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Innate Immune Responses to LCMV Armstrong and Clone 13 and Their Influence on the Development of Acquired Immunity

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By

Brian A. Norris
B.S., University of Virginia, 2002

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An abstract of
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Graduate Division of Biological and Biomedical Sciences
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ABSTRACT

Innate Immune Responses to LCMV Armstrong and Clone 13 and Their Influence on the Development of Acquired Immunity

By

Brian A. Norris

Infection of mice with LCMV Armstrong (ARM) or Clone 13 (C13), results in acute and chronic infections respectively. However the early cellular and molecular mechanisms that mediate these disparate outcomes are poorly understood. We have characterized the innate immune responses that occur during acute and chronic LCMV infection, with a view to identifying early correlates and mechanisms of the later immune responses and viral loads. Two temporally distinct phases of innate immune response were observed: early DC responses during the first 72 hours of infection, and later myeloid cell expansion peaking at day 14 post infection.

During the first 72h of infection with ARM or C13, dendritic cells (DCs) upregulated activation markers, and produced innate cytokines independent of viral strain. $CD8\alpha^+$ and $CD8\alpha^-$ DC populations decreased following activation, with $CD8\alpha^+$ DC nearly absent by 72h post infection. Finally, DCs and myeloid cells enriched during the first two days of ARM and C13 infection similarly stimulated OT-1 T cells *in vitro*.

In the second phase, by day 7, myeloid cells were expanded in the spleen and the blood during both infections. However, while myeloid cells contracted during ARM infection, C13 infection increased and sustained high numbers myeloid cells, peaking at day 14. $Ly6C^{hi}$ monocytic cells expanded during C13 infection had the morphology and phenotype of myeloid-derived suppressor cells (MDSC) and potently suppressed T cell proliferation. Blocking iNOS or IFN-γ abrogated the suppressive function of these cells. $CCR2^{-/-}$ mice showed impaired mobilization of myeloid cells during C13 infection, and had enhanced anti-viral T cell function. By administering anti-Gr-1 antibody to C13 infected mice we were able to temporarily deplete myeloid cells, leading to increased cytokine production by LCMV-specific $CD8^+$ T cells. Early innate immune responses to ARM and C13 are indistinguishable by classical methods of measuring activation and T cell stimulatory function. However, during chronic infection myeloid cells become massively expanded and are able to inhibit T cell proliferation and function, contributing to viral persistence.

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LIST OF ABBREVIATIONS

ADCC:	antibody-dependent cellular cytotoxicity
APC:	antigen presenting cell
ARG1:	arginase-1
ARM:	LCMV Armstrong 53b strain
ASC:	apoptosis-associated speck-like protein containing a CARD
BM:	bone marrow
C13:	LCMV Clone 13 strain
CARD:	caspase activation and recognition domain
cDC:	conventional DC
COX2:	cyclooxygenase-2
CSFR-1:	colony-stimulating factor receptor 1
DC:	dendritic cell
dsRNA:	double stranded RNA
ECM:	extracellular matrix
GCN2:	general control non-depressible 2
GMCSF:	granulocyte macrophage colony-stimulating factor
GP:	glycoprotein
i.p.:	intraperitoneal
i.v.:	intraveinously
ICAM-1:	intercellular adhesion molecule 1
iNOS:	inducible nitric oxide synthase
IPS-1:	IFN-β promoter stimulator

IRF3:	interferon regulatory factor 3
JAK:	Janus kinase
KLRG-1:	killer cell lectin-type receptor G1
L:	LCMV polymerase
LCMV:	lymphocytic choriomeningitis virus
LDC:	lymphoid DC
LGP2:	laboratory of genetics and physiology 2
LPS:	lipopolysaccharide
LRR:	leucine rich repeat
MCMV:	murine cytomegalovirus
MCSF:	macrophage colony-stimulating factor
MDA5:	melanoma differentiation associated factor 5
MDC:	myeloid DC
MDSC:	myeloid derived suppressor cells
MHC:	major histocompatibillity complex
MMP:	matrix metalloproteinase
MPO:	myeloperoxidase
MTOR:	mammalian target of rapamycin
MZ:	marginal zone
NADPH:	nicotinamide adenine dinucleotide phosphate
NET:	neutrophil extracellular trap
NK:	natural killer cell
NKT:	natural killer T cell

NLR:	NOD-like receptor
NP:	nucleoprotein
PAMP:	pathogen associated molecular pattern
PD-1:	programmed death-1
pDC:	plasmacytoid DC
PDGF:	platelet-derived growth factor
P.F.U.	plaque forming units
PRR:	pattern recognition receptors
PSGL-1:	P-selectin glycoprotein ligand 1
PYD:	pyrin domain
RIG-I:	retinoic acid-inducible gene-I
RLR:	RIG-I-like receptor
RNOS:	reactive nitrogen-oxide species
ROS:	reactive oxygen species
RP:	red pulp
SCF:	stem cell factor
ssRNA:	single stranded RNA
STAT:	signal transducer and activator of transcription
TCR:	T cell receptor
TGFβ:	tumor growth factor
T_H1:	T helper 1
T_H17:	T helper 17
T_H2:	T helper 2

TIR:	Toll/IL-1R homology
TIRAP:	TIR domain containing adaptor protein
TLR:	Toll-like receptor
TNF:	tumor necrosis factor
TRAM:	TRIF-related adaptor molecule
T_{reg}:	regulatory T cell
TRIF:	TIR-domain-containing adapter-inducing interferon-b
VEGF:	vaso-endothelial growth factor
WP:	white pulp

CHAPTER 1

INTRODUCTION

Part I: Innate Immune Receptors and Innate Immune Cells

The innate immune system is comprised of a variety of cell types equipped with a vast array of molecular sensors, which quickly detect the presence of non-self antigens, microbes, and damaged tissue. The diversity of these molecular sensors, coupled with the multitude of different innate cells, confers a broad arsenal of protective capabilities early during infection. The diversity and plasticity of innate immune responses also initiates and conditions a plethora of adaptive immune responses required for the resolution of infection and protection against further infection. Determining which innate immune cells, receptors and signaling pathways are involved in the recognition of pathogens and how they relate to the clearance or persistence of microbial infection is essential for our understanding of the pathogenesis of infection and central to the development of protective vaccines.

Pattern Recognition Receptors (PRR) and Modulating Adaptive Immunity

The innate immune system recognizes the presence of pathogens by a set of germline-encoded receptors with limited specificity, the PRRs. These receptors bind vital components of bacteria and viruses (pathogen associated molecular patterns, PAMPs), initiating signaling cascades that activate NFκB, MAP kinases, and type I interferons

(IFNs), among others, producing inflammation and mobilizing the innate and adaptive arms of the immune response (*1, 2*). Professional antigen presenting cells (APCs) in general, and dendritic cells (DCs) in particular, integrate diverse signals delivered from PRR and modulate the quantity, quality and longevity of the adaptive immune response (*3-6*).

The first discovered and most well characterized family of PRRs are the Toll-like receptors (TLRs). The TLR family of PRRs share homology with the *Drosophila* protein Toll, which is involved the dorso-ventral patterning of fly embryos and in inducing the secretion of antimicrobial peptides following signaling (*1, 7*). All TLRs contain leucine-rich-repeats (LRRs), which are involved in recognizing PAMPs, and a cytosolic Toll/IL-1R homology (TIR) domain that is involved in signaling. Although all TLR have a similar basic structure, they recognize a wide array of PAMPs. TLRs 1, 2 and 6 recognize glycolipids; TLR5 recognizes flagellin; TLRs 3 (dsRNA), 7 (ssRNA), and 9 (unmethylated CpG motifs) recognize nucleic acids, and TLR4 recognizes multiple types of ligands including LPS, membrane proteins and stress proteins (*1*). The signals elicited by TLR ligation also exhibit significant diversity due to the use of different combinations of common adaptor proteins (*8, 9*).

All TLRs bind the adaptor molecule MyD88 through their intracellular TIR domain, with the exception of TLR3. MyD88 signaling leads to NF-κB and MAP kinase activation, resulting in the production of multiple inflammatory cytokines (*10*). TLR3 and TLR4 bind with intracellular TIR-domain-containing adaptor-inducing interferon-β

(TRIF) and induce type I-IFN production via interferon regulatory factor 3 (IRF3) in addition to engaging the alternate pathway of NF-κB activation (*10*). A third adaptor protein utilized by TLRs is TIR domain containing adaptor protein (TIRAP). Both TLR2 and TLR4 use TIRAP to recruit MyD88 while only TLR4 uses TRIF-related adaptor molecule (TRAM) to associate with TRIF (*11*). By utilizing multiple adaptor molecules, each capable of activating at least one signaling pathway, TLRs are imbued with the capacity for diverse down-stream signaling outcomes.

TLRs are expressed on a large number of cells, including DCs, macrophages, endothelial cells, B cells and some T cells. Macrophages and all DCs, including the primary type I IFN producing DCs, plasmacytoid DCs (pDCs), express TLRs 1-9 (*12*). The responses elicited by TLR activation depend heavily on the type of cell activated. DCs stimulated by TLR generally become activated, increasing expression of MHC Class II and costimulatory molecules, increasing production of inflammatory cytokines, and altering expression of chemokine receptors, inducing migration of DCs to secondary lymphoid organs (*12, 13*). The type of TLR signal also influences the type of immune responses initiated by the activated APC. Different individual TLR signals can skew the type of T cell responses that DCs elicit; TLR2 signals stimulate DCs to secrete IL-10 and low IL-12p70, skewing $CD4^+$ T cells towards a T-helper 2 (T_H2) response, while TLRs 4, 5, and 9 individually cause DCs to produce large amounts of IL-12p70 and type I IFN, biasing the responding $CD4^+$ T cells to become T-helper 1 (T_H1) cells (*8, 9, 14-16*). Interestingly, while many TLRs are expressed on the surface membrane, nucleic acid recognizing TLRs such as TLR3, TLR7/8 and TLR9 are localized internally in the

endoplasmic reticulum or endosomes (*17-20*). Localization to these internal vesicles positions these cells to quickly interact with viral nucleic acids and initiate innate immune signaling while avoiding interaction with self-nucleic acids.

In addition to surface or endosomal PRR, there are several classes of cytosolic PRR that have been shown to recognize viral-, bacterial- and fungus-specific components within infected cells. The RIG-I like receptor (RLR) family of cytoplasmic proteins contains DExD/H box RNA helicases and recognizes PAMPs within viral RNA. The RLRs retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation associated factor 5 (MDA5) both contain a N-terminal caspase activation and recognition domain (CARD), a central DExD/H helicase domain and a C-terminal repressor domain (*21*). RIG-I has been found to preferentially recognize dsRNA and ssRNA sequences that contain a 5'-triphosphorylated end (*22-24*). MDA5 is similar in structure to RIG-I but shows a preference for high-molecular-weight poly(I:C) fragments, in contrast to the smaller RNA preference of RIG-I (*25, 26*). A third RLR, laboratory of genetics and physiology 2 (LGP2), has not been shown to recognize nucleic acids, but has been implicated as a positive regulator of the other RLRs (*27, 28*). All three RLRs are fairly ubiquitously expressed in most cell types, typically at a low level that is increased with exposure to IFN or viruses (*27, 29, 30*). RLR recognition and binding of nucleic acids drives conformational changes that in turn allows them to associate with IFN-β promoter stimulator 1 (IPS-1), leading to the formation of downstream signalsomes that drive IFN production (*31-33*). Similar to TLR signaling, NF-κB, IRF3 and IRF7 signaling pathways are engaged by RLR signaling (*34*).

In addition to RLRs, the family of NOD-like receptors or nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs) are cytosolically expressed innate receptors. This family contains over 20 discovered members, each containing a LRR near the c-terminus and a NACHT nucleotide-binding domain (*35*). NLRs can be further subdivided by their N-terminal domains, which are important for associating with accessory and signaling molecules (*36*). NLRs containing an N-terminal pyrin domain (PYD) are termed NLRP. Other NLRs contain CARDs, acidic transactivation domains, or baculoviral inhibitory-repeat domains. Some NLRs can form large cytoplasmic, multimolecular complexes termed inflammasomes. NLRP1, NLRP3 and NLRC4 have been shown to form these inflammasomes in response to different diverse factors such as PAMPs from bacteria, viruses and fungi (*37-43*). Inflammasome formation is also catalyzed by danger-associated signals like ATP released from dying cells, extracellular matrix (ECM) proteins, and the formation of crystals by exogenous compounds or typically soluble endogenous molecules (*44-46*).

Upon activation the CARDs within NLRs associate with pro-caspase-1 and catalyze its cleavage. Cleaved and activated caspase-1 can in turn catalyze the cleavage of pro-IL-1β and pro-IL-18 into active, secretable forms (*47-49*). PYD-containing NLRs can associate with the accessory protein ASC (apoptosis-associated speck-like protein containing a CARD) and also initiate caspase-1 signaling cascades (*50*). Both IL-1-faimly cytokines IL-1β and IL-18 are potent inflammatory proteins, activating lymphocytes and endothelial cells. IL-1β signaling induces production of TNF, IL-6, inducible nitric oxide

synthase (iNOS), cyclooxygenase-2 (COX2) and many adhesion molecules involved in the recruitment and trafficking of leukocytes (*51*). Furthermore, because IL-1 family receptors contain TIR domains and associate with MyD88, there is much overlap between these signaling pathways and those of TLRs. NLR activation can also lead to a distinct form of cell death resulting from the caspase-1 and possibly caspase-7 apoptosis-associated signaling cascades (*52, 53*). This process is called pyroptosis and demonstrates characteristics of apoptosis and necrosis.

The intersection of pathogens containing multiple PAMPs with diverse PRRs and cytokine signaling pathways on varied APC subsets in different tissues produces an innate response distinctive to the pathogen. This innate response in turn gives rise to a specific adaptive immune response. Knowledge of how these patterns of activation and response by innate cells during natural infection contributes to adaptive immune response and infection outcome are currently deficient. A more detailed understanding of how specific APCs and specific PRRs interact with pathogens to create different adaptive responses would vastly increase our capacity to design vaccines to elicit desired protective immunity.

Innate Immune Cells

Innate immune responses are the critical frontline defense against pathogens; quickly recognizing the presence of pathogens, beginning to combat them, and alerting the rest of the body to the threat. These responses involve both non-immune cells and cells of the

innate immune system. Innate immune cells are highly diverse and include innate effector cells such as neutrophils, eosinophils and basophils, lymphocytes like natural killer (NK) and natural killer T cells (NKT), and professional APCs which include monocytes, macrophages, and DCs. Additionally, each population can be subdivided by phenotype and function. Non-immune cells can produce inflammatory cytokines following infection; type I IFNs in particular are produced by infected cells, initiating an antiviral response within the cell and neighboring cells, as well as activating innate cells (*54*). Innate immune responses are critical to initiating, directing and maintaining humoral and cellular adaptive immune responses.

Granulocytes

Granulocytes are some of the earliest cells to be involved in innate immune responses. The major granulocyte cells are the eosinophils, basophils, mast cells and neutrophils. Granulocytes have distinct and well-characterized effector functions determined by their granule contents, which are rapidly disgorged upon activation. In addition, there is increasing evidence of a role for these cells in polarizing and sustaining T_H1 and T_H2 $CD4^+$ T cell responses. Eosinophils release large quantities of cationic proteins, ECM remodeling enzymes, and immunomodulatory cytokines and chemokines upon activation (*55*). Basophils are the rarest granulocytes and share many characteristics with tissue-resident mast cells, such as high IgεRI expression (*56*). Both eosinophils and basophils can secrete large amounts of IL-4 and IL-13, quintessential T_H2 polarizing cytokines (*57-59*). Recent reports have demonstrated that both cells can directly activate $CD4^+$ T cells via MHC Class II and indirectly influence T cells by modulating DC function with

cytokines (*60-63*). Eosinophils, basophils and mast cells play central roles in the initiating and sustaining T_H2 type responses to helmith infections and are key players in the tissue remodeling, fibrosis and inflammation found during asthma and allergic reactions (*55, 56*).

Neutrophils are the most abundant leukocyte and are essential granulocytes that promote inflammation, engulf and destroy microbes, recruit further innate and adaptive immune cells, and influence the polarization of adaptive immune responses (*64*). These cells actively survey the vasculature in a rolling adhesion manner mediated by L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils and P-selectin and E-selectin expression on endothelial cells (*65, 66*). Upon encountering activation signals, β2 integrin proteins expressed on neutrophils become activated and bind with high affinity to endothelial-expressed intercellular adhesion molecule 1 (ICAM-1) (*67*). Following this tight adhesion, neutrophils begin the process of diapedesis, migrating through the endothelium and into the underlying tissue (*68*). This process is aided by the expression of ECM specific proteolytic enzymes, which allow neutrophils to degrade the surrounding tissue and migrate towards chemotactic gradients (*64, 69*).

Upon reaching the site of infection, neutrophils have multiple mechanisms to combat microbes, further activate immune responses and promote the recruitment of additional innate cells. Neutrophils contain three types of granule; in order of size they are the azurophilic, the specific, and the gelatinase granules. Azurophilic granules contain multiple antimicrobial proteins such as defensins, lysozyme, several serine proteases and

myeloperoxidase (MPO). The specific granules lack MPO but contain chelating proteins like lactoferrin and several anti-microbial compounds (*70, 71*). The gelatinase granules lack MPO and have fewer anti-microbials than other granules. Instead they are enriched for matrix metalloproteinases (MMP), particularly gelatinase (*72*). Fusion of granules occurs at the plasma membrane or at the phagosomal membrane, which is particularly important in the destruction of phagocytosed microbes (*73*). The antimicrobial compounds released by neutrophils function by many different mechanisms including both direct and indirect killing. Defensins and cathelicidins are cationic peptides implicated in inhibiting bacterial cell wall synthesis and potentiating DC activation (*74-76*).

Neutrophils produce large amounts of reactive oxygen species (ROS) upon their activation in a process called respiratory burst. The NADPH oxidation complex is formed at the plasma membrane or phagosomal membrane and leads to the production of superoxide. Superoxide can dismutate to hydrogen peroxide or combine with other reactive nitrogen molecules, leading to many different downstream effects (*77-79*). Hypohalous acids can be produced in the phagosome by MPO reacting with hydrogen peroxide (*80*). These ROS have direct anti-microbial effects and contribute to the function of multiple neutrophil-derived molecules, including the regulation of caspases, metalloproteinases, and phosphatases (*81-84*).

Neutrophils also are major phagocytic cells essential for controlling and killing pathogens in addition to removal of cellular debris. Microbes or debris are internalized in

active processes via direct and indirect mechanisms. Antibody and complement opsonized microbes are actively internalized by Fcγ-receptor- and complement-receptor-mediated phagocytosis. Additionally, neutrophils can directly recognize PAMPs via TLRs and initiate engulfment. Mouse neutrophil cell lines express TLRs 1, 2, 3, 4, 5, 6, 7 and 9 while human primary neutrophils express all TLRs except for TLR3 (*85-87*). Granule fusion with the nascent phagosome and initiation of the respiratory burst at the phagosome membrane are key to establishing an environment toxic to microbes (*77*). Neutrophil phagosomes remain neutral in pH for extended periods of time to facilitate the function of many of their unique serine proteases (*88, 89*).

A novel method of pathogen control was recently described for neutrophils. Following activation neutrophils can undergo a form of cell death that induces the release of chromatin to form a microbe-entrapping milieu called a neutrophil extracellular trap, or NET (*90-92*). These NETs contain large concentrations of antimicrobial proteins as well as histones and cellular proteins. Neutrophil activation also causes the release many cytokines with immunomodulatory effects. Multiple monocyte/macrophage and DC attracting molecules, e.g. CCL2, CCL3, CCL20, and CCL19, are produced by activated neutrophils (*93*). Due to the potentially destructive capacity of neutrophils their function is tightly controlled. Upon elimination of microbes, neutrophils undergo apoptosis and release factors that inhibit further neutrophil recruitment and express signals that make them targets for phagocytosis. Moreover, macrophages that ingest apoptotic neutrophils have been shown to produce the anti-inflammatory cytokines IL-10 and TGFβ, promoting the resolution of infection (*94*).

Natural Killer Lymphocytes

Natural killer cells are adept at surveying self-cells for signs of viral infection and then mediating their destruction through the release of preformed lytic granules or engagement of death receptors (*95-97*). In addition to killing target cells, NK cells also release large quantities of inflammatory cytokines such as GM-CSF, MCSF, TNF, IL-5, IL-10, and IL-13 (*98*). NK cells can produce large amounts of IFN-γ during some viral infections, inhibiting viral replication and activating the immune response (*99-101*). Utilizing multiple receptors that recognize MHC Class I, non-classical MHC, and other receptors that associate with the health of a cell, NK cells are able to determine if a cell is suffering from infection. The balance of inhibitor receptor and activating receptor signaling determines NK cell function. The absence of MHC Class I on infected or cancerous cells reduces inhibitory signals from NK receptors specific for MHC molecules while recognition of stress markers or viral proteins by NK activating receptors can trigger the release of cytotoxic granules to kill the infected cell (*102-105*). NK cells can also lyse target cells via antibody-dependent cellular cytotoxicity (ADCC); NK cells release cytolytic granules upon recognition of antibodies bound to infected cells, via FcγRIII, independent of MHC expression (*106*).

