Immune Surveillance of Cancer-

Molecular and Translational Aspects of Cytotoxic Effector : Target Cell Interactions

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

an der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Nikola Baschuk

aus Bochum

Köln 2009

Berichterstatter: Prof. Dr. Martin Krönke

Prof. Dr. Jonathan Howard

Tag der Disputation: 29.Juni 2009

Table of Contents

3. Results I

Results II

Results III

Abbreviations

mAb	monoclonal antibody
CPM	counts per minute
CTL	cytotoxic T lymphocytes
DC	dendritic cell
E:T ratio	effector : target ratio
EB	embryoid body
ELISA	enzyme linked immunosorbent assay
ES cell	embryonic stem cell
Fig.	figure
FCS	fetal calf serum
gzm	granzyme
HLA	human leukocyte antigen
ICM	inner cell mass
IFN-γ	Interferon-gamma
IL-4	interleukin-4
IL-10	interleukin-10
ko	knock out
IU	infectious unit
LCM virus	lymphocytic choriomeningitis virus
LIF	leukaemia inhibitory factor
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger RNA
NK cells	natural killer cells
Oct	octamer-binding transcription factor
PBS	phosphate buffer saline
PEC	peritoneal exudate cells
PFU	plaque forming unit
RT-PCR	reverse transcriptase PCR
Serpin	serine protease inhibitor

shRNA	small hair pin RNA
siRNA	small interfering RNA
SPI	serine protease inhibitor
SSEA	stage specific embryonic antigens
T-Ag	SV40 large tumor antigen
TAL	tumor associated lymphocytes
TIL	tumor infiltrating lymphocytes.
TCR	T cell receptor
TU	transducing unit
wt	wild type

1.Introduction

1.1 Principles of antigen specific immunotherapy of tumors

A thorough understanding of the immuno-regulatory pathways supporting or suppressing the generation of $CD8^+$ CTL is of utmost relevance for the implementation of immunotherapeutic approaches against tumors for several reasons: (i) $CD8^+$ T cells are presumably the most important effector cells for immunotherapeutic approaches against cancer; (ii) endogenous as well as vaccine-driven immune responses of $CD8^+$ T cells against tumor-specific antigens are often rather weak, such that they can be measured only after several rounds of restimulation *in vitro* (Muul et al. 1987), (iii) tumor patients often suffer from immunosuppressive effects directly and indirectly caused by the tumor. If it were possible to counteract adverse immunoregulatory effects and to promote endogenous immunostimulatory mechanisms in tumor patients, the use of tumor-specific $CD8^+$ CTL could be established as a potent additional modality in tumor therapy.

Massive clonal expansion and acquisition of effector functions of $CD8^+$ T cells requires induction by fully matured DC which are able to provide to naïve $CD8^+$ T cells beyond the inductive signals 1 and 2 an additional signal 3 (reviewed in Mescher et al.). So far, as signal 3 the cytokines IL-12 and type I interferons (IFN) have been identified. DC can be induced to secrete these cytokines in either an antigen-specific or a non-specific fashion. Antigen-specific "licensing" of DC to produce IL-12 can be achieved by ligation of CD40 on DC by CD40L (CD154) on $CD4^+$ T helper cells. As a consequence of this insight, a recent improvement in tumor-specific vaccination was to mix MHC class II-restricted peptides with MHC class I-restricted peptides so that specific $CD4^+$ T helper cells are induced in parallel to $CD8^+$ T cells. The strength and quality of the $CD8^+$ T cell response was further improved by linking MHC class I- and II-restricted epitopes so that processing and presentation of both epitopes by the same antigen-presenting cell (APC) was warranted.

Despite these improvements, even T cell responses against definitely foreign, virus-derived tumor-associated antigens were mostly too weak or short-lived to achieve control of tumor cells. One approach to strengthen the antigen-specific $CD8^+$ CTL is to optimize the quality of the $CD4^+$ T helper response, e.g. by enhancing their Th1

and suppressing their Th2 activities (Pardoll et al., 2002; Rosenberg et al., 2008; Figdor et al., 2004).

But even antigen-specific, fully competent effector CD8$^+$ CTL induced by fully inflammation-matured DC did not necessarily achieve effective control of tumor cells, because these CTL accumulated largely in the lymph nodes draining the vaccination site instead of patrolling through the systemic circulation (van der Burg et al., 2006; Welters et al., 2007). Finally, even if antigen-specific effector CD8$^+$ CTL might infiltrate the tumor tissue, they can be blocked or inactivated by tumor cells via various mechanisms (Blattman and Greenberg, 2004; Melief, 2008). One of the most recently discovered is the expression of specific inhibitors of cytotoxic effector molecules (Abdullah et al., 2007; Bots et al., 2005).

1.2. Expression of specific inhibitors of cytotoxic effector molecules in tumor cells

Cells of various murine tumor types express the serine protease inhibitor 6 (SPI-6), the specific inhibitor of granzyme B (grzB). Serpin PI-9, the human homolog of SPI-6, was previously shown to be expressed by dendritic cells, which prevents premature destruction of these professional antigen-presenting cells (APC), and by cells at immune-privileged sites, where degranulation of CTL is potentially deleterious (Bladergroen et al., 2001; Medema et al., 2001). In addition, murine SPI-6 was reported to play a crucial role in the protection of CTL against the suicidal effects of their own grzB (Zhang et al., 2006). Expression of the human serpin PI-9 and its murine homologue SPI-6 has been detected in various types of melanoma, carcinomas, and lymphomas (David-Watine et al., 1987; Medema et al., 2001; Bots etal., 2005) and was realized to be linked with the escape of tumor cells from immunosurveillance.

Altogether, the experiences acquired in tumor immunology within the previous two decades indicate that an improved understanding of the function and regulation of T cells within the microenvironment during induction, homing and execution of effector functions is a prerequisite for establishing protocols for reliable and sustained control of tumors by the immune system.

1.3. The experimental system to study immuno-regulatory mechanisms

To understand immunoregulatory mechanisms in weak tumor-specific immune responses, we study the simian virus 40 (SV40) large tumor antigen (TAg)-specific rejection of syngeneic TAg-expressing mKSA tumor cells in BALB/c mice (J. Zerrahn et al., 1996; Utermöhlen et al., 2001) The mKSA cells form in syngeneic mice fibrosarcomas after s.c. injection and produce severe peritoneal carcinosis after i.p. inoculation. We chose this murine tumor model because it exhibits features resembling the clinical situation with regard to the extremely weak reactivity of the immune system towards tumor-specific antigens. With respect to the generation of TAg-specific CD8$^+$ CTL, BALB/c mice were characterized in the literature as low- or even non-responders (Bright et al., 1996; Gooding et al.,1977; Knowles et al., 1997; Newmaster et al., 1998, Schirmbeck et al., 1996; Tevethia et al., 1990, Pfizenmaier et al., 1980) This is nicely documented by the fact that so far only a single subdominant H-2K^d-restricted epitope of TAg was identified after several rounds of restimulation *in vitro* of T cells from SV40-immune BALB/c mice (Newmaster et al., 1998), while in C57BL/6 mice, known to mount a strong CTL response against TAg, five immunodominant MHC class-I-restricted epitopes have been identified (Tevethia el., 1990).

However, our group detected a small but highly cytotoxic population of TAg-specific CD8$^+$ CTL among the peritoneal exudate cells of TAg-immune BALB/c mice challenged i.p. with TAg-expressing mKSA tumor cells (Baschuk et al., 2007). These TAg-specific CTL depend on the support of CD4$^+$ T helper cells over the whole course of tumor cell rejection (Utermöhlen et al., 2001). In TAg-immunized BALB/c mice challenged with mKSA cells, CD4$^+$ tumor-associated lymphocytes (TAL) secrete both typical Th1 cytokines as IFN-γ and IL-2 and typical Th2 cytokines, e.g. IL-4 and IL-10, over the whole course of tumor rejection.

1.4. Effects of IL-4 on CD8$^+$ CTL

Interleukin-4 (IL-4) is a prototypic type 2 T helper (Th2) cytokine. Its major effects are to promote differentiation of Th2 cells, thereby supporting humoral immune responses. Moreover, IL-4 is a strong antagonist of Th1 cells and thus of cellular

immune responses (Seder et al.,1994). Despite these clearly defined functions of IL-4, the effects of this cytokine upon antigen specific CTL are still ambiguous, because IL-4 has been reported to either promote or inhibit the generation of antigen-specific CD8^{+} cytotoxic T cells in different systems (Utermöhlen et al., 2001; J. Zerrahn et al., 1996, Miller et al., 1990; Erard et al., 1993; M. Croft et al., 1994; Noble et al., 1995; Moran et al., 1996; Aleman et al., 2002; Bright et al 1996; Gooding et al., 1977; Knowles et al., 1979; N. Kienzle et al 2002, 2005; Schuler et al., 2001, 1999). The most prominent example of unpredicted effects of IL-4 on CTL was reported by Jackson et al. who generated an ectromelia virus encoding murine IL-4 (Jackson et al., 2001). This IL-4-expressing virus caused acute mousepox accompanied by high mortality even in mouse strains which are resistant to wild type ectromelia virus. The massive increase in pathogenicity was found to be due to suppression of the CTL response by IL-4. Less dramatic suppressive effects of IL-4 upon CTL function during infection were observed in other systems when mice were treated either with exogenous IL-4 or with recombinant vaccinia virus expressing IL-4 (Aung et al., 2000). Mice treated with mAb neutralizing IL-4 during immunization mounted an enhanced CTL response following challenge with RSV (Tang et al., 1994). During Sendai virus infection IL-4 deficient (IL-4$^{-/-}$) mice showed a potent response of cytotoxic T lymphocyte (CTL) recovered by bronchoalveolar lavage, while in the same animals the prevalence of virus-specific CTL precursors was consistently diminished in the spleen and in regional lymph nodes (Mo et al., 1997). On the other hand, no alterations in T cell activation or virus clearance could be detected in IL-4$^{-/-}$ mice during infection with vaccinia virus or Lymphocytic Choriomeningitis (LCM) virus (Bachmann et al., 1995). Additionally puzzling was the observation that IL-4 can act as a growth factor for CD8^{+} T cells *in vitro* (Miller et al., 1990; Noble et al., 1990)].

1.5. Effects of IL-10 on CD8^{+} CTL

IL-10 is of outstanding interest with regard to the modulation of CD8^{+} CTL, because this cytokine acts in many different systems as an inhibitor of cellular immune responses, but it has also been reported to be a maturation factor for CTL in other systems (Chen et al., 1991; Wang et al., 1994; Goillot et al., 1994; Giovarelli et al.,1995; Sharma et al., 1999; Santin et al., 2000; Fujii et al., 2001).

Interleukin 10 (IL-10) acts strongly anti-inflammatory on cells of both the innate and the acquired immune system. It is secreted not only by various cell types of the innate and adaptive immune system but also by keratinocytes and intestinal epithelial cells (for review (Moore et al., 2001)). This puts IL-10 as a major player into the highly complex regulatory networks of immune responses. The most important function of IL-10 seems to be the inhibition of the production of pro-inflammatory cytokines by macrophages and dendritic cells. Thereby IL-10 acts at the interface between innate and adaptive immune responses as a regulator suppressing or down-modulating immune responses to microorganisms. This is most prominently proven by the phenotype of IL-10 deficient mice: IL-10$^{-/-}$ mice develop severe immunopathology in response to colonization of their intestines by the physiologic gut microflora. Conventionally housed IL-10$^{-/-}$ mice develop chronic enterocolitis within the entire intestinal tract, with the duodenum, proximal jejunum, and proximal colon being most severely affected by mucosal inflammation with extensive lymphoplasmocytic and histiocytic infiltration of the lamina propria (Kuhn et al., 1993). IL-10$^{-/-}$ mice kept under specific pathogen free (SPF) conditions lack inflammatory alterations in the small intestine and show lesions mainly in the proximal colon which are less severe than in conventionally housed mice (Kuhn et al., 1993). These data suggest that the inflammatory disease is triggered by enteric microorganisms. Importantly, IL-10$^{-/-}$ mice do not show spontaneous lymphoproliferative or inflammatory activity without contact to microorganisms or their products, as observed for example in FoxP3-deficient mice lacking regulatory T cells (Treg) (Fontenot et al., 2003). Thus, IL-10 is a main factor that fine tunes the delicate balance between sufficiently effective immune responses against microorganisms and immunopathologic damage of host tissues.

On T lymphocytes, IL-10 can act in at least two fashions: In an indirect mode, IL-10 inhibits in immature DC the expression of costimulatory and MHC class II molecules. The resulting lack of inflammatory DC may inhibit Th1 inflammatory responses or even end in the induction of anergy in T cells. In a direct mode, IL-10 can inhibit production of cytokines and chemokines as well as chemotaxis of T lymphocytes. However, even some stimulatory effects of IL-10 on T cells have been described (Groux et al., 1998; Santin et al., 2000), so that the net effect of IL-10 or of IL-10 inhibition cannot easily be predicted in a given immunologic situation.

1.6. The experimental system to study the effects of inhibitors of cytotoxic effector molecules

Target cell resistance against CTL or NK cell mediated cytotoxicity of embryonic stem ES cell-derived teratomas is of particular interest for several reasons: For example, ES cell derived teratomas can be viewed as a model for the cancer stem cell hypothesis which became accepted for a growing list of tumors over the last few years (Andrews, et al. 2005). Thus, a stem cell-dependent tumor-model appears more physiologic than a model relying on a virus-transformed tumor cell line. Teratomas are tumors that comprise disorganized tissue derived from all three embryonic germ layers. Undifferentiated ES cells were reported to form teratomas not only in immuno-defficient mice, but also in immunocompetent syngeneic and in immunocompetent allogeneic recipients (Koch, et al. 2008).

While transplants of undifferentiated ES cells will probably not be used for therapeutic purposes, a few contaminating undifferentiated ES cells in transplants of more differentiated ES cell-derivatives cannot be neglected, because as few as two ES cells (Lawrenz et al., 2004) can give rise to teratomas. Cancer stem cells are defined by the capacity for self-renewal (Thomson et al., 1998; Evans et al., 1981) and the potential to differentiate into any cell type of the specific tumor-type. Cancer stem cells are supposed to persist in tumors and survive conventional tumor therapy, giving rise to relapsing tumors. Therefore, development of specific therapies targeted at cancer stem cells may eradicate the truly tumorigenic cell type, preventing relapse. We also resorted to this system, because our group already detected the expression of cathepsin B, the specific inhibitor of perforin, and SPI-6, the specific inhibitor of granzym B, in ES cells, and characterized the effects of these inhibitors in the interaction of ES cells and CTL in vitro (Abdullah et al., 2007). In the present study the interaction of ES cells with NK cells, i.e. the innate cytotoxic effector cells type, in vitro and in vivo, was analysed. A better understanding of NK cell interactions with tumors, recently attracted fast growing interest for several reasons. The most obvious explanations are: (i) Use of NK cells against tumor cells does not require identification of tumor-specific antigens, or antigen-specific immunization. (ii) Being triggered by recognition of cells with low surface expression of MHC class I molecules, NK cells might become the therapeutic approach to eliminate tumor cells that lost MHC class I due to selective pressure of antigen-specific CTL therapy.

ES cells are known to express none or low levels of MHC class I molecules on their surface (Drukker et al., 2002, 2006; Bonde et al., 2006; Abdullah et al., 2007). According to the "missing self hypothesis" of NK cell activation (Ljunggren et al., 1990) ES cells should be ideal targets for NK cells. However, human (Drukker et al., 2002, 2006) or murine ES cells (Bonde et al., 2006) were reported to be poorly lysed by NK cells in vitro. Our group recently reported that ES cells, despite being fully recognized, are not susceptible to cytolysis by CD8^{+} CTL. Having revealed that ES cells express high levels of cathepsin B and SPI-6, the specific endogenous inhibitors of the cytotoxic effector molecules perforin and granzyme B, respectively, protecting them against lysis by CD8^{+} CTL.

This project aimed at revealing the involvement of SPI-6 and cathepsin B in the low susceptibility of ES cells to NK cells.

1.7. Objective

The aim of this project was to elucidate different approaches to enhance the effectiveness of tumor-specific immune responses and to further characterize molecular and translational aspects of cytotoxic effector : target cell interactions concerning the immune surveillance of cancer.

2. Materials and Methods I

2.1.Material

2.1.1. Cell lines

CGR8 ES cells were obtained from the European collection of cell culture (ECACC), ECACC No: 95011018.

HEK293T cells, L929 cells NCTC clone L13, C57SV and BALB/cSV fetal fibroblasts, SV40-transformed mKSA tumor cells and methylcholanthrene-induced MethA tumor cells are from the Institute for Medical Microbiology, Immunology and Hygiene, Medical Centre, University of Cologne.

2.1.2. Mice

Six to eight weeks old C57BL/6 and BALB/c mice were obtained form Charles River (Sulzfeld, Germany). 129P2/OlaHsd and Beige Mice were obtained form Harlan Laboratories (the Netherlands). Breeding pairs of IL-10 deficient (IL-10$^{-/-}$) mice of the BALB/c strain were kindly provided by Werner Müller, Institute for Genetics, Cologne, Germany, and Donna Rennick, DNAX, Palo Alto, California (Kuhn et al., 1993). The mice were heterozygously mated so that wt littermates were used as controls. Breeding pairs of perforin-deficient mice on the C57BL/6 background were a kind gift of Hans Hengartner, Institute of Experimental Immunology, University Hospital Zürich, Switzerland (Kagi et al., 1994). Mice with a T cell-specific inactivation of the IL-10 gene (IL-10$^{FL/FL}$CD4-Cre$^+$) were described previously (Roers et al., 2004). Perforin-deficient and IL-10$^{FL/FL}$CD4-Cre$^+$ mice were backcrossed ten times to the BALB/c background in the animal facilities of the Center for Molecular Medicine Cologne. All mice were bred and kept strictly under barrier conditions and were used when 8 to 12 weeks old. Mice were housed under specific pathogens free conditions at the animal facility of IMMIH and fed with standard pellet food. Animal experiments were approved by the ethics committee of the Bezirksregierung Köln and were performed in accordance with the German animal protection law.

2.1.3. Plastic ware

Cell culture dishes	Nunc
Pipettes	CELLSTAR
Pipette tips	CELLSTAR

Centrifugation tubes   Falcon and Eppendorf

Glass materials   Schott

2.1.4. Technical equipment

Appliance	Name	Supplier
Centrifuge	5417 R	Eppendorf
Centrifuge	Megafuge 1.oR	Heraeus
ELISA reader	MRX TC	Dynex
Fluorescence microplate reader	victor 1420	Wallc
Flowcytometer	FACSCalibur	BD
Gama counter	COBRA II	Canberra-P
Gel chamber	Agagel Midi	Biometra
Gel documentation apparatus	Chemidoc	BioRad
Incubator	Heracell	Heraeus
Microscopes	TC SL	Leica
Photometer	Smart	BioRad
Power supply	EPS-3501 XL	Pharmacia
Shaker	Bühler Schüttler	Johanna Otto
Sterile hood	Herasafe (vertical)	Heraeus
Thermocycler	T3-Thermocycler	Biometra
Ultracentrifuge	Discovery 90SE	Sorvall

2.1.5. Chemicals and reagents

   All chemicals were off research grade. All Buffers and solutions were prepared by using deionised or bidistilled water from an EASYpure UV/UV water purification unit (Werner Reinstwassersysteme. Leverkusen), sterilized by autoclaving or sterile filtered with a 0,2 μm filter if necessary and stored at room temperature.

Acetic acid   Sigma

Agar   Neuform

Agarose   BMA

CA-074   Bachem

CA-074Me   Bachem

Crystal violet	Merck
CTL	BioRad
Dithiothreitol (DTT)	Sigma
DMEM (Dulbecco MEM)	Invitrogen
DMSO (dimethylsulfoxide)	Sigma
DNA ladder 1kb plus	Fermentas
DNA loading buffer (6x)	Fermentas
dNTPs	Peqlab
EDTA	Sigma
EGTA	Merck
Ethanol	Merck
Ethidiumbromide	Sigma
Fetal calf serum (FCS)	Invitrogen
G418	PAA Lab
Gelatine type A	Sigma
Glycerin	Merck
Glycerol	Sigma
Hanks' Balanced Salt Solution	Sigma
HEPES	Serva
Hoechst 33258	Sigma
Isopropanol	Sigma
KCl	Sigma
L-glutamine	Invitrogen
LIF	ESGRO; Chemicon
Methyl cellulose	Merck
$MgCl_2$	Invitrogen
Moninsin	Sigma
mrIFN-γ	R&D
Na_2HCO_3	Sigma
NaCl	Merck
$NaHCO_3$	Sigma
Non-essential amino acids	Invitrogen
Paraformaldehyde	Sigma
PBS (for cell culture)	Invitrogen

Penicillin/Streptomycin	Invitrogen
Polybren	Sigma
Reverse Transcriptase	Roche
RNAse inhibitor	Roche
RT-buffer	Roche
SDS	Sigma
Sodium pyruvate	Invitrogen
Taq DNA polymerase	Invitrogen
TEMED	Merck
Triton-X-100	Sigma
Trypsin-EDTA (10x)	Invitrogen
Trypton	Neuform
Tween	Merck
Yeast extract	Neuform
β-Mercaptoethanol	Invitrogen, 0,02 in water, stored at 4°C

2.1.6. Cell culture medium, reagents and buffers

All buffers and reagents were made using deionised double distilled water from the EASY-pure UV/UF water purification unit.

2.1.6.1. Cell culture media

Cultivation medium for ES cells Glasgow	MEM 90% (v/v)
	FCS 10% (v/v)
	L-glutamine 2 mM
	ß-mercaptoethanol 50µM
	LIF 100U/ml
Cultivation medium for EB (EB medium)	Iscove's MEM+GlutaMAX 80% (v/v)
	FCS 20% (v/v)
	β-mercaptoethanol 100 µM
	non-essential amino acids

11

Cultivation medium for C57SV,   DMEM 95% (v/v)

BALB/cSV fibroblasts, MethA tumor cells   FCS 5% (v/v)

mKSA tumor cells   Penicillin 50mg/ml

                                                                  Streptomycin 50mg/ml

Freezing medium for cells   Glasgow MEM 70% (v/v)

                                                              DMSO 10% (v/v)

                                                                FCS 20% (v/v)

2.1.6.2. Cell culture reagents

Gelatine type A for coating cell culture plastics   Gelatine type A 0.1% in $_{dd}H_2O$

                                                                Incubation: 30 min, 37°C

Trypsin 1x   10ml of Trypsin 10x made up to

                                                                100ml using $_{dd}H_2O$

2.1.6.3. Bacteria culture media and reagents

LB (Luria-Bertani) medium   10g/l trypton, 5g/l yeast extract and 5g/l

                                                     NaCl

LB agar: 10g/l aga, 10g/l trypton, 5g/l yeast extract

                                                    and 5g/l NaCl

S.O.C medium   (Invitrogen)

2.1.7. Reagents for molecular biology

    Tris-EDTA (pH 8.0)   Tris 10 mM, EDTA 1 mM in $_{dd}H_2O$

    TAE (pH 7.8)   Tris-HCl 40 mM, Acetic acid 5 mM, EDTA 1 mM,

                                   in $_{dd}H_20$

2.1.8. Antibodies

Antibody	Application	Provider
Ms. Anti SSEA-1 (clone MC-480)	FACS 1:1000	R&D
PE-anti-H-2Kb clone AF6-88.5	FACS	BD
mAb GB12 against human granzyme B	FACS	Caltag
CD3, CD8a, CD8b, TCRα/β, TCRγ/δ, CD44, CD62L, CD69, CD103	FACS	BD

2.1.9. Primers for real time PCR

GranzymeM

Grz.M Fr.   5' TTG AGA CCC AGA TCA TTG 3'

Grz.M Rev.   5' ATA CCC ACT TCC GAT GCA CA 3'

Grz.M P   (6-FAM)CAC TCC CGC CCA TAC AT(TAMRA-6-FAM)   (Operon)

Serpin 6   gene expression assay (Appliedbiosystems)

Serpin9b   gene expression assay (Appliedbiosystems)

18s rRNA   gene expression assay (Appliedbiosystems)

β-actin   gene expression assay (Appliedbiosystems)

GAPDH   gene expression assay (Appliedbiosystems)

2.1.10. Plasmids

Entry vector pENTER/siH1

Size: 4636 bp

Resistance gene: Kanamycin.

Destination vector pLPac/EGFP

Size: 9564bp

Resistance gene: Blasticidin

(all plasmids were kindly provided by B. Yazdanpanah from IMMIH)

pLPac/EGFP/sh-SPI-6   (kindly provided by Zeinab Abdullah)

Packaging vectors

pLP1, pLP2 and the envelope plasmid pLP/VSVG (Invitrogen)

2.1.11. Kits

NK1.1. cell Isolation kit   Miltany Biotec

ELISA mouse IFNγ OptEIA kit   Becton Dickinson

RNeasy Mini kit   Qiagen

Quantitect reverse transkription kit   Qiagen

RiboGreen RNA quantification kit   Molecular Probes

QIAprep spin Minipräp kit   Qiagen

Plasmid Maxi präp kit   Qiagen

PKH67 green Fluorecent Cell Linker kit   Sigma

for general cell membrane labeling

2.2. Methods

2.2.1. Cell Culture

Cell culture was performed under sterile conditions in a sterile hood with sterile media and plastics. Cells were cultivated in an incubator at 37°C, 5% CO_2 and humidified air.

CGR8 cells were maintained in tissue culture flasks coated with 0,1% gelantine in ES cell medium. Medium was changed daily and ES cells were passaged every 3-4 days.

Balb/cSV fibroblasts, C57BL/6 fibroblast , YAC cells, mKSA tumor cells and MethA tumor cells were maintained in tissue culture flasks in 5% DMEM.

2.2.2. In *vitro* differentiation of ES cells into embryoid bodies

For the in *vitro* differentiation of CGR8 ES cells into embryoid bodies, a single cell suspension of ES cells was adjusted to a final concentration of $2,5x10^4$ cells/ml in EB medium.

20µl drops containing 500 cells were placed on the cover of petri dishes. After two days the ES cells had formed cell aggregates that were transferred into bacterial petri dishes to avoid adherence.

2.2.3. Cloning efficiency assay

The elimination of mKSA tumor cells from the peritoneal cavity was assessed by a cloning efficiency assay. Briefly, peritoneal exudate cells were collected at the indicated days after challenge, adjusted to 10^6 Trypan blue excluding cells per ml and serially ten-fold diluted. Aliquots of each dilution were plated in 6-well plates and cultivated in complete medium for 8 days. Afterwards, the cells were fixed and stained with Crystal violett. Clonally expanded mKSA colonies were counted to calculate the number of proliferating mKSA cells that were contained within 10^6 peritoneal exudate cells at the day of the peritoneal lavage.

2.2.4. Production and purification of recombinant T-Ag

T-Ag was produced by infecting Sf 158 cells with recombinant baculovirus coding for full length SV40-T-Ag (Lanford et al., 1988), kindly provided by Dr. Ellen Fanning. T-Ag was purified from cell lysates by immunoaffinity chromatography using PAb 108 coupled to CNBr-Sepharose. The purified protein was dialyzed against PBS/40% glycerine and stored at -70°C.

2.2.5.Treatment of ES cells

2.2.5.1. Induction of MHC class I molecules on the surface of cells

To induce expression of MHC class I on the cell surface, cells were incubated with 20ng/ml IFNγ for 48 hours before the experiment. The expression of MHC class I molecules on the cell surface was measured by flow cytometry.

2.2.5.2. Blocking of Cathepsin B

ES cells were pre-treated with 10μM CA074 16 h prior to experiment.

2.2.6. PCR

2.2.6.1. RNA isolation

Total RNA was extracted from approximately $2x10^6$ ES cells, day 5 EB cells, Balb/cSV fibroblasts, resting NK and activated NK cells using the RNeasy mini elute kit (Qiagen) according to manufacturers instructions (spinning protocol).
For the isolation of RNA from Placenta the RNeasy midi kit was used (Qiagen).

2.2.6.2. RNA quantification

Isolated RNA was quantified using the RiboGreen RNA quantification kit (Molecular Probes) in a 96-well microplate. 5µl RNA and 95µl TE buffer were distributed per well. RiboGreen reagent was diluted 1:200 and 100µl was added per well. Plates were incubated 2 min at room temperature and then excited at 458 nm. The fluorescence emission intensity was measured at 525 nm using the fluorescence microplate reader (Wallac victor multilabel counter 1420). RNA concentrations were measured by comparing with a standard curve.

2.2.6.3. cDNA synthesis

To circumscribe RNA into cDNA the reverse ranscription kit Quantitect (Qiagen) was used according to the manufactures instructions.

2.2.6.4. Real time PCR (TaqMan)

Quantitative real-time PCR was performed with the TaqMan ABI-Prism 5700 Sequence Detection System (Applied Biosystems). Following the instructions of Applied Biosystems the optimal concentration for each primer pair and probe were determined. In each PCR-reaction negative and positive control templates were included. Reactions were carried out in 25µl mixture using the TaqMan Universal Master Mix (Applied Biosystems) according to the manufacturers instructions.

Components of the real time PCR reaction mixture were as follows:

  2,5µl cDNA (final concentration: 5 ng/ml)
  12,5µl PCR Master Mix
  250µM Forward Primer
  250µM ReversePrimer
  300µM Fluorogenic Probe
  8,5µl water
  Total volume: 25µl

Individual steps:

50°C   120sec   UDG PCR decontamination

95°C 120sec Taq start activation

40 Cycles :

95°C 15sec Denaturation
60°C 60sec Annealing and Extension

mRNA was calculated for each gene by using a standart curve. The value obtained for each gene was normalized to that of the housekeeping gene encoding GapDH and β-actin. Then the fold change was calculated using crossing threshold (Ct) values.

2.2.7.Treatment of mice

2.2.7.1. Immunization of mice with recombinant T-Ag and challenge with mKSA cells

Ten μg of purified T-Ag in PBS were injected twice i.p. in a one-week interval (days -14 and -7). Seven days after the second immunization (day 0), the animals were challenged by i.p. injection of 10^6 viable mKSA cells, which equals the 10,000-fold LD_{50} of these tumor cells (Baschuk et al., 2007).

2.2.7.2. Depleting mice of CD4$^+$ or CD8$^+$ T lymphocytes

Groups of mice were i.v. treated with either a mixture of each 250µg rat mAb YTS191.1 and YTA3.1(Qin et al., 1987), or 500 µg rat mAb YTS169.4 (Cobbold et al., 1984) to deplete CD4$^+$ or CD8$^+$ T lymphocytes, respectively. As a control purified rat IgG (Sigma, Deisenhofen, Germany) was administered in some experiments. The time of the Ab injection relative to tumor challenge is given in the results section. mAb were produced, purified, used, and efficacy-controlled as described (Gegin et al., 1992).

2.2.7.3. Activation of NK-cells

To otain activated NK cells adult mice (6-8 weeks of age) were infected with the LCMV, strain WE. Plaque forming units (PFU) were converted into infectious units (IU) by multiplying PFU with a factor of 10. Mice were infected by intravenous (i.v.)

inoculation of $1x10^5$ IU of the LCMV in 0,3 ml PBS with 5% penicillin/streptomycin (PS) and 1% fetal calf serum (FCS) into the tail vain.

Or Mice were intraperitonelly (I.p.) injected with 250 μg polyI:C in 500μl PBS. 48 hours later mice were sacrificed and activated NK cells were isolated.

2.2.8. Isolation of effector cells

2.2.8.1. Preparation of resting and activated NK cells from spleens

Mice were sacrificed and their spleens were removed. Single cell suspension was optained by passing spleens through a 70μm pore size cell mesh in cold RPMI. Cell suspensions were centrifuged at 1000 rpm for 5 min at 4°C. Cell pellet was resuspended in 10 ml cold 0,2% NaCl to lyse erythocytes and after 20 seconds the lysis was stopped with 1,6% NaCl. Cells were pelleted again by centrifugation and resuspended in RPMI. Cell number was determined using trypan blue exclusion method in a Neubauer chamber. NK cells were enriched from the splenic cell suspension using anti NK monoclonal antibodies conjugated to magnetic beats (Miltany Biotec) following manufactures instructions. Following the incubation time cells were washed 3 times and loaded on large MACS columns under a magnetic field. The columns were rinsed several times, removed from the magnetic field and NK cells were eluated in sorting buffer. Cells were pelleted in RPMI and cell number was determined using trypan blue exclusion method in a Neubauer chamber.

2.2.8.2. Isolation of Tumor Associated Lymphocytes (TAL)

T-Ag-immunized or naive mice were inoculated i.p. with 10^6 mKSA tumor cells. Peritoneal exudate cells (PEC) were recovered by rinsing the peritoneal cavities with cold PBS.

2.2.8.3. Isolation of mouse small intestinal intraepithelial lymphocytes (IEL)

IEL were prepared according to Montufar-Solis et al. with minor modifications. In brief, after removal of small intestines, Peyers patches were dissected. Fecal material was flushed off with RPMI 1640 supplemented with FCS (10% v/v), 100 U/ml penicillin-streptomycin and $5x10^{-5}$ M 2-ME. Subsequently, intestines were opened longitudinally and cut into pieces of 0.5cm length. The tissue fragments were rinsed several times in Ca^{2+}/Mg^{2+}-free PBS and stirred at 37°C for 20 min in Ca^{2+}/Mg^{2+}-free PBS containing 5mM EDTA and 2mM DTT. The resulting cell-suspensions were

vortexed for 15 seconds, and supernatant was collected. The last step was repeated and supernatants were combined before centrifugation at 1000 rpm, 4°C for 5min. Cells were filtered through a 10 ml syringe barrel containing wetted nylon wool and centrifuged again. The cell pellet was suspended in 40% isotonic Percoll, layered on top of a 70% Percoll cushion, and centrifuged for 20 min at 600xg. IELs were harvested from the Percoll interface and suspended in complete RPMI.

2.2.9. Magnetic cell sorting

NK cells, TAL or IELs were enriched from the splenic cell suspension, peritoneal exudates cells or intestinal single cell suspension using anti NK monoclonal antibodies, CD4 or CD8 mAb conjugated to magnetic beats (Miltany Biotec) following manufactures instructions. Following the incubation time cells were washed 3 times and loaded on large MACS columns under a magnetic field. The columns were rinsed several times, removed from the magnetic field and NK cells were eluated in sorting buffer. Cells were pelleted in RPMI and cell number was determined using trypan blue exclusion method in a Neubauer chamber.

2.2.10. Chromium release cytotoxic assay
2.2.10.1.Effector cells

Effector cells were magnetically enriched as described in 2.2.9. Effector cell number was adjusted to $3x10^6$ cells/ml in RPMI, 200μl were dispensed in U-bottom 96 well plates in four replicants and serial dilutions of 1:2 in RPMI were made three times.

