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A dissertation on:

Platelets, hemostasis, immunity, and pathogens: A conceptual understanding of their interactions.

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Platelets, hemostasis, immunity, and pathogens: A conceptual understanding of their interactions.

By

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Advisor: John D. Altman, Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Immunology and Molecular Pathogenesis

2011

Abstract

Platelets, hemostasis, immunity, and pathogens: A conceptual understanding of their interactions

By Gilbert David Loria

Severe arenaviral infections in humans are characterized by clinical findings common to other viral hemorrhagic fevers (VHF), including thrombocytopenia, leukopenia, skin and internal organ hemorrhages, high viral replication, splenic necrosis, and death. Host responses, rather than direct damage by the arenaviral replication, account for most of the observed pathology, but it is not known what protective roles platelets may have in each of the manifestations. To address this issue in an animal model, we compared non-depleted (100%), partially depleted (15%), and profoundly (<2,5%) platelet-depleted mice infected with the mouse arenavirus lymphocytic choriomeningitis virus (LCMV). Here, we describe that systemic bleedings and death were seen only in those animals receiving the stronger depletion treatment. Furthermore, we showed that the non-hemorrhagic but partially platelet-depleted mice were unable to control the viral replication due to generalized splenic necrosis, affecting innate and adaptive immune cells. These data suggest that, by their supportive roles in hemostasis, platelets may be preventing the severe pathology observed in human arenaviral infections.

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Chapter 1: Introduction

Human immunity relies on the circulatory system to efficiently protect the body from pathogen invasions. Therefore, alterations of hemostasis, the adequate maintenance of blood circulation, impair the correct function of the immune response. Experimentally, this has been difficult to prove due to the primordial role of hemostasis supporting life by transporting oxygen and nutrients to tissues. However, several systemic human infections induce severe hemostatic alterations that might facilitate pathogen replication and persistence. Here, we analyze how platelet defects, one of the most important components of hemostasis, are involved in immunity against a viral infection.

Viral hemorrhagic fevers (VHFs) are a group of distinct infectious diseases with similar clinical manifestations in humans. The acute phase of these infections is characterized by a flu-like syndrome accompanied by fever, headache, and general malaise. Severe or fulminant cases develop into hemorrhagic fevers (HFs) leading to mucocutaneous bleedings, thrombocytopenia, leukopenia, uncontrolled viral replication, internal-organ hemorrhages, immunosuppression, multiple organ dysfunction, shock and death. Lipid-enveloped, single stranded RNA viruses from the families Arenaviridae (Lassa [LASV], Junin, and lymphocytic choriomeningitis virus [LCMV]), Bunyaviridae (Hanta, Crimean-Congo, and Rift Valley), Filoviridae (Ebola, and Marburg), and Flaviviridae (Yellow Fever and Dengue) are the best known etiological agents of VHFs. (Marty, Jahrling, & Geisbert, 2006) Even though these viruses infect millions of individuals annually, our understanding of their pathophysiology is currently limited. Unfortunately, animal models do not fully recapitulate the clinical manifestations of infection with VHFs, and this, together

with the fact that most of these viruses must be studied under high biosafety containment, represents a major roadblock to enhanced understanding. (Chosewood & Wilson, 2007)

The two main clinical manifestations for all severe VHF cases in humans and non-human primates are defects in hemostasis that leads to a hemorrhagic/shock syndrome, high viral titers, and a suboptimal immune response. Thrombocytopenia is the most dramatic alteration in hemostasis. The mechanisms underlying its development are not fully understood but, it seems to be the combined result of a maturation arrest and/or apoptosis of megakaryocytes in the bone marrow in response to high levels of type I interferons (IFN-α/β) (Binder, Fehr, Hengartner, & Zinkernagel, 1997; Pozner et al., 2010) and a platelet consumption process in the periphery. (Cummins, 1991) As an example of the latter, evidence of disseminated intravascular coagulation has been consistently reported in Ebola and Marburg infections. (Geisbert et al., 2003; Mahanty & Bray, 2004) In addition, high serum viral titers are frequently associated with leukopenia and deficient immune responses. Lymphopenia in the arenavirus Lassa and Argentine HFs strongly correlates with disease severity and widespread necrosis in the splenic marginal zone and cortical and paracortical areas of the lymph nodes. (Moraz & Kunz, 2011) Large numbers of lymphocytes undergoing apoptosis are seen in Ebola and Marburg infections. (Mahanty & Bray, 2004; Sanchez et al., 2004) The sporadic severe human and non-human primate cases of LCMV infections resemble LASV infections, with thrombocytopenia, leukopenia, high viral titers, involvement of liver, lungs, and kidneys, and neurological abnormalities that were overshadowed by the severity of the systemic illness. (Fischer et al., 2006; Lukashevich et al., 2003; Montali, Connolly, Armstrong, Scanga, & Holmes, 1995; Zapata et al., 2011)

In mice, LCMV infection generates a completely different disease probably due to adaptations gained during the long virus/natural-host coevolution. LCMV is a non-cytolytic virus, which indicates that any sign of acute pathology is exclusively mediated by the host response against the infection. (Oldstone & Campbell, 2011) When inoculated intracranially into adult mice, a fulminant meningitis develops mediated by the migration of LCMV-specific cytotoxic T lymphocytes (CTLs) into the central nervous system (CNS). Arriving CTLs release cytokines and chemokines that attract a strong myelomonocytic infiltrate that disrupts the meningeal vasculature, causing vascular leakage, seizure and death. (Kim, Kang, Dustin, & McGavern, 2009) On the other hand, when the virus is inoculated subcutaneously, intraperitoneally (i.p.), or intravenously (i.v.), innate immune mechanisms limit the infection until the development of a CTL response that purges the infection. Lymphoid isolates (e.g. clone-13), in contrast to CNS isolates (e.g. Armstrong 53b) of LCMV, replicate persistently at high viral titers in multiple organs, with a deficient CTL response in adult mice. (Zajac et al., 1998) Extensive research has shown that specific mutations in the glycoprotein and polymerase proteins are responsible for the biological differences observed between isolates. Specifically, a glycoprotein mutation increases its affinity for the cellular receptor α-dystroglycan (α-DG), (Cao et al., 1998) which is highly expressed on dendritic cells (DCs), and a polymerase mutation probably increases its activity allowing a faster replication rate in vivo. (Bergthaler et al., 2010; Sullivan et al., 2011) In the mouse, both mutations give the lymphotropic viruses the ability to escape from their initial confinement in macrophages of the splenic marginal zone and invade the white pulp. (Sevilla et al., 2000) Once there, the virus infects large numbers of DCs and stromal cells (FRCs), (Mueller et al., 2007) and rapidly reaches blood and peripheral tissues. (Matloubian, Kolhekar, Somasundaram, & Ahmed, 1993) The end results of

these events are deficient antigen presentation by the infected DCs, destruction of infected DCs by early generated CTLs, (Borrow, Evans, & Oldstone, 1995) high levels of antigen presentation by non-professional antigen presenting cells (APCs), and the expression of immunosuppressive molecules such as interleukin (IL)-10, (Brooks et al., 2006; Ejrnaes et al., 2006) programmed death-1, (Barber et al., 2006) and transforming growth factor-β, (Tinoco, Alcalde, Yang, Sauer, & Zuniga, 2009) Together, these factors drive the CTL response into exhaustion with diminished proliferative capacity, poor cytotoxic activity, reduced cytokine production and, for certain epitopes, the complete deletion of the response. (Wherry, Blattman, Murali-Krishna, van der Most, & Ahmed, 2003)

Iannacone et al. (Iannacone et al., 2008) recently reported that platelet-depleted mice infected with LCMV Armstrong developed a syndrome similar to severe human cases of VHFs, with mucocutaneous bleedings, vascular leakage, anemia, uncontrolled viral replication, suboptimal immune responses, and subsequent death of the animals; lethal hemorrhage was dependent on IFN-α/β receptor signaling. Furthermore, Goerge et al. (Goerge et al., 2008) showed that mice rendered thrombocytopenic suffered localized hemorrhages at sites undergoing non-infectious inflammatory processes, and that low numbers of circulating platelets were able to prevent such inflammation-induced hemorrhages. Therefore, murine LCMV infections where the severity of the thrombocytopenia can be controlled experimentally, represent an attractive model to study the pathophysiology of VHFs. Here we describe such a model and show that preserving at least 15% of the circulating platelets prevented systemic hemorrhages and death of the mice. We, also show that even in the absence of life-threatening manifestations, viral control was not achieved, and this was associated with necrotic destruction of splenic innate and adaptive

immune cells. Altogether, these observations may contribute to the understanding and clinical treatment of severe VHF infections and report a previously unappreciated relationship between viral infections, platelet-mediated hemostasis, and immunosuppression.

Chapter 2: Theoretical background

The complexity of the interaction between a mammalian host and a pathogen infection requires the understanding of different fields of knowledge. The current scientific literature is fragmented into different disciplines, focusing in the specific interactions between the host factors and between each factor with the pathogen. Here we present a global review of these interactions organized in the following specific interactions: 1) immunity and pathogens, 2) immunity and hemostasis, 3) platelets and hemostasis, 4) platelets and immunity, 5) platelets and pathogens, and 6) pathogens and hemostasis. Diagram 1 illustrates these interactions.

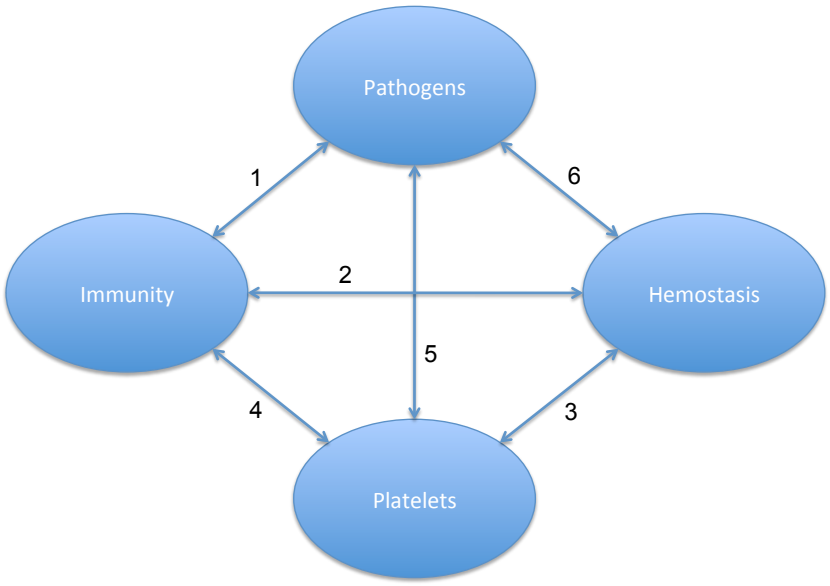


Diagram 1.

Immunity and pathogens

Humans are continuously exposed to pathogenic microorganisms and toxins but rarely develop sickness thanks to physical barriers blocking their entrance into susceptible tissues, however some times these structures are breached and

the different layers of the immune system are in place to remove the insulting agent.

Innate soluble immunity

As the first line of defense against pathogens, innate immunity is critically important in preventing microbial invasion and in alerting other components of the immune system.

Complement is a central component of the innate immunity, it consists of a group of serum and cell-surface-bound proteins that recognize foreign surfaces, disrupt lipid membranes, label pathogenic structures for immediate removal by phagocytic cells, and send activation signals to the adaptive arm of the immune system. (Morgan, Marchbank, Longhi, Harris, & Gallimore, 2005) Complement proteins serve as substrates, enzymes or modulators of a hierarchical series of extracellular proteolytic cascades. Three different pathways can achieve complement activation: the classical pathway is activated by recognition of antigens in complex with specific antibodies by a hexameric complement protein known as C1q, the lectin pathway initiates with the pattern-recognition receptors mannose-binding lectin and ficolins that recognize carbohydrates on the surface of pathogen membranes, and the third pathway, that is probably the most primitive of them, is activated by the spontaneous hydrolysis of the complement component C3 on membranes that don't express complement inhibitors. Complement activation has four major protective functions, lysis of foreign membranes by the assemblage of several complement proteins forming pores that disrupt lipid membranes, initiation of inflammation by fragments of the proteolytic cleavage of complement components that bind to endothelial cells and leukocytes that in turn release proinflammatory cytokines, opsonization of foreign

membranes by the binding of certain complement components that enhance phagocytosis, and solubilization of immune complexes by complement proteins facilitating their clearance from the circulatory system. Irrespectively of the activation mechanism that initiates it, all the pathways culminate in the cleavage of C3 into two active fragments C3a and C3b. C3b attach to lipid membrane and subsequently activates C5 into the C5 convertase that induce the assembly of the multiprotein pore complex MAC (membrane attack complex). Hydroxyl-rich pathogen surfaces facilitate recognition and attachment of C3b, which is subsequently recognized by complement receptors on leukocytes. Furthermore, the small-soluble fragments of the proteolytic cleavage of C3 and C5 (C3a and C5a) function as anaphylotoxins, triggering a series of chemotatic and pro-inflammatory responses.

Control of complement activation in self-tissues is highly regulated by a series of inhibitory soluble and membrane-bound proteins, and several pathogens have evolved virulence factors to evade recognition and removal by the complement system by mimicking complement inhibitors. (Hawlisch et al., 2005; Lambris, Ricklin, & Geisbrecht, 2008; Ricklin & Lambris, 2007; Sahu & Lambris, 2000; Song, Sarrias, & Lambris, 2000; Zhang et al., 2007)

Natural antibodies are present in the serum of humans and higher primates before encounter with antigen, and can activate the classical complement pathway leading to the rapid clearance of pathogens. The predominant source of natural antibodies is peritoneal B-1 B cells, which represent 1-5% of the total B cells in the blood of mice. Natural antibodies are predominantly of the IgM isotype, however IgG and IgA isotypes have been reported. The antigenic recognition sites of natural antibodies are encoded by germline variable genes

without extensive somatic mutations and have a wide range of binding avidities. The disaccharide α(1,3)-galactose is one of the main antigens recognized by natural antibodies, it is mainly found in the surface of pathogen glycoproteins. Humans, apes and old world monkeys lack the enzyme galactosyl-transferase, which attaches the α(1,3)-galactose. Bacteria in the gut produce this sugar and this is thought to be the reason why over 2% of serum IgM and IgG antibodies are directed against α(1,3)-galactose. Anti α(1,3)-galactose natural antibodies activation of complement is probably the reason why humans and higher primates are not frequently infected by enveloped viruses of other lower animals. (Ochsenbein et al., 1999; Takeuchi et al., 1997) However, some enveloped viruses from small rodent manage to infect humans, and interestingly, infected humans usually generate hemorrhagic fevers upon first encounter with the virus. This might be mechanistically linked to the hemorrhagic fevers seen in the secondary infections with dengue viruses, where poorly neutralizing antibodies to a different dengue serotype enhance infection of macrophages and induce an exacerbated immune response that is though to be responsible for the hemorrhages and shock syndrome.

Natural antibodies are also necessary for the immediate trapping and removal of viral particles in the blood stream into macrophage populations located in the splenic marginal zone, preventing early infection of peripheral tissues. (Ehrenstein & Notley, 2010; Matter & Ochsenbein, 2008; Ochsenbein et al., 1999; Takeuchi et al., 1997) Furthermore, natural antibodies are important in the removal of senescent erythrocytes and cellular debris from the circulation. (Ehrenstein & Notley, 2010; Matter & Ochsenbein, 2008)

Fundamental to initiation of an immune response is the recognition of the foreign particle as non-self. As explained previously complement and natural antibodies accomplish this by very different mechanisms, however there is even another mechanism for the early recognition of pathogenic molecular patterns by receptors in immune cells. These receptors are located either in the membrane or the cytoplasm, and recognize structural elements exclusively expressed by pathogens as diverse as nucleic acids, phospholipids or complex sugars. RIG-I, as an example, detects double-stranded RNA or single stranded RNA with a 5'-triphosphate, which are usually not found in the cytoplasm of uninfected cells; but are typically found in the life cycles of most viruses. When RIG-I is activated by these structures, a series of signaling events occur leading to the synthesis of cytokines with antiviral activity and also necessary for the activation of other immune cells. The membrane bound receptors are known as toll-like receptors, ligand recognition also induces the release of cytokines, although by different signaling pathways. The release of cytokines mark the initiation of the host defense against the invading organism. Over 80 different cytokines are secreted by the infected cells, and among the most studied are interferon α (more than 14 isoforms) and β (2 isoforms), tumor necrosis factor α, interleukin-6, interleukin-12, and IFN γ. Release of these cytokines act on receptors expressed on cells located in close or distant proximity. For example, IFN-α/β cytokines are recognized by a common receptor known as IF-α/βR, that signals into the cytoplasm and into the nucleus leading to the transcription of hundreds of genes with antiviral properties. (Schoggins et al., 2011)

Innate cellular immunity

A second layer of innate immunity consists of a series of sentinel cells physically and permanently located at tissues where pathogens might enter and

at the secondary lymphoid organs where they initiate the development of the adaptive immune defense. Dendritic cells and macrophages patrol the body tissues seeking signs of injure and pathogens. Locally release inflammatory cytokines are detected by the dendritic cells, which become activated, responding by releasing more cytokines to amplify the inflammatory recruitment of blood leukocytes. Neutrophils and monocytes migrate into the affected tissue to fight the infectious agent and initiate the healing process. Most of the microorganism's invasions are effectively controlled by the collective actions of the innate components, or at least its spread is restricted until the development of a pathogen specific adaptive immune response, which might take days to appear.

Adaptive Immunity B cells

Humoral immune responses are aimed to the elimination or neutralization of the insulting antigen. B cells recognize antigens in its native, unprocessed form via surface receptors. The anatomical sites in which B cells first encounter antigens depend on the route of the infection. Blood antigens are captured in the spleen, mucosal antigens are recognized in the mesenteric lymph nodes, and skin antigens in the peripheral lymph nodes. Naïve B cells recirculate through these secondary lymphoid organs to increase the likelihood of encountering their cognate antigen. The antigens are mechanically transported to the secondary lymph organs in the lymph fluid or blood, where it is capture by specifically localized macrophages and dendritic cells, which present the intact antigen to the B cells. Upon antigen recognition and B cell receptor clustering, activation of the B cells begins. B cells can differentiate along two pathways. On one hand, B cells can differentiate to form extra-follicular plasmablasts that are essential for rapid antibody production, and in the other hand, B cells can enter the germinal

centers, where they can differentiate into plasma cells, which can secrete high-affinity antibodies, following affinity maturation, or become memory B cells, which confers long-lasting protection from secondary encounters with the antigen.

Adaptive immunity T cells

Once a pathogenic structure is inside the body, it is captured by phagocytic cells, transported in the lymph fluid to the secondary lymphoid organs, processed, and presented by antigen-presenting cells into major histocompatibility complex molecules to CD8+ and CD4+ T lymphocytes. Dendritic cells are the most potent cells with capacity to activate the adaptive immune response. Captured or cytoplasmic produced antigens (in the case of direct infection of the DCs) are proteolytically degraded and short peptide sequences presented to CD4+ T lymphocytes into MHC class II molecules or to CD8+ lymphocytes into MHC class I molecules. CD4+ T lymphocytes cannot discern antigens that are exogenously delivered from those that are endogenous; CD8+ T lymphocytes sample the cytosolic environment instead.

CD4+ T lymphocytes are also known as helper T cells because their main function in a immune response is to deliver specific signals to the different effector immune cells and shape the kind of response needed to achieve the best control of the insulting pathogen. Through a series of cytokines and cellular receptors, CD4+ T lymphocytes interact with macrophages, B lymphocytes, and CD8+ T lymphocytes to acquire their fully activated state. Another primordial function of the CD4+ T lymphocytes is to deliver signals for the generation of T and B memory responses, in the absence of CD4 T cells, short in time B and T cell responses are generated but vanish in terms of weeks, and upon second encounter with the pathogen no preformed immune responses are present.

Activated CD8+ T lymphocytes proliferate massively and become effector T cells, also known as cytotoxic T lymphocytes. These cells are specialized in the destruction of cells bearing intracellular pathogens, which are protected from the action of complement, antibodies, and phagocytes. By destroying the infected cells, the immune system achieves complete clearance of most pathogens.

During certain infections or in certain pathologies, the CD8+ T lymphocytes might induce tissue damage compromising the survival of the host, inducing more damage than the one that could have been provoke by the pathogen. Under these circumstances, the immune system has certain mechanism to dampening the immune response. Of special importance is the case of chronic infection with non-cytolytic viruses, which can replicate in the host without inducing any sign of pathology, to avoid immunopathology due to the failure to control the virus the CD8 responses are turn off by a mechanism known as exhaustion.

Immune suppression by viruses

Pathogens, by definition, are able to induce disease in the host, which implies that some degree of immune evasion must be present. Certain pathogens might even be able to use the immune response to their advantage. In the most extreme cases, as for viruses able to generate persistent infections, complete subversion the immune system is in order to prevent being eliminated. On the other hand, acute viral infections might just temporarily hamper the immune response in order to reach certain levels of replication on the host, allowing the spread to the next susceptible host. Immune interference can be either termed immune evasion or immunosuppression, being immune evasion, in one hand, just able to prevent the immune response against itself, whereas

immunosuppression, on the other hand, induces such as strong functional defect of the immune response that facilitates the replication of the inducing pathogen and as well to any other pathogen that might infect the host at that time. Examples of immune evasion include latency, down regulation of immune regulatory proteins, and mimicry of host immune regulatory proteins. A series of general mechanisms shared by viruses able to induce persistent infections in humans include dysregulation of cytokine production and direct or indirect destruction of antigen presenting cells, T lymphocytes, and bone marrow progenitor cells. Hindering of antigen presentation to T cells and cytokine dysregulation seem to be responsible for short early episodes of immunosuppression, whereas bone marrow infection and disruption of hematopoiesis seem to be responsible for long term immunosuppression.

Immunosuppression-inducing viral infections in humans

Measles: Human cases of measles infection are associated with a generalized immunosuppression that can last up to six months. Even measles vaccine induced such immunosuppression although it is shorter in duration. Measles replication in humans and most animal models is acutely cleared by the immune response and it is, in general terms, a mild infection, however in countries with low vaccine coverage, the infection is associated with high morbidity and mortality due to opportunistic infections. (Burnet, 1968; Fireman, Friday, & Kumate, 1969; Halsey et al., 1985; Hirsch et al., 1981; Wesley, Coovadia, & Henderson, 1978) Therefore, this measles associated immunosuppression is difficult to explain mechanistically due to the apparently absence of the virus at the time of the immunosuppression. Analysis of lymphocytes isolated from measles patients shows limited response to mitogenic or antigenic stimulation, and the antigen presenting cells seen to be depressed

and instead promote apoptosis of the T lymphocytes. (McChesney, Altman, & Oldstone, 1988; McChesney, Kehrl, Valsamakis, Fauci, & Oldstone, 1987; Naniche, Reed, & Oldstone, 1999) (Fugier-Vivier et al., 1997; Grosjean et al., 1997; Schnorr et al., 1997) Furthermore, it have been demonstrated that virulent isolates of measles impaired the production of IL-12 and IFN-α/β by macrophages and dendritic cells in vitro, which might slow the development of the adaptive immune response, however in humans this deficiency does not affect the early production of IFN-γ and IL-2 at the onset of the cellular immune response and rash, (Fugier-Vivier et al., 1997; Griffin, Ward, Jauregui, Johnson, & Vaisberg, 1990; Karp et al., 1996; Naniche et al., 2000) but the levels of both cytokines decrease and IL-4 levels rise and are sustained for several months (Griffin & Ward, 1993). This cytokine imbalance might incline the immune response towards the production of humoral immunity instead of cellular immunity. This might explain an early immunosuppression but does not justify the long lasting immunosuppression. Profound depression of the bone marrow progenitor cells during acute infection might account for a delay in the recovery and the long-term defect due to a slowed repopulation of peripheral DCs. A recent report of measles virus infecting bone marrow progenitor cells in humans was recently published. (Mills et al., 1994) This observation might indicated that in fact direct measles infection of progenitor cells might last for longer periods of time, a previously unrecognized event that may account for the immunosuppression in certain cases. Altogether, it seems that the early events of immunosuppression may be due to perturbation of function by direct infection, (Casali, Rice, & Oldstone, 1984; McChesney, Fujinami, Lerche, Marx, & Oldstone, 1989) cell cycle block of infected T lymphocytes, apoptosis of uninfected T lymphocytes by MV-infected DCs or inhibition of IL-12 and/or IFN-

α/β synthesis. However, the long-term suppression may be due to profound effects on bone marrow progenitor cell growth and differentiation.

