

1 Anti-Asialo GM1 treatment during secondary *Toxoplasma gondii* infection is lethal and depletes T
2 cells

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4 Daria L. Ivanova,^a Steve L. Denton,^a Jason P. Gigley,^{a#}

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6 ^aDepartment of Molecular Biology, University of Wyoming, Laramie, Wyoming, USA

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8 Running Head: Anti-Asialo GM1 depletes T cells during secondary *T. gondii* infection

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10 #Address correspondence and reprint requests to: Dr. Jason P. Gigley, 1000 E. University Ave.

11 Laramie, WY 82071, USA; email: jgigley@uwyo.edu

12 ORCID: 0000-0001-8805-4561 (J.P.G.)

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21 **Abstract**

22

23 Using vaccine challenge model of *T. gondii* infection, we found that treatments with two commonly
24 used for NK cell depletion antibodies resulted in different survival outcomes during secondary
25 infection. Anti-ASGM1 resulted in 100% death and greater parasite burden at the site of infection
26 than anti-NK1.1. Anti-NK1.1 treatment resulted in increased parasite burdens, but animals did not
27 die. Further we found that anti-ASGM1 treatment depleted T cells. CD8⁺ T cells were more
28 susceptible than CD4⁺ T cells to the treatment. ASGM1 was expressed on a higher percentage of
29 CD8⁺ T cells than CD4⁺ T cells and CD8⁺ T cells. In *T. gondii*-immunized animals ASGM1 was
30 enriched on effector memory (Tem) and central memory (Tcm) CD8⁺ T cells. However, Tem were
31 more susceptible to the treatment. After secondary infection, Tem, Tcm, effector (Tef) and naïve (Tn)
32 CD8⁺ T cells were positive for ASGM1. Anti-ASGM1 treatment during reinfection resulted in
33 greater depletion of activated IFN γ ⁺, Granzyme B⁺, Tem and Tef than Tcm and Tn CD8⁺ T cells.
34 Anti-ASGM1 also depleted IFN γ ⁺ CD4⁺ T cells. Recombinant IFN γ supplementation prolonged
35 survival of anti-ASGM1 treated mice, demonstrating that this antibody eliminated IFN γ -producing T
36 and NK cells important for control of the parasite. These results highlight that anti-ASGM1 antibody
37 is not an optimal choice for targeting only NK cells and more precise approaches should be used.
38 This study uncovers ASGM1 as a marker of activated effector T cells and the potential importance of
39 changes in sialylation in lipid rafts for T cell activation during *T. gondii* infection.

40

41 **Introduction**

42 *T. gondii* is an obligate intracellular protozoan that infects ~25-30% of people worldwide
43 (Flegr et al., 2014, Jones and Dubey, 2012). Infection with the parasite in people is one of the leading
44 causes of foodborne illnesses in the U.S. resulting in hospitalization (Scallan et al., 2011). Despite
45 induction of a robust CD8⁺ T cell response, the parasite does not get completely cleared and
46 establishes a persistent infection in the brain and muscles of the host (Harker et al., 2015, Wohlfert et
47 al., 2017). Recent studies suggest that infection with the parasite correlates with schizophrenia,
48 depression, behavioral changes, and neurodegenerative disorders (Lang et al., 2018, Donley et al.,
49 2016). In the patients with weakened immune system, the *T. gondii* parasites can cause toxoplasmic
50 encephalitis and death (Luft and Remington, 1992, Kodym et al., 2015). Currently, there are no
51 vaccines or treatments that completely clear the infection (Radke et al., 2018). Understanding how
52 the immune system functions in response to the parasite is paramount for the development of novel
53 therapeutic approaches to treat this infection.

54 *T. gondii* infection induces a robust Th1 response that provides long-term protection through
55 the production of IFN γ by CD8⁺ T cells (Suzuki et al., 1988, Gazzinelli et al., 1991). However, IFN γ
56 is also produced early during infection by innate immune cells, including NK cells, ILC1 and
57 neutrophils (Sher et al., 1993, Klose et al., 2014, Sturge et al., 2013). The approaches taken to define
58 the role of different innate immune cell populations have included cell depletion studies *in vivo* using
59 antibodies to the different cell populations and animals with mutations or genetic deficiencies that
60 result in the absence of the cell of interest. REF These approaches of course are not perfect and have
61 off target effects making it difficult to fully understand the role of different cell types in a disease.
62 This situation has become more apparent for studies investigating NK cells and depends upon the
63 disease model being studied (Nishikado et al., 2011). NK cells are usually depleted using NK cell
64 specific antibodies *in vivo* (Victorino et al., 2015). However, in some disease models, antibody
65 depletion of NK cells either targeted NK cells alone, or targeted additional subsets of immune cells

66 (Nishikado et al., 2011). The conventional approach to target NK cells *in vivo* utilizes antibodies
67 against asialo GM1 (ASGM1) or the surface marker NK1.1 (Young et al., 1980). Anti-ASGM1
68 antibody reacts with a glycosphingolipid expressed on the surface NK cells, basophils, and on some
69 subsets of $\gamma\delta$ T, NKT, CD8 T cells and macrophages (Nishikado et al., 2011, Slifka et al., 2000,
70 Wiltrout et al., 1985, Kasai et al., 1980). In contrast, anti-NK1.1 targets a glycoprotein expressed on
71 NK cells and some subsets of $\gamma\delta$ T, NKT, and CD8+ T cells (Slifka et al., 2000). Anti-NK1.1
72 depletion of NK cells is limited in specific mouse strains due to an allelic variation in the NKR-P1
73 gene that encodes NK1.1. REF Therefore, anti-NK1.1 can only be used in C57BL/6, SJL, and NZB
74 and not in other strains (BALBC etc.) (Mesci et al., 2006, Carlyle et al., 2006). Anti-ASGM1 is an
75 alternative and is very commonly used for NK cell depletion because it effectively depletes NK cells
76 *in vivo* in many mouse strains and other species. REF Although there are mouse strains with genetic
77 alterations that result in NK cell deficiencies without the use of antibodies, they have additional
78 defects that make interpretation of studies difficult (Jessen et al., 2011, Eckelhart et al., 2011).
79 Therefore, when choosing between the use of antibodies for NK cell depletions and genetically
80 modified NK cell deficient mice, many investigators lean towards the use of the antibodies due to
81 their availability, ease of use, and the ability to have a temporal control of cell ablation *in vivo*.

82 Many studies have demonstrated that NK cells are essential for protection against *T. gondii*
83 infection by using anti-ASGM1 or anti-NK1.1 treatments (Denkers et al., 1993, Gazzinelli et al.,
84 1993, Goldszmid et al., 2012, Askenase et al., 2015). While there is no doubt about NK cell
85 importance, whether these experimental approaches had additional effects on the immune response to
86 the parasite is not clear. In this study we found that anti-NK1.1 and anti-ASGM1 significantly
87 differed in their impact on mouse survival and parasite burdens when administered during lethal *T.*
88 *gondii* challenge of mice vaccinated with the attenuated type I RH strain *cps1-1* (CPS {Fox, 2002
89 #390}). The parasite burdens were higher and the survival rate was reduced after the treatment with
90 anti-ASGM1 as compared to anti-NK1.1. We further explored the reason for this difference and

91 discovered that while both antibodies depleted NK cells, anti-ASGM1 also significantly depleted
92 CD8⁺ and CD4⁺ T cells both in spleen and the site of challenge infection. Anti-NK1.1 treatment did
93 not have as dramatic of an effect. Interestingly, CD8⁺ T cells were more affected by anti-ASGM1
94 treatment than CD4⁺ T cells. ASGM1 was expressed on the surface of CD4⁺ and CD8⁺ T cells in
95 CPS-vaccinated animals. Further dissection of whether ASGM1 was expressed more on memory T
96 cells after vaccination revealed that effector memory (Tem, CD62L-CD44⁺) and central memory
97 (Tcm, CD62L+CD44⁺) CD8⁺ T cells both had higher concentrations of ASGM1 on their surface.
98 However, during secondary challenge of vaccinated mice, all T cell subsets, including memory (Tem
99 and Tcm), effector (Tef) and naïve (Tn) cells increased ASGM1 on their surface. Interestingly, anti-
100 ASGM1 treatment during RH challenge of CPS-vaccinated animals effectively eliminated most Tem
101 and Tef (CD62L-) CD8⁺ T cells leading to an increase in naïve CD8⁺ T cells. Anti-ASGM1
102 significantly reduced the frequency and number of activated IFN γ ⁺, Granzyme B⁺ and
103 polyfunctional IFN γ ⁺GranzymeB⁺ CD8⁺ T cells and IFN γ ⁺ CD4⁺ T cells in RH challenged
104 animals. Recombinant IFN γ supplementation of depleted animals prolonged their survival. These
105 studies demonstrate that NK cell depletion with anti-ASGM1 during *T. gondii* infection also
106 eliminates CD8⁺ and CD4⁺ T cells in *T. gondii* vaccinated animals and that ASGM1 may be
107 important in the activation of T cells important in control of parasite infection.