2.2.10.2. Target cells

To obtain the specific cytotoxic activity of effector cells, target cells were adjusted to 10^6/ml and then incubated with 50μCi ^{51}CR for 60minutes at 37°C. Cells were washed three times to remove the extra cellular ^{51}Cr. Cell numbers were adjusted to $3x10^4$/ml and 100μl of the target cell suspensions were dispensed to the previously distributed efector cells in the 96-well plate to obtain Effector : Target cell ratios of 100:1, 50:1, 25:1and 12,5:1. The spontaneous release of each ^{51}Cr-labeled target cell was determined in 8 wells without effector cells. In these samples the spontaneous release was less than 9% of the maximum release. The maximal release was determined by the total target cell lysis in 1,6% Triton-X-100 in water.

After 4 h incubation at 37°C, cells were spun down and 100μl of the cell-free supernatants were harvestet. A probe from each well were measured in a gamma-counter to detect the amount of ^{51}Cr as counts per minute (cpm). Mean of the four replicates were calculated with correction for background lysis, as followes for esch effector to target cell ratio:

$$\% \text{ specific lysis} = (\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spontan}}) / (\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontan}}) \times 100$$

2.2.11. Flow cytometry
2.2.11.1. Surface staining

CD8$^+$ IEL, were phenotypically characterized by surface staining with mAb specific for CD3, CD8a, CD8b, TCRα/β, TCRγ/δ, CD44, CD62L, CD69 or CD103. A total of 1x10^6 cells was washed with PBS, 0,5% BSA and stained for 15 min with 1μg of fluorochrom-labeled monoclonal antibody.

2.2.11.2 Flow cytometry analysis of MHC class I expression

Flow cytometry analysis of MHC class I expression on the surface of cells. Single cell suspensions of ES or EB cells were stained with monoclonal antibody specific for H-2K^b or isotype control antibodies and were analyzed by FACScan and CellQuest software.

2.2.11.3. Flow cytometric analyses, quantification of cytokines and of cytokine-secreting T cells, and histopathology

For detection of intracellular IFN-γ, granzyme B and A CD8$^+$ T-cells were immunomagnetically enriched from PEC and were incubated on a confluent monolayer of mKSA cells in six-well plates (Nunc, Roskilde, Denmark) for 1 h. Thereafter Golgi-Stop reagent was added, and the cultures were incubated for further 5h (only for IFN-γ). The T cells were harvested by gently rinsing the plates with medium, stained with anti-CD8 mAb, fixed, permeabilized and stained for intracellular IFN-γ, granzyme B or granzyme A. The frequency of CD8$^+$ IFN-γ^+ or CD8$^+$ granzyme B$^+$ or A$^+$ T-cells was determined with a FACSCalibur (BD Biosciences, Heidelberg, Germany) flow cytometer. TAg-expressing mKSA tumor cells were visualized in specimens of intraabdominal tissues and organs by immunostaining with polyclonal rabbit antiserum R15 against Tag or CD8-specific mAb.

2.2.12. Quantification of cytokines and cells secreting cytokines

For the quantification of IFN-γ, IL-4 or IL-10 secreted by resting or activated NK cells or TAL, IFN-γ, IL-4 or IL-10 specific ELISA kits (R&D) were used following the manufacturer's instructions. Cocultures were centrifuged for 3 min at 310 *g* at 4°C and cell-free supernatants were collected and stored at -80°C if test was not performed immediately.

The fraction of CD8$^+$ TAL or IEL secreting IFN-γ in response to mKSA tumor cells was determined by an IFN-γ-specific Elispot assay (R&D systems) on day 8 or 2 after challenge of TAg-immune mice with 10^6 mKSA cells. The immunomagnetically enriched CD8$^+$ TAL or IEL were incubated at graded densities with 10^5 mKSA cells and the assays were developed according to the instructions of the manufacturer.

2.2.13. Quantifying enzymatic activity

2.2.13.1. GzmM specific activity

Enzymatic activity of granzyme M in the lysates of 5×10^5 resting NK cells, poly I:C activated NK cells and of CD8$^+$ CTL on day eight after LCMV infection was analysed by cleavage of the chromogenic substrate Boc-Ala-Ala-Met-SBzl. Substrate cleavage was followed by using absorption 405nm.

2.2.13.2. GzmA- and -B-specific enzymatic activity

In lysates of CD8$^+$ TAL the gzmA- or -B-specific activity was determined by using substrate H-D-Pro-Phe-Arg-p-nitroanilide (S2302; manufactured by Chromogenix, Lexington, MA, USA) or N-acetyl-Ile-Glu-Pro-Asp-p-nitroanilide (Biomol, Exeter, UK), respectively, according to instructions of the manufacturers.

2.2.14. Transient down regulation of Serpin-CI in ES cells by RNAi

2.2.14.1. Preparation of siRNAs

Two different siRNAs coding for SPI-CI and negative controls, including Alexa Fluor 488 at the 3' prime end of the sense stand (Qiagen) with the following Target sequences were used:

1.) TAA ATT TAA ATT TAC AAA CAA

      Sense :   r(AAU UUA AAU UUA CAA ACA A)dTdT

      Antisense : r(UUG UUU GUA AAU UUA AAU U)dTdT

2.) TGC AAC TTA TCT TGT AAC AAA

    Sense: r(CAA CUU AUC UUG UAA CAA A)dTdT

    Antisense: r((UUU GUU ACA AGA UAA GUU G) dTdT

Both siRNAs were diluted in siRNA suspension Buffer to a final concentration of $20\mu M$ and stored at -20°C according to the manufacturers instructions.

2.2.14.2. Transfection of ES cells with siRNAs

On the day of transfection 5×10^5 CGR8 cells were plated on a 6cm gelantine coated plate. Immediately $0.5\mu g$ siRNA was diluted in ES cell medium, mixed with Hyperfect (Qiagen) as a transfection reagent and incubated 10 min at room temperature. After the incubation time the transfection mix was distributed drop wise on the cell suspension.

After a 24 h incubation time the medium was changed, and 48 h later ES cells were trypsinized and analysed for the down regulation of SPI-CI by TaqMan-PCR or used as target cells in the chromium release assay.

2.2.15. Stable down regulation of Serpin-CI in CGR8 cells

For stable expression of siRNA in ES cells a Lentiviral vector was constructed.

2.2.15.1. Design of shRNA constructs

The murine SPI-CI specific and the murine specific small hairpin RNA were designed following the pSUPER RNAi system of OligoEngine. Targeted sequences are specific for murine SPI-CI and SPI-6/SPI-CI as no other matche was found using the BLAST database of the national Center for Biotechnology Information (htt://www.ncbi.nlm.nih.gov).As a negative control non specific shRNA (scrSPI-CI) was designed. The oligonucleotides were as follows:

for SPI-CI

M_Serbin9b_si-SP: 5'-GAT CCC CCA ACT TAT CTT GTA ACA AAT TCA AGA GAT TTG TTA CAA GAT AAG TTG TTT TTA-3'

M_Serbin9b_si-ASP: 5'-AGC TTA AAA ACA ACT TAT CTT GTA ACA AAT CTC TTG AAT TTG TTA CAA GAT AAG TTG GGG-3'

Serpin_scr_SP: 5'-GAT CCC CAT ACT AGA ATT CAT CAA CTT TCA AGA GAA GTT GAT GAA TTC TAG TAT TTT TTA-3'

Serpin_scr_ASP: 5'-AGC TTA AAA AAT ACT AGA ATT CAT CAA CTT CTC TTG AAA GTT GAT GAA TTC TAG TAT GGG-3'

For SPI-6/CI

M_Serbin6/9b_si-SP: 5'-GAT CCC CCT TGT GAA GTC CTC CAA ACT TCA AGA GAG TTT GGA GGA CTT CAC AAG TTT TTA-3'

M_Serbin6/9b_si-ASP: 5'-AGC TTA AAA AGA ACA CTT CAG GAG GTT TGT CTC TTG AAC AAA CCT CCT GAA TGT TTC GGG-3'

Each sense an anti-sense primer pair was annealed to generate a double-stranded oligonucleotide by mixing 3 μg of each oligo with 5μl of 10x annealing buffer and PCR water to a final volume of 50μl. The mixture was incubated 4min at 95°C, 10min at 70°C, slowly cooled down to room temperature and then stored at-20°C.

2.2.15.2. Ligation of the complementary DNA into the pENTR/siH1 plasmid

Following linearization of the pENTR/siH1 plasmid by *Bgl*II and *Hind*III restriction enzymes, the linearized plasmid was purified on 1% agarose gel (fragment size 4,400 bp) using gel extraction kit (Qiagen) and concentration was adjusted to 500 ng/μl. For the ligation of the annealed oligos into the linearized plasmid 2 μl of the annealed oligos were mixed with 1 μl T4 DNA ligase (Invitrogen), 1 μl of the ligase buffer (Invitrogen), 1 μl of the linearized plasmid and 5 μl water. Mixture was then incubated overnight at room temperature.

Recombinated vectors were then transformed into chemically competent DH5α by adding 5 μl of the ligation reaction to one aliquot (100 μl) of cells and incubated for 30 min on ice, 95 sec at 42°C and finally for 3 min on ice. Cells were centrifuged for 2 min at 1000 *g* and 500 μl of the supernatant was discarded. The pellet was resuspended in the rest of the supernatant and plated on LB agar containing 50 mg/ml kanamycin and incubated for 24 h at 37°C.

On the next day several colonies were picked and 3ml of LB broth containing 50 mg/ml kanamycin were inoculated for each colony and grown overnight at 37°C with shaking. Plasmids were purified using the mini prep kit (Qiagen), eluted in water and stored at -20°C. Positive clones were checked for the presence of the insert by

restriction digestion with the *Bgl*II and *Hind*III enzymes. Positive clone gives rise to two fragments 227 bp and 4 kb.

Fig.1. pENTR/siH1 vector
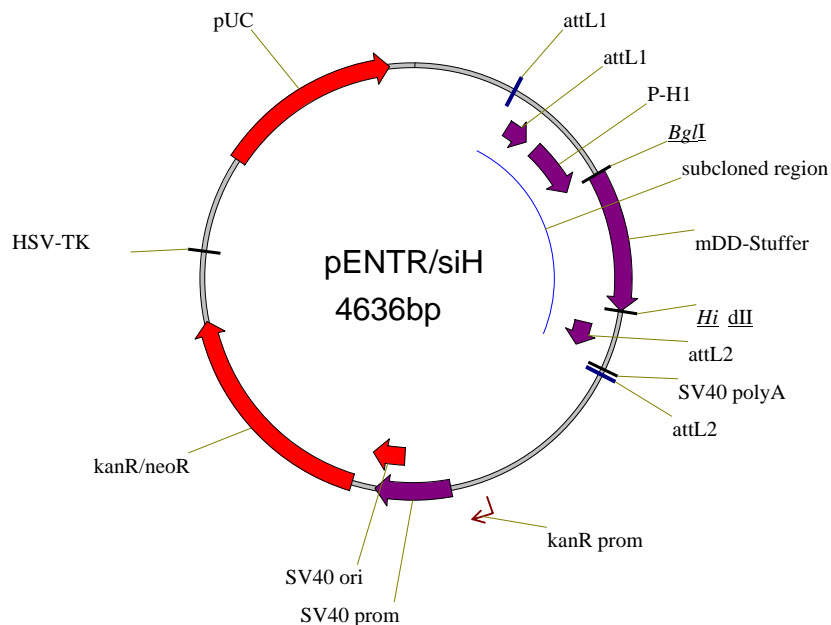
2.2.15.3. Construction of the destination vector

For the construction of the destination vector the Lentiviral vector pLenti6/EGFP was chosen. EGFP was cut out and firefly luciferase was cloned in. The oligonucleotides for firefly luciferase were as follows:

fw primer ffluc: 5 -GAT CGC TAG CGA GAA CGG TAC CAT GGA AGA - 3
rev primer ffluc: 5 -GAT CCC GCG GTA CAA TTT GGA CTT TCC GCC - 3

2.2.15.4. Recombination of the entry vector into the destination vector using the Gateway system

For a stable transcription of the SPI-CI-specific shRNA, the entry plasmids were cloned into the Lentiviral vector pLenti6 /ffluc using the Gateway system (Invitrogen). In 200 µl PCR microtubes, 1.5 µg of entry plasmid, 1.5 µg of destination vector, 1 µl of 5x LR Clonase II enzyme (Invitrogen) and 1 µl TE-buffer pH 8 were incubated overnight at room temperature. On the next day 1 µl of proteinase K was added and tubes were incubated at 37°C for 15 min.

Fig.2. destination vector
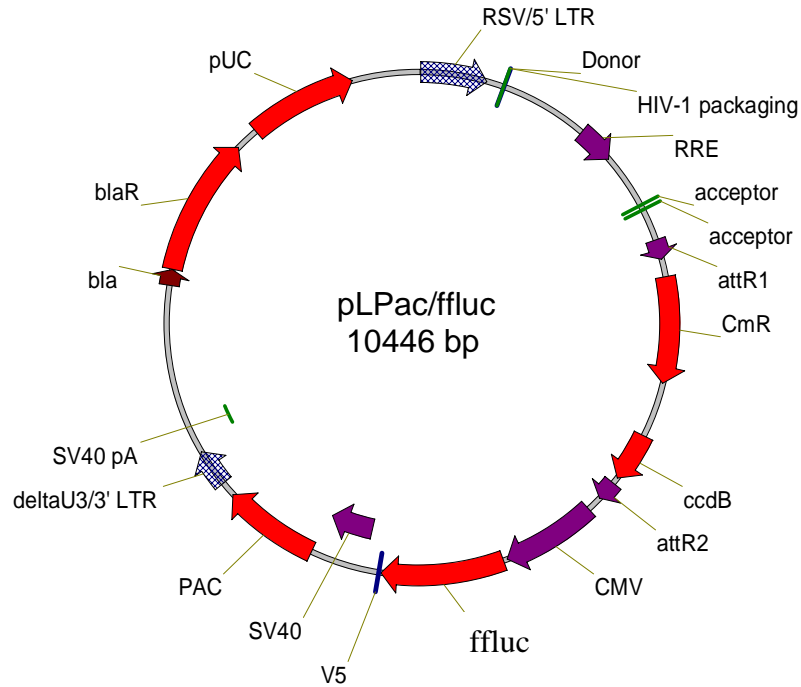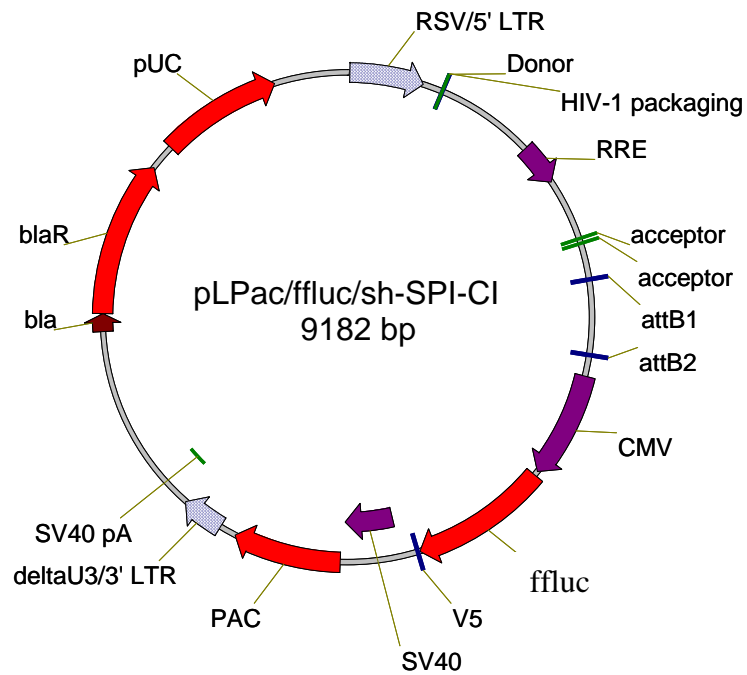[modified by B. Yazdanpanah from the pLenti/delta UBC/V5-DEST vector from Invitrogen]

Fig.3. shRNA expressing Lentiviral vector pLPac/ffluc/sh-SPI-CI

For the production of Lentivirus particles the ViraPower^{TM} Lentiviral expression system (Invitrogen) was used. HEK293T cells were detached and single cell suspension was adjusted to approximately $6x10^5$ cell/ml in DMEM (without G418). In a 10 cm petri dish, 10 ml of the cell suspension was applied and directly co transfected with 3 µg pLPac/ffluc/sh-SPI-CI or pLPac/ffluc/scr-shSPI-CI, 1 µg of each of the packaging plasmids pLP1, pLP2 and the envelope plasmid pLP/VSVG (Invitrogen).

Supernatants containing the virus were collected after 48 h and 72 h, pooled, centrifuged for 15 min at 600 g at 4°C and filtered using 0.45 µm filters to remove any cells or cell debris. To enrich for virus, supernatants were centrifuged for 2 h at 60000 g at 4°C in an ultracentrifuge (Beckman). Supernatants were discarded and pellets were resuspended in 1 ml PBS for each 20 ml start volume. Aliquots of 100 µl were then stored at -80°C.

2.2.15.5. Transduction of CGR8 ES cells

The day prior to transduction, 5×10^5 CGR8 ES cells were plated in 10 cm petri dishes coated with gelatine. The next day, Lentivirus stocks were thawed on ice and virus titers were adjusted to $1x10^5$ TU/ml in ES cell medium. 5 ml of the virus solution were added to each plate together with 10 µg/ml of polybren (Sigma). Cells were plated and incubated at 37°C for 48 h, later cells were detached and a single cell suspension of $2.5 x10^3$ cell/ml was made in ES cell medium containing 4,5 µg/ml blasticidin. In 96-well plates coated with gelatine 100 µl of the selection medium was dispensed per well. 100 µl of the neat cell suspension was dispensed in the first well 1:2 serial dilutions were made by dispensing 100 µl in each step. Single-cell colonies were isolated and plated in 6 cm plates and selected further for blasticidin resistance. After 14 days, SPI-CI expression was assessed in different clones using real time PCR.

2.2.16. *In vivo* bioluminescence imaging

mKSA cells stably expressing luciferase were generated by transduction with a recombinant lentivirus coding for luciferase of *Renilla renifares.* This virus was constructed from a PCR derived fragment encoding the luciferase (Promega GmbH, Mannheim, Germany) cloned into an entry vector (Invitrogen) and recombined with the gateway destination vector p-lenti Dest6/V5 (Invitrogen) according to the manufacturer's instructions. This construct and packaging plasmids were used to co-

transfect the HEK 293 FT producer cell-line. Supernatant containing lentiviral particles was harvested and used to tranduce mKSA tumor cells from which positive clones were selected with 10μg/ml Blasticidin (Invitrogen).

Dissemination of Renilla luciferase-expressing mKSA cells within the peritoneal cavity was monitored non-invasively by bioluminescence imaging with the in vivo imaging system IVIS (Xenogen). Mice were i.v injected with coelenterazine (Promega) at a dosage of 1,5 μg/g bodyweight, anesthetized with isoflurane and imaged for bioluminiscence for 1min exposure time. Optical images were analyzed by IVIS living image software package.

2.2.17. Cytotoxic *in vivo* assay

Wild type ES cells, shSPI-6 ES cells, shSPI-CI ES cells, shSPI-6/SPI-CI cells, scrambled control ES or YAC cells were labelled with PKH67, according to the manufacturers instruction, and adjusted to $2x10^7$ cells per ml. Activated or resting C57BL/6 mice or perforin$^{-/-}$ or beige mice were injected with $1x10^7$ cells, of one cell type in 0,5 ml per mouse, by intravenous inoculation. 30 minutes and 4 hours after inoculation, mice were killed and the total number of living fluorescent cells per spleen was ascertained by FACS-Analysis.

2.2.18. statistical Analysis

For statistical Analysis, the data were subjected to two-tailed Student's *t*-test. The survival data were analysed according to Kaplan-Meier and statistical significance was determined by log-rank tests.

3. RESULTS I

3.1. CD4$^+$ TAL from TAg immune BALB/c mice secrete IL-4 during antigen-specific rejection of mKSA tumor cells

Primary CD4$^+$ T cells, harvested by peritoneal lavage from the tumor site between days 4 to 14 after i.p. challenge of TAg-immune BALB/c mice with 10^6 viable mKSA tumor cells, secrete large amounts of IL-4 (**Fig. 1**). IL-4 secretion correlates with the immune response mounted against mKSA cells, as CD4$^+$ T cells harvested before challenge with mKSA tumor cells (**Fig. 1**, day 0 of tumor rejection) secrete only minute amounts of IL-4. Interestingly, CD8$^+$ T cells did neither spontaneously nor in response to stimulation with mKSA cells or with PMA/ionomycin secrete IL-4 as determined by ELISA (data not shown).
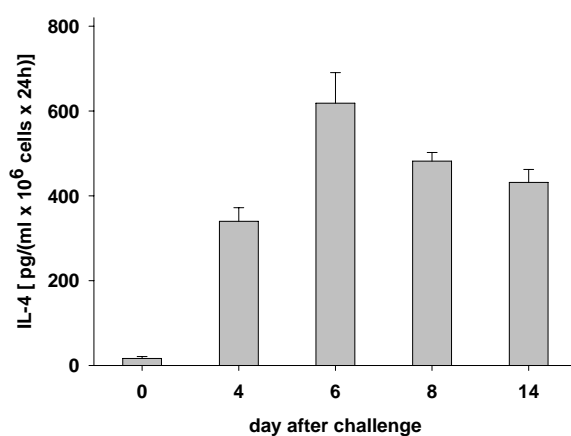
FIG. 1. Primary CD4$^+$ TAL from TAg-immunized BALB/c mice secrete IL-4 after challenge with mKSA.

BALB/c mice were immunized on days -14 and -7 by i.p. injection of 10μg TAg and challenged i.p. on day 0 with 10^6 mKSA cells. CD4$^+$ T cells were immunomagnetically enriched from peritoneal exudate cells collected prior to challenge (day 0; cells pooled from 15 mice) or on days 4, 6, 8, or 14 after challenge (pooled from 5 mice per day). The CD4$^+$ TAL were kept at a densitiy of 1 x10^6 cells/ml in complete RPMI medium without any further stimuli for 24h. The contents of IL-4 in the cell-free supernatants of these cultures were determined by ELISA. Shown are the means and standard error of the mean of three independent experiments.

3.2. IL-4 is not required for the rejection of mKSA tumor cells by TAg-immune BALB/c mice

In order to investigate the biological relevance of IL-4 secreted by CD4$^+$ TAL during TAg-specific rejection of mKSA tumor cells in BALB/c mice, the survival of TAg-immunized IL-4$^{-/-}$ and wild type (wt) BALB/c control mice was monitored. Both TAg-immunized IL-4$^{-/-}$ and wt mice survived an i.p. challenge with 10^6 mKSA tumor cells without any clinical signs of tumor growth for more than 60 days after challenge (**Fig. 2A and B**). Non-immune IL-4$^{-/-}$, as well as wt mice died between days 16 to 20 after challenge. Similarly, the maximal intraperitoneal tumor burden on day 4 after challenge as well as the kinetics of tumor cell elimination were comparable for TAg-immune IL-4$^{-/-}$ and wt mice (**Fig. 2C**). These data clearly indicate that IL-4 is not essential in TAg-immune BALB/c mice for rejection of a dose of mKSA tumor cells equalling the 10,000-fold LD$_{50}$ (data not shown) of these cells in naïve mice, although this cytokine is secreted in large amounts by CD4$^+$ TAL during this immune response.
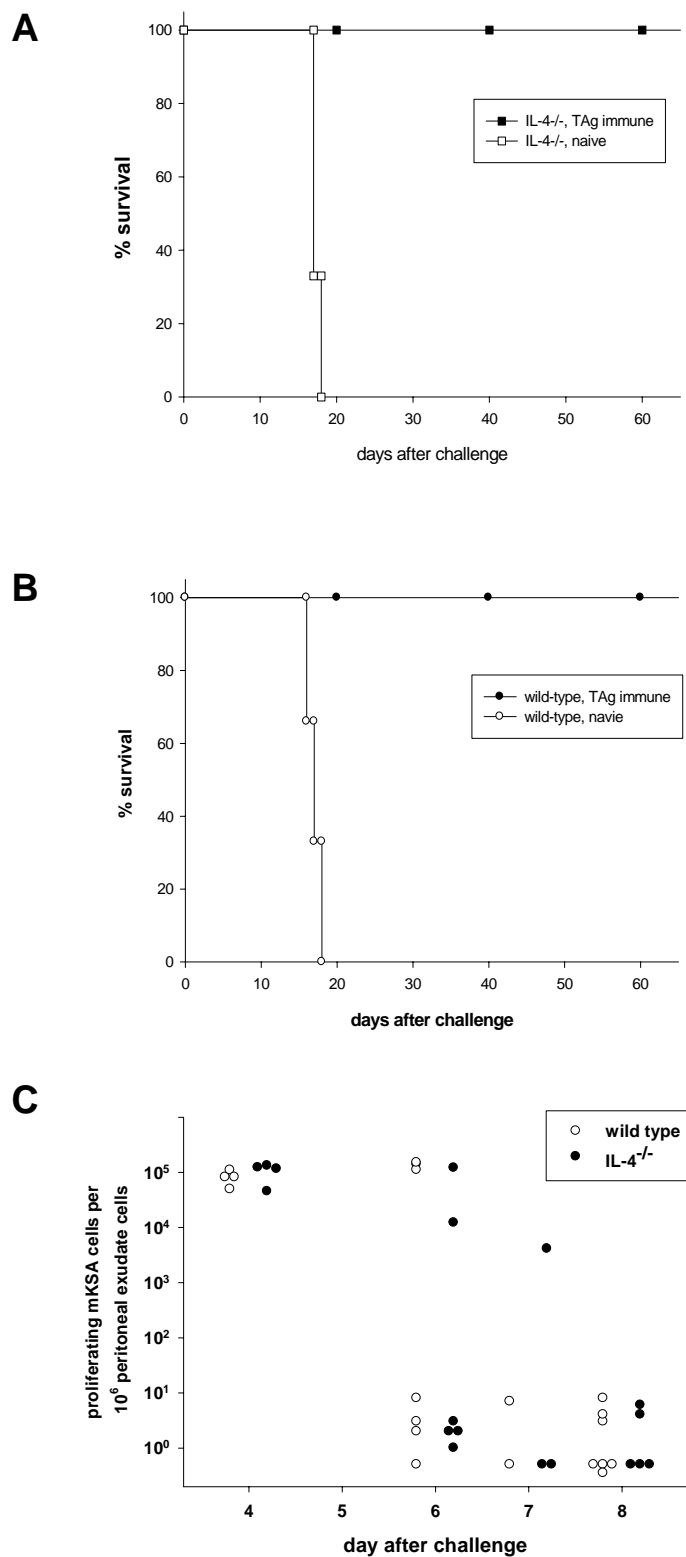
A

B

C

FIG. 2. TAg-immune IL-4$^{-/-}$ BALB/c mice are fully protected against challenge with 10^6 viable mKSA tumor cells. (legend on next page)

FIG. 2. TAg-immune IL-4$^{-/-}$ BALB/c mice are fully protected against challenge with 10^6 viable mKSA tumor cells.

Groups of IL-4$^{-/-}$ and wt BALB/c mice were immunized on days -14 and -7 by i.p. injection of 10µg TAg and challenged i.p. with 10^6 mKSA cells on day 0. Survival of (A) IL-4$^{-/-}$ and (B) wt mice (immune n=5, non-immune n=3) was monitored daily up to 65 days after challenge. (C) On the indicated days after challenge, peritoneal exudate cells harvested by peritoneal lavage were plated in ten-fold serial dilutions on 6-well plates. After cultivation for 8 days the cells were fixed and stained with crystal violett and clonal colonies of mKSA cells were counted. Shown are cumulative data from three independent experiments with each symbol representing an individual mouse.

3.3. Enhanced resistance of naive IL-4$^{-/-}$ mice to challenge with low doses of mKSA cells

To further investigate the biological relevance of IL-4 secreted during rejection of mKSA tumor cells in BALB/c mice, naive mice were challenged with low doses of mKSA cells. In preliminary experiments we established that i.p. inoculation of 100 viable mKSA cells into naive BALB/c mice is lethal for about 50% of the mice between days 30 to 50 after challenge (data not shown). In naïve IL-4$^{-/-}$ mice challenged s.c. with 10^2 mKSA tumor cells a slightly retarded tumor growth as compared to wild-type mice was observed (**Fig. 3A**). Naive IL-4$^{-/-}$ and wild type mice then were challenged i.p. with 10^3 viable mKSA cells. As shown in **Fig. 3B**, IL-4$^{-/-}$ mice challenged with this tumor cell dose had a significant advantage in survival time over wild type mice. Furthermore, depleting naive IL-4$^{-/-}$ mice of CD8$^+$ T cells by injecting a CD8-specific mAb one day prior to challenge with mKSA cells abolished this effect completely as compared to naive IL-4$^{-/-}$ mice treated with control IgG (**Fig. 3C**). This finding clearly indicates that the prolonged survival of naive IL-4$^{-/-}$ mice is dependent on CD8$^+$ T lymphocytes.

FIG. 3. Tumor growth following s.c. inoculation and survival of mice after i.p. injection of mKSA cells into naive IL-4^{-/-} and wild-type mice. (legend on next page)

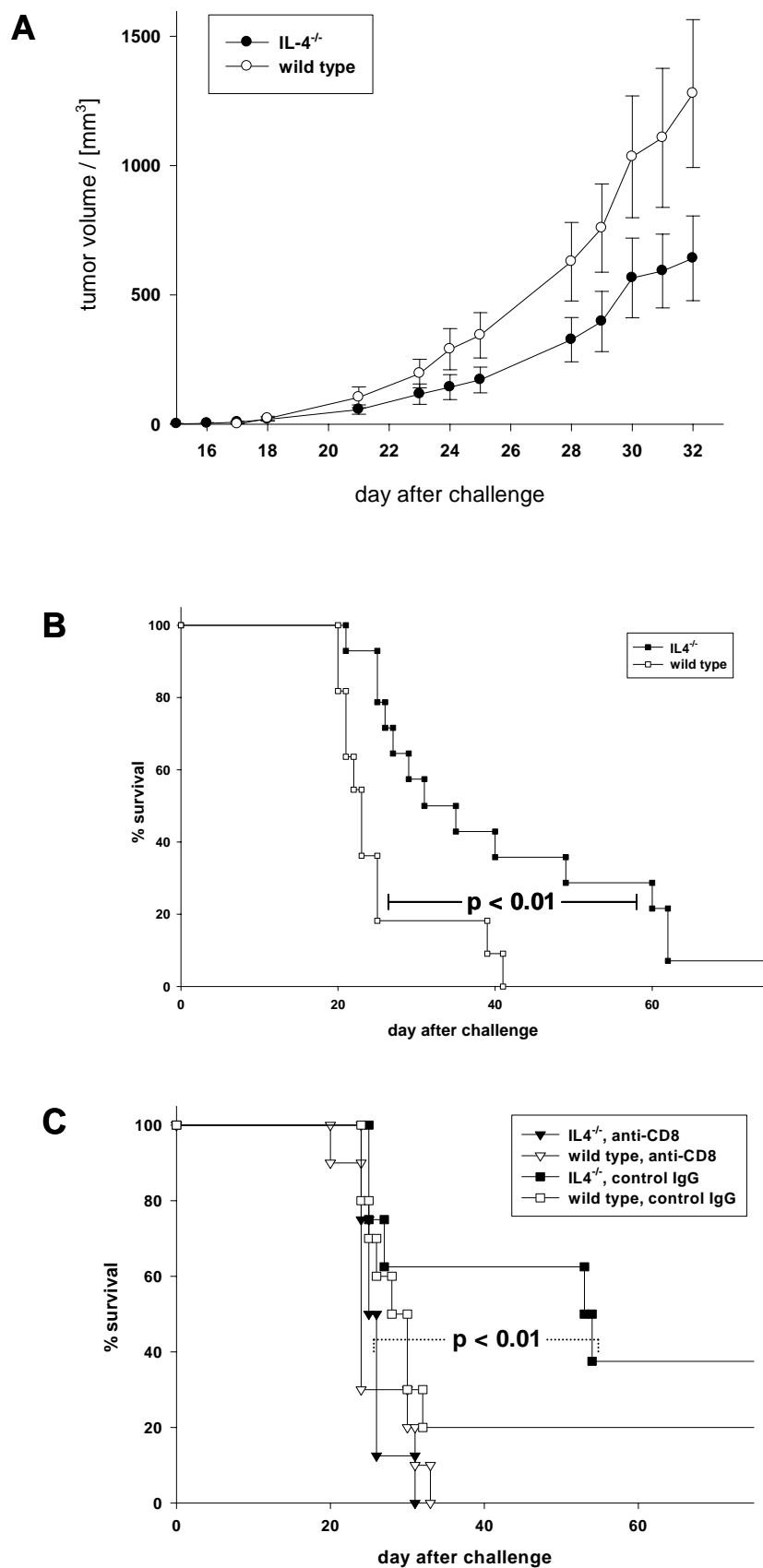**FIG. 3. Tumor growth following s.c. inoculation and survival of mice after i.p. injection of mKSA cells into naive IL-4^{-/-} and wild-type mice.**

   (A) Naïve IL-4^{-/-} (closed circles, n=11) or wild-type mice (open circles, n=12) were inoculated with 10^2 viable mKSA cells s.c. in the back of the neck. At the indicated days after challenge the diameters of the tumors were determined in three dimensions with calipers and subsequently the tumor volume was calculated. (B) Groups of naive IL-4^{-/-} (closed symbols, n=14) or wild-type mice (open symbols, n=11) were i.p. inoculated with 10^3 viable mKSA cells. The mice were monitored for the development of tumors and death daily. According to Kaplan-Meier analysis the mean survival time and the standard error were for the IL-4^{-/-} group 40.14 +/- 4.38 days, and for the wild type 25.45 +/- 2.23 days with a statistical significance of p<0.01 in log-rank test. (C) Groups of naive IL-4^{-/-} (n=8) and wt (n=10) mice were i.v. treated with 500µg of either CD8-specific mAb or of control IgG one day prior to i.p. challenge with 10^3 mKSA cells. Treatment with the antibodies was repeated 8 days after challenge. The mice were monitored for the development of tumors and death daily. According to Kaplan-Meier analysis the mean survival time in days +/- standard error and the statistical significance in the log-rank test were as follows: IL-4^{-/-}: anti-CD8 treated 25.88 +/- 0.79 versus control IgG 51.13 +/- 7.57, p<0.01; wt: anti-CD8 treated 25.80 +/- 1.29 versus control IgG 36.90 +/- 6.08, p>0.05.01 in log-rank test.