HIV: Probably the most studied immunosuppression induced by a virus is the acquired immune deficiency syndrome (AIDS), which slowly progress after the initial infection with the human immunodeficiency virus (HIV). Reactivation of latent viral infections and opportunistic infections always culminate in the dead of the patient if antiviral therapy is not administered. The virus infects mainly cells that express the CD4 molecule on its membrane, specially CD4+ T lymphocytes and the decline in its peripheral numbers indicate the progression of the immunosuppression. Although originally understood as the direct destruction of the CD4+ T lymphocytes were the explanation for the immunosuppression due to its primordial role in the development of immune responses, nowadays a more complex picture is drawn. The virus is also able to infect antigen-presenting cells, which are deficient in the capacity to stimulate T cells in vitro. (Knight, Elsley, & Wang, 1997) Furthermore, DC decreases in numbers during progression into AIDS. (Macatonia, Lau, Patterson, Pinching, & Knight, 1990) Therefore, decrease functionality and decrease numbers could explain the immunosuppression observed in patients for which the numbers of CD4+ T cells have not reach critical levels. Nevertheless, the slow progression into AIDS should allow replenishment of the dendritic cells and CD4+ T cells which is not the case, indicating that the output of the bone marrow is affected during the infection, which might underlie a third component of immunosuppression. (Marandin et al., 1996; Re, Furlini, Zauli, & La Placa, 1994) Interestingly, it was recently reported that HIV infects early progenitor cells in the bone marrow causing cell death and establishing persistent viral reservoirs. (Carter et al., 2010) Besides progenitor cells, HIV is able to infect stromal cells, endothelial

cells, and macrophages in the bone marrow. Many reports have suggested that the greatest impact of HIV on hematopoietic progenitor cell growth results from its capacity to infect these auxiliary bone marrow microvascular endothelial cells and/or macrophages. The mechanism may work via inhibition of growth factors and cytokines essential for hematopoiesis. (Bahner, Kearns, Coutinho, Leonard, & Kohn, 1997) Therefore the immunosuppression associated with the infection with HIV may thus stem from the functional impairment and or destruction of DC, macrophages, and CD4+ T cells and the incapacity to repopulate such cells due to the infection of bone marrow auxiliary cells supporting bone marrow hematopoietic growth. The collapse may arise when hematopoiesis can no longer replenish the immunologic abnormalities.

Human cytomegalovirus: HCMV infection in normal humans is characterized by an acute infection follow by latency that present a period of weeks to moths immunosuppression. As the previous pathogens discussed above, several mechanism have been proposed to explain the HCMV-induced immunosuppression. Direct inhibition of cytotoxic T lymphocytes (Schrier & Oldstone, 1986; Schrier, Nelson, & Oldstone, 1985) and decreased antigen presentation by monocytes have been shown. (Buchmeier & Cooper, 1989; Carney & Hirsch, 1981; Rinaldo, Carney, Richter, Black, & Hirsch, 1980) Virus infection of monocytes, either in vivo or infected in vitro impaired their capacity to activate T cell responses. Also, it was shown recently that the monocyte defects were associated to the high levels of IFN-α/β that HCMV induced in these cells. (Noraz, Lathey, & Spector, 1997) IFN-α/β Inactivation reversed the observed suppression. As with the other immunosuppressive infections, bone marrow hematopoiesis is present. Progenitor cells differentiation and proliferation were affected by the HCMV infection. The latency reservoir of HCMV is thought to be a

very primitive progenitor cells. Latently infected hematopoietic stem cells (CD34+ CD33-) have been described in human cases of HCMV. (Hahn, Jores, & Mocarski, 1998) HCMV infection of the bone marrow may thus be the primary mechanism of immunosuppression.

Immunosuppression in the LCMV clone 13 murine model.

LCMV is a non-cytopathic virus, which indicates that any sign of acute pathology is exclusively mediated by the host response against the infection. (Oldstone & Campbell, 2011) When CNS isolates of LCMV (e.g. Armstrong) are inoculated subcutaneously, intraperitoneally (i.p.), or intravenously (i.v.), innate immune mechanisms limit the infection until the development of a CTL response that purges the infection. In contrast, lymphoid isolates (*e.g.* clone 13) replicate persistently at high viral titers in multiple organs, with a deficient CTL response in adult mice. (Zajac et al., 1998) Extensive research has shown that specific mutations in the glycoprotein and polymerase proteins are responsible for the biological differences observed between isolates. Specifically, the glycoprotein mutation increases its affinity for the cellular receptor α-dystroglycan (α-DG), (Cao et al., 1998) which is highly expressed on dendritic cells (DCs), and the polymerase mutation probably increases its activity allowing a faster replication rate *in vivo*. (Bergthaler et al., 2010; Sullivan et al., 2011) In the mouse, both mutations give the lymphotropic viruses the ability to escape from their initial confinement in macrophages of the splenic marginal zone and invade the white pulp. (Sevilla et al., 2000) Once there, the virus infects large numbers of DCs and stromal cells (FRCs), (Mueller et al., 2007) and rapidly reaches blood and peripheral tissues. (Matloubian et al., 1993) The end results of these events are deficient antigenic presentation by the infected DCs, destruction of infected DCs by early generated CTLs, (Borrow et al., 1995) high levels of antigen

presentation by non-professional antigen presenting cells (APCs), and the expression of immunosuppressive molecules such as interleukin (IL)-10, (Brooks et al., 2006; Ejrnaes et al., 2006) programmed death (PD)-1, (Barber et al., 2006) transforming growth factor (TGF)-β, (Tinoco et al., 2009) and IL-21, (Elsaesser, Sauer, & Brooks, 2009; Fröhlich et al., 2009; Yi, Du, & Zajac, 2009) which altogether drives the CTL response into exhaustion with diminished proliferative capacity, poor cytotoxic activity, reduced cytokine production and, for certain epitopes, the complete deletion of the response. (Wherry et al., 2003) This immunosuppression observed during chronic LCMV infection extends to other pathogens and antigens inoculated in the infected mice.

Immunity and hemostasis

Mammalian immune responses are framed in the extensive collection of blood vessels that irrigate every peripheral tissue in the body and therefore, there is a tight dependence on an adequate hemostasis.

Inflammation

Rubor (redness), *calor* (hotness), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function) are the cardinal signs of inflammation, a process generated by released soluble molecules from cells recognizing pathogenic structures, inducing vasodilatation and increased vascular permeability allowing the immediate release of plasma fluids into the affected extracellular tissues. Inflammation is intended to dilute and remove the pathogenic agent towards the draining lymphoid organs, and increase complement and antibodies concentrations in the affected area.

Immune cells interaction with endothelium

Leukocytes circulating in blood barely interact with the endothelium, however endothelial activation triggers a series of changes that initiate leukocyte rolling on the endothelial bed of postcapillary venules. P-selectin (in acute injury) and E-selectin (in inflammation) expression initially allow the interaction with fucosylated syalo-glycoconjugates as sialyl Lewis X (sLex) and P-selectin glycoprotein ligand-1 (PSGL-1) decorating the surface of leukocytes. These interactions slow down the leukocytes allowing the recognition of chemokines on the endothelium by integrin receptors on the leukocyte membranes. Integrins are heterodimeric receptors formed by an α and β chains normally found in a low affinity bended conformation, recognition of the ligand induce a molecular modification increasing affinity and avidity (by clustering of activated integrins) accomplishing the complete detention of the leukocyte. All these processes selectively recruit the needed leukocytes to the inflamed tissues. Once a complete stop and activation of the leukocytes is established, the leukocytes actively migrate laterally on the venular surface, searching for the appropriate conditions for extravasation, which occurs more frequently between endothelial cells (paracellular route) involving molecules of the tight junctions, or directly through the endothelial cells (transcellular route).

Recirculation and trafficking

Blood leukocytes have evolved their migratory and circulatory properties to maximize their capacity to monitor peripheral tissues for pathogens or signs of injury. Lymphocytes continuously traffic through the body, looking for foreign antigens by recirculating from blood, through secondary lymphoid tissues, into lymph, and back to blood. Once they encounter their cognate antigen,

lymphocytes acquire a predilection, based on the chemical environment in which they first encounter the antigen, to recirculate more frequently to the tissues where the antigen was first encounter. (Mackay, 1992; Picker & Butcher, 1992) Granulocytes and monocytes are produced in the bone marrow and circulate in the blood until their death and removal in the spleen. They only leave the bloodstream in response to signals of injury or infection (inflammation). The chemical signals generated by the differential recognition of the insulting agent determine the nature of the cellular infiltrate into the tissues, exercising and exquisite specificity that control the cellular trafficking into tissues. These signals are present on vascular endothelium, which shows diversity among tissues, and alters its display of signals during inflammation. (Springer, 1994)

Platelets and hemostasis

Since Max Schultze and Giulio Bizzozero published their descriptions of platelets and platelet interactions with injured capillary walls *in vivo*, (Brewer, 2006) a plethora of clinical and experimental research have indisputably demonstrate the importance of platelets in hemostasis, and its original demonstration in 1910 by Duke (Duke, 1910) of a moribund patient with low platelet counts suffering systemic bleedings that rapidly recovered with platelet transfusions.

Platelet structure

Platelets are the smallest of the blood cells and the secondly more abundant after the erythrocytes. Averaging 2.0 to 5.0 μm in diameter, 0.5 μm in thickness, with a life span of 7 to 10 days before being removed by the reticuloendothelial system in the spleen. The platelet main and most known function is in the maintenance of the hemostasis, which is essential for host survival. The specific

structural characteristics of the platelet allow the correct platelet function in physiology and pathology.

Peripheral zone: Under the high resolution electron microscopy examination, the relatively smooth plasma membrane of resting platelets, tiny folds can be observed which provide additional membrane needed when platelets spread once activated. These openings, known as the surface connected canalicular system (SCCS), also increase the surface area of the platelet allowing a bigger interaction with the external environment. Surrounding the platelet membrane there is a thicker coat or glycocalix. The function of these two structures plays a primordial role in the acceleration of clotting reactions. The submembrane area is composed of relatively regular thin filaments resembling actin filaments, which have a function in regulating the shape of the platelet and the translocation of receptors and particles.

Organelle zone: three major organelles are found in the platelets, α granules, dense bodies (δ granules), and lysosomes. Relatively simple mitochondria are present in the platelet cytoplasm, thought to be important for energy platelet metabolism.

Platelet receptors

Platelet receptors determine weather the platelet reacts or not to an external stimuli. Unlike most of cells, platelets lack a nucleus and therefore can only respond to the different situations by the selectively release of performed proteins contained in the different granules. Although there is some evidence for residual protein translation from residual messenger RNA carried over from megakaryocytes, the impact this might have in a physiological or pathological response have not been properly addressed. Therefore, all the weaponry used

by the platelet to respond to the different situations and different agonist must be performed in the storage granules or in the different platelet receptors. Agonist interaction with the specific platelet receptor drives the mobilization and release of specific granules. Different combination of granules released might also fine-tune the responses. Most of the granule contents are proteins with a known role in hemostasis and thrombus formation.

αIIbβ3 (GPIIb-IIIa) is the major platelet integrin receptor with 50,000 to 80,000 copies per platelet and it is the uniquely expressed by platelets. Its deficiency leads to Glanzmann thrombastemia, the more frequent bleeding disorder in humans caused by a platelet receptor defect. Integrin αIIbβ3 is essential for platelet aggregation; it binds to the extracellular matrix proteins fibrinogen, fibronectin, vitronectin, thromboposdin-1, and von Willebrand factor. αIIbβ3 recognize a simple peptide sequence, Arg-Gly-Asp (RGD), present in the ligands.

GPIb-IX-V complex is the second most abundant platelet receptor (50,000 copies per platelet) and its deficiency leads to the development of the Bernard-Soulier syndrome, the second most frequent bleeding disorder associated to a platelet receptor deficiency. GPIb-IX-V is a major platelet receptor for vWF, thromboponsdin-1, the leukocyte integrin αMβ2, and P-selectin express on activated endothelial cells or platelets, ligand stimulation induces the subsequent activation of the receptors αIIbβ3 and α2β1, allowing stable platelet adhesion and aggregation, promoting platelet procoagulant activity by binding α-thrombin, factors XI and XII, and kininogen, controling platelet size and shape and regulating platelet removal by interacting with αMβ2 on liver macrophages, a

process underlying rapid clearance from the circulation of refrigerated platelets use in transfusion.

P-selectin (CD62P) function allowing transient weak interactions with carbohydrate ligands expressed on other cells, including endothelial cells and leukocytes.

C1q-Rp (CD93) is strongly expressed in platelets and megakaryocytes. It mediates interactions with collagen and immune complexes.

FcγRIIIA (CD32), is a low affinity receptor for the IgG Fc domain. FcγRIIIA has a role in immunological defense against bacteria, viruses, and parasites. Most polyclonal antibodies against platelet receptors, and many monoclonal antibodies, activate platelets by clustering this receptor.

CD40L (CD154) can interact with CD40 on endothelial cells and cause an inflammatory response. CD40L expressed by CD4+ T cells plays a central role in the development of adaptive immune response and memory generation.

Other receptors include thrombin receptors, ADP receptors, prostaglandin receptors, Platelet activating factor receptor, serotonin receptor, FcεRI, TNF receptor, and serotonin reuptake receptor. Several therapeutic strategies are directed towards the development of platelet agonist receptors inhibitors in order to prevent thrombus formation in patients with high risk of coronary thrombotic disease.

Platelet granules

Platelet granule secretion is a fundamental part of the platelet function releasing important molecules at sites of vascular injury to restore hemostasis,

induce controlled thrombosis, and initiate the vascular remodeling. Size, density, morphology and function of the different platelet granules is determine by the specific molecular content. Megakaryocytes and platelets are the only cells where α-granules and dense granules are found. Approximately, each platelet contain 80 α-granules filled with adhesion molecules P-selectin, von Willebrand factor, thrombopondin, fibrinogen, integrin αIIbβ3, integrin αVβ3, and fibronectin, the chemokines platelet factor-4, MIP-1α, RANTES, MCP-3, CCL17, CXCL1, CXCL5, and IL-18, the coagulation factors V and VIII, the growth and angiogenesis factors basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor, TGF-β, VEGF, and PDGF, and the fibrinolytic factors α2-macroglobulin, plasminogen, and plasminogen activator inhibitor-1. Dense granules are present in the platelet averaging 8-10 granules per platelet. Dense granule content is composed by the small molecules calcium, magnesium, phosphate, pyrophosphate, ATP, GTP, ADP, GDP, and serotonin, and a few proteins like CD63 and LAMP-2. P-selectin is present in both kinds of granules and is surface expression is used as an indication of platelet degranulation.

Platelet attachment and aggregation

Platelet adhesion to exposed subendotelial matrix is the main function in their supporting role in hemostasis. Specific platelet receptors recognize extracellular matrix proteins and agonists, inducing receptor cross-linking, initiating a cascade of intracellular signaling events that ultimately results in the platelet activation, adhesion, and aggregation. Upon platelet activation, conformational changes and assembly of cytoskeleton proteins results in a dramatic change in platelet shape, with extensive formation of membrane projections. Platelet activation also induces the mobilization of platelet granules towards the platelet periphery where

they ultimately fuse and release their contents. Some of the released granule contents act activating other platelets, such as ADP and serotonin. Platelet activation is associated to the receptor αIIbβ3 binding to fibrinogen, interaction that allows platelet aggregation by functioning as a molecular bridge among platelets. Once platelets have attached to the exposed subendothelium, the activated platelet surface induces procoagulant activity, thrombin generation, and formation of stable pallet-fibrin plug with subsequent clot retraction.

During certain flow conditions, platelet interaction with high molecular weight multimers plasma protein von Willebrand factor that associated with collagen of the exposed extracellular matrix, increasing the strength of the binding especially under conditions of high shear stress. Von Willebrand factor multimers posses multiple recognition sites for the GPIb-IX-V complex on the platelet leading to a strong formation of the platelet plug. Von Willebrand factor monomers are unable to cross-link and activate the platelet. In the absence of vascular damage or in conditions of low shear stress, the binding sites of the von Willebrand factor are not exposed, because a molecular change induce by the mechanical force induce by the high shear flow is needed. The antibiotic ristocetin or the snake venom toxins botrocetin and aspercetin are used to induce von Willebrand factor interaction with the GPIb-IX-V complex, this reagents associate to soluble plasma Von Willebrand and force the exposition of the binding sites, and induce platelet aggregation in the circulation. Under low shear conditions, platelet interaction with the subendothelium is mediated predominantly by collagen binding to the platelet integrin αIIbβ3.

Platelet activation leads as mentioned before to the release of the granule contents, which modulates platelet function. Several of the secretion contents

stimulate additional platelets recruiting them into the plug. ADP is the main amplifier of the initial platelet activation through interaction with P2Y-1 and P2Y-12 platelet receptors. Ligand recognition by these receptors induces a rapid calcium mobilization in the platelet cytoplasm, rapid shape change and aggregation. Control of this ADP loop is mediated by the endothelial enzyme ecto-ADPase with hydrolyzes ADP into AMP. Serotonin is a well-known vasoconstrictor, interacts with its receptor 5HT2A and amplifies platelet response as well. Serotonin also increases the procoagulant activity of the activated platelet.

Mechanistically, aggregation of platelets is the defining event preventing the release of plasma fluids into the tissues. αIIbβ3 is the main receptor in the hemostatic plug formation, linking several activated platelets through bridges of fibrinogen. Non-activated αIIbβ3 cannot bind to soluble fibrinogen, however, immobilized fibrinogen can bind to the inactive receptor. (Jurk & Kehrel, 2005; Thijs, Nuyttens, Deckmyn, & Broos, 2010; Watson, 2009)

Platelets and vascular leakage

Blocking vascular leakage by adhering to sites of injury and plugging the opening of the endothelial barrier is the main role of platelets in hemostasis. Binding to the subendothelial von Willebrand factor and collagen through platelet receptors GPIb-IX-V and α2β1 respectively is the key element in the platelet plug formation. A single platelet activation event serves to potentiate the platelet aggregation response and prevent additional blood loss in the initial recovery of hemostasis. Platelet adhesion to the damaged vasculature also constrains the subsequent procoagulant events to prevent systemic activation of coagulation. Attached platelets also actively regulate the propagation of the coagulation

reaction by releasing peptides and small molecules that recruit additional platelets to the growing thrombus, as well as coagulating factors, and expressing specific high-affinity membrane receptors for coagulation proteases, zymogens, and cofactors. The additional recruitment of platelets shields the growing thrombus from the action of inhibitors released by unaffected endothelium. Altogether, these events generate a rapid and strong plug formation that prevents excessive escape of vascular fluids into the tissues, in a strictly localize manner preventing the potential harm to the host by the formation of circulatory thrombosis.

The role of platelets during physiologic conditions, where there is not apparent vascular injure or inflammation, is a little more obscure. Patients with extremely low levels of circulating platelets as in the case of immune thrombocytopenic purpura, an autoimmune disease where the body reacts against their own platelet antigens generating antibodies that ultimately deplete them., are able to spend an entire normal life without being diagnosed, and the disease is only noticed by routine laboratory testing, whereas other patients present frequent bleeding disorder. Individual genetic or behavioral factors are thought to play a role in the different clinical observations. However, animals rendered experimentally thrombocytopenic by an anti platelet treatment of irradiation shown an increase vascular leakage over time. Danielli hypothesized that the antiperameability effect of platelets may be purely mechanical, with platelets filling the openings within the endothelial lining. Several reports by Gimbrone and colleagues (Gimbrone et al., 1969) described the presence of platelets occupying gaps in the endothelium of perfused thyroid glands and absence of vascular leakage if the perfusion was done with platelet-rich plasma, in contrast with the perfusion with platelet-poor plasma. Similar observations

were later done by ultrastructural analysis of the endothelium in patients with severe thrombocytopenia (<15,000 platelet/μL) by Kitchens and Pendergast. (Kitchens & Pendergast, 1986)

The Sphingosine 1-phosphate (S1P) role in maintenance of the vascular integrity is highly recognized by the observation that platelet removal is directly associated with decreased levels of this bioactive molecule in serum. (Gimbrone et al., 1969; Roy & Djerassi, 1972) As today, S1P is known as the major barrier-protective product present in the platelet. (Wang & Dudek, 2009) Platelets supernatants produce a similar barrier-protective effect as S1P in a Gi-dependent manner. More importantly, treatment of platelet supernatants with charcoal to deplete lipids, or intervention s that decrease endothelial expression of S1P1, significantly attenuates the barrier-enhancing effect of platelet supernatants. (Schaphorst et al., 2003)

Taken together, it seems that platelets support the resting vascular endothelium by at least five mechanism: platelets physically block potential gaps in the vascular lining, platelets and platelet components promote growth of endothelial cells, platelets help maintain the endothelium ultrastructure, platelets release soluble factors that enhance the barrier function of the endothelium, and platelets activate and regulate coagulation at the injury sites.

Platelets and immunity

Nowadays platelets are recognized as active mediators of immunity, experimental data have correlated each compartment of an immune response to a possible modulatory activity by platelets.

