Role of Arenavirus Therapy in Melanoma

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Hilal Bhat
aus Jammu and Kashmir, India

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1. Gutachter: Prof. Dr. Karl Lang

2. Gutachter: Prof. Dr. Jürgen Becker

3. Gutachter:

Vorsitzender des Prüfungsausschusses: * Prof. Dr. Ralf Küppers

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List of abbreviations

-/- knock-out
aa amino acid
ATP adenosine triphosphate
PAGE polyacrylamide gel electrophoresis

bp base pair(s)

cDNA complementary DNA
NK Natural Killer cells
IF Immunofluorescence
i.t Intratumoral
DC dendritic cell
DNA deoxyribonucleic acid
dNTP deoxy nucleotide triphosphate
ds double stranded

CCL5 Rantes
ELISA Enzyme Linked Immunosorbent Assay
Ig immunoglobulin
IHC Immunohistochemistry
LCMV Lymphocytic Choriomeningitis Virus
i.p. Intraperitoneal
i.v. Intravenous
IFN interferon
IL interleukin
VSV Vesicular Stomatitis Virus
mRNA Messenger RNA
FACS Fluorescence-activated cell sorting
mAb monoclonal antibody
NSG NOD Scid gamma mouse
NO nitric oxide
wt Wild type

o/n over night
ODN oligodeoxynucleotide
ORN oligo ribonucleotide
PCR Polymerase chain Reaction
PRR Pattern recognition Receptor
RPM revolutions per minute
RT room temperature
RNA ribonucleic acid
ss single stranded
TLR Toll-likereceptor
tRNA transfer RNA
TNF tumor necrosis factor
ICS Intracellular Cytokine Staining
EtOH ethanol

1. Introduction

1.1 The immune system

The immune system comprises cells, tissues, and organs that protect from microbial and endogenous sterile insults, thus not always distinguishing between non-self and self. The immune system branches into two subsystems, namely the evolutionary old innate immune system, which provides immediate defense, and the advanced adaptive immune system, which upon by random somatic mutation and resultant clonal individuality provides increased specificity potentially. Innate immunity thus encompasses phagocytosis and antimicrobial peptide such as defensins production.

1.1.1 Innate Immune system

The immune system has two essential components that comprises the innate immune system and the other being the adaptive immune system. Innate immune system is found in both vertebrates and invertebrates. Once the body faces the pathogen infection or once the body is attacked by some pathogenic infection our innate immune system gets activated and defends the host from the pathogenic attack. There are complex barriers that nature has provided to the innate immune system which keep the viruses, bacteria, parasites, and other foreign particles out of your body or besides these barriers can also prevent them to spread and move throughout the body. The innate immune system is comprised of different strategies to save the body from the pathogen some of them include physical barriers such as skin, the gastrointestinal tract, the respiratory tract, the nasopharynx, cilia, eyelashes and other body hair. Defence mechanisms such as secretions, mucous, bile, gastric acid, saliva, tears, and sweat is also another adaptation of the innate immune system to protect an individual from infection. The main cells types of Innate immune system include eosinophils basophils, natural killer cells, dendritic cells, neutrophils, mast cells, macrophages and monocytes.

1.1.2 Monocytes

Monocytes are one of the essential cell types of the innate immune component and belong to subset of circulating blood cells and they show remarkable plasticity. They can develop into a wide range of terminally differentiated cells and perform versatile functions during infection, tumour formation and in the setting of chronic inflammation. It is know from the literature that monocytes are a heterogeneous population and they quickly respond to stimuli including TLR signalling following exposure to pathogens and to inflammatory cytokines[1]. Murine

monocytes are divided into two categories, Ly6C^{hi} inflammatory monocytes which are expressing high levels of CC chemokine receptor 2 (CCR2) but lower of levels of CX3CR1[2] and circulate between blood and bone marrow under homeostatic conditions. Upon infection monocytes infiltrate tissue and serve as precursors for different kinds of effector cells, For instance in case tumours and chronic inflammation, monocytes differentiate into so-called myeloid-derived-suppressive cells (MDSC) or macrophages and produce a complex spectrum of cytokines and growth factors, such as IL-10, Arginase, TGF-β and M-CSF, that have the remarkable capability to suppress immune responses and promote tumour growth [3, 4] in terms of macrophages there are several clinical studies which have shown that macrophages promote tumorigenesis. It has been reported in one meta-analysis that over 80% of studies show a correlation between macrophage density and poor patient prognosis[5] and many more recent studies have further supported this conclusion. For example, there is a strong association between increased macrophage density in thyroid, lung, and hepatocellular cancers [6-8] and poor survival. But like always there are some exceptions with high macrophage densities correlating with increased survival in pancreatic cancer [9].

1.1.3 Type I Interferon

Type I interferons (IFNs) were discovered more than half a century ago as the factors underlying viral interference that is, the ability of a primary viral infection to render cells resistant to a second distinct virus[10]. There are many different classes of interferons like for instance type I IFNs, which comprise IFNα proteins (a class of homologous proteins that are encoded by 13 distinct genes in humans, IFNA1 to IFNA13), IFNβ (that is encoded by a single gene in humans and mice, IFNB1) and other, less investigated IFNs, such as IFNε, IFNκ and IFNω. Type I IFNs signal via a homodimeric IFNα/β receptor 1 (IFNAR1), which has a particularly high affinity for IFNβ, or via an IFNAR1–IFNAR2 heterodimer, which binds all type I IFNs. There is lot of data and literature available which suggest that type I IFNs have a major role to play not only in antiviral immune responses but also in the natural and the therapy-induced immunological control of virus-unrelated malignancies. Besides that antineoplastic effects against several malignancies are also mediated by Type I IFNs, which is a clinically relevant activity that has been attributed to their immunostimulatory functions [11]. Rigorous experimental data strongly suggest the existence of a process whereby the immune system, in the absence of external manipulations, protects the host against oncogenesis and controls the immunological features of developing tumour[12]. This process is known as cancer immunoediting and consists of three phases and Type 1 interferon intervene in all three phases [13, 14] From the literature point of view Type 1 interferon are known to have antitumoral

effects and it is also well know that type 1 IFNs have been shown to be inhibitors of angiogenesis and therefore could inhibit tumour growth by blocking tumor vascularization besides Type I IFNs include direct antiproliferative effects on many tumour targets[15]. It was shown in different comparative studies with the main focus on growth-inhibitory activities in melanoma cells, IFN-β exhibited has more marked growth-inhibitory activity than IFN-α2[16]. IFN-alpha in the In-vitro experimental settings blocks endothelial cell migration ,inhibits lymphocyte- and tumour-induced angiogenesis in vivo, and causes regression of experimentally induced iris neovascularization in primates[17, 18].

1.1.4 Natural Killer cells

Natural killer cells are an important component of the innate immune system that comprises 10% to 20% of circulating lymphocytes and have the capacity to control several types of tumours and microbial infections by limiting their spread and subsequent tissue damage. Originally, NK cells were described as large granular lymphocytes with natural cytotoxicity against tumour cells, It was later also know that NK is the separate lymphocyte lineage, with both cytotoxicity and cytokine-producing effector functions[19]. The effector function of natural killer cell are tightly regulated by a complex network of receptors that activate or inhibit NK cells upon stimulation[20,21]. Recent advances in the field of Immunology have highlighted the importance of NK cells and their receptors in the immune response to viral infections and bone marrow transplantation as well as in case of malignancy. There are several subsets of NK cells in mouse and in humans that have been detected so far. Like in mouse three subsets of NK cells differing in expression of CD11b and CD27 have been described. NK cell differentiate from $CD11b^{dull}CD27^{+}$ NK cells(23) by way of $CD11b^{+}CD27^{+}$ double-positive NK cells, to the most mature $CD11b^{+}CD27^{dull}$ NK cells. The killing capacity to target cells and to secrete IFN-γ in a broad range of in vitro stimulation conditions seems to be very much comparable between double-positive and $CD11b^{+}CD27^{dull}$ NK cells, while as $CD11b^{+}CD27^{dull}$ NK cells are in replicative senescence[22]**.** All these mentioned three subsets of NK cells differ widely in their tissue distribution**,** Double-positive NK cells are more homogeneously distributed while as $CD11b^{dull}CD27^{+}$ NK cells are predominantly found in bone marrow and lymph node and $CD11b^{+}CD27^{dull}$ NK cells are more abundant in blood, spleen, lung and liver[23].

In case of humans, NK cells are divided into $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, which differ in their homing properties[24]. Majority of the NK cells that means around 90% of peripheral blood and spleen NK cells are $CD56^{dim}CD16^{+}$ and express perforin. This subset of NK cells called $CD56^{dim}$ are cytotoxic and produce IFN-γ upon interaction with tumor

cells *in* vitro [25]. In contrast, most NK cells in lymph nodes and tonsils are $CD56^{bright}CD16^{-}$ and lack perforin [26] These cells also have the potential to readily produce cytokines such as IFN-γ in response to stimulation with interleukin (IL)-12, IL-15 and IL-18(24). Natural killer (NK) cells have a key role to play in host defense against viral infections**.** NK cells destroy virus-infected cells that have a downregulated expression of MHC class I this is what is knowns as 'missing self' hypothesis. NK cells can efficiently eliminate virus-infected cells that maintain expression of the inhibitory MHC class I under different situations [27, 28]. The first NK cell-activating receptor identified to be critical for viral control in vivo was Ly49H, which is necessary to clear MCMV infection [29] Ly49H, a C-type lectin-like receptor, specifically recognizes the m157 open reading frame of MCMV. Activation of Ly49H by m157 is required for NK cell-mediated clearance in MCMV-resistant mice. Besides their role in virus infection NK cells have the tremendous role to play in different cancers and also they prevent the dissemination of metastatic tumours in mice[30]. It was described in the mutant mice that there was increased tumour growth and metastasis with impaired NK cell activity [31]also the mice treated with an NK cell-depleting antibody showed enhanced tumour growth [32]**.**

It was confirmed from numerous studies that were carried from time to time that there was decreased NK cell function in cancer patients[33-35]or their families [36, 37] including one of the landmark long-term epidemiological study showing a very clear link between low NK cell activity and a very higher risk of developing various types of cancers[38]besides from several studies there was shown a strong correlation between poor NK cell function and the development of metastases for instance in case of head and neck [39, 40] non small cell lung carcinoma [41] and other solid tumours. Absence of NK cells or NK cell function or in other words, deficiencies of NK cells caused by genetic mutations in genes such as GATA2 or MCM4 lead to higher rates of malignancy. The growth and metastasis of transplantable tumours in numerous tumour models have been shown to be controlled by NK cells by antibody depletion of NK cells**.** Better NK cell infiltrate in human cancers especially in case of squamous cell lung, gastric and colorectal may be associated with a better prognosis.

1.2 Adaptive Immune System

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.

1.2.1 T cells

These cells originate from haematopoietic compartment of bone marrow and then migrate to thymus for maturation Like B-cells, T cells also express unique antigen binding receptor called the T-cell receptor (TCR). However, compared to B-cells which can detect soluble or particulate antigen by membrane bound antibodies, T-cells receptors recognize only processed antigens by APC's bound to their MHC molecules. T cells are generally divided in two major types either T helper cell (TH) or T cytotoxic (TC) cell. Both cell types can be differentiated by presence of CD4 or CD8 molecules on their surface respectively. CD4 T cells generally function as TH cells whereas CD8 T cells function as TC cells recognising antigen in complex with MHC-II and MHC-I respectively.

1.2.2 CD8 T cells

CD8 T cell or cytotoxic T lymphocyte (CTL) T-killer cells, killer T cells responses are an important branch of the immune system in the fight against viral infections. CD8 T cells destroy virus infected cells in acute and chronic viral infections[42]. For instance in case of human pathogens where the role of CTL have been studied in detail include human immunodeficiency virus[43, 44] hepatitis B[45, 46]and heptatitis C virus[47, 48]. Mice infection is one of the most valuable tool for investigating the dynamics of CTL responses against viral infections, and lymphocytic choriomeningitis virus (LCMV) has played an especially important role in this aspect[49-51]. Anti-viral CTL response besides being beneficial for the organism and controlling the infection it can also negatively impact the host through a phenomenon called CTL-induced pathology[52]. This occurs if the anti-viral CTL response damages the tissue sufficiently to cause disease. The correlation of CTL-mediated control and CTL-induced pathology have been studied in a great detail[53]**.** Besides having a remarkable role in the control of viral infection CD8 T cells also have a great role to play in case of many different cancers. T cells in tumours also known as tumour infiltrating lymphocytes (TIL) have been studied intensively over the past years. It is now well known that CD8+T cells have a tremendous antitumor effect and this effect is dependent on two crucial factors: firstly, by CD8+ T cell differentiation; and secondly by the infiltration of CD8+ T cells into the tumour site and it primarily occurs by trafficking of CD8+ T cells into the tumour microenvironment[54]. Keeping this as basis there are many factors that have a prominent role to play regarding the

trafficking of CD8+ T cells in the tumour microenvironment Fig. 1 summarises it in detail. There are many reports where scientists have linked elevated levels of cytotoxic CD8+T cells in the tumour microenvironment with positive anti-tumor effects for instance in case of breast [55] colorectal, glioblastoma[56]and cervical cancers[57]that means elevated CD8+T in tumour microenvironment can be concluded as the good prognosis for different cancer types. But the tumour microenvironment particularly solid tumours impede CD8+ T cell trafficking and function by different mechanisms like chemokine secretion abnormal tumour angiogenesis[58] [59] and the activation of inhibitory checkpoint pathways[60]. Naive CD8+ T cells once they infiltrate in the tumour bed they differentiated into effector CD8+ T cells and further differentiated and activated into cytotoxic and memory CD8+ T cells in order to perform their targeted functions at the tumour site[61, 62] and these activated cytotoxic CD8+ T cells classically secrete cytotoxic cytokines and kill the tumour cells upon their first encounter with the foreign agents. Once the CD8+T cells face the antigen they go into memory CD8+Tcells and then they remain there to perform further specialized functions. From the literature point of view and tremendous research in this field, it is now well established that CD8+ T cells have remarkable antitumor effects and harnessing this potential cell types has unparalleled benefits for the overall patient survival.

1.2.3 CD4 T cells

$CD4^+$T cells play a significant role in achieving effective immune response to pathogens. The activation of naive $CD4^+$T cells takes place upon their interaction with antigen-MHC complex and differentiate into specific subtypes depending on the presence of different cytokine milieu of the microenvironment. Based on the characteristic cytokine profile and continued studies identified new subsets of CD4+ Cells for instance T-helper 17 (Th17), follicular helper T cell (Tfh), induced T-regulatory cells (iTreg), and the regulatory type 1 cells (Tr1) apart from the classical T-helper 1 and T-helper 2 cells . The $CD4^+$T cells carry out diverse functions whether it is activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as nonimmune cells, besides that CD4+ T cells also also play critical role in the suppression of immune reaction. Besides their critical role in the different virus infections, CD4+ Th cells Also play a significant contribution in the development of an effective antitumor response in several murine tumour models[63, 64]. There are lot many accumulating evidences that hint towards the anitumoral properties of CD4+T cells. CD4 effector T cell subsets may have a more direct role to play in inhibiting tumour growth and progression. Two distinct mechanism are used by this cell types to lyse the target cell or infected cell production of cytokines, most notably IFN-γ and TNF and through direct cytolytic activity this is mediated by degranulation

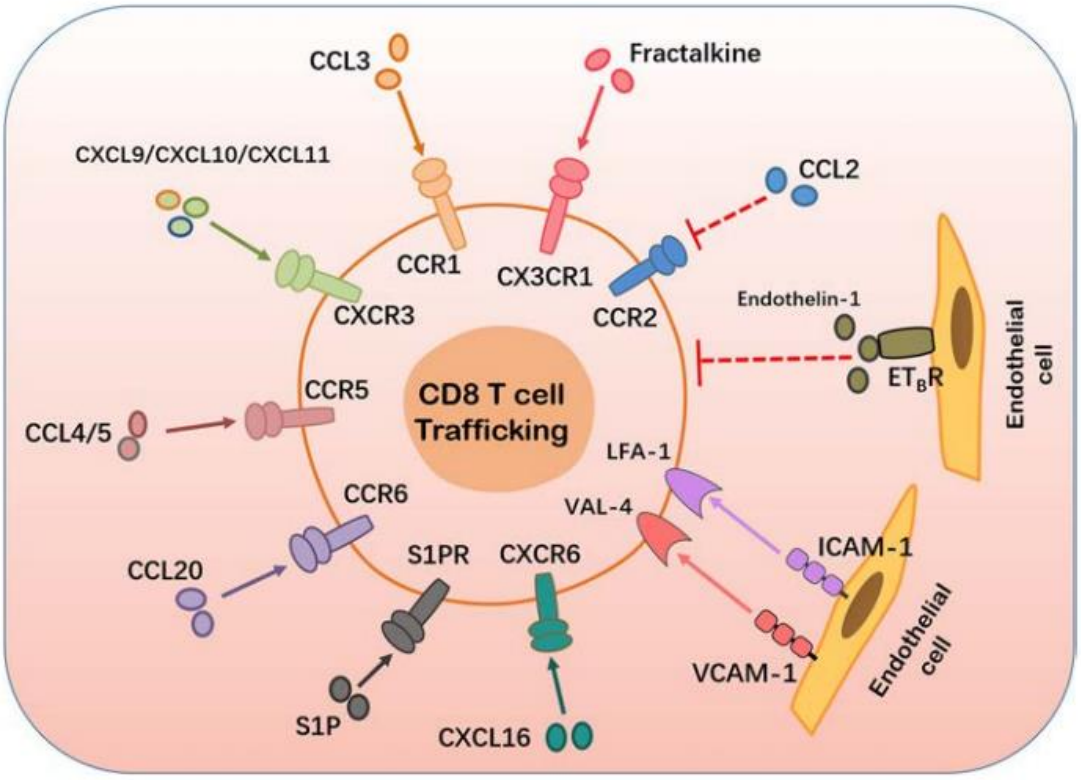

Figure 1: Factors responsible for CD8+T cells trafficking and localisation (N.R. Maimela et al. / Computational and Structural Biotechnology Journal 17 (2019) 1–13

of cytotoxic granules containing toxic effector molecules (i.e., perforin and granzyme) From the adoptive transfer studies it was shown that noncytolytic $CD4^+$ T cells were individually capable of eradication of disseminated leukemia in tumor-bearing mice[65]. There are many studies which show that CD+4 T cells have a significant antitumor property one of this kind of study showed that upon CD4+ T cell depletion by mAb before the tumour challenge there was the complete loss of tumour rejection [66] also a growing body of evidence indicate that tumour antigen-specific $CD4^+$ T cells play a pivotal role in orchestrating tumour eradication[67].

1.2.4 B cells

B-lymphocytes can be described as the population of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes. B cells are the main antibody secreting cells that generate and mature in the bone marrow. These cells are found to secret large variety of cytokines and are major players of adaptive immune response. Many studies have proved that B cells have an essential role to play in modulating tumour responses cancers. It is estimated that B cells account for up to 25% of all cells in some tumours. In some breast cancer studies it was shown that 40% of TILs are B cells[68, 69] besides that it was also shown that 40% of high-grade serous ovarian cancers contain infiltrating CD20+ B

cells[70] Apart from the different human cancers in some mouse models of cancer, about a third of tumour-draining lymph nodes cells are B cells [71] All these studies hints towards the critical role played by B cells in modulating tumour responses.

Another class of B cells also known as regulatory B cells (Bregs) have been known to promote tumour growth by inhibiting effector cells such as CD8+CTLs and NK cells[72]. Bregrs are the heterogeneous population of cells that supress inflammatory response and their role in cancer is well established[72, 73]**.** They produce many different anti-inflammatory cytokines including IL-10, TGF-beta and also IL-35 [74, 75]. So based on the cytokine production the phenotypic and functional properties of these cells types varies for instance IL-10 producing Bregs support the cancer growth[76] and B cells that inhibit CD4+ T cell responses[77]and so on.

1.3 Lymphocytic choriomeningitis virus (LCMV)

The discovery of LCMV took place back in 1934 by Charles Armstrong. It's a noncytopathic arenavirus responsible for aseptic meningitis and encephalitis. The LCMV genome consist of two negative sense-single stranded RNA designated L and S. LCMV infection is initiated by attachment of virus to the host receptors through the glycoproteins. It is then endocytosed and fusion of virus and vesicle membrane is formed followed by release of the ribonucleocapsid into the cytoplasm. There are different strains of LCMV known so far around four strains have been described. Neurotropic ''Clone 13'' is a derivative of ''Armstrong'' strain and hepatotropic ''docile'' is a derivative of WE strain WE and Armstrong infection cause acute infection where the viral particles are cleared within few days. On the other hand, docile and clone 13 cause chronic infection and it can take up to few weeks to clear the viral particles. After the virus infection there is a expansion of antigen-specific $CD8^+$cytotoxic T lymphocytes (CTLs) after they encounter antigen-presenting cells in the lymphoid tissues and their subsequent redistribution to nonlymphoid tissues to deal with the pathogen. For many viral infections, $CD8^+$ T cells form a crucial arm of the immune response through the actions of effector cytokines and cytolysis[78] and in addition to that Cd4 T cells Provide enough help for the CD8 T cells and B cells to mount the sufficient immune response[79]the activation of T cells proceeds to the differentiation into effector T cells that play a significant role for the resolution of the infection. So in the context of the LCMV infection, Like upon low dose infection of LCMV Docile in the mice there is very strong and broadly directed virus-specific $CD8^+$ T-cell response that is readily detectable in the spleen, resulting in the efficient clearance of virus within 2 weeks after infection. But however the scenario completely changes when you use the high dose of LCMV Docile which leads to the disseminating infection , because of the

higher viral load there is higher antigenic stimulation at the onset of the infection which leads to the transient $CD8^+$ T-cell response by which antigen-specific $CD8^+$ T cells are induced, proliferate, and initially exhibit antiviral functions but progressively lose this ability as a result of this protective immunity there is persistent infection[49, 80]. Disease progression in LCMV-docile or LCMV-clone13 infection is due to dysfunction of T cells. Recently there is a lot of research going on in the field of T cells exhaustion it is the state where the ability to produce cytokines such as IL-2 and higher proliferative and ex vivo capacities are lost. The production of antiviral cytokines such as TNF-α and IFN-γ are abolished and in final stage the CD8 T cells are deleted. Because of high activation due to viral load, the hepatocytes in liver are targeted by CD8 T cells, which results in high levels of immunopathology in the infected organism. Similarly, with other strains of LCMV like WE and Armstrong, upon their infection in the adult mice there is the massive virus multiplication that leads to the robust expansion of antigen specific CD8 + T cells[81] and this massive immune activation occurs 7 to 10 days after virus infection[82] and it is essential for the virus control, Besides the CTLs response that are very significant in the control of LCMV infection Neutralising antibodies (nAbs) play a pivotal role in the control of LCMV WE infection whether low or high dose in the mice[83].

1.4 Immune Sensitivity of Cancer Cells

Cancer for long is known as not a single disease but as the group of diseases, which basically involve abnormal cell growth and finally their spread to other parts of the body with an exception of the benign tumour which do not spread. It has been proposed from time to time about the hallmarks of cancer Fig. 2 illustrates it better the six main hallmarks of cancer. Cancer cells are different from their normal cells types in many different perspectives first they show uncontrolled division. There are many ways by which cancer cells can maintain their proliferative signals they can produce the growth factor ligand on their own besides that cancer cells may stimulate the normal cells in the tumour stroma that in turn will help to produce some growth factors that will nourish the growth of these cells[84] another striking property of the tumour cells is that they use different strategies to limit apoptosis and one of the most common is the loss of TP53 tumour suppressor function, apart from that tumours increase the expression of anti-apoptotic regulators like Bcl-2or survival signals (Igf1/2) besides they also promote the downregulation of Pro-Apoptotic factors which includes (Bax, Bim, Puma). Besides the property of the cancer cells to limit apoptosis, another remarkable property of the cancer cells is their incredible capability to make new blood vessels also known as angiogenesis . In case of tumours this angiogenic switch is always activated leading to the continuous formation of the new blood vessels that bring the nourishment to the tumour cells.

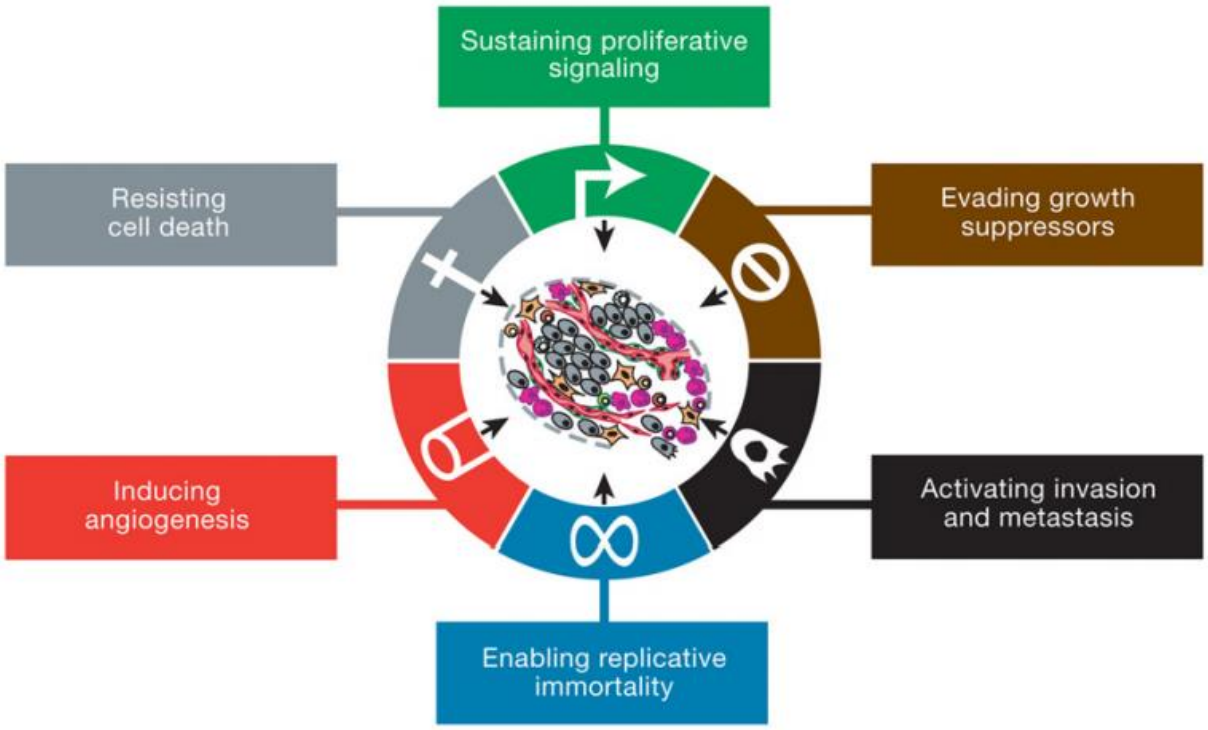

Figure 2: Hallmarks of Cancer: The next generation (Cell 144, March 4, 2011 Elsevier Inc. (Douglas Hanahan and Robert A. Weinberg)

1.5 Melanoma

Melanoma is the most dangerous form of skin cancer that develops in the cells (melanocytes) that make our skin colour. Melanoma is considered highly aggressive and metastatic type of cancer and metastatic melanoma is a fatal disease with a rapid systemic dissemination. The most common and frequent sites that become the target of melanoma are Liver, Bone and brain but in the long term, almost all the organs are involved. Melanoma is the fifth most common cancer after breast, lung, prostate and colorectal cancer, Irrespective of the advances in the field of melanoma research the number of melanoma cases diagnosed annually is increasing faster than for any other cancers making it still one of the deadliest cancer when it comes to the progression of the melanoma like other different cancers it occurs with the step wise acquisition of genetic abnormalities. It starts with the very first step that is activation of the oncogene, and it leads to the induction of benign neoplasm and the growth of this neoplasm is limited and controlled by the oncogene-induced senescence driven by activated suppressors. Malignant lesions are mainly characterised by the accumulation of additional abnormalities or by the complete loss of relevant suppressors which together count for the features of malignancy such

as invasion and metastasis, but many of these steps in this course of progression can be skipped by some alternate pathways.