A considerable amount of crosstalk between DCs and NK cells has been observed *in vivo* with DC produced IL-12 or IL-18 inducing high levels of IFN-γ in NK cells (*107, 108*). Type I IFN produced by other innate cells, particularly DCs and pDCs can also activate NK cells, leading to blastogenesis, proliferation and enhancement of killing

ability (*109-111*). In addition to their well-described innate effector functions, NK cells have been ascribed memory-like characteristics. In mice infected with murine cytomegalovirus, MCMV, NK cells expressing Ly49H expand and contract in a manner similar to T cells, and respond to further viral infection with substantial secondary expansion capable of protective immunity (*112*).

There are also subsets of lymphocytes that express many NK cell markers and αβ-T cell receptors (TCR) that have limited diversity. These NKT cells are derived from thymocytes and diverge into their distinct lineage by TCR engagement by CD1d expressed on thymic cortical thymocytes (*113-115*). CD1d is a member of a family of MHC-like proteins and binds a variety of lipids recognized by NKT cell TCRs. These lipids include α-galactosylceramide (α-GalCer) derived from marine sponges, microbial glycosphingolipids such as GSL-1, 2, 3 and 4, and the self-lipid isoglobotrihexosylceramide (iGb3) (*116-120*). In mice, NKT TCRs are comprised predominantly of Vα14-Jα18 and either Vβ8, Vβ7, or Vβ2, all of which recognize αGalCer and iGb3 (*121*). NKT cells are typically found in low numbers in the blood and lymphoid organs but are enriched in the liver (*122*). Upon TCR stimulation, NKT cells can release large quantities of T_H1 and T_H2 cytokines, IFN-γ and IL-4 (*123, 124*). Upon recognition of CD1d expression on DCs, NKT cells also upregulate CD40L. NKT cells then reciprocally activate DCs to produce IL-12 and increase expression of MHC Class I, Class II, and costimulatory molecules (*125-127*). By reacting to glycosphingolipids from the cell wall of *Sphingomonas*, a ubiquitous bacterium that is both Gram-negative and

LPS-negative, NKT cells provide a TLR-independent pathway for initiating innate and adaptive responses (*117-119*).

Macrophages

Macrophages are a heterogeneous population of cells with multiple subsets that can be defined by tissue location, surface marker diversity, and function (*128*). Although macrophages become specialized in each tissue microenvironment they inhabit, the key common function of macrophages is as the major phagocytic cell type in the body, engulfing apoptotic self cells as well as opsonized bacteria and pathogens to destroy them (*128, 129*). In the absence of immune signals or tissue damage, macrophages are essential to the clearance and recycling of ~$2x10^{11}$ erythrocytes from the blood as well as removing cellular debris and ECM components (*130, 131*). In addition to this critical steady-state role in maintaining tissue and recycling components from cell death, macrophages are poised to rapidly respond to the presence of microbes and produce pro-inflammatory cytokines. The wide array of surface receptors that define macrophages populations also define the specific function of the cells by enabling macrophages to recognize protein and carbohydrate motifs on cells and microbes and induce their internalization (*128*).

Receptors for phosphatidyl serine, thrombospondin, complement and multiple scavenger receptors are involved in the homeostatic removal of debris by macrophages, and typically do not induce inflammation or induce inhibitory signals and cytokines (*132*). Macrophages also express multiple PRRs, including TLRs, and quickly recognize

exogenous and endogenous indicators of tissue injury and infection. These cells also express Fc receptors and receptors for multiple cytokines and chemokines that act to activate and influence the function of macrophages. Because of their location and receptor expression, macrophages have a heightened ability to internalize pathogens. Macrophages are also professional APCs, and are particularly adept at digesting foreign bodies and proteins and presenting them in the context of MHC Class II to $CD4^+$ T cells, initiating adaptive T cell responses (*133*).

Consequent to the diverse repertoire of surface receptors expressed by macrophages, recognition of different endogenous an exogenous molecules can lead a spectrum of activation responses in macrophages. Three broad categories have been defined for macrophage activation, i.e. classical, alternative/wound-healing and regulatory macrophages (*131*). Each type of activation typically requires two types of innate or adaptive signal to elicit activation. Classical activation, sometimes referred to as M1 activation, was originally described as the response to IFN-γ and TNF. Macrophages treated with these cytokines produce large amounts of anti-microbials, many pro-inflammatory mediators, cytokines, and large quantities of ROS and reactive nitrogen products (*134, 135*). These macrophages are highly adept at finding and destroying microbes but also result in tissue damage. Early during infection, NK cell produced IFN-γ or infected-cell-produced IFN-β can activate macrophages when combined with TNF produced following TLR signaling. The innate IFN signaling is generally transient, and full, sustained activation of macrophages requires the production of IFN-γ by T_H1 T cells (*135*). Classically activated macrophages can produce large quantities of IL-1, IL-6, and

IL-23, which can attract neutrophils as well as induce T_H17 T cells and promote more neutrophil recruitment (*136-138*).

Wound-healing, or M2, macrophages are elicited by IL-4 produced by innate or adaptive immune cells in response to a variety of situations (*139*). They are thought to be part of T_H2 type responses to fungi, parasites and helminths as well as important mediators of the resolution of T_H1 type responses to viruses and bacteria (*140-142*). Early during infection, IL-4 is produced by basophils or mast cells, though, adaptive T_H2 produced IL-4 is needed to sustain this form of macrophage activation (*139, 143*). These macrophages produce very few pro-inflammatory cytokines or toxic free radicals but produce precursors and components of the ECM and secrete a variety of MMPs and inhibitors of MMPs (*144*). Furthermore, these macrophages secrete factors like platelet-derived growth factor (PDGF), which promote fibroblast proliferation and differentiation (*145*). Wound-healing macrophages are believed to be essential for the repair of tissue damaged by classically activated macrophages and neutrophils but have also been implicated in fibrosis and airway remodeling during asthma and other diseases (*146*).

Under certain conditions macrophages can gain a regulatory function, characterized by production of the potent anti-inflammatory cytokine IL-10 (*147*). These macrophages typically fail to prime T cells, or prime $CD4^+$ T cells to adopt a T_H2 or T_{reg} phenotype (*129, 148*). As with other types of macrophage activation, regulatory macrophages require two signals to gain their immunomodulatory function. Diverse stimuli that signal tissue damage and stress, such as glucocorticoids, Ig immune complexes, adenosine, and

histones can induce IL-10 production my macrophages when coupled with TLR signaling (*149*).

Dendritic Cells

The most potent innate APCs are the conventional DCs (cDCs), which are present in nearly every tissue to guard against infection (*5, 150*). cDCs are essential gate keepers between innate and adaptive immune responses, mediating tolerance by initiating the death of thymocytes that react to DC-presented self antigens, and initiating adaptive responses by surveying tissues and migrating to lymphoid tissues to prime T cells (*150-154*). Under normal conditions most cDCs are immature, maintaining low expression of costimulatory markers and localizing most MHC Class II molecules intracellularly (*8, 150*). Some of these immature DCs migrate to lymphoid tissues in the absence of activation signals, present food antigens or self-antigens from apoptotic cells, and induce tolerance (*8, 155-157*). DCs in tissues alter their expression of chemokine receptors and home to draining lymph nodes upon exposure to inflammatory cytokines, following an encounter with a pathogen, or upon sensing cellular damage (*158, 159*). During this migration, cDCs mature, increasing their expression of MHC Class II, costimulatory markers such as CD80 and CD86, and become capable of secreting immunomodulatory cytokines like IL-12, TGFβ, TNF and IL-10 (*160-162*). Within secondary lymphoid organs, DCs migrate to lymphoid regions, secrete chemokines to attract B and T cells and prime $CD8\alpha^+$ and $CD4^+$ T cells that recognize peptide:MHC Class I or II (*163-166*). The potent immunogenicity that DCs exhibit can also be induced by CD40 signaling.

Activated $CD4^+$ helper T cells upregulate CD40L and can induce functional activation in DCs, potentiating immune responses (*167, 168*).

The cDC subsets that reside in the spleen during steady state are derived from blood precursors, termed pre-DCs, which lack a monocyte intermediate stage and can also divide *in situ* to prolong antigen presentation in lymphoid organs (*169, 170*). Three of the main murine splenic DC subsets are the lymphoid DCs (LDCs) ($CD11c^{hi}$, $CD8\alpha^+$, $CD11b^-$, DEC-205^+, $33D1^-$), myeloid DCs (MDCs) ($CD11c^{hi}$, $CD8\alpha^-$, $CD11b^+$, DEC-205^-, $33D1^+$), and pDCs ($CD11c^{lo}$, $B220^+$, $Ly6C^+$, PDCA-1^+) (*3, 8, 165, 171, 172*). Lymphoid DCs can be activated to produce large amounts of bioactive IL-12p70, stimulating T_H1 responses, and are found in the T cell areas of secondary lymphoid organs such as the spleen and lymph nodes (*164, 165*). MDCs produce less IL-12p70 than LDCs, are concentrated in the marginal zones (MZ) of the spleen and drive a more T_H2 response in $CD4^+$ T cells than lymphoid DC (*164, 165*). Plasmacytoid DCs are prodigious producers of IFN-α in response to some viruses and other stimuli, and can also differentiate into $CD8\alpha^+$ DC under certain circumstances (*172-174*). While both LDCs and MDCs are able to prime efficient CTL responses, in non-infectious systems it has been demonstrated that only LDCs can capture exogenous antigen for cross presentation on MHC Class I molecules (*175-177*).

The two major subsets of human DCs are the myeloid DCs and plasmacytoid DCs. Human blood MDCs are generally equivalent to mouse cDCs and can be subdivided in to $CD141^+$ $CD11c^{lo}$ and $CD11c^{hi}$ $CD1c^+$ subsets. Circulating DCs expressing high CD141

have recently been described as being potent antigen-cross presenting DC and functionally resemble CD8α$^+$ LDC in mice (*178-181*). CD1c$^+$ DC in humans resemble mouse CD8α$^-$ MDC. Furthermore, human pDCs are potent producers of type I IFNs, and are very functionally close to mouse pDCs (*173, 182*). The identification and characterization of human DCs has primarily been done in the skin and blood, or through *in vitro* studies of monocyte-derived DCs (*183, 184*). Correlating mouse and human DC subset function has been difficult due to the paucity of human lymphoid tissue studies, and the nearly 10-fold lower numbers of circulating DC in mice relative to humans (*185*).

Monocytes

Monocytes are circulating mononuclear cells that are adept at phagocytosis, possess the capacity to differentiate into macrophages and, under the right circumstances, DCs. In mice, monocytes can be identified by their expression of CD115 (CSFR1), CD11b, F4/80 and CX_3CR1 (*186-188*). Monocytes have been further subdivided by phenotypic and functional differences. The major blood population of monocytes are termed 'inflammatory' monocytes, and express high levels of Ly6C, CCR2 and CD62L (*189*). These cells are rapidly recruited from the blood to inflamed tissues by chemokines that bind CCR2 or CX_3CR1 and can produce large amounts of inflammatory cytokines (*190-192*). Less abundant are the Ly6C^{lo}, CX_3CR1^{hi}, CCR2$^-$, and CD62L$^-$ monocytes. These are often referred to as 'patrolling' or 'resident' monocytes due to observations of these cells clinging to the endothelial lining of blood vessels (*193*). These cells are believed to be some of the first monocytes to extravasate following injury or inflammation due to their close association with the endothelium.

Human monocytes have been described as having similar functional properties, but are defined by the expression patterns of CD14 (a co-receptor with TLR4 and MD-2 for LPS) and CD16 (the high affinity FcγRIII). In healthy people, 'classical' $CD14^{+}CD16^{-}$ monocytes are the predominant subset, and express high levels of CCR2 and low CX_3CR1, phenotypically resembling $Ly6C^{hi}$ 'inflammatory' monocytes in mice (*189, 194, 195*). However, these monocytes appear to be functionally different from mouse cells, and produce more IL-10 than TNF or IL-1 following stimulation with LPS. Two types of $CD16^{+}$ monocytes have been described. 'Intermediate,' $CD14^{+}CD16^{+}$, monocytes are highly phagocytic and produce large amounts of TNF and IL-1 when treated with LPS (*195, 196*), The third subset of human monocytes are the 'non-classical' monocytes, $CD14^{low}CD16^{+}$, and are poorly phagocytic and produce little TNF following LPS stimulation (*197*).

As mentioned previously, during steady state the majority of tissue-resident DCs are replenished by circulating pre-DCs (*198, 199*). During infection and inflammation, the lymphoid architecture can become disrupted and innate cells like DCs and macrophages can be depleted by host and pathogen mechanisms. $Ly6C^{+}$ cells transferred to irradiated mice were able to replenish both cDC populations and macrophages in the spleen. Furthermore, $Ly6C^{+}$ cells were also able to replenish macrophages and DCs in the gut mucosa, the skin, and the lungs of recipient mice (*200-203*). During systemic inflammation these monocytes can also differentiate to TNF and iNOS producing DC-like cells, called Tip-DC (*204*). In the *Listeria monocytogenes* model of parasitic

infection, intraperitoneal (i.p.) inoculation leads to rapid egress of $Ly6C^-$ cells, which transiently produce pro-inflammatory cytokines within 2h after infection (*193*). After this early phase, approximately 8h post infection, $Ly6C^+$ monocytes extravasate and differentiate into DCs producing TNF, iNOS and ROS. $Ly6C^-$ monocytes continue to enter the peritoneum, but begin expressing genes associated with wound healing macrophages, such as Arg1, Fizz1, and IL-4Rα (*205, 206*). Sequential recruitment and differential function of monocyte subsets was also observed in models of myocardial infarction (*202, 207, 208*). Monocytes can be recruited during inflammation or infection to perform both pro-inflammatory, anti-microbial functions and tissue repairing, immunomodulatory functions. These different functions are key to induction of immune responses as well as resolving inflammation and repairing damage incurred.

Innate Cells During Chronic Inflammation: Myeloid Derived Suppressor Cells

During cases of chronic inflammation or infection the differentiation of myeloid cells into neutrophils, macrophages and DCs can become altered. Instead of maturing, these myeloid cells remain in an immature state and upregulate a variety of immunomodulatory factors that suppress T cell responses. This population of cells, called myeloid-derived suppressor cells (MDSCs), has been described in cases chronic inflammation including cancer, sepsis, trauma, autoimmune disease and many infections (*209, 210*).

MDSCs are a heterogeneous population of myeloid cell precursors that have become prevented from fully differentiating during chronic inflammation. In mice, these cells can be broadly identified by their expression of the CD11b, Ly6C and Gr-1 antigens. MDSCs can be broken down phenotypically into cells with neutrophilic ($CD11b^{+}$, $Ly6C^{lo}$, $Gr\text{-}1^{hi}$) or monocytic ($CD11b^{+}$, $Ly6C^{hi}$, $Gr\text{-}1^{lo/-}$) characteristics (*211, 212*). These populations generally lack characteristics of mature cells, such as high MHC Class II, CD86 or CD11c (*213, 214*). Expression of CD115, CD80 and IL-4Rα have been observed to be higher in populations of MDSC, but on a per cell basis expression of these molecules has not correlated with increased suppressive function (*211, 215-217*). MDSC have also been described in humans, particularly in cancer patients. Human MDSC have been described as $CD11b^{+}CD33^{+}CD14^{-}HLA\text{-}DR^{-}$ or $CD14^{+}HLA\text{-}DR^{lo/-}$ with significant variability in the expression of multiple other markers (*218-222*). Because of the high level of morphological heterogeneity, MDSC populations must be defined by their suppressive function, either *in vitro* or *in vivo*.

MDSCs are expanded in the bone marrow (BM) during pathogenic conditions and accumulate in lymphoid tissues in their immature, undifferentiated state (*210*). A variety of signals have been associated with the expansion and accumulation of myeloid cells and are summarized in many reviews (*209, 210, 223*). These factors include inflammatory cytokines, inflammatory mediators and growth factors such as IL-1β, IL-6, COX2, prostaglandins, stem cell factor (SCF), M-CSF, GM-CSF, and VEGF(*209, 210, 223*). The signal transducer and activator of transcription 3 (STAT3) signaling and transcription pathway is the most prominent pathway associated with MDSC expansion

(*224*). Activated STAT3, in association with Janus kinase (JAK) family proteins, promotes proliferation and survival of myeloid progenitors upon GM-CSF signaling (*225*). High levels of STAT3 signaling can induce aberrant and uncontrolled growth by altering the expression of cell division genes such as cyclin D1, c-Myc, Bcl-xL, Mcl-1, and survivin (*226*). Additionally, STAT3 induces the expression of S100 calcium-binding proteins A8 (S100A8) and S100A9, which are associated with inflammation, but are also implicated in the promotion of MDSCs (*227*). Genetic inhibition of STAT3 signaling or blocking of S100A8 and S100A9 receptors have been shown to prevent the expansion and accumulation of MDSCs in tumor-bearing mice (*228-231*). Furthermore, the IL-10 receptor utilizes STAT3 signaling and IL-10 has long been implicated in T cell anergy and, in conjunction with TGFβ, the induction of T_{reg} helper cells (*232, 233*). IL-10 signaling has also been shown to suppress DC-mediated T cell activation and has a direct anti-inflammatory effect on macrophages (*234, 235*).

In concert with the expansion of myeloid cells, the gain of suppressive function by MDSCs is believed to require additional signals. Factors from stromal cells, tumor cells, T cells, PAMPs or dead cells have all been implicated in MDSC function. STAT1 signaling from IFN-α or IFN-γ receptor activation is key to the regulation of iNOS and arginase 1 (ARG1), two key proteins involved in antimicrobial responses and MDSC suppression of T cells. T cells prevented from secreting IFN-γ were unable to induce iNOS, and consequently MDSC-suppression was abrogated (*215, 236*). MDSC function is also induced by STAT6 signaling downstream of the IL-4Rα chain, common to IL-4 and IL-13 receptors. STAT6 activation can induce ARG1 and TGFβ production and

mediate suppression by MDSCs, and STAT6 deficient mice are blocked from ARG1 production and suppression (*237-239*).

Prostaglandins are potent inflammatory mediators produced from arachadonic acid by the COX2 enzyme. In tumor systems, production of prostaglandin PGE_2 was associated with increased tumor proliferation, angiogenesis, and metastasis (*240*). It was also found that PGE_2 and COX2 increase ARG1 expression in myeloid cells as well as induce myeloid precursors to become MDSCs (*241*). Loss of the PGE_2 receptor, EP4, on myeloid cells or the use of COX2-inhibitors reduced the suppressive capacity of MDSCs and delayed tumor progression, respectively (*242*).

NF-κB-mediated signaling pathways have also been implicated in the induction of suppression in myeloid cell populations. In models of polymicrobial sepsis, MyD88-dependent signaling through TLRs or IL-1R activates NF-κB, and drives MDSC accumulation (*243*). LPS-induced TLR4 signaling in conjunction with IFN-γ signaling can block myeloid cell differentiation to DC and promote MDSC accumulation (*244, 245*). Inflammatory tumor environments producing IL-1β promote longer-lived MDSCs, independent of T cells, B cells and NK cells, and the inhibition IL-1β using IL-1R antagonist slowed tumor growth (*246, 247*). Most of the signaling pathways associated with MDSC expansion and induction of their suppressive function are also commonly associated with acute infection. It is highly likely that only under pathologic conditions do multiple factors act in concert to divert the normal differentiation of myeloid cells and

drive an alternate function for these cells, likely to combat excessive inflammation by both innate and adaptive arms of the immune system.

The suppressive function of MDSCs has long been linked to the metabolism of the conditionally essential amino acid L-arginine by both ARG1 and iNOS (*248*). ARG1 metabolizes L-arginine into urea and L-ornithine, the latter of which serves as a precursor to the production of cell growth promoting polyamines and L-proline, a critical component for ECM production (*249, 250*). iNOS catalyzes the conversion of L-arginine to L-citruline and NO via a redox reaction. T_H1 and T_H2 T cell responses typically competitively control these two metabolic pathways. ARG1 is a key enzyme associated with 'alternatively' activated and wound-healing macrophages and is induced by STAT6-mediated signaling and IL-4, IL-10, IL-13 and TGFβ signaling (*237, 251, 252*). Conversely, proinflammatory stimuli such as LPS, IL-1, TNF and STAT1 signaling by IFN-α, IFN-β and IFN-γ are potent inducers of iNOS, a hallmark of 'classically' activated macrophages (*253-255*). During pathologic conditions that elicit MDSCs, myeloid populations express both ARG1 and iNOS. Monocytic MDSCs typically express high levels of iNOS while neutrophilic MDSCs express ARG1 (*211, 256, 257*).

