SPI-6 Expression Protects Embryonic Stem Cells from Lysis by Antigen-Specific CD8$^+$ Cytotoxic T Lymphocytes

Dissertation

zur
Erlangung des Doktorgrades (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Zeinab Abdullah

aus
Bagdad/Irak

2007

Institut für Medizinische Mikrobiologie, Immunologie und Hygiene
der
Universität zu Köln

Anfertigung mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

1. Referent: Prof. Dr. med. M. Krönke

2. Referent: Prof. Dr. N. Koch

Tag der Promotion: 14. Dezember 2007

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

Erscheinungsjahr: 2008

dedicated to

my parents

Thomas Grosner

my brother Hussein

Table of Contents

Abbreviation

αPIG	α-Myosin Heavy Chain-Puromycine-IRIS-GFP
mAb	monoclonal antibody
AFC	7-amino-4-trifluoromethyl coumarin
APC	antigen presenting cell
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
FCS	fetal calf serum
HLA	human leukocyte antigen
ICM	inner cell mass
IFN-γ	Interferon-gamma
IU	infectious unit
Lamp	lysosome-associated membrane protein
LCM virus	lymphocytic choriomeningitis virus
LIF	leukaemia inhibitory factor
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
mHC	minor histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger RNA
NK cells	natural killer cells
Oct	octamer-binding transcription factor
PBS	phosphate buffer saline
PFA	paraformaldehyde
PFU	plaque forming unit
PI	propidium iodide
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
TCR	T cell receptor
TU	transducing unit
UV	ultra violet

1. Abstract

Embryonic stem (ES) cells are defined by their capacity of self renewal and the potential to give rise to any differentiated cell type of the entire organism. These properties have made ES cells promising tools for future applications of cell replacement therapy. The possible use of ES cells in replacement therapy clearly demands a comprehensive understanding of the immunological properties of ES cells and their differentiated derivatives the embryoid body cells (EB cells). To date, the immune response to ES cells is still poorly understood. This study addresses the adaptive cellular immune response to undifferentiated ES and EB cells infected with the Lymphocytic Choriomeningitis (LCM) virus, a vertically transmitted pathogen in mice and humans. In contrast to the prevailing view, this study shows that murine ES and EB cells express MHC class I molecules, although at low levels. Interestingly, both ES and EB cells infected with the LCM virus are able to present viral antigens at the cell surface and are effectively recognized by LCM virus-specific cytotoxic T lymphocytes (CTL) in an antigen-specific and MHC class I-restricted manner despite the low level-expression of MHC class I molecules. However, unlike control fibroblasts, LCM virus-infected ES or EB cells are not effectively killed by the highly cytotoxic LCM virus-specific $CD8^+$ CTL.

Quantitative real time PCR and Western blot analysis show that both ES and EB cells express high levels of Cathepsin B as well as the serine protease inhibitor (SPI)-6, inhibitors of the CTL-derived cytotoxic effector molecules perforin and granzyme B, respectively. Whereas pharmacological inhibition of Cathepsin B did not increase the susceptibility of ES or EB cells to lysis by CTL, down-regulation of SPI-6 by RNA interference rendered ES cells highly sensitive for CTL-induced cell death.

The results of this study show that LCM virus-infected ES and EB cells present viral antigens and are of sufficient immunogenicity to be effectively recognized by antigen-specific $CD8^+$ CTL in an antigen-specific and MHC class I-restricted manner. However, ES and EB cells resist CTL-mediated lysis due to high-level expression of cytoprotective molecules. The immunoprivileged properties of ES cells and their derivatives demands further detailed analysis because they might pose the risk of persistence infection within ES cell-derived transplants or of the non-immune-surveilled growth of malignancies.

2. Introduction

2.1. Embryonic stem cells

Stem cells are defined by the capacity of self-renewal and the potential to differentiate into various cell types (1, 2). Embryonic stem (ES) cells are pluripotent cells capable of differentiating into every cell type of the entire body, can renew continuously in culture and be maintained in their undifferentiated status for extended periods of time (1, 3). In contrast, somatic stem cells are tissue-specific, have a restricted differentiation potential and have only limited self-renewing capacity(4)

Generally, ES cells are derived from the inner cell mass (ICM) of the blastocysts, an early stage of the embryo (1, 3). To maintain ES cells in an undifferentiated state, they are usually cultivated in the presence of leukaemia inhibitory factor (LIF) on a layer of feeder cells, namely mitotically inactivated mouse embryonic fibroblasts (2).

ES cells express characteristic surface antigens which are considered to be markers of their undifferentiated state. These are the stage specific embryonic antigens SSEA (SSEA1 in murine and SSEA3 and SSEA 4 in human ES cells) which play a role in cell-cell adhesion and recognition in developing embryos (1, 5, 6). ES cells also express the transcription factor Nanog-1 and the octamer-binding transcription factor (Oct) (Oct3 in human and Oct 4 in murine ES cells) which are required for maintaining the pluripotency and self-renewal capacity of ES cells (1, 3).

2.2. Multi-lineage differentiation of ES cells in vitro

When ES cells are cultured in suspension in the absence of feeder cells and LIF, they spontaneously aggregate into spherical structures called embryoid bodies (EB). These multi-cellular aggregates resemble early post-implantation embryos and contain differentiating cells of endodermal, ectodermal and mesodermal lineages (7). Comparably, transplantation of undifferentiated ES cells into either syngeneic or immunocompromised

allogeneic mice leads to the formation of disorganized embryonic tumors – teratomas - containing cells derived from all three germ cell lineages (3, 8, 9).

Under defined culture conditions, providing certain combination of growth factors, cytokines and matrix elements, ES cells can differentiate into almost all cell lineages of the body *in vitro* (7). Over the last years, ES cells have been successfully used to generate several cell types, including cardiomyocytes (10), neurons (11, 12), pancreatic beta cells (13), hepatocytes (14), osteoblasts and hematopoeitic precursors (15). This progress have made ES cells an attractive source for cell replacement therapy of non-curable diseases (16), e.g. Parkinson's disease (17), spinal cord injury (18), myocardial infarctions (19) or diabetes mellitus (20).

2.3. Problems encountered in ES cells-based cell replacement therapy

Several important complications might arise from using ES cells in a clinical setting. One foreseeable obstacle in the treatment with ES-derived transplants that was largely neglected until recently, is the immune rejection of allogeneic, i.e. cells from a donor belonging to the same species but not being genetically identical with the recipient, ES cells (21). Transplants derived from ES cells will most probably be rejected by the very same mechanisms of immune rejection as any allogeneic cells. Therefore, grafts derived from ES cells should meet the constraints of transplantation medicine, namely require immunosuppressive therapy to prevent rejection by alloresponsive host cytotoxic T lymphocytes (CTL). Another danger that can hinder the therapeutic applications of ES-derived transplants is the ability of pluripotent ES cells to form teratomas.

2.4. Immunological properties of ES cells

The immunological properties of ES cells is a controversial issue. On the one hand, ES cells have been shown to trigger strong immune reactions after transplantation in xenogeniec or allogeneic recipients (22, 23). On the other hand, undifferentiated ES cells as well as their early differentiated derivatives

were reported to be of low immunogenicity (8). Moreover, ES cells were also shown to be resistant to killing by activated natural killer (NK) cells (24). A previous study have reported that immune incompetent mice reconstituted with human peripheral blood mononuclear cells did not reject undifferentiated or differentiated human ES cells transplanted under the kidney capsule. Even immunization of the reconstituted mice with irradiated human ES cells did not result in the rejection of subsequent transplants (25). Beyond this, intraportal injection of mismatched major histocompatibility complex (MHC) undifferentiated rat ES cell-like cells induced immunologic tolerance in rats allowing the subsequent long-term acceptance of second-set cardiac allografts (26). Li et al. have shown that human ES cells and their derivatives evade both *in vivo* xenogeneic and *in vitro* allogeneic immune responses despite normal levels of MHC I (27). The authors have suggested that ES cells possess properties of immune-privileged tissues that might be due to their embryonic nature.

Importantly, while low immunogenicity of ES cells and their derivatives may prevent rejection of transplanted ES cells, it can compromise the immunosurveillance of the transplant, thus favouring expansion and spread of malignantly transformed cells (e.g. cells of teratoma). Furthermore, immunologically uncontrolled ES cells can provide a niche for intracellular infectious agents. Thus, as a source of systemic seeding of pathogens, ES cells could contribute to the transmission of infectious diseases.

2.5. Cytotoxic T lymphocytes-mediated immune response

$CD8^+$ cytotoxic T lymphocytes have a pivotal role in pathogen eradication and in the immune response towards allografts. Antigen-specific CTL recognize and lysis cells expressing peptide antigens, which are displayed by self MHC class I molecules on the surface of the cells. These antigens are derived from viruses or bacteria that infect the cell but they may also be derived from altered self proteins e.g. mutated proteins in tumors. Thus, target cell killing by CTL is antigen-specific and MHC class I-restricted. Beyond this, alloreactive CTL recognize and kill target cells expressing foreign MHC molecule as found in MHC-incompatible tissue transplants (28, 29).

Cytotoxic T cells induce apoptotic death of their targets via two independent pathways. The first and normally dominant pathway involves the exocytosis of cytotoxic granules that induce apoptosis by the combined action of the pore-forming molecule perforin and the cytotoxic protease granzyme B (28, 30). After secretion of the cytotoxic granules by CTL, perforin monomers insert into the membrane of the target cell and polymerize to form large trans-membrane channels. These channels are essential for the delivery of granzyme B into the cytoplasm of the target cell where it triggers the apoptotic signalling cascade (31). The second pathway involves the interaction of the Fas ligand on the CTL with the Fas receptor on the target cells. This interaction also triggers the apoptotic death in the target cell (32).

Studies with knockout mice lacking perforin, granzyme B or Fas ligand indicate that the cytotoxic granule pathway is the principal mediator of cytolysis by $CD8^+$ CTL. Fas ligand is more important for the cytolytic activity of $CD4^+$ T cells (33-36).

2.6. ES cells provide a system to investigate the immune response to the early embryo

One of the major immunological conundrums is the acceptance of the embryo over the time of pregnancy despite being a true intrauterine semi-allogeneic transplant (37, 38). In general, the survival of transplanted cells correlates with the grade of differences in MHC antigens between the donor and recipient which triggers T-cell responses and rejection of cells with disparate MHC profiles (39-41). One exception to this rule is maternal tolerance of the fetus expressing paternal antigens (38). In spite of our growing understanding of the immune system, the mechanisms warranting the acceptance of the fetus remain largely unknown.

ES cells are derived from the ICM of the blastocysts and thus resemble the early embryonic stages during invasion and implantation. Therefore, in addition to their clinical application, embryonic stem cells can provide an experimental system for studying immune responses to the embryo that are inaccessible *in vivo*.

2.7 Objectives

The main objective of this study is to investigate the immunological properties of undifferentiated murine ES cells and their differentiated derivatives, the EB cells. This study aimed at characterizing the interaction between antigen-specific $CD8^+$ CTL and murine ES and EB cells *in vitro* by using the well established experimental system of acute murine infection with the Lymphocytic Choriomeningitis (LCM) virus.

The susceptibility of antigen presenting ES and EB cells to cytolysis by LCM virus-specific $CD8^+$ CTL was determined. Furthermore, antigen-specific and MHC I-restricted recognition of antigen presenting ES and EB cells by LCM were investigated. Moreover this study investigated the expression of cytoprotective molecules in ES and EB cells and the functional relevance of these cytoprotective molecules in the immunological properties of ES and EB cells.

3. Materials and Methods

3.1. Materials

3.1.1. Plastic ware

Cell culture dishes	Nunc
Pipettes	CELLSTAR
Pipette tips	CELLSTAR
Centrifugation tubes	Falcon and Eppendorf
Glass materials	Schott

3.1.2. Technical equipment

Appliance	Name	Supplier
mini trans-Blot cell		Bio Rad
Criterion Blotter	BR 8.7x13 cm	BioRad
Centrifuge	5417 R	Eppendorf
Centrifuge	Megafuge 1.oR	Heraeus
Electroporator	Gene-Pulser® II	BioRad
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
Invertoscop	IX81	Olympus
Leica confocal software		Leica
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

3.1.3. Chemicals and reagents

Product	Supplier
Acetic acid	Sigma
Acrylamid-bisAcrylamid	BioRad
Agar	Neuform
Agarose	BMA
Ammoniumpersulfate	AppliChem
Bromophenolblue	Sigma
BSA factor V	Merck
CA-074	Bachem
CA-074Me	Bachem
Complete protease inhibitor cocktail	Roche
Crystal violet	Merck
CTL	BioRad
DAPI (4,6-diamino-2-phenylindole)	Merck
DEVD-AFC	BioProbe
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
mrCathepsin B	R&D
mrIFN-γ	R&D
Na_2HCO_3	Sigma
NaCl	Merck
NaF	Sigma
$NaHCO_3$	Sigma
NaV	Sigma
Non-essential amino acids	Invitrogen
Paraformaldehyde	Sigma
PBS (for cell culture)	Invitrogen
Penicillin/Streptomycin	Invitrogen
PIPES	Sigma
PMSF	Sigma
Polybren	Sigma
Prolonged Gold anti-fade	Invitrogen
Reverse Transcriptase	Roche
RNAse inhibitor	Roche
RNeasy Mini Kit	Qiagen
RT-buffer	Roche
Saponin	Sigma
SDS	Sigma
Skimmed milk	Neuform
Sodium pyruvate	Invitrogen
Super-script III kit	Invitrogen
Staurosprin	Alexis
Taq DNA polymerase	Invitrogen

TEMED	Merck
Tris	Sigma
Triton-X-100	Sigma
Trypsin-EDTA (10x)	Invitrogen
Trypton	Neuform
Tween	Merck
Yeast extract	Neuform
β-Mercaptoethanol	Invitrogen

3.1.4. Cell culture medium, reagents and bufferes

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

3.1.4.1. 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 EBs (EB medium):

- Iscove's MEM+GlutaMAX 80% (v/v)
- FCS 20% (v/v)
- β-mercaptoethanol 100 µM
- non-essential amino acids 1X

Cultivation medium for C57SV and BALB/cSV fibroblasts

- DMEM 95% (v/v)
- FCS 5% (v/v)
- Penicillin 50mg/ml
- Streptomycin 50mg/ml

Freezing medium for ES cells

- Glasgow MEM 70% (v/v)
- DMSO 10% (v/v)
- FCS 20% (v/v)

3.1.4.2. Cell culture reagents

Gelatine type A for coating cell culture petri dishes

- 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 $_{dd}H_2O$

3.1.4.3. 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)	

3.1.4.4. Western blot reagents and buffers

Lysis buffer 20x	20 mM Tris, 10% Triton X-100, 10 mM EDTA, 10 mM NaF, 4 mM NaVa, protease inhibitor cocktail
Sample buffer 5x	0.5 M Tris pH 6.8, 0.5 % SDS, 0.1 % Glycerol, 0.05% Bromophenol blue, 0.5 µM ß-Mercaptoethanol
5x Running buffer	60 mM Tris, 7.2 % Glycine, 0.5% SDS
Transfer buffer	25 mM Tris, 0.6 % Glycine. 20% Methanol
10x TBS (pH 7.4)	0.2 M Tris, 8.7% NaCl
TBST (pH 7.4)	TBS 1x, 10% Tween
Stacking gel	4% Acrylamide-bis Acrylamide(37.5:1), 10 mM Tris, pH 6.8, 0.1% SDS, 0.001%TEMED, 0.05% Ammonium persulfate
Separation gel	10% Acrylamide-bis Acrylamide, 0.35 M Tris, pH 8.8, 0.1% SDS, 0.001%TEMED, 0.05% Ammonium persulfate

3.1.4.5. Immunocytochemistry reagents and buffers

Fixation buffer	Paraformaldehyde 4% in PBS
Blocking buffer	FCS 5% in PBS
Saponin 0.1%	Saponin 1 mg/ml in PBS
Hepes Buffer	HEPES 10 mM in $_{dd}H_2O$ (pH 7.5 – 8.0)
Prolong Gold antifade	

3.1.4.6. 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 $_{dd}H_20$

3.1.5. Antibodies

Ms. = mouse, Rb. = rabbit, Rt.= rat, Go. = goat

Antibody	Application	Provider
Rb. anti Ms. Caspase-3	immunofluorescence 1:100	Cell signalling
Ms. Anti LCM virus	immunofluorescence 1:500	Progen
Granzyme A	immunofluorescence 1:500	R&D
Ms. anti SSEA-1(clone MC-480)	immunofluorescence 1:100, FACS 1:1000, Western blot 1:500	R&D
Ms. anti SPI-6	Western blot 1:100	MBL
Ms. anti Cathepsin B	Western blot 1:200	R&D
Rb. anti mouse ß-actin	Western blot 1:1.000	Santa Cruz
Rt. anti mouse GAPDH	Western blot 1:1.000	Amersham
Rt. Oct3/4	Western blot 1:200	Santa Cruz
Rb α-fetoprotein	Western blot 1:500	Abcam
Go. anti-myosin heavy chain	Western blot 1:1000	Abcam
Rb. β2M	Western blot 1:100	Santa Cruz
PE-anti-H-2Kb clone AF6-88.5	FACS	BD
HRP anti-mouse IgG	Western blot 1:125	Dianova
HRP anti-rabbit	Western blot 1:200	Dianova
HRP anti-rat IgG	Western blot 1:200	Amersham
HRP anti- β-actin	Western blot 1:500	Sigma
FITC anti-mouse IgG	FACS 1:200	
Cy3 anti-rabbit	Immunofluorescence 1:200	Amersham
PE-anti-CD8a(Ly-2)	FACS 1:1000	BD
FITC-anti-CD107a	FACS 1:1000	BD

FITC anti-mouse IgG	FACS 1:1000	BD
FITC anti-rabbit IgG	FACS 1:1000	BD
PE anti-rat IgG	FACS 1:1000	BD
PE goat anti-mouse IgG	FACS 1:1000	BD

Anti H-2K^b heavy chain serum used in the Western blot analysis was provided by Jacques Neefjes (Netherlands Cancer Institute)

3.1.6. Primers

β_2-microglobulin: *Fw*: 5'- GTCTTTCTGGTGCTTGTCTC -3'
Rv: 5'- GGCGTATGTATCAGTCTCAG -3'
product size: 276 bp, Annealing temp.: 60°C , $MgCl_2$: 4 mM

Oct4: *Fw*: 5'- ATGAGGCTACAGGACACCTTTC -3'
Rv: 5'- CCCTCAGGAAAAGGGACTGA -3'
product size: 129 bp, Annealing temp.: 60°C, $MgCl_2$: 2 mM

α-fetoprotein: *Fw*: 5'- CCTATGCCCCTCCCCCATTC -3'
Rv: 5'- CTCACACCAAAGCGTCAACACATT -3'
product size: 324 bp., Annealing temp.: 60°C , $MgCl_2$: 2 mM

α-myosin HC: Fw: 5'-GATGGCACAGAAGATGCTGA -3'
Rv: 5'-CTGCCCCTTGGTGACATACT -3'
product size: 120 bp, Annealing temp.: 60°C , $MgCl_2$: 2 mM

H-2K^b: *Fw*: 5'- GCGGCTCTCACACTATTCAGGT -3'
Rv: 5'- TTCCCGTTCTTCAGGTATCTGC -3'
product size: 259 bp, Annealing temp.: 60°C, $MgCl_2$: 2 mM

β-actin: *Fw*: 5'- GATGACCCAGATCATGTTTGAG -3'
Rv: 5'- CCATCACAATGCCTGTGGTA -3'
product size: 107 bp, Annealing temp.: 60°C , $MgCl_2$: 4 mM

for real time PCR

mSPI-6-1: *Fw:*.5'- GTGCCTCTTCTGTCCCAACATT -3'
Rv: 5'- GTTTGCTTTGTTGTGCCTGATG -3'
Prb: 5'-TGTGCTGACCACCCCTTCCTTTTCT-3'[6~Fam-Tamra~Q]

mSPI-6-2: *Fw:* 5'- AGGCACTTGGTTTGAATAAAGAGG-3'
Rv: 5'- TGTCTGGCTTGTTCAGCTTCC-3'
Prb: 5'-CATCCATCAGGGTTTCCAGTTGCTT-3'[6~Fam-Tamra~Q]

18s rRNA gene expression assay (Appliedbiosystems)

β-actin gene expression assay (Appliedbiosystems)

3.1.7. Cell lines

The murine ES cell line αPIG was cultivated and provided by Dr. T. Saric´ from the Institute for Neurophysiology, Medical Centre, University of Cologne.

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 are from the Institute for Medical Microbiology, Immunology and Hygiene, Medical Centre, University of Cologne.

3.1.8. Animals

Six to eight weeks old C57BL/6 and BALB/c mice were obtained form Charles River (Sulzfeld, Germany). 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.

3.1.9. Plasmids

Entry vector pENTER/siH1

Size: 4636 bp

Resistance gene: Kanamycin.

Destination vector pLPac/EGFP

Size: 8133 bp

Resistance gene: Puromycin

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

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

3.2. Methods

3.2.1. Cell Culture

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

CGR8 ES cells were maintained in tissue culture plates coated with gelatine in ES cell medium and αPIG ES cells were cultivated on a layer of Mitomycin C-inactivated mouse embryonic fibroblasts (MEF) at a ratio of 1:4 in 60 cm culture dishes in ES cell medium. Medium was changed daily and ES cells were passaged every 4 days.

C57SV and BALB/cSV fibroblasts were maintained in tissue culture plates in 5% DMEM. Medium was changed every second day and cells were passaged every 4 days.

For passaging ES cells, medium was removed and the cells were incubated in 3 ml of 1x trypsin for 3 min. Subsequently, cells were rinsed off and centrifuged in a 15 ml-centrifugation tube (310 *g*, 3 min, 21°C). The pellet was resuspended and cells were plated on non treated plates, fresh MEF or gelatine coated plates depending on the cell type.

