VISUALIZATION OF TYPE I IMMUNITY USING BICISTRONIC IFN-γ REPORTER MICE *IN VITRO* AND *IN VIVO*

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Der Beginn aller Wissenschaften ist das Erstaunen, dass die Dinge sind, wie sie sind.

Aristoteles

CONTENT **PAGE**

Ab	**A**nti**b**ody
ABF	**A**nimal **b**reeding **f**acility
AIDS	**A**cquired **I**mmune **D**eficiency **S**yndrome
APC	**A**ntigen-**p**resenting **c**ell
ATF	**A**ctivating **t**ranscription **f**actor
B6 WT	C57BL/6 mouse strain
BAL	**B**ronchoa**l**veolar **l**avage
BM	**B**one **m**arrow
BSA	**B**ovine **s**erum **a**lbumin
CCL	**CC** motif chemokine **l**igand
CCR	**CC** motif chemokine **r**eceptor
CD	**C**luster of **d**ifferentiation
cJUN/JUN	v-jun sarcoma virus 17 oncogene homolog (avian)
CREB1	**C**yclic AMP-**r**esponsive-**e**lement-**b**inding protein **1**
cRPMI	**C**omplete **RPMI**
Ct	**C**ycle **t**hreshold
CTL	CD8+ **c**ytotoxic **T** **l**ymphocyte
CXCL	**CXC** motif chemokine **l**igand
CXCR	**CXC** motif chemokine **r**eceptor
DAPI	4',6-**D**i**a**midino-2-**p**heny**l**indole)
DC	**D**endritic **c**ell
DNA	**D**eoxyribo**n**ucleic **a**cid
EID_{50}	50% **e**gg **i**nfectious **d**ose
ELISA	**E**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay
EMCV	**E**ncephalo**m**yo**c**arditis **v**irus
ERK	**E**xtracellular signal-**r**egulated MAP **k**inase
eYFP	**E**nhanced **y**ellow **f**luorescent **p**rotein
eYFPhi	high eYFP
eYFPint	intermediate eYFP
eYFPneg	eYFP negative
FACS	**F**luorescence-**a**ctivated **c**ell **s**orting
FcεRI	**Fc** **e**psilon **r**eceptor I
FcγRIII	**Fc** **g**amma **r**eceptor III
FCS	**F**etal **c**alf **s**erum

Fyn	Proto-oncogene protein c-**fyn**
GADD45	**G**rowth **a**rrest and **D**NA-**d**amage inducible protein **45**
GAF	**G**amma-**a**ctivating **f**actor
GAPDH	**G**lycer**a**ldehyde-3-**p**hospate **d**e**h**ydrogenase
GAS	**G**amma-**i**nterferon **a**ctivation **s**ite
GATA-3	**GATA** binding protein **3**
GKO	B6.129S7-*Ifng*^{tm1Ts}/J mouse strain
GM-CSF	**G**ranulocyte/**m**acrophage **c**olony-**s**timulating **f**actor
H	Hemagglutinin
HLX	**H**2.0-**l**ike homeobo**x** 1
HN	**H**emagluttinin-**n**euraminidase
hPIV1	**H**uman **p**ara**i**nfluenza **v**irus type **1**
HVJ	**H**emagglutinating **v**irus of **J**apan (Sendai Virus)
IACUC	**I**nstitutional **A**nimal **C**are and **U**se **C**ommittee
Ig	Immunoglobulin
IGTP	**I**nducible expressed **GTP**ase
IFN-γ	**I**nter**f**eron **g**amma
IFN-γR	**I**nter**f**eron **g**amma **r**eceptor
IFNs	**I**nter**f**erons
IL	**I**nter**l**eukin
IL-12R	IL-12 receptor
i.n.	**I**ntra**n**asal
iNOS	Inducible **n**itric **o**xide **s**ynthase
i.p.	**I**ntra**p**eritoneal
IP-10	IFN-**i**nducible **p**rotein10, CXCL10
IRAK	**I**L-1 **r**eceptor-**a**ssociated **k**inase
IRES	**I**nternal **r**ibosomal **e**ntry **s**ite
IRF	**I**nterferon **r**egulatory **f**actor
ISRE	**I**nterferon-**s**timulated **r**esponse **e**lement
i.v.	**I**ntra**v**enous
JAK	**Ja**nus **k**inases
JNK	c-**J**un NH_2-terminal **k**inase
kDa	**K**ilo **Da**lton
KO	**K**nock **o**ut

LCMV	**L**ymphocytic **c**horio**m**eningitis **v**irus
LRG-47	Alias for "immunity related GTPase family, M" (IRGM)
mAb	**M**onoclonal **a**nti**b**ody
MAPK	**M**itogen **a**ctivated **p**rotein **k**inase
MBCF	**M**olecular **B**iology **C**ore **F**acility
MCM	**M**ini**c**hromosome **m**aintenance
MCP-1	**M**onocyte/macrophage **c**hemoattractant **p**rotein-1; CCL2
medLN	**Med**iastinal **l**ymph **n**ode
mesLN	**Mes**enteric **l**ymph **n**ode
MFI	**M**edian **f**luorescence **i**ntensity
MHC	**M**ajor **h**istocompatibility **c**omplex
MIP-1α/β	**M**acrophage **i**nflammatory **p**rotein-1α and β
mRNA	**M**essenger **RNA**
MyD88	**My**eloid **d**ifferentiation primary response gene-**88**
N	**n**euraminidase
NFAT	**N**uclear **f**actor of **a**ctivated **T** cells
NF-κB	**N**uclear **f**actor **k**appa **B**
NK	**N**atural **k**iller cell
NKT	**N**atural **k**iller **T** cell
NLS	**n**uclear **l**ocalization **s**equence
NO	**N**itric **o**xide
NP	**N**ucleo**p**rotein
NPI-1	Importin-α-1
OCT1	**Oct**amer binding **t**ranscription factor-**1**
OVA	**Ova**lbumin
PA	**A**cid **p**olymerase
PBS	**P**hosphate-**b**uffered **s**aline
PE	**P**hyco**e**rythrin
PerCP	**Per**idinin **c**hlorophyll **p**rotein
PHA	**P**hyto**h**em**a**gglutinin
PI	**P**ropidium **i**odide
PIVs	**P**ara**i**nfluenza **v**iruses
PLC	**Pl**eural exudates **c**ells
PMA	**P**horbol **m**yristate **a**cetate
PR8	Influenza strain A/**PR8**/34/H1N1

Pyk2	**P**rolin-rich t**y**rosine **k**inase 2
RANTES	**R**egulated on **a**ctivation, **n**ormal **T** **e**xpressed and **s**ecreted
RNA	**R**ibo**n**ucleic **a**cid
RSV	**R**espiratory **s**yncitial **v**irus
RT	**R**oom **t**emperature
RT-PCR	**R**everse **t**ranscription-**p**olymerase **c**hain **r**eaction
SA	**S**trept**a**vidin
SDF-1	**S**tromal cell-**d**erived **f**actor-1
SenHN	**Sen**dai virus hemagluttinin-neuraminidase epitope $\mathbf{HN}_{419\text{-}433}/A^b$
SenNP	**Sen**dai virus nucleoprotein epitope $\mathbf{NP}_{324\text{-}332}/K^b$
SOCS	**S**uppressors **o**f **c**ytokine **s**ignaling
STAg	**S**oluble ***T****oxoplasma* **a**nti**g**en
STAT	**S**ignal **t**ransducers and **a**ctivators of **t**ranscription
T-bet	**T**-**b**ox **e**xpressed in **T** cells
Tc1	Cytotoxic T cell type I
TCR	**T**-**c**ell **r**eceptor
TCRβδKO	B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J mouse strain
TGF-β	**T**ransforming **g**rowth **f**actor beta
Th1	**T** **h**elper cell type 1
tk	**T**hymidine **k**inase
TNF	**T**umor **n**ecrosis **f**actor
TRAF	**T**NF-**r**eceptor **a**ssociated **f**actor
WHO	**W**orld **H**ealth **O**rganization
X31	Influenza strain A/HK-**x31**/H3N2
Yeti	**Y**ellow **e**nhanced **t**ranscript for **I**FN-γ

