CD4+ T cells are trigger and target of the glucocorticoid response that prevents lethal immunopathology in toxoplasma infection

David G. Kugler,[1,3] Paul R. Mittelstadt,[2] Jonathan D. Ashwell,[2] Alan Sher,[1] and Dragana Jankovic[1]

[1]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases; [2]Laboratory of Immune Cell Biology, Center for Cancer Research, National Cancer Institute; and [3]The Johns Hopkins University/National Institutes of Health Graduate Partnership Program; National Institutes of Health, Bethesda, MD 20892

Synthetic glucocorticoids (GCs) are commonly used in the treatment of inflammatory diseases, but the role of endogenous GCs in the regulation of host-protective immune responses is poorly understood. Here we show that GCs are induced during acute *Toxoplasma gondii* infection and directly control the T cell response to the parasite. When infected with toxoplasma, mice that selectively lack GC receptor (GR) expression in T cells ($GR^{lck\text{-}Cre}$) rapidly succumb to infection despite displaying parasite burdens indistinguishable from control animals and unaltered levels of the innate cytokines IL-12 and IL-27. Mortality in the $GR^{lck\text{-}Cre}$ mice was associated with immunopathology and hyperactive Th1 cell function as revealed by enhanced IFN-γ and TNF production in vivo. Unexpectedly, these CD4+ T lymphocytes also overexpressed IL-10. Importantly, CD4+ T cell depletion in wild-type or $GR^{lck\text{-}Cre}$ mice led to ablation of the GC response to infection. Moreover, in toxoplasma-infected $RAG^{-/-}$ animals, adoptive transfer of CD4+ T lymphocytes was required for GC induction. These findings establish a novel IL-10–independent immunomodulatory circuit in which CD4+ T cells trigger a GC response that in turn dampens their own effector function. In the case of *T. gondii* infection, this self-regulatory pathway is critical for preventing collateral tissue damage and promoting host survival.

CORRESPONDENCE
Dragana Jankovic:
DJankovic@niaid.nih.gov
OR
Alan Sher:
ASher@niaid.nih.gov

Abbreviations used: Ab, antibody; Ag, antigen; AST, aspartate aminotransferase; BFA, brefeldin A; CK, creatine kinase; Dex, dexamethasone; GC, glucocorticoid; GR, GC receptor; HPRT, hypoxanthine guanine phosphoribosyltransferase; ICS, intracellular cytokine staining; MFI, mean fluorescence intensity; PEC, peritoneum exudate cell.

The protective antimicrobial immune response, in addition to generating appropriate effector functions, must also incorporate mechanisms for self-regulation to prevent bystander damage to host tissue. Several mechanisms have been identified that participate in effector CD4 T cell regulation. These are thought to be mediated primarily by immunosuppressive cytokines and/or inhibitory surface molecules (Bluestone, 2011).

An excellent example of host-protective negative regulation of CD4 T cell function occurs during the Th1 response to *Toxoplasma gondii*, an intracellular protozoan parasite. This IL-12–driven response results in production of high levels of IFN-γ and TNF that efficiently control parasite replication in both hematopoietic and nonhematopoietic cells (Yap and Sher, 1999). Nevertheless, the exuberant cytokine production occurring during *T. gondii* infection can also be host-detrimental, an outcome first documented in acutely infected $IL\text{-}10^{-/-}$ mice that while successfully controlling parasite growth succumb to cytokine storm–mediated immunopathology (Gazzinelli et al., 1996). Subsequent studies have revealed similar pathological sequelae in *T. gondii*–infected $IL\text{-}27R^{-/-}$ animals (Villarino et al., 2003) and in chronically infected mice deficient in 5-lipoxygenase (Aliberti et al., 2002). The common feature of these three genetic deficiencies is that each leads to uncontrolled Th1-type responses. IL-10 and lipoxin A4 act directly on APCs to suppress IL-12 secretion, whereas IL-27 acts primarily on CD4+ T cells by promoting their IL-10 expression and at the same time suppressing other components of T cell activation (Hunter and Kastelein, 2012). Importantly, these three regulatory circuits originally described in *T. gondii* infection have now been documented in several parasitic, viral, and

The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 10 1919-1927
www.jem.org/cgi/doi/10.1084/jem.20122300