3.2.1.1. Freezing and thawing of murine ES cells

Prior to freezing of ES cells, cells were treated with trypsin, rinsed off and centrifuged as previously described (3.2.1). Afterwards, pellet was carefully re-suspended in ES cell medium (0.5 ml) then freezing medium (0.5 ml) was added, re-suspended and transferred into cryo-vial. Vials were frozen overnight in an isopropanol-filled freezing container at -80°C. The following day, frozen cells were transferred to liquid N_2 tank.

ES cells were thawed in a 37°C water bath until only a small clump of frozen cells remained. After thawing, cells were immediately dispensed in ES cell medium in centrifugation tube and centrifuged (310 *g*, 3 min, 21°C). Pellet was re-supended in 2 ml ES cell medium. Cells of one cryo-vial were placed onto fresh MEF in 60 mm plates or in gelatine coated plates.

3.2.1.2. *In vitro* differentiation of ES cells into embroyid bodies

For the *in vitro* differentiation of CGR8 ES cells into embryoid bodies, ES cells were detached by trypsin-treatment as described in 3.2.1. Cell pellet was resuspended in EB medium to final concentration of $2.5x10^4$ cells/ml. 20 µl drops containing 500 cells were placed on the cover of petri dishes. After two days, cell aggregates were transferred into 6 cm-bacterial petri dishes to avoid adherence. Medium was changed every 2 days by transferring the EBs into 50 ml centrifugation tube. After sedimentation of the aggregates, supernatant was replaced with fresh EB medium.

For the *In vitro* differentiation of αPIG ES cells into embryoid bodies, ES cells were detached by trypsin-treatment as described (3.2.1). Single cell suspension was transferred into 6 cm-bacterial petri dishes to avoid adherence and placed on a horizontal shaker. Medium was changed every 2 days by transferring the EBs to a 50 ml centrifugation tube. After sedimentation of the aggregates, the supernatant was replaced with fresh EB medium.

3.2.2. Immunocytochemistry

Immunocytochemical analysis of cells was performed using primary antibodies and appropriate secondary antibodies labeled with CY3 or FITC (see 3.1.5). Cells were fixed in 4% PFA for 10 min and washed in PBS. Cells were then blocked for 10 min in blocking solution containing 10% FCS. For

permeablisation, cells were incubated with 0.1% Saponin for 1 h at room temperature and washed in PBS. Cells were then incubated with primary antibodies diluted in blocking solution for 1 h at room temperature. Secondary antibodies were diluted in blocking solution and incubated for 2 h at room temperature. Cells were washed in PBS, subsequently cells were embedded in Prolong Gold anti-fade reagent and covered with a cover-slip.

For immunocytochemical staining of embryoid bodies (EB), spheres were fixed in 4% PFA and embedded in paraffin. Spheres were sliced with a microtome (4 µm) and subsequently stained as above. Immunocytochemical analysis of ES and EB cells was performed in cooperation with Dr. Tomo Saric´ from the Institut für Neurophysiologie, Universität zu Köln.

3.2.3. PCR

3.2.3.1. RNA isolation

Total RNA was extracted from approximately $2x10^6$ cells ES and EB cells of different stages and $CD8^+$ T cells using TRIzol reagent (Invitrogen) and extracted RNA was further cleaned up using the RNeasy Mini elute kit (Qiagen) according to manufacturer's instructions (spinning protocol). For the isolation of RNA from placenta and testes, the RNeasy midi kit was used (Qiagen) according to manufacturer's instructions. After homogenising 2 mg tissue in 300 µl RLT buffer lysates were treated with 25 mg/ml proteinase K (Qiagen) to digest proteins.

3.2.3.2. RNA quantification

Isolated RNA was quantified using the RiboGreen RNA quantification Kit (Molecular Probes) in a 96-well microplate. 95 µl TE buffer and 5 µl RNA were distributed per well. RiboGreen reagent was diluted 1:200 and 100 µl was added per well. After 2 min incubation at room temperature, samples were excited at 485 nm then 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.

3.2.3.3. cDNA synthesis

For this purpose the Reverse-IT RTase Blend Kit (ABgene) was used following the manufacturer's instructions. 1 µg RNA in water and 1 µl oligo (dT) primer were filled to a final volume of 10.5 µl in water and incubated in a thermocycler for 10 min at 65°C. After that, 4 µl RT-buffer, 2 µl DTT, 0.5 µl RNAse inhibitor, 1 µl Reverse transcriptase and 2 µl dNTPs were added and again incubated in the thermocycler for 60 min at 37°C, followed by incubation for 5 min at 93°C.

3.2.3.4. RT-PCR

2 µl of the generated cDNA was used for each PCR-reaction. The compositions of the components in the PCR-reaction were as follows:

2 µl cDNA
0.1 mM dNTPs
2 µl PCR-buffer (10x)
250 nM of each forward und reverse primer
0.1 units Taq polymerase
$MgCl_2$ (varies depending on primer, see (3.1.6))
Water (varies depending on the amount of $MgCl_2$)
Total volume: 20 µl

PCR-reaction was performed in a thermocycler, with varying annealing temperatures depending on the primer. Negative and positive control templates were included in each PCR-reaction. Individual steps were performed as follows:

94°C for 4 min for initial denaturising followed by 30 cycles of 94°C for, 30 sec X°C (X = annealing temperature of the primer in the reaction) and 72°C for 1 min with a final extension of 72°C for 10 min. Reaction mixture was then cooled down to 4°C

After the PCR-reaction, 3.5 µl of 6x loading buffer were added per tube. Samples were electrophoretically separated on 1.5% agarose-gel in TAE buffer with 1 µl ethidiumbromide/10 ml buffer at 100 Volts for 30 min. The agarose gels were exposed to UV-light in a gel documentation system to visualize DNA-bands.

3.2.3.5. real time PCR (TaqMan)

Quantitative real-time PCR was carried out on the TaqMan ABI-Prism 5700 Sequence Detection System (Applied Biosystems). For an optimal performance of real-time PCR, the optimal concentration for each primer pairs and probe were determined following the instructions of Applied Biosystems. Negative and positive control templates were included in each PCR-reaction.
To quantify mRNA expression of SPI-6 two different sets of primers and probes were used. Reactions were performed in 25 µl mixture using the Taq-Man Universal Master Mix (Applied Biosystems) following manufacturer's instructions.

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

2.5 µl cDNA (final concentration: 5 ng/ml)
12.5 µl PCR Master Mix
250 µM Forward Primer
250 µM Reverse Primer
300 µM Fluorogenic Probe
8.5 µl water
Total volume: 25 µl

Individual steps were performed as follows:

50°C	120 sec	UDG PCR decontamination
95°C	120 sec	Taq start activation

40 Cycles with

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

Two internal controls were amplified in each run in order to assess reproducibility of the results. mRNA was calculated for each gene by using a standard curve (cDNA ranged from 0 ng to 400 ng). The value obtained for each gene was normalized to that of the housekeeping gene encoding 18s rRNA and β-actin before the fold change was calculated using crossing threshold (Ct) values.

3.2.4. Infection of cells and mice with the LCM virus

In this study the LCM virus from the strain WE was used for the infection of cells and mice (42).

Mice were infected intravenously (i.v.) with 10^5 infectious units (IU) of the LCM virus in PBS. Cells were infected with same strain of the LCM virus at a multiplicity of infection (MOI) of 0.01. For the infection of adherent target cells like ES cells and fibroblasts, about $0.5x10^6$ cells were plated and on the next day medium was discarded and replaced with 10 ml fresh medium containing 10^4 plaque forming units (PFU) of the LCM virus then incubated for 48 h at 37°C. For the infection of the Embryiod bodies, about 100 EBs were transfered into 50 ml centrifugation tube and centrifuged at 120 *g* for 1 min. After sedimentation of EBs, supernatant was replaced with 1 ml medium containing $3x10^4$ PFU of the LCM virus and tubes were incubated at 37°C. After 30 min EBs were placed in petri dishes and 10 ml of fresh medium was added and further incubated for 48 h.

3.2.5. Titration of the LCM virus

LCM virus was titrated as plaque-forming units (PFU) as previously described (43). Briefly, 6-well plates were seeded with 2.5×10^5 L929 cells in 2 ml of growth medium and incubated 24 h at 37°C. On the following day supernatants were collected and serially diluted in 10 fold steps to 10^{-4} using dilution buffer. Old media was aspirated carefully from the wells and 100 µl from the last 3 dilutions of each sample was applied per well. Samples were allowed to adsorb for 30 min at 37°C and cells were then covered with 2 ml of overlay medium. After 3 days of incubation at 37°C growth medium (1.5 ml) was added carefully on the top of the overlay medium. After incubation for another day, the medium was discarded and 1 ml of staining solution was added per well. After 15 min incubation, plates were rinsed with water and dried to be evaluated.

Growth medium:	20% 10x DMEM, 220mg/l Na pyruvate, 7.4g/l $NaHCO_3$, 1.16g/l glutamine, 2% penicillin/streptomycin and 10% FCS in $_{dd}H_2O$
Overlay medium:	0.9% methyl cellulose in growth medium
Dilution buffer:	FCS 2% and 1% penicillin/streptomycin in PBS

Staining solution: 0.2% crystal violet, 2% Ethanol and 11% formaldehyde in $_{dd}H_2O$

3.2.6. Loading of target cells with the LCM virus peptide

Target cells were loaded with LCM virus peptide gp_{33-41} as following: 10^6 cells were incubated with 1 µM of the peptide in a final volume of 1 ml for 1 h at 37°C. To avoid aggregation of the cells they were frequently resuspended by pipetting. Cells were washed once with 5 ml medium at room temperature and further used in different assays.

3.2.7. Preparation of LCM virus specific $CD8^+$ CTL from spleens

On day 8 after infection with the LCM virus, mice were sacrificed and their spleens were removed under sterile conditions and were ruptured in ice cold RPMI medium containing 10% FCS. Single cell suspension was obtained by passing cell suspensions through a cell mesh of 100 µm pore size. Cells were centrifuged at 310 *g* for 3 min at 4°C and cell pellet was resuspended in 5 ml of cold 0.2% NaCl to lyse erythrocytes. After 30 sec 5 ml of cold 1.6% NaCl was added to the cell suspension on ice. Cells were then centrifuged at 310 *g* for 3 min at 4°C, pellet resuspended in 1 ml sorting buffer and counted in Trypan blue solution using Neubauer chamber. $CD8^+$ CTL were enriched from the splenic cell suspension using anti CD8 monoclonal antibodies conjugated to magnetic beads (Meltenyi Biotec) following manufacturer's instructions. After 20 min cells were washed 3 times with 30 ml cold sorting buffer and once with PBS. Pellet was then resuspended in sorting buffer and the magnetically loaded $CD8^+$ cells were loaded on the Mini-MACS columns under a magnetic field at 4°C. After several rinses, columns were removed from the magnetic field and cells were eluted by rinsing the columns with sorting buffer. Cells were then centrifuged, pellet was resuspended in RPMI medium and cells counted in Trypan blue solution using a Neubauer chamber.

3.2.8. Chromium release cytotoxic assays

3.2.8.1. Effector cells

LCM virus specific $CD8^+$ effector T cells were magnetically enriched as described in (3.2.7). Effector cell number was adjusted to $3x10^6$cell/ml in RPMI medium and 200 µl was dispensed in U-bottom 96-well plates in replicates of four. To obtain reducing number of the effector cells, serial dilutions of 1:2 in RPMI medium were made three times.

3.2.8.2. Target cells

For the assessment of the LCM virus specific cytotoxicity of the $CD8^+$ CTL, target cells were either loaded with the LCM virus specific peptide or were infected with LCM virus. As negative control non-treated target cells were used. On the day of experiment, infected and control target cells were detached by trypsin-treatment as described in (3.2.1). Part of the non-infected target cells were loaded with virus peptide as described in (3.2.6). Approximately 10^6 of each cell type were incubated with 50 µCi ^{51}Cr for 60 min at 37°C. Cells were then washed thoroughly to remove the extra-cellular ^{51}Cr. Cell number was then adjusted to $3x10^4$ cell/ml. 100 µl/well of the target cell suspension was dispensed per well to the previously distributed effector cells in the 96-well plate. Thus, an Effector:Target cell ratios (E:T ratio) of 100:1, 50:1, 25:1 and 12.5:1 were reached. Eight wells containing only target cells and medium and another eight wells containing target cells with 1.6% Triton X100 in $_{dd}H_2O$ represent the spontaneous and maximal ^{51}Cr-release for each target cell type. After 4 h incubation at 37°C, plates were centrifuged. Using 8-channel pipettes 100 µl of the supernatants from each well were collected and transfered into small test tubes that were placed in the racks of the Gamma counter (Canberra-Packard). The amount of ^{51}Cr released by the killed target cells was measured as counts per minute (CPM).

The cytotoxic activity of the CTL was calculated as percentage of specific lysis for each E:T ratio using the mean CPM for each replicate:

3.2.9. Western blot analysis

3.2.9.1. Cell-lysate preparation

Cells were lysed in ice-cold lysis buffer containing protease inhibitors and were centrifuged at 3300 *g* for 5 min at 4°C and supernatants were collected. Protein concentration was determined by BCA kit (Pierce) following manufacturer's instructions. Cell lysates were mixed with 5x sample buffer to a final concentration of 20 µg/µl and boiled for 7 minutes.

3.2.9.2. SDS-PAGE electrophoresis

Equal volumes (20-50 µg protein) of cell extracts in sample buffer were loaded per well on 10% SDS-PAGE gels. Electrophoresis was performed for 60-90 min under constant voltage (100V). Sizes were determined by comparing to the molecular weight standard, High Range 45-200 kD (Bio Rad) or to the Pre-stained Protein Ladder 10-180 kD (MBI).

3.2.9.3. Blotting

Proteins were transferred to nitrocellulose membranes (0.2 µm; Bio Rad) with constant voltage (100 V) for 60 min using mini trans-Blot cell (Bio Rad) filled with Blotting buffer. Membranes were incubated with blocking buffer for 1 h and subsequently incubated overnight at 4°C with primary antibodies shown in (3.1.5). After three times washing with TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibodies (3.1.5) for 1 h at room temperature. Blots were washed three times with TBS-T and once with TBS for 5 min. The immune complex was then visualised using TCL reagent (BioRad) following the manufacturer's instructions. Signals were detected by autoradiography using hyper film (Bio Rad).

3.2.10. Apoptosis assays

3.2.10.1. Induction of apoptosis in ES cells

ES cells (10^6) were left untreated or irradiated for 1 h with 20 mJ/cm^2 UV (253-255 nm), incubated for 1 h with 0.5 µM staurosporine (Alexis, Grünberg, Germany) or with 2 µg/ml anti-mouse Fas antibody (clone Jo2) (BD Bioscience Germany).

3.2.10.2. Detection of Caspase-3 activation in ES cells

a. Detection of activated Caspase-3 by immunofluorescence

To assess the activation of Caspase-3 in apoptotic cells, 10^5 cells were treated as described in (3.2.10.1) or left untreated. Cells were fixed, blocked and permeabilised as in 3.4. Nuclei were visualized by counterstaining with 10 µg/ml in PBS Hoechst 33258 (Sigma). Cells were then washed 2 times for 20 min and then incubated with rabbit anti-Caspase-3 mAb for 1 h at room temperature. Subsequently, cells were incubated with the secondary antibody (anti rabbit IgG antibodies conjugated to Cy3) for 1 h at room temperature, washed in PBS, mounted on glass slides in Prolong Gold anti-fade and examined under fluorescence microscope.

b. Detection of enzymatic activity of Caspase-3

Enzymatic Caspase-3 activity was quantified in the cytosolic extracts of apoptotic cells using the caspase substrate DEVD conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (AFC) DEVD-AFC as previously described (44). Cleavage of DEVD-AFC by Caspase-3 releases the fluorescent fluorochrome AFC which can be monitored fluorimetrically at 500-510 nm with excitation at 400 nm.

To quantify the enzymatic activity of Caspase-3 in apoptotic cells, about 10^6 cells treated as described in (3.2.11.1) or left untreated were centrifuged and cell pellet was resuspended in 100 µl of HEB buffer (20 mM PIPES, 50 mM KCl, 5 mM EGTA, 1 mM DTT, pH 7) and allowed to swell for 20 min on ice. After the addition of 100 µM PMSF, cells were lysed and homogenized by passing through 27-gauge needle and pelleted at 14,000 *g* for 20 min at 4°C. The resulting supernatant (cytosolic extract) was collected and protein concentration was determined by using the BCA kit following manufacturer's instructions (Pierce). In 96-well plate 1 µl of the cytosolic extracts, 99 µl Caspase buffer and 100 µl of 200 µM DEVD-AFC were added per well. Fluorescence was measured using continuous-reading plate reader (Wallac victor ^{2}TM multilabel counter 1420) at 30°C and 400 nm and 505 nm excitation and emission, respectively. Level of Caspase-3 enzymatic activity in the cell lysate is directly proportional to the fluorescence signal detected.

3.2.11. Cytokine measurement by ELISA

For the quantification of IFN-γ secreted by LCM virus specific $CD8^+$ cells IFN-γ specific ELISA kit (R&D) was used following the manufacturer's instructions. Cocultures were centrifuged for 3 min at 310 *g* at 4℃ and cell-free supernatants were collected and stored at -80℃ if test was not performed immediately.

3.2.12. CD107a staining

C57SV fibroblasts, ES and EB cells were either infected with the LCM virus at a MOI of 0.01 for 48 h prior to the experiment as described in (3.2.4) or they were loaded with the LCM virus peptide gp_{33-41} as described in (3.2.6). LCM virus specific $CD8^+$ CTL cells enriched from C57BL/6 mice on day 8 after infection were prepared as described in (3.2.8.1). Effector and Target cell number was adjusted in RPMI medium to $3x10^6$ cell/ml and $6x10^6$ cell/ml respectively. In U-bottom 96-well plate 1 µl of 2 mM Moninsin,100 µl of the effector cell suspension and 100 µl of the target cell suspension or medium for the control wells. Subsequently, 1 µl of FITC-conjugated anti CD107a was added per well except to the control wells where 1 µl/well of the FITC-conjugated isotype control was added. After 1, 3 and 5 h cells were collected and washed once using EDTA-containing PBS (to dissociate effector-target cell aggregates). Pellets were then stained with APC- conjugated anti CD8 or FITC-conjugated anti CD107a or with both of the antibodies and finally were fixed using FACS fixation buffer (BD). Exposure of CD107a on the cell surface of $CD8^+$ cells was monitored by flow cytometry analysis using FACSCalibur (BD).

3.2.13. Construction of shRNA expressing vector

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

3.2.13.1. Designing the shRNA constructs: (this part is under re-construction)

Two different murine SPI-6 specific small hairpin RNA (designated as shRNA #1 and #2) were designed following the pSUPER RNAi system of OligoEngine. Targeted sequences are specific for murine SPI-6 as no other

matches were found using the BLAST database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)
As a negative control, non-specific shRNA (designated as shRNA scr.) was designed. The oligonucleotides specific for the murine *spi-6* gene were as follows:

shRNA#1 sense primer:
5´-gatccccGCTATTGATTGATAGCCTAttcaagagaTAGGCTATCAATCAATAGCttttta-3´
antisense primer:
5´-agcttaaaaaGCTATTGATTGATAGCCTAtctcttgaaTAGGCTATCAATCAATAGCggg-3´
shRNA#2, sense primer:
5´- gatccccCAATGGACATGCCCTTTAAttcaagagaTTAAAGGGCATGTCCATTGttttta-3´
antisense primer:
5´-agcttaaaaaCAATGGACATGCCCTTTAAtctcttgaaTTAAAGGGCATGTCCATTGggg-3´
The oligonucleotides for the negative control were as follows:
shRNA scr. sense primer:
5´-gatccccGGATTACTTGATAACGCTAttcaagagaTAGCGTTATCAAGTAATCCttttta-3´
antisense primer:
5´-agcttaaaaaGGATTACTTGATAACGCTAtctcttgaaTAGCGTTATCAAGTAATCCggg-3´

Sense and antisense primers of each shRNA were annealed to generate a double-stranded oligonucleotides by mixing 3 µg of each oligo, 5 µl of 10x annealing buffer and filled with PCR water to a final volume of 50 µl. The mixture was incubated for 4 min at 95°C, 10 min at 70°C then removed from the thermo-block and left to cool slowly to room temperature. Annealed oligos were stored at -20°C.

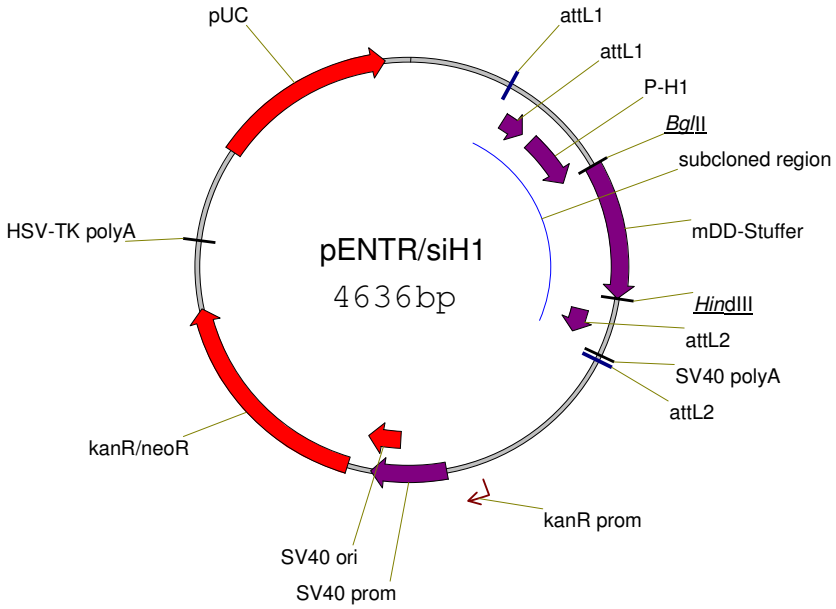
3.2.13.2. Ligation of the complementary DNA into the pENTR/siH1 plasmid

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 E. coli XL1 blue 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℃ 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℃.

