B-Myb is a crucial proviral host-factor and is involved in arenavirus mediated tumor suppression

Inaugural-Dissertation
zur
Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Piyush Sharma

aus Harpalpur, Hardoi, India

Essen, April 2017

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Immunologie der Universität Duisburg-Essen durchgeführt.

1. Gutachter: Prof. Dr. med. Karl Sebastian Lang
2. Gutachter: Prof. Astrid Westendorf

Vorsitzender des Prüfungsausschusses: Prof. Ralf Küppers

Tag der mündlichen Prüfung : 24.07.2017

CONTENTS

List of Figures:

SUMMARY

Viruses have minimal survival requirements thus ensuring their survival in harsh conditions but are unable to replicate independently. To overcome this, they hijack host resources and exploit host machinery for their benefit. These factors enable virus replication in the first place thus infecting the host but perhaps rather counterintuitively they are also necessary to ensure virus replication in antigen presenting cells (APCs) to ensure virus antigen processing and presentation ensuring immune activation such that the infection can be cleared.

Innate and adaptive immune system works side-by-side in order to effectively combat infections. We have shown that enforced virus replication in splenic $CD169^{+}$ macrophages is crucial for immune activation. In this study, we have identified a novel function of a transcription factor known for its crucial role in cell replication and proliferation. The present study thus deals with elaborating the role of B-Myb in virus replication and exploring the therapeutic effect of LCMV replication in tumors.

B-Myb is an important transcription factor for cell proliferation, member of DREAM complex and is also known to induce expression of multiple genes. Here, we show that B-Myb is also a major transcription factor activated during virus replication and helps the virus by maintaining the expression of various host-factors required for virion production and release. We observed that activation of B-Myb on viral replication is not cell-specific. We show that B-Myb dependent early virus replication in spleen is crucial for adaptive immune activation and thus anti-viral response in both RNA and DNA viruses. We also show here that B-Myb dependent replication is also a requirement for human viruses HSV and HIV. In the second portion of the thesis we explored the effect of B-Myb dependent LCMV replication in tumors. Interestingly, we show the potential of LCMV as anti-tumor virotheray which when compared to oncolytic viruses, is comparable or more effective. We demonstrated that LCMV treatment induces evasive immune infiltration in tumors. We show that LCMV treatment leads to tumor regression via IFN-I producing $Ly6C^{+}$ monocytes which acts as anti-angiogenesis.

In conclusion, we have identified a new function of B-Myb as host factor facilitating the replication of multiple viruses. We demonstrated the importance of B-Myb in early virus replication for immune activation and infection control. We also established LCMV as an IFN-I dependent anti-tumor therapy.

ZUSAMMENFASSUNG

Viren besitzen eine minimale Überlebensausstattung, die ihren Fortbestand in widrigen Umständen sichert, aber sie nicht zur unabhängigen Reproduktion befähigt. Letzteres zu kompensieren, berauben sie die Resourcen ihres Wirts und beuten die Wirts-Maschinerie zu ihrem Vorteil aus. Diese Faktoren ermöglichen in erster Linie die Virus-Replikation und führen daher zur Infektion des Wirts. Sie sind aber auch - vielleicht eher kontraintuitiv – nötig zur Sicherung der Virusreplikation in antigen-präsentierenden Zellen (*antigen presenting cells*; APCs), wodurch es zur Antigen-Prozessierung und -Präsentation kommt, welche widerum die Immunaktivierung auslöst, damit die Infektion beseitigt werden kann.

Das angeborene und das adaptive Immunsystem arbeiten nebeneinander, um Infektionen effektiv zu bekämpfen. Wir haben gezeigt, dass eine verstärkte Virusreplikation in den CD169+ Makrophagen der Milz wichtig für eine Immunaktivierung ist. In dieser Arbeit haben wir eine neue Funktion eines Transkriptions-Faktors identifiziert, der bereits für seine bedeutende Rolle in Zellreplikation und -proliferation bekannt ist. Die vorliegende Arbeit erarbeitet Rolle von B-Myb in der Virus-Replikation und untersucht den therapeutischen Effekt einer LCMV-Infektion bei Tumoren.

B-Myb ist ein wichtiger Transkriptionsfaktor für Zellproliferation, Mitglied des DREAM-Komplexes und bekannt dafür, die Expression vielzähliger Gene zu induzieren. Hier wird gezeigt, dass B-Myb auch ein bedeutender Faktor ist, der während der Virus-Replikation aktiviert wird und dem Virus hilft, indem er die Expression verschiedener Wirts-Faktoren erhält, die für die Virion-Produktion und -Freisetzung benötigt werden. Wir haben beobachtet, dass die Aktivierung von B-Myb bei der Virus-Replikation nicht zell-spezifisch ist. Wir zeigen, dass B-Myb-abhängige frühe Virus-Replikation in der Milz wichtig für die Aktivierung des adaptiven Immunsystems und somit der anti-viralen Antwort ist und das sowohl bei RNA- als auch DNA-Viren.

Zudem wird gezeigt, dass die B-Myb-abhängige Replikation eine Bedingung für die humanen Viren HSV und HIV ist. Im zweiten Teil der Arbeit wird der Effekt von B-Myb abhängiger LCMV-Replikation bei Tumoren untersucht. Interessanterweise, decken wir das Potential von LCMV als Anti-Tumor-Therapeutikum auf, dass verglichen mit onkolytischen Viren vergleichbar oder sogar effektiver ist. Wir demonstrieren, dass eine LCMV-Behandlung ein Eindringen von Immunzellen in Tumore bewirkt. Darüberhinaus wird

gezeigt, dass eine LCMV-Behandlung zur Tumor-Regression aufgrund von IFN-I produzierenden Ly6C+ Monozyten führt, welches anti-angiogenetisch wirkt.

Zusammenfassend haben wir eine neue Funktion von B-Myb als Wirtsfaktor identifiziert, der die Replikation mehrerer Viren vereinfacht. Wir zeigten die Wichtigkeit B-Myb's für die frühe Virus-Replikation, Immun-Aktivierung und Infektionskontrolle auf. Zudem etablierten wir LCMV als eine IFN-I-abhängige Anti-Tumor-Therapie.

CHAPTER-1

INTRODUCTION

Organisms are constantly evolving in order to adapt and survive the changing environment and survival needs. Humans (*Homo sapiens sapiens*) are constantly surrounded by different kinds of pathogens that range from viruses to bacteria to parasites. Among these, viruses present an interesting scenario with their dependence on the host for replication and propagation. Viruses use host resources in order to replicate and to evade immune response in order to facilitate survival. The host relies on its immune system to extradite pathogens.
Immune system represents an organized set of cells and molecules that function as a defence against the pathogens, allergens and various other foreign elements. The immune system functions in two fundamentally different responses namely innate (humoral) response and acquired (adaptive) response. The innate response behaves in the same way every time it encounters an antigen whereas acquired immune response improves upon repeated exposure to the antigen.

1.1 Immune System

1.1.1 Innate immunity:

Living organisms have constantly been threatened by different pathogens, which in turn use them for their survival. With the constant evolution of pathogens and emergence of new pathogens, both invertebrates and vertebrates have also evolved multiple ways to tackle them. The first line to response that both the invertebrates and vertebrates have is the innate immune response.

Innate immune system is the ancient form of host response against invading pathogens and it is universal. It's present in all vertebrates and invertebrates and thus can be conferred that it evolved before divergence into kingdoms. This part of immune system covers quite an extensive area of host defence which includes recognition of pathogen associated molecular patterns (PAMP), and also lies behind most of the inflammatory response. Functionality of innate immune response relies on innate immune receptors or germline-encoded recognition receptors which in-turn sense various microbial metabolites produced by pathogens. Innate immune system comprises of many cells, except for B and T lymphocytes of the adaptive system. To be more specific, the cells which functions as the base of innate immune system are macrophages, dendritic cells (DC), mast cells, neutrophils, eosinophils and NK cells. Activation of these cells during an inflammatory response can be

considered as a sign of pathogenic infection. These cells, upon encountering a pathogen divide and differentiate rapidly into effector cells for elimination of infection. Considering a scenario where innate immune system fails to control the infection, it instructs the adaptive immune system about the infection and nature of pathogenic challenge.

This is done by expression of co-stimulatory molecules such as CD80, CD86 on the surface of specialized antigen presenting cells, dendritic cells. The innate immune system is composed of many factors that all together results in effective functioning of this system.

1.1.1.1 Pattern Recognition Receptors:

Innate immune system employs a variety of receptors that are expressed on the cell surfaces, intracellular compartments, secreted into the body to recognize and neutralize antigen/antigen infected cells. These receptors are called pattern recognition receptors (PRR). The functional aspect of these includes opsonisation, phagocytosis, complement system and proinflammatory pathway activation and induction of apoptosis.

Secretory PRR*:* These include Mannan-binding protein (MBP), C-reactive protein (CRP) and serum amyloid protein (SAP). These are produced by liver during infection. Primary roles of these include opsonisation and activation of classical complement pathway. With the different binding affinities, these covers a wide array of pathogens. CRP and SAP bind to phosphorylcholine whereas MBL binds specifically to terminal mannose residues.

Intracellular PRR: Intracellular access of several pathogens, while evading the immune system requires an effective recognition of these in cellular compartments. Protein Kinase (PKR) are group of proteins, which are activated upon dsRNA binding[1], during viral infection. PKR further activates NF-κB and MAP kinase pathways leading to type-I interferon dependent response and also inactivates eIF2α which blocks cellular role in viral protein synthesis. Other PRR includes 2'-5'-oligoadenylate synthase (OAS)/RNaseL[2], which also gets activated upon dsRNA binding and acts by degrading the viral RNA and cellular RNA of infected cells leading to apoptosis. NOD family acts as a PRR in multiple ways. It contains N-terminal CARD domain, nucleotide binding domain (NBD) and C-terminal leucine rich element (LRR) region[3, 4]. They act by RIP2 which further activates NF-κB and MAP kinase pathways[3-5].

Toll Like receptors*:* TLR's or Toll Like receptors functions by sensing conserved molecular motifs known as PAMPs. These PAMPs are found in variety of pathogens which include bacteria, protozoa, fungi and viruses[6-9]. More than ten TLRs have been discovered in

mammals till now and differs in expression patterns and ligand specificities. Several TLRs are expressed on pDC to encounter and recognize viral components such as viral RNA and DNA. Among these, TLR2, 3, 4, 7 and 9 recognize viral components whereas TLR2 and 4 are critical for recognition of bacterial components and are present on plasma membrane[10]. All TLRs except for TLR3 acts via myeloid differentiation factor 88 (MyD88)[10] which further goes down to activate NF-κB signalling pathway whereas TLR3 acts via recruitment of TIR-domain containing adaptor inducing IFN-β (TRIF)[11, 12]. All together viral nucleotides recognition by TLR is controlled as per their localization as well as their ligands.

1.1.1.2 Cellular components:

Innate immune compartment consists of cells from the myeloid lineage. These cells include mononuclear and polymorphonuclear phagocytes. Mononuclear phagocytes include macrophages and dendritic cells whereas polymorphonuclear phagocytes consist of neutrophils, basophils and eosinophils[13].

1.1.1.2a Macrophages:

These mononuclear phagocytes were discovered in late 1800 by Elie Metchnikoff and named as "Big eaters" in Greek for their astonishing ability of phagocytosis. These are resident cells in lymphoid and non-lymphoid tissues. The role of macrophages is not limited to an immune effector cell but also acts as by removing the cellular debris during the tissue remodelling and apoptosis of the cells. These cells are present in virtually every tissue and arise from blood-circulating monocytes. The tissue macrophages are long-lived and are involved in the early host immune response. Functional role of macrophages can be circled into three main categories host-defence, wound healing and immune regulation[14]. Broadly they are functionally classified into M1 and M2 macrophages. The former is considered as "killer" macrophages and secrete high levels of IL-12 on activation, whereas latter are more anti-inflammatory and are involved in tissue repair and wound healing.

Tissue resident macrophages are group of heterogeneous populations of cells that functions in tissue-specific way. These acquire different name based on their residing location. These macrophages are seeded into different organs during the embryonic stage and then differentiate into different resident macrophages guided by the tissue microenvironment cytokines and metabolites.

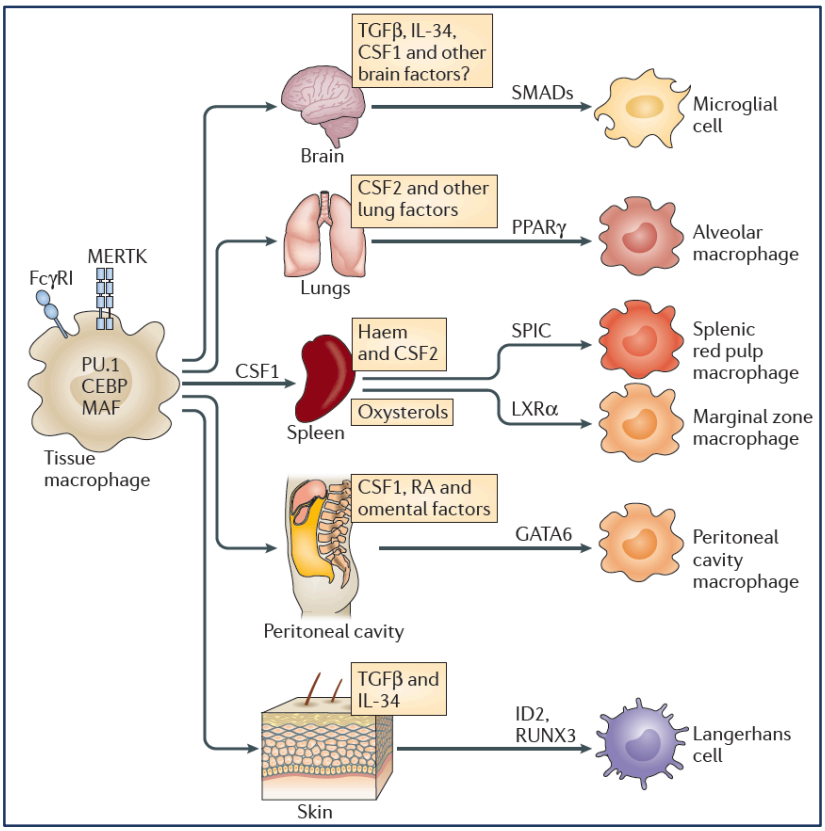


Figure 1: Tissue resident macrophages and their differentiation determinants (Lavin, Y. et. al., *Nat. Rev. Immunol.* 2015)

Among these, are the marginal zone macrophages of the spleen and LN which are characterized by the surface marker CD169. These cells are shown to be important for early virus replication[15] and thus in processing and presentation of antigen to adaptive immune cells.

1.1.1.2b Dendritic cells:

These cells were discovered by Ralph Steinman in 1973[16] and named after Greek word "Dendron" meaning tree. DC's are specialized in antigen processing and excellent stimulators of T-cells. Further discovery led to another major milestone showing expression of both major histocompatibility complex (MHC)[17, 18] by them as well as to initiate and antigen-specific immune response by processing antigen protein. DCs have differentiated into different subsets depending on their location and function. These subsets basically include classical DC(cDC), plasmacytoid DC (pDC) and monocyte-derived DC (moDC)[19, 20]. These

all play different role during an immune response. cDC are found in lymphoid organs as well as in most of the non-lymphoid organs. These are basically involved in antigen processing and presentation to adaptive immune cells. These then migrate as well as depending on the maturation stage express/overexpress various co-stimulatory molecules such as CD40, CD80, CD86, MHC-II. pDCs on the other hand are characterized by their constant presence in blood and in peripheral organs[21]. These have a constituent expression of IRF7 and thus act as potent stimulator and producer of type-I interferon upon infection[22, 23]. moDCs or imflammatory DC originate from monocytes infiltrates during inflammation. These possess similar expression profile as of cDC, differing in CD64 and Fc-gamma receptor 1 (FcγR1) expression[24, 25]. These cells efficiently stimulate T cells like cDC but requires more elaborate research on their distinct expression profile.

1.1.1.2c Polymorphonuclear phagocytes:

These granular players play key roles in the containment of infection. These cells mature in bone marrow and then enter blood circulation in their fully differentiated form. They remain in the G0 stage for their short life span, unless triggered by external stimuli. Neutrophils acts by expanding rapidly during course of infection and possesses a vast array of effector molecules to eliminate the infection. These are thus often known as specialized killers. Basophils and eosinophils particularly respond to the cytokines secreted by adaptive system and thus helps in shaping of immune milieu[13]. Mechanism of function of these granulocytes is also via release of these granules which comprises of proteolytic enzymes, cytotoxins, antimicrobial peptides, etc. along with production of cytokines, chemokines and pro-inflammatory molecules[26-28]. Neutrophils and eosinophils also produces ROS as an immune response[29, 30]. These cells migrate to the inflammation site in response to chemoattractants. Granulocytes can be identified with the expression of Gr-1, which can be sub divided into Ly6G and Ly6C. Ly6G markers on them along with CD11b. Reports have shown that F4/80 antibody also binds to Ly6G presenting population. Ly6C is enriched in monocytic myeloid lineage[31].

1.1.2 Adaptive Immunity:

Adaptive immunity is the second type of immune response constituted by the host. All the vertebrates harbour this type of immunity along with innate immunity. This part of

immune system allows the host to embark an immune response in case of new pathogens and to make a memory against them. This is an important feature as it enables the host to launch a heightened response against the pathogen upon re-encounter.

Adaptive immune system comprises of two primary sets of cells; T cells and B cells. These cells are further divided into many subsets which arises on functionality and situational needs.

1.1.2.1 T cells:

These cells originate from haematopoietic compartment of bone marrow and then migrate to thymus for maturation. These double negative cells (DN) (neither CD4, nor CD8) are also known as committed T-cells precursor. These DN cells further give rise to either γδ or αβ TCR-expressing cells. In case of TCR- αβ, re TCR-α and β undergo sets of somatic rearrangements under the control of recombination-activating gene-1 and 2 (RAG1, RAG2)[32, 33], deficiency of which leads to immature T-lymphocyte[34] and eventually elimination. Post rearrangements, thymocytes begin to express CD8 and CD4 receptors as double positive cells (DP cells). DP cells then undergo stringent selection process based on their ability of interact with self-peptide-MHC I and II presented by cortical epithelial cells. These processes can be characterized as positive and negative selection. Finally cells which can recognize self-ligands are further differentiated into mature cytotoxic T cells (CD8+) or T-helper cells (CD4+)[35]. T-cell recognizes antigen presented by APCs via MHC-I and II complex.

1.1.2.1a Cytotoxic T-cells:

These are involved in elimination of infection, when triggered they undergo clonal proliferation into effector cells which produce perforin, granzyme and granulysin[36]. First shown as a subset of T-lymphocytes involved in *in-vitro* cell mediated lysis of target cells[37, 38], these cells were then identified to present dual specificity against viral antigen plus self-MHC[39, 40]. Activation of $CD8^+$ cells in done via APC interaction, upon which naïve $CD8^+$ T-cell migrate to peripheral regions of the tissue leading to its differentiation into effector and memory cells[41, 42]. This rapid expansion of cells in associated with enhanced metabolic activity involving rapid aerobic glycolysis, which is shown to be triggered by CD28-induced PI-3K-mTOR, TCR-ERKsignalling[43, 44]. The antiviral response of these cell can be accounted by production of IFNγ and TNFα. The differentiation of naïve $CD8^+$ cell to effector and memory cells in governed by expression of multiple genes as well as cytokines after antigen

primimg[45]. IL12 promotes effector T-cell development via T-bet induction whereas IL-2 cytokine promotes expression of transcriptional repressor Blimp-1, which in-turn leads towards the effector cell formation decreasing the memory cell formation[46] (Fig.2). Memory $CD8^+$ cell differentiation is promoted by factors such as Ecf-1, Bcl-6 and Eomes. Memory cell are long-term persisting cells which arises during the late infection phase. These cells persist in antigen and TCR-independent but cytokine-dependent manner[47, 48]. The role of memory $CD8^+$ T-cell is to provide rapid proliferation and expression of effector molecules during re-encounter of the antigen to launch an immediate effective response.

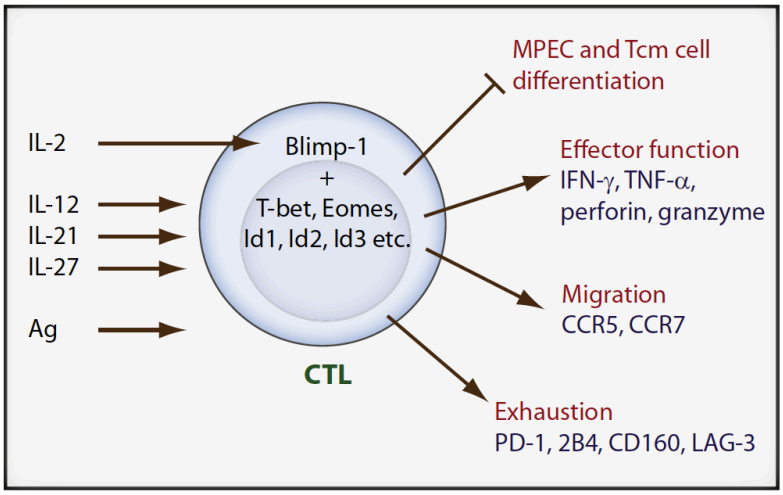


Figure 2: Effector of $CD8^+$ T-cells Differentiation

1.1.2.1b Th cells:

These cells possess no cytotoxic or phagocytic characteristic but rather they play a crucial role in enhancing and establishing an immune response. CD4+ T cells differentiate into numerous subsets which are characterized by their cytokine profile and CD expression profile. These include Th1, Th2, Th9, Th17, Th22, Tfh (Follicular helper T-cells) and T_{regs} (Regulatory T-cells)[49]. Once activated via recognition of antigen bound to MHC-II, they act via secretion and regulation of different cytokines. Each subset is differentiated via specific cytokines[49, 50] (Fig. 3). Th1 response can be recognized by production of interferon-gamma (IFN-γ) and TNF which further enhances bactericidal activities of macrophages and induction of B-cell response in neutralizing antibodies. Th2 on the other hand releases cytokines (Interleukin-4, 5 and 13) which further activates and recruits IgE producing B cells, mast cells and eosinophils[36]. Th9 produces IL9, Th17 produced IL17 thereby promoting host

defense in bacterial and fungal infection. Post clearance of infection, effector T cells and Th cells are cleared by phagocytosis with few remaining as memory T-cells which can rapidly transform into effector upon relapse of infection[51].

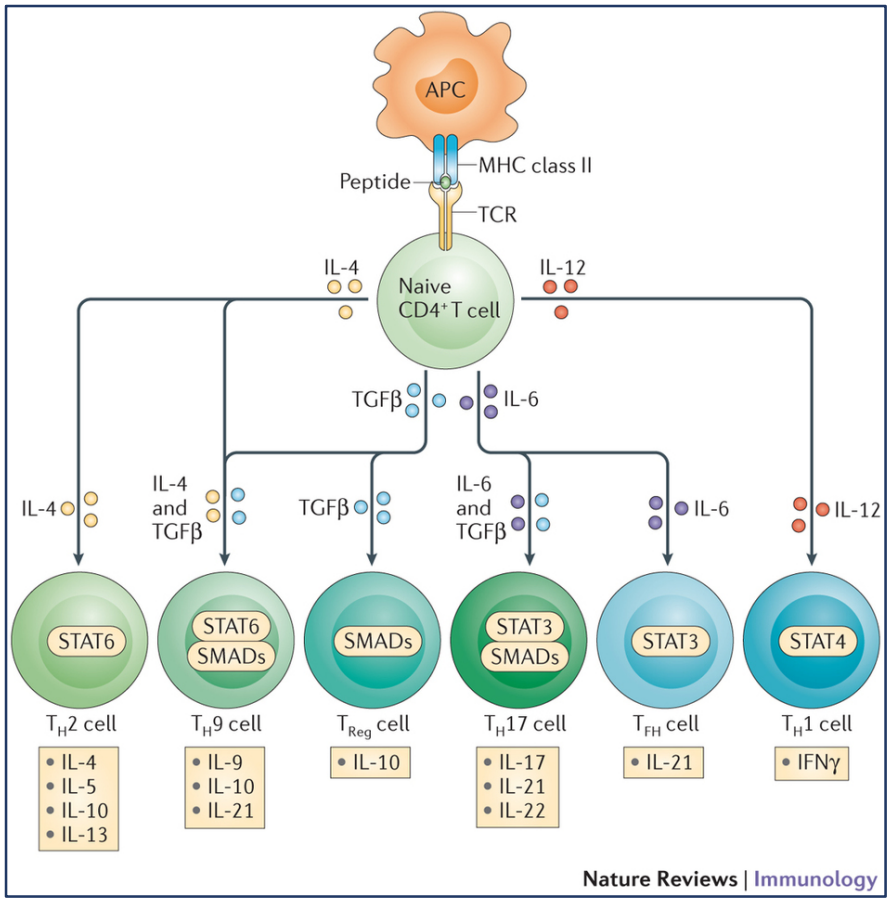


Figure 3: Th differentiation upon antigen recognition. (Kaplan, M.H., et. al., *Nat. Rev. Immunol.* 2015)

1.1.2.2 B-cell:

With the early indication of existence of these cell in 1890 with the recognition of proteins secreted by B-cells then known as serum globulins, B-cells were formally introduced in 1965 by M.D. Cooper[52]. This is the population of cells which can be defined by its expression of clonally diverse cell surface protein known as immunoglobulins (Ig) receptors recognizing specific antigenic epitopes. In addition, B-cell have other function which includes antigen presentation and regulatory cytokine production[53]. These cells are continuously produced in the bone marrow[54] with a short life span. Early development involves rearrangement of H and L chains of Ig, leading to Pre-BCR (in murine) or Pre-B-

cell (in humans), these arise from progenitor B-cells (pro B-cell) which lacks expression of both pre-BCR and surface Ig. Later development of B-cell occurs in an antigen-independent manner via process of ordered combinatorial rearrangement of V, D and J segments in H chain locus and V and J segment in L chain loci[55] which results in the very diverse repertoire of VDJ_H and VJ_L functional rearrangements[56], collectively forming B-cell receptor (BCR). These rearrangements are triggered/stimulated by variety of stimuli such as marrow stromal-cell derived IL-7[57], FLT3 ligand, E2A, EBA and Pax5 transcription factors[58]. BCR expression during the development of B-cells presents a crucial objective for B-cell development and their survival in the periphery[59]. On the other hand, antigen induced B-cell development, differentiation and activation occurs in secondary lymphoid organs governed by dynamic changes in gene expression forming germinal center (GC) reaction. GC reaction plays a crucial role in B-cell mediated immunity as they contain rapidly proliferating cells generates diverse high affinity-Ig secreting plasma cells and memory B-cells[60] against the antigen. The signature of GC reaction can be characterized by clonal expansion, somatic hypermutation at V_H genes, recombination in class switching at IgH locus and affinity maturation of a BCR for its unique antigenic epitope.

1.2 B-Myb *(Mybl2)*:

The myb gene is a proto-oncogene and was initially isolated in 1941 by W.J. Hall. It is a family of three conserved homologous proteins A-Myb, B-Myb and C-Myb. Among these, B-Myb is a powerful molecule with a plethora of interacting partner with which it exerts its effects on crucial cellular functions such as cell proliferation, apoptosis, senescence, cell metabolism and mitosis. This vast spectrum of its function has led to the interest in this molecule and its family.

After decades of modifications, molecular cloning and analysis of v-myb revealed the evolutionary conserved proto-oncogene c-myb[61]. In the late 1980s', A-Myb and B-Myb was discovered by screening of human T-cell cDNA library which was probed using C-Myb probe at low strigency[62, 63]. This family is mainly characterized by the presence of an evolutionary conserved DNA-binding domain of 50 amino acids[64] and binds to the same consensus sequence (PyAAC(G/T)G)[61, 65]. Thus, collectively myb family represents a group of functionally diverse transcriptional activators, which are found in both plants and animals.

In animals, myb family members are transcriptional activators which are basically involved in cell proliferation[66].

1.2.1 Structure:

B-Myb, encoded by *mybl2* gene and is ubiquitously expressed[62]. B-Myb shares an unique homology with A and C-Myb. It is composed as R1, R2 and R3 which are three tandem repeat sequence of 50 amino acids[67] and marks the DNA binding site of the protein. Specifically, R1 is required for stabilization of DNA-R2R3 complex[68] in which R2 and R3 binds to DNA cooperatively. These are followed by the acidic amino acid region responsible for transcription activation. The carboxy terminal of B-Myb is a negative regulatory domain and is involved in its transactivation function[69] (Fig. 4).

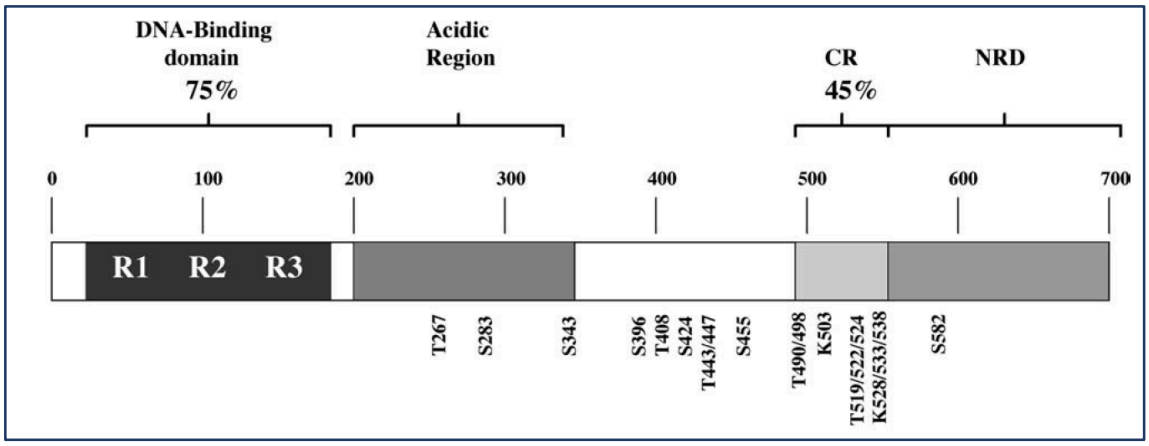


Figure 4: Schematic presentation of different domains of B-Myb protein (Jaoquin, M. and Watson, R.J. *Cell Mol Life Sci,* 2003)

1.2.2 Function:

B-Myb is widely accepted as the transcription factor having a role in cell proliferation and growth[70]. The expression of B-Myb is regulated via its E2F transcription family by binding to its E2F binding site at its promoter region[71]. Binding to E2F4 during G0/earlyG1-phase have been shown to repress B-Myb transcription, whereas binding of E2F1/3 activates it in mid-late G1-phase[72]. B-Myb transcription starts at the onset of G1/S-phase which is gradually declined till G2M-phase and is functionally activated by cyclinA2-CDK2 complex[73, 74]. B-Myb is different from its other family members as it is expressed in all replicating cells and shows strong association and functional relevance in cell cycle

progression[75] and proliferation[76-78]. Activated state of B-Myb is denoted by its phosphorylation which also triggers its degradation via ubiquitin-dependent Cdc34-SCF pathway[79]. Crucial role of B-Myb in cell cycle regulation was shown by many reports along with the early stage embryonic lethality in B-Myb gene knockout mice[80].

Phosphorylation of B-Myb can be observed at its maximal level at the S-phase[81] which has been shown to be conducted via Cyclin-A2/Cdk2. Cyclin-E1 showed minimal or no effect whereas cyclin-B1 and D1 had no effect on B-Myb phosphorylation[82, 83]. Due to presence of multiple probable phosphorylation sites, various studies were done in order to identify the sites corresponding to Cyclin-A2/Cdk2 binding. These reports have identified numerous sites (T447, T490, T497, T524 and S581) which represent a preference to a consensus site (S/T-P-X-K/R) of Cyclin-A2/Cdk2[84, 85]. Further studies identified 10 more phosphorylation sites each containing the consensus sequence S/T-P, which is known to be consistent with phosphorylation with Cdks. Phosphorylation of B-Myb causes two important functional triggers: (1) Crucial enhancement of its transcriptional activation properties and (2) it flags the protein for its degradation. Upon phosphorylation, B-Myb is localized to nuclear compartment which is triggered by multiple nuclear localization signals (NLS)[86]. Here B-myb acts binding directly to the Myb-binding sites in the genomic DNA and also via its interaction with transcription factors which further to certain genes[87]. Along with its own function of transcriptional activation of genes, B-Myb have been shown to interact with multiple partners in order to affect gene expression. One such scenario is of LINC/DREAM complex, in which B-Myb associates directly with MuVB (Dream) complex which comprises of E2F, p130, p107 and FOXM1[88]. The dream complex acts as a master coordinator of the cell cycle dependent gene expression which when disturbed leads to shifting towards proliferation stage with increased mitotic gene expression level frequently leading to tumor development. Also, overexpression of B-Myb enables cells to escape the routine stages of cell cycle and arrest[77] leading to uncontrollable growth[65, 89].

1.2.3 B-Myb in tumors:

As discussed above, B-Myb overexpression is associated with oncogenesis and poor prognosis[65, 90]. It has been shown that repression of B-Myb can inhibit the proliferation of both normal and tumor cells[76, 78, 87, 91]. This effect of B-Myb can also be contributed to its effect of increased expression of certain anti-apoptotic genes namely Bcl2, survivin,

clusterin, etc[92-94]. A number of reports have shown association of B-Myb in multiple tumors including hepatocellular carcinoma[95], breast cancer[96], colorectal cancer[97] and lung cancer[98].

1.3 Viruses

1.3.1 LCMV

LCMV is a prototypic member of arenaviridae family, discovered in 1933 by Armstrong and colleagues from the samples of an encephalitis epidemic[99]. It is a negative – strand RNA virus with a cytoplasm restricted life-cycle with a bi-segmented genome. With spherical or pleomorphic virions ranging from 40-200nm, it has viral glycoprotein (GP) as spike like structure on lipid envelope arranged subjacent to Z-protein and ribonucleoproteins[100] containing circular helical nucleocapsid with 400-1300nm in length. The genome consists of two negative ss-RNA molecules; small (3.5kb, S) and a large (7.2kb, L) segment, where small segment encodes the major components including viral nucleoprotein (NP) and glycoprotein (GP) precursors and Large segment encode viral RNA dependent polymerase and small zinc-finger structural protein Z. LCMV entry into the cell is initiated by binding to cellular receptors (α-Dystroglycan) with its GP-1 and GP-2 glycoprotein[101] and then endocytosed via uncoated endocytic vesicles.

LCMV infection: LCMV represents a cytopathic virus whose infection outcome differs from different strains[102]. There is a total of four strains that are present and being used for research purposes. Docile is the aggressive strains variants of strain UBS originally derived from WE strain and are hepatotropic in nature whereas clone-13 is derived from Armstrong and is neurotropic in nature[103]. Infection with WE or Armstrong causes an acute infection which is cleared by the host within 10-15 days. On the other hand, infection with Docile or Clone-13 establishes a persistent chronic infection, which takes 8-12 weeks to clear. The infection also varies with the initial infective titer. Infection with lower plaque forming unit (PFU) of 10^2-10^5 PFU LCMV mounts a massive $CD8^+$ T-cell expansion with neutralizing antibodies present by day 7. With higher PFU of $>10^6$, T-cell exhaustion[104] occurs after a weak CTL response. Infection with an intermediate PFU of 10^4 of Chronic LCMV, mounts a strong CTL response, which further leads to severe immunopathology.