These metabolic enzymes can indirectly impair T cell responses by depleting L-arginine. Low levels of this amino acid and high levels of urea from ARG1 activity have been shown to activate the general control non-depressible 2 (GCN2) and mammalian target of rapamycin (MTOR) pathways (*258*). These pathways are involved in sensing amino acid availability and can block transcription in times of stress, effectively

inhibiting T cell proliferation (*259, 260*). ARG1 depletion of L-arginine by MDSCs has been shown to impair T cell signaling by inhibiting the re-expression of the TCR subchain CD3ζ after antigen recognition-dependent internalization (*261*).

Immune suppression from MDSC-produced NO occurs through the direct S-nitrosylation of cysteine residues on intracellular proteins that mediate IL-2R signaling. NO thus blocks the phosphorylation of key activating residues in the JAK1, JAK3 and STAT5 pathways, reducing and blocking signal two in T cell activation (*262, 263*). NO can also directly impair the stability of IL-2 mRNA and has a direct pro-apoptotic effect on T cells through TNF-family receptors or caspase independent pathways (*264, 265*).

When both ARG1 and iNOS are expressed and concentrations of L-arginine are reduced in the environment, iNOS activity shifts away from producing NO and towards the production of superoxide (*266, 267*). These ions then react with other molecules to produce reactive nitrogen-oxide species (RNOS) and ROS. These molecules have direct effects on the ability of T cells to signal and function, and are key mediators of T cell suppression by MDSC. Reaction of NO with superoxide leads to the production of peroxynitrites ($ONOO^-$), which can readily enter cells and react with tyrosine residues within T cells and initiate apoptosis (*268, 269*). Nitration of tyrosine residues on the TCR and CD8 molecules diminishes their ability to bind with peptide:MHC and thus limits signaling (*270*). The potent ROS hydrogen peroxide, H_2O_2, produced by the reaction of two superoxide molecules with hydrogen, is associated with MDSC

suppression in tumor models. H_2O_2 can impair CD3ζ expression and induce apoptosis in T cells (*271, 272*).

An additional mechanism of MDSC-mediated immunosuppression is through the induction of T_{reg} cells by MDSCs expanded in some mouse tumor models. In one model of mouse tumor immunity, IL-10 and TGFβ production by MDSC promoted the development of Fox-$P3^+$ T cells while another model showed MDSC-mediated induction of T_{reg} in an ARG1 dependent and TGFβ-independent manner (*216, 273*). Through these multiple ARG1 and iNOS produced mediators as well as anti-inflammatory cytokines, MDSCs are able to directly and indirectly reduce the ability of T cells to become activated and also reduces their viability.

Part II: Lymphocytic Choriomeningitis Virus (LCMV) as a Model for Acute and Chronic Viral Infections

Basic Virology of LCMV

Arenaviruses have received considerable attention for their use as model systems for studying acute and persistent viral infections and for their clinical relevance as causative agents of severe hemorrhagic fever (*274, 275*). The arenavirus LCMV is a natural pathogen in mice that has been used to elucidate many aspects of T cell immunity (*276,*

277). LCMV is particularly useful as a model system because infection can lead to acute or chronic infections depending upon host (mouse strain and age) and virus (different virus isolates, route of infection and infectious dose) factors (*274, 278*).

Arenaviruses are enveloped viruses with bi-segmented, ssRNA genomes that encode proteins in an ambisense manner with some genes encoded in sense and some in antisense (*274, 279, 280*). The genome contains two genes on each of the two segments, termed L (7.2 kb) and S (3.4 kb), separated by a unique hairpin structure (*279, 281*). The L segment contains the viral RNA dependent RNA polymerase (L) and a small RING-finger protein (Z) which acts a matrix protein linking the viral nucleoprotein and membrane proteins and is the major protein involved in viral budding (*282-284*). The S segment contains the nucleoprotein (NP) and glycoprotein polyprotein (GPC) that becomes cleaved post-translationally into SSP (signal peptide), GP1 and GP2 (*285-287*). Following cleavage, the GP complex is responsible for the initial binding of virions to cells and affecting their entry into cells.

α-Dystroglycan (α-DG) is the cellular receptor protein for LCMV and the majority of known arenaviruses (*288, 289*). α-DG is a peripheral protein that when complexed with β-DG, a trans-membrane protein, associates with multiple ECM proteins such as laminin-1, laminin-2, agrin or perlecan with high affinity (*290-292*). The ECM-protein-specific interactions with α-DG influence the ability of viral GP to mediate binding; large ECM proteins with high affinity for α-DG, which are found in lymphoid organs, are more difficult for GP to displace (*289, 290, 293*). Some derived strains of LCMV with

sequence changes in the GP complex have acquired a higher affinity for binding α-DG, and as a consequence have a different cellular tropism than parental strains. This difference in tropism also alters the course and outcome of infection, with strains with high affinity for α-DG frequently resulting in persistent infection (*289, 294*).

The persistently infectious LCMV strain Clone 13 (C13) is a genetic variant derived from the Armstrong 53b (ARM) strain. Inoculation with ARM leads to an acute infection, with virus being eliminated within 7-10 days. Infection with C13, leads to persistently high viremia for up to three months with some tissues never clearing virus (*295-297*). C13 was isolated from lymphoid tissue of maternally infected BALB/c WEHI mice that showed no functional adaptive immune response to the virus following the thymic deletion of viral reactive T cells during development (*295, 298-300*). The C13 genome differs from its parental strain by 5 nucleotides, resulting in two amino acid differences. GP residue 260 is mutated from Phe to Leu and L protein residue 1079 is mutated from Phe to Leu in C13 (*301-303*). Either of these mutations been shown to be sufficient to confer persistence to LCMV infection (*288, 301, 302*). The mutation in the GP polyprotein of C13 was shown to increase the affinity of GP for α-DG up to 100 fold and increase C13 binding 2.5 log over ARM binding (*278, 288-290, 294*). The 1079L mutation of LCMV C13 increases the replication rate of LCMV and increases early viremia (*304*).

The MZ and white pulp (WP) of the spleen have particularly high expression of the α-DG-binding laminin, and also contain high concentrations of macrophages and DCs (*290,*

305). Fluorescent microscopy of infected spleens demonstrated the co-localization of C13 strains with the MZ and WP of the spleen, while virus was restricted to primarily to the red pulp (RP) of the spleen during ARM infection (*289, 294*). The higher affinity of C13 for α-DG increased its infectivity, particularly infecting DEC-205$^+$ DC, CD11c$^+$ cells, the interdigitating DC in T cell areas, and nests of cells near the MZ (*278, 306*). C13 and other persistent LCMV strains have also been shown to have higher infectivity of pDC (*304*). ARM virions, in contrast, primarily infect F4/80$^+$ macrophages and some DCs, but less so than C13 (*278*). In addition to infecting macrophages and DCs, the mutations in C13 confer greater infectivity of follicular reticular cells (FRCs) (*307*). These cells are part of a complex microstructure within lymphoid organs that support and organize the migration, recruitment and activation of lymphocytes (*308, 309*), and the disruption of the conduit network these cells create is believed to be detrimental to the maintenance of a robust adaptive immune response.

The Cellular Immune Response to LCMV

Clearance of LCMV ARM infection is associated with robust CD8α$^+$ T cell expansion, specific lysis of infected cells and IFN-γ production. C13 infection, in contrast, generates a CD8α$^+$ T cell response that is detectable one week following infection but subsequently shows diminished effector function, and eventually complete functional exhaustion (*297, 310*). During acute ARM infection, CD8α$^+$ T cell responses are directed at the immunodominant LCMV peptides $NP_{396\text{-}404}$, $GP_{33\text{-}41}$, and to a lesser extent $GP_{276\text{-}286}$ in the context of MHC Class I H-2D^b (*311-315*). CD8α$^+$ T cells reactive to these epitopes

expand rapidly and can reach a peak expansion of approximately 10^7 cells per spleen by day 8 following infection and total viral-reactive CD8α^+ T cells can constitute up to 50% of the cells in the spleen (*297, 316, 317*). Following this expansion and viral clearance >90% of these cells apoptose leaving a small population from which a stable pool of memory CD8α^+ T cells is generated (*318-321*).

During the expansion phase of LCMV ARM infection, CD8α^+ T cells express effector proteins and begin to increase expression of the genes for IFN-γ, TNF, IL-2, perforin and granzyme B and which allow them to even more quickly produce cytokines (*322-324*). T cell contraction following expansion is believed to limit the immunopathology that results from excessive cytolysis and cytokine production, and also resets the immune system following infection to become more able to respond to new infections (*320*). Several models exist to describe what controls contraction, including contraction due to scarcity of survival cytokines in the absence of infection, engagement of multiple TNF-family receptors that promote death, and potential toxicity due to repeated release of perforin and IFN-γ (*320, 325-328*).

The size of the effector population of CD8α^+ T cells during the expansion phase is directly related to the size of the memory pool that survives contraction (*320*). Two distinct but related lineages of CD8α^+ T cells emerge following infection, termed central- and effector-memory (*329*). The effector-memory cells maintain expression of granzyme B and do not express CD62L or CCR7 and are thus found in peripheral tissues. Central-memory cells localize to secondary lymphoid tissues owing to their expression of CD62L

and CCR7. Effector-memory cells patrol peripheral tissues to rapidly lyse infected cells while central-memory cells, which have a greater proliferative capacity and can become effector cells, primarily survey APCs in lymphoid tissues and react somewhat more slowly than effector-memory cells (*329, 330*). Expression of the α-chain of the IL-7 receptor has been shown to characterize both naïve cells and memory precursor cells (*331-333*). During acute viral infection, all virus specific $CD8\alpha^+$ T effector cells downregulate IL-7Rα. Following the clearance of antigen, a subset of T cells re-expresses IL-7Rα, suggesting the generation of memory cells from within the total effector population of virus-specific T cells. Those T cells within the total $IL\text{-}7R\alpha^{lo}$ effector population that become predisposed to remain short-lived effectors can be identified by high expression of killer cell lectin-type receptor G1 (KLRG-1) during the effector expansion phase of the immune response. Cells with intermediate expression of KLRG-1 preferentially become long-lived memory cells with high proliferative capacity (*334, 335*).

Although the $CD8\alpha^+$ T cell response to LCMV is the essential adaptive component for clearance of infection, there is a robust $CD4^+$ T cell response. Virus-specific $CD4^+$ T cells expand early during infection, though in significantly lower numbers and with slower kinetics than $CD8\alpha^+$ T cells, reaching a peak expansion 1-2 days after $CD8\alpha^+$ T cells (*318, 336*). The $CD4^+$ T cell responses are primarily T_H1 in nature, producing IFN-γ and IL-2 (*318, 337, 338*). Experiments in $CD4^+$ deficient mice demonstrated that $CD4^+$ T cells were dispensable to the clearance of acute infections, but in the absence of $CD4^+$ T cell help, $CD8\alpha^+$ T cells became functionally impaired and failed to maintain a stable

memory population (*319, 339, 340*). The activation of $CD4^+$ T cells has been shown to have a much higher dependence on signaling through costimulatory molecules such as CD28, CD154, and OX40 (*341-343*).

Unlike LCMV ARM infection, C13 infection does not lead to a robust $CD8\alpha^+$ and $CD4^+$ T cell response capable of clearing virus and protecting against future infection. The expansion of $CD8\alpha^+$ T cells is diminished in C13 infected mice and the hierarchy of immunodominance is altered (*329*). Although they are the immunodominant responders during ARM infection, $CD8\alpha^+$ T cells specific for $NP_{396\text{-}404}$ become nearly undetectable following C13 infection (*297, 310, 344*). Responses to GP epitopes are less affected and normally subdominant $GP_{276\text{-}286}$ specific $CD8\alpha^+$ T cells become immunodominant during C13 infection (*310*). Multiple chronic viral infections appear to support a decreasing-potential hypothesis whereby chronic antigen exposure leads to diminishing function and eventual deletion of cells that most frequently recognize viral-peptide:MHC Class I (*310, 345-347*). Because NP is the most abundantly expressed viral antigen, $NP_{396\text{-}404}$-reactive clones are more likely to be stimulated and eventually exhausted, while clones recognizing the less abundant GP will be present and functional longer (*319, 320*).

In addition to the physical deletion of virus specific $CD8\alpha^+$ T cells, infection with C13 also leads to diminished and eventual absence of T cell function. Virus specific $CD8\alpha^+$ T cells lose their functionality in a hierarchical manner during persistent C13 infection, first losing lytic capacity and the production of IL-2, then TNF and finally losing the ability to produce IFN-γ (*297*). The functional potential of $CD8\alpha^+$ T cells is inversely related to

the antigen load and directly related to the quality of $CD4^+$ T cell help (*297, 310, 345*). $CD4^+$ T cells have recently been shown to be defective in IL-2 and TNF cytokine production early during infection with persistent viral infections including C13 (*348, 349*). During C13 infection, multiple virus-specific $CD4^+$ T cells that are found during ARM infection are absent or diminished in number and function (*350*). Responses to non viral epitopes were also shown to be impaired within 12 days of infection with C13, reflecting a general impairment of the immune system (*350*).

Much research has been dedicated to deciphering the molecular mechanisms of T cell exhaustion during chronic infection. Major inroads were made following the discovery that programmed death-1 (PD-1) expression was a hallmark of $CD8\alpha^+$ T cell exhaustion and that by blocking the PD-1/PD-L1 inhibitory signaling pathway T cell function could be restored and chronic LCMV infection could be resolved (*351*). Further gene array, surface molecule expression, and functional analysis of T cells during chronic LCMV infection revealed several other inhibitory molecules that characterized T cell exhaustion, including LAG-3, Tim-3 and 2B4 (*352-355*). These data revealed a complex pattern of regulation of $CD8\alpha^+$ T cells by many co-expressed inhibitory receptors during chronic viral infection.

In addition to the multiple surface markers implicated in the exhaustion of $CD8\alpha^+$ T cells multiple cytokines have been shown to contribute to the loss of T cell function during chronic infection. Production of the immunoregulatory cytokine IL-10 has been strongly linked to the suppression of T cell responses and persistence of virus during

chronic LCMV infection in mice (*356, 357*) as well as HIV and HCV infection of humans (*358, 359*). Blocking antibodies specific for IL-10 or its receptor have successfully led to resolution of chronic LCMV infection and enhanced $CD8\alpha^+$ T cell function. Both $CD4^+$ and $CD8\alpha^+$ T cells have been shown to produce high levels IL-10 during persistent viral infection (*357*) and recently both DC and macrophages have been implicated as producing IL-10 (*360*). The immunosuppressive cytokine TGFβ is also an important mediator of $CD8\alpha^+$ T cell deletion during C13 infection. Persistently infected mice show high levels of Smad-2 phosphorylation and TGFβ production. Attenuation of this signaling pathway in T cells prevented the reduction of $CD8\alpha^+$ T cells during C13 infection, enhanced CTL responses and increased cytokine anti-viral cytokine production leading to rapid viral clearance and establishment of a protective memory T cell pool (*361*).

The Innate Immune Response to LCMV

The infection, activation and function of DCs, macrophages and other APCs during LCMV ARM and C13 infection are critical parameters in determining the outcome of infection. Differences in ARM and C13 interaction with the innate immune system are likely to play a large role in initiating the adaptive immune responses that clear ARM and hinder the ability of the immune system to clear C13. Although much of the adaptive immune responses to LCMV ARM and C13 have been elucidated, currently there is a deficit of understanding of how innate immune activation during these infections contributes to infection outcome. A thorough understanding of the interaction of these

virus strains with the innate immune system will give us insight into how some viral infections can become chronic.

Innate responses to acute LCMV have been detected within the first day of infection. Following inoculation with ARM there is a rapid activation of splenic DC subsets within 24 hours, characterized by upregulation of costimulatory markers CD40, CD80, and CD86 as well as higher surface expression of MHC Class I and Class II (*362*). It has been demonstrated that expression of pro-inflammatory cytokines human IL-8 and mouse IL-6 during exposure to LCMV requires MyD88 and TLR2 signaling in peritoneal macrophages and transfected human cells (*363*).

DC-produced IL-12p70, TNF and type I IFNs in conjunction with surface activation molecules are crucial to stimulating CTL and T_H1 responses necessary for clearing LCMV (*362, 364-366*). Mice lacking DCs are unable to mount an effective CTL response, despite the presence of macrophage and B cell APCs (*367*). During ARM infection, activated DCs achieve an optimal ability to prime T cells *ex vivo* during the first three days of infection, and are able to prime T cells as early as 24 hours post infection (*362*). This window of optimal activation capacity was framed by the decline in the numbers of splenic DC subsets and their diminishing expression of costimulatory markers by the third day of infection (*362*). This decrease in numbers and activity of DCs correlates with the peak serum IFN-α levels 2-3 days after infection. Type I IFN inducing some of the functional activation of DCs but also drastically increases the apoptosis of DCs (*362, 368*). Thus far there have not been any direct comparisons of the

stimulatory capacity of DCs and macrophages during the first 3 days of LCMV ARM or C13 infection.

Type I IFNs have been implicated as having conflicting roles in the outcome of LCMV infection. Although LCMV-specific CD8α+ T cell expansion is highly dependent upon type I IFN signaling (*364*), IFN signaling can also impair innate cells and their priming of adaptive immune responses. The production of type I IFNs following ARM infection is not primarily from pDCs, but SIGN-R1^{+} marginal zone macrophages (MZM), cells important for trapping virus and restricting dissemination (*369*). Multiple studies have shown that intact splenic architecture is necessary for control and clearance of virus, and that DCs are essential to the control of infection (*367, 369, 370*). DC production of IFN-α, as measured by mRNA, is elevated 3-5 days after infection with ARM, but can be detected in higher levels out to day 50 during C13 infection (*371*). There is also evidence that the greater infection of DCs by C13 leads to this higher production of IFN-α. The persistent presence of IFN during C13 infection can retard the development of DCs in bone marrow impairing the replacement of cells lost during infection in a STAT2 dependent manner. DCs during C13 infection have also been reported to be impaired in the expression of MHC Class I and II and costimulatory markers in a type I IFN/STAT2 dependent manner (*371, 372*). Viral proteins also can directly interfere with the ability of cells to respond to antiviral innate responses. LCMV NP effectively retards the ability of IRF-3, a critical signaling element of the type I IFN pathway, to translocate to the nucleus and initiate transcription, antagonizing type I IFN production (*373*). Thus a balance between innate activation and viral immune evasion

techniques ultimately influence the generation of robust, pathogen clearing adaptive immune responses.

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CHAPTER 2

Chronic, but not acute viral infections, induce sustained expansion of myeloid suppressor cells that inhibit viral-specific T cell immunity

Innate immunity to acute & chronic viral infection

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Summary

Resolution of acute and chronic viral infections requires activation of innate cells to initiate and maintain adaptive immune responses. Here we report that infection with acute Armstrong (ARM) or chronic Clone 13 (C13) strains of lymphocytic choriomeningitis virus (LCMV) led to two distinct phases of innate immune response. During the first 72hr of infection, dendritic cells upregulated activation markers, and stimulated anti-viral $CD8^+$ T cells, independent of viral strain. Seven days after infection, there was an increase in $Ly6C^{hi}$ monocytic and $Gr\text{-}1^{hi}$ neutrophilic cells in lymphoid organs and blood. This expansion in cell numbers was enhanced and sustained in C13 infection, whereas it occurred only transiently with ARM infection. These cells resembled myeloid-derived suppressor cells, and potently suppressed T cell proliferation. The reduction of monocytic cells in *Ccr2*$^{-/-}$ mice or after Gr-1 antibody depletion enhanced anti-viral T cell function. Thus, innate cells have an important immunomodulatory role throughout chronic infection.

Introduction

A fundamental feature of chronic viral infections, such as during HIV or HCV infection, is the generalized suppression of the host immune response. Innate immune responses to viral infections are essential for initiating the adaptive immune responses required for the resolution of infection and generation of long lasting immunity. Some viral infections fail to elicit sufficient immune responses or subvert host defenses, thus allowing their spread and eventual persistence. The ARM and C13 strains of LCMV have been studied for decades as models for acute and chronic infections, respectively (*1*). These strains are very closely related genetically, but the ARM strain leads to a strong $CD8^+$ T cell response that rapidly clears the virus and provides long lasting immunity (*2, 3*). Conversely, infection with the C13 strain of LCMV impairs virus-specific T cells, diminishing their function and enabling the persistence of the virus (*4*). Infection with C13 also leads to the suppression of additional immune responses to viral infections (*5, 6*).