Leukocyte extravasation

Rolling, attachment, an extravasation of leukocytes during inflammation or in the HEVs of the lymph nodes is accomplished by interactions with the endothelial cell monolayer. Under trauma or certain infections this monolayer is destroyed, which should result in reduced leukocyte infiltration and therefore a deficient control of the infection or slow recovery from the trauma, but this is not the case; upon exposition of the subendothelium (Diacovo, Puri, Warnock, Springer, & von Andrian, 1996) platelets attached to it firmly, forming a thin carpet that blocks the escape of plasma fluids, over this layer leukocytes can roll and transmigrate thanks to the high expression of P-selectin in the activated platelets

Platelets and Toll-like receptors (TLRs)

TLR on professional phagocytes such as neutrophils, macrophages, and dendritic cells. (Janeway, 1989; Medzhitov & Janeway, 2000a; Medzhitov & Janeway, 2000b; Medzhitov & Janeway, 1997a; Medzhitov & Janeway, 1997b; Medzhitov, Preston-Hurlburt, & Janeway, 1997) are germline-encoded proteins that recognize a variety of infectious molecular patterns, and are critical for stimulating immune mechanisms. (Janeway & Medzhitov, 1998; Medzhitov & Janeway, 1997b; Medzhitov & Janeway, 1998a; Medzhitov & Janeway, 1998b; Medzhitov et al., 1997; Medzhitov et al., 1998) However, TLRs are also express by cell not known to be involved in pathogens recognition, like platelets, that was discovered that both murine and human platelets express TLR 1, 2, 4, and 9. (Aslam et al., 2006; Shiraki et al., 2004) This observation suggested that platelets might act as bridges between pathogen recognition and inflammation. (Aslam et al., 2006; Shiraki et al., 2004) Since then, TLR expression on platelets has been shown to be functional and responsible for the LPS-induced thrombocytopenia

and TNF-α production in in vivo experimental models. (Cognasse et al., 2006; Cognasse et al., 2005; Coppinger et al., 2004; Jayachandran et al., 2007; Kuckleburg, McClenahan, & Czuprynski, 2008; Kuckleburg, Tiwari, & Czuprynski, 2008; Ma & Kubes, 2008; Patrignani et al., 2006; Scott & Owens, 2008; Semple, Aslam, Kim, Speck, & Freedman, 2007; Ståhl et al., 2006; Tremblay, Aubin, Lemieux, & Bazin, 2007; von Hundelshausen & Weber, 2007; Ward et al., 2005) This suggested that platelets may be the main player responsible for reactivity against bacterial products, acting as circulating sentinels that recognize pathogenic molecules, neutralizing their effects, and transferring them to macrophages and dendritic cells, initiating the immune response against the pathogen. (Aslam et al., 2006; Clark et al., 2007; Kuckleburg et al., 2008; Kuckleburg et al., 2008)

Platelets and CD40L

CD40 ligand, also known as CD154, is central to the development of memory cellular and humoral immune responses. The recent description of CD40 ligand expression on platelets was a shocking observation that increases the interest of the possible role of platelets in the development or regulation of immune responses. It is known that CD40 ligand plays an important role in the interaction of the platelet with the endothelium and in the development of thrombotic disease. Platelet CD40 ligand interaction with CD40 on endothelial cells triggers inflammation and the systemic release of ICAM, VCAM, and MCP-1. (Gawaz et al., 1998; Henn et al., 1998; Hollenbaugh et al., 1995) After platelet activation, CD40 ligand is exposed in the surface by the degranulation process, and after a period of time it is sheared into the plasma. The functionality of the CD40 ligand in its soluble form still being debated. (Anand, Viles-Gonzalez, Badimon, Cavusoglu, & Marmur, 2003; Hammwöhner et al., 2007; Henn, Steinbach,

Büchner, Presek, & Kroczek, 2001; Inwald, McDowall, Peters, Callard, & Klein, 2003; Prasad et al., 2003) (Anand et al., 2003; Hammwöhner et al., 2007; Henn et al., 2001; Inwald et al., 2003; Prasad et al., 2003) In vitro studies have shown that the soluble form is capable of activate and endothelial monolayer, inducing the expression of adhesion molecules as E-selectin, P-selectin, and the subsequent release of inflammatory cytokines as IL-6 and tissue factor. (Henn et al., 2001; Prasad et al., 2003; Slupsky et al., 1998; Zhou et al., 1998) During infection, most of the soluble CD40 ligand detected in serum originates from platelets, indicating platelets are being activated. (Grewal & Flavell, 1998; Henn et al., 2001)

Elzey et al. (Elzey et al., 2003; Sprague et al., 2008) have proposed an association between platelet expression of CD40 ligand and modulation of adaptive immunity, and demonstrated that this communication is possible and may play a relevant role in immune responses against pathogens in vivo. Such studies also shown that platelets, via CD40L, induce dendritic cell (DC) maturation, (Elzey et al., 2003) and induction of antibody isotype switch of B cells.

Platelets modulation of immune responses

The presence of several molecules on the platelets with well established immune-modulatory functions have encouraged research in the potential role of platelets might play in the development and shaping of immune responses against blood invading pathogens. The largest reservoir of TGF-β in the body is the platelet and changes in the levels of platelets affects the TGF-β levels in serum. (Andersson, Olsson, & Wadenvik, 2002; Andersson, Stockelberg, Jacobsson, & Wadenvik, 2000; Assoian, Komoriya, Meyers, Miller, & Sporn,

1983) Clinical relevance of this finding came from the observation that patients with immune thrombocytopenia have reduced levels of T regulatory cells circulating in blood and upon effective treatment these cells recover to normal levels. (Ling, Cao, Yu, & Ruan, 2007; Liu et al., 2007; Olsson, Ridell, Carlsson, Jacobsson, & Wadenvik, 2008; Stasi et al., 2008; Yu et al., 2008) Deeper studies are needed to clarify this association but due the fact that TGF-β is required for T regulatory cell differentiation is highly encouraging.

As mentioned before, expression of CD40 ligand in the surface of activated platelets has been shown to be able to activate dendritic cells, and to support B cell differentiation and isotype switching in vitro. (Elzey et al., 2003; von Hundelshausen & Weber, 2007) In vivo studies of viral and bacterial infections of CD154 deficient mice that received transfusions of CD40 ligand competent platelets showed increased IgG antibody and CD8 T cell specific responses and the generation of protective memory upon rechallenge. (Elzey et al., 2008; Iannacone et al., 2005; Sprague et al., 2008) Mechanistically, these observations might be through the increased activation of the antigen presenting dendritic cells or direct signaling on the B and T cells is not fully understood but it clearly shows the compensatory effect of CD154 expression on the platelet in the modulation of adaptive immunity. (Kissel et al., 2006) Furthermore, P-selectin promotes the Th-1-like immune response, (Austrup et al., 1997) potentially relevant in immune-related inflammation. On the other hand, platelets affect the development of immunopathology by enhancing tissue infiltration by cytotoxic T cell into the liver in a model of murine hepatitis. (Iannacone et al., 2005)

There has been other recent recognition of the role platelets may play in the immune response; they facilitate lymphocyte homing and trafficking through

secondary lymphoid organs by expressing copious amounts of S1P, which regulates the lymphocyte exit from the lymph exit from these organs (Diacovo et al., 1996)

Platelet leukocyte aggregates and extracellular traps

Septicemia is the clinical term use to define the systemic manifestations observed in patients with bacteria or the bacterial endotoxin LPS in blood. One of the main characteristics of septicemia is a significant neutrophil activation that provokes neutrophil accumulation in the lung capillaries and in the sinusoids of the liver; that ultimately leads to dysfunction and total organ failure. (McClenahan, Evanson, Walcheck, & Weiss, 2000; Welbourn & Young, 1992) It has been suggested that neutrophil sequestration prevent them from reaching other infected tissues favoring the infectious agent. (Clark et al., 2007; Kuckleburg et al., 2008; Kuckleburg et al., 2008). Other researchers have proposed the opposite scenario, where activated neutrophils trapped in small capillaries are a host defense mechanism relevant for the rapid removal of the invading pathogens. (Andonegui et al., 2005; Kuckleburg et al., 2008). Interestingly, platelets and platelet-antigens are consistently found at the sites of neutrophil accumulation, and when neutrophils have been experimentally removed in animal models, platelet accumulations are not seen, suggesting that the neutrophils are essential for the platelet recruitment. (Andonegui et al., 2005; Kuckleburg et al., 2008) Several mechanisms can be drawn to explain such observation, but the more probable are a direct interaction of the platelet with the activated neutrophils or that platelet recruitment activates the endothelial layer, which became adhesive to platelets. Several experimental and human observations indicate that platelet specific agonists induce the formation platelet-neutrophil aggregates and that in septicemic patients such aggregates can be

detected by flow cytometry in circulating blood. (Larsen et al., 1989; Peters, Heyderman, Hatch, & Klein, 1997; Zarbock, Polanowska-Grabowska, & Ley, 2007) (Andonegui et al., 2005; Gawaz, Dickfeld, Bogner, Fateh-Moghadam, & Neumann, 1997; Mavrommatis et al., 2000) In an recent and interesting publication, Clark and colleagues observed that LPS or plasma from septic patients were able to interact and activate platelets through the TLR on the platelet and induce platelet attachment to immobilized neutrophils. (Clark et al., 2007; Kuckleburg et al., 2008) Such platelet-neutrophil interaction in the contest of human septicemia is though to increase the capacity to remove bacteria from the blood stream and potentiate the release of inflammatory cytokines. (Nathan, 2006; Youssefian, Drouin, Massé, Guichard, & Cramer, 2002) (Nathan, 2006) The proposed mechanism of the enhance bacterial removal by the platelet–neutrophil interaction is that platelets give to the neutrophil necessary activation signals that induce complete stimulation that in addition to neutrophil degranulation, the stimulated neutrophils release their DNA content to form DNA-structures known as neutrophil extracellular traps (NETs) (Brinkmann et al., 2004) that extend out into the small lumen of the capillaries where the platelet–neutrophil aggregates were recruited. The NET formation in liver sinusoids and lung capillaries where platelets and neutrophils bound trapped and removed circulating bacteria. (Clark et al., 2007) This process was beneficial for the host initially, although prolonged NETs formation may injure the host. Platelet or neutrophil removal reduced endothelial damage in the lungs. (Clark et al., 2007)

Platelet and organ rejection

Platelet aggregates are commonly found in the vasculature of transplanted organs undergoing acute rejection. More than half of renal biopsies of acutely rejected transplanted kidneys stained positive for the platelet marker CD61 and

von Willebrand factor inside the capillaries. (Meehan et al., 2003; Nakashima, Qian, Rahimi, Wasowska, & Baldwin, 2002; Ota et al., 2005; Wasowska et al., 2001) Furthermore, in a mouse model of allograft vascularized-heart transplant platelet-derived CD40 ligand or purified CD40 ligand trimers could induce rejection in CD40 ligand knockout mice. (Xu, Zhang, Mannon, & Kirk, 2006) Additionally, antibody-mediated blockade of platelet derived CD40 ligand prevented allostimulation of platelets. Interestingly, CD40 ligand expressing platelets infused at the time of surgery were shown to traffic to the transplanted organ and the spleen accelerating rejection, while platelets infused 30 days after engraftment fail to induce rejection. Therefore, it was proposed that the surgical trauma promoted platelet activation with expression and release of CD40 ligand, which mediated the effects on the cell-mediated alloimmune responses. Recent evidence suggested that CD40 ligand could influence cellular immunity through interaction with Mac-1, an integrin expressed on monocytes, neutrophils, and NK cells. Mac-1 is a complement receptor known to facilitate macrophage migration, leukocyte diapedesis, enhance phagocytosis, and cytotoxicity. Furthermore, activated platelets express and secrete many other chemotatic factors that can guide lymphocytes to sites undergoing inflammation. Released of small vasoactive molecules like ADP, calcium, histamine, serotonin, and epinephrine promote leukocyte infiltration, stimulate vasoconstriction, and leukocyte diapedesis. (Kirk, Morrell, & Baldwin, 2009; Léveillé et al., 2007; Li et al., 2008; Ross, 2000; Xu et al., 2006; Zirlik et al., 2007)

Platelets and pathogens

Platelets are the second most abundant cell population in blood with human levels in the range of 150,000 to 350,000/μL, even though, only a small fraction (>10,000/μL) of these are sufficient to prevent bleedings in non-surgical or

severe trauma conditions. Platelets appeared in nature as a division of functions from thrombocytes in early vertebrates, into immune cells and hemostatic cells, explaining the presence of important immune molecules in human platelets. Therefore, several groups have proposed that platelets may have an unappreciated sentinel function of pathogen invasion of the systemic circulation.

Platelets and pathogen interactions

Bacteria

More than forty years ago was reported in the scientific literature the first experimental association of a bacterial infection with platelets, where it was shown that bacteria were able to induce platelet aggregation and degranulation. (Clawson, 1973; Clawson & White, 1971a; Clawson & White, 1971b; Clawson, Rao, & White, 1975) Released inflammatory cytokines, and direct or indirect platelet activation by the bacteria or by bacterial secreted toxins, were proposed in those early observations as the mechanism responsible. Nowadays it is known that platelet interaction with bacteria could be by a direct mechanism where platelet receptors interact with the bacterial surface or indirectly, by bacterial interaction with serum proteins that are the ligands for platelet receptors. (Fitzgerald, Foster, & Cox, 2006) The mechanism driving the platelet bacterial interaction determines the kinetics and strength of the platelet activation and aggregation, but it is also controlled by the amount of bacteria or bacterial components present in the platelet suspension, which determines if platelet activation is reached or it insufficient, there is not intermediate responses. However, once the activation process has begun, difference in the bacterial composition determines the speed of platelet aggregation, which varies greatly from isolate to isolate. Streptococcus sanguinis and Staphylococcus aureus

induce a short lag time for platelet aggregation and directly attach to platelets, Helicobacter pylori have a short lag time and support indirect platelet interactions, S. pneumoniae (tigr caps 4) and S. sanguinis (B10.18) strains induce platelet aggregation with short lag time and are non-adhesive, strains that induce platelet aggregation with long lag time and support direct adhesion include Streptococcus gordonii (DL1) and S. sanguinis (M108), strains of bacteria that induce platelet aggregation with a long lag time and are non-adhesive are S. gordonii (M99), S. sanguinis (NCTC7863) and S. pneumoniae (R6x)], strains that do not induce platelet aggregation but do support direct platelet adhesion are S. gordonii (Black- burn), strains that do not induce platelet aggregation but do support platelet adhesion by an indirect interaction as H. pylori (J104) does, and finally strains that do not induce platelet aggregation or support platelet adhesion S. sanguinis (SK96), S. gordonii (Channon). (Ford et al., 1997; Herzberg, Brintzenhofe, & Clawson, 1983a; Kerrigan et al., 2002; Kerrigan et al., 2007; O'Brien et al., 2002; Plummer et al., 2005)

Streptococci and S. aureus are the most common etiological agents of infective endocarditis. Oral cavity trauma introduces the bacteria in the blood stream and colonizes the heart valves. (Ford et al., 1997; Kerrigan et al., 2002; Kerrigan et al., 2007; O'Brien et al., 2002; Pampolina & McNicol, 2005; Siauw et al., 2006)Streptococcus M protein is able to interact with antibodies and complement inducing platelet aggregation. (Beachey & Stollerman, 1971) (Herzberg & Brintzenhofe, 1983; Herzberg et al., 1983a; Herzberg, Brintzenhofe, & Clawson, 1983b; Kurpiewski, Forrester, Campbell, & Barrett, 1983; Sullam, Payan, Dazin, & Valone, 1990) Most studies of bacterial interaction with platelets have been carried out in vitro, a common finding in human thrombi are streptococci bacteria trapped within platelet aggregates. Furthermore, it has been

described the streptococcal platelet associated activating glycoprotein (PAAP) which contains several collagen-like binding motives, inducing platelet activation and aggregation through a yet unknown platelet receptor. An interesting observation is that platelet from patients with Bernard-Soulier syndrome, which lacks expression of GPIbα fail to aggregate in response to S. sanguinis. (Plummer et al., 2005) Mutants of the serine-rich bacterial glycoprotein SrpA showed prolonged aggregation lag times, which might suggest that perhaps, antibody binding and complement assembly might be required. (Ford et al., 1997; Ford, Douglas, Heath, Rees, & Preston, 1996; Kerrigan et al., 2002) Depleting S. sanguinis specific antibodies from plasma significantly inhibited platelet aggregation and decreased FcRIIa phosphorylation. (McNicol et al., 2006) (Pampolina & McNicol, 2005)

S. gordonii induce platelet aggregation through GspB, Has, SspA, and SspB through the recognition of GPIbα. (Bensing & Sullam, 2002; Bensing, López, & Sullam, 2004; Jakubovics et al., 2005; Kerrigan et al., 2007; Takahashi, Sandberg, Ruhl, Muller, & Cisar, 1997; Takamatsu et al., 2005) (Nobbs, Shearer, Drobni, Jepson, & Jenkinson, 2007; Takamatsu et al., 2005; Yajima, Takahashi, & Konishi, 2005) Deletion of GspB, SspA, SspB, and Hsa from S. gordonii reduces platelet adhesion by 50% but abolishes platelet aggregation, suggesting a multifactorial mechanism (Kerrigan et al., 2007)

Several other bacteria induce platelet aggregation in an antibody-dependent or fibrinogen manner (Kurpiewski et al., 1983; Zimmerman & Spiegelberg, 1975) The M1 protein of S. pyogenes binds fibrinogen in the serum forcing its recognition by the platelet GPIIb/IIIa, anti-M1 antibodies bring to close proximity to the FcRIIa then induce the platelet activation. (Shannon et al., 2007) Strong

aggregation induced by S. pyogenes proteins, fibrinogen and antibodies are strictly necessary to accomplish thrombi formation under conditions of high shear stress. (Sjöbring, Ringdahl, & Ruggeri, 2002) Study platelet aggregation by Staphylococcus bacteria has been difficult to study due to the expression of toxins that lyses the platelets, however it is known that the protein A being recognized by antibodies activate platelets through interaction with the FcRIIa (Hawiger et al., 1979) and fibrinogen bridges through the bacterial clumping factor A (ClfA) (Herrmann, Lai, Albrecht, Mosher, & Proctor, 1993; Sullam, Bayer, Foss, & Cheung, 1996) The expression of platelet aggregation factors by the bacteria is regulated by the life cycle of the bacteria where ClfA is the dominant protein mediating platelet aggregation during the stationary phase whereas fibronectin binding proteins (FnBPA and FnBPB) are the most dominant during the exponential growth phase. However the expression of these factors in vivo are not yet known. (Loughman et al., 2005) Additionally, the protein A of Staphylococcus bacteria (SpA) is able to directly bind to the platelet complement receptor C1qR/p33, although this receptor is located inside resting platelets (Nguyen, Ghebrehiwet, & Peerschke, 2000) Helicobacter pylori infection is associated with cardiovascular disease (Kurose et al., 1994), and clinical strains of H. pylori induce platelet aggregation in vitro by binding plasma vWF that in turns bind to platelet GPIbα triggering activation, that was not observed if platelets from Bernard-Soulier patients were used. (Byrne et al., 2003; Corcoran et al., 2007)

In addition, platelets contain microbicidal substances such as the bactericidal peptide PMP within their granules (Koo, Bayer, Sahl, Proctor, & Yeaman, 1996), and molecules similar to PMP called thrombocidins have also been shown in the granules of human platelets. (Koo et al., 1996)

Virus

1959 seems to be the year that the first physical association of platelets with viruses was described in the scientific bibliography, it consisted of an electron microscopy analysis of human platelets incorporating influenza virus particles in the platelet surface connected canalicular system (SCCS), Since then additional studies demonstrated that microorganism are engulfed directly into the specific platelet compartments, immunologically distinct form the SCCS. (Youssefian et al., 2002) However, it is not yet clear whether these interactions favor the transport and spread of the infectious agent or instead, platelets are playing a protective role by removing the virus and taking it to the immune cells in the spleen. The current thinking is that platelet interactions with viruses are protective but certain pathogens have exploited the interaction to increase its virulence. More recent research has extended this observation to several other viral pathogens as vaccinia, dengue and HIV. (Bik, Sarov, & Livne, 1982; Noisakran et al., 2009; Youssefian et al., 2002)

In vitro ultrastructural studies of human platelets incubated with HIV infected PBMCs showed attachment and internalization of HIV like particles to the inside of platelets, but not a single image showed fusion of the virus and platelet lipid membranes. The viral particles were mostly observed in small endocytic vacuoles and in the SCCS, however, particles present in the SCCS had an altered morphology, being swollen and poorly delimited, with irregular shape. (Youssefian et al., 2002) Further analysis showed similar interactions in platelets from HIV seropositive patients with high viral titers. (Youssefian et al., 2002) Platelet association with viral particles induced platelet activation as demonstrated by surface expression of P-selectin. The authors proposed that platelet endocytosis of HIV virions protects the virus form the attack of the

immune response, and may transport the virus through the entire body, and by inducing platelet activation and attachment to blood leukocytes and endothelia in peripheral tissues facilitates its spread. Recently, a specific virus receptor, coxsackie-adenovirus receptor (CAR), has been identified on platelets. (Othman, Labelle, Mazzetti, Elbatarny, & Lillicrap, 2007)

Furthermore, evidence of indirect interactions of platelet with viruses is abundant in the literature, mainly mediated by the recognition of virus-IgG immune complex by the platelet receptor FcgR2A (CD32). Intravenous injection of large quantities of adenovirus serotype 5 in humans (for gene therapy) or mice induces a transient thrombocytopenia, and even though the virus does not induce platelet aggregation in vitro, it binds to platelets in vivo causing their activation, aggregation, and sequestration by kupffer cells in the liver sinusoids. (Eggerman, Mondoro, Lozier, & Vostal, 2002; Stone et al., 2007) It was later probed that the platelet removal induced by the adenovirus was dependent on scavenger receptors, natural antibodies and complement. (Xu, Tian, Smith, & Byrnes, 2008) Epstein Barr virus is also able to bind and activate platelets in vitro inducing the release of TGF-β. Human platelets express the CR2 complement receptor, which besides recognizing C3d acts as the receptor for Epstein Barr virus. (Ahmad & Menezes, 1997; Nunez, Charriaut-Marlangue, Barel, Benveniste, & Frade, 1987) Furthermore, it has been proposed that hepatitis C virus indirectly binds GPVI, potentially playing a role in viral transport and persistence of the infection. (Zahn, Jennings, Ouwehand, & Allain, 2006) Another important virus receptor present on the platelet membrane is DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin) which mediates HIV infection of dendritic cells, by recognizing pathogenic mannose-type carbohydrates from commonly found on viruses. (Boukour, Massé, Bénit, Dubart-Kupperschmitt, &

Cramer, 2006; Feinberg, Mitchell, Drickamer, & Weis, 2001) DC-SIGN cell expression mediates HIV-1 internalization and infection, however, its interaction preserves HIV infectivity of the viral particle until a permissive CD4 T lymphocytes is infected. (Geijtenbeek et al., 2000a) (Moris et al., 2004) DC-SIGN binding to ICAM-3 plays an important role in establishing the first contact between dendritic cells and resting T cells. (Geijtenbeek et al., 2000b) Megakaryocytes express the HIV receptor CD4 and the co-receptors CXCR1, 2, 4, and CCR3, however platelets only express the co-receptors. (Basch, Kouri, & Karpatkin, 1990; Rivière et al., 1999) Recently, it was reported that by blocking DC-SIGN and CLEC-2 with antibodies, in vitro association of HIV-1 particles with the platelets was avoided, strongly supporting a role for these receptors in the platelet interaction with HIV-1. (Chaipan et al., 2006)

Parasites

Malaria parasites bind to GPIV (CD36), which is a scavenger receptor present in macrophages, dendritic cells, endothelial cells, platelets, and several other cell types. GPIV was shown to be important in the interaction of the *Plasmodium falciparum* infected erythrocytes with the endothelium and in platelets where it may induce aggregation. (Barnwell et al., 1989; Ockenhouse, Magowan, & Chulay, 1989; Ockenhouse, Tandon, Magowan, Jamieson, & Chulay, 1989) In a recent report a platelet clustering around *Listeria major* migrating towards the peritoneum was observed in mice, this platelet activation was shown by platelet depletion studies attracts a GR1+ monocytes population. (Goncalves, Zhang, Cohen, Debrabant, & Mosser, 2011)

Malaria infections in endemic areas represents a challenge for the adaptive immune response because reinfections are usual with isolates that share poor

cross-reactivity, therefore innate control of the parasite, specially its erythrocytic phase, is of high relevance for the severity of the disease. In a recent report by McMorran and colleagues (McMorran et al., 2009) was shown that animals with one tenth the normal levels of circulating platelets (*Mpl*$^{-/-}$) were more susceptible to death that normal mice. The further demonstrate that platelets bind preferentially to infected erythrocytes, which interaction induced the destruction of the intraerythrocytic parasites. (McMorran et al., 2009)

Platelets and LPS shock syndrome

The Gram-negative bacterial endotoxin LPS (lipopolysaccharide) in high concentrations in blood induces a toxic shock syndrome, characterized by a massive dysregulation of coagulation. (Stevens, 2001) This results in deposition of thrombi in the microvasculature and a consumption of coagulation factors and platelets, with a secondary risk for bleeding. Thrombocytopenia is a central finding, and the severity of disease correlates with lower platelet counts. (Baughman, Lower, Flessa, & Tollerud, 1993; Mavrommatis et al., 2000) Leukocyte and platelet activation are frequently reported, and speculated to contribute to the development of disseminated intravascular coagulation (DIC) and multiple organ failure, as blood flow and oxygen delivery is reduced. (Gawaz et al., 1997; Gawaz, Fateh-Moghadam, Pilz, Gurland, & Werdan, 1995a; Gawaz, Fateh-Moghadam, Pilz, Gurland, & Werdan, 1995b). CD62P and platelet microparticles as markers of recent platelet activation are increased on septic patients. (Jacoby et al., 2001; Ogura et al., 2001) However, less platelet–neutrophil adhesion was observed in patients with multiorgan failure than in septic patients without organ failure; probably due to sequestration of the conjugates in the vasculature, thereby contributing to the development of organ failure (Gawaz et al., 1995a; Gawaz et al., 1995b). Furthermore, platelet

aggregation during sepsis is reduced. (Vincent, Yagushi, & Pradier, 2002) Bacterial and bacterial products have different effects on platelets in vitro and some know agents of sepsis do not affect platelet function in vitro. (Boldt, Menges, Wollbrück, Sonneborn, & Hempelmann, 1994; Isogai et al., 1989; Matera, Falzarano, Berrino, & Rossi, 1992; Saba, Saba, Morelli, & Hartmann, 1984; Sheu et al., 2000). This points out to a complex defect exerted on the platelet during septic shock, where in some infections the bacteria affects the platelets whereas in other infections the main player in the platelet dysfunction seen to be host factors responding to the invading agent.