1.3.2 VSV

VSV belongs to the genus Vesiculovirus in family of Rhabdoviridae and is a cytolytic virus. There are multiple variants have been isolated of VSV. The two principal strains which are used for research and medical purposes are VSV-New Jersey (VSV-NJ) and VSV-Indiana (VSV-IN). The former was isolated in New Jersey, USA in 1926 whereas the latter was isolated in Indiana, USA in 1925. VSV is a negative sense, ss-RNA, bullet shaped encapsulated virus with virion size of 70 × 200nm. VSV RNA consists of five genes that further encodes five major viral proteins namely the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large polymerase protein (L). The phospholipid capsule of VSV mediates the entry into the cell in a clatherin-dependent fashion.

VSV has been a model virus for its use in fields of molecular biology, virology and immunology. The simple structure, rapid replication rate and disease course, its broad tissue tropism all accounts for it favourable use in research[105]. VSV also exhibits neurotropism which represents the cause of host death in case of immune evasion by the virus[106]. In mouse models, if the VSV infection is not controlled efficiently enough during its early replication then virus escapes to CNS and then eventually breach blood-brain barrier reaching brain and causing paralysis with in 6-7 days of infection[107]. VSV is an interferon responsive virus whose control is majorly dependent on the antiviral antibodies. Infection of VSV leads to a major interferon secretion and is often used as to study the interferon response/to induce interferon response in pathogenesis. T-cell response on the other hand is quite limited in VSV infection, and T cells usually help in controlling the virus spread to vital organs.

1.4 Interferon:

Interferon (IFN) was the first cytokine which was discovered in late 1950s by Isaacs and Lindenmann[108] in their study of a secreted factor in influenza-virus infected chick cells. Being discovered as an antiviral agent, it has been named Interferon because of its ability to interfere with the viral replication. IFNs are a multigene family, categorized into Type-I and Type-II IFN (Fig. 5). The former is also known as viral IFNs which includes IFN-α (Leukocyte) and IFN-β (fibroblast) and are induced by virus infection, while the latter is also known as immune IFN and consists of IFN-γ and are induced by mitogenic or antigenic stimuli. Type-I IFN is synthesized by most of the virally infected cell whereas Type-II IFN is only synthesized by special cells such as NK cells, $CD4^+$ Th-1 and $CD8^+$ T-cells. Viral IFN

are coded by 13 IFN-α and 1 IFN-β genes present on chromosome 9 in human, whereas in mouse it is coded by 14 IFN-α and 1 IFN-β (including 3 IFN-α pseudogenes) genes present on chromosome 4[109]. Induction of IFN are known to be done by two distinct ways; one is antigen processing and capture in endosomes or specialized mediator cells mediated by TLRs[110] and the other is ubiquitously expressed cytosolic receptors sensing mechanism mediated by RIG-I[111], MDA5 and DNA sensors. Presence of both interferons in the system ensure proper IFN induction despite of vast range of viruses and pathogens present.

IFN exert their actions via their cognate receptor complex expressed on all cell types. Type-I IFN receptor is IFNAR-1 and IFNAR-2 while Type-II IFN receptor is IFNGR-1 and IFNGR-2. The signalling involves the dimerization of IFNAR-1/2 with IFN-α/β and of IFNGR-1/2 with IFN-γ. IFN-mediated cellular signalling is done via JAK-STAT pathway as shown in Fig. 5.

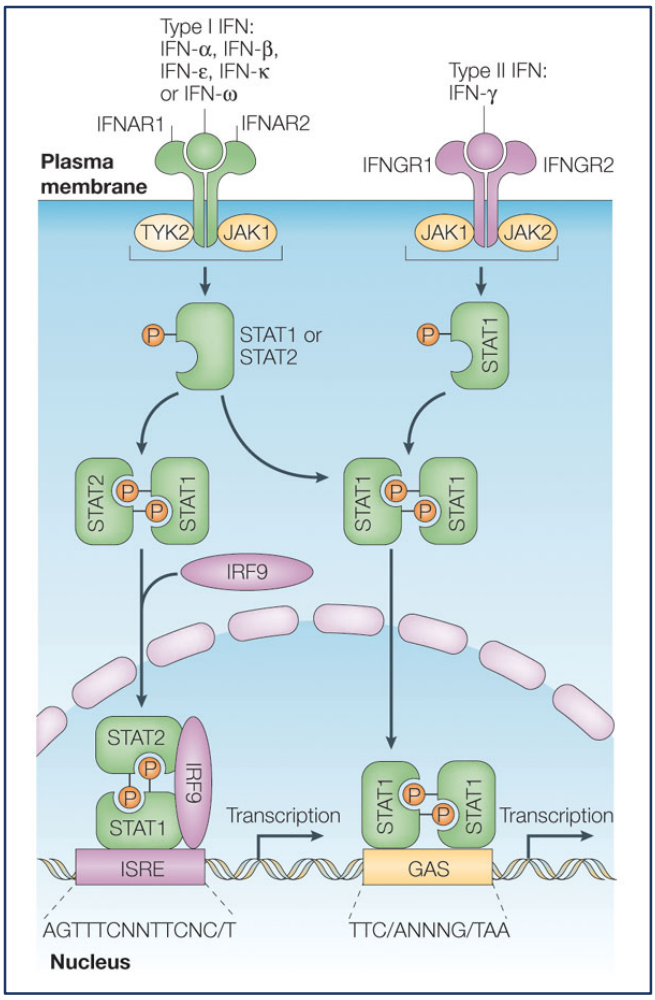


Figure 5: Type-I and II IFN signalling via JAK-STAT. (Platanias, L.C., *Nat. Rev. Immunol.* 2005)

1.4.1 Antiviral effect of Interferon:

Interferon is the primary step for the antiviral defense for a wide range of DNA and RNA viruses. IFN inhibition of viral multiplication cycles varies from early to late steps in different viruses. IFN induces proteins that further acts in anti-viral response, these are PKR, 2',5'-oligoadenylate synthetase (Oas), RNase L, RNA-specific adenosine deaminase and Mx protein GTPases[112]. Along with these IFN also induces nitric oxide synthase (iNOS2) and MHC-I and II, which plays crucial role in other anti-viral immune responses. Both IFN-α/β along with IFN-γ are able to induce MHC-I thus promoting CTL recognition of antigen and NK cell, whereas IFN-γ increases MHC-II expression on APCs, promoting antigen presentation to Th cells. IFN-I contributes via chemokines that results in activation and recruitment of NK and CTL, also they can activate NK cells directly[113].

1.4.2 Anti-tumoral effect of Interferon

IFN has been exploited for a long time for its anti-tumor capabilities. As discussed, IFN-I regulates activity of multiple cell types including DCs, macrophages, T-cells, B-cells, etc. IFN-I acts anti-tumor in a multi-faceted way. It has been shown to directly upregulate the expression of tumor-associated antigens (TAAs)[114] along with activation of APCs to cross-present TAA to T-lymphocytes[115]. Further IFN-I have been shown promote the recruitment of mature effector $CD8^+$ T-cells along with negatively regulating proliferation of T_{regs} reducing the suppressive function on CTL activity[116]. Along with this, IFN-I also expresses an extrinsic effect on tumors via regulation of angiogenesis, where it inhibits the formation of new vessels by mediating a negative effect on expression of pro-angiogenic factors[117].

1.5 Host Factors

Viruses are formed of a nucleic acid component with a protein shell in a miniscule size with rare lipid bilayer that they use to bind to their host cell. These are infectious agents that completely rely on the host resources for their replication. Despite of the simplicity of the viral structure, they have evolved complex mechanisms to hijack cellular functions in order to ensure their replication[118]. Upon infection, viruses hijack the basic machinery of

transcription and translation to manufacture viral proteins. All these factors which are provided by the host cell and are consumed by virus are known as host-factors.

Mammalian cells have a multitude of complex pathways and mechanisms to ensure the continuous and efficient functioning. This results in the precise activation and degradation of molecules to fulfill cellular energy and metabolic requirements along with transportation of molecules in and out of the cell. Viruses have been shown to utilize the cellular reserves and transport system to their advantage[119, 120]. Study of viral replication cycle proves to be crucial for the understanding of the infection and cellular response. Many such host factors for HIV[121], HCV[122], influenza[123], dengue[124], ebola[125] virus etc. have been identified via genomic and proteomic analysis of infected host cells, involved in viral entry, transcription and translation and assembly of viruses. One such report identifying an kinase CDK-2 dependent transcription of HIV-I[126] elaborates the evasive exploitation of cellular resources by viruses. Identification of these host factors contributes to the potential target therapeutics[122, 127] for the control of viral infection. As these cellular factors are of importance for cell functioning and they are mostly evolutionary conserved across the species. Thus, despite of high mutation rates in many viruses which helps them escape the immune system, one can target cell-specific host-factors to inhibit/reduce viral replication. Therefore, the identification of viral dependences on host factor provides us with the extensive details of viral life cycle along with the host-cell components exploited by them, which might be used for future therapeutics.

CHAPTER-2

MATERIALS and METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

All the chemicals were acquired by scientific reagents providers.

Chemical	Company
2-β mercaptoethanol	Sigma, Munich
Brefeldin A	eBioscience
Carboxyfluorescein Succinimidyl Ester (CFSE)	Invitrogen, Darmstadt
Cell Lysing Solution	BD Bioscience, Heidelberg
Citric Acid	Sigma, Munich
DEPC-Treated water	ThermoFisher Scientific
dNTPs	Promega
DPBS w/o Mg^{2+}, Ca^{2+}	Pan Biotec, GmBH
Ethanol	Merck
Ethylenediaminetetracetic acid (EDTA)	Sigma, Munich
Fetal Calf Serum (FCS)	Biochrom AG, Berlin
Fluorescent Mounting medium	DAKO
Formaldehyde	Sigma, Munich
L-Glutamine	Sigma, Munich
Lipopolysaccharides of *Escherichia coli* (LPS)	Sigma, Munich
Methanol	Sigma, Munich
Methylcellulose	Merck Fluka
Penicillin-Streptomycin	Sigma, Munich
Polyinosinic-polycytidylic acid (Poly I:C)	Sigma, Munich
o-Phenylenediamine dihydrochloride	Sigma, Munich
Saponin	Sigma, Munich
Sodium Bicarbonate	Sigma, Munich
Tissue Tek	Sakura
Taq Polymerase	Promega
TRIzol Reagent	ThermoFisher Scientific

2.1.2 Medium

Reagent	Company
Dulbecco's Modified Eagle's Medium (DMEM)	Pan Biotec GmBH
Iscove's Modified Dubelcco's Medium (IMDM)	Sigma, Munich
Very Low Endotoxin (VLE)-DMEM	Biochrom AG, Berlin

2.1.3 Kits

Reagent	Company
IFN-α ELISA Kit	PBL
QuantiTect Reverse Transcription Kit	Qiagen

2.1.4 Antibodies

Antibody	Company
Anti-CD169 (CD274)	eBioscience
Anti-B220	eBioscience
Anti-CD11b	eBioscience
Anti-CD11c	eBioscience
Anti-CD3	eBioscience
Anti-CD4	eBioscience
Anti-CD8	eBioscience
Anti-CD19	eBioscience
Anti-CD90.2	eBioscience
Anti-Cleaved Caspase-3	Abcam
Anti-F4/80	eBioscience
Anti-GFP	Abcam
Anti-Gr-1 (clone RB6-8C5)	Bio X Cell
Anti-IFNγ	eBioscience
Anti-LCMV-NP (VL4)	Lab produced
Anti-Ly6C	eBioscience
Anti-Ly6G (clone 1A8)	Bio X Cell
Anti-mPDCA	eBioscience
Anti-PDL1	eBioscience
Anti-VSV-GP	Lab produced

Anti-Rabbit Cy5	Jackson ImmunoResearch
Goat anti-Rabbit Biotin	Jackson ImmunoResearch
Rabbit monoclonal Anti-Phospho B-Myb	Origene
Streptavidin (Fluorescent labelled)	eBioscience

2.1.4 Primers

Name	Sequence/Catalog	Company
LCMV-GP Forward	CGA GCA TCA AAG CTG TGT ACA AT	Biomers
LCMV-GP Reverse	AAA AGG AAG CTG ACC AGT GCT AA	Biomers
VSV-GP Forward	TGA TAG TAC CGG AGG ATT GAC GAC	Biomers
VSV-GP Reverse	CCT TGC AGT GAC ATG ACT GCT CTT	Biomers

2.1.4a TaqMan Probes

Name	Catalog	Company
Mybl2	Mm00485340_m1	ThermoFisher Scientific
Atp6ap2	Mm00510396_m1	ThermoFisher Scientific
Atp6v0b	Mm01193846_g1	ThermoFisher Scientific
Atp6v0d1	Mm00442649_m1	ThermoFisher Scientific
Arcn	Mm00524375_m1	ThermoFisher Scientific
Copb1	Mm00446330_m1	ThermoFisher Scientific
Copa	Mm00550231_m1	ThermoFisher Scientific
Mat2a	Mm00728688_s1	ThermoFisher Scientific
Eif3a	Mm00468721_m1	ThermoFisher Scientific
Eif3g	Mm00469383_m1	ThermoFisher Scientific
Nop56	Mm00458467_m1	ThermoFisher Scientific
MMP14	Mm00485054_m1	ThermoFisher Scientific
MMP2	Mm00439498_m1	ThermoFisher Scientific
MMP9	Mm00442991_m1	ThermoFisher Scientific
PDGFß	Mm00440677_m1	ThermoFisher Scientific
VEGFA	Mm00437306_m1	ThermoFisher Scientific
VEGFB	Mm00442102_m1	ThermoFisher Scientific
VEGFC	Mm00437310_m1	ThermoFisher Scientific
FGFR	Mm00438930_m1	ThermoFisher Scientific

FGFR2	Mm01269930_m1	ThermoFisher Scientific
FGFR3	Mm00433294_m1	ThermoFisher Scientific
EGF	Mm00438696_m1	ThermoFisher Scientific
ANGPT1	Mm00456503_m1	ThermoFisher Scientific
SELL	Mm00441291_m1	ThermoFisher Scientific
CCL5	Mm01302427_m1	ThermoFisher Scientific
CXCL3	Mm01701838_m1	ThermoFisher Scientific
Csf3	Mm00438334_m1	ThermoFisher Scientific
CXCL15	Mm00441263_m1	ThermoFisher Scientific
CXCL1	Mm04207460_m1	ThermoFisher Scientific
Gapdh	Mm03302249_g1	ThermoFisher Scientific

2.1.4b SYBR Green Probes

Name	**Catalog**	**Company**
IFNα4	QT01774353	Qiagen
IFNß	QT00249662	Qiagen
IRF7	QT00245266	Qiagen
OAS1	QT01056048	Qiagen
ISG15	QT02274335	Qiagen
Ly6C	QT00247604	Qiagen
CCR2	QT02276813	Qiagen
CCL2	QT00167832	Qiagen

2.1.5 **Equipment**

Equipment	**Company**
Cryostat CM 3050S	Leica
ChemiDoc MP Imaging system	BioRad
FLUOstar Omega ELISA Reader	BMG Labtech
FACS Fortessa	Becton Dickinson (BD)
Fluorescence Microscope HS BZ-9000	Keyence GmBH
LightCycler 480 realtime PCR machine	Roche
SP8 gSTED Confocal microscope	Leica
Nanodrop	Peqlab

Thermocycler PCR machine	Applied Biosystems
TissueLyser II	Qiagen
Micro-centrifuge	Hettich
Centrifuge 2210R	Eppendorf

2.1.6 Plastic wares

Items	Company
1.5 ml tubes	Eppendorf
2.0 ml	Eppendorf
5 ml Polystyrene tubes	BD Falcon
10 ml syringe	Braun
15 ml Falcon tubes	Corning
50 ml Falcon Tubes	Corning
10µl Pipettes tips	STAR Labs
200µl Pipettes Tips	STAR Labs
1ml Pipettes Tips	STAR Labs
5 ml Pipettes	Corning
10 ml pipettes	Corning
25 ml Pipettes	Corning
70µ Cell strainer	BD
6-well Plate	TPP
24- well Plate	TPP
96-well Plate	TPP
MicroAmp 384-well Plate	Applied Biosystems
Reservoirs	VWR
Tissue-culture Flasks	TPP

2.1.7 Cells:

Various murine and human cells have been used in the present study. These cell line have been either purchased from companies or gifted from our collaborators. All cell lines were maintained mycoplasma free and grown in a humidified 37 ^{o}C chamber with 5% CO_2.

Cell Lines	Source	Cell-type	Company/Collaborator
MCF-7	Human	Breast carcinoma	ATCC
MOPC	Murine	Oropharyngeal carcinoma	Dr. H.J. Lee, University of Iowa
EL4-OVA	Murine	Lymphoma	Dr. B. Huard, UMC, Geneva
B16F10	Murine	Melanoma	ATCC
B16-OVA	Murine	Melanoma	Prof. P. Knolle, TU, Munich
MC38	Murine	Colon carcinoma	Dr. B. Huard, UMC, Geneva
SW480	Human	Colon carcinoma	ATCC
FaDu	Human	Oropharyngeal carcinoma	ATCC
HepG2	Human	Hematoma	ATCC
HeLa	Human	Cervix carcinoma	ATCC
FHC-CRL183	Human	Primary Colon cells	ATCC
NBHE CC2540	Human	Primary Bronchial Epithelial	LONZA
HRE CC2556	Human	Primary Hepatocytes	LONZA
NHEM C-12453	Human	Primary Melanocytes	PromoCell

2.1.8 Mice

All the mice used in the study were maintained on the C57BL6/J background and housed in single ventilated cages under standard temperature and pressure conditions. All experiments done were authorized by Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein Westfalen (Recklinghausen, Germany) and were performed under the German animal protection law.

Besides C57BL6/J mice which were used as WT, multiple other mice have been used in this study.

Cag$^{+/-}$ *Mybl2*$^{fl/fl}$

These mice have a cag promoter with cre recombinase fused with estrogen receptor (ER) gene and *Mybl2* gene floxed on both prime ends with LoxP^{128}. Upon induction with tamoxifen, CreER gets activated by dissociation of Hsp90 and translocate to nucleus. Cre recognizes LoxP sites and cleaves them excising the region flanked by them. This leads to deletion of the gene/gene functionality. These mice were used as Cag+ which when injected with tamoxifen leads to deletion of B-Myb and Cag- (control) which doesn't lead to B-Myb deletion and acts as WT.

Ifnar$^{-/-}$ mice

These mice lack IFNα/β receptor and thus lacks IFN signaling[129]. These mice suffer from uncontrolled virus replication and eventually succumb to the infection

Tcrb$^{-/-}$ mice

Tcrb$^{-/-}$ mice are genetically engineered immunodeficient mice which lack the T cell receptor beta chain[130].

Vi10 × CD45.1 mice

These mice bear a knock-in construct of VSV-neutralizing ab in the Ig locus. This in-turn produce VSV-specific BCR and thus results in rapid B-Cell response on VSV infection[131]. The Vi10 mice is further crossed with CD45.1 mice to produce a constitutive expression of CD45.1 on its lymphocytes.

Tg7 Thy1.1

These mice have knock-in of gene coding for VSV glycoprotein peptide. Thus, these mice express MHC-II TCR specific for VSV-GP[132]. These mice were then crossed with Thy1.1 mice to exchange the expression of CD90.2 to CD90.1.

Map3k14$^{aly/aly}$

These mice carry a mutated *aly* gene resulting in absence of well-developed lymphoid follicles in spleen and distorted/absence of cortical-medullary regions in thymus. The mutant homozygotes display deficiency in both humoral and cell-mediated immune response[133].

Jh$^{-/-}$

These mice harbors deletion of JH segments and intron enhancer in IgH locus. This leads to complete absence of functional B-cells[134].

Rag1$^{-/-}$

These mice are generated by introducing a mutation in *Rag1* gene which renders it non-functional. These mice fail to perform V(D)J recombination during T and B-cell development

and differentiation. Therefore, they have small lymphoid organs devoid of mature T and B lymphocytes[32].

Irf3$^{-/-}$ × *Irf7*$^{-/-}$

These mice lack the gene expression and function of *Irf3* and *Irf7* gene, resulting in the absence of interferon induction[135]. Lack of interferon signaling and pathway leads to the uncontrolled virus replication and defective immune response.

2.2 Methods

2.2.1 Bone Marrow derived macrophage and dendritic cell generation:

Mice were sacrificed by cervical dislocation. Abdomen and hind legs were sterilized, afterwards femur and tibia was isolated. After removing all muscle tissue, bones were stored in cold PBS. Bones were then disinfected with 70% ethanol for 5 min and then transferred to ice-cold PBS. After thorough cleaning of the bone, epiphyses were carefully cut from both ends. Bone marrow was then flushed from one end with cold VLE-DMEM via 24G syringe into a 50ml falcon and then centrifuged at 1400 rpm for 5 min at 4°C. Supernatant was discarded and cells were lysed with erythrocyte lysis buffer (ELB) for 2 min at RT. Lysis was stopped with 10%FCS (LPS free) containing VLE-DMEM and passed through a cell strainer. The cells were then again centrifuged at 1400 rpm for 5 min at 4°C. Supernatant was discarded and cells were re-suspended in 5 ml VLE-DMEM. Cell number was determined using haemocytometer and were plated in 10mm dishes at density of $3x10^6$ cells in MCSF containing media for macrophages and GMCSF containing media for dendritic cells, and kept at 37°C. At day 3, 10 ml of fresh media was added and at day 6 complete media was changed with fresh media. Cells were harvested at day 6 and plated into 6/24 well plates for experiment with appropriate medium.

Reagents:

Macrophage Medium	**Dendritic Cell medium**
VLE-DMEM (450ml)	VLE-DMEM (450ml)
20% (v/v) M-CSF	2% (v/v) GMCSF
10% FCS (LPS free)	10% FCS (LPS free)
0.1% (v/v) 50mM ß-me	0.1% (v/v) 50mM ß-me

Erythrocyte Lysis Buffer (ERB)

0.5M NH_4Cl_2

10mM $KHCO_3$

0.1mM EDTA (pH 7.2)

2.2.2 B-myb FACS staining:

For detection of phospho-B-Myb, spleen samples were dissociated in 1ml chilled phosphate-buffered saline(PBS). 100ul of each sample was then stained with α-CD169**,** α-CD8, α-Ly6C, α-Ly6G, α-CD4 and B220 at 4^{o} C for 30 min, samples were then washed with PBS and fixed with 10% PFA for 10 min and then permeabilized with 2% saponin/PBS for 10 min at RT. After permeabilization, samples were blocked with 2% mouse serum/PBS for 20 min, stained with phospho-B-Myb for 60 min followed by α-Rb IgG biotin for 30 min and then FITC-streptavidin for 30 min at 4^{o}C.

2.2.3 VSV and LCMV Production

VSV virus and LCMV virus (WE and Docile) were propagated on BHK-21 cells and L929 cells respectively. The cells were grown in DMEM medium containing 10% FCS, 1% penicillin, streptomycin (P/S) and L-glutamine (Glut) at 37^{o}C until 70% confluency is reached. Then cells were infected with either VSV or LCMV at a multiplicity of infection (MOI) of 0.0001 in a total volume of 5 ml and incubated for 1 hour at 37^{o}C. 25ml of the same DMEM medium were added and incubated for 48 hours. Medium was collected and subjected to centrifugation at 3000 rpm at 4^{o}C to remove large debris. Supernatant was then collected in cyro-tubes and stored at -80°C. The virus was administrated into the animals by intravenous (i.v.) injection.

2.2.4 VSV Plaque Assay:

For detection of VSV viral titers, assay was done using vero cells. Number of samples were determined and accordingly 24-well plates were taken. Vero cells were plated as 3x10^{4} cells/well and incubated at 37 ^{o}C incubator overnight. Samples were prepared and titrated as explained in LCMV plaque assay. 24-well plates containing confluent monolayer of vero cells were taken out and media was discarded. Titrated sample were then added to the plates and incubated for 2 hours at 37 ^{o}C after which overlay was added to the wells and again incubated at 37 ^{o}C for 24 hours or until plaques were visible under the microscope.

2.2.4a Staining:

Medium was discarded from the plates. Crystal violet staining solution was added to the plates and incubated at RT for 20-30 min. Plates were then washed with running water and dried inverted on paper towels. Clear plaques were counted and analyzed accordingly.

Reagents:

Overlay Medium	**Crystal Violet Staining solution**
VLE-DMEM (450ml)	VLE-DMEM (450ml)
20% (v/v) M-CSF	3% (v/v) GMCSF
10% FCS (LPS free)	10% FCS (LPS free)
0.1% (v/v) 50mM ß-me	0.1% (v/v) 50mM ß-me

2.2.5 LCMV Titer assay:

For detection of viral titers in organs and cell cultures, plaque assay was done with MC57 cells. The organs were homogenized in tissuelyser and centrifuged at 1500 rpm for 10 min. Supernatant was then titrated as 1:3 over 12 rows in a 12-well plate in 2% FCS/DMEM supplemented with 1% penstrep and glutamine. Titrated samples were then plated in 24 well plates along with 200µl of MC57 cells at the density of $9x10^5$ cells/ml. Plates were kept at 37 ^oC for 3 hours and then overlay was added and incubated again for 72 hours at 37 ^oC.

2.2.5a Staining:

Plates content was discarded and plates were incubated at RT with 4%(v/v) formaldehyde for 30 min. Formaldehyde was then replaced with 1%(v/v) Triton-X and incubated for 20 min at RT. Plates were then washed with PBS twice and blocking solution of 10% (v/v) FCS in PBS was added and incubated for 60 min at RT. Primary antibody VL4 was diluted in 1% (v/v) FCS/PBS and was added as 200µl/well and incubated for 60 min at RT. After washing twice in PBS, secondary antibody HRP linked α- rat IgG was diluted in 1% (v/v) FCS/PBS and was added as 200µl/well and kept at RT for 60 min. Plates were again washed with PBS twice and staining solution was added and incubated for 20-30 min at RT. Once the plaques were visible, plates were washed with water and plaques were counted.

Reagents:

Overlay Medium

50% (v/v) 2x IMDM (pH 7.4)

50% (v/v) 2% Methyl-cellulose

Staining Solution for LCMV

25%(v/v) 0.2M Na_2HPO_4

25% (v/v) 0.1M Citric acid

20 mg o-Phenylenediamine dihydrochloride

50 µl of 30% H_2O_2

50 ml ddH_20

2.2.6 Immunohistochemistry:

For the detection of cellular protein, immunohistochemistry was performed. Organs were harvested and stored immediately in histological tubes with tissue-tek and put in liquid nitrogen. Embedded tissues were then taken to cryotome and mounted on loading discs. Tissue slices were then cut at 8µ in size on glass slides. Slides were stored at 4 ^oC until use. All the histological cutting was done below -10 ^oC.

2.2.6a Immunostaining:

Slides were put in acetone for 10 min and then dried for few seconds. Tissue sections were then outlined with an oil-pen and let it dry for few seconds. Samples were then blocked with 2% FCS/PBS for 10 min at RT and then mounted on staining chamber. Primary antibodies were added diluted in 2% FCS/PBS on to the samples and incubated for 60 min at RT. Then samples were washed with 2% FCS/PBS and appropriate secondary antibody and then tertiary (if required) was added and again incubated for 60 min at RT. Samples were then washed again with 2% FCS/PBS and mounted with DAKO mounting solution and stored at 4^oC until microscopy was performed.

2.2.6b B-Myb immunohistostaining:

Slides were brought to RT and then fixed with 10% NBF. Tissue sections were then marked with oil-pen and were blocked with 2% FCS/PBS for 10 min at RT. After blocking, slides

were mounted on staining chamber and primary antibody α-phsopho B-Myb was added in a dilution of 1:100 in 2% FCS/0.5% saponin in PBS. Slides were incubated for 60 min at RT. Slides were washed and then exposed to secondary antibody PE labelled α-Rb IgG in a dilution of 1:200 in 2% FCS/PBS for 30 min at RT. Slides were washed, mounted with DAKO and stored at 4^{o}C until microscopy.

2.2.7 RNA isolation:

RNA was isolated using TRIzol (Thermo Fisher) according to the manufacturer's protocol. Organs were homogenized in 1ml TRIzol and kept at RT for 10 min. 200µl of chloroform was added to each sample and mixed thoroughly by inverting the tubes multiple times. Samples were then centrifuged at 13000 rpm for 15 min at 4^{o}C. Upper aqueous layer was then collected in a separate tube and equal volume of iso-propanol was added and mixed thoroughly. Samples were then incubated in ice for 10 min and then centrifuged at 13000 rpm for 15 min at 4 ^{o}C. Next, samples were washed twice with 70% ethanol in DEPC water by centrifugation for 10 min each. Supernatant was taken out via vacuum pump and samples were air dried further for 5-10 min. Samples were then dissolved in DEPC water at 56 ^{o}C for 10 min, quantified using nanodrop and then stored at -20 ^{o}C.

2.2.8 cDNA Synthesis:

cDNA was synthesized from the isolated RNA samples using Qiagen QuantiTect Reverse Transcription kit as per manufacturer's protocol. Briefly, 1000ng of RNA was taken and any DNA contamination was removed by gDNA wipeout reagent and then a mastermix of QuantiTect reverse transcriptase, RT-buffer and RT-primer mix was added and incubated at 42 ^{o}C for 30 min. Enzyme reaction was then inactivated by heating the samples at 92 ^{o}C for 2 min. Samples were stored at -20 ^{o}C until further use.

2.2.9 Intracellular cytokine staining:

ICS is performed to detect the cytokine levels produced by T-cells. Organs were harvested and smashed in DMEM. Cells were then transferred to 96 well plate and washed again with ice-cold DMEM by centrifugation as 1400 rpm for 5 min at 4 ^{o}C. 100µl of vial peptide was added for stimulation of T-cells (VSV peptide: p52 for $CD8^{+}$ and p8 for $CD4^{+}$ T-cells; LCMV peptide: gp33 for $CD8^{+}$ and gp64 for $CD4^{+}$ T-cells) in a dilution of 1:200 in 2% FCS/DMEM and incubated at 37 ^{o}C for 1 hour. 20µl BFA was then added to each sample in a

dilution of 1:200 in 2% FCS/DMEM with 1% P/S and Glut and incubated at 37 °C for 5 hours. Samples were then washed with FACS buffer and fluorescent antibodies against CD8 and CD4 diluted as 1:100 in FACS buffer were added to each sample and incubated at 4 °C for 30 min. After washing with FACS buffer, samples were fixed with 2% (v/v) formalin in FACS buffer for 10 min at RT and then washed again followed by permeabilization with 1% saponin (v/v) in FACS buffer for 10 min at RT. Antibody cocktail was of fluorescent α-IFNγ and α-TNFα in 1% saponin/FACS buffer and incubated at 4 °C for 30 min. Samples were then finally washed with FACS buffer and analyzed.

2.2.10 Cell culture

All the cells were maintained in humidified incubator at 37 °C with 5% CO_2 with the desired growth medium. Cells were split using trypsin treatment for 2-4 minutes at 37 °C, which then was inactivated by FCS containing medium. Different medium was used for different cell lines as per provider's protocol.

2.2.11 RT-PCR

Gene expression was performed using Roche LightCycler 480 with either SYBR-Green or Taqman probes. For analysis, the observed expression levels of all target genes were normalized to either GAPDH or 18s rRNA expression (ΔCt). Gene expression values were then calculated based on the ΔΔCt method. Relative quantities (RQ) were determined with the equation: RQ=2^ (-ΔΔCt). The values were wither plotted multiplied to a constant or with-relative to the non-infected control.

2.2.12 siRNA knockdown

siRNA complexes were obtained from Origene for the transient knockdown in cells. 20μM stock of each siRNA duplex was made by adding 100μl of the RNase-free buffer and heated at 72 °C for 2 min and then cooled down to RT. Working stocks of 5μM of each duplex were made and stored in aliquots at -80 °C.

Transfection with siRNA was done in MCF-7 cells. $2x10^5$ cells /well were plated in a 6-well plate and grown at 37 °C until they reach 50-60% confluency. The medium was then replaced with 2.38 ml of fresh medium. Working siRNA complex was prepared by adding 5μl from working siRNA stock to 95μl of serum and antibiotic-free DMEM along with 20μl of HiperFect and mixed by vortexing. The mixture was then incubated for 10 min at RT and

added to the cells dropwise with constant shaking of plates. The final concentration of siRNA is 10 nM.

2.2.13 *In-vivo* siRNA knockdown

In-vivo siRNA kit was purchased from Ambion, Thermo Fisher Scientific. The scrambled control and B-Myb specific siRNA was prepared in invivofectamine (Thermo Fisher Scientific) as per manufacturer's protocol. Prepared siRNA complex was injected intravenously.

2.2.14 *In-vivo* cell depletion

Depletion of in-vivo was carried out by injecting the cell-specific antibodies. For NK-cell depletion, 50 μg of NK1.1 (produced in-house) in injected i.p. at day -10 and -3. For collective depletion of $Ly6C^+$ and $Ly6G^+$ cells Gr-1 antibody was given whereas for depletion of specifically $Ly6G^+$ cells, αLy6G (500 μg) was given. Both these antibodies were given i.p thrice at day -2, 2 and 7.

2.2.15 Morphometric analysis of tumor vessels and hypoxia

The morphometric analysis was carried out by analysis of CD31 staining on consecutive cryosections of tumors. MVD was calculated as number of vessels per tumor area in top, middle and bottom section of tumor. Vessel-to –vessel distance was calculated by using the middle sections of the three sections (upper, middle and bottom) by Cell P software (Olympus, Germany). For hypoxic tumor areas analysis, Pimonidazole was injected 30 min prior to killing of mice. Hypoxic areas were detected by formation of pimonidazole adducts, and sections were stained with hydroxyprobe-1 Plus Kit.

2.2.16 SDS- electrophoresis

SDS-gel electrophoresis was carried for the protein expression analysis using BioRad Mini PROTEAN system. 10% resolving poly-acrylamide gel was cast in glass plates and left for 30-40 minutes at RT for polymerization. Iso-propanol was used as overlay to keep the gel from drying out. Then 4% poly-acrylamide stacking gel was made and cast on top of the resolving gel with combs inserted. The gel was allowed to polymerize for 20-30 min at RT. Gel cast was removed and put it to the loading chamber along with SDS-running buffer. Samples were pre-heated at 95 ^oC before loading. Equal amount of protein was loaded in the

gel and was initially electrophoresis was performed at 80 V for complete stacking of proteins. Once the proteins are stacked, voltage was turned up to 100 V to separate the proteins according to their molecular weight. Samples were then run up to desired molecular weight and processed further.

