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Modulation of Extramedullary Hematopoiesis during Cytomegalovirus Infection

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Erklärung

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Ehrenwörtliche Versicherung

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München, 11.11.2011

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Stefan Jordan

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0. Summary

The immune response against bacterial, parasitic and viral pathogens can be associated with reactivation of hematopoiesis at fetal sites of blood formation, a process termed extramedullary hematopoiesis. For example, congenital infection with human cytomegalovirus (HCMV) can induce extramedullary hematopoiesis in the skin of neonates visible as dark-blue macules – a prominent clinical picture called 'blueberry muffin baby'. Furthermore, acute infection with HCMV can lead to an enlargement of the spleen at all ages.

The cellular and molecular mechanisms governing extramedullary hematopoiesis after infection are poorly understood. Mouse cytomegalovirus (MCMV) is a reliable model for HCMV in many regards. Here, MCMV infection was used to study extramedullary hematopoiesis in the spleen.

C57BL/6 mice infected with MCMV developed splenic extramedullary hematopoiesis peaking at day 6 post infection. Both natural killer (NK) cells and dendritic cells (DCs) were found to play a central role in the modulation of extramedullary hematopoiesis upon infection with MCMV.

NK cells were essential for the establishment of extramedullary hematopoiesis. On the molecular level, extramedullary hematopoiesis required recognition of infected cells via the activating NK cell receptor Ly49H. Surprisingly, the development of extramedullary hematopoiesis was not induced by NK cell-derived cytokines but fully dependent on perforin-mediated cytotoxicity. In fact, NK cell cytotoxicity became dispensable after infection with spread-deficient ΔM94-MCMV, showing that NK mediated control of virus spread to secondary target cells is a prerequisite for extramedullary hematopoiesis. Furthermore, virus spread to secondary target cells suppressed inflammatory extramedullary hematopoiesis induced by CpG-oligodesoxynucleotides (CpG-ODN). Hence, whereas MCMV suppresses

inflammation induced extramedullary hematopoiesis, NK cells confine virus spread by direct lysis of infected cells, and thus support extramedullary hematopoiesis.

DCs were identified to be the secondary target cell that becomes a dominant suppressor of extramedullary hematopoiesis upon infection. Infection of DCs resulted in profound changes in the serum cytokine profile and constriction of stem cell proliferation in the spleen.

Notably, suppression of extramedullary hematopoiesis upon infection of DCs was not restricted to MCMV but was a general phenomenon exploited by DC-tropic viruses and bacteria such as lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes*, respectively.

0. Zusammenfassung

Die Immunantwort auf bakterielle, parasitäre und virale Erreger ist zur Reaktivierung der Hämatopoiese an fötalen Orten der Blutbildung imstande, der sogenannten extramedullären Hämatopoiese. Nach einer kongenitalen Infektion mit dem humanen Zytomegalievirus (HCMV) kann extramedulläre Hämatopoiese zum Beispiel in der Haut von Neugeborenen auftreten, welche in Form von dunkelblaue Flecken sichtbar wird – ein bekanntes klinisches Bild, das ‚blueberry muffin baby' genannt wird. Darüber hinaus führt in jedem Lebensalter die akute Infektion mit HCMV häufig zu einer Vergrößerung der Milz.

Die zellulären und molekularen Mechanismen, welche die extramedulläre Hämatopoiese nach einer Infektion mit HCMV regulieren, sind bislang kaum verstanden. Das murine Zytomegalievirus (MCMV) ist in vielerlei Hinsicht ein geeignetes Modell für die HCMV Infektion. In dieser Studie wurde die Infektion mit MCMV genutzt, um die extramedulläre Hämatopoiese in der Milz zu untersuchen.

Nach einer Infektion mit MCMV entwickelten C57BL/6 Mäuse extramedulläre Hämatopoiese in der Milz, welche ihren Höhepunkt an Tag 6 erreichte. Natürliche Killerzellen (NK) und dendritische Zellen (DCs) spielten eine zentrale Rolle in der Modulation der extramedullären Hämatopoiese nach einer Infektion mit MCMV.

Die extramedulläre Hämatopoiese trat ausschließlich in Anwesenheit von NK Zellen auf. Auf der molekularen Ebene erforderte das Auftreten der extramedullären Hämatopoiese die Erkennung infizierter Zellen durch den aktivierenden NK Zellrezeptor Ly49H. Überraschenderweise wurde die extramedulläre Hämatopoiese nicht durch Zytokine der NK Zellen induziert, sondern bedurfte ihrer durch Perforin vermittelten zytotoxischen Funktion. Die zytotoxische Funktion wurde jedoch nicht benötigt, wenn die Infektion mit der Virusmutante ΔM94-MCMV erfolgte, die nach einmaliger Infektion keine weiteren Zellen

infizieren kann. Dies zeigte, dass die Kontrolle der Virusausbreitung durch NK Zellen die Voraussetzung für das Auftreten der extramedullären Hämatopoiese ist. Virusausbreitung unterdrückte auch extramedulläre Hämatopoiese, die zuvor mit CpG-Oligodesoxynukleotiden (CpG-ODN) induziert worden war. Während MCMV also entzündungsbedingte extramedulläre Hämaopoiese unterdrückt, besteht die Rolle der NK Zellen darin, die Virusausbreitung zu verhindern und so extramedulläre Hämatopoiese zu ermöglichen.

DCs wurden als diejenige sekundäre Zielzelle identifiziert, welche nach Infektion zum dominanten Suppressor der extramedullären Hämatopoiese wird. Die Infektion von DCs führte zu tiefgreifenden Veränderungen im Zytokin-Profil des Blutserums und zu einer reduzierten Anzahl von Stammzellen in der Milz.

Die Suppression der extramedullären Hämatopoiese erfolgte nicht nur nach einer Infektion mit MCMV, sondern stellte ein generelles Phänomen nach einer Infektion mit DC-tropischen Viren und Bakterien dar, wie zum Beispiel dem Lymphozytären Choriomeningitis Virus (LCMV) und *Listeria monocytogenes*.

1. Introduction

1.1 Immune responses alter steady state hematopoiesis

It is a rather trivial insight that hematopoiesis (from Ancient Greek αμα = blood and ποιεν = to make) is fundamental for both innate and adaptive immunity as it constantly generates the cellular constituents of the immune system. More interestingly is the fact that the generation of new immune cells is regulated by the already existing white blood cells. Upon detection of an invading pathogen, activated leukocytes produce a plethora of so-called hematopoietins, a group of cytokines active in hematopoiesis including colony-stimulating factors (CSF) (e.g. granulocyte-macrophage-CSF), interleukins (e.g. IL-3, IL-4, IL-5, IL-7) and chemokines (e.g. CXCL1 / keratinocyte chemoattractant (KC)) resulting in the specific production of effector cells adjusted to the nature of the invading pathogen (Silverthorn 2009; COPE 2011). Upon bacterial infection, for example, leukocytes secrete cytokines stimulating the proliferation of neutrophil granulocytes and monocytes which fight the pathogens by phagocytosis. Virus infections, on the contrary, lead to a cytokine profile that often reduces the number of neutrophil granulocytes but increases the amount of lymphocytes lysing infected cells or producing protective antibodies. Parasites induce the proliferation of eosinophil granulocytes that secret anti-microbial mediators (Andreesen and Heimpel 2009). Hence, characteristic changes in the absolute numbers of leukocytes as well as the relative proportions of the different subgroups of leukocytes circulating with the bloodstream indicate the kind of the infectious agent. The differential blood count (differential hemogram) is therefore still an important diagnostic tool (Silverthorn 2009).

1.2 Extramedullary hematopoiesis and the ‘blueberry muffin baby’

In the developing embryo, hematopoiesis first occurs in the yolk sac and later in spleen, liver and lymph nodes (Palis *et al.* 2010). In adults, the bone marrow is the main blood forming

tissue (medullary hematopoiesis). Interestingly, the immune response against various bacterial (MacNamara *et al.* 2009; Piseddu *et al.* 2011), parasitic (Villeval *et al.* 1990; Cotterell *et al.* 2000; Giordanengo *et al.* 2002) and both acute and chronic viral infections (Lucia and Booss 1981; Costantini *et al.* 2009) does not only influence the medullary hematopoiesis but can also reactivate blood formation at sites of fetal hematopoiesis - a process termed

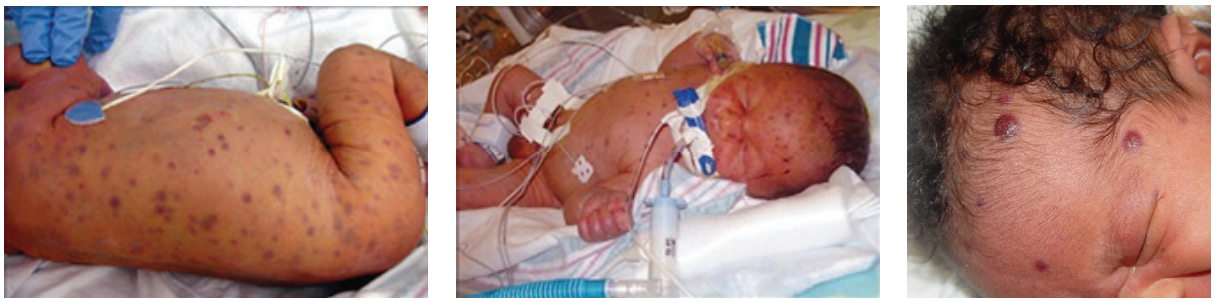

Figure 1.2 ‚Blueberry muffin baby‘.
Macules and papules are due to dermal extramedullary hematopoiesis upon congenital infection with human cytomegalovirus (HCMV) (Mehta *et al.* 2008)**.**

extramedullary hematopoiesis. In most cases liver and spleen resume their hematopoietic function resulting in a substantial increase in size of these organs (hepato-splenomegaly). In addition, extramedullary hematopoiesis can also be observed in lymph nodes, thymus, renal capsule, dura and skin.

For example, intrauterine infection can induce the unusual occurrence of extramedullary hematopoiesis in the skin of neonates which results in the characteristic clinical picture of the ‘blueberry muffin baby’. The newborns exhibit dark blue to purpuric macules or firm, dome-shaped papules predominantly favoring the trunk, head and neck (Mehta *et al.* 2008). The macules and papules start to resolve soon and are usually cleared by 3 to 6 weeks after birth (Mehta *et al.* 2008). The first virus described to cause the ‘blueberry muffin baby’ was rubella virus. In most cases, however, the causative agent is human cytomegalovirus (HCMV) (Groark and Jampel 1989; Hodl *et al.* 2001; Shaffer *et al.* 2005; Gaffin and Gallagher 2007).

1.3 Cytomegalovirus – history, epidemiology and clinical manifestation

The occurrence of gigantic, 'protozoa-like' cells with characteristic inclusion bodies in histologic samples from autopsies of dead children was noted more than 100 years ago (Jesionek and Kiolemenoglou 1904; Ribbert 1904). Although already in 1921 Goodpasture interpreted these histopathological changes as caused by virus infection (Goodpasture and Talbot 1921), the aetiologic agens was not discovered until 1956, when at the same time three US-American research groups isolated a herpesvirus of the β-subfamily (Rowe *et al.* 1956; Smith 1956; Weller *et al.* 1956). One year later, the first detailed German description of the pathology of the 'cytomegalic disease' was published (Seifert and Oehme 1957).

	Herpesvirus		Disease
α	Herpes simplex (HSV-1)	HHV-1	cold sore
	Herpes simplex 2 (HSV-2)	HHV-2	genital herpes
	Varicella-Zoster-Virus (VZV)	HHV-3	chickenpox / herpes zoster
β	**Cytomegalovirus (CMV)**	**HHV-5**	**diverse clinical manifestations**
	Reseolovirus	HHV-6 HHV-7	'sixth [childhood] disease' with rash
γ	Epstein-Barr-Virus (EBV)	HHV-4	infectious mononucleosis = 'Kissing disease', Burkitt lymphoma
	Karposi's Sarcoma-associated virus (KSHV)	HHV-8	Karposi's Sarcoma

Table 1.3 Human herpesvirus family.
Human herpesviruses (HHV) are classified in α-, β- and γ-subfamilies. α-Herpesviruses preferentially infect neuronal tissue, γ-herpesviruses lymphocytes. β-Herpesviruses can infect all kinds of tissue and thus cause various clinical outcomes. Original Roseolovirus was found to be two different herpesviruses. Adapted from (Hamprecht and Jahn 2007).

Human cytomegalovirus (HCMV; also classified as human herpesvirus 5 (HHV-5)), is highly prevalent worldwide and reaches up to an infection rate of 100% in developing countries. In the Western World the infection rate depends on the ethnic origin as well as the socio-economic status: for example 50% to 80% of the adult population are infected in the United

States (CDC 2011); in Germany no data are collected, but local studies suggest an infection rate of around 50% (Hamprecht and Jahn 2007).

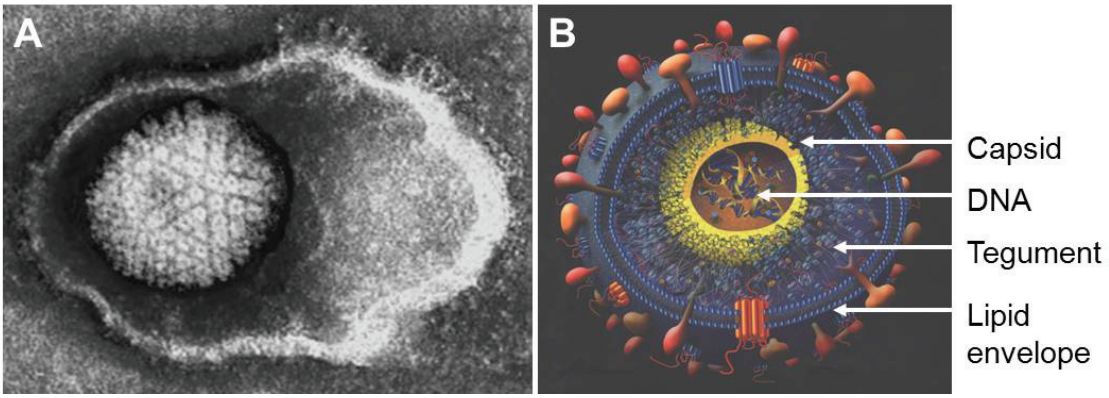

Figure 1.3 Structure of the herpesviruses.
The icosahedral capsid (100 nm in diameter) contains a double stranded DNA-genome. The tegument between capsid and envelope harbors host and viral RNA and proteins. The lipid envelope is host-derived with embedded viral glycoproteins. **A)** Electron micrograph of a herpesvirus virion (Stannard 1995). **B)** Schematic representation of a HCMV virion (Streblow *et al.* 2006).

Of all pathogens, HCMV is the most frequently transmitted virus during pregnancy. In Germany, estimated 0.5% of all newborns are already infected at the time of delivery (i.e. around 3700 children each year) and 20% of those ultimately suffer from severe neurological damages, which can lead to mental retardation and deafness (Hamprecht and Jahn 2007; Mosca and Pugni 2007; Grosse *et al.* 2008). In fact, congenital HCMV infection is the leading cause of deafness in Germany (Hamprecht and Jahn 2007). Furthermore, congenital HCMV infection is responsible for around 400 deaths of neonates in the United States and estimated 37 deaths in Germany per year (Hamprecht and Jahn 2007).

After infection, HCMV establishes lifelong latency. In the case of immunosuppression, as it for instance occurs in transplant recipients or patients with Acquired Immune Deficiency Syndrome (AIDS), virus replication frequently reactivates and causes severe disease (Britt 2006; Mocarski *et al.* 2007). Since virus replication can reactivate in various organs, the clinical manifestations of HCMV disease are diverse, including pneumonitis, myocarditis, diabetes, enteritis, retinitis, hepatitis, esophagitis, colitis, nephritis, encephalitis and graft loss

(Drew 1992). Retinitis caused by HCMV is the leading cause of blindness in AIDS patients (Hamprecht and Jahn 2007).

Up to date, there is no vaccine available for preventing HCMV disease. The US-American Institute of Medicine of the National Academies has ranked the development of a HCMV vaccine as highest priority since preventing HCMV-related disabilities would save considerable resources for the society otherwise required for lifelong health care (Stratton *et al.* 2000).

As described above, congenital HCMV infection may result in 'blueberry muffin babies' due to extramedullary hematopoiesis in the skin. Apart from the characteristic hemorrhagic-purpuric looking skin eruptions these newborns also present with hepato-splenomegaly (Groark and Jampel 1989; Hodl *et al.* 2001; Shaffer *et al.* 2005; Gaffin and Gallagher 2007). In general, hepato-splenomegaly is one of the distinctive features detectable by ultrasound diagnostics that indicate a HCMV infection *in utero* (Chaoui *et al.* 2002; Hamprecht and Jahn 2007). In childhood and in adults, most cases of HCMV infection are subclinical. Only occasionally HCMV infection induces a mononucleosis-like syndrome with sore throat, fever, malaise, muscle pain (myalgia), lymphadenopathy and also hepato-splenomegaly associated with the risk of rupture of the splenic capsule and severe hemorrhage (Rogues *et al.* 1994; Alliot *et al.* 2001; Duarte *et al.* 2003; Gorgone *et al.* 2005; Amathieu *et al.* 2007). It has not been investigated in patient samples, however, whether hepato-splenomegaly can be attributed to extramedullary hematopoiesis. To investigate the causative relationship between CMV infection, splenic extramedullary hematopoiesis and splenomegaly, an animal model is required.

1.4 Mouse cytomegalovirus (MCMV) induces extramedullary hematopoiesis

One common characteristic of the β-herpesviruses is their pronounced species specificity (Britt 2006). Mouse cytomegalovirus (MCMV) has an extensive sequence homology with HCMV (Rawlinson *et al.* 1996) and provides a model to study the biology of CMV infection in the living host (Mocarski and Kemble 1996; Kern 1999). MCMV resembles its human counterpart with respect to organ- and cell tropism, pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression (Mocarski and Kemble 1996; Reddehase *et al.* 2002; Krmpotic *et al.* 2003). Interestingly, as for HCMV, acute MCMV infection induces splenomegaly (Loh and Hudson 1981; Loh and Hudson 1982; Leung *et al.* 1991). For MCMV, splenomegaly could be attributed to an enlargement of the hematopoietic islands in the red pulp, i.e. to extramedullary hematopoiesis (Lucia and Booss 1981).

Thus, MCMV is the model system of choice to get insights into the mechanisms of HCMV pathogenesis. In addition, a wide range of genetically engineered mice offers the opportunity to study the molecular mechanisms of CMV infection. In this study MCMV was used to elucidate virus and host genetic factors that determine extramedullary hematopoiesis in the spleen.

1.5 The role of the spleen during cytomegalovirus infection

Although the spleen has aroused interest for more than 2000 years now – it is mentioned in the Talmud, the Midrash and was already studied by Hippocrates, Plato, Aristotle and Galen - its anatomy and physiology are still not completely understood (McClusky *et al.* 1999). In the 17th century Marcello Malpighi, the founder of microscopic anatomy, created a comprehensive description of the histology of the spleen (McClusky *et al.* 1999). Yet, it was still not clear what purpose the spleen served – and until today there is room for speculations.

While aged blood cells are degraded in the red pulp, the white pulp of the spleen is compartmentalized in T cell and B cell areas and therefore offers all structural requirements to enable efficient interactions between the different cells of the immune system which are necessary for a successful immune response (Karrer *et al.* 1997). Viruses and bacteria that

Figure 1.5 Splenomegaly upon infection.
Sketch by German painter Albrecht Dürer (1471-1528). Dürer suffered from splenomegaly probably due to Malaria infection. The text reads: do der gelb Fleck is vnd mit dem finger drawf dewt, do is mir we ("Where the yellow spot is and the finger points, I'm sore") (Kunsthalle Bremen).

have been opsonized by antibodies are cleared in the spleen from the circulation. Therefore, the spleen is generally regarded as the most important lymphoid organ for the initiation of an immune response against blood-borne antigens. Splenectomized patients, however, present a higher susceptibility only to infections with certain encapsulated bacteria, for example *Klebsiella pneumonia*, *Streptococcus pneumonia* or *Haemophilus influenzae* (Murphy *et al.* 2009), and the definitive role of the spleen during virus infection is still a matter of debate.