Pathogens and thrombocytopenia

Lymphocytic choriomeningitis virus

LCMV induced thrombocytopenia is probably the best-understood infectious animal model so far. In a series of studies by Binder and colleagues demonstrated that upon murine infection with LCMV, a failure of the bone marrow production of several hematopoietic cells was the consequence of direct virus infection of progenitor cells and cell dead by an interferon mediated mechanism. LCMV infection of mouse deficient in interferon signaling recovered all the cellular blood parameters, including platelets, reticulocytes, and leukocytes. The initial bone marrow aplasia was directed by the action of IFN-α/β, whereas the deficiency in chronic infections was dependent on the effects of IFN-γ, TNF-α, and lymphotoxin-α secreted by cytotoxic T lymphocytes. Subsequent exhaustion of the CD8+ T lymphocytes abrogated the bone marrow aplasia. (Binder et al., 1997; Binder et al., 1998) Even though, these studies were compelling, there are several indications that there might be other factors influencing the thrombocytopenia, as platelet function is decreased and signs of

intravascular platelet activation is observed. (Iannacone et al., 2005; Iannacone et al., 2008; Lang et al., 2008)

HIV

HIV infection is frequently associated with thrombocytopenia in the presence or absence of AIDS, affecting up to 40-50% of patients at some time during infection. (Karpatkin, Nardi, & Hymes, 1988; Liebman, 2008; Savona, Nardi, Lennette, & Karpatkin, 1985) (Murphy et al., 1987; WALSH, KRIGEL, LENNETTE, & KARPATKIN, 1985) Several observations indicate that the main mechanism responsible for the platelet drop is the formation of platelet-antibody complexes. Patients strongly respond to common treatments for autoimmune thrombocytopenia like corticosteroids, IV gammaglobulin, and splenectomy. Presence of immune complexes in the blood, increased megakaryocytes in the bone marrow, no splenomegaly, negative antinuclear antibodies, and non-clinical indications of other disorders known to cause thrombocytopenia further confirm its etiology. (Auch et al., 1987; Ehmann, Rabkin, Eyster, & Goedert, 1997; Gessain et al., 1985) The mean platelet survival time in these patients is less that a day compared to 8 to 10 days in control-infected groups. (Bel-Ali, Dufour, & Najean, 1987) Treatment of thrombocytopenic HIV seropositive patients with antiretroviral drugs drastically improves the platelet counts and induces a 64-fold drop in the levels of HIV genomes in the blood. (Arranz Caso, Sanchez Mingo, & Garcia Tena, 1999; Bel-Ali et al., 1987; Hymes, Greene, & Karpatkin, 1988) This last observation might as well indicate that HIV abrogates platelet production in the bone marrow by infecting precursor cells, megakaryocytes or stromal cells. (Louache et al., 1992; Stella, Ganser, & Hoelzer, 1987; Zauli et al., 1996; Zauli et al., 1992)

Dengue

Concomitant with endothelial permeability in dengue infection is a marked thrombocytopenia. However, the exact cause for the drop in platelet numbers, seen often in dengue fever and always in dengue hemorrhagic fever, is unclear. It has been proposed that dengue infection results in the transient suppression of hematopoiesis, presumably to limit the damage to progenitor cells during the elimination of the infected cells. (La Russa & Innis, 1995) This is supported by the finding that growth and division of cord blood mononuclear cells was inhibited by dengue infection, via the effects of macrophage inflammatory protein-1. (Murgue, Cassar, Deparis, Guigon, & Chungue, 1998) However, it has not been confirmed that this also involves megakaryocytes and the production of platelets. In a small study of biopsy and autopsy samples from dengue patients, megakaryocytes were not infected with dengue. (Jessie, Fong, Devi, Lam, & Wong, 2004) Alternatively, it has been demonstrated that dengue virus binds to platelets in the presence of virus specific antibodies, suggesting that immune mediated clearance may account for the observed thrombocytopenia. (Wang, He, Patarapotikul, Innis, & Anderson, 1995)

A similar mechanism of immune- mediated clearance, initiated by anti-virus antibodies cross-reacting with platelets, has also been proposed. (Lin et al., 2001) An analysis of patient sera from a Taiwanese outbreak revealed the presence of anti-NS1 antibodies, IgM but not IgG, which cross-reacted with human platelets. (Lin et al., 2001) The antibodies were shown to not only be able to induce complement-mediated lysis but also to inhibit platelet aggregation. (Lin et al., 2001) The levels of these autoantibodies were higher in DHF patients than in DF patients and persisted for several months after illness. (Lin et al., 2001) Other studies have demonstrated that anti-platelet antibody was also

produced in a mouse model of dengue infection and thrombocytopenia. (Huang et al., 2000) Anti-dengue antibodies which cross-react with other components of the coagulation system including plasminogen (Chungue et al., 1994; Markoff, Innis, Houghten, & Henchal, 1991) and fibrinogen (Falconar, 1997) and also endothelium (Lin et al., 2002) have been described. Immune-mediated clearance, and other mechanisms, could result in the disruption of the coagulation system as suggested by the higher tissue-type plasminogen activator to plasminogen activator inhibitor ratio seen in DHF patients. (Huang et al., 2001; Rothman, 2011)

Together, these studies suggest an immune-mediated clearance of platelets similar to that in the autoimmune disease thrombocytopenic purpura. In this disease antibody against platelets leads to their clearance via Fc receptor mediated phagocytosis by macrophages, particularly in the spleen. (Semple, 2003) Interestingly, chronic autoimmune thrombocytopenic purpura is associated with elevated concentrations of IL-2, IL-10, and IFN-γ in the serum. (Semple et al., 1996) While anti-platelet antibodies in dengue patients appear to persist beyond illness, it is perhaps only in the presence of high concentrations of proinflammatory cytokines and mediators and activated complement, as in DF and DHF, that they lead to platelet clearance. The clearance of platelets may then contribute to vascular leakage following cytokine and mediator-induced endothelial permeability.

Malaria

Cerebral malaria is the most serious form of the disease; responsible for 80% of the 1 million annual deaths reported. *Plasmodium falciparum* infections are responsible for the majority of the cerebral cases. The exact causes are not

known but it occurs when infected red blood cells occlude cerebral blood vessels. In a recent study, human brain blood vessels stained positive for platelet specific markers, showing that there is significantly higher platelet accumulation in the brains of patients with cerebral malaria compare with the uninfected control, this indicates that platelets may be probably part of the pathogenesis of malaria. In fact, a drop in platelet count is a common feature in malaria and considered diagnostic in febrile suspects, and the extension of the thrombocytopenia is a predictor of outcome and severity in children. The causes of the thrombocytopenia are obscure but seem to be due to a decreased production and increase consumption of platelets. Because the effect of a decrease production will take time to develop it is understood instead as ineffectiveness to replenish the platelet lost by the platelet activation, as indicated by elevated levels of soluble P-selectin, platelet microparticles, platelet factor-3, and platelet aggregates present in patients with malaria, or by immune mediated consumption as antibodies associated to the platelets are frequently found.

VHFs

Most viral infections in humans present an acute mild thrombocytopenia. In the case of viral hemorrhagic fever infections thrombocytopenia is always present. The magnitude of the thrombocytopenia varies depending on the pathogen and the severity of the infection. Decrease platelet production or increased platelet consumption are thought to be the leading mechanisms towards low platelet counts. The analysis of bone marrow from infected patients supports the decrease production and increased platelet consumption whereas platelet kinetic analysis indicate a decrease platelet survival in the periphery. (BIERMAN & NELSON, 1965; La Russa & Innis, 1995; Mitrakul, Poshyachinda, Futrakul, Sangkawibha, & Ahandrik, 1977; Na-Nakorn, Suingdumrong, Pootrakul,

& Bhamarapravati, 1966) For example, studies of human cases of hanta fever with renal syndrome showed decreased survival times of platelets, increased giant platelets in the circulation, and decreased numbers of bone marrow megakaryocytes. Bone marrow megakaryocytes from Argentine hemorrhagic fever cases shown evidence of direct virus infection and decreased platelet production by high levels of IFN-α/β. The early development of thrombocytopenia in humans suggests that adaptive immune mechanisms are not directly responsible for the thrombocytopenia. (de Bracco et al., 1978; Carballal et al., 1981) Lassa infections also suggest the presence of the previously mentioned mechanisms but also suggest the presence of a platelet-function inhibitor in serum, most likely an inflammatory cytokine. (Lange et al., 1985) Bunyavirus infections of humans show a dramatic bone marrow aplasia. (Swanepoel et al., 1989; Tikriti et al., 1981) The collective data for intravascular coagulation during VHFs indicates that this process is not a significant factor leading to the early platelet consumption but it is more likely the result of subsequent bacterial infections.

Even though the human cases of these special pathogens are limited to draw accurate conclusions, a common mechanistic picture can be drawn to explain the thrombocytopenia. These zoonotic viruses may infect the bone marrow (directly the megakaryocytes or stromal cells) inducing a strong inflammatory reaction that induce the arrest of the megakaryocyte development, secondly, the viruses may induce platelet sequestration in the periphery by activating the endothelium, or inducing platelet activation or destruction by deposition of immune complexes, and third, a soluble molecule released in serum during these infections with platelet inhibitory potential induce the shortening of the half life of the circulating platelets. (Chen & Cosgriff, 2000)

Pathogens and hemostasis

The amount of scientific literature is not equally distributed among these interactions as illustrated in the diagram number 2, where the weight of the connecting arrows represents the amount of literature found. Therefore, the amount of research involving the interactions between pathogens and hemostasis are highly limited.

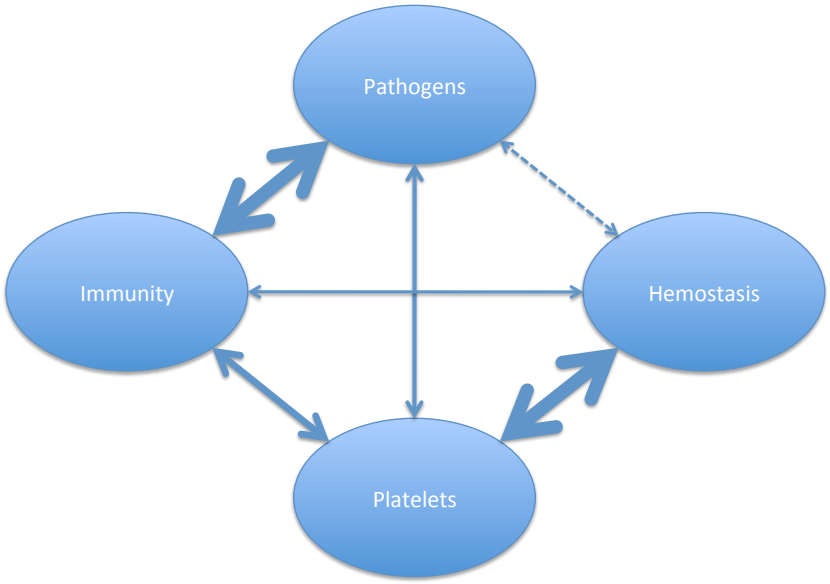


Diagram 2.

Inflammation and hemorrhages

Inflammatory processes during thrombocytopenia are strong inducers of hemorrhages. (Ho-Tin-Noé, Demers, & Wagner, 2011; Ho-Tin-Noé, Goerge, & Wagner, 2009; Wagner & Frenette, 2008) Experimentally induced thrombocytopenic mice exposed to inflammatory agents show a break of the endothelial support of the hemostatic confinement of plasma fluids and erythrocytes into the interstitial tissues at the specific anatomical sites were the

inflammation was being developed. Control of the bleeding reaction was recovered after a mild platelet transfusion able to replenish 10% of the circulating platelets. (Goerge et al., 2008) Similar situations are constantly observed in humans were patients with a tenth of circulating platelets show no sign of spontaneous bleedings and under local inflammation, as in the case of sunburn, develop localized petechial bleedings. (Carbo, del Conde, & Duerschmied, 2009) Bleedings appeared as fast as 20 minutes after the application of the inflammatory process indicating the continuous need for platelets to maintain the vascular integrity. During inflammation several bioactive molecules with potential hemorrhagic activity are released, pro inflammatory cytokines, matrix metalloproteases, serine proteases, and reactive oxygen species can alter the vascular integrity. Platelet may prevent hemorrhages by directly plug the openings in the vasculature or through the release of their granule contents release during the rolling process on the inflamed endothelium. Some of the granule contents, which potentially may mediate the protective activity, include serpins, tissue inhibitors of metalloproteinases (TIMS) and reactive oxygen species scavengers, S1P, serotonin and vasoendothelial growth factor. (Ho-Tin-Noé, Goerge, Cifuni, Duerschmied, & Wagner, 2008)

Tumor growth in thrombocytopenia

During solid tumor growth, adequate nutrient and oxygen supply are critically regulated in order to satisfy the enhanced tumor metabolism by means of angiogenic development of new blood vessels. These newly formed blood vessels are fragile in terms of vascular permeability, due to its immature state, flow irregularity, constant tumor remodeling, and constant leukocyte infiltration. Therefore, platelet support of vascular permeability in a tumor environment is of vital relevance for the continuous growth of the tumor. Experimentally, solid

tumor growth in mice rendered severely thrombocytopenic suffered massive bleedings exclusively at the tumor sites, which translated in tumor cell dead and necrosis. (Ho-Tin-Noé et al., 2009; Ho-Tin-Noé et al., 2008) Interestingly, tumor-bleeding protection by platelets was independent of their main aggregating receptors, suggesting a mechanism independent of thrombi formation to prevent hemorrhages. Supporting this, intra vital microscopy of tumor vessel with fluorescently labeled platelets, did not reveal thrombi formation, and degranulated platelets transfused into thrombocytopenic animals fail to prevent the development of the bleedings. Once a platelet is activated, soluble and membrane bound bioactive molecules are released or exposed on the platelet membrane, which could protect the newly formed blood vessels. (Ho-Tin-Noé et al., 2009; Ho-Tin-Noé et al., 2011; Ho-Tin-Noé et al., 2009; Ho-Tin-Noé et al., 2008)

Hemorrhages in VHFs

Our results

In this dissertation, we are the first ones proposing the pathogen strategy of dismantling the hemostasis in order to escape control by the immune system due to the necrosis of the secondary lymphoid organs by uncontrolled release of fluids into the interstitial tissues and poor oxygen supply.

Chapter 3: Materials, methods, and systems optimization

Mice, viruses, and infections

Five to eight week old female C57BL/6J mice were purchased from The Jackson Laboratory. LCMV Armstrong and clone-13 isolates were obtained from the laboratory of Dr. Rafi Ahmed and triple plaque purified; viral stocks were prepared from infected (0.01 m.o.i.) BHK-21 cell supernatants 72 hours after infection. Vesicular stomatitis virus (VSV) was obtained from Dr. David Masopust and grown in BHK-21 cells. Titers from supernatants, serum, or homogenized tissue samples were determined by standard plaque assays on Vero cells. All infections were done intravenously with 2x106 p.f.u. of the specific virus, as indicated in the results section. All infections were carried out under ABSL-3 confinement at Yerkes National Primate Center and performed in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

Platelet depletions, platelets counts, and thrombopoietin treatment

Platelets were depleted by intraperitoneal injection into the lower left abdominal quadrant of 0.2 mL of antibodies against GPIIb (MWReg30, BD Biosciences), GPIbα (R300, Emfret, Germany), or Aspercetin (isolated as previously described) (Rucavado et al., 2001) diluted to concentrations indicated in the results section. Capillary blood was collected directly from the tip of the tails of the mice into a BD Unopette System Test to count platelets in a hemocytometer according to the manufacturer's instructions. Recombinant mouse thrombopoietin (TPO) (R&D) produced in murine myeloma cells was injected intravenously at 5 days and 1 day prior to infection.

Flow cytometry and live/dead discrimination

Spleens were collected from animals and, following collagenase IV digestion and lysis of red blood cells, single cell suspensions were counted and stained with fluorochrome-conjugated antibodies obtained from BD Pharmingen, eBioscience, and Biolegend. Flow cytometric analysis was performed on LSR-II or FACSCalibur flow cytometers (BD Biosciences), and data was analyzed using FlowJo software (TreeStar). Live/dead discrimination was performed using the amine-reactive dye Alexa Fluor 430 carboxylic acid-succinimidyl ester, using protocols adapted from the manufacturer (Invitrogen). Splenocytes from naïve mice were used to set the gates at zero percent necrosis.

Histology and Immunofluorescence microscopy

Tissues were collected from sacrificed animals, formaldehyde-fixed, sectioned, and stained with H&E. Spleens were removed and frozen in OCT (Tissue Tek). Six µm cryostat sections were fixed in ice-cold acetone, air dried and stained with monoclonal antibodies to F4/80, B220, Thy1.2, and CD11c from eBioscience to ER-TR9, ER-TR7, and MOMA-1 from Acris GmbH (Germany), and to β3 integrin from BD Pharmigen. Guinea pig polyclonal sera anti-LCMV was used to visualize viral antigens. Images were obtained using a Zeiss Axioscope Z.1 microscope equipped with a Zeiss Imager 2.1 camera.

Peptide stimulations, tetramer staining, and VSV-neutralizing antibodies

Intracellular staining for IFNγ and TNFα after 6 hrs in vitro stimulation with 2.5 µM GP33, GP276, or NP396 peptide in the presence of 1µL/mL of Brefledin A was performed using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmigen). MHC class I tetramers were prepared and used as described. (Murali-Krishna et al., 1998) VSV-neutralizing antibody responses

(total and IgM-depleted) were determined in a plaque reduction assay on Vero cells as described before. (Charan & Zinkernagel, 1986)

Cytokine analysis

Measurement of IFN-α/β serum bioactivity was performed as previously described. (Vogel, English, & O'Brien, 1982) RayBio Mouse Cytokine Antibody Array-1 (RayBiotech) was used as instructed by the manufacturer.

Statistical analysis

Statistical analysis was performed with an unpaired t-test using Graphpad Prism.

Chapter 4: Results

Low levels of circulating platelets prevent severe systemic hemorrhage and lethality during acute LCMV infection

To investigate the role of platelets in the control of LCMV infections, we developed a depletion protocol that induces partial or complete depletion of platelets in mice. Zero, 40, or 80μg of anti-GPIIb monoclonal antibody were injected intraperitoneally in the mice and 24 hours later blood was collected for platelet quantification in the absence of infection. To simplify titration of the platelet depleting reagents, we chose a single anti-GPIIb monoclonal antibody instead of the more commonly used anti-GPIbα antibody cocktail, and the intraperitoneal route for a slower diffusion and depletion rate, resulting in a more controlled study. Under these conditions we managed to preserve approximately 15-20% of the circulating platelets when the animals received the 40μg treatment, but saw an almost complete depletion (±2.5%) in mice receiving the 80μg dose (Fig 1A).

Next, we proceeded to infect mice with LCMV Armstrong 12 hours after initiating the platelet depletion. Subsequent doses of anti-GPIIb were administered at days 2 and 4 postinfection (p.i.) and survival of the animals was monitored daily for 16 days. All animals treated with the partial depletion dose (40μg) survived the infection until the end of the experiment, as did mice in the PBS control group. In contrast, approximately 25% of mice receiving the complete depletion treatment (80μg) succumbed to infection around days 7 to 8 postinfection (Fig 1B). The experiment was repeated with the same treatment groups, and animals were sacrificed 8 days postinfection and dissected to look for signs of hemorrhage in the skin, brain, gut, and lungs. Mice without depletion

and mice with partial depletion of platelets showed no signs of hemorrhage induced by the infection in any of the tissues analyzed. On the other hand, all mice that were completely depleted of platelets had signs of systemic hemorrhage in all organs, as indicated by hemorrhagic spots (Fig 1C). Then, to better characterize the thrombocytopenic model, we analyzed the platelet kinetics of the anti-GPIIb 40µg treatment (12hrs before infection and at day +2 and +4 p.i.) in the presence or absence of infection. As shown in figure 1D, the treatment accomplishes a significant platelet drop throughout the course of the study.

Thus, it seems that highly thrombocytopenic mice suffered severe systemic hemorrhages and were more likely to succumb to LCMV infections. This is consistent with previously published data in which mice given a stronger platelet depletion protocol (±0.7% remaining platelets) showed massive hemorrhages and higher (±60%) mortality. (Iannacone et al., 2008) In contrast, it seems that a small fraction of circulating platelets were enough to completely prevent both manifestations.

Mice with reduced platelet numbers are unable to control LCMV Armstrong replication

Although partial depletion of platelets followed by LCMV Armstrong infection did not lead to lethal hemorrhages, it did impair clearance of the viral infection and generated exhausted T cell responses. Mice receiving three 40µg doses of anti-GPIIb (at -0.5, +2, and +4 days postinfection) were infected with LCMV Armstrong, and 8 days later viral titers were determined in serum, liver, and kidneys. PBS treated animals did not have detectable virus, whereas mice with reduced levels of platelets had significant levels of virus replication in all organs examined (Fig 2A). Splenocytes recovered from these animals were restimulated

in vitro with three different LCMV immunodominant epitopes (GP33, GP276, and NP396), and analyzed for the capacity of CD8+ T cells to produce cytokines. Figure 2B shows flow cytometry plots of gated CD8+ T cells for untreated, and platelet-depleted mice. The percentage of cells capable of producing a single cytokine (IFNγ+) or double (IFNγ+, TNFα+) cytokines was severely reduced for all the epitopes tested in the infected mice that were subjected to the anti-GPIIb treatment (Fig 2B). A fraction of the same splenocytes was directly stained with fluorochrome-conjugated MHC class I tetramers specific for the same epitopes as above. As shown in figure 2C, the percentage of CD8+ T cells that stained positive for each of the tetramers was reduced in the treated mice, with the NP396 population being the most affected. The defect became more significant when the total numbers of tetramer specific populations per spleen were calculated, showing an almost tenfold reduction in each of the populations (Fig 2C). Thus, under conditions of reduced circulating platelets but in the absence of lethal hemorrhage, mice fail to clear LCMV Armstrong from serum and peripheral tissues and show significant lower and functionally impaired LCMV-specific CD8+ T cell responses.

