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

Yamada, Douglas

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

A dissertation submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology & Molecular Genetics

by

Douglas Yamada

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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 Fcγ-Receptor (FcγR) 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 FcγR activity was not due to inhibitory FcγRs or high concentrations of free antibody, and proper FcγR 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.
The dissertation of Douglas Yamada is approved.

Sherie L. Morrison
Jerome A. Zack
John M. Timmerman
David G. Brooks, Committee Chair

University of California, Los Angeles
2015
This work is dedicated to my teachers, mentors, family, and friends.
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Douglas Yamada

EDUCATION

University of California, Los Angeles, CA USA 2009 - Present

• Doctoral Candidate
• Department of Microbiology, Immunology, and Molecular Genetics
  Laboratory: David G. Brooks, Ph.D.

NORTHEASTERN UNIVERSITY, Evanston, IL USA 2000 - 2004

• Degree: B.A. – Chemistry with a Biochemistry Emphasis (June 2004)
  Laboratory: Richard I. Morimoto, Ph.D.
• Thesis: Identification of Potential Protein Interactors with Microtubule Organizing Protein Mbo1

WORK EXPERIENCE

UNIVERSITY OF CALIFORNIA, Los Angeles, CA USA 2009 - Present

  Dr. David G. Brooks Laboratory
  Dept. Microbiology, Immunology, and Molecular Genetics, UCLA

UNIVERSITY OF CALIFORNIA, LOS ANGELES, CA USA 2011

  Teaching Assistant: MIMG 101 Undergraduate Microbiology (Fall 2011)
  Teaching Assistant: MIMG 185A Undergraduate Immunology (Winter 2011)

NORTHWESTERN UNIVERSITY, Evanston, IL USA 2007 - 2009

  Center for Innovation in Global Health Technologies (CIGHT)
  Laboratory of Dr. David M. Kelso
  Sponsored by the Bill and Melinda Gates Foundation

THE JAPAN EXCHANGE AND TEACHING (JET) PROGRAM 2004 - 2007

  Mie Prefecture, Matsusaka City, Japan

  Assistant Language Teacher Matsusaka Science High School

DR. RICHARD I. MORIMOTO LABORATORY 2002 - 2004

  Northwestern University, Evanston, IL USA

  Independent Undergraduate Student Researcher
AWARDS / HONORS / SERVICE

2014  UCLA Dissertation Year Fellowship Award
2014  The American Association of Immunologists (AAI) Trainee Abstract Award
2014  UCLA Dept. Microbiology, Immunology, and Molecular Genetics Travel Award
2012  UCLA AIDS Institute Seed Grant Recipient (AI28697)
2011  TA for microbiology and immunology undergraduate courses
2011  Patent: (South Africa No. 2011/02195)
2010  UCLA Biological/Biomedical Sciences Graduate Program Recruitment Leader
2010  Patent: (USA 8,148,071 B2)
2003  Northwestern University Undergraduate Research Fellowship
2000  Japanese American Citizens League – Chicago Chapter – University Scholarship

PRESENTATIONS / ABSTRACTS

2014  American Association of Immunologists (AAI Annual Meeting), Pittsburgh, PA (selected oral presentation and poster)
2014  Immunology LA, Los Angeles, CA (selected poster)
2013  Midwinter Conference of Immunologists, Asilomar, CA (selected poster)
2013  Immunology LA, Los Angeles, CA (selected oral presentation)
2012  Immunology LA, Los Angeles, CA (selected poster)
2011  UCLA MIMG Departmental Retreat (selected poster)

PUBLICATIONS


CHAPTER 1

Introduction
GENERAL INTRODUCTION

The World Health Organization estimates that over half a billion people are currently infected with hepatitis C virus (HCV), hepatitis B virus (HBV), and HIV; accounting for millions of deaths each year. In order to reduce the global burden of persistent viral infections, a primary aim of medical research must be to investigate how persistent infections evade host immune responses. It is known that persistent virus infections result from a combination of factors mediated by both the virus and the host. To maintain their continued survival and propagation, persistent viruses must be able to replicate inside host cells while causing little to no damage and in turn, the host immune response must be sufficiently inadequate to completely purge those virus-infected cells. Viruses can employ many strategies to evade the immune response including latency/dormancy, high rates of genetic mutation, altered viral tropism, and viral replication in sites of immune privilege (1). Host-mediated contributions to viral persistence include the dysregulation of T cell and B cell responses, the disorganization of lymphoid architecture, and the generation of suppressive factors or the deletion of high affinity T cell responses in order to avoid excessive immune activation and immunopathology (2, 3). Our understanding of these highly complex virus-host interactions is improving everyday, and to find success against these devastating diseases, virologists and immunologists must continue to identify the fundamental mechanisms of viral pathogenesis to design therapeutic strategies that enhance immune resolution of persistent infections.

The first viruses were discovered in the 1890s when Dmitri Ivanovski in Russia and Martinus Beijerinck in the Netherlands observed that material passed through a Pasteur-Chamberland filter could cause a disease in tobacco plants without losing infectivity (4, 5). Previous to this observation, scientists like Louis Pasteur and Robert Koch were developing the germ theory of disease and showing that cultured bacteria
were retained in the small pore sizes of these filters and that specific bacteria could be linked to particular diseases (6). In Germany, Friedrich Loeffler and Paul Frosch also identified that material responsible for causing foot-and-mouth disease in cows was able to pass through the small pore filters and maintain pathogenicity when inoculated into previously healthy cattle (5, 7). In the decades following, a wide variety of viruses and virus families would be discovered, and with the invention of the electron microscope in the 1930s, scientists were finally able to visualize these subcellular pathogens responsible for such diverse diseases (8). It would take much longer for scientists to begin to understand how our bodies respond to viral infections, why some infections are quickly and effectively resolved, and the complex immunological mechanisms allowing other viruses to persist indefinitely; a continuously evolving picture that we are still refining today. This dissertation describes a novel mechanism of immunosuppression whereby immune complexes generated during persistent viral infection affect antibody-mediated effector functions required for killing virus-infected cells and cross-presenting antigens to prime T cell responses.

AN OVERVIEW OF IMMUNE RESPONSES TO VIRAL INFECTION

**Innate immunity**

Upon viral infection, the host immune system coordinates the activities of both the innate and adaptive immune responses in order to effectively control virus replication and eliminate the infecting pathogen. The innate immune system is responsible for the initial detection of viral infection and assisting in the activation of adaptive immunity. Innate immune cell subsets including natural killer (NK) cells, monocytes/macrophages, dendritic cells (DCs), and NK-T cells possess pattern recognition receptors (PRRs) such as NK cell receptors, Toll-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors, and
mannose-binding receptors (9-11). These PRRs recognize pathogen-associated molecular patterns (PAMPs) like viral proteins, CpG DNA, double-stranded RNA (dsRNA), and single-stranded RNA (ssRNA) (12, 13). When PRRs sense viral infection, these cells release chemokines to recruit other cells to the site of infection and secrete type 1 interferon (IFN-I) and other proinflammatory cytokines that provide immediate antiviral defense as well as activate macrophages, NK cells, and DCs (11, 14, 15). NK cells can recognize virus-infected cells through innate PRRs and produce high levels of the antiviral cytokine interferon-γ (IFN-γ), which can also activate DCs and help initiate adaptive immune responses. Activated DCs gather viral antigen at the site of infection, increase antigen presentation on major histocompatibility complex (MHC) class I and class II, increase the expression of costimulatory molecules (i.e., CD40, CD80, and CD86), and then migrate to the lymph nodes where they serve as antigen presenting cells (APCs) to initiate T cell and B cell responses (1). Alternatively, tissue resident DCs in the draining lymph nodes can capture antigens and express the peptide fragments on MHC molecules along with costimulatory molecules for effective antigen presentation (16).

**Adaptive immunity: T cells (naive)**

In contrast to the generalized sensing of PAMPs by the innate immune system, adaptive immunity provides highly specific immune responses to an incredible variety of pathogens through germline-encoded diversity in their antigen recognition receptors. CD8⁺ and CD4⁺ T cells are generated from hematopoietic stem cells and matured in the thymus where they develop their antigen specificity to become mediators of cellular immunity (17). Inside the thymus each T cell achieves a genetically diverse T cell receptor (TCR) through successful V(D)J recombination and becomes CD8⁺ or CD4⁺ based on its ability to recognize self-peptide bound to MHC class I (CD8⁺) or MHC class
II (CD4$^+$) in a process called positive selection (18). Before exiting the thymus and entering the periphery, these T cells must undergo a process of negative selection where T cells that bind too strongly to self-peptide/MHC complexes are deleted to prevent autoimmunity by highly self-reactive T cells (19). The resulting repertoire of T cells displays a tremendous amount of diversity in antigen recognition needed for effective responses to the vast variety of pathogens encountered during the lifespan of the host.

After leaving the thymus, naïve T cells circulate throughout the body and enter peripheral lymphoid organs such as the spleen and lymph nodes where they survey APCs in order to detect their cognate antigens. In the case of CD8$^+$ T cells, antigen recognition is restricted to MHC class I complexes that display peptide fragments of 8-10 amino acids in length (20, 21). CD4$^+$ T cells are restricted to MHC class II complexes that display peptide fragments around 13-17 amino acids in length (21, 22). The peptide fragments displayed on MHC class I and class II molecules are a representation of the proteins being produced inside those cells or that the APCs have picked up from the extracellular environment (23, 24). Under healthy conditions, cells will display a wide variety of self-peptides that will not engage T cells since self-reactive TCRs were eliminated during negative selection (25). However, virally infected APCs or ones that have taken up extracellular viral antigen, processed the peptides, and loaded them into their MHC molecules will present these non-self peptides to potentially reactive CD8$^+$ and CD4$^+$ T cells (26, 27).

APCs that have been matured by innate immune responses to infection can help to activate naïve T cells by supplying three important signals (28). Signal 1 is the TCR recognition of a pathogen-derived peptide displayed on an MHC molecule of the APC. Signal 2 is achieved when costimulatory molecules on T cells are triggered by the ligands that have been upregulated on mature APCs (i.e., the interaction of CD28 on T
cells and CD80/CD86 on APCs) (29-31). Signal three is provided by inflammatory cytokines like IL-12 and type I interferons that help produce productive T cell responses (32, 33).

**Adaptive immunity: T cells (activated)**

CD8\(^+\) T cells undergo a massive clonal burst of proliferation following activation by APCs and become cytotoxic T lymphocytes (CTLs) capable of killing infected target cells (34-36). Since MHC class I molecules are expressed on nearly every cell type in the body, after expanding in the lymphoid organs CD8\(^+\) CTLs home to sites of infection where they can kill infected cells that are displaying pathogen-derived peptides on their MHC class I complexes (37, 38). After TCR recognition of infected cells, CTLs mediate their killing by releasing perforin proteins that insert into the target cell membrane forming pores through which the CTLs release apoptosis-inducing granzyme proteins (39-42). In addition, CTLs release antiviral cytokines IFN\(_{\gamma}\) and TNF\(_{\alpha}\), and can induce apoptosis of target cells through Fas/FasL interactions (39, 43, 44). Through these mechanisms, CD8\(^+\) T cells can mount highly specific responses to quickly and effectively control viral infections.

CD4\(^+\) T cells are known as T helper (Th) cells because they can support a wide variety of immune activity depending on the response needed at the time of activation. Unlike CD8\(^+\) T cells that can recognize MHC class I complexes expressed on most cells, the TCR of CD4\(^+\) T cells is restricted to MHC class II molecules that are only present on APCs (e.g., macrophages, dendritic cells, and B cells) (45, 46). Upon activation, CD4\(^+\) T cells have historically been described to differentiate into functionally distinct T helper subsets defined by specific transcription factors and the expression of signature cytokines. T helper 1 (Th1) cells express the transcription factor T-bet and produce high amounts of cytokines IFN\(_{\gamma}\), TNF\(_{\alpha}\), and IL-2 that support macrophage activation and
CD8$^+$ T cell responses (32, 33). T helper 2 (Th2) cells express the transcription factor Gata3 and produce IL-4, IL-5, and IL-13 to provide B cell support as well as protection against helminthes and extracellular pathogens (47-49). Th17 cells are driven by the transcription factor RORγt and produce IL-17, IL-21, and IL-22 to provide help against bacterial and fungal infections, but these cells have also been implicated in autoimmune disorders (50-52). Regulatory T cells (Tregs) are a subset identified by the transcription factor FoxP3 and thought to secrete cytokines IL-10 and TGF-β that suppress self-reactive T cell responses and dampen existing immune responses to limit immunopathology (53). One of the most recently identified T helper lineages is the T follicular helper (Tfh) subset that expresses the transcription factor Bcl6 and provides help to B cells by homing to B cell follicles using the chemokine receptor CXCR5 and secreting high amounts of the effector cytokine IL-21 (54-56).

Although these T helper cells are distinguished by transcription factors commonly referred to as master regulators, the emerging picture is that activated T helper subsets are not terminally differentiated lineages (57, 58). Instead, researchers have demonstrated that CD4$^+$ T cells can co-express combinations of transcription factors and there is a great deal of overlap and plasticity between the helper responses (59, 60). It has recently been shown that throughout the course of a persistent viral infection CD4$^+$ T cell help shifts away from Th1 responses and progressively develops into Tfh responses to assist in virus-specific antibody production (61). In this way, CD4$^+$ T cells can provide optimal help that is tailored to the changing immune environment throughout the course of infections.