3.4. IL-4-deprivation results in enhanced TAg-specific cytotoxic activity, accompanied by an increase in the fraction of tumor associated CD8^{+} T cells expressing granzymes A and B

In search for the mechanism by which endogenous IL-4 impairs resistance of BALB/c mice against mKSA tumor cells, we monitored the TAg-specific cytotoxic activity of CD8^{+} TAL in IL-4^{-/-} and wild-type mice at days 5, 7, and 8 after challenge of TAg-immunized mice with mKSA cells (**Fig. 4**). On day 5 after challenge, CD8^{+} TAL did not exert any TAg-specific cytotoxicity regardless whether these TAL were prepared from IL-4^{-/-} or wild-type mice (**Fig. 4A**). However, starting from day 7 after challenge, CD8^{+} TAL from IL-4^{-/-} mice exhibited a three- to five-fold enhanced TAg-specific cytotoxic activity as compared to TAL from wt mice as judged by horizontal comparison of the effector: target cell ratio required to achieve a given percentage of specific target cell lysis (**Fig. 4B-C**). Additionally, treatment of immunized wt mice at the day of tumor challenge and three days thereafter with neutralizing IL-4-specific monoclonal antibody augmented the TAg-specific activity of CD8^{+} TAL to an extent comparable to that observed in IL-4^{-/-} mice (**Fig. 4C**). Treatment of mice with control IgG did not enhance the TAg-specific cytotoxicity of CD8^{+} TAL (data not shown). The enhanced cytotoxic activity of CD8^{+} T cells from IL-4^{-/-} mice was both TAg-specific and perforin-dependent as shown by use of TAg-negative, syngeneic MethA target cells and of the perforin-inhibitor concanamycin A, respectively (**Fig. 4D**).

Further characterization of the CD8^{+} T cell response revealed comparable total numbers of CD8^{+} TAL recovered from the peritoneal lavage of TAg-immune IL-4^{-/-} or wt mice on day 8 after i.p. challenge with 10^6 mKSA tumor cells (**Fig. 5A**) and comparable expression levels of CD8 on the plasma membrane of these cells (Fig. 5B). Flow cytometry confirmed that CD8^{+} T cells of both genotypes were CD3^{+} CD8^{+} T cells that did not differ with regard to surface expression levels of the activation markers CD44 and CD62L (data not shown).

The fraction of TAg-specific CD8^{+} TAL from IL-4^{-/-} and from wt mice was determined by flow cytometry of intracellular IFN-γ and by IFN-γ-specific ELIspot. The percentage of CD8^{+} TAL responding to co-incubation with TAg-expressing BALB/c-SV40 fibroblasts *in vitro* by producing IFN-γ was comparable, regardless whether determined by flow cytometry or by an ELISpot assay (**Fig. 5C and D**). We also attempted to quantify the number of TAg-specific CD8^{+} TAL by the use of a H-2K[d]-

pentamer with the only H-2^d-restricted TAg-epitope identified so far, the subdominant epitope encompassing aa499-507 of TAg (TAg$_{499-507}$) (Newmaster et al., 1998). However, as might have been expected from the report of Newmaster and colleagues that TAg$_{499-507}$-specific CTL can only be detected after repeated *in vitro* restimulation of lymphocytes from BALB/c mice immunized with SV40 (Newmaster et al., 1998), TAg$_{499-507}$-specific cells were not detectable in significant frequencies among the CD8$^+$ TAL of TAg-immune IL-4$^{-/-}$ or wild-type mice challenged with mKSA cells (data not shown).

Although the percentage of CD8$^+$ T cells expressing the cytotoxic effector molecule granzyme A, as determined by flow cytometry, was significantly enhanced for IL-4$^{-/-}$ CD8$^+$ TAL as compared to wt cells on day 8 after challenge (Fig. 5E),the gzmA-specific enzymatic activity in lysates of these cells was not different between IL-4$^{-/-}$ and wt mice (data not shown). However, both the frequency of CD8$^+$ T cells expressing gzmB as well as the gzmB-specific enzymatic activity were clearly enhanced for IL-4$^{-/-}$ CD8$^+$ T cells (**Fig. 5F**). The data suggest that IL-4 deprivation increases the fraction of mature, i.e. lysis-competent CD8$^+$ T cells expressing cytotoxic effector molecules in the CD8$^+$ TAL population.

In search for the mechanism behind the enhanced tumor resistance of naïve IL-4$^{-/-}$ mice, CD8$^+$ TAL from naïve mice challenged with 10^3 mKSA cells were analyzed on days 8, 14, and 21. The total number of CD8$^+$ TAL was slightly increased in both wt and IL-4$^{-/-}$ mice on day 14 as compared to days 8 and 21 without showing significant differences between the genotypes (**Fig. 6A**). The low numbers of CD8$^+$ TAL recovered on any of these days did not exert significant TAg-specific cytotoxic activity against mKSA cells as determined by chromium release assays (data not shown). However, in IL-4$^{-/-}$ mice the frequency of CD8$^+$ TAL expressing gzmA significantly ($p<0.01$) increased between days 8 and 14 and dropped to background levels by day 21 (**Fig. 5B**). In contrast, the increase in the percentage of CD8$^+$ TAL expressing gzmA was less pronounced ($p>0.05$) in wt mice. The percentages of CD8$^+$ TAL expressing gzmB did not differ between wt and IL-4$^{-/-}$ mice on day 14 after challenge (**Fig. 6C**). Together with the dependence of increased resistance of naïve IL-4$^{-/-}$ mice against mKSA tumor cells on CD8$^+$ T cells (**Fig. 3C**) the strong increase in the frequency of CD8$^+$ T cells expressing gzmA in IL-4$^{-/-}$ mice suggests that after challenge with low doses of mKSA cells naïve IL-4$^{-/-}$ mice generate a CD8$^+$ T cell response of some anti-tumor activity leading to prolonged survival of the mice.

However, the steep decline in the total numbers (**Fig. 6A**) and in the percentage of gzmA-expressing CD8^{+} T cells between days 14 and 21 (**Fig. 6C**) and the delayed death of these mice from about day 20 on, indicate that this endogenous T cell response is too weak to eradicate the fast growing (cell cycle time about 12h) and highly malignant mKSA tumor cells.
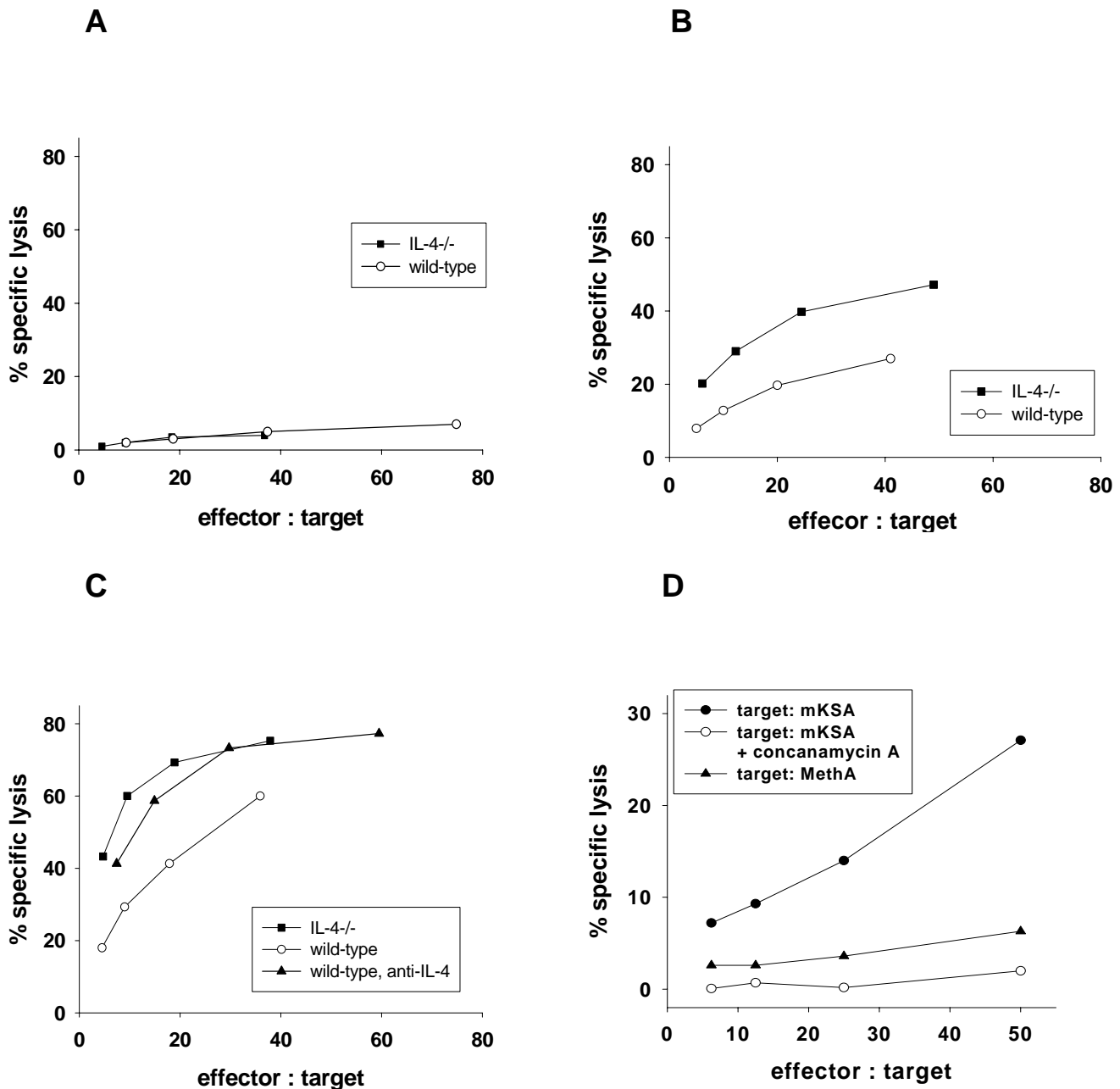
A

B

C

D

FIG. 4. Primary cytotoxicity of CD8^{+} TAL from Tag-immunized IL-4^{-/-} or wild-type mice at different times after challenge.

(legend on next page)

FIG. 4. Primary cytotoxicity of CD8^{+} TAL from Tag-immunized IL-4^{-/-} or wild-type mice at different times after challenge.

IL-4^{-/-} or wild-type mice were immunized on days -14 and –7 by i.p. injection of 10μg TAg and challenged i.p. with 10^6 mKSA cells on day 0. CD8^{+} TAL were immunomagnetically enriched from the pooled peritoneal exudate cells of groups of five mice on day 5 (A), day 7 (B), or day 8 (C) after challenge. On day 8 CD8^{+} TAL from a group of wt mice (n=5) that were i.v. treated with 0.5 mg of IL-4 specific mAb 11B11 on days 0 and 3 after challenge was included . The TAg-specific cytotoxic activity of the CD8^{+} TAL was determined in a standard 4h-chromium release assay using mKSA cells as targets. (D) On day 8 after challenge, CD8^{+} TAL were immunomagnetically enriched from the pooled peritoneal exudate cells of 10 TAg-immune wild-type mice. TAg-expressing mKSA cells and TAg-negative MethA cells, both derived from BALB/c mice and expressing H-2K[d] molecules, were used as target cells to prove antigen-specificity of the CTL. To prove dependence of cytotoxicity on perforin, in an additional set-up CD8^{+} effector cells were pre-incubated with concanamycin A at a final concentration of 200 nM for 2 h [55].

Variable effector : target cell ratios are due to varying yields of CD8^{+} TAL in the different groups. Data shown are representative for three comparable experiments.
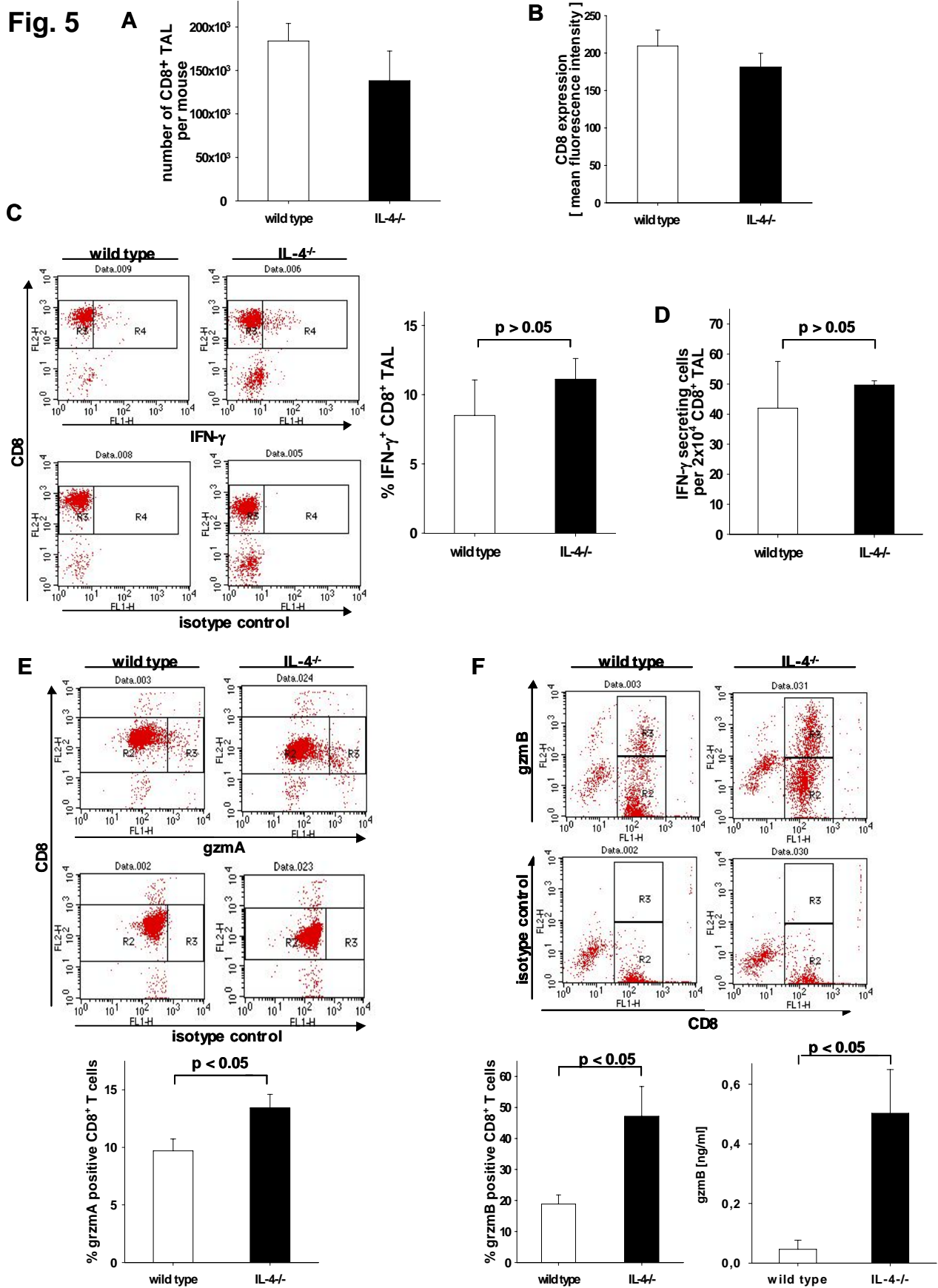
Fig. 5

FIG. 5. Characterization of the CD8+ TAL of TAg-immune IL-4-/- and wild type BALB/c mice on day 8 after challenge with mKSA cells.

(legend on next page)

FIG. 5. Characterization of the CD8$^+$ TAL of TAg-immune IL-4$^{-/-}$ and wild type BALB/c mice on day 8 after challenge with mKSA cells.

   IL-4$^{-/-}$ (black bars) or wild-type (white bars) mice were immunized on days -14 and –7 by i.p. injection of 10μg TAg and challenged i.p. on day 0 with 10^6 mKSA cells. CD8$^+$ TAL were immunomagnetically enriched from the peritoneal exudate cells of individual mice on day 8 after challenge. (A) Numbers of CD8$^+$ TAL recovered from the peritoneal cavity. Shown are the mean and standard error for n= 14 wild type and IL-4$^{-/-}$ mice. (B) Surface expression level of CD8 determined as mean fluorescence intensity by flow cytometry of CD8$^+$ TAL on day 8 after challenge (n=17 for each genotype). (C) Percentage of TAg-specific CD8$^+$ TAL as determined by intracellular detection of IFN-γ by flow cytometry in CD8$^+$ TAL after co-incubation   with TAg-expressing BALB-SV40 fibroblast in the presence of Golgi-stop for 6h.. Shown are dot plots from one representative experiment (left panel) and the mean and standard error cumulated from 3 independent experiments with n=9 wild types and n=8 IL-4$^{-/-}$ mice. (D) Enumeration of TAg-specific CD8+ TAL by an IFN-γ-specific Elispot assay (R&D systems). CD8$^+$ TAL were incubated at a density of 2x10^4 cells per well with 10^5 mKSA cells for 24h before developing the assay according to the instructions of the manufacturer and enumerating the IFN-γ positive spots by an AID EliSPOT reader system ELA 02. Shown are the mean and standard error for n=3 wild type and IL-4$^{-/-}$ mice. The data of IL-4$^{-/-}$ and wild type cells in (A) to (D) do not differ statistically significant as determined by Student´s t-test. (E) Granzyme A was detected by flow cytometry after intracellular staining of the cells with a rabbit anti mouse granzyme A serum. Shown are representative dot plots (upper panel) and the mean and standard error for n=10 wild type and IL-4$^{-/-}$ mice (lower panel). The statistical significance of IL-4$^{-/-}$ versus wild types is p<0.05 according to Student´s t-test. (F) Measurement of Granzyme B-expressing cells and of gzmB-specific enzymatic activity in CD8$^+$ TAL on day 8 after challenge. Granzyme B was detected by flow cytometry after intracellular staining of the cells with the mAb GB12 specific for human granzyme B, which was shown to cross-react with murine granzyme B previously [39, 40]. Shown are representative dot plots (upper panel) the mean and standard error for n=5 wild types and IL-4$^{-/-}$ mice (lower left panel). The statistical significance of IL-4$^{-/-}$ versus wild types is p<0.05 according to Student´s t-test. GzmB-specific enzymatic activity in lysates of CD8$^+$ TAL was determined in a colorimetric assay with the substrate N-acetyl-Ile-Glu-Pro-Asp-p-nitroanilide (lower right panel). Shown are representative the mean and standard error for n=4 wild type and IL-4$^{-/-}$ mice. The statistical significance of IL-4$^{-/-}$ versus wild type is p<0.05 according to Student´s t-test.

FIG. 6
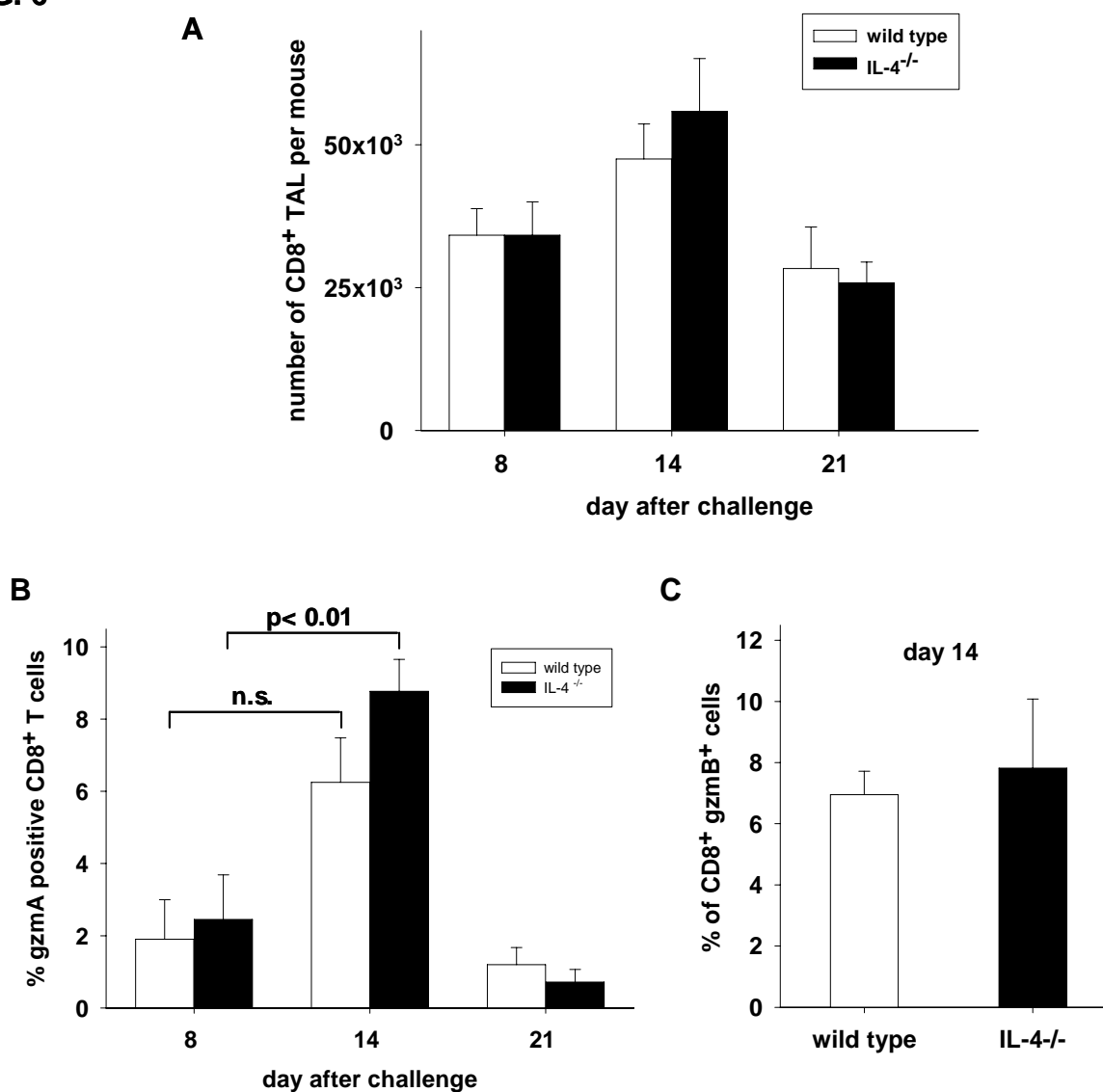
FIG. 6. **Characterization of the CD8^{+} TAL of naive IL-4^{-/-} and wild type BALB/c mice challenged with 10[3] mKSA cells.**

Navie IL-4^{-/-} or wild-type mice were challenged i.p. on day 0 with 10[3] mKSA cells. CD8^{+} TAL were immunomagnetically enriched from the peritoneal exudate cells of individual mice at the indicated times after challenge. (A) Numbers of CD8^{+} TAL recovered from the peritoneal cavity. Shown are means and standard errors (day 8: n=3; day 14: n=6; day 21: n=3). (B) Granzyme A was detected by flow cytometry after intracellular staining of the cells with a rabbit anti mouse gzmA serum. Shown are the means and standard errors (day 8 and 21: n=3; day 14: n=14). Percentage of CD8^{+} TAL expressing gzmB as determined by flow cytometry on day 14 after challenge (n=6).

3. Results II

3.1. Accelerated rejection of mKSA tumor cells by TAg-immune IL-10$^{-/-}$ mice

Naïve IL-10-deficient (IL-10$^{-/-}$) or wt BALB/c mice succumb to i.p. challenge with 1×10^6 Simian Virus 40 TAg-expressing mKSA tumor cells between day 15 and 25 after challenge. In contrast, previous immunization of mice with recombinant TAg leads to immunoprotection against the tumor (**Supplemental Fig.1**). Notably, the kinetics of tumor cell elimination from the peritoneal cavity differs markedly between TAg-immunized IL-10$^{-/-}$ and wt mice. Within 4 days after challenge, proliferating mKSA cells completely vanished among the peritoneal exudate cells of TAg-immune IL-10$^{-/-}$ mice. In contrast, wild type mice harbored clearly detectable proliferating mKSA cells (**Fig. 1A**).

This rapid kinetics of tumor cell eradication was confirmed by *in vivo*-bioluminescence imaging and by immunohistology. mKSA cells stably transduced with a lentiviral vector coding for Renilla luciferase rapidly formed tumors in naïve IL-10$^{-/-}$ and wt mice (**Fig. 1B**). In TAg-immune IL-10$^{-/-}$ mice, the mKSA cells expanded less than in wt mice between days 2 and 4 after challenge. By day 7 after challenge, IL-10$^{-/-}$ mice were almost devoid of abdominal bioluminiscence signals as opposed to strong signals over large adbominal areas in wt mice.

Histomorphological and immunohistochemical analysis of the abdominal cavity revealed knotty and diffuse TAg-positive tumor infiltrates along the visceral peritoneum. As early as 2 days post challenge, peritoneal TAg-positive mKSA tumor aggregates in IL-10$^{-/-}$ mice were significantly smaller and less frequent than those in wt mice (**Fig. 2A-B**). On day 4, tumor cell aggregates were only rarely detectable in IL-10$^{-/-}$ mice but in wt mice mKSA cells were present in large numbers. On day 6, in IL-10$^{-/-}$ mice the few remaining tumor cells were detectable by immunohistochemistry only. Eight days after challenge, only single TAg-expressing mKSA cells were detectable along the visceral peritoneum in IL-10$^{-/-}$ mice while tumor nodes were still large and frequent in wt mice.

In IL-10$^{-/-}$ mice, marked infiltrates of CD8$^+$ lymphocytes were detectable in the tumor nodules already on day 4 after challenge while in wt mice noteworthy amounts of CD8$^+$ lymphocytes were not detectable before day 8 after challenge (**Fig. 3**).

Taken together, three independent experimental approaches document a more rapid reduction of the tumor load in TAg-immune IL-10$^{-/-}$ mice than in corresponding wt controls.
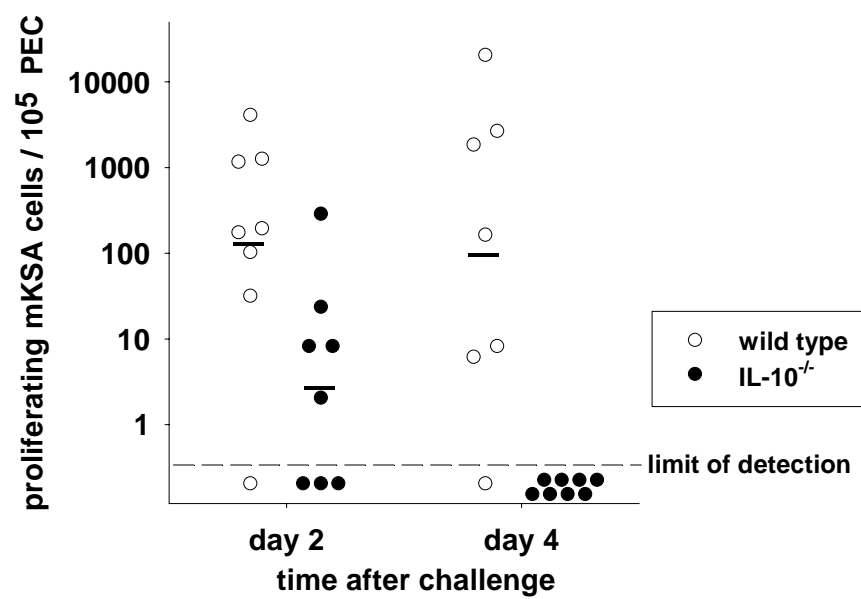
A

Figure 1. Accelerated rejection of mKSA tumor cells by TAg-immunized IL-10$^{-/-}$ BALB/c mice.
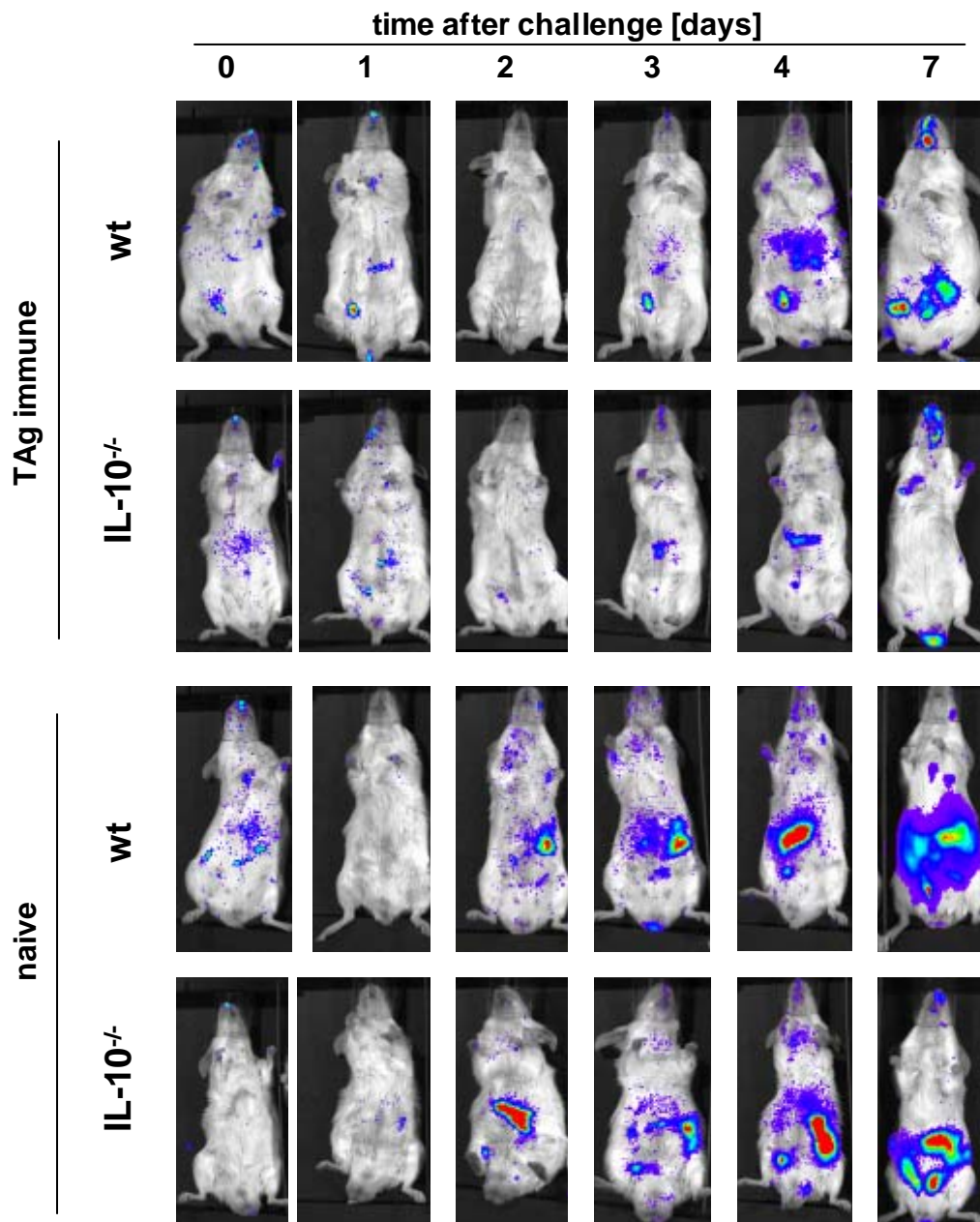
(legend on next page)

B

Figure 1. Accelerated rejection of mKSA tumor cells by TAg-immunized IL-10$^{-/-}$ BALB/c mice.

Groups of IL-10$^{-/-}$ and wt BALB/c mice were immunized on days -14 and -7 by i.p. injection of 10μg TAg or left untreated. On day 0, i.e. seven days after the second immunization, the mice were challenged i.p. with 10^6 mKSA cells. (A) On days 2 and 4 after challenge, peritoneal exudate cells (PEC) harvested by peritoneal lavage were plated in ten-fold serial dilutions. After cultivation for 8 days, the cells were fixed and stained with crystal violett before clonal colonies of mKSA cells were counted and expressed as mKSA cells per 10^5 PEC. Shown are cumulative data of two independent experiments as values for individual mice (n=8 for all groups except for wild type, day 4 n=7) and means for each group (horizontal bars). (B) On day 0, immune or naïve mice were challenged i.p. with 10^6 mKSA cells stably expressing Renilla luciferase. At the indicated days, mice were i. v. injected with the luciferase substrate coelenterazine, anesthesized and analyzed by transcutaneous luminescence imaging for the distribution and signal strength of bioluminescent mKSA cells. All images were taken with identical instrument settings. One representative mouse out of 5 mice per group analyzed in three independent experiments is shown.

