MECHANISMS OF REJECTION OF HIGH GRADE B CELL LYMPHOMA IN MICE

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A. Synopsis

The incidence of high grade B cell lymphoma in western countries has increased over the last decades. Improvement of conventional chemotherapy regimens has significantly contributed to prolonged 5-year survival rates which currently reach around 60%. However, relapse after conventional chemotherapy is an important challenge, especially in high grade B cell lymphomas. The potential benefit of immunological approaches for the elimination of such lymphomas still remains unclear. In this study, we attempted to address whether the forced expression of foreign antigens in a tumor of B cell origin leads to immune recognition and elimination of the tumor and to assess the potential role of IFN-gamma (IFN-γ) in tumor rejection. To this end, we used a transgenic mouse lymphoma model, where the human proto-oncogene *c-myc* (a foreign antigen for the mouse host) is under the control of regulatory elements of the immunoglobulin lambda locus, thereby recapitulating the important features of a t(8;22) translocation as found in human Burkitt's lymphoma. From these spontaneously developing tumors, lymphoma cell lines were established that either express (line 291) or are deficient (line 9) in Stat1- a key signaling molecule in the response to interferons. We found that the expression of foreign antigens such as chicken ovalbumin (OVA) and green fluorescent protein (GFP) in Stat1-competent 291 cells led to immune responses that delayed tumor progression and improved survival of wild-type animals. Consistent with this, loss of foreign antigen inevitably led to accelerated tumor progression upon transfer into immunocompetent wild-type mice. Transfer of immunogenic 291-OVA-GFP lymphoma cells led to increased tumor progression without loss of foreign antigen upon transfer into IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ mice indicating that no selection of antigen loss-variants occurred in these mice. The rejection of 291-OVA-GFP cells in wild-type mice was at least in part mediated by CD8+ T cells as measured by enrichment of the OVA antigen-derived MHC class I-restricted SIINFEKL epitope-specific cells in wild-type recipients.. Interestingly, Stat1-deficient lymphoma cells (9-GFP and 9-OVA-GFP) were rejected by immunocompetent UBQ-GFP transgenic wild-type C57BL/6 mice irrespectively of the presence of a foreign antigen, indicating the existence of immunosurveillance against these Stat1-deficient lymphomas. To evaluate the key players behind lymphoma rejection, we transferred 9-GFP cells into IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients. This led to enhanced tumor growth indicating that endogenous IFN-γ production and Stat1 signaling are critical for tumor rejection. To gain an insight into the mechanistic aspects of innate immunosurveillance against the Stat1-competent and Stat1-deficient lymphomas, NK cell functionality was evaluated. We found that NK cells could

efficiently lyse both Stat1-competent and Stat1-deficient lymphoma cell lines *in vitro*. Treatment with IFN-γ increased the susceptibility of Stat1-deficient lymphoma cells to NK cell killing, but decreased that of Stat1-competent cells, presumably by upregulating MHC class I expression. The results of this work show that host IFN-γ and Stat1 signaling are important for tumor clearance, and that paradoxically, the absence of Stat1 within the lymphoma is required for rejection. The data suggest a dual role for Stat1 in the tumor cells: it is required for T cell mediated rejection of tumors expressing foreign antigens. Yet, if no foreign antigen is expressed, it acts as an oncogene and is required for outgrowth of a tumor in an immunocompetent host, presumably by allowing NK cells to be efficiently inactivated by the tumor cells.

B. List of Abbreviations

BL	Burkitt's lymphoma
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CDK	Cyclin-dependent kinases
CFSE	Carboxy fluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DMEM	Dulbecco's modified eagle medium
E:T	Effector:Target ratio
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetraacetic acid
ELISPOT	Enzyme-linked immunosorbent spot
GFP	Green fluorescent protein
IFN	Interferon
IFNγR	Interferon-γ receptor
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-10	Interleukin-10
IMDM	Iscove's modified Dulbecco's medium
IRES	Internal ribosome entry site
JAK	Janus-associated kinase
kDa	Kilodalton
LCL	Lymphoblastoid cell line
LDH	Lactate dehydrogenase
LL	Lymphoblastic lymphoma
MACS	Magnetic cell separation
MART-1	Melanoma-associated antigen recognized by T cells
MCA	3-Methycholanthrene
MCS	Multiple cloning site
NA	Not available

NK	Natural killer cell
NKT	Natural killer T cell
OVA	Ovalbumin
PBS	Phosphate buffered saline
RAG-1	Recombination activating gene 1
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell

1. Introduction

1.1 Lymphomas of B cell origin

Disruption of the physiological balance between cell death and proliferation is a common feature of cancers. According to the mechanisms through which the imbalance is achieved, human B-cell lymphomas can be grouped into low and high growth fraction lymphomas. Lymphomas and leukemia are a group of different types of cancers of the lymphoid system, in which distinctive molecular alterations are observed. Low growth fraction B cell lymphomas including follicular lymphoma (FL), marginal zone lymphomas (MZLs), mantle cell lymphoma (MCL), and B-cell chronic lymphocytic leukemia (B-CLL), are distinguished by small cell size, low proliferative index, formation of large tumoral masses in lymph nodes and clinical stages associated with low clinical aggressivity (Seng and Peterson, 1997; Capello and Gaidano, 2000). A different picture is commonly observed in high-growth fraction B cell lymphomas such as large B cell lymphoma (LBCL) and Burkitt's lymphoma (BL). These tumors are associated with an increased proliferative index and larger cell size and are clinically more aggressive as a consequence of alterations in key cell cycle regulators such as BCL6 or c-MYC (Sanchez-Beato et al., 2003). The different B cell lymphomas and their distinct alterations in the molecular patterns are indicated in Table 1.

Lymphoma	Gene	Function	Alteration	Frequency (%)	Reference
FL	*BCL2*	Apoptosis inhibition	t(14;18)	70-90	(Yunis et al., 1987)
MALT lymphoma	*BCL10*	Apoptosis inhibition through NF-kB	t(1;14)	50	(Willis et al., 1999)
MCL	*Cyclin D1*	G1/S cell cycle transition	t(11;18)	90	(Levine et al., 1989)
B-CLL	*Unknown*	Unknown	13q14 deletion	50-60	(Kalachikov et al., 1997)
LPL	*PAX5*	B-cell differentiation	t(9;14)	50	(Iida et al., 1996)
LBCL	*BCL6*	B-cell activation and differentiation, cell cycle control	t(3;N)	30-40	(Lo Coco et al., 1994)
BL	*c-myc*	Differentiation, cell cycle control, apoptosis induction, Adhesion	t(8;14) t(8;22) t(2;8)	80 10 10	(Taub et al., 1982; Boxer and Dang, 2001)

Table 1 B cell lymphomas and their defining molecular characteristics
FL-'Follicular lymphoma', MALT-'Mucosa associated lymphoid tissue lymphoma', MCL-'Mantle cell lymphoma', B-CLL-'B cell chronic lymphocytic leukemia', LPL-'lymphoplasmacytic lymphoma', LBCL-'large B-cell lymphoma' and BL-'Burkitt's lymphoma'.

1.2 Burkitt's Lymphoma

Burkitt's lymphoma (BL), first described by Denis Burkitt as a clinical entity in African children, is the first human tumor whose pathogenesis could be linked both to an oncogenic virus, Epstein-Barr Virus (EBV), and to the activation of a specific cellular oncogene namely *c-myc*. Three epidemiologically distinct forms of BL are now recognized. The high-incidence

'endemic' form typically presents as a jaw and/or abdominal tumor in children in tropical areas of equatorial Africa and Papua New Guinea. Elsewhere, BL occurs in 'sporadic' form mainly in children at intermediate to low incidence. Remarkably, a third, adult form of the tumor, AIDS-BL proved to be very common among HIV-infected individuals. All forms of BL show a similar histological appearance, with malignant population of round monomorphic B cells, that gives the tumor histology a 'starry sky' pattern.

1.2.1 Translocations

Regardless of its geographical origin, Burkitt's lymphoma is invariably characterized by chromosomal translocations juxtaposing the *c-myc* protooncogene (on chromosome 8) either with regulatory sequences of immunoglobulin (Ig) heavy (H) chain on chromosome 14 leading to the translocation t(8;14) or with regulatory sequences of Ig light (L) chain genes, either Ig kappa (Igκ) on chromosome 2 leading to t(2;8) or Ig lambda (Igλ) on chromosome 22 leading to translocation t(8;22). All these translocations result in deregulated expression of *c-myc* which is a hallmark of Burkitt's lymphoma (Dalla-Favera et al., 1982). Accordingly, the World Health Organization Classification of Hematologic Malignancies indicates that the presence of one of the *c-myc*/Ig translocations should be considered the gold standard for the diagnosis of BL (Harris et al., 1999).

1.2.2 EBV and Burkitt's lymphoma

Epstein-Barr virus (EBV), a gamma 1-herpesvirus carried by the vast majority of individuals worldwide as a lifelong asymptomatic infection, has growth-transforming potential and is linked with a variety of B cell, T/NK cell and epithelial malignancies (Kelly and Rickinson, 2007). The high-incidence 'endemic' BL form occurring in children of Africa is almost always EBV genome-positive whereas in the 'sporadic' form, different degrees of EBV association are observed. 30-40% of AIDS-BL tumors carry EBV. EBV preferentially infects B lymphocytes through the binding of viral envelope glycoprotein gp350 to the CD21 receptor on the surface of B cells and through the binding of the second glycoprotein gp42 to human leukocyte antigen (HLA) MHC class II molecule as a co-receptor (Young and Rickinson, 2004). The frequency of EBV's association with BL (particularly endemic) and the fact that all EBV+ BLs (whether endemic, sporadic or AIDS-associated) carry the virus in every cell of the malignant clone, imply a definitive role for the virus in the tumor pathogenesis. Importantly, EBV has the ability to transform resting B cells into immortalized, latently infected lymphoblastoid cell lines (LCLs), which provide an in vitro system for the lymphomagenic potential of the virus (Young and Rickinson, 2004)

1.2.3 c-myc function in normal cell growth and cancer development

The *c-myc* gene was identified as the cellular homologue of the retroviral v-myc oncogene. *c-myc* belongs to the family of myc genes which includes *B-myc*, *L-myc*, *N-myc* and *s-myc* although only *c-myc*, *L-myc* and *N-myc* were found to be involved in neoplasmic diseases (Dang, 1999). c-MYC protein is overexpressed in a variety of human cancers including approximately 80% of breast cancers, 70% of colon cancers, 90% gynecological cancers, and 50% of hepatocellular carcinomas. A variety of hematological malignancies also express abnormal *c-myc* levels. The gene is located on human chromosome 8 band q24, consists of three exons that give rise to a 64 kDa protein. It has been suggested that both activation and repression of genes through *c-myc* are important for neoplastic transformation (Dang, 1999)

Targeted deletion of the murine *c-myc* gene leads to embryonic lethality with growth abnormalities in the heart, pericardium, neural tube and a delay or failure in embryonic turning, suggesting its role in the normal development (Davis et al., 1993). In normal cells, expression of *c-myc* is tightly-regulated at transcriptional and post-transcriptional level by growth factors and external signals. In general, resting cells express very low amounts of c-MYC protein and the levels increase in response to growth factors leading to entry into the cell cycle. The levels return to the basal quiescent state in the resulting daughter cells. Ectopic overexpression of *c-myc* in primary cells leads to the activation of failsafe mechanisms which involve the p19/p14ARF and p53-dependent pathways and switch on apoptosis programs to prevent uncontrolled growth (Lowe et al., 2004).

1.2.4 Models for studying Burkitt's lymphoma

The involvement of EBV and *c-myc* in determining disease pathogenesis and progression on the one hand and the immunogenicity of the tumor on the other hand has been a subject of intensive investigations. To gain insights into the changes imposed by different EBV genes and the oncogene *c-myc*, an *in vitro* model system that recapitulates important features of LCLs and BL cells *in vitro* has been established which is described in detail in section 1.6.4.

Previous efforts to develop a mouse model of BL centered on generation of transgenic mice that express the *c-myc* gene under the control of IgH or IgL regulatory sequences. The classic experiment from Adams et al., showed that transgenic mice, expressing *c-myc* gene under the control of the intronic immunoglobulin (Ig) heavy chain enhancer, develop monoclonal or oligoclonal early pre-B cell lymphoid malignancies (Adams et al., 1985). These constructs induced primarily precursor B cell as well as some surface Ig (sIg) positive B cell lymphomas. However, the pathological features of the neoplasms were consistent with the diagnosis of lymphoblastic lymphoma (LL) rather than BL. In 2000, Kovalchuk et al., have

shown that mice transgenic for a human *c-myc* protooncogene driven by regulatory elements of Igλ locus develop lymphomas with striking similarities to human BL. Monoclonal tumors with the characteristic starry sky appearance of BL developed from an initially polyclonal population of B cells. Lymph nodes and spleens of tumor bearing mice were populated by uniform populations of IgM+CD19+CD5-CD23- cells, which is characteristic phenotype of human BL (Gerbitz et al., 1999; Kovalchuk et al., 2000).

1.3 Immune response to tumors

An increased understanding of the complex interaction of transformed cells and cells of the immune system in the late 1950s led to the formulation of the cancer immunosurveillance theory by Burnet and Thomas. However, as Burnet and Thomas commented, the caveat with the idea of cancer immunosurveillance was that it could not be shown to exist in experimental animals (Burnet et al., 1957; Thomas L et al., 1982). This led others to explore the existence of cancer immunosurveillance, although the initial studies were inconclusive and failed to prove or disprove the existence of cancer immunosurveillance (Klein, 1973; Stutman, 1975). With the availability of the mouse models and development of monoclonal antibodies, the concept of cancer immunosurveillance has recently been validated (Smyth et al., 2001b; Dunn et al., 2002; Dunn et al., 2004).

Additional studies demonstrated that the immune system may not only be involved in tumor elimination but also promote the development of primary tumors with reduced immunogenicity that allows escape from immune recognition (Shankaran et al., 2001). This led to the formation of another, yet more refined hypothesis termed as "cancer immunoediting" encompassing host-protective and tumor-sculptive actions of the immune system during tumorigenesis.

1.3.1 Cancer immunoediting

The ability of the immune system to control and shape tumors, termed cancer immunoediting, is the result of three processes that function either independently or in a sequence of three phases namely, elimination (cancer immunosurveillance, in which the immune system functions as extrinsic tumor suppressor in the naïve host), equilibrium (expansion of transformed cells is held in check by the immune system) and escape (emergence of tumor cell variants with impaired immunogenicity which later grow into macroscopic tumors).

1.3.2 Elimination phase

One approach for testing the role of the immune system in controlling tumor development is to remove specific components of the mouse immune system and monitor the mice for the

development of tumors. Predominantly through the use of gene-targeted mice, such an approach has demonstrated that a number of immune cells including those of the innate and adaptive system are important for the suppression of tumors. For example, studies on BALB/c and C57BL/6 mice revealed that natural killer (NK) and natural killer T (NKT) cells also participate in cancer immunosurveillance (Smyth et al., 2001a). Studies in spontaneous tumor models of B cell lymphomas in mice lacking perforin and beta 2 microglobulin (β2m) have revealed that such lymphomas can be rejected by NK cells and γδ T cells following transplantation into WT mice. These studies demonstrated that cell surface expression of MHC class I molecules by tumor cells is important in determining which effector cells mediate protective effects (Street et al., 2004). Many of the hypotheses of cancer immunosurveillance were validated in models of chemical carcinogenesis. The most commonly used carcinogen-induced tumor models are fibrosarcomas induced using MCA (methylcholanthrene). Recombination activating gene ($Rag2^{-/-}$) and Severe Combined Immunodeficiency (SCID) mice, both of which lack an adaptive immune system display an increased incidence of tumor formation indicating that both αβ T cells and γδ T cells are found to be important in tumor suppression (Shankaran et al., 2001). Studies on IFN (Dighe et al., 1994; Kaplan et al., 1998; Shankaran et al., 2001) demonstrated that deficiencies in key immunological cytokines and signal transducers such as IFN-γ and Stat1 resulted in higher susceptibility to chemical carcinogens. The different mouse models which were used to study immunosurveillance against tumors are shown in table 2.

Mice	Immune phenotype	Tumor phenotype	Reference
$Rag1^{-/-}$ or $Rag2^{-/-}$	lack T cells, B cells, NKT cells but posses functional NK cells	↑ MCA induced sarcomas	(Shankaran et al., 2001; Dunn et al., 2006)
$Rag2^{-/-}\gamma c^{-/-}$	lack T cells and B cells, NKT and NK cells	↑ B cell tumors	(Kovacic et al., 2006)
IFN-$\gamma^{-/-}$	Lack IFN-γ	↑ MCA induced sarcomas, ↑ Spontaneous disseminated lymphomas ↑ B16-OVA melanoma formation	(Kaplan et al., 1998; Shankaran et al., 2001; Schuler and Blankenstein, 2003)
IFN-γR1$^{-/-}$	Insensitive to IFN-γ	↑ MCA-induced sarcomas	(Qin and Blankenstein, 2000)
$Stat1^{-/-}$	Insensitive to IFN-α, IFN-β and IFN-γ	↑ MCA induced sarcomas, Wider tumor range in $Stat1^{-/-}Tp53^{-/-}$ mice	(Kaplan et al., 1998; Shankaran et al., 2001; Kovacic et al., 2006)
$Rag2^{-/-}Stat1^{-/-}$	Lacks T cell, B cells, NKT cells. Insensitive to IFN-α, IFN-β and IFN-γ	↑ MCA induced sarcomas, ↑ Spontaneous intestinal and mammary neoplasms	(Shankaran et al., 2001; Dunn et al., 2006)

Table 2 Increased susceptibility of immunodeficient mice to carcinogen-induced and spontaneous tumors

Various contemporary studies of mice with molecularly defined inactivating defects in innate and adaptive immunity have shown that immunodeficient mice do develop more spontaneous and carcinogen-induced tumors than their immunocompetent counterparts. This table depicts the various immunodeficent mice used to examine the cancer immunoediting hypothesis. MCA (methylcholanthrene) is widely used as chemical carcinogen in the above mentioned studies. B16-OVA is a mouse melanoma tumor engineered to express chicken ovalbumin as foreign antigen.$Stat1^{-/-}$ $Tp53^{-/-}$ is Stat1 and p53 tumor suppressor double knock out mice

1.3.3 Equilibrium phase

The elimination phase of the cancer immunoediting process leads to the eradication of a significant proportion of transformed cells but the tumor cells exist in a period of latency extending from the end of the elimination phase until the start of the escape phase which ultimately results in the emergence of detectable malignant disease. It is also hypothesized that during the equilibrium phase, the tumor cells acquire enormous plasticity which allows them to grow in immunocompetent animals (Lengauer et al., 1998). However, the whole process is terminated if the elimination process in immunoediting is successful at destroying the developing tumor. In a recent study of mouse models of primary carcinogenesis, Schreiber and colleagues have demonstrated that neoplastic cells in equilibrium are transformed but proliferate poorly *in vivo*. Furthermore, they showed that the elimination phase requires the action of both innate and adaptive immunity and the equilibrium is maintained solely by the adaptive immune system (Koebel et al., 2007).The three phases of the cancer immunoediting process are shown in figure 1.

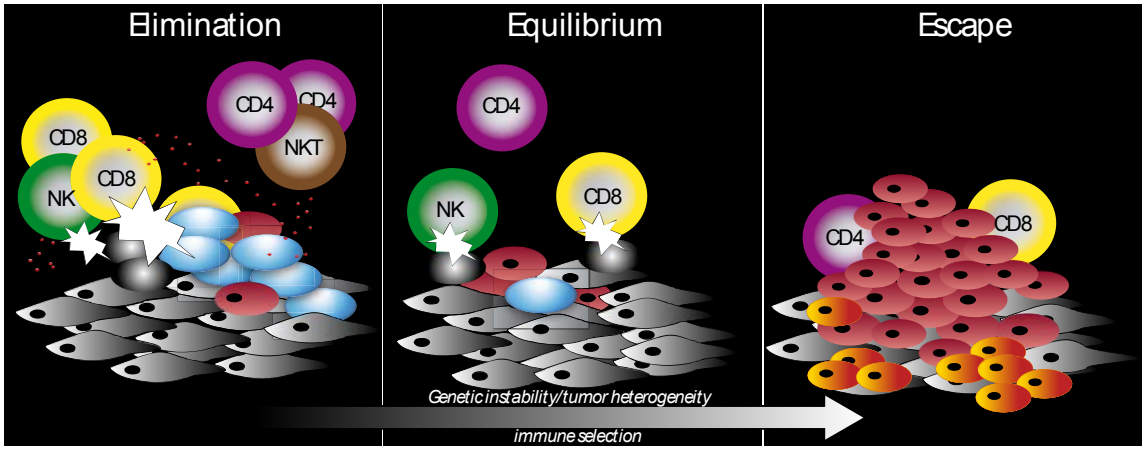


Figure 1 Three Es of cancer immunoediting

Cancer immunoediting comprises three phases. a) Elimination generally indicates the immunosurveillance where different immune cells encounter the tumor and control tumor growth. b) Equlibrium refers to the process in which the immune system selects and promotes the generation of tumor cells or antigen loss variants with increased capacity to survive the immune attack. c) Escape is the process wherein the immunologically sculpted tumor expands in an uncontrollable manner. In a and b, developing tumor cells (blue), tumor cell variants (red) and underlying stroma and non-transformed cells (grey) are shown; in c, additional tumor variants (orange) that have formed as a result of the equilibrium process are shown. Different lymphocyte populations are as marked. The small orange circles represent cytokines and the white flashes represent cytotoxic activity of lymphocytes against tumor cells (Adapted from Dunn GP et al., 2002).

1.3.4 Escape phase

When malignant growth is tightly prevented due to immune recognition, tumors should be eliminated. Their persistence in immunocompetent hosts demonstrates the beginning of an escape process - the third phase in the cancer immunoediting model. Many studies have shown that tumor escape can be a direct consequence of alterations occurring in edited tumors themselves. During the escape period it is envisaged that tumor cells accumulate further changes (such as DNA mutations and changes in gene expression) that can modulate the expression of tumor-associated antigens (TAA) and as this process continues, the immune system exerts a selective pressure by eliminating susceptible tumor clones. The pressure exerted by the immune system is often sufficient to control tumor growth. But if the immune response fails to completely eliminate the tumor, this results in the selection of tumor cell variants that are able to resist, avoid or suppress the anti-tumor immune response leading to tumor escape. In this line it has been shown that malignancies develop due to either direct or indirect alterations in antigen processing and presentation that render them invisible for T cells (Seliger et al., 2000a; Seliger et al., 2000b). In addition, loss of tumor-specific antigen as escape mechanisms was seen not only in naturally occurring but also in the therapeutic vaccination settings. Although adoptive transfer of T cells specific for tumor antigens such as

Melanoma antigen recognized by T cells (MART-1)/Melan A or gp100 together with IL-2 leads to better clinical responses in metastatic melanoma, investigators have observed loss of antigens in progressive melanomas (Yee et al., 2002). In recent years, NK cell-mediated tumor surveillance has been shown to be important for tumor rejection and a recent study demonstrated that human tumors secrete soluble forms of MHC class I chain related (MIC) NKG2D ligands leading to downregulation of NKG2D receptor on effector cells and subsequently resulting in attenuated cytotoxicity functions (Groh et al., 2002). In addition, studies have shown that depletion of CD4+CD25+ regulatory T cells (Tregs) by using an anti-CD25 monoclonal antibody enabled mice to reject tumors that grew progressively in control mice (Shimizu et al., 1999) indicating that tumors might induce regulatory T cell phenotypes and exploit this to escape immune responses.

1.4 Foreign antigen-mediated tumor rejection

Tumors are caused by the progressive growth of the progeny of single transformed cells. Treatment of tumors requires that all malignant cells be either removed or destroyed without affecting normal cells. An attractive way of achieving this goal is to induce an immune response against the tumor that would discriminate between tumor cells and their normal counterparts. Immunological approaches to the treatment of tumors have been attempted over several years but the success rates are variable and often inconsistent. In general, during the development of T cells, only those thymocytes whose receptors interact weakly with self antigen-MHC complexes receive a signal that enables them to survive. This is called positive selection. Thymocytes whose receptors bind and react strongly to self antigens receive signals that lead to their death, a process called ‘negative selection’. These two selection processes allow for the establishment of cellular immunity that can recognize foreign antigens presented by self MHC molecules and at the same time eliminate potentially self-reactive thymocytes, thereby establishing immunological tolerance to ubiquitous self antigens. In spontaneously developing tumors, the immune system is challenged with a dilemma, namely that proteins present in cancerous cells are largely normal self proteins. Some of these proteins, however, are altered self proteins. As such mutations in proteins might give rise to peptides that are not presented in normal self tissues; the immune system may selectively recognize tumor cells. Furthermore, in some tumors normal proteins are overexpressed thereby allowing the immune system to discriminate between normal and aberrant cells by the mere fact that aberrant cells produce and therefore present those overexpressed proteins to higher levels than normal cells. The recognition of self proteins presented in abnormal levels or in abnormal forms is believed to be due to the fact that the negative selection of thymocytes is not absolute. Indeed the

success stories of adoptive T cell transfer for the treatment of malignant melanoma demonstrate that self antigens expressed in abnormal levels can be efficiently targeted by the immune system. Nevertheless, tumors expressing foreign antigens are better targeted by the immune system as exemplified by numerous virus-associated tumors. T cell reactivity against foreign antigens is clearly stronger than against self antigens. It is widely believed that the presence of foreign antigens is crucial for eliciting strong immune responses against tumors.

The role of different components of the host immune system involved in suppression of tumor progression has been under intense investigation. The CD8+ T cells recognize fragments of intracellular proteins in the form of peptides of 8-10 amino acids complexed with MHC class I molecules (Townsend et al., 1986; Townsend and Bodmer, 1989). Studies from Mc Cabe et al have shown that recombinant vaccinia virus expressing the minimal determinant as well as the full length ovalbumin protein elicited the specific lytic response in C57BL/6 mice against EG.7OVA, a transfectant of the murine thymoma EL4 cell line expressing ovalbumin (Zhou et al., 1992; Minev et al., 1994; McCabe et al., 1995). Stimulating these effectors in vitro with $OVA_{257-264}$ peptide induced H-$2K^b$ restricted CD8+ T cells that not only lysed but also specifically secreted IFN-γ in response to the antigen. Furthermore, the growth of established ovalbumin transfected tumors in a pulmonary metastasis model system was significantly reduced upon adoptive transfer of anti-$OVA_{257-264}$ CD8+ T cells (McCabe et al., 1995). Moreover, using the B16 melanoma model, which expresses OVA as a surrogate tumor antigen, Schüler et al. have shown that CD8+ effector T cells recognize the antigens directly on tumor cells and that tumor rejection requires IFN-γ (Schuler and Blankenstein, 2003). Despite the cancer cell's expression of immunogenic molecules, the host does not always mount an effective immune response to these antigens. A number of groups have conducted experiments in which highly immunogenic foreign antigens such as hemagglutinin from influenza A virus (Staveley-O'Carroll et al., 1998), the β-galactosidase (β-gal) from E.coli (Wang et al., 1995) and the ovalbumin protein from chicken (McCabe et al., 1995) were expressed in tumor cells. The results obtained using different foreign antigens are fairly uniform; tumors tend to grow progressively, retaining their lethality despite of the expression of foreign and highly immunogenic proteins by the tumor cells (Restifo et al., 2002). It is not yet known whether targeting 'self' or foreign antigens will be more successful in the immunotherapy for cancer. Some researchers have asserted that mutated tumor-associated antigens (TAA) are superior targets for vaccine design because immune cells will not be tolerized to these antigens. However, recent work from Hy Levitsky's and Linda Sherman's groups has shown that even the most immunogenic 'foreign' antigen, such as hemagglutinin

(HA) antigen from influenza A virus can be tolerizing when expressed peripherally (i.e. outside the thymus) either in normal cells or in tumor cells (Staveley-O'Carroll et al., 1998; Morgan et al., 1999). The different foreign antigens used in studies are shown in table 3.