Reagents

10x SDS-Running Buffer
30 gm Tris base
144 gm Glycine
10 gm SDS
1000 ml ddH_20

	10% Resolving Gel	3.5% Stacking Gel
30% Acrylamide	3.3 ml	0.45 ml
1M Tris-Cl (pH 8.0)	3.6 ml	-
1M Tris-Cl (pH 6.8)	-	0.38 ml
10% SDS (w/v)	98 µl	30 µl
10% APS (w/v)	98 µl	30 µl
TEMED	8.1µl	5 µl

2.2.17 Western blotting

Western blotting is done to detect the presence and to quantify the protein in sample. Proteins were transferred using wet blot method. Polyacrylamide gel was taken out of the electrophoretic chamber and transferred to western transfer buffer after the removal of stacking gel along with nitrocellulose membrane. Western blot chamber cassette was then arranged from anode to cathode; first with two whatman paper soaked in western transfer buffer followed by the polyacrylamide gel containing proteins. Then wet nitrocellulose membrane was put carefully avoiding any bubbles and finally whatman papers were put on top. The stack was pressed with botting roller gently to remove any trapped air and cassette was closed. The blotting cassette was then transferred to the chamber containing ice-cold western transfer buffer. Ice pack was added to the chamber in order to maintain the temperature of the buffer during transfer. Transfer was done as 90 V for 1.5 hours. After transfer, membrane was carefully taken out and blocked with blocking buffer for 1 hour at RT to avoid non-specific binding of antibodies. Then incubation with primary antibody was done as 1:1000 dilution of the desired antibody in blocking buffer at 4 ^{o}C on a shaker overnight. Next day the membrane was washed thrice for 5 min each with 0.05 % Tween-20/PBS (PBS-T) and incubated with HRP-linked secondary antibody diluted in blocking

buffer for 1 hour at RT on shaker. The blots were then washed with PBS-T thrice for 10 min each and were finally stored in PBS.

DEVELOPMENT OF WESTERN BLOTS: Blots were incubated with thermo scientific femto chemiluminescent reagents for detection of phospho protein and with thermo scientific chemiluminescent reagents for total proteins for 5 min at RT. Blots were then developed and photographed using BioRad ChemiDoc.

Reagents

Transfer Buffer (10x)	**1x Transfer buffer**
30 gm Tris	10x transfer Buffer (100 ml)
144 gm Glycine	Methanol (200 ml)
ddH_2O (1000 ml)	ddH_2O (700 ml)

PBS-T	**Blocking Buffer**
1x PBS	Non-fat dried milk (5% w/v)
0.05% Tween-20	PBS-T

2.2.16 FACS

FACS acquisition was performed on BD FACS Fortessa and analysis was performed on FlowJo 10. Organs were harvested and then crushed in cold PBS. Cells were collected after washing with PBS once in a BD FACS tube. Antibody cocktail was made with desired antibodies in FACS buffer in a dilution of 1:100/ sample. Samples were then incubated at 4 ^{o}C for 30 min and then washed with FACS buffer via centrifugation at 1500rpm for 5 min. Cells were re-suspended in FACS buffer and were analyzed.

Virus-Specific $CD8^{+}$ T-cell staining.

Samples were taken in FACS tubes and were incubated with fluorescent Labelled (APC) GP33 tetramer for 15 min at 37 ^{o}C. Cocktail of CD8 along with other desired markers was made in FACS buffer as 1:100/sample and added to each sample. Samples were then incubated at 4 ^{o}C for 30 min and then washed afterwards with FACS buffer. In case of blood samples, erythrocytes were lysed with BD Cell Lysing buffer for 7 min and then washed with FACS buffer. Samples were then re-suspended in FACS buffer and were analyzed.

2.2.17 Genotyping

In order to determine the transgenic state of the mice, genotyping was performed. Mice were ear-marked and 2-4 mm of tail was cut and put into 600 µl digestion buffer. Proteinase-K was then added at the concentration of 20 ug/ml. Samples were then incubated at 56^{o}C till the tails were dissolved properly. Samples were then spun down to collect tail hairs and supernatant was transferred to a fresh tube. Equal amount of iso-propanol was added to the samples and mixed properly. Samples were then centrifuged at 12000 rpm for 10 min at 4 ^{o}C and then washed twice with 70% ethanol. Samples were then air-dried and dissolved in DEPC water at 60 ^{o}C, quantified and stored at 4 ^{o}C. For genotyping PCR, 50-100 ng of DNA was taken and added with the optimized concentration of PCR mix and primers and then run in PCR machine. Amplified samples were then visualized by agarose gel electrophoresis.

2.2.18 Statistical Analysis

All the results were analyzed from minimum of three biological replicates (n = 3), unless indicated otherwise. The level of significance was calculated using the Student's t-test. In case of presence of more than two groups, 1-way ANOVA followed by a Tukey post-test was carried out. Survival analysis was compared with log-rank (Mantel-Cox) tests. All the data are presented as mean ± S.E.M. Observed p-values are indicated as "*" ($p \leq 0.05$), "**" ($p \leq 0.01$), "***" ($p \leq 0.001$), "****" ($p \leq 0.0001$).

CHAPTER-3

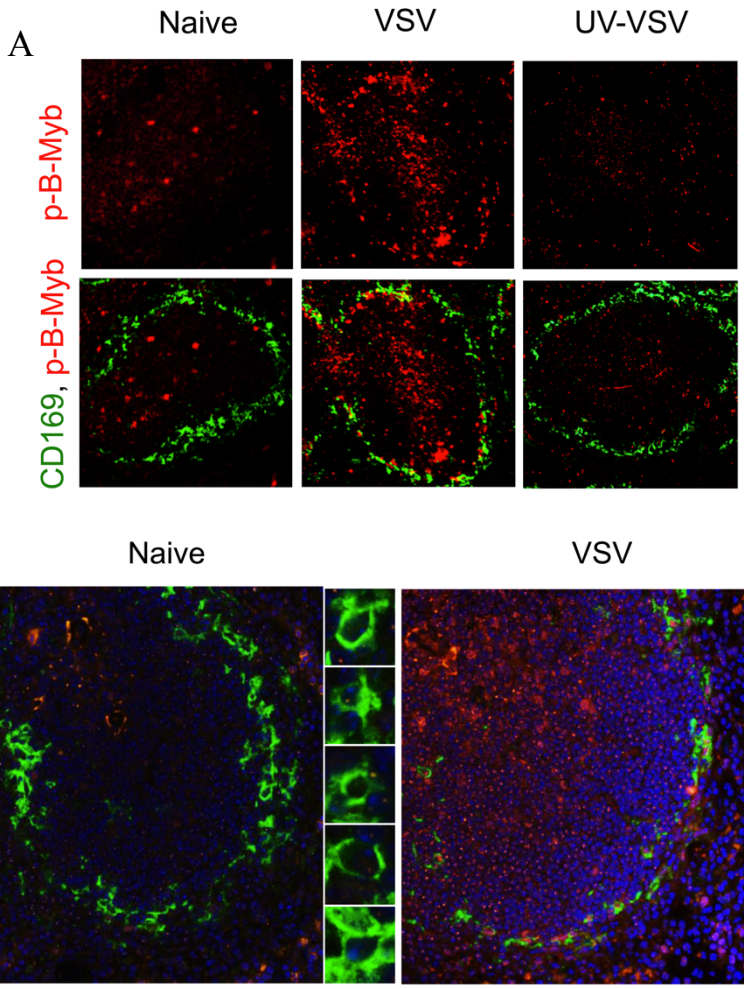
RESULTS

RESULT 1: B-Myb is a major host factor for viral replication

3.1.1 Active VSV replication induces B-Myb phosphorylation.

For certain viruses such as VSV, early virus replication occurs in $CD169^+$ macrophages also known as marginal zone macrophages which are present in the spleen[15]. Upon infection, virus is taken up via APCs into the spleen from blood stream. The spleen is an important site for viral processing due to presence of T and B-cells in white pulp surrounded by APCs and it represents an ideal special layout of cells for effective processing of viral antigens and immune activation. Upon virus uptake, many genes are up-regulated by the host in the marginal zone macrophages of the spleen in order to facilitate early viral replication.

To address the activation of B-Myb during VSV replication, we infected C57BL6/J (WT) mice with $2x10^8$ PFU VSV intravenously. Mice were sacrificed after 7 hours of infection and spleen were harvested for immunohistochemistry and FACS for detection of phospho-B-Myb (p-B-Myb). We found out that the B-Myb is phosphorylated specifically in VSV replicating $CD169^+$ macrophages (Fig. 6A). To further verify whether phosphorylation of B-Myb is specific to infected $CD169^+$ macrophages on early VSV replication, we performed confocal analysis of histological samples as well as FACS evaluation of phospho-B-Myb expression. In confocal analysis, we demonstrated that indeed phosphorylation of B-Myb occurs specifically in $CD169^+$ macrophages (Fig. 6B). Next, we performed FACS analysis in different immune cell types present in the spleen to quantify p-B-Myb expression. The analysis revealed distinct and significant upregulation of p-B-Myb expression in infected $CD169^+$ macrophages compared to naïve, whereas other infected cells expressed the same amount of p-B-Myb compared to their naïve counterparts (Fig. 6C, Fig. A1). Next, we wondered whether active VSV replication is indeed required for B-Myb phosphorylation, so we inactivated VSV under UV-light twice for duration of 5 min each and infected WT mice with $2x10^8$ PFU. Spleens were harvested for immunohistochemical analysis, which showed no phosphorylation of B-Myb upon UV-inactivated VSV (Fig 6A). We also wondered whether other molecules that are activated along with B-Myb are also involved in VSV replication. One such molecule is CyclinD1, which is activated alongside of B-Myb. Upon analysis, we found out that there was no effect on the expression of Cyclin-D1 during VSV infection (Fig. 6D).


A
Naive
VSV
UV-VSV
p-B-Myb
CD169, p-B-Myb
B
Naive
VSV
DAPI
CD169
p-B-Myb
C
Naïve
B6/VSV
CD169
Ly6C
Ly6G
B220
CD4
CD8
Normalized to Mode
p-B-Myb
**

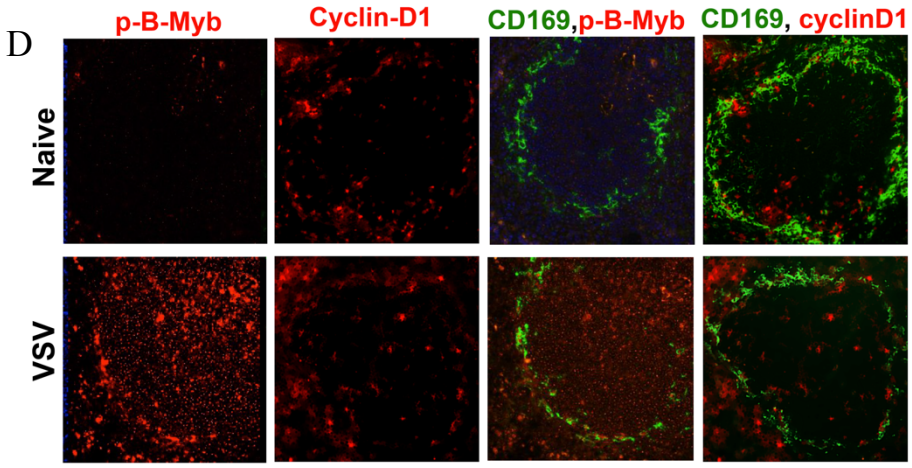


Figure 6: VSV replication phosphorylates B-Myb in CD169^{+} macrophages

A: Immunohistochemistry of phospho B-Myb of spleen sections from C57BL6/J mice infected with 2x10^{8} PFU VSV, 8 hrs p.i.(n=6) B: Confocal analysis of spleen of C57BL/6J mice infected with 2x10^{8} PFU VSV, 8 hrs p.i.(n=6). C: FACS histograms and quantification analysis of phospho-B-Myb in different cells from spleen of C57BL/6J mice infected with 2x10^{8} PFU VSV, 8 hrs p.i.(n=5). D: Immunohistochemistry of phospho B-Myb and cyclin D1 of spleen sections from C57BL6/J mice infected with 2x10^{8} PFU VSV, 8 hrs p.i.(n=4)

3.1.2 *In-vitro* and *in-vivo* knockdown of B-Myb reduces virus replication.

Next, we checked whether the knockdown of B-Myb has functional consequences on virus replication. We performed siRNA knockdown of B-Myb *in-vitro* and *in-vivo*.

For *in-vitro* analysis, we used origene siRNA directed against human B-Myb and performed knockdown in MCF7 breast cancer cells. The cells were transfected and were collected for western blot analysis for 24 and 48 hours post-transfection. Protein analysis revealed the level of B-Myb was robustly reduced 24 and 48 hours post transfection (Fig. 7A). As B-Myb is a crucial cell proliferation regulation transcription factor, we checked whether the transient knockdown induced apoptosis via propidium-iodide staining. We found no significant apoptosis when compared to scrambled control with one siRNA (Fig. 7A, right panel). Next, we checked for virus replication using LCMV and VSV as viral models. MCF7 cells were grown in 24 well plates and on cover-slips and were then transfected with one control and the non-apoptotic inducting B-Myb directed siRNA. The cells were then infected with an MOI 1.0 of both viruses 24 hours post-transfection. Supernatant was taken at 24 and 48 hours post infection of LCMV and coverslips were processed for immunocytochemistry (ICC) for detection of LCMV-NP and VSV-GP after 24 and 7 hours respectively. Upon analysis of

infective viral titers after LCMV infection via plaque assay, we found significant reduction in the LCMV viral titers in B-Myb knockdown cells as compared to scrambled controls (Fig. 7B). This showed that B-Myb has an impact on virus replication and the production of active viral particles. ICC analysis confirmed this as we found reduced LCMV-NP and VSV-GP expression in B-Myb knockdown cells (Fig. 7C).

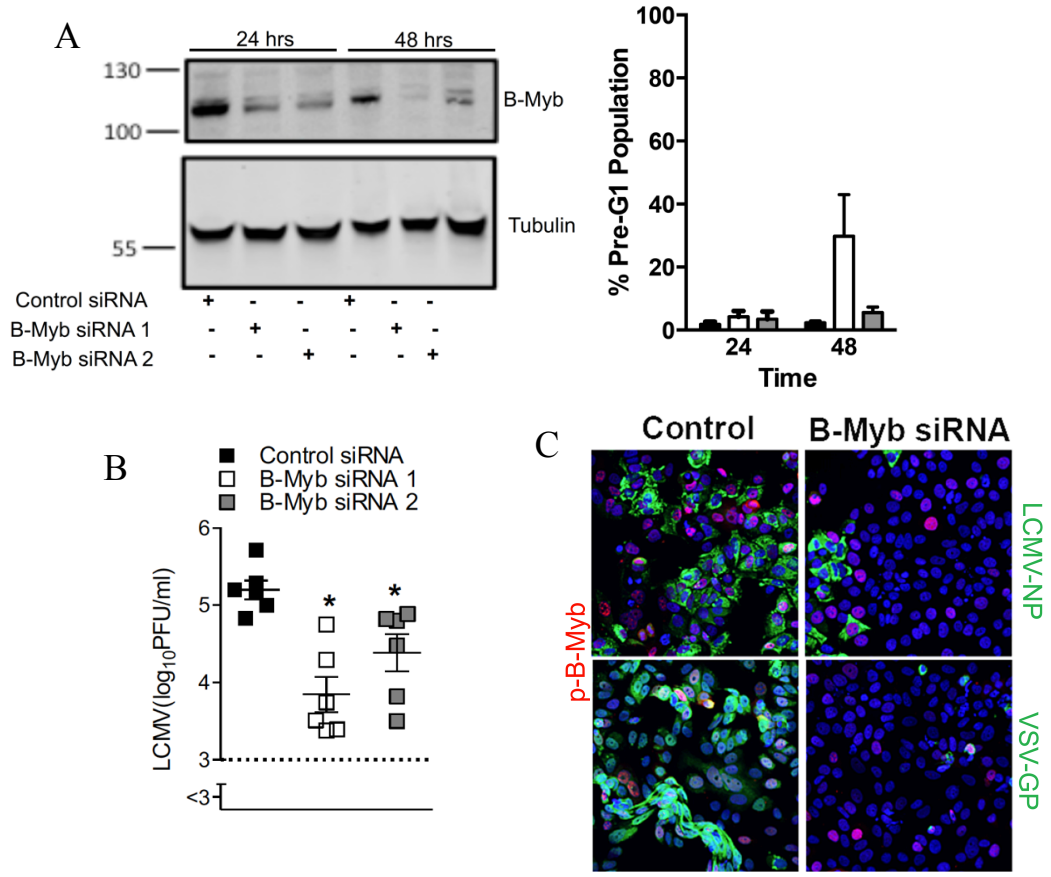


Figure 7: Transient knockdown of B-Myb abrogates LCMV and VSV replication *in-vitro*

A: representative immunoblot of control and B-Myb siRNA transfected MCF7, 24 and 48 hours post transfection (n=3-4) and Pre-G1analysis of MCF7 cells transfected with control and 2 independent siRNA (n=3) B: LCMV infective viral titers from MCF7 cells transfected with control and B-Myb siRNA, 24 hrs p.i.(n=5). C: Immunocytochemistry analysis of LCMV-NP and VSV-GP from LCMV and VSV MOI 1.0 infected, control and B-Myb specific siRNA treated MCF7 cells, 24 hrs (LCMV) 7hrs (VSV) p.i. (n=3).

Next, to further test that B-Myb dependent virus replication is not limited to a specific cell type but rather a global phenomenon we used ambion *in-vivo* siRNA technology to knockdown B-Myb specifically in liver hepatocytes. This siRNA is specifically designed to

target hepatocytes when injected intravenously (Fig. 8A). VSV, as discussed earlier is a cytolytic virus and interferon responsive. In a control scenario, when infected with VSV and immune response is launched and the virus is controlled with in spleen with little or no virus escape to vital organs. However, when macrophages are depleted and then infected, VSV is not controlled in the spleen and escapes to liver and other vital organs. We used this strategy in this experiment. First the mice were treated with *in-vivo* control and B-Myb directed siRNA intravenously and 12 hours later treated with clodronate-liposomes to deplete macrophages from the host. The mice were then infected with $2x10^6$ VSV, and livers were harvested for immunohistochemistry and RNA analysis 18 hours post infection. Analysis revealed severe liver damage in scrambled control siRNA treated mice as compared to B-Myb siRNA treated (Fig. 8B). This correlates with increased viral replication in control mice in histological analysis (Fig. 8C). Taken together, we showed that B-Myb depletion abrogates virus replication *in-vitro* and *in-vivo* and the observed phenomenon is not cell specific.

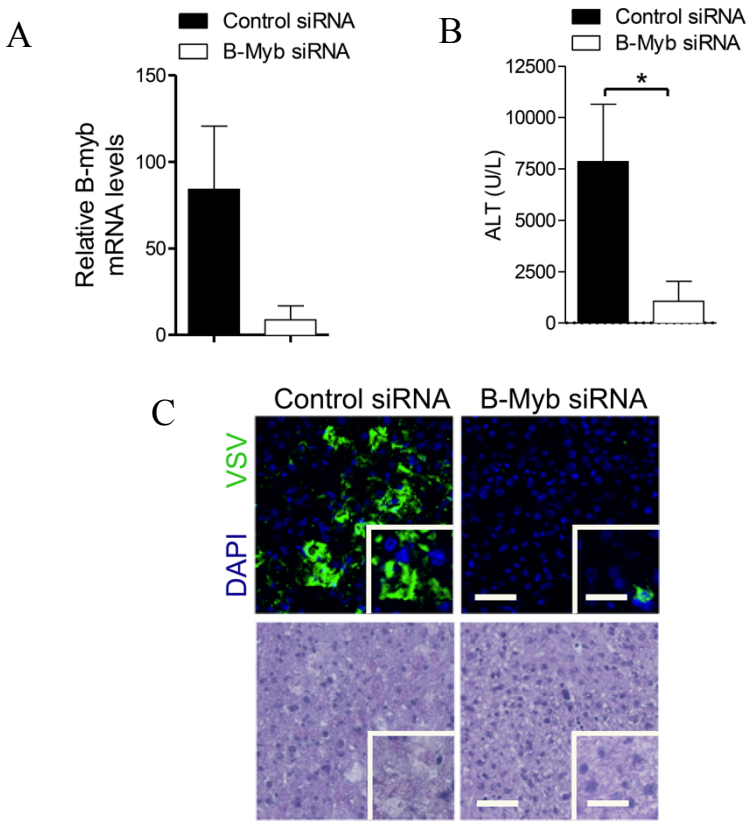


Figure 8: *In-vivo* liver depletion of B-Myb reduces VSV replication and damage in liver

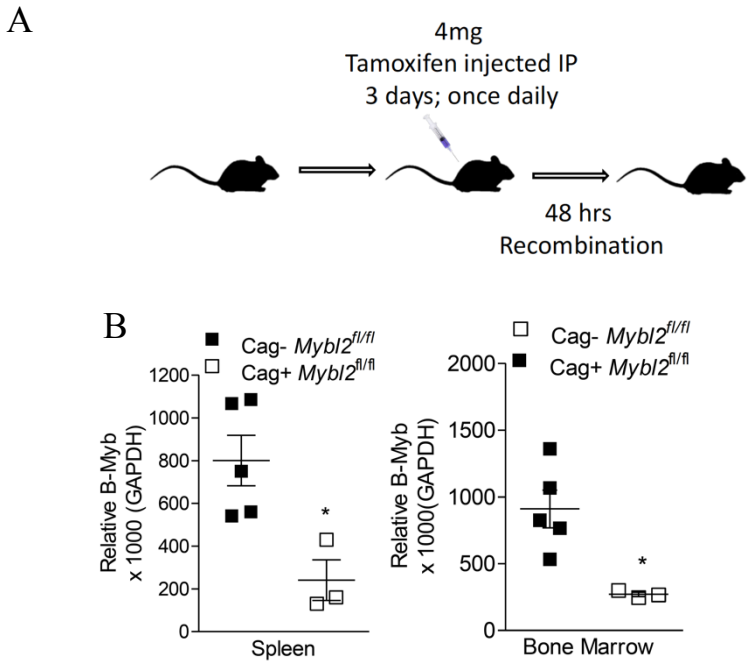
A: Quantitative mRNA analysis of B-Myb from liver of clodronate treated C57BL6/J mice transfected with Ambion *in-vivo* control and B-Myb specific siRNA, infected with $2x10^6$ PFU VSV, 18 hours p.i. (n=4) B: Serum alanine-aminotransferase (ALT) levels of clodronate treated C57BL6/J mice transfected with Ambion *in-vivo* control and B-Myb specific siRNA,

infected with 2x10^6 PFU VSV, 18 hours p.i. (n=5) C: Immunohistochemistry and H&E analysis of VSV-GP from 2x10^6 PFU VSV infected, control and B-Myb specific siRNA treated C57BL6/J mice (n=5).

3.1.3 B-Myb depletion abrogates VSV replication *in-vivo.*

To study the role of B-Myb in VSV infection, we made use of tamoxifen induced mouse model in which the B-Myb allele is floxed. This leads to excision of B-Myb when tamoxifen is administered. In the control mice, the absence of the Cag promoter ensures that any non-specific effects of tamoxifen treatment are taken into account (Cag-*Mybl2*$^{fl/fl}$ control mice), whereas in the B-Myb conditional transgenic mice, Cag promoter is present at the start of B-Myb allele denoted as Cag+*Mybl2*$^{fl/fl}$ (B-Myb transgenic mice).

We optimized the tamoxifen dosage for knockdown of B-Myb. Optimized dose is obtained at 4mg/mouse, injected intra-peritonealy for three consecutive days. The mice were left for recombination for 48 hours and then used for experimentation (Fig. 9A). RT-PCR analysis revealed that B-Myb is reduced by >60% in spleen and 80% in bone marrow after tamoxifen treatment (Fig. 9B). As B-myb depletion is also known to induce apoptosis[90], we checked if our dosage of tamoxifen is inducing apoptosis in mice spleen. Both the control Cag-*Mybl2*$^{fl/fl}$ and transgenic Cag+*Mybl2*$^{fl/fl}$ mice were treated with tamoxifen and sacrificed 2 days after last treatment. Spleen samples were analyzed for cleaved caspase-3 expression via immunohistochemistry showing no difference in the in the expression of the same n both control and B-Myb deficient mice (Fig. 9C).



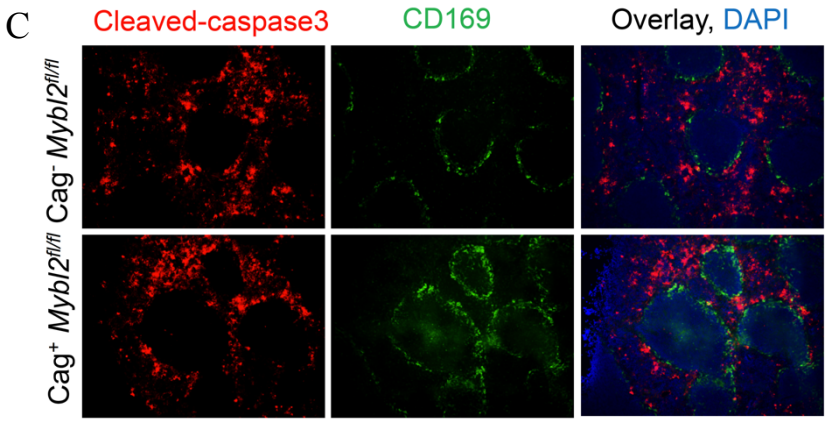


Figure 9: *In-vivo* depletion of B-Myb via tamoxifen

A: Experimental schematic of tamoxifen treatment for B-Myb depletion. B: Quantitaive mRNA analysis of B-Myb from spleen and bone marrow of Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ treated with tamoxifen (n= 3-5) C: Immunohistochemistry analysis of cleaved caspase-3 from tamoxifen Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice (n=4-5).

Further, to determine whether the depletion of B-Myb has any effect on early virus replication, we treated control Cag-*Mybl2*$^{fl/fl}$ and transgenic Cag+*Mybl2*$^{fl/fl}$ mice with 2x10^8 PFU VSV and sacrificed them 8 hour post infection. Spleen was harvested for immunohistology, viral titers and RNA analysis. Immunohistological analysis revealed significantly reduced virus replication in CD169$^+$ macrophages (Fig. 10A) which is also reflected in reduced infective viral titers in B-Myb depleted mice (Fig. 10B). Further analysis confirmed significantly diminished VSV replication at the transcription level (Fig. 10C). Tamoxifen is an established drug which is used in cancer therapy[136]. Reports have also shown effects of tamoxifen in regulating viral replication in-vitro[137, 138]. To exclude any effects of tamoxifen on VSV replication, we treated WT mice with corn oil and tamoxifen and infected them with 2x10^8 PFU VSV. Mice were then sacrificed 8 hours later and analyzed for infective viral titers. Results showed no effect of tamoxifen on VSV titers between both groups (Fig. 10D). Collectively, we show here that B-Myb plays an important role in ensuring early/initial VSV replication in CD169$^+$ macrophages.

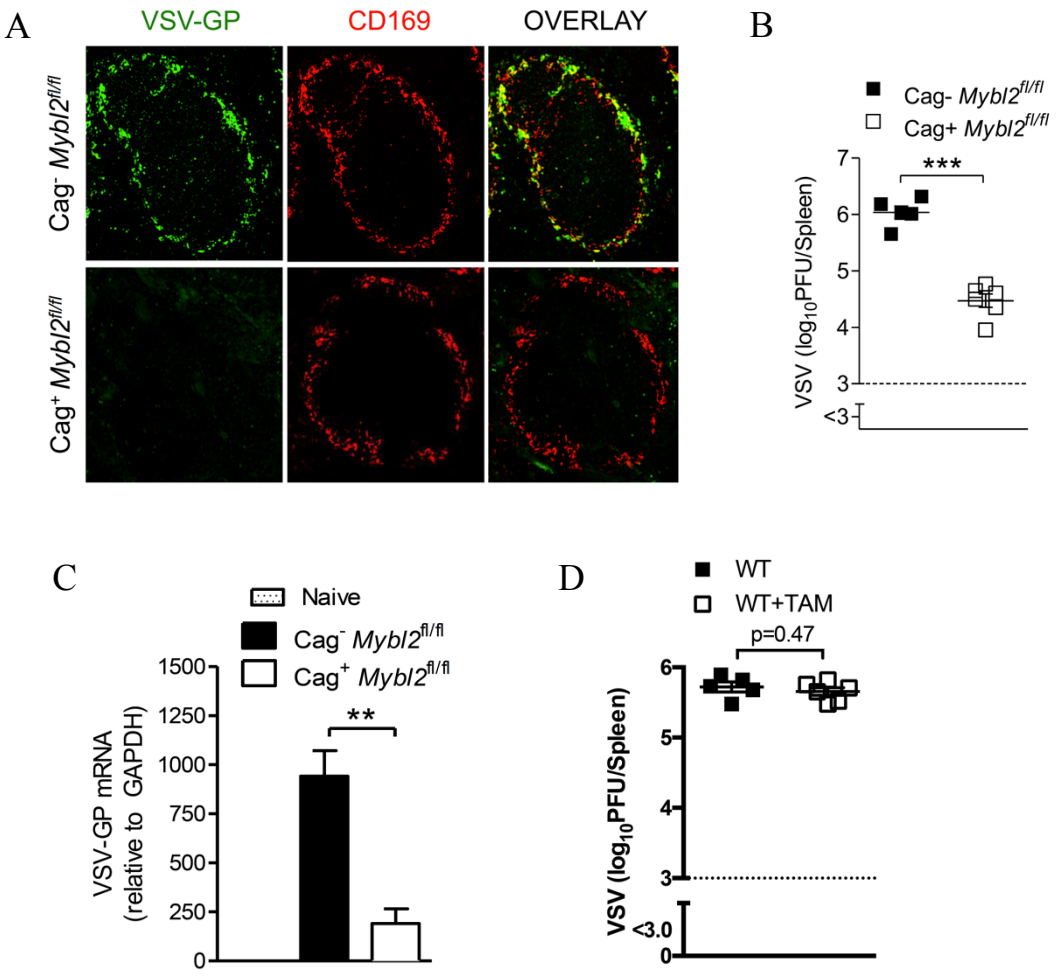


Figure 10: *In-vivo* B-Myb depletion abrogates VSV replication

A: Immunofluorescence of spleen from tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected with 2x10^{8} PFU VSV, 8 hours p.i. (n=6) B: Infective VSV titers from spleen of tamoxifen treats Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected with 2x10^{8} PFU VSV, 8 hours p.i. (n=5-6) C: Quantitative mRNA analysis of VSV-GP from tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected with 2x10^{8} PFU VSV, 8 hours p.i. (n=3-4). D: Infective VSV titers of control mice treated with or without tamoxifen, infected with 2x10^{8} PFU VSV, 8 hours p.i. (n=4-5).

3.1.4 Effect of B-Myb deficiency on interferon responses.

Interferon is a key player in antiviral defenses, especially Type-I interferon. Type-I interferon which includes IFN-α and β are known to be potent antiviral cytokines and also modulators of adaptive immune responses[139]. Viral replication in CD169^{+} macrophages is essential to initiate antiviral response as well as IFN-I production. In previous results, we showed that B-Myb deficiency impairs VSV replication. Also, VSV is known to be a highly

interferon responsive virus[129] and thus tends to abrogates it for its successful replication[140]. Therefore, we checked for IFN-I induction in control and B-Myb deficient mice after 2x10^8 PFU VSV infection. We found significantly lower IFN-I production in B-Myb deficient mice (Fig. 11A). To correlate the reduced interferon production with the observed effect of virus replication, we analyzed interferon-stimulated genes (ISGs) in the same experimental settings via RT-PCR. Results showed significant reduction in the gene expression level of the ISGs (Fig. 11B). The reduced IFN-I production and ISGs gene expression is a direct effect of reduced early VSV replication in CD169$^+$ macrophages.

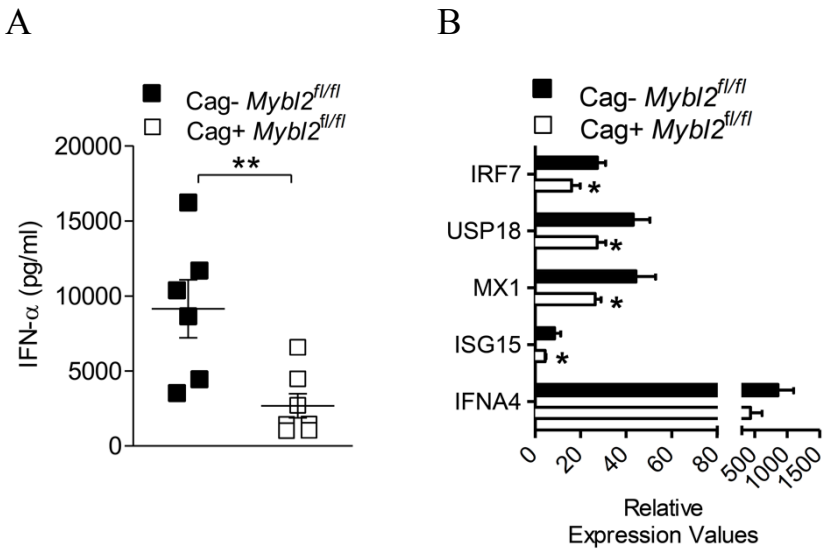


Figure 11: Reduced IFN response results from abrogated VSV replication

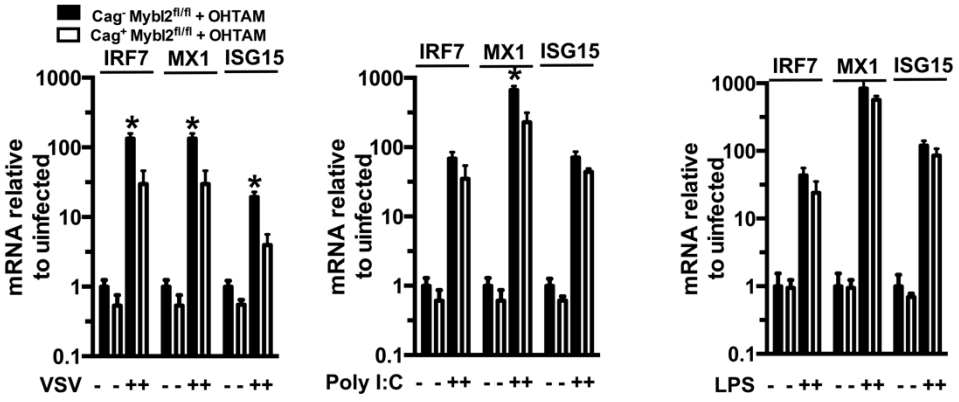
A: Quantification of IFN-α in serum of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected with 2x10^8 PFU VSV, 8 hours p.i. (n=5-6) B: Quantitative mRNA analysis of interferon induced genes from spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected with 2x10^8 PFU VSV, 8 hrs p.i. (n= 3).