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116 **Results**

117 ***Anti-NK1.1 and anti-ASGM1 have different effects on control of T. gondii infection***

118 The importance of NK cells for early control of *T. gondii* via IFN γ is well established
119 (Denkers et al., 1993, Sher et al., 1993, Combe et al., 2005, Goldszmid et al., 2012). We recently
120 demonstrated that NK cells are also important in controlling secondary parasite infection in a
121 vaccine-challenge model of *T. gondii* (Ivanova D. et al. in prep.). Our experimental approach to
122 testing the importance of NK cells in this model involved the use of NK cell specific antibodies anti-
123 NK1.1 and anti-ASGM1 to deplete the cells *in vivo*. C57BL6 mice were immunized with the
124 attenuated type I strain *cps1-1* (CPS) and five weeks later challenged with a lethal dose of the CPS
125 parental parasite strain RH and mouse survival and parasite burdens by real time semi quantitative
126 PCR measured. We observed that anti-ASGM1 treatment resulted in the death of all of the animals
127 within 11 days of type I RH challenge and 31 days after type II ME49 challenge (Fig. 1A). However,
128 Anti-NK1.1 treatment did not result in death after type I RH and type II ME49 challenge (Fig. 1A).
129 We also observed that parasite burdens were higher in immunized then challenged mice after anti-
130 ASGM1 treatment (Fig. 1B-C, Supplementary Fig. 1A-C). In addition, the parasite burdens were
131 greater in the mice treated with anti-ASGM1 compared to anti-NK1.1 (Supplementary Fig. 1B-C). In
132 a separate set of experiments mice were chronically infected with ME49 cysts and then challenged
133 with RH tach. in the presence or absence of anti-ASGM1. Anti-ASGM1-treated animals died much
134 sooner compared to untreated controls (Fig. 1D). These results showed that, in comparison with anti-
135 NK1.1 treatment, the administration of anti-ASGM1 during secondary *T. gondii* infection had a more
136 robust effect on the parasite dissemination and the death of mice. Since both antibody treatment
137 regimens effectively depleted NK cells (Supplementary Fig. 1D), this result suggested that anti-
138 ASGM1 may have depleted additional cell subsets necessary for the protection during secondary *T.*
139 *gondii* infection.

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141 *Anti-ASGM1 treatment depletes CD8+ and CD4+ T cells after secondary challenge of T. gondii*
142 *immunized mice*

143 *In vivo* antibody depletion of cells can have unexpected off target effects on other immune
144 cells (Nishikado et al., 2011). Several studies showed that T cell numbers were reduced after anti-
145 ASGM1 treatment during certain viral infections, including vaccinia virus, LCMV, reovirus and RSV
146 (Stitz et al., 1986, Doherty and Allan, 1987, Parker et al., 1988, Moore et al., 2008). One study
147 showed T cell numbers were reduced and the parasite burdens were increased after anti-ASGM1
148 treatment, however, no death was observed and it was not clear whether the parasite burdens were
149 increased due to the depletion of NK, T or both cell types (Moore et al., 2008). Based on these
150 previous findings, increased susceptibility of *T. gondii* immunized and anti-ASGM1 treated during
151 reinfection mice could be associated with the depletion of T cells. To address whether anti-ASGM1
152 depleted not only NK cells, but also T cells in CPS-immunized animals, the CD8+ and CD4+ T cell
153 frequencies and absolute numbers were measured by flow cytometry in the peritoneum (PEC) and
154 spleens of antibody treated or not treated immunized mice. Anti-ASGM1 treatment did not
155 significantly affect T cells in the peritoneum, but the numbers of CD4+ and CD8+ T cells were
156 significantly reduced in the spleen (Fig. 2A-E), demonstrating that in the absence of secondary
157 challenge anti-ASGM1 also depleted T cells. To address whether this treatment also depleted CD4+
158 and CD8+ T cells during secondary challenge, anti-ASGM1 treated or non-treated CPS-immunized
159 mice were challenged with RH and T cell numbers measured. Anti-ASGM1 administered upon RH
160 challenge significantly reduced CD8+ T cell frequency and numbers in both peritoneum and the
161 spleen compared to non-treated animals (Fig. 2A-E). In contrast, CD4+ T cells were not significantly
162 affected by anti-ASGM1 treatment during challenge infection. In contrast to anti-ASGM1, anti-
163 NK1.1 treatment did not reduce CD8+ and CD4+ T cell frequency and numbers in both peritoneum
164 and spleen (Supplementary Fig. 2). This result suggests that the increased susceptibility of mice

165 during anti-ASGM1 treatment upon secondary *T. gondii* infection could be associated with the
166 depletion of CD8+ and CD4+ T cells.

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168 ***ASGM1 is differentially expressed on CD8+ and CD4+ T cells in T. gondii immunized mice***

169 The previous result showed that anti-ASGM1 treatment depleted CD8+ and CD4+ T cells
170 with potentially CD8+ T cells being targeted more than CD4+ T cells. Previous studies indicate that
171 CD8+ and CD4+ T cells have higher levels of ASGM1 on their surface after viral infections (Slifka
172 et al., 2000, Moore et al., 2008) possibly making them susceptible to anti-ASGM1 depletion.
173 Therefore, we measured by flow cytometry whether ASGM1 was expressed on CD8+ and CD4+ T
174 cells after *T. gondii*-immunization in mice and how ASGM1 levels changed on T cells after challenge
175 infection. ASGM1 was detected on both cell types in immunized mice (Fig. 3A-F). After secondary
176 parasite infection, the frequency and number of ASGM1+ CD4+ T cells and number of ASGM1+
177 CD8+ T cells increased at the challenge site (Fig. 3C-E). The frequency of ASGM1+ CD8+ T cells in
178 peritoneum and spleen did not increase (Fig. 3 A-D). Interestingly, ASGM1 was expressed on a
179 much higher percentage of CD8+ T cells than CD4+ T cells in both PEC and spleen.

180

181 ***Anti-ASGM1 treatment depletes effector and effector memory CD8+ T cell subsets***

182 Previous studies reported that ASGM1 was differentially enriched among different CD8+ T
183 cell subsets. In naïve unimmunized mice central memory phenotype (CD44^{high}, CD62L+, CCR7+ and
184 CD122+) CD8+ T cells expressed more ASGM1 + than other T cell subsets (Kosaka et al., 2006,
185 Kosaka et al., 2007). Since the mice in our studies were immunized and the protection was lost when
186 the animals were treated with anti-ASGM1, this could suggest that ASGM1 might be enriched on *T.*
187 *gondii* specific memory T cell populations. Therefore, we next determined whether ASGM1 was
188 expressed on certain subsets of CD8+ T cells and whether these same subsets were preferentially
189 depleted by anti-ASGM1 treatment. CPS-immunized mice were treated with anti-ASGM1 in the

190 absence or presence of RH infection and T cell phenotype was assayed based on the expression of
191 CD44, a marker of antigen-experienced T cells (Budd et al., 1987) and CD62L, a marker of cells that
192 home to lymph nodes (Lefrancois, 2006). In CPS-immunized mice CD8⁺ T cells were distributed
193 between Tcm (central memory, CD44⁺CD62L⁺) > Tn (naïve, CD44⁻CD62L⁺) > Tem (effector
194 memory, CD44⁺CD62L⁻) > Tef (effector, CD44⁻CD62L⁻) (Fig. 4A) (Srivastava et al., 2018).
195 ASGM1 was mainly expressed on antigen-experienced CD44^{high} Tcm and Tem CD8⁺ T cells in CPS
196 immunized mice (Fig. 4B). After infection of CPS-immunized mice with RH, ASGM1 was also
197 expressed on Tef and Tn CD8⁺ T cells (Fig. 4B). Anti-ASGM1 reduced the frequency of all of the
198 CD8⁺ T cell subsets in CPS-immunized animals without RH challenge, except for the Tn subset,
199 which increased after the depletion. After RH challenge, there was an expected increase in Tef.
200 However, anti-ASGM1 treatment during this challenge infection significantly reduced effector T cell
201 populations, including Tef and Tem, while the frequency of naïve T cells increased. Interestingly,
202 among all T cell subsets, Tcm cells seemed resistant to anti-ASGM1 treatment. Our results indicate
203 that ASGM1 is enriched on different CD8⁺ T cell subsets after CPS immunization and secondary
204 infection. We also observe that anti-ASGM1 treatment preferentially depletes effector populations of
205 CD8⁺ T cells including Tem and Tef.