Adaptive immunity: B cell and antibody early history and activation

B cells and antibody responses are the last major arm of the adaptive immune system. Unlike CD4$^+$ and CD8$^+$ T cells that can only recognize foreign antigens once the
pathogens have already infected cells and their peptide fragments have been displayed on MHC complexes, B cells and antibodies can detect and potentially neutralize extracellular infectious agents. However, in contrast to CD8⁺ CTLs and some CD4⁺ T cells (62) that can directly kill target cells upon recognizing foreign antigen, B cells and antibodies do not possess direct killing capacity.

The first experiments indicating the importance of B cells and antibodies to adaptive immunity have been credited to Emil von Behring and Shibasaburo Kitasato in 1890 (63). Their studies demonstrated that cell-free serum from an animal that had been immunized against diphtheria toxin (DT) could be transferred to a naive animal and provide protection from a fatal dose of DT (64). The specificity of this protection was also shown when they repeated these results using serum from tetanus toxin immunized animals that could neutralize a virulent form of tetanus, but this immune serum had no effect against challenge with diphtheria toxin (63-65). While studying the antimicrobial activity of immune serum, Paul Ehrlich coined the term “antibodies,” and in 1897 theorized that toxin receptors could be found on the surface of blood cells and that after these “side chains” engage their specific toxins, more receptors would be released into the blood as antibodies (66). Arne Tiselius and Elvin Kabat later demonstrated in the 1930s that the antitoxin effects provided by serum transfer resided in a protein fraction called gammaglobulin that could be isolated using electrophoresis (67). The cellular source of antibodies was not demonstrated until the late 1940s when Astrid Fagraeus correlated antibody production with the growth in numbers of immature plasma cells following secondary infection with Salmonella typhi (63, 68).

It is now known that B cells possess surface immunoglobulins called B cell receptors (BCRs) that maintain the same antigen specificity as the antibodies they produce (69). During viral infections, BCRs recognize extracellular virions or viral proteins and upon cross-linking of the receptors, activation signals are initiated causing
the BCRs to internalize the foreign antigens (70). In most cases, BCR stimulation alone is not enough to generate optimal antibody responses, so antigen-specific CD4⁺ T cell help provides accessory signals and cytokines necessary for B cell proliferation and differentiation into antibody producing plasma cells (71, 72). Once BCRs have internalized the foreign antigens, these proteins are degraded into peptide fragments that are loaded into MHC class II complexes and displayed on the surface of B cells (73). Activated CD4⁺ T helper cells responding to the same virus infection will recognize and bind to the peptide:MHC class II complex on B cells and supply CD40 costimulatory signals to the B cells by expressing CD40-Ligand (CD40L) (74, 75). These interactions drive the proliferation and expansion of both B cell and CD4⁺ T helper cell immune responses with additional bi-directional costimulatory interactions including molecules such as 4-1BB or ICOS on T cells and 4-1BBL or B7RP-1 (ICOSL) on B cells (76, 77).

In addition to costimulation, CD4⁺ T helper cells provide necessary cytokines to B cells such as IL-4, IL-5, IFNγ, and TGF-β that help to determine the type of antibody produced by the B cell in order to generate the correct effector function to fight the specific infection (78).

**Adaptive immunity: Antibody responses**

The prototypical antibody secreted by plasma cells in response to viral infection is Immunoglobulin G (IgG). IgG antibodies consist of two identical heavy chains connected by disulphide bonds, and each heavy chain is associated by disulfide bonds with an identical light chain to form a Y-shaped structure (79). Studies into the structure of IgG established that enzymatic cleavage with papain produced two separate fragments; one stable component composed of heavy chains only that could be easily crystallized, but did not interact with antigen (fragment crystallizable; Fc), and one composed of heavy chain fragments with associated light chains that retained antigen
binding capacity (fragment antigen binding; Fab) (80, 81). The separate or combined interactions of the immunoglobulin variable regions in both the heavy chains and light chains confer the antigen binding specificity of the Fab (82, 83). Antibodies are able to achieve their incredible diversity in antigen binding capacity by germline-encoded differences in these variable regions as well as an affinity maturation process that further alters these variable regions called somatic hypermutation (84). Both the heavy chain and light chain contain three hypervariable loops called complementarity determining regions (CDR1-3) encoded by either the combination of V, D, and J gene segments for the heavy chain or the V and J gene segments for the light chain (85, 86). It is these globular hypervariable loops extending from the antibodies that make highly specific contacts with foreign antigens, thus resulting in direct neutralization of viruses and toxins or highly effective targeting of pathogens for immune mediated clearance/destruction.

In contrast to the genetic diversity needed for the Fab portion of the antibody to bind to a wide variety of pathogens, the Fc antibody component is genetically constant in order to interact with evolutionarily conserved defense strategies utilized by the host immune system. There are five main classes of antibody Fc components (IgM, IgD, IgG, IgE, and IgA) that have variations in their heavy chain constant regions such as the number of domains, glycosylation sites, disulphide bonds, and hinge region flexibility, which all confer specialized functionality in the immune responses that they elicit (87, 88). Some main Fc class differences allow active transport receptors to deliver those antibodies to specialized anatomical locations like IgA to mucosal layers, tears, and breast milk or maternal IgG delivery across the placenta to protect a developing fetus (89, 90). Other Fc portions (IgM and IgG) are highly effective at activating complement cascade proteins that enable targeted lysis of pathogens and the inflammatory recruitment of phagocytic cells that recognize complement-opsonized microbes (91, 92). In addition to class differences, IgG antibodies also vary in subclass (IgG1, IgG2, IgG3,
and IgG4 in humans and IgG1, IgG2a, IgG2b, and IgG3 in mice) and these variations confer distinctive functional properties through their interactions with other components of the immune system (93). A major way that antibodies are able to instruct immune responses is through their interactions with Fc receptors on the surfaces of various immune cells (94, 95). The constant regions of IgG antibodies that have bound to a pathogen can bind to Fcγ-receptors (FcγRs) on NK cells causing them to release cytotoxic perforin and granzyme proteins in a process called antibody-dependent cell-mediated cytotoxicity (ADCC) (96). Similarly, phagocytic cells possess FcγRs and can bind the IgG constant regions stemming from antibody-opsonized pathogens to initiate phagocytic clearance in a process known as antibody-dependent cell-mediated phagocytosis (ADCP) (97, 98). The Fc portion of IgE can bind to Fce-receptors on mast cells, basophils, and eosinophils inducing the release of histamine and other inflammatory mediators thought to be beneficial in immune responses to parasites, but also involved in allergic responses and some autoimmune disorders (99-101). Thus, antibodies are critical mediators of effective pathogen control by the adaptive immune system. The generation of highly specific binding by the Fab can neutralize viruses or toxins to prevent further propagation of disease, and the Fc portion can instruct multiple effector functions leading to direct killing of antibody-opsonized pathogens or antibody-mediated clearance by phagocytic cells.

**Summary: Immune function and dysfunction**

Together, the innate and adaptive immune responses to viral infections produce a coordinated effort to potently and effectively control viral replication while causing minimal damage to the host. Innate immune cells initially sense pathogens through pattern recognition receptors and in turn produce inflammatory cytokines to directly inhibit viral replication, recruit further innate immune defenses, and begin to activate
adaptive immune responses. Activated dendritic cells in secondary lymphoid organs can present viral antigens to initiate virus-specific CD8$^+$ and CD4$^+$ T cell responses. After homing to sites of inflammation and infection, CD8$^+$ CTLs can directly kill virus-infected cells that are presenting viral peptides in their surfaces MHC class I molecules. CD4$^+$ T cells provide helper functions in the form of cytokine help and costimulation for the generation and survival of effective CD8$^+$ T cell responses as well as B cell proliferation and virus-specific antibody production. Immediately following viral infection, a race begins between the virus’s ability to replicate and prolong infection versus these combined efforts by the host immune system to completely purge the virus (3). Some viral infections are effectively controlled leading to an acute infection and resulting in complete viral resolution. Other viruses have evolved multiple methods to outpace and evade the developing immune response resulting in incomplete resolution and continued viral persistence (2, 102).

Persistent viral infections have become one of the greatest threats to global health, and hundreds of millions of people continue to suffer their effects everyday. Two common strategies used by persistent viruses to evade immune resolution are (1) the generation of viral latency and (2) the ability to achieve high levels of replication thereby inducing immune dysregulation and immunosuppression (103, 104). Viruses such as the herpes simplex viruses can avoid immune detection by going through long periods of latency where no viral activity can be detected in host cells (103, 105). Upon virus reactivation during sporadic lytic stages of infection, viral replication and shedding resumes and these outbreaks must be quickly controlled by the host immune system. In contrast to latent viruses where intermittent viremia is generally well controlled, highly replicative viruses such as HCV, HBV, HIV, and mouse lymphocytic choriomeningitis virus (LCMV) maintain persistence by causing alterations in the host immune environment resulting in increased expression of immunosuppressive cytokines,
negative regulatory signaling factors, lymphoid disorganization, and exhausted or dysfunctional T cell and B cell responses (106-111). The focus of this dissertation is on host immune responses generated against these persistent viral infections, and we use the LCMV model of murine infection to examine novel mechanisms of immune evasion utilized by highly replicative viruses.

USING THE LCMV MODEL TO STUDY PERSISTENT VIRAL INFECTION

**LCMV: Early history**

Since its discovery in 1933 by Charles Armstrong, the lymphocytic choriomeningitis virus (LCMV) murine model of viral infection has been involved in many fundamental advances in viral immunology (112, 113). The exceptional amount of preexisting literature, transgenic mice, reagents, and easily adaptable murine experimental system make LCMV an incredibly powerful model to study host:virus interactions. Even from the early days of LCMV research, scientists have been interested in elucidating the immunological differences between acute and persistent viral infections. In 1936, Erich Traub observed that mice infected with LCMV in utero did not mount an immune response to the infection and the virus persisted indefinitely in these animals; a sharp contrast to adult mice infected with LCMV whose immune response clears the virus within 8-10 days (114). In these LCMV carrier mice, it was thought that the existence of infection during the immune system’s development made them immunologically tolerant of these foreign viral antigens. Interestingly, in the late 1960s studies by Michael Oldstone and Frank Dixon identified virus:antibody immune complexes (ICs) in the glomeruli of the kidneys, blood vessels, and choroid plexus of these persistently infected mice, thus identifying active antibody responses and IC-mediated tissue injury similar to those found in human persistent infections (112, 115).
In the early 1980s, Rafi Ahmed used the carrier model of persistent infection to show that genetic variants of LCMV with altered viral tropism could be isolated from the spleens of persistently infected mice and these variants could suppress LCMV-specific CTL responses (116). When adult mice were challenged with these viral variants they mounted poor CTL responses and were unable to clear the virus whereas adult mice infected with the parental strain (LCMV Armstrong) mounted productive CTL responses and effectively cleared the virus. One viral variant (LCMV clone 13) persists for 60-90 days in the periphery of adult mice and indefinitely in the central nervous system (CNS) and kidneys (116, 117). Compared to the parental LCMV Armstrong strain, LCMV clone 13 was subsequently shown to have three amino acid substitutions (118) and only two were found to be responsible for its prolonged persistence (119). An F→L substitution in the glycoprotein at position 260 confers higher affinity binding of LCMV clone 13 to its cellular receptor (α-dystroglycan) on the surface of dendritic cells and macrophages, thus increasing infectivity (120). The second amino acid difference is a K→Q substitution at position 1079 in the viral polymerase conferring a higher rate of viral replication following infection (119-121). Since LCMV Armstrong and clone 13 have very few genetic differences, they share identical CD4⁺ and CD8⁺ T cell epitopes allowing for direct comparison of T cell responses in acute (Armstrong) and persistent (clone 13) viral infections. Studies using these viral infections have been instrumental in identifying the immunological factors leading to productive versus abortive T cell immune responses, many of which have been later observed in human persistent infections including HIV, HCV, and HBV (122-124).

**LCMV: Immunosuppression and immune restoration**

In contrast to acutely cytopathic viruses such as smallpox virus, rabies virus, neurotropic poliovirus, and murine vesicular stomatitis virus (VSV) that can cause
excessive pathology to infected tissues, poorly or non-cytopathic viruses such as HBV, HCV, HIV, and LCMV do not directly induce cellular damage. Instead, disease in these viral infections is caused by the host immune responses, and this immunopathology can result in complications including liver disease, meningitis, or disruption of lymphoid tissue architecture (125-129). Interestingly, in addition to CTL and immune complex-mediated tissue damage, researchers have shown that infiltrating pathogenic monocytes and neutrophils cause fatal CNS vascular lethality in LCMV meningitis studies (130). It is therefore necessary that immune responses be the combined efforts of aggressive host antiviral tactics generated to provide swift killing of virus-infected cells, but also host-derived negative regulators to prevent excessive immunopathology.