For HCMV it has been reported that splenectomy can facilitate virus infection, increases the severity of the systemic (mononucleosis) or localized (for example retinitis) disease and can ultimately lead to fatal cases of HCMV infection (Baumgartner *et al.* 1982; de Gorgolas

Hernandez-Mora *et al.* 2001; Han *et al.* 2005; Vote *et al.* 2005; Assy *et al.* 2007; Han *et al.* 2010). Thus, the spleen contributes to the immune response against HCMV and this contribution might manifest in the enlargement of the organ.

Detailed studies of CMV infection in the spleen have been done using MCMV. Endothelial cells of the red pulp are the first infected cells (Benedict *et al.* 2006). Only afterwards, virus can be found in the white pulp (Bekiaris *et al.* 2008; Hsu *et al.* 2009). Around 72 to 96 hours post infection the virus replication in the spleen reaches its peak. At the same time, the compartmentalization between the T cell and the B cell areas in the white pulp is dissolved (Benedict *et al.* 2006). This destruction of the microarchitecture of the white pulp can particularly be observed in the spleen of mice which are more susceptible for MCMV infection due to reduced Natural Killer (NK) cell function. In fact, restoration of NK cell function protects the microarchitecture of the splenic white pulp (Bekiaris *et al.* 2008).

1.6 Natural Killer (NK) cells in CMV infection

NK cells are lymphocytes that express only a limited repertoire of germline-encoded receptors in contrast to B cells and T cells. Therefore, they are classified to be part of the innate immune system. NK cells are important for the early control of virus infection, particularly for herpesviruses. They possess cytotoxic function and can - unlike T cells - induce the death of infected cells without previous immunization. Furthermore, NK cells are important producers of antiviral cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Vivier *et al.* 2011). Thereby, NK cells help to shape the adaptive immune response.

NK cells express surface receptors that can either stimulate (activating receptors) or dampen (inhibitory receptors) their activity (Vivier *et al.* 2004; Bryceson *et al.* 2006). Inhibitory receptors mostly measure major histocompatibility (MHC) class I molecules on target cells. MHC class I molecules present intracellular peptides to the outside and are scanned by

cytotoxic T cells for non-self determinants. Consequently, down-regulation of MHC class I molecules is a mechanism used by many pathogens and tumor cells to evade T cell recognition and lysis. Thus, lack of MHC class I expression on the cell surface indicates danger and leads to the activation of patrolling NK cells. NK cell activation due to a lack of ligation of inhibitory receptors has been named 'missing self' recognition (Karre *et al.* 1986).

Activating receptors have either cellular partners, as NK group 2D (NKG2D) that binds to several surface molecules overexpressed upon cellular distress, or directly recognize virus gene products. For example, NKp46 recognizes hemagglutinins from influenza and parainfluenza virus (Arnon *et al.* 2001; Mandelboim *et al.* 2001). Ly49H which is expressed in C57BL/6 mice recognizes the MCMV encoded m157 glycoprotein on the surface of infected cells (Arase *et al.* 2002; Smith *et al.* 2002; Bubic *et al.* 2004) leading to specific proliferation of $Ly49H^+$ NK cells and perforin-mediated cytotoxicity (Dokun *et al.* 2001; Hsu *et al.* 2009). Unlike C57BL/6 mice, MCMV susceptible mouse strains lacking the Ly49H receptor are unable to mount an effective NK cell control of this virus (Scalzo *et al.* 2007).

In fact, several CMV genes have been identified that interfere with NK cell recognition. HCMV UL16 down-regulates the NKG2D ligands MICB, ULBP-1 and ULBP-2 (Dunn *et al.* 2003; Rolle *et al.* 2003; Welte *et al.* 2003; Wu *et al.* 2003). MICB is also targeted by the HCMV-encoded microRNA hcmv-miR-UL112 (Stern-Ginossar *et al.* 2007). UL142 affects the expression of NKG2D ligand MICA (Chalupny *et al.* 2006).

MCMV encodes m04 that escorts specific MHC class I molecules to the surface in order to prevent 'missing self' recognition (Babic *et al.* 2010). Furthermore, at least four MCMV genes have been found that down-regulate cellular ligands of the activating NKG2D receptor. Genes *m145*, *m152* and *m155* are responsible for the down-regulation of MULT-1, RAE-1 and H60, respectively (Krmpotic *et al.* 2002; Lodoen *et al.* 2003; Lodoen *et al.* 2004; Hasan

et al. 2005; Krmpotic *et al.* 2005). In addition, the m138 protein affects the surface expression of MULT-1, H60 and RAE-1 isoforms (Lenac *et al.* 2006; Arapovic *et al.* 2009).

Nevertheless, NK cells depend on factors produced by other immune cells to proliferate, mature and acquire full effector potential. One of the most important cells for the development and activation of NK cells is the dendritic cell (DC) (Walzer *et al.* 2005; Zitvogel *et al.* 2006; Castillo *et al.* 2009).

1.7 Dendritic cells (DCs) and herpesviral immune evasion

DCs were discovered in 1973 when Ralph M. Steinman and Zanvil A. Cohn "during the course of observations on the cells of mouse spleen that adhere to glass and plastic surfaces […] noticed a large stellate cell with distinct properties" (Steinman and Cohn 1973). Up to

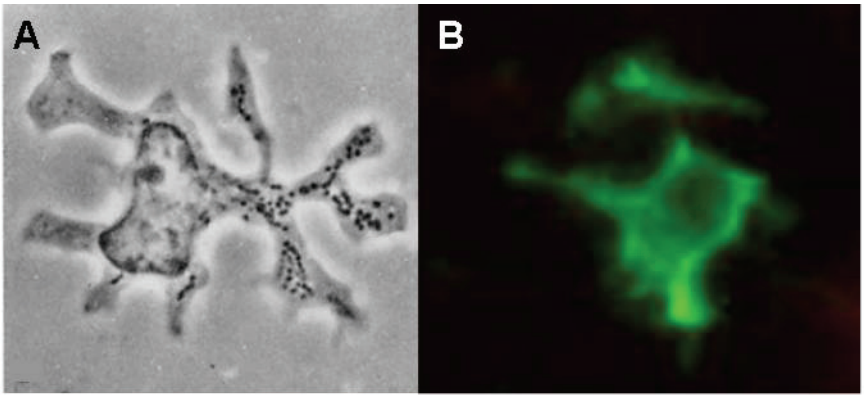

Figure 1.7 Dendritic cells.
A) The first photograph taken of a dendritic cell (Steinman and Cohn 1973).
B) Dendritic cells in the lymph node identified by CD11c expression (picture taken by the author).

date it has become evident that DCs are specialized for the uptake, transport, processing and presentation of antigens to naïve T cells and therefore play a key role for the initiation of adaptive immune responses (Kushwah and Hu 2011). Furthermore, DCs are major cytokine producers and are thus important for the orchestration of innate immunity. Due to the importance of DCs Ralph M. Steinman was awarded with the Nobel Prize in Physiology or Medicine for his discovery in 2011. DCs, however, were found to be a very heterogeneous

cell population. At least four DC subsets have been identified in mouse lymphoid organs (Henri *et al.* 2001; Kushwah and Hu 2011). All of these subsets express the surface molecule CD11c which is therefore used as a general DC marker. Plasmacytoid DCs (pDCs) are defined by the expression of CD45RA (B220) in contrast to conventional DCs (cDCs) that do not express this surface marker. cDCs can further be subdivided in subsets by the expression of CD8 and CD4.

Functionally, there are important differences between the DC subtypes. pDCs, for instance, produce high amounts of IFN-α. $CD8^+$ cDCs have the unique ability to cross-present non-self antigens on MHC class I molecules (Schulz and Reis e Sousa 2002). In addition, they are the major source of IL-12 triggering antiviral Th1 immune responses (Hochrein *et al.* 2001; Shortman and Liu 2002). Hence, these two DC subsets are particularly important during virus infection.

Many bacteria (e.g. *Salmonella enterica*, *Yersinia enterolytica*, *Helicobacter pylori*) (Bedoui *et al.* 2010) and viruses (e.g. Measles virus, Human Immunodeficiency virus (HIV), Herpesviruses) (Naniche and Oldstone 2000; Andrews *et al.* 2001; Raftery *et al.* 2001) specifically target DCs to prevent the initiation of the immune response.

CMV employs an array of mechanisms to interfere with immune stimulatory DC functions, for example the down-modulation of MHC class I and class II complexes or of co-stimulatory molecules, the up-regulation of apoptosis-inducing ligands or the alteration of inflammatory cytokine secretion profiles. This leads to an overall functional paralysis of the CMV-infected DC (Andrews *et al.* 2001; Raftery *et al.* 2001; Rolle and Olweus 2009).

1.8 Aim of the study

Ongoing immune reactions have a profound impact on the hematopoietic system leading to the specific production of leukocytes according to the kind of pathogen. Furthermore, infectious agents can reactivate the blood formation at fetal sites of hematopoiesis, i.e. in spleen, liver and skin. The ‘blueberry muffin baby’ is a prominent clinical manifestation of unusual postnatal extramedullary hematopoiesis in the skin. In most cases it is caused by HCMV infection.

HCMV is the most frequently transmitted virus during pregnancy. It is highly prevalent worldwide and causes severe disease in individuals with immature or compromised immune systems. A frequent indication for HCMV disease is the enlargement of liver and spleen, which can already be observed in the infected fetus by ultrasound.

MCMV is a reliable mouse model for HCMV in many regards. Splenomegaly upon MCMV infection could be attributed to extramedullary hematopoiesis.

The mechanisms governing extramedullary hematopoiesis during inflammation or infection, however, are poorly understood. As such, the role of the antiviral immune response in the induction of extramedullary hematopoiesis and vice-versa has not been studied.

The aim of this study was the identification of cellular and molecular determinates of MCMV-induced extramedullary hematopoiesis in the spleen.

Here, both NK cells and DCs were identified to play a central role in the modulation of extramedullary hematopoiesis. Similar to other inflammatory stimuli, MCMV infection induced extramedullary hematopoiesis in the spleen. Surprisingly, establishment of extramedullary hematopoiesis required NK cell control of virus spread independent of cytokines but dependent on perforin-mediated cytotoxicity.

In absence of NK cells, NK cell activation via Ly49H or NK cell cytotoxicity, virus spread resulted in suppression of extramedullary hematopoiesis.

DCs were responsible for the suppression of extramedullary hematopoiesis. Extramedullary hematopoiesis was not suppressed after depletion of DCs in addition to NK cells. Furthermore, direct transfer of *in vitro* infected DCs suppressed extramedullary hematopoiesis *in vivo*.

Notably this suppression of hematopoiesis upon infection of DCs was not restricted to the β-herpesvirus model but was also observed after infection with other DC-tropic pathogens. Hence, it is conceivable that regulation of extramedullary hematopoiesis is a mechanism of immune evasion exploited by DC-tropic pathogens.

2. Material and Methods

2.1 Material

2.1.1 Equipment

Device	Company
Analytical balance BP210 D	Sartorius, Göttingen
Balance Kern 470	Kern & Sohn GmbH, Balingen-Frommern
CantoII™	Becton Dickinson, Heidelberg
Centrifuge Beckmann Coulter J-20 XP	Beckman Coulter, Krefeld
Centrifuge Beckmann Coulter J-26 XPI	Beckman Coulter, Krefeld
Centrifuge Sorvall Evolution RC	Thermo Fisher Scientific Inc., Waltham, USA
CO_2-Incubator HeraCell 150	Thermo Fisher Scientific Inc., Waltham, USA
Douncer	Sartorius, Göttingen
Eickemeyer NarKoVet	Eickemeyer, Tuttlingen
FACS Calibur™	Becton Dickinson, Heidelberg
FACSAria™	Becton Dickinson, Heidelberg
Freezer Forma -86C, Model 8695	Thermo Fisher Scientific Inc., Waltham, USA
Fridge “öko plus”	Siemens, München
Fridge easy store	Siemens, München
Fridges (4°C)	Liebherr, Ochsenhausen
Gene Pulser™	Bio-Rad, München
GeneAmp®PCR System 9700	Applied Biosystems, Foster City, USA
Incubation shaker Certomat® BS-1	Sartorius, Göttingen
Incubation shaker ISF-1-W	Kühner, Adolf AG, Birsfelden, Switzerland
Incubator B5060E	Heraeus, Hanau
Incubator B6420	Heraeus, Hanau

Infra Red light IR 11	Petra electric, Burgau
LightCycler 2.0	Roche Diagnostics, Indianapolis, USA
Magnetic Stirrer RCT basic	IKA Labortechnik, Staufen
Microscope, Axiovert 25	Zeiss, Jena
Microwave	Panasonic, Osaka, Japan
Midi MACS Magnets and Stand	Miltenyi Biotec, Bergisch Gladbach
Multifuge 3 S-R	Hereaus, Hanau
Multipipette	Eppendorf, Hamburg
Nanodrop®ND-1000 Spectrophotometer	Nanodrop, Steinfurt
OXYMAT®3	Weinmann, Hamburg
PCR machineTGradient	Biometra, Göttingen
pH meter 430	Corning, Miami, USA
Pipette Helper	Hirschmann Laborgeräte, Eberstadt
Pipettes	Eppendorf, Hamburg
Pipettes	Gilson, Middleton, USA
Power supply EPS 200	PharmaciaBiotech, Freiburg
Pulse Controller	Bio-Rad, München
Table centrifuge 5417C	Eppendorf, Hamburg
Table centrifuge 5417R	Eppendorf, Hamburg
Table centrifuge Biofugepico	Heraeus, Hanau
Thermomixer 5436	Eppendorf, Hamburg
TissueLyser	Qiagen, Hilden
Ultracentrifuge Optima l-80 XP	Beckman Coulter, Krefeld
Universal Hood II	Biorad, Segrate, Italy
UV-cross-linker	Stratagene, Amsterdam, Netherlands
Vortex Mixer	Bender/Hobein, Bruchsal

Water bath GFL 1002	GFL, Burgwede
Water bath GFL 1092	GFL, Burgwede

2.1.2 Expandable items

Item	Company
96-well V-bottom dish	Nunc, Langenselbold
Cell culture dishes (15 cm, 10 cm, 6 cm)	Becton Dickinson, Heidelberg
Cell culture well plates (6-, 48-, 96-well)	Becton Dickinson, Heidelberg
Cell scrapers (25 cm, 39 cm)	Sarstedt, Nümbrecht
Cell strainer	Becton Dickinson, Heidelberg
Combitips plus (2.5 ml, 5 ml, 10 ml)	Eppendorf, Hamburg
Cuvettes	Brand, Wertheim
Electroporation cuvettes, 2 mm	Bio-Rad, München
Examination gloves	Unigloves, Troisdorf
Falcons conical tubes (15 ml, 50 ml)	Becton Dickinson, Heidelberg
Inoculationloops	Nunc, Langenselbold
Parafilm	Roth, Karlsruhe
PCR Softstrips	Biozym, Oldendorf
Pipettes (2 ml, 5 ml, 10 ml, 25 ml)	Sarstedt, Nümbrecht
Reaction tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg
SafeSeal-Tips®	Biozym, Oldendorf
Sterile injection-needles, Microlance™	Becton Dickinson, Heidelberg
Syringe Injekt® Solo 20 ml	B. Braun Melsungen AG, Melsungen
Syringe TBC 1 ml	Dispomed Witt oHG, Gelnhausen
Tubes for Ultracentrifugation	BeckmanCoulter, Krefeld
Polysterene round-bottom tube w/ cell strainer cap	Becton Dickinson, Heidelberg

2.1.3 Chemicals and biochemicals

2.1.3.1 Chemicals

Chemical	Company
Agarose	Invitrogen, Karlsruhe
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Taufkirchen
Ammonium sulphate ($(NH_4)_2SO_4$)	Baker, Deventer, Netherlands
Boric acid (H_3BO_3)	Roth, Karlsruhe
Bromphenol blue	Sigma-Aldrich, Taufkirchen
Carboxymethylcelullose	Sigma-Aldrich, Taufkirchen
D-(+)-Galactose	Sigma-Aldrich, Taufkirchen
D-(+)-Saccharose	Merck, Darmstadt
D-Biotine	Sigma-Aldrich, Taufkirchen
DMSO (Dimethyl sulfoxide)	Merck, Darmstadt
DOG (2-deoxy-galactose)	Sigma-Aldrich, Taufkirchen
EDTA (Ethylenediaminetetraacetic acid)	Sigma-Aldrich, Taufkirchen
Ethanol	Merck, Darmstadt
Ethidiumbromide	Roth, Karlsruhe
FTY720 (Fingilimod)	Merck, Darmstadt
Glutamate	Invitrogen, Karlsruhe
Glycerol	Roth, Karlsruhe
Hydrochloric acid (HCl)	Roth, Karlsruhe
Iron(II) sulfate ($FeSO_4 * 7H_2O$)	Sigma-Aldrich, Taufkirchen
Isopropanol	Merck, Darmstadt
Korsolex®	Bode Chemie, Hamburg
L-Leucin	Sigma-Aldrich, Taufkirchen
Magnesium sulfate ($MgSO_4 * 7H_2O$)	Merck, Darmstadt

Methanol	Roth, Karlsruhe
Orange G	Sigma-Aldrich, Taufkirchen
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich, Taufkirchen
Potassium chloride (KCl)	Sigma-Aldrich, Taufkirchen
Potassium dihydrogenphosphate (KH_2PO_4)	Merck, Darmstadt
Potassium hydroxid (KOH)	Merck, Darmstadt
Roti®-Phenol/C/I (Phenol/Chloroform/Isoamylalcohol 25/24/1)	Roth, Karlsruhe
Sodium bicarbonate ($NaHCO_3$)	Pan Biotech GmbH, Aidenbach
Sodium chloride (NaCl)	Merck, Darmstadt
Sodium hydrogenphosphate (Na_2HPO_4)	Merck, Darmstadt
Tris-HCl (Tris-(hydroxymethyl)-aminomethane)	Roth, Karlsruhe
β-Mercaptoethanol	Pan Biotech GmbH, Aidenbach

2.1.3.2 Biochemicals

Biochemical	Company
1 kb ladder GeneRuler™	Fermentas, St. Leonroth
6x Agarose gel loading buffer	Fermentas, St. Leonroth
Ampicillin	Sigma-Aldrich, Taufkirchen
Bacillol AF	Bode Chemie, Hamburg
Bacto™ YeastExtract	Becton Dickinson, Heidelberg
Bacto™Agar	Becton Dickinson, Heidelberg
Bacto™Tryptone	Becton Dickinson, Heidelberg
BSA (albumin from bovine serum)	Sigma-Aldrich, Taufkirchen
Chloramphenicol	Sigma-Aldrich, Taufkirchen
Difco MacConkey Agar Base	Becton Dickinson, Heidelberg
Diphteria toxin (DT)	Merck, Darmstadt

dNTPs, Roti-Mix® PCR3	Roth, Karlsruhe
Gentamycin	Invitrogen, Karlsruhe
Granulocyte / macrophage colony stimulating factor (GM-CSF)	PeproTech, Hamburg
Isofluran CP	CP Pharma, Burgdorf
Kanamycin	Sigma-Aldrich, Taufkirchen
Pertussis toxin (PTX)	Sigma-Aldrich, Taufkirchen
Stem cell factor (SCF)	PeproTech, Hamburg
RNAlater®	Sigma-Aldrich, Taufkirchen
SuperFect®	Qiagen, Hilden
Trypan blue	Sigma-Aldrich, Taufkirchen
Trypsin/EDTA	Invitrogen, Karlsruhe

2.3.1.3 Enzymes

Enzyme	Company
ApaLI	NEB, Ipswich, USA
AseI	NEB, Ipswich, USA
BamHI	NEB, Ipswich, USA
DNaseI	Roche Diagnostics, USA
DpnI	NEB, Ipswich, USA
EcoRI	NEB, Ipswich, USA
EcoRV	NEB, Ipswich, USA
HindIII	NEB, Ipswich, USA
NcoI	NEB, Ipswich, USA
PvuII	NEB, Ipswich, USA
RNase	Sigma-Aldrich, Taufkirchen
SalI	NEB, Ipswich, USA

ScaI	NEB, Ipswich, USA
SuperScript® III Reverse Transcriptase	Invitrogen, Karlsruhe
Taq DNA Polymerase High Fidelity	Roche Diagnostics, Grenzach-Wyhlen

2.1.4 Buffer and media

2.1.4.1 Buffer

DPBS (Dulbecco's phosphate buffered saline) was purchased from Invitrogen, Karlsruhe.