1.5.1 Natural killer cells and melanoma

Despite of the many advances in the field of cancer immunotherapy melanoma is largely resistant to current therapies whether it is chemotherapy or radiotherapy[85]. Metastatic melanoma is highly aggressive cancer constituting the most lethal skin cancer[86]. Apart from that there are a lot of young people who die because of the aggressive metastatic melanoma. Innate immune responses has a very prominent role to play against melanoma. Over the years natural killer (NK) cells have came to the lime light in case of melanoma therapy and they constitute the first line of defense against transformed cells as tumours or virus-infected cells. From the literature, it is also proved and well established that that NK cells can recognize and destroy melanoma cell lines [87, 88]. It has been shown in different murine models that NK cells have a significant role to play against melanoma in the in vivo experimental model [89]. NK cells after the activation lead to the robust production of cytokines such as interferon (IFN)-Gamma and tumor necrosis factor (TNF) or they can directly kill target cells by releasing perforins and granzymes [90]. NK cells mainly kill the target which have low levels of MHC class I expression or have lost the expression of 1 or more MHC class I alleles. Virus infected or tumour transformation may lead to the downregulation of MHC class I molecule that makes them the prime target of the NK cell mediated killing. NK cells have this remarkable ability to discriminate between self, normal and transformed or infected target cells due to the expression of MHC class I-specific inhibitory receptors[91]. The interaction of the NK cells with the melanoma cells has been widely studied, but the presence of wide range of NK cell activating receptors make it difficult to pin point the participation of the different NK cell receptors in the lysis of melanoma cells. It is quiet possible that an individual receptor may be involved in the lysis of some melanoma cells or the mutual engagement of different receptors may be required for the NK cell mediated lysis of most of the melanoma cells. Like it is well explained and shown in the Fig. 3 NK cells express many receptors and one of them is NKG2D. Human NKG2D is a C-type lectin-like receptor which is expressed on NK cells, CD8 Alpha-beta T cells, Tδ T cells also in NKT cells and CD4 T cells . It is considered as the activating receptor for the NK cells and it helps the NK cells to kill the target cells and also also release the cytokine which further help in the efficient killing of the target cells. There are several ligands for the human NKG2D which includes the MHC class I chain related antigen (MICA) and (MICB) proteins and UL-16 binding proteins 1–5 (ULBP1–5). The ligands for NKG2D are

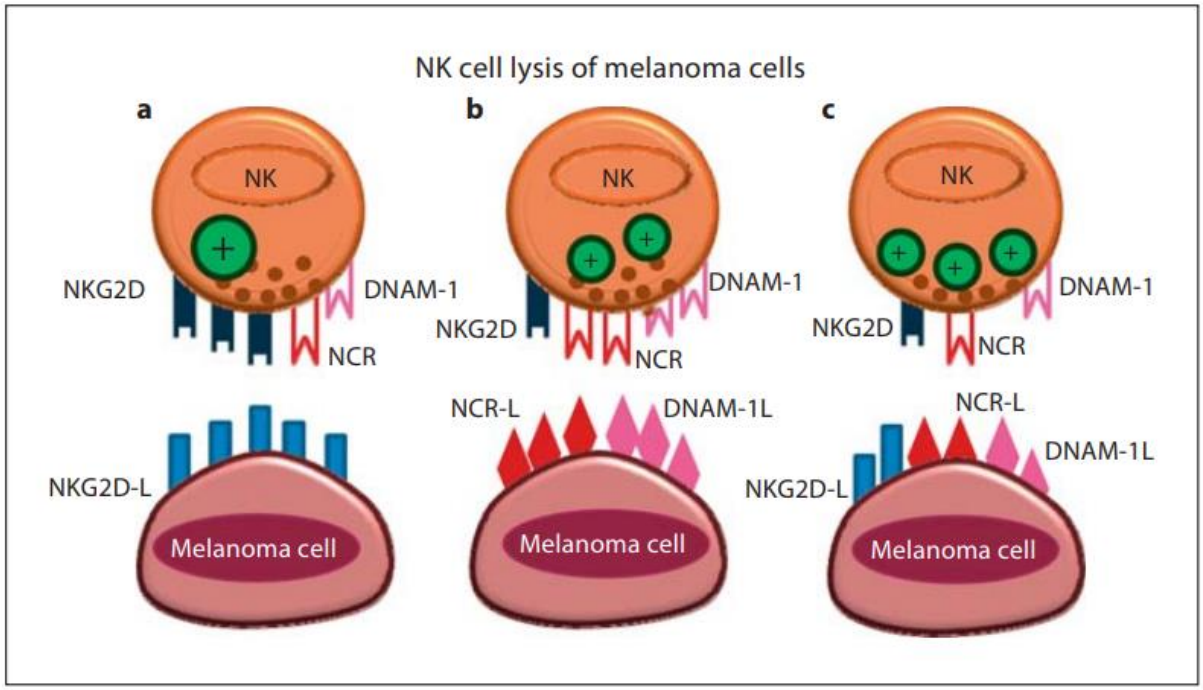

Figure 3: Interaction of different receptors on NK cells with melanoma cells (J Innate Immun 2011; 3:365–373 Review (Sara Morgado and Raquel Tarazona)

either absent or expressed at very low levels on healthy cells but during the tumour formation or under stress conditions the ligands for the NKG2D ligands can be upregulated[92]. The expression of the ligands for the NKG2D on cancer cells especially the melanoma cells showed that the majority do expressed its ligands, which further confirms the NK cell mediated cytotoxicity of melanoma cells. But these ligands for the human and the murine NKG2D are somehow different and show different binding affinities towards NKG2D. For instance murine NKG2D ligands include members of the retinoic acid early (RAE) family of proteins, as well as the minor histocompatibility antigen H60 and the murine ULBP like transcript 1 (MULT1) The association rates and the binding affinity of murine NKG2D towards RAE-1 and H60 is different for instance NKG2D binds to H60 with ~25-fold higher affinity than to RAE1. There is the competition between RAE1 and H60 for the binding and for the occupancy of NKG2D, and, thus, NKG2D can be occupied by only one ligand at a time. Besides the binding affinity of the ligands for the NKG2D that might influence the NK cell activation and effector function There are several factors that have a significant role to play for the activation of the NK cells which furthermore plays a role in the melanoma tumour cell killing but at the site of the tumour there are lot many things going on one hand there are many cytokines and soluble molecules that influence the activation state of NK cells for instance cytokines like

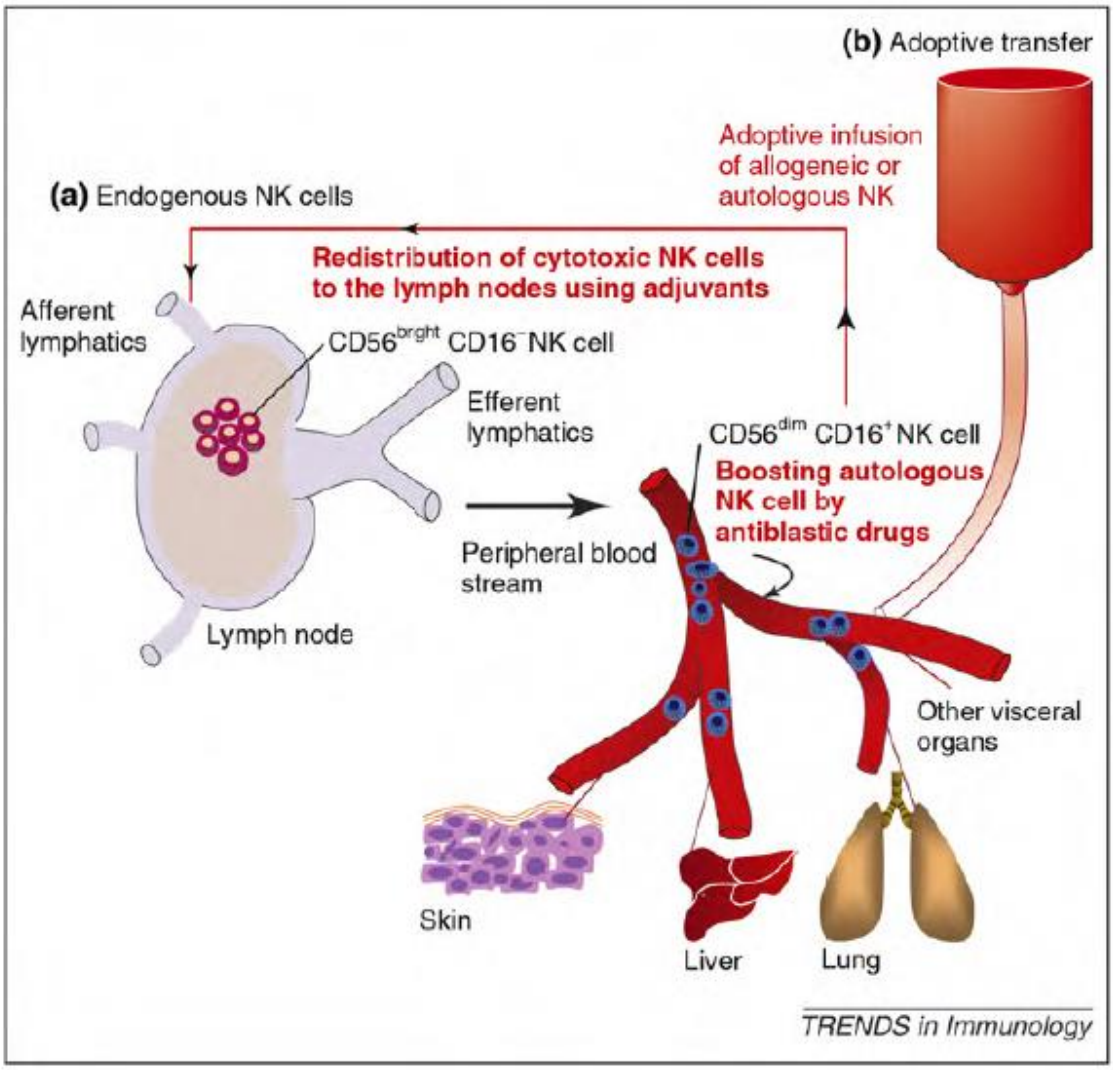

Figure 4: Manipulating NK cells to target melanoma (Trends in Immunology 31 (2010) 339–345 (Shannon Burke and Ennio Carbon)

IL-2, IL-12, IL-15, IL-18, and IL-21 can enhance the function of the NK cells In different ways [93]. There are several ways that NK cells use to overcome the melanoma tumour growth and promote its regression and clearance. New strategies and interventions based on NK cells that can help to better target the visceral metastasis include to employ adoptive transfer of high numbers of autologous or allogeneic NK cells Fig. 4 summarises it in detail. Melanoma cells are very sensitive to the NK cell mediated killing but over time they have also developed some smart strategies to overcome this NK cell mediated effect and escape the killing. This immune escape of melanoma cells is the great strategy to proliferate and metastasise there are many recent studies where they have investigated the link between the NK cell infiltrate and the melanoma phenotype resistance for instance, Depending on the NK cell infiltrate in the site of the tumour like presence of low number of NK cells, there is an enhanced upregulation of HLA-1 on the NK-susceptible melanoma cells that are not eliminated and this cell population acquire resistance to NK-cell-mediated killing and show enhanced proliferation in the long term co-

culture studies[87]. This Protective phenotype that melanoma cells acquire is basically related to the upregulation of both classical and nonclassical HLA-I molecules. However upon the HLA upregulation the melanoma cells can more likely become the target of the T cells, interestingly higher expression of nonclassical HLA-I molecules have been investigated and concluded that it favours the tumour resistance against the NK cell activity [94]. The Protective phenotype of different tumour cells is also acquired by IFN-γ that is released upon interaction of the NK and melanoma cells[95] it is suggested that IFN-γ exposure to the tumour cells leads to the upregulation of HLA-E and HLA-G. HLA-G upregulation has been correlated with the survival benefits of different cancer types[96]. The diagrammatic representation of NK cell mediated melanoma Immune editing is shown clearly in the Fig.5, Thus the explore of tumour cells to the IFN-γ leads to the upregulation of HLA that further hinths that T cells also have a immense role to play in case of melanoma. CD8+ (cytotoxic) T cells are generated in the thymus and express the T-cell receptor (TCR) these cells types express a dimeric co-receptor. This subset of cells is usually composed of mainly two chains one CD8α and one CD8β chain. CD8+ T cells recognise peptides presented by MHC class I molecules which is found on all nucleated cells. These cells recognize the target by binding to the antigen associated with MHC-I molecule on antigen presenting cells. After antigen challenge these cells differentiate into stem cell memory cells (TSCM), T central memory cell (TCM), T effector memory cell (TEM) and T effector cells (TEFF). Cytotoxic T cells are very important component of adaptive immune system and have an important role to play against intracellular pathogens, which includes both bacteria and viruses. CD8+ T cell (CTL) responses play an important role in the clearance of acute and control of chronic viral infections During acute virus infection there is the significant activation and dramatic expansion of the antigen-specific CD8 T cells during the first 1–2 weeks post infection. During this period of expansion, there are extensive cellular changes as T cells become activated and acquire effector functions, including the rapid production of antiviral cytokines such as IFN-γ and TNF-α and granzyme/perforin-mediated cytotoxicity [97] once the CD8 T cells reach at the peak during the virus infection around 90–95% of the effector CD8 T cell population dies by apoptosis. The surviving CD8 T cells differentiate into a pool of long-lived memory T cells and adopt a resting phenotype while as the memory CD8 T cells retain the ability to rapidly activate effector functions more quickly than naive CD8 T cells[98]. During chronic LCMV infection in mice virus specific CD8 T cells are present but they failed to produce the effector cytokine upon antigen stimulation[99]this effect is known as T cell exhaustion this phenemonenon of T cell inactivation occurs in the hierarchical manner[100]. The kinetics of T cell during the virus infection is shown in a detail in Fig. 6,In case of melanom

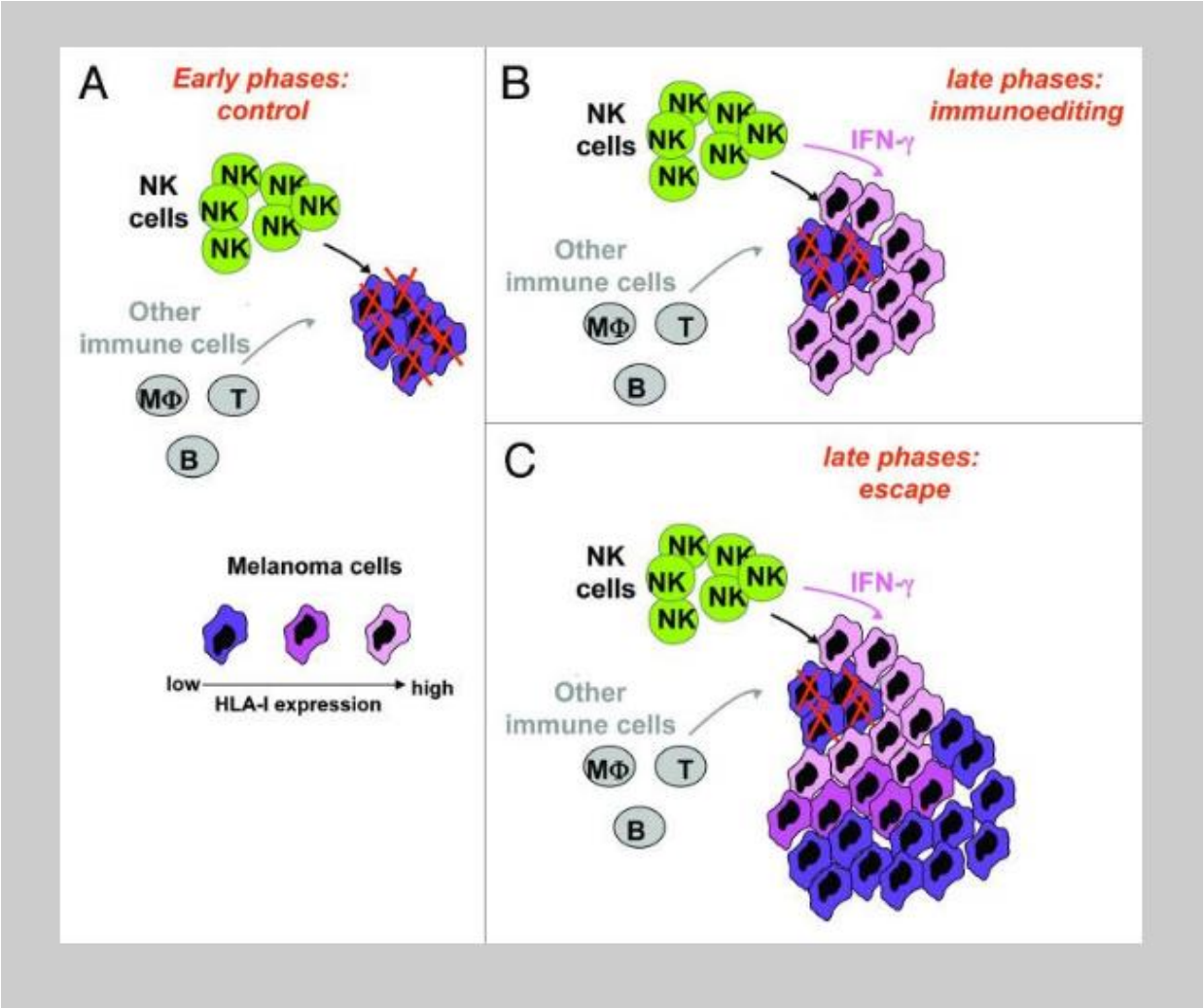

Figure 5: NK cell mediated melanoma immune editing (Oncoimmunology 2012 Dec 1; 1(9): 1607–1609. **(**Mirna Balsamo and Massimo Vitale**)**

-a T cells have a significant role to play there are different immune cells which infiltrate in the site of melanoma so the immune cell infiltration into the metastatic melanoma microenvironment (MME) is associated with better patient survival. T cells are the predominant immune cells in the MME and several studies have proven that those patients who have the highest $CD8^+$ T cell infiltration (not CD4) show the best clinical outcome[101]. There are several therapies available for the melanoma from immune check point blockers, CART cell therapy, Adoptive cell therapy and oncolytic virus therapy, so far only one oncolytic virus TVEC has been approved for the melanoma therapy worldwide. All of these approaches have some good benefits for the melanoma patients but until now there is no proper cure nor there is any drug with 100% efficacy there is still a lot to be done and explored in this field.

1.6 Interferons and Cancer

The interferons (IFNs) are a family of cytokines that perform many different functions they protect against disease by direct effects on target cells or indirectly by activating immune

responses. The production and actions of IFNs are tightly regulated and finely tuned to achieve maximal protection and avoid the potential toxicity associated with excessive responses. As IFNs can be produced and have the potential to act on both both tumour cells and immune cells so it is important to understand this reciprocal interaction will help and enable in future to design single of combination therapy for the better disease control also it will give us a chance to exploit IFN pathways and new 'omics'-based biomarkers to indicate responsive patients too.

A New understanding how different immune cells and immune components against the different tumours and how it is regulated by the different types of IFN provides exciting opportunities for redesigning when and how IFNs can be used in the clinic. Since tumour are extremely heterogeneous structures and are composed of different cells types and these cells types also Produce IFNs , so whether this antitumoral effect can be directly by interferon acting on the tumour cells or indirect effects via modulation of the immune response. Interferons (IFNs) are long known as pleiotropic cytokines with their confirmed and well established involvement in the development and treatment of cancer. There are three major types of IFN which can be primarily distinguished by their sequence identity, the nature and distribution of cognate receptors and to a lesser extent their inducing stimulus and cell of origin[102]. The human type I IFN genes encode a family of 17 distinct proteins (including 13 subtypes of IFNα, plus IFNβ, IFNε, IFNκ and IFNω) that bind to their cognate receptor, which is composed of the IFNα/β receptor 1 (IFNAR1) and IFNAR2 subunits. There is only a single type II IFN, IFNγ, which binds to a receptor composed of IFNγ receptor 1 (IFNGR1) and IFNGR2 subunits. The type III IFNs consist of IFNλ1, IFNλ2 and IFNλ3 (also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively) and IFNλ4, which bind the IFNλ receptor 1 (IFNLR1) and IL-10 receptor subunit-β (IL-10Rβ) heterodimeric receptor. Most of the cell types have the tendency to produce Type I IFNs in our body depending on array of different stimulus. Plasmacytoid dendritic cells (pDCs) are considerd to be one of the main cells types that produce IFNα because of their high basal expression of IFN regulatory factor 7 (IRF7), other cells may require priming through IRF3-regulated IFNβ expression. The *IFNB1* (which encodes IFNβ) promoter contains nuclear factor-κB (NF-κB) and activating protein 1 (AP1) as well as IRF binding sites, which is important in understanding the different circumstances of IFNβ production. As we know from the literature and that many cells express type I IFNs that can act in an autocrine or paracrine manner. In case of tumours infiltrating innate immune cells and adaptive immune cells including lymphocytes as well as the tumour cells themselves are capable of producing type

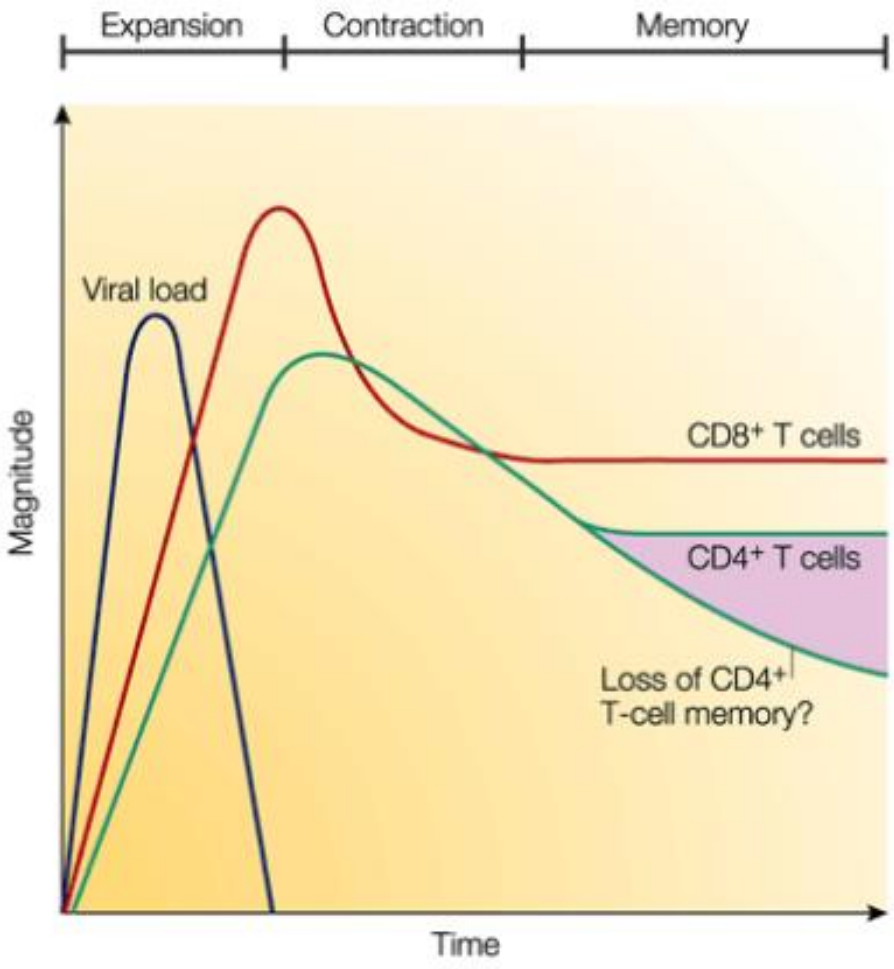

Figure 6: Antiviral CD8+ and CD4+ T cell response (Nature reviews Immunology volume2, pages251–262 (2002) (Susan M. Kaech and Rafi Ahmad)