Leukocyte numbers and locations are not affected by platelet removal

To evaluate if the removal of platelets was affecting the blood or secondary lymphoid organ (SLO) composition and structure, we performed multicolor flow cytometry and immunohistochemistry of those tissues. Mice were sacrificed 24 hours after injection of PBS or 40μg of anti-GPIIb intraperitoneally, and blood, spleens, and peripheral lymph nodes (PLNs) were collected and processed as indicated in materials and methods. Flow cytometric analysis of blood leukocytes of control and platelet-depleted mice as shown in figure 3A, showed no significant changes among the populations analyzed between the two groups.

Single cell suspensions of collagenase-digested SLOs were analyzed and are presented in figure 3B as the total numbers of each individual population per organ. No difference could be detected in any of the populations analyzed in the treatment group compared to the control group. Immunostaining of splenic sections with antibodies against T, B, and DCs (left panels), or metallophillic macrophages (MM), red pulp macrophages (F4/80), and marginal zone macrophages (MZM) (right panels) showed that, all the cells were properly positioned at their specific locations in the spleen. B cells were located at the B cell follicles and marginal zones, DCs were at the bridging channels and T cell zones, and T cells were at the T cell zones. Likewise, all macrophage populations were present and identically distributed in the treated and untreated spleens (Fig 3C). Thus, the partial depletion of platelets with the anti-GPIIb antibody did not change the composition of the blood or SLOs nor did it alter the splenic microarchitecture.

Early innate control of LCMV requires platelets

To understand the mechanisms affecting the ability to control LCMV infection by platelet removal, a kinetic analysis of the depletion was performed. Three groups of mice were treated with anti-GPIIb at different time points. The first group received three doses (at days -0.5, +2, and +4 postinfection), the second group skipped the first dose (at days +2 and +4), and the third group received only the last dose (at day +4). When LCMV serum titers were measured at 8 days postinfection, only the group that received all three doses of antibody had detectable levels of virus in the serum (Fig 4A). CD8+ T cells from these mice showed a marked decrease in their cytokine production only in the group that received the three depleting doses and had circulating virus in serum (Fig 4B). Then, we measured viral titers at 48 hours postinfection in spleen, liver, and

lungs, and observed that the levels of virus replication were comparable in the spleen and liver (sites of massive virus production), but viral replication in the lungs was significantly higher in the treated groups compared to undetectable levels in the control group at this early time point (Fig 4C). The previous results point to an early defect in the control of LCMV infections, which is highly associated to IFN-α/β activity. However, when the production and release of IFN-α/β was measured following infection, the removal of platelets showed no appreciable changes in serum levels of these cytokines (Fig 4D). Together, these results indicate that, under our experimental conditions, platelets need to be present at the beginning of the infection to constrain the early virus replication, by a mechanism independent of IFN-α/β production.

CD8 T cell responses are affected by platelet depletion independently of high viral titers

Study of the effect of platelet depletion on CD8+ T cells is complicated due to the early loss of control of the virus. The observed CD8+ T cell exhaustion may be solely due to increased viral replication rather than a direct effect of platelet removal on these cells. However, the observation that subsequent treatments with anti-GPIIb (40μg) enhanced the defect seen after one dose (Fig 5A) suggested that platelets are required for late control of LCMV Armstrong infection. To gain a better understanding of this issue, we designed a set of experiments wherein early control of the virus is lost and CD8+ T cell responses become exhausted even in the presence of platelets. We used the LCMV clone-13 strain as an established system where the early control of the virus is deficient. Mutations in this viral strain allow the virus to infect DCs and FRCs in the splenic white pulp (WP), reach higher viral titers in peripheral tissues and blood earlier than its parental strain Armstrong, and induce the expression of

immunosuppressive molecules that, altogether, contribute to the exhaustion of CD8+ T cells that fails to control the infection. (Bergthaler et al., 2010; Matloubian et al., 1993; Mueller et al., 2007; Sevilla et al., 2000; Sullivan et al., 2011) Platelet removal after day 4 postinfection allowed us to evaluate if platelets were directly required in the accumulation of the CD8+ T cells independently of their exhausted status (Fig 5B). The late removal of platelets during LCMV clone-13 infections did not affect the high levels of viral replication reached 15 days postinfection (Fig 5C). This may correspond to a saturation of the capacity of the mice to produce virus, as suggested by the levels reached in CD8+ deficient mice. (Recher et al., 2004) No differences were observed in the exhausted CD8+ T cell responses when we analyzed them by tetramer staining as percentages of total CD8+ T cells (top panel, Fig 5D); however, the total number of the two tetramer positive populations analyzed per spleen was statistically reduced in the platelet-depleted group (bottom panel, Fig 5D), indicating that the platelet removal affected the CD8+ T cell responses against LCMV by non-specifically affecting the total population of CD8+ T cells. Thus, even if a defect in the innate control of the infection drives the exhaustion of the CD8+ T cells, platelet removal further affects the generation of a protective adaptive response.

Splenic necrosis in platelet-depleted mice affects innate and adaptive immune components

The data presented in figures 4 and 5 indicate that removing platelets during an LCMV infection results in a generalized deficiency in elicited immune responses against the virus. To further analyze this defect, 8 days following infection with LCMV Armstrong we quantified different immune cells in the spleens of partially platelet-depleted and control mice by flow cytometry. Under partial platelet depletion, shown in figure 6A, numbers of all innate and adaptive

immune cells, with the exception of B cells, were reduced in the spleens of anti-GPIIb treated mice, confirming our previous observations. The size of the spleens and the number of total splenocytes quantified by light microscopy were not reduced in the platelet-depleted mice (data not shown); however, most of the cells in the treated group stained positive by a live/dead flow cytometry based assay that detects disrupted cellular membranes in necrotic cells (Fig 6B). Several reasons could explain why the removal of large amount of platelets induces necrosis in the spleen. The possibility of a cytokine storm was discarded by a cytokine array performed 24 hours postinfection (36 hrs after platelet depletion), which showed no difference in any of the 21 cytokines analyzed (Fig 6C). Cross-reactivity of the anti-GPIIb was excluded by depleting platelets with anti-GPIbα, which recognizes a protein in a different molecular complex on the platelet (left panel, Fig 6D). Finally, possible damage by deposition of large quantities of immune complexes in the spleen was ruled out by removing platelets from the blood by aggregating them in the circulation with a toxin (aspercetin) (right panel, Fig 6D). Thus, it seems that the deficient immune response against LCMV infection is the result of a necrotic process that unspecifically affects the immune cells of the spleen by the reduction of functional platelets *per se*.

Platelet depletion during LCMV infection disrupts the splenic architecture

Structural analysis of the spleen of day 8 treated and untreated mice by light and immunofluorescence microscopy shows that the platelet depletion protocol during the LCMV infection induced an absolute disruption of the splenic morphology as indicated by the absence of discernable areas of white versus red pulp in the H&E staining, and the disintegration of the follicular structures by immunofluorescence. Furthermore, immunostaining of platelets with an antibody

against integrin β3 (exclusively expressed on platelets) shows a reorganization of the platelets in the spleen during the LCMV infection towards structures resembling capillaries and sinusoids. Analysis of viral antigen and FRCs indicated an extensive and homogenous viral replication throughout the splenic parenchyma in the platelet-depleted mice (Fig. 7).

Thrombopoietin treatment does not improve chronic LCMV viral clearance

Mice infected with the LCMV clone-13 strain suffer an acute thrombocytopenia that begins 3-4 days postinfection and an immunosuppression that leads to the chronic replication of the virus. To determine if there is any association between thrombocytopenia and immunosuppression in this infection, we treated mice 5 and 1 days before LCMV clone-13 infection with 5 μg (10 μg per mouse total) of TPO intravenously to increase the numbers of circulating platelets, and 15 days later we measured viral titers in serum. Even though we were able to considerably increase the numbers of circulating platelets before (approximately 2 fold) and at the nadir of the thrombocytopenia (approximately 1.6 fold at day +4), the viral titers obtained at day 15 postinfection were not statistically different to the control group (data not shown); indicating that thrombocytopenia is not associated with the immunosuppression seen in the LCMV clone-13 infections.

Control of VSV infection is also affected in the absence of platelets

Next we asked whether the described observations were exclusive to LCMV infection or whether partial platelet depletion affects protection to other pathogens. For this purpose, we treated mice with the depleting antibody and infected the mice with VSV intravenously. Approximately 10 days postinfection, some mice that received the anti-platelet treatment begin to show signs of

paralysis and were sacrificed (Fig 7A). Determination of VSV brain titers on those animals showed high levels of replication (Fig 7B). Next we determined the levels of protective neutralizing anti-VSV antibodies (IgM and IgG) in serum at 4 and 8 days postinfection. Neither death nor CNS replication in the platelet-depleted mice was correlated with a deficient production of neutralizing antibodies (Fig 7C). Thus, platelets seem to be required in the control of viral infections other than LCMV.

Figures

Figure 1. Approximately 15% of the total platelet count is sufficient to prevent lethality and peripheral organ hemorrhage during LCMV Armstrong infection.

A. Circulating platelet counts 24 hours after i.p. injection of PBS, 40µg, or 80µg of anti-GPIIb monoclonal antibody. Groups of 5 animals per treatment, of at least two independent experiments. Error bars are SEM. **B. Survival of LCMV Armstrong infection after platelet depletion treatment.** Mice were injected i.p. with PBS, 40µg, or 80µg of anti-GPIIb monoclonal antibody and infected 12 hours later with 2x106 p.f.u. of LCMV Armstrong i.v. Depletion treatment was repeated 2 and 4 days post infection and survival was monitored daily for 16 days. The plot shows at least 8 animals per group from the combination of two experiments. **C. Signs of hemorrhage observed in infected animals treated with 80µg of anti-GPIIb antibody.** Animals treated as in B. were sacrificed at day 8 p.i. Skin, brains, and guts were dissected and photographed and lungs were histologically processed, H&E stained, and micrographs were taken (100× and 40× inserts). Arrows indicate hemorrhagic spots. **D. Kinetics of the thrombocytopenia induced by the anti-GPIIb treatment and the LCMV Armstrong infection (40µg of anti-GPIIb, 12 hrs before and, 2 and 4 days post LCMV Armstrong infection or PBS injection).** Platelet counts were determined at the indicated time points for mice non treated and non infected (black bars), mice treated and non infected (open bars), mice non treated and infected (grey bars), and mice treated and infected (checkered bars). Error bars represent SEM. *, $P < 0.05$. Data of 2 separate experiments, with 5 animals per group per experiment.

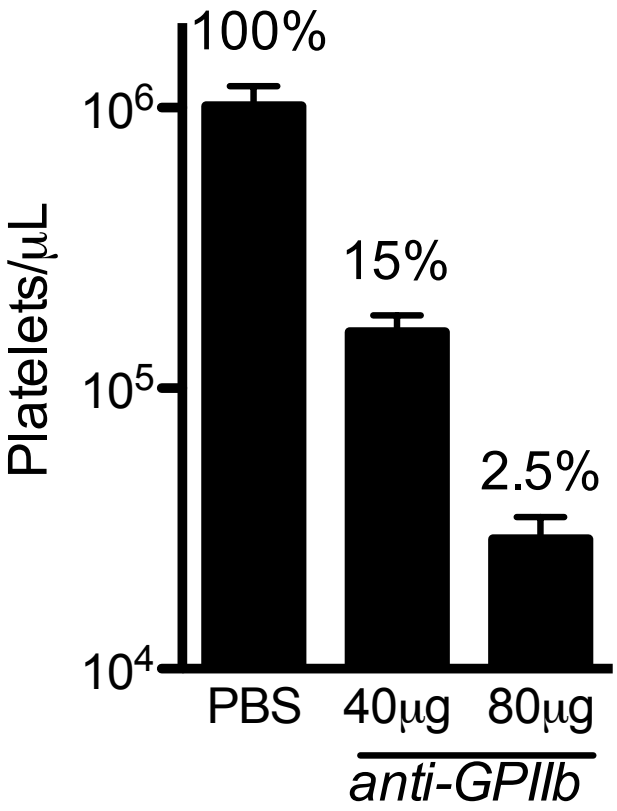


Figure 1A

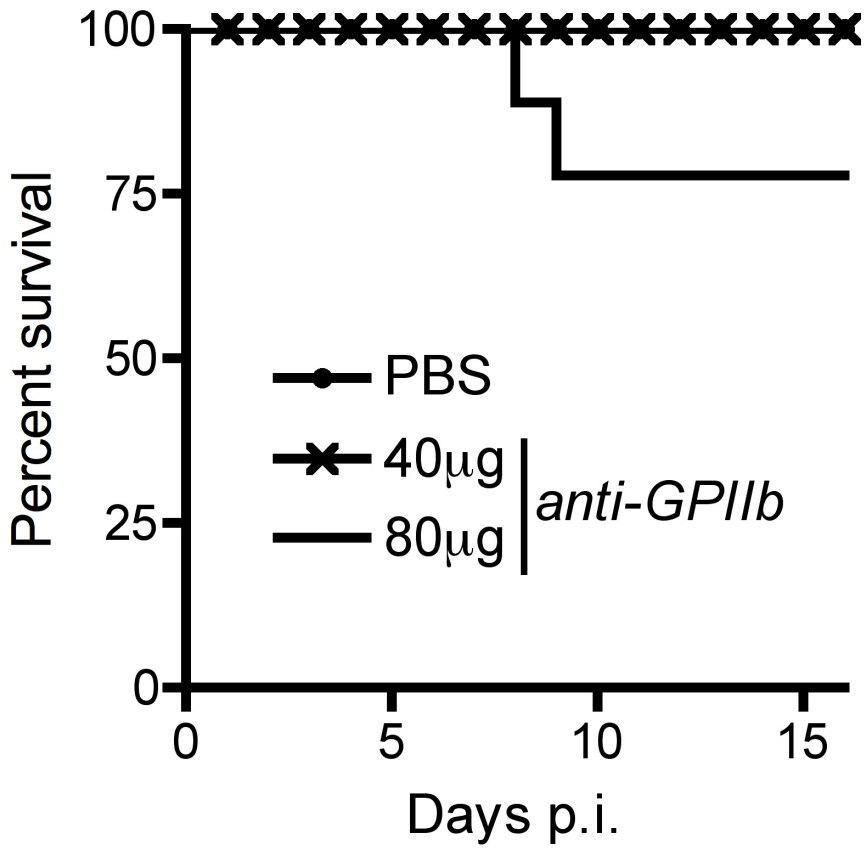

100
75
50
25
0
Percent survival
0
5
10
15
Days p.i.
PBS
40μg
80μg
anti-GPIIb

Figure 1B

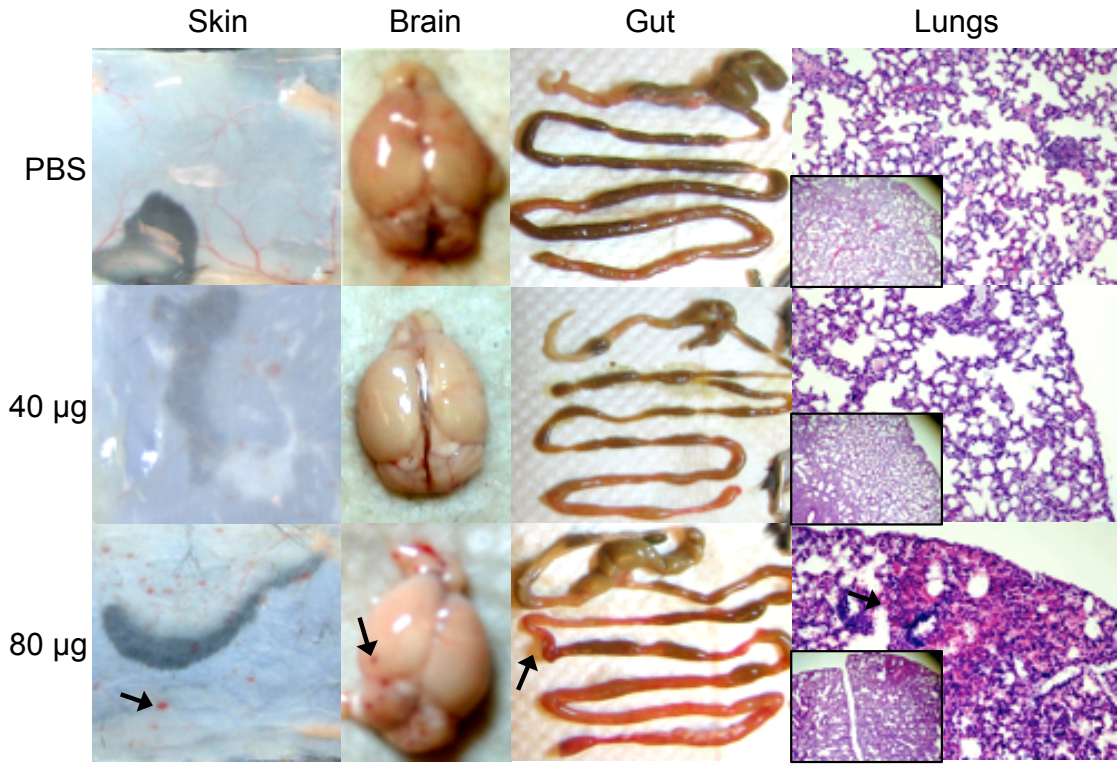


Figure 1C

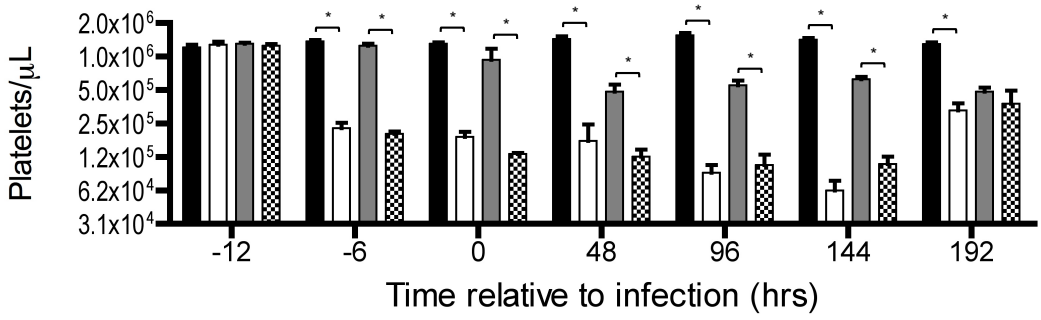

Platelets/μL
2.0x10^6
1.0x10^6
5.0x10^5
2.5x10^5
1.2x10^5
6.2x10^4
3.1x10^4
-12
-6
0
48
96
144
192
Time relative to infection (hrs)

Figure 1D

Figure 2. Partially platelet-depleted mice fail to control an acute LCMV Armstrong infection and have exhausted LCMV-specific CD8+ T cell responses.

Mice were treated with PBS or 40μg of anti-GPIIb antibody 12 hrs before and, 2 and 4 days post LCMV Armstrong infection. **A. Viral titers found in serum, liver, and kidneys.** Serum and organs of infected mice were recovered 8 days p.i.. **B. Cytokine production by splenic virus-specific CD8+ T cells.** IFNγ and TNFα production by splenocytes 8 days p.i. following in vitro stimulation with the indicated peptides (cells gated on CD8+ events, numbers indicate percentage of cells in each region). **C. Total numbers of LCMV-specific CD8+ T cells in the spleen.** Day 8 p.i. tetramer positive populations (as indicated in the graphs) expressed as percentage of CD8+ cells (top panel) and as total cells per spleen (bottom panel). (A-C: Open bars PBS treated controls, black bars anti-GPIIb treated mice. Error bars represent SEM. *, $P < 0.05$. One representative experiment of at least 2 is shown, with 5 animals per group per experiment).

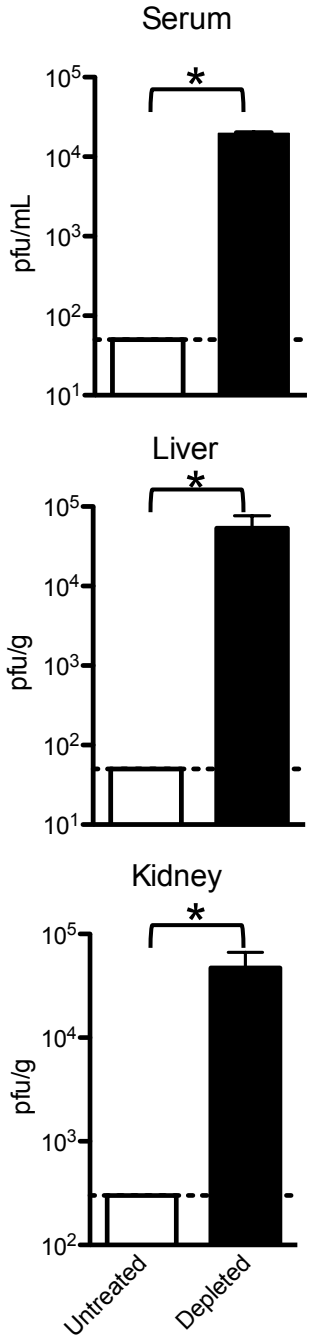

Serum
*
10^5
10^4
10^3
10^2
10^1
pfu/mL
Liver
*
10^5
10^4
10^3
10^2
10^1
pfu/g
Kidney
*
10^5
10^4
10^3
10^2
pfu/g
Untreated
Depleted

Figure 2A

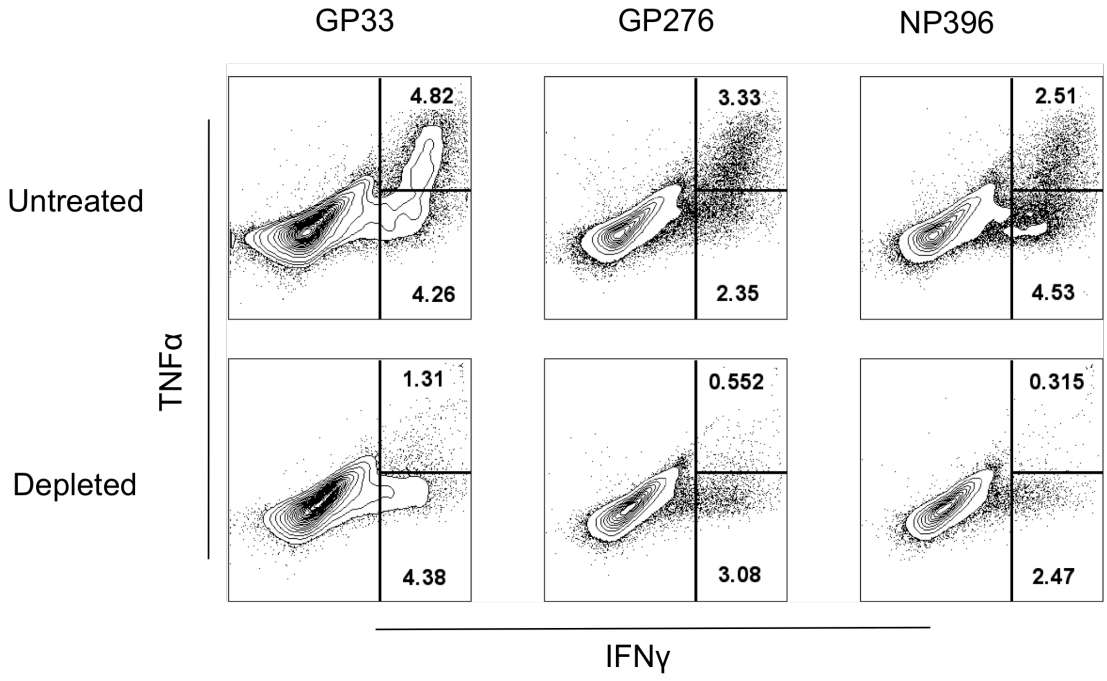

GP33
GP276
NP396
Untreated
Depleted
TNFα
IFNγ
4.82
4.26
3.33
2.35
2.51
4.53
1.31
4.38
0.552
3.08
0.315
2.47

Figure 2B

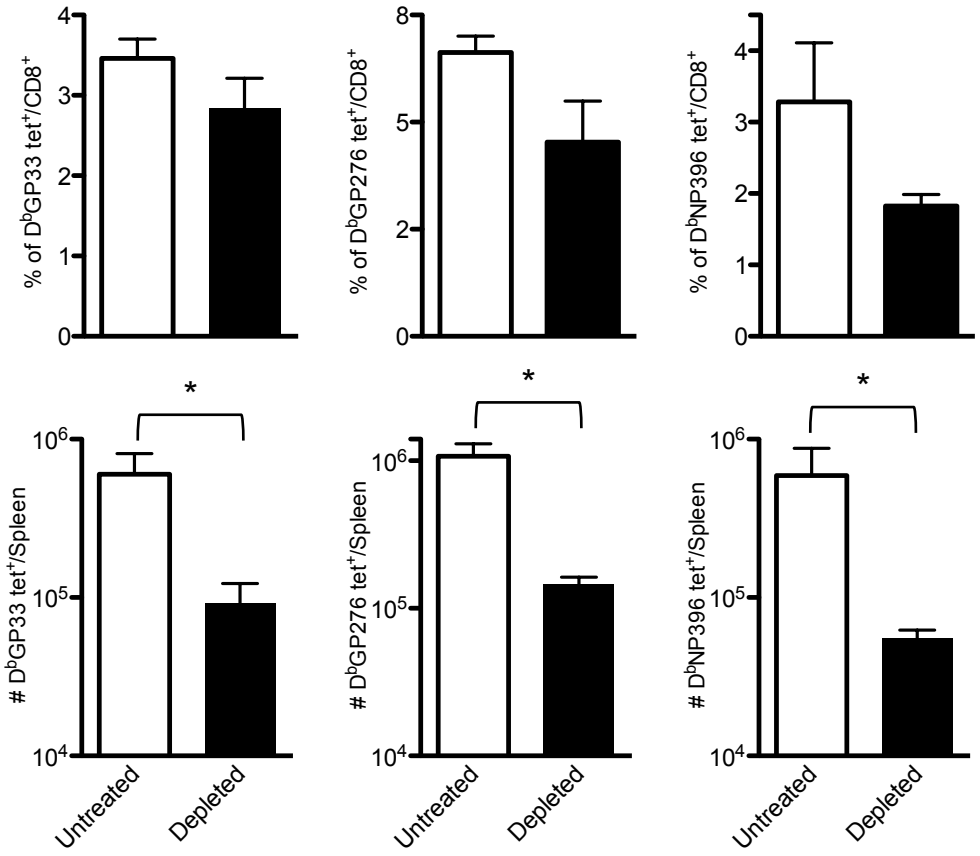

% of DbGP33 tet+/CD8+
% of DbGP276 tet+/CD8+
% of DbNP396 tet+/CD8+
# DbGP33 tet+/Spleen
# DbGP276 tet+/Spleen
# DbNP396 tet+/Spleen
*
Untreated
Depleted

Figure 2C

Figure 3. Platelet removal in the absence of infection does not affect the blood and secondary lymphoid organ (SLO) compartments.