The Journal of Experimental Medicine

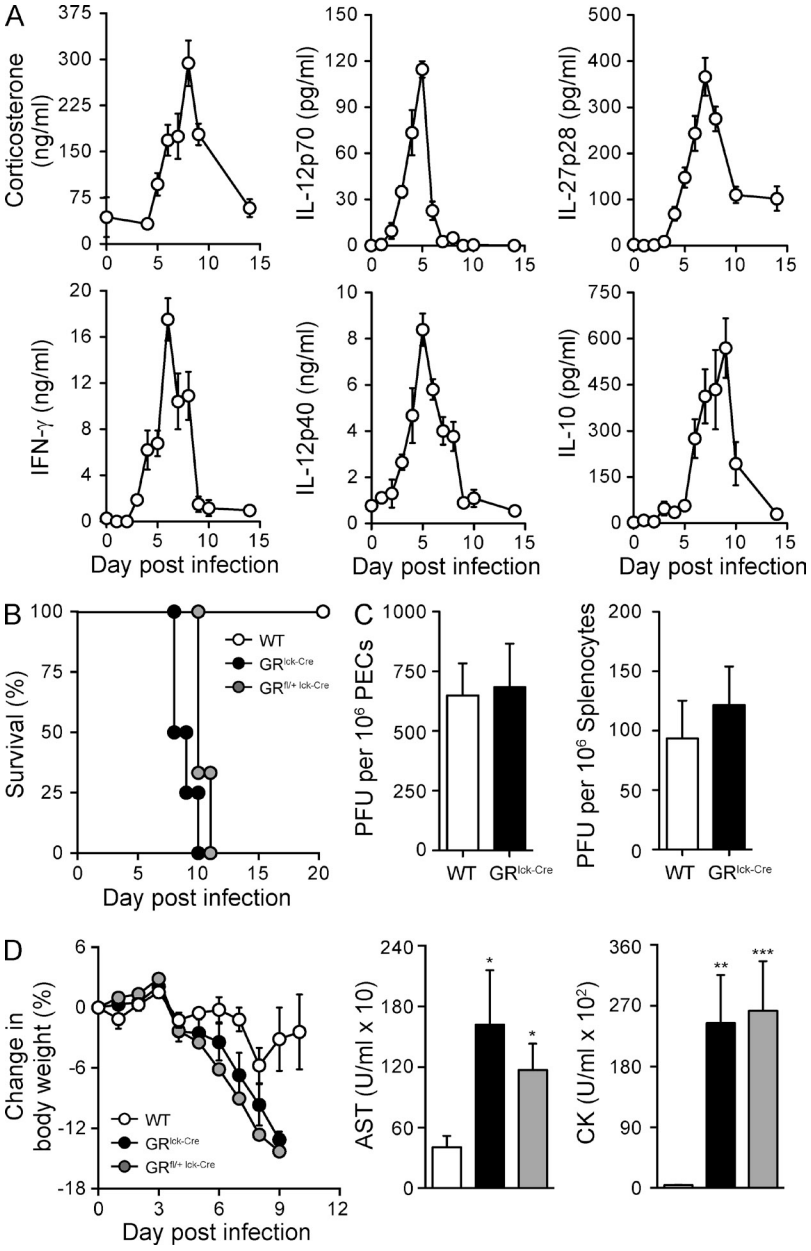

Figure 1. *T. gondii* infection elicits a GC response, and the lack of GR signaling in T cells results in acute mortality of toxoplasma-infected mice. (A) C57BL/6 mice were infected i.p. with an average of 15 ME49 cysts, and serum corticosterone, IFN-γ, IL-12p70 and p40, IL-27p28, and IL-10 levels were measured on the days indicated. Symbols represent mean ± SEM of the ELISA values for the individual animals (n = 3–12) at each time point pooled from three independent experiments. (B) Survival of homozygote $GR^{lck\text{-}Cre}$, heterozygote $GR^{fl/+lck\text{-}Cre}$, and littermate control animals after infection. The survival curves shown are from one representative of 10 experiments performed, two of which included $GR^{fl/+lck\text{-}Cre}$ mice. (C) Parasite burdens in PECs and spleen on day 8 after infection as determined by plaque assay. Bars represent mean ± SEM number of PFU per organ (n = 3–5 mice). (D) Weight loss and serum levels of AST and CK in infected animals. Results shown are means ± SEM for values for the individual mice (n = 4–5). No distinguishing histopathological changes were detected in lung, heart, liver, and kidney at this day 8 time point. Data presented in C–E are representative of two experiments performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

bacterial experimental models (Jankovic et al., 2010; Cyktor and Turner, 2011).

In the present study, we identify the endogenous glucocorticoid (GC) response as an additional pathway that plays a critical role in regulating CD4 T cell effector function during *T. gondii* infection. GCs are steroid hormones driven by the hypothalamic-pituitary-adrenal axis that are known to exert pleiotropic effects on immune cells and are frequently induced in response to infection (Sternberg, 2006; Jamieson et al., 2010; Pérez et al., 2011). Here, we demonstrate that CD4 T lymphocytes are both the target and trigger of the *T. gondii*–induced GC response. This pathway of self-regulation appears to play a critical role in host resistance by preventing the pathological consequences of Th1 hyperresponsiveness.

RESULTS AND DISCUSSION

GC receptor (GR) signaling in T cells is required for host survival during acute *T. gondii* infection

Although able to control infection, WT mice inoculated with nonlethal *T. gondii* strains undergo transient weight loss and display a hunched and scruffy appearance suggestive of a GC-mediated stress response. To determine whether toxoplasma infection triggers GC production, we measured corticosterone by ELISA in the sera of C57BL/6 mice challenged i.p. with cysts of the ME49 strain while simultaneously assaying IL-12, IFN-γ, IL-10, and IL-27. Serum GC levels increased sixfold during acute infection with kinetics that closely resembled those determined for the antiinflammatory (IL-10 and IL-27) as opposed to proinflammatory (IL-12 and IFN-γ) cytokines