Fig. 2A

wt

overview (4x)   insert (10x magnified)

d2

d4

d6

d8

Figure 2. Immunohistochemical detection of TAg-expressing mKSA tumor cells in intraabdominal tissues of TAg-immune mice at various times after challenge.   (legend on next page)
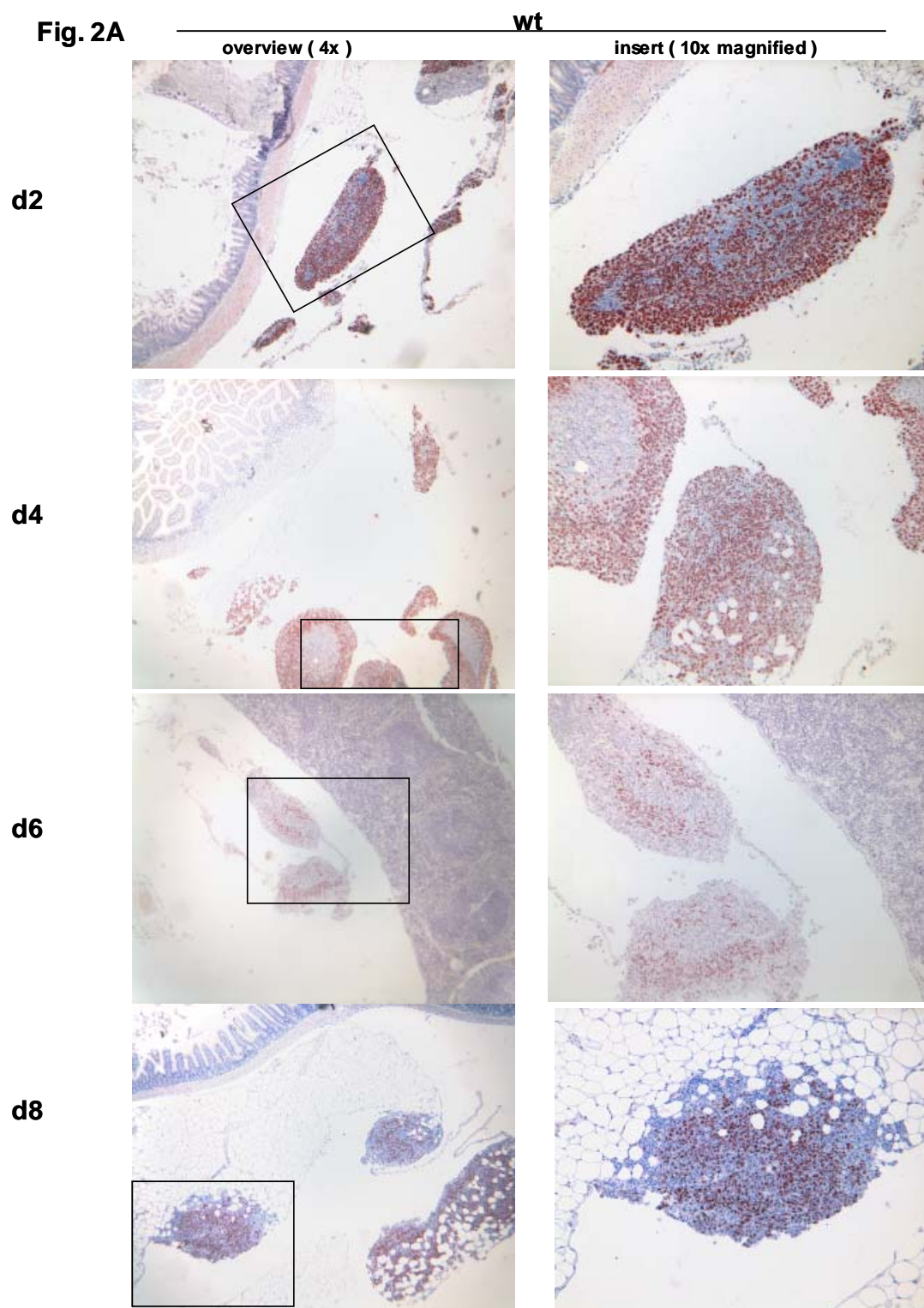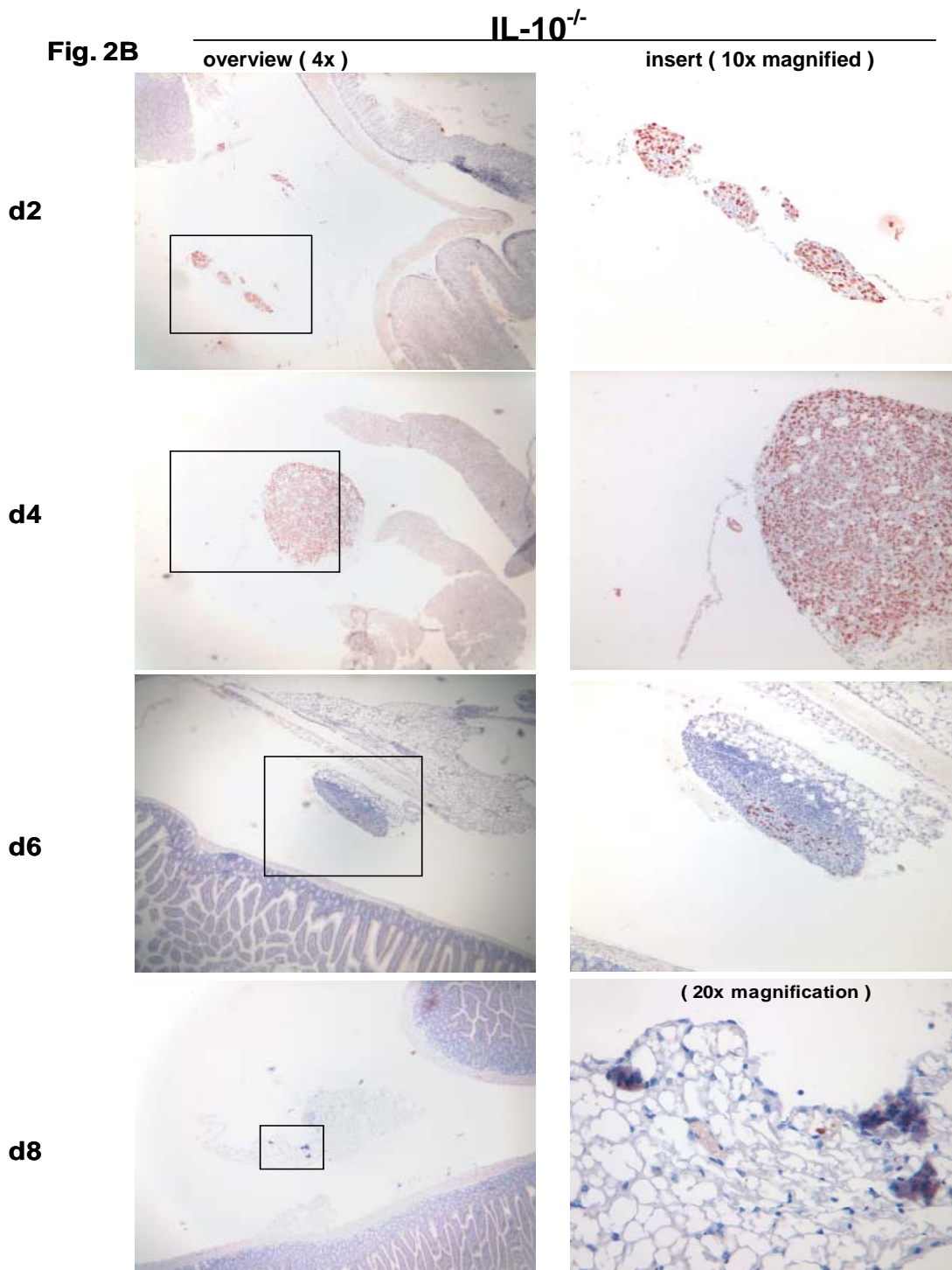
Figure 2. Immunohistochemical detection of TAg-expressing mKSA tumor cells in intraabdominal tissues of TAg-immune mice at various times after challenge.

Groups of IL-10$^{-/-}$ and wt BALB/c mice were immunized as described for Fig. 1. On day 0, immune and naive mice were challenged i.p. with 10^6 mKSA cells. At the indicated days after challenge, intestinal organs were removed, fixed and stained with TAg-specific rabbit antiserum R15 according to standard procedures. Representative mesenterial segments from (A) wt and (B) IL-10$^{-/-}$ mice are shown as overviews (4x magnification) and enlargened views (10x magnification) of the indicated inserts. The islands of mKSA tumor cells can be easily recognized by their intense red staining of the nuclei, while nuclei of infiltrating immune cells appear blue. To allow assessment of the size of tumor nodes, micrographs of sections including the intestinal wall were selected.

46

Fig. 3

infiltrating CD8$^+$ cells

| wt | IL-10$^{-/-}$ |

d4

d6

d8

d12
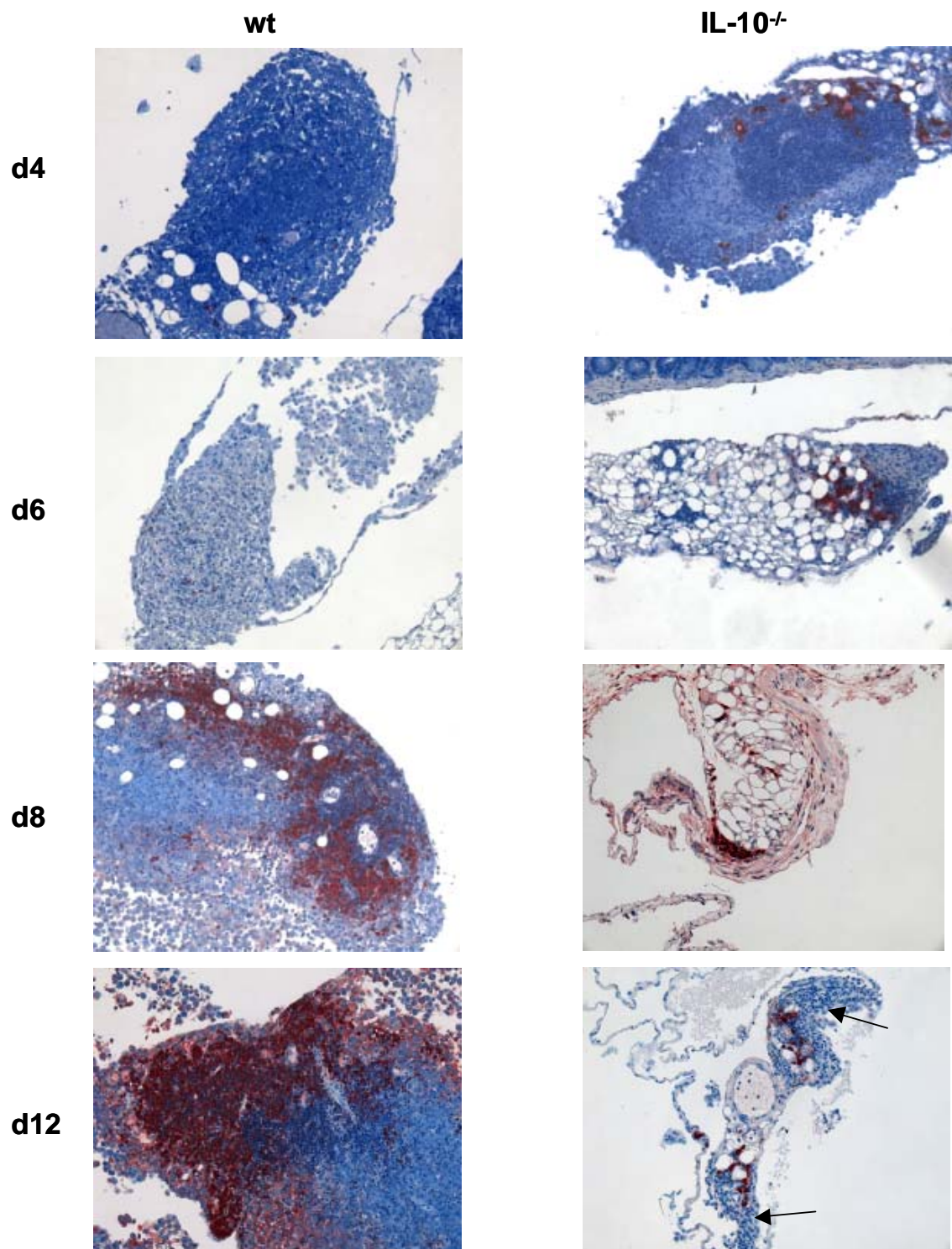
Figure 3. Immunohistochemical detection of infiltrating CD8+ lymphocytes in intraabdominal tumor nodules of TAg-immune mice at various times after challenge.

Sections of the samples described in Fig. 2 were stained with CD8-specific mAb according to standard procedures. Representative mesenterial segments from wt and IL-10$^{-/-}$ mice are shown (10x magnification). Please note on day 12 in IL-10$^{-/-}$ mice the cell-dense areas of reactive mesothelial cells (arrows) which must not be mistaken for tumor cell aggregates. Immunohistochemical analysis has been done in cooperation with Prof. Dr. med Claudia Wickenhauser, Universitätsklinikum Leibzig.

3.2. Absence of TAg-specific CD8$^+$ CTL in the peritoneal cavity of TAg-immune IL-10$^{-/-}$ mice

To identify the cell type mediating accelerated elimination of mKSA tumor cells from TAg-immunized IL-10$^{-/-}$ mice, immunized mice were depleted of CD8$^+$ cells by i.v. injection of monoclonal antibodies prior to i.p. challenge with mKSA cells. TAg-immunized IL-10$^{-/-}$ and wt mice depleted of CD8$^+$ cells succumbed to the tumor with similar kinetics, while most immune, but non-depleted mice survived the challenge (**Fig. 4A**). These data suggest that CD8$^+$ T cells mediate the eradication of mKSA cells both in IL-10$^{-/-}$ and wt mice.

The effector mechanism by which mKSA cells are eradicated in IL-10$^{-/-}$ mice was further characterized by using mice double deficient for IL-10 and the cytotoxic effector molecule perforin (IL-10$^{-/-}$/perforin$^{-/-}$). Despite prior immunization with TAg, IL-10$^{-/-}$/perforin$^{-/-}$ as well as perforin$^{-/-}$ mice died as rapidly as naïve mice after challenge with mKSA cells (**Fig. 4B**). Apparently, the TAg-specific protection is dependent on perforin as expected.

As shown previously in our earlier studies (Zerrahn et al., 1996; Utermöhlen et al., 1996; Baschuk et al., 2007), cytolytically active CD8$^+$ CTL were detectable among the peritoneal exudate cells of TAg-immunized wt mice from day 6 after challenge on (**Fig. 4C**). Surprisingly, CD8$^+$ T cells were mostly absent in the peritoneal cavity of TAg-immunized IL-10$^{-/-}$ mice. The few CD8$^+$ T cells recovered from IL-10$^{-/-}$ mice did not lyse mKSA cells to a significant degree, regardless whether they were harvested on day 4, 6, or 8 after tumor cell challenge. It was conspicuous that lower numbers of CD8$^+$ T cells were recovered from IL-10$^{-/-}$ than from wt mice (**Fig. 4D**). The numbers of peritoneal CD8$^+$ T cells specific for mKSA cells were determined by measuring the intracellular accumulation of IFN-γ in T cells stimulated with mKSA tumor cells using flow cytometry. In IL-10$^{-/-}$ mice lower numbers of tumor-specific CD8$^+$ T cells were detectable than in wt mice (**Fig. 4E**). In light of the accelerated kinetics of tumor cell eradication in IL-10$^{-/-}$ mice, the lack of CD8$^+$ T cells with significant TAg-specific cytotoxic activity in peritoneal exudates was puzzling. This led to the hypothesis that TAg-specific CD8$^+$ CTL reside in some other tissue of IL-10$^{-/-}$ mice.
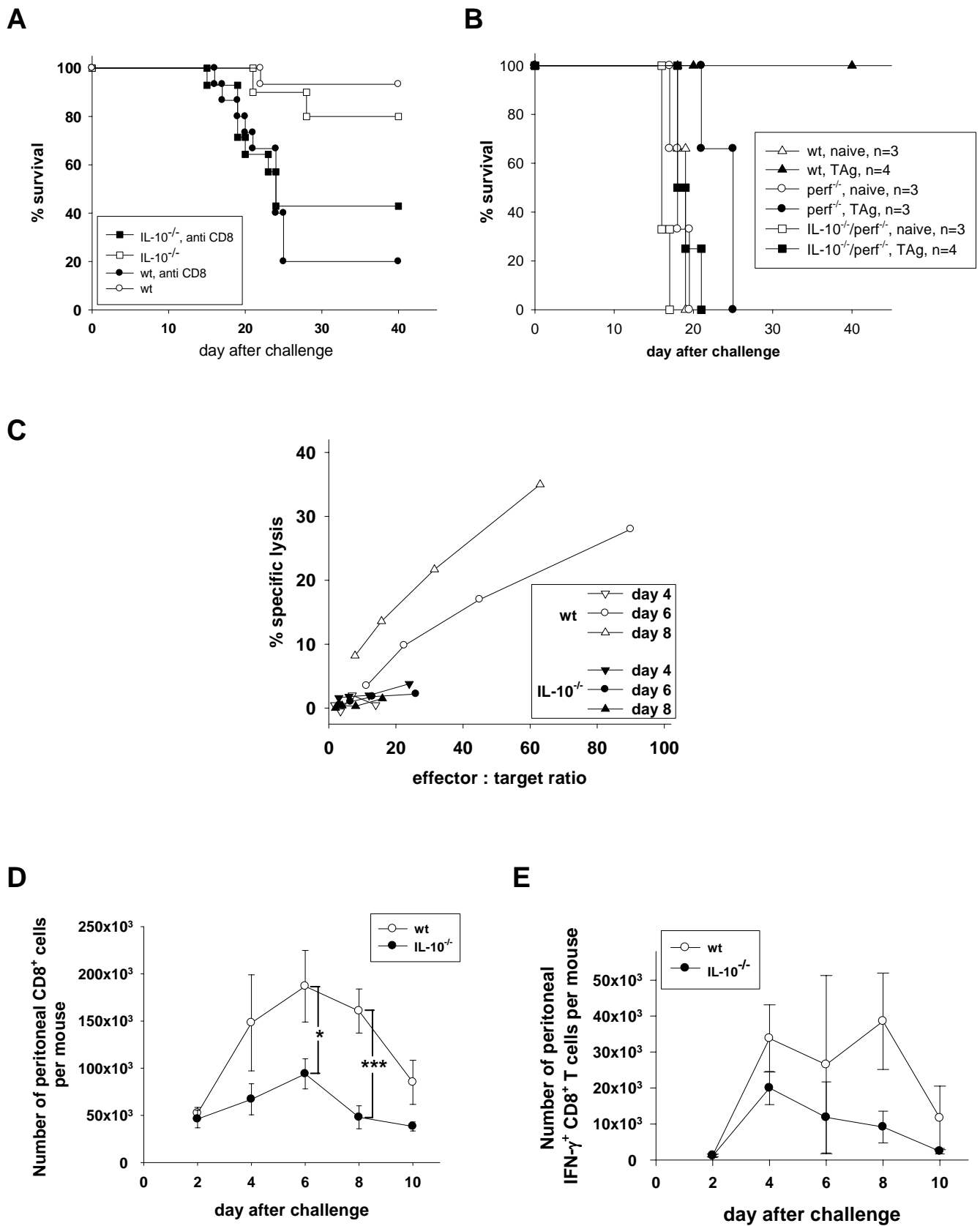
Figure 4. Characterization of the TAg-specific CD8$^+$ T cell response of IL-10$^{-/-}$ and wt mice during rejection of i.p. injected mKSA cells. (legend on next page)

49

Figure 4. Characterization of the TAg-specific CD8^{+} T cell response of IL-10^{-/-} and wt mice during rejection of i.p. injected mKSA cells.

Groups of mice were immunized twice by i.p. injection of 10µg TAg at a weekly interval. (A) Six days after the second immunization, groups of IL-10^{-/-} (n=14) or wt (n=15) mice were treated by i.v. injection of 500 µg CD8-specific mAb while the control groups (wt: n=15; IL-10^{-/-}: n=10) received no mAb treatment. One day after T cell depletion, mice were challenged by i.p. inoculation of 10[6] mKSA cells. Cumulative data from three independent experiments are shown as a Kaplan Meier graph of the survival of mice. (B) Naïve or TAg-immune wt mice, mice deficient for perforin (perf^{-/-}), or mice deficient for both IL-10 and perforin (IL-10^{-/-}/perf^{-/-}), were i.p. challenged with 10[6] mKSA cells and monitored for tumor-dependent

death daily. (C) On days 4, 6, or 8 after challenge, CD8^{+} TAL were immunomagnetically enriched from pooled peritoneal exudate cells of 5 mice per group (purity of CD8^{+} cells >95%). The TAg-specific cytotoxic activity of these CD8^{+} TAL was determined in a standard 4h-chromium release assay using mKSA cells as targets. Variable E:T ratios are due to various yields of CD8^{+} TAL in the groups and are an indicator for the low numbers of CD8^{+} TAL in particular in IL-10^{-/-} mice. Shown is one representative series of cytotoxicity assays out of three. (D) Absolute numbers of CD8^{+} TAL that were harvested by immunomagnetic enrichment from PEC of individual mice. Cumulative data of at least 3 experiments per day including 6 to 15 mice are shown as means and SEM. Statistical significance of the increase of CD8^{+} TAL: IL-10^{-/-}: day 6 versus day 2: $p>0.05$; wt: day 6 versus day 2: $p<0.05$; day 8 versus day 2: $p< 0.001$. (E) The CD8^{+} TAL described in (D) were incubated on a confluent layer of mKSA cells for 6 hours and processed according to standard procedures for the detection of intracellular IFN-γ by flow cytometry. CD8^{+} TAL incubated in the presence of TAg-negative, H-2[d] MethA tumor cells did not produce IFN-γ (data not shown). The number of CD8^{+} TAL per mouse TAg-specifically producing IFN-γ was calculated from the number of CD8^{+} TAL per mouse and the frequency of IFN-γ producing CD8^{+} TAL. Shown are mean and SEM. Statistical significance: d8: wt versus IL-10^{-/-} $p=0.056$.

3.3. Cytotoxicity of TAg-specific CD8$^+$ intestinal intraepithelial lymphocytes in TAg-immunized IL-10$^{-/-}$ mice

Like conventional CTL, intestinal intraepithelial lymphocytes (IEL) express the CD8$^+$ phenotype. One of the hallmarks of CD8$^+$ IEL is their spontaneous high cytotoxicity against epithelial tumor cells detectable *ex vivo* (Roberts et al., 1993). Because intestinal CD8$^+$ IEL were shown to exert during viral infection systemic antigen-specific protection (Corazza et al., 2000), we tested whether IEL contribute to antitumoral immunity in IL-10$^{-/-}$ mice. Intestinal CD8$^+$ IEL harvested from TAg-immunized IL-10$^{-/-}$ mice on day two after i.p. challenge with mKSA are highly cytotoxic against mKSA cells at moderate effector to target ratios, while CD8$^+$ intestinal IEL from wt mice do not lyse mKSA cells at all (**Fig. 5A**). CD8$^+$ intestinal IEL prepared from IL-10$^{-/-}$ mice on day 2 after challenge are specific for TAg, because they lyse the H2^d-positive, TAg-expressing mKSA cells but not the syngeneic, TAg-negative MethA tumor cells (**Suppl. Fig. 2**).

The frequency of CD8$^+$ intestinal IEL responsive to mKSA cells was determined by flow cytometry and ELISpot assays. On day 2 after challenge, about 17% of IL-10$^{-/-}$ versus 8% of wt CD8$^+$ intestinal IEL accumulated intracellular IFN-γ after non-specific stimulation with PMA/ionomycin (data not shown). In response to specific stimulation with mKSA cells almost 2% of IL-10$^{-/-}$ versus about 0.7% of wt CD8$^+$ intestinal IEL became positive for IFN-γ (**Fig. 5B**). The frequency of IL-10$^{-/-}$ CD8$^+$ intestinal IEL secreting IFN-γ in response to mKSA cells was about threefold higher than that of wt CD8$^+$ intestinal IEL as determined by ELISpot (**Fig. 5C**).

These data show that as early as 2 days after i.p. challenge with mKSA cells in TAg-immune IL-10$^{-/-}$ mice the frequency of CD8$^+$ intestinal IEL specifically reacting to mKSA cells is at least twofold higher than in wt controls. More importantly, only IL-10$^{-/-}$, but not wt CD8$^+$ intestinal IEL are highly cytotoxic against mKSA cells on day 2 after challenge.

Further characterization by flow cytometry revealed that the IEL prepared from IL-10$^{-/-}$ or wt mice were CD3$^+$ CD8$^+$ T cells (**Suppl. Fig. 3A**). These CD8$^+$ intestinal IEL from IL-10$^{-/-}$ and wt mice did not differ with respect to the frequencies or expression levels of surface markers widely used to characterize IEL, i.e. CD44, CD62L, CD69, or CD103 (**Suppl. Fig. 3B**). Moreover, CD8$^+$ IEL from IL-10$^{-/-}$ or wt mice did not differ with regard to expression of CD8α, CD8β, TCRα/β, or TCRγ/δ (**Suppl. Fig. 3C**).

Together, CD8^{+} intestinal IEL from TAg-immune IL-10^{-/-} versus wt mice are phenotypically undistinguishable with regard to activation markers or components of the TCR complex, but differ functionally in the frequency of TAg-specific cells and their TAg-specific cytotoxic activity.
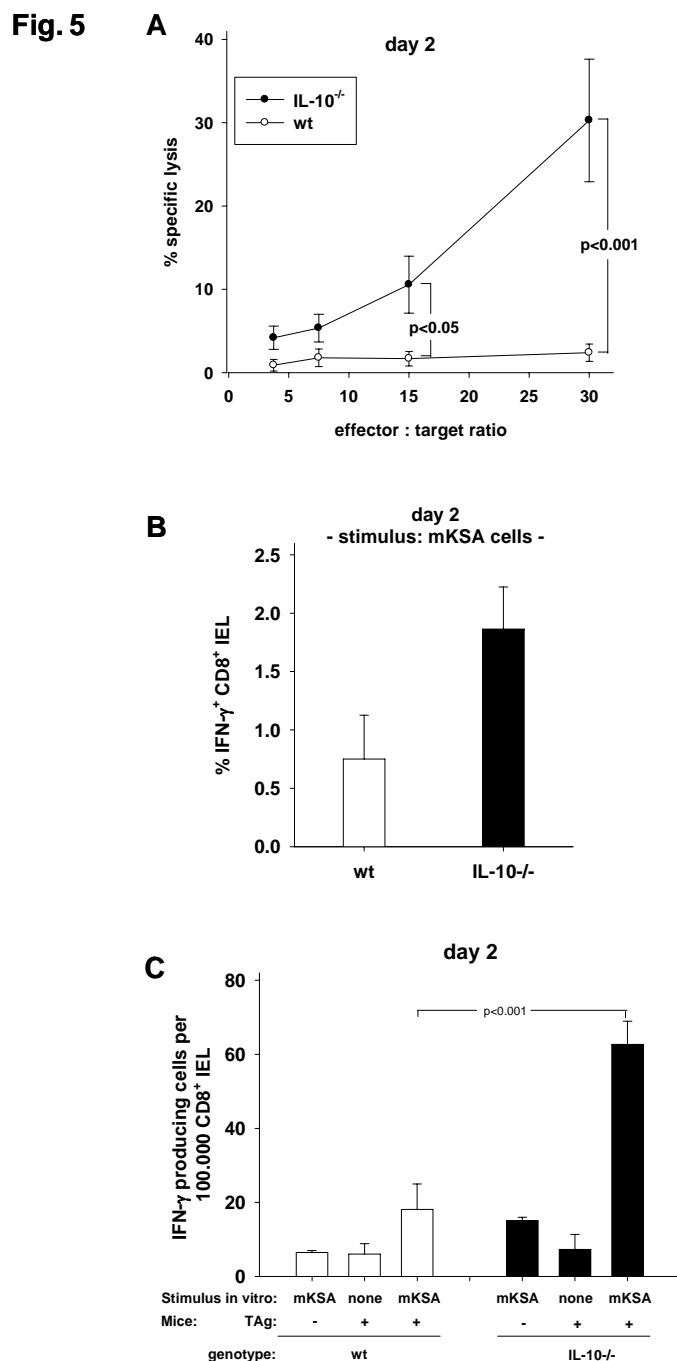

Figure 5. Functional *ex vivo* characterization of the mKSA-specific activity of CD8^{+} intestinal intraepithelial lymphocytes from TAg immunized IL-10^{-/-} and wt mice early after challenge with mKSA cells. (legend on next page)

Figure 5. Functional *ex vivo* characterization of the mKSA-specific activity of CD8^{+} intestinal intraepithelial lymphocytes from TAg immunized IL-10^{-/-} and wt mice early after challenge with mKSA cells.

Mice were immunized on days -14 and -7 by i.p. injection of 10μg TAg and challenged i.p. with 10[6] mKSA on day 0. CD8^{+} cells were immunomagnetically enriched from intestinal intraepithelial lymphocytes of mice at the indicated days after challenge. (A) Primary cytotoxic activity of the CD8^{+} IEL was determined in a 5h ^{51}Cr-release assay against mKSA cells on day 2 after challenge. Shown are the means and SEM of cumulative data from 5 independent experiments including 5 IL-10^{-/-} and 7 wt mice. The statistical significance was determined for the indicated E:T ratios with Student´s t-test. (B) The frequency of CD8^{+} IEL expressing IFN-γ was determined by flow cytometry after specific stimulation with mKSA target cells. Shown are the means and SEM of cumulative data from 2 independent experiments including n=3 mice per group. (C) The frequency of CD8^{+} IEL secreting IFN-γ in response to mKSA cells was determined by ELISpot assays according to standard procedures. Shown are the means and SEM of cumulative data from 3 independent experiments including n=6 mice per genotype.

3.4. Intravenously transferred CD8⁺ intestinal IEL from TAg-immunized IL-10^{-/-} mice protect naïve wt mice against lethal challenge with mKSA cells

The *in vivo* relevance of the *ex vivo* highly cytotoxic CD8$^+$ intestinal IEL from IL-10$^{-/-}$ mice was assessed by adoptive transfer experiments. On day 2 after challenge of TAg-immunized mice, CD8$^+$ intestinal IEL were immunomagnetically enriched and transferred into naïve wt acceptor mice that were previously challenged with mKSA cells. After intravenous transfer of CD8$^+$ intestinal IEL from IL-10$^{-/-}$ donor mice, 89% (8 of 9) of naïve acceptor mice survived the challenge with mKSA cells whereas only 11% (1 of 9) acceptor mice survived that received CD8$^+$ intestinal IEL from wt donors (**Fig. 6A**). To exclude the remote possibility that this significant difference might be due to the transfer of contaminating tumor cells among the CD8$^+$ intestinal IEL from wt donors, groups of naïve acceptor mice received IEL from donors of either genotype without being subsequently challenged. None of these mice died, indicating that the preparations of IEL were not contaminated by mKSA cells. Strikingly, neither IL-10$^{-/-}$ nor wt CD8$^+$ intestinal IEL protected naïve acceptor mice against challenge with mKSA cells when the IEL were transferred by intraperitoneal injection (**Fig. 6B**).

Unlike intestinal CD8$^+$ IEL, adoptively transferred conventional CD8$^+$ T cells prepared from tumor-associated lymphocytes (TAL) from TAg-immune IL-10$^{-/-}$ or wt mice did not protect naïve recipients against mKSA tumor cells regardless whether the CD8$^+$ TAL were transferred i.v. or i.p. (**Fig. 6C and D**). This underscores the outstanding antitumoral effector activity of IL-10$^{-/-}$ CD8$^+$ IEL versus conventional CD8$^+$ CTL *in vivo*.

The data suggest that CD8$^+$ intestinal IEL of TAg-immunized IL-10$^{-/-}$ mice not only are highly cytotoxic *ex vivo*, but are also able to protect *in vivo* naïve acceptor mice against mKSA cells.
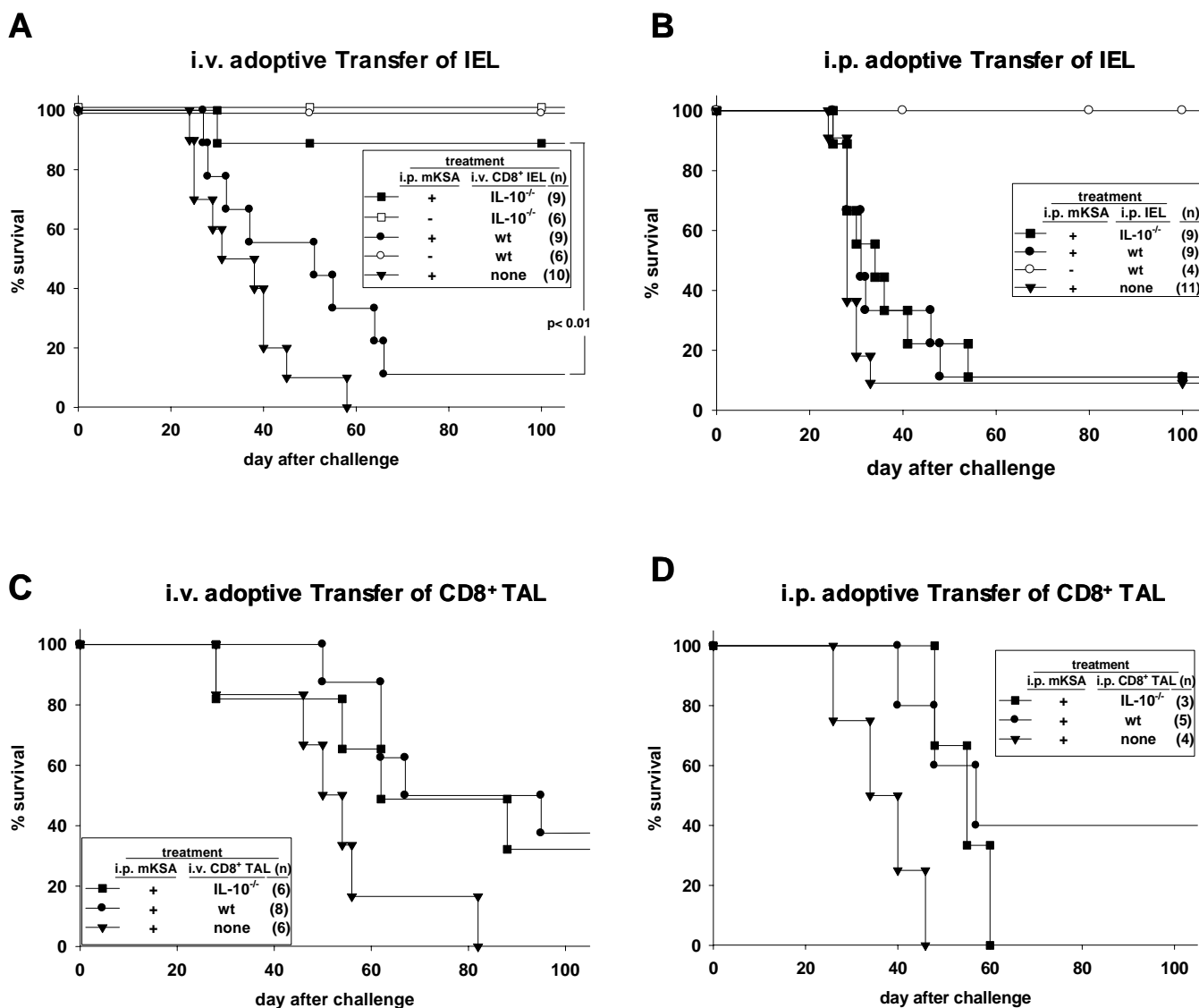
Fig. 6

A

i.v. adoptive Transfer of IEL

B

i.p. adoptive Transfer of IEL

C

i.v. adoptive Transfer of CD8$^+$ TAL

D

i.p. adoptive Transfer of CD8$^+$ TAL

Figure 6. Protection of naïve BALB/c mice against mKSA cells by i.v. transfer of CD8$^+$ IEL from TAg-immunized IL-10$^{-/-}$ but not from wt mice.

IL-10$^{-/-}$ and wt donor mice were immunized on days -14 and -7 by i.p. injection of 10μg TAg and challenged i.p. with 10^6 mKSA cells on day 0. Two days after challenge, CD8$^+$ IEL were prepared and transferred (A) i.v. or (B) i.p. at doses of 1x10^6 cells per mouse into naïve BALB/c recipients that had been challenged i.p. with 10^3 mKSA cells 1 h previously. To exclude that contaminating mKSA cells were transferred with enriched CD8$^+$ IEL, control mice received CD8$^+$ IEL but no challenge inoculum of mKSA cells. Shown are cumulative data for each i.v. or i.p. transfer from three independent experiments. Kaplan-Meier curves were analyzed for statistical significance by log-rank test.

Eight days after challenge, CD8$^+$ tumor-associated lymphocytes (TAL) were prepared from peritoneal exudate cells and transferred (C) i.v. or (D) i.p. at doses of 1x10^6 cells per mouse into naïve BALB/c recipients that had been challenged i.p. with 10^3 mKSA cells 1 h previously. Shown are cumulative data of three (C) or two (D) independent experiments.

3.5. T cell-derived IL-10 is not significantly involved in suppressing TAg-specific CD8$^+$ intestinal IEL

CD4$^+$ T cells harvested from the peritoneal cavity of TAg-immunized wt mice secrete considerable amounts of IL-10 from day 4 after challenge onwards (**Fig. 6A**), raising the question whether T cell-derived IL-10 suppresses the generation of TAg-specific CD8$^+$ intestinal IEL in wt mice.