Mice were treated with PBS or 40µg of anti-GPIIb antibody and 24 hours later sacrificed. Blood, spleen, and peripheral lymphoid nodes (PLN) were recovered and analyzed as described in the materials and methods. **A. Flow plots of circulating immune cells in blood.** Numbers indicate percentage of cells in each gate. **B. Number of immune cells in SLOs. Spleen and PLN cells populations after collagenase digestion, were counted, stained, and analyzed by flow cytometry.** Open bars PBS treated controls, black bars anti-GPIIb treated mice. Error bars represent SEM. NS: non significant. **C. Conserved splenic microarchitecture.** Spleens were frozen in OCT medium, cut and stained with antibodies to Thy1.2 (T cells, blue), B220 (B cells, green), and CD11c (Dendritic cells, red) or Moma-1 (marginal zone metallophilic macrophages, blue), F4/80 (red pulp macrophages, green), and ER-TR9 (marginal zone macrophages, red).

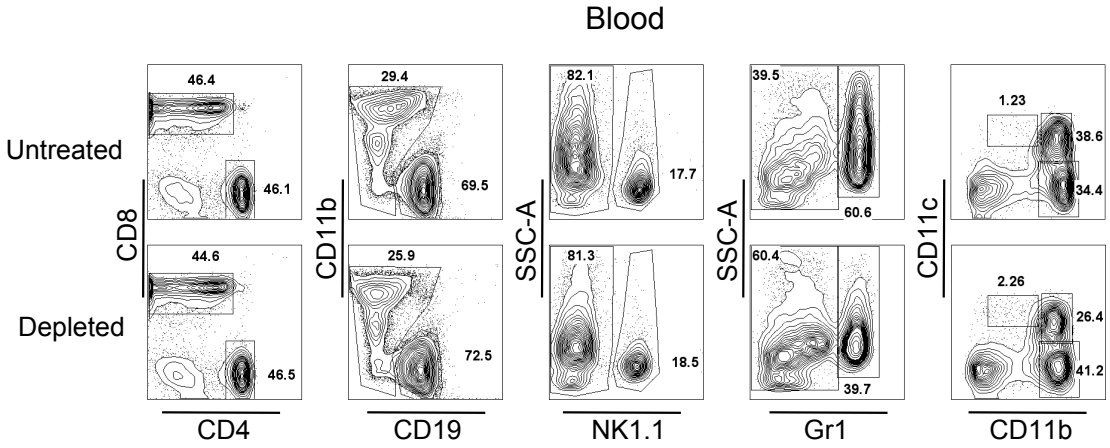

Blood
Untreated
Depleted
CD8
CD4
46.4
46.1
44.6
46.5
CD11b
CD19
29.4
69.5
25.9
72.5
SSC-A
NK1.1
82.1
17.7
81.3
18.5
SSC-A
Gr1
39.5
60.6
60.4
39.7
CD11c
CD11b
1.23
38.6
34.4
2.26
26.4
41.2

Figure 3A

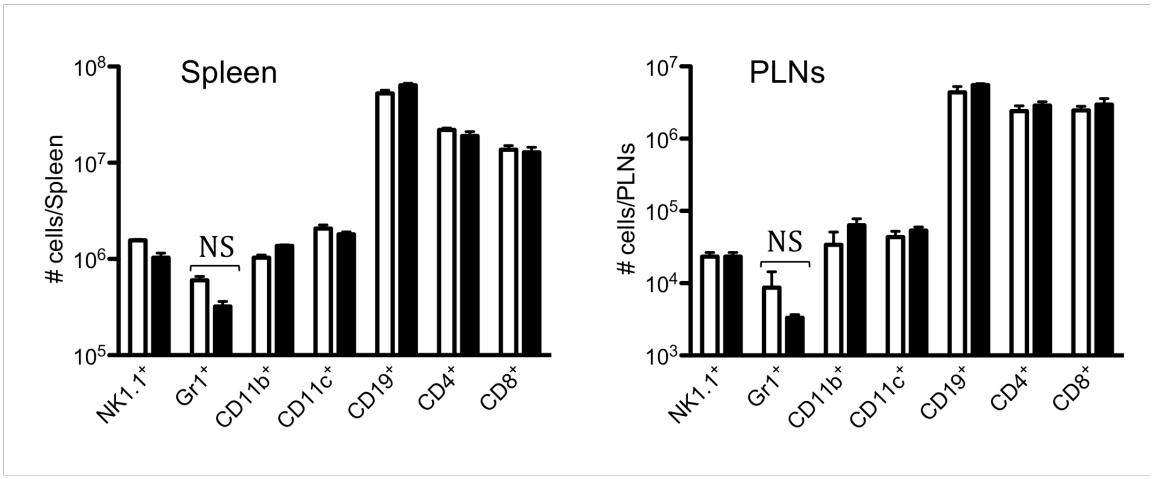

Spleen
cells/Spleen
10^8
10^7
10^6
10^5
NS
NK1.1+
Gr1+
CD11b+
CD11c+
CD19+
CD4+
CD8+
PLNs
cells/PLNs
10^7
10^6
10^5
10^4
10^3
NS
NK1.1+
Gr1+
CD11b+
CD11c+
CD19+
CD4+
CD8+

Figure 3B

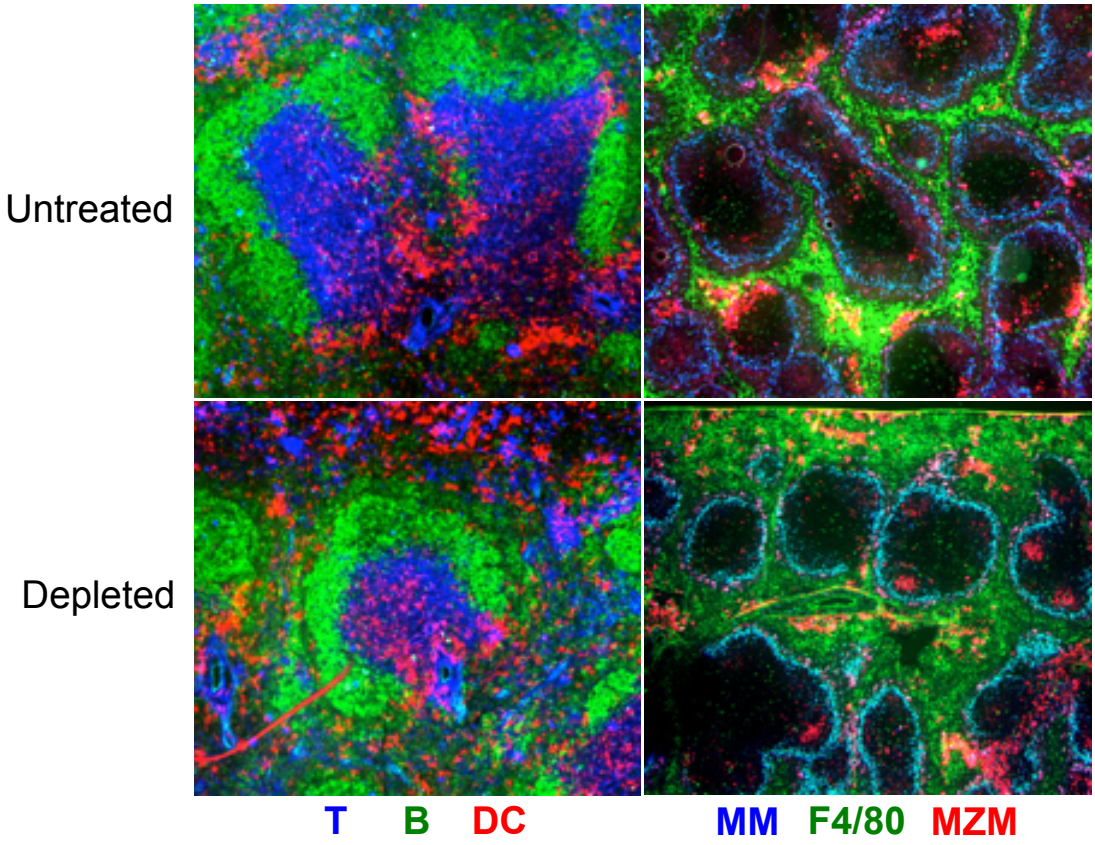


Figure 3C

Figure 4. Early control of the virus is affected by partial platelet depletion independent of type I IFN production.

Mice were treated with three doses (12 hrs before and 2 and 4 days p.i.), two doses (2 and 4 days p.i.), or one dose (4 days p.i.) of 40µg of anti GPIIb and infected with LCMV Armstrong i.v. (A and B). **A. Platelet presence in the first two days of infection is needed to control the infection.** Eight days post infection viral titers in serum were determined. **B. Functional exhaustion of LCMV specific CD8 T cells is restored if platelets are present in the first two days of infection.** Intracellular IFNγ and TNFα cytokine production by day 8 splenocytes after in vitro stimulation (Numbers in the flow plots indicate percentage of cells inside the gates).

Mice were treated with 40µg of anti GPIIb and infected i.v. 12 hrs later with LCMV Armstrong (C and D). **C. Equivalent LCMV replication in spleen and liver but higher titers in lungs 48 hours post infection in the absence of platelets.** Viral titers in spleen, liver, and lung tissue homogenates. Open bars PBS treated controls, black bars anti-GPIIb treated mice. Error bars represent SEM. *, $P < 0.05$. One representative experiment of at least 2 is shown). **D. LCMV induced anti-viral IFN-α/β levels.** Mice were bled at 6, 12, 24 and 48 hours p.i. and IFN-α/β bioactivity was determined in serum (n=4-6 animals, in two independent experiments, Error bars represent SEM.)

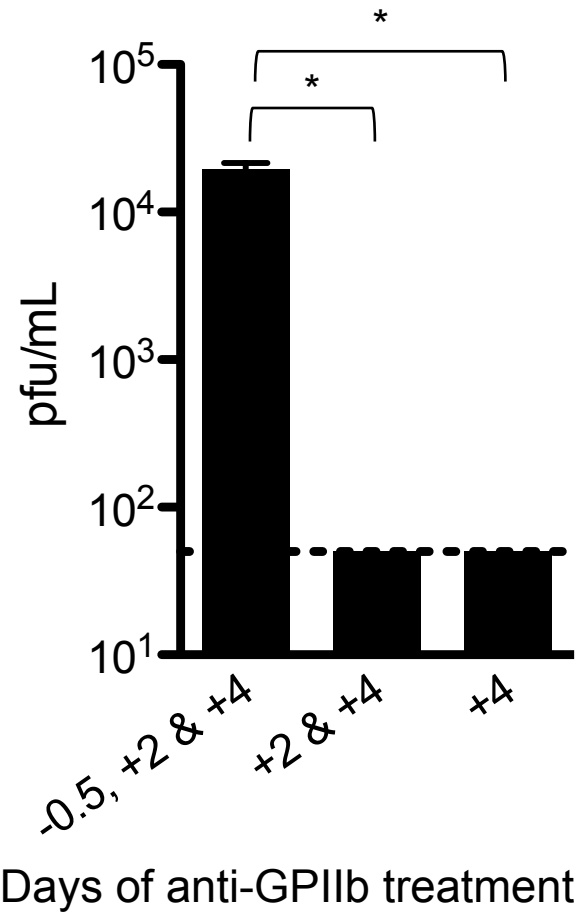

*
*
10^5
10^4
10^3
10^2
10^1
pfu/mL
-0.5, +2 & +4
+2 & +4
+4
Days of anti-GPIIb treatment

Figure 4A

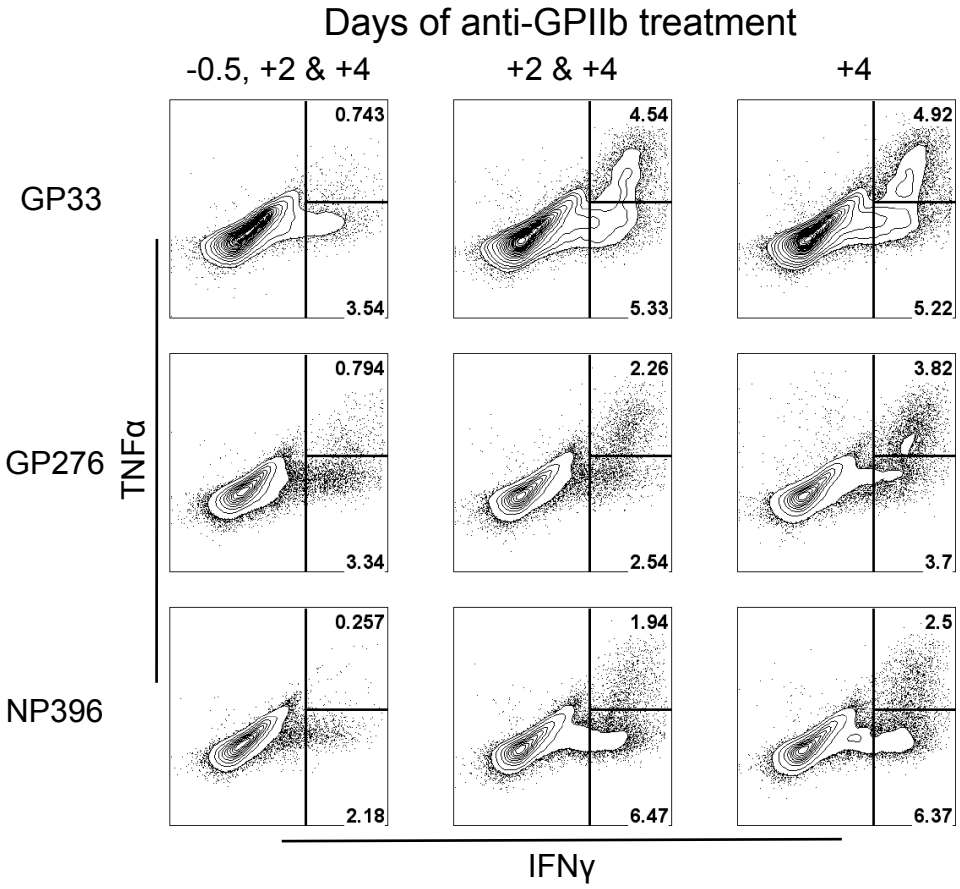

Days of anti-GPIIb treatment
-0.5, +2 & +4
+2 & +4
+4
GP33
GP276
NP396
TNFα
IFNγ
0.743
3.54
4.54
5.33
4.92
5.22
0.794
3.34
2.26
2.54
3.82
3.7
0.257
2.18
1.94
6.47
2.5
6.37

Figure 4B

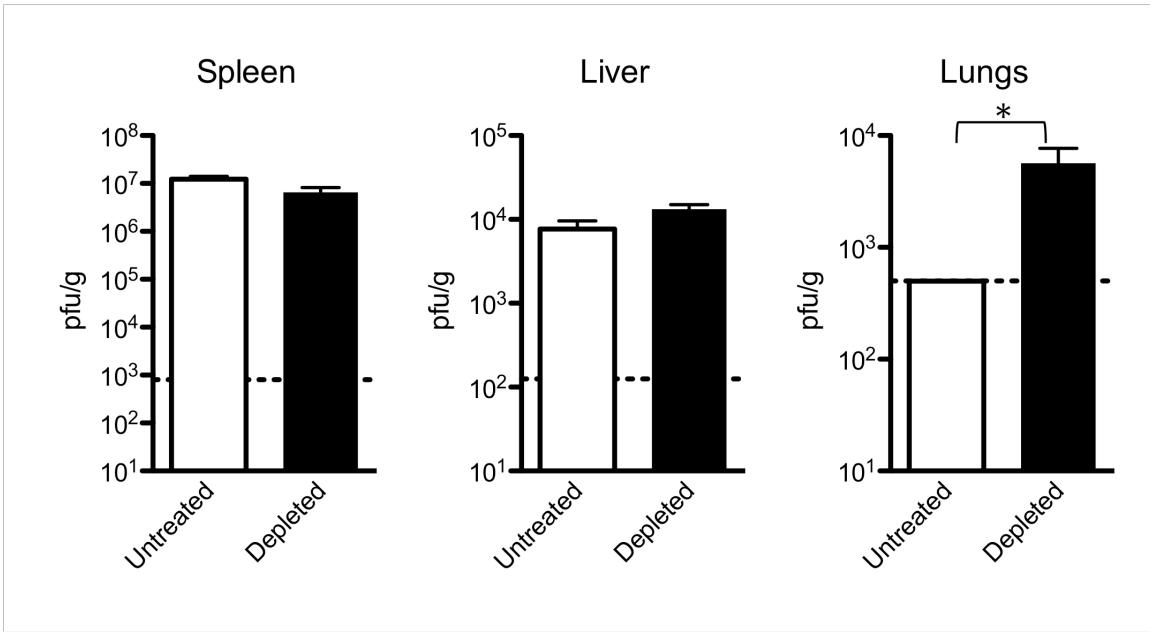

Spleen
Liver
Lungs
pfu/g
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Untreated
Depleted
*

Figure 4C

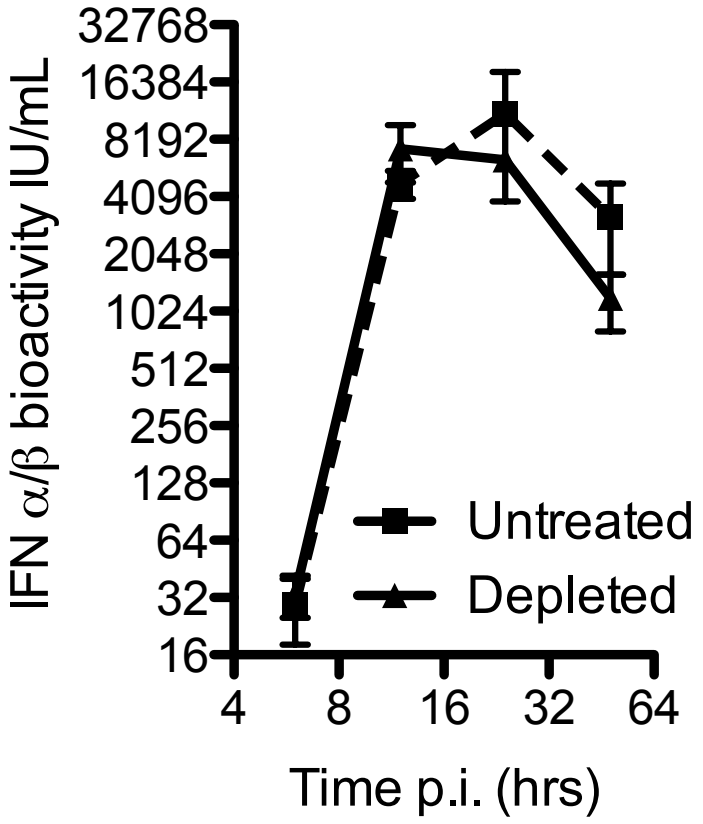

32768
16384
8192
4096
2048
1024
512
256
128
64
32
16
IFN α/β bioactivity IU/mL
Untreated
Depleted
4
8
16
32
64
Time p.i. (hrs)

Figure 4D

Figure 5. CD8 T cell responses are directly affected by partial platelet depletion independent of the levels of circulating viral antigen.

A. Continuous platelet removal enhances LCMV Armstrong replication. Mice were treated with one dose (12 hrs before infection), two doses (12 hrs before and 2 days p.i.), or three doses (12 hrs before and 2 and 4 days p.i.) of 40μg of anti GPIIb and infected with LCMV Armstrong i.v. Serum viral titers were determined at day 8 p.i. **B. Effect of platelet removal on exhausted CD8 T cells.** Mice were infected with the LCMV chronic strain clone-13, which in adult mice infects the white pulp (WP), reaches higher titers in blood and peripheral tissues by day 4, and generates exhausted T cells responses (C and D). Groups of mice were infected with the chronic LCMV strain clone-13 and treated with PBS or 40μg of anti GPIIb 4, 6, and 8 days post infection. **C. Platelet depletion of LCMV clone-13 infected animals does not affect viral replication.** Viral serum titers were determined after 15 days of infection. D. LCMV-specific CD8+ T cell responses during the course of a chronic infection are further affected by the absence of platelets. Two different CD8+, tetramer+ populations (DbGP33 and DbGP276) were quantified on day 15 LCMV clone-13 infected splenocytes. (C and D. Open bars PBS treated controls, black bars anti-GPIIb treated mice. Error bars represent SEM. *, $P < 0.05$. One representative experiment of at least 2 is shown, with 6 animals per group).