I interferon and eliciting antitumor immune responses via tumour cell intrinsic or extrinsic mechanisms . It is described in a detail In the Fig. 7. There are many new and exciting insights regarding the endogenous and exogenous activation of type I interferons in the tumour and its microenvironment that has paved the path for the drug discovery and patient evaluation of interferon-directed strategies. Type I interferons have many key functions to play besides their know role in pro-apoptotic, anti-angiogenic, and immunomodulatory actions for many of these benefits they were expected to be the ultimate therapy against malignancies and infectious diseases. For instance In case of chronic myeloid leukaemia's (CML), complete cytogenetic response was achieved in 20–30% of the cases and increased survival was also seen[103]. Other clinical trials have indicated that the combination of IFN-α with Imatinib is more effective for the chronic myeloid Leukaemia patients in comparison to Imatinib alone. Breast cancer mouse models have shown decrease in tumour progression and metastasis to the bone and prolonged metastasis free survival upon the systemic administration of Type I

interferon and this effect was seen to be mediated through the NK cell mediated antitumor function[104]. There is adequate data available that is the indication that Type I interferon can be used for the treatment of different cancers. There are many genes which are regulated by IFNs that directly affect tumour cell growth, proliferation, differentiation, survival, migration and other specialized functions. It is well represented by the Fig. 8. There are many pathways that got implicated upon the Type 1 Interferon treatment for instance in case of prostate cancer cell lines upon IFNα treatment endogenous inhibitors of cyclin-dependent kinases, such as p21, and stalls cell cycle progression are upregulated. Similarly in case of patients with polycythaemia vera which is the proliferative disorder of the bone marrow, IFNα induced activation of the p38 MAPK pathway and growth suppression of ex vivo haematopoietic progenitor cells[105]. The family of CRK proteins is another pathway that has been implicated in type I IFN-dependent growth arrest, so use of Interferon could definitely modulate these pathways. Besides Type I interferon and their use in different cancers models there are lot of studies about Type II interferon and cancer. Interferon-gamma (IFN-γ) is a pleiotropic molecule with associated antiproliferative, pro-apoptotic and antitumor mechanisms. This effector cytokine has been used in the treatment of diseases and has gained a lot of success and promising results that might be the reason it is often termed as major effector of immunity. Interferon-gamma is secreted predominantly by lymphocytes such as CD4 T helper type 1 (Th1) cells and CD8 cytotoxic T[106] and also natural killer (NK) cells[107] and to a less extent by natural killer T cells (NKT) once upon the activation. It is shown by several studies that IFN-γ has a tremendous anti-cancer potential, for Instance FN-γ-deficient mice spontaneously develop lung epithelial malignancies and lymphoma[108] Also it is well documented that exogenous administration of IFN-γ is used for treatment of different cancer patients like ovarian cancer adult T cell leukemia and also in case of malignant melanoma[109]. It is also very interesting not only the exogensous administration has the anti-tumoral effect but the IFN-γ that is secreted by CTLs, NK cells, NKT cells acts as a potent anticancer cytokine[110]. once the IFN-γ is stimulated p21 and p27 are expressed to arrest the cell cycle by attenuating the stability of cyclin/cyclin-dependent kinase complexes There are direct and indirect ways through with IFN-γ induces apoptosis, either by directly downregulating the Bcl-2 or by activation of cathepsin and massive generation of reactive oxygen species (ROS) and induction of endoplasmic reticulum (ER) stress these are all included in the IFN-γ induced apoptosis[111]. Besides that IFN-γ has the immunomodulatory actions through which it can lead to the anticancer immunity. It can been seen in case of lung cancer patients that there is massive decreased expression of granzyme B, perforin, and IFN-γ clearly visible in infiltrating

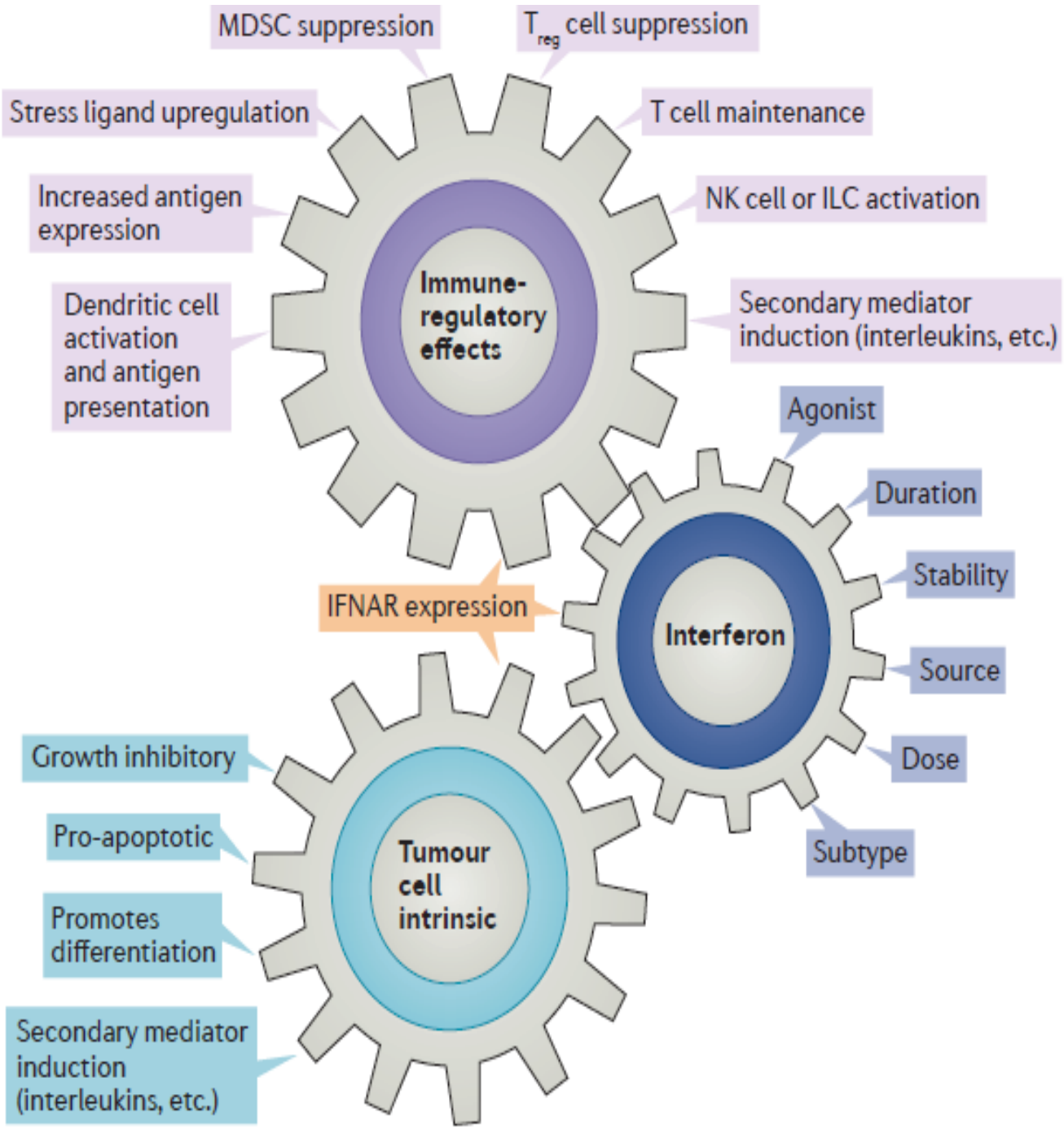

Figure 7: Cross talk of the intrinsic and extrinsic antitumour actions of interferons (Nature reviews. cancer volume 16 .March 2016 (Belinda S. Parker and Paul J. Hertzog)

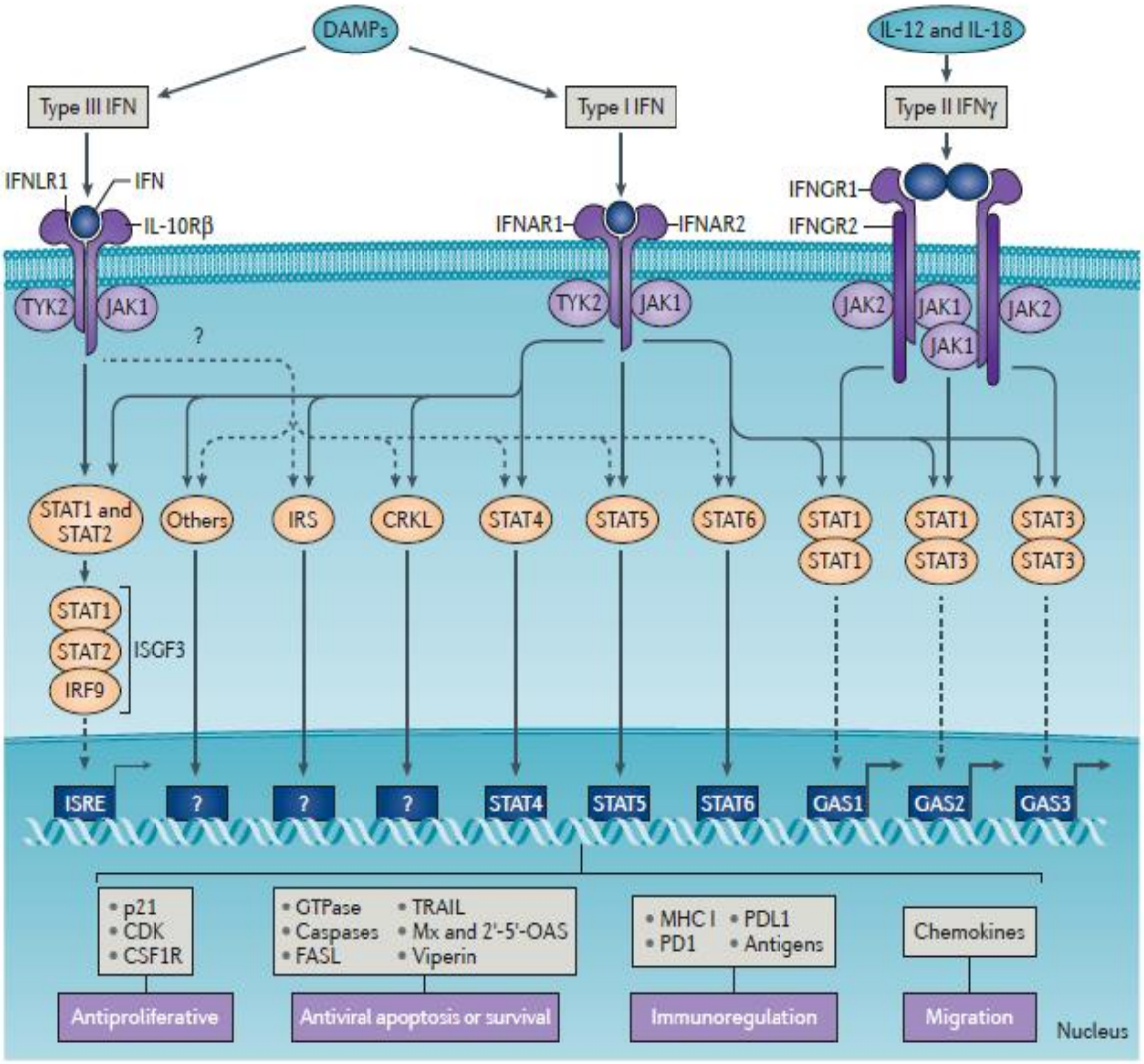

Figure 8: Signalling pathways of the interferons that mediate antitumor responses (Nature review Cancer volume 16. March 2016 (Belinda S. Parker and Paul J. Hertzog)

T cells, NK cells, and NKT cells [112]. In order to have a better antitumoral effects IFN-γ may increase the mRNA expression levels of perforin, granzyme B that will help clear the target cells/infected cells or tumour cells much efficiently[113]. IFN-γ has several strategies to be an effective antitumoral agent one of the important strategies is that it inhibits angiogenesis that further leads to the impaired proliferation and survival of endothelial cell also promoting the ischemia in the tumour stroma [114] Since this cytokine has also immense role to play regarding several immune cell activation it also play a main role in in the trafficking of T cell, NK and NKT cells into the site of tumour by inducing several chemokines like CXCL9, CXCL10, and CXCL11[115]. In case of the mice that is deficient of IFN-γ T cells failed to migrate in the site of the tumour[116]. Some of them chemokines that are produced upon the IFN-γ release from different immune cells also have the potential to prevent tumour angiogenesis for instance

CXCL10 prevents the endothelial cell proliferation thus inhibiting angiogenesis, since IFN-γ promotes the apoptosis of the endothelial cells that ultimately leads to the decreased blood flow within the tumour vasculature leads to the tumour regression[117]. This is considered as an indirect effect of IFN-γ where IFN-γ targets the tumour vasculature and leads to the massive tumour regression by inhibiting angiogenesis. So based on literature and many exciting results of IFN-γ mediated therapy in different cancers it has predictive values in cancer immune phenotypes. Based on the versatility of IFN-γ and its incredible results on cancer it has the potential therauptic benefits and some clinical trials have shown its great potential as the key immunotherauptic candidate for many cancers, For instance in cases of metastatic melanoma around 75% patients who were non-responders to anti-CTLA-4 therapy have shown that this was mainly associated with the genomic defects of IFN-γ signalling genes on tumours[118] similarly in case of ovarian cancer

Patients who have received the subcutaneously injection of interferon-gamma (IFN-γ)showed an enhanced overall survival with very mild toxicity, so IFN- gamma turned to be the first line chemotherapy for the for the patients with the ovarian cancer[119]. IFN-γ therapy also shows significant results when it comes to bladder cancer, it is very effective against stage different stages of bladder tumour recurrence like stage Ta, T1, grade 2 and this antitumor effect seems to be dependent on the significant activation of intramural leukocytes[120]. Thus from many In-Vivo studies from mice as well as the data from the humans IFN-γ seems to be the potential candidate for the treatment of different cancers.

1.7 Cancer therapy via the modulation of Immune system.

By Immune modulation in relation to cancer we primarily refer how we can harness the patient's immune system for the better treatment of different cancers their control, prevention and their complete eradication immune checkpoint-blocking antibodies are a tremendous success in the field of cancer immunotherapy and that is one of the prime example how we can harness the immune system to overcome different diseases. After the US FDA approval, Immune checkpoint blockade therapies are now being used for the treatment of a broad range of tumour types. The inhibitory signals of T cell activation that makes them non functional in case of chronic infection, these non functional T cell revive their function by the use of Immune checkpoint blockade that basically removes inhibitory signals on these cells[121] so basically these Immune checkpoint blockade inhibits T cell–negative costimulation which further helps to unleash antitumor T-cell responses that recognize tumour antigens. One of the primary biological functions of PD-1 is the maintenance of peripheral tolerance and keep the T-cell responses within a desired normal range. Because the PD-1/PD-L1 regulatory system is induced

by immune responses, which keeps an eye and control over the T cell response and that further minimises tissue damage so it is a kind of negative feedback loop. PD-1 is expressed upon activation of T and B lymphocytes (35). Once the T and B cells are activated they begin to express PD-1[122], the ligands of the PD1 are widely distributed and expressed on nonlymphoid tissues so what PD-1 basically does it dampens the T cell activation in the periphery[123]. Inflammatory cytokines such as IFNγ leads to the enhanced expression of PD-L1and to much lesser extend the expression of PD-L2[124]. Cytolytic and effector T cell function leads to the PD-1 regulation of T-cell activity**.** Once during the immune activation there is the mutual engagement of PD-L1 and PD-L2, PD-1 somehow transmits the negative signal which is mediated through tyrosine phosphatase SHP2 which further attenuates T cell activation or it can be the direct attenuation of the TCR signalling Via dephosphoralation of vicinity signalling elements through the recruitment of the SHP2.

In contrast to CTLA4, PD-1 leads to the attenuation of T cell activity but in a more direct way, but recent evidences have highlighted that CD28 is the primary target for PD-1 mediated T cell signal attenuation, so basically one can say that the suppression of T cell function that is mediated by PD-1 occurs through inactivation of CD28 signalling [125]. One of the main point is that PD-1 is often used as a marker of exhaustion but on the same hand PD-1 is a marker of activated T cells and exhausted T cells being one of the subset among them. Apart from PD-1 there are other molecules that are also coexpressed on the exhausted T cells and it includes LAG3 and TIM3. However the essential difference is that exhausted T cells are still functionally active but they have very much reduced capacity. For Instance, CD8 T cells which are exhausted are still able to contribute for the antitumour immune response but are somehow very less potent. T-cell exhaustion is an important phenomenon where you have enhanced expression of PD-1 on T cells which further limits the activity of the T cells in the presence of chronic antigen stimulation and it helps to preserve T- Cell clones that would otherwise get eliminated and die. Consistent with this idea elevated PD-1 signalling during the T cell exhaustion lead to the metabolic changes which drives the T cell exhaustion. Once PD-1 is ligated, it limits the glycolysis but at the same time it enhances the fatty acid oxidation and lipid catabolism so there is change in the energy production that takes place but in case of CTLA4 the ligation leads to glycolysis attenuation but it is primarily independent on the regulation of lipid metabolism. Upon the chronic infection where you have chronic antigen stimulation this leads to many dramatic changes in gene regulation and stable epigenetic reprogramming of T cells [126]. Taken together the Exhausted T cell state can be better explained by transcriptional, epigenetic and metabolic changes, which takes place during the cell activation. From many recent papers,

it become clear that such epigenetic changes can prevent rescue of exhausted state by checkpoint blockade and attenuate tumour response.

Immune check point blockers have a great success in the control of different cancer and many different phase wise clinical trials are done with the antibodies for the treatment of many different kinds of cancers too. In case of monoclonal antibody against PD-1 has shown a great clinical responses in multiple tumour types including melanoma, renal cell carcinoma, non-small cell carcinoma[127] hodgkin's lymphoma[128]and head and neck cancers [129] Quiet recently in 2013 a study was published with a large Phase I clinical trial with an anti-PD-1 antibody previously known as MK-3475 and it showed response rates of ~37–38% in case of advanced melanoma patients[130]. The co-receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed on T cells and is a central inhibitory regulator of T-cell proliferation and expansion It has a dampening effect on the activation process that helps in the termination of the T cell response. Its effect and striking phenotype was first seen in CTLA4 knock out mice these mice showed enhanced T cell activation/ auto proliferation that finally leads to the lethality of the mice. The expression and function of CTLA-4 is intrinsically linked with T-cell activation. The expression of CLTA-4 reaches to peak on day 2 and day 3 following the T cell –receptor (TCR) engagement [131]**.** TCR signalling can be dampened by CLTA-4 through the competition with the the costimulatory molecule CD28 for the B7 ligands B7-1 (CD80) and B7-2 (CD86) the reason being CLTA-4 has high affinity and avidity for it. Since both B7-1 and B7-2 provide positive costimulatory signals through CD28, so for the effective T cell attenuation there should be the inhibition of both molecules by CTLA-4. Once the T cell is activated there is significant upregulation of CTLA-4 and later CTLA-4 is trafficked from intracellular vesicles to the immunological synapse. But the accumulation of CTLA-4 at the synapsis will depend on how strong is the TCR signal so in could also mean that the cells who have the chance to receive strong stimulus will be inhibited by CTLA-4 faster [132]. Once the CTLA-4 is trafficked to the immunological synapses it is stabilized by b7 ligand binding that further helps it to outcompete CD28, this is one way where CTLA4 attenuates positive costimulation by CD28 and thus limits CD28 downstream signalling, which is primarily mediated by PI3K and AKT. There are two separate mechanism through CTLA-4 works and influences T cell activity one is by cell intrinsic mechanism through which CTLA-4 primarily attenuates T cell activity and other by cell extrinsic mechanism while cell-extrinsic effects basically relate with the regulation of function Via distal cell or cytokine especially the majority of cell extrinsic effects of CTLA-4 are mediated by Tregs[133]. Tregs also do express CTLA-4 this can further lead to the attenuation of T cell activation in a cell extrinsic manner by

restricting the B7 ligand availability for CD28 mediated positive costimulation of nearby effector T cells. The mice which are deficient of CTLA-4 and FoxP3 have multiorgan lymphocytic infiltration (lymphoproliferation), enhanced tissue destruction and autoimmune like syndromes that makes these mice to scum very fast [134] when there is the loss or you delete CTLA-4 during adulthood it leads to the activation and expansion of both conventional and regulatory T cells (Tregs) but surprisingly loss of CTLA-4 on Tregs gives the protection against experimental autoimmune encephalomyelitis (EAE)[135] which hints that enhanced expansion of Treg or their increased activation has the possibility to prevent autoimmunity.

Blockade of CTLA-4 with Anti-CTLA-4 monoclonal antibody results in the direct activation of $CD4^+$ and $CD8^+$ effector cells and it is well established from clinical and preclinical data. The graphicial representation showing in Fig.9 explains how use of blockade of Immune checkpoints enhance T cell response. The monoclonal anti-CTLA-4 antibody has sown successful results in different cancers models. There are several clinical trials with the Anti-CTLA-4 antibody in case of advanced-stage melanoma and renal cell carcinoma it has shown very good response rates in the range of 12%–19% [136]. Many of the clinical trials the response last from 18 months to >35 months which is longer. In many other cases the blocking antibody enhances the immunological activity such as immune cell infiltrates into the tumours and tumour necrosis. Monotherapy of Anti-CTLA-4 showed clinical responses in patients with melanoma (22), renal cell carcinoma [136]prostate cancer[137] urothelial carcinoma[138] and ovarian cancer[139]. Phase III clinical trials were done in patients with advanced melanoma the patients treated with Ipilimumab alone showed the median overall survival of about 10 months [140]. A recent study showed that the survival of around 10 years in a subset of patients [141] combined Immunotherapy for Cancer treatment. Besides a great success in the monotherapy of PD-1 and CTLA-4 but there is the great need of the combination therapy. Since there is the dynamic and robust immune response against Tumours also upon activation there is expression of multiple immune-check points and their ligands it is somehow very difficult to completely rely on one biomarker and then select the patients based on it that further concerns the need for the combination therapies Tumour microenvironment is comprised of multiple components so it is very essential to access all the components in the tumour bed that can help to distinguish between immunogenic tumours (hot tumours) that encompasses T cells, Cytokines , granzyme B while as in non-immunogenic tumour (Cold tumour) these components are missing. So the patients who have immunogeneic tumours will be treated with immune checkpoint therapy while as the patients who have non immunogenic tumours combination therapy will be a better alternative to have good results. The already data that is available in relation with different can

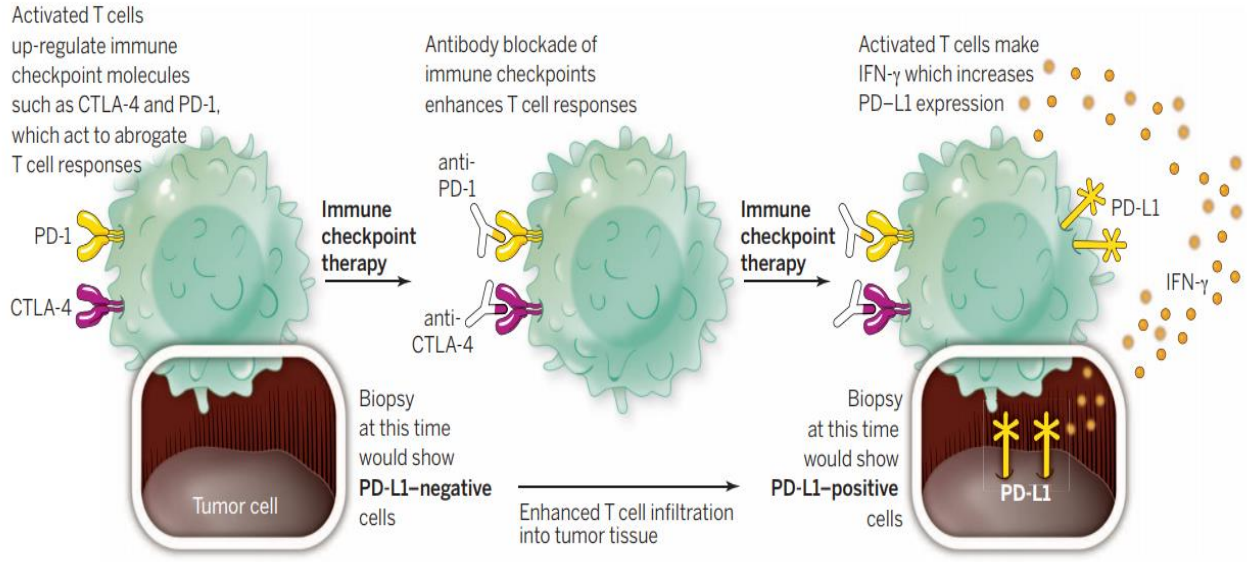

Figure 9: Blockade of immune checkpoints to enhance T cell responses (Science 3 April 2015. Vol 348 issue 6230 (Padmanee Sharma and James P. Allison)

-cer models hint that to overcome the limitations of monotherapy of anti-CTLA-4 and anti-PD-1/PD-L1 combination have been effective. In case of monotherapy of anti-CTLA-4 there is a significant increase of number of T cells and increase of IFN-gamma which has subsequently induce the expression of PD-L1 in the tumour microenvironment that leads to the inhibition of anti-tumour T cell response which can further enhance the beneficial effects from anti-PD-1 and anti-PDL1 therapies. Thus, the combination therapy of both anti-CTLA4 and anti-PD-1/PD-L1 helps in the immune infiltrate makes the tumour more or less immunogenic that further leads to the better survival effects and better clinical outcomes. Use of conventional cancer therapies which includes chemotherapy, radiotherapy, oncolytic therapy has the chances to lead to the tumour cell death and release of antigens to initiate activation of T cells and these cells then start to infiltrate in the site of tumour, there the combination therapy which may also include these agents plus in addition to immune check point therapies will further help to create Immunogenic tumour environment that will give further clinical benefits to patients. There are several clinical trails going on where radiation therapy is combined with immune check point blockers . For instance patients with metastatic castration-resistant prostate cancer responded to the combination therapy of Ipilimumab plus radiation therapy[142]several other combinations strategies also showed beneficial results like the combination of GM-CSF cell-based vaccines (GVAX with immune check point blockers like CTLA-4 in showed a better effect in the patients with pancreatic cancer [143] and this tumour type has been considered as the cold tumour type. Several other combinations therapies are also developed to enable

blockade of multiple inhibitory pathways like LAG-3, TIM-3, VISTA and BTLA to have better effects for instance use of Tim-3 blockade together in combination with PD-1/PDL1 blockade to reverse the exhaustion of CD8 T cells in the advanced melanoma [144] has been also effective. This there is the need for the further development of these kind of combination therapies and several others so that we have better anti-tumoral control and that will help for the better survival of cancer patients. Combination therapy is a new hope for many patients

1.7.1 Viruses to treat cancer

Viruses and their use to cancer has been in debate since a long time now and from many years it has achieved tremendous success with many phase I and Phase II clinical trials done with incredible results and that has led to the foundation of many companies that make the recombinant virus for the better treatment of many different cancers. Since viruses have the immense potential to interact with the immune system and can activate the innate and adaptive immune system very efficiently, Virus medicated immune activation can be explained as follows once the virus enters in the body it introduces new antigens to the immune system which are recognised as the foreign entity by our immune system[145] second interesting thing is virus carry ligands for pattern recognition receptors which have the capacity to trigger the innate immune system[146] another mechanism that virus uses for the activation of immune system follows the pattern like this, once virus invades the body after entering into the peripheral site it is drained to lymphatic system where they undergo spatiotemporally restricted replication in antigen presenting cells and thereby specifically activate innate and adaptive immune cells. [147]. Based on this idea of using viral therapy for different cancers, Oncolytic viruses have achieved some great results. Cancer cells are specifically targeted by oncolytic viruses because they are able to exploit the very same cellular defects that promote tumour growth. Some oncolytic viruses have been selected or designed to take advantage of frequent tumour-specific mutations in antiviral defence programmes. Since in the tumour there are many signalling pathways and transcriptional programmes that are activated and some oncolytic viruses are engineered to be dependent on these pathways. Another approach for the effective clearance of tumour cells is that to restrict the virus entry into the cells especially tumor cells based on the idea that many antigens that are overexpressed on the tumour cell surface.