206 CD11a and CD49d on CD8⁺ T cells can be used as surrogate markers of *T. gondii*-specific
207 CD8⁺ T cells (Hwang et al., 2016). We therefore further explored whether *T. gondii*-specific
208 CD11a⁺CD49d⁺ CD8⁺ T cells were depleted by anti-ASGM1 treatment and expressed ASGM1. The
209 majority of CD8⁺ T cells were CD11a⁺CD49d⁺ and anti-ASGM1 treatment resulted in a depletion
210 of these cells in CPS-immunized mice (Fig. 4C). Anti-ASGM1 also depleted these cells during
211 secondary RH infection. Similarly to the previously observed increase in naïve CD8⁺ T cells, non-*T.*
212 *gondii* specific CD11a⁻CD49d⁻ CD8⁺ T cells increased after the depletion. In accordance to the
213 depletion effect of anti-ASGM1, CD11a⁺CD49d⁺ CD8⁺ T cells were highly enriched in ASGM1
214 expression in CPS-immunized mice and in the presence of RH challenge (Fig. 4D). The result

215 suggested that ASGM1 is enriched on *T. gondii*-specific CD8⁺ T cells and these cells were depleted
216 by anti-ASGM1 treatment.

217

218 ***Anti-ASGM1 treatment depletes activated CD8⁺ and CD4⁺ T cells***

219 The above data identified that ASGM1 was expressed on a majority of CD8⁺ T cells that
220 expressed surrogate markers of *T. gondii* specificity (CD11a and CD49d) and effector CD8⁺ T cells
221 were depleted the most by anti-ASGM1 treatment. One study showed that ASGM1⁺ CD8⁺ T cells
222 produced more IFN γ than ASGM1⁻ CD8⁺ T cells upon stimulation with IL-12 (+IL-2) *in vitro*
223 (Kosaka et al., 2006). IL-12 is produced after *T. gondii* infection and is essential for protection *via*
224 induction of IFN γ (Gazzinelli et al., 1993, Hunter et al., 1995b, Yap et al., 2000). To find whether the
225 increased susceptibility to the secondary infection during anti-ASGM1 treatment could be associated
226 with the depletion of IFN γ -producing T cells, CPS-immunized mice were challenged with RH and
227 their CD8⁺ and CD4⁺ T cell functionality was assessed after treatment with anti-ASGM1 compared
228 to non-treated controls. Peritoneal CD8⁺ T cells were stimulated with TLA (Fig. 5A-B) or
229 PMA/ionomycin (Fig. 5C-D) and stained intracellularly for IFN γ and Granzyme B and single
230 function as well as polyfunctional responses were measured by flow cytometry. Anti-ASGM1
231 treatment significantly reduced the frequency and numbers of IFN γ ⁺, Granzyme B⁺ and
232 polyfunctional IFN γ ⁺GranzymeB⁺ CD8⁺ T cells in the peritoneum (Fig. 5A-D). In addition, there
233 was a significant reduction in frequency and number of IFN γ ⁺ CD4⁺ T cells after anti-ASGM1
234 treatment at the site of infection. Thus, anti-ASGM1 treatment depletes activated CD4⁺ and CD8⁺ T
235 cells during secondary challenge of immunized mice.

236

237 ***IFN γ supplementation prolongs the survival of reinfected and anti-ASGM1 treated mice***

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239 Anti-ASGM1 treatment during reinfection increased the rate of mouse death and depleted
240 IFN γ + CD8+ and CD4+ T cells. To address whether the lethal effect of the antibody could be
241 counterbalanced by IFN γ supplementation, recombinant IFN γ was administered during anti-ASGM1
242 treatment of CPS-immunized and RH infected mice. The cytokine supplementation had a therapeutic
243 effect and significantly prolonged the survival of anti-ASGM1 treated mice (Fig. 6). Therefore, the
244 detrimental effect of anti-ASGM1 treatment on the health and survival of mice could be associated
245 with the depletion of IFN γ -producing CD8+ and CD4+ T cells in addition to IFN γ -producing NK
246 cells.
247

248 **Discussion**

249 Two commonly used for NK cell depletion antibodies exhibited different effects on the
250 survival of mice infected with *T. gondii*. In contrast to anti-NK1.1, *in vivo* anti-ASGM1 treatment
251 during secondary *T. gondii* infection led to a higher increase in parasite burdens and was lethal. This
252 suggested that anti-ASGM1 could have an off-target effect, such as depletion of a cell type other than
253 NK cells necessary for protection. In line with that, we found that anti-ASGM1 treatment resulted in
254 the depletion of T cells. This is important because the anti-ASGM1 antibody is widely used to
255 deplete NK cells (Ferreira et al., 2018, Haspesslagh et al., 2018). Many studies on the role of NK cells
256 in *T. gondii* infection also relied on the use of anti-ASGM1 (Hunter et al., 1994, Haque et al., 1999,
257 Goldszmid et al., 2012). Our data showed that this treatment is not a specific approach to address the
258 role of NK cells during *T. gondii* infection. Our findings emphasize that anti-ASGM1 should be used
259 with caution and in combination with other NK cell-specific methods.

260 ASGM1+ T cells could be essential for protection against *T. gondii* infection. A few previous
261 studies showed that anti-ASGM1 treatment did not deplete T cells in naïve mice (Nishikado et al.,
262 2011), but negatively affected T cells in the mice challenged with viruses (reovirus, lymphocytic
263 choriomeningitis virus (LCMV), vaccinia, respiratory syncytial virus (RSV)) and alloantigens (Stitz
264 et al., 1986, Slifka et al., 2000, Moore et al., 2008). While the treatment was shown to deplete viral-
265 activated T cells (Stitz et al., 1986, Slifka et al., 2000), none of those studies reported the death of
266 mice after the treatment. A single study showed T cell depletion by anti-ASGM1 and also addressed
267 the effect of the treatment on the health outcome (Moore et al., 2008). There, anti-ASGM1 treatment
268 was associated with a higher parasite burden after infection with RSV; however, all mice survived
269 and had lower weight loss than control mice, suggesting that ASGM1+ cells also contributed to RSV-
270 induced illness (Moore et al., 2008). Whether this health outcome was caused by depletion of
271 ASGM1+ T cells or NK cells was not specified. The lethality of anti-ASGM1 treatment during

272 secondary *T. gondii* infection suggested that ASGM1⁺ T cells could be more essential for protection
273 against infection with *T. gondii* parasites than viruses.