10x DNA running buffer:	50 mg	Bromphenol blue
	3 ml	150 mM Tris pH 7.6
	60 ml	Glycerol
	7 ml	H_2Odd
FACS-buffer:	1 %	FCS in DPBS
10x Orange G running buffer:	50 mg	Orange G
	500 µl	1 M Tris
	15 ml	Glycerol
	35 ml	H_2Odd
Red Blood Cell Lysis Buffer:	4.14 g	Ammonium chloride (NH_4Cl)
	0.5 g	Potassium bicarbonate ($KHCO_3$)
	0.1 ml	0.5 M EDTA, pH 8.0
		1 N Hydrochloric acid (HCl) for adjusting to pH 7.2-7.4
	ad 500 ml	H_2O
50x TAE:	42 g	Tris-HCl

		100 ml	0.5 M EDTA, pH 8.0
	ad	1 l	H_2O
1x TBE		10.8 g	Tris-HCl
		5.5 g	Boric acid (H_3BO_3)
		0.7 g	EDTA, pH 8.0
	ad	1 l	H_2O
10x TE-Buffer:		10 ml	1M Tris-HCl, pH 7.5
		2 ml	0.5 M EDTA, pH 8.0
	ad	1 l	H_2O
1x Virusstock-buffer:		6.055 g	Tris-HCl
		0.895 g	Potassium chloride (KCl)
		1.86 g	EDTA
			1 N Hydrochloric acid (HCl) for adjusting to pH 7.8
	ad	1 l	H_2O
1x Virusstock-Buffer 15% Succrose:		75 g	D-(+)-Saccharose
		500 ml	Virusstock-buffer

2.1.4.2 Media for the culture of prokaryotic cells

LB-medium:		10 g	Bacto™Tryptone
		5 g	Bacto™YeastExtract
		10 g	Sodium chloride (NaCl)
	ad	1 l	H_2Odd

M9 medium:		6 g	Sodium hydrogenphosphate (Na_2HPO_4)
		3 g	Potassium dihydrogenphosphate (KH_2PO_4)
		1 g	Ammonium chloride (NH_4Cl)
		0.5 g	Sodium chloride (NaCl)
	ad	1 l	H_2O
5x M63 salts:		10 g	Ammonium sulphate ($(NH_4)_2SO_4$)
		68 g	Potassium dihydrogenphosphate (KH_2PO_4)
		2.5 mg	Iron(III) sulphate ($FeSO_4 * 7H_2O$)
			Potassium hydroxide (KOH) for adjusting to pH 7
	ad	1 l	H_2O
LB-agar plates:		1 l	LB-medium
		15 g	Bacto™Agar
MacConkey plates:		10 g	Difco MacConkey agar base
		225 ml	H_2Odd
		25 ml	10% Galactose
M63 minimal plates:		4 g	Bacto™Agar
		200 ml	H_2Odd
		50 ml	5x M63 salts
		0.5 ml	1 M Magnesium sulphate ($MgSO_4$)
		1.25 ml	0.2 mg / ml D-Biotine
		1.1 ml	10 mg / ml L-Leucine
		5 ml	10% Glycerol

5 ml 10% DOG (2-deoxy-galactose)

Liquid medium as well as plates were stored at 4°C.

Plates and media containing antibiotics were prepared by diluting the antibiotics to final concentrations as listed below:

Antibiotic	stock concentration	final concentration
Ampicillin	100 mg / ml	100 µg / ml
Chloramphenicol	25 mg / ml	25 µg / ml
Kanamycin	100 mg / ml	50 µg / ml

2.1.4.3 Media for the culture of eukaryotic cells

RPMI 1640 medium, DMEM medium, Fetal Calf Serum (FCS), Penicillin / Streptomycin, 10x Minimal essential medium (10x MEM) and 10x Non-essential amino acids (10x NEAA) were purchased from Invitrogen, Karlsruhe. Media were prepared as follows:

RPMI++:
500 ml RPMI 1640
50 ml FCS
5 ml Penicillin / Streptomycin

RPMI++++:
500 ml RPMI 1640
50 ml FCS
5 ml Penicillin / Streptomycin
5 ml Glutamate
500 µl β-Mercaptoethanol

DMEM++:
500 ml DMEM
50 ml FCS

	5 ml	Penicillin / Streptomycin
Methylcellulose medium:	3.75 g	Carboxymethylcellulose
	388 ml	H_2Odd
	25 ml	FCS
	50 ml	10x Minimal essential medium (10x MEM)
	5 ml	Glutamine
	2.5 ml	10x Non-essential amino acids (10x NEAA)
	5 ml	Penicillin / Streptomycin
	24.7 ml	Sodium bicarbonate ($NaHCO_3$)

2.1.5 Kits

Name	Company
Expand High Fidelity PCR System	Roche Diagnostics, Grenzach-Wyhlen
GFX PCR DNA and Gel Band Purification Kit	GE Healthcare, Freiburg
Mouse erythrocyte lysing kit	R&D systems, Minneapolis, USA
NK cell isolation kit	Miltenyi Biotech, Bergisch Gladbach
NucleoBond™Xtra Midi Kit	Macherey-Nagel, Düren
QuantiTect SYBR Green PCR Kit	Qiagen, Hilden
QuantiTect SYBR Green PCR Master Mix	Qiagen, Hilden
Quick Ligation™ Kit	NEB, Ipswich, USA
RNeasy Mini Kit	Qiagen, Hilden
Venor®GeM – Mycoplasma detection kit	Minerva biolabs, Berlin

2.1.6 Bacterial artificial chromosomes (BACs) and vectors

The vector pgalK-Kn was present in the laboratory (EMBL Acc. number FR832405).

All MCMV mutants were generated on the basis of BAC pSM3fr originally published by Messerle and colleagues (Messerle *et al.* 1997).

2.1.7 Primer and oligonucleotides

All primers and oligonucleotides were purchased from 'Metabion international AG', Planegg-Martinsried.

2.1.7.1 Primer and oligonucleotides for generation of the BAC targeting construct

Name	Sequence
P-5'-Oligo-Hm157	5'-TCGACTGGCCACACACGTGGTCAAGCCGGTCGTGTTGTACCAGAACTCGACTTCGGTCGCGTTGATATCAAGCGGCCGCGTCAAGAGGTACTGAATATCGGGGTACACTTTCTCAAATATCGGGGTTCAGCTGG-3'
P-3'-Oligo-Hm157	5'-GATTCCCAGCTGAACCCCGATATTTGAGAAAGTGTACCCCGATATTCAGTACCTCTTGACGCGGCCGCTTGATATCAACGCGACCGAAGTCGAGTTCGTGTACAACACGACCGGCTTGACCACGTGTGTGGCCAG-3'
P-5'-Oligo-m157-loxP	5'-ATCAATAACTTCGTATAGCATACATTATACGAAGTTATTATCAACCATGGATAACTTCGTATAGCATACATTATACGAAGTTATGC-3'
P-3'-Oligo-m157-loxP	5'-GGCCGCATAACTTCGTATAATGTATGCTATACGAAGTTATCCATGGTTGATAATAACTTCGTATAATGTATGCTATACGAAGTTATTGAT-3'
P-5'-m157ampl	5'-TAAGTACTCCATGGTCAAACGACCAGACGCATAAA-3'
P-3'-m157ampl	5'-AGGATCCCCATGGATGGTCATCGTCCCCCTAGT-3'
P-5'-Cterm-m157	5'-TAAGTACTCAGCTGCCGAAGTCACGACCGTCAGT-3'
P-3'-Cterm-m157	5'-AGATATCAACGCGACCGAAGTCGAGTT-3'
P-5'-not recombined	5'-CGAACTGACATCCGGACAG-3'
P-5'-recombined	5'-TTGCCGGGAAGCTAGAGTAA-3'

P-3'-universal	5'-ATGGCTCATAACACCCCTTG-3'

2.1.7.2 Primer for BAC targeting

Complementary sequences for the GalK-kan cassette are italicized.

Name	Sequence
P-5'-m156/157-GalK	5'-CCATTATCACCAAGATAGTTCCCACCATAATTCCCATC GTCACTAGAGTC*CCTGTTGACAATTAATCATCGGCA*-3'
P-3'-Δm157-GalK	5'-CCCGATATTTGAGAAAGTGTACCCCGATATTCAGTAC CTCTTGACTAA*GCCAGTGTTACAACCAATTAACC*-3'
P-5'-m157sequ	5'-AGATAGTTCCCACCATAATT-3'
P-3'-m157sequ	5'-GTGTGAAACGCAGGAGAATC-3'

2.1.7.3 Primer for quantitative RT-PCR

Name	Sequence
Cxcl12_fwd	5'-CGCCAAGGTCGTCGCCG-3'
Cxcl12_rev	5'-TTGGCTCTGGCGATGTGGC-3'
Kitl_fwd	5'-CCCTGAAGACTCGGGCCTA-3'
Kitl_rev	5'-CAATTACAAGCGAAATGAGAGCC-3'
Vcam1_fwd	5'-GACCTGTTCCAGCGAGGGTCTA-3'
Vcam1_rev	5'-CTTCCATCCTCATAGCAATTAAGGTG-3'
LBR_for	5'-GGAAGTTTGTTGAGGGTGAAGTGGT-3'
LBR_rev	5'-CCAGTTCGGTGCCATCTTTGTATTT-3'
S1PL_for	5'-TCTGCTGATAGTCTGGGTGTATGAG-3'
S1PL_rev	5'-CCAATAAATGGCATCTTCCTGATA-3'

S1PR-for	5’-CGGTGTAGACCCAGAGTCCT-3’
S1PR-rev	5’-AGCTTTTCCTTGGCTGGAG-3’

2.1.7.4 Immunostimulatory oligonucleotides

Immunostimulatory oligonucleotides were purchased as thioates from TIB MOLBIOL, Berlin. CpG motifs marked in bold.

Name	Sequence
CpG-ODN 1826	5’-TCC ATG A**CG** TTC CTG A**CG** TT-3’

2.1.8 Antibodies

2.1.8.1 Antibodies for flow cytometry

Antigen	Recognized species	Isotype	Hybridoma	Conjugate	Dilution used	Company
B220	Mouse / human	Rat IgG2a, κ	RA3-6B2	FITC	1:200	eBioscience
B220	Mouse / human	Rat IgG2a, κ	RA3-6B2	PE	1:200	eBioscience
CD117	mouse	Rat IgG2b, κ	2B8	PerCP-eFluor710	1:200	eBioscience
CD11b	mouse	Rat IgG2b, κ	M1/70	FITC	1:200	eBioscience
CD11c	mouse	Armenian Hamster IgG	N418	APC	1:500	eBioscience
CD19	mouse	Rat IgG2b, κ	eBio1D3	FITC	1:200	eBioscience
CD3e	mouse	Armenian Hamster IgG	145-2C11	FITC	1:200	eBioscience
CD8a	mouse	Rat IgG2b, κ	53-6.7	PerCP-Cy5.5	1:200	eBioscience

FcRII/III	mouse	Rat IgG2b, κ	2.4G2	Fc-Block	1:100	BD Pharmingen
Gr-1	mouse	Rat IgG2b, κ	RB6-8C5	FITC	1:200	eBioscience
keyhole limpet hemo-cyanin	nil	Isotype-control rat IgG2a		PE	1:200	eBioscience
keyhole limpet hemo-cyanin	nil	Isotype-control rat IgG2b, κ		PE	1:200	eBioscience
MHC class II	mouse	Rat IgG2b, κ	M5/114.15.2	FITC	1:200	eBioscience
NK1.1	mouse	Mouse IgG2a, κ	PK136	PE	1:200	eBioscience
Sca-1	mouse	Rat IgG2b, κ	D7	APC	1:200	eBioscience
TER119	mouse	Rat IgG2b, κ	TER119	PE	1:200	eBioscience

2.1.8.2 Antibodies for *in vivo* treatments

Name	Company
Anti-asialo GM1	Wako Chemicals, Neuss
Enbrel (Etanercept)	Wyeth Pharma, Münster

2.1.9 Organisms and viruses

2.1.9.1 Bacteria

Engineering of BACs was performed in *E. coli* strain SW102 (Warming *et al.* 2005).

Listeria monocytogenes strain EGD and *Listeria monocytogenes* ΔactA were a kind gift of Werner Göbel, Max von Pettenkofer-Institute, Ludwig-Maximilians-Universität München.

2.1.9.2 Cells

For reconstitution of virus particles murine embryonal fibroblasts (MEFs) of BALB/c mice were transfected (Serrano *et al.* 1997).

For further virusstock preparation the cell line M2-10B4 (bone marrow stromal cells) from (C57BL/6J x C3H/HeJ) F1 mice was used (ATCC® number: CRL-1972, LGC Standards GmbH, Wesel).

The M94-complementing cell line NT/M94-7 was used to propagate ΔM94-MCMV (Mohr *et al.* 2010).

All cells were grown at 37°C, 7% CO_2.

2.1.9.3 Mice

Female C57BL/6 mice were purchased from Elevage Janvier (Le Genest Saint Isle, France). Cre-transgenic strains Tie2-cre (Constien *et al.* 2001), Alb-cre (Postic *et al.* 1999), CD19-cre (Rickert *et al.* 1997) and CD11c-cre (Caton *et al.* 2007), as well as $IFNGR^{-/-}$ animals (Huang *et al.* 1993) were bred at the Max von Pettenkofer-Institute, ΔDC mice (Ohnmacht *et al.* 2009) at the Institute for Immunology (Ludwig-Maximilians-Universität München), SCID and $Prf1^{-/-}$ mice (Kagi *et al.* 1994) at the Department for Histology and Embryology (University of Rijeka). NKp46-DTR → C57BL/6 bone marrow chimeras were generated at the Centre d'Immunologie de Marseille-Luminy (Université de la Méditerranée).

All transgenic and knockout mice were maintained on the C57BL/6 background, except $IFNGR^{-/-}$ mice that were bred on 129 background.

2.1.9.4 Viruses

The construction of Δm157-MCMV and ΔM94-MCMV mutants as well as pSM3fr with a restored full-length MCK-2/m129 open reading frame (ORF) (3.3) was described elsewhere (Bubic *et al.* 2004; Mohr *et al.* 2010; Jordan *et al.* 2011).

Lymphocytic choriomeningitis virus (LCMV) strain WE and murine herpesvirus 68 (MHV-68) were obtained from David Voehringer, University of Erlangen, and Heiko Adler, Helmholtz Center Munich, respectively (Lehmann-Grube 1971; Flach *et al.* 2009).

2.1.10 Computer Software

Software	Company
BD CellQuest Pro™	BD Bioscience, Heidelberg
BD FACSDiva™	BD Bioscience, Heidelberg
EndNote X4	Thomson Reuters, New York City, USA
Flow Jo	Tree Star Inc., Ashland, USA
Microsoft Office	Microsoft, Unterschleißheim
Prism 5	GraphPad Software, La Jolla, USA
Vector NTI Suite 8	Invitrogen, Karlsruhe

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Basic tools for molecular genetic approaches

DNA-purifications were performed using the kits from GE Healthcare or Macherey-Nagel following the manufacturer's instructions. The DNA concentration of plasmid vector and BAC preparations was determined with a Nanodrop®ND-1000 spectrophotometer.

2.2.1.2 Generation of conditional virus mutant MCMV-floxed-m157 and control MCMV-recΔm157

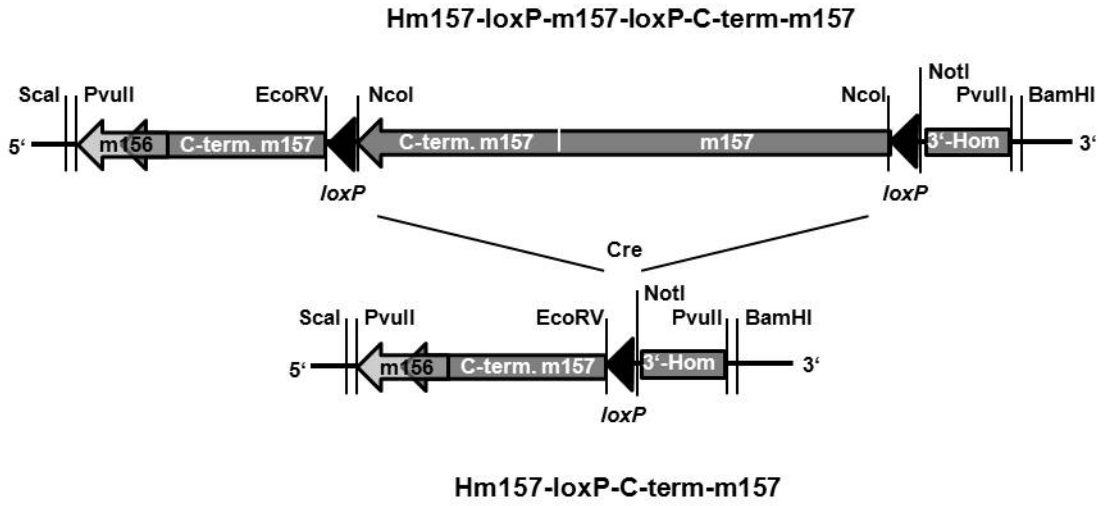

Figure 2.2 Scheme of the genetic constructs generated for BAC targeting.
ORF *m157* was flanked with two *loxP*-sites in direct orientation. The C-terminus of m157 was doubled since it probably contains promoter elements of ORF *m156*. Cre-mediated recombination results in deletion of m157 except the C-terminus. 3'-Hom = sequence homology 3' of *m157* for homologous BAC recombination.

MCMV open reading frames (ORFs) *m157* and *m156* are partly overlapping and promoter elements of *m156* are probably located within ORF *m157*. To preserve the expression of m156, 200 bp of the C-terminus of m157 were doubled and two *loxP*-sites flanking the ORF were introduced in direct orientation to allow cre-mediated excision of *m157*. Cre-recombination of the MCMV-floxed-m157 virus genome would therefore result in deletion of m157, except its C-terminal coding region (Figure 2.2) (Sacher *et al.* 2008a).

2.2.1.2.1 Generation of constructs for BAC targeting

Synthetic oligonucleotides P-5'-Oligo-Hm157 and P-3'-Oligo-Hm157 containing 50 bp homology each upstream and downstream of the *m157*-sequence to be replaced were annealed and inserted between the *ScaI* and *BamHI* sites of vector pgalK-Kn giving rise to vector pGK-Hm157. pGK-Hm157 was digested with NotI and EcoRV and two *loxP*-sites in direct

orientation were introduced using the synthetic oligonucleotides P-5'-Oligo-m157-loxP and P-3'-Oligo-m157-loxP resulting in the vector pGK-Hm157-loxP-loxP.

ORF *m157* was amplified from BAC pSM3fr using the primers P-5'-m157ampl and P-3'-m157ampl containing the restriction sites for NcoI. The 990 bp fragment was inserted into the corresponding site of pGK-Hm157-loxP-loxP (pGK-Hm157-loxP-m157-loxP). Finally, a 995 bp fragment encompassing 200 bp of the C-terminus of *m157* was amplified using the primers P-5'-Cterm-m157 and P-3'-Cterm-m157 and cloned into pGK-Hm157-loxP-m157-loxP via *Sca*I and *EcoR*V, resulting in the vector pGK-Hm157-loxP-m157-loxP-C-term-m157. To generate the already recombined construct for MCMV-recΔm157, pGK-Hm157-loxP-m157-loxP-C-term-m157 was incubated with cre-recombinase. Bacterial colonies after transformation were checked by PCR with primers P-3'-universal, P-5'-not recombined (product 460 bp) and P-5'-recombined (product 979 bp) for successful cre-recombination (pGK-Hm157-loxP-C-term-m157).