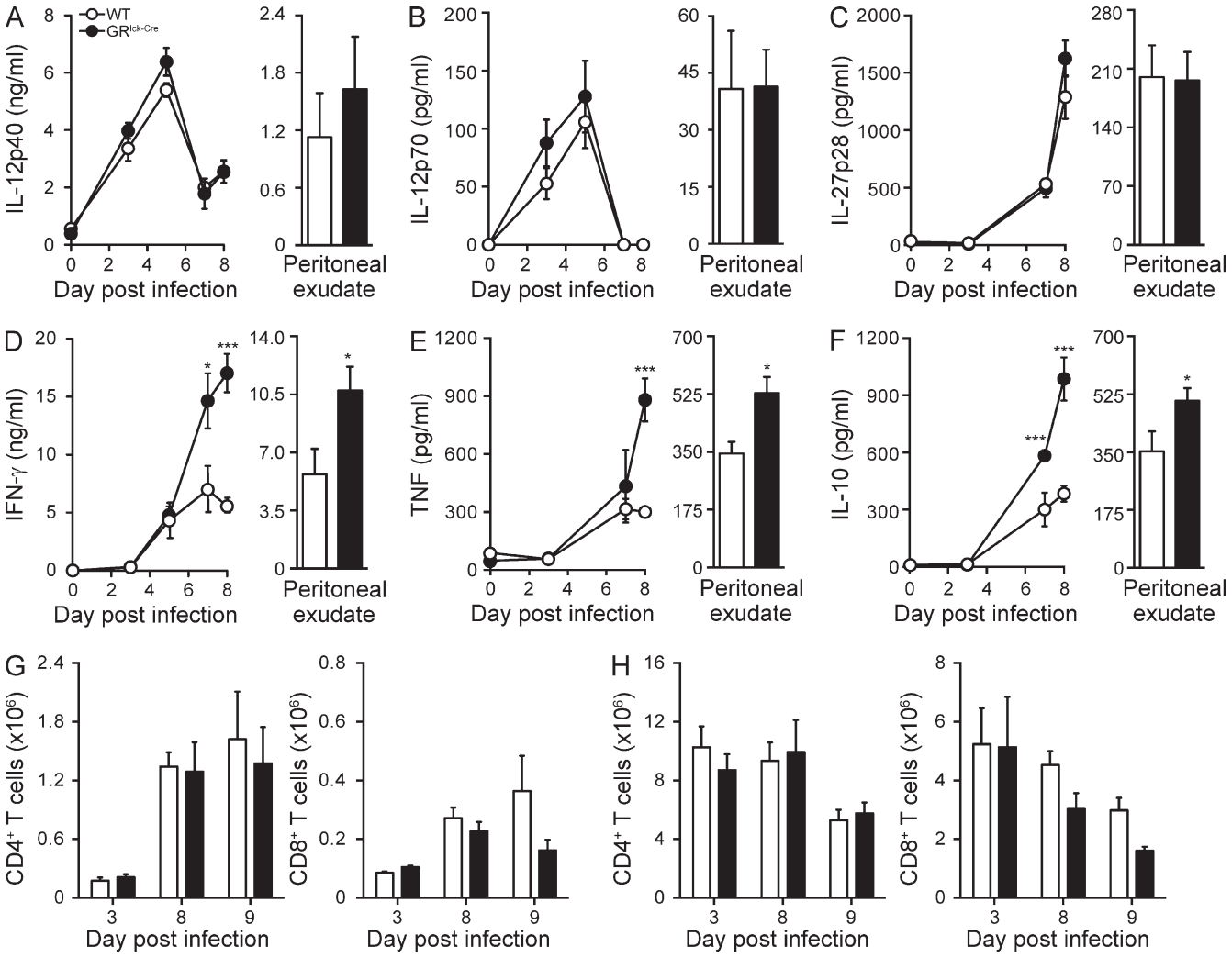

Figure 2. *T. gondii*–infected GR$^{lck\text{-}Cre}$ mice display enhanced IFN-γ, TNF, and IL-10 production, despite normal levels of innate cytokines and CD4$^+$ T cell numbers. (A–F) WT and GR$^{lck\text{-}Cre}$ littermates were infected i.p. with 15 ME49 cysts, and serum IL-12p40 (A), IL-12p70 (B), IL-27p28 (C), IFN-γ (D), TNF (E), and IL-10 (F) were measured on the days indicated as well as cytokine levels in peritoneal exudates on day 8 or 4 in the case of IL-12p70 (bar graphs). Each symbol or bar represents mean ± SEM of values for individual animals (n = 3–22). (G and H) Kinetics of total numbers of CD4$^+$ and CD8$^+$ T cells in PECs (G) or spleen (H) in *T. gondii*–infected animals. Bars represent mean ± SEM of values for individual animals (n = 3–28 per time point). Data presented are pooled from 10 independent experiments performed. *, $P < 0.05$; ***, $P < 0.001$.

(Fig. 1 A). Thus, whereas IL-12 (p40 and p70) and IFN-γ reached peak levels on days 5 and 6 after infection, respectively, GCs together with IL-10 and IL-27 displayed minor increases at these time points and did not peak until day 8.

Because GCs promote IL-10 production by T cells (Barrat et al., 2002) and dampen IFN-γ production by Th1 lymphocytes (Franchimont et al., 2000; Liberman et al., 2007), we asked whether GCs exert a similar regulatory role during *T. gondii* infection by acting on T cells. To this end, we infected mice that selectively lack GR expression in T cells (GR$^{lck\text{-}Cre}$; Mittelstadt et al., 2012). In contrast to littermate control animals, GR$^{lck\text{-}Cre}$ mice rapidly succumbed during the acute phase of infection with similar kinetics to those previously described for both IL-10$^{-/-}$ and IL-27R$^{-/-}$ *T. gondii*–infected animals (Fig. 1 B). Interestingly, mice heterozygous for GR deletion (GR$^{fl/+lck\text{-}Cre}$) displayed the same mortality as homozygous-deficient animals (Fig. 1 B), consistent with prior findings (Wüst et al., 2008; Mittelstadt et al., 2012), arguing for a threshold requirement in GR signaling in T cells. Together these results demonstrate that *T. gondii* infection triggers GC production and that this response plays a critical and nonredundant T cell–dependent host-protective function.

T. gondii–infected GR$^{lck\text{-}Cre}$ mice display evidence of enhanced tissue pathology despite unaltered parasite burdens

To determine whether the increased mortality in *T. gondii*–exposed GR$^{lck\text{-}Cre}$ mice is caused by an ineffective immune response, we measured parasite burdens on day 8 after infection. Despite their distinct survival outcomes, GR$^{lck\text{-}Cre}$ and control animals displayed indistinguishable frequencies of