In mice with a T cell-specific inactivation of the IL-10 gene (IL-10$^{FL/FL}$CD4-Cre$^+$) (Roers wt al., 2004) the tumor load in the peritoneal cavity was similar to that in immune IL-10$^{FL/FL}$CD4-Cre$^-$ mice, which were used as wt controls (**Fig. 6B**). In contrast, the tumor load in whole-body IL-10-deficient mice was about 70-fold lower. Additionally, only CD8$^+$ intestinal IEL from IL-10$^{-/-}$ mice but not from IL-10$^{FL/FL}$CD4-Cre$^+$ mice were highly cytotoxic against mKSA cells (**Fig. 6C**).

The data suggest that T cell-derived IL-10 is not significantly involved in suppressing the generation of TAg-specific CD8$^+$ intestinal IEL in mice.

A

B

C

Figure 6. Specific deletion of IL-10 in T cells does not result in the generation of TAg-specific CD8^{+} cytotoxic IEL or in the hyperacute rejection of mKSA cells. (legend on next page)
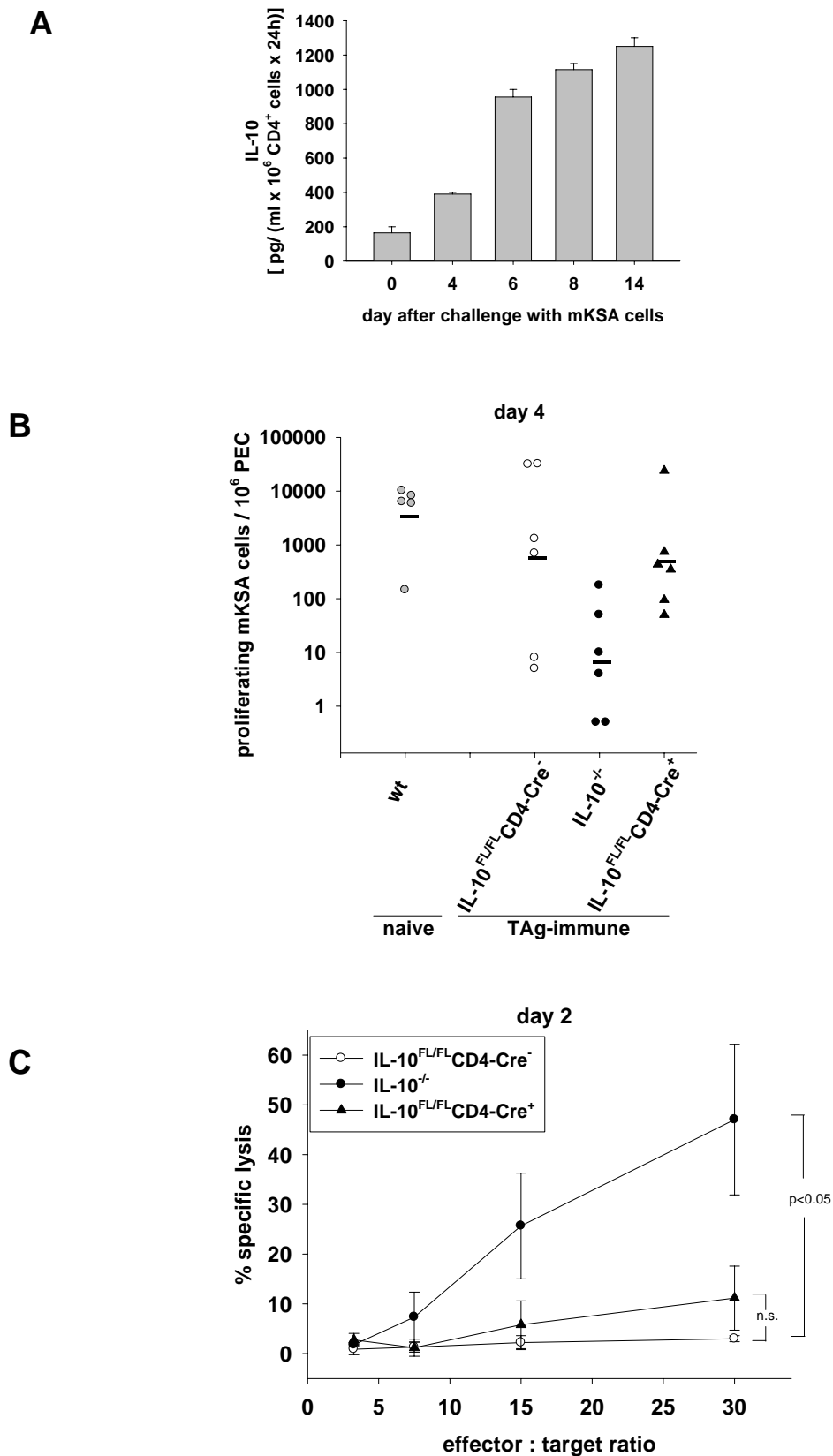
57

Figure 6. Specific deletion of IL-10 in T cells does not result in the generation of TAg-specific CD8^{+} cytotoxic IEL or in the hyperacute rejection of mKSA cells.

Groups of wt, IL-10$^{FL/FL}$CD4-Cre^{-} , IL-10$^{-/-}$ or IL-10$^{FL/FL}$CD4-Cre^{+} mice were immunized on days -14 and -7 by i.p. injection of 10mg TAg and challenged i.p. with 10[6] mKSA cells on day 0. (A) CD4^{+} TAL cells were immunomagnetically enriched from peritoneal exudate cells collected from wt mice prior to challenge (day 0; cells pooled from 15 mice) or on days 4, 6, 8, or 14 after challenge (pooled from 5 mice per day) and kept at a densitiy of 1 x10^{6} cells/ml in complete RPMI medium without any further stimuli for 24h. IL-10 in the cell-free supernatants of these cultures were determined by ELISA. Shown are the means and standard error of the mean of two independent experiments. (B) The numbers of proliferating mKSA cells recovered from the peritoneal cavity of mice on day 4 after challenge were determined as described for Fig. 1. Shown are cumulative data of two independent experiments as values for individual mice and means (horizontal bars) for each group. (C) On day 2 after challenge, cytotoxic activity of CD8^{+} IEL from IL-10$^{FL/FL}$CD4-Cre^{-}, IL-10$^{-/-}$ or IL-10$^{FL/FL}$CD4-Cre^{+} mice was determined with mKSA cells as target cells in chromium release assays. Shown are cumulative data from three independent experiments as means and SEM.
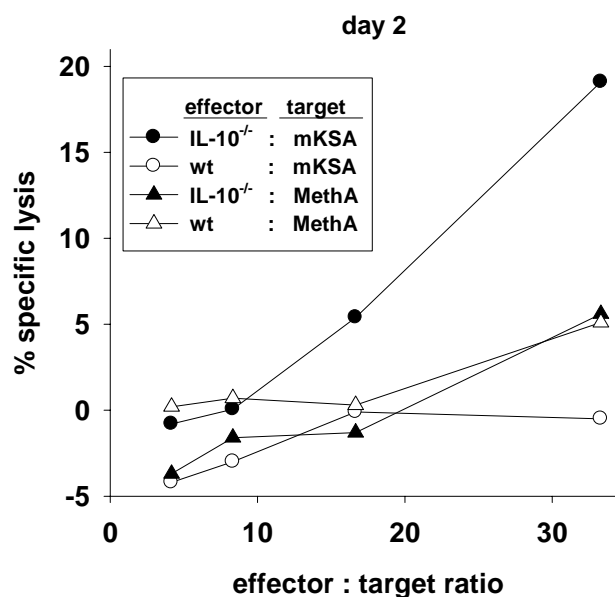
Suppl. Fig. 1
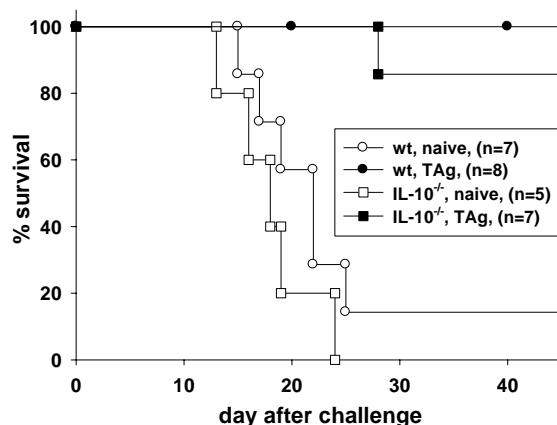
suppl. Figure 1. Survival of TAg-immunized IL-10$^{-/-}$ and wt BALB/c mice after i.p. challenge with mKSA tumor cells.

Groups of IL-10-/- and wt BALB/c mice were immunized on days -14 and -7 by i.p. injection of 10mg TAg or left untreated. On day 0, i.e. seven days after the second immunization, the mice were challenged i.p. with 106 mKSA cells. Survival of the mice was monitored daily up to 50 days after challenge.

Suppl. Fig. 2

Suppl. Fig. 2. CD8+ IEL prepared from TAg-immune IL-10$^{-/-}$ mice specifically lyse TAg-expressing syngeneic tumor cells.

Mice were immunized on days -14 and -7 by i.p. injection of 10mg TAg and challenged i.p. with 106 mKSA on day 0. CD8+ cells were immunomagnetically enriched from intestinal intraepithelial lymphocytes of mice at day 2 after challenge. Specificity of CD8+ IEL for TAg was assessed by using either TAg-negative, H-2d-positve MethA tumor cells or TAg-expressing, H-2d-positive mKSA cells as target cells in standard chromium release assays. One representative experiment out of three is shown.

Suppl. Fig. 3
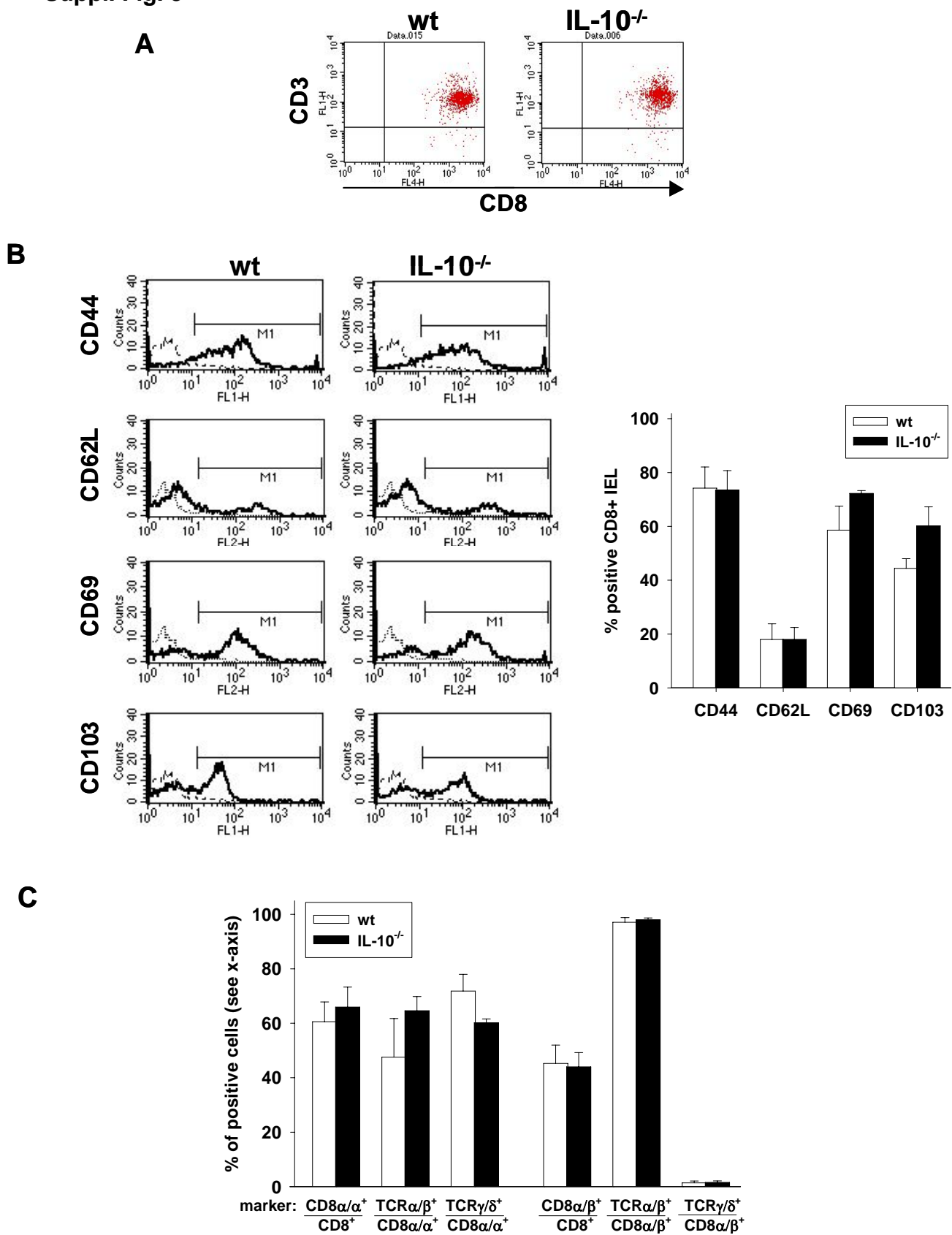
Suppl. Fig. 3. Phenotypic characterization of CD8+ IEL prepared from TAg-immune IL-10$^{-/-}$ and wt mice on day 2 after challenge with mKSA cells. (legend on next page)

Suppl. Fig. 3. Phenotypic characterization of CD8+ IEL prepared from TAg-immune IL-10^{-/-} and wt mice on day 2 after challenge with mKSA cells.

IL-10^{-/-} and wt mice were immunized on days -14 and -7 by i.p. injection of 10mg TAg and challenged i.p. with 10$6$ mKSA on day 0. Two days after challenge, CD8+ cells were immunomagnetically enriched from IEL and analyzed for expression of surface markers and TCR-components. (A) Immunomagnetically enriched IEL are CD3+ CD8+ T cells. (B) Expression profile of CD44, CD62L, CD69, or CD103 on CD8+ IEL shown in representative histograms for each staining (left panel) and as mean +/- SEM of cumulative data (right panel) from 3 experiments including measurements from 4 mice per marker. (C) Frequency analysis of the expression of CD8a, CD8b, TCRa/b, or TCRg/d by CD8+ IEL. Shown are mean +/- standard errors of cumulative data from 3 experiments including measurements from 3 to 6 mice per marker.

61

3. Results III

3.1. Murine embryonic stem cells are lysed less efficiently than YAC cells by resting or activated natural killer cells

We have previously shown that embryonic stem cells (ES cells) are resistant to lysis by CD8^{+} cytotoxic T-lymphocytes due to the expression of SPI-6, the specific inhibitor of granzyme B (Abdullah et al., 2007). The present study aims at characterizing the interaction between ES cells and natural killer (NK) cells in as the second important cytotoxic effectorcell type. For this study CGR8 ES cells, derived from H-2^b expressing 129Ola/Hsd mice were chosen, because these cells can be cultivated *in vitro* without feeder cells. In contrast to studies using feeder cell dependent ES cell lines, the use of CGR8 ES cells precludes any interference of feeder cells in immunologic assays.

NK cells were prepared from C57BL/6 mice, also expressing the MHC class I haplotype H2^b. NK cells were immunomagnetically enriched from splenic single cell suspensions of naïve mice or of mice pre-treated with poly I:C to generate activated NK cells. In standard 4h ^{51}Cr-release assays, resting or activated NK cells were used as effector cells against CGR8 ES cells. YAC cells, the wildly used murine standard target cell for NK cells or allogeneic BALB/c-SV fibroblasts were used as positive or negative control target cells, respectively.

Resting NK cells lysed ES cells to about 20% at an 50:1 E:T ratio. YAC cells were lysed two fold more effective than ES cells. As shown in **Fig. 1A** ES cells required a two fold increased effector : target cell ratio, compared to YAC cells, to be lysed to the same degree by naïve NK cells. This equals a two fold reduced susceptibility of ES cells to NK cell mediated lysis. In contrast, BALB/c-SV fibroblasts expressing high levels of allogeneic MHC class I molecules were lysed to minimal extents by resting NK cells.

Compared to the cytotocix activity NK cells, poly I:C activated NK cells exerted higher cytotoxic activity by lysing ES cells to about 60% and YAC cells to about 80% at an 100:1 E:T ratio (**Fig. 1B**). Importantly activated NK cells, lysed ES cells two fold less effectively than YAC cells. Embryoid body cells (EB cells) at day five of differentiation

were lysed to comparable extends like undifferentiated ES cells by activated NK cells (**Fig. 1B**).

   To asses the lysis of ES and day 5 EB cells by NK cells activated by a physiologic stimulus, NK cells were prepared from mice on day four after intravenous (i.v.) infection with the LCM-virus. Virus induced NK cells lysed undifferentiated ES cells and day five EB cells to the same degree as poly I:C activated NK cells. Additionally, YAC control cells, were also lysed comparable by LCMV induced NK cells as by poly:IC activated NK cells (**Fig. 1C**). This result excludes the possibility that the reduced lysis of ES and EB cells by resting or poly I:C activated NK cells is due to poor activation of NK cells by the non physiological stimulus poly I:C.

A
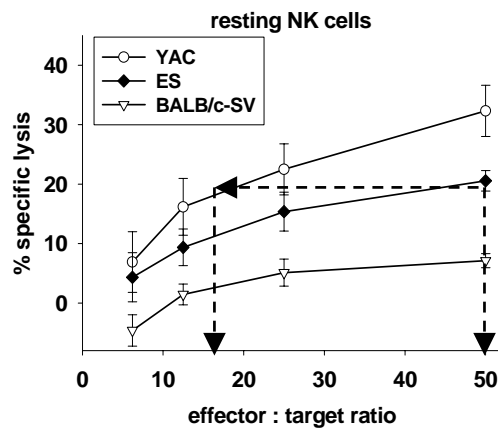
B
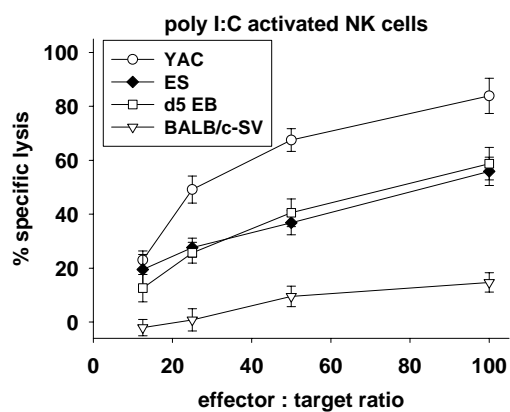
C
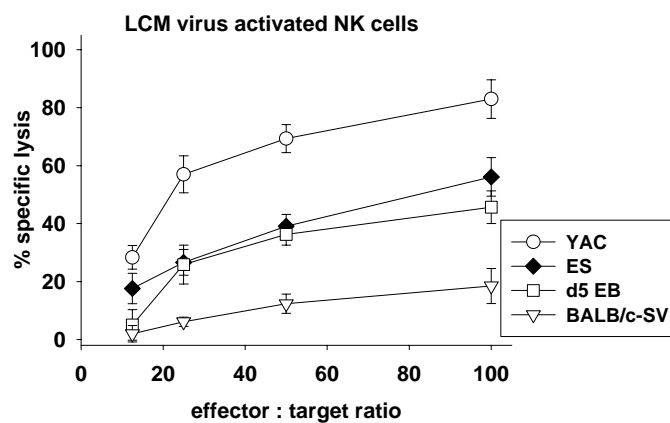
Fig. 1   ES cells and EB cells are lysed less effectively than YAC cells by resting and activated NK cells

Resting, poly I:C, or LCMV activated NK cells were prepared from B6 mice. NK cells were immunomagnetically enriched from splenic single cell suspensions and used as effector cells against undifferentiated ES cells, day five EB cells, YAC cells or Balb/c-SV fibroblasts in a 4 h ^{51}Cr release
(A) Cytotoxic activity of resting NK cells from naive B6 mice, against ES cells, YAC cells and Balb/c-SV fibroblasts.(B) Cytotoxic acivity against ES cells, day five EB cells, YAC cells or Balb/c-SV fibroblasts of NK cells activated two days prior to experiment with poly I:C. (C) Cytotoxic activity against ES cells, day five EB cells, YAC cells and Balb/c-SV fibroblasts of f NK cells activated four days prior to experiment with LCMV.

3.2. Expression of MHC class I molecules by ES and EB cells

NK cells are triggered by integration of signals from inhibitory and activating receptors. Engagement of inhibitory receptors by MHC I molecules on target cells blocks early activation signals. Vice versa, lack of MHC class I on target cells results in prevailing of activating signals and triggering of cytotoxic activity, which has been formulated as the "missing self hypothesis" of NK cell activation (Ljunggren et al., 1990). Murine ES cells were reported to be devoid of MHC class I molecules (Tian et al., 1997; , Draper et al., 2002 , Drukker et al., 2002; Bonde et al., 2006; Abdullah etal., 2007), which was confirmed by flow cytometry for the CGR8 ES cells used in this study. After induction of differentiation, MHC I is expressed at low levels by day five EB cells, while B6SV fibroblast as positive controls, express high levels of MHC class I molecules (**Fig.2A**).

IFNγ enhances the expression of MHC class I molecules in adult (Hobart et al., 1997; Niederwieser et al.,1988) as well as in embryonic cells that express IFNγ receptors on their surface (Li et al., 2004; Hobart et al., 1997).

To asses whether IFNγ enhances the level of MHC I expression on the ES and EB cells used in this study, the cells were incubated with 20ng/ml IFNγ for 48 hours. IFNγ treatment did not enhance MHC class I expression on ES cells. However, a fraction of day five EB cells responded to IFNγ with enhanced expression of MHC I, albeit not as strong as B6SV fibroblast cells (**Fig.2A**). The absent or low expression of MHC class I molecules on ES and EB cells, respectively, should render these cells ideal targets for NK cells. Although EB cells express higher levels of MHC class I molecules than ES cells, EB and ES cells were lysed to the same degree by poly I:C activated NK cells (**Fig. 1B**).

This suggests that expression levels of MHC class I on ES and EB cells might not be crucial for the susceptibility of these cells to NK cell mediated lysis.

To address this point in more detail, ES and day five EB cells were left untreated or pre-treated with IFNγ and incubated with poly I:C activated NK cells in 4h standard ^{51}Cr-release assays. The susceptibility of ES and day five EB cells pre-treated with IFNγ was indistinguishable from that of untreated cells (**Fig. 2B**). These results strongly indicate that low levels of surface MHC class I molecules are not a decisive trigger for NK cell mediated lysis of ES and EB cells.

A

B
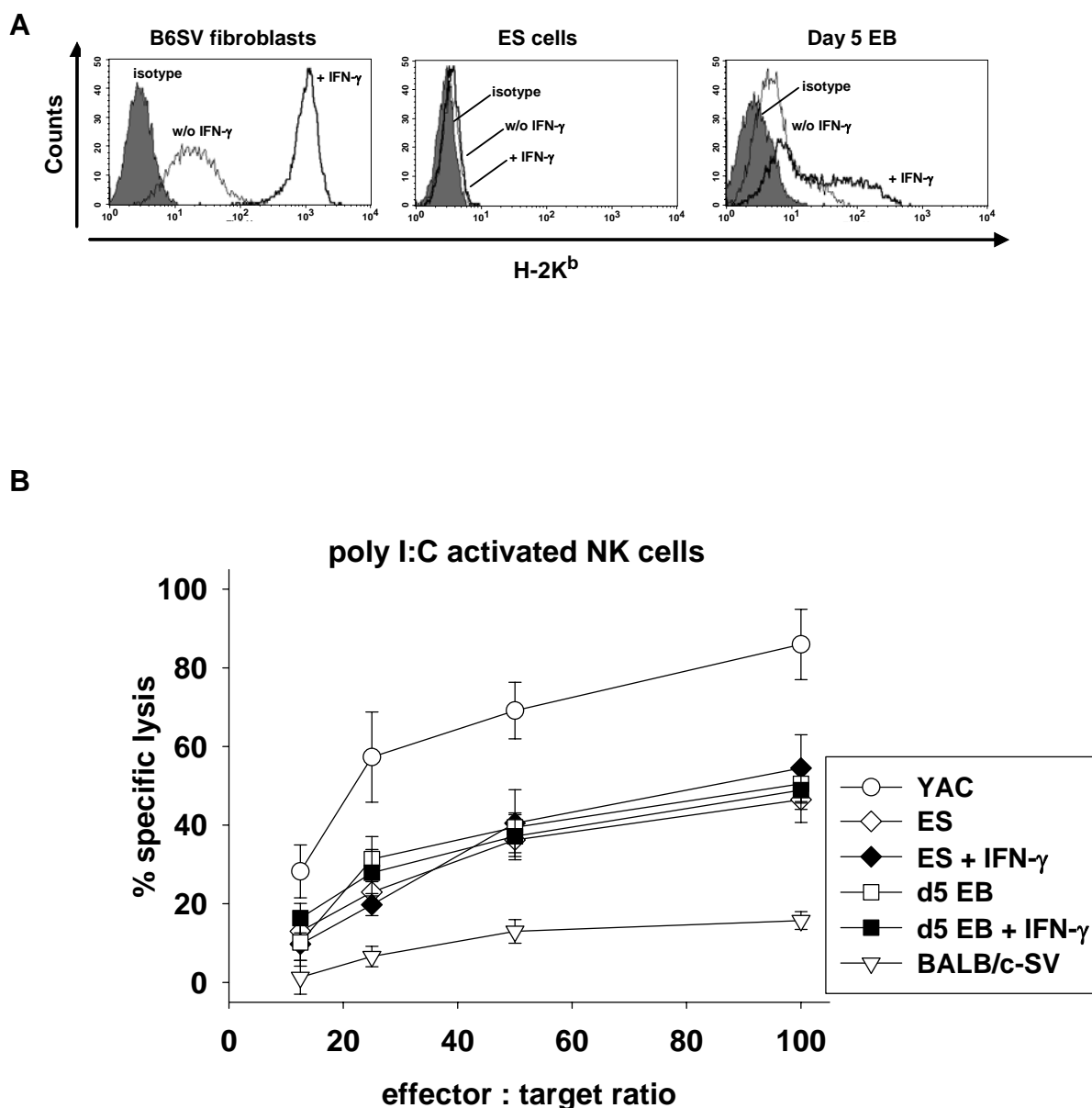
Fig. 2   Expression of MHC class I molecules on the surface of ES and day five EB cells and influence of pre-treatment of ES cells with IFNγ on susceptibility to NK cell mediated lysis.

   (A) Flow cytometric analysis of MHC class I molecules on the surface of undifferentiated ES cells and day five EB cells. B6SV fibroblasts (from H-2b C57BL/6 mice) were used as positive control. To induce expression of MHC class I molecules, IFNγ was added to a final concentration of 20ng/ml for 48 h. Single cell suspensions of ES or EB cells were stained with the monoclonal antibody specific for H2K^b (clone AF6-88.5, BD Pharmingen) or isotype control antibody. Results were analysed by FACScan and CellQuest software.

(B) Cytotoxic activity of polyI:C activated NK cells against untreated ES cells, EB cells, YAC cells and Balb/c-SV fibroblasts left untreated (white symbols), or with a IFNγ pre-treated ES cells (black symbols). Shown are cumulative data of three experiments.

3.3. Murine ES cells and EB cells are properly recognized by resting or activated NK cells

The observation that undifferentiated ES and day five EB cells are lysed less effectively than YAC cells by resting or activated NK cells (**Fig.1**) raised the question, whether NK cells do properly recognize ES and EB cells as target cells. To address this question the secretion of IFNγ by immunomagnetically enriched resting, poly I:C or virus activated NK cells of C57BL/6 mice in response to ES cells, day five EB cells, YAC cells as positive and BALB/cSV fibroblasts as negative control was determined. After a 24 h incubation time, IFNγ was measured in the supernatants by ELISA, in order to assess the recognition of the target cells by NK cells. Within 24 h, resting, poly I:C- or LCM virus- induced NK cells secreted similar high amounts of IFNγ in response to either ES cells, EB cells or YAC cells, while in response to BALB/cSV fibroblasts only background levels of IFNγ were secreted. These results indicate that NK cells recognize ES cells or day five EB cells as well as YAC cells. However, despite proper recognition, NK cells do not lyse ES and day five EB cells as effectively as YAC cells.
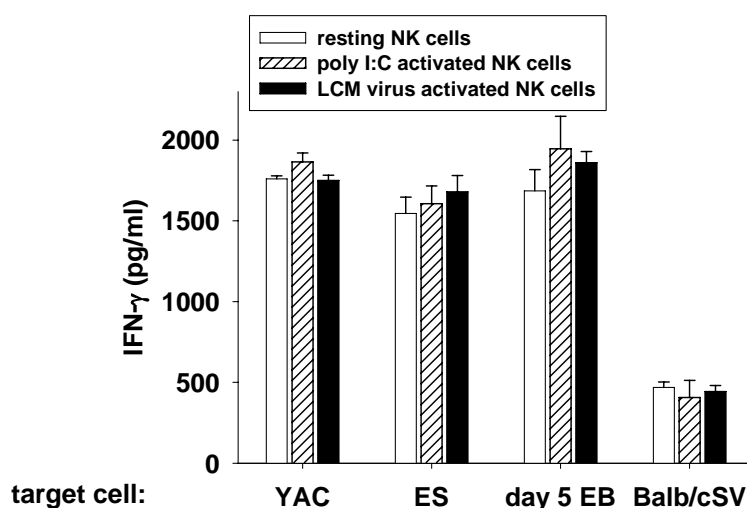
Fig. 3   Secretion of IFNγ by NK cells in response to ES and EB cells

NK cells from naive, poly I:C or LCMV activated mice were immunomagnetically enriched from splenic single cell suspensions and used as effector cells. The effector cells were incubated with undifferentiated ES cells, day five EB cells, or YAC cells (positive control) and Balb/c-SV fibroblasts (negative control). After 24 hours supernatants of the cultures were collected and IFNγ was quantified by ELISA.

3.4. Lysis of ES cells is perforin dependent

The discrepancy between full recognition but reduced cytolysis of ES and EB cells by resting or activated NK cells raised the question, whether ES cells might be resistant to the cytotoxic effector molecules used by NK cells. NK cells induce cell death via two distinct pathways: secretion of cytotoxic granules or binding and oligomerization of death receptors. The cytotoxic granules contain several cytotoxic effector molecules, including the pore-forming protein perforin and granzymes. Perforin is indispensable for the induction of target cell lysis (Trapani et al., 2002; Voskoboinik et al., 2006).

To determine if lysis of ES and EB cells is perforin-dependent, activated NK cells from perforin deficient mice (perforin$^{-/-}$) were used as effector cells in cytotoxicity assays against ES cells, YAC cells or BALB/cSV fibroblasts. Neither YAC cells nor ES cells were lysed by NK cells from perforin$^{-/-}$ (**Fig. 4**), suggesting that the lysis of ES cells by NK cells relies on the perforin dependent cytotoxic pathway.
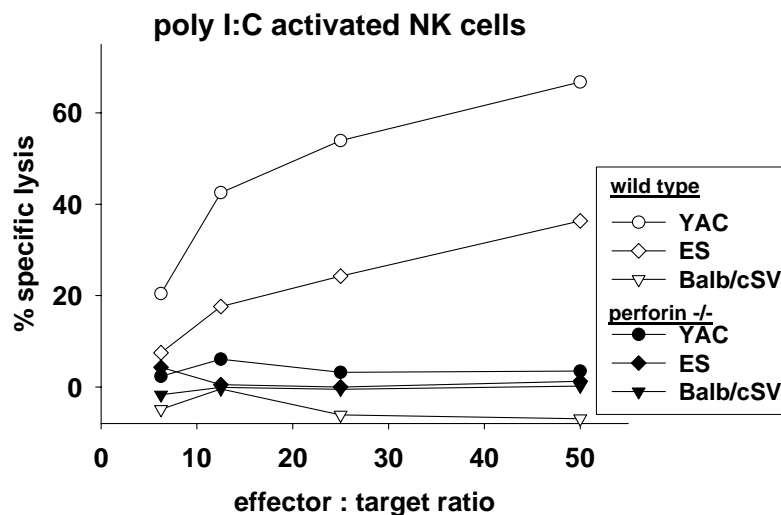
Fig. 4   Lysis of ES is perforin dependent

C57BL/6 mice and C57BL/6 perforin$^{-/-}$ mice were left untreated or activated by i.p. injection of poly I:C two days prior to the experiment. Resting and activated NK cells were immunomagnetically enriched from splenic single cell suspensions and used as effector cells in a 4h ^{51}Cr release assay against ES cells. YAC cells were used as positive controls and Balb/cSV fibroblasts as negative controls.

Lysis of ES cells, YAC cells and Balb/cSV fibroblasts by activated NK cells from C57BL/6 (white symbols) and or C57BL/6 perforin$^{-/-}$ mice (black symbols).

3.5. Expression of cathepsin B and Serpin 6 does not contribute to reduced susceptibility of ES cells to NK cell mediated lysis

We have previously shown that ES and EB cells express Cathepsin B, the specific inhibitor of perforin (Abdullah et al., 2007). To test whether Cathepsin B might be involved in the reduced susceptibility of ES cells to NK cell mediated lysis, ES cells were pre-treated with the specific inhibitor of Cathepsin B, CA074, for 16 h prior to experiment. Inhibition of Cathepsin B did not render ES cells significantly more susceptible to lysis by resting (**Fig. 5 A**) or polyI:C activated (**Fig. 5 B**) NK cells.

In addition to Cathepsin B, ES and EB cells express high levels of SPI-6, the specific inhibitor of Granzyme B. Knock down of SPI-6 expression by specific shRNA rendered ES cells fully susceptible to lysis by antigen-specific CD8^{+} CTL (34).

To determine whether low susceptibility of ES cells for NK cell mediated lysis is mediated by SPI-6, ES cells stably expressing SPI-6 specific shRNA (SPI-6-shRNA) or scrambled shRNA (scrRNA) were used as target cells for resting or activated NK cells in ^{51}Cr release assays. Knock down of SPI-6 did not enhance the susceptibility of ES cells to lysis either by resting (**Fig. 5C**) or by activated (**Fig. 5D**) NK cells. The scr ES control target cells, were lysed to the same degree as SPI-6 knock down ES cells (**Fig. 5C, D**).

Even combined inhibition of Cathepsin B and SPI-6 knock down did not enhance lysis of ES cells by either resting (**Fig. 5E**) or activated (**Fig. 5F**) NK cells.

