Immunotherapy of chronic virus infections: Exhausted CD8+ T cell subsets are differentially regulated by XCR1+ DC

Eva Domenjó Vila

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DIRECTORS DE LA TESI

Dr. Andreas Meyerhans i Dr. Jordi Argilaguet

Biologia de la Infecció

DEPARTAMENT DE CIÈNCIES EXPERIMENTALS
I DE LA SALUT

Universitat Pompeu Fabra Barcelona

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Abstract

The contribution of cross-presenting XCR1+ dendritic cells (DC) in maintaining T cell function during exhaustion and immuno-therapeutic interventions of chronic infections remains poorly characterized. Using the mouse model of chronic LCMV infection, we found that XCR1+ DC were more resistant to infection and highly activated compared to SIRPα+ DC. Exploiting XCR1+ DC via Flt3L-mediated expansion or XCR1-targeted vaccination notably reinvigorated CD8+ T cells and improved virus control. Upon PD-L1 blockade, XCR1+ DC were not required for the proliferative burst of progenitor exhausted CD8+ T (T_{PEX}) cells, but proved indispensable to sustain the functionality of exhausted CD8+ T (T_{EX}) cells. Combining anti-PD-L1 therapy with increased frequency of XCR1+ DC improved functionality of T_{PEX} and T_{EX} subsets, while increase of SIRPα+ DC dampened their proliferation. Together, this demonstrates that XCR1+ DC are crucial for the success of checkpoint inhibitor-based therapies through differential activation of exhausted CD8+ T cell subsets.

Resum

La participació de les cèl·lules dendrítiques (DC) cros-presentadores d'antígens, XCR1+ DC, en el manteniment de funció de les cèl·lules T durant el seu esgotament progressiu ("T cell exhaustion" en anglès) en infeccions cròniques i càncer, i la reactivació d'aquestes promoguda per immunoteràpies no està totalment caracteritzada. Emprant el model d'infecció crònica amb LCMV en ratolins, en aquest estudi hem pogut determinar que les XCR1+ DC són més resistents a la infecció i mantenen alts nivells d'activació en comparació amb les SIRPα+ DC. Hem demostrat que augmentar el número de XCR1+ DC mitjançant la injecció de Flt3L o utilitzar vacunes dirigides al receptor XCR1 són estratègies capaces de reactivar les cèl·lules T CD8+, resultant en un major control del virus. Durant el tractament d'immunoteràpia basat en el bloqueig de PD-L1, hem observat que les XCR1+ DC no són necessàries per l'increment de proliferació de les cèl·lules T CD8+ progenitores (T_{PEX}) però, en canvi, són indispensables per sostenir la funcionalitat de la seva progènie (T_{EX}). La combinació del bloqueig de PD-L1 i l'augment del número de XCR1+ DC resulta en una major reactivació de la funció tant en les T_{PEX} com en les T_{EX}, mentre que augmentar el número de SIRPα+ DC en dificulta la proliferació. En conjunt, aquest estudi evidencia el paper crucial de les XCR1+ DC en les intervencions d'immunoteràpia mitjançant l'activació diferencial dels diferents tipus de cèl·lules T CD8+ esgotades.

Preface

The outcome of a viral infection is determined by the dynamic interplay between the expanding virus and the concomitantly induced immune response. Viral infections can be categorized as either acute or chronic depending on temporal virus-host relationships. In humans, acute infections are usually resolved within a few weeks. In contrast, chronic infections are not resolved and, instead, develop when innate and adaptive immune responses are not sufficient to eliminate the invading virus. Once a chronic infection is established, the medical challenge becomes to either eliminate the virus or keep it sufficiently controlled to minimize its pathogenic consequences.

A hallmark of chronic virus infections is the downregulation of immune effector mechanisms to prevent immunopathology, amongst which is CD8+ T cell exhaustion. Exhausted CD8+ T cells are a heterogeneous population covering diverse differentiation states critical to restrict virus expansion and have been detected in a variety of chronic virus infections, including infections with lymphocytic choriomeningitis virus (LCMV) and simian immunodeficiency virus (SIV) in animal models as well as in human infections with HIV, HBV, HCV and human cancers.

Checkpoint inhibitors like anti-PD-1 or anti-PD-L1 antibodies that block inhibitory receptor functioning have been shown to partly restore exhausted CD8+ T cell function. They have evolved as a greatly promising immunotherapeutic approach in the treatment of cancers and are potentially advantageous against chronic infections. However, many challenges still limit further development

of these immunotherapies as high numbers of patients fail to respond. A major contributor to anti-PD-1/PD-L1 resistance is the status of CD8+ T cell priming. Thus, to achieve maximum therapeutic efficacy it is essential for CD8+ T cells to be optimally primed. Mounting evidence points at cross-presenting XCR1+ dendritic cells (XCR1+ DC) as the preferred antigen-presenting cells to choreograph and successfully prime CD8+ T cells. XCR1+ DC are highly specialized at presenting exogenous and endogenous antigens to T cells, which can induce protective cytotoxic responses.

Recent studies in our lab reported the existence of a communication axis between virus-specific exhausted CD8+ T cells and XCR1+ DC as a crucial component in virus control during the initial phase of a chronic infection. However, it remained unclear to what extent it contributes to virus control during the chronic infection steady-state, whether DC function is maintained during infection, and how the interplay between XCR1+ DC and exhausted CD8+ T cell subsets can be manipulated for host benefit. Results presented in this thesis not only propose different immunotherapy options to treat chronic virus infections but also highlight the key role of XCR1+ DC and their differential effects on exhausted CD8+ T cell subsets for therapy success.

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1. INTRODUCTION

1.1. Chronic viral infections

1.1.1. Chronic viral infections and their implications for human health

A myriad of chronic virus infections exist in humans, some of them so common that are considered an integral component of our physiology. A rough estimate is that each individual harbors ~8-12 chronic infections, many of them not associated with disease or only harmful in a small fraction of the population (*Virgin et al., 2009*). Such is the case of members of the herpesvirus and adenovirus families, among others. Nevertheless, chronic infections can lead to medically important diseases right after primary infection or months, years and even decades later.

Despite the success of recent antiviral therapies, chronic infections continue to be a major health concern and remain a financial burden to healthcare systems. Pathogens associated with high rates of morbidity and mortality include the human immunodeficiency virus (HIV), causative agent of the acquired immune deficiency syndrome (AIDS), and hepatitis B and C virus (HBV, HCV), major causes of viral hepatitis leading to cirrhosis and hepatocellular carcinoma. These viruses can continuously replicate in the host and the constant presence of viral products triggers drastic and sustained alterations in the immune system. This influences the susceptibility to secondary infections, and contributes to the development of certain cancers, as well as to diabetes and atherosclerosis, and other inflammatory disorders

(*Zuniga et al., 2015*). Therefore, further understanding of the unique and sophisticated adaptation of immune cells to a chronic infectious environment is key to develop new therapies and vaccination strategies to treat and prevent chronic viral infections.

1.1.2. Immunological dysregulation and suppression during chronic viral infections

The host immune system is a highly potent mechanism of defense. It is composed of a network of cell types that reciprocally regulate each other to determine the scope and direction of the immune response. Continuous immune activation and inflammation during chronic infections, however, lead to multiple immunologic dysfunctions. These include aberrant activation, cell exhaustion and depletion of T cells, dysfunctional B cell responses, alterations in innate immune capacity and disruption of lymphoid architecture (**Figure I1**).

Control of viral infections depends on effective antiviral CD8+, CD4+ T cell and B cell responses (*Ng et al., 2013*). CD8+ T cells express inflammatory and antiviral cytokines and lyse infected cells. CD4+ T cells (i.e. helper T cells) have many roles that comprise expression of inflammatory cytokines, dendritic cell (DC) licensing, optimal activation, and maintenance of CD8+ T cells responses, and generation of B cell and antibody responses. However, during chronic viral infections CD8+ T cells are physically deleted or succumb a dysfunctional state known as exhaustion whereby functions are typically inferior (see section 1.2). Similarly, CD4+ T cells progressively lose the ability to produce key antiviral

Th1 cytokines (*Fahey et al., 2011*), are transformed into follicular helper T cells and accumulate to contribute to the development of late-arising neutralizing antibodies (*Greczmiel et al., 2017; Kräutler, et al., 2020*). Early and throughout the infection, however, B cells produce a collection of virus-specific non-neutralizing and non-specific antibodies (hypergammaglobulinemia) that cannot prevent the infection (*Hunziker et al., 2003; Hangartner et al., 2006*).

Other important elements that participate in the downregulation of the antiviral effector responses are immunoregulatory cytokines, regulatory T cells (Treg) and myeloid derived suppressor cells (MDSC). A key immunoregulatory cytokine is interleukin-10 (IL-10). Multiple cell types can express it, although the main producers in chronic infections are cDC2, macrophages and CD4+ T cells. IL-10 directly limits T cell proliferation, functional differentiation and effector activity maintaining the exhausted phenotype (*Wilson and Brooks, 2011*). Additionally, and in conjunction with other immuno-suppressive factors (e.g. PD-L1, indoleamine 2,3-dioxygenase [IDO]), IL-10 acts on antigen presenting cells to decrease stimulatory molecule expression (i.e. MHC-II, CD80, C86), alter cytokine production and prevent maturation, ultimately dampening T cell activation (*Sevilla et al. 2004*). In parallel, chronic infection leads to the accumulation of large numbers of Treg and MDSC that also contribute to T cell exhaustion. MDSC suppress T cell activity by direct contact and through production of suppressive cytokines and other factors such as nitric oxide and reactive oxygen species (*Gabrilovich and Nagaraj, 2009, Norris et al., 2013*). They also promote differentiation of Treg cells, which in turn contribute to limit T cell functions in order to protect from tissue damage (*Veiga-Parga et al., 2013*).

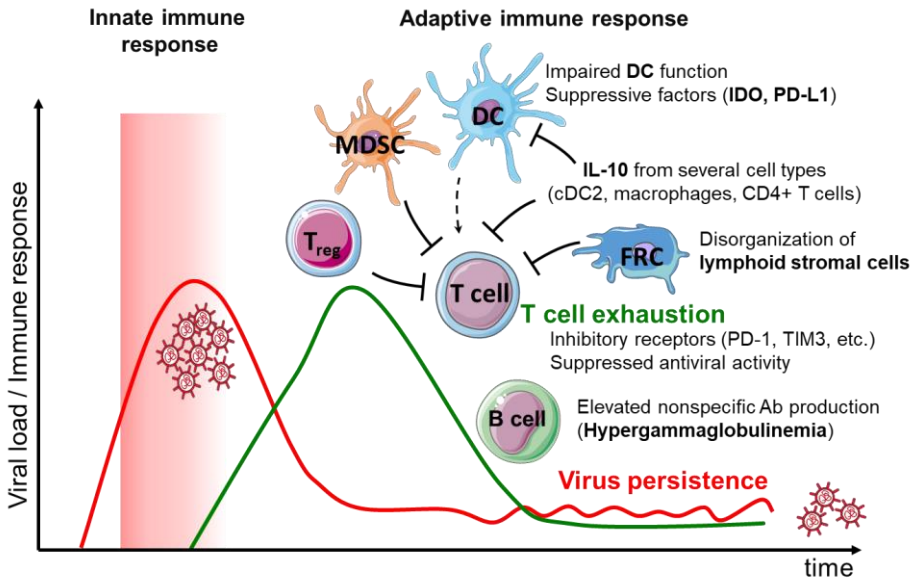


Figure I1. Main cell subsets contributing to immunological dysregulation and suppression during chronic infections.
Schematic representation of the fundamental features of a chronic infection. At the center is T cell exhaustion manifested by progressive loss of effector functions, co-expression of inhibitory receptors and altered expression and use of transcription factors. Pathways implicated in regulating T cell exhaustion include excessive levels of soluble factors such as interleukin-10, altered cell-to-cell signals due to impaired antigen-presenting cell functions including DC and B cells and expansion of regulatory cell subsets, namely MDSC and Treg. Tissue destruction and altered lymphoid organization also have a major role and other immune cell types and stromal cells such as FRC could be the source of many of the changes occurring during T cell exhaustion.

Immunological abnormalities also affect other components of the immune system. For example, plasmacytoid dendritic cell (pDC) that secrete high levels of type I interferon at the onset of an infection, decrease their capacity as the infection progresses. Moreover, type I interferon signaling shifts from an early antiviral role, promoting viral clearance and control of the infection, to a detrimental role during chronic infections, negatively impacting DC-

mediated T cell activation and contributing to lymphoid tissue disorganization (*Wilson et al., 2013; Teijaro et al, 2013*). Disruption of lymphoid architecture results in the loss of fibroblast reticular cell (FRC)/follicular DC networks, and/or defined areas for T, B, and other lymphocytes. This severely affects lymphocyte trafficking and survival and can result in the development of tissue fibrosis, impairing the overall ongoing immune response and the generation of new responses (*Schacker et al. 2006; Scandella et al., 2008*).

1.2. T cell exhaustion

1.2.1. Development of exhaustion

Exhaustion is a broad term used to define a state of cellular dysfunction that occurs after persistent antigen exposure and/or inflammation (*McLane et al., 2019; Pauken and Wherry 2015; Wherry, 2011*). It is a common feature of chronic infections and cancer that is mainly studied in CD8+ T cells, although exhaustion can also occur in other immune cell populations namely CD4+ T cells, B cells and natural killer (NK) cells. In chronic infections, exhausted CD8+ T cells are not effective in eradicating the invading virus, but they retain some residual functionality that limits ongoing virus replication (*Kahan et al., 2015*). Therefore, exhaustion might be an adaptation to reduce the sensitivity of virus-specific T cells to antigen and promote their survival in an antigen-rich environment. Like this, the immune response could balance control of the virus and immunopathology, while maintaining the adaptability of T cell responses to subsequent viral bursts

(*Radziewicz et al., 2009; Barnaba and Schinzari 2013; Pauken and Wherry 2015*).

T cell exhaustion is associated with an altered metabolism and epigenetic landscape, and a unique transcriptional program when compared with functional effector (T_{EFF}) and memory (T_{MEM}) T cells. Early after the onset of chronic infections, high antigen stimulation and calcium influx alter the activity of several transcription factors including IRF4, BATF and nuclear factor of activated T cells (NFAT) (*Martinez et al., 2015; Man et al.,2017*). In its monomeric form, NFAT drives the expression of the thymocyte selection-associated high mobility group box (TOX) and nuclear receptor NR4A family proteins (*Alfei et al., 2019; Khan et al., 2019; Mann and Kaech, 2019; Seo et al., 2019; Liu et al., 2019*). These initiate and sustain widespread epigenetic changes that lead to loss of effector functions and upregulation of PD-1 and other inhibitory receptors. Network analyses revealed that exhausted T cells often reuse transcription factors used by functional T cells, but they operate in a distinct manner. This is the case for the T-box family transcription factors, T-bet and Eomes (*Pauken and Wherry, 2015*). Together, T-bet and Eomes cooperate to promote differentiation of naïve T cells into T_{EFF} cells and T_{MEM}. During T cell activation, higher amounts of T-bet lead to terminal T_{EFF} differentiation and higher amounts of Eomes foster the development of T_{MEM}, quiescence and homeostasis. Conversely, within the pool of exhausted T cells, high expression of Eomes drives terminal differentiation of the exhausted T cells while high expression of T-bet is associated with maintenance of proliferative and functional capabilities. Similarly, TCF1, a transcription factor involved in initial T cell development and memory formation, plays a central role in

the development of precursor exhausted CD8+ T cells (T_{PEX}) (see section 1.2.3) and is crucial to preserve their proliferation capacity (*Chen et al. 2019; Utzschneider et al., 2016; Raghu et al., 2019*).

1.2.2. Cellular and functional features of exhausted T cells

Throughout exhaustion, T cell dysfunction develops in a progressive manner and there is a hierarchical loss of the effector functions. Some are lost in an early stage, for instance high proliferative capacity and IL-2 production, and other functions cease in a more advanced stage of exhaustion, such as cytotoxicity and TNFα and IFNγ production (*Virgin et al. 2009; Wherry, 2011*). Moreover, exhausted T cells are also unable to proliferate in response to IL-7 and IL-15, thus depending on antigen signals to drive their proliferation (*Shin et al., 2007*). The severity of T cell dysfunction during chronic infection correlates directly with the level of infection and expression of inhibitory receptors, ultimately ending with apoptosis of the exhausted cell (*Kahan et al., 2015*).

Typically, inhibitory receptors are transiently expressed by T_{EFF} and their function is to attenuate T cell activation and restrain immune function to prevent immunopathology and autoimmunity. In contrast, exhausted T cells display higher and sustained expression of inhibitory receptors (**Table I1**). The extent of coexpression and specific patterns define the degree of dysfunction, and they differ between CD4+ and CD8+ T cells, as well as the type of infection (*McLane et al., 2019; Wykes and Lewin, 2018*). Among the most relevant inhibitory receptors is PD-1, as it was demonstrated that blockade of PD-1 signaling leads to

reinvigoration of exhausted T cells and reduced viral load (*Barber et al., 2006;* see section 1.4). Other inhibitory receptors include CTLA4, TIM-3, LAG3, CD244 (2B4) and others (**Table I1**) (*Crawford and Wherry 2009; Nguyen and Ohashi 2014*).

Table I1. Main inhibitory receptors expressed by exhausted CD8+ T cells, their ligands and mechanisms of action.
Adapted from *Odorizzi and Wherry, 2009.*

Receptor	Ligands	Mechanism of action
CD244 (2B4)	CD48	ITIM/ITSM
CD94-NKG2A	HLA-E	
GP49B	Integrins	
KLRG-1	E-cadherin	
Lair-1	Collagen	
Ly49 family	MHCI	
PD-1	PD-L1, PD-L2	
PECAM/CD31	PECAM1, integrin, CD38	
CD200R	CD200	Unconventional signalling
LAG3	MHCII	
TIM-3	Galectin9, phosphatidylserine	
CD160	HVEM, MHCI	Receptor competition
CTLA4	CD80, CD86	
BTLA	HVEM	Other
TIGIT	CD226, CD115, CD112	

1.2.3. Differentiation and subset dynamics of exhausted T cells

Exhausted CD8+ T cells are a heterogeneous population covering diverse differentiation states. In early fate decisions, TCF1 governs the development of progenitor exhausted (T_{PEX}) cells and its expression is maintained by FOXO1 (*Chen et al., 2019, Utzschneider et al., 2018*). Unlike T_{MEM}, T_{PEX} maintain their proliferative capacities independently of CD4+ T cell help (*Kanev et al., 2019*). They acquire a level of autonomous function and their differentiation and expansion is driven at least partly by cytokine inputs. It has been shown that intrinsic IL-27 signaling promotes amplification of the T_{PEX} population and prevents programmed cell death (*Huang et al., 2019*), while type I interferon and IL-12 facilitate effector-like differentiation by inducing TCF1 downregulation (*Danilo et al., 2018*).