1. ZUSAMMENFASSUNG

Typ I Immunantworten, wie z.B. gegen Influenza Virus, Sendai Virus aber auch gegen intrazelluläre Erreger wie *Toxoplasma gondii* sind klassischerweise durch robuste IFN-γ Expression gekennzeichnet. Th1 und CD8+ Effektor T Zellen zählen zu den Hauptproduzenten von IFN-γ. Im Zusammenhang mit Autoimmunerkrankungen, Immunpathologie aber auch Impfstoffentwicklung, ist es überaus wichtig die Regulierung von IFN-γ zu verstehen. In der vorliegenden Arbeit wurde die IFN-γ Expression von CD4+ und CD8+ T Zellen detailliert charakterisiert. Des Weiteren wurde die Rolle des IFN-γ Rezeptors für die IFN-γ Expression von T Zellen untersucht. Unter Zuhilfenahme von bicistronischen IFN-γ-eYFP Reporter Mäusen, welche die direkte Identifizierung und Isolierung von vitalen IFN-γ exprimierenden Zellen ermöglichen, wurde die Expression von IFN-γ *in vitro* und *in vivo,* nach Infektion mit den bereits erwähnten Erregern, visualisiert.

Die Expression des IFN-γ-eYFP Reporters zeichnete sich, sowohl *in vitro* als auch *in vivo* nach Infektion, durch ein äußerst heterogenes Fluoreszenzspektrum aus. Die Helligkeit der Reporter Fluoreszenz korrelierte positiv mit der Menge an IFN-γ Transkripten und mit der Menge des sekretierten IFN-γ Proteins nach Stimulierung. Die Helligkeit des Reporters reflektierte das Potenzial zur IFN-γ Produktion, die eigentliche Sekretion war jedoch weitgehend abhängig von zusätzlicher Stimulierung durch Antigen. Des Weiteren korrelierte die Helligkeit des Reporters mit der zunehmenden Produktion von weiteren proinflammatorischen Zytokinen und Chemokinen. Hoch fluoreszente Zellen exprimierten zudem vermehrt Marker auf ihrer Oberflache, die auf akute Aktivierung hinweisen. Die am hellsten eYFP fluoreszierenden Zellen waren im Allgemeinen weiter ausdifferenziert und ihre Präsenz war auf bestimmte Organe beschränkt. Die anatomische Begrenzung wurde durch den Erreger bestimmt.

IFN-γ exprimierende Zellen wurden nach Infektion mit Sendai Virus oder *Toxoplasma gondii* in IFN-γ Rezeptor defizienten Reporter Mäusen generiert. Die Frequenz und die Helligkeit der eYFP Reporter Expression waren jedoch verändert. Experimente mit dualen Knochenmarks-Chimären Mäusen, welche mit Wild-Typ und IFN-γ Rezeptor defizientem

Knochenmark rekonstituiert wurden, ergaben eine T Zell-intrinsische Abhängigkeit von IFN-γ Rezeptor vermittelten Signalen für die Expression von IFN-γ. Die Helligkeit des Reporters dagegen wurde unabhängig von dem IFN-γ Rezeptor reguliert. Abschließend wurde ein Modell für die Expression von IFN-γ in CD4+ und CD8+ T Zellen entwickelt.
Zusammenfassend führen diese Ergebnisse zu dem Schluss, dass die Expression von IFN-γ in CD4+ und CD8+ T Zellen und nach viraler oder parasitärer Infektion unterschiedlich reguliert wird. Zusätzlich wurde gezeigt, dass der IFN-γ Rezeptor an der Modulation der IFN-γ Expression beteiligt ist.

2. SUMMARY

IFN-γ is the signature cytokine of Th1 and CD8+ effector cells generated in type I immune responses against pathogens, such as Influenza virus, Sendai virus and the intracellular protozoan parasite *Toxoplasma gondii*. Understanding the regulation of IFN-γ is critical for the manipulation of immune responses, prevention of immunopathology and for vaccine design. In the present thesis, IFN-γ expression by CD4+ and CD8+ T cells was characterized in detail and the requirement of IFN-γ receptor mediated functions for IFN-γ expression was assessed. Bicistronic IFN-γ-eYFP reporter mice, which allow direct identification and isolation of live IFN-γ expressing cells, were used to visualize IFN-γ expression *in vitro* and *in vivo* after infection with the afore mentioned pathogens.

Expression of the IFN-γ-eYFP reporter by CD4+ and CD8+ T cells was broadly heterogeneous *in vitro* and *in vivo* after infection. Increased expression of the reporter correlated positively with the abundance of IFN-γ transcripts and IFN-γ protein production upon stimulation. eYFP reporter brightness reflected the potential for IFN-γ production, but actual secretion was largely dependent on antigenic stimulation. Increased expression of the reporter also correlated with enhanced secretion of additional proinflammatory cytokines and chemokines and cell surface expression of markers that indicate recent activation. Highly eYFP fluorescent cells were generally more differentiated and their anatomical distribution was restricted to certain tissues. The anatomical restriction depended on the pathogen.

IFN-γ expressing CD4+ and CD8+ T cells were generated in IFN-γ receptor deficient reporter mice after infection with Sendai virus or *Toxoplasma gondii*. However, in the absence of IFN-γ receptor mediated functions, the frequency and brightness of the eYFP reporter expression was altered. Dual BM chimeric mice, reconstituted with wild-type and IFN-γ receptor deficient reporter BM, revealed a T cell-intrinsic requirement for the IFN-γ receptor for optimal IFN-γ expression. Reporter fluorescence intensities were regulated independently of IFN-γ receptor mediated functions. Finally, we propose a model for IFN-γ expression by CD4+ and CD8+ T cells.

In summary, the expression of IFN-γ is differentially regulated in CD4+ and CD8+ T cells and after viral or protozoan infections. Additionally, the role of IFN-γ receptor mediated functions for the expression of IFN-γ was determined.

3. INTRODUCTION

3.1. Cytokines and the immune system

Cytokines are small protein messengers, secreted mainly by cells of the immune system, which transfer vital information within the immune systems delicate network. They are crucial for communication between cells as they mediate and regulate immunity, inflammation, and hematopoiesis in response to immune stimuli. Although cytokines are heterogeneous in both their molecular structure as well as their effector functions, they have several characteristics in common. They are produced in relatively small quantities but nonetheless display potent biological activity. The effector properties of cytokines, pleiotropic and partially redundant in nature, are exerted by binding to their respective cytokine receptors on target cells that are in close spacial proximity. To band these different immune mediators together S. Cohen and colleagues introduced the term “cytokine” in 1974 (Cohen et al., 1974). Cytokines are now subdivided into five families. The type I cytokines include many of the interleukins (IL) as well as certain hematopoietic growth factors. The type II cytokines are constituted of the interferons and IL-10. The tumor necrosis factor (TNF) related molecules encompass TNF, the lymphotoxins and Fas ligand. IL-1 and IL-18 are members of the Ig super family. Chemokines, small chemoattractant proteins constitute another growing cytokine family (Janeway et al., 2004; Paul, 2003).