274 ASGM1 could be a marker of specific T cell subsets. In naïve unimmunized mice ASGM1
275 was expressed on Tcm (defined as CD44^{high}, CD62L⁺, CCR7⁺) CD8⁺ T cells (Kosaka et al., 2007).
276 We found that in *T. gondii*-immunized mice ASGM1 was expressed on memory phenotype CD8⁺ T
277 cells, including both Tem (CD44^{high} CD62L⁻) and Tcm (CD44^{high} CD62L⁺) (Srivastava et al., 2018).
278 After reinfection, ASGM1 was distributed between all CD8⁺ T cell subsets, including Tef and Tn.
279 Importantly, effector phenotype CD8⁺ T cells (Tef and Tem) were more susceptible to anti-ASGM1
280 treatment than Tn and Tcm. A previous study also suggested that effector or memory T cells could be
281 more susceptible to the treatment than naïve T cells (Stitz et al., 1986). Virus-specific T cell activity
282 was reduced when anti-ASGM1 was administered after and not before infections with vaccinia or
283 LCMV (Stitz et al., 1986). Furthermore, we found that anti-ASGM1 treatment selectively eliminated
284 CD8⁺ T cells that provided effector functions (IFN γ and GranzymeB) in response to reinfection.
285 Similar to our findings, the majority of ASGM1⁺ CD8⁺ T cells produced IFN γ and were virus-
286 specific after viral infections (Slifka et al., 2000, Moore et al., 2008). It is possible that ASGM1
287 could be expressed on *T. gondii*-specific CD8⁺ T cells. In support of this, we found that ASGM1 was
288 enriched on CD8⁺ T cells that expressed surrogate markers of *T. gondii*-specificity (CD11a and
289 CD49d) (Hwang et al., 2016). Whether or not ASGM1 is a marker *T. gondii*-specific CD8⁺ T cells
290 would be important to confirm using MHC tetramer staining (Marple et al., 2017). Alternatively,
291 ASGM1 could be expressed on cytokine-activated T cells. ASGM1⁺ CD8⁺ T cells from naïve mice
292 were shown to produce more IFN γ than ASGM1⁻ CD8⁺ T cells after IL-12 (+IL-2) stimulation
293 (Kosaka et al., 2007). After *T. gondii* infection IL-12 is abundantly produced (Gazzinelli et al., 1993,
294 Khan et al., 1994, Hunter et al., 1994, Hunter et al., 1995b). Therefore, IL-12 could induce activation
295 of ASGM1⁺ CD8⁺ T cells after *T. gondii* infection. Elucidation of the stimuli necessary for induction

296 of ASGM1 expression on T cells could provide the cues on its functional importance and reveal its
297 potential in use for immunotherapies.

298 CD4⁺ T cells were negatively affected by anti-ASGM1 treatment of *T. gondii*-immunized
299 mice. In addition to CD8⁺ T cells, total splenic and IFN γ -producing peritoneal CD4⁺ T cells were
300 reduced after the treatment. This is in accordance to an early study on NK cell role in protection
301 against *T. gondii* reinfection in the absence of CD8⁺ T cells (Denkers et al., 1993). *T. gondii*-
302 immunized CD8⁺ T cell deficient mice ($\beta 2m^{-/-}$) survived lethal reinfection and died after anti-NK1.1
303 or anti-ASGM1 treatments. Interestingly, mice died faster after anti-ASGM1 treatment as compared
304 to anti-NK1.1. This result implied that these treatments could have different effects on the cells other
305 than CD8⁺ T cells and NK cells, potentially CD4⁺ T cells (Denkers et al., 1993).

306 The functional role of ASGM1-mediated signaling in T cells is not known. One possibility is
307 that ASGM1 could be functionally relevant for T cell biology as a part of lipid rafts. ASGM1 is a
308 glycolipid that is enriched in lipid rafts, small dynamic ordered domains that compartmentalize
309 cellular processes (Pike, 2006). Upon T cell activation, TCR engagement leads to an aggregation of
310 these domains (Dinic et al., 2015). This aggregation then facilitates TCR signaling by bringing
311 molecules needed for TCR closer to each other. It was found earlier that engagement of GM1 by
312 cholera toxin leads to an activation of T cells (Janes et al., 1999). ASGM1 is a derivative of GM1 and
313 colocalizes with GM1 in the lipid rafts on T cells (Moore et al., 2008). ASGM1 could also be
314 involved in TCR signaling and T cell activation. Whether the presence and quantity of ASGM1 in
315 lipid rafts affects T cell functionality is not known. In addition, whether T cells with enriched
316 ASGM1 lipid raft content have a superior ability for TCR signaling and activation as compared to
317 ASGM1 negative T cells remains to be investigated. That would be important to address as it could
318 be useful for the future design of T cell-based immunotherapies.

319 As a further point, the presence of ASGM1 could be advantageous for T cell survival during
320 *T. gondii* infection. This is because ASGM1 is an asialylated derivative of GM1 (Moore et al., 2008).

321 Due to an abundance of sialic acids on the cell surfaces, many viruses and bacteria use host-sialylated
322 structures for binding and recognition (Schwegmann-Wessels et al., 2011, Neu et al., 2011).
323 Apicomplexan parasites, including *T. gondii* and *Plasmodium falciparum*, also employ recognition of
324 sialic acids as part of interactions with the hosts (Monteiro et al., 1998, Blumenschein et al., 2007,
325 Gaur and Chitnis, 2011). The *T. gondii* microneme proteins bind to sialylated glycoconjugates on
326 host cells and facilitate invasion (Blumenschein et al., 2007, Friedrich et al., 2010). As any other
327 nucleated cells, T cells can get *T. gondii*-infected and killed by parasites or lymphocytes. Therefore,
328 one possible mechanism by which ASGM1+ CD8+ T cells could have an advantage in survival over
329 ASGM1- CD8+ cells during *T. gondii* infection could be their reduced sialic acid content and thus
330 resistance to being infected by the parasites.

331 In summary, our data showed that anti-ASGM1 antibody treatment is not a specific approach
332 to study the role of NK cells during *T. gondii* infection. This method could be informative when used
333 with caution and in combination with other NK cell specific methods. We demonstrated that in *T.*
334 *gondii*-immunized mice certain subsets of CD8+ and CD4+ T cells express ASGM1 and get depleted
335 by anti-ASGM1. Elimination of T cells correlated with increased susceptibility to secondary *T.*
336 *gondii* infection.

337

338 **Materials and Methods**

339

340 **Mice.** C57BL/6 (B6) mice were purchased from The Jackson Laboratory. Animals were housed in
341 specific pathogen-free conditions at the University of Wyoming Animal Facility.

342 **Ethics Statement.** This study was carried out in strict accordance following the recommendations in
343 the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The
344 protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the
345 University of Wyoming (PHS/NIH/OLAW assurance number: A3216-01).

346 ***T. gondii* parasites, infections.** Tachyzoites (tach.) of RH and RH Δ *cpsII* (*cpsI-1*, CPS) strains
347 (kindly provided by Dr. Bzik, Dartmouth College, NH) were cultured by serial passage in human
348 fetal lung fibroblast (MRC5) cell monolayers in complete DMEM (supplemented with 0.2 mM uracil
349 for *cpsI-1* strain). The parasites were purified by filtration through a 3.0- μ m filter (Merck Millipore
350 Ltd.), then washed with phosphate-buffered saline (1 X PBS) and administered intraperitoneally (i.p.)
351 1×10^3 RH tach. or 1×10^6 *cpsI-1* tach. The brains of 5 wk ME49 infected CBA mice were harvested
352 and 100 ME49 cysts were administered intragastrically (i.g.).

353 **Real-time PCR for parasite burden.** DNA was extracted from entire PECs and spleens harvested
354 from infected mice using a Qiagen DNeasy Blood & Tissue Kit (Qiagen Sciences). Parasite DNA
355 from 600 ng of PECs, 800 ng of splenic or brain tissue DNA was amplified using primers specific for
356 the *T. gondii* B1 gene (forward primer GGAAGTGCATCCGTTTCATG and reverse primer
357 TCTTTAAAGCGTTCGTGGTC) at 10 pmol of each per reaction (Integrated DNA Technologies) by
358 real-time fluorogenic PCR using SsoAdvancedTM Universal IT SYBR® Green SMx (BIO-RAD) on a
359 CFX ConnectTM Real-Time System cycler (BIO-RAD). Parasite equivalents were determined by
360 extrapolation from a standard curve.

361

362 ***In vivo treatments.*** Anti-NK1.1 (clone PK136, Bio X cell) was diluted in 1X PBS and administered
363 i.p. at 200 µg one day before (d -1), the day of infection (d 0) and every other day after for the
364 maximum duration of 21 d. Anti-Asialo GM1 (Rabbit, FUJIFILM Wako) was diluted in 1 mL sterile
365 H₂O and administered i.p. at 50 µl on d -1, 0 and every third day after.