The genome of C13 differs from ARM by two coding mutations, one in the glycoprotein (GP) and one in the polymerase protein L (*7, 8*). The GP mutation enhances the affinity of this protein for the cellular receptor, α-dystroglycan (αDG), leading to greater infection of αDG expressing cells such as macrophages, dendritic cells (DCs) and fibroblastic reticular cells (FRC) (*7, 9, 10*). The mutation in the L protein correlates with higher levels of viral replication in plasmacytoid DCs (pDCs) and increased early viremia (*11*). These mutations as well as similar mutations in related LCMV strains have been implicated as causing the persistence of chronic LCMV strains.

Acute LCMV infection rapidly induces DCs to upregulate the expression of costimulatory markers and gain T cell stimulatory capacity in a largely type I interferon (IFN-I) dependent manner (*12*). LCMV-specific T cell responses are also highly dependent on IFN-I with maximal $CD8^+$ T cell proliferation requiring IFN-I signaling (*13*). However, LCMV nucleoprotein (NP) inhibits IFN-I production in infected cells and persistent LCMV infection inhibits IFN-I production by pDCs (*14, 15*). IFN-I-induced STAT2 signaling inhibits the development and proliferation of DCs (*16, 17*). PD-1-PD-L1 signaling, IL-10 and TGF-β are additional immune regulatory mechanisms linked to the persistence of C13 infection and the exhaustion of the $CD8^+$ T cell response (*18-20*). Despite these advances, the innate immune events that occur during the very earliest hours of infection with LCMV ARM or C13 have not been studied. Furthermore, although there have been reported defects in antigen presenting cell (APC) function during the chronic stages of C13 infection, whether such defects precede and cause dysfunctional T cell responses, and enable persistence is unclear.

We hypothesized that ARM or C13 induce distinct types of innate immune responses within the first few hours of infection, which result in the differential induction of T cell immunity and viral persistence. To address this issue, we undertook a detailed characterization of the kinetics of the innate immune responses to chronic and acute LCMV infection with a view to identifying early correlates and mechanisms of adaptive immune exhaustion and viral persistence. Two temporally distinct phases of innate immune response were observed: an early DC response during the first 72hr of infection and a later myeloid cell proliferation peaking at day 14 post infection (p.i.). We found the

innate response within the first 72hr, as assessed by the numbers and activation status of DC and APC subsets and cytokine production, was largely independent of the strain of LCMV. Later during infection, as $CD8^+$ T cell responses begin to peak, there was a large expansion of myeloid cell numbers. This expansion was identical between acute and chronic strains of LCMV until about day 7. After this period, there was a rapid decline in the numbers of myeloid cells in ARM infected mice. During C13 infection, however, there was a sustained expansion of myeloid cells that exhibited the phenotype and suppressive function of myeloid-derived suppressor cells (MDSCs). Diminished recruitment of these cells or the elimination of these cells during chronic infection by depleting antibody enhanced the function of exhausted T cells. Our findings demonstrate different phases of innate cell function during viral infection and highlight an important role for innate cell inhibition of adaptive immune responses during chronic infection.

Results

Innate cell activation during first 72hr is strain independent

The innate immune responses during the first few hours of acute and chronic LCMV infection remain to be described. To address this we performed a detailed kinetic analysis of the various sub-populations of APCs, as well as induction of innate immune cytokines, within the first 72hr of infection. We directly compared innate activation to infection outcome following ARM and C13 intravenous infection at $2x10^6$ p.f.u. Utilizing an 11-parameter flow cytometry panel, we identified multiple innate cell populations and gauged their activation by expression of costimulatory and inhibitory markers.

After gating on live, singlet-discriminated cells we then identified lymphocytes by expression of NK1.1, CD19, B220, and Thy1.2 (Figure S1A). In accord with previous reports (*12*), there was a trend for the cellularity of LCMV infected spleens to decrease during the first 3 days of infection with both LCMV strains (Figure S1B). There was also a trend for the numbers of B, $CD4^+$ and $CD8^+$ T cells to decrease during the first 72hr.

Having removed the lymphocyte lineage from our gating we characterized the various DC populations (Figure S1C-D). pDCs can produce high amounts of IFN-I in response to viral infections or exposure to foreign nucleic acids (*21, 22*) and during the first few days of LCMV infection pDCs produce prodigious amounts of IFN-α (*15*). In mice, pDC can be identified as $CD11c^{lo}$, $B220^+$, $Ly6C^+$ and $CD11b^-$(*23*) (Figure S1C). We observed a reduction in pDCs between 12-25hr post LCMV infection but pDC numbers typically

rebounded around 42hr p.i. (Figure S1E). There was no difference in pDC numbers between ARM and C13 infection.

The two major murine splenic conventional DC (cDC) populations, $CD8^-$ and $CD8^+$, were identified from total non-lymphocytes by high expression of CD11c and lack of Ly6C (Figure S1D). $CD8^-$ DCs were reduced to about ¼ their initial number during the first 72hr of infection (Figure S2C). $CD8^+$ DCs cross present antigens phagocytosed from the environment via MHC Class I, and are potent initiators of Th1 cell responses (*24, 25*). In the spleen, LCMV infection leads to a substantial reduction in the numbers of these cells by day 3 of infection with either strain (Figure S2C).

Total myeloid cells were identified as $CD11c^{lo/-}$ and $CD11b^+$ and were sub-divided into three main populations; Gr-1^{lo} $Ly6C^{lo}$ $F4/80^+$ SSC^{hi} eosinophils, Gr-1^{lo} $Ly6C^{hi}$ $F4/80^+$ SSC^{lo} monocytic and Gr-1^{hi} $Ly6C^{int}$ $F4/80^-$ SSC^{int} neutrophilic cells (Figure S1F). Over the first two days, there was a trend for eosinophil, monocytic and neutrophilic cell numbers to be lower during the first 24-48hr of infection before rebounding by day 3 (Figure S1H).

We then determined the activation status of the DC subsets. cDCs expressed higher levels of CD80, CD86 and MHC Class II molecule I-A^b 25hr p.i. compared to pDCs (Figure 1A). $CD8^+$ DCs and $CD8^-$ DCs were both robustly activated, and expressed high CD80 and I-A^b. However, activation was transient and substantially reduced by day 3. In contrast, pDCs did not upregulate CD80, or I-A^b, and expression of CD86 was less than

that of cDC expression. Amongst the major myeloid cell populations, only the monocytic cells showed any appreciable expression of the costimulatory marker CD86 (data not shown). Activation marker expression on all cell types was independent of the viral strain.

Furthermore, the inhibitory receptors PD-L1 and PD-L2 (*18, 26, 27*) were also induced by infection. PD-L1 expression was increased on most cell types examined following LCMV infection. The kinetics of PD-L1 upregulation was indistinguishable between ARM and C13. The expression of PD-L2 was restricted to $CD8^+$ DCs, but was only present at very low levels on these cells.

Using intracellular cytokine staining, IL-12p40 and TNF were readily detectible in cDC populations over the first day of LCMV infection (Figure 1B and data not shown), consistent with previous studies (*12*). DC-produced IL-12 was detectible between 12-48hr p.i., with $CD8^+$ DCs having the highest amounts of IL-12. TNF was transiently produced by $CD8^+$ DCs during infection with similar kinetics as IL-12 (data not shown). Cytokine production by cDCs was not significantly different between acute and chronic infection.

We then assessed cytokines in the serum of LCMV infected mice. Large amounts of IFN-α were readily detected in the sera, as early as 24hr, in mice infected with either ARM or C13 (Figure 1C). In contrast, IFN-γ was only detectable at 72hr (Figure 1C). Furthermore, at 25hr p.i. IFN-induced chemokines CCL2, CXCL9, and CXCL10 were

readily detectable (Figure 1C). Again, induction of these innate cytokines and chemokines was nearly identical in both ARM and C13 infection. Thus, these results indicate, as assessed by the aforementioned parameters, that innate responses to LCMV infection during the first 72hr were independent of viral strain.

Myeloid cells from C13 infected mice show impaired $CD8^+$ T cell priming

Next, we determined the stimulatory capacity of APCs from LCMV infected spleens. $CD11c^+$ DC and $CD11c^-CD11b^+$ myeloid cells isolated from the spleens 24 or 48hr p.i. were cultured with OVA-specific $CD8^+$ cells from OT-I mice and SIINFEKL peptide for 3 days. Both populations stimulated T cell proliferation with as little as 0.5 pg/mL peptide (Figure 2A and data not shown). DCs were more potent stimulators than myeloid cells and were capable of inducing similar T cell proliferation with 1/10 the number of APCs. Importantly, APCs from ARM and C13 infection demonstrated nearly identical stimulatory ability.

DCs and myeloid cells from day 7 p.i. induced equivalent T cell proliferation, regardless of LCMV strain (Figure 2B). Unexpectedly, at day 14 p.i., whereas DCs from all groups were fully capable of stimulating T cells, myeloid cells from C13 infection were unable to effectively stimulate T cell proliferation. In fact, virtually no $CD8^+$ T cells seemed to survive culture, regardless of the concentration of antigenic peptide used. In contrast, myeloid cells from ARM infected spleens induced slightly lower proliferation than myeloid cells from uninfected mice, although this was evident in only in some of the

experiments (Figure 2C). This deficit in proliferation was overcome by adding higher concentrations of peptide.

We measured the viral loads in $CD11c^+$ and $CD11c^-CD11b^+$ cells day 14 post C13 infection to determine whether the reduced immune stimulatory capacity of the myeloid cells was a consequence of enhanced viral loads in these cells compared to DCs. We found that both populations contained measurable infectious virus, though DCs contained roughly 20x more virus than an equivalent number of myeloid cells from day 14 p.i. with C13 (data not shown). Thus, despite both DCs and myeloid cells containing virus during chronic infection, only myeloid cells lose their ability to stimulate T cells.

Chronic LCMV infection induces enhanced and sustained myeloid cell expansion

To better understand the impaired stimulatory capacity of the $CD11c^-CD11b^+$ myeloid cell population during C13 infection, we determined the cellular composition of this population during infection. At day 7 p.i., when the ARM and C13 derived myeloid cells were equally stimulatory (Figure 2), the composition of the myeloid cells was virtually identical between infections (Figure S2A). However, at day 14 p.i. the ratio of eosinophils to monocytic cells was substantially greater in ARM infected mice relative to myeloid cells from C13 infected mice (Figure S2B). The weakly stimulatory myeloid cell population isolated at day 14 during C13 infection consisted almost entirely of monocytic and neutrophilic cells.

We then evaluated myeloid cell populations over ~3 weeks of infection. Total splenic $CD11b^+$ myeloid cells, defined as described in Figure S1, increased during ARM and C13 infection between day 3-7, reaching about a 4 fold expansion above what was observed in uninfected mice (Figure 3A). Myeloid cell numbers in ARM infected mice began contracting around day 10, reaching baseline numbers by day 21. In contrast, these cells continued to increase with C13 infection, peaking between day 14 and 21 with nearly 10 fold more myeloid cells than in naïve mice (Figure 3A).

Both monocytic and neutrophilic myeloid cell populations were expanded during ARM and C13 infection (defined in Figure S1). Monocytic cell expansion peaked between days 5-7 in the spleen and blood (Figure 3B and data not shown). After day 10 of ARM infection the monocytic cells began contracting, reaching naïve levels by day 14. C13 infection led to sustained numbers of monocytic cells (about 10-fold higher than naive mice), which persisted for up to 3 weeks. Neutrophilic cells also peaked in the spleen and blood day 7 p.i. before contracting in ARM infected animals (Figure 3B and data not shown). In contrast, neutrophilic cells continued to increase during C13 infection reaching numbers more than 20 fold above naïve levels before contracting.

On day 14 p.i. the number of both monocytic and neutrophilic cells were significantly higher in the spleen, blood, and peripheral lymph nodes (pLN) of C13 infected mice compared to naïve or ARM infected mice (Figure 3C and data not shown). Histology of spleens from the peak of myeloid cell expansion during C13 infection shows a higher

proportion of F4/80^{+} myeloid cells and a corresponding loss of lymphoid-follicle structure consistent with previous reports (Figure 3D) (*28*).

Monocytic cells expanded during C13 infection resemble MDSCs

The expression of phenotypic and activation markers on myeloid cells was examined during C13 and ARM infections. Spleen monocytic cells did not show differences in expression of CD86 and I-A^{b}, however, PD-L1 expression remained elevated in C13 infected mice whilst subsiding during ARM infection. CD80 expression was also elevated on monocytic cells in lymphoid tissues and in the blood at the peak of the myeloid cell expansion during chronic infection (Figure 4A-B).. In addition, the CSF-R (CD115), which has been shown to be expressed on MDSCs in tumor models (*29, 30*), was expressed on the monocytic cells in C13 infected mice (Figure 4C). Neutrophilic cells increased expression of PD-L1 but not CD80, CD86, I-A^{b}, or CD115 during ARM or C13 infection (data not shown).

Sorted monocytic cells from day 14 post C13 infection were very similar in morphology to naïve sorted monocytes (Figure 4D). Neutrophilic cells from day 14 C13 infected spleens showed less lobation and were predominantly in band form, representing a less mature state and further suggesting that these cells are immature myeloid cells (Figure 4D).

We then performed microarray gene expression analysis on monocytic cells sorted from naïve and day 14 ARM or C13 infected spleens to obtain insights into the regulatory

networks that control the functions of these cells. Monocytic cells from C13 infected mice expressed many genes, such as *Trem1, Cd33, Ly6c1, Ptgs1, Ptgse* and *Sell* (encoding CD62L), which are known to be associated with MDSCs (*31*) (Figure 4E). In addition, there was increased expression of *Cd274* (PD-L1), *Kit*, *S100a8, S100a9,* and *Birc5* during both LCMV infections.

We then sought to profile the expression of genes that would give insight to the function of monocytic cells during C13 infection. Transcripts for inflammatory chemokines CXCL9 and CXCL10 were increased during both ARM and C13 infection but increases in CCL2, IL-7, CSF-1, and IL-27 cytokine transcripts above naïve levels were unique to C13 infection (Figure 4E). ARM and C13 infection also increased expression of genes that encode CCR5, IL-1R2, IL-28R, and IL-18R and decreased CX_3CR1, IL-6, IL-10, CSF-1 and VEGF receptor transcription in monocytic cells. Only C13 infection increased transcripts for the receptors for IL-8, IL-15, IL-12, IL-20 and GM-CSF. Monocytic cells during C13 infection showed differential expression of activation induced markers, myeloid-macrophage markers, homing and recruitment genes and functional markers.

Infection with either ARM or C13 induced expression of genes related to IFN responses. Induction of 2'-5' oligoadenylate synthetase anti-viral genes and many other IFN stimulated genes were not unique to C13 infection, however there were more of these types of genes upregulated during chronic infection. Monocytic cells also upregulated several genes related to extracellular matrix breakdown and remodeling such

as matrix metallopeptidases, cathepsins and lamin. These cells also showed differential expression of approximately 80 genes related to the mitochondrial respiratory burst, including genes involved in the regulation of oxidative stress, both in genes whose products promote reactive oxygen species (ROS) production and those that mitigate ROS-related tissue damage. These ROS-related genes were predominantly induced during chronic infection. Increased ROS production has been shown to be one of the main identifiers of MDSCs in multiple tumor and infection models (*32, 33*). These data suggest that whilst monocytic cells from C13 infected mice express many genes that encode proinflammatory mediators; they also express genes that encode molecules involved in oxidative stress, which is implicated in tolerogenic responses.

Furthermore, monocytic cells showed a significant increase in molecules related to the processing and presentation of peptides on Class I MHC. Transcripts for proteosome subunits, peptide transporters and MHC Class I molecules were all increased in monocytic cells from C13 infected mice, relative to cells from naïve mice. Conversely, multiple genes related to MHC Class II antigen presentation were down regulated during C13 infection; transcripts for multiple MHC Class II, invariant peptide, and HLA-DM molecules were all decreased. In contrast, monocytic cells from acute infection increased transcription for only a few Class I genes and upregulated some Class II related genes.

Overall these data reveal a distinctive molecular signature of monocytic cells isolated from C13 infected mice, relative to those from ARM infected or naïve mice. Taken

together, the phenotypic, morphological and transcriptional signatures suggest that myeloid cells from C13 infected mice resemble MDSCs.

Myeloid cells suppress T cell proliferation *ex vivo*

MDSCs expand during multiple tumor models can potently suppress T cell responses (*34*). The substantial increase of myeloid cells during chronic LCMV infection coincided with the functional exhaustion of LCMV-specific $CD8^+$ T cells (Figure 5A), raising the possibility that myeloid cells suppressed LCMV-specific $CD8^+$ T cells.

To determine if these cells were immunosuppressive we sort purified monocytic cells and co-cultured them with total splenocytes from OT1 mice and stimulated with SIINFEKL peptide. Monocytic cells from day 14 post-C13 infection suppressed OT-I T cell proliferation in a cell number dependent manner (Figure 5B). Naïve and ARM derived myeloid cells had no effect on the proliferation of the T cells even at high ratios. Monocytic cells from C13 infected mice were also able to suppress T cell responses in a different system using plate bound anti-CD3 and anti-CD28 stimulation of T cells from OT-1 mice (Figure 5C-D). Interestingly, however, undivided $CD8^+$ T cells seem to survive culture, when plate bound stimulation with anti-CD3ε and anti-CD28 was used, presumably because of survival signals delivered via these receptors to T cells. Neutrophilic cells could suppress T cell responses when OT-1 cells were used in the presence of SIINFEKL peptide, but were unable to induce suppression in the system using plate bound anti-CD3 and -CD28 stimulation of T cells (data not shown).

MDSCs from tumor and other models systems have been reported to suppress T cell proliferation by metabolism of L-arginine by inducible nitric oxide synthase (iNOS) and arginase1 (ARG1). To determine the mechanism by which monocytic cells suppress T cell proliferation, these cells were cultured with $CD8^+$ T cells from OT-1 mice, with or without inhibitors against the aforementioned molecules. With the addition of the iNOS-specific inhibitor L-NIL or total NOS inhibitor L-NMMA the proliferation of T cells was restored. Additionally, blocking antibodies to IFN-γ, which is known to induce iNOS (*35*), alleviated suppression. Neither the addition of ARG1 inhibitor, nor-NOHA, or blocking antibodies to IL-10R, TGF-β, or PD-1 were able to restore suppression. Monocytic MDSCs derived from chronic LCMV infection limit T cell proliferation via IFN-γ induced iNOS production of NO.

Mobilization of monocytic suppressor cells is dependent on CCR2

The two major monocytic cell subsets in mice are typically distinguished by their differential expression of chemokine receptors and other surface markers. 'Classical' monocytes have been described as $CCR2^+$, $Ly6C^{hi}$, CX_3CR1^+, and $CD62L^+$ while 'non-classical' monocytes are $CCR2^-$, $Ly6C^{Int}$, $CX3CR1^{++}$, and $CD62L^-$ (*36*). CCL2 is known to be a ligand for CCR2 and previous studies have demonstrated that the recruitment of MDSCs to tumors was mediated by CCR2-CCL2 signaling and depletion of $CCR2^+$ cells enhanced antigen specific $CD8^+$ T cell responses (*37, 38*). We therefore determined whether recruitment of MDSCs during C13 infection was dependent on CCR2.

We first measured the serum concentrations of CCL2 throughout acute and chronic LCMV infection (Figure 6A). Both ARM and C13 infection led to a high amount of CCL2 24hr p.i. (Figure 1C). CCL2 concentrations were still significantly elevated day 7 p.i. but by day 10 the chemokine concentration had dropped during ARM infection to near baseline levels. In contrast, CCL2 concentrations following C13 infection remained markedly elevated over baseline and ARM levels as late as 28 days p.i.

Upon infection of WT and *Ccr2*$^{-/-}$ mice with C13, we observed that *Ccr2*$^{-/-}$ mice showed significantly increased concentrations of CCL2 in the serum 14 days p.i. (Figure 6B). Monocytic cells expanded in the spleen, pLN, blood and liver were predominantly CCR2^{+} at both day 7 and day 14 post C13 infection (Figure 6C and data not shown). The numbers of monocytic cells in the blood at these times were significantly lower in C13 infected *Ccr2*$^{-/-}$ mice than infected controls (Figure 6D), despite the elevated levels of CCL2. This is consistent with previous reports that *Ccr2*$^{-/-}$ monocytes are defective at migrating from the bone marrow to the blood (*39*). Lower numbers of monocytic cells in the blood of *Ccr2*$^{-/-}$ mice were evident even 14 days p.i., although the numbers of such cells in both *Ccr2*$^{-/-}$ and WT mice had increased relative to day 7 (Figure 6D).