The broad field of cytokine research is providing new insights into the regulatory elements that orchestrate immune responses. Therefore, it is not surprising that manipulation of the cytokine network represents a promising strategy in the fight against cancer, autoimmunity and viral infections.

3.2. Interferons

In 1957, Alick Isaacs and Jean Lindemann discovered an antiviral substance able to induce biological interference between chick cells and Influenza virus (Isaacs and Lindenmann, 1957). The responsible substance was then termed "interferon" after the Latin words for *inter* (between) and *ferire* (to strike). Interferons (IFNs) were the first cytokine family to be identified and then cloned. As early as the 1960s separate classes of interferons could be distinguished based upon their secretion by different cell types (i.e. IFN-α by leukocytes and IFN-β by fibroblasts), and later upon their biochemical behavior and induction by viruses (IFN-α and IFN-β) or antigens (IFN-γ). Today IFNs are defined as cytokines that induce cells to resist viral replication and are divided into three subfamilies, type I and type II IFNs and IFN-like cytokines (De Maeyer and De Maeyer-Guignard, 1998; Farrar and Schreiber, 1993; Janeway et al., 2004; Pestka et al., 2004; Stark et al., 1998). The division into type I and type II IFNs is based upon differences in structure, signaling through the respective receptors as well as differences in effector function. Type I IFNs are subdivided into seven classes, encompassing IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ and IFN-ω. Type I IFNs share structural homologies and they all bind to the same heterodimeric receptor, composed of the IFN-αR1 and IFN-αR2 chains. Although originally associated with antiviral properties and despite signaling through a common receptor, type I IFNs exhibit a wide breadth of effector functions, including an important role in the host response to bacterial infections (Decker et al., 2005; Perry et al., 2005). They affect a variety of cellular functions, such as cell growth (antiproliferative effects), viability and antigen presentation (up regulation of major histocompatibility complex (MHC) class I antigens) (De Maeyer and De Maeyer-Guignard, 1998; Decker et al., 2005; Stark et al., 1998). Additionally, type I IFNs induce cytotoxic activity in macrophages, dendritic cells, natural killer (NK) cells, and T cells (De Maeyer and De Maeyer-Guignard, 1998,Pestka, 2004 #103; Stark et al., 1998). Recently, IFN-like cytokines have been reported; so far they include IL-29, IL-28A, IL-28B (also known as IFN-λ1-3). Their function is similar to the type I IFNs, although IL-28 and IL-29 signal through a unique receptor complex composed of the IL-28R1 and IL-10R2 subunits (Kotenko et al., 2003; Sheppard et al., 2003). IFN-γ, also known as immune IFN, represents the sole known member of the type II IFN category.

3.3. IFN-γ

Similar to the type I IFNs, IFN-γ was first recognized on the basis of its antiviral properties after phytohemagglutinin (PHA) stimulation of leukocytes in 1965 (Wheelock, 1965). However, IFN-γ is structurally unrelated to the type I IFNs; it binds to a different receptor, and is encoded by a separate chromosomal locus as a single gene on the human chromosome 12 and the murine chromosome 10, respectively (Pestka et al., 2004). In humans and in mice, IFN-γ is encoded by a single-copy gene, which generates one mRNA-species (1.2 kb) and a polypeptide of 166 residues including a cleaved hydrophobic signal sequence of 23 residues (Derynck et al., 1982; Rinderknecht et al., 1984). In its biologically active form, the IFN-γ protein presents itself is a 34 kDa homodimer, stabilized by noncovalent forces through self-association, and variable N-glycosylation gives rise to a mature form with a predominant molecular mass of 50 kDa (Ealick et al., 1991; Walter et al., 1995). Moreover, specific antigens and mitogens, such as staphylococcal enterotoxin A, B, or the combination of PHA and phorbol esters or ionophores induce IFN-γ expression, while type I IFNs can be directly induced by viruses. Although IFN-γ displays most of the properties that have been associated with type I IFNs, though to a much lesser extent, IFN-γ has now been recognized as a very potent immunomodulatory cytokine beyond its antiviral activity. IFN-γ, a classical pleiotropic cytokine, exerts its multiple effector functions on a wide variety of different cell types through its ubiquitously expressed receptor. The major cellular sources of IFN-γ are CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic T lymphocytes (CTLs), NK and natural killer T (NKT) cells. However, there is some evidence that other cell types, such as B cells, mast cells, macrophages and professional antigen-presenting cells (APCs) are also capable of expressing IFN-γ (Frucht et al., 2001; Gessani and Belardelli, 1998; Gupta et al., 1996; Harris et al., 2005; Harris et al., 2000; Yamaguchi et al., 2005). Production of IFN-γ by NK cells is likely to be important in the innate host defense against infection, whereas T cells represent the major source of IFN-γ in the adaptive immune response (Farrar and Schreiber, 1993; Frucht et al., 2001). IFN-γ is the signature cytokine of type I immune responses, which are induced after infection with intracellular bacteria (e.g. *Listeria monocytogenes*, *Mycobacteria spec.*), protozoa (e.g. *Toxoplasma gondii*) and viruses (e.g. Influenza virus; Sendai virus; lymphocytic choriomeningitis virus, LCMV)

(Abbas et al., 1996; Farrar and Schreiber, 1993). The expression of a variety of genes in different cell types, such as the diverse cells of the immune system but also fibroblasts and epithelial cells, is regulated by IFN-γ. It is conservatively estimated that IFN-γ directly regulates at least 200-500 genes in cells expressing its receptor (Boehm et al., 1997; Ramana et al., 2002). The diversity of target genes is mirrored by the vast diversity of biological functions of IFN-γ (see Table I in appendix). Table I represents by no means a complete account of regulated genes but merely demonstrates the variety of genes regulated by IFN-γ in a broader context of immunological functions. Besides these genes, IFN-γ also regulates certain oncogenes, thyroid-specific genes, genes encoding protein-tyrosine kinases, extracellular matrix and cytoskeletal elements, acute phase reactants, ribosomal RNAs and proteins, lipids and steroids and genes involved in tryptophan and iron metabolism (Boehm et al., 1997).

Within the immune system's network, however, IFN-γ has multiple important functions (Table I). Besides its antiviral activity, it enhances antigen-presentation by professional APCs and conventional cell types through up-regulation of both antigen processing and presentation by MHC class I and II molecules (Decker et al., 2002; Mach et al., 1996). The important role of IFN-γ in viral immune defense is further highlighted by the fact that several viruses encode proteins designed to interfere with IFN-γ signaling (Alcami and Smith, 1995; Khan et al., 2004). In B cells IFN-γ induces class switch recombination towards IgG2a and IgG3 (Boehm et al., 1997). One of the most important effects of IFN-γ is the activation of macrophages and their microbicidal effector functions, including nitric oxide (NO) production (Cooper et al., 2002; Decker et al., 2002). Moreover, IFN-γ displays antiproliferative properties and has been tightly linked with induction of apoptosis in macrophages and T cells (Dalton et al., 2000; Farrar and Schreiber, 1993; Pestka et al., 2004). Additionally, IFN-γ induces and enhances other cytokines and cytokine receptors involved in immunomodulatory and proinflammatory mechanisms, including the IL-4 receptor, IL-12, TNF-α and its receptor, RANTES (**r**egulated on **a**ctivation, **n**ormal **T** **e**xpressed and **s**ecreted, CCL5) and IP-10 (IFN-inducible protein10, CXCL10)(Boehm et al., 1997; Schroder et al., 2004).