Tumor model	Foreign antigen	Experimental outcome	Reference
Colon carcinoma CT26	β-galactosidase from *E . coli*	Delay in tumor progression and improved survival	(Wang et al., 1995)
A20 B cell lymphoma	Hemagglutinin from influenza	Induction of T cell anergy during tumor progression	(Staveley-O'Carroll et al., 1998)
EG7-Thymoma	Ovalbumin from chicken	Reduction of growth of established tumors	(McCabe et al., 1995)

Table 3 Different foreign antigens used in tumor models and their experimental outcome.

1.5 Role of interferons in cancer surveillance

Interferons (IFNs) are a family of proteins originally identified by their ability to protect cells from viral infections. Depending on the structural basis and the stimuli that induce their expression, IFNs have been divided into type I and type-II interferons. Type I IFNs are produced in response to viral infections and are subclassified into IFN-α and IFN-ß (Pestka et al., 1987). The only type-II interferon, IFN-γ, is produced by T lymphocytes, NKT cells and NK cells (Farrar and Schreiber, 1993; Boehm et al., 1997). The interferon signaling pathway is shown in figure 2.

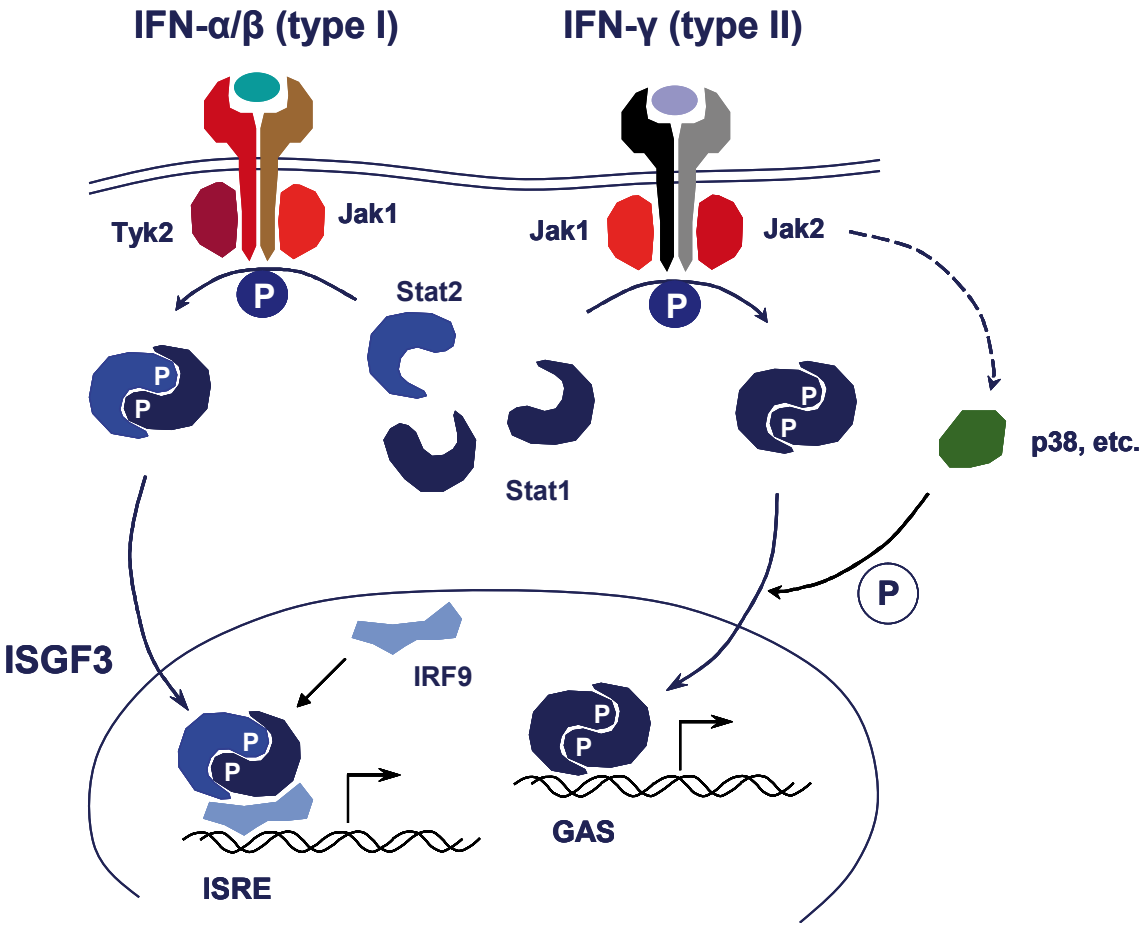


Figure 2: Overview of Interferon Signaling
Interferon-γ (IFN-γ; type II IFN) induces the dimerization of the IFN-γ receptor subunits, IFNGR1 and IFNGR2, which leads to the activation of the Janus kinases Jak1 and Jak2 that are constitutively associated with these subunits. The Jaks phosphorylate a crucial tyrosine residue of IFNGR1, forming a Stat1 (signal transducer and activator of transcription 1)-binding site; they then tyrosine phosphorylate receptor-bound Stat1. Stat1 homodimers enter the nucleus and bind promoters at IFNγ -activated sites (GASs) and induce gene transcription. The type I IFNs (IFNα and IFNβ) bind a receptor that consists of the subunits IFNAR1 and IFNAR2, which are constitutively associated with tyrosine kinase 2 (Tyk2) and Jak1, respectively. Activated Tyk2 and Jak1 phosphorylate Stat1 or Stat2. Stat1 and Stat2 associate with the cytosolic transcription factor IFN-regulatory factor 9 (IRF9), forming a trimeric complex known as IFN-stimulated gene factor 3 (ISGF3). On entering the nucleus, ISGF3 binds IFN-stimulated response elements (ISREs). Courtesy of Mario Köster, HZI Braunschweig.

1.5.1 IFN-γ in tumor immunity

Recent experiments performed in various animal models lacking either IFN-γ (Dalton et al., 1993), IFN-γ receptor (Huang et al., 1993; Lu et al., 1998), or IFN-γ signaling components have emphasized the importance of IFN-γ in tumor rejection. In most of these animal models, IFN-γ was shown to be involved in the tumor rejection as blocking endogenous IFN-γ by administrating neutralizing antibodies inhibited tumor rejection (Dighe et al., 1994). Moreover, in adoptive transfer tumor models, the efficiency of CD8+ T cells in mediating

tumor rejection was directly proportional to the production of IFN-γ (Barth et al., 1991; Becker et al., 2001). It has been shown that the injected CD8+ T cells can eradicate established melanoma tumors and that rejection was mediated by IFN-γ producing T cells. Interestingly, the release of IFN-γ had two crucial functions. First, it inhibited tumor angiogenesis and second, it increased MHC class I expression on tumor cells (Meunier et al., 2005).

Many of the functions of IFN-γ on tumor growth were identified in animal models using chemical carcinogenesis such as MCA. Experiments performed in the interferon unresponsive host ($Stat1^{-/-}$ mice) showed that these animals were highly sensitive to MCA-induced primary tumors. These tumors grew progressively when transplanted into $IFN\gamma^{-/-}$ animals but were rejected when transplanted into a WT immunocompetent host (Shankaran et al., 2001). These experiments demonstrated that IFN-γ responsiveness on the host side is a prerequisite for tumor elimination. The experiments did not address the question whether the tumor cells were direct targets of IFN-γ. Immunogenic MCA-induced tumor cells derived from $Rag2^{-/-}$ mice were rejected when injected into naïve immunocompetent recipients (Shankaran et al., 2001). Surprisingly, when the IFN-γ responsiveness of these cells was abolished by expressing the dominant negative IFNγR1 mutant, then the cells grew progressively in wild-type mice (Dighe et al., 1994). It has been shown that tumors derived from $IFN\gamma R1^{-/-}$ mice are poorly immunogenic. These tumors become immunogenic when interferon signaling was restored by expression of IFNγR1 (Kaplan et al., 1998). Moreover $IFN\gamma R1^{-/-}$ tumor cells were rendered immunogenic by expressing IFN-γ inducible components involved in antigen processing and presentation (Shankaran et al., 2001). The data argue that IFN-γ exerts its function by directly acting on the tumor by increasing the immunogenicity and/or exerting anti-proliferative effects. Conflicting data with respect to the action of IFN-γ derives from Qin and Blankenstein. When MCA-induced fibrosarcomas derived from $IFN\gamma R1^{-/-}$ mice were reconstituted with IFNγR1, the tumor cells were rejected but only in IFNγR competent hosts. The expression of IFNγR in the tumor cells was not of relevance. The authors concluded that IFNγR expression in the tumor cells is not necessary for an antitumor response whereas the IFNγR expression within the host cells is important for the generation of an antitumor response (Qin and Blankenstein, 2000).

As shown in figure 2, the binding of the cytokine to its cell surface receptor results in receptor dimerization and the subsequent activation of Stat proteins. After dimer formation, Stat proteins translocate to the nucleus, where they induce or modulate the expression of target genes (Bromberg and Darnell, 2000; Levy and Darnell, 2002). The binding of IFN-α to its

receptor activates two receptor-associated Janus kinases, Jak1 and Tyk2, which phosphorylate the cytoplasmic tails of the interferon receptor subunits IFNAR-1 and IFNAR-2 on specific tyrosine residues. These phospho-tyrosine residues provide docking sites for the transcription factors of the Stat family which are phosphorylated after ligand binding (Haque and Williams, 1998; Leonard and O'Shea, 1998). IFN-α signaling leads to the formation of interferon stimulated gene factor 3 (ISGF3), a DNA binding complex that consists of Stat1-α (or Stat1-β, an inactive form), Stat2 and p48 DNA binding protein known as interferon regulatory factor 9 (IRF-9) (Darnell et al., 1994).

Increasing evidence suggests that Stat1 acts as a tumor suppressor and seems to play an important role in the regulation of tumor surveillance (Kaplan et al., 1998; Shankaran et al., 2001; Lesinski et al., 2003; Badgwell et al., 2004). Stat1$^{-/-}$ mice are prone to develop solid tumors and the tumor suppressing activity of Stat1 was attributed to its role as a key transcription factor in the interferon signaling pathway. Mice deficient in Stat1 display no developmental abnormalities but completely lack responsiveness to type I and type II interferons (Durbin et al., 1996; Meraz et al., 1996). Stat1 is found to be inactive or deficient in many human tumors (Wong et al., 1997; Abril et al., 1998; Sun et al., 1998; Pansky et al., 2000). Other studies have shown that Stat1 is constitutively expressed in tumors (Bowman et al., 2000). Stat1 is not just activated by IFNs but also by a variety of cytokines such as IL-6, IL-10, growth hormone, leukemia inhibitory factor (LIF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Ruff-Jamison et al., 1993; Silvennoinen et al., 1993; Raz et al., 1994). It is evident that cytokines and growth factors might interfere with tumor growth. It has been shown that Stat1 expression by tumor cells is a negative regulator for tumor angiogenesis and proliferation (Huang et al., 2002). Using fibrosarcoma RAD-105 cells derived from Stat1$^{-/-}$ mice, the reconstitution of Stat1 expression results in a reduction of pro-angiogenic molecules such as bFGF, MMP-2 and MMP-9, suggesting that Stat1 negatively regulates tumor angiogenesis.

1.5.2 Stat1 as tumor promoter for leukemia development

In a recent study, this tumor-suppressive role of Stat1 was challenged. Using a model of v-abl induced B-cell leukemia, it was shown that Stat1 functions as a tumor promoter and induces hematopoietic malignancies (Kovacic et al., 2006). This study suggests that low MHC class I expressing tumor cells enable NK cell lysis and account for increased tumor clearance, but paradoxically they found that Stat1$^{-/-}$ tumor cells acquired increased MHC class I expression upon leukemia progression (Kovacic et al., 2006).

1.6 Immunosuppressive strategies of tumor cells

Despite increasing knowledge of the regulation of anti-tumoral immune responses, tumor immunotherapy is still unsuccessful. Studies from pre-clinical mouse models have shown that cells of the innate and adaptive arm of the immune system can mediate anti-tumoral functions. However, tumors have developed strategies to counteract immunological recognition or effector functions.

1.6.1 Abnormalities in antigen presentation machinery

A major mechanism that allows tumor cells to evade T cell recognition is impaired antigen presentation (Marincola et al., 2000). This is achieved by defects in antigen processing and involves components of the immunoproteasome (LMP2 and LMP7) or the transporter associated with antigen processing. Moreover, low expression, selective loss of individual HLA alleles or complete absence of MHC class I expression due to mutations in the ß2-microglobulin gene, are considered hallmarks in tumor progression.

1.6.2 Secretion of immunosuppressive factors

Transforming growth factor ß (TGF-ß), an immunosuppressive cytokine inhibits T cell activation, differentiation and proliferation (Li et al., 2006), and blockade of TGF-ß signaling allows the generation of otherwise repressed tumor-specific T cells (Gorelik and Flavell, 2001). Recent experiments have shown that TGF-ß acts on CTLs to specifically repress the expression of different cytolytic gene products such as perforin, granzyme A, granzyme B, Fas-L and IFN-γ, which are collectively responsible for CTL mediated cytotoxicity (Thomas and Massague, 2005). In addition to TGF-ß, other biologically active agents such as IL-10 present within the tumor microenvironment impair immune cell functions, including dendritic cells (Gerlini et al., 2004), and it has been demonstrated that IL-10 protects the tumor from CTL mediated cytotoxicity by downregulating TAP-1 and TAP-2 (Kurte et al., 2004).

1.6.3 Tumor-induced antigen-specific CD8+ T cell tolerance

Immunological tolerance is established not only in the thymus but also in the periphery. Several mechanisms have been proposed for the establishment of peripheral tolerance including the presentation of antigen in the absence of co-stimulatory molecules or in the presence of T cell suppressive cytokines. Furthermore, T cells in the periphery are known to be rendered unresponsive against their cognate antigen, which is also referred to as T cell anergy. It has long been speculated that tumors might escape immunological surveillance through the induction of peripheral T cell tolerance and anergy. In support of this concept, the transfer of antigen-specific T cells into tumor-bearing mice caused phenotypic changes in the

transferred T cells as determined by their poor cytolytic and proliferative capacities (Otten and Germain, 1991; Lee et al., 1999). .

1.6.4 MYC overexpression imposes non-immunogenic phenotype on human B cells

When primary EBV infection leads to virus-driven proliferation of LCL-like cells *in vivo*, as seen in patients with infectious mononucleosis, these cells elicit strong EBV specific CD8+ T cell response (Young and Rickinson, 2004). EBV-positive BL and derived cell lines differ dramatically from LCLs in their cellular phenotype, growth behavior, and immunogenicity. EBV-positive BL cells do not express the activation markers, adhesion molecules, and costimulatory molecules typical of LCLs (Gregory et al., 1988; Billaud et al., 1990) and they grow as single cell suspension rather than in clumps. Since a major difference between EBV-positive BLs and LCLs is the expression of *c-myc*, these findings pointed to the possibility that *c-myc* directly imposes the non-immunogenic phenotype seen in BL cells. For this, LCLs were established in which the function of the Epstein-Barr nuclear antigen 2 protein, a key transcriptional activator involved in the B cell transformation program, is regulatable by estrogen. In the presence of estrogen the cells grow with a LCL phenotype and stop proliferating when estrogen is withdrawn (ER/EB2 cells, (Kempkes et al., 1995)). These cells were then stably transfected with a constitutively active, Igκ-enhancer driven c-myc gene (A1 cells, (Polack et al., 1996) or with a tetracycline-regulatable c-myc expression construct (P493-6 cells, (Pajic et al., 2000)). In both cell lines *c-myc* overexpression rendered proliferation independent of estrogen and the viral proliferation program, and imposed the cell surface phenotype of BL cells. Studies in these conditional cell lines revealed that MHC class I, activation markers, and co-stimulatory molecules are highly downregulated during *c-myc* driven proliferation. These *c-myc* driven cells had not only lost the allostimulatory capacity to T cells of the parental ER/EB2 cells in a mixed lymphocyte reaction, they also could not be recognized by antigen-specific CD8+ T cell clones even when they were forced to express the cognate antigen by infection with recombinant vaccinia virus (Staege et al., 2002). Consecutive studies using expression profiling revealed that *c-myc* not only downregulates MHC class I, costimulatory and adhesion molecules, by negatively regulating the NF-κB and interferon response it also impairs antigen processing and presentation by downregulating components of the immune proteasome and the peptide transporter TAP1 and TAP2 (Schlee et al., 2007a; Schlee et al., 2007b). Thus, in this *in vitro* system, B cells driven into proliferation through constitutive or conditional up-regulation of *c-myc* are immunologically silenced in the absence of any immune selection. This implies that non-immunogenicity of tumor cells may not necessarily be the result of stepwise immune escape, it may also be

directly imposed by the oncogenic transformation process. These findings are consistent with the observation that EBV-positive and EBV-negative BL show essentially the same non-immunogenic phenotype. This is a very important feature of BL cells as the viral antigens expressed in EBV-positive BLs are foreign antigens and should therefore be easily recognized. The key differences between the LCLs and Burkitt's lymphoma cells are listed in figure 3

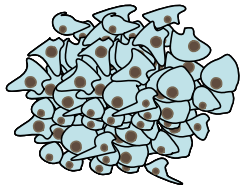
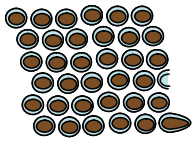
		EBV-LCLs	EBV-Burkitt's lymphoma
			
Growth pattern		clumps	single cells
c-myc/Ig translocation		-	+
Expression of viral antigens			
	EBNA1	+++	+++
	EBNA2	+++	-
	EBNA-LP	+++	-
	EBNA 3A, 3B, 3C	+++	-*
	LMP1	+++	-
	LMP2	+++	-
Antigen processing & presentation			
	MHC class-I and class-II	+++	+
	peptide transporters (TAP1 & TAP2)	+++	+
	components of immunoproteasome		
	PA28α & PA28β	+++	-
	Lmp2 & Lmp7	+++	-
Allo-stimulation		+++	-
Susceptibility to CTL killing		+++	-

Figure 3
Compared to LCLs the immunogenicity of BL cells is severely impaired
EBV-infected lymphoblastoid cell lines and EBV-positive Burkitt's lymphoma cells display differences in viral antigen expression, cell phenotype and immunological recognition. As indicated in the figure, the T cell-mediated elimination of Burkitt's lymphoma is impaired because of low expression of MHC class I, impaired response to interferons, and defects in antigen presentation compared to the highly immunogenic LCLs. '+++' indicates high, '+' indicates low and '–' indicates no expression levels.
** In about 15% of African Burkitt's lymphomas the highly immunogenic EBNA3A, -3B, and -3C proteins are expressed.*

1.7 Aim of the Study

The previous studies have revealed unequivocally that rapid proliferation and reduced immunogenicity of BL tumors are direct consequences of the activation of the proto-oncogene c-myc *in vitro*, but it remains to be elucidated if the observed effects of c-myc overexpression *in vitro* can be recapitulated *in vivo*. In this study we aimed to establish a murine model to elicit immune responses directed against foreign antigens in high grade B cell lymphomas and we addressed the following questions:

1) Can high-grade B-cell-lymphomas be rejected when a foreign antigen is expressed? And if yes:
2) What is the role of the interferon system in the rejection of such lymphomas?
3) What are the mechanisms of rejection of high-grade B-cell lymphomas?

2. Materials and Methods

Materials

2.1 Surgical related reagents and equipment

Formalin: 10% solution of formaldehyde (Sigma-Aldrich) in water.

Sterile syringes: BD Plastipak 1 ml syringe (BD Biosciences).

Sterile needles: 0.5 x 25 mm for injection of cell in mice i.v. and 0.55 x 25 mm (BD Microlance).

Erythrocyte lysis buffer: 0.8% NH_4Cl with 0.1 mM EDTA (Stem Cell Technologies)

Heparinized capillaries: (Microvette CB 300) plastic capillaries for collection of blood.

2.2 Mammalian cell lines

Phoenix: A packaging cell line expressing gag, pol, and env viral proteins (a kind gift from Clemens Schmitt, Humboldt University, Berlin).

2.3 Oligonucleotides

All nucleotides were synthesized by Metabion AG, Martinsried.

Oligonucleotide Sequence 5´ to 3´

Ova-*Xho*I 5´-GCG TCT CGA GGC CAC CAT GGG CTC CAT CGG CG 3´

Ova-*Eco*RI 5´-GAG TGG AAT TCT TAA GGG GAA ACA CAT CTG CC 3´

2.4 Plasmids

MSCV-IRES-GFP: A modified form of the MSCV vector, it contains a bi-cistronic GFP expression cassette with an internal ribosomal entry site.

pcDNA-Chicken Ovalbumin: Vector encoding for the chicken ovalbumin.

2.5 Antibodies

Stain	Company	Clone	Fluorochrome
CD19	BD Pharmingen	1D3	PE
H-2K^b	BD Pharmingen	AF6-88.5	PE
IA-^{b}	BD Pharmingen	AF6-120.1	PE
CD80	BD Pharmingen	16-10A1	PE
CD86	BD Pharmingen	GL-1	PE
CD54	BD Pharmingen	3E2	PE
CD3e	BD Pharmingen	145-2C11	PE

CD4	BD Pharmingen	(L3T4)(RM4-5)	FITC/PE
CD8	BD Pharmingen	(Ly-2)(53-6.7)	FITC/PE
Vα2-TCR	BD Pharmigen	(B20.1)	PE
Vβ5-TCR	BD Pharmigen	(MR9-4)	FITC
H-2K^b-SIINFEKL	Proimmune	not available	PE

The control pentamer is not available and not included in experiment.

2.6 Reagents, media and apparatus

2.6.1 Molecular biology

Agarose: Molecular biology tested (Sigma-Aldrich).

Polybrene: cell culture tested (Sigma-Aldrich).

Small-scale plasmid preparation: GFX miniprep kit for the isolation of plasmid DNA from bacteria (Amersham Biosciences).

Gel Elution of DNA or DNA cleanup: GFX gel elution and PCR purification kit for DNA elution from gels and clean up of PCRs (Amersham Biosciences).

Western blot: ECL Western blotting analysis system (Amersham Biosciences).

Molecular weight markers: Nucleic acid size standards, 1 kb ladder, 1 kb plus ladder and 100 bp ladder (Invitrogen).

Enzymes: Ligase, calf intestine phosphatase, *Xho*I, *Eco*RI from New England Biolabs.

PCR: Platinum Taq DNA polymerase kit, PCR soft tubes (0.2ml) (Biozym Scientific).

dNTP mix: 10 mM each of dATP, dTTP, dCTP and dGTP (Invitrogen).

2.6.2 Cell and tissue culture

Filtration units: Millex syringe-driven 0.22 μm and 0.45 μm filter units (Millipore).

Cell strainer: BD Falcon 40 μm nylon strainer for macerating the spleen and filtering the tissue (BD Biosciences).

Cell scrapers: 25 cm sterile cell scrapers (Sarstedt).

Cell culture pipettes (2, 5, 10 and 25 ml): Sterile disposable pipettes (Corning Inc.).

Cell culture plates and dishes: Sterile 96 well, 24 well, 6 well plates (Sarstedt) 100 mm x 20 mm dishes for adherent cells (Corning Inc.), and Petri dishes for suspension cells (Becton Dickinson) 150 mm x 20mm dishes for adherent cells (Greiner Bio One).

Calcium chloride solution for transfection: 2.5 M $CaCl_2$ (Sigma-Aldrich) solution in water.

Hepes buffered saline: (HBS) (Invitrogen).

Media: **Dulbecco's Modified Eagle Medium (DMEM)** (PAN Biotech).

Fetal bovine serum (FBS): 0,2 μm-filtered mycoplasma screened (PAN Biotech).

Dulbecco's phosphate buffered saline (DPBS) (PAN Biotech).

Iscove's Modified Dulbecco media (IMDM) for lymphoma cell culture (Invitrogen)

Trypsin – EDTA in 1 x HBS (Invitrogen).

Penicillin/Streptomycin: Antibiotic solution with 10,000 U/ml Penicillin G sodium and 10,000µg/ml Streptomycin sulfate in 0,85% saline. Used at a final concentration of 1:100 (Invitrogen).

Murine cytokines: m-IFN-α and m-IFN-γ (lyophilized) (BD Biosciences)

Flow cytometry: BD FACS Calibur System (BD Biosciences).

Fluorescence activated cell sorting: BD FACSVantage SE System (BD Biosciences).

Microscope: Leitz Diavert Inverted Microscope (Leitz)

Supplementation of Iscove's Modified Dulbecco media (IMDM) for lymphoma cell culture

500 ml of IMDM (Invitrogen)

10% heat inactivated FBS (Invitrogen)

2 mM L-glutamate (Invitrogen)

2 mM penicillin-streptomycin (Invitrogen)

1 mM sodium pyruvate (Invitrogen)

1 % fungizone (Invitrogen)

Supplementation of Dulbecco's modified eagle's media (DMEM) for phoenix cell culture

500 ml of DMEM-high glucose (Invitrogen)

10% heat inactivated fetal bovine serum (Invitrogen)

2 mM L-glutamate (Invitrogen)

2 mM penicillin-streptomycin (Invitrogen)

1 mM sodium pyruvate (Invitrogen)

1 % fungizone (Invitrogen)

2.7 Mice

The different mouse strains used in this study are shown in table 4.

Name	Vendor
C57BL/6	Charles River Laboratories
UBQ-GFP tg C57BL/6	Jackson Laboratories
C57BL/6 λ-hu-c-myc tg	Gift from HC Morse (NIAID,NIH)
Stat1$^{-/-}$	From David E Levy (Durbin et al., 1996)
C57BL/6-OT1Tg(TcraTcrb)1100Mjb/J	Jackson Laboratories
C57BL/6IFN-γ$^{-/-}$(C57BL/6.129S7-*Ifng*^{tm1Ts}/J)	Jackson Laboratories

Table 4 Different mouse strains used for the study is shown

Mice lines used in this study

C57BL/6 mice are wild-type recipients. Around 8-10 week old mice were used as recipients in all our lymphoma transfer experiments.

UBQ-GFP tg C57BL/6 mice is a transgenic mice expressing GFP ubiquitously all over the tissues and therefore T cells developed in these mice are tolerant to GFP antigen. Upon transfer of lymphomas expressing GFP (291-GFP or 9-GFP), GFP does not act as antigen and we can rule out the immune response elicited by GFP antigen by transferring in UBQ-GFP Tg recipients and in wild-type recipients.

C57BL/6 λ-hu-*c-myc* tg mice are transgenic for human *c-myc* mice lead to the development of spontaneous lymphomas six months post birth. Lymphomas with striking similarities to Burkitt's lymphoma are developed in these mice. These mice bear a mutated human *c-myc*

gene controlled by reconstructed Ig λ locus. Lymphomas are established in culture from these mice and transferred in both wild-type immunocompetent and immunodeficient mice in our studies.

Stat1$^{-/-}$ and IFN-γ$^{-/-}$ mice are deficient in type-I, type II and type II IFN respectively. To explore the contribution of IFN signaling, these mice were used as recipients in our transfer experiments.

C57BL/6-OT-ITg (TcraTcrb) mice are T cell receptor transgenic mice recognizing SIINFEKL epitope of ovalbumin antigen $OVA_{257-264}$. Adoptive transfer of either naïve or primed antigen specific CD8+ T cells is generated from these mice. These mice are generally used to study the CD8+ T cell response in anti-tumor immunity.

All experiments were performed according to the guidelines and approval of the local animal protection committee (application: 55.2-1-54-2531-8-04 and 209.1/211-2531-8/04, Government of Bavaria, Munich, Germany).