A third approach is to restrict virus entry into cells based on the expression of antigens that are unique or overexpressed on the tumour cell surface. So oncolytic virus is ultimately killing the tumour cell and then its slowly takes over the cellular translational and

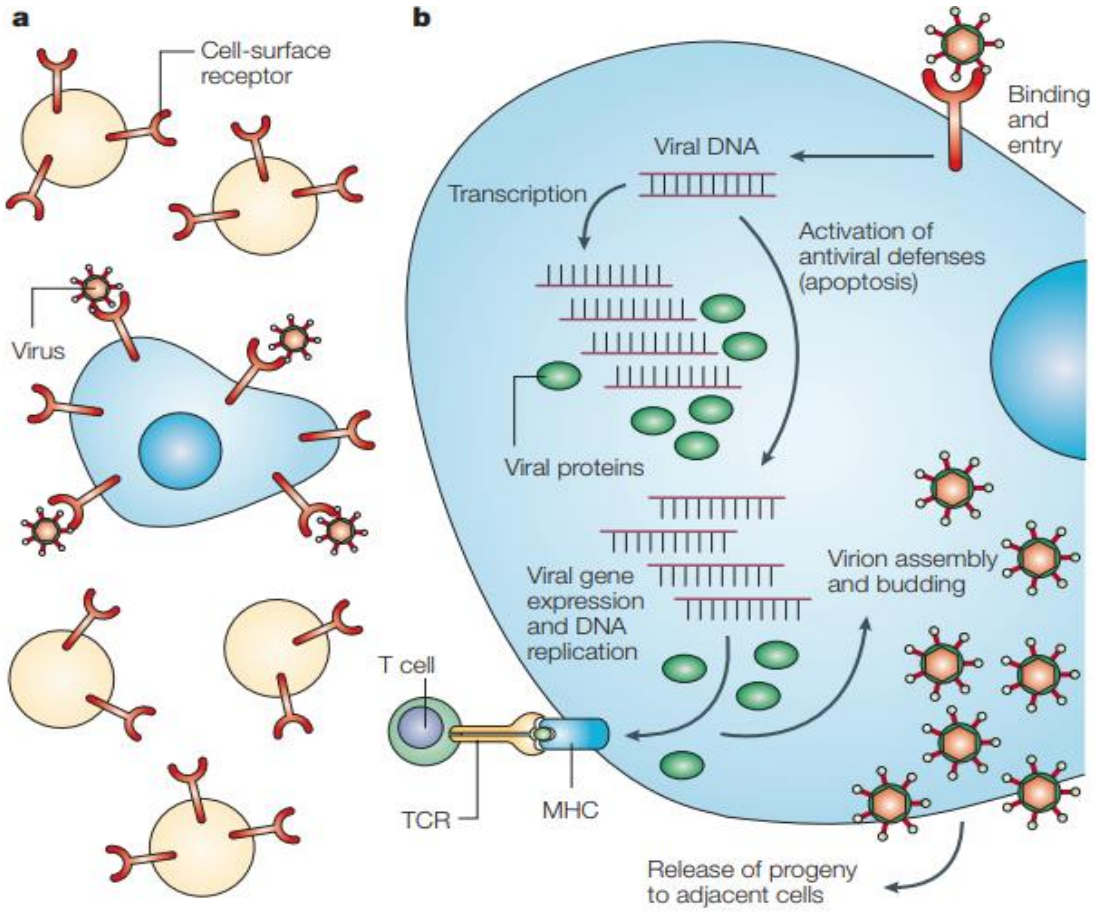

Figure 10: Infection and killing of tumour cells by an oncolytic virus (Nature Reviews | Cancer. December 2005 | VolumeE 5 (Kelley A. Parato and John C. Bell)

transcriptional machinery and finally leads to apoptosis as is indicated in detail in the Fig. 10 Although anticancer drug therapy has some success but oncolytic viral therapy offers several better advantages.one big advantage being using viruses as an anticancer agent is it can be modified by using recombinant DNA technology that helps for the creation of designer viruses another interesting property of the viruses is that they promote tumour specific inflammation and immune infiltrate at the site of tumour bed for instance in case of ovarian cancer[148] that makes oncolytic therapy multimodal therapeutics. Oncolytic viruses have the ability to replicate in the cancer cell and considering their genetic make up and their diverse properties, oncolytic viruses are thought to be some kind of miniature biological machines that can be programmed to specifically target and finally kill the tumour cell. Until now there are many viral species in their different stages of investigation and many efforts have been made to bring them to the clinic for the better outcomes when it comes to patients treatment especially for the treatment of different cancer types. One of the most important thing about viruses is that each one of the viral species have specific cellular tropism which further indicates which tissue they will infect and ultimately which disease they will cause for instance hepatitis B virus damages hepatocytes, HIV damages helper T lymphocytes, influenza virus damages airway epithelium, and rabies

virus damages neurons. What is also interesting about using viruses in the cancer treatment is by this modern tool of genetic engineering viruses can be genetically modified to more better and effective tumour cell lysis and killing, among many of them the most widely studies viruses in the perspective of their use in different tumour models herpes viruses seems to be most widely investigate, some of them have been found to have tumour cell tropism while others have been modified and engineered also. For instance genetically engineered and neuroattenuated herpes simplex viruses (HSVs) expressing different cytokines can improve survival when used in case of murine brain tumors[149], similarly it is not only confined to murine brain tumour but others cancers also,Talimogene laherparepvec (T-VEC), is a genetically modified herpes simplex virus, type 1, and is the first oncolytic virus therapy that has been approved for the treatment of advanced melanoma by the US FDA. In phase III clinical trials with the patients, suffering from advanced melanoma T-Vec has shown amazing results with durable response rates and progression free survival. Also in case of prostate cancer using poxviral vaccine along with the combination of immune check point blockers especially ipilimumab have seen to upregulate prostate specific antigen and expression of three costimulatory factors involved in antigen presentation and T cell activation[150]. Vesicular stomatitis virus is another oncolytic virus that also has been using in murine tumour models, it is a prototypic non-segmented, negative-strand RNA virus with inherent OV qualities. It has been seen that most of the cancer cells have impaired antiviral response induced by type I interferon that makes them more prone to the VSV than normal cells. Many groups have been trying to use again genetic engineering and develop recombinant VSV and further try in different cancer models. VSV-GP has been also very much effective in case of malignant melanoma[151]similarly rhabdovirus VSV-GP has also been used in case of murine prostate cancer. It was shown that in case of prostate cancer long term remission has been seen upon intratumour and also upon intravenous treatment of subcutaneous and bone metastasis[152] these findings regarding the VSV-GP in relation to murine prostate cancer model have urged the need for further investigation and rigorous experiments to expand this therapy for other tumour models too.

Besides oncolytic viruses being used to treat different cancers there are many attempts where people have tried to work with arenaviruses and check if they have antitumor properties especially LCMV have been a target candidate in this race for better antitumor effects. Arenaviruses are enveloped, bipartite with two single-stranded RNA genome segments with a diameter of 60–300nm. These non-cytopathic arenavirus have the tendency to propagate rapidly without directly harming susceptible tissues but what is most likely happens is the immune

response that is generated against the infection causes the severe tissue damage [153]arenavirus can infest humans and cause a wide spectrum of disease and disease outcome depends on the specific strain. Lassa virus and Junin virus are responsible for the Lassa and Argentine haemorrhagic fever[154]. The majority of these viruses are rodent-borne and the arenavirus family can be divided into two groups: the Lassa-Lymphocytic choriomeningitis serocomplex and the Tacaribe serocomplex, Of the arenavirus-animal model research, the majority of work has focused on Lymphocytic choriomeningitis virus (LCMV) and LASV pathogenesis. LCMV was the first arenavirus discovered during an outbreak of St. Louis encephalitis in 1933. The natural host of this virus is house mouse and for that reason it is distributed all over the world, which is important since most people infected are likely exposed to infectious rodents. Until now many different arenavirus are known LASV is also one of them and it causes lassa fever, Lassa fever was first described in 1969 in Nigeria during an investigation of a hemorrhagic disease in missionaries . Currently it is estimated that 100,000 to 300,000 LASV infections occur annually primarily in west Africa. The reservoir for the LASV is multimammate mouse (Mastomys natalensis). The main symptoms and clinical signs include chest pains, gastrointestinal illness, and pharyngitis human infection with the arenavirus strains lymphocytic choriomeningitis virus (LCMV, strain WE) and Candid#1, which is a clinically applied vaccine virus to protect against Argentine haemorrhagic fever and is usually asymptomatic or causes nonspecific symptoms such as fever, body aches, dehydration and malaise[155]. There are some prophylactic and therapeutic treatments available for arenavirus-induced symptoms. But most of the arenavirus induced diseases are treated with Supportive care and ribavirin, until now they are actually the most predominant ones. Upon LCMV-WE there is the strong immune activation especially it induces the strong T- Cell response which has a great antitumor effects in cancer models[156] recombinant single –cycle LCMV is considered a vaccine virus with the potential to immunize against tumour antigens[157]. So using on non-oncolytic arenavirus in advantageous in inducing sustained immune surveillance , Arenavirus such as LCMV will not kill the host cell by direct cytopathic effects thus the virus has immense chances to maintain its replication until the immune response is provoked within the tumour tissue, besides that it is also known that arenavirus replication cannot be limited only by strong type I interferon response[158] another advantage in using the LCMV is that it usually fails to induce rapid neutralising antibodies[159]. So the control of arenavirus is mostly dependent on CD8 +T cells so as long as the virus specific CD8+ T cells do not infiltrate in the site of the tumour, arenavirus will keep replicating for several days or even weeks even if the tumour cells tend to respond to the Type I interferon. Thus LCMV is an efficient model since it stimulates the immune response

thus enhanced replication will promote the strong tumour regression. It was shown is a study that LCMV works better than in comparison with oncolytic viruses even VSV-GP and rVACV in case of murine head and neck cancer (MOPC) and Sw480 [160]. Based on the data that we have generated and the literature available LCMV is one of the emerging virus that has the displayed great antitumoral activities

1.7.2 Chemokines and cancer

Chemokines are the key molecules that promote the migration of leukocytes and their induction is facilitated by inflammatory cytokines, growth factors and pathogenic stimuli[161]. The transcription of many target genes that are involved in cell invasion, motility and interaction with the extracellular matrix is also mediated through the chemokine signalling[161]During the inflammation there are many processes that chemokine signalling can coordinate which primarily involves homeostatic transport of haematopoietic stem cells (HSCs), lymphocytes and dendritic cells, until so far there are around more than 50 chemokines that have been discovered, Fig. 11 explains it in great detail besides that there are at least 18 human seven-transmembrane-domain chemokine receptors. There are many factors that determine the chemokine receptor expression on individual cells like lineage, differentiation stage, microenvironment factors for instance chemokine concentration, presence of inflammatory cytokines and hypoxia have a significant role to play. During the infection, tissue leukocytes are the first cell type that produce chemokines besides that endothelial cells, fibroblasts and epithelial cells also do produce chemokines and can generate chemokine gradient. There is a great variety of chemokines that have been detected in neoplastic tissues as products of either tumour cells or stromal elements, like mentioned previously chemokines have been associated with leukocyte recruitment in tumours[162] in different cancer types CCL2 and CCL5 levels are correlated with high numbers of intra-tumour myeloid cells [163] Further which have the tendency to differentiate into mature tumour-associated macrophages (TAM) in the local microenvironment, in case of cancer stroma these cells are the key inflammatory components and have the tendency to affect different aspects of neoplastic tissue. One of the pro-tumoral function of TAM in the establishment of tumours is the suppression of adaptive anti-tumour immune response One of the characteristic of this cell type is the IL-12 low and IL-10 high phenotype and higher production of TGFβ, prostaglandins and indole amine dioxygenase. On the top of that TAM themselves are the potent producers of chemokines. The immune suppressive activity of TAM is exerted indirectly by the release of chemokines such as CCL17 and CCL22 since these chemokines attract T cell subsets devoid of cytotoxic functions. chemok

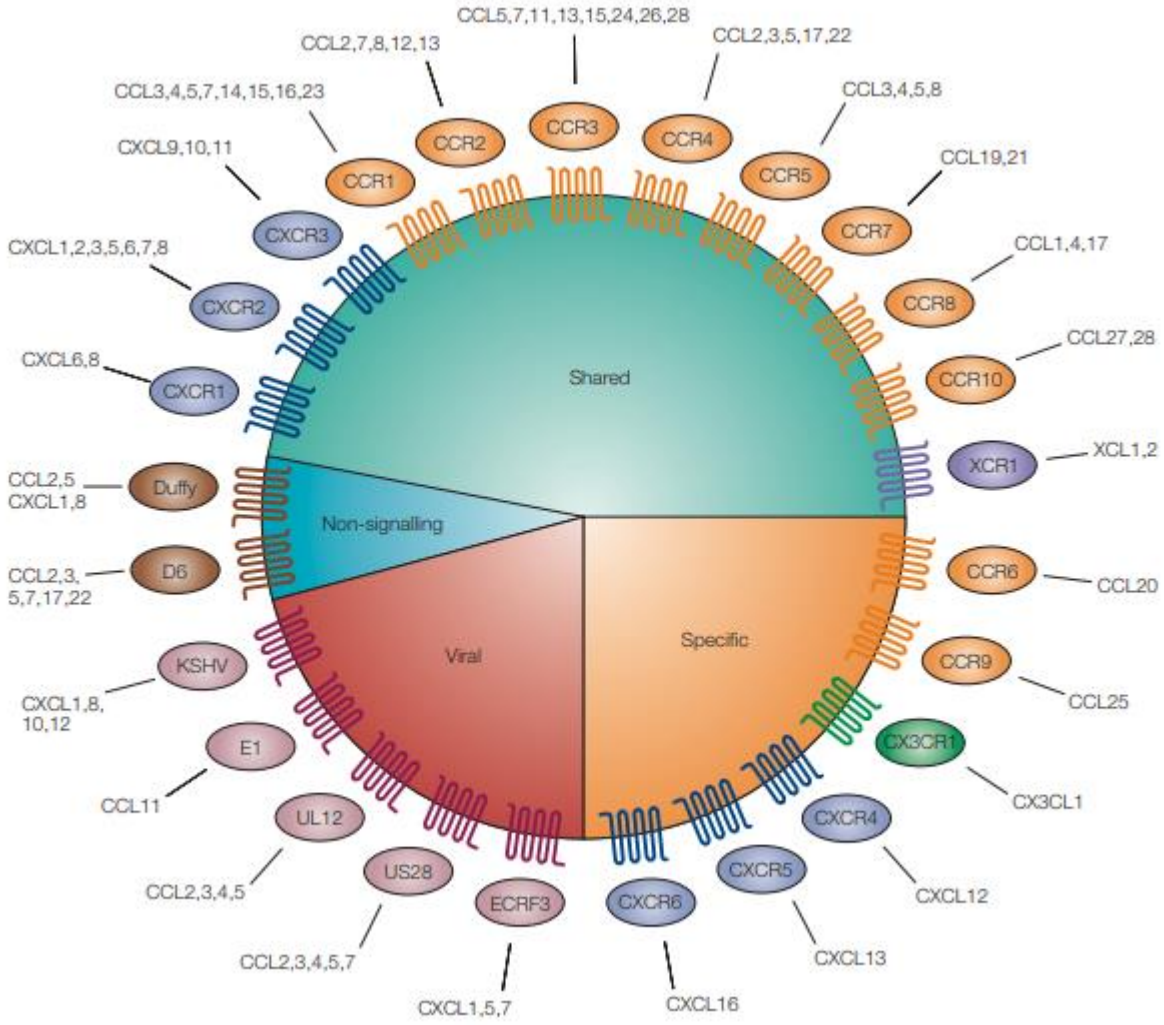

Figure 11: Cancer and chemokine network (Nature Review Cancer 2004 (Fran Balkwill)

-ines can also boost the neoplastic progression by shaping the leukocyte infiltrate of human tumors and one of a good example of this mechanism is oncogenic virus HHVB, the genome of the HHVB encodes three CC chemokines (vMIPI II and III) that attract th2 lymphocytes and Treg cells that are lacking anti-viral activity [164].

1.7.3 Chemokines and metastasis.

Once tumour cells undergo metastasis they have the remarkable ability to invade and survive in ectopic tissue, lymphatic environments as well as ability to reside and proliferate at distal site. It is still not well understood whether malignant cells metastasize to environments that are favourable for their growth or whether organs are endowed to attract the specific malignant cell types a phenomenon called homing theory , Many different cancers and their progression is facilitated by chemokines for instance it was shown by Muller and colleagues That breast cancer

metastases is in part governed by specific interactions between CXCR4 and its ligand SDF-1/CXCL12, It was reported in different studies that metastatic breast cancers selectively express CXCR4 and migrate to organs that express high levels of its respective ligand CXCL12, also known as SDF-1. In case of many different types of metastatic cancer cell lines like breast cancer cell lines, lymph node metastases and liver metastases chemokine receptor CXCR4 is consistently upregulated while as in case of normal epithelial cells the levels of this chemokine are undetectable[165]. While as its ligand CXCL12 is expressed in the most common sites of breast cancer metastasis, liver, bone marrow and lung [165]. This hints towards the fact that metastatic breast tumor cells selectively express CXCR4 which leads them to organs with high expression levels of CXCL12. It was also shown that if you block the CXCR4-CXCL12 interactions this leads to the significant reduction of metastasis of breast tumor cells to the lymph node and lungs. There are many different studies which have proved that CXCR4 is not confined to breast cancer but it has a significant role to play in any different cancers which includesbladder[166]prostate[167]pancreatic[168]melanoma[169]glioblastoma[170] oesophageal[171]acute lymphoblastic leukaemia [172]ovarian[173] and neuroblastoma[174]. Thus you can say that many different independent studies have confirmed the role of CXCR4 in metastasis, Besides CXCR4, CCR7 has been documented to play a significant role in case of leukaemia's and lymphomas. Many leukaemia's and lymphomas highly express CCR7 and experience frequent metastasis to the lymph nodes [175] Besides that there are different studies that have proved that CCR7 mediates metastasis of T-cell leukaemia to the central nervous system (CNS). In case of breast cancer higher expression of CCR7 is also correlated with lower survival and worse prognosis[176] but it is not only confined to breast cancer but also to different other cancer types which include melanoma, gastric , head and neck and colorectal cancer . besides that there are other chemokines that have been documented to play a significant role in different cancer types CCR9 and CCL25 are equally important. Thus chemokines play a vital role in the metastasis.

1.7.4 CCL5 and Cancer

CCL5 also known with its second name rantes (regulated on activation normal T cell expressed and secreted) is encoded by CCL5 gene in humans. The CCL5 gene was discovered in the year 1988 and later on the isolation and characterisation of the protein was done in the year 1990. CCL5 belongs to C--C chemokine subfamily the chemokines of this class present adjacent cysteines and comprises the majority of the chemokines[177] the chemokines of this class present adjacent cysteines and comprises the majority of the chemokines. Rantes was initially considered as the T cell specific cytokine but later on several independent studies have shown

that it induces the in vitro migration and recruitment of dendritic cells, T cells , NK cells, mast cells and basophils[178]. There are many different cell types that produce CCL5 like fibroblasts, macrophages, platelets, eosinophils,epithelial and endometrium, since being by different cell types it has many different role to play from pathogen control, amplification of inflammation in several disorders like atherosclerosis and cancer[179]. Chemokines interact with their receptors that are located on cell surface that is the mechanism how their biological functions are mostly mediated. There are some known receptors for CCL5 through which this chemokine shows its effect it includes CCR1, CCR3 and CCR5[177]. Fig. 12 shows clearly the interaction of CCL5 with its different receptors**,** besides that the binding affinity of CCL5 for it different receptors is very different, CCL5 binds strongly with CCR5 followed by CCR1 and least with the CCR3. Since CCL5 is very important chemokine responsible for cell recruitment in vivo CCL5 oligomers are immobalised endothelial glycosaminoglycans (GAGs) and interact with the receptors expressed by incoming leukocytes in the blood vessels. For the in vivo activity, oligomerisation has an important role to play as well as glycosaminoglycans (GAGs) binding [180] the biological function of CCL5 is enhanced after the heterodimerization with CXCL4, it is well know that chemokine receptors are fully functional as monomers but they have the tendency to form homo or heterodimers which helps them with new properties. Oligomers of CCL5 interact with the the receptors at the three extracellular loops and the aminoterminal portion[181]. Once you have the receptor ligand complex it leads to the conformational change in the receptor that activates the subunits $G_{\beta\gamma}$ and $G\alpha i$ of G protein, leading to an increase in levels of cyclic AMP, inositol triphosphate and intracellular calcium, Furthermore these signalling events lead to the polarization and translocation of NF-kB, which results in the increase of phagocytic ability, transcription of proinflammatory genes and cell survival. CCL5 can induce both G protein dependent and independent signalling once G proten dependent signalling takes place CCL5 receptor complex is internalised via chlathrin mediate endocytosis associated to the adapter molecules Adaptin-2 and Beta-arrestin. It is assumed that CCL5 is associated with chronic inflammation and might play a direct role in angiogenesis and could also lead to progression of tumours, angiogenesis is a phenomenon of sprouting of new vessels from pre-existing activated endothelial cells, which migrates and proliferates to form new vessels. It has been shown by several studies that chemokines contribute to angiogenesis by balancing the stimulation or inhibition of endothelial cell proliferation and branching In contrast different studies have concluded that CCL5 may be a proangiogenic factor, the angiogeneic effect of CCL5 is mediated though CCR1 and CCR5 but for that reason CCL5 oligomerization and binding with GAGs is very indispensable[182] as angiogenesis is one of

the main property of cancer cells this helps them to get the nourishment , continue to grow and to promote potential routes for metastasis. CCL5 is expressed in different cancers for instance in breast cancer it is highly expressed in breast tumor cells at primary tumor site, metastatic site and regional lymph nodes that actually hints CCL5 expression is acquired in the course of malignant transformation[183] it is know that enhanced expression of CCL5 in case of breast cancer promotes metastasis and progression of the disease and even relapse[184], in case of melanoma both CCL5 and CCR5 are expressed on melanoma cells as compared to normal melanocytes that is an indication of a state of higher malignancy and progression of disease[185]. Primary melanomas and cutaneous metastases have higher expression of CCR5[186] but on the other hand there are some studies that have proved that enhanced expression of CCL5 on melanoma is protective and leads to disease free progression, enhanced expression of CCL5 on melanoma cells leads to the massive infiltration of natural killer cells (NK cells) to the site of the tumour that has the cytotoxic effects on melanoma cells and lead to their cytolysis[187], In case of human gastric cancer cell lines increased levels of CCL5 is an indication of enhanced metastatic potential[188] which again hints for the tumour promoting role of CCL5 in gastric cancer. Many studies have proved that there is the correlation between the enhanced CCL5 expression and formation of metastasis. This leads to the possibility that CCL5 could be used as the tool to predict metastasis and circulatin CCL5 levels were check before the anticancer treatment. It was seen that CCL5 levels were higher in patients than in healthy controls and the levels were higher stage IV patients than in stages I or II-III[189]. Thus in case of gastric cancer enhanced CCL5 levels can be used as the prognostic marker for the disease progression, similarly in case of colon cancer enhanced CCL5 expression promotes the tumor growth, Chang et al recently showed that CCL5 mediated immune escape in case of mouse colon cancer CT26 knockdown of CCL5 decreases the apoptosis of tumor infiltrating CD8+ T and thus reduces the tumour growth in mice[190]. In case of ovarian cancer there are also many reports in correlation with CCL5, malignant ovarian biopsies show detectable level of CCL5 but also normal biopsies show minimal expression [191]. CCL5 is also involved in prostate cancer, both CCL5 and CCR5 are expressed in human prostate cancer (PCa) cell lines and CCL5 stimulates human prostate cancer cell proliferation and invasion[192] chemokines also have a key role to play in case of multiple myeloma (MM), in case of MM there is the accumulation of cancer cells in bone marrow this leads to the dramatic alterations in the bone marrow (BM) microenvironment that ultimately is detrimental and leads to tumour progression , bone destruction and resistance to therapies[193]. There are several reports that have found CCR-5 ligand CCL3in MM cell line and freshly isolated MM cells [194]. The patients with

multiple bone lesions secrete higher amounts of CCL3 and CCL4 than those with less advanced bone involvement. The serum levels of MM patients are elevated and correlate with the bone disease and progression[195] and also the increased levels of CCL3 in case of bone biopsies has a significant correlation with the increased angiogenesis and enhanced bone diseases[196]. The multiple myeloma cell lines and cells derived from patients have been seen to express CCR5 and CCR1[194]. The migration of the MM cells depends on the CCL5 and the extend to which they can migrate will depend on the CCR5 expression levels[197]. Thus the CCL5 and CCL3 that is realised from the tumour tissues and their receptors support MM progression. Similarly in case of classical Hodgkin lymphoma the growth and the survival is dependent of many chemokines. It is assumed that chemokines in case of HL can lead to the immune suppression by supressing the cytotoxic immune response that further might also lead to the increased cell survival, enhanced proliferation and tumour progression[198]classical Hodgkin lymphoma cells express and secrete different types of cytokines/chemokines besides that they do also express different types of chemokine or cytokine receptors. CCL5 and CCR5 are expressed by cHL derived cell lines, tumor cells from cHL lymph node tissues and macrophages and lymphocytes including bystander cells too[199]

