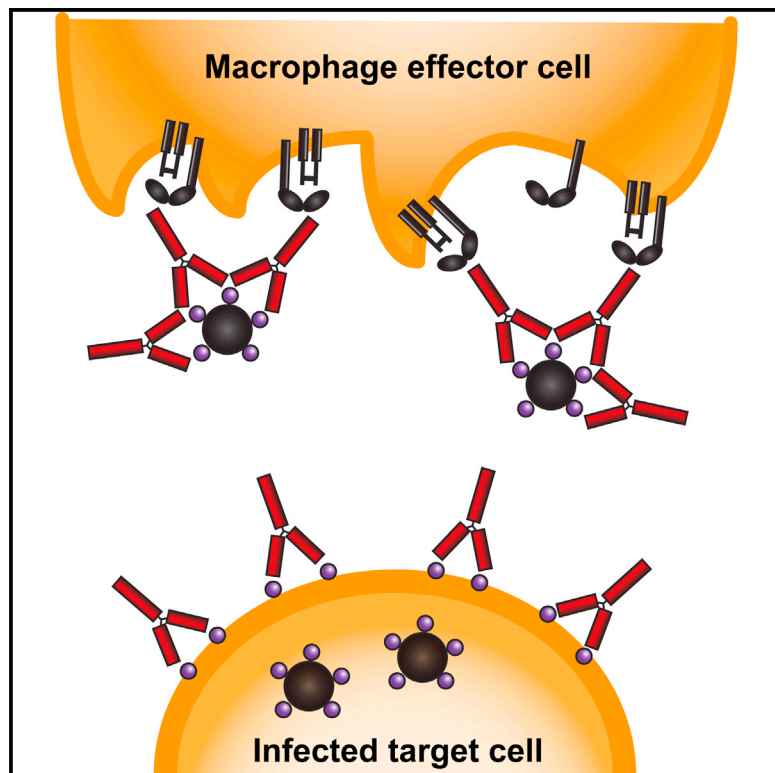


Immunity

Suppression of Fc γ -Receptor-Mediated Antibody Effector Function during Persistent Viral Infection

Graphical Abstract



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In Brief

Suppression of antibody effector function could hinder critical parameters of immunity during persistent viral infection. Brooks and colleagues demonstrate that high amounts of immune complexes generated during persistent viral infection suppress multiple antibody-mediated Fc γ -receptor effector functions.

Highlights

- Multiple Fc γ R-mediated Ab functions are suppressed by persistent viral infection
- Immune complexes inhibit Fc γ Rs to suppress killing of virus infected cells
- Immune complexes suppress DC-mediated cross-presentation



Suppression of Fc γ -Receptor-Mediated Antibody Effector Function during Persistent Viral Infection

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SUMMARY

Understanding how viruses subvert host immunity and persist is essential for developing strategies to eliminate infection. T cell exhaustion during chronic viral infection is well described, but effects on antibody-mediated effector activity are unclear. Herein, we show that increased amounts of immune complexes generated in mice persistently infected with lymphocytic choriomeningitis virus (LCMV) suppressed multiple Fc γ -receptor (Fc γ R) functions. The high amounts of immune complexes suppressed antibody-mediated cell depletion, therapeutic antibody-killing of LCMV infected cells and human CD20-expressing tumors, as well as reduced immune complex-mediated cross-presentation to T cells. Suppression of Fc γ R activity was not due to inhibitory Fc γ Rs or high concentrations of free antibody, and proper Fc γ R functions were restored when persistently infected mice specifically lacked immune complexes. Thus, we identify a mechanism of immunosuppression during viral persistence with implications for understanding effective antibody activity aimed at pathogen control.

INTRODUCTION

Antibodies are critical for eliminating viral infection, preventing re-infection, and controlling persistent virus infections (Hangartner et al., 2006). Antibodies (Abs) prevent de novo cell infection by neutralizing the interaction between a virus and its cellular receptor (neutralizing; nAbs), opsonizing free virions, and targeting viral proteins on the surface of infected cells to facilitate cell-killing by either NK cells (Ab-dependent cellular cytotoxicity; ADCC) or phagocytic cells (Ab-dependent cellular phagocytosis; ADCP) (Nimmerjahn and Ravetch, 2008). Persistent virus replication is associated with multiple T cell and B cell dysfunctions that impede control of infection, but little is known about potential Ab defects during viral persistence (Hangartner et al., 2006; Moir and Fauci, 2009). Considering the importance of antiviral Abs

in maintaining control of persistent infections and their potential therapeutic value, diminished Ab efficacy could substantially impact productive immune responses (Burton et al., 2012). In many persistent virus infections such as human immunodeficiency virus (HIV) and lymphocytic choriomeningitis virus (LCMV) in mice, nAbs are low and late to emerge (Hangartner et al., 2006), yet non-nAbs can still exhibit immune pressure, suggesting that non-nAbs can perform functions important for Ab-mediated control of persistent virus infection. Consistent with this notion, the contribution of non-nAbs toward control of infection is beginning to emerge, and although their exact mechanism of antiviral activity is unclear, it is surmised that ADCP and/or ADCC are critical (Burton, 2002; Chung et al., 2014). Thus, a defect in Ab function could have a detrimental impact on the immune system's ability to target and kill infected cells, further contributing to immunosuppression and ineffective viral control.

In addition to their endogenous antiviral roles, therapeutic administration of Abs (particularly nAbs) has shown efficacy to limit virus replication in multiple models of persistent infection (Barouch et al., 2013; Burton et al., 2012; Klein et al., 2012; Law et al., 2008; Shibata et al., 1999; Trkola et al., 2005). Separate from neutralization capacity, both nAbs and non-nAbs can inhibit viral replication at its source by targeting and killing virus-infected cells through Fc γ -receptor (Fc γ R)-dependent effector mechanisms. Further, antigen-presenting cells (APCs) internalize antigen-Ab immune complexes (ICs) via Fc γ Rs to then initiate T cell responses against virus escape mutants and co-infecting pathogens. Similarly, Fc γ Rs on follicular dendritic cells (FDCs) help to retain ICs for B cell selection and affinity maturation to produce highly effective antibodies (Guilliams et al., 2014). Although dysfunctions in T cell and B cell responses are hallmarks of persistent viral infections (Moir and Fauci, 2009; Wherry, 2011), whether Ab effector functions such as ADCC, ADCP, or cross-presentation are affected remains unclear. We previously observed that substantially higher amounts of cell-depleting Abs were required to deplete target cells during persistent LCMV infection compared to in naive mice (Fahey et al., 2011), suggesting a functional suppression of Ab activity during viral persistence. Further, in initial experiments using less effective nAbs than used now, it was observed that nAbs could not control HIV infection in humans despite their ability to neutralize patient samples ex vivo (Mehandru et al., 2007; Poignard et al., 1999; Trkola et al., 2005). Upon subsequent analysis, it was

estimated that at least 10× more Ab would be required to achieve a 50% response in HIV-infected patients (Huber et al., 2008; Trkola et al., 2008), suggesting diminished Ab effector activity during persistent infections of humans as well. Herein, we demonstrate that high amounts of ICs generated during viral persistence suppress FcγR-dependent, Ab-mediated effector functions including the killing of infected cells and antigen presentation for T cell activation, thus compounding the overall immunosuppression that potentiates persistent viral infection.