Methods

2.8 Establishment of cell lines

The cell lines were established either from the λ-hu-*c-myc* transgenic mice or from the tumor transplanted recipient mice. Mice were sacrificed by CO_2 euthanasia when peripheral lymph node enlargements were well palpable and 10mm in diameter. The lymph nodes were removed and the tissue was minced into a single cell suspension by using 80 μm cell strainers. After preparation of the single cell suspensions, the erythrocytes were removed by incubating the lymphoma cells in ice cold ammonium chloride lysis buffer for 5 to 10 minutes. After incubation with lysis solution, the cells were washed twice with ice cold PBS or with 10% IMDM. The establishment of lymphoma cells grown in the absence of any irradiated feeder cells was unsuccessful. The cells were initially grown in Iscove's modified Dulbecco's medium (IMDM) in the presence of irradiated MRC-5 (human pulmonary fibroblast) as feeder cells. After 4-8 weeks, the cells grew independently of the feeder layers and aliquots of the cells were frozen in 10% DMSO / 90% FCS and stored short-term at -80˚C and subsequently in liquid nitrogen.

Ammonium Chloride lysis buffer

This solution is used to lyse the red blood cells from peripheral blood, spleen and tumors and bone marrow.

Ammonium chloride 10 X lysing concentrate solution

a) NH_4Cl (1.5 M) 80 g

b) $KHCO_3$ (100 nM) 10 g

c) Na_4 EDTA (10 nM) 3.7 g

d) distilled H_20 1000 mL

1N HCl or 1 M NaOH

Adjust the pH to the range of 7.2-7.4. Add distilled water to final volume of 1000 mL.

To prepare the working solution, dilute the concentrate 1: 10 (10 mL concentrate + 90 mL distilled water).

2.9 Cloning of chicken ovalbumin

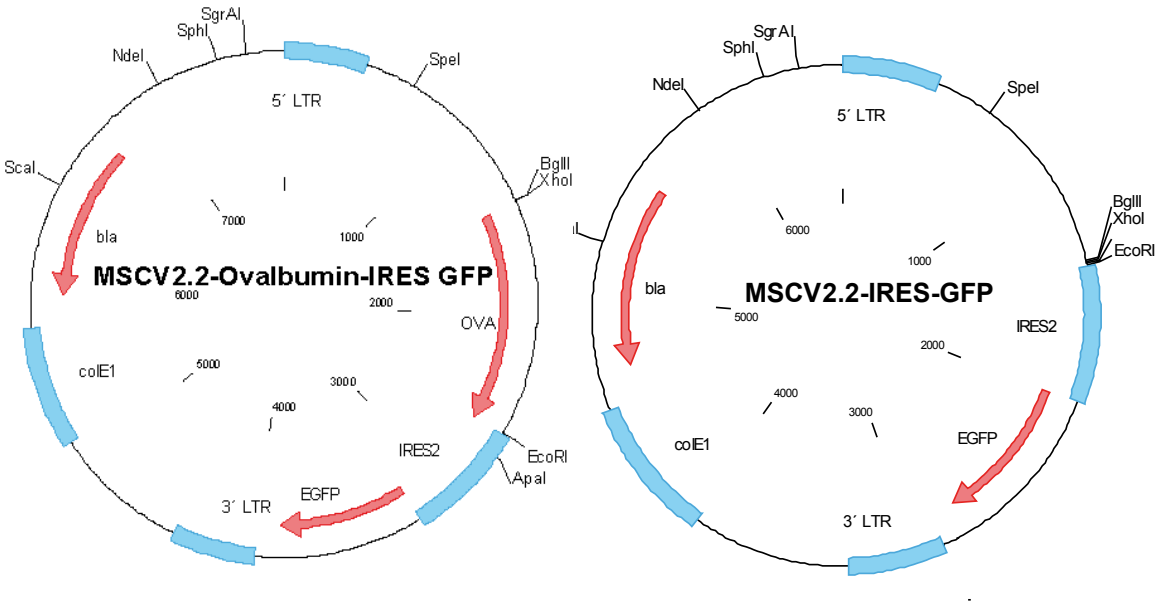


Figure 4

Map of MSCV-GFP and MSCV-OVA-GFP retroviral vector

A 1.1 kb ovalbumin cDNA fragment covering the coding sequence of the gene was amplified by PCR using pcDNA3 as template (a kind gift from Prof. Thomas Brocker, Institute of Immunology, LMU, Munich). The PCR fragment was digested with *Eco*RI and *Xho*I and ligated into the *Eco*RI and *Xho*I digested murine stem cell virus vector (MSCV) 5´ of the internal ribosome entry site (IRES) and the enhanced GFP fluorescent protein gene. After ligation, competent bacteria were transformed with the ligation products and plated on agar plates containing ampicillin as selection marker. Single bacterial colonies were used to

inoculate liquid bacterial cultures and the contained plasmids analyzed by restriction enzyme digestion. The maps of the plasmids are shown in figure 4.

2.10 Retroviral production and transduction

Phoenix cells (a kind gift from Prof. Thomas Brocker, Ludwig-Maximilians-University, Munich), an ecotrophic packaging cell line for retroviral production, were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The components for complete DMEM are described later in this chapter. In order to achieve high titer virus, phoenix cells were plated 12 hours before transfection at a density of 5 million cells in a 10 cm cell culture dish. Immediately before transfection, the media was removed and replenished with media supplemented with 25 µM chloroquine. The transfection mix was prepared by mixing 60 µg of plasmid DNA, 75 µl of 1.5 M $CaCl_2$ and water to a final volume of 750 µl. After adding 750 µl of 2X HBS buffer, the mixture was kept at room temperature for 15 minutes and then added at the sides of the culture dish. The plates were incubated for 6 hours at 37 º C, then the media was removed and the cells were washed twice with PBS before addition of IMDM media. 48 hours later, the retroviral supernatants were collected and passed through a 0.45 µm filter. The retroviral supernatants were either snap frozen and stored at -80 º C or used immediately for the transduction. One million primary lymphoma cells were incubated with 3 ml of retroviral supernatants supplemented with 4 µg/ml of polybrene (Sigma) in a 6 well plate and the plates were centrifuged at 1200 rpm for 60 minutes at 32 º C. The infected cells were kept at 37 º C in an incubator for additional 12 hours. After two cycles of infection, the cells were washed with PBS and supplemented with IMDM media. In order to determine the efficiency of retroviral transduction, GFP expression was assessed by FACS.

After the retroviral transduction using either GFP or OVA-GFP, the transduced cells were analszed for GFP expression. We obtained initial transduction efficiency of about 10 to 15 %. However, we sorted the high GFP populations and after the first round of sorting we could increase the GFP populations to about 60 %. After 1 to 2 week, a second sorting was performed to enrich the GFP positive lymphomas. We found that after sorting the high GFP expressing cells, we could maintain around 95 % of GFP in primary lymphomas after retroviral transduction.

2.11 Western blotting

For whole cell lysate preparation, cells were lysed in LCW lysis buffer, containing protease inhibitor cocktail (Roche Diagnostics) and incubated for 15 minutes on ice. The cell debris was removed by centrifugation for 15 minutes at 4 º C at 10,000 g. The protein concentration of the supernantant was determined by the DC protein assay (Bio-Rad), according to the manufacturer's instructions. Equal amount of protein lysates were mixed with sample loading buffer, boiled for 5 minutes at 95 º C, and the proteins were separated by 12 % SDS-PAGE at 160 V in a mini-Protean 3 electrophoresis cell. Proteins were transferred from the gel onto a Hybond-C super nitrocellulose membrane in blotting buffer with a trans-blot semi-dry blotter at 20 V for 30 minutes. Membranes were blocked with 5 % skim milk powder in TBST for 60 minutes and hybridized with a primary antibody at 4 º C for atleast 12 hours. Membranes were washed three times in TBST for 5 minutes, following HRP-conjugated secondary antibody incubation for 90 minutes. The nitrocellulose membrane was repeatedly washed in TBST (3 X 5 minutes) and proteins were visualized by ECL detection on hyperfilm. Efficient stripping of Hybond-C super membranes was performed with 0.4 M NaOH for 10 minutes.

Blotting buffer: 10 % Running buffer (10 X), 20% methanol.

Western analysis of MYC protein expression was performed using a polyclonal antibody (c-Myc (A-14)-G: sc-789-G; Santa Cruz Biotechnology.

Sample loading buffer (2 X): 100 nM Tris, 4 % SDS, 20 % glycerol, 0.1 % bromophenol blue, 100 mM DTT, pH 6.8

LCW lysis buffer: 0.5 % Triton X-100, 0.5 % sodium deoxycholate salt, 150 mM Nacl, 20 mM TRIS, 10 mM EDTA, 30 mM Na-Pyrophosphate, pH 7.5

Running buffer (10 X): 250 mM TRIS-Base, 1 % SDS, 2.5 M glycine

TBST: 25 mM TRIS, 125 mM NaCl, 0.1 % Tween-20, pH 8.0

Ponceau Stain:

Following transfer to nitrocellulose, the membrane was quickly placed in the ponceau stain and was shaken in a roller for approximately 5 to 10 minutes. Later, the membrane was transferred into Ponceau destain solution and was shaken until the bands are visible.

1 X Ponceau stain

0.1 % Ponceau S – 0.5 g Ponceau-S

1 % acetic acid 5ml acetic acid and made upto 500 ml

Ponceau Destain

1 % acetic acid

2.12 Tumor transfer and monitoring

For lymphoma transfer experiments, 8-10 week old mice were used for experiments. Preliminary experiments for the cell number for tumor cells injected indicated that 100,000 cells resulted in tumor formation in 100% of animals. Therefore, using 100,000 cells as lethal dose LD_{100} for lymphoma transfer experiments, we performed the transfer experiments in both immunocompetent and immunodeficient recipient mice. 100,000 cells from cultured lymphomas were freshly split 48 hours prior to transfer. Cells were washed in cold PBS and injected s.c. at the site of the upper flank of the recipient mice. Lymphoma growth was measured at the site of injection at least 3 times per week using calipers. The animals were killed when the tumor burden became excessive to avoid pain and suffering. The animals were regarded as tumor free when surviving for longer than 100 days.

2.13 Immunization protocol and transfer of naïve and activated OT-I CD8+ T cells

To generate the activated SIINFEKL-specific CD8+ T cells, OT-I mice were immunized with 5 to 10 million irradiated (20 Gy) 291-OVA-GFP lymphoma cells. The 291-OVA-GFP lymphoma cells were injected subcutaneously in C57BL/6 OT-I Tg animals and one week later, the mice were boosted with same dose of irradiated 291-OVA-GFP. Two weeks follwed by the immunization with irradiated OVA cells leads to the activation of CD8+ SIINFEKL-specific T cells in OT-I transgenic mice. The transfer of unvaccinated naïve OT-I CD8+ T cells served as controls. Naïve or primed OT-I CD8+ T cells were injected in $Stat1^{-/-}$ recipients at day 1. We used three cell dose ranging from 0.1 million, 1.0 million and 10 million OT-I CD8+ T cells were transferred in $Stat1^{-/-}$ recipients at day 1. The 291-OVA-GFP lymphoma cells were challenged at day 0 in all groups. Lymphoma group without OT-I CD8 T cells were also included as control groups. The persistence of naïve and primed transferred OT-I CD8+ T cells were evaluated in $Stat1^{-/-}$ recipients by TCR staining using Vα2 and Vβ5.

2.14 SIINFEKL pentamer staining

Single cell suspensions from the spleens of diseased 291-GFP and 291-OVA-GFP challenged mice were stained for the presence of SIINFEKL-specific CD8+ T cells. One million spleen cells were washed twice with wash buffer (0.1% sodium azide, 0.1% BSA in PBS) and subsequently resuspended in the residual volume of about 50 µl. The concentration of the pentamer was not supplied by the manufacturer. According to the instructions from manufacturer, 10 µl of pentamer was used for each staining condition.The fluorescently labeled pentamer (Proimmune) was spun down for 5 minutes at 14000 rpm prior to use. One million splenocytes were incubated at room temperature with 10 µl pentamer in 50 µl total

volume for 15 minutes in the dark. The cells were washed once with wash buffer and incubated on ice for 30 minutes following addition of titrated amounts of anti-CD8 antibody. Cells were washed twice in wash buffer and analyzed by flow cytometry.

2.15 Flow cytometric analysis

Surface expression levels of various CD markers including CD4, CD8 and TCR staining on T cells and MHC class I, MHC class II, co-stimulatory molecules on tumor cells was performed by flow cytometry. First, around 0.5 million to 1.0 million lymphoma cells or the T cells were incubated with blocking antibody 2.4 G2 in a reaction volume of 50 µl for 30 minutes on ice to block Fc receptors. All staining of using fluorchrome labeled monoclonal antibodies were used according to the manufacturer's instructions. After blocking the Fc recptors, the FITC labeled or PE-labelled mAb was added to the reaction mixture. Generally 1:100 dilution of antibody was used for all CD markers. After 30 minutes incubation on ice, the cells were washed with 10 % FCS supplemented in PBS. After washing step, the supernantants were discarded and the pellet was resuspended in 400 µl PBS supplemented with 10% FCS. The dead cells were excluded from the measurement using propidium iodide staining. Flow cytometry analysis was performed on a FACScan using CellQuest (BD Biosciences). The stained cells were analyzed and the data were processed by CellQuest program.

2.16 CFSE staining

The proliferation of the C57BL/6 Eλ-hu-c-Myc-Stat1$^{+/+}$ and C57BL/6 Eλ-hu-c-Myc-Stat1$^{-/-}$ lymphoma cells and the proliferation of the adoptively transferred OT-I T cells was measured by labeling the cells with CFSE. A 5mM stock solution of CFSE in PBS was prepared. In general, the cells were washed with PBS, centrifuged, and the supernatant aspirated. Working concentration of about 2 µM CFSE concentration was prepared by diluting CFSE in phosphate buffered saline buffer. About 5 million to 10 million T cell were resuspended in prewarmed PBS containing CFSE and incubated at room temperature for 15 minutes. Cells were washed in PBS, resuspended in fresh prewarmed medium and incubated for another 30 minutes to ensure complete CFSE labeling. The CFSE labeled cells were then analyzed by FACS.

2.16 LDH cytotoxicity assay

The cytotoxicity of effector immune cells was measured using the non-radioactive LDH (lactate dehydrogenase) release assay (Clontech). The LDH cytotoxicity detection kit provides a simple and precise colorimetric method to measure LDH release. In principle, the LDH is a stable cytoplasmic enzyme present in all cells and released upon cell death. 25,000 lymphoma

cells were taken as target cells. Different effector to target (E:T) ratios ranging from 0,1:1; to 10;1 were used in these experiments. The purified populations of Effector cells (either NK cells or T cells) were carried out according to the manufacturer's instruction. The effector and target cells were incubated for 8 hrs in a reaction volume of 200 µl and after incubation, 100 µl of the supernatant was transferred into new 96-well plate and then 100 µl of reaction mix consisting of catalyst and dye solution was added to the supernatant and incubated for 30 minutes at room temperature in the dark. The lymphoma cells with medium were taken to calculate the minimal release, and lymphoma cells in the presence of 5 % Triton X-100 for maximal release. After incubation, the absorbance at 492 nm wavelength was measured in an ELISA reader (Tecan). The percentage of cell-mediated cytotoxicity was calculated as follows.

$$\text{Cytotoxicity (\%)} = \frac{(\text{effector:test} - \text{minimal release})}{(\text{maximal release} - \text{minimal release})} * 100$$

3. Results

We aimed to establish primary lymphoma cells from λ-hu-c-myc transgenic mice in culture. Because lymphoma cells often fail to grow *ex vivo* on their own, they were initially grown for 4-6 weeks on irradiated human pulmonary fibroblast (MRC-5). Cultivated lymphoma cells grew independently of feeder cells after 4-6 weeks and showed high apoptosis rates as observed in forward scatter/side scatter profiles in flow cytometry (data not shown). All cells showed CD19 positivity confirming the B cell origin of the tumors. The lymphoma cells were cultivated in complete 10 % complete Iscove's Modified Dulbecco Media (IMDM).

3.1 Determination of lethal dose for lymphoma transfer

In preliminary experiments we conducted dose escalation studies of cells inoculated subcutaneously (s.c.) to determine the LD_{100} (lethal dose) of the cell line used. The lowest number of 291 cells to reach LD_{100} was 0.1 million. The implanted lymphoma cells grow at the site of injection and growth could be monitored using a caliper. We almost always observed systemic growth in lymph nodes and spleen after s.c. injection. We selected the cell line 291 for transfer experiments because of the following reasons. As shown in Figure 5, the cell line 291 expresses high amounts of c-MYC in comparison to other established lymphoma cells. Moreover we found that MHC class I ($H\text{-}2K^b$) expression was low and MHC class II (IA^b) expression was negative when compared to other lymphoma cell lines established in culture (data not shown).

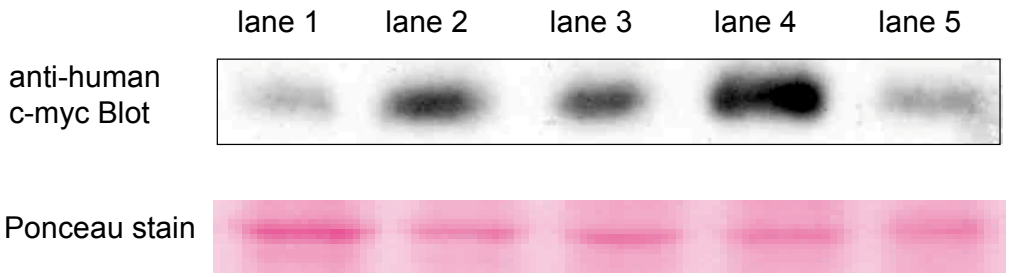


Figure 5
Western blot of human c-MYC expression in primary lymphoma cells
The expression of human c-myc was evaluated in 5 different lymphoma cells established in culture. Line 291 (lane 4) expressed higher c-myc levels compared to other tested lymphoma cells. Lane 1 = line 265; lane 2 = line 288; lane 3 = line 58; lane 4 = 291; lane 5 =307. 20 µg of protein was loaded in each lane. Western analysis of MYC protein expression was performed using a polyclonal antibody c-Myc (A-14)-G. Equal loading of proteins was detected by Ponceau stain. The equal protein loading of corresponding c-myc band is shown by ponceau stain

3.2 Introduction of foreign antigens and establishment of stable cell lines of λ-hu-c-myc lymphoma cells

We hypothesized that tumor progression of c-myc induced lymphomas could be due to the lack of expression of a strong lymphoma specific antigen. Therefore we sought to introduce chicken ovalbumin to the primary lymphomas by retroviral transduction. We choose chicken ovalbumin as a foreign antigen because of the following reasons:

- Chicken ovalbumin is a well characterized T cell antigen.
- TCR transgenic adoptive transfer approaches are feasible because of the availability of OT-I and OT-II TCR transgenic mice on C57BL/6 background.
- Fluorescent MHC class I pentamers allow the enumeration of antigen-specific CD8+ T lymphocytes because the multimeric MHC-peptide complexes bind to T cell receptors (TCRs) of a particular specificity.
- Vaccination with low dose of OVA derived MHC class I binding peptide SIINFEKL can result in activation of CD8+ effector T cells.

In order to establish stable cell lines from 291 cells expressing foreign antigens we used retroviral transduction. High GFP expressing cells were sorted by flow cytometry and approximately 95% purity was achieved. In the retroviral system, GFP in the vector is linked via an IRES site and expression of GFP is coupled to the expression of OVA. Thus GFP expression in flow cytometry was used as a read out system for antigen expression. The expression of GFP after sorting remained identical over time in culture. In order to rule out the possibilities of differences in expression of H-2K^b and IA^b molecules after retroviral infection, the cell lines were analyzed for the B cell specific marker CD19, H2Kb and IAb molecules. As shown in figure 6, the expression of H-2K^b and IA^b remained unaltered in 291-non transduced (291-Parental cells), 291-GFP (Mock virus infected) and in 291-OVA-GFP cell lines (Ovalbumin virus infected).

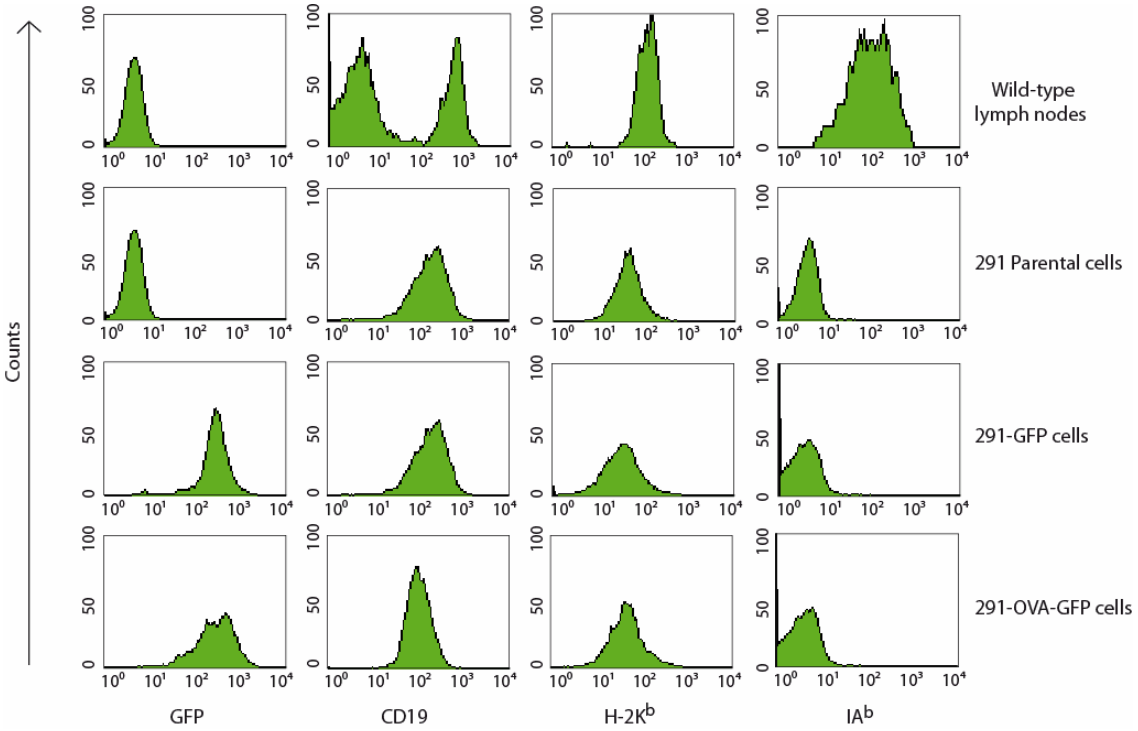


Figure 6
Expression of CD19, H-2K^b and IA^b is not altered after retroviral transduction of lymphoma cell line 291
Chicken ovalbumin was introduced into the 291 cell line by retroviral transduction. The unmanipulated 291 parental cells, 291-GFP cells and 291-OVA-GFP cells after retroviral transduction were analyzed for GFP expression, CD19, H-2K^b and IA^b surface expression. The H-2K^b and IA^b expression was determined on CD19 positive cells. The retroviral transduction did not alter the surface expression of H-2K^b and IA^b molecules. The CD19, H-2K^b and IA^b expression of 291 cells was compared with cells isolated ex vivo from lymph nodes (LN) of wild-type C57BL/6 mice.

3.3 Introduction of OVA into 291 lymphoma cells leads to delay in tumor formation

The parental 291 cell line exhibits one possible foreign antigen, the human c-myc gene product. When transduced with GFP containing mock vector, GFP will only serve as an antigen when transferred into C57BL/6 mice but not into UBQ-GFP-C57BL/6 mice. Similarly, 291-OVA-GFP transduced cells exhibit 3 possible antigens namely MYC, GFP and OVA, when transferred into C57BL/6 mice, and two antigens when transferred into UBQ-GFP-C57BL/6 mice. Because transfer of 100,000 cells resulted in 100% mortality, human MYC expression alone does not induce antitumor immunity in this experimental setting. With the transduction using OVA-GFP containing vector, we introduced 2 new foreign antigens into 291 lymphoma cells, GFP and ovalbumin, respectively. This model for lymphoma transfer was chosen such that we can eliminate GFP as antigen by transferring transduced cells into GFP transgenic mice which are tolerant towards GFP. We therefore can use the system to explore the rejection mediated by introduction of GFP and OVA, or OVA antigen

alone. Table 5 displays the different antigens upon transfer of cell line 291 into C57BL/6 and UBQ-GFP C57BL/6 mice in our lymphoma transfer model.

	C57BL/6 Wild-type 	UBQ-GFP-Tg 
291 Parental Line (Non transduced)	**MYC**	**MYC**
291- GFP	**MYC** **GFP**	**MYC**
291- OVA-GFP	**MYC** **GFP** **OVA**	**MYC** **OVA**

Table 5 Foreign antigens in 291 cell lines
Foreign antigens expressed in 291 parental cells, 291-GFP and 291-OVA-GFP lymphoma cell lines when transferred in both wild-type C57BL/6 recipients and in UBQ-GFP-transgenic recipients. Since the GFP is expressed ubiquitously in UBQ-GFP-Tg mice, GFP does not act as antigen upon transfer of GFP expressing lymphomas in these recipients.

To test the *in vivo* efficiency of OVA-mediated rejection, 291 parental cells, 291-GFP and 291-OVA-GFP cells were injected subcutaneously into immunocompetent wild-type-C57BL/6 and UBQ-GFP transgenic C57BL/6 mice. As determined in chapter 3.1, we injected 100,000 cells and the tumor size following lymphoma injection was monitored 3 times a week. As shown in figure 7, parental lymphoma bearing animals developed lymphoma at day 16 and tumor grew progressively over time. This observation is consistent in both wild-type-C57BL/6 and UBQ-GFP transgenic animals as recipients. When 291-GFP cells were injected into wild-type C57BL/6 animals, we observed a delay of lymphoma onset of approximately 4-5 days compared to UBQ-GFP recipients which are tolerant to GFP. 291-GFP cells grew as fast in UBQ-GFP transgenic mice as 291 parental cells. This suggests that introduction of GFP into lymphoma cells elicits detectable anti-tumoral activity. Most interestingly, there was a consistent delay in lymphoma growth in case of 291-OVA-GFP cells in both wild-type C57BL/6 and UBQ-GFP transgenic animals indicating that ovalbumin response mediated the delay in tumor development. The 291-OVA-GFP cells developed lymphoma at day 26 with

significant delay of 10 days when compared with 291 parental cells in wild-type C57BL/6 mice and 291-GFP cells in UBQ-GFP transgenic animals. Although there was delay in tumor development in case of 291-OVA-GFP lymphomas, 291-OVA-GFP lymphomas grew progressively in some WT-C57BL/6 and UBQ-GFP transgenic animals.

