Universidade do Minho
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Immune Suppression During Chronic Lymphocytic Choriomeningitis Virus Infection and Response to Unrelated Ectromelia Virus Infection

Julho de 2020

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Tese de Doutoramento em Medicina

Trabalho efetuado sob a orientação de
Professor Doutor Luis J. Sigal
Professora Doutora Margarida Correia-Neves

Julho de 2020

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Supressão imunológica durante a infeção crónica com o vírus da coriomeningite linfocítica e resposta à infeção não relacionada pelo vírus ectromelia

Infeções virais persistentes, incluindo as causadas por vírus pouco patogénicos como o citomegalovírus, mas também outros mais virulentos para o hospedeiro, como o vírus da hepatite B (VHB) ou da hepatite C (VHC) podem resultar em disfunção imunitária e aumentar a suscetibilidade a infeções não relacionadas ou a cancro. No trabalho aqui apresentado utilizámos o modelo de infeção crónica em murganhos pelo vírus da coriomeningite linfocítica (LCMV) clone 13 (CL13) para estudar a resposta à co-infeção pelo vírus ectromelia (ECTV), o agente da *mousepox*.

Os nossos resultados mostram que murganhos cronicamente infetados com CL13 são mais suscetíveis à infeção por ECTV, do que animais previamente saudáveis ou recuperados de infeção pela estirpe Armstrong (Arm) do LCMV. A infeção crónica por CL13 traduz-se numa redução acentuada dos números e frequências de células *Natural Killer* (NK), que apresentam um fenótipo marcadamente imaturo e que são incapazes de responder à infeção subsequente por ECTV, com níveis reduzidos de maturação, ativação e citotoxicidade, com produção reduzida de granzima (Gzm) B e interferão (IFN) γ, em contraste com as células NK de murganhos saudáveis ou recuperados após infeção por Arm.

Murganhos infetados cronicamente com CL13 apresentam células T $CD8^+$ que respondem insuficientemente a uma infeção secundária por ECTV, com níveis de ativação fracos, frequências reduzidas e alterações fenotípicas do compartimento de células $CD44^+GzmB^+$, bem como com reduzida expansão de células TCR-epítopo especificas. No entanto, é possível induzir uma resposta T $CD8^+$ TCR-epítopo especifica forte aquando da infeção secundária de murganhos infetados cronicamente com CL13 com um vírus atenuado ECTVΔ36, conferindo resistência à infeção posterior com ECTV.

O nosso trabalho sugere que a suscetibilidade a infeções oportunistas em indivíduos previamente persistentemente infetados pode estar associada a defeitos das células NK e T $CD8^+$. Portanto, será possível que doentes imunodeprimidos por estas razões possam ser protegidos de infeções oportunistas desde que vacinas efetivas sejam utilizadas.

Palavras chave: Infeção viral crónica; Infeção viral aguda; LCMV; ECTV; células NK, células T

Immune suppression during chronic lymphocytic choriomeningitis virus infection and response to unrelated ectromelia virus infection

Persistent viral infections, from the relatively nonpathogenic cytomegalovirus, to others that severely impact the host, including the human immunodeficiency virus (HIV), even under antiretroviral therapy (ART), hepatitis B virus (HBV) or hepatitis C virus (HCV), can result in immune dysfunction that predisposes to severe infections with unrelated pathogens and to cancer. Here we use the mouse model of infection with the Clone 13 (CL13) strain of lymphocytic choriomeningitis virus (LCMV) to study how the immune system of the persistently infected wild-type (WT) mouse host responds to unrelated ectromelia virus (ECTV) infection, the agent of mousepox in the mouse.

We show that WT mice chronically infected with CL13 are fully susceptible to ECTV infection and succumb to mousepox, contrasting with previously naïve mice or convalescent from acute LCMV infection with the Armstrong strain (Arm), due to a multitude of defects in their immune. We show that CL13 infection leads to a severe reduction of the numbers and frequencies of Natural Killer (NK) cells, that bear a highly immature phenotype and fail to respond to secondary ECTV infection, with impaired maturation and activation, and decreased cytotoxic function, due to reduced production of granzyme B and interferon (IFN) γ, in contrast with NK cells in Arm-convalescent or previously naïve mice.

Moreover, in CL13 chronically infected mice, $CD8^+$ T cells respond poorly to secondary infection with the ECTV, with impaired activation, reduced frequencies and phenotypic changes of highly functional $CD44^+$ $GzmB^+$ cells and reduced clonal expansion of TCR epitope specific $CD8^+$ T cells. We show that it is possible to induce a potent ECTV-specific $CD8^+$ T cell response if CL13 infected mice are challenged with a highly attenuated ECTVΔ36 virus strain, resulting in full resistance to subsequent ECTV infection.

Our work suggests that susceptibility to opportunistic infections in the persistently infected individuals may be linked to NK cell and $CD8^+$ T cell dysfunction. Moreover, it is possible that immunocompromised persistently infected individuals may be protected from opportunistic infections provided effective vaccines are used.

Keywords: Chronic Viral Infection; Acute Viral Infection; LCMV; ECTV; NK cells; T cells.

Abbreviations

AIDS – Acquired Immune Deficiency Syndrome

APC – Antigen Presenting Cell

Arm – Armstrong strain

BrdU – Bromodeoxyuridine

BTLA – B and T Lymphocyte Attenuator

CCL – Chemokine (C-C motif) Ligand

CCL – Chemokine Ligand

CCR – C-C Chemokine Receptor

CL13 – clone 13

CMV – Cytomegalovirus

CTL – Cytotoxic T Lymphocytes

CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4

CXCL – Chemokine (C-X-C motif) Ligand

CXCR – C-X-C Chemokine Receptor

DCs – Dendritic Cells

DG – dystroglycan

dLN – draining Lymph Node

dpi – days post infection

E – Glutamate

EBV – Epstein-Barr Virus

ECTV – Ectromelia Virus

EOMES – Eomesodermin

F – Phenylalanine

GP – Glycoprotein

Gzm – Granzyme

HAART – Highly Active Antiretroviral Therapies

HBV – Hepatitis B Virus

HCMV – Human Cytomegalovirus

HCV – Hepatitis C Virus

HHV – Human Herpesvirus

HIF – Hypoxia-Inducible Factors

HIV – Human Immunodeficiency Virus

HSV – Herpes Simplex Virus

IAV – Influenza A virus

IFN – Interferon

IFNAR – Interferon-α/β Receptor

Ig – Immunoglobulin

IL – interleukin

IL-10R – IL-10 Receptor

iMOs – inflammatory Monocytes

ISG – interferon Stimulated Gene

K – Lysine

KLRG1 – Killer cell Lectin-like Receptor subfamily G member 1

L – Leucine

LAG – Lymphocyte Activation Gene protein

LCMV - Lymphocytic Choriomeningitis Virus

MCMV – Mouse Cytomegalovirus

MHC – Major Histocompatibility Complex

NK cells – Natural Killer cells

NP – Nucleoprotein

OPV – orthopoxvirus

PD1 – Programmed cell Death protein 1

pDCs – plasmocytoid DCs

PD-L1 – Programmed Death-Ligand 1

PFU – plaque forming units

Prf - Perforin

PV- Pichinde virus

SIRPα – Signal Regulatory Protein α

TCF – Transcription factor T cell factor

TCR – T cell receptor

TF- Transcription factor

TGF-β – Tumour Growth Factor β

TIGIT - T cell immunoreceptor with Ig and ITIM domains

TIM-3 – T-cell limmunoglobulin and mucin-domain containing protein 3

TLR – Toll-Like Receptor

TNF – Tumour Necrosis Factor

VHL – Von Hippel–Lindau Tumor Supressor

VSV– Vesicular Stomatitis Virus

VZV – Varicella Zoster Virus

WHO – World Health Organization

WT – Wild Type

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General thesis outline

The current dissertation has seven chapters. The introduction consists of the first 3 chapters. The results are presented in chapters IV, V and VI. The last chapter integrates the previous literature with the results generated during this thesis.

In the first chapter, we demonstrate the relevance of the study-subject of this thesis. To this, we overview many of the chronic infections currently affecting humans. Many features of those are detailed, including epidemiology and general mechanisms of chronicity. We also discuss the occurrence of viral co-infection in humans, notably the Human Immunodeficiency Virus (HIV) concurrent with Human Hepatitis C Virus (HCV). Finally, we present chronic infection models in the mouse that we can use to model those occurring in humans, as well as the mouse models for viral co-infections.

In chapter II, we explain in detail the most relevant features of the lymphocytic choriomeningitis virus – clone 13 virus (CL13) chronic infection model. We start by a brief historical presentation of the emergence of this model. Then, we show how CL13 infection becomes chronic. Therefore, we elaborate on the changes observed in the innate and adaptive immune response to CL13, as compared to lymphocytic choriomeningitis virus – clone Armstrong, when scientific data is available.

Chapter III presents the acute infection model used in this dissertation. First, we present an historical overview of the emergence of smallpox in humans. Then, we present the analogous model in the mouse, with ectromelia virus infection and mousepox. The remaining of the chapter covers briefly the innate and adaptive immune response to ectromelia virus infection. There, we explain in greater detail the main immune players that I will further discuss in the results section, namely IFN-I and NK cells as well as T cells, respectively.

Chapter IV is the first chapter including experimental work performed in the context of this dissertation. It consists of the report “Chronic Lymphocytic Choriomeningitis infection causes susceptibility to mousepox and impairs Natural Killer cell maturation and function” as it was published in the Journal of Virology. Briefly, we show how CL13 or Arm infection in the mouse differently impact the NK cell immune response to unrelated ectromelia virus infection.

Chapter V is a manuscript currently being prepared for submission into publication. There, we go into greater detail on how different IFN-I molecules modulate NK cells maturation, activation, and effector function during early of late phases of CL13 infection.

In chapter VI, we include the full report "Loss of resistance to mousepox during chronic lymphocytic choriomeningitis virus infection is associated with impaired T-cell responses and can be rescued by immunization" as it was published in the Journal of Virology. Here, we show that CL13 or Arm infection modulate T-cells response to unrelated mousepox. We also show how boosting T-cells response using an attenuated ectromelia virus vaccine provides full protection from mousepox in the chronically infected mouse.

The general discussion of the introductory section and subsequent results is shown in in the III part of the thesis.

Part I – Introduction

CHAPTER I. Chronic viral infections: epidemiology and overview

It is estimated that every person in the world carries 8-12 long-lasting infections, at any given time. Currently thousands of millions of people are infected with a chronic virus, regardless of their pathogenicity to the host (**Figure 1**). Some of these chronic viral infections are associated with obvious increased morbidity and mortality and received strong attention like the human immunodeficiency virus (HIV) among a few others. Yet, most of the chronic viral infections cause a less severe and obvious effect and consequently receive relatively low attention. However, all these chronic infections impact the host immune system and need to be carefully addressed.

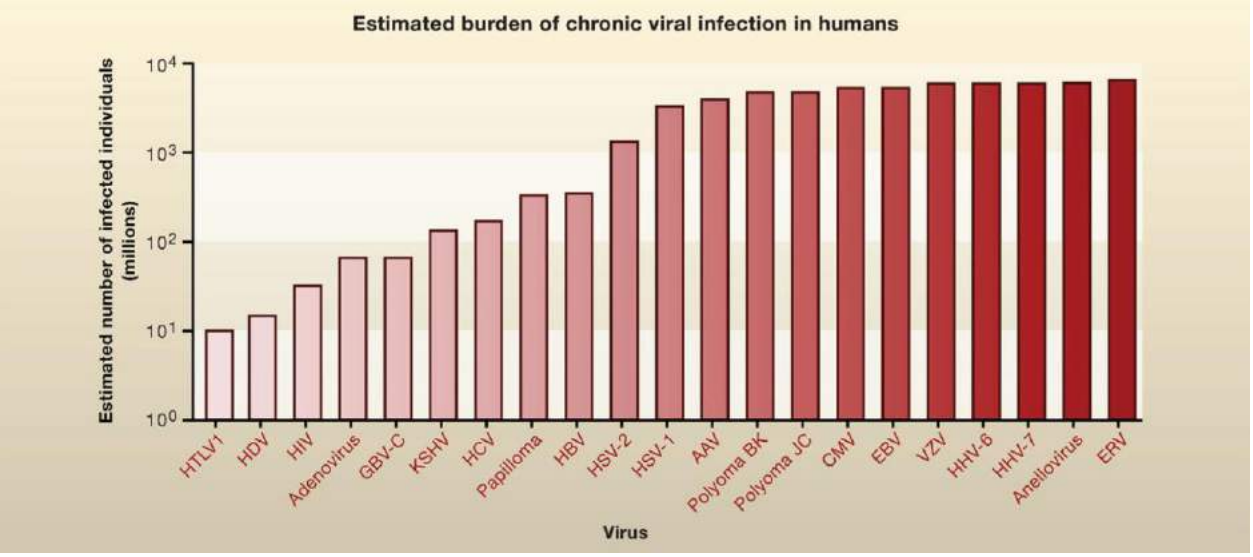

Figure 1. The estimated global prevalence of chronic infections.

Estimates consider a world population of 6.75 billion people. Extracted from (1).

Human immunodeficiency virus (HIV)

Since the 1980s, when HIV was described and the first acute immunodeficiency syndrome (AIDS) victims perished to the destructive effects of HIV, much has changed in the treatments used. The emergence of highly active antiretroviral therapies (HAART) in the 1990s transformed HIV infection from an eminently lethal disease against which little could be done, to a chronic infection that can be controlled, with patients having a lifespan very close to the population average, despite premature aging, drug treatment toxicity, decreased quality of life and increased frequency of comorbidities, including increased susceptibility to opportunistic infections and cancer (**Table 1** and (2)).

In 2018, there were 37.9 million people living with HIV, and of those, 1.7 million were newly infected cases. Therefore, the number of people infected with HIV is still increasing, with a 20% increase in the period between 2010 and 2018. Despite current treatment therapies, 0.8 million people perished due to HIV-related diseases, a problem that is aggravated by the fact many people have limited access to treatment or have it in an intermittent manner. However, this still represents a 33% decrease in mortality from 2010 (2).

Infection with HIV leads to severe immunosuppression and ultimately death of the host, if left without treatment. HIV infects cells by binding to the CD4 receptor in $CD4^+$ T cells, with the help of the CCR5 and CXCR4 co-receptors. After an initial period of relative viral control, the numbers of $CD4^+$ T cells progressively plummet (3). Moreover, the remaining $CD4^+$ T cells have lower potential to respond to new *stimuli* and produce a polyfunctional response (-4). HIV also induces severe dysfunction of $CD8^+$ T cells, B cells and natural killer cells (NK cells) (3). All of this results in severe immunosuppression (3) and contributes to the increased susceptibility to opportunistic infections, cancer and comorbidities, including metabolic diseases, higher levels of inflammation and hyperstimulation of the immune system, together with higher frailty levels at later timepoints in life (5–7). Unfortunately, despite the effectiveness of current HAART in recovering $CD4^+$ T cell numbers in most patients, the higher risks of development of disease in HIV infected individuals is not fully eliminated (5).

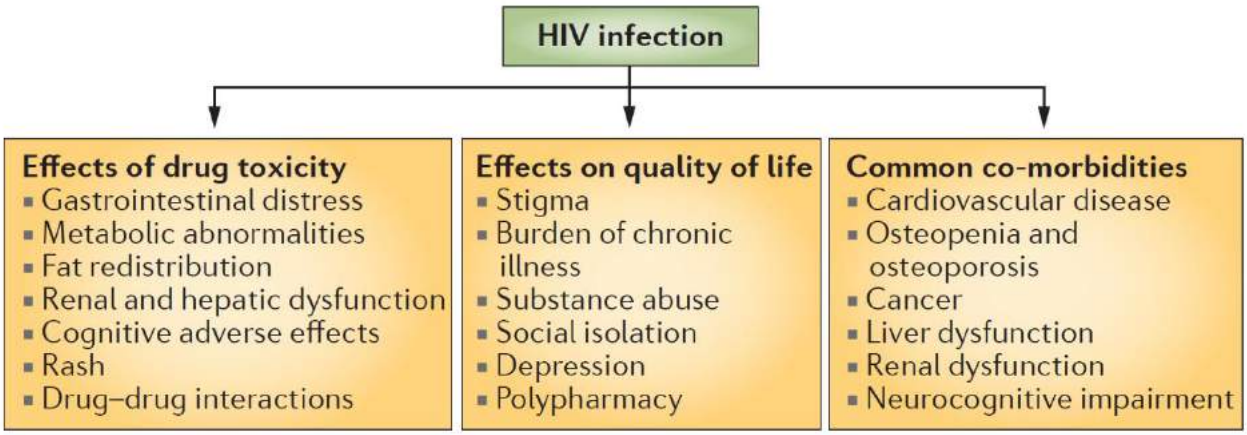

Table 1. Quality of life in HIV infected individuals.

Extracted from (5).

Viral hepatitis

Chronic viral hepatitis virus infections are a major burden worldwide, despite an effective vaccine for Hepatitis B (HBV), and curative treatment for Hepatitis C (HCV). In 2015, the World Health Organization (WHO) estimated that 257 million people were living with HBV and 71 million with HCV infection. Together the two infections were responsible for 1.34 million fatalities. To this scenario, new cases are added every year. In 2015, the WHO estimated 1.75 million people were newly diagnosed with HCV infection (8).

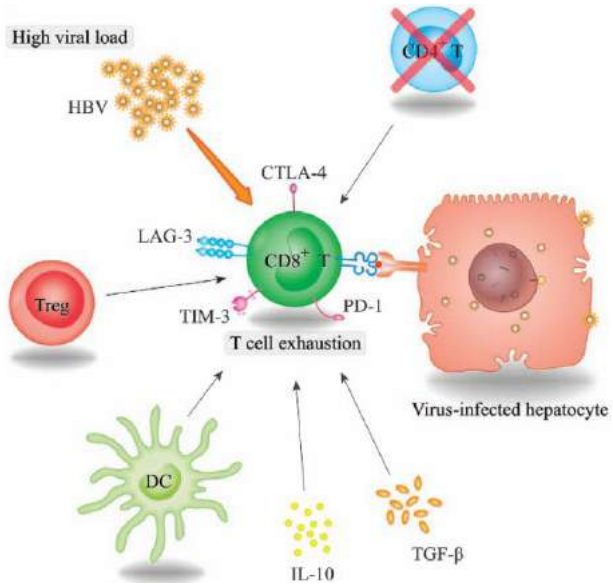

Figure 2. Mechanisms of suppression of T cells in chronic HBV infection.

Extracted from (9).

Hepatitis viruses can induce severe immune suppression (**Figure 2** and (10)). For instance HBV chronic infection leads to impairment of several immune cells, including monocytes, macrophages, dendritic cells (DCs), NK cells and T cells (10). HBV drives DCs to produce tumor growth factor β (TGF-β) and interleukin (IL) 10 (11,12), potent immune suppressors, which contributes to exhaustion of $CD4^+$ T cells and $CD8^+$ T cells (9,11) and weakened function of NK cells (13). NK cells show reduced production of Interferon (IFN) γ and increased IL10 production (10). In infection with either HBV or HCV, $CD8^+$ T cells increased expression of several suppressing receptors, including programmed cell death protein 1 (PD1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), 2B4 and

T-cell immunoglobulin and mucin-domain containing protein 3 (TIM-3) (14,15). In hepatitis, $CD8^+$ T cells have reduced production of IFN-γ, tumour necrosis factor (TNF) α and IL21, and reduced proliferative capability (9).

There are 2.7 million people co-infected with HIV and HBV and 2.3 million with HIV and HCV (8). These co-infections are associated with worse prognosis of HIV infection progression (16). Among those coinfected with both, HIV and either HBV or HCV, the prevalence of AIDS complications, including several bacterial infections, mycosis and toxoplasmosis, is higher than those only infected with HIV (16).

Chronic HCV infection is reported to impact the immune response to vaccination against unrelated pathogens. For example, it severely decreases immunoglobulin (Ig) G seroconversion following anti-Hepatitis A and anti-Influenza vaccination and also reduces the duration of protection (17–19).

Herpesviridae

Herpesviridae is a family of DNA viruses known to become latently present in different subsets of differentiated cells. There are three different subfamilies of Herpesviridae capable of infecting humans: alpha Herpesviridae, including herpes simplex virus (HSV) -1, HSV-2 and varicella zoster virus (VZV); beta Herpesviridae, including human cytomegalovirus (HCMV), human herpesvirus (HHV) -6A and HHV-6B, HHV-7; gamma Herpesviridae, that include Epstein-Barr virus (EBV) and Kaposi sarcoma associated herpesvirus (20). The most relevant virus of the Herpesviridae family will be next briefly described.

Cytomegalovirus

Human infection with cytomegalovirus (HCMV) is complex since it is difficult to have a clear epidemiologic profile of this infection. In most instances, primary HCMV infection is asymptomatic. HCMV infection invariably becomes chronic in the infected host, with viral latency and gene expression occurring persistently at low levels. Moreover, there is no vaccination implemented against this viral infection. Together, this helps to explain why the seropositivity is so high, ranging from 45% to 100% IgG seropositivity for the virus in different regions of the world (21,22). In Portugal, the 2001-2002 second National Serological Survey showed a seropositivity of 72.3% in men and 80.2% in women (23).

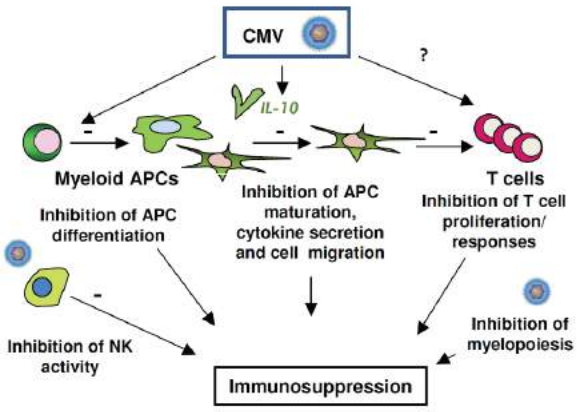

Figure 3. Mechanisms of immunosuppression during CMV infection.

Extracted from (24).

HCMV evolved together with the human being for millions of years. It shows specificity to the species and adapted itself to override the host immune response. It encodes several proteins in its

genome that can evade the host defense. Metanalysis show that anti-CMV prophylaxis in transplanted-organ recipients is linked to decreased incidence of opportunistic infections. CMV is thought to induce immunosuppression of the host through a variety of mechanisms, including the induction of IL10 production and the suppression of antigen presenting cells (APCs), T cells and NK cells responses (**Figure 3** and (24)). In young human adults, CMV-seropositivity is associated with improved antibody production, $CD8^+$ T cell response and circulating levels of IFN-γ following anti-flu vaccination (25).

Varicella Zoster virus

VZV primary infection (chickenpox) occurs most often during infanthood, with up to 95% of adults over the age of 50 being IgG seropositive. In most cases, it resolves without sequelae. However, the virus can stay in the host in a latent state in the spinal and cranial sensorial ganglia. Later in life, and particularly in immunocompromised patients, Varicella Zoster reactivation can occur (shingles). Between 25-30% of all adults are at risk of developing secondary Herpes Zoster, with people over the age of 80 having a 50% risk of developing viral reactivation (26).

Epstein-Barr virus

EBV is the etiological agent of infectious mononucleosis. This is a relatively benign and self-limiting disease. However, the virus is not fully cleared in most individuals, persisting in in the cell nuclei of oropharyngeal epithelial cells and in memory B cells (27). Moreover, it is estimated that 20-30% of adults are shedding live viral particles at any given time (27). In addition, the virus can live outside the human host for a considerable time. Hence, it is very contagious, with some estimates considering that up to 95% of the human population is seropositive (27). In most cases, this represents no threat to the host. Yet, in some individuals it can reactivate in later periods of life, especially those immunocompromised. Of note, this was also the first virus associated with malignant tumors in humans, when virus particles were first collected from jaw sarcomas tissues (27).

Herpes simplex virus I and II

In the human species, there are two strains of HSV, HSV-1 and HSV-2. It is estimated that 67% to 90% of the world population is infected with either of the two viruses. HSV-1 is the most frequent, with 3.7 billion people below the age of 50 living with the virus, either in its active or latent stage. For

HSV-2, it is estimated that 417 million people are living with the infection. In most cases, symptoms are minor and self-limiting. Yet, particularly HSV-2 is linked to malignant tumors (28).

HSV downregulation of mucosal immunity of the vagina and infection reactivation are known to be associated with increased rates of HIV infection (29,30). Also, viral reactivation in mucosal tissues results in local recruitment of activated $CD4^+$ T cells, possibility facilitating HIV transmission (31).

Viral co-infections

During our life, we can be infected with multiple different viruses. In fact, co-infection may be the real-world scenario in which most infections develop. Yet, we have limited knowledge on how our immune system handles multiple simultaneous infections. There are many confounding factors that limit scientific research in this area. For instance, the study subjects may be infected with pathogens other than those we are evaluating. It can even occur the pathogens with which they are infected cannot be readily recognized by the diagnostic tools available at that time. It is also difficult to pinpoint precisely at what time one was infected with each pathogen; each infection may modulate, positively or negatively the clinical course of the concurrent infections, which does not facilitate epidemiologic investigation (32).

HIV infections concurrent with hepatitis virus infections, either HBV or HCV, are the most studied viral co-infections occurring in humans. It is currently known HIV infection accelerates the development of clinical complications of hepatitis infections, including liver failure (33). On the other hand, hepatitis virus infections contribute to slower immunological recovery, including $CD4^+$ T cell counts, in HIV infected individuals (34).

Mouse models of single chronic viral infection

There are several viruses that can naturally infect mice and persist for long periods of time, including mouse Cytomegalovirus (MCMV) and lymphocytic choriomeningitis virus (LCMV). There are also other viral pathogens that can persist for longer periods of time in the mouse, but of which our knowledge is more limited, including parvovirus type 1, mouse thymic virus, lactate dehydrogenase-elevating virus, and the FL1 adenovirus strain (35).

MCMV infection is the archetype model of latent infection (36). Following a period of rapid proliferation, MCVM evolves to latency, with virions detectable only in the salivary glands and nasal mucosa (37). This occurs because the virus avoids recognition by NK cells and T cells, using several different mechanisms that impair cell proliferation or suppress their natural responses (36).

LCMV can cause acute as well as persistent infections in the mouse, but in opposition to MCMV infection, viable virions are detectable in several organs, including the brain, spleen and kidneys, as well as blood (38). There are several different strains of LCMV, that can be used as a models of acutely resolved or chronically persistent infections in the mouse (38). I will talk in detail about the LCMV infection models in Chapter II.

Mouse models of viral co-infections

In the laboratory, typically one studies the immune response to viral infection by a single agent. Yet, in nature, co-infections are frequent. Depending on the viral infections occurring simultaneously, one can have increased or decreased pathology and clinical symptomatology. Moreover, the immune response established targeting a primary viral infection may alter, positively or negatively the immune response to a secondary viral infection (**Figure 4** and **5** and (39)). Current evidence is conflicting, yet most studies report overall increased pathology when the host is infected with two or more pathogens simultaneously (**Table 1** and **2** and (40).

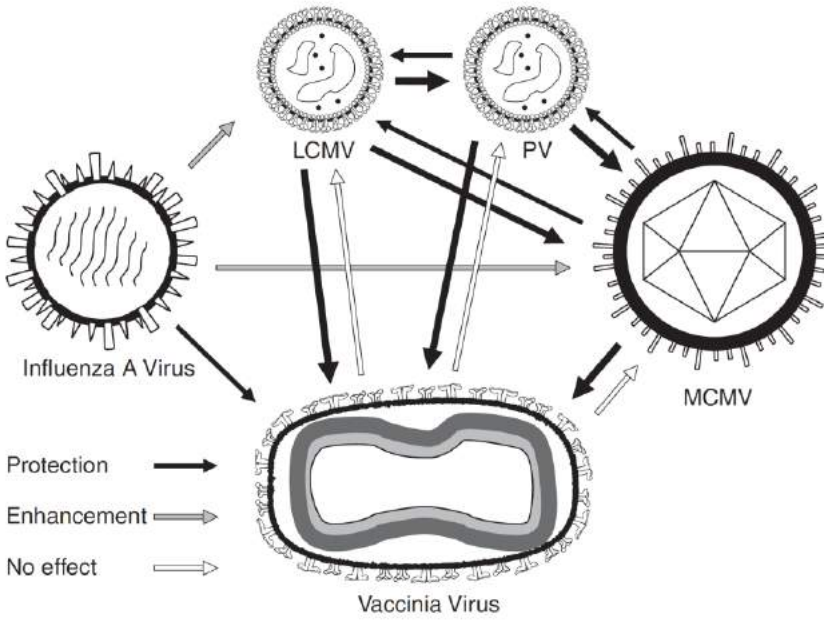

Figure 4. Heterologous immunity outcomes of viral infections in mice.

Extracted from (41)

Heterologous viral infections may lead to improved innate and adaptive immune activation and response to unrelated stimuli. For instance, models of EBV and cytomegalovirus infections in mice show that the primary viral infection translates into increased levels of IFN-γ production and activation of macrophages, which contributes to improved survival to secondary bacterial infection (42). Also, mice infected with MCMV show improved Lymphocytic Choriomeningitis virus (LCMV) – Armstrong (Arm) specific $CD8^+$ T cell response (43). Similarly, previous infection with Armstrong, MCMV or Influenza A virus (IAV) improves the immune control of viral infection with Vaccinia Virus (VV) (41,44).

There is increased IFN-γ and IL-6 production following VV infection in previously MCMV infected mice, which suggests an increased pro-inflammatory immune response in such instances (41,44). In the case of Armstrong immune mice, the response to unrelated VV, but not to Pichinde virus (PV), is improved, with increased T-cell associated IFN-γ production, and accelerated VV infection clearance, suggesting the existence of T-cell cross-reactivity between LCMV and VV (45).

Heterologous viral infections may be deleterious to the host immune response to unrelated pathogens: previous IAV infection is deleterious to the control of both Armstrong or MCMV (41,44). Moreover, mice infected with Armstrong show decreased T-cell activation following MCMV infection, with increased viral loads and pathology (43). Previous Armstrong infection, despite the protection rendered to VV infection, it also increases pathology, with extensive necrosis of adipose tissue of the host (46).

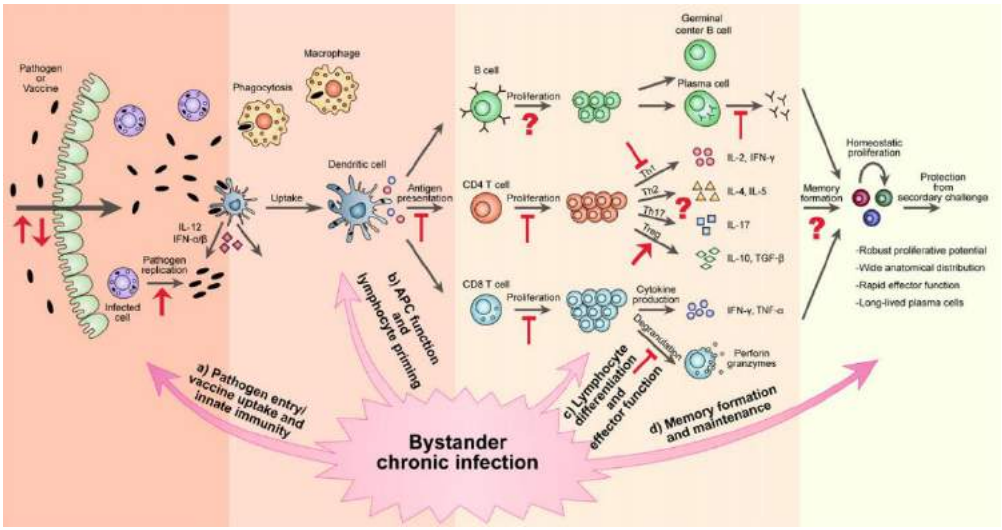

Figure 5. Immunological outcomes of heterologous viral infections.

Adapted from (47).

Altogether, the information presented in this chapter indicates that we still need to further study the role of bystander viral infections in the host immune response to unrelated viral infections. Current literature suggests that long-lasting viral infections can improve the response towards non-related viral infections, but they can also have the opposite result (7,25,50–52,39,41,43–45,47–49). This points out to the possibility that there is a wide spectrum of possible unexpected outcomes in those individuals receiving new viral stimuli when already having chronic exposure to another one. In

later chapters of this thesis, I will present novel results that provide some novel insight into these issues.

Confecting viruses	Outcome	Method(s) of detection	Remark(s)
DNV and CHIKV	Accommodation	Nucleic acid	CHIKV neither triggered nor suppressed DNV replication; mosquitoes with DNV infection were equally susceptible to infection by CHIKV
DNV and DENV	Interference/ enhancement	Nucleic acid	Reduced DENV replication concomitant with increased DNV replication
DNV and DENV	Accommodation	Nucleic acid	Persistent DENV and DNV coinfection
DNV, DENV, and JEV	Accommodation	Immunofluorescence	Stable infection of all three viruses without any CPE
CHIKV and JEV	NA	Antibody	Prevalence of antibodies against dual infection
IBV and avian pneumovirus	Interference	Viral titers, nucleic acid, antibody	IBV interfered with replication of pneumovirus vaccine strain in fowl
IBV and NDV	Interference	Viral titers, nucleic acid	IBV interfered with NDV replication
Sylvatic and endemic DENV strains	Interference	Viral titers	Primary virus suppressed secondary virus infection
NDV and HPAIV	Interference	Viral titers	NDV blocked HPAIV replication
WMV and ZYMV	Interference	Nucleic acid	ZYMV inhibited WMV replication
Henipavirus and rubulavirus	NA	Nucleic acid	Evidence of dual virus infection in bats
SINV and LACV	Interference	Viral titers	Replication of both viruses suppressed
SINV and LACV	Interference	Viral titers	LACV titers suppressed but no effect on SINV titers if SINV infection was 2 h before LACV infection
DENV2 and DENV4	Interference	Viral titers	Suppression of both viruses but greater suppression of DENV2
SINV, SFV and SINV, RRV	Exclusion	Viral titers	Persistently SINV-infected cells excluded superinfecting heterologous alphaviruses
SINV and YFV	Accommodation	Viral titers	Persistently SINV-infected cells did not impair YFV replication
CxFV and WNV	Enhancement	Viral titers/nucleic acid	Enhanced WNV transmission in mosquitoes
CxFV and WNV	Accommodation	Viral titers/nucleic acid	CxFV had no impact on WNV replication
IPNV and VHSV	Interference/ accommodation	mRNA	Accommodation on coinfection and primary VHSV and secondary IPNV infection but interference on primary IPNV and secondary VHSV infection
NDV and LPAIV	Interference	Viral titers	Coinfection decreased LPAIV shedding and transmission but had no impact on clinical signs.
NDV and HPAIV	Interference	Viral titers	Coinfected ducks survived for shorter duration
HSV and VZV	Exclusion	Immunofluorescence	Exclusion of each other
BHV-1 gD and HSV-1/BHV-1/PRV	Interference	Viral titers	Expression of BHV-1 gD inhibited HSV-1, PRV, and BHV-1 replication
TTSuV1a and ASFV	NA	Nucleic acid	NA
hMPV and hRSV	NA	NA	Increased hospitalization rates in humans
HCV and TTV	NA	Nucleic acid (HMA)	NA
Fowlpox virus and ILTV	NA	Viral titers/nucleic acid	NA
WSSV and IIHNV	NA	Nucleic acid/ histopathology	Increased mortality in Pacific white shrimp
Influenza A/H1N1 and A/H3N2 viruses	NA	Nucleotide sequencing	Demonstrated ability of these two influenza virus subtypes to coinfect humans and a potential risk of influenza virus reassortment
CIAV and IBDV	Interference/ enhancement	Nucleic acid/cytofluorometric analysis	Enhanced CIAV titers in bursa and thymus but diminished IBDV-induced lymphocyte disorder
Cytopathic and noncytopathic BVDV	NA	Radioimmunoprecipitation/ polypeptide profile	Induced different polypeptide profiles
Multiple coronaviruses (BtCoV HKU2, BtCoV HKU8, BtCoV HKU1, BtCoV HKU7, BtCoV HKU10)	NA	Nucleotide sequencing	Evidence of multiple coronavirus infections in bats (zoonoses)
SINV and DENV4	Interference	Viral titers/nucleotide sequencing	SINV infection resisted DENV infection
ZIKV, CHIKV, and DENV	NA	Nucleic acid/antibody	Cross-reactivated antibodies against ZIKV/DENV may lead to misleading serological conclusions

Table 2. Co-infections and outcomes in the host (1).

