Mechanisms involved in regulation of MHC class I molecules in murine embryonic stem cells

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Abbreviations

°C	degree Celsius
α	alpha
β	beta
γ	gamma
μL	microliter
μg	microgram
bp	base pair
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
cpm	counts per minute
cDNA	complimentary deoxyribonucleic acid
CTLs	cytotoxic T lymphocytes
CTSB	cathepesin B
CMs	cardiomyocytes
D	day
DNA	deoxyribonucleic acid
DMSO	dimethyl sulpfoxide
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
DPBS	Dulbecco's phosphate buffered saline
Dnmt	DNA methyl transferase
ESCs	embryonic stem cells
ESCMs	ES cell derived cardiomyocytes
EBs	embryoid bodies
FBS	fetal bovine serum
GFP	green fluorescent protein
GMEM	Glasgow minimal essential medium
h	hour
IMDM	Iscove's modified Dulbecco's medium
INDO	Indoleamine 2,3-deoxygenase
IRES	Internal ribosomal entry site
IRF	interferon regulatory factor
IFNγ	interferon gamma
iPSCs	induced pluripotent stem cells
JAK	Janus activating kinase
kb	kilobase
L	liter
LIF	leukemia inhibitory factor
mg	miligram
min	minute
ml	mililiter
mM	milimolar
MACS	magnetically assisted cell sorting
mRNA	messenger ribonucleic acid

MEFs	murine embryonic fibroblasts
MHC	major histocompatibility complex
NEAA	non-essential amino acids
NK cells	natural killer cells
OD	optical density
Ova	ovalbumin
PCR	polymerase chain reaction
PBS	phosphate buffer saline
PE	phycoerythrin
PTP1B	protein-tyrosine phosphatase 1B
αPIG	alpha-myosin heavy chain-puromycin-IRES-GFP
RT	room temperature
RNA	ribonucleic acid
rpm	revolution per minute
rcf	relative centrifugal force
RPMI	Roswell Park memorial Institute
RT-PCR	reverse transcription-PCR
SOCS	suppressor of cytokine signaling
STAT	signal transducers and activators of transcription
S8L	SIINFEKL
SIINFEKL	Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu
sec	second
TCR	T cell receptor
TSA	trichostatin A
2,5-aza	2,5-azacytidine
KD	knock down
KO	knock out
PI(3)K	phosphatidylinositol 3-kinase
Con-A	Concanavalin- A
NKT	Natural killer T cells

Abstract

Major histocompatibility complex (MHC) is at the center of immune responses that support survival, fitness and adaptation of mammalian species to the environment. These molecules are not only crucial for adaptive and innate immune responses against microorganisms and cancer cells but also play an important role in reproduction process and development of embryo during the preimplantation period. In the present study, we use murine embryonic stem cells (ESCs) as model to dissect the molecular mechanism involved in the regulation of MHC class I molecules during differentiation *in vitro*. MHC class I molecules are expressed at very low levels on murine ESCs and they are not induced by the immunomodulatory cytokine interferon gamma (IFNγ) despite the presence of IFNγ receptors on their cell surface. First, we showed that removal of leukemia inhibitory factor (LIF), a standard component of murine ES cell culture media required for the maintenance of a pluripotent state, did not result in up regulation of MHC class I expression in murine ESCs, presumably due to incomplete inactivation under these experimental conditions of STAT3 signaling pathway, which is used by LIF. However, the addition of LIF to differentiated cells in embryoid bodies strongly suppressed the expression of these molecules. Down regulation of STAT3 in undifferentiated ESCs cultured in the presence of LIF significantly increased the expression of MHC class I molecules and this was further enhanced by IFNγ treatment. Flow cytometric analysis revealed that STAT1 is phosphorylated by IFNγ in STAT3 knockdown (KD) ESCs, whereas there was only weak or no phophorylation detected in mock siRNA 647-treated ESCs exposed to IFNγ. Luciferase reporter assay also indicated that GAS promoter responded to IFNγ much strongly in STAT3-depleted ESCs than in intact cells, suggesting that ESCs do not respond to IFNγ at least partially due to inhibitory effects of STAT3-signaling components on STAT1-phosphorylation. Moreover, the down regulation of suppressor of cytokine signaling 3 (SOCS3) in STAT3 KD ESCs increases the possible regulation of STAT1 phosphorylation by SOCS3. No effect in

MHC class I molecules induction was observed in STAT3 KD ES-derived cardiomyocytes (ESCMs) indicating the differential regulation of these molecules during the course of differentiation from undifferentiated stage (ESCs) to differentiated stage (ESCMs).

An additional mechanism by which STAT3 regulates MHC class I expression may involve epigenetic modification of MHC class I gene expression since Dnmt knockout (KO) murine ESCs showed upregulation of MHC class I molecules. Chromatin modifying gene Eed1 but not Dnmt1 and Jmjd1 was significantly downregulated in STAT3 KD murine ESCs. Additionally, murine ESCs showed increased MHC class I expression and enhanced response to IFNγ after treatment with the DNA-methyltransferase inhibitor 2,5-azacytidine and histone deacetylase inhibitor Trichostatin A.

Modulation of MHC class I expression by STAT3 KD in ESCs reduced their lysis by activated syngeneic NK cells and increased their lysis by cytotoxic T cells compared to mock siRNA 647-treated ESCs. These data indicate that STAT3 pathway plays a dual role in modulating the MHC class I expression in ESCs. Interfering with the inhibitory pathways that suppress MHC class I expression in pluripotent ESCs may help to control teratoma formation from contaminating ESCs in therapeutic cell transplants and may also help to eradicate cancer cells and virus-infected cells that are known to frequently evade immune recognition by down regulating the MHC class I expression.

Zusammenfassung

Der Haupthistokompatibilitätskomplex (MHC) ist der Mittelpunkt der Immunantwort, welche Überleben, Fitness und Anpassung der Säugetiere an die Umgebung gewährleistet. Die MHC Moleküle sind nicht nur für die adaptive und die angeborene Immunantwort verantwortlich, sondern spielen außerdem eine wichtige Rolle im Reproduktionsprozess und der Entwicklung des Embryos während der Präimplantationsphase. In der vorliegenden Arbeit nutzen wir murinen embryonale Stammzellen (mESCs), um die molekularen Mechanismen, die an der Regulation der MHC Klasse I Moleküle während der *in vitro* Differenzierung der Stammzellen beteiligt sind, zu untersuchen. MHC Klasse I Moleküle werden in murinen ESCs nur schwach exprimiert und werden trotz entsprechender Rezeptoren auf ihrer Zelloberfläche nicht vom immunmodulatorischen Zytokin Interferon Gamma (IFNγ) induziert. Zunächst zeigten wir, das in Abwesenheit vom Leukemia inhibierenden Faktor (LIF), welcher als Standardmediumkomponente muriner ESC Kultur zur Erhaltung der Pluripotenz eingesetzt wird, nicht zur Hochregulierung der MHC Klasse I Molekülexpression führte. Dieser Umstand könnte auf eine unvollständige Inaktivierung des STAT3 Signalweges, über welchen LIF reguliert, unter den verwendeten experimentellen Konditionen zu erklären sein. Allerdings führte die Zugabe von LIF zu differenzierenden embryonalen Körpern (EB) zu einer starken Unterdrückung der MHC-Moleküle. Abregulierung von STAT3 in undifferenzierten ESCs, welche in Anwesenheit von LIF kultiviert wurden, erhöhten ihre MHC Klasse I Molekülexpression signifikant. IFNγ-Behandlung verstärkte diese Expression. Mittels Durchflusszytometrie konnte gezeigt werden, dass STAT1 in ESCs von IFNγ phosphoryliert wird, wenn STAT3 in der Zelle ausgeschaltet wird. Im Gegensatz dazu war nur eine schwache bis keine Phosphorylierung in ESCs unter IFNγ-Einfluss detektierbar, welche mit Kontroll-siRNA-647 behandelt wurden. Der Luziferasetest ergab ebenfalls, dass der GAS promotor in Zellen ohne STAT3-Expression weitaus stärker auf IFNγ reagierte, als in Zellen mit STAT3-Expression. Diese Daten

lassen darauf schließen, dass ein Grund, warum ESCs nicht auf IFNγ reagieren, auf inhibierende Effekte von Komponenten aus dem STAT3-Signalweg auf die STAT1-Phosphorylierung zurückzuführen sind. Darüberhinaus führte die Abregulierung des Unterdrückerzytokinsignal 3 (SOC3) in STAT3-defizienten ESCs zu einem Anstieg der möglichen Regulation der STAT1-Phosphorylierung durch SOCS3. In STAT3-defizienten Kardiomyozyten, welche aus ESCs generiert wurden (ESCMs), war keine Induktion der MHC Klasse I Moleküle zu beobachten. Dies weist auf eine verschiedene Regulierung dieser Moleküle vom undifferenzierten zum differenzierten Zustand hin. Der Mechanismus durch den STAT3 direkt auf die MHC Klasse I Expression wirkt mag auch epigenetische Modifikationen mit einschließen, da murine DNMT Knockout (KO) ESCs ebenfalls eine Hochregulierte MHC Klasse I Expression zeigten. In STAT3 KD murinen ESCs wurde das Gen Eed1 signifikant herabreguliert, während die Expreession von Dnmt1 und Jmjd1 nicht signifikant verändert wurde. Außerdem zeigten murine ESCs erhöhte MHC Klasse I Expression und eine verstärkte Antwort auf IFNγ nach Behandlung mit dem DNA-methyltransferase Inhibitor 2,5-azacytidine und dem Histondeacetylase Inhibitor Trichostatin A. Im Vergleich zu mock siRNA 647-treated ESCs wurden STAT3 KD ESCs auch weniger effizient von aktivierten sygenen NK Zellen und effizienter von zytotoxischen T-Zellen lysiert. Diese Daten weisen darauf hin, dass STAT3 eine duale Rolle bei der Modulation der MHC Klasse I Expression in ESCs spielt.

Die Auseinandersetzung mit den inhibierenden Signalwegen, welche die MHC Klasse I Molekülexpression in ESCs unterdrücken, kann hilfreich sein bei der Kontrolle von Tumorbildung durch kontaminierende ESCs in therapeutischen Zelltransplantationen, sowie bei der Vernichtung von Krebs- und virusinfizierten Zellen, die bekannt dafür sind der Immunerkennung durch Abregulierung der MHC Klasse I Moleküle zu entkommen.

I. Introduction

I.1. Organization of the immune system

The major role of the immune system is to protect against disease by recognizing and killing pathogens such as bacteria, viruses, fungi and parasites as well as by eliminating abnormal, transformed somatic cells. If immune system is defective, the organism will die due to infection by microorganisms or development of cancer. For protection of our body, various immune mechanisms evolved that recognize and kill infectious agents. The primitive forms of the immune system in bacteria, invertebrates, plants and insects are based on antimicrobial enzymes, toxins and peptides called – defensins. Humans have typical vertebrate immune system that is composed of many types of proteins, cells, tissue and organs that work and interact in a more complex and sophisticated way to defend against various types of pathogens (Beck et al. 1996). The human immune system is divided into two main parts: humoral and cellular. Humoral immunity deals with the infectious agents in blood and body tissues and is mediated by the action of different soluble factors such as lysosome, complement system, cytokines, acute phase proteins and antibodies. The cellular part of the immune system is responsible for phygocytosis, processing of pathogens, secretion of soluble mediators of immune reactions and elimination of body cells that have been infected with intracellular pathogens. Both humoral and cellular branches of the immune system have their unspecific (innate, native) and specific (adaptive, acquired) components (Table1).

Table 1. The characteristics of innate and adaptive immune responses

Characteristic	**Innate (native)**	**Adaptive (acquired)**
Species distribution	Nearly all forms of life	Only in jawed vertebrates
Pathoden specificity	Low	Very high
Diversity	Limited	High (~ 10^9)
Memory	None	Yes
Secondary response	None	Yes
Clonality	None	Yes
Kinetic of response	Immediate	Delayed

Innate immunity

The innate immune system refers to the first line of defense against infection that a species possesses as basic resistance to disease. The responses in this system are non-specific and have no memory or long lasting protective immunity. These responses are phylogenetically old (found in plants, fungi, insects and primitive multicellular organisms) and have a limited repertoire of recognition molecules (Litman et al. 2005). They encounter possible pathogens and destroy in daily life and the disfunctions of specific components in this system lead to rare diseases such as leukocyte adhesion deficiencies, congenital neutropenia, chronic granulomatous disease, various complement deficiencies and others.

The cells of the innate immune system include natural killer cells, basophils, eosinophils including phagocytic cells such as neutrophils, dendritic cells and macrophages. The main function of these cells is to recognize and eliminate the pathogens from the body. (Guermonprez et al. 2002; Middleton et al. 2002; Kariyawasam et al. 2006; Krishnaswamy et al. 2006). In addition, professional antigen-presenting cells (macrophages, dendritic cells and B cells) brake down pathogens in a process of antigen processing and presentation, which is required for activation of cells of the adaptive immune system.

Adaptive immunity

Adaptive immunity is acquired in jawed vertebrates including human and is activated by the innate immune system. When an immune system encounters foreign molecules (antigens), the cells and other components of adaptive immunity attack and process each antigen. Intracellular antigens (e.g. foreign viral proteins) are presented by MHC class I molecules to $CD8^+$ T cells. Extracellular antigens are processed in the endolysosomal system and presented by MHC class II molecules to $CD4^+$ T cells (more details about antigen presentation are found in next chapter). This type of immunity takes time to develop after exposure to a new antigen. But once memory is formed, the immune response is mounted in a more effective and rapid way against previously encountered antigens (Pancer et al. 2006).

The fundamental characteristics of the acquired immune system are its specificity, diversity, ability to learn and, by retaining the memory of a previous encounter with a pathogen, prepare the body against the future challenges by the same pathogen. Four distinct but related cell-membrane molecules are responsible for a highly specific antigen recoginition. These are membrane-bound antibodies on B cells, T-cell receptors, class I MHC molecules and class II MHC molecules. These molecules play a unique role in antigen recoginition, ensuring that the immune system can recognize and respond to the various types of antigen that it encounters (see detailed information in the text below).

The main cells of acquired immunity are lymphocytes which are a type of white blood cells. They enable body's immune system to discriminate self from non-self and to remember antigens. Lymphocytes migrate without any restriction in the blood stream and lymphatic system and infiltrate into tissues as needed. Lymphocytes are divided into two classes: B cells and T cells.

B cells develop in the adult bone marrow or the fetal liver. The antigenic specificity of each B cell is determined by the membrane-bound antigen-receptor (antibody) expressed by the cell. The B cell antigen receptor (BCR) is composed of membrane immunoglobulin (mIg) molecules (antibody) and associated Igα/Igβ (CD79a/CD79b) heterodimers (α/β). The mIg subunits bind antigen, resulting in receptor aggregation, while the α/β subunits transduce signals to the interior of the cell. The complexity of BCR signaling permits many distinct outcomes, including survival, tolerance or apoptosis, proliferation, and differentiation into antibody-producing cells or memory B cells. The outcome of the response is determined by the maturation state of the cell, the nature of the antigen, the magnitude and duration of BCR signaling, and signals from other receptors such as CD40 and BAFF-R. The antibody on a B cell can recognize epitopes on macromolecules with incredible precision. The random gene rearrangements during B-cell maturation in the bone marrow generate an enormous number of different antigenic specificities. Other

functions for B cells include antigen presentation, cytokine production and lymphoid tissue organizations.

T cells develop in the thymus where they learn how to differentiate self from non-self (Zinkernagel 1978). T cells which ignore self antigen molecules are allowed to mature and leave the originating place. T cells are abundantly present in lymphatic system and migrate to secondary lymphoid organs such as spleen, lymph nodes, tonsils, appendix and Payer's patches in the small intestine. There are several classes of T cells that can be distinguished by expression of specific molecules on their cell surface: $CD8^+$-expressing cytotoxic T cells, $CD4^+$-expressing helper T cells, and $CD4^+CD25^+$-expressing regulatory T cells. $CD8^+$ T cells are involved directly in the killing of infected cells, while helper T (Th) cells augment the responses of other lymphocytes by secreting various cytokines (Abbas et al. 1996; Harty et al. 2000; Radoja et al. 2006). CD4 helper T cells produce two types of cytokines - Th1-type and Th2-type. Th1-type cytokines mainly include IFNγ which tend to produce the proinflammatory responses responsible for killing intracellular pathogens and for perpetuating auoimmune responses. The Th2-type cytokines include interleukins 4, 5 and 13, which are associated with promotion of IgE and eosinophilic responses in atopy, and also interleukin-10, which has an anti-inflammatory properties. In excess, Th2 responses will counteract the Th1 mediated microbicidal action. Regulatory T cells produce molecules that help to end the immune responses (Sakaguchi 2005). An additional type of T cells are gamma/delta ($\gamma\delta$) T cells which share the characteristics of helper T cells, cytotoxic T cells and NK cells. They are considered to be linker of adaptive and innate immunity (Holtmeier et al. 2005). T cells are especially important in cell-mediated immunity, which is the defense against tumor cells and pathogenic organisms inside body cells and are also involved in graft rejection reaction.

I.2. Major Histocompability Complex (MHC) and their role in immunorecognition

All vertebrate cells except erythrocytes express histocompatibility molecules on their surfaces. They are mostly responsible for immune responses to protein antigens are encoded by a region of highly polymorphic genes, called ther major histocomaptibility complex. Human MHC molecules are known as human leckocyte antigens (HLA) and mouse as histocompatibility 2 (H-2) antigens (Gill et al. 1978).

Their genetic loci H-2^k, H-2^d and H-2^L are present on chromosome 17 in mice while HLA-A, HLA-B and HLA-C are present on chromosome 6 in humans. Each MHC class I gene encodes a single transmembrane polypeptide chain known as alpha (α), which is folded into three extracellular globular domains (α1, α2, α3), of which the domains α1 and α2 are highly polymorphic. Each α-chain is non-covalently associated with a protein called β_2-microglobulin. MHC class I molecules present peptide fragments of endogenous protein antigens to $CD8^+$ T cells. These MHC class I/peptide complex is recognized by T cell receptor (TCR). The random rearrangement of the TCR genes is capable of generating about 10^9 unique antigenic specificities. The TCR is composed of two different heterodimeric protein chains. The α/β receptor chains are present in 95% of T cells (CD4 and CD8 T cells) while γ/δ receptor chains consist of only 5% of T cells ($\gamma\delta$ T cells). CD3 molecules (CD3-γ, δ, ε, and ζ), which are assembled together with the TCR heterodimer, possess a characteristic sequence motif for tyrosine phosphorylation, known as ITAMs (Immunoreceptor Tyrosine-based Activation Motifs). The TCR polypeptides themselves have very short cytoplasmic tails, and all proximal signaling events are mediated through the CD3 molecules. The interaction of an antigenic peptide with the TCR-CD3 complex generates the initial signal 1. TCR activation is regulated by various costimulatory receptors/molecules such as CD28, B7-1, B7-2 and CD45. CD28 provides an essential co-stimulatory signal (signal 2) during T cell activation, which augments the production of interleukin-2 (IL-2) cytokine, increases T cell proliferation and prevents the induction of anergy and cell death. On the

other hand, engagement of CTLA-4, a close relative of CD28, by B7 inhibits T-cell activation. Engagement of the TCR with MHC class I/peptide complex initiates positive (signal-enhancing) and negative (signal-attenuating) cascades that ultimately result in cellular proliferation, differentiation, cytokine production, and/or activation-induced cell death.

MHC molecules play a role in intercellular recognition and in discrimination between self and nonself. They determine whether transplanted tissue is accepted as self (histocompatible) or rejected as foreign (histoincompatible). The MHC plays a critical role in antigen recognition by T cells. The phenomenon that a given T cell recognizes a peptide antigen only when it is bound to a host own MHC molecules, is known as the MHC restriction phenomenon. MHC restriction is due to positive selection of thymocytes bearing receptors capable of binding self-MHC molecules. On the other side, negative selection by elimination of thymocytes bearing high-affinity receptors for self-MHC molecules alone or self-antigen presented by self-MHC results in self-tolerance. Both MHC restriction and self-tolerance are necessary to generate mature T cells that are self-MHC restricted and self-tolerant.

Allorecognition refers to the T-cell recognition of genetically encoded polymorphisms within the members of the same species. T-cell recognition involves both the MHC molecules and its associated peptide ligand. The polymorphic residues located within the peptide binding groove of the MHC and inaccessible to the T cell receptor can greatly affect selection and recognition of bound peptides. The antigenic impact of MHC polymorphisms is profoundly amplified by these peptide differences between histoincompatible individuals and result in the high frequency of alloreactive cells. Moreover, the frequency of T cells responding to any conventional foreign antigen presented on self-MHC molecules usually amonts to 0.01-0.001% of all T cells. However, T cell-mediated allorecognition is a rapid and vigorous process because up to 1% of peripheral T cells in each individual can cross-react with allogeneic MHC antigens (Lindahl et al. 1977; Suchin et al. 2001).

MHC molecules are highly polymorphic. Each individual has multiple genetic loci encoding MHC molecules, so it is very rare for two individuals

to have same set of MHC proteins leading to the problem of tissue matching. But on the other hand, MHC molecule polymorphism is very important from the point of view of disease resistance because allelic forms of MHC genes may encode molecules that serve as receptors for pathogens including viruses and bacterial toxins. The MHC genes are divided into three classes: the MHC class I and class II genes, which encode the antigen-presenting MHC molecules, and the class III genes, which encode the genes with both known or unknown immune functions (1999; Shiina et al. 2004; Trowsdale et al. 2004; Deakin et al. 2006).

Antigens bound by MHC class I molecules are generated by proteolytic degradation of pathogen-encoded (e.g. by viruses) or normal host proteins in the cytosol or nucleus of cells. The major protease involved in this process is the proteasome, which cleaves all intercellular proteins into short peptides (Figure 1). Peptides generated in the cytosol are further cleaved by aminopeptidases to a length of 8-9 amino acid residues optimal for binding to MHC class I molecules. Longer proteasome products as well as correctly trimmed antigenic peptides are transported into rough endoplasmic reticulum (ER) by heterodimeric transmembrane protein channel called transporter associated with antigen processing (TAP). In the ER, longer peptides can be further edited by ER-aminopeptidases ERAP1 and ERAP2 (Saric et al. 2002). Peptides of 8-9 residues in length are then captured by empty MHC heterodimers in the lumen of the ER with the help of several ER resident proteins such as calnexin, tapasin and protein disulfide isomerase. Finally, the stabilized MHC class I-antigenic peptide complex is carried out to the cell membrane via Golgi complex and is recognized on the cell surface by $CD8^+$ T cells (Li et al.; Rock et al. 1999; Hansen et al. 2009).

MHC class II molecules are mainly present on professional antigen-presenting cells (APCs) such as dendritic cells, macrophages and B lymphocytes. They are also expressed on thymic epithelial cells. In mice, their genetic loci are called I-A and I-E and in humans HLA-DP, HLA-DQ and HLA-DR. Each MHC class II gene encodes heterodimeric chains (α and β) with two conserved immunoglobulin-like domains ($\alpha 2$ and $\beta 2$) close

to the membrane and two antigen-binding polymorphic amino-terminal domains (α1 and β1) farthest from the membrane. MHC class II molecules bind and present peptide fragments of exogenous, extracellular protein antigens to $CD4^+$ helper T cells. Exogenous antigens that are engulfed by APCs by means of phagocytosis or endocytosis are processed in the endosomal-lysosomal antigen processing pathway (Sant et al. 1994; Lennon-Dumenil et al. 2002; Gelin et al. 2009) (Figure 1).

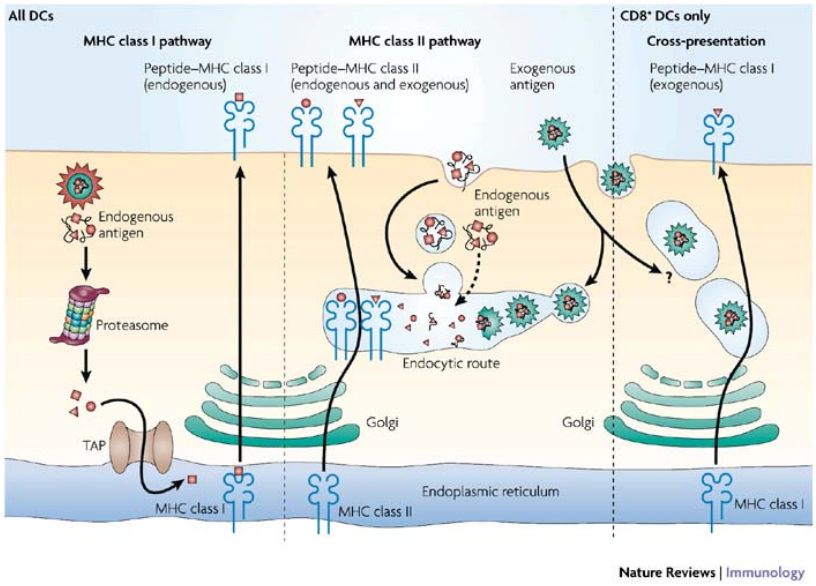


Figure 1. The antigen presentation pathways in dendritic cells. "All dendritic cells (DCs) have functional MHC class I and MHC class II presentation pathways. MHC class I molecules present peptides that are derived from proteins degraded mainly in the cytosol, which in most DC types comprise almost exclusively endogenous proteins (synthesized by the cell itself). MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. $CD8^+$ DCs have a unique ability to deliver exogenous antigens to the MHC class I (cross-presentation) pathway, although the mechanisms involved in this pathway are still poorly understood. The bifurcated arrow indicates that the MHC class II and the MHC class I cross-presentation pathways may compete for exogenous antigens in $CD8^+$ DCs, or that the endocytic mechanism involved in internalization of a given antigen may determine whether it is preferentially delivered to MHC class II pathway or the MHC class I cross-presentation pathway. TAP, transporter associated with antigen processing". (*Villadangos and Schnorrer, Nature Reviews Immunology,* 2007)

I.3. Interferon gamma (IFNγ) and regulation of MHC molecules

Interferons (IFNs) are proteins made and released by host cells in response to the presence of pathogens or tumor cells. They are typically divided into type I (alpha and beta) and type II (gamma) classes. IFNs are

multifunctional secreted proteins having similar functions activating partly overlapping pattern of induced genes related to cell growth regulation and anti-viral and immune defense (Stark et al. 1998). Interferon-gamma (IFNγ), a 34 KDa protein, was discovered in 1965 as an antiviral protein (Boehm et al. 1997). IFN activity may be divided into acid stable and acid labile activity. All cells produce the acid stable activity while acid labile is only produced by lymphocytes, and this is known as IFNγ. Besides antiviral function, IFNs have broader range of anti-proliferation and pro-inflammatory activities. IFNγ exposure to cells can up-regulate MHC class I expression on their cell surface and enhance cytotoxic T-lymphocytes (CTLs) recognition (Rosa et al. 1988). In this way, CTLs destroy bacteria and virus infected cells via CTL epitope/MHC class I complexes on the surface of target cells (Boehm et al. 1997). The components of MHC class I antigen processing machinery are up-regulated by IFNγ through JAK/STAT signal transduction pathway (Kohlhuber et al. 1997; Wu et al. 1997; Fruh et al. 1999). IFNγ performs its biological functions through binding to IFNγ-receptor (IFNGR). IFNGR is composed of two subunits (α and β) and is present on all nucleated cells. IFNGR-α chain associates with Janus active kinase-1 (JAK1) and signal transducer and activator of transcription 1 (STAT1) while IFNGR-β chain binds to JAK2 (Figure 2). IFNγ bound to its receptor leads to the trans-phosphorylation and reciprocal activation of JAKs which subsequently activate STAT1 by phosphorylation at tyrosine 701 (Y701) and serine 727 (S727) DNA-binding site. After activation the phosphorylated STAT1 dimerizes in the cytosol and is translocated into the nucleus to activate IFNγ-inducible genes by binding to the gamma interferon activation site (GAS) elements in their promoter regions. The nuclear transport of STAT1 dimers occurs through nuclear pore complexes and is dependent on heterodimeric importin receptors, which, on one side, bind to the nuclear localization signal of the cargo (importin α subunit) and, on the other side, enable docking of the protein cargo on the cytoplasmic site of the nuclear pore complex (importin β subunit). There are six different α-importin (α1-α6) and one β-importin (β1). Various α-importins are differentially expressed in

different tissues and are responsible for nuclear import of different cargo proteins. Nuclear import of activated STAT1 occurs through importin-α1 (McBride et al. 2002). In the nucleus, phosphorylated STAT1 dimers activate IFNγ inducible target genes including MHC class I heavy chain and light chain molecules and several components of the MHC class I antigen processing and presentation pathway (Fruh et al. 1999). IFNγ also activates some important transcription factors from interferon regulatory factor (IRF) family (especially IRF-1 and IRF-8). They have a capacity to bind to IFNγ regulated response elements in promoter region of genes and stimulate the transcription of IFNγ stimulated genes (ISGs) such as LMP2, LMP7, TAP1, TAP2 and tapasin (Taniguchi et al. 2002). IRF-1 expression is induced by IFNγ through STAT1-dependent fashion over a period of hours (Harada et al. 1994; Pine et al. 1994) and it is not directly activated by IFNγ. IRF-1 cooperates with STAT1 to transcribe ISGs which require intact IRF-1 and STAT1 binding sites to be optimally transcribed. IRF-1 plays an important role in regulating MHC class I gene expression (Mori et al. 1999). However, other IFNγ -induced factors such as p48, also up-regulate MHC class I gene expression in STAT1 independent manner. This describes the diversity of regulation of MHC class I expression mediated by IFNγ (Bluyssen et al. 1996; Kimura et al. 1996; Majumder et al. 1998).