In a context with prolonged antigen exposure, T_{PEX} sustain cytotoxic CD8+ T cell function, they produce the conventional DC1 chemoattractant XCL1 (*Argilaguet et al., 2019; Andreatta et al., 2021*) and they are crucial for inhibitory receptor blockade interventions (i.e. anti-PD-1/PD-L1). Upon PD-1/PD-L1 blockade, T_{PEX} massively proliferate and replenish the pool of effector-like exhausted CD8+ T cells, which restrict virus expansion (see section 1.4; *Utzschneider et al., 2016*). Besides, T_{PEX} might also perform specialized functions due to their localization in, or proximal to, B cell follicles. Likely, T_{PEX} participate in controlling follicular helper T cell and B cell infection by HIV as well as the regulation of antibody responses, but the mechanisms remain unclear (*Leong et al., 2016; Yu and Ye; 2018*).

T_{PEX} are a precursor population that give rise to a more effector-like yet terminally exhausted (T_{EX}) population displaying higher levels of inhibitory receptors, including TIM-3, and downregulated TCF1 (*Im et al., 2016*). Ly108 is a surrogate of TCF1 expression, which mostly overlaps with the expression of CXCR5 chemokine receptor (*Beltra et al., 2020*). Thus, in mice, T_{PEX} can be identified as Ly108+/CXCR5+ TIM3- and T_{EX} as Ly108-/CXCR5- TIM3+ exhausted CD8+ T cells. Furthermore, T_{PEX} and T_{EX} have been further subdivided according to their accessibility to the blood circulation defined by CD69 expression (*Beltra et al., 2020*). These subsets were named T progenitor exhausted 1 (CXCR5+ CD69+; $T_{PEX}1$) and 2 (CXCR5+ CD69-; $T_{PEX}2$), and T exhausted intermediate (CXCR5- CD69-; T_{EX}INT) and terminal (CXCR5- CD69+; T_{EX}TER) (**Figure I2**). They define four developmental stages among which there is some degree of conversion. $T_{PEX}1$ and $T_{PEX}2$ appear as interchangeable states despite transcriptional, phenotypic, functional and anatomical differences. The conversion is related to the shift from quiescence and residence in lymphoid tissues to blood access and proliferation-driven transition into T_{EX}INT. Although epigenetically different from T_{EFF}, T_{EX}INT resemble effector cells and they ultimately convert to T_{EX}TER, which egress blood circulation and accumulate in tissues (*Beltra et al., 2020*).

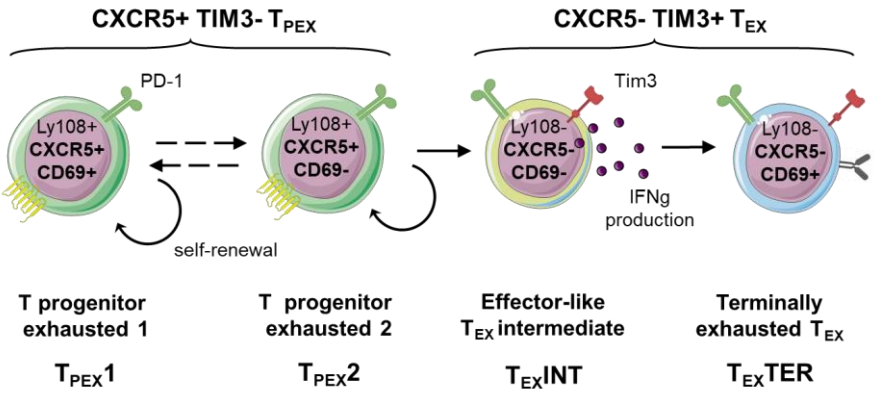


Figure I2. Exhausted CD8+ T cells subsets.
Schematic representation of exhausted CD8+ T cell population dynamics and marker expression. T_{PEX} express TCF1 (Ly108) and the CXCR5 receptor, and include two interchangeable states $T_{PEX}1$ (CXCR5+, CD69+) and $T_{PEX}2$ (CXCR5+, CD69-). T_{PEX} downregulate TCF1 (Ly108) and CXCR5 and transition to more exhausted TIM-3+ T_{EX} which can be subdivided into effector-like T_{EX}INT (CXCR5-, CD69-) and terminally exhausted T_{EX}TER (CXCR5-, CD69+).

Exhausted CD8+ T cells including T_{PEX} have been detected in chronic virus infections i.e. with lymphocytic choriomeningitis virus (LCMV) (*Im et al., 2016; Sandu et al., 2020*) and simian immunodeficiency virus (SIV) in animal models (*Mylvaganam et al., 2017*) as well as in human infections with HIV (*He et al., 2016; Leong et al., 2016; Petrovas et al., 2017*), HBV (*Li et al., 2020*), HCV (*Wieland et al., 2017*) and human cancers (*Brummelman et al., 2018, Miller et al., 2019; Siddiqui et al., 2019*). In proof-of-concept experiments using chronic LCMV infection in mice, adoptive transfer of LCMV-specific T_{PEX}, but not T_{EX}, drastically reduced viral loads in chronically infected recipient mice (*He et al. 2016*). Thus, targeting T_{PEX} has been particularly regarded as an important component of cure strategies for chronic infections. Given that antigen-specific T_{PEX} might be present at a low frequency in human infections, immunotherapeutic approaches

capable of boosting T_{PEX} numbers as well as potentiating their function may represent a promising direction.

1.3. Dendritic cells

1.3.1. Dendritic cell diversity, phenotype and function

Dendritic cells (DC) are professional antigen presenting cells critical for initiation and orchestration of immune responses. They arise from unique DC-restricted bone-marrow progenitors known as common DC progenitors, which give rise to DC precursors (pre-DC) and plasmacytoid DC (pDC). Pre-DC migrate out of the bone marrow into peripheral tissues and eventually differentiate into conventional DC (cDC) (*See et al., 2017, Balan et al., 2019*). Their differentiation heavily depends on the cytokine Fms-like tyrosine kinase 3 ligand (Flt3L) and several transcription factors (i.e. E2-2, Zbtb46, BATF3, ID2, IRF4, ZEB2 and others) drive subset differentiation (**Figure I3**; *Guilliams et al., 2016*). Dendritic cells can be identified in mice by the lack of expression of well-known lineage specific markers for T (CD3), B (CD19) and NK (NK1.1) cells, high levels of major histocompatibility complex class II (MHC-II) and the expression of integrin alpha X, CD11c, the function of which is not well characterized. pDC possess a unique morphology of secretory cells, lacking the typical veiled DC morphology, and they are major producers of type I interferons. Critical markers for murine pDC are CD45R/B220, Bone Marrow Stromal Cell Antigen 2 (BST2) and sialic acid binding Ig-like lectin H (SiglecH), and they do not express any markers found on cDC. Two subsets exist within cDC: conventional DC1 (referred to as cDC1 or XCR1+ DC),

characterized by the unique expression of C type lectin receptor Clec9A and chemokine receptor XCR1, and conventional DC2 (referred to as cDC2 or SIRPα+ DC), expressing the signal-regulatory-protein SIRPα. These signatures are conserved across species and establish useful markers for identifying homolog cDC subsets (*Bachem et al., 2006; Crozat et al., 2010; Dutertre et al., 2014; Alcántara-Hernández et al., 2017 Guilliams et al., 2016*).

cDC are especially adept at presenting exogenous and endogenous antigens to T cells and regulating T cell proliferation, survival, and effector function. Generally, XCR1+ DC are associated with recognition of intracellular pathogens and the initiation responses that require early activation of ILC1 and NK cells, as well as Th1 polarization and antigen cross-presentation to CD8+ T cells (see section 1.3.2; *Mashayekhi et al., 2011, Yamazaki et al., 2013*). On the other hand, some SIRPα+ DC are more specialized in mounting responses against parasites, in which they activate ILC2 and favor CD4+ T cell polarization towards Th2; while other SIRPα+ DC sense extracellular bacteria and initiate responses by activating ILC3 and inducing Th17 dfferentiation (*Tussiwand et al., 2015; Satpathy et al., 2013*). Additionally, DC also control Treg cell numbers primarily by regulating their proliferation rather than their induction and are critical in establishing tolerance to self-antigens, contributing to general homeostasis (*Leventhal et al., 2016*).

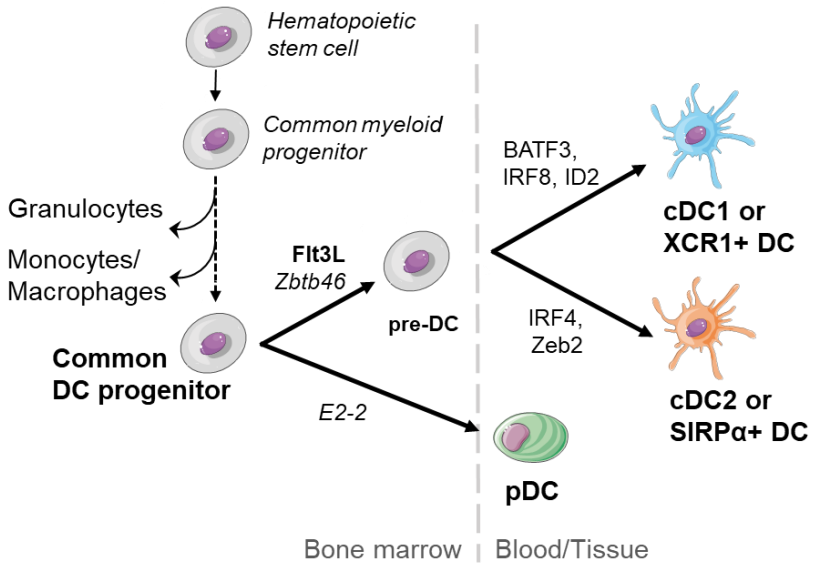


Figure I3. Dendritic cell lineage, differentiation and markers.
DC develop from bone-marrow common myeloid progenitors, diverge at the point of emergence of pre-DC and pDC potential, and culminate in maturation of both lineages in the blood. The pre-DC compartment further differentiates into functionally and phenotypically distinct subpopulations, cDC1/XCR1+ DC and cDC2/SIRPα+ DC through corresponding transcription factors.

1.3.2. Cross-presenting XCR1+ DC are critical orchestrators of adaptive immunity

Despite XCR1+ DC are very scarce, they are a unique DC subset highly specialized at priming cytotoxic CD8+ T cells markedly due to their ability to cross-present antigens. Cross-presentation is the process by which exogenous antigen is uptaken, processed and loaded on major histocompatibility complex class I (MHC-I) molecules to be presented to CD8+ T cells (*Alloati et al., 2016*). Antigens can be delivered into DC via multiple routes, including

macropinocytosis, endocytosis, and phagocytosis. Compared to other antigen presenting cells, DC have limited lysosomal proteolysis, preventing internalized antigens from being degraded prior to encountering antigen-specific T cells in lymph nodes (*Delamarre et al., 2005*). Cross-presentation is favored when endocytic receptors, such as Clec9A, Clec4A, DC-SIGN or DEC-205, deliver antigen into early/late endosomes and it is subject to regulation by extracellular cues (*Blander, 2018*). Two major antigen-processing pathways, vacuolar and cytosolic, have been described to explain how MHC-I molecules are loaded with peptides derived from extracellular sources. In the vacuolar pathway, internalized proteins are degraded by endosomal or phagosomal proteases. Alternatively, the cytosolic pathway involves translocation of the internalized proteins into the cytoplasm and proteasomal degradation into peptides that are imported back via transporter associated with antigen processing (TAP) into phagosomes/endosomes (*Joffre et al., 2012*). Rather than peptide-loading occurring in the endoplasmic reticulum, mounting evidence suggests that MHC-I molecules trafficking between the plasma membrane and endosomal recycling compartments (ERC) are recruited to these phagosomes/ endosomes where the antigen is found (*Blander et al., 2018*). Of note, XCR1+ DC have been shown to harbor large pools of MHC-I molecules in the ERC (*Nair-Gupta et al., 2014*) and might have other unique adaptations of the subcellular pathways that grant their superior ability in cross-presentation. For example, a selective role was recently reported for the vesicle trafficking regulating protein WDFY4 in XCR1+ DC-mediated cross-presentation (*Theisen et al., 2018*). Together with Clec9A receptor, WDFY4 might route internalized antigen directly into ERC

bypassing help from inflammatory signals, thus facilitating antigen processing and loading on MHC-I molecules (*Barbet and Blander, 2019*). However, complete understanding of the mechanisms of cross-presentation is still lacking. Further research may elucidate previously unknown components of the pathway and thereby offer therapeutic targets as well as inform effective vaccine design.

During a viral infection, different immune cell subsets cooperate to trigger antiviral immunity establishing dynamic cellular networks (**Figure I4**). Activated CD8+ T cells at the site of infection, produce the chemokines CCL3 and CCL4 and recruit pDC in a CCR5-dependent manner (*Brewitz et al., 2017*). They also secrete the chemokine XCL1 that promotes XCR1+ DC recruitment (*Argilaguet et al., 2019; Im et al., 2016; Dorner et al., 2009*). Such reorganization of the local DC network enables the type I interferon produced by pDC to optimize cross-presentation by XCR1+ DC, thereby supporting optimal CD8+ T cell responses (*Brewitz et al., 2017*). This network also facilitates the transmission of information to XCR1+ DC that have an innate resistance to viral infection by enveloped viruses (*Silvin et al., 2017*). Initial activation of CD4+ and CD8+ T cells is spatially segregated and mediated by different DC subsets (*Calabro et al., 2016; Hor et al., 2015*). Later during infection, the two T cell subsets migrate to interact with XCR1+ DC that are able to present antigen to CD4+ and CD8+ T cells via both the MHC-II and MHC-I pathways, respectively (*Eickhoff et al., 2015*). Like this, non-infected XCR1+ DC serve as a platform to mediate communication between CD4+ and CD8+ T cells, shaping their differentiation and activation and becoming essential regulators of the elicited antiviral immune response.

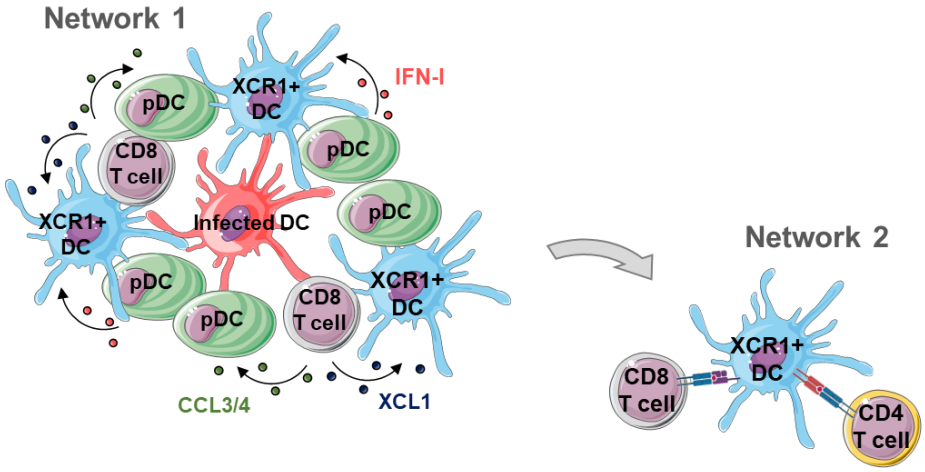


Figure I4. Reorganization of the dendritic cell – T cell networks during antiviral immunity.
Activated CD8+ T cells recruit pDC via CCL3 and CCL4 as well as XCR1+ DC via XCL1 to the site of infection (Network 1). Production of type I interferon (IFN-I) by pDCs optimizes antigen cross-presentation and facilitates the transmission of information to XCR1+ DC. XCR1+ DC then migrate and mediate the communication between activated CD4+ and CD8+ T cells (Network 2).

1.4. Emerging immunotherapeutic interventions

1.4.1. Reversing exhaustion

Many pathogens as well as cancers promote inhibitory interactions between immune cells through checkpoint proteins (i.e. inhibitory receptors) to escape immune control (see section 1.2.2). Interfering with these inhibitory interactions has proven effective in enhancing CD8+ T cell function and holds promising therapeutic advantages. It was first shown using the LCMV mouse model system that administration of antibodies blocking PD-1 signaling partially restored the ability of CD8+ T cells to undergo proliferation, secrete

cytokines, kill infected cells and decrease viral load during chronic infection (*Barber et al., 2006*). This observation was rapidly extended to HIV and HCV infections *in vitro* (*Barber et al., 2006; Day et al., 2006; Trautmann et al., 2006 and Urbani et al., 2006*), (2) to infection models such as SIV in macaques, HCV in chimpanzees and HIV or HBV in mice (*Velu et al., 2008; Dyavar Shetty et al., 2012; Seung et al., 2013*) and (3) to clinical trials for several cancers (*Yao et al., 2013; Topalian et al., 2015*) and more recently for chronic infections (*Gardiner et al., 2013; El-Khoueiry et al., 2020; Gonzalez-Cao et al., 2020*). To date, therapeutic use of the monoclonal antibodies nivolumab (anti-PD1; Bristol-Myers Squibb), pembrolizumab (anti-PD1; Merck), atezolizumab (anti-PD-L1; Genentech), avelumab (anti-PD-L1; EMD Serono) and durvalumab (anti-PD-L1; AstraZeneca) has been approved for various cancers.

Despite being one of the most effective immunotherapeutic approaches, the mechanisms underlying PD-1/PD-L1 blockade continue to be incompletely understood. Recent observations determined that PD-1 suppresses T-cell activation at least in part through the inhibition of CD28 signaling, a major co-stimulatory pathway required for optimal activation of T cells (*Hui et al., 2017; Kamphorst et al., 2017*). The receptor PD-1 has two ligands, PD-L1 and PD-L2. Many cell types express PD-L1 constitutively and virus infections lead to upregulation of PD-L1 levels in response to type I IFN, IFNγ and other cytokines (*Schönrich and Raftery, 2019*). During chronic infections, PD-L1 is widely expressed on both hematopoietic (including T cells, B cells, DC and macrophages) and non-hematopoietic cells in different tissues (*Blackburn et al., 2010*). On hematopoietic cells, PD-L1 was shown to inhibit CD8+ T

cell function, while PD-L1 expression on non-hematopoietic cells limited viral clearance and immunopathology in infected tissues (*Mueller et al., 2010*). In contrast, PD-L2 expression is inducible and much more restricted, predominantly found on DC, macrophages and B cell populations (*Blackburn et al., 2010*). PD-L2 has overlapping functions with PD-L1, which seem less potent and may only become apparent when the effects of PD-L1 are abrogated (*Latchman et al., 2001*). In fact, blocking PD-L2 had almost no effect in a murine tumor model, although synergistic effects were observed when combining anti-PD-L2 with anti-PD-L1 (*Umezu et al., 2019*). In the cancer context, despite being vastly outnumbered by PD-L1+ macrophages, DC represent a major source of PD-L1 and selective elimination of PD-L1 on DC greatly restricts tumor growth and enhances antitumor responses (*Oh et al., 2020*). In addition to PD-L1, DC also express the CD28 ligands, CD80 and CD86. CD80 physiologically interacts with PD-L1 in *cis* on primary activated DC, which interferes with PD-L1/PD-1 binding and subsequently abrogates the function of PD-1 (*Sugiura et al., 2019*). Apart from disrupting PD-L1/PD-1 signaling, PD-L1 blocking antibodies also promote the release of the PD-L1–CD80 association, making CD80 available to ligate CD28 and induce T cell priming (*Mayoux et al., 2020*). Moreover, IL-12 signaling together with IFNγ abre critical for effective anti-PD-1/PD-L1 therapy of tumors (*Garris et al., 2018*). A particular source of IL-12 in mice are cDC1, which have been proven to be essential for the success of antitumor immunotherapies (*Roberts et al., 2016; Salmon et al., 2016; Sánchez-Paulete et al., 2016; Böttcher and Reis e Sousa, 2018*). Whether this holds true for immuno-therapeutic interventions during chronic infections and the extent to which DC maintain their functions remains to be elucidated.