Fig.1. pENTR/siH1 vector



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℃ with shaking. Plasmids were purified using the mini prep kit (Qiagen), eluted in water and stored at -20℃. 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.

3.2.13.3. Transient transfection of ES cells

CGR8 ES cells plated one day earlier were co-transfected with 2 µg of the entry plasmid pENTR/siH1-SPI-6#1 or pENTR/siH1-SPI-6#2 together with 20 µg of the puromycin resistant plasmid pPGK (Invitrogen) via electroporation. ES cells transfection was performed in 4 mm electroporation cuvets using Gene Pulser Xcell nucleofection device (BioRad) under following parameters: 950 µF, 200 Ohms and 250 V.

After transfection, CGR8 cells were plated in 10 ml culture medium and were allowed to recover for 24 h. Positive cells were then selected for 72 h with 30 µg/ml of puromycin.

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

For a stable transcription of the SPI-6-specific shRNA, the entry plasmids were cloned into the Lentiviral vector pLPAC/EGFP 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 protienase 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]

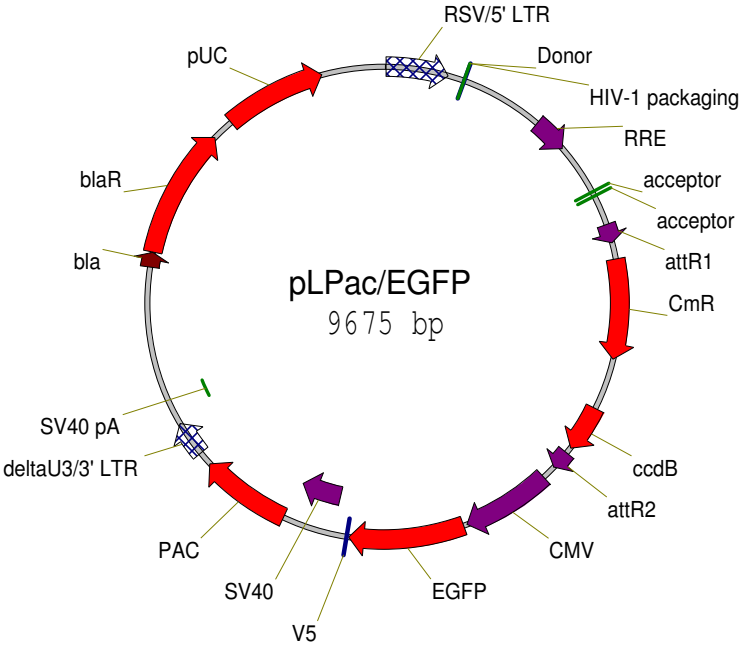
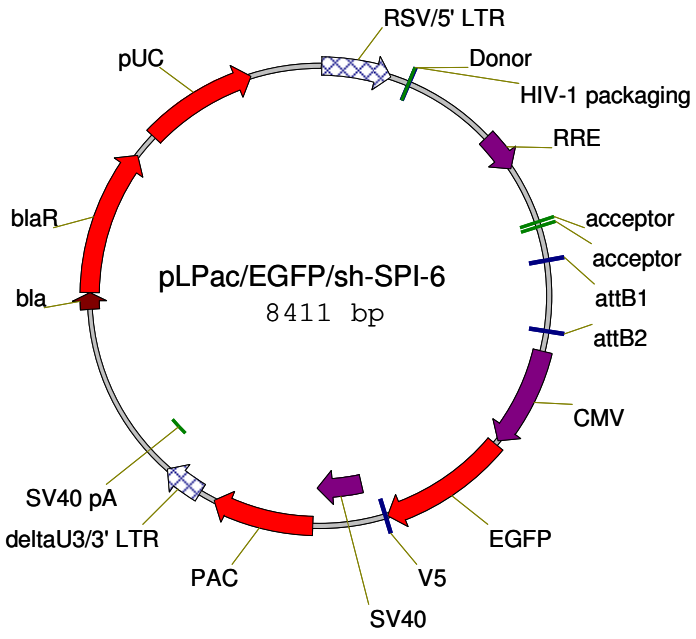


Fig.3. shRNA expressing Lentiviral vector pLPac/EGFP/sh-SPI-6



For the production of Lentivirus particles the ViraPower™ 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 cotransfected with 3 µg pLPac/EGFP/sh-SPI-6 (either pLPac/EGFP/sh-SPI-6#1, pLPac/EGFP/sh-SPI-6#2 or pLPac/EGFP/sh-scr), 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.

3.2.13.5. Titration of the Lentivirus

To determine the transducing unit (TU) of the produced Lentivirus the HT1080 cells were used. Cells were detached and a single cell suspension was adjusted to $2x10^5$ cells/ml in DMEM. Lentivirus stocks were thawed on ice and serially diluted 1:10 for 5 times in 1 ml DMEM medium. To each of the dilutions 1 ml of the cell suspension and 6 µg/ml polybren (Invitrogen) were added. Plates were incubated at 37°C for 14 h. After incubation, medium was changed and plates were incubated for 36 h. Infected cells were then selected with 10 µg/ml puromycin for 10 days. Medium was changed every second day. After 10 days medium was discarded and plates were washed twice with PBS. Colonies were visualised by Crystal Violet staining for 20 min and subsequently washed with water. Number of colonies was counted and the virus titer TU/µl was calculated from the mean of colonies of different dilutions.

3.2.13.6. 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 gelatin. 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℃ 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 10 µg/ml puromycin. 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 puromycin resistance. After 14 days puromycin concentration was reduced to 3 µg/ml and SPI-6 expression was assessed in different clones using real time PCR.

4. Results

4.1. Characterization of murine embryonic stem cells and embryoid body cells

Two widely used and independently generated ES cell lines αPIG (α-Myosin Heavy Chain-Puromycin-IRIS-GFP) (45) derived from D3 cells (46) and CGR8 (47) were used in this study to avoid any artefacts that might result from specific properties of a single cell line. CGR8 and αPIG ES cell lines are derived from the 129P2/Ola and 129S2/SvPas mouse strains, respectively. Both strains express H-2^{b} haplotype of the MHC class I molecules.

The developmental stages of ES cells used in this study were verified by monitoring the expression of several markers. Undifferentiated ES cells were identified by expression of the stem cell marker stage specific embryonic antigen-1 (SSEA-1), the pluripotency marker Oct4 and the absence of lineage specific markers, i.e. α-fetoprotein for endodermal tissues and α-myosin heavy chain for mesodermal tissues. Both undifferentiated CGR8 as well as αPIG cells showed high expression of SSEA-1 (Fig. 4B) and Oct4 (Fig. 4C) but no detectable expression of α-fetoprotein or the α-myosin heavy chain (Fig. 4A).

Differentiation of ES cells was achieved by the hanging drop method with 500 cells/20 µl-drop and withdrawal of LIF from the culture medium which results in the formation of embryoid bodies (EB) (14). The differentiation stages of cells derived from the EBs were assessed at the mRNA and protein level by using RT-PCR, Western blot and flow cytometric analysis. Expression of SSEA-1 and Oct4 in EB cells declined over time and reached almost a background level at about day 7 post induction of differentiation (Fig 4B and 4C). In contrast, expression of α-fetoprotein and α-myosin heavy chain increased progressively from day 4 of differentiation onwards (Fig 4A).

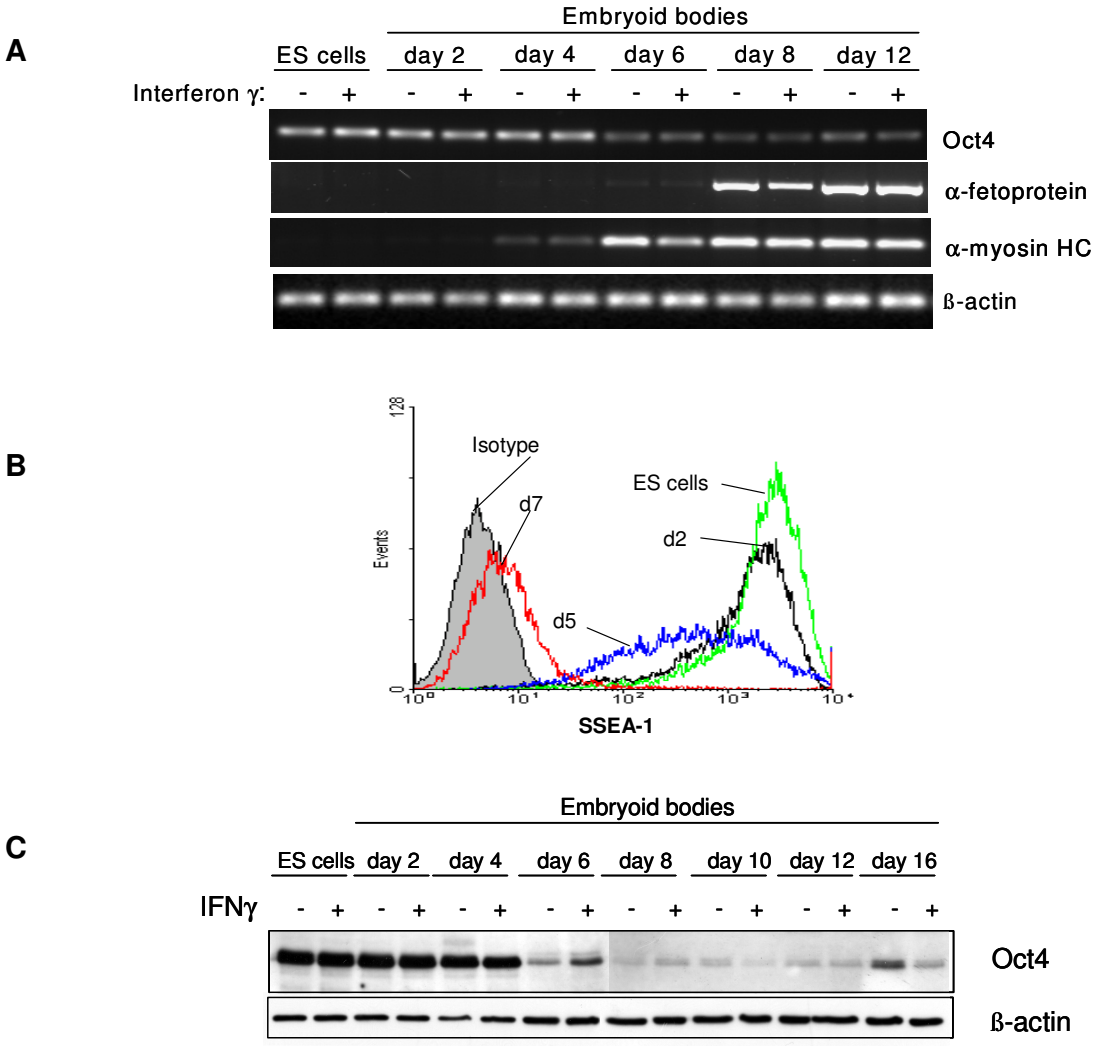


Figure 4: Characterization of mouse embryonic stem cells and embryoid body cells

(A) Expression of mRNA coding for the pluripotency marker Oct 4 and the lineage specific markers α-myosin heavy chain (α-Myosin HC) and α-fetoprotein in non-treated αPIG ES cells, EB cells or cells treated with 20 ng/ml IFN-γ for 48 h. The absence of α-fetoprotein and α-myosin HC expression indicates the undifferentiated phenotype of the ES cells and increasing expression of α-fetoprotein and α-myosin HC indicates the differentiation of EB derived cells.

(B) Flow cytometry analysis of the expression of the stem cell marker SSEA-1 on the cell surface of undifferentiated αPIG ES and EB cells on day 2, 5 and 7 post induction of differentiation. Single cell suspensions of ES or EB cells were stained with SSEA-1 specific antibody or isotype control. SSEA-1 is expressed at high level on the surface of undifferentiated ES cells and progressively decreases over time and reaches almost control levels at about day 7 post induction of differentiation.

(C) Western blot analysis of Oct4 in αPIG ES and EB cells. Whole cell lysates were prepared from non-treated ES and EB cells or cells treated with 20 ng/ml IFN-γ for 48 h. β-actin was amplified as a loading control. Expression of Oct4 progressively decreased over time reaching background levels on day 8 post induction of differentiation.

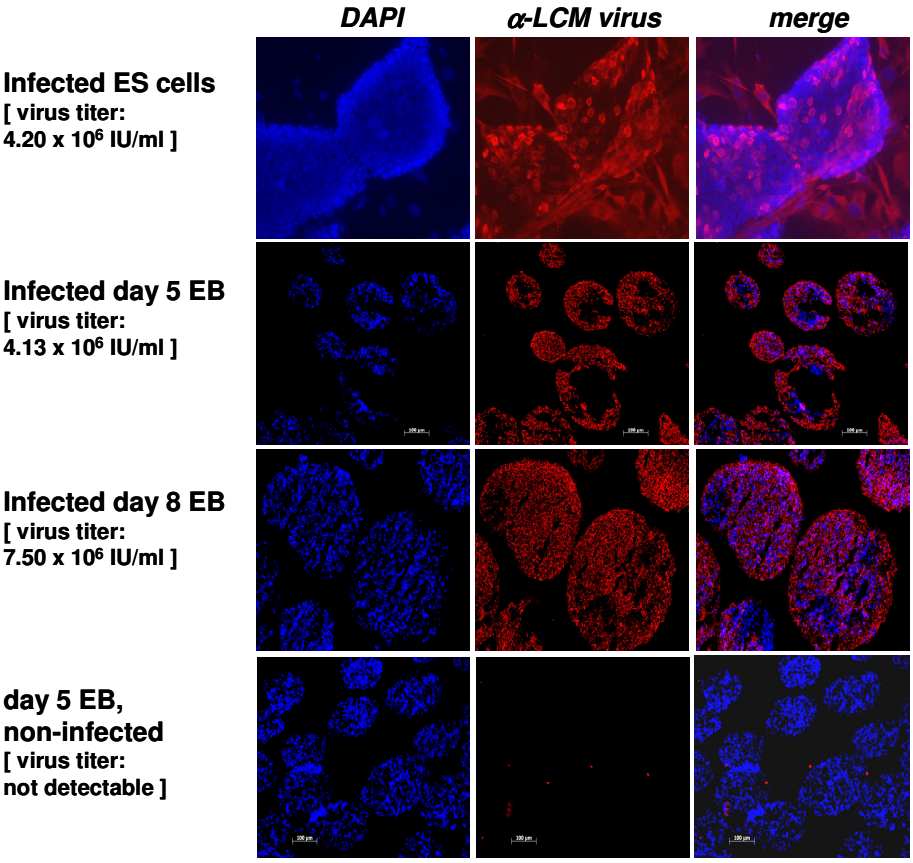
Similar results were obtained for CGR8 ES and EB cells.

4.2. ES and EB cells can be productively infected by the LCM virus

The Lymphocytic Choriomeningitis virus (LCM virus) infects almost any somatic cell type of mice with the exception of T lymphocytes (48). However, it was so far unknown whether murine ES and EB cells can be infected with this virus. Therefore, undifferentiated αPIG and CGR8 ES cells as well as EB cells derived from αPIG cells at day 5 or 8 of differentiation were incubated with the LCM virus at a multiplicity of infection (MOI) of 0.01. Undifferentiated αPIG and CGR8 ES cells as well as EB cells of both differentiation stages were readily infected by the LCM virus as illustrated by immunofluorescence microscopical analysis of cells stained with LCM virus-specific monoclonal antibodies (Fig. 5A). Productive infection was proven by quantification of infectious virus particles in the supernatants of the infected cells by a plaque assay using L929 cells. While $3.5x10^3$ PFU/ml of LCM virus were inoculated into the cultures, viral titers above $1x10^5$ PFU/ml were measured in the culture medium of ES and EB cells at 24 h post infection (Fig. 5B). At 48 h post infection viral titers had further increased to reach levels of about $7x10^6$ PFU/ml (Fig. 5B) proving a productive infection of the ES and EB cells.

Altogether, both ES cells as well as EB cells can be productively infected by the LCM virus. Productive infection of ES and EB cells with the LCM virus is an important prerequisite for the processing and presentation of antigenic viral peptides via the MHC class I restricted pathway.

A



B

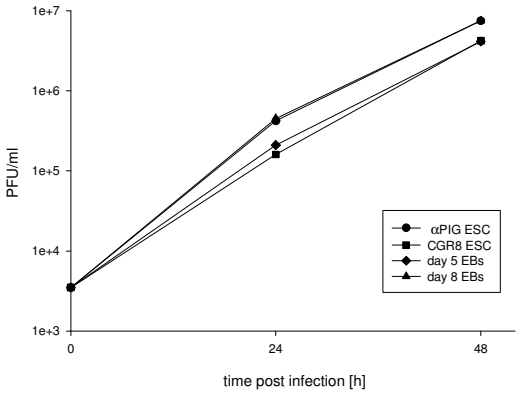


Figure 5: The LCM virus productively infects murine ES and EB cells.

(A) Immunofluorescence microscopy images of undifferentiated αPIG ES or EB cells at day 5 or day 8 of differentiation infected with the LCM virus at a MOI of 0.01. Cells were fixed 48 h after infection and stained in cryosectioned samples using LCM virus specific mAb.

(B) Quantification of infectious LCM virus particles in the culture-supernatants of infected ES and EB cells. Samples were collected at the indicated time points and virus titer was determined by plaque assay. One representative experiment out of four is shown.

4.3. Despite productive infection with the LCM virus neither ES nor EB cells are lysed by LCM virus-specific $CD8^+$ CTL

Having shown that ES and EB cells are productively infected with the LCM virus, we asked whether and to what extend LCM virus infected ES or EB cells are lysed by LCM virus-specific $CD8^+$ T cells. To obtain highly active LCM virus specific $CD8^+$ cytotoxic T cells, C57BL/6 mice were infected intravenously (i.v.) with 10^5 IU of the LCM virus. This mouse strain was chosen because it expresses the same MHC I haplotype, i.e. H-2^b, as that of the ES cell lines used in this study. Eight days after infection, when $CD8^+$ cytotoxic T cell activity reaches its peak level in the C57BL/6 mice, $CD8^+$ T cells were immunomagnetically enriched from splenic single cell suspensions. In standard 4 h ^{51}Cr-release assays, these $CD8^+$ T cells were used as effector cells against non-infected syngeneic C57BL/6-SV fibroblasts (B6SV) or B6SV fibroblasts infected 48 h earlier with the LCM virus as negative or positive control target cells, respectively. LCM virus infected B6SV fibroblasts were lysed to about 65% at an effector : target ratio (E:T) of 100:1, proving that the primary $CD8^+$ CTL were highly cytotoxic *in vitro* (Fig. 6). Despite their high cytotoxic activity, these $CD8^+$ T cells did not significantly lyse LCM virus infected ES cells, as indicated by approximately 10% lysis at an E:T ratio of 100:1 which is close to the background levels of non infected B6SV fibroblasts (Fig. 6). Moreover, LCM virus infected EB cells at day 5 or 8 of differentiation were also poorly lysed, with less than 15% target cell lysis at an E:T ratio of 100:1.

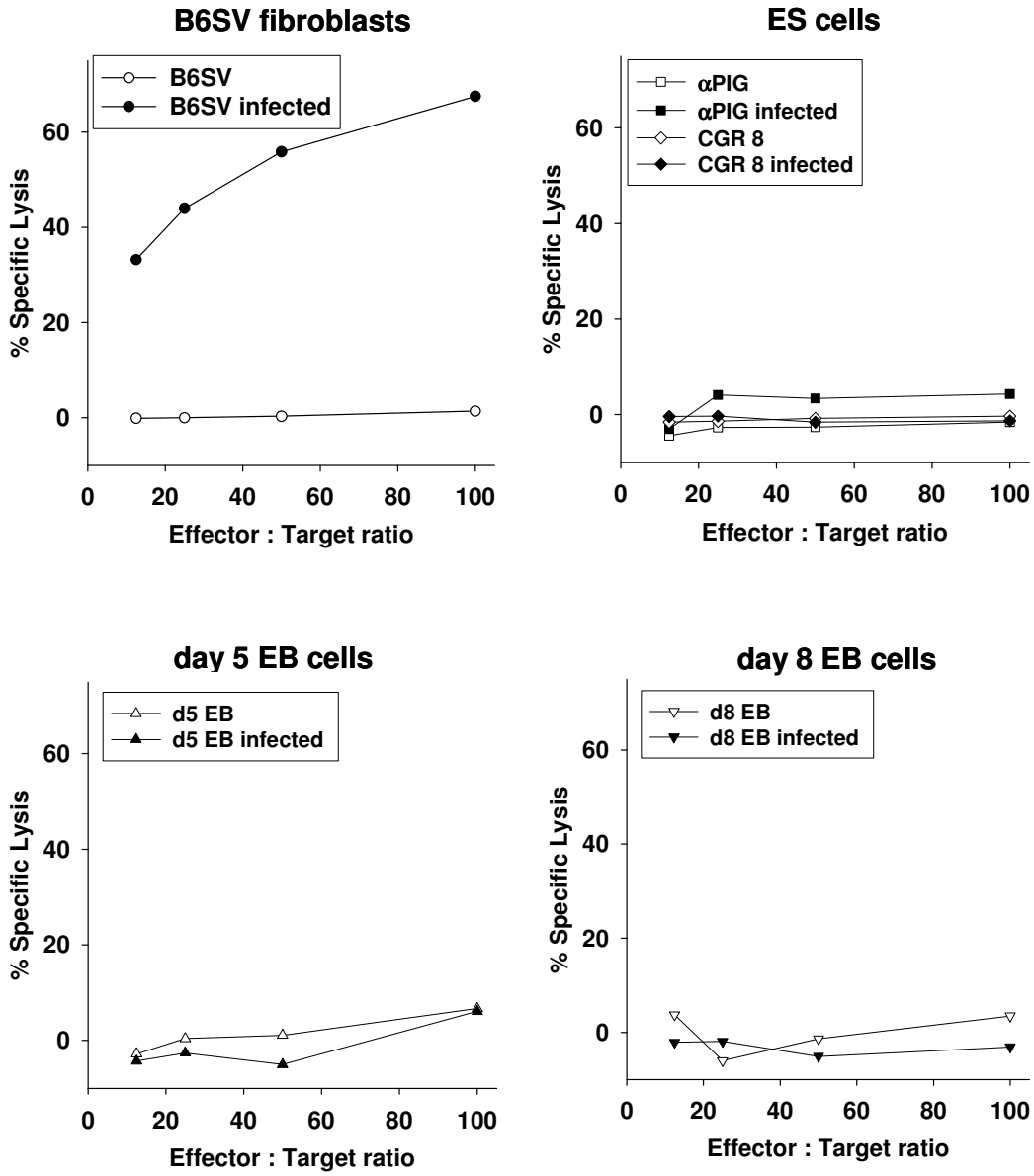


Figure 6: ES and EB cells are resistant against lysis by LCM virus-specific primary $CD8^+$ T cells

C57BL/6 mice were infected i.v. with 10^5 IU of the LCM virus. After eight days $CD8^+$ T cells were immunomagnetically enriched from the splenic single cell suspensions and used as effector cells in 4 h standard ^{51}Cr-release assays against αPIG or CGR8 ES cells and day 5 or 8 EB cells derived from αPIG cells. B6SV fibroblasts were used as controls. Target cells were infected at a MOI of 0.01 with the LCM virus 48 h prior to the assay. The experiment was repeated four times with similar results.