Extracted from (39).

Confecting viruses	Outcome	Method(s) of detection	Remark(s)
CHIKV and DENV	NA	Nucleic acid/antibody	CHIKV and DNV were successfully purified by plaque purification and antibody neutralization, respectively
DENV3 and CHIKV	Interference	Cell culture/nucleic acid	Interference depended on virus dose
DENV and CHIKV	NA	Antibody	NA
DENV1 and DENV3	Interference	Antibody	Higher DENV3 prevalence
GPV and ORFV	NA	Nucleic acid	NA
Human adenovirus, human enterovirus, RSV, and human rhinovirus	NA	Nucleic acid	NA
HIV-1 and influenza virus	Enhancement	NA	High risk of influenza infection in HIV-1-infected individuals
PCV2 and CSFV	NA	NA	Induction of stress response and apoptotic signaling pathways
DENV and HIV-1	NA	Nucleic acid	NA
DENV, CHIKV, and ZIKV	NA	Nucleic acid	Concurrent circulation of DENV, CHIKV, and ZIKV and their coinfection
PPRV and FMDV	Interference	Viral titers/nucleic acid	Reciprocal replicative suppression in BHK-21 and Vero cells, respectively
Aura virus, SFV, RRV, and flaviviruses	Interference	Viral titers/nucleic acid	Insect cells persistently infected with SINV resisted infection of both homologous (SINV) and heterologous (Aura virus, SFV, and RRV) alphaviruses but had no effect on flaviviruses
IAV and RSV	Interference	Viral titers/ immunofluorescence	IAV competitively suppressed RSV at the level of viral protein synthesis and budding
IAV and hPIV2	Enhancement	Viral titers/ immunofluorescence	hPIV2 facilitated IAV replication
Wild-type and oseltamivir-resistant IAV strains	Interference	Viral titers/nucleotide sequencing	H3N2 and H1N1 differed in their ability to suppress replication and in transmissibility of the respective drug-resistant viral mutants
Swine influenza virus and PRRV	Interference	Viral titers/nucleic acid	Primary virus infection interfered with replication of the secondary virus
Group 1 and group 2 Brazilian vaccinia viruses	Interference/ enhancement	Viral titers/nucleic acid	Higher titers in lungs, lower titers in spleen; greater disease severity in mice
HBV and HCV	Coexistence	Viral titers/nucleic acid	Coexistence in Huh-7 cells without any interference
WNV (different strains)	Interference	Viral titers	Block in transmission of superinfecting virus
WNV. SLEV	Interference	Viral titers/nucleic acid	Block in transmission of superinfecting virus
HBV and HCV	Enhanced disease severity	Antibody	HBV-exposed individuals experienced enhanced HCV-associated disease severity

Table 3. Co-infections and outcomes in the host (2).

Extracted from (39).

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CHAPTER II. The CL13 mouse model of chronic infection

The persistence of a clinically silent viral infection in mice was first reported in 1935, by Erich Traub (1). In his work, he injected naïve mice intracranially with a preparation containing smashed organs from mice apparently clinically well and symptom free. Following infection, previously naïve mice showed neurological clinical symptoms, including somnolence, photophobia, tremors of the legs and tonic muscle spasms (1). With this, Traub hypothesized that donor mice should be infected with an unknown virus, able to persist in the host for long periods of time without overt symptomology. Later, Traub identified this virus as lymphocytic choriomeningitis virus (LCMV), which naturally infects mice vertically *in utero* or shortly after birth, without clinical signs of infection in most mice, yet persisting at high viral titers in several organs in many animals (2,3).

This model was insufficient to replicate viral infections in humans that install later in life such as cytomegalovirus (CMV) or hepatitis C virus (HCV). The team of Michael Oldstone first described a mouse model of chronic infection of LCMV with adulthood onset (4). Clone 13 (CL13) is a viral strain of LCMV that was originated from the parent virus strain LCMV-Armstrong (Arm) (4). The CL13 strain was first isolated from 2 months old BALB/C WEHI carrier mice, infected at birth with Arm (4). CL13 differs from the parent virus Arm in 5 nucleotide, that result in two amino acid substitutions in the viral glycoprotein (GP) (phenylalanine to leucine, F→L, GP260) and in the polymerase (lysine to glutamate, K→E, L1079) (5–7) that promotes extensive proliferation within dendritic cells (DCs) and fibroblastic reticular cells (8,9). In contrast to Arm, CL13 produces chronic infection with persistent viremia in blood and several organs, and impaired cytotoxic T lymphocytes (CTL) response for several months (4,10).

Establishment of chronicity and innate immunity

The innate immune response is pivotal in the early control of Arm or CL13 infection.

The higher levels of replication within DCs allows for increased antigen presentation to T cells while altering the homing of multiple immune players (11). This coincides with an increased peak of interferon (IFN)-I production compared to Arm (12). Simultaneously, the inhibitory programmed death-ligand 1 (PD-L1) is upregulated in DCs and there is higher production of interleukin (IL) 10 (13). Together, this contributes to reduced T cell effector activity.

The elevated infection of fibroblastic reticular cells results in severe, long-lasting, disruption of the fibroreticular mesh of several lymphoid organs, including the spleen (9,14). Moreover, CL13 results in increased expression of PD-L1 in the fibroblastic reticular cells, which further contributes to impair the cytotoxic activity of $CD8^+$ T cells, disturbs the production of chemokine ligand (CCL) 19 and CCL21, important for T cell recruitment, and IL-7 production, which provides survival signals to T cells (9).

When CL13 infection is established, there is also extensive deletion of several clones of T cells. It is hypothesized that natural killer cells (NK cells) may have a role in this process. Following rapid NK cell differentiation and activation in the early phases of CL13 infection, NK cells show extensive changes in their phenotype and apparently contribute to the killing of $CD4^+$ T cells, hence impairing $CD8^+$ T cell activation and differentiation (15,16).

How each of these innate immune players contributes to the establishment of chronic CL13 infection is described below.

Dendritic Cells

CL13 also replicates to higher levels than Arm in plasmacytoid DCs (pDCs) (8,17). CL13 infections results in higher levels of infection of antigen presenting cells (APCs) in the white pulp of the spleens (18). Splenic DCs have high levels of expression of α-dystroglycan (α-DG) which serves as a receptor for LCMV entry into cells (17,19). Interestingly, LCMV strains that produce chronic infections, such as CL13, bind strongly to α-DG, whereas the acutely resolved Arm does not. This is particularly evident in the white pulp of the spleen, where high infection rates of DCs are observed (19). This enhances killing of DCs mediated by $CD8^+$ T cells (18).

Therefore, as CL13 infection progresses into chronicity, the number of DCs decreases (12). Furthermore, the remaining DCs bear an exhausted phenotype and respond poorly to *stimuli* (12,20). The remaining DCs localize along the red pulp and the marginal zone and produce high levels of IL-10. As the infection progresses, they become more disperse, co-localizing with $CD4^+$ T cells and B cells (17). In addition, during CL13 infection, DCs produce a wide range of cytokines, including IFN-I, IL-10, IL-12, tumor necrosis factor (TNF) α, IL-1α, and chemokines, such as CCL2, CCL4, and CCL5 (17,21,22). suggesting a role for DCs in the regulation of $CD4^+$ T cells and B cells (23).

Interferon-I

IFN-I signaling is pivotal in the establishment of CL13 chronic infection and arguably one of the most debated figures in the field (24). Infection with LCMV induces IFN-I (both IFN-α and IFN- β) production, mostly by pDCs, that peaks at 12-48 h of infection (**Figure 6** and (21,22)). Absence of IFN-I signaling in this period results in increased LCMV viral loads in the spleen of infected mice (22).

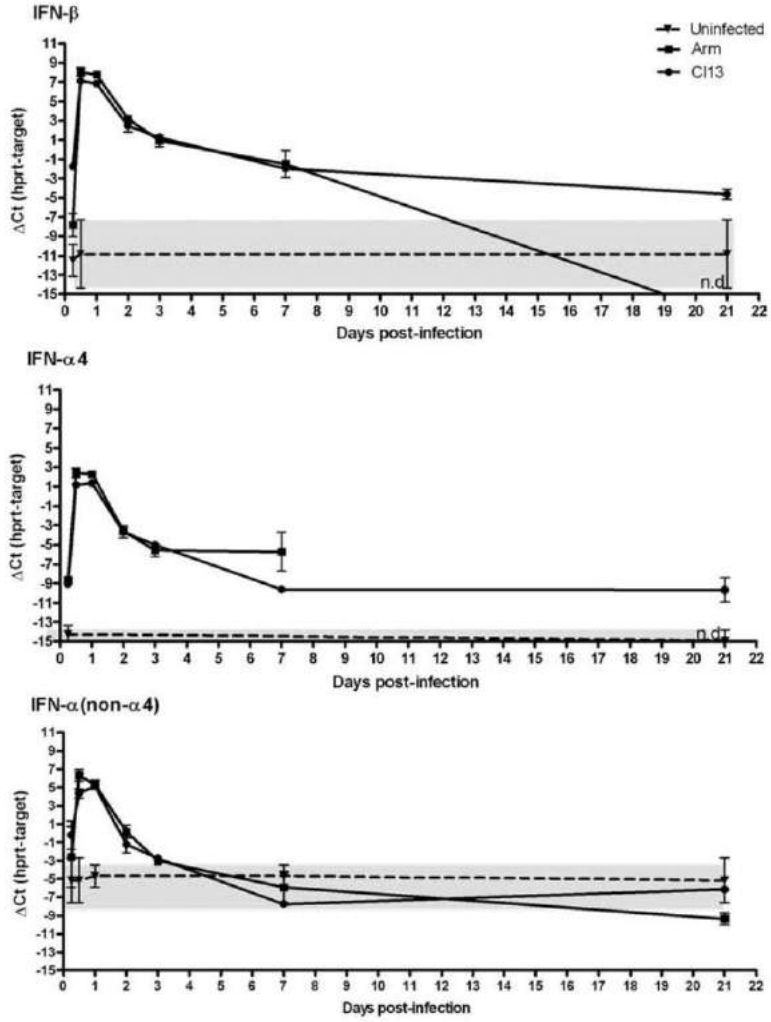

Figure 6. IFN-I Splenic levels of IFN-I mRNA over time after infection with Arm or Cl13.

Extracted from (12). Results are expressed as the difference between the Ct values of the housekeeping gene HPRT and that of the target gene of interest (ΔCt HPRT-target) in each sample.

Following the initial peak of IFN-I, the levels of IFN-I bioactivity and of RNA transcripts start to decrease, reaching values that may be lower than in non-infected mice (12). This reduction in IFN-I production is difficult to revert by different additional stimuli, including TLR ligands or secondary

unrelated infection (25). Surprisingly, the activity of IFN-I persists far longer throughout CL13 infection. In fact, at one month post infection, the expression of several IFN-I receptor inducible genes including Mx2, Oas1a, Oas1g, Oas2, Oas3, and others remains increased in CL13 infected mice (21).

Current evidence suggests IFN-I production during CL13 infection occurs following the activation of the transcription factor (TF) TLR7 which promotes the transcription of IFN-I genes (22,23). The activation of other pathways, via TLR3 or TLR9 ligands, also results in increased IFN-I production (12,20,26–28). There is also evidence that the recognition of viral RNA by the MDA5 RNA sensor is also important for the activation of the TLR7 pathway (22).

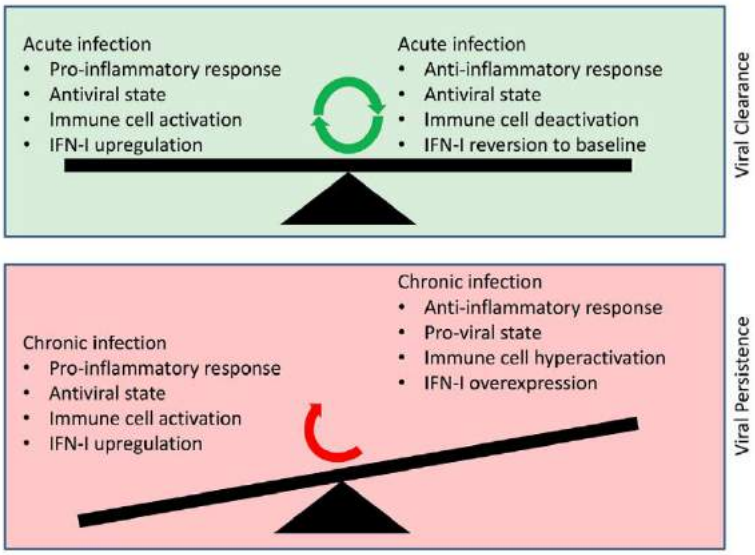

Figure 7 IFN-I role in clearance/ persistence of LCMV infection.

Extracted from (1)

In the LCMV WE acute infection model, another LCMV virus strain that results in acute infection, used by many research labs, IFN-I signaling has a preponderant role in the clonal proliferation of LCMV specific $CD8^+$ T cells. When transferring LCMV specific P14 T $CD8^+$ T cells into LCMV WE infected recipients, they proliferate extensively, making up to 60% of $CD8^+$ T cells in the recipients around 10 days post infection (dpi), with increased IFN-γ production and expression of the maturation markers KLRG1, CD62L and CD44. In opposition, *Ifnar1*$^{-/-}$ P14 T cells do not proliferate and have a severely reduced IFN-γ response, with decreased maturation. This indicates that IFN-I signaling is required for the development of a $CD8^+$ T cell response to LCMV (22,29). Interestingly, the impaired proliferation of transferred *Ifnar1*$^{-/-}$ P14 T cells appears to be infection specific, because they proliferate

and expands normally *Ifnar1*$^{-/-}$ in mice infected recombinant vaccinia virus carrying the GP33 epitope (29). Oxenius and colleagues showed that IFN-I signaling protects LCMV specific P14 T cells from the action of NK cell mediated killing (30). Transferred P14 *Ifnar1*$^{-/-}$ T cells are preferentially killed by NK cells, compared to the WT counterparts (30). Interestingly, this appears to be dependent on NKp46 signaling in NK cells, because similar to after NK cell depletion, the preferential killing of *Ifnar1*$^{-/-}$ P14 cells does not occur in Ncr1$^{icre/icre}$ mice, which lack NKp46 (31). Moreover, Diefenbach and colleagues showed that P14 T cells co-cultured with Prf$^{-/-}$ NK cells, lacking perforin production, are not preferentially killed when compared to the WT counterparts (32). Diefenbach and colleagues also suggested the possible mechanism of protection induced by IFN-I signaling in T cells. A micro-array of isolated WT T cells compared with *Ifnar1*$^{-/-}$ T cells, shows that IFN-I signaling upregulates several MHC-I molecules that bind to several inhibitory receptors on NK cells (32).

Considering that most of the early IFN-I comes from DCs, one would imagine that the absence of this source of IFN-I would lead to a severe impairment of the CD8^{+} T cells response. Yet, this is not the case. The IFN-I produced by DCs has a limited impact in the CD8^{+} T cells response to LCMV (17). In addition to this, infected CD8α^{-} DCs alone are unable to stimulate LCMV-specific CD4^{+} and CD8^{+} T cell proliferation (8). Furthermore, their absence does not translate into lower levels of LCMV-specific CD8^{+} T cell response (22).

As CL13 progresses into chronicity, IFN-I signaling also contributes to the reduction in the number of pDCs, because it reduces pDCs precursors in the BM in a STAT2-/-, but not STAT1-/- depended way (20,33). Therefore, blocking IFNAR signaling increases BM precursors, to numbers similar to those observed in non-infected mice (20). Yet, it does not restore the numbers of splenic pDCs (20). Current evidence suggests that IFN-I signaling is also required for the proliferation of pDCs (20). In this process, IFN-I induces TLR7 signaling and stimulates the proliferation of pDCs in a cell-intrinsic way (20). However, this catalyzes a negative feedback mechanism in which IFN-I production is suppressed and pDCs become unresponsive to new stimuli (20).

IFN-I signaling likely also contributes to increased expression of immunosuppressor mediators. In CL13 infected *Ifnar1*$^{-/-}$ mice, the expression of PD-L1, the ligand for the inhibitory receptor Programmed cell death protein 1 (PD1) in DCs and macrophages, is significantly decreased. Infected *Ifnar1*$^{-/-}$ mice also show a markedly decreased production of IL-10. The same is observed when

blocking IFNAR with a blocking antibody. Interestingly, the absence of IFN-I signaling, during the establishment of chronic CL13 infection, either by absolute absence, as is the case of *Ifnar1*$^{-/-}$ mice, or reduced activity using blocking antibodies, results in increased viremia. These observations suggest that IFN-I has an important role in controlling infection. However, by 15 dpi with CL13, the effect of blocking IFNAR is attenuated, with no changes in IL-10 production. In the long term, blocking IFNAR signaling may contribute to improved viral control, with improved APC activity of DCs, that henceforward express PD-L1 in similar levels to naïve mice (21).

It is yet to be explained why IFN-I production relates to chronicity of CL13 infection when its levels progressively decrease as the infection progresses (**Figure 7** summarizes the main key points of this debate). To answer this, Oldstone and colleagues infected mice in which either the IFNAR receptor or IFN-β were blocked: In mice where the IFNAR receptor was blocked, LCMV early replication was increased, with higher rates of infection of DCs and monocytes. However, this did not occur in mice were IFN-β was blocked. Moreover, blockade of IFN-β did not result in an increased inflammatory response in the spleen. Also, when blocking IFNAR, there was increased inflammation of the spleen. Intriguingly, blocking IFN-β translated into improved specific T cell response and decreased viral titers, similarly to when blocking only IFNAR. Therefore, we may conjecture that IFN-β contributes to decreased pathology early in LCMV infection but promotes chronicity in the long term due to the inherent reduction of the T cell responses (34).

Interleukin 10

IL-10 signaling is particularly critical to the establishment of CL13 chronic infection (35). Production of IL-10, mostly by macrophages and DCs, increases rapidly following CL13 infection, peaking before 2 dpi (36,37). As the infection progresses, $CD4^+$ T cells also become IL-10 producers (36). There is also some production of IL-10 by NK cells, $CD8^+$ T cells, and B cells, yet with limited impact in the outcome of CL13 infection (36,38,39).

Current evidence suggests that IL-10 is detrimental to the control of CL13 infection. IL-10 contributes to the suppression of the $CD4^+$ and $CD8^+$ T cells effector responses, leading to reduced TNF-α and IL-2 production (39). In addition, IL-10 reduces the avidity of $CD8^+$ T cells to antigens by impairing the co-localization of the T cell receptor (TCR) and the CD8 co-receptor, leading to decreased peptide/MHC complex avidity (11). Moreover, IL-10 decreases TCR signaling (11). Together this contributes to decreased virus-specific $CD8^+$ T cell responses (11). Blockade of IL-10 results in improved viral control, increased lymphocytes counts and better $CD8^+$ T cell response (37–39). Following IL-10 blockade, $CD8^+$ T cells increase IFN-γ production and decrease PD1 expression (37–39).

As CL13 infection progresses, the phenotypic changes in $CD8^+$ T cell effector function becomes persistent and independent of IL-10 signaling, since blockade of the IL-10R following 30 days of infection with CL13 does not result in improved CTL activity nor LCMV viral control (37). Possibly other mechanisms act synergistically to induce $CD8^+$ T cell exhaustion. Thereafter, *Il10*$^{-/-}$ mice infected with CL13 show improved $CD8^+$ T cell effector response at 8 dpi but not following 30 dpi with CL13 (37).

IL-10 and PD-L1 are classically associated with the establishment of LCMV infection chronicity, yet they probably do not regulate each other expression. When IL-10 deficient mice are infected with CL13, expression of PD-L1 is overt and indistinguishable from infected WT mice. On the other hand, PD-L1 deficient mice do not show decreased *Il10* expression. Moreover, blocking both IL-10 and PD-L1 signaling improves T cells response significantly more than blocking any of the two pathways independently and rescues exhausted T cells to produce more IFN-γ and TNF-α. Ultimately, blocking both pathways improves viral control (40).

Tumor growth factor β

Tumor growth factor (TGF) β signaling has been arguably associated with the establishment of chronic CL13 infection. It has been suggested that it contributes to $CD8^+$ T cell exhaustion. The team of Zuñiga and colleagues described increased levels of TGF-β production during CL13 infection, and increased TGF-βR mediated downstream activity, with increased levels of p-Smad2 (41). Moreover, dnTGFBRII mice, in which TGF-βR mediated downstream activity is attenuated specifically in T cells, show rapid clearance of CL13 infection and increased frequencies of LCMV virus-specific $CD8^+$ T cells (41). Then, LCMV virus-specific $CD8^+$ T cells show increased levels of effector activity, with increased production of IL-2, TNF-α and IFN-γ, with many of the cells showing increased polyfunctionality, together with improved cytolytic activity, since the expression of the degranulation marker CD107α is increased (41). Two possible explanations could explain this: 1 - apoptosis of virus-specific $CD8^+$ T cells is decreased, since surface expression of annexin V, associated with death of cells, is decreased, whereas the incorporation of bromodeoxyuridine (BrdU), signaling duplication of DNA chains, is not increased; 2- improved $CD4^+$ T cell help, since the frequency of virus-specific $CD4^+$ T cells is increased, while also showing increased polyfunctional activity, with simultaneous production of IL-2, TNF-α and IFN-γ (41).

In contrast, the daily blockade of TGF-β with anti-TGF-β antibodies or the adoptive transfer of memory cells, from dnTGFBRII naive mice, failed to protect WT mice from chronic CL13 infection, despite improved T cell response, with increased frequencies of LCMV virus-specific $CD8^+$ T cells (42). Therefore, McGavern and colleagues suggest that the rapid clearance of CL13 infection in dnTGFBRII mice is not a function of TGF-β impaired signaling in T cells, *per se*. Instead, they suggest it is due to the *a priori* abundance of activated $CD8^+$ T cells, because expression of CD122 and CD44 on those dnTGFBRII mice is increased. Moreover, when transferring P14 cells to CL13 infected dnTGFBRII mice, they also expand more than those transferred to C57BL/6 mice, while retaining full TGF-β signaling. Altogether, the current evidence on the role of TGF-β in the establishment of chronicity of CL13 infection is still sparse. Yet, it suggests that it has as role, at least at impairing the persistence of functional T cells while chronic infection develops.

Natural killer cells

NK cells have a crucial role in the establishment of chronic CL13 infection (**Figure 8**). The work of Welsh and colleagues illustrates this in a very elegant manner. They infected mice with three different doses of CL13 (high dose: $2x10^6$ plaque forming units (PFU), the one used in the chronic infection model; medium dose: $2x10^5$ PFU; low dose: $5x10^4$ PFU) and evaluated the effect of depleting NK cells in each scenario (15):

When depleting NK cells in the context of a low dose of CL13, the effect on mice survival was neglectable, as both mice groups survived in the long run, and cleared the viral infection within 15 days (15);

In the context of a medium dose of CL13, however, depleting NK cells resulted in increased susceptibility to CL13, yet with surviving mice able to clear the infection and assemble proper $CD4^+$ and $CD8^+$ T cell responses, with increased levels of polyfunctional (IFN-γ^+, TNF-α^+, IL-2^+) LCMV specific cells (15). Interestingly, depleting NK cells in the context of a medium dose of infection was beneficial up to 5 dpi with CL13 (16).

When depleting mice of NK cells before a high dose of CL13, mice showed increased pathology and death, inflammation and pulmonary edema, with lower viremia in surviving mice in the long run (15). Mice also showed improved GP33 and NP396 specific $CD8^+$ T cell responses with increased production of IFN-γ, TNF-α, and IL-2, increased frequencies of lymphocyte-activation gene protein (LAG) 3 and 2B4 positive cells and decreased expression of the inhibitory receptor PD1 (43,44). Frequencies of GP66 specific cells $CD4^+$ T cells were also increased (45).

Finally, depletion of NK cells prior to a very high dose of CL13 ($4x10^6$ PFU) did not improve viral control. However, it did not cause increased mortality as in the case of the high dose infection (16).

Therefore, current evidence suggests that NK cells can modulate several steps in the innate and acquired immune response to CL13 infection. For instance, Whitmire and colleagues showed that the depletion of NK cells at the onset of CL13 infection, though not increasing the numbers of splenic DCs, did not lead to improved performance of APCs (44). Moreover, the deleterious role of NK cells in the control of LCMV infection seems to correlate to a significant extent with their activation status.

When inhibiting NKG2D signaling, an activation receptor for NK cells, with a blocking antibody the $CD8^+$ T cell response was improved to a significant extent in LCMV WE infection. Ohashi and colleagues also demonstrated that when $Prf^{-/-}$ mice were infected with LCMV WE, the specific $CD8^+$ T cell response was improved suggesting that NK cells may exert their suppressive activity by killing other cells (43). Welsh and colleagues suggested that NK cells exert their activity by actively lysing LCMV-specific activated $CD4^+$ T cells, hence impairing their helper activity on $CD8^+$ T cells: when depleting simultaneously NK cells and $CD4^+$ T cells, in the context of a medium dose of infection, the improved $CD8^+$ T cell observed with NK cell depletion alone did not occur. Moreover, when they adoptively transferred activated T cells into recipient mice, infected with a medium dose of CL13, and thereafter depleted of NK cells, they showed decreased lysis of the donor $CD4^+$ T cells, but not of the donor $CD8^+$ T cells (15).

NK cells are also critical in the maintenance of the chronic infection with CL13. Depletion of NK cells late during chronicity (30 dpi) in the context of a high dose of CL13 improves $CD8^+$ and $CD4^+$ T cells responses, with higher IFN-γ expression. Therefore, NK cells continue to actively suppress the immune response to CL13 even at later timepoints, possibly by impairing the response of $CD4^+$ T cells because simultaneous depletion of NK cells and $CD4^+$ T cells at 30 dpi with CL13, the protective effect seen before was no longer evident (16).

The dual role of NK cells in the regulation of the T cell response can possibly be explained by the existence of tissue specific populations that specialize into the different functions associated with NK cells. While conventional NK cells positively regulate the T cell response against viral infection, liver resident NK cells promote the suppression of such responses in a PD1/PD-L1 dependent manner (46).

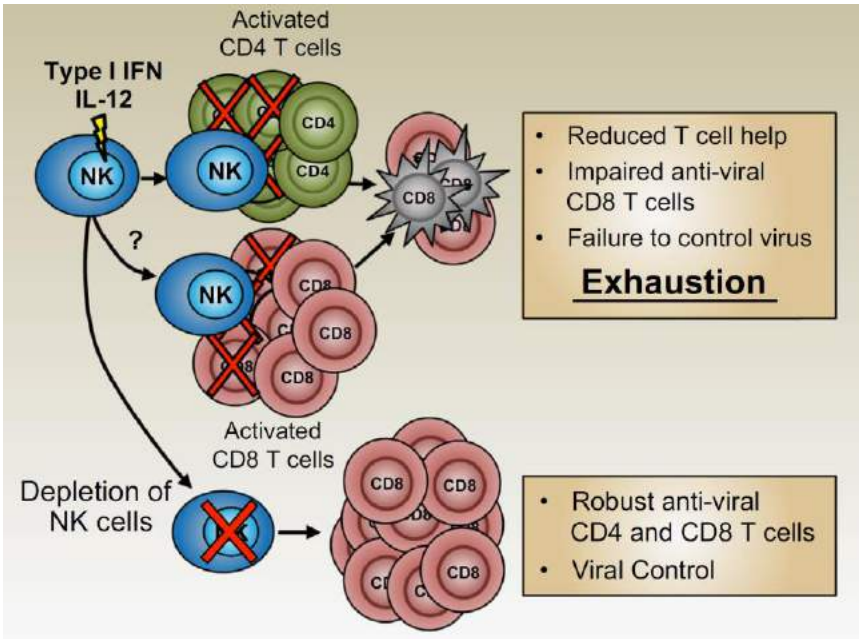

Figure 8. Contribution of NK cells to CL13 infection chronicity.

Extracted from (47).

Adaptive immunity and establishment of chronicity

The adaptive immune response to CL13 must be critically altered for chronicity to get established. This mouse infection model allowed the uncovering of many of the mechanisms behind T cell exhaustion in many long-lasting infections, including those with HIV in humans. Below I explain many of the ways in which T cells are modulated and their response is shaped to allow for CL13 chronicity to establish.

T cells

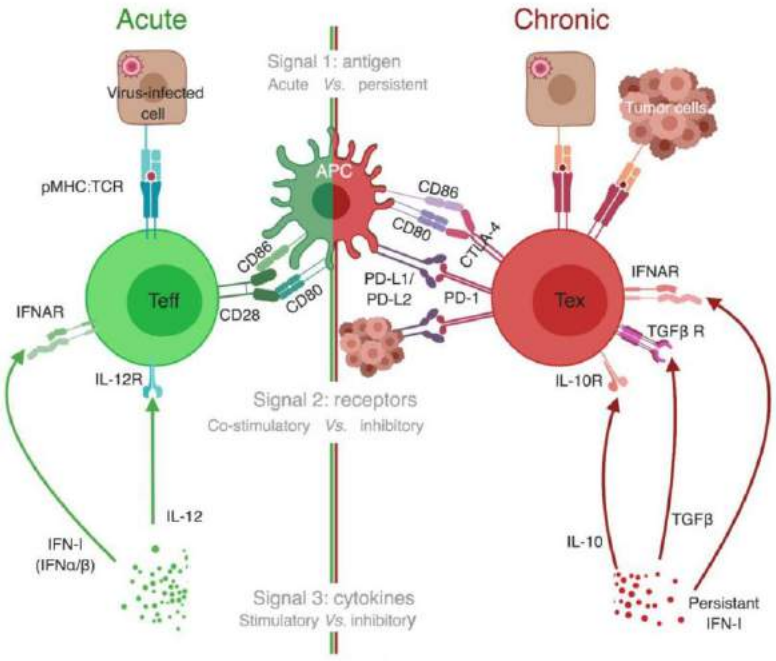

Figure 9. Different signals required for the establishment of exhaustion in CD8⁺ T cells.

Extracted from (48).

Both CD4⁺ T and CD8⁺ T cells are induced shortly after chronic LCMV infection, yet they become unresponsive and reduced numbers 6 weeks after infection (49,50). When the infection becomes chronic, the induced LCMV specific cytotoxic CD8⁺ T cells are rapidly clonally deleted or functionally exhausted within 2 weeks of infection (49,51).

CD8+ T cells

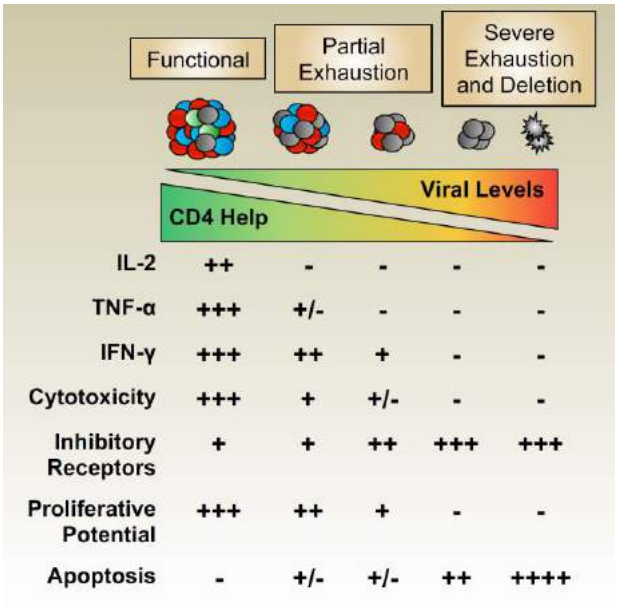

Figure 10. Establishment of CL13 chronicity and CD8+ T-cell exhaustion.

Extracted from (47).

The epitope-specific CD8+ T cell response to CL13 differs from that to Arm (10). For CL13, CD8+ T cells specific to some LCMV epitopes are eliminated, while others maintain an exhausted phenotype throughout the infection, with increased expression of CD44 and CD69, and decreased expression of CD62L, but without antiviral effector activity (52). The reason for the persistence of some exhausted CD8+ T cells clones and the deletion of others has been strongly debated (**Figure 9** and **10**). Ahmed and colleagues suggested that the presence of continued antigen presentation leads to deletion of a significant part of the antigen specific CD8+ T cells and exhaustion of the remaining cells (53).

In mice that recover from acute Arm, a pool of LCMV specific CD127hi, CD122hi memory CD8+ T cells remains long after infection resolution (54). In contrast, in chronically CL13 infected mice, this same LCMV-specific population has reduced expression of both markers (54). Moreover, and as exhaustion is being established, the production of IL-2, TNF-α and IFN-γ in CD8+ T cells progressively decreases. Also, CD8+ T cells gradually decrease polyfunctionality, motility and metabolic activity (10,55–58). Even if stimulated with adjuvants such as IFN-I or TLR7 ligands, in CL13 infected mice, the CTL response is small (12). Interestingly, suppression of cytolytic activity in exhausted CD8+ T cells

is not as startling as it is with the other effector functions (59). This suggests that some $CD8^+$ T cell activity remains and exerts residual virus control.

The expression of inhibitory receptors in $CD8^+$ T cells is directly correlated with the establishment of $CD8^+$ T cells exhaustion (56). During CL13 infection, $CD8^+$ T cells express multiple inhibitory receptors, including PD1, LAG-3, CD160, 2B4, CTLA-4, PIR-B, GP49, KLRG1, NKG2A, Tim-3, BTLA and TIGIT (56,60–63). Moreover the differentiation of effector $CD8^+$ T cells into memory cells is impaired, with reduced expression of CD62L, CD27, and CXCR3 (64). Interestingly, as chronicity is established, the vast majority of $CD8^+$ T cells express at least 3 of these receptors (60). Between 65% to 80% of LCMV specific $CD8^+$ T cells co-expressing Tim-3 and PD1, while producing the immune suppressor IL-10 (61). Notably, the larger the number of inhibitory receptors that $CD8^+$ T cells express during CL13 infection, the lower the IL-2, TNF-α and IFN-γ production (60,61)

This exhaustion phenotype of $CD8^+$ T cells can be somewhat reverted by blocking PD1 signaling. Blocking PD-L1 or PD1 decreases the exhaustion of $CD8^+$ T cells, which show increased proliferation and polyfunctional activity, with improved TNF-α and IFN-γ simultaneous production, increased degranulation and CD107α expression, increased metabolic activity and motility, and results in improved viral control (57,58,60,63). Blockade of PD1 signaling results in TF T cell factor 1 (TCF 1) dependent proliferation of $PD1^+$ $CD8^+$ T cells. These cells co-localize with naïve T-cells in lymphoid tissues and bear a gene profile that resembles stem cells and express several co-stimulatory molecules, including ICOS and CD28 (65,66). In contrast, the blockade of LAG-3, Tim-3 or TIGIT alone does result in improved viral control or T-cell function, yet it potentiates the effects from PD1 blockade (60–62,67). Interestingly, the effects of PD1 blockade therapy may be absent if stimulating other inhibitory receptors simultaneously. When blocking Tim-3 alone, that does not result in increased exhaustion of $CD8^+$ T cells but is enough to impair the response to PD1 blockade therapy (68).

Considering the sharp exhaustion phenotype associated with the expression of inhibitory receptors, it is possible to hypothesize that impeding the expression of such receptors early in infection, $CD8^+$ T cell exhaustion would be averted and CL13 persistence avoided. However, CL13 infection is lethal to $PD\text{-}L1^{-/-}$ due to the severe immunopathology (63). This suggests that the expression of inhibitory receptors is necessary to protect the infected mice from $CD8^+$ T cell cytotoxicity during persisting infections such as CL13. $PD1^{-/-}$ $CD8^+$ T cells transferred to C57BL/6 mice proliferate more

and show improved cytotoxicity between 8-14 dpi with CL13 yet later become even more exhausted than the host $CD8^+$ T cells (69), suggesting that other mechanisms counteract the absence of PD1 signaling to induce of $CD8^+$ T cell exhaustion.