HCV, HIV, and LCMV clone 13 quickly establish highly viremic infections due to their fast speed of replication that outpaces the developing immune response, thus inducing host-mediated immunosuppression and further contributing to viral persistence (Figure 1) (122, 131-134). Studies in LCMV first described the phenomenon of T cell exhaustion where virus-specific CD8$^+$ CTLs were deleted or remained in a state of reduced functionality during viral persistence in order to limit immunopathology (104, 135). It was later shown that both CD4$^+$ and CD8$^+$ T cells can become exhausted and that exhaustion results from continuous antigen presence since effector responses (e.g., IL-2, TNF$\alpha$, and IFN$\gamma$ antiviral cytokine production) are initially similar between acute and persistent infections, but become suppressed as the virus persists (136, 137). Many host-mediated immunosuppressive factors combine to induce T cell exhaustion, but the most widely known are IL-10 and PD-1 (109, 110, 138). By blocking these suppressive pathways, researchers have observed restored polyfunctional T cells producing multiple antiviral cytokines leading to enhanced control of persistent infection. Similarly, chronic type I interferon signaling has recently been shown to be associated with hyperimmune activation and immunosuppression during viral persistence. Blockade of the type I
interferon signaling reduced the levels of chronic immune activation, facilitated the decreased expression of immunoregulatory molecules, restored the lymphoid architecture, and ultimately enabled virus clearance (139, 140). Multiple regulatory pathways control T cell responses and researchers are now beginning to elucidate the synergistic effects of therapeutically targeting these inhibitory molecules for the restoration of T cell immunity during persistent viral infections as well as various types of cancer (141-145).

**LCMV: B cell and antibody responses**

Less is known about the suppression of B cell and antibody responses during persistent viral infection. Mice genetically deficient of B cells that therefore cannot produce antibodies are still able to control LCMV Armstrong infection (acute), but cannot clear persistent LCMV strains (146, 147). However, B cell deficient mice were also found to have distorted splenic architecture and altered CD4+ and CD8+ T cell functionality (148). Researchers therefore infected mice with normal splenic architecture, but whose B cells recognized a non-LCMV antigen and they again observed protracted viremia as well as a failure to resolve persistent LCMV infection, thus confirming the necessity for LCMV-specific antibody production for eventual viral control (147, 149).

Antiviral antibodies can be either neutralizing or non-neutralizing (Figure 2). Neutralizing antibodies bind to viral surface proteins and are able to prevent cellular entry by either blocking virus attachment to its receptor or preventing conformational changes in the viral protein required for productive infection (150-152). In addition, neutralizing and non-neutralizing antibodies can bind to free virus or virus-infected cells and promote viral clearance through Fc-mediated effector mechanisms like complement-mediated lysis, NK-mediated ADCC, and phagocyte-mediated ADCP (Figure 3) (153-
The biological relevance of non-neutralizing antibodies was shown in LCMV infection of CTL-deficient mice where non-neutralizing monoclonal antibodies could bind to early viral variants, but no binding was observed later during infection due to selective immune pressure and distinct escape variants (156).

The induction of neutralizing antibodies is one of the best correlates of vaccine efficacy against several viral diseases (157). Passive antibody transfer has also been shown to provide protection in humans against viral diseases such as HBV, measles, polio, and respiratory syncytial virus (RSV) (158). However, since the neutralizing antibody titers following vaccination or passive antibody transfer typically do not reach levels required for sterilizing immunity, the exact mechanism(s) behind the increased protection are unknown. Researchers believe that the initial presence of antiviral antibodies helps to sufficiently blunt infection allowing for the development of productive adaptive immune responses capable of eventually controlling the infection (153). Similarly, transgenic mice that generate high levels of LCMV-specific neutralizing antibodies early during infection enhanced virus clearance (159, 160) and neutralizing antibody-producing B cells helped to clear high viral loads in the carrier model of persistent infection, but these effects all required CD4+ and CD8+ T responses (161). These researchers showed that virus-specific antibodies in combination with productive T cell responses effectively maintain control of certain viral infections leading to reduced host-derived suppressive factors and eventual antiviral immunity.

Virus neutralizing antibodies are generated following acute LCMV infection (162), but are poorly produced and often appear late during persistent viral infections such as HCV, HIV, and LCMV clone 13 (163-165). In contrast, virus-specific yet non-neutralizing antibodies appear early following infection with persistent LCMV strains, but these antibodies remain at very low titers and are insufficient to blunt infection (162). Although virus-specific antibodies remain at low titers, total antibody production is greatly
increased during infections like HIV, HCV, and LCMV clone 13 that exhibit polyclonal B cell activation and hypergammaglobulinemia (166-168). This process was shown in the LCMV system to rely on uncontrolled virus-specific CD4+ T cell help to B cells that present LCMV peptides on their MHC class II molecules regardless of their BCR specificity (169). Thus, persistent viral infections are associated with multiple B cell and antibody abnormalities leading to insufficient early viral control and leading to impaired adaptive immune responses.

IgG ANTIBODY EFFECTOR FUNCTIONS

IgG response to virus

IgG antibodies are the most abundant immunoglobulins found in the serum likely due to their long half-lives (170), and these antibodies are increased close to 10-fold during persistent infections (166, 169, 171). There are four isotypes of IgG found in the serum; IgG1, IgG2, IgG3, and IgG4 in humans and IgG1, IgG2a, IgG2b, and IgG3 in mice. IgG1 and IgG3 are the predominant IgG isotypes generated in humans to viral infections including persistent viral infections like HIV (172), whereas the IgG2a subclass is most commonly elicited to viral infections in mice (173). IgG1, IgG3, and IgG2a are all antibody isotypes that effectively activate complement and FcγR-mediated effector functions (93, 174) and many studies have indicated the importance of these isotypes in viral disease (175-177).

The complement system

Complement proteins are effector molecules of the innate immune system that can be classically activated by antibodies of the adaptive immune responses. Once initiated, a cascade of complement proteins becomes successively activated resulting in lysis or phagocytosis of complement-targeted cells or bacteria (Figure 3). When
neutralizing or non-neutralizing IgG antibodies bind to the surface of virus-infected cells (or free virus), complement C1q proteins bind to the Fc portions of the IgG antibody complexes (178). Following the C1q – antibody Fc interaction, a series of pathway events occurs initially resulting in deposition of C3b proteins on to the surface of infected cells (179). Macrophages and neutrophils have complement receptors (CRs) that can bind to C3b molecules and initiate phagocytic clearance of those infected cells (180, 181). In addition, another cascade of complement pathway proteins initiated by surface C3b molecules results in deposition of C5b proteins onto the surface of the infected cells. C5b proteins then initiate the formation of a membrane attack complex that disrupts the lipid bilayer by forming pores and results in a loss of cellular homeostasis and eventual cell death (182).

**NK cells (ADCC)**

Another effector pathway mediated by the antibody Fc domain is ADCC by NK cells. NK cells are members of the innate immune system since they lack antigen-specific cell surface receptors (183). However, NK cells can mediate targeted destruction of virus-infected cells because they express FcγRIII, which binds to the Fc portions of virus-specific IgG antibodies coating those cells (Figure 3). After binding, NK cells release cytotoxic granules containing perforin and granzyme B to mediate apoptotic cell death (184). For apoptosis to occur, granzyme B molecules enter through perforin-formed holes in the cellular membrane and they can directly activate caspases leading to DNA damage or granzyme B molecules can cleave Bid proteins leading to mitochondrial damage, caspase activation, and eventual cell death (185).

**Phagocytic cells (ADCP)**
Phagocytic cells also express FcγRs that can recognize the Fc domains of antibody-opsonized infected cells leading to ADCP (Figure 3). In the early 1970s Frixos Paraskevas coined the term Fc receptor to describe the observation that spleen cells bearing no pre-existing antibody on their surface could bind antigen-antibody complexes (186). The Fc receptor genes were first identified in the mid-1980s when Jeffrey Ravetch and Jay Unkeless partially sequenced an Fc receptor protein and screened a cDNA library to identify the corresponding gene sequences (63). This screen produced two Fc receptor genes that they were then able to put into receptor negative cells to validate the functionality of the receptor proteins (187). Since their initial characterization, four murine and five human FcγR classes have been identified. Murine receptors include the high affinity FcγRI that is capable of binding monomeric IgG, and low-to-medium affinity FcγRIIB, FcγRIII, and FcγRIV that preferentially bind antibody immune complexes. Humans have FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA as well as the FcγRIIIB, which does not have a signaling domain and may function as a decoy receptor (94, 188). Human and mouse FcγRI and FcγRIIB have similar sequence homology and functionality, human FcγRIIA and mouse FcγRIII are believed to be closely related, and human FcγRIIIA is considered a functional ortholog of murine FcγRIV (93).

FcγRs can be further classified by whether clustering and cross-linking of the receptors will send an activating or inhibitory signal. Activating receptors (FcγRI, FcγRIII, and FcγRIV) contain or are associated with molecules that contain an immunoreceptor tyrosine-based activation motif (ITAM) and the inhibitory FcγRIIB receptor contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) (94). Since phagocytic cells express both activating and inhibitory FcγRs, immune complexes and antibody-opsonized virus-infected cells can cross-link multiple receptors at the same time. The ratio of activating and inhibitory signals received by the cell determines whether
phagocytosis will proceed, and this balance can shift based on factors such as the amount of antibody opsonized to the target or preferential binding of certain IgG subclasses to certain FcγRs (189). Dendritic cells also express all FcγR and can become activated into mature APCs upon immune complex binding and internalization. DCs can then activate CD4⁺ and CD8⁺ T cell responses because antigens internalized through FcγR can be presented on MHC class II molecules as well as cross-presented on MHC class I (190, 191). In addition to the complex signaling ratios of FcγRs determining cellular function, the individual expression of FcγRs can be regulated by environmental cytokines. Researchers have shown that IFNγ regulates the transcription of the FCGR1 gene and cytokines like IL-4 and IL-13 can increase the expression of the inhibitory FcγRIIB receptor, thus they showed that bacterial phagocytosis was increased in the presence of IFNγ whereas IL-4 and IL-13 reduced antigen uptake (192, 193).

Persistent viral infections have evolved mechanisms to outpace and evade innate and adaptive immunity resulting in various immune dysfunctions. If early immune responses are not sufficient to blunt infection, host-mediated immunosuppressive factors are induced to limit the immunopathology caused by hyperimmune activation. Although persistent infections have been associated with polyclonal B cell activation and a delay in neutralizing antibody formation, less is known about inherent antibody function. Here we investigate the effects of the altered immune environment of persistent viral infection on antibody effector functions necessary to elicit effective antiviral activity.

AIMS OF THE DISSERTATION

The experiments described in this dissertation investigate the altered immune environment during an established persistent viral infection and its potential impact on
the antibody effector functions of antiviral antibodies. In chapter 2, we attempt to characterize how high levels of antibodies and immune complexes generated during a persistent viral infection affect the efficacy of several target cell depleting antibodies. Chapter 3 assesses the mechanism behind our observation that these target cell depleting antibodies are substantially suppressed during persistent infection. We explore several common IgG effector pathways and their contribution to this antibody effector suppression, and we examine whether other antibody-dependent immune functions are similarly affected. These studies offer a better understanding of how antibody functions other than virus neutralization are affected during prolonged viral persistence.
CHAPTER 1

FIGURES
Figure 1: Lymphocytic Choriomeningitis Virus Model of Acute and Persistent Infection

Schematic representation of viral kinetics following acute LCMV Armstrong infection (black) and persistent LCMV clone 13 infection (red). LCMV Armstrong causes an acute infection that is controlled by effector CD4^+ and CD8^+ T cells that secrete high levels of antiviral cytokines IFNγ, TNFα, and IL-2. Infection with LCMV clone 13 leads to high viral titers that quickly suppress effector T cell responses leading to protracted viremia and persistent infection.
**FIGURE 1**

- Loss of T cell function
- Failure to clear infection

**Virus rapidly cleared (8-10 days)**
- ↑CD8 and CD4 T cell responses
- ↑IFNγ, TNFα & IL-2
- ↑CTL activity

**Virus persists**
- ↓CD8 and CD4 T cell responses
- ↓IFNγ, TNFα & IL-2
- ↓CTL activity
- ↓high-affinity T cells
Neutralizing antibodies can bind to viral surface proteins and physically block the interaction of the virus and its cellular receptor, thus preventing infectivity. Other neutralizing antibodies prevent conformational changes in viral proteins necessary for virus attachment or insertion into the surface of target cells. Non-neutralizing antibodies are those that retain binding to viral surface proteins, but are unable to prevent cellular infection.
Target Cell

Virus

Neutralizing Ab.