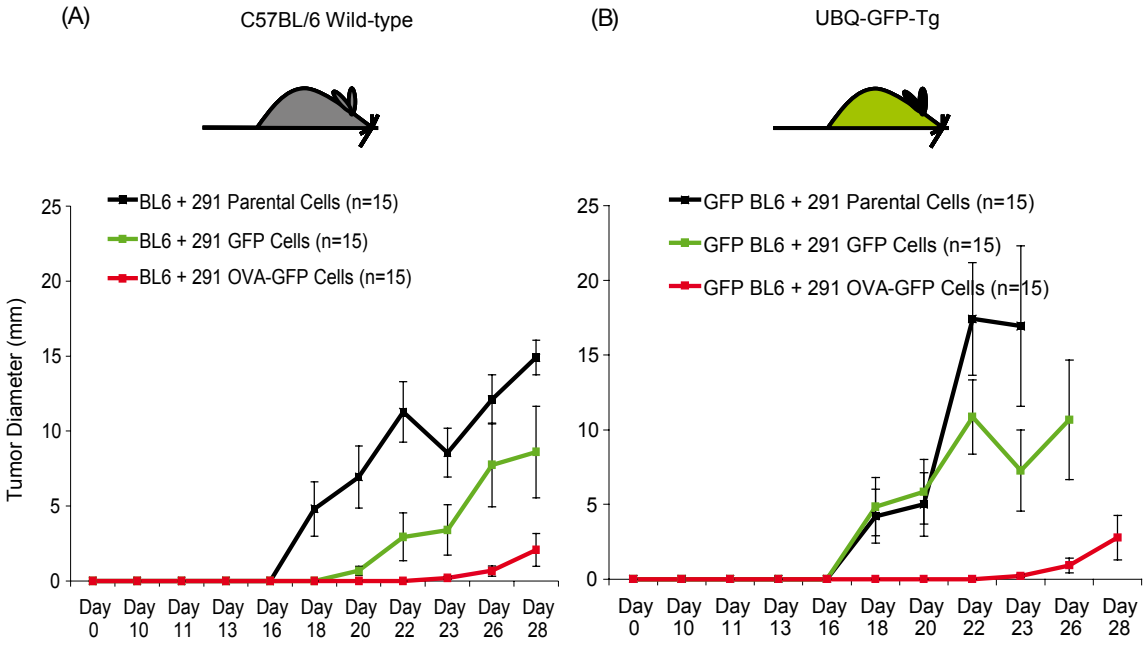


Figure 7
Introduction of ovalbumin delays lymphoma growth in wild-type C57BL/6 and C57BL/6 UBQ-GFP transgenic recipients
100,000 cells each for 291 Parental cells, 291-GFP, and 291-OVA-GFP cells were injected s.c. in both wild-type C57BL/6 and UBQ-GFP transgenic mice. Upon lymphoma progression, tumor size at the site of injection was measured 3 times per week using calipers. The 291 parental cells grew progressively in both wild type (A) and UBQ-GFP transgenic (B) recipients, whereas the 291-OVA-GFP lymphomas display 10 days delay in lymphoma growth. The immune response against ovalbumin bearing lymphoma is not due to the presence of GFP antigen, as 291-GFP cells grew progressively in UBQ-GFP transgenic recipients. The graph shows combined data from four independent experiments with similar results. The lymphoma growth of 291 parental cells is shown in black, that of 291-GFP cells in green, and that of 291-OVA-GFP cells in red.

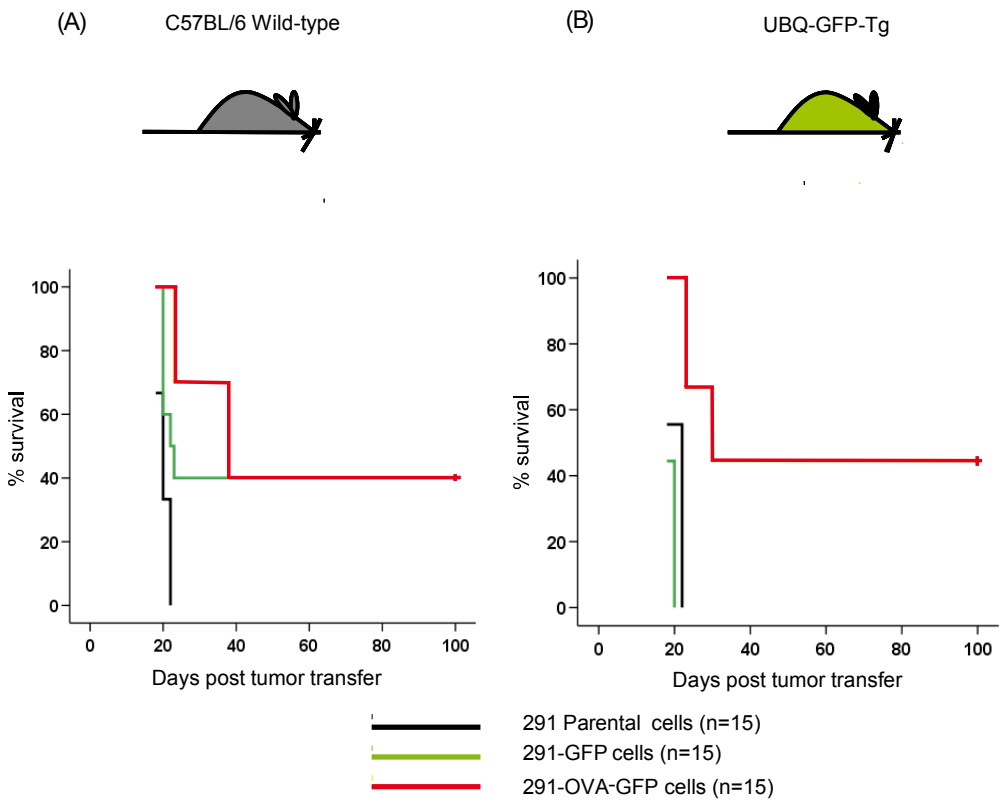


Figure 8
Overall survival of recipient mice is increased when challenged with a tumor expressing a foreign antigen
The immune response against ovalbumin bearing lymphomas were monitored for long term survivors after tumor challenge. All the mice challenged with 291 parental cells grew progressively and killed the mice within 20 days after challenge in both wild-type C57BL/6 (A) and in UBQ-GFP transgenic (B) recipients (black line). In contrast, introduction of OVA-GFP led to improved survival in both recipient strains (291-OVA-GFP cells) (red line). Around 45% of animals challenged with 291-OVA-GFP survived for more than 100 days without any signs of lymphoma. The introduction of GFP displayed protection only in wild-type animals (291-GFP cells) (green line) leading to long term survival for more than 100 days. All the animals challenged with 291-GFP developed tumors and killed the mice within 20 days in GFP tg recipients.

Corresponding to the delay in lymphoma growth, 40-45 % of the animals rejected lymphoma cells completely when the OVA-GFP had been introduced into the cells. As shown in figure 8, all animals harboring unmanipulated 291 parental cells (black line) died because of progressive lymphoma. Comparable results were observed in wild-type C57BL/6 and UBQ-GFP transgenic mice. By contrast, 40-45 % of the animals inoculated with lymphoma cells harbouring 291-OVA-GFP (red line) rejected the lymphoma cells and remained tumor free after 100 days without any signs of lymphoma. Similar results were observed when UBQ-GFP transgenic mice were used as recipients for 291 parental and OVA-GFP transduced cells. However, when the cells had been transduced with GFP only, survival of 291-GFP lymphoma bearing animals in wild-type C57BL/6 animals was nearly identical to that of wild-type C57BL/6 animals receiving 291-OVA-GFP lymphoma cells, and 40 % of the animals remained tumor free after 100 days without any sign of lymphoma. This effect was not

observed in GFP-transgenic mice. This data clearly indicates that GFP acts as an antigen and increases the immunogenicity of lymphomas.

3.4 Loss of antigen in outgrowing lymphomas

As shown in the figure 8, approximately 60 % of the recipient animals developed lymphoma despite the presence of OVA. We therefore analyzed outgrowing tumors for their expression of GFP as a surrogate marker for antigen expression. As shown in the figure 6, we obtained approximately 95 % purity of GFP expressing cells after FACS sorting and addressed the question whether outgrowing lymphomas were selected for loss of antigen. The GFP expression of the 291-GFP and 291-OVA-GFP cell lines was determined prior to injection and compared to the GFP expression on lymphomas arising in wild-type C57BL/6 and UBQ-GFP transgenic mice. When the tumor burden was critical (above 10 mm), outgrowing lymphomas were harvested and subsequently analyzed for GFP expression by flow cytometry. As shown in figure 9, we observed partial loss of GFP expression in wild-type recipient mice challenged with 291-GFP cells. Striking differences emerged when GFP expression was compared in developing tumors in wild-type C57BL/6 and UBQ-GFP transgenic mice. *Ex vivo* analysis of GFP expression confirmed that 291-GFP lymphomas arising in GFP transgenic mice display high GFP expression as shown in figure 9. However, expression of GFP was dramatically reduced in lymphomas transduced with OVA-GFP that were arising in wild type C57BL/6 (A) and in UBQ-GFP transgenic mice (B). We conclude that introduction of ovalbumin delays the tumor formation, and developing lymphoma displays a loss of antigen.

However, differences emerged when the GFP expression of 291-OVA-GFP lymphomas arising in wild type C57BL/6 and UBQ-GFP transgenic mice was investigated. The GFP expression of developing 291-OVA-GFP lymphomas in wild type C57BL/6 displayed 25 % compared with the 35 % for 291-OVA-GFP lymphomas developing in C57BL/6 UBQ-GFP transgenic mice. Thus from this experiment, we conclude that introduction of ovalbumin delays the tumor formation and, developing lymphoma display a loss of antigen.

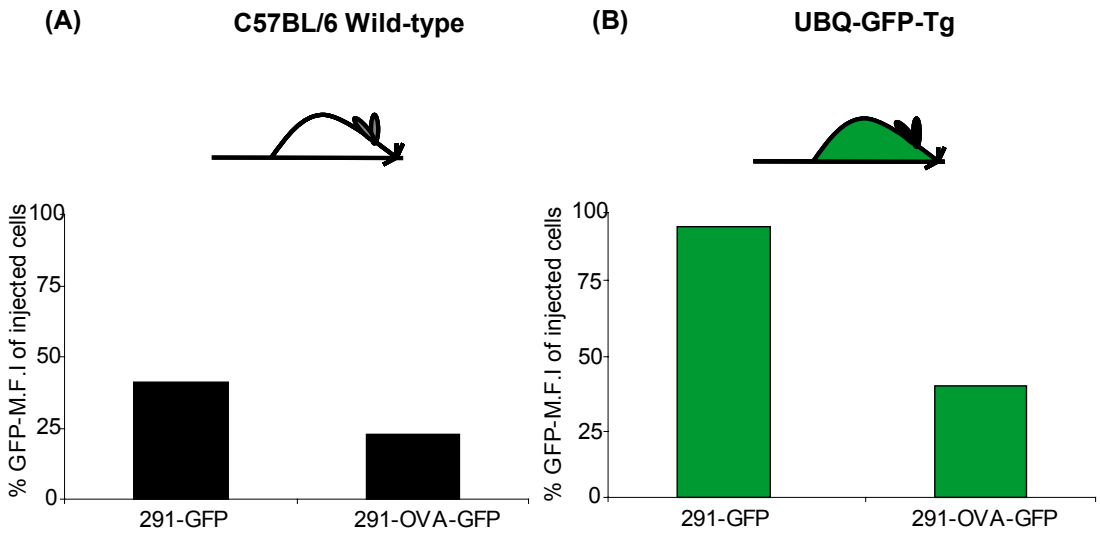


Figure 9
Loss of antigen in lymphomas developing in recipient wild-type and UBQ-GFP transgenic mice after inoculation of 291 cells transduced with GFP or OVA-GFP
After the challenge of 100,000 cells of 291-GFP and 291-OVA-GFP,GFP expression of 291-GFP and 291-OVA-GFP cells was compared after injection into C57BL/6 wild type (A) and C57BL/6 UBQ-GFP transgenic mice (B). Since GFP antigen is tolerant in UBQ-GFP-Tg mice, we observed almost 90% of GFP expression in developing lymphomas after challenge with 291-GFP indicating no loss of antigen variants. However, 25 % of GFP expression is observed in developing 291-OVA-GFP lymphomas in wild-type recipients and 35% of GFP expression is observed in developing 291-OVA-GFP lymphomas in UBQ-GFP Tg recipients indicating emergence of loss of antigen variants after 291-OVA-GFP challenge. Graphs display the % mean fluorescence intensity of GFP expression of injected cells. P<0.05 in wild-type C57BL/6 recipients and P=0.01in UBQ-GFP transgenic recipients. n=8 for 291-GFP and 291-OVA-GFP cells in both wild-type C57BL/6 and UBQ-GFP-Tg recipients.

3.5 Detection of SIINFEKL specific T cells in splenocytes of mice that have rejected lymphoma cells expressing ovalbumin and GFP

As shown in the figure 8, 40-45 % of the recipient animals rejected OVA bearing lymphomas. We hypothesized that elicitation of an ovalbumin specific immune response led to the rejection of 291-OVA-GFP lymphoma cells. The analysis of an antigen-specific CD8+ T cell response has been greatly facilitated by the recent introduction of tetramer/pentamer technology that allows accurate enumeration and phenotypic characterization of antigen specific T cells by flow cytometry. With available SIINFEKL-specific pentamers, we determined the presence of antigen-specific CD8+ T cells in mice during ovalbumin mediated lymphoma rejection. As shown in the figure 10, the animals injected with 291-GFP cells displayed background staining of about 0.3 %, whereas the splenocytes of 291-OVA-GFP challenged animals displayed around 1,6 % of SIINFEKL-specific CD8 T cells. We conclude

from this experiment that inoculation of mice with ovalbumin expressing lymphoma cells leads to the generation of SIINFEKL-specific CD8+ T cells which could eventually mediate rejection.

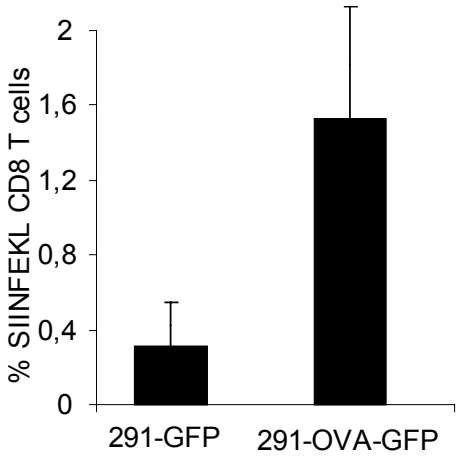


Figure 10
Detection of SIINFEKL-specific CD8+ T cells in splenocytes of wild-type mice that have rejected ovalbumin-GFP expressing lymphoma cells
The percentage of SIINFEKL- pentamer positive T cells in splenocytes of wild type C57BL/6 mice that have received either 100,000 cells of 291-GFP or 291-OVA-GFP cells was determined by flow cytometry. Rejected mice after challenge of 291-GFP and 291-OVA-GFP at day 40 was sacrificed and splenocytes was analyzed for the presence of SIINFEKL positive cells. Graphs display the pentamer positive cells ± standard deviation by Mann-Whitney non-parametric test. P=0.008 compared to the control group.n=6 for 291-GFP and 291-OVA-GFP.

3.6 Stat1 and IFN-γ deficient mice fail to reject OVA-GFP cells

We have shown that expression of the foreign antigen OVA in lymphoma cells enhanced the immunogenicity of the cells and resulted in partial rejection and selection of antigen loss variants. To investigate whether rejection requires the endogeneous production of IFN-γ by the host, we transferred the immunogenic 291-OVA-GFP cells in IFN-$\gamma^{-/-}$ recipients. As described in chapter 1.5, Stat1 is instrumental for the biological actions of both type I and type II interferons. Therefore, Stat1 deficient mice were also employed as tumor recipients. As control, we transferred the 291-OVA-GFP cells in wild-type C57BL/6 mice. As shown in Figure 11, the 291-OVA-GFP cells start to develop palpable tumors at day 22 in IFN-$\gamma^{-/-}$ mice in comparison with lymphoma development at day 26 in wild type control recipients. Striking differences emerged when 291-OVA-GFP cells were transferred in syngeneic Stat1$^{-/-}$ recipients. As shown in figure 11, the 291-OVA-GFP cells begin to develop lymphoma at day 10 in Stat1$^{-/-}$ recipients.

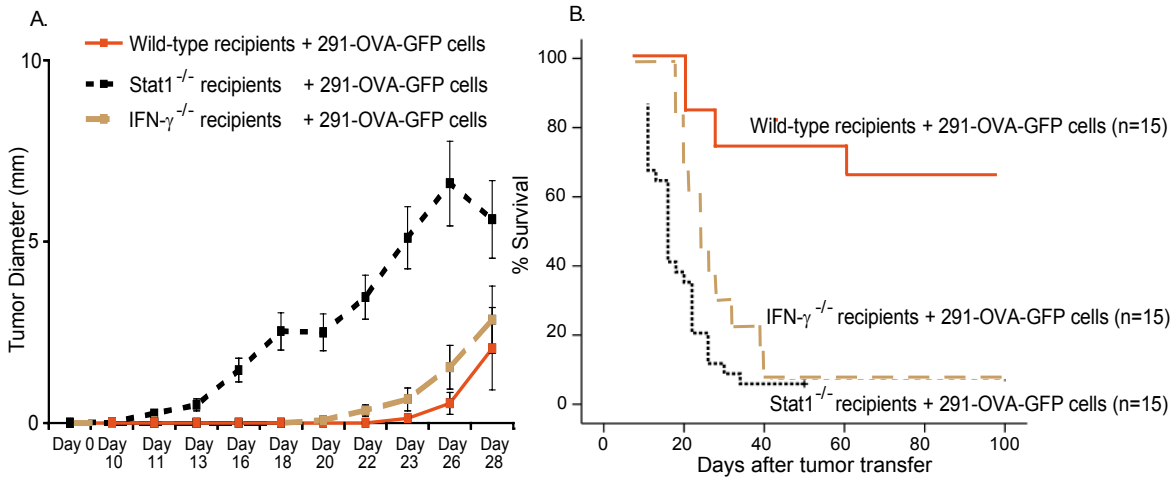


Figure 11
Accelerated tumor growth (A) and decreased overall survival (B) of Stat1$^{-/-}$ and IFNγ$^{-/-}$ recipient mice challenged with OVA-GFP expressing lymphoma cells
0.1 million cell dose of 291-OVA-GFP lymphoma were transferred in WT-C57BL/6 (solid red line), Stat1$^{-/-}$ (dashed black line) and IFN-γ$^{-/-}$ recipients (dashed light brown line). (A) Tumor growth was assessed 3 times/week using calipers and is depicted as mean +/- S.D. (B) Kaplan-Meier plots showing overall survival of different recipients after challenge with 291-OVA-GFP cells. 291-OVA-GFP cells develop lymphoma at day 26, day 10 and day 22 in wild-type, Stat1$^{-/-}$ and IFN-γ $^{-/-}$ recipients respectively. 65% of animals survived for long term after 291-OVA-GFP challenge in wild-type recipients whereas 93% of IFN-γ $^{-/-}$ and Stat1$^{-/-}$ recipients died because of tumor development.
P value was calculated using log rank test for survival. P < 0.01 for all groups.

The differences among the recipients with different genetic background became much more obvious when the overall survival was analyzed instead of tumor growth. As shown in figure 11, the growth kinetics and tumor size was also reflected when survival was analyzed. Stat1$^{-/-}$ recipients displayed a significantly faster lymphoma growth compared to IFN-γ$^{-/-}$ or C57BL/6 recipients and approximately 93 % of the animals died due to lymphoma progression. In contrast, tumor growth kinetics of IFN-γ$^{-/-}$ host were comparable to C57BL/6 mice. However, most interestingly the difference was reflected by survival since 90 % of the IFN-γ$^{-/-}$ recipients died of progressive lymphoma whereas 65 % of C57BL/6 mice successively rejected 291-OVA-GFP lymphomas and survived long term.

The IFN-γ$^{-/-}$ mice develop lymphomas at the same kinetics compared to lymphoma development in C57BL/6 mice. By contrast, Stat1$^{-/-}$ display much faster tumor growth and fail to reject 291-OVA-GFP lymphomas. This could give us the hint for the early involvement of NK cells in tumor rejection, since Stat1$^{-/-}$ mice exhibit defects in NK function. Thus from this experiment, it is evident that rejection of ovalbumin transduced lymphoma requires host interferon gamma production and Stat1 signaling within the host.

3.7 Organ infiltration by 291-OVA-GFP cells is higher in Stat1 and IFN-γ deficient mice

Since we found that 291-OVA-GFP cells grew progressively in IFN-γ and Stat1 deficient mice, we sought to determine the mechanism behind tumor progression. The tumor infiltration of 291-OVA-GFP cells within different organs of IFN-$\gamma^{-/-}$, Stat1$^{-/-}$ and wild-type C57BL/6 recipients was determined by GFP fluorescence staining. As shown in figure 12, we detected increased tumor infiltration in the spleen of tumor bearing animals in IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients and most importantly, the tumor did not infiltrate the spleen of wild-type recipients. Thus the experiment indicates that the failure to reject 291-OVA-GFP cells by IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients was due to increased infiltration of tumors in their spleens indicating escape from immunological recognition.

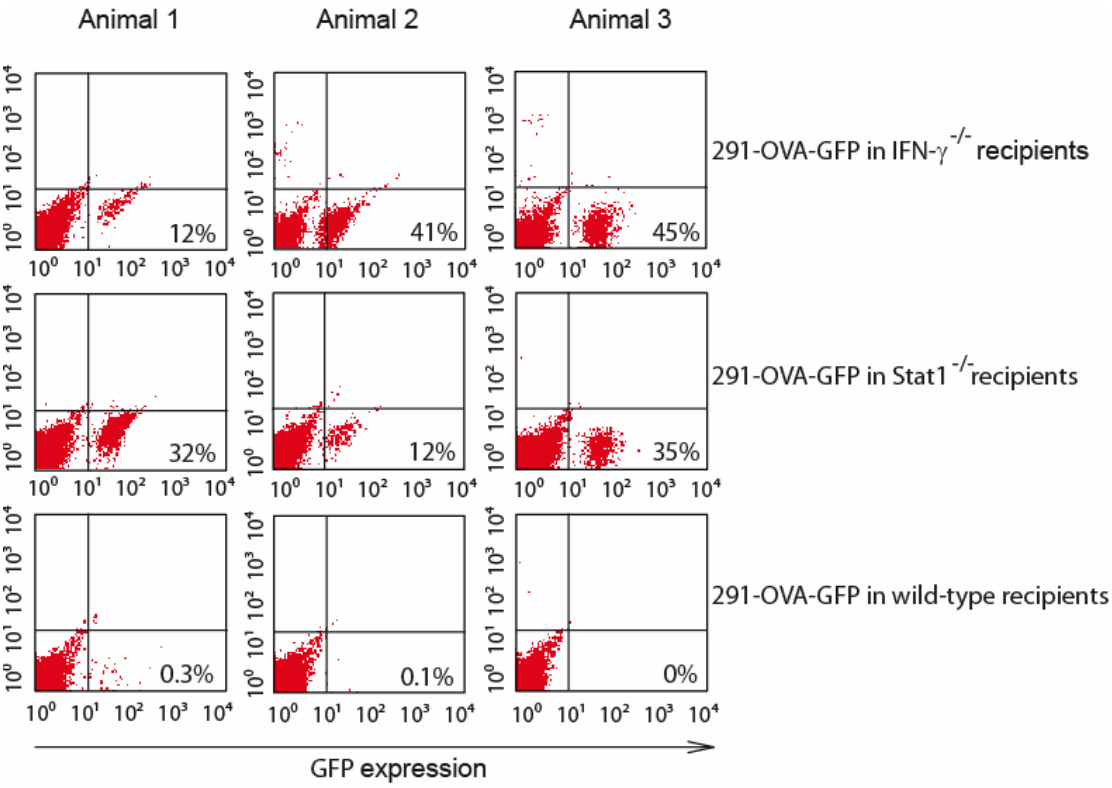


Figure 12
IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipient mice showed increased splenic infiltration with 291-OVA-GFP cells as compared to wild-type recipients
Wild-type, Stat1$^{-/-}$ and IFN-$\gamma^{-/-}$ recipients were challenged with 100,000 cells of 291-OVA-GFP .Splenic infiltration by 291-OVA-GFP cells in IFN-$\gamma^{-/-}$, Stat1$^{-/-}$ and in wild-type C57BL/6 mice is analyzed. When the tumor burden is huge and when the animals suffer, the animals were killed at day 28 and the tumor infiltration was evaluated by the presence of GFP-expressing cells in the spleen of tumor bearing animals. The presence of GFP-expressing lymphoma cells were indicated as % cells and tumor cells infiltrate at higher numbers in IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients but not in wild-type recipients. . The analysis shows three animals from each group.

3.8 Expression of antigen is preserved in IFN-γ$^{-/-}$ and Stat1$^{-/-}$ mice

Similarly to the analysis presented in figure 9, we analyzed GFP expression in outgrowing 291-OVA-GFP lymphomas not only in wild-type C57BL/6, but also in IFN-γ$^{-/-}$ and Stat1$^{-/-}$ mice. As shown in figure 13, we observed a reduction of GFP-expression in wild-type C57BL/6 host as described earlier. In contrast, GFP was expressed at about 10 fold higher levels when Stat1$^{-/-}$ mice were used as recipients (p=0.001). Although 90 % of IFN-γ$^{-/-}$ mice receiving 291-OVA-GFP cells failed to reject the lymphoma, we observed a significant reduction of GFP expression when compared to Stat1$^{-/-}$ recipients (p=0.003). This GFP expression in developing lymphomas in IFN-γ$^{-/-}$was still significantly higher as compared to lymphomas from C57BL/6 recipients (p=0.034). This indicates that loss of IFN-γ can partially be compensated, presumably by type-I interferons and maintenance of selective pressure. Thus from this experiment it is evident that increased lymphoma growth and lack of rejection of highly immunogenic 291-OVA-GFP lymphomas in IFN-γ$^{-/-}$ and Stat1$^{-/-}$ mice was not due to the loss of antigen as tumor escape mechanism.

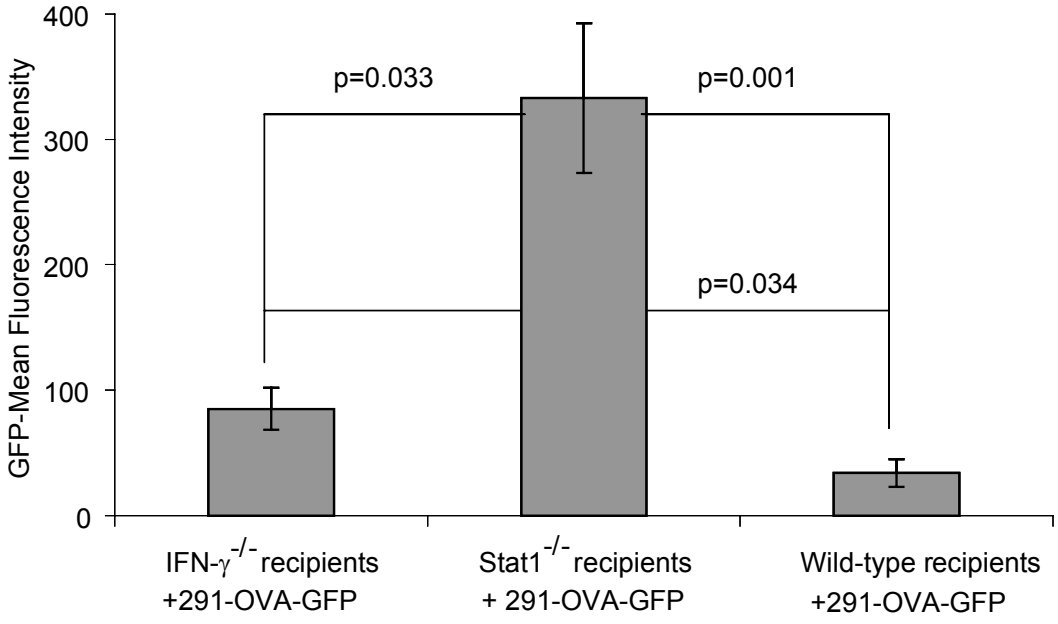


Figure 13
Selection for antigen-loss variants in developing lymphomas arising in wild-type, but not in IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipients upon challenge with 291-OVA-GFP cells
Wild-type, IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipients were challenged with 100,000 cell dose of 291-OVA-GFP lymphoma cells. GFP expression of 291-OVA-GFP cells was compared after injection into wild-type (n=8), IFN-γ$^{-/-}$ (n=10) and Stat1$^{-/-}$ (n=10) recipient mice. The animals were killed at day 28 and the GFP expression was evaluated in developing lymphomas. The GFP expression of lymphomas from Stat1$^{-/-}$ recipients was 3 to 4 fold higher than that of IFN-γ$^{-/-}$ recipients indicating that antigen expression is preserved in developing lymphomas from Stat1$^{-/-}$ recipients and IFN-γ $^{-/-}$ recipients. In case of wild-type recipients, developing lymphomas are selected for antigen loss variants. P values were determined by Mann Whitney test.