In the spleen, there was a marked reduction in monocytic cell numbers in *Ccr2*$^{-/-}$ mice at day 7 p.i., although at day 14 monocytic cell numbers in *Ccr2*$^{-/-}$ mice had expanded relative to WT mice (Figure 6E). This suggests that while CCR2 plays a key role in the exit of monocytic cells from the bone marrow to the blood, it plays little or no role in the migration of such cells from the blood to the spleen (*40*). When multiple

lymphocyte and DC populations were measured day 14 p.i. we observed that T cell, pDC, and cDC numbers were equivalent in the spleen and pLN of B6 and *Ccr2*$^{-/-}$ mice (Figure S3A-B). Only B cells in the spleen of *Ccr2*$^{-/-}$ mice were slightly diminished compared to WT.

Given the reduced numbers of monocytic cells in the spleens of C13 infected *Ccr2*$^{-/-}$ mice at day 7, we assessed the magnitude of antigen-specific CD8^{+} T cell responses. Cytokine expression by CD8^{+} T cells stimulated ex vivo with three immunodominant MHC Class I peptides were not significantly different in *Ccr2*$^{-/-}$ and B6 mice day 7 p.i. (Figure 6F and Figure S3C). However, by day 14 the cytokine responses of LCMV-specific T cells were significantly elevated in frequency and absolute number in the *Ccr2*$^{-/-}$ mice (Figure S3C). Taken together, these data demonstrate a decreased mobilization of monocytic cells from the bone marrow into circulation during chronic infection of *Ccr2*$^{-/-}$ mice. This diminished pool of blood monocytic cells delays their recruitment to the spleen, thereby reducing the number of MDSCs in lymphoid tissues, and decreasing the opportunity for T cell suppression.

Antibody depletion of myeloid cells enhances LCMV-specific T cell responses *in vivo*

To test specifically whether myeloid cells during C13 infection inhibit T cell responses and contribute to T cell exhaustion we depleted total myeloid cells with an antibody against Gr-1. We treated mice with 100 μg of anti-Gr-1 for three days prior to the peak of myeloid cell expansion on day 14. It has been previously reported that anti-Gr-1 does not completely deplete Gr-1^{+} cells and leaves a population with the antibody on the surface

for more than four days following treatment (*41*). Staining with a secondary antibody recognizing the depleting antibody confirmed that there was significant occlusion of the Gr-1 epitope (data not shown) that blocked fluorophore-conjugated RB6-8C5 (Ly6C/G) and 1A8 (anti-Ly6G) antibody staining. Because of this we measured total non-lymphocyte, non-DC, myeloid cells. Treatment with anti-Gr-1 significantly reduced the total numbers of myeloid cells in the spleen and blood (Figure 7A-B) to levels seen in naïve mice. On average, relative to untreated mice, antibody treated mice had 1/10 and 1/6 the number of myeloid cells in the blood and spleen, respecitively. Using expression of F4/80 by monocytic cells and DEC-205 by neutrophilic cells it was possible to subdivide the monocytic populations independent of Gr-1 expression. In the blood and spleen there was a significant reduction in monocytic cells, and in the blood, spleen and pLN there was significant reduction of neutrophilic cells (Figure S4A-B).

The depletion was specific to myeloid cells with there being no significant reduction in total lymphocyte and DC numbers in the spleen or pLN (Figure S4C-D). The depletion of myeloid cells correlated with a significant increase in the frequency of virus-specific functional T cell responses. Depleted mice showed increased frequencies and absolute numbers of IFN-γ, TNF, and IL-2 producing LCMV-specific T cells in the spleen (Figure 7C and Figure S4E). These cells were also more polyfunctional than T cells from untreated mice, having increased proportions of IFN-γ and TNF double positive cells (data not shown). Myeloid cells were also reduced by half in the pLN (Figure 7D) and had a corresponding increase in $CD8^+$ T cell function (Figure 7E and Figure SF). There was trend for viral loads to be reduced following depletion of myeloid cells, although this

was not statistically significant (data not shown). Our data suggests that by reducing the numbers of suppressive myeloid cells we were able to boost $CD8^+$ T cell function.

Discussion

Diminished $CD8^+$ T cell number and function has been attributed to the persistence of viral infections in both humans and mice (*42, 43*). In this study we show that during the T cell priming phase of LCMV infection both ARM and C13 strains activate innate APCs and induce phenotypic and functional changes with virtually identical kinetics. Following the induction of T cell responses, C13 infection leads to a massive expansion and accumulation of myeloid cells with T cell suppressive function.

Persistently infecting strains of LCMV initially infect marginal zones by 24hr p.i., then progress to the interdigitating DC of the white pulp by day 3 p.i. In contrast, acutely infecting strains are restricted to the red pulp of the spleen (*6, 10*). This difference in tropism has been attributed to the higher affinity of the viral GP for its cellular receptor, αDG, which is highly expressed on DCs, and results in a much higher viral burden in splenic DCs (*5*). Our data suggests that despite these differences, during the first three days of infection persistent and chronic strains of LCMV similarly affect the activation and function of innate APCs. The upregulation of activation markers and pro-inflammatory cytokines by DC and myeloid populations during the first three days is independent of viral strain.

Both DC and myeloid cells are capable of stimulating T cell proliferation and differentiation during the first few days of infection with acute or chronic LCMV. Addition of exogenous peptide to APC cultures leads to nearly identical proliferation T cells. It is important to note that these data do not preclude the possibility that APCs

during acute or chronic infection have different antigen loads or specific impairments in presenting viral antigens on MHC molecules.

Although we could not demonstrate any functional deficit in DC purified from acute or chronically infected mice, we observed that $CD11b^+$ myeloid cells from chronically infected mice 14 days p.i. were not only unable to stimulate T cells, but they actively suppressed the priming of T cells. Both the monocytic and neutrophilic cells, profoundly expanded during C13 infection, had the appearance of MDSCs as described in multiple models of chronic infection (*32, 44, 45*).

It is important to emphasize that while myeloid cells are expanded during day 7 post both ARM and C13 infection, these cells were not observed to be suppressive at this time. Only after this point to do we observe suppression of T cell proliferation by C13 derived $CD11c^-CD11b^+$ myeloid cells. We cannot formally exclude the possibility that early on both infections induce some suppressive activities in myeloid cells, but C13 infection clearly leads to the sustained and enhanced suppressive function of these cells.

Although CCR2 is important to the egress of monocytes from the bone marrow into the blood, the entry of monocytes into inflamed tissues is not CCR2-dependent and can happen by a variety of other receptors, including CCR5 (*39*). Indeed, our gene chip data suggests that CCR5 is upregulated on monocytic cells during infection and may be an alternative mechanism for MDSC accumulation in tissues. The diminished level of circulating monocytic cells and alternative egress mechanisms may cause $Ccr2^{-/-}$ MDSC

entry into the spleen with slightly delayed kinetics, delaying their suppressive effect on T cells.

Currently there are no known markers specific to murine MDSC. We have used an antibody recognizing one of the predominant markers expressed by MDSC, Gr-1, to deplete myeloid cells expanded during C13 infection. The RB6-8C5 antibody clone has been used for years to deplete neutrophils, monocytes and MDSC in a variety of settings (*46-48*). We used a dose and regimen of antibody delivery that reduced both neutrophilic and monocytic cell populations in the blood, spleen and pLN, while not significantly decreasing other cell types. The loss of myeloid cells correlated with enhanced $CD8^+$ T cell function, but surprisingly did not significantly impact viral loads. The failure eliminate virus may be due to multiple factors. Although myeloid cells are infected by C13, they are but one of many cell types, including DCs and FRCs, which are highly infected by C13. Thus it was not surprising that the depletion of myeloid cells did not in itself lower viral loads. We administered antibody during the window when myeloid cells began to diverge in ARM and C13 infection, for only 3 days. This is likely insufficient time for the $CD8^+$ T cells to significantly impact viral loads and eliminate the signals leading to further MDSC expansion and function. Additionally, RB6-8C5 can also transmit signals via STAT1, STAT3 and STAT5 and consequently induce BM myelopoeisis, induce apoptosis in granulocytes and cause transient loss of MDSC suppressive function (*41*). These factors which complicate the use of this antibody as a treatment for chronic LCMV infection. Furthermore, MDSC may be just one of several

factors (e.g. PD1-PDL1, IL-10, and TGF-β) involved in the suppression of T cell responses and persistence of viral infection during C13 infection.

It seems that there is no inherent defect in the initial priming of $CD8^+$ T cells during chronic LCMV infection compared to acute infection. However, the increased infectivity of DC, myeloid cells and FRC by persistent strains of LCMV and their faster replication likely outpaces the ability of the innate and adaptive immune responses to contain the infection. In addition to increasing the opportunity for T cells to receive antigenic stimulation which drives T cell exhaustion (*49*), widespread C13 infection increases the number and dissemination of infected targets for CTL killing. CTL lysis of infected cells is the cause of immunopathology during C13 infection and can risk the survival of the infected animal. Following the loss of infected accessory and hematopoietic cells to CTL killing, and in response to stimulatory signals such as CCL2, myeloid progenitor cells are mobilized from the bone marrow to replace the dead cells. Upon entering tissues experiencing prolonged inflammation, with both innate and adaptive effector cytokines present, myeloid precursors gain a suppressive function. IFN-γ produced upon recognition of viral antigens can induce iNOS production in monocytic cells, and in turn inhibit T cell function. MDSCs, in concert with multiple suppressive mechanisms counter the excessive activation of the innate immune system and immunopathology caused by the persistence of pathogens during chronic infections (*50*), but consequently hinder viral clearance.

The appearance of large numbers of MDSCs during chronic inflammation appears to be a common feedback mechanism in both mice and humans. Because of this, many different avenues of combating MDSC number, function and generation are being actively pursued (*31*). Having implicated MDSCs in the suppression of T cell responses during LCMV infection, and demonstrating that depletion of these cells can enhance T cell function, we believe that counteracting MDSC effects is likely to be an important additional way to confront difficulties in treating chronic human infections like HIV and HCV. Taken together, the data presented in this study highlight the polyphasic nature of the innate responses to viral infections, and reveal a continuing role for the innate immune system throughout the course of persistent viral infections.

Experimental Procedures

Mice and Viruses

C57BL/6 (Charles River Laboratory), OT-I(Rag1/2$^{+/+}$) and *Ccr2*$^{-/-}$ B6.129S4-Ccr^{tm1Ifc}/J (The Jackson Laboratory), B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1(OT-I(Rag1$^{-/-}$)(Taconic) mice were maintained under specific pathogen-free conditions in the Emory Vaccine Center vivarium. All of the animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Emory University. LCMV strains ARM and C13 from Rafi Ahmed and Joshy Jacob (Emory Vaccine Center, Emory University, Atlanta, GA) were grown and quantified as described (*1, 6*).

Flow Cytometry

Spleens from naïve and LCMV infected mice were collagenase digested as described (*51*). Collagenase digested splenocytes were incubated with blocking mAb 2.4G2 anti-FcγRIII/I and Alexa Fluor 430 (live/dead stain) (Invitrogen). Cells were then stained with labeled mAb. From BD Pharmingen, CD11b (clone M1/70), B220 (RA3-6B2), Ly6C (AL-21), CD80 (16-10A1), CD86 (GL-1), I-A^b (AF6-120.1), CD70 (FR70), Isotype IgG1 (R3-34), IgG2a (R35-95), and IgG2b (A95-1) antibodies were used. From eBioscience, CD19 (MB19-1), Thy1.2 (53-2.1), F4/80 (BM8), CD11c (N418), CD8α (53-6.7), Gr-1 (RB6-8C5), PD-L1 (MIH5), PD-L2 (TY25), PanNK (DX5). Some samples were also stained with NK1.1 (PK136) (Biolegend) or CCR2 (475301) (R&D Systems). Cells were then stained with Streptavidin conjugated QDot655 (Invitrogen) before fixation. Samples were acquired on a BD Biosciences LSR II and analyzed using FlowJo (TreeStar, Inc). Geometric mean fluorescence intensities of activation markers were normalized to non-

specific isotype controls. The normalization was calculated as ($gMFI_{marker} - gMFI_{isotype}$) / $gMFI_{isotype}$. Further details are in supplementary data.

For innate cell intracellular staining, collagenase digested splenocytes were incubated in medium containing Brefeldin A and monensin (BD Pharmingen) for 6hr then surface stained with mAb. Cells were then permeablized with cytofix/cytoperm (BD Pharmingen) and intracellularly stained. Intracellular staining of T cells was performed as previously described following 5hr of stimulation with 0.2μg/ml LCMV $NP_{396-404}$, $GP_{33-41,}$ and $GP_{276-286}$ peptides (Emory Univ. Microchemical Peptide Facility) (*4*). Some naïve samples were also cultured with 5μg/ml R848 (Enzo Life Sciences) or 50μg/ml Zymosan (Sigma-Aldrich) as positive controls. Cells were harvested, live/dead stained, FcR blocked and surface stained as described above. Cells were then permeabliized with cytofix/cytoperm (BD Pharmingen) and intracellularly FcR blocked. Samples were then stained with mAb for TNF (eBioscience) or IL-12 p40/p70 (BD Pharmingen).

Serum Cytokine Analysis

Serum pooled from three mice was assayed with Bio Rad and Invitrogen multi-cytokine detection panels. Data were acquired using the Luminex 100 reader and analyzed with Masterplex Quantitation software (Miraibio). ELISAs were performed for of IFN-γ (eBioscience), IFN-α (PBL InterferonSource), and CCL2 (R&D Systems).

APC Functional Assays

Total DC and myeloid cells were purified from the collagenase digested spleens of infected mice 24hr, days 7 and day 14 p.i. Splenocytes were depleted with anti-CD19 coated microbeads (Miltenyi) then positively selected by anti-$CD11c^+$ microbeads. Total myeloid cells were purified from the $CD11c^-$ fraction by using anti-$CD11b^+$ microbeads. Total DC and myeloid cell populations were determined as >95% pure by flow cytometry.

Purified DC or myeloid cells were cultured with 10^5 CFSE-labeled $CD8^+$ OT-I T cells at a ratio of 1:10 or 1:1 respectfully. Cells were cultured with SIINFEKL peptide (a generous gift from Dr. Jan Pohl, Biotechnology Branch, CDC, Atlanta, GA) for three days before being stained with anti-CD8 and anti-Thy1.2 (BD Pharmingen) and analyzed on an LSR II flow cytometer.

T Cell Suppression Assay

Total $CD11b^+$ cells enriched from the spleens of 5 naïve or day 14 LCMV infected mice using MACS beads before being sorted on a FACS Aria II (BD Biosciences) into $Ly6C^{hi}$, $Gr\text{-}1^{int}$ and $Ly6C^{int}$, $Gr\text{-}1^{hi}$ populations. The average purity of monocytic cell populations from 3 experiments was >93%. Myeloid cells were added to 10^5 OTI responder splenocytes and cultured for 24h with SIINFEKL or control Vaccinia peptide (B8R) before [^{3}H]thymidine was added and proliferation was evaluated as previously described (*34*). In other assays, sort purified monocytic cells from day 14 C13 infection were cultured 1:2 with 10^5 purified and CFSE labeled $CD8^+$ cells and stimulated with plate

bound anti-CD3ε and anti-CD28 for three days. Inhibitors L-NIL (0.5 mM, Sigma-Aldrich), nor-NOHA (0.5 mM, Cayman Chemical Company), L-NMMA (5 mM, Calbiochem), and blocking antibodies anti-IFN-γ (XMG1.2, 10μg/mL), anti-IL-10R (1B1.3a, 20μg/mL, Biolegend), anti-PD1 (J43, 10μg/mL, eBioscience) and anti-TGF-$\beta_{1,2,3}$ (1D11, 10μg/mL, R&D Systems) were added at the beginning of culture.

Gene expression analyses

Total RNA from sorted splenic monocytic cells of naïve and day 14 ARM or C13 infected mice were purified using Trizol (Invitrogen). All RNA samples were checked for purity using a ND-1000 spectrophotometer (NanoDrop Technologies) and for integrity by electrophoresis on a 2100 BioAnalyser (Agilent Technologies). The RNA was processed using the Illumina TotalPrep RNA Amplification Kit Protocol according to manufacturer's protocol. Briefly, 150-500ng of total RNA was reverse transcribed with an oligo(dT) primer bearing a T7 promoter to generate first strand cDNA. The cDNA then underwent second strand synthesis and cleanup. Next, this cDNA was used as a template for in vitro transcription with biotin-UTP to generate biotinylated cRNA. The biotinylated cRNA was quantitated and run on the Agilent Bioanalyzer to ensure adequate yield with the appropriate size distribution was obtained. Biotinylated cRNA was then normalized to 150ng/ul and 10ul were used to hybridize onto Illumina MouseWG-6 v2.0 Expression BeadChips. Scanning was performed by the Illumina iScan. Arrays were analyzed by the Illumina GenomeStudio Gene Expression Module with default parameters to generate standard data tables with no background subtraction or normalization. Next, the expression levels were background-corrected by Maximum

Likelihood Estimation (MLE) and normalized by quantile using MBCB R package (*52*). Genes were filtered out from the analysis if the their expression was below the detection limit (p-value < 0.05) in at least 2 out of 3 samples in both naïve and LCMV infected mice. Data was then converted to non log values and differential expression was tested using VAMPIRE (*53*) (FDR < 0.05; post-filtering mean fold-change > 1.4). All microarray data was deposited in NCBI GEO website (GSE43896).

Myeloid Cell Depletion

Mice infected with C13 were given intraperitoneal injections of 100 μg of anti-Gr-1 (RB6-8C5) (Bio X Cell) or isotype control antibody at days 11, 12 and 13 p.i. Spleens, LN, blood were collected on day 14.

Immunofluorescence Microscopy

Spleens from naïve and LCMV infected mice were frozen in OCT, then cut into 6μm sections and fixed with %100 acetone for 10 min. Slides were permeablized with PBS +0.5% Triton X-100 +1% BSA, blocked with normal rat serum and anti-FcRIII/I antibodies and then incubated with anti-B220-AF488, Thy1.2-PE, and F4/80-biotin. Tissues were incubated with Streptavidin-APC and mounted with Prolong-GOLD (with DAPI) (Invitrogen). Images were captured using the x10 objective on a Zeiss Axioscope (Carl Zeiss) and analyzed using ImageJ (National Institute of Mental Health) and DoubleTake (Echo One, Denmark) software.

Geimsa Staining

Sorted monocytic and neutrophilic cells were spun onto glass slides using a Cytospin (Shandon) centrifuge, air-dried, fixed in 100% methanol, and stained using Modified Giemsa Stain (Sigma-Aldrich).

Statistics

Statistical significance was determined by Mann-Whitney tests using Prism (GraphPad Software). Probability values of $p \leq 0.05$ were considered significant.