RESULTS

Suppression of Ab-Mediated Cell Killing during Persistent Virus Infection

To determine whether persistent virus infection suppresses Ab effector activity, we utilized the LCMV model of murine infection. Infection with the Armstrong (Arm) variant of LCMV induces a robust immune response that clears the virus within 2 weeks after infection. Infection with the LCMV variant Clone 13 (Cl13) establishes a persistent infection due to increased virus replication and receptor affinity that help to outcompete the developing immune response, thereby inducing immunosuppression, T cell exhaustion, and B cell dysfunctions (Ahmed et al., 1984; Fahey and Brooks, 2010; Hangartner et al., 2006). To examine Ab suppression, we treated naive and LCMV-Cl13 persistently infected mice with high and low doses of two different monoclonal anti-CD4⁺ T cell-depleting Abs (both of the rat IgG2b isotype). Whereas a low Ab dose resulted in nearly complete CD4⁺ T cell depletion in naive mice, Ab-mediated depletion was suppressed even at the highest dose administered during persistent infection (Figures 1A and 1B). Although both Abs were equally effective at CD4⁺ T cell depletion in naive mice, only the YTS191 Ab showed some, but decreased efficacy in LCMV persistently infected mice (Figures 1A and 1B). Together, these data indicate that, similar to CD8⁺ T cell exhaustion during persistent virus infection wherein some antiviral function is retained (Wherry, 2011), Ab depleting activity is not completely lost but suppressed.

To further explore the suppression of effector Ab function, we evaluated the efficacy of multiple cell-depleting monoclonal Abs in naive and LCMV persistently infected mice using Abs of different isotypes and targeting a variety of cell surface antigens. Unlike naive mice wherein Ab-mediated depletion was highly efficacious, Ab effector function was suppressed in LCMV persistently infected mice treated with CD8⁺ T cell, B cell, or platelet-depleting Abs (Figure 1C, Table S1, and data not shown). Similarly, Ab immunotherapy with Rituximab to target huCD20-expressing murine tumor cells was protective in naive mice at the dose given, whereas four out of five persistently infected mice rapidly formed tumors (Figure 1C). Thus, the consistently observed decrease in cell-depleting capacity of Abs from various species, of multiple isotypes, and affecting various targets indicates that inherent Ab effector activity is suppressed during persistent virus infection.

High Concentrations of ICs Impede Ab Effector Activity during Viral Persistence

To address whether the suppression of Ab function also occurs during acute viral infection, we infected mice with LCMV-Arm (acute) or LCMV-Cl13 (persistent) and then treated with anti-

CD4⁺ T cell depleting Abs. Compared to naive mice, low suppression of Ab effector function was observed at day 8 in both LCMV-Arm and Cl13 infections (Figure 2A). At day 45 of LCMV-Arm infection (30 days post viral clearance), Ab-mediated depletion was effective, whereas it remained substantially suppressed in persistently infected mice (Figure 2A), indicating that Ab effector suppression is present early in viral infection but increases only during the course of persistent infection.

Persistent viral infections such as HIV, hepatitis B and C viruses (HBV, HCV), and LCMV are characterized by high amounts of ICs and non-virus-specific immunoglobulin G (IgG) (often termed hypergammaglobulinemia; hyperIgG; Figure 2B) (Hangartner et al., 2006; Hunziker et al., 2003; Moir and Fauci, 2009). Although LCMV-specific Abs are detected in the plasma of animals with acute and persistent infections by day 8, they remain at much lower concentrations during persistent infection despite high titers of virus present (Figure 2B). In contrast, as the infection progresses, total plasma IgG and IC concentrations are higher in animals with persistent compared to acute infections (Figures 2B and 2C).

To address the role of hyperIgG-ICs in the suppression of Ab effector function, we tested cell-depleting activity in three separate cohorts of mice that maintain the same high titers of persistent viral replication, but have greatly reduced Ab concentrations (termed low Ab mice; Figure S1A and S1B). We used: (1) B cell-deficient mice (μMT) that cannot produce Ab; (2) mice expressing a transgenic non-LCMV-specific B cell receptor against hen egg lysozyme (HEL-tg mice) that do not see cognate antigen and thus do not produce Ab; and (3) mice that are CD4⁺ T cell-depleted prior to infection and lack sufficient B cell help to produce Ab (Figures S1A and S1B). For the mice that were CD4⁺ T cell-depleted prior to infection to achieve low Ab concentrations, anti-CD8⁺ T cell Abs were used for subsequent depletion. In contrast to persistently infected wild-type (WT) mice wherein Ab effector function is suppressed (termed high Ab mice), Ab-mediated depletion was effective in all low Ab mice despite the same amount of virus replication (Figure 3A and S1A), indicating that the high amount of Ab and/or ICs lead to the suppression of Ab effector activity.

To confirm that the failure to deplete cells in persistent infection was not due to intrinsic resistance of the target cells themselves or an inability of antibodies to reach their targets, we transferred CD4⁺ T cells from persistently infected high Ab mice into infection matched low Ab mice. Transferred CD4⁺ T cells from high Ab mice were effectively targeted and depleted in low Ab mice, highlighting that the lack of depletion in high Ab mice is not due to cell-intrinsic differences (Figure 3B). Further, despite accelerated Ab catabolism in persistently infected high Ab mice (Figure 3C), target cells were completely coated with depleting Ab for at least 5 days following treatment (Figure S1C), and depletion did not occur early then reconstitute in high Ab mice nor was depletion enhanced by multiple treatments with depleting Abs (Figure S1D). Therefore, binding defects, increased target turnover from the cell surface, or rapid repopulation of target cells were not responsible for the observed decrease in immune-mediated cell depletion.

Interestingly, early (day 8) in acute LCMV-Arm infection, the amount of ICs is increased compared to persistent LCMV-Cl13 infection corresponding to a ~5%–10% decrease in Ab