MHC class II trans-activator (CIITA) is a transcriptional factor that regulates the expression of MHC class II (Chang et al. 1996). CIITA is also efficiently induced by IFN-γ through a complex consisting of upstream factor 1 (USF-1) and STAT1 (Muhlethaler-Mottet et al. 1997; Muhlethaler-Mottet et al. 1998). This is an additional example of co-operation of STAT1 with other transcriptional factors. In contrast, the transcription factors STAT3, STAT5, NF-κB and AP-1 are induced by IFN-γ without the cooperation of STAT1. In some cases, STAT3 and STAT5 are activated by IFNγ to compensate the absence of STAT1 (Meinke et al. 1996; Ramana et al. 2000; Ramana et al. 2005) but during anti-viral response, STAT3 can not compensate the loss of STAT1 (Horvath et al. 1996). Because, biologically active STAT1 must be full length and phosphorylated on both

tyrosine and serine residues for full IFNγ antiviral activity. Furthermore, these closely related STAT proteins (STAT1 and STAT3) are not functionally redundant in their ability to establish the antiviral effect.

Previous reports demonstrated that the expression and phophorylation of STAT3 was increased in cells deficient of STAT1 (Ramana et al. 2005). But, this was not true in all STAT1-deficient strains (Gough et al. 2007). The reason for this discrepancy is still unclear and might be dependent on animal strain.

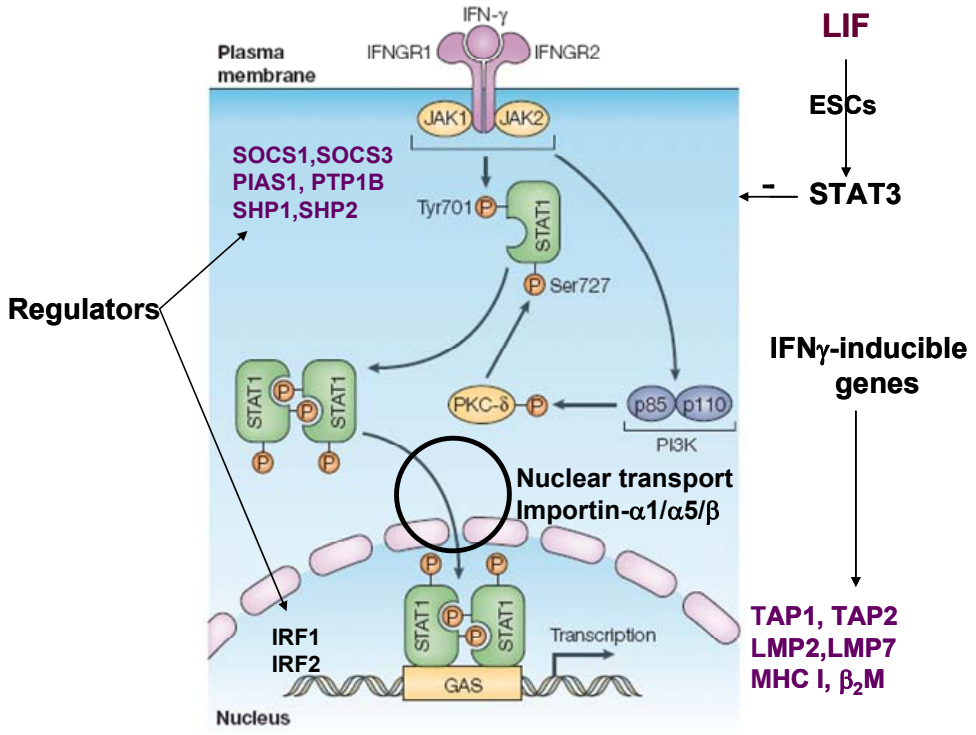


Figure 2. The IFNγ signaling pathway in all nucleated cells. IFNγ binds to its receptor IFNGR1/R2 and leads to activation of JAK1/2 which phosphorylates STAT1 on Tyr701 or Ser727 residues. Active STAT1 dimerizes and goes into the nucleus by nuclear import proteins importin α1/β. Active STAT1 binds to the IFNγ-activated site (GAS) in promoter region leading to the induction of IFNγ-inducible genes including TAP1,TAP2, LMP2, LMP7, MHC I heavy chain and β2M. SHP1, IRF1 and IRF2 act as positive regulators while SOCS1, PIAS1, PTP1B and SHP2 act as negative regulators of this pathway. LIF/IL-6 inhibits through STAT3 pathway the IFNγ induced STAT1 signaling. *(Leonidas C. Platanias, Nature Reviews Immunology, 2005)*

The significance of the NF-κB pathway in IFNγ signaling is not known so far. However, IFNγ can induce DNA binding of NF-κB in the absence of STAT1 in some primary fibroblasts. This cell restricted phenomenon reveals that this may not be the main mechanism of STAT1 independent

transcriptional effects of IFNγ (Deb et al. 2001). The activator protein 1 (AP-1) is the family of dimeric transcription factors, which are rapidly activated by IFNγ and required for transcription of several IFNγ stimulated genes (Clarke et al. 2003; Gough et al. 2007). AP-1 DNA binding activity is increased rapidly by IFNγ independently of JAK1/2 or STAT1. AP-1 activity is also required for transcription of several ISGs with or without co-operating with STAT1 (Gough et al. 2007).

I.4. Role of STAT1:STAT3 signaling in biological systems

Interferons and gp130 family of cytokines play a major role in regulating immune responses and in mediating cellular decisions during growth and development. Signal transducer and activators of transcription, especially STAT1 and STAT3, are the main targets of type I and type II interferons and cytokines belonging to the gp130 family such as interleukin 6 (IL-6) and leukemia inhibitory factor (LIF). STAT3 promotes cell proliferation/survival and immune tolerance and inhibits inflammation, while STAT1 counteracts proliferation and supports innate and adaptive immune responses. IFNγ mainly activates prolonged STAT1 activation through JAK1 and JAK2 while type I IFNs activate the distinct transcriptional complex including STAT1, STAT2 and ISGF3. The cytokines of IL-6 family activate JAK1, JAK2 and TYK2 through specific leukemia inhibitory factor receptor (LIFR) leading to the prolonged phosphorylation of STAT3. LIF acts through a high-affinity receptor complex composed of a low affinity LIF binding chain (LIFR, also known as CD118) and high-affinity converter subunit gp130.

STAT1 pathway activates the expression of apoptotic genes like caspases, death receptors and ligands, and nitric oxide synthase (Allione et al. 1999; Fulda et al. 2002). On the other side, it negatively regulates the expression of prosurvival genes such as Bcl-xL, and Bcl-2 (Stephanou et al. 2000). STAT3 target genes differ according to cell type and environmental circumstances. In general, STAT3 induces anti-apoptotic genes of Bcl family to prevent apoptosis and it promotes proliferation

through the induction of oncogenes and cell cycle regulatory genes such as cyclin D1, c-myc and pim-1 (Chin et al. 1996; Dimberg et al. 2003; Xiao et al. 2006). STAT1 and STAT3 exert opposing roles in inflammation. IFN-mediated STAT1 activation acts as a proinflammatory factor by inducing tissue apoptosis and a number of genes that favor activation and recruitment of immune cells to the side of inflammation. STAT1 activation by type I and II IFNs advocates antigen presentation by enhancing the expression of MHC class I and MHC class II molecules and the components of antigen-processing machinery (Lee et al. 1996; Brucet et al. 2004; Marques et al. 2004; Rouyez et al. 2005). In contrast, STAT3 mediates the functions of major anti-inflammatory cytokine IL-10 which maintains the balance between activation and deactivation of mononuclear cells and downregulates the surface expression of MHC molecules (Donnelly et al. 1999). STAT3 activated IL-10 also directly inhibits IFN-induced gene transcription partly by downregulating STAT1 activation due to inhibition of its tyrosine phosphorylation (Ito et al. 1999). Interestingly, STAT1 is usually considered as a tumor suppressor while STAT3 is known as an oncogene since it is constitutively expressed in almost 70% of solid and hematological tumors (Turkson et al. 2000; Turkson 2004; Kim et al. 2007). STAT1 directly controls tumor cell expansion by up regulating many pro-apoptotic and anti-proliferative genes in cancer cells. In contrast, STAT3 constitutive activity is necessary for survival and proliferation of many different kinds of established or primary cancer cells (Turkson et al. 2004; Kim et al. 2007) and STAT3 allows cancer cells to escape from cell-mediated immune system by enhancing the secretion of dendritic cell inhibitors such as IL-10 and VEGF (Wang et al. 2004). Therefore, the balance between STAT3 and STAT1 activity is important for homeostasis and physiology of normal cells (Figure 3).

Although STAT1 and STAT3 play opposing roles in cell survival, proliferation, apoptotic death or inflammation, the counterbalance between STAT1 and STAT3 may decide the result of cytokine treatment and pathological responses. In fact, previous reports demonstrated that STAT1 or STAT3-deficient cells have reciprocal STAT1:STAT3 regulatory mechanisms and relative affluence of STAT3 or STAT1 may play a role in

shaping the biological effects of their main activating cytokines (Costa-Pereira et al. 2002; Qing et al. 2004; Gimeno et al. 2005; Tanabe et al. 2005). For example, in absence of STAT3, gp130 cytokines strongly modify STAT1 activation profiles and trigger prolonged phosphorylation of STAT1 upregulating multiple IFNγ inducible genes in a number of cell types (Costa-Pereira et al. 2002).

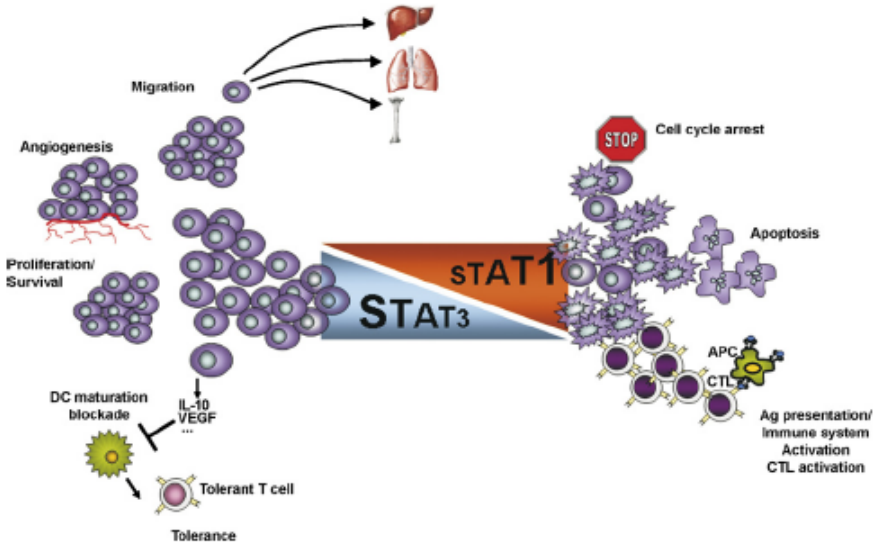


Figure 3. The importance of balanced expression/activation of STAT1 and STAT3 in tumor setting. When STAT3 activation and/or expression overwhelm, tumor development and maintenance are favored. The presence of soluble factors such as IL-10 induces tolerance in immune cells; tumor cell proliferation and survival are favored not only directly, but also indirectly, by the enhancement of angiogenesis. On contrary, the prevelance of STAT1 activation directly blocks cell cycle progression and induces apoptosis of cancer cells. Moreover, STAT1 favors the generation of an adequate immune response against the tumor. (*Regis et al, Seminars in Cell and Developmental Biology,* 2008)

These findings indicate that STAT3 downregulates STAT1 activity (IFNγ responses) and allow LIF/IL-6 specific responses in normal cells. Each specific cell type has characteristic STAT protein levels. The proteins of the suppressor of cytokine signaling (SOCS) family are induced as immediate early genes downstream of different STATs and inhibit STATs phosphorylation as a negative feedback mechanism (Chen et al. 2000). Fascinatingly, both SOCS1 and SOCS3 work not only as feedback mechanisms to quantitatively regulate STAT1 and STAT3 activation

respectively, but they can also finely adjust STAT1 or STAT3 mediated responses. For example, STAT3-dependent SOCS3 induction can prevent STAT1 activation and vice versa by inhibiting the JAKs in Con-A induced heapatitis in hepatocytes (Hong et al. 2002; Wormald et al. 2006).

I.5. Allorecognition of embryo and expression of MHC molecules in the course of embryonic development

Allorecognition attributes to T-cell recognition of genetic polymorphisms between members of same species. MHC molecules are the main targets of the immune responses to allogeneic tissues which are present on the donor cells. There are two pathways of allorecognition: direct and indirect (Lechler et al. 1982). Direct recognition involves the recognition by recipient T cells of intact donor MHC molecules complexed with peptide on donor antigen presenting cells. On contrary, the requirement of indirect recognition is that recipient antigen presenting cells (APCs) process the donor-MHC antigen prior to presentation to recipient T cells in a self-restricted manner. Therefore, the direct and indirect alloresponses are governed by different APCs and differ in their cellular mechanisms (Figure 4).

Allorecognition is a basic system that animals use to preserve individuality. Although embryos are usually semiallogeneic with their mother, viviparous animals including human beings are required to allow these embryos to develop inside the mother's body eliminating an 'invasion' by nonself. The embryo is directly exposed to the maternal immune system during pregnancy and embryonic cells can be found in maternal organs and blood in mice and humans (Liegeois et al. 1981; Guetta et al. 2003). This provides ample opportunities for the maternal immune system to recognize fetal alloantigens. However, embryo is not destroyed indicating that the immune system is tolerant of the fetal presence. It has been shown that maternal tolerance of the embryo in allogeneic pregnancy is dependent upon $CD25^+$ regulatory T (Treg) cells but $CD25^+$ Treg cells play no role during late stages of pregnancy (Shima et al. 2010; Aluvihare et al. 2004), indicating the involvement of other mechanisms. Interestingly, maternal

and fetal cells are involved in the establishment of the tolerogenic environment having overlapping molecular mechanisms. The maternal endometrium expresses the immunosuppressive factors TGF-β, Galectin-1 (GAL-1) and thymic stromal lymphopoietin (TSLP) (Simpson et al. 2002; von Wolff et al. 2005). They induce Treg cells and Th2 responses and inhibit Th1 response providing tolerogenic environment. Both the maternal decidua and fetal trophoblast express indoleamine 2,3-dioxygenase (IDO) (Kamimura et al. 1991) which induces Treg cells and inhibits the activation of T cells and NK cells (Frumento et al. 2002). Crry is also expressed by fetal and maternal cells and it stops deposition of the complement proteins C3 and C4 to stop the formation of the cytosolic membrane attack complex (Weigle et al. 1983; Molina et al. 1992; Kim et al. 1995). Human villous cytotrophoblast cells also express non-classical HLA class Ib HLA-G molecules which help to evade cytotoxic NK activity and induce the tolerogenic phenotype of dendritic cells. Fas-L expression on these cells also helps to kill activated T cells via apoptosis (Le Bouteiller et al. 1999; Fournel et al. 2000; Hviid 2006; Carosella et al. 2008).

In mammals the expression of MHC genes is developmentally programmed. After fertilization, a hierarchical order of gene expression takes place including the MHC genes. In order to understand how the developing embryo avoids rejection, it is essential to know if, and when, MHC class I expression occurs during embryonic development. It is well postulated that a possible route of escape of the embryo from maternal rejection is by down-modulating the MHC. This down-modulation of MHC genes occurs either at the feto-maternal interface and/or on the surface of other embryo cells. HLA class I and II are absent while HLA-G and β2M are present in human blastocyts and preimplantation embryos (Roberts et al. 1992; Jurisicova et al. 1996). Human trophoblasts are resistant to NK cell attack, possibly as a consequence of the presence of HLA-G, which ensures that NK cells detect the trophoblast as normal self. It is possible, that expression of certain MHC products is advantageous for the survival of the early embryo. The selective expression of MHC products during development is potentially subject to modulation, by a fluctuating pattern of

Th1-type and Th2-type cytokines, maternal hormones and other cellular interactions. Unexplained human reproductive failure is quite common and has often associated with MHC products (Christiansen et al. 1997). If MHC determinants were to be expressed in vivo, then cytotoxic T-cell attack could occur, resulting in spontaneous abortion.

The onset of expression of MHC class Ia, class Ib, β2M, TAP1 and TAP2 in murine embryonic development is shown in Table 2 (Sprinks et al. 1993; Cooper et al. 1998).

Table 2. Detection of MHC and transporter associated with antigen processing (TAP) mRNA and protein products during preimplantation mouse embryonic development

	1-cell stage		2-cell stage		8-cell stage		Blastocyst	
	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA
MHC class Ia ($H\text{-}2D^b$)	+	-	+	+	+	+	+	+
MHC class Ib (Q7/9)	+	+	+	+	+	+	+	+
β_2M	-	-	+	-	+	+	+	+
TAP1	-	+	-	+	-	+	+	+
TAP2	-	-	-	-	-	-	-	-

Note: symbols in parentheses indicate presence (+) or absence (-) of mRNA transcripts, as detected by reverse transcription-polymerase chain reaction, and protein products, as detected by indirect immunofluoresence. Based on data from: Sprinks *et al,* (1993), sprinks (1994), Cooper *et al.* (1998) and Cooper (1998).

Taken together these data suggest that, at least for mice, MHC class I complexes composed of heavy chain and β2M are present at the surface of embryos at very early stages of development. However, they are unlikely to be functional in the normal sense of antigen presentation, due to the lack of a functional peptide transporter. Therefore, during pregnancy the developing fetus is protected from immunological attack potentially by both a physical barrier, such as negatively charged, sialic acid-rich mucopolysaccharide, and also an immunologically inert layer of placental

trophoblast tissue, on which MHC class I antigens are very weakly distributed and from which MHC class II molecules are absent.

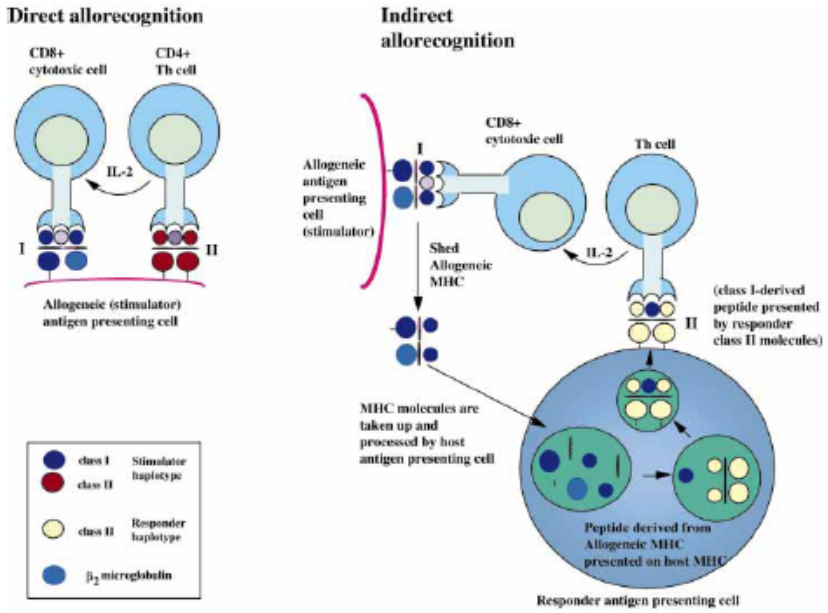


Figure 4. Diagrammatic representation of direct and indirect allorecognition In direct allorecognition pathway, intact donor Class-I and –II MHC molecules present on the surface of donor antigen presenting cell (APC) are recognized directly by recipient $CD8^+$ and $CD4^+$ T cells, respectively. In the indirect allorecognition pathway, donor Class-I and –II MHC molecules are taken up, processed and presented in the context of recipient MHC molecules to recipient $CD8^+$ and $CD4^+$ T cells, respectively. (*Rogers and Lechler, American Journal of Transplantation,* 2001).

I.6. Embryonic stem (ES) cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts (Evans et al. 1981; Martin 1981); (Thomson et al. 1998). Human ESCs are maintained in undifferentiated stage in the presence of basic fibroblasts growth factor (bFGF). The human ESC colonies have typical round shape compact morphology and cells express SSEA-4, Tra-1-60 and Tra-1-81 pluripotent markers on their cell surface. They are passaged every 5-7 days either by mechanical cutting or by using collagenase IV or dispase in the form of small clumps. Single cell passaging of these ESCs can be done in presence of Rho kinase inhibitor (ROCKi), thiazovivin or neurotrophins using trypsin-EDTA (Pyle et al. 2006; Watanabe et al. 2007). Murine ESCs are maintained in undifferentiated stage in the presence of LIF. In comparison to human

ESCs, the colonies are smaller in size having round or oval shape structures. They express SSEA-1 as pluripotency marker on their cell surface but not the SSEA4 or Tra antigens.They are passaged as single cells every 2-3 days using trypsin-EDTA. The characteristics of murine and human ESCs are summarized in Table 3.

Table 3. Characteristics of murine and human ESCs

Marker	Mouse ESCs	Human ESCs
Oct-4	+	+
Alkaline Phosphatase	+	+
SSEA-1	+	-
SSEA-4	-	+
Tra-1-60	-	+
Tra-1-81	-	+
Telomerase activity	+	+
Factors aiding in self-renewal	LIF+MEFs or LIF+gelatin-coated plastic	MEFs+serum or MEFs+bFGF
Morphology	Tight, rounded, multilayer colonies, high nucleus to cytoplasm ratio	Flatter colonies with tight border, high nucleus to cytoplasm ratio
Rate of division	Fast, passage every 2-3 days	Slower, passage every 3-7 days
Teratoma formation	+	+
Germ line competent	+	Unknown

Note: Presence (+) and absence (-)**,** SSEA = stage specific embryonic antigen, LIF = leukemia inhibitory factor, bFGF = basic fibroblast growth factor; MEFs = murine embryonic fibroblasts. Table adapted from NIH Stem Cell report (NIH, 2006).

I.7. Immunological properties of ES cells (ESCs)

ESCs express MHC molecules on their cell surface but the constitutive expression of MHC I molecules as well as in their response to IFNγ differ greatly in human and murine ESCs. MHC class I molecules are present on the cell surface of undifferentiated human ESCs at low levels and are

inducible by IFNγ (Drukker et al. 2002; Grinnemo et al. 2006). There was no MHC induction by IFN-α and IFN-β due to the missing expression of these cytokine receptors on human ES cells (Drukker et al. 2002). On other side, the expression of MHC class I molecules was not detectable by flow cytometry on undifferentiated murine ES cells (Tian et al. 1997; Magliocca et al. 2006; Abdullah et al. 2007; Nussbaum et al. 2007). However, transcripts of MHC class I molecules, β2-microglobulin and several components of antigen presentation pathway were present in detectable amount in murine ESCs (Magliocca et al. 2006; Abdullah et al. 2007). Using a highly sensitive and specific method for detection of rare peptide-MHC class I complexes utilizing the lacZ-inducible, antigen/MHC-specific T cell hybridomas it was possible to provide the evidence that murine ES cells express MHC class I molecule but at very low levels that are not detectable by flow cytometry (Abdullah et al. 2007). The murine ESCs do not respond to IFN-γ and only after differentiation, they respond to IFNγ and strongly up regulate the expression of MHC class I molecules on a significant fraction of cells and components of antigen presenting pathway (Abdullah et al. 2007). Interestingly, MHC class II transcripts were present in human (Grinnemo et al. 2006) and murine (Magliocca et al. 2006) ESCs that were inducible by IFNγ.

Previous reports showed that murine as well as human ES cells may possess immune-privileged properties. Bonde and Zavazava have demonstrated that murine ESCs can not be lysed by naïve allogenic NK (Bonde et al. 2006) or polyI:C activated NK cells (Koch et al. 2008). However, other reports showed that murine ESCs can be lysed by NK cells in presence of IFNγ (Bonde and Zavazava 2006). The recognition of ESCs by NK cells appears to be mediated by ligands of activating natural-killer group 2 member D (NKG2D) molecules and intercellular adhesion molecule-1 (ICAM-1) expressed on ESCs (Bonde et al. 2006; Frenzel et al. 2009). Human ES cells also showed limited lysis by NK cells possibly due to low expression of activating NK cell ligands such as NKp46 and CD16 on these ES cells (Drukker et al. 2002). Similarly, murine ES cells were not lysed by *in vitro* generated alloreactive CTLs (Bonde et al. 2006).

Additionally, lymphocytic choriomeningitis (LCM) virus-infected or peptide–loaded murine ES cells were also resistant to killing by activated LCM virus-specific syngeneic CTLs (Abdullah et al. 2007). The expression of the granzyme B inhibitor serine protease inhibitor 6 (SPI-6) in undifferentiated murine ES cells appears to protect ESCs from CTL killing *in vitro* (Abdullah et al. 2007). This study also provided the evidence for functional recognition of ES cells by cytotoxic $CD8^+$ T cells *in vitro*. Another study showed that ES cells suppress T cell proliferation in a contact-independent manner by secreting TGF-β and expressing Fas-L on their surface to block alloreactive T cell apoptosis (Koch et al. 2008).

Induced pluripotent stem cells (iPSCs) are ES-like cells made first time by retroviral reprogramming of murine somatic cells using combination of four transcription factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al. 2006). These iPSCs have highly similar transcriptional and epigenetic features to those of ESCs (Gupta et al. 2010; Takahashi et al. 2007; Wernig et al. 2007; Yu et al. 2007). After this major findings, lot of different groups reprogrammed different murine and human somatic cells into iPSCs using viral and non-viral methods (Takahashi et al. 2007; Wernig et al. 2007; Stadtfeld et al. 2008; Kaji et al. 2009; Yu et al. 2009). In first stance, iPS technology opened new avenues for autologus transplantation but there are still lots of challenges of immunogenicity regarding the use of iPSCs and its derivatives in regenerative medicine. Additionally, researchers also found the epigenetic differences between ES and iPS cells (Kim et al. 2010; Polo et al. 2010; Doi et al. 2009).