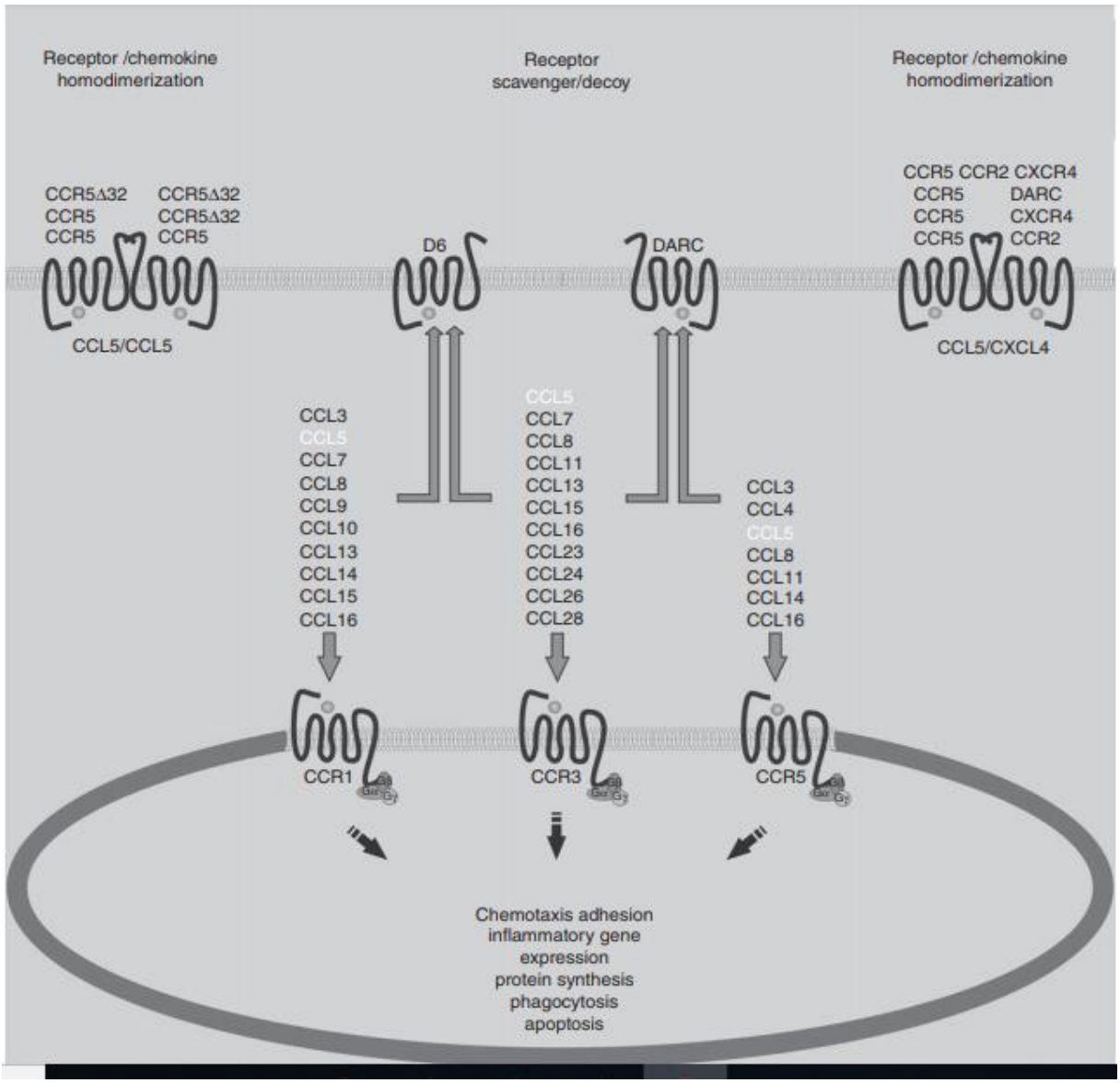

Figure 12: CCL5 biology (Targeting CCL5 in inflammation, Expert Opin. Ther. Targets (2013) 17(12) (Rafael Elias Marques)

1.8 Aims of the thesis

Cancer is the term which is used for the collection of related diseases. In almost all different types of cancers some cells begin to divide without any control and then spread into sorrunding tissues. Cancer can start almost anywhere in the human body once the cancer deveops then the cells become more and more abnormal, some cancerious tumors are malignant they are the ones which have the tendency to migrate to different regions of the body through the blood or lymphatic system and there they form new tumorus, There are different types of cancers known until now from our previuos publication it was shown murine squamous oropharynx carcinoma cell line (MOPC) showed the massive tumor regression upon intratumor LCMV injection and once the MOPC tumor bearing BL/6 mice were followed after the LCMV injection these mice showed better survival as compared to the untreated mice. Based on this study the first scientfic objective of my thesis was to check the effect of arenavius on genetically diverse tumor entities. So we took different types of human cancers ranging from the most deadliest cancers like anaplatsic thyroid carcinoma,lung adenocarcinoma, and melanoma. It was clear that arenavirus therapy (LCMV-WE) showed the massive antitumot effects not only in one tumor type but in the broad range of tumors. The sccond scientific objectve of my thesis was to find out why some tumor enteties showed massive resistantce to arenavirus based immune tharepay. So we looked for the underlying molecular mechanism responsible for the resistance and upon investigation we found out the tumors that responded very well to the viral therapy have the enhanced upregulation of CCL5, We performed many in vivo and in-vitro studies where we investigated tumor sections taken from tumor bearing NodScid mice and also from the tumor cells that we cultured it become quiet clear that upon LCMV infection there is significant CCL5 production.

The third scientific objective of my study was to find out what are the immune cell infiltrates that migrate to the tumor site and lead to this massive antitumor effects. Since CCL5 being a chemokine and it is know that it can lead to the infiltration of many different immune cells but in our study since we use NodScid the easy guess was NK cells and when we check it was clear that upon LCMV infcetion there is much more NK cells infiltrate in the responding tumor as compared to the non responding one. Our last question of interest was how is CCL5 being produced upon LCMV infection, to further investigate it and explore the mechanism we use different inhibitors that block the downstream molecules and one of the most interesting for us

was PI3K inhibitor, upon PI3K inhibition there is significant decreease of CCL5 production, Thus even after virus infection there is no significant CCL5 production by melamoma cells.

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-b mercaptoethanol	Sigma, Munich
Brefeldin A	eBiosciences
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 Mg2+, Ca2+	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
Methanol	Sigma, Munich
Methylcellulose	Merck Fluke
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

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
Rosewell Park Memorial Institute (RPMI1640)	Biochrom AG, Berlin
Minimum Essential Medium (MEM)	Sigma Aldrich

kits

Reagent	**Company**
QuantiTect Reverse Transcription Kit	Qiagen

Antibodies

Antibody	**Company**
Anti-F4/80	eBioscienes
Anti-CD11c	eBiosciences
Anti-CD3	eBiosciences
Anti-CD11b	eBiosciences
Anti-CD4	eBioscienes
Anti-CD8	Abcam

Anti-IFNg	eBiosciences
Anti-LCMV-NP (VL4)	Lab produced
Anti-Ly6C	eBiosciences
Anti-Ly6G (clone 1A8)	eBioscienes
Anti-PDL1 eBiosciences	Bio X Cell
Anti-VSV-GP	Lab produced
Goat anti-Rabbit Biotin	Jackson
Streptavidin (Fluorescent labelled)	eBiosciences
Anti-Rantes/CCL5	Abcam
Anti-rat IgG1 Fitc	Abcam
donkey anti-Rabbit IgG	Jackson
Anti-Rabbit Cy5	Jackson
Goat anti-Rabbit Biotin	Jackson

2.1.2 SYBR GREEN PROBES

Name	**Catalog**	**Company**
HsCCL5	QT00090083	Qiagen
HsCCL3	QT01008063	Qiagen

HsCCL2	QT00212730	Qiagen
HsCXCL10	QT01003065	Qiagen
HsCCL4	QT01008070	Qiagen
HsCCL1	QT00203154	Qiagen
HsMX1	QT00090895	Qiagen
HsIRF1	QT00063917	Qiagen
HsIRF3	QT00012866	Qiagen
HsIRF5	QT00092736	Qiagen
HsIRF2	QT00056455	Qiagen
HsIRF6	QT00059745	Qiagen
HsIRF7	SI00448693	Qiagen
HsOAS1	QT00099134	Qiagen
HsAIM2	QT00042273	Qiagen
HsIFIT2	QT00219345	Qiagen
HsIFIT3	QT00100030	Qiagen

Hs STAT1	QT01008945	Qiagen
HsNOXA1	QT00074438	Qiagen
HsIFIT3	QT00100030	Qiagen
HsIFI16	QT00066675	Qiagen
HsNUB1	QT00033950	Qiagen
HsIFITM2	QT01883413	Qiagen
HsGAPDH	QT02504271	Qiagen

2.1.3 EQUIPMENTS

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

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

2.1.4 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°C chamber with 5% CO2.

Cell lines	Source	Cell-type	Company/Collaborator
Mamel-86A	Human	Melanoma Lymph node	Prof. Annette Paschen Uniklinic Essen.
Mamel-51	Human	Melanoma	Prof. Annette Paschen Uniklinic Essen
Mamel-86C	Human	Melanoma	Prof.Annette Paschen Uniklinic Essen
H1975	Human	Lung	ATCC
A549	Human	Epithelial	ATCC
B16-OVA	Murine	Melanoma	Prof.P.Knolle, TU,Munich
C643	Human	Anaplastic	Prof. Lars Möller, Uniklinic Essen.
RT4	Human	Urinary Bladder	ATCC

2.1.5 MICE

Among many mice strains that were used in the study some of them 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.

Tcrb-/- mice

Tcrb–/– mice are genetically engineered immunodeficient mice which lack the T cell receptor beta chain [200]

NodScid

The SCID mutation has been transferred onto a non-obese diabetic background. Animals homozygous for the SCID mutation have impaired T and B cell lymphocyte development. All these mice were breed in our animal facility and housed in single ventilated cages under standard temperature and pressure conditions.

NSG

This is the new generation of severely immunodeficient mouse also known as the nonobese diabetic (NOD)-scid gamma mice. These mice have a background of SCID mice and IL-2 receptor gamma chain deficiency. These mice are severely immunodeficient due to the lack of mature B, T, and NK cells; in addition, they lack cytokine signalling such as IL-2, IL-4, IL-7, IL-9, and IL-15. NSG mice live longer than some other immune-deficient mice because unlike SCID mice, they do not develop thymic lymphomas . These mice were breed in our animal facility (breeding room) and housed in single ventilated cages under standard temperature and pressure conditions.

2.2 Methods

2.2.1 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°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°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°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 intratumour route. But most of the experiments were done by using LCMV (WE) strain.

2.2.2 LCMV titer Assay

For detection of viral tiers in organs and cell cultures, plaque assay was done with MC57 cells. The organs were homogenized in tissue lyser 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 37^0C for 3 hours and then overlay was added and incubated again for 72 hours at 37^0C.

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 Na2HPO4

25% (v/v) 0.1M Citric acid

20 mg o-Phenylenediamine dihydrochloride

50 µl of 30% H2O2

50 ml ddH20

2.2.3 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 °C until use. All the histological cutting was done below -10°C. All the histological cutting was done below -10°C

2.2.4 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°C until microscopy was performed.

2.2.5 LCMV and CCL5 staining

Immunocytochemistry was performed on cells grown on coverslips. Different human cancer cells like melanoma cells (Mamel-86A, Mamel-51 and Mamel-86C) also human lung carcinoma (A549) and H1975 ($2x10^5$ cells per well) were grown in 24 well plate on the cover slips. Then 200 µL of triton X solution was added and incubated for 20min room temperature after that two washing with PBS containing 2% FCS. Then 200 µL of PBS with 10% FCS was added per well just to block non-specific binding followed by one hour incubation. Primary antibody VL-4 RAT Anti-LCMV mab in addition Anti-rantes antibody (ab9679) was added and samples were incubated for 1-hour room temperature after that two times washing with PBS containing 2% FCS. Secondary antibody Anti-rat IgG1 Fitc and PE conjugated donkey anti-Rabbit IgG (Lot 131220 Jackson) were added (1:100 dilution) followed by one hour incubation and two times washing with PBS containing 2% FCS and finally Coverslips were mounted on microscope slides using mounting medium (S3023, Dako). Images were acquired with a fluorescence microscope (KEYENCE BZ II analyser).

2.2.6 CCL5 staining by Flow cytometry

Human melanoma cells (Mamel-86A) and lung carcinoma (A549) cells were grown in 24 well plates overnight, afterwards cells were treated with different inhibitors like LY294002 (Sigma Catalog Number S1105 Conc10µmol) BX795 (Catalog SML0694 Sigma Conc 2µmol) MG131(Catalog474787, Sigma, Conc 2.1 µmol) SP600125(Catalog number S5567 Sigma Conc.10µmol),U0126 (Catalog number U120 company Sigma, concentration 1µmol) and after one hour cells were infected with LCMV WE (MOI-1),after four hours of incubation add 50

microliters of BFA to each well (1:200 dilution) incubate the cells for 45 hours .Wash the plate with PBS (1X). Add 200ul of trypsin to each well at room temperature (RT) mix the well properly and don't leave the cells in trypsin longer than 3 minutes and then transfer the suspension into FACS tubes, centrifuge 1500 rpm for 5 minutes and afterwards decantate the supernatant and resuspend in 200 microliters of 4% formalin and incubate 15 minutes at room temperature then wash with 1% saponin in FACS buffer decantate the supernatant and add primary antibody cocktail 50 microliter per sample of CCL5/Rantes (abcam 9679 Lot GR5419-63) 1:100 dilution and VL4 (1:50 dilution) incubate the samples for 1 hour at 4 degrees. After incubation wash the samples with facs buffer containing saponin centrifuge the samples at 1400 rpm for 5 minutes and decantate the supernatant, Now add the secondary antibody (50 µl per sample), Anti-rat IgG1 Fitc and PE conjugated donkey anti-Rabbit IgG (Lot 131220 Jackson) in 1:100 dilution and incubate at 4 degrees for one hour now was the samples once with facs buffer in saponin centrifuge for 1400 rpm for 5 minutes discard the supernatant and resuspend the pellet in facs buffer and then read the samples.

2.2.7 Tumor induction

Patient derived melanoma cells early-stage III lymph node metastasis (Mamel-86A) which was excised 2 months after diagnosis late recurrent lymph node lesions excised in years 1.5 and at stage IV of the disease (Mamel-86C) another patient derived melanoma cell line (Mamel-51) , lung carcinoma A549 and H1975, anaplastic thyroid carcinoma(C643)and bladder cancer (RT4) were maintained at 37℃ with 5% CO2 in DMEM medium supplemented with 10% heat-in-activated fetal calf serum, penicillin, streptomycin and Glutamine. 2×10^6 cells were injected subcutaneously in 200ul Medium on the left flank in the mice. Tumor size was determined by the formula L×W×W /2 where L=length, W=width, on the indicated days.

2.2.8 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°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°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°C for 10 min, quantified using nanodrop and then stored at -20°C .

2.2.9 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°C for 30 min. Enzyme reaction was then inactivated by heating the samples at 92°C for 2 min. Samples were stored at -20°C until further use.

2.2.10 Cell culture

All the cells were maintained in humidified incubator at 37°C with 5% CO2 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 SYBRGreen 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 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°C before loading. Equal amount of protein was loaded in the gel and was initially electrophoresis was performed at 80V for complete stacking of proteins. Once the proteins are stacked, voltage was turned up to 100V 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

30gm Tris base

144 gm Glycine

10 gm SDS

1000 ml ddH20

	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)	98µl	30µl
10% APS (w/v)	98µl	30µl
TEMED	8.1µl	5µl

2.2.13 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 90V 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°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 blocking3buffer 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**
30gm Tris	10x transfer Buffer (100ml)
144 gm Glycine	Methanol (200ml)
ddH2O (1000 ml)	ddH2O (700ml)

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

2.2.14 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°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.

2.2.15 Genotyping

In order to determine the transgenic state of the mice, genotyping was performed. Mice were ear-marked and 2-4mm of tail was cut and put into 600 µl digestion buffer. Proteinase-K was then added at the concentration of 20ug/ml. Samples were then incubated at 56°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°C and then washed twice with 70% ethanol. Samples were then air-dried and dissolved in DEPC water at 60°C uantified and stored at 4°C For genotyping PCR, 50- 100ng 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.

2.2.16 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 "*" ($p \leq 0.05$), "**" ($p \leq 0.01$), "***" ($p \leq 0.001$), "****" ($p \leq 0.0001$).

2.2.17 Enzyme Linked Immunosorbent Assay (ELISA)

CCL5 levels in the supernatant were analyzed by enzyme linked immunosorbent assay with enzyme-mediated calorimetry. ELISA plates were coated with capture antibody (diluted in PBS, 100 µl/well), placed on plate shaker o/n at RT (over the weekend at 4°C). Plates were washed (ELISA-washer, 3x 300 µl PBT/well). Blocking buffer was applied to prevent unspecific binding to the plastic surface for 1h (150 µl/well, at RT). The plates were washed, samples and standard curve dilutions of the respective chemokine was applied,H2O as blank; samples were diluted in RD if applicable). Plates were incubated on plate shaker (65 rpm) for 90' at 37°C (or 2h at RT). Plates were washed. Biotin-coupled detection Ab was added (diluted in RD, 50 µl/well) followed by 90' of incubation at 37°C with shaking (or 2h at RT). Plates were washed. Streptavidin-coupled horse raddish peroxidase (HRP) was added (1:200, RD, 50 µl/well, 5 µl Streptavidin in 1 ml RD). The plates were shaken for 20' at 37°C in the dark. After

washing, freshly prepared TMB substrate (reagent A : reagent B equally mixed, BD) was applied to the plates (50 µl/well). Plates were developed by incubating in dark at room temperature (blue colored product). The reaction was stopped by H_2SO_4 (25 µl/well, color turns to yellow). chemokine concentration was measured with a micro plate spectrophotometer at an absorption of 450nm (Epoch BioTek) and analyzed with Gene% software

3. Results

3.1 Arenavirus therapy induces massive tumour regression in genetically diverse tumours

There are several therapies for cancer treatment but most of them have little effects that is why cancer immuno-therapy has emerged as a new promising strategy to fight different types of cancers and it is based on the idea of activation and arming of the immune system against tumours. There are many approaches among which arenavirus based therapy is one of the most encouraging. LCMV-WE induces a strong T-cell response which can be antitumoural in cancer models[156, 201]. Therefore, recombinant single-cycle LCMV is considered a vaccine virus with potential to immunize against tumour antigens[157]. Now to determine immunological signals by virotherapy we tested different tumour cell lines for their response to LCMV Including A549 (lung carcinoma), Anaplastic Thyroid carcinoma (C643), Pharynx (FADU) and Bladder Cancer (RT4) as shown in Figure (Fig. 13A), (Fig. 13B) and (Fig.13D) LCMV has a massive antitumor properties and leads to the complete tumour regression while as LCMV therapy showed limited antitumour effect in case of bladder cancer (Fig. 13C)

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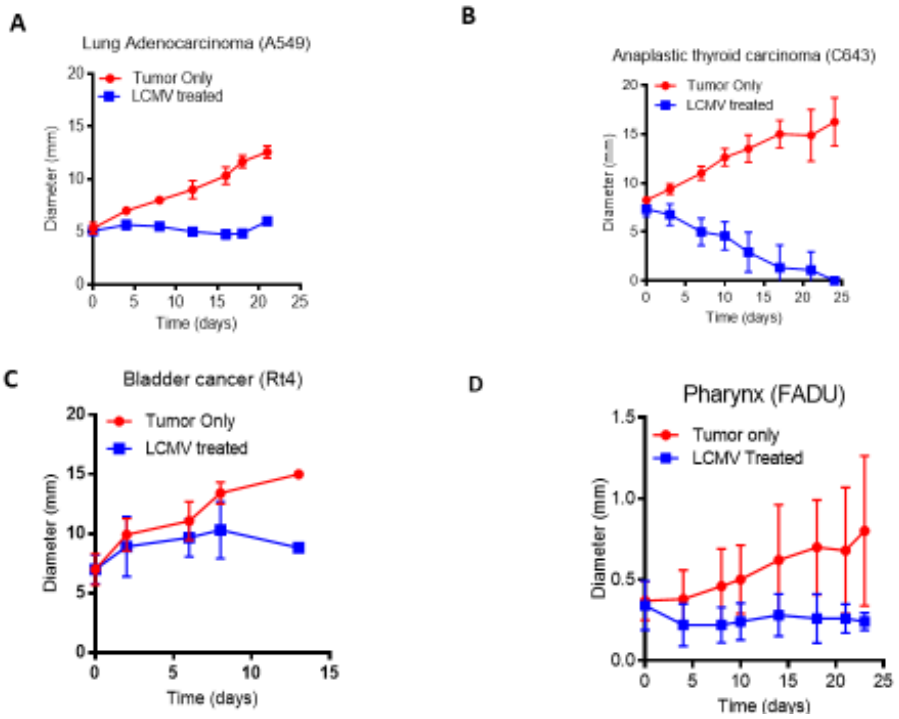


Figure 13: Arenavirus leads to the massive tumor regression.

(Fig. 13A) Human lung adenocarcinoma cells (2 x 10^6 cells per mice) were injected in the flank of the NodScid mice and once tumour was 5mm LCMV-WE (2 x 10^6 PFU) was injected intraumorally in one group (n=6) and another group was left untreated (control n=6) and tumour growth was followed (Fig. 13B) Human anaplastic thyroid carcinoma cells We (2 x 10^6 cells per mice) were injected in NodScid mice and once tumor was 5mm LCMV WE was injected intratumorally in one group (n=3) and another group left untreated as control (N=3) and tumour growth was followed. (Fig. 13C) Similarly human bladder cancer cells We (2 x 10^6 cells per mice) cells were injected in the flank of the NodScid mice and once tumour was 5mm approximately LCMV-WE(2 x 10^6 PFU)was injected intratumorally in one group (n=6) and another group was left as untreated (n=6) tumor growth was followed (Fig. 13D) Human pharynx cancer cells We (2 x 10^6 cells per mice) were injected in the flank of NodScid mice and once tumor is 5mm LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=5) and another group left untreated as control (n=5)

3.2 A single shot of LCMV prevents tumour relapse and leads to better survival of mice

Viral therapy showed effective tumour clearance since we inject LCMV WE in small tumours so we were very much curious to check whether we can see such antitumor effects not only in small tumours but also big tumours. So we raised several questions first to check effectiveness of LCMV WE therapy in big tumours, Second to investigate the long term survival and relapse of the LCMV-WE treated mice. It was clear anaplastic thyroid carcinoma tumour bearing NodScid mice when treated with LCMV-WE showed better survival (Fig. 14A) and when these mice were followed for the long term survival to check for any signs of relapse it was a clear observation that all these mice after one time intratumor LCMV-WE injection did not show any sign of tumour relapse (Fig. 14C) To address this question whether arenavirus therapy can be applicable to big tumours we injected tumour bearing NodScid mice with LCMV-WE intratumorally and followed the tumour regression to our surprise LCMV showed massive tumour regression and the mice at one point where completely tumour free (Fig. 14B).

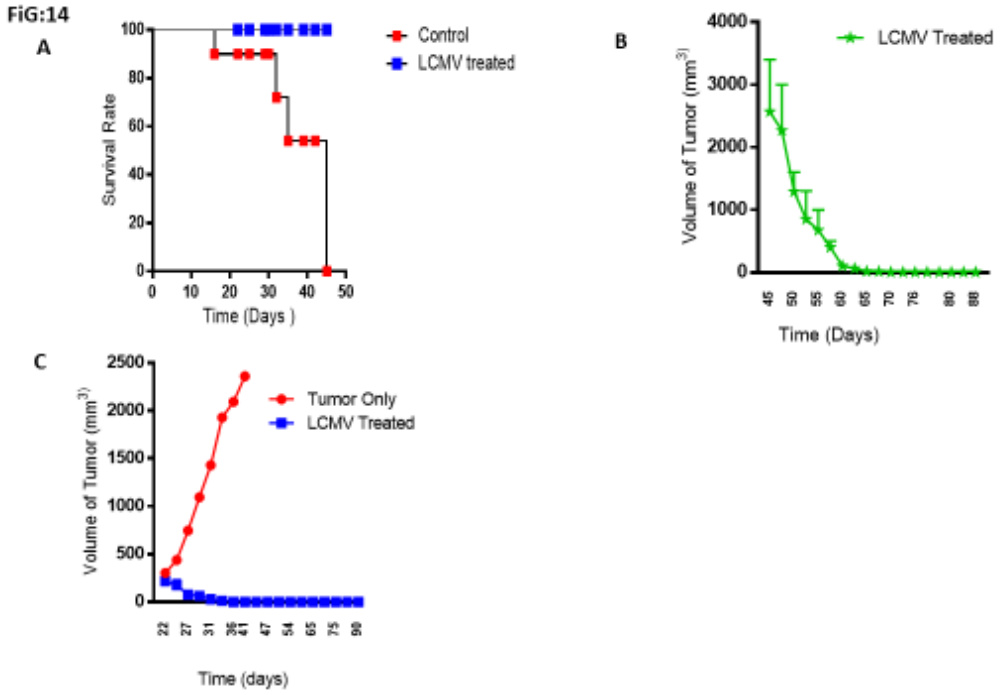


Figure 14: Arenavirus therapy leads to fast tumour clearance and better survival.

