

**Aktivierung der angeborenen und adaptiven Immunantwort und deren
Bedeutung bei der Immunantwort gegenüber malignen Gliomen**

**Activation of the innate and adaptive immune response and its role in the
immune surveillance of malignant glioma**

DISSERTATION

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2005

vorgelegt von

Steffen Alexander Aulwurm

Tag der mündlichen Prüfung: 18. April 2005

Dekan: Professor Dr. S. Laufer

1. Berichterstatter: Professor Dr. Hans-Georg Rammensee
2. Berichterstatter: Professor Dr. Michael Weller

Accordingly, the projects summarized in this thesis sought to explore the activation of the innate and adaptive immunity and possibly overcome human glioma cells resistance to apoptosis as well as their escape from host immune surveillance. Work from other areas to which I also contributed has been omitted (see attached reference list).

Proprietary Declaration

The work presented here has been performed under the guidance of Prof. Dr. Michael Weller from April 2000 until December 2004 in the Department of Neurology at the University of Tübingen Medical School. I hereby declare that this PhD thesis is the product of my own work and has been written autonomously. Wherever further resources have been used, this has been indicated. In all sections that contain work from my colleagues, I have specified the extent of my contribution. Parts of this work have already been published and are referenced accordingly, others have been submitted for publication.

Table of Contents

List of abbreviations	9
Introductory remarks	14
I. Introduction	15
Glioma biology	15
Prognosis, current treatment and perspectives	15
Tumor immunology and immunotherapy	22
Basic immunology	22
Innate immunity	22
Toll-like receptors	23
Induced-self and missing-self recognition	26
Adaptive immunity	28
CD27/CD70 costimulation	28
Immunosuppression by TGF- β	30
Experimental overview	31
II. CD70/CD27 interactions and the immune response to malignant glioma: balancing immune effector function	35
Introduction	36
Materials and methods	38
Results	41
Balancing effector lymphocyte formation <i>via</i> CD70/CD27 interaction: increased immune cell apoptosis <i>in vitro</i> but enhanced tumor cell immunogenicity	41
CD70 inhibits the growth of SMA-560 gliomas in CD1 ^{nu/nu} mice	44
CD70 mediates tumor regression in immunocompetent VM/Dk mice	46
Mouse CD70 gene transfer promotes immune cell infiltration in the SMA-560 syngeneic mouse glioma model	48
Disruption of the CD27 gene in C57BL/6 mice nullifies CD70-mediated anti-tumor responses	50
Discussion	53

III. Anti-tumor immunity induced by injections of immune stimulatory RNA in tumor-bearing mice	56
Introduction	57
Materials and methods	59
Results	62
Bone marrow dendritic cells and B cells are activated by stabilized mRNA	62
Stabilized mRNA has an as good anti-tumor effect as CpG-DNA	63
Intratumoral injection of stabilized mRNA leads to memory immune response	64
No tumor-specific, IFN- γ -producing cells are detectable in the spleen	65
Intratumoral injection of stabilized mRNA leads to a specific immune response and tumor infiltrating cells	65
Protamine-condensed mRNA is nontoxic	66
Discussion	68
IV. SD-208, a novel TGF- β receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells <i>in vitro</i> and <i>in vivo</i>	70
Introduction	71
Materials and methods	72
Results	78
SD-208 is a functional TGF- β_1 and TGF- β_2 antagonist <i>in vitro</i>	78
SD-208 abrogates autocrine TGF- β -dependent signal transduction in glioma cells	81
SD-208 inhibits constitutive and TGF- β -evoked migration and invasion .	83
SD-208 enhances allogeneic immune responses to glioma cells <i>in vitro</i> ..	84
SD-208 prolongs the survival of SMA-560 intracranial experimental glioma-bearing syngeneic mice.....	87
Histological changes in gliomas of SD-208-treated animals	88
Discussion	92

V.	Processing of immunosuppressive pro-TGF-beta 1,2 by human glioblastoma cells involves cytoplasmic and secreted furin-like proteases	94
	Introduction	95
	Materials and methods	96
	Results	99
	Human malignant glioma cells express furin and exhibit FLP activity	99
	TGF- β synthesis and release by glioma cells.....	101
	Glioma cells release furin into the cell culture supernatant	103
	FLP activity is required for the processing of TGF- β in glioma cells	104
	PDX gene transfer reduces glioma-associated TGF- β bioactivity.....	105
	Discussion	107
VI.	MICA/NKG2D-mediated immunogene therapy of experimental gliomas	109
	Introduction	110
	Materials and methods	111
	Results	116
	Expression of NKG2DL and MHC class I antigens by human glioma cells.....	116
	Stable transfection and adenoviral gene transfer of MICA into glioma cells stimulates NK cytolytic activity.....	117
	Costimulatory functions of MICA-transfected glioma cells	119
	MICA expression delays growth of glioma xenografts in nude mice.....	122
	NKG2DL expression by mouse glioma cell lines.....	124
	MICA expression in SMA-560 glioma cells delays tumor growth.....	126
	Subcutaneous vaccination with MICA-expressing glioma cells after tumor inoculation delays tumor growth	127
	Discussion	130
	Summary	132
	Zusammenfassung.....	137
	References	143
	List of publications.....	167

Acknowledgements	169
Academic teachers.....	171
Curriculum vitae.....	172

List of Abbreviations

Ad-dE1	adenovirus delta exon 1 (control virus)
Ad-eGFP	adenovirus encoding enhanced green fluorescent protein
Ad-MICA	adenovirus encoding MHC class I chain-related protein A
ADCC	antibody-dependent cellular cytotoxicity
AnxV	annexinV
APC	antigen presenting cell
BAK	BCL2-antagonist/killer2
BAX	BCL2-associated X protein
BCL-2	B cell lymphoma/leukemia-2
BCL-XL	B cell lymphoma mutant, extra long
BH3	Bcl homology 3
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumin
caspase	cysteine aspartyl protease
CD	cluster of differentiation
CD95L	CD95 ligand
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CHX	cycloheximide
CMV	cytomegalovirus
CPT	camptothecin
CTL	cytotoxic T lymphocytes
CTLA-4	common T lymphocyte antigen 4
DAB	diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DC	dendritic cell(s)
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DR4/5	death receptor 4/5
dsRNA	double-stranded ribonucleic acid
E:T ratio	effector target ratio

EC ₅₀	50% effective concentration, i.e. the concentration at which the half-maximal effect is achieved
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
eGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ER	endoplasmatic reticulum
Fab	antigen-binding fragment
FasL	Fas ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FLP	furin-like protease
GAP-DH	glyceraldehyde-3-phosphate dehydrogenase
GARG	glucocorticoid-attenuated response gene
GCV	ganciclovir
GM-CSF	granulocyte macrophage colony stimulating factor
HCL	hydroxymethylcellulose
HE	hematoxylin, eosin
HIV-1	human immunodeficiency virus-1
HLA	human leukocyte antigen
HRP	horseradish peroxidase
hsp70	heat shock protein 70
HSV-TK	herpes simplex virus thymidine kinase
ICOS	inducible costimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRAK	IL-1R-associated kinases
IRG	immunoresponsive gene
KIR	killing inhibitory receptor
LAK	lymphokine-activated killer
LDH	lactate dehydrogenase

LPS	lipopolysaccharide
LRR	leucine-rich repeat
mAb	monoclonal antibody
MAGE	melanoma-associated antigen
Mdm2	mouse double minute 2
Melan-A	melanocyte differentiation antigen (MART-1)
MEM	modified Eagle's medium
MΦ	macrophages
MHC class I	major histocompatibility complex class I
MICA/B	MHC class I chain-related proteins A and B
MLR	mixed lymphocyte reaction
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mt	mutant
MyD88	myeloid differentiation primary-response protein (88)
NAC	N-acetylcysteine
NF-κB	nuclear factor-κB
NIH	National Institute of Health (USA)
NK	natural killer
NKG2D	natural killer group 2D
NKG2DL	NKG2D ligand
ODN	oligodeoxynucleotide
PAMP	pathogen-associated molecular patterns
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride
PRR	pattern recognition receptors
p-Smad	phosphorylated Smad

pSUPER	plasmid for suppression of endogenous RNA
PTEN	phosphatase and tensin homolog deleted on chromosome ten
QPCR	quantitative reverse transcription polymerase chain reaction
RAG	recombinase activating gene
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SBE	Smad-binding element
sCD27	soluble CD27
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
serpin	serine protease inhibitor
SEREX	serological analysis of tumor antigens by recombinant cDNA expression cloning
SFI	specific fluorescence index
siRNA	short interfering RNA
SMA	spontaneous murine astrocytoma
sMICA	soluble MICA
SN	supernatant
ssRNA	single-stranded ribonucleic acid
TAB	TAK1-binding protein
TAP	transporter associated with antigen processing
TAK	transforming growth factor- β (TGF- β)-activated kinase
TBS	Tris-buffered saline
TCR	T cell receptor
TGF- β	transforming growth factor- β
TGF- β RI	TGF- β receptor I
TIR	Toll/IL-1R
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand

TRAILR	TRAIL receptor
TRIF	TIR domain-containing adaptor protein inducing IFN- β
ULBP	UL-16-binding proteins
VEGF	vascular endothelial growth factor
WHO	World Health Organisation
wt	wild-type
zVAD-fmk	N-tert-butoxy-carbonyl-Val-Ala-DL-Asp-fluoromethylketone

Introductory remark

In order to give this PhD thesis more structured contours, I decided to split it into various chapters by projects, each consisting of an introduction, a material and methods section, the results paragraph and a discussion. All references as well as a summary are provided at the end. The introductory paragraphs on glioma biology and immunology are intended to provide the background for the understanding of the work that follows. This format should enable readers who are interested in a specific part of the work outlined here to find all relevant information within a concise standard format. All sections represent the state-of-the-art when the respective project was prepared for publication and have been supplemented with the most relevant recent findings.

I. Introduction

Glioma biology

Prognosis, current treatment and perspectives

Prognosis. Glioblastomas are primary brain tumors of unknown origin. Based on the cancer stem cell hypothesis by Singh *et al.* CD133-expressing stem cells generate later progenitor cells (CD133⁻) that give rise to the mature forms of brain cells like neurons, astrocytes and oligodendrocytes (Fig. 1.1.a), which are all CD133⁻ (1). Neoplastic glioma clones (Fig. 1.1.b) are maintained exclusively by a rare fraction of cells with stem cell properties, expressing CD133, which possibly could recruit for the different grades of gliomas (2): pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma (grade IV). In 2004, the prognosis of glioblastoma patients remains poor, with less than 5% of patients living beyond 2 years after diagnosis. While tumor removal, irradiation, and chemotherapy have somewhat improved patient survival in the last 30 years, current treatment results are still unsatisfactory (3). The reason for this failure is inherent to the biological properties of malignant gliomas. Malignant glioma cells are highly infiltrative in the brain, which prevents total tumor resection; they are also intrinsically resistant to the doses of radiotherapeutic and chemotherapeutic therapies that can be safely applied to the brain.

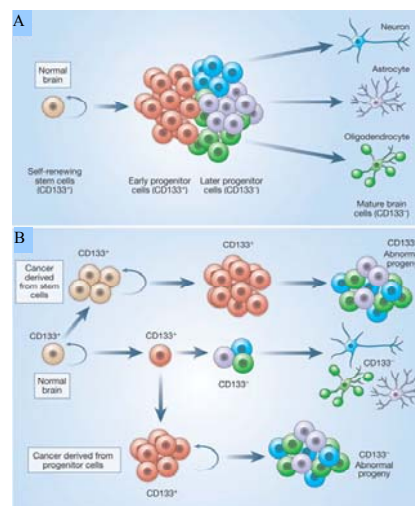


Fig. 1.1.A,B **Brain-cell hierarchy.** A. In the normal brain, stem cells, which express the CD133 marker and are designated CD133⁺, can generate new stem cells by the process of self-renewal. They can also produce early progenitor cells (CD133⁺) and later progenitor cells (CD133⁻) that give rise to the mature forms of brain cells (neurons, astrocytes and oligodendrocytes, all CD133⁻). Unlike the stem cells, progenitor cells have limited ability to replicate. B. Singh *et al.* (1) have

identified cancer stem cells. Such cells could arise from CD133⁺ brain stem cells, when loss of normal constraints results in expansion of the abnormal stem cell pool, or from early CD133⁺ progenitor cells as a result of mutations that make these cells self-renewing. In either case, the cancer stem cells also generate abnormally differentiated CD133⁻ progeny that cannot self-renew and thus cannot form new tumors. Figure modified from (1).

Current treatment. On the positive side, major strides have been made in the last decade in understanding the genetic, immunologic and biologic mechanisms that underlie the initiation and malignant progression of gliomas. We have gained a much clearer understanding of the pathways leading to glioma development. These advances are already being exploited for the development of new treatment approaches targeting the key molecular effectors of these tumorigenic pathways, regaining tumor cell cycle control, inducing glioma cell death, antagonizing angiogenesis, and immunotherapies.

Cell cycle control. Tumor cells display uncontrolled proliferation. Genetic alterations underlie this disruption in cell cycle control. Activation of protooncogenes by mutational events can promote mitotic signalling while loss of tumor suppressors can remove regulatory checkpoints. Interference with oncogenic signalling and restoration of tumor suppressor function are two therapeutic approaches aiming at regaining cell cycle control.

Protooncogenes. Protooncogenes can be modified by genetic alterations into genes that promote tumor formation (oncogenes). Protooncogenes are activated by several mechanisms, including overexpression caused by gene amplification, point mutations, modification of regulatory elements leading to increased transcription, and gene rearrangements. Protooncogene products can be growth factors or their receptors, like epidermal growth factor (EGF), EGF receptor (EGFR), signal transducing proteins (ras, phosphatidylinositol 3-kinase), transcription factors (myc, fos, jun), and suppressors of apoptosis B cell lymphoma/leukemia-2 (BCL-2), B cell lymphoma mutant, extra long (BCL-XL). In gliomas (4), protooncogene activation occurs as the result of gene amplification with or without gene rearrangement. Examples include *EGFR*, *murine double minute 2 (MDM2)*, *cyclin-dependent kinase 4 (CDK)4*, *CDK6*, and the platelet-derived growth factor receptor- α . Persistent oncogenic signals can augment the proliferation of cells and render them resistant to irradiation (5) and apoptosis (6, 7). Because of these properties, EGFR (8, 9) and the tyrosine kinase signalling have become a major focus of antiproliferative therapy.

While many of these antiproliferative strategies have great potential, they are unlikely to succeed on their own because gliomas are genetically unstable and because of intertumor and intratumor heterogeneity.

Tumor suppressor genes. Tumor suppressor genes hold their name because they are instrumental in protecting a cell from becoming transformed. Typically, these genes are lost in two steps, one allele is deleted and the other acquires an inactivating mutation. A number of target genes for these deletions have been identified: Phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) (10q) (10-12) and others directly regulate the cell cycle (*TP53*, *p16INK4A*, *p14ARF*) (13-15). Therapeutic strategies aiming at restoring tumor suppressor function in gliomas have essentially focused on p53. Preclinical models have shown that restoration of p53 function in glioma cells using retroviral or adenoviral *TP53* delivery induces cell cycle arrest and apoptosis (16). Other strategies are based on the restoration of p53 function using small molecules such as CP-31398 stabilizes the active conformation of p53 and promotes p53 activity in cancer cell lines with mutant or wild-type p53 (17).

Prodrug/suicide gene therapy. Gene-directed enzyme prodrug therapy is a two step therapeutic approach for cancer gene therapy. In the first step, the transgene is delivered into the tumor and expressed. In the second step a prodrug is administered and is selectively activated by the expressed enzyme. The first system described was the thymidine kinase gene of the Herpes Simplex virus (*HSV-TK*) in combination with the prodrug Ganciclovir (*GCV*). The enzyme phosphorylates the prodrug to *GCV* triphosphate, an inhibitor of DNA synthesis that leads to cell death. Prodrug/suicide gene therapies lead to the killing of infected as well as nontransduced tumor cells. This useful feature, coined “bystander effect”, is believed to be mediated by the cell-to-cell transfer of phosphorylated ganciclovir through gap junctions as well as an immune response (18). Phase I/II clinical trials with the *GCV/TK* system showed low toxicity and preliminary indications of tumor response. Unfortunately, when efficacy was tested in newly diagnosed gliomas in a phase III randomized trial, no significant advantage over standard therapy was found(19). One of the limitations of the *GCV/TK* system is poor blood-brain barrier penetration of *GCV* and insufficient *HSV-TK* gene transduction of tumor cells. Local infusion of ganciclovir to the tumor area or the use of different prodrug/suicide gene systems (cyclophosphamide/cytochrome P450 2B1), in which the prodrug can cross the blood-brain barrier, are currently being developed (20, 21).

Apoptosis induction. Apoptosis induction is an attractive therapeutic approach that tries to trigger the natural mechanism of programmed cell death in tumor cells while sparing normal cells. This has been tried in different ways that all take advantage of the fact that tumor cells show a degree of apoptosis “priming” as the result of cell transformation. For example, glioma cells express death receptors of the tumor necrosis factor (TNF) receptor family while normal astrocytes do not. Their ligands include TNF- α , FasL and TRAIL. While all three can induce apoptosis in tumor cells, TRAIL shows the lowest toxicity to normal tissue, principally the liver (22, 23), and is therefore a promising new therapeutic agent for cancer (7). TRAIL induces apoptosis by binding to death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2). These are type I transmembrane receptors containing an intracellular death domain (24) that is involved in the assembly of a death-inducing signal complex. This induces caspase-8 cleavage, activation of downstream effector caspases (caspase-3 and others), and leads to programmed cell death. Malignant glioma cells mostly express DR5 and are sensitive to TRAIL-induced apoptosis (25). While this is a novel and promising strategy to kill tumor cells, concerns regarding potential hepatotoxicity have so far prevented its testing in the clinic.

Inhibition of angiogenesis. Tumor angiogenesis, the process by which a tumor attracts endothelial cells to form a tumor-specific vascular network, has raised considerable interest as a therapeutic target. The rationale is that as a consequence of every microvascular cell killed an additional 50 to 100 tumor cells will die because they depend on the vascular supply of oxygen and nutrients (26). Furthermore, it is believed that because vascular cells are nontransformed they will not easily develop resistance to anti-angiogenesis treatment like genetically instable tumor cells do. Angiogenesis is triggered by the release of angiogenic stimulators by the tumor cells. This can occur as the result of genetic alterations as well as through the activation of the physiologic response to hypoxia. Hypoxia, a reduction in partial oxygen pressure, is a characteristic feature of a growing tumor and results from both tumor outgrowth of pre-existing co-opted vascular supply and through tumor-induced normal vascular regression events. Hypoxia activates hypoxia-inducible factor, a transcription factor inducing the transcription of genes encoding angiogenic stimulators like the vascular endothelial growth factor (VEGF) family. Expression of VEGF is particularly evident in regions surrounding necrosis in gliomas where hypoxia is most severe (27). A great number of therapeutic strategies aim at inhibiting the pro-angiogenic forces operating in the tumor as well as modulating the expression of angiogenesis inhibitors. This is achieved through small molecular compounds or antibodies that block multiple stages of the neovascularization

process, the use of soluble endogenous inhibitors as well as gene therapy approaches. A large number of pharmacotherapeutic agents are tested in phase I and II clinical trials for the treatment of newly diagnosed or recurrent malignant gliomas, including agents that target the positive regulators of angiogenesis (28). Endogenous inhibitors of angiogenesis (IFN- γ , IL-12, angiostatin, endostatin) are also evaluated in phase I/II trials for gliomas (29).

Immunotherapy. The extensive spread of tumor cells in the brain presents a particular challenge. Surgical resectability is a major limitation and radiotherapy and chemotherapy have their limits in moderate efficacy and neurotoxicity. Therefore, immunotherapy has for a long time been particularly attractive as a “microsurgical tool” to eliminate single infiltrating cancer cells while sparing normal brain. This putative highly specific antitumor cytotoxicity depends on the identification of specific tumor antigens. While only a limited number of glioma-associated antigens were identified over the last 20 years (Table 1), the recent advances in screening for new brain tumor-associated antigens has been more successful. Besides the classical antibody based serological screening of cDNA expression libraries (SEREX) or screening for cytolytic T cell clones via eucaryotic expression of such libraries, several novel methods are available today (30). Our progress in the understanding of the molecular mediators of the immune response will also help overcome the well known but incompletely understood immune escape mechanisms used by tumors (31). Several routes of administration for immunologic medicine will have to be evaluated once identification of immunologically active glioma peptides has been achieved. Immunotherapy includes molecules, that can be antibodies or ssRNA, or cells like T cells or lymphokine activated killer cells, and activated tumor infiltrating lymphocytes. A number of antibodies coupled to radioisotopes or immunotoxins have been tested in clinical trials (32, 33).

Active (vaccine) immunotherapy augments an existing or creates a new antitumor immune response in the host. These cancer “vaccine” approaches have initially focused on cytokine gene therapy (IL-2, IL-4, IL-12, GM-CSF and interferons) to generate or augment antitumor immunity (34-37). Many of the recent efforts have capitalized on our better understanding of the role of antigen-presenting cells in the initiation of the immune response. Professional antigen-presenting cells (dendritic, microglial, and Langerhans cells) will capture tumor antigens, process them, and present them on major histocompatibility complex molecules to T cell receptors. In addition, T cell activation requires a second stimulatory signal provided by co-stimulatory molecules (e.g., B7 or CD70 on antigen-presenting cells engaging CD28 or CD27 on T cells). Once properly activated, these T cells will launch a potent tumor-specific immune response. Recent approaches have attempted to prime dendritic cells with antigen in

vitro and to reinject these cells into the patient to activate a T cell response. This peripheral immunization has been shown to elicit immune responses against central nervous system tumors *in vivo* (38).

Category of antigen	Antigen	Clinical trials
Unique mutant antigens (Ag)	EGFRvIII Mutated ras Mutated p53 Mutated p16	Anti-EGFR MAb Farnesyltransferase inhibitors ONYX-015 No
Cancer-testis Ag	melanoma-associated antigen (MAGE-1) MAGE-3 B-melanoma antigen (BAGE) G-antigen (GAGE) IL-13R Sox6	No No No No IL-13 PE38QQR No
Tissue of origin-related Ag (differentiation antigens)	gp100 Tyrosinase Tyrosinase-related protein-1 Tyrosinase-related protein-2 MART-1	No No No No No
Normal proteins with selective or over-expression in brain cancer	Wild-type EGFR Her-2/ <i>neu</i> Tenascin gp240 Proteoglycan chon. sulfate Ag SART-1 Granulin	Anti-EGFR Mab Herceptin MAb 81C6 No No No No No

Table 1. Tumor-associated antigens in human gliomas

Perspectives. The extensive development of central nervous system tumor research in the last decade may provide new clinical tools that will improve survival in patients with malignant gliomas. Current radiotherapy and chemotherapy regimens may have reached their limits in improving survival. Novel strategies for treating gliomas must be brought into clinical trials and will likely function best in combination with standard therapies. These advances are made possible by progress in the understanding of tumor genesis, tumor biology and immunology, including adaptive immunity as well as innate immunity, and their combination and the availability of novel tumor-antigens.

While this progress is clearly exciting, it is sobering to keep in mind the challenges that remain. Malignant gliomas are a very heterogeneous group of tumors, as is evidenced by the number and diversity of genetic mutations involved in their genesis. Genetic diversity is also found within a single tumor, probably a reflection of genetic instability, and likely contributes to the development of resistance to radiotherapy and chemotherapy by clonal evolution. Furthermore, the highly infiltrative nature of these tumors is a tremendous challenge and the main reason for tumor recurrence after surgery and focal radiotherapy. Because of this heterogeneity and propensity to therapeutic resistance, simultaneous targeting of multiple components essential to the tumor's growth and survival will maximize the chances to control the disease. Therapies based on immune system activation, angiogenesis inhibition, restoration of cell cycle control, induction of programmed cell death, and others, combined with the standards of radiotherapy and chemotherapy may have a significant impact on the disease. A further advantage of such integrated therapeutic strategies may be reduced toxicity and possible synergistic effects. To make this possible, additional mechanisms need to be put in place to foster transition of exciting preclinical work into clinical trials.

Tumor immunology and immunotherapy

Basic immunology

The latin word *munia* can be translated as responsibilities or merits. As the roman senators were free of responsibilities, or later free of legal prosecution, their status was called *immunitas* (negation forming prefix, lat.: *im*). Nevertheless it was not a roman but a greek historian, namely Thukydides (460-400 B.C.) who first circumscribed immunological incidents during a siege in “The Peloponnesian War”. People who suffered from “pestilential plague” (with Perikles as its most famous victim, 500-429 B.C.) and survived did not fall ill again. He did not know that he described one of the major features of the adaptive immune system: immunological memory. The immune system defends our body against infections. In the late 19th century Robert Koch proved that infectious diseases could be evocated from microorganisms, causing specific illnesses with a particular etiopathology. There are three major columns of the immune system: physical and chemical barriers, the innate immune system and the adaptive immune system. The first line of active defense against microorganisms is called innate immunity. However, innate immunity is unable to protect against a reinfection, as immunological memory is unique to adaptive immunity based on the “clonal selection” of lymphocytes of an existing repertoire with a multiplicity of highly specific receptors. Thus the immune system is able to cope with nearly any foreign antigen, as antigen specific lymphocytes proliferate and differentiate to “effector cells”, which in turn destroy these pathogens. These immune defense mechanisms require different recognition systems and a broad variety of effector functions to search and destroy foreign pathogens. Adaptive immunity is also able to build up a repertoire of memory cells differentiated by clonal selection, each specific for a certain pathogen. This allows for a more rapid and effective reaction during a reinfection.

Innate Immunity

Innate immunity can be defined as the components of the immune system with a constant specificity during an individual lifetime. The first component comprises a barrier function of the epithelia of the body (39), as linings and covering on all external and internal body surfaces pose a barrier to infection. The barriers can be divided into physical and chemical ones, which are dermis, mucosa, mucus and cilia for the physical barriers and hydrochloric acid (pH=1-2) and lysozyme (degrading bacterial murein), for the chemical barriers. Pathogens penetrating this epithelial defense are facing a stronghold of cells and molecules

confining and destroying pathogens. Most importantly tissue macrophages patrol on body boundaries while the complement system of proteins mediates the second line of defense in the interstitia and blood. Other important innate cell types are granulocytes, monocytes, mast cells, natural killer and $\gamma:\delta$ T cells. The latter have some effector function besides being assisted by antibodies or T cells. Innate immune cells recognize pathogen-associated molecular patterns (PAMP), i.e. structural motifs that are conserved on pathogenic microorganisms (lipopolysaccharide, mannose, bacterial cell wall) and absent from eukaryotic cells via pattern recognition receptors (PRR). Endocytic pattern recognition receptors (mannose or scavenger receptors) are found on the surface of phagocytes like macrophages or neutrophilic granulocytes, dendritic cells and natural killer (NK) cells. Their activation promotes the engulfment and destruction of microorganisms (40) and the release of cytokines. Signalling PRR include CD14 and the group of *toll-like receptors* (TLR). Importantly, expression of TLR is not restricted to innate immune cells (41) and will be addressed in detail in the next subchapter.

Toll-like receptors. The discovery of the TLR as sensors of microbial molecules transformed the views of discrimination between self and non-self, a key requirement of any immune system. It turns out that much of microbial recognition is served by only a handful of TLR. As evolutionary successful processes are conserved, the Toll gene is found in mice, sharks, nematodes and plants. Toll-like receptors are regarded as a link between innate and adaptive immunity because they recognize PAMP like LPS, unmethylated DNA or single-stranded RNA and in addition are able to trigger immunological adaptive responses via dendritic cells (42). TLR are type I integral membrane glycoproteins. On the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin1 receptors (IL-1Rs). By contrast, the extracellular region of TLR and IL-1Rs differs markedly: the extracellular region of TLR contains leucine-rich repeat (LRR) motifs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains (Fig. 1.2.a).

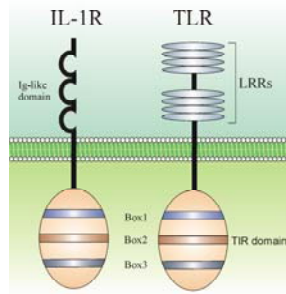


Fig. 1.2.a **TLR structure and signalling.** Toll-like receptors (TLR) and interleukin-1 receptors (IL-1Rs) have a conserved cytoplasmic domain, known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions known as boxes 1, 2 and 3. Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLR have tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR) whereas IL-1Rs have three immunoglobulin (Ig)-like domains. Figure modified from (43).

The subcellular localization of different TLR correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. By contrast, TLR3, TLR7 and TLR9, all of which are involved in the recognition of nucleic acid-like structures, are not expressed on the cell surface (44-46). For example, TLR9 is expressed in the endoplasmic reticulum and recruited to endosomal/lysosomal compartments after stimulation with CpG-containing DNA (47). Double-stranded ribonucleic acid (dsRNA) serves as a danger signal associated with viral infection and leads to stimulation of innate immune cells. In contrast, the immunostimulatory potential of single-stranded RNA (ssRNA) was poorly understood and innate immune receptors for ssRNA were unknown until Bauer *et al.* (48) reported that guanosine (G)- and uridine (U)-rich ssRNA oligonucleotides derived from human immunodeficiency virus-1 (HIV-1) stimulated DC and macrophages to secrete interferon-alpha and proinflammatory as well as regulatory cytokines. By using Toll-like receptor (TLR)-deficient mice and genetic complementation, these authors showed that murine TLR7 and human TLR8 mediate species-specific recognition of GU-rich ssRNA. After ligand binding, TLR/IL-1Rs dimerize and undergo the conformational change required for the recruitment of downstream signalling molecules. These include the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor- β (TGF- β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (49, 50). MyD88-deficient mice do not produce TNF or IL-6 when exposed to IL-1 or microbial

components that are recognized by TLR2, TLR4, TLR5, TLR7 or TLR9 (50, 51) . Thus, MyD88 is essential for responses against a broad range of microbial components. However, closer study of MyD88-deficient cells has revealed the

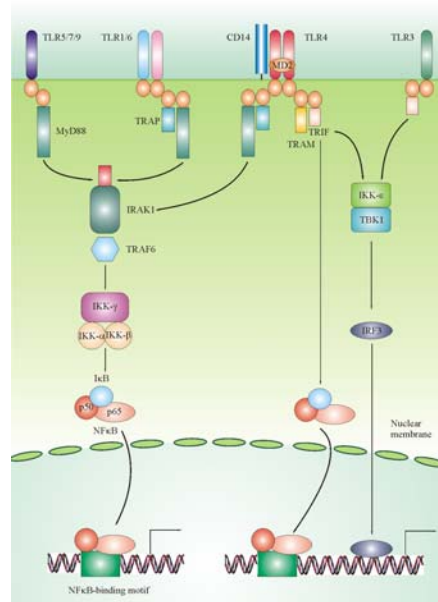


Fig. 1.2.b. **Involvement of TIR domain-containing adaptors in TLR signalling pathways.** The Toll/IL-1-receptor (TIR)-domain-containing adaptor molecule MyD88 mediates the TLR-signalling pathway that activates IRAKs and, and leads to the activation of the IKK (inhibitor of nuclear factor- κ B ($\text{I}\kappa\text{B}$)-kinase) complex which consists of IKK- α , IKK- β and IKK- γ . This pathway is used by TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 and releases NF- κ B from its inhibitor. NF- κ B then translocates to the nucleus and induces the expression of inflammatory cytokines. TIRAP, a second TIR-domain-containing adaptor protein, is involved in the MyD88-independent pathway. The non-typical IKKs, IKK- ϵ and TBK1 mediate activation of IRF3 downstream of TRIF. A fourth TIR-domain-containing adaptor, TRAM, is specific to the TLR4-mediated, MyD88-independent/TRIF-dependent pathway. Modified from (43).

existence of MyD88-dependent and -independent pathways, both of which mediate signalling in response to LPS. The MyD88-independent pathway was further characterized by determining the genes expressed in MyD88-deficient macrophages following exposure to LPS (52). A number of genes known to be interferon (IFN)-inducible genes were identified, such as glucocorticoid-attenuated response gene 16 (GARG16), immunoresponsive gene 1 (IRG1) and the gene encoding CXC-chemokine ligand 10. In addition to inducing the expression of IFN-inducible genes, the MyD88-independent pathway leads to the LPS-mediated maturation

of dendritic cells (53). When cultured with LPS, MyD88-deficient bone marrow-derived DC upregulate the cell surface expression of co-stimulatory molecules, such as CD40, CD80 and CD86 and induce the proliferation of T cells. In contrast, TLR4-deficient DC fail to mature in response to LPS, indicating that DC maturation proceeds in a MyD88-independent manner (53). The discovery of the MyD88-independent pathway led to the characterization of adaptor proteins: TIRAP (TIR-domain-containing adaptor protein; also known as MyD88-adaptor-like protein, MAL); TRIF (TIR-domain-containing adaptor protein inducing IFN- β ; also known as TIR-domain-containing molecule 1, TICAM1); and TRAM (TRIF-related adaptor molecule; also known as TIR-domain-containing molecule 2, TICAM2) (Fig. 1.2.b).

Induced-self and missing-self recognition. NK cells mediate cellular cytotoxicity and produce chemokines and inflammatory cytokines such as IFN- γ and TNF (54). They are important in attacking pathogen-infected cells, especially during the early phases of an infection. They are also efficient killers of tumor cells and are believed to be involved in tumor surveillance (55). The hypothesis that NK cells exert an immunoregulatory effect is supported by recent evidence of “crosstalk” with DC (56). Unlike B and T cells, receptor genes of NK cells do not undergo a variable-diversity-joining (VDJ) recombination or sequence diversification in somatic cells (57-59). NK cells use a multiple receptor recognition strategy whereby an individual NK cell can be triggered through various receptors independently or in combination, depending on the ligands presented by the target cell (58, 60). Besides recognition of pathogen-encoded molecules, NK cells use the so called “induced-self” recognition strategy, exemplified by the NK group 2D (NKG2D) receptor. NKG2D recognizes self proteins that are upregulated on the surface of most tumors and many infected cells. NKG2D, like Ly49H, is encoded in the NK gene complex and a lectin-like type 2 transmembrane receptor (61). The receptor is expressed by all NK cells and by certain T cell subsets. The NKG2D ligands are type I transmembrane proteins encoded by the host genome: they include MHC class I chain-related A chain (MICA) or B chain (MICB) in humans (62) and a diverse family of ligands shared by human and mice called the retinoic acid early transcripts (RAET1) family, which includes Rae-1, H-60 and murine UL16-binding protein-like transcript 1 (Mult1) (63-66) in mice and the UL16-binding protein (ULBP) or human Rae-1 proteins in humans (67). All the ligands are distant relatives of MHC class I molecules and adopt a MHC class I-like structure. However, none associate with β_2 -microglobulin and, with the exception of human MICA and MICB, most of the corresponding genes are located in a gene cluster separate from the MHC. The expression patterns of different NKG2D ligands

differ and are complex. In general, however, it seems that ligands are expressed poorly on the surface of normal cells but are often upregulated in tumor cells (68) or infected cells. Ligand mRNAs are upregulated in cells infected with cytomegalovirus (69). It seems that cells use intrinsic signals associated with tumorigenesis as well as extrinsic and/or intrinsic signals associated with infections to regulate expression of NKG2D ligands. Ligand expression by target cells provokes NK cells to attack and can enhance antigen-specific CD8⁺ T cell responses. *In vivo*, ligand expression by subcutaneously transferred tumor cells incites immune rejection of the tumor (70, 71). Thus, the ligand upregulation that occurs normally in tumorigenesis may activate immune responses that can in some cases eliminate the tumor cells or impede their growth or spread. In the case of certain human tumors evasion may take place by shedding of a soluble form of the MICA ligand into the circulation where it binds to NKG2D on lymphocytes and desensitizes the cells to subsequent stimulation via NKG2D (72). At least three other stimulatory receptors have been linked to NK-mediated lysis of tumor cells. NKp46, NKp44 and NKp30 are all members of the immunoglobulin superfamily (73-75). Not the induced- but the missing-self recognition was the first strategy for NK cells being discovered (76). The principle is that the NK cell is inhibited by receptors specific for proteins expressed on the surface of normal cells. Strong inhibitory interactions can even prevent the lysis of target cells that express ligands for stimulatory NK receptors. Loss of the self protein, which can occur as a result of infection or transformation, unleashes the NK cell to attack the target cell. It is likely that missing-self recognition operates only when the target cell also expresses ligands for stimulatory receptors expressed by NK cells. In this sense, missing-self recognition occurs in concert with various forms of stimulatory recognition, rather than as a completely independent form of recognition. The mechanism of missing-self recognition was elucidated by the discovery of MHC-recognizing inhibitory receptors, including Ly49 receptors (77), the killer immunoglobulin-like receptors (KIRs) (78, 79), the leukocyte immunoglobulin-like receptors (LIRs) (80, 81) and the CD94-NKG2 receptors (82-84). The Ly49 and CD94-NKG2 receptors are lectin-like type II transmembrane proteins encoded in the NK gene complex whereas the KIR and LIR genes are immunoglobulin-like type I transmembrane proteins and are encoded in the leukocyte receptor gene complex. Ly49 receptors have a prominent function in mouse NK cells but are not functionally expressed in humans whereas KIR and LIR are important in humans but not in mice. Genes encoding CD94-NKG2A are transcribed in both species. It seems that the activation state of NK cells is regulated by the equilibrium between the input received from stimulatory (NKp, NKG2D) and inhibitory (NKG2A, KIR) NK cell receptors (57). Cellular stress like viral infection or

malignant transformation induces MHC class I chain-related proteins A and B (MICA/B) and UL-16 binding proteins (ULBP1-4), all of which are absent from normal somatic cells. Some expression is found on intestinal epithelia, most likely due to the continuous presence of symbiotic bacteria on these tissues. Activation of the NKG2D receptor by these ligands (62, 67) may overcome the inhibitory effect of “self recognition”. Activated NK cells would then induce apoptosis in the recognized target cell by releasing cytotoxic granules containing perforin and granzyme B and by upregulating death ligands (85-87). This NK cell response may therefore clear malignant cells before they can form a tumor (88).

Adaptive Immunity

The five major features of the adaptive immune system are specificity, diversity, memory, self restriction and the “self/non-self” discrimination. Adaptive immunity is mediated by B and T cells. Their receptors, BCR on B cells (with their secreted form Ig) and TCR on T cells, are able to adapt their specificity during their lifetime. Ig and TCR are highly variable molecules with a great variety in the variable (V)-region of the molecule which is binding the antigen. Ig are binding many chemically different antigens. $\alpha:\beta$ -TCR recognize peptide fragments of foreign proteins when presented by MHC molecules on cell surfaces. The stem cells of the innate and adaptive immune system originate in the bone marrow.

In chicken a special gland, the *bursa fabricii* was eponymous for the B cells (in humans one can bear in mind “B cells” for “bone marrow”, as the “T lymphocytes” mature in the “thymus”, although alike originating in the bone marrow).

CD27/CD70 costimulation. Effective activation of T cells not only requires signals from TCR after its interaction with peptide-MHC complexes but also costimulatory signals. Costimulatory molecules affect the level of T cell activation by enhancing T cell receptor signalling (89) or providing additional signals that increase expansion of T cell populations and their responses. The best defined costimulatory signals are mediated by interactions between CD28 and B7 as well as ICOS and ICOSL (90) and between TNF receptor family members and their ligands (91), exemplified by CD27 and CD70, OX40 and OX40L and 4-1BB and 4-1BBL (Fig. 1.3).

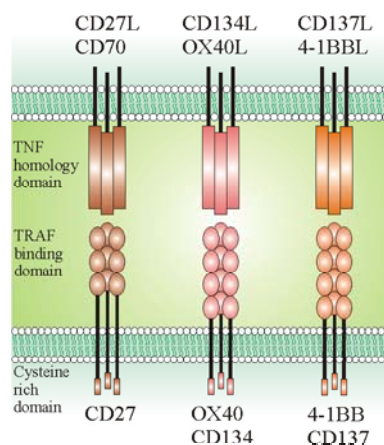


Fig. 1.3. **Representation of the costimulatory ligands of the TNF family and their receptors.** Ligands of the TNF family are homotrimeric type II transmembrane proteins with a carboxy-terminal extracellular domain and an amino-terminal intracellular domain. The C-terminal extracellular domain, also known as the TNF homology domain, has 20-30% amino acid identity between the superfamily members. The TNF receptor family molecules are type I transmembrane monomers (extracellular N-terminus and intracellular C-terminus) and are thought to trimerize when interacting with their ligands. The receptors are characterized by cysteine rich repeats in the extracellular domain. Soluble forms of receptors have been identified. Modified from (92).

The constitutive expression pattern of CD27 is at low-to-medium levels (93) whereas expression of OX40 and 4-1BB is inducible several hours after recognition of antigen (94, 95). CD70 seems not to be constitutively expressed, neither by naïve T cells (96) nor by resting or immature APC (Table 1), but is inducible 24 hours to several days after activation, largely coinciding with the peak level of CD27 expression by T cells (97). Since CD27 is not fully expressed until after activation and since CD70 has to be induced, this interaction might indicate a second round of costimulation during the clonal expansion phase of T cell responses.

Table 1. **Expression characteristics of CD27 and CD70 molecules by T cells and APCs.**
Table modified from (92).

Molecule	T cells			APC	
	Naïve	Effector	Memory	Resting	Activated
CD27	++	+++	++/-	-	B
CD70	-	+++	-	-	B,DC,MΦ

CD27 is a costimulating receptor of mature T and B cell responses. Clinical data revealed that CD27 can be used as a marker for human lymphoid malignancies (98, 99). Several *in vitro* blocking studies have indicated that CD27 is also important in the initial stages of a T cell response: proliferation and cytokine secretion were inhibited when the CD27-CD70 interaction is disrupted (100-102). T cells that lack CD27 initially divide normally, but then proliferate poorly 3 or more days after activation (103). This indicates that CD27 participates in promoting the initial expansion of the naive T cell population, by either early suppression of T cell death or by acting on the cell cycle to allow sustained division 2-3 days after activation. This is reinforced by *in vivo* studies of CD27-deficient mice, in which lower numbers of antigen-specific CD4⁺ and CD8⁺ T cells are seen at the peak of primary responses (days 4-8) and fewer memory T cells develop over 3 or more weeks (103). These observations have been supported by the analysis of mice that express a CD70 transgene in B cells (104), and from studies in which mice were immunized with CD70-transfected tumor cells (105, 106). In the former, widespread expression of CD70 leads to increased numbers of memory/effector T cells and, in the latter, priming of tumor-reactive CD8⁺ and CD4⁺ T cells is augmented. However, definitive mechanistic studies are still lacking, and it is unclear exactly when and how CD27-CD70 interactions influence T cell priming. Because expression of CD27 is upregulated early after T cell activation, it might mainly deliver signals that maintain early proliferation, prior to the peak of the effector response.

Immunosuppression by TGF- β . Secretion of TGF- β has been found in a variety of cancer types including malignant glioma, breast cancer, prostate cancer and leukemia. TGF- β is one of the most potent immunosuppressive cytokines yet characterized. It is capable of affecting the proliferation, activation and differentiation of cells participating in both the innate and acquired immune response. The proliferation of thymocytes, T cells, B cells, natural killer (NK) cells, monocytes and macrophages is inhibited by TGF- β . In addition to suppressing proliferation, TGF- β has been shown to induce apoptosis in B and T cells. Of particular importance is its effect on cytotoxic T lymphocytes (CTLs) which are probably required for anti-tumor immunity. TGF- β down-regulates many of the processes necessary for CTL activation. This is done by shifting the Th1-Th2 balance towards Th2, inhibiting Th1 cytokines including IL-12, downregulating IL-2 receptors on T cells, inhibiting antigen presentation on MHC class II molecules and downregulating adhesion and costimulatory molecules. Inhibiting antigen presentation prevents T cells from recognizing the cancer cells

as foreign and downregulating adhesion molecules prevents T cells from even getting to the tumor site. If the T cells do manage to get to the tumor site and recognize the tumor, a lack of costimulation can cause the Th cells to become anergic instead of activated. Shifting the Th balance towards Th2 changes cytokines produced to ones such as IL-4 and IL-10 that mediated a humoral response appropriate for intercellular invaders, not cancer cells. Inhibiting Th1 cytokine production further prevents the appropriate, cell-mediated immune response from taking place. TGF- β activates a serin/threonine kinase complex that phosphorylates Smad2 and Smad3. Once activated, Smads accumulate in the nucleus and form transcriptional complexes with Smad4 and different DNA binding partners, coactivators, and corepressors. These genes target different genes, depending on their composition (107). Carcinoma and glioblastoma cells that avert TGF- β -mediated cytostasis may use this factor with impunity to exacerbate their own proliferative, invasive, and metastatic behavior (108). The TGF- β cytostatic program involves transcriptional activation of the cyclin-dependent kinase inhibitors *p21Cip1* and *p15Ink4b* and repression of the growth-promoting transcription factors *c-myc* and *Id1-Id3*. Cooperatively, these gene responses mediate cell cycle arrest at G1. Many types of cancers have taken advantage of the regulatory role of cytokines to down-regulate appropriate immune responses targeted at destroying cancer cells. They do this by secreting immunosuppressive cytokines that induce generalized and specific inhibition of immune responses. Cancer patients often fail to mount sufficient or appropriate immune responses to their cancers.

Experimental overview

Restoring immune surveillance via CD70/CD27 interactions mediates glioma eradication

The first part of the results section of this PhD thesis describes a novel strategy for the reinduction of immune surveillance *via* CD70/CD27 interactions against mouse glioma cells. In this context, delivery of co-activatory signals *via* CD70 was investigated. Showing the imbalance of effector cell formation by immunosuppressive properties of glioma cells, the *in vitro* micromilieu proved to be apoptotic for immune cells (109), whereas the stimulatory capacity of CD70 outweighed its potential to transmit apoptotic stimuli *in vivo* as outlined in **chapter 2**. Furthermore, in the presence of apoptotic tumor cells, CD70 may trigger protective anti-tumor memory against wildtype glioma cells inoculated intracranially in the immunocompetent SMA-560 VM/Dk mouse model. The present work also sought to

characterize the classes of cell types involved in this anti-tumor immunity, showing participation of NK cells in the innate phase of defense *via* CD1^{nu/nu} mice, whereas during adaptive phases cytotoxic T cells seem to predominate. Another question was if TGF- β could interfere whilst tumor formation. This approach was addressed using a novel TGF- β -RI kinase inhibitor, SD-208, which abrogates TGF- β signalling (110). Cormary *et al.* have also shown strong anti-tumor effects in a mouse model using a soluble form of CD70 (111) evading the need of a genetic approach to transduce the whole tumor. This is relevant since gliomas are thought to relapse from transmigrated single cells.

ssRNA generates potent danger signals and elicits immunological memory

Bacterial DNA (112) or its synthetic version, CpG ODN (OligoDeoxyNucleotide) generate danger signals through Toll-like-receptor (TLR) 9 in the context of vaccinations and immunotherapies. *In vitro*, CpG ODN trigger a strong activation of human plasmacytoid DC and B cells. *In vivo*, intra-tumor injection of CpG ODN in mice results in tumor regression (113). This immunity is mediated by the release of Th1-type cytokines, the production of antibodies, and the induction of CTL and T helper cells. On the other hand, CpG ODN are very stable and trigger severe side effects in mice (114). Such uncontrolled overstimulation may prevent the generalization of CpG ODN as an immune stimulatory tool. Like DNA, RNA has also been shown to generate potent danger signals depending on TLR-signalling. Unlike CpG ODN, ssRNA is very sensitive to degradation by ubiquitous RNases and must be protected *in vitro* by encapsulation in liposomes (48, 115), condensation to a cationic peptide (Scheel *et al.*, unpublished data) or chemical modification by phosphothioate (ORN) backbones (42) to show immune stimulatory potency. ssRNAs are recognized by TLR7 and -8 (48). TLR7 is expressed in mice in the cytosole of DC. In humans, TLR7 is expressed in the cytosole of PDC, B cells, monocytes (41) and eosinophils (13), TLR8 mainly in the cytosole of monocytes (41). TLR7 and 8 are not only expressed on different cell types, but also trigger different responses. The activation of cells by ssRNA is MyD88-dependent (42) whereas stimulation with dsRNA is partially MyD88-independent (Hilf *et al.*, submitted). Thus, although the adjuvant and immunotherapeutic capacities of dsRNA were reported earlier, it is of interest to study the immune stimulatory properties of ssRNA. Ultimately, the interesting characteristics of these two types of RNA molecules may be combined in a potent RNA-only adjuvant or therapeutic tool. Based on this rationale ssRNA was used to generate an anti-tumor treatment in mice and compared to CpG-DNA (**chapter 3**). Our promising pre-clinical

evaluation of ssRNA as an anti-tumor treatment is currently being translated from the laboratory to the clinic.