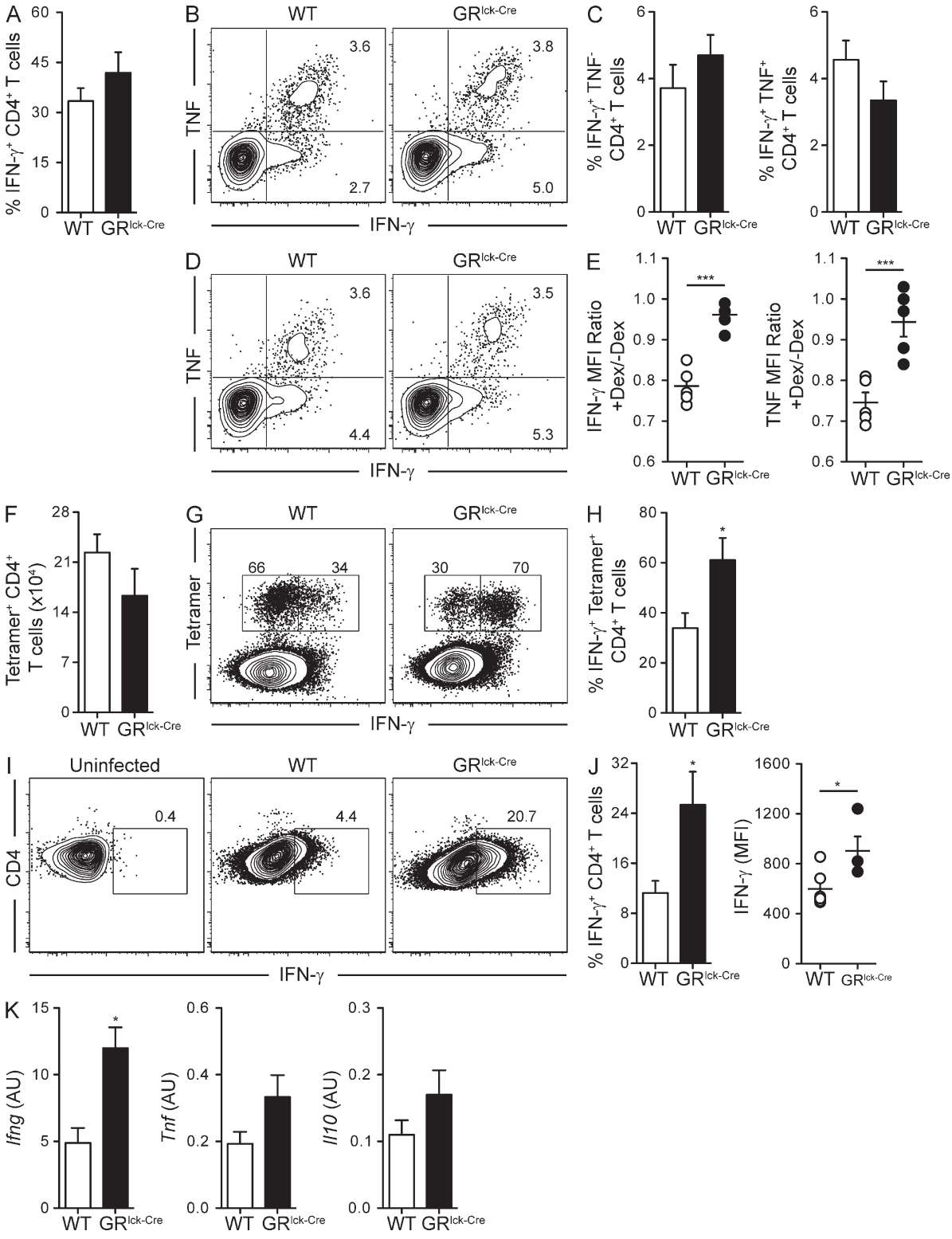

Figure 3. GCs regulate the magnitude of the Th1 response in vivo in *T. gondii*–infected mice. (A) WT and $GR^{lck\text{-}Cre}$ mice were infected i.p. with 15 ME49 cysts, and frequencies of IFN-γ^+ $CD4^+$ $CD44^+$ T cells were determined on day 8 in splenocytes stimulated in vitro by plate-bound anti-CD3 mAb. Bars represent mean ± SEM of values for individual animals (n = 5–11) determined by ICS. (B–E) Bulk splenocytes, described in A, were stimulated in vitro with AS15 peptide alone (B and C) or in the presence of Dex (D and E), and frequencies of IFN-γ^+ and TNF^+ cells were assayed by ICS. (B and D) The dot plots shown were gated on $CD44^+$ $CD4^+$ T cells. (C) Bars represent mean ± SEM of values determined from cultures of individual animals (n = 5) shown in B. In E, each symbol indicates the ratio of cytokine MFI for IFN-γ^+ TNF^+ $CD4^+$ T cells determined by ICS in parallel cultures from individual mice (n = 5)

infected cells (not depicted), as well as total numbers of parasites in peritoneum (the site of infection; peritoneum exudate cells [PECs]) and spleen (Fig. 1 C). Instead, the rapid mortality of infected $GR^{lck\text{-}Cre}$ (and heterozygous $GR^{fl/+lck\text{-}Cre}$) mice was preceded by enhanced weight loss and increased levels of serum aspartate aminotransferase (AST) and creatine kinase (CK), major biomarkers for hepatic dysfunction and muscle damage, respectively (Fig. 1 D).

$GR^{lck\text{-}Cre}$ mice infected with *T. gondii* exhibit a cytokine expression profile distinct from IL-$10^{-/-}$ and IL-$27R^{-/-}$ animals

The phenotype observed for $GR^{lck\text{-}Cre}$ animals was reminiscent of that previously described in *T. gondii*–infected IL-$10^{-/-}$ and IL-$27R^{-/-}$ mice, which display increased levels of serum IL-12p40 and IFN-γ as a result of their deficiencies in IL-10 production. Because in our model GR deletion is restricted to T cells, an effect on innate cytokine production by APCs seemed unlikely. Nevertheless, lack of GR expression in T cells could indirectly cause increased IL-12p40 if GC signaling is required for IL-10 production by $CD4^+$ T lymphocytes. However, levels of IL-12p40 or p70 and IL-27p28 in serum and peritoneum were indistinguishable between control and $GR^{lck\text{-}Cre}$ animals (Fig. 2, A–C), a finding inconsistent with either altered APC function or defective IL-10 production.

In contrast to IL-12 and IL-27, dramatic increases in IFN-γ levels were observed in the same infected $GR^{lck\text{-}Cre}$ mice. Although the initial phase of the response (before day 5) was similar to that seen in WT animals, on days 7–8 after infection, when GC levels peak and IFN-γ levels are decreasing in WT mice, $GR^{lck\text{-}Cre}$ animals exhibited an uncontrolled increase in the cytokine (Fig. 2 D). Interestingly, infected $GR^{lck\text{-}Cre}$ animals also displayed a dramatic elevation in TNF and IL-10 (Fig. 2, E and F), a phenotype clearly distinct from that of infected IL-10– or IL-27R–deficient mice. Moreover, the delayed onset and combined overexpression of IFN-γ, TNF, and IL-10, which are known Th1 products, support the concept that the defective host resistance in $GR^{lck\text{-}Cre}$ mice results from a perturbation of the adaptive, rather than innate, immune system.