Non-neutralizing Ab.
Figure 3: Graphical Summary of Common Antibody Fc Domain-Mediated Effector Mechanisms

Antibody Fc domains can utilize three major pathways to facilitate the effective clearance of virus-infected cells. (1) Complement proteins bind to the Fc domain of IgG antibodies and initiate a cascade of protein activation leading to eventual target cell lysis or engulfment by phagocytes possessing receptors to surface-deposited complement proteins. (2) NK cells possessing FcγRs can bind to the Fc domain of IgG on the surface of infected cells and initiate antibody-dependent cell-mediated cytotoxicity. (3) Phagocytic cells possessing FcγRs can bind to IgG antibodies to mediate the engulfment and removal of antibody-coated virus-infected cells.
FIGURE 3

CDC
Complement Activation

C1q

C3b

Target Cell

Virus

Effector Cell

FcγR

ADCC (Lysis):
- NK Cells

ADCP (Phagocytosis):
- Neutrophils
- Dendritic cells
- Macrophages

Cell Death/Removal
CHAPTER 2

Suppression of Antibody-Mediated Target Cell Depletion during Persistent Viral Infection
ABSTRACT

Persistent viral infections are a worldwide health concern and therapies to treat these diseases have been disappointingly unsuccessful because the mechanisms utilized by viruses to evade host immunity are still largely unknown. T cell dysfunctions have been well characterized during viral persistence, but less is known about abnormalities in antibody-mediated immune control. Using the lymphocytic choriomeningitis virus (LCMV) model of persistent viral infection, we show that increased concentrations of immune complexes (ICs) generated by host immune responses suppress the efficacy of multiple target cell depleting antibodies (Abs). The altered of Ab activity was detected in all organs analyzed and did not depend on target cell type or Ab isotype, suggesting a global suppression of Ab effector function during persistent viral infection. Importantly, Ab effector activity was restored in persistently infected mice lacking ICs and could be again suppressed upon transfer of ICs, but not free Ab. Thus, we identify a novel mechanism of immunosuppression during persistent viral infection with potential implications toward impaired antiviral Ab-mediated immunity resulting in continued viral persistence.
INTRODUCTION

Antibodies are critical for eliminating viral infection, preventing re-infection, and controlling persistent virus infections (194). Antibodies (Abs) prevent de novo cell infection by opsonizing free virions and neutralizing the interaction between the virus and its cellular receptor (neutralizing; nAbs). In addition, Abs can facilitate infected cell-killing by targeting viral proteins on the cell surface and either activating complement dependent cytotoxicity (CDC), recruiting NK cells (Ab-dependent cell-mediated cytotoxicity; ADCC), or attracting phagocytic cells (Ab-dependent cell-mediated phagocytosis; ADCP) (94). 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 (194, 195). 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 (196).

In many persistent virus infections such as human immunodeficiency virus (HIV) and lymphocytic choriomeningitis virus (LCMV) in mice, nAbs emerge only late during infection and remain at low concentrations (194). In contrast, non-nAbs have been observed to develop early during infection and may exhibit early immune pressure important for the eventual control of persistent virus infection. Consistent with this notion, the contribution of non-nAb 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 (153, 197). 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 (196, 198-202). Separate from neutralization capacity, both nAbs and
non-nAbs can inhibit viral replication at its source by targeting and killing virus-infected cells. Although dysfunctions in T cell and B cell responses are hallmarks of persistent viral infections (195, 203), whether inherent Ab effector functions 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 naïve mice (61), 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 (198, 204, 205). Upon subsequent analysis, it was estimated that at least 10x more Ab would be required to achieve a 50% response in HIV-infected patients (206, 207), suggesting diminished Ab effector activity during persistent infections of humans as well.

Globally, nearly one in six cancers arise due to persistent infections, and tumor-specific immunity has been shown to be severely compromised during persistent infections such as Hepatitis B and C virus or HIV (208). Antibody immunotherapy using Rituximab to target CD20 on B cells has revolutionized our ability to treat B cell lymphomas, but recent studies have shown conflicting results regarding the utility and efficacy of Rituximab therapy in HIV-infected patients (209, 210). Considering that the delicate balance between the control and outgrowth of cancers relies heavily on functional immune systems (211), a suppression of immune-mediated Ab effector activity could dramatically affect anti-cancer immunotherapies.

Herein, we demonstrate that high amounts of ICs generated during viral persistence suppress Ab-mediated depletion of multiple cellular subsets as well as Rituximab-mediated cancer immunotherapy. This Ab effector suppression was independent of cellular target, Ab isotype, or Ab species, and was suggestive of a larger level of global Ab immunosuppression that potentiates persistent viral infection.
MATERIALS AND METHODS

Mice and virus

C57BL/6 mice were purchased from The Jackson Laboratory or the rodent breeding colony at UCLA. B cell-deficient [\(Ighm^{-/-}\); (\(\mu\)MT)] and B cell transgenic [hen egg lysozyme (HEL) Ab specific] were purchased from The Jackson Laboratory. Mouse handling conformed to the experimental protocols approved by the UCLA Animal Research Committee (ARC). In all experiments, the mice were infected i.v. via the retroorbital sinus with 2x10^6 PFU of LCMV-Arm or LCMV-Cl13. Virus stocks were prepared and viral titers were quantified as previously described (108).

In vivo cell-depletions

Target cell depletion was performed by i.p. or i.v. injection of 100\(\mu\)g or 1mg (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 (BioXcell); anti-mouse CD20 (clone MB20; 50\(\mu\)g/mouse) and anti-platelet (clone 6A6; 4\(\mu\)g/mouse); anti-human CD20 (Rituximab; 250\(\mu\)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 5x10^5 EG7 tumor cells expressing human-CD20. At day 1 and 3 after tumor injection, mice were given 250\(\mu\)g of Rituximab (i.p.). Tumor growth was monitored until tumor diameter reached the 10mm endpoint. To reconstitute Ab/IC levels in low Ab mice, mice were treated daily with 25mg/mouse i.p. (in PBS) of human IVIG (Privigen) obtained from the UCLA Pharmacy.
**IC precipitation and in vivo treatment**

ICs were precipitated from mouse plasma as performed in (212). 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 x g for 30 min at 4°C, washed once with PEG6000, again isolated at 2000 x g, and resuspended in warm PBS. Precipitated IgG was then quantified by ELISA.

In vivo ICs were generated by incubating 1mg of rabbit anti-chicken egg albumin Ab (Sigma) and 1mg of ovalbumin (Ova) protein for 30 minutes in PBS at 37°C. Low Ab mice were treated i.p. with 1mg Ab or 1mg Ova-Ab ICs daily for 5 days. One day after the first immune complex treatment, mice received 100μg of anti-CD8 Ab and cell-depletion was quantified 5 days later.

**Flow cytometry**

Cells were stained for the expression of CD4 (clone GK1.5 or RM4-4), CD8α (clone 53.6.72), CD8b.2 (52-5.8), NK1.1, Thy1.1, Thy1.2, CD41, CD61, B220, CD45.1, and CD45.2 (Biolegend); Flow cytometric data were collected on the FacsVerse (BD) and analyzed using FlowJo software (Tree Star).

**ELISA**

Plasma total IgG and LCMV-specific IgG virus-binding studies were performed as in (61). Briefly, ELISA plates were coated overnight with either goat anti-mouse IgG or 2x10^6 PFU LCMV-C113 prepared from supernatants of infected BHK cells. Serial dilutions of mouse plasma samples or the anti-LCMV KL25 Ab were incubated with either goat anti-mouse IgG-coated plates or LCMV-coated plates, washed, and then plate-bound mouse IgG (from the plasma) was detected with HRP-labeled goat anti-mouse IgG. Antibody concentration was quantified by interpolating plasma dilutions.
falling within a standard curve generated from serial dilution of purified mouse IgG (Invitrogen; 500-0.245ng). Plasma Rituximab concentrations were determined by coating ELISA plates with purified goat anti-human IgG (Caltag Laboratories) overnight. Plates were incubated with the mouse plasma samples containing human IgG, washed, and human-IgG concentration was detected with HRP-conjugated goat anti-human IgG (Caltag Laboratories). Quantification was achieved by comparing to known dilutions of a human IgG1 standard (Sigma).

Cell transfer to demonstrate that T cells are not inherently undepletable in persistent infection

Splenocytes isolated on day 21 after persistent LCMV-CI13 infection were B cell and CD8⁺ T cell-depleted using CD19 and CD8 magnetic beads (Miltenyi Biotec). 3.5x10⁵ CD45.1⁺ CD4⁺ T cells were transferred into LCMV-CI13 infected high or low Ab mice that were then treated with 100µg of isotype or anti-CD4 (clone GK1.5) Ab. Total number of CD45.1⁺ CD4⁺ T cells following Ab treatments was quantified by flow cytometry.

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, Inc.).
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 two 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 (116, 194, 213). To examine Ab suppression, we treated naïve 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 naïve mice, Ab-mediated depletion was suppressed even at the highest dose administered during persistent infection (Figure 1A and B). Although both Abs were equally effective at CD4+ T cell-depletion in naïve mice, only the YTS191 Ab showed some, but decreased efficacy in LCMV persistently infected mice (Figure 1A and B). Together, these data indicate that similar to CD8+ T cell exhaustion during persistent virus infection wherein some antiviral function is retained (203), Ab depleting activity is not completely lost, but suppressed.

To assess whether depletion occurs earlier in persistently infected mice followed by rapid repopulation of target cells, we examined the kinetics of depletion in naïve and persistently infected mice. Following Ab treatment, target cells in naïve mice rapidly deplete in the blood by 24 hours and remain depleted for five days, but no depletion was observed during persistent infection (Figure 1C). In addition, repeated treatments to sustain higher concentrations of depleting Ab had no effect on target cells in persistently infected mice (Figure 1D). Thus, the suppression of otherwise highly effective cell-
depleting Abs during persistent infection is not an artifact of observation timing and is not easily overcome.

To further explore the suppression of effector Ab function, we evaluated the efficacy of multiple cell-depleting monoclonal Abs in naïve and LCMV persistently infected mice using Abs of different isotypes and targeting a variety of cell surface antigens. Unlike naïve 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 (Table 1, Figure 2A-C). Similarly, Ab immunotherapy with Rituximab to target huCD20-expressing murine tumor cells was protective in naïve mice at the dose given, whereas 4 out of 5 persistently infected mice rapidly formed tumors (Figure 2D). 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.

**Suppression of Ab Effector Activity is Maintained in Mice with High Antibody and Immune Complex levels 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 naïve mice, low suppression of Ab effector function was observed at day 8 in both LCMV-Arm and Cl13 infections (Figure 3A). 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 3A), 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 IgG (often termed hypergammaglobulinemia; hyperIgG; Figure 3B) (169, 194, 195). 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 3B). In contrast, as the infection progresses, total plasma IgG and IC concentrations are higher in animals with persistent compared to acute infections (Figure 3B and C).

To address the role of hyperIgG and immune complexes 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 4A and B). We used: (1) B cell-deficient mice (\(\mu\)MT) 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 (Figure 4A and B). 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 wildtype (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 4C and 4A), 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 4D). In addition, Ab-mediated cell-depletion was again inhibited in persistently infected low Ab mice given intravenous IgG (human IVIG; 25mg/mouse) to reconstitute mice with levels of Ab and ICs similar to those observed in persistently infected high Ab mice (Figure 4E and F), indicating that the high levels of Ab and/or ICs lead to the suppression of Ab effector activity.

**High Concentrations of ICs Impede Ab Effector Activity during Viral Persistence**

High serum Ab levels in persistently infected mice could lead to reduced half-lives of target-depleting Abs or greater competition to find their target. We observed accelerated Ab catabolism in persistently infected high Ab mice (Figure 5A) that was dependent on high Ab concentration and not merely persistent infection since naïve and infected low Ab mice had similar Ab catabolism kinetics (Figure 5A). Despite differences in antibody turnover, target cells were completely coated with depleting Ab for at least 5 days following treatment (Figure 5B), thus binding defects or increased target turnover from the cell surface 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-C13 infection corresponding to a ~5-10% decrease in Ab depletion efficacy in the acute infection (Figure 3A and C). 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 (Figure 3A and C), suggesting that ICs are responsible for the suppression of Ab depletion activity. To conclusively differentiate the relative contributions of free Ab alone vs. 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 5C). Even treating low Ab mice with 10-times more free Ab still was not sufficient to inhibit Ab effector activity (Figure 5D) and ICs, but not free Ab readily prevented cell-depletion in ex vivo assays (Figure 5E). Together, these data indicate that it is the high concentration of ICs and not free Ab during persistent infection that suppresses effector Ab activity.
DISCUSSION

Despite the many immune dysfunctions associated with persistent viral infections (3), a specific impact on inherent Ab 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 Ab-mediated target cell depletion. This Ab effector suppression was not solely due to the presence of high viral titers observed during persistent infection since persistently infected mice that only generate low Ab levels did not display impaired Ab activity. In addition, no suppression of Ab effector activity was observed when persistently infected low Ab mice were reconstituted with high levels of free Ab, whereas target cell depletion was again suppressed when mice were given high doses of immune complexes (Figure 5C). Thus, high concentrations ICs generated by aberrant host immune responses suppress inherent Ab effector activity during persistent viral infection.

We show that the defect in Ab-mediated target cell depletion was not due to cell intrinsic resistance mechanisms since target cells from persistently infected high Ab mice (depletion resistant) were effectively depleted after being transferred into persistently infected low Ab mice (depletion permissive). Also, the suppression of Ab effector function was observed in multiple organs and found to be independent of antibody isotype and target cell type arguing that this is a global defect impacting Ab activity during persistent viral infection.