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Figures

Figure 1

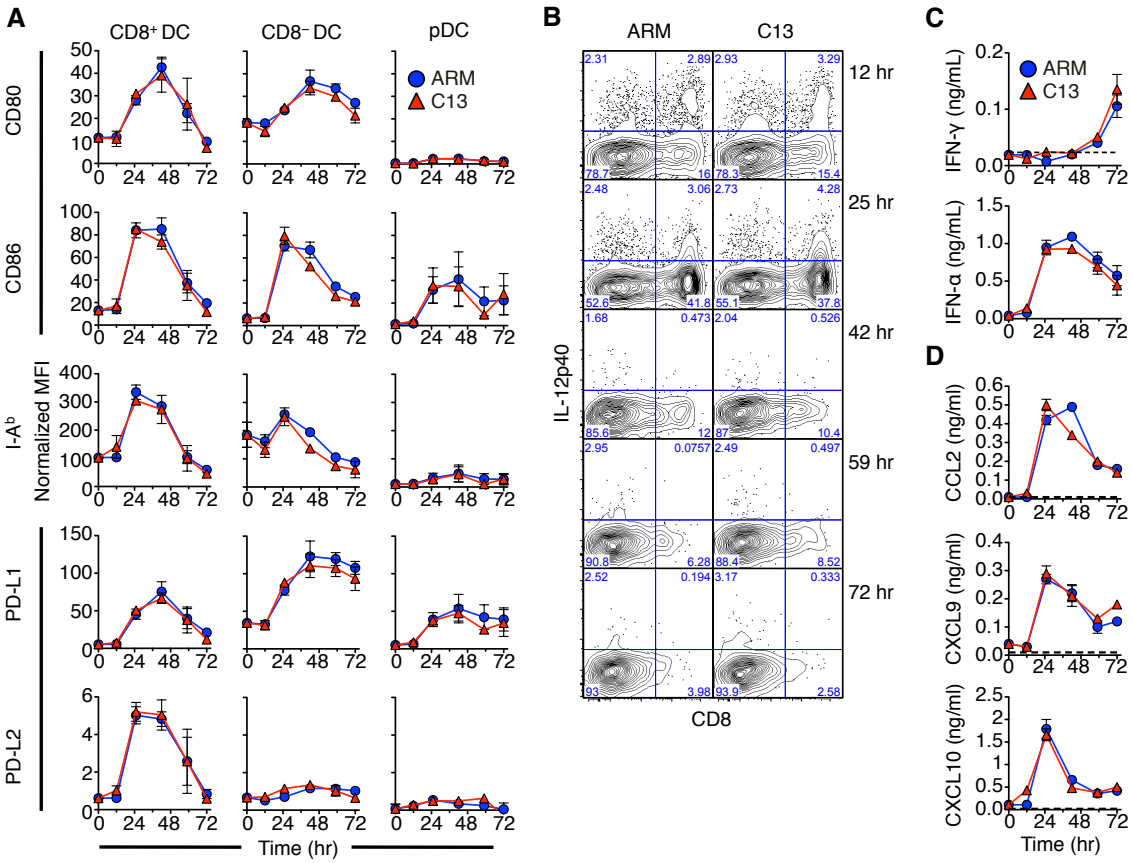
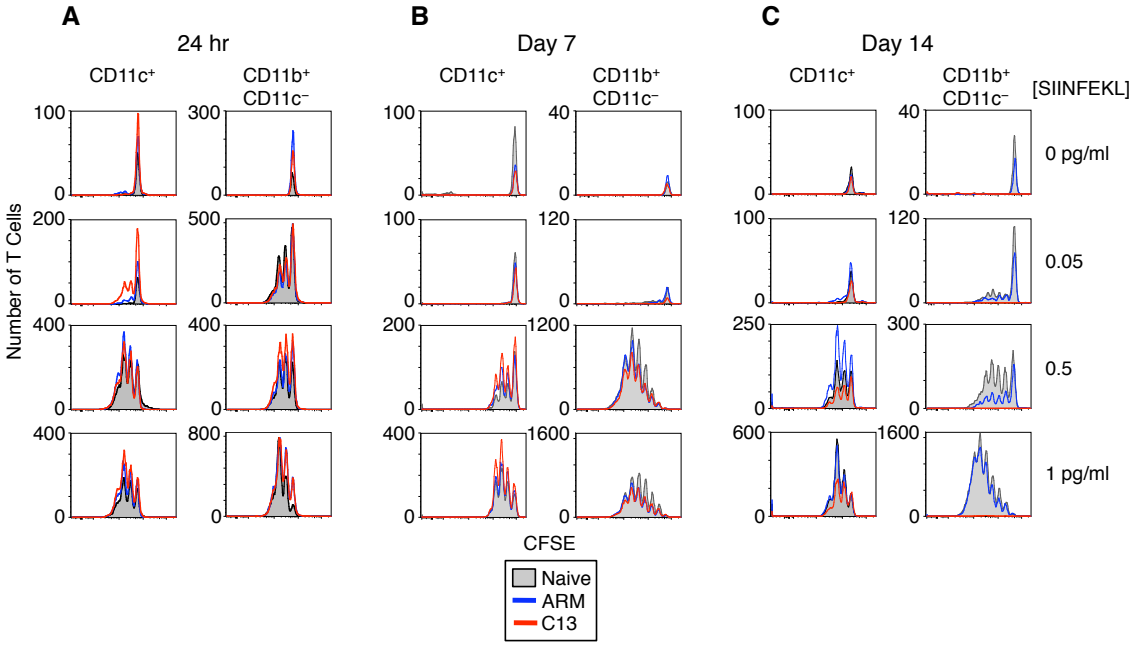


Figure 2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24 hr
B
Day 7
C
Day 14
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CD11b+ CD11c−
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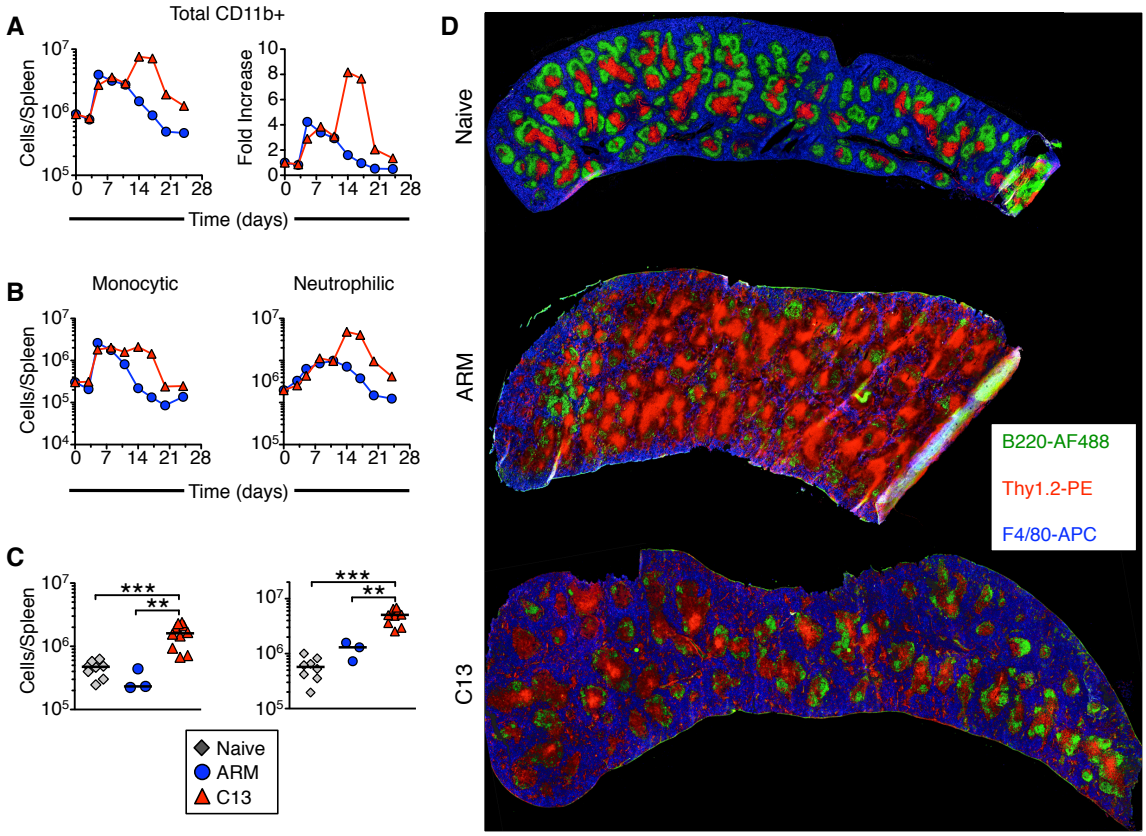
0.05
0.5
1 pg/ml
Number of T Cells
CFSE
Naive
ARM
C13

Figure 3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Total CD11b+
Cells/Spleen
Fold Increase
Time (days)
B
Monocytic
Neutrophilic
Cells/Spleen
Time (days)
C
Cells/Spleen
***
**
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ARM
C13
D
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C13
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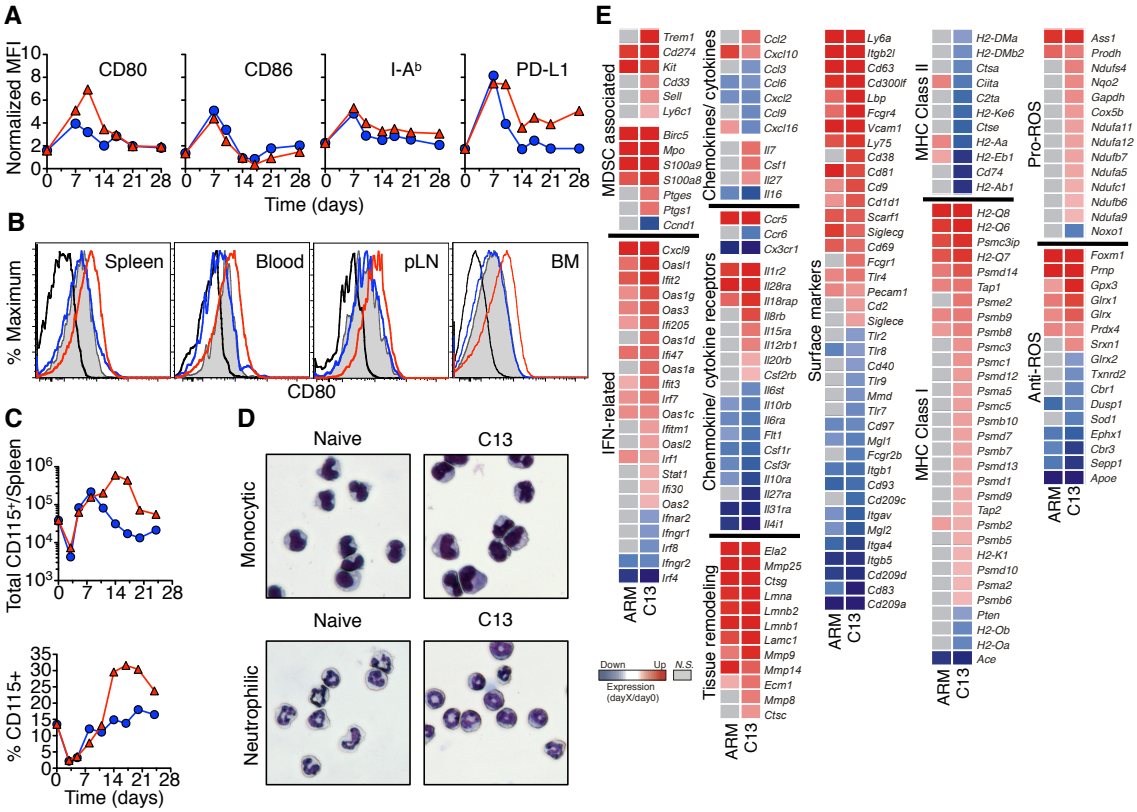
Thy1.2-PE
F4/80-APC

Figure 4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% CD115+
Time (days)
D
Naive
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Monocytic
Neutrophilic
E
MDSC associated
Trem1
Cd274
Kit
Cd33
Sell
Ly6c1
Birc5
Mpo
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Mmp9
Mmp14
Ecm1
Mmp8
Ctsc
Surface markers
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Itgb2l
Cd63
Cd300lf
Lbp
Fcgr4
Vcam1
Ly75
Cd38
Cd81
Cd9
Cd1d1
Scarf1
Siglecg
Cd69
Fcgr1
Tlr4
Pecam1
Cd2
Siglece
Tlr2
Tlr8
Cd40
Tlr9
Mmd
Tlr7
Cd97
Mgl1
Fcgr2b
Itgb1
Cd93
Cd209c
Itgav
Mgl2
Itga4
Itgb5
Cd209d
Cd83
Cd209a
MHC Class II
H2-DMa
H2-DMb2
Ctsa
Ciita
C2ta
H2-Ke6
Ctse
H2-Aa
H2-Eb1
Cd74
H2-Ab1
MHC Class I
H2-Q8
H2-Q6
Psmc3ip
H2-Q7
Psmd14
Tap1
Psme2
Psmb9
Psmb8
Psmc3
Psmc1
Psmd12
Psma5
Psmc5
Psmb10
Psmd7
Psmb7
Psmd13
Psmd1
Psmd9
Tap2
Psmb2
Psmb5
H2-K1
Psmd10
Psma2
Psmb6
Pten
H2-Ob
H2-Oa
Ace
Pro-ROS
Ass1
Prodh
Ndufs4
Nqo2
Gapdh
Cox5b
Ndufa11
Ndufa12
Ndufb7
Ndufa5
Ndufc1
Ndufb6
Ndufa9
Noxo1
Anti-ROS
Foxm1
Prnp
Gpx3
Glrx1
Glrx
Prdx4
Srxn1
Glrx2
Txnrd2
Cbr1
Dusp1
Sod1
Ephx1
Cbr3
Sepp1
Apoe
ARM
C13
Down
Up
N.S.
Expression
(dayX/day0)

Figure 5

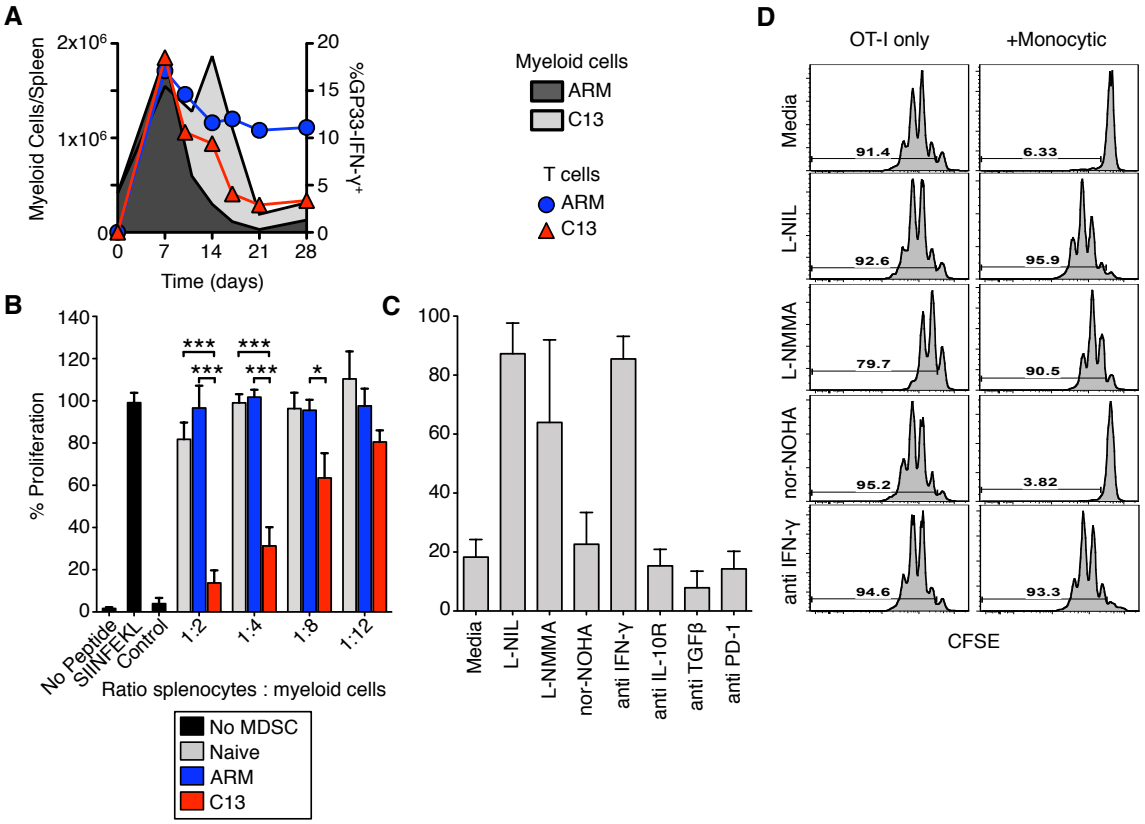

A
Myeloid Cells/Spleen
2x10^6
1x10^6
0
%GP33-IFN-γ+
20
15
10
5
0
0 7 14 21 28
Time (days)
Myeloid cells
ARM
C13
T cells
ARM
C13
B
% Proliferation
140
120
100
80
60
40
20
0
***
***
***
***
*
No Peptide
SIINFEKL
Control
1:2
1:4
1:8
1:12
Ratio splenocytes : myeloid cells
No MDSC
Naive
ARM
C13
C
100
80
60
40
20
0
Media
L-NIL
L-NMMA
nor-NOHA
anti IFN-γ
anti IL-10R
anti TGFβ
anti PD-1
D
OT-I only
+Monocytic
Media
L-NIL
L-NMMA
nor-NOHA
anti IFN-γ
91.4
6.33
92.6
95.9
79.7
90.5
95.2
3.82
94.6
93.3
CFSE

Figure 6

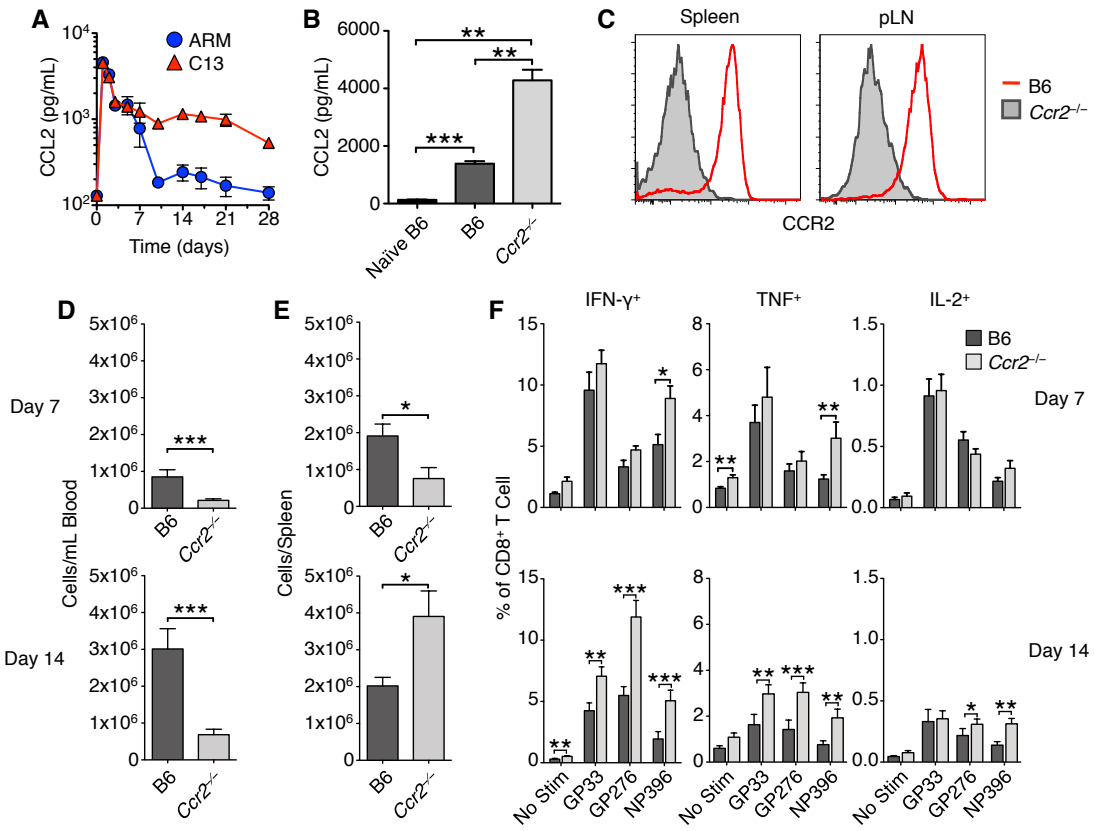
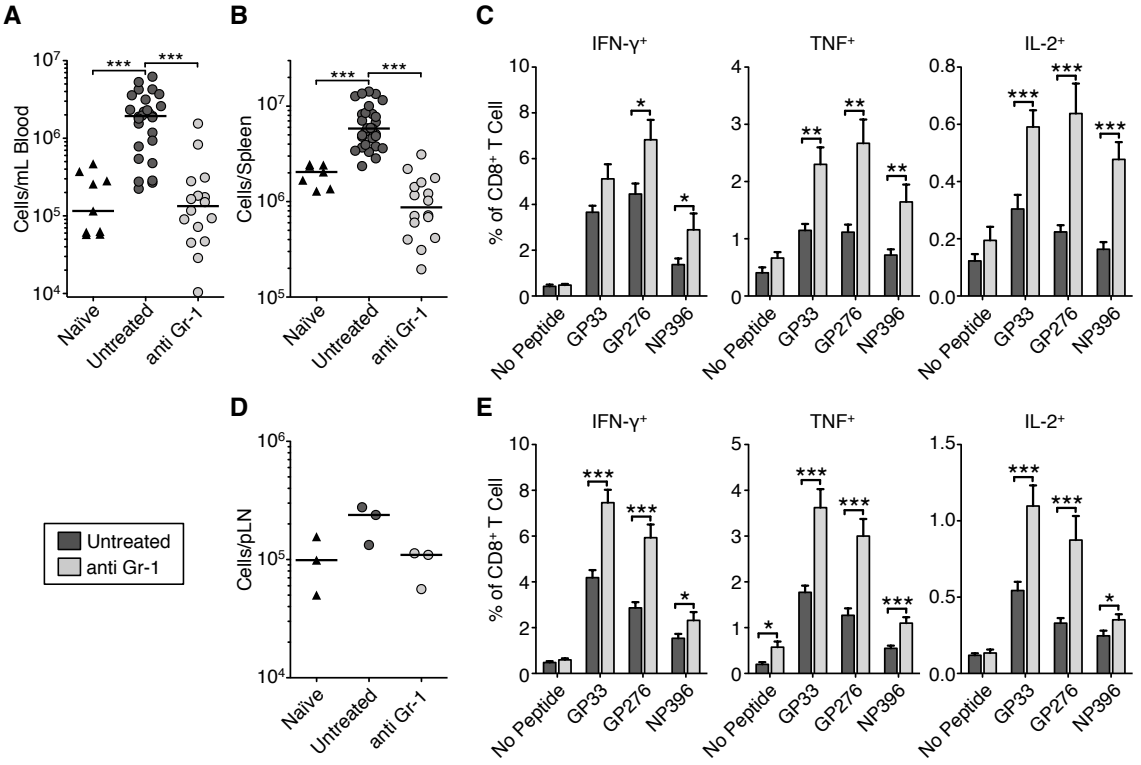
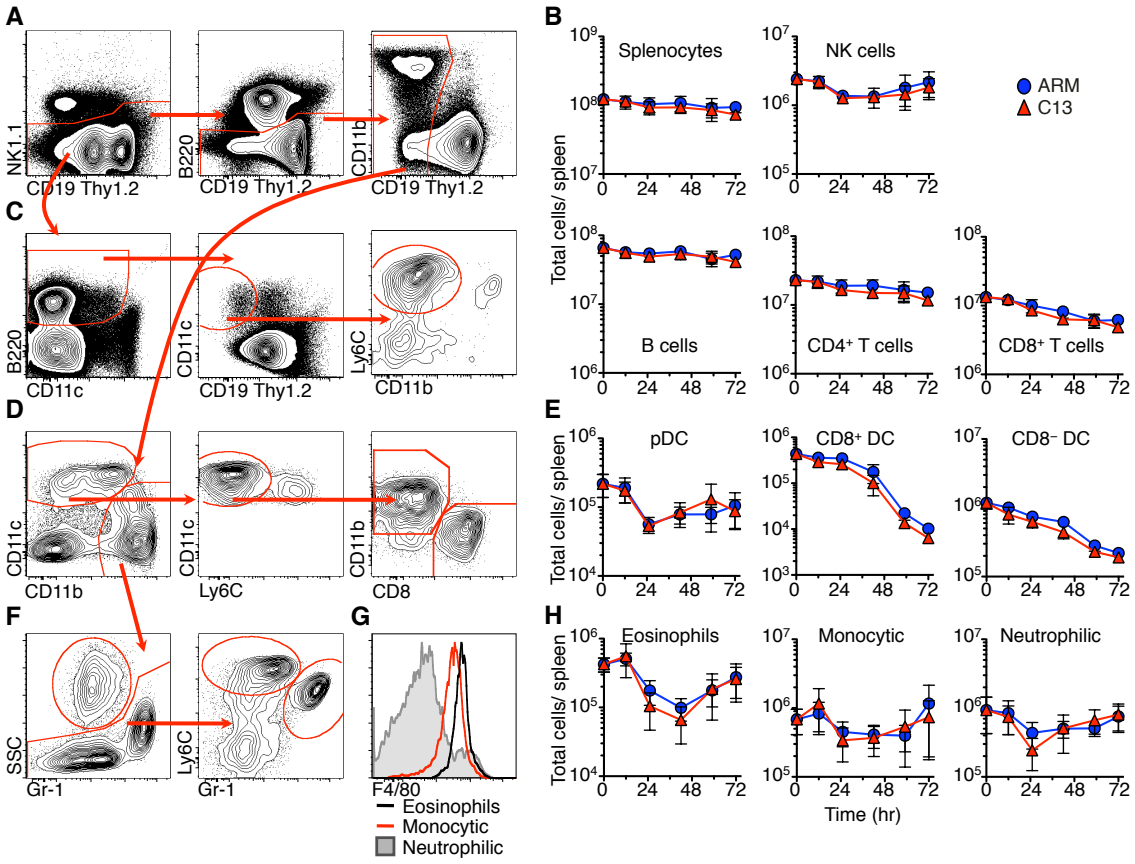