Th1 cells from *T. gondii*–infected $GR^{lck\text{-}Cre}$ mice display hyperactivity in vivo but not in vitro

The increased production of IFN-γ, TNF, and IL-10 in $GR^{lck\text{-}Cre}$ mice could result from either the expansion of activated T cells or the enhanced responsiveness of cells within an unchanged pool size. Although the first hypothesis is consistent with the known role of GC in T cell apoptosis (Sionov et al., 2006), no significant increases were observed in the total number of $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 2, G and H) in $GR^{lck\text{-}Cre}$ versus control mice during acute infection. In addition, no differences were detected in the frequencies of Ki-67^+ versus Annexin-V^+ T cells in the two mouse strains (not depicted).

To test the second possibility, spleen cells isolated from $GR^{lck\text{-}Cre}$ and WT mice on day 8 after infection were stimulated in vitro with either anti-CD3 mAb or the MHC class II–restricted parasite peptide AS15 (Grover et al., 2012). No differences were observed in IFN-γ or IL-10 responses (not depicted). Moreover, frequencies of IFN-γ^+–producing cells, which at this time point are restricted to the $CD4^+$ $CD44^+$ T-bet^+ $Foxp3^-$ population (Jankovic et al., 2007), were comparable between $GR^{lck\text{-}Cre}$ and WT animals (Fig. 3, A–C).

Although $CD4^+$ T lymphocytes from both groups displayed indistinguishable cytokine responsiveness in vitro, their behavior in vivo was clearly distinct. Whereas the *T. gondii*–specific $CD4^+$ T population recognized by the AS15 tetramer (Grover et al., 2012) expanded equally in $GR^{lck\text{-}Cre}$ and control mice (Fig. 3 F), after injection of AS15 peptide i.v. on day 8 of infection, significantly higher frequencies of Th1 cells producing IFN-γ were detected in $GR^{lck\text{-}Cre}$ animals (Fig. 3, G and H). To extend this in vivo analysis to a broader range of T cell specificities, we next analyzed infected mice injected i.p. with brefeldin A (BFA). We found that $CD44^+$ T-bet^+ $Foxp3^-$ cells in PECs represent 79% versus 80% of CD4 T lymphocytes in control and $GR^{lck\text{-}Cre}$–infected animals, respectively. Nevertheless, the frequency of IFN-γ–producing cells was significantly higher in $GR^{lck\text{-}Cre}$ mice as was their overall IFN-γ mean fluorescence intensity (MFI; Fig. 3, I and J). In addition, increased levels of *Ifng* and *Tnf* as well as *Il10* mRNA were detected by RT-PCR in FACS-sorted PEC $CD4^+$ $CD44^+$ T lymphocytes from $GR^{lck\text{-}Cre}$ versus control animals (Fig. 3 K). However, no differences in expression of the co-stimulatory molecules CD28, CD69, ICOS, and GITR, as well as cytokine receptors CD25, CD119, and CD127 were detected in the same populations (not depicted).

Together these experiments demonstrated that despite similar antigen (Ag) load and comparable innate cytokine milieu, Th1 effectors in *T. gondii*–infected $GR^{lck\text{-}Cre}$ animals respond more vigorously to Ag stimulation than the equivalent cell population in WT mice. However, when removed from GC

stimulated with AS15 peptide in the presence versus absence of Dex (shown in D and B). (F) Numbers of AS15 $tetramer^+$ $CD4^+$ T cells in spleen on day 8 after infection. Bars represent mean ± SEM of values for individual animals (n = 13–16). (G and H) Day 8 toxoplasma-infected mice were injected i.v. with AS15 peptide, and 6 h later, frequencies of IFN-γ^+ $tetramer^+$ cells were determined by ICS in splenocytes prepared from individual mice. The dot plots shown were gated on $CD44^+$ $CD4^+$ T cells (G), and bars represent mean ± SEM of values for individual mice (n = 6; H). (I and J) Uninfected WT and day 8 infected mice were injected i.p. with BFA and ICS was performed. (I) The representative dot plots shown are gated on $CD44^+$ $CD4^+$ T lymphocytes. (J) Bars represent mean ± SEM of frequencies of IFN-γ^+ $CD44^+$ $CD4^+$ T cells for individual mice (n = 5), whereas symbols indicate IFN-γ MFI values for $CD44^+$ $CD4^+$ T cells from one mouse. The experiments shown in A–J are representative of two to four performed. (K) *Ifng*, *Tnf*, and *Il10* mRNA expression in $CD4^+$ $CD44^+$ T cells purified from peritonea of day 8 infected mice (n = 3–5). Bars represent mean ± SEM of cytokine expression relative to *Hprt* from three independent experiments. AU, arbitrary units. *, $P < 0.05$; **, $P < 0.01$.

pressure by in vitro culture, cells from both groups exhibited equal responsiveness. When GC pressure was reintroduced in vitro by addition of dexamethasone (Dex), cytokine production by Th1 cells from infected WT, but not $GR^{lck\text{-}Cre}$, mice was suppressed (not depicted). Interestingly, in WT cultures stimulated with AS15 peptide in the presence of Dex, the frequency of responding $CD4^+$ T cells remained unchanged (Fig. 3, B vs. D), but their MFIs for IFN-γ^+ and TNF^+ were reduced (Fig. 3 E).

CD4 T lymphocytes drive mortality in *T. gondii*–infected $GR^{lck\text{-}Cre}$ mice

The above experiments suggest that Th1 cells are a major target of GC-mediated suppression during toxoplasma infection. In support of this hypothesis, anti-CD4, but not anti-CD8, mAb treatment attenuated acute mortality in $GR^{lck\text{-}Cre}$ mice (Fig. 4 A). Importantly, CD4 T cell depletion also prevented the increase in pathological markers observed in the latter group (Fig. 4 B). As predicted (Jankovic et al., 2007), although serum IL-12p40 levels were similarly increased, the systemic IFN-γ and IL-10 responses in sera of CD4-depleted *T. gondii*–infected mice were reduced to a comparable level in both $GR^{lck\text{-}Cre}$ and WT animals (Fig. 4 C). To directly test the pathological role of $CD4^+$ T lymphocytes lacking GR, we reconstituted RAG KO mice with either WT or $GR^{lck\text{-}Cre}$ $CD4^+$ T cells from naive mice. In agreement with the depletion experiments, all RAG KO animals that received $GR^{lck\text{-}Cre}$ $CD4^+$ T lymphocytes succumbed to infection, whereas the majority of recipients of WT cells survived (Fig. 4 D). Moreover, serum levels of IFN-γ, alanine aminotransferase (ALT), and AST were significantly higher in mice reconstituted with $GR^{lck\text{-}Cre}$ versus WT cells (IFN-γ: 11.6 ± 1.5 vs. 7.2 ± 1.0; ALT: 353 ± 73 vs. 162 ± 28; AST: 662 ± 122 vs. 310 ± 46).