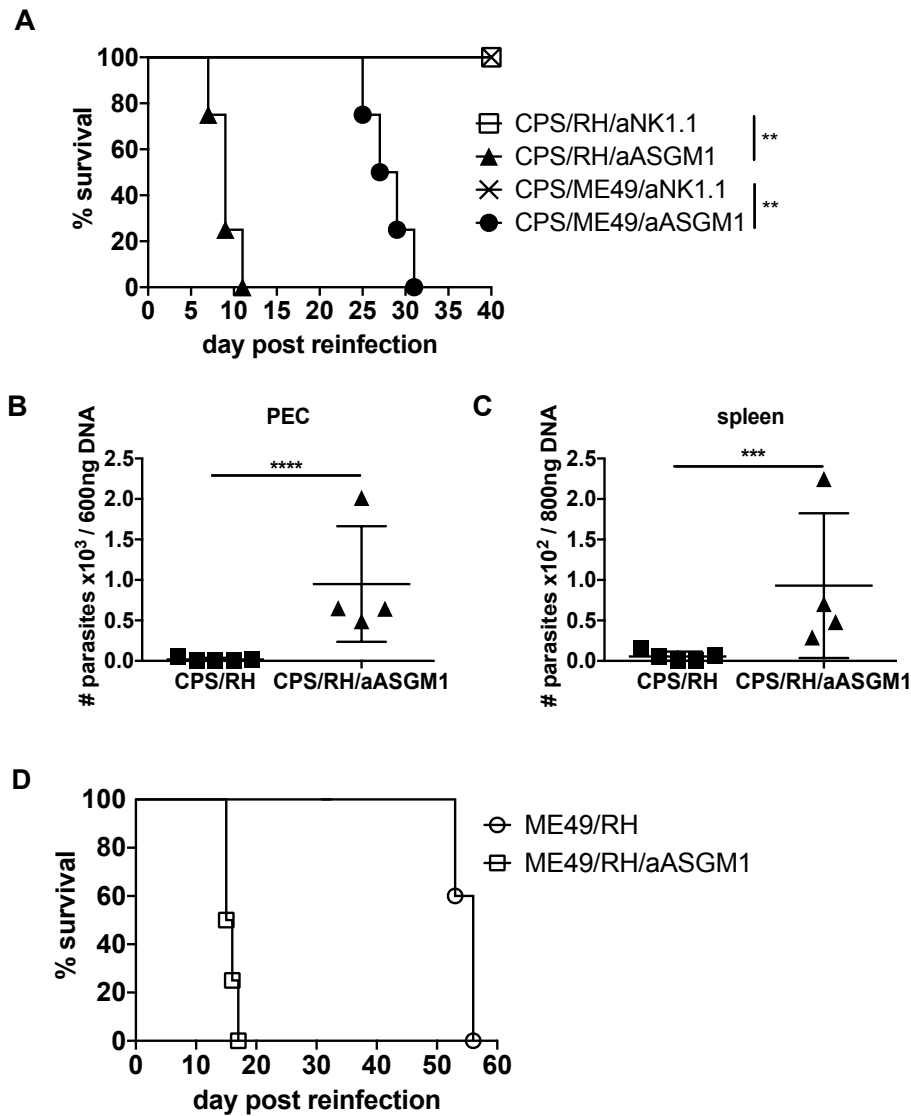
366 ***Flow cytometry.*** Peritoneal exudate cells (PEC) and splenocytes were harvested as previously
367 described (Ivanova et al., 2016) and plated at 0.5-1.5x10⁶ cells/well. For surface staining, cells were
368 washed twice with 1X PBS and stained for viability in 1 X PBS using Fixable Live/Dead Aqua dye
369 (Invitrogen) for 30 min. After washing with 1 X PBS, surface staining was performed using
370 antibodies diluted in stain wash buffer (SWB, 2% FBS in 1 X PBS and EDTA) for 25 min on ice. For
371 functional assays, cells were stimulated with 20 µg/mL Toxoplasma Lysate Antigen (TLA) for 8 h in
372 complete Isclove's media and then treated with 0.7 X PTIC for 4 h. For additional functional assays,
373 cells were incubated for 8 h in complete Isclove's media and then treated with 0.7 X Cell Stimulation
374 Cocktail containing PMA/ionomycin and PTIC (eBioscience, ThermoFisher) for 4 h. After live dead
375 and surface staining, cells were fixed and permeabilized for 1 h on ice (BD bioscience, Fix/Perm
376 solution) followed by intracellular staining in 1 X permeabilization wash buffer (BD) with anti-IFN γ ,
377 anti-TNF α , anti-Granzyme B antibody for 40 min. After washing twice with 1X PBS, cells were
378 resuspended in 1X PBS and acquired using Guava easyCyte HT (Millipore). All samples were
379 analyzed with FlowJo software (Tree Star). The antibodies purchased from Biolegend were: CD3
380 (17A2), CD4 (RM4-5), CD8b (YTS156.7.7), NK1.1 (PK136), Asialo-GM1 (Poly21460), CD44
381 (IM7), CD62L (MEL-14), CD11a (M17/4), CD49d (R1-2). The antibodies purchased from
382 eBioscience/ThermoFisher were: IFN γ (XMG1.2), TNF α (Mab11), Granzyme B (NGZB).

383 ***Statistical analysis.*** Statistical analysis was performed using Prism 7.0d (GraphPad) or Microsoft
384 Excel 2011. Significant differences were calculated using either unpaired Student's t-test or analysis
385 of variance (ANOVA). The log-rank (Mantel-Cox) test was used to evaluate survival rate.
386 Significance is denoted where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

387 **Acknowledgments**

388 We thank Rida Fatima, Tiffany Mundhenke, Sally Murray and Ryan Krempels for assistance.

389 **Figure legends**



390

391 **Figure 1. Anti-ASGM1 treatment during secondary *T. gondii* infection leads to rapid death and**

392 **increased parasite burdens. (A-C)** Immunized i.p. with 1 X 10⁶ CPS tach. C57BL/6 mice were five

393 to six weeks later infected i.p. with 1 X10³ RH tach. (A-C) or i.g. with 100 ME49 brain cysts (A) and

394 treated with anti-NK1.1 (PK136) (A) or anti-ASGM1 (A-C) during secondary infections. (A) The

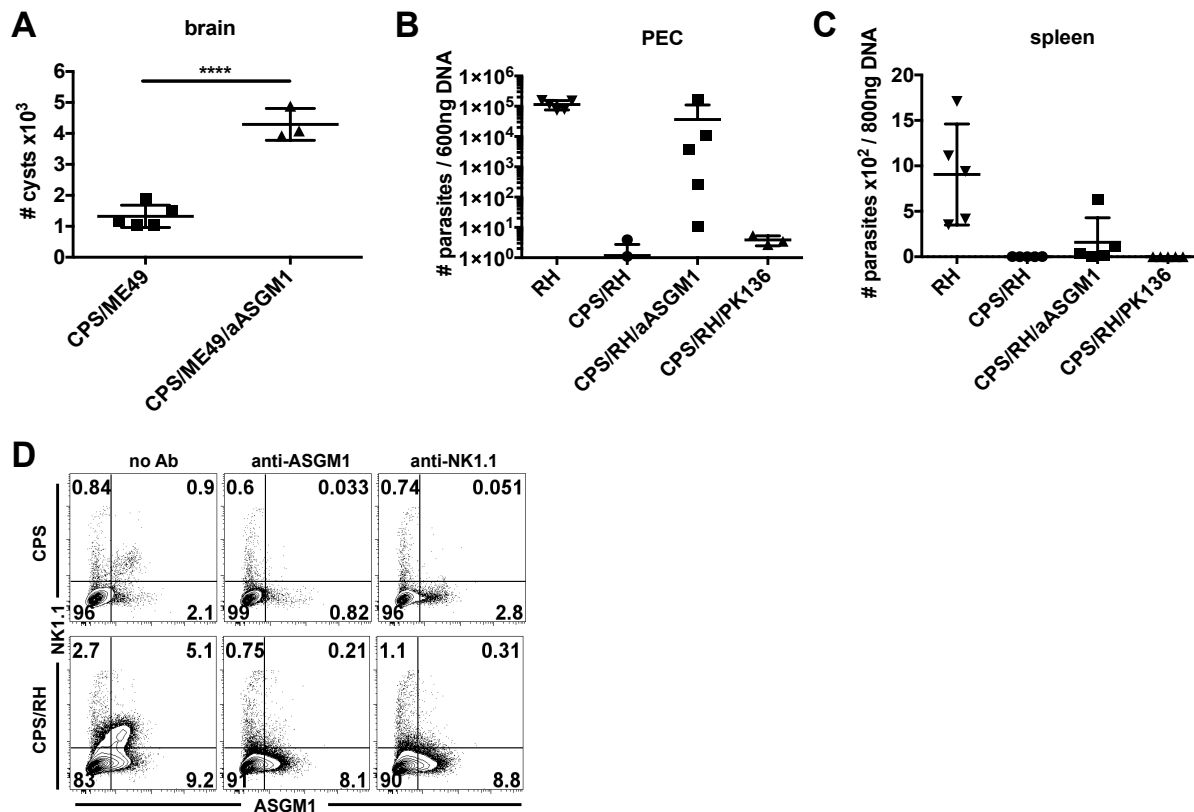
395 survival curve represents one of three to four experiments, n=4 to 5. (B and C) The parasite burdens

396 measured by real time PCR for *T. gondii* B1 gene 8 days after RH infection in PEC (B) and spleen

397 (C). The data represent one of two experiments, n=4 to 5. (D) Infected with 10 ME49 cysts i.p.