Interestingly, the ability of injected Abs to find and bind to their cellular targets was not altered, and complete epitope masking was confirmed during the five-day observation period. Since neutralizing Abs are known to play key roles in controlling and treating viral infections (194), these data argue that neutralization capacity of Abs may not be altered during persistent viral infection. However, the elevated total IgG concentration during persistent infection may increase competition for the neonatal Fc
receptor (FcRn) responsible for salvaging Abs from degradation, thus accounting for the increased Ab turnover we observe in persistently infected high Ab mice. This may then explain both the low LCMV-specific Ab titers maintained throughout infection and the shorter duration of vaccination specific antibodies observed in HIV-infected patients (89, 214, 215). Future studies are needed to investigate the efficacy of Ab-mediated virus neutralization during persistent viral infection, and whether higher turnover rates lead to variations in nAb-mediated virus resolution during persistent viral infection.

Since virus-neutralizing Abs only develop late during infection and require extensive somatic hypermutation (216), the suppression of IgG effector functions mediated by the Fc domain is likely highly detrimental to Ab-mediated antiviral immunity. Complement activation by Ab Fc domains can result in the clearance of Ab-coated virus-infected cells by specialized effectors expressing complement receptors that bind to deposited complement proteins on the infected-cell surface (217). Activation of complement components can also result in the formation of membrane attack complexes that form holes in the surface of virus-infected cells leading to lytic death (182). IgG Ab ICs are known to be highly effective complement activators and the high levels of ICs may suppress Ab effector functions by depleting essential serum complement components needed for effective cellular phagocytosis or lysis. More studies are needed to investigate the importance of complement activity in the suppressive activity of ICs generated during persistent viral infection.

IgG Fc domains also engage FcγRs on NK cells and phagocytic cells to mediate ADCC and ADCP, respectively. NK cells express high levels of FcγRIII that upon binding to IgG trigger the release of perforin and granzyme molecules to mediate cytotoxic killing of antibody-coated virus-infected cells (184). Phagocytic cells express all activating FcγRs (FcγRI, FcγRIII, and FcγRIV) as well as the inhibitory receptor
FcγRIIB (93, 94). When IgG Abs engage activating FcγRs, they signal cells to initiate active phagocytosis whereas engagement of the inhibitory FcγR sends signals that prevent phagocytic activation. During persistent viral infection, high concentrations of ICs could suppress IgG effector activity by blocking the interaction of FcγRs on NK cells or activating FcγRs on phagocytic cells, thus preventing the recognition of Ab-opsonized virus-infected cells. Alternatively, ICs could engage the inhibitory FcγRIIB on phagocytic cells, thus preventing the cellular engulfment. In addition, immunosuppressive cytokines such as IL-10 and TGF-β are highly present during persistent viral infection and known to downregulate the expression of activating FcγRs as well as enhance the expression FcγRIIB (218, 219). Further investigation into how high concentrations of ICs may suppress FcγR-mediated effector functions of IgG Abs during persistent viral infections may provide mechanistic insight into the critical regulation of FcγR immunity.

Administration of higher doses of Ab improved 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 (210). Moreover, the multiple mechanisms through which Rituximab can kill B cells in addition to ADCP may also contribute to the efficacy of Rituximab in the presence of persistent infections (220). 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.
Polyclonal B cell activation and hyperIgG production during persistent viral infections are associated with the accumulation of auto-antibodies (169, 221, 222). 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 (223, 224). Thus, it is interesting to speculate that the suppression of Ab effector activity is not entirely detrimental during viral persistence, but that an important function of increased ICs might be to suppress excessive immunopathology and potential autoimmunity from self-reactive Abs that are generated during the response to persistent viruses. Indeed, researchers have observed antibody responses generated against molecules such as thyroglobulin, double-stranded DNA, single-stranded DNA, and insulin proteins following persistent infection with LCMV (169), yet these mice do not display symptoms of overt autoimmunity.

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 may 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.

We have identified a novel suppression of Ab effector activity mediated by high concentrations of ICs generated during persistent viral infection. Many important questions still remain to be answered into the various mechanisms by which ICs can suppress inherent Ab effector function, whether Ab-mediated antiviral immunity is similarly suppressed, and whether this is another host-derived protective measure to prevent severe immunopathology. Importantly, as immune responses are typically the
sum total of stimulatory and suppressive mechanisms, future studies will be necessary
to characterize the extent to which Ab effector suppression contributes to overall
dysfunctional antiviral responses to persistent viral infections.
CHAPTER 2

FIGURES
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; 100µg) or high dose (HD; 1mg) 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 +/- standard deviation (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 remaining +/- SD of the number of splenic CD4^+ T cells 5 days after Ab treatment relative to isotype treated mice. Percent remaining 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) Naïve and persistently infected mice (day 21 after LCMV-Cl13 infection) received 100µg of isotype or anti-CD4 Ab (clone GK1.5). Graphs represent the number of CD4^+ T cells +/- SD detected in the blood on the indicated days following treatment.

(D) Flow plots represent the percentage of CD4^+ T cells detected in the blood of LCMV-Cl13 infected high Ab mice after repeated treatments with 100µg of isotype or anti-CD4 Ab (clone GK1.5). Anti-CD4 Ab treatment was initiated on day 21 after infection. In this experiment, infected mice received Ab treatments on days 21, 23, and 25 after infection and cell depletion in the blood was assessed the day after each treatment.

*p<0.05. **p<0.05 compared to the same condition in naïve mice. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 1

A.

B.

C.

D.

Wildtype Mice (High Ab)

Day 1 (1st Tx)  Day 3 (2nd Tx)  Day 5 (3rd Tx)

Isotype  αCD4 Tx  Isotype  αCD4 Tx  Isotype  αCD4 Tx

CD8

CD4
TABLE 1: Antibodies Used for *In Vivo* Depletion Experiments

<table>
<thead>
<tr>
<th>Target</th>
<th>Clone</th>
<th>Species</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cells</td>
<td>GK1.5</td>
<td>Rat</td>
<td>IgG2b</td>
</tr>
<tr>
<td>CD4 T cells</td>
<td>YTS191</td>
<td>Rat</td>
<td>IgG2b</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td>53.6.72</td>
<td>Rat</td>
<td>IgG2a</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td>2.43</td>
<td>Rat</td>
<td>IgG2b</td>
</tr>
<tr>
<td>B cells</td>
<td>MB20</td>
<td>Mouse</td>
<td>IgG2a/c</td>
</tr>
<tr>
<td>Platelets</td>
<td>6A6</td>
<td>Mouse</td>
<td>IgG2a/c</td>
</tr>
<tr>
<td>huCD20</td>
<td>Rituximab</td>
<td>Human</td>
<td>IgG1</td>
</tr>
</tbody>
</table>
Figure 2: Multiple Target-Depleting Abs Suppressed During Persistent Infection

(A) Graph represents the percent remaining +/- SD of the number of CD8\(^+\) T cells in the various organs 5 days after Ab treatment relative to isotype treatment in naive mice (left) versus persistently infected mice (right; day 21 of LCMV-Cl13 infection). Percent remaining was calculated by dividing the number of CD8\(^+\) T cells following depletion by the average number of CD8\(^+\) T cells present in isotype treated mice. Isotype is set to 100.

(B) Uninfected and mice persistently infected with LCMV-Cl13 for 21 days received isotype or anti-CD20 B cell-depleting Ab (clone MB20; mouse IgG2a/c). Flow plots represent the percent of B cells (B220\(^+\)) in the blood and graphs represent the number of B cells remaining in the blood and spleen following antibody treatments.

(C) Uninfected (black) and mice persistently infected with LCMV-Cl13 (red) for 21 days received isotype or platelet-depleting Ab (clone 6A6; mouse IgG2a/c). Graph represents the number of platelets determined as CD41\(^+\), CD61\(^+\) per mL blood at the various timepoints following Ab treatment.

(D) Uninfected (black) and mice persistently infected with LCMV-Cl13 (red) for 21 days received huCD20\(^+\) EG7 tumor cells followed by anti-huCD20 (Rituximab; human IgG1). 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 (not shown).
*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 2

A. Bone
   Spleen  Blood  Liver  Marrow

   CD8+ T cells
   (Percent of isotype)

   Iso  αCD8
   Iso  αCD8
   Iso  αCD8
   Iso  αCD8
   Iso  αCD8

B. B cell depletion

   Naive
   Isotype  αCD20
   62  2

   Infected
   Isotype  αCD20
   50  46

C. Platelet depletion

   Platelets (No. x10^9 / mL)
   Time after Ab treatment (hours)

   - Naive
   - Infected

D. Tumor burden

   Percent Survival
   Time after tumor injection (days)

   - Naive
   - Infected

p<0.05
Figure 3: Suppressed Ab-Mediated Cell-Depletion in Persistent, but not Acute LCMV Infection

(A) Naïve mice or mice infected with LCMV-Arm or LCMV-Cl13 8 or 45 days earlier received 100 µg of isotype or anti-CD4 (clone GK1.5) Ab. Graphs represent the number +/- SD of CD4⁺ T cells 5 days after Ab treatment. The number above each bar represents percent depletion vs. isotype.

(B) Graphs represent the concentration +/- 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-Cl13 (red). Assay limit of detection (dotted line).

(C) Graph represents the concentration +/- SD of ICs (black) or total IgG (Ab+IC; grey) detected in the plasma of naïve mice (N) and at the indicated time points after LCMV-Arm (A) or Cl13 (Cl) infection.

*p<0.05 Amount of ICs Arm versus Cl13. **p<0.05 total Ab+IC Arm versus Cl13. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 3

A.

LCMV-Armstrong

Day 8

Blood

Spleen

CD4+ T cells

(No. x 10^4 / million PBMC)

Naive

Blood

Spleen

CD4+ T cells

(No. x 10^4 / million PBMC)

Day 45

Blood

Spleen

CD4+ T cells

(No. x 10^4 / million PBMC)

LCMV-Clone 13

Day 8

Blood

Spleen

CD4+ T cells

(No. x 10^4 / million PBMC)

Day 45

Blood

Spleen

CD4+ T cells

(No. x 10^4 / million PBMC)

B.

Total IgG

Plasma IgG (mg/ml)

Time after infection (days)

LCMV-specific IgG

Time after infection (days)

LCMV Titer (spleen)

Time after infection (days)

△ LCMV-Arm (acute)

● LCMV-C13 (persistent)

C.

Precipitated IgG (mg/mL)

A

Cl

D8

D21

D45

N

Ab + IC

IC

**

**

*
Figure 4: High Antibody Immune Environments Suppress Ab Effector Function

(A) High Ab mice (WT) and multiple low Ab mice [B cell-deficient, B cell Hen Egg Lysozyme (HEL) Tg, and CD4+ T cell-depleted prior to infection] were infected with LCMV-CI13. The graph demonstrates plasma virus titers on the indicated day.

(B) Total plasma IgG in naive wildtype mice, LCMV-CI13 infected (day 21 after infection) high Ab mice (WT), and the indicated low Ab mice.

(C) LCMV-CI13 infected wildtype, B cell-deficient (μMT), B cell transgenic (HEL-tg) mice, or mice CD4+ T cell-depleted prior to infection received 100μg of isotype, anti-CD4 (clone GK1.5) or anti-CD8 (clone 53.6.72) depleting Ab 21 or 30 days after infection. The mice that were CD4+ T cell-depleted before infection received anti-CD8 Ab at the 30 day time point. Flow plots indicate the percent and graphs represent the number +/- SD of CD4+ or CD8+ T cells in the blood 5 days following Ab treatment.

(D) CD19, CD11b, and CD8-depleted splenocytes from LCMV-CI13 persistently infected CD45.1+ mice (D21 after infection) were transferred into infection matched CD45.2+ low Ab mice (CD4+ T cell-depleted prior to infection) or high Ab mice. Mice received 100μg of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and graphs represent the number +/- SD of donor CD4+ T cells (B220+, Thy1.2+, CD4+, CD45.1+) remaining in the blood 2 days following Ab treatment.

(E) B cell-/− mice at day 21 of LCMV CI13 infection (low Ab) were left untreated or treated with 25mg of IVIG daily for 5 days. Graphs represent the concentration +/- SD of
ICs (black) or total IgG (Ab+IC; grey) detected in the IVIG pre-transfer and from the serum of B cell-/− mice reconstituted with IVIG.

(F) B cell-/− mice at day 21 of LCMV CI13 infection (low Ab) were left untreated or treated with 25mg of IVIG daily for 5 days. Mice received 100µg of isotype or anti-CD4 (clone GK1.5) Ab 1 day after the first IVIG treatment. Flow plots indicate the percent and graphs represent the number +/- SD of CD4+ T cells in the blood 5 days after anti-CD4 Ab treatment.

*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 4

A. Virus Titer

- High Ab
  - WT
  - Low Ab
  - B cell-deficient (μMT)
  - B cell Tg (HEL-Tg)
  - αCD4 WT

Time after infection (days)

B. Plasma IgG (mg/L)

- WT
- HEL-Tg
- αCD4 WT

C. Cytokine Response

- High Ab
  - WT
  - B cell-deficient
  - B cell Tg
  - Pre-CD4 depletion

D. CD4/CD8 Ratio

E. Pre-transfer

F. Serum IgG

- αCD4
- IVIG
Figure 5: High Concentrations of Immune Complexes Impede Ab Effector Activity during Viral Persistence

(A) Naive (black) or LCMV-CI13 infected (day 21 after infection; red) wildtype mice (left) and B cell-/- mice (right) received 1.5mg of Rituximab. Mice were bled at the indicated times after treatment and human IgG (Rituximab) concentration +/- SD was measured in the plasma by ELISA.