The major cell subset responding to PD-1/PD-L1 blockade are T_{PEX} (*Im et al., 2016; He et al., 2016; Kallies et al., 2020*). PD-1/PD-L1 pathway blockade promotes T_{PEX} proliferation and rebalances the exhausted T cell population distribution, resulting in the preferential amplification of the $T_{PEX}2$ and $T_{EX}INT$ subsets (*Beltra et al., 2020; Petrovas et al., 2017*) as well as increased expression of effector molecules (**Figure I5**). In numerous mouse models of cancer and chronic infection, PD-1/PD-L1 blockade suppresses tumor growth and reduces viral load (*Hashimoto et al., 2018*). However, only some advanced cancer patients (up to 30% depending on the cancer type) experienced reduced tumor burden and improved survival following PD-1-targeted therapy. Similarly, and due to the use of single and lower dose regimens to avoid potential toxicities of anti-PD-1/PD-L1 treatment for chronic infections, very few patients with HBV, HCV or HIV displayed significant reductions in viral load (*Gay et al., 2017*). Furthermore, it was shown that resistance to anti-PD-1/PD-L1 treatment and subsequent therapeutic failure occurs when PD-1/PD-L1 blockade is administered in suboptimally primed CD8+ T cell conditions and results in the generation of dysfunctional and unresponsive CD8+ T cells (*Verma et al., 2019*). Thus, evaluation of treatments that combine the use of anti-PD-1 or anti-PD-L1 with immune-activating agents should be further evaluated.

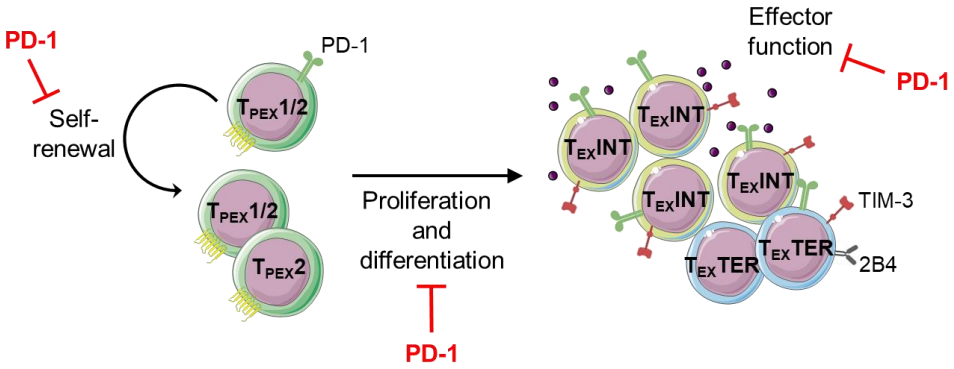


Figure I5. PD-1/PD-L1 regulation of exhausted CD8+ T cells.
The PD-1/PD-L1 pathway regulates proliferation/differentiation of T_{PEX} cells. Differentiated T_{EX} cells migrate to sites of infection or tumor. The PD-1 pathway dampens effector function of T_{EX}, hampering control of infections and tumors.

Drugs targeting other immune checkpoints (CTLA4, LAG3, TIM-3, TIGIT) exist and are currently in clinical trials. Despite the effects of monotherapy are limited, co-blockade of these inhibitory receptors synergized with PD-1 pathway blockade in chronic viral infection and tumor models (*Larkin et al., 2015; Anderson et al., 2016*). Combination of PD-1 with therapeutic vaccine strategies has also shown promising results in chronic viral infections with LCMV, Friend retrovirus and SIV (*Ha et al., 2008; Knuschke et al., 2021; Rahman et al., 2021*). Typically, therapeutic vaccines are delivered with adjuvants, such as agonists for the CD40/CD40L pathway or TLR agonists, to induce activation and improve efficacy (*Barr et al. 2006*); and actively targeting antigens to highly specialized antigen presenting cells like DC has the potential to further enhance immunogenicity.

1.4.2. Targeting DC: a promising strategy

The first attempts to use DC as immunotherapy in humans involved the adoptive transfer of monocyte- or stem cell-derived DC differentiated *ex vivo* and loaded with antigens. Although they proved to be safe, antigen-loaded DC vaccines have elicited only limited clinical responses and their use is labor-intensive, expensive and needs to be individualized to each patient (*Coelho et al., 2016; Kastenmüller et al., 2014*). Instead, techniques that target DC *in vivo* aim to manipulate the DC within the host, through administration of DC-activating factors or vaccination with antigens selectively targeted to DC-specific endocytic receptors. Both the route of vaccine administration as well as the targeted DC subset(s) can substantially affect clinical outcomes. Albeit engaging multiple DC subsets may be superior to targeting only one DC subset, it has the inherent risk of inducing a deleterious cytokine storm (*Saxena et al., 2018*). Besides, the choice of targeting receptor, even on the same dendritic cell subpopulation, may also strongly influence the resulting immune response (*Macri et al., 2016; Fossum et al., 2020*). As cDC1 are superior in cross-presentation, targeting this DC subset could be advantageous for inducing CD8+ T cell responses.

A number of studies have focused on delivering antigens to the lectin receptor DEC-205 on cDC1 and have observed induction of Th1 responses, especially when using adjuvants or in combination with other vaccine approaches to boost T cell responses (*Idoyaga et al., 2011; Flynn et al., 2011*). DEC-205 is, however, also expressed on other cells types, such as B cells and cDC2, which likely affect the resulting immune responses. Conversely, the

expression of the receptors Clec9A and XCR1 is more restricted to cDC1 both in mice and in humans (*Guilliams et al., 2016*), making them perhaps the most promising candidates for the induction of CD8+ T cell responses. Targeting antigens conjugated to anti-Clec9A antibodies has shown positive results both as prophylaxis and immunotherapy, leading to cytotoxic T lymphocyte and particularly strong humoral responses together with the induction of T follicular helper cells (*Sancho et al., 2008; Joffre et al., 2010; Idoyaga et al., 2011*). XCR1 is a chemokine receptor uniquely expressed on cDC1 with only one exclusive ligand XCL1 (*Yamazaki et al., 2013*). XCL1 can successfully be employed as a carrier for vaccines intended to elicit potent antigen-specific T cell cytotoxicity *in vivo* (*Kroczek et al., 2018; Fossum et al., 2015*). The use of chemokines as targeting units has the advantage of inducing chemotaxis of the DC. Several groups have reported the benefits of XCL1-targeted vaccines in antitumor immunotherapy (*Terhorst et al., 15; Botelho et al., 2019*) which are enhanced by the combination with PD-1/PD-L1 checkpoint blockade (*Chen et al., 2020; Mizumoto et al., 2020*). Since cDC1 are rare, the effects of expanding their numbers using the differentiation factor Flt3L has also been evaluated in tumors. Administration of supra-physiological concentrations of Flt3L results in selective clonal expansion of hematopoietic stem cells that are primed to produce cDC1 without compromising the development of other lineages (*Lin et al., 2021*). This strategy has also shown synergies both with XCL1-targeted vaccines and anti-PD-1 immunotherapy (*Salmon et al., 2016; Sánchez-Paulete., 2018; Hammerich et al., 2019; Lai et al., 2020*). Thus, therapeutic vaccines targeting XCR1+ DC are a very promising strategy which might also hold potential benefits for the treatment of chronic infections.

1.5. Lymphocytic choriomeningitis virus (LCMV) infection in mice: a model for chronic infections

1.5.1. The virus

One of the best-studied model systems of viral infections is that of the lymphocytic choriomeningitis virus (LCMV) in mice. LCMV is an enveloped RNA virus of the *Arenaviridae* family and it is noncytopathic *in vivo*. It is able to replicate without causing direct damage to cells or tissues and thus, any damage that appears in the course of an infection can be related to host responses against the virus (*Zinkernagel et al., 2002*). The mouse is its natural host, but LCMV can also infect a wide range of other animals. Humans can be infected with LCMV by inhaling particles contaminated with rodent excreta, during organ transplantation or congenitally during pregnancy. Although there is no quantitative data on the relative threats of the various LCMV virus strains for humans, the symptoms range from a mild respiratory infection to encephalitis or meningitis. Death from LCMV infection is rare, and patients usually recover without any sequelae (*Farmer and Janeway, 1942; Vilibic-Cavlek et al., 2021*).

LCMV has a bisegmented negative single-stranded RNA genome and its life cycle is restricted to the cytoplasm of the infected cell (**Figure I6**). Each of the RNA genome segments, designated as large (L, 7.3kb) and small (S, 3.5kb), uses an ambisense coding strategy to produce two viral gene products in opposite orientation, and is separated by a non-coding intergenic region that folds into a

stable hairpin structure (*De la Torre, 2009*). The L RNA segment encodes for the viral RNA-dependent RNA polymerase (RdRp, also referred to as L polymerase), and a small RING finger protein Z that localizes in the plasma membrane. The Z protein is a structural component of the virion that interacts with host proteins, inhibits RNA synthesis by the RdRp, and is the main driver of LCMV budding (*De la Torre, 2009*). The S RNA segment encodes the immature viral glycoprotein precursor (GPC) and the nucleoprotein (NP), the most abundantly produced protein during infection. The GPC is co- and post-translationally cleaved into GP1 and GP2 and the stable signal peptide (SSP). These three subunits form the mature glycoprotein spike complex (referred to as GP) on the viral surface. GP1 is involved in receptor engagement and entry into host cells, while GP2 and SSP are responsible for stabilizing receptor-GP complexes and viral fusion within host cell membranes (*Buchmeier et al., 2007; Hastie et al., 2016*). The NP is the main structural element, with key roles in viral replication and encapsidation of the viral genome (*West et al., 2014*). It is also involved in host immunosuppression acting as a type I interferon antagonist (*Martínez-Sobrido et al. 2009; Martínez-Sobrido et al. 2007*).

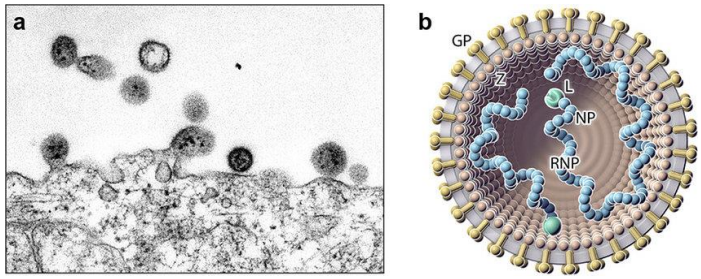


Figure I6. LCMV virion structure.
(A) Electron micrograph of LCMV particles, showing dark internal inclusion bodies, budding from an infected cell. (B) Schematic

representation of a viral particle. Shown is the spherical and enveloped (grey) particle that is spiked with glycoproteins (GP, gold) around a layer of Z proteins (Z, brown). The small and large ribonucleoprotein (RNP) complexes inside the particle consist of nucleoprotein (NP, blue) and RNA-dependent RNA polymerase (L, green). From Radoshitzky et al., 2019.

The only known receptor for LCMV is α-dystroglycan (α-DG). α-DG is a highly conserved, ubiquitous cell surface molecule that links the extracellular matrix with the cytoskeleton (*Cao, 1998; Kunz et al., 2002; De la Torre, 2009*). Within immune cell populations, α-DG is mainly expressed on DCs (*Sevilla et al. 2003*). Virus strains and variants that bind α-DG with high affinity are associated with virus replication in the white pulp of the spleen with preferential replication in CD8- DC (which mostly overlap with cDC2) and pDC (*Oldstone and Campbell, 2011*). Interaction of LCMV with α-DG is dependent on specific glycosylation mediated by the glycosyltransferase LARGE (*Kunz et al., 2003*). After interaction with the viral GP1, LCMV virions are endocytosed. The subsequent fusion between the viral and cell membranes is triggered by the acidic environment of the late endosome and GP2 (*Gallaher et al., 2001*). Upon release of viral genomic RNA, protein synthesis and genomic RNA replication starts. Formation and budding of arenavirus infectious progeny requires assembly of the viral ribonucleoproteins (RNPs) and enrichment of the cellular membranes with viral GPs. The final steps are assembly and release of the infectious virions (*Kunz et al., 2002; Perez and de la Torre, 2003*).

1.5.2. Contributions of LCMV towards understanding antiviral immunity

In 1936, Traub found that mice infected with LCMV *in utero* or shortly after birth, neither died nor eliminated the virus. Instead, they became lifelong carriers, with high virus titers in most of their organs. This was defined as persistent or chronic viral infection (*Traub 1936a; Traub 1936b*). In that time, three different LCMV isolates were identified: the Armstrong strain isolated from monkeys, the Traub strain isolated from a laboratory colony of persistently infected mice, and the WE strain, isolated from a human after exposure to persistently infected mice. Many different variants of these strains exist, but the most commonly used in laboratories are Clone13, which derives from the Armstrong strain, and Docile, a derivative of the WE strain (*Welsh and Seedhom, 2008*). LCMV infection fate varies dramatically depending on the virus strain, age and genetic background of mice, route of infection, as well as the dose used for infection (*Spiropoulou et al., 2002; Zinkernagel et al., 2002*). Intraperitoneal injection with LCMV Armstrong leads to acute infection while intravenous injection with LCMV Clone13 is widely used to establish chronic infection. Of particular interest is the LCMV Docile strain ($LCMV_{DOC}$) strain. Intraperitoneal infection with an $LCMV_{DOC}$ low dose results in viral clearance within 8-10 days post-infection (p.i), while infection with an $LCMV_{DOC}$ high dose results in T cell exhaustion and viral persistence (*Cornberg et al. 2013; Suprunenko and Hofer, 2019*). This feature enables direct comparison of two different immunological outcomes: acute and chronic infections (*Klenerman and Hill, 2005; Wilson and Brooks, 2010*).

Several aspects of the immune response triggered against acute and chronic LCMV have been extensively studied. At the peak of the primary phase of LCMV infection in C57BL6/J mice, ~70% of the CD8+ T cells are LCMV-specific. The strongest response is against $NP_{396-404}$ and GP_{33-41}, the two dominant epitopes, followed by responses to $GP_{276-286}$, $NP_{205-212}$ and GP_{92-101} (*Murali-Krishna et al., 1998*). In an acute infection, the majority of the activated epitope-specific CD8+ T cells die by apoptosis and a stable pool of memory T cells persists during the lifetime of the mouse. Instead, prolonged antigenic stimulation during a chronic infection leads to deletion of $NP_{396-404}$-specific CD8+ T cells and functional inactivation of GP_{33-41}-specific CD8+ T cells, resulting in altered immunodominance and unresponsiveness of the T cells (*Wherry et al, 2003, van der Most et al., 2003*). Unlike for CD8+ T cells and despite antiviral B cells are largely depleted at the onset of infection, chronic LCMV infection drives a delayed but functional, productive and protective humoral response. Neutralizing antibodies targeting GP1 are detectable 40-60 days post-infection (*Fallet et al., 2020; Kräutler et al., 2020*) and non-neutralizing antibodies have occasionally been reported to exhibit antiviral activity if combined with other effector mechanisms of the immune system (*Hangartner et al., 2006; Stoycheva et al., 2021*).

For decades, the LCMV mouse model system has proven an extremely useful exploratory tool to investigate fundamental immunological mechanisms of viral persistence and basic concepts of virus-induced immunity and immunopathology (*Wilson and Brooks, 2010; Kahan and Zajac. 2019*). The importance is evidenced by its roles in the award of the 1996 Nobel Prize for Medicine for the discovery of MHC-restriction (*Zinkernagel and*

Doherty, 1974; Doherty and Zinkernagel, 1975) and the 1960 Nobel Prize for Medicine for the discovery of immune tolerance (*Burnet, 1957*). Other key findings include the understanding of perforin-based cytotoxicity of T cells to destroy LCMV-infected target cells (*Masson and Tschopp, 1985*), the aforementioned quantitation of adaptive immune responses and T cell memory (*Murali-Krishna et al., 1998*), and the identification of NK cell central role in regulating CD4+ T cell support to antiviral CD8+ T cell responses during infection (*Waggoner et al., 2011*). The concept of immunopathology, that is, the damage of tissues and organs due to the antiviral immune response rather than the infecting virus itself, was also established using LCMV (*Cole et al., 1972*). First revealed by Zajac et al. (*Zajac et al., 1998*) and Gallimore et al. (*Gallimore et al., 1998*), the state of dysfunction of CD8+ T cells responding to chronic LCMV infection known as T cell exhaustion, has been thoroughly studied using this model system. Exhausted CD8+ T cells are now targets of immunotherapies, such as PD-1/PD-L1 blockade, placing them at the center of a paradigm shift in the ability to target the immune system for therapeutic benefit (see section 1.4.1). Furthermore, the LCMV infection model system offers sufficient experimental data to develop mathematical models, which can provide with quantitative predictions of the outcomes of immune system perturbations and help understand infection fate regulation in general terms (*Bocharov et al., 2015*). In all, LCMV has illuminated multiple breakthroughs since its discovery and it will continue to contribute to the field of viral immunology (*Zhou et al., 2012*).