(Fig. 14A) Human anaplastic thyroid carcinoma cells (2 x 10^6 cells) were injected in NodScid mice in the flank and one group was treated with LCMV-WE (2 x 10^6 PFU)) (n=8) and another group left untreated as control (n=8). (Fig. 14B) To check the effect of viral therapy in big tumours we treated the tumor bearing NodScid mice with LCMV-WE (2 x 10^6 PFU) and followed the tumor growth. (Fig. 14C) Anaplastic thyroid carcinoma cells (2 x 10^6 cells per mice) were injected in the NodScid mice in the flank and once tumor was 5mm size one group was treated with LCMV WE (2 x 10^6 PFU) and another group left untreated (n=3) and long term tumor growth and relapse was followed.

3.3 LCMV therapy showed massive antitumor effects in melanoma.

Like we discussed that LCMV therapy showed the massive antimoral effects in genetic diverse tumour entities then we go one step further and look for different human melanoma types and investigated if LCMV therapy leads to some promising results. We analysed three human melanoma cell lines that have been taken from patients at different stages of the disease progression. Upon intratumor LCMV injection in Mamel-86C tumour bearing NodScid mice there was a massive regression as compared to untreated tumours (Fig. 15A). Similarly NodScid mice were injected with Mamel-86A cells and later one group was treated with LCMV- WE and upon viral therapy there was the complete elimination of the tumour and mice were tumour free (Fig. 15B), similarly we also injected the NodScid mice with the Mamel-51 tumour cells and later treated the tumor bearing mice with LCMV-WE but surprisingly the tumour growth

didn't show any significant sign of regression upon viral therapy (Fig. 15C) this we further investigated what is the underlying mechanism for this cell line to show the resistance to viral therapy, this made us curious to look for the different immune infiltrates, interferons and different chemokines to check if there is some special pattern that we might see in this tumour type. Thus it become very clear that viral therapy is a powerful new emerging tool that has a great potential for tremendous anticancer properties.

Fig:15

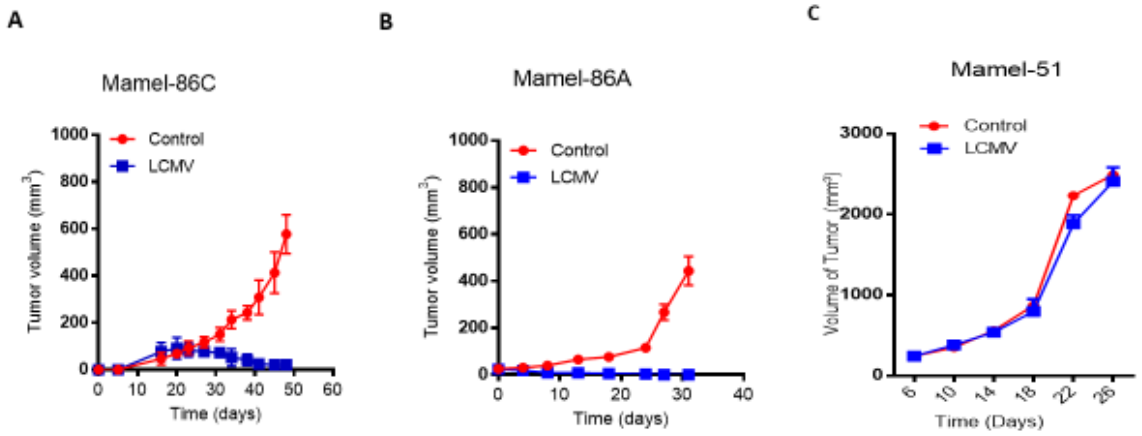


Figure 15: Human melanoma responded very well to the arenavirus therapy

(Fig. 15A) Human melanoma mamel-86C cells (2 x 10^6 cells) were injected in the flank of NodScid mice and once the tumour was 5mm LCMV WE (2 x 10^6 PFU) was injected intratumorally in the NodScid mice (n=3) and another group was left untreated as control (N=3). (Fig. 15B) Melanoma cells, Mamel-86A (2 x 10^6 cells) were injected in the NodScid mice subcutaneously in the flank and once tumor was of 5mm approximately LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=4) and another group left untreated as controls (n=4) tumor growth was followed. (Fig. 15C) Similarly melanoma cells Mamel-51 (2 x 10^6 cells per mice) were injected subcutaneously in the flank of the NodScid mice and once the tumor was approximately of the size of 5mm LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=4) and another group was left untreated as control (n=5) tumor growth was followed.

3.4 LCMV WE replicates very well in all three human melanoma cancer cell types.

Next we tried to address this question of massive tumour regression shown by melanoma especially Mamel-86A, Mamel-51 and Mamel-86C upon arenavirus therapy and also tried to investigate the reason for the resistance to LCMV therapy shown by Mamel-51. At first we thought it could be possibly that virus does not replicate in Mamel-51 compared to other melanoma cells types. So we took all the three human melanoma cell types grow them in-vitro

and once cells were confluent we treated them with LCMV-WE and after 24 hours the cells were stained to check for the LCMV-NP staining and it was clear that there was no defect in the virus replication, In fact the virus replicated almost equally in all melanoma cell types (Fig. 16) that leads us to this conclusion that there is no defect in the viral replication and that hints towards the fact that there are possibly some other immune components or cell types that have the role to play and furthermore contribute to this massive antitumor effects that we observed in our In-Vivo animal models.

Fig:16

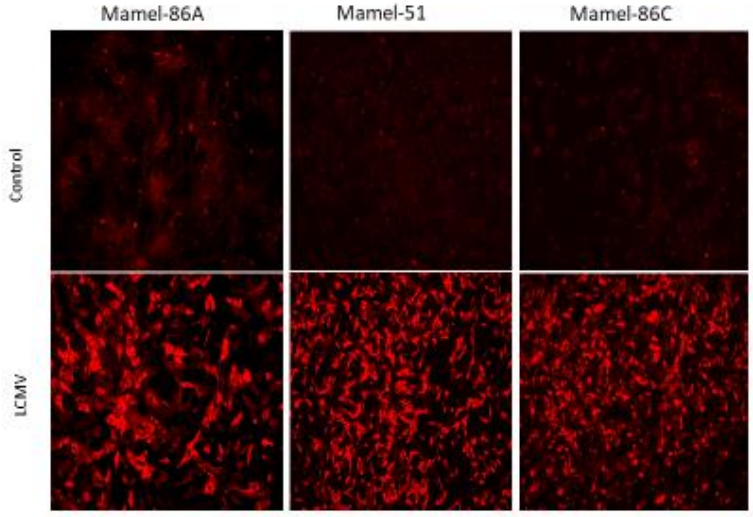


Figure 16: Viral replication is same in all the human melanoma cells.

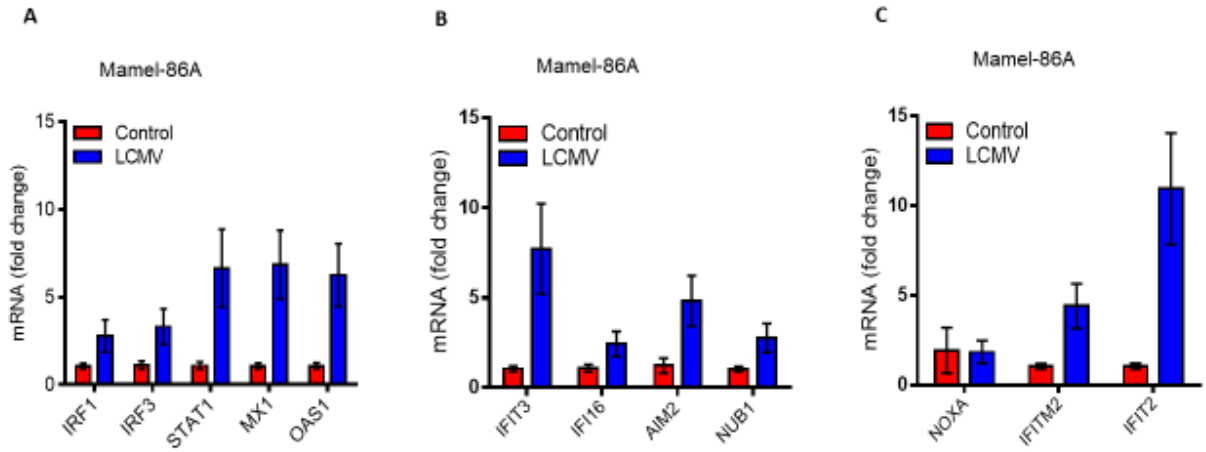
Fig: 16 Different human different human melanoma cell types Mamel-86A, Mamel-51 and Mamel-86C (2 x 10^6 cells per well) were cultured in 24 well plate on cover slips and once the cells were confluent we treated them with LCMV WE (MOI-1) and after 24 hours the cells were stained to check for LCMV NP replication. From the staining results, it becomes clear that LCMV WE replicates very well in all the human cancer cell lines.

3.5 Arenavirus therapy promotes the upregulation of interferon and pro-apoptotic genes in human melanoma.

Next, we were wondering although the replication of the virus is almost the same in all the three different melanoma cells types so what could be the possible explanation for our In-Vivo antitumoral effects. To further look for better explanation for our phenotype , We injected the NodScid mice with Mamel-86A cells subcutaneously in the flank and later on treated the tumours with LCMV-WE intratumor and finally we made the RTPCR from the RNA extracted from the tumour and investigated the profile of different interferon (Fig.17A) and pro-apoptotic genes (Fig.17B and Fig.17C). It was clear that LCMV-WE injection in Mamel-86A tumour bearing mice leads to the upregulation of most of these genes, Next we analysed whether we

can see the similar effects in case of non-responding melanoma tumour model (Mamel-51). Like previously we again injected the melanoma cells in the flank of NodScid mice but this time with mamel-51 cells and subsequently injected LCMV-WE intratumorally and another group was left untreated, RTPCR was done to check for the expression of interferon genes (Fig. 17D) and Pro-apoptotic genes (Fig. 17E and Fig. 17F), thus we conclude upon intratumor LCMV injection in mamel-86A tumor bearing NodScid mice leads to the massive upregulation of interferon and pro-apoptotic genes while as we don't see such a robust and strong upregulation in case of Mamel-51.

Fig. 17



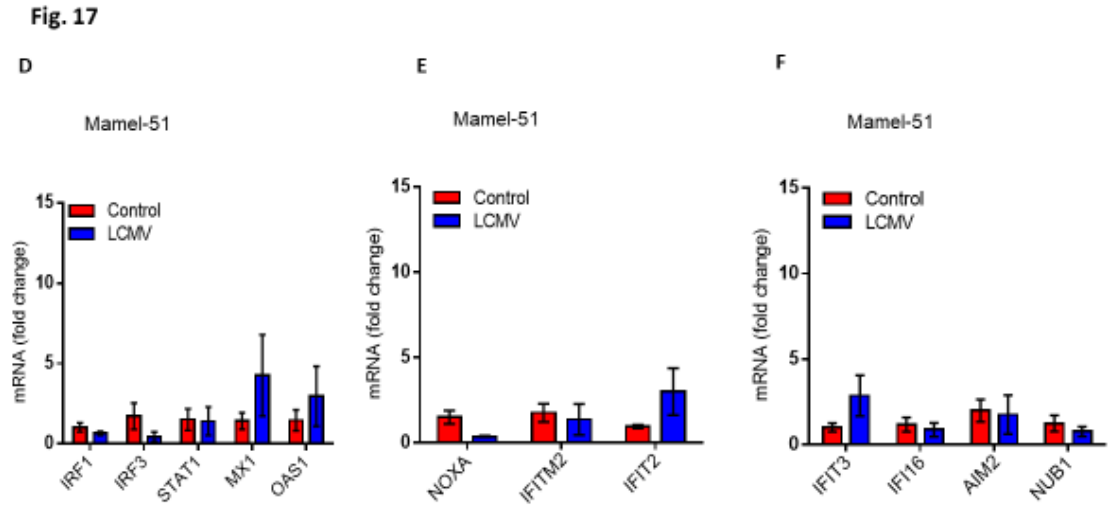


Figure 17: upon virus infection there is significant upregulation of interferon genes

(Fig. 17A**)** Mamel-86A cells (2 x 10^6 cells per mice) were injected in NodScid mice and once tumor was 5mm LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group and another group was left untreated and tumor growth was followed, Mice were sacrificed on day 10 after LCMV Injection and RTPCR was done to look for interferon genes and pro-apoptotic gene profile (Fig. 17B and 17C). Similarly Mamel-51 tumour cells (2 x 10^6 cells per mice) were injected in NodScid mice subcutaneously in the flank and once tumour was approximately of the size of 5mm LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group and another group was left untreated as control and tumor growth was followed, On day 10 after LCMV injection tumour sections were collected and RTPCR was made to check for the expression of interferon genes(Fig. 17D) and Pro-apoptotic genes(Fig. 17E and 17F)

3.6 LCMV promotes the upregulation of IRF1 in melanoma cells at translational level.

Next, we tried to investigate whether we can see some differences at translational level upon LCMV-WE infection in both mamel-86A and mamel-51 cell lines. We infected the human melanoma cells both mamel-86A and mamel-51 with LCMV-WE and performed the western blot we interestingly found that upon LCMV-WE infection there was a significant difference in the upregulation of IRF1 in case of mamel-86A while as in mamel-51 this effect was not visible (Fig. 18A). Since we see the differences in the IRF1 expression in both melanoma cell types then we thought maybe it might be one of the reasons why they respond differently towards the LCMV therapy, It is already know from the literature (Peter K M Kim and John H

YimFeb 2004) that overexpression of IRF1 in the mouse breast cancer lines promotes the apoptosis, they showed in different experimental settings that suppression of growth for breast cancer cell lines in vivo by intratumoral injection of Ad-IRF-1 into established tumours in their natural host. They concluded that IRF1 has antitumoral properties and use of potential agents that can increase the expression of IRF-1 in breast cancer and enhance apoptosis[202]. Since we find out that IRF1 is strongly upregulated in our mamel-51 while as in case of mamel-86A it seems downregulated and then we thought to compare the individual tumour growth of both mamel-86A and Mamel-51 and to our surprise we find out that mamel-51 tumour grows slower than mamel-86A(fig. 18B) pointing out that IRF-1 is one of the key factors that has an indispensable role to play in our tumor model.

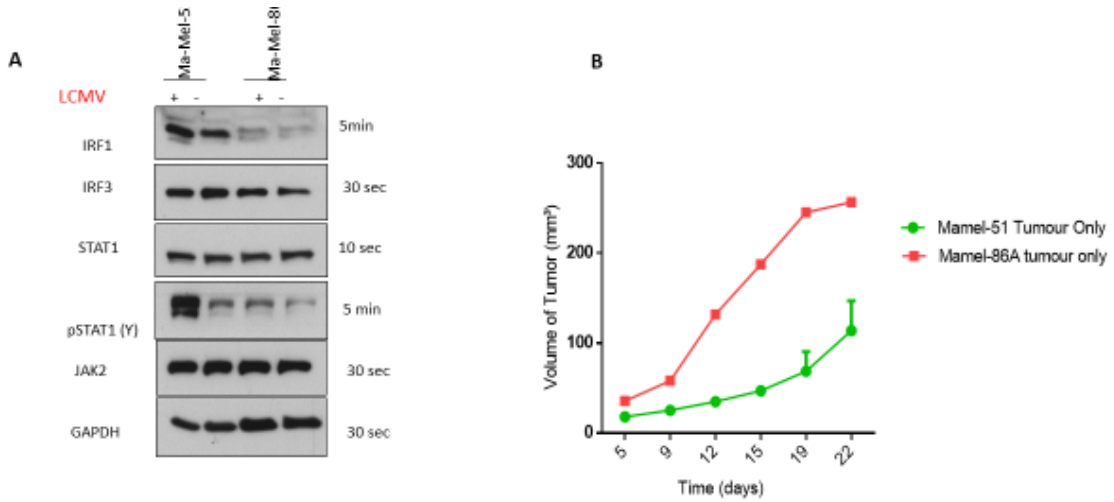


Figure 18: IRF1 is upregulated in mamel-51 upon LCMV infection.

(Fig. 18A) Mamel-86A and Mamel-51 cells were grown (2 x 10^5 cells) in 24 well plate and once they are confluent they were treated with LCMV WE (MOI-1) and after 24 hours supernatant was collected and western blot was performed and various downstream molecules were analysed which includes IRF1,IRF3, STAT1, pSTAT and JAK2. (Fig. 18B) Mamel-86A and Mamel-51 cells (2 x 10^6 Cells per mice) were injected subcutaneously in the flank of the NodScid mice (n=3) and tumour growth was followed.

3.7 Intratumor LCMV injection promotes CCL5 upregulation in human melanoma

Since upon virus infection there is the activation of many different cells types, cytokine release and many different chemokines are also released upon LCMV infection. Chemokines are small proteins that work as the chemotactic cytokines by directing cell migration. Chemokines sometimes work as signboards or one can say they are road sings that help the different cell

types like leukocytes etc into the body. Chemokine gradient stimulates the cellular migration and changes in chemokine-receptor expression by leukocytes are a key mechanism for regulating the homing of these cells to various tissues. It is also known that chemokines are also important to traffic leukocytes into CNS, It is also well know that resident population of CNS synthesis and secrete different types of chemokines[203]. Upon LCMV infection astrocytes and microglia are the main source of chemokines production, neurons also produce chemokines in relation to HIV and West Nile Virus[204]. Many different in-vivo and In vitro studies have proved that CNS viral infection leads to the distinct chemokine signature pattern thus it encouraged us to further investigate the role of chemokine in relation to LCMV infection and to address our central question what cells types or factors mediate the massive tumor regression in relation to LCMV infection. We injected Mamel-86A and Mamel-51 cells in NodScid mice and subsequently treated them with LCMV and later mice were sacrificed and RNA extracted, RTPCR was made to check for the expression of CCL5. It was clear that upon LCMV injection in the mamel-86A tumour bearing NodScid mice CCL5 was upregulated (Fig. 19A)while as in case of Mamel-51 tumour bearing NodScid mice we didn't see this effect (Fig. 19B). Thus, it proved that in case of our responding tumour cell line CCL5 is significantly upregulated upon LCMV-WE injection.

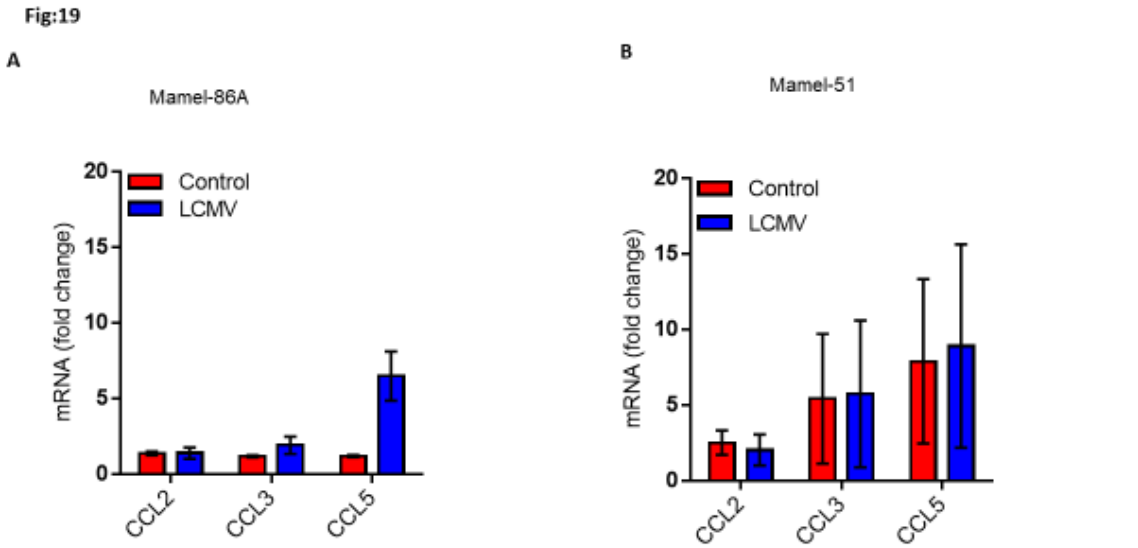


Figure 19: Human melanoma showed upregulation of CCL5 upon virus infection.

Fig. 19A Mamel-86A cells (2 x 10^6 cells per mice) were injected in NodScid mice and once tumor was 5mm LCMV WE(2 x 10^6 PFU) was injected intratumorally in one group(n=5) and another group was left untreated as control (n=5) tumor growth was followed, mice were sacrificed on day 10 after LCMV injection and RNA was

extracted and RTPCR was made to check for the expression of different chemokines. Similarly Mamel-51 cells (2 x 10^6 cells per mice) (Fig. 19B) were injected subcutaneously in the flank of the NodScid mice and once tumor was 5mm approximately LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group and another group was left untreated (n=5) tumor growth was followed and mice were sacrificed on day 10 after LCMV injection, RNA was extracted and RTPCR was made to check for the expression of different chemokines.

3.8 In vitro LCMV infection in human melanoma cells promotes CCL5 upregulation.

CCL5 also known as RANTES is one of the very important chemokine when it comes to the field of cancer biology. This chemokine plays an indispensable role in recruiting different leukocytes into inflammatory site, which includes basophils, macrophages, eosinophils and T cells. CCL5 also induces activation and proliferation of particular natural killer cells to generate C-C chemokine killer cells that happens in response to some cytokines that are secreted by T cells like for instance IL-2 and IFN-γ, [183]. CCL5 also binds to its receptor CCR5 one of the human immunodeficiency virus cell entry receptors on $CD4^+$ T cells (Ward and Westwick 1998) this further makes it so important in the field of HIV research. In case of cancer, the role of CCL5 is still controversial some group claim that CCL5 has antitumor properties while has some believe in the idea that it promotes cancer growth and progression. Tumour CCL5 has been described to attract T cells [205] and macrophages in tumour bed [206]. There is the strong correlation with the CCL5 expression and NK cell migration, It was also shown recently that CCL5 also attracts the NK cell to the site of the tumour especially melanoma which finally clears the tumour[187]. All these studies have proved that CCL5 is the potent chemokine that has the important role to play in the field of cancer. Next after In-vivo we tried to investigate whether CCL5 is directly coming from the tumour cells, so we grow both melanoma cells types in-vitro and treated them with LCMV- WE and afterwards collected the RNA and made RTPCR it turns out that in case of mamel-86A upon LCMV infection there is the significant upregulation of CCL5(Fig. 20A) while as in case of Mamel-51 there is no significant upregulation of CCL5(Fig.20B). Besides that we also used another approach apart from RTPCR to validate the expression of CCL5 and to be sure that CCL5 is basically coming and also expressed by the tumor cells in itself and not from the other immune components. We grow different tumour cells in vitro on cover clips and let the cells to grow and subsequently treated the cells with the LCMV-WE and once we stained all the different tumor cell types including lung adenocarcinoma (Fig. 20C) and melanoma (Fig. 20C) it was clear that CCL5 was significantly upregulated in case of Mamel-86A than in Mamel-51 and H1975 cells .

Fig. 20

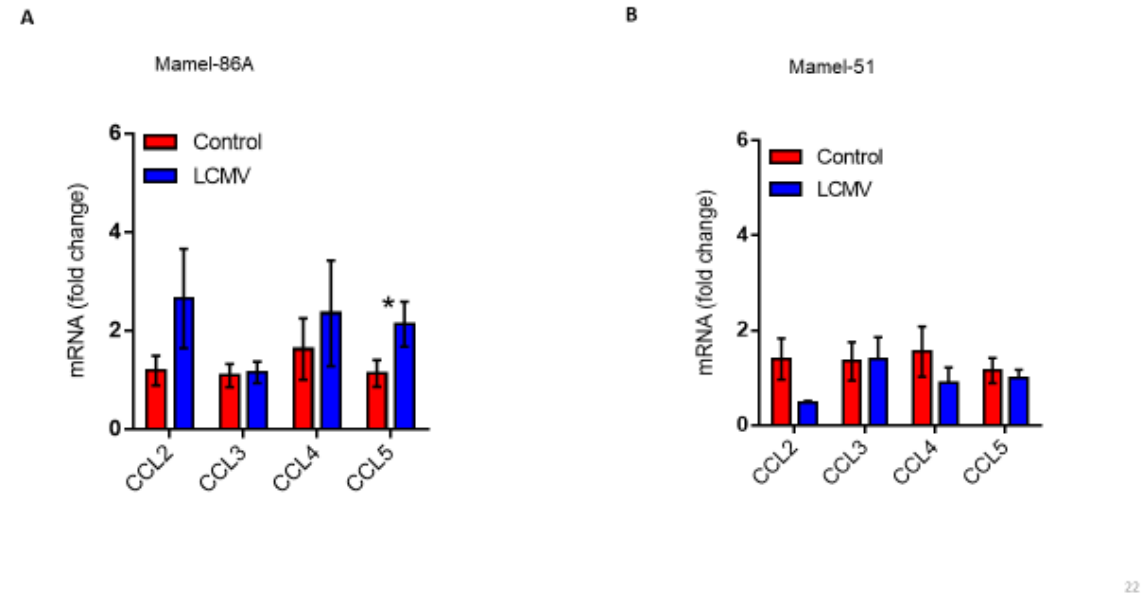


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Fig. 20

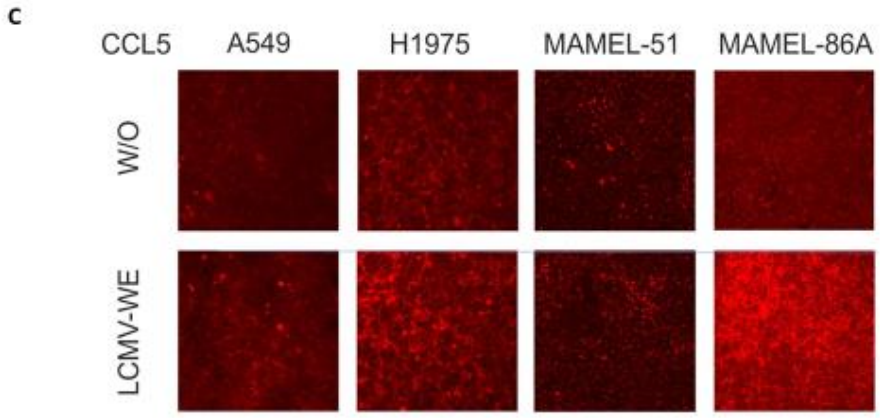

Figure 20: CCL5 levels are enhanced upon LCMV infection in Vitro

Fig.20A Mamel-86A cells were grown in 24 well plate (2 x 10^5 cells per well) and after they are confluent they were treated with LCMV WE (MOI-1) and after 24 hours of virus infection the cell pellet was collected followed by rna extraction and finally rtpcr was made to check for the chemokine profile. (Fig. 20B) Mamel-51 cells were grown in 24 well plate (2 x 10^5 cells per well) and once they are confluent they were treated with LCMV-WE (MOI-1) and after 24 hours of incubation the cell were collected which was followed by RNA collection and finally RTPCR was made to look for the chemokine profile.(Fig.20C) Similarly different human cancer cells like melanoma cells (mamel-86A, Mamel-51 and Mamel-86C) also human lung carcinoma (A549) and H1975 (2x10^5 cells per well) were grown in 24 well plate on the cover slips. Then 200 µL of triton X solution was added and

incubated for 20min room temperature after that two washing with PBS containing 2% FCS. Primary antibody VL-4 RAT Anti-LCMV mab in addition Anti-rantes antibody (ab9679) was added and samples were incubated for 1-hour room temperature after that two times washing with PBS containing 2% FCS. Secondary antibody Anti-rat IgG1 Fitc and PE conjugated donkey anti-Rabbit IgG (Lot 131220 Jackson) were added (1:100 dilution) followed by one hour incubation and two times washing with PBS containing 2% FCS and finally Coverslips were mounted on microscope slides using mounting medium (S3023, Dako). Images were acquired with a fluorescence microscope (KEYENCE BZ II analyser).