A
ARM
C13
CCL2 (pg/mL)
Time (days)
B
CCL2 (pg/mL)
Naïve B6
B6
Ccr2−/−
C
Spleen
pLN
B6
Ccr2−/−
CCR2
D
Day 7
Day 14
Cells/mL Blood
E
Cells/Spleen
F
IFN-γ+
TNF+
IL-2+
% of CD8+ T Cell
No Stim
GP33
GP276
NP396

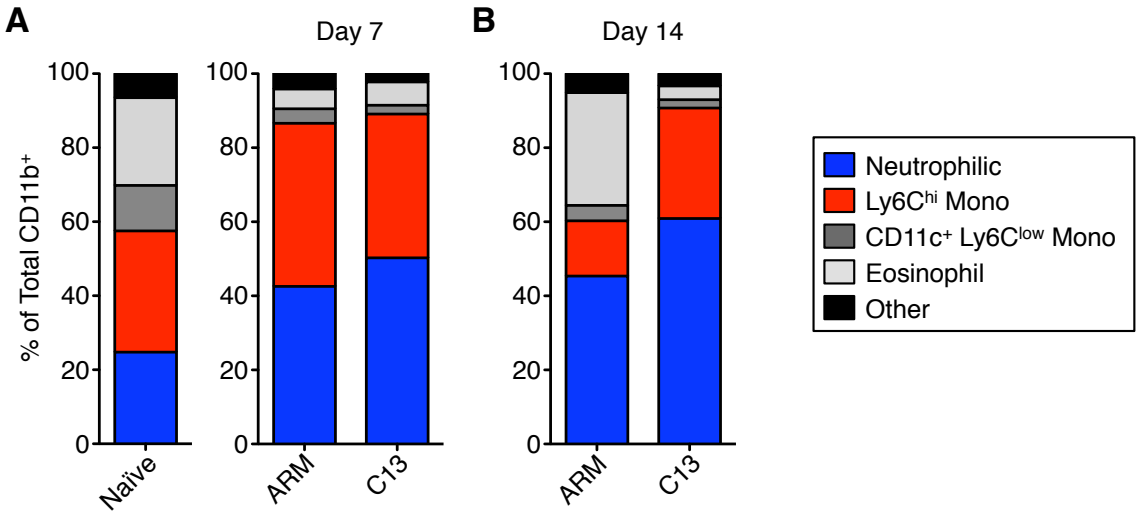
Figure 7


A
B
C
D
E
Cells/mL Blood
Cells/Spleen
Cells/pLN
% of CD8+ T Cell
IFN-γ+
TNF+
IL-2+
Naïve
Untreated
anti Gr-1
No Peptide
GP33
GP276
NP396
Untreated
anti Gr-1

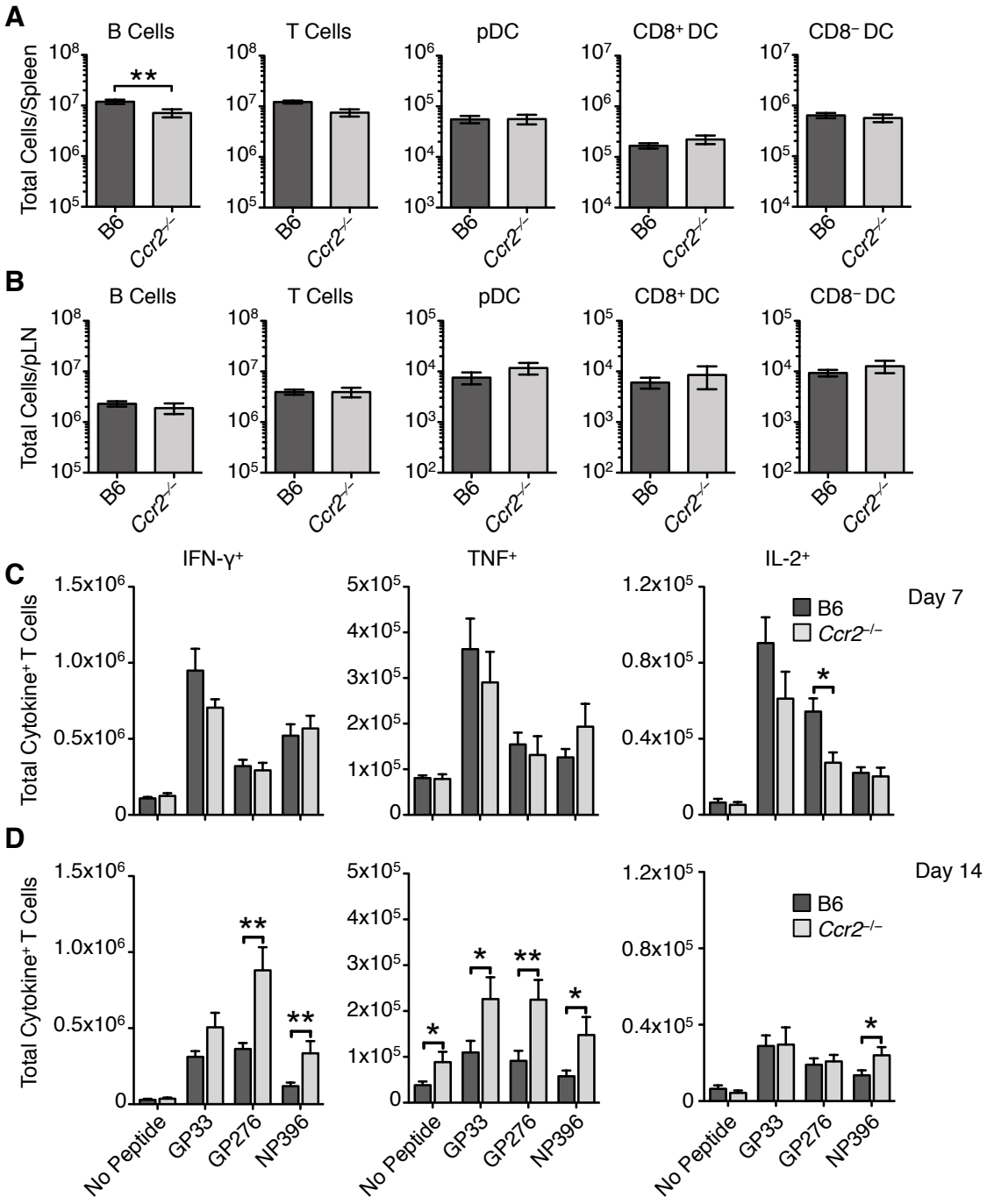
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

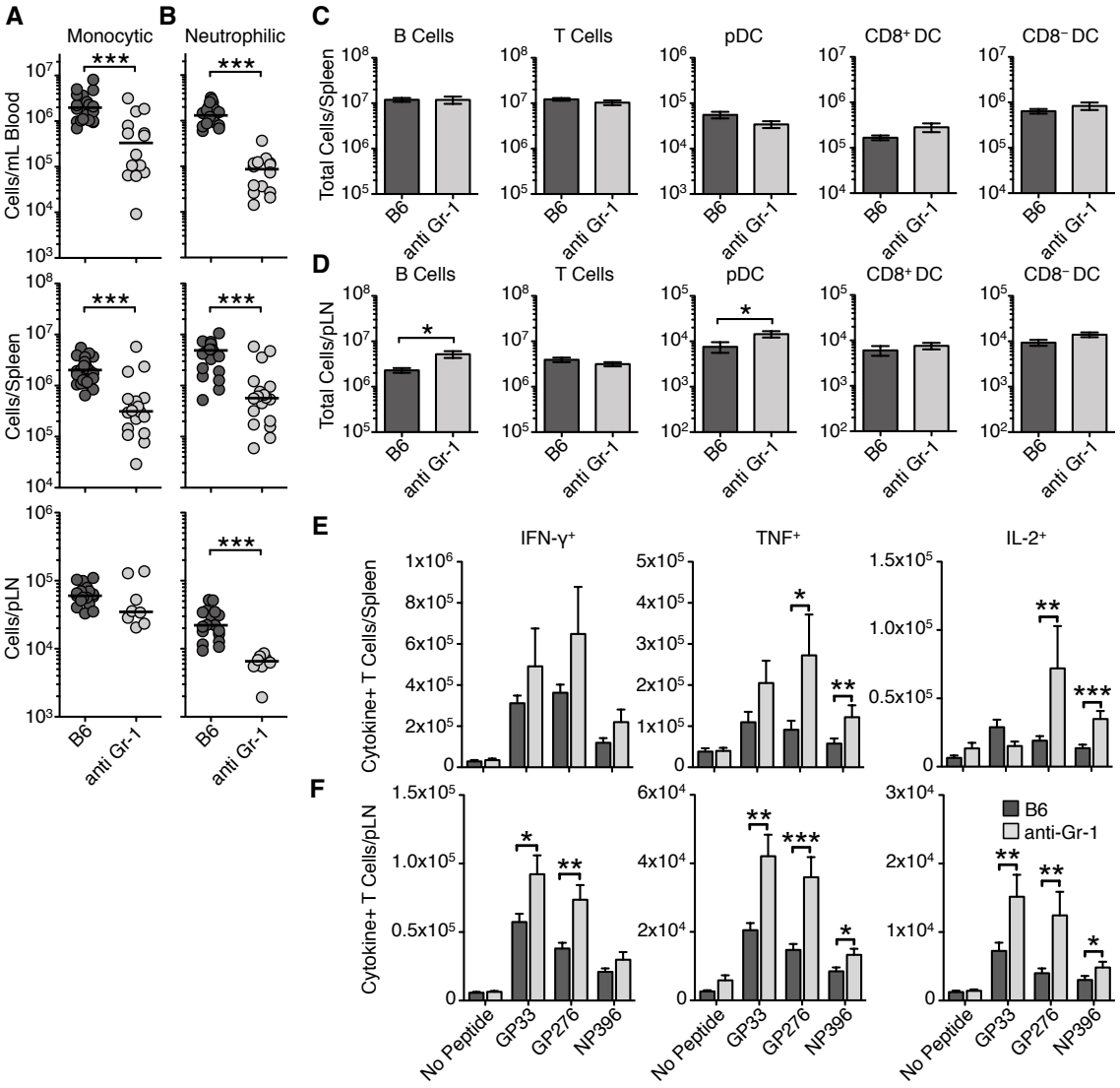


Figure Legends

Figure 1: The kinetics of APC activation and innate cytokine production are LCMV strain independent

(A) The normalized MFI of activation markers on DC populations relative to isotype control during early LCMV infection. Data are represented as mean ± SEM of 3 experiments with 3 pooled spleens for each time point.

(B) Representative intracellular staining for IL-12p40 of splenocytes over indicated time. Numbers in the quadrant represent percent cytokine expression in cDC from LCMV infected mice.

(C) Serum IFN concentrations in naïve and LCMV infected mice as quantified by ELISA. Data are represented as mean ± SEM of 3 independent experiments with 3 mice per time point.

(D) Serum chemokine levels in naïve and LCMV infected mice as quantified by luminex bead assay. Data are represented as mean ± SEM and are representative of two independent luminex assays of serum pooled from 3 mice and analyzed in duplicate. See also Figure S1.

Figure 2: $CD8^+$ T Cell stimulatory capacity of APCs from LCMV infected mice

(A) Total proliferating CFSE-labeled OT-I T cells following culture with $CD11c^+$ DC or $CD11c^-CD11b^+$ myeloid cells 24hr p.i. Proliferation was measured by flow cytometry after 3 days of culture with different amounts of SIINFEKL peptide.

(B) Total DC and myeloid cells from day 7 p.i. or day 14 p.i. (C) were bead purified and cultured with purified OVA-specific OT1 T cells as in (A).

Data are representative of 3 (A) or 2 (B-C) experiments.

See also Figure S2.

Figure 3: Enhanced and sustained expansion of myeloid cells during chronic LCMV infection

(A) Total splenic $CD11b^+$ myeloid cells during the first 24 days of ARM and C13 infection were enumerated by flow cytometry and the fold increase over naïve levels was determined. Data are representative of 3 experiments and each point represents 3 pooled spleens.

(B) The number of monocytic and neutrophilic cells in the spleen of LCMV infected mice. Data are representative of at least 3 experiments and each point is the mean ± SEM of 3 pooled spleens.

(C) Total number of monocytic and neutrophilic myeloid cells in the spleen of naïve and day 14 LCMV infected mice. Data are individual mice from 4-6 experiments and the bar represents the median. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

(D) Representative histology of mouse spleens from naïve and day 14 LCMV infected mice. $B220^+$ B cells are green, $Thy1.2^+$ T cells are red, and $F4/80^+$ myeloid cells are blue.

Figure 4: Myeloid cells expanded during chronic infection resemble MDSC

(A) Expression of phenotypic markers on monocytic myeloid cells following LCMV infection.

(B) Expression of CD80 by monocytic cells from multiple organs 14 days p.i.

(C) Kinetics of CD115 expression on total $CD11b^+$ cells and myeloid cells.

Data are representative of 3 experiments with 3 pooled spleens per group.

(D) Geimsa staining of sorted monocytic and neutrophilic cells from naïve and day 14 C13 infected mice. Data are representative of 3 independent experiments.

(E) Relative expression of genes in monocytic cells from ARM or C13 infected mice compared to naïve mice, as determined by Illumina BeadChips. Genes are ranked by relative expression and represent monocytic cells purified from three independent experiments (mean fold-change).

Figure 5: Monocytic cells from C13 infected mice suppress $CD8^+$ T cell proliferation

(A) Total spleen monocytic cells from ARM or C13 infected mice were compared to the frequency of IFN-γ^+ GP33-specific $CD8^+$ T cells.

(B) Flow cytometry sorted monocytic cells from day 14 post LCMV infection were cultured with OT-I splenocytes in the indicated ratios. Cultures were stimulated with no peptide, SIINFEKL or vaccinia (B8R) peptide. Proliferation was determined by [^{3}H] Thymidine incorporation. Mean ± SEM are performed with 4 experiments performed in triplicate.

(C and D) Sorted MDSCs from C13-infected mice were cultured 1:2 with purified OT-I T cells and stimulated in the presence of NOS or ARG1 inhibitors or blocking antibodies.

(C) Percent proliferation was calculated relative to stimulated OT-I splenocytes without added MDSC and mean ± SEM are performed on 3 independent experiments.

(D) Representative proliferation was measured by CFSE dilution after 3 days of culture.

Figure 6: CCR2-deficient mice have enhanced T cell responses to C13

(A) Serum concentrations of CCL2 were measured during ARM and C13 infection.

(B) Serum concentrations of CCL2 in WT and *Ccr2*$^{-/-}$ mice day 14 post C13 infection.

(A and B) Serum cytokine concentrations are presented as mean ± SEM from ≥3 mice per group.

(C) Representative flow cytometry CCR2 staining on monocytic cells from WT and *Ccr2*$^{-/-}$ mice day 14 post C13 infection.

(D and E) Blood (D) and spleen (E) levels of monocytic cells on days 7 and 14 post C13 infection were determined by flow cytometry. Data are represented as mean ± SEM of 3-5 experiments, each with 4-6 mice per group.

(F) Spleen CD8^{+} T cell cytokine production following stimulation with indicated LCMV peptides day 7 and day 14 p.i. Data are represented as mean ± SEM of 3-4 experiments, each with ≥4 mice per group. *p<0.05, **p<0.01, ***p<0.001.

See also Figure S3

Figure 7: Depletion of myeloid cells enhanced LCMV-specific T cell cytokine responses

C13 infected mice received 100μg anti-Gr-1 (RB6-8C5) or isotype control antibody per day starting day 11 p.i.

(A and B) Total myeloid cells (CD11b^{+}CD11c$^{lo/-}$) in the blood (A) and spleen (B) were measured by flow cytometry on day 14 p.i. Data are individual mice from 3 independent experiments and the bar represents the median.

(C) Frequency of cytokine production in spleen $CD8^+$ T cells following stimulation of splenocytes with indicated LCMV peptides for 5hr and intracellular flow cytometry cytokine staining. Data are represented as mean ± SEM from at least 3 experiments with ≥5 mice per group.

(D) Total myeloid cells in the pooled total pLN. Data are averages of total pLN from 3 mice in 3 independent experiments and the bar represents the median.

(E) Frequency of cytokine production in pLN $CD8^+$ T cells following stimulation with indicated LCMV peptides for 5hr and intracellular flow cytometry cytokine staining. Data are represented as mean ± SEM from 3 experiments with ≥5 mice per group.

*p<0.05, **p<0.01, ***p<0.001.

See also Figure S4

Figure S1, related to Figure 1: Identification of multiple innate APC subsets by 10-color flow cytometry

(A) Live singlet cells from collagenase-digested spleens were lineage gated by expression of NK1.1, Thy1.2, B220 and CD19.

(B) Total numbers splenocytes and lymphocyte populations during the first 72hr of LCMV ARM and C13 infection.

(C) pDC were identified from $B220^+$, $Ly6C^+$, $CD19^-$, $Thy1.2^-$, $CD11c^{int}$, and $CD11b^-$ cells.