Next, considering B-Myb is an important transcription factor, we checked whether B-Myb deficiency itself have any effect on the immune signature of the cell with the use of immune stimulants such as LPS and Poly I:C. LPS is a lipopolysaccharide, which is expressed on the cell wall of gram-negative bacteria. LPS is recognized as PAMPs by PRRs and by TLRs[141, 142]. LPS forms a complex with LPS-binding protein (LBP) which further binds to CD14 which is either available in blood plasma or bound to the cell surface. The immune activation by LPS is mediated via its binding to TLR4[143, 144], which includes activation of Nf-κB, ERK1/2, SAPK/JNK pathways. Poly I:C is a synthetic analog of

dsRNA, which mimic the molecular patterns associated with viral infection. As it has been shown that dsRNA is been recognized by TLR3[145], poly I:C acts via two ways once recognized by PRRs; endocytosed poly I:C activates TLR3 while cytosolic one activates RIG-I/MDA-5. Activation by poly I:C results in the induction of IL-12 and type-I IFNs which in-turn enhance the T and B cell immunity. Poly I:C has been reported to be used as an adjuvant as it also improves MHC-II expression and antigen cross-presentation[146].

For this experiment, we generated bone marrow derived macrophages (BMDM) and dendritic cells (BMDC) and treated then with 7.5μM and 5μM 4-hydroxy tamoxifen for 48 hours respectively. At first, we compared ISG expression in between VSV infection and other immune stimulants LPS and Poly I:C. Upon mRNA expression analysis, we found no effect of B-Myb deficiency on the immune signature in case of LPS and Poly I:C, whereas when infected with VSV, the expression of evaluated ISGs was significantly less (Fig. 12A). Next, we analyzed the phosphorylation status of up-stream molecules of immune-regulation pathways. We treated BMDC with LPS in a time dependent manner and Poly I:C for 7 hours and harvested for western blot analysis. Western blot was done for total Nf-κB p102, Nf-κB p65, iKKβ and their phosphorylated forms. Analysis shows no significant difference in the activation of these molecules in B-Myb deficient BMDC when compared to control BMDC with both Poly I:C (Fig. 12B) and LPS (Fig. 12C) treatment. Taken all together, we showed that B-Myb deficiency has no effect on the immune signature of the cell when stimulated by non-viral agents.

A



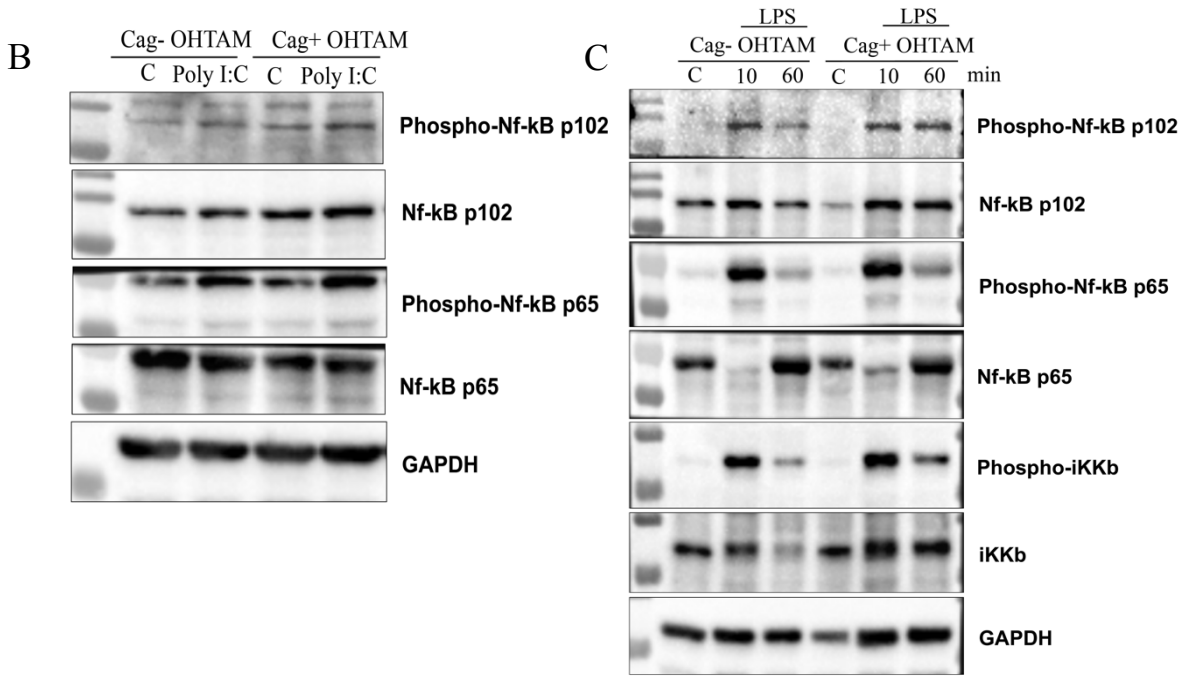


Figure 12: B-Myb depletion doesn't alter cell immune signature

A: Quantification mRNA analysis of 4-Hydroxy tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ BMDM, stimulated with Poly I:C (50µg/ml), LPS (50ng/ml) and VSV (MOI 1.0) (n=5-6) B,C: Representative immunoblots of IFN-responsive molecules in OHTAM treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ BMDCs' stimulated with Poly I:C (50µg/ml) for 7 hours (B) and LPS (50ng/ml) for 10 and 60 min (C) (n= 3).

3.1.5 Adaptive antiviral response is dependent on B-Myb dependent VSV replication.

The adaptive immune system presents us with the next line of defense in an infection scenario. Successful activation of the adaptive immune system depends on the efficient presentation of the antigen by APCs, which further require viral replication in APCs specially in CD169^{+} macrophages[15]. Activation of adaptive immunity can be measured by various methods, one such method is measurement of IFNγ producing CD8^{+} and CD4^{+} T-cells. We infected control and B-Myb deficient mice with 2x10^{6} PFU VSV and harvested the spleen at day 8 post infection. The spleen cells were then stimulated with VSV- peptide p52 (CD8^{+} T-cell specific) and p8 (CD4^{+} T-cell specific) for 1 hour and then processed for intracellular IFNγ staining. B-Myb deficiency led to significantly impaired viral responses in both CD8^{+} (Fig. 13A) and CD4^{+} T-cells. (Fig. 13B). As in VSV infection, the B-cell response plays an

important role in the clearance of the virus via production of neutralizing antibodies. We checked the B-cell response in control and B-Myb-deficient mice. We infected control and B-Myb deficient mice with $2x10^6$ PFU VSV and then took serum samples at the mentioned days for neutralizing antibody analysis. Upon analysis, we found significantly reduced VSV-specific total neutralizing antibodies in B-Myb deficient mice as compared to control mice (Fig. 13C). To further verify if the antibody production was completely virus dependent and not B-Myb dependent, we made use of UV-inactivated VSV virus. We infected both control and B-Myb deficient mice with the higher PFU of UV-inactivated VSV as of active VSV virus i.e. $2x10^8$ PFU and then measured the total neutralization antibodies from serum samples. We found no significant difference in the production of total neutralizing antibodies in B-Myb deficient mice (Fig. 13C, right panel) which confirmed that B-Myb doesn't affect the B-cell response by itself rather by limiting early virus replication. This is in-line with our observed phenotype of reduced early VSV replication in $CD169^+$ macrophages, which in-turn presents less antigen thus reducing significantly the adaptive immune response.

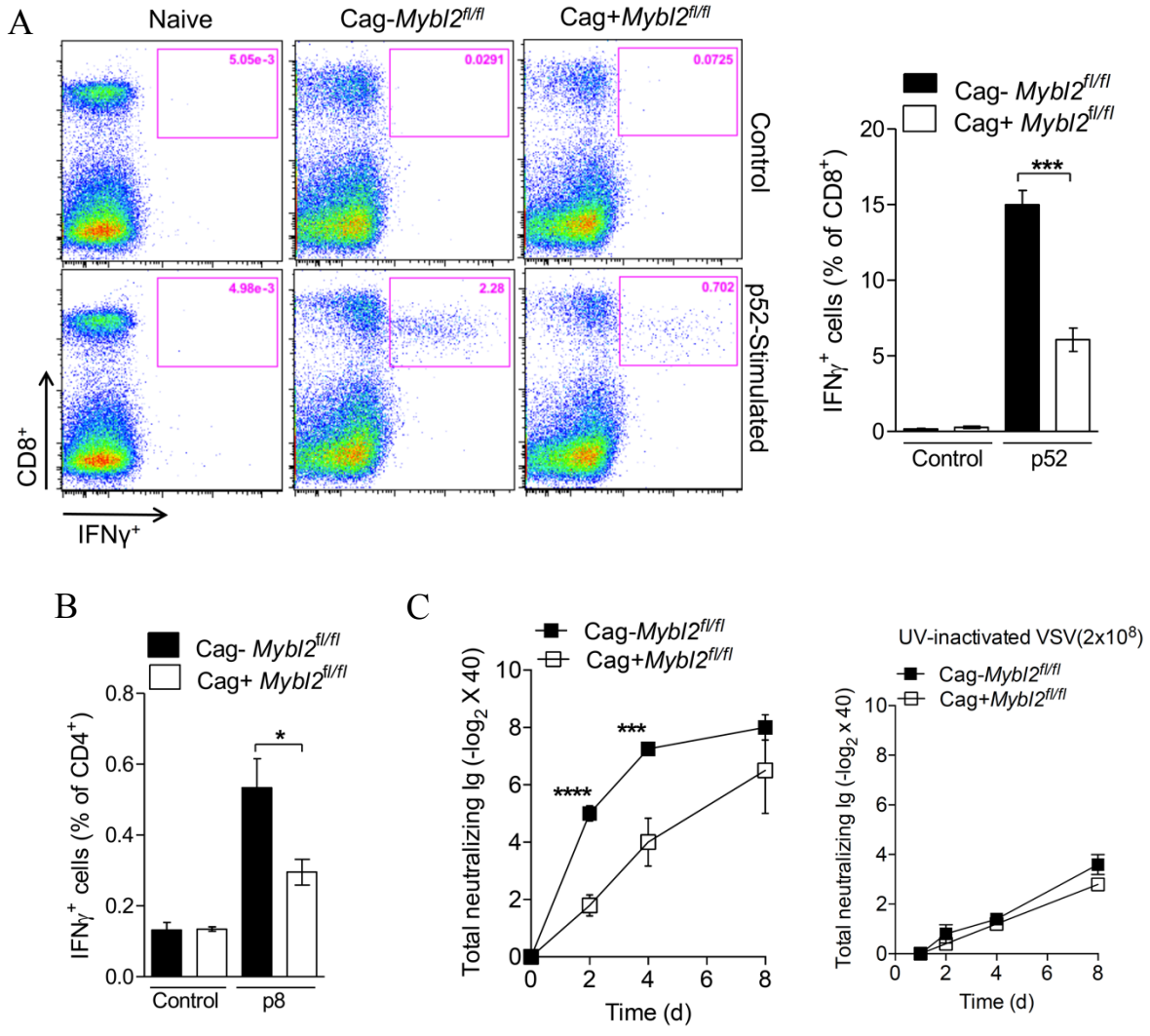


Figure 13: B-Myb dependent VSV replication is crucial for adaptive immune activation

A: Original FACS blot and quantification of intracellular IFN-γ producing $CD8^+$ T-cells from spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected i.v. with $2x10^6$ PFU VSV, 8 days p.i. (n=6) B: Intracellular IFN-γ producing $CD4^+$ T-cells from $2x10^6$ PFU VSV i.v. infected Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, 8 days p.i. (n=6) C: VSV-neutralizing total Ig from serum of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice infected with either $2x10^6$ PFU VSV or $2x10^8$ PFU UV-inactivated VSV (n=5-6).

3.1.6 Antiviral adaptive response is independent of B-Myb.

As mentioned earlier, B-Myb is an important transcription factor which regulates cellular proliferation and differentiation. We wondered whether B-Myb deficiency has an effect on adaptive cell expansion upon antigen presentation and thus on adaptive immune response. To answer this, we made use of two transgenic mice with VSV-specific $CD4^+$ T-cell and B-cells (Fig. 14A). As these mice are not transgenic for B-Myb, thus expression of B-Myb in these mice remains as that of control. Tg7 Thy1.1 mouse have a VSV-specific $CD4^+$ T-cell antigen receptor and can be differentiated with the expression of CD90.1 instead of CD90.2 on lymphocytes. Due to presence of the transgene, $CD4^+$ T-cells expands rapidly once presented with VSV-antigen and thus were made use in the present study. We compared the expansion of VSV-specific $CD4^+$ T cells in both control and B-Myb deficient mice after infection. We transferred splenocytes from Tg7 Thy1.1 into control and B-Myb deficient mice and infected with $2x10^6$ PFU VSV 24 hours later. Mice were then sacrificed at day 4 post-infection and spleen was harvested for FACS analysis. Results showed significantly reduced expansion of VSV-specific $CD4^+$ T-cell (Fig. 14B). This implied that the reduced T-cell effect in B-Myb deficient mice is the result of the abrogated early VSV replication.

Next, we evaluated the effect of B-Myb on B-cell expansion and thus in-turn antibody production. For this we made use of the transgenic mouse model Vi10 x CD45.1 expressing VSV-specific B-cell receptor as knock-in, thus this mouse when presented with VSV results in rapid expansion of B-cells. For the experiment, we harvested the splenocytes from Vi10 x CD45.1 mice and labeled them with CFSE for 10 min in PBS. CFSE is a chemical which, when added to cells, binds to the cellular phospholipid bilayer. Thus, the cellular division can be tracked as the intensity of CFSE reduces each time a cell divides. We then adoptively transferred these splenocytes into control and B-Myb deficient mice and infected with $2x10^6$

PFU VSV 24 hours later. Mice were then sacrificed 4 days post-infection and spleen were harvested for FACS analysis. Results showed significantly less expansion of VSV-specific B-cells in B-Myb deficient mice (Fig. 14C). These results suggest that B-cell response to the antigen is dependent on virus replication rather on B-Myb.

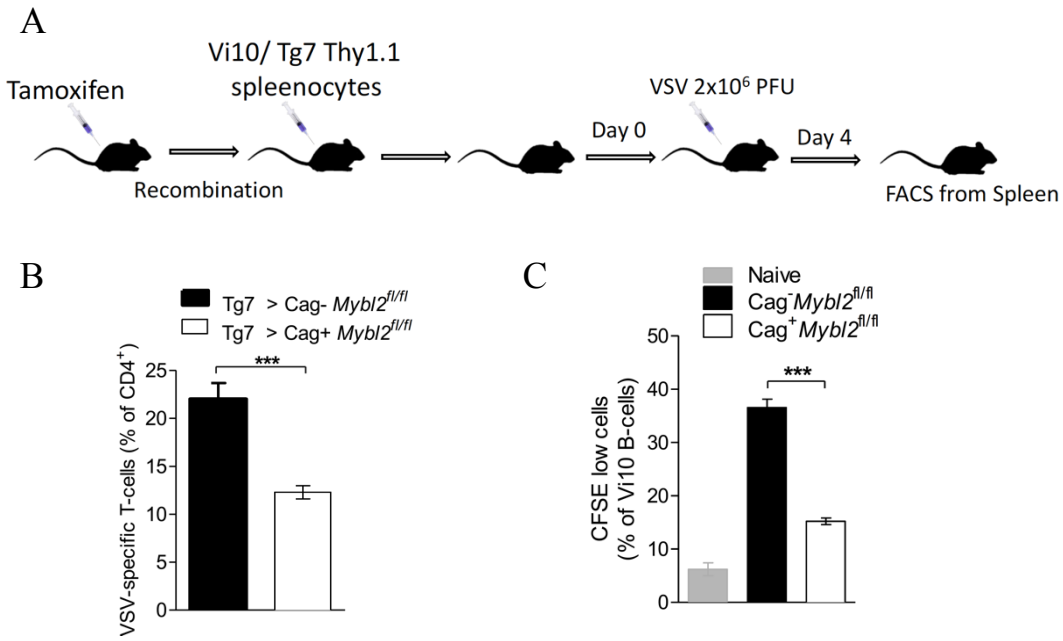


Figure 14: Antigen-dependent T and B-cell proliferation is independent of B-Myb

A: Experimental schematic of Tg7Thy1.1 and Vi10 CD45.1 splenocytes transfer and proliferation analysis. B: Frequency of adoptively transferred VSV-specific $CD4^+$ T-cells in tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with $2x10^6$ PFU VSV i.v. analyzed 4 days p.i. (n=6-7) C: Quantification of CFSE labelled CD45.1 VSV-specific B-cells transferred in tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with $2x10^6$ PFU VSV i.v. analyzed 4 days p.i. (n=5) (naïve n=3).

3.1.7 B-Myb dependent early VSV replication is crucial for host survival.

Early virus replication in splenic APCs is crucial for an effective immune response. During the early infection cycle, viruses replicate in spleen APCs which then process these pathogens and presents them to adaptive immune system for their activation along with the release of pro-inflammatory cytokines. Failure of early virus replication leads to reduced immune activation and thus further escape of virus to vital organs.

In B-Myb deficient mice, we have shown till now that the early virus response is abrogated and thus so the adaptive immune response as well. With the inability of VSV to replicate in the spleen, the virus then tries to escape to other organs for its survival and

replication. We checked in our experimental model, if the VSV escapes to other organs and replicates. As the tamoxifen induced knockdown is not a total body knockdown and our optimized dose effect spleen and bone marrow the most, the virus has multiple other tissues to facilitate its replication. Thus, we infected control and B-Myb deficient mice with $2x10^6$ PFU VSV and sacrificed the mice 8 days post infection. Brain and spinal cord were harvested for viral titer analysis via plaque forming assay. Results revealed significantly high viral titers in both brain and spinal cord in B-Myb deficient mice than control mice (Fig. 15A). This indicates the escape of virus to the CNS of the B-Myb deficient mice due to its inability to replicate in spleen. As mentioned earlier that VSV is a cytolytic virus and thus leads to cell lysis upon infection, we checked for the survival of our mice models. We infected control and B-Myb deficient mice with non-lethal VSV PFU of $2x10^6$. More than 80% of B-Myb deficient mice developed paralysis in both legs by day 8-10 and were sacrificed. whereas all the control mice were able to control VSV and didn't develop paralysis (Fig. 15B).

Next, we wondered if conditional B-Myb knockdown has an effect on the survival of the host. Therefore, we treated control and B-Myb transgenic mice with the optimized dose of tamoxifen and were kept under analysis for up to 30 days. Both the control and B-Myb transgenic mice showed no signs of stress or pain and survived till 30 days (Fig. 15B). Taken together, we showed that B-Myb is crucial for early virus replication and thus in-turn enabling the virus control and effective immune priming and survival of the host.

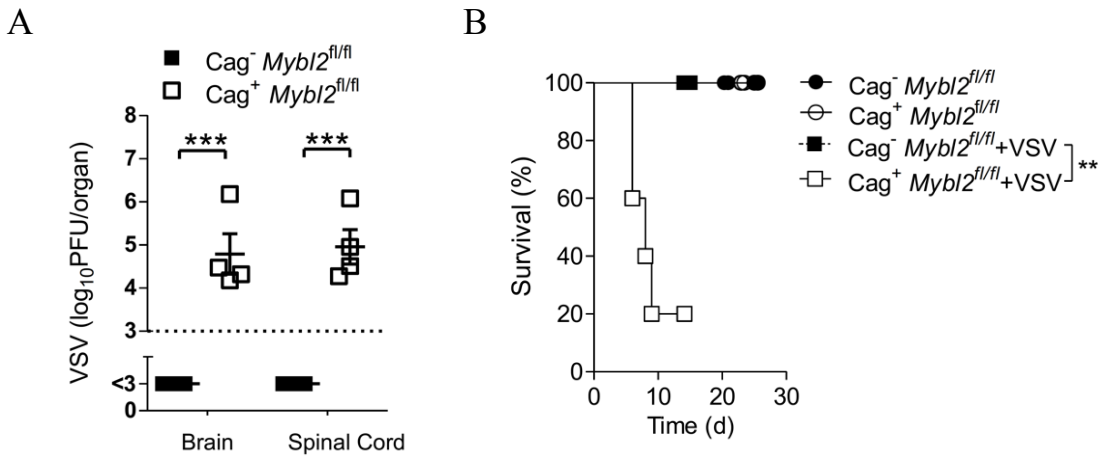


Figure 15: B-Myb dependent early VSV replication is crucial for host survival.

A: Infective VSV titers from brain and spinal cord from tamoxifen treates Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with $2x10^6$ PFU VSV i.v. analyzed 8 days p.i. (n=4) B: Survival of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, either non-infected or infected with $2x10^6$ PFU VSV i.v. (n=5-6).

3.1.8 Global requirement of B-Myb by viruses for replication.

We have shown that VSV replication is dependent on B-Myb which is important for the immune activation and thus in-turn virus control. As B-Myb is a ubiquitously expressed protein which is conserved evolutionary and is a major transcription factor, we wondered if the observed phenomenon of B-Myb dependent viral replication is globally recapitulated in different RNA and DNA viral models. To check this, we made use of two more murine virus models LCMV and MCMV.

LCMV is a cytopathic RNA virus. It also replicates in $CD169^+$macrophages during its initial replication and its control is majorly T-cell dependent. To check if B-Myb is also crucial for early LCMV replication, we infected control and B-Myb deficient mice with $2x10^6$ PFU LCMV intravenously. Mice were then sacrificed 24 hours later and spleen was harvested for immunohistochemistry and viral titer analysis. Upon staining of the spleen tissue to check the viral nucleoprotein NP, we found significantly reduced replication of LCMV in $CD169^+$ macrophages in B-Myb deficient mice as compared to control mice (Fig. 16A) which is also reflected by reduced infective viral titers as analyzed by plaque forming assay (Fig. 16B). Reduced early LCMV replication in spleen leads to ineffective priming of T-cells. To check this, we infected control and B-Myb deficient mice with $2x10^4$ PFU LCMV and sacrificed at day 8 post-infection. Both liver and spleen were harvested and intracellular cytokine staining was performed to check for the activated $CD8^+$ T-cells stimulated with gp33 peptide. We found that B-Myb deficient mice showed reduced IFNγ producing $CD8^+$ T-cells than compared to control (Fig. 16C). Thus, reduced LCMV replication led to ineffective priming of T-cells in B-Myb deficient mice.

MCMV is a DNA virus and also cytopathic. Infection and successful pathogenesis of MCMV is not completely dependent on $CD169^+$ macrophages. Rather reports have shown its replication in marginal zone macrophages and eventually in red pulp during the early stages of replication[147]. To check whether MCMV replication is also dependent on the expression of B-Myb, we infected control and B-Myb deficient mice with $5x10^5$ PFU of MCMV-GFP and sacrificed 24 hours later. Spleen was harvested for immunohistochemical analysis. Upon staining with GFP for MCMV-GFP, we found significantly reduced MCMV infected cells in B-Myb deficient mice (Fig. 16D). Clearance of MCMV is majorly T-cell dependent[148, 149]. To study this, we infected control and B-Myb deficient mice with $1x10^5$ PFU MCMV-GFP. The mice were sacrificed 8 days post-infection and liver and spleen was isolated. Organs were then crushed in medium and cells were subjected to intracellular cytokine staining after

stimulation with H-9-I peptide for $CD8^+$ T-cells. Results showed significantly less IFNγ producing $CD8^+$ T-cells in B-Myb deficient mice (Fig. 16E).

Next, we wondered whether the dependence on B-Myb for replication also applies to human viruses. We made use of HSV-1 and HIV luciferase reporter particles. Here we transfected MCF-7 cells with control scrambled or B-Myb specific siRNA and infected with HSV or HIV (MOI 1.0) particles. Viral titers were analyzed 24 hrs p.i. (Fig. 16F)

Compiling all the results in these experiments, we show that B-Myb is a major host factor important for the replication of a broad range of viruses. The dependence on B-Myb is a crucial viral replication step which further leads to the activation of adaptive immune system.

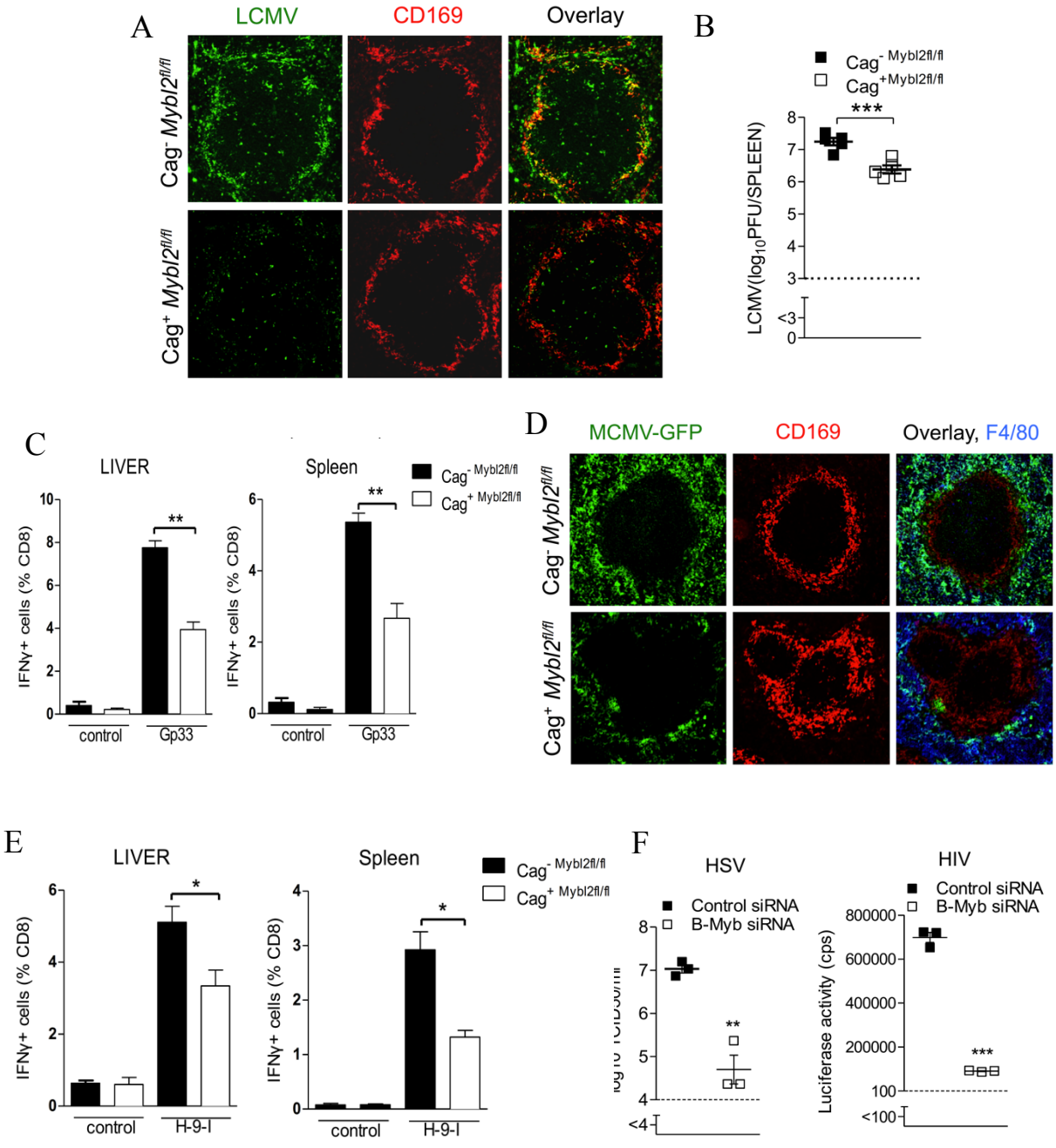


Figure 16: B-Myb has broad pro-viral capacity

A,B: Immunofluorescence of LCMV-NP(A) along with infective LCMV titers (B) from spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with 2x10^6 PFU LCMV i.v. analyzed 24 hrs p.i. (n=5) C: Intracellular IFN-γ staining of CD8$^+$ T-cells from liver and spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with 2x10^6 PFU VSV i.v, analyzed 8 days p.i. (n=5-6). D: Immunohistochemical staining of MCMV-GFP in spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with 5x10^5 PFU MCMV i.v, analyzed 24 hrs p.i. (n=5) E: Intracellular IFN-γ staining of CD8$^+$ T-cells from liver and spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with 1x10^5 PFU MCMV i.v, analyzed 8 days p.i. (n=4). F: HSV plaque assay and HIV luciferase assay on control and B-Myb siRNA treated MCF7 cells (day -1), infected with MOI 1.0 HSV or HIV luciferase particles (day 0), analyzed 24 hrs p.i. (n=3).

3.1.9 B-Myb depletion leads to down-regulation of multiple gene expression upon VSV infection.

As discussed before, B-Myb is an important transcription factor. Deletion of B-Myb leads to embryonic lethality due to abrogated expression of multiple genes required for cellular differentiation and proliferation. Here we wondered the effect on B-Myb on the cellular gene signature after VSV infection. We infected control and B-Myb depleted mice with 2x10^8 PFU VSV and harvested the spleen 8 hours later. mRNA was isolated from the whole spleen and was subjected to microarray analysis. We performed a transcriptome profiling with the whole spleen in both control and B-Myb depleted mice and also compared to naïve. In the Fig. 17A, we can see that the expression of the majority of genes is down-regulated with the depletion of B-Myb.

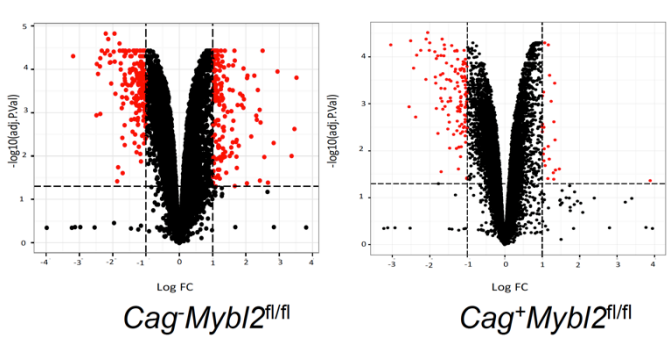
3.1.10 B-Myb dependent host-factors are required for successful viral replication.

Host factors are the cellular factors which are required by the virus for its successful replication. These host factors vary in molecular functions ranging from endocytosis to transcription and translation all the way towards the budding of the virions[150]. Being a part of the host cellular machinery, these factors are mostly evolutionary conserved and transcriptionally regulated. As B-Myb regulates and modulates a variety of cellular pathways, we investigated the effect of B-Myb on the host-factors required for virus replication.

We identified the genes which were regulated by B-Myb as transcription factor, we analyzed the probable binding sites of B-Myb using MatInspector suite of Genomatix software[151]. We ran the identification of probable genes with the default parameters of the MatInspector and obtained the probable gene hits. Then the gene list was sorted with a matrix similarity cutoff value of >0.8. The sorted gene list was then enriched for their biological processes using GO enrichment analysis based on PANTHER classification system[152]. The results revealed B-Myb regulation of transcriptional activity of RNA polymerase in a very significant manner along with DNA transcription and RNA metabolic activity (Fig. 17B). These results come in the agreement of our observed results of reduced viral RNA and thus impaired viral replication.

Here, we showed that B-Myb is regulating variety of genes including that of transcription and translation related ones. So, we wondered the direct effect of B-Myb on the known host factors for VSV replication. Many reports have identified the host factors which are crucial for VSV replication[153, 154]. Therefore, we analyzed selective host factors which plays role in transcription and translation, viral uncoating, vesicular transport during viral entry and processing, etc. We generated bone marrow derived macrophages (BMDM) from control and B-Myb floxed mice and treated them with 7.5µM OHTAM for 48 hours. BMDM were then harvested for mRNA expression analysis for various host factors. We found significant down-regulation in the expression of genes associated with transcriptional and translational activities (Fig. 17C). Next, we harvested spleen from control and B-Myb deficient mice and RNA was isolated for expression analysis of the host factors. The results obtained were similar to our *in-vitro* results and showed significantly reduced expression of multiple host factors required for RNA processing and translation along with vesicular transport and uncoating (Fig. 17D). We found that B-Myb is not only crucial for a regulated successful cell proliferation but also regulates multiple factors that aids in viral replication, ensuring successful viral replication.

A



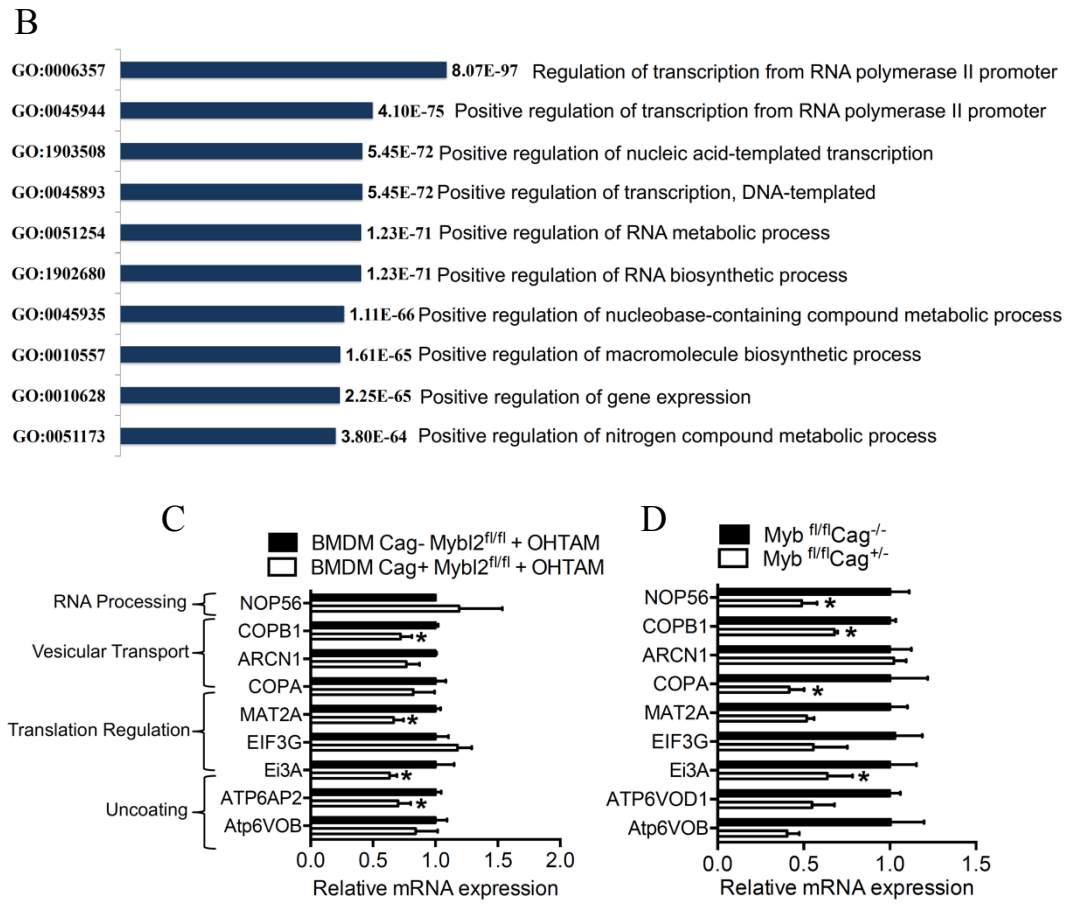


Figure 17: B-Myb facilitates expression of pro-viral host factors

A: Volcano plot illustrating differential expressed genes analyzed from microarray of spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with 2x10^{8} PFU VSV i.v. analyzed 8 hrs p.i. (n=3) B: Major pathways regulated by B-Myb analyzed by gene enrichment of B-Myb binding genes extracted from MatInspector Suite. C,D: Quantitative mRNA analysis of essential host factors from OHTAM treated BMDMs (C) and tamoxifen treated spleen (D) of Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice E: Intracellular IFN-γ staining of CD8^{+} T-cells from liver and spleen of tamoxifen treated Cag-*Mybl2*$^{fl/fl}$ and Cag+*Mybl2*$^{fl/fl}$ mice, infected with 1x10^{5} PFU MCMV i.v, analyzed 8 days p.i. (n=4).