1.5.3. Insights from chronic LCMV infection for HIV immunology

Despite HIV and LCMV are inherently different viruses regarding genetic composition, replicative strategies and mechanisms of infection, immunologically speaking they elicit similar antiviral responses in many aspects. Initially, both viruses trigger productive T cell responses but are unable to clear the infection, and other commonalities exist in host-derived immunoregulatory strategies. Exhausted CD8+ T cell responses in LCMV are comparable to those against HIV. In particular, the progressive loss of proliferation and ability to produce cytokines (*Virgin et al., 2009*), the elevated expression of PD-1 (*Blackburn et al., 2010; Day et al., 2006; Petrovas et al., 2006*) and the presence of precursor exhausted CD8+ T cells expressing CXCR5 (*He et al., 2016, Leong et al, 2016*). PD-1/PD-L1 blockade restores CD8+ T cell functionality in both viral infections and has become a highly promising immunotherapeutic intervention (*Barber et al., 2006; Wykes and Lewin, 2018; Rajdev et al., 2018; Uldrick et al., 2019; Blanch-Lombarte et al., 2019; Gonzalez-Cao et al., 2020*). In addition, multiple cell types show increased IL-10 production during LCMV infection and neutralization of IL-10 activity results in increased virus-specific T cell responses (*Clerici et al., 1994; Landay et al., 1996*). In HIV infection, serum levels of IL-10 are also elevated and this correlates with diminished T cell activity and increased virus replication (*Klenerman and Hill, 2005*). One particularly debilitating feature of HIV, leading to the progression to AIDS and death, is its capacity to infect and deplete the host pool of CD4+ T cells. In LCMV as well, depletion of CD4+ T cells leads to drastically enhanced deletion and functional exhaustion of virus-specific CD8+

T cells and anti-LCMV specific antibody responses are not mounted (*Battegay et al., 1994; Matloubian et al., 1994*). Common-gamma chain family of cytokines like IL-2 and IL-21, are also critical in supporting differentiation and proliferation of effector and memory T cells. During chronic LCMV and HIV infections, IL-2 expression by both CD4+ and CD8+ T cells is suppressed, diminishing the expansion and generation of lasting memory CD8+ T cells (*Aiuti and Mezzaroma, 2006; Pipkin et al., 2010*). Similarly, IL-21 deficiency leads to severe exhaustion and the inability to control the infection (*Frohlich et al., 2009; Yi et al., 2009; Yue et al., 2010*). Given all these similarities, the LCMV model is a very useful system to explore the therapeutic potential of manipulating these pathways ultimately aiming to enhance HIV vaccination strategies.

2. OBJECTIVES

A fundamental unresolved issue is the contribution of cross-presenting XCR1+ dendritic cells (XCR1+ DC) in maintaining T cell function during exhaustion and immunotherapeutic interventions (i.e. anti-PD-L1) in chronic virus infections.

The main objective of this thesis was to explore the interplay between XCR1+ DC and exhausted CD8+ T cell subsets and how it could be manipulated for host benefit.

The specific objectives were:

1. To characterize the phenotype of XCR1+ and SIRPα+ DC during the chronic infection steady-state

2. To test whether targeting antigens specifically to XCR1+ DC or expanding XCR1+ DC numbers can improve virus control.

3. To analyze the role of XCR1+ DC in immunotherapeutic interventions (i.e. anti-PD-L1) during chronic LCMV infection.

4. To evaluate the effects of combining XCR1-targeted vaccine or expansion of XCR1+ DC with anti-PD-L1 immunotherapy.

3. METHODS

3.1. Media, buffers and solutions

Complete RPMI (cRPMI): RPMI 1649 with L-glutamine (Sigma-Aldrich), 10% heat inactivated fetal bovine serum (FBS), 1 U/mL penicillin, 1 µg/mL streptavidin, 0.05 mM β- Mercaptoethanol, 1mM sodium pyruvate.

PMA/Ionomycin: cRPMI containing 30ng/ml PMA and 500ng/ml Ionomycin

Lysing solution: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA pH 7.2-7.4.

FACS buffer: Phosphate buffered saline (PBS), 5% FCS, 0.5% Bovine serum albumin, 0.07% sodium azide

FACS Fix buffer: Deionized water, 1% paraformaldehyde, 150 mM NaCl, pH7.4.

Perm Wash buffer: Phosphate buffered saline (PBS), 1% fetal bovine serum (FBS), 0.1% NaN_3, 0.1% Saponin.

LB medium: Deionized water**,** 25g/L LB broth powder, 100 µg/ml Ampicillin (100mg/ml).

LB agar plates: Deionized water**,** 25g/L LB broth powder (Invitrogen), 15g/L Agar, 100 µg/ml Ampicillin (100mg/ml)

Freezing media for cell lines: 10% dimethyl sulfoxide (DMSO), 90% media (DMEM or IMDM).

3.2. Mice

C57BL/6J mice (RRID:IMSR_JAX:000664) were purchased from Charles River Laboratories and XCR1-DTRvenus mice were obtained from Dr. Tsuneyasu Kaisho (*Yamazaki et al. 2013*), bred and maintained under specific pathogen-free conditions at in-house facilities. The number of animals for each experiment was determined based on previous experience with the model system. All animal work was conducted according to the guidelines from Generalitat de Catalunya approved by the ethical committees for animal experimentation at Parc de Recerca Biomèdica de Barcelona (CEEA-PRBB, Spain).

3.3. Cell lines and culture

MC57 and L929 cells were maintained in DMEM, supplemented with 10% heat-inactivated FBS, 1 U/mL penicillin and 1 µg/mL streptavidin (P/S).

Anti-LCMV-NP (VL-4) antibody-producing hybridoma cells (kindly provided by Dr. Burkhard Ludewig) were maintained in suspension in IMDM supplemented with 10% FBS, 1 U/mL penicillin and 1 µg/mL streptavidin (P/S), 7.5% Sodium Bicarbonate (Gibco) and 0,05 mM 2- Mercaptoethanol.

3.4. Viruses and infections

LCMV Docile ($LCMV_{Doc}$) was grown in L929 cells and titrated using focus forming assay on MC57 cells (*Battegay et al. 1991*). Mice were infected intraperitoneally with either a low-dose ($2x10^2$ plaque-forming units) or a high-dose ($2x10^6$ plaque-forming units) of $LCMV_{Doc}$ to induce an acute or chronic infection, respectively.

3.5. *In vivo* treatments in mice

3.5.1. *In vivo* anti-PD-L1 antibody administration

Where indicated, groups of mice were injected with 200µg of anti-PD-L1-specific mAb (10F.9G2, BioXCell) intraperitoneally three times, every third day starting at the indicated time points. As a control, physiological serum was administered.

3.5.2. *In vivo* cell depletion

For depletion of XCR1+ dendritic cells, groups of heterozygote XCR1-DTRvenus mice were injected with 25ng/g body weight of Diphtheria Toxin (DT) intraperitoneally three times, every third day starting at the indicated time points. Mice were weighed one day prior to DT injection. As control, physiological serum was administered.

3.5.3. *In vivo* transfection of human Flt3L

Human Flt3L (GenBank: NM_001459.3) gene was previously subcloned into the pEF-BOS-bsr plasmid (*Iwabuchi et al, 2018*). Plasmid DNA was transformed into *E. coli DH5α* (New England Biolabs) and purified using the NucleoBond Xtra Maxi EF Kit (Macherey-Nagel GmbH & Co). For hydrodynamic gene delivery, 50µg plasmid DNA was diluted in 2mL TransIT-QR Hydrodynamic Delivery Solution (Mirus Bio LLC) and rapidly injected intravenously (tail vein) in 3-5s using a 27-gauge needle. As control, 50µg of the empty vector pEF-BOS-bsr plasmid was administered.

3.5.4. Immunization with Xcl1-targeted fusion vaccines

The fusion vaccines are dimeric molecules where each monomer consists of a targeting unit, an antigenic unit, and a dimerization unit (*Fredriksen et al, 2006*). Construction of the XCL1-targeted fusion vaccines has been described previously (*Grodeland et al, 2013 and Fossum et al, 2015*). Nucleotide sequences encoding the LCMV nucleoprotein were obtained from GenScript, with added 5' BsmI and 3' BsiWI sites and cloned into the vaccine construct. Fusion vaccines containing a single chain variable fragment specific to the hapten NIP were used as non-targeted controls and vaccines expressing the Influenza virus hemagglutinin as negative controls. All plasmids were transformed into *E. coli DH5α* (New England Biolabs) and purified using the QIAGEN Endofree MegaPrep Kit according to the manufacturer's instructions. The indicated groups of mice were anesthetized with isoflurane and

after shaving the lower back, 25µl of DNA vaccine (0.5µg/µL in physiological serum) was injected intradermally on the left flank followed by electroporation using the ECM 830 Electroporation System (BTX Molecular Delivery Systems) with 2 pulses of 450 V/cm × 2.5µs and 8 pulses of 110 V/cm × 10ms. The procedure was repeated on the right flank.

3.6. Virus load quantification

Viral titers from spleens of infected mice were quantified by focus forming assay on MC57 cells as described previously (*Battegay et al. 1991*). Briefly, spleens were frozen at -80ºC right after collection. Tissue was mechanically disrupted and 500µL of DMEM 2% FBS were added to the homogenization. One in ten-fold dilutions were prepared and overlaid onto MC57 cell monolayers in 24-well plates at $4x10^5$ cells/well. A 1:1 mixture of 3% Methocel (Sigma-Aldrich) and 2x DMEM 25% FBS was added after the first 5h of incubation. After a total of 48h at 37C 5% CO_2, cells were fixed with 37% formaldehyde, permeabilized with 1% TritonX solution (Sigma-Aldrich) for 20 minutes and incubated with PBS 10% FBS to block non-specific binding. Staining was performed using monoclonal rat anti-LCMV antibody (VL-4) for 1h, Peroxidase Anti-Rat IgG Polyclonal Ab (Jackson ImmunoResearch) for 1h and DAB Peroxidase substrate kit (Vector Laboratories) for 2-5 minutes. Plaque forming units (pfu) were manually counted.

3.7. Cell preparation, *ex vivo* stimulation and flow cytometry

3.7.1. Splenocyte isolation

Spleens were mechanically disrupted onto a 40µM cell strainer using the plunger of a 1mL syringe and incubated in 5mL of 0.15M Ammonium chloride buffer for 5 min at room temperature (RT) for red blood cell lysis. Cell suspensions were washed in RPMI supplemented with 10% FBS, 1% penn/strep, 0.05mM β-Mercaptoethanol and 1mM Sodium Pyruvate (cRPMI).

3.7.2. Cell staining

For flow cytometric analysis, equal number of cells were stained with Live/Dead Fixable Violet Cell Stain (ThermoFisher Scientific) or Fixable Viability Stain 780 (BD Biosciences) in PBS for 15 min at RT followed by staining with extracellular antibodies for 20 min on ice in FACS buffer. Cells were then fixed for 20 min on ice with 2% Formaldehyde and stained with antibodies for intracellular proteins (XCL1, LCMV-NP, IFNγ, IL-12(p40), CXCL9, Ki67) for 20 min on ice in Perm/Wash buffer (PBS 1% FCS, NaN_3 0.1%, Saponin 0.1%). All antibodies were purchased from either BD Biosciences, eBioscience, BioLegend, Miltenyi or R&D Systems. Samples were acquired on an LSR Fortessa (BD Biosciences), a SP6800 Spectral (Sony) or an Aurora (Cytek) analyzer.

FACS data was analyzed using FlowJo 10 software (Tree Star Inc). Stain index was calculated by subtracting the mean fluorescence intensity (MFI) of the unstained or fluorescence minus one (FMO) controls to the MFI of the stained samples and dividing the subtraction by two times the standard deviation of the unstained or FMO population.

Table M1. Flow cytometry panels.

Markers, fluorochromes and antibody titers are listed below for all flow cytometry panels used in this study. Suppliers, identifiers and specific clones are listed in Table A1.

Exhausted CD8+ T cell subsets

Marker	Fluorochrome	Antibody titer
Surface markers		
Fixable Viability dye 780	-	1:1,000
CD3	BV785	1:20
CD8	PerCPCy5.5	1:160
CD44	eF450	1:80
PD-1	BV605	1:40
CXCR5	PEDazzle594	1:80
TIM-3	APC	1:25
CD69	PECy7	1:160

XCL1-production by exhausted CD8+ T cell subsets

Marker	Fluorochrome	Antibody titer
Surface markers		
Fixable Viability dye 780	-	1:1,000
CD3	BV785	1:20
CD8	PerCPCy5.5	1:160
CD44	eF450	1:80
PD-1	BV605	1:40
CXCR5	PEDazzle594	1:80
TIM-3	APC	1:25
CD69	PECy7	1:160
Intracellular markers		
XCL1	AF488	1:20

Functionality of exhausted CD8+ T cell subsets

Marker	Fluorochrome	Antibody titer
Surface markers		
Fixable Viability dye 780	-	1:1,000
CD3	BV785	1:20
CD8	PerCPCy5.5	1:160
CD44	eF450	1:80
PD-1	BV605	1:40
CXCR5	PEDazzle594	1:80
TIM-3	APC	1:25
CD69	PECy7	1:160
Intracellular markers		
IFNG	FITC	1:1,600

Proliferation of exhausted CD8+ T cell subsets

Marker	Fluorochrome	Antibody titer
Surface markers		
Fixable Viability dye 780	-	1:1,000
CD3	BV785	1:20
CD8	PerCPCy5.5	1:160
CD44	eF450	1:80
PD-1	BV605	1:40
CXCR5	PEDazzle594	1:80
TIM-3	APC	1:25
CD69	PECy7	1:160
Intracellular markers		
Ki67	BV510	1:10

Dendritic cell subsets

Marker	Fluorochrome	Antibody titer
Surface markers		
Live/Dead Violet	-	1:5,000
CD3	PacBlue	1:100
CD19	PacBlue	1:200
Nk1.1	PacBlue	1:50
MHC-II	FITC	1:800
CD11c	PerCP-Cy5.5	1:10
CD45R (B220)	PECF594	1:320
SiglecH	PE	1:80
XCR1	BV510	1:20
SIRPα	APC	1:80

LCMV infection in dendritic cell subsets

Marker	Fluorochrome	Antibody titer
Live/Dead Violet	-	1:5,000
CD3	PacBlue	1:100
CD19	PacBlue	1:200
Nk1.1	PacBlue	1:50
MHC-II	AF647	1:80
CD11c	PerCP-Cy5.5	1:10
CD45R (B220)	PECF594	1:320
XCR1	BV510	1:20
SIRPα	PE	1:80
Intracellular markers		
LCMV-NP	AF488	1:20

Activation and inhibition markers on dendritic cell subsets

Marker	Fluorochrome	Antibody titer
Live/Dead	BioLegend	1:5,000
CD3	PacBlue	1:100
CD19	PacBlue	1:200
Nk1.1	PacBlue	1:50
MHC-II	AF647	1:80
CD11c	PerCP-Cy5.5	1:10
CD45R (B220)	PECF594	1:320
XCR1	BV510	1:20
CD40	APCFire750	1:40
CD80	BV711	1:50
CD86	FITC	1:20
PD-L1	PE	1:1,600

Functionaity of dendritic cell subsets

Marker	Fluorochrome	Antibody titer
Live/Dead	BioLegend	1:5,000
CD3	PacBlue	1:100
CD19	PacBlue	1:200
Nk1.1	PacBlue	1:50
MHC-II	FITC	1:800
CD11c	PerCP-Cy5.5	1:10
CD45R (B220)	PECF594	1:320
XCR1	BV510	1:20
Intracellular markers		
IL-12p40	PE	1:20
CXCL9	AF647	1:100

3.7.3. Splenocyte stimulation and intracellular cytokine staining

For determination of IFNγ-producing T cells, splenocytes (1-2x10^6) were stimulated with GP_{33-41}(1µg/mL) or $NP_{396-404}$ (1µg/mL) peptides for 5h at 37C 5% CO_2 in cRPMI in the presence of Brefeldin A (BFA, Sigma) before antibody staining. Complete RPMI and PMA/ionomycin were used as negative and positive stimulation controls, respectively For determination of XCL1-producing T cells, Ki67+ T cells and IL-12(p40) and CXCL9-producing dendritic cells, spleens were harvested in media containing 10µg/ml BFA and antibody staining was performed without additional stimulation.

3.8. Quantification and statistical analysis

Flow cytometry data analysis was performed using FlowJo (BD). Graphs were compiled and statistical analyses were performed with Prism software (GraphPad). Statistical significance was evaluated with the unpaired t-test when comparing two groups and one-way ANOVA when comparing more than two groups. Non-significant differences were indicated as “ns”. P-values below 0.05 were considered significant and were indicated by asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

4. RESULTS

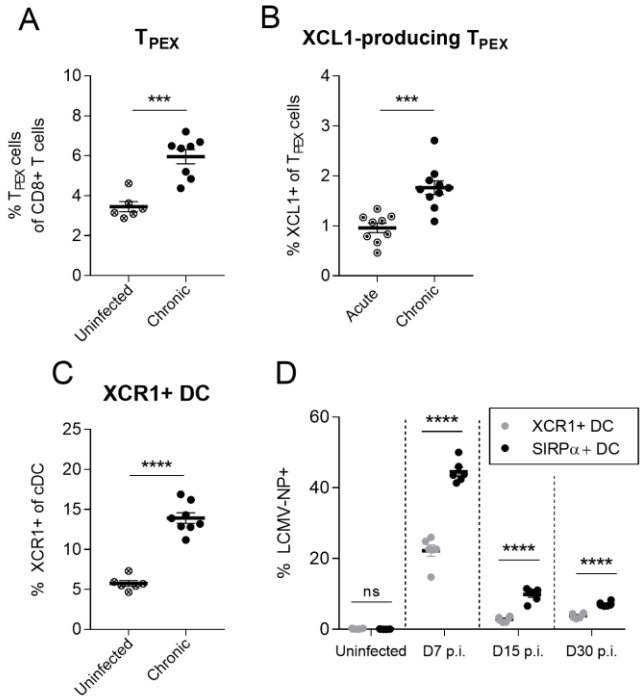
4.1. XCR1+ DC but not SIRPα+ DC maintain an activation phenotype during chronic LCMV infection

We have previously demonstrated the importance of XCR1+ DC in virus control during the initial phase of chronic LCMV infection (*Argilaguet et al., 2019*). Here we aimed to further investigate the role of XCR1+ DC in the established chronic infection phase. C57BL/6J mice were chronically infected with a high-dose (2×10^6 plaque-forming units; pfu) of LCMV strain Docile ($LCMV_{Doc}$), and CD8+ T cell and DC populations were analyzed 30 days post-infection (p.i.) (**Figures R1 and S1**). Percentage of progenitor exhausted CD8+ T cells (CXCR5+ TIM-3-; T_{PEX}) in the spleen as well as their production of XCL1 were elevated in chronically infected mice compared to uninfected mice or mice that had recovered from an acute LCMV infection (**Figures R1A and R1B**). Concomitantly, chronically infected animals also had a higher percentage of splenic cross-presenting DC expressing the XCL1 receptor (XCR1+ DC) (**Figure R1C**), demonstrating the maintenance of the XCL1-XCR1 axis during the course of chronic virus infection.