CL13 infection can suppress the $CD8^+$ T cell response by inducing the death of virus-specific cells. Decaluwe and her colleagues showed that during CL13 infection, the differentiation marker CD122 is significantly increased, coinciding with other classical exhaustion markers CD160, 2B4, LAG-3, TIM-3 and PD1. Interestingly, those cells expressing higher levels of PD1 are also the ones expressing the higher levels of the death markers 7AAD and annexin V.

Not all remaining $CD8^+$ T cells become exhausted during chronic CL13 infection. Hasenkrug's team described a population of $CD8^+$ T cells that expresses the inhibitory receptor SIRPα (70). Surprisingly, in this subset of cells, the expression of multiple inhibitory and stimulatory receptors is increased, including CD122, Tim3, Lag3, CD95, CD43, CD44, CD40, CD728, KLRG1, CD62L, CD47 and CXCR31(70). Also, the SIRPα^+ $CD8^+$ T cells have increased proliferative capability, IFN-γ production and cytolytic activity than the SIRPα^- counterparts (70).

Oldstone and colleagues studied the early suppression of $CD8^+$ T cell response upon CL13 infection. When wild-type mice were primed with Arm up to 8 hours prior to CL13 infection, chronicity did not occur. Instead, GP33 specific $CD8^+$ T cells were fully functional, with increased IFN-γ and TNF-α production. Moreover, NP396 specific $CD8^+$ T cells, which, typically become virtually undetectable as CL13 infection becomes chronic, were increased and functional (71).

During chronic infection, CL13-specific $CD8^+$ T cells respond poorly to proliferative stimuli. 30 dpi with CL13, $CD8^+$ T cells proliferated poorly following in vitro IL7 and IL-15 stimulation, whereas those from Arm infected mice proliferated extensively (72). Interestingly, exhausted CL13 specific $CD8^+$ T cells can partially respond to a secondary Arm infection. When CL13-specific $CD8^+$ T cells from mice chronically infected with CL13 are transferred to naïve mice, which are subsequently infected with Arm, they expand at similar levels as virus-specific $CD8^+$ T cells isolated from Arm infected mice. Yet, $CD8^+$ T cells from chronically infected mice show reduced cytokine production, including TNF-α, and retain high PD1 expression (73). This suggests the exhaustion of $CD8^+$ T cells persists even in daughter cells, which implies the differentiation process of $CD8^+$ T cells is irreversibly altered with prolonged virus exposure and antigen overload.

The establishment of exhaustion of $CD8^+$ T cells in chronic LCMV infection is also regulated by several TFs. Wherry and colleagues observed that in CL13 infected mice, virus-specific $CD8^+$ T cells overexpress Blimp-1, a transcriptional repressor of IFN-I related genes. Moreover, the expression of this repressor gradually increases as the infection progresses. The expression of Blimp-1 is also associated with the expression of the inhibitory receptors PD1, LAG3, CD160, and 2B4. When conditionally eliminating Blimp-1 in $CD8^+$ T cells, the expression of those inhibitory receptors was significantly decreased. Moreover, those $CD8^+$ T cells expressed a more memory-like phenotype, with increased levels of CD127 and CD62L. Ultimately, the expansion of LCMV specific cells was significantly improved (74). Blimp-1 expression is also more abundant in exhausted $CD8^+$ T cells that have low expression of the TF TCF-1. Interestingly, TCF-1 overexpressing P14 $CD8^+$ T cells persisted much longer and showed a less exhausted phenotype than the C57BL/6 counterparts, including decreased Blimp-1 expression. Therefore TCF-1 expression is associated with increased frequencies of memory-like $CD8^+$ T cells, that have reduced signs of suppression and exhaustion and improved effector activity, with increased GzmB production (75,76).

Many other TFs are important for the establishment of $CD8^+$ T cell exhaustion. For instance, PD1 expression requires the modulation by the TF FoxO1 (77). T-bet is more associated with the differentiation of effector $CD8^+$ T cells following acute infection, whereas EOMES is more associated with exhausted $CD8^+$ T cells (78). The tumor suppressor VHL negatively downregulates HIF expression, decreasing $CD8^+$ T cell effector functions but protecting chronically infected mice from increased $CD8^+$ T cell induced immunopathology. $VHL^{-/-}$ $CD8^+$ T cells have reduced expression of the inhibiting receptor KLRG1, expand to higher numbers in infected mice and express increased levels of GzmB and other activation markers (79).

Absence of the TFs IRF4 or BATF results in limited $CD8^+$ T cell responses in mice infected with with $2x10^6$ PFUs of LCMV WE (80). In contrast, work by Kallies and colleagues showed that exhausted $CD8^+$ T cells have increased expression of IRF4 and BAFT. Compared to WT P14 cells $IRF4^{+/-}$ P14 cells transferred into LCMV infected mice were more poly-functional and had decreased expression of several inhibitory receptors, including PD1, TIGIT, Tim-3, Lag3, 2B4 and CTLA-4 (81). Together, these results suggest that $CD8^+$ T cells require optimal levels of IRF4 and BAFT to overcome exhaustion during the early and later phases of LCMV infection. In addition, the TF NFAT promotes $CD8^+$ T cell exhaustion but also effector phenotype when interacting with the TF AP-1 (82).

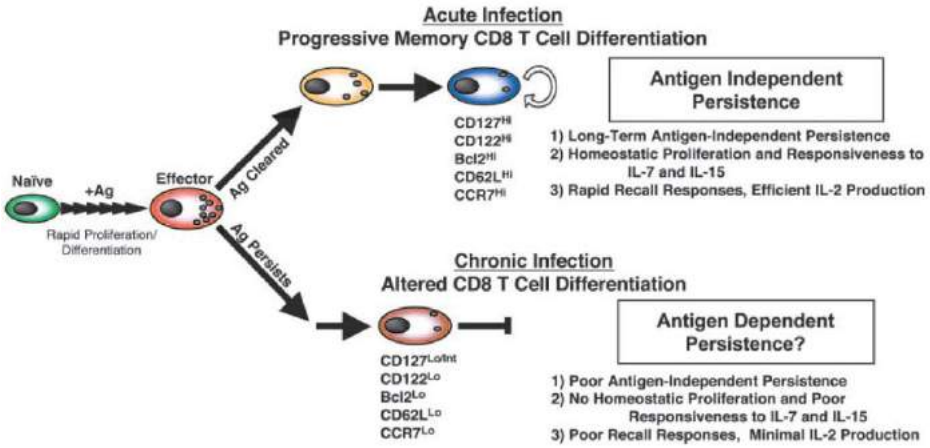

Figure 11. LCMV-depended antigen stimulation and CD8+ T cell differentiation.

Extracted from (72).

With the establishment of chronicity, the continued antigen overload of $CD8^+$ T cells appears to suppress the progression of effector $CD8^+$ T cells into the memory pool, possibly resulting in reduced life span (**Figure 11**). Ultimately, this results in fewer and less responsive memory cells. When LCMV-specific $CD8^+$ T cells, obtained from CL13 infected mice at 8 dpi, were transferred into mice that had been infected with Arm also for 8 days, were still present at 30 dpi and expressed CD127 (83). This suggests that in the early phase of CL13 infection, virus-specific $CD8^+$ T cells retain the potential to become memory cells. Yet, when the LCMV-specific CD8 T cells were obtained from mice infected with CL13 for 30 days, they persisted at lower levels than in Arm infected control mice, with reduced upregulation of CD127, indicating they were unable to survive and become protective memory cells (83).

CD4+ T cells

CD4+ T cells are pivotal in controlling LCMV infection and provide crucial help for CD8+ T cells responses (**Figure 12**) by inducing IL-12 production in DCs through CD40L-CD40 interactions IL-12. In addition, CD4+ T cells produce IL-2, which promotes CD8+ T cell survival (84,85).

During LCMV infection, the CD4+ T helper response is dependent on IL-6 signaling. IL-6-/- mice have persistent and increased serum viremia (86). Absence of IL6 signaling, including in a CD4+ T cell restricted manner, results in reduced numbers of GP66 virus-specific CD4+ T helper cells and decreased production of IFN-γ and IL-21 in a per cell basis (86,87). In the absence of IL-6 signaling, there are also reduced numbers of GP33-specific CD8+ T cells and persistent serum viremia and increased viral loads in several organs, including the spleen, the liver and the lungs (87). In vitro stimulation of LCMV-specific SMARTA CD4+ T cells with IL-6 resulted in upregulation of STAT1 and STAT3 and increased production of IL-21. Together, this suggests that IL-6 stimulates T helper cells, via the activation of STAT1 and STA2, with increased production of IL-21, which promotes the expansion of virus-specific CD8+ T cells.

In the absence of CD4+ T cells, LCMV-specific CD8+ T cell clones expand at reduced levels and show a more exhausted phenotype, with reduced cytotoxic activity and potential to respond to new viral stimuli, resulting in increased CL13 loads and impaired viral control (52,55,60,88,89). Transfer of LCMV-specific SMARTA CD4+ T cells into CL13 chronically infected mice rescues exhausted CD8+ T cell effector function, proliferation and cytokine production, including IFN-γ, leading to improved viral control, with reduced serum LCMV viremia (90). Together, this clearly shows a role for CD4+ T cells in the modulation of CD8+ T cells following viral infection.

Similarly to CD8+ T cells, CD4+ T cells progressively lose effector activity and become exhausted with the establishment of chronic CL13 infection. In CL13 chronically infected mice CD4+ T cells show reduced production of IL-2, TNF-α and IFN-γ (91,92). Moreover, GP66 virus-specific CD4+ T cells increase expression of several inhibitory receptors, including PD1, LAG-3 and CTLA4, with a markedly exhausted molecular signature (92).

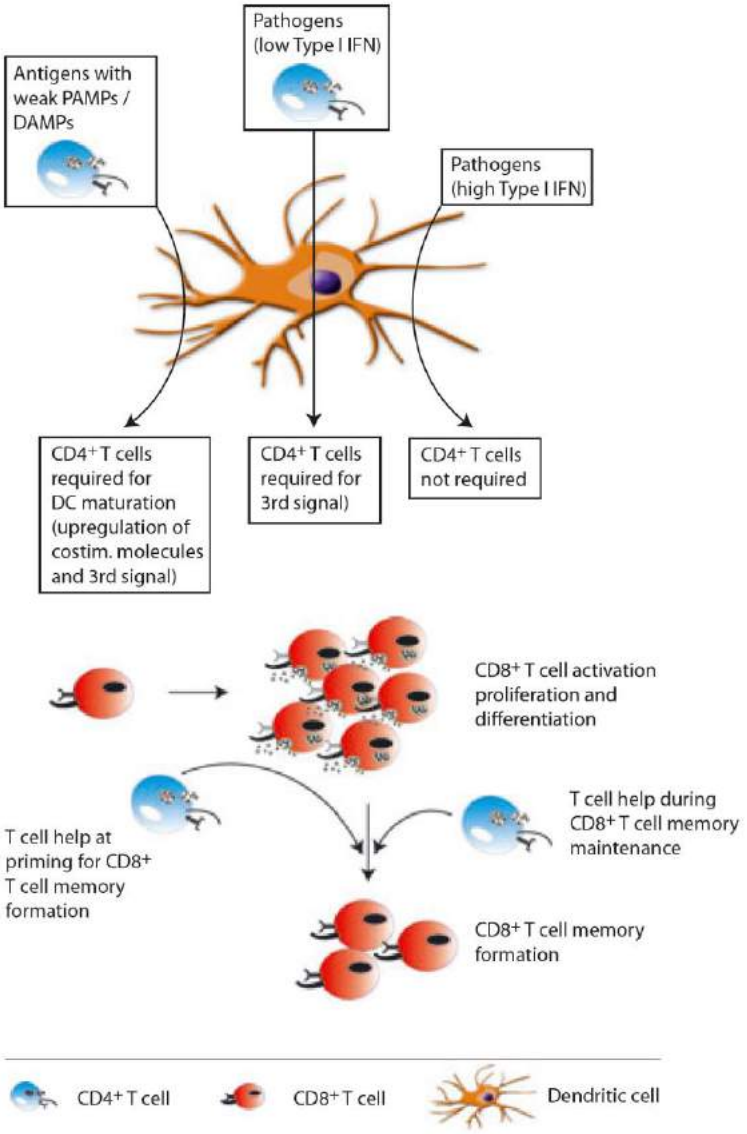

Figure 12. CD4+ T cell help for CD8+ T cell differentiation in LCMV infection.

Extracted from (93).

Interestingly, a population of CD4+ T cells persists during infection and shows increased IL-10 production and FoxP3 expression, as well as CTLA-4, CD39, GzmB, ICOS, PD1. PD-L1, CD44 and CD69, which suggests they bear a regulatory phenotype (91,92,94). Using Foxp3^{DTR} mice, Ahmed and colleagues demonstrated a role for regulatory T cells in suppressing CD8+ T cells (94). In mice, after ablation of regulatory T cells in Foxp3^{DTR}, LCMV specific CD8+ T cells displayed increased expression of the proliferation marker Ki67 and were highly activated, with increased expression of CD127, CD44 and GzmB (94). Moreover, following *ex vivo* stimulation with multiple LCMV peptides, they produced

larger amounts of IFN-γ (94). Yet, the ablation of regulatory T cells alone did not result in improved viral control (94). This was possibly linked to continued PD1 expression in $CD8^+$ T cells. Hence, when blocking PD1 and ablating regulatory T cells, there was improved viral control, comparing to any of the two alone (94). Altogether, these data suggest that with the establishment of chronic CL13 infection, a population of $CD4^+$ regulatory T cells emerges, with high levels of IL-10 production, and actively suppresses $CD8^+$ T cell effector function.

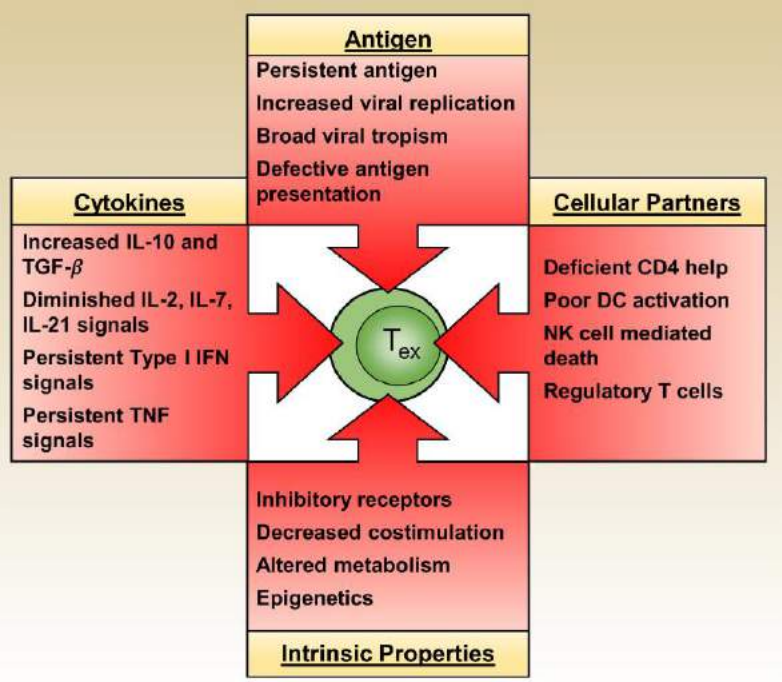

Figure 13. Drivers of T cell exhaustion in CL13 infection.

Extracted from (95).

In summary, CL13 infection produces an immunosuppressed state within the host, with many $CD8^+$ T cell clones deleted or exhausted. The many possible reasons for this to occur, are shown in **Figure 13**, including alterations in APCs, the structure of the lymphoid organs, pro and anti-inflammatory cytokines, as well as changes in $CD4^+$ T and NK cells. Despite being detrimental for virus control, this immunosuppression may be beneficial to the host. It is hypothesized that without exhaustion of T-cells, the immune response of the host would be so strong that could lead to death (95).

B cells

Many virus-specific B cells are deleted at the onset of CL13 infection, showing increased incorporation of 7AAD and surface expression of Annexin V (96). Also, during infection with LCMV-DOCILE, another LCMV virus strain that results in chronic infection, there is significant impairment of the neutralizing antibody response, even in the setting of an unrelated subsequent infection with Vesicular stomatitis virus – New Jersey strain (VSV-NJ) (97). Lamarre and colleagues described a similar outcome following CL13 infection, with reduced specific IgG response to CL13 (50). Moreover, as the infection progresses, the B cell numbers are reduced, together with a disrupted spleen architecture (50).

Deletion of B cells is time dependent and likely related to the peak of IFN-I that follows shortly after the onset of CL13 (50,96,98). IFN-I probably acts on B cells early during the infection because at 1-week post infection, the viral loads are high in CL13 infected mice but undetectable in Arm infected mice. Despite current conflicting evidence, it is likely that IFN-I signaling acts indirectly on B cells, modulating other immune cells that respond to CL13 infection, but also directly, likely via modulation of interferon stimulated genes (ISGs) (50,96,98). This hypothesis still requires further studies to be fully uncovered.

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CHAPTER III. The mousepox mouse model of acute infection

Variola and smallpox

The eradication of smallpox is one of the greatest achievements of public health. This was a disease that affected humankind for thousands of years. The first biological evidences of the disease were collected in ancient Egypt mummies from the 3rd century BC. The first written reports of the disease only emerged much later in the 4th century AD (1).

Smallpox was caused by the variola virus. Variola virus is a poxvirus from the *Poxviridae* family, subfamily *Chordopoxvirinae*, genus orthopoxvirus (OPV). There are several other orthopoxviruses, such as vaccinia virus, cowpox virus, monkeypox viruses and other serologically cross-reactive animal-borne viruses (2,3).

OPVs are amongst the largest viruses infecting mammals in nature, measuring 200 x 400 nm and show a brick-like structure. OPVs have and envelope that encases the other viral structures: an outer lipidic membrane; several lateral bodies, which contain several enzymes required for hijacking the infected cell; and the core membrane, surrounding the viral double-stranded, linear DNA and all the proteins necessary for mRNA synthesis to occur in the infected cell cytoplasm (2,3).

Infections with Poxviruses occur through 2 main routes: respiratory and contact trough skin abrasions. From the local of entry, the virus proliferates and propagates rapidly reaching the draining lymph nodes (dLN). In the case of smallpox, a short period of viremia is followed by a low-symptomatic incubation period in which the virus is mostly restricted to the reticuloendothelial system. After 4 to 14 days, a new period of viremia occurs, and the virus propagates to the mucous membranes in the mouth and pharynx and the capillary epithelium of the dermal layer of the skin. During this prodromal phase, patients abruptly develop severe headaches and backaches and become feverish, with temperature rising over 40 °C. Concomitantly, they show severe enanthem over the tongue, mouth, and oropharynx. Two days later, the infected individuals develop the characteristic rash lesions, first in body extremities and then throughout the whole body. This is the period in which infected individuals are the most contagious, with large amounts of viable virus being shed in urine and other body secretions. At this stage, the virus is also present in the spleen and liver, lymph nodes, bone marrow, kidneys and other viscera. The ablation of the infection is mostly cell-mediated, with macrophages, cytotoxic T cells and B cells taking major roles. However, up to 30% of the virus genome encodes for

proteins which functions are to evade and counteract the immune response of the host. Death from smallpox occurs in 1/3 of the infected individuals and is associated with toxemia from immune complexes and hypotension (3).

In the late XVIII century, Edward Jenner pioneered the discovery of the first effective vaccine against smallpox, simultaneously the first reported vaccine in the history of mankind. Jenner observed milkmaids who milked cows affected with peculiar eruptions on their teats, would contract a relatively benign disease, cowpox and possibly eliciting protection from mousepox. Jenner confirmed this association and developed a protocol, ethically questionable by current standards, of inoculating children in succession with a variant of cowpox and then exposing them to smallpox, to which they all resisted (4–6). From that time onwards, this new strategy was widely adopted to eradicate smallpox, but that was only attained during the XX century, when the joint efforts of the WHO and thousands of public health organizations around the globe drove the eradication of the disease. From the last reported case in Somalia, in 1977, to the official declaration of eradication in 1980 and cessation of widespread public vaccination, today there are only two reported stocks, both at WHO facilities, in Atlanta, USA, and in Novosibirsk, Russian Federation (1).Yet, the risk of biological warfare and climate change, which is exposing contaminated biological tissues from permafrost grounds, still threatens the great achievement of smallpox eradication (1,7).

The mousepox model of acute infection

Ectromelia virus (ECTV) is the agent of mousepox, an acute lethal viral disease of the mouse, very similar to human smallpox. It was first suggested as a model to study human smallpox since it is closely related to the variola and vaccinia virus (8).

Besides being the optimal mouse-specific model to study the human-specific variola infection, ECTV infection model provides an excellent model to study other viruses that spread lymphohematogenously, including those that responsible for smallpox, chickenpox, West Nile fever, Dengue fever, yellow fever, rubella and measles (9).

In the mouse, ECTV infection occurs in the skin of the footpad, trough skin abrasions (10). From there, the virus proliferates and propagates reaching the dLNs. Following 2-3 dpi with ECTV, the virus spreads lymphohematogenously to the liver and spleen, the primary infection sites of ECTV. By 4 dpi it is already possible to detect placable ECTV from the spleen and liver (11).

Following footpad infection, Mice can be either genetically susceptible and succumb to mousepox or resistant to the disease. BALB/C, DBA/2J and A/J mice succumb following ECTV infection, whereas C57BL/6J and 129 mice recover from disease (12). In resistant strains, virus loads peaks in the footpad and dLNs at 5-6 days following infection, and at 7 dpi in the liver and spleen. After that, most resistant strains show a progressive decrease in viral load, that is mostly undetectable following 10 dpi with ECTV, whereas susceptible strains continue to have progressively higher viral loads, until the moment they succumb to the disease (12), arguably due to liver necrosis (9).

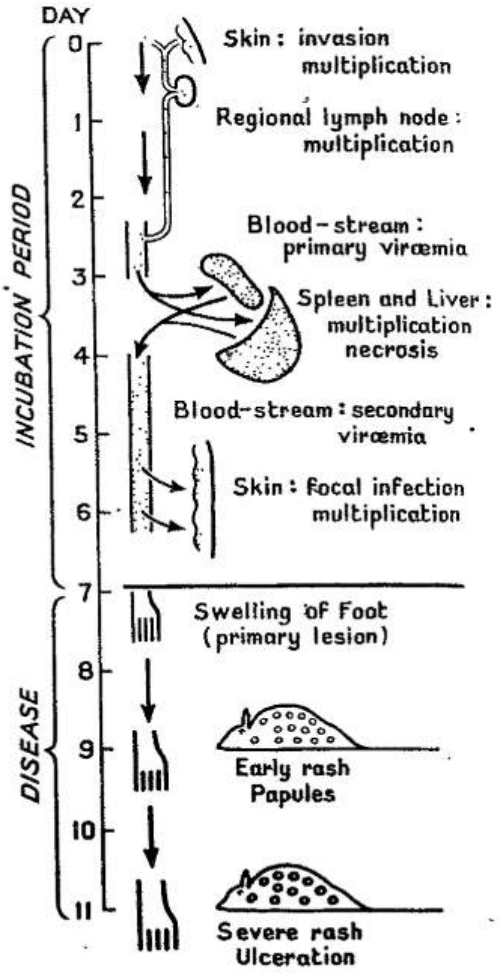

Figure 14. The pathogenesis of mousepox.

Extracted from (11).

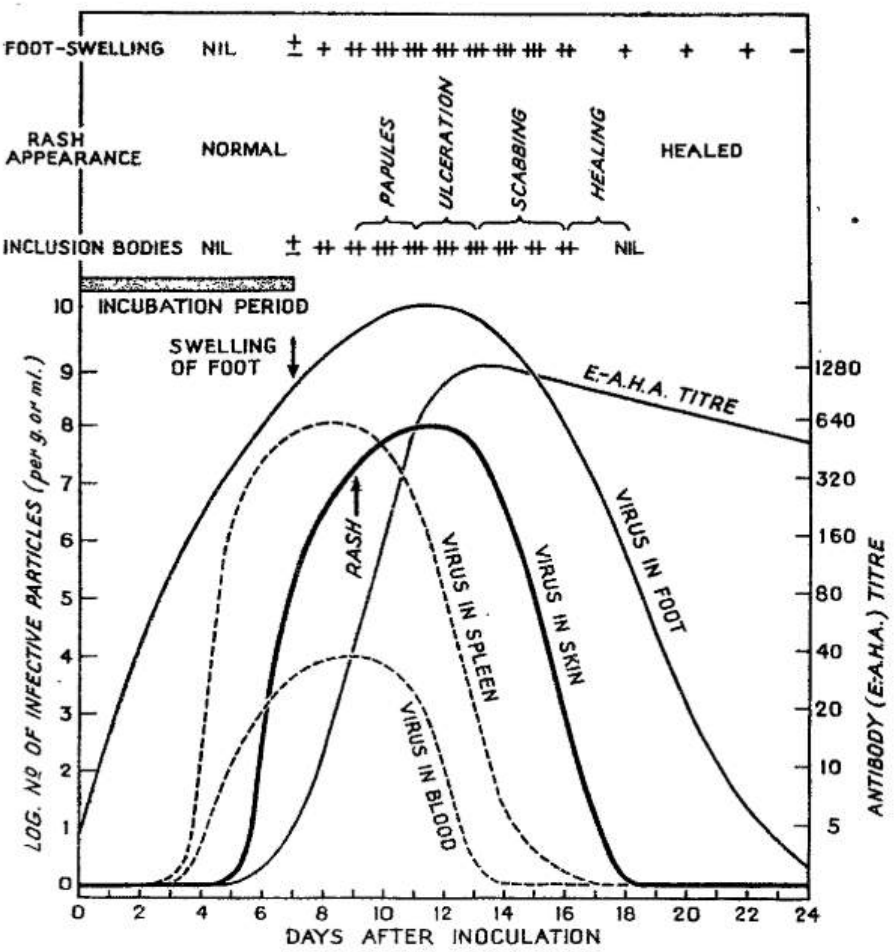

Figure 15. Clinical features and ECTV virus load in organs during mousepox in resistant mice.

Extracted from (11).

Innate immunity to ECTV

The immune response to ECTV starts right in the footpad skin. Infected MHC-II^{hi} DCs rapidly migrate from the footpad skin to the dLN, where their numbers and response peak at 24 post-infection. These DCs upregulate the expression of CCL2, CCL7, CXCL9, several interleukins, including IL18, TNF-α and IFN-I. Together, this contributes to the accumulation of iMOs and NK cells in the dLN (13). iMOs are able to modulate the recruitment and activation of several immune players, including NK and T cells, as they are major sources of IFN-I and CXCL9 (9). NK cells can modulate the recruitment and activation of several immune cells, including T cells, as well as acting directly in eliminating infected cells. The detail of some of these pathways is detailed ahead.

IFN-I

IFN-I stands out as one of the most prominent players of the innate immune response in protection from mousepox. Depletion of IFN-I signaling in C57BL/6 mice, using anti- IFN-I antibodies, results in increased mortality following ECTV infection, with faster viral spread from the dLNs to the liver and spleen, and overall higher ECTV viral titers (14). Similarly, the absence of *Ifnar1* signaling in *Ifnar1*$^{-/-}$ 129S7 mice results in full susceptibility to ECTV infection, whereas WT 129S7 mice are fully resistant to this infection (15).

The role of IFN-I is more evident early in ECTV infection. Its levels increase at the dLNs as early as 1-3 dpi with ECTV. This IFN-I production is highly functional, promoting the expression of downstream interferon induced genes (ISGs), such as MX1, IRF-7, and ISG-15 (16).

Interestingly, ECTV developed a decoy receptor for IFN-I that sequesters IFN-I molecules, likely impairing its bioactivity (17). When infection occurs with the ECTV-Δ166 strain, that does not express this decoy receptor, the EVM166 protein is no longer observable on the cell surfaces of infected cells, with overall improved viral control. Hence when infecting BALB/C mice with the ECTV-Δ166, the mice are fully resistant to infection, in stark contrast to what occurs following WT ECTV infection (18).

NK cells

NK cells are lymphoid cells that, differently from T cells, do not bear antigen specific T cell receptors (TCRs). Instead, they express a pool of activating and inhibitory receptors that modulate their effector activity (**Table 4** and (19,20))

Several cytokines modulate NK cell activation including TNF-α, IL2 +, IL12, IL15, IL18 and IFN-I (21–24). IL12 and TNF-α promote IFN-γ production (24). IL15 induces NK cell proliferation (24,25). IL2, IL12 and IL18 promotes the development of NK cytolytic activity (26,27). IL2, IL12 and IL18 can also promote NK cell suppressive activity and IL10 production (21,28–30).

Other than cytokine receptors, NK cells have receptors that directly sense danger signals from infected cells, such as the homodimeric NKG2D receptor, which is upregulated in infection, as it was described in ECTV infection (31). NKG2D senses several ligands that are only expressed in infected cells, such as RAE-1, MULT-1 and several H60 proteins isoforms, all of them capable of inducing similar levels of cytotoxicity in NK cells (32). Blocking of NKG2D during ECTV infection impairs NK cells function and leads to increased viral loads and lethality (31).

CD94 and NKG2A form a heterodimeric inhibitory receptor that binds to Qa-1^b, a non-classical MHC I molecule, to restrain unwanted NK cell activation (33). CD94 also forms activating heterodimeric receptors, with NKG2E and NKG2C that also binds with Qa-1^b. While CD94 is necessary for optimal NK cell mediated resistance to mousepox (34), NKG2C and E seem to be dispensable (Ferez-Ruiz, in preparation and (34)).

In the mouse, there is also another family of activating and inhibitory receptors, the Ly49 family, absent in humans, that senses MHC-I receptors. Those with inhibitory functions include Ly49A, Ly49C, Ly49I and Ly49P. Most of them signal trough ITIM. Those with activating functions include Ly49D and Ly49H. Most of them signal through DAP12 (35–37). Ly49H is particularly interesting because it is the only one to which viral specificity is known. It binds specifically to m157, a viral glycoprotein encoded my MCMV, which in turn activates NK cells (37,38). After an early non-specific proliferation period, $Ly49H^+$ NK cells expand and proliferate, generating a memory like population against MCMV, in what is suggested to be an adaptive immune response from NK cells (39,40).

Despite the discovery of antigen specificity in NK cells targeting MCMV, no other specific viral antigens that would activate or inhibit NK cells has been uncovered so far.

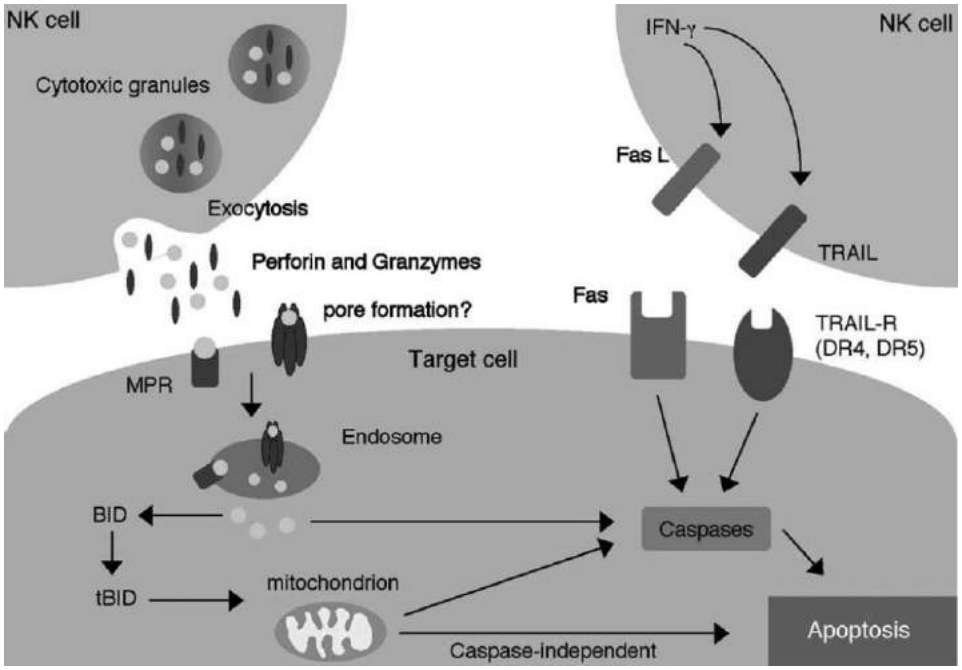

Figure 16. NK cell mediated killing.

Extracted from (41).

NK cells act by killing the target cell (**Figure 16**). The main mechanism of NK cell killing is granule exocytosis whereby they release perforin, that open pores in target cells, and granzymes, which are serine proteases that induce apoptosis after entering the cytosol of the target cells through the pores generated by perforin (27,42). NK cells can also induce apoptosis of target cells via tumor necrosis factors (TNF), with the production of the death-receptor ligands FasL and TRAIL (41).

The action of NK cells also involves the modulation of other immune cells. They produce several cytokines, including IFN-γ, TNF-α, GM-CSF and several chemokines, including CCL1, CCL2, CCL3, CCL4 and CXCL8, which modulate other innate and adaptive immune responses (43). NK cells can contribute to the suppression of immune responses, including with production of IL10 (21,44).

Receptor family	*Species*	*Ligands*	*Activation/ inhibitory*
Ly49	M	MHC class I	ACT/INHIB
Ly49A		H-2D^{d,k,p}	Inhib
Ly49C		H-2K^{b,d}, H-2D^{b,d,k}	Inhib
Ly49D		H-2D^{d}	Act
Ly49H		m157	Act
Ly49I		H-2K/D^{b,d,s,q,v}	Inhib
Ly49P		H-2D^{d}	Inhib
KIR	H	HLA-A/-B/-C	ACT/INHIB
KIR2DL1		HLA-C2	Inhib
KIR2DL2/3		HLA-C1	Inhib
KIR2DL4		HLA-G	Act
KIR2DL5		?	Inhib
KIR3DL1		HLA-Bw4	Inhib
KIR3DL2		HLA-A3, -A11	Inhib
KIR2DS1		HLA-C2	Act
KIR2DS2		HLA-C1	Act
KIR2DS3		?	Act
KIR2DS4		?	Act
KIR2DS5		?	Act
KIR3DS1		HLA-Bw4	Act
CD94-NKG2	H/M	H: HLA-E M: Qa1b	ACT/INHIB
NKG2A			Inhib
NKG2C			Act
NKG2E			Act
NKG2D	H/M	H: MIC-A/-B, ULBP1/2/3/4 M: RAE-1, MULT-1, H60	ACT
NCRs	H/M	Viral HA	ACT
NKp30		BAT-3, HSPG, B7-H6	Act
NKp44		Viral HA	Act
NKp46		Viral HA, HSPG	Act
NKp80		AICL	Act
LILR	H/M	MHC class I, UL18	INHIB
2B4	H/M	CD48	ACT/INHIB
KLRG1	H/M	Cadherins	INHIB
NKR-P1	M	Ocil/Clr-b	ACT/INHIB
DNAM-1	H/M	PVR, CD122	ACT
PILR	M	CD99	ACT

Abbreviations: ACT, activation; BAT-3, HLA-B-associated transcript 3; H, human; HA, hemagglutinin; HLA, human leukocyte antigen; INHIB, inhibitory; KIR, killer immunoglobulin-like receptor; KLRG1, killer cell lectin-like receptor G1; LILR, leukocyte immunoglobulin-like receptor; M, mouse; MHC, major histocompatibility complex; MULT-1, mouse UL16-binding-like transcript-1; NCR, natural cytotoxicity receptor; NK, natural killer; PVR, polio virus receptor; RAE-1, retinoic acid early transcript-1.

Table 4. Inhibitory and activating receptors in NK cells.

Extracted from (37).