A recent study demonstrated that few cells derived from iPSCs can be immunogeneic even in syngeneic settings (Zhao et al. 2011).This group reprogrammed C57BL6 (B6) mouse embryonic fibroblasts (MEFs) into iPSCs using either retroviral approach (ViPSCs) or an episomal approach (EiPSCs) that causes no permanent genomic integration of vector sequences. Later on, they transplanted B6 ESCs, ViPSCs and EiPSCs into the B6 recipients. Teratomas were formed by ESCs while teratomas formed by B6 ViPSCs were mostly immune-rejected by B6 recipients mice. Additionally, B6 EiPSCs teratomas were also immunogenic showing tissue

damage and regression with T cell infiltration. Since iPSCs and ESCs have shown to have subtle yet apparent epigenetic differences, this could be the basis for the expression of abnormal expression of antigens (minor antigens) on iPSCs but not on ESCs who have normal development and differentiation (Kim et al. 2010; Polo et al. 2010; Chin et al. 2009; Doi et al. 2009). The expression of minor antigens on iPSCs makes them susceptible for T-dependent immune rejection. Moreover, iPSCs have shown mutations in their coding sequences that could further contribute to the immunogenicity of iPSCs derivetives (Gore et al. 2011). Recently, iPSCs have generated from patients with genetically inherited as well as sporadic diseases for *in vitro* disease modeling, gene corrections of defected gene and transplantation of corrected cell types to patients (Howden et al. 2011; Liu et al. 2011; Zou et al. 2011). However, successful replacement of cell types may elicit rejection of the grafted cells themselves enduring to recognition of the processed gene product in a MHC-restricted manner, likewise to recognition of minor histocompatibility antigens (Figure 5). To circumvent these limitations, one can capitalize the pluripotency of iPSCs to provide a source of immature dendritic cells (DCs) expressing the alloantigens to which tolerance is required (Fairchild 2010).

Pearl and coworkers reported that short time blockade treatment of costimulatory molecules is enough to induce engraftment of allogeneic mouse ESCs and iPSCs as well as xenogeneic human ESCs and iPSCs (Pearl et al. 2011). They showed that short term blockade of three costimulatory receptors: CTL-associated antigen 4 (CTLA4)-Ig, anti-CD40 ligand (anti-CD40L), and anti-lymphocyte function-associated antigen 1 (anti-LFA-1) - could induce long-term allogeneic and xenogeneic ESC engraftment by decreasing the expression of proinflammatory cytokines and increasing the establishment of a proapototic phenotype. These results indicated that short term blockade of costimulatorly molecules may also overcome the problem of minor histocompatibilty antigens in cellular engraftment of iPSCs.

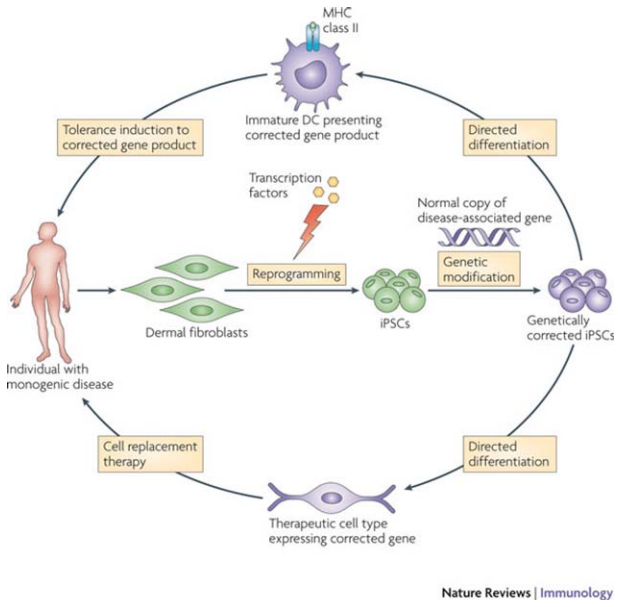


Figure 5. Induction of tolerance to the products of corrected genes. "The generation of induced pluripotent stem cells (iPSCs) from individuals with diseases that are under monogenic control might enable correction of the genetic defect. Successful replacement of cell types *in vivo* could, however, induce neutralizing immune responses to products of the corrected gene or provoke rejection of the grafted cells themselves owing to recognition of the processed gene product in an MHC-restricted manner, similarly to the recognition of minor histocompatibility antigens. One approach to circumventing such immunogenicity might be the directed differentiation of immature dendritic cells (DCs) from the disease-corrected iPSCs, which endogenously express the gene product to which tolerance is required. Administration of such DCs in advance of cell replacement therapy in a non-inflammatory, tolerogenic context that does not promote DC maturation could enable the induction of a repertoire of regulatory T cells specific for the therapeutic gene product. Tolerance can subsequently be reinforced by the allograft itself, which functions as a continuous source of antigen". *(Fairchild,PJ, Nature Reviews Immunology, Dec. 2010).*

II. Aims

In mammals the expression of MHC genes are developmentally programmed. The MHC class I molecules play crucial role not only in fertilization and development of embryos but also in reinforcement of innate and adaptive immune responses against infectious agents or tumor cells. Since ESCs are excellent models to study development and differentiation into various types of cells, we used it to analyze the regulation of MHC class I molecules in these cells. Our group has previously shown that murine ESCs express very low levels of MHC class I molecules, which are not inducible by IFNγ. I, therefore, wished to investigate the reason of low expression of MHC class I molecules in ESCs and their unresponsiveness to IFNγ.

The specific aims of this thesis are:

1. To elucidate the mechanisms responsible for low expression of MHC class I molecules in undifferentiated murine ESCs
2. To determine the mechanism for IFNγ unresponsiveness in ESCs.
3. To determine whether same mechanisms are involved in regulation of expression of MHC class I molecules in differentiated cells
4. To determine whether modulation of expression of MHC class I molecules affects the susceptibility of ESCs to immune cells in *vitro*.

III. Results

III.1. Regulation of MHC class I molecules in ESCs

III.1.1. Expression of MHC class I molecules on ESCs

Human and murine ESCs grow on feeders and need, respectively, bFGF or LIF as crucial additives in culture media to maintain their pluripotency. Human ESCs show typical flat, large and compact colony expressing Tra-1-81 as a pluripotency marker on their surface (Figure 6A,B). Murine ESCs are round, oval shaped, smaller in size and express SSEA1 as a pluripotency marker on their surfaces (Figure 6C,D).

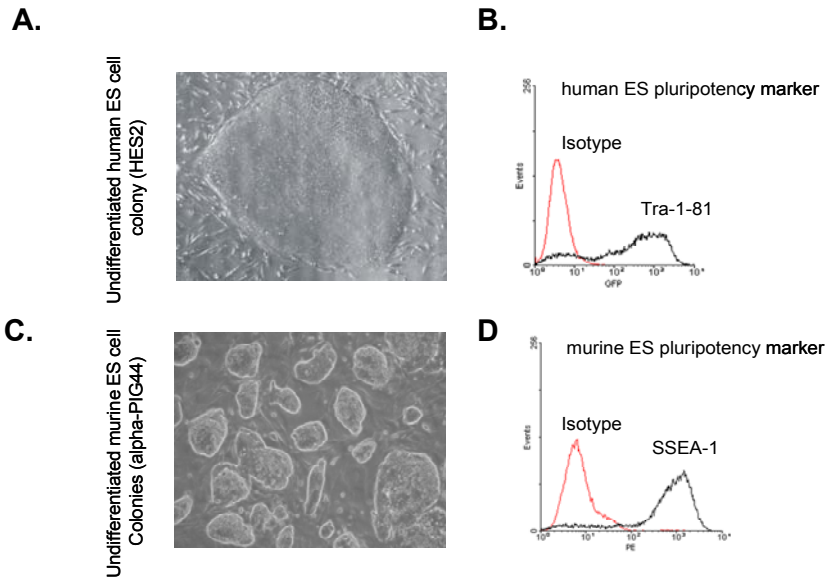


Figure 6. Characterization of human and murine ESCs. Typical morphology of human (A) and murine (C) ESCs. The expression of pluripotency markers Tra-1-81 (B) and SSEA1 (D) on human and murine ES cells, respectively, was determined by flow cytometry.

To set conditions for our further mechanistic studies we used flow cytometry to determine expression of MHC class I molecules on ESCs and validate previously published results. We showed that human ESCs stained with pan-HLA-ABC antibodies W6/32 express MHC class I molecules on their surface (93.2% of positive cells, MFI=103.9) and that their expression is enhanced 7.8-fold after stimulation with IFNγ (98.4% of positive cells, MFI=812.3) (Figure 7A). In contrast, staining for H-2K^b MHC

class I molecules on two murine ESC lines (αPIG44 and CGR8) revealed that they were not detectable by flow cytometry and also not induced by IFNγ (Figure 7C). Same result was also obtained with murine induced pluripotent stem cell (iPSC) line AT25 (Figure 7C).

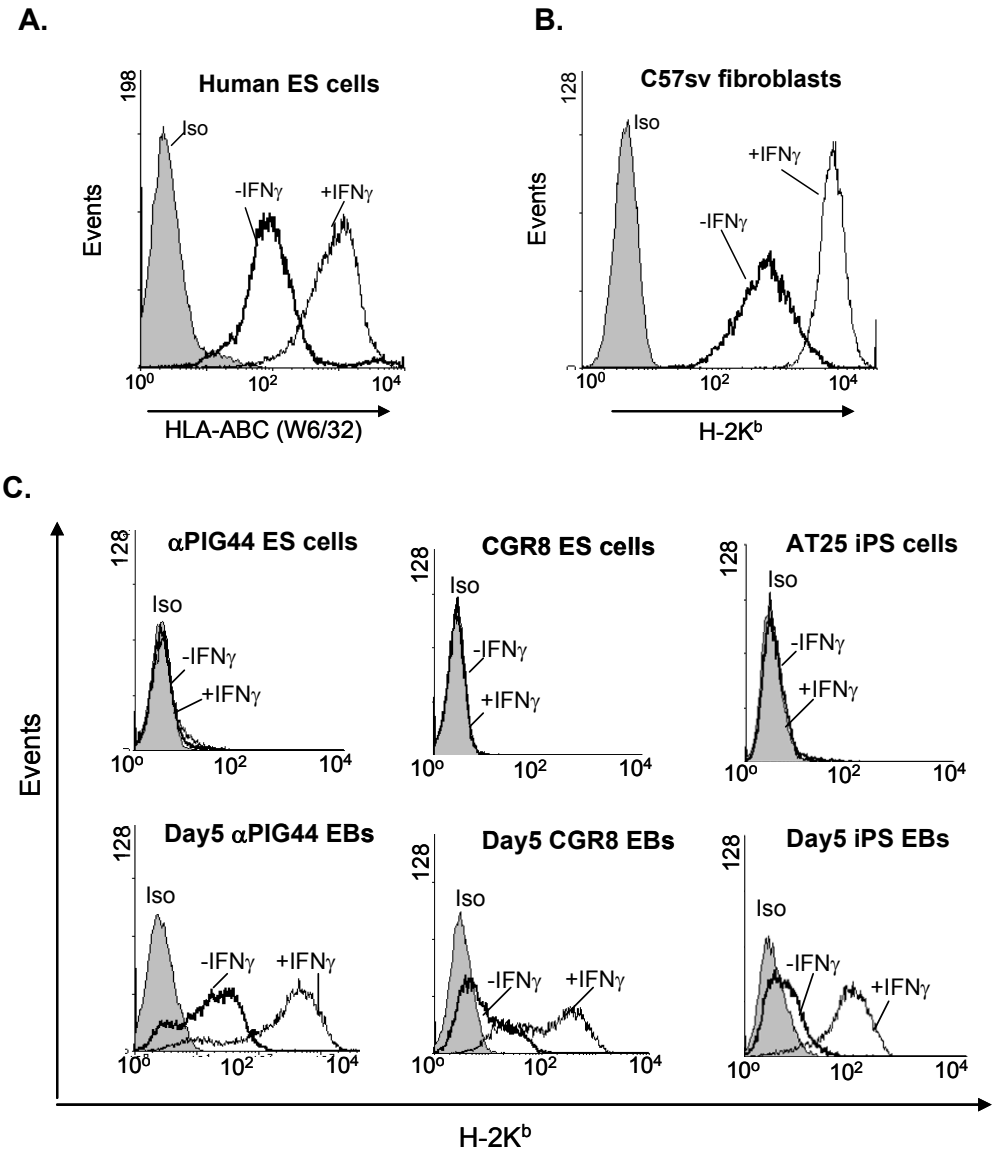


Figure 7. Expression of MHC class I molecules in murine and human ES cells and their regulation by IFNγ. Murine ES cells (αPIG44 and CGR8), murine iPS cells (AT25), human ES cells (HES2) and C57sv fibroblasts were cultured with or without 100 ng/ml IFNγ for 2 days. Cells were trypsinized and MHC class I molecules were analyzed using flow cytometry by acquiring 10,000 events. C57sv fibroblasts and murine ESCS/iPSCs were labeled with PE-conjugated anti-H-2K^b monoclonal antibody using PE-conjugated IgG2a antibody as isotype control (B,C). Human ES cells were labeled with primary pan-HLA class I (W6/32) antibody and visualized with secondary anti-murine IgG-PE antibodies (A). Dead cells were excluded by gating or PI staining and histograms were generated by gating on viable cells.

In contrast to undifferentiated cells, ESCs and iPSCs undergoing differentiation in day 5 embryoid bodies (EBs) express flow cytometrically detectable levels of H-2K^b MHC class I molecules which were induced by IFNγ. αPIG44 ESC-, CGR8 ESC- and AT25 iPSC-derived EBs expressed H-2K^b molecules on 58.4% (MFI=35.1), 27.4% (MFI=32.1) and 23.3% (MFI=14.9) of cells, respectively. After stimulation with IFNγ these molecules were induced in these three cell lines by 9.7, 4.7- and 5.1-fold, respectively, increasing the percentage of positive cells to 89.0% (MFI=339.1), 88.8% (MFI=149.3) and 91.5% (MFI=76.1) (Figure 7C). For comparison, we also used as a positive control for expression of H-2K^b molecules the murine embryonic fibroblast cell line C57sv that was derived from C57BL/6 inbread mouse strain (MHC class I haplotype H-2K^b) and immortalized by stable expression of SV40 large T antigen. In these cells, H-2K^b molecules were constitutively expressed at very high levels (99.7% of positive cells, MFI=305) and were upregulated 7.9-fold after stimulation by IFNγ (99.6% of positive cells, MFI=2424) (Figure 7B).

III.1.2. Expression of components of MHC class I processing machinery in ESCs and their differentiating derivatives

MHC class I molecules are presented on the cell surfaces with the help of several components of the antigen processing and presentation pathway. The absence of any component of this pathway may result in reduced expression of these molecules on the cell surface and subsequent modulation of their recognition by CD8$^+$ T cells or NK cells. To determine whether murine ESCs and day 5 EBs express the antigen presenting pathway components we determined the levels of transcripts encoding for MHC class I heavy (H-2K^b) and light (β2M) chain molecules, transporters associated with antigen processing *(TAP)-1* and *-2*, as well as two immunoproteasomal subunits *LMP2* and *LMP7* by real-time PCR. The transcript for MHC class I heavy and light chain molecules were expressed at detectable levels in undifferentiated ES cells and day 5 EBs but this expression was two orders of magnitute lower compared to C57sv fibroblasts (Figure 8A). In agreement with flow cytometric analyses, these

transcripts were upregulated by IFNγ only in fibroblasts (2-fold for *H-2K*^{b} and 2-fold for *β2M* with a p<0.04) and day 5 EBs (9.6-fold for *H-2K*^{b} and 4.5-fold for *β2M* with a p<0.001) but not in undifferentiated ESCs (1.1-fold for *H-2K*^{b} and 1.08-fold for *β2M* with p>0.39) (Figure 8A). The transcripts for *TAP-1*, *TAP-2*, *LMP2* and *LMP7* were present at transcript level in CGR8 ES cells as well as in day 5 EBs but they were only inducible by IFNγ in day 5 EBs (Figure 8B). Compared to fibroblasts, undifferentiated ESCs expressed lower levels of these transcripts and *LMP7* was hardly detectable in untreated ESCs but was strongly induced by IFNγ (Figure 8B).

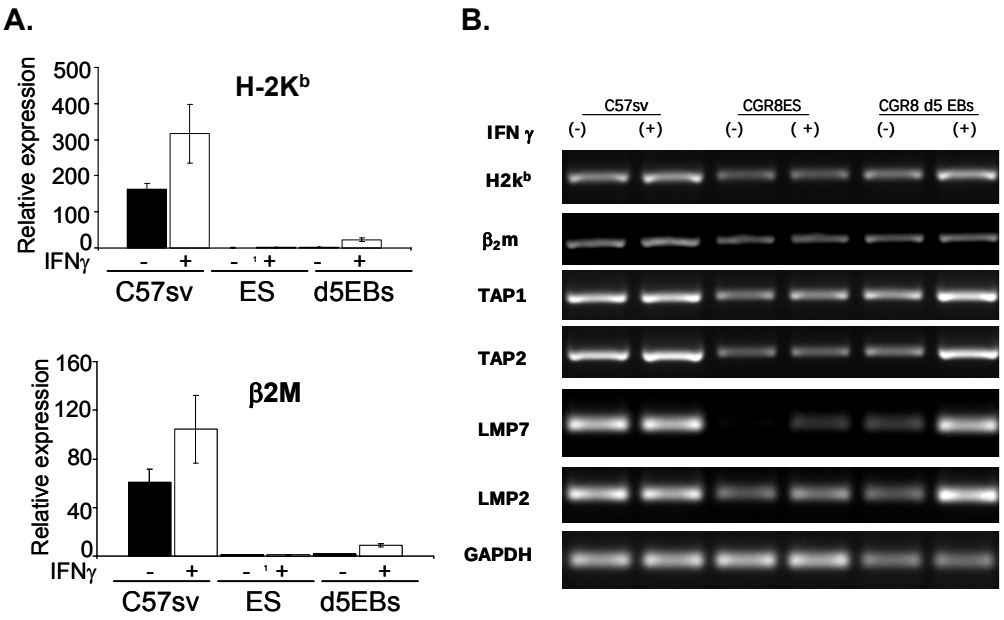


Figure 8. Expression of MHC class I molecules and several components of antigen processing machinery. RNA was isolated from all the samples using Trizol method and cDNA was amplified from the same amount of RNA from all cell types with or without IFNγ treatment (100 ng/ml for 2 days). Real-time PCR analysis (A) showed the H-2K^b and β2M expression and induction by IFNγ in C57sv fibroblasts and ESC-derived day 5 EBs but very low expression and no induction in αPIG44 ESCs. Semiquantitative RT-PCR analysis of genes involved in MHC class I antigen presenting pathway. Data are given as mean ± s.e.m. of three replicates. These data are representative of three independent experiments. (B) In fibroblasts and CGR8 ESCs showed the expression of almost all analyzed genes (*TAP1, TAP2, LMP7* and *LMP2*) in every cell type. However, a very low level of *LMP7* was detected in CGR8 ES cells without IFNγ treatment. GAPDH gene used as endogenous control.

Comparing the data for MHC class I expression in C57sv fibroblasts at protein and transcript levels after IFNγ stimulation it is of note that the expression of MHC class I molecules on the cell surface is upregulated by IFNγ to a much higher extent (7.9-fold) than the expression of heavy and light chain transcripts (upregulation by only about 2-fold). However, in αPIG ESC-derived day 5 EBs IFNγ induced MHC class I expression at the protein and transcript level to a similar extent (9.7 vs 9.6 fold, respectively). This data indicate that different mechanisms regulate the extent of induction of MHC class I molecules on the surface of C57sv fibroblasts and day 5 EB cells by IFNγ and that stronger induction of MHC class I molecule expression at the protein than at the transcript level in fibroblasts but not in ESCs is presumably governed by other mechanisms (e.g. enhancement of MHC class I loading in the ER and transport to the cell surface).

III.1.3. Expression of IFNγ receptors in ESCs and their differentiating derivatives

IFNγ exerts its effect by interacting with the IFNγ-receptors (IFNGR) which are present on the surfaces of nearly all cells and leads to the downstream signaling activating IFNγ-inducible genes. We next assessed whether the lack of murine ESCs to respond to IFNγ was due to absence of IFNGR on their cell surface. The expression of IFNγ-R1 (αR) was determined at the transcript and protein level in undifferentiated ESCs, differentiated day 6 EBs and C57sv fibroblasts cultured with and without IFNγ (100 ng/ml) for 2 days). At the transcript level, the *IFNγ-R1(αR)* was expressed in C57sv fibroblasts, CGR8 ESCs and day 6 EBs at similar levels (Figure 9A). These transcripts were induced by IFNγ in fibroblasts and EBs but not in the ESCs. Flow cytometric analysis revealed that IFNγ-αR and IFNγ-βR subunits are equally expressed in undifferentiated ESCs as well as in differentiating day 5 EBs (Figure 9B). The expression of IFNGR was reduced by IFNγ treatment on both ES and EB cells, presumably as a consequence of receptor downregulation (Figure 9B). These data indicate

that the inability of ESCs to upregulate MHC class I expression after IFNγ stimulation is not due to missing expression of IFNGR on their surface.

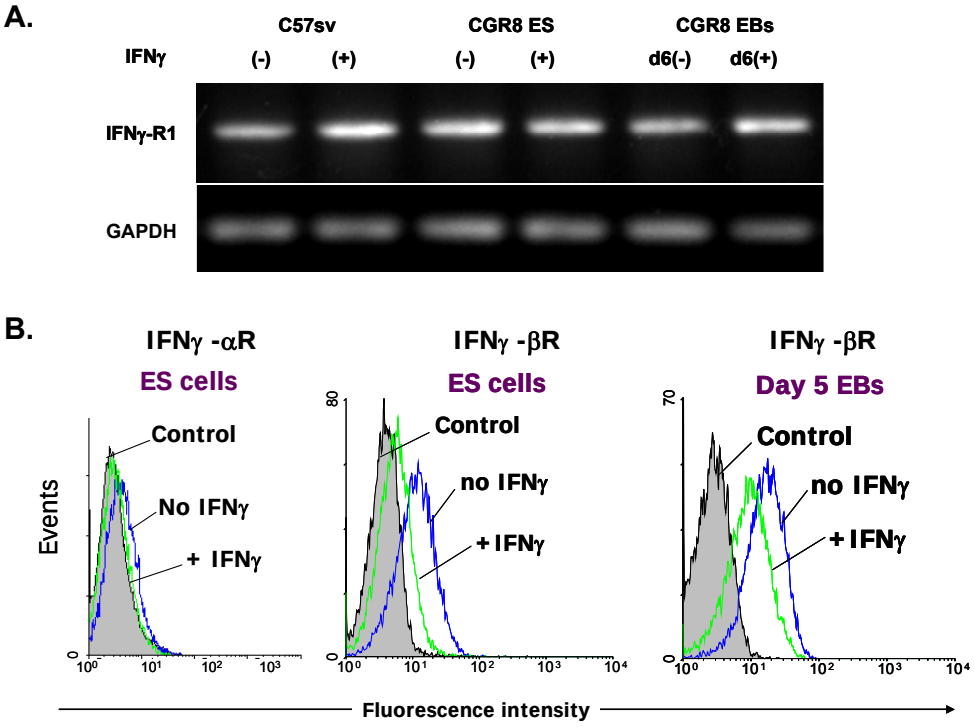


Figure 9: Expression of IFNγ receptors in undifferentiated and differentiated ES cells. A. The expression of *IFNγ-R1* in fibroblasts, CGR8 ES cells and day 6 EBs was determined by semiquantitative RT-PCR analysis (product size = 208 nt). GAPDH was used as housekeeping gene in this analysis (product size = 111). B. Flow cytometry was done to determine the protein level expression of IFNγ-αR and IFNγ-βR in αPIG-ESCs and ESC-derived EBs in the presence and absence of IFNγ (100 ng/ml for 2 days). Cells were dissociated by trypsin-EDTA and stained with monoclonal antibodies specific for IFNγ-αR (Isotype Rat IgG2a) and IFNγ-βR (Isotype ArmHam IgG).

III.1.4. Expression and activity of STAT1 in ESCs

The biological actions of IFNγ are mediated through the STAT1 signaling cascade. Binding of IFNγ to IFNGR induces activation of receptor complex which leads to the transphosphorylation of Janus-activated kinases 1 and 2 (JAK1, JAK2) that, in turn, phosphorylate STAT1 at tyrosine residue 701. Activated STAT1 dimerizes and translocates into the nucleus to induce the expression of IFNγ-inducible genes. Therefore, we asked whether lack of STAT1 expression or phosphorylation in ESCs may be the reason for their unresponsiveness to IFNγ. Real-time PCR analyses revealed that *STAT1* transcripts were expressed at much lower levels in ESCs (relative

expression=1) and day 5 EBs (relative expression=1.08) as compared to fibroblasts (relative expression=20.9) (Figure 10A). *STAT1* transcripts were upregulated by IFNγ only in fibroblasts (3.5-fold, p<0.005) and day 5 EBs (20-fold, p<0.001) but not in undifferentiated ESCs (1.4-fold, p<0.19), which is in agreement with data obtained for MHC class I molecules and other genes in this study.

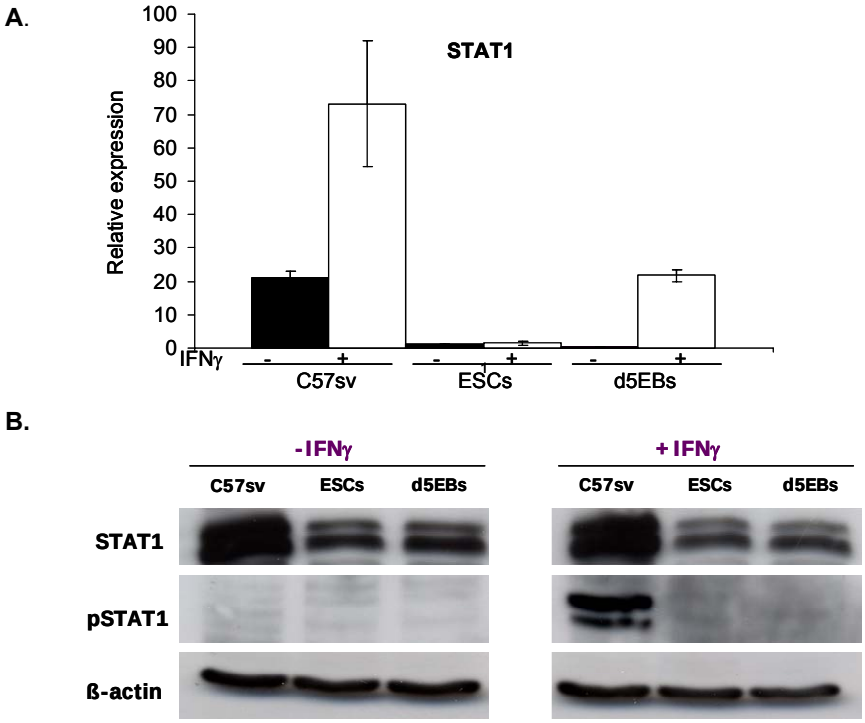


Figure 10: Expression of STAT1 at transcript and protein level in ESCs and EBs. A. C57sv fibroblasts, CGR8 ES cells and day 5 EBs were cultured with or without IFNγ (100 ng/ml for two days) and the *STAT1* gene expression was determined by real-time PCR. The *STAT1* expression in each sample was normalized with the housekeeping gene GAPDH. Data are presented by assigning the expression value of 1 to ESCs. Data were statistically analyzed using Student's t-test. Data are given as mean ± s.e.m. of three replicates. These data are representative of two independent experiments. B. C57sv fibroblasts, CGR8 ES cells and day 5 EBs were treated with or without IFNγ (100 ng/ml for 15 minutes) and the same amount of protein (15 μg) was loaded for each sample for analysis of total STAT1 and STAT1 phophorylated at tyrosine residue 701 by immunoblotting. The signal was visualized using alkaline phosphatase-based chemiluminescence detection system.