(B) LCMV-CI13 infected mice (day 21 after infection) received 100µg of isotype or anti-CD4 Ab (clone GK1.5). PBMC were stained for CD4+ T cells using an anti-CD4 detection Ab that recognizes a different epitope than the GK1.5 depleting Ab (clone RM4-4) or with the same anti-CD4 GK1.5 clone used for depletion.

(C) LCMV-CI13 infected low Ab mice (CD4+ T cell-depleted prior to infection) were left untreated, treated daily with 1mg of anti-OVA Ab alone or 1mg of Ova protein/ anti-OVA ICs. One day after the first treatment, mice received 100µg of isotype or anti-CD8 Ab (clone 2.43). Graph represents the number +/- SD of CD8+ T cells in the spleen 5 days after depletion.

(D) LCMV-CI13 infected low Ab mice (CD4+ T cell-depleted prior to infection) received 100ug of isotype Ab (Iso), 100ug of anti-CD8 Ab (clone 2.43; αCD8 Tx), or anti-CD8 Ab plus daily treatment with 10mg monomeric Rituximab Ab (Ab+αCD8 Tx). In the latter, anti-CD8 Ab was administered 1 day after the first Rituximab treatment. Graphs represent the number +/- SD of CD8+ T cells in the blood and spleen 4 days after depletion.
(E) Peritoneal macrophages from LCMV-Cl13 infected low Ab mice (CD4+ T cell-depleted prior to infection) were incubated with CD45.1+ CD4+ T cells and isotype (light grey) or anti-CD4 Ab (clone GK1.5) in the presence of increasing concentrations of anti-Ova Ab alone (black) or anti-Ova/OVA ICs (grey). Graph represents the number of CD45.1+ CD4+ T cells one day later.

*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 5

A. Wildtype Mice (High-Ab.)  B Cell KO Mice (Low-Ab.)

* Naive  Red Persistently Infected

Days Post Infection

Plasma Rituximab (µg/mL)

C.

B. Wildtype Mice (High Ab)

Detection w/ different Ab clone (unblocked)

CD8

Isotype  αCD4 (GK1.5)

Detection w/ same Ab clone (blocked)

CD4 (RM4-4) → CD4 (GK1.5)

CD4 (GK1.5)

No. CD8 T cells (x10^6)

D.

Blood

Spleen

CD8+ T cells (No. x10^4/million PBMC)

αOVA Ab  αOVA IC

No. CD4 T cells (No. x10^5)

E.

αCD4 T x

Ab conc. (µg/mL)

0 10 20 40 80

n.s.

αOVA Ab  αOVA IC
CHAPTER 3

Suppression of Fcγ-Receptor-Mediated Antibody Functions during Persistent Viral Infection
ABSTRACT

Persistent viruses have evolved strategies to subvert and evade host immunity, and a greater understanding of these mechanisms is needed if we are to eventually eliminate these infections. In this chapter, we demonstrate a novel mechanism of immunosuppression whereby increased amounts of immune complexes generated in mice persistently infected with lymphocytic choriomeningitis virus (LCMV) suppressed multiple Fcγ-Receptor (FcγR) functions. Increased concentrations of immune complexes physically blocked FcγRs on macrophages and dendritic cells thereby suppressing antibody-mediated target cell depletion, therapeutic antibody-killing of LCMV infected cells, and reduced immune complex-mediated cross-presentation for T cell activation. Suppression of FcγR activity was not due to increased signaling of inhibitory FcγRs, and proper FcγR functions were restored when immune complexes were washed from the cell surface. Thus, we identify a mechanism of immunosuppression during viral persistence with implications for understanding effective antibody activity aimed at pathogen control.
INTRODUCTION

During persistent viral infections such as HIV in humans, SIV in primates, and lymphocytic choriomeningitis virus (LCMV) in mice, virus-specific CD4⁺ T helper cells differentiate into T follicular helper cells (Tfh) and give rise to increased levels of germinal center B cells, plasma cells, and high concentrations of IgG antibodies (Abs) (61, 225, 226). These persistent viral infections are associated with polyclonal B cell activation, hypergammaglobulinemia, and immune complex (IC) accumulation resulting from non-virus-specific B cells displaying exogenously acquired viral peptides on major histocompatibility complex class II molecules (MHC II) and receiving help from CD4⁺ Tfh cells (166, 169, 227, 228). We have recently observed a suppression of Ab-mediated target cell depletion dependent on increased concentrations of ICs during persistent viral infection (Chapter 2). Given the importance of antiviral Abs, we investigated the mechanism(s) involved in Ab effector suppression and its impact on overall Ab immune activity during persistent viral infection.

Effective antibody responses are essential for protective immunity against many pathogens and for eventual control of persistent viral infections (194). Vaccine-induced antiviral Abs can provide protective immunity against several viral diseases (157), and rather than providing complete sterilizing immunity, it is believed that these Abs sufficiently blunt infections allowing for the initiation of productive innate and adaptive immune responses to rapidly clear the pathogen (153). While neutralizing Abs (nAbs) mediate pathogen inhibition through their antigen-specific variable domains (Fab), the antibody constant domains (Fc) of both virus-specific nAbs and non-nAbs can initiate potent antiviral properties by recruiting a wide spectrum of proteins and effector cells of the innate immune system.

Both nAbs and non-nAbs can bind to the surface of virus-infected cells and mediated infected cell killing by complement-dependent cytotoxicity (CDC), antibody-
dependent cell-mediated cytotoxicity (ADCC) by NK cells, or antibody-dependent cell-mediated phagocytosis (ADCP) by phagocytic cells (229). Effector cells of the innate immune system including NK cells, monocytes, macrophages, dendritic cells, neutrophils, and others express Fcγ-receptors (FcγRs) that engage the Fc domain of these virus-specific IgG Abs and mediate killing/clearance of infected cells (230). Therefore, increased concentrations of ICs present during persistent viral infection (Chapter 2), may interfere with complement proteins or FcγRs to suppress CDC, ADCC, and/or ADCP activity. Further, antigen presenting cells (APCs) internalize antigen-Ab ICs via FcγRs to then initiate T cell responses against virus escape mutants and co-infecting pathogens. Similarly, FcγRs on follicular dendritic cells (FDCs) help to retain ICs for B cell selection and affinity maturation to produce highly effective antibodies (231). Although dysfunctions in T cell and B cell responses are hallmarks of persistent viral infections (195, 203), whether Ab effector functions such as CDC, ADCC, ADCP, or cross-presentation are affected remains unclear.

In this study, we continue to expand on the finding that Ab-mediated target cell depletion is highly suppressed by increased concentrations of ICs generated during persistent viral infection (Chapter 2). Herein, we investigate the impact of ICs on complement proteins, NK cells, and phagocytic cells such as dendritic cells, neutrophils and macrophages to establish their contribution to Ab-dependent effector functions. We demonstrate that FcγR-mediated Ab activity including the killing of virus-infected cells and cross-presentation for T cell activation are suppressed by high concentrations of ICs, thus compounding the overall immunosuppression that potentiates persistent viral infection. These findings are highly suggestive that human persistent infections associated with IC accumulation such as hepatitis B and C virus as well as HIV infection
may display a similar suppression of Ab effector activity, and that therapeutic intervention to restore Ab efficacy could enhance overall antiviral immune control.
MATERIAL AND METHODS

Mice and virus

C57BL/6 mice were purchased from The Jackson Laboratory or the rodent breeding colony at UCLA. B cell-deficient \([ \text{Ighm}^{-/-}; (\mu\text{MT})] \), FcγRIIB-deficient \((\text{Fcgr}2b^{-/-})\), B cell transgenic [hen egg lysozyme (HEL) Ab specific], and CD11c-DTR mice were purchased from The Jackson Laboratory. FcγR-deficient mice \((\text{Fcer}1g^{-/-}; \text{lacking FcγRI, III, and IV, but expressing FcγRIIB})\) 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 i.v. via the retroorbital sinus with \(2 \times 10^6\) PFU of LCMV-Cl13 or LCMV-M1. LCMV-M1 was generated as described in (232) through a reverse genetics approach to rescue a recombinant Cl13 virus containing mutations within the GP1 coding region at I118L and S119N. Virus stocks were prepared and viral titers were quantified as previously described (108).

In vivo cell-depletions

Target cell depletion was performed by i.p. or i.v. injection of 100µg or 1mg (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). For analysis of CD4+ or CD8+ T cell depletion, cells were gated on NK1.1-, Thy1.2+ cells unless otherwise stated.

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 (not shown). The LCMV-M1 infected DC were then transferred into LCMV-Cl13 infected mice that had been CD8+ T cell depleted prior to infection (to prevent killing of transferred DCs by LCMV-specific CTL). 250µg of isotype or anti-
LCMV KL25 Ab were administered 1 hour after LCMV-M1 infected DC transfer. In vivo depletion was quantified 6 hours after transfer.

**Ex vivo phagocytosis assay**

Splenocytes isolated on day 21 of LCMV-CI13 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 1hr 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, naïve CD45.1 CD4 T cell targets at a 100:1 splenocyte:T cell or 50:1 peritoneal macrophage:T cell ratio. 30μg/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 two 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 (233). CD45.1\(^+\) LCMV-CI13 infected high and low antibody mice received 2x10\(^6\) CFSE-labeled CD45.2\(^+\) OT-I T cells i.v. on day 30 after infection. One day later, mice received i.v. 150μg of isotype Ab or rabbit anti-OVA IgG followed by 2.5μg of OVA protein 2 hours later.

**Flow cytometry**
Cells were stained for the expression of CD4 (clone GK1.5 or RM4-4), CD8α (clone 53.6.72), CD8b.2 (52-5.8), NK1.1, Thy1.1, Thy1.2, Ly6C, Ly6G, CD64 (FcγRI), CD16/32 (FcγRII/III), B220, F4/80, CD11c, CD11b, CD45, CD45.1, CD45.2, and mouse IgG (Biolegend); mouse FcγRIV (F. Nimmerjahn); fixable viability dye (Zombie Aqua; Biolegend). Carboxyfluorescein succinimidyl ester (CFSE) dilution analysis was performed by incubating naïve OT-I cells with 2.5µM CFSE (Life Technologies). Flow cytometric data were collected on the FacsVerse (BD) and analyzed using FlowJo software (Tree Star).

ELISA

LCMV-specific KL25 virus-binding studies were performed as in (61). Briefly, ELISA plates were coated overnight with either goat anti-mouse IgG or 2x10^6 PFU LCMV-CI13 or LCMV-M1 prepared from supernatants of infected BHK cells. Serial dilutions of the anti-LCMV KL25 Ab were incubated with LCMV-coated plates, washed, and then plate-bound mouse IgG (from the plasma) was detected with HRP-labeled goat anti-mouse IgG. Antibody concentration was quantified by interpolating plasma dilutions falling within a standard curve generated from serial dilution of purified mouse IgG (Invitrogen; 500-0.245ng).

In vivo phagocytic cell, dendritic cell, and complement depletion

To deplete all phagocytic cells, mice received 200µl i.v. of clodronate-filled liposomes or control liposomes (Encapsula Nano Sciences) at day -1, day +2, +4. Phagocytic cell depletion was confirmed by flow cytometric analysis. To deplete dendritic cells, wildtype mice were lethally irradiated with 1100 rads then reconstituted with 2x10^7 bone marrow cells from CD11c-iDTR mice. Recipient mice were treated with antibiotics
(Sulfamethoxazole and Trimethoprim in the drinking water) for 3 wks to prevent infection and to allow for immune reconstitution. Eight weeks later, mice were treated i.p. with PBS control or 100ng of diptheria toxin (DT) in PBS (List Biological Laboratories) 1 day before performing cell-depletion assay. CD11c+ cell depletion was confirmed by flow cytometry.

For complement depletion, mice were treated i.p. with 15U of cobra venom factor (CVF; Quidel) diluted in PBS on days -3, -1, +2, and +4 of cell-depletion assay to effectively deplete C3 and C5 complement components (234). Complete inactivation of serum complement activity was confirmed using an *in vitro* red blood cell lysis assay (235).

**KL25 Ab isolation and neutralization assays**

Mouse IgG1 KL25 Ab was isolated from a KL25 B cell hybridoma (236) that was obtained from the European Virus Archive. To determine KL25 neutralization activity against LCMV-Cl13 and LCMV-M1, each virus was pre-incubated with serial dilutions of KL25 antibody for 30 min and plaque assay performed on Vero cells. Assays were performed in triplicate. For *in vivo* neutralization, mice were infected with 2x10⁶ PFU of LCMV-M1 and then at day 21 after infection treated with 250µg of mouse IgG1 isotype or KL25 virus neutralizing Ab.