Vectors pGK-Hm157-loxP-m157-loxP-C-term-m157 and pGK-Hm157-loxP-C-term-m157 were both digested with PvuII to generate the targeting constructs.

2.2.1.2.2 BAC targeting

BAC targeting was performed with a modified method based on homologous BAC recombination (Warming *et al.* 2005).

In a first step, endogenous ORF *m157* had to be replaced by a GalK-kan cassette. Therefore, a 2197 bp GalK-kan sequence was amplified from pgalK-Kn with primers P-5'-m156/157-GalK and P-3'-Δm157-GalK using the following touchdown PCR conditions:

1.	94°C	5 min.	
2.	94°C	1 min.	18x, every cycle the temperature in step 3 decreased by 1°C
3.	62°C	2 min.	
4.	68°C	1.5 min.	
5.	95°C	1 min.	16x
6.	45°C	2 min.	
7.	68°C	1.5 min.	
8.	68°C	10 min.	
9.	4°C		

The PCR-product was purified and digested with DpnI for at least 2 hours at 37°C to remove template vector sequences.

Prior to BAC targeting, safety pipet tips, double destilled water, 2 ml reaction tubes and electroporation cuvettes were precooled at 4°C. 500 µl of an overnight culture were used to inoculate 25 ml LB-medium containing chloramphenicol with SW102 bacteria harbouring BAC pSM3fr. The culture was grown at 32°C to an OD_{600} 0.55-0.6. Then, 10 ml culture was transferred into a new flask and incubated at 42°C in a shaking water bath for 15 minutes to induce expression of the recombinase. Afterwards, the culture was immediately cooled on ice and all following steps were performed at 4°C. 2 ml of bacteria were pelletized at 5,500xg for 5 minutes, the supernatant was discarded and the step repeated. Next, the bacteria were carefully re-suspended in 1 ml ice-cold water by snapping against the tube. 1 ml water was added and the bacteria were pelletized again. This washing step was performed 3 times. Finally, the bacterial pellet was taken up in 60 µl water and the precooled GalK-kan PCR-product was added. The mixture was transferred to an electroporation cuvette and pulsed with a 2.5 kV, 200 Ω and 25 µF electric shock. The bacteria were transferred into 1 ml LB-medium and grown for 1 hour at 32°C. 100 µl and 10 µl of the culture were then plated on agar plates containing chloramphenicol and kanamycin. Growing colonies were used to inoculate 10 ml LB-medium containing chloramphenicol and kanamycin. The BAC-DNA was prepared and successful recombination and BAC-integrity were checked by restriction

enzyme digestion with AseI and analysis on a 0.8% agarose gel in TBE buffer. The whole procedure gave rise to BAC pSM3fr-Δm157-GalK-kan.

In a second step, the GalK-kan cassette was replaced by the 1624 bp and 634 bp PvuII fragments of pGK-Hm157-loxP-m157-loxP-C-term-m157 and pGK-Hm157-loxP-C-term-m157, respectively. To this aim, the same protocol as for the introduction of the GalK-kan cassette was used. After washing the electrocompetent bacteria, 1.5 µg of the purified restriction enzyme digests of the targeting constructs were added, the bacteria pulsed and grown in 10 ml LB-medium in a 50 ml flask for 4.5 hours at 32°C. 1 ml culture was pelletized at 14,000xg for 15 seconds, the supernatant was carefully removed by pipetting and the pellet washed twice in 1 ml M9 Medium. Serial dilutions were plated on M63 minimal dishes and incubated at 32°C for 3 to 4 days. Colonies were then streaked on MacConkey agar plates and incubated for at least 2 days. White colonies, i.e. GalK-negative clones, were streaked on LB-agar plates to remove passenger colonies. This step was repeated at least twice. Then, 10 ml LB-cultures were inoculated to check for replacement of the GalK-kan cassette with the targeting constructs by restriction digestion. Finally, the successful construction of pSM3fr-Hm157-loxP-m157-loxP-C-term-m157 (= MCMV-floxed-m157) and pSM3fr-Hm157-loxP-C-term-m157 (= MCMV-recΔm157) was confirmed by sequencing with primers P5'-m157sequ and P3'-m157sequ.

2.2.1.2 RNA isolation, reverse transcription and quantitative RT-PCR

Immediately after removal of the spleen from the animal a small piece was cut and stored in RNAlater at 4°C until RNA preparation was performed. Prior to RNA preparation, the piece of spleen was removed from RNAlater® and disrupted using a TissueLyser device. RNA was extracted from spleens using the RNeasy mini kit according to the manufacturer´s instruction. 500 ng of total RNA was reverse transcribed using SuperScript III and oligo-dT primers

following the manufacturer´s instructions. PCR was performed on a LightCycler. Each reaction was carried out using 5 µl of cDNA (1:10 dilution), 15 µl reaction mixtures of Quantitect SYBR Green PCR master mix and primers at a concentration of 0.5 µM. PCRs were subjected to 10 minutes of 95°C hot-start and Sybr Green incorporation was monitored for 45 cycles of 95°C denaturation for 10 seconds, 58°C annealing for 3 seconds, and 72°C elongation for 10 seconds. The data was analyzed using the ΔΔCt method and laminin B receptor (LBR) as an endogenous reference, and the mock-infected sample as a calibrator.

2.2.2 Microbiology

2.2.2.1 Bacteriology

2.2.2.1.1 Culture of *Listeria monocytogenes* for *in vitro* and *in vivo* infection

20 ml BHI medium were inoculated with 400 µl overnight culture and bacteria were grown to an OD_{600} 1.0. Cells were pelletized at 7,000xg for 10 minutes and washed twice with ice-cold DPBS. Finally, the bacteria were re-suspended in DPBS with 20% glycerol, aliquoted and frozen at -80°C. To determine the colony forming units (CFU) / ml, a serial dilution was prepared and 100 µl of each dilution was streaked in duplicates on BHI-plates. Plates were incubated overnight at 37°C. CFU were calculated from plates with more than 100 colonies.

2.2.2.2 Virology

2.2.2.2.1 Virus reconstitution from BACs

Mouse embryo fibroblasts (MEFs) were transfected with pSM3fr-Hm157-loxP-m157-loxP-C-term-m157 and pSM3fr-Hm157-loxP-C-term-m157 to reconstitute MCMV-floxed-m157 and MCMV-recΔm157. MEF cells from a 10 cm dish were split onto six 6 cm dishes one day prior to transfection. All safety-tips were cut to avoid shearing of the BAC DNA. 1,500 ng BAC DNA were added to 150 µl DMEM (without FCS and Penicillin / Streptomycin) and

mixed carefully. 10 µl ice-cold SuperFect® were added and the mixture was incubated for 10 to 15 minutes at room temperature. Meanwhile, MEF cells were washed with 3 ml DPBS. 1 ml DMEM++ was added to the transfection mix and distributed on the MEF cells. The cells were grown for 2.5 h at 37°C. Afterwards, the transfection medium was replaced by fresh DMEM++. 3 days after transfection the cells were split onto 10 cm dishes. Thereafter, the culture was split every three days until complete lysis due to virus replication.

2.2.2.2.2 Preparation of virusstocks

10 ml supernatant from virus reconstitution were mixed with M2-10B4 cells and plated on a 15 cm dish. After lysis of the cells, the supernatant was harvested and titrated.

For virusstock production, 10^8 M2-10B4 cells were infected with an MOI 0.1. After 4 days remaining cells and supernatant were harvested on ice and centrifuged for 15 minutes at 5,500xg, 4°C. The supernatant was stored on ice and an aliquot was taken to test for contamination with mycoplasma by PCR. The pellet was re-suspended in 2 ml of the stored supernatant and three freeze-thaw cycles were performed. Afterwards, this fraction was centrifuged at 12,000 rpm for 10 minutes. Both virus particle containing supernatants were combined and centrifuged for 3.5 to 4 hours at 20,000xg and 4°C. The supernatant was discarded and the pellet re-suspended in 2 ml virusstock-buffer using a douncer. After further virus purification over a 15%-Sucrose cushion at 20,000 rpm for 1:20 hours, the pellet was covered with 400 µl virusstock-buffer and stored over night at 4°C. The next day, the pellet was re-suspended again using a douncer and remaining cell debris was removed by repeated centrifugation at 3,500 rpm for 2 minutes. The virus suspension was aliquoted and stored at -80°C.

2.2.2.2.3 Virus quantification by standard plaque titration assay

To determine the virus titer, MEF cells were plated on a 48-well dish one day prior to the assay. The virus samples were diluted serially ($10^{-1}$to 10^{-6}) in DMEM++ on 48-well plates in quadruplicades and 200 µl of each dilution was transferred onto the MEFs. After incubation for one hour at 37°C the medium were removed and the MEFs were covered with 500 µl methylcellulose medium. Plaques were counted 4 days later and viral titer was calculated:

$$\text{Virus titer } (\text{PFUml}^{-1}) = \frac{\text{counted plaques x dilution factor}}{\text{volume of virus dilution}}$$ (Dulbecco and Vogt 1953).

2.2.2.2.4 UV inactivation of viruses

Virus was inactivated by exposure to 1.5 kJ/cm^2 UV-light at a distance of 5 cm in a UV-cross-linker at 4°C. Inactivation was controlled by titration on MEFs.

2.2.3 Immunology

2.2.3.1 Cellular Immunology

2.2.3.1.1 Generation of bone marrow derived dendritic cells (BMDC)

Bone marrow-derived dendritic cells (BMDC) were prepared on the basis of a protocol by Lutz and colleagues (Lutz *et al.* 1999). Mice were sacrificed by cervical dislocation, femora and tibiae were removed and cleaned of muscle tissue, disinfected for 3 minutes in 70% ethanol and then rinsed with DPBS. The bones were cut with scissors at the epiphyses and the marrow was flushed with DPBS using a sterile 27-gauge needle. After passing the marrow through a cell strainer, cells were pelletized at 1,300 rpm for 7 minutes and erythrocytes lysed with sterile Red Blood Cell Lysing Buffer for one minute at room temperature. The cells were washed once and plated at a density of 5x10^5 cells / ml in 10 ml RPMI++++ supported with 200 U / ml GM-CSF on 10 cm diameter Petri dishes. At day 3 of culture another 10 ml

medium with 200 U / ml GM-CSF were added. After six days 10 ml of cell culture were removed, cells pelletized at 1,300 rpm for 7 minutes, re-suspended in 10 ml fresh medium containing 200 U / ml GM-CSF and retransferred to the remaining culture. At day 7 cells were usually used for further experiments. Successful generation of BMDC was controlled by staining for CD11c and MHC class II expression and subsequent flow cytometry (Fluorescence Activated Cell Sorting, FACS).

2.2.3.1.2 *In vitro* infection of BMDC with *Listeria monocytogenes*

BMDC were infected with *Listeria monocytogenes* following a protocol by Neuenhahn and colleagues (Neuenhahn *et al.* 2006). BMDC were harvested and washed extensively by three times repeated centrifugation and re-suspension in antibiotic-free RPMI++++. Subsequently, the cells were incubated for another 4 to 5 hours in antibiotic-free RPMI++++ before they were infected for 60 minutes with *Listeria monocytogenes* ΔactA at an MOI 10. Afterwards, infected BMDC were washed and incubated for another hour in medium containing Gentamycin (50 mg / ml) in order to kill remaining extracellular bacteria. Then, cells were washed twice in DPBS and $2x10^6$ infected BMDC were injected per mouse. Uninfected control BMDC were treated as BMDCs to be infected, but DPBS was added instead of bacteria.

2.2.3.1.3 Analysis of cell surface antigens by flow cytometry

Cell populations in the murine spleen were identified on the basis of surface marker expression by FACS. Cells were kept at 4°C unless indicated otherwise. Single cell solutions were prepared by passing the spleen through a 100 µm cell strainer and washing the strainer with 5 ml DPBS. For surface staining, 75 µl cell suspension were transferred to a 96-well V-bottom plate and pelletized. Red blood cells were removed by re-suspending each cell pellet

in 100 µl red blood cell lysis buffer followed by incubation for 3 minutes at room temperature. Remaining cells were collected at 1,300xg for 2 minutes in FACS-buffer. Fcγ-receptors were blocked with anti-FcRII/III monoclonal antibodies for at least 10 minutes to avoid unspecific binding of the staining antibodies. Afterwards, cells were washed and re-suspended in 100 µl FACS-buffer containing the specific fluorochrome-conjugated antibodies (see 2.1.8.1) at the respective dilution and incubated for 20 minutes in the dark. Finally, cells were washed twice and re-suspended in 200 µl FACS-Buffer. FACS acquisition was performed on Becton Dickinson flow cytometers FACSCalibur™ and CantoII™ using the CellQuest Pro™ and the FACSDiva™ software, respectively, for immediate detection and compensation. The obtained FACS-data were analysed using the FlowJo software.

2.2.4 *In vivo* methods

2.2.4.1 Animal housing

Mice were purchased at the age of 7 weeks. They were housed at the animal facility of the Max von Pettenkofer-Institute (Ludwig-Maximilians-Universität München) for at least one week before the experiments.

Wild-type, cre-transgenic and knockout animals were kept under specified-pathogen-free conditions in air-conditioned rooms (45-50% air humidity, 20-22°C) with a 12 hours day-night rhythm. They were housed in individually ventilated cages (IVC) with autoclaved litter and free access to extruded phyto-oestrogen-low germfree feed and autoclaved water. Microbiologic examinations according to the Federation of Laboratory Animal Science Associations (FELASA)-guidelines ensured hygienic standards.

Experiments were performed with sex- and age-matched groups. Animal experiments were approved by the State of Bavaria or by the Ethics Committee of the respective universities.

2.2.4.2 Infection of mice

If not indicated otherwise, infections with MCMV and MCMV mutants were performed with 10^5 PFU, LCMV with 200 PFU, MHV-68 with $5x10^4$ PFU and *Listeria monocytogenes* strain EGD as well as *Listeria monocytogenes* ΔactA with $5x10^3$ CFU injected into the tail vein.

2.2.4.3 CpG-ODN treatment of mice

10 nmol / mouse of CpG-ODN 1826 were dissolved in a volume of 25 µl PBS and injected subcutaneously after anesthesia with isofluran.

2.2.4.4 *In vivo* application of antibodies, toxins and small molecules

NK cells were depleted in C57BL/6 mice by i.p. application of 25 µg anti-asialo GM1 antibody 24 hours before and 3 days after infection. In NKp46-DTR bone marrow chimeras NK cells were depleted by i.p. application of 1 µg diphtheria toxin (DT) one day before and 3 days after infection.

TNF-α was depleted by i.p application of 300 µg Enbrel (Etanercept) 2 hours before and 3 days after infection as described (Plater-Zyberk *et al.* 2009).

Egress of stem cells from the spleen was examined by i.p. application of 1 µg Pertussis toxin (PTX) at day one p.i. or 20 µg FTY720 at days 1, 2 and 3 p.i. (Matloubian *et al.* 2004; Massberg *et al.* 2007).

1 µg stem cell factor (SCF) was applied two times a day i.p. at days 1, 2 and 3.

2.2.4.5 Adoptive transfer of cells

For adoptive transfer of NK cells, spleens from mice infected for 48 hours were harvested and homogenized. Splenocytes were enriched in NK cells up to 80% using an NK cell isolation kit according to the manufacturer's instructions. 10^6 NK cells were transferred at 48 hours p.i.

For adoptive transfer of *Listeria monocytogenes* infected BMDC $2x10^6$ cells were used.

2.2.4.6 Organ harvest

Mice were euthanized in a vessel containing a saturated CO_2 atmosphere. Afterwards, spleens were removed and stored in 1 ml ice-cold PBS for following preparation steps. For quantitative RT-PCR analysis, a small part of the spleen was cut immediately after removal of the spleen and stored in RNAlater® at 4°C until RNA preparation.

2.2.5 Statistical analysis

Statistical analyses were done using Prism 5. For all experiments, the mean values and the standard deviations were calculated. To test for significance either a two-tailed Student's t-test or ANOVA with Bonferroni's test were used according to the data set.

3. Results

3.1 The cytotoxic function of NK cells is required for extramedullary hematopoiesis

3.1.1 MCMV induces extramedullary hematopoiesis in the spleen - experimental conditions

Already in 1981 Lucia and colleagues described extramedullary hematopoiesis in the spleen of mice upon MCMV infection (Lucia and Booss 1981). The animals used in their study, however, were hybrids of the strains C3H/HeJ and DBA/2. To circumvent the breeding of F1 hybrids for this study, it was tested whether extramedullary hematopoiesis occurs in the commonly used C57BL/6 strain as well. Furthermore, studying extramedullary hematopoiesis

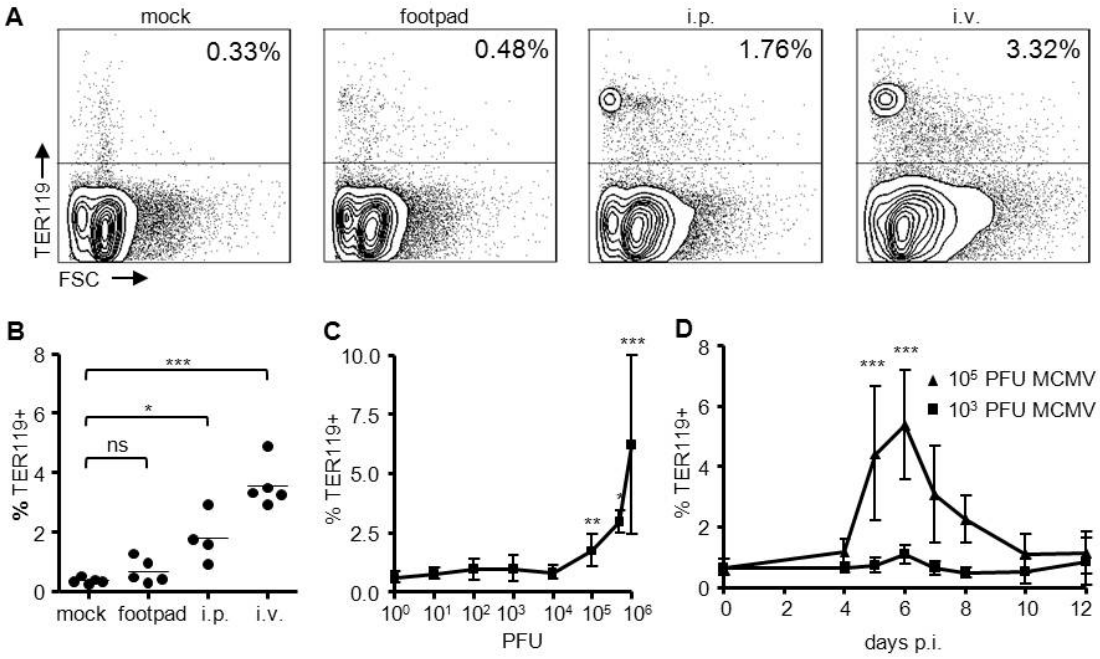

Figure 3.1.1 MCMV induces extramedullary hematopoiesis in C57BL/6 mice.
(A) TER119 expression on splenocytes at day 7 post infection (p.i.) with 10^6 PFU MCMV injected into the footpad, intraperitoneally (i.p.) and intravenously (i.v.). Dot plot of the animal representing the median of n=5 animals is shown. **(B)** Percentage of $TER119^+$ splenocytes in mock, footpad, i.p. and i.v. infected animals at day 7 p.i. Each dot represents an individual animal. Horizontal bar = mean value. **(C)** Percentage of $TER119^+$ splenocytes in mice infected with indicated doses of MCMV i.v. at day 7 p.i. **(D)** Kinetics of extramedullary hematopoiesis in C57BL/6 mice infected with MCMV i.v. Asterisks indicate significant values, * = $p < 0.05$, ** = $p < 0.03$, *** = $p < 0.001$, ns = not significant (one-way ANOVA with Bonferroni's test).

in C57BL/6 mice would allow the use of genetic tools generated on this background.