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

$CD8^+$ CTL recognize with their T cell receptor (TCR) short peptides derived from intracellular antigens presented by MHC I molecules on the surface of nucleated cells. Therefore, the expression of MHC I molecules on the cell surface is an absolute prerequisite for the recognition and elimination of cells infected with intracellular pathogens by antigen specific CTL. In previous studies, murine ES cells were found to be devoid of MHC I molecules while human ES cells expressed low levels (26, 49, 50).

The expression level of MHC class I molecules on the ES cell lines and on EB cells used in this study was measured both at the mRNA and the protein level. PCR analysis revealed that undifferentiated ES cells express readily detectable levels of mRNA coding for the heavy chain of H2-K^b MHC class I molecules. In EB cells, expression of MHC I mRNA was enhanced between days 2 to 12 of differentiation (Fig. 7A). Expression of mRNA coding for β_2-microglobulin (β_2M) which is required for the stabilisation of the complex of MHC heavy chain and antigenic peptide was detected in both ES and EB cells (Fig. 7A).

Western blot analysis revealed that in lysates of undifferentiated ES cells, only a faint signal for H2-K^b molecules was detectable. However, H2-K^b was detectable already on day 2 of differentiation in EB cells and the signal strength increased further over the next days of differentiation (Fig. 7B).

IFN-γ enhances the expression of MHC class I molecules in somatic (51, 52) as well as in embryonic cells that express IFN-γ receptors on their surface (27, 50). To assess whether IFN-γ enhances the level of MHC I expression in the ES and EB cells from various differentiation stages used in this study, these cells were incubated with 20 ng/ml IFN-γ for 48 h. As shown in figure 7A and 7B, treatment with IFN-γ did neither enhanced the expression of MHC class I mRNA nor MHC I protein in undifferentiated ES cells as assessed by RT-PCR and Western blot analysis, respectively. However, from day 2 of differentiation, EB derived cells treated with IFN-γ exhibited strongly enhanced expression of MHC I heavy chain at the protein level (Fig. 7B).

Western blot analysis detects the total contents of cellular MHC class I protein without allowing differentiation between the fractions of MHC class I

molecules present on the cell surface, i.e. the fraction relevant for recognition by T cells, and within intracellular compartments. Therefore, surface expression of MHC class I molecules on ES and EB cells was determined by flow cytometry. As expected from the Western blot data, no significant expression of MHC class I molecules on undifferentiated ES cells was detected by flow cytometry (Fig. 7C). However, surface expression of MHC class I molecules was detectable in EB cells from day 2 of differentiation onwards. Treatment with IFN-γ strongly enhanced the expression of MHC class I molecules in EB derived cells from day 4 of differentiation onwards to a level almost comparable to that of the B6SV fibroblasts serving as positive controls (Fig. 7C).

Finally, MHC I expression on the surface of ES and EB cells infected with LCM virus was assessed to exclude that infection with LCM virus might interfere with the expression of MHC I molecules. As shown in figure 7D, infection with LCM virus did neither in undifferentiated ES cells nor in EB cells influence the expression of MHC class I molecules as detected by flow cytometry (Fig. 7D).

Spontaneous expression of low level of MHC I molecules and strong induction of MHC I expression on EB cells by IFN-γ are, in addition to the productive infection of these cells with the LCM virus, essential prerequisites for recognition of target cells by antigen specific CTL. Nevertheless, the poor lysis of ES and EB cells infected with the LCM virus by highly active LCM virus specific $CD8^+$ CTL might be due to low expression of MHC class I molecules or an immature antigen processing machinery in these cells.

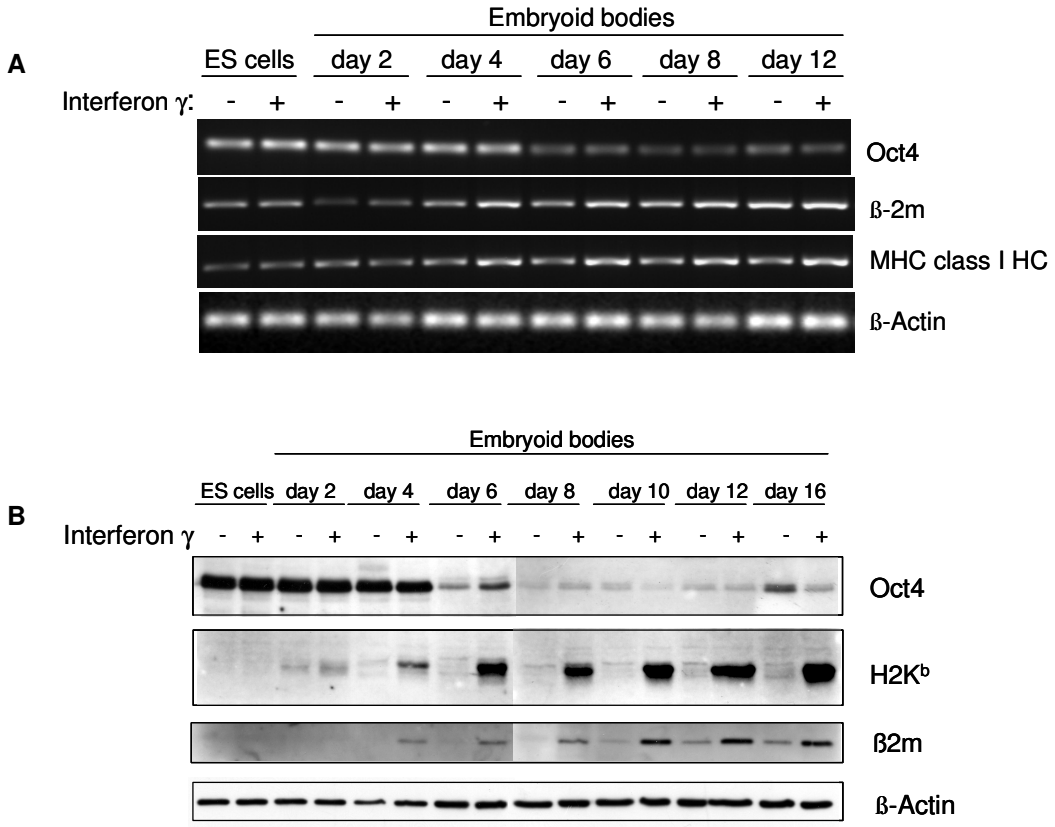


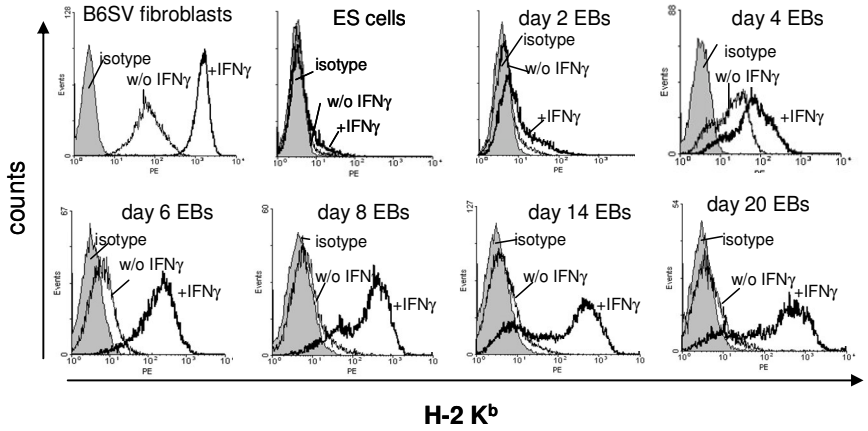
Figure 7: MHC class I expression in ES and EB cells

(A) Expression of mRNA coding for the MHC class I heavy chain, β_2-microglobulin, and Oct4 in IFN-γ-treated and control CGR8 ES and EB cells as detected by RT-PCR. IFN-γ was added to a final concentration of 20 ng/ml for 48 h prior to mRNA isolation. β-actin was amplified as a loading control.

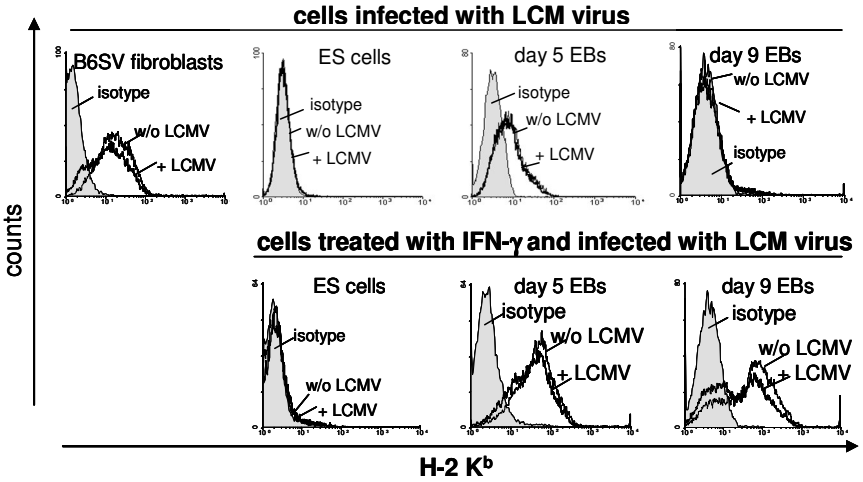
(B) Western Blot analysis of MHC I heavy chain, β_2-microglobulin and Oct4 proteins in CGR8 ES and EB cells from various differentiation stages. ES and EB cells were left untreated or incubated with 20 ng/ml IFN-γ for 48 h prior to preparation of cell lysates. H-2K^b heavy chain was detected by using anti-H-2K^b heavy chain-specific serum. β_2-microglobulin, Oct4 and β-actin were detected using specific monoclonal antibodies.

(to be continued)

C



D



(C) Flow cytometry analysis of MHC class I expression on the surface of undifferentiated αPIG ES and EB cells at different stages of differentiation. B6SV fibroblasts (from H-2^{b} C57BL/6 mice) were used as a positive control. IFN-γ was added to a final concentration of 20 ng/ml for 48 h. Single cell suspensions of ES or EB cells were stained with monoclonal antibody specific for H-2K^{b} (clone AF6-88.5, BD Pharmingen) or isotype control antibodies and were analyzed by FACScan and CellQuest software.

(D) Flow cytometry analysis of MHC I expression on the surface of LCM virus-infected undifferentiated αPIG ES and EB cells at different stages of differentiation. B6SV fibroblasts were used as a positive control. Cells were infected with LCM virus at a MOI of 0.01. After 48 h, single cell suspensions of ES or EB cells were stained with monoclonal antibody specific for H-2K^{b} (clone AF6-88.5, BD Pharmingen) or isotype control antibodies and analyzed by FACScan and CellQuest software.

All data are representative of at least three independent experiments and were also reproduced with CGR8 ES cells.

4.5. ES and EB cells loaded with a peptide resembling LCM virus-derived immunodominant epitope are not lysed by antigen specific $CD8^+$ CTL

Productive infection with LCM virus in combination with expression of cell surface MHC I molecules does not warrant presentation of viral peptides on the plasma membrane of ES and EB cells because the complex machinery of antigen processing and presentation might not be operational in these cell types. To bypass possible defects in the MHC I restricted antigen processing and presentation pathway in ES and EB cells, MHC I molecules were loaded externally with a peptide representing the H-$2D^b$ restricted immunodominant epitope gp_{33-41} derived from the glycoprotein of LCM virus (53). Control B6SV fibroblasts, ES or EB cells were left untreated or loaded with gp_{33-41} at a concentration of 1 µM for 1 h and used as target cells in 4 h ^{51}Cr-release assays. $CD8^+$ T cells immunomagnetically enriched from C57BL/6 mice on day 8 after i.v. infection were used as effector cells. As shown in figure 8, control fibroblasts loaded with gp_{33-41} were lysed to about 40% by the virus specific $CD8^+$ CTL at an E:T ratio of 100:1. In contrast, undifferentiated ES cells loaded with gp_{33-41} were lysed just slightly above the background level regardless of whether αPIG or CGR8 cells were used. Furthermore, EB cells at day 5 and 8 of differentiation loaded with gp_{33-41} were not specifically lysed by the virus specific CTL.

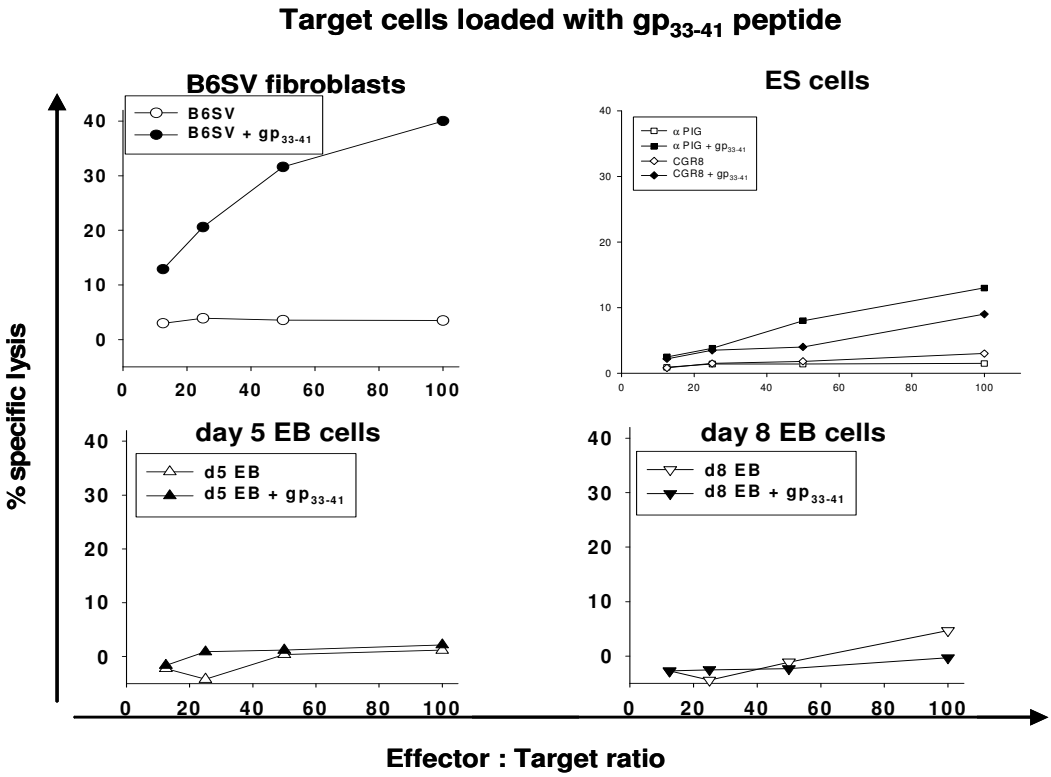


Figure 8: Despite loading with synthetic peptide, ES and EB cells are resistant against CTL mediated cytolysis

Mice were infected i.v. with 10^5 IU of the LCM virus. After 8 days $CD8^+$ T cells were immunomagnetically enriched from the spleens and used as effector cells in 4 h ^{51}Cr-release cytotoxicity assays against αPIG ES cells and day 5 or 8 EB cells. B6SV fibroblasts served as positive controls in this assay. Target cells were loaded with the synthetic LCM virus epitope gp_{33-41} at a concentration of 1 µM for 1 h and non-bound peptide was thoroughly washed away. Experiment repeated five times with similar results.

Since spontaneous expression of MHC I molecules was low in ES and EB cells, these experiments were repeated after induction of MHC I expression with 20 ng/ml IFN-γ for 48 h. Although IFN-γ strongly enhanced MHC I expression in both day 5 and 8 EB cells (Fig. 7B and 7C), only day 8 EB cells were lysed to about 20% at an E:T ratio of 100:1 (Fig. 9A). Thus, the cytolysis level of day 8 EB-derived target cells treated with IFN-γ remained more than four-fold reduced as compared to that of control fibroblasts, as judged by horizontal comparison of the effector to target ratios required to achieve a given percentage of cytolysis (Fig. 9B).

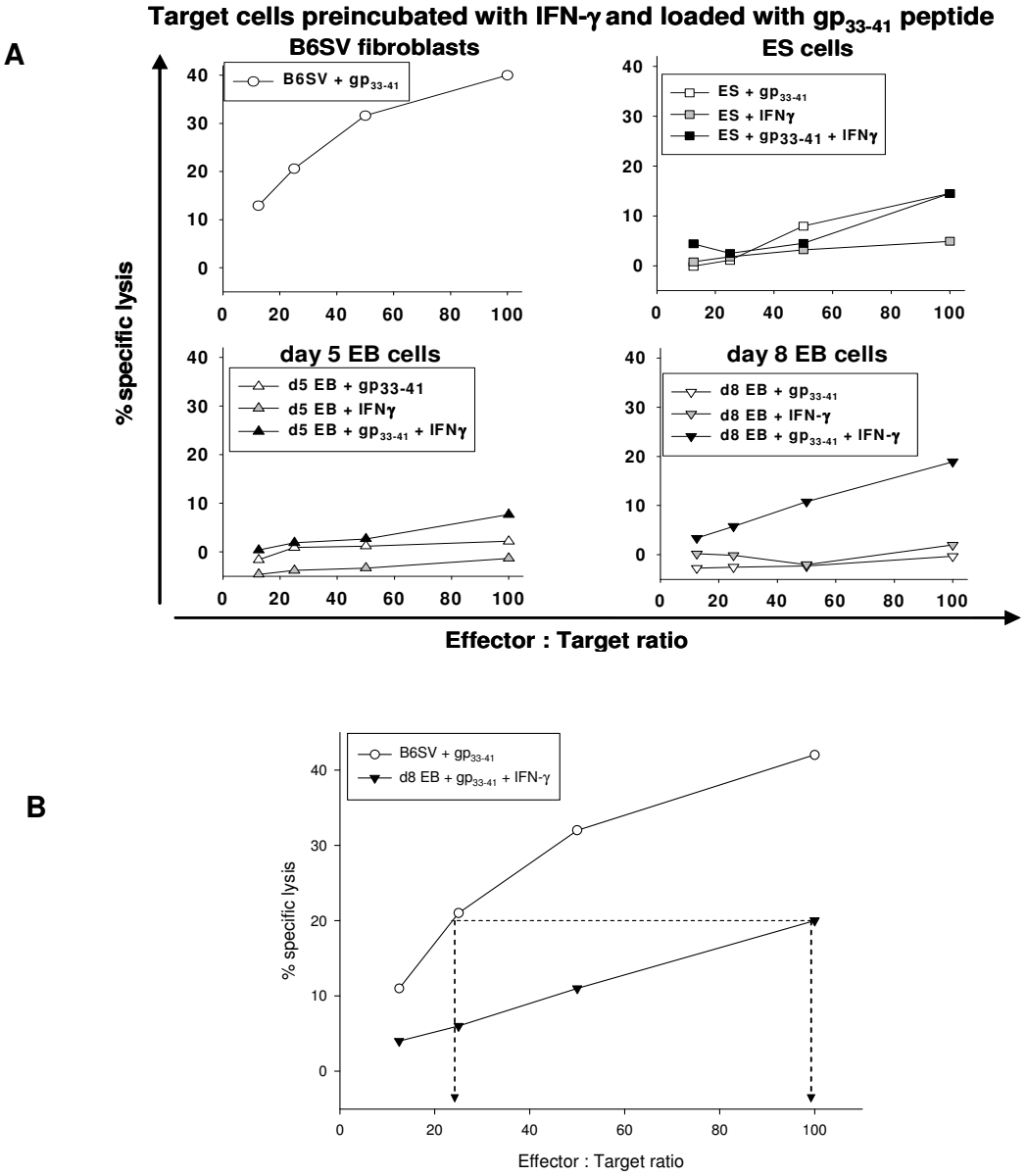


Figure 9: Despite induction of MHC I expression with IFN-γ, ES and EB cells are resistant against CTL mediated cytolysis

(A) Mice were infected i.v. with 10^5 IU of the LCM virus. After 8 days $CD8^+$ T cells were immunomagnetically enriched from the spleens and used as effector cells in 4 h ^{51}Cr-release cytotoxicity assays against αPIG ES cells and day 5 or 8 EB cells. B6SV fibroblasts served as positive controls. Target cells were pretreated with 20 ng/ml IFN-γ for 48 h prior to loading with gp_{33-41}. These assays were repeated three times with similar results.