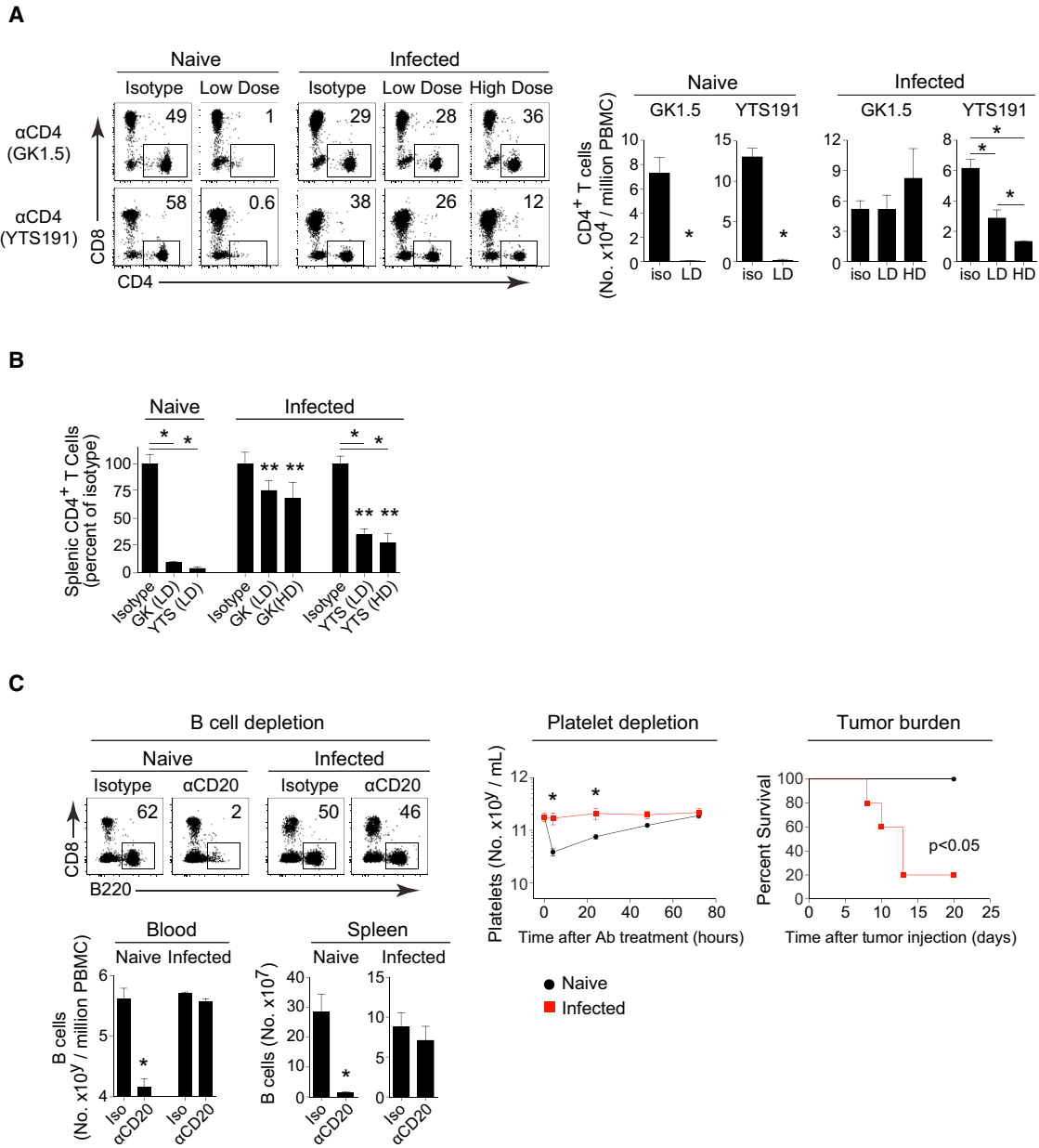


Figure 1. Persistent Virus Infection Suppresses Ab-Mediated Cell Depletion

(A) Uninfected and mice persistently infected with LCMV-Cl13 for 21 days received low dose (LD; 100 μg) or high dose (HD; 1 mg) of isotype or anti-CD4⁺ T cell-depleting Ab (clone GK1.5 or clone YTS191; both rat IgG2b). Flow plots represent the frequency and graphs indicate the number ± SD of CD4⁺ T cells in the blood 5 days after Ab treatment. Ab clones recognizing separate epitopes than the depleting antibodies were used for staining.

(B) Graph represents the percent depletion ± SD of the number of splenic CD4⁺ T cells 5 days after Ab treatment relative to isotype-treated mice. Percent depletion was calculated by dividing the number of CD4⁺ T cells following depletion by the average number of CD4⁺ T cells present in isotype treated mice. Isotype is set to 100.

(C) Uninfected (black) and mice persistently infected with LCMV-Cl13 (red) for 21 days received isotype or anti-CD20 B cell-depleting Ab (clone MB20; mouse IgG2a/c), platelet-depleting Ab (clone 6A6; mouse IgG2a/c) or huCD20⁺ EG7 tumor cells followed by anti-huCD20 (Rituximab; human IgG1). Flow plots represent the percent of B cells (B220⁺) in the blood, platelets determined as CD41⁺, CD61⁺ per mL blood, and tumor burden represented as the percent of mice protected from tumor formation following treatment (survival). Note, all mice treated with non-tumor-specific isotype-matched Ab (Herceptin; human IgG1) developed tumors (data not shown).

*p < 0.05. **p < 0.05 compared to the same condition in naive mice. Data are representative of two or more independent experiments using three to five mice per group. See also Table S1.

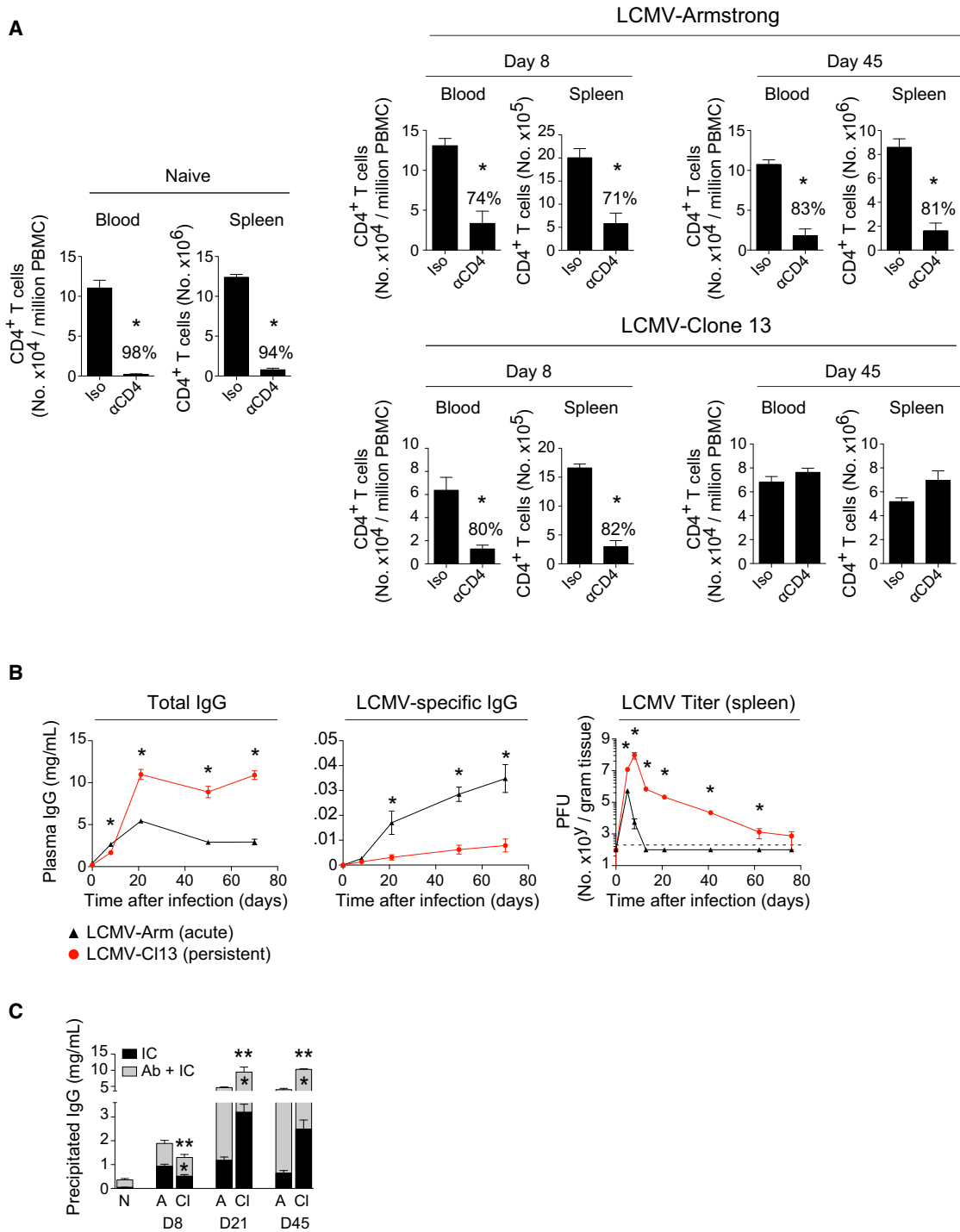


Figure 2. Suppressed Ab-Mediated Cell Depletion in Persistent, but Not Acute LCMV Infection

(A) Naive mice or mice infected with LCMV-Arm or LCMV-C13 8 or 45 days earlier received 100 μ g of isotype or anti-CD4 (clone GK1.5) Ab. Graphs represent the number \pm SD of CD4⁺ T cells 5 days after Ab treatment. The number above each bar represents percent depletion versus isotype.