In agreement with the *STAT1* transcript levels, the total STAT1 protein was expressed at significantly higher amounts in fibroblasts than in ESCs

and EBs (Figure 10B). In the absence of IFNγ the amount of STAT1 in ESCs and EBs was comparable. In the presence of IFNγ the total STAT1 protein levels did not change in IFNγ-treated ESCs but, surprisingly, also in IFNγ-treated EBs. This was not expected because the expression of *STAT1* transcripts was strongly upregulated by IFNγ in EBs (see Figure 10A). Without IFNγ, STAT1 was not phosphorylated in any cell type analyzed (Figure 10B, left panel). IFNγ treatment induced strong phosphorylation of STAT1 only in C57sv fibroblasts but not in ES cells and day 5 EBs (Figure 10B, right panel). The inability to detect phosphorylated STAT1 in ESCs and EBs might be due to low expression levels of this protein in these cells. Therefore, with this method it is not possible to assess whether ESCs and EBs exhibit any differential STAT1 phosphorylation, which could explain the differences in MHC class I induction by IFNγ in these cells.

III.1.5. Expression of regulatory components of STAT1 signaling pathway and their activation by IFNγ in murine ESCs

The activity of the STAT1 signaling pathway is also regulated by other components of this pathway such as obove mentioned kinases JAK1 and JAK2 as well as regulators such as suppressor of cytokine signaling 1 (SOCS1), non-receptor type protein tyrosine phosphatase PTP1B (encoded by *PTPN1*) and Src homology region 2 domain-containing phosphatases 1 and 2 (SHP-1 and SHP-2 encoded by *PTPN6* and *PTPN11* genes, respectively). SHP-1 enhances STAT1 activation while SHP-2, PTP1B and SOCS1 negatively regulate STAT1 activation. The transactivating activity of STAT1 dimers is also regulated by their import from the cytosol into the nucleus through importin α. Since all these components of STAT1 signaling pathway are important for its proper activity, we checked the transcript level expression of these genes in C57sv fibroblasts, murine CGR8 ESCs and day 5 EBs. *JAK1, JAK2, SOCS1, PTP1B, SHP-1* and *SHP-2* were expressed at similar levels in all analyzed cells both in the presence and absence of IFNγ (Figure 11A).

IFNγ upregulated only the expression of JAK2 and SOCS1 in C57sv fibroblasts and day 5 EBs but not in ESCs further confirming our finding of unresponsiveness of ESCs to IFNγ.

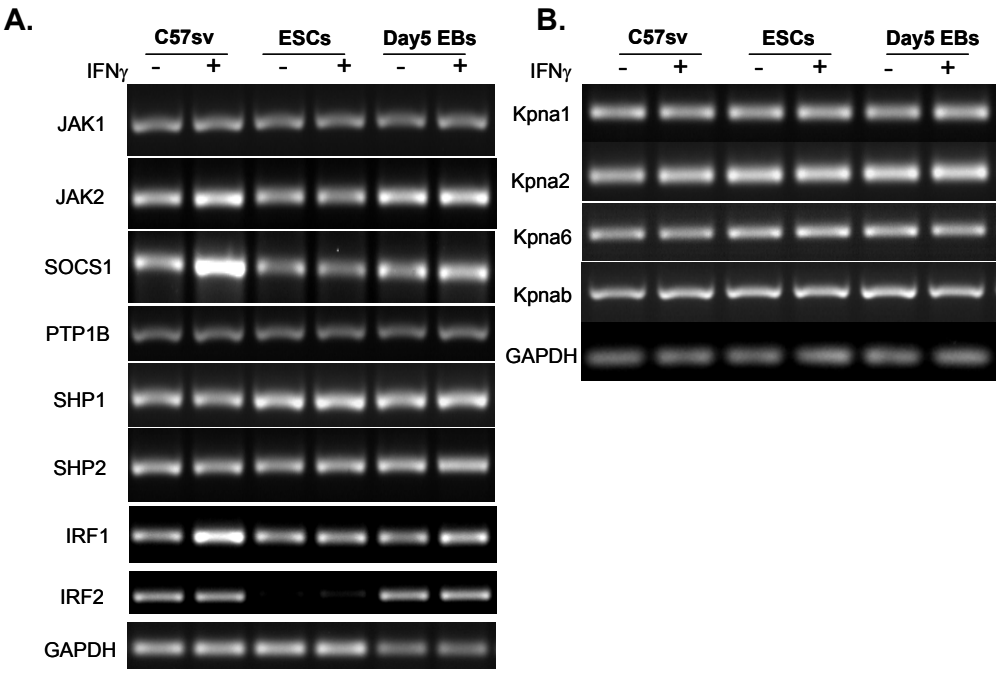


Figure 11. Expression of components related to STAT1 signaling pathway in murine ES cells. A. Expression of JAK1, JAK2, SOCS1, PTP1B, SHP1, SHP2, IRF1 and IRF2 transcripts as determined by semiquantitative RT-PCR analysis in C57sv fibroblasts, CGR8 ESCs and day 5 EBs. B. Expression of transcripts encoding for importin subunits Kpna1, Kpna2, Kpna6 and Kpnb in the same cell lines.

The IFNγ regulatory factors 1 and 2 (encoded by *IRF1* and *IRF2* genes, respectively) mediate transcriptional effects of IFNγ in STAT1 dependent or independent manner depending on a target gene. IRF-1 generally functions as a transcriptional activator or repressor of a variety of target genes that are activated by type I IFN signaling, while IRF2 is a transcriptional suppressor that competitively inhibits the IRF1-mediated transcriptional activity. IRF1 transcripts were expressed in ESCs and day 5 EBs at the same level as in C57sv fibroblasts but IFNγ induced the IRF1 expression only in fibroblasts and EBs (Figure 11A). IRF2 transcripts were expressed in day 5 EBs and fibroblasts at comparable levels and were not

regulated by IFNγ. Interestingly, IRF2 transcripts were not detectable in ESCs both in the presence or absence of IFNγ (Figure 11A). Absence of IRF2 in ESCs could be one of the reasons for unresponsiveness of ESCs to IFNγ (Rouyez et al. 2005).

The importins play a crucial role in the nuclear localization of activated STAT1 inside the nucleus to induce IFNγ regulated genes. The importin subunits α-5, α-1, α-6 (also known as karyopherin α-1, α-2, α-6) are encoded by genes *KPNA1* and *KPNA2*, *KPNA6* respectively. The importin subunit β (also known as karyopherin β) is encoded by gene *KPNB*. Activated STAT1 goes into the nucleus by importin-α5 (KPNA1) with the help of importin-β (KPNB) and it was previously reported that importin-α1 (KPNA2) switches to importin-α5 when ESCs differentiate. The *KPNA1* and *KPNB* were expressed in ESCs at levels similar to those in fibroblasts and day 5EBs. None of these genes was inducible by IFNγ in any of the cell lines tested (Figure 11B). These data suggest that the inability of IFNγ to induce the expression of IFNγ responsive genes in ESCs may not be due to lack of translocation of STAT1 dimers to the nucleus.

III.1.6. The role of LIF in control of MHC class I expression in murine ESCs and its derivatives

Pluripotency of murine ESCs is maintained by LIF, which is the standard component of murine, but not human ESC culture media. Therefore, we tested if LIF is responsible for low expression levels of MHC class I molecules on murine ES cells as compared to their human counterparts. In addition, we were interested in determining if LIF signaling through STAT3 pathway interferes with the activation of STAT1 pathway by IFNγ and in this way negatively affects the induction of MHC class I expression in murine ESCs. To test this hypothesis, ESCs were first cultured with or without LIF in the presence or absence of IFNγ and H-2K^b expression was measured by flow cytometry after 48 h of treatment. These analyses revealed that in the absence of IFNγ transient LIF removal for 2 days did not increase the expression of H-2K^b molecules on ESCs (Figure 12A).

IFNγ treatment of ESCs cultured in the absence of LIF only insignificantly enhanced the expression of these molecules (9.1% positive cells with LIF, MFI=52; 18.6% of positive cells without LIF, MFI= 61.3) (Figure 12B).

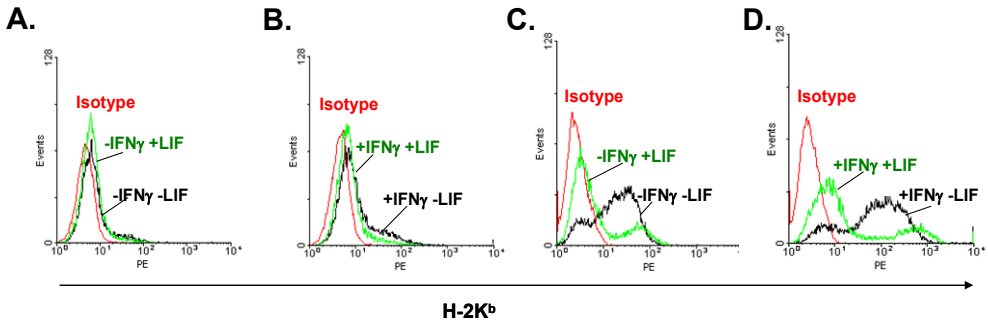


Figure 12. Regulation of MHC class I expression on murine ES cells (A,B) and EBs (C,D) by LIF in the presence and absence of IFNγ. αPIG ES cells were cultured for two days with or without IFNγ and/or LIF and the expression of H-2K^b molecules was determined by flow cytometry (A,B). To test the effect of LIF on MHC class I expression on differentiating cells, the formation of EBs was initiated for three days without LIF. LIF was then added to day 3 EBs for additional 2 days in the presence or absence of 100 ng/ml IFNγ and the expression of H-2K^b molecules was determined in EBs on day 5 of differentiation (C,D). For each sample 10000 events were acquired and all analyses were done by gating on viable cells.

The failure to detect a major effect of LIF on MHC class I expression in undifferentiated ES cells could be due to incomplete inactivation of LIF signaling pathway under these culture conditions. Therefore, ES cells that were differentiated to EBs in the absence of LIF for 5 days may represent more appropriate model to investigate the role of LIF in regulating the expression of MHC class I molecules, because the LIF pathway may be sufficiently inactivated under these conditions. To this end, we have first induced the differentiation of ES cells by allowing formation of EBs without LIF for 3 days. At day 3 of differentiation, the EBs were then divided into two groups, which were cultured for additional two days, one without and another with LIF. The expression of H-2K^b molecules was determined at day 5 of differentiation by flow cytometry. As already shown in Figure 7C, cells in day 5 EBs differentiated without LIF expressed high amounts of MHC class I molecules on their cell surface (68.9% of positive cells, MFI=30.9) and their levels were upregulated 3.7-fold (in reference to MFI) by IFNγ increasing the fraction of positive cells to 84.2% and MFI to 115.3 (Figure 12C,D). However, compared to cells cultured without LIF, EBs

treated with LIF in the absence of IFNγ showed expression of MHC class I molecules only on 26.2% cells (MFI=46), which represents a 2.6-fold reduction in reference to % positive cells cultured in the absence of LIF (Figure 12C). In addition, LIF also strongly reduced the expression of MHC class I molecules in EBs treated with IFNγ (42.3% of positive cells, MFI=95.4) (2-fold reduction in reference to positive cells without LIF) (Figure 12D). Interestingly, H-2K^b expressing day 5 EBs maintained the same MFI value even in the presence of LIF, indicating that LIF completely abrogated the MHC class I expression rather than simply decreased their levels on the surface of these cells. Since LIF mediates its effects through STAT3 pathway, these data indicate that LIF/STAT3 signaling may play a negative role in regulating MHC class I molecules not only in EBs but also in undifferentiated murine ES cells.

III.1.7. The role of STAT3 in regulation of MHC class I molecules in undifferentiated ESCs

The LIF removal experiments with EBs provided strong indication that LIF/STAT3 pathway may play an important role in keeping the expression of MHC class I molecules in ESCs at low levels and preventing their induction by IFNγ. In order to provide additional evidence for the role of this pathway in control of MHC class I expression in undifferentiated ESCs, we used STAT3-specific small interfering RNAs (STAT3 siRNA) to transiently downregulate the expression of STAT3 in these cells.

III.1.7.1. Validation of STAT3 knockdown (KD) by siRNA

To validate the efficiency of transfection of ES cells with siRNA, we used control scrambled siRNA conjugated to AlexaFLour 647 (siRNA647). Flourescence microscopy of these cells one day after lipofection showed that the great majority of cells were efficiently labeled by these molecules (Figure 13A). In the next step, the optimal siRNA concentration required for maximal STAT3 KD was determined. To this end, ES cells were treated for 48 h with different concentrations of STAT3-siRNA or control

scrambled siRNA647 and STAT3 transcript levels were analyzed by real-time PCR and immunoblotting. Compared to control cells, *STAT3* mRNA expression in ES cells treated with STAT3-siRNA was reduced by up to 80-90% at all used STAT3-siRNA concentrations (Figure 13B).

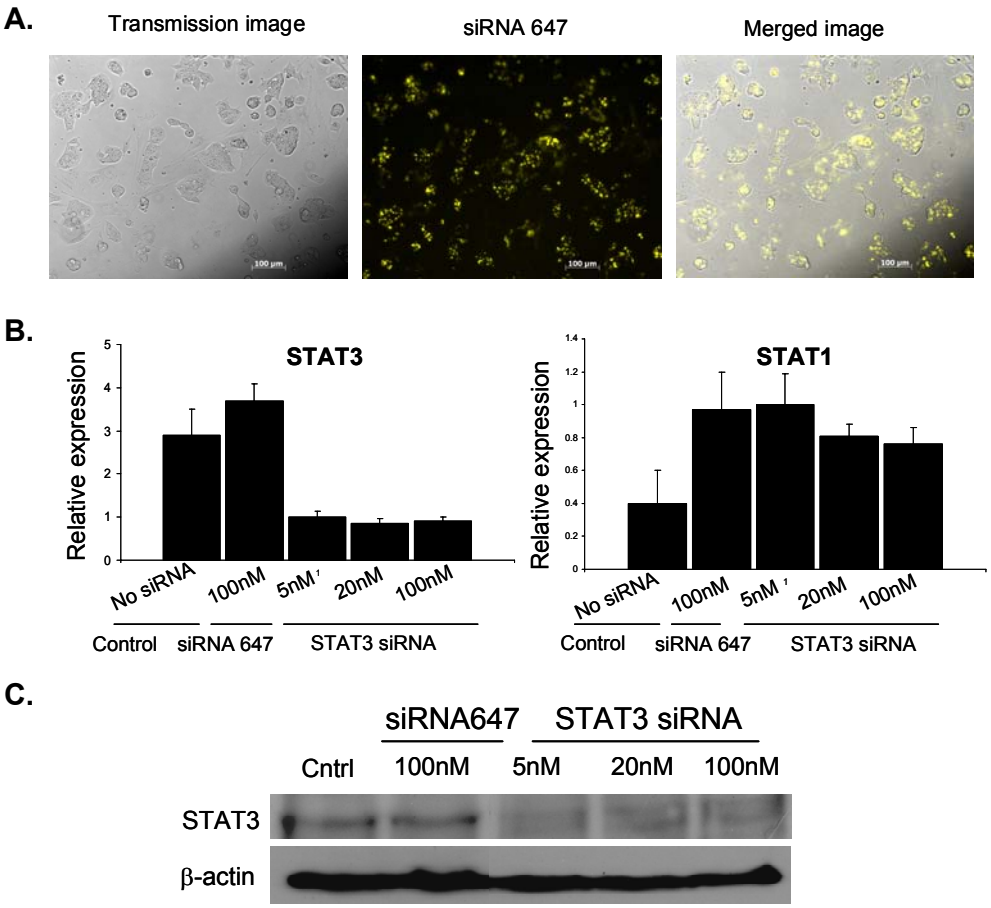


Figure 13: The validation of transient STAT3 KD in murine ES cells. A. Murine ESCs were transfected with 20 nM scrambled siRNA conjugated to AlexaFluor 647 (yellow) for 2 days. Merged image showed successful transfection of siRNA647 into murine CGR8 ESCs.. B. RNA was isolated from STAT3 KD and control murine ES cells. Real time PCR was performed using SybrGreen and primers for *STAT3* and *STAT1*. *GAPDH* was used as endogenous control. Data are given as mean ± s.e.m. of three replicates. These data are representative of two independent experiments. C. Validation of STAT3 KD by immunoblotting. The same amount of protein (15 μg) was loaded for immunoblot analysis. STAT3 polyclonal antibody was used to detect the protein level expression and β-actin antibody was used as housekeeping control.

In contrast, the expression of *STAT1* transcripts was enhanced both in siRNA647 and STAT3 siRNA treated ESCs as compared to untreated cells, suggesting a non-specific and concentration independent effect of siRNA treatment on expression of this gene in ESCs. The immunoblot

analysis showed that *STAT3* protein level was strongly dowregulated in STAT3 siRNA treated ESCs at all concentrations used and was not affected by siRNA647 compared to untreated control (Figure 13C). For all further knock down experiments we used both siRNAs at the concentration of 20 nM.

III.1.7.2. STAT3 KD ESCs maintain expression of pluripotency markers

Since LIF/STAT3 plays a major role in keeping undifferentiated state of ESCs, it was essential to determine whether the pluripotency of ESCs was affected by transient STAT3 KD. If STAT3 KD also stimulated the differentiation of ESCs, it would be more difficult to discern whether STAT3 KD itself or other processes induced by differentiation are responsible for upregulation of MHC class I molecules in these cells. Semiquantitative RT-PCR analysis showed that the core pluripotency genes *Sox2*, *Nanog* and *Oct4* were expressed at similar levels in control and STAT3 KD murine ES cells (Figure 14A). Quantitative RT-PCR assessment of *Oct4* and *Nanog* mRNA expression confirmed these data (Figure 14B). Another marker, the T-box transcription factor 3 (Tbx3), which maintains pluripotency of ES cells by PI(3)K pathway, was also not affected by transient STAT3 downregulation. However, the expression of Krueppel-like factor 4 (Klf4), which is directly regulated by LIF/STAT3 pathway, was significantly reduced in STAT3 KD ES cells (Figure 14B). In addition, cell surface marker of pluripotent murine ESCs SSEA-1 was also expressed at similar levels on both cell populations (Figure 14C). Collectively, these data suggest that STAT3 KD ESCs remain in their undifferentiated state despite the transient reduction of STAT3 levels. This may be possible because LIF signals to the core circulatory network of pluripotency not only through the STAT3/Klf4 but also the PI(3)K/Tbx3 pathway (Niwa H et al 2009), which was not affected by out experimental conditions.

III.1.7.3. STAT3 KD ESCs form teratomas at the same kinetics as control cells

Undifferentiated ESCs routinely develop into teratomas when injected into different anatomical sites of syngeneic or immunodeficient recepients. Teratoma formation by ESCs is another important proof to show ES cell's pluripotency and is an essential part of a routine quality control of ES cells propagated in cell culture. Therefore, in order to provide further evidence for the pluripotency of STAT3 KD ESCs we injected one million of control siRNA- and STAT3 siRNA-treated ESCs subcutaneously into scapular

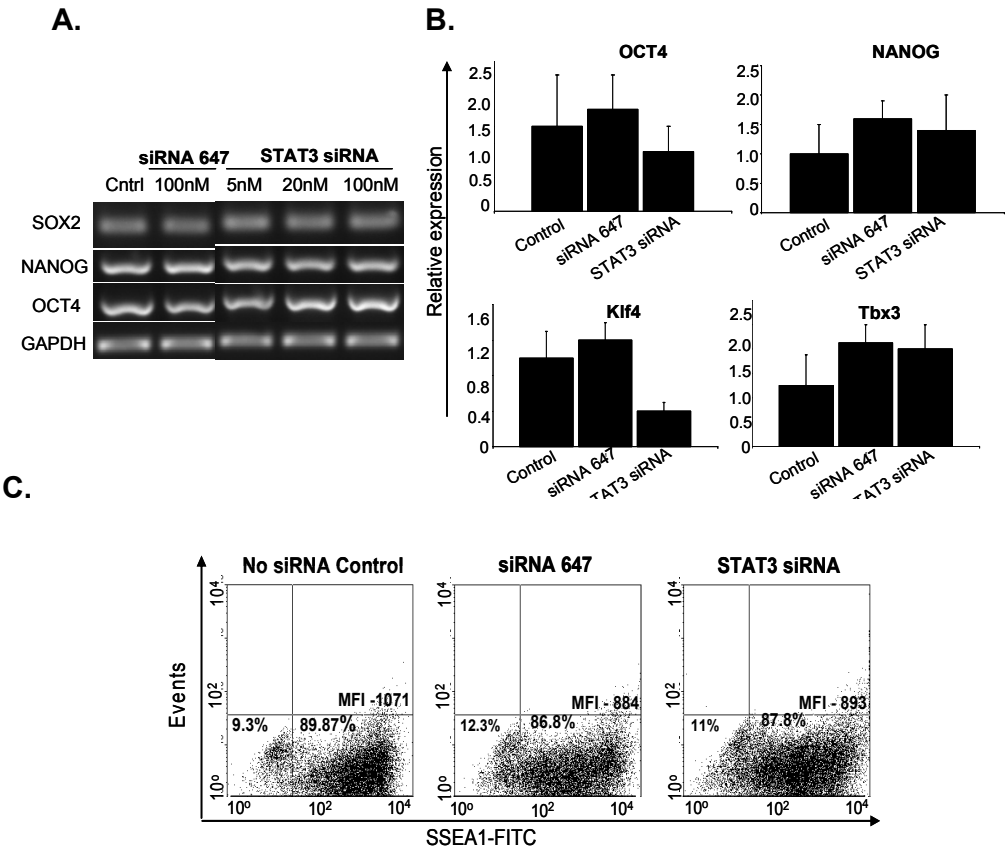


Figure 14: STAT3 KD murine ES cells maintain their pluripotency.RNA was isolated from controls and STAT3 KD ES cells. Pluripotency markers *Oct4, Nanog, Sox2*, *Klf4* and *Tbx3* were measured by RT-PCR and real time PCR analysis in control and STAT3 KD ES cells. A. RT-PCR analysis showed similar expression levels of *Oct4*, *Sox2* and *Nanog* in control and STAT3 KD ESCs. Data are given as mean ± s.e.m. of three replicates. These data are representative of two independent experiments. B. Expression of *Oct4, Nanog* and *Tbx3* were not changed (p-value> 0.05) while expression of Klf4 was changed significantly in STAT3 KD ES cells in comparison to controls (p< 0.05)..C. The expression of SSEA-1 pluripotency marker as determined by flow cytometry on control and STAT3 KD ES cells.

region of immunodeficient Rag2$^{-/-}\gamma_c^{-/-}$ mice and teratoma growth was measured over the period of one month. No significant difference was observed in the growth kinetics of teratoma derived by control and STAT3 siRNA-treated αPIG ESCs (Figure 15). Taken together, these results confirmed that transient STAT3 KD doesn't affect the pluripotency of murine ESCs and that the upregulation of MHC class I molecules on STAT3 siRNA-treated ESCs can be attributed to direct effect of STAT3 and not other processes that may be induced by differentiation of these cells.

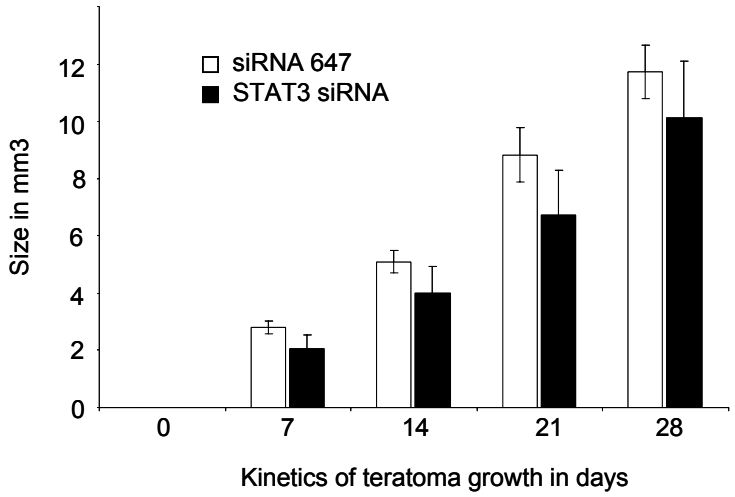


Figure 15. Teratoma growth kinetics of STAT3 KD and siRNA647 control αPIG ES cells. ESCs (1x 10^6 cells) were injected subcutaneously into immunodeficient Rag2$^{-/-}\gamma_c^{-/-}$ mice (N=3). In every mouse, both types of KD cells were injected into each side. The size of teratomas was measured every week up to 1 month. In all time points, no significant difference in growth kinetics were observed ($p>0.1$).

III.1.7.4. Effect of PI(3)K-inhibition on pluripotency of STAT3 KD ESCs

LIF acts through STAT3 and PI(3)K pathways and maintains the pluripotency of murine ESCs. Since STAT3 KD murine ESCs retained their pluripotency, we asked whether inhibition of PI(3)K pathway would compromise the expression of core pluripotency markers in ESCs so as to provide the indirect evidence for our notion that STAT3 KD ESCs maintain pluripotency througt this pathway.

To this end, STAT3 KD ESCs were treated with PI(3)K inhibitor LY294002. for 48 h and the expression of pluripotency markers *Oct4* and *Nanog* was determined by quantitative RT-PCR. As compare to control and siRNA 647 treated ESCs, *OCT4* expression was maintained but *NANOG* expression was down regulated significantly in LY294002 treated STAT3 KD ESCs (Figure 16). This data is in agreement with the fact that *NANOG* is a direct target of PI(3)K pathway. The maintenance of *Oct4* expression in LY94002 treated STAT3 KD ESCs might be due to incomplete inhibition of STAT3 and PI(3)K pathways or other mechanisms. This data suggest that the activation of the PI(3)K pathway and *Nanog* by LIF at least partially supports the undifferentiated state in STAT3 KD ESCs.

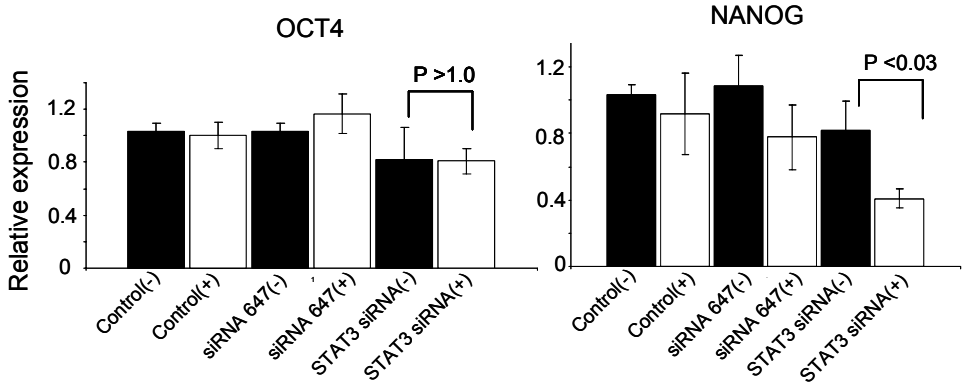


Figure 16. Effect of pluripotency in LY294002 treated STAT3 KD αPIG44 ES cells
STAT3 KD αPIG ES cells were treated with 10 μM of PI(3)K inhibitor LY294002 for 48 h. Oct4 and Nanog mRNA expression was determined by real-time PCR using SyberGreen dye. GAPDH was used as endogenous control. LY294002 treated STAT3 KD ES cells showed down regulation of Nanog ($p<0.05$) while Oct4 mRNA expression was affected neither by STAT3 KD nor by LY294002 ($p>0.05$). Black columns represent the treatment of PI(3)K inhibitor LY294002 while white columns represent no treatment of this inhibitor. Data are given as mean ± s.e.m. of three replicates. These data are representative of three independent experiments.