NK cells are major innate immunity mediators in the response to ECTV infection. Their depletion in mousepox resistant C57BL/6 mice results in full susceptibility to disease (45). NK cells help control virus replication in the spleen and liver, without the help of T cells, up to 6 dpi with ECTV

(46). They are recruited to the site of infection by inflammatory monocytes, proliferate and produce IFN-γ, eliciting an anti-viral state, upon surrounding non-infected cells, and producing granzymes, that directly kill infected cells in lymph nodes, liver, and spleen (46).

Upon infection with ECTV, the number of NK peaks at 2 dpi in the draining lymph nodes and 5-6 dpi in the spleen and liver (46). After 6 dpi, the NK cell activity is no longer required for optimal recovery from mousepox, since their depletion does not result in increased mortality (31).

The current understanding of NK cells shows that they help curb ECTV infection by exerting cytotoxic activity, through granule exocytosis and by producing IFN-γ. This IFN-γ production helps in promoting a pro-inflammatory state in the infection site that recruits more NK cells and promotes T cell activation (13,31,46). These functions require mostly the more mature NK cells. Sigal and colleagues showed that C57BL/6 aged mice, that succumb to mousepox, have decreased frequencies of the R3 ($CD27^{-}CD11b^{+}$) subset on NK cells, which is the most mature subsets and increased frequency of the more immature R1 ($CD27^{+}CD11b^{-}$) NK cell subset. This seems to be a broad defect in aged mice, as this phenotype is observable both in the spleen and in the bone marrow (25).

Adaptive immunity to ECTV and T cells

In opposition to NK cells, T cells bear a large pool of TCR receptors that confers them antigen specificity. However, they also require antigen presentation from antigen presenting cells, such as DCs, macrophages and monocytes, and B cells. Moreover, they also need the presence of pro-inflammatory cytokines to get recruited to the infection site, to get activated, proliferate and respond to the infection. This helps explaining why, despite the large magnitude of the adaptive T cells response, this takes more time to take place. Upon stimuli by antigens, presented by MHC molecules, T cells become activated, proliferate and kill infected cells providing an essential second layer of virus control (47,48). The requirement for T cells for resistance to ECTV infection was first uncovered in the 1970s, when Blanden treated C57BL/6 mice with anti-thymocyte serum, resulting in full susceptibility to disease (49).

$CD8^+$ T cells

$CD8^+$ T cells recognize peptide antigen sequences bound to the MHC-I molecules. Simultaneously, $CD8^+$ T cells benefit from the priming by APCs, including DCs, macrophages and monocytes, and possibly B cells. APCs, together with NK cells, produce a pool of pro-inflammatory cytokines and chemokines that contribute to the activation, differentiation, and recruitment of cytotoxic $CD8^+$ T cells, commonly known as CTL cells. They display antigen specificity and are highly cytolytic to infected cells. CTLs exert their function through the production and release of perforin and granzymes, similarly to what occurs with NK cells.

In ECTV infection, $CD8^+$ T cells are pivotal in the response against infection, and without them C57BL/6 mice succumb to disease (50,51). Following innate immune response, the numbers of $CD8^+$ T cells peak in the dLN at 5 dpi and in the spleen at 7 dpi with ECTV. At 7 dpi with ECTV, 60-80% of the total pool are specific to ECTV, with 5-10% being restricted to the immunodominant Kb restricted TSYKFESV epitope from the IFN-γ decoy receptor coded in the EVM158 sequence from ECTV (52,53). In fact, after 6 dpi with ECTV, the response against ECTV rely mostly in $CD8^+$ T cells, since the depletion of NK cells has a limited effect in viral control after this timepoint (31). In addition, $CD4^+$ T cells show smaller levels of proliferation and granzyme B production than their $CD8^+$ T cell counterparts (54).

$CD4^+$ T cells

$CD4^+$ T cells are not required for the generation of a primary $CD8^+$ T cell response to ECTV (53). Yet they are required for the control of ECTV infection. They recognize peptide sequences bound to MHC-II receptors. Mice devoid of MHC-II signaling succumb to ECTV infection (55). $CD4^+$ T cells must have a unique role on their own, since MHC-II -/- mice succumb to mousepox later than mice without $CD8^+$ T cells but earlier than B cell deficient mice (55). In fact $CD4^+$ T cells proliferate following ECTV infection, showing GzmB production and the absence of perforin restricted to $CD4^+$ T cells results in impaired ECTV infection control (54,55).

In addition, activated $CD4^+$ T cells provide essential help to B cells, which produce anti-viral antibodies essential for the long-term control of ECTV (56). The importance of such processes became clear when mice that lack $CD4^+$ T cells revealed a much higher susceptibility than wild-type mice (31).

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Part II – Results

CHAPTER IV. Chronic Lymphocytic Choriomeningitis infection causes susceptibility to mousepox and impairs Natural Killer cell maturation and function

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PATHOGENESIS AND IMMUNITY

Chronic Lymphocytic Choriomeningitis Infection Causes Susceptibility to Mousepox and Impairs Natural Killer Cell Maturation and Function

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ABSTRACT Chronic viral infections. like those of humans with cytomegalovirus, human immunodeficiency virus (even when under antiretroviral therapy), and hepatitis C virus or those of mice with lymphocytic choriomeningitis virus (LCMV) clone 13 (CL13), result in immune dysfunction that predisposes the host to severe infections with unrelated pathogens. It is known that C57BL/6 (B6) mice are resistant to mousepox, a lethal disease caused by the orthopoxvirus ectromelia virus (ECTV), and that this resistance requires natural killer (NK) cells and other immune cells. We show that most B6 mice chronically infected with CL13 succumb to mousepox but that most of those that recovered from acute infection with the LCMV Armstrong (Arm) strain survive. We also show that B6 mice chronically infected with CL13 and those that recovered from Arm infection have a reduced frequency and a reduced number of NK cells. However, at steady state, NK cells in mice that have recovered from Arm infection mature normally and, in response to ECTV, get activated, become more mature, proliferate, and increase their cytotoxicity *in vivo*. Conversely, in mice chronically infected with CL13, NK cells are immature and residually activated, and following ECTV infection, they do not mature, proliferate, or increase their cytotoxicity. Given the well-established importance of NK cells in resistance to mousepox, these data suggest that the NK cell dysfunction caused by CL13 persistence may contribute to the susceptibility of CL13-infected mice to mousepox. Whether chronic infections similarly affect NK cells in humans should be explored.

IMPORTANCE Infection of adult mice with the clone 13 (CL13) strain of lymphocytic choriomeningitis virus (LCMV) is extensively used as a model of chronic infection. In this paper, we show that mice chronically infected with CL13 succumb to challenge with ectromelia virus (ECTV; the agent of mousepox) and that natural killer (NK) cells in CL13-infected mice are reduced in numbers and have an immature and partially activated phenotype but do respond to ECTV. These data may provide additional clues why humans chronically infected with certain pathogens are less resistant to viral diseases.

KEYWORDS chronic infection, ectromelia virus, lymphocytic choriomeningitis virus, natural killer cells, poxvirus, viral pathogenesis

Chronic viral infections in humans, such as cytomegalovirus, human immunodeficiency virus (HIV), even under antiretroviral therapy (ART), or hepatitis C virus (HCV), are major problems to public health. Despite the widespread use of prevention strategies, the numbers of people infected with HIV or HCV continue to increase (1, 2), and even with effective new treatments that reduce viremia, people infected with HIV

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and HCV remain more susceptible to opportunistic infections and cancer (3–5). This indicates that the persistence of the virus, even at low levels, results in an immune dysfunction that prevents the proper control of other pathogens and cancer, yet the details of this immune dysfunction remain poorly understood.

Lymphocytic choriomeningitis virus (LCMV) is commonly used as a model to study acute and chronic viral infections. In adult mice, the LCMV Armstrong (Arm) strain replicates to moderate levels and is cleared in about a week by effective T-cell responses. In contrast, the LCMV clone 13 (CL13) strain, a mutant variant of Arm, causes chronic infection (6). It has been shown that CL13 replicates to high levels in dendritic cells (DCs), causing the functional exhaustion and/or deletion of virus-specific T cells. The consequence of a lack of virus control by the T cells is viral persistence (7). Virus is found in serum and other tissues at variable levels as late as 80 days postinfection (dpi) or more and is found indefinitely in the kidneys and the central nervous system (8–15). Of note, virus-specific T-cell exhaustion (7, 16, 17) also occurs during HIV and HCV infection and cancer progression in humans (18, 19), demonstrating the translation usefulness of the CL13 model.

The orthopoxvirus ectromelia virus (ECTV) is a natural pathogen of the laboratory mouse whose biological route of entry is through microabrasions of the skin, mostly in the footpad, from which it disseminates systemically and infects the liver and the spleen (20). After natural infection or experimental inoculation in the footpad, the outcome of ECTV infection is genetically controlled. Susceptible strains of mice, such as BALB/c and DBA/2 mice, succumb to mousepox (the mouse homolog of human smallpox) due to uncontrolled viral replication in the liver, while resistant strains, such as C57BL/6 (B6) mice, survive with almost no signs of disease because they delay virus spread and better control virus replication in the liver and spleen (20, 21). Many immune mechanisms contribute to this resistance, including natural killer (NK) cells and T cells (21), yet the effect of chronic viral infection on the resistance of B6 mice to ECTV has not been studied.

NK cells are lymphocytes of the innate immune system that serve as a first line of defense against certain viral infections and tumors (22–27). For example, NK cell-deficient humans become sick or succumb to normally non-life-threatening infections with human cytomegalovirus (HCMV) or varicella-zoster virus (28, 29). In addition, B6 mice, which are normally resistant to mouse cytomegalovirus (MCMV) and ECTV, become susceptible when NK cells are depleted (30–32).

After maturing in the bone marrow (BM), NK cells enter the circulation and migrate to lymphoid and nonlymphoid tissues, where they may be present at relatively low numbers, acting as early sentinels of infection. During infection, additional NK cells are recruited to tissues from the circulation and become activated by cytokines and/or by virus-induced changes in infected cells, such as the downregulation of major histocompatibility complex (MHC) class I (MHC-I) molecules and/or the upregulation of NK cell-activating ligands (33). Activation triggers NK cell effector functions, such as the production of interferon gamma (IFN-γ) and the ability to kill virus-infected cells, by releasing granules that contain perforin and granzymes, such as granzyme B (GzmB) (25, 34–37), which help control viruses. In addition to their role in virus control, NK cells are also important for the control of many tumors (22, 37–40).

NK cells develop in the BM from a common lymphoid progenitor (CLP). As CLPs develop into NK cell-committed progenitors (NKPs), they express the receptor β chain for interleukin-15 (IL-15), a cytokine that is essential for NK cell maturation, and the tumor necrosis family receptor CD27, a marker of NK cell intermediate maturation (41–43). NKPs continue to mature into NK cells by first expressing the activating receptor NK1.1 (NK1.1$^+$), followed by NKp46 and then the integrin CD49b and the Ly49 family of inhibitory and activating receptors (43, 44). These NK cells, which are CD27$^+$ CD11b$^-$ and which are referred to as R1 NK cells (41), do not have effector functions and express high levels of the chemokine receptor CXCR3. R1 NK cells further mature to express the integrin CD11b and intermediate levels of CXCR3 to become R2 NK cells (CD27$^+$ CD11b$^+$) which already have a functional capacity,

mainly the ability to produce IFN-γ (41). Finally, R2 NK cells lose the surface expression of CD27 and CXCR3 to become fully mature R3 NK cells ($CD27^-$ $CD11b^+$), which have the strongest cytolytic capacity and which acquire the inhibitory receptor KLRG1 when mature; hence, they have a slower turnover (45). Of note, R3 NK cells are important for the control of ECTV infection and the survival of B6 mice after ECTV infection (26).

It has been shown that during the acute phase of Arm and CL13 infection, NK cells are more mature in infected mice than in uninfected controls, as determined by an increase in the frequencies of $CD11b^+$ $KLRG1^+$ cells. At this stage, NK cells are also more cytolytic *in vitro*, with a corresponding increase in the production of GzmB (46, 47). It has also been shown that soon after Arm is cleared and when infection with CL13 becomes chronic, the frequencies of NK cells and their expression of GzmB decrease (47), yet the long-term impact of cleared acute Arm infection and chronic CL13 infection in the development of NK cells and their ability to respond to other infections has not been fully investigated.

In this report, we show that ECTV is mostly lethal to mice chronically infected with CL13 but not to mice that have recovered from Arm infection. We also show that mice recovered from Arm infection and those chronically infected with CL13 have reduced frequencies of NK cells in several organs. Notably, mice that have recovered from Arm infection have NK cells that mature normally, proliferate, become activated, and increase their cytotoxicity *in vivo* in response to ECTV infection. On the contrary, NK cells in mice chronically infected with CL13 are immature, have a phenotype consistent with intermediate activation, and do not proliferate, become more activated, or increase their cytotoxicity *in vivo* following ECTV infection. Thus, in addition to T-cell exhaustion, long-term exposure to the chronically infected environment promotes an NK cell dysfunction that, together with CD8 T-cell dysfunction (78), can potentially contribute to the high susceptibility of CL13-infected mice to lethal mousepox.

RESULTS

Chronic CL13 infection causes susceptibility to mousepox. It is well established that young B6 mice are resistant to mousepox, and the L. J. Sigal laboratory has been working with ECTV for many years. With an interest in determining whether chronic infection or convalescence from infection with an unrelated virus affects resistance to mousepox, we established the models of chronic infection with LCMV CL13 and acute infection with LCMV Arm in the L. J. Sigal laboratory. To determine whether these models perform as expected in our hands, we first infected mice with CL13 and Arm and determined the presence of infectious virus in their kidneys using a plaque assay. We found that most mice infected with CL13 (CL13 mice) but none of those infected with Arm (Arm mice) had infectious virus in their kidneys at 8, 15, and also 35 days postinfection (dpi). On the other hand, infectious virus was not detected at any of these time points in Arm mice (Fig. 1A). Thus, in our hands, at 35 dpi, CL13 mice were chronically infected with LCMV, while Arm mice were convalescent. As expected, when previously naive (∅) B6 mice were infected with ECTV in the footpad (∅+ECTV mice), they survived, and most mice convalescent from Arm infection and infected with ECTV (Arm+ECTV mice) also survived. On the other hand, most CL13 mice infected with ECTV at 30 dpi with LCMV (CL13+ECTV mice) succumbed to mousepox at 9 to 11 dpi with ECTV (Fig. 1B), with comparatively high ECTV titers being found in the spleen (Fig. 1C). Thus, chronic CL13 infection renders B6 mice susceptible to mousepox, while convalescence from Arm infection results in an intermediate phenotype.

Chronic CL13 infection causes altered anti-ECTV NK cell responses and reduced ECTV loads in the dLN. We and others have shown that B6 mice deficient in NK cells succumb to mousepox at 9 to 11 dpi (31, 32). We have also shown that at 2 to 3 dpi with ECTV, NK cells are recruited to the draining lymph node (dLN) of B6 mice and become activated to control systemic ECTV spread (31, 32). Thus, we determined whether the susceptibility of CL13+ECTV mice to mousepox correlated with deficient NK cell

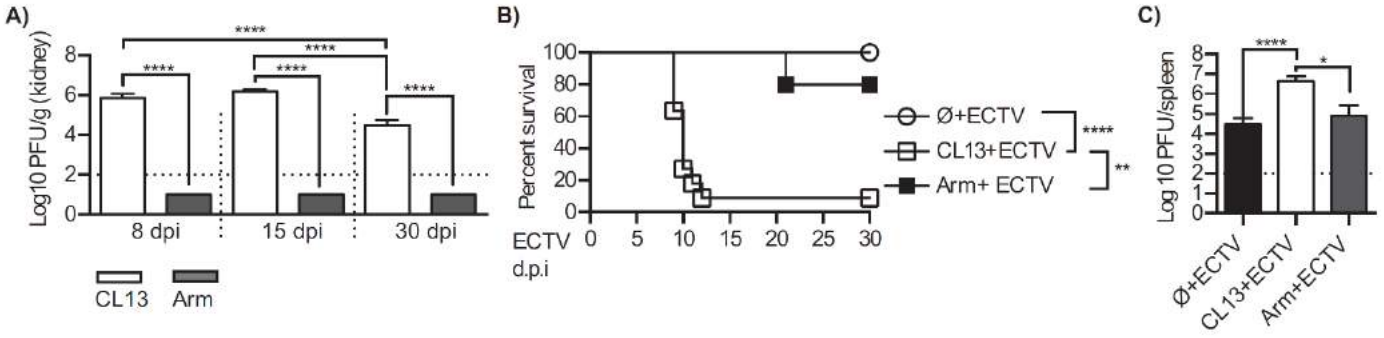


FIG 1 Chronic CL13 infection causes susceptibility to mousepox. (A) LCMV titers in the kidneys of B6 mice at 8, 15, and 35 dpi with CL13 or Arm, as determined by plaque assay. (B) Survival of the indicated mice following ECTV infection with 5 to 10 mice per group. The results are representative of those from at least 3 independent experiments. (C) ECTV titers, determined by plaque assays, in the spleen following 7 dpi with ECTV. Each graph displays data pooled from at least 2 similar and independent experiments with 5 to 10 mice per group, with the data being shown as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

responses and/or virus control in the dLN. At 30 dpi, ∅, CL13, and Arm mice were infected in the footpad with ECTV, and NK cells in the dLN and in the nondraining LN (ndLN; the popliteal LN of the uninfected limb) were analyzed 2.5 days later (32.5 dpi with LCMV, 2.5 dpi with ECTV). In the ndLN, the frequency and total numbers of NK cells and the frequency of NK cells expressing the effector molecules interferon gamma (IFN-γ) and granzyme B (GzmB) were not different between ∅+ECTV, CL13+ECTV, and Arm+ECTV mice (Fig. 2A to D). Compared to the findings for the ndLNs, the dLNs of ∅+ECTV and Arm+ECTV mice had increased frequencies and numbers of NK cells (Fig. 2B), indicating NK cell recruitment. Arm+ECTV mice recruited significantly fewer NK cells to the dLNs than ∅+ECTV mice, suggesting some impairment. On the other hand, there was no increase in the frequency or total numbers of NK cells in CL13+ECTV mice (Fig. 2B). The frequencies of IFN-γ-expressing (IFN-γ$^+$) (Fig. 2C) and GzmB-expressing (GzmB$^+$) (Fig. 2D) NK cells were increased in the dLNs of ∅+ECTV, CL13+ECTV, and Arm+ECTV mice compared to those in their ndLNs; however, CL13+ECTV mice had a significantly higher frequency of IFN-γ$^+$ NK cells and a significantly lower frequency of GzmB$^+$ NK cells than ∅+ECTV and Arm+ECTV mice, yet when ECTV titers were determined at 2.5 dpi with ECTV, CL13+ECTV mice had significantly lower ECTV titers in the popliteal draining lymph nodes (dLNs) than ∅+ECTV and Arm+ECTV mice, suggesting that the inflammation induced by chronic infection or the IFN-γ produced by the NK cells contributed to the initial control of ECTV in the footpad or the dLNs (Fig. 2E). Thus, these experiments were inconclusive because it was not possible to distinguish if NK cells in CL13+ECTV mice were intrinsically different or whether their anti-ECTV response was different because the ECTV titers in their dLNs were lower.

LCMV infection decreases NK cell frequency and numbers for an extended time. In addition to the dLN response, we and others have previously shown that at 5 dpi with ECTV, NK cells proliferate and become activated in the spleens and livers of B6 mice, helping control ECTV until the T-cell response becomes sufficiently strong (31, 32). Thus, we hypothesized that LCMV could be affecting NK cells and their response to ECTV in the spleen. ECTV titers in the spleen at 5 dpi with ECTV were similar in ∅+ECTV and CL13+ECTV mice and somewhat elevated in Arm+ECTV mice (Fig. 3A), indicating similar early systemic spread in ∅+ECTV and CL13+ECTV mice, despite early reduced loads in the dLNs of CL13+ECTV mice. Thus, we next focused on the frequency and the total number of NK cells in the spleens of mice infected only with LCMV or with LCMV and ECTV. The frequencies and total numbers of NK cells in the spleen were reduced at 8, 15, and 35 dpi in CL13 mice and at 15 and 35 dpi in Arm mice compared to those in the spleens of ∅ mice. This indicates that LCMV infection, whether it is cleared or not, results in a long-term loss of NK cells and could explain why CL13+ECTV and Arm+ECTV mice recruited fewer NK cells to the dLN. Of note, the frequency of NK cells was also reduced in the livers and BM of CL13 and Arm mice at 35 dpi (but was

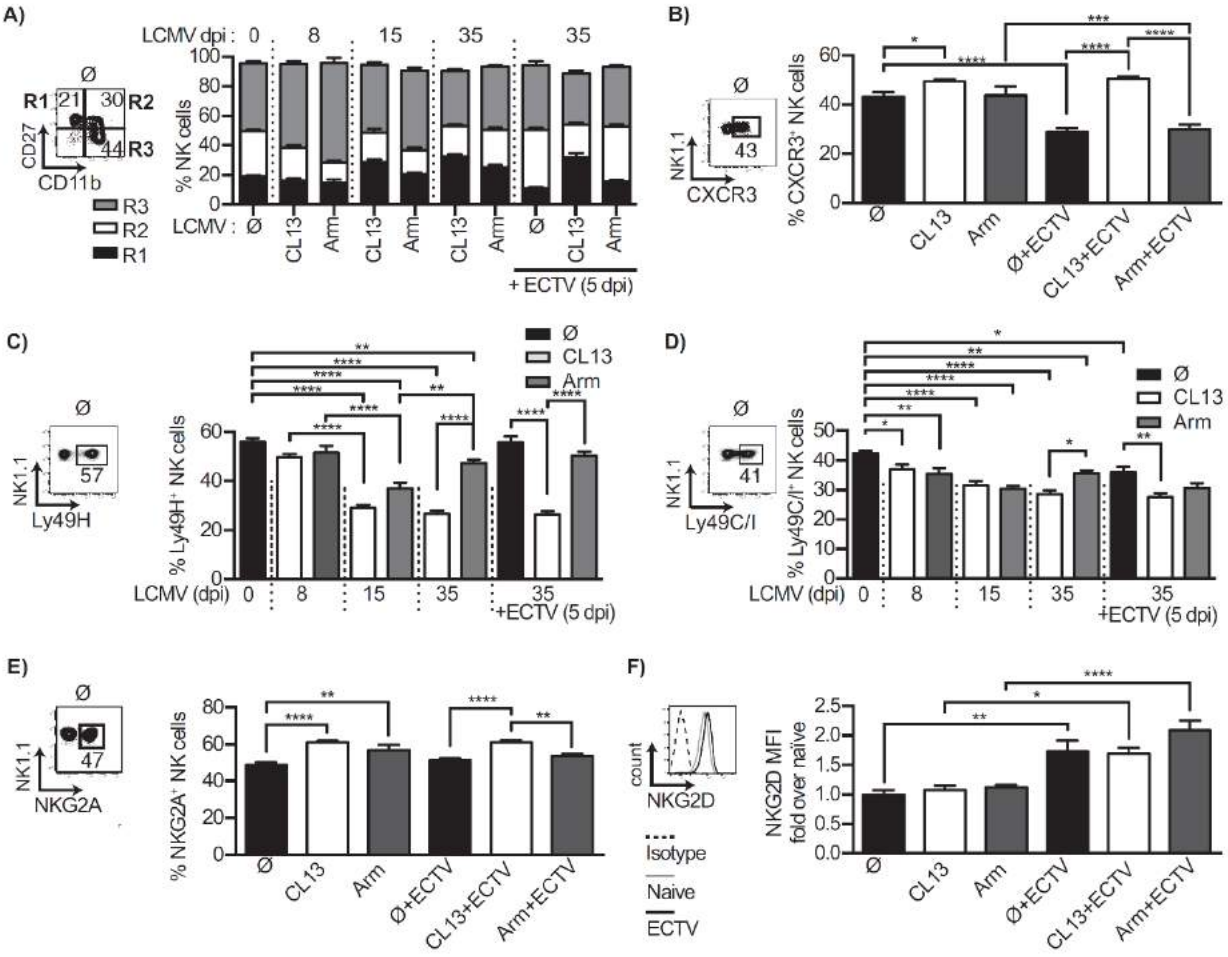


FIG 4 Chronic CL13 infection impairs NK cell maturation. (A) NK cells (TCR-β^-, NK1.1$^+$) in the spleen at the indicated time points according to their expression of CD11b and CD27, with the frequencies of the R1 (CD11b$^-$ CD27$^+$), R2 (CD11b$^+$ CD27$^+$), and R3 (CD11b$^+$ CD27$^-$) NK cell subpopulations being shown. (B) NK cells in the spleen according to their expression of CXCR3. (C) As described in the legend to panel B but for Ly49H expression. (D) As described in the legend to panel B but for Ly49C/I expression. (E) As described in the legend to panel B but for NKG2A expression. (F) MFI of NKG2D in NK cells in the spleens of the indicated mice. All data were collected at 5 dpi with ECTV or at the indicated time points for LCMV infection. The graphs display the results of at least 2 similar and independent experiments, each with 6 to 10 mice per group. Data are shown as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

to the findings for CL13 and Arm mice, CL13+ECTV and Arm+ECTV mice had a significant reduction in the frequency and/or absolute number of NK cells in the spleen (Fig. 3B). Hence, the loss of NK cells caused by LCMV was maintained upon unrelated ECTV infection.

Chronic CL13 infection impairs NK cell maturation. Given the findings that NK cells are reduced in numbers in the spleens of CL13, Arm, CL13+ECTV, and Arm+ECTV mice but that most CL13+ECTV mice succumb while most Arm+ECTV mice survive, we looked at NK cells in the spleen in more detail. First, we determined the maturation of NK cells in different groups of mice using CD27 and CD11b as markers (Fig. 4A). Compared to the frequency in ∅ mice, CL13 and Arm mice at 8 dpi had an increased frequency of mature R3 NK cells ($P \leq 0.0001$) and a decreased frequency of intermediate R2 NK cells ($P \leq 0.01$ and $P \leq 0.0001$, respectively), with no changes in the frequency of immature R1 NK cells being found between ∅ mice and CL13 and Arm mice. This suggests that acute LCMV infection induces R2 NK cell maturation. However, at 15 and 35 dpi with LCMV, CL13 mice had an increased frequency of R1 NK cells ($P \leq 0.01$) and a decreased frequency of R2 NK cells compared to those in ∅ mice ($P \leq 0.001$ and $P \leq 0.0001$, respectively). The frequency of mature R3 NK cells was also reduced at 35 dpi ($P \leq 0.01$). In contrast, in Arm mice the frequency of R2 and R3 NK cells at 15 dpi with LCMV remained decreased ($P \leq 0.0001$) and increased ($P \leq 0.01$),

CL13 mice. In contrast, in ∅+ECTV and Arm+ECTV mice, there was a significant increase in the numbers of GzmB$^+$ CD8 T cells compared to the numbers in ∅ and Arm mice, respectively (Fig. 3A).

Next, we analyzed the expression of CD44, which is present in activated and memory T cells (Fig. 3B). CL13 mice had higher frequencies of CD44$^+$ CD8$^+$ T cells than ∅ and Arm mice. Following ECTV infection, ∅+ECTV and Arm+ECTV mice had increased frequencies of CD44$^+$ CD8$^+$ T cells compared to those in ∅ and Arm mice, respectively. In contrast, the frequency of CD44$^+$ CD8$^+$ T cells was not increased in CL13+ECTV mice compared to that in CL13 mice.

We also examined the expression of CD62L, which is rapidly downregulated in activated T cells. CL13 mice and, to lesser extent, Arm mice had a significantly reduced frequency of CD62L$^+$ CD8$^+$ T cells compared to that in ∅ mice. After ECTV infection, ∅+ECTV and Arm+ECTV mice had a lower frequency of CD62L$^+$ CD8$^+$ T cells than ∅ and Arm mice, respectively. In contrast, the frequency of CD62L$^+$ cells in CL13+ECTV mice was not decreased from that in CL13 mice. Together, the data presented above indicate that while CD8$^+$ T cells are activated/exhausted in CL13 mice, they do not get further activated after ECTV infection.

Next, we evaluated the expression of PD1 and KLRG1, two inhibitory receptors that are upregulated in activated T cells (36, 37). It is known that during chronic CL13 infection PD1 persists at high levels in exhausted T cells (38), while KLRG1 is progressively downregulated (36, 39). As expected, we found significantly higher levels of PD1 expression in the CD8$^+$ T cells of CL13 mice than in those of ∅ or Arm mice. After ECTV infection, ∅+ECTV and Arm+ECTV mice had increased frequencies of PD1$^+$ CD8$^+$ T cells compared to those in ∅ and Arm mice, respectively. In contrast, the frequency of PD1$^+$ CD8 T cells was not increased in CL13+ECTV mice compared to that in CL13 mice (Fig. 3D). For KLRG1, we found that CL13 and Arm mice had increased KLRG1 expression compared to that in ∅ mice, likely representing the expression by short-lived effector cells induced by LCMV that persisted during convalescence from the Arm infection. After ECTV infection, ∅+ECTV and Arm+ECTV mice had increased frequencies of KLRG1$^+$ CD8 T cells compared to those in ∅ and Arm mice, respectively. Of note, KLRG1 upregulation mostly occurred in GzmB$^+$ CD8 T cells (not shown). In contrast, the frequency of KLRG1$^+$ CD8 T cells was not increased in CL13+ECTV mice compared to that in CL13 mice (Fig. 3E). Similar profiles were observed for CD4$^+$ T cells with respect to the expression of CD44, CD62L, and PD1 but not with respect to the expression of KLRG1, as the CD4$^+$ T cells did not express KLRG1 (data not shown). Together, these data indicate that, in addition to preventing T-cell proliferation, chronic CL13 infection impairs overall T-cell activation.

Next, we analyzed the relationship between cell cycle progression and effector differentiation in CD8$^+$ T cells by analyzing the expression of GzmB together with the expression of Ki67 or the incorporation of BrdU (Fig. 3F and G). We found that in ∅+ECTV and Arm+ECTV mice the vast majority of Ki67$^+$ CD8$^+$ T cells were effectors because most of the Ki67$^+$ cells (~90%) also expressed GzmB, while only ~50% of the Ki67$^+$ cells in CL13+ECTV mice were GzmB$^+$. Of note, virtually all BrdU$^+$ cells in ∅+ECTV and Arm+ECTV mice and also in CL13+ECTV mice were GzmB$^+$, indicating that in mice chronically infected with CL13, the anti-ECTV CD8$^+$ T-cell response is severely reduced but is not nonexistent.

Impaired expansion of ECTV-specific CD8$^+$ T cells in mice chronically infected with CL13. Our data thus far indicate reduced T-cell proliferation and effector T-cell

FIG 3 Legend (Continued)
mice (right). (B to E) Representative flow cytometry histograms of the mean fluorescence intensity (left) and frequencies of CD44$^+$ (B), CD62L$^+$ (C), PD1$^+$ (D), or KLRG1$^+$ (E) CD8 T cells (right) in the indicated mice. (F and G) Representative flow cytometry dot plots with the mean ± SEM proportion of CD8$^+$ T cells in the spleens of the indicated mice for the expression of GzmB by CD8$^+$ T cells, together with Ki67 (F) or BrdU (G) incorporation, at 3 h following intraperitoneal injection of BrdU. All data were collected at 7 dpi with ECTV in the spleens, and the graphs present the results of at least 3 comparable and independent experiments with an accumulated total of 6 to 12 mice per group. Data are shown as the mean ± SEM. **, $P < 0.01$; ****, $P < 0.0001$.

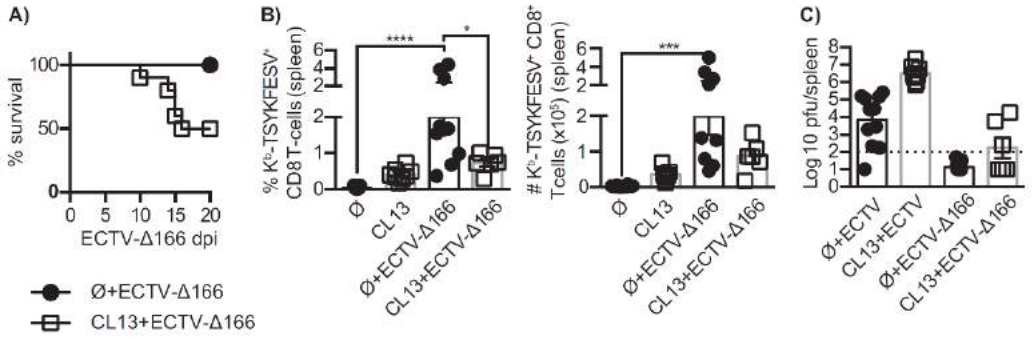


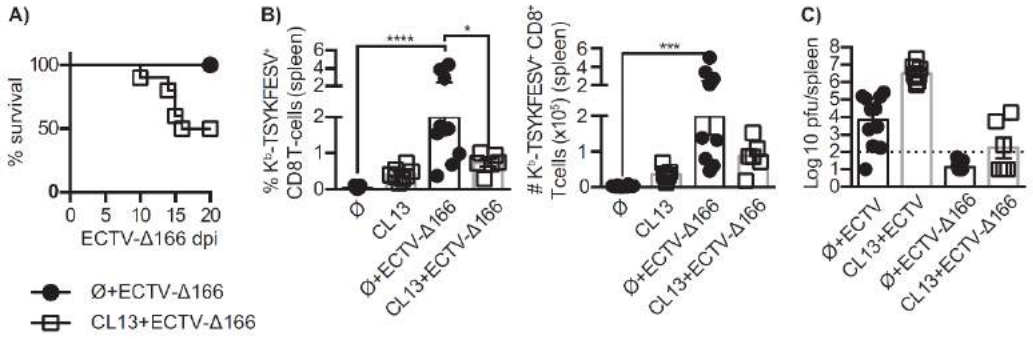
FIG 5 Mice chronically infected with CL13 remain susceptible to highly attenuated ECTV-Δ166. (A) Survival of ∅+ECTV-Δ166 and CL13+ECTV-Δ166 mice. (B) Frequency (left) and absolute numbers (right) of K^b-TSYKFESV-specific $CD8^+$ T cells in the spleens of the indicated mice. (C) ECTV-Δ166 titers at 7 dpi in the spleens of the indicated mice. The dotted line indicates the limit of ECTV detection. Graphs present the results of at least 2 independent and comparable experiments with 6 to 10 mice per group. Data are presented as the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

in ∅+ECTV, CL13+ECTV, and Arm+ECTV mice were $CD44^+$ and PD1 positive ($PD1^+$) (data not shown). However, the frequencies of K^b-TSYKFESV$^+$ $CD8^+$ T cells that were also $GzmB^+$ or $KLRG1^+$ were significantly lower in CL13+ECTV mice than in ∅+ECTV and Arm+ECTV mice. Moreover, in CL13+ECTV mice a significantly higher frequency of K^b-TSYKFESV$^+$ $CD8^+$ T cells remained $CD62L^+$. Thus, chronic CL13 infection drastically reduced the expansion of antigen-specific $CD8^+$ T cells, and those that expanded had a dimmed effector phenotype.

To further test the role of the chronic infection environment on antigen-specific $CD8^+$ T-cell expansion, we transferred 10,000 T-cell receptor (TCR)-transgenic OT-I $CD8^+$ T cells (CD45.1), which are specific for the ovalbumin (OVA) epitope SIINFEKL, into ∅ and CL13 mice (B6-CD45.2) and challenged them with ECTV expressing OVA (ECTV-OVA). Seven days later, we determined the expansion of the OT-I cells in the spleen. We found that, in agreement with the results of experiments with polyclonal T cells presented in Fig. 2, OT-I cells expanded 5-fold more in ∅ mice challenged with ECTV-OVA (∅+ECTV-OVA mice) than in CL13 mice challenged with ECTV-OVA mice (CL13+ECTV-OVA mice) (Fig. 4C). Moreover, the virus loads were not reduced in CL13+ECTV-OVA mice to which OT-I cells were transferred compared to those in CL13+ECTV-OVA mice (Fig. 4D). These data confirm that the CL13 chronic infection environment reduces the ability of antigen-specific $CD8^+$ T cells to expand and that the few expanded cells do not provide antiviral protection.