(B) Graphs represent the concentration \pm SD of total IgG (left) and LCMV-specific IgG (middle) in the plasma, and splenic virus titers (right) are represented at the indicated time points after infection with LCMV-Arm (black) and LCMV-C13 (red). Assay limit of detection (dotted line).

(C) Graph represents the concentration \pm SD of ICs (black) or total IgG (Ab+IC; gray) detected in the plasma of naive mice (N) and at the indicated time points after LCMV-Arm (A) or C13 (CI) infection.

* $p < 0.05$ Amount of ICs Arm versus C13. ** $p < 0.05$ total Ab+IC Arm versus C13. Data are representative of two or more independent experiments using three to five mice per group.

depletion efficacy in the acute infection (Figures 2A and 2C). However, as persistent infection progresses, the concentration of ICs increases and Ab effector activity is lost. In contrast, depletion efficacy remains high following the resolution of acute LCMV infection where IC concentrations are low (Figures 2A and 2C), suggesting that ICs are responsible for the suppression of Ab depletion activity. To conclusively differentiate the relative contributions of free Ab alone versus ICs to the suppression of depleting Ab effector function, we treated persistently infected low Ab mice with anti-Ovalbumin (Ova) Ab alone or anti-Ova-Ova protein ICs. Although in these experiments the amount of total Ab achieved was much lower than that observed in high Ab mice, the ICs suppressed Ab-mediated depletion, whereas the Ab alone at these low concentrations had little inhibitory effect (Figure 3D). Even treating low Ab mice with 10 times more free Ab still was not sufficient to inhibit Ab effector activity (Figure S1E) and ICs, but not free Ab readily prevented cell depletion in ex vivo assays (Figure 3E). Together, these data indicate that it is the high concentration of ICs and not free Ab during persistent infection that suppresses effector Ab activity.

High Amounts of ICs Impede Macrophage-Mediated Phagocytosis

Ab-mediated depletion can occur via complement-dependent cytotoxicity, ADCC by NK cells, or ADCP by macrophages, dendritic cells (DC), and neutrophils (Jiang et al., 2011; Nimmerjahn and Ravetch, 2008). We used the low Ab mice where cell depletion is efficacious despite persistent virus replication (Figure 2C) to determine the effector mechanisms impeded by the high concentrations of ICs during viral persistence. Neither inactivation of complement components using cobra venom factor (CVF) nor the removal of NK cells or neutrophils led to a decrease in Ab-mediated cell depletion (Figures S2A–S2C), indicating that the complement pathway, ADCC by NK cells, or ADCP by neutrophils are not involved. On the other hand, treatment of persistently infected low Ab mice with clodronate-filled liposomes to deplete phagocytic cells (i.e., macrophages and DC) suppressed Ab-depleting activity (Figure 4A and S2D), indicating that the high concentration of ICs primarily suppress Ab-mediated phagocytic activity during persistent virus infection.

Both macrophages and DC can be phagocytic (Guilliams et al., 2014) and were depleted by clodronate treatment (Figure S2D). To differentiate the role of macrophages and DC, we performed ex vivo phagocytosis assays with sorted splenocytes from persistently infected low Ab mice. Ab-mediated depletion was observed using total splenocytes (Figure 4B) or DC-depleted splenocytes (Figure S3A), but was lost when macrophages were depleted (Figure 4B). Similarly, no effect on Ab-mediated depletion was observed when DC were selectively depleted in vivo (Figure S3B). Taken together, these data demonstrate that macrophage-mediated phagocytosis is suppressed by the high amounts of ICs generated during persistent viral infection.

Fc γ R Blockade Is Responsible for Suppression of Macrophage-Mediated Phagocytosis

We next determined whether the decreased ADCP was due to fundamental alterations in macrophage function or a physical blockade of phagocytic activity. ADCP is mediated by Ab

engagement with activating Fc γ R on macrophages (Fc γ RI, Fc γ RIII, Fc γ RIV in mice; Fc γ RI, Fc γ RIIA, Fc γ RIIC, Fc γ RIIA in humans), while engagement with the inhibitory receptor Fc γ RIIB inhibits phagocytic activity (Nimmerjahn and Ravetch, 2008). Similar numbers of monocytes and macrophages were observed in the blood and spleens of persistently infected high and low Ab mice (Figure S3C). Further, no decrease (and in most cases an increase) in activating Fc γ R expression was observed in both high and low Ab mice compared to naive controls (Figure 4C), indicating that the suppression was not due to decreases in Fc γ R expression. Interestingly, although inhibition mediated by Fc γ RIIB is an immunosuppressive mechanism in some situations (Nimmerjahn and Ravetch, 2008), persistent infection of Fc γ RIIB-deficient (high Ab) mice did not rescue the suppression of Ab function (Figure 4D), indicating that macrophage suppression during persistent viral infection is not due to a decreased ratio of activating to inhibitory receptors or increased Fc γ RIIB signaling.

We observed increased amounts of IgG on the surface of macrophages in persistently infected high Ab mice compared to low Ab or naive mice (Figure 5A) suggesting that surface-bound Ab physically impedes effector Ab activity. Importantly, the amount of surface IgG during persistent infection compared to naive mice is not solely due to increased Fc γ R expression because low Ab mice also have greatly increased Fc γ R expression compared to naive mice (and similar/higher than high Ab mice), but maintain low amounts of circulating and surface-bound IgG similar to naive mice. To address whether the suppression of macrophage activity was due to permanent cellular inhibition or a physical blockade of Fc γ R interactions, splenocytes and peritoneal macrophages were isolated from high Ab mice, washed to remove Fc γ R-bound Ab-ICs, and allowed time to recover (Figure 5B and data not shown). Despite the inhibition in vivo, splenic and peritoneal macrophages from the high Ab mice now exhibited similar effector activity to macrophages from low Ab mice ex vivo (Figures 5B and 5C). Importantly, the Ab effector function was lost when these phagocytic cells lacked activating Fc γ Rs (Figure 5C). Thus, the suppression of Ab effector function is not a permanent functional macrophage defect but instead results from blockade of surface Fc γ Rs by the high amount of ICs present during persistent infection.