398 C57BL/6 mice were four weeks later infected with 1 X10³ RH tach. i.p. and treated with anti-

399 ASGM1 upon reinfection. The survival curve represents one of two experiments, n=3 to 5. The log-
400 rank (Mantel-Cox) test was used to evaluate survival rates. Ordinary one-way ANOVA was used to
401 evaluate the parasite burdens. Data are mean \pm SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



402

403 **Figure 2. Anti-ASGM1 and anti-NK1.1 treatments during secondary *T. gondii* infection. (A-D)**

404 Immunized i.p. with 1×10^6 CPS tach. C57BL/6 mice were five to six weeks later infected i.g. with

405 20 ME49 brain cysts (A) or i.p. with 1×10^3 RH tach. (B-D) and treated with anti-ASGM1 (A-D) or

406 anti-NK1.1 (PK136) (B-D) during secondary infection. (A) The brain cysts were counted after ME49

407 reinfection at 35 dpi in control mice and immediately after the death in anti-ASGM1 treated mice.

408 The data represent 1 experiment, n=3 to 5. (B-C) The parasite burdens were measured by real time

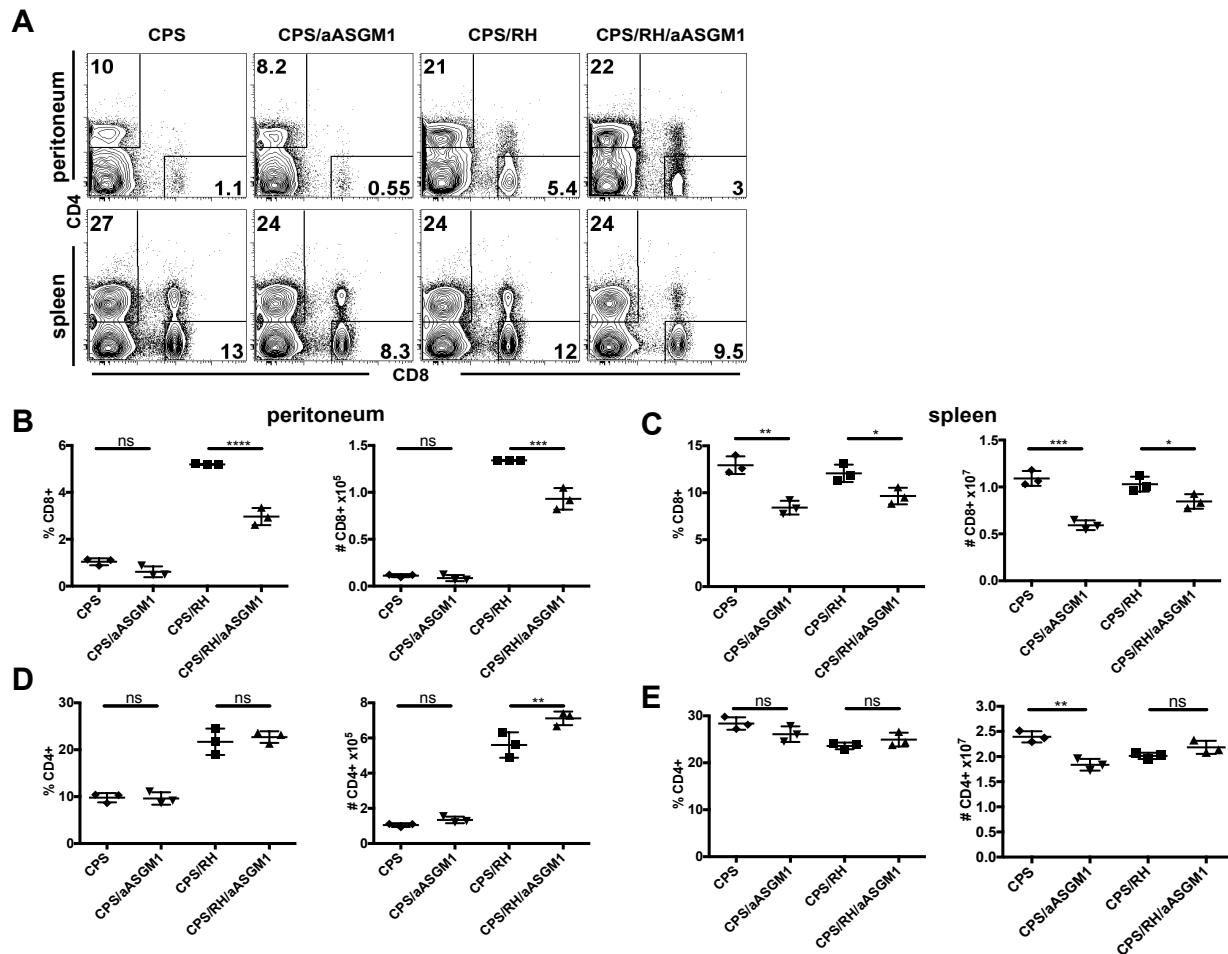
409 PCR for *T. gondii* B1 gene 8 days after RH reinfection in PEC (B) and spleen (C). The data represent

410 one experiment, n=3 to 5. (D) NK cell (gated as NK1.1+ASGM1+ live lymphocytes) depletion after

411 anti-ASGM1 or anti-NK1.1 treatment was measured by flow cytometry. Representative contour plots

412 from one of two experiments, n=3 to 4. Ordinary one-way ANOVA, data are mean \pm SD,

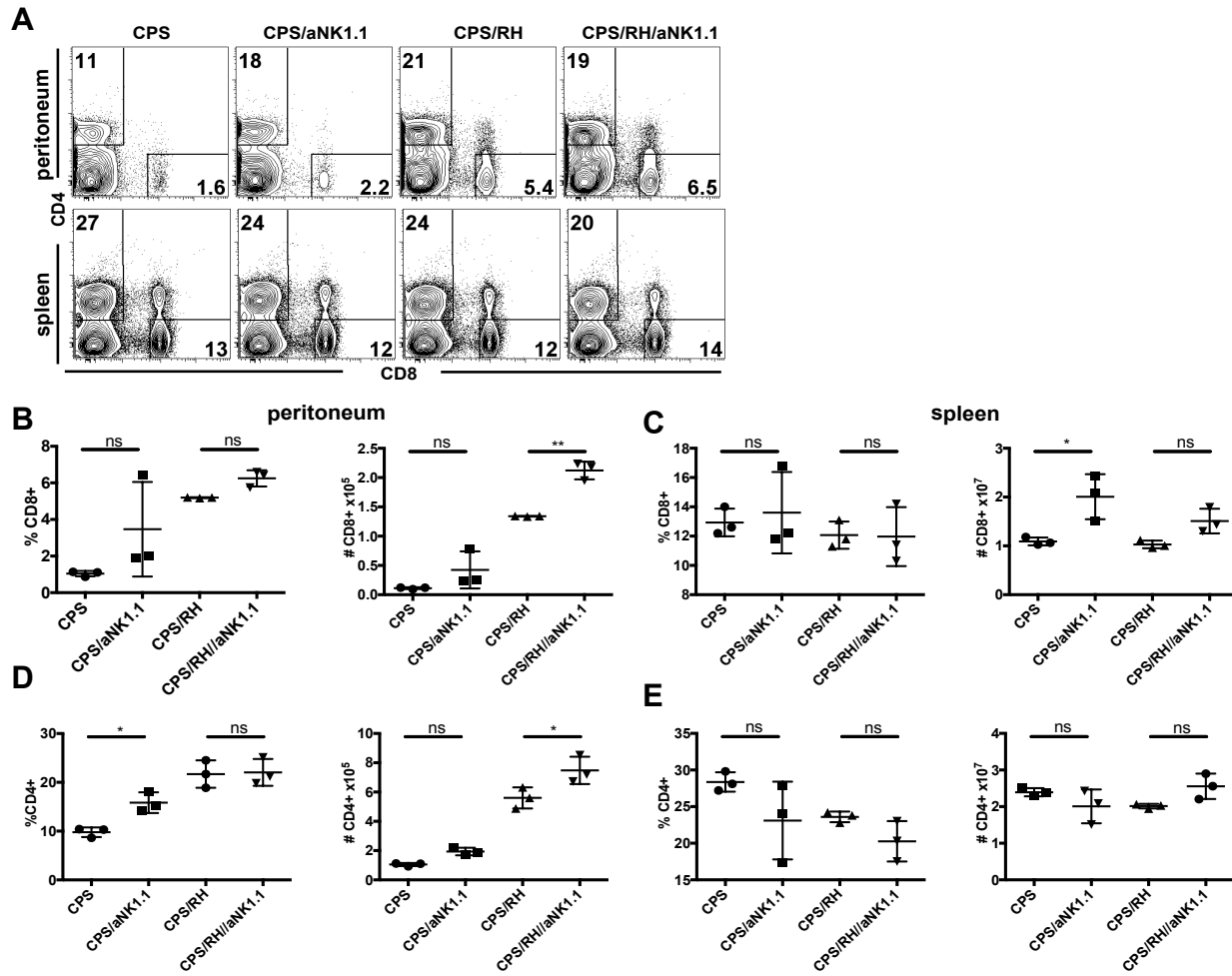
413 ****p<0.0001.