(B) Horizontal comparison of E:T ratio required to achieve the same percentage of specific lysis in B6SV fibroblasts (taken form figure 9A, upper left panel) and IFN-γ-treated EB cells (taken form figure 9A, lower right panel) which were loaded with gp_{33-41} epitope at a concentration of 1 µM. For about 20% target cell lysis an E:T ratio of about 25:1 is required for B6SV fibroblasts whereas, an E:T ratio of 100:1 is required for IFN-γ-treated EB cells.

4.6. ES cells are susceptible to well established apoptotic stimuli

The observation that ES and EB cells are resistant to lysis by highly cytotoxic $CD8^+$ T cells raised the question whether ES and EB derived cells might be resistant to apoptosis in general. To assess the susceptibility of ES cells to apoptosis, undifferentiated ES cells were incubated for 5 h with 0.5 µM staurosporine (STS) or were irradiated with UV light (253-255 nm, 20mJ/cm^2) for 5 h. Subsequently, cells were labelled with Annexin V, a probe for the early phase of apoptosis and the percentage of apoptotic cells in the samples was determined by flow cytometry. As shown in figure 10A, more than 75% of ES cells were positive for Annexin V after treatment with staurosporine or irradiation with UV light. This indicates that ES cells are fully susceptible to two independent apoptotic stimuli.

One of the molecular features of apoptosis is the activation of the proapoptotic caspase-3 in response to death signals. Caspase-3 activation was assessed by staining ES cells with mAb specific for the activated form of caspase-3 and subsequent immunofluorescence microscopy analysis (Fig 10B). Furthermore, enzymatic activity of caspase-3 was measured in total cytosolic extracts of ES cells by fluorometry using DEVD-AFC as a substrate (44). Both approaches revealed that caspase-3 activity in ES cells following treatment with staurosporine or irradiation with UV light was almost as high as in control fibroblasts (Fig. 10B and 10C). In agreement with previous data for murine and human ES cells (49, 54-60), these data indicate that ES cells are responsive to different apoptotic stimuli. This argues strongly against resistance of ES cells to CTL mediated lysis due to general defects in the proapoptotic signalling pathways.

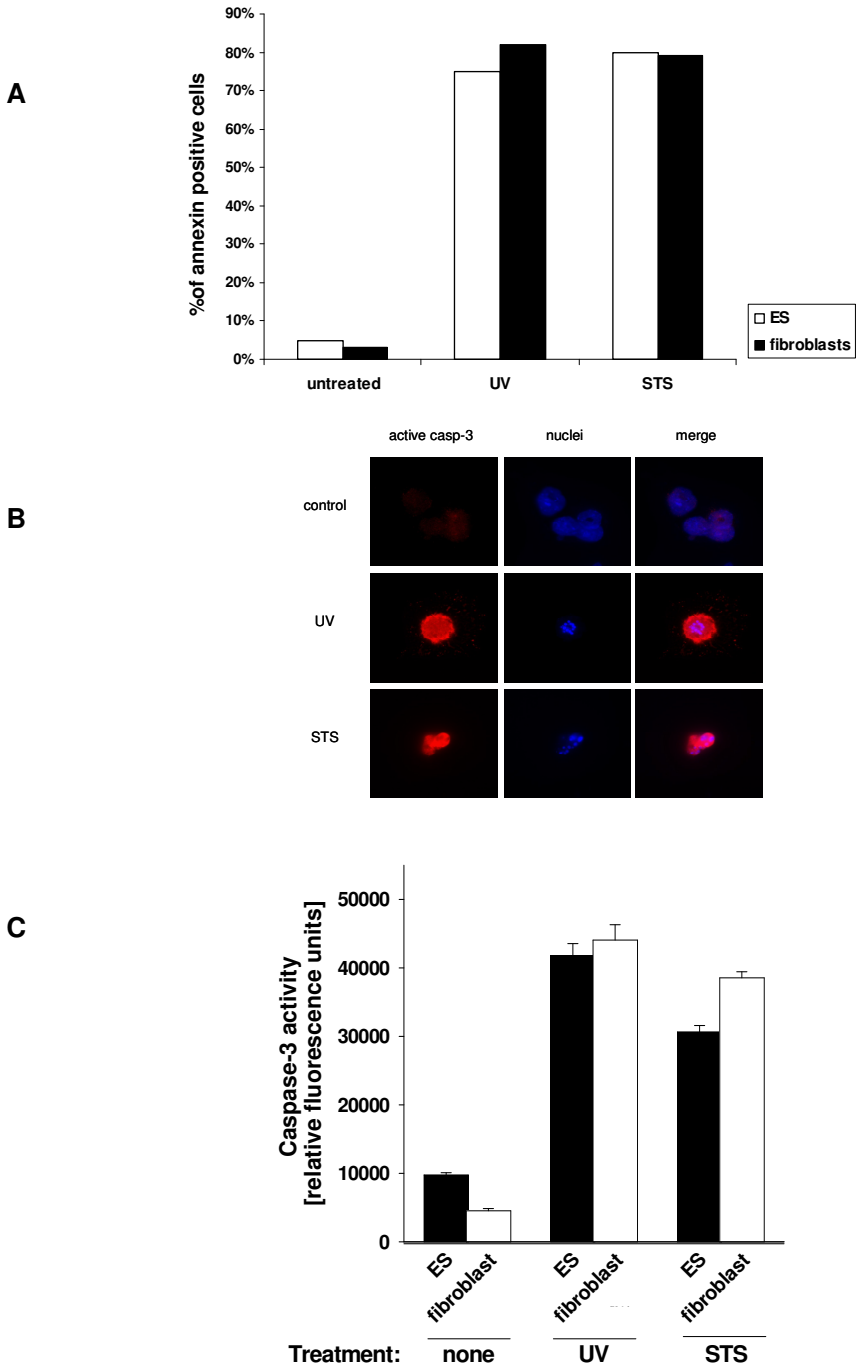


Figure 10: ES and EB cells are not resistant against apoptosis induced by UV-irradiation or staurosporine

Apoptosis of ES cells was induced by UV irradiation (20 mJ/10 cm^2) or treatment with 0.5 µM staurosporine (STS) for 5 h.

(A) The percentage of apoptotic cells detected by flow cytometry analysis of ES cells or control B6SV fibroblast after staining with Annexin V.

(B) Activated caspase-3 was detected by immunofluorescence microscopy using monoclonal antibody specific for the activated form of caspase-3.

(C) Caspase-3 specific enzymatic activity was analyzed in the cytosolic extracts using DEVD-AFC after 12 h. One fluorescence unit is equivalent to 0.65 pmol of released AFC (44).

4.7. ES cells and EB cells presenting viral epitopes are recognized by LCM virus-specific CD8$^+$ CTL in antigen-specific and MHC class I restricted manner

The observation that ES cells infected with the LCM virus or loaded with a peptide resembling an immunodominant viral epitope are not lysed by LCM virus specific CTL in spite of functional apoptotic machinery, led to the question whether ES and EB cells are recognized by antigen specific CD8$^+$ T cells. To address the question whether LCM virus specific CD8$^+$ T cells recognize ES and EB cells presenting the LCM virus derived epitope gp_{33-41}, ES and EB cells were infected with LCM virus 48 h prior to the experiment at a MOI of 0.01 or they were loaded with the LCM virus-derived peptide gp_{33-41} for 1 h. B6SV and BALB/cSV fibroblasts were used as syngeneic or allogeneic control target cells, respectively. LCM virus specific CD8$^+$ CTL cells immunomagnetically enriched from C57BL/6 or BALB/c mice on day 8 of LCM virus infection were incubated with the target cells at an E:T ratio of 100:1.

After 5 h incubation, IFN-γ was measured in the supernatant of the cocultures by ELISA in order to assess the recognition of target cells by CTL. As shown in figure 11, CTL from C57BL/6 mice secreted in response to ES and EB cells infected with LCM virus or loaded with gp_{33-41} almost as large quantities of IFN-γ as in response to syngeneic B6SV fibroblasts. The high amount of IFN-γ secreted by CTL in response to ES and EB cells indicates an effective recognition of the ES and EB cells by the antigen specific CTL. MHC I restriction was confirmed by using allogeneic CTL derived from BALB/c mice that did not respond to H-2^b expressing ES and EB cells while they strongly responded to virus infected syngeneic BALB/cSV fibroblasts. These lines of evidence suggest that ES and EB cells presenting antigenic peptides in a MHC I-restricted fashion are actively recognized by antigen specific CTL despite low surface expression of MHC I in these cells.

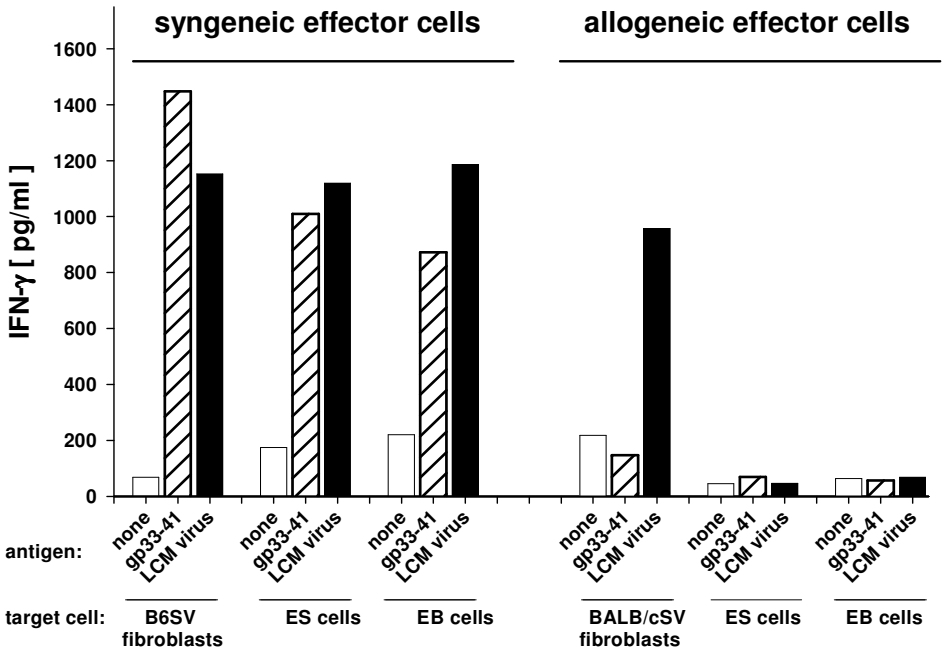


Figure 11: Secretion of IFNγ by LCM virus-specific CD8$^+$ T cells in response to ES and EB cells presenting a viral epitope

B6SV or BALB/cSV fibroblasts, αPIG ES cells and day 8 EB target cells were left non-treated, loaded with gp_{33-41} peptide (1 µM for 1 h prior to the assay) or infected with the LCM virus at MOI 0.01. Syngeneic (C57BL/6) or allogeneic (BALB/c) CD8$^+$ T cells were immunomagnetically enriched from splenic cells of LCM virus-infected mice and were used as effector cells. Target and effector cells were cocultured at an E:T ratio of 100:1. After 5 h supernatants of the cultures were collected and IFNγ was quantified by ELISA.

The use of high concentrations of antigenic peptide in the above described assays raised the concern that subsequent to washing away unbound peptide, some peptide might diffuse from the MHC molecules of ES and EB cells and bind to the MHC molecules on $CD8^+$ T cells which are present at high densities. Thus, $CD8^+$ T cells could be rendered with stimulatory potential for neighbouring $CD8^+$ CTL. To estimate the possibility that $CD8^+$ T cells present viral peptide, LCM virus specific CTL obtained from C57BL/6 mice, ES and EB cells from day 5 and 8 of differentiation were loaded with gp_{33-41} at different concentrations and used as target cells. As shown in figure 12, in comparison to ES or EB cells, $CD8^+$ target cells required about 100-fold increased concentrations of gp_{33-41} to induce secretion of comparable amounts of IFN-γ by virus specific CTL. Thus, the remote possibility that $CD8^+$ effector CTL might pick up peptide from ES or EB target cells and subsequently function themselves as antigen presenting cells was largely ruled out.

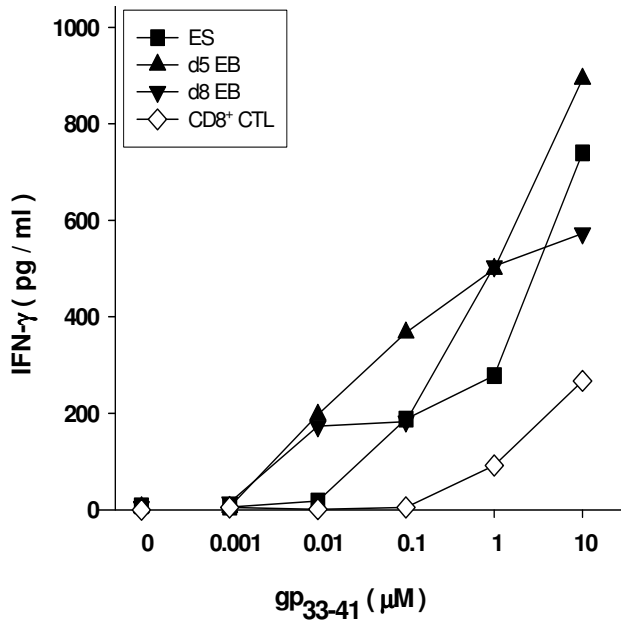


Figure 12: $CD8^+$ target cells require about 100-fold higher peptide concentration than ES cells to induce the secretion of comparable amounts of IFN-γ

$CD8^+$ T cells obtained form LCM virus-immune C57BL/6 mice, ES and day 5 and day 8 EB cells were loaded with the LCM virus peptide gp_{33-41} at the indicated concentrations for 1 h and were used as target cells. LCM virus specific $CD8^+$ T cells were immunomagnetically enriched from splenic cells of LCM virus-infected mice and were used as effector cells. Target and effector cells were cocultured at an E:T ratio of 100:1. After 5 h supernatants of the cultures were collected and IFNγ was quantified by ELISA.

4.9. ES and EB cells infected with LCM virus induce polarization and fusion of cytotoxic granules at the immunological synapse of LCM virus-specific CD8$^+$ CTL

The recognition of target cells by cytotoxic T lymphocytes via their TCR triggers the polarization of the cytotoxic granules containing the cytotoxic effector molecules, i.e. the pore forming perforin and the proapoptotic granzymes, towards the immunologic synapse (61). It is well known that secretion of cytokines and degranulation of cytotoxic granules by CTL have different stimulatory thresholds (62). Therefore the observation that ES and EB cells infected with LCM virus or loaded with gp_{33-41} epitope are not lysed although they were recognized by LCM virus specific CD8$^+$ CTL, raised the question whether interaction between CTL and these target cells is strong enough to trigger the polarization of the cytotoxic granules towards the immunologic synapse and their subsequent exocytosis.

LCM virus specific CD8$^+$ T cells enriched from splenic cells of C57BL/6 mice on day 8 post infection with LCM virus were used as effector cells. ES cells were loaded with the LCM virus specific epitope gp_{33-41} for 1 h and were incubated with the CD8$^+$ cells at an E:T ratio of 2:1 for 10 min. Afterwards, cells were processed for the visualization of cytotoxic granules in the CD8$^+$ T cells by mAb specific for granzyme A and counter staining of ES cells with mAb specific for SSEA-1. As shown in figure 13, virus specific CD8$^+$ T cells in contact with ES target cells rapidly and quantitatively polarized their cytotoxic granules towards the immunologic synapse within 10 min.

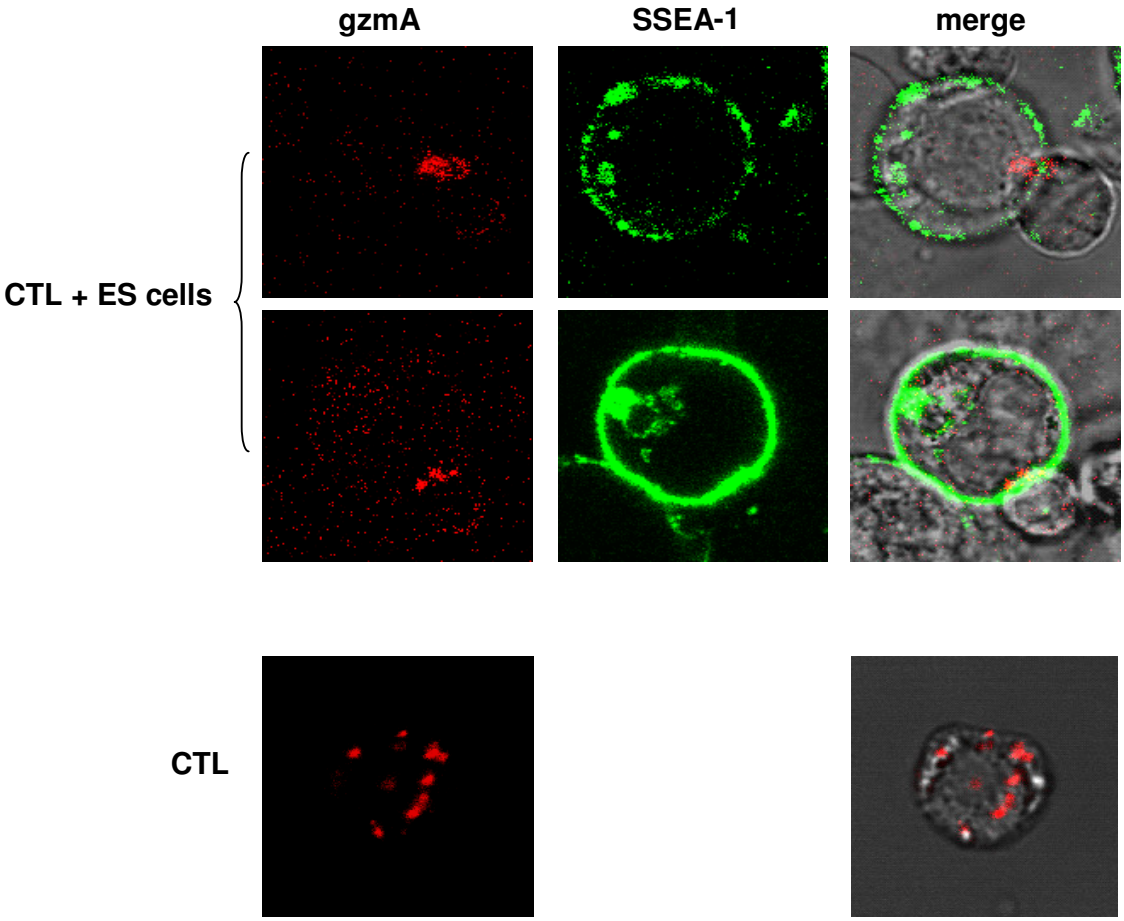


Figure 13: LCM virus infected ES cells induce the polarization of the cytotoxic granules of CD8$^+$ CTL towards the immunologic synapse

CGR8 ES cells infected with LCM virus at MOI 0.01 for 48 h were used as target cells. CD8$^+$ T cells from LCM virus-immune mice were either incubated with target cells at a ratio of 2:1 for 10 min or left alone. Subsequently cells were stained with anti-granzyme A antibody to visualize the cytotoxic granules in the CD8$^+$ CTL and with SSEA-1 specific mAb to identify the ES cells. Polarization of the cytotoxic granules towards the immunologic synapse was determined by confocal microscopy.

To assess whether the polarized cytotoxic granules fuse properly with the plasma membrane of the $CD8^+$ T cells at the immunologic synapse, an assay detecting the exposure of CD107a on the plasma membrane of CTL was used (63). CD107a, i.e. lysosome-associated membrane protein 1 (Lamp1), localizes in resting CTL to the inner leaflet of the lysosomal membrane. Upon fusion of the lysosomal membrane with the plasma membrane, CD107a is exposed on the outer leaflet of the plasma membrane so that it can be quantified by flow cytometry analysis. ES or EB cells were infected with the LCM virus 48 h prior to the experiment at a MOI of 0.01 or they were loaded with LCM virus-specific epitope gp_{33-41} for 1 h. B6SV fibroblasts treated in the same way were used as positive control target cells. $CD8^+$ CTL cells enriched from C57BL/6 mice on day 8 after infection with LCM virus were incubated with these target cells at an E:T ratio of 2:1. Exposure of CD107a on the cell surface of $CD8^+$ cells was monitored by flow cytometry analysis. Both, the percentage of $CD8^+$ CTL expressing surface CD107a in response to ES or EB cells as well as the strength of the signal were comparable to that observed in response to B6SV control fibroblasts (Fig. 14).

These findings indicate a proper fusion of the cytolytic granules with the plasma membrane of the CTL, which is the ultimate prerequisite for the release of cytotoxic effector molecules.

Taken together, despite low level expression of MHC I molecules on their surface, undifferentiated ES or EB cells induce virus specific $CD8^+$ T cells in an antigen specific and MHC class I-restricted fashion to form immunological synapses, to secrete IFN-γ, to polarize the cytotoxic granules towards the synapse and to fuse the membrane of these granules with the plasma membrane. However, highly effective cytotoxic lymphocytes do not lyse ES or EB target cells. This might be due to a specific resistance of ES or EB cells against the cytotoxic mechanisms of the CTL.