Mice chronically infected with CL13 remain susceptible to highly attenuated ECTV-Δ166, suggesting altered IFN-I production and/or sensing. WT ECTV is extremely virulent: the dose that kills 50% of genetically susceptible BALB/c mice (the 50% lethal dose [LD_{50}]) is ~1 PFU (29). Given that CL13+ECTV mice generated mild T-cell responses to WT ECTV, it was of interest to determine whether they could control highly attenuated ECTV mutants. The gene EVM166 encodes a type I interferon (IFN-I) decoy receptor in ECTV. We have previously shown that ECTV-Δ166 was not lethal for BALB/c mice at doses as high as 5×10^7 PFU (29). However, it is fully lethal to mice deficient in the IFN-I receptor (29). When CL13 mice were infected with 3,000 PFU of the ECTV-Δ166 mutant (CL13+ECTV-Δ166 mice), 50% succumbed (Fig. 5A) and mounted a variable small or absent K^b-TSYKFESV-specific CD8 T-cell response (Fig. 5B), and in contrast to ∅ mice infected with ECTV-Δ166 (∅+ECTV-Δ166 mice), CL13+ECTV-Δ166 mice had virus in their spleens at 7 dpi (Fig. 5C), indicating a surprisingly poor attenuation of ECTV-Δ166 in CL13 mice and suggesting that chronic CL13 infection results in inefficient IFN-I production and/or sensing.

Mice chronically infected with CL13 resist ECTV-Δ036 and mount an immune response that is protective against WT ECTV challenge. Next, we tested survival in response to infection with ECTV deficient in EVM036 (ECTV-Δ036), which encodes a protein required for the formation of mature enveloped virions and viral plaques and



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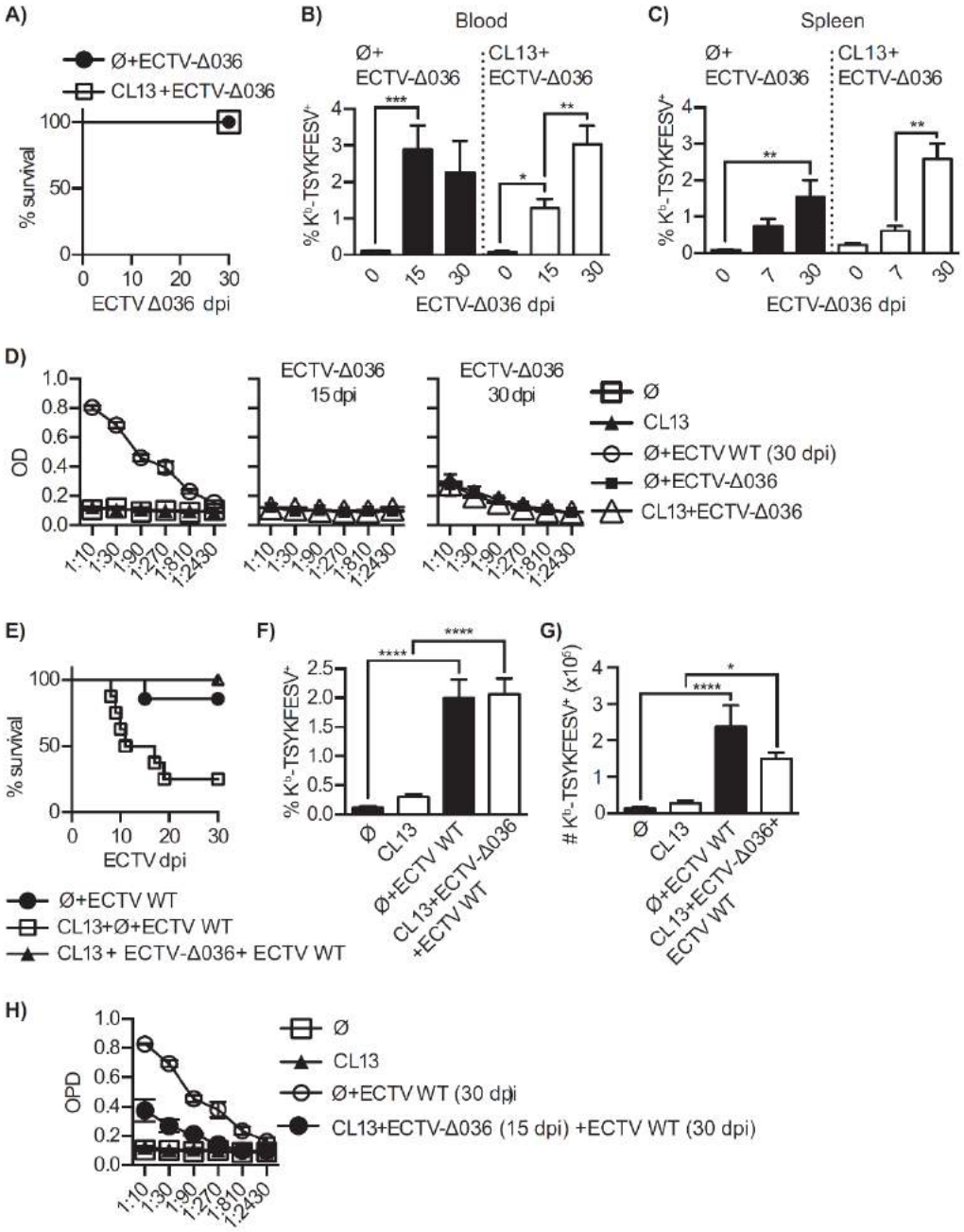

FIG 6 Mice chronically infected with CL13 resist ECTV-Δ036 and mount an immune response that is protective against WT ECTV challenge. (A) Survival of the indicated mice. (B) Frequency of K^b-TSYKFESV-specific $CD8^+$ T cells in the blood of the indicated mice on the indicated days after ECTV-Δ036 infection. (C) As for panel B but in the spleen. (D) ECTV-specific total IgG in the blood of the indicated mice, represented as the optical density (OD) at 450 nm. All ELISAs were performed simultaneously, and data are representative of those from at least 2 independent and similar experiments. (E) Survival of the indicated mice. (F) Frequency of K^b-TSYKFESV-specific $CD8^+$ T cells in the spleen at 30 dpi with WT ECTV in the indicated mice. (G) As for panel F but displaying the calculated absolute numbers. (H) ECTV-specific total IgG in the blood of the indicated mice. ELISAs were performed together with those for panel D, and the results for ∅ mice infected with WT ECTV are the same as those shown in panel D and are shown for comparison. The graphs present the results of at least 2 independent and comparable experiments with 6 to 10 mice per group. Data are presented as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

for efficient systemic virus spread from the footpad and which is not lethal at 5×10^7 PFU in BALB/c mice but which is lethal to SCID mice, which are deficient in adaptive immunity (40). We found that, like ∅ mice, CL13 mice survived ECTV-Δ036 challenge (Fig. 6A), with no signs of disease or detectable virus in the spleen being seen at 7 or 30 dpi (data not shown).

Given that mice chronically infected with CL13 survived ECTV-Δ036 challenge with no signs of disease, we tested whether they generated an anti-ECTV immune response. Notably, compared to ∅ mice, ∅ mice challenged with ECTV-Δ036 (∅+ECTV-Δ036 mice) and CL13 mice challenged with ECTV-Δ036 (CL13+ECTV-Δ036 mice) had significantly increased frequencies of K^b-TSIKFESV$^+$ CD8$^+$ T cells in blood at 15 and 30 dpi (Fig. 6B) and in the spleen at 7 and 30 dpi (Fig. 6C) with ECTV-Δ036. On the other hand, ∅+ECTV-Δ036 and CL13+ECTV-Δ036 mice had low but detectable levels of anti-ECTV IgG in their sera at 30 dpi but not at 15 dpi with ECTV-Δ036 (Fig. 6D).

Given that CL13+ECTV-Δ036 mice survived infection and mounted an immune response, we next tested whether this response could be sufficient for protection against WT ECTV challenge. Thus, we infected ∅, CL13, and CL13+ECTV-Δ036 mice with WT ECTV at 15 dpi with ECTV-Δ036. Strikingly, all CL13+ECTV-Δ036 mice infected with WT ECTV (CL13+ECTV-Δ036+ECTV-WT mice) survived infection, whereas most CL13 mice infected with WT ECTV succumbed and most ∅+ECTV mice survived (Fig. 6E). When their CD8$^+$ T-cell responses were analyzed at 30 dpi with WT ECTV, CL13+ECTV-Δ036+ECTV-WT mice had frequencies (Fig. 6F) and numbers (Fig. 6G) of K^b-TSYKFESV$^+$ CD8 T cells similar to those in ∅ mice infected with WT ECTV, had IgG specific to ECTV in their sera (Fig. 6H), and had no detectable ECTV titers in the spleen or liver (data not shown). Because the challenge with WT ECTV was done at 15 dpi with ECTV-Δ036 and at this time K^b-TSYKFESV$^+$ CD8$^+$ T cells but not anti-ECTV IgG was detected in blood, these data strongly suggest that mice chronically infected with CL13 mount a CD8$^+$ T-cell response to a highly attenuated ECTV mutant that can protect them from subsequent challenge with virulent ECTV.

DISCUSSION

It is well established that, in addition to viral persistence, chronic infection with LCMV causes immune suppression, as revealed by impaired immune responses to other infectious agents. For example, chronic infection with LCMV causes defective antibody (Ab) responses and increased lethality to vesicular stomatitis virus (VSV) and decreased T-cell responses to vaccinia virus (VACV) after intravenous (i.v.) inoculation at high doses. However, chronic LCMV infection does not increase VSV lethality following footpad infection (23, 41). Moreover, VSV and VACV are not natural mouse pathogens and the i.v. route of inoculation bypasses all the barriers imposed on the virus during natural entry. It has also been shown that chronic infection with CL13 also results in increased lethality following i.v. infection with *Histoplasma capsulatum* (20), reduced CD8 T-cell responses to influenza A virus (IAV), VACV, and herpes simplex virus (HSV) (42), and defective IFN-I and NK cell responses to mouse cytomegalovirus (MCMV) (22). Some proposed mechanisms for the immunosuppression caused by CL13 are the killing of dendritic cells (DCs) and impaired antigen presentation (23, 43), the infection of fibroblastic reticular cells in secondary lymphoid organs (44), quantitative and qualitative defects of plasmacytoid DCs (pDCs) (22), and, more recently, an impaired Fc gamma receptor-mediated Ab effector function (45), yet the effect of chronic infection on intrinsic and acquired resistance to a highly pathogenic host-specific virus has not been thoroughly studied, and an in-depth analysis of the T-cell response has not been performed.

We have recently shown that B6 mice chronically infected with CL13 lose their natural resistance to mousepox following ECTV infection in the footpad, while mice convalescent from Arm infection are mostly resistant. We have also shown that CL13 and Arm mice have reduced numbers of NK cells, but only CL13 mice have NK cells with maturational defects. Moreover, we have demonstrated that, different from the findings for NK cells from ∅+ECTV and Arm+ECTV mice, NK cells do not respond to ECTV in the spleen or liver of CL13+ECTV mice at 5 dpi, yet the NK cell defect is not the single culprit for the susceptibility of CL13+ECTV mice to lethal mousepox because the adoptive transfer of NK cells from naive mice does not restore resistance to mousepox (35). Given that CD8$^+$ and CD4$^+$ T-cell responses are critical for ECTV control (33, 46), it was important to test whether defective T-cell responses during chronic LCMV infection could also contribute to the loss of resistance to mousepox.

In this study, we first confirmed that CL13+ECTV mice are susceptible to mousepox and that Arm+ECTV mice are mostly resistant and then thoroughly compared the anti-ECTV T-cell responses in ∅+ECTV, CL13+ECTV, and Arm+ECTV mice, with a particular emphasis on $CD8^+$ T cells. Our data show that at 7 dpi with ECTV, $CD8^+$ and $CD4^+$ T cells prominently expanded in the spleens of ∅+ECTV and Arm+ECTV mice but not in those of CL13+ECTV mice. We also showed that the failure of $CD4^+$ and $CD8^+$ T cells to expand properly in CL13+ECTV mice was due to their inability to enter the cell cycle, with many more cells being in G_0 phase (Ki67-negative cells) in CL13+ECTV mice than in ∅+ECTV and Arm+ECTV mice. Also, a much lower proportion of the $Ki67^+$ cells in CL13+ECTV mice than those in ∅+ECTV and Arm+ECTV mice were $BrdU^+$ (i.e., synthesized DNA), indicating that the rate of proliferation of the cycling cells was much lower in CL13+ECTV mice. Of note, before ECTV infection, both CL13 and Arm mice had increased frequencies of apparently slowly proliferating T cells or T cells stuck in G_2 phase ($Ki67^+$ $BrdU^-$), likely representing LCMV-specific cells. Despite this similitude, after ECTV infection, T cells strongly increased their proliferation in Arm mice that had recovered from infection but very poorly in mice chronically infected with CL13.

Our data also indicate that the failure of $CD8^+$ and $CD4^+$ T cells to expand in response to ECTV in CL13+ECTV mice was likely due to T-cell-extrinsic factors, as $CD8^+$ and $CD4^+$ T cells from ∅ mice proliferated much less in CL13+ECTV mice than in ∅+ECTV mice. This is consistent with previous reports indicating inefficient antigen presentation (23, 43), excessive interleukin-10 (IL-10) production (9, 47), or an altered IFN-I response (10, 48–51). Of note, while there was some proliferation of the transferred T cells in CL13+ECTV mice, they did not control the virus, even though the number of $CD8^+$ T cells transferred was sufficient to rescue $CD8^+$ T-cell-deficient ($CD8^{-/-}$) mice from mousepox (33).

Consistent with their expansion and proliferation, GzmB, CD44, CD62L, PD1, and KLRG1 staining showed that large numbers of bulk $CD8^+$ and $CD4^+$ T cells differentiated into effectors in ∅+ECTV and Arm+ECTV mice but not in CL13+ECTV mice, further emphasizing their defective response. Of note, combining differentiation into $GzmB^+$ cells with Ki67 and BrdU staining indicated that the differentiation of $CD8^+$ T cells into effector cells in CL13+ECTV mice was greatly diminished but not nil.

In addition to analyzing bulk $CD8^+$ and $CD4^+$ T-cell responses, we also examined the response to the K^b-restricted immunodominant epitope TSYKFESV and found that it was somewhat decreased in Arm+ECTV mice compared to its level in ∅+ECTV mice and severely curtailed, but not nil, in CL13+ECTV mice. Notably, in addition to their numbers being decreased, the few K^b- $TSYKFESV^+$ $CD8^+$ T cells found in CL13+ECTV mice did not properly differentiate to full-fledged effectors. On the other hand, while Arm+ECTV mice had a reduction in the numbers of K^b-$TSYKFESV^+$ $CD8^+$ T cells, their differentiation into effectors was normal. Of note, the bulk of the $CD8^+$ T-cell response between ∅+ECTV and Arm+ECTV mice was similar. While the reason for the decreased number of K^b-TSYKFESV-specific $CD8^+$ T cells in Arm+ECTV mice is not clear, we speculate that it may be due to a shift in immunodominance to another ECTV epitope cross-reactive with one in LCMV, as suggested by reports showing that LCMV and VACV have cross-reactive epitopes (52, 53). The adoptive transfer of SIINFEKL-specific OT-I CD8 T cells and infection with ECTV-OVA confirmed the reduced but not nil expansion of antigen-specific $CD8^+$ T cells and the negative effect of the chronic infection environment in CL13+ECTV mice.

Because the results of our experiments indicate that the reason for the inability of T cells to respond in CL13+ECTV mice is cell extrinsic, we tested the effects of blocking PD1 and TIGIT, which are inhibitory and which have been implicated in the T-cell exhaustion and chronicity of CL13 (38, 54, 55). However, we did not observe any improvement in survival (data not shown). Also, the adoptive transfer of splenocytes deficient in the receptor for IL-10, an immunosuppressive cytokine also shown to participate in the persistence of CL13 (9, 56, 57), conferred only a slight increase in the life span but did not confer an increase in survival in response to mousepox in CL13+ECTV mice (data not shown).

Given the high susceptibility of CL13 mice to mousepox, it was of interest to determine whether they were also susceptible to highly attenuated ECTV mutants. One important mutant to test was ECTV-Δ166, which lacks a decoy receptor that inactivates mouse alpha interferon (IFN-α) but not IFN-β (29, 58). Of note, the role of IFN-I during CL13 infection is complex. IFN-I is important for the maintenance of chronic LCMV infection and T-cell dysfunction, because treatment with Abs to IFNAR accelerates recovery from chronic CL13 infection (10, 59). The main culprit is IFN-β and not IFN-α because Abs to IFN-β but not Abs to IFN-α protected CL13-infected mice from splenic disorganization, reduced DC infection, increased $CD8^+$ T-cell responses, and accelerated CL13 clearance (60), yet CL13 still establishes chronic infection with enhanced titers in mice deficient in the interferon receptor (*Ifnar1*$^{-/-}$ mice) and in IFN-β (57, 61). Moreover, IFN-I is barely detectable in CL13 mice after the acute phase, and CpG treatment induces IFN-I in ∅ mice but not in CL13 mice (22). Furthermore, treatment of CL13-infected mice at 3 and 5 dpi with a mixture of IFN-α and IFN-β enhances the anti-CL13 $CD8^+$ T-cell response (62). For ECTV, IFN-α is critical for resistance to mousepox (29, 63, 64), but IFN-β is not because IFN-β-deficient mice are resistant to mousepox (not shown). Notably, 50% of CL13+ECTV-Δ166 mice succumbed to mousepox, suggesting poor IFN-α production or signaling in CL13+ECTV mice. This will be an important avenue of future exploration.

It was also of interest to test ECTV-Δ036, which is as attenuated as ECTV-Δ166, but the reason for its attenuation is its deficient spread. Of note, despite its attenuation, ECTV-Δ036 induces an immune response in BALB/c mice (40). Notably, CL13+ECTV-Δ036 mice were fully resistant to mousepox and eliminated ECTV-Δ036. Moreover, they mounted a relatively strong K^b-TSYKFESV-specific $CD8^+$ T-cell response that was comparable to that in ∅+ECTV-Δ036 mice at 7, 15, and 30 dpi. The Ab response to ECTV-Δ036 was also similar in ∅+ECTV-Δ036 and CL13+ECTV-Δ036 mice, but it was relatively weak and still not detectable at 15 dpi. Notably, CL13+ECTV-Δ036 mice challenged with WT ECTV were resistant to mousepox, maintained anti-ECTV $CD8^+$ T cells and Abs, and eliminated WT ECTV. These results indicate that while chronic infection results in profound immunosuppression, resistance to some, but not all, types of attenuated viruses is possible. Moreover, the data also indicate that protective immunization is achievable in highly immunosuppressed individuals, provided that an adequate stimulus is used. We have previously shown that mice susceptible to mousepox can be protected by preexisting Abs or memory $CD8^+$ T cells (65–67). Our results suggest that the mechanism of protection by ECTV-Δ036 is through $CD8^+$ T cells because at the time of WT ECTV challenge (15 dpi with ECTV-Δ036), CL13+ECTV-Δ036 mice had already mounted a $CD8^+$ T-cell response but not an Ab response. However, to reach a definitive conclusion, this hypothesis will have to be thoroughly tested. Unfortunately, a deficiency or depletion of CD8 T cells or B cells in Ab- or $CD8^+$ T-cell-immune mice results in susceptibility to mousepox. Thus, future experiments will require T-cell- or Ab-specific immunization, which could be challenging in the context of chronic infection.

In summary, our data indicate that despite increased basal activation levels, chronic infection with CL13 results in strongly impaired $CD8^+$ T-cell responses to ECTV and susceptibility to mousepox, yet immunization with an attenuated virus deficient in spread induces an immune response that prevents mousepox and eliminates WT ECTV. Together, these results highlight some of the mechanisms involved in immunosuppression during chronic viral infection, provide evidence for further avenues of exploration, and demonstrate that protective immunization against highly pathogenic viruses of individuals immunosuppressed by viral infection is possible, provided that the appropriate immunogen is used.

MATERIALS AND METHODS

Mice. All experiments were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC). C57BL/6 (B6-CD45.2$^+$) and NCI B6-Ly5.1/Cr (B6-CD45.1$^+$) mice were purchased from Charles River or bred in-house from breeders from Charles River. B6-CD45.1/2 mice were F1 hybrids from B6 and B6-CD45.1 mice. OT-I mice (Jackson) were bred with *Rag*$^{-/-}$ mice (The Jackson

Laboratory) and then with B6 Thy1.1 mice (The Jackson Laboratory) and were thereafter bred in-house. Males and females 6 to 10 of age weeks were used. After infection, the animals were observed for signs of disease and distress (lethargy, ruffled hair, weight loss, skin rash, and eye secretions) and euthanized if they were unresponsive to touch or without voluntary movements.

Viruses. LCMV CL13 and LCMV Arm were gifted by E. John Wherry (University of Pennsylvania, Philadelphia, PA) and propagated, and their titers were determined as described previously (7, 11). ECTV Moscow was propagated and titers were determined as described previously (17, 18). LCMV CL13 infections were performed intravenously with 2×10^6 PFU, and LCMV Arm infections were performed intraperitoneally with 2×10^5 PFU. ECTV, ECTV-Δ166, and ECTV-Δ36 infections were performed in the footpad with 3.0×10^3 PFU.

Flow cytometry. Flow cytometry was performed as previously described (17, 18). We used the following Abs: anti-CD45.1 (clone A20, phycoerythrin [PE]-Cy7; BioLegend), anti-CD45.2 (clone 104, allophycocyanin [APC]; BioLegend), anti-BrdU (clone PRB-1, fluorescein isothiocyanate [FITC]; eBioscience), anti-T-cell receptor beta (anti-TCR-β; clone H57-597 [Brilliant Violet 605 {BV605}; BioLegend] and clone H57-597 [BV786; BD]), anti-CD8 (clone 53-6.7, BV711 or peridinin chlorophyll protein-Cy5.5; BioLegend,), anti-CD4 (clone RM4-5, BV650 or BV785; BioLegend), anti-KLRG1 (clone 2F1/KLRG1, PE-Cy7 or APC; BioLegend), anti-granzyme B (clone GB11, Pacific Blue; BioLegend), anti-IFN-γ (clone XMG1.2, PE-Cy7; BioLegend), anti-CD44 (clone IM7, Brilliant Ultraviolet 395 [BUV395]; BD), anti-CD62L (clone MEL-14, FITC or PE-Cy7; BioLegend), and anti-PD1 (clone 29F.1A12, FITC; BioLegend). For TSYKFESV staining, we conjugated 7.5 μg of the TSYKFESV peptide (GenScript) with 20 μl of the K^b dimer (BD Dimer X) in 72.5 μl of phosphate-buffered saline (PBS) overnight at 37°C and on the following day incubated the solution with 20 μl of PE-conjugated IgG1 (clone RMG1-1; BioLegend) at room temperature for 1 h. Briefly, all organs were made into single-cell suspensions. Red blood cells were lysed with 0.84% NH_4Cl, washed in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, brefeldin A (20 μg/ml; Sigma-Aldrich), and 2 μg/100 μl of TSYKFESV peptide (GenScript), and resuspended at 2.0×10^6 cells/100 μl. After 5 h of incubation, Ab 2.4G2 (anti-Fc gamma II/III receptor; American Type Culture Collection) was added together with the stains for the extracellular molecules. After 25 min or after 60 min, if staining for TSYKFESV was also performed, the cells were fixed, permeabilized, and stained for 30 min for the cytoplasmic or nuclear molecules (with a BD Cytofix/Cytoperm kit and an Invitrogen Fixation/Permeabilization kit, respectively) according to the manufacturers' instructions. Data were acquired with a BD LSRFortessa cytometer and analyzed with FlowJo software (v10; TreeStar).

BrdU incorporation assay. We intraperitoneally injected mice with 2 mg of BrdU labeling reagent (Life Technologies). Following 3 h of incubation in the host, we collected their spleens and made them into single-cell suspensions. The cells were then stained for cell surface molecules, fixed, permeabilized for nuclear molecules, and incubated with RNase-free DNase (Qiagen) at 37°C for 1 h, and anti-BrdU antibody was added to evaluate incorporation.

CellTrace violet labeling and adoptive transfers. Splenocytes from ∅ B6 mice were isolated in DMEM supplemented with 10% fetal bovine serum and brefeldin A (Sigma-Aldrich). Red blood cells were lysed with 0.84% NH_4Cl. Cells were counted, washed with phosphate-buffered saline (PBS)–0.1% bovine serum albumin (BSA), filtered with a 40-μm-mesh-size sterile mesh, resuspended at a concentration of 5×10^7 cells/ml in PBS–0.1% BSA, and labeled with 4 μM CellTrace violet stain (Thermo Fisher). Mice were injected intravenously with 2×10^7 splenocytes and immediately infected with ECTV. For OT-I cell adoptive transfers, splenocytes were isolated in a similar manner and injected intravenously with 1.0×10^4 OT-I $CD8^+$ T cells.

Anti-ECTV IgG enzyme-linked immunosorbent assay (ELISA). On the day prior to the assay, enzyme immunoassay/radioimmunoassay 96-well round-bottom 3366 Costar plates were coated with 5×10^4 PFU of vaccinia virus in 100 μl of PBS/well and incubated overnight at 4°C. On the following day, the liquid in each well was removed by flicking the plates over bleached water. The plates were blocked by the addition of 200 μl of 5% (wt/vol) instant nonfat dry milk (Signature Select) diluted in PBS and incubated for 2 h at 37°C. The plates were washed 3 times with 200 μl of 5% (vol/vol) Tween 20 (Fisher Scientific) diluted in PBS, and the liquid was forcefully removed by flicking each time. Serum samples were serially diluted in 5% BSA–5% Tween 20, and 100 μl of each dilution was added to each well of the plates. The plates were incubated for 2 h at 37°C and washed 3 times as described above. After this, 100 μl of affinity-purified antibody consisting of peroxidase-labeled goat anti-mouse IgG (gamma globulin) human serum-adsorbed reagent diluted (1:2,000) in 5% Tween 20 diluted in PBS was added, and the plates were incubated for 0.5 h at 37°C. The plates were washed 3 times as described above. Peroxide tablets and *o*-phenylenediamine dihydrochloride (OPD) substrate tablets (Sigmafast OPD) were diluted according to the manufacturer's instructions, and 200 μl of the dilution was added to each well. The plates were developed in the dark at room temperature for 0.5 h. The reaction was stopped by the addition of 50 μl/well of a 3 M solution of HCl. The plates were read at 450 nm under the OPD setting in the KCjunior program utilizing a BioTek Instruments μQuant plate reader.

Statistical analysis. Data were analyzed using Prism (v6) software. For the analysis between 2 independent groups, we used a parametric unpaired *t* test. For the difference between 2 or more groups, we used one-way analysis of variance. For the *post hoc* analysis, we used Tukey's multiple-comparison test. To study differences in survival, we used the log-rank (Mantel-Cox) test for the comparison of survival curves. All experiments were repeated a minimum of 2 times with similar results each time.

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P.A.-P. and L.J.S. conceived of and designed the experiments, analyzed the results, and cowrote the paper. P.A.-P. performed most of the experiments. L.J.S. conceived of the initial idea and supervised the work. M.C.-N. supervised the work and contributed intellectually. M.F., C.J.K., C.S., C.R.M.-S., and E.B.W. helped with some of the experiments.

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CHAPTER V. Role of IFN-I in NK cell maturation and activation during acute and chronic LCMV CL13 infection

Running title: **IFN-I signaling in NK cells during infection**

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Abstract

The important role of Natural killer (NK) cells in the control of acute viral infections can become dysfunctional during chronic viral infections. Whether the Type I interferons (IFN-Is) IFN-α and IFN-β play a role in this dysfunction is unknow. Using the lymphocytic choriomeningitis virus clone 13 (CL13) model of chronic infection, we show that at 8 days post infection (dpi) with CL13, the large increase in the frequency of NK cells expressing the activation markers CD11b, GzmB, and KLRG1 was highly impaired in mice deficient in the IFN-I receptor (Ifnar1$^{-/-}$) while mice deficient in IFN-β(*Ifnb1*$^{-/-}$) had an intermediate phenotype. This suggests that IFN-α and IFN-β have a synergistic role during acute infection. In contrast, at 30 dpi with CL13, the maturation and activation defects of NK cells were more pronounced in *Ifnb1*$^{-/-}$ than in *Ifnar1*$^{-/-}$ mice suggesting that during chronic infection IFN-β promotes maturation and activation and IFN-α restrains them. Our data also suggests that IFN-I is important to preserve late-stage GzmB production by NK cells. Altogether, our data suggests that IFN-α and IFN-β may have opposing roles during chronic infections and this may have implications for the use of IFN-I as therapies.

Introduction

Natural killer (NK) cells are bone marrow-derived lymphocytes, critical in the innate immune response and surveillance of viral infections and cancer (1–3). When NK cells recognize infected cells or tumor cells, they promptly release cytotoxic mediators, such as granzyme B (GzmB) and perforins, and produce pro-inflammatory cytokines such as interferon-γ (IFN-γ) (1–3). The maturation of NK cells begins in the bone marrow and continues as they migrate to the periphery (4,5).(6,7). In the mouse, NK cells can be classified into four distinct maturation groups according to their expression of CD27 and CD11b. R0 ($CD27^{-}CD11b^{-}$) NK cells are the most immature, with no effector ability. R1 ($CD27^{+}CD11b^{-}$) NK cells have some IFN-γ production capacity, but limited cytotoxicity and are also considered immature. Transitional R2 ($CD27^{+}CD11b^{+}$) produce high amounts of IFN-γ but mid-levels of GzmB. Finally, mature R3 ($CD27^{-}CD11b^{+}$) NK cells have the highest levels of GzmB and cytolytic capacity (6,7). In addition, highly mature NK cells also upregulate the expression of the inhibitory receptor KLRG1. Viral infections, including HIV and HCV infections, can have significant effects on the maturation status of NK cells (8–17). For example, at 8 days post infection (dpi) of C57BL/6 (B6) mice with CL13, NK cells become highly activated and mature, with increased frequencies of GzmB+ KLRG1+ R3 NK cells (8–10). In contrast, at 30 dpi with CL13, NK cells become highly immature, with reduced expression of CD11b, and poor responses to new stimuli (8,10).

Type I interferon (IFN-I) comprises IFN-α and IFN-β cytokines, which are promptly produced following viral infection. They can interfere with viral replication, initiate, and enhance antigen presentation and broadly prime innate and adaptive immune responses. IFN-I signals trough a heterodimeric IFN-I receptor (IFNAR) comprises two subunits, the IFNAR1 (IFN-α/β receptor α chain) and IFNAR2 (IFN-α/β receptor β chain), which together are expressed in most cells. When IFN-I binds to IFNAR, this leads to its dimerization and downstream signaling, ultimately inducing the expression of hundreds of interferon stimulated genes (ISGs) (18).

During CL13 infection, IFN-I production peaks at 0.5-1 dpi (19–21). This early production of IFN-I contributes to viral infection control (20,21). However, IFN-I can also play a detrimental role during the chronic phase of CL13 infection, because it is associated with elevated expression of immunosuppressive IL-10 and PDL-1 in infected DCs and blockade of IFN-I can hasten the clearance of the virus (20,21). Yet, to what extent IFN-I contribute to the effects of viral infection on the maturation of

NK cells is not known. Here we aimed to understand how IFN-I modulates NK cells during the acute and chronic phases of CL13 infection. With this purpose, we analyzed the impact of IFNAR1 or IFN-β deficiency on NK cells maturation, activation and effector function at 8 or 30 dpi with CL13 by measuring CD11b, CD27, KLRG1 and GzmB expression. Our results indicate that IFN-I impacts NK cell maturation during CL13 infection and that IFN-α and IFN-β may provide additive maturational cues to NK cells during the acute phase of infection but may have opposing effects during the chronic phase of the infection, with IFN-β promoting and IFN-α restraining maturation.

Materials and methods

Mice

All experiments were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC). C57BL/6 (B6, CD45.2) mice breeders were purchased from Charles River. NCI B6-Ly5.1/Cr B6 CD45.1 (B6-CD45.1) mice (Charles River). *Ifnar1*$^{-/-}$ mice (CD45.2), which do not express the IFNAR1 chain of the IFNAR receptor, (22,23) backcrossed to the B6 strain were a gift from Dr. Thomas Moran, Mount Sinai School of Medicine, New York, NY (24). C57BL/6-*Ifnb1*$^{tm1(lacZ)}$ mice (herein *Ifnb1*$^{-/-}$ mice), which lack IFN-β, were obtained from the Kumamoto University Animal Facilities – Institute of Resource Development and Analysis Center for Animal Resources and Development (25). All mouse strains were bred in house. Male and female mice were 6-10 weeks old when used for experiments.

Viruses

The CL13 virus strain was a kind gift of Dr. E. John Wherry (University of Pennsylvania, Philadelphia, PA). CL13 was propagated and organ viral loads were quantified as described previously (26). CL13 infections were performed intravenously with $2x10^6$ plaque forming units (pfu).

Flow cytometry

Cell staining for flow-cytometry was performed as previously described (10). The following antibodies were used: anti-NK 1.1 (clone PK136, APC or BV605, Biolegend), anti-TCR-β (clone H57-597, BV605 or BV421 Biolegend; clone H57-597, BV786, BD), anti-CD45.1 (clone A20, PE or PE-Cy7, Biolegend), anti-CD45.2 (clone 104, APC or PercP C5.5, Biolegend) anti-CD11b (clone M1/70,

BUV395, BD), anti-CD27 (clone LG.3A10, PerCP-Cy5.5, Biolegend), anti-KLRG1 (clone 2F1/KLRG1, PE-Cy7 or APC, Biolegend), anti-granzyme B (clone GB11, Pacific Blue, Biolegend). Data were acquired with a BD LSRFORTESSA™ cytometer and analyzed with FlowJo™ version 10 (Treestar).

Mixed bone marrow chimeras

To generated mixed bone marrow chimeras where we could distinguish the cells derived from either of the two donors or from the host, 6-8 weeks old B6-CD45.1 ($CD45.1^+$ $CD45.2^-$) mice were irradiated with 900 Rad and reconstituted with $5x10^6$ bone marrow cells from F1[B6-CD45.1x B6] mice ($CD45.1^+$ $CD45.2^+$) and *Ifnar1*$^{-/-}$ ($CD45.1^-$ $CD45.2^+$) or B6 ($CD45.1^-$ $CD45.2^+$) mice in a 1:1 ratio to generate CD45.1+*Ifnar1*$^{-/-}$→ F1[B6CD45.1x B6] or CD45.1+B6→ F1[B6CD45.1x B6] chimeric mice. Mice were rested for two months and given acidified water for the first 20 days.