414

415 **Figure 3. Anti-ASGM1 treatment depletes CD8+ and CD4+ T cells in *T. gondii*-immunized**
 416 **mice.** (A-E) C57BL/6 mice were immunized i.p. with 1×10^6 CPS tach. and infected i.p. with 1
 417 $\times 10^3$ RH tach. and treated with anti-ASGM1 antibody. PECs and spleens were analyzed by flow
 418 cytometry at day 3 post RH infection. (A) Representative contour plots of CD8+ and CD4+ T cells
 419 (gated on NK1.1- live lymphocytes). The percentages and numbers of CD8+ T cells per PEC (B) or
 420 spleen (C). The percentages and numbers of CD4+ T cells per PEC (D) or spleen (E). Data are
 421 representative of one of three experiments, n=3 to 4. Data are mean \pm SD. *p<0.05, **p<0.01,
 422 ***p<0.001, ****p<0.0001, ordinary one-way ANOVA.

423



424

425 **Figure 4. Anti-NK1.1 treatment does not deplete CD8+ and CD4+ T cells in *T. gondii*-**

426 **immunized mice.** C57BL/6 mice were immunized i.p. with 1×10^6 CPS tach. and infected i.p. with

427 1×10^3 RH tach. six weeks later and treated with anti-NK1.1 antibody. PECs and spleens were

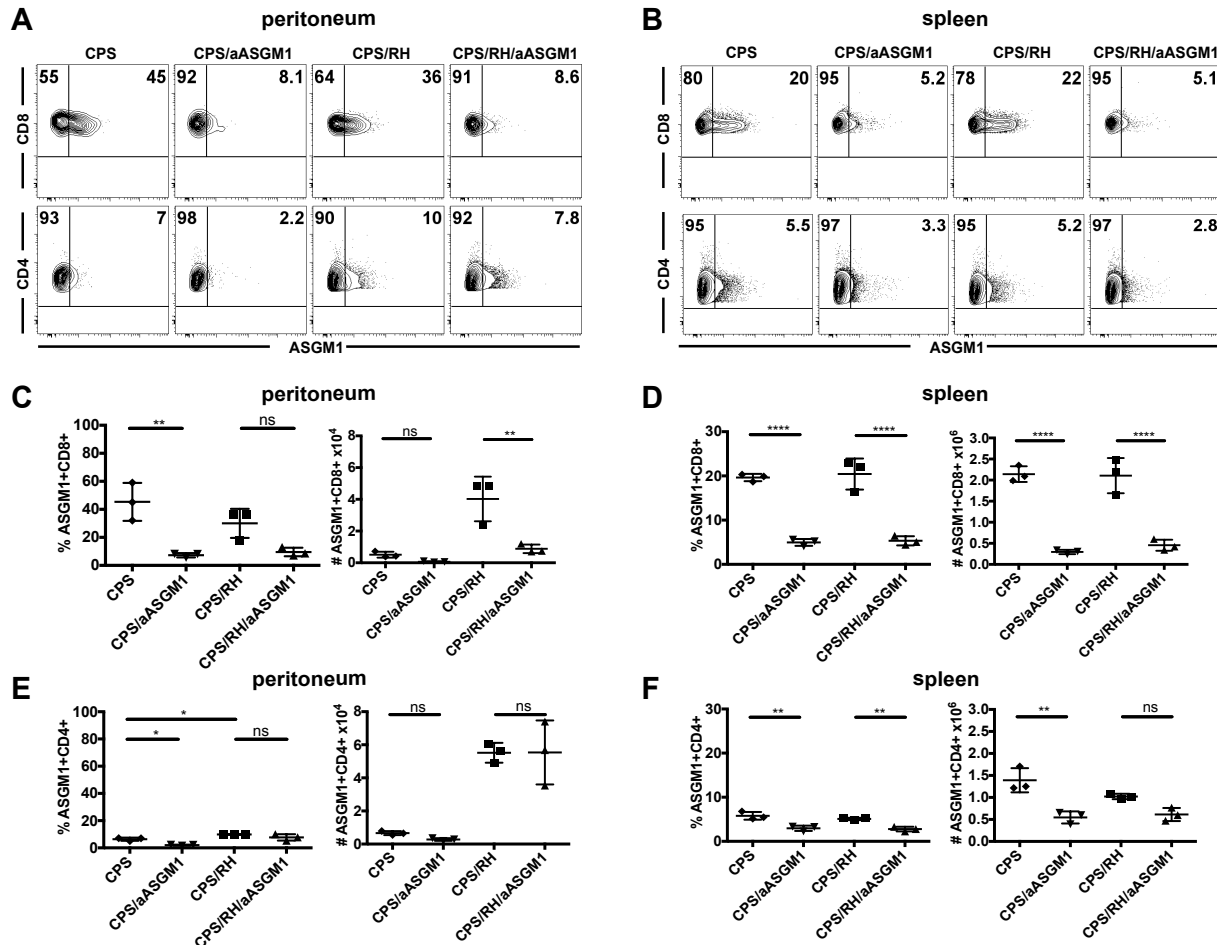
428 analyzed by flow cytometry at day 3 post RH infection. (A) Representative contour plots of CD8+

429 and CD4+ T cells (gated on NK1.1- live lymphocytes). The percentages and numbers of CD8+ T

430 cells per PEC (B) or spleen (C). The percentages and numbers of CD4+ T cells per PEC (D) or

431 spleen (E). Data are representative of one of three experiments, n=3 to 4. Data are mean \pm SD.

432 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ordinary one-way ANOVA.



433

434 **Figure 5. CD8⁺ and CD4⁺ T cells differentially express ASGM1 in *T. gondii* immunized mice.**

435 (A-D) C57BL/6 mice were immunized i.p. with 1 X 10⁶ CPS tach. and infected i.p. with 1 X 10³ RH

436 tach. six weeks later. PECs and spleens were analyzed by flow cytometry at day 3 post RH infection.

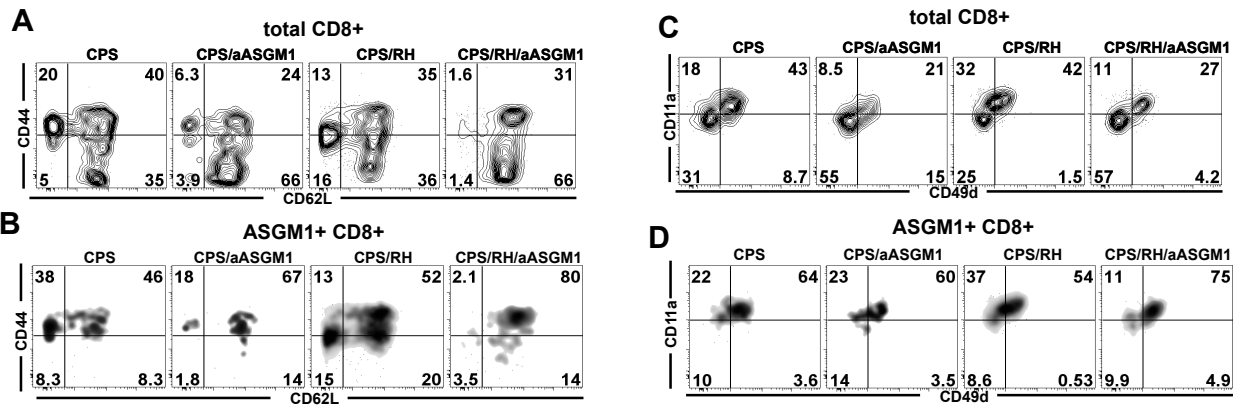
437 (A) Representative contour plots of ASGM1 expression on CD8⁺ and CD4⁺ T cells (gated on

438 NK1.1- live lymphocytes) in peritoneum (A) or spleen (B). The percentages and numbers of CD8⁺ T

439 cells per peritoneum (C) or spleen (D). The percentages and numbers of CD4⁺ T cells per

440 peritoneum (E) or spleen (F). Data are representative of one of three experiments, n=3 to 4. Data are

441 mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ordinary one-way ANOVA.