TGF- β as a principal target for immunotherapy of malignant gliomas

The strongest mediator of glioma-induced immunosuppression is TGF- β . TGF- β_2 was named “glioblastoma-derived immunosuppressive factor” when it was discovered by Fontana *et al.* in 1987 (116, 117). TGF- β is the cytokine with the strongest known immunosuppressive activity. The direct effects of TGF- β on immune effector cells are well-documented (118) and have been further characterized in our group using the novel receptor kinase inhibitor SD-208 (**chapter 4**). TGF- β is synthesized as a pre-pro-TGF- β polypeptide that contains a signalling peptide (pre) (residues 1-29), the pro-region (residues 30-278) and the mature TGF- β moiety (residues 279-390) (119). Activation to the mature 12.5 kDa TGF- β_1 , that needs to dimerize to exert its biological effects, depends on the action of subtilisin-like proprotein convertases such as furin, as shown for purified proteins obtained from cell culture supernatants in a cell-free system (120). Subtilisin-like proprotein convertases such as furin are thought to mediate TGF- β processing. In **chapter 5** we report that human malignant glioma cell lines express furin mRNA and protein, exhibit furin-like protease (FLP) activity and release active furin into the cell culture supernatant. FLP activity is not modulated by exogenous TGF- β or by neutralizing TGF- β antibodies. Exposure of LN-18 and T98G glioma cell lines to the furin inhibitor, dec-RVKR-cmk, inhibits processing of the TGF- β_1 and TGF- β_2 precursor molecules and consequently the release of mature bioactive TGF- β molecules. Ectopic expression of PDX, a synthetic antitrypsin analog with antifurin activity, in the glioma cells inhibits FLP activity, TGF- β processing and TGF- β release. Thus, subtilisin-like proprotein convertases may represent a novel target for the immunotherapy of malignant glioma and other cancers or pathological conditions characterized by enhanced TGF- β bioactivity

MICA/NKG2D-mediated immunogene therapy of experimental gliomas

The failure of conventional cancer therapy renders glioblastoma an attractive target for immunotherapy. Tumor cells expressing ligands of the activating immunoreceptor NKG2D stimulate tumor immunity mediated by NK and CD8⁺ T cells. **Chapter 6** reports that human glioma cells express the NKG2D ligands MICA, MICB and members of the ULBP family constitutively. However, glioma cells resist NK cell cytotoxicity due to high MHC class I antigen expression. We investigated if plasmid-mediated or adenovirus-mediated (Ad-MICA)

overexpression of MICA enhances glioma cell sensitivity to NK and T cell responses *in vitro*. Furthermore we analyzed if the growth of subcutaneous and intracerebral LN-229 human glioma cell xenografts in nude mice and of SMA-560 gliomas in syngeneic VMDk mice is affected by MICA expression. Glioma cells forming progressive tumors after implantation of stably MICA-transfected human LN-229 cells have lost MICA expression, indicating a strong selection against MICA expression *in vivo*. Rejection of MICA-expressing SMA-560 cells in VMDk mice results in protective immunity to a subsequent challenge with wild-type tumor cells. Finally, the growth of syngeneic intracerebral SMA-560 tumors is inhibited by the peripheral vaccination with Ad-MICA-infected irradiated tumor cells, and vaccination results in the immune cell activation in the NK and T cell compartments *in vivo*. These data commend MICA immunogene therapy as a novel experimental treatment for human malignant gliomas. By targeting TGF- β (chapters 4 and 5) the relief of glioma-induced immunosuppression could be combined with the active induction of NK and T cell responses against cancer cells.

II. CD70/CD27 interactions and the immune response to malignant glioma: balancing immune effector formation

A related manuscript has been submitted to Journal of Immunology, 2005 January

I want to thank René A.W. van Lier (Department of Haematology, Academic Medical Centre, University of Amsterdam, The Netherlands) for the purified polyclonal anti-mCD70 antibodies (3B9, 6D9) and polyclonal anti-mCD70 F(ab')₂ fragments (3B9, 6D9), further thank goes to Jannie Borst (Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands) who supplied the C57BL/6 CD27^{-/-} mice. She also gave helpful advice during many stages of this project. Jörg Wischhusen who had published a previous study on CD70 contributed with valuable advice and discussion on CD70 interactions. Manuel Friese introduced me to the NK immunological assays and accompanied the project immunologically.

Introduction

The protein encoded by the CD70 gene (121) is a type II transmembrane protein physiologically expressed on mature DC and activated T and B cells, but not on resting lymphocytes. CD70 belongs to the tumor necrosis factor family. Its receptor, CD27 (TNFRSF7) (122), a type I glycoprotein and member of the TNF receptor family, is expressed constitutively by T and NK cells and acquired by B cells after activation (123). CD27 counteracts apoptosis in primed T cells and thereby promotes the accumulation of cytolytic effector cells as well as T cell memory (124, 125). Deliberate constitutive CD70 expression on B cells in transgenic mice thus allows the rejection of poorly immunogenic tumors (125). CD70/CD27 interactions also play a role in regulating B cell expansion and consequent plasma cell formation (126, 127), and the cytotoxic function of NK cells (124).

Soluble CD70-secreting cells induce T cell-mediated inhibition of wild-type tumor growth (111) in immunocompetent mice. In a RMA-S tumor model (128), CD70/CD27 interactions provide a key link between innate NK-mediated and adaptive T cell tumor rejection. However, chronic stimulation of CD27 in CD70 transgenic mice results in a progressive conversion of naïve T cells into effector-memory cells. This has no effect on immunity towards a challenge with vaccinia virus, but leads to progressive B (104) and T cell depletion, rendering the mice susceptible to opportunistic infections (129).

Immune stimulatory signals transduced *via* CD27 are mediated by the adaptor proteins TRAF2 and TRAF5 (130) and result in the activation of NF- κ B and MAPK8/JNK. In addition, Siva, a pro-apoptotic protein, can bind to the cytoplasmic tail of CD27 and has been implicated in apoptosis induction *via* CD27. Mouse Siva-1 and Siva-2 both bind to CD27 but differentially transmit apoptotic signals. The shorter alternate splice form Siva-2 has no death domain homology region (131) and thus cannot induce apoptosis.

The major focus of our previous studies was the immune privilege created by human glioblastoma cells which involves the release of soluble factors such as TGF- β , IL-10 and prostaglandins, as well as cell surface molecules such as CD95L and HLA-G (132-134). A cDNA array analysis of irradiated glioma cells led to the identification of CD70 as an aberrantly expressed molecule in glioma cells.

Unexpectedly, in contrast to the presumed role of CD70/CD27 interactions in lymphoid cell survival, CD70 expressed on glioma cells induced immune cell apoptosis rather than immune stimulation (109). The present work illustrates that CD70 expressed on glioma cells exhibits

opposing immune inhibitory and activating properties *in vitro*, but clearly overwhelming immune activating properties in mouse glioma models *in vivo*.

Materials and methods

Reagents and cell lines. Purified polyclonal anti-mCD70 Abs (3B9, 6D9) and polyclonal anti-mCD70 F(ab')₂ fragments (3B9, 6D9) were a kind gift of R.A.W. van Lier (Amsterdam, The Netherlands). Mouse IL-2 and IL-10 and human TGF-β₂ were from Peprotech (London, UK). Concanavalin A and camptothecin (CPT) were from Sigma-Aldrich Chemie (Taufkirchen, Germany). zVAD-fmk was from Bachem (Weil am Rhein, Germany). The flow cytometry reagents including FITC-labeled AnxV were from Pharmingen unless indicated otherwise. [*methyl*-³H] thymidine (925 GBq/mmol) and ⁵¹Cr (Sodium chromate/18.5 GBq/mg) were from Amersham Biosciences (Freiburg, Germany). SMA-560 cells (135) were kindly provided by D.D. Bigner (Durham, NC), GL-261 cells by X.O. Breakefield (Boston, MA). The cells were grown in DMEM supplemented with 10% heat-inactivated FCS (Biochrom KG, Berlin, Germany), 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). To generate glioma cell lines expressing CD70, a mouse CD70 cDNA (InvivoGen, San Diego, CA) was cloned into pIRES2-hygro (Clontech, Palo Alto, CA) to yield pIRES-mCD70. pIRES-mCD70 was transfected into SMA-560 or GL-261 cells by lipofection with FuGENE6 (Roche, Mannheim, Germany). The cells were subsequently screened for expression of mouse CD70 by flow cytometry using the FR70 mAb. Pan-specific TGF-β-neutralizing antibody was purchased from R&D (Wiesbaden, Germany). SD-208 was kindly provided by SCIOS (Fremont, CA) (110).

Animal experiments. C57BL/6 mice and CD1^{nu/nu} mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Inbred VM/Dk mice were obtained from the TSE Resource Centre (Berkshire, UK). The generation of CD27^{-/-} mice has been described (10). They were backcrossed for eight generations to a C57BL/6 background. The experiments were performed according to the German animal protection law. For the intracranial glioma model, groups of 5-6 mice aged 6-12 weeks were anesthetized and placed in a stereotaxic fixation device (Stoelting, Wood Dale, IL). A burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe (Darmstadt, Germany) was introduced to a depth of 3 mm. Five x 10³ SMA-560 cells or 2 x 10⁴ GL-261 cells resuspended in a volume of 2 μl PBS were injected into the right striatum. The mice were observed twice daily and, in the survival experiments, sacrificed when developing neurological symptoms, or sacrificed as indicated in the other experiments. For the s.c. glioma model, groups of 5 mice were injected 10⁶ glioma cells suspended in 0.1 ml PBS into the right flank. The mice were examined

regularly for tumor growth using a metric caliper and killed when tumors reached a diameter of more than 12 mm diameter. SD-208 was administered by oral gavage twice every second day (15 mg/kg) from day 3-7 post inoculation (110).

Flow cytometry. The adherent glioma cells were detached nonenzymatically using cell dissociation solution (Sigma). Cell cycle analysis was performed using fixed and ethanol (70%)-permeabilized B, T and NK cells. RNA was digested with RNase A (Gibco). DNA was stained with PI (50 µg/ml). B, T and NK cell apoptosis was also analyzed by AnxV staining for phosphatidylserine translocated to the outer cell membrane. Fluorescence was measured in a Becton Dickinson FACSCalibur. SFI were calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with control antibody.

Isolation of NK, B and T cells. Spleen cells from VM/Dk and C57BL/6 mice were depleted of erythrocytes by hypoosmolaric (NH₄Cl 1.66% (w/v)) treatment for 5 min. T and B cells were isolated by negative selection using mouse Lineage Panel (Pharmingen, San Diego, CA) monoclonal biotinylated antibodies and streptavidin MicroBeads with the corresponding column system (Miltenyi Biotech, Bergisch Gladbach, Germany) to avoid nonspecific activation. Purity of T and B cells was > 95% CD3ε⁺ or B220⁺ by flow cytometry. Splenocytes, T, B and DX5⁺-purified NK cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. For coculture experiments, purified splenocytes, B, T or NK cells (10⁶/well) were cultured with SMA-560 CD70 or hygro cells (10⁵/well) in 24 well plates for 24-72 h.

RT-PCR. Total RNA was extracted using the RNeasy RNA purification system (Qiagen, Hilden, Germany). RT-PCR for the detection of CD70 mRNA expression was performed according to standard procedures. The following mouse CD70 specific primers were used with an annealing temperature of 50.5 °C: 5'-GTAGCGGACTACT CAGTAAG-3' (119-138), 5'-CAAGGGCATATCCACTGAAC-3' (587-568), yielding a 469 bp product.

QPCR. Gene expression was measured in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers (B&G Biotech, Freiburg, Germany) were selected with the Husar Genius software (DKFZ, Heidelberg, Germany) to span exon-intron junctions to prevent amplification of genomic DNA and to obtain amplicons of <150 bp to enhance efficiency of PCR amplification whenever possible. The sequences of the primers were as follows: mouse Siva-1: 5'-GGCCTAT AGAGATCACATATCGAG-3'(153-176), 5'-CCTTCTGAGGCTGAGCTTG-3' (311-292), yielding a 159 bp product, mouse Siva-2: 5'-GGTCTTCGGCCTTCCC AGG-3' (111-129), 5'-CTCAGGCTTCAAACATAGCACAG-

3' (334-312), yielding a 224 bp product. Product specificity of each PCR product was further confirmed by agarose gel electrophoresis or by dissociation curve analysis. Amplification of specimens and serial dilutions of the amplicon standards were carried out in a total volume of 15 μ l using 2x SYBR Green Master Mix (Applied Biosystems) and primers at optimized concentrations. Standard curves were generated for each gene and the amplification was 90-100% efficient as determined by the slopes of the standard curves. Relative quantification of gene expression was done using the comparative $\Delta\Delta C_T$ method. 18S rRNA was used as housekeeping gene.

Cytotoxicity assay. Mouse NK cells were prepared from splenocytes of VM/Dk, C57BL/6 or C57BL/6 CD27^{-/-} mice and cultured with mouse IL-2 (5000 U/ml) for at least 10 d before use. Glioma cell targets (10⁴/well) were labeled using ⁵¹Cr (50 μ Ci, 90 min) and incubated with effector cells at E:T ratios of 5-40. The maximum ⁵¹Cr release was determined by the addition of NP40 (1%). After 4 h the supernatants were transferred to a Luma-Plate TM-96 (Packard, Dreieich, Germany) and measured in a Wallac 1450 Microbeta Plus Liquid Scintillation Counter (Turku, Finland). The percentage of ⁵¹Cr release was calculated as follows: 100 x [experimental release – spontaneous release]/[maximum release – spontaneous release].

Immunohistochemistry. Glioma or mouse brain cryosections (0.7 mm) were prepared at days 5 or 12 after s.c. glioma cell inoculation, fixed in acetone and blocked with 2% normal rabbit serum and 2% BSA. Tissue sections were stained with antibodies (1:50) to CD70 (FR70), CD8 (53-6.7), Ly49G2 (detecting NK cells) and CD11b (M1/70, detecting macrophages and microglial cells) (BD Pharmingen, Heidelberg, Germany). A biotinylated anti-rat secondary antibody (Zymed, San Francisco, CA) was used at 1:150. Avidin conjugate (ABC-Elite Kit Vector, Wiesbaden, Germany) was added, and the staining was developed with diaminobenzidine. Mouse spleen (Ly49G2, CD11b and CD8) and thymus (CD70) served as positive controls. Cells were counted in high power fields (x 40 in an area of 400 μ m x 400 μ m).

Statistical analysis. The experiments were performed at least three times. Significance was tested by Student's *t*-test. P values are derived from two-tailed *t*-tests.

Results

Balancing effector lymphocyte formation *via* CD70/CD27 interaction: increased immune cell apoptosis *in vitro* but enhanced tumor cell immunogenicity. The glioma cell line SMA-560 was derived from a SMA syngeneic in VM/Dk mice and does not express CD70 constitutively. To assess the biological effects of CD70 expression by glioma cells in a syngeneic tumor model, we cloned mouse CD70 into pIRES and generated stably transfected polyclonal SMA-560 sublines expressing high levels of mouse CD70 (Fig. 2.1.A,B). The ectopic expression of CD70 in SMA-560 cells had no effect on doubling time and clonogenicity *in vitro* (data not shown). Further, mouse GL-261 glioma cells, which are syngeneic to C57BL/6 mice, were transfected with the pIRES-CD70 plasmid. The overexpression of CD70 in the polyclonal glioma sublines reached a SFI of 7.1 in SMA-560 and 6.9 in GL-261 cells.

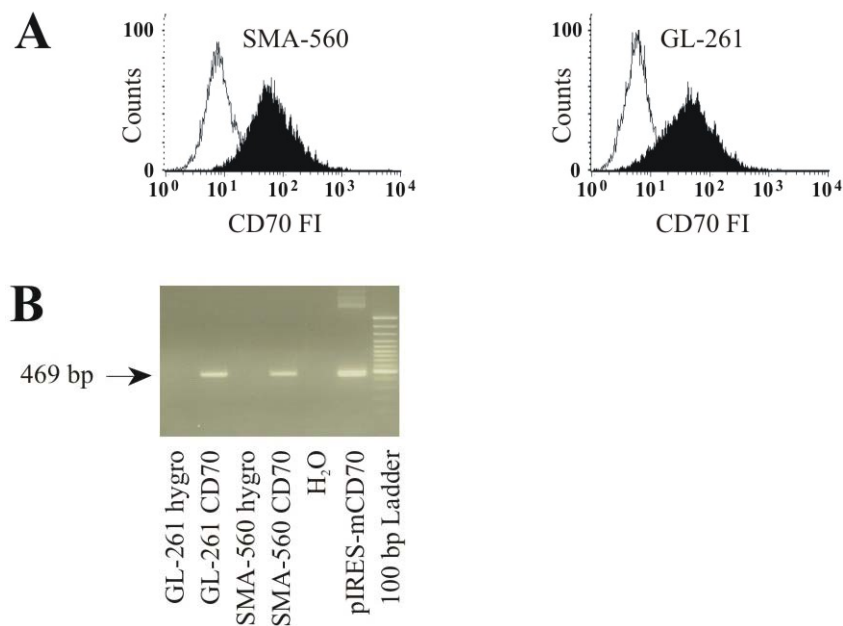


Fig. 2.1.A,B **CD70 expression in mouse glioma cells.** A,B. SMA-560 or GL-261 cells were transfected with pIRES-hygro (open histogram) or pIRES-mCD70 (black histogram) and assessed for transgene expression by flow cytometry using CD70 (FR70) antibody (A) and RT-PCR (B).

To confirm our previous finding that CD70 expressed on glioma cells induces T cell apoptosis in alloreactivity assays (109), we characterized the fate of various negatively selected immune cell populations cocultured with syngeneic SMA-560 hygro or CD70 cells. There was a significant increase in the proportion of apoptotic splenocytes, T, B and NK

cells defined by PI flow cytometry for hypodiploid cells (Fig. 2.1.C,D) or by AnxV staining (data not shown) in cocultures containing SMA-560 CD70 cells compared with SMA-560 hygro cells. The pro-apoptotic effect of CD70 appeared to depend on caspase activation since the broad-spectrum caspase inhibitor zVAD-fmk was protective (Fig. 2.1.D).

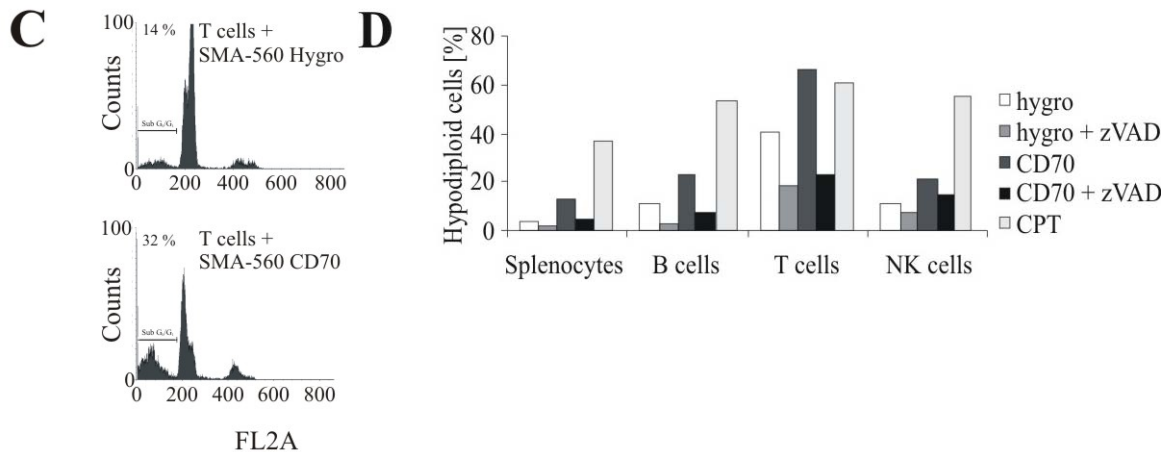


Fig. 2.1.C,D **CD70 promotes immune cell apoptosis.** Syngeneic VM/Dk splenocytes, B, T or NK cells were co-cultured with SMA-560 hygro or CD70 cells in the absence or presence of zVAD-fmk (100 μ M) for 72 h. Exposure to CPT (4 μ M) for 6 h served as a positive control. Immune cells were fixed, permeabilized and stained with PI. Representative flow cytometry profiles for T cells are shown in C, depicting an increase in cell death from 14% to 32% in cocultures with hygro vs. CD70 cells. In D, data for splenocytes, B, T or NK cells are expressed as percentages of cells with hypodiploid DNA content (sub G₀/G₁ fraction).

CD27-dependent apoptosis has been proposed to require the pro-apoptotic adaptor protein Siva-1 whereas its truncated form Siva-2 is thought to block apoptosis (131, 136). In cocultures with CD70-transfected glioma cells, an almost complete loss of Siva-1 mRNA as well as an increase in Siva-2 mRNA expression were observed (Fig. 2.1.E), suggesting that splenocytes surviving the coculture with CD70-positive glioma cells are selected for resistance to pro-apoptotic CD27 signalling or adapt to the CD70 challenge or both. This adaptation required cell-cell contact, and thus probably CD70/CD27 interactions, since it was not mimicked by glioma cell supernatant. Of note, a weaker alteration of the respective Siva-1 and Siva-2 levels was also observed in T cells cocultured with control-transfected CD70-negative cells which may reflect an adaptation to CD27-independent pro-apoptotic pathways

involving Siva-1 (137-139). Moreover, no changes in Siva-1 or Siva-2 mRNA expression were noted in response to IL-2 or TGF- β (data not shown).

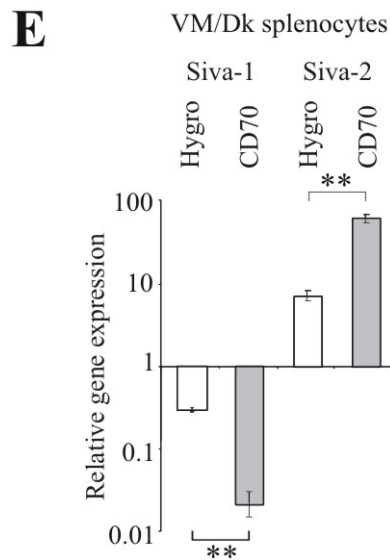


Fig. 2.1.E. **CD70 modulates Siva expression.** Syngeneic splenocytes were cocultured with SMA-560 hygro (open bars) or CD70 (gray bars) cells for 72 h. Siva-1 and Siva-2 mRNA expression was assessed by QPCR and is expressed relative to untreated splenocytes (** $p < 0.01$, t -test, CD70 vs. hygro, $n=3$).

T cell apoptosis induced by coculture with SMA-560 hygro or CD70 cells (Fig. 2.1.C) was inhibited by exogenous IL-2 in a concentration-dependent manner (Fig. 2.1.F).

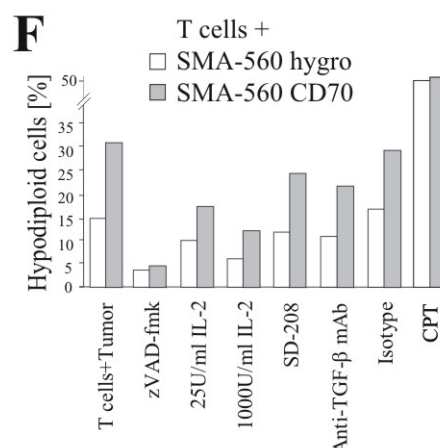


Fig. 2.1.F. **IL-2 and TGF- β contribute to the apoptotic milieu *in vitro*.** Cocultures (48 h) of syngeneic T cells and SMA-560 hygro (open bars) or CD70 (grey bars) cells were supplemented with zVAD-fmk (100 μ M), mouse IL-2 (25 or 1000 U/ml), SD-208 (0.1 μ M), TGF- β mAb (10 μ g/ml), isotype control mAb (10 μ g/ml) or CPT (4 μ M).

Further, apoptosis was facilitated by endogenous TGF- β since either exposure to neutralizing TGF- β mAb or to the TGF- β receptor I kinase antagonist, SD-208, reduced apoptosis. Appropriate control experiments showed that the ectopic expression of CD70 in SMA-560 or GL-261 glioma cells did not affect the expression of TGF- β_1 or TGF- β_2 mRNA as assessed by QPCR (data not shown). Altogether, these data corroborate in a syngeneic mouse system previous data from alloreactivity assays in the human system (109). However, in apparent contrast to the immune paralytic effects of CD70 expressed on glioma cells summarized in (Fig. 2.1.C-F), NK cells nevertheless lysed SMA-560 CD70 cells significantly better than SMA-560 hygro cells. NK lytic activity was abolished by a neutralizing CD70 antibody F(ab')₂ fragment, but not by an isotype F(ab')₂ fragment (Fig. 2.1.G).

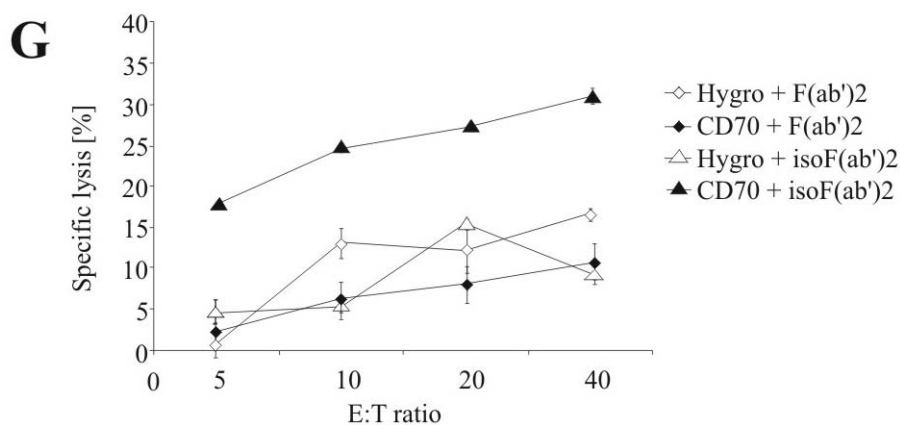


Fig. 2.1.G. **CD70 enhances tumor cell immunogenicity.** The cytotoxicity of syngeneic VM/Dk DX5⁺-selected (10 d, 5000 U/ml IL-2) NK cells was tested against ⁵¹Cr-labeled SMA-560 hygro (open) or CD70 cells (black) in the presence of control F(ab')₂ fragment (triangles) or CD70 F(ab')₂ fragment (diamonds) (clone 3B9, 10 μ g/ml).

CD70 inhibits the growth of SMA-560 gliomas in CD1^{nu/nu} mice. Athymic CD1^{nu/nu} mice are deficient in T and B cells and therefore a suitable model to assess the role of NK cells in the response to tumor allo- or xenografts. The growth of s.c. SMA-560 gliomas expressing CD70 was significantly delayed compared with gliomas formed by hygro control cells (Fig. 2.2.A). Similarly, there was a delay in the development of neurological symptoms and thus an increase in overall survival in CD1^{nu/nu} mice inoculated i.c. with CD70-expressing SMA-560 glioma cells (Fig. 2.2.B).

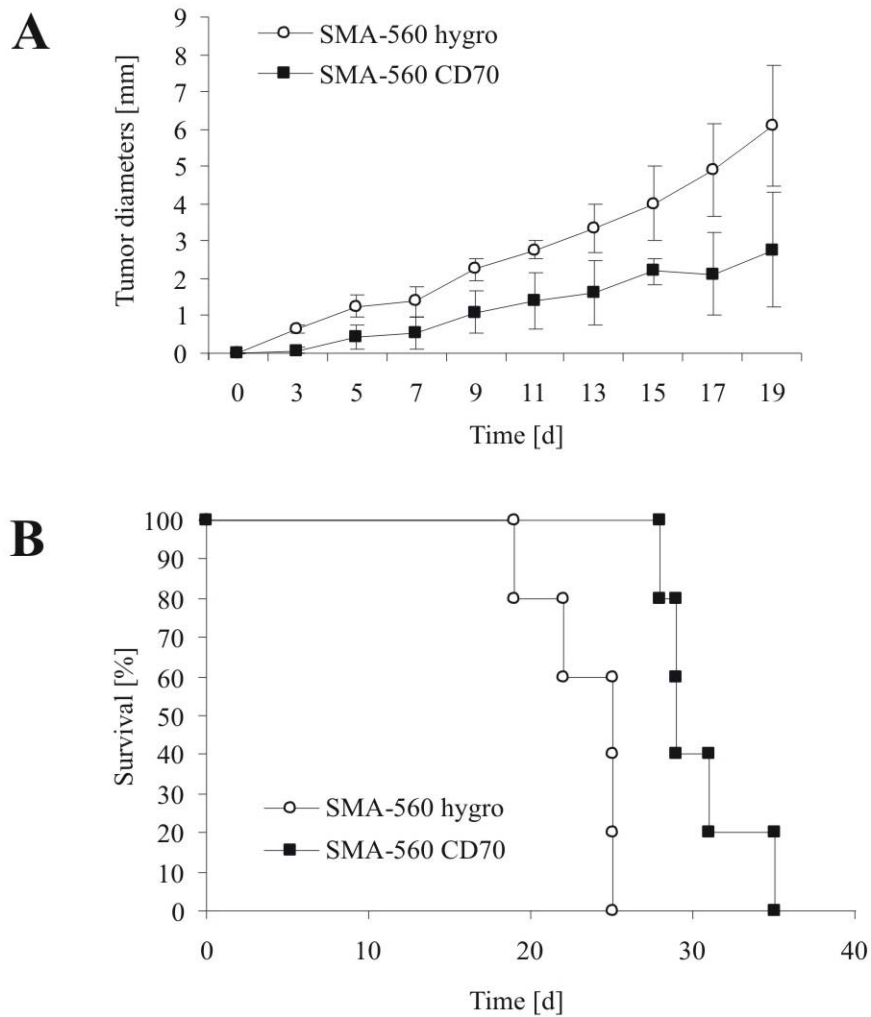


Fig. 2.2.A,B **CD70 delays the growth of s.c. and i.c. SMA-560 gliomas in nude mice.** A. 10^6 SMA-560 hygro (open circles) or CD70 (black squares) cells were inoculated s.c. into $CD1^{nu/nu}$ mice. Tumor growth was examined every second day with a caliper. Data are expressed as mean diameters and SEM (n=5). B. 5×10^3 SMA-560 hygro (open circles) or CD70 cells (black squares) were inoculated i.c. into $CD1^{nu/nu}$ mice. The mice were monitored for survival.

The mean survival was prolonged from 23.2 ± 2.5 days (median 25) in control animals to 30.4 ± 2.6 days (median 29) in animals carrying CD70-expressing tumors. The survival rate at 27 days was 100% in the latter, but 0% in the control mice. Maintenance of transgene expression *in vivo* was ascertained by immunocytochemistry on day 5 (Fig. 2.2.C-F). These data strongly suggest that CD27-expressing NK cells of $CD1^{nu/nu}$ mice interact with CD70 expressed on glioma cells *in vivo*, that NK cells gain access to glioma-bearing mouse brain and that the CD70-mediated increase in NK cell susceptibility probably outweighed the proapoptotic effect on immune cells *in vivo* in mice.

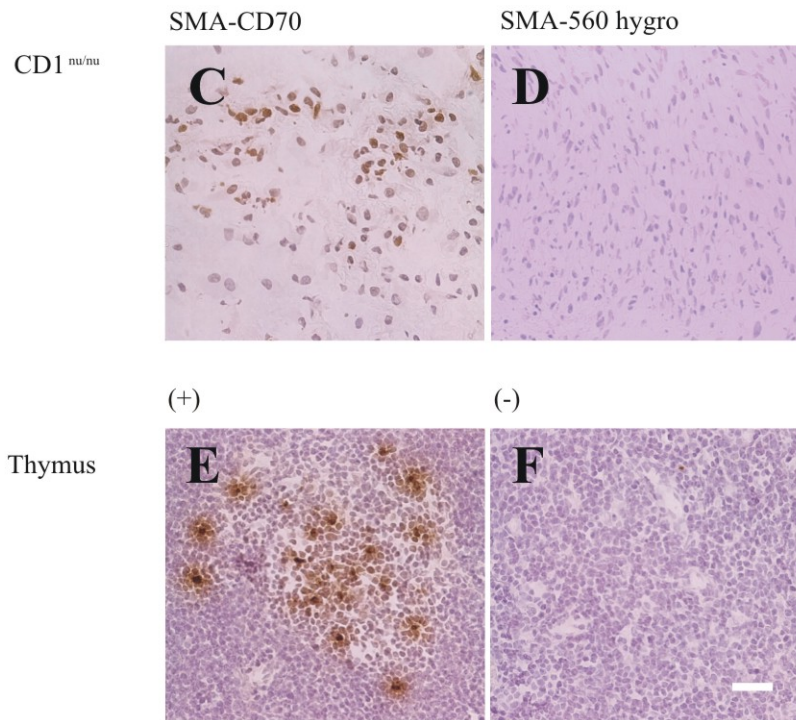


Fig. 2.2.C-F. **CD70 expression was assessed by immunocytochemistry using CD70 (FR70) antibody on day 5 (size bar in F: 40 μ m):** s.c. SMA-560 CD70 (C) or hygro (D). Thymus stained with specific antibody served as a positive control (E), staining with isotype control antibody was the negative control (F).

CD70 mediates tumor regression in immunocompetent VM/Dk mice. We next explored the immune response to CD70-expressing SMA-560 gliomas in immunocompetent syngeneic VM/Dk mice. SMA-560 hygro cells inoculated s.c. formed rapidly growing tumors whereas CD70-expressing SMA-560 gliomas showed a delayed growth which peaked around day 12, followed by tumor regression evolving over a few days (Fig. 2.3.A). Previous work has demonstrated that immune responses to glioma cells are counteracted by large quantities of TGF- β released by glioma cells (140, 141). Here we observed that tumor formation by CD70-expressing glioma cells was abrogated when the animals bearing CD70-expressing gliomas were treated with the TGF- β RI kinase antagonist, SD-208. SD-208 also delayed the growth of s.c. SMA-560 hygro tumors. Moreover, VM/Dk mice challenged i.c. with CD70-expressing SMA-560 cells never developed neurological symptoms, and their survival was 100% with a median observation of more than 60 days. In contrast, control animals experienced a median survival time of 18.8 ± 1.2 days (Fig. 2.3.B).

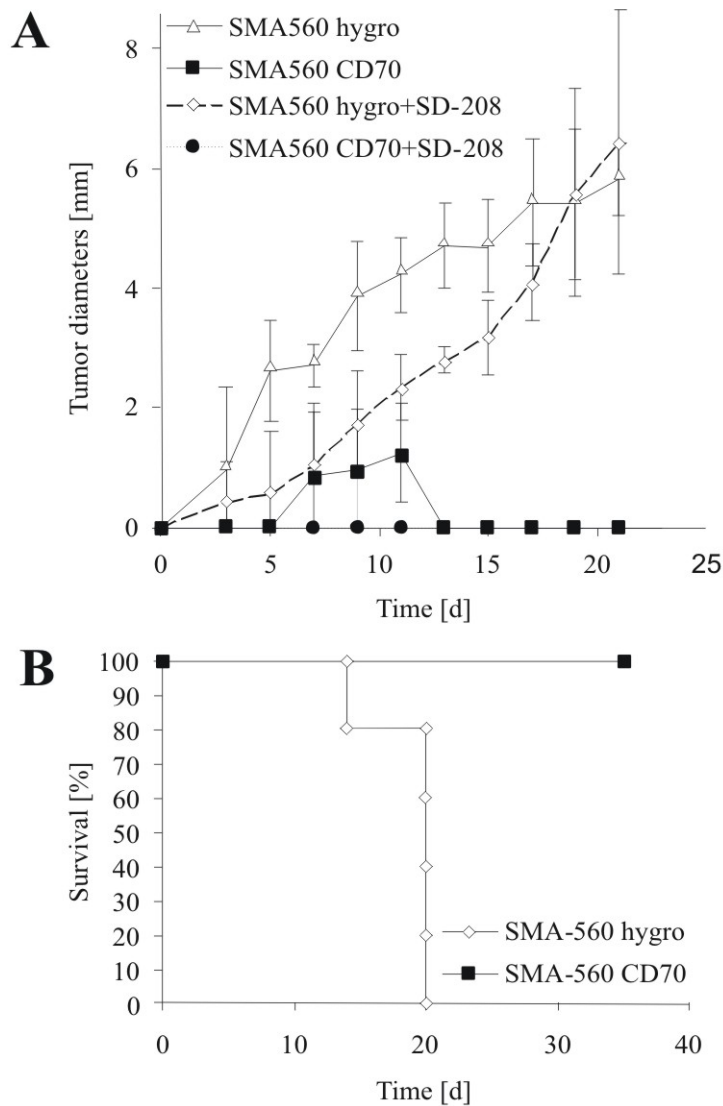


Fig. 2.3.A,B CD70 promotes the rejection of s.c. and i.c. SMA-560 gliomas in syngeneic mice.
 A. 10^6 SMA-560 hygro or CD70 cells were inoculated s.c. into VM/Dk mice. The mice were treated with vehicle or SD-208 (15 mg/kg) on Days 3, 5 and 7 twice a day. Tumor growth was examined every second day with a caliper. Data are expressed as mean diameters and SEM (n=5).
 B. 5×10^3 SMA-560 hygro or CD70 cells were inoculated i.c. into VM/Dk mice. The mice were monitored for survival.

Immunocytochemistry confirmed persistent transgene expression in CD70-transfected SMA-560 glioma cells whereas control tumors were negative (Fig. 2.3.C, D). Tumor-free s.c. challenged mice were rechallenged i.c. with 5×10^3 wild-type SMA-560 glioma cells and remained symptom-free for more than 60 days (Fig. 2.3.E, F). Thus CD70 expression on tumor cells apparently allowed for the recruitment of a tumor-specific T cell repertoire and the establishment of tumor-specific memory.

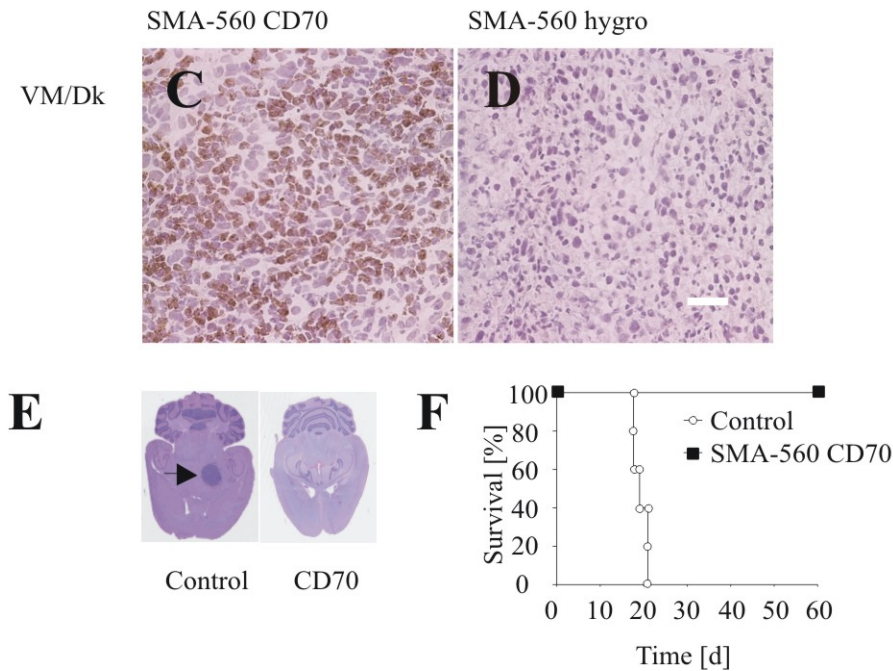


Fig. 2.3.C-F. **In vivo CD70 expression and wt i.c. rechallenge of s.c. SMA-560 CD70 challenged mice.** CD70 expression was assessed by immunocytochemistry in s.c. SMA-560 CD70 (C) or hygro (D) tumors on day 5 (size bar: 40 μ m). E,F. SMA-560 CD70 s.c. surviving or untreated control mice (n=3) were (re)challenged 60 days later i.c. with 5×10^3 SMA-560 wild-type glioma cells (E), representative brain sections at day 12, arrow denotes tumor in control animal, (F), survival curve).

Mouse CD70 gene transfer promotes immune cell infiltration in the SMA-560 syngeneic mouse glioma model. The infiltration of the experimental gliomas by immune cells was examined in s.c. tumors at day 12 after tumor inoculation using specific mAb for NK cells (Ly49G2), macrophages/microglia (CD11b) or T cells (CD8). A similar extent of NK cell infiltration was observed in CD70-negative and CD70-positive tumors of CD1^{nu/nu} and VM/Dk mice (Fig. 2.4.A-D, Table 1). There also was no difference in the extent of macrophage infiltration between SMA-560 hygro or CD70 tumors in either s.c. mouse model (Fig. 2.4.G-J). However, there was a significant increase in CD8 T cells in SMA-560 CD70 compared with hygro tumors in VM/Dk mice (Fig. 2.4.M, N, Table 1).

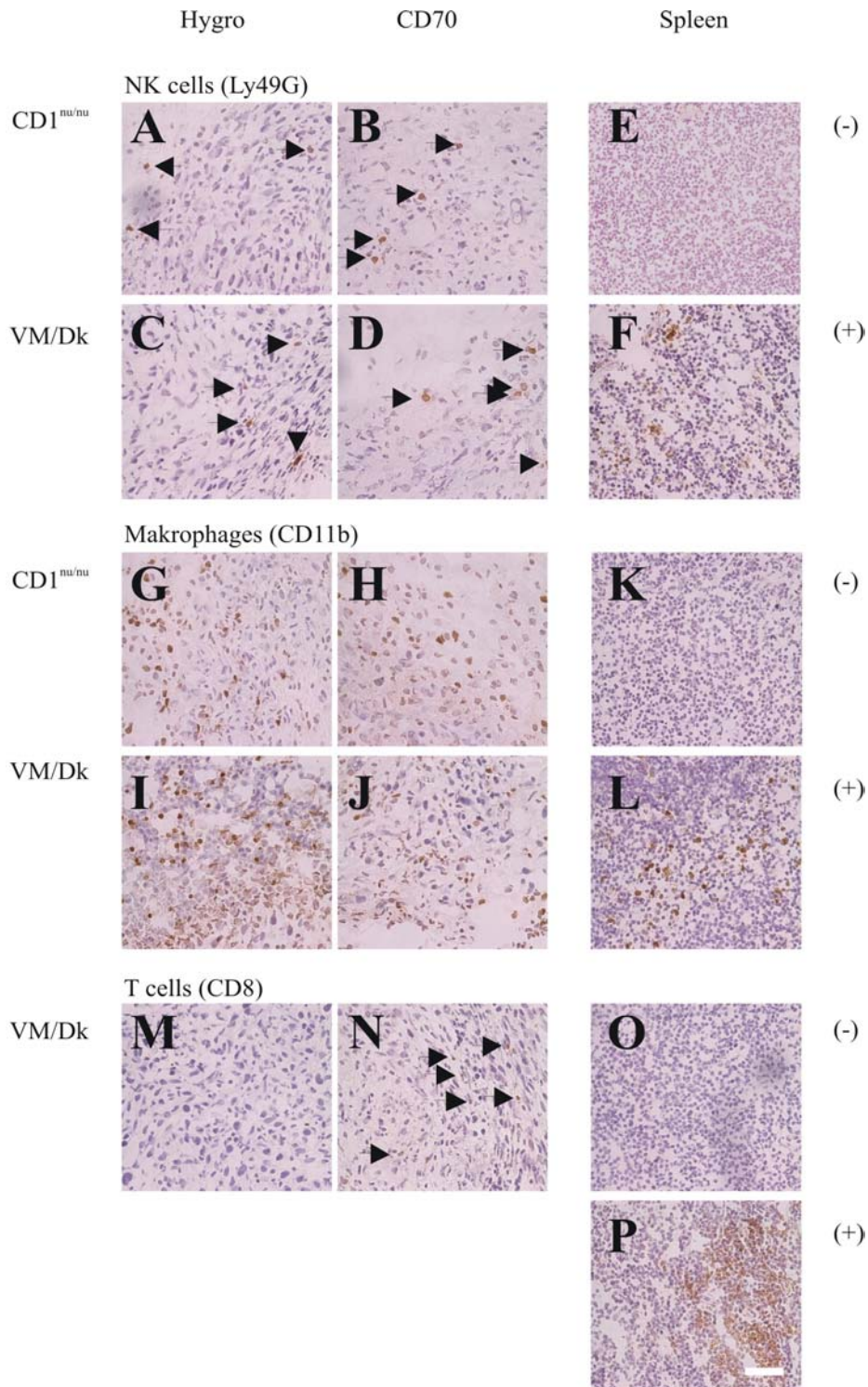


Fig. 2.4.A-P. **Immune cell infiltration in CD70-negative and CD70-transfected SMA-560 tumors in the s.c. VM/Dk syngeneic mouse glioma model.** SMA-560 hygro (left column) or CD70 (middle column) tumors grown in the right flank of CD1^{nu/nu} (A,B,G,H) or VM/Dk (C,D,E,F,I,J,K,L,M,N,O,P) mice were assessed for infiltrating Ly49G⁺ NK cells (A-D), macrophages (G-J) or CD8⁺ T cells (M and N) on day 12. Spleen served as a positive control (+) with specific antibody and negative control (-) with isotype antibody (right column) (size bar: 40 μ m).

Table 1. Patterns of immune infiltration in control and CD70 s.c. SMA-560 tumors on day ¹

CD1nu/nu	NK cells	SMA-560 hygro	8.0±1.0
		SMA-560 CD70	9.5±2.5
	Macrophages	SMA-560 hygro	17.0±3.0
		SMA-560 CD70	16.0±4.0
VM/Dk	NK cells	SMA-560 hygro	7.0±1.0
		SMA-560 CD70	3.5±1.5
	Macrophages	SMA-560 hygro	36.0±3.0
		SMA-560 CD70	41.0±3.0
	T cells	SMA-560 hygro	0.5±0.5
		SMA-560 CD70	26.5±1.5*

¹Data are expressed as mean percentages and S.E.M. of labeled cells within a high power field (*p<0.05, *t*-test)

Disruption of the CD27 gene in C57BL/6 mice nullifies CD70-mediated anti-tumor responses. To demonstrate the unique requirement for CD70/CD27 interactions mediating tumor rejection as shown in Fig. 2.2. and Fig. 2.3., we reproduced the observations made in the VM/Dk model using the GL-261 glioma cell line in syngeneic C57BL/6 mice. Like SMA-560 cells, GL261 cells did not express CD70 constitutively, but expressed high levels after pIRES-CD70 transfection (Fig. 2.1.A). Similar to SMA-560 cells, CD70 enhanced the susceptibility of GL-261 to NK cell lysis. In contrast, there was no difference in the lytic activity of NK cells from CD27^{-/-} mice against GL-261 CD70 or hygro cells (Fig. 2.5.A).

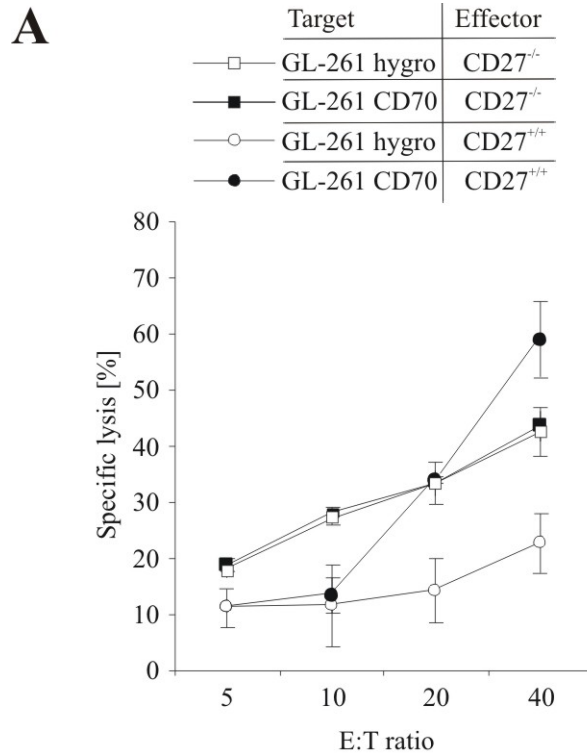


Fig. 2.5.A. **CD70-mediated tumor regression requires CD27 interaction.** A. The cytotoxicity of syngeneic C57BL/6 DX5⁺-selected (10 d, 5000 U/ml IL-2) NK cells from wild-type (circles, dashed lines) or congenic CD27^{-/-} C57BL/6 mice (squares, solid lines) was tested against ⁵¹Cr-labeled GL-261 hygro (open symbols) or CD70 cells (filled symbols).