3.9 Intratumor staining reveals that LCMV promotes CCL5 upregulation within tumour cells

To further explain and understand at the mechanistic level in relation to LCMV therapy in both mamel-86A and mamel-51 tumour cells. CCL5 is extremely important chemokine and has a great role in the cancer biology, next we injected the mice with the Mamel-86A and Mamel-51 tumour cells subcutaneously and once the tumour was of desired size we injected the LCMV WE intratumorally and followed the tumour growth, mice were sacrificed at different time point after LCMV injection and tumour sections were stained for the CCL5 expression. It was clear from the immunohistochemistry that in case of Mamel-86A tumour bearing NodScid mice upon LCMV injection there is the massive upregulation of CCL5(Fig. 21A)while as in case of mamel-51 tumour bearing NodScid mice there is no significant upregulation of CCL5as is clearly seen in the figure (Fig. 21B). Thus, we conclude from here that LCMV promotes the upregulation of CCL5 in human melanoma cell type that is mamel-86A

Fig. 21

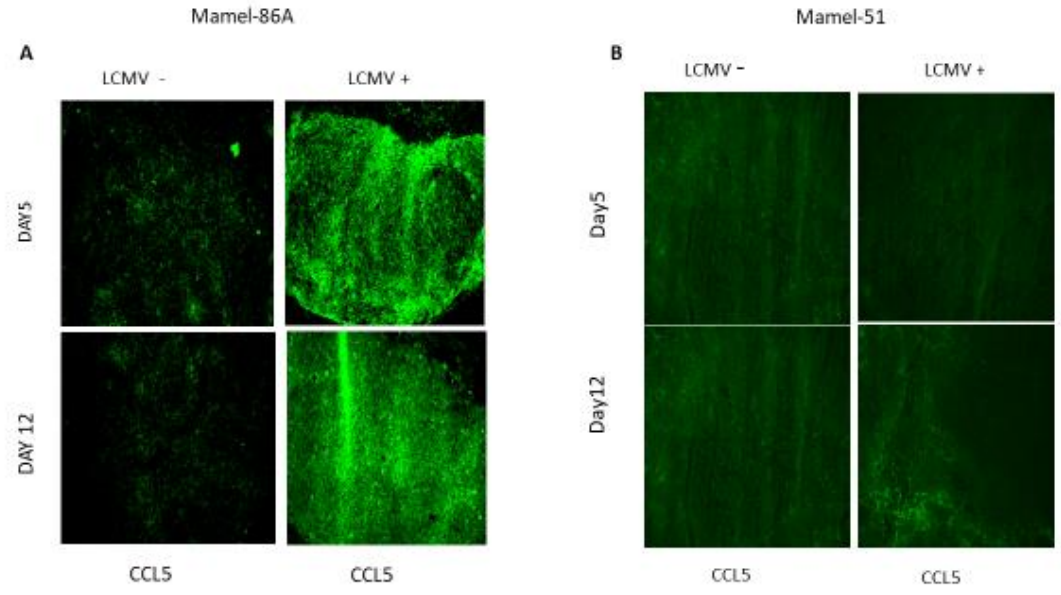

Figure 21: Mamel-86A tumours have enhanced CCL5 expression upon LCMV infection.

(Fig. 21A) Mamel-86A cells were injected (2 x 10^6 cells per mice) in NodScid mice and once tumor was of the size of 5mm LCMV-WE (2 x 10^6 PFU) was infected intratumorally in one group and another group was left untreated, Mamel-86A tumor bearing NodScid mice were sacrificed on Day 5 and day 12 after LCMV-WE injection, tumors sections were stained for the expression of CCL5. (Fig. 21B) Similarly Mamel-51 tumour cells were injected in the flank of NodScid mice (2 x 10^6 cells per mice) and once tumour was of the size of 5mm approximately LCMV-WE (2 x 10^6 PFU) was injected intratumorally in one group and another group was left untreated a control, Mamel-51 tumor bearing mice were then sacrificed at different time points like on day 5 and day 12 and CCL5 expression was checked.

3.10 upon LCMV infection cancer cells secrete CCL5

It became clearer from our In-vivo and In-vitro data that LCMV infection in the melanoma cells promotes the upregulation of CCL5, Then the next question we asked was whether the viral infection leads to the secretion of CCL5 in the supernatant. So again we took the human melanoma cancer cells and lung adenocarcinoma and grow them in vitro and infected them with the LCMV-WE, We collected the supernatant and analysed the CCL5 production it was quiet clear that upon LCMV-WE infection CCL5 is secreted by mamel-86A cells (Fig. 22C) and lung adenocarcinoma cells also showed the significant secretion of CCL5 upon LCMV-WE infection (Fig. 22B) but mamel-51 even after the LCMV infection did not show the significant secretion of CCL5 (Fig. 22A). Thus we reach to this conclusion that upon LCMV infection different cancer cell types lead to the plentiful secretion of CCL5 and this enhanced CCL5 has the promising antitumor effects in melanoma since it drives NK cells to the tumour site.

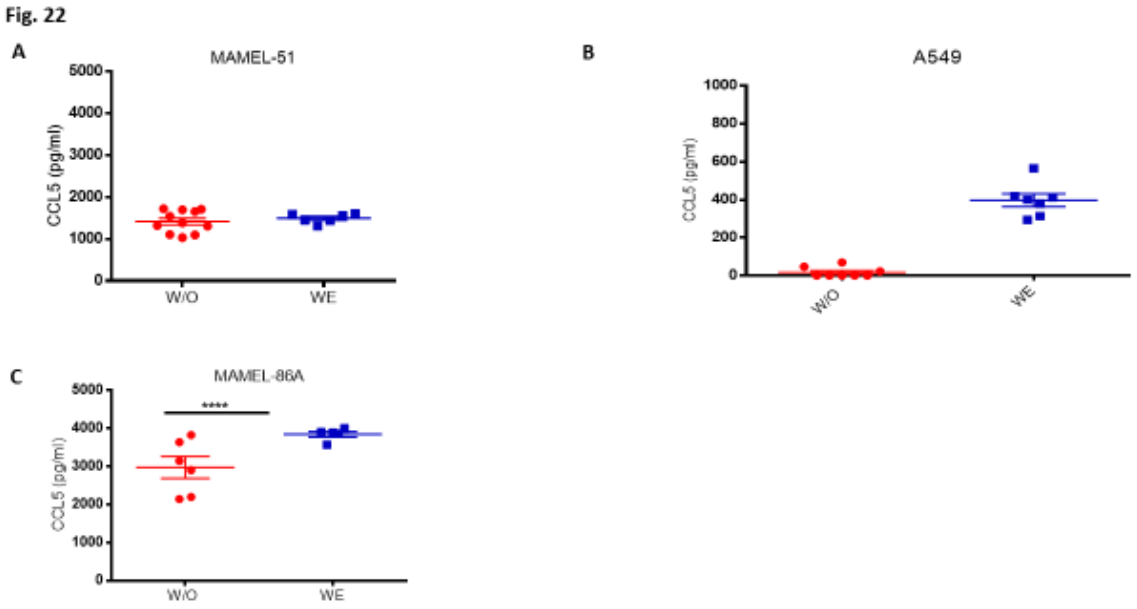

Figure 22: CCL5 production is cancer cell intrinsic and is enhanced by LCMV infection.

(Fig. 22A) Mamel-51 Cells were grown in 24 well plate and then treated with LCMV-WE high dose (2 X10^6 PFU) with an MOI-1 and after 24 hours of the virus infection supernatant was collected and Elisa was made. (Fig.22B and Fig. 22C) similarly we grow lung adenocarcinoma (A549) and melanoma (Mamel-86A) cells in the in-vitro and infected the cells with the LCMV-WE (2 X10^6 PFU) with an MOI-1 and collected the supernatant and check for the CCL5 levels after 2 hours of virus infection.

3.11 Enhanced CCL5 drives massive NK cell infiltrate in the tumour bed.

Chemokines have been addressed from time to time in different studies as key molecules that have a significant role to play not only in case of inflammation, immune surveillance but also in cancer progression too. Chemokines can also behave as growth factors too especially the one secreted by tumour cells metastatic sites or even by normal cells[198] or they can also induce the formation of an immunosuppressive microenvironment. A variety of chemokines and chemokine receptors has been detected in neoplastic tissues. Among many different chemokines our main concern is CCL5 being as the potent chemokine and having a significant role to play in different cancer models, CCR5 is a seven-transmembrane G-protein-coupled receptor, mediating diverse signalling cascades in response to its ligands. CCL5 belongs to the C-C chemokine family whose members also include CCL3 and CCL4. CCL5 is a target gene of NF-κB activity and is expressed by T lymphocytes, macrophages, platelets, synovial fibroblasts, tubular epithelium, and certain types of tumour cells [207]. CCL5 production is also stimulated by NF-κB activation by different stimuli such as CD40L and IL-15. Since CCL5 is

the chemokine, thus it has a significant role to play in case of recruiting a variety of leukocytes into inflammatory sites, which includes T cells, macrophages, eosinophils, and basophils. In association with different types of cytokines that are released by T cells such as IL-2 and IFN-γ, CCL5 also induces the activation and proliferation of particular natural killer cells to generate C-C chemokine activated killer cells. It is also know from the literature that there is the link between NK cells and CCL5, (Takouhie Mgrditchian and Bassam Janji) have shown that higher the expression of CCL5 in the melanoma tumour sections more is the infiltrate of the NK cells, thus there seems to be the direct correlation between CCL5 expression and NK cell infiltrate and CCL5 is driving the NK cells into the tumour microenvironment that further promotes the killing of tumour cells, since from the literature it is also proved and well established that that NK cells can recognize and destroy melanoma cell lines [87]. NK cells after the activation lead to the robust production of cytokines such as interferon (IFN)Gamma and tumour necrosis factor (TNF) or they can directly kill target cells by releasing perforins and granzymes**.** NK cell mainly kill the target which have low levels of MHC class I expression or have lost the expression of 1 or more MHC class I alleles. NK cells have this remarkable ability to discriminate between self, normal and transformed/ infected target cells due to the expression of MHC class I-specific inhibitory receptors. NK cells express different receptors and one of them being NKG2D The ligands for NKG2D are either absent or expressed at very low levels on healthy cells but during the tumour formation or under stress conditions the ligands for the NKG2D ligands can be upregulated. The expression of the ligands for the NKG2D on cancer cells especially the melanoma cells showed that the majority do expressed its ligands, which further confirms the NK cell mediated cytotoxicity of melanoma cells, Thus all these studies and reports suggested that NK cells are an important cell type in case of melanoma, we investigated the role of NK cells in our experimental model since we use the human melanoma cells thus it was a hint that NK cells might be playing a great role since melanoma cells with time downregulate the MHC and NK cells specially target the cells that have MHC downregulated. Thus we designed an experiment where we infected the NodScid mice with Mamel-86A and Mamel-51 human melanoma cells and once the tumor was of appropriate size we injected the LCMV WE intratumorally and followed the tumor growth , subsequently the mice were sacrificed at different time points after the intratumor LCMV infection and intratumor NK cell were checked in both Mamel-86A (Fig. 23A) and Maml-51 tumor sections (Fig. 23B). Thus it was clear that there was the massive NK cell infiltrate in Maml-86A tumor

as compared to Mamel-51 tumor sections. This somehow correlates with the higher CCL5 expression

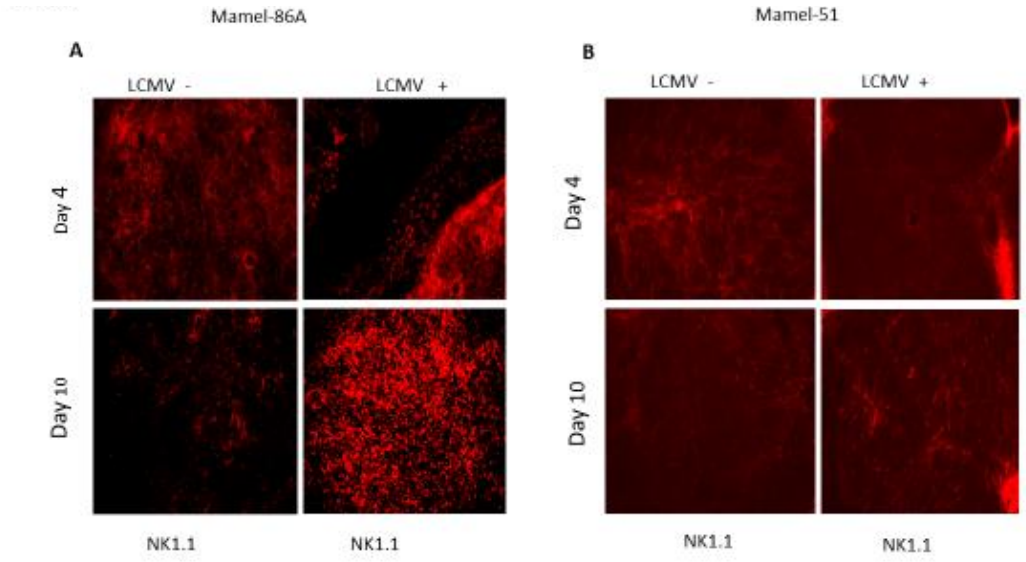

Figure 23: Upon LCMV infection there is massive NK cell infiltrate in the tumour site.

(Fig.23A) NodScid mice were injected with Mamel-86A tumour cells (2 x 10^6 cells per mice) and once tumour was of the size of around 5mm approximately LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=5) and another group was left untreated as control (n=5), Mamel-86A tumour bearing NodScid mice were sacrificed on day 4 and day 10 after intratumor LCMV injection and histology was made to check the immune infiltrate especially NK cell infiltrate. (Fig. 23B) NodScid mice were injected with Mamel-51 tumour cells subcutaneously in the flank (2 x 10^6 cells per mice) and once tumour was approximately of the size of 5mm LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=5) and another group was left untreated as control (n=5) and tumour growth was followed, mice were sacrificed on day 4 and day 10 after LCMV injection , immunohistochemistry was made to check the infiltrate of different immune cells especially NK cells but the histology showed that there was poor NK cells infiltrate in case of Mamel-51 tumour sections.

3.12 NK cells are the potent cell type that are responsible for massive tumour regression.

Thus from our experiments and data it was clear that CCL5 is responsible for driving NK cells into the battle field that is tumour, since it was clear from the kinetics experiment that NK cells are the main cell types that migrate to the tumour bed, to further address this question and strengthen our hypothesis we have to either deplete NK cells or look for NK cell knockout mice that would further help to solve our scientific question. Next we took NodScid and NSG (triple knockout mice) and injected mamel-86A in both the groups and followed the tumour growth it

was clear that tumour was growing much faster in NSG mice than in the NodScid mice (Fig. 24A) which was the proof that NK cells have a significant role to play, Next to further rule out that the effect is primarily because of NK cells and not with the other cells types like dendritic cells, monocytes, granulocytes etc to further pin point this we make another experimental plan where we took the NSG (triple knockout) mice and injected them subcutaneously with the Mamel-86A tumour cells and once the tumour was of approximate size one group of the Mamel-86A tumour bearing NodScid mice were treated with LCMV-WE and tumour growth was followed it was quiet clear that even after LCMV infection in absence of NK cells there is no antitumor effect (Fig. 24B) that means LCMV induced antitumor effects are dependent on the presence of NK cells. Thus the intratumor LCMV injection leads to CCL5 upregulation that further leads to massive NK migration to the tumour site which ultimately promotes the better tumour clearance.

Fig. 24

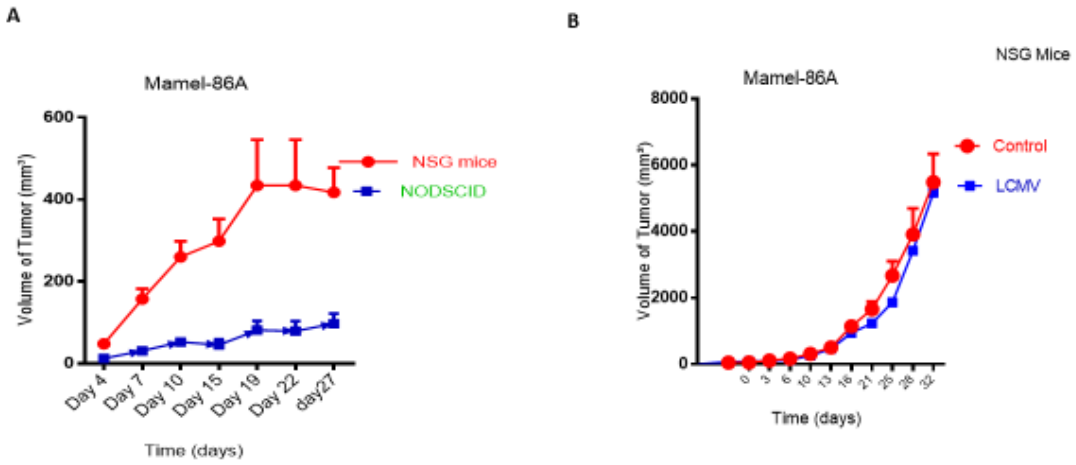

Figure 24: NK are the efficient cell types that lead to the better clearance of tumor mass

(Fig. 24A) NodScid and NSG mice were injected with Mamel-86A tumour cells (2 x 10^6 cells per mice) subcutaneously in the flank and tumour growth was followed. (Fig. 24B) Mamel-86A tumour cells (2 x 10^6 cells per mice) were injected subcutaneously in the flank of the NSG mice and tumour growth was followed, once the tumour was of 5mm in size LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=4) and another group was left untreated as control (n=4) and tumour growth was followed.

3.13 Mamel-51 do not respond to the LCMV therapy because of poor NK cell infiltrate.

Next to further, address this question why Mamel-51 is resistant to LCMV therapy we again have to go into the In-vivo mouse model and check whether in absence of NK cells the scenario is same is somewhat different, Again we took the NSG (triple knockout mice) and NodScid mice and injected the mamel-51 cells subcutaneously in the flank (Fig. 25A). Thus it proved that whether there is presence or absence of NK cells mamel-51 tumor growth is not effected at all that hints in the direction that not enough NK cells migrate in the tumor microenvironment, but when we next injected the mamel-51 cells in the flank of NodScid mice and once tumor was of appropriate size we injected LCMV WE intratumorally and followed the tumor growth as you can see in (Fig. 25B) tumor growth is not influenced even after LCMV-WE injection that clearly indicates that NK cells are not migrating into the tumor microenvironment.

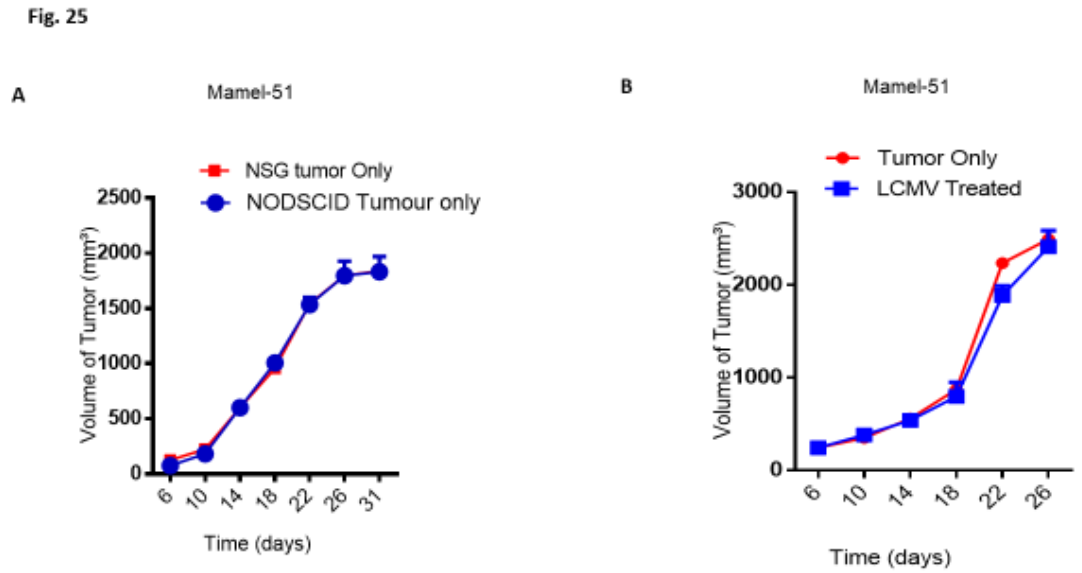

Figure 25: There is poor NK cell infiltrate in case of mamel-51

Fig. 25A Mamel- 51 cells were injected in the NodScid (n=3) and NSG (n=3) mice (2 x 10^6 cells per mice) subcutaneously in their flank and the tumor growth was followed. (Fig. 25B) NodScid mice were infected with mamel-51 cells (2 x 10^6 cells per mice) and once tumor was of the appropriate size LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=3) and another group was left untreated as control (n=3) tumor growth was followed twice a week.

3.14 Blockade of CCL5 by maraviroc promotes the massive melanoma tumor growth

Next we asked whether CCL5 production is linked to anti-tumoral activity and how anti-tumoral activity is mediated. Therefore, we blocked CCL5 with Maraviroc. Maraviroc is the first licensed representative of the class of chemokine receptor type 5 (CCR5) inhibitors used for the treatment of human immunodeficiency virus (HIV) infection. Maraviroc binds in the transmembrane pocket of CCR5 and is a slow-offset functional antagonist that prevents internalization[208, 209]. It has potent antiviral activity against a wide-range of HIV-1 isolates [208]. Cell based assays were used to establish the mechanism of action of maraviroc where it blocked binding of viral envelope, gp120, to CCR5 to prevent the membrane fusion events necessary for viral entry. Maraviroc did not affect CCR5 cell surface levels or associated intracellular signaling, confirming it as a functional antagonist of CCR5. Besides that, it is confirmed from many different studies that maraviroc has no detectable in vitro cytotoxicity and is highly selective for CCR5 and is confirmed against a wide range of receptors and enzymes. Many in vivo and In Vitro studies have predicted maraviroc to have human pharmacokinetics consistent with once or twice daily dosing following oral administration. Maraviroc has become important which has further lead to several clinical trials for the treatment of HIV-1 infections and AIDS. Next we asked the question whether blockade of CCL5 by maraviroc could affect the tumor growth, we designed the experiment where we orally injected the mice with the maraviroc twice a week and followed the tumor growth , it was clear that upon CCL5 blockade by maraviroc there is an enhanced tumor growth (Fig. 26) while as the mice which were which were treated with LCMV WE intratumorally again showed the faster tumor clearance, but when we combined maraviroc with LCMV the effect of LCMV is limited that further proves our hypothesis that while once you block the CCL5 LCMV shows very limited antitumoral effect (Fig. 26) thus from this experiment we conclude that blockade of CCL5 leads to enhanced growth of melanoma.

Fig. 26

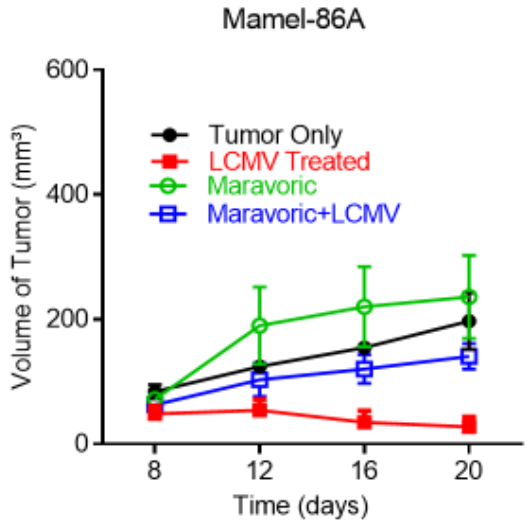

Figure 26: CCL5 blockade enhances the melanoma tumour growth

(Fig. 26) Nodscid mice were injected with mamel-86A tumour cells (2 x 10^6 cells per mice) subcutaneously in the flank and once tumor was of the size of 5mm in one group LCMV WE (2 x 10^6 PFU) was injected intratumorally in one group (n=4) and another group was treated with maraviroc (50mg/kg body weight/mice) only twice a week for more than two weeks (n=4) , another group was treated with the combination of maraviroc and LCMV WE (n=3) , tumour growth was taken twice a week.