Several viruses including LCMV can directly infect DC and interfere with their maturation and functioning (*Richter et al., 2013; Ng and Oldstone, 2012; Sevilla et al., 2004; Macal et al., 2012; Ng et al., 2015*). However, it was recently reported that human cross-presenting DC have an innate resistance to infections by

enveloped viruses including HIV and Influenza virus, and thus preserve the host capacity to elicit an antiviral response (*Silvin et al., 2017*). Therefore, we examined whether XCR1+ DC remain uninfected and functional during chronic $LCMV_{Doc}$ infection. Throughout the different stages of infection, the percentage of XCR1+ DC containing intracellular LCMV nucleoprotein (LCMV-NP) was lower than that of SIRPα+ DC (**Figures R1D and S2A**).

To characterize their functional state, we measured the expression of activation markers (CD40, CD80, CD86) and inhibitory receptors (PD-L1) on both DC subsets (**Figures R1E, R1F and S2B**). XCR1+ DC exhibited an activation phenotype with a major increase of CD40+ cells already at day 15 p.i., a steady high level of CD80 and a slight reduction of CD86 while maintaining low PD-L1 expression at all time points post-infection.



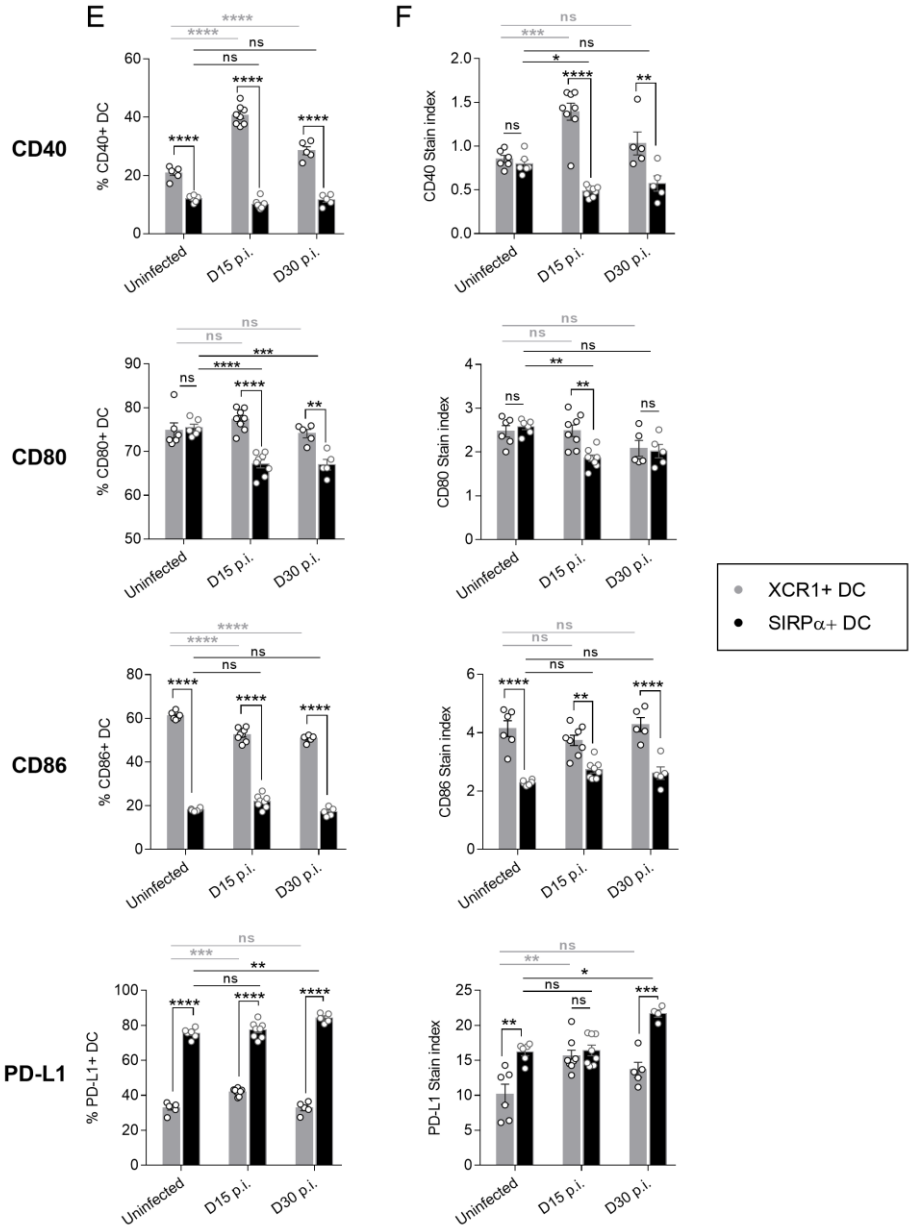


Figure R1. Phenotypic characterization of CD8+ T cells, XCR1+ and SIRPα+ DC in chronic LCMV infection.

Mice were chronically infected with a high-dose of LCMV strain Docile (LCMVDoc), acutely infected with a low -dose of LCMV or left uninfected, and splenic CD8+ T cells and DC populations were analyzed by flow cytometry. Percentages of progenitor exhausted CD8+ T cells (CXCR5+ TIM-3-; T_{PEX}) (A), XCL1-producing T_{PEX} (B) and XCR1+ DC (C) at day 30

p.i. are shown. (D) Percentages of LCMV-NP+ XCR1+ and SIRPα+ DCv at the indicated time points. Percentages (E) and stain index (F) of CD40, CD80, CD86 and PD-L1 proteins expressed by XCR1+ and SIRPα+ DC at the indicated time points. Data shown are the mean ± SEM from 5-10 mice per group. Statistical analysis was performed using unpaired t-test (ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001).

Conversely, SIRPα+ DC showed an inhibitory phenotype with up to 90% positive staining for PD-L1 at day 30 p.i., displayed a low level of CD40 and CD86, and a decreasing CD80 expression as the infection progressed. Hence, the relative resistance of XCR1+ DC to infection while maintaining their functionality, makes them preferential candidates for immunotherapeutic strategies against chronic virus infections.

4.2. XCR1+ DC are critical for the therapeutic enhancement of antiviral CD8+ T cell responses

In order to assess whether XCR1+ DC can be exploited therapeutically to improve CD8+ T cell function and restrain LCMV replication during a chronic infection, we first evaluated the effects of augmenting XCR1+ DC numbers by systemic Flt3L administration. Chronically infected and uninfected control mice were transfected *in vivo* with the pEF-BOS-Flt3L-bsr plasmid encoding the human Flt3L gene (**Figure R2**) (*Iwabuchi et al., 2018*). This procedure dramatically expanded the numbers of XCR1+ DC and SIRPα+ DC in uninfected (44-fold and 7-fold, respectively) and chronically infected mice (18-fold and 5-fold, respectively).

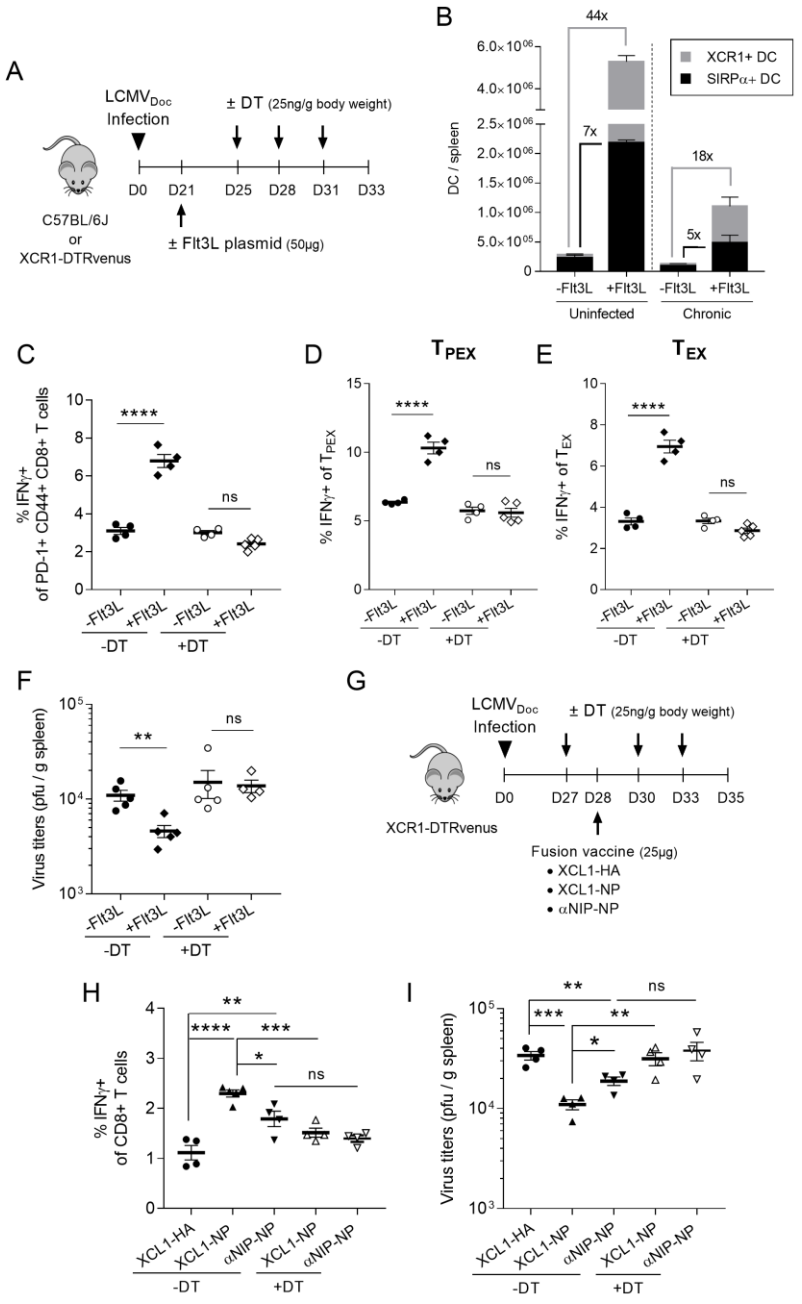


Figure R2. XCR1+ DC are critical for enhancing antiviral CD8+ T cell immunity in chronic infections.

(A) Schematic representation of Flt3L and DT treatment regimens in chronic LCMV-infected C57BL/6J and XCR1-DTRvenus mice. (B) Number of XCR1+ (grey) and SIRPα+ (black) DC in the spleen from uninfected or

chronically infected C57BL/6J mice 7 days after transfection with empty vector pEF-BOS-bsr plasmid (-Flt3L) or pEF-BOS-Flt3L-bsr plasmid (+Flt3L). Data shown are the mean ± SEM, and the fold change over empty vector pEF-BOS-bsr plasmid transfection (n= 2-6 mice). (C-E) Frequency of GP33-41-specific IFNγ-producing PD-1+ CD44+ CD8+ T cells (C), T_{PEX} (D) and T_{EX} (E) cells, and viral loads (F) in spleens from Flt3L-treated (+Flt3L) or untreated (-Flt3L) mice at 33 days p.i.. (G) Schematic representation of fusion vaccine and DT treatment regimens in chronically infected XCR1-DTRvenus mice. (H-I) Frequency of NP396-404-specific IFNγ-producing CD8+ T cells (H) and viral loads (I) in spleen from DT-treated or untreated vaccinated mice at day 35 p.i.. Data shown are the mean ± SEM from 4-6 mice per group. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons (ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

The lesser increase in DC chronically infected mice is likely due to the previously described type-I interferon-mediated inhibition of DC maturation (*Sevilla et al., 2004*). Considering absolute cell numbers per spleen, we observed a shift in the ratio of XCR1+ to SIRPα+ DC from about 1:7 to 1:1.5, thus resulting in roughly equal absolute numbers of both cell subsets 7 days after *in vivo* transfection (**Figures R2B and S3A**). DC expansion led to an increase in virus-specific CD8+ T cell activity that was pronounced in both, T_{PEX} and their progeny effector exhausted CD8+ T cells (CXCR5- TIM-3+; T_{EX}) (**Figures R2C-E and S3B**). This was accompanied by a considerable reduction in virus titers in the spleen (**Figure R2F**) although the number of virus target SIRPα+ DC was higher. To then validate that the observed antiviral effects were promoted by XCR1+ DC, we transfected Flt3L in chronic LCMV-infected XCR1-DTRvenus mice that allow depletion of XCR1+ DC by diphtheria toxin (DT) treatment (**Figure R2A**) (*Yamazaki et al., 2013*). In the absence of XCR1+ DC, CD8+ T cells remained exhausted and virus titers remained high (**Figures R2C-F and S3B**). Altogether,

these results highlight the substantial ability of XCR1+ DC to invigorate virus-specific exhausted CD8+ T cells and indicate their therapeutic potential during chronic virus infections.

To exploit XCR1+ DC as therapeutic targets, fusion vaccine constructs encoding XCL1 and the LCMV nucleoprotein (XCL1-NP) were generated. These vaccines consist of dimeric XCL1-antigen fusion constructs that specifically target XCR1+ DC and can generate protective immunity *in vivo* (*Fossum et al., 2015*). Chronically infected XCR1-DTRvenus mice were vaccinated with XCL1-NP. Non-targeting anti-NIP-NP (αNIP-NP) and fusion vaccines encoding influenza virus hemagglutinin (XCL1-HA) were used as controls (**Figure R2G**). XCL1-NP induced a higher frequency of functional virus-specific CD8+ T cells than αNIP-NP with corresponding reductions in viral loads (**Figures R2H, R2I and S3C**). This induction of functional T cells and their antiviral effect were dependent on the presence of XCR1+ DC (**Figures R2H, R2I and S3C**) demonstrating that this DC subset is critical for an efficient vaccine response in an established chronic infection

4.3. XCR1+ DC are indispensable for increasing functionality of $T_{PEX}2$, $T_{EX}INT$ and $T_{EX}TER$ during anti-PD-L1 treatment but not for their proliferation

Checkpoint inhibitors like anti-PD-L1 antibodies have been successfully used to reinvigorate exhausted CD8+ T cells (*Barber et al., 2006*). To determine whether the beneficial effects derived

from anti-PD-L1 immunotherapy are orchestrated by the XCL1-XCR1 communication axis, we first analyzed functional changes in T_{PEX} and XCR1+ DC in chronically infected C57BL/6J mice treated with anti-PD-L1. After treatment, more T_{PEX} produced XCL1 and XCR1+ DC were present at higher frequency in the spleen (**Figures R3A, R3B and S4A**). Moreover, anti-PD-L1 treatment led to functional activation of XCR1+ DC measured by IL-12(p40) and CXCL9 cytokine production, and thus promoted T cell activation and recruitment (**Figures R3C, R3D and S4B**).

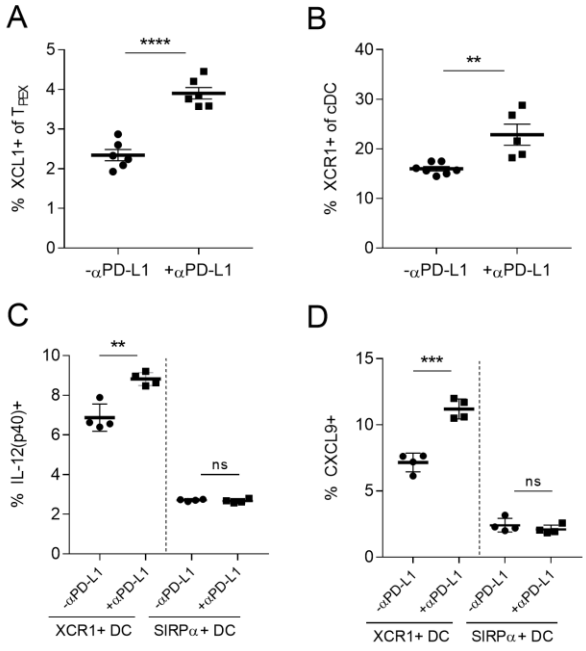


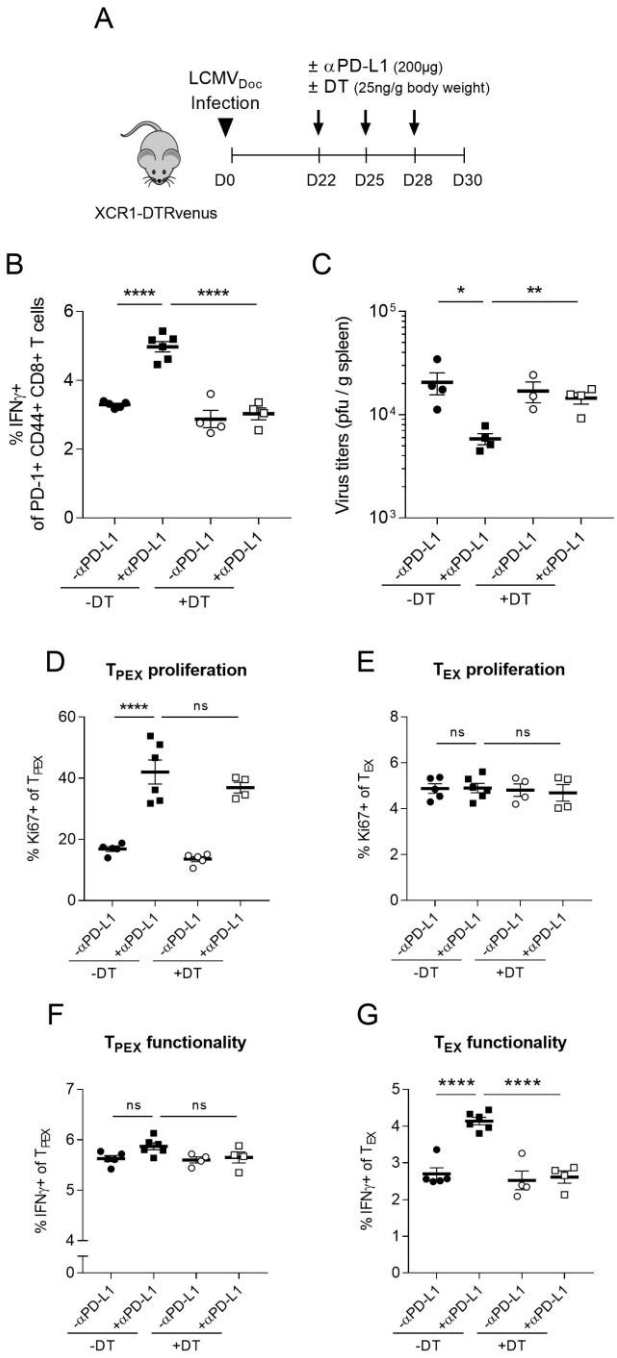
Figure R3. Anti-PD-L1 treatment enforces the XCL1-XCR1 communication axis.

Chronically LCMV-infected mice were treated with anti-PD-L1 antibody (+αPD-L1) at days 22, 25 and 28 p.i. or left untreated (-αPD-L1). At day 30 p.i., frequencies of XCL1-producing T_{PEX} (A), XCR1+ DC (B), IL-12(p40)-producing (C) and CXCL9-producing (D) XCR1+ and SIRPα+ DC were assessed by flow cytometry. Data shown are the mean ± SEM from 4-7 mice per group. Statistical analysis was performed using unpaired t-test (ns = not significant; **p < 0.01; ***p < 0.001; ****p < 0.0001).