3.9 IFN-γ-dependent induction of MHC class I and MHC class II

Successful T-cell mediated rejection by foreign antigens is considered to require several features. In particular, the antigen must be processed and presented in the context of MHC. Furthermore, the amount of MHC must be sufficient on the surface to induce T-cell response. Moreover, IFN-γ mediated immunosurveillance plays an important role in tumor rejection (Shankaran et al., 2001). Therefore we sought to determine the possibility of IFN-γ mediated immunogenicity in developing 291-OVA-GFP lymphomas in wild-type, IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ mice. The surface expression of H-2K^b and IA^b in the 291-OVA-GFP cell lines was determined before injection and compared with developing 291-OVA-GFP lymphomas in control wild-type C57BL/6, IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients. As shown in figure 14, lymphomas developing in C57BL/6 mice exhibited about 3 fold higher H-2K^b and approximately 100 fold higher IA^b expression than the parental 291-OVA-GFP cells cultivated before inoculation *in vitro*. This induction of H-2K^b and IA^b expression was not observed in Stat1$^{-/-}$ and IFN-$\gamma^{-/-}$ recipients and both strains failed to reject the lymphoma. From this experiment, we conclude that induction of MHC-class I and class II is associated with lymphoma rejection in our experimental system.

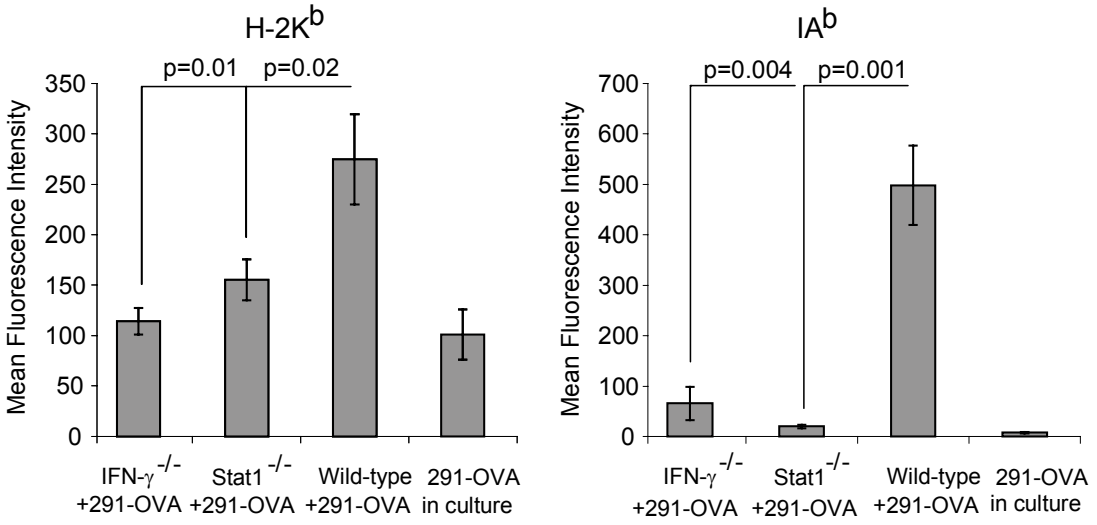


Figure 14
MHC expression was upregulated on the cell surface of 291-OVA-GFP lymphoma cells after transfer into wild-type mice, but not after transfer into IFNγ$^{-/-}$ and Stat1$^{-/-}$ mice
H-2K^b and IA^b expression of 291-OVA-GFPcells was determined prior to injection and compared to that in developing 291-OVA-GFP lymphomas in wildtype, IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipient mice. The 291-OVA-GFP bearing lymphomas in wild-type, IFN-$\gamma^{-/-}$ amd Stat1$^{-/-}$ recipients were analysed at day 30. H-2K^b expression was induced only in tumors arising in wild-type recipients but not in those arising in IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients. Similarly, IA^b expression was induced only in lymphomas arising in wild-type C57BL/6 mice and this upregulation was completely abolished in lymphomas arising in IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients. H-2K^b and IA^b expression in cultured 291-OVA-GFP served as controls. n=10 for IFN-$\gamma^{-/-}$ and Stat1$^{-/-}$ recipients and n=8 for wild-type recipients.

As shown in figure 14, the 291-OVA-GFP lymphomas arising in C57BL/6 animals displayed higher H-2K^b and IA^b expression levels than the parental 291-OVA-GFP cell line. We wished to evaluate the underlying mechanisms and addressed the question whether MHC class I and MHC class II upregulation is cell autonomous or requires the *in vivo* tumor environment. To this end, 291-OVA-GFP lymphomas arising in C57BL/6 mice were placed back in culture, cultivated for 60 days *in vitro*, and were then analyzed again for H-2K^b and IA^b expression. As shown in figure 15, H-2K^b and IA^b expression of explanted lymphomas was reduced back to basal levels after long term cultivation *in vitro*. Treatment with murine IFN-γ could partially restore MHC expression as shown in figure 15. Treatment with murine IFN-γ led to a 2-3 fold increase in MHC class I (H-2K^b) and 10 to 15 fold increase in MHC class II expression in 291-OVA-GFP lymphomas seeded into culture from C57BL/6 and GFP-transgenic C57BL/6 recipients.

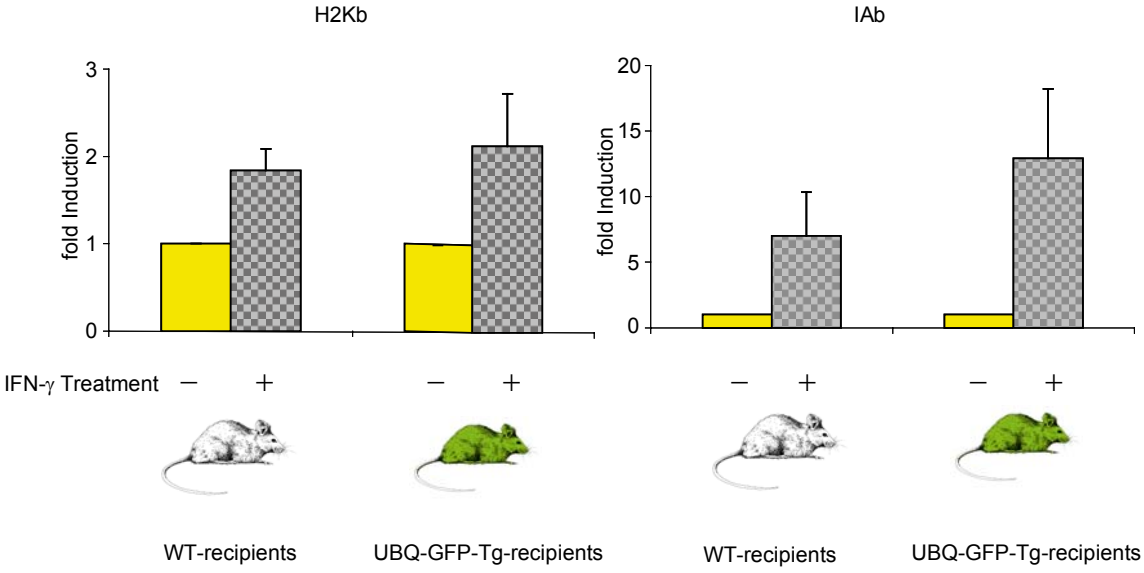


Figure 15
291-OVA-GFP lymphomas arising in wild-type or UBQ-GFP transgenic mice and cultivated ex vivo responded to IFN-γ by upregulation of H-2K^b and IA^b expression
291-OVA-GFP lymphomas arising in C57BL/6 and UBQ-GFP transgenic mice were placed back in culture and cultivated for two months. The in vitro cultivation led to down regulation of H-2K^b and IA^b on the cell surface of 291-OVA-GFP lymphoma cells as shown in yellow bars. Treatment of 291-OVA cells with murine IFN-γ led to 2-3 fold increase in H-2K^b expression and 10-15 fold increase in IA^b expression. The expression levels of IFN-γ treated 291-OVA-cells are shown in grey bars. n=5 for both untreated cultivated cells and IFN-γ treated cells.

3.10 OT-I T cells fail to reject 291-OVA-GFP lymphomas in Stat1$^{-/-}$ recipients

The previous results demonstrated that Stat1$^{-/-}$ mice provide a platform to allow 291OVA-GFP cells to grow without loss of antigen indicating no selection pressure as opposed to IFN-$\gamma^{-/-}$ mice (Figure 11 and 13). In order to further address rejection mechanisms, we used Stat1$^{-/-}$ recipients as host to transfer OT-I CD8+ T cells that harbor an ovalbumin-specific transgenic T cell receptor (TCR). CD8+ T cells from OT-I mice express a transgenic TCR that recognises H-2Kb and the immunodominant $OVA_{257-264}$ peptide SIINFEKL. The fast growth of 291-OVA-GFP lymphoma in Stat1$^{-/-}$ recipients prompted us to investigate whether adoptive transfer of primed OT-I CD8+ T cells is able to mediate tumor rejection. We therefore used CD8+ T cells from vaccinated and unvaccinated mice in a dose range from 10,000 to 1 million CD8+ T cells. C57BL/6 OT-I transgenic mice were vaccinated with irradiated 291-OVA-GFP cells one week prior to harvest of CD8+ T cells. The lymphoma cells (0.1 million) were injected s.c. at day 0 and the OT-I CD8+ T cells were injected i.v. at day 1. As shown in figure 16, neither transfer of naïve OT-I CD8+ T cells, nor of activated OT-I CD8+ T cells prevented lymphoma growth, even when the number of activated OT-I T cells exceeded the number of lymphoma cells by a factor of 10.

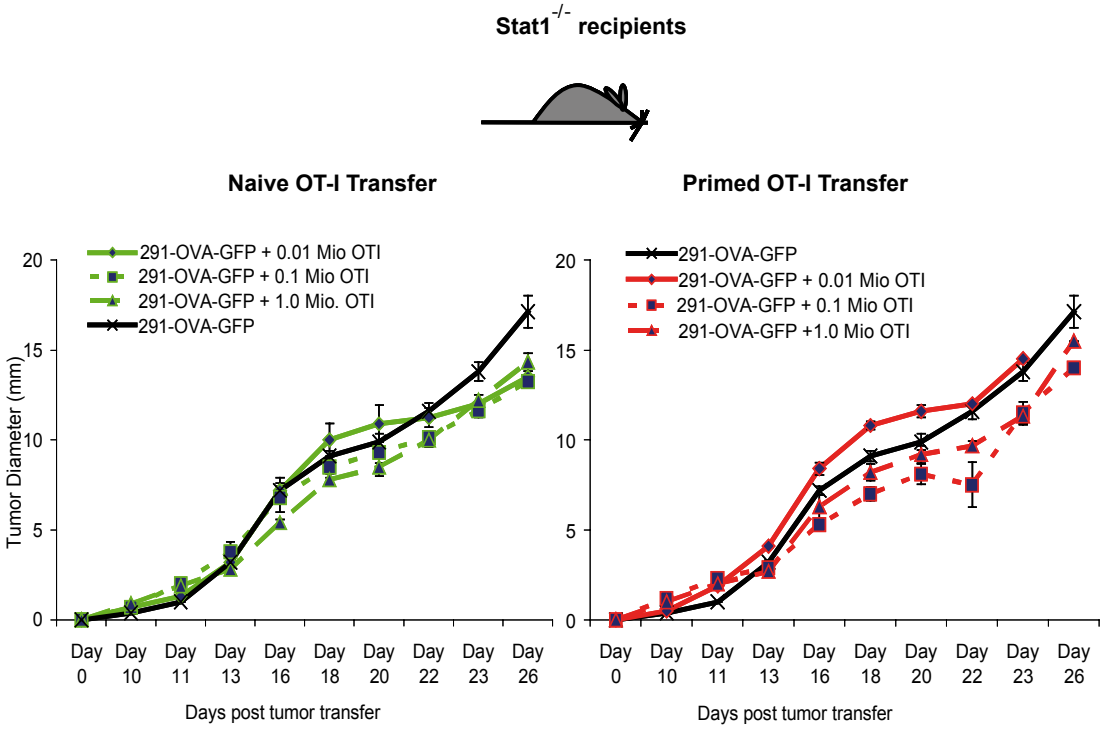


Figure 16
OT-I CD8+ T cells failed to reject 291-OVA-GFP cells in Stat1$^{-/-}$ recipients
The lymphoma cells is injected s.c at day 0 and either naïve or primed OT-I CD8+ T cells is injected i.v at day 1. Lymphoma growth after transfer of either naïve or primed OT-I CD8+ T cells following inoculation of 291-OVA-GFP cells into Stat1$^{-/-}$ recipients are monitored. Lymphoma growth at the site

of injection is measured using calipers. Lymphoma growth was observed in both groups of mice that received either naïve or primed OT-I CD-8+ T cells. Two independent experiments were performed with identical results. n=10 per group.

The results of this experiment show that OT-I CD8+ T cells fail to reject the lymphoma regardless of their priming. Tumor growth was not even delayed after transfer of primed OT-I CD8+ T cells despite the fact that the number of OVA-specific T cells was by a factor of 10 higher in the primed as compared to the unprimed group.

3.11 Primed OT-I T cells persist in tumor bearing Stat1$^{-/-}$ animals

The failure of naïve or primed OT-I CD8+ T cells to control tumor growth could be due to the lack of persistence or expansion post-transfer. The presence of OT-I CD8+ T cells can be evaluated by the staining of T cell receptor Vα2 and Vβ5 CD8+ T cells. The TCR staining was performed when the recipient mice were bearing tumor size of about 10 mm. As shown in figure 17, twenty three days after transfer of the T cells, we could detect large numbers of OT-I CD8+ T cells only in the group of mice that had received primed OT-I T cells but not the group that had received naïve T cells.

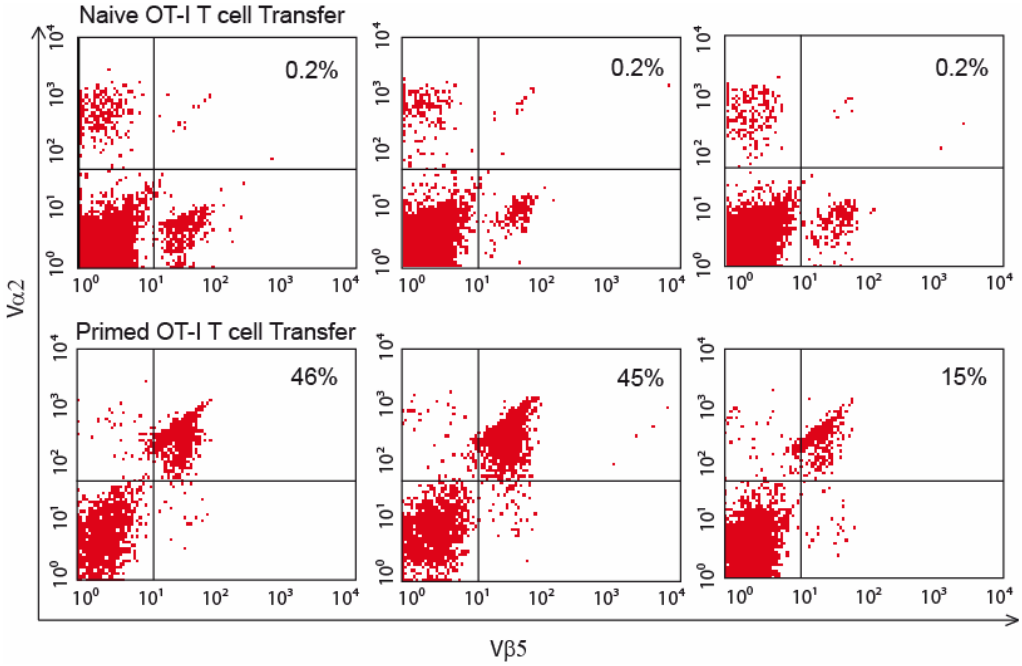


Figure 17
Ovalbumin-specific OT-I CD8+ T cells persisted in Stat1$^{-/-}$ recipients challenged with 291-OVA-GFP cells
The persistence of naïve or primed transferred OT-I CD8+ T cells in the spleen of Stat1$^{-/-}$ recipients were evaluated by TCR staining for Vα2 and Vβ5. After the tumor challenge and T cell transfer in Stat1$^{-/-}$ recipients, all diseased animals were killed and the TCR staining was performed at day 26 in the splenocytes of lymphoma bearing animals. The Vβ5-TCR stain is shown in the X- and the Vα2-TCR stain in the Y-axis. Results are shown from three mice of each group that had received either naïve or primed OT-I T cells. Persistence of transferred OT-I CD8+ T cells is shown in quadrant plot as % cells positive for both Vα2 and Vβ5.

3.12 Generation of IFN unresponsive lymphoma cells

Since our data so far had demonstrated the importance of MHC upregulation and IFN-γ for the rejection of 291-OVA-GFP cells, we hypothesized that unresponsiveness towards IFNs would render cells invisible to T-cells and therefore promote tumor growth despite the presence of foreign antigen. For the generation of Stat1-deficient lymphoma cells, we crossbred λ-hu-c-myc transgenic mice with Stat1$^{-/-}$ animals maintained on C57BL/6 background. The survival of λ-hu-c-myc transgenic Stat1$^{-/-}$ mice compared to that of λ-hu-c-myc transgenic mice is shown in figure 18. Loss of Stat1 led to a slight, but significant acceleration of tumor development.

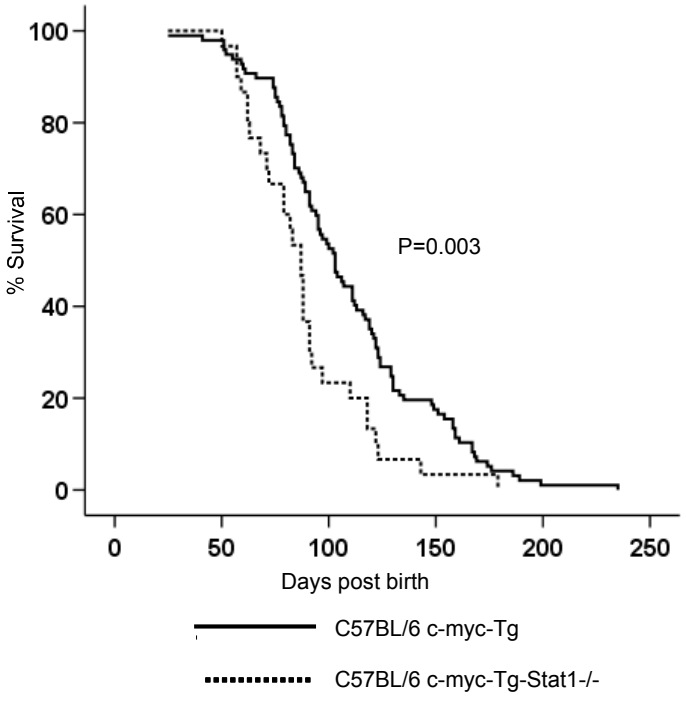


Figure 18
Loss of Stat1 slightly accelerated λ-hu-c-myc-induced lymphoma development in vivo
C57BL/6 λ-hu-c-myc Tg mice were cross bred with Stat1$^{-/-}$ mice to generate λ-hu-c-myc Tg-Stat1$^{-/-}$ mice. Kaplan-Meier plot of the survival of λ-hu-c-myc Stat1$^{+/+}$ and λ-hu-c-myc Stat1$^{-/-}$ transgenic mice. The median survival of λ-hu-c-myc Stat1$^{+/+}$ mice was 107 days and that of λ-hu-c-myc Stat1$^{-/-}$ mice 87 days (P=0.003). Loss of Stat1 within c-myc overexpressing lymphomas accelerated lymphoma development compared with Stat1 competent λ-hu-c-myc Tg mice.

3.13 Stat1$^{-/-}$ lymphomas are unresponsive to IFN-γ

After establishing the Stat1-deficient lymphomas in culture, we studied the action of murine IFN-γ on Stat1-competent and Stat1-deficient lymphoma cell lines *in vitro*. As shown in figure 19, H-2K^b and IA^b upregulation was observed only in Stat1-competent cells (line 291) cells, but the effect of murine IFN-γ on MHC class I and class II expression was completely abolished in Stat1-deficient lymphoma cells (line 9). It is thus evident from this experiment that MHC-upregulation through IFN-γ is indeed dependent on Stat1, as expected.

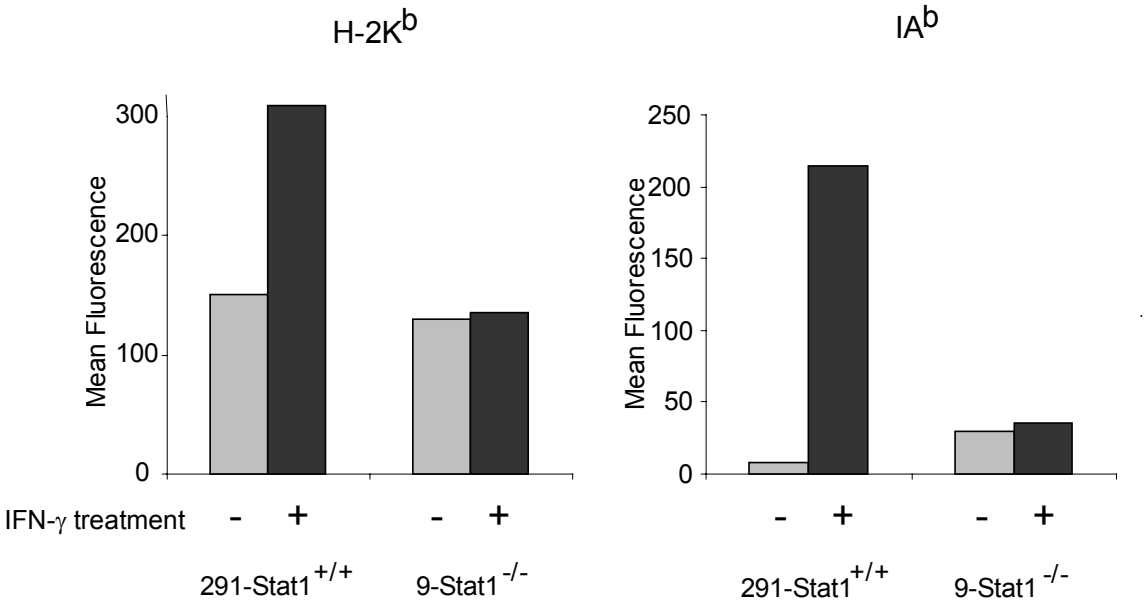


Figure 19
Stat1$^{-/-}$ lymphomas did not respond to IFN-γ
Stat1-competent (line 291) and Stat1-deficient cells (line 9) were analyzed for their sensitivity to murine IFN-γ. The cells were treated with murine IFN-γ for 48 hours and cell surface expression of H-2K^b and IA^b was determined. H-2K^b and IA^b were upregulated in Stat1 competent cells and this effect was completely abolished in Stat1 deficient lymphoma cells.

Similarly to the Stat1-competent 291-Stat1$^{+/+}$ cell line, we introduced chicken ovalbumin and GFP into the Stat1 deficient lymphoma cell line (9-Stat1$^{-/-}$). After retroviral transduction, the infected cells were monitored for GFP expression by flow cytometry. In order to establish a Stat1-deficient cell line that expresses the foreign antigens, high GFP expressing cells were sorted. As shown in figure 20, about 95% of 9-Stat1$^{-/-}$-GFP and 9-Stat1$^{-/-}$-OVA-GFP cells expressed GFP. Since we wished to study the immune response against GFP and ovalbumin expressing lymphomas, we monitored the stability of foreign antigen expression in lymphoma cells *in vitro* by measuring GFP expression as read out. We found that GFP expression

remained virtually unaltered in culture for more than 100 days. In order to rule out differences in the expression of MHC molecules after retroviral transduction, the cell lines were analyzed for the surface expression of H-2K^b, IA^b and CD19 markers. As shown in figure 20, the level of H-2K^b, IA^b and CD19 expression remained unaltered in 9-Stat1$^{-/-}$-GFP and 9-Stat1$^{-/-}$-OVA-GFP cells as compared to 9-Stat1$^{-/-}$ parental cells confirming that retroviral transduction procedures did not alter the surface phenotype of the cells.

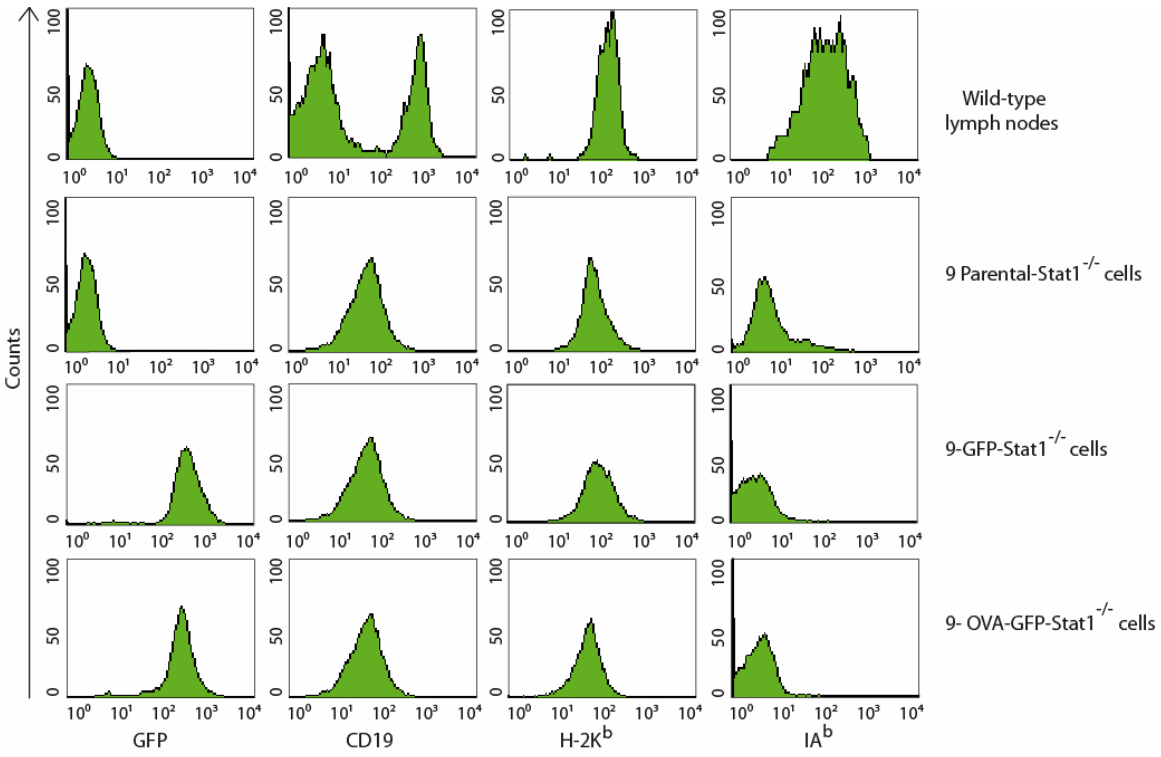


Figure 20
No alteration of CD19, H-2K^b, and IA^b expression after retroviral transfer of chicken ovalbumin and GFP into 9-Stat1-/- lymphoma cells
9-Stat1$^{-/-}$ cells were retrovirally transduced and GFP, CD19, H-2K^b and IA^b expression in 9-GFP-Stat1$^{-/-}$, 9-OVA-GFP-Stat1$^{-/-}$, and unmanipulated parental 9-Stat1$^{-/-}$ cells monitored by flow cytometry. Retroviral transduction did not alter the surface expression of H-2K^b and IA^b molecules. Expression of CD19, H-2K^b and IA^b of 9-Stat1$^{-/-}$ lymphoma cells was compared with that of cells in lymph nodes of wild-type C57BL/6 mice.