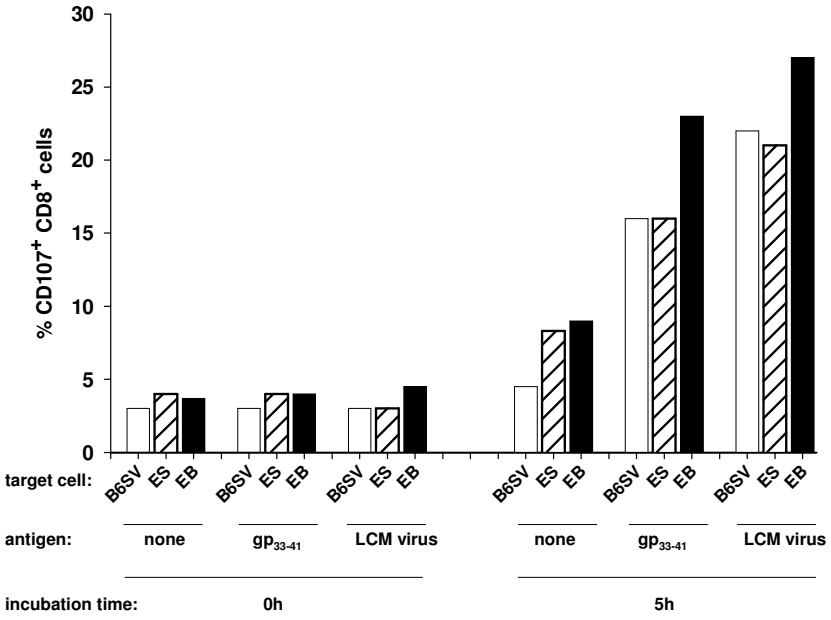


Figure 14: ES cells induce the fusion of cytotoxic granules with the plasma membrane of CD8$^+$ T cells as effectively as B6SV fibroblasts

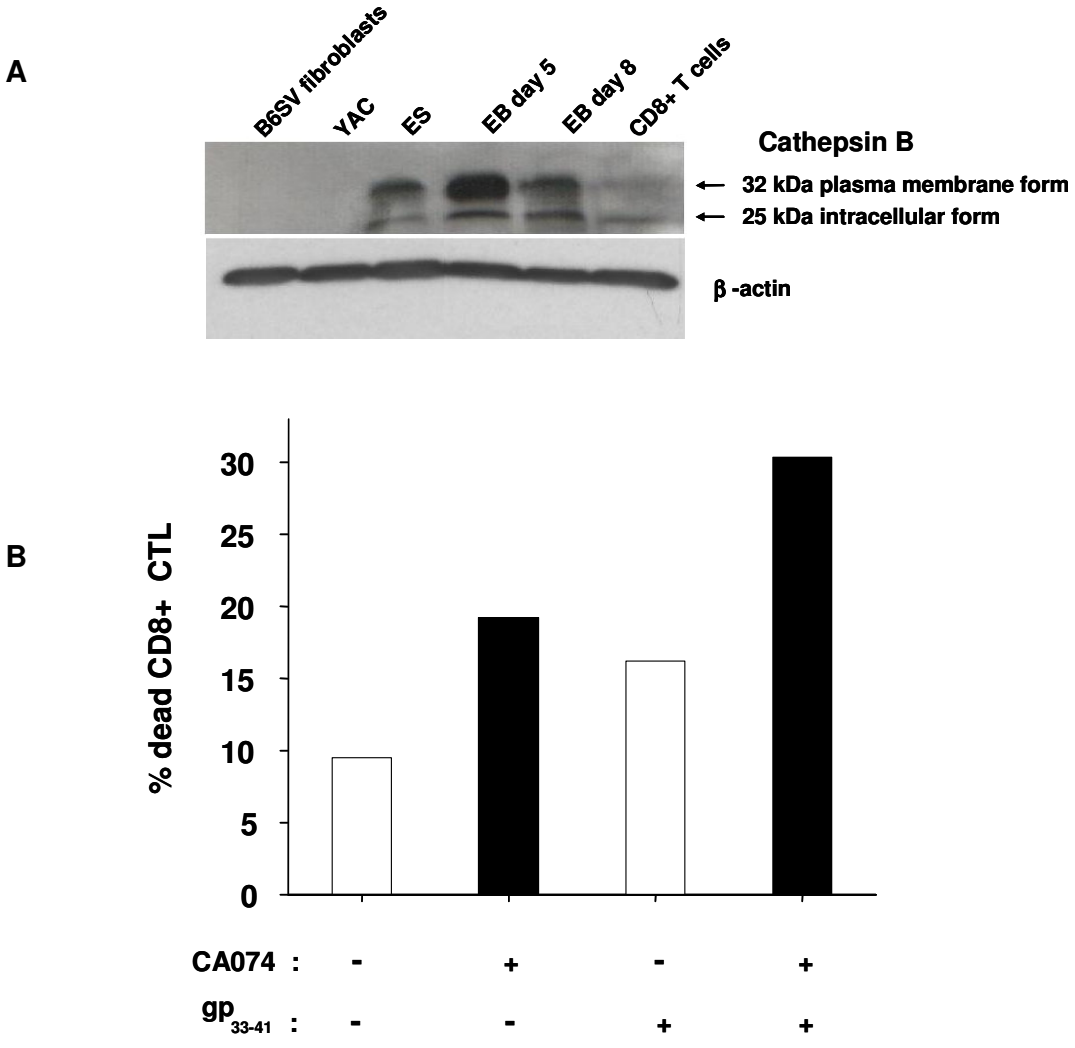
CD8$^+$ T cells from LCM virus-immune mice were incubated with syngeneic fibroblasts (B6SV), undifferentiated ES cells and day 8 EB cells derived from αPIG ES cells. The target cells were non-infected, loaded with 1 µM of the epitope gp_{33-41} for 1 h prior to the assay or infected with the LCM virus. Exposure of CD107a/Lamp1 on the plasma membrane of the CD8$^+$ T cells was determined by flow cytometry at the indicated time points after staining the cells for CD8 and CD107a.

4.10. Undifferentiated ES and EB cells express high levels of Cathepsin B, a specific inhibitor of perforin

Cytotoxic lymphocytes lyse their target cells mainly by virtue of the pore forming protein perforin together with granzymes. To avoid self destruction by these highly cytotoxic effector molecules stored at high concentrations within the cytotoxic granules, CTL express several specific inhibitors. The observed resistance of ES and EB cells against lysis by CTL led to the hypothesis that ES and EB cells might also employ these anti-cytotoxic molecules to protect themselves against lysis by CTL.

Cathepsin B, a lysosomal cystein protease, was recently reported to protect CTL against suicide by perforin via hydrolytic inactivation of perforin (64-66). As shown in figure 15A, lysates of undifferentiated ES as well as EB cells contained high levels of both forms of Cathepsin B, the cell surface-attached 25 kDa form and the intracellular 32 kDa form, as detected by Western blot analysis. Remarkably, the level of Cathepsin B protein observed in ES and EB cells is even higher than that observed in activated $CD8^+$ T cells.

Before studying the functional significance of Cathepsin B in ES and EB cells, an assay proving specific inhibition of Cathepsin B had to be established. Therefore, the LCM virus specific $CD8^+$ effector cells were left untreated or pretreated with 10 µM of the Cathepsin B inhibitor CA074 for 4 h. B6SV target fibroblasts loaded with the LCM virus specific epitope gp_{33-41} or left untreated were coincubated with the $CD8^+$ effector cells at an E:T ratio of 50:1 to stimulate the $CD8^+$ cells to secrete cytotoxic granules. After 4 h cells were stained for CD8 and propidium iodide (PI) and analysed by flow cytometry for the percentage of dead $CD8^+$ cells as determined by PI uptake. As shown in figure 15B, about 10% of untreated $CD8^+$ cells incubated with target cells not presenting the viral epitope gp_{33-41} were dead. Both, pretreatment of $CD8^+$ cells with CA074 alone or incubation of untreated $CD8^+$ cells with target cells loaded with the viral epitope resulted in a slightly decreased percentage of 15-20% dead $CD8^+$ cells. Finally, coincubation of $CD8^+$ cells pretreated with CA047 with target cells presenting the viral epitope gp_{33-41} resulted in an increased fraction of 30% dead $CD8^+$ cells, confirming that CA074 inhibits Cathepsin B.



Figue 15: ES and EB cells express Cathepsin B, an inhibitor of perforin mediated suicide in CD8$^+$ CTL

(A) Western blot analysis of the expression of Cathepsin B in αPIG ES cells, EB cells, B6SV fibroblasts, YAC cells, and LCM virus-immune day 8 CD8$^+$ CTL. Cathepsin B isoforms were detected by using anti-murine Cathepsin B monoclonal antibody and β-actin was detected using specific monoclonal antibody.

(B) Proof of the effectiveness of Cathepsin B inhibitor CA074. LCM virus specific CD8$^+$ cells were left untreated or incubated with 10 µM CA074 for 4 h prior to the assay. B6SV fibroblast target cells loaded with the epitope gp_{33-41} or left untreated were incubated with the CD8$^+$ effector cells at E:T ratio of 50:1. After 4 h coincubation, the percentage of PI$^+$ CD8$^+$ cells was determined by flow cytometry.

To test whether Cathepsin B protects ES and EB derived target cells against CTL mediated lysis, these cells were treated with 10 µM CA074 for 16 h prior to the experiment. Subsequently, ES and EB cells were loaded with the viral peptide gp_{33-41} at a concentration of 1 µM for 1 h. B6SV fibroblasts treated in the same way were used as control target cells. LCM virus specific CTL were immunomagnetically enriched from C57BL/6 mice on day 8 after infection were used as effector cells in 4 h ^{51}Cr-release assays. As shown in figures 16A and 16B, pretreatment with the Cathepsin B inhibitor CA074 did not render ES and EB cells susceptibility to lysis by virus specific CTL.

Thus, although ES and EB cells express high levels of Cathepsin B no contribution of Cathepsin B to the resistance of these cells against lysis by CTL was detectable. It remains to be studied whether Cathepsin B might protect ES and EB cells under different conditions.

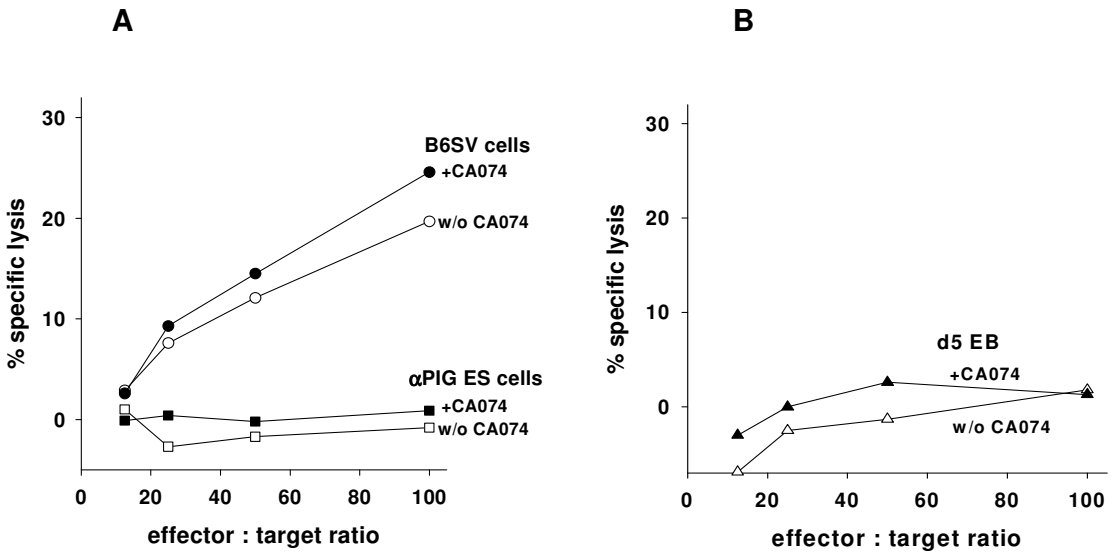


Figure 16: Treatment of ES and EB cells with the Cathepsin B inhibitor C074 does not render these cells susceptible to lysis by CTL

B6SV fibroblast or αPIG ES cells (A) or day 5 EB cells (B) were left untreated or pretreated with the Cathepsin B inhibitor CA074 at a concentration of 10 µM for 16 h before being loaded with 1 µM of gp_{33-41} peptide for 1 h. These cells were used as target cells in a 4 h standard ^{51}Cr-release assay. LCM virus specific $CD8^+$ CTL effector cells from mice infected 8 days before with the LCM virus were incubated with the target cells at different E:T ratios.

4.12. Undifferentiated ES and EB cells express high levels of Serpin-6, a specific inhibitor of granzyme B

The murine serine proteinase inhibitor 6 serpin-6 (SPI-6) and its human homologue PI9 were reported to inactivate granzyme B and thus protect CTL against suicide or fratricide (67, 68). Beyond CTL, dendritic cells and several tumor cells were shown to protect themselves against CTL mediated cytolysis by expressing SPI-6 or PI9 (67-76).

To investigate whether SPI-6 might be involved in the resistance of ES and EB cells against lysis by LCM virus specific CTL, expression of SPI-6 in ES and EB cells was assessed by real time PCR and Western blot analysis. Real time PCR revealed that both ES cell lines CGR8 and αPIG as well as EB cells express significant amounts of mRNA coding for SPI-6 although slightly less than activated $CD8^+$ cells (Fig. 17A). Western blot analysis revealed that expression levels of SPI-6 protein in both ES cell lines and in EB cells are as high as those observed in activated $CD8^+$ cells (Fig. 17B and 17C) and just slightly below those detected in placenta and dendritic cells (Fig. 17B).

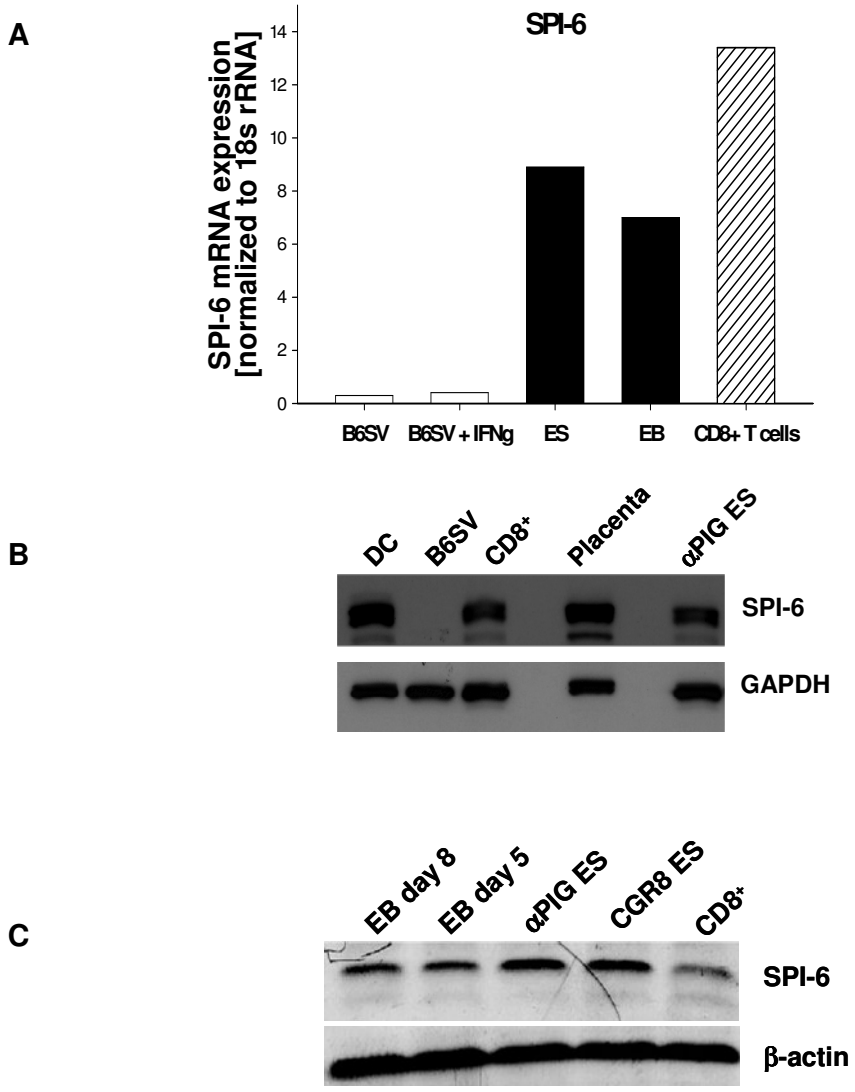


Figure 17: ES and EB cells express SPI-6, an inhibitor of granzyme B

(A) Expression of mRNA coding for murine SPI-6 was determined by real time PCR in undifferentiated αPIG and CGR8 ES cells, day 8 EB cells differentiated from αPIG ES cells, B6SV fibroblasts, and $CD8^+$ CTL from day 8 LCM virus-infected C57BL/6 mice.

(B) Western blot analysis for the expression of SPI-6 in undifferentiated αPIG ES cells, placenta, LCM virus-immune day 8 $CD8^+$ CTL, B6SV fibroblasts and DC. SPI-6 was detected by using anti-SPI-6/PI9 monoclonal antibody and GAPDH was detected using specific monoclonal antibody.

(C) Western blot analysis for the expression of SPI-6 in LCM virus-immune day 8 $CD8^+$ CTL, CGR8 and α-PIG undifferentiated ES cells, day 5 and day 8 EB cells derived from CGR8 ES cells. SPI-6 was detected by using anti-SPI-6/PI9 monoclonal antibody and β-actin was detected using specific monoclonal antibody.

4.13. SPI-6 protects ES cells against cytolysis by LCM virus-specific $CD8^+$ cells

To determine whether SPI-6 is involved in the resistance of ES cells against CTL mediated cytotoxicity, RNA interference was used to knock down the expression of SPI-6 in ES cells. As shown in figure 18A, effective down-regulation of SPI-6 mRNA was achieved with the two independent shRNA expressing Lentiviral vectors pLPac/EGFP/sh-SPI-6#1 and pLPac/EGFP/sh-SPI-6#2 designated as SPI-6-#1 and #2. Transduction of ES cells with each of these shRNA vectors that target different sequences of SPI-6 mRNA significantly reduced the expression of SPI-6 mRNA to less than 10% of untreated control cells (Fig. 18A). A vector coding for scrambled sequence (pLPac/EGFP/sh-scr) showed only a moderate non-specific effect. Consistent with the down-regulation of SPI-6 mRNA, Western blot analysis revealed that both specific vectors induced a strong reduction in the expression of SPI-6 protein in ES cells (Fig. 18B and 18C), while the control vector showed no effect.

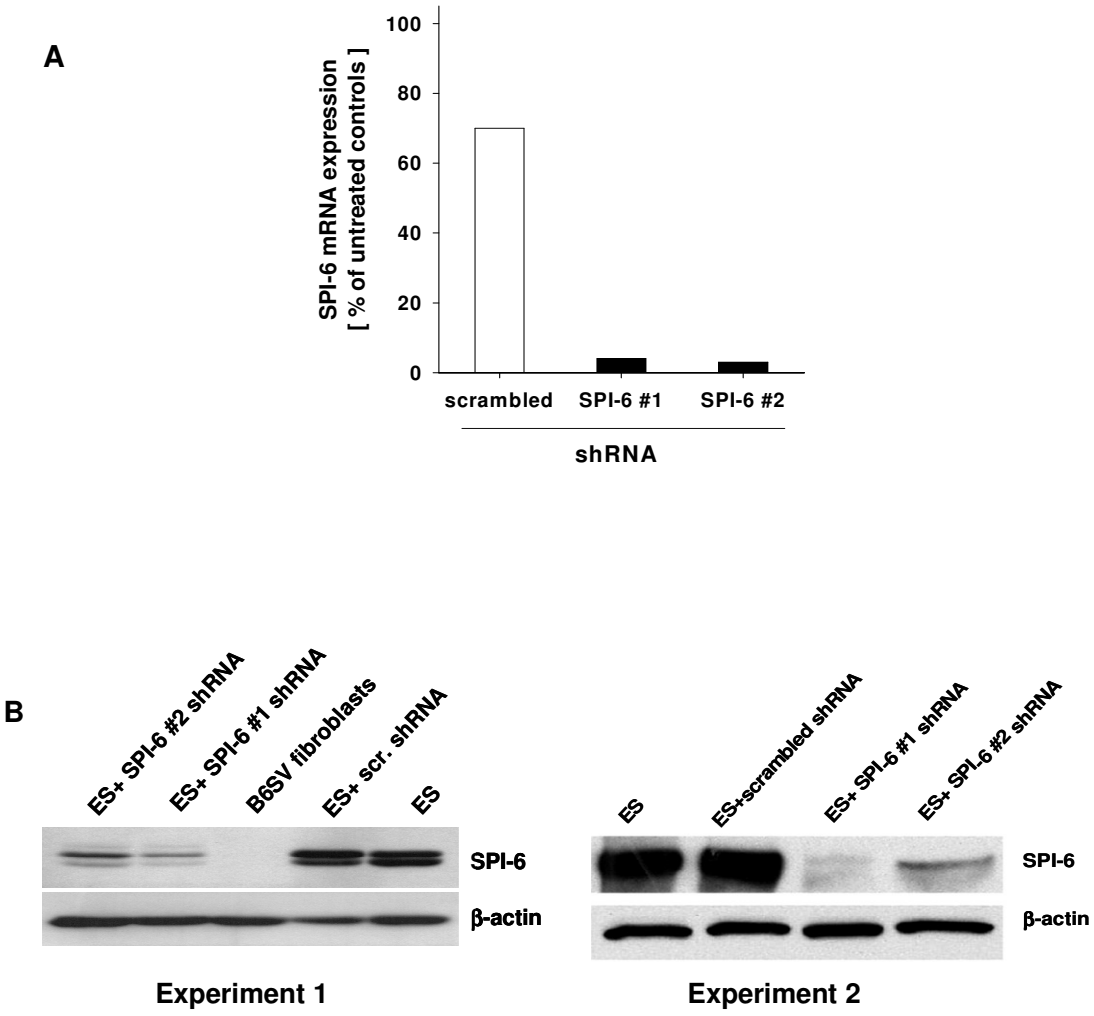


Figure 18: Down regulation of SPI-6 on mRNA and protein level by RNA interference

(A) Quantification of SPI-6 mRNA in CGR8 ES cells after transduction with vectors expressing shRNA targeting two distinct regions of murine *spi*-6, SPI-6 #1 and SPI-6 #2, or a non-specific scrambled shRNA (scr) and selection for puromycin resistance for four days. The residual expression was normalized to the level measured in non-treated control cells.