Further, immunocompetent C57BL/6 mice challenged s.c. with GL-261 hygro cells developed large tumors whereas CD70-expressing GL-261 gliomas regressed within 15 days (Fig. 2.5.B). In contrast, CD27^{-/-} mice developed large s.c. tumors irrespective of whether CD70 was expressed or not (Fig. 2.5.B). Finally, C57BL/6 mice challenged i.c. with CD70-expressing GL-261 cells developed neurological symptoms, but 50% of the mice survived. In contrast, control animals experienced a median survival time of 38.5±2.5 days (Fig. 2.5.C).

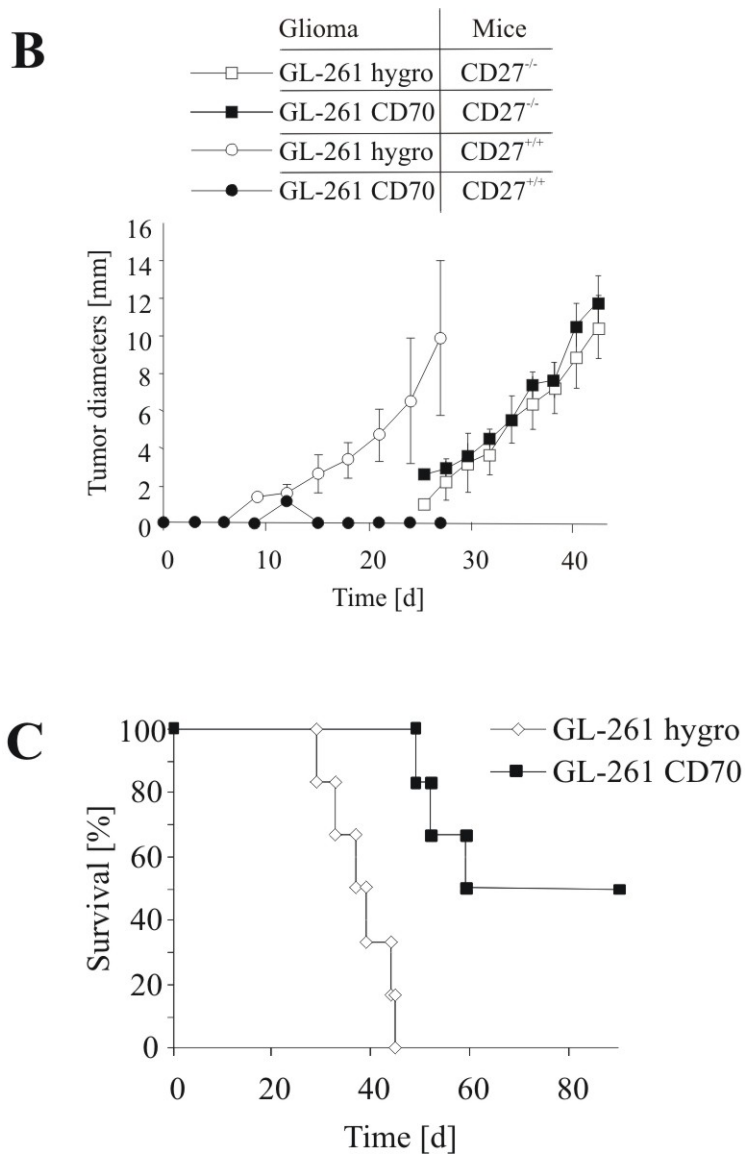


Fig. 2.5.B,C. **CD70 promotes the rejection of s.c. and partially i.c. GL-261 gliomas in syngeneic mice.** 10^6 GL-261 hygro or CD70 cells were inoculated s.c. into wild-type (circles) or CD27^{-/-} (squares) C57BL/6 mice. Growth was assessed by determining mean diameters and SEM (n=5). C. 2×10^4 GL-261 hygro or CD70 cells were inoculated i.c. into C57BL/6 mice which were then monitored for survival.

Discussion

Glioblastoma is a paradigmatic type of cancer which exerts multiple effects, mostly inhibitory, on various aspects of immune cell function. Among the growing list of molecules with potential immune inhibitory function expressed by glioma cells, we previously identified CD70, the ligand for CD27 (109). Although CD70/CD27 interactions in the lymphoid system support expansion and differentiation by promoting survival (127), CD70 expressed on human glioma cells strongly inhibited alloreactivity and promoted apoptosis in the immune compartment *in vitro* (109). The induction of immune cell apoptosis *via* CD70/CD27 interactions was confirmed in syngeneic glioma mouse models here when CD70 was expressed in the mouse glioma cell lines SMA-560 and GL-261 (Fig. 2.1.C-F, data not shown).

Interestingly, mRNA levels of the pro-apoptotic adaptor molecule Siva-1 and its anti-apoptotic counterpart Siva-2 changed in splenocytes challenged with CD70-positive tumor cells. The minor effects observed for SMA-560 hygro cells, which are necessarily CD70-independent, may be due to changes in transcription mediated by p53 or E2F1 (137) or interactions of Siva and Bcl-2 family proteins (138). The massive induction of Siva-2 and the almost complete loss of Siva-1 (Fig. 2.1.E) may represent a mechanism by which mouse splenocytes adapt to chronic stimulation *via* CD27 and escape from CD70-mediated apoptosis. Fig. 2.1.F demonstrates that immune cell apoptosis evolving in cocultures with glioma cells is a complex process regulated at several levels, including CD70/CD27 interactions. Thus, IL-2 inhibits apoptosis whereas TGF- β promotes apoptosis, both in the absence and presence of CD70. In conclusion, one might assume that the net effect of CD70 expressed on glioma cells acting on CD27 expressed on immune cells *in vivo* might depend on the absence or presence of further immune activating or inhibitory signals operating in microenvironment of human gliomas.

In fact, despite the confirmation of immune cell apoptosis mediated by CD70 in the mouse system (Fig. 2.1.C-F), SMA-560 CD70 glioma cells were lysed significantly better by NK cells than control cells (Fig. 2.1.G). We therefore set out to determine which of these opposing effects of CD70 expressed on glioma cells would dominate *in vivo*. To this end, we used three different model systems of s.c. and i.c. glioma growth: (i) SMA-560 gliomas grown in nude mice, (ii) SMA-560 gliomas grown in syngeneic VM/Dk mice and (iii) GL-261 gliomas grown in wild-type and CD27^{-/-} syngeneic C57BL/6 mice.

The growth delay of s.c. and i.c. CD70-expressing gliomas in nude mice suggests a role for CD27 expressed on NK cells as a pathway of immune surveillance (Fig. 2.2). This is because CD1^{nu/nu} mice possess innate immunity only since they are athymic and therefore devoid of T and B cells. Enhanced tumor rejection mediated by NK cells may reflect the ability of CD70 to stimulate the lytic activity of NK cells by increasing proliferation and IFN- γ production (142) as well as effector and target conjugate formation (124). Consequently, CD70-transfected tumor cells are cleared more efficiently than control transfectants.

Compared with the nude mice model, the syngeneic model provided evidence for cures and immunological memory when CD70 was expressed, attributing a key role to T cell-mediated immunity in rejecting CD70-expressing gliomas (Fig. 2.3). There was no significant difference in macrophage infiltration, but a CD70-dependent increase in CD8 T cell infiltration (Table 1). The growth inhibition presumably mediated by NK cells in nude mice together with the cures achieved in immunocompetent mice are fully consistent with a role for CD70 in the cooperation between NK and T cell immunity (128).

We have previously demonstrated the pivotal role of TGF- β as an immune inhibitory molecule and therefore promising molecular target in glioblastoma (110, 140, 141, 143). Here we observed an additive effect of CD70-mediated immune stimulation and the abrogation of TGF- β signalling, mediated by the TGF- β receptor I kinase antagonist, SD-208 (Fig. 2.3.A). These results are consistent with a central role for glioma-derived TGF- β in the priming phase of cytotoxic T lymphocytes or in the potential to suppress innate immunity or both. Alternatively, TGF- β may play a role in the initial seeding of gliomas in the host tissue, given its role in invasion and angiogenesis (144).

The net immune stimulatory effect of CD70 expressed on glioma cells was then confirmed in another syngeneic model, GL-261 glioma cells grown in C57/BL6 mice (Fig. 2.5) which had the advantage that CD27^{-/-} mice were available (145) to confirm the specificity of the observations made in the VM/Dk model (Fig. 2.3, 2.4). In fact, the disruption of CD27 expression nullified the immune stimulatory properties of CD70 gene transfer in GL-261 glioma cells (Fig. 2.5.A, B).

The expression of CD70 in human glioblastomas (109) which ultimately kill their host clearly shows that the suppressed immune system of human glioma patients cannot eliminate CD70-expressing glioma cells. Nevertheless, the present study shows that CD70, a cell surface protein and ligand for the TNF receptor-related CD27 protein, which was identified as a radio-inducible gene in glioma cells (109), has the potential to mediate strong immune responses in several *in vivo* glioma models, suggesting that CD70/CD27-interactions could

be exploited in the context of future strategies of active immunotherapy for glioblastoma, notably those relying on the antagonism of TGF- β and the reinforcement of IL-2-dependent immune activation.

III. Anti-tumor immunity induced by injections of immune stimulatory RNA in tumor-bearing mice

A related manuscript is under preparation for submission in Cancer Research

I want to thank Birgit Scheel (at that time Ph. D. student at Tübingen University) who contributed to this project as an equal author, and Dr. Steve Pascolo (CureVac) who initiated this project and made the ssRNA synthesized by CureVac GmbH (Paul-Ehrlich-Str. 15, 72076 Tübingen, Germany) available. Hopefully ssRNA turns out to be as potent as in our project in the ambitious clinical phase I study.

Introduction

Glioblastomas are the most common malignant intrinsic brain tumors in adults (146). Despite surgery, radiation therapy and chemotherapy, affected patients experience a median survival of only 12 months. Although they express a variety of antigens (147), glioblastomas do not seem to be recognized by the immune system. A reason for this apparent immune ignorance may be that glioma cells induce active immunosuppression through the release of TGF- β (148, 149). Immunological ignorance may be a frequent phenomenon in tumor patients. *In situ* immunostimulation is a method to overcome immunosuppression and to signal tumor cells to the immune system. This phenomenon was observed more than 100 years ago when W. Coley injected bacterial products at the tumor site. Coley found that this treatment was associated to protection against sarcoma in more than 10% of patients (150). The injected prokaryotic components are now known to be immune stimulatory due to danger signals such as unmethylated CpG motifs in bacterial DNA, endotoxins and flagellin. A danger signal in the context of vaccinations and immunotherapies appears to be bacterial DNA (112) or its synthetic version, CpG ODN (OligoDeoxyNucleotide). By mimicking the conserved immune stimulatory feature of bacterial genomic DNA, CpG ODN activate immune cells through Toll-like-receptor (TLR) 9. *In vitro*, CpG ODN trigger a strong activation of human plasmacytoid DC and B cells. *In vivo*, intra-tumor injection of CpG ODN in mice results in tumor regression (113). This immunotherapy activates a local immunity that kills tumor cells and triggers a systemic immunity. This preventing the formation of metastases and providing long-term anti-tumor immune memory. This immunity is mediated by the release of Th1-type cytokines, the production of antibodies and the induction of CTL and T helper cells. Still, the transfer of the pre-clinical results to the clinic may be limited by the different pattern of TLR9 expression in mouse and human immune cells. More importantly, CpG ODN are very stable and trigger severe side effects in mice (114). Such uncontrolled overstimulation may prevent the clinical value of CpG ODN as a immune stimulatory tool.

Other danger signals originating from viruses include double-stranded RNA or stabilized single-stranded RNA (ssRNA). Like DNA, RNA has also been shown to be a potent danger signal dependent on TLR-signalling. Unlike CpG ODN, ssRNA is very sensitive to degradation by ubiquitous RNases and must be protected *in vitro* by encapsulation in liposomes (48, 115), condensation to a cationic peptide (Scheel *et.al.*, unpublished data) or chemical modifications (phosphorothioate ORN) (42) to show immune stimulatory potency. Protamine-stabilised mRNA can readily activate human immune cells whereas

phosphorothioate ORN must be encapsulated in liposomes to be immune stimulatory *in vitro* for human cells. ssRNAs are recognized by TLR7 and -8 (48). TLR7 is expressed in mice in the cytosole of DC. In humans, TLR7 is expressed in the cytosole of PDC, B cells, monocytes (41) and eosinophils (13), TLR8 mainly in the cytosole of monocytes (41). TLR7 and 8 are not only expressed on different cell types, they also trigger different responses. The activation of cells by ssRNA is MyD88 dependent (42) whereas stimulation with dsRNA is partially MyD88-independent (Hilf *et.al.*, submitted). Thus, although the adjuvant and immunotherapeutic capacities of dsRNA were reported earlier, it is of interest to study the immune stimulatory properties of ssRNA. Ultimately, the interesting characteristics of these two types of RNA molecules may be combined in a potent RNA-only adjuvant or therapeutic tool.

Here, we investigated the possibility to use ssRNA as an anti-tumor treatment in mice and compare it to CpG-DNA. We found that intratumor or subcutaneous injections of ssRNA are as potent as injections of CpG-DNA as an anti-tumor immunotherapy. As opposed to CpG-DNA, repeated ssRNA injections were not associated with side effects such as splenomegaly. This pre-clinical evaluation of ssRNA as an anti-tumor treatment is currently being translated from the laboratory to the clinic.

Material and methods

Mouse strains and animal experiments. Inbred VM/Dk mice were obtained from the TSE Research Centre (Berkshire, UK). The experiments were performed according to the German animal protection law. For the subcutaneous glioma model, groups of 5 mice aged 6-12 weeks were injected s.c. in the right flank with 10^6 SMA-560 cells resuspended in a volume of 100 μ l PBS. The mice were observed daily and, in the survival experiments, sacrificed when developing neurological symptoms, or sacrificed as indicated in the other experiments. The mice were examined every second day for tumor growth using a metric caliper and killed when tumors reached ≥ 12 mm diameter. Treatment started on day 3 post inoculation. 50 μ l PBS containing 25 μ g RNA alone, protamine-condensed RNA or CpG-DNA 1826 were injected in the vicinity of the tumor or into the contralateral flank every other day.

Cell lines. SMA-560 cells (135) were kindly provided by D.D. Bigner (Durham, NC). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom KG), 2 mM L-glutamine (Gibco Life Technologies), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Gibco). The cells were routinely screened for mycoplasma contamination.

RNA and oligonucleotides. Nucleic acids were produced by CureVac. For the production of messenger RNA, linearized DNA templates containing the β -galactosidase gene under a phage promoter were transcribed for two hours using the T7 or the SP6 RNA polymerase (CureVac). Then, DNase (CureVac) was added to the reaction and after 30 min at 37°C, the RNA was precipitated by LiCl and washed with ethanol 75%. The RNA pellet was resuspended in water, quantified by OD260, extracted by phenol/chloroform and re-precipitated by ethanol/ammonium acetate. After a wash of the pellet with ethanol 75%, the RNA was re-suspended in water at 1 mg/ml, checked on a formaldehyde/agarose gel, aliquoted and stored at -20°C until utilization. Protamine-protected RNA was produced by mixing 1 mg/ml RNA with an equal volume of a 1 mg/ml solution of protamine sulfate (Leo Pharma, Ballerup, Denmark). The content of endotoxins in the preparations was tested using the Limulus Amebocyte Lysate (LAL) assay (Cambrex Bio Science, East Rutherford, NJ) being consistently below 10 IU/mg RNA. Injection preparations were supplemented with 10x PBS to a concentration of 500 μ g/ml RNA in 1x PBS. CpG ODN was synthesized by CureVac following standard protocols. In all ODN the phosphate group was substituted by a phosphorothioate group desensitizing the nucleic acid backbone against RNase degradation. The sequence used was 1826 5'-TCCATGACGTTCCCTGACGTT-3'.

Stimulation of splenocytes. Splenocytes of BALB/c mice were isolated. After red blood cell lysis, cells were incubated in 96-well U-bottom plates at 2×10^5 cells/well in 200 μ l of medium RPMI with 10% heat inactivated FCS (both from PAA, Wien, Austria), 2 mM L-Glutamine (Bio Whittaker), 10 μ g/ml Streptomycin, 10 U/ml Penicillin (PEN-STREP, Bio Whittaker) with protamine-condensed mRNA at 10 μ g/ml for 16 h. Cells were stained with CD 86 PE and B220 FITC (BD Pharmingen) and analyzed by flow cytometry with a FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Cytokine ELISA. Seventeen hours after addition of the particular stimulus, 100 μ l of the splenocyte (DC) supernatant was removed. ELISA plates (Nunc, Maxisorb) were coated overnight with capture antibody in binding buffer (NaN_3 0.02%, Na_2CO_3 15 mM, NaHCO_3 15 mM, pH 9.7), blocked for one hour with PBS containing 1% BSA, and then incubated with 100 μ l of DC supernatant for 4 hours at 37°C, 7.5% CO_2 in a humidified atmosphere. Biotinylated antibody (BD Pharmingen) at a concentration of 0.5 μ g/ml in blocking solution was added after 4 washing steps with PBS 0.05% Tween and incubated for 2 hours at room temperature. HRP-Streptavidin (BD Pharmingen) was diluted 1:1000 in blocking solution and added in the wells (100 μ l/well). After three washing steps, 100 μ l ABTS (300 mg/l 2,2'-Azino-bis-(3-ethylbenzthiazoline-6)-sulfonic acid (Sigma) in 0.1 M citric acid, pH 4.35) per well were added, and the levels of cytokines were quantified by OD_{405} and calculated according to a standard curve made in the ELISA by titrating amounts of recombinant cytokines (BD Pharmingen).

ELISPOT. For *ex vivo* ELISPOT the ELISPOT plates were coated over night with IFN- γ antibody in coating buffer at 4°C. After 2 h of blocking at 37°C and 3 washing steps with serum-containing medium, splenocytes of the mice were taken and counted after red blood cell lysis by Gey's solution (7.0 g/l NH_4Cl , 0.37 g/l KCl , 0.3 g/l $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.024 g/l KH_2PO_4 , 1.0 g/l glucose, 10.0 mg/l phenol red, 8.4 mg/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 7.0 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.8 mg/l CaCl_2 and 45 mg/l NaHCO_3). Different cell numbers were then coincubated in ELISPOT plate (Millipore) with 5000 irradiated (28 Gy) SMA-560 cells per well. After 24 h incubation at 37°C and 6 washing steps with PBS Tween 0.05% the second antibody was added at 1 μ g/ml in PBS Tween containing 1% BSA. Incubation was performed at 37°C for 2 h.

Immunohistochemistry. Glioma cryosections (8 μ m) were prepared after the mice were sacrificed. For staining, they were fixed in PFA and blocked with 2% normal rabbit serum, 2% BSA using the biotin blocking system (Dako Cytomation, Hamburg, Germany). Tissue sections were stained with antibodies (1:50) to CD8 (53-6.7) and CD11b (M1/70, detecting

macrophages and microglial cells) and NK cells (Ly49G2) (BD Pharmingen). A biotinylated anti-rat secondary antibody (Zymed) was used at 1:150. Avidin (Vector Labs) conjugate was added, and the staining was developed with diaminobenzidine (Vector Labs). Mouse spleens served as positive controls, normal mouse brain as a negative control.

Crystal violet staining. The SMA-560 cells were seeded in 96 well plates at 10^4 cells/well and allowed to attach for 24 h. Protamin-condensed β -Gal mRNA at different concentrations was added in fresh serum-free medium for 24 h, camptothecin served as a positive control (4 μ M for 6 h). After staining with crystal violet for 15 minutes at room temperature, unspecific staining was washed away with H₂O. Viable cell counts were obtained by crystal violet staining analysed by absorption at $\lambda=550\text{ nm}$.

Statistical analysis. The experiments reported herein were usually performed at least three times with similar results. Significance was tested by Student's *t*-test. P values are derived from two-tailed *t*-tests.

Results

BMDC and B cells are activated by stabilized mRNA. Synthetic ssRNA oligonucleotides stabilized by a phosphorothioate backbone have immune stimulating properties in mice (115). Condensation of *in vitro* transcribed mRNA to the cationic peptide protamine is another method to generate stabilized ssRNA capable of stimulating mouse DC (Scheel *et.al.*, unpublished data). In order to check whether protamine-mRNA stimulates other cell types than DC in mice, we incubated splenocytes for 16 h with protamine-condensed mRNA at 10 μ g/ml. This results in the production of IL-6 (Fig. 3.1.A) and IL-12 (data not shown). After 2 days of culture, B220⁺ splenocytes (B cells) were evaluated for the up-regulation of CD86. The baseline CD86 expression detected at the surface of B cells cultured in medium without additional molecules or in the presence of protamine, is strong up-regulated when splenocytes are cultured in the presence of poly I:C, CpG-DNA or protamine-condensed mRNA (Fig. 3.1.B). Thus, protamine-mRNA complexes not only activate myeloid mouse DC but also stimulate B cells in fresh mouse spleen.

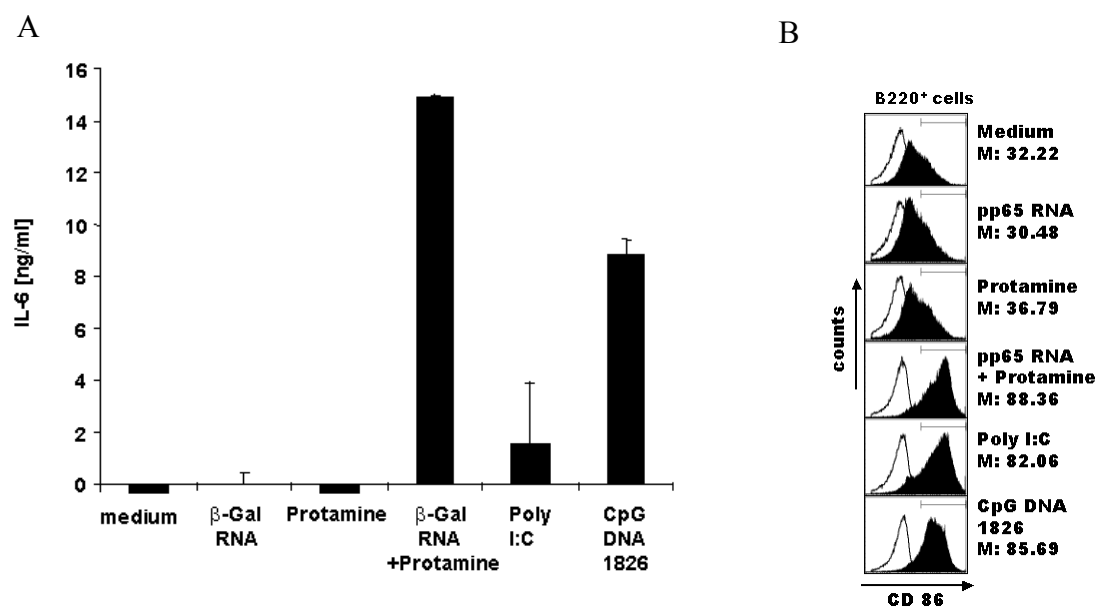


Fig. 3.1.A,B. **Activation of mouse splenocytes.** A. Release of IL-6 by BMDC after 16 h co-culture with poly I:C, CpG-DNA, protamine, uncapped β -Gal mRNA or protamine-condensed uncapped β -Gal mRNA. Cytokine release was measured by ELISA. B. Up-regulation of CD86 on B220-positive cells. Splenocytes were co-incubated for 16 h with poly I:C, CpG-DNA, protamine, uncapped pp65 mRNA or protamine-condensed pp65 mRNA. The surface expression CD86 was measured by flow cytometry following multicolour staining: B220 FITC and CD86 PE.

Stabilized mRNA exhibits anti-tumor properties comparable to CpG-DNA. Since danger signals such as CpG-DNA or poly I:C are potent *in situ* immune stimulatory molecules, we were interested to see whether ssRNA injected into solid tumors induces tumor regression. Mice were inoculated with 10^6 SMA-560 glioma cells subcutaneously (s.c.) in the right flank. The treatment involved injections into the tumor of 50 μ g RNA alone, 50 μ g protamine alone, 50 μ g mRNA condensed to 50 μ g protamine or 50 μ g CpG ODN 1826. One group of tumor-implanted mice were injected s.c. with 50 μ g mRNA condensed to 50 μ g protamine into the non-tumor-bearing flank. The treatment was started on day 3 after tumor inoculation and performed every second day. Each group consisted of 5 mice. In all mice tumors grew rapidly and were palpable 3 days after implantation. Mice had to be sacrificed when tumor sizes reached more than 12 mm in diameter. Injection of mRNA alone or protamine-protected mRNA into the tumor led to a noticeable delay in tumor growth, as did the injection of CpG-DNA (Fig. 3.2).

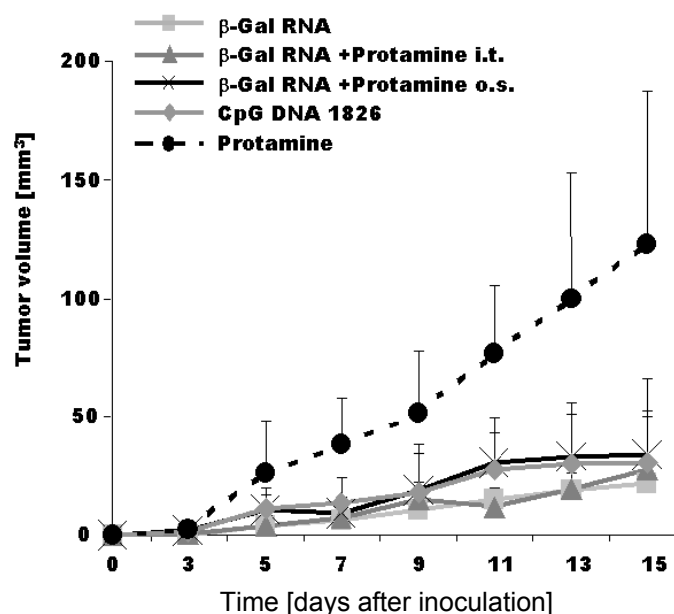


Fig. 3.2. **Course of tumor development.** Mice were inoculated with 10^6 SMA-560 glioma cells s.c. in the flank and injected every other day with β -Gal mRNA, protamine-condensed β -Gal mRNA, CpG-DNA or protamine into the tumor (i.t.) or into the opposite flank (o.s.). Tumor size was measured every second day using a metric caliper. Tumor volume is given in mm^3 .

Interestingly the same effect on tumor growth could be observed when the protamine-condensed mRNA was injected into the contralateral flank. Two of 5 mice in the CpG ODN-treated group were tumor-free after 21 days, and 1 of 5 mice in the group injected with

mRNA protamine into the tumor and into the other flank. In the group treated with mRNA alone, tumor growth was delayed but not impeded whereas protamine alone treated mice experienced aggressive tumor growth and had to be sacrificed.

Intratumoral injection of stabilized mRNA leads to memory immune response. At the end of the experiment all mice that were tumor-free (two in the CpG treated group, one each in the mRNA protamine intra tumor and contralaterally injected group) were re-challenged with an injection of 10^6 glioma cells into the opposite flank. As a control, 2 mice that had not received tumor cells in the first round of the experiment were inoculated with 10^6 tumor cells in the right flank. The tumor size was measured every second day and mice were sacrificed when tumor sizes reached more than 12 mm in diameter. Whereas the control mice developed fast growing tumors and had to be sacrificed, none of the mice that had recovered from the primary tumor challenge showed any palpable tumors (Fig. 3.3). Mice were observed for 60 days after re-challenge and remained tumor-free. Thus, immunotherapy of solid tumors with mRNA may trigger long-term anti-tumor immunity.

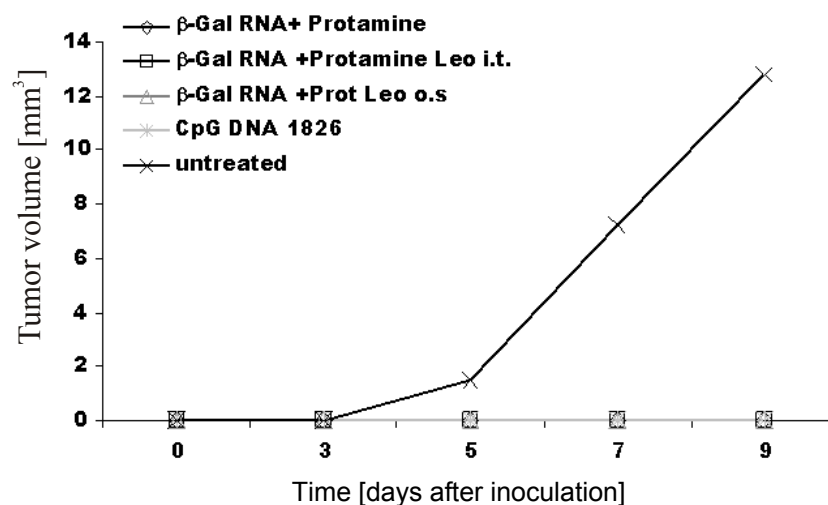


Fig. 3.3. **Rechallenge of tumor-free mice.** 4 weeks after the beginning of the treatment, the mice that were by then tumor-free were again inoculated with 10^6 SMA-560 glioma cells s.c. in the other flank. Tumor size was measured using a metric caliper every second day.

No tumor-specific, IFN- γ -producing cells are detectable in the spleen. We performed immunomonitoring studies using the spleens of the tumor challenged mice. In an ELISPOT assay, fresh splenocytes were mixed with SMA-560 cells (Fig. 3.4.). Although the average number of spots may grossly correlate with the clinical outcome, in several animals, the detected immune response was lower in cured mice compared to mice with progressive disease.

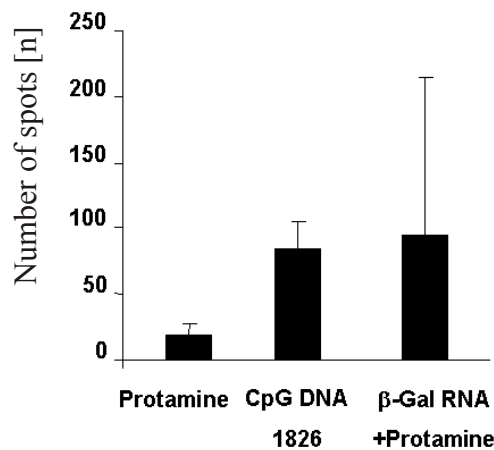
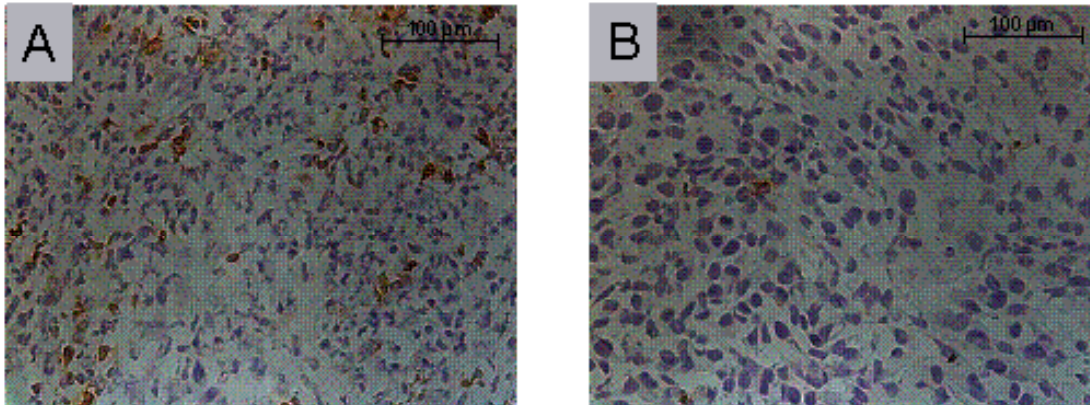


Fig. 3.4. **IFN- γ ELISPOT of splenocytes.** After the mice were sacrificed, splenocytes were coincubated overnight in an ELISPOT plate with SMA-560 cells as targets. IFN- γ release was measured by ELISPOT. The number of spots given is the mean of three experiments .

Intratumoral injection of stabilized mRNA leads to a specific immune response and tumor infiltrating cells. We tested whether immune cells infiltrated regressing or progressing tumors in mice treated with CpG-DNA, stabilized mRNA or protamine alone. When the mice were sacrificed, tumors were surgically removed and cryopreserved. Cryosections of tumors treated with CpG-DNA (A), protamine-condensed mRNA (B) and protamine (C) were stained with antibodies against cytotoxic T cells (CD8) (Fig. 3.5) and NK cells (Ly49G2) (data not shown). Spleen sections of healthy mice were used as positive control. In all regressive tumors (injected with CpG ODN or protamine-condensed mRNA) an infiltration of CD8+ cells was observed. Few NK cells were also detected. Progressive as well as regressive tumors contained many CD11b positive cells (data not shown).



mRNA + Protamine

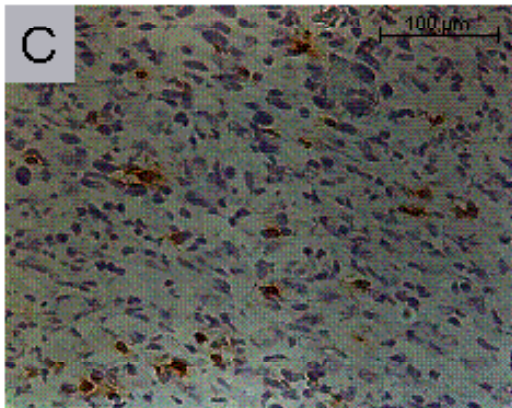


Fig. 3.5.A-C. **Antibody staining of tumor sections.** Glioma cryosections were stained with CD8 antibody (53-6.7) and examined by light microscopy to analyse infiltrating cells (200X, Axioplan II Carl Zeiss). A. CpG-treated tumor. B. Protamine mRNA-treated tumor. C. Protamine-treated tumor.

Protamine-condensed mRNA is nontoxic. One of the well-known side effects induced by repeated CpG injections is uncontrolled B cell proliferation evidenced by enlarged spleens. To screen for splenomegaly, spleens from treated tumor-implanted mice were weighed. As shown in Fig. 3.6.A, the spleen weight of mice treated with protamine-condensed mRNA remained normal, i.e. similar to the one of controlled mice (untreated, not shown, or protamine-injected mice). In contrast, the spleens of the mice that received CpG ODN were approximately 3-fold larger than these of protamine or protamine-mRNA treated mice. Thus,

whereas repeated injections of CpG ODN induce splenomegaly, this is not the case for repeated injections of protamine-mRNA.

To investigate a possible direct toxicity of stabilized mRNA on tumor cells, we also performed an *in vitro* toxicity experiment. The tumor cells were co-incubated with different concentrations of protamine-condensed mRNA, stained with crystal violet and retention of crystal violet by viable cells was measured by absorption. There was no direct toxicity even at high mRNA-protamine concentrations (Fig. 3.6.B). Thus, stabilized mRNA does not induce a direct lysis of tumor cells but rather, according to experiments shown in Fig. 3.3 and 3.4 triggers an anti-tumor immune response that is responsible for tumor regression *in vivo*.

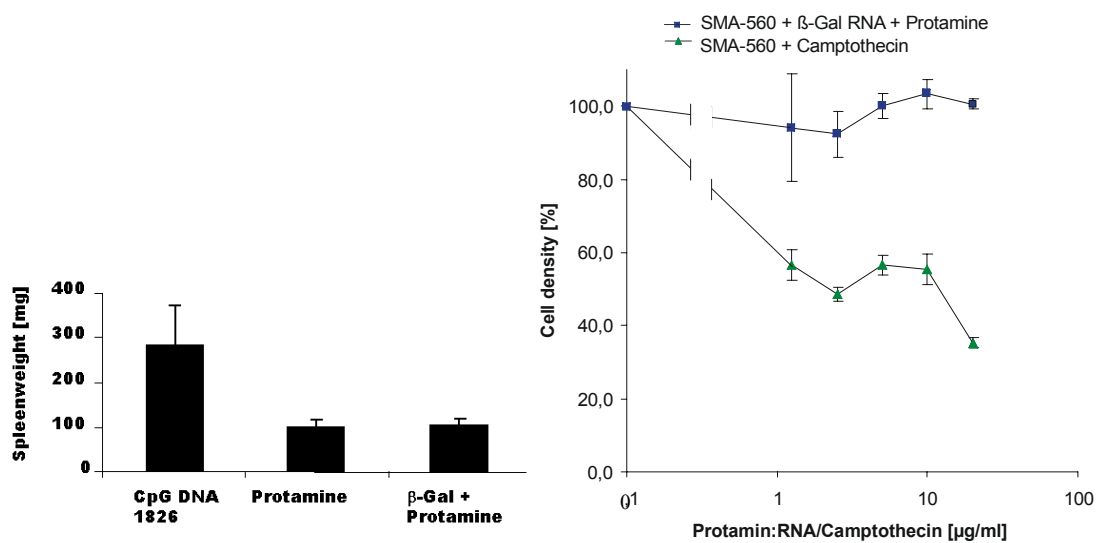


Fig. 3.6.A,B. **Toxicity assays with protamin-condensed mRNA.** A. Spleen weight after tumor treatment. After the end of the experiment, the spleens of the mice were taken and weighed. Weight is given in grams. B. *In vitro* toxicity of protamine. SMA-560 cells were co-incubated with different concentrations of uncapped β -Gal mRNA condensed with protamine or camptothecin. After 24 h, viable cell counts were obtained by crystal violet staining. The cytotoxic agent camptothecin was used as a positive control.

Discussion

Our data show that the injection of protamine-stabilized mRNA into solid tumors results in a growth arrest, regression or in 20% of the cases to a complete remission of the tumors. It is likely, that this effect is due to several mechanisms. ssRNA (48, 151, 152) as well as dsRNA (153) may serve as a danger signal which leads to the activation of various cell types *via* TLR. Thus, the presence of a danger signal at the tumor site triggers DC to incorporate tumor antigens, migrate to regional lymph nodes and present them to T cells. Furthermore, protamine-stabilized mRNA activates DC and B cells in mice (42), which release cytokines and upregulate costimulatory molecules. The local release of IL-6 and IL-12 by mouse DC in turn leads to a recruitment of T cells to the site of the tumor (Fig. 3.5.). Besides, the presence of cytokines can have a direct effect on the tumor. Kishima *et al.* showed, that the injection of IL-12 i.p. in glioma-bearing mice can lead to tumor eradication and gliomas in rats that are injected peripherally with IL-12 along with irradiated tumor cells promote an immunological rejection of flank 9L gliomas (154). The fact that a rechallenge in cured mice did not lead to development of a tumor and that invasion of CD8⁺ cells was seen in tumor sections of RNA-treated mice strongly indicates that the treatment of tumors with protamine-complexed RNA triggers a specific immune response against tumor cells. The sequence of the RNA may have no effect on the activity of the treatment, since 2 different sequences of mRNA and additionally a Phosphorothioate RNA Oligonucleotide which all had the same activity (data not shown). The stabilized RNA itself had no direct toxic effect on the tumor cells in *in vitro* assays.

Structural similarities of imidazoquinoline which activates TLR7 and TLR8 (155) and nucleic acids suggest that nucleic acids might be the natural ligand for these TLR and indeed, short ssRNA derived from HIV-1 stimulate mouse DC *via* TLR7 and human DC *via* TLR8 (10). Also, ssRNA viruses are recognized *via* TLR7 by pDC and B cells (14) and lead to the production of cytokines and activation of costimulatory molecules.

Since that the TLR 7,8 and 9 form a subfamily according to genomic structure and sequence homology (156, 157), it is not unexpected that ssRNA, the natural ligand for TLR 7 and 8 has a similar effect on tumor growth as the TLR9 ligand, CpG-DNA, that is a strong anti-tumor agent.

Regarding the severe side effects that have been observed after treatment with CpG-DNA (8), stabilized mRNA could be an alternative, being as potent in stimulating a specific immune response against the tumor, but exhibiting fewer side effects due to its much shorter half life.

mRNA can be a vaccine either loaded on DC (158) or by direct injection (159) and give rise to a specific immune response. We propose that mRNA can be used as a tumor treatment with a double effect, functioning as a danger signal and coding for tumor antigens at the same time.

IV. SD-208, a novel TGF-beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells *in vitro* and *in vivo*

A related manuscript has been published in Cancer Research, 2004 Nov 1;64(21):7954-61

This project was a collaborative effort of Scios Inc., Fremont, CA and our laboratory. Scios scientists (Ramona Almirez, Ruban Mangadu, Yu-Wang Liu, Alison Murphy, Darren H. Wong and Linda S. Higgins) developed the drug and characterized it for receptor kinase specificity and bioavailability. In our laboratory, most experiments were carried out by Dr. Martin Uhl. My contribution involved animal experiments, mainly intracranial and subcutaneous inoculation, survival screening and oral gavage as well as functional immunological assays.

Introduction

Human glioblastoma patients experience a median survival of little more than one year with the standard treatment of surgery, radiotherapy and nitrosourea-based chemotherapy (160). For many years immunotherapy has been explored as an alternative approach for these tumors because human glioma patients exhibit specific deficits in their cellular immune response *ex vivo* (161) and because human glioma cells are paradigmatic for the property of cancer cells to express immunosuppressive molecules. These include soluble factors such as TGF- β (148), prostaglandins (162) or IL-10 (163), as well as cell surface molecules such as CD70 (109) or HLA-G (134). Among these, TGF- β has attracted most interest (132), resulting in experimental therapeutic approaches using antisense strategies (164), gene transfer of TGF- β antagonists such as decorin (165), inhibition of TGF- β -processing proteases of the furin family (141) or drugs such as tranilast (166). The undesirable effects of TGF- β in malignant glioma are not restricted to the induction of immunosuppression in the host, but include a critical role of TGF- β in migration and invasion (167). We here examine a novel therapeutic principle of TGF- β antagonism in malignant glioma, defined by SD-208, a pharmacological agent which blocks TGF- β RI signalling, similar to SB-505124, an inhibitor of activin-like kinase receptors 4, 5 (=TGF- β RI) and 7 (168).

Materials and methods

Materials and cell lines. PHA was from Biochrom (Berlin, Germany). [Methyl-³H]-thymidine was obtained from Amersham (Braunschweig, Germany). ⁵¹Cr was purchased from New England Nuclear (Boston, MA). Human recombinant TGF- β_1 , TGF- β_2 and mouse IL-2 were obtained from Peprotech (London, UK). Neutralizing pan-anti-TGF- β antibody was purchased from R&D (Wiesbaden, Germany). Specific ELISA kits (R&D) were used for the detection of human and murine TGF- β . The human malignant glioma cell line LN-308 was kindly provided by N. de Tribolet (Lausanne, Switzerland). The murine glioma line SMA-560 was a kind gift of D.D. Bigner (Durham, NC). CCL64 mink lung epithelial cells were obtained from the American Type Culture Collection (Rockville, MD).

Cell culture. The glioma cells and CCL64 cells were maintained in DMEM supplemented with 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), 10% FCS (Biochrom) and penicillin (100 IU/ml)/streptomycin (100 μ g/ml) (Gibco). Growth and viability of the glioma cells were examined by crystal violet staining, LDH release (Roche, Mannheim, Germany) and trypan blue dye exclusion assays. For crystal violet staining, the cell culture medium was removed and surviving cells were stained with 0.5% crystal violet in 20% methanol for 10 min. The plates were washed extensively under running tap water, air-dried and optical density values were read in an ELISA reader at 550 nm wave length. Human PBMC were isolated from healthy donors by density gradient centrifugation (Biocoll, Biochrom). Monocytes were depleted by adhesion and differential centrifugation to obtain PBL. To obtain purified T cells, PBMC were depleted of B cells and monocytes using LymphoKwik TTM reagent (One Lambda, Canoga Park, CA). The purity of this population was > 97% verified by flow cytometry using anti-human CD3-PE antibody (Becton Dickinson, Heidelberg, Germany). Human polyclonal NK cell populations were obtained by culturing PBL on irradiated RPMI 8866 feeder cells for 10 days (169). Murine NK cells were prepared from splenocytes from VM/Dk mice by positive selection using DX5 monoclonal antibody-coupled magnetic beads with the corresponding column system (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured with mouse IL-2 (5000 U/ml) for at least 10 days before use. The human polyclonal NK cell cultures, PBL, T cells and mouse NK cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-

glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol and penicillin (100 IU/ml)/streptomycin (100 μ g/ml).

Characterization of SD-208. SD-208 is a TGF- β RI kinase inhibitor developed by Scios Inc. (Fremont, CA). To assess the specificity of SD-208 for TGF- β RI, various kinase activities were assayed by measuring the incorporation of radiolabeled ATP into a peptide or protein substrate. The reactions were performed in 96 well plates and included the relevant kinase, substrate, ATP and appropriate co-factors. The reactions were incubated and then stopped by the addition of phosphoric acid. Substrate was captured onto a phosphocellulose filter which was washed free of unreacted ATP. The counts incorporated were determined by counting on a microplate scintillation counter (TopCount, Perkin Elmer Corporation, Boston, MA). The ability of SD-208 to inhibit the respective kinase was determined by comparing counts incorporated in the presence of compound to those incorporated in the absence of compound.

TGF- β bioassay. The levels of bioactive TGF- β were determined using the CCL64 bioassay. Briefly, 10^4 CCL64 cells were adhered to 96 well plates for 24 h and then exposed to recombinant TGF- β_1 , TGF- β_2 or glioma cell culture SN diluted in complete medium for 72 h. Growth was assessed by crystal violet staining at 72 h. Glioma cell SN were harvested from subconfluent cultures maintained for 48 h in serum-free medium and heat-treated (5 min, 85°C) to activate latent TGF- β (141).

Proliferation. Glioma cells were cultured in the absence or presence of SD-208 (1 μ M) for 48 h. The cells were pulsed for the last 24 h with [methyl- 3 H]-thymidine (0.5 μ Ci), harvested (Tomtec, Hamden, CT), and incorporated radioactivity was determined in a Liquid Scintillation Counter (Wallac).

Flow cytometry. The adherent glioma cells were detached nonenzymatically using cell dissociation solution (Sigma, Taufkirchen, Germany). Cell cycle analysis of glioma or immune effector cells was performed on fixed and 70% ethanol-permeabilized glioma or immune effector cells. RNA was digested with RNase A (Life Technologies, Inc.). DNA was stained with propidium iodide (50 μ g/ml). Fluorescence was measured in a Becton Dickinson FACScalibur (Heidelberg, Germany).

TGF- β reporter assays. Intracellular TGF- β signalling was assessed by reporter assays using pGL2 3TP-Luc (170) or pGL3 SBE-2 Luc (171) reporter gene plasmids kindly provided by J. Massagué (New York, NY) and B. Vogelstein (Baltimore, MD). The pGL2 3TP-Luc construct contains a synthetic promoter composed of a TGF- β -responsive plasminogen activator inhibitor 1 promoter fragment inserted downstream of

three phorbol ester-responsive elements. The pGL3 SBE-2-Luc reporter contains two copies of the Smad-binding element GTCTAGAC. LN-308 and SMA-560 cells were transfected using FuGene (Roche). At 24 h after transfection the cells were pretreated in serum-containing medium with SD-208 for 12 h (1 μ M). TGF- β_1 (5 ng/ml) was then added for another 16 h. The cells were lysed and transferred to a LumiNunc™ plate (Nunc, Roskilde, Denmark) and luminescence was measured in a LumimatPlus (EG&G Berthold, Pforzheim, Germany), using Luciferase assay substrate (Promega, Mannheim, Germany). For T cell assays, 5 x 10⁶ freshly isolated PBL were co-transfected with 4.5 μ g pGL2-3TP-Luc or pGL3-SBE-2 Luc reporter gene plasmid and 0.5 μ g pRL-CMV (Promega), using the Nucleofector™ device and the cell type-specific human T cell nucleofector™ kit (Amaxa, Cologne, Germany). IL-2 (50 U/ml) was added 4 h after nucleofection and the cells were pretreated with SD-208 for 1 h before TGF- β_1 (5 ng/ml) was added for another 16 h. The respective activities of firefly and renilla reniformis luciferase were determined sequentially using the firelite dual luminescence reporter gene assay (Perkin-Elmer, Rodgau-Jügesheim, Germany). Counts obtained from the measurement of firefly luciferase were normalized with respect to pRL-CMV.