3.4. IFN-γ receptor mediated signaling

IFN-γ exerts its biological functions through binding to its specific receptor (IFN-γR), a heterodimer comprised of a 90 kDa α chain (IFN-γR1, CD119) and a smaller 65 kDa β chain (IFN-γR2; accessory factor-1, AF-1) (Aguet et al., 1988; Aguet and Merlin, 1987; Hemmi et al., 1994; Soh et al., 1994). Both IFN-γR1 and IFN-γR2 belong to the class II cytokine receptor family. The functional IFN-γR complex consists of two high affinity ligand-binding IFN-γR1 chains and two signal-transducing IFN-γR2 chains (Walter et al., 1995). The IFN-γR2 chains do not interact directly with IFN-γ but are believed to stabilize the interaction of IFN-γ with IFN-γR1. Moreover, two IFN-γR complexes are assumed to interact with two IFN-γ homodimers, forming a tetramer (Langer et al., 1994). Binding of homodimeric IFN-γ with IFN-γR1 facilitates the dimerization of IFN-γR1. However, in contrast to previous assumptions, the IFN-γR1 and IFN-γR2 chains are pre-assembled prior to presence of the ligand (Krause et al., 2002). Additionally, the IFN-γ/IFN-γR1 and IFN-γR1/IFN-γR2 interactions are species-specific and conferred by the receptor extracellular domains (Gibbs et al., 1991; Hemmi et al., 1992).

The IFN-γR is expressed on all nucleated cells, and is most highly expressed outside the lymphoid system (i.e. fibroblasts, endothelial cells) (Farrar and Schreiber, 1993; Valente et al., 1992). Though ubiquitously expressed, IFN-γR expression is not uniform on all cell types. Expression of the two IFN-γR chains is differentially regulated. While both chains are expressed constitutively, with IFN-γR1 at moderate levels and IFN-γR2 at extremely low levels on the cell surface, the expression of IFN-γR2 is tightly regulated on certain cell types by cell-extrinsic stimuli. Naïve T cells and CD4+ Th2 cells express IFN-γR2 at relatively high levels. However, CD4+ Th1 cells are unresponsive to IFN-γ stimulation due to the lack of IFN-γR2 expression on developing Th1 but not Th2 cells (Gajewski and Fitch, 1988; Groux et al., 1997; Pernis et al., 1995). However, the lack of IFN-γR2 expression on the surface of Th1 cells is not linked to T helper cell differentiation, but rather to the presence of IFN-γ, which induces down-regulation of its own IFN-γR2 chain. Accordingly, adding exogenous IFN-γ to Th2 cultures similarly results in ligand-induced down-regulation of the IFN-γR2 chain (Bach et al., 1995; Sakatsume and Finbloom, 1996). The loss of IFN-γR2 is therefore a characteristic of the CD4+ T cell response to IFN-γ exposure, rather than the result of a specific genetic

program during Th1 development. This ligand-dependent down-regulation of IFN-γR2 is thought to represent a key mechanism for protecting T lymphocytes from the antiproliferative effects of IFN-γ. This is supported by the fact that other cell types, like fibroblasts and B cells do not lose IFN-γR2 on their surface after exposure to IFN-γ (Bach et al., 1997). Stimulation of T cells with certain phorbol esters or α-CD3 antibodies has been shown to induce up-regulation of IFN-γR2 mRNA expression (Sakatsume and Finbloom, 1996). In contrast to murine CD4+ T cell clones, human T cells appear to regulate IFN-γR2 expression in a ligand-independent manner (Rigamonti et al., 2000). Resting and PHA-stimulated human $CD3^+$ T cells maintain large cytoplasmic stores of IFN-γR2 whereas surface expression remains low. The intracellular pools of IFN-γR2 are the result of constitutive recycling of IFN-γR2 between the cell surface and the cytoplasm. This process has been demonstrated to be ligand-independent, because recycling of IFN-γR2 still occurs in the absence of surface IFN-γR1 or in the presence of neutralizing Abs for IFN-γ (Rigamonti et al., 2000). Allospecific $CD8^+$ T cell lines also maintain expression of IFN-γR1, but are unresponsive to IFN-γ due to down-regulation of IFN-γR2 at the mRNA level (Tau et al., 2001). Transient down-regulation of IFN-γR1 in transgenic CD4+ T cells after exposure to antigen or TCR ligation with antibody *in vitro* has also been reported (Skrenta et al., 2000). Other reports, describe a modest transient down-regulation on transgenic antigen-specific CD8+ T cells within 24 hrs after infection with *L. monocytogenes*-OVA or LCMV *in vivo* (Haring et al., 2005b; Whitmire et al., 2005). However, the *L. monocytogenes* OVA-specific cells remained IFN-γ responsive and these observations are yet to be confirmed in a non-transgenic setting.

IFN-γR signaling is under tight control. Binding of IFN-γ to its receptor causes internalization and dissociation of the complex (Boehm et al., 1997; Farrar and Schreiber, 1993). In most cells, including murine macrophages, internalized IFN-γ is quickly degraded, and IFN-γR1 is efficiently recycled back to the cell surface (Celada and Schreiber, 1987). Upon binding of their cognate ligands, cytokine receptors are responsible for transmitting signals from the cell surface to the inside. Many of the class II cytokine receptors lack intrinsic tyrosine kinase domains. Signaling through the IFN-γR is therefore achieved by phosphorylation of proteins already present and constitutively associated with the receptor α and β chains. It is noteworthy, that studies on the IFN-γR revealed fundamental mechanisms of cytokine signaling. These include the recognition

that tyrosine phosporylation of the cytokine receptor intracellular domain represents the mechanism that links the activated receptor to its signal transduction system. They further include the discovery of a novel pathway of signal transduction that mediates the biological functions of a variety of different cytokines (namely the JAK-STAT-pathway) and the molecular basis of the observed specificity of cytokine–induced cellular responses (Bach et al., 1997; Ivashkiv, 1995). The fundamental principle of the JAK-STAT-pathway lies in the activation of specific members of two protein families, the Janus kinases (JAK) and the signal transducers and activators of transcription (STAT). This pathway is employed by a at least 50 different cytokine receptors of the type I and type II families (Brierley and Fish, 2005; Igarashi et al., 1994; Ihle, 1996; Kisseleva et al., 2002; O'Shea et al., 2002). Four JAKs (JAK1-4) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) have been described in mammals. JAKs are directly associated with the intracellular domains of the respective cytokine receptor chains. Upon ligand binding of the receptor, JAKs "trans-activate" each other through phosphorylation and subsequently phosphorylate the receptor. STATs, constitutively present in the cytosol, bind to the phosphorylated receptor, and are in turn activated by tyrosine phosphorylation through JAKs. After activation they can form homodimers or heterodimers. These translocate to the cell nucleus where they either directly bind to DNA or act together with other DNA-binding proteins or transcription factors to form multiprotein transcription complexes that regulate gene expression. Accordingly, IFN-γR1 and IFN-γR2 are constitutively associated with their respective Jamus kinases; IFN-γR1 with JAK1 and IFN-γR2 with JAK2 (Bach et al., 1997) (Figure 1). Although pre-assembled, the IFN-γR complex becomes only activated upon binding of IFN-γ with IFN-γR1 and the intracellular domains of the receptor chains change structurally to allow association of JAK1 and JAK2. Additionally, binding of IFN-γ to IFN-γR1 induces autophosporylation and activation of JAK2, which in turn transphosphorylates JAK1 (Igarashi et al., 1994). The activated JAK1 then phosphorylates a functionally critical tyrosine on residue 440 of each IFN-γR1 chain, which function as the docking sites for the latent cytosolic STAT1. STAT1, which is already dimerized prior to activation (Braunstein et al., 2003; Ota et al., 2004), is recruited to the receptor where it is then phosphorylated at a tyrosine on residue 701 by JAK2 (Briscoe et al., 1996) (Figure 1). The tyrosine phosphorylation induces the dissociation of the now activated STAT1 homodimer from the receptor complex (Greenlund et al., 1995). In addition to the phosphorylation at