To further investigate to which degree XCR1+ DC participate in the immunotherapeutic effect elicited by anti-PD-L1 blockade, we compared the anti-PD-L1-induced CD8+ T cell responses and viral titers in the presence or absence of XCR1+ DC in chronically infected XCR1-DTRvenus mice (**Figure R4A**). Anti-PD-L1 treatment significantly increased the frequency of IFNγ+ virus-specific CD8+ T cells and reduced virus loads (**Figures R4B and R4C**). Both effects were dependent on the presence of XCR1+ DC thus demonstrating the crucial contribution of XCR1+ DC in anti-PD-L1 immunotherapy.

To analyze the dependence of progenitor exhausted T_{PEX} and terminally exhausted T_{EX} populations on XCR1+ DC during anti-PD-L1 blockade in chronic infection, we quantified their proliferation by Ki67 expression and functionality by IFNγ production. Anti-PD-L1 treatment induced a massive proliferation of T_{PEX} that was not altered by XCR1+ DC depletion (**Figures R4D and R4E**). In contrast, the increase in IFNγ+ T_{EX} cells was strictly dependent on XCR1+ DC (**Figures R4F and R4G**). We then performed the same analysis using the newly established classification of exhausted CD8+ T cell subsets: T progenitor exhausted 1 (CXCR5+ CD69+; $T_{PEX}1$) and 2 (CXCR5+ CD69-; $T_{PEX}2$), and T exhausted intermediate (CXCR5- CD69-; T_{EX}INT) and terminal (CXCR5- CD69+; T_{EX}TER) (*Beltra et al., 2020*). Their relative frequencies upon different treatments are shown in **Figure S4C**. Anti-PD-L1 induced the proliferation of $T_{PEX}2$ and T_{EX}INT that was independent on XCR1+ DC (**Figures R4H and S4D**). The functional gain observed in $T_{PEX}2$, T_{EX}INT and T_{EX}TER however was dependent on XCR1+ DC (**Figures R4I and S4E**). Taken together these results demonstrate that XCR1+ DC are indispensable to promote T_{EX}

antiviral activity during anti-PD-L1 immunotherapy but not important for the proliferation burst of T_{PEX} stem-like progenitors.



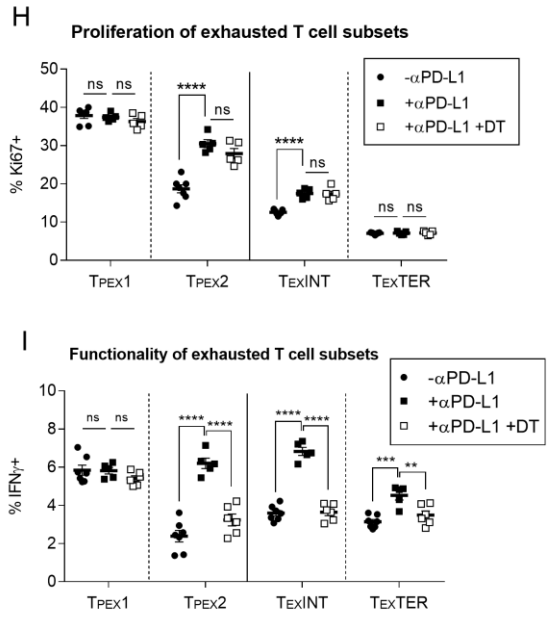
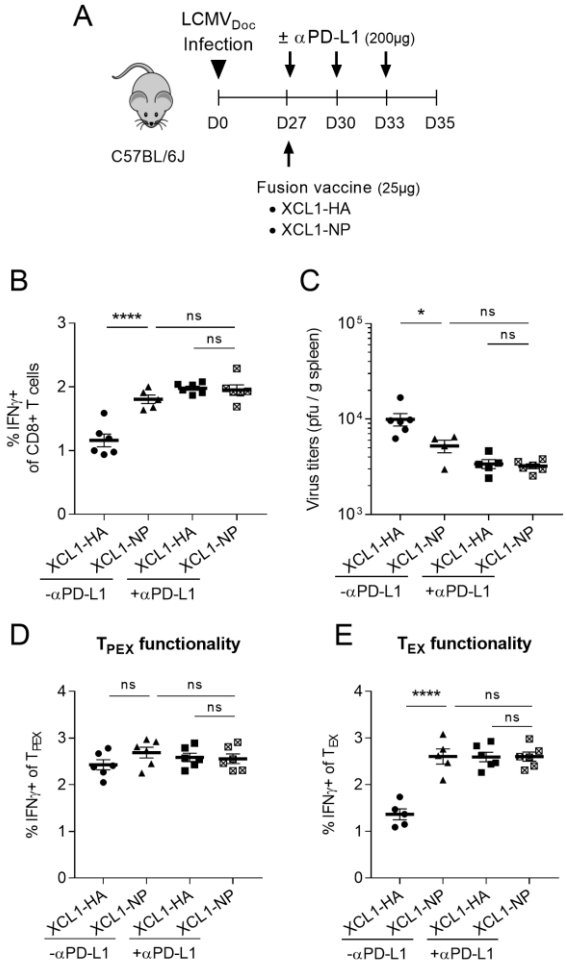


Figure R4. XCR1+ DC are essential to maintain functionality of effector TEX subsets during anti-PD-L1 treatment.
(A) Schematic representation of anti-PD-L1 and DT treatment regimens in chronic LCMV-infected XCR1-DTRvenus mice. Chronic infected anti-PD-L1 treated (+αPD-L1) or untreated (-αPD-L1) mice were sacrificed at day 30 p.i., and spleens were harvested to quantify the percentage of GP_{33-41}-specific IFNγ-producing PD-1+ CD44+ CD8+ T cells (B), viral loads (C), percentage of proliferating (D-E and H) and IFNγ-producing (F-G and I) exhausted T cell subsets. Data shown are the mean ± SEM from 4-6 mice per group. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons (ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

4.4. Dichotomy of XCR1+ DC and SIRPα+ DC for CD8+ T cell function and proliferation during anti-PD-L1 treatment

To examine whether anti-PD-L1 immunotherapy could be further improved by targeting viral antigens to XCR1+ DC, we treated

chronic LCMV-infected C57BL/6J mice with anti-PD-L1, and simultaneously vaccinated them with XCL1-NP or the negative control XCL1-HA (**Figure R5A**). The massive increase of virus-specific IFNγ-producing CD8+ T cells and subsequent virus reduction upon anti-PD-L1 treatment could not be further enhanced by XCL1-NP vaccination (**Figures R5B and S5A**). The effect of XCL1-NP vaccination alone could be slightly but non-significantly increased by addition of anti-PD-L1, demonstrating that anti-PD-L1 treatment alone already results in maximal immune enhancement under these conditions.



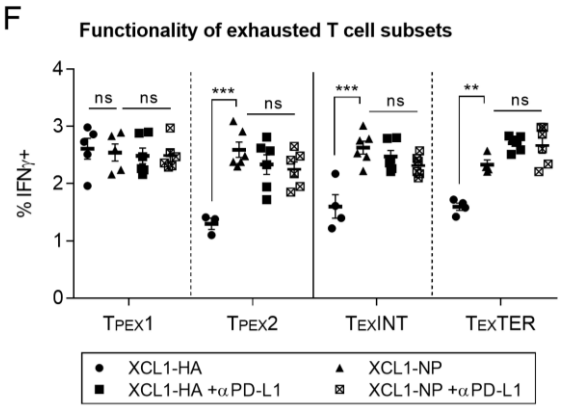
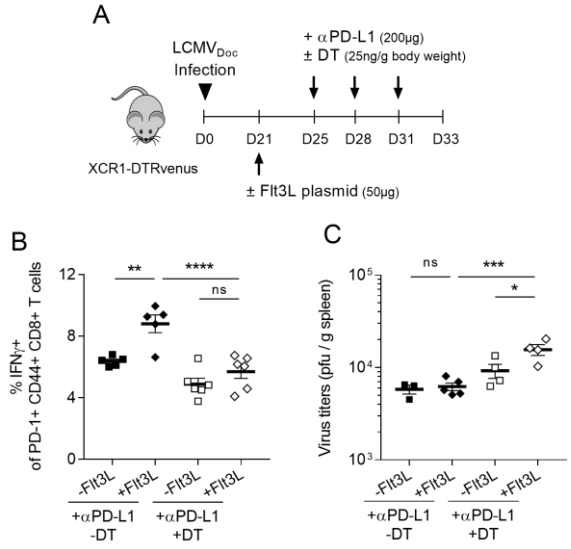


Figure R5. Combination of XCR1+ DC antigen targeting and anti-PD-L1 treatment in chronic LCMV infection.
(A) Schematic representation of fusion vaccine and DT treatment regimens in C57BL/6J mice. Chronic infected and anti-PD-L1 treated (+αPD-L1) or untreated (-αPD-L1) mice were vaccinated with the corresponding fusion vaccines and sacrificed at day 35 p.i.. Spleens were harvested to quantify the percentage of $NP_{396-404}$-specific IFNγ-producing CD8+ T cells (B), viral loads (C), and frequencies of IFNγ-producing exhausted T cell subsets (D-F). Data shown are the mean ± SEM from 6 mice per group. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons (ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

In all cases, virus load reductions corresponded inversely to the CD8+ T cell responses (**Figure R5C**). Heightened CD8+ T cell function was observed in T_{EX} cells, but not in T_{PEX} cells (**Figures R5D and R5E**). Similarly, increased T cell functionality was observed in the T_{EX} INT, T_{EX}TER and $T_{PEX}2$ populations, but not the $T_{PEX}1$ population (**Figure R5F**). Together this demonstrated that anti-PD-L1 treatment alone or single XCL1-NP vaccination lead to similar immunotherapeutic effects in chronic virus infection.

We next tested whether the lack of immune enhancement by the combination of anti-PD-L1 and XCL1-NP vaccines could be explained by insufficient numbers of XCR1+ DC. For this we combined anti-PD-L1 and Flt3L treatments in chronic LCMV-infected XCR1-DTRvenus mice and evaluated the contribution of XCR1+ DC by DT-mediated depletion (**Figure R6A**). Coupling anti-PD-L1 therapy with XCR1+ DC expansion led to a significant increase in IFNγ-producing CD8+ T cells compared to anti-PD-L1 treatment alone (**Figure R6B**). However, there was no reduction in virus loads (**Figure R6C**). Depletion of XCR1+ DC by DT abolished the increase of CD8+ effector T cells and resulted in increased viral loads that was highest in Flt3L-transfected mice (**Figure R6C**). Together this demonstrated that PD-L1-expressing SIRPα+ DC can interfere with anti-PD-L1 immunotherapy especially in the absence of XCR1+ DC, and suggested that their Flt3L-mediated expansion (**Figure R2B**) counterbalances virus control possibly by increasing the number of target cells available for the virus (**Figure R1D**).



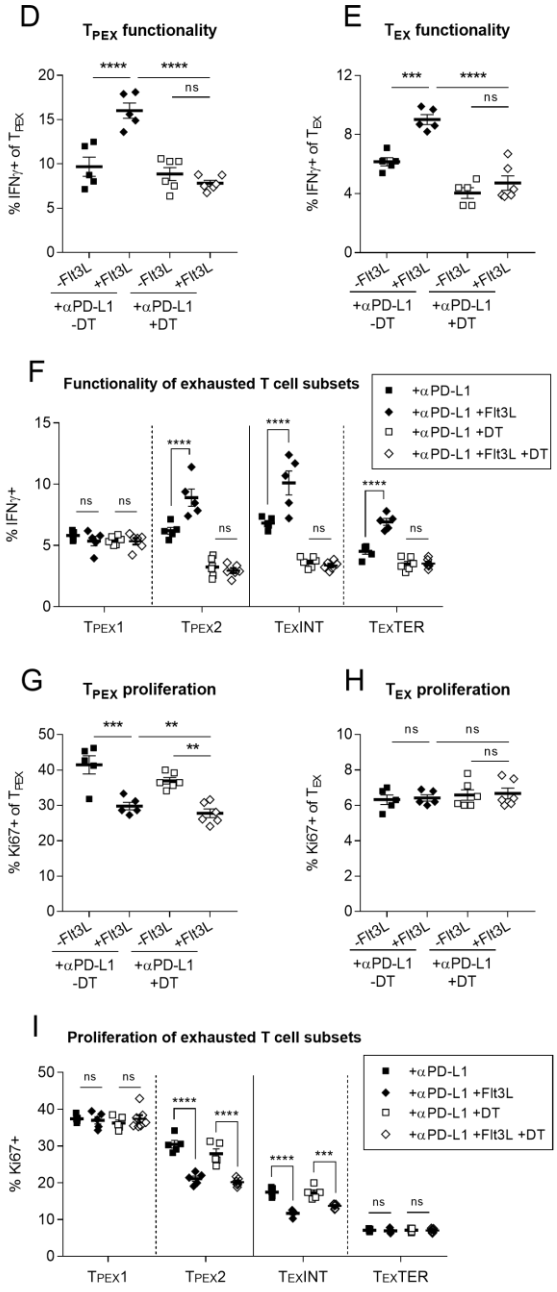


Figure R6. Improvement of anti-PD-L1 treatment by Flt3L-mediated expansion of XCR1+ DC.

(A) Schematic representation of Flt3L and DT treatment regimens in XCR1-DTRvenus mice during anti-PD-L1 immunotherapy. Chronic

infected and anti-PD-L1 treated mice were transfected with Flt3L-expressing plasmid in the presence (-DT) or absence (+DT) of XCR1+ DC, and sacrificed at day 33 p.i.. Spleens were harvested to quantify the percentage of GP33-41-specific IFNγ-producing PD-1+ CD44+ CD8+ T cells (B) viral loads (C), and frequencies of GP33-41-specific IFNγ-producing (D-F) and proliferating (G-I) exhausted T cell subsets. Data shown are the mean ± SEM from 5-7 mice per group. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons (ns = not significant; *p>0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Exhausted CD8+ T cell subset analysis showed that the combination of anti-PD-L1 and Flt3L resulted in significantly higher frequencies of IFNγ+ T_{EX} and T_{PEX} cells which was promoted exclusively by XCR1+ DC (**Figures R6D, R6E and S5B**). Of the four exhausted subpopulations, the most substantial frequency increase was with the T_{EX}INT subpopulation that almost doubled (**Figure R6F**). In contrast, the anti-PD-L1-induced T_{PEX} proliferation was partially abrogated by Flt3L treatment, possibly due to negative signaling from expanded PD-L1+ SIRPα+ DC (**Figures R6G-I and S5C**). This was reflected in the relative frequency of the exhausted CD8+ T cell subsets (**Figure S5D**). Taken together, while antigen delivery to XCR1+ DC can increase IFNγ-producing CD8+ T cells, there is no significant additional improvement when combined with anti-PD-L1. Only when expanding XCR1+ DC, the anti-PD-L1-induced functional responses of T_{PEX} and T_{EX} are further improved.

5. SUPPLEMENTAL FIGURES

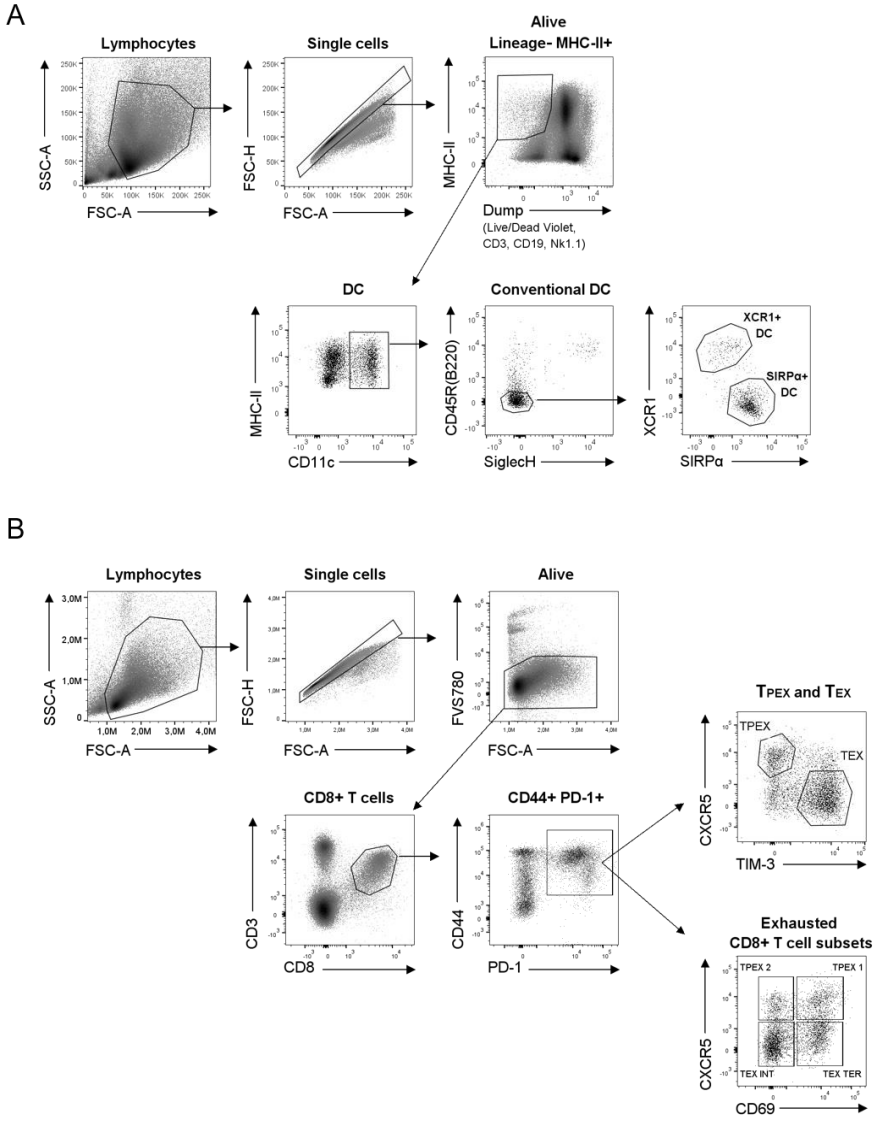


Figure S1. Identification of DC and exhausted CD8+ T cell subsets in splenocytes from chronic LCMV-infected mice using flow cytometry.