III.1.7.5. STAT3 KD leads to induction of H-2K^b molecules in ESCs

The analyses described above strongly suggest that the inability to induce H-2K^b molecules by IFNγ in murine ESCs might be due to active LIF/STAT3 pathway. To determine if this is the case, STAT3 gene was transiently downregulated in ES cells, which were cultured either in the presence or absence of IFNγ. Using real-time PCR analysis we found that in comparison to no-siRNA- and scrambled siRNA-treated cells, the STAT3 KD significantly increased the levels of *H-2K^b* and *β2M* transcripts

both in IFNγ-untreated and IFNγ-treated cells (Figure 17A). Compared to control siRNA treated cells, STAT3 KD increased the expression of MHC class I α and β chain mRNAs in IFNγ-treated cells, respectively, by 1.9- and 1.6-fold, and in IFNγ-treated cells by 2.4- and 2.5-fold (Figure 17A).

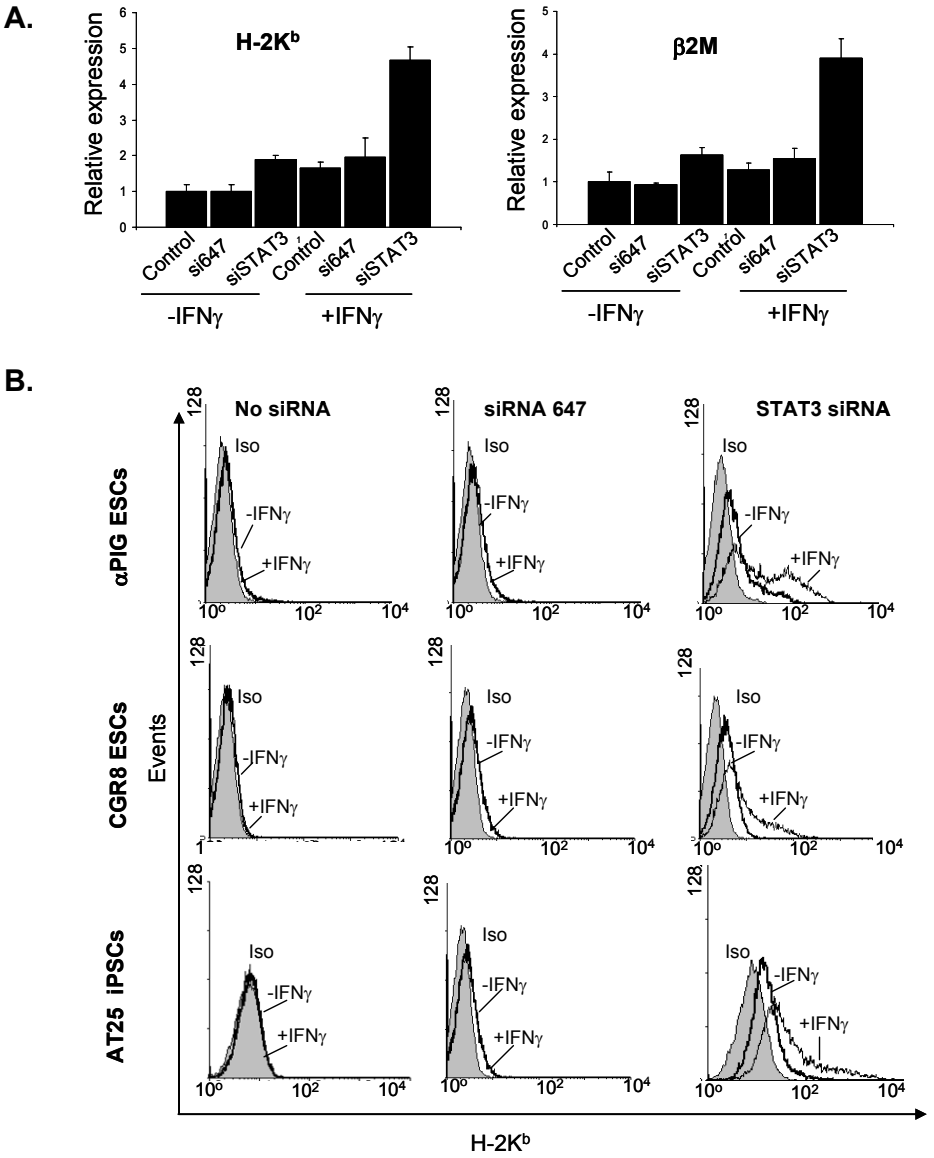


Figure 17. Transient STAT3 KD leads to the induction of H-2K^b in murine ESCs. A. The expression of H-2K^b and β2M at transcript level was determined by real time PCR in in control and STAT3 KD CGR8 ESCs cultured in the presence or absence of IFNγ (100 ng/ml). GAPDH gene was used as internal control and the expression in control ESCs was set at value of 1. Data are given as mean ± s.e.m. of three replicates. These data are representative of three independent experiments. B. The expression of H-2K^b molecules on αPIG and CGR8 ESCs and AT25 iPSCs was determined by flow cytometry. Cells were treated for 2 days (100 ng/ml) with or without IFNγ and indicated siRNAs. Cells were stained with the indicated antibodies or with the corresponding isotype controls (gray shaded histograms). All histograms were generated by gating on viable cells.

To determine whether the STAT3 KD mediated increase of *H-2K^b* and *β2M* transcript levels also lead to higher expression of mature MHC class I molecules on the cell surface, STAT3 KD ES cells were stained with monoclonal antibody specific for H-2K^b molecules and analyzed by flow cytometry. STAT3 KD increased the fraction of H-2K^b-positive αPIG ESCs 5.9-fold to 23.6% (MFI=19.3) as compared to control siRNA treatment yielding only 4.0% positive cells (MFI=13.2) (Figure 17B, top row). Importantly, the fraction of H-2K^b–positive ESCs further increased to 62.0% (MFI=41.1) by treatment with IFNγ.

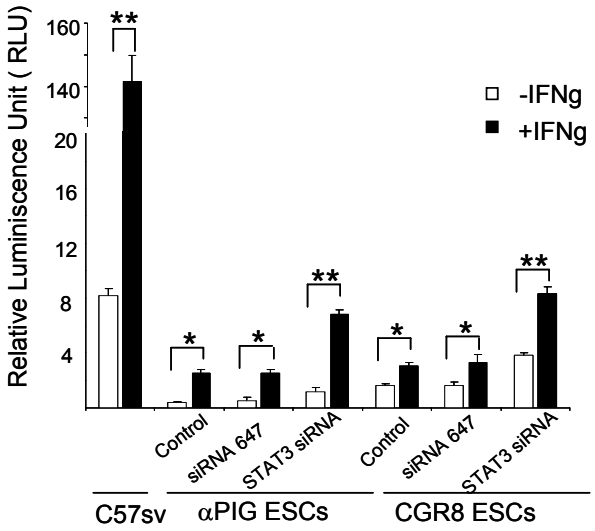
STAT3 KD CGR8 ESCs also increased 2.7-fold the fraction of H-2K^b positive cells (11%, MFI=10.7) as compared to control siRNA647-treated cells (4%, MFI= 10.5) (Figure 17B, middle row). Like in αPIG ESCs, the H-2K^b expression was further induced on 42.8% of CGR8 ESCs (MFI=23.7) by treatment with IFNγ. Additionally, STAT3 KD AT25 iPSCs also showed 24.4% enhancement of H-2K^b positive cells (MFI=26.6) as compared to control si RNA 647 treated cells (5.6% positive cells with MFI= 44), and this was further increased by addition of IFNγ (46.3%, MFI=41.4). No change in H-2K^b expression was observed in control or scrambled siRNA-treated iPSCs (Figure 17B, bottom row). These analyses clearly demonstrate that STAT3 plays an important role in regulation of H-2K^b molecules in ES and iPS cells and both in the presence and absence of IFNγ.

III.1.7.6. STAT3 KD enhances STAT1 activity in IFNγ treated and untreated ESCs

IFNγ is a chief cytokine that phosphorylates STAT1 leading to its dimerization, translocation into the nucleus and the activation of a number of IFNγ inducible genes including the MHC class I α and β molecules. Transcriptionally active STAT1 dimers recognize specific DNA sequences, called Interferon-Gamma Activated Sites (GAS), in promoter regions of Interferon Stimultaed Genes (ISGs). To determine the transcriptional activity of STAT1, we performed the dual luciferase reporter assay system where we transiently co-transfected the firefly and renilla luciferase vectors

into C57sv fibroblasts, and control and STAT3 KD αPIG and CGR8 ESCs. Firefly luciferase activity (encoded by *luc* gene) was under the control of GAS promoter while constitutive renilla luciferase activity (encoded by *hRluc* gene), was under the control of ubiquitious CMV promoter used as internal control.

A.



B.

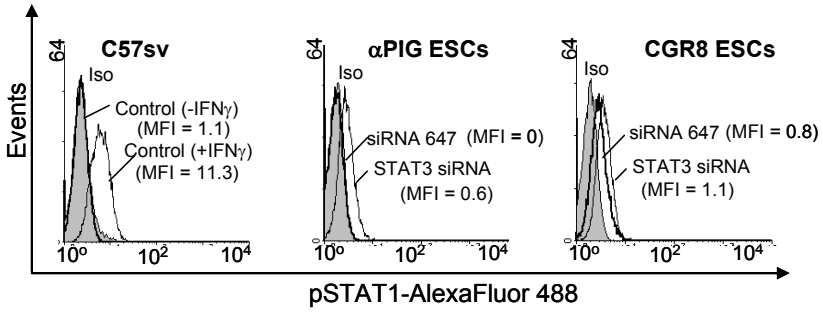


Figure 18. Effect of STAT3 KD on transcriptional activity and phosphorylation of STAT1 gene in murine ESCs. A. Luciferase reporter assay was performed using IFNγ responsive GAS element. The C57sv fibroblasts and ESCs were transiently transfected with vectors in which firefly luciferase was under the control of GAS promoter and renilla firefly luciferase under the control of CMV promoter. In parallel, cells were treated with or without indicated siRNAs After 48hrs, the ESCs were treated with 100 ng/ml IFNγ for 6 hrs and the luciferase activity was measured in luminometer. .The asterisk (*) indicates a p value < 0.01 and asterisks (**) indicate a p-value< 0.001. Data are given as mean ± s.e.m. of three replicates. B. Intracellular staining was used to detect the phosphorylation of STAT1 at the tyrosine residue 701 in C57sv fibroblasts, control and STAT3KD ESCs. Cells were fixed in cold methanol, stained with phopho-STAT1 antibody conjugated to AlexaFluor488 and measured by flow cytometry.

In C57sv fibroblasts, that were used as our reference cell line, IFNγ induced 17.3-fold the low constitutive transcriptional activity of STAT1 by increasing the luminescence intensity from 8.1 relative luminescence units

(RLU), in untreated cells to 139.8 RLU in IFNγ-treated cells (n=3, p<0.001) (Figure 18A).

In IFNγ-untreated control ESCs, the transcriptional activity of STAT1 was very low (o.38 RLU in αPIG ESCs and 1.6 RLU in CGR8 ESCs) (Figure 18A). STAT3 KD slightly increased its transcriptional activity by about 2-fold (1.1 RLU in αPIG ESCs and 3.8 RLU in CGR8 ESCs). However, in IFNγ-treated STAT3 KD αPIG and CGR8 ESCs the STAT1 was trancriptionally more active reaching the values similar to those measured in siRNA- and IFNγ-untreated C57sv fibroblasts (6.8 RLU in αPIG and 8.3 RLU in CGR8 ESCs as compared to 8.1 RLU in C57sv fibroblasts) (Figure 18A). In untreated or siRNA647-treated control cells IFNγ enhanced the binding of STAT1 to GAS (2.5 RLU in siRNA647 treated αPIG ESCs and 3.3 RLU in CGR8 ESCs, p<0.01), but this was presumably not sufficient for any significant induction of H-2K^b molecules on the cell surface (compare to Figure 17B). These data demonstrate that STAT3 is an important regulator of STAT1 transcriptional activity in ESCs and that STAT3 in ESCs prevents, at least in part, the induction of MHC class I molecules by IFNγ by blocking STAT1 activation.

To provide experimental evidence for this conclusion, we used flow cytometry to measure the phosphorylation of STAT1 at residue 701 in our cell lines after short exposure to IFNγ. We found that STAT1 was phosphorylated only in IFNγ-treated C57sv fibroblasts (MFI=11.1) but not in untreated cells (MFI=1.1) (Figure 18B). In agrrement with our luciferase reporter assay, STAT3 KD led to the STAT1 phosphorylation in IFNγ-treated αPIG ESCs (MFI=0.6) and CGR8 ESCs (MFI=1.1) but not in IFNγ-untreated αPIG ESCs (MFI=0) and CGR8 ESCs (MFI=0.8) (Figure 18B). In conclusion, these results indicate that STAT3 signaling has negative impact on STAT1 activity thus preventing the efficient induction of MHC class I molecules by IFNγ.

III.1.7.7. STAT3 KD downregulates the expression of suppressor of cytokine signaling 3 (SOCS3) in ESCs

The members of the suppressor of cytokine signaling (SOCS) family play an important role in regulating the activity of STATs. SOCS1 negatively regulates the STAT1 while SOCS3 negatively regulates the STAT3. It was previously reported that STAT3 induced SOCS3 plays negative role in the activation of STAT1 (Hong et al. 2002). Therefore, we used quantitative RT-PCR to determine if STAT3 KD affects *SOCS1* and *SOCS3* mRNA levels in ESCs. *SOCS1* mRNA expression was reduced by 33% without statistical significance ($p<0.06$, n=3). In contrast, *SOCS3* mRNA expression was statistically significantly downregulated in STAT3 KD ESCs by 50% ($p<0.05$, n=3) (Figure 19). These findings raise the possibility that blockade of STAT1 phosphorylation by STAT3 is mediated by SOCS3 in murine ESCs.

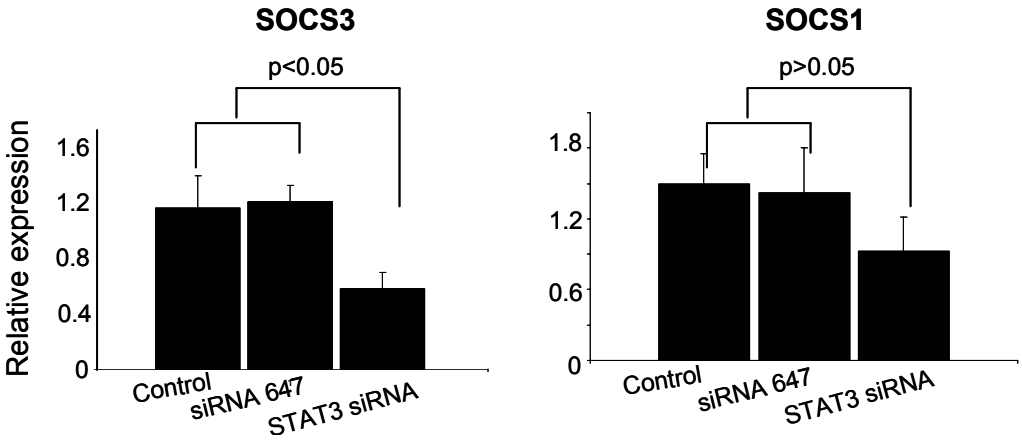


Figure 19. Effect of STAT3 KD on expression of suppressor of cytokine signaling -1 and 3 (SOCS1 and 3) mRNAs in murine CGR8 ESCs. The expression of SOCS1 and SOCS3 transcripts was determined by real time RT-PCR. *SOCS3* mRNA was significantly downregulated ($p<0.05$, n=3) while *SOCS1* was not significantly changed in STAT3 depleted ES cells ($p>0.05$, n=3, number of independent experiments =2). Data are given as mean ± s.e.m. of three replicates.

III.2. Epigenetic regulation of MHC class I molecules in murine ESCs

The downregulation of STAT3 leads to increase of MHC class I expression also in the absence of IFNγ. Therefore, this effect of STAT3 is not mediated through STAT1 signaling, but rather through direct effect of STAT3 on transcription of genes for MHC class I molecules or genes encoding epigenetic modifiers, which then indirectly effect the expression of MHC class I (Suarez-Alvarez et al. 2010). Therefore, we decided to check the expression of epigenetic modifier genes in STAT3 KD ESCs and the expression level of MHC class I molecules and their response to IFNγ in Dnmt KO ESCs. Addtionally, the effect of 2,5-aza and TSA (potent epigenetic modifiers) were checked on the expression of MHC class I molecules and their response to IFNγ in murine ESCs.

III.2.1. STAT3 regulates the expression of epigenetic modifier genes in ESCs

Dnamt1 is the most abundant methyltransferase in mammals and plays a major role in establishment and regulation of tissue specific patterns of methylated cytosines residues. Eed1 is a member of polycomb (PcG) protein family. It interacts with two other proteins (immunodeficiency virus type 1 (HIV-1) protein and histone deacetylase proteins). This complex serves as a platform to recruit DNA methyltransferases. JmjD1 is a jumonji-domain containing protein which encodes lysine-specific demethylase. This enzyme is supposed to be involved in posttranslational histone modifications.

Since Dnamt1, Eed1 and JmjD1 are target genes of STAT3 (Zhang et al. 2006; Ura et al. 2008; Bourillot et al. 2009), we asked whether their expression in ESCs was affected by transient STAT3 KD. *Dnamt1*, *Eed1* and *JmjD1* mRNAs were all expressed at lower levels in STAT3 KD ES cells. However, only *Eed1* was significantly downregulated in comparison to control ESCs (Figure 20). This data suggests that in ESCs STAT3 maintains the promoter regions of MHC class I α and β chain genes in

highly methylated state through recruitment of DNA methyltransferases thus preventing their efficient transcription.

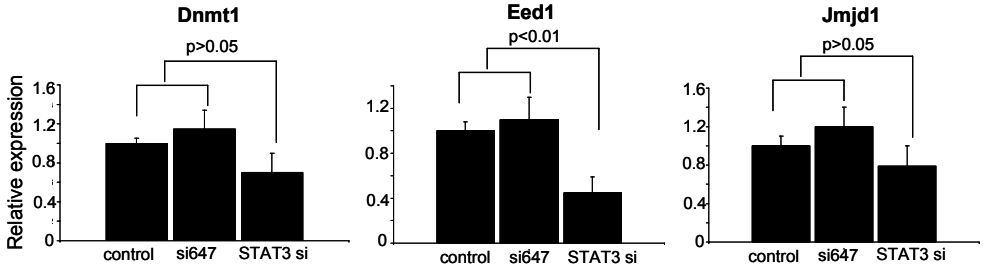


Figure 20. Expression of epigenetic modifiers in STAT3 KD ES cells. RNA was isolated from controls and STAT3 KD CGR8 ES cells and cDNA was amplified with the same amount of RNA. Real time PCR analysis was performed using primers specific for indicated genes. Data are given as mean ± s.e.m. of three replicates. These data are representative of two independent experiments.

III.2.2. Dnamt knockout murine ES cell lines show enhanced MHC class I expression

DNA methyltransferase Dnamt-1, Dnamt-3A and Dnamt-3B is a group of three enzymes, which take part in the methylation of DNA and are present in all mammalian cells. Dnamt-1 knock out (KO), Dnamt-3 (KO), Dnamt-all KO ES cell lines, in which all three enzymes are missing, were obtained from Prof. Hans R. Schöler (Max Planck Institute for Molecular Biomedicine, Münester). These cell lines were originally generated in Prof. Rudolf Jaenisch group, Whitehead Institute, MIT, USA. Wild type E14 and OG2 murine ES cell lines were also obtained from Prof. Schöler group ans served as controls. All Dnamt mutant ES cells were viable and contained a small percentage of methylated DNA and methyltransferase activity (Li et al. 1992). The OG2 and Dnamt-all KO ES cell lines express GFP under the Oct4 promoter, which is convenient for visualizing the pluripotent ESCs.

Flow cytometric analysis of these cells cultured in the presence of LIF showed that H-2K^b molecules in Dnamt-1 KO (17.7%), Dnamt-3 (20.2%) and Dnamt-all KO (21.2%) ES cell lines were expressed at significantly higher fraction of cells compared to wild type E14 (1.1%) and OG2 (3.5%) ESCs. Dnamt-1, 3 and all KO cells stimulated with IFNγ exhibited only

slightly higher enhancement of H-2K^b expression compared to untreated KO cells (25.3%, 25.8% and 23.2%, respectively) (Figure 21). No induction of H-2K^b molecules was observed in E14 and OG2 ES cell lines, in which, respectively, only 2.8% and 3.5% of cells expressed H-2K^b molecules. These data indicate that Dnamt are only partially responsible for regulation of MHC class I expression and that they are not essential for their induction by IFNγ in ESCs cultured in the presence of LIF. Most likely, other mechanisms or chromatin modifying factors are responsible for maintaining MHC class I expression at low levels in undifferentiated ESCs.

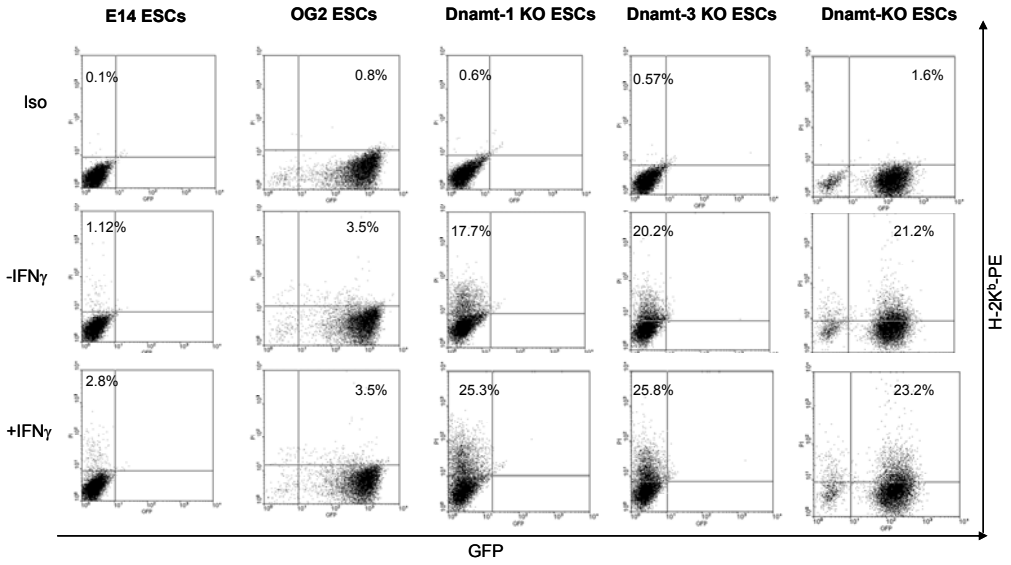


Figure 21. Expression of MHC class I molecule in Dnamt knockout murine ES cell lines. Dnamt-1, Dnamt-3 and Dnamt-all knockout (KO) and wild type E14 and OG2 murine ES cell lines were used to determine the expression of cell surface H-2K^b molecules in the presence or absence of IFNγ (100 ng/ml for 2 days). OG2 and Dnamt-all KO cells are transgenic lines that express EGFP under the control of Oct4 promoter. Analysis was performed by flow cytometry using H-2K^b-specific monoclonal antibody.by gating on viable cells. Data are given as means ± SEM of at least two independent biological experiments.

III.2.3. Epigenetic modifiers 2,5-azacytidine and Trichostatin A increase the expression of MHC class I molecules in ESCs

2,5-Azacytidine (2,5-aza) is a potent inhibitor of DNA methyltransferases leading to the reduction of DNA methylation. Demethylation activates genes by preventing gene silencing mechanisms. Trichostatin A (TSA) is used as an antifungal antibiotic and inhibits histone deacetylase (HDAC) family of enzymes. 2,5-aza and TSA act in a non-specific manner.

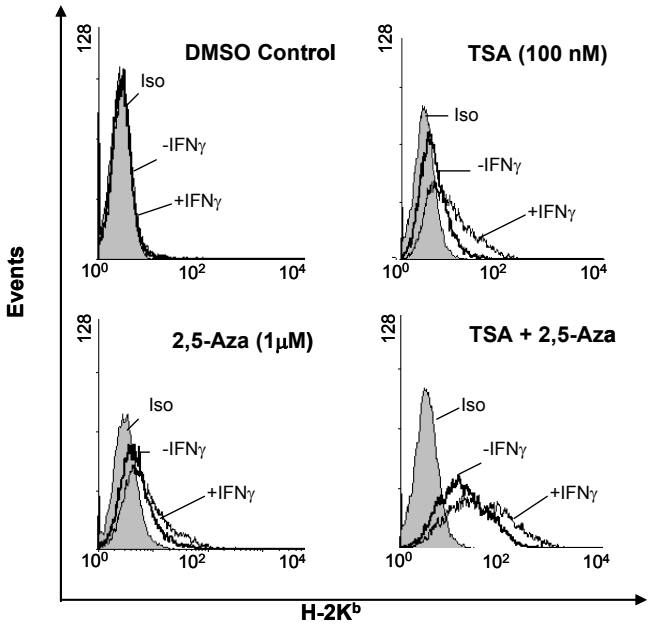


Figure 22. Synergistic effect of Trichostatin A and 2,5-Azacytidine on MHC class I induction in murine ES cells. CGR8 ESCs were treated with TSA (100 nM) and 2,5-aza (1 μM) individually and together for 2 days without or with IFNγ (100 ng/ml). Control cells were treated with DMSO because TSA and 2,5-aza were dissolved in DMSO. After 2 days of treatment, cells were stained with the MHC class I antibodies and proper isotype controls (grey shaded histogramsand analyzed by flow cytometry by gating on viable cells. Data are representative of at least two independent biological experiments.

To determine the effect of these agents on the expression of MHC class I molecules in ESCs, we treated the CGR8 ES cells with 2,5-aza (1 μM) and TSA (100 nM) separately or in combination for 2 days in presence or absence of 100 ng/ml IFNγ. Flow cytometric analysis demonstrated that the fraction of H-2K^b positive ESCs was slightly increased when cells were treated with TSA alone (12.5%, MFI=14.1) and 2,5 aza alone (16.0%; MFI=18.9) (Figure 22). In combination with IFNγ, TSA induced the H-2K^b expression on 42.8% of cells (MFI=21.9) and 2,5 Aza on 32.7% of cells (MFI=24.5). Control cells treated with DMSO did not express H-2K^b molecules above the background levels irrespective of IFNγ treatment. Interestingly, treatment of ESCs with both 2,5-aza and TSA resulted in even stronger induction of MHC class I molecules in the absence (58.82%, MFI=26.6) as well as in the presence of IFNγ (74.49%, MFI=45.1) (Figure 22). These results suggest that epigenetic mechanisms involving both

DNA methylation and histone deacetylation participate in regulation of MHC class I molecules in ESCs.