tyrosine 701, STAT1 becomes phosphorylated at a serine residue on 727, which is essential for maximal ability to activate transcription of target genes (Wen et al., 1995; Zhang et al., 1995). The phosphorylated STAT1 homodimer forms a complex known as the gamma-activating factor (GAF), which translocates to the nucleus and controls the transcription of IFN-γ regulated genes. This migration through the cytoplasm was shown to be the result of a random walk movement, rather than liberation from a cytoplasmic anchor or transport by microtubules (Lillemeier et al., 2001). While the nuclear membrane forms an efficient barrier to inactivated STAT1, the entry requires a special amino acid sequence known as the nuclear localization sequence (NLS). Moreover, since GAF does not display NLS function, and IFN-γ itself has been shown to posses a NLS sequence, required for full biological activity, a model has been proposed in which STAT1 translocates with requirement for intracellular IFN-γ (Lillemeier et al., 2001; Lundell et al., 1991; Subramaniam et al., 2000; Subramaniam et al., 1999). Nuclear accumulation of

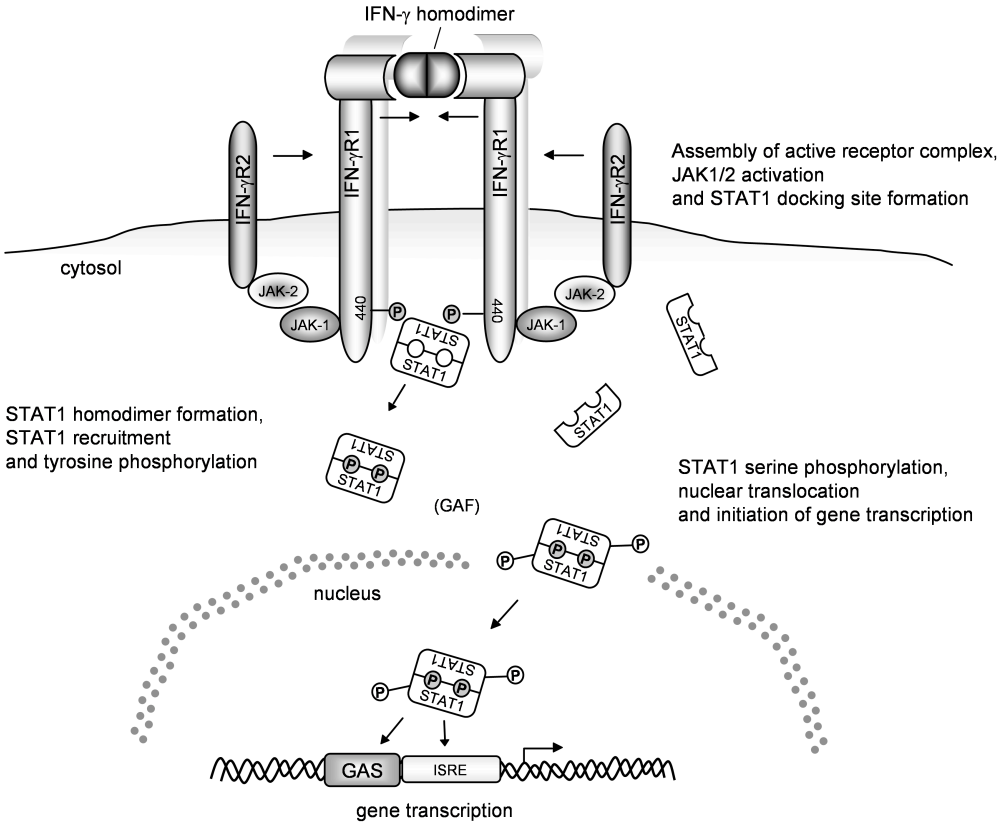


Figure 1:
Schematic signaling mechanism of IFN-γ. The details of the model are described in the text.

IFN-γ was first observed 20 years ago and since then has been linked to induce MHC I expression on macrophages (Bader and Weitzerbin, 1994; MacDonald et al., 1986; Smith et al., 1990). According to the model, STAT1 forms a cytosolic complex with IFN-γ, internalized IFN-γR1 and importin-α-1 (NPI-1) which is then utilizing the NLS sequence of IFN-γ to translocate into the nucleus (Subramaniam et al., 2001). Once STAT1 crosses the nuclear membrane it binds to specific sequences, the gamma-interferon activation site (GAS) and the interferon-stimulated response element (ISRE), in the promoter regions of IFN-γ regulated genes to initiate or suppress transcription (Figure 1) (Darnell et al., 1994; Ramana et al., 2002). Recently, cofactors of the minichromosome maintenance (MCM) family, assumed to act mainly as essential helicases for DNA replication, were found to associate with STAT1 at the promoter of IFN-γ target genes to induce optimal transcription (Snyder et al., 2005).

IFN-γ signaling through homodimeric STAT1 represents the classical pathway after receptor complex activation. However, there are several additional STAT1-independent signaling pathways activated by IFN-γ, which play important roles in IFN-γ-induced biological responses and regulation of gene expression (Ramana et al., 2001). These pathways involve the activation of signal-transduction proteins such as mitogen-activated protein kinases (MAPKs) Pyk2 and ERK1/2, the Src-family kinase Fyn, the adapter protein Vav and the SH2-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 (English et al., 1997; Ramana et al., 2002; Takaoka et al., 1999; Uddin et al., 1997; You et al., 1999). Using microarray-technology, STAT1-independent regulated genes were identified in macrophages, including monocyte chemoattractant protein-1 (MCP-1; CCL2), macrophage inflammatory protein-1α and β (MIP-1α/β; CCL3/4) and IL-1β (Gil et al., 2001).

Dysfunction of IFN-γ mediated signaling has been extensively studied in patients, where loss-of-function mutations in the IFN-γR chains lead to increased susceptibility to bacterial, parasitic and viral infections (Jouanguy et al., 1997; Roesler et al., 1999; Rosenzweig and Holland, 2005). Similarly, IFN-γ and IFN-γR1 knockout mice display deficiencies in natural resistance to bacterial infection with low virulence mycobacteria species, *Listeria monocytogenes* (*L. monocytogenes*), parasitic infections with *Leishmania major* (*L. major*), *Toxoplasma gondii* (*T. gondii*) and viral infections such as vaccinia virus and LCMV (Dalton et al., 1993; Huang et al., 1993; van den Broek et al., 1995).