Statistical analysis

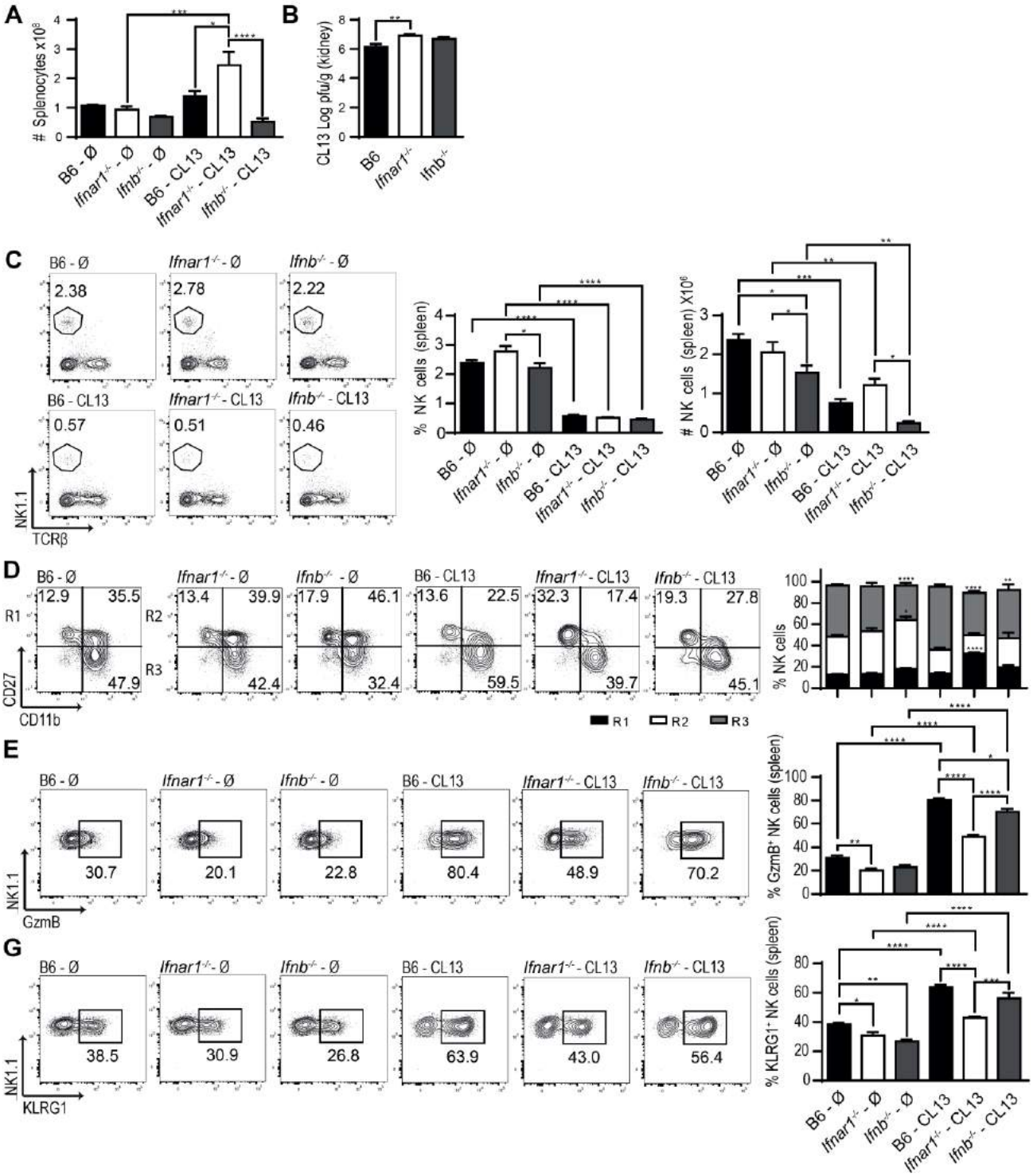
Data were analyzed using Prism v6. We used a parametric unpaired t-test to study difference between 2 independent groups or One-Way ANOVA test if more than 2 independent groups. For the Post-Hoc analysis, we used the Tukey's multiple comparisons test. Experiments were repeated at least twice. For all, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Results and discussion

At steady state, B6, *Ifnar1*$^{-/-}$ and *Ifnb1*$^{-/-}$ mice had comparable numbers of splenocytes (**Figure 1A**). At 8 dpi with CL13, the number of splenocytes were similar to those in naïve mice in B6 and *Ifnb1*$^{-/-}$mice but were significantly increased in *Ifnar1*$^{-/-}$ mice (**Figure 1A**). Also, compared to B6 mice, the virus titers in the kidneys of *Ifnar1*$^{-/-}$ mice were slightly but significantly increase (**Figure 1B**) supporting previous observations that IFN-I signaling helps control CL13 (20,27). On the other hand, *Ifnb1*$^{-/-}$ mice did not have increased virus indicating that IFN-α alone can contribute to virus reduction.

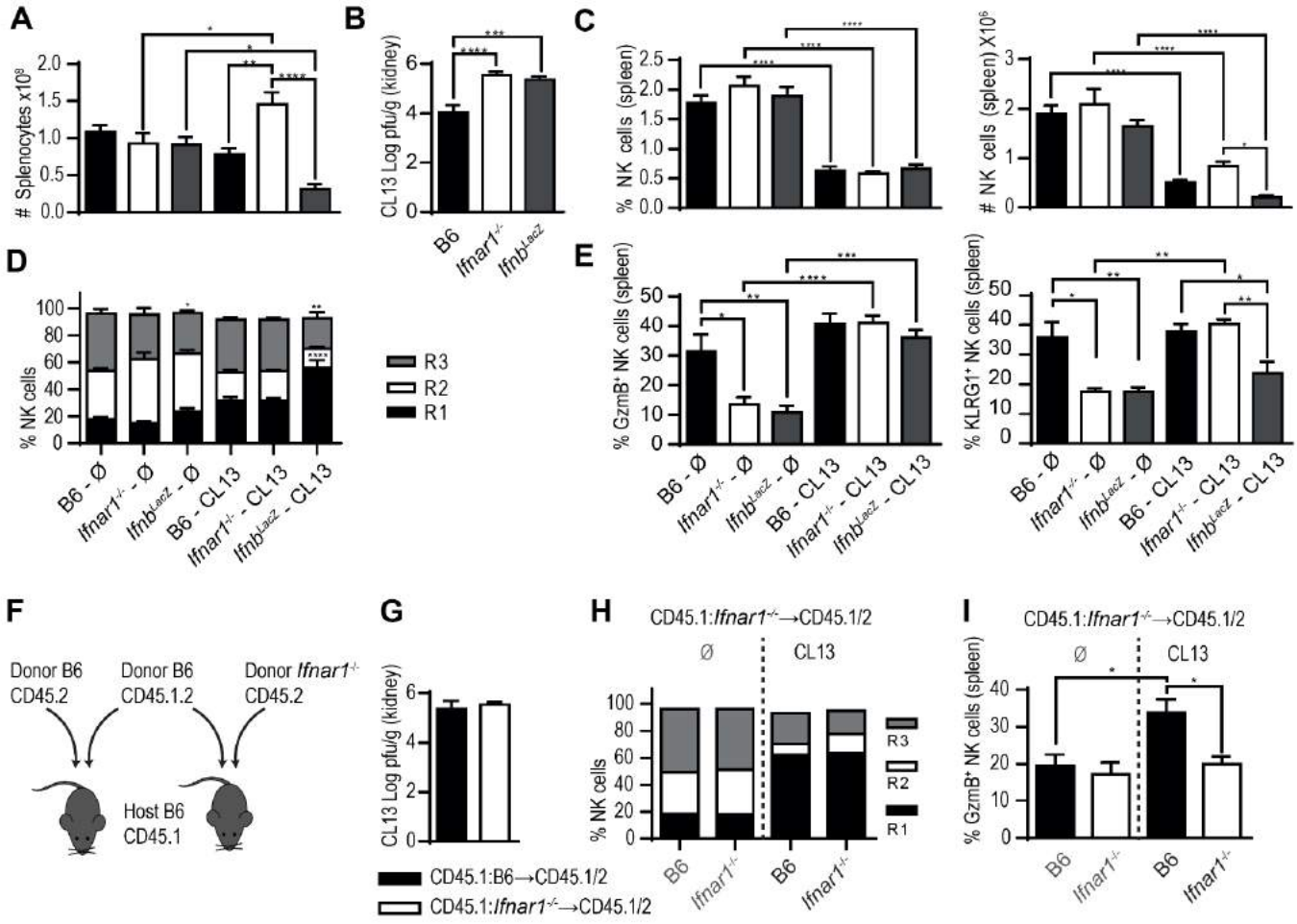
We and others have shown that B6 mice have reduced frequencies and numbers of NK cells at 8 dpi with CL13, (8,10). Here, we observed a comparable phenotype in infected *Ifnar1*$^{-/-}$ and *Ifnb*^{lacZ} mice (**Figure 1C**), suggesting that the reduction in NK cells is not because of IFN-I. Of note, *Ifnar1*$^{-/-}$ mice had higher numbers of NK cells in the spleen compared to *Ifnb1*$^{-/-}$ mice, which most likely reflect splenocyte number differences between these mice (**Figure 1A**).

Figure 1. IFN-I signaling and NK cells in early chronic CL13 infection



A) Number of splenocytes in the indicated mice. **B)** LCMV titers in the kidneys of the indicated mice at 8 dpi with CL-13 **C)** Flow cytometry dot-plots representing the gating strategy utilized for the definition of NK cells (TCRNK 1.1^{+}) (left) with the frequencies and numbers in the spleen (right). **D)** Flow-cytometry dot-plots with the gating strategy used to define the R1, R2 and R3 NK cell subpopulations, and cumulative frequencies of R1, R and R3 NK cells in the spleens of the indicated mice. Flow-cytometry dot-plots indicating the gating strategy used to define GzmB^{+} (**E**) or KLRG1^{+} (**G**) NK cells, and cumulative frequencies of GzmB^{+} or KLRG1^{+} NK cells in the spleen. All data was collected following 8 dpi with CL13. Graphs display at least 2 similar and independent experiments, with 6-13 mice per group each. In all graphs, data is shown as mean ± SEM.

Figure 2. IFN-I signaling and NK cells in established chronic CL13 infection



A) Number of splenocytes in the indicated mice. **B)** LCMV titers in the kidneys of the indicated mice at 30 dpi with CL-13. **C)** NK cell frequencies and calculated total numbers in the spleen of the indicated mice. **D)** Frequency of R1, R2 and R3 NK cell subpopulations in the spleen of the indicated mice. **E)** Frequency of NK cells expressing GzmB (left) or KLRG1 (right) in the spleen of the indicated mice. **F)** Schematic representation of the process involved in creating F1[B6CD45.1x B6] +*Ifnar1*-/-→ CD45.1 or F1[B6CD45.1x B6] +B6→ CD45.1 chimeric mice. The gating strategy for each NK cell compartment within a mouse is represented on the right **G)** CL13 virus titers in the kidneys of the indicated mouse chimeras at 30 dpi. **H)** Frequency of R1, R2, and R3 NK cell subpopulations, within the B6 or *Ifnar1*-/- NK cell compartments in naïve or CL13 infected F1[B6CD45.1x B6] +Ifnar1-/-→ CD45.1 chimeras. **I)** Frequency of NK cells of B6 or *Ifnar1*-/- origin expressing GzmB in the spleens of F1[B6CD45.1x B6] +*Ifnar1*-/-→ CD45.1 mice. All data was collected at 30 dpi with CL13. Each graph displays data from at least 2 similar and independent experiments, with 4-10 mice per group and data is shown as mean ± SEM.

We next assessed the maturation status of NK cells in these mice using CD27 and CD11b as markers. At baseline, naïve B6 and *Ifnar1*$^{-/-}$ mice showed comparable frequencies of R1, R2 and R3 NK cells, while naïve *Ifnb1*$^{-/-}$ mice showed increased frequencies of R2 NK cells (ANOVA, $p<0.05$), and decreased frequencies of R3 NK cells (ANOVA, $p<0.0001$) (**Figure 1D**). These observations suggest that IFN-I may have a role in the steady state maturation of NK cell and that IFN-β and IFN-α may exert opposing effects in this process.

Previously, we and others have shown that compared to naive mice, the NK cells at 8 dpi with CL13 become more mature with increased frequencies of mature R3 at the expense of transitional R2 cells, and also become activated as indicated by increased frequencies of GzmB^{+} and KLRG1^{+} NK cells. Notably, we now found that CL13 infection in *Ifnar1*$^{-/-}$ mice increased the frequencies of immature R1 ($p<0.001$) while reducing the transitional R2 NK cells ($p<0.001$). This did not occur in *Ifnb1*$^{-/-}$ mice were NK cells became more mature with increased frequencies of R3 cells ($p<0.05$), but significantly less than in B6 mice ($p<0.01$). Thus, IFN-I has a critical role in the maturation of NK cells induced by acute viral infection Also, while the frequencies of GzmB^{+} (**Figure 1E**) and KLRG1^{+} (**Figure 1G**) NK cells increased significantly in *Ifnar1*$^{-/-}$ mice, these increases were significantly smaller than in B6 mice indicating that IFN-I plays a role but is not absolutely necessary for the activation of NK cells during acute infection. On the other hand, *Ifnb1*$^{-/-}$ mice had a slightly reduced frequency of GzmB^{+} and normal frequency of KLRG1^{+} NK cells.

Our data thus far indicate that IFN-I signaling is pivotal for optimal NK cell response during th acute phase of CL13 infection. The early peak of IFN-I production following CL13 infection occurs ~12-24h post CL13 infection (21,28). As the infection progresses, IFN-I decreases to baseline levels (19). Yet, current evidence suggests there are still remnants of IFN-I activity during the chronic phase of the infection, with increased expression of IFN-I inducible genes (ISGs, including *Mx2*, *Oas1a*, *Oas1g*, *Oas2*, *Oas3*, and others (20). Therefore, we next analyzed how IFN-I affects the NK cell phenotype of NK cells during the chronic phase of CL13 infection (30 dpi). We found that CL13 chronically infected B6 and *Ifnb1*$^{-/-}$ mice had decreased numbers of splenocytes compared to naïve mice (t-test, $p<0.05$) while *Ifnar1*$^{-/-}$ mice had increased numbers of splenocytes when compared to all other groups (**Figure 2A**). Others have shown that the blockade of IFNAR or IFN-β alone during chronic CL13 infection, results in reduced virus loads in several organs (20,29). In contrast, we found that in *Ifnar1*$^{-/-}$ mice and *Ifnb1*$^{-/-}$

mice had increased and comparable virus burden (**Figure 2B**), indicating that IFN-I contribute to virus control in chronic infections.

Compared to naïve mice and similar to the acute phase of the infection, the frequencies and absolute numbers of NK cells were reduced in B6, *Ifnar1*$^{-/-}$ and *Ifnb1*$^{-/-}$ (**Figure 2C** and (10)) with *Ifnar1*$^{-/-}$ mice having slightly higher absolute numbers possibly due to the splenomegaly.

NK cells from all infected groups displayed an immature phenotype with significantly increased frequencies of R1 at the expense of R2 NK cells, but the frequency or R1 was significantly much higher and the frequency of R2 NK cells was significantly much lower in *Ifnb1*$^{-/-}$ mice than in B6 and *Ifnar1*$^{-/-}$ mice (**Figure 2D**) This suggests that IFN-β is critical to restrain a shift to an immature phenotype in NK cells during the chronic phase of the infection and that the roles of IFN-α and IFN-β are non-synergistic during chronic infection. Because the defects were more pronounced in the maturation and activation of NK cells in *Ifnb1*$^{-/-}$ mice, one would assume that IFN-β signaling becomes more important in activating NK cells as the CL13 infection progresses.

Chronic CL13 infection also resulted in increased frequencies of GzmB^{+} and KLRG1^{+} NK cells in B6, *Ifnar1*$^{-/-}$ and *Ifnb1*$^{-/-}$ mice, with *Ifnb1*$^{-/-}$ mice being slightly less efficient at upregulating KLRG1 (**Figure 2E**). This indicates that the activation of NK cells and their production of GzmB is independent of IFN-I during chronic CL13 infection. These results were unexpected, because IFNAR blockade during chronic CL13 infection results in decreased NK cell cytotoxicity (9).

In the experiments in **Figure 2E**, it was possible that the increase virus titers in the absence of IFN-I signaling resulted in NK cell activation through alternative pathways. Thus, we next tested the role of intrinsic IFN-I signaling for NK cell maturation and activation during the chronic phase of the infection using F1[B6 CD45.1x B6] +*Ifnar1*$^{-/-}$→ CD45.1 and control F1[B6CD45.1x B6] +B6→ CD45.1 chimeric mice (**Figure 2F**). Both groups had comparable virus loads in their kidneys (**Figure 2G**) which allowed us evaluating the intrinsic requirement for IFN-I signaling in NK cells maturation and activation independently of virus loads. Both IFNAR-sufficient and IFNAR-deficient NK cells from infected F1[B6CD45.1x B6] +*Ifnar1*$^{-/-}$→ CD45.1 mice had increased frequencies of R1 at the expense of R2 NK cells and R3 NK cells (ANOVA, $p<0.0001$ for all) (**Figure 2H**). Also, the WT NK cells from infected F1[B6CD45.1x B6] +*Ifnar1*$^{-/-}$→ CD45.1 had increased frequency of GzmB^{+} NK cells as compared to similar cells from naïve chimeras while *Ifnar1*$^{-/-}$ NK cells did not (**Figure 2I**). This indicates that IFN-I

signaling can directly controls GzmB expression in NK cells during chronic CL13 infection and is in agreement with previous reports demonstrating reduced NK cell cytotoxicity during chronic CL13 infection or after MCMV infection (9,30).

In summary, we show that IFN-I plays an important role in the modulation of NK cells during CL13 infection. In the acute phase of CL13 infection, both IFN-α and IFN-β contribute to the maturation of NK cells, with increased expression of CD11b. Interestingly, during the chronic phase of CL13 infection, the actions of IFN-α and IFN-β on NK cells are not synergistic because in absence of IFN-β alone results in higher accumulation of immature NK cells than in the absence of IFNAR.

Our data suggest that during the acute phase of CL13 infection optimal upregulation of GzmB depends on the additive of multiple activation mechanism including but not limited to IFN-αand IFN-β. The IFN-I independent mechanisms of GzmB induction become dominant during chronic CL13 infection likely due to increased virus loads, because when virus loads were the same in bone marrow chimeras the induction of GzmB was fully dependent on IFN-I.

The finding that IFN-α and IFN-β have opposite effects in maturation of NK despite signaling through the same receptor was unsuspected. These results are important an worth of further exploration because they could have major impact for the use of different IFN-I subtype in the treatment of infectious diseases and cancer.

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Author Contributions

PAP, CM-S and LJS conceived and designed experiments, analyzed results and co-wrote the paper. PAP performed most of the experimental work. CM-S helped with some of the experiments. LJS conceived the initial idea and supervised the work.

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CHAPTER VI. Loss of resistance to mousepox during chronic lymphocytic choriomeningitis virus infection is associated with impaired T-cell responses and can be rescued by immunization

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PATHOGENESIS AND IMMUNITY

Loss of Resistance to Mousepox during Chronic Lymphocytic Choriomeningitis Virus Infection Is Associated with Impaired T-Cell Responses and Can Be Rescued by Immunization

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ABSTRACT It is well established that chronic viral infections can cause immune suppression, resulting in increased susceptibility to other infectious diseases. However, the effects of chronic viral infection on T-cell responses and vaccination against highly pathogenic viruses are not well understood. We have recently shown that C57BL/6 (B6) mice lose their natural resistance to wild-type (WT) ectromelia virus (ECTV) when chronically infected with lymphocytic choriomeningitis virus (LCMV) clone 13 (CL13). Here we compared the T-cell response to ECTV in previously immunologically naive mice that were chronically infected with CL13 or that were convalescent from acute infection with the Armstrong (Arm) strain of LCMV. Our results show that mice that were chronically infected with CL13 but not those that had recovered from Arm infection have highly defective ECTV-specific $CD8^+$ and $CD4^+$ T-cell responses to WT ECTV. These defects are at least partly due to the chronic infection environment. In contrast to mice infected with WT ECTV, mice chronically infected with CL13 survived without signs of disease when infected with ECTV-Δ036, a mutant ECTV strain that is highly attenuated. Strikingly, mice chronically infected with CL13 mounted a strong $CD8^+$ T-cell response to ECTV-Δ036 and survived without signs of disease after a subsequent challenge with WT ECTV. Our work suggests that enhanced susceptibility to acute viral infections in chronically infected individuals can be partly due to poor T-cell responses but that sufficient T-cell function can be recovered and resistance to acute infection can be restored by immunization with highly attenuated vaccines.

IMPORTANCE Chronic viral infections may result in immunosuppression and enhanced susceptibility to infections with other pathogens. For example, we have recently shown that mice chronically infected with lymphocytic choriomeningitis virus (LCMV) clone 13 (CL13) are highly susceptible to mousepox, a disease that is caused by ectromelia virus and that is the mouse homolog of human smallpox. Here we show chronic CL13 infection severely disrupts the expansion, proliferation, activation, and cytotoxicity of T cells in response due at least in part to the suppressive effects of the chronic infection milieu. Notably, despite this profound immunodeficiency, mice chronically infected with CL13 could be protected by vaccination with a highly attenuated variant of ECTV. These results demonstrate that protective vaccination of immunosuppressed individuals is possible, provided that proper immunization tools are used.

KEYWORDS T cells, ectromelia virus, immunization, lymphocytic choriomeningitis virus, poxvirus

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Chronic human viral infections frequently result in grave or lethal disease after acute infection with unrelated viruses. Moreover, despite a reduction in virus loads, people undergoing treatment for immunodeficiency virus (HIV) and hepatitis C virus (HCV) infection continue to present increased susceptibility to viral infections (1–5). This indicates that the presence of a chronic viral infection, even at low levels, results in poorly understood immune dysfunction that prevents the proper control of other pathogens.

The lymphocytic choriomeningitis virus (LCMV) Armstrong (Arm) strain replicates to moderate levels in the mouse and is cleared in about a week by effective T-cell responses, serving as an excellent model to study T-cell responses to nonpathogenic acute viral infections. In contrast, the Arm substrain clone 13 (CL13) persists in the blood and several other tissues at variable levels for as long as 80 days postinfection (dpi) or more and indefinitely in the kidneys and the central nervous system (6–13). A main reason for this is that CL13, but not Arm, replicates preferentially in dendritic cells (DCs), first inducing very strong virus-specific T-cell responses, followed by their functional exhaustion and/or deletion (9, 14–16). Of note, exhaustion of virus-specific T cells also occurs following HIV and HCV infection and during cancer progression in humans (17–19).

In addition to deletion and/or exhaustion of LCMV-specific T cells and similar to what happens during various human chronic infections, chronic infection of mice with LCMV results in increased infectivity and/or a deficient immune response to secondary infections or vaccination with unrelated pathogens. For example, chronic LCMV infection of mice results in high mortality following intravenous (i.v.) infection with the fungus *Histoplasma capsulatum*, to which they are normally resistant (20); reduced control of intraperitoneal attenuated *Salmonella enterica* serovar Typhimurium (21); increased virus loads in the spleen but not in the liver due to defective type I interferon (IFN-I) responses after intraperitoneal inoculation with mouse cytomegalovirus (MCMV) (22); decreased antibody responses to intravenous vesicular stomatitis virus (VSV) (23) and inactivated influenza A virus (IAV) vaccine (21); and decreased T-cell responses to intravenous infection with vaccinia virus (23). Nevertheless, how chronic LCMV infection affects the immune response and immune-mediated protection from a highly pathogenic mouse-specific virus introduced through its natural route is not well understood.

Ectromelia virus (ECTV), the agent of mousepox, is a poxvirus that naturally infects mice and that is very similar to variola virus, the causative agent of human smallpox. In nature, ECTV infects mice through microabrasions of the skin, most commonly in the footpad (24), which in the laboratory can be replicated by inoculation at this site. In some mouse strains, such as BALB/c and DBA2/J mice, footpad inoculation of ECTV leads to acute mousepox and death at 7 to 12 dpi (25), yet young, immunologically naive (∅) C57BL/6 (B6) mice infected with ECTV in the footpad survive without major signs of disease, except for the inflammation of the footpad (26, 27). This resistance requires a cascade of innate and adaptive immune cells and molecules, such as dendritic cells (DCs), chemokines, inflammatory monocytes (iMOs), type I interferons (IFN-I), natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells, and antibodies (Abs), that successively control the systemic spread of ECTV from the draining lymph node (dLN) and viral replication in the spleen, liver, and other organs. The timely participation of these immune mediators is revealed by the loss of resistance to lethal mousepox at a particular dpi when each of them is removed (28–34).

We have recently reported (35) that most B6 mice convalescent from Arm infection survive when infected with ECTV in the footpad at 30 dpi with LCMV. On the other hand, most mice chronically infected with CL13 for 30 days succumb to mousepox, which correlates with absent NK cell responses, yet because NK cell transfer did not rescue mice that had been chronically infected with CL13 for 30 days (CL13 mice) and that were infected with ECTV in the footpad (CL13+ECTV mice) from their susceptibility to lethal mousepox, the data suggested that, in addition to the NK cell defects, other immune deficiencies, most likely, defective T-cell responses, should contribute to the susceptibility of CL13 mice to mousepox. Here we compared the T-cell responses to

ECTV in previously immunologically naive mice with those in mice chronically infected with CL13 or convalescent from acute Arm infection. We show that the ECTV-specific T-cell response in mice chronically infected with CL13 but not in mice convalescent from Arm infection is severely reduced. Our study suggests that the defective T-cell response is, at least in part, cell extrinsic and due to the chronic infection environment because naive T cells adoptively transferred into chronically infected mice display an impaired response similar to that shown by host T cells. We also show that chronically infected mice are still highly susceptible to a highly attenuated mutant ECTV strain that lacks a type I interferon evasion molecule (ECTV-Δ166) but are resistant to infection to another attenuated ECTV mutant with compromised spread (ECTV-Δ036). Notably, mice infected with ECTV-Δ036 mount an anti-ECTV T-cell response and are protected from wild-type (WT) ECTV challenge.

RESULTS

Impaired CD8 T-cell expansion in response to ECTV in mice chronically infected with CL13. Recapitulating the findings presented in our recent report (35), mice that had been chronically infected with CL13 for 30 days (CL13 mice) and that were infected with ECTV in the footpad (CL13+ECTV mice) succumbed to mousepox, while most mice that had been infected with Arm 30 days before (Arm mice) and that were infected with ECTV (Arm+ECTV mice) and all previously naive (∅) mice that were infected with ECTV (∅+ECTV mice) survived ECTV infection without signs of mousepox (Fig. 1A). Also, as before and coherent with the survival results, ECTV titers in the spleen at 7 dpi with ECTV were significantly higher in CL13+ECTV mice than in ∅+ECTV and Arm+ECTV mice, with Arm+ECTV mice having intermediate virus titers (Fig. 1B). Compared to the numbers of splenocytes in ∅ mice and Arm mice, ∅+ECTV and Arm+ECTV mice, respectively, had significantly increased numbers of splenocytes, while those in CL13+ECTV mice were not significantly different from those in CL13 mice and were significantly lower than those in ∅+ECTV and Arm+ECTV mice (Fig. 1C). When T cells were analyzed (Fig. 1D), ∅+ECTV mice but not CL13+ECTV mice had increased total numbers of T cells (Fig. 1E), CD8 T cells (Fig. 1F), and $CD4^+$ T cells (Fig. 1G) in their spleens. Arm+ECTV mice displayed an intermediate phenotype. Also, the numbers of $CD4^+$ and $CD8^+$ T cells in CL13+ECTV mice were significantly lower than the numbers in ∅+ECTV and Arm+ECTV mice. Together, these data suggest that $CD4^+$ and $CD8^+$ T cells expand in response to ECTV infection in ∅+ECTV and Arm+ECTV mice but not in CL13+ECTV mice.

The proliferation of T cells during ECTV infection is impaired in mice chronically infected with CL13. In light of the previous results, we determined the expression of Ki67 and the incorporation of bromodeoxyuridine (BrdU) at 7 dpi with ECTV in the spleen to evaluate the cell cycle status of $CD8^+$ T cells (Fig. 2A) and $CD4^+$ T cells (not shown). Before ECTV infection, a larger proportion of $CD8^+$ T cells were Ki67 positive ($Ki67^+$) in CL13 and Arm mice than in ∅ mice, yet the vast majority of the $Ki67^+$ cells were BrdU negative ($BrdU^-$), suggesting that a significant proportion of T cells in CL13 and Arm mice were stuck in G_2 phase and/or synthesized DNA and divided slowly. After ECTV infection, there was a dramatic increase in the frequencies of $Ki67^+$ $CD8^+$ T cells in ∅+ECTV and Arm+ECTV mice but not in CL13+ECTV mice compared to those in ∅, Arm, and CL13 mice, respectively. In ∅, CL13, and Arm mice, the frequency of BrdU-expressing ($BrdU^+$) $CD8^+$ T cells was low (~1 to 3%) and not significantly different, yet compared to the frequencies in their specific controls, the frequencies of $BrdU^+$ $CD8^+$ T cells were significantly increased in ∅+ECTV and Arm+ECTV mice and also, but to a much lower degree, in CL13+ECTV mice. Indeed, the frequency of $BrdU^+$ cells was significantly lower in CL13+ECTV mice than in ∅+ECTV and Arm+ECTV mice. Of note, about two-thirds of the $Ki67^+$ cells in ∅+ECTV and Arm+ECTV mice were $BrdU^+$, but only one-third of the $Ki67^+$ cells in CL13+ECTV mice were $BrdU^+$ (compare the numbers in Fig. 2A). Similar results were obtained for $CD4^+$ T cells (not shown). When total numbers were calculated, we found that in ∅, CL13, and Arm mice, the numbers of $Ki67^+$ or $BrdU^+$ $CD8^+$ and $CD4^+$ T cells expressing Ki67 or incorporating BrdU were

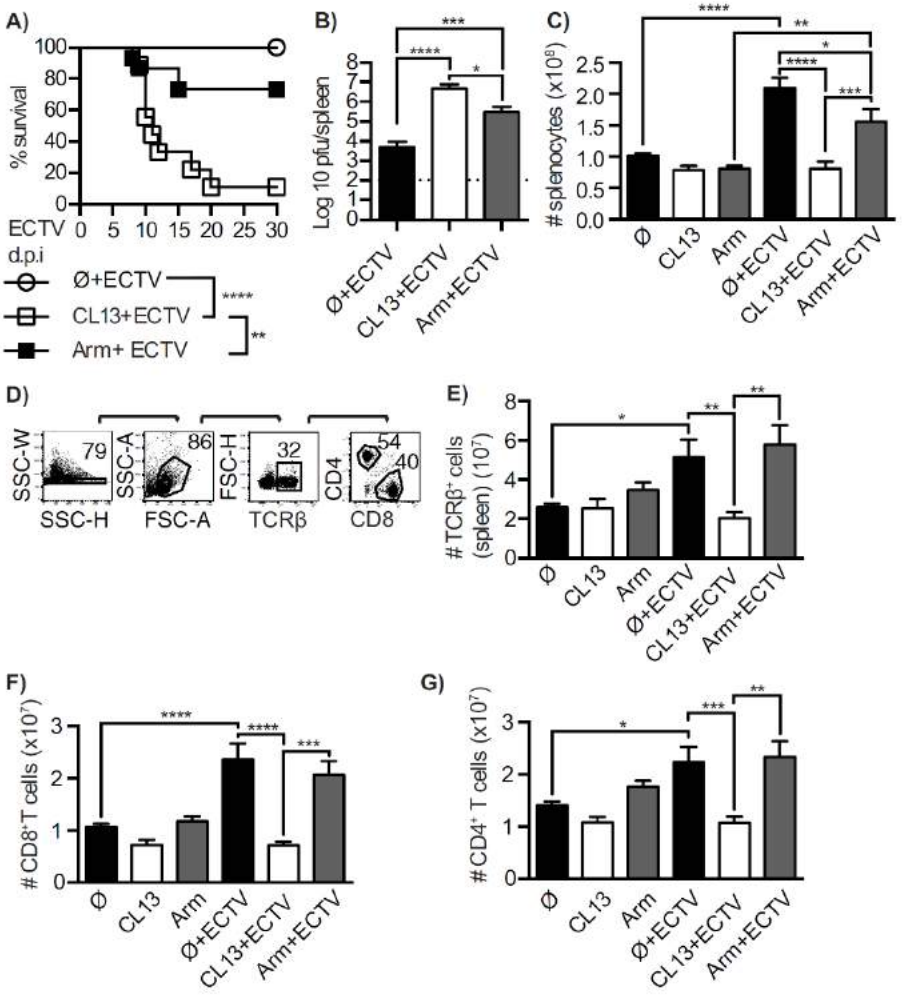

FIG 1 Impaired CD8 T-cell expansion in response to ECTV in mice chronically infected with CL13. (A) Survival of the indicated mice after infection with 3,000 PFU of ECTV. (B) ECTV titers in the spleens (in numbers of PFU per organ) of the indicated mice at 7 dpi with ECTV. The dotted line indicates the limit of detection of the number of ECTV PFU. (C) Number of splenocytes in the indicated mice at 7 dpi with ECTV. (D) Flow cytometry dot plots representing the gating strategy utilized for the definition of TCR-β^+ T cells, CD8$^+$ T cells, and CD4$^+$ T cells. SSC-W, side scatter width; SSC-H, side scatter height; SSC-A, side scatter area; FSC-H, forward scatter height; FSC-A, forward scatter area. Numbers indicate the frequency of the gated populations. (E) Number of T cells (TCR-β positive) in the spleen. (F and G) Percentage and number of CD8$^+$ T cells (F) and CD4$^+$ T cells (G) in the spleens of the indicated mice. All graphs present the results from at least 2 similar and independent experiments with 6 to 10 mice per group. Data are shown as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

relatively small and not significantly different, yet following ECTV infection, significantly larger numbers of CD8$^+$ T cells and, to a lesser extent, of CD4$^+$ T cells became BrdU$^+$ and/or Ki67$^+$ in ∅+ECTV and Arm+ECTV mice but not in CL13+ECTV mice (Fig. 2A to E). Together, these results indicate that in ∅+ECTV and Arm+ECTV mice, most CD4$^+$ and CD8$^+$ T cells were cycling and more than 50% were in the S phase, while most T cells remained quiescent in G_0 phase in CL13+ECTV mice.