Multiple Fc γ R-Mediated Effector Functions Are Suppressed during Viral Persistence

Abs mediate multiple antiviral functions during infection including virus neutralization, the opsonization/killing of infected cells, and Fc γ R-mediated IC internalization by APCs for cross-presentation and T cell activation. Thus, suppression of Ab effector activity could have many ramifications toward ongoing and de novo immune responses. To determine the impact of Ab suppression toward virus neutralization, we used the M1 variant of LCMV (LCMV-M1) in which a two amino acid change engineered into the glycoprotein (GP) of LCMV-C113 (Osokine et al., 2014) renders it susceptible to neutralization by the anti-LCMV KL25 antibody, unlike the parental LCMV-C113 strain that is not neutralized or bound by KL25 (Figure S4). High Ab and low Ab cohorts of mice were generated using persistent infection with LCMV-M1 and then treated with KL25 Ab 21 days after infection. Seven days following KL25 Ab treatment,

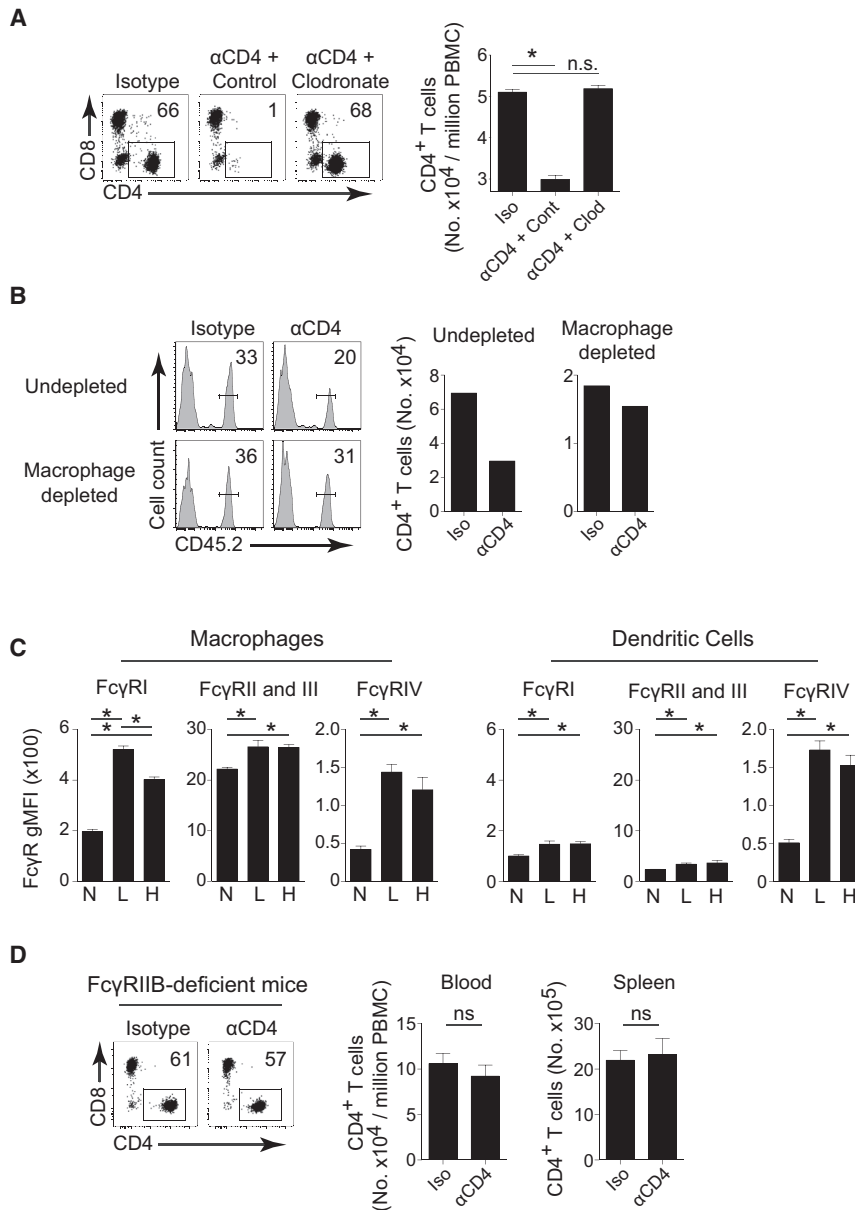


Figure 4. Macrophages Mediate Cell-Depleting Ab Function

(A) LCMV-CI13 infected low Ab (HEL-tg) mice were treated 21 days after infection with clodronate-filled (Clod) liposomes or control (Cont) liposomes. One day later, mice received 100 μ g of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and the graph represents the number \pm SD of CD4⁺ T cells in the blood 5 days after Ab treatment.

(B) T cell and B cell-depleted splenocytes pooled from low Ab (CD4⁺ T cell-depleted before infection), CD45.1⁺ mice containing macrophages or further depleted of macrophages were mixed with CD45.2⁺ CD4⁺ T cells for an ex vivo phagocytosis assay. Cultures were given isotype or anti-CD4 Ab (clone GK1.5) and the frequency and number of CD45.2⁺ CD4⁺ T cells was quantified in the cultures 2 days later.

(C) Graphs represent the gMFI of the indicated Fc γ R on macrophages [B220⁻, Thy1.2⁻, Ly6G⁻, NK1.1⁻, CD11c^(mid-lo), F4/80⁺, CD11b⁺] and dendritic cells [B220⁻, Thy1.2⁻, Ly6G⁻, CD11c^(hi)] from naive (N), and day 21 after LCMV-CI13 infection of mice that were CD4⁺ T cell-depleted prior to infection (low Ab; L) or left undepleted (high Ab; H).

(D) LCMV-CI13 infected Fc γ RIIB-deficient mice were treated 21 days after infection with 100 μ g of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and graphs represent the number \pm SD of CD4⁺ T cells in the blood and spleen 5 days following Ab treatment.

**p* < 0.05. Data are representative of two or more independent experiments using three to five mice per group. See also Figures S2 and S3.

viremia was undetectable in both high Ab and low Ab mice (Figure 6A), demonstrating that virus neutralization with the KL25 Ab is effective in persistent virus infection.

Another main function of virus-specific Abs is the binding of viral proteins expressed on the surface of infected cells to initiate killing via Fc γ R interactions. To address whether the high amount of ICs generated during persistent virus infection suppress Ab-mediated killing of infected cells, we infected DC with LCMV-M1, transferred the infected cells into LCMV-CI13 persistently infected low Ab or high Ab mice, and then treated the mice with isotype control or KL25 Ab. In this way, the transferred cells were targeted by the KL25 Ab while not affecting endogenous titers of LCMV-CI13. The mice were also CD8⁺ T cell-depleted prior to infection to prevent cytotoxic lymphocyte (CTL)-mediated killing of the transferred infected cells. Within 6 hr after transfer, KL25 Ab treatment reduced the number of in-

fecting DC by 50% in low Ab mice, but was ineffective in the high Ab mice (Figure 6B). Since the KL25 Ab effectively binds and neutralizes LCMV-M1 in persistently infected high Ab mice (Figure 6A and S4), these data further demonstrate that even highly potent Abs exhibit suppression of ADCP activity and are diminished in their ability to kill infected

cells when high concentrations of ICs are present during persistent viral infection. Interestingly, elevated concentrations of IgG are also detected on DC in persistently infected high Ab mice (Figure 6C), suggesting that their Fc γ R-mediated functions might also be suppressed. A major Fc γ R-mediated function of DC is cross-presentation of antigen to prime and sustain T cell activity (Platzer et al., 2014; Regnault et al., 1999). To address the suppression of Fc γ R-mediated cross-presentation by APC during persistent infection, we transferred CFSE-labeled, OVA-specific CD8⁺ T cells (OT-I) into high or low Ab mice persistently infected with LCMV-CI13 and then primed them with either Ova protein alone or Ova-anti-Ova ICs. In both cohorts, Ova alone failed to notably prime CD8⁺ T cell responses despite the ongoing inflammation of the persistent virus infection (Figure 6D). In contrast, Ova-anti-Ova ICs effectively primed OT-I T cell