Immunoblot analysis. p-Smad2 levels in glioma cells were analyzed by immunoblot using 20 μ g protein per lane on 12% SDS polyacrylamide gels. PBMC were analysed using 100 μ g per lane and 10% gels. After transfer to a polyvinylidene difluoride membrane (Amersham), the blots were blocked in PBS containing 5% skim milk and 0.05% Tween 20 and incubated overnight at 4°C with p-Smad2 antibody (2 μ g/ml) (Cell Signaling Technology, Beverly, MA). Visualization of protein bands was accomplished using horseradish peroxidase-coupled secondary antibody (Sigma) and enhanced chemiluminescence (Amersham). Total Smad2/3 levels were assessed using a specific Smad2/3 antibody (1 μ g/ml) (Becton Dickinson).

Matrigel invasion assay (Boyden Chamber). Invasion of glioma cells was measured by the invasion of 10,000 cells through Matrigel-coated transwell inserts (Becton Dickinson). Briefly, transwell inserts with 8 μ m pore size were coated with Matrigel and preincubated SMA-560 cells were applied to the upper wells and allowed to transmigrate through the membrane towards conditioned medium derived from NIH-3T3 fibroblasts which was added to the lower wells. Migrated cells on the lower side of the membrane were fixed, stained in toluidine blue solution (Sigma) and counted in 5 microscopic high power fields using a microgrid.

Spheroid collagen invasion assay. Multicellular SMA-560 glioma cell spheroids were cultured in 25 cm² culture flasks base-coated with 1% Noble Agar (Difco Laboratories, Detroit, MI). Briefly, 4 x 10⁵ cells were suspended in 10 ml medium, seeded onto 1% agar plates and cultured until spheroids had formed. Spheroids of about 200 µm diameter were selected for the experiments. Preincubated spheroids were seeded into collagen I and fibronectin containing wells. Spheroid radius, which is determined by the invasion of single cells into the matrix (172), was analyzed by morphometry using the MCID digitalization system (IMAGING Research, Ontario, Canada) at 24, 48 and 72 h.

Alloproliferation. HLA-A2-mismatched human PBL (10⁵/well) were cocultured with 10⁴ irradiated (30 Gy) LN-308 glioma cells in 96 well plates in triplicates for 5 days. Some cocultures received PHA (5 µg/ml). The cells were pulsed for the last 24 h with [methyl-³H]-thymidine (0.5 µCi), harvested (Tomtec), and incorporated radioactivity was determined in a Liquid Scintillation Counter (Wallac).

Lysis assay. HLA-A2-mismatched PBL or T cells (10⁷/25 cm² flask) were cocultured with 10⁶ irradiated (30 Gy) LN-308 glioma cells for 5 days. Glioma cell targets were labeled using ⁵¹Cr (50 µCi, 90 min) and incubated (10⁴/well) with effector PBL harvested from the cocultures at E:T ratios of 100:1 to 3:1. The maximum ⁵¹Cr release was determined by addition of NP40 (1%) (Sigma). After 4 h the SN were transferred to a Luma-Plate TM-96 (Packard, Dreieich, Germany) and measured. The percentage of ⁵¹Cr release was calculated as follows: 100 x [experimental release – spontaneous release]/[maximum release – spontaneous release].

Cytokine release. IFN-γ, TNF-α and IL-10 release by immune effector cells was assessed by ELISPOT assay in multiscreen-HA 96 well plates (Millipore, Eschborn, Germany) coated with corresponding anti-human capture antibodies (Becton Dickinson). Briefly, 5 x 10⁴ glioma cells were cocultured for 24 h with 10⁵, 2.5 x 10⁵ or 5 x 10⁵ HLA-A2-mismatched, prestimulated (5 days) PBL. The cells were removed using double-distilled water and captured cytokines were visualized using biotinylated antibodies and streptavidin-alkaline phosphatase (Becton Dickinson). Spots were counted on an ELISPOT reader system (AID, Straßberg, Germany).

Ex vivo p-Smad2/3 ELISA. Male mice (BALB/c, Jackson Labs, Bar Harbor, ME) were studied in 6 groups of 8 animals each. For each drug group, a single volume of SD-208 was administered by oral gavage 1 h prior to dosing with TGF-β₁ (R&D Systems, Minneapolis, MN) diluted in 100 µl 0.1% BSA / 4 mM HCL/PBS by i.v. injection. The mice were sacrificed by cervical dislocation 1 h later. Tissues were removed and lysed

in 20 mM Tris, pH 7.5, containing 1 mM EDTA, 0.5% TX-100, 0.5% NP-40, 150 mM NaCl and 1x protease inhibitor cocktail (Roche) and 1x phosphatase inhibitor cocktail set II (Calbiochem, San Diego, CA). Tissue was homogenized using an Ultra-turrax T8 (Reyom Instruments, Brabcova, Czech Republic). Tissue homogenates were clarified by centrifugation and the supernatant fraction was collected. Protein concentrations were determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL). The levels of p-Smad were determined by sandwich ELISA. Briefly, 96 well ELISA plates were coated with an anti-Smad2/3 monoclonal antibody (100 ng/well, Becton Dickinson) for 18 h at 4°C. Excess antibody was removed and the wells were treated with blocking buffer (0.3% BSA/PBS) for 2 h at room temperature. Tissue lysates (125-150 µg total protein) were added to each well and incubated overnight at 4°C. Wells were rinsed before adding a polyclonal anti-p-Smad 2/3 antiserum diluted in 2% BSA/0.5% Tween-20/PBS. Following a 2 h incubation at room temperature, the wells were washed and secondary antibody was applied (horseradish peroxidase-conjugated goat-anti-rabbit IgG, Southern Biotech, Birmingham, AL). After 1 h the wells were developed with TMB (Sigma). The plate was incubated 5-30 min before the reaction was stopped with 0.5 N H₂SO₄ and read at 450 nm in a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA).

Survival studies *in vivo*. VM/Dk mice were purchased from the TSE Research Center (Berkshire, UK). Mice of 6-12 weeks of age were used for the survival experiments. The experiments were performed according to the German animal protection law. Groups of 8 mice were anesthetized before all intracranial procedures and placed in a stereotaxic fixation device (Stoelting, Wood Dale, IL). A burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm. Five x 10³ SMA-560 cells (135) resuspended in a volume of 2 µl PBS were injected into the right striatum. Three days later the mice were allowed to drink SD-208 at 1 mg/ml in deionized water. The mice were observed daily and, in the survival experiments, sacrificed when developing neurological symptoms.

Histology. Glioma-bearing mice were sacrificed 10 days after tumor implantation by cardiac puncture, perfused and post-fixed in 4% paraformaldehyde (Sigma) overnight. Five µm paraffin sections were cut at 150 µm intervals from each brain. The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (Harri's, American Master Tech, Lodi, CA), rat anti-mouse monoclonal CD34 IgG_{2a} (1:100,

CL8927AP, Cedarlane, Hornby, Canada), rabbit polyclonal anti-Ki67 (1:100, ab833-500, Novus Biologicals, Littleton, CO), rabbit anti-mouse caspase 3 active (1:400, AF835, R&D), anti-mouse CD8 (1:50, 53-6.7, BD Biosciences, Heidelberg, Germany), anti-mouse CD11b (1:50, M1/70, BD Biosciences) or anti-Ly-49G2 (1:50, 4D11, BD Biosciences). Biotinylated secondary antibodies (1:150) Zymed, San Francisco, CA were used for detection. Streptavidine alkaline phosphatase (1:100) was added, and the staining was developed with naphtol as substrate and levamisole as inhibitor of endogeneous alkaline phosphatase (Fast Red Tablets, Roche). The negative control for CD34 was normal rat IgG_{2a} (CBL605, Chemicon International, Temecula, CA). The negative control used for Ki67 and caspase 3 was normal rabbit IgG (SC-2027, Santa Cruz Biotechnology, Santa Cruz, CA). The negative control for CD8, Ly-49G and CD11b was rat IgG_{2a} or rat IgG_{2b}. Murine spleen served as a positive control, normal murine brain as a negative control in these assays. The total number of CD34+ microvessels was counted in an area of 0.63 mm² corresponding to 2 high power fields in 2 non-consecutive sections in the tumor center of the tumor. To assess the percentage of proliferating cells, Ki67-positive nuclei were counted. At least 600 nuclei were counted in 4 high power fields in 2 non-consecutive sections in the tumor center. To assess the degree of apoptosis, caspase-3-positive cells were counted in the tumor center in 2 two non-consecutive sections.

***Ex vivo* immune effector assays.** Glioma-bearing mice were sacrificed 10 days after tumor cell injection. Splenocytes were isolated and used in 24 h IFN- γ ELISPOT assays as described above. Further, these cells were stimulated with IL-2 (5000 U/ml) for 10 days to generate LAK cells which were used in ⁵¹Cr release assays against SMA-560 glioma cells as targets.

Statistical analysis. The experiments were usually performed at least three times with similar results. Significance was tested by Student's t-test. P values are derived from two-tailed t-tests.

Results

SD-208 is a functional TGF- β_1 and TGF- β_2 antagonist *in vitro*. The initial characterization of SD-208 in cell-free assays led to its identification as a potent inhibitor of TGF- β RI. SD-208 exhibited an *in vitro* specificity for TGF- β RI kinase of >100-fold compared with TGF- β RII kinase and at least 20-fold over members of a panel of related protein kinases (Table 1).

Table 1. **Specificity of SD-208 for TGF- β RI kinase activity¹**

TGF- β RI	0.048
TGF- β RII	> 50
Epidermal growth factor receptor	68%
mutant <i>Drosophila</i> p38- α	0.867
p38 kinase γ	> 50
c-jun N-terminal kinase	> 50
Extracellular signal-regulated kinase-2	> 50
Mitogen-activated protein kinase-activated protein-2	87%
Mitogen-activated protein kinase kinase 6	> 50
Protein kinases A and C	> 50
Protein kinase D	70%

¹Data are expressed as EC₅₀ [μ M] or percentage inhibition at 5 μ M SD-208 in cell-free systems *in vitro*.

The differential patterns of release of TGF- $\beta_{1/2}$ by human glioma cell lines have been reported (141). The TGF- β release by the cell lines examined here was ascertained by ELISA. The levels of TGF- β released into the supernatant were unaffected by SD-208 within the concentration and time frame of the ensuing experiments (Fig. 4.1.A).

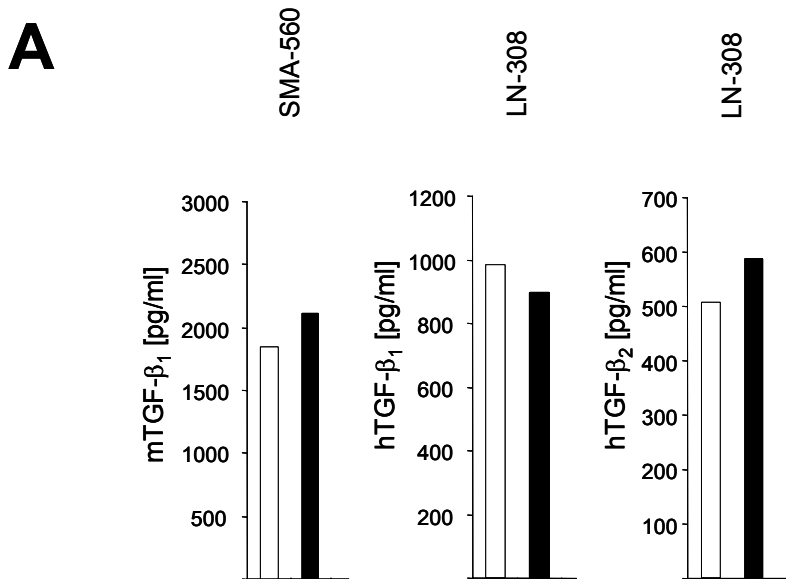
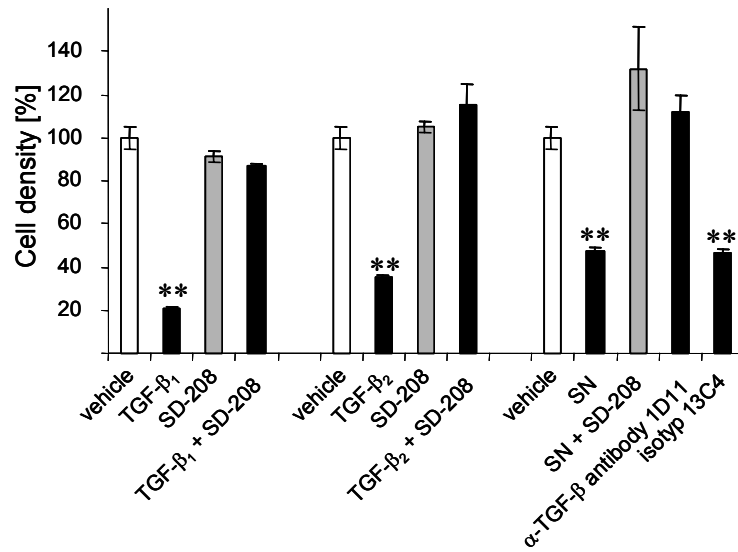
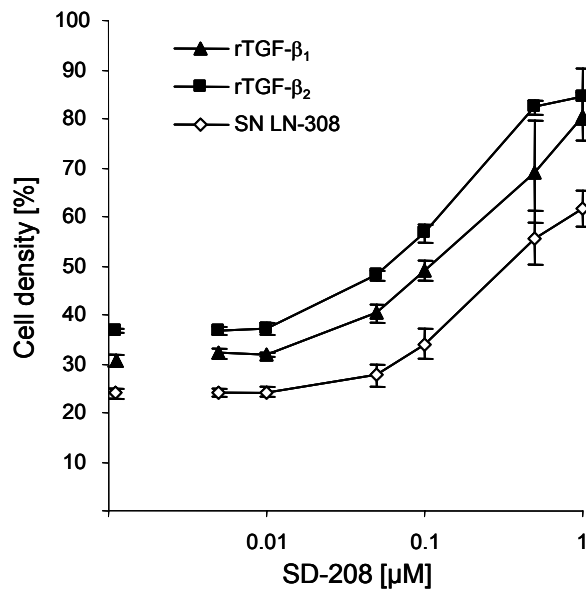


Fig. 4.1.A. **Prevention of the growth inhibitory effects of recombinant and glioma-derived TGF- β_1 and TGF- β_2 in the CCL64 bioassay by SD-208.** A. Murine SMA-560 or human LN-308 glioma cells were evaluated for the release of TGF- β into the SN by ELISA. SN was generated in the absence (open bars) or presence (filled bars) of SD-208 (1 μ M) for 48 h.

CCL64 mink lung epithelial cells are sensitive to the growth inhibitory effects of human TGF- β_1 and TGF- β_2 at EC_{50} concentrations of 0.5 ng/ml. The inhibitory effects of recombinant TGF- β as well as those of TGF- β -containing glioma cell SN were abrogated by specific TGF- β antibodies (141). The CCL64 bioassay was used here to verify the TGF- β -antagonistic properties of SD-208 (Fig. 4.1.B). SD-208 rescued the inhibition of growth mediated by TGF- β_1 or TGF- β_2 (10 ng/ml) or diluted SMA-560 (not shown) or LN-308 glioma cell SN in a concentration-dependent manner, with an EC_{50} concentration of 0.1 μ M (Fig. 4.1.C). When these bioassays were performed in the absence of serum in the CCL64 medium, the EC_{50} for SD-208 was 0.03 μ M (data not shown), corresponding to the data in Table 1.

B**C**

4.1.B,C. **SD-208 abrogates bioactive TGF- β in CCL64 assays.** B. CCL64 cells were exposed to human TGF- β_1 , TGF- β_2 or LN-308 SN (1:2) in the absence or presence of SD-208 (0.5 μ M) and cell density was assessed 72 h later. Neutralizing pan-TGF- β and isotype control antibodies (10 μ g/ml) were also included (* p <0.05, ** p <0.01, t-test, relative to vehicle). C. CCL-64 cells were cultured with TGF- β_1 (filled triangles), TGF- β_2 (filled squares) (10 ng/ml) or heat-activated glioma cell SN (1:2) (open rhomboids) in the absence or presence of SD-208 for 72 h. Cell density was assessed by crystal violet assay (mean and SD, n =3).

SD-208 abrogates autocrine TGF- β -dependent signal transduction in glioma cells.

We next examined the biological effects of SD-208 on murine and human glioma cells *in vitro*. The concentrations required to block the growth inhibitory effects of TGF- β in the CCL64 bioassay had no effect on the proliferation of either glioma cell line. SD-208 did not reduce proliferation assessed by [methyl- 3 H]-thymidine incorporation or viability assessed by LDH release or trypan blue dye exclusion assays at concentrations up to 1 μ M for 48 h in SMA-560 (Fig. 4.2.A) or LN-308 cells (data not shown). Further, neither exogenous TGF- β nor neutralizing TGF- β antibodies had such an effect. Accordingly cell cycle analysis showed no difference in both cell lines after exposure to SD-208 for 48 h (Fig. 4.2.B,C).

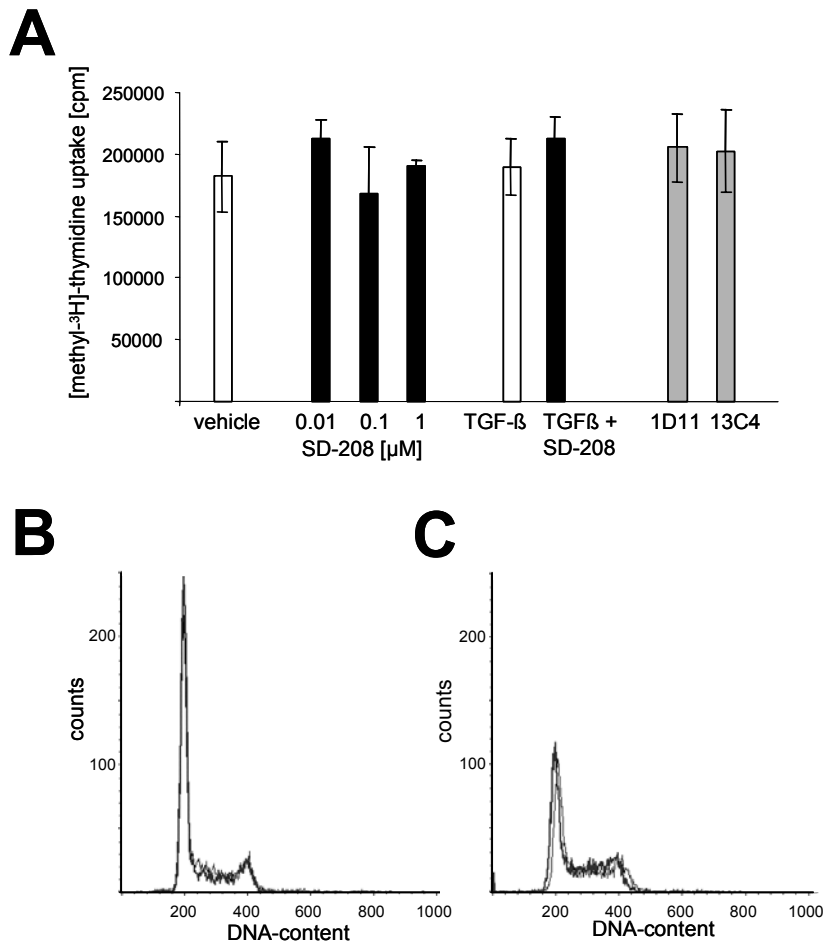


Fig. 4.2.A-C. **Absence of toxicity and abrogation of TGF- β signalling in glioma cells by SD-208.** A. SMA-560 cells were treated with SD-208, TGF- β_1 (10 ng/ml), their combination (1 μ M SD-208) or neutralizing TGF- β (1D11) or isotype control (13C4) antibodies (10 μ g/ml) for 48 h. Proliferation was assessed by [methyl- 3 H] thymidine incorporation. B,C. Cell cycle analysis of untreated or SD-208 (1 μ M, 48 h)-treated LN-308 (B) or SMA-560 cells (C) was performed using flow cytometry. The profiles overlap because SD-208 has no effect.

The inhibition of TGF- β signalling transduced by endogenous or exogenous TGF- β was ascertained by demonstrating that SD-208 interfered with Smad2 phosphorylation without altering total cellular Smad2/3 levels (Fig. 4.2.D). Similarly, two different reporter assays revealed a strong inhibition of TGF- β signalling when the glioma cells were treated with SD-208 (Fig. 4.2.E).

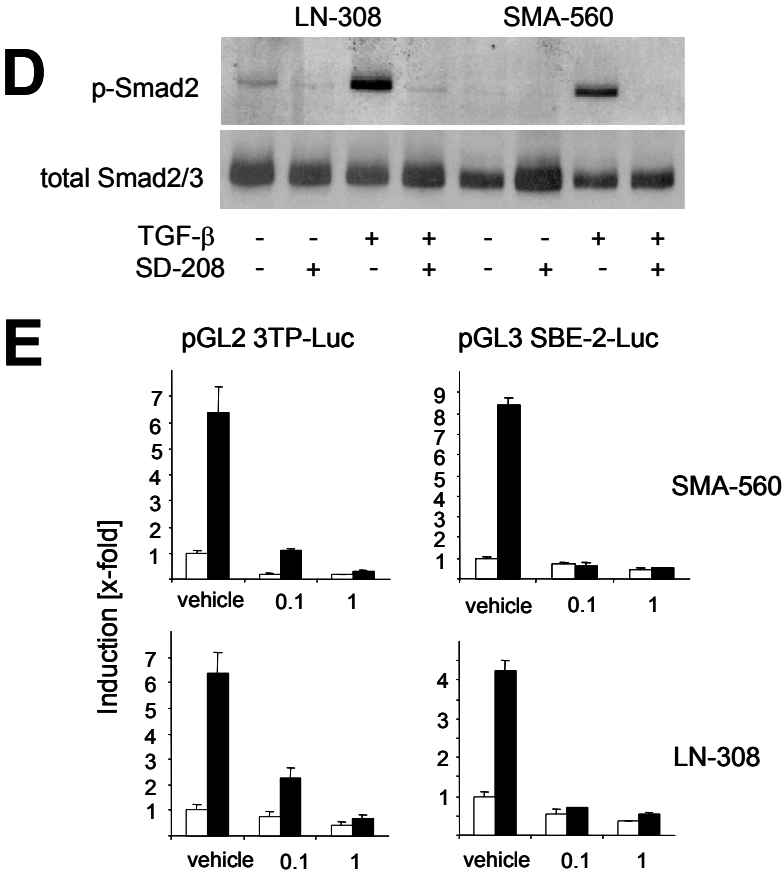


Fig. 4.2.D,E. **SD-208 abrogates TGF- β signalling via p-Smad2 and reduces TGF- β promoter activity.** D. Lysates from untreated glioma cells or cells preexposed to SD-208 (1 μ M) for 24 h or exposed to TGF- β_2 (5 ng/ml) for 1 h or both were assessed for the levels of p-Smad2 or total Smad2/3. Note that the antibodies are specific for p-Smad2 and total Smad2 and 3, respectively. E. The cells were untreated (open bars) or treated with TGF- β_1 (5 ng/ml, filled bars) for 16 h in the absence or presence of SD-208 at 0.1 or 1 μ M and assessed for TGF- β reporter activity in serum-containing medium.

SD-208 inhibits constitutive and TGF- β -evoked migration and invasion. We next examined the biological effects of SD-208 on SMA-560 cells in two independent migration and invasion paradigms *in vitro*. SD-208 reduced the invasion of glioma cells from a spheroid into a three dimensional collagen I gel and also the transmigration of glioma cells in a Boyden

chamber assay. Moreover, the proinvasive effect of exogenous TGF- β was neutralized by SD-208 in either assay (Fig. 4.3.A,B).

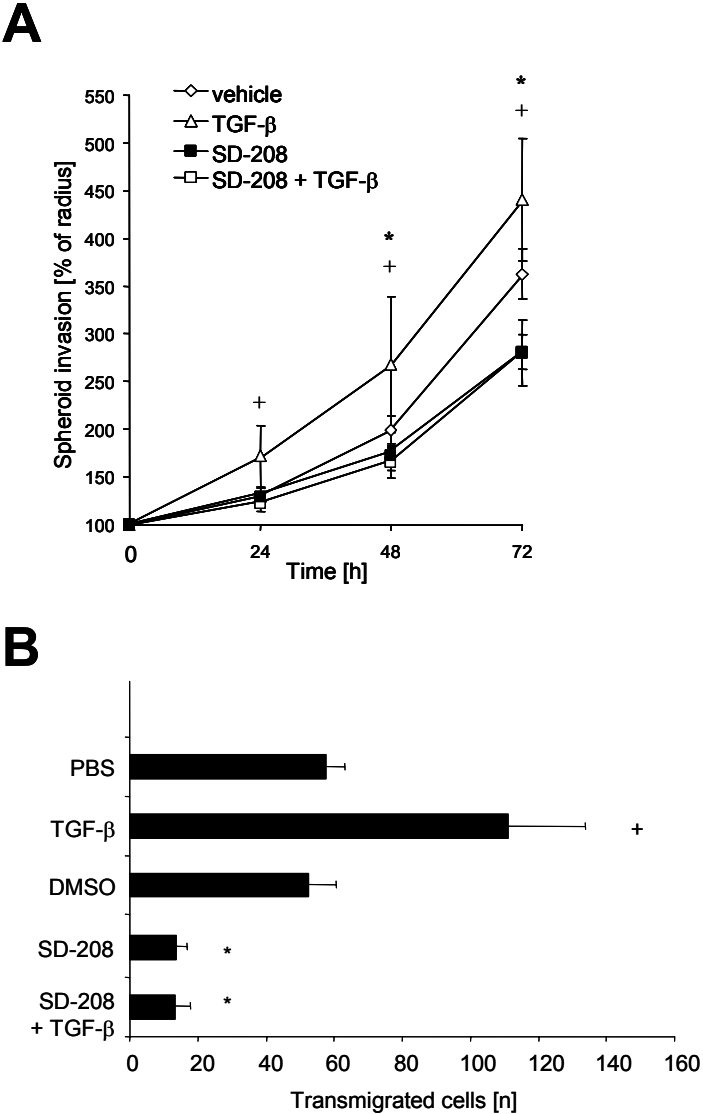


Fig. 4.3.A,B. **Inhibition of constitutive and TGF- β -induced invasion of glioma cells by SD-208.** A. SMA-560 spheroids were untreated or treated with SD-208 (1 μ M), TGF- β_2 (5 ng/ml) or both for 24 h prior to placement into the collagen I gel and during the experiment and spheroid diameter was determined every 24 h. Data are expressed as mean radius in percent of the spheroid radius at 0 h set to 100% (n=3, *p<0.05, t-test, effect of SD-208; ⁺p<0.05, t-test, effect of TGF- β_2). The concentration of DMSO required to dissolve SD-208 had no effect in this assay. B. Invasion was analyzed with matrigel-coated membranes in a Boyden chemotaxis chamber assay applying 10⁴ SMA-560 cells, untreated or treated with SD-208 (1 μ M), TGF- β_2 (5 ng/ml) or both for 24 h prior to and during the experiment, in the upper chamber. Invaded cells were counted at 24 h. Data are expressed as mean cell counts (n=3, *p<0.05, t-test, effect of SD-208, ⁺p<0.05, t-test, effect of TGF- β_2).

SD-208 enhances allogeneic immune responses to glioma cells *in vitro*. Consistent with the effects of SD-208 on TGF- β -mediated signalling in glioma cells (Fig. 4.2.D,E), immunoblot analysis and reporter assays also revealed a strong resistance to TGF- β signalling in the presence of SD-208 in immune cells (Fig. 4.4.C,D). Again, SD-208 did not affect cell cycle distribution, apoptosis or proliferation (Fig. 4.4.A,B).

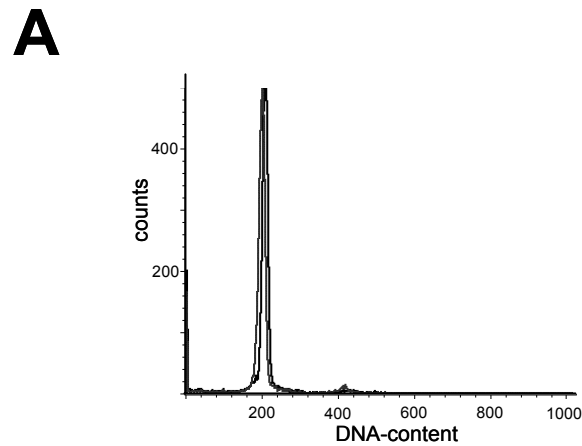


Fig. 4.4.A. **SD-208 inhibits TGF- β signalling in immune effector cells.** A. Flow cytometric cell cycle analysis of untreated (grey line) and SD-208 (1 μ M, 48 h)-treated (black line) PBMC show overlapping profiles.

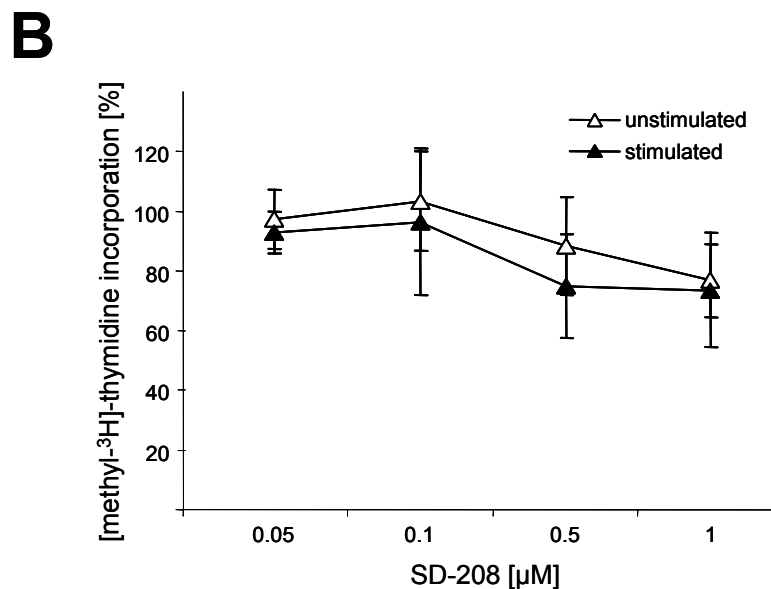


Fig. 4.4.B. **Proliferation of PBMC.** B. The proliferation of unstimulated (open symbols) or PHA-stimulated (filled symbols) PBMC was assessed by [methyl- 3 H]-thymidine incorporation in the presence of increasing concentrations of SD-208. Vehicle-treated PBMC were normalized to 100%. These cpm values were 6500 for unstimulated PBMC and 36000 for PHA-stimulated PBMC.

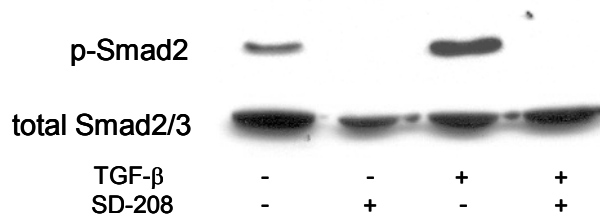
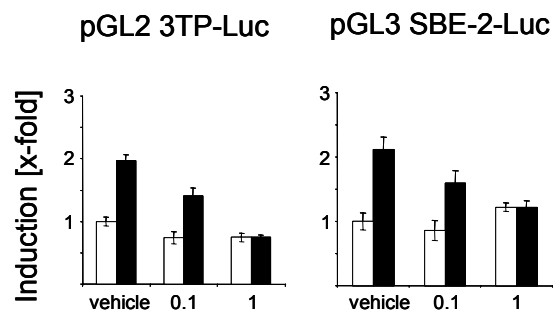
C**D**

Fig. 4.4.C,D. **P-Smad expression and TGF- β -reporter gene activity.** C. PBMC were treated with TGF- β_1 (5 ng/ml) in the absence or presence of SD-208 (1 μ M) for 30 min and then subjected to immunoblot analysis for Smad2 phosphorylation (upper panel). Total Smad2 expression was assessed as a loading control (lower panel). D. PBL were untreated or pretreated with SD-208 (0.1, 1 μ M) for 1 h and then untreated (open bars) or treated with TGF- β_1 (5 ng/ml) (filled bars) for 16 h and subjected to the firelite reporter assay.

The next series of experiments was designed to examine whether SD-208 restores allogeneic immune cell responses to cultured human glioma cells. When HLA-A2-mismatched PBL or purified T cells were cocultured with irradiated glioma cells in the presence of SD-208, their lytic activity in a subsequent 4 h ^{51}Cr release assay was significantly enhanced (Fig. 4.5.A). Similar effects were obtained using neutralizing TGF- β antibodies (10 μ g/ml, added every two days) (data not shown). The release of IFN- γ by HLA-mismatched PBL was strongly inhibited when the priming had taken place in the presence of glioma cells. SD-208 restored the IFN- γ release to levels comparable to PBL pre-cultured in the absence of LN-308 cells (Fig. 4.5.B). Similar results were obtained for TNF- α (Fig. 4.5.C). In contrast, IL-10 release was stimulated after coculturing with LN-308 cells, and SD-208 reduced the release of IL-10 by immune effector cells generated both from unstimulated and glioma cell-primed cultures (Fig. 4.5.D). The lytic activity of polyclonal NK cells against LN-308 targets was inhibited by

exogenous TGF- β , and TGF- β -mediated inhibition was relieved by SD-208 (Fig. 4.5.E). Similarly, LN-308 SN inhibited NK cell activity, and this inhibition was also blocked by SD-208 (Fig. 4.5.F) or neutralizing TGF- β antibodies (data not shown). Note that while the absolute lytical activity was donor-specific, the effect of SD-208 was consistent through all experiments.

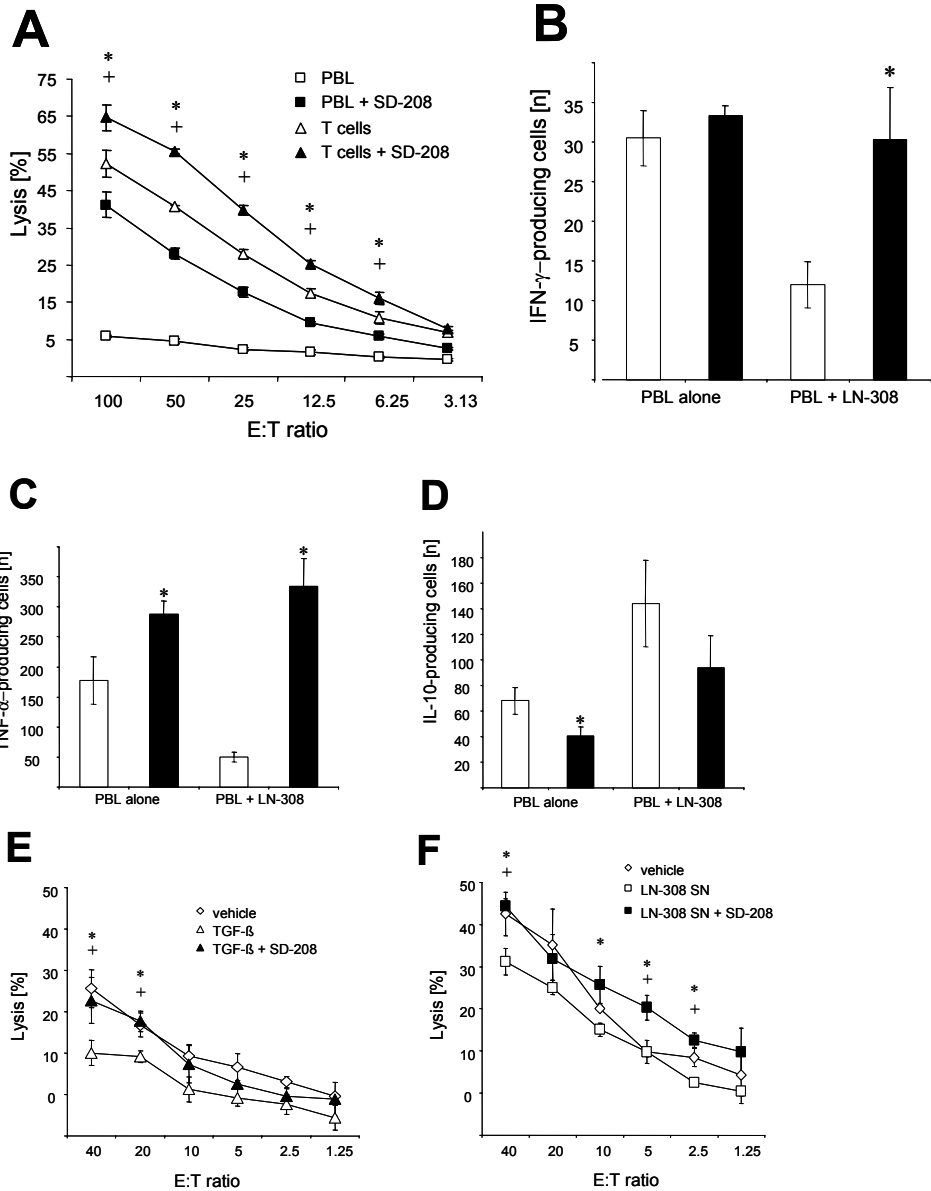


Fig. 4.5.A-F. **Modulation of allogeneic anti-glioma immune responses by SD-208 involves TGF- β antagonism.** A. The lytic activity of PBL (squares) or purified T cells (triangles) preincubated with irradiated LN-308 cells in the absence (open symbols) or presence (filled symbols) of SD-208 (1 μ M) was determined in 51 Cr release assays using LN-308 cells as targets ($^+$ p<0.05, t-test, effect of SD-208 on PBL; *p<0.05, t-test, effect of SD-208 on T cells). B-D. PBL

were cultured in the absence (left) or presence (right) of irradiated LN-308 cells for 5 days. The cultures contained SD-208 (1 μ M) (filled bars) or not (open bars). Subsequently these effector cells were cocultured for 24 h with fresh non-irradiated LN-308 cells in the absence of SD-208. The release of IFN- γ (B), TNF- α (C) or IL-10 (D) was assessed by ELISPOT assay. Data are expressed as cytokine-producing cells per 5×10^5 effector cells ($n=3$, $*p<0.05$, t-test, effect of SD-208). E,F. Polyclonal NK cell cultures were exposed to TGF- β_1 (5 ng/ml) (E) or diluted (1:4) glioma cell SN (F) without or with SD-208 (1 μ M) for 48 h and subsequently used as effectors in ^{51}Cr release assays using LN-308 cells as targets. SD-208 alone or TGF- β antibody alone had no effect on NK cell activity in these assays (data not shown, E: $^+p<0.05$, effect of TGF- β ; $*p<0.05$, effect of SD-208; F: $^+p<0.05$, effect of glioma SN; $*p<0.05$, effect of SD-208).

SD-208 prolongs the survival of SMA-560 intracranial experimental glioma-bearing syngeneic mice. To verify the bioavailability of orally administered SD-208, we ascertained that SD-208 inhibited the TGF- β -dependent *in vivo* phosphorylation of Smad2/3 in spleen and brain (Fig. 4.6.A,B). Note that exogenous TGF- β was a more potent inducer of Smad2/3 phosphorylation in spleen than brain, but that SD-208 was an equally effective antagonist of TGF- β in both tissues. The therapeutic effects of SD-208 administered *via* the drinking water (1 mg/ml) were assessed in the syngeneic SMA-560 mouse glioma paradigm. The development of neurological symptoms was delayed by SD-208-treated mice and the mean survival was prolonged to 25.1 ± 6.5 days (median 23) compared with 18.6 ± 2.1 days (median 18) in vehicle-treated animals (Fig. 4.6.C) ($p=0.004$, t-test). The survival rate at 30 days was 29% in SD-208-treated animals, but 0% in control animals. Preliminary evidence also indicated that SD-208 modulated the immune response of the glioma-bearing animals. ELISPOT assays for IFN- γ release by splenocytes harvested at day 7 after the initiation of SD-208 treatment revealed an increase over background in 3 of 5 SD-208-treated animals, but only in 1 of 5 control animals (data not shown). Further, LAK cells generated from the splenocytes of SD-208-treated animals showed an enhanced lytic activity against SMA-560 as targets (Fig. 4.6.D).

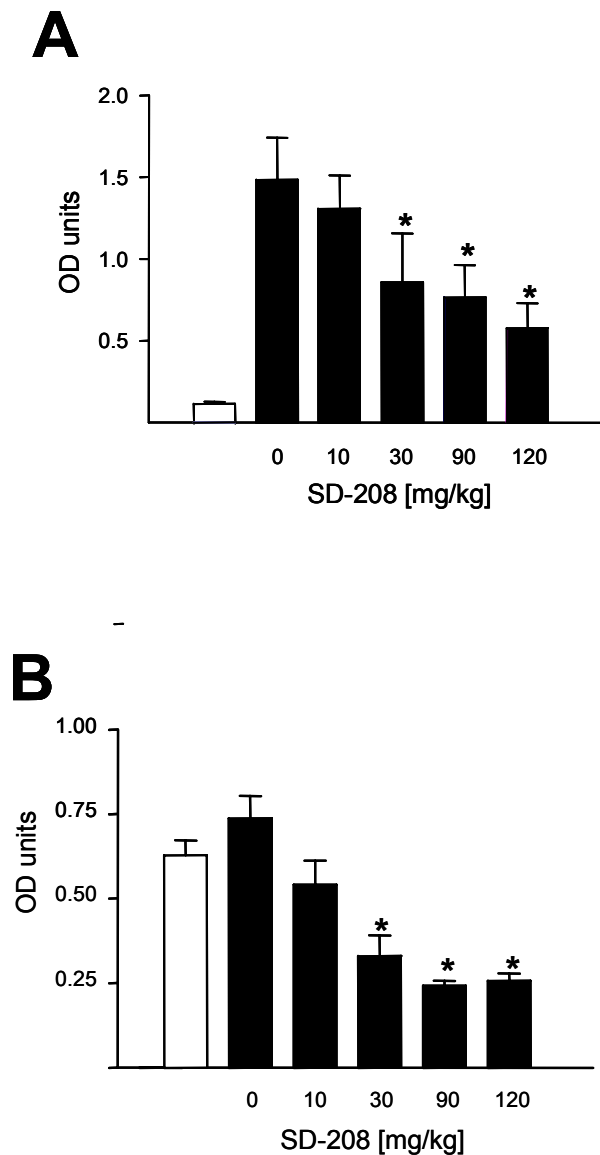


Fig. 4.6.A,B. **SD-208 blocks TGF- β signalling in the brain and inhibits the growth of syngeneic SMA-560 experimental gliomas *in vivo*.** A,B. Non-tumor-bearing animals were untreated (open bars) or treated with 150 ng TGF- β_1 i.v. (filled bars) at 1 h after oral exposure to SD-208 at increasing doses. The mice were sacrificed 1 h later and analyzed for Smad2/3 phosphorylation by ELISA in spleen (A) or brain (B) (* $p < 0.01$, Bonferroni's test, effect of SD-208 on TGF- β).

Histological changes in gliomas of SD-208-treated animals. The tumor volumes at day 10 showed a trend towards a lower tumor burden in SD-208-treated animals, but these changes were not significant (data not shown), consistent with the clinical course at day 10 shown in Fig. 4.6.C. Immunohistochemistry revealed no significant difference in blood vessel formation (CD34), tumor cell proliferation (Ki67) or apoptosis (active

caspase 3) between the gliomas of untreated and SD-208-treated animals (Table 2). There was little tumor infiltration by immune or inflammatory cells in the vehicle group as detected by HE staining. However, HE staining revealed major differences in the sizes and the histology of the gliomas of SD-208-treated animals: as indicated by the variable clinical course (Fig. 4.6.C), there appeared to be “non-responder” and “responder” animals. Animals with small tumors had stronger immune cell infiltration of their tumors than mice bearing larger tumors. Typical staining patterns for CD8 T cells, NK cells (Ly49G) and macrophages/neutrophils (CD11b) in a vehicle-treated, a SD-208-treated non-responder and a SD-208-treated responder animal are presented in Fig. 4.7.

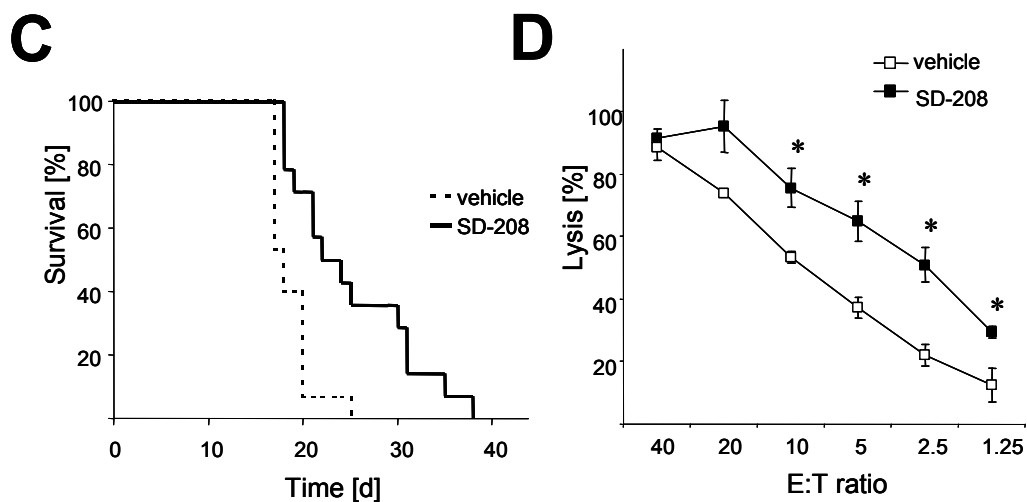
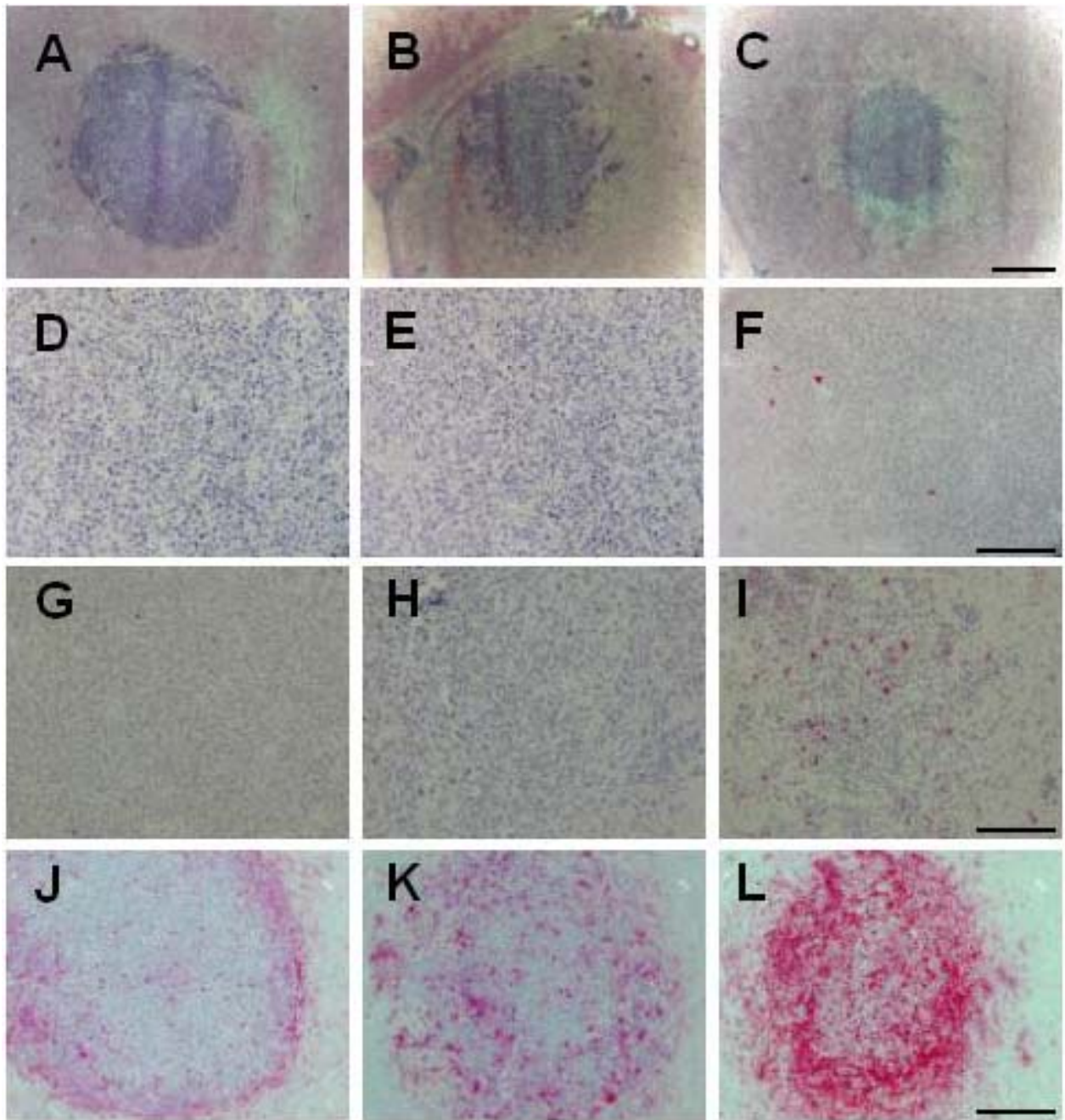


Fig. 4.6.C,D. **SD-208 promotes the rejection of i.c. SMA-560 gliomas in syngeneic mice.**

C. VM/Dk mice received an intracranial injection of 5×10^3 SMA-560 cells. Three days later SD-208 treatment was initiated, and survival was monitored. Two experiments involving 7-8 animals per group were pooled. D. Animals were treated as in the survival experiments and sacrificed on day 10 to obtain splenocytes which were stimulated with IL-2 for 10 days to generate LAK cells. Their lytic activity was measured by ^{51}Cr release using SMA-560 as target cells (vehicle, open squares; SD-208, filled squares; * $p < 0.05$, t-test, effect of SD-208).



vehicle

non-responder

responder

Fig. 4.7.A-L. **Immune cell infiltration in SD-208-treated tumor-bearing mice.** Gliomas from a vehicle-treated animal (A,D,G,J), a SD-208-treated non-responder animal (B,E,H,K) and a SD-208-treated responder animal (C,F,I,L) were stained with HE (A-C) or analysed by immunohistochemistry for NK cells (D-F), CD8 T cells (G-I) or macrophages (J-L) (magnification: A-C 5x, D-I 20x and J-L 10x. Size bar in C: 500 μm ; in F and I: 100 μm ; in L: 200 μm).