The appearance of $TER119^+$ cells in the spleen is a characteristic hallmark of extramedullary hematopoiesis in the mouse model (MacNamara *et al.* 2009; Jackson *et al.* 2010). TER119 is a marker commonly found on the surface of late stages of the erythroid lineage from early proerythroblasts to mature erythrocytes (Kina *et al.* 2000). The extent of extramedullary hematopoiesis in the spleen could therefore be measured by quantification of $TER119^+$ splenocytes after MCMV infection using flow cytometry.

C57BL/6 mice were probed for extramedullary hematopoiesis upon MCMV infection using different routes of virus application. MCMV was applied into the footpad, intraperitoneally and intravenously. Intraperitoneal and intravenous infection expanded the $TER119^+$ compartment in the spleen while the infection via the footpad did not lead to a significant increase in the proportion of $TER119^+$ cells (Figure 3.1.1A, B). The highest increase in the percentage of $TER119^+$ cells was observed after intravenous infection. Next, intravenous infections with 1 to 10^6 PFU MCMV were performed. At low infection doses (1 to 10^4 PFU) no significant increase in $TER119^+$ cells could be detected at day 7 post infection (p.i.) (Figure 3.1.1C). At high doses (10^5 to 10^6 PFU), however, the proportion of $TER119^+$ cells in the spleen increased remarkably. Then, the proportion of $TER119^+$ cells in the spleen was followed over time after low dose (10^3 PFU) or high dose (10^5 PFU) infection with MCMV. After low dose infection, no significant increase in $TER119^+$ cells in the spleen could be detected (Figure 3.1.1D). After high dose infection a rapid induction of $TER119^+$ cells between day 4 and day 5 and a peak around day 6 was seen which contracted after 10 days. In summary, MCMV infection induced extramedullary hematopoiesis in C57BL/6 mice in a route-, dose- and time-dependent manner. In subsequent experiments intravenous (i.v.) infection with 10^5 PFU MCMV was carried out and the analysis of splenocytes was done at day 6 p.i. unless indicated otherwise.

3.1.2 The virus' ability to replicate in the salivary gland is not associated with enhanced extramedullary hematopoiesis

Virus derived from BAC pSM3fr shows a selective growth defect the in salivary gland due to a frameshift mutation within ORF MCK-2/m129 that results in a truncated MCK-2 protein (Jordan *et al.* 2011). MCK-2 is a viral CC(β) chemokine homologue which is thought to be involved in the recruitment of leukocyte subsets that serve as vehicles for viral dissemination (Fleming *et al.* 1999; MacDonald *et al.* 1999; Saederup *et al.* 2001).

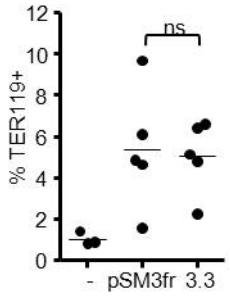

Figure 3.1.2 Virus growth in the salivary gland does not enhance extramedullary hematopoiesis.
Percentage of TER119$^+$ cells in the spleen after infection with MCMV derived from BAC pSM3fr (pSM3fr) with a truncated and BAC 3.3 (3.3) with a restored MCK-2 ORF, respectively. Each dot represents an individual animal. Horizontal bar = mean value, ns = not significant (t-test).

To investigate whether this organ-specific growth defect affects extramedullary hematopoiesis, a BAC-derived MCMV mutant with a restored MCK-2/m129 ORF was used to infect mice (BAC 3.3) (Figure 3.1.2). Both viruses derived from BAC pSM3fr and BAC 3.3 induced extramedullary hematopoiesis to the same extent. Therefore, restoration of virus growth in the salivary gland does not enhance extramedullary hematopoiesis. Since most virus mutants available were generated on the pSM3fr background, this BAC-derived virus was used in further experiments. The use of pSM3fr as 'wild-type' is further justified by the fact that the MCMV strain Smith preparation commercially available compromises a mixture of

both virus populations with an full-length and shortened MCK-2/m129 ORF (Jordan *et al.* 2011).

3.1.3 NK cells are essential for extramedullary hematopoiesis upon MCMV infection

During the adaptive immune response against MCMV, T cells proliferate with a peak around day 6 p.i. (Mohr *et al.* 2010). Remarkably, the kinetics of extramedullary hematopoiesis after MCMV infection parallels the expansion and contraction of T cells (Figure 3.1.1D). In addition, cells of the adaptive immune system produce many cytokines with hematopoietic activity. For example, IL-10 produced by B cells has been shown to at least partially account

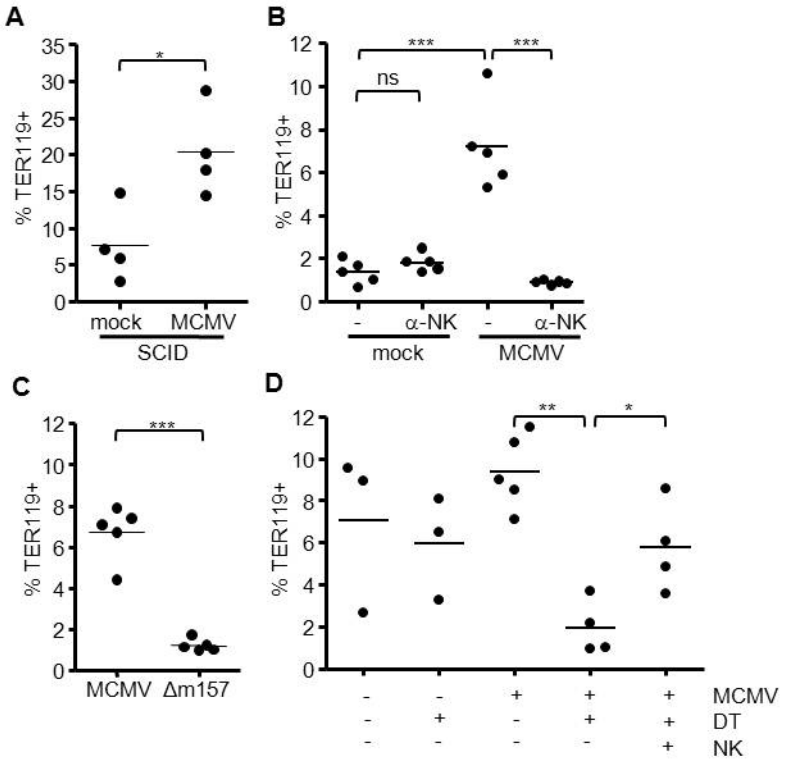

Figure 3.1.3 NK cells are required for extramedullary hematopoiesis.
Percentage of TER119$^+$ splenocytes after infection with MCMV in **(A)** SCID mice, **(B)** C57BL/6 mice depleted of NK cells (α-NK), **(C)** C57BL/6 mice infected with MCMV and Δm157-MCMV (Δm157), **(D)** NKp46-DTR bone marrow chimeras after depletion of NK cells (DT) and transfer of splenocytes enriched in NK cells (NK). Each dot represents an individual animal. Horizontal bar = mean value. Asterisks indicate significant values, * = $p < 0.05$, ** = $p < 0.03$, *** = $p < 0.001$, ns = not significant (t-test (A, C, D), one-way ANOVA with Bonferroni's test (B)).

for the splenomegaly observed after injection of immunostimulatory CpG-oligodeoxynucleotides (CpG-ODN) (Miyazaki *et al.* 2009). Therefore, it was tested whether T or B cells are required for the induction of extramedullary hematopoiesis. Upon infection of severe combined immunodeficiency (SCID) mice that lack T and B cells, a robust increase of $TER119^+$ splenocytes was observed (Figure 3.1.3A) indicating that cells of the adaptive immune response are dispensable for the induction of extramedullary hematopoiesis following MCMV infection.

In the acute phase of an MCMV infection, innate immunity, in particular the NK cell response, is important for the containment of virus replication (Vivier *et al.* 2011). To examine whether NK cells contribute to the regulation of extramedullary hematopoiesis, NK cells were depleted using anti-asialo GM1-antibody. Surprisingly, depletion of NK cells completely abolished the expansion of $TER119^+$ cells after MCMV infection (Figure 3.1.3B).

In C57BL/6 mice, the $Ly49H^+$ subset of NK cells is able to directly recognize MCMV infected cells due to expression of the viral m157 protein on the cell surface (Arase *et al.* 2002; Smith *et al.* 2002; Bubic *et al.* 2004). To investigate whether NK cell activation via Ly49H is important for the development of extramedullary hematopoiesis, an m157-deletion virus (Δm157-MCMV) was used (Bubic *et al.* 2004). Indeed, the proportion of $TER119^+$ cells was significantly reduced after infection with Δm157-MCMV compared to wild-type MCMV (Figure 3.1.3C). These data demonstrated that recognition of infected cells and activation of NK cells via m157-Ly49H-interaction was essential to establish extramedullary hematopoiesis.

To formally prove that NK cells were required for the establishment of EMH, we performed adoptive transfer of splenocytes enriched in NK cells into NK cell-depleted mice. In order to selectively deplete host NK cells but leave the transferred NK cells unaffected, we generated

bone marrow chimeras using a graft from NKp46-DTR mice (Walzer *et al.* 2007). After bone marrow reconstitution, NK cells in these mice express the human diphtheria toxin receptor (DTR) and can be selectively depleted by injection of diphtheria toxin (DT). Of note, higher percentages of $TER119^+$ cells were already present in mock-infected chimeras irrespective of DT-treatment, most likely due to a higher background level of extramedullary hematopoiesis during the reconstitution phase of the hematopoietic system following bone marrow transfer (Figure 3.1.3D) (Hodek *et al.* 2008). Nevertheless, NK cell depletion by DT-injection decreased the number of MCMV-induced $TER119^+$ cells in these mice. Splenocytes enriched in NK cells from MCMV infected wild-type mice were then transferred into infected and NKp46-DTR depleted chimeric mice. Consequently, this transfer restored $TER119^+$ cell expansion.

In summary, these data revealed an unsuspected, crucial role of NK cells in the establishment of extramedullary hematopoiesis after MCMV infection.

3.1.4 NK cell-mediated cytotoxicity is essential for extramedullary hematopoiesis

Next, the mechanism by which activated NK cells expand $TER119^+$ cells was examined. NK cells are important producers of pro-inflammatory cytokines, for example IFN-γ and TNF-α, which might induce extramedullary hematopoiesis. In fact, it was reported that IFN-γ can activate quiescent hematopoietic stem cells during infection (Baldridge *et al.* 2010). $TER119^+$ cells, however, also expanded after infection of $IFNGR^{-/-}$ mice indicating that IFN-γ-signalling is dispensable for induction of extramedullary hematopoiesis (Figure 3.1.4A). TNF-α has also been shown to act on hematopoietic stem cells after bone marrow transplantation (Pearl-Yafe *et al.* 2010). Yet, depletion of TNF-α had no effect on MCMV-induced extramedullary hematopoiesis (Figure 3.1.4B).

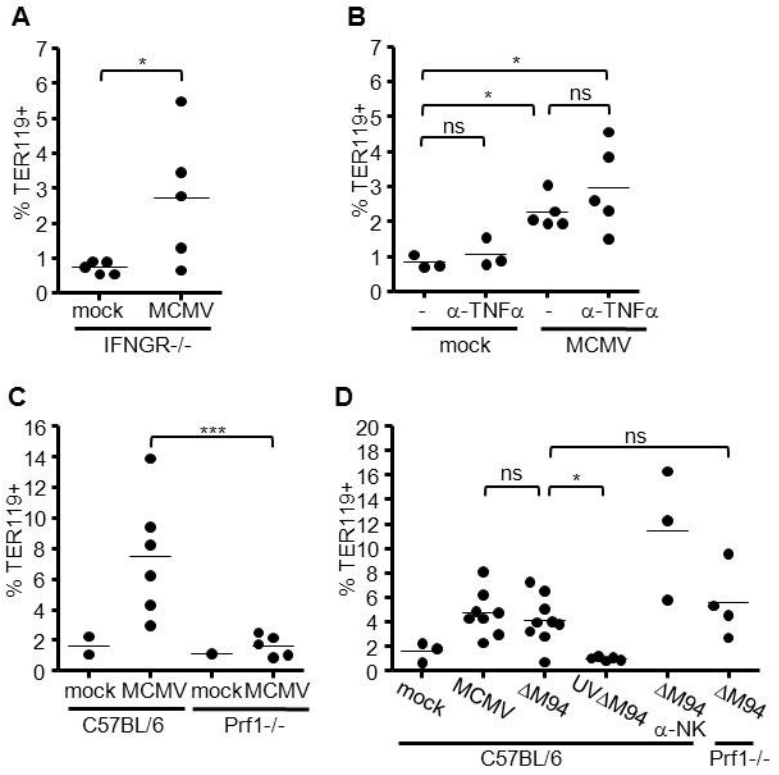

Figure 3.1.4 NK cell-mediated containment of virus spread is required for MCMV-induced extramedullary hematopoiesis.
Percentage of TER119$^+$ splenocytes after infection with MCMV in **(A)** IFNGR$^{-/-}$ mice, **(B)** C57BL/6 mice after depletion of TNF-α (α-TNFα), **(C)** Prf1$^{-/-}$ mice, **(D)** C57BL/6 mice infected with MCMV, ΔM94-MCMV (ΔM94) or UV-irradiated ΔM94-MCMV (UVΔM94) after depletion of NK cells (α-NK) and Prf1$^{-/-}$ mice infected with ΔM94-MCMV. Each dot represents an individual animal. Horizontal bar = mean value. Asterisks indicate significant values, * = $p < 0.05$, *** = $p < 0.001$, ns = not significant (t-test (A,C), one-way ANOVA with Bonferroni's test (B, D)). (C) Data were pooled from two experiments.

Besides cytokine production, perforin-mediated cytotoxicity is the most prominent function of NK cells. Therefore, perforin-knockout mice (Prf1$^{-/-}$) lacking NK cell-mediated cytotoxicity were tested (Kagi *et al.* 1994). Remarkably, expansion of TER119$^+$ cells was completely abrogated in Prf1$^{-/-}$ mice after MCMV infection (Figure 3.1.4C). Hence, it is the cytotoxic function of NK cells rather than cytokine production which is required for the establishment of extramedullary hematopoiesis. This demonstrated that NK cell-mediated killing of MCMV infected cells and thus control of MCMV infected targets was required for the establishment of EMH.

NK cell-mediated cytotoxicity results in the elimination of infected cells and thereby control of virus replication and spread. To dissect whether virus replication in first target cells or spread to secondary target cells affects the development of extramedullary hematopoiesis, mice were infected with ΔM94-MCMV. This virus can fully replicate in cells but virus morphogenesis is interrupted at the stage of secondary envelopment which blocks virion export from cells and spread (Mohr *et al.* 2010; Maninger *et al.* 2011).

Interestingly, infection with ΔM94-MCMV resulted in expansion of $TER119^+$ cells to a similar extent as seen for the wild-type virus (Figure 3.1.4D). Yet, in contrast to wild-type virus infection, NK cell-depletion following infection with ΔM94-MCMV did not abrogate the expansion of $TER119^+$ cells. Also, in $Prf1^{-/-}$ mice infected with ΔM94-MCMV the expansion of $TER119^+$ cells was not reduced. Hence, NK cell-mediated cytotoxicity is required to restrict MCMV spread in order to allow for extramedullary hematopoiesis.

3.1.5 CpG-ODN induced extramedullary hematopoiesis is suppressed by virus spread

The previous data indicated that virus spread in absence of NK cell control might suppress extramedullary hematopoiesis. To formally proof this hypothesis, mice were treated with synthetic CpG-motif containing oligodesoxynucleotide (CpG-ODN). CpG-ODN is a TLR9 agonist which activates cells of the adaptive and the innate immune system, and also strongly induces inflammatory extramedullary hematopoiesis (Sparwasser *et al.* 1999). CpG-ODN pre-treated mice were infected with wild-type MCMV, UV-irradiated wild-type MCMV, spread-deficient ΔM94-MCMV or Δm157-MCMV. Interestingly, infection with Δm157-MCMV completely abrogated CpG-ODN-induced expansion of $TER119^+$ cells (Figure 3.1.5A), demonstrating that MCMV escaping NK cell control suppresses inflammation-induced extramedullary hematopoiesis. It is important to note that infection with wild-type MCMV also led to a lesser, but nevertheless significant suppression of CpG-ODN-induced expansion

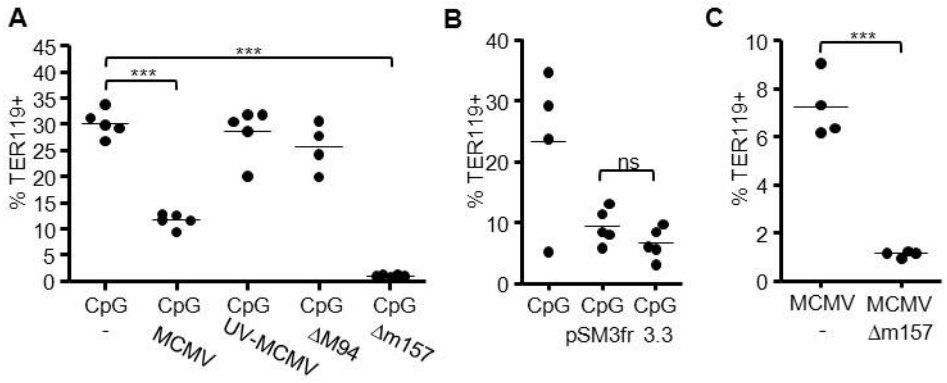

Figure 3.1.5 MCMV spread suppresses CpG-ODN-induced extramedullary hematopoiesis.

(A) Percentage of TER119$^+$ splenocytes in mice pre-treated with CpG-ODN and infected with MCMV, UV-irradiated MCMV (UV-MCMV), ΔM94-MCMV (ΔM94) or Δm157-MCMV (Δm157). Data were pooled from two experiments. **(B)** Percentage of TER119$^+$ splenocytes in mice treated with CpG-ODN and infected with virus derived from BAC pSM3fr (pSM3fr) used as wild-type virus in this study and virus derived from BAC 3.3 with a restored MCK-2/m129 ORF (3.3). **(C)** Percentage of TER119$^+$ splenocytes in mice infected with10^5 PFU MCMV and 10^5 PFU MCMV + 10^5 PFU Δm157-MCMV. Each dot represents an individual animal. Horizontal bar = mean value. Asterisks indicate significant values, *** = $p < 0.001$, ns = not significant (one-way ANOVA with Bonferroni's test (A), t-test (B, C)).

of TER119$^+$ cells. After infection with non-replicating UV-irradiated wild-type MCMV or spread-deficient ΔM94-MCMV neither additional expansion nor contraction of TER119$^+$ cells could be observed, providing further evidence that suppression of inflammatory extramedullary hematopoiesis depends on viral spread.

In addition, it was tested whether virus with a restored MCK-2/m129 ORF and therefore enhanced virus dissemination to the salivary gland has an increased suppression capacity (Figure 3.1.5B). But also in this assay no MCK-2 related differences could be observed.

These data indicated two consequences of an acute MCMV infection with regard to extramedullary hematopoiesis. On the one hand, the inflammation associated with acute MCMV infection induced extramedullary hematopoiesis in a similar fashion as treatment with TLR-agonists like CpG-ODN. On the other hand, the virus was able to suppress this inflammation-induced extramedullary hematopoiesis. This suppressive effect was dependent on virus spread and was controlled by efficient NK cell killing.

To formally clarify the interplay between induction and suppression of extramedullary hematopoiesis by MCMV double infections with MCMV and Δm157-MCMV were performed (Figure 3.1.5C). As seen for CpG-ODN-induced extramedullary hematopoiesis, Δm157-MCMV efficiently suppressed wild-type MCMV-induced expansion of $TER119^+$ cells demonstrating that suppression of extramedullary hematopoiesis is the dominant phenotype. Hence, MCMV suppresses inflammatory extramedullary hematopoiesis and NK cells limit this property.