The severe hyporesponsiveness of T cells described above could be due to T-cell-intrinsic or -extrinsic deficiencies in mice chronically infected with CL13. To distinguish between these possibilities, we adoptively transferred CellTrace violet-labeled splenocytes from ∅ B6-CD45.2 mice into B6-CD45.1 mice that were ∅ or chronically infected with CL13 and then immediately infected them or not with ECTV. The proliferation of CD8$^+$ and CD4$^+$ T cells was determined by the dilution of the CellTrace violet signal at 7 dpi with ECTV. We found that the transferred CD8$^+$ T cells proliferated significantly more in ∅+ECTV and CL13+ECTV mice than in ∅ and CL13 mice, respectively, but significantly more in ∅+ECTV mice than in CL13+ECTV mice, resulting in a 5-fold higher number of proliferated cells in ∅+ECTV mice than in CL13+ECTV mice

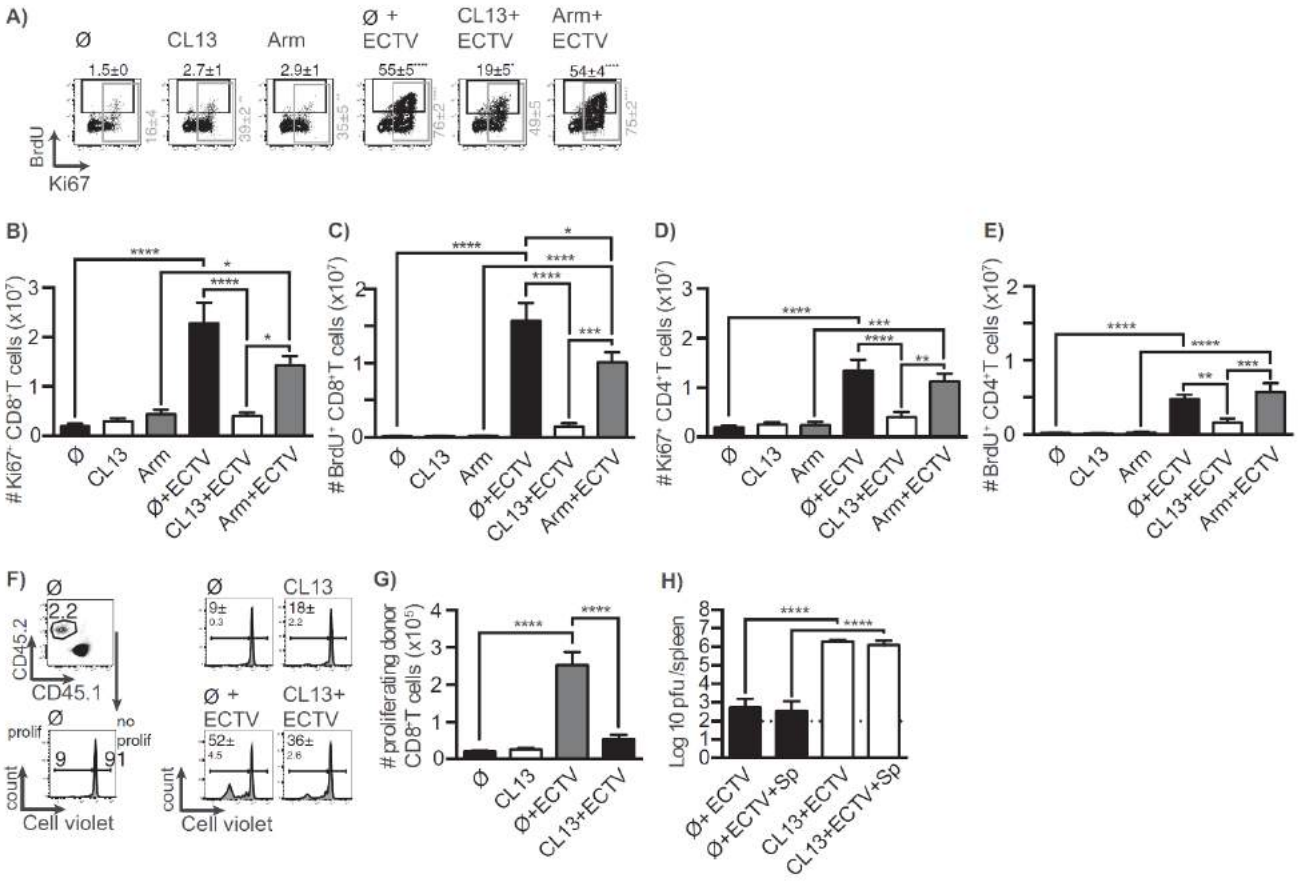

FIG 2 The proliferation of T cells during ECTV infection is impaired in mice chronically infected with CL13. (A) Representative flow cytometry dot plots of the expression of Ki67 and the incorporation of BrdU 3 h after intraperitoneal injection in $CD8^+$ T cells in the spleens of the indicated mice. Numbers indicate the frequency of the gated populations. (B and C) Numbers of $Ki67^+$ (B) and $BrdU^+$ (C) $CD8^+$ T cells in the spleens of the indicated mice. (D and E) As for panels B and C, respectively, but for $CD4^+$ T cells. (F) Representative flow cytometry dot plots of CellTrace violet staining in the $CD8^+$ T cells of the adoptively transferred ∅ mouse splenocytes, with the percent proliferation (prolif; CellTrace violet low) being given. (G) Numbers of proliferating donor cells in the indicated mice. (H) ECTV titers in the indicated mice that received 2×10^7 splenocytes (+Sp) or that did not receive splenocytes intravenously prior to ECTV infection. The dotted line indicates the limit of ECTV detection. The graphs present the results of at least 2 independent and comparable experiments with 6 to 10 mice per group following 7 days of ECTV infection in the spleen. Data are represented as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

(Fig. 2F and G). Similar results were observed for $CD4^+$ T cells (data not shown). These results indicate that cell-extrinsic factors are at least partly responsible for the inability of T cells to fully respond in mice chronically infected with CL13. Also, of note, the adoptively transferred splenocytes did not protect CL13+ECTV mice from virus proliferation, as ECTV titers were similar in CL13+ECTV mice that did or did not receive ∅ mouse splenocytes (Fig. 2H).

Effector T-cell differentiation during ECTV infection is impaired in mice chronically infected with CL13. Having found defective T-cell expansion in response to ECTV in mice chronically infected with CL13, we next looked at overall effector differentiation. First, we analyzed granzyme B (GzmB) expression (Fig. 3A). Compared to the frequencies in ∅ mice, CL13 mice had increased frequencies of GzmB-expressing ($GzmB^+$) $CD8^+$ T cells ($P \leq 0.0001$), while no significant changes were observed in Arm mice, confirming that some of the $CD8^+$ T cells in CL13 mice were activated (data not shown), yet this did not translate into significantly higher numbers of $GzmB^+$ $CD8^+$ T cells in CL13 mice than in ∅ and Arm mice. Following ECTV infection, ∅+ECTV, Arm+ECTV, and CL13+ECTV mice had increased frequencies of $GzmB^+$ $CD8^+$ T cells compared to ∅ ($P \leq 0001$), Arm ($P \leq 0001$), and CL13 ($P \leq 0.01$) mice, respectively, but they were significantly higher in ∅+ECTV and Arm+ECTV mice than in CL13+ECTV mice ($P \leq 0.0001$ for both comparisons) (data not shown), yet the absolute numbers of $GzmB^+$ $CD8^+$ T cells increased little in CL13+ECTV mice compared to the numbers in

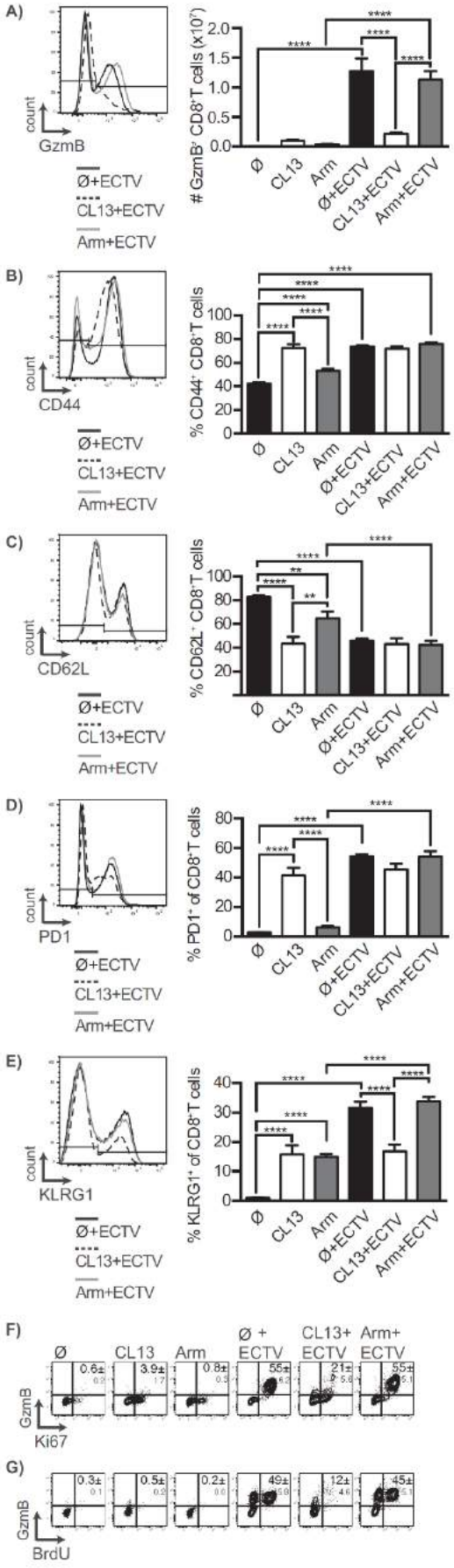

FIG 3 Effector T-cell differentiation during ECTV infection is impaired in mice chronically infected with CL13. (A) Representative flow cytometry histograms of the mean fluorescence intensity of the expression of GzmB by CD8$^+$ T cells in the spleen (left) and the numbers of GzmB$^+$ CD8$^+$ T cells in the indicated

(Continued on next page)

CL13 mice. In contrast, in ∅+ECTV and Arm+ECTV mice, there was a significant increase in the numbers of GzmB$^+$ CD8 T cells compared to the numbers in ∅ and Arm mice, respectively (Fig. 3A).

Next, we analyzed the expression of CD44, which is present in activated and memory T cells (Fig. 3B). CL13 mice had higher frequencies of CD44$^+$ CD8$^+$ T cells than ∅ and Arm mice. Following ECTV infection, ∅+ECTV and Arm+ECTV mice had increased frequencies of CD44$^+$ CD8$^+$ T cells compared to those in ∅ and Arm mice, respectively. In contrast, the frequency of CD44$^+$ CD8$^+$ T cells was not increased in CL13+ECTV mice compared to that in CL13 mice.

We also examined the expression of CD62L, which is rapidly downregulated in activated T cells. CL13 mice and, to lesser extent, Arm mice had a significantly reduced frequency of CD62L$^+$ CD8$^+$ T cells compared to that in ∅ mice. After ECTV infection, ∅+ECTV and Arm+ECTV mice had a lower frequency of CD62L$^+$ CD8$^+$ T cells than ∅ and Arm mice, respectively. In contrast, the frequency of CD62L$^+$ cells in CL13+ECTV mice was not decreased from that in CL13 mice. Together, the data presented above indicate that while CD8$^+$ T cells are activated/exhausted in CL13 mice, they do not get further activated after ECTV infection.

Next, we evaluated the expression of PD1 and KLRG1, two inhibitory receptors that are upregulated in activated T cells (36, 37). It is known that during chronic CL13 infection PD1 persists at high levels in exhausted T cells (38), while KLRG1 is progressively downregulated (36, 39). As expected, we found significantly higher levels of PD1 expression in the CD8$^+$ T cells of CL13 mice than in those of ∅ or Arm mice. After ECTV infection, ∅+ECTV and Arm+ECTV mice had increased frequencies of PD1$^+$ CD8$^+$ T cells compared to those in ∅ and Arm mice, respectively. In contrast, the frequency of PD1$^+$ CD8 T cells was not increased in CL13+ECTV mice compared to that in CL13 mice (Fig. 3D). For KLRG1, we found that CL13 and Arm mice had increased KLRG1 expression compared to that in ∅ mice, likely representing the expression by short-lived effector cells induced by LCMV that persisted during convalescence from the Arm infection. After ECTV infection, ∅+ECTV and Arm+ECTV mice had increased frequencies of KLRG1$^+$ CD8 T cells compared to those in ∅ and Arm mice, respectively. Of note, KLRG1 upregulation mostly occurred in GzmB$^+$ CD8 T cells (not shown). In contrast, the frequency of KLRG1$^+$ CD8 T cells was not increased in CL13+ECTV mice compared to that in CL13 mice (Fig. 3E). Similar profiles were observed for CD4$^+$ T cells with respect to the expression of CD44, CD62L, and PD1 but not with respect to the expression of KLRG1, as the CD4$^+$ T cells did not express KLRG1 (data not shown). Together, these data indicate that, in addition to preventing T-cell proliferation, chronic CL13 infection impairs overall T-cell activation.

Next, we analyzed the relationship between cell cycle progression and effector differentiation in CD8$^+$ T cells by analyzing the expression of GzmB together with the expression of Ki67 or the incorporation of BrdU (Fig. 3F and G). We found that in ∅+ECTV and Arm+ECTV mice the vast majority of Ki67$^+$ CD8$^+$ T cells were effectors because most of the Ki67$^+$ cells (~90%) also expressed GzmB, while only ~50% of the Ki67$^+$ cells in CL13+ECTV mice were GzmB$^+$. Of note, virtually all BrdU$^+$ cells in ∅+ECTV and Arm+ECTV mice and also in CL13+ECTV mice were GzmB$^+$, indicating that in mice chronically infected with CL13, the anti-ECTV CD8$^+$ T-cell response is severely reduced but is not nonexistent.

Impaired expansion of ECTV-specific CD8$^+$ T cells in mice chronically infected with CL13. Our data thus far indicate reduced T-cell proliferation and effector T-cell

FIG 3 Legend (Continued)
mice (right). (B to E) Representative flow cytometry histograms of the mean fluorescence intensity (left) and frequencies of CD44$^+$ (B), CD62L$^+$ (C), PD1$^+$ (D), or KLRG1$^+$ (E) CD8 T cells (right) in the indicated mice. (F and G) Representative flow cytometry dot plots with the mean ± SEM proportion of CD8$^+$ T cells in the spleens of the indicated mice for the expression of GzmB by CD8$^+$ T cells, together with Ki67 (F) or BrdU (G) incorporation, at 3 h following intraperitoneal injection of BrdU. All data were collected at 7 dpi with ECTV in the spleens, and the graphs present the results of at least 3 comparable and independent experiments with an accumulated total of 6 to 12 mice per group. Data are shown as the mean ± SEM. **, $P < 0.01$; ****, $P < 0.0001$.

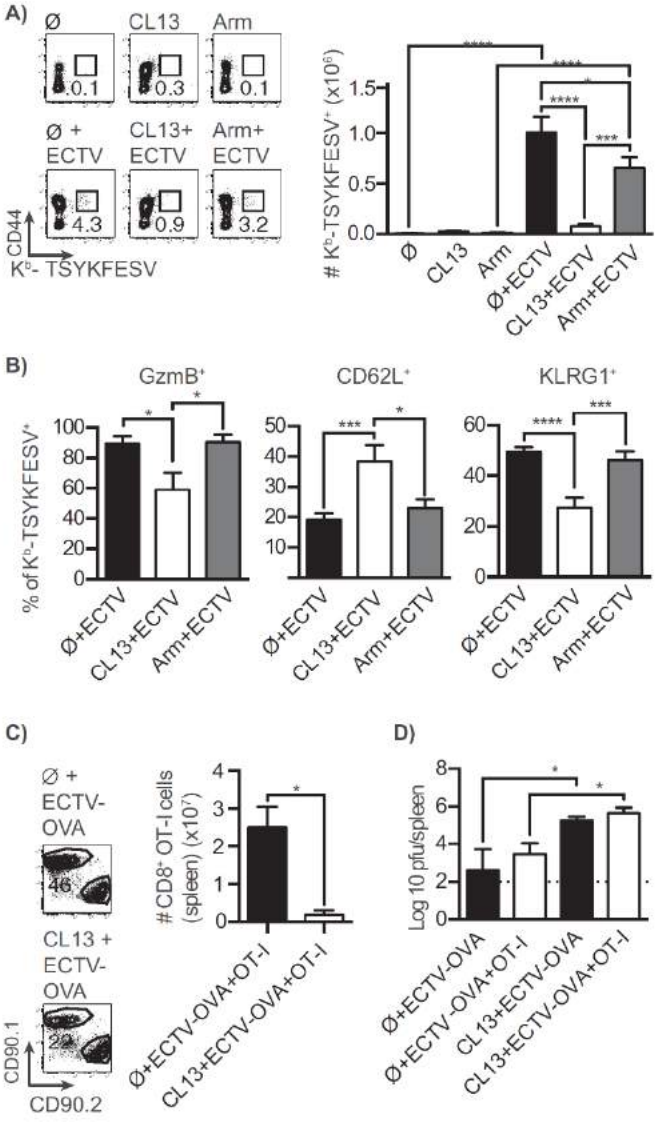

FIG 4 Impaired expansion of ECTV-specific CD8 T cells in mice chronically infected with CL13. (A) Representative flow cytometry dot plots of the expression of CD44 and the T-cell receptor specific for K^b-TSYKFESV in $CD8^+$ T cells, with the frequencies of the gated populations (left) and the number of K^b-TSYKFESV-specific $CD8^+$ T cells (right) in the spleens of the indicated mice. (B) Frequency of K^b-TSYKFESV-specific $CD8^+$ T cells expressing GzmB, CD62L, or KLRG1 in the indicated mice. (C and D) A total of 10,000 OT-I cells were transferred into the mice before ECTV-OVA infection. (C) Representative flow cytometry dot plots gated on $CD8^+$ T cells for OT-I cells (CD90.1) and endogenous $CD8^+$ T cells (CD90.2) at 7 dpi with ECTV (the indicated frequencies are for OT-I cells) (left) and the calculated numbers of OT-I cells (right) in the spleens of the indicated mice. (D) ECTV-OVA titers in the spleens of the indicated mice at 7 dpi. The dotted line indicates the limit of detection of the numbers of ECTV PFU. Data were collected from the spleen at 7 dpi with ECTV, are from at least 2 independent experiments with 7 to 12 mice per group, and are presented as the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

differentiation in response to ECTV in mice chronically infected with CL13, yet it was important to determine the effects of chronic infection in antigen-specific T cells. Thus, we next determined the numbers of $CD8^+$ T cells specific for the K^b-restricted peptide TSYKFESV, which is the immunodominant major histocompatibility complex class I-restricted epitope in ECTV, using K^b-TSYKFESV dimers. As expected, K^b-TSYKFESV-expressing (K^b-TSYKFESV$^+$) $CD8^+$ T cells were virtually absent in ∅, CL13, and Arm mice. After ECTV infection, K^b-TSYKFESV$^+$ $CD8^+$ T cells dramatically expanded in ∅+ECTV and Arm+ECTV mice to ~1.0×10^6 and ~0.65×10^6, respectively (the reason for the difference between the two groups of mice is not clear), while they expanded to only ~0.075×10^6 in CL13+ECTV mice (Fig. 4A). We also evaluated the quality of the K^b-TSYKFESV-specific $CD8^+$ T cells. We found that all K^b-TSYKFEV$^+$-specific $CD8^+$ T cells

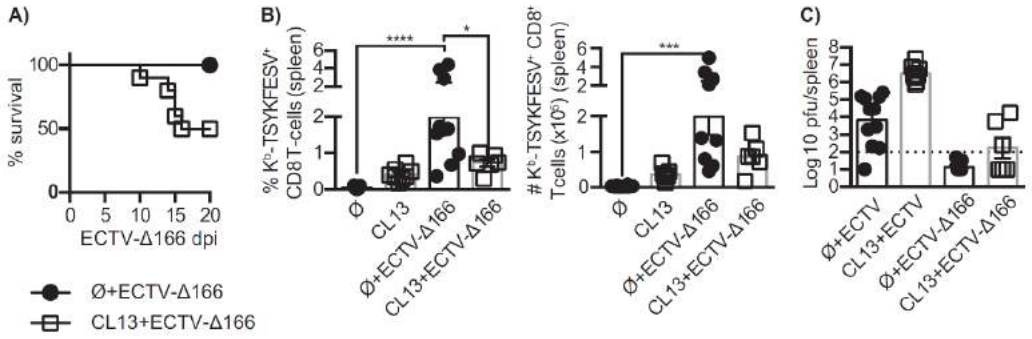

FIG 5 Mice chronically infected with CL13 remain susceptible to highly attenuated ECTV-Δ166. (A) Survival of ∅+ECTV-Δ166 and CL13+ECTV-Δ166 mice. (B) Frequency (left) and absolute numbers (right) of K^b-TSYKFESV-specific $CD8^+$ T cells in the spleens of the indicated mice. (C) ECTV-Δ166 titers at 7 dpi in the spleens of the indicated mice. The dotted line indicates the limit of ECTV detection. Graphs present the results of at least 2 independent and comparable experiments with 6 to 10 mice per group. Data are presented as the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

in ∅+ECTV, CL13+ECTV, and Arm+ECTV mice were $CD44^+$ and PD1 positive ($PD1^+$) (data not shown). However, the frequencies of K^b-TSYKFESV$^+$ $CD8^+$ T cells that were also $GzmB^+$ or $KLRG1^+$ were significantly lower in CL13+ECTV mice than in ∅+ECTV and Arm+ECTV mice. Moreover, in CL13+ECTV mice a significantly higher frequency of K^b-TSYKFESV$^+$ $CD8^+$ T cells remained $CD62L^+$. Thus, chronic CL13 infection drastically reduced the expansion of antigen-specific $CD8^+$ T cells, and those that expanded had a dimmed effector phenotype.

To further test the role of the chronic infection environment on antigen-specific $CD8^+$ T-cell expansion, we transferred 10,000 T-cell receptor (TCR)-transgenic OT-I $CD8^+$ T cells (CD45.1), which are specific for the ovalbumin (OVA) epitope SIINFEKL, into ∅ and CL13 mice (B6-CD45.2) and challenged them with ECTV expressing OVA (ECTV-OVA). Seven days later, we determined the expansion of the OT-I cells in the spleen. We found that, in agreement with the results of experiments with polyclonal T cells presented in Fig. 2, OT-I cells expanded 5-fold more in ∅ mice challenged with ECTV-OVA (∅+ECTV-OVA mice) than in CL13 mice challenged with ECTV-OVA mice (CL13+ECTV-OVA mice) (Fig. 4C). Moreover, the virus loads were not reduced in CL13+ECTV-OVA mice to which OT-I cells were transferred compared to those in CL13+ECTV-OVA mice (Fig. 4D). These data confirm that the CL13 chronic infection environment reduces the ability of antigen-specific $CD8^+$ T cells to expand and that the few expanded cells do not provide antiviral protection.

Mice chronically infected with CL13 remain susceptible to highly attenuated ECTV-Δ166, suggesting altered IFN-I production and/or sensing. WT ECTV is extremely virulent: the dose that kills 50% of genetically susceptible BALB/c mice (the 50% lethal dose [LD_{50}]) is ~1 PFU (29). Given that CL13+ECTV mice generated mild T-cell responses to WT ECTV, it was of interest to determine whether they could control highly attenuated ECTV mutants. The gene EVM166 encodes a type I interferon (IFN-I) decoy receptor in ECTV. We have previously shown that ECTV-Δ166 was not lethal for BALB/c mice at doses as high as 5×10^7 PFU (29). However, it is fully lethal to mice deficient in the IFN-I receptor (29). When CL13 mice were infected with 3,000 PFU of the ECTV-Δ166 mutant (CL13+ECTV-Δ166 mice), 50% succumbed (Fig. 5A) and mounted a variable small or absent K^b-TSYKFESV-specific CD8 T-cell response (Fig. 5B), and in contrast to ∅ mice infected with ECTV-Δ166 (∅+ECTV-Δ166 mice), CL13+ECTV-Δ166 mice had virus in their spleens at 7 dpi (Fig. 5C), indicating a surprisingly poor attenuation of ECTV-Δ166 in CL13 mice and suggesting that chronic CL13 infection results in inefficient IFN-I production and/or sensing.

Mice chronically infected with CL13 resist ECTV-Δ036 and mount an immune response that is protective against WT ECTV challenge. Next, we tested survival in response to infection with ECTV deficient in EVM036 (ECTV-Δ036), which encodes a protein required for the formation of mature enveloped virions and viral plaques and

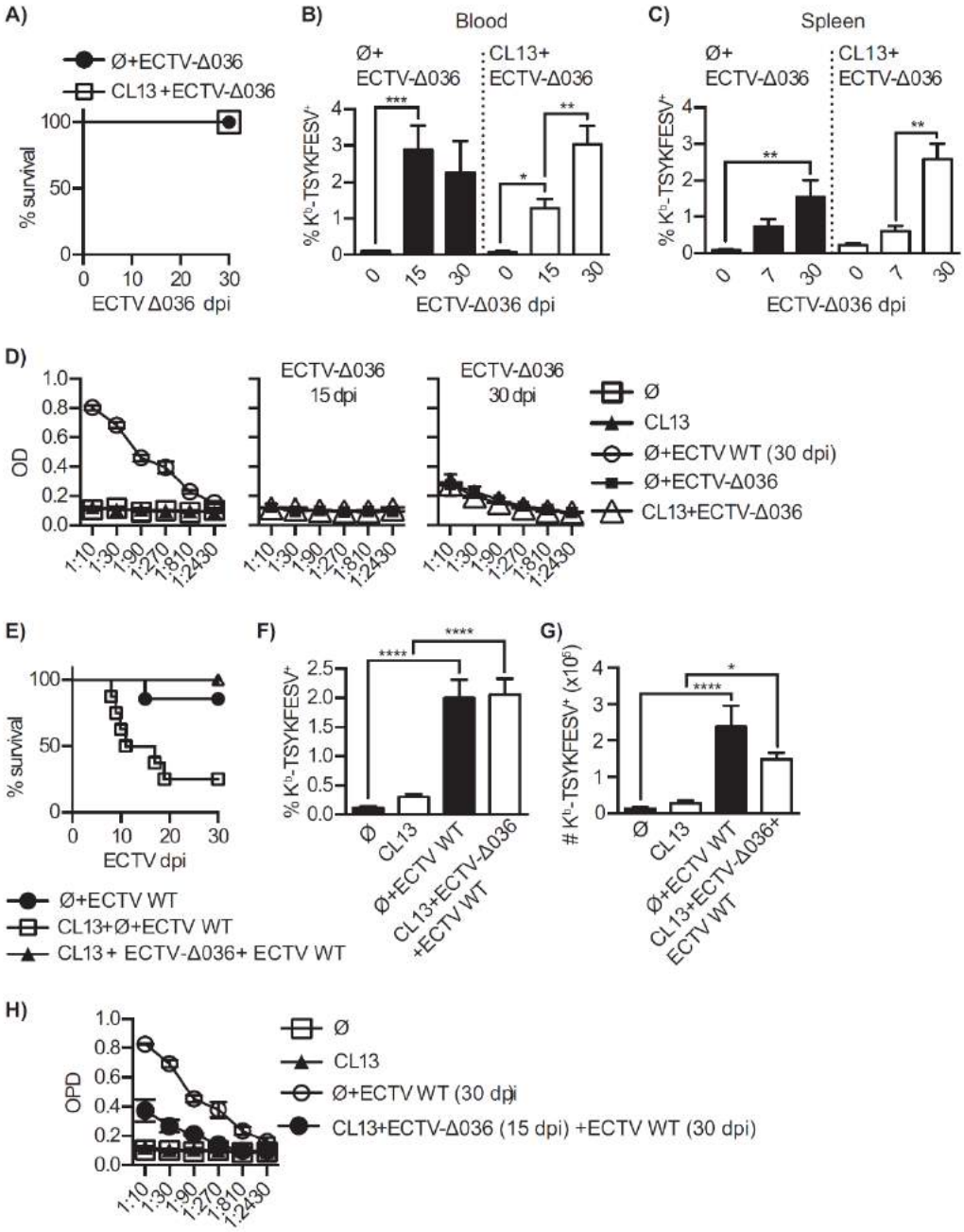

FIG 6 Mice chronically infected with CL13 resist ECTV-Δ036 and mount an immune response that is protective against WT ECTV challenge. (A) Survival of the indicated mice. (B) Frequency of K^b-TSYKFESV-specific CD8$^+$ T cells in the blood of the indicated mice on the indicated days after ECTV-Δ036 infection. (C) As for panel B but in the spleen. (D) ECTV-specific total IgG in the blood of the indicated mice, represented as the optical density (OD) at 450 nm. All ELISAs were performed simultaneously, and data are representative of those from at least 2 independent and similar experiments. (E) Survival of the indicated mice. (F) Frequency of K^b-TSYKFESV-specific CD8$^+$ T cells in the spleen at 30 dpi with WT ECTV in the indicated mice. (G) As for panel F but displaying the calculated absolute numbers. (H) ECTV-specific total IgG in the blood of the indicated mice. ELISAs were performed together with those for panel D, and the results for ∅ mice infected with WT ECTV are the same as those shown in panel D and are shown for comparison. The graphs present the results of at least 2 independent and comparable experiments with 6 to 10 mice per group. Data are presented as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

for efficient systemic virus spread from the footpad and which is not lethal at 5×10^7 PFU in BALB/c mice but which is lethal to SCID mice, which are deficient in adaptive immunity (40). We found that, like ∅ mice, CL13 mice survived ECTV-Δ036 challenge (Fig. 6A), with no signs of disease or detectable virus in the spleen being seen at 7 or 30 dpi (data not shown).

Given that mice chronically infected with CL13 survived ECTV-Δ036 challenge with no signs of disease, we tested whether they generated an anti-ECTV immune response. Notably, compared to ∅ mice, ∅ mice challenged with ECTV-Δ036 (∅+ECTV-Δ036 mice) and CL13 mice challenged with ECTV-Δ036 (CL13+ECTV-Δ036 mice) had significantly increased frequencies of K^b-TSIKFESV$^+$ CD8$^+$ T cells in blood at 15 and 30 dpi (Fig. 6B) and in the spleen at 7 and 30 dpi (Fig. 6C) with ECTV-Δ036. On the other hand, ∅+ECTV-Δ036 and CL13+ECTV-Δ036 mice had low but detectable levels of anti-ECTV IgG in their sera at 30 dpi but not at 15 dpi with ECTV-Δ036 (Fig. 6D).

Given that CL13+ECTV-Δ036 mice survived infection and mounted an immune response, we next tested whether this response could be sufficient for protection against WT ECTV challenge. Thus, we infected ∅, CL13, and CL13+ECTV-Δ036 mice with WT ECTV at 15 dpi with ECTV-Δ036. Strikingly, all CL13+ECTV-Δ036 mice infected with WT ECTV (CL13+ECTV-Δ036+ECTV-WT mice) survived infection, whereas most CL13 mice infected with WT ECTV succumbed and most ∅+ECTV mice survived (Fig. 6E). When their CD8$^+$ T-cell responses were analyzed at 30 dpi with WT ECTV, CL13+ECTV-Δ036+ECTV-WT mice had frequencies (Fig. 6F) and numbers (Fig. 6G) of K^b-TSYKFESV$^+$ CD8 T cells similar to those in ∅ mice infected with WT ECTV, had IgG specific to ECTV in their sera (Fig. 6H), and had no detectable ECTV titers in the spleen or liver (data not shown). Because the challenge with WT ECTV was done at 15 dpi with ECTV-Δ036 and at this time K^b-TSYKFESV$^+$ CD8$^+$ T cells but not anti-ECTV IgG was detected in blood, these data strongly suggest that mice chronically infected with CL13 mount a CD8$^+$ T-cell response to a highly attenuated ECTV mutant that can protect them from subsequent challenge with virulent ECTV.

DISCUSSION

It is well established that, in addition to viral persistence, chronic infection with LCMV causes immune suppression, as revealed by impaired immune responses to other infectious agents. For example, chronic infection with LCMV causes defective antibody (Ab) responses and increased lethality to vesicular stomatitis virus (VSV) and decreased T-cell responses to vaccinia virus (VACV) after intravenous (i.v.) inoculation at high doses. However, chronic LCMV infection does not increase VSV lethality following footpad infection (23, 41). Moreover, VSV and VACV are not natural mouse pathogens and the i.v. route of inoculation bypasses all the barriers imposed on the virus during natural entry. It has also been shown that chronic infection with CL13 also results in increased lethality following i.v. infection with *Histoplasma capsulatum* (20), reduced CD8 T-cell responses to influenza A virus (IAV), VACV, and herpes simplex virus (HSV) (42), and defective IFN-I and NK cell responses to mouse cytomegalovirus (MCMV) (22). Some proposed mechanisms for the immunosuppression caused by CL13 are the killing of dendritic cells (DCs) and impaired antigen presentation (23, 43), the infection of fibroblastic reticular cells in secondary lymphoid organs (44), quantitative and qualitative defects of plasmacytoid DCs (pDCs) (22), and, more recently, an impaired Fc gamma receptor-mediated Ab effector function (45), yet the effect of chronic infection on intrinsic and acquired resistance to a highly pathogenic host-specific virus has not been thoroughly studied, and an in-depth analysis of the T-cell response has not been performed.

We have recently shown that B6 mice chronically infected with CL13 lose their natural resistance to mousepox following ECTV infection in the footpad, while mice convalescent from Arm infection are mostly resistant. We have also shown that CL13 and Arm mice have reduced numbers of NK cells, but only CL13 mice have NK cells with maturational defects. Moreover, we have demonstrated that, different from the findings for NK cells from ∅+ECTV and Arm+ECTV mice, NK cells do not respond to ECTV in the spleen or liver of CL13+ECTV mice at 5 dpi, yet the NK cell defect is not the single culprit for the susceptibility of CL13+ECTV mice to lethal mousepox because the adoptive transfer of NK cells from naive mice does not restore resistance to mousepox (35). Given that CD8$^+$ and CD4$^+$ T-cell responses are critical for ECTV control (33, 46), it was important to test whether defective T-cell responses during chronic LCMV infection could also contribute to the loss of resistance to mousepox.

In this study, we first confirmed that CL13+ECTV mice are susceptible to mousepox and that Arm+ECTV mice are mostly resistant and then thoroughly compared the anti-ECTV T-cell responses in ∅+ECTV, CL13+ECTV, and Arm+ECTV mice, with a particular emphasis on $CD8^+$ T cells. Our data show that at 7 dpi with ECTV, $CD8^+$ and $CD4^+$ T cells prominently expanded in the spleens of ∅+ECTV and Arm+ECTV mice but not in those of CL13+ECTV mice. We also showed that the failure of $CD4^+$ and $CD8^+$ T cells to expand properly in CL13+ECTV mice was due to their inability to enter the cell cycle, with many more cells being in G_0 phase (Ki67-negative cells) in CL13+ECTV mice than in ∅+ECTV and Arm+ECTV mice. Also, a much lower proportion of the $Ki67^+$ cells in CL13+ECTV mice than those in ∅+ECTV and Arm+ECTV mice were $BrdU^+$ (i.e., synthesized DNA), indicating that the rate of proliferation of the cycling cells was much lower in CL13+ECTV mice. Of note, before ECTV infection, both CL13 and Arm mice had increased frequencies of apparently slowly proliferating T cells or T cells stuck in G_2 phase ($Ki67^+$ $BrdU^-$), likely representing LCMV-specific cells. Despite this similitude, after ECTV infection, T cells strongly increased their proliferation in Arm mice that had recovered from infection but very poorly in mice chronically infected with CL13.

Our data also indicate that the failure of $CD8^+$ and $CD4^+$ T cells to expand in response to ECTV in CL13+ECTV mice was likely due to T-cell-extrinsic factors, as $CD8^+$ and $CD4^+$ T cells from ∅ mice proliferated much less in CL13+ECTV mice than in ∅+ECTV mice. This is consistent with previous reports indicating inefficient antigen presentation (23, 43), excessive interleukin-10 (IL-10) production (9, 47), or an altered IFN-I response (10, 48–51). Of note, while there was some proliferation of the transferred T cells in CL13+ECTV mice, they did not control the virus, even though the number of $CD8^+$ T cells transferred was sufficient to rescue $CD8^+$ T-cell-deficient ($CD8^{-/-}$) mice from mousepox (33).

Consistent with their expansion and proliferation, GzmB, CD44, CD62L, PD1, and KLRG1 staining showed that large numbers of bulk $CD8^+$ and $CD4^+$ T cells differentiated into effectors in ∅+ECTV and Arm+ECTV mice but not in CL13+ECTV mice, further emphasizing their defective response. Of note, combining differentiation into $GzmB^+$ cells with Ki67 and BrdU staining indicated that the differentiation of $CD8^+$ T cells into effector cells in CL13+ECTV mice was greatly diminished but not nil.

In addition to analyzing bulk $CD8^+$ and $CD4^+$ T-cell responses, we also examined the response to the K^b-restricted immunodominant epitope TSYKFESV and found that it was somewhat decreased in Arm+ECTV mice compared to its level in ∅+ECTV mice and severely curtailed, but not nil, in CL13+ECTV mice. Notably, in addition to their numbers being decreased, the few K^b- $TSYKFESV^+$ $CD8^+$ T cells found in CL13+ECTV mice did not properly differentiate to full-fledged effectors. On the other hand, while Arm+ECTV mice had a reduction in the numbers of K^b-$TSYKFESV^+$ $CD8^+$ T cells, their differentiation into effectors was normal. Of note, the bulk of the $CD8^+$ T-cell response between ∅+ECTV and Arm+ECTV mice was similar. While the reason for the decreased number of K^b-TSYKFESV-specific $CD8^+$ T cells in Arm+ECTV mice is not clear, we speculate that it may be due to a shift in immunodominance to another ECTV epitope cross-reactive with one in LCMV, as suggested by reports showing that LCMV and VACV have cross-reactive epitopes (52, 53). The adoptive transfer of SIINFEKL-specific OT-I CD8 T cells and infection with ECTV-OVA confirmed the reduced but not nil expansion of antigen-specific $CD8^+$ T cells and the negative effect of the chronic infection environment in CL13+ECTV mice.

Because the results of our experiments indicate that the reason for the inability of T cells to respond in CL13+ECTV mice is cell extrinsic, we tested the effects of blocking PD1 and TIGIT, which are inhibitory and which have been implicated in the T-cell exhaustion and chronicity of CL13 (38, 54, 55). However, we did not observe any improvement in survival (data not shown). Also, the adoptive transfer of splenocytes deficient in the receptor for IL-10, an immunosuppressive cytokine also shown to participate in the persistence of CL13 (9, 56, 57), conferred only a slight increase in the life span but did not confer an increase in survival in response to mousepox in CL13+ECTV mice (data not shown).