Mice that were genetically engineered to constitutively express IFN-γR2 (IFN-γR2 transgenic mice) were unable to mount productive Th1 immune responses to *L. monocytogenes* or *Leishmania*, and thus resembled IFN-γ deficient mice (Tau et al., 2000). Allospecific $CD8^+$ T cell lines made from these mice had impaired cytotoxic capabilities in vitro despite being able to produce IFN-γ and proliferate in response to antigen (Tau et al., 2001). These studies suggest that regulation of IFN-γ responsiveness is required for normal T cell function.

3.5. Regulation of IFN-γ expression in effector T lymphocytes

3.5.1. IFN-γ expression in T helper type 1 lymphocytes

Strategies of resistance towards pathogens include both innate and adaptive immune responses. Adaptive immunity can further be divided into antibody-mediated (also known as humoral immunity) or a cell-mediated forms of immunity (i.e. activation of cytolytic and phagocytic pathways). Despite the variety of cell types partaking in the fight against unwanted invaders, CD4+ effector T cells play pivotal roles in determining the outcome of infections. Especially the development of an appropriate CD4+ T helper (Th) response during infections is critical for mounting an effective immune response against pathogens. Thus, adaptive immune responses against pathogens are usually driven towards either a cell-mediated response with a predominant CD4+ T helper type 1 (Th1) phenotype (type I immune response) or a humoral immune response where CD4+ T helper type 2 (Th2) cells are most abundant (type 2 immune response). The Th1 phenotype dominates and directs the immune response against intracellular pathogens such as *Mycobacterium tuberculosis*, *T. gondii* as well as viral infections. The Th1 phenotype is characterized by intensive production of IFN-γ. In contrast, the Th2 phenotype is associated with immune responses against extra-cellular pathogens, such as parasitic helminth infections, and is prominent in allergic responses, with IL-4, IL-5 and IL-13 as signature cytokines (Figure 2) (Glimcher, 2001; Mosmann et al., 1986; Mosmann and Sad, 1996; Murphy and Reiner, 2002b). The cytokine expression profile of each subset is regulated by cell-specific transcription

factors. In Th1 cells, IFN-γ production and lineage commitment is largely controlled by T-bet (T-box expressed in T cells), whereas in Th2 cells, specific cytokine production is mediated mainly by the transcription factor GATA-3 (Grogan and Locksley, 2002; Murphy and Reiner, 2002a). Generally, Th1/Th2 cytokines promote their respective phenotype in an autocrine fashion, while suppressing the differentiation of the other phenotype (Figure 2) (Grogan and Locksley, 2002). T helper cells "help" other cell types to exert their effector functions, according to the type of CD4 T helper lymphocyte. In type I immunity, Th1 cells provide help for activation of macrophages, CTLs and IFN-γ dependent Ig class switch of B cells to IgG2a and IgG3 (Abbas et al., 1996; Finkelman et al., 1990; Paul, 2003; Paul and Seder, 1994). The majority of the effects of Th1 help are due to the effects IFN-γ itself (see Table I). In contrast, during a type 2 immune response, Th2 cells provide help for the maturation and degranulation of eosinophils and basophils, the activation of B cells and IL-4 dependent Ig class switch to IgE and IgG1 (Abbas et al., 1996; Finkelman et al., 1990; Paul, 2003; Paul and Seder, 1994). Differentiated Th1 and Th2 cells derive from a common precursor cell (Thp) (Figure 2) that produces little amounts of IL-2, IL-4 and IFN-γ (Constant and Bottomly, 1997; Kamogawa et al., 1993; Swain, 1995). Factors that influence T helper differentiation include the cytokine microenvironment during priming of naïve T cells (especially IL-12 and IL-4), the signal strength and duration of APC-T cell interaction, type of APC and its activation status, co-

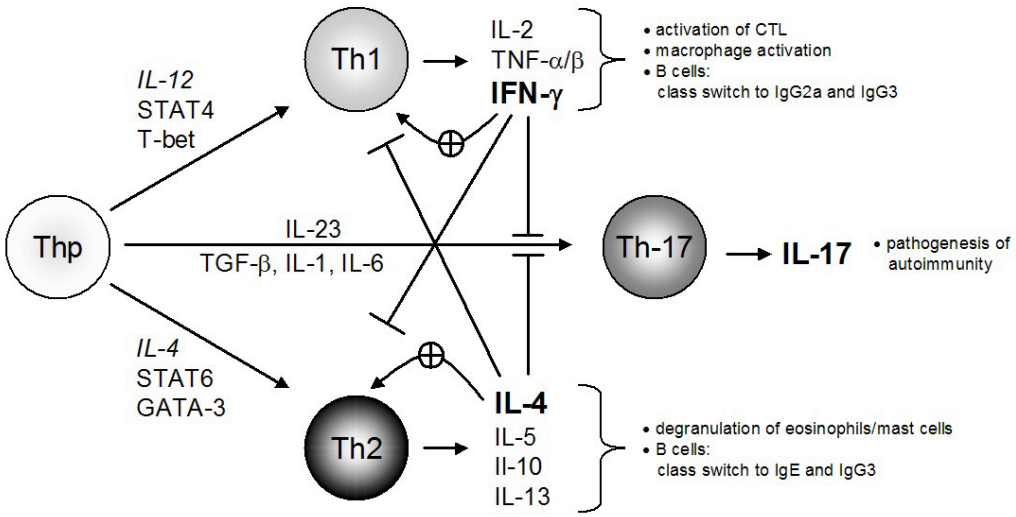


Figure 2:
Lineage decisions of T helper cells (simplified model). Details of the model are described in the text.

stimulatory molecules and cell cycle (Amsen et al., 2004; Bird et al., 1998; Constant and Bottomly, 1997; Kuchroo et al., 1995; Reiner and Seder, 1995; Seder, 1994). However, the Th1/Th2 classification is based upon the assumption, that CD4+ T cells are able to acquire stable and polarized cytokine producing phenotypes. Recently, another Th subset was identified on the basis of these criteria. This new subset, termed Th-17 cells, secrets predominantly IL-17 and no IFN-γ, and plays an important role in the development of autoimmune pathogenesis (Figure 2) (Cua et al., 2003; Hunter, 2005; Nakae et al., 2002; Nakae et al., 2003). IL-23, TGF-β, IL-6 and IL-1 have been associated with Th-17 development (Harrington et al., 2005; Park et al., 2005; Veldhoen et al., 2006). The importance of the Th1/Th2 paradigm on the outcome of infections has been well established *in vivo*. First by seminal experiments characterizing CD4 T cell responses in mouse strains that are either naturally susceptible (Th2, Balb/c) or resistant (Th1, C57BL/6) against the parasite *L. major*, and later in humans with studies on leprosy (Heinzel et al., 1989; Scott et al., 1988; Yamamura et al., 1991,Reiner, 1995 #353).