The GC response in *T. gondii* infection is triggered by CD4 T lymphocytes

The above results demonstrated that the GC response induced during the acute phase of infection plays an essential host-protective role by modulating the responsiveness of CD4 T lymphocytes. Because the interaction between the endocrine and immune system is known to be bidirectional, we next tested whether depletion of $CD4^+$ T cells might influence GC production during toxoplasma infection. Unexpectedly, serum GC levels were dramatically reduced in infected mice depleted of $CD4^+$, but not $CD8^+$, T cells (Fig. 4 E). In contrast, GC responses were not compromised in toxoplasma-infected mice lacking the signaling molecules MyD88, IFN-γR, or IL-27R and cytokines TNF, IL-12p40, or IL-10 that are associated with both Th1 responses and have been previously implicated in driving GC responses (Fig. 4 F). Moreover, antibody (Ab) neutralization of IFN-γ, TNF, and IL-6 failed to inhibit the GC response in infected WT mice (Fig. 4 G).

Consistent with the depletion studies, $RAG^{-/-}$ mice failed to display significant increases in GC levels after toxoplasma infection (Fig. 4 H). In addition, serum GC levels remain unchanged in infected OT-II Tg $RAG^{-/-}$ mice that possess only OVA-specific $CD4^+$ T cells (Fig. 4 H). However, RAG-deficient mice reconstituted with polyclonal WT $CD4^+$ T cells showed a full restoration of GC levels, confirming that CD4 T lymphocytes recognizing parasite and/or self-Ags are necessary and sufficient to drive the GC response during acute *T. gondii* infection (Fig. 4 I).

GCs have multiple and sometimes opposing effects on T lymphocytes. They promote apoptosis but can also induce IL-7R expression and inhibit synthesis of some while enhancing production of other cytokines (Ashwell et al., 2000). In $GR^{lck\text{-}Cre}$ mice, the GR is deleted at the stage of double-negative thymocytes. As a consequence of thymic selection in the absence of GC influence, $GR^{lck\text{-}Cre}$ mice display blunted responses after immunization with soluble Ag (Mittelstadt et al., 2012). One would therefore predict that $GR^{lck\text{-}Cre}$ animals would be unable to control *T. gondii* infection. However, $GR^{lck\text{-}Cre}$ mice mount a highly effective Th1 immune response against the parasite and, indeed, are hyperresponsive. Thus, challenge with an antigenically complex intracellular pathogen, and the strong pro-inflammatory response that it induces, appears to completely reverse the immunocompromised state of $GR^{lck\text{-}Cre}$ mice. The nonbiased overproduction in $GR^{lck\text{-}Cre}$ mice of IFN-γ, TNF, and IL-10, three distinct cytokines secreted by Th1 cells, suggests that in *T. gondii* infection GCs function as a general damper of TCR signaling.

Based on these observations, we propose that the same phenomenon of decreased Ag threshold for TCR activation in the absence of GC signaling, which during thymocyte maturation drives the appearance of hyporeactive T cells, paradoxically, also underlies the hyperreactivity of Th1 cells in vivo during *T. gondii* infection. Although it is as yet unclear whether GR is a component of the TCR complex (Löwenberg et al., 2007), the blunted TCR response occurring in WT Th1 cells is consistent with the known inhibitory effects of GCs on AP-1, NF-κB, and NFAT (Ashwell et al., 2000). Superimposed on this mechanism may be additional suppressive effects of GCs on the STAT4 and/or T-bet signaling pathways (Franchimont et al., 2000; Liberman et al., 2007).

Although the induction of GCs has been previously observed in a variety of viral, bacterial, and parasitic infections, the signals responsible for triggering this response are poorly defined. Cytokines, and in particular IL-1, have been shown to be potent inducers of GC production, arguing for a strong association with the innate immune system (Sternberg, 2006). Nevertheless, the IL-1 pathway is not involved in the *T. gondii* system described here as infected $MyD88^{-/-}$ mice display an uncompromised GC response. Instead, our depletion and adoptive transfer experiments clearly implicate CD4 T lymphocytes themselves as drivers of GC production. Although $CD4^+$ T cell–derived cytokines are obvious candidate mediators, our current findings argue against the role of IFN-γ, TNF, and IL-10. Nevertheless, the involvement of other soluble T cell products has not been ruled out. Alternatively, as a stress response, GC production in *T. gondii* infection could be the indirect result of $CD4^+$ T cell–mediated tissue damage (Jamieson et al., 2010).