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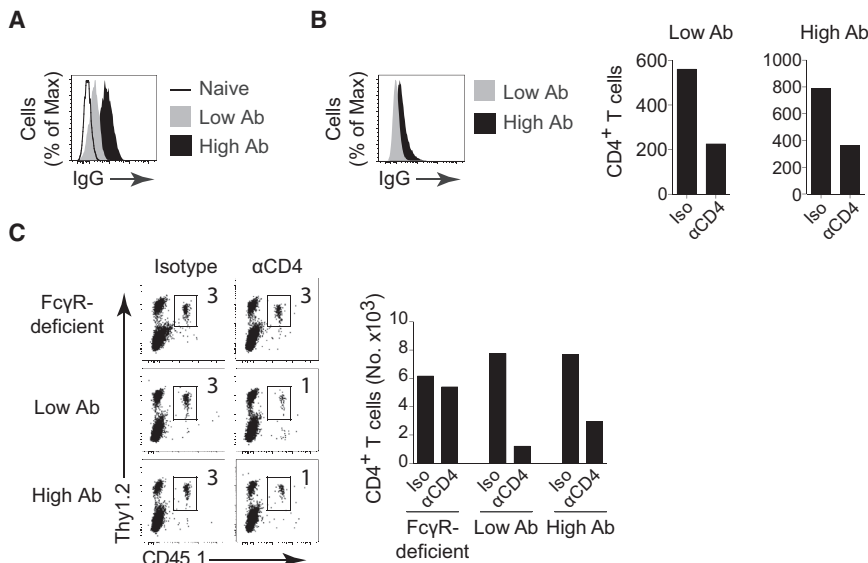


Figure 5. High Amounts of ICs Impede Fc γ R-Mediated Phagocytosis

(A) Surface-bound mouse IgG on macrophages of naive mice (black line) and day 21 after LCMV-CH13 infection of mice that were CD4⁺ T cell-depleted prior to infection (low Ab; gray) or left undepleted (high Ab; black).

(B) Histogram shows surface bound mouse-IgG ex vivo on splenic macrophages from low Ab and high Ab mice following enrichment by B cell and T cell depletion. These sorted splenocytes from low Ab and high Ab mice were mixed with CD45.1⁺ CD4⁺ T cells in the presence of isotype or anti-CD4 Ab (clone GK1.5) for an ex vivo phagocytosis assay. Graphs represent the number of CD45.1⁺ CD4⁺ T cells 2 days after culture.

(C) Peritoneal macrophages from CD45.2⁺ persistently infected Fc γ R-deficient and WT mice (both high Ab) and low Ab WT mice (CD4⁺ T cell-depleted prior to infection) were mixed with CD45.1⁺ WT CD4⁺ T cells in the presence of isotype or anti-CD4 Ab (clone GK1.5). Flow plots show the percent and graphs represent the number of CD45.1⁺ CD4⁺ T cells in the culture 1 day later. Data are representative of two or more independent experiments using three to five mice per group.

expansion in persistently infected low Ab mice, whereas priming was almost entirely suppressed in high Ab mice (Figure 6D). Thus, the high concentrations of ICs generated during persistent infection suppress Fc γ R-mediated cross-presentation by APCs to limit de novo CD8⁺ T cell responses to secondary antigens.

DISCUSSION

Despite the many immune dysfunctions associated with persistent viral infections, a specific impact on inherent Ab effector activity has not been observed. Herein, we identify a previously unrecognized mechanism of immunosuppression during persistent virus infection in which the elevated concentrations of ICs directly impede macrophages and DCs to suppress multiple Fc γ R-dependent Ab effector functions. Similar to naive mice (Biburger et al., 2011), cell-depletion capacity during persistent infection was primarily dependent on phagocytosis by macrophages and not other Ab-mediated mechanisms of cell killing. Macrophage suppression was reversed when cells were removed from their high IC environment and could be inhibited when ICs were added back, demonstrating that the macrophages were not permanently dysfunctional, and that high concentrations of ICs were continually blocking surface Fc γ Rs and inhibiting their activity in vivo. Importantly, we did not observe a notable suppression of other Ab functions such as effective target binding, receptor blockade (Barber et al., 2006; Brooks et al., 2006; Teijaro et al., 2013; Wilson et al., 2013), or neutralization of infectious virus, suggesting that the non-Fc γ R-mediated functions of Abs might not be affected. On the other hand, functions that rely on Fc γ Rs (including a component of the neutralizing capacity; DiLillo et al., 2014) may be compromised by ICs generated during ongoing persistent virus replication and affect both endogenous and therapeutic immune control of infection.

The elevated total IgG concentration during persistent infection might increase competition for the neonatal Fc receptor (FcRn) responsible for salvaging Abs from degradation, accounting for the increased loss of non-replenished, adoptively transferred antibody or outcompeting other antibodies present in relatively low amounts. This might then contribute to both the low LCMV-specific Ab titers maintained throughout infection and the shorter duration of vaccination specific antibodies observed in HIV-infected patients (Miotti et al., 1989; Nielsen et al., 1998; Roopenian and Akilesh, 2007). Our results indicate that high doses of potent nAbs can still be efficacious during persistent infections, but their ADCP activity is suppressed rendering them less effective at killing infected cells. Administration of higher doses of Ab enhanced cell-depleting activity during persistent infection (although they were still suppressed). Consistent with this, Rituximab immunotherapy is administered at high doses over multiple treatments and is generally effective in HIV-infected individuals (Sparano et al., 2010). Moreover, the multiple mechanisms through which Rituximab can kill B cells in addition to ADCP might also contribute to the efficacy of Rituximab in the presence of persistent infections (Jiang et al., 2011). Thus, our data demonstrate that not all Ab functions are lost and depleting activity is not entirely absent, just substantially diminished. This similar immunosuppression is observed with CD8⁺ T cells during many persistent virus infections wherein they still retain some antiviral function, but are insufficient to purge the infection. As a result, in conjunction with diminished T cell function, decreased Ab effector activity could contribute to the overall immune suppression and dysfunction that enables viral persistence.

Our data indicate that the broad spectrum of Ab functions that rely on Fc γ R interactions will be compromised during persistently viremic infections that generate high concentrations of ICs. Not only is targeting and killing of infected cells suppressed, but Ab-mediated cross-presentation to prime CD8⁺ T cell