3.14 Stat1 deficient lymphoma is rejected upon transfer into immuno-competent mice

To test the immunological recognition of Stat1$^{-/-}$ tumors, GFP- and OVA-GFP transduced 9-Stat1$^{-/-}$ lymphoma cells were inoculated into immunocompetent UBQ-GFP transgenic C57BL/6 mice. As shown in figure 21, partial lymphoma growth was observed upon transfer of 9-GFP-Stat1$^{-/-}$ cells and 9-OVA-GFP-Stat1$^{-/-}$ cells in UBQ-GFP transgenic C57BL/6

recipients but growth was discontinuous and the tumor started to regress over time in the majority of cases. Corresponding to this discontinuous tumor growth, approximately 85% of the animals rejected the lymphoma cells completely, irrespective of the presence of OVA antigen. Thus both 9-GFP-Stat1$^{-/-}$ cells and 9-OVA-GFP-Stat1$^{-/-}$ cells were rejected efficiently upon transfer into immunocompetent UBQ-GFP transgenic mice. Thus, this experiment suggests that loss of Stat1 signaling does not lead to enhanced tumorigenesis as one might have expected, it rather leads to efficient rejection independent of the presence of strong foreign antigen. The growth kinetics of the tumor and the overall survival of UQB-GFP transgenic mice upon challenge with 9-GFP-Stat1$^{-/-}$ and 9-OVA-GFP-Stat1$^{-/-}$ cells are shown in figure 21.

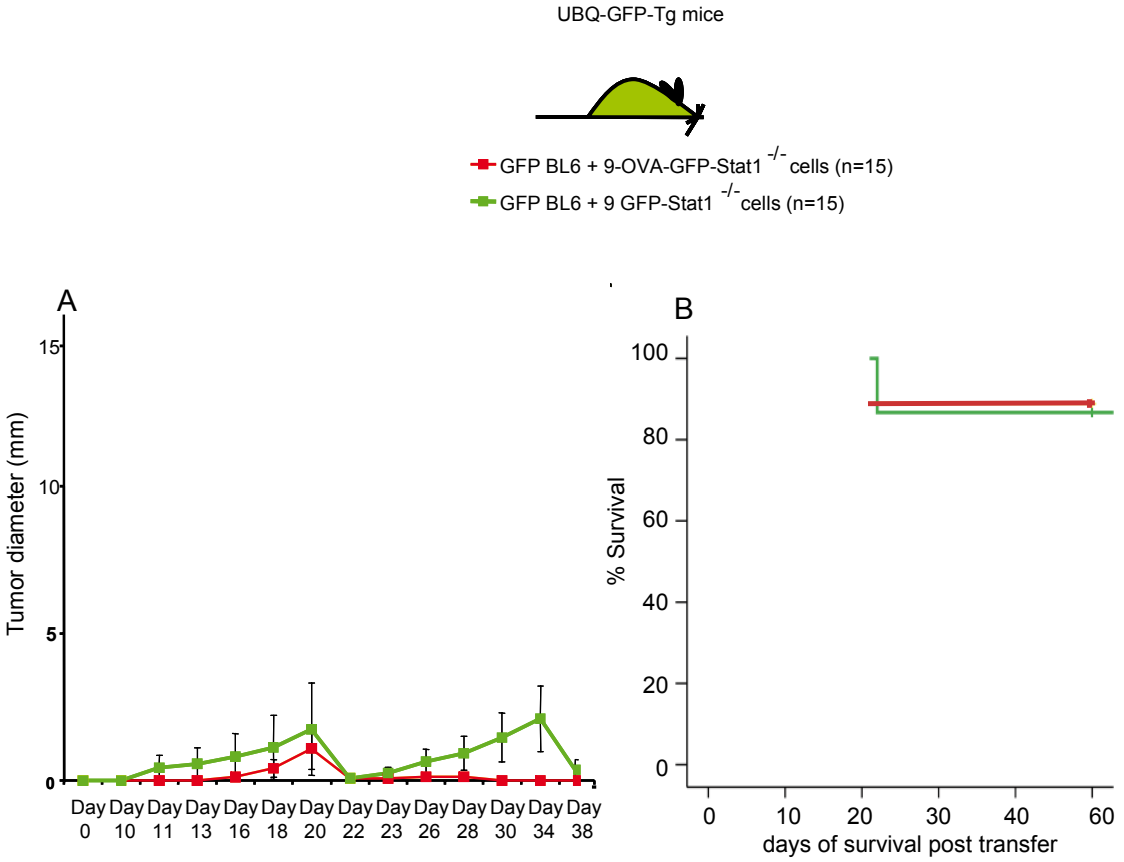


Figure 21
Stat1-deficient lymphomas were rejected in UBQ-GFP-transgenic recipients irrespective of foreign antigen expression in the tumor
UBQ-GFP-Tg recipients were challenged with 100,000 cells of 9-GFP-Stat1$^{-/-}$ and 9-OVA-GFP-Stat1$^{-/-}$ lymphoma cells. A) 9-GFP-Stat1$^{-/-}$ (green line) and 9-OVA-GFP-Stat1$^{-/-}$ lymphoma cells (red line) were injected s.c. in UBQ-GFP transgenic wild-type C57BL/6 recipients and the tumor size at the site of injection was measured 3 times a week using calipers. Three independent experiments were performed with reproducible results. B) Overall survival of UBQ-GFP mice challenged with Stat1-deficient lymphoma cells expressing GFP (green) or GFP and OVA (red). Loss of Stat1 by the lymphoma cells led to efficient rejection in UBQ-GFP-transgenic recipients irrespective of the expression of OVA antigen.

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3.15 IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipients fail to reject Stat1 deficient lymphomas

The rejection of 9-GFP-Stat1$^{-/-}$ and 9-OVA-GFP-Stat1$^{-/-}$ cells in GFP-transgenic immunocompetent mice provided evidence for antigen-independent rejection. Since 80-85% of GFP-transduced Stat1-deficient lymphomas were rejected in GFP-transgenic mice, we investigated the mechanisms involved in the process. Furthermore, we knew already from previous experiments that rejection of Stat1 competent, foreign antigen expressing cells (line 291) requires production of IFN-γ and Stat1 signaling by the host. To address the question whether rejection of Stat1-deficient lymphomas also requires host IFN-γ and Stat1, we transplanted Stat1$^{-/-}$ lymphoma cells into IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipients. As rejection of Stat1-deficient lymphomas was independent of foreign antigen, we used 9-GFP-Stat1$^{-/-}$ cells and UBQ-GFP transgenic mice as recipients for further experiments. As shown in figure 22, transfer of 9-GFP-Stat1$^{-/-}$ cells into UBQ-GFP transgenic as well as wild-type C57BL/6 mice led to efficient rejection. However, striking differences emerged when 9-GFP-Stat1$^{-/-}$ cells were transferred into IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipients. Transfer of 9-GFP-Stat1$^{-/-}$ cells into IFN-γ$^{-/-}$ and Stat1$^{-/-}$ mice led to early lymphoma onset at day 10 and continuous tumor progression, whereas they were rejected in wild-type C57BL/6 mice. The results indicate that lack of IFN responsiveness within the lymphoma does not affect induction of an immune response, however, lack of IFN-γ and of Stat1 signaling within the host increases tumorigenicity. Thus, it is important to note that rejection of both, Stat1 competent cells (line 291) in an antigen-dependent manner and rejection of Stat1 deficient cells (line 9) in an antigen-independent manner requires production of IFN-γ and Stat1 signaling by the host.

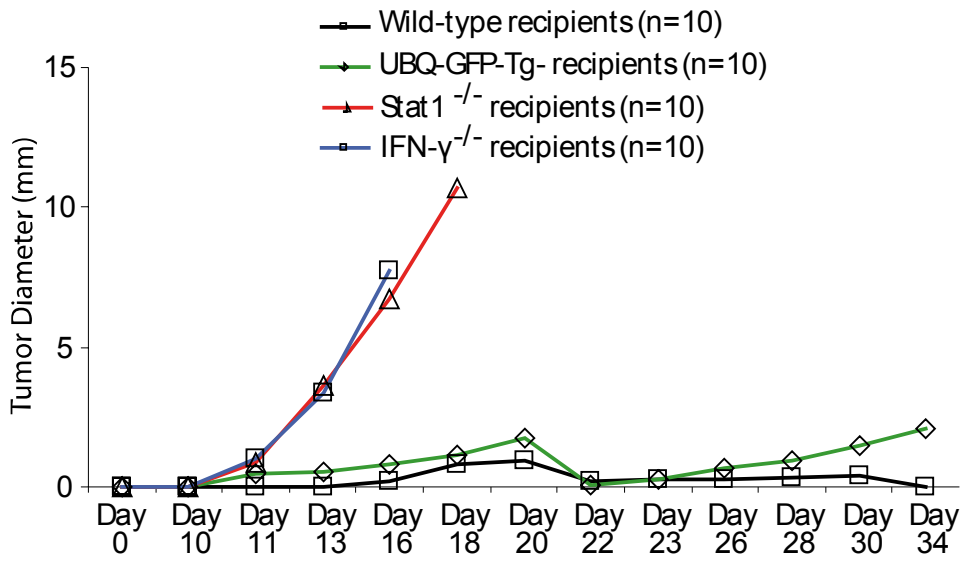

Figure 22
Host IFN-γ and Stat1 were required for antigen-independent rejection of Stat1-deficient lymphoma cells
9-GFP-Stat1$^{-/-}$ lymphoma cells were injected s.c. into wild-type (black line), UBQ-GFP transgenic (green line), IFN$^{-/-}$ (blue line), and Stat1$^{-/-}$ recipients (red line). 0.1 million cells were injected in each recipient mice and the tumor size at the site of injection was measured 3 times a week using calipers. 9-GFP-Stat1$^{-/-}$ cells were rejected in both, wild-type C57BL/6 and UBQ-GFP transgenic mice, but not in IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipients.

3.16 Lymphoma cells are lysed by NK cells in vitro

Tumor cell killing by NK cells has been studied in various tumor models and has invariably been found to be dependent on absent or low MHC class I expression. As low MHC class I and class II expression is a hallmark of λ-hu-c-myc-induced lymphomas, we sought to determine whether there is any NK cell recognition in our model system. Most importantly, we found that Stat1 deficient lymphoma cells fail to upregulate MHC class I and class II molecules in the presence of IFN-γ, whereas Stat1-competent cells do respond to IFN-γ by upregulating MHC class I and class II as shown in figure 19. Therefore, we addressed the question whether the Stat1-competent (cell line 291) and the Stat1-deficient lymphoma cells (cell line 9) can serve as targets for NK cell lysis. 291-OVA-GFP Stat1-competent and 9-OVA-GFP-Stat1-deficient cells were treated with murine IFN-γ and co-cultured with NK cells for 8 hours. YAC cells are mouse lymphoma cells and were described to be sensitive to the cytotoxic activity of NK cells. YAC cells were used as the positive control for NK cell lysis. As shown in figure 23, 65% of lysis was observed in 291-OVA cells in the absence of IFN-γ and lysis was reduced to 30 % upon treatment of 291-OVA cells with murine IFN-γ. In contrast, IFN-γ treated Stat1-deficient 9-OVA cells were lysed significantly more efficiently than untreated 9-OVA cells. IFN-γ treatment of Stat1-competent lymphoma cells thus

diminished NK cell killing, whereas NK cell killing of Stat1-deficient lymphoma target cells was enhanced by IFN-γ treatment. However, it is important to note that both Stat1 competent (line 291-OVA) and Stat1 deficient (line 9-OVA) lymphoma cells were targets of NK cells and spontaneously sensitive to NK lysis.

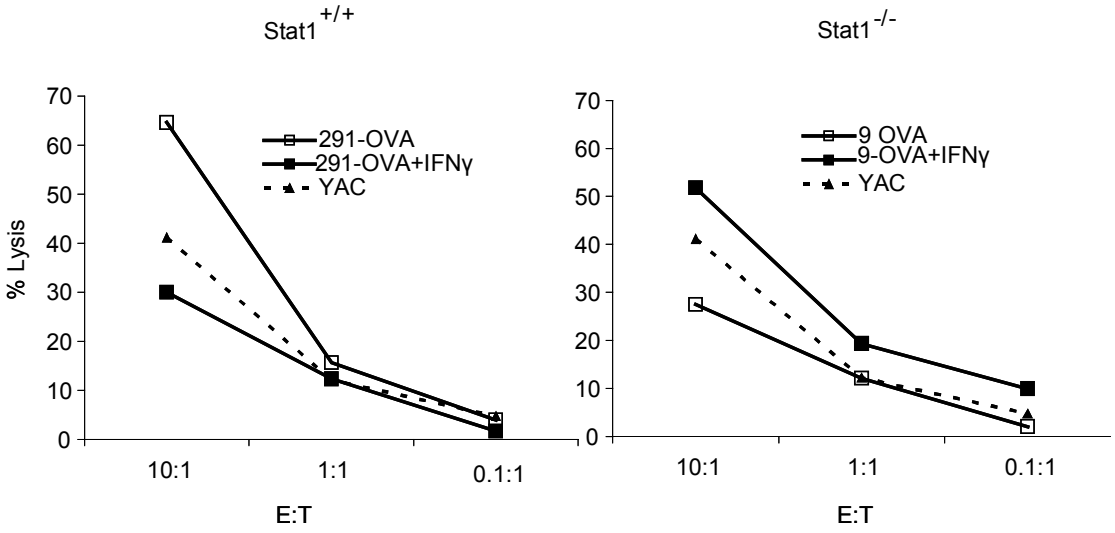

Figure 23
291-OVA and 9-OVA lymphoma cells were both targets of spontaneous NK cell lysis. IFN-γ diminished NK cell killing in Stat1-competent cells and increased killing in Stat1 deficient lymphoma cells
Stat1-competent 291-OVA-GFP cells and Stat1-deficient 9-OVA-GFP cells were treated with murine IFN-γ for 48 hours and co-cultured with NK cells. After 8 hours of co-culture with effector and target cells, the supernatants were removed and analyzed for LDH activity. IFN-γ treatment of 291-OVA-GFP cells led to diminished NK cell killing whereas IFN-γ treatment of 9-OVA-GFP cells increased NK cell killing of target cells.

4. Discussion

4.1 MYC overexpression in human B cells imposes a non-immunogenic phenotype in vitro

A major difference between EBV-positive BLs and LCLs is the level of expression of *c-myc.* Studies by Staege et al. pointed to the possibility that *c-myc* directly imposes the non-immunogenic phenotype observed in BL cells (Staege et al., 2002). Besides the marked restriction of EBV antigen expression, there exists a selective down-regulation of some HLA class I alleles such as HLA-A11 (Imreh et al., 1995) and these cells exhibit a reduced capacity to process and present antigens in a HLA-restricted manner (Rowe et al., 1995; Frisan et al., 1998). In cell lines stably transfected with a constitutively active, Igκ-enhancer driven *c-myc* gene (A1 cells) (Polack et al., 1996) or with a tetracycline-controlled *c-myc* expression construct (P493-6 cells) (Pajic et al., 2000), overexpression of *c-myc* rendered proliferation independent of the viral proliferation program, and imposed the cell surface phenotype of BL cells. Studies from these conditional cell lines revealed that MHC class I, activation markers, and co-stimulatory molecules are highly downregulated during *c-myc* driven proliferation. Interestingly, c-myc driven cells had not only lost the allostimulatory capacity of the parental ER/EB2 cells in a mixed lymphocyte reaction, they also could not be recognized by antigen-specific CD8+ T cell clones even when they were forced to overexpress the cognate antigen by infection with recombinant vaccinia virus (Staege et al., 2002). Furthermore, the peptide transporters such as TAP1 and TAP2 were also downregulated upon *c-myc* overexpression. The authors concluded that, in their *in vitro* system, B cells driven into proliferation through constitutive or conditional up-regulation of *c-myc* are immunologically silenced in the absence of any immune selection. The immunological silencing could be due to the cellular changes caused by *c-myc* up-regulation. These findings suggested that the outgrowth of poorly immunogenic tumors may not only be the consequence of an immunoediting process, but also may be caused by genetic alterations occurring during the transformation process. It was therefore important to know whether the effects of *c-myc* overexpression seen in the in vitro model can be recapitulated in an in vivo setting.

4.2 Mouse model to evaluate the immune response against a B cell lymphoma expressing a foreign antigen in vivo

To elucidate if the observed effects of *c-myc* overexpression in vitro can be recapitulated in an in vivo setting, we took advantage of mice that are transgenic for *c-myc*. We wished to look for any evidence of immunological silencing by expressing foreign antigens in lymphomas established from these mice. Previous efforts to develop a mouse model centered on the generation of transgenic mice that express *c-myc* under the control of IgH regulatory sequences (Adams et al., 1985). However it is important to note that these constructs induced primarily precursor B cell as well as some surface Ig-positive B-cell lymphomas and in addition the transgenic mice displayed features of lymphoblastic lymphoma (LL) rather than of BL. Studies from Kovalchuk et al report that lymphomas with striking similarities to BL develop in mice bearing a MYC gene controlled by reconstructed Igλ locus (Kovalchuk et al., 2000). Monoclonal IgM+ CD5- CD23- tumors developed and therefore these lymphoma bearing mice provide a better system for studying the pathogenesis of Burkitt's lymphoma. Transgenic mice described above are usually considered as reliable mouse models of human cancer in comparison to carcinogen-induced cancer models which may not accurately mimic the spontaneous human tumor model (Prehn, 1975).

We wished to address the immunological recognition of high grade B-cell lymphoma expressing the foreign antigens in vivo by transferring the lymphomas in immunocompetent mice. We selected chicken ovalbumin as a model foreign antigen because the introduction of ovalbumin does not alter the cell's physiological condition. In addition, the availability of OVA-specific T cell tools makes it possible to study and quantify the T cell response against this antigen. After having established that transfer of 100.000 unmanipulated cells from a cell line (291) established from a spontaneous lymphoma (non transduced) led to 100% mortality, we analyzed whether or not the retroviral introduction of ovalbumin as foreign antigen leads to better immunological recognition and tumor rejection. Importantly retroviral transduction did not produce any notable effects in the expression of the major histocompatibility antigens.

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4.3 Rejection of wild-type lymphoma cells is antigen dependent

The observation that GFP- or OVA-GFP-transduced lymphoma cells were efficiently rejected upon transfer into wild-type mice implicated a protective role of the immune response against either GFP or ovalbumin protein. We found that the introduction of ovalbumin-bearing lymphomas led to delay in tumor development by ten days and to an increased survival compared to inoculation of unmanipulated parental lymphoma cells. The results of efficient

lymphoma rejection from OVA-bearing lymphomas were consistent both in wild-type mice and UBQ-GFP transgenic mice. It is also noteworthy that the introduction of GFP antigen alone led to an improved rejection when transferred in wild-type recipients but not in UBQ-GFP transgenic mice which clearly indicated that GFP can act as an antigen and increases the immunogenicity of the lymphoma cells. GFP has been used as a potential marker in human clinical trials (Aran et al., 1998; Levenson et al., 1998). The mechanism for immune detection of cytoplasmic proteins like GFP is based on the ability of MHC to present processed antigenic peptides at the cell surface (Germain and Margulies, 1993). T cell receptors recognize antigen fragments associated with MHC. Studies from Stripecke et al. have demonstrated that the immune response to GFP may affect the outcome of gene transfer in an immunocompetent murine model (Stripecke et al., 1999). Their studies have shown that engraftment of leukemia and lymphoma cells in a murine host are significantly reduced when GFP is co-expressed. In addition, GFP expressing leukemia cells grew normally in Nu/Nu mice lacking functional T cells indicating the existence of a T cell response against GFP in wild-type mice. Furthermore, rejection of GFP-positive cells correlated with the appearance of CTL reacting specifically against cells expressing GFP. These results, together with our observation of rejection of GFP positive lymphomas, demonstrate the existence of potential antigenic peptides derived from GFP that could mediate lymphoma rejection.

In contrast to GFP, ovalbumin has been widely used as model antigen in a number of experimental mouse tumor models. In most of these tumor models ovalbumin was a weak antigen that did not lead to tumor rejection unless the mice were treated with stimulated OVA-specific T cells. Using EL4 thymoma as a tumor model, where EG7-OVA cells differ from the parental EL4 cells by expressing OVA, Zhou et al. demonstrated that OVA-specific CTLs elicited by vaccination can protect the mice against a subsequent tumor challenge (Zhou et al., 1992; Minev et al., 1994). Likewise, in a EL4 thymoma and a B16 melanoma lung metastasis model, transfer of antigen-stimulated ovalbumin-specific effector T cells was a prerequisite for tumor rejection (McCabe et al., 1995; Dobrzanski et al., 1999; Dobrzanski et al., 2000; Dobrzanski et al., 2004; Hollenbaugh et al., 2004). Given the low antigenicity of ovalbumin in these models, it was unforeseeable and surprising that ovalbumin as well as GFP acted as rejection antigens in this high grade B cell lymphoma model resulting in a 40-45% increase in overall survival in recipient mice challenged with 291-OVA-GFP lymphoma cells. The role of antigen-specific T cells in lymphoma rejection was underlined by two additional observations: (i) we found increased numbers of SIINFEKL-specific CD8+ T cells in the splenocytes of wild type and UBQ-GFP mice challenged with OVA-GFP-expressing

tumor cells as compared to mice challenged with GFP-expressing tumor cells, (ii) mice, that developed delayed fatal lymphomas after inoculation of OVA-GFP expressing lymphoma cells, invariably displayed an antigen-loss phenotype, indicating that tumor cell variants were selected in outgrowing lymphomas. This may explain why approximately 55% of recipient mice develop tumors upon challenge with OVA-GFP bearing lymphoma cells. From these results it may be inferred that immunosurveillance can play an active role in suppressing or preventing the growth of tumors expressing a foreign antigen.

In our B cell lymphoma model, the lymphoma cells expressed also the human c-MYC protein as a potential foreign antigen. According to peptide prediction algorithms potentially immunogenic epitopes could be predicted for the human c-MYC protein (data not shown). Yet, irrespective of potential epitopes in the human c-MYC protein, the inoculated unmanipulated 291 parental lymphoma cells grew progressively in both wild-type and UBQ-GFP transgenic recipient mice and led to fatal tumor progression in 100% of the animals within 20 days. Thus in our model, the presence of human c-MYC protein appears not to be sufficient to induce an efficient immune response in an immunological competent host. Alternatively, it is also conceivable that the tumor cells grew too fast and outpaced a weak immune response against the human c-MYC protein when inoculated in numbers of 100.000 lymphoma cells. Below the threshold of 100.000 cells injected, we observed variable engraftment of lymphoma, and below 10.000 cells all animals rejected the cells. Several reasons might account for the necessity to inoculate high cell numbers for tumor engraftment: (i) c-myc driven lymphomas exhibit a high degree of spontaneous apoptosis rendering tumor engraftment a potentially inefficient process, (ii) it is not known whether only a minor fraction of the cells or many or all cells in the tumor may act as tumor stem cells, (iii), in case that the majority of tumor cells has cancer stem cell properties, c-MYC protein may act as a weak antigen, and finally (iv) other mechanisms of rejection like the action of NK cells may be operating as well rendering the inoculation of a high number of tumor cells necessary. To address the potential immunogenicity of the human c-MYC protein, it will be necessary to study the efficiency of lymphoma engraftment/rejection in wild type mice that have been pre-vaccinated with the human c-MYC protein. Such studies are in progress.

The question remains why ovalbumin is such a weak antigen in the EL4 thymoma and B16 melanoma models that can be recognized only after antigen-specific T cell stimulation ((McCabe et al., 1995; Dobrzanski et al., 1999) and such a potent rejection antigen in our c-

myc driven B cell lymphoma model. It is well known that membrane anchorage strongly increases the antigenicity of ovalbumin (Kurts et al., 1996). But this cannot account for the differences observed. In the EL4, B16, as well as in our c-myc driven lymphoma model it is the cytosolic version of ovalbumin that has been expressed. The most likely explanation for the differences observed in the various tumor models is that ovalbumin is expressed at much higher level after retroviral transduction as compared to the transfected EL4 and B16 cell lines expressing OVA at low level.

In summary, our experiments have provided evidence that expression of foreign antigen (either GFP alone or OVA-GFP) in this B-cell lymphoma model led to immunological recognition, efficient tumor clearance, and increased survival in about half of the animals, and in delayed outgrowth of antigen-loss variants in the remaining mice. We have not yet defined the effector cell population that is mediating rejection of OVA-GFP expressing lymphomas. Given the increase in SIINFEKL-specific T cells after tumor inoculation, it is most likely that these CD8+ T cells also represent the effector cells mediating tumor rejection since SIINFEKL is the dominant peptide derived from OVA. This is also is supported by the notion that two different populations of OVA-specific cytotoxic CD8+ effector T cells mediate increased survival in the EL4 thymoma and the B16 melanoma models, one that secretes IFN-γ (McCabe et al., 1995) and one that secretes IL-4, IL-5, and IL-10 (Dobrzanski et al., 2000). Whether the rejection of ovalbumin bearing lymphomas is dependent only on CD8+ T cells or requires additional immune effector cells, e.g. CD4+ T and/or NK cells, needs further attention and studies to address these questions are under investigation.

4.4 Rejection requires the presence of IFN-γ and Stat1 signaling in the host

The biology of IFN-γ and its role in transplantation immunity, tumor development and in cancer immunoediting processes are well documented (Ikeda et al., 2002; Blankenstein and Qin, 2003). IFN-γ contributes to the rejection of transplantable tumors and the inhibition of MCA-induced carcinogenesis by different mechanisms. To study the role of IFN-γR in tumor rejection, Blankenstein and co-workers used MCA-induced fibrosarcomas generated from IFN-γR KO mice and reconstituted IFN-γR expression in these cells. These studies demonstrated that IFN-γ receptor expression on tumor cells is not necessary for the generation of anti-tumor immunity, neither during the priming nor the effector phase (Qin and Blankenstein, 2000). Schreiber and colleagues reported that endogenously produced IFN-γ forms the basis of the tumor surveillance system that controls both chemically induced and

spontaneously arising tumors in mice. Using tumor cells derived from MCA-treated IFN-γ unresponsive mice, the authors found that IFN-γ directly impinges on the immunogenicity of the tumor (Kaplan et al., 1998; Shankaran et al., 2001; Dunn et al., 2004). The importance of IFN-γ is emphasized by the finding that the lack of IFN-γ sensitivity predisposes the mice to enhanced tumor development (Shankaran et al., 2001). Therefore, one of the roles played by IFN-γ is at the level of the tumor cell, presumably by up-regulating the MHC molecules and/or antigen processing machinery. It is also important to note that in the human c-myc overexpressing cell line A1, interferon inducible components of the proteasome, namely lmp2, lmp7, PA28α, and PA28β, are significantly down-regulated as compared to the parental EREB2-5 cells. Furthermore, both components of the peptide transporter complex TAP1 and TAP2 are expressed at very low level in these cells indicating that c-myc overexpression impairs antigen processing and presentation which – in addition to downregulation of MHC, adhesion and costimulatory molecules - might contribute to the non-immunogenic phenotype of c-myc overexpressing cells (Staege et al., 2002). Consistent with this, the proteasomal composition is altered and the proteasomal activity decreased and shifted towards chymotrypsin-like substrates in these cells (Frisan et al., 1998; Gavioli et al., 2001).