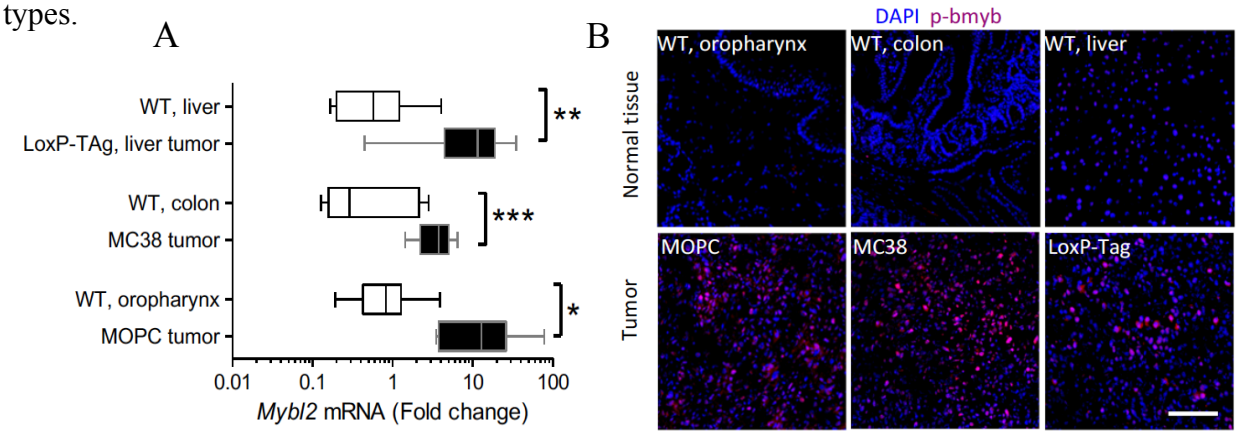
RESULT 2: B-Myb dependent LCMV replication suppress tumor growth

3.2.1B-Myb facilitates replication of LCMV in tumors.

We have shown that B-Myb is important for virus replication both *in-vitro* and *in-vivo*. It is well-established that many tumors express high levels of B-Myb, which increases

their oncogenic potential[90]. Mutation of B-Myb alleles results in enhanced B-Myb expression in tumors and further it also acts in up-regulation of another oncogene retinoblastoma. It has been shown that viruses preferentially replicate in tumors[155, 156], due to multiple factors which are higher in tumor such as availability of metabolic resources, cellular transcriptional machinery and enhanced expression of viral binding factors on cell surface.

We compared the expression of host-factors in engrafted tumor cells as well as in a spontaneous HCC model and their primary counterparts via RT-PCR and immunohistochemistry. The experiment revealed significantly higher expression levels of both B-Myb (Fig.18A) and host-factors (Fig. A2) in RT-PCR as well as much higher presence of phosphorylated B-Myb in tumor cells of subcutaneously established tumors (Fig. 18B). Next, we wondered about the viral replication capacity in primary untransformed cells versus tumor cells. We cultured various different tumor cells along with their primary counterparts and infected them with MOI 1.0 LCMV for 48 hours to perform immunocytochemistry of LCMV-NP. Analogously, cells were grown in 24-well plates and infected them with LCMV MOI 1.0 to collect supernatant for infective viral titer analysis for three consecutive days. Immunocytochemistry showed clear presence of LCMV-infection of most tumor cells when compared to primary cells (Fig. 18C). Confirmingly, plaque forming assay revealed significantly higher replication of LCMV in tumor cells than their primary non-transformed counterparts (Fig. 18D). Here we showed that B-Myb is highly expressed in tumor cells which is one of the factors required for their strong proliferation. It has been long established that virus preferentially replicates in tumor cells. Our data reveal, that indeed knockdown of B-Myb in a tumor cell disrupts the virus replication and thus B-Myb could be considered as one of the major factor regulating virus replication in tumor cells. This is in line with our data showing rapid infection and enhanced replication of LCMV in cancer cells when compared to their primary untransformed cells types.



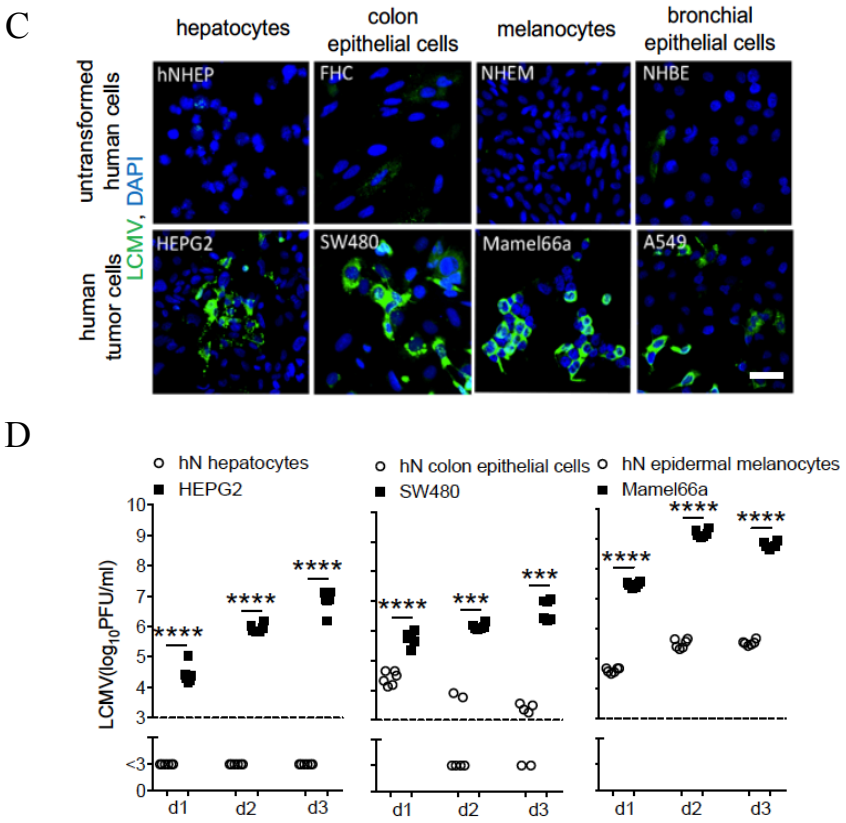


Figure 18: B-Myb-dependent LCMV replication in tumors

A,B: Quantification mRNA expression analysis of B-Myb (A) and immunocytochemistry of phospho-B-Myb (B) in mentioned tumors compared to respective healthy tissue (n=9-10) (scale-200µm). C: Immunofluorescence of LCMV-NP of human tumor cells and tissue matched un-transformed cells, infected with LCMV MOI 1.0, analyzed 48 hrs p.i. (n=3). D: Infective LCMV titers from human normal untransformed cell and respective tumor cells, infected with LCMV MOI 1.0 (n=6).

3.2.2 Preferential replication of LCMV leads to tumor regression.

Viruses have long been proposed as therapeutics for tumor treatment as they can selectively infect cancer cells causing damage to cancerous tissues with limited collateral damage to normal host tissue. Various ways have been employed to use viruses as tumor therapy such as modified and attenuated or as adjuvants. Viruses have been sought as a major therapeutic agent for tumor therapies, especially oncolytic viruses[157-159]. The specific mechanism of virotherapy have long been considered to be directly oncolytic. In cancer therapies with oncolytic viruses, this is considered to be a limited collateral effect. Arenaviruses on the other hand have been explored less frequently in terms of tumor therapy[160]. Thus, we wondered the replication of LCMV in tumor and its effect on tumor

growth. For this we injected a oropharyngeal tumor cells MOPC to establish subcutaneous tumors in mice. Once the tumor reached a specific size, we injected $2x10^4$ PFU LCMV peritumorally along with the medium as control in the other mice. Following the course of the tumor growth, we saw complete regression of tumor in LCMV-treated mice than compared to the control (Fig. 19A) leading to longer survival and being relapse-free during the observational time (Fig. 19A). Upon checking if the time of LCMV infection has any effect on the tumor regression using more advanced tumor, we found significant reduction of tumor sizes in LCMV treated mice (Fig. 19B). In line with decreased tumor burden, again LCMV-treated mice showed better survival than MOCK-treated mice. Next, we checked whether LCMV is can be used systemically to effect distant tumors. To mimic metastatic stage, we used tumors grafted in abdominal flank and shoulder and treated the mice with $2x10^4$ PFU LCMV intravenously. We found that the systemic treatment of LCMV results in tumor suppression in both shoulder and flank (Fig. 19C). As viruses display preferential proliferation and anti-tumoral activity after local and systemic delivery, we expanded our observations on different engrafted and spontaneous tumor models. We cultured MC38 colon carcinoma cells and B16 lung carcinoma and engrafted them into the flanks of WT mice. Once the tumors were established, we treated one group with LCMV peritumorally and the other with medium. With the course of tumor growth, we observed similar effect of LCMV as both MC38 (Fig. 19D) and B16 (Fig. 19E) tumor growth was significantly inhibited in the LCMV treated mice.

As engrafted models of cancer still don't represent the normal scenario of disseminated malignant disease, we made use of spontaneous mouse tumor models. These are the transgenic mice which develop endogenous tumors during the duration of their life cycle. MT/*ret* melanoma model develop visual endogenous cutaneous malignant melanoma. We injected MT/*ret* mice with LCMV i.v. once the tumors were palpable. On day 15, mice were sacrificed and tumor nodules in the skin were counted. We found that the LCMV-treated MT/*ret* mice had reduced macroscopically detectable tumor nodules compared to controls (Fig. 19F). Next, we used LoxP-Tag mice, which develop endogenous hepatocellular carcinoma with age. In this experiment, we used 9-10 months old mice with palpable liver tumors and infected them with $2x10^6$ PFU LCMV i.v. The mice were then observed and sacrificed 6 and 20 days post-infection. Liver was taken out and macroscopic nodules were counted. We found significant early reduction in the tumor nodules in the LCMV treated mice (Fig. 19H) at day 6 which can be seen by the overall architecture of the liver (Fig. 19G).

The reduction in the tumor nodules continued to decrease significantly as seen by the analysis of the liver at day 20 (Fig. 19H).

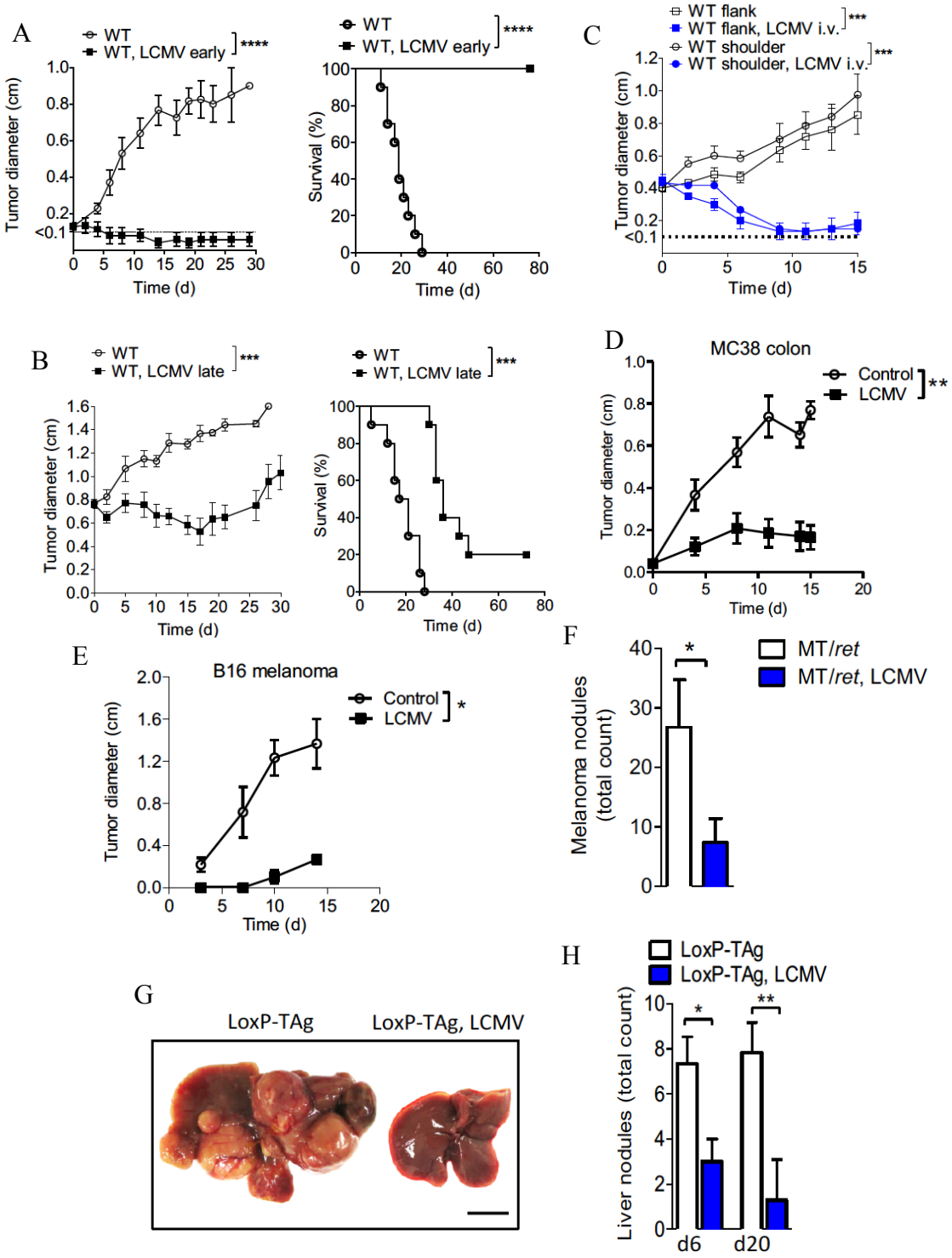


Figure 19: LCMV treatment leads to tumor regression and host survival

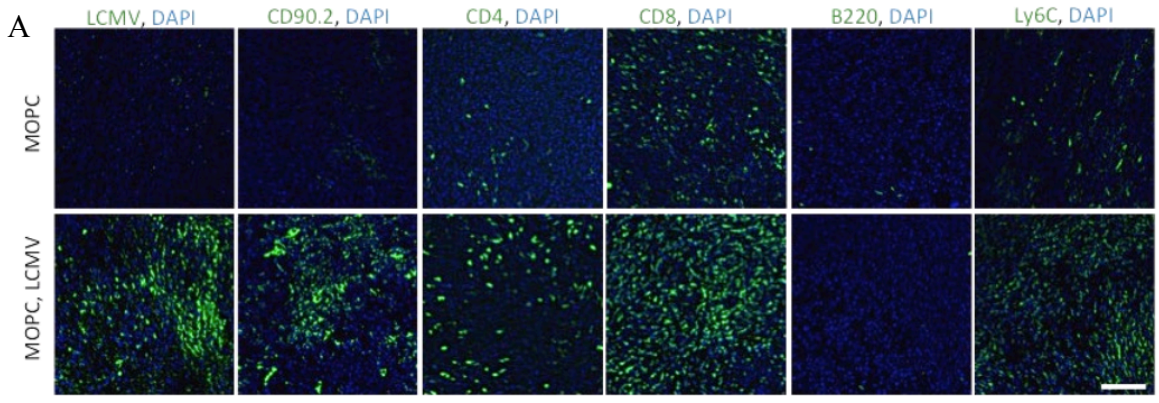
A: Tumor size and survival of MOPC tumor bearing mice (day -3) treated with $2x10^4$ PFU LCMV peritumorally (n=10). (B) tumor size and survival of advanced MOPC-tumor bearing mice (day-10), treated with $2x10^4$ PFU LCMV peritumorally (n=10). C: Tumor size of

shoulder and flank of MOPC-tumor bearing mice on shoulder and abdominal flank, injected with $2x10^6$ PFU LCMV intravenously (n=6). D,E: Tumor size of WT mice bearing MC38 (D) and B16F10 (E) tumor (day-3) treated with $2x10^4$ PFU LCMV peritumorally. (n=6-7). F: Number of melanomas in MT/*ret* (day 15), treated with $2x10^4$ PFU LCMV systemically (n=3-4) G,H: Representative picture (day 6 p.i., n=3) and macroscopic nodule quantification (day 6 n=3, day 20 n=6) of/in livers of LoxP-Tag mice bearing spontaneous HCC, treated with $2x10^6$ PFU LCMV systemically (scale- 0.5cm)

3.2.3 LCMV treatment leads to the immune infiltration in tumors.

Various reports have shown that the major cell types to infiltrate the tumor are T-cells specifically $CD8^+$ T-cells. It has been recognized that induction of inflammatory response is crucial to virus mediated tumor regression. Presence of immune infiltrates determines the progression of the tumor as well as the survival of the host. Studies have correlated the immune infiltration with the prognosis of the tumors[161-163].

LCMV infection leads to activation of both innate and adaptive immune system. This results in the infiltration of the immune cells to the site of infection. We therefore checked for the immune cells in our model of LCMV induced tumor regression. We engrafted $5x10^5$ cells subcutaneously on the right flank of the mice and injected $2x10^4$ PFU LCMV peritumorally once the tumors are palpable. Tumors were then harvested 10 days post infection and were subjected to immunofluorescence analysis for the presence of various immune infiltrates. Results displayed high infiltration of $CD8^+$ T-cells along with $CD4^+$ T-cells in LCMV-infected tumors. Interestingly, high amount of $Ly6C^+$ monocytes were also found in LCMV-infected tumors. However, we didn't observe any presence of B-cells (Fig. 20A). We also analyzed draining lymph nodes for the presence of immune cells and found strong infiltration of inflammatory $Ly6C^+$ monocytes in LCMV-treated mice (Fig. 20B). These results cumulatively show the strong activation and accumulation of inflammatory monocytes as well as T-cells during the LCMV-mediated tumor regression.



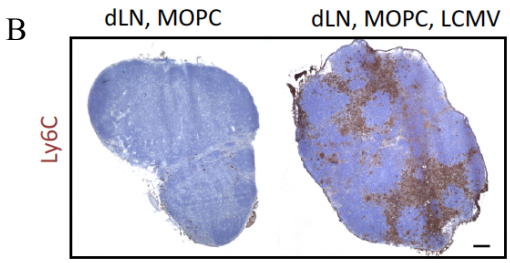


Figure 20: LCMV treatment leads to immune infiltration

A: Immunohistochemistry analysis of MOPC tumors of C57BL6/J (day -10) treated with $2x10^4$ PFU LCMV peritumorally, analyzed 10 days p.i. (n=3) (scale 200µm). B: Immunohistochemistry of draining lymph nodes from MOPC tumor bearing C57BL6/J mice treated with $2x10^4$ PFU LCMV peritumorally (n=3) (scale 200µm).

3.2.4 LCMV-induced Tumor regression is dependent on immune infiltration.

As shown by the above results, LCMV treatment leads to immune infiltration in the tumors. Thus, we wondered whether the tumor regression is due to the LCMV-induced immune infiltration or solely depends on LCMV replication. To answer this query, we made use of *Map3K14*$^{aly/aly}$ mice which lack both humoral and cell-mediated immune response and lymph nodes due to the defects in Nf-κB signaling[133]. We engrafted WT and *Map3K14*$^{aly/aly}$ mice with MOPC cells and treated one group with $2x10^4$ PFU LCMV peritumorally. Tumor growth was measured with the course of time. Results showed that LCMV-treated tumor bearing *Map3K14*$^{aly/aly}$ mice were unable to control the tumor whereas LCMV-treated WT mice showed efficient tumor regression (Fig. 21A), indicating that LCMV-mediated tumor regression depended on the activation of immune defenses.

3.2.5 Presence of IFN-I leads to LCMV-induced tumor regression.

Our previous results have established the presence of monocytes and adaptive immune infiltrates in the tumor when treated with LCMV along with the importance of innate immune response in tumor regression. Monocytes have been long known as the strong inducer and mediator of IFN-I[164, 165] upon innate sensing and vice versa. Monocytes are found in blood and are associated with tumor as myeloid-derived suppressor cells (MDSC). MDSC in mice are basically composed for Ly6C^{hi} monocytes and Ly6G^{hi} granulocytes. Among these, Ly6C^{hi} cells display high plasticity and contribute to inflammatory monocytic MDSC population associated with tumor suppression[166, 167].

To investigate the role of IFN-I in LCMV-mediated tumor regression, we used *ifnar*$^{-/-}$ mice. IFNAR mice lack IFNAR1 and IFNAR2 interferon receptors and thus IFN signaling is abolished. We injected 5x10^5 MOPC cells subcutaneously in WT and *ifnar*$^{-/-}$ mice and either left the mice un-treated or treated them with 2x10^4 PFU LCMV injected peritumorally. Tumors were measured during the course of time. Analysis revealed no significant difference in tumor bearing *ifnar*$^{-/-}$ mice when compared to LCMV treated tumor bearing WT mice (Fig. 21B), indicating that IFNAR expression on host cells was not crucial for LCMV- cancer therapy. To explore further the involvement of IFN in the LCMV-mediated tumor response, we made use of *Irf3*$^{-/-}$x *Irf7*$^{-/-}$ double knockout mice. These mice lack *Irf3* and *Irf7* genes which are the regulatory factors for the transcription of *Interferon*. We engrafted WT and *Irf3*$^{-/-}$x *Irf7*$^{-/-}$ mice with MOPC tumors and injected 2x10^4 PFU LCMV peritumorally. The tumor size was analyzed within the course of tumor growth in both groups. The anti-tumoral effect LCMV was blunted in tumor-bearing *Irf3*$^{-/-}$x *Irf7*$^{-/-}$ mice treated compared to WT control mice (Fig. 21C). Taken together, the results displayed the crucial role of IFN-I in LCMV-treatment of tumors, acting rather directly on tumor cells than on host cells.

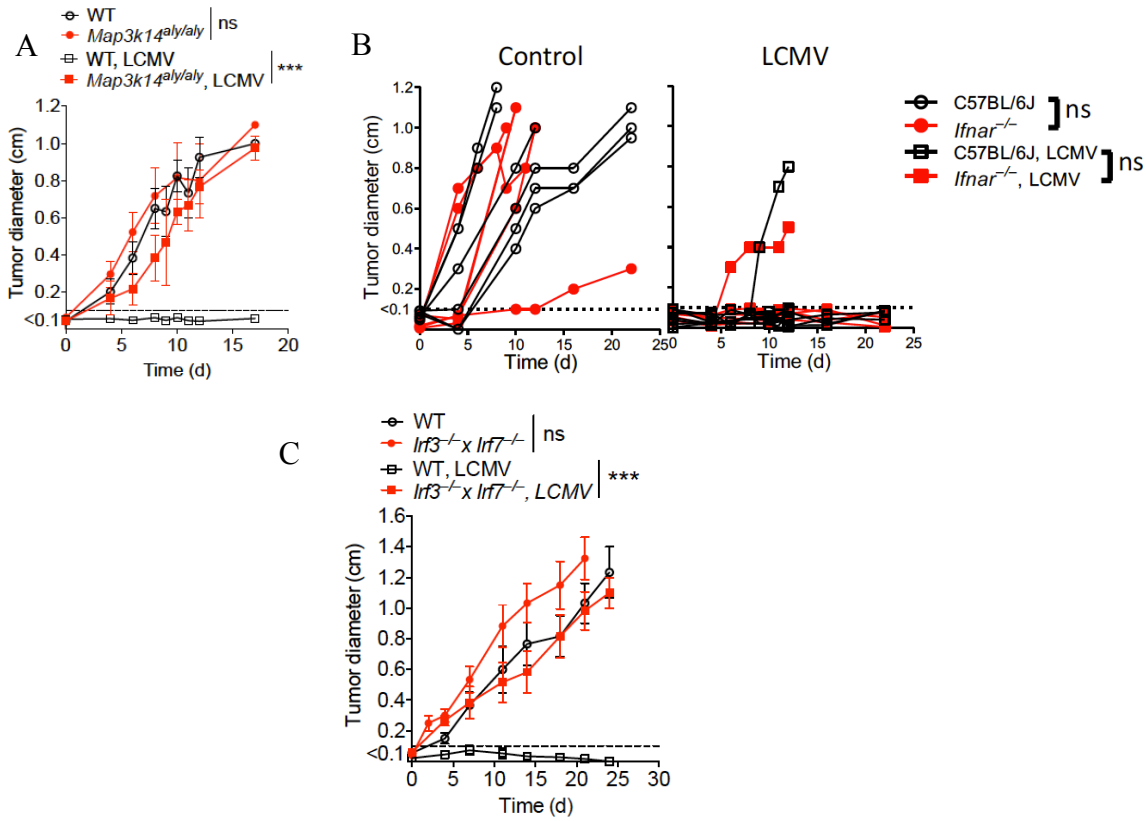


Figure 21: Anti-tumor LCMV effect is dependent on immune infiltration and host IFN.
A: Tumor measurement of MOPC-tumor bearing WT or *Map3K14*$^{aly/aly}$ (day -3) treated with (n=8 WT; n=6 *Map3K14*$^{aly/aly}$) or without (n=7 WT; n=6 *Map3K14*$^{aly/aly}$) 2x10^4 PFU LCMV

peritumorally. B: Tumor measurement of MOPC-tumor bearing WT and *Ifnar*$^{-/-}$ mice either untreated (left) or treated (right) with 2x10^4 PFU LCMV peritumorally (n=6-7).

3.2.6 Monocytic IFN production leads to tumor regression with LCMV treatment.

As IFN-I is produced by many cell types, we wondered if MDSC monocytes have a role in the current model. Thus, we made use of IFN-β reporter mice established as IFNβ$^{mob/mob}$ mice[168], these mice have a GFP reporter tagged IFN-β gene and thus expression of the same can be detected. We established tumors in WT and IFNβ$^{mob/mob}$ mice and infected them with 2x10^4 PFU LCMV peritumorally, once the tumors were palpable. Draining lymph nodes were harvested on 2 days post-infection and analyzed for GFP signals via FACS. Results showed that the IFN-β was produced by Ly6C$^+$ monocytes in LCMV infected tumor bearing mice with the molecular signature as mPDCA^{hi}, CD11c^{med} and B220^{med} which suggests their differentiation into interferon producing cells (Fig. 22A). To strengthen further our results of IFN dependence in LCMV mediated tumor reduction, we analyzed various IFN-I associated genes expression from draining lymph nodes of LCMV-treated tumor bearing mice, sacrificed on 3 days post 2x10^4 PFU LCMV infection along with the analysis of blood serum IFN-α levels. We found significantly high expression of IFN related genes along with high serum IFN-α levels in LCMV-treated tumor-bearing mice as compared to tumor-bearing WT mice (Fig. 22B). These results prove conclusively the high expression of type-I IFN in our LCMV-tumor model. To further investigate the role of monocytes in our observed tumor regression, we used different antibodies for depletion of various monocytic populations. For this experiment, we used two different antibodies; α-Gr-1 (clone RB6-8C5) which binds to both Ly6G$^+$ and Ly6C$^+$ cells[169, 170] and α-Ly6G (clone 1A8) antibody which specifically binds to Ly6G$^+$ cells. Mice were treated with three consecutive treatments of 200μg of α-Gr-1 antibody at day -2, 2 and then 7 of 5x10^5 MOPC cells injection. Second group of mice were treated with 500μg of α-Ly6G antibody at day -2, 2 and 7 of MOPC cell injection. In both experiments isotype-treated mice were used as control. All the groups were then injected with either control or 2x10^4 PFU LCMV peritumorally. The mice were observed for their tumor growth and tumor size was analyzed. In the group treated with α-Gr-1 antibody resulting in depletion of both Ly6G$^+$ and Ly6C$^+$ cells, LCMV showed no effect on tumor growth as compared to isotype treated mice (Fig. 22C). Whereas mice treated with α-Ly6G antibody showed tumor reduction with LCMV treatment same as that of isotype treated WT control (Fig. 22D). To investigate whether the recruitment of monocytes is necessary for

the IFN mediated tumor regression, we employed *Ccr2*$^{-/-}$ mice. *Ccr2* (chemokine receptor 2) is a major receptor for monocyte chemoattractant proteins (MCPs)[171], which is one of the potent chemoattractant for multiple cell types including monocytes[172], immature dendritic cells[173], memory T-cells[174] and NK cells[175]. Thus *Ccr2*$^{-/-}$ mouse presents impaired recruitment of monocytes and reduced monocyte number. We injected WT and *Ccr2*$^{-/-}$ mice with 5x10^5 MOPC cells subcutaneously and once the tumors were palpable, 2x10^4 PFU LCMV was injected peritumorally. The tumor growth was then measured for 28 days. We observed significantly impaired anti-tumoral effect of LCMV in *Ccr2*$^{-/-}$ mice as compared to WT mice (Fig. 22E). Taken together we show that Ly6C$^+$ monocytes play an important role in the control of tumors in LCMV treated mice via the induction and release of type-I IFN.

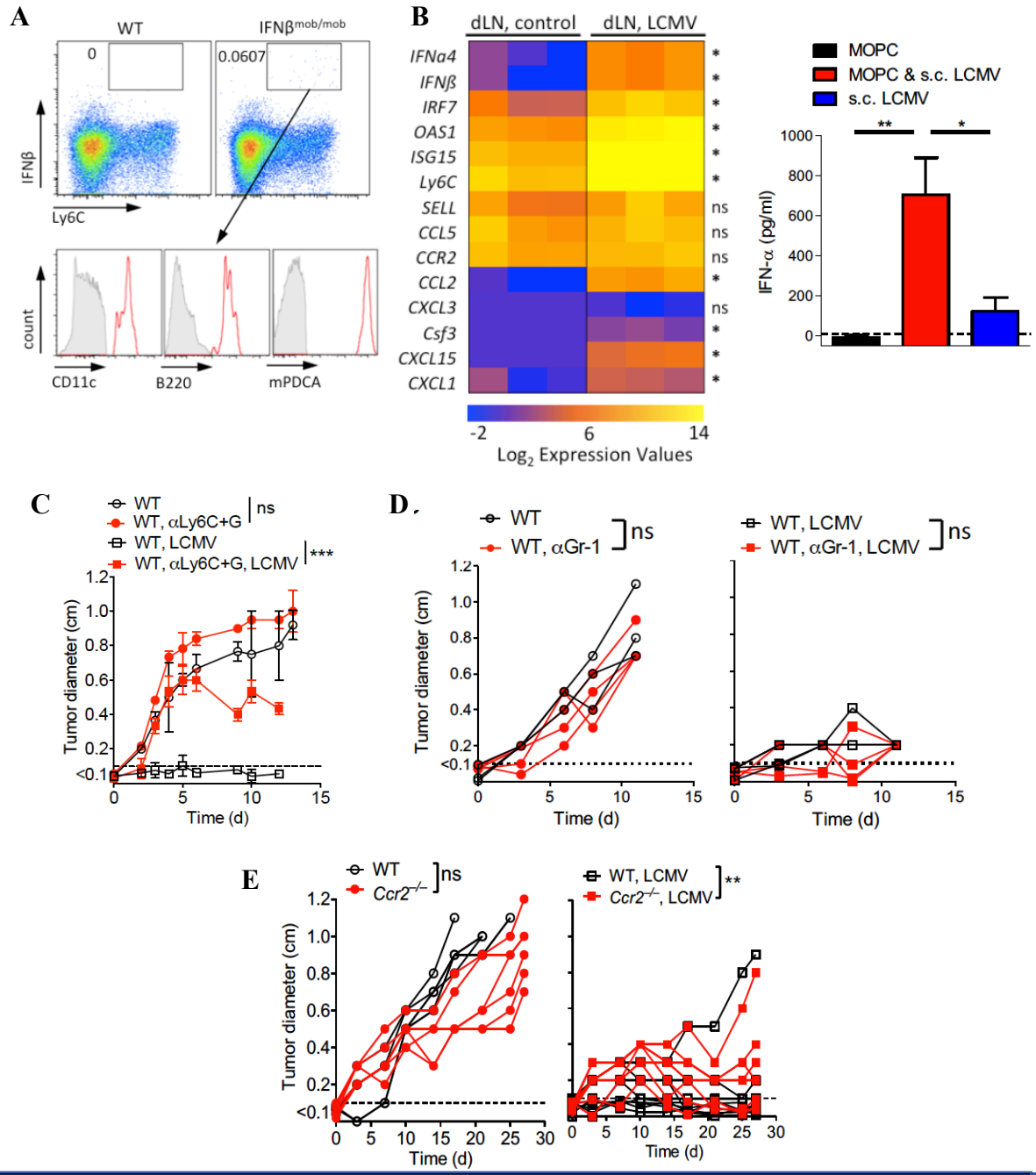


Figure 22: Monocytic Ly6C$^+$ IFN-I drives LCMV mediated tumor regression

A: Representative FACS blots from draining lymph nodes of MOPC-tumor bearing WT and IFNβ$^{mob/mob}$ mice treated with 2x10^4 PFU LCMV peritumorally, analyzed day 2 p.i. (n=4); Grey area-isotype control. B: qRT-PCR analysis from draining lymph nodes (left; n=3) and serum IFN-α levels (right; n=4) from MOPC-tumor bearing mice either treated or untreated with 2x10^4 PFU LCMV peritumorally, analyzed 3 days p.i. C: Tumor measurement of MOPC-tumor bearing WT mice injected with or without α-Ly6G+C antibody (200μg), treated with (n=6) or without (n=6-7) 2x10^4 PFU LCMV peritumorally. D: Tumor measurement of WT mice injected with or without α-Gr-1(Clone1A-D)(500μg), treated without (left0 or with (right) 2x10^4 PFU LCMV peritumorally (n=3-4). E: Tumor measurement of MOPC-tumor bearing WT and *Ccr2*$^{-/-}$ mice, treated without (n=6; left) or with (n=9; right) 2x10^4 PFU LCMV peritumorally.