3.2 Dendritic cells suppress extramedullary hematopoiesis

3.2.1 Identification of DCs as the suppressive cell type in extramedullary hematopoiesis

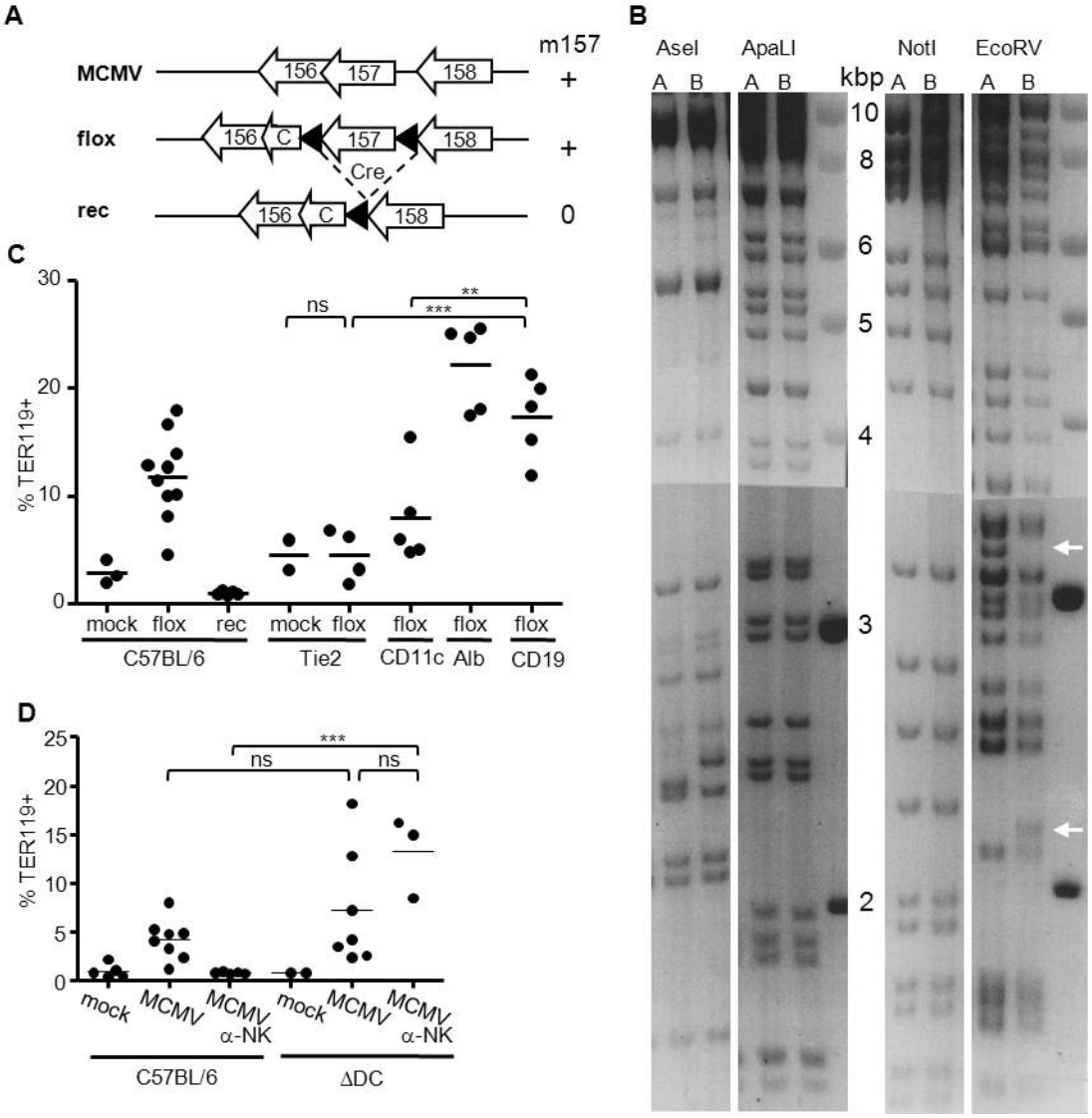

Figure 3.2.1 DCs are dominant suppressors of extramedullary hematopoiesis.

(A) Schematic representation of the *m156*, *m157* and *m158* gene locus (arrows) and principle of conditional *m157*-gene deletion by cre-recombination from virus MCMV-floxed-m157 (flox) resulting in MCMV-recΔm157 (rec). The C-terminus of *m157* was doubled and two *loxP*-sites were inserted (◄). Cre-mediated recombination resulted in deletion of the *m157*-sequence between both *loxP*-sites while the *m156* locus is maintained. **(B)** Successful generation of the BACs pSM3fr-Hm157-loxP-m157-loxP-C-term-m157 (= MCMV-floxed-m157 [A]) and pSM3fr-Hm157-loxP-C-term-m157 (= MCMV-recΔm157 [B]) was verified by restriction enzyme digestion. Arrows mark expected losses and gains of fragments in the EcoRV restriction pattern. **(C)** Percentage of $TER119^+$ splenocytes after infection with MCMV-floxed-m157 (flox) and MCMV-recΔm157 (rec) in C57BL/6, Tie2-cre (Tie2), CD11c-cre (CD11c), albumin-cre (Alb) and CD19-cre (CD19) mice. **(D)** Percentage of $TER119^+$ splenocytes after infection with MCMV in C57BL/6 and ΔDC mice depleted of NK cells (α-NK). Each dot represents an individual animal. Horizontal bar = mean value. Asterisks indicate significant values, ** = $p < 0.03$, *** = $p < 0.001$, ns = not significant (one-way ANOVA with Bonferroni's test).

In order to identify the cell type which suppresses inflammation-induced extramedullary hematopoiesis upon MCMV infection, a conditional virus mutant was created which escapes NK cell control after replication in a certain cell type. To this end, we flanked the *m157* gene in the viral genome with *loxP*-sites (MCMV-floxed-m157). Accordingly, if this virus infects a cell that expresses cre-recombinase the *m157* gene will be deleted from the genome (Figure 3.2.1A). Thus, infection of cre-transgenic mice with MCMV-floxed-m157 virus should result in a cell type-specific deletion of m157 and subsequent uncontrolled replication and spread, since the recombined genome is stably maintained in the virus progeny (Sacher *et al.* 2008a; Sacher *et al.* 2008b). Also, a control virus with an already inactivated *m157* locus (MCMV-recΔm157) was generated (Figure 3.2.1B). Both viruses were tested in C57BL/6 mice. As expected, infection with MCMV-floxed-m157 induced the NK cell response and TER119$^+$ cells expanded, whereas MCMV-recΔm157 did not activate the Ly49H$^+$ NK cell subset resulting in suppression of extramedullary hematopoiesis (Figure 3.2.1C). Then, mice were infected that selectively expressed cre-recombinase in either hepatocytes (Alb-cre), B cells (CD19-cre), endothelial cells (Tie2-cre) or DCs (CD11c-cre). In both Alb-cre and CD19-cre mice cre-mediated deletion of m157 did not result in the suppression of TER119$^+$ cells. B cells are not infected by MCMV and thus CD19-cre mice served as negative controls. Hepatocytes, however, are important targets for MCMV and produce the majority (55%) of the individual's whole body virus load during the first three days of infection (Sacher *et al.* 2008b). Nevertheless, potent virus replication within liver left the expansion of TER119$^+$ cells in the spleen unaffected. Our group has recently reported that hepatocyte-derived virus is not able to disseminate from liver to other organs even under conditions of severe immunosuppression, e.g. NK cell depletion (Sacher *et al.* 2008b). Obviously, uncontrolled virus replication of MCMV only in liver is not sufficient to suppress extramedullary hematopoiesis in spleen.

In contrast, infection of Tie2-cre and CD11c-cre mice resulted in reduced expansion of $TER119^+$ cells. $Tie2^+$ endothelial cells are efficiently infected and represent the dominant and ubiquitous cell type infected first by MCMV (Benedict *et al.* 2006; Sacher *et al.* 2008b; Hsu *et al.* 2009). Thus, it is likely that endothelial cells are only indirectly related to the suppression of extramedullary hematopoiesis because endothelial cell-derived virus progeny spreads and infects another cell type that suppresses extramedullary hematopoiesis.

DCs are secondary targets during MCMV infection (Hsu *et al.* 2009). Regarding the data obtained in CD11c-cre mice, the most plausible cells responsible for suppression of extramedullary hematopoiesis were $CD11c^+$ DCs. Therefore, ΔDC mice which are devoid of DCs by constitutive expression of DT in $CD11c^+$ cells (Ohnmacht *et al.* 2009) were infected with wild-type MCMV. In ΔDC mice expansion of $TER119^+$ cells was not different from wild-type mice (Figure 3.2.1D). In contrast to what is observed in C57BL/6 mice, after depletion of NK cells in ΔDC mice $TER119^+$ cells still expanded. These data implicate DCs in the suppression of extramedullary hematopoiesis during MCMV-induced inflammation, since suppression of extramedullary hematopoiesis upon uncontrolled virus spread only occurred in presence of DCs.

3.2.2 Viral and bacterial DC-tropic pathogens induce suppression of extramedullary hematopoiesis

Suppression of extramedullary hematopoiesis by MCMV could be either a hallmark of MCMV pathogenesis or may characterise those pathogens in general which infect the DC compartment. To address this question, mice were infected with other DC-tropic pathogens. First, lymphocytic choriomeningitis virus (LCMV), a DC-tropic arenavirus with an RNA-genome, was tested. Interestingly, similar to the DNA-virus MCMV, LCMV infection actively suppressed CpG-ODN-induced extramedullary hematopoiesis (Figure 3.2.2A).

Next, infection with the intracellular bacteria *Listeria monocytogenes* was tested. Also *Listeria monocytogenes* efficiently suppressed the CpG-ODN-induced expansion of $TER119^+$ cells (Figure 3.2.2B). Notably, the ability *of Listeria monocytogenes* to move within the cytosol of infected cells and to spread cell-to-cell depends on interaction of the bacterial actA protein with the host's actin microfilaments (Domann *et al.* 1992). This opens the opportunity to study the role of pathogen spread, similar to the situation in MCMV. Remarkably, just as the spread-deficient mutant ΔM94-MCMV, the *in vivo* spread-deficient actA null mutant of *Listeria monocytogenes* lost the ability to suppress the expansion of $TER119^+$ cells, too. To prove formally that infection of DCs is sufficient for suppression of extramedullary hematopoiesis one could take advantage of the ability of the spread-deficient mutant to efficiently infect DCs *in vitro.* Accordingly, ΔactA *Listeria monocytogenes* infected DCs were transferred into CpG-ODN-stimulated mice. While transfer of uninfected DCs did not reduce the proportion of $TER119^+$ cells, there was a complete suppression of $TER119^+$ cells upon transfer of DCs infected with ΔactA *Listeria monocytogenes* (Figure 3.2.2C, D).

β–herpesviruses like MCMV are able to infect many different cell types including DCs, while the closely related γ-herpesviruses preferentially infect lymphocytes and epithelial cells

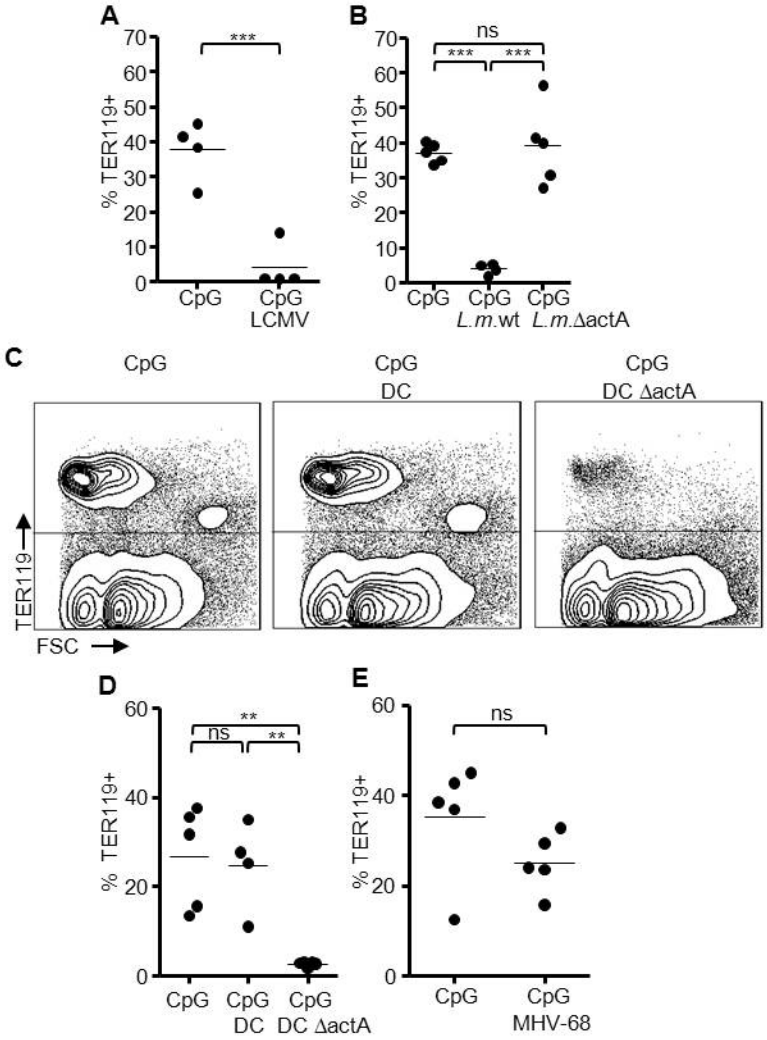

Figure 3.2.2 DC-tropic pathogens suppress extramedullary hematopoiesis.

Percentage of TER119$^+$ splenocytes in mice stimulated with CpG-ODN and infected with **(A)** LCMV, **(B)** *Listeria monocytogenes* (L.m.wt) and actA-deficient *Listeria monocytogenes* (L.m.ΔactA). **(C)** and **(D)** TER119$^+$ splenocytes after transfer of DCs not infected (DC) or infected with *Listeria monocytogenes*-ΔactA (DC ΔactA) into CpG-ODN-stimulated mice. (C) Dot plot of the animal representing the median of n=5 animals is shown, (D) percentage of TER119$^+$ splenocytes. **(E)** Percentage of TER119$^+$ splenocytes in mice stimulated with CpG-ODN and infected with MHV-68 (MHV-68). Each dot represents an individual animal. Horizontal bar = mean value. Asterisks indicate significant values, ** = $p < 0.03$, *** = $p < 0.001$, ns = not significant (t-test (A, E), one-way ANOVA with Bonferroni's test (B, D)).

(Barton *et al.* 2011). MHV-68 is the mouse model for γ-herpesvirus infection. As expected and in contrast to infection with the more DC-tropic pathogens, infection with MHV-68 did not significantly suppress CpG-ODN-induced expansion of TER119$^+$ cells (Figure 3.2.2E).

Taken together, these data demonstrated that - with respect to the examples provided - only infection of DCs by DC-tropic pathogens suppresses inflammation-induced extramedullary hematopoiesis.

3.2.3 Inflammation-induced expansion of stem cells in the spleen is constricted after DC-infection

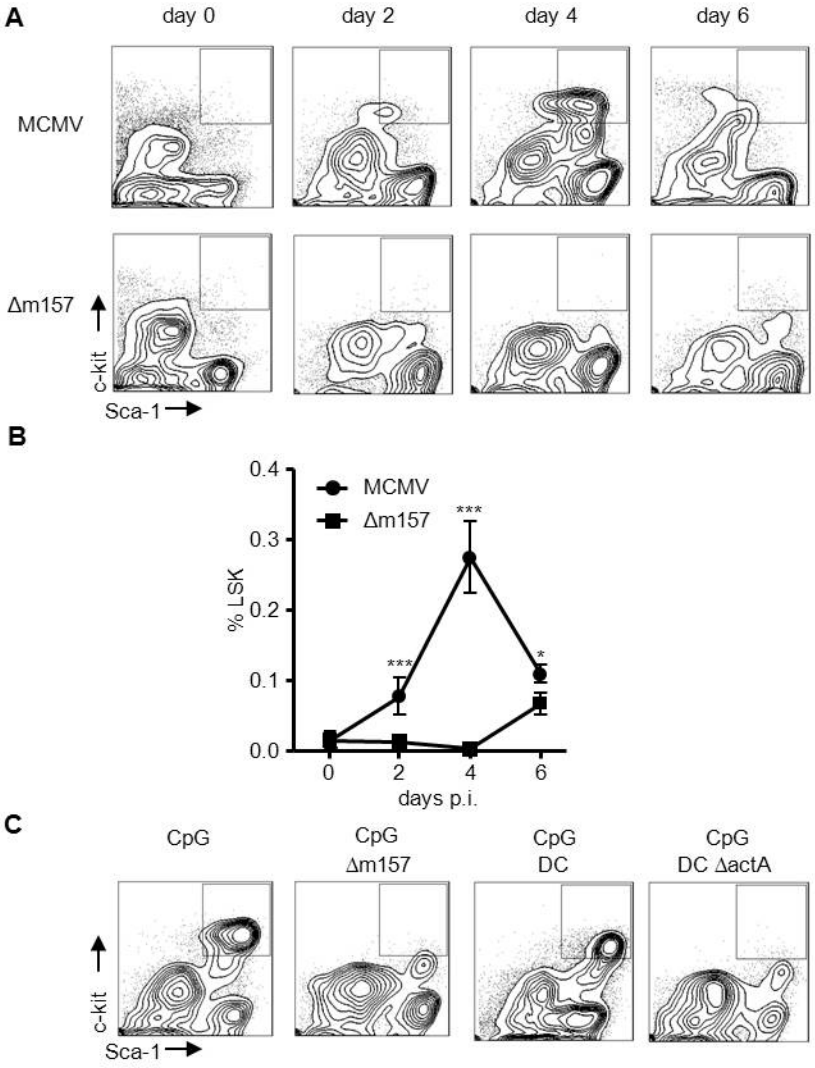

Figure 3.2.3 Constricted stem cell numbers in the spleen upon infection of DCs.
lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells in mice infected with MCMV or Δm157-MCMV (Δm157) at the indicated time points in the spleen **(A)** Dot plot of the animal representing the median of n=5 animals is shown, **(B)** percentage of LSK cells. Asterisks indicate significant values, * = $p < 0.05$, *** = $p < 0.001$ (one-way ANOVA with Bonferroni's test). **(C)** Expansion of LSK cells in the spleen of mice stimulated with CpG-ODN and infected with Δm157-MCMV (Δm157) or after transfer of DCs (DC) and *Listeria monocytogenes* ΔactA infected DCs (DC ΔactA). Dot plot of the animal representing the median of n=5 animals is shown.

Finally, the mechanism of DC-mediated suppression of extramedullary hematopoiesis was investigated. Since all hematopoietic lineages descend from lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) stem cells, LSK cells in the spleen following infection with MCMV and Δm157-MCMV were examined (Figure 3.2.3A, B). The proportion of LSK cells was increased after MCMV infection with a peak at day 4 p.i. followed by a rapid decrease. Interestingly, after infection with Δm157-MCMV no increase of LSK cells in the spleen could be observed. Decreased LSK cell numbers in the spleen were also seen after previous induction of extramedullary hematopoiesis with CpG-ODN and subsequent infection with Δm157-MCMV or transfer of *Listeria monocytogenes* ΔactA infected DCs (Figure 3.2.3C), i.e in the situation of suppressed extramedullary hematopoiesis.