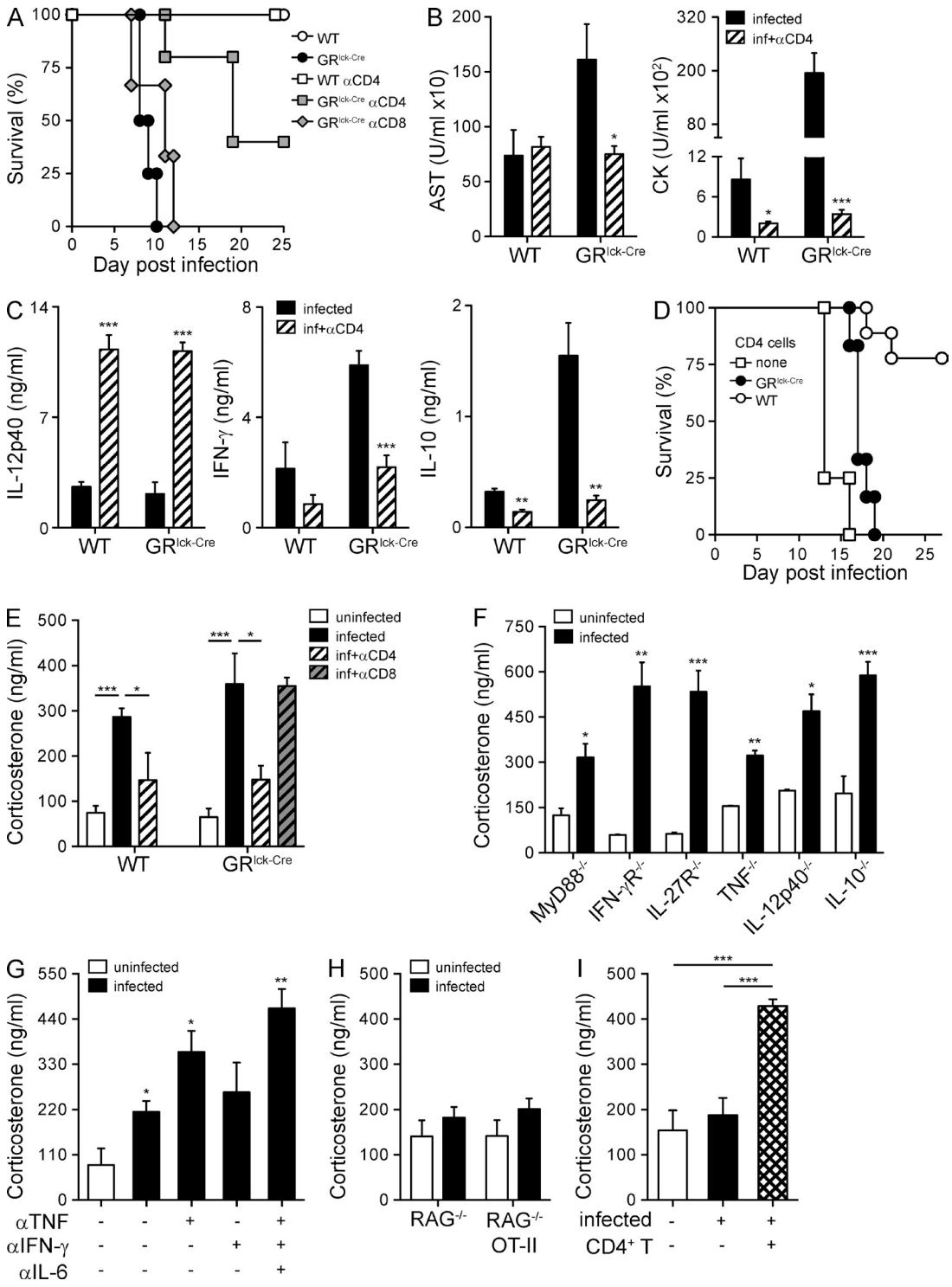

Figure 4. Mortality in *T. gondii*–infected GR$^{lck\text{-}Cre}$ mice is dependent on CD4$^+$ T cells that also drive the induction of the GC response. (A–C) *T. gondii*–infected mice were treated i.p. with anti-CD4, anti-CD8, or control mAb on days −1, 3, 5, 8, and 12 (n = 3–5 mice). Survival was monitored daily (A), and serum levels of AST and CK (B) and IL-12p40, IFN-γ, and IL-10 (C) were measured on day 8. Bars represent mean ± SEM of values for individual animals (n = 3–12). (D) Survival of *T. gondii*–infected RAG KO mice (n = 4) and those adoptively transferred with naive GR$^{lck\text{-}Cre}$ (n = 6) or WT (n = 9) CD4$^+$ T cells. (E) Serum corticosterone levels in animals described in A. The results shown in A–E are representative of three experiments performed. (F–H) Serum corticosterone levels in naive and toxoplasma-infected mice deficient in the genes indicated (F), WT animals treated with anti-TNF and anti–IFN-γ Ab alone or in combination with anti–IL-6 Ab (G), and RAG$^{-/-}$ and OT-II RAG$^{-/-}$ mice (H). Bars represent mean ± SEM of the ELISA values for individual mice (n = 4–10) pooled from two independent experiments. (I) Corticosterone levels in uninfected and 8-d *T. gondii*–infected RAG$^{-/-}$ animals, one group of which received naive WT CD4$^+$ T cells. Bars represent mean ± SEM of ELISA values for individual mice (n = 5–8) from one representative out of three performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The findings reported here establish GC signaling as a fourth major pathway (along with IL-10, IL-27, and lipoxin production) for dampening the Th1 response during *T. gondii* infection to protect the host against lethal immunopathology. Such a multitargeted regulatory mechanism may be necessary to adequately brake the Th1 pathway, which also incorporates a positive feedback-loop through IFN-γ production (Lugo-Villarino et al., 2003; Schulz et al., 2009). In turn, each of these circuits represents a potential target for exploitation by the parasite in enhancing its virulence and/or promoting its own survival (Butcher et al., 2005; Bradley and Sibley, 2007). In this regard, it is of interest that the virulence of avian influenza virus has been associated with induction of the endogenous GC response (Tian et al., 2012).

MATERIALS AND METHODS

Experimental animals. C57BL/6 mice were purchased from Taconic, and IL-10$^{-/-}$, IL-12p40$^{-/-}$, and RAG OT-II animals were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) contract facility maintained by the same supplier. IFNγR$^{-/-}$, TNF$^{-/-}$ (The Jackson Laboratory), IL-27R$^{-/-}$ (TCCR$^{-/-}$; Chen et al., 2000), MyD88$^{-/-}$ (Adachi et al., 1998), GR$^{lck\text{-}Cre}$, and GR$^{fl/+lck\text{-}Cre}$ (Mittelstadt et al., 2012) mice were bred and housed in our facilities. All animals were maintained at an American Association for the Accreditation of Laboratory Animal Care–accredited and specific pathogen–free facility at the NIAID/National Institutes of Health (NIH) or the National Cancer Institute/NIH. All procedures were performed in accordance with the protocols outlined in the Guide for the Care and Use of Laboratory Animals and described in an animal study proposal approved by the NIAID Animal Care and Use Committee. Age- and sex-matched GR$^{lck\text{-}Cre}$ and littermate control mice were used in all experiments.