(B) Western blot analysis for the expression of SPI-6 protein in cell lysates of CGR8 ES cells expressing shRNA against murine *spi*-6, SPI-6 #1 and SPI-6 #2, or a non-silencing scrambled shRNA (scr) or B6SV fibroblasts as negative control. Blots were subjected to mAb specific for SPI-6 or β-actin as loading control. Shown are results from two independent experiments.

To test the retrace of SPI-6 for the resistance of ES cells against the cytotoxicity of virus specific $CD8^+$ cells, ES cells expressing SPI-6 specific shRNA or scrambled shRNA were loaded with the LCM virus specific epitope gp_{33-41} and used as target cells. LCM virus specific $CD8^+$ effector cells were immunomagnetically enriched from C57BL/6 mice infected 8 days earlier with LCM virus and were used in 4 h ^{51}Cr-release assay B6SV fibroblasts were left untreated or loaded with gp_{33-41} were used as control target cells. As shown in figure 19, control ES cells or ES cells expressing non-specific scrambled shRNA that are loaded with gp_{33-41} were not lysed by the LCM virus specific $CD8^+$ cells. In contrast, ES cells expressing SPI-6 specific shRNA that are loaded with gp_{33-41} epitope were lysed almost as effectively as control fibroblasts by the virus specific CTL.

These data indicate that SPI-6 down-regulation completely sensitizes ES cells for lysis mediated by virus specific $CD8^+$ CTL. This provides a strong functional proof for the hypothesis that expression of SPI-6 protein confers resistance to ES cells against lysis by virus specific $CD8^+$ CTL.

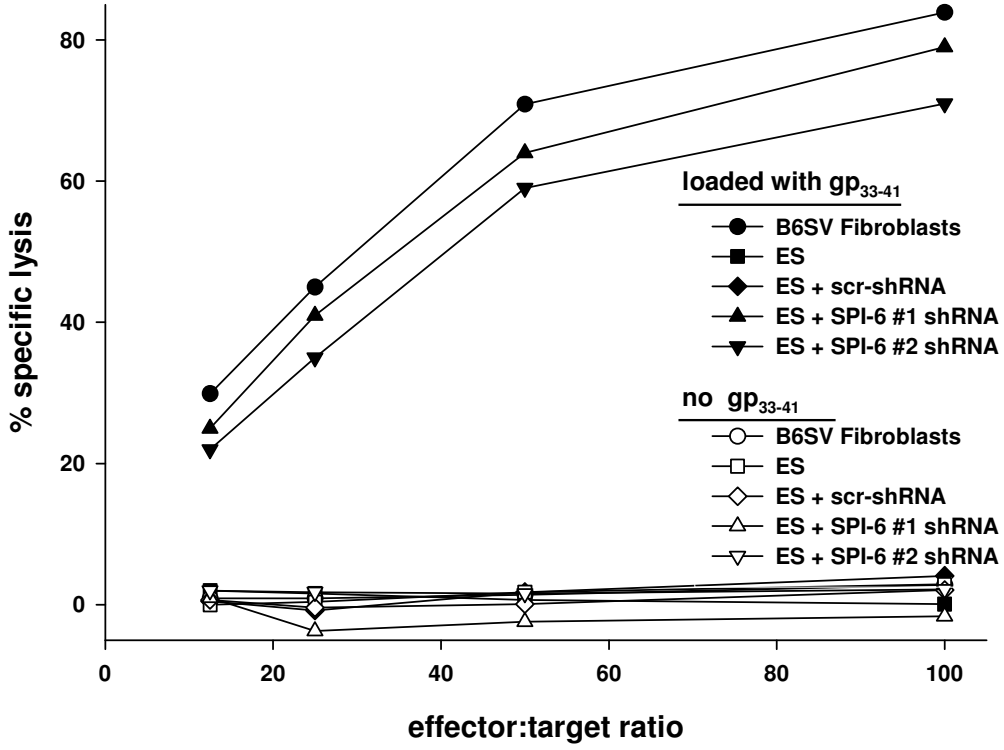


Figure 19: Down-regulation of SPI-6 renders ES cells fully susceptible for lysis by $CD8^+$ CTL

CGR8 ES cells expressing either SPI-6-specific shRNA, SPI-6 #1, SPI-6 #2 or non-specific scrambled shRNA were loaded with 1 µM of gp_{33-41} peptide for 1 h were used as target cells in ^{51}Cr-release cytotoxicity assay. LCM virus specific $CD8^+$ effector cells were immunomagnetically enriched from splenic single cell suspensions of C57BL/6 mice infected eight days earlier with 10^5 IU of the LCM virus. Effector and target cells were incubated for 4 h at different E:T ratios. B6SV fibroblasts were used as positive or negative control target cells.

5. Discussion

In regenerative medicine, transplants of embryonic stem cells (ES) or their differentiated derivatives will be confronted by the same immune responses as any conventional organ transplant. To date, little is known about the immunologic properties of ES cells and several assumptions have just been extrapolated from findings with other cell types without being formally proven for ES cells. Therefore, to exploit the promising potential of ES cells in regenerative medicine, more should be known about the immunological characteristics of ES cells and their differentiated derivatives.

In principle, allogeneic transplants are rejected due to incompatibility in one or more of the following three classes of transplant antigens: ABO blood group antigens, minor histocompatibility complex (mHC) antigens and major histocompatibility complex (MHC) antigens.

Incompatibility in the ABO blood group antigens expressed by most cell types throughout the body triggers complement activation and leads to hyper-acute graft rejection. Yet, it is not clear whether human ES cells express ABO blood group antigens, but it is reasonable to assume that their differentiated cell products certainly will express these antigens (4). Therefore, ABO incompatibility should be avoided when transplanting ES cell-derived tissues. Indeed, due to the limited variety in ABO blood group antigens this demand can be easily fulfilled.

In contrast, minor histocompatibility complex (mHC) antigens are much more polymorphic alloantigens. Minor histocompatibility antigens are peptides derived from polymorphic proteins, other than MHC molecules that can induce T cell mediated immune responses when they are presented by host antigen presenting cells (APC) to host T cells (39, 41). Undoubtedly, these antigens are also expressed by ES-derived tissues, however, they are less important than ABO and MHC antigens because they induce usually weak and slow rejection responses which can be controlled by mild immunosuppressive drugs (40).

Mismatches in major histocompatibility complex (MHC) antigens have to be considered more seriously, however, because they elicit vigorous immune responses of cytotoxic T cells that lead to acute rejection of the transplant. Due to the high polymorphism of MHC antigens, accurately match donors and recipients is a major limitation in organ transplantation.

5.1. ES cells and their differentiated derivatives fulfil all prerequisites for an efficient recognition by CTL

In previous studies about the immunogenicity of ES cells, murine ES cells were found to be devoid of MHC class I expression at any passage number (77, 78). Human ES cells were found to express low levels of MHC I which rise during differentiation to embryoid bodies *in vitro* or to teratoma cells *in vivo* (24). Human and murine ES cells and their derivatives, except for haematopoietic cells were negative for MHC class II antigens (24) which is not surprising in view of the limited spectrum of cells expressing MHC class II. In early derivatives of human and mouse ES cells, expression of MHC I was shown to increase in response to IFN-γ. This means that ES cells or transplants derived from ES cells might express higher levels of MHC class I after transplantation, because inflammatory cytokines, including IFN-γ, will be presumably secreted at the site of transplantation due to the operation trauma.

This project aimed at revealing the functional sequences of low level expression of MHC class I molecules by ES and EB cells and to enhance our knowledge about the immunological characteristics of ES cells and their derivatives. The wider scope is to avoid rejection of ES cell-derived transplants in regenerative medicine.

To avoid artefacts due to unique properties of a single ES cell line, two independently generated, well characterized murine ES cell lines were used: CGR8 cells (47) and the αPIG cell line transgenic for α-Myosin Heavy Chain - Puromycin-IRIS-GFP (45), which was generated from D3 cells (46). CGR8 and αPIG ES cell lines are derived from the 129P2/Ola and 129S2/SvPas mouse strains, respectively. As differentiated derivatives of ES cells, embryoid bodies (EB) which contain cells of the three germ layers ectoderm, endoderm and mesoderm (14) were generated *in vitro.* Cells were derived form the embryoid bodies on days 2-20 of differentiation and used in the experiments.

To characterize the T cell-mediated adaptive immune response to murine ES cells and their differentiated derivatives in detail, the well established murine Lymphocytic Choriomeningitis (LCM) virus infection was used as an experimental system. This virus was chosen because infection with the LCM virus results in the expression of large quantities of viral antigen in almost any

murine cell type. Moreover, infection of mice with the LCM virus induces a vigorous activation and expansion of virus specific $CD8^+$ T cells. These cells can be easily harvested from spleens and can be used as highly effective CTL in *ex vivo* assays (48, 79, 80). Besides, infections with LCM virus are of clinical relevance. Infection of the human fetuses with LCM virus during the first trimester leads to abortions, while infection at later times during pregnancy is an increasing cause of early neonatal death, hydrocephalus and chorioretinitis in infants (81, 82). Furthermore, fatal transmission of LCM virus by organ transplantation has been recently reported (83).

Initial experiments revealed that both murine ES and EB cells can be productively infected by the LCM virus. These findings extend results of previous studies in which it was shown that human ES cells can be infected by the LCM virus (84). Thus, as a first prerequisite of this study it was proven that ES and EB cells infected with LCM virus produce viral antigen as a model antigen for the endogenous pathway of antigen processing and presentation by MHC class I molecules.

Further prerequisite for the presentation of endogenous antigens is the expression of MHC class I molecules, which was assessed in ES cell lines by three different approaches. First, by RT-PCR it was shown that undifferentiated ES cells as well as EB cells express mRNA coding for the heavy chain of MHC class I molecules. These findings confirm recently reported results for the murine ES cell lines R1, YC5 and D3 and their differentiated derivatives (85). Second, the total amount of cellular MHC class I protein was determined by Western blot analysis. While MHC I protein was not detectable in undifferentiated ES cells by Western blot, EB cells were found to express increasing amounts of MHC class I protein with progressing differentiation states. In undifferentiated ES cells, the lack of detectable MHC I proteins despite the presence of mRNA coding for MHC I heavy chain might be due to either very low level of MHC I protein expression or to immature post-translational modification machinery of MHC I molecules e.g. glycosylation. As it was beyond the scope of this study, this topic was not further addressed.

For the MHC I-restricted recognition of target cells by CTL, only those MHC class I molecules present on the plasma membrane of the target cell are relevant. Therefore, as a third approach, surface expression of MHC class I was

assessed by flow cytometry. Consistent with the previous approach, no expression of MHC class I was detectable on the surface of undifferentiated ES cells. However, from day 2 of differentiation onwards, MHC class I molecules were detectable on the surface of EB cells. In few experiments a peak of MHC I expression was observed on day 4 of differentiation (see Fig. 4C). However, since this observation was not consistently reproducible it was not investigated in more detail.

In addition to MHC I heavy chain, ES and EB cells were found to express high levels of β_2 microglobulin (β_2M) at the mRNA and protein levels. Since β_2M is an essential component of the ternary MHC class I-peptide complex (86), EB cells, at least from day 2 of differentiation onwards, appear to be fully equipped to present antigens in a MHC class I-restricted way.

The proinflammatory cytokine IFN-γ is a potent inducer of MHC class I expression in somatic cells (51, 52). We asked whether IFN-γ also enhances MHC class I expression in ES and EB cells. We have observed that IFN-γ did not induce the expression of MHC class I in undifferentiated ES cells at the mRNA or at the protein level. However, IFN-γ strongly enhanced the expression of MHC class I in EB cells from day 2 of differentiation onwards at both mRNA and protein levels. Thus, up to this point our data fully confirm similar data reported previously for human (24) and murine ES cells (49). However, a low immunogenicity of ES cells or an inability of CTL to recognize ES cells should not be inferred from these findings without formal proof.

After having established that ES and EB cells can be productively infected with the LCM-virus, allowing the accumulation of large amounts of viral endogenous antigens and that MHC I molecules are expressed at least in early differentiated EB cells we asked whether ES and EB cells infected with the LCM virus are lysed by LCM virus-specific CTL. Splenic $CD8^+$ T cells prepared from MHC class I compatible mice on day 8 after infection were used in standard ^{51}Cr-release cytotoxicity assays to assess the susceptibility of ES and EB cells to lysis by virus specific CTL. Remarkably, neither ES cells nor EB cells infected with the LCM virus were lysed to a significant degree by LCM virus-specific $CD8^+$ T cells although these effector cells were shown to be highly cytotoxic against infected control fibroblasts.

Recently, Drukker et al have shown that undifferentiated human ES cells infected with the Influenza virus are not effectively lysed by Influenza virus-specific human CTL generated *in vitro* (50). The authors hypothesised that processing and presentation of viral peptides via MHC class I molecules might be inefficient in ES and EB cells. To date, it is largely unknown whether ES and EB cells possess the intact machinery for the processing and presentation of endogenous antigens which is an absolute prerequisite for the recognition and lysis of target cells by CTL. To bypass possible defects in the antigen processing and presentation pathways in ES and EB cells, these target cells were loaded externally with the LCM virus specific peptide gp_{33-41} at a concentration of 1µM. This peptide resembles an immunodominant epitope of the LCM virus and is restricted by the H-2D^{b} MHC molecules (53). In ^{51}Cr-release assays the susceptibility of ES and EB cells loaded with gp_{33-41} to lysis by LCM virus-specific CTL was assessed. Despite the high cytotoxicity of the LCM virus specific CTL against control targets, neither ES cells nor EB cells were significantly lysed after loading with gp_{33-41}. The concentration of 1 µM gp_{33-41}, used in this assay to load ES and EB cells should provide high levels of peptide/MHC I complexes on target cells far beyond those can be achieved by physiological antigen processing and presentation. The most obvious explanation for the observed lack in lysis of ES and EB cells loaded with viral peptide resides in the fact that expression of MHC I molecules is rather low on ES and EB cells. Since this study has previously shown that IFN-γ enhances the expression of MHC class I at least on EB cells and it has been previously shown that IFN-γ restored a defect in the presentation of viral antigens in cell lines with impaired MHC class I assembly (87), cytotoxicity assays with ES and EB cells after treatment with IFN-γ was performed. However, neither ES nor EB cells loaded with gp_{33-41} were significantly lysed by virus specific CTL, although MHC I expression was strongly enhanced by IFN-γ in EB cells.

Drukker and colleagues have reported that human ES cells loaded with the antigenic peptide of the Influenza virus type A were not lysed by human virus-specific CTL. The authors suggested that lack of killing of ES cells might be due to low expression of MHC I molecules (50). In contrast, even a 50-fold enhanced expression of MHC I after treatment with IFN-γ did not result in significant lysis of ES cells by these CTL. Drukker and colleagues reasoned that

viral infection might be more efficient than external loading of MHC I molecules with viral peptides, because a slight cytolysis was observed for IFN-γ-treated ES cells infected with the influenza virus in comparison to that of ES cells externally loaded with the viral peptide. However, these explanations appear not fully convincing for the following reasons: first, lysis of IFN-γ-pretreated ES cells infected with the influenza virus was only slightly above background level. Second, the high concentration of peptide used by Drukker et al exceeds by 50-fold the generally accepted upper limit of 1 µM, therefore it is presumably at least as efficient as endogenous loading. Third, recognition of antigen presenting ES cells by CTL was not formally proven.

In search for the cause of the lack of lysis by highly effective CTL, recognition of antigen presenting ES and EB cells by antigen specific CTL was assessed. CTL when coincubated with ES and EB cells either infected with LCM virus or loaded with the gp_{33-41} epitope, secrete almost as high levels of IFN-γ as in response to infected control fibroblasts. This suggests that ES cells as well as EB cells are readily recognized by LCM virus-specific CTL. Antigen specificity and MHC I restriction of this recognition were proven by using non infected target cells and virus specific CTL derived from the allogeneic BALB/c mice, respectively.

At first glance, recognition of ES cells by antigen specific CTL might be surprising due to the low level of MHC I expression on ES and EB cells. However, it is well documented in the literature that T cells can recognize as few as 10 specific MHC/peptide complexes on a target cell (29, 88, 89).

It has to be emphasized that secretion of high amounts of IFN-γ by LCM virus specific CTL in response to ES and EB cells infected with the LCM virus suggests that ES and EB cells are fully competent to process and present viral antigens via the endogenous pathway. These findings are supported by a study showing that undifferentiated murine ES cells and their early derivatives express mRNA coding for critical components of the antigen processing and presentation machinery including: β_2M, the peptide transporter proteins TAP1 and TAP2 and components of the proteasome LMP2 and LMP7 (85). Taken together, although levels of MHC class I expression on the surface of ES cells are below the detection limit by Western blot analysis and flow cytometry, these cells are effectively recognized by virus specific $CD8^+$ CTL in an antigen specific

and MHC class I restricted fashion. Nevertheless, despite effective recognition of antigen presenting ES and EB cells by CTL, no significant lysis of these target cells was observed.

It is known that CTL have different stimulatory thresholds for the secretion of cytokines and the degranulation of cytotoxic granules (62). Therefore it had to be addressed, whether ES and EB cells can trigger the exocytosis of cytotoxic granules by CTL. Upon target cell recognition, CTL rapidly polarize their lytic granules towards the site of contact with the target cells, i.e. the immunological synapse (90). These lytic granules, containing the cytotoxic effector molecules, fuse with the CTL plasma membrane and release their contents towards the target cell (91). This study revealed that LCM virus-specific CTL in response to contact with ES and EB cells loaded with gp_{33-41} peptide polarize their cytotoxic granules within just 10 minutes towards the immunological synapse. Furthermore, it was shown that CTL fuse their cytotoxic granules with their plasma membrane in response to contact with antigen presenting ES cells as judged by the exposure of the lysosomal marker CD107 on their plasma membrane. Since CD107 localizes in CTL to the inner leaflet of the membrane of cytotoxic granules and is exposed on the outer leaflet of plasma membrane of the CTL only after fusion of the granule membrane with the plasma membrane at the immunologic synapse, exposure of CD107 is accepted as a measure for degranulation of CTL (63, 92). These findings revealed that LCM virus specific CTL expose CD107 on the plasma membrane in response to antigen presenting ES cells with comparable kinetics and densities as in response to control fibroblasts. Hence, polarization of cytotoxic granules and exposure of CD107 strongly suggest that the recognition of antigen presenting ES cells by CTL is so effective that cytotoxic functions of the CTL are fully triggered, including exocytosis of the cytotoxic granules. In the view of this full blown CTL response to infected ES cells, it remains enigmatic, why infected ES cells are not lysed as effectively as infected control target cells.

One explanation for the resistance of ES and EB cells to lysis by CTL might be due to a generally reduced susceptibility of ES cells to pro-apoptotic stimuli. However, in agreement with previous studies (54-60, 93) these data demonstrated here that ES cells readily undergo apoptosis in response to several exogenous apoptotic stimuli, e.g. UV irradiation or treatment with

staurosporine. Thus, ES and EB cells are not immortal due to general defects in the proapoptotic signalling pathways. Taken together, the above discussed characteristics of the interaction between CTL and ES cells suggest that ES cells are specifically resistant to the cytotoxic effector mechanisms of CTL.

5.2. Resistance of ES and EB cells against cytolysis by $CD8^+$ CTL is due to the expression of cytoprotective molecules

Cytotoxic T cells lyse virus infected cells via exocytosis of cytotoxic granules, which contain the pore-forming molecule perforin and an array of cytotoxic proteases known as granzymes (28, 30). Perforin monomers insert into the membrane of the target cell and polymerize to form pores. These pores are essential for the delivery of the granzymes into the cytoplasm of the target cells where they trigger the apoptotic signalling cascade (31).

More recently, the longstanding conundrum of how CTL are protected against their own cytotoxic effector molecules has been solved. Henkart and colleagues reported that CTL protect themselves against the toxic effect of perforin by the expression of Cathepsin B (64). Cathepsin B, a lysosomal cysteine protease, was reported to cleave perforin before its assembly within the cell membrane thereby impairing its pore forming activity (64). This study reveals that both ES and EB cells express levels of Cathepsin B protein even higher than those observed in $CD8^+$ CTL. To characterize the functional relevance of Cathepsin B in the resistance of ES and EB cell against lysis by CTL, we specifically inhibited Cathepsin B by using the compound CA074.

The use of the specific Cathepsin B inhibitor CA074 results in significantly enhanced suicide/fratricide among $CD8^+$ CTL, however it did not enhance the susceptibility of ES and EB cells to lysis by LCM virus specific $CD8^+$ CTL. This observation might be due to inherent problems of pharmacologic inhibitors, e.g. incomplete inhibition of Cathepsin B by CA074. Alternatively, Cathepsin B expressed by ES and EB cells might not be involved in protection of ES cells against cytolysis but rather take part in tissue degradation during invasion of the uterine stroma by the embryonic trophoblast as suggested by Afonso and

colleagues (94, 95). Hence, the biological function of Cathepsin B expression in ES and EB cells remains to be elucidated.

Besides, Cathepsin B, other cytoprotective molecules have been identified in CTL during the recent years. Among these are the serine protease inhibitor (serpin) SPI-6 and its human homologue PI-9 which inactivate granzyme B irreversibly *in vitro* and *in vivo* (96). Several studies demonstrate that SPI-6 is expressed by dendritic cells, in which it prevents the destruction of these professional antigen-presenting cells (APC) during interaction with CTL and at immune-privileged sites, where degranulation of CTL is potentially deleterious (71, 96). More recently, murine SPI-6 was reported to play a crucial role in the protection of CTL themselves against the suicidal effects of their own granzyme B (76). Moreover, an aberrant expression of the human serpin PI-9 and its murine homologue SPI-6 has been detected in various types of melanoma, carcinoma and lymphoma and that is linked with the escape of tumor cells from immunosurveillance (71, 97, 98).