III.3. Biological consequences of STAT3 KD in ESCs

Previous studies reported that ESCs were killed by NK cells and they were resistant to killing by cytotoxic T cells *in vitro* (Dressel et al. 2010; Drukker et al. 2002; Abdullah et al. 2007). In our results, we showed that STAT3 KD leads to the induction of MHC class I molecules in murine ESCs without changing their pluripotency. Since MHC class I molecules play an important role in modulating killing by NK cells and CTLs, we checked the susceptibility of STAT3 KD murine ESCs towards NK cells and CTLs in syngeneic settings.

III.3.1. Downregulation of STAT3 in murine ESCs reduces their lysis by syngeneic NK cells

According to "missing self" hypothesis a very low or no expression of MHC class I molecules on target cells allows activation of NK cells by interaction with their activating ligands and leads to lysis of such target cells (Karre 2008). We have earlier demonstrated that murine ES cells expressing very low levels of MHC class I molecules, were lysed by NK cells because they express the activating NKG2D ligands (Frenzel et al. 2009). Previous *in vivo* studies also demonstrated that low MHC class I expression and NK cells play vital role in controlling the survival of vascular progenitors derived from ES cells after syngeneic transplantation (Ma et al 2009). Therefore, it is expected that increasing the expression of MHC class I molecules on ES cells would reduce their killing by NK cells. Indeed, using a ^{51}Cr-release cytotoxicity assay we found that upregulation of MHC class I molecules on ES cells after STAT3 KD significantly diminished (more than 50%) the lysis on αPIG ESCs (Figure 23A) and CGR8 ESCs (Figure 23B) by poly(I:C) activated NK cells. YAC cells that were used as a positive control were lysed up to 80% at E:T ratio of 1:100 while C57sv fibroblasts serving as a negative control were not lysed by activated NK

cells. These data indicate that STAT3 maintains the expression of MHC class I molecules in native ES cells at sufficiently low levels to permit their efficient killing by NK cells.

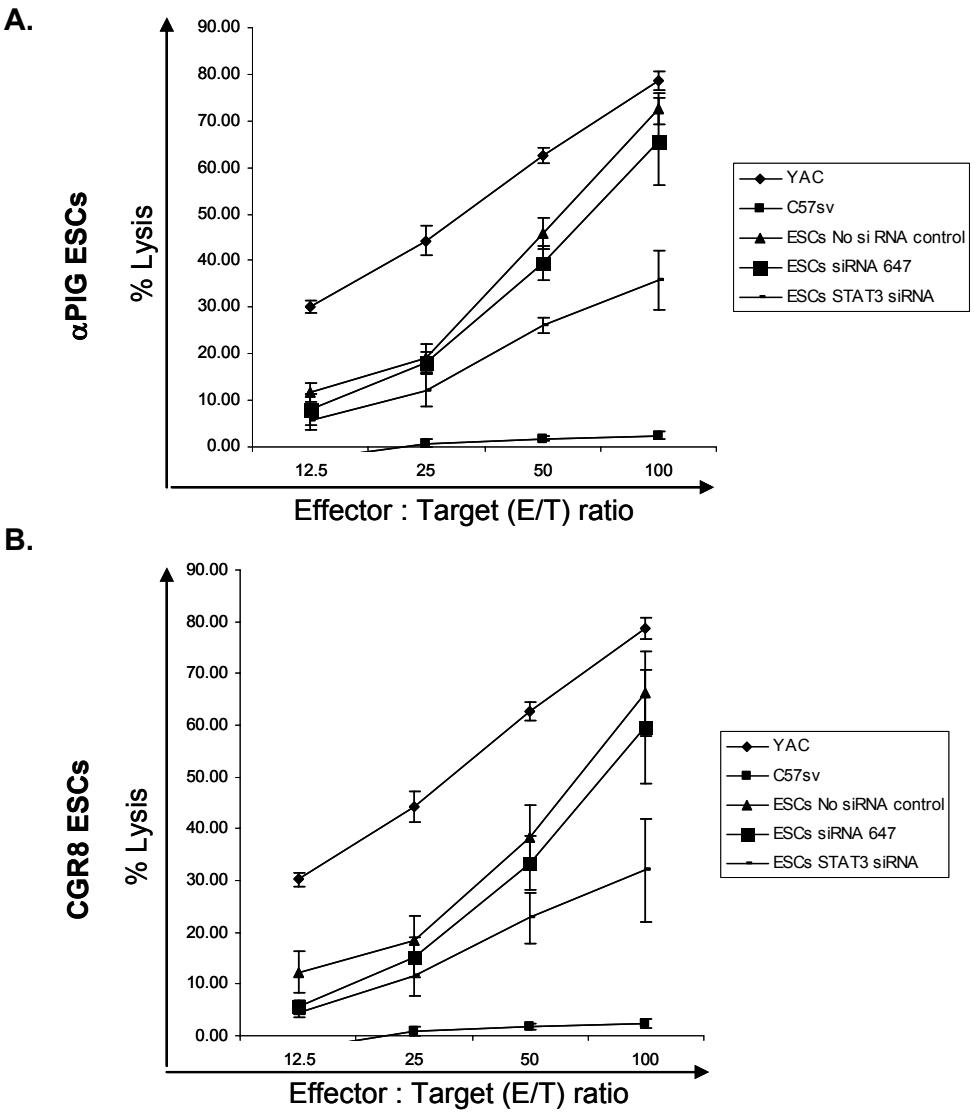


Figure 23. STAT3 KD murine ES cells show reduced susceptibility to lysis by activated syngeneic NK cells *in vitro*. NK cell cytotoxicity assay was performed with STAT3 KD aPIG (A) and CGR8 (B) ES cells. In comparison to wild type and mock siRNA-treated controls, both STAT3 KD murine ES cell lines showed approximately 50% reduced lysis. NK-sensitive YAC cell line was used as a positive control and murine C57sv fibroblasts served as a negative control in this assay. Data are given as means ± SEM of at least three independent biological measurements.

III.3.2. STAT3 partially inhibits the lysis of murine ESCs by CTLs

The expression of H-2K^b molecules on ESCs at very low levels might be the reason for their relative protection from killing by CTL. To determine whether the upregulation of H-2K^b molecules by STAT3 KD in ES cells enhances their lysis by CTLs, ovalbumin (OVA)- expressing transgenic aPIG-CytOVA (clone A4) ESCs and C57sv-CytOVA fibroblasts were generated to serve as targets for cytotoxic syngeneic T cells in an *in vitro* cytotoxicity assay. OVA-specific effector T cells were generated by immunizing the OT1 transgenic mice with OVA protein and subsequent stimulation of splenocytes *in vitro* by synthetic ovalbumin-derived immunodominant SIINFEKL peptide. OT1 mice express on all T cells a T cell receptor, which recognizes the ovalbumin-derived T cell epitope SIINFEKL in a complex with H-2K^b MHC class I molecules.

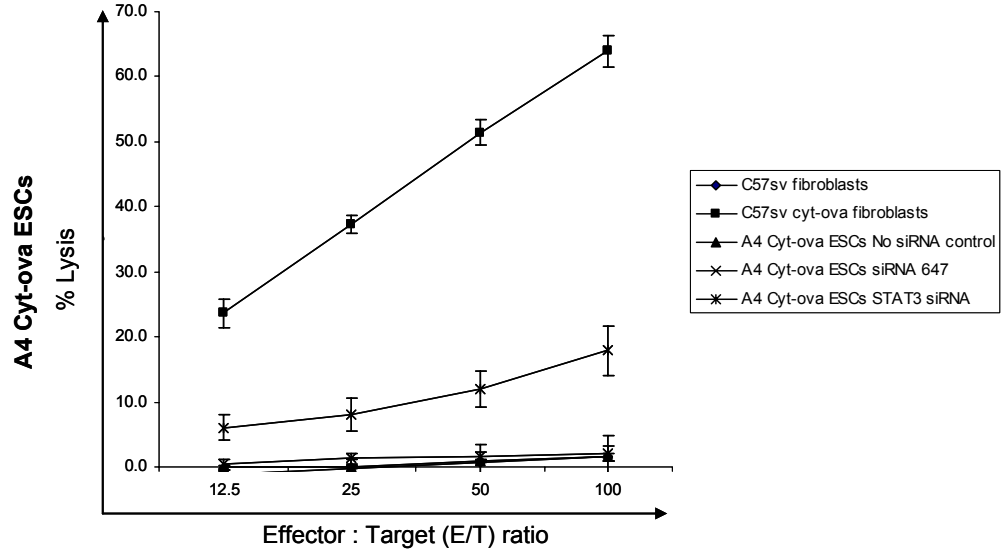


Figure 24. STAT3 KD in murine ES cells enhances their lysis by syngeneic cytotoxic CD8$^+$ T lymphocytes *in vitro*. Ovalbumin (OVA)-specific cytotoxicity assay was performed as described in the Methods section. OVA–expressing ES cells (clone A4) and C57sv fibrobalsts were generated to stably express OVA in the cytosol. Wild type murine C57sv fibroblasts served as negative control. Data are given as means ± SEM of at least three independent biological measurements.

Mouse C57sv fibroblasts that did not express OVA were not lysed by OT-1 CTLs. In contrast, C57sv fibroblasts expressing OVA were strongly lysed (63.9% lysis at the E:T ratio of 100) (Figure 24). In contrast, control and

scrambled siRNA treated ES cells were not lysed by CTLs at any E:T ratio. However, after STAT3 KD the OVA-expressing ES cells were moderately lysed (17.9% lysis at the E:T ratio of 100), (Figure 24). These data indicate that STAT3 pathway plays an important role in regulating the susceptibility of ES cells to CTL-mediated lysis by downregulating the expression of MHC class I molecules.

III.4. Regulation of MHC class I expression in ES cell-derived cardiomyocytes (ES-CMs)

ES-CMs were used as a model for differentiated cells with the aim to determine to what extent the regulatory mechanisms of MHC class I in ESCs have changed in the course of differentiation and diversification of cell types**.** That is why, we decided to KD STAT3 gene in ES-CMs and checked the expression of MHC class I molecules in these differentiated cell-types.

III.4.1. STAT3 KD in ES-CMs does not induce the expression of MHC class I molecules on their surface

We have previously shown that, like undifferentiated ESCs, differentiated murine ES-CMs express flow cytometrically undetectable amounts of MHC class I molecules on their surface (Frenzel et al. 2009). To elucidate whether STAT3 plays a role in regulation of H-2K^b expression in ES-CMs, CMs were treated with STAT3 siRNA for 48 h and analyzed for the expression of MHC class I molecules at the transcript and protein level. Quantitative RT-PCR analysis confirmed that *STAT3* mRNA expression in ES-CMs was strongly reduced by 85% in STAT3 siRNA treated cells as compared to control siRNA-treated cells (Figure 25A). In contrast, the expression of MHC class I heavy chain and STAT1 mRNA was not affected by any of these treatments (Figure 25A). Flow cytometric analysis revealed that H-2K^b molecules could not be detected on intact CMs and that no induction of these molecules could be elicited by treatment with STAT3 siRNA or scrambled siRNA647 (Figure 25B). These findings indicate that mechanisms responsible for the maintenance of low

expression levels of H-2K^b molecules diversified in the course of differentiation from undifferentiated ESCs to highly specialized cells, such as CMs.

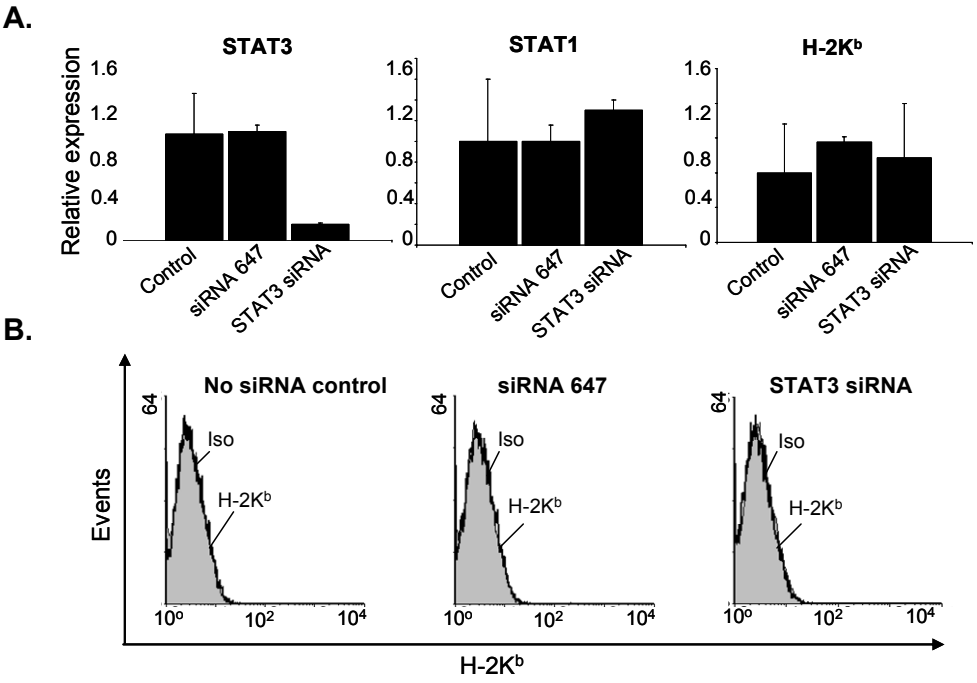


Figure 25. The effect of STAT3 KD on expression of MHC class I molecules in murine ES cell-derived cardiomyocytes (CMs). A. RNA was isolated from STAT3 KD day 16 CMs and control cells cultured in the absence of IFNγ. Real-time PCR analysis was performed for STAT3, STAT1 and H-2K^b genes. The expression levels were shown relative to siRNA647-treated ESCs, which was set to value of 1. GAPDH gene was used as internal control. Data are given as mean ± s.e.m. of 6 replicates. These data are representative of two independent experiments. B. Flow cytometric analysis was performed using H-2K^b specific antibody with control, siRNA647 and STAT3 siRNA KD day16 CMs. This analysis was performed in two independent experiments.

III.4.2. Effect of IFNγ on expression of components of MHC class I antigen processing machinery in ES-CMs

We have previously shown that, unlike undifferentiated ESCs, ES-CMs are highly responsive to IFNγ and highly upregulate the MHC class I molecules after stimulation with this cytokine (Frenzel et al. 2009). Here we extend these data and show that in addition to transcripts for MHC class I α and β chains, IFNγ also stongly upregulates the expression of transcripts encoding various components of antigen processing machinery such as

LMP2, *LMP7*, *TAP1* and *TAP2* (Figure 26). As expected, these transcripts were not affected by IFNγ in undifferentiated αPIG ESCs.

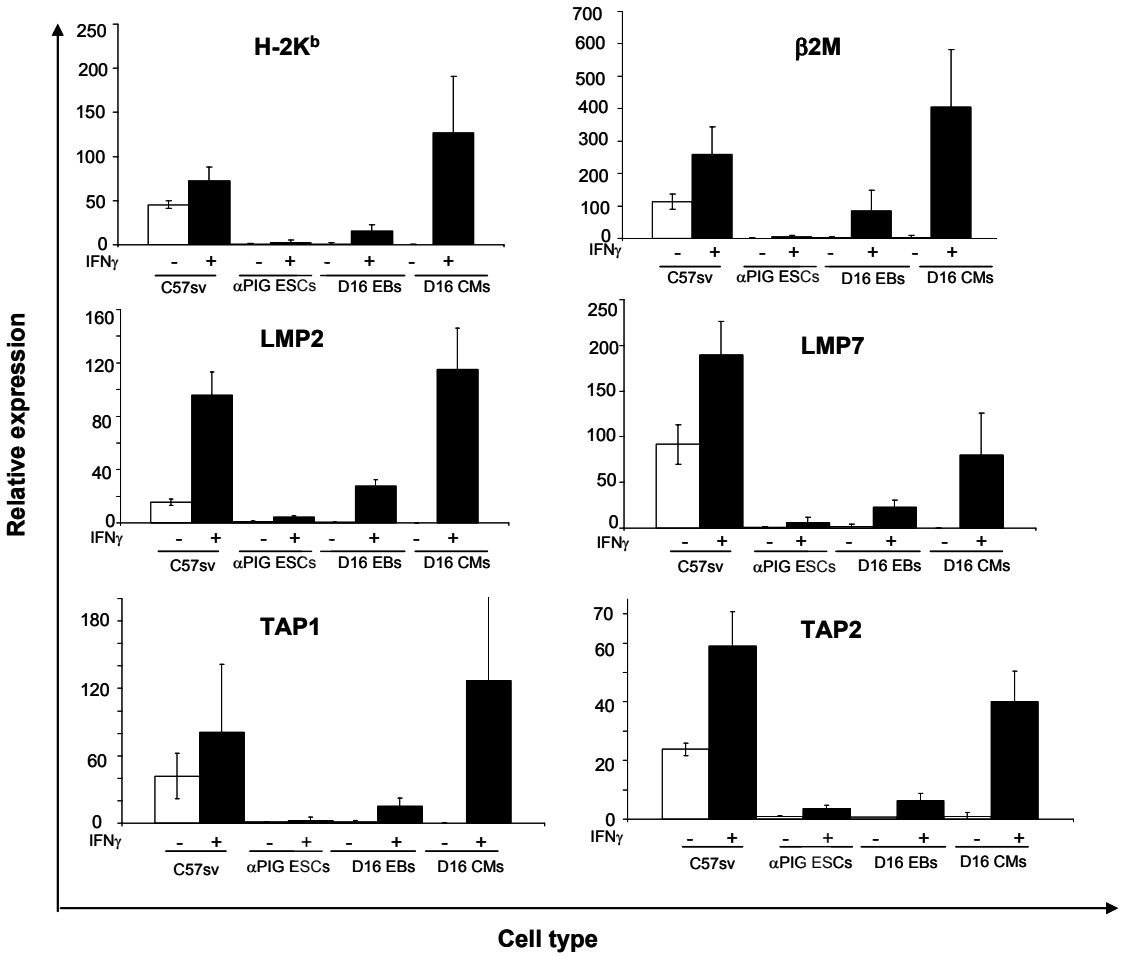


Figure 26. Comparison of expression of various MHC class I transcripts in undifferentiated ESCs, EBs and pure ES-CMs. RNA was isolated from C57sv fibroblasts, aPIG ESCs, day 16 EBs, and purified day 16 ES-CMs cultured in the presence or absence of IFN-γ (100 ng/ml) for 2 days. Real time PCR analysis of *H-2K^b*, *β2M, LMP2, LMP7, TAP1* and *TAP2* mRNA was performed using Syber green dye and GAPDH used as endogenous control.

Interestingly, the induction of these transcripts by IFNγ was much stronger in ES-CMs than in C57sv fibroblasts and unpurified EBs at day 16 of differentiation. Surprisingly, under basal conditions transcripts for MHC class I molecules and selected antigen processing factors were expressed in ES-CMs at the same level like in undifferentiated ESCs (Figure 26). However, the expression of these molecules was highly inducible by IFNγ

in only in ES-CMs (MHC class I α chain by 126-fold, *β2M* by 404-fold, *LMP2* by 115-fold, *LMP7* by 80-fold, *TAP1* by 82-fold and *TAP2* by 40-fold). In contrast, these transcripts were induced to a much lower extent in C57sv fibroblasts (MHC class I α chain by 1.6-fold , *β2M* by 2.29-fold, *LMP2* by 6.1-fold, *LMP7* by 2.06-fold, *TAP1* by 3.04-fold and *TAP2* by 2.47-fold) and EBs (MHC class I α chain by 14.2-fold , *β2M* by 44.7-fold, *LMP2* by 55.6-fold, *LMP7* by 14.2-fold, *TAP1* by 20.2-fold and *TAP2* by 9.2-fold).

III.4.3. ES-CMs are partially lysed by CTLs only after induction of MHC class I molecules by IFNγ

IN previous chapters we have shown that ESCs were not killed by specific CTLs under basal conditions. Moderate lysis could be achieved only when the concentrations of MHC class I molecules on their surface was increased by STAT3 KD. Under basal conditions, ES-CMs are also not killed by CTLs. We have shown above that IFNγ dramatically upregullates all components of the MHC class I antigen processing pathway in ES-CMs, but not in ESCs. Therefore, we asked whether ES-CMs treated with IFNγ could be killed by CTLs more efficiently than ESCs.

Using standard ^{51}Cr-release cytotoxicity assay and ovalbumin system we show that positive control cells EG7OVA and C57sv-CytOVA were lysed by up to 40-50% by OVA specific CTLs while wild type C57sv fibroblasts, EL4 and αPIG ES-CMs, which did not express OVA, were not lysed by OVA specific OT1 CTLs, irrespective of IFNγ treatment (Figure 27A). Similarly, OVA expressing αPIG-CytOVA ES-CMs (clone A4) were also not lysed by OT1 CTLs at any E:T ratio. However, partial lysis was observed with αPIG-CytOVA ES-CMs (up to 20%) that were pretreated with IFNγ (Figure 27A).

Interaction of active CTLs with their targets cells leads to secretion of IFNγ by cytotoxic $CD8^+$ T cells. This phenomenon can be used to assess whether ES-CMs can be recognized by CTLs or not. For this, we

performed ELISA from samples that were collected in cytotoxicity assays shown above. As shown in figure 27B, OT1 CTLs did not secrete IFNγ when they were cocultured with C57sv fibroblasts, EL4 cells and aPIG ES-CMs. Prestimulation of these cells by IFNγ did not affect this result. In contrast, OT1 CTLs secreted IFNγ in cocultures with control cells expressing OVA such as EG7OVA, C57sv-CytOVA and OVA-expressing ES-CMs. These results suggest that OVA-expressing ES-CMs were recognized by OVA-specific CTLs independently of the MHC class I levels on their cell surface but CTLs were able to partially kill only OVA-expressing ES-CMs that expressed high levels of H-2K^b molecules after induction by IFNγ. Weak lysis of ES-CMs even after a dramatic induction of MHC class I molecules sugest that these cells possess specific mechanisms to protest them from killing by CTLs.

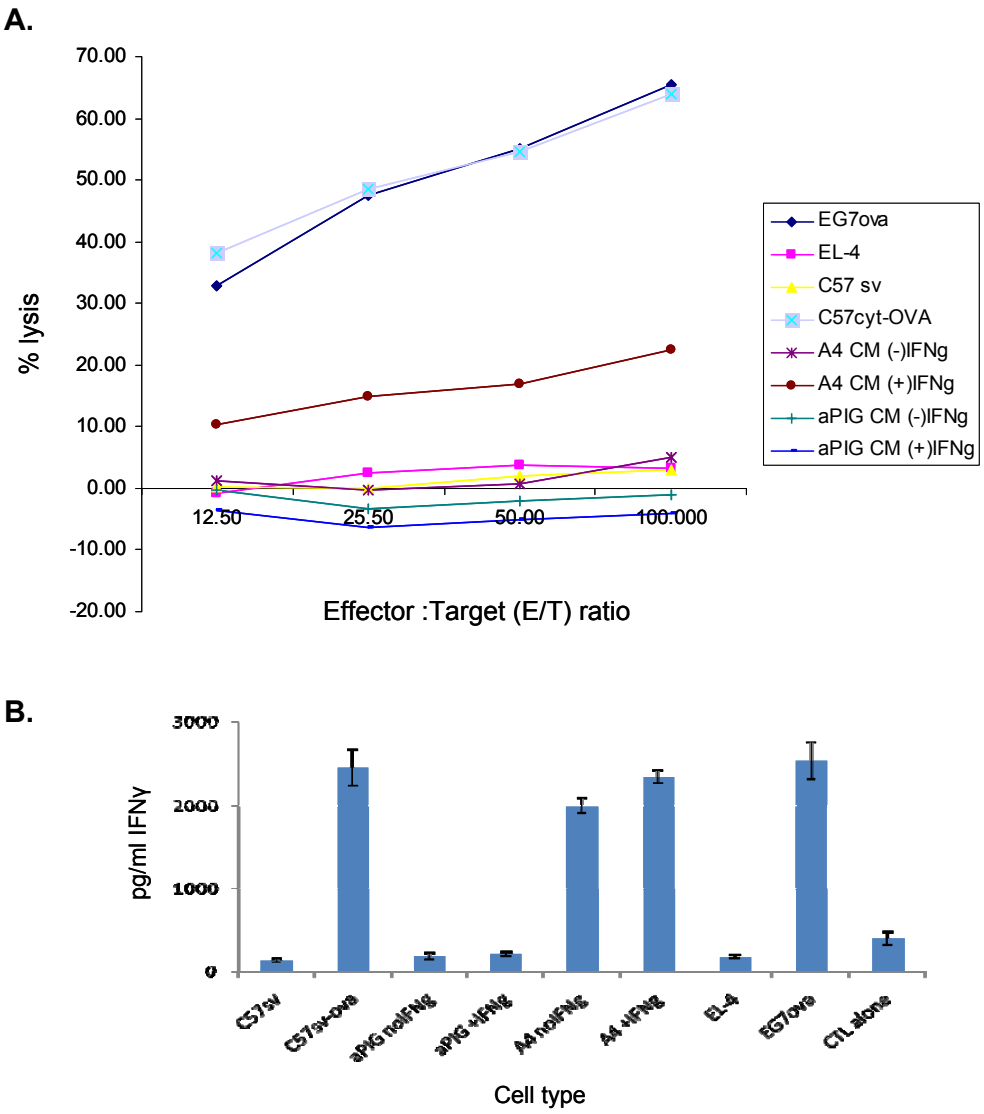


Figure 27. Interaction of ,urine ES-CMs and OVA-specific syngeneic cytotoxic CD8+ T lymphocytes (CTLs). A. CTLs were generated in syngeneic mice by injecting ovalbumin. Splenocytes were taken from ovalbumin immunized mice and restimulated with SIINFEKL *in vitro*. ES-CMs were treated with 100 ng/ml interferon gamma for two days prior to ^{51}Cr-release assay. EG7OVA is an ovalbumin-expressing lymphoblastoid cell line, derived from parental EL4 cells. B. Samples were collected after 51Cr-release assay for ELISA assay. ELISA was performed to measure the IFNγ release using anti-IFNγ antibody during CTLs interactions with different cell types in syngeneic settings.

IV. Discussion

ESCs present a unique model to study early embryonic development and to study different genetic disorders. These cells also provide a platform to investigate developmentally programmed genes during embryonic progression. MHC class I genes, which are biologically relevant, are activated during embryonic development, whereas at birth most somatic cells display the antigens on their surface (Johnson 1993; Fiszer et al. 1997). MHC class I genes play an unequivocally central role in the immune response (Li et al. 2010; Hansen et al. 2009), but have also been shown to influence reproductive behaviour and the development of the embryo (Warner et al. 1991; Tian et al. 1992; Hviid 2006). Therefore, it is essential to understand the immune privileged properties and the regulation of MHC class I molecules in ESCs. The aim of present study was to elucidate the mechanisms that regulate the expression of MHC class I molecules in murine ESCs at undifferentiated stage and after their differentiation to cardiomyocytes, which served as a model for a highly specialized cell type. Similar to an embryo capable of evading maternal immune system, ESCs possess unique immunological properties compared to typical transplantation grafts (Swijnenburg et al. 2005; Bonde et al. 2006; Magliocca et al. 2006; Abdullah et al. 2007; Saric et al. 2008). Consistent with previous findings, I showed that, in contrast to human ESCs, their murine counterparts express very low levels of MHC class I molecules and do not respond to IFNγ by upregulation of IFNγ responsive genes. My results indicate that MHC class I molecules in murine ESCs are regulated at different levels. The dominant LIF/STAT3 pathway negatively regulates the MHC class I molecules in murine ESCs and iPSCs as demonstrated by STAT3 knock-down experiments which significantly increased the expression of these molecules at basal state as well as in response to IFNγ. As compared to fibroblasts, the expression of STAT1 was very low in murine ESCs and IRF2 was either absent or present at negligible amount in these cells. STAT3 KD in murine ESCs demonstrated the dowregulation of epigenetic modifier genes Eed1, Dnamt1 and Jmjd1 while Dnmt KO murine ESCs showed the partial upregulation of MHC

class I molecules on their surface. Moreover, the treatment of 2,5-aza and TSA to murine ESCs enhanced the MHC class I expression on their surface. Strikingly, STAT3 KD murine ESCs showed STAT1 phosphorylation and more STAT1 transcriptonal activity as compared to control ESCs indicating the inhibitory role of STAT3 on IFNγ signaling. I have shown that relatively modest modulation of MHC class I molecule expression on the surface of ESCs affects their susceptibility towards NK cell and CTL cytotoxicity *in vitro.* Similar effect was also observed after more dramatic increase of MHC class I expression in ES-CMs that slightly increased their lysis by CTLs.

