel single-cell RNA sequencing using Chromium Single Cell 3’ Reagent Kits as per manufacturer’s instructions (10x Genomics). The libraries were sequenced using a HiSeq3000 instrument (Illumina).

Sample demultiplexing, barcode processing, and single-cell 3’ counting was performed using the Cell Ranger Single-Cell Software Suite (10x Genomics). Cellranger count was used to align samples to the mm10 reference genome, quantify reads, and filter reads with a quality score below 30.
The Seurat package in R was used for subsequent analysis (Butler et al., 2018). Genes expressed in fewer than 3 cells and cells that expressed less than 400 or greater than 3500 genes were removed for downstream analysis. Data was normalized using a scaling factor of 10,000 and nUMI was regressed with a negative binomial model. Principal component analysis was performed using the top 3000 most variable genes and t-SNE analysis was performed with the top 40 PCAs. Clustering was performed using a resolution of 0.8. For heatmaps, the mean expression by all cells within the cluster was used.

5.11 Quantification and Statistical Analysis
Prism (GraphPad) was used for statistical analysis. Student’s t-test was used for comparison of 2 groups and 2-way ANOVA with Bonferroni correction was used in analyses involving multiple comparisons. P-values are shown in figures and figure legends. In all graphs, points represent biological replicates, bar position represents the mean, and error bars represent +SEM.
References