3.15 CCL5 seems to be upregulated through PI3K

Next we wanted to look for the molecular mechanism responsible for the CCL5 production in case of our tumour models. Since CCL5 is an essential chemokine that performs many different functions it has an important role to play for the migration of different cell types for instance in case of virus infection CCL5 directs lymphocytes and monocytes to the site of the infection, apart from that it also plays a vital role in the progression of breast cancer but in case of melanoma enhanced CCL5 promotes the NK cell migration which further leads to the antitumor activity and inhibits melanoma growth, There are many signalling pathways through which CCL5 is upregulated it involves PI3K/Akt, IKK1 etc. so next we thought of using different inhibitors that are downstream and upstream to CCL5 and we assumed that blocking either one or many of them should inhibit the CCL5 production, Thus we planned an experiment where we took different human cancer cell types like lung adenocarcinoma (Fig. 27C) and melanoma (Fig. 27A and Fig. 27B) and later infected them with LCMV-WE and finally treated them withPI3K inhibitor and looked for the CCL5 expression, LY294002 (PI3K inhibitor) leads to

the massive downregulation of CCL5 in all melanoma (Fig. 27A and 27B) and also in case of lung adenocarcinoma there is the massive downregulation of CCL5 (Fig. 27C)

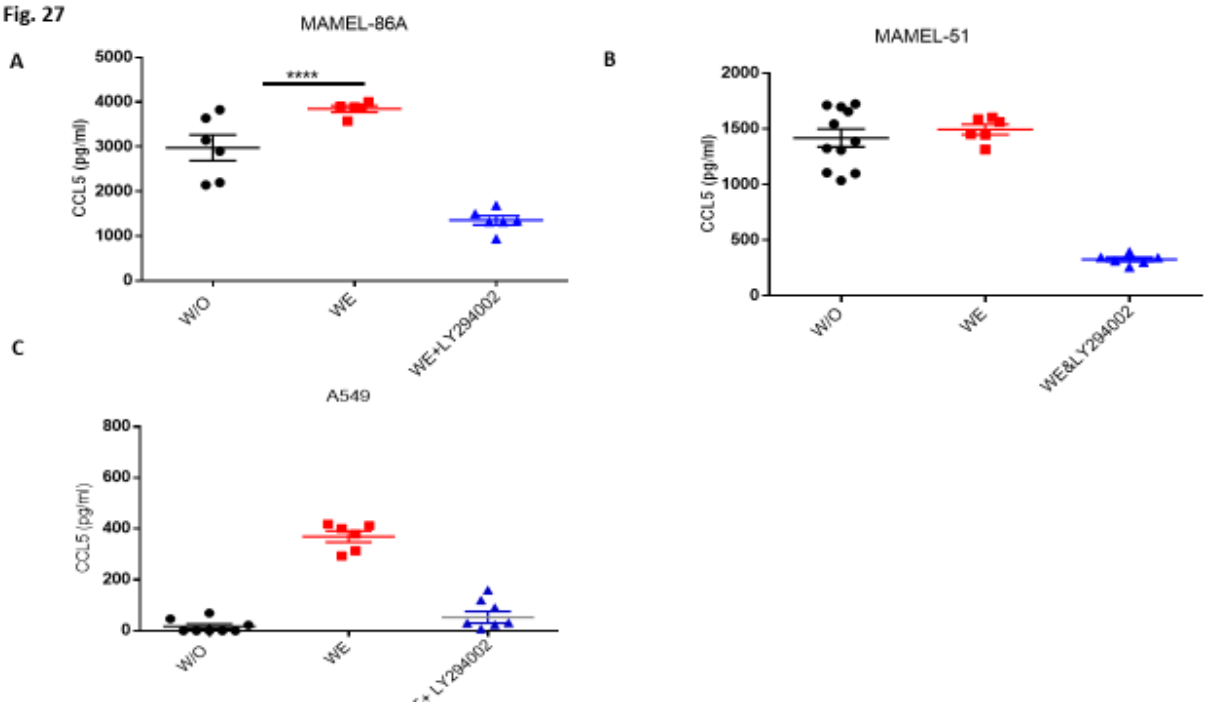

Figure 27: Blockade of PI3K/Akt significantly reduces the CCL5 production.

Fig. 27A Human melanoma cancer cells were mamel-86A ($2x10^5$cells per well) cultured in 24 well plate and then treated with LY294002 and subsequently some cells were treated with LCMV-WE and after 48 hours of virus infection supernatant was collected and Elisa was made to check the CCL5 profile. Fig. 27B and 27C similarly mamel-51 and A549 cells were also grown ($2x10^5$cells per well) in 24 well and later treated with LY294002 and LCMV-WE, after 24 hours supernatant was collected and CCL5 profile was checked by ELISA

4 Discussion

Viruses have been extensively studied now from a very time and presents an interesting and exciting field to explore. There are many different type of viruses and they range from being non-pathogenic to pathogenic causing mild to severe damage to their host. Multiple disease-causing viruses such as HIV, HBV,HCV, 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.

Many different studies with bacterial viruses and animal viruses has helped a lot in understanding the structure of the virus, Since viruses have the tendency to infect different cell types these virus infected cells have proved a better model system for the study of basic aspects of cell biology. Most of the viruses utilise normal cellular ribosomes, translational factors, tRNAs for the synthesis of proteins. Once the virus enters into the host then there starts this complex interaction between virus and host this has been understood largely by the study of viral life cycle. But viruses are obligate pathogens which are indigent compared to their hosts. Viruses need minimal required components for their survival and replication and mostly they rely on the host for their replication which further will depend on the immune response towards the virus, since keep on evolving and new viruses keep on emerging it is quiet very much necessary to dissect the basic cellular replication of the virus and to understand the cellular dependence of viral replication.

The present study mainly deals with the role of the arenavirus therapy in tumour regression especially with the main interest in melanoma. We have also identified CCL5 as the potent chemokine that plays a significant role in arenavirus therapy induced tumour regression and drives NK cell infiltrate into the site of the tumour. Thus we identified LCMV based approach for the better clearance of different cancer types, rather LCMV as anti-tumour virotherapy.

4.1 Intratumor LCMV injection leads to massive tumour regression in multiple tumour models.

There are different kind of treatments available for different kinds of cancer types ranging from chemotherapy, radiotherapy, immunological approaches and in the end surgery, but once the cancer reaches in its advances stage it is difficult to treat and the cure is very rear. Many attempts were made to improve the early detection and treatment of advanced-stage cancers but until

now most of these attempts are unsuccessful. Even the advanced chemo and radiotherapy that is available so far have failed to improve the overall survival of patients with locally advanced or metastatic disease. That means there is still a lot do in this ever-changing field and more focus should be in developing innovative therapeutic approaches for the control of advanced-stage cancer. There are many attempts that people have made in investigating various immunotherapeutic strategies to enhance the immune response against tumour-associated antigens (TAA). Recombinant virus is one of the easy ways to deliver your gene of interest to the targeted cancer cells because they have good transduction efficiency and better oncolytic properties. There are different oncolytic viruses that have been tested so far like influenza virus, vesicular stomatitis virus, measles virus, Newcastle disease virus and herpes simplex virus and they have some promising anticancer properties. Vaccinia virus has the property that it infects the tumour cells but not the normal cells . It was shown quiet recently by Hung et al that injecting the vaccinia virus to the mice intraperitoneal (i.p) can preferentially infect ovarian tumour cells but surprisingly not the normal cells and not only that it generates the massive antitumor effects[210]besides that vaccinia has been used as the efficient mode for the vaccine delivery and has a tremendous potential to generate antigen specific immune response [211]. In our study we used the LCMV as the model arenavirus, since LCMV is noncytopathic so it will not kill the host cells but LCMV generates strong CD8 T cell response which is also one of the many factors that contribute to the massive tumour regression. We used the NOdScid mouse model and further investigated the effect of intratumor injection of LCMV WE in different cancer types and to our surprise, LCMV works very much effective in different cancers and show tremendous antitumor properties. Next we also investigated whether LCMV-WE injection could help to prolong the survival of the mice and the second question was whether one shot of LCMV–WE high dose was sufficient to prevent the relapse of the tumour. It was quiet remarkable that a single injection of LCMV-WE was sufficient enough not only for the effective clearance of the tumour but these mice who have received the intratumor LCMV-WE infection these mice show the better survival (Fig. 13 and Fig. 14) and after the long term monitoring of these mice they don’t show any sign of tumour relapse which clearly means that a single shot of LCMV-WE has the multiple benefits from tumour clearance to better survival.

4.2 LCMV replicates very well in different human melanoma cancer cells.

Next question that we asked was whether LCMV-WE can replicate in the different human melanoma cells. Since it is know that viruses can replicate very well in different cells types including dendritic cells, macrophages, CD169 marcophages etc. we have found that fast

replication of arenavirus in dendritic cells led to massive activation of the innate and adaptive immune system[146]. Since cancer cells are completely different from the normal cells with the altered cell cycle, translation and metabolism[212]thus it was quiet logical to check the replication of LCMV-WE in different melanoma cancer cells and correlate it whether this might have some affect the antitumoral immune response. So we took three different human melanoma cancer cell types and we found out that virus was replicating very well in all the three cells types (Fig. 16)

4.3 Upon Intratumor LCMV injection there is significant upregulation of interferon and pro-apoptotic genes.

Since decades, LCMV has been the virus of interest for many immunologist worldwide and has been central to the discovery of wide range of ground breaking immunological concepts. LCMV is non-cytopathic in nature so the killing of the target cells is not mediated by the virus itself but by the immune infiltrate in the targeted tumour tissue. There are different strains of LCMV available and these strains differ significantly in their biology and pathogenicity in mice and the other factors include dosage and route of administration. Depending on the strain of LCMV the outcome of the infection also will vary for instance LCMV Armstrong strain (LCMV –Arm) and LCMV-WE will establish the acute infection in mice while as LCMV-Cl13 and LCMV – Docile will establish the chronic infection. There is also the difference in the interferon response followed by the LCMV infection with IFN-α and IFN-β responses following LCMV- Cl13 infection reaching to several folds as compared with to the LCMV-Arm infection[213] but upon IV injection LCMV-Arm leads to the stronger and more prolonged IFN-α response as compared to the LCMV-Cl13[214]. We used LCMV-WE in all our tumour models and we investigated the role of LCMV-WE and the interferon and pro-apoptotic gene profile upon intratumor LCMV-WE injection, it was clear that upon intratumor LCMV –WE infection there was the massive upregulation of interferon and pro-apoptotic genes in case of Mamel-86A (Fig. 17A, Fig. 17B and Fig. 17C) while as in case of mamel-51 we did not see such a big effect although the route of the LCMV–WE injection was the same in both tumour models. This individual differences in the interferon gene upregulation upon LCMV injection is very much prominent in case of mamel-86A than in mamel-51.

4.4 CCL5 levels are significantly upregulated upon intratumor LCMV WE injection

Virus infection generates the proinflammatory response including expression of cytokines and chemokines. After the virus infection there is the strong host response that gets started. It is known for different viruses that the interaction of viral surface proteins with the cellular surface proteins starts a cellular reaction that ultimately leads to the first wave of cytokine production, Also during the course of infection many virus particle that are present in the virus life cycle are able to effect the cellular signalling that also leads to the cytokine production, Apart from cytokine production upon virus infection a large family of small secreted proteins known as chemokines are also secreted during the course of virus infection. Among many different chemokines CCL5 is one of the very important chemokine and plays an active role in recruiting a variety of leukocytes into inflammatory sites including T cells, macrophages, eosinophils, and basophils. This chemokine in participation with other cytokines like IL-2 and IFN-γ, lead to the activation and proliferation of natural killer cell [183]**,** Besides that CCL5 has a significant role to play to overcome different viral infections like west Nile virus[215]. Rabies virus and tick borne encephalitis thus making it an indispensable chemokine in terms of virus infection. From the cancer point of view CCL5 plays much bigger role as compared to virus infection there are several studies which prove that CCL5 promotes multiple myeloma progression [197] Hodgkin lymphoma, colorectal cancer and several independent studies have also proved that CCL5 promotes breast cancer metastasis[216]. So from the literature point of view we investigated whether LCMV promotes the upregulation of CCL5 in different melanoma cells although many different cancer types in itself produce the CCL5 but from our investigation LCMV promotes the massive upregulation of CCL5 in different melanoma cell types (Fig. 19A). Thus we can say that intratumor LCMV-WE (high dose) injection leads to the upregulation of CCL5, since CCL5 is the chemokine from that point it hints that it might be helping for the recruitment of different immune cells types to the site of the tumour. This clearly hints for different cells types and next we were wondering what are the most important cells types that CCL5 can drive to the tumour battlefield.

4.5 CCL5 upregulation upon LCMV infection is tumour cell intrinsic

Different independent studies from many groups have suggested that many different cancer cell types express CCL5 for instance in case of ovarian cancer CCL5 expression is detected not only in malignant ovarian biopsies, but also in normal biopsies[191]besides that CCL5 is also

expressed in ovarian cancer stem cells[217]. There is the strong correlation even with the patient point of view CCL5 levels are enhanced in the ovarian cancer patients as the disease progress like in stages III-IV of ovarian cancer there is much higher level of CCL5 compare to stages I-II. Similar observations have been made in case of breast cancer that hints somehow that cancer cells do produce CCL5 and that was the question we next we asked whether LCMV infection leads to much stronger CCL5 production from the In-Vivo data that we mention before it was clear that intratumor LCMV injection leads to the CCL5 upregulation but tumour in itself being a heterogeneous structure and besides on virus infection there is also massive immune infiltrate like NK cells, monocytes, granulocytes start to migrate to the tumour bed so to rule out and to be more specific that CCL5 is coming from tumour cells itself we designed another experiment in vitro where we showed that LCMV leads to the robust CCL5 upregulation in the human melanoma cells and also in human lung adenocarcinoma in itself (Fig. 20B and Fig. 20C). Thus our in-vivo study corresponds with the in-vitro study and confirms that CCL5 is extremely important chemokine that tumour cell secrete upon LCMV-WE infection

4.6 CCL5 drives NK cells to the site of the tumour

Since CCL5 in itself is a chemokine and chemokines have the significant role to play in the migration of different cell types. CCL5 can lead to the recruitment of the leukocytes in case of herpes simplex encephalitis, CCL5 also has the significant role to play in case of CD8 T cells during the chronic virus infection in fact CCL5 can also influence the functionality of the T cells in case of RANTES$^{-/-}$ mice, virus-specific CD8 T cells had poor cytokine production. CD8 T cells which are deficient of rantes rather RANTES$^{-/-}$ CD8 T undergo much severe exhaustion during the chronic virus infection since they do express much higher levels of inhibitory receptors[218]Similarly,CCL5 is also important in case of macrophages functionality, CCL5 is important for the protection of tissue macrophages against the cell mediated by the virus thus further helps them in the better clearance of infection. This protective effect of CCL5 will basically depend on the activation of the CCL5 ligand which is CCR5 and Parallel activation of $G_{\alpha i}$-PI3K-AKT and $G_{\alpha i}$-MEK-ERK signalling pathways [219]**.** CCL5 also has the significant role to play in driving NK cells to the site of the tumour, mouse models of breast cancer and melanoma have shown that if you target autophagy gene Beclin 1 it significantly reduces the tumor growth and enhances the NK cell mediated antitumor immune response but the migration of the NK cells to the tumour site[220]. Since our model is NodScid mouse model so the easy guess for us was to either it could be NK cells, monocytes or macrophages, but we found out that upon LCMV-WE infection mamel-86A tumour bearing

NodScid mice showed much more massive NK cell infiltrate which further proves that tumour cells which show higher expression of CCL5 upon LCMV-WE infection also have more NK cell in the tumour microenvironment (Fig. 23A)and the tumours which have less CCL5 expression showed poor NK cell infiltrate, This whole investigation further hints that Mamel-86A NodScid mice upon LCMV infection showed massive tumour regression (Fig. 15B) while as Mamel-51 was resistant to the LCMV-WE therapy (Fig. 15C) that further explains higher the CCL5 expression better the response to the LCMV-WE therapy and vice versa.

4.7 Depletion of NK cells limits the antitumor effects of LCMV WE in melanoma

The next interesting question that we were interested was since having a NOdScid mice also can hint about the macrophages or monocytes or dendritic cells. Thus to figure out this possibility that NK cells are the prime cells which have the most indispensable role to play in the arenavirus based therapy we thought of the depletion of NK cells or the use of the mice which are tripple deficient for B, T and NK cells. It became very clear once we used the mice deficient of NK cells and treated the tumour bearing mice with the LCMV-WE, we didn't see any antitumor effect after the viral therapy and the follow up of the Mamel-86A tumour bearing NSG mice treated with LCMV –WE didnt show regression of tumour mass (Fig. 24B)**.** This clearly proves that strong antitumor effects upon LCMV-WE infection are mediated via NK cells.

4.8 Blockade of CCL5 promotes the melanoma growth

Since from our data it become more and more clear enhanced CCL5 expression limits the growth of melanoma by promoting more NK cell infiltrate in the tumour, so next question that popped up was that whether blockade of CCL5 will promote the growth of melanoma. So we used maraviroc, it is the first licensed representative of the class of chemokine receptor type 5 (CCR5) inhibitors used for the treatment of human immunodeficiency virus (HIV) infection. Maraviroc binds in the transmembrane pocket of CCR5 and is a slow-offset functional antagonist that prevents internalization[208, 209]. It has potent antiviral activity against a wide-range of HIV-1 isolates[208]. There are several studies in case of breast cancer where they have reported that enhanced CCL5 promotes breast cancer progression. It has been also studied that using maraviroc in case of metastatic breast cancer model impairs CCL5-stimulated cytoplasmic calcium waves and that further reduces basal breast cancer cells. Besides In -Vivo studies on metastatic breast cancer using the maraviroc has shown that it significantly reduces

the number and the size of pulmonary metastasis[221]. In our experimental settings using human melanoma in NodScid mouse model we have shown that blockade of CCR5 by using the maraviroc significantly enhances the growth of melanoma that further proves the significance of CCL5 in melanoma tumour model, similarly when we use LCMV-WE alone it leads to the complete regression of tumour(Fig. 26)which further signifies that CCL5 overexpression has the protective effect on melanoma. Thus we further believe that we can use CCL5 as one of the biomarkers for the melanoma in the near future.

4.9 CCL5 upregulation upon LCMV infection seems to be dependent on PI3K/Akt pathway.

Now another question that was interesting for us was the signalling pathways involved in the CCL5 upregulation upon LCMV-WE infection. When we look for the different up stream molecules that might be involved in the CCL5 upregulation, we found out PI3K/AkT as one of the main targets, this pathway is required to induce growth, proliferation and differentiation of adult stem cells and also is responsible for the transformation in different tissues[222]. It is quite possible that non-intervention of the PI3K communication pathway could be an important event in the cancer process that can be owed to the oncogenic activating mutations and inactivation of the tumour suppressor regulating pathways[223]. The PI3K/AKT signalling cascade has been reported to be extremely decisive in cancer because it advances cell growth and survival[224]for instance in case of breast cancer this pathway is highly activated through the change in copy number or by mutations or deletions of several other gene**s** [225]. Epigenetic modulators have been reported to play a significant role in the oncogenicity of PI3K in different cancers[223]. In many different human cancers PI3K signalling is activated via several different mechanisms[226] mutational activation or the amplification of the genes that actually encode important components of the PI3K pathway lead to an enhanced PI3K signalling[227]. According to different reports genetic mutations or amplifications of RTKs can lead to the PI3K activation, there might be a mechanism of P13K activation in a individual cancer cell type that further hints for the effective inhibition of this pathway by different inhibitors or therapeutics. This pathway in the recent years has emerged as the goal for the cancer treatment, since tumours do exist in the stressful conditions and the role of PI3K/Akt becomes crucial and many different drugs that inhibit different components of this pathway is also now in clinical trials[228]. So according to our hypothesis blocking of the PI3K will further limit the production of CCL5 from the melanoma cell types, to further test our hypothesis we tested different inhibitors that

block the whole signalling pathway among them we got the best results with the PI3K inhibitor, when we block it there is significant reduction of CCL5 production from the melanoma cells this effect was highly visible in both Mamel-86A and mamel-51 cells (Fig. 27A and Fig. 27B**).** Thus we conclude that upon LCMV-WE infection there is the significant activation of the PI3K pathways which further leads to the activation of the downstream signalling molecules and finally this whole signalling cascade lead to the massive CCL5 production that ultimately leads to the NK infiltrate to the tumour bed and finally the efficient tumour clearance.

5 Summary

Immune activation within the tumor is one promising approach to induce immune-mediated tumor regression. Viruses are promising approaches to induce tumor-specific immune activation, however not all tumor types respond to virotherapy and mechanisms explaining such differences remains to be defined. Here, by using the lymphocytic choriomeningitis virus (LCMV) in different tumor cell lines, we found that some human melanoma cell lines responded strongly with CCL5 production especially mamel-86A responded very well to the arenavirus therapy, when the mamel-86A tumor bearing NodScid mice were treated with the LCMV there was the complete tumor regression and mice were tumor free at one point, but some tumors like mamel-51 showed massive resistance to the arenavirus therapy, To further address this question of robust tumor regression in case of mamel-86A and strong resistance towards LCMV therapy by mamel-51. We performed both in-vivo and in-vitro experiments and we discovered that the tumors that responded well to the LCMV therapy showed enhanced CCL5 production upon LCMV infection while as in case of non-responding tumors there was indigent CCL5 production, this variation in the chemokine profile upon the viral therapy further gave us the clue that there must be different immune cells infiltrate in the tumor microenvironment both in responding and non-responding tumor models and upon further investigation we discovered that there is massive NK cell infiltrate to the tumor site upon LCMV infection but this scenario only happens in case of responding tumor (Mamel-86A) while as in case of non-responding tumor there was very poor NK cell infiltrate, so higher CCL5 drives more NK cells to the site of the tumor and vice versa. The next interesting finding in our study was that the production of CCL5 in the Mamel-86A tumor cells is dependent on PI3K pathway; blockade of PI3K by using the inhibitors abrogates the production of CCL5. To further, address this massive antitumor effects we used NK cell deficient mice, these mice showed faster tumor growth and poor antitumor effects even after LCMV therapy but since the antitumor effects in our experimental model is also linked to the CCL5, this further prompted us to look how the tumors will be behave upon the blockage of CCR5, upon in-vivo experimental settings we found out that blockade of CCL5 leads to the enhanced growth of tumor Thus we can say depletion of NK cells or CCL5 abolished anti-tumoral effects of virotherapy. In conclusion, we identified CCL5 and NK cell mediated cytotoxicity as a new mechanism leading to regression of melanoma and explaining differences in the immunological response to virotherapy.

6 Zusammenfassung

Die Immunaktivierung innerhalb des Tumors ist ein vielversprechender Ansatz, um eine immunvermittelte Tumorregression zu induzieren. Viren sind vielversprechende Ansätze zur Induktion einer tumorspezifischen Immunaktivierung, jedoch sprechen nicht alle Tumortypen auf eine Virotherapie an und die Mechanismen, die diese Unterschiede erklären würden, müssen noch identifiziert werden. Hier fanden wir unter Verwendung des lymphozytären Choriomeningitis-Virus (LCMV) in verschiedenen Tumorzelllinien heraus, dass einige humane Melanomzelllinien wie MaMel-86a sehr gut auf die Arenavirus- Therapie ansprachen. Während die LCMV Behandlung MaMel-86a Tumor tragender NodScid Mäuse, die mit einer starken CCL5-Produktion reagierten, zu einer vollständigen Tumorregression und sogar teilweise tumorfreien Mäusen führten, zeigten manche Tumoren wie das MaMel-51 eine massive Resistenz gegen die Arenavirus Therapie. Um die Fragestellung sowohl um diese starke Tumorregression der MaMel 86a Tumoren, als auch die starke Resistenz der MaMel-41 Tumoren gegen die LCMV Therapie weiter zu untersuchen führten wir sowohl in-vivo als auch in-vitro Experimente durch und entdeckten, dass die Tumoren, die gut auf die LCMV-Therapie ansprachen, eine erhöhte CCL5-Produktion nach LCMV-Infektion zeigten, während im Falle von nicht ansprechenden Tumoren eine schwache CCL5-Produktion auftrat. Ferner ergab das Chemokinprofil nach der Virustherapie den Hinweis, dass sowohl in ansprechenden als auch in nichtansprechenden Tumormodellen unterschiedliche Immunzellen die Tumormikroumgebung infiltrieren. Nach weiteren Untersuchungen stellten wir fest, dass die Tumoren nach LCMV-Infektion massiv mit NK-Zellen infiltriert sind, dieses Szenario aber nur im Falle eines ansprechenden Tumors (Mamel-86A) auftritt, während im Falle eines nicht ansprechenden Tumors eine sehr schlechte NK-Zell- Infiltration vorlag, so dass ein höheres CCL5 mehr NK-Zellen in den Tumor lockt und umgekehrt. Der nächste interessante Befund in unserer Studie war, dass die Produktion von CCL5 in den Mamel-86A-Tumorzellen vom PI3K-Signalweg abhängt. Die Blockade von PI3K durch Verwendung von Inhibitoren unterbindet die Produktion von CCL5. Um diesem massiven antitumoralen Effekten entgegenzuwirken verwendeten wir Mäuse ohne NK- Zellen. Diese Mäuse zeigten auch nach LCMV-Therapie ein schnelleres Tumorwachstum und schwache Antitumoreffekte. Da die antitumoralen Effekte in unserem Versuchsmodell jedoch auch mit CCL5 in Verbindung standen, führte uns das dazu, sich anzuschauen, wie sich die Tumoren bei einer Blockade von CCL5 verhalten. In vivo fanden

wir heraus, dass eine Blockade von CCL5 zu einem verstärkten Wachstum des Tumors führt. Man kann also sagen, dass die Depletion von NK-Zellen oder CCL5 die antitumoralen Wirkungen der Virotherapie aufhob. Zusammenfassend identifizierten wir die CCL5- und NK-Zell-vermittelte Zytotoxizität als neuen Mechanismus, der zur Regression des Melanoms und zur Erklärung der Unterschiede in der immunologischen Reaktion auf Virotherapie führt.

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8 List of figures

9 Curriculum vitae

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Thank you

Hilal Bhat

11 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 „Role of arenavirus therapy in melanoma“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Hilal Ahmad Bhat befürworte.

Essen, den _____ _____Prof. Dr. Karl Sebastian Lang

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