Regulation of MHC class I in IFNγ treated state in ESCs

The pathway of STAT1 activation is fundamental for IFNγ mediated pro-apoptotic responses including expression and induction of MHC class I molecules in different cell types (Schroder et al. 2004). IFNγ is a significant cytokine that orchestrates many distinct cellular programs through transcriptional control over large number of genes including STAT1, MHC class I molecules and IFNγ-induced effects resulting in heightened immune surveillance and immune system function during infection. Several studies showed the impairment of STAT1/IFNγ pathway due to various mechanisms. One report demonstrated that the downregulation of IFNγ-R2 chain impaired the responsiveness to IFNγ in T lymphocytes (Regis et al. 2006). The authors showed that accumulation of IFNγ-R2 induces IFNγ-induced apoptosis, whereas IFNγ-R2 chain internalization induces T cell unresponsiveness to IFNγ signaling. We showed that IFNγ receptors are expressed at the comparable levels on murine ESCs and EBs and were downregulated by IFNγ. However, despite the presence of its receptor, IFNγ did not seem to transduce the signal and induce its responsive genes in ESCs (Figure 9A,B).

Previous reports demonstrated that the downregulation of IFNγ-inducible gene expression in human cancer cell lines is due to phosphatase mediated inhibition of JAK/STAT1 pathway (Choi et al. 2007; Rodriguez et

al. 2007). Choi and coworkers 2007 reported that IFNγ-induced expression of multiple genes is significantly reduced in human trophoblast-derived choriocarcinoma cells relative to Hela epithelial or fibroblasts cells due to the compromised tyrosine phosphorylation of JAK2 and STAT1. They successfully showed that treatment of choriocarcinoma cells with the tyrosine phosphatase inhibitor pervanadate significantly enhanced the IFNγ-inducible tyrosine phosphorylation of JAK and STAT1 and expression of IFNγ-inducible genes. Rodriguez et al elucidated that some melanoma cell lines did not induce MHC class I in response to IFNγ due to the absence of STAT1 phosphorylation. However, they did not find out the role of phosphatases in STAT1 phosphorylation. Many reports also described the unresponsiveness to IFNγ due to the absence of IFNγ regulatory factor 1 and 2 (IRF1 and IRF2) or due to the defect in antigen processing machinery molecules (Harada et al. 1990; Nozawa et al. 1998; Dovhey et al. 2000; Rodriguez et al. 2007). Haradha H et al. described the absence of type I IFN system in murine embryonic carcinoma (EC) cells and showed that transcriptional activator (IRF1) and repressor (IRF2) were developmentally regulated in EC cells. They described that IRF1 and IRF2 become functional only after cell differentiation. Rodriguez et al showed that ESTDAB-159 melanoma cells did not have IFNγ-mediated HLA class I expression due to epigenetic blocking of IRF1 transactivation, while Nozawa et al found functionally inactivating point mutation in tumor suppressor IRF1 gene in human gastric cancer. Dovhey et al reported that some cancer cell lines have abnormally low expression of TAP1 and LMP2 and loss of their induction leads to the defect in IFNγ signaling pathway resulting in the inability of cancer cell lines to upregulate MHC class I antigen-processing pathway. In the present study, we have shown that IFNγ receptors are expressed on the surface of ESCs and that their absence can not be the reason for the unresponsiveness of ESCs towards IFNγ. However, as compared to C57sv fibroblasts, STAT1 protein levels as well as transcript levels were very low in ESCs and EBs (Figure 10A,B). It might be possible that each cell type requires minimum threshold expression of STAT1 for IFNγ response and low expression of STAT1 may

contribute to unresponsiveness of ESCs to IFNγ. Furthermore, phosphatases SHP1, SHP2, PTPB1 and molecules of the antigen processing machinery TAP1, TAP2, LMP2, LMP7, JAK1, JAK2 are also expressed at the transcript level on these cells (Figure 8B; Figure 11A), but were not induced by IFNγ. Interestingly, IRF1 was expressed but IRF2 was not detectable at the transcript level in ESCs (Figure 11A). Therefore, it is possible that the inability of ESCs to respond to IFNγ may be based on several factors: low level of STAT1 expression, lack of IRF2 expression, and blockade of STAT1 activation by STAT3 signaling pathway. It is unlikely that the unresponsiveness to IFNγ is due to dysfunctional antigen processing and presentation pathway, because all analyzed components of this pathwas were expressed in ESCs, EBs and fibroblasts at similar levels.

Another check point that may play a role in regulation of IFNγ signaling is the transport of phosphorylated STAT1 dimers into the nucleus that occurs through nuclear import proteins, known as importins. Yasuhara and coworkers demonstrated that mouse brain predominantly expresses importin-α5 (also known as Kpna1) while murine ESCs predominantly express importin-α1 (also known as Kpna2) but not importin-α5 (Yasuhara et al. 2007). This raises the possibility that importin-α undergoes subtype switching during neural differentiation. Indeed, they found that importin-α1 switches to importin-α5 when ESCs differentiate into neural cells (Yasuhara et al. 2007). Another study conducted by McBride and colleagues reported that nuclear import of STAT1 was regulated by importin-α5 in HeLa cells (McBride et al. 2002). This group proposed that the importin-α5 shuttling receptor recognizes and binds to the nuclear localization signal (NLS) on the activated STAT1 dimer and effects translocation into the nucleus in association with importin-β (also known as Kpnb). In this study, we found that all importins were expressed at the transcript level in murine ESCs as well as in differentiating EBs (Figure 11B), suggesting that STAT1 import may not be the reason for unresponsiveness to IFNγ in ESCs. This conculsion is indirectly supported by the finding that the responsiveness to IFNγ could be established in

ESCs by downregulation of STAT3 expression that led to enhanced STAT1 phosphorylation. However, we can not exclude the possibility that importin-α5 is absent at the protein level in ESCs and thus reduces the nuclear import of STAT1 and expression of STAT1 responsive genes, such as MHC class I molecules.

LIF/STAT3 signaling maintains pluripotency of ES/iPS cells and is a dominant pathway, which might inhibit STAT1 pathway in these cells. A number of studies showed the contrasting role of STAT1 and STAT3 signaling in different cell types (Hong et al. 2002; Qing et al. 2004). Hong and coworkers have shown that STAT1 plays a damaging role in Con A-mediated hepatitis by activation of $CD4^+$ T cells and NKT cells and directs hepatocytes towards death. On contrary, STAT3 protects against liver injury by suppression of IFNγ signaling and induction of antiapoptotic gene Bcl-X_L. Furthermore, their results showed the SOCS3-mediated inhibitory cross-talk between STAT1 and STAT3 in hepatocytes in Con A-induced hepatitis. They demonstrated that STAT1-mediated induction of SOCS1 gives negative feedback for STAT1 activation and attenuates activation of STAT3 in Con A-induced hepatitis, whereas, STAT3 play a more important role than STAT1 in inducing SOCS3 in the model of hepatitis. Taken together, these findings propose that T-cell-induced hepatiis is tightly controlled by mutual antagonism of IFNγ/STAT1 and IL6/STAT3 pathways, which regulate each other negatively through the induction of SOCS. Consistent with these results; our data showed that expression of SOCS3 was reduced significantly in STAT3 KD murine ESCs while SOCS1 remained unchanged (Figure 19B). This showed that SOCS3 but not SOCS1 was regulated by STAT3 in murine ESCs and may mediate inhibition of STAT1 phosphorylation. Interestingly, some groups reported that SOCS3 has been shown to negatively regulate STAT3 and STAT1 activation by inhibition of JAKs (Naka et al. 1999; Nicola et al. 1999). Forrai and colleagues described that absence of SOCS3 reduces self-renewal and promotes differentiation in murine ESCs (Forrai et al. 2006).

Our results were also in line with previous studies indicating the opposing role of STAT3 activation on IFNγ/STAT1 signaling in hepatocytes. In

general, murine ESCs were differentiated into EBs in absence of LIF. Expectedly, LIF addition to ES-derived EBs drastically reduced the basal and IFNγ induced MHC class I expression, because LIF addition activates the STAT3 pathway. Most likely, the activation fo STAT3 pathway by readdition of LIF to EBs negatively affected the basal expression of MHC class I molecules in the absence of IFNγ as well as the STAT1 phosphorylation and transcriptional activity in the presence of IFNγ leading to the reduced expression of MHC class I molecules.

Regulation of MHC class I in basal state in ESCs

Of interest, the dominant LIF/STAT3 pathway in ES/iPS cells might be possible reason for the low expression of MHC class I molecules at basal state in the absence of IFNγ. The MHC class I molecules were upregulated in ES/iPS cells also when STAT3 transcripts were knocked down in the absence of this cytokine. Since MHC class I expression was induced by STAT3 KD even without IFNγ, other mechanisms unrelated to STAT1 signaling pathway must be responsible for this phenomenon. Epigenetic modifier genes Dnamt1, Eed1 and Jmjd1 were downregulated in STAT3 KD ESCs, with Eed1, a crucial gene for Dnamt enzyme recruitment, being the most significantly downregulated and therefore may act as a potentially responsible mediator of STAT3-suppressive action on MHC class I expression (Figure 20). Interestingly, Dnamt KO murine ESCs showed enhanced MHC class I expression on a small fraction of cells, which was slightly inducible by IFNγ (Figure 21). Therefore, Dnamt enzymes do not seem to play a crucial role of regulation of MHC class I expression in ESCs. However, the involvement of epigenetic mechanisms in regulation of MHC expression in ESCs was reported recently by showing hypermethylation for MHC class II genes and demethylation for MHC class I genes in human ESCs/iPSCs (Suarez-Alvarez et al. 2010). They showed that treatment of murine ESCs with 2,5-aza and trichostatin A not only increased the expression of classical class I and class II and non-classical MHC molecules but also genes related to antigen processing machinery

such as TAP-2, TPN, and LMP-7. In this report, they also demonstrated that combined effect of epigenetic modifiers enhanced the expression of MHC class I molecules and β2M. Consistent with this, I also showed that expression of MHC class I molecules increased in murine ESCs by treatment with 2,5-aza and trichostatin A. Combined effect synergistically increased the expression of MHC class I molecules which were further inducible by IFNγ (Figure 22). Therefore, STAT3 pathway in ESCs most likely keeps the expression of MHC class I molecules by modulating the expression and activity of specific epigenetic factors, such as Eed1 (Ura et al. 2008).

STAT3 KD ES cells maintained their pluripotency and showed similar teratoma growth kinetics in RAG2$^{-/-}\gamma_c^{-/-}$ immunodeficient mice (Figure 14D). Expression of Oct4, Nanog, Sox2 and Tbx3 did not change in STAT3 KD ESCs while expression of Klf4 was reduced significantly, which is in accordance with Klf4 being the immediate downstream target to STAT3 (Figure 14B). These findings were in the line with evidence of other reports. Niwa and coworkers demonstrated that LIF signals enter into the cells through STAT3 and PI(3)K pathway and maintain the pluripotency of ESCs (Niwa et al. 2009). They have shown that the LIF signal is integrated into the core regulatory circuitry of pluripotency of murine ES cells via these two parallel pathways: The JAK-STAT3 activates Klf4 and then Sox2 but not Nanog, and the PI(3)K-Akt pathway activates Tbx3 and then preferentially Nanog (Figure 28). Since some studies demonstrated that PI(3)K pathway also plays an important role in maintaining the pluripotency of murine ESCs (Chen et al. 2010; Storm et al. 2009), the possible reason for the undisturbed pluripotency of STAT3 KD ES cells could be the retention of a fully active PI(3)K pathway by LIF. Previous reports have demonstrated that constitutive expression of STAT3 is sufficient to stop differentiation of murine ES cells even in the absence of LIF (Matsuda et al. 1999), whereas dominant negative form of mutant STAT3 could abolish the pluripotency of mouse ES cells even if maintained in the presence of LIF (Niwa et al. 1998; Raz et al. 1999). This indicates that STAT3 is essential for maintaining the pluripotency of ESCs.

Another possible reason for the inability of transient STAT3 KD to affect the pluripotency of murine ES cells in the presence of LIF is the partial retention of STAT3 mRNA expression in ESCs (at about 20% of normal levels).

Furthermore, Xie and coworkers reported that murine ESCs retain active STAT3 for up to 4 to 6 days upon removal of LIF (Xie et al. 2009). The imaging of STAT3 signaling pathway during mouse ES cell differentiation revealed that STAT3 activation is required in both undifferentiated and differentiated stages because of STAT3's ability to generate a diversity of biological outcomes in different cell types (Katoh et al. 2007). The prolonged retention of active STAT3 upon LIF removal in ESCs may explain the failure to induce MHC class I molecules in undifferentiated ESCs under this experimental condition in this study.

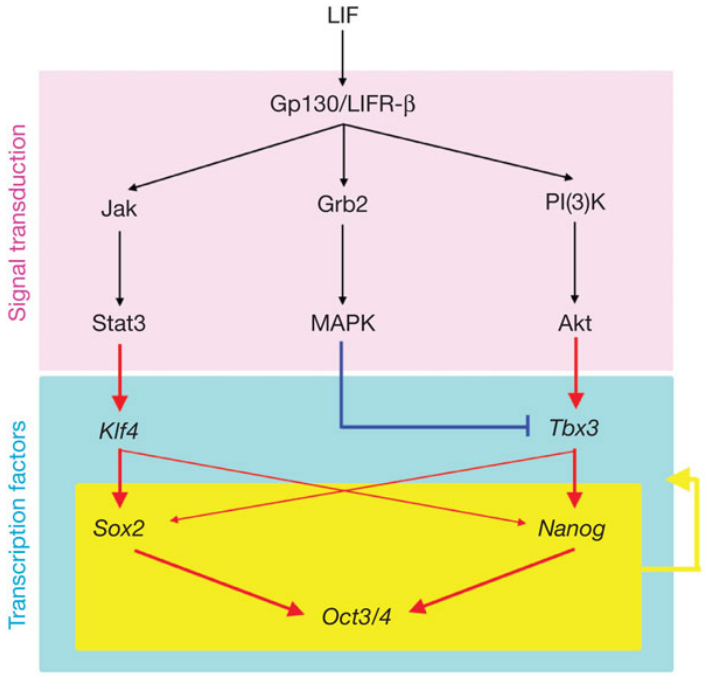


Figure 28. Diagram of the parallel circuitry of the LIF signal pathway integrating into the transcription factor network. The JAK-STAT3 pathway activates Klf4, whereas the PI(3)K-Akt pathway stimulates the transcription of Tbx3. The MAPK pathway antagonizes the nuclear localization of Tbx3. Klf4 and Tbx3 mainly activates Sox2 and nanog, respectively, and maintain expression of Oct3/4. Transcription of all these transcription factors is positively regulated by Oct3/4, Sox2 and Nanog, which may confer robustness and stable expression in the absence of all signals. (*Niwa, H, Nature, 2009*)

Differential regulation of MHC class I in ESCs and ES-CMs

The role of MHC class I in the initiation and extent of NK cell-dependent killing has been extensively investigated and debated as the concept of 'missing self 'hypothesis (Lanier 2005; Yokoyama et al. 2008). Most of the studies reported that undifferentiated ESCs, having a very low level of MHC class I, were recognized and lysed by natural killer (NK) cells *in vitro* (Dressel et al. 2010; Drukker et al. 2002; Frenzel et al. 2009). Ma and coworkers described that MHC class I expression on ES cell-derived vascular progenitor cells is critical for syngeneic transplant survival (Ma et al. 2010). Their study clearly pointed out that IFNγ-induced expression of MHC class I by ESC-derived progenitor cells was linked with attenuated host NK cell attack, enhanced graft survival, and the further differentiation of transplanted ESC-derived cells into vascular smooth muscle cells and endothelial cells. Our group revealed the role of natural-killer group 2 member D (NKG2D) ligands and intracellular adhesion molecule 1 (ICAM1) in NK cell-mediated lysis of murine ES cells and ES-CMs (Frenzel et al. 2009). They showed that NKG2D and ICAM1 play key role in lysis of ESCs by activated NK cells, on contrary ES-CMs are not lysed by activated NK cells due to the lack of detectable amount of NKG2D and ICAM1 on their surfaces. This differential sensitivity of ES cells and ES-CM to NK cells could be useful for selective elimination of residual ESCs in grafts of differentiated cells to prevent teratoma formation. In the present study, we have shown that STAT3 KD murine ESCs have an enhanced expression of MHC class I molecules resulting in the reduced lysis by activated syngeneic NK cells. Our results suggest, at least in a syngeneic setting, that the induction of MHC class I after transient KD of STAT3 gene during in vitro differentiation of ESCs may improve the subsequent survival and immune integration of ESCs- derived differentiated derivatives after transplantation.

Previous reports demonstrated that murine ESCs lack MHC class I expression at any passage number (David-Watine et al. 1987; Tian et al. 1997). Our group showed that MHC class I molecules are expressed at

transcript level in undifferentiated murine ESCs and its derived EBs, however the protein level expression of these molecules were below the level of detection by flow cytometry in murine ESCs (Abdullah et al. 2007). They reported that murine ESCs and EBs express cytoprotective molecules cathepsin B and serine protease inhibitor-6 (SPI-6) and revealed that SPI-6 expression protects murine ESCs from lysis by antigen-specific CTLs. Consistent with these reports, I have confirmed the low level expression of MHC class I molecules and presence of other immunomodulatory molecules in ESCs and its derived EBs (unpublished data). Moreover, the data presented in this thesis showed that the increased expression of MHC class I molecules allows for the partial lysis of murine ESCs by antigen-specific CTLs after STAT3 KD (Figure 24).

In this study, it was very interesting to note that unlike ESCs, MHC class I molecules and their genes were up regulated in ES-CMs to a much higher extent than in fibroblasts or EBs by IFNγ treatment (Figure 26). However, basal level expression of MHC class I molecules was same in ES-CMs and undifferentiated ESCs. Unlike in ESCs, downregulation of STAT3 did not induce MHC class I molecules in ES-CMs. Drug selected murine ES-CMs were resistant to lysis by antigen-specific syngeneic cytotoxic $CD8^+$ T cells *in vitro*, despite their recognition by T cells as shown by IFNγ release in an ELISA assay (Figure 27). However, upregulation of MHC class I molecules on ES-CMs by IFNγ led to their partial lysis by CTLs. Taken together, our findings demonstrated that in both cases either STAT3 KD in murine ESCs or IFNγ treatment in ES-CMs lead to the induction of MHC class I molecules on their surface and partial lysis by antigenic-specific CTLs.

Cancer evades immune system by down regulating the MHC class I expression on the surface of cells and most of the studies showed the apoptotic role of STAT1 and pro-apoptotic role of STAT3 gene in different tumor cell lines (Choi et al. 2007; Rodriguez et al. 2007; Regis et al. 2008). Immunotherapy is an important approach to cancer treatment, therefore, it

is essential to understand the mechanisms by which these tumors circumvent IFNγ signaling, thereby representing the potential mechanism of cancer cytokine-resistance to therapy. Indeed, suppression of STAT3 gene and activation of STAT1 gene can be also useful for killing the cancer cells by CTLs and dissecting the STAT1 pathway and opposing role of STAT1-STAT3 in ESCs can give insight into cancer biology.

Further effort should be made to determine the interaction partners between these two essential transcriptional factors (STAT1 and STAT3) of biological system that would further help to design stratiges for drug development against tumor cells. Nevertheless; this present study could be helpful in designing the strategies to prevent teratoma by ESCs which is a great hurdle in cell transplantation study. Understanding the complex regulatory mechanisms that govern pluripotency, differentiation and immune interactions of ESCs and its derivatives will undoubtedly be an obligatory hurdle that will need to be overcome for the successful implementation of cell-based regenerative therapies.

Previous studies demonstrated the crucial role of MHC class I molecules in reproduction and development of embryos (Warner et al. 1991; Tian et al. 1992; Boyson et al. 1997; Christiansen et al. 1997; Tarin 1997; Fernandez et al. 1999). Warner and Tian and co-workers demonstrated that Qa-2, a nonclassical MHC antigen, has been shown to influence dramatically the timing and rate of preimplantation cleavage division, clearly indicating a developmental role both before and after implantation. Intriguingly, MHC genes, in particular, appear to play a similar role in determining and influencing longevity, since mice with faster cleavage rates have shorter life spans than their slower congenic counterparts (Tarin 1997). However, in humans the situation is considerably harder to study. Because, not only is the arrangement of the MHC genes quite different from that in the mouse (Trowsdale 1995), but also the physiological and anatomical characteristics of pregnancy are not the same. MHC class 1b genes and their products (HLA-E,-F,-G) have been identified in human embryos, and many investigators believe that they play a role in reproductive success (Johnson 1993; Fiszer et al. 1997).

However, HLA protein expression and function does not appear essential for fetal survival, since an individual with a rare deletion resulting in failure of HLA-G protein expression has been reported. This present study is the first study which uses murine ESCs and their differentiated derivatives as a model to dissect the regulation of MHC class I molecules and demonstrates that STAT3 negatively regulates the expression of these molecules. I believe that ESCs represent an excellent model framework within which to study immunological tolerance and allorecognition, as mechanisms involved in the prevention of the rejection of these cells are likely to overlap with those involved in tolerance induction per se.

Proposed model of regulation of MHC class I molecules in murine ES cells

In the present study, I have explored the role of STAT3 signaling pathway in the regulation of MHC class I molecules on murine ESCs at basal state and in the presence of immunomodulatory cytokine IFNγ. The LIF/STAT3 pathway keeps the expression of MHC class I molecules low under native conditions. The STAT3 signaling also prevents the stimulatory effect of IFNγ on MHC class I molecule expression by inhibiting the phosphorylation of STAT1 and its transcriptional activity in ESCs (Figure 29).

However, IFNγ is also unable to strongly induce the MHC class I expression in ESCs because they contain very low levels of STAT1 protein as compared to good IFNγ-responder cells, such as fibroblasts. Interestingly, IRF-2 is absent in murine ESCs and could be another reason for low expression of MHC class I molecules and their unresponsiveness to IFNγ (Figure 29).

STAT3 also appears to mediated downregulation of MHC class I molecules at the epigenetic level. STAT3 regulates the expression of several epigenetic modifiers, especially the Eed1 and possible the JmjD1 and Dnamt1, that could affect the methylation of promoter regions controlling the expression of MHC class I heavy and light chain transcripts in ESCs. Indirect evidence for this assumption is provided by the observation that the expression of MHC class I molecules on Dnmt1 KO

and TSA/2,5-aza-treated ESCs is higher than in wild type or untreated cells, respectively (Figure 29).

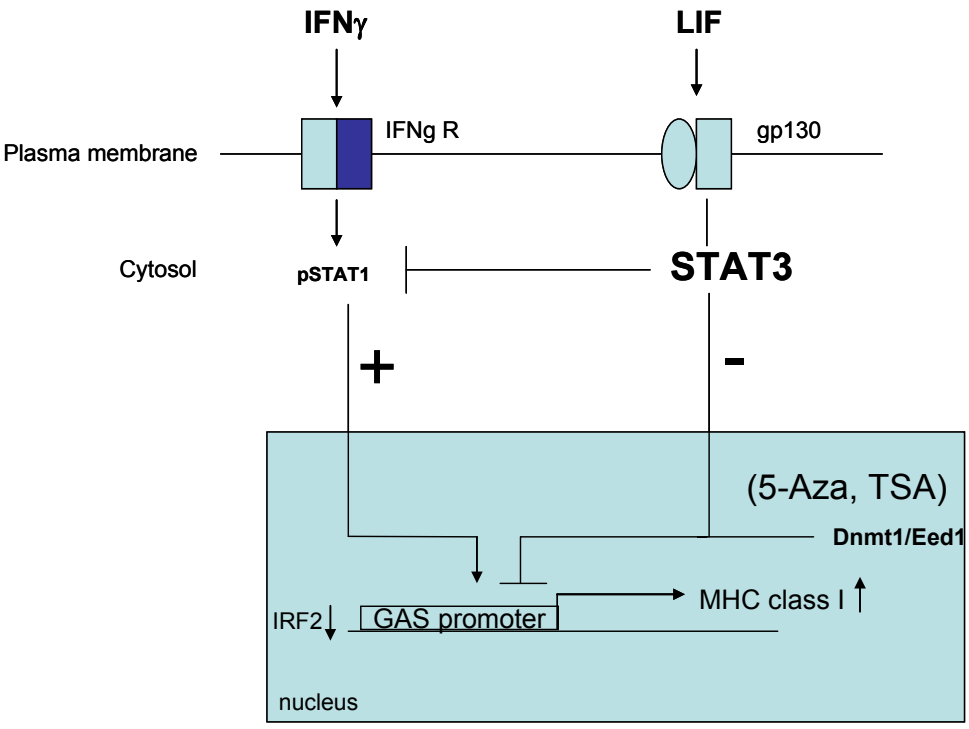


Figure 29. Schematic representation of model of regulation of MHC class I molecules in murine ES cells. In normal cell type, IFNγ binds to IFNγ receptors and LIF binds to gp130 receptors to activate STAT1 and STAT3 pathways, respectively. However, STAT3 KD experiments demonstrated that STAT1 transcriptional activity and its phosphorylation were negatively regulated by STAT3 signaling-a dominant pathway in murine ES cells. Epigenetic mechanisms also seem to negatively regulate the expression of MHC class I molecules in murine ESCs.

V.1. Materials and Methods

V.1.1. Cell Lines

Cell line	Description
HES2	Human ES cell line (WiCell Research Institute (Madison, WI, USA)
αPIG44-ES/aPIG44-ES	Transgenic murine embryonic stem cell line, expressing the puromycin N-acetyl transferase and IRES-linked green fluorescent protein (GFP) under control of the cardiac α-MHC promoter (made by Eugen Kolossov)
A4 ova	aPIG44 trangenic ES cell line expressing ova (generated by Charis Satrazami in AG Saric)
CGR8-ES	Murine ES cell line (provided by Prof. Sachinidis lab, Institute for Neurophysiology)
AT25	Transgenic murine iPS cell line (provided by Rudolf Jänisch lab, USA), expressing the green fluorescent protein (GFP) under control of the cardiac α-MHC promoter (generated by Azra Fatima in AG Saric)
EG7ova	OVA-expressing EL4 cells, mouse strain C57BL/6N (ATCC)
EL4	Mouse lymphoma cell line, mouse strain C57BL/6N (ATCC)
Dermal fibroblasts (C57sv)	Mouse strain C57BL/6 (Millipore)
CF-1 MEFs	Mouse embryonic fibroblasts (MEFs)

	isolated from CF-1 mouse strain
MEF-Neo^R	Neomycin resistant MEFs
Dnamt-1, Dnamt-3 and Dnamt-all KO	DNA methyl transferase enzyme (Dnamt) 1, 3 and all knockout murine ES cell lines (generated by Rudolf Jänisch lab at MIT, USA, and provided by Hans Schöler, Max Planck Institute, Münster)
OG-2	Murine ES cell line expressing GFP under Oct4 promoter (provided by Hans Schöler, Max Planck Institute, Münster)

V.1.2. Mouse Strains

C57BL/6, 129sv, and OT-1 mice were originally obtained from Charles River Laboratories (Sulzfeld, Germany). Rag2$^{-/-}\gamma_c^{-/-}$ mice were obtained from Mamoru Ito (Central Institute for Experimental Animals, Tokyo, Japan) and were bred and maintained in a pathogen-free animal facility. OT-1 mouse is on a C57BL/6 background (H-2K^b) and expresses a transgenic T-cell receptor specific for the SIINFEKL peptide of ovalbumin (OVA) in a complex with H-2K^b MHC class I molecules. Rag2$^{-/-}\gamma_c^{-/-}$ mice are immunodeficient mice lacking B cells, T cells and NK cells in their immune system. Animal experiments were approved by the Ethics committee of the Government of Cologne and performed in accordance to the German animal protection law.