We investigated the role of adaptive immune cells and also NK-cells in the LCMV dependent tumor suppression. As the adaptive immune system plays a crucial role in the immunity against cancer, we were wondering if these cells play a crucial role in our observed suppression of tumors. We use *Tcrb*$^{-/-}$, *Jh*$^{-/-}$ knockout mice to study the role of T cells and B cells. *Tcrb*$^{-/-}$ mice lack the TCR-α/β chains of their receptors. This leads to the major reduction in total number of cells in thymus which can be seen by reduction of CD4$^+$CD8$^+$ cells by upto 90% than that of WT. The T-cells differentiation and development is blocked at an early stage. *Jh*$^{-/-}$ mice harbor deletion of endogenous J segments of Ig heavy chain locus. This leads to the severely impaired development of B-cell lineage resulting in almost negligible presence of mature B-lymphocytes and complete absence of IgM and IgG. Here, we injected MOPC cells subcutaneously into both *Tcrb*$^{-/-}$ and *Jh*$^{-/-}$ along with WT as control and once tumors were palpable, medium or 2x10^4 PFU LCMV was injected peritumorally. The tumors were monitored for their growth. We found no significant difference in tumor growth in *Tcrb*$^{-/-}$ (Fig. 23A) and *Jh*$^{-/-}$ (Fig. 23B) mice when compared to their respective controls. Next, we analyzed role of NK-cells in LCMV-dependent tumor suppression. For this we used α-NK1.1 depletion antibody which selectively binds and depletes NK cells. We injected 400μl α-NK1.1 and control antibody at day -3 and -1 in WT mice leading to complete depletion of NK-cells (Fig. 23C) and injected with 5x10^5 MOPC cells subcutaneously. The mice were either treated with 2x10^4 PFU LCMV or left un-treated. Tumor growth was observed for the duration of experiment. Results showed no significant

difference in the reduction of tumor between WT and NK-cell depleted mice with LCMV treatment (Fig. 23D). Together here we couldn't find any relevance of T, B or NK-cells in arenavirus mediated tumor suppression.

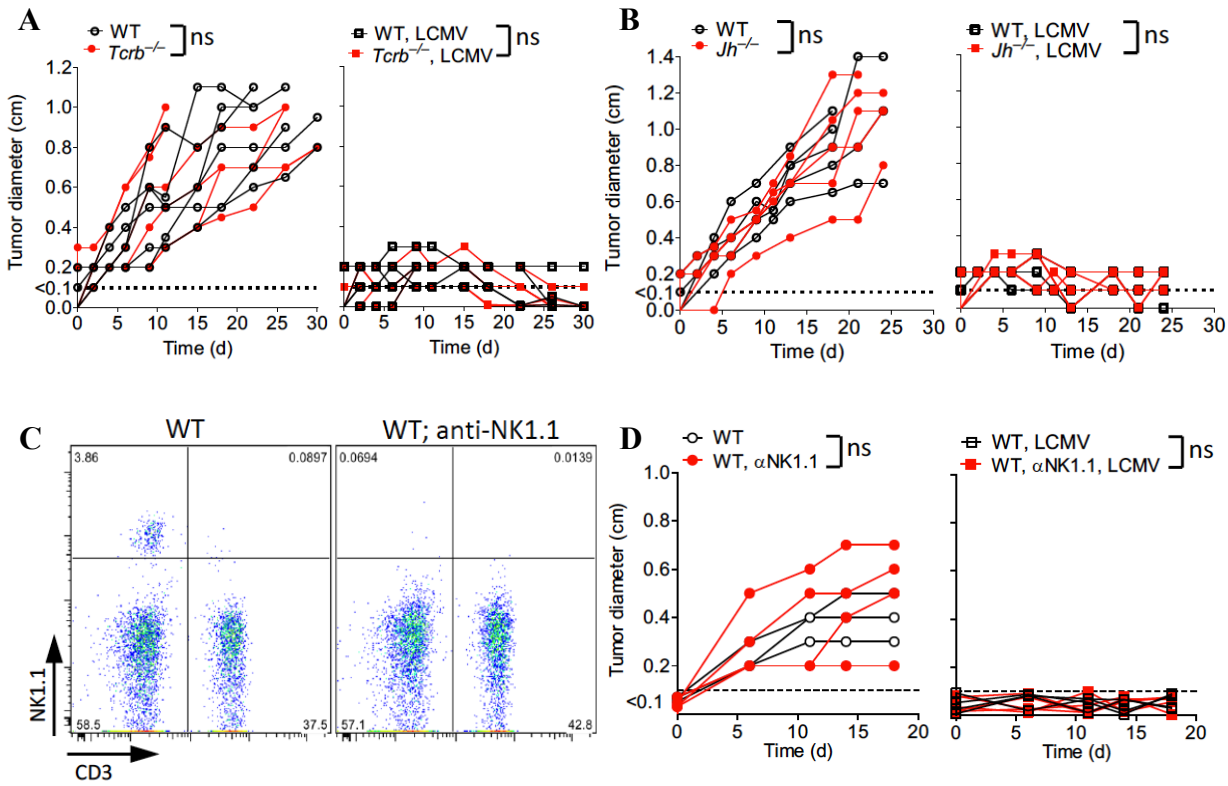


Figure 23: Monocytic Ly6C$^+$ IFN-I drives LCMV mediated tumor regression

A: Tumor measurement of MOPC-tumor bearing WT and *Tcrab*$^{-/-}$ mice (day -3) treated with (left) or without (right) 2x10^4 PFU LCMV peritumorally on day 0 (n=5) B: Tumor measurement of MOPC-tumor bearing WT and *Jh*$^{-/-}$ mice (day -3) treated with (left) or without (right) 2x10^4 PFU LCMV peritumorally on day 0 (n=5) C,D: Representative FACS dot plot from peripheral blood (C) and Tumor measurement (D) of MOPC-tumor bearing WT mice injected with or without α-NK1.1 antibody, treated with or without 2x10^4 PFU LCMV peritumorally (n=4).

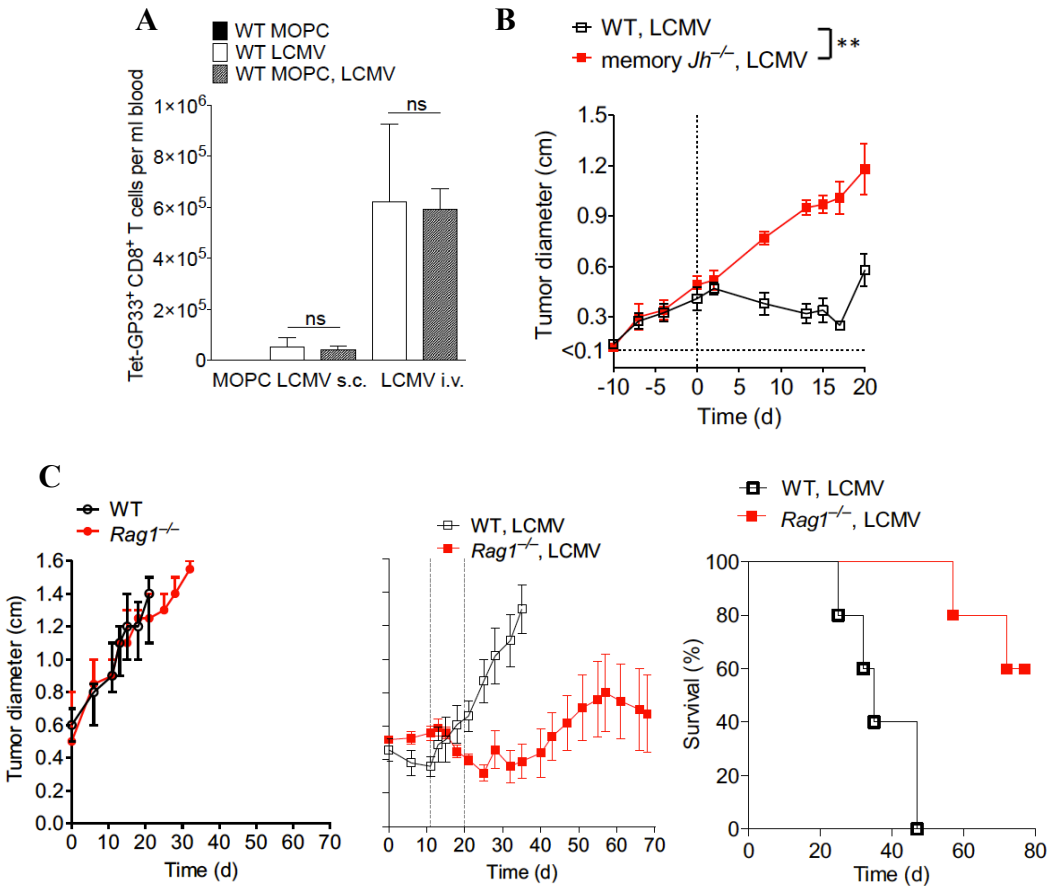
3.2.7 Role of CD8$^+$ T-cells in anti-tumor arenavirus virotherapy.

T cells play a central role in the design of an anti-tumor therapy[176]. Due to their ability to recognize antigen, infiltrate and to effectively remove the target cells, these are one of the preferred cells that are taken into consideration for the therapy. Specifically, CD8$^+$ T-cells are of crucial interest in virotherapy. They aid in the removal of virus from the host infected

tissue which including tumor tissue but virus treatment also leads to effective tumor-specific $CD8^+$ T cells leading to efficient control of tumor growth. As we have shown in the previous results (Fig. 20A), LCMV treatment led to significantly increased $CD8^+$ T-cell infiltration, and we further examined the presence of virus specific $CD8^+$ T-cell in WT mice infected with $2x10^4$ PFU LCMV and MOPC-tumor bearing WT mice infected with same PFU of LCMV. Upon analysis, we found that indeed virus-specific $CD8^+$ T-cell were induced in both the groups but no difference in their numbers were observed (Fig. 24A). T-cell memory plays a crucial role in viral infections as to initiate an effective response on the re-encounter of the antigen. Same T-cell memory might be able to play a negative role in the arenavirus dependent tumor therapies. To explore this hypothesis, we infected *Jh*$^{-/-}$ mice with 200 PFU LCMV i.v for 100 days to induce memory T-cell generation. We then injected WT and memory *Jh*$^{-/-}$ mice with $5x10^5$ MOPC subcutaneously and then injected $2x10^4$ PFU LCMV i.v. once the tumors reached desired size. Results showed that the memory *Jh*$^{-/-}$ mice were unable to effectively reduce tumor growth as compared to WT mice (Fig. 24B). This indicates that the presence of virus-specific $CD8^+$ T-cells have a potentially negative impact on arenavirus mediated tumor therapy. Further we wondered the effect of T cells as such in the arenavirus mediated tumor suppression. To examine this, we used *Rag1*$^{-/-}$ mice which lack mature T-cells due to the lack of V(D)J recombination gene *Rag1*[32]. We injected $5x10^5$ MOPC cells in WT and *Rag1*$^{-/-}$ mice and were infected with $2x10^4$ PFU LCMV intratumorally once the tumor reached advanced size. We observed enhanced tumor suppression and longer host survival in LCMV treated *Rag1*$^{-/-}$ mice than compared to WT mice (Fig. 24C). Next, we checked whether the effect of IFN is correlated with T-cell activity. We depleted IFN signaling in WT and *Rag1*$^{-/-}$ mice by blocking IFN receptors using α-IFNAR antibody, injected 10 days post MOPC cells injection. Upon LCMV treatment we found that the absence of T-cells prolongs the effect of LCMV-mediated tumor regression (Fig. 24D). Together, we show that the presence of virus-specific $CD8^+$ T-cell mounts an early antiviral response resulting in the elimination of LCMV and thus impairing the LCMV-dependent tumor suppression. On the other hand, absence of T-cells leads to the prolonged and more efficient tumor suppression with LCMV treatment.

Further, we went on to analyze the role of LCMV on the anti-tumor activity of tumor-specific $CD8^+$ T-cells. For this we made use of OT-1 mice, which harbor transgenic inserts in TCR region thus recognizing ovalbumin[177]. We transferred $5x10^6$ splenocytes from OT-1 mice into WT mice 10 days after the injection of B16-OVA melanoma tumor cells. The mice were

infected with $2x10^4$ PFU LCMV and observed for 8 days. Mice were then sacrificed on day 8 and tumors were isolated and analyzed for different survival markers for tumor-specific T-cells. We observed enhanced expression of IL-2Rβ (CD122) and IL-7Rα (CD127) on tumor -specific $CD8^+$ T-cells with LCMV treatment as compared to controls (Fig. 24E). Expression of these factors signals the effectiveness and survival of the cytotoxic T-cells. Next, we investigated the effect of LCMV treatment in the tumor growth and survival of host with and without tumor-specific $CD8^+$ T-cells. We injected B16-OVA cells and EL4-OVA lymphoma cells to establish tumors in the mice. Once the tumors were established, the mice received $5x10^6$ splenocytes from OT-1, transferred i.v. 24 hours later, mice were either left un-treated or infected with $2x10^4$ PFU LCMV and observed for tumor growth. We observed significant reduction in tumor growth in presence of tumor-specific $CD8^+$ T-cells along with LCMV treatment than alone with LCMV treatment. The results correlated with the survival of the host in both the cases i.e. B16-OVA melanoma tumors (Fig. 24F) as well as in EL4-OVA lymphoma tumors (Fig. 24G) Compiling all the results, we found out that LCMV dependent tumor suppression is enhanced effectively in the presence of tumor-specific $CD8^+$ T-cells.



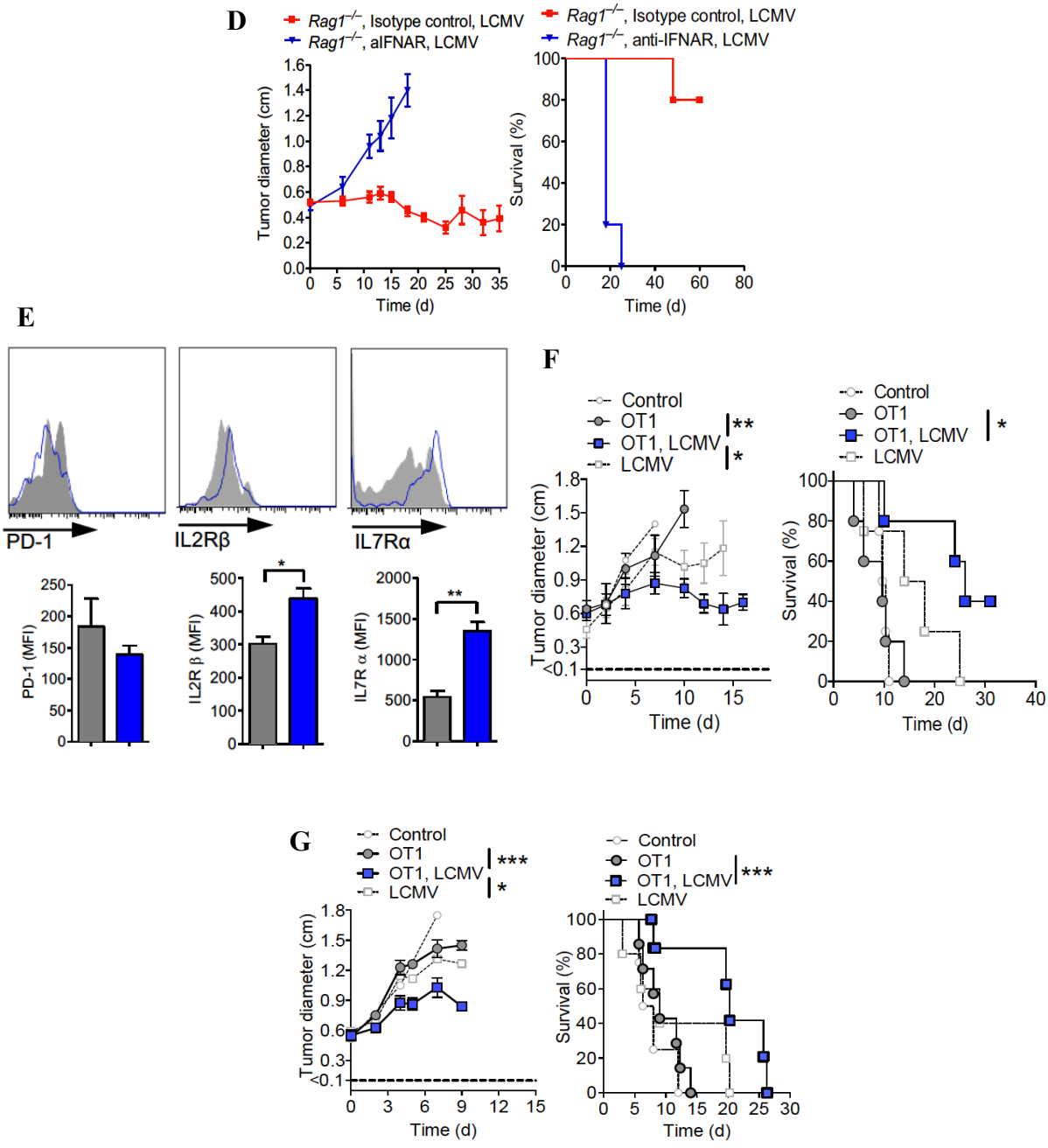


Figure 24: Tumor-specific CD8$^+$ cells enhances LCMV mediated tumor suppression

A: LCMV-specific CD8$^+$ T-cells of MOPC-tumor bearing WT or control mice treated with 2x10^4 PFU LCMV (n=3). B: Tumor measurement of MOPC-tumor bearing WT and memory *Jh*$^{-/-}$ mice treated with 2x10^4 PFU LCMV intravenously on day 0 (n=5). C: Tumor measurement and survival (extreme right) of MOPC-tumor bearing WT and *Rag1*$^{-/-}$ mice (day -10), treated with (middle) or without (extreme left) 2x10^4 PFU LCMV intratumorally on Day 0 (n=5). D: Tumor measurement and survival of MOPC-tumor bearing *Rag1*$^{-/-}$ mice

(day -10) injected with α-IFNAR1 antibody or isotype control, treated with $2x10^4$ PFU LCMV peritumorally (n=5). E: Expression of PD-1, IL2Rβ and IL7Rα on tumor-specific $CD8^+$ T cells (OT1) of B16-OVA-tumor bearing WT mice (day -10) transferred with $5x10^6$ OT1 splenocytes (day -1) and additionally treated with (n=5) or without (n=3) $2x10^4$ PFU LCMV i.t. F: Tumor measurement and survival of B16-OVA-tumor bearing WT mice (day -10) transferred with (n=4) or without (n=5) $5x10^6$ OT-1 splenocytes, treated with or without $2x10^4$ PFU LCMV i.t. G: Tumor measurement and survival of EL4-OVA-tumor bearing WT mice (day -6) transferred with (n=7) or without (n=4-5) $5x10^6$ OT-1 splenocytes, treated with or without $2x10^6$ PFU LCMV i.t.

3.2.8 IFN-I mediated suppression of angiogenesis leads to tumor suppression.

Angiogenesis refers to the formation of new blood vessels which is crucial for the growth and progression of solid tumors. Angiogenesis provides much needed oxygen and essential nutrients for the tumor development[178]. Various factors are secreted by tumor cells in order to continuously remodel the tumor micro-environment to sustain regular growth. Some of these factors include vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP). Inhibition of angiogenesis inhibits the tumor development as the tumor cells are deprived of nutrients and succumb to apoptosis. IFN-α is known for its multiple effects amongst them its anti-angiogenic function[117, 179]. Studies have shown that treatment of IFN-α, have resulted the reduction of tumors *in-vivo* and *in-vitro*[180, 181]. IFN treatment results in the reduced secretion of various angiogenic factors such as FGF and VEGF.

As we have shown that the LCMV dependent tumor regression is dependent on the infiltration of $Ly6C^+$ monocytes and host IFN-I production, we next wondered the mechanism of this anti-tumor activity. We injected $5x10^5$ MOPC cells in to WT mice flank and then injected $2x10^4$ PFU LCMV on day 3 of cells injection. Tumors were harvested on day 6 post-infection for immunofluorescence analysis and also analyzed for the expression of angiogenic regulators via RT-PCR. We found that the expression of almost all the angiogenic regulators have been significantly reduced upon LCMV treatment (Fig. 25A). Next, we analyzed the expression of CD31 also known as PECAM (platelet endothelial cell adhesion molecule-1), which is expressed by the early endothelial cells during angiogenesis. The harvested tumors were subjected to CD31 immunostaining. We found significantly reduced expression of CD31 in LCMV treated tumors as compared to un-treated tumors (Fig. 25B)

Quantification of the same reveals the reduced micro-vessel density (MVD) along with increased vessel-to-vessel distance in LCMV treated tumors (Fig. 25B Lower panel). Reduction in angiogenesis leads to reduced supplement of the soluble oxygen and essential nutrients to the tumor. This further results in the occurrence to increased hypoxic areas and apoptosis. We next harvested MOPC-bearing mice infected with $2x10^4$ PFU LCMV, on day 9 and stained for pimonidazole which is a hypoxia marker[182]. Results showed increased hypoxic areas in the LCMV treated tumors (Fig. 25C) resulting in increased apoptosis as observed by cleaved caspase-3 staining (Data not shown).

Together, we showed that monocytic IFN-I dependent tumor suppression is the result of reduced tumor vasculature due to the anti-angiogenic activities of IFN-I.

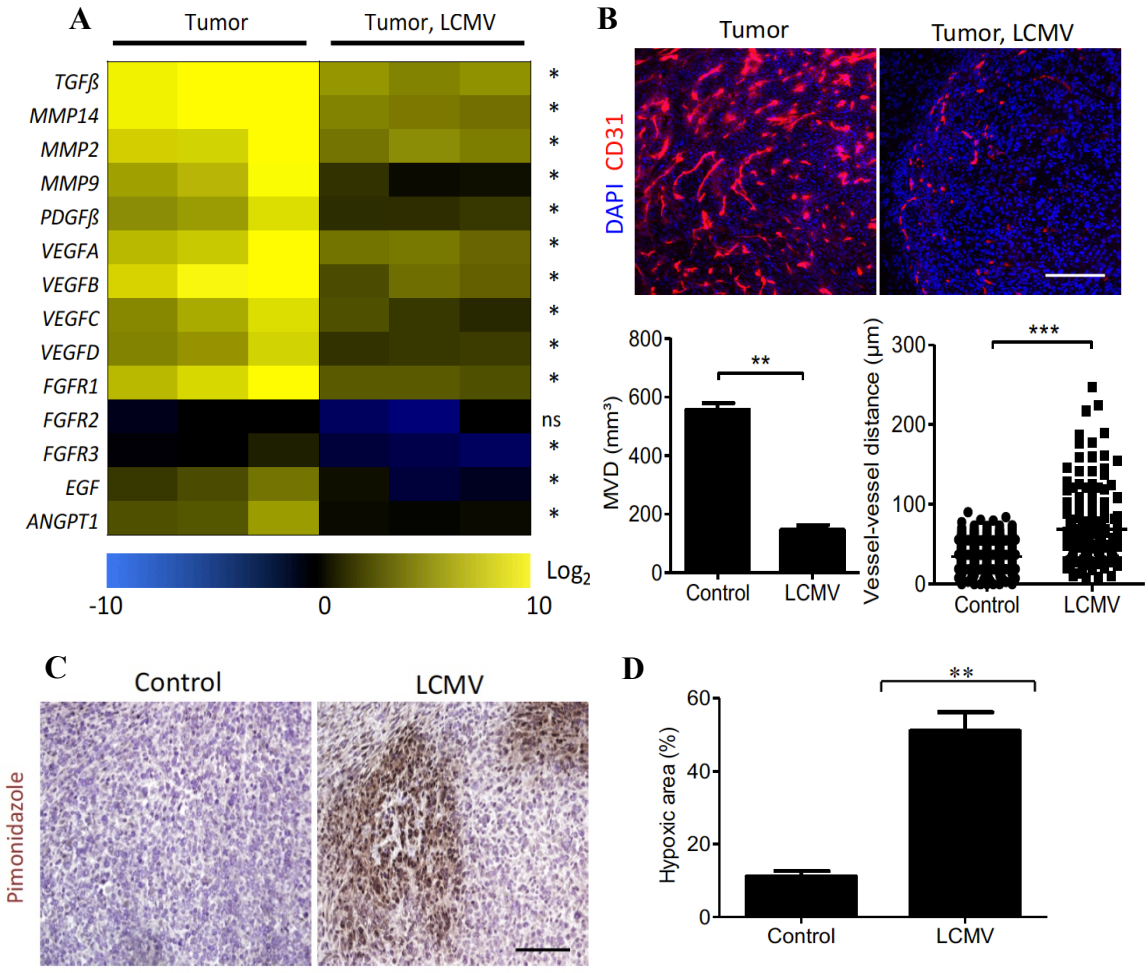


Figure 25: LCMV treatment results in IFN-I mediated suppression of angiogenesis

A: Quantitative mRNA analysis from MOPC-tumor bearing WT or control mice (day -3) treated with $2x10^4$ PFU LCMV peritumorally on day 0, analyzed on day 6 (n=3). B: Immunofluorescence of CD31 (top) and quantification of microvessel density (MVD)

(bottom left) and vessel-vessel distance (bottom right) from MOPC-tumor bearing WT or control mice (day -3) treated with $2x10^4$ PFU LCMV peritumorally on day 0, analyzed on day 6 (n=3). C,D: Immunohistochemistry (day 6; C) and quantification of hypoxic areas (day 9; D) from MOPC-tumor bearing WT or control mice (day -3) treated with or without $2x10^4$ PFU LCMV peritumorally on day 0(n=3).

3.2.9 LCMV mediated anti-tumor effect is more effective than other oncolytic viruses

Oncolytic viruses have been into multiple anti-tumor research and also in pre-clinical stages. These viruses have been long studied and have found their niche in anti-tumor treatment. Oncolytic viruses have been modified and mutated to be better suited as therapeutic agents. However, in comparison, arenaviruses have minimal side-effects due to their cytopathic nature, restrictive tissue tropism and immune cell mediated control. So, here we wondered about the anti-tumor efficacy of the oncolytic viruses as compared to our LCMV virus in tumor suppression and host survival.

For these experiments, we employed two different oncolytic viruses which are either currently in clinical or pre-clinical trials. A chimeric VSV variant (VSV-GP)[183] is currently being developed as an oncolytic therapy for tumors (ww.viraltherapeutics.com), but a similar variant is in Phase-I trial of hepatocellular carcinoma (HCC). Another virus is a recombinant TK-depleted vaccinia virus with GFP/LacZ integration (TK^- VACV-GFP/LacZ, rVACV), which is tested as a double mutant in phase-I study of patients with advanced solid tumors[184]. We established MOPC tumors in WT mice by injecting $5x10^5$ cells subcutaneously and 10 days after injected LCMV, rVSV and rVACV in the indicated dose either intratumorally (Fig. 26A) or intravenously (Fig. 26B). We observed limited tumor suppression with rVSV and rVACV when compared to LCMV treatment in line with better survival of LCMV treated mice. We then also injected 100-fold increased dosage of rVSV and rVACV, but the observed anti-tumor effect of these oncolytic viruses was significantly lower than that of with LCMV treatment (Fig. 26B). Together here we show that the treatment of tumors with non-cytolytic arenaviruses have better efficacy than compared to oncolytic viruses, which also leads to better host survival.

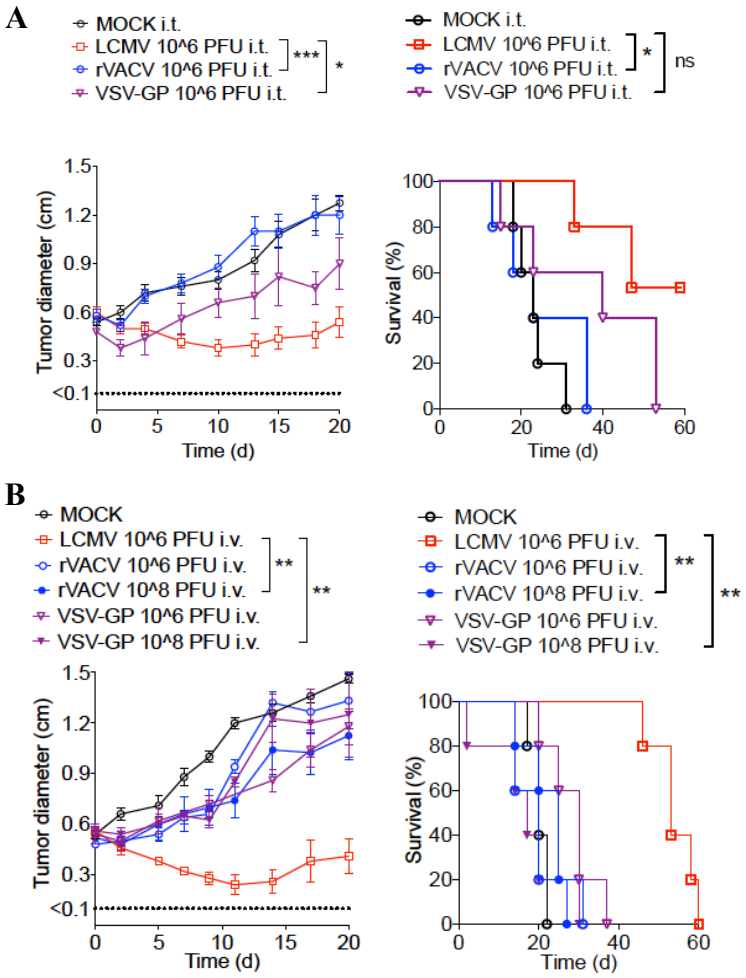


Figure 26: LCMV shows better efficiency and efficacy compared to oncolytic viruses

A: Tumor measurement and survival of MOPC-tumor bearing WT mice (day -10) treated with mentioned PFU of LCMV and oncolytic viruses intratumoraly (n=5). B: Tumor measurement and survival of MOPC-tumor bearing WT mice (day -10) treated with mentioned PFU of LCMV and oncolytic viruses intravenously (n=5).

CHAPTER-4

DISCUSSION

Viruses have been extensively studied and presents an interesting and exciting field to explore. They range from being non-pathogenic to pathogenic causing mild to severe damage to their host. Multiple disease-causing viruses such as HIV, HBV, Ebola, etc. have posed difficult problems through the infection of the human population. Their ability to infect, replicate and integrate into the host genome has been explored and exploited in many situations. They have been used as therapeutic agents for multiple diseases including cancer as modified and inactivated forms.

Study of viral life cycle has given us the understanding of the complex interactions between virus and host. Viruses are the obligate pathogens which are indigent compared to their hosts. They carry only minimal required components for their survival and replication and thus rely on their host for their replication. Understanding of those host factors provides us with the various host cellular contributions towards viral replication. This then in-turn is related to the outcome of the immune response towards the virus. They are of continuous interest to the research community. With the evolution and emergence of new viruses, it has been become quite important to dissect the basic cellular replication of the virus and to understand the cellular dependence of viral replication.

The present study deals with the novel functional identification of a transcription factor known for its role in cell cycle and cellular proliferation. We have identified B-Myb as the major regulator of viral replication which acts via its transcriptional regulation activity of multiple host factors. We have also identified that B-Myb assisted viral replication also acts as tumor suppression in an IFN dependent fashion, thus establishing LCMV as anti-tumor virotherapy.

4.1 Identification of B-Myb role in viral replication

Multiple factors have been identified till now for viral replication. Viruses also modulate and induces expression of many host factors. Due to their rapid replication, viruses require high expression of these host-factors. They make this sure by usually restricting cell to a certain phase or targeting the master switches of cell cycle regulation. Studies have shown that viruses also modulate cell cycle to prioritize their replication[185, 186]. In other words, virus dependence on certain factors might also be related to certain phases of cell cycle and thus have developed multiple strategies to manipulate the cell cycle by associating with cyclins and cyclin-dependent kinases (CDK)[187].

One such master cell cycle regulator is B-Myb which regulates the cell cycle through G1/S phase towards G2/M phase[72]. B-Myb is a widely-accepted regulator of cellular proliferation by being involved in DREAM complex regulating multiple gene expression during cell cycle. B-Myb activity is induced by CyclinA2/CDK2 complex and is phosphorylated to be activated[81].

We here discovered that B-Myb phosphorylation is associated with viral replication which raised further question of this association. With subsequent experiments, we revealed that the phosphorylation of B-Myb occurs during active virus replication (Fig. 6). B-Myb phosphorylation is associated with enhancement of its transactivation properties during cell cycle via Cyclin A/CDK2[85]. Multiple studies have identified various phosphorylation sites, however here we have explored only one phosphorylation site on residue threonine 487 (T487). In our study, T487 is shown to be phosphorylated during active virus replication. The possibility of B-Myb being phosphorylated at other sites owing to its activity during virus replication still needs to be explored as does the functional importance of phosphorylation. It is unclear whether phosphorylation is merely a surrogate marker for B-Myb expression. With this we raised a novel relation of virus replication with B-Myb activity. Next, we wondered the importance of B-Myb for viral replication, as it was specifically phosphorylated in virus infected cells. We found that not only virus replication triggers B-Myb phosphorylation but also transient knockdown of B-Myb abrogates virus replication *in-vitro*. This presented us with the importance of B-Myb for a successful viral replication.

B-Myb is a ubiquitously expressed protein but expression of B-Myb is high in dividing and differentiating cells such as those of the bone marrow. Terminally differentiated cells express little or no B-Myb as the requirement of the same for day-to-day cellular function is very minimal. Therefore, infection of terminally differentiated cells answers the need of B-Myb activation during viral replication. We performed an *in-vivo* transient knockdown of B-Myb in the liver hepatocytes to check whether viral dependent B-Myb activation is cell specific. We made use of VSV model in the absence of macrophages, thus making VSV migrate to liver and infect hepatocytes. Interestingly we found that depletion of B-Myb from hepatocytes severely impaired VSV replication in liver (Fig. 8). The collective results showed that the B-Myb activation is very much dependent on active virus replication in a cell-independent manner. This showed that activation of B-Myb is a crucial requirement by viruses to establish cellular infection and propagate. However, B-Myb is classically activated during cell cycle in a cyclin dependent fashion. The mechanism of viral phosphorylation of B-Myb can be a consequence of viral hijacking of cellular transcriptional

machinery to manipulate the cell cycle. The relation between viral replication and B-Myb phosphorylation still needs to be elucidated.

4.2 Control of viral replication by B-Myb in early virus replication.

Viruses replicates primarily in specific organs/cells in order to ensure their successful propagation. VSV and LCMV as shown from our previous studies[15], replicates in $CD169^+$ macrophages in spleen during initial infection and early replication. This early replication in spleen is enforced by the host and is called enforced virus replication. Replication in $CD169^+$ macrophages ensures viral processing and presentation to adaptive immune system for their activation and thus abrogation of the same results in impaired immune activation and viral control. We studied the role of B-Myb in early virus replication and their respective adaptive immune response using VSV as primary model virus and inducible knockout mice model for B-Myb. We induced knockdown of B-Myb via tamoxifen administered intraperitoneally. Upon infection of B-Myb depleted mice with VSV, we found that the VSV replication is abrogated in spleen (Fig. 10). This was in line with the observed results *in-vitro* and thus revealed the importance of B-Myb in viral replication.