Given the high susceptibility of CL13 mice to mousepox, it was of interest to determine whether they were also susceptible to highly attenuated ECTV mutants. One important mutant to test was ECTV-Δ166, which lacks a decoy receptor that inactivates mouse alpha interferon (IFN-α) but not IFN-β (29, 58). Of note, the role of IFN-I during CL13 infection is complex. IFN-I is important for the maintenance of chronic LCMV infection and T-cell dysfunction, because treatment with Abs to IFNAR accelerates recovery from chronic CL13 infection (10, 59). The main culprit is IFN-β and not IFN-α because Abs to IFN-β but not Abs to IFN-α protected CL13-infected mice from splenic disorganization, reduced DC infection, increased $CD8^+$ T-cell responses, and accelerated CL13 clearance (60), yet CL13 still establishes chronic infection with enhanced titers in mice deficient in the interferon receptor (*Ifnar1*$^{-/-}$ mice) and in IFN-β (57, 61). Moreover, IFN-I is barely detectable in CL13 mice after the acute phase, and CpG treatment induces IFN-I in ∅ mice but not in CL13 mice (22). Furthermore, treatment of CL13-infected mice at 3 and 5 dpi with a mixture of IFN-α and IFN-β enhances the anti-CL13 $CD8^+$ T-cell response (62). For ECTV, IFN-α is critical for resistance to mousepox (29, 63, 64), but IFN-β is not because IFN-β-deficient mice are resistant to mousepox (not shown). Notably, 50% of CL13+ECTV-Δ166 mice succumbed to mousepox, suggesting poor IFN-α production or signaling in CL13+ECTV mice. This will be an important avenue of future exploration.

It was also of interest to test ECTV-Δ036, which is as attenuated as ECTV-Δ166, but the reason for its attenuation is its deficient spread. Of note, despite its attenuation, ECTV-Δ036 induces an immune response in BALB/c mice (40). Notably, CL13+ECTV-Δ036 mice were fully resistant to mousepox and eliminated ECTV-Δ036. Moreover, they mounted a relatively strong K^b-TSYKFESV-specific $CD8^+$ T-cell response that was comparable to that in ∅+ECTV-Δ036 mice at 7, 15, and 30 dpi. The Ab response to ECTV-Δ036 was also similar in ∅+ECTV-Δ036 and CL13+ECTV-Δ036 mice, but it was relatively weak and still not detectable at 15 dpi. Notably, CL13+ECTV-Δ036 mice challenged with WT ECTV were resistant to mousepox, maintained anti-ECTV $CD8^+$ T cells and Abs, and eliminated WT ECTV. These results indicate that while chronic infection results in profound immunosuppression, resistance to some, but not all, types of attenuated viruses is possible. Moreover, the data also indicate that protective immunization is achievable in highly immunosuppressed individuals, provided that an adequate stimulus is used. We have previously shown that mice susceptible to mousepox can be protected by preexisting Abs or memory $CD8^+$ T cells (65–67). Our results suggest that the mechanism of protection by ECTV-Δ036 is through $CD8^+$ T cells because at the time of WT ECTV challenge (15 dpi with ECTV-Δ036), CL13+ECTV-Δ036 mice had already mounted a $CD8^+$ T-cell response but not an Ab response. However, to reach a definitive conclusion, this hypothesis will have to be thoroughly tested. Unfortunately, a deficiency or depletion of CD8 T cells or B cells in Ab- or $CD8^+$ T-cell-immune mice results in susceptibility to mousepox. Thus, future experiments will require T-cell- or Ab-specific immunization, which could be challenging in the context of chronic infection.

In summary, our data indicate that despite increased basal activation levels, chronic infection with CL13 results in strongly impaired $CD8^+$ T-cell responses to ECTV and susceptibility to mousepox, yet immunization with an attenuated virus deficient in spread induces an immune response that prevents mousepox and eliminates WT ECTV. Together, these results highlight some of the mechanisms involved in immunosuppression during chronic viral infection, provide evidence for further avenues of exploration, and demonstrate that protective immunization against highly pathogenic viruses of individuals immunosuppressed by viral infection is possible, provided that the appropriate immunogen is used.

MATERIALS AND METHODS

Mice. All experiments were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC). C57BL/6 (B6-CD45.2$^+$) and NCI B6-Ly5.1/Cr (B6-CD45.1$^+$) mice were purchased from Charles River or bred in-house from breeders from Charles River. B6-CD45.1/2 mice were F1 hybrids from B6 and B6-CD45.1 mice. OT-I mice (Jackson) were bred with *Rag*$^{-/-}$ mice (The Jackson

Laboratory) and then with B6 Thy1.1 mice (The Jackson Laboratory) and were thereafter bred in-house. Males and females 6 to 10 of age weeks were used. After infection, the animals were observed for signs of disease and distress (lethargy, ruffled hair, weight loss, skin rash, and eye secretions) and euthanized if they were unresponsive to touch or without voluntary movements.

Viruses. LCMV CL13 and LCMV Arm were gifted by E. John Wherry (University of Pennsylvania, Philadelphia, PA) and propagated, and their titers were determined as described previously (7, 11). ECTV Moscow was propagated and titers were determined as described previously (17, 18). LCMV CL13 infections were performed intravenously with 2×10^6 PFU, and LCMV Arm infections were performed intraperitoneally with 2×10^5 PFU. ECTV, ECTV-Δ166, and ECTV-Δ36 infections were performed in the footpad with 3.0×10^3 PFU.

Flow cytometry. Flow cytometry was performed as previously described (17, 18). We used the following Abs: anti-CD45.1 (clone A20, phycoerythrin [PE]-Cy7; BioLegend), anti-CD45.2 (clone 104, allophycocyanin [APC]; BioLegend), anti-BrdU (clone PRB-1, fluorescein isothiocyanate [FITC]; eBioscience), anti-T-cell receptor beta (anti-TCR-β; clone H57-597 [Brilliant Violet 605 {BV605}; BioLegend] and clone H57-597 [BV786; BD]), anti-CD8 (clone 53-6.7, BV711 or peridinin chlorophyll protein-Cy5.5; BioLegend,), anti-CD4 (clone RM4-5, BV650 or BV785; BioLegend), anti-KLRG1 (clone 2F1/KLRG1, PE-Cy7 or APC; BioLegend), anti-granzyme B (clone GB11, Pacific Blue; BioLegend), anti-IFN-γ (clone XMG1.2, PE-Cy7; BioLegend), anti-CD44 (clone IM7, Brilliant Ultraviolet 395 [BUV395]; BD), anti-CD62L (clone MEL-14, FITC or PE-Cy7; BioLegend), and anti-PD1 (clone 29F.1A12, FITC; BioLegend). For TSYKFESV staining, we conjugated 7.5 μg of the TSYKFESV peptide (GenScript) with 20 μl of the K^b dimer (BD Dimer X) in 72.5 μl of phosphate-buffered saline (PBS) overnight at 37°C and on the following day incubated the solution with 20 μl of PE-conjugated IgG1 (clone RMG1-1; BioLegend) at room temperature for 1 h. Briefly, all organs were made into single-cell suspensions. Red blood cells were lysed with 0.84% NH_4Cl, washed in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, brefeldin A (20 μg/ml; Sigma-Aldrich), and 2 μg/100 μl of TSYKFESV peptide (GenScript), and resuspended at 2.0×10^6 cells/100 μl. After 5 h of incubation, Ab 2.4G2 (anti-Fc gamma II/III receptor; American Type Culture Collection) was added together with the stains for the extracellular molecules. After 25 min or after 60 min, if staining for TSYKFESV was also performed, the cells were fixed, permeabilized, and stained for 30 min for the cytoplasmic or nuclear molecules (with a BD Cytofix/Cytoperm kit and an Invitrogen Fixation/Permeabilization kit, respectively) according to the manufacturers' instructions. Data were acquired with a BD LSRFortessa cytometer and analyzed with FlowJo software (v10; TreeStar).

BrdU incorporation assay. We intraperitoneally injected mice with 2 mg of BrdU labeling reagent (Life Technologies). Following 3 h of incubation in the host, we collected their spleens and made them into single-cell suspensions. The cells were then stained for cell surface molecules, fixed, permeabilized for nuclear molecules, and incubated with RNase-free DNase (Qiagen) at 37°C for 1 h, and anti-BrdU antibody was added to evaluate incorporation.

CellTrace violet labeling and adoptive transfers. Splenocytes from ∅ B6 mice were isolated in DMEM supplemented with 10% fetal bovine serum and brefeldin A (Sigma-Aldrich). Red blood cells were lysed with 0.84% NH_4Cl. Cells were counted, washed with phosphate-buffered saline (PBS)–0.1% bovine serum albumin (BSA), filtered with a 40-μm-mesh-size sterile mesh, resuspended at a concentration of 5×10^7 cells/ml in PBS–0.1% BSA, and labeled with 4 μM CellTrace violet stain (Thermo Fisher). Mice were injected intravenously with 2×10^7 splenocytes and immediately infected with ECTV. For OT-I cell adoptive transfers, splenocytes were isolated in a similar manner and injected intravenously with 1.0×10^4 OT-I $CD8^+$ T cells.

Anti-ECTV IgG enzyme-linked immunosorbent assay (ELISA). On the day prior to the assay, enzyme immunoassay/radioimmunoassay 96-well round-bottom 3366 Costar plates were coated with 5×10^4 PFU of vaccinia virus in 100 μl of PBS/well and incubated overnight at 4°C. On the following day, the liquid in each well was removed by flicking the plates over bleached water. The plates were blocked by the addition of 200 μl of 5% (wt/vol) instant nonfat dry milk (Signature Select) diluted in PBS and incubated for 2 h at 37°C. The plates were washed 3 times with 200 μl of 5% (vol/vol) Tween 20 (Fisher Scientific) diluted in PBS, and the liquid was forcefully removed by flicking each time. Serum samples were serially diluted in 5% BSA–5% Tween 20, and 100 μl of each dilution was added to each well of the plates. The plates were incubated for 2 h at 37°C and washed 3 times as described above. After this, 100 μl of affinity-purified antibody consisting of peroxidase-labeled goat anti-mouse IgG (gamma globulin) human serum-adsorbed reagent diluted (1:2,000) in 5% Tween 20 diluted in PBS was added, and the plates were incubated for 0.5 h at 37°C. The plates were washed 3 times as described above. Peroxide tablets and *o*-phenylenediamine dihydrochloride (OPD) substrate tablets (Sigmafast OPD) were diluted according to the manufacturer's instructions, and 200 μl of the dilution was added to each well. The plates were developed in the dark at room temperature for 0.5 h. The reaction was stopped by the addition of 50 μl/well of a 3 M solution of HCl. The plates were read at 450 nm under the OPD setting in the KCjunior program utilizing a BioTek Instruments μQuant plate reader.

Statistical analysis. Data were analyzed using Prism (v6) software. For the analysis between 2 independent groups, we used a parametric unpaired *t* test. For the difference between 2 or more groups, we used one-way analysis of variance. For the *post hoc* analysis, we used Tukey's multiple-comparison test. To study differences in survival, we used the log-rank (Mantel-Cox) test for the comparison of survival curves. All experiments were repeated a minimum of 2 times with similar results each time.

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P.A.-P. and L.J.S. conceived of and designed the experiments, analyzed the results, and cowrote the paper. P.A.-P. performed most of the experiments. L.J.S. conceived of the initial idea and supervised the work. M.C.-N. supervised the work and contributed intellectually. M.F., C.J.K., C.S., C.R.M.-S., and E.B.W. helped with some of the experiments.

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Part III - Discussion

CL13 infection renders NK cells unable to promptly respond to unrelated ECTV infection

We used chronic CL13 infection and acute Arm infection to understand how chronic viral infection and convalescence from acute infection shapes the NK cell phenotype and response to a subsequent infection with an unrelated virus. Before our work, Whitmire and colleagues had shown that both Arm and CL13 infections lead to reduced numbers of NK cells during the acute phase of the infection (1). Here we show that low NK cell numbers persist in the long-term not only in the case of chronic CL13 infection, but also in mice convalescent from Arm, where virus is no longer detectable by plaque assay as early as 8dpi. This is puzzling and suggests that viral infection with LCMV impacts NK cells in a long-lasting manner independently of chronicity. The numbers of NK cells reach a nadir at 15 dpi with either CL13 or Arm infection, with a lower value in CL13 infected mice. Interestingly, at 30-35 dpi NK cell express Ki67 and their numbers are higher than at 15 dpi, suggesting that they can eventually recover.

At 8 dpi with CL13 or ARM, NK cells show a strongly activated and mature phenotype. The frequencies of R3 NK cells dramatically increase, while there are clear signs of strong activation, with high levels of NK cells producing GzmB and expressing KLRG1. Again, the magnitude of this response is higher with Arm than with CL13 infection, particularly regarding the expansion of the R3 NK cell subset. As both infections progress, NK cells develop an immature phenotype, with progressively lower frequencies of R3 NK cells and increased frequencies of the highly immature R1 NK cells, reaching a peak at 15 dpi with Arm. Later, the profile of NK cells begins to improve in Arm infected mice, with increased frequencies of more mature and functional R2 NK cells. In contrast, CL13 infected mice show growing frequencies of R1 NK cells, and limited recovery of R2 NK cell frequencies. Interestingly, the changes in maturation correlated with decreased expression of activating Ly49H and inhibitory Ly49C/I receptors which reach their nadir at 15 dpi with Arm but persist with CL13 at 30-35 dpi. This is surprising because NK cells acquire expression of Ly49 receptors at very early stages of maturation in the bone marrow following interaction with stroma cells (2,3). Altogether, we hypothesize that following Arm and CL13 infection, NK cells respond promptly to infection and then a negative feedback mechanism enters into action: the expression of the inhibitory receptor KLRG1 increases, suppressing NK cells effector activity. After that, terminally differentiated R3 NK cells may die and be replaced by a pool of immature NK cells that migrates from the bone marrow.

While this process may be effective after Arm infection, in which there is a NK cell repopulation of more mature cells in the periphery in the absence of virus at 30-35 dpi, during CL13 infection, the persisting viral *stimuli* does not allow an interruption of the negative feedback loop, resulting in immaturity and reduced numbers of NK cells in the periphery as late as 30-35 dpi. Our data supports this theory to a limited extent. We have found CL13 enters the bone marrow, because we detected viral RNA there (data not shown), even at 30-35 dpi. Hence, virus stimuli is widespread, and wherever we found virus, we also detected reduced numbers of NK cells, and an immature phenotype in those that remain.

To fully prove this theory, we could evaluate to what extent CL13 infection in the bone marrow affects the interaction of NK cells with bone marrow stromal cells. It is known that CL13 infection induces persistent structural changes of the stroma of lymphoid organs in the periphery (4,5). It is therefore possible that similar structural changes also occur in the bone marrow. However, Arm infection is not known to induce changes in the stroma of periphery lymphoid organs (4,5). Also, we have previously reported that alteration in the stroma of the bone marrow leads to defective NK cells maturation in old wild type (WT) mice and that leads to the accumulation of immature NK cells in the periphery (6). Microscopy imaging of the bone marrow of CL13 and Arm infected mice, at various timepoints post infection should help address this question. Also, we could evaluate NK cell death, such as by the incorporation of PI and the binding of Annexin V. Furthermore, proliferation assays should be optimized to evaluate incorporation of BrdU at several timepoints post infection and in several organs, including bone marrow and spleen. Finally, we could evaluate migration of NK cells from the bone marrow to the periphery, possibly by optimizing a technique in which cells from a CD45.1 implanted femur are exposed to infection within a CD45.2 host and then assess their migration to the periphery after CL13 infection.

Despite their immaturity, NK cells in chronically infected mice have a relatively activated phenotype, with increased expression of GzmB, IFN-γ and KLRG1. A recent report showed similar data regarding persistent activation but did not identify the immature phenotype. Interestingly, they showed that the pre-activated NK cells were more potent at eliminating B16 melanoma tumors than NK cells in naïve mice, indicating that the pre-activation may be beneficial to combat tumors (7). Yet, in the case of ECTV infection, persistent activation was detrimental as NK cells in CL13+ECTV mice failed to become

fully activated as demonstrated by the lack of further GzmB, IFN-γ and KLRG1 upregulation and did not protect from mousepox.

NK cells have a crucial role in the intrinsic resistance of B6 mice to mousepox (6,8). Here we demonstrated that B6 mice chronically infected with CL13 lose their intrinsic resistance to ECTV, whereas mice that recover from Arm mostly survive. Following 5 dpi with ECTV, NK cells from previously naïve mice or convalescent from acute Arm infection, show extensive proliferation, particularly among the highly functional R2 NK cells, as indicated by increased Ki67 expression and abundant incorporation of administered BrdU. This was particularly notorious in Arm convalescent mice, possibly due to their lower numbers of NK cells. In contrast NK cells from CL13+ECTV mice showed minimal proliferation and expansion.

At 5 dpi with ECTV, there were very high frequencies of NK cells in the spleens of ARM+ECTV and Ø+ECTV mice that produced GzmB and IFN-γ. Notably, the negative feedback loop that we observed after LCMV infection was also apparent after ECTV infection. In both Arm+ECTV and Ø+ECTV there was a sharp upregulation of KLRG1, possibly indicating high levels of NK cell activation that need to be suppressed to prevent immunopathology (9,10). In contrast, NK cells from CL13+ECTV infected mice showed impaired GzmB production and minimal increases in IFN-γ production. To test whether this would translate into decreased NK cell cytolytic activity *in vivo*, we co-transferred *tap1*$^{-/-}$ and B6 splenocytes into the different mouse groups. Full fledge effectors NK cells, can recognize the lower MHC-I expression of *tap1*$^{-/-}$ splenocytes compared to WT and preferentially kill them (11,12). We observed that NK cells from naïve, Arm or CL13 infected B6 mice preferentially kill *tap1*$^{-/-}$ splenocytes. This is quite surprising, since numbers of NK cells are much lower in LCMV infected mice than in naïve mice. However it coincides with recent experiments that showed that NK cells from chronically CL13 infected mice have increased *in vitro* killing activity (7). Yet, after ECTV infection, which activates NK cells, preferential killing of *tap1*$^{-/-}$ cells increased in the Ø+ECTV and in the Arm+ECTV groups but not in CL13+ECTV mice. This was likely due to the inability of their NK cells to become activated and not to the reduced NK cell numbers because Arm recovered mice had reduced NK cell numbers too.

In summary, our data indicate that despite increased basal activation levels, chronic infection can result in impaired NK cell maturation and activation in response to secondary infections, which can contribute to increased susceptibility to opportunistic infections in chronically infected individuals.

The recruitment of NK cells to respond to ECTV infection in chronically CL13 infected mice is likely reduced

CXCL9 is an important chemokine for the recruitment of NK cells (13). Following ECTV infection, DCs and monocytes produce large amounts of CXCL9 that is recognized by immature NK cells via the CXCR3 receptor (13–15). This leads to the recruitment of fully fledged cytolytic R3 NK cells, critical in the control of ECTV infection (8,9). Simultaneously in this process, they downregulate the expression of the CXCR3 receptor (16). One may hypothesize that NK cells may develop some level of tissue residency following infection, and therefore should not be recruited elsewhere. This was already described to a certain degree to occur during LCMV infection, with the persistence of a population of liver-resident NK cells (17).

Interestingly, we see that NK cells downregulate the expression of the CXCR3 receptor in Ø+ECTV and Arm+ECTV infected WT mice but fail to do so in CL13+ECTV infected mice. This further strengthens our hypothesis in which CL13 renders NK cells arrested in an immature state. Yet, it also supports the hypothesis that the process leading to the recruitment of NK cells in CL13+ECTV infected mice is impaired. In fact, we observed that the numbers of monocytes and DCs, powerful producers of the recruiting chemokine CXCL9, did not increased in Ø+ECTV and Arm+ECTV but not in CL13+ECTV mice (data not shown). Moreover, the frequency of both DCs and monocytes producing CXCL9 was much lower in CL13+ECTV infected mice than in Ø+ECTV or Arm+ECTV, where CXCL9 production increased substantially following ECTV infection (data not shown).

IFN-I signaling is required for normal NK cell development during CL13 infection

There is plenty of research about the role of IFN-I signaling in the establishment of chronic CL13 infection. IFN-I production peaks shortly after CL13 infection, yet progressively decreases from there to values that may be lower than in previously naïve mice (18). Despite this fact, many associate the presence of IFN-I signaling to the persistence of chronic CL13 infection (19,20). Others consider IFN-I to be pivotal in eliciting effective anti-viral responses (21).

It was previously shown that IFN-I signaling is important for NK cell maturation after viral infection (22). Moreover, NK cells have been associated for a long time with the establishment of chronic CL13 infection (23–25). Therefore, one of the hypothesis currently defended is that NKG2D-mediated activation of NK cells results in the killing of $CD4^+$ T cells (23–25).

With this in mind, we hypothesized that IFN-I signaling could lead to the expansion of dysfunctional NK cells. To test for this, we infected with CL13 *Ifnar1*$^{-/-}$ and *Ifnb*$^{-/-}$ mice, in which both IFN-α and IFN-β, or only IFN-β signaling is absent and observed the NK cell phenotype at different timepoints. We found that both mouse strains had increased virus loads at 8 dpi, suggesting this IFN-I production is critical to the early control of CL13 infection. Interestingly, both strains showed impaired NK cell maturation, activation and effector activity. At 8 dpi with CL13, NK cells of *Ifnar1*$^{-/-}$ mice had higher frequencies of immature R1 NK cells, minimal frequencies of differentiated R3 NK cells, and reduced frequencies of $GzmB^+$ and $KLRG1^+$ cells as compare with WT mice with *Ifnb*$^{-/-}$ mice having an intermediate phenotype . This suggests that both IFN-α and IFN-β contribute to the proper maturation and activation of NK cell at 8 dpi with CL13.

Our data also suggests that IFN-I signaling is required for the long term control of CL13 because both, *Ifnar1*$^{-/-}$ and *Ifnb*$^{-/-}$ mice had increased CL13 virus loads at 30 dpi and reduced numbers of NK cells. Interestingly, maturation of NK cells in infected *Ifnar1*$^{-/-}$ and WT mice was similarly impaired, with higher frequencies of immature R1 NK cells and lower frequencies of functional R2 NK cells as compared to naïve mice. Interestingly, *Ifnb*$^{-/-}$ mice had more severe maturation defects and lower frequencies of $GzmB^+$ NK cells suggesting that as CL13 infection progresses, IFN-β becomes increasingly pivotal in preserving some of the NK cell function.

We also showed that IFN-I signaling is important for NK cells to preserve effector function at 30 dpi with CL13. In B6+*Ifnar1*$^{-/-}$→B6 bone marrow chimeras NK cells have comparable maturation defects in the B6 and in the *Ifnar1*$^{-/-}$ compartments at 30 dpi with CL13. Yet, production of GzmB was higher in the B6 than in the *Ifnar1*$^{-/-}$ NK cell populations.

We could complement our work in the role for IFN-I signaling for NK cell maturation, activation and function with several experiments. First, we could evaluate what happens with NK cells from the bone marrow chimeras above at 8 dpi with CL13. Also, we could determine how IFN-α and IFN-β differ in their roles in shaping NK cells. Unfortunately, there is not yet an IFN-α$^{-/-}$ mouse strain, which complicates our assessments. Alternatively, we could design new experiments in which either IFN-α or IFN-β are blocked at different timepoints of CL13 infection, and then assess how that changes the NK cell profile and their ability to kill protective CD4^{+} T cells.

ECTV-specific T-cell maturation and activation are impaired during CL13 infection, but response is possible following priming with attenuated ECTV-Δ36

CL13 induces strong exhaustion of T cells, yielding them unresponsive to infection, with decreased polyfunctional activity and deletion of many of the LCMV specific T-cell clones (26–30). We now reveal a previously unknown facet in which the $CD8^+$ T cells response towards an unrelated pathogen is impaired. At 7 dpi with ECTV, we observed the expansion of T cells in Ø+ECTV and Arm+ECTV but not in CL13+ECTV (31–33). We also showed that following ECTV infection, T cells in CL13+ECTV infected mice proliferate less, with reduced expression of Ki67 and reduced incorporation of BrdU, than in Ø+ECTV and Arm+ECTV infected mice. Yet, some of the effects of LCMV infection should persist following clearance of infection, because expression of Ki67 in Arm+ECTV infected mice was lower than in Ø+ECTV.

Notably, when we adoptively transferred total splenocytes from naïve WT mice, theoretically sufficient to curb ECTV infection (32), the transferred T cells expanded less in CL13+ECTV than in Ø+ECTV. This occurred despite that the CL13+ECTV had higher antigen load than Ø+ECTV infected group. Similar results were obtained after transfer of OVA-specific cells OT-I CD8 T cells and challenge with ECTV-OVA. This data suggests that cell-extrinsic contribute to the impaired T cell expansion in CL13+ECTV mice.

We also found that the activation of T cells following ECTV infection was significantly diminished in CL13+ECTV mice. As showed above, $CD8^+$ T cells in CL13 infected mice had increased frequency of T cells expressing the maturation marker CD44 and decreased frequency of cells expressing the lymph node homing marker CD62L (28). Yet, after ECTV infection, the frequency of GzmB+ $CD8^+$ T cells in CL13+ECTV increased to much lower levels than in Ø+ECTV or Arm+ECTV mice. We hypothesize that the unresponsiveness to ECTV infection was due to the suppressive effects of inhibitory receptors. As described before, we showed that $CD8^+$ T cells in CL13 infected mice had increased frequencies of cells expressing the inhibitory receptors KLRG1 and PD1 (34–38). Since blockade of inhibitory receptors is associated with rescue of T-cell function during CL13 infection (35–40), we blocked PD1 signaling with anti-PD1 monoclonal antibodies, either alone or together with anti-TIGIT monoclonal antibodies and we saw minimal improvement effects in ECTV virus control (data not shown).

The expansion of CD8$^+$ T cells specific for the immunodominant ECTV epitope TSYKFESV was also reduced in CL13+ECTV as compared to Ø+ECTV and Arm+ECTV mice. Notably, the few TSYKFESV specific CD8$^+$ T cells that did expand in CL13+ECTV also had defective activation, with decreased production of GzmB, increased expression of CD62L and decreased expression of KLRG1. Since these are *de novo* differentiated CD8$^+$ T cells, we hypothesized that circulatory factors should be suppressing the development of effector activity in CD8$^+$ T cells. IL-10 has been abundantly described has pivotal in the establishment of chronicity during CL13 infection; its levels remain increased throughout CL13 chronic infection, and it is associated with decreased TCR signal transduction (19,41–45). To bypass the effects of IL-10 mediated immunosuppression, we adoptively transferred *IL10R*$^{-/-}$ total splenocytes to CL13+ECTV infected mice. This provided some increase in time-to-death but not in survival time compared to CL13+ECTV receiving WT splenocytes. This suggests that various mechanisms contribute to the T-cell unresponsiveness to unrelated pathogens in CL13 infected mice.

There is abundant literature suggesting that persistent IFN-I leads to T cell unresponsiveness during CL13 infection (19,20). Yet, *Ifnar*$^{-/-}$ splenocytes adoptively transferred into CL13+ECTV mice had limited effects on virus control and did not expand TSYKFESV-specific CD8$^+$ T cells at 7 dpi with ECTV (data not shown). In contrast, following ECTV Δ166 infection, which is severely attenuated because it lacks an IFN-I decoy receptor (46), 50% of mice previously infected with CL13 survived and mounted TSYKFESV specific CD8$^+$ T cell responses that were as strong as those in previously naïve mice. This suggests that optimizing IFN-I signaling may help to promoted ECTV virus control in the setting of chronic CL13 infection.

Since CL13+ECTV mice mounted limited but clear TSYKFESV-specific CD8$^+$ T cell responses, we hypothesized that if they had more time to respond, they would be able to effectively respond and protect mice from ECTV infection. To test this, we infected mice with the highly attenuated ECTV virus strain ECTV-Δ36 (47). and found that the number of TSYKFESV-specific CD8$^+$ T cells in Ø+ECTV-Δ36 and CL13+ECTV-Δ36 were similar at 7, 15 and 30 dpi with ECTV-Δ36. At this later timepoint, and in contrast to 7 and 15 dpi, there was also an effective antibody response to ECTV-Δ36.

We then challenged CL13+ECTV-Δ36 mice at 15 dpi ECTV-Δ36 with ECTV WT and found that they survived ECTV infection without overt signs. Protection was likely due to CD8 T cells, because at

the time of challenge CL13+ECTV-Δ36 lacked an Ab response but had a T cell response. In addition, their CD8 T cell response to ECTV WT was comparable to that in Ø+ECTV mice.

In summary, our data suggest that despite increased basal activation levels, chronic CL13 infection causes impaired T-cell cell responses, with impaired activation, differentiation and clonal expansion against unrelated pathogens. Hence, this work may contribute to explain why chronically infected individuals are more susceptible to opportunistic infections. We also provide evidence of the importance of slowing the progression of infection and how that may protect chronically infected individuals from from opportunistic infections.

Adequacy of the CL13 chronic infection model to study the effect of long-lasting infections in the immune system of humans

In this thesis, we have used the model of chronic infection of mice with CL13 to study how chronic viral infections alter the ability of the immune system to protect from an unrelated viral infection, which in this case was ectromelia virus. We compared the immune response to ectromelia virus infection in the setting of CL13 chronic infection, convalescence from Arm or in previously naïve mice. Of interest, the mouse strain we chose, the WT, is resistant to any of these infections when infected with only one of the pathogens.

CL13 is a great model to study how chronic infections alter the host immune system (30,48–50). We not only have convalescence of Arm as a control group, but also one of the most studied viral infection models in the mouse. We have a good understanding of the genetics that render CL13 infection chronic and Arm infection to acutely resolve. CL13 was originally derived from Arm, hence the two virus strains are almost genetically identical (51). Only 5 mutations were identified in CL13, with only 2 resulting in changes on how the virus behaves when infecting the host (52–54).

It is important to note the many similarities between chronic infection with CL13 in the mouse and long-lasting infections of the human. For instance, CL13 infection is known to lead to significant and long last changes in the architecture of many lymphoid organs (4,5). which also occurs in HIV infection of humans, where the lymphoid structure and the follicular reticular cells are severely disrupted, impairing the reception of survival signals in T-cells, which leads to their deletion (55).

Many arms of the innate immune response are shared between chronic CL13 infection in the mouse and long-lasting infections in humans. Both CL13 infection in the mouse and HIV infected humans with uncontrolled viremia show increased IFN-I signaling (19,20,56). In both settings, blockade of IFN-I signaling improves the immune response of the host, with decreased viral loads (19,20,56). Therefore, with the help of the chronic CL13 infection model, a bulk of evidence is emerging showing the benefits of the modulation of IFN-I to the control of long-lasting infections in humans (57).

T-cell exhaustion is an hallmark of CL13 infection (30). This also occurs with many long-lasting viral infections of humans, including those with HIV, HBV and HCV (58–60). Moreover, the profile of T-cell exhaustion in CL13 infection shares many of the features observed in HIV, HBV or HCV infections,

in which T cells increase the expression of the inhibitory receptor PD1 (58,61,62). In CL13 chronically infected mice, exhausted T cells express multiple inhibitory receptors, including PD1, LAG-3, CD160, 2B4, CTLA-4, PIR-B, GP49, KLRG1, NKG2A, Tim-3, BTLA and TIGIT (34–38). Moreover, the higher the expression of inhibitory receptors, the higher is the exhaustion of T-cells (34). In fact, the potential of modulating the activity of inhibitory receptors such as PD1 was vastly successfully explored using the CL13 infection model (35,37–40,63). More recently, extensive efforts have been made to optimize the effectivity of immune checkpoint inhibitor blockade therapies using the CL13 infection model. For instance, individual blockade of several inhibitory receptors, including TIGIT, LAG-3 and TIM-3 is ineffective at controlling chronic infections (35–37,63). Yet, the co-blockade of PD1 with an of these receptors is much more effective at controlling viral infection than PD1 blockade alone (35–37,63). Also, the CL13 infection model allows the exploration of potential new adjuvant therapies to improve checkpoint inhibitory blockade therapies, which is currently ongoing in many laboratories. Findings using the CL13 infection model have been remarkably translational, particularly as powerful tools in therapies against several cancers in humans (64). So far, the success of the modulation of checkpoint inhibitors to treat viral infections in humans has been very limited (65). Further studies in the mouse using the CL13 infection model may help to solve this problem, since it will be possible to test adjuvant therapies which may boost the response to checkpoint blockade therapies when treating infections in humans.

One of the reasons CL13 infection becomes chronic is the persistent antigenic stimulation of T cells, because the virus remains proliferating to high levels in several immune cells, remarkably DCs. This ultimately leads to T-cell exhaustion and deletion of many of the T-cell clones specific to various LCMV derived antigens, which also contributes to ineffective viral control (29,66–68). This mechanism of viral persistence may be present in some human infections, particularly HIV, in which discontinuation of HAART leads to increased plasma viremia and antigenic loads, subsequently contributing to $CD8^+$ T cells functional exhaustion (69).

The benefits in the control of CL13 infection arising from IL-10 signaling blockade therapy at several timepoints of CL13 infection have been abundantly explored (19,42,70). IL-10 production and signaling contributes to the establishment of chronicity of CL13 infection. During CL13 infection, IL-10 is produced continuously in varying levels during the infection, particularly by DCs and $CD4^+$ T cells, and contributes to the suppression of T-cell effector activity (41–43,45). Since IL-10 is elevated in several

long-lasting infections in humans, including EBV, HIB, HBV and HCV infection, one may expect that IL-10 should have similar suppressive effects in the immune system of the human (71–74).

However, there are several limitations of the CL13 chronic infection model that threaten translational conclusions from research with this virus strain. Firstly, it is quite artificial, since chronicity is only obtained when inoculating mice with large amount of viable virus PFUs. The naturally occurring vertical transmission of LCMV-Armstrong to the progeny from infected mouse mothers has limited comparison to what occurs in humans, since today it is infrequent for humans to become chronically infected before or at birth. Moreover, the virus infection control of CL13 shows opposing kinetics to those of long-lasting infections in the human. CL13 viral titers progressively decrease, with many laboratories, including ours, not being able to detect viable virus plaques in the serum, with the virus persisting only in limited locations, such as the kidneys and brain (19,42,53,75–79). In contrast, several of the long-lasting infections in humans show increasing levels of virus in the serum, as the infection progresses uncontrolled by effective therapy, including HIV, HBV and HCV infections.

In conclusion, studying how the immune system responds to new *stimuli* when the mouse host is chronically infected with CL13 may help understanding the multivariate immune response that is elicited following new unrelated viral infections in such hosts: how does the previously persisting antigenic *stimuli* shapes the potential for leukocytes to act towards different pathogens; how can one revert cell exhaustion and improve effectiveness of the immune response to curb the new unrelated viral infection.

Concluding remarks

Our work suggests that there are various possible mechanisms that render CL13 chronically infected WT mice fully susceptible to mousepox, in opposition to previously naïve WT mice that usually survive ECTV infection without overt signs of disease. CL13 infection alters the immune response to ECTV from very early in the infection, in possible dichotomous ways. Uncovering a single culprit for mice chronically infected with CL13 being susceptible to mousepox is incredibly challenging, since our data suggests that multiple stages of the immune response towards ECTV are impaired. In this thesis, we report severe defects in NK cells and an ECTV-specific T-cell response during ECTV infection.

In this thesis we show that the CL13 infection model provides a good system to test new strategies to improve resistance to unrelated viral infections. We show that survival to ECTV can be attained in the chronically infected mice, by priming with attenuated versions of ECTV. This apparently elicits enough T-cell response to curb the infection. In the future, one can use the CL13 chronic infection model to test the response to vaccines to unrelated virus infections, including ECTV, as well as to develop new treatment strategies targeting multiple facets of the immune response.

At the end, this is the most striking conclusion: chronic infections can impair the immune system of the host in a multitude of different ways and one should evaluate all of them, either together or in association, when assessing responses to secondary unrelated infections. This is particularly important in individuals infected with long-lasting infections such as those of HIV, HBV, HCV, when trying to develop new treatment strategies aiming to improve the outcome of unrelated opportunistic infections.

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