***T. gondii* infection and determination of parasite burden.** Type II avirulent strain ME49 cysts were obtained from the brains of chronically infected C57BL/6 mice. Cyst preparations were pepsin treated to eliminate potential contamination with host cells and mice were inoculated i.p. with an average of 15 cysts. Parasite burden was assessed in PECs and spleen tissues by plaque assay that quantitates live parasites (Roos et al., 1994).

In vivo mAb treatment. To deplete CD4 or CD8 T cells, mice were injected i.p. with 250 µg mAb on days −1, 2, 5, 8, and 12 with either anti-CD4 (GK1.5) or anti-CD8 (2.43), respectively. FACS analysis on blood samples from the treated animals performed with a noncompeting mAb (anti-CD4 [RM4-4] and anti-CD8 [53-6.7]) confirmed a >90% depletion of the targeted population. To block TNF, IFN-γ, or IL-6, mice were injected i.p. with 1 mg of neutralizing Ab XT3-11, XMG-1.2, or MP5-20F3 (Bio X Cell) on days −1, 1, 3, 5, and 7. Control groups of animals received an equivalent amount of rat mAb GL113.

In vivo cytokine response. On day 8 after infection, mice were injected i.p. with BFA (Sigma-Aldrich; 250 µg/0.5 ml PBS; Liu and Whitton, 2005) or i.v. with the *T. gondii*–specific AS15 peptide (25 µg/0.2 ml PBS). In both cases, mice were euthanized 6 h later, and cell isolation and all subsequent steps of FACS staining were performed in BFA-containing medium until samples were fixed with 2% paraformaldehyde.

Body weight and hepatic enzyme measurements. Individual mice were weighed before and each day after infection, and the percent change in body weight was calculated for each animal. Serum levels of AST and CK were determined using a commercial kit (Boehringer Ingelheim) in an automatic analyzer (model 917; Hitachi).

Cytokine ELISA and corticosterone measurements. Serum samples were collected between 8:30–9:30 a.m. the day before or on indicated days after toxoplasma infection. Peritoneal lavage was performed with 3 ml PBS, cells were spun, and supernatants were stored. Levels of IL-12p40, IL-12p70, IL-27p28, IFN-γ, IL-10, TNF, and IL-10 were assayed using commercial ELISA kits (BD or R&D Systems). Serum corticosterone levels were measured by an EIA ELISA (Enzo Life Sciences).

Cell preparation and culture conditions. Single-cell suspensions were prepared from PECs or spleens from individual naive and infected animals. Cells (1–2 × 10^6/ml) were cultured in 24-well plates in RPMI complete medium alone or in the presence of plate-bound anti-CD3 mAb (Jankovic et al., 2007) or 5 µg/ml AS15 peptide for 1 h before the addition of 10 µg/ml BFA. After an additional 5–6 h of incubation time, cells from each well were harvested and intracellular cytokine staining (ICS) was performed. Where indicated, 100 nM Dex (Sigma-Aldrich) was added to the cultures stimulated with anti-CD3 mAb or AS15 peptide.

Cell purification for adoptive transfer. CD4$^+$ T cells were isolated from spleens and lymph nodes by negative selection (Miltenyi Biotec) from either naive WT or GR$^{lck\text{-}Cre}$ mice. For adoptive transfer, RAG recipient received 10 × 10^6 CD4$^+$ T cells i.v. (Jankovic et al., 2007).

Flow cytometry and ICS. Splenocytes and PEC samples from individual animals were stained with Fixable Viability Dye (eBioscience), and an appropriate combination of mAbs specific for CD4 (RM4-5), CD8 (53–6.7), CD44 (IM7), TCRβ (H57-597), T-bet (O4-46), Foxp3 (MF23), IFN-γ (XMG1.2), TNF (MP6-XT22), or IL-10 (JES5-16E3) was purchased from eBioscience, BioLegend, or BD. Parasite-specific CD4$^+$ T cells were determined by fluorescently labeled MHC class II tetramer bound to *T. gondii* antigenic peptide AS15 (Grover et al., 2012) provided by the NIH Tetramer Core Facility. Analysis of intracellular cytokine expression and Foxp3/T-bet staining were performed after cells were fixed and permeabilized using Perm-Fix solution (eBioscience). All samples were acquired on a LSR Fortessa flow cytometer (BD), and data were analyzed with FlowJo software (Tree Star).

Cell sorting. Activated CD44$^+$ CD62L$^-$ CD4$^+$ T lymphocytes were isolated from pooled peritonea from day 8 infected mice (n = 3–5) stained with mAbs specific for CD4 (RM4-5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), I-A/I-E (M5/114.15.2), and B220 (RA-6B2) by sorting on a FACSAria III (BD).

Quantitative RT-PCR. Total RNA was isolated (RNeasy Mini kit; QIAGEN) and reverse transcribed (SuperScript II Reverse transcription; Invitrogen). Gene expression analysis was performed using SYBR Green–based real-time quantitative PCR on an ABI Prism 7900HT analyzer (Applied Biosystems). Arbitrary units represent the ratio of cytokine mRNA levels compared with hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA levels. The following primer pairs were used: *Hprt*, 5′-AGCCTAAGATGAGCGCAAGT-3′ (forward) and 5′-TTACTAGGCAGATGGCCACA-3′ (reverse); *Ifng*, 5′-ATGAACGCTACACACTGCATC-3′ (forward) and 5′-CCATCCTTTTGCCAGTTCCTC-3′ (reverse); *Tnf*,5′-GCCTCTTCTCATTCCTGCTTGT-3′ (forward) and 5′-GGCCATTTGGGAACTTCTCAT-3′ (reverse); and *Il10*, 5′-GCTCTTACTGACTGGCATGAG-3′ (forward) and 5′-CGCAGCTCTAGGAGCATGTG-3′ (reverse).

Statistical analysis. The statistical significance of differences between data means was evaluated using a paired, two-tailed Student's t test.

We thank Calvin Eigsti for performing FACS sorting. We also thank Drs. Carl G. Feng and Daniel L. Barber for invaluable discussions and Drs. Jacques Peschon and Carl G. Feng for critical reading of the manuscript.

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and in part by an Intramural AIDS Research Fellowship (to D.G. Kugler).

The authors have no conflicting financial interests.

Submitted: 11 October 2012
Accepted: 1 August 2013

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