In this study, ES and EB cells were found to express SPI-6 on both the mRNA and protein levels. In fact, expression of SPI-6 in ES and EB cells was almost as high as in $CD8^+$ CTL. The biological relevance of SPI-6 expression in ES cells for their resistance against lysis by CTL was determined by down-regulating SPI-6 expression by specific RNA interference. Remarkably, by down-regulation of SPI-6 expression, ES cells become as susceptible to lysis by CTL as fibroblasts. Thus, murine ES and EB cells establish an immune privileged state by expression of SPI-6 that prevents destruction of these cells by highly cytotoxic CTL.

Thus far, the resistance of ES cells to lysis by CTL was assumed by several groups (8, 24, 49, 50) to be due to the lack of target cell recognition as a consequence of low MHC I expression in ES cells without formal proof of this hypothesis. By demonstrating that ES and EB cells express high levels of Cathepsin B and SPI-6, this study significantly extends our knowledge about the immunological properties of ES cells and their differentiated derivative.

Obviously, protection of ES cells, which are early embryonic cells, against lysis by CTL is of great biological importance, because from an immunologic point of view the embryo is a true intrauterine semi-allogeneic transplant. This means that during the implantation of the early embryo into the decidua, it might

be a target for maternal allospecific CTL. To date, mechanisms by which the embryo might be protected against rejection by the maternal immune system were explained on the one hand by low expression of MHC molecules resulting in low immunogenicity and on the other hand by active immunosuppression through high expression of HLA-G impairing lysis by CTL and natural killer (NK) cells (99, 100). However, in view of the high sensitivity of T cells for very low levels of MHC I molecules on target cells, the low MHC I expression of ES and EB cells does not warrant the acceptance of embryonic cells. Apparently, instead of trusting to be ignored by T cells, ES and EB cells rather have evolved mechanisms to actively counteract cytotoxic effector molecules of CTL. However, the functional *in vivo* relevance of the cytoprotective molecules SPI-6 and Cathepsin B for preventing the rejection of the embryo remains to be investigated.

Beyond their physiological significance during pregnancy it remains to be investigated what consequences the expression of the cytoprotective molecules in ES cells have for regenerative medicine that relies on ES cell-derived tissues. An obvious advantage of the endogenous cytoprotective molecules in ES cells and their derivatives is that they might prevent rejection of ES cell-derived grafts after therapeutic implantation. The flipside of this coin, however, could be that implanted ES cells not differentiating into the desired cell types rather than develop into teratoma or teratocarcinoma and escape from immune-surveillance. This danger would apply if differentiated derivatives of ES cells were transplanted, because just a single remaining undifferentiated ES cell or a differentiated cells reverting to a less differentiated state is sufficient to induce teratoma or teratocarcinoma formation.

Even if ES cells do not give rise to tumors, immune-privileged cells within ES cell-derived transplants pose another risk by providing a niche for intracellular pathogens. Within such a niche beyond the immune surveillance pathogens might be able to establish persistence.

This study of stem cell immunobiology reveals molecular mechanisms which contribute to the unique immunological properties of embryonic stem cells and which would have an impact on our basic understanding of CTL-mediated immune response towards ES cells, the immunologically privileged state of the embryo and on future approaches of ES cell-based cell replacement therapy.

6. References

1. 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.

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

3. Smith, A. G. 2001. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* 17:435-462.

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

5. Toyooka, Y., N. Tsunekawa, R. Akasu, and T. Noce. 2003. Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci U S A* 100:11457-11462.

6. Koestenbauer, S., N. H. Zech, H. Juch, P. Vanderzwalmen, L. Schoonjans, and G. Dohr. 2006. Embryonic stem cells: similarities and differences between human and murine embryonic stem cells. *Am J Reprod Immunol* 55:169-180.

7. Itskovitz-Eldor, J., M. Schuldiner, D. Karsenti, A. Eden, O. Yanuka, M. Amit, H. Soreq, and N. Benvenisty. 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6:88-95.

8. Fändrich, F., B. Dresske, M. Bader, and M. Schulze. 2002. Embryonic stem cells share immune-privileged features relevant for tolerance induction. *J Mol Med* 80:343-350.

9. Stevens, L. C. 1970. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev Biol* 21:364-382.

10. 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.

11. 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.

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

13. 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.

14. 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.

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

16. Draper, J. S., and P. W. Andrews. 2002. Embryonic stem cells: advances toward potential therapeutic use. *Curr Opin Obstet Gynecol* 14:309-315.

17. Kim, J. H., J. M. Auerbach, J. A. Rodriguez-Gomez, I. Velasco, D. Gavin, N. Lumelsky, S. H. Lee, J. Nguyen, R. Sanchez-Pernaute, K. Bankiewicz, and R. McKay. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50-56.

18. 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.

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

20. Soria, B., E. Roche, G. Berna, T. Leon-Quinto, J. A. Reig, and F. Martin. 2000. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 49:157-162.

21. Lafferty, K. J., S. J. Prowse, S. Babcock, and R. Gill. 1986. The allograft response. *The Surgical clinics of North America* 66:1231-1253.

22. Swijnenburg, R. J., M. Tanaka, H. Vogel, J. Baker, T. Kofidis, F. Gunawan, D. R. Lebl, A. D. Caffarelli, J. L. de Bruin, E. V. Fedoseyeva, and R. C. Robbins. 2005. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. *Circulation* 112:I166-172.

23. Kofidis, T., D. R. Lebl, R. J. Swijnenburg, J. M. Greeve, U. Klima, and R. C. Robbins. 2006. Allopurinol/uricase and ibuprofen enhance engraftment of cardiomyocyte-enriched human embryonic stem cells and improve cardiac function following myocardial injury. *Eur J Cardiothorac Surg* 29:50-55.

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

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

26. Fandrich, F., X. Lin, G. X. Chai, M. Schulze, D. Ganten, M. Bader, J. Holle, D. S. Huang, R. Parwaresch, N. Zavazava, and B. Binas. 2002. Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning. *Nat Med* 8:171-178.

27. Li, L., M. L. Baroja, A. Majumdar, K. Chadwick, A. Rouleau, L. Gallacher, I. Ferber, J. Lebkowski, T. Martin, J. Madrenas, and M. Bhatia. 2004. Human

embryonic stem cells possess immune-privileged properties. *Stem Cells* 22:448-456.

28. Jenne, D. E., and J. Tschopp. 1988. Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. *Immunol Rev* 103:53-71.

29. George, A. J., J. Stark, and C. Chan. 2005. Understanding specificity and sensitivity of T-cell recognition. *Trends Immunol* 26:653-659.

30. Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell, and T. J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* 76:977-987.

31. Trapani, J. A. 1998. Dual mechanisms of apoptosis induction by cytotoxic lymphocytes. *Int Rev Cytol* 182:111-192.

32. Nagata, S., and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol Today* 16:39-43.

33. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 369:31-37.

34. Smyth, M. J., K. Y. Thia, S. E. Street, D. MacGregor, D. I. Godfrey, and J. A. Trapani. 2000. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med* 192:755-760.

35. Mullbacher, A., P. Waring, R. Tha Hla, T. Tran, S. Chin, T. Stehle, C. Museteanu, and M. M. Simon. 1999. Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. *Proc Natl Acad Sci U S A* 96:13950-13955.

36. Riera, L., M. Gariglio, M. Pagano, O. Gaiola, M. M. Simon, and S. Landolfo. 2001. Control of murine cytomegalovirus replication in salivary

glands during acute infection is independent of the Fas ligand/Fas system. *New Microbiol* 24:231-238.

37. Jiang, S. P., and M. S. Vacchio. 1998. Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft". *J Immunol* 160:3086-3090.

38. Mellor, A. L., and D. H. Munn. 2000. Immunology at the maternal-fetal interface: lessons for T cell tolerance and suppression. *Annu Rev Immunol* 18:367-391.

39. Goulmy, E., R. Schipper, J. Pool, E. Blokland, J. H. Falkenburg, J. Vossen, A. Gratwohl, G. B. Vogelsang, H. C. van Houwelingen, and J. J. van Rood. 1996. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* 334:281-285.

40. Warrens, A. N., G. Lombardi, and R. I. Lechler. 1994. Presentation and recognition of major and minor histocompatibility antigens. *Transpl Immunol* 2:103-107.

41. Goulmy, E., J. Pool, E. Van Lochem, and H. Volker-Dieben. 1995. The role of human minor histocompatibility antigens in graft failure: a mini-review. *Eye* 9 (Pt 2):180-184.

42. Weidt, G., O. Utermohlen, J. Zerrahn, J. Reimann, W. Deppert, and F. Lehmann-Grube. 1994. CD8+ T lymphocyte-mediated antiviral immunity in mice as a result of injection of recombinant viral proteins. *J Immunol* 153:2554-2561.

43. Lehmann-Grube, F., and J. Ambrassat. 1977. A new method to detect lymphocytic choriomeningitis virus-specific antibody in human sera. *J Gen Virol* 37:85-92.

44. Kashkar, H., C. Haefs, H. Shin, S. J. Hamilton-Dutoit, G. S. Salvesen, M. Krönke, and J. M. Jurgensmeier. 2003. XIAP-mediated caspase inhibition in Hodgkin's lymphoma-derived B cells. *J Exp Med* 198:341-347.

45. Kolossov, E., T. Bostani, W. Roell, M. Breitbach, F. Pillekamp, J. M. Nygren, P. Sasse, O. Rubenchik, J. W. Fries, D. Wenzel, C. Geisen, Y. Xia, Z. Lu, Y. Duan, R. Kettenhofen, S. Jovinge, W. Bloch, H. Bohlen, A. Welz, J. Hescheler, S. E. Jacobsen, and B. K. Fleischmann. 2006. Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. *J Exp Med* 203:2315-2327.

46. Doetschman, T. C., H. Eistetter, M. Katz, W. Schmidt, and R. Kemler. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* 87:27-45.

47. Nichols, J., E. P. Evans, and A. G. Smith. 1990. Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. *Development* 110:1341-1348.

48. Oldstone, M. B. 2002. Biology and pathogenesis of lymphocytic choriomeningitis virus infection. *Curr Top Microbiol Immunol* 263:83-117.

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

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

51. 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.

52. 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.

53. Klavinskis, L. S., J. L. Whitton, E. Joly, and M. B. Oldstone. 1990. Vaccination and protection from a lethal viral infection: identification, incorporation, and use of a cytotoxic T lymphocyte glycoprotein epitope. *Virology* 178:393-400.

54. Zou, G. M., M. F. Reznikoff-Etievant, A. Leon, V. Verge, F. Hirsch, and J. Milliez. 2000. Fas-mediated apoptosis of mouse embryo stem cells: its role during embryonic development. *Am J Reprod Immunol* 43:240-248.

55. Zou, G. M., M. F. Reznikoff-Etievant, F. Hirsch, and J. Milliez. 2000. IFN-gamma induces apoptosis in mouse embryonic stem cells, a putative mechanism of its embryotoxicity. *Dev Growth Differ* 42:257-264.

56. Nishitai, G., N. Shimizu, T. Negishi, H. Kishimoto, K. Nakagawa, D. Kitagawa, T. Watanabe, H. Momose, S. Ohata, S. Tanemura, S. Asaka, J. Kubota, R. Saito, H. Yoshida, T. W. Mak, T. Wada, J. M. Penninger, N. Azuma, H. Nishina, and T. Katada. 2004. Stress induces mitochondria-mediated apoptosis independent of SAPK/JNK activation in embryonic stem cells. *J Biol Chem* 279:1621-1626.

57. Koyanagi-Katsuta, R., N. Akimitsu, N. Arimitsu, T. Hatano, and K. Sekimizu. 2000. Apoptosis of mouse embryonic stem cells induced by single cell suspension. *Tissue Cell* 32:66-70.

58. Duval, D., M. Trouillas, C. Thibault, D. Dembele, F. Diemunsch, B. Reinhardt, A. L. Mertz, A. Dierich, and H. Boeuf. 2006. Apoptosis and differentiation commitment: novel insights revealed by gene profiling studies in mouse embryonic stem cells. *Cell Death Differ* 13:564-575.

59. Chan, W. H. 2006. Ginkgolide B induces apoptosis and developmental injury in mouse embryonic stem cells and blastocysts. *Hum Reprod* 21:2985-2995.

60. Bieberich, E., J. Silva, G. Wang, K. Krishnamurthy, and B. G. Condie. 2004. Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for

neural precursors in ES cell-derived neural transplants. *J Cell Biol* 167:723-734.

61. Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. *Cell* 76:241-251.

62. Faroudi, M., C. Utzny, M. Salio, V. Cerundolo, M. Guiraud, S. Muller, and S. Valitutti. 2003. Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. *Proc Natl Acad Sci U S A* 100:14145-14150.

63. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 281:65-78.

64. Balaji, K. N., N. Schaschke, W. Machleidt, M. Catalfamo, and P. A. Henkart. 2002. Surface Cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *J Exp Med* 196:493-503.

65. Baran, K., A. Ciccone, C. Peters, H. Yagita, P. I. Bird, J. A. Villadangos, and J. A. Trapani. 2006. Cytotoxic T lymphocytes from Cathepsin B-deficient mice survive normally in vitro and in vivo after encountering and killing target cells. *J Biol Chem* 281:30485-30491.

66. Catalfamo, M., and P. A. Henkart. 2003. Perforin and the granule exocytosis cytotoxicity pathway. *Curr Opin Immunol* 15:522-527.

67. Sun, J., C. H. Bird, V. Sutton, L. McDonald, P. B. Coughlin, T. A. De Jong, J. A. Trapani, and P. I. Bird. 1996. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. *J Biol Chem* 271:27802-27809.

68. Bird, C. H., V. R. Sutton, J. Sun, C. E. Hirst, A. Novak, S. Kumar, J. A. Trapani, and P. I. Bird. 1998. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol Cell Biol* 18:6387-6398.

69. Barrie, M. B., H. W. Stout, M. S. Abougergi, B. C. Miller, and D. L. Thiele. 2004. Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6. *J Immunol* 172:6453-6459.

70. Edwards, K. M., J. E. Davis, K. A. Browne, V. R. Sutton, and J. A. Trapani. 1999. Anti-viral strategies of cytotoxic T lymphocytes are manifested through a variety of granule-bound pathways of apoptosis induction. *Immunol Cell Biol* 77:76-89.

71. 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.

72. Bladergroen, B. A., C. J. Meijer, R. L. ten Berge, C. E. Hack, J. J. Muris, D. F. Dukers, A. Chott, Y. Kazama, J. J. Oudejans, O. van Berkum, and J. A. Kummer. 2002. Expression of the granzyme B inhibitor, protease inhibitor 9, by tumor cells in patients with non-Hodgkin and Hodgkin lymphoma: a novel protective mechanism for tumor cells to circumvent the immune system? *Blood* 99:232-237.

73. Rowshani, A. T., S. Florquin, F. Bemelman, J. A. Kummer, C. E. Hack, and I. J. Ten Berge. 2004. Hyperexpression of the granzyme B inhibitor PI-9 in human renal allografts: a potential mechanism for stable renal function in patients with subclinical rejection. *Kidney Int* 66:1417-1422.

74. Bots, M., V. A. N. B. L, M. T. Rademaker, R. Offringa, and J. P. Medema. 2006. Serpins prevent granzyme-induced death in a species-specific manner. *Immunol Cell Biol* 84:79-86.

75. Bots, M., R. Offringa, and J. P. Medema. 2006. Does the serpin PI-9 protect tumor cells? *Blood* 107:4974-4975; author reply 4975.

76. 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.

77. 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.

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

79. Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. Oldstone. 1980. The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Adv Immunol* 30:275-331.

80. Zinkernagel, R. M. 2002. Lymphocytic choriomeningitis virus and immunology. *Curr Top Microbiol Immunol* 263:1-5.

81. Deibel, R., J. P. Woodall, W. J. Decher, and G. D. Schryver. 1975. Lymphocytic choriomeningitis virus in man. Serologic evidence of association with pet hamsters. *Jama* 232:501-504.

82. Enders, G., M. Varho-Gobel, J. Lohler, E. Terletskaia-Ladwig, and M. Eggers. 1999. Congenital lymphocytic choriomeningitis virus infection: an underdiagnosed disease. *Pediatr Infect Dis J* 18:652-655.

83. Fischer, S. A., M. B. Graham, M. J. Kuehnert, C. N. Kotton, A. Srinivasan, F. M. Marty, J. A. Comer, J. Guarner, C. D. Paddock, D. L. DeMeo, W. J. Shieh, B. R. Erickson, U. Bandy, A. DeMaria, Jr., J. P. Davis, F. L. Delmonico, B. Pavlin, A. Likos, M. J. Vincent, T. K. Sealy, C. S. Goldsmith, D. B. Jernigan, P. E. Rollin, M. M. Packard, M. Patel, C. Rowland, R. F. Helfand, S. T. Nichol, J. A. Fishman, T. Ksiazek, and S. R. Zaki. 2006. Transmission of lymphocytic choriomeningitis virus by organ transplantation. *N Engl J Med* 354:2235-2249.

84. Smelt, S. C., P. Borrow, S. Kunz, W. Cao, A. Tishon, H. Lewicki, K. P. Campbell, and M. B. Oldstone. 2001. Differences in affinity of binding of lymphocytic choriomeningitis virus strains to the cellular receptor alpha-

dystroglycan correlate with viral tropism and disease kinetics. *J Virol* 75:448-457.

85. Magliocca, J. F., I. K. Held, and J. S. Odorico. 2006. Undifferentiated Murine Embryonic Stem Cells Cannot Induce Portal Tolerance but May Possess Immune Privilege Secondary to Reduced Major Histocompatibility Complex Antigen Expression. *Stem Cells Dev* 15:707-717.

86. Sege, K., L. Rask, and P. A. Peterson. 1981. Role of beta2-microglobulin in the intracellular processing of HLA antigens. *Biochemistry* 20:4523-4530.

87. Sibille, C., K. Gould, G. Hammerling, and A. Townsend. 1992. A defect in the presentation of intracellular viral antigens is restored by interferon-gamma in cell lines with impaired major histocompatibility complex class I assembly. *Eur J Immunol* 22:433-440.

88. Harding, C. V., and E. R. Unanue. 1990. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* 346:574-576.

89. Demotz, S., H. M. Grey, and A. Sette. 1990. The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* 249:1028-1030.

90. Yannelli, J. R., J. A. Sullivan, G. L. Mandell, and V. H. Engelhard. 1986. Reorientation and fusion of cytotoxic T lymphocyte granules after interaction with target cells as determined by high resolution cinemicrography. *J Immunol* 136:377-382.

91. Peters, P. J., H. J. Geuze, H. A. Van der Donk, J. W. Slot, J. M. Griffith, N. J. Stam, H. C. Clevers, and J. Borst. 1989. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *Eur J Immunol* 19:1469-1475.

92. Betts, M. R., and R. A. Koup. 2004. Detection of T-cell degranulation: CD107a and b. *Methods Cell Biol* 75:497-512.

93. Kircheis, R., L. Kircheis, H. Oshima, C. Kohchi, G. Soma, and D. Mizuno. 1996. Selective lysis of early embryonic cells by the alternative pathway of complement--a possible mechanism for programmed cell death in embryogenesis. *In Vivo* 10:389-403.

94. Afonso, S., L. Romagnano, and B. Babiarz. 1997. The expression and function of cystatin C and Cathepsin B and cathepsin L during mouse embryo implantation and placentation. *Development* 124:3415-3425.

95. Afonso, S., L. Romagnano, and B. Babiarz. 1999. Expression of cathepsin proteinases by mouse trophoblast in vivo and in vitro. *Dev Dyn* 216:374-384.

96. Medema, J. P., J. de Jong, L. T. Peltenburg, E. M. Verdegaal, A. Gorter, S. A. Bres, K. L. Franken, M. Hahne, J. P. Albar, 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.

97. 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.

98. Bots, M., I. G. Kolfschoten, S. A. Bres, M. T. Rademaker, G. M. de Roo, M. Kruse, K. L. Franken, M. Hahne, C. J. Froelich, C. J. Melief, R. Offringa, and J. P. Medema. 2005. SPI-CI and SPI-6 cooperate in the protection from effector cell-mediated cytotoxicity. *Blood* 105:1153-1161.

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

100. Ponte, M., C. Cantoni, R. Biassoni, A. Tradori-Cappai, G. Bentivoglio, C. Vitale, S. Bertone, A. Moretta, L. Moretta, and M. C. Mingari. 1999. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc Natl Acad Sci U S A* 96:5674-5679.

Declaration

I hereby declare that the work in this thesis is original and has been carried out by me (with the exception of the parts mentioned below) at the Institute for Medical Microbiology, Immunology and Hygiene, Medical Centre, University of Cologne, under the supervision of Prof. Dr. M. Krönke and in partial fulfilment of the requirements of the Doctor of Philosophy degree of the Bonn University. I further declare that this work has not been the basis for the awarding of any degree of any university or institute.

Cultivation of the ES cell line αPIG, *In vitro* differentiation of ES cells into embroyid bodies and Immunocytochemical analysis of ES and EB cells after infection with the LCM virus were performed by Dr. Tomo Saric´ in the Institute of Neurophysiology, University of Cologne

Zeinab Abdullah

September 2007

Publications

Abdullah Z*, Saric T*, Kashkar H, Baschuk N, Yazdanpanah B, Fleischmann BK, Hescheler J, Krönke M, Utermöhlen O. *Serpin-6 expression protects embryonic stem cells from lysis by antigen-specific CTL*. J Immunol. 2007 Mar 15;**178**(6):3390-9.

Popov A*, **Abdullah Z***, Wickenhauser C*, Saric T, Driesen J, Hanisch FG, Domann E, Raven EL, Dehus O, Hermann C, Eggle D, Debey S, Chakraborty T, Krönke M, Utermöhlen O, Schultze JL. *Indoleamine 2,3-dioxygenase-expressing dendritic cells form suppurative granulomas following Listeria monocytogenes infection*. J Clin Invest. 2006 Dec;**116**(12):3160-70. Epub 2006 Nov 16.