Table 2 **Immunolabeling for microvessels, proliferation and apoptosis in vivo**¹

	Vehicle	SD-208
CD34	149 ± 27	132 ± 28
Ki67	29 ± 7	26 ± 6
Caspase 3	13 ± 9	9 ± 7

¹Sections from 7 animals were analysed by immunochemistry as outlined in the *Methods* section.

Discussion

Antagonizing the biological effects of TGF- β has become a very attractive experimental strategy to combat various types of cancer including malignant gliomas. Current rationales for anti-TGF- β strategies include its putative role in migration and invasion (167), metastasis (173) and tumor-associated immunosuppression (132, 174). All of the TGF- β -based therapeutic approaches evaluated in experimental gliomas so far appear to have limitations with regard to their transfer into the clinic. Antisense oligonucleotides pose severe problems in terms of delivery to the desired site of action. The same applies to gene therapy strategies based e.g. on the transfer of the decorin gene (165). Inhibition of furin-like proteases aiming at limiting TGF- β bioactivity at the level of TGF- β processing (141) may not be achieved with acceptable specificity at present since a whole variety of molecules require processing by such enzymes (175). More specificity may result from the use of soluble TGF- β receptor fragments which act to scavenge bioactive TGF- β before it may reach the target cell population (173, 174). However, this approach may find its limits in the complex pathways of storage and activation of TGF- β . These considerations suggest that specific small molecules designed to protect cells from the actions of TGF- β at the level of receptor-dependent intracellular signal transduction are a particularly promising alternative for antagonizing TGF- β (168).

Here we characterize the activity of one such candidate agent, SD-208, against murine and human glioma cells *in vitro* and *in vivo*. Human LN-308 cells were chosen because they are paradigmatic for their prominent TGF- β synthesis (148),(141) (Fig. 4.1.A). SMA-560 cells transplanted in syngeneic VM/Dk mice probably represent the best model for the immunotherapy of rodent gliomas (135). We show that SD-208 is a potent TGF- β RI kinase inhibitor (Table 1) which blocks the biological effects of TGF- β_1 and TGF- β_2 as well as glioma cell SN in the CCL64 mink lung epithelial assay (Fig. 4.1.B,C). Since SD-208 did not modulate glioma cell proliferation at concentrations up to 1 μ M (Fig. 4.2.A), we did not confirm a negative growth regulatory effect of TGF- β on SMA-560 cells (176). Smad2 phosphorylation is induced by TGF- β in a SD-208-sensitive manner (Fig. 4.2.C), indicating that TGF- β signalling is not abrogated constitutively in glioma cells, but may not play a role in the modulation of glioma cell proliferation. Moreover, as expected (167), the antagonism of autocrine and paracrine

signalling by TGF- β in SD-208-treated glioma cells, as confirmed by reporter assay (Fig. 4.2.E) resulted in a potent inhibition of migration and invasion (Fig. 4.3).

We then focused on the desired immune modulatory effect of SD-208 which should result in an enhanced immunogenicity of glioma cells as a consequence of reduced TGF- β bioactivity. As predicted, human PBL and purified T cells developed enhanced lytic activity against LN-308 glioma cell targets when prestimulated with glioma cells in the presence of SD-208 (Fig. 4.5.A). This was paralleled by an enhanced release of proinflammatory cytokines such as IFN- γ and TNF- α and a reduced release of the immunosuppressive cytokine IL-10 in SD-208-treated cells (Fig. 4.5.B-D). Similarly SD-208 restored the lytic activity of polyclonal NK cell cultures cocultured with TGF- β or LN-308 SN (Fig. 4.5.E,F).

The strong reduction of Smad phosphorylation in the unlesioned mouse brain indicates that SD-208 may reach sufficient levels beyond the intact blood brain barrier to counteract the biological effects of tumor-derived TGF- β (Fig. 4.6.B). Accordingly, SD-208 prolonged the median survival of SMA-560 glioma-bearing mice significantly (Fig. 4.6.C). No dose-limiting toxicity was reached in these experiments, but higher doses could not be administered by the drinking water because of poor solubility of SD-208, suggesting that the therapeutic effect of SD-208 or related agents might even be improved in that glioma model. The therapeutic effect of SD-208 might be mediated by the inhibition of glioma cell migration and invasion (167) or the promotion of anti-glioma immune responses (132) or both. An immune contribution is suggested by the histological analyses (Fig. 4.7) which delineated an interrelation between tumor shrinkage and the degree of immune cell infiltration.

The present data strongly suggest a role for SD-208 or related molecules in the treatment of gliomas. Such a systemic treatment with TGF- β RI kinase inhibitors might well be combined with local approaches to limit the bioavailability of TGF- β , e.g., TGF- β antisense oligonucleotides which are already evaluated clinically.

**V. Processing of immunosuppressive pro-TGF-beta 1,2 by human glioblastoma cells
involves cytoplasmic and secreted furin-like proteases**

*A related manuscript has been published in Journal of Immunology. 2001 Jun
15;166(12):7238-43*

Most of the work has been carried out by Dr. Jens Leitlein who is the first author of this project. I designed and performed the northern blot analysis, together with Dr. Robert Waltereit, and was involved in the overall conception of the immunoblot analysis and the TGF- β bioassays.

Introduction

Deficient T cell function is commonly found in human patients with glioblastoma (177). This state of immunosuppression is attributed to the release of various soluble factors, notably transforming growth factor- β (TGF- β) type 2 (147). Consistent with a pivotal role of TGF- β in the growth of malignant gliomas, expression of TGF- β_2 antisense mRNA in 9L (164) or in C6 rat gliomas (178) induced their regression. Similarly, gene transfer-mediated ectopic expression of the functional TGF- β antagonist, decorin, promoted the regression of rat C6 gliomas (165).

TGF- β is synthesized as a pre-pro-TGF- β polypeptide that contains a signalling peptide (pre) (residues 1-29), the pro-region (residues 30-278) and the mature TGF- β moiety (residues 279-390) (119). Activation to the mature 12.5 kDa TGF- β_1 , that needs to dimerize to exert its biological effects, depends on the action of subtilisin-like proprotein convertases such as furin, as shown for purified proteins obtained from cell culture supernatants in a cell-free system (120). In fact, it has been suggested that TGF- β promotes furin gene expression as part of an amplification cascade of its own activation in fibroblasts and rat hepatocytes (179, 180). The subtilisin-like proprotein convertases of mammalian cells constitute a family of proprotein convertases related to bacterial subtilisins and yeast Kex2p which process multiple protein precursors, including growth factors, proteases of the coagulation and complement cascades, glycoproteins of viral envelopes and bacterial exotoxins at multibasic recognition sites (181-183). Furin seems to be universally expressed in mammalian cells and localizes mainly to the trans-Golgi network.

In the present study, we examined the role of FLP in the processing of TGF- β in malignant glioma cells. Since TGF- β_2 is the predominant isoform of TGF- β secreted by human malignant glioma cells and since TGF- β_2 has the same cleavable consensus site for furin (R-X-R/K-R) as TGF- β_1 , we were specifically interested in examining whether human glioma cells express furin and whether inhibition of furin may result in the inhibition of the processing of both isotypes of TGF- β in intact glioma cells.

Materials and methods

Reagents. The synthetic furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk), as well as the furin substrate for the fluorescence assays, N-t-butoxycarbonyl-Arg-Val-Arg-Arg-7-amido-4-methylcoumarine (boc-RVRR-amc), were purchased from Bachem (Heidelberg, Germany). [Methyl-³H]thymidine and 5'-[α-³²P]desoxycytidine triphosphate were from Amersham (Braunschweig, Germany). The following antibodies were purchased: rabbit polyclonal antibody to TGF-β₁ from Promega (Mannheim, Germany), rabbit polyclonal antibody to TGF-β₂ from Santa Cruz (Santa Cruz, CA). Anti-furin antiserum was raised as previously described (184). Human recombinant TGF-β₁ and TGF-β₂ were purchased from Roche (Mannheim, Germany). ELISA assays for total TGF-β₁ and TGF-β₂ were obtained from R&D (Minneapolis, MN).

Cell culture. sv-FHAS is a SV40 large T-antigen immortalized fetal human astrocytic cell line that was generously provided by A. Muruganandam and D. Stanimirovic (Institute of Biological Sciences, National Research Council of Canada, Ottawa, Canada). The human glioma cell lines were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) and maintained in DMEM supplemented with 10% FCS and penicillin (100 IU/ml)/streptomycin (100 µg/ml) (185). Transfections were carried out by electroporation (7), using a PDX expression plasmid (186) and a human furin expression plasmid kindly provided by J. Creemers (187).

TGF-β bioassay. Levels of bioactive TGF-β were determined using a modification of the CCL64 bioassay (165). Briefly, 5000 CCL64 cells were adhered to 96 well plates for 24 h. After removal of regular medium, the cells were exposed to cell culture supernatants diluted in serum-free medium for 56 h and then labeled with [methyl-³H]-thymidine (1 µCi/well) for additional 16 h. Cell culture supernatants were obtained by seeding 10⁶ cells in a 25 cm² culture flask. After 24 h, the cells were washed with PBS and then incubated with serum-free medium. After further 48 h, the conditioned medium was harvested, cell debris was removed by centrifugation, and the supernatant stored at -20°C. Cell counts obtained at the end of supernatant generation were used to normalize the supernatants for cell culture density. Latent TGF-β was activated by heating of the supernatants to 85°C for 5 min (188).

RT-PCR. RT-PCR for the detection of furin mRNA expression was performed according to standard procedures. The following primer sequences were used: furin sense (nucleotides corresponding to NotI site printed in italics plus nucleotides -7 to 11 containing the ATG), 5'-TTTTTTG-*CGGCCG-C-CCCCCATGGAGCTGAA*-3'; furin antisense (nucleotides

EcoRI in italics plus 428-409), 5'-*TTTTGAATTC*GTGTAGCCCTGCGCCCAGGC-3'; 35 cycles, 40 sec/94°C, 60 sec/56°C, 60 sec/72°C; β -actin sense (nucleotides 409-429), 5'-TGTTTGAGACCTTCAACACCC-3'; β -actin antisense (nucleotides 937-918), 5'-AGCACTGTGTTGGCGTACAG-3'; 35 cycles, 40 sec/94°C, 60 sec/53°C, 60 sec/72°C; PDX sense (nucleotides 552-571 of the α_1 -antitrypsin mRNA), 5'-CGTGGAGAAGGGTACTCAAG-3', PDX antisense (nucleotides 1164-1145 of the α_1 -antitrypsin mRNA but with the last two nucleotides complementary to the changes made to generate PDX, in italics) (17), 5'-GACCTCGGGGGGATAGATC-3', 30 cycles, 45 sec/94°C, 45 sec/60°C, 60 sec/72°C.

Northern blot analysis. Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Four micrograms of total RNA per lane were separated on 1.2% agarose gels and blotted onto nylon membranes (Amersham). The filters were hybridized according to standard procedures with ³²P-labelled full-length human cDNA probes for furin (189), kindly provided by V. van de Ven (Leuven, Belgium), TGF- β_1 (190) and TGF- β_2 (117), generous gifts of A. Fontana (Zürich, Switzerland). All blots were re-hybridized with a β -actin probe (191). Autoradiography signals were quantified with a phosphor-imager (Fuji BasReader 1500, Raytest, Staubenhardt, Germany), and the signals of interest were normalized against β -actin expression.

Immunoblot analysis. The levels of TGF- β and furin protein expression were assessed by immunoblot analysis. The general procedure has been described (192). Briefly, cells were lysed in 50 mM TRIS-HCl (pH 8) containing 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 2 μ g/ml aprotinin, 10 μ g/ml leupeptin and 100 μ g/ml phenylmethylsulfonylfluoride (PMSF). For the detection of TGF- β in the supernatant, serum-free supernatants were concentrated (because of the low protein production of some of the cell lines) with the Centriplus® centrifugal filter device YM-3 (3,000 Da cut-off) (Millipore, Eschborn, Germany). This concentrated supernatant was also used for the immunoblot detection of furin in the supernatant. The furin antiserum was used at a dilution of 1:1000 in TS-TMBSA (TRIS-HCl, pH 7.5, 10 mM; NaCl 150 mM; Tween 20 0.1%; skim milk 5%; BSA 2%; sodium azide 0.01%), the antibodies to TGF- β_1 and TGF- β_2 were used at 1:2000 and 1:3000. Anti-rabbit IgG (Santa Cruz, CA) at a dilution of 1:3500 in PBS/0.05% Tween-20/1.3% skim milk was used as a secondary antibody to detect TGF- β , anti-mouse IgG (Amersham, Braunschweig, Germany) diluted 1:2000 was used to detect furin. Labeling was visualized using enhanced chemiluminescence (ECL system, Amersham). Equal protein loading was ascertained by Ponceau S staining for all blots.

FLP activity. To determine cellular FLP activity, 15,000 cells were adhered to 96 well plates for 24 h. Growth medium was replaced by 50 μ l of DMEM without phenol red, but containing 0.25% Triton X-100 for permeabilization and boc-RVRR-amc (100 μ M) as a substrate. Because of the stable FLP activity over a range of 48 h in that assay, the data shown were measured at 4 h after substrate addition. Fluorescence was measured at 380 nm excitation and 460 nm emission wavelengths. The data were normalized to cell density by parallel staining of another 96 well plate with crystal violet. For the determination of FLP activity in the supernatant, cells were grown in a 75 cm² culture flask. At nearly confluent stage, normal growth medium was changed to 7 ml of DMEM without phenol red. The supernatant was harvested 48 h later, cleared of cell debris by centrifugation and stored until further use at -20°C . The assay was performed with 0.25% Triton X-100 and 100 μ M substrate in a total volume of 100 μ l. The data were normalized to the protein concentration in the supernatant.

Statistical analysis. All experiments reported herein were performed at least three times with similar results. A correlation between various sets of data was examined by linear correlation analysis as outlined below.

Results

Human malignant glioma cells express furin and exhibit FLP activity. To assess whether glioma cells express furin, RNA was prepared from 12 different human malignant glioma cell lines and analysed for furin mRNA by Northern blot (Fig. 5.1.A). A 4.2 kB furin mRNA species was detected in all cell lines. The highest levels of furin mRNA were detected in U138MG, LN-428, U373MG and LN-308 cells. Low levels were detected in U87MG, T98G and LN-319 cells. RT-PCR confirmed that all glioma cell lines expressed furin mRNA (data not shown).

Next, we determined the levels of furin protein by immunoblot analysis. The 98 kD form is thought to be the major form of furin (183). Cleavage of furin in the cysteine-rich region is thought to result in a minor 60 kD form (193). Furin protein was detected in soluble protein lysates from all cell lines at differing levels. Both the 98 kD and 60 kD forms of furin were detected. Rather low levels of furin protein were visualized in the sv-FHAS nonneoplastic astrocyte cell line (Fig. 5.1.B). Overall, mRNA and protein levels appeared to correlate. For instance, LN-308, the cell line showing the highest mRNA expression, also yields the strongest protein signal on the immunoblot.

FLP activity was determined by measuring conversion of a fluorogenic substrate, boc-RVRR-*amc* (Fig. 5.1.C). Overall, there was little variation in FLP activity among the cell lines. FLP activity correlated less well with mRNA and protein levels, suggesting that the enzyme assay does not only detect furin activity but also the activity of related furin-like proteases which are probably expressed by glioma cells. Of note, the astrocytic cell line sv-FHAS exhibited lower FLP activity than each of the glioma cell lines, consistent with the low levels of furin protein (Fig. 5.1.B).

Since furin has been reported to be regulated by TGF- β in fibroblasts and rat hepatocytes (10, 11), we examined whether exogenous TGF- β_1 or TGF- β_2 modulate FLP activity. LN-18 or T98G exposed to either isoform of TGF- β for 4 or 24 h failed to exhibit a significant change in FLP activity. Moreover, neutralizing TGF- β antibodies failed to modulate FLP activity in these cells (data not shown), suggesting that endogenous TGF- β does not modulate FLP activity.

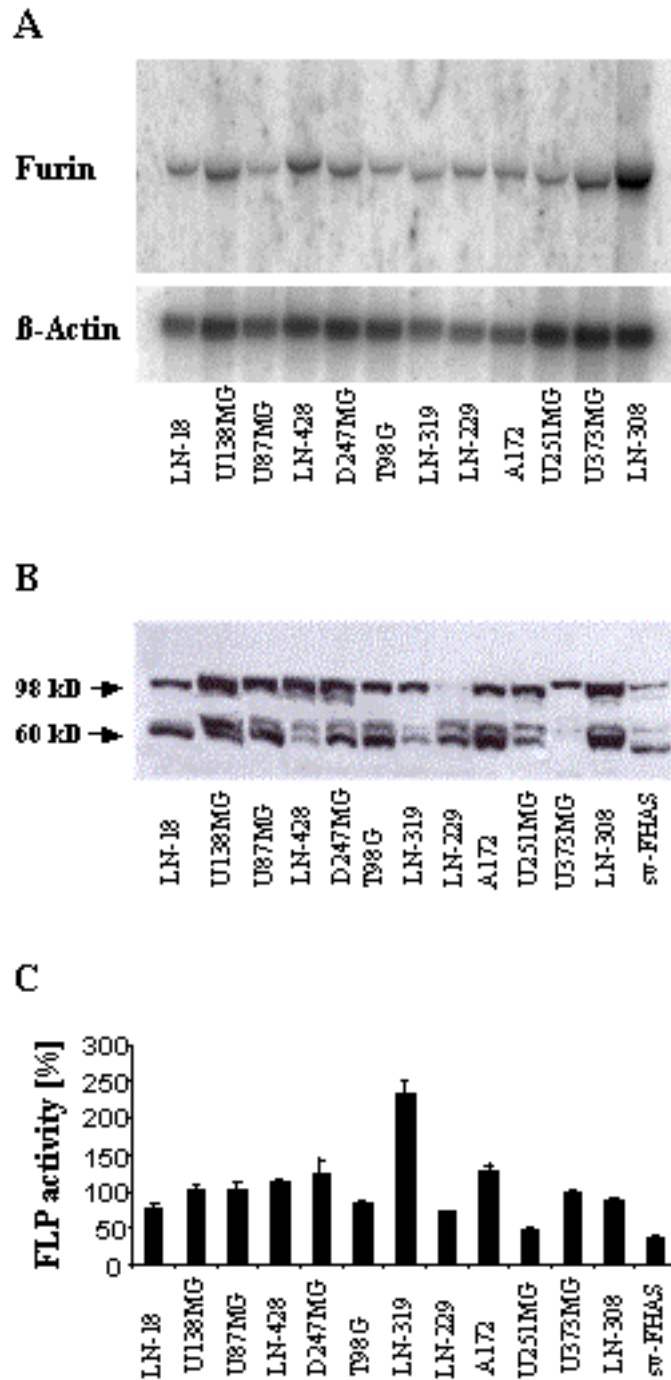


Fig. 5.1.A-C. **Furin expression and FLP activity in human malignant glioma cells.** A. Furin mRNA expression was examined by Northern blot analysis. A 4.2 kB furin mRNA species was detected in all cell lines. Equal loading was confirmed by Northern blot analysis for β -actin. B. Cellular furin protein levels were determined by immunoblot analysis. The putative furin band was identified by transient transfection of LN-229 cells with a furin expression plasmid (data not shown). C. FLP activity was determined by monitoring boc-RVRR-amc cleavage. Data are expressed as mean relative decrease or increase and SEM of activity in percentages of the mean values for all cell lines for each single assay (n=6).

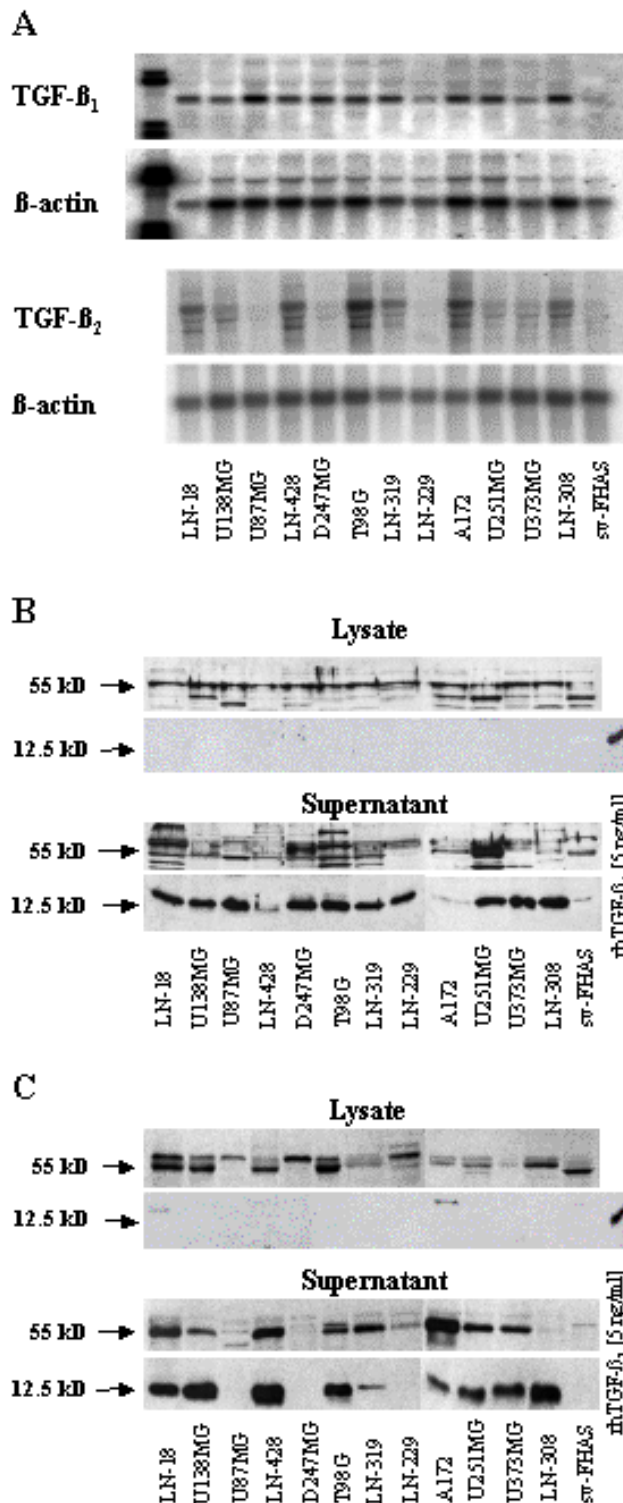


Fig. 8.2.A-C. **TGF- β expression and bioactivity in malignant glioma cells.** A. TGF- β_1 and TGF- β_2 mRNA expression were examined by Northern blot. Equal loading was ascertained by rehybridization of the filters to β -actin. B,C. Levels of TGF- β_1 and TGF- β_2 in cellular lysates and total secreted protein were assessed by immunoblot. The 12.5 kD TGF- β_1 and TGF- β_2 forms were not detected in the cellular lysates. 5 ng recombinant human TGF- β_1 or TGF- β_2 served as a positive control.

TGF- β synthesis and release by glioma cells. TGF- β_1 and TGF- β_2 mRNA and protein expression and TGF- β bioactivity were assessed in the same panel of glioma cell lines. Northern blot analysis revealed that most glioma cell lines expressed a 5.1 kB mRNA for TGF- β_1 and TGF- β_2 (Fig. 5.2.A). Immunoblot analysis was performed with total cellular soluble protein lysates and with supernatant protein (Fig. 5.2.B,C). Cellular lysates contained

comparable levels of 55 kD TGF- β_1 but greatly differing levels of 55 kD TGF- β_2 . The 12.5 kD active fragment was not detected in the cellular lysate (Fig. 5.2.B,C). Both the 55 kD and the 12.5 kD fragments for TGF- β_1 and TGF- β_2 were detected in the supernatant of both cell lines. These data suggest that TGF- β is mainly released as the 55 kD form and further processed following release. Appropriate control experiments were performed to assure that the TGF- β_1 antibody did not recognize human recombinant TGF- β_2 , and *vice versa* (data not shown). The levels of TGF- β_1 and TGF- β_2 in the supernatant were also quantified by ELISA (Fig. 5.2.D) and by CCL64 bioassay which determines both TGF- β_1 and TGF- β_2 but allows to differentiate latent and active TGF- β (Fig. 5.2.E). Overall, there was fairly good correlation between the levels of mRNA and protein expression determined by the different methods. For instance, U87MG, D247MG and LN-229 cells, which showed very low TGF- β_2 mRNA expression on Northern blot analysis, also showed correspondingly low levels of TGF- β_2 protein on immunoblot analysis or in the ELISA. Statistical analysis revealed that the levels of active TGF- β determined by bioassay correlated with the sum of TGF- β_1 and TGF- β_2 levels in the ELISA ($r=0.716$, $p=0.009$), and the levels of total TGF- β in the bioassay with TGF- β_1 and TGF- β_2 levels in the ELISA as well ($r=0.675$, $p=0.016$). There was no clearcut correlation between furin mRNA and protein levels (Fig. 5.1) and the activation of TGF- β (Fig. 5.2), suggesting that furin is not the only mediator of TGF- β processing in glioma cells. However, some of the data fit rather well, e.g., the very efficient processing of TGF- β_1 and TGF- β_2 in LN-308 cells which are strongly furin-positive.

D

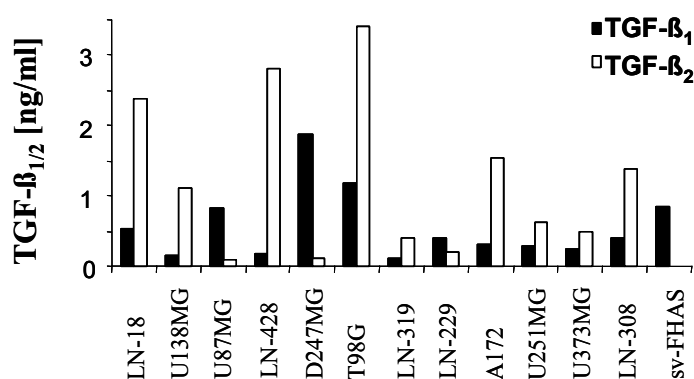


Fig. 5.2.D. **TGF- β ELISA.** Levels of total TGF- β_1 (black bars) and TGF- β_2 (open bars) were measured by ELISA. Data are expressed in pg/ml, mean of duplicate values, per 10^6 cells, released in 48 h.

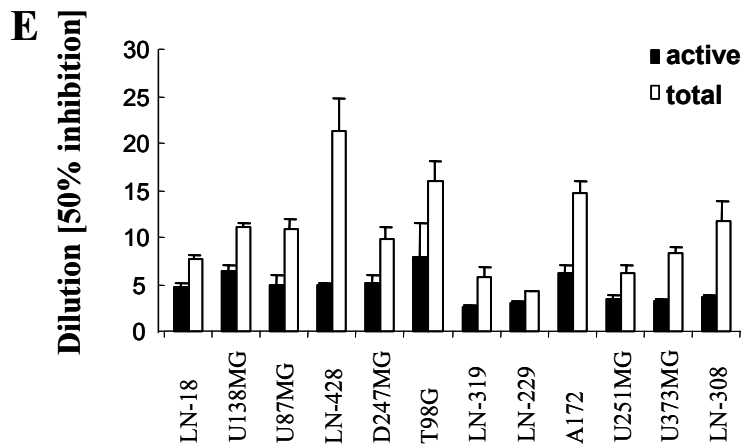


Fig. 5.2.E. **TGF- β -CCL64 bioassay.** Levels of active (black bars) and total (open bars) TGF- β were measured by CCL64 bioassay. Data are expressed as the dilution of supernatant required for 50% growth inhibition of CCL64 cells for native (black bars) and heat-activated (open bars) supernatants.

Glioma cells release furin into the cell culture supernatant. Since (i) furin is thought to be required for the processing of 55 kD TGF- β to active 12.5 kD TGF- β , since (ii) the 12.5 kD TGF- β is not detected in the cell lysate, but in the supernatant, and since (iii) high levels of 55 kD TGF- β are released into the cell culture supernatant where 12.5 kD is also detected, we concluded that significant TGF- β processing must take place in the cell culture supernatant or at the extracellular aspect of the cell membrane. First, to examine whether FLP activity localizes to the extracellular aspect of the cell membrane, we performed the fluorescent FLP activity assay in parallel *with* (standard) and *without* cellular permeabilization (see Methods). These experiments revealed that the FLP activity of nonpermeabilized cells did not exceed 10% of the activity measured in permeabilized cells, consistent with previous studies which indicate that some furin is exposed at the cell surface (194-196). Yet, the residual 10% may also originate from minor penetration during the assay of the substrate into the cells or from furin release into the supernatant (see below).

We thus went on to test the hypothesis that furin is released into the supernatant to convert 55 kD TGF- β to 12.5 kD TGF- β . Fig. 5.3.A shows that immunoreactive furin can be detected in the supernatant of most glioma cell lines. Particularly high levels were detected in LN-308 cells, corresponding to the high intracellular levels of furin in these cells (Fig. 5.1.B). Interestingly, there was very little release of 60 kD furin into the cell culture supernatant. Moreover, glioma cell supernatants exhibited significant FLP activity (Fig. 5.3.B). As observed for cellular furin and cellular FLP (Fig. 5.1), there was no strong

correlation of furin levels and FLP activity in the supernatant (Fig. 5.3).

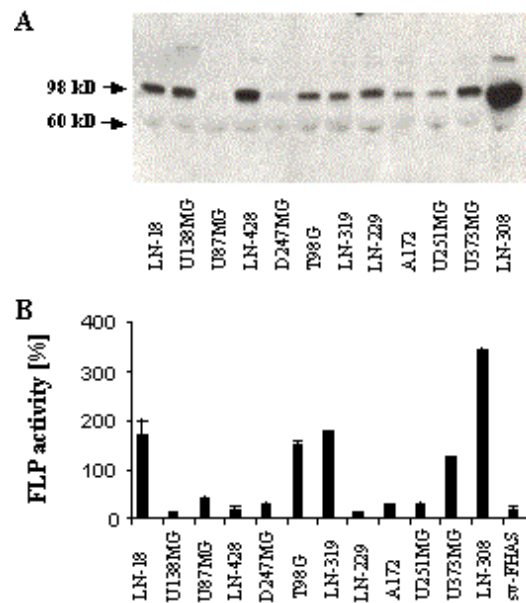


Fig. 5.3.A,B. **Furin release by cultured glioma cells.** Cell culture supernatants were assessed for immunoreactive furin by immunoblot (A) or FLP activity by boc-RVRR-amc cleavage (B) as in Fig. 5.1.

FLP activity is required for the processing of TGF- β in glioma cells. To assess whether furin-like enzymes mediate TGF- β processing in glioma cells, we monitored changes in TGF- β release induced by an inhibitor of furin, dec-RVKR-cmk. Fig. 5.4.A shows that dec-RVKR-cmk inhibited the formation of active (12.5 kDa) TGF- β_1 and TGF- β_2 in a concentration-dependent manner. The loss of active TGF- β after exposure to the furin inhibitor was confirmed at the level of ELISA (Fig. 5.4.B,C) and bioassay (Fig. 5.4.D). In parallel, there was an increase in the 55 kDa proform of TGF- β_1 and TGF- β_2 in the supernatant (Fig. 5.4.A). In contrast, the levels of intracellular 55 kDa pro-TGF- β were unaffected by inhibition of FLP activity (data not shown), confirming that most TGF- β conversion by furin takes place extracellularly. The inhibitor was equally effective in inhibiting TGF- β synthesis in the glioma cell lines which express both isoforms of TGF- β (Fig. 5.4) and in sv-FHAS astrocytes which express only TGF- β_1 (data not shown). Altogether, these observations, notably the loss of 12.5 kD TGF- β and the relative increase of 55 kD TGF- β , support the hypothesis that released soluble furin plays a prominent role in the processing of TGF- β by glioma cells.

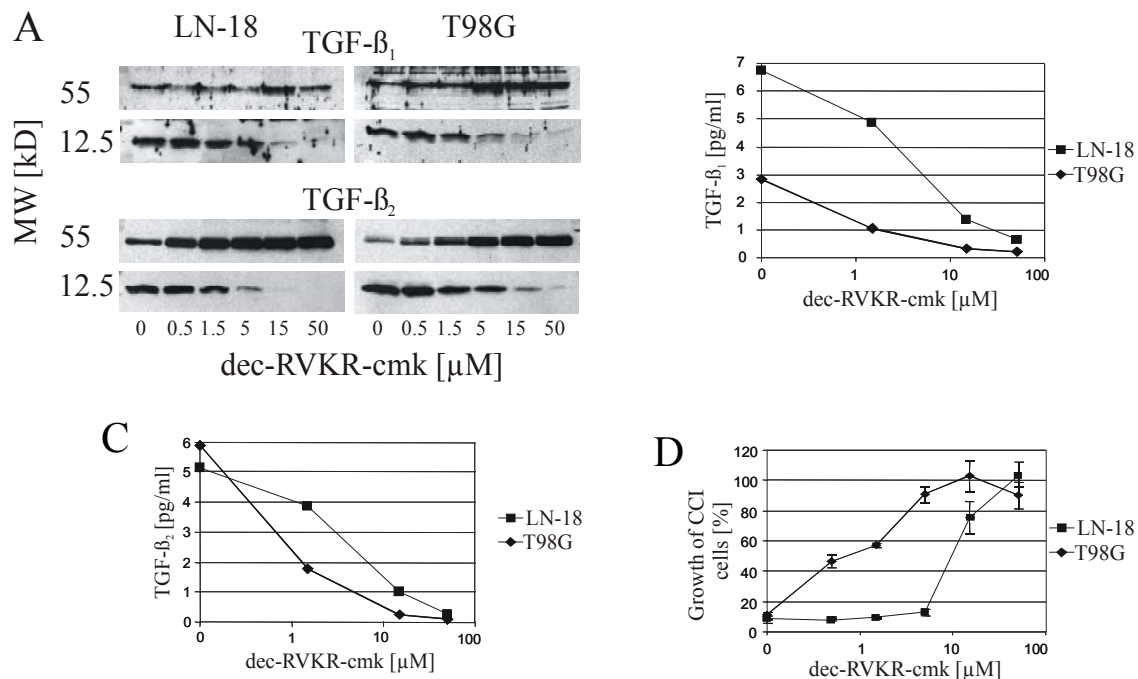


Fig. 5.4.A-D. **Furin-like proteases process TGF- β in malignant glioma cells.** A. LN-18 or T98G cells were untreated or treated with increasing concentrations of dec-RVKR-cmk for 48 h. The inhibitor was reapplied every 12 h because of its unstable nature ((34); W. Garten, unpublished observation). Immunoblot analysis for TGF- β_1 and TGF- β_2 in the supernatant was performed as in Fig. 5.2.B-D. The glioma cells were exposed to increasing concentrations of dec-RVKR-cmk for 48 h as above. Supernatants were assessed for TGF- β_1 (B) or TGF- β_2 (C) by ELISA or total TGF- β by CCL64 bioassay (D). In D, supernatants were activated and diluted 1:10.

PDX gene transfer reduces glioma-associated TGF- β bioactivity. PDX is a synthetic serine protease inhibitor (serpin) designed to selectively inhibit furin (17). The LN-18 and T98G glioma cell lines were transfected with a PDX expression plasmid (17). PDX transgene expression was verified by RT-PCR (Fig. 5.5.A). PDX-transfected glioma cell sublines exhibited a minor, but significant reduction in cytoplasmic FLP activity (Fig. 5.5.B). However, although the reduction of FLP activity did not exceed 20%, there was strong inhibition of TGF- β processing, as assessed by the levels of 12.5 kD TGF- β in the supernatant (Fig. 5.5.C). Quantification of the immunoblots for 12.5 kD TGF- β revealed a reduction of TGF- β_1 to less than 60% in T98G cells, and of TGF- β_2 to less than 70% in LN-18 and to less than 40% in T98G cells (Fig. 5.5.D). PDX transgene expression was not very

prominent in the glioma cell lines and was lost within few passages *in vitro*, suggesting strong selection pressure against furin inhibition *in vitro* (data not shown).

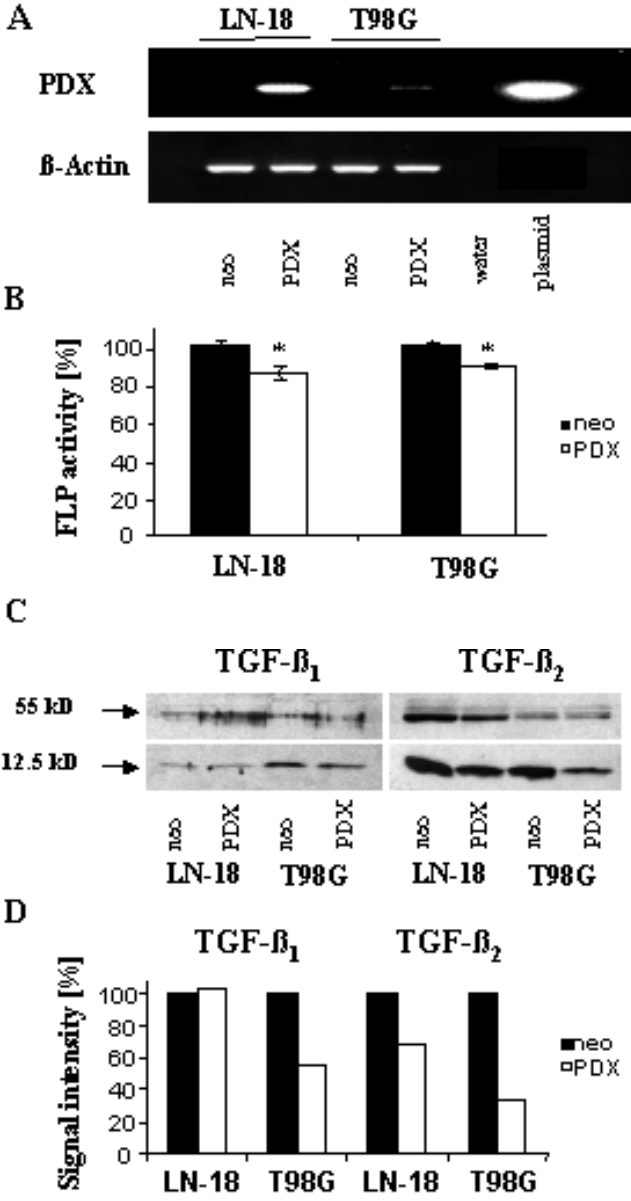


Fig. 5.5.A-D. **PDX gene transfer inhibits FLP activity and TGF-β processing.** A. Expression of the PDX transgene was confirmed by RT-PCR. B. Cytoplasmic FLP activity of PDX transfectants was assessed by boc-RVRR-ame cleavage and is expressed relative to neo control cells (*p<0.05, t-test). C. Immunoblot analysis for TGF-β₁ and TGF-β₂ in the supernatant was performed as in Fig. 5.2.D. The representative 12.5 kD TGF-β blot shown in C was quantified by densitometry. Signal intensity is expressed as percentage of PDX transfectants compared with neo controls.

Discussion

TGF- β is a cytokine that is released by glioma cells in large quantities *in vitro* and *in vivo* and that has been considered central to the malignant progression of glial tumors and to the immune dysfunction in human patients with glioblastoma (4). This is because TGF- β promotes tumor angiogenesis, enhances migration and invasion, and inhibits T cell-mediated immune responses. Experimental therapeutic efforts to neutralize TGF- β activity associated with glioblastoma include antisense strategies (197) and antagonism by decorin (198).

Here, we examined the biochemical pathways of TGF- β synthesis and processing in malignant glioma cells and asked whether proteases of the subtilisin-like proprotein convertase family such as furin are involved in TGF- β maturation and might therefore become a target for immunotherapy in these tumors. We find that glioma cells express furin mRNA and protein and exhibit FLP activity in a fluorometric enzyme assay. Cellular FLP activity does not correlate well with furin protein levels, indicating that this enzyme assay detects the activity of other furin-related proteases as well.

As indicated above, there is an increasing number of subtilisin-like proprotein convertases which are now designated SPC1-8 and which are candidate enzymes for TGF- β processing (182). There were also significant differences in the levels of released furin and the levels of FLP activity in the supernatant among the cell lines (Fig. 5.3). A definite physiological role of released furin has not been characterized. However, previous studies have postulated a role for furin release in the processing of cellular substrates (199). The present study suggests that the release of furin may play a crucial role in creating an immunosuppressive microenvironment in human gliomas *via* enhanced TGF- β processing.

We confirm that the synthesis and release of large levels of TGF- β_1 and TGF- β_2 are a feature of cultured glioma cell lines (Fig. 5.2). Numerous previous studies have confirmed that glioblastoma cells produce TGF- β *in vivo*, too (reviewed in (147)). Here, we identify one or more furin-like proteases as the enzyme(s) responsible for TGF- β activation in glioma cells (Fig. 5.4). Furin has so far been characterized as a TGF- β_1 -processing enzyme in cell-free systems (120). For the first time ever, we show that furin inhibitors abrogate TGF- β_1 processing in intact cells and extend these observations to the processing of TGF- β_2 , too.

The present study does not allow firm conclusions as to whether FLP activity is pathologically elevated in glioma cells and thus primarily responsible for the high level of TGF- β release from human gliomas *in vivo*. In contrast to other cell types (179), TGF- β

appears not to promote furin mRNA expression or activity to enhance its own release in a positive autocrine loop in glioma cells (data not shown).

At present, there is little information on the expression and activity of furin-like proteases in other cell types *in vitro* and *in vivo* (200). Our study indicates that differences in furin expression or FLP activity alone do not account for most of the variation in the levels of TGF- β_1 or TGF- β_2 that are processed and released in a panel of human glioma cell lines. Further, the phenotype obtained after targeted disruption of the furin gene in mice revealed that furin is probably not the only pathway that regulates the processing of TGF- β family members (200). Yet, the effects of the synthetic pseudosubstrate furin inhibitor, dec-RVKR-cmk (Fig. 5.4) and the antitrypsin derivative, PDX (Fig. 5.5), demonstrate that furin may be a suitable target to reduce TGF- β bioactivity associated with glioma cells. Of note, since subtilisin-like proprotein convertases other than furin may be chiefly responsible for the inhibition of TGF- β processing observed here, inhibition of furin alone may not be sufficient for successful immunotherapy of malignant glioma. Thus, the present study defines a novel strategy for the pharmacotherapy or somatic gene therapy, e.g., using PDX, of conditions associated with pathological levels of TGF- β bioactivity, including glioblastoma.

VI. MICA/NKG2D-mediated immunogene therapy of experimental gliomas

A related manuscript has been published in Cancer Research, 2003 Dec 15;63(24):8996-9006

This project is based on previous work from my colleague Dr. Manuel Friese who also carried out most of the experiments. Dr. Alexander Steinle generated and supplied MICA and MICB-specific antibodies. Yasmin Breithardt and Brigitte Frank provided expert technical assistance. Dr. Ulrike Naumann generated the adenoviral constructs, Dr. Hans-Jörg Bühring was responsible for the cell sorting. I contributed to this project by establishing the syngeneic murine astrocytoma SMA-560 mouse glioma model, which served and still serves as a precious immunological read out tool for glioma immunotherapy approaches in our laboratory, further I planned and characterized the immunological animal experiments together with Manuel Friese.

Introduction

Glioblastoma, the most frequent malignant primary brain tumor, carries a poor prognosis, with a median survival after resection, radiotherapy and chemotherapy of 12 months (201). The lack of effective immune responses to glial tumors inside the CNS has been attributed to the immune-privileged status of the brain conferred by the blood-brain barrier, the lack of conventional lymphatics and the local release of immunosuppressive factors (132, 202, 203). However, both lymphocytes and macrophages infiltrate malignant gliomas, indicating the potential for lymphocyte homing and presentation of processed tumor antigens (132).

NKG2D is a C-type lectin-like homodimeric receptor expressed by human NK, $\gamma\delta$ T and CD8⁺ $\alpha\beta$ T cells (204). In the mouse, NKG2D expression is found on NK cells, activated CD8⁺ T cells and LPS-stimulated macrophages (205). Ligation of NKG2D may be a critical activating pathway to provide tumor surveillance (206-208). NKG2D interacts with ligands that are not constitutively, but inducibly expressed, including human MICA and MICB, distant cell stress-inducible homologs of MHC class I (209-211). The tissue distribution of MIC molecules is highly restricted to intestinal epithelia, but they are frequently expressed in epithelial tumors (210, 212). A second family of human NKG2D ligands (NKG2DL), designated UL16-binding proteins (ULBP)1, ULBP2 and ULBP3, has been characterized (213-215). Although no murine MIC molecules have been found, there are mouse orthologs of ULBP, the retinoic acid early inducible gene-1 products (RAE-1). The minor histocompatibility antigen H60 is another murine NKG2DL (205, 216-218). H60 and RAE-1 are upregulated on tumor cells and in response to genotoxic stress and induce tumor immunity (206-208).

Activation of NK and effector T cells is inhibited by interactions of MHC class I antigens on $\alpha\beta$ and $\gamma\delta$ T cells. These inhibitory NK cell receptors include isoforms of the killer cell immunoglobulin (Ig)-like receptors (KIR) and Ig-like transcript/leukocyte Ig-like receptors (ILT/LIR) that interact with HLA-A, -B, -C or -G antigens, and a heterodimer formed by the C-type lectin like CD94 and NKG2A that interacts with HLA-E (57, 219-222). Since a decrease or loss of MHC class I antigen expression may accompany neoplastic transformation, NK cells represent a natural defense to sense and selectively eliminate abnormal cells (223-225). This NK-mediated cytolytic activity must be down-regulated in the brain to prevent autoimmune damage because of low expression levels of MHC class I antigens on glial cells and a lack of MHC class I antigen expression on neurons (226, 227).