Upon infection, antigen is acquired by APCs for processing. Antigen presentation by APCs during a viral infection is the crucial step towards the successful immunity[188]. Any defect in antigen presentation leads to impaired T and B-cell response[189, 190]. As discussed before, viral replication in $CD169^+$ macrophages is important for antigen presentation and adaptive immune activation. During a viral infection, T-cells play the role of both cytotoxic T-cell portrayed by $CD8^+$ T-cell and helper T-cells portrayed by $CD4^+$ T cells. Both are required for an effective and complete eradication of virus as well as the virus-infected cells. B-cells on the other hand require their differentiation into antibody secreting plasma cells. They are activated by APC and also via Th-1 cells in order to secrete antigen specific antibodies and further neutralize the infection. In case of VSV, B-cells plays a major role along with IFN-I in the control of the infection[191]. With our results of reduced VSV replication, we analyzed the activation of both T and B-cells in control and B-Myb depleted mice with VSV infection. We observed that in B-Myb depleted mice, due to impaired VSV replication $CD8^+$ and $CD4^+$ T-cells antiviral response was significantly reduced as seen by IFNγ production upon re-stimulation with their respective viral peptide. Upon analysis of B-cell response via the measurement of VSV-neutralizing antibodies, we found significantly reduced response as compared to WT (Fig. 13).

T and B-cells undergo rapid clonal expansion post antigen recognition. Studies have shown that c-Myb is required for differentiation of B-cells[192]. Thus, requirement of B-Myb in clonal expansion leading to the observed defect in adaptive immunity was questioned. We showed that VSV-specific $CD4^+$ T-cells and B-cells when adoptively transferred into B-Myb depleted mice and challenged with VSV undergoes normal proliferation than compared to the host $CD4^+$ T-cells and B-cells. This showed that B-Myb depletion has no adverse effect on adaptive immunity during viral infection and is crucial for immune activation by ensuring viral replication in APCs (Fig. 14).

VSV is a cytolytic virus, thus it leads to the lysis of the infected cell. If not controlled by innate and adaptive immune cells, VSV escapes to vital organs of the host, eventually causing death. As discussed VSV, possesses broad tissue tropism and is able to penetrate the blood-brain barrier[106]. In the case of its impaired replication in spleen during early replication, it escapes to CNS and brain causing cell death resulting in paralysis of the host. Our model of B-Byb is a conditional knockdown model and utilized knockdown of the desired (B-Myb) gene using a chemical entity (tamoxifen). Tamoxifen has been used in research for a long time, still its dose for mouse application varies from study to study. It has been shown that tamoxifen has the ability to cross blood-brain barrier in a dose-dependent manner which also depends on the age and weight of the mice[193]. Although even with the higher dose, the level of active tamoxifen metabolites in brain were observed only till day 4-5 and were completely degraded by day 6 after the injection[193]. VSV at the dose of $2x10^6$ PFU is controlled by the WT mice and thus is not found in CNS and brain of the host. However in B-Myb depleted mice, failure of virus control and antiviral immune activation leads to VSV escape to CNS by day 5-6 post tamoxifen injection (Fig. 15). Due to degradation of tamoxifen, B-Myb knockdown in CNS and brain, if it is even expressed there, is potentially ineffective, resulting in replication of VSV. This lead to the lysis of infected cells in CNS and brain resulting in observed paralysis in mice starting from hind legs and then spreading to the fore limbs.

We showed the importance of B-Myb dependent early VSV replication in the activation of immune system leading to effective control of the virus and survival of the host.

4.3 Immune signature of cell is independent of B-Myb

Interferon is a major cytokine produced during an antiviral response and is released by most of the cells and has a broad role in varying from direct antiviral to immune

modulatory. Lack of IFN response or the disruption of IFN signaling pathways leads to uncontrolled virus replication and host death[129, 194]. Many factors are known to contribute towards the secretion of IFN in an infection, which further ensures controlled early viral replication in order to mount an effective immune response. We have shown previously[15] that IFN-I is involved in the enforced virus replication via *usp18* ensuring immune activation. In the current study, we also observe reduced interferon production in B-Myb deficient mice on infection. This can be associated with the reduced viral replication in the spleen. Although as we discussed earlier the functional broad range of B-Myb, we wondered if B-Myb depletion have any adverse effect on immune signature of the cell. To ensure this, we checked with multiple immune stimulants the activation of signaling molecules involved in an antiviral immune signaling (Fig. 11, 12). We found that the immune signature remains intact with B-Myb depletion. Here we show that despite B-Myb is a major transcription role, it doesn't regulate the immune pathways activation and response. Thus, here we identify IFN-independent role of B-Myb in early virus replication.

4.4 Global dependence of viral replication on B-Myb

We have shown that VSV replication is dependent on the activation and expression of B-Myb. Which is also crucial for the host to mount an efficient antiviral immune response. VSV in a ssRNA virus and their replication cycle is completely different from that of DNA viruses. RNA viruses consist of single or double strand of RNA and thus require RNA polymerase called RNA-replicase and thus bypass the need for having a DNA copy, whereas DNA viruses needs the transcription of the DNA genome into mRNA in order to synthesize proteins required for virion assembly. Thus, both kinds of viruses opt for different host factors and rely on different host machinery for their complete replication. With B-Myb being a major transcription factor, its requirement by other RNA and DNA viruses was explored.

We made use of two murine and two human virus models. In the case of murine viruses, we used another RNA virus LCMV and a DNA virus MCMV which have been shown to replicate in spleen during their early replication[147]. In line with our results of VSV replication, we observed abrogated early LCMV replication in $CD169^+$ macrophages as well as reduced MCMV replication in marginal zone macrophages and $CD11b^+$ cells in B-Myb depleted mice. With their reduced early replication, we investigated the adaptive immune response against both LCMV and MCMV in B-Myb deficient mice and significant reduction

in the $CD8^+$ T-cell response in both the viruses (Fig. 16). These results validate that the B-Myb dependent virus replication in both RNA and DNA viruses. Next, we investigated replication of human viruses HSV and HIV in B-myb knockdown cells. Interestingly we found similar results as that of murine viruses. The viral titers were significantly reduced in both HSV and HIV.

These results established the crucial importance of B-Myb in a broad range of virus replication.

4.5 B-Myb assists viral replication via multiple host factors.

Host factors are the important tools hijacked by the viruses. As discussed before these vary in function from the entry of virus all the way to the budding of virions. As these are provided by the host, they need to be transcribed by transcription factors. We hypothesized that B-Myb regulated some of these factors. B-Myb, a member of a transcription factor family and its association with the cell cycle G1/S phase signifies its broad transcriptional importance. In the *Myb* family, the binding sites of all three family member is conserved and thus specific target gens for B-Myb is still needs to be explored extensively. To check our hypothesis, we quantified the transcriptional expression of multiple known host factors for VSV and LCMV in B-Myb depleted mice and found that indeed their expression was significantly reduced. We found that mostly expression of genes required for translational activity was significantly reduced. These results postulate the crucial transcriptional role of B-Myb for genes involved in translation. However, B-Myb is not known as the transcriptional activator of these genes and thus raises the question whether the observed host factor expression is a direct or indirect effect of B-Myb depletion. This requires more in-depth study of B-Myb relation with host factors by transcription factor binding analysis and functional analysis amongst others.

4.6 B-Myb facilitates LCMV replication in tumors

We have shown B-Myb is an important regulator of virus replication and is involved in broad range of viruses. It is known that B-Myb is also expressed at high levels in multiple tumors, where it helps in tumor progression and proliferation.

Viruses have been researched for their use in anti-tumor therapies for quite long now. Various viral models have been used, but the most accepted are the oncolytic models which have shown promising results in pre-clinical and clinical trials. Initially oncolytic viruses

have been sought as tumor lysing therapeutics but with the research they have shown to induce a systemic anti-tumor immune response[195]. These oncolytic viruses have been modified to adhere to the host requirement but still needs host factors for their replication[158]. These host factors as discussed earlier have important role in metabolism, cellular proliferation etc. In tumors, metabolic processes along with cell proliferation processes are up-regulated to ensure the energy requirements of the cell. Thus, this presents viruses with the abundance of cellular resources for their replication[196, 197].

With our findings of B-Myb dependent viral replication, we explored the ability of cytopathic arenavirus to replicate in tumors. We observed that indeed our tumor models expressed high amount of phosphorylated B-Myb. Upon intravenous or peritumoral injection of LCMV, we found that LCMV preferentially replicated in tumor cells (Fig. 18) which leads to tumor suppression (Fig. 19) in multiple engrafted and spontaneous tumor models. Further analysis of tumor cells showed abundant expression of host-factors required for LCMV replication[154] (Fig. A2). These host-factors are important for viral RNA transcriptional and translational process along with viral assembly and budding. These tumor cells thus act as treasure reserves for viruses.

4.7 LCMV induces strong immune infiltration and IFN-I response

Effective immune activation is known to be the pre-requisite for effective anti-tumor virotherapy[198]. Expression of tumor antigens known as tumor-associated antigens (TAA) helps in the recognition of tumor cells by the immune system. However, tumor in-turn devises multiple ways to escape from immune recognition like by reducing expression of co-stimulatory molecules, delivering inhibitory signals to immune cells, etc[199, 200]. Once the TAA are presented and recognized, it leads to infiltration of immune cells known as tumor infiltrating leukocytes (TIL). Enhancement of anti-tumor immunity can be divided into two aspects; 1. Increased efficient presentation of TAAs and production of local cytokines to attract more TILs, 2. Use of foreign antigen to attract TIL to tumor and enhance anti-tumor mediated immunity[201]. Thus, use of foreign antigen to attract TILs into tumors results in release of cytokines presents an effective and efficient strategy for anti-tumor therapy.

Arenaviruses are known to be efficient inducer of both humoral and cell-mediated immune response[202]. B-Myb dependent LCMV replication in tumors showed effective replication which further lead to increased immune infiltration in the tumors. LCMV treatment lead to significantly more T-cell and monocyte infiltration in tumors and well as

increased monocyte accumulation in draining lymph nodes (Fig. 20). This triggered an efficient interaction of TILs in the tumor microenvironment resulting in efficient recognition of TAAs. LCMV has an additional advantage over oncolytic viruses as it persists in tumor cells until an antiviral immune response is successfully established within the tumor.

Interferons are pleotropic cytokines which mediate anti-tumor effect in a wide array of ways[203]. Among major types of IFN present, Type-I IFN proficiently secreted by most of the cells including tumor cells. IFN-I types IFN-α and IFN-β have shown to have important function in cellular homeostasis and mediation of anti-tumor activity[204, 205]. IFN-mediate tumor immunotherapies have shown promising results, although it's hard to predict the patient sensitivity towards IFN-I. Multiple reports have shown that IFN-I shows direct effect on tumor cells, when used alone or in combination with chemotherapy leads to better patient survival and have immunomodulatory effects[206, 207]. Use of viruses thus presents us a way to initiate and activate IFN-I response within the tumor cells, thus activating various anti-tumor signaling pathways[208]. We observed that indeed LCMV dependent tumor regression is IFN-I mediated (Fig. 21).

Oncolytic viruses possess some limitations as they are highly IFN-I responsive and thus so presence of IFN activity limits their replication in the tumor cells[209]. On the other hand, arenaviruses (LCMV) replication have shown not to be solely on IFN-I[210] leading to its continuous replication in the tumor as observed. We show that LCMV replicates in the tumor for a longer extent leading to a prolong immune response and presence of immune cells with in the tumor. We observed that LCMV treatment leads to a high infiltration of monocytes which are one of the major inducer of IFN-I. Further investigation led to the identification of $Ly6C^+$ monocytes which are basically responsible for the suppressive effect of LCMV on tumors via IFN-I dependent manner (Fig. 22). As discussed, IFN-I plays its anti-tumor role in variety of ways. Here we showed that LCMV infection indeed led to immune infiltration and resulted in high IFN response.

4.8 LCMV induced IFN-I leads to reduced angiogenesis and apoptosis of tumor cells

Angiogenesis plays a critical role in the tumor growth and progression. This leads to the formation of new blood vessels for oxygen and nutrients delivery. Initiation of angiogenesis is done by various pro-angiogenic factors secreted by tumor cells, such as EGF, VEGF, FGF, etc. This process ensures tumor cell proliferation and continuous formation of

new vessels leading to metastasis[178]. Inhibition of angiogenesis specifically within the tumor is one anti-tumor strategy. IFN have known to have anti-angiogenesis function, thereby inhibiting/suppressing formation of new vessels and thus limiting tumor growth[179]. We showed here that LCMV induces a strong IFN-I upon which leads to suppression of tumor. Further investigation revealed that the tumor suppression observed was related to the down-regulation of many pro-angiogenetic factors. Reduction of these factors results in the reduced MVD because of lack of formation of new vessels and thus increasing the vessel-vessel distance with-in the tumor with LCMV treatment. These findings were further verified with the reduction in CD31 expression in tumor which is majorly expressed by newly formed epithelial cells. Reduction if vessels in tumor microenvironment leads to the deficiency of oxygen and nutrients resulting in cell death which was observed in LCMV treated tumors (Fig. 25). Thus, here we show that IFN-I induced by LCMV treatment acts as anti-tumor by suppressing the angiogenetic factors and thus restricting tumor growth.

4.9 Role of T-cells in LCMV-mediated tumor therapy.

T-cells immunity holds one of the important role in the anti-tumor immunity[176]. T-cell infiltrates tumor microenvironment and acts by recognition of TAAs. Infiltration of T-cells leads to CTL mediated killing of tumor cells along with Th mediated activation of B-cells. Although tumor cells usually escape the T-cell mediated killing by down-modulation of MHC-I and MHC-II along with TAAs[211]. One alternative to improve adaptive immune/T-cell mediated anti-tumor activity is to selectively activates T-cells or to enhance the recruitment of T-cells in the tumor via chemical or biological way. Viruses have been used to modulate the recruitment of T-cells into tumors and also to enhance their activation[212]. Viral infection leads to the identification of viral antigen by immune system which then results in an anti-viral response with-in the tumor. Killing of infected tumor cells results in antigen presentation of viral particles along with TAAs leading to anti-tumor T-cell activity. We show here that LCMV infection indeed results in infiltration of various immune cells including both $CD8^+$ and $CD4^+$ T-cells. Infiltration of $CD8^+$ T-cells tends to be a worthy marker for the T-cell mediates anti-tumor effect. Although our further investigation proved that LCMV-dependent tumor suppression was largely independent of T-cells as analyzed by various functional T-cell deficient mice models (*Tcrb*$^{-/-}$ and *Rag*$^{-/-}$)$^-$ and was even worse in presence of virus-specific T-cells as it controlled the virus replication before it can assist in anti-tumor immunity (Fig. 23, 24). To recognize the role of tumor specific T-cells, we used

OVA-specific T-cells which showed enhanced anti-tumoral effect with LCMV treatment (Fig. 24). These results showed the ambiguous role of T-cells in the LCMV-mediated anti-tumor activity and that it is majorly based on recruitment IFN-I producing $Ly6C^+$ cells. However, we show that combination of tumor-specific T-cells and LCMV therapy have better anti-tumor effect and thus LCMV can be combined with existing anti-tumor therapies for improved results.

4.10 LCMV virotherapy presents better immune modulation and anti-tumor function

Oncolytic viruses have been studied and used in the anti-tumoral activities. This is majorly based on the activity of viruses to infect and lyse the cell. Use and development of oncolytic viruses as anti-cancer agent is recognized throughout the world. This can be seen by the approval of an oncolytic herpesvirus (Talimogene Iaherparepvec, Amgen) for melanoma patients[213]. Tumor regression by oncolytic viruses is an attribute by their lysis ability along with the induction of inflammatory response[214]. Although many studies are being performed using oncolytic viruses, these still present slight advantage over tumor immunity and need to be modified to a greater extent to prevent non-tumor host-cell damage. We used non-oncolytic arenavirus LCMV as anti-tumor viral therapy. Arenaviruses are cytopathic in nature and induce immune response which is majorly regulated by cell-mediated immunity. In this study, we observed that LCMV treatment leads to tumor regression over a prolong period of time. This observed tumor regression was observed in many tumor models we investigated and was based on humoral and cell-mediated immunity. Upon comparison with other pre-clinical/clinical oncolytic viruses; rVSV, rVACV, we found that LCMV has better efficiency and efficacy in suppression of tumor. The reduced effect of oncolytic viruses can be explained by their need of defects in IFN-I pathway for replication in tumors[215]. Viral treatment in oncolytic viruses also depends on the dose and site of administration. Upon increasing the dose of compared oncolytic viruses to over 100-fold, they still showed significantly lower tumor regression and host survival compared to LCMV (Fig. 26). We show that LCMV is therapeutically much more potent and efficient anti-tumor virotherapy than rVSV and rVACV. However, the application of LCMV as a therapeutic intervention in the clinic is still in the early preclinical stages. We have used the laboratory strain of LCMV (LCMV-WE) and its impact on human beings can still be quite harmful. Arenaviruses have been related to hemorrhagic fevers and thus LCMV needs to be carefully

manipulated in order to have no or minimal effect on host health, before being used in clinical scenario.

In this study, we have identified B-Myb as one of the major host-factor which is activated (phosphorylated) during active virus replication and that it is required by majority of DNA and RNA viruses. B-Myb expression is crucial for viral replication as virus shows severely reduced replication in absence of B-Myb. B-Myb dependent viral replication is a result of B-Myb dependent expression of multiple host factors responsible for viral replication. We show that B-Myb dependent early virus replication is important for the host to mount an efficient innate and adaptive immune response. In the further study, we have identified B-Myb assisted LCMV replication as an efficient and effective anti-tumor treatment. We show that LCMV treatment results in immune infiltration with in the tumor. We also showed that arenavirus treatment results in better tumor control and host survival than oncolytic viruses and the observed tumor regression is largely dependent on IFN-I producing $Ly6C^{+}$ produced.

CHAPTER-5

BIBLIOGRAPHY

1. Clemens MJ, Elia A. The double-stranded RNA-dependent protein kinase PKR: structure and function. *J Interferon Cytokine Res* 1997; **17**(9)**:** 503-524. doi: 10.1089/jir.1997.17.503

2. Kumar M, Carmichael GG. Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol Mol Biol Rev* 1998; **62**(4)**:** 1415-1434.

3. Bertin J, Nir WJ, Fischer CM, Tayber OV, Errada PR, Grant JR *et al.* Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J Biol Chem* 1999; **274**(19)**:** 12955-12958.

4. Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S *et al.* Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem* 1999; **274**(21)**:** 14560-14567.

5. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 2001; **276**(7)**:** 4812-4818. doi: 10.1074/jbc.M008072200

6. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; **17**(1)**:** 1-14. doi: 10.1093/intimm/dxh186

7. Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu Rev Immunol* 2005; **23:** 307-336. doi: 10.1146/annurev.immunol.23.021704.115843

8. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001; **2**(8)**:** 675-680. doi: 10.1038/90609

9. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003; **21:** 335-376. doi: 10.1146/annurev.immunol.21.120601.141126

10. Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev* 2009; **227**(1)**:** 75-86. doi: 10.1111/j.1600-065X.2008.00737.x

11. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H *et al.* Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; **301**(5633)**:** 640-643. doi: 10.1126/science.1087262

12. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 2003; **4**(2)**:** 161-167. doi: 10.1038/ni886

13. Beutler B. Innate immunity: an overview. *Mol Immunol* 2004; **40**(12)**:** 845-859.

14. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; **8**(12)**:** 958-969. doi: 10.1038/nri2448

15. Honke N, Shaabani N, Cadeddu G, Sorg UR, Zhang DE, Trilling M *et al.* Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus. *Nat Immunol* 2011; **13**(1)**:** 51-57. doi: 10.1038/ni.2169

16. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 1973; **137**(5)**:** 1142-1162.

17. Nussenzweig MC, Steinman RM, Gutchinov B, Cohn ZA. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med* 1980; **152**(4)**:** 1070-1084.

18. Steinman RM, Kaplan G, Witmer MD, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J Exp Med* 1979; **149**(1)**:** 1-16.

19. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 2003; **19**(1)**:** 59-70.

20. Mildner A, Jung S. Development and function of dendritic cell subsets. *Immunity* 2014.

21. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol* 2011; **29:** 163-183. doi: 10.1146/annurev-immunol-031210-101345

22. Nakano H, Yanagita M, Gunn MD. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* 2001; **194**(8)**:** 1171-1178.

23. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999; **284**(5421)**:** 1835-1837.

24. Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W *et al.* Conventional and monocyte-derived CD11b(+) dendritic cells

initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013; **38**(2)**:** 322-335. doi: 10.1016/j.immuni.2012.10.016

25. Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ *et al.* CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol* 2012; **42**(12)**:** 3150-3166. doi: 10.1002/eji.201242847

26. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; **11**(8)**:** 519-531. doi: 10.1038/nri3024

27. Karasuyama H, Obata K, Wada T, Tsujimura Y, Mukai K. Newly appreciated roles for basophils in allergy and protective immunity. *Allergy* 2011; **66**(9)**:** 1133-1141. doi: 10.1111/j.1398-9995.2011.02613.x

28. Geering B, Stoeckle C, Conus S, Simon HU. Living and dying for inflammation: neutrophils, eosinophils, basophils. *Trends Immunol* 2013; **34**(8)**:** 398-409. doi: 10.1016/j.it.2013.04.002

29. Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E *et al.* Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat Med* 2008; **14**(9)**:** 949-953. doi: 10.1038/nm.1855

30. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004; **303**(5663)**:** 1532-1535. doi: 10.1126/science.1092385

31. Rose S, Misharin A, Perlman H. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry A* 2012; **81**(4)**:** 343-350. doi: 10.1002/cyto.a.22012

32. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 1992; **68**(5)**:** 869-877.

33. Shinkai Y, Koyasu S, Nakayama K, Murphy KM, Loh DY, Reinherz EL *et al.* Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science* 1993; **259**(5096)**:** 822-825.

34. Falk I, Potocnik AJ, Barthlott T, Levelt CN, Eichmann K. Immature T cells in peripheral lymphoid organs of recombinase-activating gene-1/-2-deficient mice. Thymus dependence and responsiveness to anti-CD3 epsilon antibody. *J Immunol* 1996; **156**(4)**:** 1362-1368.

35. Germain RN. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* 2002; **2**(5)**:** 309-322. doi: 10.1038/nri798

36. Warrington R, Watson W, Kim HL, Antonetti FR. An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol* 2011; **7 Suppl 1:** S1. doi: 10.1186/1710-1492-7-S1-S1

37. Cantor H, Boyse EA. Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J Exp Med* 1975; **141**(6)**:** 1376-1389.

38. Golstein P, Wigzell H, Blomgren H, Svedmyr EA. Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. *J Exp Med* 1972; **135**(4)**:** 890-906.

39. Zinkernagel RM, Doherty PC. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* 1974; **251**(5475)**:** 547-548.

40. Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. *Immunity* 2011; **35**(2)**:** 161-168. doi: 10.1016/j.immuni.2011.07.010

41. Gerlach C, van Heijst JW, Swart E, Sie D, Armstrong N, Kerkhoven RM *et al.* One naive T cell, multiple fates in CD8+ T cell differentiation. *J Exp Med* 2010; **207**(6)**:** 1235-1246. doi: 10.1084/jem.20091175

42. Stemberger C, Huster KM, Koffler M, Anderl F, Schiemann M, Wagner H *et al.* A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity* 2007; **27**(6)**:** 985-997. doi: 10.1016/j.immuni.2007.10.012

43. Michalek RD, Rathmell JC. The metabolic life and times of a T-cell. *Immunol Rev* 2010; **236:** 190-202. doi: 10.1111/j.1600-065X.2010.00911.x

44. Carr EL, Kelman A, Wu GS, Gopaul R, Senkevitch E, Aghvanyan A *et al.* Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. *J Immunol* 2010; **185**(2)**:** 1037-1044. doi: 10.4049/jimmunol.0903586

45. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* 2010; **33**(2)**:** 153-165. doi: 10.1016/j.immuni.2010.08.004

46. Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E *et al.* Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 2009; **31**(2)**:** 296-308. doi: 10.1016/j.immuni.2009.05.014

47. Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J, Ahmed R. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 1999; **286**(5443)**:** 1377-1381.

48. Becker TC, Wherry EJ, Boone D, Murali-Krishna K, Antia R, Ma A *et al.* Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med* 2002; **195**(12)**:** 1541-1548.

49. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* 2015; **74**(1)**:** 5-17. doi: 10.1016/j.cyto.2014.09.011

50. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H *et al.* Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* 2009; **10**(11)**:** 1178-1184. doi: 10.1038/ni.1791

51. Murphy K, Travers P, Walport M, Janeway C. *Janeway's immunobiology*, 8th edn Garland Science: New York, 2012.

52. Cooper MD, Peterson RD, Good RA. Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. *Nature* 1965; **205:** 143-146.

53. *Drugs targeting B-cells in autoimmune diseases*, Springer: New York, 2013.

54. Hardy RR, Kincade PW, Dorshkind K. The protean nature of cells in the B lymphocyte lineage. *Immunity* 2007; **26**(6)**:** 703-714. doi: 10.1016/j.immuni.2007.05.013

55. Brack C, Hirama M, Lenhard-Schuller R, Tonegawa S. A complete immunoglobulin gene is created by somatic recombination. *Cell* 1978; **15**(1)**:** 1-14.

56. Komori T, Okada A, Stewart V, Alt FW. Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. *Science* 1993; **261**(5125)**:** 1171-1175.

57. Milne CD, Paige CJ. IL-7: a key regulator of B lymphopoiesis. *Semin Immunol* 2006; **18**(1)**:** 20-30. doi: 10.1016/j.smim.2005.10.003

58. Nutt SL, Kee BL. The transcriptional regulation of B cell lineage commitment. *Immunity* 2007; **26**(6)**:** 715-725. doi: 10.1016/j.immuni.2007.05.010

59. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 1997; **90**(6)**:** 1073-1083.

60. Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. *Nature* 1991; **354**(6352)**:** 389-392. doi: 10.1038/354389a0

61. Oh IH, Reddy EP. The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* 1999; **18**(19)**:** 3017-3033. doi: 10.1038/sj.onc.1202839

62. Nomura N, Takahashi M, Matsui M, Ishii S, Date T, Sasamoto S *et al.* Isolation of human cDNA clones of myb-related genes, A-myb and B-myb. *Nucleic Acids Res* 1988; **16**(23)**:** 11075-11089.

63. Roussel M, Saule S, Lagrou C, Rommens C, Beug H, Graf T *et al.* Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation. *Nature* 1979; **281**(5731)**:** 452-455.

64. Rosinski JA, Atchley WR. Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. *J Mol Evol* 1998; **46**(1)**:** 74-83.

65. Thorner AR, Hoadley KA, Parker JS, Winkel S, Millikan RC, Perou CM. In vitro and in vivo analysis of B-Myb in basal-like breast cancer. *Oncogene* 2009; **28**(5)**:** 742-751. doi: 10.1038/onc.2008.430

66. Introna M, Luchetti M, Castellano M, Arsura M, Golay J. The myb oncogene family of transcription factors: potent regulators of hematopoietic cell proliferation and differentiation. *Semin Cancer Biol* 1994; **5**(2)**:** 113-124.

67. Nakagoshi H, Takemoto Y, Ishii S. Functional domains of the human B-myb gene product. *J Biol Chem* 1993; **268**(19)**:** 14161-14167.

68. Tanikawa J, Yasukawa T, Enari M, Ogata K, Nishimura Y, Ishii S *et al.* Recognition of specific DNA sequences by the c-myb protooncogene product: role of three repeat units in the DNA-binding domain. *Proc Natl Acad Sci U S A* 1993; **90**(20)**:** 9320-9324.

69. Sala A, Watson R. B-Myb protein in cellular proliferation, transcription control, and cancer: latest developments. *J Cell Physiol* 1999; **179**(3)**:** 245-250. doi: 10.1002/(SICI)1097-4652(199906)179:3<245::AID-JCP1>3.0.CO;2-H

70. Martinez I, Dimaio D. B-Myb, cancer, senescence, and microRNAs. *Cancer Res* 2011; **71**(16)**:** 5370-5373. doi: 10.1158/0008-5472.CAN-11-1044

71. Lam EW, Robinson C, Watson RJ. Characterization and cell cycle-regulated expression of mouse B-myb. *Oncogene* 1992; **7**(9)**:** 1885-1890.

72. Hanada N, Lo HW, Day CP, Pan Y, Nakajima Y, Hung MC. Co-regulation of B-Myb expression by E2F1 and EGF receptor. *Mol Carcinog* 2006; **45**(1)**:** 10-17. doi: 10.1002/mc.20147

73. Lefebvre C, Rajbhandari P, Alvarez MJ, Bandaru P, Lim WK, Sato M *et al.* A human B-cell interactome identifies MYB and FOXM1 as master regulators of proliferation in germinal centers. *Mol Syst Biol* 2010; **6:** 377. doi: 10.1038/msb.2010.31

74. Down CF, Millour J, Lam EW, Watson RJ. Binding of FoxM1 to G2/M gene promoters is dependent upon B-Myb. *Biochim Biophys Acta* 2012; **1819**(8)**:** 855-862. doi: 10.1016/j.bbagrm.2012.03.008

75. Ness SA. Myb protein specificity: evidence of a context-specific transcription factor code. *Blood Cells Mol Dis* 2003; **31**(2)**:** 192-200.

76. Arsura M, Introna M, Passerini F, Mantovani A, Golay J. B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic cell lines. *Blood* 1992; **79**(10)**:** 2708-2716.

77. Lin D, Fiscella M, O'Connor PM, Jackman J, Chen M, Luo LL *et al.* Constitutive expression of B-myb can bypass p53-induced Waf1/Cip1-mediated G1 arrest. *Proc Natl Acad Sci U S A* 1994; **91**(21)**:** 10079-10083.

78. Sala A, Calabretta B. Regulation of BALB/c 3T3 fibroblast proliferation by B-myb is accompanied by selective activation of cdc2 and cyclin D1 expression. *Proc Natl Acad Sci U S A* 1992; **89**(21)**:** 10415-10419.

79. Charrasse S, Carena I, Brondani V, Klempnauer KH, Ferrari S. Degradation of B-Myb by ubiquitin-mediated proteolysis: involvement of the Cdc34-SCF(p45Skp2) pathway. *Oncogene* 2000; **19**(26)**:** 2986-2995. doi: 10.1038/sj.onc.1203618

80. Tanaka Y, Patestos NP, Maekawa T, Ishii S. B-myb is required for inner cell mass formation at an early stage of development. *J Biol Chem* 1999; **274**(40)**:** 28067-28070.

81. Robinson C, Light Y, Groves R, Mann D, Marias R, Watson R. Cell-cycle regulation of B-Myb protein expression: specific phosphorylation during the S phase of the cell cycle. *Oncogene* 1996; **12**(9)**:** 1855-1864.

82. Sala A, Kundu M, Casella I, Engelhard A, Calabretta B, Grasso L *et al.* Activation of human B-MYB by cyclins. *Proc Natl Acad Sci U S A* 1997; **94**(2)**:** 532-536.

83. Ziebold U, Bartsch O, Marais R, Ferrari S, Klempnauer KH. Phosphorylation and activation of B-Myb by cyclin A-Cdk2. *Curr Biol* 1997; **7**(4)**:** 253-260.

84. Bartsch O, Horstmann S, Toprak K, Klempnauer KH, Ferrari S. Identification of cyclin A/Cdk2 phosphorylation sites in B-Myb. *Eur J Biochem* 1999; **260**(2)**:** 384-391.

85. Saville MK, Watson RJ. The cell-cycle regulated transcription factor B-Myb is phosphorylated by cyclin A/Cdk2 at sites that enhance its transactivation properties. *Oncogene* 1998; **17**(21)**:** 2679-2689. doi: 10.1038/sj.onc.1202503

86. Takemoto Y, Tashiro S, Handa H, Ishii S. Multiple nuclear localization signals of the B-myb gene product. *FEBS Lett* 1994; **350**(1)**:** 55-60.

87. Gualdrini F, Corvetta D, Cantilena S, Chayka O, Tanno B, Raschella G *et al.* Addiction of MYCN amplified tumours to B-MYB underscores a reciprocal regulatory loop. *Oncotarget* 2010; **1**(4)**:** 278-288. doi: 10.18632/oncotarget.100808

88. Sadasivam S, DeCaprio JA. The DREAM complex: master coordinator of cell cycle-dependent gene expression. *Nat Rev Cancer* 2013; **13**(8)**:** 585-595. doi: 10.1038/nrc3556

89. Raschella G, Cesi V, Amendola R, Negroni A, Tanno B, Altavista P *et al.* Expression of B-myb in neuroblastoma tumors is a poor prognostic factor independent from MYCN amplification. *Cancer Res* 1999; **59**(14)**:** 3365-3368.

90. Sala A. B-MYB, a transcription factor implicated in regulating cell cycle, apoptosis and cancer. *Eur J Cancer* 2005; **41**(16)**:** 2479-2484. doi: 10.1016/j.ejca.2005.08.004

91. Sala A, Casella I, Bellon T, Calabretta B, Watson RJ, Peschle C. B-myb promotes S phase and is a downstream target of the negative regulator p107 in human cells. *J Biol Chem* 1996; **271**(16)**:** 9363-9367.

92. Knight AS, Notaridou M, Watson RJ. A Lin-9 complex is recruited by B-Myb to activate transcription of G2/M genes in undifferentiated embryonal carcinoma cells. *Oncogene* 2009; **28**(15)**:** 1737-1747. doi: 10.1038/onc.2009.22

93. Cervellera M, Raschella G, Santilli G, Tanno B, Ventura A, Mancini C *et al.* Direct transactivation of the anti-apoptotic gene apolipoprotein J (clusterin) by B-MYB. *J Biol Chem* 2000; **275**(28)**:** 21055-21060. doi: 10.1074/jbc.M002055200

94. Grassilli E, Salomoni P, Perrotti D, Franceschi C, Calabretta B. Resistance to apoptosis in CTLL-2 cells overexpressing B-Myb is associated with B-Myb-dependent bcl-2 induction. *Cancer Res* 1999; **59**(10)**:** 2451-2456.

95. Calvisi DF, Simile MM, Ladu S, Frau M, Evert M, Tomasi ML *et al.* Activation of v-Myb avian myeloblastosis viral oncogene homolog-like2 (MYBL2)-LIN9 complex contributes to human hepatocarcinogenesis and identifies a subset of hepatocellular carcinoma with mutant p53. *Hepatology* 2011; **53**(4)**:** 1226-1236. doi: 10.1002/hep.24174

96. Forozan F, Mahlamaki EH, Monni O, Chen Y, Veldman R, Jiang Y *et al.* Comparative genomic hybridization analysis of 38 breast cancer cell lines: a basis for interpreting complementary DNA microarray data. *Cancer Res* 2000; **60**(16)**:** 4519-4525.

97. Ren F, Wang L, Shen X, Xiao X, Liu Z, Wei P *et al.* MYBL2 is an independent prognostic marker that has tumor-promoting functions in colorectal cancer. *Am J Cancer Res* 2015; **5**(4)**:** 1542-1552.