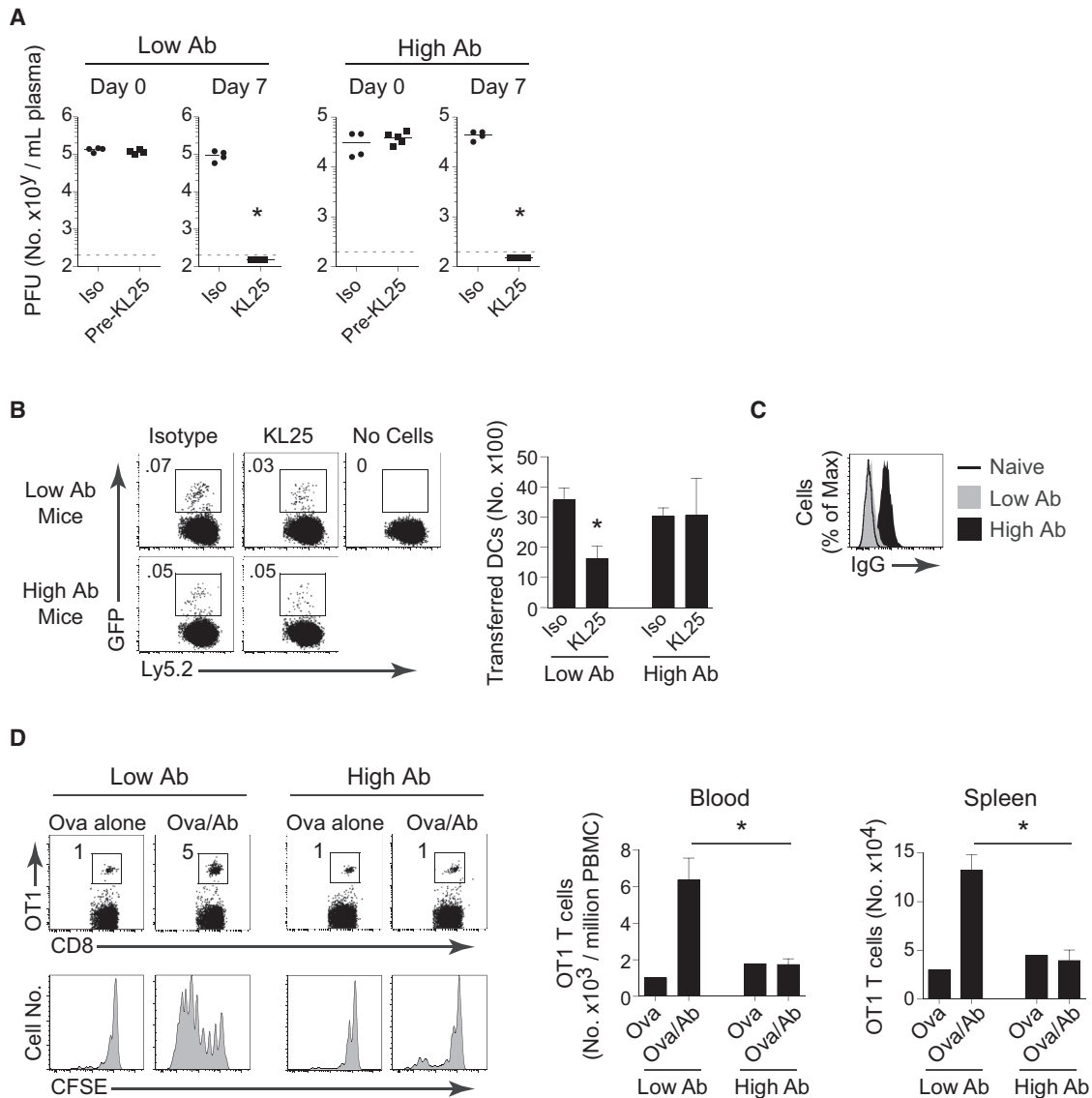


Figure 6. Suppression of Ab Effector Activity Diminishes Killing of Infected Cells and IC-Mediated Cross-Priming of T Cells

(A) High Ab mice (WT) and low Ab mice (CD4⁺ T cell-depleted prior to infection) were persistently infected with the KL25 neutralization-sensitive LCMV-M1 (see Figure S4). On day 21 after infection, mice received 250 μ g of isotype or anti-LCMV KL25 Ab. Graphs represent infectious virus from the plasma before (day 20) and 7 days after isotype or KL25 treatment.

(B) LCMV-M1-infected, GFP⁺ DC (KL25 targets) were transferred into LCMV-C13 persistently infected high Ab mice and low Ab mice (CD4⁺ T cell-depleted prior to infection). All mice were CD8⁺ T cell-depleted prior to infection. Transferred DC were 99% infected (data not shown). Mice received 250 μ g of isotype Ab or anti-LCMV-M1 KL25 Ab and the graph represents the number \pm SD of targets in the spleen 6 hr following Ab treatment.

(C) Surface-bound mouse IgG on dendritic cells in naive mice (black line) and LCMV-C13 persistently infected low Ab mice (B cell-deficient; gray) and high Ab mice (black).

(D) CFSE-labeled Ova-specific OT-I CD8⁺ T cells (CD45.2⁺) were transferred into CD45.1⁺ LCMV-C13 persistently infected (day 21) high Ab mice or low Ab mice (CD4⁺ T cell-depleted prior to infection). One day later, mice were given Ova protein alone or anti-Ova Ab followed by Ova protein (to generate ICs). Flow plots show the percent of OT-I cells in the blood and histograms indicate cell division by CFSE dilution. Graphs represent the number \pm SD of OT-I cells measured in the blood and spleen 3 days after antigen delivery.

* $p < 0.05$. Data are representative of two or more independent experiments using three to five mice per group. See also Figure S4.

responses against secondary antigens is also affected. Since dendritic cells and macrophages act as APCs following IC internalization through Fc γ R (Guilliams et al., 2014), the blockade of Fc γ R could further limit the development of de novo T cell responses against virus escape mutants or secondary co-infec-

tions. Antigen-Ab ICs bound by Fc γ R on FDC are critical for B cell selection, hyper-mutation, and de novo recognition of Ab-escape viruses by naive B cells (Heesters et al., 2014). Thus, decreased ability to bind to FDC could affect the developing and sustained B cell response to persistent viruses. Further,

pre-existing vaccine-induced immunity is compromised during many persistent infections (De Milito et al., 2004; Stelekati and Wherry, 2012) and the suppression of Fc γ R-dependent effector mechanisms such as killing infected cells, opsonizing virions, or cross-presenting antigens to T cells could contribute to their decreased protective capacity. Therefore, the suppression of Ab-mediated effector activity could undermine many critical immune functions, thereby compounding immunosuppression and facilitating viral persistence and co-infections.

It is interesting that although suppression of Ab function was observed for all Abs analyzed, some Abs worked better than others during persistent virus infection. Both murine and non-murine Abs were functionally suppressed during persistent infection despite being highly effective in naive mice, thus arguing against species-specific differences in Fc γ R affinity to explain their loss of efficacy. Restored function of these same depleting Abs in persistently infected low Ab mice and the ability to effectively deplete cells transferred from high to low Ab mice indicate that neither factors secreted during persistent infection nor intrinsic changes in cell resistance to ADCP are responsible for the reduced Ab activity. The continued ability of the anti-*LCMV* Ab to effectively neutralize virus in vivo, yet lose ADCP efficacy, further demonstrates that Abs remain intact during persistent infection and that Ab effector functions can be differentially impacted. Although it is important to note that all Abs analyzed exhibited decreased activity during viral persistence, differences in glycosylation, affinity, target-epitope, or ability to generate ICs might explain why some Abs are more suppressed than others. Considering the desire to elicit broadly effective Abs capable of exhibiting multiple effector functions as preventative and therapeutic vaccines, it will be important in the future to determine why certain Abs are better able to resist suppression.