Material and methods

Cell lines and transfectants. The human Sv-FHAS astrocytic cell line was provided by D. Stanimirovic (Institute of Biological Sciences, National Research Council of Canada, Ottawa, Canada). The human malignant glioma cell lines were provided by Dr. N. de Tribolet (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Primary glioblastoma cells were established from freshly resected tumors, cultured in monolayers and used between passages 4 and 9 (228). The murine glioma cell lines GL261 and SMA-560 were gifts of X. O. Breakefield (Harvard Medical School, Boston, MA) and D. D. Bigner (Duke University Medical Center, Durham, NC). The cells were maintained in DMEM supplemented with 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), 10% FCS (Biochrom KG, Berlin, Germany) and penicillin (100 IU/ml)/streptomycin (100 µg/ml) (Gibco). NKL cells, kindly provided by M. J. Robertson (Indiana University School of Medicine, Indianapolis, IN), COS-7, YAC-1 and K562 cells (ATCC, Rockville, MD) were grown in RPMI 1640 medium supplemented with 15% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin (100 IU/ml)/streptomycin (100 µg/ml). Transfections with cDNA clones, RSV.5neo, RSV.5neo-MICA*01 (209, 210) or RSV.5neo-PatrmIC1 (229) were conducted using FuGENE 6™ (Roche, Mannheim, Germany) and selection with G418 (1 mg/ml). PatrmIC1 is the chimpanzee homolog of MICA which show 87% homology and was used as a negative control in some experiments (229). Stable positive isolates were identified by flow cytometry and, to speed up the selection process sorted using a FACSvantage (Becton Dickinson, Heidelberg, Germany).

RT-PCR. Total RNA was prepared using RNAeasy (Quiagen, Hilden, Germany) and transcribed according to standard protocols. The conditions for all standard PCR were: 5 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C, and 10 min at 72°C, using the following PCR primers: H60: sense, 5'-GATGGTACAGACTCTCTAAGTTGT-3'; antisense, 5'-CAGACCCTGGTTGTCAGAATTATG-3' (554 bp); RAE-1: sense, 5'-ATGGCCAAGGCAGCAGTGACCAAG-3'; antisense, 5'-CACATCGCAAATGCAAATGCAAATAAT-3' (748 bp); MICA: sense, 5'-GTGCCCCAGTCCTCCAGAGCTCAG-3'; antisense, 5'-GTGGCATCCCTGTGGTCACTCGTC-3' (635 bp); MICB: sense, 5'-GGCGTCAGGATGGGGTATCTTTGA-3'; antisense, 5'-GGCAGGAGCAGTCGTGAGTTTGCC-3' (690 bp); ULBP1: sense, 5'-CTGCAGGCCAGGATGTCTTGTGAG-3'; antisense, 5'-TGAGGGTGGTGGCCATGGCCTTGG-3' (319 bp); ULBP2: sense, 5'-CTGCAGGCAAGGATGTCTTGTGAG-3'; antisense, 5'-TGAGGGTGGTGGCTGTGGCCCTGA-3' (327

bp); ULBP3: sense, 5'-CTGCAGGTCAGGATGTCTTGTGAG-3'; antisense, 5'-TGAGGGTGGTGGCTATGGCTTTGG-3' (321 bp); β -actin: sense, 5'-AGCCATGTACGTAGCCAT-3'; antisense, 5'-TTTGATGTCACGCACGATTT-3' (232 bp).

Generation of monoclonal antibodies. The mouse P815 mastocytoma cell line was transfected using FuGENE 6TM with full length cDNA of NKG2DL MICA*01, MICA*04, MICB*02, ULBP1, ULBP2 or ULBP3, in RSV.5 neo (214). Transfectants were selected with G418. BALB/c mice were immunized either with a mixture of MICA*01-, MICA*04-, and MICB*02-, or ULBP1-, ULBP2-, and ULBP3- expressing P815 cells. Splenocytes of immunized mice were fused with P3X63Ag8.653 myeloma cells. Hybridoma supernatants were tested by indirect immunofluorescence using a FACSCalibur (Becton Dickinson) for selective binding to P815-NKG2DL transfectants and to COS cells transiently transfected with the various NKG2DL cDNAs. Hybridomas producing NKG2DL-specific mAbs were subcloned twice and immunoglobulins were isotyped using an isotyping kit (Roche). BAMO-1 (IgG₁) and BAMO-2 (IgG_{2a}) recognize MICA and MICB, AMO-1 (IgG₁) is MICA-specific, BMO-1 (IgG₁) MICB-specific, AUMO-1 (IgG₁) ULBP1-specific, BUMO-2 (IgG₁) ULBP2-specific and CUMO-1 (IgM) ULBP3-specific (230).

Production of soluble mNKG2D in insect cells. Recombinant soluble mNKG2D lacking the amino-terminal cytoplasmic region and transmembrane domain was produced in Sf9 insect cells (Invitrogen, Karlsruhe, Germany) using the BAC-to-BAC system (Gibco Life Technologies, Paisley, UK). The expression construct included an amino-terminal FLAG/hexahistidine (214). Soluble mNKG2D was purified from culture supernatant by affinity chromatography on Ni²⁺-charged chelating sepharose (BD Pharmingen, Heidelberg, Germany) and size-exclusion filtration.

Monoclonal antibodies and flow cytometry. Cell surface expression of MHC class I antigens and NKG2D was assayed with the following monoclonal antibodies: W6/32, IgG_{2a} pan anti-HLA-A, -B, -C, -E, -G (Biozol, Echingen, Germany); anti-H-2K^b (BD Pharmingen, Heidelberg, Germany); anti-H-2D^b (Caltag Laboratories, Hamburg, Germany); M585, IgG₁ anti-NKG2D (kindly provided by Immunex, Seattle, WA). IgG₁, IgG_{2a} and IgM isotype-matched antibodies were used as controls (BD Pharmingen). For the mNKG2D binding assays, mAb anti-FLAG M2 (5 μ g/ml) (Sigma) was mixed with soluble mNKG2D (3 mg/ml) prior to addition to cells. COS-7 cells were transiently transfected with neo-control, MICA*01 or PatrMIC1. MICA/B and PatrMIC1 expression levels were detected by flow cytometry using the mAb AMO-1. Interactions of human MICA*01 and chimpanzee PatrMIC1 with mouse NKG2D was analyzed by staining with flagged soluble mouse NKG2D and anti-

FLAG mAb. Glioma cells were detached using Accutase (PAA, Wien, Austria), preincubated in PBS with 2% BSA and incubated with the specific antibody or matched mouse Ig isotype (10 µg/ml) as a control for 30 min on ice. Specific binding was detected with PE-conjugated goat anti-mouse IgG (Sigma). Fluorescence was measured in a Becton Dickinson FACScalibur. Specific fluorescence indexes (SFI) were calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with control antibody.

Adenoviral constructs and transduction. Replication-deficient recombinant adenoviruses (Ad5dE1,3) that had the E1A region replaced by the full length MICA*01 cDNA (Ad-MICA) were constructed using the AdEasy System, kindly provided by Bert Vogelstein (The Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD). Recombinant adenoviral particles were produced in 293 cells (ATCC CRL-1573) and transgene expression was assessed by RT-PCR. Infection of target cells with recombinant viruses was accomplished by exposing cells to different concentrations of adenovirus in PBS containing 10% glycerol for 15 min followed by addition of serum-containing medium for 2 days.

Purification of PBL and isolation of NK and T cells. PBMC were derived from healthy donors by density gradient centrifugation (Biocoll, Biochrom KG) and depletion of plastic-adherent cells. PBL were cultured on irradiated RPMI 8866 feeder cells in order to obtain polyclonal NK cell populations. To further enrich NK cells, PBL were sorted by immunomagnetic depletion using Dynabeads (NK Cell Negative Isolation Kit, Dynal, Oslo, Norway). CD3⁻ CD56⁺ cells were used for cytotoxicity assays. To obtain purified T cells, fresh PBL were depleted of B cells and monocytes using the LymphoKwik TTM reagent (One Lambda Inc., Canoga Park, CA).

Mouse lymphocyte isolation. Murine NK cells were prepared from splenocytes from CD1-deficient BALB/c nude mice or VMDk mice. NK cells were positively selected using DX5 mAb-coupled magnetic beads with the corresponding column system (Miltenyi Biotech, Bergisch Gladbach, Germany). Polyclonal mouse NK cells were cultured with mouse IL-2 (5000 U/ml, Preprotech, London, UK) for at least 10 days before use in cytotoxicity assays.

Cytotoxicity assay. Cytotoxicity was assessed in 4 h ⁵¹Cr release assays in the absence or presence of various mAb or soluble mNKG2D. The concentrations for the masking experiments were 10 µg mAb/ml and 20 µg/ml for soluble mNKG2D. The ⁵¹Cr release assay was performed using 2000 ⁵¹Cr-labeled targets per well. Effector and target cells were incubated at various effector:target (E:T) ratios for 4 h. Spontaneous release of ⁵¹Cr was

determined by incubating the target cells with medium alone. Maximum release was determined by adding NP40 to a final concentration of 2%. The percentage of ^{51}Cr release was calculated as follows: $100 \times ([\text{experimental release} - \text{spontaneous release}] / [\text{maximum release} - \text{spontaneous release}])$.

T cell reaction against allogeneic glioma cells. To suppress LN-229 glioma cell proliferation, the cells were irradiated at 100 Gy. HLA-A2-positive T cells or PBMC (10^5 /well) were cocultured with 10^4 irradiated LN-229 HLA-A2-negative glioma cells in 96 well plates in triplicates. After 4 days the cells were pulsed for 24 h with [methyl- ^3H]-thymidine (0.5 μCi , Amersham, Braunschweig, Germany) and harvested with a cell harvester (Tomtec, Hamden, CT). Incorporated radioactivity was determined in a Wallac 1450 Microbeta Plus Liquid Scintillation Counter. For the priming assays, non-irradiated glioma cells were incubated with PBMC or T cells for specific priming and clonal expansion. Lymphocytes were harvested on day 5 and used as effectors in a 4 h ^{51}Cr release cytotoxicity assay. Mock-transfected LN-229.neo cells or LN-229 cells transfected with MICA*01 or PatrMIC1 were used as targets.

IL-2 release assay. T cells (1×10^6 /well) isolated at day 10 after tumor inoculation from spleens of vaccinated or control animals were stimulated *ex vivo* with irradiated SMA-560 cells (1×10^5). T cell supernatants from triplicate wells were collected and pooled 48 h later. IL-2 release was determined by ELISA (R&D Systems, Minneapolis, MN).

Mice and animal experiments. Athymic CD1-deficient BALB/c nude mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and VMDk mice from the TSE Research Center (Berkshire, UK). Mice of 6-12 weeks of age were used in all experiments. The experiments were performed according to NIH guidelines "Guide for the Care and Use of Laboratory Animals". Groups of 4-6 mice were injected subcutaneously in the right flank with transfected LN-229 tumor cells, or uninfected or infected SMA-560 glioma cells (1×10^6 cells) in 0.1 ml of PBS, as indicated. Mice were examined regularly for tumor growth using a metric caliper and killed when tumors reached >12 mm diameter. Mice were anesthetized by intraperitoneal injection of 7% chloral hydrate before all intracranial procedures. For intracranial implantation the mice were placed in a stereotactic fixation device (Stoelting, Wood Dale, IL) and a burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm. Five $\times 10^4$ LN-229 glioma cells or 5×10^3 SMA-560 cells in a volume of 2 μl PBS were injected into the right striatum. The mice were observed daily and sacrificed when developing neurological symptoms.

Immunohistochemistry. Cryosections (0.7 mm) of SMA-560 gliomas, prepared at day 10 after glioma cell inoculation, were fixed in acetone and blocked with 2% normal rabbit serum and 2% BSA. Tissue sections were stained with antibodies (1:50) to CD8 (53-6.7) (CD8 T cells) and CD11b (M1/70) (macrophages/microglia) (BD PharMingen, Heidelberg, Germany). A biotinylated anti-rat secondary antibody (Zymed, San Francisco, CA) was used at 1:150. Avidin biotin complex was added, and the staining was developed with diaminobenzidine. Murine spleens served as positive controls. Normal murine brain served as a negative control.

Results

Expression of NKG2DL and MHC class I antigens by human glioma cells. To investigate the expression of the NKG2DL MICA, MICB, ULBP1, ULBP2 and ULBP3 by glioma cells, 12 glioma cell lines, a non-neoplastic astrocyte line, Sv-FHAS and five primary *ex vivo* cultured glioma cells, were assessed by RT-PCR (Fig. 6.1. A,B).

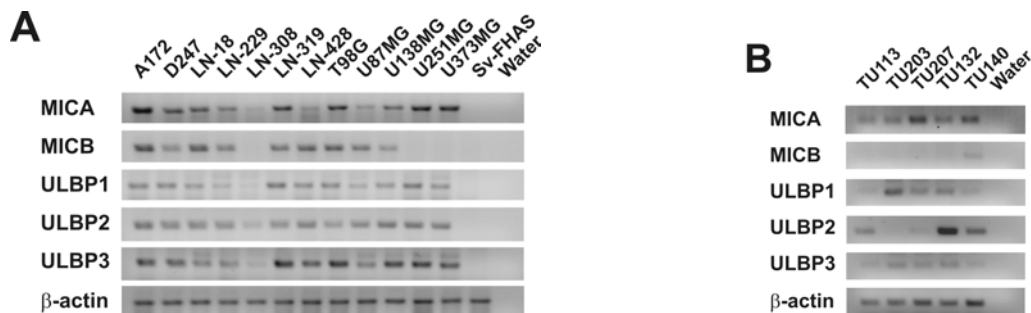


Fig. 6.1.A,B **NKG2DL expression in human glioma cells.** A, B. NKG2DL (MICA, MICB, ULBP 1, 2, 3) mRNA expression was assessed by RT-PCR in human glioma cell lines (A) and primary glioma cells (B). β-actin was used as a standard.

MICA and ULBP1, 2 and 3 mRNA were expressed by almost all cell lines and most of the primary glioma cell cultures. MICB was not detectable in U251MG or U373MG cells and several primary glioma cell cultures. In contrast, none of the NKG2DL were detected in Sv-FHAS cells. We next monitored the cell surface expression of NKG2DL and MHC class I antigens using monoclonal antibodies. All glioma cell lines expressed MICA, ULBP2 and ULBP3. MICB was expressed by 9 of 12 cell lines while ULBP1 was only detectable for 5 of 12 cell lines. All of the primary gliomas expressed MICA and ULBP2, and the other NKG2DL at various levels (Table 1). MICB was expressed by TU132, TU140 and 203 while TU113 and TU 207 did not (Table 1). All cell lines and primary cultures showed high levels of MHC class I antigen expression. The non-neoplastic Sv-FHAS cell line did not express any NKG2DL, as determined by RT-PCR and flow cytometry. Sv-FHAS cells showed low MHC class I antigen expression (Table 1).

Cell line	MHC class I (W6/32)	MICA (AMO-1)	MICB (BMO-1)	MICA/B (BAMO-1)	ULBP1 (AUMO-1)	ULBP2 (BUMO-2)	ULBP3 (CUMO-1)
A172	145	13.0	2.3	11.2	1.8	4.1	1.8
D247	113	8.2	1.5	6.5	1.0	1.6	1.4
LN-18	85	6.1	3.6	7.3	1.0	2.4	1.9
LN-229	215	6.9	2.0	5.2	1.1	5.8	6.7
LN-308	153	4.7	1.8	5.0	1.1	3.1	2.6
LN-319	112	9.7	2.2	8.9	1.3	3.7	4.4
LN-428	129	5.9	1.6	6.3	1.2	3.1	2.2
T98G	480	12.3	5.0	10.3	1.6	6.7	8.6
U87MG	351	8.6	2.2	6.4	1.3	5.9	8.4
U138MG	346	11.7	1.2	11.2	2.4	3.6	3.4
U251MG	216	14.5	1.0	9.2	1.0	7.6	5.3
U373MG	358	11.6	1.0	7.0	1.0	11.8	6.0
Sv-FHAS	90	1.0	1.0	1.0	1.0	1.1	1.2
TU113	29	4.0	1.0	3.9	1.0	1.7	1.4
TU203	23	3.8	1.3	3.5	1.3	1.7	1.1
TU207	38	1.4	1.1	1.4	1.1	1.7	1.1
TU132	95	6.2	1.4	4.4	1.0	1.3	1.0
TU140	100	9.1	2.5	7.4	1.3	1.8	1.0

Table 1. Flow cytometric analysis of MHC class I antigens and NKG2DL expression by human malignant glioma cell lines, non-neoplastic Sv-FHAS astrocytes and primary glioma cell cultures. The cells were stained with the indicated mAb or isotype-matched Ig. Expression was quantified as SFI values (mean fluorescence_{specific mAb}/mean fluorescence_{isotyp control}).

Stable transfection and adenoviral gene transfer of MICA into glioma cells stimulates NK cytolytic activity. To determine whether increased NKG2DL expression confers additional stimulatory signals for NK cells, we generated stable MICA*01 and PatrMIC1 (chimpanzee MIC homolog (229)) transfectants of the LN-229 and Sv-FHAS cell lines (Fig. 6.2. A,B). PatrMIC1 was included in this study to serve as a negative control for the nude mouse model in vivo (see below). Enhanced MICA or PatrMIC1 expression rendered cells susceptible to lysis by human NKL effector cells, suggesting that ectopic MIC expression overcomes the inhibitory effect of MHC class I antigens (Fig. 6.2.A,B, lower panels).

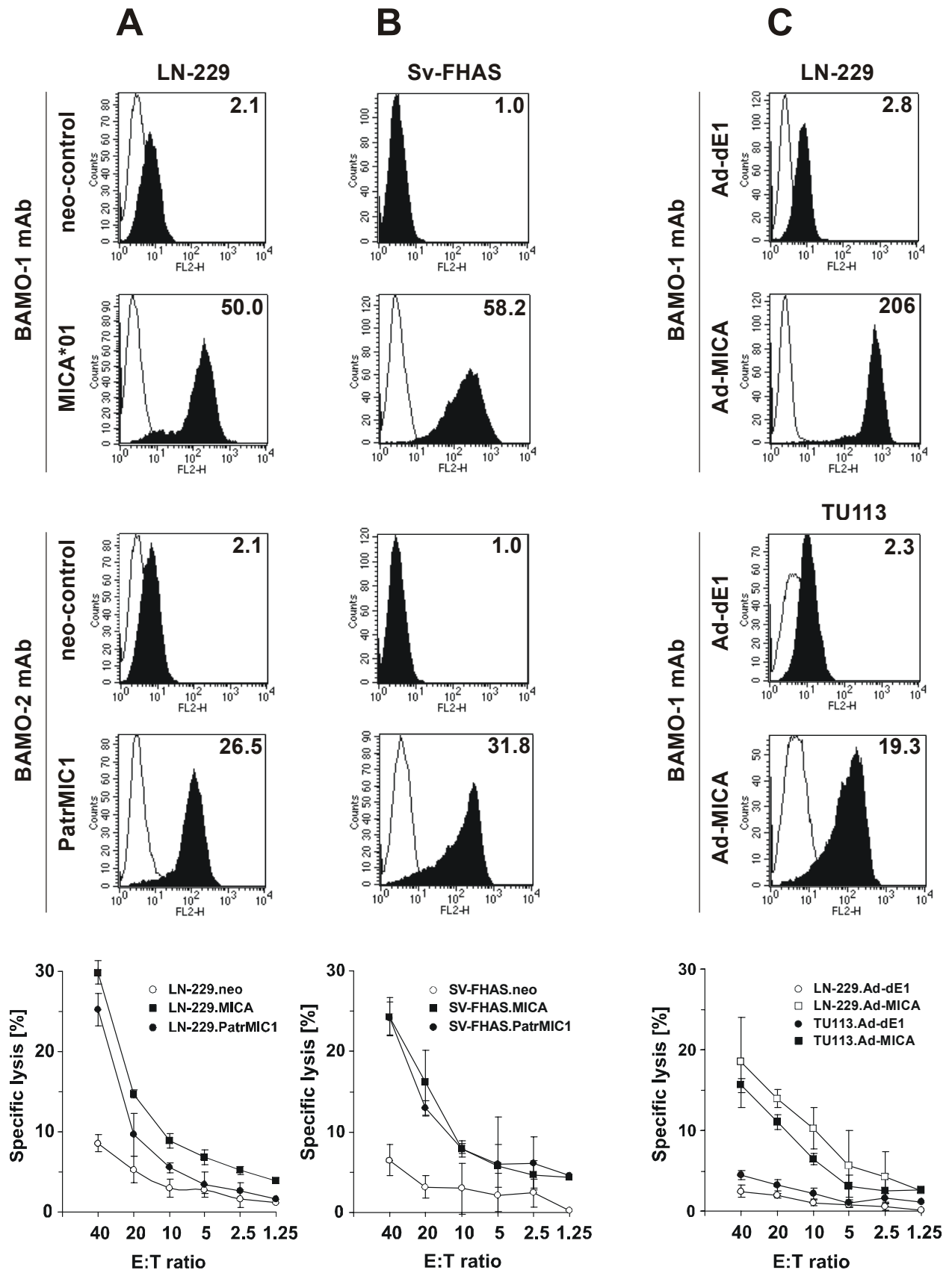


Fig. 6.2.A-C. Ectopic MICA*01 and PatrMIC1 expression by glioma cells promote NK cell-mediated cytotoxicity. A, B. LN-229 (A) or Sv-FHAS (B) cells transfected with MICA*01 or PatrMIC1 were assessed for MICA (mAb BAMO-1) or PatrMIC1 (mAb BAMO-2) expression

(filled profiles) as compared to mock-transfectants (neo-control) by flow cytometry. Open profiles show the labeling with isotype-matched Ig. The SFI values are indicated in the upper right of each panel. (C) LN-229 or TU113 primary glioma cells were infected with Ad-MICA or Ad-dE1 (300 MOI) and assessed for MICA expression accordingly. In the lower panels of A-C, the cell lines were tested as targets for human NKL cells in ^{51}Cr release assays. Data are expressed as specific lysis at different E:T ratios.

Similar results were obtained with freshly isolated polyclonal NK cells (data not shown). For a gene therapeutic approach, we generated an adenoviral vector encoding MICA*01 (Ad-MICA). Infection of LN-229, primary glioma cells (Fig. 6.2.C) or other glioma cell lines (data not shown) with Ad-MICA resulted in a marked increase in MICA expression. Adenoviral MICA gene transfer also promoted NK-mediated cytotoxicity (Fig. 6.2.C, lower panel).

LN-229.neo transfectants were sensitized to NK cytotoxicity by treatment with the anti-MHC class I mAb W6/32 (Fig. 6.3.A), confirming that classical MHC class I antigens protect LN-229.neo cells from NK cytotoxicity. Anti-MICA/B (BAMO-1) or -NKG2D (M585) mAb had no effect on cytotoxicity in LN-229.neo cells. However, these mAb either attenuated (anti-MICA) or blocked (anti-NKG2D) the sensitization mediated by anti-MHC class I mAb. The superior effect of anti-NKG2D compared with anti-MICA reflects the composition of the system of a single receptor (NKG2D) in NK cells, but multiple ligands (MIC and ULBP molecules) expressed by LN-229 cells (Fig. 1, Table 1). Soluble mouse NKG2D (mNKG2D) attenuated the anti-MHC class I mAb-mediated sensitization as effectively as anti-MICA, suggesting that murine NKG2D interacts with some human NKG2DL. NK cytotoxicity of LN-229.MICA targets pre-treated with anti-MICA or anti-NKG2D mAb was reduced (Fig. 6.3.B), demonstrating that enhanced cytotoxicity of LN-229.MICA cells is due to MICA overexpression overruling MHC class I antigen inhibitory signals. Similar results were obtained with LN-229.PatMICA1 transfectants (Fig. 6.3.C)

Costimulatory functions of MICA-transfected glioma cells. Allostimulation assays were performed to analyze whether MICA/NKG2D interactions promote the induction of T cell responses. LN-229.MICA or LN-229.PatMICA1 cells were substantially more potent stimulators (3-fold) of T cell proliferation than LN-229.neo cells (Fig. 6.4.A). mAb masking of MICA abrogated the augmentation of T cell proliferation mediated by MICA, but had no effect when T cells were coincubated with LN-229.neo cells (Fig. 6.4.B). Additional evidence

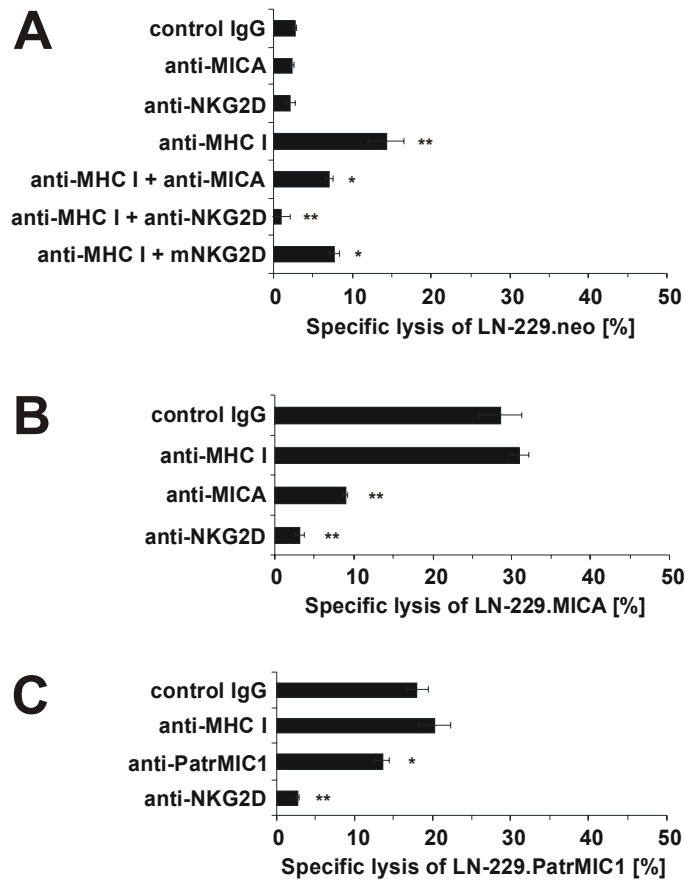


Fig. 6.3.A-C. **NKG2DL and MHC class I antigen expression determines NK cytotoxicity.** A-C. Different target cell lines were pretreated with control Ig or anti-MICA BAMO-1 mAb, anti-PatrMIC1 BAMO-2 mAb, anti-MHC class I W6/32 mAb or soluble mNKG2D. NKL effector cells were pretreated with control Ig or anti-NKG2D M585 mAb for 30 min before use in ^{51}Cr release cytotoxicity assays. LN-229.neo (A), LN-229.MICA (B) or LN-229.PatrMIC1 (C) were used as targets. The specific lytic activities are given for an E:T ratio of 40:1 (mean \pm SD, t-test, * $P < 0.05$, ** $P < 0.01$, compared with isotype control Ig).

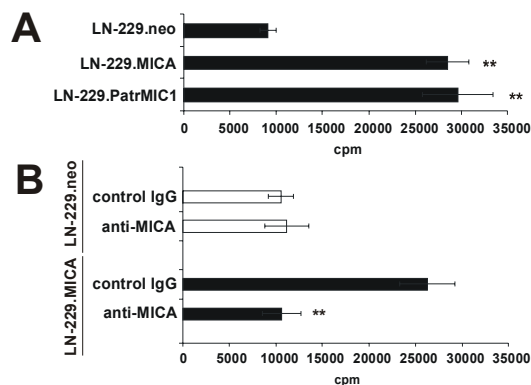


Fig. 6.4.A,B. **Modulation of alloreactivity to glioma cells by MICA and PatrMIC1.** A. Irradiated LN-229.neo, LN-229.MICA or LN-229.PatrMIC1 cells were coincubated with HLA-A2-mismatched T cells. [^3H]-thymidine was added on day 4 for 16 h and incorporation was measured by liquid scintillation counting. (B) Irradiated glioma cells were preincubated with

isotype control ab or anti-MICA (BAMO-1) and processed as in A. Data are expressed as cpm \pm SD (t-test, ** $P < 0.01$, compared with LN-229.neo in A or isotype control ab in B).

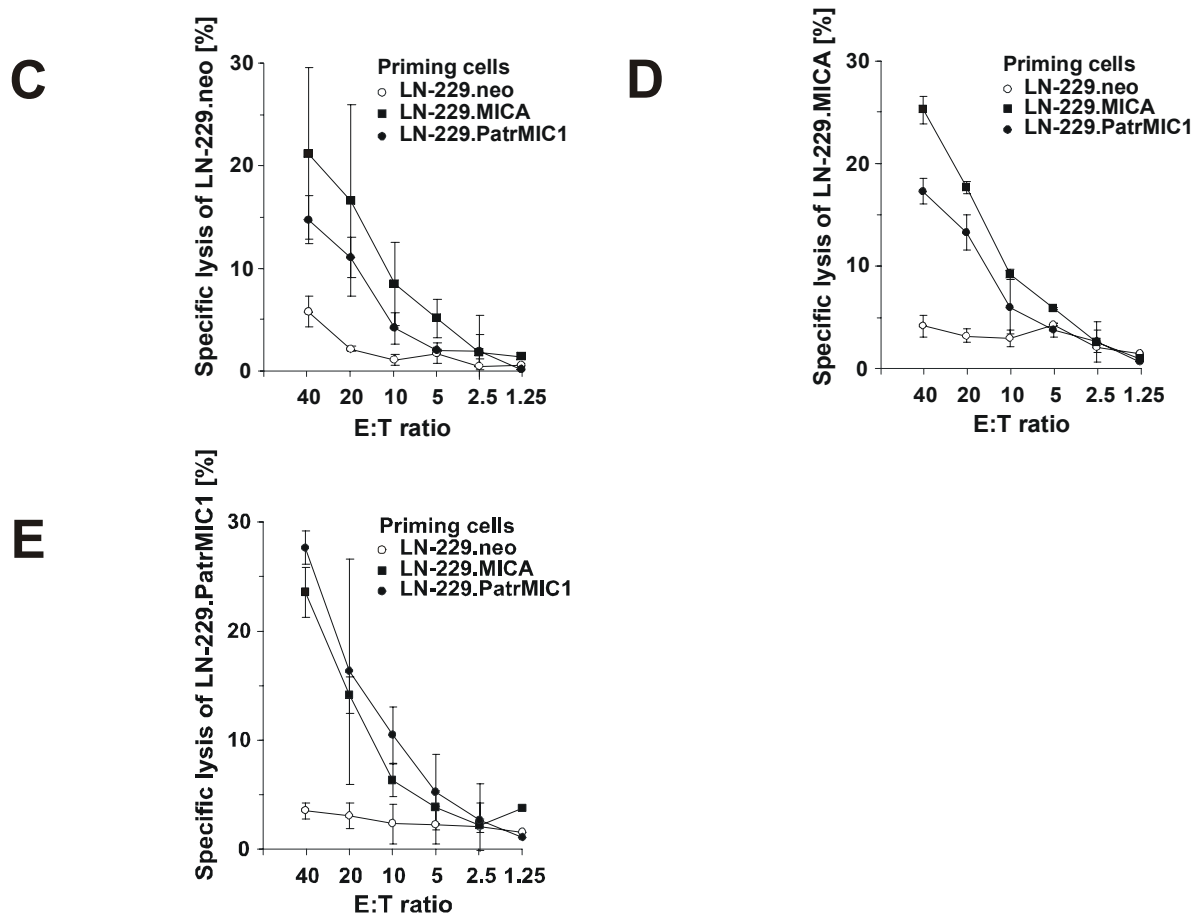


Fig. 6.4.C-E. T cells were incubated with glioma cells, harvested on day 4 and used as effectors in a 4 h ^{51}Cr -release assay at the indicated E:T ratios.

for the costimulatory potential of NKG2D for T cells was obtained by rechallenging primed T cells. T cells were primed for 4 days with LN-229.neo, LN-229.MICA or LN-229.PatrMIC1 cells and then used as effectors against fresh LN-229.neo, LN-229.MICA or LN-229.PatrMIC1 cells. No target cell lysis was seen when LN-229.neo cells, LN-229.MICA or LN-229.PatrMIC1 cells were used as targets for LN-229.neo-primed T cells (Fig. 6.4.C-E). In contrast, LN-229.MICA- or LN-229.PatrMIC1-primed T cells efficiently killed LN-229.neo, LN-229.MICA or LN-229.PatrMIC1 cells with comparable efficacy, indicating that NKG2D ligation by MICA or PatrMIC1 acts as a costimulatory signal in the priming phase of T cells (Fig. 6.4.C-E).

MICA expression delays growth of glioma xenografts in nude mice. We next sought to examine the immune-stimulatory capacity of MICA-overexpressing glioma cells in murine *in vivo* models. We first tested the physical and functional engagement of MICA*01 and PatrMIC1 by mouse NKG2D (mNKG2D). Interaction of MICA*01 and PatrMIC1, respectively, with mNKG2D, was assessed by using soluble mNKG2D. COS-7 cells transiently transfected with either MICA*01 or PatrMIC1 cDNA stained with the mAb AMO-1, but only MICA*01-transfected COS cells bound soluble mNKG2D (data not shown). We concluded that mNKG2D interacts with human MICA*01, but not with PatrMIC1. PatrMIC1 differs from MICA and MICB in several positions directly involved in NKG2D interaction, which may selectively interfere with binding of mNKG2D (209-211). Accordingly, NK cell cytotoxicity assays using polyclonal mouse NK cells as effector cells revealed marked lysis of human MICA glioma cell transfectants, but not of mock or PatrMIC1 transfectants (Fig. 6.5.A), indicating an activation by MICA*01 of the mouse NKG2D receptor. PatrMIC1 transfectants served hereafter as negative controls in the ensuing nude mice studies.

The immune stimulatory potential of MICA transfected LN-229 cells for the NK cell mediated anti-glioma response *in vivo* was examined in subcutaneous and intracerebral xenograft glioma models in nude mice which have NK cells, but lack T cells. LN-229 glioma cells were injected subcutaneously and the tumor sizes were measured every two days. LN-229. PatrMIC1 cells grew rapidly to form compact tumors whereas LN-229.MICA tumor growth was significantly delayed (Fig. 6.5.B). Tumor volumes differed significantly (t-test, $P < 0.01$) from day 17 to the end of the experiment. When LN-229 cells were implanted stereotactically into the brains of nude mice, animals carrying LN-229.PatrMIC1 tumors developed neurological symptoms and had to be sacrificed at day 20-21. In contrast, animals carrying LN-229.MICA tumors showed significantly prolonged survival (Fig. 6.5.C) (t-test, $P < 0.01$). Appropriate control experiments disclosed no difference between the proliferation of LN-229.MICA and LN-229.PatrMIC1 cell lines *in vitro* (Table 2).

	Proliferation [cpm]	
	LN-229	SMA-560
Parental	1802 ± 401	3078 ± 523
PatrMIC1 (stable)	1766 ± 431	-
MICA (stable)	1722 ± 386	-
Ad-dE1 (300 MOI)	-	3153 ± 500
Ad-MICA (300 MOI)	-	3122 ± 489

Table 2. MICA gene transfer does not modulate glioma cell growth as assessed by [³H]-thymidine incorporation *in vitro*. [³H]-thymidine incorporation was measured after 24 h by liquid scintillation counting. Data are expressed as cpm ± SD.

Since MICA gene transfer delayed glioma growth, but did not lead to tumor rejection, we characterized the glioma cells forming progressive tumors in nude mice. To this end, we isolated the tumor cells, which had grown subcutaneously and analyzed the expression of MICA and PatrMIC1 by flow cytometry. These experiments yielded two findings which support a proficient role for MICA as a potent molecule inhibiting the growth of gliomas. First, glioma cells recovered from mice that had received LN-229.PatrMIC1 cells, showed unaltered levels of high PatrMIC1 expression before and after tumor inoculation, consistent with the failure of PatrMIC1 to interact with mNKG2D (Fig. 6.5.D, left); secondly, all tumor cells harvested from mice which had received LN-229.MICA*01 cells were essentially devoid of MICA. This is probably due to tumor formation by a small non-MICA expressing population among the LN-229.MICA cells (Fig. 6.5.D, right). Endogenous MICA expression in LN-229.PatrMIC1 cells was also reduced after glioma cell passaging *in vivo*: the SFI decreased from 2.6 prior to inoculation to 1.4 after inoculation. MHC class I antigen expression measured by flow cytometry did not change after MICA transfection or *in vivo* passaging (data not shown).

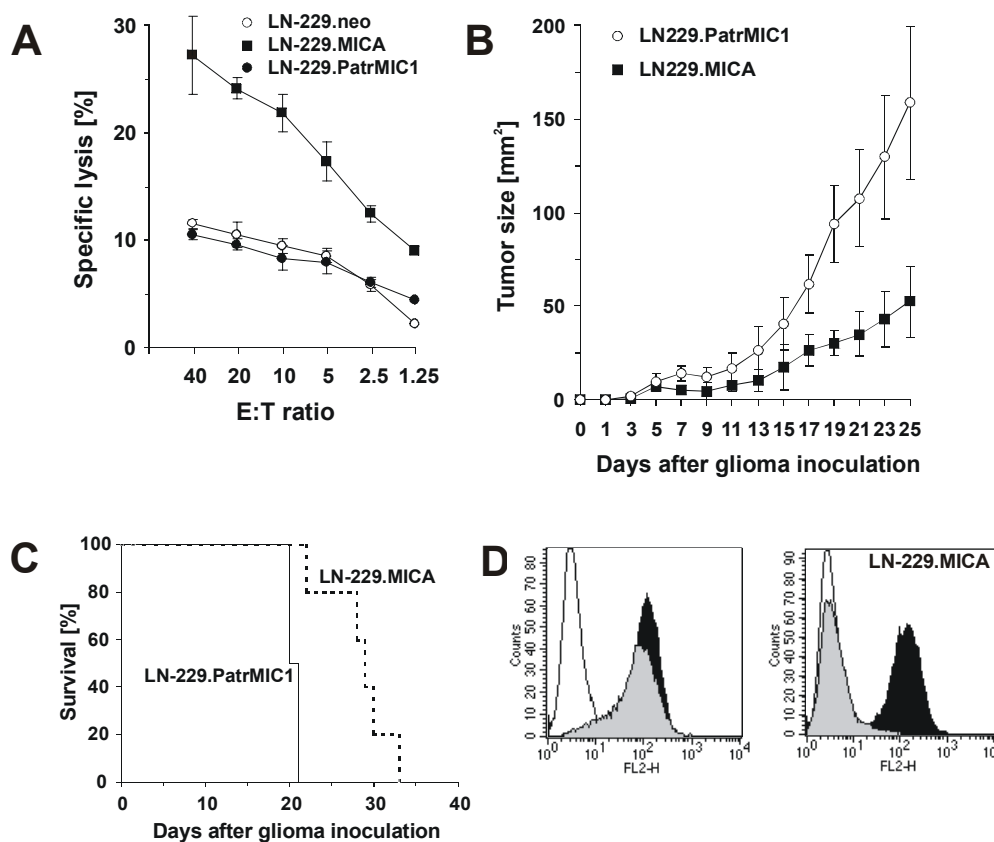


Fig. 6.5. Ectopic MICA expression delays the growth of subcutaneous and intracerebral human glioma xenografts in nude mice. (A) LN-229 transfectants were tested as targets for BALB/c nude mice NK cells in a ⁵¹Cr release assay. (B) The growth of subcutaneous LN-229.PatrMIC1 or LN-

229.MICA tumors was monitored every 2 days. (C) LN-229.PatrMIC1 (solid line) or LN-229.MICA*01 (broken line) cells (5×10^4) were inoculated intracerebrally in Balb/c nude mice. Survival data for five animals per group are shown (t-test, r^2 0.59, $P < 0.01$). (D) At 25 days after injection of tumor cells, the mice were killed, and freshly isolated tumor cells from subcutaneous tumors were analyzed for PatrMIC1 in PatrMIC1 tumors (left) or MICA in MICA tumors (right) by flow cytometry. Representative tumor isolates are shown as grey profiles, the preinoculation levels as black profiles, and an isotype control as open profiles.

NKG2DL expression by mouse glioma cell lines. We evaluated two syngeneic mouse glioma models, SMA-560 cells in VMDk mice (spontaneous tumor) and GL261 cells in C57BL/6 mice (3-methylcholanthrene-induced), for a therapeutic trial of MICA gene transfer *in vivo*. The mNKG2DL RAE-1 β was prominently expressed by SMA-560 cells while GL261 showed only a weak expression. H60 was only expressed by SMA-560 and not by GL261 cells, consistent with the report that C57BL/6 mice do not express H60 (207) (Fig. 6.6.A).

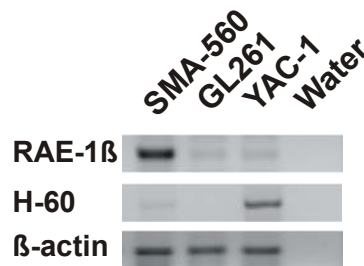


Fig. 6.6.A. **SMA-560 glioma cells in VMDk mice as syngeneic model for MICA gene transfer.**

(A) Expression of the murine NKG2DL RAE-1 β and H-60 mRNA was assessed by RT-PCR.

YAC-1 cells were used as a positive control. β -actin was used as a standard.

Soluble mNKG2D was more prominently bound by SMA-560 than GL261 cells (Fig. 6.6.B), indicating higher expression levels of mNKG2DL in SMA-560 cells. Whereas SMA-560 cells expressed both MHC class I antigens H-2K^b and H-2D^b which are potential ligands for inhibitory Ly49 receptors, GL261 cells showed only low level H-2D^b expression (Fig. 6.6.B). SMA-560 cells thus resemble the phenotype of human glioma cell lines and were used in further animal experiments. When SMA-560 cells were infected with Ad-MICA, MICA expression increased in a multiplicity of infection (MOI)-dependent manner: the SFI values were 6.6 at 100 MOI, 12.2 at 300 MOI and 22.8 at 1000 MOI (Fig. 6.7.B). Similar to LN-229 cells, the growth of SMA-560 cells was unaffected by forced MICA expression (Table 2). The functional significance of increased MICA expression on mouse glioma cells after Ad-MICA infection was confirmed in cytotoxicity assays using syngeneic VMDk NK cells as effectors

(Fig. 6.6.C). Blocking experiments with anti-MICA mAb and soluble mNKG2D demonstrated that the increased killing of Ad-MICA infected SMA-560 cells was due to MICA expression. Attenuation of the constitutive lysis of control-infected SMA-560 cells by approximately 50% by mNKG2D probably reflects the activation by endogenous NKG2DL like RAE-1 and H60 (Fig. 6.6.D). Accordingly, neutralization of MICA was less effective in inhibiting lysis of Ad-MICA-infected cells than mNKG2D.

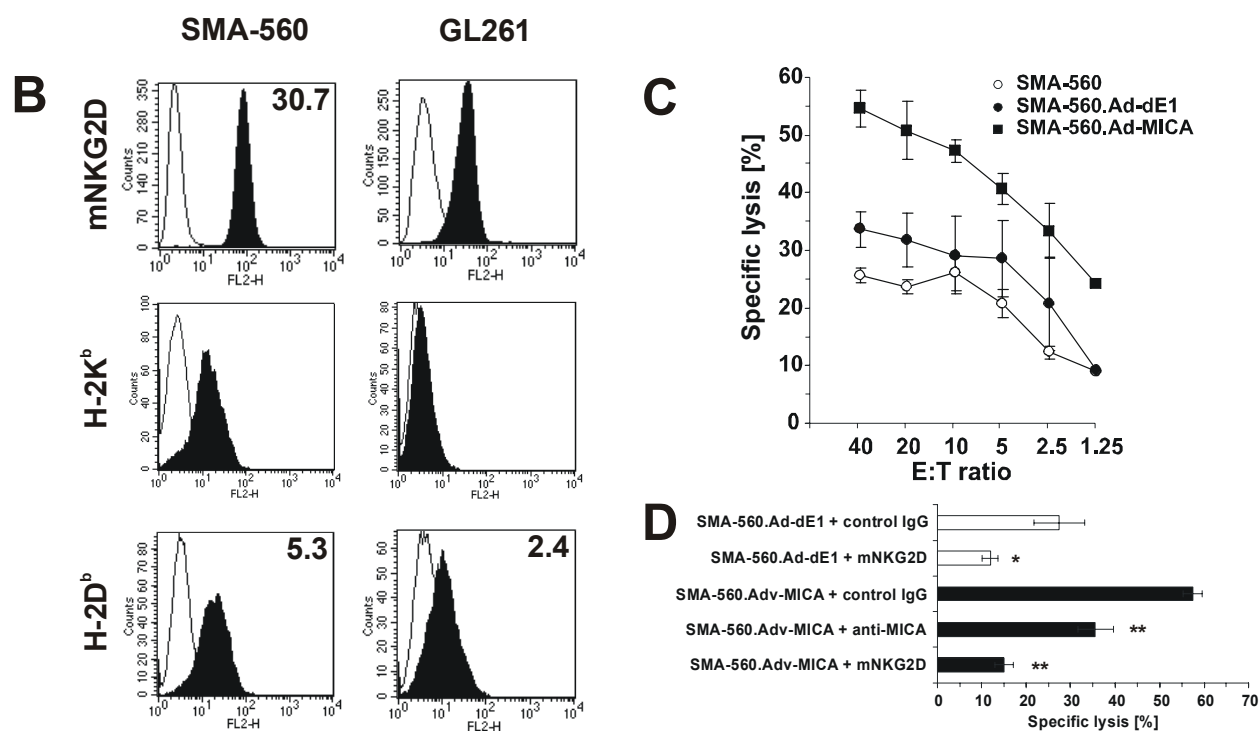


Fig. 6.6.B. NKG2DL and MHC class I antigen expression (H-2K^b, H-2D^b) was assessed by flow cytometry. The cell lines were stained with soluble mNKG2D/anti-FLAG or the indicated mAb (solid profiles) or with isotype-matched control ab (open profiles). SFI values are indicated in the upper right corner of each panel. (C) Uninfected, Ad-dE1 (300 MOI)-infected or Ad-MICA (300 MOI)-infected SMA-560 cells were tested as targets for NK cells isolated from VMDk mice in ⁵¹Cr release assays at 48 h. (D) Ad-dE1- or Ad-MICA-infected (300 MOI) SMA-560 cells were untreated or pretreated with soluble mNKG2D, or control ab or anti-MICA, and tested as targets in ⁵¹Cr release assay, using VMDk NK cells as effectors. Data are expressed as specific lytic activities at an E:T ratio of 40:1 (mean ± SD, t-test, * *P* < 0.05, ** *P* < 0.01).

MICA expression in SMA-560 glioma cells delays tumor growth. To analyse the immune stimulatory capacity of MICA-transduced SMA-560 tumor cells *in vivo*, VMDk mice were inoculated subcutaneously with syngeneic tumor cell transductants. There was no difference between the *in vivo* growth of Ad-dE1-infected and uninfected SMA-560 cells. Conversely, MICA*01 expression by SMA-560 glioma cells resulted in a substantial delay of tumor growth in a MOI-dependent manner (Fig. 6.7.A). Of note, two out of six animals in the groups challenged with 300 and 1000 MOI-infected glioma cells, respectively, developed no tumor. Irrespective of the initial level of MICA expression prior to inoculation, glioma cells harvested from progressive tumors at day 10 after inoculation were devoid of MICA (Fig. 6.7.B). To address whether prior immunization with glioma cells that express MICA induces protective immunity to wild-type glioma cells, mice that had previously rejected MICA-transduced tumor cells were rechallenged with wild-type SMA-560 cells 10 weeks after the first exposure in the opposite flank. Wild-type SMA-560 cells grew progressively in naïve

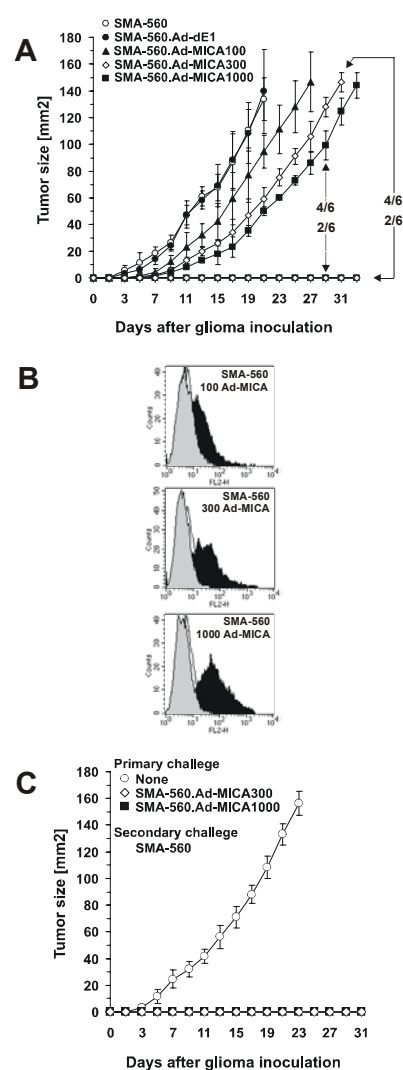


Fig. 6.7. MICA delays SMA-560 glioma growth in syngeneic mice. A. The growth of subcutaneous tumors formed by SMA-560 cells infected *in vitro* with Ad-dE1 (1000 MOI) or Ad-MICA (100, 300 or 1000 MOI) was monitored every 2 days. Two of 6 animals challenged with Ad-MICA-infected cells at 300 and 1000 MOI did not develop a measurable tumor. (B) SMA-560 cells infected with Ad-MICA at increasing MOI were assessed for MICA expression before subcutaneous inoculation (black profiles). Open profiles show the labeling with isotype-matched Ig. Further, glioma cells were freshly isolated from subcutaneous tumors at 10 days after inoculation and re-analyzed for MICA expression (grey profiles). (C) VMDk mice that had previously rejected MICA-transduced SMA-560 cells were inoculated subcutaneously with wild-type SMA-560 glioma cells in the opposite flank. Primary exposure preceded the challenge by 10 weeks.