Representative gating strategy of DC (A) and exhausted CD8+ T cell subsets (B) from splenocytes isolated from an LCMV-infected C57BL6/J mouse at day 30 p.i.. (A) DC were selected as alive, lineage negative

(CD3-, CD19- and Nk1.1-), MHC-II+ and CD11c+. Conventional DC (cDC) were selected as CD45R- (B220-) and SiglecH-. XCR1+ and SIRPα+ DC were selected from cDC by their respective markers. (B) Exhausted CD8+ T cells were selected as alive, CD3+, CD8+, CD44+ and PD-1+. T progenitor exhausted cells (T_{PEX}) were selected as CXCR5+ TIM-3-, and T exhausted cells (T_{EX}) as CXCR5- TIM-3+. T progenitor exhausted 1 cells ($T_{PEX}1$) were selected as CXCR5+ CD69+, T progenitor exhausted 2 cells ($T_{PEX}2$) as CXCR5+ CD69-, T exhausted intermediate cells (T_{EX}INT) as CXCR5- CD69-, and T exhausted terminal cells (T_{EX}TER) as CXCR5- CD69+.

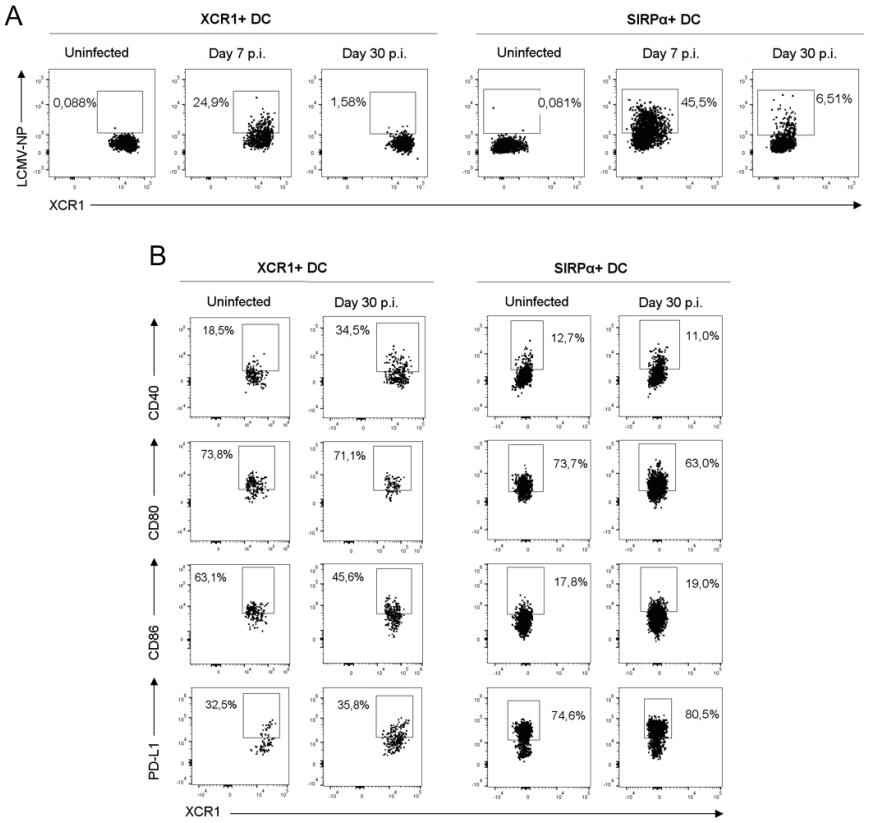


Figure S2. LCMV-NP levels, expression of activation marker (CD40, CD80 and CD86) and inhibition marker (PD-L1) on XCR1+ and SIRPα+ DC.

Representative dot plots for intracellular LCMV-NP staining (A), and surface expression of CD40, CD80, CD86 and PD-L1 (B) on XCR1+ and SIRPα+ DC measured by flow cytometry in uninfected and chronic LCMV-infected mice at the indicated time points.

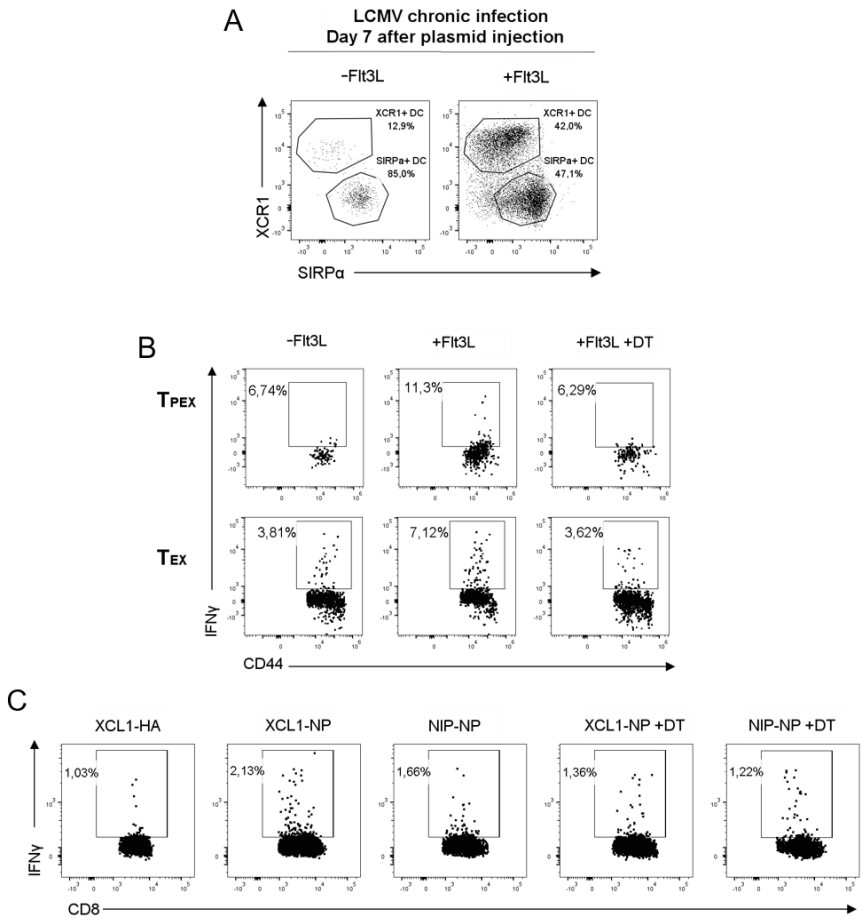


Figure S3. Increase of exhausted CD8+ T cell functionality after Flt3L-mediated expansion of XCR1+ DC or vaccination with XCR1-targeted fusion constructs.

(A-B) Chronic LCMV-infected mice were transfected with empty vector pEF-BOS-bsr plasmid (-Flt3L) or pEF-BOS-Flt3L-bsr plasmid (+Flt3L), and treated with Diphtheria Toxin (+DT) or left untreated. (A) Representative dot plots of Flt3L-mediated DC expansion at day 7 after plasmid transfection. (B) GP_{33-41}-specific IFNγ-producing T_{PEX} and T_{EX} at day 33 p.i.. (C) Chronic LCMV-infected mice were vaccinated with XCL1-HA, XCL1-NP or αNIP-NP, and treated with DT (+DT) or left untreated. Representative dot plots of $NP_{396-404}$-specific IFNγ-producing CD8+ T cells at day 35 p.i. are shown.

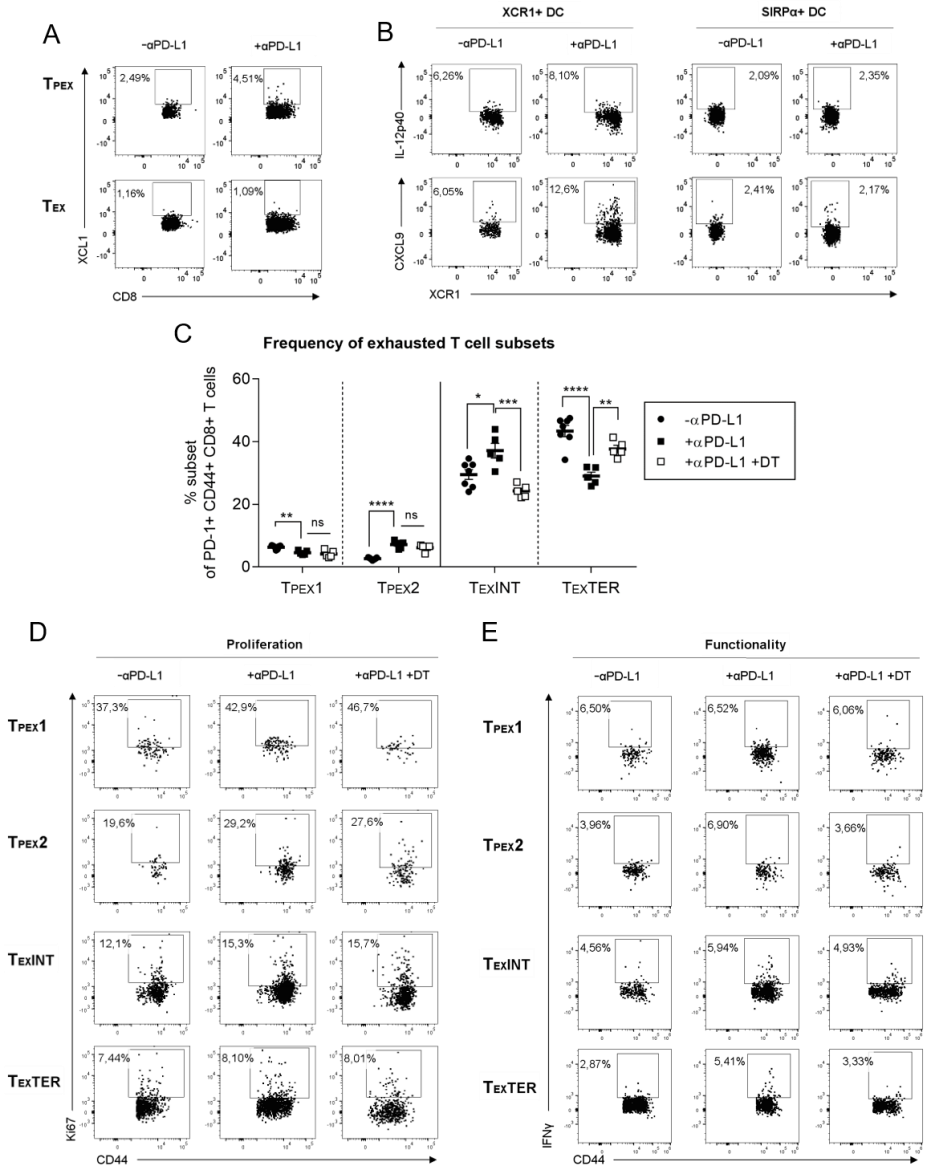


Figure S4. Functional changes of exhausted CD8+ T cells upon anti-PD-L1 treatment in the presence or absence of XCR1+ DC.

Chronic LCMV-infected mice were treated with anti-PD-L1 antibodies in the presence or absence (+DT) of XCR1+ DC, and sacrificed at day 30 p.i.. Representative dot plots of intracellular expression of XCL1 by T_{PEX} cells (A), and IL-12 and CXCL9 by XCR1+ and SIRPα+ DC (B) from mice chronically infected with LCMV and treated with anti-PD-L1 (+αPD-L1) or

left untreated (-αPD-L1). (C) Percentages of $T_{PEX}1$, $T_{PEX}2$, $T_{EX}INT$ and $T_{EX}TER$ analyzed at day 30 p.i.. (D) Representative dot plots of proliferating Ki67+ (D) and GP_{33-41}-specific IFNγ-producing (E) exhausted CD8+ T cell subsets.

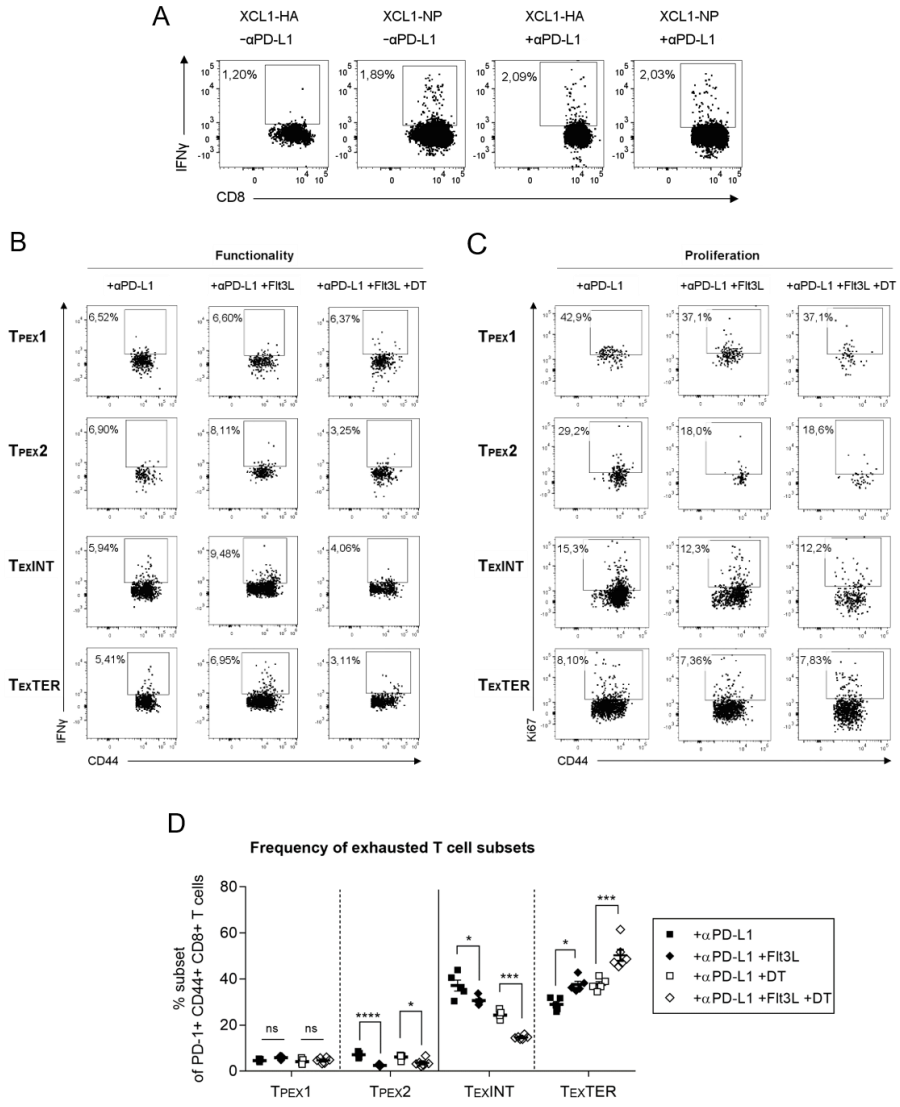


Figure S5. Combination of anti-PD-L1 treatment with XCR1-targeted fusion construct vaccination or Flt3L-mediated expansion of DC.

(A) Chronic LCMV-infected and anti-PD-L1 treated (+αPD-L1) or untreated (-αPD-L1) mice were vaccinated with XCL1-HA or XCL1-NP

fusion vaccines. Representative dot plots of $NP_{396-404}$-specific IFNγ-producing CD8+ T cells are shown. (B-D) Chronic infected and anti-PD-L1 treated mice were transfected with Flt3L-expressing plasmid (+Flt3L) in the presence or absence (+DT) of XCR1+ DC, and sacrificed at day 33 p.i.. Representative dot plots of GP_{33-41}-specific IFNγ-producing (B) and proliferating Ki67+ (C) exhausted CD8+ T cell subsets. (D) Percentages of $T_{PEX}1$, $T_{PEX}2$, $T_{EX}INT$ and $T_{EX}TER$ are shown.

6. DISCUSSION

In the present study we demonstrate the role of XCR1+ DC in maintaining the chronic state of a virus infection. Chronic infections are characterized by a dynamic equilibrium of virus expansion and CD8+ T cell mediated control. Perturbing this equilibrium by (i) targeting viral antigens to XCR1+ DC, (ii) blocking inhibitory checkpoints by anti-PD-L1 antibodies and/or (iii) increasing XCR1+ DC to SIRPα+ DC ratios by Flt3L administration all led to an increase of functional virus-specific CD8+ T cells with enhanced virus control. Importantly, the functional gain of CD8+ T cells, mainly $T_{PEX}2$, $T_{EX}INT$ and $T_{EX}TER$, was dependent on XCR1+ DC in all cases. In contrast, SIRPα+ DC with their high level of PD-L1 expression appeared to have suppressive function and were readily infectable thus contributing to virus expansion. These data not only propose different immunotherapy options to treat chronic virus infections but also highlight the key role of XCR1+ DC and their differential effects on exhausted CD8+ T cell subsets for therapy success.

XCR1+ and SIRPα+ DC behave differently with respect to virus susceptibility and immune regulatory functionality during chronic LCMV infection. In the chronic infection phase, XCR1+ DC expressed the activation markers CD40, CD80 and CD86 as well as the cytokines CXCL9 and IL-12(p40) demonstrating that they are capable to attract T cells and promote their differentiation towards an effector phenotype (*Sung et al., 2012; Danilo et al., 2018; Keppler et al., 2009*). In contrast, SIRPα+ DC had lower levels of activation markers but higher levels of PD-L1 and were more susceptible to infection. When being expanded by Flt3L in the

absence of XCR1+ DC, they inhibited T_{PEX} expansion triggered by anti-PD-L1. Together this suggests that both DC subtypes are part of the homeostatic control mechanism within lymphatic tissue that balances immune stimulatory and immune suppressive activities. It implies that either expansion of XCR1+ DC or inhibition of SIRPα+ DC could be of therapeutic use. Evidence for both strategies have been suggested in the context of cancer immunotherapy (*Sánchez-Paulete et al., 2016; Lai et al., 2020; Willingham et al., 2012; Veillette and Chen, 2018*) and they seem to apply for chronic infection control as well.

The observation that two separate DC subsets regulate division and effector function of antigen-specific CD8+ T cells in chronic LCMV infection is consistent with the concept of “balance of growth and differentiation” by Grossman and Paul (*Grossman et al., 2004*). This concept describes the feedbacks that regulate the intensity of proliferation, differentiation and death of specific T cells and explains their expansion upon immunization (*Quiel et al., 2011; Bocharov et al., 2011*). It was hypothesized that these T cell responses might be driven as well as limited by competition for cytokines or by the action of specialized suppressive elements while clustered around antigen-presenting DC. Our study here may suggest cross-presenting XCR1+ DC and PD-L1-expressing SIRPα+ DC to represent those biological control elements that implement the intra-cluster feedback regulation. Further studies along this concept deserve systematic analyses.

Our findings that XCR1+ DC are indispensable for anti-PD-L1 immunotherapy during a chronic viral infection are in line with prior studies in the context of cancer therapies (*Salmon et al., 2016;*

Spranger et al., 2017). Importantly, this requirement for XCR1+ DC was only linked to the functional gain of the exhausted CD8+ T cell subpopulations but not their proliferation. Anti-PD-L1 therapy in the absence of XCR1+ DC only induced CD8+ T cell proliferation without a gain in functionality and without better virus control. This indicates that XCR1+ DC numbers may limit treatment success in certain conditions and that an increase in that DC subset would be beneficial. Indeed, this was observed in cancer treatment when combining Flt3L, radiotherapy and a TLR3/CD40 agonist by in-situ administration (*Hammerich et al., 2019; Oba et al., 2020*). However, in our experiments with chronic LCMV infection and systemic Flt3L delivery, the functional gain of CD8+ T cells was counterbalanced by the expansion of SIRPα+ DC that act as virus target cells and contribute to virus increase.