**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, Inc.).
RESULTS

CDC, ADCC, and ADCP by Neutrophils are not Major Mechanisms of Ab-Mediated Cell-Depletion during Persistent Viral Infection

Ab-mediated depletion can occur via complement-dependent cytotoxicity (CDC), ADCC by NK cells, or ADCP by macrophages, dendritic cells (DC), and neutrophils (94, 220). We used low Ab mice where cell-depletion is efficacious despite persistent virus replication (Chapter 2; Figure 4C) to determine the effector mechanisms impeded by the high concentrations of ICs during viral persistence. Using cobra venom factor to deplete serum concentrations of C3 and C5 proteins, we investigated the contribution of CDC to antibody-mediated cell-depletion. If the loss of CDC activity in depletion-permissive low Ab mice also results in a suppression of Ab effector activity, then the high concentrations of ICs generated during viral persistence exert their suppressive function by affecting the CDC pathway. Following CVF treatment and an observed loss of serum complement activity (Figure 1A), Ab-mediated cell-depletion was still highly effective in low Ab mice (Figure 1B). Similar to our CDC studies, the removal of NK cells (Figure 1C and D) or neutrophils (Figure 1E and F) also did not impact the efficacy of target-cell depletion in low Ab mice. These results indicate that the complement pathway, ADCC by NK cells, and ADCP by neutrophils are not involved in Ab-mediated cell depletion.

High Amounts of ICs Impede Macrophage-Mediated Phagocytosis

We next investigated the contribution of ADCP by other phagocytic cell populations to Ab effector activity by treating persistently infected low Ab mice with clodronate-filled liposomes to deplete macrophages and DCs. The removal of these phagocytic cells from depletion-permissive low Ab mice (Figure 2A) resulted in a suppression of Ab-mediated target cell depletion similar to that observed in persistently infected high Ab mice (Figure 2B). Thus, high concentrations of ICs generated during
persistent viral infection suppress the Ab-mediated cell clearance activity by phagocytic cells.

Both macrophages and DC can be phagocytic (231) and were depleted by clodronate treatment (Figure 2A). 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 2C) or DC-depleted splenocytes (Figure 2D and E), but was lost when macrophages were depleted (Figure 2C). Similarly, no effect on Ab-mediated depletion was observed when DC were selectively depleted in vivo (Figure 2F and G). Taken together, these data demonstrate that macrophage-mediated phagocytosis is suppressed by the high amounts of ICs generated during persistent viral infection.

**FcγR 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 FcγR on macrophages (FcγRI, FcγRII, FcγRIII, FcγRIIV in mice; FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA in humans), while engagement with the inhibitory receptor FcγRIIB inhibits phagocytic activity (94). Similar numbers of monocytes and macrophages were observed in the blood and spleens of persistently infected high and low Ab mice (Figure 3A). Further, no decrease (and in most cases an increase) in activating FcγR expression was observed in both high and low Ab mice compared to naïve controls (Figure 3B), indicating that the suppression was not due to decreases in FcγR expression. Interestingly, although inhibition mediated by FcγRIIB is an immunosuppressive mechanism in some situations (94), persistent infection of
FcγRIIB-deficient (high Ab) mice did not rescue the suppression of Ab function (Figure 3C), indicating that macrophage suppression during persistent viral infection is not due to a decreased ratio of activating to inhibitory receptors or increased FcγRIIB signaling.

We observed increased amounts of IgG on the surface of macrophages in persistently infected high Ab mice compared to low Ab or naïve mice (Figure 4A) suggesting that surface bound Ab physically impedes effector Ab activity. We also observed higher concentrations of human IgG on the surface of macrophages in IVIG-reconstituted B cell−/− mice (Figure 4A) that show a similar suppression of Ab-mediated effector activity (Chapter 2; Figure 4F). Importantly, the amount of surface IgG during persistent infection compared to naïve mice is not solely due to increased FcγR expression since low Ab mice also have greatly increased FcγR expression compared to naïve mice (and similar/higher than high Ab mice; Figure 3B), but maintain low amounts of circulating and surface-bound IgG similar to naïve mice (Figure 4A). To address whether the suppression of macrophage activity was due to permanent cellular inhibition or a physical blockade of FcγR interactions, splenocytes and peritoneal macrophages were isolated from high Ab mice, washed to remove FcγR-bound Ab-ICs, and allowed time to recover (Figure 4B). 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 (Figure 4B and C). Importantly, the Ab effector function was lost when these phagocytic cells lacked activating FcγRs (Figure 4C). In addition, CD4+ T cell depleting Ab clone YTS191 displays some, but suppressed Ab effector activity during persistent viral infection, and this activity critically depends on FcγRs, but not other IgG effector pathways (Chapter 2; Figure 1A and Chapter 3; Figure 4D). Thus, the suppression of Ab effector function is not a permanent macrophage defect, but instead results from blockade of surface FcγRs by the high amount of ICs
present during persistent infection, which affects some target-depleting Abs more than others.

**Multiple FcγR-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 FcγR-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-Cl13 (232) renders it susceptible to neutralization by the anti-LCMV KL25 antibody, unlike the parental LCMV-Cl13 strain that is not neutralized or bound by KL25 (Figure 5A). 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, viremia was undetectable in both high Ab and low Ab mice (Figure 5B), 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 FcγR 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-Cl13 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-Cl13. 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 hours after transfer, KL25 Ab treatment reduced the number of infected DC by 50% in low Ab mice, but was ineffective in the high Ab mice (Figure 5C). Since the KL25 Ab effectively binds and neutralizes LCMV-M1 in persistently infected high Ab mice (Figure 5A and B), 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 and in IVIG-reconstituted B cell+ mice (Figure 6A), suggesting that their FcγR-mediated functions may also be suppressed. A major FcγR-mediated function of DC is cross-presentation of antigen to prime and sustain T cell activity (190, 237). To address the suppression of FcγR-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-C113 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 6B). In contrast, Ova-anti-Ova ICs effectively primed OT-I T cell expansion in persistently infected low Ab mice, whereas priming was almost entirely suppressed in high Ab mice (Figure 6B). Thus, the high concentrations of ICs generated during persistent infection suppress FcγR-mediated cross-presentation by APCs to limit de novo CD8+ T cell responses to secondary antigens.
DISCUSSION

T cell dysfunctions during persistent viral infection have been well characterized using the LCMV murine model, and many of these observations have later been confirmed in human persistent infections (124, 134, 203, 238). Herein, we demonstrate that high concentrations of ICs generated during the host immune response to persistent LCMV infection suppress FcγR-mediated Ab effector activity. In addition, we show that during persistent infection, Ab-mediated target cell depletion is not affected by the loss of complement activity, NK cells, neutrophils, or dendritic cells, but critically relies on FcγR-expressing macrophage populations. This finding is similar to that observed in naïve mice (239-241) where cell-depletion capacity was primarily dependent on the mononuclear phagocyte system and not other Ab-mediated mechanisms of cell killing. Importantly, macrophage suppression was reversed when cells were removed from their high IC environment and could be again inhibited when ICs were added back (Chapter 2), demonstrating that the macrophages were not permanently dysfunctional, and that high concentrations of ICs were continually blocking surface FcγRs to inhibit their activity in vivo.

Interestingly, we did not observe a notable suppression of other Ab functions such as effective target binding, receptor blockade (109, 110, 139, 140), or in vivo neutralization of high concentrations of infectious virus suggesting that the non-FcγR-mediated functions of Abs may not be affected. On the other hand, functions that rely on FcγRs, including a component of the neutralizing capacity (242) or agonistic anti-CD40 Ab activation of DCs for effective T cell stimulation (243, 244), may be compromised by ICs during an ongoing persistent infection. 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.
These data indicate that the broad spectrum of Ab functions relying on FcγR 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 responses against secondary antigens is also affected. Since dendritic cells and macrophages act as APCs following IC internalization through FcγR (231), the blockade of FcγRs could further limit the development of de novo T cell responses against virus escape mutants or secondary co-infections. Antigen-Ab ICs bound by FcγRs on FDC are critical for B cell selection, hypermutation, and de novo recognition of Ab-escape viruses by naïve B cells (245). 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 (166, 246) and the suppression of FcγR-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. Here we show that the enhanced Ab efficacy was completely abrogated in mice lacking FcγRs, indicating that complement or other non-FcγR effector mechanisms are not responsible for their increased depleting activity. More studies are needed to determine the factor(s) responsible for the improved Ab function including differences in glycosylation, FcγR affinity, target affinity, epitope binding orientation, as well as many others. Further understanding of these Abs that show less effector suppression during persistent viral
infection may improve the efficacy of therapeutic monoclonal Abs used to target and treat cancer and other diseases.

We also show that both murine and non-murine Abs were functionally suppressed during persistent infection despite being highly effective in naïve mice, thus arguing against species-specific differences in FcγR 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.

However, in response to IC-mediated suppression of FcγR function during persistent infection, we did not observe a rescue of antibody activity in persistently infected FcγRIIB-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 (247-249). Although SLE is an autoimmune disorder propagated by pathogenic Abs and IC deposition, the suppression of phagocytic macrophage function may attenuate further disease through other mechanisms (247). Thus, the suppression of FcγR-mediated function may not be entirely detrimental during viral persistence, and the decreased Ab activity may help to avoid excessive immunopathology and autoimmunity from self-reactive Abs generated during the polyclonal B cell activation and hypergammaglobulinemia exhibited in persistent infections (169).
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 may 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.
CHAPTER 3

FIGURES
Figure 1: Complement, NK Cells, and Neutrophils are not Required for Ab-Mediated Cell-Depletion during Persistent Viral Infection

(A) LCMV-CI13 infected low Ab mice (B cell-deficient; day 21 after infection) received cobra venom factor (CVF) daily for 6 days. Graph represents the level of complement activity in the serum of CVF-treated mice as measured by lysis of Ab-opsinized sheep red blood cells.

(B) LCMV-CI13 infected low Ab mice (B cell-deficient; day 21 after infection) received cobra venom factor (CVF) daily for 6 days. One day following the first CVF treatment, mice were given 100µg of isotype or anti-CD4 Ab (clone GK1.5). Flow plots represent the percent and graphs the number +/- SD of CD4⁺ T cells in the blood and spleen 5 days after Ab treatment.

(C) LCMV-CI13 infected low Ab mice (B cell-deficient; day 21 after infection) received 250µg of isotype or anti-NK1.1 Ab to deplete NK cells. Graphs represent the number +/- SD of NK cells present in the blood and spleen following depletion (cell depletion was observed in the blood throughout the experiment; not shown).

(D) LCMV-CI13 infected low Ab mice (B cell-deficient; day 21 after infection) received 250µg of isotype or anti-NK1.1 Ab to deplete NK cells. Isotype and NK cell-depleted low Ab mice subsequently received 100µg of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and the graph represent the number of CD4⁺ T cells in the spleen 5 days after anti-CD4 Ab treatment.
(E) LCMV-CI13 infected low Ab mice (B cell-deficient; day 21 after infection) were treated with 250µg of isotype or anti-Ly6G Ab (clone 1A8) to deplete neutrophils. Graph represents the number +/- SD of neutrophils present in the blood following depletion (cell depletion was observed throughout the experiment; not shown).

(F) LCMV-CI13 infected low Ab mice (B cell-deficient; day 21 after infection) were treated with 250µg of isotype or anti-Ly6G Ab (clone 1A8) to deplete neutrophils. Neutrophil-depleted mice subsequently received 100µg of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and graphs (right) represent the number +/- SD of CD4⁺ T cells in the blood and spleen 5 days after Ab treatment.

*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
Figure 2: Macrophage Suppression Responsible for Impaired Antibody Effector Function during Persistent Viral Infection

(A) LCMV-Cl13 infected low Ab mice (B cell tg; day 21 after infection) were treated with control liposomes or clodronate-filled liposomes. Graphs represent the number +/- SD of macrophages and dendritic cells present in the blood 1 day following liposome treatment.

(B) LCMV-Cl13 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 100μg of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and the graph represents the number +/- SD of CD4+ T cells in the blood 5 days after Ab treatment.

(C) 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.

(D) T cell and B cell-depleted splenocytes from low Ab mice (CD4+ T cell-depleted prior to infection) containing dendritic cells (Undepl) or further depleted of dendritic cells (DC depl). Graphs indicate the number of DC and macrophages in the DC undepleted or DC depleted fractions.

(E) T cell and B cell-depleted splenocytes from low Ab mice (CD4+ T cell-depleted prior
to infection) containing dendritic cells (Undepl) or further depleted of dendritic cells (DC depl) were mixed with CD45.1+ CD4+ T cells for an \textit{ex vivo} phagocytosis assay. Cultures were mixed with isotype or anti-CD4 Ab (clone GK1.5) and the frequency and number of CD45.1+ CD4+ T cells were assessed in the cultures 2 days later.

\textbf{(F)} LCMV-CI13 infected low Ab CD11c-iDTR mice (CD4+ T cell-depleted prior to infection) were treated with PBS (control) or diphtheria toxin (DT; dendritic cell depletion) every three days. DCs were depleted by day 1 following DT treatment (not shown) and throughout the experiment. Graph represents the number +/- SD of DC present in the spleen following PBS or DT treatment at the end of the experiment.

\textbf{(G)} LCMV-CI13 infected low Ab CD11c-iDTR mice (CD4+ T cell-depleted prior to infection) were treated with PBS (control) or diphtheria toxin (DT; dendritic cell depletion) every three days. DCs were depleted by day 1 following DT treatment (not shown) and throughout the experiment. PBS and DT treated mice received 100\(\mu\)g of isotype or anti-CD8 Ab (clone 53.6.72) one day after the first DT treatment. Flow plots represent the percent and the graph (right) represents the number of CD8+ T cells present in the spleens of these mice at the day 5 endpoint.

*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 2

A. 

B. 

C. 

D. 

E. 

F. 