Most of the experiments validating the cancer immunoediting hypothesis have been performed using MCA-induced chemical carcinogenesis. The finding that ovalbumin transduced lymphomas are rejected by the immune system raises the following questions: Firstly, which are the molecular mechanisms leading to the rejection of ovalbumin-expressing lymphomas, and secondly, what is the contribution of cells of the innate and of the adaptive immune system in tumor rejection? It is noteworthy that type I interferons are also involved in upregulation of MHC molecules. Recently, an important role of type I interferons has been demonstrated in the rejection of highly immunogenic unedited MCA-induced sarcomas which required IFN-α/β responsiveness at the level of hematopoetic cells (Dunn et al., 2005).
In order to address the possible contribution of interferon signaling in tumor rejection, we took advantage of both Stat1$^{-/-}$ and IFN-γ$^{-/-}$ recipient mice in tumor transfer experiments. We further dissected the cellular and molecular mechanisms behind OVA-GFP lymphoma rejection. Consistent with the previous findings, we found that transfer of OVA-GFP lymphoma cells in IFN-γ$^{-/-}$ and Stat1$^{-/-}$ recipient mice increased tumor growth and decreased overall survival, indicating that lymphoma rejection is associated with host IFN-γ production and Stat1 signaling. One potential reason for the failure of Stat1$^{-/-}$ mice to recognize ovalbumin transduced lymphomas was that antigen loss variants were selected (measured by

GFP expression). However, GFP expression and therefore ovalbumin expression was preserved in developing lymphomas from Stat1$^{-/-}$ and IFN-γ$^{-/-}$ mice. Thus, unlike in wild-type recipient mice, no selection of antigen-loss variants appeared to occur in Stat1$^{-/-}$ and IFN-γ$^{-/-}$ mice. This is consistent with a previous study in which Stat1$^{-/-}$ mice failed to reject immunogenic tumors and did not support regression of poorly immunogenic tumors when treated with an IL-12 based vaccine (Fallarino and Gajewski, 1999). The authors concluded that the lack of rejection was due to decreased production of IFN-γ from T cells of Stat1$^{-/-}$ mice and that these T cells lacked the cytolytic activity seen in wild-type mice. The authors reported furthermore, that NK cells are not functional in Stat1$^{-/-}$ mice. Lack of NK cell function may further contribute to the fast outgrowth of lymphomas inoculated into Stat1$^{-/-}$ mice, but this possibility has not been further pursued in this work.

To assess the possible reason for the failure to reject OVA-GFP expressing tumors in Stat1$^{-/-}$ and IFN-γ $^{-/-}$ mice, we evaluated expression of H-2K^{b} (MHC class I) and IA^{b} (MHC class II) in developing ovalbumin-transduced lymphomas from wild-type, Stat1$^{-/-}$ and IFN-γ$^{-/-}$ recipient mice. We found that H-2K^{b} and IA^{b} expression is increased only in developing lymphomas from wild-type but not from Stat1$^{-/-}$ and IFN-γ $^{-/-}$ recipient mice. This supports the notion that IFN-γ mediated upregulation of MHC class I and MHC class II molecules is associated with lymphoma rejection. A recent study has highlighted the importance of IFN-γ mediated upregulation of MHC for the rejection of a minor antigen-expressing tumor. Meunier et al. showed that injection of CD8+ T cells primed against immunodominant H7a minor H antigen could cure established melanomas in mice. These studies demonstrated IFN-γ released into the tumor is crucial for tumor rejection for two reasons: it increased MHC class I expression on tumor cells and inhibited angiogenesis (Meunier et al., 2005). Whether IFN-γ also suppresses angiogenesis in our tumor model needs further investigation. In our model, it is evident that upregulation of MHC expression in lymphoma cells is associated with tumor rejection. The importance of MHC class I upregulation for rejection of OVA-GFP expressing lymphomas in wild type mice was further supported by the finding that OVA-GFP expressing lymphomas exhibited consistently higher levels of MHC class I and II expression when analysed ex vivo as compared to the parental OVA-GFP expressing cells cultured in vitro. Importantly, MHC class I and II expression decreased to the initial level when the tumors were explanted and cultivated again in vitro. Upregulation of MHC class I and II expression could be recapitulated by addition of IFN-γ to cultured OVA-GFP expressing 291 lymphoma cells in vitro. Taken together, our data demonstrate that host IFN-γ and Stat1 signaling within

host immune cells play an important role in the rejection of foreign antigen expressing lymphomas.

Having demonstrated unequivocally that c-myc driven murine lymphomas expressing OVA-GFP or GFP are recognized and rejected by the adaptive immune system the question remains why murine lymphoma cells expressing foreign antigens are efficiently recognized by the adaptive immune system whereas Burkitt's lymphoma cells expressing Epstein-Barr viral antigens are not. The data from the murine in vivo model clearly show that rejection is associated with upregulation of MHC class I on the tumor cells in vivo. Murine lymphoma cells cultivated in vitro have furthermore retained the ability to respond to IFN-γ by upregulation of MHC molecules. In this regard it is interesting to note that human Burkitt's lymphoma cells have lost the ability to upregulate MHC class I molecules in response to IFN-γ. Many EBV-positive Burkitt's lymphoma lines have furthermore developed resistance to IFN-γ taking STAT1 tyrosine 701 phosphorylation as a readout. In many of these cell lines the molecular basis for this partial or complete IFN-γ resistance has been molecularly defined and found to be linked to JAK2 downregulation (Judith Mergler, Ph.D. thesis, 2008, Department of Biology of the LMU Munich). Downregulation of JAK2 has also been found to be a hallmark of the Burkitt's lymphoma gene expression signature in two studies comparing gene expression profiles of Burkitt´s lymphoma (BL) and diffuse large B cell lymphomas (Dave et al., 2006; Hummel et al., 2006). These findings suggest that loss of IFN-γ responsiveness is a prerequisite for development of lymphomas expressing foreign antigens. We will address this question experimentally by studying engraftment versus rejection of OVA-GFP expressing lymphomas that have either retained or lost the ability to respond to IFN-γ. To this end, λ-hu-cmyc transgenic mice are being crossed to IFNGR-/- mice.

4.5 Induction of antigen-specific CD8+ T cell tolerance in B cell lymphoma

The priming of tumor antigen-specific T cells is an important prerequisite for tumor rejection (Fallarino and Gajewski, 1999). It is known that adoptive transfer of antigen-specific T cells prior to tumor establishment can result in efficient tumor rejection (Cheever et al., 1980; Gattinoni et al., 2006). Studies from mouse models involving immunization against OVA antigen (Minev et al., 1994) or adoptive transfer of CD8+ T cells directed against ovalbumin (McCabe et al., 1995) indicated that the transfer of ovalbumin specific CD8 + T cells results in significant reduction of tumor burden in mice. We observed, however, in our tumor model that ovalbumin-transduced lymphoma cells growing in $Stat1^{-/-}$ mice were not affected by the adoptive transfer of either naïve or primed antigen-specific OT-I cells. The finding that

transfer of either naïve or primed antigen-specific OT-I CD8+ T cells failed to induce protective immune response against ovalbumin-transduced lymphoma cells was surprising. Considering the experimental details, it is important to note that the number of transferred primed OT-I CD8+ T cells was 10-fold higher then the injected tumor cells. This finding strongly suggests that the tumor is able to induce T cell anergy or tolerance. Additional experiments in our model provided evidence that a large population of transferred primed OT-I T cells persisted in vivo whereas naive OT-I T cells did not. Paradoxically, the presence of transferred OT-I CD8+ T cells did not lead to tumor rejection in Stat1$^{-/-}$ recipients. Similar results have been reported in the A20 B cell lymphoma and in a metastatic melanoma model. Naïve CD4+ T cells specific for an antigen expressed by the tumor cells were rendered anergic early during tumorigenesis in the A20 B cell lymphoma model (Staveley-O'Carroll et al., 1998). Likewise, transfer of high numbers of antigen-specific CD8+ T cells into the tumor-bearing host did not induce an efficient immune response against the tumor indicating that transfer of antigen specific CD8+ T cells alone is not enough to induce anti-tumor immunity (Overwijk et al., 2003). An alternative explanation for these findings is the induction of antigen-specific T cell unresponsiveness early in the course of tumor-T cell interactions.

A similar state of T cell anergy has been demonstrated in animals and patients harboring large tumor burdens as demonstrated by the impaired response against recall antigens in vivo. The identification of antigen-specific T cell anergy as an early event in tumor progression has clear implications for the future development of cancer immunotherapy. For the design of efficacious immunotherapeutic approaches it remains a major challenge to identify the molecular basis of lymphoma-induced T cell anergy and to develop strategies to restore T cell responsiveness.

4.6 Generation of Stat1-deficient B cell lymphomas

In the next part of our studies, we focussed on lymphoma cells that are devoid of Stat1, a key protein in IFN signaling. Experiments performed in different experimental models have provided evidence that tumors are the targets of IFN-γ and have validated the concept of IFN-γ as an extrinsic tumor suppressor (Kaplan et al., 1998; Shankaran et al., 2001). It has, however, remained open whether the tumor cells themselves or host cells are the targets of IFN-γ mediating an antitumoral effect. IFN-γ insensitive but not IFN-responsive Meth A tumors grew progressively when transferred into syngeneic recipients (Dighe et al., 1994) suggesting that the increased tumorigenicity of IFN-γ insensitive Meth A tumor cells is due to

decreased immunogenicity of the tumor cells. Of note, IFN-γ insensitive Meth A tumors failed to prime anti-Meth A immunity in naïve mice and were not rejected in mice that had been vaccinated with wild type tumor cells (Dighe et al., 1994). These studies highlighted that tumor cells are targets of IFN-γ. However, studies by several groups have stressed the point that not the tumor cells, but host stroma or phagocytic cells, are the important target cells for IFN-γ to mediate an antitumor response (Mumberg et al., 1999; Pulaski et al., 2002; Segal et al., 2002; Qin et al., 2003). Surprisingly, the question of whether tumors are targets of interferons has been addressed almost exclusively in MCA induced carcinogenesis models and not in oncogene induced tumor settings.

Since type I as well as type II interferons can upregulate MHC class I and class II and thereby augment tumor immunogenicity, we have addressed (i) whether *c-myc* over-expressing high grade lymphomas are direct targets of IFNs, and (ii) whether Stat1 competence is a prerequisite for rejection of OVA-GFP expressing lymphoma cells. To this end, we have crossed the λ-hu-c-myc transgene onto Stat1$^{-/-}$ mice and established Stat1-deficient c-myc-induced lymphomas. Lymphomas developed slightly faster on Stat1-deficient as compared to wild-type background, and the effect appeared to be significant.
To study the role of Stat1-deficiency exclusively at the level of the tumor cell and not the host, Stat1-competent (IFN responsive) and Stat1-deficient lymphoma cell lines (IFN unresponsive) were established from lymphoma bearing mice, in which both, the type I and type II interferon signaling pathways are either functional or abrogated. As expected, treatment of Stat1$^{-/-}$ lymphoma cells with IFN-γ did not increase MHC class I and MHC class II expression *in vitro*.

4.7 Stat1-deficient B cell lymphomas are rejected in wild type recipient mice and this rejection is antigen independent

If rejection of an antigen expressing tumor requires IFN-responsiveness of the tumor cell, the transfer of OVA-GFP expressing Stat1$^{-/-}$ lymphomas into wild-type mice should lead to accelerated tumor growth. To test this hypothesis, Stat1-competent cells (cell line 291) and Stat1-deficient cells (line 9) were transduced with OVA-GFP and injected into UBQ-GFP transgenic recipient mice. Paradoxically, antigen expressing Stat1-deficient cells were efficiently rejected in wild-type mice and, upon longer follow up, no antigen loss variants grew out. This unexpected finding raised two questions: Firstly, is the rejection of Stat-1-

deficient lymphoma cells antigen-dependent? And secondly, what is the mechanism responsible for the rejection of IFN unresponsive lymphomas? To address the first question, GFP-transduced Stat1-deficient lymphoma cells were inoculated into UBQ-GFP transgenic mice and equally rejected in the same manner as the OVA-GFP expressing lymphoma cells. These results imply that the rejection of Stat1-deficient lymphoma cells in UBQ-GFP transgenic mice is antigen-independent. As Stat1-competent lymphomas form tumors in the same host, and Stat1-deficient lymphomas are rejected, Stat1 may be regarded as an oncogene in this experimental setting.

In a recent study Kovacic et al. came to a similar conclusion using two different murine myeloid leukemia models. These authors demonstrated that lack of Stat1 decreased v-abl- or Jak2/Tel-induced myeloid leukemogenesis and provided evidence that retrovirally transduced $Stat1^{-/-}$ cells, but not $Stat1^{+/-}$ cells, have been efficiently eliminated in immunodeficient $Rag2^{-/-}$ mice by NK cells. The authors demonstrated that efficient NK cell lysis and tumor clearance is dependent on low MHC class I protein levels of $Stat1^{-/-}$ tumor cells and can be efficiently inhibited by exogenous MHC class I expression(Kovacic et al., 2006).

The similarities between the two experimental systems strongly suggests that rejection of OVA-GFP or GFP-expressing $Stat1^{-/-}$ lymphoma cells in wild type or UBQ-GFP transgenic mice is mediated by NK cell lysis.

4.8 Rejection of Stat1-deficient B cell lymphomas is dependent upon IFN-γ

We next addressed the question whether Stat1-deficient lymphoma cells have lost their oncogenic potential or whether they have remained oncogenic when inoculated into $Stat1^{-/-}$ and IFN-$\gamma^{-/-}$ recipient mice. Inoculation of Stat1-deficient lymphoma cells in $Stat1^{-/-}$ and IFN-$\gamma^{-/-}$ recipient mice resulted in efficient and rapid tumor formation. This ruled out the possibility that Stat1 deficient cells engrafted less efficiently in vivo. Rather, Stat1 deficient cells are targets for immune attack. Importantly, both the rejection of OVA-GFP expressing Stat1 competent lymphomas in an antigen-dependent manner and rejection of Stat1 deficient lymphoma cells in an antigen-independent manner required host IFN-γ signaling. This supports the notion of an extrinsic tumor suppressor function of IFN-γ. Mice with disruptions in genes encoding proteins that play a critical role in either IFN-γ receptor signaling (IFNGR1 or Stat1) or lymphocyte development (Rag2) displayed increased incidence and kinetics of MCA-dependent tumor induction compared to wild-type counterparts (Shankaran et al., 2001). Taken together, our results revealed that IFN-γ played an indispensable role in tumor rejection. Although the concept of IFN-γ mediated tumor immunosurveillance is widely

accepted, it is still not known which cells are responsible for producing IFN-γ during the anti-tumoral immune response. Different immune cells, specifically NKT cells, γδ T cells and NK cells may be the first to recognize the developing tumors. Many of these cells involved in innate immunity can also produce higher amounts of IFN-γ during early recognition before tumor recognition by the adaptive immune system is initiated.

The finding that Stat1-deficient lymphomas are rejected in an antigen-independent manner in wild type mice raised two important questions: Firstly, are Stat1-deficient lymphoma cells direct targets for NK cell lysis? And secondly, is there a role for NK cells in the development of spontaneous lymphomas in wild type mice?

4.9 Lymphomas are sensitive to NK-cell mediated cytolysis in vitro, but IFN-γ decreases NK cell sensitivity through induction of MHC-class I expression on lymphoma targets

The finding that Stat1-deficient cells are unresponsive to IFN-γ, fail to induce MHC class I expression and are rejected in wild type mice gave the first indication that NK cells might play an important role in tumor surveillance in our experimental model system. This conclusion was strongly supported by the recent data of Kovacic et al. who showed that Stat1$^{-/-}$ leukemic cells are cleared by NK cells due to low MHC class I expression (Kovacic et al., 2006). This raised the possibility that NK cells might play a role also in the immunosurveillance of spontaneously arising lymphomas in λ-hu-cmyc transgenic mice. The following points support this notion: (i) c-MYC strongly downregulates MHC class I expression, (ii) MHC class I expression appears to be higher on tumor cells ex vivo than on the parental cells in culture that have been inoculated to generate the tumor.

We therefore asked whether Stat1-competent and Stat1-deficient cells can be directly recognized by NK cells in vitro, and if so, whether and how cytolysis is affected by IFN-γ treatment. We showed that Stat1-competent as well as Stat1-deficient lymphoma cells were direct targets of NK cells and could be lysed efficiently in vitro. IFN-γ treatment increased MHC expression and decreased NK cell mediated lysis of Stat1-competent lymphoma cells in vitro, whereas IFN-γ - without affecting MHC class I expression on Stat1-deficient tumor cells - increased the lysis of these cells, by either activating the cytotoxic potential of NK cells, by inducing the expression of NK activating ligands on the tumor cells or both. These results suggest that NK cells may play an important role in the rejection of Stat1-competent as well as of Stat1-deficient lymphoma cells not only *in vitro* but also *in vivo*. IFN-γ might play a dual role in this experimental setting depending on whether the tumor expresses foreign antigens and is immunogenic or not. If the tumor is immunogenic, upregulation of MHC class

I molecules on Stat1-competent lymphomas by IFN-γ might facilitate the recognition and rejection of the lymphoma cells by the adaptive arm of the immune system in wild-type mice. If the tumor cells are not immunogenic, they might be initially recognized by NK cells. The IFN-γ produced by activated NK cells might upregulate MHC class I expression on remaining tumor cells in a paracrine fashion thus protecting the tumor cells from further NK cell attack. Such a model is compatible with the finding that (i) Stat1-competent lymphoma cells are recognized by NK cells *in vitro*, although they form tumors efficiently *in vivo*, and (ii) that Stat1 deletion does not dramatically accelerate tumor development in vivo although the NK barrier is missing. A testable prediction of this concept would be that λ-hu-myc transgenic lymphoma cells might be less oncogenic in IFN-$\gamma^{-/-}$ than in wild type mice.

4.10 NK cell-dependent innate immunosurveillance in B cell lymphomas in vivo

The concept of cancer immunoediting has mostly focused on the adaptive arm of immune system. A potential involvement of innate immune cells has only recently emerged. Studies performed using B cell lymphomas spontaneously arising in immunocompromised mice (IFN-γ-, β2-microglobulin and/or perforin deficient) have shown that the rejection of the tumors is mediated by NK and γδ T cells (Street et al., 2002; Street et al., 2004). Most importantly, depletion of either CD4+ T cells or CD8+ T cells or both populations in wild-type mice did not prevent the rejection of spontaneously arising B cell lymphomas (Street et al., 2002). These elegant studies have demonstrated the potential involvement of NK cells in tumor surveillance. A more recent study has shown that low expression of MHC class I on Stat1$^{-/-}$ tumor cells causes efficient lysis and enhanced clearance of Stat1$^{-/-}$ leukemias by NK cells (Kovacic et al., 2006). To demonstrate that rejection of Stat1$^{-/-}$ leukemic cells depended on NK cells, the authors increased MHC class I levels on tumor cells by retroviral transduction and found that the tumor cells became resistant to NK mediated rejection. As a consequence, Stat1$^{-/-}$ tumor cells transduced with MHC class I gave rise to leukemic disease that progressed almost as rapidly as Stat1-expressing tumor cells (Kovacic et al., 2006). This observation is also consistent with earlier studies showing that B cell malignancies are predominantly under NK cell-mediated innate immunosurveillance (Cerwenka et al., 2000; Stoiber et al., 2004; Street et al., 2004).

The susceptibility of tumor cells to NK cell-mediated killing is thought to be determined by the relative expression of ligands for NK-activating and inhibitory receptors (Vivier and Anfossi, 2004; Vivier et al., 2004). NKG2D is an activating receptor expressed on human and

mouse NK cells that recognizes the Rae-1 family of proteins, H60 and MULT1 in mice, and MICA proteins in humans (Cerwenka et al., 2001; Jamieson et al., 2002; Raulet, 2003). NKG2D ligands are not expressed on the surface of normal cells but are upregulated on many primary tumors, many tumor cell lines and some carcinogen-induced tumors (Diefenbach et al., 2000). A hallmark of many tumor cells is genomic instability and one pathway that has been implicated in the induction of these genes is the DNA-damage response (Gasser et al., 2005). Consistent with these findings, transfected tumor cell lines expressing NKG2D ligands were rejected in vivo in an NKG2D-dependent manner (Cerwenka et al., 2001; Diefenbach et al., 2001; Diefenbach and Raulet, 2001). However, examples in which tumors have evaded NKG2D surveillance have also been described (Groh et al., 2002; Coudert et al., 2005; Oppenheim et al., 2005).

A recent study has demonstrated that NKG2D-mediated immunoediting occurs in c-myc overexpressing B cell lymphomas (Gasser et al., 2005). This study has provided genetic evidence for the recognition of primary tumors by NK cells. Using transgenic NKG2D-deficient TRAMP mice, Guerra et al. demonstrated that NKG2D plays an important role in tumor immunosurveillance in a model of prostate adenocarcinoma and in Eμ-myc transgenic mice. Oncogene-induced stress as a result of c-myc overexpression is known to activate the DNA-damage response pathway (Dominguez-Sola et al., 2007; Reimann et al., 2007), which might cause up-regulation of NKG2D ligands during early tumorigenesis and may cause recognition of developing tumor cells in a NKG2D-dependent fashion. Another study has provided evidence that NKG2D ligands are induced on spontaneously arising tumors in a murine model of lymphomagenesis and that c-myc is involved in this up-regulation (Unni et al., 2008). Thus, the above two recent studies suggest that up-regulation of NKG2D ligands by oncogenic transformation sensitizes the cells for immune recognition by NK cells.

4.11 How is the NK cell barrier overcome in naturally arising tumors?

If NK cells indeed do play such an important role, the question remains how this barrier is overcome in the course of spontaneously arising tumors, including those developing in Eμ-myc or λ-hu-c-myc transgenic mice. One apparent mechanism appears to be that constitutive expression of NK activating ligands on tumor cells will induce downregulation of NKG2D receptors on NK cells and will thus lead to desensitization of NK cells (Groh et al., 2002; Oppenheim et al., 2005; Coudert et al., 2005, 2008; Unni et al., 2008). Interestingly, although constitutive encounter of activating ligands significantly impaired the cytotoxicity of NK cells in the NK-sensitive RMA-S T cell lymphoma model, it led to constitutive IFNγ production of NK cells (Coudert et al., 2008).

Our data suggested an important role for STAT1 not only in T cell-mediated rejection of tumors expressing foreign antigens, STAT1 acted also as an oncogene and was required for outgrowth of a tumor in an immunocompetent host. The finding that STAT1-deficient lymphomas were rejected in an antigen-independent manner strongly suggested an important role for NK cells in this experimental setting. Yet, as STAT1-competent lymphomas were not rejected in wild type recipients, a possible role of NK cells in the control of STAT1-competent lymphomas was much less evident and has emerged only recently when STAT1 – deficient and STAT1-competent lypmphomas were compared. Outgrowth of STAT1-competent lymphomas was associated with MHC class I upregulation in vivo. It is tempting to speculate that increased MHC class I expression has been selected for in vivo to inactivate NK cells. It will be important to repeat these tumor inoculation experiments with Rag1-/- mice that lack B and T cells but harbor functional NK cells, and after inoculation into wild type mice in which distinct cell populations like e.g. NK cells have been deleted by monoclonal antibodies.

It will be particularly interesting to address te question whether constitutive upregulation of MHC class I in λ-hu-c-myc transgenic mice will accelerate spontaneous lymphoma development. Likewise, it will be most interesting to see whether host IFNγ is required for outgrowth of STAT1-competent lymphomas by increasing MHC class I expression on the tumor cells above a critical threshold that is required to inactivate NK cells. To address these questions two reciprocal experimental approaches will be taken: (i) inoculation of STAT1-competent lymphomas into IFNγ-/- recipient mice and (ii) conversely, inoculation of IFNγ-unresponsive (IFNGR-/-) lymphomas into wild type recipient mice. Likewise, spontaneous tumor development will be monitored in λ-hu-c-myc transgenic mice that lack either IFNγ or the IFNγ receptor.

5. References

Abril, E., Real, L.M., Serrano, A., Jimenez, P., Garcia, A., Canton, J., Trigo, I., Garrido, F. and Ruiz-Cabello, F. Unresponsiveness to interferon associated with STAT1 protein deficiency in a gastric adenocarcinoma cell line. *Cancer Immunol Immunother* **47** (1998), pp. 113-20.

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D. and Brinster, R.L. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318** (1985), pp. 533-8.

Aran, J.M., Gottesman, M.M. and Pastan, I. Construction and characterization of bicistronic retroviral vectors encoding the multidrug transporter and beta-galactosidase or green fluorescent protein. *Cancer Gene Ther* **5** (1998), pp. 195-206.

Badgwell, B., Lesinski, G.B., Magro, C., Abood, G., Skaf, A. and Carson, W., 3rd The antitumor effects of interferon-alpha are maintained in mice challenged with a STAT1-deficient murine melanoma cell line. *J Surg Res* **116** (2004), pp. 129-36.

Barth, R.J., Jr., Mule, J.J., Spiess, P.J. and Rosenberg, S.A. Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8+ tumor-infiltrating lymphocytes. *J Exp Med* **173** (1991), pp. 647-58.

Becker, C., Pohla, H., Frankenberger, B., Schuler, T., Assenmacher, M., Schendel, D.J. and Blankenstein, T. Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. *Nat Med* **7** (2001), pp. 1159-62.

Billaud, M., Rousset, F., Calender, A., Cordier, M., Aubry, J.P., Laisse, V. and Lenoir, G.M. Low expression of lymphocyte function-associated antigen (LFA)-1 and LFA-3 adhesion molecules is a common trait in Burkitt's lymphoma associated with and not associated with Epstein-Barr virus. *Blood* **75** (1990), pp. 1827-33.

Blankenstein, T. and Qin, Z. Chemical carcinogens as foreign bodies and some pitfalls regarding cancer immune surveillance. *Adv Cancer Res* **90** (2003), pp. 179-207.

Boehm, U., Klamp, T., Groot, M. and Howard, J.C. Cellular responses to interferon-gamma. *Annu Rev Immunol* **15** (1997), pp. 749-95.

Bowman, T., Garcia, R., Turkson, J. and Jove, R. STATs in oncogenesis. *Oncogene* **19** (2000), pp. 2474-88.

Boxer, L.M. and Dang, C.V. Translocations involving c-myc and c-myc function. *Oncogene* **20** (2001), pp. 5595-610.

Bromberg, J. and Darnell, J.E., Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* **19** (2000), pp. 2468-73.

Capello, D. and Gaidano, G. Molecular pathophysiology of indolent lymphoma. *Haematologica* **85** (2000), pp. 195-201.

Cerwenka, A., Bakker, A.B., McClanahan, T., Wagner, J., Wu, J., Phillips, J.H. and Lanier, L.L. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* **12** (2000), pp. 721-7.

Cerwenka, A., Baron, J.L. and Lanier, L.L. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A* **98** (2001), pp. 11521-6.

Cheever, M.A., Greenberg, P.D. and Fefer, A. Specificity of adoptive chemoimmunotherapy of established syngeneic tumors. *J Immunol* **125** (1980), pp. 711-4.

Coudert, J.D., Zimmer, J., Tomasello, E., Cebecauer, M., Colonna, M., Vivier, E. and Held, W. Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells. *Blood* **106** (2005), pp. 1711-7.

Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C. and Croce, C.M. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci U S A* **79** (1982), pp. 7824-7.

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A. and Stewart, T.A. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* **259** (1993), pp. 1739-42.

Dang, C.V. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol* **19** (1999), pp. 1-11.

Darnell, J.E., Jr., Kerr, I.M. and Stark, G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264** (1994), pp. 1415-21.