Thus, although expressed at high levels by ES cells, neither SPI-6 nor Cathepsin B do contribute to the low susceptibility of ES cells for lysis by NK cells.
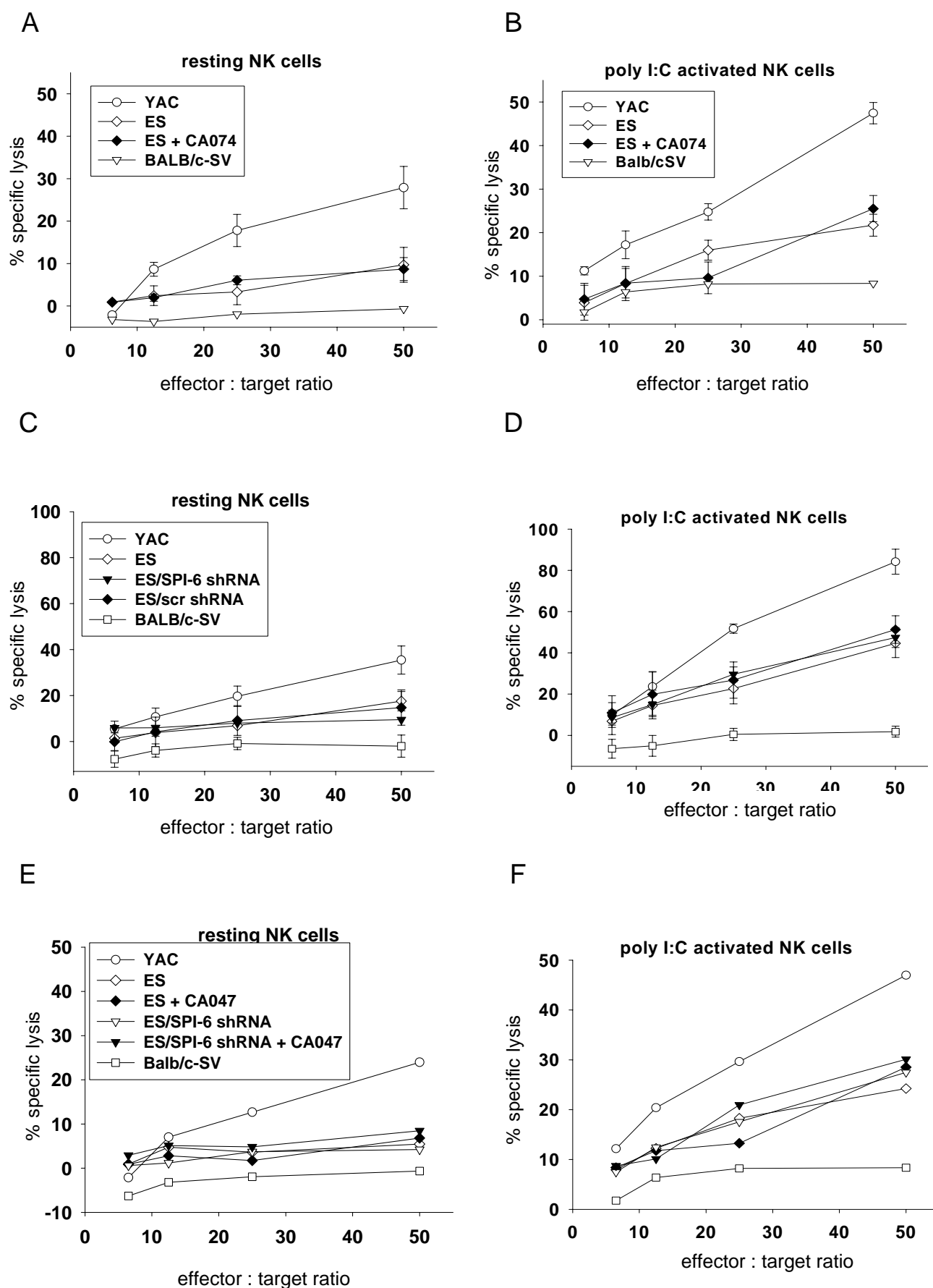
A **resting NK cells**

B **poly I:C activated NK cells**

C **resting NK cells**

D **poly I:C activated NK cells**

E **resting NK cells**

F **poly I:C activated NK cells**

Fig. 5 Expression of cathepsin B and Serpin 6 does not contribute to reduced susceptibility of ES cells for NK cell mediated lysis. (legend on next page)

70

Fig. 5 Expression of cathepsin B and Serpin 6 does not contribute to reduced susceptibility of ES cells for NK cell mediated lysis

   C57BL/6 mice were left untreated or activated by i.p. injection of poly I:C two days prior to experiment. Resting and activated NK cells were immunomagnetically enriched from splenic single cell suspensions and used as effector cells in a 4h ^{51}Cr release assay against target cells.

Resting (A, C, E) or poly I:C activated (B, D, F) NK cells were used against the following target cells:

(A, B) ES cells pre-treated with 10μM CA074 16 h prior to experiment (black symbol), untreated (white symbols) or of YAC cells or Balb/cSV fibroblasts.

(C, D) shSPI-6 ES cells, scrSPI-6 control cells (black symbols), wild type (wt) ES cells, YAC cells and Balb/cSV fibroblasts (white symbols).

(E, F) shSPI-6 ES cells, wt ES cells pre-treated with 10μM CA074 16 h prior to experiment (black symbols) and untreated control cells (white symbols)

3.6. Resting and activated NK cells express Granzyme M

Since both, NK cells and CTL, use cytotoxic granules to kill target cells, it was surprising that high level expression of Cathepsin B and SPI-6 apparently does not contribute to the low susceptibility of ES cells to NK cells. However, despite similarities in their cytotoxic effector molecules, NK cells and CTL differ considerably in the usage of specific granzymes as cytotoxic effector molecules (Sayers et al., 2001; Smyth et al.,1995). Most prominently, NK cells express high levels of granzyme M (Sayers et al., 2008; Chowdhury,et al., 2008; Janice et al., 2004). Granzyme M is involved in cytotoxicity although its exact mode of action has not yet been elucidated (Chowdhury,et al., 2008; Janice et al., 2004). The resting and activated NK cells used in this study contain large amounts of granzyme M specific mRNA and granzyme M specific enzymatic activity, as determined by quantitative real time PCR and granzyme M-specific substrate cleavage (**Fig. 6A,B**).

Resting NK cells and activated CD8$^+$ CTL harvested from mice on day eight post LCM virus infection express comparable low amounts of granzyme M-specific mRNA, while poly I:C activated NK cells express four-fold increased levels of granzyme M-specific mRNA. The granzyme M enzymatic activity of resting NK cells is already higher than that of CD8$^+$ CTL, and in activated NK cells even higher activities are detected. Although the exact role of granzyme M in NK cell mediated cytotoxicity is not yet known, differential expression and use of granzyme M by NK cells and CTL might be responsible for the differential susceptibility of ES cells to the two cytotoxic effector cell types.

A

B
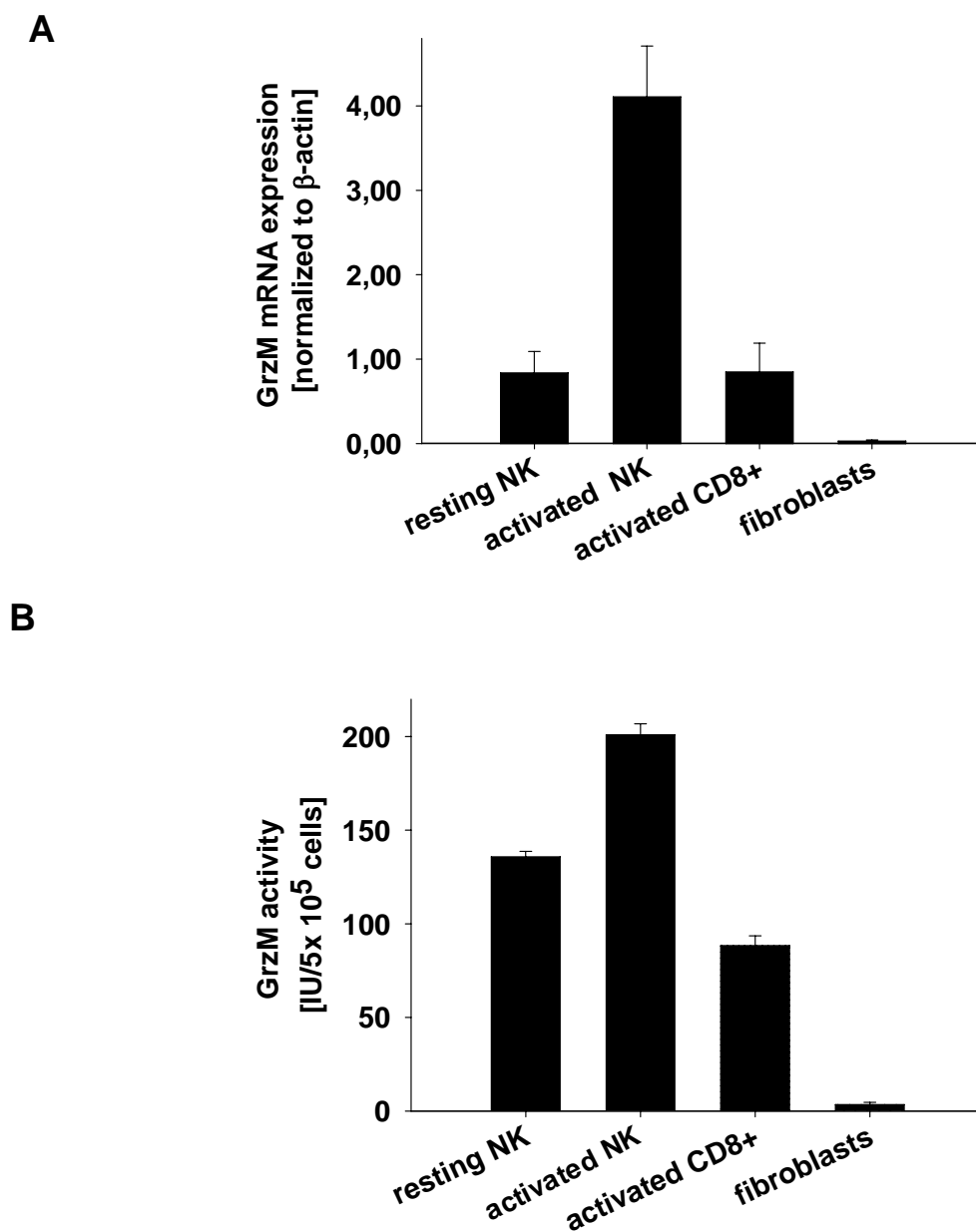
Fig. 6   Expression of granzyme M in resting and activated NK cells

Resting and activated NK cells were prepared from naïve or poly I:C activated C57BL/6 mice and were immunomagnetically enriched from spleens.

(A) Expression of mRNA coding for murine granzyme M was determined by real time PCR in resting NK cells, poly I:C activated NK cells, CD8^{+} CTL from day 8 LCM virus-infected C57BL/6 mice and B6SV fibroblasts.

(B) Granzyme M enzymatic activity of lysates from 5×10^5 resting NK cells, poly I:C activated NK cells, CD8^{+} CTL from day 8 LCM virus-infected C57BL/6 mice and B6SV fibroblasts by cleavage of the chromogenic substrate Boc-Ala-Ala-Met-SBzl .

3.7 Expression of the Serine Protease Inhibitor Involved in Cytotoxic Inhibition (SPI-CI), a specific inhibitor of granzyme M

The murine serine protease inhibitor involved in cytotoxic inhibition (SPI-CI) is a chymotrypsin-specific inhibitor of granzyme M (Bots et al., 2005), comparable to SPI-6 as the specific inhibitor of granzyme B. SPI-CI is expressed by cytotoxic effector cells, especially by NK cells. Furthermore, SPI-CI is highly expressed in immune-privileged sites e.g. placenta, testis and brain (Bots et al., 2005).

Remarkably, SPI-CI specific mRNA is expressed in ES cells, albeit at lower levels than in resting or activated NK cells (**Fig. 7**).

To determine, whether SPI-CI contributes to the reduced susceptibility of ES cells for NK cell mediated-cytotoxicity, SPI-CI was down-regulated by RNA interference in ES cells. Effective down regulation of SPI-CI mRNA was achieved with a Lentiviral vector expressing SPI-CI specific shRNA (shSPI-CI). Transduction of ES cells with this shRNA lenti-virus significantly reduced the expression of SPI-CI mRNA to about 15% of untreated control cells (**Fig.8**). In ES cells transduced with a control vector coding for a scrambled sequence (scrSPI-CI), expression of SPI-CI was almost not effected. The influence of SPI-CI on the susceptibility of ES cells for the cytotoxicity of NK cells was assessed by using shSPI-CI ES cells as target for NK cells.

shSPI-CI ES cells were lysed by, resting (**Fig. 9A**) and activated (**Fig. 9B**) NK cells, as effectively as YAC cells, while wt ES cells or scrSPI-CI ES cells were less susceptible to lysis by resting and activated NK cells. This result indicates that SPI-CI partially protects ES cells against lysis by NK cells.

To elucidate whether SPI-CI and SPI-6 cooperate in the protection of ES cells against NK cell mediated lysis, wt ES cells, ES cells expressing specific shRNA for SPI-C,I or SPI-6, or combined down regulation of SPI-CI and SPI-6 (shSPI-6/SPI-CI) were used as target cells in 4h standard ^{51}Cr release assays. ES cells that are deficient for both serpins were lysed by resting (**Fig. 10A**) and activated (**Fig. 10B**) NK cells comparable to ES cells expressing specific shRNA for SPI-CI, exclusively. Thus, the combined knock down of SPI-CI and SPI-6 was not more effective than the single knock down of SPI-CI. Therefore, these results indicate that SPI-CI plays a critical role in the reduced susceptibility of ES cells for NK cell mediated lysis, while SPI-6 does not contribute.
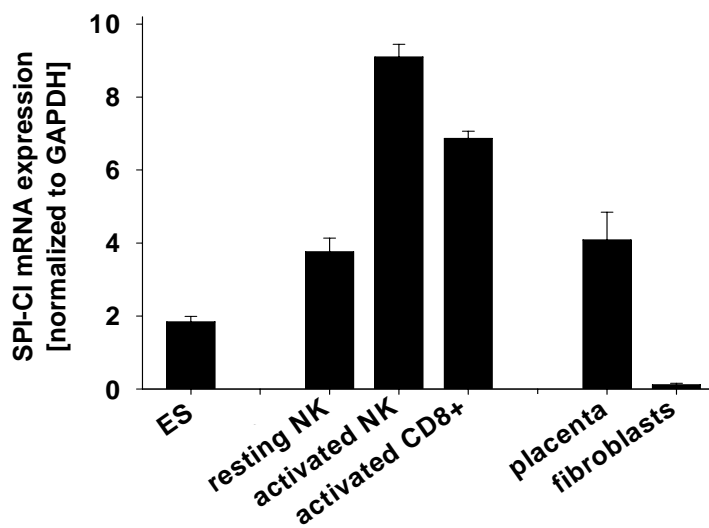
Fig. 7   ES cells express SPI-CI, the specific inhibitor of granzyme M

Expression of mRNA coding for murine SPI-CI was determined by real time PCR in undifferentiated CGR8 ES cells, resting NK cells, polyI:C activated NK cells, B6SV fibroblasts, placenta and CD8^{+} CTL from day 8 LCM virus-infected C57BL/6 mice. Expression levels were normalized to GAPDH.
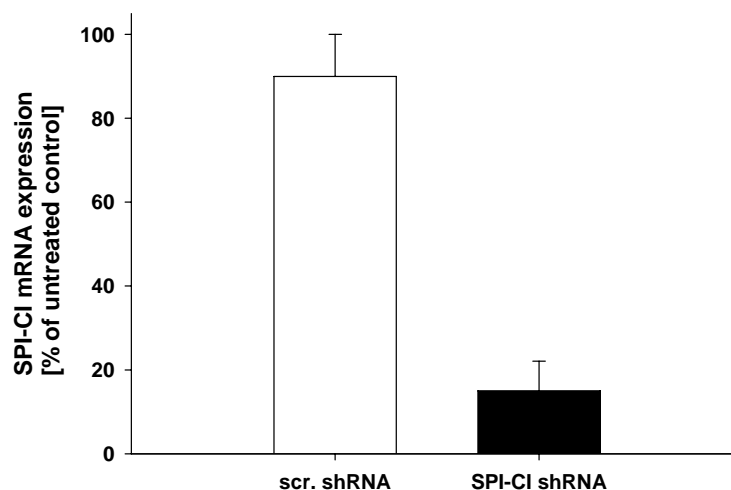
Fig. 8   Down regulation of SPI-CI on mRNA level by specific shRNA

Quantification of SPI-CI mRNA in CGR8 ES cells after transduction with vectors expressing shRNA targeting the specific region of murine SPI-CI (SPI-CI shRNA), or a non-specific scrambled shRNA (scr. shSPI-CI) after selection with blasticidin and expansion of single cell clones . The expression level was normalized to the level measured in non-treated control cells.

A

B
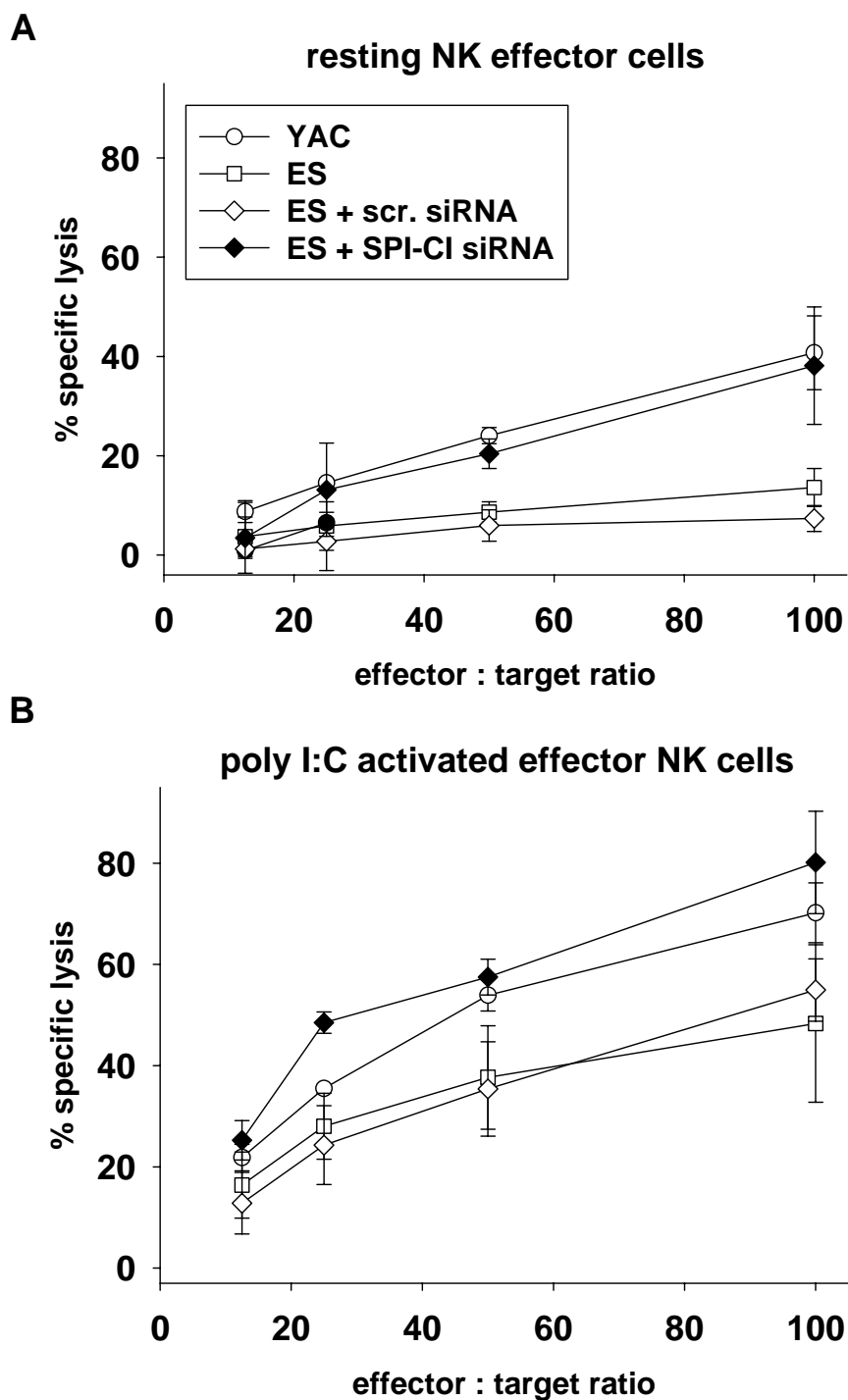
Fig. 9 SPI-CI partially protects ES cells against cytolysis by resting or activated NK cells

C57BL/6 mice were left untreated or were activated i.p. with poly I:C two days prior to experiment. Resting and activated NK cells were immunomagnetically enriched from splenic single cell suspensions and used as effector cells in a 4h ^{51}Cr release assay against target cells.

(A) Susceptibility of shSPI-CI ES cells, scrSPI-CI control cells, wt ES cells and YAC cells to resting NK cells from naïve C57BL/6.

(B) Susceptibility of shSPI-CI ES cells, scrSPI-CI control cells, wt ES cells and YAC cells to polyI:C activated NK cells from B57BL/6. Shown are the cumulative data of three experiments.

A

B

resting NK cells

poly I:C activated NK cells
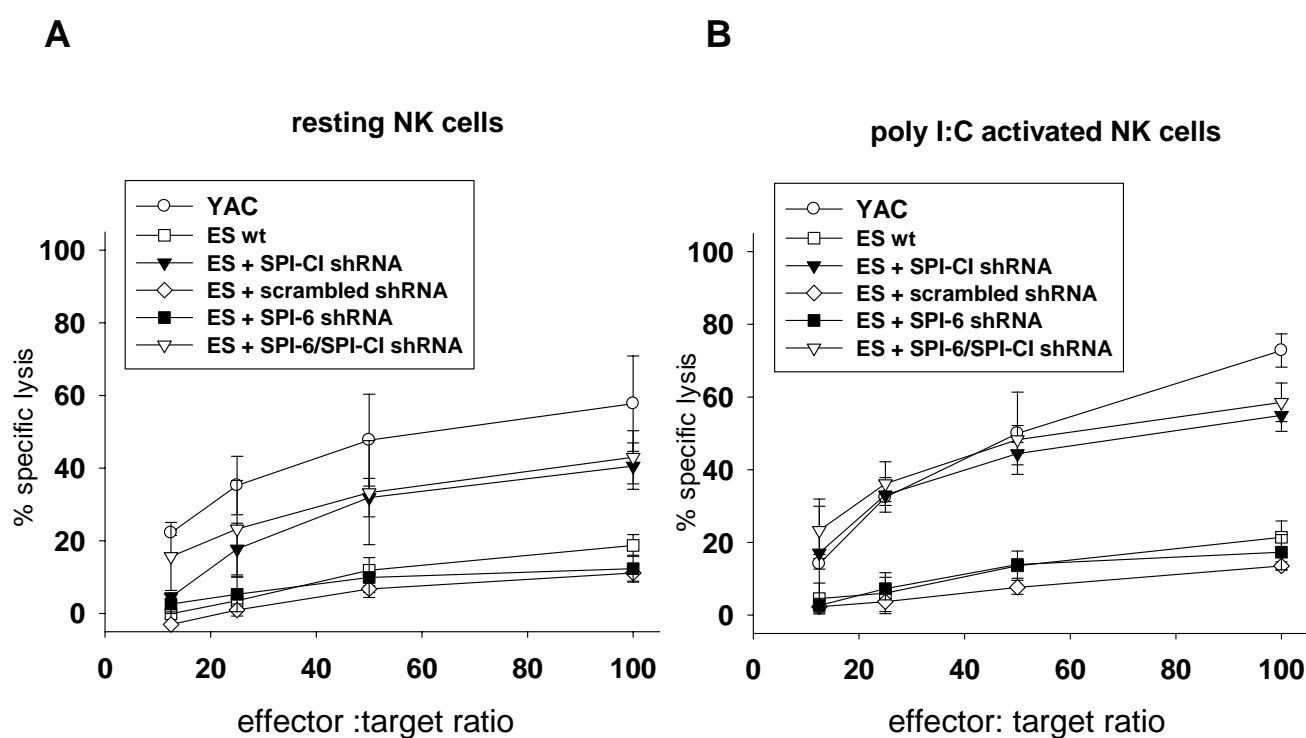
Fig. 10   SPI-CI and SPI-6 do not cooperate in the protection of ES cells from NK cell mediated lysis

C57BL/6 mice were left untreated or activated i.p. with poly I:C two days prior to experiment. Resting and activated NK cells were immunomagnetically enriched from splenic single cell suspensions and used as effector cells in a 4h ^{51}Cr release assay against target cells.

(A) Susceptibility of shSPI-CI ES cells, shSPI-6 ES cells, scrSPI-CI control cells, wt ES cells, YAC cells and shSPI-6/SPI-CI cells to resting NK cells.

 (white symbols) to resting NK cells from naïve B57BL/6.

(B) Susceptibility of shSPI-CI ES cells, shSPI-6 ES cells, scrSPI-CI control cells, wt ES cells, YAC cells and shSPI-6/SPI-CI cells to polyI:C activated NK cells.

Shown are the cumulative data of three experiments.

77

3.8. SPI-CI protects ES cells from NK-cell mediated lysis *in vivo*

SPI-CI reduces susceptibility of ES cells for NK cell mediated lysis *in vitro.* The effects of SPI-CI on the susceptibility of ES cells to NK cells were assessed *in vivo* by monitoring the accumulation of PKH labelled wild type ES cells, shSPI-6 ES cells, shSPI-CI ES cells, shSPI-6/SPI-CI cells, scrambled control ES cells or YAC cells in the spleens. Following i.v. inoculation of $1x10^7$ cells, the numbers of fluorescent cells accumulating in the spleens of naïve or poly I:C pre-treated mice were determined after 30 minutes and 4 hours. Within 30 minutes after injection about $1x10^5$ to $2x10^5$ fluorescent cells were detectable per spleen, regardless whether SPI-CI or SPI-6 were expressed or not. By four hours after inoculation, numbers of wild type and scrambled shRNA control ES cells in the spleens increased up to $8x10^5$ cells per spleen, while the numbers of SPI-CI shRNA ES cells remained at about the level of 30 minutes (**Fig. 11A**). SPI-6 shRNA cells accumulated as well as wt control cells, while SPI-6/SPI-CI double knock down cells behaved like SPI-CI shRNA cells. YAC cells were at 30 minutes and 4 hours after inoculation only detectable in minimal numbers below $0,5x10^5$ per spleen.

In vitro, ES cells were more susceptible to lysis by activated NK cells than by resting NK cells. By monitoring the accumulation of fluorescent cells in poly I:C pre-treated mice, it was assessed whether SPI-CI influences the susceptibility of ES cells for the cytotoxicity of activated NK cells. Similar to the observations in naïve mice, wt ES cells accumulated in the spleens of poly I:C treated mice between 30 minutes and 4 hours after injection of the cells, albeit the increase was lower than in naïve mice (**Fig. 11B**). In contrast, after 4 hours the number of SPI-CI shRNA ES cells even decreased compared to 30 minutes.

The decreased accumulation of shSPI-CI ES cells in naïve or poly I:C treated mice might be due to either altered migration of the cells within the acceptor mice or to destruction of the cells by NK cells. To differentiate between these two explanations the above described experiments were performed in mice with an impaired granule mediated cytotoxicity due to inactivation of the perforin gene (perforin$^{-/-}$). In sharp contrast to wt acceptor mice, in perforin$^{-/-}$ mice shSPI-CI ES cells or as well control ES cells, accumulated to high numbers in the spleens within 4 hours after injection (**Fig. 11C**). This suggests that the failure of shSPI-CI ES cells to accumulate in

spleens of wt mice is due to the elimination of these cells by granule mediated cytotoxicity.

To confirm these findings, we assessed the susceptibility of these cells in beige mice. Beige mice are profoundly deficient for NK cells and CTL mediated lysis (50) and are an accepted model to examine the role of NK cells *in vivo*. Like in perforin $^{-/-}$ mice, and in contrast to wt mice, shSPI-CI ES cells or as well control ES cells accumulated to high numbers in the spleens after 4 hours (**Fig. 11D**). The high amounts of YAC cells in the spleens of beige mice after 4 hours suggests that the susceptibility of shSPI-CI ES cells is dependent on NK cells.

These *in vivo* results suggest, that in line with the *in vitro* data, SPI-CI protects ES cells *in vivo* from perforin dependent NK cell mediated lysis.
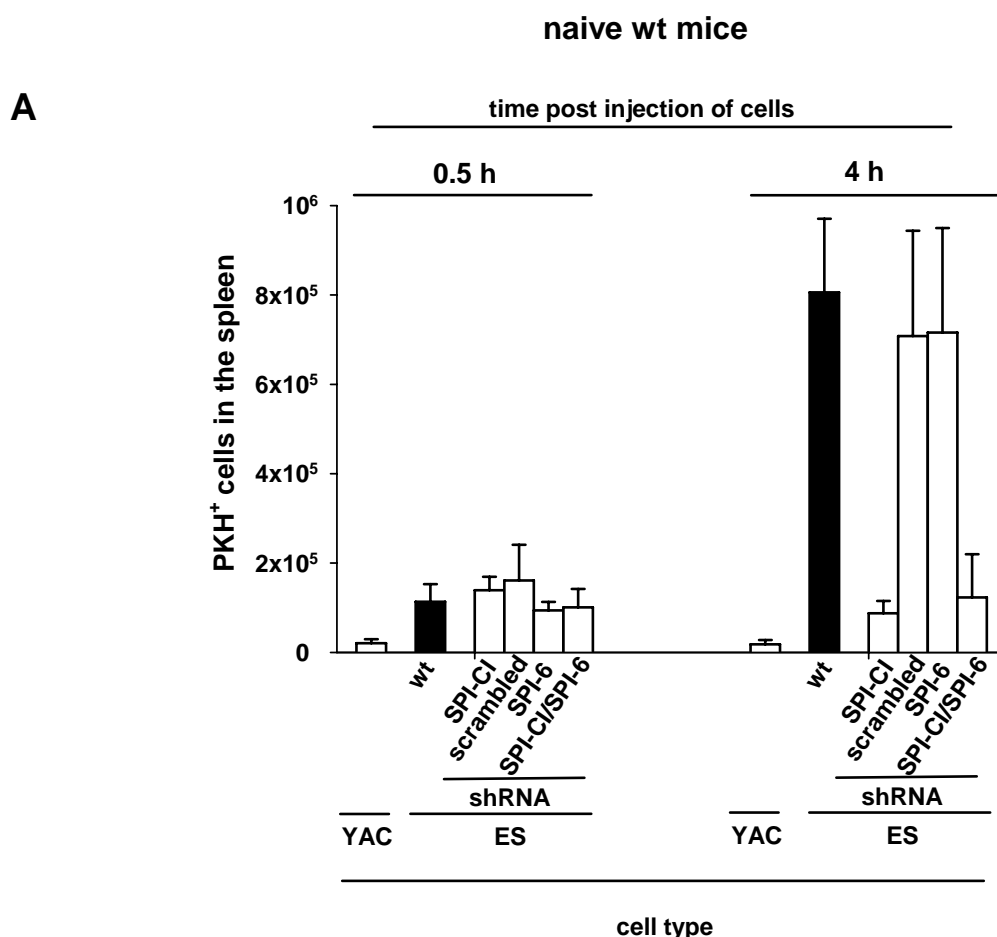
Fig. 11 SPI-CI protects ES cells from NK-cell mediated lysis *in vivo.*
   (Legend on page 81)

poly I:C treated wt mice

B

naive perforin -/- mice

C
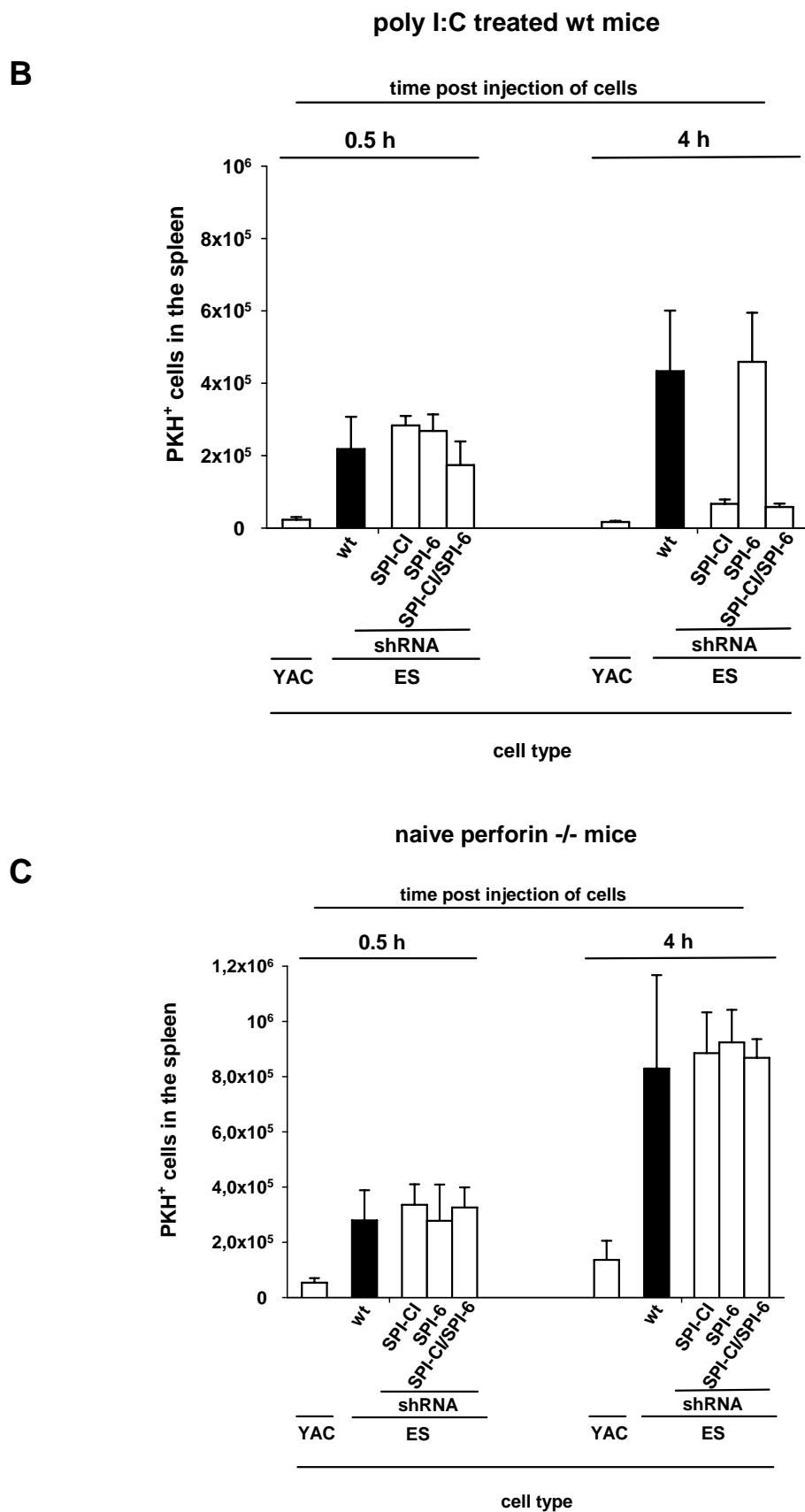
Fig. 11 SPI-CI protects ES cells from NK-cell mediated lysis *in vivo*. (legend on next page)

D

naive beige mice
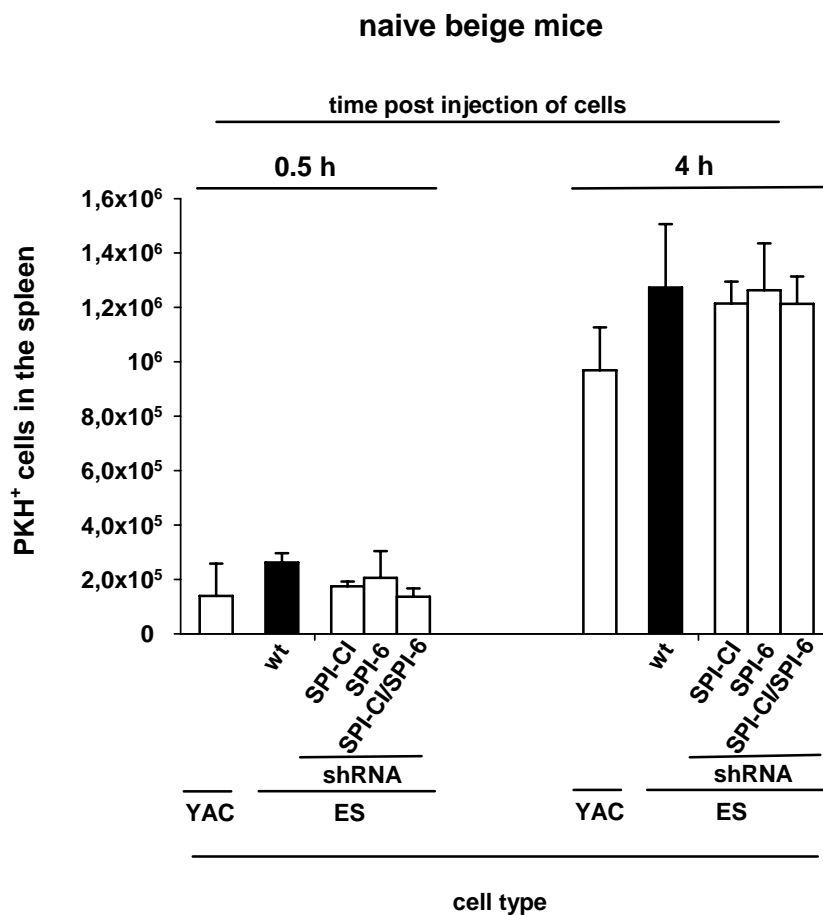
Fig. 11   SPI-CI protects ES cells from NK-cell mediated lysis in vivo

WT ES cells, shSPI-6 ES cells, shSPI-CI ES cells, shSPI-6/SPI-CI cells, scrSPI-CI control cells and YAC cells were labeled with PKH. 1×10^7 of PKH labeled cells were i.v. inoculated into naïve or two day prior to experiment poly I:C activated C57BL/6 mice. 30 minutes and 4 hours later the total number of living fluorescent cells per spleen was ascertained by FACS-Analysis. Total number of inoculated, fluorescent cells in the spleens of: (A) naïve C57BL/6 mice, (B) poly I:C activated C57BL/6 mice, (C) naïve perforin [-/-] mice and (D) naïve beige mice. Every bar represents a minimum of four individuals.