442

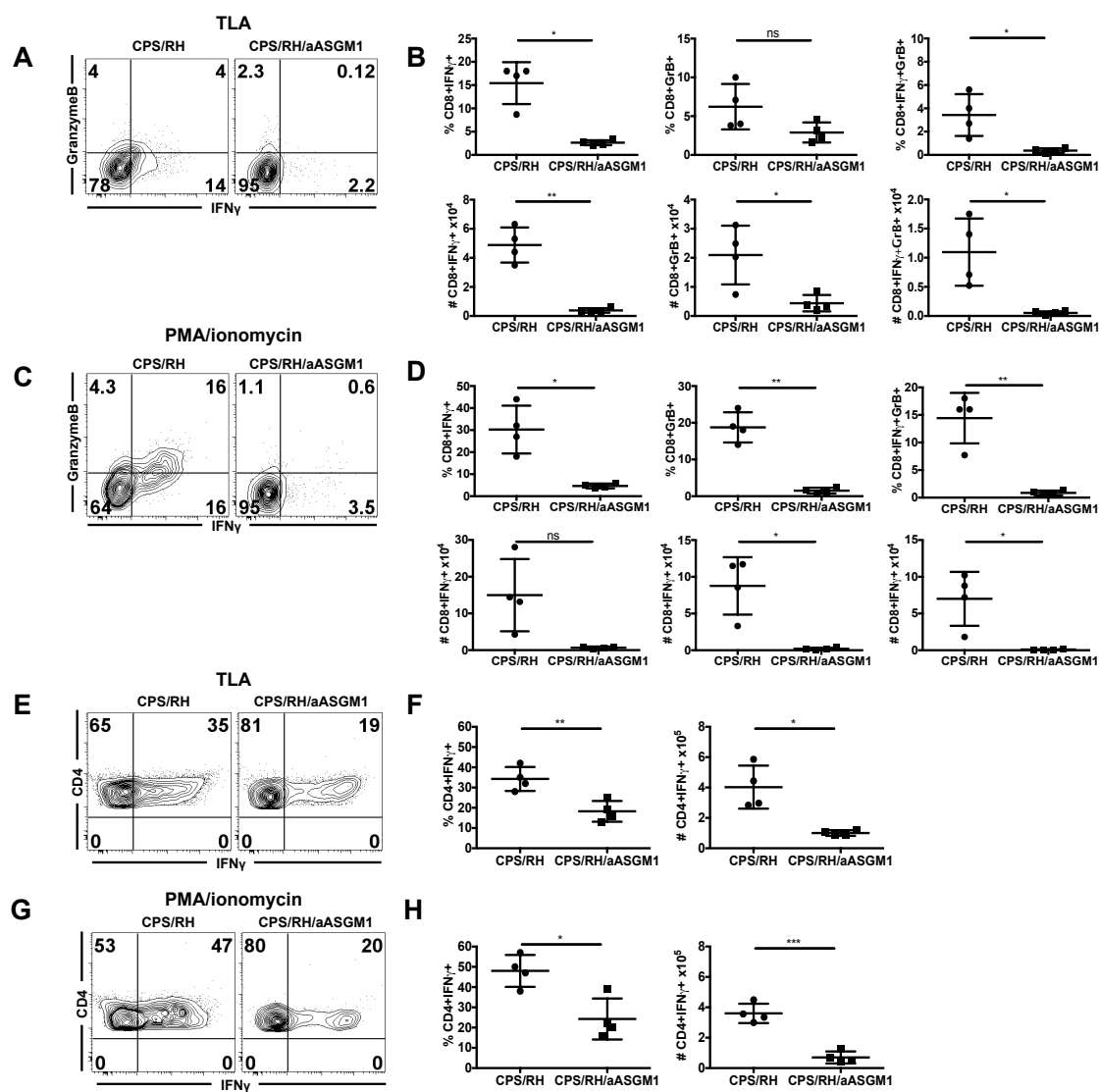
443 **Figure 6. Anti-ASGM1 treatment depletes effector and effector memory CD8+ T cell subsets.**

444 (A-D) C57BL/6 mice were immunized i.p. with 1×10^6 CPS tach. and infected i.p. with 1×10^3 RH
 445 tach. six weeks later. PECs were analyzed by flow cytometry at day 3 post RH infection.

446 Representative contour plots of CD44 and CD62L expression on total CD8+ T cells (gated on
 447 NK1.1- live lymphocytes) (A) and on ASGM1+ CD8+ T cells (B). Representative contour plots of

448 CD11a and CD49d expression on total CD8+ T cells (gated on NK1.1- live lymphocytes) (C) and on
 449 ASGM1+ CD8b+ T cells (D). Data are representative of one of two experiments, n=3 to 4. Data are

450 mean \pm SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ordinary one-way ANOVA.



451

452 **Figure 7. Anti-ASGM1 treatment depletes activated CD8+ and CD4+ T cells. (A-H) C57BL/6**

453 mice were immunized i.p. with 1×10^6 CPS tach. and infected i.p. with 1×10^3 RH tach. six weeks

454 later. PECs were analyzed by flow cytometry at day 3 post RH infection. Representative contour

455 plots of intracellular staining for IFN γ and Granzyme B on CD8+ T cells (gated on CD8b+ live

456 lymphocytes) after TLA stimulation (A) and in the presence of PMA/ionomycin stimulation (C). The

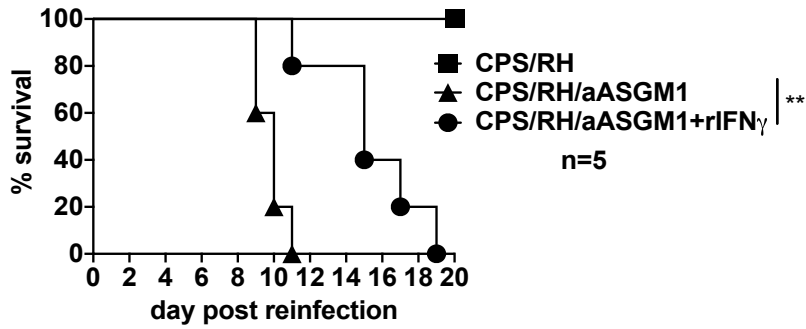
457 percentages and numbers of IFN γ +, GranzymeB+ and IFN γ +GranzymeB+ CD8+ T after TLA (B) or

458 PMA/ionomycin stimulation (D). Representative contour plots of intracellular staining for IFN γ on

459 CD4+ T cells (gated on live lymphocytes) after TLA stimulation (E) and in the presence of

460 PMA/ionomycin (G). The percentages and numbers of IFN γ + CD4+ T after TLA (F) or

461 PMA/ionomycin stimulation (**H**). Data are representative of 1 of 2 experiments, n=4 to 3. Data are
462 mean \pm SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ordinary one-way ANOVA.



463

464 **Figure 8. IFN γ supplementation prolongs the survival of *T. gondii* reinfected and anti-ASGM1**
465 **treated mice.** Immunized i.p. with 1×10^6 CPS tach. C57BL/6 mice were five to six weeks later
466 infected i.p. with 1×10^3 RH tach. and treated with anti-ASGM1 during secondary infection in the
467 presence of recombinant IFN γ supplementation. The survival curve represents 1 experiments, n=5.
468 The log-rank (Mantel-Cox) test was used to evaluate survival rates, **p<0.01.

469

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