98. Hibi K, Liu Q, Beaudry GA, Madden SL, Westra WH, Wehage SL *et al.* Serial analysis of gene expression in non-small cell lung cancer. *Cancer Res* 1998; **58**(24)**:** 5690-5694.

99. Laposova K, Pastorekova S, Tomaskova J. Lymphocytic choriomeningitis virus: invisible but not innocent. *Acta Virol* 2013; **57**(2)**:** 160-170.

100. Neuman BW, Adair BD, Burns JW, Milligan RA, Buchmeier MJ, Yeager M. Complementarity in the supramolecular design of arenaviruses and retroviruses revealed by electron cryomicroscopy and image analysis. *J Virol* 2005; **79**(6)**:** 3822-3830. doi: 10.1128/JVI.79.6.3822-3830.2005

101. Borrow P, Oldstone MB. Mechanism of lymphocytic choriomeningitis virus entry into cells. *Virology* 1994; **198**(1)**:** 1-9. doi: 10.1006/viro.1994.1001

102. Zinkernagel RM, Leist T, Hengartner H, Althage A. Susceptibility to lymphocytic choriomeningitis virus isolates correlates directly with early and high cytotoxic T cell activity, as well as with footpad swelling reaction, and all three are regulated by H-2D. *J Exp Med* 1985; **162**(6)**:** 2125-2141.

103. Fox JG. *The mouse in biomedical research*, 2nd edn Elsevier, AP: Amsterdam ; Boston, 2007.

104. Ng CT, Sullivan BM, Oldstone MB. The role of dendritic cells in viral persistence. *Curr Opin Virol* 2011; **1**(3)**:** 160-166. doi: 10.1016/j.coviro.2011.05.006

105. Letchworth GJ, Rodriguez LL, Del cbarrera J. Vesicular stomatitis. *Vet J* 1999; **157**(3)**:** 239-260. doi: 10.1053/tvjl.1998.0303

106. Nair S, Michaelsen-Preusse K, Finsterbusch K, Stegemann-Koniszewski S, Bruder D, Grashoff M *et al.* Interferon regulatory factor-1 protects from fatal neurotropic infection with vesicular stomatitis virus by specific inhibition of viral replication in neurons. *PLoS Pathog* 2014; **10**(3)**:** e1003999. doi: 10.1371/journal.ppat.1003999

107. Hangartner L, Zinkernagel RM, Hengartner H. Antiviral antibody responses: the two extremes of a wide spectrum. *Nat Rev Immunol* 2006; **6**(3)**:** 231-243. doi: 10.1038/nri1783

108. Isaacs A, Lindenmann J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 1957; **147**(927)**:** 258-267.

109. van Pesch V, Lanaya H, Renauld JC, Michiels T. Characterization of the murine alpha interferon gene family. *J Virol* 2004; **78**(15)**:** 8219-8228. doi: 10.1128/JVI.78.15.8219-8228.2004

110. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**(4)**:** 783-801. doi: 10.1016/j.cell.2006.02.015

111. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004; **5**(7)**:** 730-737. doi: 10.1038/ni1087

112. Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev* 2001; **14**(4)**:** 778-809, table of contents. doi: 10.1128/CMR.14.4.778-809.2001

113. Nguyen KB, Salazar-Mather TP, Dalod MY, Van Deusen JB, Wei XQ, Liew FY *et al.* Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol* 2002; **169**(8)**:** 4279-4287.

114. Greiner JW, Hand PH, Noguchi P, Fisher PB, Pestka S, Schlom J. Enhanced expression of surface tumor-associated antigens on human breast and colon tumor cells after recombinant human leukocyte alpha-interferon treatment. *Cancer Res* 1984; **44**(8)**:** 3208-3214.

115. Schiavoni G, Mattei F, Gabriele L. Type I Interferons as Stimulators of DC-Mediated Cross-Priming: Impact on Anti-Tumor Response. *Front Immunol* 2013; **4:** 483. doi: 10.3389/fimmu.2013.00483

116. Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. *Nat Rev Cancer* 2016; **16**(3)**:** 131-144. doi: 10.1038/nrc.2016.14

117. Albini A, Marchisone C, Del Grosso F, Benelli R, Masiello L, Tacchetti C *et al.* Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: A gene therapy approach. *Am J Pathol* 2000; **156**(4)**:** 1381-1393. doi: 10.1016/S0002-9440(10)65007-9

118. Wang RY, Li K. Host factors in the replication of positive-strand RNA viruses. *Chang Gung Med J* 2012; **35**(2)**:** 111-124.

119. Pelkmans L. Viruses as probes for systems analysis of cellular signalling, cytoskeleton reorganization and endocytosis. *Curr Opin Microbiol* 2005; **8**(3)**:** 331-337. doi: 10.1016/j.mib.2005.02.018

120. Miller S, Krijnse-Locker J. Modification of intracellular membrane structures for virus replication. *Nat Rev Microbiol* 2008; **6**(5)**:** 363-374. doi: 10.1038/nrmicro1890

121. Zhou H, Xu M, Huang Q, Gates AT, Zhang XD, Castle JC *et al.* Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe* 2008; **4**(5)**:** 495-504. doi: 10.1016/j.chom.2008.10.004

122. Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L *et al.* EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med* 2011; **17**(5)**:** 589-595. doi: 10.1038/nm.2341

123. Karlas A, Machuy N, Shin Y, Pleissner KP, Artarini A, Heuer D *et al.* Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature* 2010; **463**(7282)**:** 818-822. doi: 10.1038/nature08760

124. Rodenhuis-Zybert IA, Wilschut J, Smit JM. Dengue virus life cycle: viral and host factors modulating infectivity. *Cell Mol Life Sci* 2010; **67**(16)**:** 2773-2786. doi: 10.1007/s00018-010-0357-z

125. Reid SP, Shurtleff AC, Costantino JA, Tritsch SR, Retterer C, Spurgers KB *et al.* HSPA5 is an essential host factor for Ebola virus infection. *Antiviral Res* 2014; **109:** 171-174. doi: 10.1016/j.antiviral.2014.07.004

126. Leng J, Ho HP, Buzon MJ, Pereyra F, Walker BD, Yu XG *et al.* A cell-intrinsic inhibitor of HIV-1 reverse transcription in CD4(+) T cells from elite controllers. *Cell Host Microbe* 2014; **15**(6)**:** 717-728. doi: 10.1016/j.chom.2014.05.011

127. Konig R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S *et al.* Human host factors required for influenza virus replication. *Nature* 2010; **463**(7282)**:** 813-817. doi: 10.1038/nature08699

128. Garcia P, Berlanga O, Watson R, Frampton J. Generation of a conditional allele of the B-myb gene. *Genesis* 2005; **43**(4)**:** 189-195. doi: 10.1002/gene.20170

129. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM *et al.* Functional role of type I and type II interferons in antiviral defense. *Science* 1994; **264**(5167)**:** 1918-1921.

130. Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafaille JJ *et al.* Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* 1992; **360**(6401)**:** 225-231. doi: 10.1038/360225a0

131. Hangartner L, Senn BM, Ledermann B, Kalinke U, Seiler P, Bucher E *et al.* Antiviral immune responses in gene-targeted mice expressing the immunoglobulin heavy chain of virus-neutralizing antibodies. *Proc Natl Acad Sci U S A* 2003; **100**(22)**:** 12883-12888. doi: 10.1073/pnas.2135542100

132. Maloy KJ, Burkhart C, Freer G, Rulicke T, Pircher H, Kono DH *et al.* Qualitative and quantitative requirements for CD4+ T cell-mediated antiviral protection. *J Immunol* 1999; **162**(5)**:** 2867-2874.

133. Miyawaki S, Nakamura Y, Suzuka H, Koba M, Yasumizu R, Ikehara S *et al.* A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur J Immunol* 1994; **24**(2)**:** 429-434. doi: 10.1002/eji.1830240224

134. Gu H, Zou YR, Rajewsky K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 1993; **73**(6)**:** 1155-1164.

135. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K *et al.* Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* 2000; **13**(4)**:** 539-548.

136. Jameera Begam A, Jubie S, Nanjan MJ. Estrogen receptor agonists/antagonists in breast cancer therapy: A critical review. *Bioorg Chem* 2017. doi: 10.1016/j.bioorg.2017.02.011

137. Watashi K, Inoue D, Hijikata M, Goto K, Aly HH, Shimotohno K. Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with

viral RNA polymerase NS5B. *J Biol Chem* 2007; **282**(45)**:** 32765-32772. doi: 10.1074/jbc.M704418200

138. Laurence J, Cooke H, Sikder SK. Effect of tamoxifen on regulation of viral replication and human immunodeficiency virus (HIV) long terminal repeat-directed transcription in cells chronically infected with HIV-1. *Blood* 1990; **75**(3)**:** 696-703.

139. Stetson DB, Medzhitov R. Type I interferons in host defense. *Immunity* 2006; **25**(3)**:** 373-381. doi: 10.1016/j.immuni.2006.08.007

140. Faul EJ, Lyles DS, Schnell MJ. Interferon response and viral evasion by members of the family rhabdoviridae. *Viruses* 2009; **1**(3)**:** 832-851. doi: 10.3390/v1030832

141. Hancock RE, Scott MG. The role of antimicrobial peptides in animal defenses. *Proc Natl Acad Sci U S A* 2000; **97**(16)**:** 8856-8861.

142. Ginsburg I. Role of lipoteichoic acid in infection and inflammation. *Lancet Infect Dis* 2002; **2**(3)**:** 171-179.

143. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; **282**(5396)**:** 2085-2088.

144. Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P *et al.* Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 1999; **189**(4)**:** 615-625.

145. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001; **413**(6857)**:** 732-738. doi: 10.1038/35099560

146. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity* 2010; **33**(4)**:** 492-503. doi: 10.1016/j.immuni.2010.10.002

147. Hsu KM, Pratt JR, Akers WJ, Achilefu SI, Yokoyama WM. Murine cytomegalovirus displays selective infection of cells within hours after systemic administration. *J Gen Virol* 2009; **90**(Pt 1)**:** 33-43. doi: 10.1099/vir.0.006668-0

148. Walton SM, Torti N, Mandaric S, Oxenius A. T-cell help permits memory CD8(+) T-cell inflation during cytomegalovirus latency. *Eur J Immunol* 2011; **41**(8)**:** 2248-2259. doi: 10.1002/eji.201141575

149. Jeitziner SM, Walton SM, Torti N, Oxenius A. Adoptive transfer of cytomegalovirus-specific effector CD4+ T cells provides antiviral protection from murine CMV infection. *Eur J Immunol* 2013; **43**(11)**:** 2886-2895. doi: 10.1002/eji.201343690

150. Nagy PD, Pogany J. The dependence of viral RNA replication on co-opted host factors. *Nat Rev Microbiol* 2011; **10**(2)**:** 137-149. doi: 10.1038/nrmicro2692

151. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A *et al.* MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 2005; **21**(13)**:** 2933-2942. doi: 10.1093/bioinformatics/bti473

152. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc* 2013; **8**(8)**:** 1551-1566. doi: 10.1038/nprot.2013.092

153. Lee AS, Burdeinick-Kerr R, Whelan SP. A genome-wide small interfering RNA screen identifies host factors required for vesicular stomatitis virus infection. *J Virol* 2014; **88**(15)**:** 8355-8360. doi: 10.1128/JVI.00642-14

154. Panda D, Das A, Dinh PX, Subramaniam S, Nayak D, Barrows NJ *et al.* RNAi screening reveals requirement for host cell secretory pathway in infection by diverse families of negative-strand RNA viruses. *Proc Natl Acad Sci U S A* 2011; **108**(47)**:** 19036-19041. doi: 10.1073/pnas.1113643108

155. Parato KA, Senger D, Forsyth PA, Bell JC. Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* 2005; **5**(12)**:** 965-976. doi: 10.1038/nrc1750

156. Liu TC, Kirn D. Gene therapy progress and prospects cancer: oncolytic viruses. *Gene Ther* 2008; **15**(12)**:** 877-884. doi: 10.1038/gt.2008.72

157. Csatary LK, Gosztonyi G, Szeberenyi J, Fabian Z, Liszka V, Bodey B *et al.* MTH-68/H oncolytic viral treatment in human high-grade gliomas. *J Neurooncol* 2004; **67**(1-2)**:** 83-93.

158. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nat Biotechnol* 2012; **30**(7)**:** 658-670. doi: 10.1038/nbt.2287

159. Zamarin D, Holmgaard RB, Subudhi SK, Park JS, Mansour M, Palese P *et al.* Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. *Sci Transl Med* 2014; **6**(226)**:** 226ra232. doi: 10.1126/scitranslmed.3008095

160. Molomut N, Padnos M, Papperman TW, Pevear DC, Pfau CJ. Immune recognition of tumor cells in mice infected with Pichinde virus. *Cancer Immunol Immunother* 1984; **17**(1)**:** 56-61.

161. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005; **353**(25)**:** 2654-2666. doi: 10.1056/NEJMoa051424

162. Azimi F, Scolyer RA, Rumcheva P, Moncrieff M, Murali R, McCarthy SW *et al.* Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *J Clin Oncol* 2012; **30**(21)**:** 2678-2683. doi: 10.1200/JCO.2011.37.8539

163. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**(9)**:** 942-949. doi: 10.1038/nm1093

164. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. *Nat Rev Immunol* 2015; **15**(2)**:** 87-103. doi: 10.1038/nri3787

165. Han SJ, Melichar HJ, Coombes JL, Chan SW, Koshy AA, Boothroyd JC *et al.* Internalization and TLR-dependent type I interferon production by monocytes in response to Toxoplasma gondii. *Immunol Cell Biol* 2014; **92**(10)**:** 872-881. doi: 10.1038/icb.2014.70

166. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science* 2010; **327**(5966)**:** 656-661. doi: 10.1126/science.1178331

167. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* 2009; **27:** 669-692. doi: 10.1146/annurev.immunol.021908.132557

168. Scheu S, Dresing P, Locksley RM. Visualization of IFNbeta production by plasmacytoid versus conventional dendritic cells under specific stimulation conditions in vivo. *Proc Natl Acad Sci U S A* 2008; **105**(51)**:** 20416-20421. doi: 10.1073/pnas.0808537105

169. Dunay IR, Fuchs A, Sibley LD. Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice. *Infect Immun* 2010; **78**(4)**:** 1564-1570. doi: 10.1128/IAI.00472-09

170. Wojtasiak M, Pickett DL, Tate MD, Londrigan SL, Bedoui S, Brooks AG *et al.* Depletion of Gr-1+, but not Ly6G+, immune cells exacerbates virus replication and

disease in an intranasal model of herpes simplex virus type 1 infection. *J Gen Virol* 2010; **91**(Pt 9)**:** 2158-2166. doi: 10.1099/vir.0.021915-0

171. Peters W, Dupuis M, Charo IF. A mechanism for the impaired IFN-gamma production in C-C chemokine receptor 2 (CCR2) knockout mice: role of CCR2 in linking the innate and adaptive immune responses. *J Immunol* 2000; **165**(12)**:** 7072-7077.

172. Valente AJ, Graves DT, Vialle-Valentin CE, Delgado R, Schwartz CJ. Purification of a monocyte chemotactic factor secreted by nonhuman primate vascular cells in culture. *Biochemistry* 1988; **27**(11)**:** 4162-4168.

173. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR *et al.* Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 1998; **28**(9)**:** 2760-2769. doi: 10.1002/(SICI)1521-4141(199809)28:09<2760::AID-IMMU2760>3.0.CO;2-N

174. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A* 1994; **91**(9)**:** 3652-3656.

175. Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. *J Immunol* 1996; **156**(1)**:** 322-327.

176. Guinn BA, Kasahara N, Farzaneh F, Habib NA, Norris JS, Deisseroth AB. Recent advances and current challenges in tumor immunology and immunotherapy. *Mol Ther* 2007; **15**(6)**:** 1065-1071. doi: 10.1038/sj.mt.6300138

177. Didierlaurent AM, Collignon C, Bourguignon P, Wouters S, Fierens K, Fochesato M *et al.* Enhancement of adaptive immunity by the human vaccine adjuvant AS01 depends on activated dendritic cells. *J Immunol* 2014; **193**(4)**:** 1920-1930. doi: 10.4049/jimmunol.1400948

178. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**(6801)**:** 249-257. doi: 10.1038/35025220

179. Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci U S A* 1994; **91**(4)**:** 1198-1205.

180. Sgonc R, Fuerhapter C, Boeck G, Swerlick R, Fritsch P, Sepp N. Induction of apoptosis in human dermal microvascular endothelial cells and infantile hemangiomas by interferon-alpha. *Int Arch Allergy Immunol* 1998; **117**(3)**:** 209-214.

181. Dinney CP, Bielenberg DR, Perrotte P, Reich R, Eve BY, Bucana CD *et al.* Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res* 1998; **58**(4)**:** 808-814.

182. Varia MA, Calkins-Adams DP, Rinker LH, Kennedy AS, Novotny DB, Fowler WC, Jr. *et al.* Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma. *Gynecol Oncol* 1998; **71**(2)**:** 270-277. doi: 10.1006/gyno.1998.5163

183. Muik A, Stubbert LJ, Jahedi RZ, Geibeta Y, Kimpel J, Dold C *et al.* Re-engineering vesicular stomatitis virus to abrogate neurotoxicity, circumvent humoral immunity, and enhance oncolytic potency. *Cancer Res* 2014; **74**(13)**:** 3567-3578. doi: 10.1158/0008-5472.CAN-13-3306

184. Zeh HJ, Downs-Canner S, McCart JA, Guo ZS, Rao UN, Ramalingam L *et al.* First-in-man study of western reserve strain oncolytic vaccinia virus: safety, systemic spread, and antitumor activity. *Mol Ther* 2015; **23**(1)**:** 202-214. doi: 10.1038/mt.2014.194

185. Davy C, Doorbar J. G2/M cell cycle arrest in the life cycle of viruses. *Virology* 2007; **368**(2)**:** 219-226. doi: 10.1016/j.virol.2007.05.043

186. Dove B, Brooks G, Bicknell K, Wurm T, Hiscox JA. Cell cycle perturbations induced by infection with the coronavirus infectious bronchitis virus and their effect on virus replication. *J Virol* 2006; **80**(8)**:** 4147-4156. doi: 10.1128/JVI.80.8.4147-4156.2006

187. Nascimento R, Costa H, Parkhouse RM. Virus manipulation of cell cycle. *Protoplasma* 2012; **249**(3)**:** 519-528. doi: 10.1007/s00709-011-0327-9

188. Getts DR, Chastain EM, Terry RL, Miller SD. Virus infection, antiviral immunity, and autoimmunity. *Immunol Rev* 2013; **255**(1)**:** 197-209. doi: 10.1111/imr.12091

189. Harvey BP, Gee RJ, Haberman AM, Shlomchik MJ, Mamula MJ. Antigen presentation and transfer between B cells and macrophages. *Eur J Immunol* 2007; **37**(7)**:** 1739-1751. doi: 10.1002/eji.200636452

190. Baron S. *Medical microbiology*, 4th edn University of Texas Medical Branch at Galveston: Galveston, Tex., 1996.

191. Bachmann MF, Hengartner H, Zinkernagel RM. T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? *Eur J Immunol* 1995; **25**(12)**:** 3445-3451. doi: 10.1002/eji.1830251236

192. Fahl SP, Crittenden RB, Allman D, Bender TP. c-Myb is required for pro-B cell differentiation. *J Immunol* 2009; **183**(9)**:** 5582-5592. doi: 10.4049/jimmunol.0901187

193. Valny M, Honsa P, Kirdajova D, Kamenik Z, Anderova M. Tamoxifen in the Mouse Brain: Implications for Fate-Mapping Studies Using the Tamoxifen-Inducible Cre-loxP System. *Front Cell Neurosci* 2016; **10:** 243. doi: 10.3389/fncel.2016.00243

194. van den Broek MF, Muller U, Huang S, Aguet M, Zinkernagel RM. Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. *J Virol* 1995; **69**(8)**:** 4792-4796.

195. Prestwich RJ, Errington F, Diaz RM, Pandha HS, Harrington KJ, Melcher AA *et al.* The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon. *Hum Gene Ther* 2009; **20**(10)**:** 1119-1132. doi: 10.1089/hum.2009.135

196. Mahoney DJ, Lefebvre C, Allan K, Brun J, Sanaei CA, Baird S *et al.* Virus-tumor interactome screen reveals ER stress response can reprogram resistant cancers for oncolytic virus-triggered caspase-2 cell death. *Cancer Cell* 2011; **20**(4)**:** 443-456. doi: 10.1016/j.ccr.2011.09.005

197. Hay S, Kannourakis G. A time to kill: viral manipulation of the cell death program. *J Gen Virol* 2002; **83**(Pt 7)**:** 1547-1564. doi: 10.1099/0022-1317-83-7-1547

198. Melcher A, Parato K, Rooney CM, Bell JC. Thunder and lightning: immunotherapy and oncolytic viruses collide. *Mol Ther* 2011; **19**(6)**:** 1008-1016. doi: 10.1038/mt.2011.65

199. Kurnick JT, Ramirez-Montagut T, Boyle LA, Andrews DM, Pandolfi F, Durda PJ *et al.* A novel autocrine pathway of tumor escape from immune recognition: melanoma cell lines produce a soluble protein that diminishes expression of the gene encoding the melanocyte lineage melan-A/MART-1 antigen through down-modulation of its promoter. *J Immunol* 2001; **167**(3)**:** 1204-1211.

200. Pandolfi F, Cianci R, Lolli S, Dunn IS, Newton EE, Haggerty TJ *et al.* Strategies to overcome obstacles to successful immunotherapy of melanoma. *Int J Immunopathol Pharmacol* 2008; **21**(3)**:** 493-500. doi: 10.1177/039463200802100302

201. Pandolfi F, Cianci R, Pagliari D, Casciano F, Bagala C, Astone A *et al.* The immune response to tumors as a tool toward immunotherapy. *Clin Dev Immunol* 2011; **2011:** 894704. doi: 10.1155/2011/894704

202. Koma T, Huang C, Kolokoltsova OA, Brasier AR, Paessler S. Innate immune response to arenaviral infection: a focus on the highly pathogenic New World

hemorrhagic arenaviruses. *J Mol Biol* 2013; **425**(24)**:** 4893-4903. doi: 10.1016/j.jmb.2013.09.028

203. Platanias LC. Interferons and their antitumor properties. *J Interferon Cytokine Res* 2013; **33**(4)**:** 143-144. doi: 10.1089/jir.2013.0019

204. Gough DJ, Messina NL, Clarke CJ, Johnstone RW, Levy DE. Constitutive type I interferon modulates homeostatic balance through tonic signaling. *Immunity* 2012; **36**(2)**:** 166-174. doi: 10.1016/j.immuni.2012.01.011

205. Hwang SY, Hertzog PJ, Holland KA, Sumarsono SH, Tymms MJ, Hamilton JA *et al.* A null mutation in the gene encoding a type I interferon receptor component eliminates antiproliferative and antiviral responses to interferons alpha and beta and alters macrophage responses. *Proc Natl Acad Sci U S A* 1995; **92**(24)**:** 11284-11288.

206. Hervas-Stubbs S, Perez-Gracia JL, Rouzaut A, Sanmamed MF, Le Bon A, Melero I. Direct effects of type I interferons on cells of the immune system. *Clin Cancer Res* 2011; **17**(9)**:** 2619-2627. doi: 10.1158/1078-0432.CCR-10-1114

207. de La Salmoniere P, Grob JJ, Dreno B, Delaunay M, Chastang C. White blood cell count: a prognostic factor and possible subset indicator of optimal treatment with low-dose adjuvant interferon in primary melanoma. *Clin Cancer Res* 2000; **6**(12)**:** 4713-4718.

208. Kaufman HL, Kohlhapp FJ, Zloza A. Oncolytic viruses: a new class of immunotherapy drugs. *Nat Rev Drug Discov* 2015; **14**(9)**:** 642-662. doi: 10.1038/nrd4663

209. Westcott MM, Liu J, Rajani K, D'Agostino R, Jr., Lyles DS, Porosnicu M. Interferon Beta and Interferon Alpha 2a Differentially Protect Head and Neck Cancer Cells from Vesicular Stomatitis Virus-Induced Oncolysis. *J Virol* 2015; **89**(15)**:** 7944-7954. doi: 10.1128/JVI.00757-15

210. Moskophidis D, Battegay M, Bruendler MA, Laine E, Gresser I, Zinkernagel RM. Resistance of lymphocytic choriomeningitis virus to alpha/beta interferon and to gamma interferon. *J Virol* 1994; **68**(3)**:** 1951-1955.

211. Bubenik J. Tumour MHC class I downregulation and immunotherapy (Review). *Oncol Rep* 2003; **10**(6)**:** 2005-2008.

212. Oelkrug C, Ramage JM. Enhancement of T cell recruitment and infiltration into tumours. *Clin Exp Immunol* 2014; **178**(1)**:** 1-8. doi: 10.1111/cei.12382

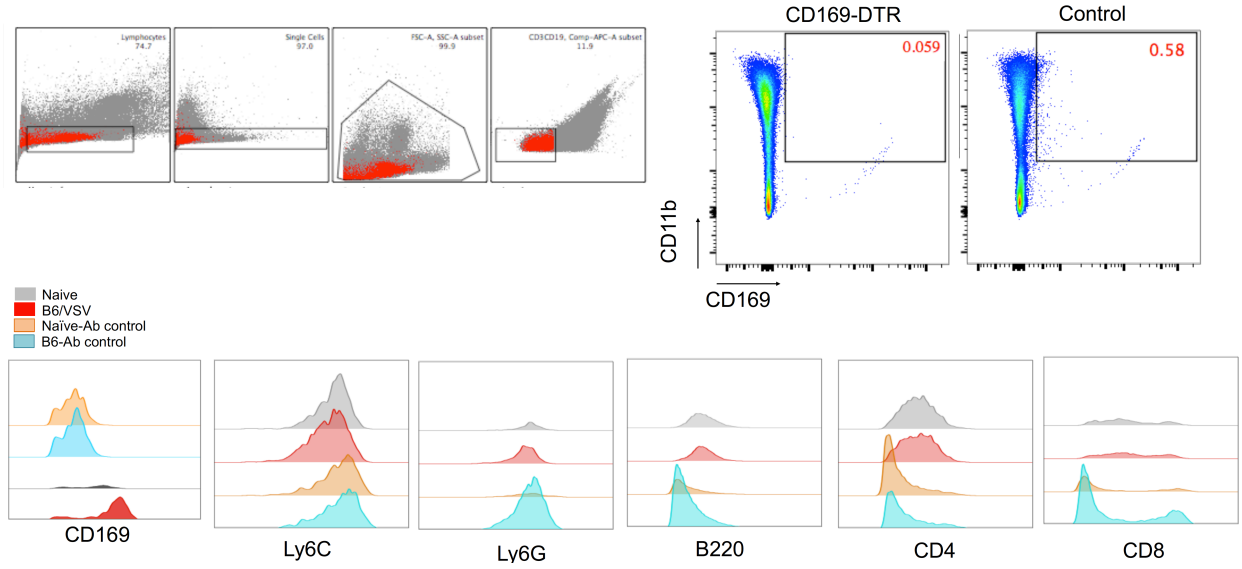
213. Pol J, Kroemer G, Galluzzi L. First oncolytic virus approved for melanoma immunotherapy. *Oncoimmunology* 2016; **5**(1)**:** e1115641. doi: 10.1080/2162402X.2015.1115641

214. Lichty BD, Breitbach CJ, Stojdl DF, Bell JC. Going viral with cancer immunotherapy. *Nat Rev Cancer* 2014; **14**(8)**:** 559-567. doi: 10.1038/nrc3770

215. Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N *et al.* Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* 2000; **6**(7)**:** 821-825. doi: 10.1038/77558

APPENDIX

A1



A2

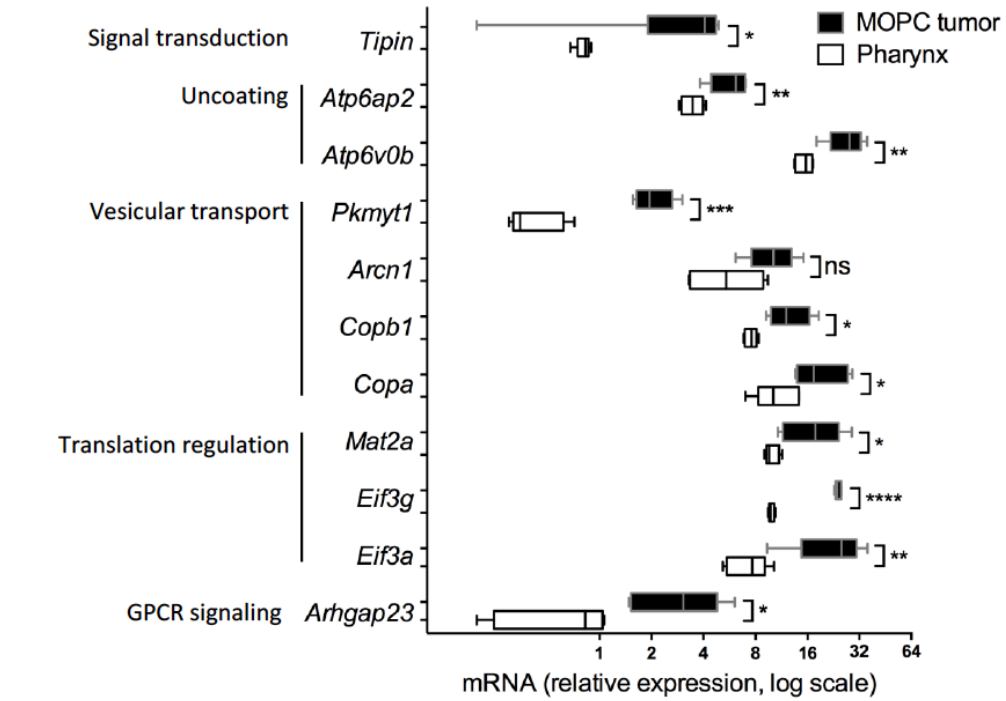


Figure A1: Dot-plot of CD169 staining from CD-169 DTR and Control mice treated with diphtheria toxin (30µg/kg) (n=3) (top panel). Representative histograms of phospho-B-Myb staining of different cell types from spleen of naïve and $2x10^8$ PFU VSV treated C57BL6/J mice analyzed 8 hrs p.i. (n=5).

Figure A2: Quantitative analysis of mRNA expression of viral host factors from MOPC tumors and control pharynx tissue (n=5)

CONTRIBUTIONS:

All the experiments were carried out in collaboration with Dr. Aleksandra Pandyra and Dr. Halime Kalkavan.

HSV infection studies were carried out in Dr. Mirko Trilling's lab, Universitätsklinikum Essen. HIV infection studies were carried out in Dr. Carsten Münk's lab, Heinrich Heine University, Düsseldorf. Microarray was performed by Dr. Rene Deenen, Heinrich Heine University, Düsseldorf. OT-1 transfer experiments were carried out in Prof. Percy Knolle lab, TU Munich. Angiogenesis studies were done by Dr. Iris Helfrich, Universitätsklinikum Essen.

Declaration:

Part of the thesis can also be used by MD student Mr. Tim Brandenbrug for his thesis.

ABBREVIATIONS

Ab	Antibody
ALT	Alanine aminotransferase
APC	Antigen Presenting Cells
BMDM	Bone marrow derived macrophages
BMDC	Bone marrow derived dendritic cells
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immune sorbent assay
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
GP	Glycoprotein
HSV	Herpes Simplex virus
HIV	Human immuno-deficiency virus
i.p.	intra-peritoneal
i.v.	intra-venous
i.t.	intra-tumoral
IFN	Interferon
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
LPS	Lipo-polysaccharide
MCMV	Murine cytomegalovirus
MVD	Micro-vessel density
NP	Nucleoprotein
PBS	Phosphate buffered saline
p.i.	post-infection
p.t.	post-treatment
PCR	Polymerase chain reaction
PFU	plaque forming units
RNA	Ribonucleicacid
RPM	Rotations per minute
RT	Room temperature
OVA	Ovalabumin
SDS	Sodium dodecyl sulphate
TCR	T-cell receptor
TLR	Toll-like receptor
UV	Ultraviolet
VSV	Vesicular stomatitis virus
LCMV	Lymphocytic choriomeningitis virus

ACKNOWLEDGEMENT

After a period of four years, I would like to thank many people who supported me professionally and personally. My work here has been a period of learning and growth in the scientific world. It would not have been possible alone and thus I am deeply thankful to everyone.

Living and staying away from home is always tough. This makes me learn who I am and grow into what I was raised to be. I am really thankful to my parents and my brother for their continuous belief and support in all my decisions and ventures.

I would like to express my sincerest gratitude towards my advisor Prof. Karl S. Lang for giving me a great opportunity to work in his lab and for his patience, motivation and continuous support. His constructive criticism and immense knowledge has helped me to achieve my research goals. He has been a wonderful mentor who has helped me grow professionally into this world of science.

Besides my mentor, I am in debt to Dr. Aleksandra Pandyra and Dr. Halime Kalkavan for their professional and emotional support. These wonderful ladies have been my second mentors, friends and colleagues. My work has always been in collaboration with them, which endorsed me with vast experimental and technical expertise. They have truly made my stay here a great learning experience with lots of fun.

My sincere thanks to Prof. Astrid Westendorf for being my second supervisor throughout my research work. She has been a great advisor and help in my projects. I am also thankful to Prof. Philipp Lang, who has always been there for project discussions and support. His lab has been a great place to collaborate and has provided me with the tools for my research work.

I express my gratitude to a very helpful and kind lady, Ms. Daniela Catrini who took care of all the formalities for the RTG1949 graduate school, so that we all can work. It's her hard work which made this graduate school a wonderful experience, be it at a regular seminar or an annual retreat presentation. Also, I would like thank Ms. Konstanze Schättel and Patricia

Spieker for the great help with official formalities and laboratory work at the beginning of my Ph.D.

Friends in a new country makes you feel at home. I am really thankful to my colleagues Vishal, Vikas, Katja, Asmae, Namir, Nadine, Sarah and to my friends Chiru, Paul, Nidhi and Rutuja for making my work and my personal life here a great experience filled with fun and adventure. I would like to acknowledge everyone who has helped me personally and professionally.

I am deeply thankful to DFG for providing me an opportunity to join the RTG1949 graduate school and by providing me with the financial means for my stay and research. This has been a great graduate school with tremendous learning opportunities.

Lastly, I would just like to say thank you to all for believing in me, supporting me and making this a memorable time.

Thank you

Piyush Sharma

Curriculum Vitae

The biography is not included in the online version for reasons of data protection

Erklärung

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *"B-Myb is a crucial proviral host-factor and is involved in arenavirus mediated tumor suppression"* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *Piyush Sharma* befürworte.

Essen, den 12/04/2017 _____ _____

Name des wissenschaftl.s Betreuers/Mitglieds der Universität Duisburg-Essen	Unterschrift d. wissenschaftl. Betreuers/ Mitglieds der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den 12/04/2017 _____

Unterschrift des/r Doktoranden/in

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, e und g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den 12/04/2017 _____

Unterschrift des/r Doktoranden/in