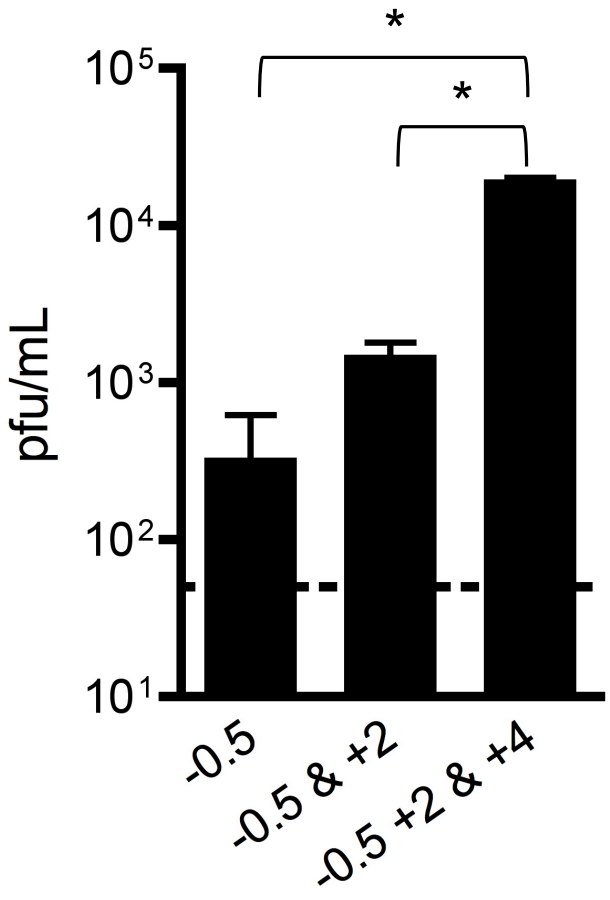


Figure 5A

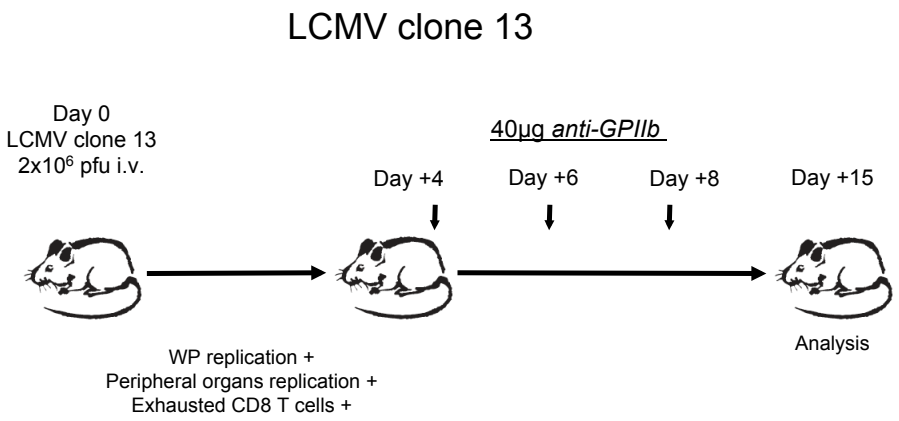

LCMV clone 13
Day 0
LCMV clone 13
2x10^6 pfu i.v.
40µg anti-GPIIb
Day +4
Day +6
Day +8
Day +15
WP replication +
Peripheral organs replication +
Exhausted CD8 T cells +
Analysis

Figure 5B

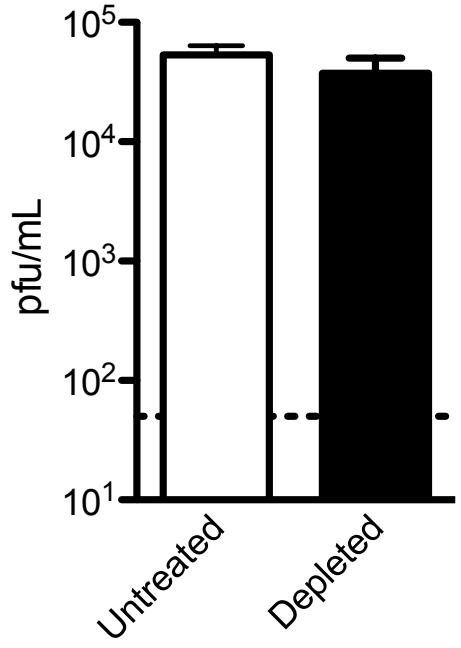


Figure 5C

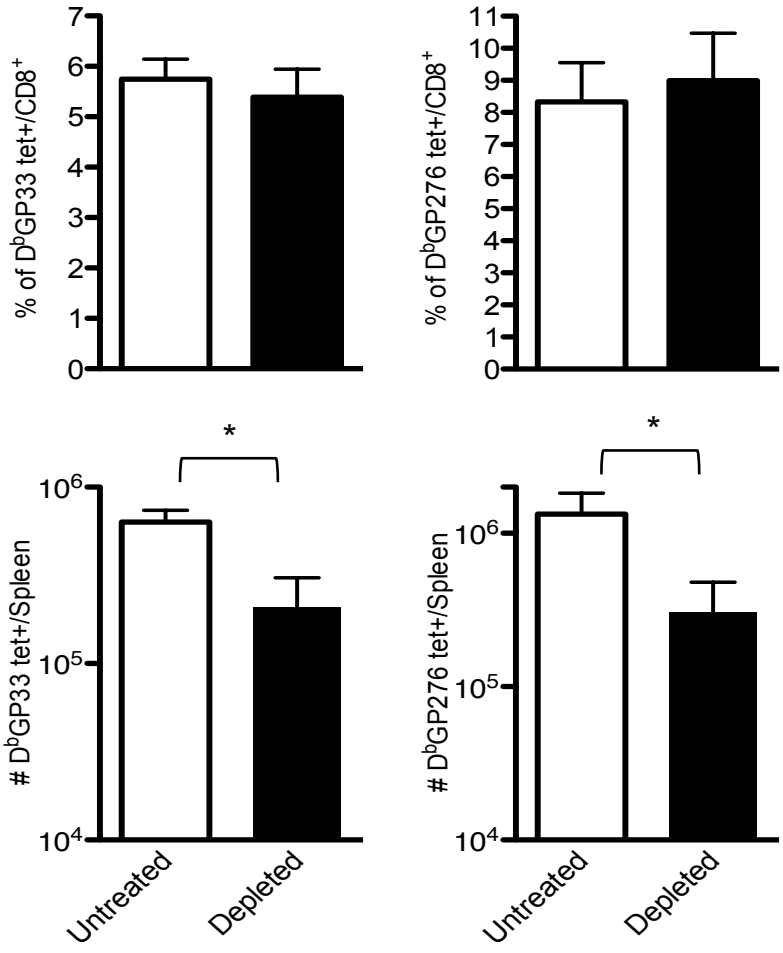

% of DbGP33 tet+/CD8+
% of DbGP276 tet+/CD8+
# DbGP33 tet+/Spleen
# DbGP276 tet+/Spleen
*
*
Untreated
Depleted
Untreated
Depleted

Figure 5D

Figure 6. Reduced innate and adaptive immune cell populations in spleens of platelet-depleted infected animals correlates with an increase of necrotic splenocytes.

Groups of mice were treated with PBS or 40μg of anti-GPIIb antibody 12 hrs before and, 2 and 4 days post LCMV Armstrong infection. **A. Splenic quantification of immune cells by flow cytometry.** Collagenase digested spleens of day 8 infected animals were quantified. **B. Detection and quantification of dead splenocytes.** Flow cytometry analysis of day 8 p.i. splenocytes that were collagenase digested and stained with an in-house Alexa Fluor 430 dye protocol that detects disruptions of cell membranes. Top panel shows a representative histogram of the analysis gated on total splenocytes (gray histogram: untreated; empty histogram: depleted; number indicate percentage of positive cells). Bottom panel shows numbers of dead splenocytes. **C. Serum cytokine levels.** Untreated and depleted animals were bled 24 hours after infection. Sera from each group were pooled and levels of 21 different circulating cytokines determined by a commercial immunoassay method with each cytokine detected in duplicate (n=6). Bottom table shows the arrangement of the cytokines on the membranes, and three relevant cytokines (IL-17, IFNγ, and TNFα) were marked. **D. The absence of platelets, not the anti GPIIb depletion protocol, is the defining factor.** Mice were treated with PBS or with two different platelet-depleting reagents (anti GPIbα or aspercetin), and serum viral titers were determined 8 days after infection. (A, B, and D. Open bars PBS treated controls, black bars platelet-depleted mice. Error bars represent SEM. *, $P < 0.05$. NS: non significant).

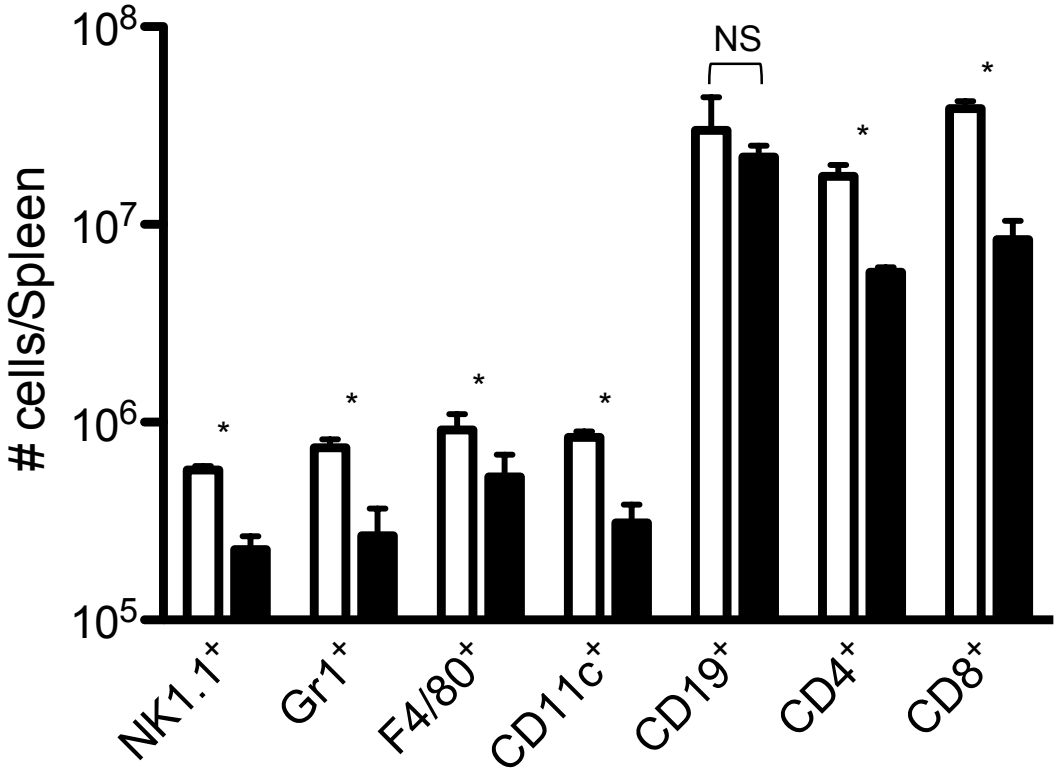

10^8
10^7
10^6
10^5
cells/Spleen
NS
*
NK1.1+
Gr1+
F4/80+
CD11c+
CD19+
CD4+
CD8+

Figure 6A

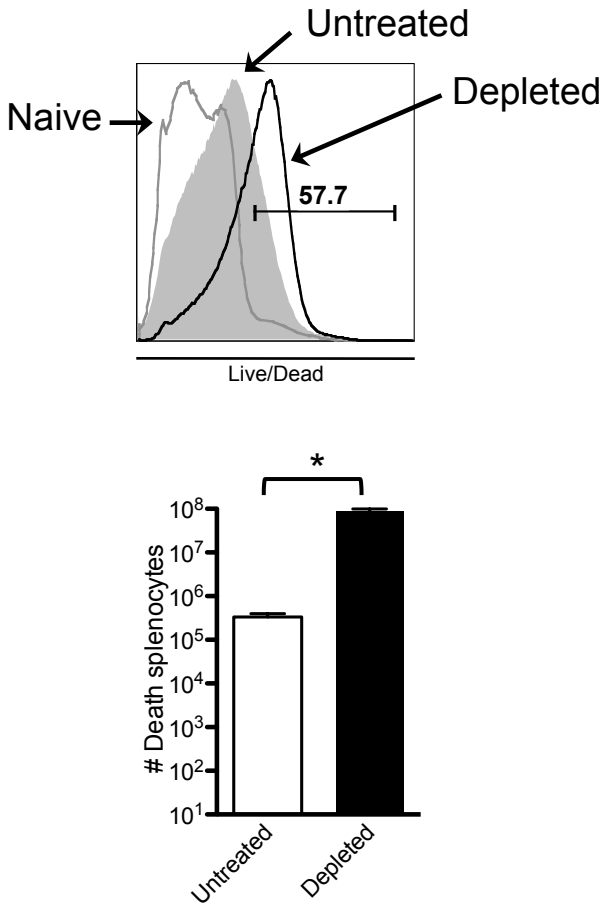
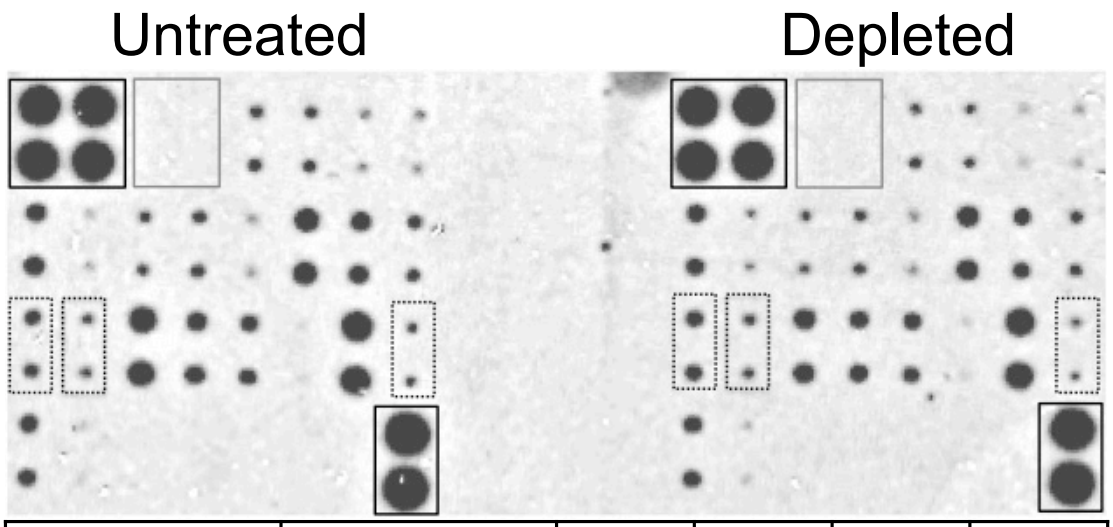

Untreated
Naive
Depleted
57.7
Live/Dead
*
Death splenocytes
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Untreated
Depleted

Figure 6B



Positive		Negative		GCSF	GM-CSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12 p70	IL-13
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
TPO	VEGF	Blank					Positive

Figure 6C

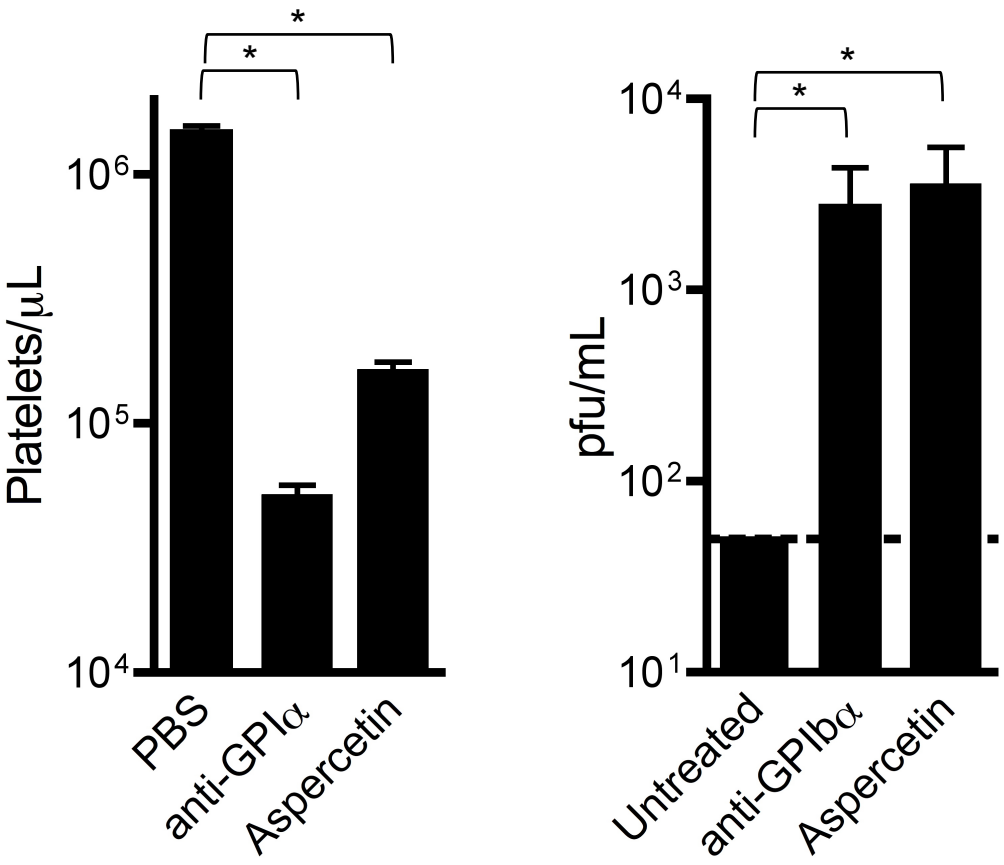

*
*
Platelets/μL
10^6
10^5
10^4
PBS
anti-GPIα
Aspercetin
*
*
pfu/mL
10^4
10^3
10^2
10^1
Untreated
anti-GPIbα
Aspercetin

Figure 6D

Figure 7. Organization of the splenic structure is disrupted by the LCMV infection in platelet-depleted mice.

Four groups of mice were treated with PBS or 40µg of anti-GPIIb antibody 12 hrs before and, 2 and 4 days post LCMV Armstrong infection or PBS injection (group 1: non treated and non infected, group 2: treated and non infected, group 3: non treated and infected, and group 4: treated and infected). Spleens were collected from mice sacrificed 8 days later and thin tissue sections were stained for light or immunofluorescence microscopy analysis. **Figure shows representative photomicrographs of H&E (40×, top row); T, B, and DCs (Thy1.2, B220, CD11c; 50×, second row); platelets (β3 integrin; 50×, third row); or fibroblastic reticular cells and LCMV (ER-TR7 and anti-LCMV, 50×, bottom row); (n=5).**

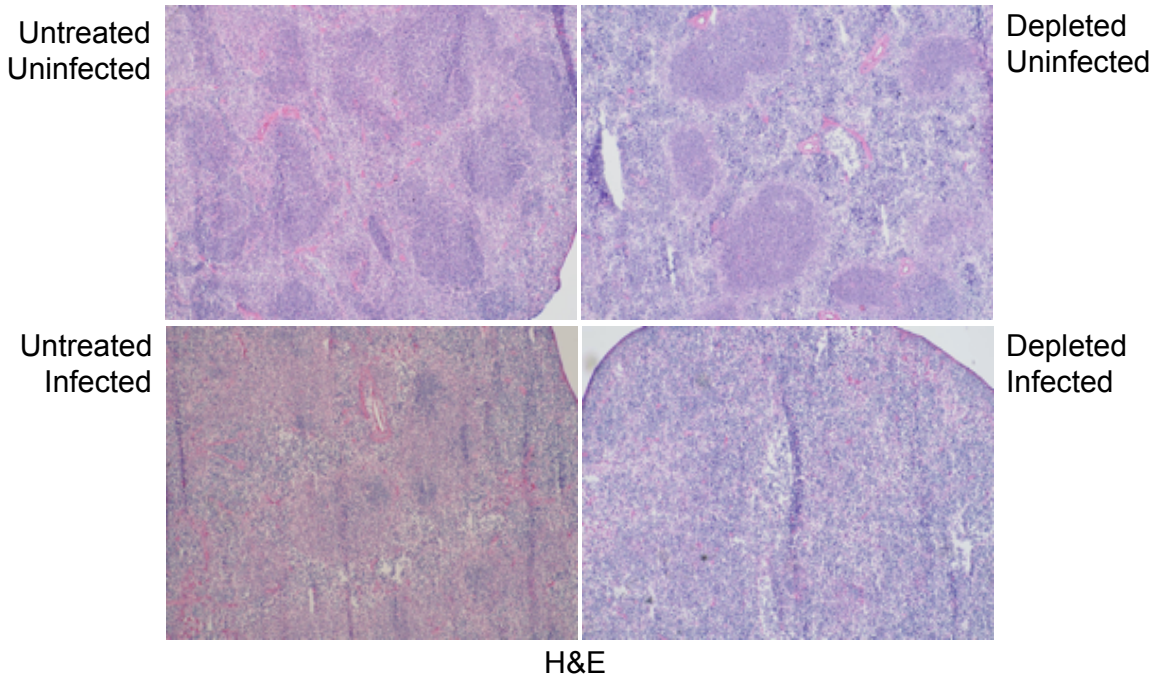

Untreated
Uninfected
Depleted
Uninfected
Untreated
Infected
Depleted
Infected
H&E

Figure 7A

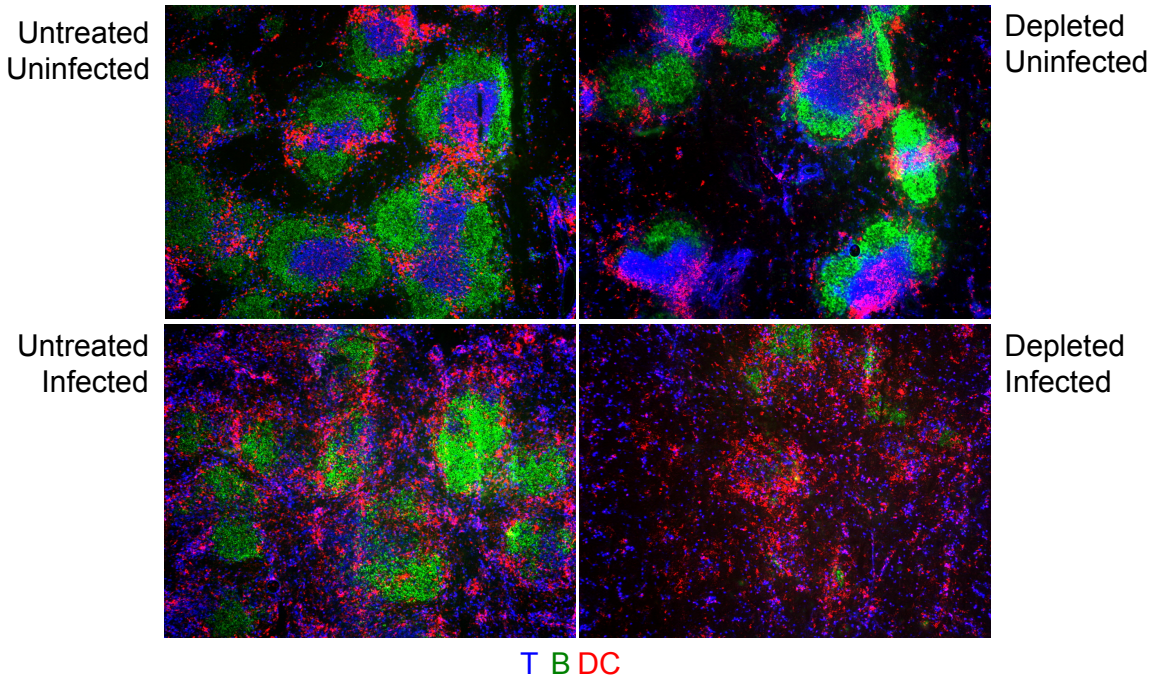

Untreated
Uninfected
Depleted
Uninfected
Untreated
Infected
Depleted
Infected
T B DC