VMDk mice, but were rejected by mice that had been exposed previously to the MICA-transduced tumor cells (Fig. 6.7.C)

Subcutaneous vaccination with MICA-expressing glioma cells after tumor inoculation delays tumor growth. To address whether subcutaneous vaccination with tumor cells expressing MICA induces protective immunity to pre-established SMA-560 gliomas, mice were intracerebral inoculated with SMA-560 wild-type cells and then immunized with irradiated wild-type cells or irradiated Ad-dE1 (1000 MOI) or Ad-MICA (1000 MOI) -infected SMA-560 cells at days 3 and 8 (Fig. 6.7.D). Peripheral vaccination with Ad-MICA-infected irradiated cells resulted in a significant prolongation of survival with 2 of 6 animals still alive and asymptomatic after 90 days (t-test, $P < 0.01$). NK cells isolated from splenocytes from mice vaccinated with Ad-MICA-transduced SMA-560 cells at day 10 showed a substantially enhanced cytotoxic activity against YAC-1 target cells compared with NK cells from animals vaccinated with control-transduced SMA-560 cells (Fig. 6.7.E). Evidence for T cell stimulation by MICA was obtained by re-stimulating T cells isolated at day 10 after tumor cell inoculation. Only T cells from animals vaccinated with Ad-MICA-infected SMA-560 cells released high levels of IL-2 after restimulation with SMA-560 wild-type cells, while no such effect was seen with control-transduced tumor cells (Fig. 6.7.F).

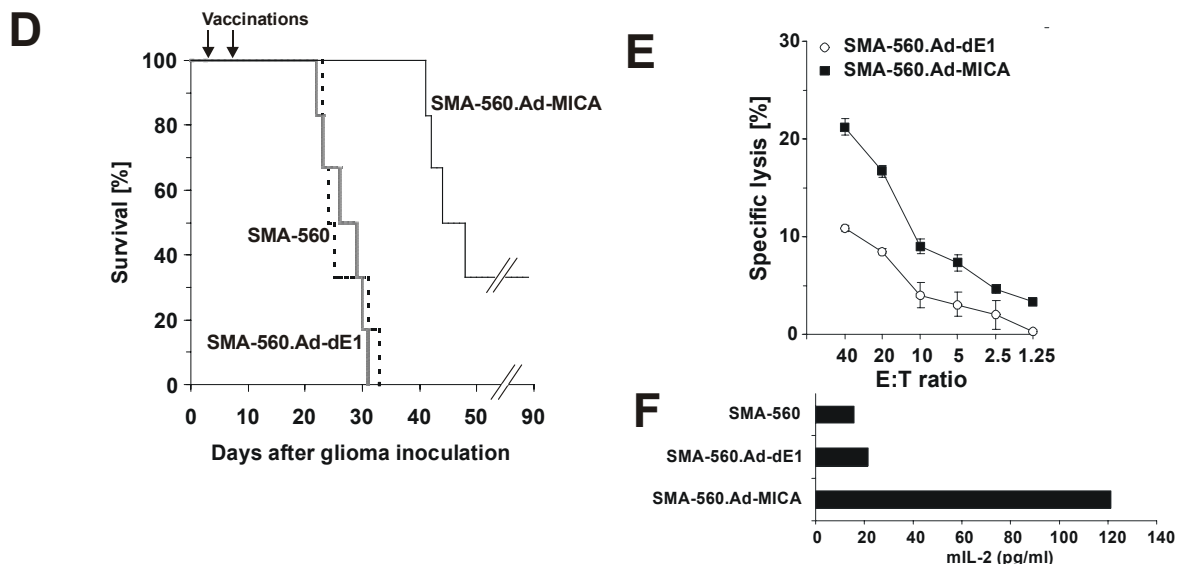


Fig. 6.7.D-F. 5×10^3 SMA-560 cells were inoculated intracerebrally in syngeneic VMDk mice (day 1). D. At days 3 and 8 the animals were vaccinated subcutaneously with 1×10^6 irradiated Ad-dE1- or Ad-MICA-infected (1000 MOI) or uninfected SMA-560 cells. The graph shows survival data for 6 animals per group (t-test, $P < 0.01$). (E, F) At day 10, splenocytes were

recovered from the differently vaccinated animals and T and NK cells were isolated. (E) NK cells were used as effector cells in a ^{51}Cr release assay using YAC-1 cells as targets. (F) Isolated T cells were restimulated with irradiated wild-type SMA-560 cells, and IL-2 release was measured by ELISA 48 h later.

To determine the *in situ* distribution of glioma-infiltrating immune cells, we analyzed brain sections at day 10 post tumor inoculation by immunohistochemistry (Fig. 6.8). Using specific mAbs for CD8⁺ T cells (CD8) and macrophages/microglia (CD11b), we observed an increase in number of infiltrating immune cells in SMA-560 gliomas after SMA-560.Ad-MICA vaccination compared with SMA-560 or SMA-560.Ad-dE1 vaccinated animals. We were not able to stain murine NK cells with the currently available antibodies (231). An antibody recognizing Ly-49G2 (4D11) which has been reported to stain murine NK cells in immunohistochemistry provided no signal in our hands probably because VMDk mice do not express Ly-49G2 (231).

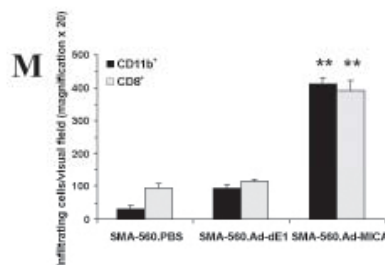
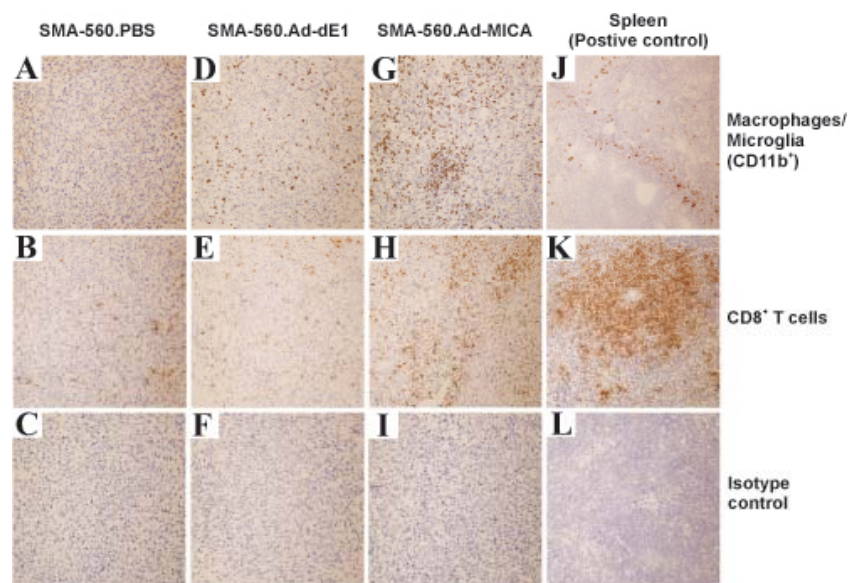


Fig. 6.8.A-M. **Ad-MICA gene transfer promotes immune cell infiltration in the SMA-560 syngeneic mouse glioma model.** VMDk mice were inoculated intracerebrally with SMA-560 wild-type cells and vaccinated subcutaneously with SMA-560 (A, B, C), SMA-560.Ad-dE1 (D, E, F) or SMA-560.Ad-MICA (G, H, I) at days 3 and 8 post-inoculation. Cryosections were generated

from brains at day 10 following tumor inoculation and stained with mAb directed against macrophages/microglia (A, D, G) or CD8⁺ T cells (B, E, H), or with isotype-matched control mAb (C, F, I). Spleen sections from untreated animals were used as positive controls (J, K, L). Magnification for all sections was x 20. (M) Quantification of positively stained cells per visual field in glioma sections in x 20 magnification. Each column represents the average number (\pm SD, t-test, ** $P < 0.01$) of positively stained cells from at least three different brain sections.

Discussion

This study investigates the potential of NKG2DL to promote an immune response to glioblastoma. We show that NKG2D-mediated NK cell triggering induced by NKG2DL expressed on glioma cells is overruled by MHC class I molecules which engage inhibitory receptors on NK cells. Gene transfer-mediated ectopic expression of MICA on human and mouse glioma cells permits their *in vitro* immune recognition and lysis and *in vivo* growth inhibition despite high level MHC class I antigen expression. These observations provide a rationale for MICA-based immunogene therapy of gliomas.

Malignant transformation of glial cells is associated with an NKG2DL expression, this is, of MICA/B and ULBP molecules, at the cell surface (Fig. 6.1.A,B, Table 1). However, the NK cell inhibitory potential of MHC class I antigens expressed on human glioma cells masks the activating signals of NKG2DL. The interaction of effector NKG2D with endogenous tumor MIC and ULBP molecules promotes NK cell-mediated tumor cell lysis *in vitro* when MHC class I antigens are masked (Fig. 6.3.A). NK cell-mediated tumor cell lysis was also achieved when MICA expression was enhanced *via* plasmid transfection or adenoviral gene transfer (Fig. 6.2 and 6.3.B) (225). NKL cells do not express the Fc γ RIII (CD16) (232) which mediates antibody-dependent cellular cytotoxicity (ADCC), ruling out that the lysis seen in the presence of mAbs is due to ADCC. Thus, glioma cells out-balance activating ligand expression by inhibitory MHC class I antigens and prevent NK cell lysis. This concept is supported by a highly significant correlation between MHC class I antigen expression and NKG2DL expression on human glioma cells when the SFI values for the NK cell inhibitory MHC class I antigens were correlated with a sum score of the SFI values for MICA, MICB, ULBP1, 2 and 3, (t-test, r^2 0.6523, P 0.0015).

One way of immune escape for glioma cells may be the expression of MHC class I antigens at a level that allows sufficient binding of NK inhibitory receptors and escape from NK-mediated killing. High expression of MHC class I antigens in human glioblastomas *in vivo* has been confirmed by several studies (233, 234). MHC class I down-regulation, conversely, is a hallmark of many peripheral cancers and decreases the susceptibility of tumor cells to lysis by cytotoxic T cells (224, 235). Possibly only those glioma cells which show an upregulation of MHC class I antigens expression to compensate for the acquisition of activating NKG2DL expression during malignant transformation will survive. This selection may render tumor cells more vulnerable towards T cell-dependent elimination through tumor-associated antigen detection (224, 235). Since the brain lacks specific brain-associated lymphoid tissue, antigens which are introduced directly into the brain parenchyma exclusively

elicit transient innate inflammatory immune responses, but no adaptive immunity (236-240). However, if immunization is elicited by injecting the identical antigen outside the brain, the adaptive immune system becomes primed, and antigenic epitopes anywhere within the brain will be recognized as targets of either activated effector T cells, B cells or antibodies (236, 238, 240). This immunological peculiarity may explain high expression levels of MHC class I antigens on glioma cells which could effectively present tumor-associated antigens to primed T cells. Since intracerebral T cell priming is not found, the MHC class I antigens may only serve to suppress NK cell activation by NKG2DL.

The highly lethal nature of glioblastoma suggests that the levels of activating NKG2DL expressed by glioma cells are too low to induce anti-tumor immunity. Thus, we concluded that immunity to gliomas may be boosted by engineering cells expressing high levels of activating NKG2DL. We noted that MICA gene transfer induces NK and T cell responses to glioma cells *in vitro* (Fig. 6.2-4) and delays growth in a human xenograft and syngeneic mouse glioma model *in vivo* (Fig. 6.5-7). In this model, the *in vivo* selection for low MICA expressing glioma cells strikingly demonstrated the NK cell activating potential of the NKG2D/NKG2DL system (Fig. 6.5.D, 6.7.B). Previously it was shown that ligation of NKG2D on NK and T cells can promote subsequent T cell immunity to parental tumors which lack NKG2DL, although T cell priming was NK cell-independent (207). However, a similar study did not detect such T cell-mediated memory against the parental tumor in mice (206). Our data support that NKG2D engagement costimulates human CTL responses since prior immunization with tumor cells expressing the NKG2DL MICA induces protective immunity against a challenge with wild-type glioma cells in mice that had previously rejected MICA-transduced glioma cells (Fig. 6.7.C) (207, 241). Finally, the peripheral vaccination with irradiated Ad-MICA-infected cells *after* the intracerebral implantation of wild-type tumor cells delayed the growth of syngeneic gliomas and promoted immune activation in the NK and T cell compartments (Fig. 6.7.E,F, 6.8). NKG2DL expression and consequent activation of NK cells and T cells may thus provide a novel therapeutic approach for the treatment of human gliomas. If the magnitude of the effect achieved with peripheral vaccination, this is, a median prolongation of survival from 27.5 in control animals to 46 days in SMA-560.Ad-MICA vaccinated animals, with 2 of 6 animals (33%) alive and asymptomatic after 90 days (Fig. 6.7.D), could be transferred into the human situation, this would be a major advance, compared, e.g., with all chemotherapy trials performed in the recent 20 years (160).

Summary

The immune system is essential in guarding the body against pathogens and tumor cells. Immunity has many facets, the dichotomy separating innate and adaptive immunity in the last decades, nowadays emerges as evolutionary consecutive with interdepending pathways and continuative receptor evolution. The complexity of the somatic rearrangement of TCR genes guarantees virtually infinite variability in the specificity of TCRs expressed on naïve T cells, i.e. costimulated via CD27/CD70 interaction, but relies on the body's rapid innate defense mechanisms where receptors with consistent PRR recognise PAMP transmitting valuable signals to the adaptive immune system, i.e. via TLR, activated by ssRNA. Thus it is not astonishing that TLR are accounted as the interface between innate and adaptive immunity (242). It actually becomes clear that the innate arm of immunity links with its adaptive counterpart in tumor immune surveillance also via NK cells, i.e. with NKG2D as a receptor of an activatory pathway. Proteomic analysis of unactivated and activated human NK cell membrane-enriched fractions demonstrated that activated NK cells can efficiently stimulate T cells, since NK cells upregulate MHC class II molecules and multiple ligands for TCR costimulatory molecules (243). Combining these findings with our improved understanding of cancer biology has helped to understand how to configure immunological strategies against the “hallmarks of cancer” (244). Encounter the escape of immune surveillance and the immunosuppressive properties of glioma cells (**chapter 1**), i.e. by TGF- β , are the major challenges in our laboratory and were the main topics in my Ph.D. work.

In **chapter 2** we describe the approach of overcoming immunosuppression by stimulating the immune system via CD70 (CD27 ligand) which promotes the expansion of primed lymphocytes by enhancing cell survival. Surprisingly, we had previously observed that CD70 aberrantly expressed on human glioma cells promoted immune cell apoptosis and inhibited alloreactive lysis (109). In the mouse system we found that the ectopic expression of CD70 in mouse glioma cells enhanced apoptosis of T, B and NK cells in coculture, but nevertheless promoted glioma cell lysis by NK cells *in vitro*. In nude mice, CD70 expression in SMA-560 gliomas delayed their growth upon s.c. or intracerebral (i.c.) inoculation, suggesting a role for CD70/CD27-dependent NK cell activity in tumor surveillance. In syngeneic immunocompetent mice, CD70 allowed the rejection of s.c. and i.c. implanted SMA-560 tumors. The tumorigenicity of CD70-expressing glioma cells was altogether abrogated when TGF- β signalling was blocked. Moreover, mice surviving the s.c. CD70 glioma challenge

subsequently also rejected wild-type glioma cells administered i.c.. Similarly, CD70-expressing GL-261 gliomas were rejected in syngeneic C57BL/6 mice while glioma growth was restored in CD27^{-/-} mice, suggesting that the CD70/CD27 interaction recruited a tumor-specific T cell repertoire and induced tumor-specific memory. Altogether, these observations indicate that the net effect of aberrant CD70 expression in gliomas was immune stimulatory rather than immune paralytic and encourage its application in tumor immunotherapy.

Activating the immune system on the basis of activatory information at the level of innate immunity being transduced to adaptive immunity were the efforts described in **chapter 3**. Stabilized synthetic RNA oligonucleotides (ORN) and protected messenger RNA (mRNA) such as protamine-condensed mRNA were recently discovered to be immune stimulatory through their recognition by TLR7 and TLR8. We tested whether these danger signals would be capable of triggering anti-tumor immunity when injected locally into an established tumor as it has been shown for other danger signals. Using the mouse glioma tumor cell line SMA-560 in syngeneic VM/Dk mice, we showed that intra-tumor injections of protamine-stabilized mRNA induced tumor regression and lasting anti-tumor immunity. Residual RNA-injected tumors showed CD8 infiltration. Injections of protamine-protected mRNA and intra-tumor injection of naked mRNA also resulted in anti-tumor immunity. RNAs are labile molecules with a short half live. They do not trigger side effects such as splenomegaly in treated mice. These observations indicate that mRNAs are potent and safe danger signals that are relevant in the context of active immunotherapy strategies for the eradication of solid tumors.

As one of the major aspects in tumor immune escape mechanisms, TGF- β immunosuppression and its abrogation is one of the most pursued aims in our laboratory. In **chapter 4** we characterized the effects of a novel TGF- β RI kinase inhibitor, SD-208, on the growth and immunogenicity of mouse SMA-560 and human LN-308 glioma cells *in vitro* and the growth of, and immune response to, intracranial SMA-560 gliomas in syngeneic VM/Dk mice *in vivo*. SD-208 inhibited the growth inhibition of TGF- β -sensitive CCL64 cells mediated by recombinant TGF- β_1 or TGF- β_2 , or of TGF- β -containing glioma cell supernatant, at an EC₅₀ of 0.1 μ M. SD-208 blocked autocrine and paracrine TGF- β signalling in glioma cells as detected by the phosphorylation of Smad2 or TGF- β reporter assays and strongly inhibited constitutive and TGF- β -evoked migration and invasion, but not viability or proliferation. PBL or purified T cells, cocultured with TGF- β -releasing LN-308 glioma cells

in the presence of SD-208, exhibited enhanced lytic activity against LN-308 targets. The release of IFN- γ and TNF- α by these immune effector cells was enhanced by SD-208 whereas the release of IL-10 was reduced. SD-208 restored the lytic activity of polyclonal NK cells against glioma cells in the presence of recombinant TGF- β or of TGF- β -containing glioma cell supernatant. The oral bioavailability of SD-208 was verified by demonstrating the inhibition of TGF- β -induced Smad phosphorylation in spleen and brain. Systemic SD-208 treatment initiated three days after the implantation of SMA-560 cells into the brains of syngeneic VM/Dk mice prolonged their median survival from 18.6 to 25.1 days. Histological analysis revealed no difference in blood vessel formation, proliferation or apoptosis. However, animals responding to SD-208 showed an increased tumor infiltration by NK cells, CD8 T cells and macrophages. These data define TGF- β RI kinase inhibitors such as SD-208 as promising novel agents for the treatment of human malignant glioma and other conditions associated with pathological TGF- β activity.

Subtilisin-like proprotein convertases such as furin are thought to mediate TGF- β processing and thus enhance its bioavailability. In **chapter 5** we report that human malignant glioma cell lines express furin mRNA and protein, exhibit furin-like protease activity and release active furin into the cell culture supernatant. FLP activity was not modulated by exogenous TGF- β or by neutralizing TGF- β antibodies. Exposure of LN-18 and T98G glioma cell lines to the furin inhibitor, dec-RVKR-cmk, inhibited processing of the TGF- β_1 and TGF- β_2 precursor molecules and consequently the release of mature bioactive TGF- β molecules. Ectopic expression of PDX, a synthetic antitrypsin analog with antifurin activity, in the glioma cells inhibited FLP activity, TGF- β processing and TGF- β release. Thus, subtilisin-like proprotein convertases may represent a novel target for the immunotherapy of malignant glioma and other cancers or pathological conditions characterized by enhanced TGF- β bioactivity.

The failure of conventional cancer therapy renders glioblastoma an attractive target for immunotherapy. Tumor cells expressing ligands of the activating immunoreceptor NKG2D stimulate tumor immunity mediated by NK and CD8⁺ T cells. In **chapter 6** we report that human glioma cells express the NKG2D ligands MICA, MICB and members of the ULBP family constitutively. However, glioma cells resist NK cell cytotoxicity due to high MHC class I antigen expression. Plasmid-mediated or adenovirus-mediated (Ad-MICA) overexpression of MICA in glioma cells enhanced their sensitivity to NK and T cell lysis *in vitro* and markedly delayed the growth of subcutaneous and intracerebral LN-229 human glioma cell xenografts

in nude mice, and of SMA-560 gliomas in syngeneic VM/Dk mice. Glioma cells forming progressive tumors after implantation of stably MICA-transfected human LN-229 cells have lost MICA expression indicating a strong selection against MICA expression *in vivo*. Rejection of MICA expressing SMA-560 cells in VMDk mice resulted in protective immunity to a subsequent challenge with wild-type tumor cells. Finally, the growth of syngeneic intracerebral SMA-560 tumors was inhibited by the peripheral vaccination with Ad-MICA-infected irradiated tumor cells, and vaccination resulted in the immune cell activation in the NK and T cell compartments *in vivo*. These data commend MICA immunogene therapy as a novel experimental treatment for human malignant gliomas.

In my Ph.D. research I have thus explored several novel approaches aiming at engaging immune surveillance via direct activation of adaptive immunity or by more indirect ways via innate pathways and at an improved understanding of the immune-paralysing capacities of glioma cells. While the connections between these various aspects of glioma biology may not be apparent at first sight, there are good reasons for taking a combined look at the complex interactions of innate and adaptive immunity and tumor immunology. Tumor immune surveillance is mediated by immune effector cells that selectively kill cancer cells. Activated T and NK attack their targets by the expression of death ligands (245, 246) and by secretion of cytotoxic granules (87, 247) and thus seek to activate both the death receptor and the intracellular apoptotic pathway. An apoptosis-resistant target cell is therefore less susceptible to immune-mediated lysis. Sensitization of tumor cells for apoptotic stimuli could therefore enhance the efficacy of ongoing anti-tumor immune responses. Further, activation of the costimulatory pathways, e.g. using CD70 transgenes or ssRNAs may be beneficial when the tumor may strike back and induce apoptosis in tumor-infiltrating lymphocytes by various mechanisms, including the expression of CD95/FasL, HLA-G or TGF- β (140, 248-250). Finally, therapeutic induction of apoptosis via these mechanisms is thought to provide a source of antigens that can be taken up and presented by professional APC, resulting in an anti-tumor immune response. The mounting of a productive anti-tumor immune response may be prevented by immune-inhibitory signals sent out by the tumor itself. Therefore, a costimulation-based reduction of large tumor masses may require the simultaneous relief of tumor-dependent immunosuppression in order to be followed by a productive immune response that can finally clear residual glioma cells dispersed in the brain.

Whether the emerging concepts outlined in this Ph.D. thesis have the potential to improve current glioma treatments will, of course, have to be explored in further models. Thus, the present work finally was driven by what is expressed by Camille Flammarion's woodcut from: "L'Atmosphère, et la Météorologie populaire", Paris 1888", which gives a symbolical description of the myth of the destruction of the ancient world view by the Copernican Revolution and the ensuing loss of the central position of man in cosmos.



Man penetrating the celestial spheres reminds me of how encouraging it is to find out a new immunological tool, knowing how far it is from being transferred from “bench to bedside”, imposingly documented by this woodcut: “how deep is the urge (...) to break the familiar horizon, and how independent it is from the question of its practical realisability”.

Zusammenfassung

Das Immunsystem schützt unseren Körper vor Pathogenen und Tumorzellen, als Spezialfall. Die Immunität hat viele verschiedene Facetten, wobei die in den letzten Jahrzehnten propagierte Zweiteilung der angeborenen und adaptiven Immunität heute vielmehr als evolutionär aufeinander aufbauend, mit voneinander abhängigen Signaltransduktionspfaden und ontogenetisch verwandten Rezeptoren, angesehen wird. Die Komplexität der somatischen Rekombination der TCR-Gene ermöglicht eine nahezu unendliche Variabilität der Spezifität von TCR. Diese werden auf naiven T-Zellen exprimiert. Diese Zellen können u.a. durch kostimulatorische Interaktionen via CD27/CD70 stimuliert werden, sind aber von einer schnellen Antwort der angeborenen Immunität abhängig. Rezeptoren mit konsistenten PRRs erkennen PAMP und leiten Signale an das adaptive Immunsystem weiter. Die Familie der TLR spielt hier eine wichtige Rolle. In dieser Arbeit waren vor allem die Interaktionen der TLR7-9 mit ihren jeweiligen Liganden bei der Abwehr von Tumorzellen von Bedeutung. Es ist deshalb nicht erstaunlich, dass die TLR als Schnittstelle zwischen angeborener und adaptiver Immunantwort angesehen werden (242). Bei der Immunüberwachung des Körpers sind nicht nur wie bisher bekannt T- und B-Zellen, sondern auch NK-Zellen beteiligt, die z.B. über den aktivatorischen Rezeptor NKG2D aktiviert werden. Proteomanalysen von angereicherten nicht aktivierten und aktivierten NK-Zellmembranen zeigten, dass aktivierte NK-Zellen T-Zellen sehr effizient stimulieren können, da sie MHC-Klasse II-Moleküle und multiple TCR-kostimulatorische Liganden hochregulieren (243). Die Kombination dieser Befunde mit unserem verbesserten Verständnis der Tumorbiologie hilft uns zu verstehen, wie wir immunologische Strategien konfigurieren müssen, um den Mechanismen, die einen Tumor kennzeichnen, etwas entgegenzusetzen können (244). Gliomzellen entziehen sich der Immunüberwachung durch immunsuppressive Mechanismen. Dabei werden das angeborene und adaptive Immunsystem, u.a. TGF- β -vermittelt supprimiert. Die Überwindung solcher immunsuppressiver Mechanismen waren die Hauptpunkte meiner Doktorarbeit.

In einem Ansatz wurde versucht, diese Immunsuppression durch Stimulation des Immunsystems über CD70 (CD27 Ligand) zu durchbrechen, welches die Expansion geprimter Lymphozyten dadurch unterstützt, dass es deren Überlebensrate erhöht. Interessanterweise konnten wir in früheren Untersuchungen feststellen, dass aberrant auf Gliomzellen exprimiertes CD70 die Apoptose von Immunzellen begünstigt und die alloreaktive Lyse inhibierte. Dennoch fanden wir heraus, dass die ektope Expression von

CD70 auf murinen Gliomzellen in einer Langzeit-Kokultur mit T-, B- und NK-Zellen zu einer erhöhten Apoptoserate dieser Immunzellen führte. Während einer kurzen Kokultivierung in einem Lyseassay wurden jedoch auch die Gliomzellen von NK-Zellen lysiert. In Nacktmäusen verzögerte eine CD70-Expression auf SMA-560-Gliomzellen deren subkutanes und intrakranielles Wachstum, was eine Beteiligung der CD70/CD27-abhängigen NK-Zellaktivität bei der Immunüberwachung gegen Tumorzellen suggeriert. In syngenem immunkompetenten Mäusen vermittelt CD70 eine Abstoßung von subkutan und intrakraniell implantierten SMA-560-Tumoren. Die Tumorigenität der CD70-exprimierenden Gliomzellen wurde durch eine Blockade des TGF- β -Signaltransduktionsweges unterbunden. Zudem stiessen Mäuse, die subkutane CD70-exprimierende Tumoren abgestossen hatten, später selbst intrakraniell implantierte Wildtyp-Gliomzellen ab. Gleichermassen wurden CD70-exprimierende GL-261-Gliome in syngenem C57BL/6-Mäusen abgestossen, während das Gliomwachstum in CD27^{-/-} Mäusen wiederhergestellt werden konnte. Dies suggeriert, dass die CD70/CD27-Interaktion ein Tumor-spezifisches T-Zellrepertoire und Tumor-spezifisches immunologisches Gedächtnis induziert hat. Zusammengefasst zeigen diese Beobachtungen *in vivo* einen deutlichen immunstimulatorischen Beitrag von CD70, dies ermutigt zu einer Anwendung von CD70 in zukünftigen Immuntherapien.

Auf eine andere Weise haben wir versucht, das adaptive Immunsystem indirekt über eine Aktivierung der angeborenen Immunität zu stimulieren. Vor Kurzem wurde die immunstimulatorische Bedeutung von stabilisierter, synthetischer RNA und geschützter Boten-RNA (mRNA), wie auch Protamin-kondensierter RNA, mit deren Rezeptoren TLR7 und TLR8, erkannt. Wir haben überprüft, ob diese Danger-Signale anti-Tumor-Immunität vermitteln können, wenn man sie lokal in einen bereits etablierten Tumor injiziert, so wie es für andere Danger-Signale bereits gezeigt wurde. Die intratumorale Injektion von Protamin-stabilisierter mRNA bewirkt einen Tumorrückgang und eine anhaltende anti-Tumor-Immunität in unserem syngenem SMA-560-Gliom-VM/Dk-Mausmodell. Noch nicht abgeräumtes, RNA-behandeltes Tumorgewebe zeigte eine Infiltration durch CD8-T-Zellen. Injektionen von Protamin-geschützter mRNA und intra-Tumor-Injektionen von nackter mRNA führten ebenfalls zu einer anti-Tumor-Immunität. Da RNA-Moleküle sehr instabil sind und eine kurze Halbwertszeit besitzen vermitteln sie auch keine unerwünschten Nebenwirkungen wie etwa Splenomegalien oder eine Verminderung von Immunzellen in Mäusen. Diese Beobachtungen prädestinieren mRNAs als potente und nebenwirkungsfreie

Danger-Signale im Kontext einer aktiven immuntherapeutischen Strategie gegen etablierte Tumoren.

Die Immunsuppression durch TGF- β ist einer der grundlegenden Mechanismen, durch die Tumoren einer Immunüberwachung entkommen, die Aufhebung dieser Immunsuppression ist eines der Hauptziele in unserer Arbeitsgruppe. In unseren Arbeiten beschreiben wir einen neuen TGF- β RI-Kinase-Inhibitor, SD-208, seinen Effekt auf Wachstum und Immunogenität von murinen SMA-560 und humanen LN-308 Gliomzellen *in vitro* und das Wachstum und die Immunantwort auf intrakraniell inokulierte SMA-560-Gliome in syngenem VM/Dk-Mäusen *in vivo*. SD-208 hebt die TGF- β -vermittelte (rekombinantes TGF- β_1 oder TGF- β_2 , oder TGF- β -haltiger Gliomzellüberstand) Wachstumsinhibition von TGF- β -sensitiven CCL64-Zellen mit einem EC₅₀-Wert von 0.1 μ M auf. Dabei blockiert SD-208 autokrine und parakrine Signaltransduktionswege in Gliomzellen, was durch die Phosphorylierung von Smad2 und in TGF- β -Reporter-Assays gezeigt wurde. Weiterhin wurde auch die konstitutive und TGF- β -evozierte Migration und Invasion stark gehemmt, die Viabilität und Proliferation jedoch nicht beeinflusst. PBL oder aufgereinigte T-Zellen zeigten eine erhöhte lytische Aktivität gegenüber LN-308-Targetzellen, wenn sie mit TGF- β -sezernierenden LN-308-Gliomzellen in der Anwesenheit von SD-208 kokultiviert wurden. Die Freisetzung von IFN- γ und TNF- α durch Immuneffektorzellen wurde durch SD-208 erhöht, wohingegen die Freisetzung von IL-10, das als immuninhibitorisch gilt, reduziert wurde. SD-208 war in der Lage, die lytische Aktivität polyklonaler NK-Zellen gegenüber Gliomzellen in Anwesenheit von rekombinantem TGF- β oder von TGF- β -haltigem Gliomzellüberstand wiederherzustellen. Die orale Bioverfügbarkeit von SD-208 wurde durch die Inhibition einer TGF- β -vermittelten Smad-Phosphorylierung in der Milz und im Gehirn gezeigt. Eine systemische Gabe von SD-208 verlängerte die mittlere Überlebensspanne von VM/Dk-Mäusen, die mit syngenem, intrakraniell 3-Tage-präimplantierten SMA-560 Zellen inokuliert wurden, von 18,6 auf 25,1 Tage. Histologische Untersuchungen ergaben keine Beeinflussung der Blutgefäßbildung, Proliferation oder Apoptose durch SD-208. Jedoch zeigten Tiere, die auf die SD-208-Behandlung ansprachen, eine Erhöhung an Tumor-Infiltration durch NK-Zellen, CD8⁺-T-Zellen und Makrophagen. Diese Daten beschreiben TGF- β RI-Kinase-Inhibitoren wie SD-208 als vielversprechende neue Agenzien zur Behandlung humaner maligner Gliome.

Es wird vermutet, dass TGF- β durch Subtilisin-ähnliche Proprotein-Konvertasen wie Furin prozessiert wird. Humane maligne Gliomzelllinien exprimieren Furin-mRNA und -Protein, weisen Furin-ähnliche Protease-Aktivität auf und sezernieren Furin in den Extrazellulärraum, respektive den Zellkulturüberstand. Die Furin-Aktivität wurde durch die exogene Zugabe von TGF- β oder neutralisierendem TGF- β Antikörper nicht verändert. Die Freisetzung von bioaktivem TGF- β und die Prozessierung von TGF- β_1 und TGF- β_2 wurde in den Gliomzelllinien LN-18 und T98G durch den Furin-Inhibitor dec-RVKR-cmk verhindert. Die ektopische Expression von PDX, einem synthetischen Antitrypsin-Analogon mit anti-Furin-Aktivität, hemmt die Furin-Aktivität in Gliomzellen, wie auch die TGF- β -Prozessierung und die TGF- β -Freisetzung. Demnach könnten Subtilisin-ähnliche Proprotein Konvertasen ein neues Zielmolekül für die Immuntherapie maligner Gliome und anderer Krebsarten so wie anderer Erkrankungen mit erhöhter TGF- β -Bioaktivität darstellen.

Das Versagen der herkömmlichen Standardkrebstherapien beim Glioblastom lässt dieses als einen attraktiven Modelltumor der Immuntherapie erscheinen. Tumorzellen, die den Liganden des aktivatorischen Immunrezeptors NKG2D exprimieren, stimulieren die Tumormunität, die durch NK- und CD8⁺-T-Zellen vermittelt wird. Humane Gliomzellen exprimieren die NKG2D-Liganden MICA, MICB und Mitglieder der ULBP-Familie konstitutiv. Jedoch widerstehen Gliomzellen einer NK-Zell-vermittelten Zytolyse aufgrund einer hohen Expression von MHC-Klasse I-Molekülen. Die transgene Überexpression von MICA durch Plasmide oder Adenoviren (Ad-MICA) in Gliomzellen erhöht deren Sensitivität gegenüber NK- und T-Zell-Antworten *in vitro* und verzögert das Wachstum subkutaner und intrazerebraler humaner LN-229-Gliomzellen in einem Nacktmaus Xenograft- und einem syngenem SMA-560-VM/Dk-Maus-Modell. Gliomzellen, die progressive Tumoren nach einer Inokulation stabil MICA-transduzierter humaner LN-229-Gliomzellen bilden, verlieren ihre MICA-Expression vermutlich durch einen starken Selektionsdruck *in vivo*. Die Abstossung MICA-exprimierender SMA-560-Zellen in VM/Dk Mäusen führte zu protektiver Immunität, selbst nach einer anschließenden Reexposition mit Wildtyp-Tumorzellen. Abschliessend wurde das Wachstum syngener intrakranieller SMA-560-Tumoren untersucht. Das Wachstum dieser Tumoren wurde nach einer peripheren Vakzination mit Ad-MICA-infizierten bestrahlten Tumorzellen gehemmt und führte zu einer Immun-Aktivierung in den NK- und T-Zellkompartimenten *in vivo*. Diese Daten legen die MICA-Immunogen-therapie als eine neue experimentelle Behandlungsstrategie für humane maligne Gliome nahe.

Während meiner Doktorarbeit habe ich an verschiedensten neuen Ansätzen zur Therapie maligner Gliome mitgewirkt. Diese basierten nicht nur auf der Wiederherstellung einer Tumor-Immunüberwachung durch direkte Aktivierung der adaptiven Immunantwort, sondern auch auf Mechanismen der angeborenen Immunität, die schliesslich auch zu einer Aktivierung der adaptiven Immunität führten. Während die Verbindungen zwischen diesen verschiedenen Aspekten der Gliombiologie nicht sofort ersichtlich sind, ist es dennoch unumgänglich, sich mit den komplexen Zusammenhängen zwischen angeborener und adaptiver Immunantwort und der Tumorummunologie zu befassen. Die Tumor-Immunüberwachung wird von Immuneffektorzellen vermittelt, die selektiv apoptotischen und nicht-apoptotischen Zelltod in Krebszellen induzieren können. Werden diese Zellen jedoch in ihren Mechanismen supprimiert, etwa durch TGF- β , so können Mechanismen die zu einer Erhaltung eines T-Zellpools, z.B. nach einer Aktivierung durch CD70, dienen, dennoch eine Immunantwort erzeugen, die es erlaubt bereits etablierte Tumoren zu vernichten. Weiterhin können Aktivierungsprozesse induziert werden, die der angeborenen Immunität angehören, wie es die Toll-ähnlichen Rezeptoren 7 und TLR8 durch einen Kontakt mit ihrem Liganden, der Einzelstrang-RNA, oder MICA als Ligand von NKG2D, vermögen. Jedoch können Tumoren auch diesen Mechanismen dadurch entgegenwirken, dass sie in Lymphozyten Apoptose induzieren, etwa durch CD95/FasL-Expression oder diese immunsupprimieren durch HLA-G oder TGF- β (140, 248-251). Schliesslich führt die Abstossung von Tumorgewebe zu einer Quelle von Antigenen, die von professionellen Antigen-präsentierenden Zellen, wie es DC sind, aufgenommen und präsentiert werden können (252-256).

Ob die in dieser Doktorarbeit aufgezeigten Konzepte das Potenzial haben, die bestehenden Gliom-(Immun)-therapien zu verbessern, muss in weiteren Modellen untersucht und verifiziert werden. Deshalb möchte ich die der vorliegenden Arbeit zugrundeliegende Motivation mit einem Holzschnitt von Camille Flammarion's beschreiben, "L'Atmosphère, et la Météorologie populaire", Paris 1888": dieser Holzschnitt stellt den Mythos von der Zertrümmerung des antiken Weltbildes durch die Kopernikanische Revolution und dem darauffolgenden Verlust der zentralen Stellung des Menschen im Kosmos symbolisch dar.



Der Mensch durchbricht die Himmelsphären erinnert mich daran, wie ermutigend es ist ein neues immunologisches Werkzeug zu entdecken und wie fern dessen Umsetzung in die alltägliche Praxis einer therapeutischen immunologischen Behandlung in der Klinik dennoch ist. Dieser Holzschnitt aus dem 16. Jahrhundert dokumentiert eindrucksvoll: „Wie tief der Drang (...) zur Durchbrechung des gewohnten Horizontes verwurzelt ist, und wie unabhängig er ist von der Frage seiner praktischen Realisierbarkeit“.

References

1. Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., and Dirks, P. B. Identification of human brain tumour initiating cells. *Nature*, *432*: 396-401, 2004.
2. Kleihues, P. and W.K., C. *Pathology and Genetics of Tumours of the Nervous System*. Lyon: IARC Press, 2000.
3. Weller, M., Thomas, D.G.T. Primary tumors of the central and peripheral nervous system. *In*: C. L. Brandt T, Dichgans J, Diener HC, Kennard C (ed.), *Course and Treatment of Neurological Disorders*, 2nd edition edition, pp. 827-863. San Diego, CA, USA: Academic Press, 2003.
4. Reifenberger, G., Liu, L., Ichimura, K., Schmidt, E. E., and Collins, V. P. Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res*, *53*: 2736-2739, 1993.
5. Wild-Bode, C., Weller, M., Rimmer, A., Dichgans, J., and Wick, W. Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. *Cancer Res*, *61*: 2744-2750, 2001.
6. Steinbach, J. P., Supra, P., Huang, H. J., Cavenee, W. K., and Weller, M. CD95-mediated apoptosis of human glioma cells: modulation by epidermal growth factor receptor activity. *Brain Pathol*, *12*: 12-20, 2002.
7. Fulda, S., Wick, W., Weller, M., and Debatin, K. M. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med*, *8*: 808-815, 2002.
8. Steinbach, J. P., Eisenmann, C., Klumpp, A., and Weller, M. Co-inhibition of epidermal growth factor receptor and type 1 insulin-like growth factor receptor synergistically sensitizes human malignant glioma cells to CD95L-induced apoptosis. *Biochem Biophys Res Commun*, *321*: 524-530, 2004.
9. Steinbach, J. P., Klumpp, A., Wolburg, H., and Weller, M. Inhibition of epidermal growth factor receptor signaling protects human malignant glioma cells from hypoxia-induced cell death. *Cancer Res*, *64*: 1575-1578, 2004.
10. Li, D. M. and Sun, H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res*, *57*: 2124-2129, 1997.

11. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet*, 15: 356-362, 1997.
12. Mollenhauer, J., Wiemann, S., Scheurlen, W., Korn, B., Hayashi, Y., Wilgenbus, K. K., von Deimling, A., and Poustka, A. DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. *Nat Genet*, 17: 32-39, 1997.
13. Giani, C. and Finocchiaro, G. Mutation rate of the CDKN2 gene in malignant gliomas. *Cancer Res*, 54: 6338-6339, 1994.
14. He, J., Olson, J. J., and James, C. D. Lack of p16INK4 or retinoblastoma protein (pRb), or amplification-associated overexpression of cdk4 is observed in distinct subsets of malignant glial tumors and cell lines. *Cancer Res*, 55: 4833-4836, 1995.
15. Nishikawa, R., Furnari, F. B., Lin, H., Arap, W., Berger, M. S., Cavenee, W. K., and Su Huang, H. J. Loss of P16INK4 expression is frequent in high grade gliomas. *Cancer Res*, 55: 1941-1945, 1995.
16. Fulci, G., Ishii, N., and Van Meir, E. G. p53 and brain tumors: from gene mutations to gene therapy. *Brain Pathol*, 8: 599-613, 1998.
17. Wischhusen, J., Naumann, U., Ohgaki, H., Rastinejad, F., and Weller, M. CP-31398, a novel p53-stabilizing agent, induces p53-dependent and p53-independent glioma cell death. *Oncogene*, 22: 8233-8245, 2003.
18. Uhl, M., Weiler, M., Wick, W., Jacobs, A., Weller, M., and Herrlinger, U. Migratory neural stem cells for improved thymidine kinase-based gene therapy of malignant gliomas. *Biochem. Biophys. Res. Commun. In Press*
19. Rainov, N. G., Kramm, C. M., Banning, U., Riemann, D., Holzhausen, H. J., Heidecke, V., Burger, K. J., Burkert, W., and Korholz, D. Immune response induced by retrovirus-mediated HSV-tk/GCV pharmacogene therapy in patients with glioblastoma multiforme. *Gene Ther*, 7: 1853-1858, 2000.
20. Nanda, D., Driesse, M. J., and Sillevs Smitt, P. A. Clinical trials of adenoviral-mediated suicide gene therapy of malignant gliomas. *Prog Brain Res*, 132: 699-710, 2001.
21. Wei, M. X., Tamiya, T., Chase, M., Boviatsis, E. J., Chang, T. K., Kowall, N. W., Hochberg, F. H., Waxman, D. J., Breakefield, X. O., and Chiocca, E. A. Experimental

- tumor therapy in mice using the cyclophosphamide-activating cytochrome P450 2B1 gene. *Hum Gene Ther*, 5: 969-978, 1994.
22. Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H., and Okumura, K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med*, 7: 94-100, 2001.
 23. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med*, 5: 157-163, 1999.
 24. Ashkenazi, A. and Dixit, V. M. Death receptors: signaling and modulation. *Science*, 281: 1305-1308, 1998.
 25. Hao, C., Beguinot, F., Condorelli, G., Trencia, A., Van Meir, E. G., Yong, V. W., Parney, I. F., Roa, W. H., and Petruk, K. C. Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in human malignant glioma cells. *Cancer Res*, 61: 1162-1170, 2001.
 26. Stacker, S. A., Williams, R. A., and Achen, M. G. Lymphangiogenic growth factors as markers of tumor metastasis. *Apmis*, 112: 539-549, 2004.
 27. Brat, D. J., Kaur, B., and Van Meir, E. G. Genetic modulation of hypoxia induced gene expression and angiogenesis: relevance to brain tumors. *Front Biosci*, 8: d100-116, 2003.
 28. Tremont-Lukats, I. W. and Gilbert, M. R. Advances in molecular therapies in patients with brain tumors. *Cancer Control*, 10: 125-137, 2003.
 29. Kerbel, R. and Folkman, J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer*, 2: 727-739, 2002.
 30. Kawakami, Y., Fujita, T., Matsuzaki, Y., Sakurai, T., Tsukamoto, M., Toda, M., and Sumimoto, H. Identification of human tumor antigens and its implications for diagnosis and treatment of cancer. *Cancer Sci*, 95: 784-791, 2004.
 31. Igney, F. H. and Krammer, P. H. Immune escape of tumors: apoptosis resistance and tumor counterattack. *J Leukoc Biol*, 71: 907-920, 2002.
 32. Quang, T. S. and Brady, L. W. Radioimmunotherapy as a novel treatment regimen: 125I-labeled monoclonal antibody 425 in the treatment of high-grade brain gliomas. *Int J Radiat Oncol Biol Phys*, 58: 972-975, 2004.

33. Kawakami, M., Kawakami, K., and Puri, R. K. Interleukin-4-Pseudomonas exotoxin chimeric fusion protein for malignant glioma therapy. *J Neurooncol*, *65*: 15-25, 2003.
34. Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R. C. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A*, *90*: 3539-3543, 1993.
35. Sampson, J. H., Archer, G. E., Ashley, D. M., Fuchs, H. E., Hale, L. P., Dranoff, G., and Bigner, D. D. Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8⁺ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system. *Proc Natl Acad Sci U S A*, *93*: 10399-10404, 1996.
36. Ewend, M. G., Thompson, R. C., Anderson, R., Sills, A. K., Staveley-O'Carroll, K., Tyler, B. M., Hanes, J., Brat, D., Thomas, M., Jaffee, E. M., Pardoll, D. M., and Brem, H. Intracranial paracrine interleukin-2 therapy stimulates prolonged antitumor immunity that extends outside the central nervous system. *J Immunother*, *23*: 438-448, 2000.
37. Okada, H. and Pollack, I. F. Cytokine gene therapy for malignant glioma. *Expert Opin Biol Ther*, *4*: 1609-1620, 2004.
38. Okada, H., Tahara, H., Shurin, M. R., Attanucci, J., Giezeman-Smits, K. M., Fellows, W. K., Lotze, M. T., Chambers, W. H., and Bozik, M. E. Bone marrow-derived dendritic cells pulsed with a tumor-specific peptide elicit effective anti-tumor immunity against intracranial neoplasms. *Int J Cancer*, *78*: 196-201, 1998.
39. Ouellette, A. J. IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. *Am J Physiol*, *277*: G257-261, 1999.
40. Medzhitov, R. and Janeway, C., Jr. Innate immune recognition: mechanisms and pathways. *Immunol Rev*, *173*: 89-97, 2000.
41. Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., and Hartmann, G. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol*, *168*: 4531-4537, 2002.
42. Scheel, B., Braedel, S., Probst, J., Carralot, J. P., Wagner, H., Schild, H., Jung, G., Rammensee, H. G., and Pascolo, S. Immunostimulating capacities of stabilized RNA molecules. *Eur J Immunol*, *34*: 537-547, 2004.