3.2.4 Constriction of stem cells in the spleen is not due to diminished recruitment or enhanced egress

The spleen, as other peripheral organs, satisfies an increased demand of hematopoietic stem cells either by local expansion of organ-resident stem cells or by recruiting bone marrow-derived stem cells from the blood (Massberg *et al.* 2007; Schulz *et al.* 2009; Morita *et al.* 2011). Induction of inflammation using CpG-ODN increased the number of LSK cells in the blood (Figure 3.2.4A). The numbers of circulating stem cells, however, were also increased after CpG-ODN stimulation and subsequent Δm157-MCMV infection. Therefore, lower stem cell numbers in the spleen in the situation of suppressed extramedullary hematopoiesis could not be linked to diminished supply by the bone marrow. Nevertheless, spleen intrinsic mechanisms regulating the recruitment of stem cells from the blood might account for the difference in stem cell numbers. The chemokine CXCL12 (SDF-1) is the major chemoattractant for hematopoietic stem cells and recruits stem cells to the spleen via binding to CXCR4 (Zhao *et al.* 2010; Magnon *et al.* 2011). The cell adhesion molecule VCAM-1 can

also recruit stem cells to the spleen (Papayannopoulou *et al.* 1995). Using quantitative RT-PCR the expression of *Cxcl12* and *Vcam1* mRNA at day 2 p.i. was analysed and groups of mice with extramedullary hematopoiesis (MCMV, CpG-ODN) and suppressed extramedullary hematopoiesis (Δm157-MCMV, CpG-ODN + Δm157-MCMV) were compared side by side (Figure 3.2.4B). *Cxcl12* and *Vcam1,* however, were both not

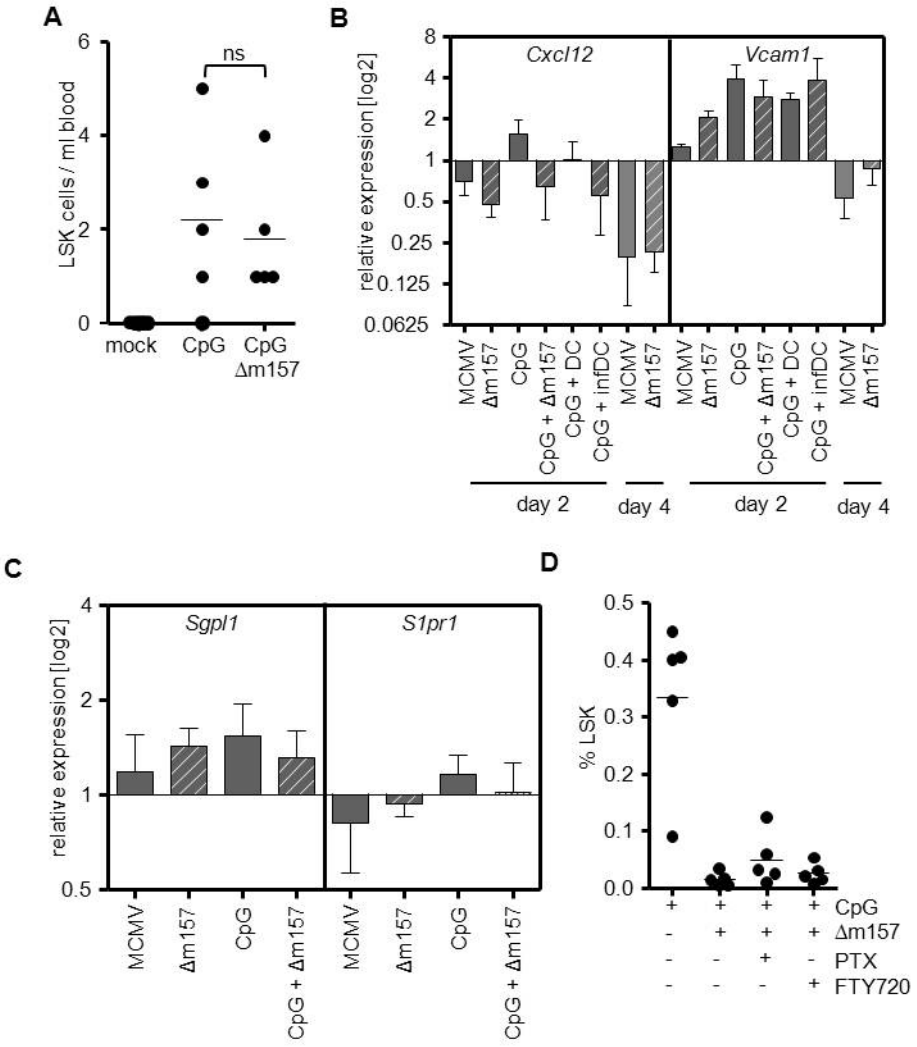

Figure 3.2.4 Constriction of stem cells in the spleen is not due to diminished recruitment or egress.

(A) LSK cells in the blood of mice stimulated with CpG-ODN and infected with Δm157-MCMV (Δm157). Each dot represents an individual animal. Horizontal bar = mean value. **(B)** Relative expression of *Cxcl12* and *Vcam1* in the spleen compared to uninfected mice. **(C)** Relative expression of Sphingosine-1-phosphat lyase (*Sgpl1)* and S1P1 (*S1pr1)* in the spleen compared to uninfected mice. **(D)** Percentage of LSK cells in spleens of mice stimulated with CpG-ODN, infected with Δm157-MCMV (Δm157) and treated with pertussis toxin (PTX) or FTY720. Each dot represents an individual animal. Horizontal bar = mean value.

differentially expressed in the spleen in groups with extramedullary hematopoiesis and suppressed extramedullary hematopoiesis. Therefore, differences in stem cell numbers in the spleen might not be due to differential recruitment from the blood.

Once migratory stem cells are recruited to extramedullary tissues, they must be precluded from leaving the organ to the draining lymphatic vasculature (Massberg *et al.* 2007; Schulz *et al.* 2009). Thus, inhibition of stem cell egress could account for the observed accumulation of stem cells. Similar to lymphocytes, circulating stem cells are attracted into the draining lymphatics by following gradients of Sphingosine-1-phosphate (S1P) (Massberg *et al.* 2007). In contrast to blood and lymph, S1P concentrations in lymphoid and non-lymphoid tissues are low due to rapid degradation by S1P lyase (Schwab *et al.* 2005). Stem cell sensing of S1P and migration are mediated mainly by S1P1, a $G\alpha_i$-coupled receptor that has been shown to be down-regulated upon inflammatory stimuli leading to accumulation of stem cells in peripheral tissues (Massberg *et al.* 2007). To investigate whether reduced expression of S1P lyase or S1P1 might play a role in accumulation of stem cells in the spleen during inflammation, we performed quantitative RT-PCR (Figure 3.2.4C). However, no differential expression of *Sgpl1* (S1P lyase) and *S1pr1* (S1P1) in groups of mice with extramedullary hematopoiesis and suppressed extramedullary hematopoiesis could be detected at day 2 p.i..

On the other hand, stem cell numbers could also be reduced due to enhanced egress from the spleen. Egress can pharmacologically be blocked by either inhibiting $G\alpha_i$-coupled receptor signaling using pertussis toxin (PTX) or by blocking S1P receptors specifically using the immunosuppressant drug FTY720 (Papayannopoulou *et al.* 2003) that sequesters lymphocytes in secondary lymphoid organs (Chiba *et al.* 1998; Mandala *et al.* 2002; Matloubian *et al.* 2004). However, no significant increase of stem cells in the pharmacologically treated groups could be observed (Figure 3.2.4D). Altogether, neither diminished expression of factors regulating accumulation of stem cells during extramedullary hematopoiesis nor enhanced

egress of stem cells from the spleen when extramedullary hematopoiesis was suppressed could account for differences in splenic stem cell numbers.

3.2.5 Expression of stem cell factor (SCF) is reduced upon virus spread

Since a significant role of stem cell recruitment or egress for the number of stem cells in the spleen upon infection could not be found, further experiments focused on factors regulating organ-resident stem cell proliferation and differentiation. One of the most prominent factors regulating stem cell proliferation is stem cell factor (SCF; also steel, KIT ligand). Notably,

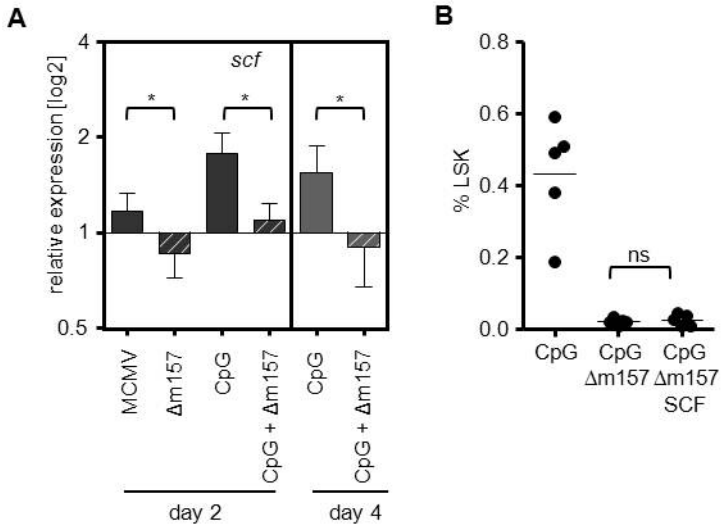

Figure 3.2.5 Expression of stem cell factor is reduced upon infection with Δm157-MCMV.

(A) Relative expression of *scf* in the spleen compared to uninfected mice. **(B)** LSK cells in the spleen of mice stimulated with CpG-ODN, infected with Δm157-MCMV (Δm157) and injected with SCF every day until analysis at day 4 p.i. Each dot represents an individual animal. Horizontal bar = mean value. Asterisks indicate significant values, * = $p < 0.05$, ns = not significant (t-test).

quantitative RT-PCR revealed that *scf* expression in the spleen was enhanced upon MCMV infection or CpG-ODN stimulation (Figure 3.2.5A). Interestingly, the expression of SCF in groups infected with Δm157-MCMV was reduced. Therefore, injection of SCF in mice stimulated with CpG-ODN and infected with Δm157-MCMV should restore the proliferation

of stem cells. However, restoration of stem cell proliferation was not observed (Figure 3.2.5B).

3.2.6 Infection of DCs causes profound changes in the serum hematopoietin profile

Application of SCF alone was not sufficient to restore stem cell proliferation in mice stimulated with CpG-ODN and infected with Δm157-MCMV. It is conceivable that more factors with hematopoietic activity are affected upon infection of DCs. To get a global view on the hematopoietins present in the serum of mice under condition of extramedullary hematopoiesis (MCMV, CpG-ODN, CpG-ODN + DCs) or suppressed extramedullary hematopoiesis (Δm157-MCMV, CpG-ODN + Δm157-MCMV, CpG-ODN + infected DCs) multi cytokine profiling was performed (Figure 3.2.6). A total of 13 growth factors, interleukins and chemokines with hematopoietic activity were assayed, including factors with lineage differentiating (IL-3, IL-5, GM-CSF, M-CSF), stem cell mobilizing (CXCL1, G-CSF) and hematopoiesis inhibiting (CXCL2, CCL2, CCL3, LIF, FGF-2) activities, as well as factors previously implicated in the occurrence of extramedullary hematopoiesis (VEGF, PDGF) (Ghinassi *et al.* 2010). IL-3, GM-CSF, M-CSF, LIF, FGF-2 and VEGF did not show consistent differences between groups with extramedullary hematopoiesis and suppression of extramedullary hematopoiesis at any time point. However, some chemokines with known inhibitory functions on hematopoiesis, i.e CXCL2 (MIP-2), CCL2 (MCP-1) and CCL3 (Mip-1α) (Broxmeyer 2001), displayed selectively elevated serum levels in groups with suppressed extramedullary hematopoiesis particularly at day 2 p.i.. Interestingly, also factors with stem cell mobilizing (CXCL1 / KC and G-CSF (Nardini *et al.* 2005)) and lineage differentiating (IL-5) capacity were dramatically increased when extramedullary hematopoiesis was suppressed. PDGF, which supports hematopoiesis by maintaining stromal

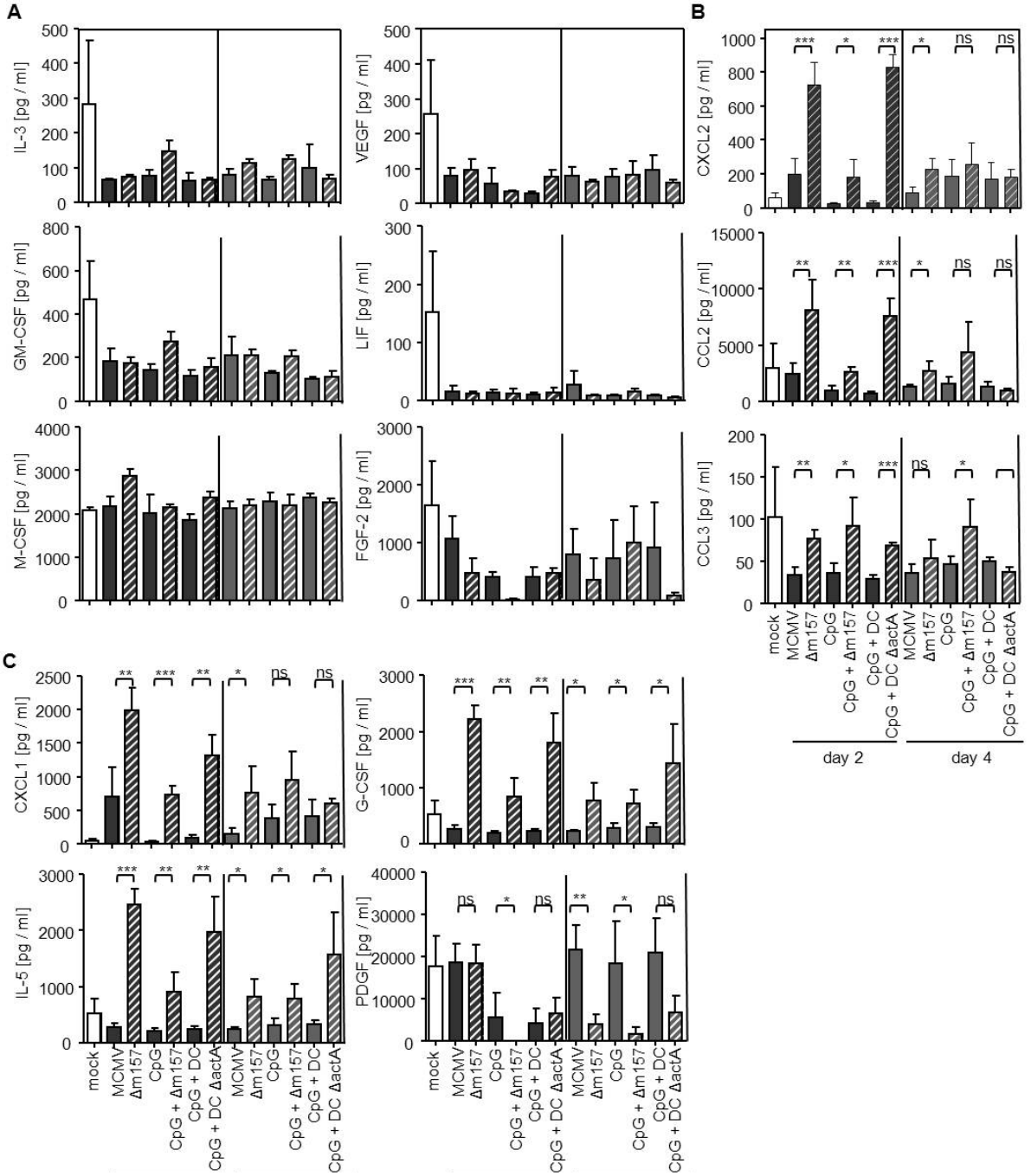

Figure 3.2.6 Infection of DCs results in alterations of the serum hematopoietin profile.

Multi cytokine profiling of mouse blood sera. Mice were either infected with MCMV (MCMV), Δm157-MCMV (Δm157), stimulated with CpG-ODN (CpG), transferred with DCs (DC), *Listeria monocytogenes* ΔactA infected DCs (DC ΔactA) or treatments were combined as indicated. **(A)** Factors that do not show differences between groups of mice with extramedullary hematopoiesis and with suppressed extramedullary hematopoiesis. **(B)** Factors with known suppressive function on hematopoiesis that show differences between groups of mice with extramedullary hematopoiesis and with suppressed extramedullary hematopoiesis. **(C)** Factors with stem cell mobilizing or differentiating or hematopoiesis supporting function that show differences between groups of mice with extramedullary hematopoiesis and with suppressed extramedullary hematopoiesis. Asterisks indicate significant values, * = $p < 0.05$, ** = $p < 0.03$, *** = $p < 0.001$, ns = not significant (column pairs were compared by t-test).

microenvironments (Duhrsen *et al.* 2001), was the only factor examined that showed reduced levels in groups with suppressed extramedullary hematopoiesis at day 4. Hence, infection of DCs resulted in profound alterations of the serum hematopoietin profile creating a suppressive environment for hematopoiesis in extramedullary tissues.

4. Discussion

Extramedullary hematopoiesis during the course of acute or chronic infections is a widely recognized phenomenon. The underlying mechanisms, however, have been enigmatic. In this study, the cellular and molecular determinants of extramedullary hematopoiesis after CMV infection were investigated. Both NK cells and DCs were found to play an unexpected role in the modulation of extramedullary hematopoiesis.

The immune system adjusts hematopoiesis to the optimal cellular response against a specific invading pathogen. Inflammatory stimuli can also reactivate hematopoiesis at extramedullary sites of fetal blood formation, mainly in liver, spleen and skin. Congenital HCMV infection can result in the clinical presentation of the 'blueberry muffin baby' due to dermal extramedullary hematopoiesis. One indication for intrauterine infection with HCMV is the enlargement of liver and spleen which can be detected by ultrasound (Chaoui *et al.* 2002). Splenomegaly also occurs in 30-50% of symptomatic HCMV infections in adults (Britt 2006) and can lead to severe complications as for example rupture of the splenic capsule (Rogues *et al.* 1994; Alliot *et al.* 2001; Duarte *et al.* 2003; Gorgone *et al.* 2005; Amathieu *et al.* 2007). Dermal extramedullary hematopoiesis in the 'blueberry muffin baby' suggests that the enlargement of the spleen in infected fetuses or adults is also due to extramedullary hematopoiesis. However, this has not yet been examined in patient samples.

MCMV is a reliable model for HCMV with regard to organ- and cell tropism, pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression (Mocarski and Kemble 1996; Reddehase *et al.* 2002; Krmpotic *et al.* 2003). It is known since the early 1980s that splenomegaly in the murine model is due to extramedullary hematopoiesis (Lucia and Booss 1981). The mechanisms that regulate extramedullary hematopoiesis, however, have not been investigated.

Here it was observed that the kinetics of extramedullary hematopoiesis developed in parallel to the adaptive immune response, although B and T cells were dispensable for the establishment of ectopic blood formation. Instead, a striking dependence of extramedullary hematopoiesis on NK cells became apparent. Both depletion of NK cells with anti-Asialo GM1 antibody in wild-type mice or with DT in NKp46-DTR bone marrow chimeras led to the reduction of extramedullary hematopoiesis during infection. This excludes the possibility that any other cell population, which might also be depleted by anti-Asialo GM1 antibody (Slifka *et al.* 2000), can be responsible for the induction of extramedullary hematopoiesis. In addition, transfer of splenocytes enriched in NK cells into NK cell depleted NKp46-DTR bone marrow chimeras reconstituted extramedullary hematopoiesis.

Cytokines produced by NK cells were proposed to have an effect on hematopoiesis (Trinchieri 1995; Murphy and Longo 1996). NK cell-derived IFN-γ and TNF-α for example act directly on hematopoietic stem cells (Baldridge *et al.* 2010; Pearl-Yafe *et al.* 2010). Yet, MCMV-induced extramedullary hematopoiesis, however, did not depend on these cytokines but on perforin-mediated cytotoxicity. This demonstrates a specific role of a defined NK cell effector function, namely cytolysis, in this reaction.

Why was perforin-mediated lysis of infected cells required? Cell lysis eliminates infected target cells and thereby prevents viral spread to secondary targets. Using ΔM94-MCMV, it was possible to dissect whether virus replication in the first target or virus spread affects extramedullary hematopoiesis since ΔM94-MCMV's intracellular replication capacity is comparable to wild-type virus but this mutant is not able to spread (Mohr *et al.* 2010; Maninger *et al.* 2011). It is important to note that ΔM94-MCMV induced extramedullary hematopoiesis to the same extent as wild-type MCMV. In contrast, the presence of NK cells or NK cell-mediated cytotoxicity was no longer required, showing that it is virus spread that

impairs the development of extramedullary hematopoiesis. This is the first demonstration that virus spread *per se* has a systemic consequence.