(D) cDC were identified from non-lymphocytes by high expression of CD11c then $CD8^+$ and $CD8^-CD11b^+$ DC were identified from $CD11c^{hi}$ $Ly6C^-$ cells.

(E) Total pDC, $CD8^+$ and $CD8^-CD11b^+$ DC were measured during ARM and C13 infection.

(F) $CD11b^{hi}$ myeloid cells were identified from non-lymphocytes and subdivided into eosinophils, monocytic and neutrophilic cells by Gr-1, Ly6C, SSC and (G) F4/80 expression.

(H) Eosinophils, monocytic and neutrophilic cells were measured during ARM and C13 infection.

Data are represented as mean ± SEM of 3 experiments with 3 pooled spleens for each time point.

Figure S2, related to Figure 2: Myeloid cell proportions diverge day 14 post LCMV infection

(A) The frequencies of the major splenic myeloid cell sub-populations day 7 and day 14 (B) of ARM and C13 infection were compared to naïve mice.

Data are represented as mean ± SEM of 4 experiments with 3 pooled spleens for each time point.

Figure S3, related to Figure 6: CCR2-deficient mice have enhanced T cell numbers during C13 infection

(A) Total spleen and pLN (B) B cells, T cells, pDC, $CD8^+$ DC, and $CD8^-$ DC, day 14 post C13 infection. Data are mean ± SEM and represent 3 experiment with 3 pooled spleens for each time point. $^{**}p<0.01$.

(C) Total number of cytokine expressing splenic $CD8^+$ T cells in WT and *Ccr2*$^{-/-}$ mice day 7 and day 14 p.i. (D) as determined by intracellular staining (ICS) following 5hr of stimulation with the indicated LCMV peptides. Data are mean ± SEM and represent 1 experiment with 5 mice per group (day 7) and 4 experiments with ≥4 mice per group (day 14). *p<0.05, **p<0.01.

Figure S4, related to Figure 7: Anti-Gr-1 depletion of monocytic and neutrophilic myeloid cells enhances $CD8^+$ T cell responses to C13

(A) Total numbers of monocytic and neutrophilic cells (B) in the blood, spleen and pLN following anti-Gr-1 treatment. Monocytic and neutrophilic cells were distinguished by F4/80 and DEC-205 expression, respectively.

(C) Total spleen and pLN (D) B cells, T cells, pDC, $CD8^+$ DC, and $CD8^-$ DC, day 14 post C13 infection. *p<0.05. Data are mean ± SEM and represent 3 experiments with 3 pooled spleens for each time point. **p<0.01.

(E) Absolute number of cytokine expressing $CD8^+$ T cells in the spleen and pLN (F) day 14 post C13 infection and determined by ICS following 5hr of stimulation with the indicated LCMV peptides.

Data are represented as mean ± SEM from 3 experiments with ≥5 mice per group. *p<0.05, **p<0.01, ***p<0.001.

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CHAPTER 3

Discussion and Future Directions

Multiple phases of innate immune function during acute and chronic viral infection

The LCMV model of acute and chronic infection of mice has been an essential tool to investigate and elucidate the adaptive immune responses to viral infection. The functional exhaustion that accompanies the persistence of virus during chronic infections has been well characterized in ARM and C13 infection. Additionally, the LCMV system has been critical to the discovery of the markers that characterize the generation of protective memory and has facilitated the discovery of methods for determining the quality of memory responses. The usefulness of this system has been expanded even further with the investigation of innate immune responses during acute and chronic infections. Determining the correlates of innate activation that lead to protective immune responses is critical to the development of vaccines and treatments for the multitude of chronic viral infections afflicting humans.

Initially we began our research with an eye to identify the early innate correlates to the adaptive outcome of ARM and C13 infection. We generated a multicolor flow cytometry panel capable of identifying activation and cytokine production by multiple innate cell subsets. We hypothesized that different LCMV strains would produce distinct activation profiles that could then be correlated to infection outcome. Despite our expectations, the major innate cell subsets responsible for activating and priming the T cell responses were

virtually identical between acute and chronic strains of LCMV. All three of the major mouse DC subsets increased expression of costimulatory and inhibitory receptors with kinetics that were indistinguishable between virus strains. Furthermore, the numbers of LDC and MDC were severely reduced during both acute and chronic infection. Innate cytokine production by these cells also occurred with kinetics that were independent of LCMV strain and total innate cytokine levels in the serum of infected mice did not differ between acute and chronic infection. Total DC populations isolated 1 or 2 days post ARM and C13 infected mice were able to prime T cell proliferation with similar efficacy. Thus we have shown that by classical methods of determining DC activation and stimulatory capacity, DC activation during acute and chronic LCMV infection occurs nearly identically.

We expanded our investigation of innate cells to later phases of LCMV infection and observed that myeloid cells from chronic infection showed a profound deficiency in T cell stimulation. DCs purified from 1 or 2 weeks post infection with ARM or C13 were identical in their stimulatory capacity, as they had been earlier during the priming phase. Total myeloid cells from both infections could stimulate T cell one week post infection, but when these cells were isolated two weeks post infection, T cells failed to proliferate when they were cultured with C13-derived myeloid cells. Both ARM and C13 infection led to large expansion of myeloid cells in the blood and lymphoid tissues day 7 p.i. Only C13 infection, however, led to the continued increase and persistence of monocytic and neutrophilic cells, which peaked day 14 p.i. before contracting.

The monocytic cells sustained at high levels during C13 infection expressed high levels of CD115, suggesting that they were in an immature state. Gene array analysis revealed that monocytic cells from C13 infection had increased transcripts for multiple genes associated with myeloid cell development and MDSCs when compared to naïve cell gene expression. These cells also increased gene expression of more innate cytokines and chemokines, IFN-related genes, MHC-Class I processing and presentation related genes, tissue remodeling genes, and ROS-related genes than monocytic cells from day 14 post ARM infection. Thus, at the peak of C13-induced expansion of monocytic cells, these cells expressed multiple genes associated with inflammation and tissue damage. When these cells were purified and added to T cell cultures, only C13-derived monocytic cells inhibited the proliferation of T cells in an iNOS and IFN-γ-dependent manner. Mice deficient in CCR2, a chemokine receptor expressed by monocytic cells and associated with MDSC recruitment, showed a diminished pool of circulating monocytic cells during C13 infection. The reduced circulation of monocytes delayed the accumulation of these cells in lymphoid tissues between day 7 and 14 leading to increased $CD8\alpha^+$ T cell function. Administration of an antibody specific for the Gr-1 antigen expressed on monocytic and neutrophilic cells, we significantly depleted circulating and lymphoid tissue myeloid cells during C13 infection, leading to enhanced anti-viral T cell function.

Innate cell activation during the T cell priming phase

The innate phenotyping panel and innate cytokine staining protocols we generated are powerful tools that we have used to characterize innate immune responses to LCMV infection. We focused on innate responses to LCMV primarily in the spleen, where we determined the kinetics of DC subset activation to be strain-independent. The innate immune responses in other secondary lymphoid tissues such as peripheral LN, primary lymphoid tissues, or non-lymphoid tissues were not as thoroughly evaluated. LCMV-specific T cells may receive different priming signals in the LN, which could impact their function or survivability. Because LCMV is a systemic infection, and C13 virus can readily be detected in vital organs such as the kidneys, liver and lungs, it is important to address the potential role of innate immune responses within these tissues. How LCMV infection affects the composition and activation of innate APC populations within these tissues has yet to be determined. There may be differences in the kinetics of infection and, consequently, innate activation in these tissues between acute and chronic LCMV strains. High levels of antigen presentation by innate and non-innate cells of the periphery may functionally exhaust T cells during C13 infection. Furthermore, the recruitment of myeloid cells and MDSCs might be different during ARM and C13 infections, which could impact T cell function and viral persistence.

Our approach to investigating innate immune responses was limited, however, by the choice of 'classical' activation and inhibitory markers we investigated. The activation markers CD80, CD86 and I-A^b and the inhibitory markers PD-L1 and PD-L2 have long been associated with inflammation induced maturation and activation of APCs (*1, 2*).

We demonstrated that inflammation induced by both ARM and C13 infection led to the upregulation of these markers independent of viral strain. These markers constitute the tip of the iceberg of possible immunomodulatory molecules that could potentially differently affect T cell responses during acute or chronic LCMV infection. We did evaluate the expression of additional activation markers; expression of CD40 and CD70 was induced on DC subsets during the first 72h of infection but there were no significant differences between ARM or C13 infection (unpublished data). Our flow cytometry panel can easily be modified to accommodate all manner of additional activation or inhibitory markers as well as cytokines. Additionally, our innate panel can be used to investigate innate responses to all manner of infectious and inflammatory models of disease in mice.

Flow cytometry evaluation of innate immune responses provides aggregate information of activation throughout the organ being evaluated. Information such as where APCs are located within tissues and how closely they associate with T cells cannot be determined by flow cytometry. The uniformity of the population wide DC activation we observed during ARM and C13 may mask differences in function related to presence of antigen within APCs, antigen processing capacity and location of antigen presenting cells. Immunofluorescent histology staining of spleens over the first 3 days of LCMV infection that identifies DCs as well as viral antigens could help further characterize the innate responses and determine if DC containing viral antigen associate with T cells similarly in ARM and C13 infection.

It has been demonstrated that C13 is present in the WP and RP of the spleen by day 3 p.i., while ARM remains restricted to the RP. Although both infections activate DCs throughout the spleen, it is possible that DC within the WP that are infected or contain high levels of viral antigen early during C13 infection might prime T cells with different kinetics than during ARM infection. High levels of TCR stimulation have been correlated with functional exhaustion and anergy in LCMV infection and many other models. The tropism differences between these two strains may lead to increased frequency of TCR stimulation early during C13 infection. This might predispose T cells to become exhausted as infection progresses, or it might overwhelm immune responses, increase pathology and promote immunosuppression.

Our functional assays were specifically designed with exogenous OVA peptides and OVA-specific T cells to eliminate any potential effects that antigen load differences in APCs might have on T cell priming. That said, there may be differences in the amount of antigen with APCs or differences in the processing and presentation of viral antigens, which might impact T cell priming. We were not able to normalize purified DC or myeloid cells based on amount of viral antigen, and thus could not utilize LCMV-specific transgenic T cells. A reporter system that allows for quantification of antigen load independent of T cell proliferation, or allows for the purification of APC based on antigen load would help address this question.

MDSC during the T cell phase of chronic viral infection

Myeloid cells with suppressive function have been described in diverse systems ranging from cancer, autoimmunity, parasitic infections, and many models of chronic inflammation (*3, 4*). We have described for the first time a role for MDSCs specifically in persistent viral infections but not acute infections. Both acute and chronic LCMV infections lead to large expansions of myeloid cells, including both neutrophilic and monocytic cells. This expansion occurs as early as day 5 p.i. and roughly correlates with the initiation of the CD8α^+ T cell response. Myeloid cell numbers began to contract following the peak of anti-viral T cell responses day 7 post ARM infection and remained capable of stimulating naïve T cell proliferation. Infection with persistent LCMV, however, increased and sustained high levels of monocytic and neutrophilic cells following the peak of CD8α^+ T cell expansion. During this second phase of innate immune cell function, after initiation of the T cell response, myeloid cells expanded during C13 infection gain a suppressive function, reducing T cell expansion and facilitating functional exhaustion.

LCMV is not a lytic virus and the cell death and pathology associated with infection is mediated by the anti-viral CD8α^+ CTL response. Day 7, the approximate peak of LCMV-specific CD8α^+ T cell expansion (Figure 1A), marks a point of major divergence in immune responses between acute and chronic LCMV infection. In stark contrast to ARM infected mice, which experience no weight loss, mice infected with C13 begin to rapidly lose weight by day 7 p.i., and lose 20-25% of their body weight around day 10 (Figure 1B). Serum levels of CCL2 rapidly decline day 7 after ARM infection but are

maintained at significant levels for weeks in C13 infected animals (Figure 1C). Viral loads in the kidneys of LCMV infected mice are roughly equivalent up until day 5, after which ARM levels drop significantly until no virus is detectable by day 11 p.i. (Figure 1D). The amount of virus in the kidneys of C13 infected animals continues to increase from day 7 to day 14 where it peaks ~3-4 log above day 5 levels.

Monocytic cells numbers in the spleens of LCMV infected mice begin to diverge after day 7 p.i. as they contract during ARM infection and remain substantially elevated from day 7 to day 17 post C13 infection (Figure 1E). Monocytic cells purified from day 10 to day 17 after C13 infection suppressed CD8α^+ T cell priming *in vitro* (Chapter II and unpublished data). During this period of time, the frequency of Fox-P3$^+$ T_{reg} CD4$^+$ T cells increased 3-4 fold in C13 infected animals, which is consistent with previous reports implicating MDSCs in promoting T_{regs} (Figure 1F). These data portray a second phase of innate immune response, following initiation of the cellular adaptive immune response, where immunopathology coincides with innate and adaptive immune effectors and promotes an immunosuppressive environment.

We have demonstrated that IFN-γ-induced iNOS expression has a role in the mechanism of MDSC-mediated suppression of CD8α^+ T cell function. However, the conditions and signals that drive the expansion, accumulation and function of MDSCs during C13 infection are currently unknown. Myeloid cells only gain suppressive function after the peak of anti-viral T cell function, which could reflect the need for IFN-γ for MDSC suppression. There may be a role for signals produced by tissue damage and

cell lysis in sustaining MDSCs. CCL2 remains high throughout C13 infection, but suppression only occurs after around day 10, suggesting a role for this chemokine in promoting myeloid cell expansion, but not directly inducing suppressive function. IFN-α is continuously produced during C13 infection and type I IFN signaling has been linked to the expansion of myeloid progenitors in the BM (*5-7*). Type I IFN and STAT 2 signaling has also been shown to prevent DC development, which could contribute to the accumulation of myeloid progenitors that become MDSCs (*7*).

IL-10 has directly linked the immunosuppression observed during C13 infection (*8-10*). DCs and macrophages have been shown recently to be producers of IL-10 during C13 infection, and constitute a population of inhibitory APC (iAPC) (*11*). These iAPC are likely to be related to or identical to the MDSCs that we have described. Although we were not able to demonstrate any role of IL-10 in MDSC suppression of T cell priming *in vitro*, there may be immunomodulatory effects of MDSC-produced IL-10 *in vivo*. TGFβ has also been shown to drive T cell exhaustion during C13 infection, and MDSCs producing TGFβ have been described in tumor models, which may be another potential *in vivo* mechanism of MDSC suppression during chronic LCMV infection (*12, 13*).

Further studies will need to determine the full kinetics of MDSC suppression during C13 infection. We hypothesize that during C13 infection an anti-viral T cell response is mounted but unable to control viral load. These adaptive responses damage tissues and induce pathology. Myeloid progenitors are induced by inflammatory signals to

proliferate in the BM and enter lymphoid tissues where they encounter innate and adaptive inflammatory signals as well as signals of tissue damage. The confluence of these signals prevents these cells from differentiating into DCs or macrophages and causes them to adopt a suppressive function. This suppression acts to limit adaptive immune responses and limit the immunopathology but as a consequence hinders the clearance of virus and facilitates persistence.

LCMV infects myeloid cells such as monocytes and macrophages, in addition to DCs. It is possible that the massive expansion of monocytic cells during LCMV infection facilitates the increasing viral burden by expanding the number of target cells for C13 replication. It may be difficult to extrapolate our findings on the role of MDSCs in chronic infections in other systems or in human infection if persistence is due to C13 infection of myeloid cells. Our unpublished data indicates that total myeloid cells and both monocytic and neutrophilic subsets isolated from day 14 post C13 infection do contain virus, but much less than total DC populations. Treatment of C13 infected mice for three days with anti-Gr-1 antibody achieved 10- and 6-fold reductions in myeloid cell numbers in the blood and spleen, respectively. This loss of myeloid cells, however, did not correlate to a significant reduction in the viral loads in the serum, spleen, liver or kidneys. This suggests that viral burden and myeloid cell expansion are not directly linked and MDSC expansion and suppression during persistent infection may be a common compensation mechanism. Because of the consistent association of MDSC with cancers of many different mammals, it is likely that this suppressive feedback to chronic

inflammation may play a substantial role in human chronic viral diseases, and targeting MDSC may be a new front in treating chronic infections.

Figures

Figure 1

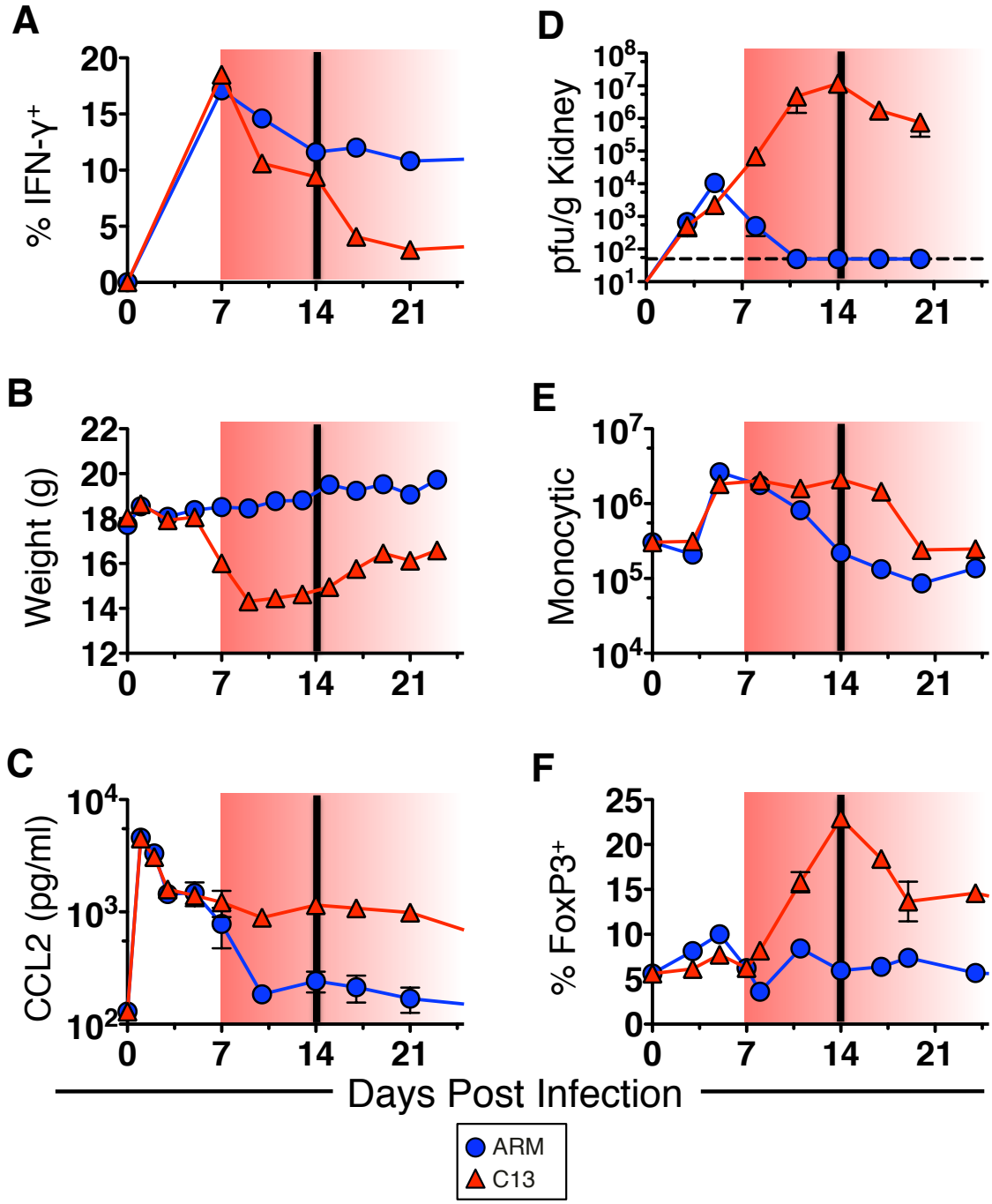

Figure Legends

Figure 1: Kinetics of immune responses to acute and chronic LCMV infection

(A) GP_{33} specific $CD8\alpha^+$ T cell IFN-γ responses in the spleens of ARM and C13 infection.

(B) Total mass of ARM and C13 infected mice.

(C) Serum levels of CCL2 were measured during ARM and C13 infection.

(D) Viral loads in the kidneys of LCMV infected mice as determined by plaque assay.

(E) The number of monocytic cells in the spleen of LCMV infected mice.

(F) Frequency of $FoxP3^+$ expression within $CD4^+$ T cells during LCMV infection.

Red shading represents $CD8\alpha^+$ T cell-mediated immunopathology and the black line represents the peak of monocytic cell expansion in the spleen.

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