Figure 7B

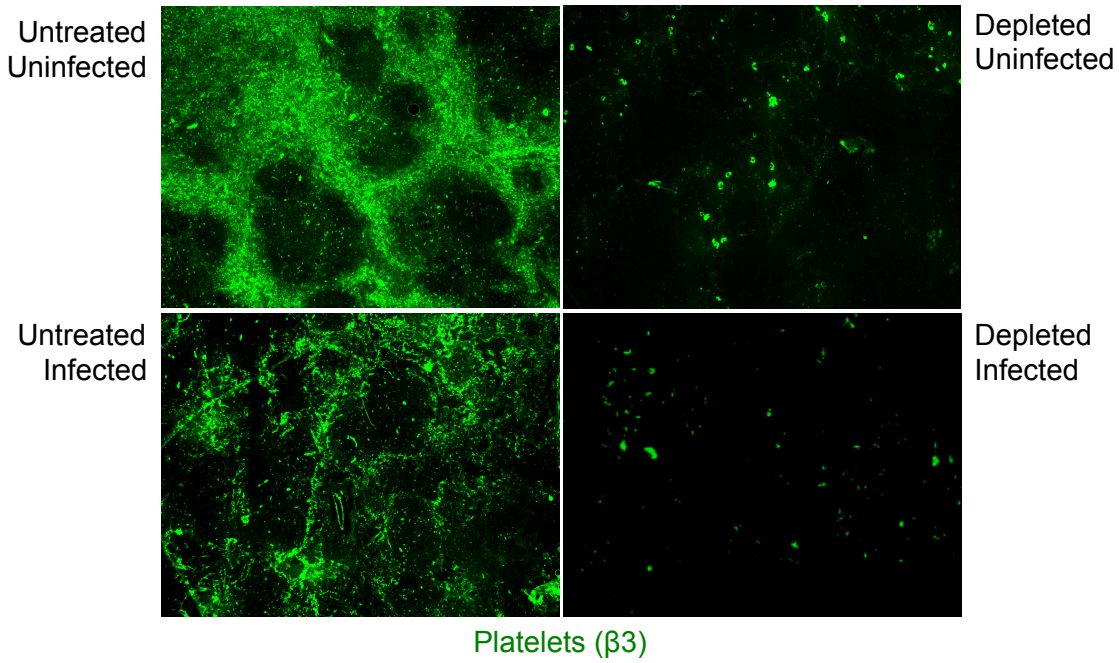

Untreated
Uninfected
Depleted
Uninfected
Untreated
Infected
Depleted
Infected
Platelets (β3)

Figure 7C

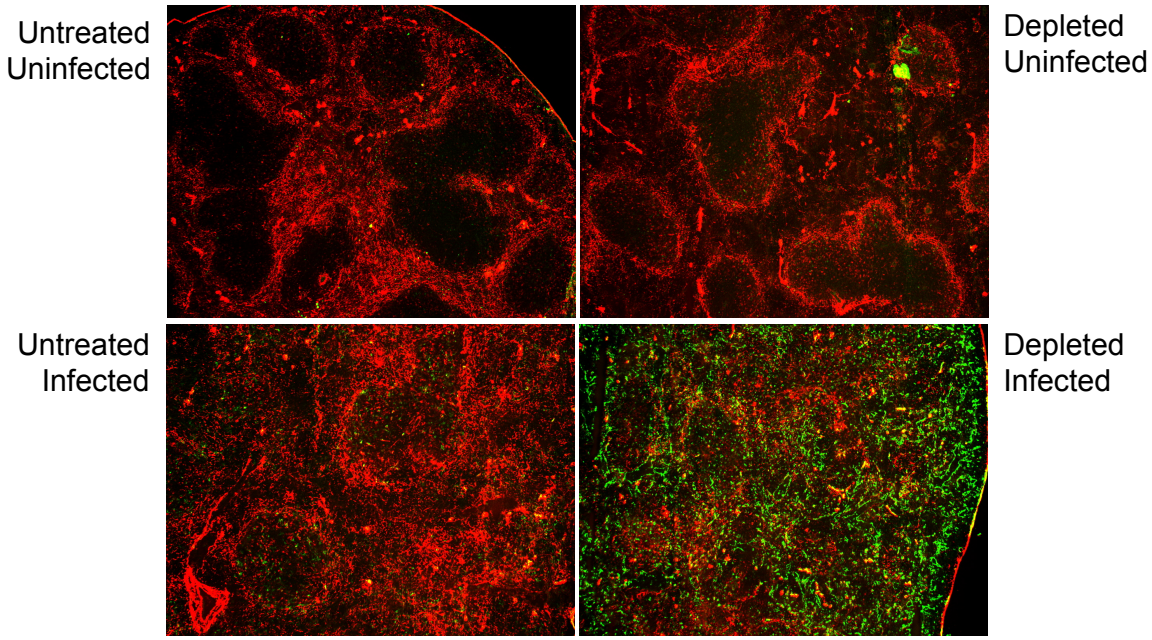

Untreated
Uninfected
Depleted
Uninfected
Untreated
Infected
Depleted
Infected
FRCs LCMV

Figure 7D

Figure 8. Vesicular Stomatitis virus (VSV) infection is also affected by the partial depletion of platelets.

Groups of mice were treated with PBS or 40µg of anti-GPIIb antibody 12 hrs before and 2 and 4 days post infection with 2x106 p.f.u. of VSV i.v. **A. Survival curves.** Mice were monitored daily for survival or signs of paralysis and sacrificed when it was noted. **B. Brain viral titers.** Brains of animals presenting signs of paralysis were homogenized and VSV titers quantified. Brains of untreated mice recovered at the end of the experiment were used as controls. (A and B, n=5). **C. Indistinguishable serum neutralizing antibodies.** Total and IgG VSV neutralizing antibody titers were determined in infected animals at days 4 and 8 p.i. (Open bars IgM plus IgG neutralizing titers, black bars IgG neutralizing titers; n=5 of two independent experiments. Error bars represent SEM).

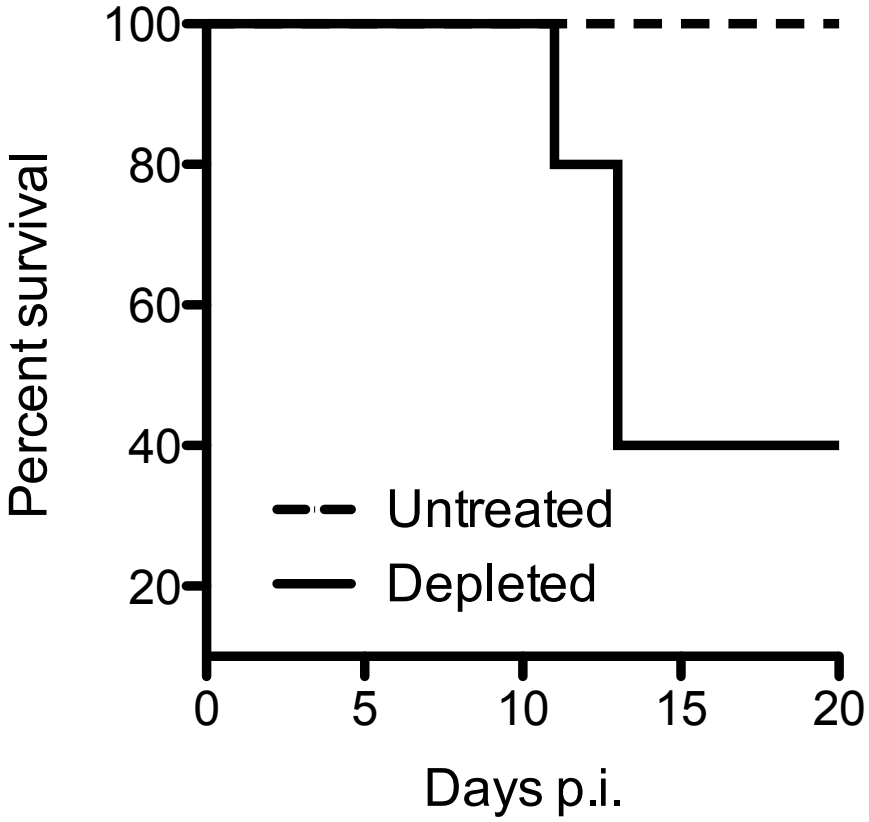

100
80
60
40
20
Percent survival
Untreated
Depleted
0
5
10
15
20
Days p.i.

Figure 8A

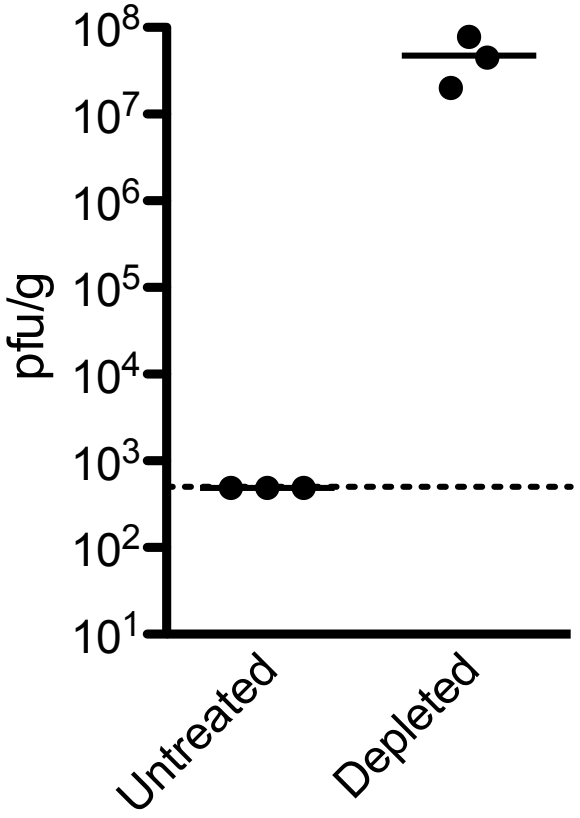


Figure 8B

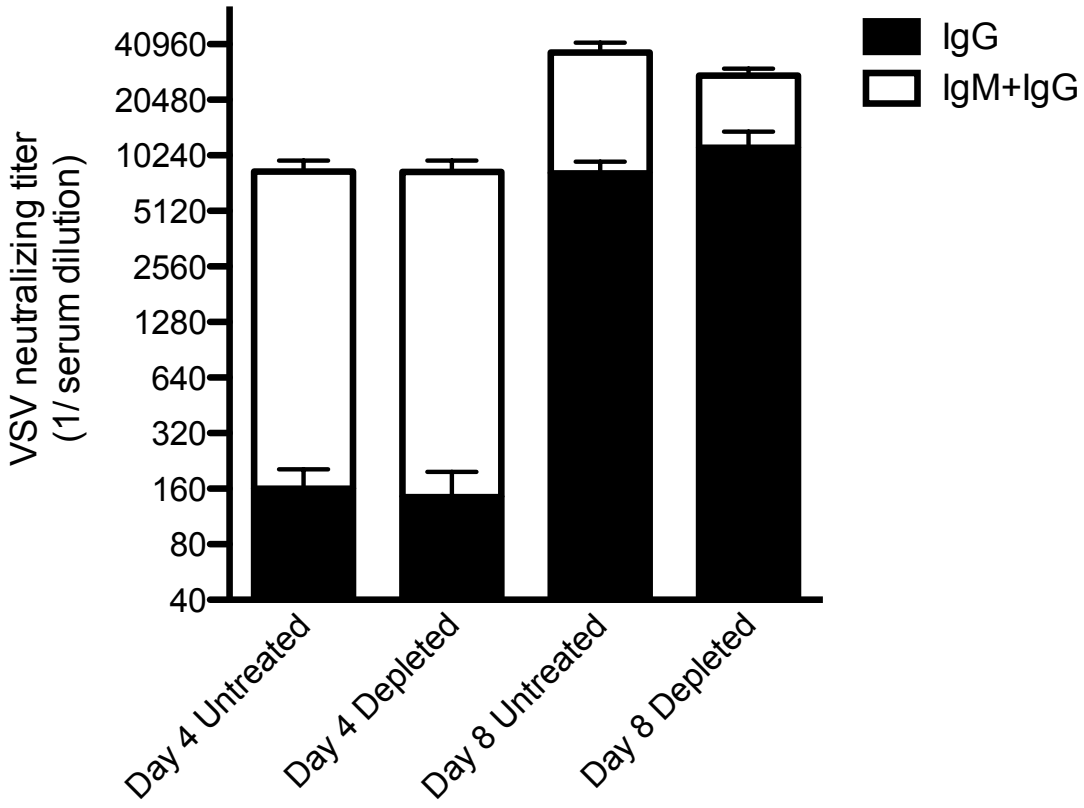

VSV neutralizing titer
(1/ serum dilution)
40960
20480
10240
5120
2560
1280
640
320
160
80
40
IgG
IgM+IgG
Day 4 Untreated
Day 4 Depleted
Day 8 Untreated
Day 8 Depleted

Figure 8C

Chapter 5: Discussion

Here we have demonstrated the importance of the physical presence of platelets in the prevention of typical clinical manifestations of severe VHFs in a murine model of LCMV infection. First we showed that systemic bleedings and death of the mice due to LCMV infection appeared only when more than 85% of platelets were depleted and second we described that even a milder removal of platelets during infection provoked a necrotic destruction of splenocytes, impairing the control of the infection. Both observations are novel and relevant to the understanding of the pathophysiology of VHFs *per se*, but they also highlight basic concepts on the role of platelets in supporting vascular integrity during the response to viral infections.

When mice were profoundly depleted of platelets and infected with LCMV Armstrong, hemorrhagic spots in lungs, skin, brain, and intestines were observed along with some degree of mortality. Interestingly, when the depletion treatment was reduced so that 15% of platelets in blood were present at the time of the infection, neither bleedings nor death were observed. This implies that platelet counts must drop to extremely low levels for hemorrhage and death to occur, and indirectly links the cause of death to the increased vascular leakage and systemic shock. Iannacone (Iannacone et al., 2008) demonstrated that severe thrombocytopenic wild type mice infected with LCMV developed lethal hemorrhages, that mononuclear infiltrates and viral antigens were not present at the hemorrhagic sites, and that development of hemorrhages was absolutely dependent upon signaling through the IFN-α/β receptor. Furthermore, it has been shown that inflammation induces hemorrhages in uninfected thrombocytopenic mice only at the specific anatomic locations were the inflammatory stimulus was

applied, and that 10% to 15% of platelets were sufficient to prevent the hemorrhages. (Goerge et al., 2008) Altogether, these data are consistent with the hypothesis that the hemorrhages and death in severely thrombocytopenic mice infected with LCMV (Iannacone et al., 2008) may be consequences of the high levels of IFN-α/β released into the serum, inducing a systemic inflammation that opens the vascular barriers, allowing the escape of fluids and erythrocytes into the tissues. The extreme efficiency of a small number of platelets at preventing these systemic manifestations suggests that physical adhesion and aggregation may not be required but, perhaps, the mechanism in place may involve transient delivery of signals to the endothelium during platelet rolling through membrane receptors or vasoactive compounds from the platelet granules. (Ho-Tin-Noé et al., 2011; Ho-Tin-Noé et al., 2008)

Next, we observed that mice with reduced levels of circulating platelets, lower than normal levels but high enough to prevent hemorrhages and death, failed to control infection with LCMV Armstrong due to necrotic destruction of innate and adaptive immune splenocytes and the subsequent exhaustion of the remaining CTLs. Infection of untreated adult mice with LCMV Armstrong induces a strong and fully protective CTL response able to clear the infection in a week. If the virus is inoculated intracranially, the migration of CTLs into the CNS to fight infection provokes a fatal meningitis. Mechanistically, this is mediated by a massive myelomonocytic infiltrate recruited by the CTLs in the meninges. (Kim et al., 2009) This large migration breaks the vascular barrier, allowing the release of fluids into the CNS interstitial tissues, which may ultimately be the event leading to the seizure and death of the mice. On the other hand, Ho-Tin-Noe et al. (Ho-Tin-Noé et al., 2009; Ho-Tin-Noé et al., 2008) showed that tumor growth is reduced in platelet-depleted mice by a tumor infiltration of innate immune cells

that elicit hemorrhages resulting in the necrotic death of tumor cells. Soluble P-selectin serum levels increase during LCMV infection of mice, indicating that platelets are activated during infection. (Lang et al., 2008) Altogether, we hypothesize that infection with LCMV causes a dramatic alteration of splenic structure due to the large recruitment, proliferation, and control of the infection by immune cells, generating a stressful environment for the endothelial cells. The enhanced transmigration of cells may open holes in the vascular wall, exposing extracellular matrix that activates the platelets and these subsequently adhere to prevent the leakage of fluids; in the absence of platelets large quantities of vascular fluids and erythrocytes may be released in the interstitial tissues inducing hypoxia and necrosis of splenic immune cells resembling a splenic infarction. The need for platelets to directly attach to the exposed subendothelial tissue would explain the requirement for higher numbers of platelets to prevent the splenic necrosis than systemic bleedings, and also explain the platelet activation and consumption observed during infection. In our partial platelet depletion model, loss of viral control seems to require accumulated defects of both early (innate) and late (adaptive) immune responses.

The previous was a compelling result because similar immunosuppression and uncontrolled viral replication are seen in untreated adult mice infected with the LCMV clone-13 strain. We then attempted to compensate for LCMV-induced thrombocytopenia by increasing the number of circulating platelets prior to infection by using the megakaryocyte growth and development factor thrombopoietin, to evaluate if the increased platelet count would improve the control of the chronic LCMV clone-13 infection in mice. Under the conditions obtained in this study, no differences were seen in the viral titers reached 15 days postinfection, indicating that the immunosuppression that leads to persistent

replication of this strain is not a consequence of the thrombocytopenia. Even though the number of platelets was increased significantly before and at the nadir of the thrombocytopenia versus the untreated infected mice, complete reconstitution could not be achieved, probably due to the destruction of megakaryocytes by the IFN-α/β response that induces the thrombocytopenia. (Binder et al., 1997) We hypothesize that the virally-induced thrombocytopenia in LCMV clone-13 infected mice may not be severe enough to cause the immunosuppression, hemorrhage, and death; instead, other mechanisms such as altered cellular tropism and enhanced replication kinetics account for the observed persistence of clone-13 and similar isolates in wild type mice, the natural host. (Bergthaler et al., 2010; Cao et al., 1998; Mueller et al., 2007; Sevilla et al., 2000; Sullivan et al., 2011) We speculate that there could be a direct link between susceptibility to severe manifestations of VHFs and platelet counts in the host before the infection, where mice are resistant ($1.2x10^6$/μL), (Schmitt, Guichard, Massé, Debili, & Cramer, 2001) monkeys are more susceptible ($0.5x10^6$/μL), (Levin, 2007) and humans are highly affected ($0.2x10^6$/μL). (Gowen & Holbrook, 2008)

We extended our observations to a different infectious model by using the rhabdovirus VSV. When thrombocytopenic mice were infected with VSV, the animals succumbed due to extensive viral replication and possible destruction of neuronal tissues by the cytopathic nature of this pathogen. Even though normal levels of neutralizing antibodies were generated during the infection, the early loss of innate control may have facilitated the infection of terminal neurons from which the virus was axonal-transported retrograde towards the CNS before neutralizing antibodies could be generated. An alternative explanation may be that platelet removal opened the blood brain barrier allowing direct infection of

the CNS. However, we do not favor this hypothesis because platelet-depleted mice infected with the CNS-tropic LCMV Armstrong isolate did not have detectable virus replication in the brain 4 days postinfection (data not shown).

Chapter 6: Conclusions and future perspectives

The data presented in this work creates several research inquiries for future studies. First, vasoactive molecules such as S1P agonists, serotonin, and VEGF could be evaluated for their ability to prevent hemorrhages and death in experimentally platelet-depleted mice. Second, our results suggest that the higher level of circulating platelets compared to other species (including humans) may explain why mice have not proven to be an adequate general experimental model for VHFs. Mice genetically deficient in TPO signaling (*TPO* or *c-mpl* knockouts), which have platelet levels similar to those found in humans and are viable, healthy, and display no signs of spontaneous bleeding, may be a better system to study its pathology. (Gurney, Carver-Moore, de Sauvage, & Moore, 1994; de Sauvage et al., 1996) Third, we hypothesize that by selectively inactivating IFN-α/β signaling in megakaryocytes but preserving its important anti-viral activity in immune and stromal cells, we would be able to completely overcome the thrombocytopenia induced by infection with LCMV clone-13 and better study if its control has any positive effect. Finally, the mechanism responsible for the splenocytes destruction should also be studied to conclude whether it is mediated by complement, oxygen/nutrient deprivation, apoptosis, or if reactive oxygen species are involved in their demise.

In conclusion, we have shown in the murine model of LCMV infection that a spectrum of clinical manifestations resembling VHFs in humans can be manipulated by careful titration of antibodies used to deplete platelets. While nearly complete depletion of platelets produces severe hemorrhages and death, mice survive infection following partial platelet depletion but fail to control viral replication due to the necrotic destruction of innate and adaptive immune cells.

Our approach opens new insights for the study of the pathophysiology of VHFs and may provide a theoretical foundation for the development of new therapies for humans. Furthermore, an unrecognized pathogenic mechanism of immune escape is proposed (diagram number 3), where the viral infection decreases platelet counts, the drop in platelets counts affects the splenic hemostasis, altered splenic hemostasis compromise the protective immune response, which ultimately allows a higher and prolonger virus replication.

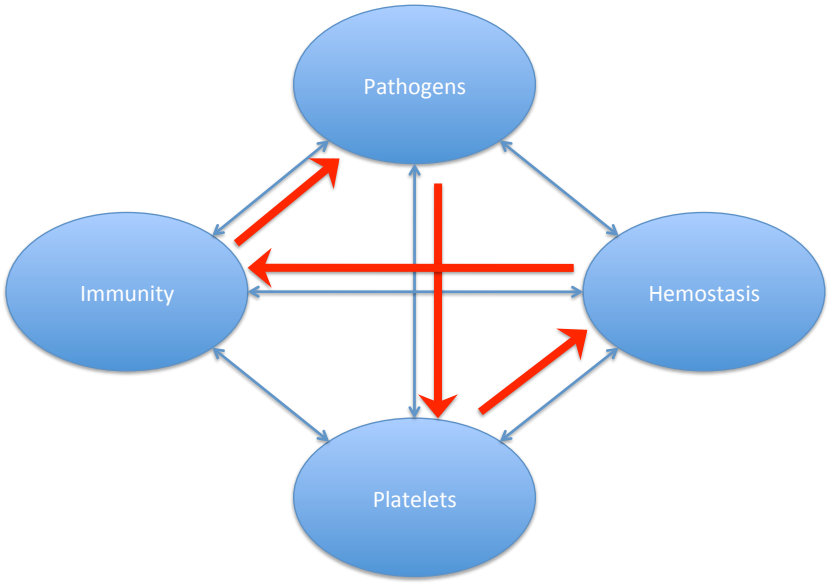


Diagram 3.

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