3.9. Expression of SPI-CI contributes to the tumorigenicity of ES cells *in vivo*

After having shown the relevance of SPI-CI expression for acute resistance of ES cells to elimination by NK cell mediated cytotoxicity *in vivo* for up to four hours, long term effects of SPI-CI remained to be evaluated. To this end, the contribution of SPI-CI to the capacity of ES cells to form teratomas in immuno-competent syngeneic hosts was assessed. Following subcutaneous (s.c.) inoculation of 2×10^6 wild type ES cells and scrambled control ES cells, teratomas were detectable from about day seven and grew rapidly with similar kinetics. In contrast, SPI-CI knock down ES cells caused only in 60 % of the recipients slow growing teratomas, measuring by day 27 below 2 mm in diameter as opposed to 10 mm resulting from wt ES cells (**Fig. 12A, B**). These results indicate that SPI-CI contributes to the tumorigenicity of ES cells in syngeneic immuno-competent hosts *in vivo*.

A

s.c. growth of teratoma in syngenic mice

B

tumor free mice
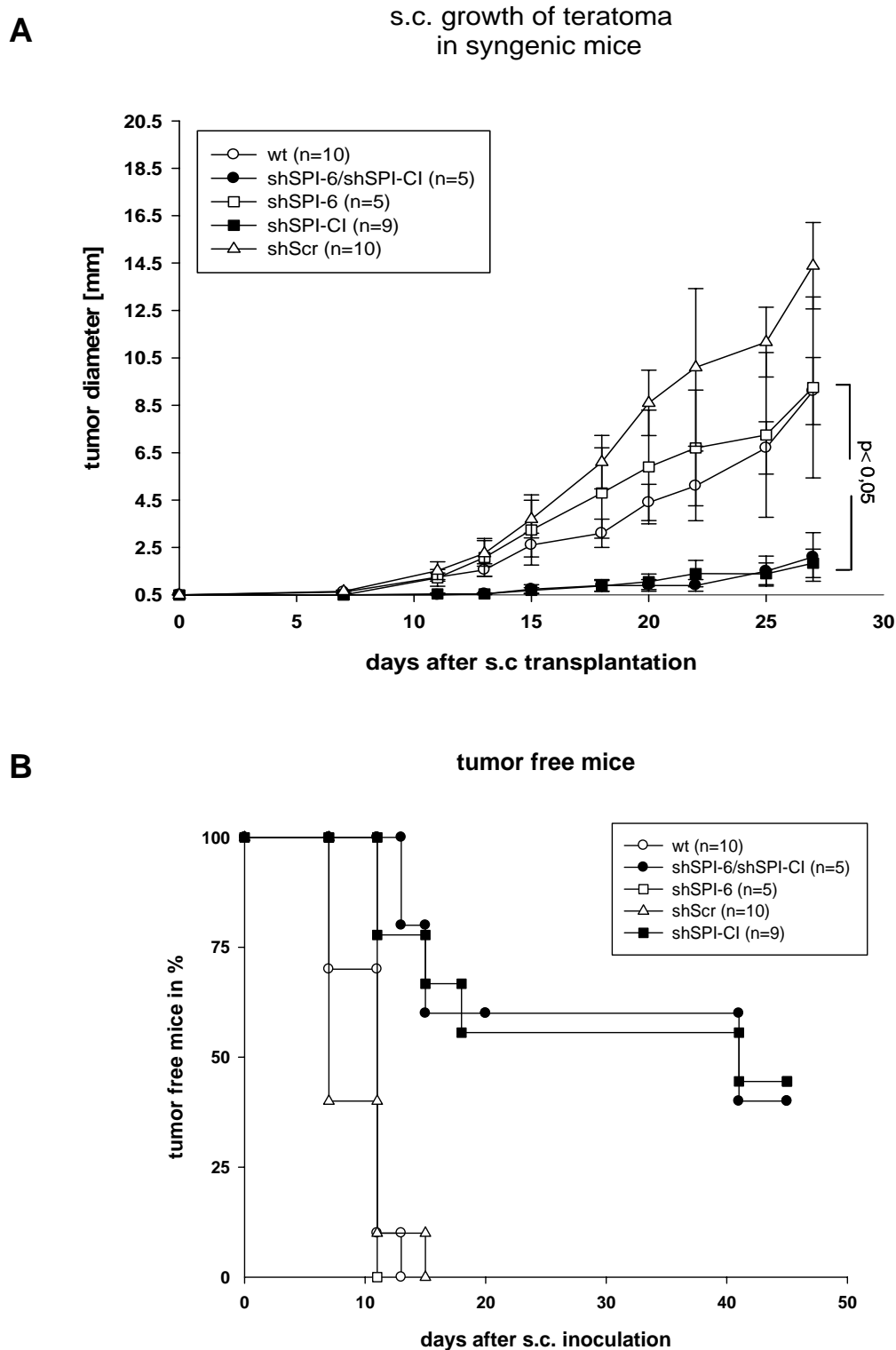
Fig. 12   Reduced *in vivo* tumorigenicity of ES cells deficient for SPI-CI

$2x10^6$ shSPI-CI ES cells, shSPI-6/SPI-CI ES cells (black symbols) wt ES cells, shSPI-6 ES cells and scrambled control ES cells (white symbols) were injected subcutaneously into the neck of complete syngeneic male 129/Ola Hsd mice. (A,B) The tumor size was recorded every second day using linear callipers. The tumor growth of the individual groups is shown

4. Discussion

In many animal models effective tumor-specific immunotherapy has been archived (Blattman and Greenberg, 2004). However, success in clinically applied tumor-immunotherapy has been rather limited. Depending on the specific situation this might be due to limited strength or duration of the immune response, or to tumor cells evading from or even counterattack against the immune system (Gabriel et al., 2006; Siqing et al ., 2006; Qiang et al., 2001; Melief 2008, Ochsenbein, 2002). From these experiences can be concluded that chances for effective and reliable immunotherapy of tumors depend on a thoroughly insight into the interactions between tumors and the immune system in the respective microenvironment during each single phase of this immune response. By detailed knowledge of the interaction of specific immune cells, of their recruitment into the tumor site, and of the delivery of their effector functions. It should be possible to further enhance desired and to block inhibitory immuno-regulatory mechanisms. In this project we revealed three quantitatively different approaches to enhance the effectiveness of tumor specific immune responses. These approaches are: (i) the inhibition of IL-4 even during the active tumor effector phase, resulting in enhanced cytotoxic activity of CD8$^+$ CTL. (ii) The inhibition of IL-10 during i.p. immunization and i.p. tumor-challenge, resulting in a shift from conventional CD8$^+$ CTL to more potent tumor-specific CD8$^+$ IEL, and (iii) the down regulation of SPI-CI in tumor stem cells in order to increase their susceptibility for cytotoxic NK cells.

These three immuno-regulatory aspects of tumor-specific immunotherapy will be consecutively discussed below.

4.I. Interleukin-4 impairs granzyme-mediated cytotoxicity of Simian virus 40 large tumor antigen-specific CTL in BALB/c mice

IL-4 is a key cytokine regulating the induction and differentiation of T helper cell subsets (Seder at al., 1994; Murphy et al., 2002; Agnello et al., 2004). IL-4 generally supports humoral immune responses and suppresses cellular immunity. Nevertheless, both, supportive or inhibitory effects of IL-4 upon CD8$^+$ T cells have been reported (Utermöhlen et al., 2001; J. Zerrahn et al., 1996, Miller et al., 1990;

Erard et al., 1993; M. Croft et al., 1994; Noble et al., 1995; Moran et al., 1996; Aleman et al., 2002; Bright et al 1996; Gooding et al., 1977; Knowles et al., 1979; N. Kienzle et al 2002, 2005; Schuler et al., 2001, 1999). In this study we analyzed the influence of endogenous IL-4 on a CTL response against a notoriously weakly immunogenic tumor antigen.

BALB/c mice are considered to be low- or even non-responders with respect to the generation of SV40 TAg-specific CTL, while C57BL/6 mice mount a strong TAg-specific CTL response (Bright et al., 1996, 1995; Gooding et al., 1977; Knowles et al., 1977; Newmaste et al., 1998; Schirmbeck et al., 1996; Tevethia etal., 1990). We previously detected a small population of highly active TAg-specific CD8$^+$ CTL in the peritoneal cavity of TAg-immunized BALB/c mice challenged i.p. with TAg-expressing mKSA tumor cells (Zerrahn et al., 1996; Utermöhlen et al., 2001), thereby providing us with a system for testing CTL activity induced by this very weakly immunogenic antigen.

Our finding that CD4$^+$ TAL secrete IL-4 during TAg-specific rejection of mKSA cells was somewhat unexpected, as we had already reported that CD4$^+$ TAL secrete the prototypic Th1 cytokines IL-2 and IFN-γ (Bright et al., 1996). Thus the tumor site at the same time harbors CD4$^+$ T cells secreting antagonistic cytokines, namely IL-4 and IFN-γ. Furthermore, it had been published that the cytokine profile in TAg-immune BALB/c mice is a typical TH1 profile (Bright et al., 1995). Specifically, Bright and colleagues detected the Th1 factors IL-2 and IFN-γ, but neither IL-4 nor IL-5. However, this apparent discrepancy can be easily explained by the different assay conditions applied. We studied cytokine secretion by CD4$^+$ TAL enriched from the peritoneal cavity during the acute rejection phase of mKSA cells without any stimulation *in vitro*, while these authors investigated the cytokine profile of crude spleen lymphocyte preparations from TAg-immunized mice after re-stimulation with TAg *in vitro*.

The decisive question about the effects of any given cytokine during tumor-specific immune responses is whether this cytokine is necessary, beneficial, futile, or even detrimental for the outcome of tumor cell elimination. In our system, TAg-immunized IL4$^{-/-}$ as well as wild-type mice survived a challenge with 10^6 viable mKSA cells, equalling the ten thousand-fold LD$_{50}$ of these tumor cells in naïve BALB/c mice, without developing any signs of tumor growth. Additionally, the kinetics of tumor cell elimination was similar in wt and IL-4$^{-/-}$ mice. These data clearly indicate that IL-4 is

not required for an effective rejection of the highly malignant mKSA tumor cells in immunized mice. However, a significant gain in survival time of naïve IL-4$^{-/-}$ mice over wild-type controls challenged with low doses of 10^3 mKSA cells was observed. CD8$^+$ TAL harvested on days 8, 14, or 21 after challenge of naïve wt or IL-4$^{-/-}$ mice did not exert significant TAg-specific cytotoxic activity in vitro. However, increases in both the total numbers of CD8$^+$ TAL and in the percentage of gzmA-expressing CD8$^+$ TAL within the peritoneal cavity of wt and IL-4$^{-/-}$ mice at day 14 after challenge suggest that mKSA cells induce a T cell response in naïve mice of both genotypes. Remarkably, in IL-4$^{-/-}$ mice the increase in gzmA-expressing CD8$^+$ TAL was much more pronounced than in wt controls. Moreover, depletion of naive IL-4$^{-/-}$ mice prior to challenge abolished their prolonged survival time while the shorter survival time of wt mice was not further reduced by depletion of CD8$^+$ T cells. These in vivo data strongly indicate that the absence of IL-4 allows the generation of a quite effective mKSA tumor-specific CD8$^+$ T cells response in naïve mice. Unfortunately, this T cell response apparently becomes insufficient for sustained control of the tumor as indicated by the steep decline in the numbers of total as well as gzmA-expressing CD8$^+$ T cells between days 14 and 21 after challenge. It remains to be elucidated wether the CD8$^+$ T cells are simply diluted out by bleeding into the peritoneal cavity from vessels eroded by mKSA cells. Alternatively, a more specific mechanism might be at work, e.g. the exhaustion of the small numbers of CD8$^+$ T cells facing the growing tumor load. This mechanism has been observed both in chronic virus infections and in tumor systems (Gallimore et al., 1998; Klein et al., 2003). A better understanding of these mechanisms might provide means to enhance and support the endogenous T cell response so that more effective and sustained tumor cell control can be achieved. A recent report by Kelso and colleagues indicates that direct effects of IL-4 on the effector functions of tumor specific CTL in vivo may be quite common (Olver et al., 2006). This group observed that transgenic expression of IL-4 by P815 tumor cells led to a reduced clearance of this tumor and to a deviated cytokine and granzyme profile of tumor associated CD8$^+$ T cells. In our system, the IL-4 produced by CD4$^+$ T cells at the tumor site in wt mice does not result in the generation of CD8$^+$ T cells producing IL-4 themselves. This might be due to the lower amounts of IL-4 secreted by endogenous CD4$^+$ T cells as compared to transgenic tumor cells.

In search for the mechanism by which IL-4 impairs TAg-specific resistance against mKSA tumor cells, a suppressive effect of IL-4 on the TAg-specific CD8$^+$ CTL response was observed. Thus, the cytotoxic activity of CD8$^+$ TAL was significantly enhanced in TAg-immune IL-4$^{-/-}$ BALB/c mice after challenge with mKSA cells as compared to wild type controls. In addition to the primary *ex vivo* CTL response during tumor cell rejection, also the secondary response of CTL from spleens of TAg-immunized and mKSA-challenged mice restimulated *in vitro* was significantly enhanced (data not shown). This suggests that in this system the effects of IL-4 are imprinted on the effector properties of CTL during the primary challenge phase.

Concerning the mechanism of the inhibitory effect of IL-4 in the mKSA system, we found that in IL-4$^{-/-}$ mice a significantly enhanced proportion of the CD8$^+$ TAL express the cytotoxic effector molecules granzyme A and B during rejection of mKSA tumor cells as compared to wt mice. More importantly, the gzmB-specific enzymatic activity in the lysates of IL-4$^{-/-}$ CD8$^+$ TAL was significantly increased. As granzyme A and B are only expressed by mature CD8$^+$ CTL, these findings indicate that IL-4 inhibits the maturation of the cytotoxic effector function of CD8$^+$ T cells directly. This conclusion is further supported by our finding that in IL4$^{-/-}$ mice neither the overall number of CD8$^+$ TAL, nor the proportion of CD8$^+$ TAL specific for TAg, as assessed by intracellular accumulation as well as secretion of IFN-γ in CD8$^+$ TAL stimulated with TAg-expressing target cells, was increased. IL-4 thus does not inhibit the proliferation of antigen-specific CD8$^+$ TAL in this system. Direct effects of IL-4 on CD8$^+$ T cells *in vitro* have been recently described by Kelso and colleagues (Kienzle et al., 2002, 2004, 2005). In a series of elegant studies these authors showed that stimulation of naïve CD8$^+$ T cells by immobilized CD3-, CD8-, and CD11a-specific mAb in the presence of IL-4 resulted in a gradual loss of CD8 expression and in reduced levels of perforin and granzyme mRNA, accompanied by reduced cytolytic activity. In our system, the expression levels of CD8 were not reduced in IL-4-competent wt mice. Nevertheless, our data further expand the findings reported by Kelso´s group to the *in vivo* situation by demonstrating that endogenous IL-4 *in vivo* has a comparable inhibitory effect on the expression of cytotoxic effector molecules by CD8$^+$ T cells.

However, when IL-4 acts indirectly on CD8$^+$ CTL, e.g. via its effects on the maturation of DC, a different picture is obtained. Blankenstein and colleagues (Schuler et al.,1999, 2002) reported that establishment of tumor specific immunity against a mammary adenocarcinoma and a colon carcinoma was severely impaired

in IL-4$^{-/-}$ mice. By using IL-4$^{-/-}$ and IL-4 receptor$^{-/-}$ mice in adoptive transfer experiments, they showed that in their system IL-4 is produced by CD8$^+$ T cells and contributes to the activation of DC, i.e. is effective during the initiation phase of the immune response. Thus, in different systems IL-4 can act either indirectly during the initiation phase, or directly during the effector phase of a CTL response, thereby leading to diverse effects in different systems.

The observation of a CD8$^+$ T cell-dependent gain of survival time of IL-4$^{-/-}$ mice challenged with low doses of mKSA cells suggests that these mice spontaneously mount a tumor-specific T cell response. However, this T cell response is insufficient to eradicate the tumor cells. In patients, CTL responses against tumor antigens also are usually rather weak, regardless of whether the CTL were elicited spontaneously or by immunization with specific vaccines. Our observations may stimulate studies aimed at enhancing the activity of tumor antigen-specific CD8$^+$ CTL by eliminating the effects of IL-4. This approach seems rather promising, since in our system both the complete lifelong absence of IL-4, as well as the neutralization of IL-4 by specific mAb during the effector phase of tumor rejection resulted in comparably enhanced TAg-specific CTL activity. The data show promising prospects to interfere with IL-4, e. g. by mAb or soluble IL-4 receptor, as a potent modality to enhance and prolong CTL responses against tumor-specific antigens in immunotherapeutic treatment of cancer.

4.II. Highly effective tumor antigen-specific cytotoxicity of intraepithelial lymphocytes unfolded in interleukin-10-deficient mice

IL-10 suppresses cellular immune responses in many systems (reviewed in Montufar-Solis et al., 2007), but IL-10 also enhances CD8$^+$ T cell responses in some instances (Chen et al., 1991; Giovarelli et al., 1995; Santin et al., 2000; Fujii et al., 2001). In this light, the effect of IL-10 on the rejection of i.p. injected, TAg-expressing mKSA tumor cells by TAg-immunized BALB/c mice appeared rather unpredictable.

We observed that IL-10 is not required by TAg-immunized BALB/c mice to mount a protective immune response against a lethal challenge inoculum of mKSA tumor cells. On the contrary, IL-10$^{-/-}$ mice rejected mKSA cells with accelerated kinetics. These data thus suggested that IL-10 suppresses the TAg-specific immune response by which wt mice reject mKSA cells.

Detailed analysis revealed, that the lack of IL-10 does not simply result in a quantitatively enhanced, but rather in a qualitatively different immune response. Instead of generating intraperitoneal CD8^{+} lymphocytes with an enhanced cytotoxic activity as observed in IL-4^{-/-} mice (Baschuk et al.,2007), IL-10^{-/-} mice were devoid of CD8^{+} T cells with TAg-specific cytotoxic actitivity in the peritoneal cavity at any time after challenge. This functional shortage was accompanied by a very limited increase of the absolute numbers of intraperitoneal CD8^{+} lymphocytes in IL-10^{-/-} as compared to wt mice. These observations could be explained as a consequence of the early elimination of mKSA cells from the peritoneal cavity of IL-10^{-/-} mice, i.e. a reduced antigenic stimulus preventing the maturation and/or recruitment of antigen-specific CD8^{+} T cells to this site.

Although CD8^{+} CTL specific for mKSA cells were not detectable in the peritoneal cavity of IL-10^{-/-} mice, CD8^{+} T cells were identified as critically required for rejection of mKSA cells in these mice by depletion of CD8^{+} T cells with CD8-specific mAb. Anatomical proximity to the site of tumor cell inoculation led us to hypothesize that CD8^{+} intestinal intraepithelial lymphocytes (IEL) may be the effector cell population that mediates accelerated rejection of mKSA tumor cells in IL-10^{-/-} mice.

Intestinal IEL are heterogeneous with respect to their phenotype, development and function (reviewed in Cheroutre 2005). The most typical common feature of intestinal IEL is their particular effector memory phenotype for which terms like "activated yet resting" (Cheroutre 2005) or "partially activated" (Roberts et al., 1993) have been coined. These terms refer mainly to the spontaneous cytotoxicity of CD8^{+} IEL in *ex vivo* assays.

The spontaneous cytotoxicity of human intestinal IEL is directed against several cancer cell lines and not against the NK cell-sensitive target cell line K-562 (Roberts et al., 1993), but the exact antigenic specificity of these IEL is largely unknown. In the murine system studied here, the highly cytotoxic CD8^{+} IEL harvested from IL-10^{-/-} mice specifically lysed TAg-expressing mKSA cells, but not the TAg-negative, syngeneic Meth A tumor cells. Moreover, mKSA-specific cytolysis was strictly dependent on prior immunization of IL-10^{-/-} mice with TAg, suggesting that the CD8^{+} IEL are specifically reacting to TAg. It appears likely that the generation of TAg-reactive IEL depends on the intraperitoneal route of immunization with TAg we have chosen. Via this route, TAg might get access to the intestine-associated lymphatic

tissues, in which IEL are primed against antigens derived from intestinal pathogens, as shown previously for the model antigen ovalbumin injected i.p. (Kim et al., 1998).

The frequency of TAg-specific CD8$^+$ IEL was found to be in IL-10$^{-/-}$ mice up to threefold higher than in wt mice, which appears surprisingly low considering the extremely rapid and effective eradication of mKSA cells in IL-10$^{-/-}$ mice and the stunning difference in the TAg-specific cytotoxic activity between IL-10$^{-/-}$ and wt CD8$^+$ IEL. It remains to be investigated in more detail, whether antigen-specific CD8$^+$ IEL from IL-10$^{-/-}$ and wt mice differ with regard to the amount or composition of their cytotoxic effector molecules.

IEL are assumed to be in constant standby mode as a first-line defense against enteral pathogens or early intestinal cancer cells. Thus, within the intestinal epithelia they are strategically positioned to ward off pathogens invading the epithelia from the luminal side or against tumor cells emerging from within the epithelia. Our data suggest that CD8$^+$ IEL are also able to respond to invaders approaching from the visceral surface of the intestine. The challenging inoculum of mKSA cells is injected intraperitoneally and our histologic analyses show micrometastases mainly within the peritoneum and occasionally attached to the visceral layers of the intestines. It is conceivable that cells of the peritoneum and of the visceral layers of the intestines respond to the invasion by mKSA cells by secreting proinflammatory cytokines or chemokines that activate and attract IEL from the epithelia to the deeper layers of the intestines.

A selective homing specificity of TAg-specific CD8$^+$ IEL is suggested by our adaptive transfer experiments: CD8$^+$ IEL transferred intravenously fully protect naïve wt mice against intraperitoneal challenge with mKSA cells, while the same effector cells transferred intraperitoneally do not protect against i.p. inoculated mKSA cells. At the first glance this behavior of the IEL appears counter-intuitive, because it might be expected that i.p. injection of IEL and mKSA cells into the same compartment should accelerate or facilitate the detection and destruction of the tumor cells by the CD8$^+$ IEL. However, considering the colonization of the peritoneum and the visceral layers of the intestines by mKSA cells, it appears more likely that the decisive encounters between tumor cells and IEL take place within the visceral layers of the intestines. Access to these sites from the bloodstream appears to resemble the physiologic route for IEL on immunosurveillance.

Another important conclusion that can be drawn from the adoptive transfer experiments regards the stage at which IL-10 exerts its decisive effect on TAg-specific CD8^{+} IEL. The protection of naïve wt mice mediated by adoptively transferred CD8^{+} IEL from IL-10^{-/-} mice indicates that the IL-10-competent milieu in wt mice does not impair the effector functions of the IEL. This suggests that IL-10 secreted either during the immunization phase or during the first 2 days after challenge of TAg-immunized mice with mKSA cells impairs the generation of TAg-specific CD8^{+} IEL in wt mice.

Prime suspects as the source of IL-10 suppressing the generation of TAg-specific CD8^{+} IEL were peritoneal CD4^{+} T cells secreting large quantities of IL-10 from early on after challenge of TAg-immune wt mice with mKSA cells. However, T cell-derived IL-10 is not vital in preventing the generation of antigen-specific CD8^{+} IEL, because in TAg-immune mice with a T cell-specific inactivation of the gene coding for IL-10 neither accelerated rejection of mKSA cells nor enhanced TAg-specific cytotoxicity of CD8^{+} IEL were observed. Likely sources of IL-10 are monocytes, macrophages, and dendritic cells. As APC they are involved in priming of antigen-specific IEL. Additionally, intestinal epithelial cells are known to secrete IL-10 into the direct environment of IEL, which might be important for immune homeostasis in the intestine.

Taken together, this study demonstrates that tumor antigen-specific, cytotoxic CD8^{+} IEL generated in an IL-10-deficient milieu are able to control highly malignant tumor cells with stunning efficacy both *in situ* and after adoptive transfer into naïve hosts.

4.III. Tumorigenicity of murine embryonic stem cells caused by serpin-Cl-mediated protection against NK cells

Embryonic stem cells are pluripotent cells that are capable to differentiate into any cell type of the organism.

The isolation of ES cells from several mammalian species, including humans, raised high expectations to apply these cells for the generation of irreversibly damaged tissues and organs. ES cells already have been successfully differentiated into specific cell types like neurons (Schuldiner et al., 2001; Svendsen et al., 1999),

endothelial cells (Levenberg et al., 2002), cardiomyocytes (Kehat et al., 2001), hematopoetic precursors (Dang et al., 2002), keratinocytes (Green et al., 2003), osteoblasts (Lerou et al., 2005), hepatocytes (Dang et al., 2002) or insulin producing pancreatic β cells (Soria et al., 2001). Thus, ES cells represent in principle an unlimited source of cells for future approaches in the treatment of hither to non curable diseases like spinal cord injury (McDonald et al., 1999), heart failure (Kehat et al., 2003), or diabetes mellitus (Axelrod et al., 1983). However, in regenerative medicine transplants of ES cells or of ES cell derived grafts faces the same immunologic barriers as conventional organ transplants. Remarkably, the immunologic implications of ES cell based therapy became only recently a focus of interest in regenerative medicine (Bradley et al., 2002).

To date little is known about the immunologic characteristics of ES cells. For a long time, basic assumptions about the interaction of ES cells and effector cells of the immune system were just deduced from simple observations. For example it was assumed that the observed low or absent lysis of ES cells by antigen-specific CD8$^+$ CTL is due to the low surface expression of MHC class I molecules on ES cells. However, recently our group revealed that ES cells are fully recognized by antigen-specific CTL but they evade cytotoxicity by expression of cathepsin B and SPI-6, the specific inhibitors of perforin and granzyme B, respectively (Abdullah et al., 2007).

4.III.1. ES cells are fully recognized by cytotoxic effector cell but poorly lysed by NK cells

ES cells were assumed to be immunoprivileged in a recipient organism for at least two major reasons (Li et al., 2004; Burt et al., 2004): First, ES cells express none or low levels of MHC class I molecules on their surface, so that they might be ignored by MHC I restricted cytotoxic T-lymphocytes. Second, ES cells are derived from pre-implantation embryos which are usually not rejected by the mothers immune system despite being true semiallogeneic implants with half of their proteins being foreign paternal antigens (Rukavina et al., 2000).

Confirming previous studies about the immunologic properties of ES cells, we show here that ES cells express none or low levels of MHC class I molecules on their surface. According to the „missing self hypothesis" of NK cell activation (Ljunggren et

al., 1990) a major trigger for NK cell mediated cytotoxicity is absent or low level expression of MHC class I molecules on cells. Thus, ES cells should be ideal targets for NK cells. However, human (Drukker et al., 2002; Drukker et al., 2006; Bonde et al., 2006) or murine ES cells (Bonde et al., 2006) were reported to be poorly lysed by NK cells in vitro.

To study the interaction of ES cells and NK cells in detail, we used CGR8 ES cells. In contrast to most other ES cell lines, these cells do not depend on feeder cells, which avoids any artefacts due to interference of feeder cells in immunologic assays. CGR8 cells were at least 2-fold less susceptible cells to NK cell mediated lysis than YAC cells, which are the standard target cells for murine NK cells. This holds true for resting as well as poly I:C induced NK cells. To exclude the possibility that the low susceptibility of ES cells to NK cells is due to the activation of NK cells by the non-physiological stimulus poly I:C, NK cells activated during infection of mice with the LCM-virus were used. Undifferentiated ES cells were lysed by LCM-virus induced NK cells as by polyI:C activated NK cells, precluding that the reduced level of cytotoxicity is due to non-physiologically or sub-optimally activated NK cells.

Remarkably, contact with either ES cells or YAC cells stimulated resting as well as activated NK cells to secrete similar high amounts of IFNγ. This observation indicates that ES cells are not ignored by NK cells, but rather fully recognized.

4.III.2. Active protection of ES cells against effector cells of the immune system

This constellation of reduced cytolysis despite full recognition is similar to the interaction of ES cells and CTL as reported by us recently (Abdullah et al., 2007). We detected in ES cells high amounts of cathepsin B and SPI-6, specific endogenous inhibitors of the cytotoxic effector molecules perforin and granzyme B, respectively. Knock down of SPI-6 by specific shRNA fully restored susceptibility of murine ES cells to lysis by CD8$^+$ CTL. Therefore, involvement of SPI-6 and cathepsin B in the low susceptibility of ES cells to NK cells was assessed. By using NK cells from perforin deficient mice as effector cells against ES cells, we confirmed that perforin is an essential effector molecule in lysis of ES cells by NK cells. Surprisingly, blocking the perforin specific inhibitor cathepsin B by CA074 in ES cells did not enhance lysis

of ES cells. Interestingly, inhibition of cathepsin B in ES cells by CA074 did also not enhance susceptibility of ES cells to perforin-mediated lysis by CTL, as reported by us previously (Abdullah et al., 2007). Thus, the biological significance of cathepsin B-expression in ES cells remains to be elucidated.

NK cells and CTL both lyse their target cells mainly by granule mediated effector mechanisms. In addition to perforin, granzymes are essential cytotoxic effector molecules. Having shown previously that ES cells resist lysis by CTL by expression of SPI-6, the specific inhibitor of granzyme B, the hypothesis was tested that SPI-6 also contributes to the low susceptibility of ES cells for NK cells.

Knock down of SPI-6 did not increase the susceptibility of ES cells to NK cells, although it rendered ES cells fully susceptible to lysis by CTL. Recently, it was realized that despite using the common granule mediated cytotoxic pathway, NK cells and CTL differ in their repertoire of cytotoxic effector molecules (Smyth et al., 1995; Sayers et al., 2001; Chowdhury et al., 2008; Janice et al., 2004). A prominent difference is the high expression of granzyme M in NK cells. Although the precise molecular function of granzyme M has not been elucidated, SPI-CI has been identified as its specific inhibitor (Bots et al., 2005). SPI-CI irreversibly interacts with purified granzyme M and was shown to protect e.g. tumor cells from granzyme M mediated lysis . We detected high levels of SPI-CI mRNA expression in murine ES cells. Stable knock down of SPI-CI by lentiviral expression of specific shRNA rendered ES cells fully susceptible to lysis not only by activated NK cells, but also by resting NK cells. After combined knock down of both SPI-CI and SPI-6, we did not observe an increased susceptibility of CGR8 ES cells for NK cell mediated lysis. In contrast in the colon carcinoma cell line CMT 93 Medema and co-workers observed that only combined knock down of SPI-CI and SPI-6 rendered these cells susceptible to lysis by activated NK-like cells (Bots et al., 2005).

These different outcomes of combined knock down of SPI-6 an SPI-CI may either be due to different properties of the target cells or differences of the cell populations used in the respective studies.