Stimulation with the immunostimulatory TLR9 agonist CpG-ODN induces a potent extramedullary hematopoiesis response (Sparwasser *et al.* 1999). Virus infection after CpG-ODN pre-stimulation demonstrated that MCMV infection, in particular in absence of NK cell control of virus spread, indeed results in suppression of extramedullary hematopoiesis.

Interestingly, in mice deficient for Natural Killer T (NKT) cells or NKT cell-activation hematopoiesis has been reported to be suppressed upon MCMV infection and adoptive transfer of NKT cells lifted suppression (Broxmeyer *et al.* 2007). Besides their production of hematopoietic growth factors (Leite-de-Moraes *et al.* 2002; Kotsianidis *et al.* 2006), it is known that NKT cells help NK cells to limit MCMV infection particularly at later time points of infection (van Dommelen *et al.* 2003). Yet, it remains to be elucidated whether cytokine production or also cell lysis is the NKT cell effector function that preserves hematopoiesis upon MCMV infection.

In C57BL/6 mice, Ly49H$^+$ NK cells govern the early immune response against MCMV (Vivier *et al.* 2011) as they are able to directly recognize the viral m157 protein on the surface of infected cells. Recognition of m157 leads to specific proliferation of Ly49H$^+$ NK cells (Dokun *et al.* 2001) which reduce of the total viral load and preserve of the microarchitecture of the splenic white pulp (Benedict *et al.* 2006; Bekiaris *et al.* 2008).

Recognition of infected cells via the m157/Ly49H interaction was critical also for the development of MCMV-induced extramedullary hematopoiesis in C57BL/6 mice. Upon infection with Δm157-MCMV that escapes activation and control of Ly49H$^+$ NK cells extramedullary hematopoiesis was suppressed. Therefore, the role of Ly49H for promotion of NK cell activation and extramedullary hematopoiesis was not redundant in C57BL/6 mice.

This is because MCMV is able to evade all the other mechanisms that could have contributed to NK cell recognition, such as NKG2D engagement by cellular stress molecules like RAE-ligands, MULT-1 or H60, or ‚missing self' recognition. Gene products m145, m152 and m155 down-regulate MULT-1, RAE-1 and H60, respectively (Krmpotic *et al.* 2002; Lodoen *et al.* 2003; Lodoen *et al.* 2004; Hasan *et al.* 2005; Krmpotic *et al.* 2005) and m138 affects the expression of MULT-1, H60 and RAE-1 isoforms (Lenac *et al.* 2006; Arapovic *et al.* 2009). Thus, MCMV tightly prevents the activation of NK cells in C57BL/6 mice, with the exception of Ly49H-dependend activation. In fact, in most field isolates of MCMV m157 evades Ly49H-dependend recognition and passage of MCMV through $Ly49H^+$ mouse strains under laboratory conditions leads to the emergence of crippling mutations in m157 (Voigt *et al.* 2003), demonstrating the selection pressure executed by Ly49H recognition on the virus.

Nevertheless, it would be interesting to delete MCMV genes known to prevent NK cell recognition, for example m145, m152, m155 and m138, on a Δm157 background, infect C57BL/6 mice with these viruses and assay for extramedullary hematopoiesis. This would elude on the role of NKG2D regulation for virus spread. Furthermore, this would help to clarify whether MULT-1, H60 and RAE-1 isoforms have redundant functions in NK cell recognition *in vivo*.

The MCMV genome harbours about 170 ORFs (Rawlinson *et al.* 1996). Large deletions in the MCMV genome proved 101 ORFs not to be essential for replication (Brune *et al.* 2006; Mohr *et al.* 2008). These genes, however, are probably involved in the modulation of the host's immune response. As discussed above, NK cell recognition in C57BL/6 mice depends exclusively on Ly49H-m157 interaction because recognition via other activating mechanisms or 'missing self' recognition is tightly blocked by MCMV. Therefore, Δm157-MCMV disseminates uncontrolled by the early NK cell activation and suppresses extramedullary hematopoiesis. Any defect in genes regulating NK cell recognition or any other gene defect

limiting virus spread in Δm157-MCMV should therefore result in restoration of extramedullary hematopoiesis. Thus, generation of additional gene knockout mutants on a Δm157 background and examination of extramedullary hematopoiesis in C57BL/6 mice should be a promising method to identify genes with immune modulatory function *in vivo* in the future. Previous *in vivo* assays were limited to the identification of genes with immune-modulatory function based on attenuated virus growth in different organs as indicator phenotype. Notably, identification on the basis of the development of extramedullary hematopoiesis is independent of virus titer but dependent on the ability of the virus to spread. For example, the application of increasing doses of controlled wild-type virus leads to enhanced extramedullary hematopoiesis, while spreading Δm157-MCMV suppresses extramedullary hematopoiesis. Hence, the suppression of extramedullary hematopoiesis may turn out to be a better indication for viral immune escape than virus titer in the organs.

Until today the capacity of CMV to regulate the host's immune system has hampered the development of a vaccine. Altogether, the *in vivo* assay for uncontrolled virus spread based on the suppression of extramedullary hematopoiesis could contribute to the identification of immune-regulatory genes and therefore help to generate safe and immunogenic CMV-vaccine vectors.

In order to elucidate the cellular mechanisms of suppression of extramedullary hematopoiesis an approach based on the Cre / *loxP*-system was chosen. The Cre / *loxP*-system is the most frequently used recombination system for the activation or silencing of genes *in vivo* (Feil and Metzger 2007). Previously, our group has applied it to identify and to quantify viral productivity of selected cell types (Sacher *et al.* 2008a; Sacher *et al.* 2008b). Here, the approach was applied to disrupt NK cell control of virus replication and spread in a cell type-

and organ-specific manner. Gene m157 was flanked by *loxP*-sites and infection of a cre-expressing cell type would result in excision of m157 from the viral genome. Virus replication in this cell type as well as virus spread from this cell cannot be controlled any more by NK cells.

Although hepatocytes produce ~55% of the total body virus load until day 3 p.i., elimination of NK cell control of hepatocyte-derived virus did not result in suppression of extramedullary hematopoiesis. Our group has demonstrated that hepatocyte-derived virus is not able to disseminate to other organs (Sacher *et al.* 2008b). Obviously, intra-hepatic spread of MCMV is not sufficient to suppress extramedullary hematopoiesis in the spleen. This concept is supported by the fact that enhanced MCK-2/m129-dependent virus dissemination to the salivary gland also does not affect extramedullary hematopoiesis (Jordan *et al.* 2011).

In contrast to hepatocytes, uncontrolled virus replication in two cell types was found to be particularly suppressive for extramedullary hematopoiesis: endothelial cells and DCs. Endothelial cells have been found to be among the first targets of MCMV (Benedict *et al.* 2006; Hsu *et al.* 2009) and are important virus distributors (Sacher *et al.* 2008b). Since endothelial cells are infected by ΔM94-MCMV (Mohr *et al.* 2010) which is not suppressive for extramedullary hematopoiesis, however, it is unlikely that endothelial cells represent the suppressive cell type. Endothelial cell-derived virus that escapes NK cell control rather spreads to the cell that suppresses extramedullary hematopoiesis upon infection. DCs are also permissive for virus infection (Andrews *et al.* 2001; Mathys *et al.* 2003). Furthermore, *in vivo* data demonstrated that MCMV first infects cells in the marginal zone and the red pulp of the spleen and then spreads to the white pulp, where infected DCs can be detected earliest at 48 hours post infection (Hsu *et al.* 2009). These data suggest that DCs are secondary targets of CMV *in vivo*. Since suppression of extramedullary hematopoiesis depended on virus spread, further investigations focused on DCs.

In fact, two sets of experiments confirmed that MCMV infected DCs were responsible for the suppression of extramedullary hematopoiesis. First, MCMV infection was not suppressive in ΔDC mice irrespective of the presence of NK cells. Most importantly, the transfer of infected DCs into CpG-ODN-stimulated mice was sufficient to install their suppressive effects on CpG-ODN-induced extramedullary hematopoiesis. These findings identify a central role for DCs in the regulation of inflammation-induced extramedullary hematopoiesis.

CMV employs an array of mechanisms to interfere with immune stimulatory DC-functions, for example the down-modulation of MHC class I and class II complexes or of co-stimulatory molecules, the up-regulation of apoptosis-inducing ligands or the alteration of inflammatory cytokine secretion profiles leading to an overall functional paralysis (Andrews *et al.* 2001; Raftery *et al.* 2001; Rolle and Olweus 2009). This study shows that CMV also renders DCs into active suppressors of extramedullary hematopoiesis which is conceptually different from the hitherto described functional paralysis of DCs upon CMV infection.

The role of DCs in active suppression of extramedullary hematopoiesis is supported by the finding of Birnberg and colleagues showing that depletion of DCs in healthy animals results in a myeloproliferative syndrome associated with a remarkable increase of $TER119^+$ cells in the spleen (Birnberg *et al.* 2008). This argues that suppression of hematopoiesis including extramedullary hematopoiesis is an intrinsic property of DCs even in the steady state.

This study suggests that DC-mediated alterations of the serum hematopoietin profile lead to suppression of extramedullary hematopoiesis by inhibiting stem cell proliferation at extramedullary sites. Preceding the peak of extramedullary hematopoiesis at day 6 p.i, increasing stem cell numbers in the spleen were found until day 4 p.i. with MCMV or stimulation with CpG-ODN. Infection of DCs, however, restricted the increase of stem cells in the spleen. Diminished recruitment of stem cells from the blood via CXCR4 / CXCL12

interaction as well as enhanced S1P-mediated egress could be excluded to be responsible for the constriction of stem cell numbers (Matloubian *et al.* 2004; Massberg *et al.* 2007; Schulz *et al.* 2009) leading to the conclusion that inhibition of stem cell proliferation is most likely the cause. Proliferation of stem cells is controlled by SCF, and SCF expression was reduced in experimental groups showing uncontrolled virus spread. Substitution of SCF, however, could not restore stem cell proliferation. Therefore, further factors must be involved in the regulation of stem cells in the spleen.

In fact, the serum hematopoietin profile was profoundly altered upon DC-infection. Levels of chemokines that negatively regulate hematopoiesis, i.e. CXCL2, CCL2 and CCL3 (Broxmeyer 2001), were elevated as early as day 2 p.i. These chemokines have been found to be produced by DCs upon infection with bacteria and viruses (Piqueras *et al.* 2006; Zaharik *et al.* 2007; Lin *et al.* 2008; Guerrero-Plata *et al.* 2009) and, remarkably, are also produced by DCs *in vitro* in the steady state but the production is abrogated upon DC-maturation (Heufler *et al.* 1992). For example the infection with MCMV or stimulation with CpG-ODN led to a reduction of CCL3 serum levels compared to uninfected animals *in vivo*, but not if the virus escaped NK control or after transfer of infected DCs. The chemokines tested here, however, are only a representative selection of a plethora of hematopoiesis suppressing factors, which perhaps must synergize or require certain combinations to exert suppressive functions (Broxmeyer 2001; Broxmeyer *et al.* 2006). Interestingly, stem cell mobilizing (G-CSF, CXCL1) and differentiating (IL-5) factors were also increased in blood samples of animals with suppressed extramedullary hematopoiesis upon DC-infection. This is probably due to existing negative feedback loops between stem cell proliferation and mobilizing as well as differentiating factors.

In summary, it is conceivable that induction and suppression of extramedullary hematopoiesis is regulated by a complex network of stimulating and inhibiting factors (Figure 4). In the

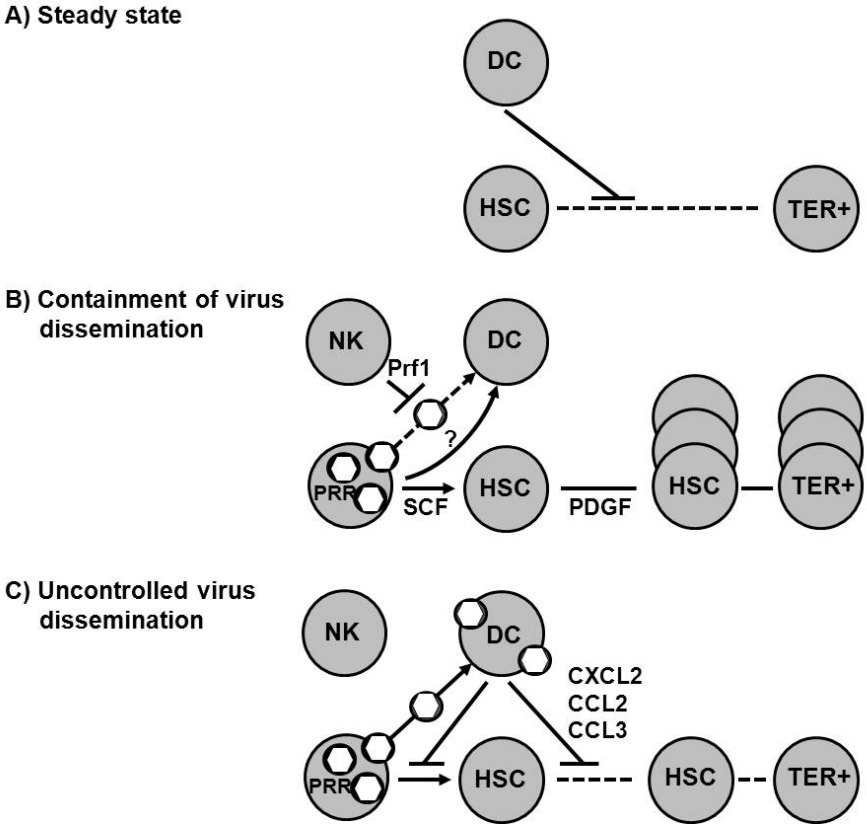

Figure 4 Modell of virus-induced extramedullary hematopoiesis and DC-mediated suppression.

A) In the steady state, DCs suppress extramedullary hematopoiesis (Birnberg *et al.* 2008). **B)** Virus infection induces extramedullary hematopoiesis through Pattern Recogniton Receptor (PRR) signaling. A yet unknown signal (?) might relieve the DC-mediated suppression. Expression of SCF leads to proliferation of hematopoietic stem cells (HSC) which differentiate into $TER119^{+}$ cells (TER+). This is supported by PDGF. NK cells prevent virus spread to DCs via perforin-1-mediated cytotoxicity (Prf1). **C)** Virus spread to DCs abrogates stem cell proliferation via inhibition of SCF and PDGF expression and shifs the serum hematopoietin profil to chemokines suppressive for hematopoiesis.

steady state, DCs suppress extramedullary hematopoiesis (Figure 4A). Upon infection (MCMV) or sterile inflammation (CpG-ODN) extramedullary hematopoiesis is induced (Lucia and Booss 1981; Sparwasser *et al.* 1999) (Figure 4B). The induction is independent of NK cells and DCs, since extramedullary hematopoiesis occurred in NK cell-depleted ΔDC mice. MCMV and also CpG-ODN are recognized by Pattern Recognition Receptors (PRRs) which leads to activation of NFκB (Krug *et al.* 2004; Zucchini *et al.* 2008). Interestingly, constitutive activation of NFκB is already sufficient for induction of extramedullary

hematopoiesis (Kool *et al.* 2011). Therefore, it is plausible that innate immune recognition mechanisms are responsible for the induction of extramedullary hematopoiesis upon MCMV-infection, maybe by delivering a signal to the DCs that relieves the suppressive function. Upon infection, DCs become dominant suppressors of extramedullary hematopoiesis, which in this respect, might simply boost their steady state suppressive function (Figure 4C). NK cells are probably required to eliminate MCMV-infected DCs or prevent virus spread to DCs in order to maintain a permissive environment for the development of extramedullary hematopoiesis upon an inflammatory stimulus.

In contrast to extramedullary hematopoiesis, the effect of MCMV infection on medullary hematopoiesis has been investigated extensively (Mutter *et al.* 1988; Reddehase *et al.* 1992; Mayer *et al.* 1997; Mori *et al.* 1999). Furthermore, bone marrow failure due to HCMV infection is a dreaded complication after allogeneic bone marrow transplantation and HCMV has long been associated with suppression of hematopoiesis (Torok-Storb *et al.* 1992; Lagneaux *et al.* 1993; Sing and Ruscetti 1995; Randolph-Habecker *et al.* 2002). *In vitro*, this was attributed either to the direct infection of early hematopoietic progenitors (Sing and Ruscetti 1990; Maciejewski and St Jeor 1999; Sindre *et al.* 2000; Goodrum *et al.* 2004) or of the supportive microenvironment (Apperley *et al.* 1989; Simmons *et al.* 1990; MacKintosh *et al.* 1993; Steinberg *et al.* 1993; Lagneaux *et al.* 1996; Smirnov *et al.* 2007). *In vivo* studies showed that NKT cells could prevent suppression of medullary hematopoiesis (Broxmeyer *et al.* 2007). The role of NK cells and DCs for medullary hematopoiesis, however, has not been studied so far. Therefore, it is worth to investigate whether the here identified cellular mechanisms also apply to medullary hematopoiesis. It is possible that the immunosuppressive regimen before the bone marrow transplantation leads to uncontrolled virus spread and infection of DCs which might in fact inhibit the reconstitution of the hematopoietic system.

Further studies are needed to examine whether deletion of DCs previous to transplantation could prevent severe and possibly lethal complications due to bone marrow failure.

Containment of virus spread is a prerequisite for extramedullary hematopoiesis. Thus, if the results can be translated to man, the ‘blueberry muffin baby’ represents an immunopathology that still indicates a certain functionality of the NK cell responses in the affected patients.

Immune reactions can reactivate blood formation at ectopic sites. The contribution of this phenomenon to antigen-specific immunity, however, is not known. Here, the expansion of the $TER119^+$ red blood cell lineage was used as an indicator for extramedullary hematopoiesis. It remains to be elucidated whether extramedullary hematopoiesis also expands other cell lineages including antiviral leukocytes. Nevertheless, the contribution of extramedullary hematopoiesis to the production of functional leukocytes during an antiviral immune response is conceivable. MCMV-induced suppression of extramedullary hematopoiesis by DCs, therefore, may constitute a new layer in the virus-host interaction. In this respect it is important to consider that suppression of extramedullary hematopoiesis is not restricted to MCMV, but was also observed upon infection with DC-tropic LCMV and *Listeria monocytogenes*. The effect of pathogen-induced suppression of extramedullary hematopoiesis on the course of these infections remains to be elucidated. Yet, it is tempting to speculate that it represents a common way of immune evasion by DC-tropic pathogens.

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6. Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
BAC	Bacterial artificial chromosome
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumine
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CMV	Cytomegalovirus
CPE	Cytopathic effect
CpG-ODN	CpG-oligodeoxynucleotide
CSF	Colony-stimulating factor
DBPS	Dulbecco´s phosphate-buffered saline
DC	Dendritic cell
dd	Double distilled
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EBV	Epstein-Barr virus
FELASA	Federation of Laboratory Animal Science Associations
GM-CSF	Granulocyte / macrophage-colony stimulating factor
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HIV	Human Immunodeficiency virus
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
i.p.	Intraperitoneal
i.v.	Intravenously
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IL	Interleukin
KC	Keratinocyte chemoattractant
KSHV	Kaposi-sarcoma associated virus
LCMV	Lymphocytic choriomeningitis virus
MCMV	Mouse cytomegalovirus

MEF	Murine embryonic fibroblasts
MHC	Major histocompabitility complex
MHV-68	Murine herpesvirus 68
MOI	Multiplicity of infection
NK	Natural killer (cell)
NKT	Natural killer T (cell)
ODN	Oligonucleotide
ORF	Open reading frame
p.i.	Post infection
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PFU	Plaque forming units
PTX	Pertussis toxin
s.c.	Subcutaneously
S1P	Sphingosine-1-phosphate
S1P1	Spingosinge-1-phosphate receptor 1
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
TNF-α	Tumor necrosis factor-α
VZV	Varicella-Zoster-Virus

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8. Publications

An updated publication list can be found on http://www.researcherid.com/rid/G-6243-2011