Dave, S.S., Fu, K., Wright, G.W., Lam, L.T., Kluin, P., Boerma, E.J., Greiner, T.C., Weisenburger, D.D., Rosenwald, A., Ott, G., Muller-Hermelink, H.K., Gascoyne, R.D., Delabie, J., Rimsza, L.M., Braziel, R.M., Grogan, T.M., Campo, E., Jaffe, E.S., Dave, B.J., Sanger, W., Bast, M., Vose, J.M., Armitage, J.O., Connors, J.M., Smeland, E.B., Kvaloy, S., Holte, H., Fisher, R.I., Miller, T.P., Montserrat, E., Wilson, W.H., Bahl, M., Zhao, H., Yang, L., Powell, J., Simon, R., Chan, W.C. and Staudt, L.M. Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* **354** (2006), pp. 2431-42.

Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R. and Bradley, A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev* **7** (1993), pp. 671-82.

Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N. and Raulet, D.H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* **1** (2000), pp. 119-26.

Diefenbach, A., Jensen, E.R., Jamieson, A.M. and Raulet, D.H. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* **413** (2001), pp. 165-71.

Diefenbach, A. and Raulet, D.H. Strategies for target cell recognition by natural killer cells. *Immunol Rev* **181** (2001), pp. 170-84.

Dighe, A.S., Richards, E., Old, L.J. and Schreiber, R.D. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* **1** (1994), pp. 447-56.

Dobrzanski, M.J., Reome, J.B. and Dutton, R.W. Therapeutic effects of tumor-reactive type 1 and type 2 CD8+ T cell subpopulations in established pulmonary metastases. *J Immunol* **162** (1999), pp. 6671-80.

Dobrzanski, M.J., Reome, J.B. and Dutton, R.W. Type 1 and type 2 CD8+ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. *J Immunol* **164** (2000), pp. 916-25.

Dobrzanski, M.J., Reome, J.B., Hollenbaugh, J.A. and Dutton, R.W. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. *J Immunol* **172** (2004), pp. 1380-90.

Dominguez-Sola, D., Ying, C.Y., Grandori, C., Ruggiero, L., Chen, B., Li, M., Galloway, D.A., Gu, W., Gautier, J. and Dalla-Favera, R. Non-transcriptional control of DNA replication by c-Myc. *Nature* **448** (2007), pp. 445-51.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. and Schreiber, R.D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3** (2002), pp. 991-8.

Dunn, G.P., Bruce, A.T., Sheehan, K.C., Shankaran, V., Uppaluri, R., Bui, J.D., Diamond, M.S., Koebel, C.M., Arthur, C., White, J.M. and Schreiber, R.D. A critical function for type I interferons in cancer immunoediting. *Nat Immunol* **6** (2005), pp. 722-9.

Dunn, G.P., Koebel, C.M. and Schreiber, R.D. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* **6** (2006), pp. 836-48.

Dunn, G.P., Old, L.J. and Schreiber, R.D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21** (2004), pp. 137-48.

Durbin, J.E., Hackenmiller, R., Simon, M.C. and Levy, D.E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84** (1996), pp. 443-50.

Fallarino, F. and Gajewski, T.F. Cutting edge: differentiation of antitumor CTL in vivo requires host expression of Stat1. *J Immunol* **163** (1999), pp. 4109-13.

Farrar, M.A. and Schreiber, R.D. The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol* **11** (1993), pp. 571-611.

Frisan, T., Levitsky, V., Polack, A. and Masucci, M.G. Phenotype-dependent differences in proteasome subunit composition and cleavage specificity in B cell lines. *J Immunol* **160** (1998), pp. 3281-9.

Gasser, S., Orsulic, S., Brown, E.J. and Raulet, D.H. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* **436** (2005), pp. 1186-90.

Gattinoni, L., Powell, D.J., Jr., Rosenberg, S.A. and Restifo, N.P. Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol* **6** (2006), pp. 383-93.

Gavioli, R., Frisan, T., Vertuani, S., Bornkamm, G.W. and Masucci, M.G. c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. *Nat Cell Biol* **3** (2001), pp. 283-8.

Gerbitz, A., Mautner, J., Geltinger, C., Hortnagel, K., Christoph, B., Asenbauer, H., Klobeck, G., Polack, A. and Bornkamm, G.W. Deregulation of the proto-oncogene c-myc through t(8;22) translocation in Burkitt's lymphoma. *Oncogene* **18** (1999), pp. 1745-53.

Gerlini, G., Tun-Kyi, A., Dudli, C., Burg, G., Pimpinelli, N. and Nestle, F.O. Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. *Am J Pathol* **165** (2004), pp. 1853-63.

Germain, R.N. and Margulies, D.H. The biochemistry and cell biology of antigen processing and presentation. *Annu Rev Immunol* **11** (1993), pp. 403-50.

Gorelik, L. and Flavell, R.A. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* **7** (2001), pp. 1118-22.

Gregory, C.D., Murray, R.J., Edwards, C.F. and Rickinson, A.B. Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's

lymphoma underlies tumor cell escape from virus-specific T cell surveillance. *J Exp Med* **167** (1988), pp. 1811-24.

Groh, V., Wu, J., Yee, C. and Spies, T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* **419** (2002), pp. 734-8.

Haque, S.J. and Williams, B.R. Signal transduction in the interferon system. *Semin Oncol* **25** (1998), pp. 14-22.

Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Vardiman, J., Lister, T.A. and Bloomfield, C.D. The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. *Ann Oncol* **10** (1999), pp. 1419-32.

Hollenbaugh, J.A., Reome, J., Dobrzanski, M. and Dutton, R.W. The rate of the CD8-dependent initial reduction in tumor volume is not limited by contact-dependent perforin, Fas ligand, or TNF-mediated cytolysis. *J Immunol* **173** (2004), pp. 1738-43.

Huang, S., Bucana, C.D., Van Arsdall, M. and Fidler, I.J. Stat1 negatively regulates angiogenesis, tumorigenicity and metastasis of tumor cells. *Oncogene* **21** (2002), pp. 2504-12.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M. and Aguet, M. Immune response in mice that lack the interferon-gamma receptor. *Science* **259** (1993), pp. 1742-5.

Hummel, M., Bentink, S., Berger, H., Klapper, W., Wessendorf, S., Barth, T.F., Bernd, H.W., Cogliatti, S.B., Dierlamm, J., Feller, A.C., Hansmann, M.L., Haralambieva, E., Harder, L., Hasenclever, D., Kuhn, M., Lenze, D., Lichter, P., Martin-Subero, J.I., Moller, P., Muller-Hermelink, H.K., Ott, G., Parwaresch, R.M., Pott, C., Rosenwald, A., Rosolowski, M., Schwaenen, C., Sturzenhofecker, B., Szczepanowski, M., Trautmann, H., Wacker, H.H., Spang, R., Loeffler, M., Trumper, L., Stein, H. and Siebert, R. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N Engl J Med* **354** (2006), pp. 2419-30.

Iida, S., Rao, P.H., Nallasivam, P., Hibshoosh, H., Butler, M., Louie, D.C., Dyomin, V., Ohno, H., Chaganti, R.S. and Dalla-Favera, R. The t(9;14)(p13;q32) chromosomal translocation associated with lymphoplasmacytoid lymphoma involves the PAX-5 gene. *Blood* **88** (1996), pp. 4110-7.

Ikeda, H., Old, L.J. and Schreiber, R.D. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev* **13** (2002), pp. 95-109.

Imreh, M.P., Zhang, Q.J., de Campos-Lima, P.O., Imreh, S., Krausa, P., Browning, M., Klein, G. and Masucci, M.G. Mechanisms of allele-selective down-regulation of HLA class I in Burkitt's lymphoma. *Int J Cancer* **62** (1995), pp. 90-6.

Jamieson, A.M., Diefenbach, A., McMahon, C.W., Xiong, N., Carlyle, J.R. and Raulet, D.H. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17** (2002), pp. 19-29.

Kalachikov, S., Migliazza, A., Cayanis, E., Fracchiolla, N.S., Bonaldo, M.F., Lawton, L., Jelenc, P., Ye, X., Qu, X., Chien, M., Hauptschein, R., Gaidano, G., Vitolo, U., Saglio, G., Resegotti, L., Brodjansky, V., Yankovsky, N., Zhang, P., Soares, M.B., Russo, J., Edelman, I.S., Efstratiadis, A., Dalla-Favera, R. and Fischer, S.G. Cloning and gene mapping of the chromosome 13q14 region deleted in chronic lymphocytic leukemia. *Genomics* **42** (1997), pp. 369-77.

Kaplan, D.H., Shankaran, V., Dighe, A.S., Stockert, E., Aguet, M., Old, L.J. and Schreiber, R.D. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A* **95** (1998), pp. 7556-61.

Kelly, G.L. and Rickinson, A.B. Burkitt lymphoma: revisiting the pathogenesis of a virus-associated malignancy. *Hematology Am Soc Hematol Educ Program* **2007** (2007), pp. 277-84.

Kempkes, B., Spitkovsky, D., Jansen-Durr, P., Ellwart, J.W., Kremmer, E., Delecluse, H.J., Rottenberger, C., Bornkamm, G.W. and Hammerschmidt, W. B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2. *EMBO J* **14** (1995), pp. 88-96.

Klein, G. Tumor immunology. *Transplant Proc* **5** (1973), pp. 31-41.

Koebel, C.M., Vermi, W., Swann, J.B., Zerafa, N., Rodig, S.J., Old, L.J., Smyth, M.J. and Schreiber, R.D. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* **450** (2007), pp. 903-7.

Kovacic, B., Stoiber, D., Moriggl, R., Weisz, E., Ott, R.G., Kreibich, R., Levy, D.E., Beug, H., Freissmuth, M. and Sexl, V. STAT1 acts as a tumor promoter for leukemia development. *Cancer Cell* **10** (2006), pp. 77-87.

Kovalchuk, A.L., Qi, C.F., Torrey, T.A., Taddesse-Heath, L., Feigenbaum, L., Park, S.S., Gerbitz, A., Klobeck, G., Hoertnagel, K., Polack, A., Bornkamm, G.W., Janz, S. and Morse, H.C., 3rd Burkitt lymphoma in the mouse. *J Exp Med* **192** (2000), pp. 1183-90.

Kurte, M., Lopez, M., Aguirre, A., Escobar, A., Aguillon, J.C., Charo, J., Larsen, C.G., Kiessling, R. and Salazar-Onfray, F. A synthetic peptide homologous to functional domain of human IL-10 down-regulates expression of MHC class I and Transporter associated with Antigen Processing 1/2 in human melanoma cells. *J Immunol* **173** (2004), pp. 1731-7.

Kurts, C., Heath, W.R., Carbone, F.R., Allison, J., Miller, J.F. and Kosaka, H. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* **184** (1996), pp. 923-30.

Lee, P.P., Yee, C., Savage, P.A., Fong, L., Brockstedt, D., Weber, J.S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P.D., Roederer, M. and Davis, M.M. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med* **5** (1999), pp. 677-85.

Lengauer, C., Kinzler, K.W. and Vogelstein, B. Genetic instabilities in human cancers. *Nature* **396** (1998), pp. 643-9.

Leonard, W.J. and O'Shea, J.J. Jaks and STATs: biological implications. *Annu Rev Immunol* **16** (1998), pp. 293-322.

Lesinski, G.B., Anghelina, M., Zimmerer, J., Bakalakos, T., Badgwell, B., Parihar, R., Hu, Y., Becknell, B., Abood, G., Chaudhury, A.R., Magro, C., Durbin, J. and Carson, W.E., 3rd The antitumor effects of IFN-alpha are abrogated in a STAT1-deficient mouse. *J Clin Invest* **112** (2003), pp. 170-80.

Levenson, V.V., Transue, E.D. and Roninson, I.B. Internal ribosomal entry site-containing retroviral vectors with green fluorescent protein and drug resistance markers. *Hum Gene Ther* **9** (1998), pp. 1233-6.

Levine, E.G., Arthur, D.C., Machnicki, J., Frizzera, G., Hurd, D., Peterson, B., Gajl-Peczalska, K.J. and Bloomfield, C.D. Four new recurring translocations in non-Hodgkin lymphoma. *Blood* **74** (1989), pp. 1796-800.

Levy, D.E. and Darnell, J.E., Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* **3** (2002), pp. 651-62.

Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K. and Flavell, R.A. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24** (2006), pp. 99-146.

Lo Coco, F., Ye, B.H., Lista, F., Corradini, P., Offit, K., Knowles, D.M., Chaganti, R.S. and Dalla-Favera, R. Rearrangements of the BCL6 gene in diffuse large cell non-Hodgkin's lymphoma. *Blood* **83** (1994), pp. 1757-9.

Lowe, S.W., Cepero, E. and Evan, G. Intrinsic tumour suppression. *Nature* **432** (2004), pp. 307-15.

Lu, B., Ebensperger, C., Dembic, Z., Wang, Y., Kvatyuk, M., Lu, T., Coffman, R.L., Pestka, S. and Rothman, P.B. Targeted disruption of the interferon-gamma receptor 2 gene results in severe immune defects in mice. *Proc Natl Acad Sci U S A* **95** (1998), pp. 8233-8.

Marincola, F.M., Jaffee, E.M., Hicklin, D.J. and Ferrone, S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* **74** (2000), pp. 181-273.

McCabe, B.J., Irvine, K.R., Nishimura, M.I., Yang, J.C., Spiess, P.J., Shulman, E.P., Rosenberg, S.A. and Restifo, N.P. Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T lymphocyte responses. *Cancer Res* **55** (1995), pp. 1741-7.

Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., Carver-Moore, K., DuBois, R.N., Clark, R., Aguet, M. and Schreiber, R.D. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84** (1996), pp. 431-42.

Meunier, M.C., Delisle, J.S., Bergeron, J., Rineau, V., Baron, C. and Perreault, C. T cells targeted against a single minor histocompatibility antigen can cure solid tumors. *Nat Med* **11** (2005), pp. 1222-9.

Minev, B.R., McFarland, B.J., Spiess, P.J., Rosenberg, S.A. and Restifo, N.P. Insertion signal sequence fused to minimal peptides elicits specific CD8+ T-cell responses and prolongs survival of thymoma-bearing mice. *Cancer Res* **54** (1994), pp. 4155-61.

Morgan, D.J., Kurts, C., Kreuwel, H.T., Holst, K.L., Heath, W.R. and Sherman, L.A. Ontogeny of T cell tolerance to peripherally expressed antigens. *Proc Natl Acad Sci U S A* **96** (1999), pp. 3854-8.

Mumberg, D., Monach, P.A., Wanderling, S., Philip, M., Toledano, A.Y., Schreiber, R.D. and Schreiber, H. CD4(+) T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-gamma. *Proc Natl Acad Sci U S A* **96** (1999), pp. 8633-8.

Oppenheim, D.E., Roberts, S.J., Clarke, S.L., Filler, R., Lewis, J.M., Tigelaar, R.E., Girardi, M. and Hayday, A.C. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol* **6** (2005), pp. 928-37.

Otten, G.R. and Germain, R.N. Split anergy in a CD8+ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. *Science* **251** (1991), pp. 1228-31.

Overwijk, W.W., Theoret, M.R., Finkelstein, S.E., Surman, D.R., de Jong, L.A., Vyth-Dreese, F.A., Dellemijn, T.A., Antony, P.A., Spiess, P.J., Palmer, D.C., Heimann, D.M., Klebanoff, C.A., Yu, Z., Hwang, L.N., Feigenbaum, L., Kruisbeek, A.M., Rosenberg, S.A. and Restifo, N.P. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* **198** (2003), pp. 569-80.

Pajic, A., Spitkovsky, D., Christoph, B., Kempkes, B., Schuhmacher, M., Staege, M.S., Brielmeier, M., Ellwart, J., Kohlhuber, F., Bornkamm, G.W., Polack, A. and Eick, D. Cell cycle activation by c-myc in a burkitt lymphoma model cell line. *Int J Cancer* **87** (2000), pp. 787-93.

Pansky, A., Hildebrand, P., Fasler-Kan, E., Baselgia, L., Ketterer, S., Beglinger, C. and Heim, M.H. Defective Jak-STAT signal transduction pathway in melanoma cells resistant to growth inhibition by interferon-alpha. *Int J Cancer* **85** (2000), pp. 720-5.

Pestka, S., Langer, J.A., Zoon, K.C. and Samuel, C.E. Interferons and their actions. *Annu Rev Biochem* **56** (1987), pp. 727-77.

Polack, A., Hortnagel, K., Pajic, A., Christoph, B., Baier, B., Falk, M., Mautner, J., Geltinger, C., Bornkamm, G.W. and Kempkes, B. c-myc activation renders proliferation of Epstein-Barr virus (EBV)-transformed cells independent of EBV nuclear antigen 2 and latent membrane protein 1. *Proc Natl Acad Sci U S A* **93** (1996), pp. 10411-6.

Prehn, R.T. Relationship of tumor immunogenicity to concentration of the oncogen. *J Natl Cancer Inst* **55** (1975), pp. 189-90.

Pulaski, B.A., Smyth, M.J. and Ostrand-Rosenberg, S. Interferon-gamma-dependent phagocytic cells are a critical component of innate immunity against metastatic mammary carcinoma. *Cancer Res* **62** (2002), pp. 4406-12.

Qin, Z. and Blankenstein, T. CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity* **12** (2000), pp. 677-86.

Qin, Z., Schwartzkopff, J., Pradera, F., Kammertoens, T., Seliger, B., Pircher, H. and Blankenstein, T. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res* **63** (2003), pp. 4095-100.

Raulet, D.H. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* **3** (2003), pp. 781-90.

Raz, R., Durbin, J.E. and Levy, D.E. Acute phase response factor and additional members of the interferon-stimulated gene factor 3 family integrate diverse signals from cytokines, interferons, and growth factors. *J Biol Chem* **269** (1994), pp. 24391-5.

Reimann, M., Loddenkemper, C., Rudolph, C., Schildhauer, I., Teichmann, B., Stein, H., Schlegelberger, B., Dorken, B. and Schmitt, C.A. The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. *Blood* **110** (2007), pp. 2996-3004.

Restifo, N.P., Antony, P.A., Finkelstein, S.E., Leitner, W.W., Surman, D.P., Theoret, M.R. and Touloukian, C.E. Assumptions of the tumor 'escape' hypothesis. *Semin Cancer Biol* **12** (2002), pp. 81-6.

Rowe, M., Khanna, R., Jacob, C.A., Argaet, V., Kelly, A., Powis, S., Belich, M., Croom-Carter, D., Lee, S., Burrows, S.R. and et al. Restoration of endogenous antigen processing in Burkitt's lymphoma cells by Epstein-Barr virus latent membrane protein-1: coordinate up-regulation of peptide transporters and HLA-class I antigen expression. *Eur J Immunol* **25** (1995), pp. 1374-84.

Ruff-Jamison, S., Chen, K. and Cohen, S. Induction by EGF and interferon-gamma of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. *Science* **261** (1993), pp. 1733-6.

Sanchez-Beato, M., Sanchez-Aguilera, A. and Piris, M.A. Cell cycle deregulation in B-cell lymphomas. *Blood* **101** (2003), pp. 1220-35.

Schlee, M., Holzel, M., Bernard, S., Mailhammer, R., Schuhmacher, M., Reschke, J., Eick, D., Marinkovic, D., Wirth, T., Rosenwald, A., Staudt, L.M., Eilers, M., Baran-Marszak, F., Fagard, R., Feuillard, J., Laux, G. and Bornkamm, G.W. C-myc activation impairs the NF-kappaB and the interferon response: implications for the pathogenesis of Burkitt's lymphoma. *Int J Cancer* **120** (2007a), pp. 1387-95.

Schlee, M., Schuhmacher, M., Holzel, M., Laux, G. and Bornkamm, G.W. c-MYC Impairs Immunogenicity of Human B Cells. *Adv Cancer Res* **97** (2007b), pp. 167-88.

Schuler, T. and Blankenstein, T. Cutting edge: CD8+ effector T cells reject tumors by direct antigen recognition but indirect action on host cells. *J Immunol* **170** (2003), pp. 4427-31.

Segal, J.G., Lee, N.C., Tsung, Y.L., Norton, J.A. and Tsung, K. The role of IFN-gamma in rejection of established tumors by IL-12 : source of production and target. *Cancer Res* **62** (2002), pp. 4696-703.

Seliger, B., Maeurer, M.J. and Ferrone, S. Antigen-processing machinery breakdown and tumor growth. *Immunol Today* **21** (2000a), pp. 455-64.

Seliger, B., Wollscheid, U., Momburg, F., Blankenstein, T. and Huber, C. Coordinate downregulation of multiple MHC class I antigen processing genes in chemical-induced murine tumor cell lines of distinct origin. *Tissue Antigens* **56** (2000b), pp. 327-36.

Seng, J.E. and Peterson, B.A. Indolent B-cell non-Hodgkin's lymphomas. *Oncology (Williston Park)* **11** (1997), pp. 1883-94, 1987; discussion 1901-2, 1.

Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J. and Schreiber, R.D. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410** (2001), pp. 1107-11.

Shimizu, J., Yamazaki, S. and Sakaguchi, S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* **163** (1999), pp. 5211-8.

Silvennoinen, O., Ihle, J.N., Schlessinger, J. and Levy, D.E. Interferon-induced nuclear signaling by Jak protein tyrosine kinases. *Nature* **366** (1993), pp. 583-5.

Smyth, M.J., Crowe, N.Y. and Godfrey, D.I. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* **13** (2001a), pp. 459-63.

Smyth, M.J., Godfrey, D.I. and Trapani, J.A. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* **2** (2001b), pp. 293-9.

Staege, M.S., Lee, S.P., Frisan, T., Mautner, J., Scholz, S., Pajic, A., Rickinson, A.B., Masucci, M.G., Polack, A. and Bornkamm, G.W. MYC overexpression imposes a nonimmunogenic phenotype on Epstein-Barr virus-infected B cells. *Proc Natl Acad Sci U S A* **99** (2002), pp. 4550-5.

Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D. and Levitsky, H. Induction of antigen-specific T cell anergy: An early

event in the course of tumor progression. *Proc Natl Acad Sci U S A* **95** (1998), pp. 1178-83.

Stoiber, D., Kovacic, B., Schuster, C., Schellack, C., Karaghiosoff, M., Kreibich, R., Weisz, E., Artwohl, M., Kleine, O.C., Muller, M., Baumgartner-Parzer, S., Ghysdael, J., Freissmuth, M. and Sexl, V. TYK2 is a key regulator of the surveillance of B lymphoid tumors. *J Clin Invest* **114** (2004), pp. 1650-8.

Street, S.E., Hayakawa, Y., Zhan, Y., Lew, A.M., MacGregor, D., Jamieson, A.M., Diefenbach, A., Yagita, H., Godfrey, D.I. and Smyth, M.J. Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. *J Exp Med* **199** (2004), pp. 879-84.

Street, S.E., Trapani, J.A., MacGregor, D. and Smyth, M.J. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med* **196** (2002), pp. 129-34.

Stripecke, R., Carmen Villacres, M., Skelton, D., Satake, N., Halene, S. and Kohn, D. Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther* **6** (1999), pp. 1305-12.

Stutman, O. Delayed tumour appearance and absence of regression in nude mice infected with murine sarcoma virus. *Nature* **253** (1975), pp. 142-4.

Sun, W.H., Pabon, C., Alsayed, Y., Huang, P.P., Jandeska, S., Uddin, S., Platanias, L.C. and Rosen, S.T. Interferon-alpha resistance in a cutaneous T-cell lymphoma cell line is associated with lack of STAT1 expression. *Blood* **91** (1998), pp. 570-6.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. and Leder, P. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc Natl Acad Sci U S A* **79** (1982), pp. 7837-41.

Thomas, D.A. and Massague, J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* **8** (2005), pp. 369-80.

Townsend, A. and Bodmer, H. Antigen recognition by class I-restricted T lymphocytes. *Annu Rev Immunol* **7** (1989), pp. 601-24.

Townsend, A.R., Rothbard, J., Gotch, F.M., Bahadur, G., Wraith, D. and McMichael, A.J. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44** (1986), pp. 959-68.

Unni, A.M., Bondar, T. and Medzhitov, R. Intrinsic sensor of oncogenic transformation induces a signal for innate immunosurveillance. *Proc Natl Acad Sci U S A* **105** (2008), pp. 1686-91.

Vivier, E. and Anfossi, N. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat Rev Immunol* **4** (2004), pp. 190-8.

Vivier, E., Nunes, J.A. and Vely, F. Natural killer cell signaling pathways. *Science* **306** (2004), pp. 1517-9.

Wang, M., Bronte, V., Chen, P.W., Gritz, L., Panicali, D., Rosenberg, S.A. and Restifo, N.P. Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. *J Immunol* **154** (1995), pp. 4685-92.

Willis, T.G., Jadayel, D.M., Du, M.Q., Peng, H., Perry, A.R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P.G. and Dyer, M.J. Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. *Cell* **96** (1999), pp. 35-45.

Wong, L.H., Krauer, K.G., Hatzinisiriou, I., Estcourt, M.J., Hersey, P., Tam, N.D., Edmondson, S., Devenish, R.J. and Ralph, S.J. Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *J Biol Chem* **272** (1997), pp. 28779-85.

Yee, C., Thompson, J.A., Byrd, D., Riddell, S.R., Roche, P., Celis, E. and Greenberg, P.D. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A* **99** (2002), pp. 16168-73.

Young, L.S. and Rickinson, A.B. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* **4** (2004), pp. 757-68.

Yunis, J.J., Frizzera, G., Oken, M.M., McKenna, J., Theologides, A. and Arnesen, M. Multiple recurrent genomic defects in follicular lymphoma. A possible model for cancer. *N Engl J Med* **316** (1987), pp. 79-84.

Zhou, F., Rouse, B.T. and Huang, L. Prolonged survival of thymoma-bearing mice after vaccination with a soluble protein antigen entrapped in liposomes: a model study. *Cancer Res* **52** (1992), pp. 6287-91.

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Research Experience

Project- "Rejection of high grade lymphoma by syngeneic and allogeneic stem cell transplantation and mechanisms of immune escape"-Institute of Clinical Molecular Biology and Tumor Genetics, Helmholtz Zentrum Munich, Germany

Using the animal model of the c-myc t (8, 22) translocation resembling Burkitt's lymphoma, the anti lymphoma activity of lymphoma specific T cells was studied and further investigated the cellular and molecular mechanisms behind tumor rejection

Junior research fellow – Design of Temperauture sensitive mutants from yeast GAL4.
2003-2004, Indian Institute of Science, Bangalore, India

Using the E.coli as model system, simple procedures for developing Temperature sensitive mutants at high frequency through site directed mutagenesis were developed and can be used for conditional expression of variety of genes

Presentations

American Society of Hematology-2005, Atlanta
Abstract selected for publication – 47th Annual Meeting of the American Society of Hematology (ASH)

American Society of Hematology-2006, Orlando
Poster presentation – 48th Annual Meeting of the American Society of Hematology (ASH)

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Publications

1. Design of temperature-sensitive mutants solely from amino acid sequence
   Ghadiyaram Chakshusmathi, Kajari Mondal, G. Santosh Lakshmi, Guramrit Singh, Ankita Roy, Ravindra Babu Ch,**S. Madhusudhanan**, and Raghavan Varadarajan
   Proc Natl Acad Sci U S A. 2004 May 25; 101(21):-7925-7930
2. Design and isolation of temperature-sensitive mutants of Gal4 in Yeast and Drosophila
   Kajari Mondal, Antara Ghosh Dastindar, Guramrit Singh, **S. Madhusudhanan**, Santosh Lakshmi Gande, K Vijay Raghavan and Raghavan Varadarajan
   J.Mol.Biol. 2007; 370; 939-950
3. Rejection of high grade B-cell lymphoma mediated by foreign antigen is Interferon dependent and requires STAT1 signalling**.(Manuscript in preparation)**
   Madhusudhanan Sukumar, Joseph Mautner, Andrea wilke, Joachim Ellwart, Hans-Jochem Kolb, Georg Bornkamm, Armin Gerbitz