V.1.3. Molecular Biology reagents

Reagent	**Catalogue No.**	**Supplier**
TRIzol reagent	15596026	Invitrogen
DNAeasy Blood and Tissue kit	69504	Qiagen
Plasmid Maxi kit	K210017	Invitrogen
JumpStart REDTaq ReadyMix	P0982	Sigma
dNTP Mix	18427013	Invitrogen
DNase I	18068015	Invitrogen
5x or 10x first strand buffer	810462	Invitrogen
Random Primers	48190011	Sigma
SuperScript II RNase H- Reverse Transcriptase	18064014	Invitrogen
Lipofectamine 2000	11668019	Invitrogen

V.1.4. Cell culture reagents

Medium	Composition	Intended use
20% DMEM F12+Glutamax (1X)	DMEM F12 + Glutamax (GIBCO), 20% KOSR, 1X NEA, 50 µM ß-Mercaptoethanol, 1X Pen-Strep and bFGF (4 ng/ml for human ES and 50 ng/ml for human iPS cells)	Human ES cell culture
KO DMEM	KO DMEM + conc? Glutamine (GIBCO), 1X NEA, 50 µM ß-Mercaptoethanol, 1X Pen-Strep	Cardiac differentiation
15% DMEM	DMEM + Glucose + conc? Glutamine (GIBCO), 15% FBS, 1X NEA, 50 µM ß-Mercaptoethanol, LIF 1000 U/ml (Invitrogen)	Murine αPIG ES cell culture
10% GMEM	GMEM + conc? Glutamine (GIBCO), 10% FBS, 1X NEA, 50 µM ß-Mercaptoethanol	Murine CGR8 ES cell culture
5% DMEM	DMEM + Glucose + conc? Glutamine + 5% FBS	Fibroblats culture
10% RPMI	RPMI, 10% FBS, 50 µM ß-Mercaptoethanol, L-Glutamin 1 mM, Sodium pyruvate 110 µg/ml, 1X NEA, IL-2 100 U/ml , SIINFEKL 10^{-9}M	In vitro stimulation of CTLs
Freezing medium	80% DMSO (GIBCO), 20% FBS	Cryopreservation of cells
Basic fibroblasts growth factor (bFGF)	Peprotech	Human ES culture
Leukemia Inhibitory factor (LIF)	Millipore	Murine ES culture
Trypan blue stain (0.4%)	GIBCO	Cell counting
Trypsin-EDTA (0.05%)	Sigma	Cell-passaging
Knockout Serum Replacer (KOSR)	Gibco	Cell culture

Fetal bovine serum (FBS)	Gibco	Cell culture

V.1.5. Radioactive material

For cytotoxicity experiments, $Na_2{}^{51}CrO_4$ were obtained from Hartmann-analytic GmbH, Germany (Activity: 74 Mbq (2 mCi), Size 400 µl, Code: Cr-RA-8, Catalogue No. 150496).

V.1.6. Common reagents

Reagent	Catalogue No.	Supplier
Agarose	16500500	Invitrogen
Albumin-Fraktion V pH 7,0 (BSA)	A1391,0250	AppliChem
CD8a (Ly-2) MicroBeads mouse	130-042-401	Miltenyi Biotec
Anti-NK (DX5) MicroBeads mouse	130-052-501	Miltenyi Biotec
16% paraformaldehyde	18814-20	Polysciences
Agar Agar	9002-18-0	Roth
DPBS	14190169	GIBCO
DTT	844789	Roth
Ethanol	A1613,1000	AppliChem
Ethidium bromide	15585011	Merck
Gelatin powder	G1890-100G	Sigma
Glycerol (99%)	G5150-1L	Sigma
Interferon-γ (IFNγ)	PMC4034	Invitrogen
Interleukin 2 (IL-2)	PHC0026	Invitrogen
Isopropanol	A0900,1000PE	AppliChem
MACS-Buffer	130-091-221	Miltenyi Biotec
Ovalbumin (albumin from chicken egg white)	A5503-5G	Sigma
SIINFEKL (OVA257-264)	S7951	ProImmune
Sodium chloride	7647-14-5	Roth
Tris base	5429.3	MERCK
Trypton	07-119	Roth
Yeast extract	07-079	Roth

CellWash	554723	BD Pharmingen
Geneticin	10131019	Invitrogen
Puromycin	P15-019	PAA
Ampicillin	1503,5	Biomol
Ethanol/Methanol absolute	500156	Applichem

V.1.7. Primers

Gene	NCBI Accession	Sequence (5' to 3')	Size (nt)	Ta, °C
mH2K^{b}	NM_001001892.2	F-GCGGCTCTCACACTATTCAGGT R-TTCCCGTTCTTCAGGTATCTGC	259	60
mβ2M	NM_009735.3	F-GTCTTTCTGGTGCTTGTCTC R-GGCGTATGTATCAGTCTCAG	276	60
mSTAT1	NM_009283	F-CCTCTTCCAGCAGCTCATTC R-TGTGTGCGTACCCAAGATGT	241	60
mSTAT3	NM_0011486	F-TCACTTGGGTGGAAAAGGAC R-TGGTCGCATCCATGATCTTA	129	60
mSOCS1	NM_009896	F-CTTAACCCGGTACTCCGTGA R-GAGGTCTCCAGCCAGAAGTG	210	60
mSOCS3	NM_007707	F-CCTTTGACAAGCGGACTCTC R-GCCAGCATAAAAACCCTTCA	216	60
mJAK1	NM_146145.2	F-CATCCCAGTCTCTGTGCTGA R-GCTCCTTGCAAGATGGAGTC	236	60
mJAK2	NM_008413.2	F-TCCTTGACGGAGAGCAAGTT R- GAAGGGAAAGGTCCCTGAAG	292	60
mPTP1B	NM_011201.3	F-GACTCGTCAGTGCAGGATCA R- GCCTGAGCACTTTGAAGACC	299	60
mSHP1	NM_001077705.1	F- AACCAGCTGCTAGGTCCAGA R-CTGCTGTGTCATGCTCCCTA	241	60

mSHP2	NM_011202.2	F-TTCTCACCAGCACACAGAGG R-CTCGCTGTCTCAAATCCACA	300	60
mKpna1	NM_008465	F-CCTGAGGCTTGGAGAACAAG R-GCTGCTGGCTAAGATCAACC	218	60
mKpna2	NM_010655	F-GGAGCACTTGCAGTCTTTCC R-CCACCGCTGGTATAGTTGGT	221	60
mKpna6	NM_008468	F-CTAAATGGTGCGACCCAACT R-CCAGCTATGGCAGAAGGAAG	244	60
mKpnab1	NM_008379	F-CTGTCCACCTGCTGTGAAGA R-GGTGCCAGGTAGACATCGTT	290	60
mTAP1	NM_013683	F-CCGCTGCTATTTGGAAGAAG R-TGTCATAGCCCTGAGGGAAC	139	60
mTAP2	NM_011530	F-AAGGTGGTGGGGCTCTACTT R-GGGGGTTGTACACCTTCTCA	109	60
mLMP2	NM_013585.2	F-CATCATGGCAGTGGAGTTTG R-ACCTGAGAGGGCACAGAAGA	136	60
mLMP7	NM_010724.2	F-CAGTCCTGAAGAGGCCTACG R-CACTTTCACCCAACCGTCTT	121	60
mIRF1	NM_008390	F-AGGGCTTAGGAGGCAGAGTC R- TCTAGGGCCAGTGCTATGCT	219	60
mIRF2	NM_008391	F-CTTATCCGAACGACCTTCCA R- ATGTCTGGCGGGTTAGTGAC	258	60
mDnmt1	NM_010066.3	F-TGAGGAAGGCTACCTGGCTA R-GTCTGCCATTTCTGCTCTCC	142	60
mEed1	NM_021876.2	F-CAACTGTGGGAAGCAACAGA R-ATAGAGGGTGGCTGGTGTTG	147	60

mJmjd1a	NM_0173001.1	F-ACCATGGTCCAGCAAAGTTC R-TTCCCACTGGTAAGGTCAGC	143	60
mIFNγ-R1	NM_010511.2	F:ATTCCTGCACCAACATTTCTG R:ACGGAGAGCTGTTCTTCCTTC	208	60
mOCT3/4	NM_013633	**F:** AGCCGACAACAATGAGAACC **R:** TGATTGGCGATGTGAGTGAT	168	61
mSOX2	NM_011443	**F:** TACCTCTTCCTCCCACTCCA **R:** TCTCCAGTTCGCAGTCCAG	212	61
mNanog	NM_028016.2	F:AGGGTCTGCTACTGAGATGTCCTG R:CAACCACTGGTTTTTCTGCCACCG	364	68
mTbx3	NM_011535.2	F: AGATCCGGTTATCCCTGGGAC R: CAGCAGCCCCCACTAACTG	200	60
mKlf4	NM_010637.2	F-CCAAAGAGGGGAAGAAGGTC R-CTGTGTGAGTTCGCAGGTGT	124	60
mINDO	NM_008324.1	F:GTACATCACCATGGCGTATG R:CGAGGAAGAAGCCCTTGTC	245	60
mCTSB	NM_007798.2	F:GGCTTTGACTGCAGGACTTC R:GGCTTTGACTGCAGGACTTC	205	60
mTGF-β	NM_011577.1	F:TGAGTGGCTGTCTTTTGACG R:TCTCTGTGGAGCTGAAGCAA	293	60
hINDO	NM_002164.5	F:GCGCTGTTGGAAATAGCTTC R:CAGGACGTCAAAGCACTGAA	234	60
hCTSB	NM_001908.3	F:CACTGACTGGGGTGACAATG R:AACCACAGGCTGGGATGTAG	293	60
hTGF-β	NM_000660.3	F:GGGACTATCCACCTGCAAGA R:CCTCCTTGGCGTAGTAGTCG	239	60

V.1.8. Antibodies used for immunoblotting

Antibody specificity	Catalogue #, clone	Provider	Working dilution
STAT1	sc-346, E-23	Santa Cruz	1:500
pSTAT1 (Y701)	sc-8394, A-2	Santa Cruz	1:1000
STAT3	sc-483, K-15	Santa Cruz	1:1000
Alkaline phosphatase conjugated anti-rabbit IgG	A-3687	Sigma	1:10,000
Alkaline phosphates conjugated anti-mouse IgG	A-3562	Sigma	1:10,000

V.1.9. Antibodies used for flow cytometry

Antibody specificity	Catalogue #, clone	Provider	Working dilution
IgG-PE	P8547	Sigma	1:100
pSTAT1 (Y701)-alexa488	612596	BD Pharmingen	1:10
IgG2a-alexa488	558055, MOPC-173	BD Pharmingen	1:10
H-2K^{b}-PE	553570, AF6-88.5	BD Pharmingen	1:100
IgG2a-PE	Sc-2867	Santa cruz	1:100
SSEA-1	sc-21702, 480	Santa Cruz	1:100
IgM	sc-3881	Santa cruz	1:100
IFNgR-α chain-biotin	558771, GR20	BD Pharmingen	1:100
Rat-IgG2a	sc-3883	Santa cruz	1:100
IFNgR-β chain-biotin	559917	BD Pharmingen	1:100
Hamaster-IgG-biotin	554010	BD Pharmingen	1:100
Tra-1-81-FITC	sc-21706	Santa Cruz	1:100
IgM-FITC	F9259	Sigma	1:100

V.1.10. siRNA sequence

STAT3 siRNA ID: s74452 (Ambion)

Sense strand (5'-3'): GAGUUGAAUUAUCAGCUUAtt

Antisense strand (5'-3'): UAAGCUGAUAAUUCAACUCag

Scrambled siRNA AlexaFluor 647

All star negative control siRNA-647 (QIAGEN)

V.1.11. Glassware and plastics

Description	Supplier
Tissue culture 6-,12-, 24- ,48- and 96-well plates	TPP
Tissue culture dishes	Falcon, BD
Tissue culture flasks	(Cellstar) Greiner
Cell-strainer (40uM)	Falcon, BD
CryoTube 1.8ml vials	NUNC
Disposable scalpel	FEATHER
Electroporation cuvette (0.4 cm, gap 50)	Bio-RAD
Pipette tips	SARSTEDT, Labomedic
PP-test tubes	(Cellstar) Greiner
Safe lock tubes	Eppendorf
Serological pipettes	(Cellstar) Greiner
LS Columns plus tubes (general items)	Miltenyi Biotec
Neubauer improved counting chamber	Laboroptik

V.1.12. Instruments and devices

Description	Supplier
FACScan (with CellQuest® software)	Becton Dickinson
Gamma counter	PerkinElmer
MACS MultiStand	Miltenyi Biotec
Centrifuge 5417R	Eppendorf
Centrifuge Varifuge RF	Heraeus Sepatech
BioVortex V1	G Kisker

Cell Chamber Neubauer Improved	Neubauer
Gel Electrophoresis Power Pac 300	Bio-RAD
Gel Imager and UV-System	Intas
Gene Pulser Electroporation System	BIO-RAD
Pipettes	GILSON, Eppendorf
Thermocycler Mastercycler personal	Eppendorf
Thermomixer Comfort	Eppendorf
Sterile Bench	Thermo Scientific
Nitrogen Tank Cryotherm	CHRONOS
Microscope Axiovert 10	ZEISS
Microscope TELAVAL 31	ZEISS
Microwave oven Easytronic	Whirlpool

V.2. Methods

V.2.1. Culture of undifferentiated ESCs/iPSCs and other cell lines

Human ES cell line HES-2 was generated by the ES Cell International (Singapore, http://www.escellinternational.com/about_esi/index.html) and obtained from the repository at the WiCell Research Institute (Madison, WI, USA, http://www.wicell.org/). The ES cells were maintained on irradiated MEFs in DMEM/F12 medium supplemented with Glutamax, 20% knockout serum replacer, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and with 4 ng bFGF (Peprotech, Rocky Hill, NJ, USA, www.peprotech.com). Culture media were changed daily and cells were passaged by manual dissection of cell clusters every five to six days.

D3 ES cell-derived transgenic clone αPIG44, E14, OG2, and Dnmt KO ES cell lines were maintained on irradiated MEFs in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1X nonessential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1,000 U/ml LIF (Chemicon). Murine CGR8 ES cells were maintained on 0.1% gelatin-coated tissue culture plates in Glasgow modified Eagle's medium supplemented with 10% FBS, 1X nonessential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 100 U/ml LIF. All ES cell lines used in present study are of H-2K^b MHC haplotype. Induced pluripotent stem cells (iPSCs) AT25 were maintained in murine ES cell culture media and originally derived from tail tip fibroblasts of mouse (kind gift from Rudolf Jänisch (Whitehead Institute, USA) and Alexander Meissner (Harvard Stem cell Institute, USA). C57sv fibroblasts were grown in DMEM containing 10% FBS and the YAC-1 lymphoma cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS. All culture media and reagents were supplied by Invitrogen (Karlsruhe, Germany). In all analyses, the cells were stimulated with 100 ng/ml interferon gamma (IFNγ) for 2 days prior to analysis.

V.2.2. RT-PCR and quantative RT-PCR

Total RNA was isolated from human ES cells, murine C57sv fibroblasts, murine ES cells, scrambled siRNA treated ES cells and STAT3 siRNA treated ES cells using TRIzol Reagent (Invitrogen). DNase I-treated total RNA (500 ng or 1 μg) was reverse-transcribed using Superscript II RTase (Invitrogen) and random hexamers for priming. cDNA was diluted 1:4 with sterile tri-destilled water and 5 μl were amplified using JumpStart^{TM} RedTaq ReadyMix^{TM} PCR Reaction Mix (Sigma). Negative controls were generated in RT reactions in which all reaction components were included except reverse transcriptase. Reactions were terminated at the exponential phase of amplification and products were analyzed by 1.5% agarose gel electrophoresis. For quantitative RT-PCR the cDNA probes were diluted 1:20 and 2 μl was amplified using SYBR Advantage qPCR Premix (Clontech Laboratories, Inc. Takara bio Company) in triplicate for each sample and each gene. Real-time PCRs were performed in a 7500 Standard System Real time Cycler (Applied Biosystems) and analyzed with SDSShell 1.4 software (Applied Biosystems). GAPDH was used for normalization of expression levels of individual genes.

V.2.3. STAT3 knock down and its validation in ESCs

STAT3 mRNA in murine ES cells was downregulated by using pre-designed small interfering RNA (siRNA) specific for STAT3 gene (Oligo ID s74452, Ambion). All-star scrambled siRNA conjugated to Alexa Fluor 647 was used as a negative control. One day before STAT3 knock down, 100,000 ES cells were plated in each 0.1% gelatine-coated well of a 6-well plate. Next day, the cells were transfected with respective siRNA using Opti-MEM serum reduced medium and Lipofectamine 2000 (Invitrogen) for 18 hrs. After 18 hrs, ES cell media was refreshed and all experiments were performed after 48 h of lipofection. STAT3 KD in ES cells was validated by qRT-PCR and immunoblotting. IFNγ (100 ng/ml) was added to ESCs.at the time of lipofection with siRNA

V.2.4. Immunoblotting

For immunoblotting, proteins were isolated using protein lysing buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 5 mM EDTA; 1% protease inhibitor cocktail (sigma) and 1% phosphatase inhibitor cocktail (sigma)). 15 µg of protein was loaded per lane of 10% seperating gel and 4% stacking gel and run by PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Bio-Rad, www.bio-rad.com). Proteins were transferred onto methanol-activated PVDF transfer membrane using wet transfer system (Schleicher and Schuell, BioScience) in transfer buffer (25mM Tris, 192mM Glycine and 10% methanol). The membrane was blocked by 5% skimmed milk for 1 h at room temperature, incubated with the primary antibodies overnight at 4°C and proteins of interest were detect by incubation with alkaline phosphatase-conjugated secondary antibodies for 30 min at room temperature. The signal was detected using chemiluminescent substrate by incubating the membrane in CDP-star (Applied Biosystems) buffer (2.5 M 2-amino 2-methyl-1-propanol,20mM $MgCl_2$) for 1-2 min and subsequent exposure of x-ray film (18x24cm, 28906836, Amersham Hyperfilm ECL). The list of antibodies used is provided in Table in Chapter V1.9.

V.2.5. Flow cytometric analyses

C57sv fibroblasts and undifferentiated ES cells were dissociated using 0.05% Trypsin-EDTA (Invitrogen) and 2-5x10^5 cells were stained for 30 minutes at 4°C in 100 µl of 0.1% FBS in PBS containing an appropriate dilution of a desired antibody for surface markers. For detection of pSTAT1, intracellular staining was performed by fixing the cells using 2% paraformaldyhyde for 10-15 min at room temperature followed by permealization using ice cold methanol for 30 min on ice. Cells were then stained with specific pSTAT1 antibody or proper isotype control. Flow cytometric analyses were performed on a FACScan (BD Biosciences), and data were analyzed using WinMDI2.8 software (BD Pharmingen). Dead

cells were excluded by gating on viable cells on the basis of staining with propidium iodide or 7-aminoactinomycin D and 10,000 events were acquired for each analysis. Primary antibodies used were phycoerythrin (PE)-conjugated anti-$H2K^b$ (clone AF6-88.5; BD Pharmingen, cat. no. 553570, 1:100), biotin conjugated anti-IFNγ-receptor α-chain (clone GR20, BD Pharmingen, cat. No. 558771, dilution 1:100), purified anti-IFNγ-receptor β-chain antibody (BD Pharmingen, cat. no. 559917, dilution 1:100), Alexa Fluor 488-conjugated anti-pStat1 (pY701) (BD Pharmingen, cat. No. 612596, dilution 1:10), Alexa Fluor 488 anti-IgG2a (clone MOPC-173, cat. No. 558055, BD Pharmingen, dilution 1:10). PE- or straptividin-conjugated secondary antibodies and all isotype control antibodies were purchased from BD Pharmingen or Santa Cruz Biotechnology Inc (Santa Cruz).

V.2.6. Luciferase reporter assay

Luciferase reporter assay was performed to determine the promoter response to IFNγ in native and STAT3 KD ES cells. For the assay, 10,000 ESCs were plated in each well of a 96-well flat-bottom white plate (Cat no.136101, Nunc) that was pre-coated with 0.1% gelatine and transiently transfected with a mixture of vectors (25ng/well). This mixture is composed of the STAT1/STAT1-responsive firefly luciferase construct encoding the firefly luciferase reporter gene under the control of a minimal (m) CMV promoter and tandem repeats of the IFNγ activation sequence (GAS) response element and an another vector with constitutively expressing Renilla luciferase, which serves as an internal control for normalizing transfection efficiencies and monitoring cell viability (Cignal GAS Reporter (luc) Kit, cat. no. CCS-009L, SA Biosciences) using Lipofectamine 2000 (Invitrogen) for 48h. In parallel, ES cells were treated with scrambled or STAT3 siRNA for the same period of time. IFNγ was added to the cells 6h before the measurement of luciferase activity. Luciferase activity was measured by Dual Luciferase Reporter assay system (Promega) as specified by the manufacturer.

V.2.7. Teratoma formation by ES cells in mice

Control si647 and STAT3 KD murine aPIG ES cells were trypsinized, washed twice with PBS, and one million ES cells were injected subcutaneously in 200 µl PBS into the interscapular region of $Rag2^{-/-}\gamma_c^{-/-}$. Both control and STAT3 KD ES cells were injected into the same mice (N=3) but opposite sides to measure the kinetic of teratoma growth. The size of teratoma formation was measured every week using digital caliper gauge.

V.2.8. Activation and isolation of ovalbumin-specific CTLs

In vivo activation of ova-specific CTLs was performed by i.p. injection of ovalbumin (Sigma-Aldrich; 2.5 µg per mouse in 2 mg/ml solution of SDA in 500µl ml PBS) into OT-1 transgenic mice. Injections were performed twice at day 0 and 7. After 14 days, spleenocytes were harvested from these mice and cultured for 1 week in RPMI medium supplemented with 10% FBS, 100 U/ml IL-2 and 10 µM SIINFEKL,. After three weeks of ova (2 weeks) and SIINFEKL (1 week) stimulation, CTLs were harvested using a positive-selection magnetic cell sorting kit (Miltenyi Biotech) with antibodies directed against CD8a molecules (clone Ly-2) specifically expressed on T cells.

V.2.9. Activation and isolation of syngenic NK cells

In vivo activation of NK cells was performed by i.p. injection of poly(I:C) (Sigma-Aldrich; 200 µg per mouse in 200 µl of PBS buffer) into wild type C57BL/6 mice 48h before mice were sacrificed and spleenocytes isolated. After lysis of erythrocytes using red blood lytic buffer, NK cells were purified using a positive-selection magnetic cell sorting kit (Miltenyi Biotech) with antibodies directed against CD49b molecules (clone DX5) specifically expressed on NK cells. The purity of 90% was achieved as assessed by flow cytometry analysis.

V.2.10. ^{51}Cr-release cytotoxicity assay

To perform NK cell or T cell cytotoxicity assays, target cells (1X 10^6) were labeled with 10 μl (1000 counts per second, cps) of $Na_2\,^{51}CrO_4$ (Hartmann-Analytic GmbH) for 1h at 37°C. NK cells or Ova-specific CTLs (effector cells) were plated at the appropriate densities to achieve the desired effector-to-target (E:T) ratios in the range of 1:12.5 -1:100. First, 100 μl culture media without any cells was added into wells for E:T ratio of 1:12.5, 1:25, 1:50 and spontaneous release wells (in triplicates) of a 96-well plate. 200 μl containing 6X 10^5 of effector cells were then added only into the wells of 1:100 E:T ratio and 100 μl of effector cells from 1:100 wells were transfered into 1:50 well containing already 100 μl of culture media. Now after mixing the cell suspension in 1:50 ratio wells, 100 μl media was transferred into 1:25 ratio wells and then into 1:12.5 ratio wells following the serial dilution principle. After pipetting the effector cells into the wells, 3X 10^3 target cells in 100 μl media were transferred into the each well. For maximum release, 100 μl of 10% Triton-X100 was transferred into the well containing only 100μl of target cells. After 4 hours of incubation, plates were centrifuged to take cell culture supernatants (100 μl per well) and the radioactivity in the supernatant was determined in a gamma counter (PerkinElmer Life and Analytical Sciences, Gamma counter). The percentage of specific ^{51}Cr-release was determined by the following equation: specic lysis (%) = [(experimental release-spontaneous release)/(maximum release-spontaneous release)] X 100. All groups were run in triplicate and all experiments were performed at least three times.

V.2.11. Interferon-γ enzyme-linked immunosorbent assay

The concentration of IFNγ released by activated CTLs into medium with various target cells at an E:T ratio of 50:1 was determined in the cell culture supernatant using DuoFlow IFNγ ELISA kit (R&D systems, Cat No. MFI00) according to the manufacturer's instructions using the 96-well flat bottom transparent plates (cat no.456537, MaxiSorp96-well, Nunc). Murine

recombinant IFNγ provided in the kit was used to generate the standard curve. According to the kit, the optical density of each well was determined using a microplate reader (GenyosPro, Tecan) and the 450 nm absorbance filter.

V.2.12. Generation of ES cell-derived cardiomyocytes

ES-CMs were generated from a transgenic clones of D3 ES cells (αPIG44) and ova-expressing αPIG44 ES cell subclone A4 by modification of a previously used mass culture protocol (Kolossov et al. 2006). Cardiomyocytes that can be obtained from these transgenic ES cell lines after puromycin selection are predominantly of atrial-like and pacemaker-like type. To initiate the ES cell differentiation, 1x 10^6 ES cells were suspended in 14 ml of differentiation medium (IMDM supplemented with 20% FBS, 10 μM 2-mercaptoethanol, and 1X nonessential amino acids) and incubated in non-adherent plates under continuous horizontal agitation to allow formation of EBs. After 2 days the EBs were diluted into fresh nonadherent plates to a density of 1,000 EBs per 14 ml of differentiation medium per plate. The differentiation of EBs continued on a horizontal shaker without medium change until EGFP-positive EBs occurred on days 8-9 of differentiation. At this time, fresh medium supplemented with puromycin (8 μg/ml) was added to select for ES-CMs. Medium containing puromycin was changed every 2-3 days until pure beating cardiac clusters were collected at day 16 of differentiation. For analyses, cardiac clusters were enzymatically dissociated into single ES-CM after 7 days of puromycin treatment.

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VIII. Erklärung

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

Luciferase reporter assay was performed in collaboration with Dr. Benjamin Yazdanpanah. Experiments on iPSCs were done in cooperation with Azra Fatima. FACS staining of IFNγ-β-receptors was performed by Dr.Dr.Tomo Saric. Ova expressing murine ESCs (A4) clone was generated by Charis Satrazami and CTLs assays on ES-CMs were done with the help of Lukas P. Frenzel.
Complete figure legends copied from other sources were put with quotation marks in this thesis.

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