G.
Figure 3: Neither FcγR Expression nor Inhibitory Signaling Responsible for Suppressed Macrophage Function during Persistent Viral Infection

(A) Graphs represent the number +/- SD of monocytes/macrophages [Thy1.2^-, B220^-, Ly6G^-, NK1.1^-, CD11c^{(mid-lo)}, F4/80^+, CD11b^+] in the blood and spleen of LCMV-CI13 infected high Ab mice and low Ab mice (CD4^+ T cell-depleted prior to infection) day 21 after infection.

(B) Graphs represent the gMFI of the indicated FcγR on macrophages [B220^-, Thy1.2^-, Ly6G^-, NK1.1^-, CD11c^{(mid-lo)}, F4/80^+, CD11b^+] and dendritic cells [B220^-, Thy1.2^-, Ly6G^-, CD11c^{(hi)}] from naïve (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).

(C) LCMV-CI13 infected FcγRIIB-deficient mice were treated 21 days after infection with 100μg of isotype or anti-CD4 Ab (clone GK1.5). Flow plots indicate the percent and graphs represent the number +/- 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.
FIGURE 3

A.

B.

C.

FcγRIIIB-deficient mice
Figure 4: High amounts of ICs impede FcγR-mediated phagocytosis

(A) (Top) Surface bound mouse IgG on macrophages of naïve mice (black line) and day 21 after LCMV-C13 infection of mice that were CD4⁺ T cell-depleted prior to infection (low Ab; grey) or left undepleted (high Ab; black). (Bottom) Surface bound human IgG on macrophages of B cell⁻ mice treated with PBS (grey) or reconstituted with IVIG (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 FcγR–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.

(D) FcγR–deficient and WT mice at day 21 of persistent infection were treated with 250ug of isotype or anti-CD4 T cell depleting antibodies. Flow plots show the percent and graphs represent the number of CD4⁺ T cells remaining in the spleens of the mice 5 days following antibody treatment.
*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
Figure 5: Ab-Mediated Depletion of Virus-Infected Cells, but Not Virus Neutralization is Suppressed during Persistent Viral Infection

(A) (Left) KL25 antibody was incubated on ELISA plates previously coated with medium alone (B) or 5x10^6 plaque forming units (PFU) of LCMV-CI13 or LCMV-M1. Antibody binding was determined by ELISA and is displayed as optical density units (ODU). (Right) Neutralization of LCMV-CI13 (grey) and LCMV-M1 (black) by the KL25 antibody. Graphs demonstrate the number +/- SD of viral plaques as a percentage of isotype antibody control.

(B) 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. On day 21 after infection, mice received 250 µg 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.

(C) LCMV-M1-infected, GFP^+ DC (KL25 targets) were transferred into LCMV-CI13 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 (not shown). Mice received 250 µg of isotype Ab or anti-LCMV-M1 KL25 Ab and the graph represents the number +/- SD of targets in the spleen 6 hours following Ab treatment.

*p<0.05. Data are representative of two or more independent experiments using three to five mice per group.
FIGURE 5

A. 

B. 

C. 

93
Figure 6: IC-Mediated Antigen Cross-Presentation is Suppressed during Persistent Viral Infection

(A) (Top) Surface bound mouse IgG and on dendritic cells in naive mice (black line) and LCMV-CI13 persistently infected low Ab mice (B cell-deficient; grey) and high Ab mice (black). (Bottom) Surface bound human IgG on dendritic cells of B cell−/− mice treated with PBS (grey) or reconstituted with IVIG (black).

(B) CFSE-labeled Ova-specific OT-I CD8+ T cells (CD45.2+) were transferred into CD45.1+ LCMV-CI13 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 +/- 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.
FIGURE 6

A.

Ms IgG

% of Max

---

Naive
Low Ab
High Ab

Hu IgG

% of Max

---

No IVIG
IVIG

B.

Low Ab

Ova alone
Ova/Ab

High Ab

Ova alone
Ova/Ab

OT1

CD8

Cell No.

CFSE

Blood

No. OT1 T cells (x10^3)

per million PBMC

---

Low Ab
High Ab

Spleen

No. OT1 T cells (x10^3)

---

Low Ab
High Ab
CHAPTER 4

Conclusions
CONCLUSIONS

Many persistent viral infections are associated with widespread immunosuppression and cellular dysfunctions that prevent virus clearance. Despite decades of research on impaired immune responses during persistent infections, a specific impact on inherent Ab effector activity has not been identified. In this dissertation, we describe a previously unrecognized mechanism of immunosuppression whereby FcγR-mediated Ab effector functions are highly suppressed resulting from increased concentrations of immune complexes commonly generated during persistent viral infections including SIV, HIV, HCV, and LCMV (61, 225, 226, 250). The efficacy of cell-depleting Abs during persistent infection does not rely on complement-mediated lysis, NK cells, neutrophils, or dendritic cells, but critically requires macrophages. Our data indicate that Ab/immune complexes directly suppress macrophages and that interestingly, cell-depletion capacity during persistent infection is completely dependent on phagocytosis and not other Ab-mediated mechanisms of cell killing. Suppression of macrophage effector function can be restored when Ab/immune complexes are washed from the cell surface, demonstrating that macrophages are not permanently dysfunctional, but instead that the high levels of Ab/immune complex physically block surface FcγRs and inhibit their activity (Chapter 4; Figure 1).

The importance of FcγRs in the regulation of innate and adaptive immune responses is a highly researched field of investigation. Although the Fc domain of IgG Abs is widely considered to display less diversity than the highly variable Fab portion, differences in Ab subclass (IgG1-4 in humans and IgG1, IgG2a, IgG2b, and IgG3 in mice) as well as N-linked glycans attached at Asn297 result in considerable heterogeneity (189, 251). These differences can both increase and decrease the binding affinity of IgG Abs to various FcγRs, thus dramatically influencing immune
responses (252, 253). Although we identify highly increased concentrations of IgG Ab immune complexes during persistent viral infection, variations in Ab subclass and glycosylation patterns were not investigated and these may offer further insights into the mechanism behind IC blockade of FcγRs. In addition, it will be interesting to determine the antigen specificity of the ICs generated during persistent viral infections since the virus-specific Ab titers are a relatively low percentage of the total IgG detected (Chapter 2; Figure 3B).

We show that FcγRs expressed by dendritic cells can help internalize and cross-present ICs composed of ovalbumin protein and anti-ovalbumin IgG to activate CD8\(^+\) T cell responses to secondary antigens during persistent infection of low Ab mice (Chapter 3; Figure 6B). In contrast, cross-presentation is highly suppressed during persistent viral infection when high concentrations of ICs are present, but it is unknown whether this is due to a lack of antigen processing or other suppressive effects by DCs inhibiting T cell activation. Studies have shown that signaling through the inhibitory FcγRIIB on DCs negatively regulates T cell priming and FcγRIIB-deficient mice are more effective at T cell activation \textit{in vitro} and \textit{in vivo} (254, 255), so ICs generated during persistent viral infection may engage FcγRIIB, thus eliciting poorer T cell activation. Indeed, T cell dysfunctions during viral persistence are thought to result from interactions with immunoregulatory antigen presenting cells (i-reg APCs) (107) and the role of ICs as well as inhibitory FcγRIIB signaling on the maintenance of i-reg APCs may further our understanding of T cell suppression during persistent infection.

Intravenous immunoglobulin (IVIG) is composed of polyclonal IgG that is pooled and purified from thousands of blood donors. One of the early uses of IVIG therapy was to prevent platelet depletion in patients suffering from autoimmune idiopathic thrombocytopenic purpura (ITP) (256). Interestingly, using the mouse model of ITP, we
observe that persistently infected mice with high concentrations of ICs are protected from Ab-mediated platelet depletion, suggesting that ICs may serve a protective function against auto-Ab induced pathology (Chapter 2; Figure 4F). However, researchers have elucidated the mechanism of IVIG-mediated immunosuppression showing that a small fraction of IVIG Abs are enriched for sialylation, resulting in decreased affinity for FcγRs and increased binding to surface DC-SIGN (human) / SIGN-R1 (mouse) on regulatory DC and macrophages. Upon engagement of DC-SIGN/SIGN-R1 regulatory DCs/macrophages secrete IL-33 causing basophils to secrete IL-4. When monocytes/macrophages receive IL-4 signals they upregulate the expression of inhibitory FcγRIIB therefore raising the threshold for monocyte/macroage activation and attenuating autoimmune responses (257-259). Although we observe a similar increase in FcγRIIB expression during persistent viral infection (Chapter 3; Figure 3B), the suppression of Ab effector activity is not rescued in FcγRIIB-deficient mice, suggesting that multiple protective mechanisms induced by high Ab concentrations may exist, further highlighting their importance in preventing immunopathology.

Another protective effect of the increased concentrations of ICs during persistent viral infection may be to limit antibody-dependent enhancement of infection. Enhanced disease is thought to occur when low avidity antibodies mediate the increased infection of monocytes and macrophages through internalization by FcγRs, resulting in rapid cytokine release and potential shock syndrome (260). An FcγR blockade by ICs could limit the spread of infection and further immunopathology caused by excessive inflammation and cytokine release. Thus, additional research into the protective mechanisms of immunosuppression by ICs during persistent viral infection may reveal an important role in antiviral immune control.
Non-FcγR-mediated Ab functions such as virus neutralization and receptor blockade are not affected by increased concentrations of ICs present during persistent viral infection (109, 110, 139, 140). Further, higher doses of Ab can eventually achieve cell-depletion during persistent infection similar to the effective administration of therapeutic Abs (particularly nAbs) to control persistent virus infections in mice, monkeys, and humans (196, 199-202, 206). For these approaches, high doses of potent nAb are necessary to control infection since initial experiments using less effective nAbs indicated a discrepancy between HIV control in humans and the ability to neutralize virus in the laboratory or in humanized mice (198, 200, 204, 205, 261). Interestingly, diminished Ab effector activity during human persistent infection was suggested after subsequent analysis estimated that at least 10x more Ab would be required to achieve efficacy in patients with chronic HIV infection (206, 207). Therefore, similar to the exhaustion of T cell responses during persistent infection wherein T cells retain some antiviral function, Ab functions are not entirely absent, and future studies focusing on improving Ab effector activity may expedite antiviral immune control.

In our studies we came across one monoclonal Ab (anti-CD4; clone YTS191) that was less suppressed during persistent viral infection than most other Abs including the anti-CD4 clone GK1.5. YTS191 and GK1.5 share the same cellular target (CD4) and are the same isotype subclass (rat IgG2b) therefore excluding major differences in Ab effector capacity. We also demonstrate that the efficacy of YTS191 during persistent viral infection still relies solely on FcγRs (Chapter 3; Figure 4D), suggesting that differences in glycosylation, binding affinity, or binding orientation may improve the interaction of the Ab Fc domain with macrophage FcγRs. Similarly, the antitumor antibody obinutuzumab targeting human CD20 has been engineered to increase binding affinity for FcγRIIIA and has been shown to enhance survival of chronic lymphocytic
leukemia patients relative to unmodified rituximab controls (262). In addition, the
efficacy of therapeutic anti-CTLA-4 Abs targeting the immunological checkpoint inhibitor
has recently been suggested to also act through FcγR-mediated depletion of intratumoral
suppressive T_{reg} cells. These data suggest that studies into the differences between
YTS191 and GK1.5 may provide clues for future Ab engineering to improve FcγR
interactions and enhance the efficacy of therapeutic Abs targeting cancer and virus
resolution.

Another method for improving Ab efficacy could be to enhance the activation and
phagocytic function of macrophage effector cells. Researchers have shown that the
interaction between macrophage signal regulatory protein α (SIRPα) and its ligand
(CD47) generates an inhibitory signal that prevents FcγR-mediated phagocytosis (263).
Blocking SIRPα increased the specific phagocytosis of Ab-opsonized target cells without
affecting non-targeted populations, thus improving macrophage-mediated target cell
depletion efficacy. Additionally, CD40 agonists can also activate macrophage function
and are being used in studies to improve cancer therapies (264, 265), but a direct
enhancement of FcγR-mediated phagocytosis has yet to be shown. Further studies into
macrophage activation during persistent viral infection may reveal methods to improve
Ab effector activity and enhance viral clearance.

In this dissertation we describe a novel mechanism of immunosuppression
during persistent viral infection affecting inherent Ab effector activity and humoral
immunity. Many persistent viral infections exhibit high concentrations of ICs generated
during aberrant immune activation leading to IC-deposition and immunopathology. Our
studies also demonstrate that the increased IC levels suppress FcγR effector functions
including macrophage-mediated ADCP and DC-mediated cross-presentation for
effective T cell activation. These observations are clinically relevant and suggest similar
defects in Ab activity may be found in other persistent IC diseases. Importantly, FcγR-mediated Ab effector suppression was not permanent, and future studies to enhance FcγR interactions may lead to improved therapeutic Ab efficacy and the resolution of persistent viral infections.
CHAPTER 4

FIGURES
FIGURE 1: Graphical Summary of Immune Complex-Mediated FcγR Blockade

The figure depicts viral immune complexes binding to FcγRs on the surface of macrophage effector cells and preventing the recognition of Ab-coated virus-infected cells. Thus, IC blockade suppresses FcγR-mediated ADCP of infected target cells.
REFERENCES