IFN-γ is the hallmark cytokine of type I immune responses and Th1 cells are classically characterized by a robust IFN-γ expression. But how exactly is IFN-γ expression induced during Th1 differentiation? IFN-γ expression is the result of a sequential combination of different mechanisms, including inter- and intrachromosomal interactions, epigenetic modifications, initiation of crucial transcription factors and up-regulation of pivotal cytokine receptors (Grogan and Locksley, 2002; Murphy and Reiner, 2002b; Spilianakis et al., 2005). In the case of Th1 differentiation, it is well established that IL-12 and its receptor, T-bet, STAT4 and IFN-γ are crucial in inducing Th1 development (Magram et al., 1996; Szabo et al., 2002; Wurster et al., 2000; Zhang et al., 2001), but the sequential hierarchy of known events leading to Th1 differentiation is still unclear and subject to vigorous investigation. However, IFN-γ, probably produced by NK cells, is thought to be among the cytokines initiating the differentiation program (Bradley et al., 1996; Lohoff and Mak, 2005; Murphy and Reiner, 2002b). Naïve Th cells activated under Th1-polarizing conditions become exposed to IFN-γ during T cell receptor (TCR) engagement, leading to the STAT1-dependent induction of the transcription factor T-bet (Afkarian et al., 2002; Lighvani et al., 2001) (Figure 3, 1). A member of the T-box family of transcription factors, T-bet represents one of the key factors required for Th1 lineage commitment (Ho and Glimcher, 2002; Szabo et al., 2000; Szabo et al., 2002). After activation, T-bet, in cooperation with the homeobox transcription factor HLX (**H**2.0-**l**ike

homeobox 1), induces chromatin remodeling and activation of the repressed *ifng* gene locus, stabilizes its own expression through an autocatalytic feedback loop and actively suppresses GATA-3 (Afkarian et al., 2002; Mullen et al., 2001; Mullen et al., 2002; Szabo et al., 2000; Szabo et al., 2002) (Figure 3). Additionally, T-bet up-regulates expression of the IL-12 receptor β2 subunit (Afkarian et al., 2002; Mullen et al., 2001) (Figure 3, 2). IL-12 is a heterodimeric cytokine, consisting of a IL-12/p40 and a IL-12/p35 subunit, which signals through the IL-12 receptor complex (IL-12R) and plays an important role in Th1 differentiation (Trinchieri, 1993). IL-12R is composed of the ligand-binding IL-12 receptor β1 chain (IL-12Rβ1; constitutively expressed) and the signaling IL-12 receptor β2 chain (IL-12Rβ2; inducible), and activates the JAK-STAT pathway of signal transduction (Trinchieri, 2003). The specific cellular effects of IL-12 are mainly due to activation of STAT4 (homodimer, but also STAT4/STAT3 heterodimers). It is

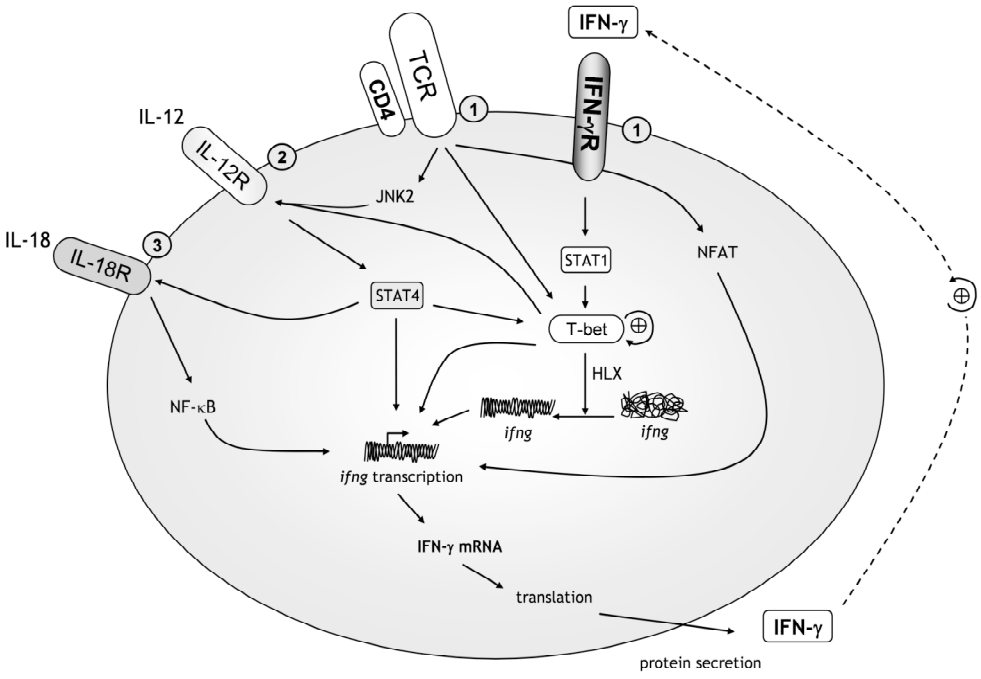


Figure 3:
Model of IFN-γ expression in CD4+ Th1 cells.

Naïve CD4+ T cells, activated under Th1 polarizing conditions are exposed to IFN-γ during T cell receptor (TCR) engagement (**1**), leading to the primarily STAT1-dependent induction of the transcription factor T-bet. T-bet in turn induces chromatin remodeling and activation of the repressed *ifng* gene locus, stabilizes its own expression through an autocatalytic feedback loop (⊕) and up-regulates functional IL-12R by inducing IL-12Rβ2 expression (**2**). STAT4 activation downstream of IL-12R signaling in turn leads to increased levels of functional IL-18R by inducing IL-18Rβα expression (**3**). The now committed Th1 cell is able to induce acute *ifng* transcription by both antigen-dependent (TCR) and cytokine-dependent (IL-12 in combination with IL-18) mechanisms. NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB.

predominately produced by activated inflammatory cells, such as macrophages, neutrophils and dendritic cells (DCs). In context of Th1 differentiation DCs appear to provide the initial IL-12 during priming, independently of IFN-γ (Gazzinelli et al., 1994; Scharton-Kersten et al., 1996; Trinchieri, 2003). STAT4 activation downstream of IL-12 signaling enhances acute transcription of *ifng* (Kaplan et al., 1998; Mullen et al., 2001; Schijns et al., 1998) and up regulation of the IL-18 receptor α chain (Nakahira et al., 2001; Yoshimoto et al., 1998) (Figure 3, 3). IL-18 is primarily produced by macrophages and dendritic cells and belongs to the IL-1 family of cytokines (Nakanishi et al., 2001). It signals through the IL-18 receptor complex (IL-18R), consisting of a ligand-binding IL-18R α chain (IL-18Rα) and a signal-transducing IL-18R β chain (IL-18Rβ) (Nakanishi et al., 2001). IL-18R utilizes signaling pathways analogous to the IL-1 receptor, including IRAK (**I**L-1 **r**eceptor-**a**ssociated **k**inase) and the adaptor protein MyD88 (Adachi et al., 1998; Nakanishi et al., 2001). Activation of IRAK leads to subsequent activation of TRAF6 and finally to the nuclear translocation of NF-κB, which can function as a regulator of IFN-γ transcription (Kanakaraj et al., 1999; Kojima et al., 1998; Matsumoto et al., 1997; Robinson et al., 1997; Sica et al., 1997) (Figure 3). IL-18 serves as a cofactor for IL-12-induced Th1 differentiation and, in synergy with IL-12, strongly enhances IFN-γ expression and production in an antigen-independent manner and induces IFN-γ production in fully differentiated and activated Th1 cells (Ahn et al., 1997; Nakahira et al., 2002; Nakanishi et al., 2001; Robinson et al., 1997; Szabo et al., 2003). Antigen-dependent transcription of IFN-γ occurs, when TCR stimulation induces the activation of NFAT (**n**uclear **f**actor of **a**ctivated **T** cells), which in turn binds to specific NFAT binding sites within in the IFN-γ promoter, regulating transcription (Campbell et al., 1996; Sica et al., 1997; Sweetser et al., 1998) (Figure 3). Therefore, there are at least two physiologically distinct pathways that can independently induce IFN-γ expression, including TCR signaling and IL12/IL18 signaling (Robinson et al., 1997; Yang et al., 1999; Yang et al., 2001). Additionally, in a positive-feedback loop, IFN-γ itself is reported to drive Th1 differentiation and IFN-γ production independently of IL-12 (Bradley et al., 1996) (Figure 3).