4.III.3. Relevance of SPI-CI for the survival and tumorigenicity of ES cells *in vivo*

After having identified a contribution of constitutively expressed SPI-CI to the low susceptibility of ES cells for NK cell mediated cytotoxicity, the relevance of SPI-CI for the survival and growth of ES cells was studied *in vivo.*

Monitoring the number of i.v. injected fluorescently labeled ES cells homing to the spleens of immuno-competent mice revealed a significant effect of the SPI-CI expression in ES cells upon the short time survival of these cells *in vivo.*

Compared to wild type ES cells five time lower numbers of shSPI-CI ES cells accumulated in the spleens of naïve as well as of poly I:C pre-treated wild type recipients, within four hours after i.v. injection

Our hypothesis to explain this differential accumulation of shSPI-CI ES cells versus wild type ES cells in the spleens was that shSPI-CI ES cells might be eliminated by NK cells more efficiently than wild type ES cells. Another remote explanation for the differential accumulation of shSPI-CI ES cells versus wild type ES cells in spleens would be altered migration of these cells *in vivo*.

To differentiate between these alternatives the fate of i.v. injected ES cells was monitored in perforin deficient and in beige mice. Perforin deficient mice and beige mice are impaired in NK and CTL mediated cytotoxicity (Baca et al., 1988; Kagi et al.,1994; Stinchcombe et al., 2000).In perforin deficient mice as well as in beige mice, shSPI-CI ES cells and wild type ES cells accumulated in the spleens to comparable numbers. Thus in mice with profound defects in cell mediated cytotoxicity, ES cells are not eliminated within four hours, while in fully immuno-competent mice, only shSPI-CI ES cells are eliminated.

In syngeneic 129 OLA/Hsd mice wild type ES cells and ES cells with scr shRNA gave rise to palpable teratomas in about eight days after transplantation, that grew within 27 days to 10 mm in diameter, so that the mice had to be euthanasised. In contrast shSPI-CI ES cells developed in only about 50% of the recipients teratomas, and if so, the tumors grew very slowly in syngeneic OLA/Hsd mice.

These results suggest, that via protection of ES cells against NK cell mediated cytotoxicity expression of SPI-CI contributes to the tumorigenicity of ES cells *in vivo.*

5. References

Abdullah, Z., Saric, T., Kashkar, H., Baschuk, N., Yazdanpanah, B., Fleischmann, B.K., Hescheler, J., Krönke, M. and Utermöhlen, O. (2007). Serpin-6 expression protects embryonic stem cells from lysis by antigen-specific CTL. *J Immunol 178*, 3390-3399.

Agnello D., C.S. Lankford, J. Bream, A. Morinobu, M. Gadina, J.J. O'Shea,D.M. Frucht, Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights, J Clin Immunol, 23 (2003) 147-161

Albar J. P., C. J. Melief, and R. Offringa. 2001. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. *Proc Natl Acad Sci U S A* 98: 11515-11520.

Aleman M., S. De La Barrera, S. Fink, M. Finiasz, M.H. Farina, G. Pizzariello,M.D. Sasiain, Interleukin-12 amplifies the M. leprae hsp65-cytotoxic response in the presence of tumour necrosis factor-alpha and interferon-gamma generating CD56+ effector cells: interleukin-4 downregulates this effect, Scand J Immunol, 51 (2000) 262-270

Andrews, P.W., Matin, M.M., Bahrami, A.R., Damjanov, I., Gokhale, P. and Draper, J.S. (2005). Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. Biochem Soc Trans *33*, 1526-1530.

Aung S., B.S. Graham, IL-4 diminishes perforin-mediated and increases Fas ligand-mediated cytotoxicity In vivo, J Immunol, 164 (2000) 3487-3493

Axelrod, H.R.1983. Embryonic stem cell lines derived from Blastocysts by a simplified Technique. *Developmental Biology 101*, 225-228.

Bachmann M.F., H. Schorle, R. Kuhn, W. Muller, H. Hengartner, R.M. Zinkernagel,I. Horak, Antiviral immune responses in mice deficient for both interleukin-2 and interleukin-4, J Virol, 69 (1995) 4842-4846

Baschuk, N. *et al.* Interleukin-4 impairs granzyme-mediated cytotoxicity of Simian virus 40 large tumor antigen-specific CTL in BALB/c mice. *Cancer Immunol Immunother* **56**, 1625-1636 (2007).

Bladergroen, B. A., M. C. Strik, N. Bovenschen, O. van Berkum, G. L. Scheffer, C. J. Meijer, C. E. Hack, and J. A. Kummer. 2001. The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites. *J Immunol* 166: 3218-3225

Blattman J.N., Greenberg P.D. Cancer Immunotherapy: A Treatment for the Masses *Science 9*, pp.200-205 (2004).

Bonde, S. and Zavazava, N. (2006). Immunogenicity and engraftment of mouse embryonic stem cells in allogeneic recipients. *Stem Cells 24*, 2192-2201.

Bots, M., et al., 2005. SPI-CI and SPI-6 cooperate in the protection from effector cell-mediated cytotoxicity. *Blood*, 105(3): p. 1153-61.

Bradley, J.A., Bolton, E.M. and Pedersen, R.A. (2002). Stem cell medicine encounters the immune system. *Nat Rev Immunol 2*, 859-871.

Bright R.K., B. Beames, M.H. Shearer,R.C. Kennedy, Protection against a lethal challenge with SV40-transformed cells by the direct injection of DNA-encoding SV40 large tumor antigen, Cancer Res, 56 (1996) 1126-1130

R.K. Bright, M.H. Shearer,R.C. Kennedy, Examination of lymphokines induced in mice following immunization with recombinant simian virus 40 large tumor antigen, Cancer Immunol Immunother, 40 (1995) 206-211

Burt, R.K., Verda, L., Kim, D.A., Oyama, Y., Luo, K. and Link, C. (2004). Embryonic stem cells as an alternate marrow donor source: engraftment without graft-versus-host disease. *J Exp Med 199*, 895-904.

Chen, W.F. & Zlotnik, A. IL-10: a novel cytotoxic T cell differentiation factor. *J Immunol* 147, 528-534 (1991).

Cheroutre, H. IELs: enforcing law and order in the court of the intestinal epithelium. *Immunological reviews* 206, 114-131 (2005).

Chowdhury, D., Lieberman J.,2008. Death by a thousand cuts: Granzyme pathways of programmed cell death. *Annu. Rev. Immunol.* 26 :389-420

Cobbold, S.P., Jayasuriya, A., Nash, A., Prospero, T.D. & Waldmann, H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature* 312, 548-551 (1984).

Cobbold S., S., Tighe, H., Benjamin, R. & Waldmann, H. CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. *European journal of immunology* 17, 1159-1165 (1987).

Corazza N., Müller S., Brunner T., Kägi D., Müller C..Differential contribution of Fas- and perforin-mediated mechanisms to the cell-mediated cytotoxic activity of naïve and in vivo primed instestinal intraeithelial Lymphocytes *The journal of immunology*, 200, 164: 398-403. (2000)

Croft M., L. Carter, S.L. Swain,R.W. Dutton, Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles, J Exp Med, 180 (1994) 1715-1728

Dang, S. M., M. Kyba, R. Perlingeiro, G. Q. Daley, and P. W. Zandstra. 2002. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol Bioeng* 78:442-453.

David-Watine, B., C. Transy, G. Gachelin, and P. Kourilsky. 1987. Tissue-specific expression of the mouse Q10 H-2 class-I gene during embryogenesis. *Gene* 61: 145-154.

Draper, J.S., Pigott, C., Thomson, J.A. and Andrews, P.W. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat 200*, 249-258.

Drukker, M., Katz, G., Urbach, A., Schuldiner, M., Markel, G., Itskovitz-Eldor, J., Reubinoff, B., Mandelboim, O. and Benvenisty, N. (2002). Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci U S A 99*, 9864-9869.

Drukker, M., Katchman, H., Katz, G., Even-Tov Friedman, S., Shezen, E., Hornstein, E., Mandelboim, O., Reisner, Y. and Benvenisty, N. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. *Stem Cells 24*, 221-229.

Evans, M. J., and M. H. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Erard F., M.T. Wild, J.A. Garcia-Sanz,G. Le Gros, Switch of CD8 T cells to noncytolytic CD8-CD4- cells that make TH2 cytokines and help B cells, Science, 260 (1993) 1802-1805

Figdor, C.G., de Vries, I.J., Lesterhuis, W.J. & Melief, C.J. Dendritic cell immunotherapy: mapping the way. *Nature medicine* **10**, 475-480 (2004).

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology *4*, 330-336.

Fujii, S., Shimizu, K., Shimizu, T. & Lotze, M.T. Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ. *Blood* 98, 2143-2151 (2001).

Gabriel A. Rabinovich,Dmitry Gabrilovich, and Eduardo M. Sotomayor Immunosupressive Strategies that are Mediated by Tumor cells. *Ann. Rev. Immunol.* 25: 267-96, (2006)

Gallimore A., A. Glithero, A. Godkin, A.C. Tissot, A. Pluckthun, T. Elliott, H. Hengartner,R. Zinkernagel, Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes, J Exp Med, 187 (1998) 1383-1393

Gegin, C. & Lehmann-Grube, F. Control of acute infection with lymphocytic choriomeningitis virus in mice that cannot present an immunodominant viral cytotoxic T lymphocyte epitope. *J Immunol* 149, 3331-3338 (1992).

Giovarelli, M. *et al.* Local release of IL-10 by transfected mouse mammary adenocarcinoma cells does not suppress but enhances antitumor reaction and elicits a strong cytotoxic lymphocyte and antibody-dependent immune memory. *J Immunol* 155, 3112-3123 (1995).

Green H., Easly K., Iuchi S., 2003. Marker succession during the development of keratinocytes from cultured human embryonic stem cells. *Proc. Natl. Acad. Sci. USA 100*, page 15625-15630.

Groux, H., Bigler, M., de Vries, J.E., and Roncarolo, M.G. (1998). Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. J Immunol *160*, 3188-3193.

Gooding L.R., Specificities of killing by cytotoxic lymphocytes generated in vivo and in vitro to syngeneic SV40 transformed cells, J Immunol, 118 (1977) 920-927

Hobart, M., V. Ramassar, N. Goes, J. Urmson, and P. F. Halloran. 1997. IFN regulatory factor-1 plays a central role in the regulation of the expression of class I and II MHC genes in vivo. *J Immunol* 158:4260-4269.

Janice M. Kelly, Nigel J. Waterhouse, Erika Cretney, Kylie A. Browne, Sarah Ellis,Joseph A. Trapani and Mark J. Smyth 2004. Granzyme M Mediates a Novel Form of Perforin-dependent Cell Death.J. *Biol. Chem.*, Vol. 279, Issue 21, 22236-22242.

Jackson R.J., A.J. Ramsay, C.D. Christensen, S. Beaton, D.F. Hall,I.A. Ramshaw, Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox, J Virol, 75 (2001) 1205-1210

Kagi, D. *et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 369, 31-37 (1994).

Kehat, I., D. Kenyagin-Karsenti, M. Snir, H. Segev, M. Amit, A. Gepstein, E. Livne, O. Binah, J. Itskovitz-Eldor, and L. Gepstein. 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 108:407-414.

Kehat, I., and L. Gepstein. 2003. Human embryonic stem cells for myocardial regeneration. *Heart Fail Rev* 8:229-236.

Kienzle N., K. Buttigieg, P. Groves, T. Kawula,A. Kelso, A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression, J Immunol, 168 (2002) 1672-1681

Kienzle N., A. Baz,A. Kelso, Profiling the CD8low phenotype, an alternative career choice for CD8 T cells during primary differentiation, Immunol Cell Biol, 82 (2004) 75-83

Kienzle N., S. Olver, K. Buttigieg, P. Groves, M.L. Janas, A. Baz,A. Kelso, Progressive differentiation and commitment of CD8+ T cells to a poorly cytolytic CD8low phenotype in the presence of IL-4, J Immunol, 174 (2005) 2021-2029

Kim, S.K. *et al.* Generation of mucosal cytotoxic T cells against soluble protein by tissue-specific environmental and costimulatory signals. *Proceedings of the National Academy of Sciences of the United States of America* 95, 10814-10819 (1998).

Klein L., L. Trautman, S. Psarras, S. Schnell, A. Siermann, R. Liblau, H. von Boehmer,K. Khazaie, Visualizing the course of antigen-specific CD8 and CD4 T cell responses to a growing tumor, Eur J Immunol, 33 (2003) 806-814

Knowles B.B., M. Koncar, K. Pfizenmaier, D. Solter, D.P. Aden,G. Trinchieri, Genetic control of the cytotoxic T cell response to SV40 tumor-associated specific antigen, J Immunol, 122 (1979) 1798-1806

Koch, C.A., Geraldes, P. and Platt, J.L. (2008). Immunosuppression by embryonic stem cells. Stem Cells *26*, 89-98.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-274 (1993).

Lanford, R.E. Expression of simian virus 40 T antigen in insect cells using a baculovirus expression vector. *Virology* 167, 72-81 (1988).

Lawrenz B, Schiller H, Willbold E, Ruediger M, Muhs A, Esser S. 2004. Highly sensitive biosafety model for stem-cell-derived grafts. *Cytotherapy 6*, 212-22.

Lerou, P. H., and G. Q. Daley. 2005. Therapeutic potential of embryonic stem cells. *Blood Rev* 19:321-331

Levenberg S., Golub J. S., Amit M., Itskovitz-Eldor J., Langer R., 2002
Endothelial cellls derived from human embryonic cells. *Proc. Natl. Acad. Sci. USA 99*, p 4391-4396.

Li, L., Baroja, M.L., Majumdar, A., Chadwick, K., Rouleau, A., Gallacher, L., Ferber, I., Lebkowski, J., Martin, T., Madrenas, J. and Bhatia, M. (2004). Human embryonic stem cells possess immune-privileged properties. *Stem Cells 22*, 448-456

Liu Y., W. Zhang, T. Chan, A. Saxena,J. Xiang, Engineered fusion hybrid vaccine of IL-4 gene-modified myeloma and relative mature dendritic cells enhances antitumor immunity, Leuk Res, 26 (2002) 757-763

Ljunggren, H.G. and Kärre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today 11*, 237-244.

McDonald, J. W., X. Z. Liu, Y. Qu, S. Liu, S. K. Mickey, D. Turetsky, D. I. Gottlieb, and D. W. Choi. 1999. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* 5:1410-1412.

Medema, J. P., D. H. Schuurhuis, D. Rea, J. van Tongeren, J. de Jong, S. A. Bres, S. Laban, R. E. Toes, M. Toebes, T. N. Schumacher, B. A. Bladergroen, F. Ossendorp, J. A. Kummer, C. J. Melief, and R. Offringa. 2001. Expression of the serpin serine protease inhibitor 6 protects dendritic cells from cytotoxic T lymphocyte-induced apoptosis: differential modulation by T helper type 1 and type 2 cells. *J Exp Med* 194: 657-667.

Medema, J. P., J. de Jong, L. T. Peltenburg, E. M. Verdegaal, A. Gorter, S. A. Bres, K. L. Franken, M. Hahne, Muul, L.M., Spiess, P.J., Director, E.P. & Rosenberg, S.A. Identification of
specific cytolytic immune responses against autologous tumor in humansbearing malignant melanoma. *J Immunol* 138, 989-995 (1987).

Melief C.J.M. Cancer Immunotherapy by Dendritic Cells.Immunity 29, 372-383. (2008)

Mescher, M.F., Agarwal, P., Casey, K.A., Hammerbeck, C.D., Xiao, Z., and Curtsinger, J.M (2007). Molecular basis for checkpoints in the CD8 T cell response: tolerance versus activation. Seminars in immunology *19*, 153-161.

Miller C.L., J.W. Hooton, S. Gillis,V. Paetkau, IL-4 potentiates the IL-2-dependent proliferation of mouse cytotoxic T cells, J Immunol, 144 (1990) 1331-1337

Montufar-Solis, D. & Klein, J.R. An improved method for isolating intraepithelial lymphocytes (IELs) from the murine small intestine with consistently high purity. *Journal of immunological methods* 308, 251-254 (2006).

Montufar-Solis, D., Garza, T. & Klein, J.R. T-cell activation in the intestinal mucosa. *Immunological reviews* 215, 189-201 (2007).

Mo X.Y., M.Y. Sangster, R.A. Tripp,P.C. Doherty, Modification of the Sendai virus-specific antibody and CD8+ T-cell responses in mice homozygous for disruption of the interleukin-4 gene, J Virol, 71 (1997) 2518-2521

Moran T.M., H. Isobe, A. Fernandez-Sesma,J.L. Schulman, Interleukin-4 causes delayed virus clearance in influenza virus-infected mice, J Virol, 70 (1996) 5230-5235

Moore, K.W., de Waal Malefyt, R., Coffman, R.L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology* 19, 683-765 (2001).

Murphy K.M.,S.L. Reiner, The lineage decisions of helper T cells, Nat Rev Immunol, 2 (2002) 933-944

Newmaster R.S., L.M. Mylin, T.M. Fu,S.S. Tevethia, Role of a subdominant H-2Kd-restricted SV40 tumor antigen cytotoxic T lymphocyte epitope in tumor rejection, Virology, 244 (1998) 427-441

Niederwieser, D., J. Aubock, J. Troppmair, M. Herold, G. Schuler, G. Boeck, J.Lotz,P. Fritsch, and C. Huber. 1988. IFN-mediated induction of MHC antigen expression on human keratinocytes and its influence on in vitro alloimmune responses. *J Immunol* 140:2556-2564.

Noble A., P.A. Macary,D.M. Kemeny, IFN-gamma and IL-4 regulate the growth and differentiation of CD8+ T cells into subpopulations with distinct cytokine profiles, J Immunol, 155 (1995) 2928-2937

Ochsenbein A.F. Princioles of tumor immunosurveillance and implications for immunotherapy. Cancer Gene Therapy 9, 1043-1055. (2002)

Olver S., P. Groves, K. Buttigieg, E.S. Morris, M.L. Janas, A. Kelso,N. Kienzle, Tumor-Derived Interleukin-4 Reduces Tumor Clearance and Deviates the Cytokine and Granzyme Profile of Tumor-Induced CD8+ T Cells, Cancer Res, 66 (2006) 571-580

Pardoll, D.M. Spinning molecular immunology into successful immunotherapy. *Nature reviews* **2**, 227-238 (2002).

Pfizenmaier K., S.H. Pan,B.B. Knowles, Preferential H-2 association in cytotoxic T cell responses to SV40 tumor-associated specific antigens, J Immunol, 124 (1980) 1888-1891

Qin, S., Cobbold, S., Tighe, H., Benjamin, R. & Waldmann, H. CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. *European journal of immunology* 17, 1159-1165 (1987).

Qiang X, Guo Y.J. Apoptosis in oncology. Cell research 11.1-7. (2001)

Roberts, A.I., O'Connell, S.M., Biancone, L., Brolin, R.E. & Ebert, E.C. Spontaneous cytotoxicity of intestinal intraepithelial lymphocytes: clues to the mechanism. *Clinical and experimental immunology* 94, 527-532 (1993)

Rosenberg, S.A., Restifo, N.P., Yang, J.C., Morgan, R.A. & Dudley, M.E. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* **8**, 299-308 (2008).

Rukavina, D. and Podack, E.R. (2000). Abundant perforin expression at the maternal-fetal interface: guarding the semiallogeneic transplant? *Immunol Today* 21,160-163.

Schirmbeck R., W. Bohm,J. Reimann, DNA vaccination primes MHC class I-restricted, simian virus 40 large tumor antigen-specific CTL in H-2d mice that reject syngeneic tumors, J Immunol, 157 (1996) 3550-3558

Schuler T., T. Kammertoens, S. Preiss, P. Debs, N. Noben-Trauth,T. Blankenstein, Generation of tumor-associated cytotoxic T lymphocytes requires interleukin 4 from CD8(+) T cells, J Exp Med, 194 (2001) 1767-1775

Sharma, S. *et al.* T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function. *J Immunol* 163, 5020-5028 (1999).

Santin, A.D. *et al.* Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific CD8(+) cytotoxic T lymphocytes. *Journal of virology* 74, 4729-4737 (2000).

Sayers, T.J., et al., 2001. The restricted expression of granzyme M in human lymphocytes. *Immunol,*. 166(2): p. 765-71.

Schuler T., Z. Qin, S. Ibe, N. Noben-Trauth,T. Blankenstein, T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice, J Exp Med, 189 (1999) 803-810

Schuler T., T. Kammertoens, S. Preiss, P. Debs, N. Noben-Trauth,T. Blankenstein, Generation of tumor-associated cytotoxic T lymphocytes requires interleukin 4 from CD8(+) T cells, J Exp Med, 194 (2001) 1767-1775

Schuldiner, M., R. Eiges, A. Eden, O. Yanuka, J. Itskovitz-Eldor, R. S. Goldstein, and N. Benvenisty. 2001. Induced neuronal differentiation of human embryonic stem cells. *Brain Res* 913:201-205.

Svendsen, C. N., and A. G. Smith. 1999. New prospects for human stem-cell therapy in the nervous system. *Trends Neurosci* 22:357-364.

Seder R.A., W.E. Paul, Acquisition of lymphokine-producing phenotype by CD4+ T cells, Annu Rev Immunol, 12 (1994) 635-673

Sharma D.P., A.J. Ramsay, D.J. Maguire, M.S. Rolph,I.A. Ramshaw, Interleukin-4 mediates down regulation of antiviral cytokine expression and cytotoxic T-lymphocyte responses and exacerbates vaccinia virus infection in vivo, J Virol, 70 (1996) 7103-7107

Siqing Wang, Jind Yang, Jianfei Qian, Michele Wezman, Larry W. Kwak,Qing Yi
Soria, B., A. Skoudy, and F. Martin. 2001. From stem cells to beta cells: new strategies in cell therapy of diabetes mellitus. *Diabetologia* 44:407-415.

Tumor evasion of the immunesystem : inhibiting p38 MAPK signalling restores the function of dendritic cells in multiple myeloma
Blood, 15 March, Vol. 107, No. 6, pp. 2432-2439 (2006)

Smyth, M.J., et al., 1995. Expression of recombinant human Met-ase-1: a NK cell-specific granzyme. *Biochem Biophys Res Commun*, 217(2): p. 675-83.

Tang Y.W.,B.S. Graham, Anti-IL-4 treatment at immunization modulates cytokine expression, reduces illness, and increases cytotoxic T lymphocyte activity in mice challenged with respiratory syncytial virus, J Clin Invest, 94 (1994) 1953-1958

Tevethia S.S., Recognition of simian virus 40 T antigen by cytotoxic T lymphocytes, Mol Biol Med, 7 (1990) 83-96

Tian, L., Catt, J.W., O'Neill, C. and King, N.J. (1997). Expression of Immunoglobulin superfamily cell adhesion molecules on murine embryonic stem cells. *Biol Reprod 57*, 561-568.

Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall, and J. M. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.

Trapani J, A., Smyth M, J.2002. Functional significance of the perforin/granzyme cell death pathway.*Nat Rev Immunol*. Oct;2(10):735-47. Review

Utermohlen O., C. Schulze-Garg, G. Warnecke, R. Gugel, J. Lohler,W. Deppert, Simian virus 40 large-T-antigen-specific rejection of mKSA tumor cells in BALB/c

mice is critically dependent on both strictly tumor-associated, tumor-specific CD8(+) cytotoxic T lymphocytes and CD4(+) T helper cells, J Virol, 75 (2001) 10593-10602

van der Burg, S.H., Bijker, M.S., Welters, M.J., Offringa, R., and Melief, C.J. (2006). Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy. Advanced drug delivery reviews *58*, 916-930.

Voskoboinik I, Smyth MJ, Trapani JA. 2006. Perforin –mediated target cell death and immune homeostasis. *Nat Rev Immunol. (12)*:940-52. Review.

Wang, L., Goillot, E. & Tepper, R.I. IL-10 inhibits alloreactive cytotoxic T lymphocyte generation in vivo. *Cellular immunology* 159, 152-169 (1994).

Welters, M.J., Bijker, M.S., van den Eeden, S.J., Franken, K.L., Melief, C.J., Offringa, R., and van der Burg, S.H. (2007). Multiple CD4 and CD8 T-cell activation parameters predict vaccine efficacy in vivo mediated by individual DC-activating agonists. Vaccine *25,* 1379-1389.

Zerrahn J., O. Utermohlen, G. Warnecke, W. Deppert,F. Lehmann-Grube, Protective immunity in BALB/c mice against the simian virus 40-induced mKSA tumor resulting from injection of recombinant large T antigen. Requirement of CD8+ T lymphocytes, J Immunol, 156 (1996) 3919-3924

Zhang, M., S. M. Park, Y. Wang, R. Shah, N. Liu, A. E. Murmann, C. R. Wang, M. E. Peter, and P. G. Ashton-Rickardt. 2006. Serine protease inhibitor 6 protects cytotoxic T cells from self-inflicted injury by ensuring the integrity of cytotoxic granules. *Immunity* 24: 451-461.

6. Abstract

6.1. Effects of IL-4 and IL-10 on CD8$^+$ CTL in a tumor antigen-specific immune response

Sustained control or even eradication of tumors by immunotherapy requires durable and highly effective tumor-specific CD8$^+$ cytotoxic T lymphocytes (CTL). In this report we analyzed the impact of interleukin-4 (IL-4) and interleukin-10 (IL-10) on tumor-associated simian virus 40 (SV40) large T-antigen (TAg)-specific CD8$^+$ cytotoxic T cells during rejection of syngeneic SV40 transformed mKSA tumor cells in BALB/c mice.

Strikingly, challenge of naïve mice with low doses of mKSA tumor cells revealed a CD8$^+$ T cell-dependent prolonged survival time of naïve IL-4$^{-/-}$ mice. In mice immunized with SV40 TAg we observed in IL-4$^{-/-}$ mice, or in wild type mice treated with neutralizing anti-IL-4 monoclonal antibody, a strongly enhanced TAg-specific cytotoxicity of tumor associated CD8$^+$ T cells. The enhanced cytotoxicity in IL-4$^{-/-}$ mice was accompanied by a significant increase in the fraction of CD8$^+$ tumor associated T-cells expressing the cytotoxic effector molecules granzyme A and B and in granzyme B-specific enzymatic activity. The data suggest that endogenous IL-4 can suppress the generation of CD8$^+$ CTL expressing cytotoxic effector molecules especially when the antigen induces only a very weak CTL response.

In IL-10-deficient mice we here describe a previously unrecognized population of tumorantigen-specific cytotoxic intraepithelial lymphocytes (IEL) whose anti-tumoral effectivity by far exceeds that of conventional CD8$^+$ CTL. TAg-immune IL-10$^{-/-}$ BALB/c mice reject intraperitoneal TAg-expressing mKSA tumor cells more rapidly than wt mice. TAg-specific conventional CD8$^+$ CTL became detectable in the peritoneal exudate of wt mice on day 6 after challenge. Unexpectedly, already on day 2 after challenge highly cytotoxic, TAg-specific CD8$^+$ small intestinal intraepithelial lymphocytes (IEL) emerged in IL-10$^{-/-}$ mice but not in wt mice. When adoptively transferred, only CD8$^+$ IEL from IL-10$^{-/-}$ mice but not conventional CD8$^+$ CTL from wt mice, conferred to naïve wt recipients full protection against mKSA cells.

6.2. Tumorigenicity of murine embryonic stem cells caused by serpin-CI-mediated protection against NK cells

Undifferentiated human and murine embryonic stem (ES) cells express very low to undetectable levels of MHCI molecules. Fulfilling the 'missing self' criterion, ES cells should be readily recognized and lysed by NK cells. However, ES cells were previously reported to be poorly lysed by NK cells, which was assumed to be due to low or missing expression of activating ligands for NK cells.

Here, we report that murine ES cells were poorly lysed by either resting NK cells or activated NK cells although these NK cell populations recognized ES cells and secreted IFN-γ in response to target cell contact.

Murine ES cells express the serine protease inhibitor involved in cytotoxicity inhibition (SPI-CI). SPI-CI works as a specific inhibitor of granzyme M, a major component of the cytotoxic effector molecules stored in cytotoxic granules of NK cells. Knock down of SPI-CI by stable lentiviral expression of SPI-CI-specific shRNA (ES/shSPI-CI) sensitized ES cells for lysis by NK cells. After i.v. injection into syngeneic mice, ES/shSPI-CI cells were eliminated from the spleen more effectively than parental wt ES cells. ES/shSPI-CI cells implanted s.c. into syngeneic mice developed in only about 60% of the recipients very slowly growing tumors, barely detectable at 21 days post injection with average diameters below 1mm. This is in stark contrast to wt ES cells that gave rise to fast growing teratoma in all recipients with a mean diameter of more than 5mm at 21 days post injection.

Our data suggest that constitutive expression of SPI-CI protects murine ES cells against lysis by NK cells in vitro and in vivo and thereby contributes to the tumorigenicity of ES cells.

6.1. Einfluss von IL-4 und IL-10 auf CD8$^+$ ZTL während der antigenspezifischen Tumor Abstoßung

Für die Abstoßung von Tumoren sind hoch aktive, tumorspezifische zytotoxische CD8$^+$ T-Lymphozyten (ZTL) unbedingt notwendig. In dieser Arbeit wurde untersucht welchen Einfluss Interleukin-4 (IL-4) und Interleukin-10 (IL-10) auf tumorassoziierte, für das große Tumor-Antigen (TAg) des Simian Virus 40 (SV40) spezifische CD8$^+$ T-Zellen, während der Abstoßung von syngenen SV40 transformierten mKSA Tumorzellen, in BALB/c Mäusen haben.

Erstaunlicherweise führte die Belastung mit wenig Tumorzellen bei naiven IL-4$^{-/-}$ Mäusen, im Vergleich zu Wildtyp Mäusen, zu einer verlängerten, CD8$^+$ T-Zell abhängigen Überlebenszeit. Bei mit SV40 TAg immunisierten IL-4$^{-/-}$ oder bei mit monoklonalem anti-IL-4 Antikörper behandelten Mäusen wurde eine stark erhöhte TAg-spezifische Zytotoxizität von tumorassoziierten CD8$^+$ T-Zellen beobachtet. Diese gesteigerte Zytotoxizität in IL-4$^{-/-}$ Mäusen ging mit einem signifikant erhöhten Anteil von Granzyme A und B, und einer erhöhten spezifischen Aktivität von Granzyme B, in den tumorassoziierten CD8$^+$ T-Zellen einher.
Somit kann IL-4 die Generierung zytotoxischer Effektor Moleküle in CD8$^+$ ZTL behindern, vor allem wenn eine eher schwache Immunantwort durch das Antigen hervorgerufen wird.

In Interleukin (IL-) 10-defizienten Mäusen haben wir eine neue Population tumorantigenspezifischer, intraepithelialer Lymphozyten identifiziert. Diese zytotoxischen, CD8$^+$ intestinalen intraepithelialen Lymphozyten (IEL) können wesentlich effektiver als konventionelle CD8$^+$ zytotoxische T-Lymphozyten (CTL) Tumorzellen eliminieren. TAg-immune IL-10$^{-/-}$ BALB/c stoßen mKSA Tumorzellen, die das große Tumorantigen des SV 40 Virus exprimieren, schneller ab als WT Mäuse. In WT Mäusen sind 6 Tage nach Tumorbelastung TAg-spezifische konventionelle CD8$^+$ CTL in der Peritonealhöhle nachweisbar. Erstaunlicherweise konnten aus IL-10$^{-/-}$ Mäusen, aber nicht aus Wildtyp Mäusen, schon 2 Tage nach Tumorbelastung TAg-spezifische CD8$^+$ intestinale intraepitheliale Lymphozyten isoliert werden. Während durch konventionelle T-Zellen kein Schutz vermittelt werden konnte, wurden naive Empfänger im adoptiven Transfer durch CD8$^+$ IEL, von IL-10$^{-/-}$ Mäusen, vollständig geschützt.

6.2. Die Expression von SPI-CI schützt ES Zellen zum Teil vor NK Zellen und trägt zu der Bildung von Teratomen bei

Undifferenzierte humane und murine embryonale Stammzellen (ES Zellen) exprimieren nur sehr wenig oder keine MHC Klasse I Moleküle auf der Oberfläche. Somit erfüllen ES Zellen, der „missing self Hypothese" zufolge, alle Vorraussetzungen als ideale Zielzellen für NK Zellen. Allerdings wurde bisher gezeigt, dass ES Zellen nur sehr schlecht von NK Zellen lysiert werden. Als Grund für die schlechte Lyse von ES Zellen durch NK Zellen wurde die geringe Expression von aktivierenden Liganden für NK Zellen auf ES Zellen vermutet.

In dieser Arbeit zeigen wir, dass ES Zellen trotz der Tatsache, dass die verwendeten NK-Zellpopulationen ES Zellen gut erkennen und nach Kontakt große Mengen von IFNγ sezernieren, nur gering von ruhenden und aktivierten NK Zellen lysiert werden.

Murine ES Zellen exprimieren den Serin Protease Inhibitor SPI-CI, den spezifischen Inhibitor von Granzyme M. Granzyme M wiederum ist ein wichtiger Bestandteil der zytotoxischen Effektormoleküle, gespeichert in den zytotoxischen Granular von NK Zellen.

Durch stabile Hemmung von SPI-CI, mit Hilfe von einem spezifisch für SPI-CI exprimierenden shRNA Vector (ES/shSPI-CI), konnte das Lyse-Niveau von ES Zellen durch ruhende oder aktivierte NK Zellen angehoben werden.

Nach intravenöser Injektion in syngene Mäuse wurden die shSPI-CI Zellen bedeutend besser als die Wildtyp ES Zellen aus der Milz eliminiert.

Auch nach subkutaner Injektion der shSPI-CI Zellen in syngene Mäuse konnte nur bei 60% der Empfänger, an Tag 21 nach Injektion, ein Tumor unter 1mm festgestellt werden. Im Gegensatz dazu entstanden aus subkutan implantierten Wildtyp ES Zellen in allen Empfängern schnell wachsende Tumore, mit einem durchschnittlichen Durchmesser von 21mm.

Die konstitutive Expression von SPI-CI schützt somit ES Zellen, *in vitro* und *in vivo,* vor der Lyse durch NK Zellen und trägt letztlich zu ihrer Tumorigenität bei.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen, Karten und Abbildungen, die in anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Disputation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie –abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss meines des Promotionsverfahren nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Arbeit ist von Herrn Prof. Dr. Martin Krönke betreut worden.

Interleukin-4 impairs granzyme-mediated cytotoxicity of Simian virus 40 large tumor antigen-specific CTL in Balb/cmice

Nikola Baschuk, Olaf Utermöhlen, Roland Gugel, Gabriele Warnecke, Ulrike Karow, Daniela Paulsen, Frank Brombacher, Martin Krönke, Wolfgang Deppert
Cancer Immunology, Immunotherapy (2007) 00012, 1625-1636

Serpin-6 expression protects embryonic stem cells from lysis by antigen-specific CTL

Abdullah, Z., Saric, T., Kashkar, H., Baschuk, N., Yazdanpanah, B., Fleischmann, B.K., Hescheler, J., Krönke, M. and Utermöhlen, O. (2007).
J Immunol 178, 3390-3399.

Immunologic hurdles of therapeutic stem cell transplantation

Olaf Utermöhlen, Nikola Baschuk, Zeinab Abdullah, Afra Engelmann, Udo Siebolts, Claudia Wickenhauser, Carol Stocking, Martin Krönke
In Revision