The differential dependence of the exhausted CD8+ T cell subsets on XCR1+ DC for their functional activation might be explained by their different localization. Activation requires direct contact of T cell receptors with epitope-loaded MHC molecules on antigen presenting cells. Thus, the exhausted CD8+ T cell subsets $T_{PEX}2$, T_{EX}INT and T_{EX}TER and XCR1+ DC should contact each other temporarily, most likely in the white pulp of the spleen (*Calabro et al., 2016*). From there, they then migrate and egress the lymphoid organs, gain access to the blood circulation (*Beltra et al., 2020*) and subsequently reach their tissue destination (*Sandu et al., 2020*). In contrast, the $T_{PEX}1$ population is not activated by XCR1+ DC. This may be due to its presence in specialized niches that help maintain their quiescent and stem-like properties both during chronic infections (*Im et al., 2020; Leong et al., 2016*) and in tumors (*Jansen et al., 2019*). However, direct evidence for this

dynamic and localized interplay between XCR1+ DC and the exhausted CD8+ T cell subsets is still lacking.

The observed functional dichotomy between XCR1+ and SIRPα+ DC may direct the options for immunotherapeutic intervention strategies against chronic virus infections. Targeting virus antigen to XCR1+DC i.e. by linking a viral protein to the DC-attracting chemokine XCL1, or expanding XCR1+DC numbers by Flt3L, augmented exhausted CD8+ T cell functions and led to better virus control. The same was achieved when blocking the PD-L1-mediated inhibitory function of SIRPα+ DC. Targeting both DC subtypes simultaneously i.e. by combining anti-PD-L1 with antigen targeting to XCR1+DC or by combining anti-PD-L1 with XCR1+DC expansion may further increase T cell functionality. However, the extent of this and its benefit for virus control may depend on the properties of the infecting virus. In the experimental infection system used here, in which LCMV infects lymphatic tissue and uses antigen-presenting cells including SIRPα+ DC as target cells, the chronic infection state may have sufficient antigen levels in the spleen so that additional targeting to XCR1+DC only had a minor effect. Likewise, the T cell functionality improvement by increasing the number of XCR1+DC by Flt3L may be compensated by the increase of SIRPα+ DC that represent new virus target cells and help virus expansion. The respective implications for the immunotherapy of chronic virus infections like those with HIV or HBV in humans are unclear but should clearly be addressed.

While the immunotherapy of virus infections is an old concept, it gained a lot of attention in recent years due to the success of checkpoint inhibitors in cancer treatment (*Ribas and Wolchok,*

2018). However, in most of these trials, HIV or chronic Hepatitis B or C virus infections were exclusion criteria due to concerns regarding safety, efficacy and tolerance of checkpoint inhibitors when combined with antiviral therapy (*Gonzalez-Cao et al., 2019*). Nonetheless, trial results with few chronic virus-infected patients did not support such concerns and suggest a benefit at least for some of the patients (*Gardiner et al., 2013; El-Khoueiry et al., 2020; Gonzalez-Cao et al., 2020*). With the data provided here in the chronic LCMV infection model in mice, especially the combination of anti-PD-L1 with virus antigen targeting to cross-presenting DC would be an interesting therapy option for HIV-infected individuals. With the virus load controlled by antiretroviral therapy, the likelihood of exhausted regulatory T cell expansion would be reduced (*Peligero et al., 2015*) and cross-presenting DC-targeting vaccines might then redirect HIV-specific cytotoxic CD8+ T cell (CTL) responses to conserved epitopes within the patient (*Mothe et al., 2020*). Given that all the elements of such a therapy strategy are in place including human cross-presenting DC-targeting constructs (*Gudjonsson et al., 2017*), conserved HIV CTL epitope immunogens (*Mothe et al., 2020*) and a variety of available checkpoint inhibitors, it seems a feasible and well supported immunotherapy approach.

These results have been submitted as a journal article that is under revision:

Immunotherapy of chronic virus infections: exhausted CD8+ T cell subsets are differentially regulated by XCR1+ DC

Domenjo-Vila E[1], Casella V[1], Iwabuchi R[2,3], Fossum E[4], Pedragosa M[1], Castellví Q[5], Kaisho T[6], Terahara K[2], Bocharov G[7,8], Argilaguet J[1,9,*], Meyerhans A[1,10,*]

Affiliations:

[1] Infection Biology Laboratory, Department of Experimental and Health Sciences (DCEXS), Universitat Pompeu Fabra, Barcelona, Spain.

[2] Department of Immunology, National Institute of Infectious Diseases, Tokyo, Japan.

[3] Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan.

[4] Department of Immunology, Division of Laboratory Medicine, Oslo University Hospital, Norway.

[5] Department of Information and Communication Technologies, Universitat Pompeu Fabra, Barcelona, Spain.

[6] Department of Immunology, Institute of Advanced Medicine, Wakayama Medical University, Wakayama, Japan.

[7] Marchuk Institute of Numerical Mathematics, Russian Academy of Sciences, Moscow, Russia.

[8] Sechenov First Moscow State Medical University, Moscow, Russia.

[9] IRTA, Centre de Recerca en Sanitat Animal (CReSA-IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, Bellaterra, Spain.

[10] Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain.

*Correspondence:
Dr. Andreas Meyerhans (andreas.meyerhans@upf.edu)
Dr. Jordi Argilaguet (jordi.argilaguet@irta.cat)

7. CONCLUSIONS

The main conclusions of the present study are the following:

1. Targeting viral antigens to the XCR1 receptor of cross-presenting DC or increasing XCR1+ DC numbers during chronic virus infections provide therapeutic benefits.

2. The increase of functionality of virus-specific CD8+ T cells, in particular $T_{PEX}2$, $T_{EX}INT$ and $T_{EX}TER$ subsets, and their activation of proliferation during anti-PD-L1 immunotherapy are uncoupled. The former requires cross-presenting XCR1+ DC while the latter is independent of them.

3. XCR1+ DC and SIRPα+ DC have opposing roles in maintaining the steady state of chronic virus infection. XCR1+ DC maintain effector function and virus control while SIRP+ DC control exhaustion to avoid immunopathology. These observations are compatible with a theoretical concept developed by Zvi Grossman and Bill Paul to describe the regulation of immune responses.

4. The numbers of XCR1+ DC may be limiting during immunotherapeutic interventions and thus may need to be increased for successful treatment.

7.1. Graphical summary

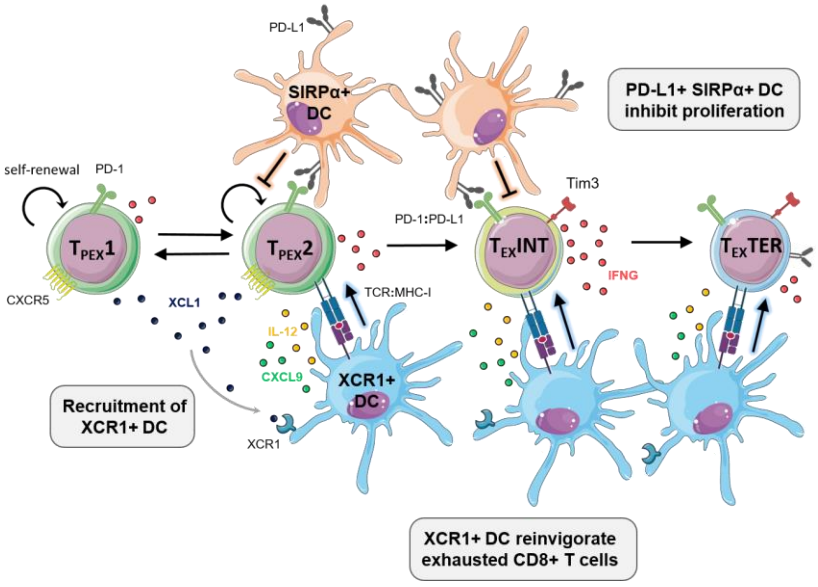


Figure C1. Role of conventional DC subsets in the regulation of the exhausted CD8+ T cell population dynamics.

Our proposed model for the regulation of the dynamics between the different populations of exhausted CD8+ T cells involves the two conventional DC subsets. XCR1+ DC or cDC1 would promote differentiation, CD8+ T cell priming and production of effector molecules (i.e. IFNγ) while SIRPα+ DC or cDC2 would limit the proliferation of the exhausted CD8+ T cell populations.

8. ANNEX

8.1. List of abbreviations

AIDS	Acquired immune deficiency syndrome
Clec9A	C type lectin domain containing 9A
CXCR5	C-X-C Motif Chemokine Receptor 5
cDC1	Convetional dendritic cell type 1
cDC2	Convetional dendritic cell type 2
DC	Dendritic cell
ERC	Endosomal recycling compartments
Flt3L	Fms-like tyrosine kinase 3 ligand
FRC	Fibroblast reticular cell
GP	Glycoprotein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIFs	Hypoxia-induced factors
HIV	Human immunodeficiency virus
IDO	Indoleamine 2,3 dioxygenase
IFN-I	Type I interferon
IFNγ	Interferon gamma
IL-10	Interleukin-10
IL-12	Interleukin-12
LCMV	Lymphocytic choriomeningitis virus
MDSC	Myeloid derived suppressor cells
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
NFAT	Nuclear factor of activated T cells
NK	Natural killer cell
NP	Nucleoprotein

PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PD-L2	Programmed death ligand 2
pDC	Plasmacytoid dendritic cell
SIRPα	Signal regulatory protein α
SIV	Simian immunodeficiency virus
T_{EFF}	Effector T cell
T_{MEM}	Memory T cell
T_{PEX}	Precursor exhausted CD8+ T cells
T_{EX}	Exhausted CD8+ T cells
TAP	Transporter associated with antigen processing
TCF1	Transcription factor T cell factor 1
TNFα	Tumor necrosis factor alpha
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TOX	Thymocyte selection-associated high mobility group box
Treg	Regulatory T cell
VHL	Von Hippel-Lindau
XCL1	X-C Motif Chemokine Ligand 1
XCR1	X-C Motif Chemokine Receptor 1
α-DG	α-dystroglycan

8.2. List of reagents and resources

Table A1. List of reagents and resources, their source and identifier.

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies (Mouse, Intracellular, *In vivo*)		
Alexa Fluor® 647 Rat Anti-Mouse I-A/I-E (clone M5/114)	BD Biosciences	Cat#562367
Alexa Fluor® 647 anti-mouse CXCL9 (MIG) (clone MIG-2F5.5)	BioLegend	Cat#515606
Anti-LCMV nucleoprotein (clone VL-4)	BioXcell	Cat#BE0106
Anti-mouse XCL1/Lymphotactin (clone 80222)	R&Dsytems	Cat#MAB486
APC Hamster Anti-Mouse CD3e (clone 145-2C11)	BD Biosciences	Cat#553066
APC Rat anti-Mouse CD172a (SIRPα) (clone P84)	BD Biosciences	Cat#560106
APC anti-mouse CD366 (Tim3) (clone RMT3-23)	Miltenyi	Cat#130-102-366
APC/Fire™ 750 anti-mouse CD40 (clone 3/23)	BioLegend	Cat#124631
BV421 Rat Anti-Mouse CD44 (clone IM7)	BD Biosciences	Cat#563970
BV510 Mouse Anti-Ki-67 (clone B56)	BD Biosciences	Cat#563462
Brilliant Violet 510 anti-mouse/rat XCR1 (clone ZET)	BioLegend	Cat#148218
BV605 Hamster Anti-Mouse CD279 (clone J43)	BD Biosciences	Cat#563059
BV711 Hamster Anti-Mouse CD80 (clone 16-10A1)	BD Biosciences	Cat#740698
Brilliant Violet 785™ anti-mouse CD3 (clone 17A2)	BioLegend	Cat#100231
eFluor 450 anti-mouse CD44 (clone IM7)	Ebioscience	Cat#48-0441-80
FITC anti-mouse CD44 (clone IM7)	Ebioscience	Cat#11-0441-82

FITC Rat Anti-Mouse CD86 (clone GL1)	BD Biosciences	Cat#553691
FITC anti-mouse CD279 (PD-1) (clone J43)	Ebioscience	Cat#11-9985-81
FITC Rat Anti-Mouse IFN-γ (clone XMG1.2)	BD Biosciences	Cat#554411
FITC anti-mouse MHC Class II (I-A/I-E) (clone M5/114.15.2)	Ebioscience	Cat#11-5321-81
InVivoMAb anti-mouse PD-L1 (B7-H1)	BioXcell	Cat#BE0101
Pacific Blue anti-mouse CD3 (clone 17A2)	BioLegend	Cat#100213
Pacific Blue anti-mouse CD19 (clone 6D5)	BioLegend	Cat#115526
Pacific Blue anti-mouse Nk1.1 (clone PK136)	BioLegend	Cat#108721
Peroxidase AffiniPure Goat Anti-Rat IgG (H+L)	Jackson ImmunoResearch	Cat#112-035-003
PE Rat Anti-Mouse CD4 (clone H129.19)	BD Biosciences	Cat#553653
PE anti-mouse CD172a (SIRPα) (clone P84)	BioLegend	Cat#144011
PE anti-mouse CD274 (B7-H1, PD-L1) (clone 10F.9G2)	BioLegend	Cat#124307
PE anti-mouse IL-12/IL-23 p40 (clone C17.8)	Ebioscience	Cat#12-7123-81
PE anti-mouse SiglecH (clone eBio440c)	Ebioscience	Cat#12-0333-82
PerCP-Cy™5.5 Rat Anti-Mouse CD4 (clone RM4-5)	BD Biosciences	Cat#550954
PerCP-Cy™5.5 Rat Anti-Mouse CD8a (clone 53-6.7)	BD Biosciences	Cat#551162
PerCP-Cy5.5 Hamster Anti-Mouse CD11c (clone HL3)	BD Biosciences	Cat#560584
PE-CF594 Rat Anti-Mouse CD45R (clone RA3-6B2)	BD Biosciences	Cat#562313
PE/Dazzle™ 594 anti-mouse CD185 (CXCR5) (clone L138D7)	BioLegend	Cat#145521
PE-Cy™7 Rat Anti-Mouse CD8a (clone 53-6.7)	BD Biosciences	Cat#552877

PE-Cy™7 Hamster Anti-Mouse CD3e (clone 145-2C11)	BD Biosciences	Cat#561100
PE-Cy7 anti-mouse CD69 (clone H1.2F3)	Ebioscience	Cat#25-0691-82
PE-Cy7 anti-mouse CD366 (Tim3) (clone RMT3-23)	Ebioscience	Cat#25-5870-82
Purified Rat anti-mouse CD16/32 (Mouse BD Fc Block™) (clone 2.4G2)	BD Biosciences	Cat#553141

Bacterial and virus strains		
LCMV Docile	Burkhard Ludewig	Grew in house
E. coli DH5alpha	New England Biolabs	Cat#C2987H

Chemicals and Peptides		
Diphtheria Toxin (DT) from Corynebacterium diphtheriae	Sigma	Cat#D0564
TransIT™-QR Delivery Solution	Mirus	Cat#MIR5240
Alexa Fluor™ 488 Antibody Labeling Kit	ThermoFisher	Cat#A20181
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit	ThermoFisher	Cat#L34955
BD Horizon™ Fixable Viability Stain 780	BD Biosciences	Cat#565388
37% Formaldehyde	Sigma	Cat#252549
Brefeldin A	Sigma	Cat#B7651
GP(33-41) peptide KAVYNFATM	David Andreu	Custom
NP(396-404) peptide FQPQNGQFI	David Andreu	Custom
Ampicillin 100 mg/mL, 0.2 µm filtered	Sigma	Cat#A5354
Luria Broth	ThermoFisher	Cat#12795027

Cell culture reagents		
RPMI-1640 medium	ThermoFisher	Cat# 21875-034
DMEM, high glucose, pyruvate	ThermoFisher	Cat#41966-052
IMDM, Hepes medium	ThermoFisher	Cat#21980-032
Fetal Bovine Serum	Sigma	Cat#F7524
Penicillin-Streptomycin	ThermoFisher	Cat#15140122
β-mercaptoethanol	Sigma	Cat#M3148
Sodium pyruvate	Sigma	Cat#S8636
Trypsin-EDTA 0.25%	ThermoFisher	Cat#25200
DMSO	Sigma	Cat#D4540

Experimental models: Cell Lines		
MC57 cell line	Grown in house	N/A
L929 cell line	Grown in house	N/A
VL-4 hybridoma	Grown in house	N/A

Experimental models: Organisms/Strains		
C57BL/6J mice	NCI/Charles River	Cat#027
XCR1DTRVenus mice	Yamazaki et al. 2013	Bred in house

Critical Commercial Assays		
DAB Substrate Kit, Peroxidase (HRP), with Nickel	Vector Laboratories	Cat#SK-4100
NucleoBond Xtra Maxi EF Kit	Macherey-Nagel GmbH & Co	Cat# 740424.10
QIAGEN EndoFree MegaPrep Kit	QIAGEN	Cat# 12381

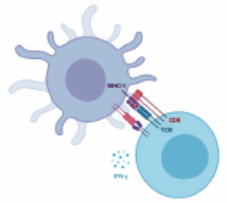
Plasmid DNA		
pEF-BOS-bsr vector	Iwabuchi et al. 2018	N/A
pEF-BOS-humanFlt3L-bsr vector	Iwabuchi et al. 2018	N/A
XCL1-HA fusion vaccine	Fossum et al. 2015	N/A
XCL1-LCMV-NP fusion vaccine	Even Fossum	N/A
αNIP-LCMV-NP fusion vaccine	Even Fossum	N/A

Software and algorithms		
FlowJo v10.7.1	TreeStar	https://www.flowjo.com/solutions/flowjo/downloads
GraphPad Prism v7	GraphPad Software	https://www.graphpad.com/scientificsoftware/prism/

8.3. Other contributions

During the time of my PhD thesis, I participated in the experimental planning, support and supervision of Ariadna's Master Thesis.

Optimization of dendritic cell-targeting vaccine constructs for immunotherapy of chronic virus infections



Ariadna Grinyó i Escuer

Final Project Master's Degree in Biomedicine

UNIVERSITAT DE BARCELONA

Academic year 2020-2021

Supervised by Andreas Meyerhans and Eva Domenjó Vila

Departament de Ciències Experimentals i de la Salut (DCEXS-UPF)

upf. Universitat Pompeu Fabra Barcelona

prbb Barcelona Biomedical Research Park

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