43. Akira, S. and Takeda, K. Toll-like receptor signalling. *Nat Rev Immunol*, 4: 499-511, 2004.
44. Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A., and Seya, T. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol*, 171: 3154-3162, 2003.
45. Heil, F., Ahmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S., Wagner, H., and Bauer, S. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur J Immunol*, 33: 2987-2997, 2003.
46. Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M., and Wagner, H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol*, 32: 1958-1968, 2002.
47. Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T., and Golenbock, D. T. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol*, 5: 190-198, 2004.
48. Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*, 303: 1526-1529, 2004.
49. Dunne, A. and O'Neill, L. A. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE*, 2003: re3, 2003.
50. Takeda, K., Kaisho, T., and Akira, S. Toll-like receptors. *Annu Rev Immunol*, 21: 335-376, 2003.
51. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity*, 9: 143-150, 1998.
52. Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P. F., Sato, S., Hoshino, K., and Akira, S. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol*, 167: 5887-5894, 2001.
53. Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol*, 166: 5688-5694, 2001.
54. Trinchieri, G. Biology of natural killer cells. *Adv Immunol*, 47: 187-376, 1989.

55. Burnet, F. M. The concept of immunological surveillance. *Prog Exp Tumor Res*, *13*: 1-2, 1970.
56. Mailliard, R. B., Son, Y. I., Redlinger, R., Coates, P. T., Giermasz, A., Morel, P. A., Storkus, W. J., and Kalinski, P. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. *J Immunol*, *171*: 2366-2373, 2003.
57. Lanier, L. L. NK cell receptors. *Annu Rev Immunol*, *16*: 359-393, 1998.
58. Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., Biassoni, R., and Moretta, L. Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu Rev Immunol*, *19*: 197-223, 2001.
59. Yokoyama, W. M. and Plougastel, B. F. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol*, *3*: 304-316, 2003.
60. Raulet, D. H., Vance, R. E., and McMahon, C. W. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol*, *19*: 291-330, 2001.
61. Raulet, D. H. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol*, *3*: 781-790, 2003.
62. Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L., and Spies, T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science*, *285*: 727-729, 1999.
63. 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*: 119-126, 2000.
64. 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*: 721-727, 2000.
65. Carayannopoulos, L. N., Naidenko, O. V., Fremont, D. H., and Yokoyama, W. M. Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J Immunol*, *169*: 4079-4083, 2002.
66. Diefenbach, A., Hsia, J. K., Hsiung, M. Y., and Raulet, D. H. A novel ligand for the NKG2D receptor activates NK cells and macrophages and induces tumor immunity. *Eur J Immunol*, *33*: 381-391, 2003.
67. Cosman, D., Mullberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N. J. ULBPs, novel MHC class I-related molecules, bind to

- CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity*, *14*: 123-133, 2001.
68. Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K. H., and Spies, T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A*, *96*: 6879-6884, 1999.
 69. Lodoen, M., Ogasawara, K., Hamerman, J. A., Arase, H., Houchins, J. P., Mocarski, E. S., and Lanier, L. L. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. *J Exp Med*, *197*: 1245-1253, 2003.
 70. 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*: 165-171, 2001.
 71. 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*: 11521-11526, 2001.
 72. Groh, V., Wu, J., Yee, C., and Spies, T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*, *419*: 734-738, 2002.
 73. Sivori, S., Vitale, M., Morelli, L., Sanseverino, L., Augugliaro, R., Bottino, C., Moretta, L., and Moretta, A. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J Exp Med*, *186*: 1129-1136, 1997.
 74. Vitale, M., Bottino, C., Sivori, S., Sanseverino, L., Castriconi, R., Marcenaro, E., Augugliaro, R., Moretta, L., and Moretta, A. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med*, *187*: 2065-2072, 1998.
 75. Pende, D., Parolini, S., Pessino, A., Sivori, S., Augugliaro, R., Morelli, L., Marcenaro, E., Accame, L., Malaspina, A., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med*, *190*: 1505-1516, 1999.
 76. Ljunggren, H. G. and Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*, *11*: 237-244, 1990.
 77. Karlhofer, F. M., Ribaldo, R. K., and Yokoyama, W. M. MHC class I alloantigen specificity of Ly-49⁺ IL-2-activated natural killer cells. *Nature*, *358*: 66-70, 1992.

78. Colonna, M. and Samaridis, J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*, 268: 405-408, 1995.
79. Wagtmann, N., Biassoni, R., Cantoni, C., Verdiani, S., Malnati, M. S., Vitale, M., Bottino, C., Moretta, L., Moretta, A., and Long, E. O. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity*, 2: 439-449, 1995.
80. Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L., and Hsu, M. L. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity*, 7: 273-282, 1997.
81. Samaridis, J. and Colonna, M. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur J Immunol*, 27: 660-665, 1997.
82. Braud, V. M., Allan, D. S., O'Callaghan, C. A., Soderstrom, K., D'Andrea, A., Ogg, G. S., Lazetic, S., Young, N. T., Bell, J. I., Phillips, J. H., Lanier, L. L., and McMichael, A. J. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*, 391: 795-799, 1998.
83. Lee, N., Llano, M., Carretero, M., Ishitani, A., Navarro, F., Lopez-Botet, M., and Geraghty, D. E. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci U S A*, 95: 5199-5204, 1998.
84. Vance, R. E., Kraft, J. R., Altman, J. D., Jensen, P. E., and Raulet, D. H. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med*, 188: 1841-1848, 1998.
85. Peters, P. J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J. W., and Geuze, H. J. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med*, 173: 1099-1109, 1991.
86. Russell, J. H. and Ley, T. J. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol*, 20: 323-370, 2002.
87. Trapani, J. A. and Smyth, M. J. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol*, 2: 735-747, 2002.
88. Parham, P. Innate immunity: The unsung heroes. *Nature*, 423: 20, 2003.
89. Viola, A. and Lanzavecchia, A. T cell activation determined by T cell receptor number and tunable thresholds. *Science*, 273: 104-106, 1996.

90. Sharpe, A. H. and Freeman, G. J. The B7-CD28 superfamily. *Nat Rev Immunol*, 2: 116-126, 2002.
91. Croft, M. Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine Growth Factor Rev*, 14: 265-273, 2003.
92. Croft, M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol*, 3: 609-620, 2003.
93. Borst, J., Sluysers, C., De Vries, E., Klein, H., Melief, C. J., and Van Lier, R. A. Alternative molecular form of human T cell-specific antigen CD27 expressed upon T cell activation. *Eur J Immunol*, 19: 357-364, 1989.
94. Gramaglia, I., Weinberg, A. D., Lemon, M., and Croft, M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol*, 161: 6510-6517, 1998.
95. Pollok, K. E., Kim, S. H., and Kwon, B. S. Regulation of 4-1BB expression by cell-cell interactions and the cytokines, interleukin-2 and interleukin-4. *Eur J Immunol*, 25: 488-494, 1995.
96. Lens, S. M., Baars, P. A., Hooibrink, B., van Oers, M. H., and van Lier, R. A. Antigen-presenting cell-derived signals determine expression levels of CD70 on primed T cells. *Immunology*, 90: 38-45, 1997.
97. Lens, S. M., de Jong, R., Hooibrink, B., Koopman, G., Pals, S. T., van Oers, M. H., and van Lier, R. A. Phenotype and function of human B cells expressing CD70 (CD27 ligand). *Eur J Immunol*, 26: 2964-2971, 1996.
98. van Oers, M. H., Pals, S. T., Evers, L. M., van der Schoot, C. E., Koopman, G., Bonfrer, J. M., Hintzen, R. Q., von dem Borne, A. E., and van Lier, R. A. Expression and release of CD27 in human B-cell malignancies. *Blood*, 82: 3430-3436, 1993.
99. Hintzen, R. Q., van Lier, R. A., Kuijpers, K. C., Baars, P. A., Schaasberg, W., Lucas, C. J., and Polman, C. H. Elevated levels of a soluble form of the T cell activation antigen CD27 in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol*, 35: 211-217., 1991.
100. Hintzen, R. Q., Lens, S. M., Lammers, K., Kuiper, H., Beckmann, M. P., and van Lier, R. A. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. *J Immunol*, 154: 2612-2623, 1995.
101. Agematsu, K., Kobata, T., Sugita, K., Freeman, G. J., Beckmann, M. P., Schlossman, S. F., and Morimoto, C. Role of CD27 in T cell immune response. Analysis by recombinant soluble CD27. *J Immunol*, 153: 1421-1429., 1994.

102. Ohshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., and Delespesse, G. Expression and function of OX40 ligand on human dendritic cells. *J Immunol*, *159*: 3838-3848, 1997.
103. Hendriks, J., Gravestien, L. A., Tesselaar, K., van Lier, R. A., Schumacher, T. N., and Borst, J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol*, *1*: 433-440., 2000.
104. Arens, R., Tesselaar, K., Baars, P. A., van Schijndel, G. M., Hendriks, J., Pals, S. T., Krimpenfort, P., Borst, J., van Oers, M. H., and van Lier, R. A. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFN γ -mediated B cell depletion. *Immunity*, *15*: 801-812., 2001.
105. Couderc, B., Zitvogel, L., Douin-Echinard, V., Djennane, L., Tahara, H., Favre, G., Lotze, M. T., and Robbins, P. D. Enhancement of antitumor immunity by expression of CD70 (CD27 ligand) or CD154 (CD40 ligand) costimulatory molecules in tumor cells. *Cancer Gene Ther*, *5*: 163-175., 1998.
106. Lorenz, M. G., Kantor, J. A., Schlom, J., and Hodge, J. W. Anti-tumor immunity elicited by a recombinant vaccinia virus expressing CD70 (CD27L). *Hum Gene Ther*, *10*: 1095-1103., 1999.
107. Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., and Miyazono, K. Two major Smad pathways in TGF- β superfamily signalling. *Genes Cells*, *7*: 1191-1204, 2002.
108. Derynck, R., Akhurst, R. J., and Balmain, A. TGF- β signaling in tumor suppression and cancer progression. *Nat Genet*, *29*: 117-129, 2001.
109. Wischhusen, J., Jung, G., Radovanovic, I., Beier, C., Steinbach, J. P., Rimner, A., Huang, H., Schulz, J. B., Ohgaki, H., Aguzzi, A., Rammensee, H. G., and Weller, M. Identification of CD70-mediated apoptosis of immune effector cells as a novel immune escape pathway of human glioblastoma. *Cancer Res*, *62*: 2592-2599, 2002.
110. Uhl, M., Aulwurm, S., Wischhusen, J., Weiler, M., Ma, J. Y., Almirez, R., Mangadu, R., Liu, Y. W., Platten, M., Herrlinger, U., Murphy, A., Wong, D. H., Wick, W., Higgins, L. S., and Weller, M. SD-208, a novel transforming growth factor { β } receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res*, *64*: 7954-7961, 2004.
111. Cormary, C., Gonzalez, R., Faye, J. C., Favre, G., and Tilkin-Mariame, A. F. Induction of T-cell antitumor immunity and protection against tumor growth by secretion of soluble human CD70 molecules. *Cancer Gene Ther*, *11*: 497-507, 2004.

112. Rothenfusser, S., Tuma, E., Wagner, M., Endres, S., and Hartmann, G. Recent advances in immunostimulatory CpG oligonucleotides. *Curr Opin Mol Ther*, 5: 98-106, 2003.
113. Carpentier, A. F., Xie, J., Mokhtari, K., and Delattre, J. Y. Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. *Clin Cancer Res*, 6: 2469-2473, 2000.
114. Heikenwalder, M., Polymenidou, M., Junt, T., Sigurdson, C., Wagner, H., Akira, S., Zinkernagel, R., and Aguzzi, A. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat Med*, 10: 187-192, 2004.
115. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science*, 303: 1529-1531, 2004.
116. Wrann, M., Bodmer, S., de Martin, R., Siepl, C., Hofer-Warbinek, R., Frei, K., Hofer, E., and Fontana, A. T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-beta. *Embo J*, 6: 1633-1636, 1987.
117. de Martin, R., Haendler, B., Hofer-Warbinek, R., Gaugitsch, H., Wrann, M., Schlusener, H., Seifert, J. M., Bodmer, S., Fontana, A., and Hofer, E. Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family. *Embo J*, 6: 3673-3677, 1987.
118. Platten, M., Wick, W., and Weller, M. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech*, 52: 401-410, 2001.
119. Derynck, R., Jarrett, J. A., Chen, E. Y., and Goeddel, D. V. The murine transforming growth factor-beta precursor. *J Biol Chem*, 261: 4377-4379, 1986.
120. Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E., and Leduc, R. Processing of transforming growth factor beta 1 precursor by human furin convertase. *J Biol Chem*, 270: 10618-10624, 1995.
121. Bowman, M. R., Crimmins, M. A., Yetz-Aldape, J., Kriz, R., Kelleher, K., and Herrmann, S. The cloning of CD70 and its identification as the ligand for CD27. *J Immunol*, 152: 1756-1761, 1994.
122. van Lier, R. A., Borst, J., Vroom, T. M., Klein, H., Van Mourik, P., Zeijlemaker, W. P., and Melief, C. J. Tissue distribution and biochemical and functional properties of

- Tp55 (CD27), a novel T cell differentiation antigen. *J Immunol*, 139: 1589-1596, 1987.
123. Lens, S. M., Tesselaar, K., van Oers, M. H., and van Lier, R. A. Control of lymphocyte function through CD27-CD70 interactions. *Semin Immunol*, 10: 491-499, 1998.
 124. Yang, F. C., Agematsu, K., Nakazawa, T., Mori, T., Ito, S., Kobata, T., Morimoto, C., and Komiyama, A. CD27/CD70 interaction directly induces natural killer cell killing activity. *Immunology*, 88: 289-293., 1996.
 125. Arens, R., Schepers, K., Nolte, M. A., van Oosterwijk, M. F., van Lier, R. A. W., Schumacher, T. N. M., and van Oers, M. H. J. Tumor Rejection Induced by CD70-mediated Quantitative and Qualitative Effects on Effector CD8+ T Cell Formation. *J. Exp. Med.*, 199: 1595-1605, 2004.
 126. Kobata, T., Jacquot, S., Kozlowski, S., Agematsu, K., Schlossman, S. F., and Morimoto, C. CD27-CD70 interactions regulate B-cell activation by T cells. *Proc Natl Acad Sci U S A*, 92: 11249-11253, 1995.
 127. Xiao, Y., Hendriks, J., Langerak, P., Jacobs, H., and Borst, J. CD27 is acquired by primed B cells at the centroblast stage and promotes germinal center formation. *J Immunol*, 172: 7432-7441, 2004.
 128. Kelly, J. M., Darcy, P. K., Markby, J. L., Godfrey, D. I., Takeda, K., Yagita, H., and Smyth, M. J. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nat Immunol*, 3: 83-90, 2002.
 129. Tesselaar, K., Arens, R., van Schijndel, G. M., Baars, P. A., van der Valk, M. A., Borst, J., van Oers, M. H., and van Lier, R. A. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. *Nat Immunol*, 4: 49-54, 2003.
 130. Akiba, H., Nakano, H., Nishinaka, S., Shindo, M., Kobata, T., Atsuta, M., Morimoto, C., Ware, C. F., Malinin, N. L., Wallach, D., Yagita, H., and Okumura, K. CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. *J Biol Chem*, 273: 13353-13358, 1998.
 131. Prasad, K. V., Ao, Z., Yoon, Y., Wu, M. X., Rizk, M., Jacquot, S., and Schlossman, S. F. CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. *Proc Natl Acad Sci U S A*, 94: 6346-6351., 1997.

132. Weller, M. and Fontana, A. The failure of current immunotherapy for malignant glioma. Tumor-derived TGF-beta, T-cell apoptosis, and the immune privilege of the brain. *Brain Res Brain Res Rev*, 21: 128-151, 1995.
133. Weller, M., Kleihues, P., Dichgans, J., and Ohgaki, H. CD95 ligand: lethal weapon against malignant glioma? *Brain Pathol*, 8: 285-293, 1998.
134. Wiendl, H., Mitsdoerffer, M., Hofmeister, V., Wischhusen, J., Bornemann, A., Meyermann, R., Weiss, E. H., Melms, A., and Weller, M. A functional role of HLA-G expression in human gliomas: an alternative strategy of immune escape. *J Immunol*, 168: 4772-4780, 2002.
135. Serano, R. D., Pegram, C. N., and Bigner, D. D. Tumorigenic cell culture lines from a spontaneous VM/Dk murine astrocytoma (SMA). *Acta Neuropathol (Berl)*, 51: 53-64, 1980.
136. Yoon, Y., Ao, Z., Cheng, Y., Schlossman, S. F., and Prasad, K. V. Murine Siva-1 and Siva-2, alternate splice forms of the mouse Siva gene, both bind to CD27 but differentially transduce apoptosis. *Oncogene*, 18: 7174-7179., 1999.
137. Fortin, A., MacLaurin, J. G., Arbour, N., Cregan, S. P., Kushwaha, N., Callaghan, S. M., Park, D. S., Albert, P. R., and Slack, R. S. The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. *J Biol Chem*, 279: 28706-28714, 2004.
138. Chu, F., Borthakur, A., Sun, X., Barkinge, J., Gudi, R., Hawkins, S., and Prasad, K. V. The Siva-1 putative amphipathic helical region (SAH) is sufficient to bind to BCL-XL and sensitize cells to UV radiation induced apoptosis. *Apoptosis*, 9: 83-95, 2004.
139. Cao, C., Ren, X., Kharbanda, S., Koleske, A., Prasad, K. V., and Kufe, D. The ARG tyrosine kinase interacts with Siva-1 in the apoptotic response to oxidative stress. *J Biol Chem*, 276: 11465-11468, 2001.
140. Weller, M., Constam, D. B., Malipiero, U., and Fontana, A. Transforming growth factor-beta 2 induces apoptosis of murine T cell clones without down-regulating bcl-2 mRNA expression. *Eur J Immunol*, 24: 1293-1300, 1994.
141. Leitlein, J., Aulwurm, S., Waltereit, R., Naumann, U., Wagenknecht, B., Garten, W., Weller, M., and Platten, M. Processing of immunosuppressive pro-TGF-beta 1,2 by human glioblastoma cells involves cytoplasmic and secreted furin-like proteases. *J Immunol*, 166: 7238-7243, 2001.

142. Takeda, K., Oshima, H., Hayakawa, Y., Akiba, H., Atsuta, M., Kobata, T., Kobayashi, K., Ito, M., Yagita, H., and Okumura, K. CD27-mediated activation of murine NK cells. *J Immunol*, *164*: 1741-1745., 2000.
143. Friese, M. A., Wischhusen, J., Wick, W., Weiler, M., Eisele, G., Steinle, A., and Weller, M. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res*, *64*: 7596-7603, 2004.
144. Wick, W., Platten, M., and Weller, M. Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta. *J Neurooncol*, *53*: 177-185, 2001.
145. Hendriks, J., Gravestein, L. A., Tesselaar, K., van Lier, R. A., Schumacher, T. N., and Borst, J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol*, *1*: 433-440, 2000.
146. Avgeropoulos, N. G. and Batchelor, T. T. New treatment strategies for malignant gliomas. *Oncologist*, *4*: 209-224, 1999.
147. Weller, M. and Fontana, A. The failure of current immunotherapy for malignant glioma. Tumor-derived TGF-beta, T-cell apoptosis, and the immune privilege of the brain. *Brain Res Brain Res Rev*, *21*: 128-151., 1995.
148. Fontana, A., Hengartner, H., de Tribolet, N., and Weber, E. Glioblastoma cells release interleukin 1 and factors inhibiting interleukin 2-mediated effects. *J Immunol*, *132*: 1837-1844, 1984.
149. Leitlein, J., Aulwurm, S., Waltereit, R., Naumann, U., Wagenknecht, B., Garten, W., Weller, M., and Platten, M. Processing of immunosuppressive pro-tgf-beta1,2 by human glioblastoma cells involves cytoplasmic and secreted furin-like proteases. *J Immunol*, *166*: 7238-7243., 2001.
150. Wiemann, B. and Starnes, C. O. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. *Pharmacol Ther*, *64*: 529-564, 1994.
151. Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A., and Flavell, R. A. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A*, *101*: 5598-5603, 2004.
152. Kariko, K., Ni, H., Capodici, J., Lamphier, M., and Weissman, D. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem*, *279*: 12542-12550, 2004.
153. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, *413*: 732-738, 2001.

154. Jean, W. C., Spellman, S. R., Wallenfriedman, M. A., Hall, W. A., and Low, W. C. Interleukin-12-based immunotherapy against rat 9L glioma. *Neurosurgery*, 42: 850-856; discussion 856-857, 1998.
155. Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol*, 3: 196-200, 2002.
156. Du, X., Poltorak, A., Wei, Y., and Beutler, B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur Cytokine Netw*, 11: 362-371, 2000.
157. Chuang, T. H. and Ulevitch, R. J. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw*, 11: 372-378, 2000.
158. Gilboa, E. and Vieweg, J. Cancer immunotherapy with mRNA-transfected dendritic cells. *Immunol Rev*, 199: 251-263, 2004.
159. Hoerr, I., Obst, R., Rammensee, H. G., and Jung, G. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. *Eur J Immunol*, 30: 1-7, 2000.
160. Stewart, L. A. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet*, 359: 1011-1018, 2002.
161. Roszman, T., Elliott, L., and Brooks, W. Modulation of T-cell function by gliomas. *Immunol Today*, 12: 370-374, 1991.
162. Fontana, A., Kristensen, F., Dubs, R., Gemsa, D., and Weber, E. Production of prostaglandin E and an interleukin-1 like factor by cultured astrocytes and C6 glioma cells. *J Immunol*, 129: 2413-2419, 1982.
163. Hishii, M., Nitta, T., Ishida, H., Ebato, M., Kurosu, A., Yagita, H., Sato, K., and Okumura, K. Human glioma-derived interleukin-10 inhibits antitumor immune responses in vitro. *Neurosurgery*, 37: 1160-1166; discussion 1166-1167, 1995.
164. Fakhrai, H., Dorigo, O., Shawler, D. L., Lin, H., Mercola, D., Black, K. L., Royston, I., and Sobol, R. E. Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci U S A*, 93: 2909-2914, 1996.

165. Stander, M., Naumann, U., Dumitrescu, L., Heneka, M., Loschmann, P., Gulbins, E., Dichgans, J., and Weller, M. Decorin gene transfer-mediated suppression of TGF-beta synthesis abrogates experimental malignant glioma growth in vivo. *Gene Ther*, 5: 1187-1194, 1998.
166. Platten, M., Wild-Bode, C., Wick, W., Leitlein, J., Dichgans, J., and Weller, M. N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) inhibits transforming growth factor-beta release and reduces migration and invasiveness of human malignant glioma cells. *Int J Cancer*, 93: 53-61, 2001.
167. Wick, W., Grimm, C., Wild-Bode, C., Platten, M., Arpin, M., and Weller, M. Ezrin-dependent promotion of glioma cell clonogenicity, motility, and invasion mediated by BCL-2 and transforming growth factor-beta2. *J Neurosci*, 21: 3360-3368, 2001.
168. DaCosta Byfield, S., Major, C., Laping, N. J., and Roberts, A. B. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol*, 65: 744-752, 2004.
169. Valiante, N. M., Rengaraju, M., and Trinchieri, G. Role of the production of natural killer cell stimulatory factor (NKSF/IL-12) in the ability of B cell lines to stimulate T and NK cell proliferation. *Cell Immunol*, 145: 187-198, 1992.
170. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massague, J. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell*, 71: 1003-1014, 1992.
171. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell*, 1: 611-617, 1998.
172. Tamaki, M., McDonald, W., Amberger, V. R., Moore, E., and Del Maestro, R. F. Implantation of C6 astrocytoma spheroid into collagen type I gels: invasive, proliferative, and enzymatic characterizations. *J Neurosurg*, 87: 602-609, 1997.
173. Yang, Y. A., Dukhanina, O., Tang, B., Mamura, M., Letterio, J. J., MacGregor, J., Patel, S. C., Khozin, S., Liu, Z. Y., Green, J., Anver, M. R., Merlino, G., and Wakefield, L. M. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest*, 109: 1607-1615, 2002.
174. Gorelik, L. and Flavell, R. A. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol*, 2: 46-53, 2002.
175. Thomas, G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol*, 3: 753-766, 2002.

176. Ashley, D. M., Kong, F. M., Bigner, D. D., and Hale, L. P. Endogenous expression of transforming growth factor beta1 inhibits growth and tumorigenicity and enhances Fas-mediated apoptosis in a murine high-grade glioma model. *Cancer Res*, 58: 302-309, 1998.
177. Roszman, T., Elliott, L., and Brooks, W. Modulation of T-cell function by gliomas. *Immunol Today*, 12: 370-374., 1991.
178. Liao, L. M., Fakhrai, H., and Black, K. L. Prolonged survival of rats with intracranial C6 gliomas by treatment with TGF-beta antisense gene. *Neurol Res*, 20: 742-747, 1998.
179. Hoshino, H., Konda, Y., and Takeuchi, T. Co-expression of the proprotein-processing endoprotease furin and its substrate transforming growth factor beta1 and the differentiation of rat hepatocytes. *FEBS Lett*, 419: 9-12, 1997.
180. Blanchette, F., Day, R., Dong, W., Laprise, M. H., and Dubois, C. M. TGFbeta1 regulates gene expression of its own converting enzyme furin. *J Clin Invest*, 99: 1974-1983, 1997.
181. Steiner, D. F. The proprotein convertases. *Curr Opin Chem Biol*, 2: 31-39, 1998.
182. Bergeron, F., Leduc, R., and Day, R. Subtilase-like pro-protein convertases: from molecular specificity to therapeutic applications. *J Mol Endocrinol*, 24: 1-22, 2000.
183. Nakayama, K. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J*, 327 (Pt 3): 625-635, 1997.
184. Schafer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M. L., Kern, H. F., Klenk, H. D., and Garten, W. Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *Embo J*, 14: 2424-2435, 1995.
185. Weller, M., Rieger, J., Grimm, C., Van Meir, E. G., De Tribolet, N., Krajewski, S., Reed, J. C., von Deimling, A., and Dichgans, J. Predicting chemoresistance in human malignant glioma cells: the role of molecular genetic analyses. *Int J Cancer*, 79: 640-644., 1998.
186. Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A. J., and Thomas, G. alpha1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: application as an antipathogenic agent. *Proc Natl Acad Sci U S A*, 95: 7293-7298, 1998.

187. Creemers, J. W., Vey, M., Schafer, W., Ayoubi, T. A., Roebroek, A. J., Klenk, H. D., Garten, W., and Van de Ven, W. J. Endoproteolytic cleavage of its propeptide is a prerequisite for efficient transport of furin out of the endoplasmic reticulum. *J Biol Chem*, *270*: 2695-2702, 1995.
188. Brown, P. D., Wakefield, L. M., Levinson, A. D., and Sporn, M. B. Physicochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3. *Growth Factors*, *3*: 35-43, 1990.
189. van den Ouweland, A. M., van Duijnhoven, H. L., Keizer, G. D., Dorssers, L. C., and Van de Ven, W. J. Structural homology between the human fur gene product and the subtilisin-like protease encoded by yeast KEX2. *Nucleic Acids Res*, *18*: 664, 1990.
190. Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B., and Goeddel, D. V. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature*, *316*: 701-705, 1985.
191. Ponte, P., Ng, S. Y., Engel, J., Gunning, P., and Kedes, L. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res*, *12*: 1687-1696, 1984.
192. Weller, M., Frei, K., Groscurth, P., Krammer, P. H., Yonekawa, Y., and Fontana, A. Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells. Induction and modulation of sensitivity by cytokines. *J Clin Invest*, *94*: 954-964, 1994.
193. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin. Characterization and kinetic parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *J Biol Chem*, *269*: 25830-25837, 1994.
194. Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J., and Thomas, G. Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. *J Cell Biol*, *111*: 2851-2859, 1990.
195. Klimpel, K. R., Molloy, S. S., Thomas, G., and Leppla, S. H. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc Natl Acad Sci U S A*, *89*: 10277-10281, 1992.
196. Dufour, E. K., Denault, J. B., Hopkins, P. C., and Leduc, R. Serpin-like properties of alpha1-antitrypsin Portland towards furin convertase. *FEBS Lett*, *426*: 41-46, 1998.

197. Jachimczak, P., Hessdorfer, B., Fabel-Schulte, K., Wismeth, C., Brysch, W., Schlingensiepen, K. H., Bauer, A., Blesch, A., and Bogdahn, U. Transforming growth factor-beta-mediated autocrine growth regulation of gliomas as detected with phosphorothioate antisense oligonucleotides. *Int J Cancer*, *65*: 332-337, 1996.
198. Munz, C., Naumann, U., Grimm, C., Rammensee, H. G., and Weller, M. TGF-beta-independent induction of immunogenicity by decorin gene transfer in human malignant glioma cells. *Eur J Immunol*, *29*: 1032-1040., 1999.
199. Vey, M., Schafer, W., Berghofer, S., Klenk, H. D., and Garten, W. Maturation of the trans-Golgi network protease furin: compartmentalization of propeptide removal, substrate cleavage, and COOH-terminal truncation. *J Cell Biol*, *127*: 1829-1842, 1994.
200. Roebroek, A. J., Umans, L., Pauli, I. G., Robertson, E. J., van Leuven, F., Van de Ven, W. J., and Constam, D. B. Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development*, *125*: 4863-4876, 1998.
201. Surawicz, T. S., Davis, F., Freels, S., Laws, E. R., Jr., and Menck, H. R. Brain tumor survival: results from the National Cancer Data Base. *J Neurooncol*, *40*: 151-160., 1998.
202. Saas, P., Walker, P. R., Hahne, M., Quiquerez, A. L., Schnuriger, V., Perrin, G., French, L., Van Meir, E. G., de Tribolet, N., Tschopp, J., and Dietrich, P. Y. Fas ligand expression by astrocytoma in vivo: maintaining immune privilege in the brain? *J Clin Invest*, *99*: 1173-1178., 1997.
203. Miller, D. W. Immunobiology of the blood-brain barrier. *J Neurovirol*, *5*: 570-578., 1999.
204. Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L., and Spies, T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science*, *285*: 727-729., 1999.
205. 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*: 119-126., 2000.
206. 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*: 11521-11526., 2001.
207. 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*: 165-171., 2001.

208. Girardi, M., Oppenheim, D. E., Steele, C. R., Lewis, J. M., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R. E., and Hayday, A. C. Regulation of cutaneous malignancy by gammadelta T cells. *Science*, *294*: 605-609., 2001.
209. Bahram, S., Bresnahan, M., Geraghty, D. E., and Spies, T. A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A*, *91*: 6259-6263., 1994.
210. Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., and Spies, T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A*, *93*: 12445-12450., 1996.
211. Li, P., Morris, D. L., Willcox, B. E., Steinle, A., Spies, T., and Strong, R. K. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. *Nat Immunol*, *2*: 443-451., 2001.
212. Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K. H., and Spies, T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A*, *96*: 6879-6884., 1999.
213. Cosman, D., Mullberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N. J. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity*, *14*: 123-133., 2001.
214. Steinle, A., Li, P., Morris, D. L., Groh, V., Lanier, L. L., Strong, R. K., and Spies, T. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics*, *53*: 279-287., 2001.
215. Sutherland, C. L., Chalupny, N. J., Schooley, K., VandenBos, T., Kubin, M., and Cosman, D. UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J Immunol*, *168*: 671-679., 2002.
216. Nomura, M., Zou, Z., Joh, T., Takihara, Y., Matsuda, Y., and Shimada, K. Genomic structures and characterization of Rael family members encoding GPI-anchored cell surface proteins and expressed predominantly in embryonic mouse brain. *J Biochem (Tokyo)*, *120*: 987-995., 1996.
217. Malarkannan, S., Shih, P. P., Eden, P. A., Horng, T., Zuberi, A. R., Christianson, G., Roopenian, D., and Shastri, N. The molecular and functional characterization of a dominant minor H antigen, H60. *J Immunol*, *161*: 3501-3509., 1998.

218. 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*: 721-727., 2000.
219. Brooks, A. G., Posch, P. E., Scorzelli, C. J., Borrego, F., and Coligan, J. E. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J Exp Med*, *185*: 795-800., 1997.
220. Carena, I., Shamshev, A., Donda, A., Colonna, M., and Libero, G. D. Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-gamma/delta stimulated by nonpeptidic ligands. *J Exp Med*, *186*: 1769-1774., 1997.
221. Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M., and Lopez-Botet, M. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med*, *186*: 1809-1818., 1997.
222. Braud, V. M., Allan, D. S., O'Callaghan, C. A., Soderstrom, K., D'Andrea, A., Ogg, G. S., Lazetic, S., Young, N. T., Bell, J. I., Phillips, J. H., Lanier, L. L., and McMichael, A. J. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*, *391*: 795-799., 1998.
223. Ljunggren, H. G. and Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*, *11*: 237-244., 1990.
224. Garrido, F., Ruiz-Cabello, F., Cabrera, T., Perez-Villar, J. J., Lopez-Botet, M., Duggan-Keen, M., and Stern, P. L. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today*, *18*: 89-95., 1997.
225. Pende, D., Cantoni, C., Rivera, P., Vitale, M., Castriconi, R., Marcenaro, S., Nanni, M., Biassoni, R., Bottino, C., Moretta, A., and Moretta, L. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *Eur J Immunol*, *31*: 1076-1086., 2001.
226. Joly, E., Mucke, L., and Oldstone, M. B. Viral persistence in neurons explained by lack of major histocompatibility class I expression. *Science*, *253*: 1283-1285., 1991.
227. Neumann, H., Cavalie, A., Jenne, D. E., and Wekerle, H. Induction of MHC class I genes in neurons. *Science*, *269*: 549-552., 1995.
228. Rieger, J., Wick, W., and Weller, M. Human malignant glioma cells express semaphorins and their receptors, neuropilins and plexins. *Glia*, *42*: 379-389, 2003.

229. Steinle, A., Groh, V., and Spies, T. Diversification, expression, and gamma delta T cell recognition of evolutionarily distant members of the MIC family of major histocompatibility complex class I-related molecules. *Proc Natl Acad Sci U S A*, *95*: 12510-12515., 1998.
230. Welte, S. A., Sinzger, C., Lutz, S. Z., Singh-Jasuja, H., Sampaio, K. L., Eknigk, U., Rammensee, H. G., and Steinle, A. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. *Eur J Immunol*, *33*: 194-203, 2003.
231. Dokun, A. O., Chu, D. T., Yang, L., Bendelac, A. S., and Yokoyama, W. M. Analysis of in situ NK cell responses during viral infection. *J Immunol*, *167*: 5286-5293, 2001.
232. Robertson, M. J., Cochran, K. J., Cameron, C., Le, J. M., Tantravahi, R., and Ritz, J. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. *Exp Hematol*, *24*: 406-415., 1996.
233. Saito, T., Tanaka, R., Yoshida, S., Washiyama, K., and Kumanishi, T. Immunohistochemical analysis of tumor-infiltrating lymphocytes and major histocompatibility antigens in human gliomas and metastatic brain tumors. *Surg Neurol*, *29*: 435-442., 1988.
234. Klein, B., Loven, D., Lurie, H., Rakowsky, E., Nyska, A., Levin, I., and Klein, T. The effect of irradiation on expression of HLA class I antigens in human brain tumors in culture. *J Neurosurg*, *80*: 1074-1077., 1994.
235. Hicklin, D. J., Marincola, F. M., and Ferrone, S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol Med Today*, *5*: 178-186., 1999.
236. Knopf, P. M., Harling-Berg, C. J., Cserr, H. F., Basu, D., Sirulnick, E. J., Nolan, S. C., Park, J. T., Keir, G., Thompson, E. J., and Hickey, W. F. Antigen-dependent intrathecal antibody synthesis in the normal rat brain: tissue entry and local retention of antigen-specific B cells. *J Immunol*, *161*: 692-701., 1998.
237. Perry, V. H. A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. *J Neuroimmunol*, *90*: 113-121., 1998.
238. During, M. J., Symes, C. W., Lawlor, P. A., Lin, J., Dunning, J., Fitzsimons, H. L., Poulsen, D., Leone, P., Xu, R., Dicker, B. L., Lipski, J., and Young, D. An oral vaccine against NMDAR1 with efficacy in experimental stroke and epilepsy. *Science*, *287*: 1453-1460., 2000.

239. Thomas, C. E., Schiedner, G., Kochanek, S., Castro, M. G., and Lowenstein, P. R. Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: toward realistic long-term neurological gene therapy for chronic diseases. *Proc Natl Acad Sci U S A*, *97*: 7482-7487., 2000.
240. Flugel, A., Berkowicz, T., Ritter, T., Labeur, M., Jenne, D. E., Li, Z., Ellwart, J. W., Willem, M., Lassmann, H., and Wekerle, H. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity*, *14*: 547-560., 2001.
241. Groh, V., Rhinehart, R., Randolph-Habecker, J., Topp, M. S., Riddell, S. R., and Spies, T. Costimulation of CD8alpha T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol*, *2*: 255-260., 2001.
242. Hoebe, K., Janssen, E., and Beutler, B. The interface between innate and adaptive immunity. *Nat Immunol*, *5*: 971-974, 2004.
243. Hanna, J., Gonen-Gross, T., Fitchett, J., Rowe, T., Daniels, M., Arnon, T. I., Gazit, R., Joseph, A., Schjetne, K. W., Steinle, A., Porgador, A., Mevorach, D., Goldman-Wohl, D., Yagel, S., LaBarre, M. J., Buckner, J. H., and Mandelboim, O. Novel APC-like properties of human NK cells directly regulate T cell activation. *J Clin Invest*, *114*: 1612-1623, 2004.
244. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. *Cell*, *100*: 57-70, 2000.
245. Rosen, D., Li, J.-H., Keidar, S., Markon, I., Orda, R., and Berke, G. Tumor Immunity in Perforin-Deficient Mice: A Role for CD95 (Fas/APO-1). *J Immunol*, *164*: 3229-3235, 2000.
246. Screpanti, V., Wallin, R. P. A., Ljunggren, H.-G., and Grandien, A. A Central Role for Death Receptor-Mediated Apoptosis in the Rejection of Tumors by NK Cells. *J Immunol*, *167*: 2068-2073, 2001.
247. Hayakawa, Y., Kelly, J. M., Westwood, J. A., Darcy, P. K., Diefenbach, A., Raulet, D., and Smyth, M. J. Cutting edge: tumor rejection mediated by NKG2D receptor-ligand interaction is dependent upon perforin. *J Immunol*, *169*: 5377-5381, 2002.
248. Weller M, W. C., Will C, Wagenknecht B, Dichgans J, Lang F, Gulbins E. CD95-dependent T cell killing by glioma cells expressing CD95 ligand: more on tumor immune escape, the CD95 counterattack, and the immune privilege of the brain. *Cell Physiol Biochem*, *7*: 282-288, 1997.

249. Fournel, S., Aguerre-Girr, M., Huc, X., Lenfant, F., Alam, A., Toubert, A., Bensussan, A., and Le Bouteiller, P. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8⁺ cells by interacting with CD8. *J Immunol*, *164*: 6100-6104, 2000.
250. Jekle, A., Obst, R., Lang, F., Rammensee, H.-G., and Gulbins, E. CD95/CD95 Ligand-Mediated Counterattack Does Not Block T Cell Cytotoxicity. *Biochemical and Biophysical Research Communications*, *272*: 395-399, 2000.
251. Igney, F. H. and Krammer, P. H. Immune escape of tumors: apoptosis resistance and tumor counterattack. *J Leukoc Biol*, *71*: 907-920, 2002.
252. den Brok, M. H. M. G. M., Suttmuller, R. P. M., van der Voort, R., Bennink, E. J., Figdor, C. G., Ruers, T. J. M., and Adema, G. J. In Situ Tumor Ablation Creates an Antigen Source for the Generation of Antitumor Immunity. *Cancer Res*, *64*: 4024-4029, 2004.
253. Goldszmid, R. S., Idoyaga, J., Bravo, A. I., Steinman, R., Mordoh, J., and Wainstok, R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4⁺ and CD8⁺ T cell immunity against B16 melanoma. *J Immunol*, *171*: 5940-5947, 2003.
254. Russo, V., Tanzarella, S., Dalerba, P., Rigatti, D., Rovere, P., Villa, A., Bordignon, C., and Traversari, C. Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc Natl Acad Sci U S A*, *97*: 2185-2190, 2000.
255. Scheffer, S. R., Nave, H., Korangy, F., Schlote, K., Pabst, R., Jaffee, E. M., Manns, M. P., and Greten, T. F. Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response in vivo. *Int J Cancer*, *103*: 205-211, 2003.
256. Nowak, A. K., Lake, R. A., Marzo, A. L., Scott, B., Heath, W. R., Collins, E. J., Frelinger, J. A., and Robinson, B. W. S. Induction of Tumor Cell Apoptosis In Vivo Increases Tumor Antigen Cross-Presentation, Cross-Priming Rather than Cross-Tolerizing Host Tumor-Specific CD8 T Cells. *J Immunol*, *170*: 4905-4913, 2003.

List of publications

Aulwurm S, Wischhusen J, Friese M, Borst J, Weller M. CD70/CD27 interactions and the immune response to malignant glioma: a double-edged sword. manuscript submitted

Aulwurm S, Scheel B, Teufel R, Carralot JP, Probst J, Rammensee HG, Weller M, Pascolo S. Protamine-stabilized RNA induces regression of malignant gliomas *in vivo*. manuscript submitted

Uhl, M., **Aulwurm, S.**, Wischhusen, J., Weiler, M., Ma, J. Y., Almirez, R., Mangadu, R., Liu, Y. W., Platten, M., Herrlinger, U., Murphy, A., Wong, D. H., Wick, W., Higgins, L. S., and Weller, M. SD-208, a Novel Transforming Growth Factor β Receptor I Kinase Inhibitor, Inhibits Growth and Invasiveness and Enhances Immunogenicity of Murine and Human Glioma Cells In vitro and In vivo. *Cancer Res*, 64: 7954-7961, 2004.

Herrlinger U, **Aulwurm S**, Strik H, Weit S, Naumann U, Weller M. MIP-1 α antagonizes the effect of a GM-CSF-enhanced subcutaneous vaccine in a mouse glioma model. *J Neurooncol*. 2004 Jan;66(1-2):147-54

Friese MA, Platten M, Lutz SZ, Naumann U, **Aulwurm S**, Bischof F, Bühring HJ, Dichgans J, Rammensee HG, Steinle A, Weller M. MICA/NKG2D-mediated immunogene therapy of experimental gliomas. *Cancer Res*. 2003 Dec 15;63(24):8996-9006

Platten M, Kretz A, Naumann U, **Aulwurm S**, Egashira K, Isenmann S, Weller M. Monocyte chemoattractant protein-1 increases microglial infiltration and aggressiveness of gliomas. *Ann Neurol*. 2003 Sep;54(3):388-92.

Weigert C, Brodbeck K, Brosius FC 3rd, Huber M, Lehmann R, Friess U, Facchin S, **Aulwurm S**, Häring HU, Schleicher ED, Heilig CW. Evidence for a novel TGF- β 1-independent mechanism of fibronectin production in mesangial cells overexpressing glucose transporters. *Diabetes*. 2003 Feb;52(2):527-35.

Leitlein J, **Aulwurm S**, Waltereit R, Naumann U, Wagenknecht B, Garten W, Weller M, Platten M. Processing of immunosuppressive pro-TGF- β 1,2 by human glioblastoma cells

involves cytoplasmic and secreted furin-like proteases. J Immunol. 2001 Jun 15;166(12):7238-43

Platten M, Wick W, Wild-Bode C, **Aulwurm S**, Dichgans J, Weller M. Transforming growth factors beta(1) (TGF-beta(1)) and TGF-beta(2) promote glioma cell migration *via* Up-regulation of alpha(V)beta(3) integrin expression. Biochem Biophys Res Commun. 2000 Feb 16;268(2):607-11.

Bublitz R, Armesto J, Hoffmann-Blume E, Schulze M, Rhode H, Horn A, **Aulwurm S**, Hannappel E, Fischer W. Heterogeneity of glycosylphosphatidylinositol-anchored alkaline phosphatase of calf intestine. Eur J Biochem. 1993 Oct 1;217(1):199-207.

Acknowledgements

With a deep sense of gratitude, I wish to express my sincere thanks to Prof. Dr. Michael Weller. The confidence and dynamism with which he guided this work requires no elaboration, demanding and inspiring at the same time, always motivating when science had its disheartening days. Your expert group leadership allowed to work in a remarkable team!

I am also indebted to Prof. Hans-Georg Rammensee who has supported the present work during many stages with helpful suggestions and discussions and who has volunteered to review this thesis. The collaboration with you and your group has been a real pleasure, especially with my fellow Ph.D. student Dipl. Biol. Birgit Scheel and your group around Dr. Steve Pascolo at Curevac: "The RNA people". Special mention also must be made to Dr. Cécile Gouttefangeas, Prof. Dr. Stefan Stevanovic and Prof. Dr. Hans-Jörg Schild, Beate Pömmerl and Dipl. Biol. Jochen Probst.

I also want to say thanks to our lab leader Dr. Ulrike Naumann who always had a good advice on molecular biology topics, voodoo incantations actually helped mostly. Close collaborators in our group were also Dr. Jörg Wischhusen and Dr. Manuel Friese. Thanks for a productive and pleasant working atmosphere characterized by a constant interchange of good ideas also go to PD Dr. Wolfgang Wick, Dr. Oliver Bähr, Dr. Marion Bohatschek, Dr. Günter Eisele, Dr. Mirjam Hermisson, Dr. Johannes Rieger, Dr. Patrick Roth, Dr. Friederike Schmidt, Dr. Joachim Steinbach, Dr. Ghazaleh Tabatabai, Dr. Robert Waltereit, Dr. Markus Weiler, Brigitte Frank, Andrea Klumpp, Simone Weit, Ulrike Obermüller and Gabriele von Kürthy and all other former and present members of our group I have had the pleasure to work with. Sorry for not mentioning all of you by name. PD. Dr. Ulrich Herrlinger and Dr. Michael Platten got me across my first steps in immunology in our lab, very special thanks for that. Further I have had the pleasure of guiding a number of M.D. and Ph.D. students, as well as first- and second-year residents in our lab. Special thanks goes to Dr. Jens Leitlein, Dipl. Biochem. Isabel Burghardt and Dr. Martin Uhl whom I taught his first immunological steps in our laboratory, he quickly found out how to run.

Further partners in the Dept. of Neurology were Dr. Heinz Wiendl and his group as well as the group of Prof. Dr. Schulz (around Dr. Ellen Gerhardt). I also want to thank all collaborators who are mentioned in the respective chapters of this thesis and apologize for everybody who would have deserved being mentioned and got forgotten (shame on me!).

Without the help of some really close friends you can manage tons of work but what for? I want to thank Cora Weigert, Hans-Jürgen Rumpf, Jörg Moelleken and Isabella Gekel for having shown me what for, your light illuminates my way.

I also want to thank my parents, who taught me the value of hard work by their own example. I would like to share this moment of happiness with my parents and brother and his family. They inspired me during the whole tenure of my research.

Academic teachers

at the University of Tübingen:

Prof. Dr. Hermann Bauer, Prof. Dr. Hans Bisswanger, Prof. Dr. Peter Bohley, Prof. Dr. Johannes Dichgans, Prof. Dr. Michael Duszenko, Prof. Dr. Karl Eisele, Prof. Dr. Kai-Uwe Fröhlich, Prof. Dr. Günter Gauglitz, Prof. Dr. Hanspaul Hagenmaier, Prof. Dr. Bernd Hamprecht, Prof. Dr. Dr. h.c. Michael Hanack, Prof. Dr. Gundram Jung, Prof. Dr. Dieter Mecke, Prof. Dr. Helga Ninnemann (†), Prof. Dr. Peter Overath, Prof. Dr. Wolfgang Pfeiffer (†), Prof. Dr. Hansgeorg Probst, Prof. Dr. Hans-Georg Rammensee, Prof. Dr. Klaus Reutter, Prof. Dr. Hans-Jörg Schild, Prof. Dr. Stefan Stevanovič, Prof. Dr. Dr. h.c. Wolfgang Voelter, Prof. Dr. Dr. h.c. Klaus Wegmann, PD Dr. Cora Weigert, Prof. Dr. Michael Weller, Prof. Dr. Ulrich Weser

external:

Prof. Dr. Hermann Bauer, Nürnberg, Prof. Dr. Ewald Hannappel, Erlangen, Prof. Dr. Werner Fischer (†), Erlangen