Polyclonal B cell activation and hyperIgG production during persistent viral infections are associated with the accumulation of auto-antibodies (Hunziker et al., 2003; Massabki et al., 1997; Zandman-Goddard and Shoefeld, 2002). Interestingly, using the platelet-depletion model of idiopathic thrombocytopenic purpura (ITP), we observe a suppression of autoAb-induced platelet reduction during persistent *LCMV* infection when high concentrations of ICs are present. Similar protection is observed with high dose IVIG in the treatment of Ab-mediated autoimmune diseases (Schwab and Nimmerjahn, 2013; Siragam et al., 2005) and is thought to work through a variety of mechanisms including the inhibitory Fc γ RIIB on macrophages (Anthony et al., 2011). However, in response to IC-mediated suppression of Fc γ R function during persistent infection, we did not observe a rescue of antibody activity in persistently infected Fc γ RIIB-deficient mice, although this type of regulation may be important for other aspects of the immune response including B cell antibody production. Interestingly, mouse models and patients with autoimmune systemic lupus erythematosus (SLE) exhibit hyperIgG and a similar defect in Ab-mediated B cell depletion (Ahuja et al., 2011; Merrill et al., 2010; Sanz and Lee, 2010). Although SLE is an autoimmune disorder propagated by pathogenic Abs and IC deposition, the suppression of phagocytic macrophage function might attenuate further disease through other mechanisms (Ahuja et al., 2011). Thus, it is interesting to speculate that the suppression of Fc γ R-mediated function is not entirely detrimental during viral persistence, but that an important func-

tion of the increased Ab observed during persistent infection might be to suppress excessive immunopathology and potential autoimmunity from the self-reactive Abs that are generated during the response to persistent viruses (Hunziker et al., 2003).

Overall, our results have important implications for understanding the ongoing immune response to persistent virus infections, for pre-existing and de novo vaccine-induced Abs aimed at controlling these viruses, and for effective responses to secondary co-infections. Our data indicate that Ab therapies relying on effector mechanisms beyond neutralization might be suppressed by persistent virus infection. Thus, therapies designed to overcome this suppression during viral persistence could increase vaccine-induced immunity and enhance immune control of both persistent infections and secondary co-infecting pathogens.

EXPERIMENTAL PROCEDURES

Mice and Virus

C57BL/6 mice were purchased from The Jackson Laboratory or the rodent breeding colony at UCLA. B cell-deficient (*Ighm*^{-/-}; μ MT), Fc γ RIIB-deficient (*Fcgr2b*^{-/-}), B cell transgenic (hen egg lysozyme [HEL] Ab specific), and CD11c-DTR mice were purchased from The Jackson Laboratory. Fc γ R-deficient mice (*Fcer1g*^{-/-}; lacking Fc γ RI, III, and IV, but expressing Fc γ RIIB) were provided by J. Ravetch (Rockefeller University). Mouse handling conformed to the experimental protocols approved by the UCLA Animal Research Committee (ARC). In all experiments, the mice were infected intravenously (i.v.) via the retroorbital sinus with 2×10^6 PFU of *LCMV*-Arm, *LCMV*-CI13, or *LCMV*-M1. *LCMV*-M1 was generated as described in Osokine et al. (2014) through a reverse genetics approach to rescue a recombinant CI13 virus containing mutations within the GP1 coding region at I118L and S119N. Virus stocks were prepared and viral titers were quantified as previously described (Brooks et al., 2005).

In Vivo Cell Depletions

Target cell depletion was performed by intraperitoneal (i.p.) or i.v. injection of 100 μ g or 1 mg (unless otherwise stated) of the following Abs: anti-CD4 clone GK1.5, anti-CD4 clone YTS191, anti-CD8 clone 53.6.72, anti-CD8 clone 2.43, anti-NK1.1 clone PK136, anti-Ly6G clone 1A8 (BioXcell); anti-mouse CD20 (clone MB20; 50 μ g/mouse) and anti-platelet (clone 6A6; 4 μ g/mouse); and anti-human CD20 (Rituximab; 250 μ g/mouse). For analysis of CD4⁺ or CD8⁺ T cell depletion, cells were gated on NK1.1⁻, Thy1.2⁺ cells unless otherwise stated. For the Rituximab protection experiments, uninfected and *LCMV*-persistently infected mice were given subcutaneous injections of 5×10^5 EG7 tumor cells expressing human-CD20. At day 1 and 3 after tumor injection, mice were given 250 μ g of Rituximab (i.p.). Tumor growth was monitored until tumor diameter reached the 10 mm endpoint.

For studies targeting *LCMV*-infected cells in vivo, the DC cell line DC2.4 was infected with *LCMV*-M1 in vitro for 3 days. Greater than 98% of the cells were infected at the end of the culture (data not shown). The *LCMV*-M1 infected DC were then transferred into *LCMV*-CI13 infected mice that had been CD8⁺ T cell depleted prior to infection (to prevent killing of transferred DCs by *LCMV*-specific CTL). 250 μ g of isotype or anti-*LCMV* KL25 Ab were administered 1 hr after *LCMV*-M1 infected DC transfer. In vivo depletion was quantified 6 hr after transfer.

IC Precipitation and In Vivo Treatment

ICs were precipitated from mouse plasma as performed in Lux et al. (2013). Briefly, mouse plasma was incubated in 8% PEG6000 (IgG IC precipitation) or 20% PEG6000 (free Ab plus IC precipitation; total IgG) overnight at 4°C. PEG precipitations were spun down at 2000 \times g for 30 min at 4°C, washed once with PEG6000, again isolated at 2000 \times g, and resuspended in warm PBS. Precipitated IgG was then quantified by ELISA.

In vivo ICs were generated by incubating 1 mg of rabbit anti-chicken egg albumin Ab (Sigma) and 1 mg of ovalbumin (Ova) protein for 30 min in PBS

at 37°C. Low Ab mice were treated i.p. with 1 mg Ab or 1 mg Ova-Ab ICs daily for 5 days. One day after the first immune complex treatment, mice received 100 µg of anti-CD8 Ab and cell depletion was quantified 5 days later.

Ex Vivo Phagocytosis Assay

Splenocytes isolated on day 21 of LCMV-Cl13 infection were pooled from multiple mice and then B cell and T cell-depleted using anti-CD19, anti-Thy1.2, and anti-CD4 magnetic beads (Miltenyi Biotec) and allowed to recover for 1 hr at 37°C in complete media. Splenocytes were pooled from multiple mice for each experiment to obtain enough cells for the ex vivo phagocytosis assay and experiments were repeated multiple times. B cell/T cell-depleted splenocytes or peritoneal macrophages were mixed with negatively selected, naive CD45.1⁺ CD4 T cell targets at a 100:1 splenocyte:T cell or 50:1 peritoneal macrophage:T cell ratio. 30 µg/ml of isotype or anti-CD4 (clone GK1.5) Ab were added to the culture and the number of target cells was assessed by flow cytometry 2 days later. In some assays, DC or macrophages were removed using anti-CD11c or F4/80-PE staining followed by anti-PE magnetic beads, respectively (Miltenyi Biotec).

In Vivo Ab Mediated Cross-Priming

Ova-specific CD8⁺ OT-I T cell cross-priming was performed as in (Li et al., 2014). CD45.1⁺ LCMV-Cl13 infected high and low antibody mice received 2×10^6 CFSE-labeled CD45.2⁺ OT-I T cells i.v. on day 30 after infection. One day later, mice received i.v. 150 µg of isotype Ab or rabbit anti-OVA IgG followed by 2.5 µg of OVA protein 2 hr later.

Statistics

Student's t tests (two-tailed, unpaired) and two-phase non-linear regression curve fit analyses were performed using GraphPad Prism 5 software (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.01.005>.

AUTHOR CONTRIBUTIONS

D.H.Y. and D.G.B. designed research; D.H.Y. and H.E. performed research; A.L., J.M.T., S.L.M., J.C.d.I.T., and F.N. contributed new reagents, analytic tools, and intellectual input; D.H.Y. and D.G.B. analyzed the data and compiled the manuscript.

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