With both IFN-γ-dependent (T-bet) and independent mechanisms (TCR stimulation is able to induce IL-12Rβ2 through JNK2 (Murphy et al., 2000)) inducing IL-12 responsiveness, IL-12 is also thought to be crucial for initiating the Th1 differentiation

program (Afkarian et al., 2002; Dong et al., 1998; Yang et al., 1998). Contrary to that conception, T-bet, primarily induced by IFN-γ, has been shown to act prior to IL-12 in Th1 differentiation (Mullen et al., 2001). Thus, the sequential roles of IFN-γ vs. IL-12 in the induction of both IFN-γ expression and Th1 differentiation by CD4+ T cells remain controversial with existing models in favor of both IFN-γ and IL-12 as the important initiating cytokine (Murphy and Reiner, 2002b; Robinson and O'Garra, 2002).

3.5.2. IFN-γ expression in CD8+ effector T cells

Among T lymphocytes, CD8+ effector cells (CTLs) are the major producers of IFN-γ besides Th1 cells. Despite being so closely related to CD4+ T cells, IFN-γ expression seems to be differentially regulated in CD8+ T cells. Both CD4+ and CD8+ T cells arise from a common progenitor in the thymus and share most of the surface receptors for cytokines and transcription factors, involved in Th1/Th2 differentiation and IFN-γ expression, respectively (Figure 4). However, surprisingly little is known about the specific differences in IFN-γ expression unique to CD8+ T cells (Glimcher et al., 2004; Ho and Glimcher, 2002; Murphy et al., 2000). Analogous to the Th1/Th2 dichotomy in CD4+ T cells, CD8+ T cells can be further divided in T cells of the cytotoxic type I (Tc1) and type II (Tc2), with Tc1 cells producing IFN-γ and Tc2 cells IL-4 (Mosmann et al., 1997). However, unlike Th2 cells, Tc2 cells produce significantly less IL-4 and retain the capacity to produce high levels of IFN-γ, although reduced when compared to Tc1 (Carter and Dutton, 1996; Croft et al., 1994; Sad et al., 1995; Seder et al., 1992). Further differences in IFN-γ expression between CD4+ and CD8+ T cells have been reported. Distal and proximal promoter elements have been identified in the 5′-flanking region of the IFN-γ gene (Penix et al., 1993). While CD4+ T cells utilize both promoter elements when expressing IFN-γ, CD8+ T cells display transcriptional activity only in the distal promoter, which requires IL-12 and IL-2 for maximum activity (Aune et al., 1997). The failure of CD8+ T cells to express transcriptional activity directed by the proximal element might provide a mechanism for differential regulation of IFN-γ expression in CD4+ and CD8+ T cells. The transcription factor T-bet is expressed in a cytokine-inducible fashion

in CTLs, but is surprisingly dispensable for both IFN-γ production and cytolytic activity, whereas IFN-γ production is absent in CD4 T cells lacking T-bet (Pearce et al., 2003; Szabo et al., 2002; Yin et al., 2002). However, one report has shown diminished IFN-γ expression by CD8+ effector cells in the absence of T-bet and failure to down-regulate surface markers, such as CD62L (Sullivan et al., 2003). In contrast to Th1 development and IFN-γ production by CD4+ T cells, Tc1 development and IFN-γ production by Tc1 cells can occur independently of STAT4 (Aronica et al., 1999; Carter and Murphy, 1999). This is due to distinct regulation of TCR-induced IFN-γ production in CD4+ and CD8+ T cells. Carter and Murphy showed that STAT4-deficient CD8+ T cells produced IFN-γ abundantly, particularly when activated via the TCR, whereas CD4+ T cells lacking STAT4 were unable to generate significant amounts of IFN-γ (Carter and Murphy, 1999). However, the IL12/IL18 pathway for induction of IFN-γ operates in CD8+ T cells as well

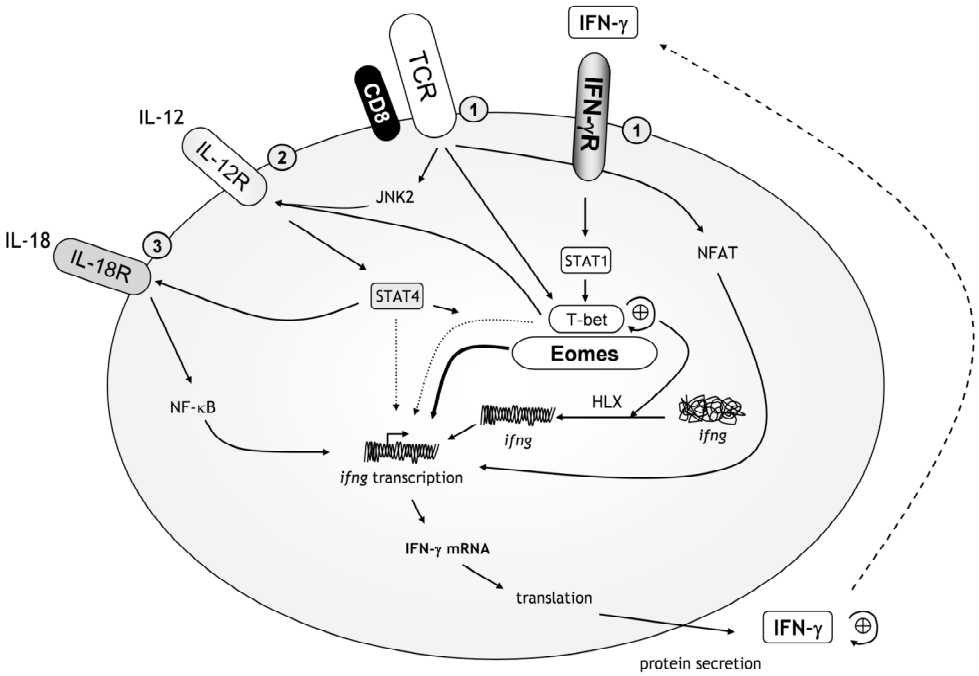


Figure 4:
Model of IFN-γ expression in CD8+ Tc1 cells.

Naïve CD8+ T cells, activated under type I polarizing conditions are exposed to IFN-γ during T cell receptor (TCR) engagement (**1**), leading to the primarily STAT1-dependent induction of the transcription factor T-bet. T-bet in turn induces chromatin remodeling and activation of the repressed *ifng* gene locus, stabilizes its own expression through an autocatalytic feedback loop (⊕) and up-regulates functional IL-12R by inducing IL-12Rβ2 expression (**2**. However, Eomes is the dominant transcription factor inducing IFN-γ expression in CD8+ T cells. STAT4 activation downstream of IL-12R signaling in turn leads to increased levels of functional IL-18R by inducing IL-18Rβα expression (**3**). Tc1 cells are able to induce acute *ifng* transcription by both antigen-dependent (TCR) and cytokine-dependent (IL-12 in combination with IL-18) mechanisms. NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB

as CD4+ T cells, and is strictly STAT4 dependent in both cases (Carter and Murphy, 1999). It has been well established that memory (CD44hi) CD8+ T cells produce IFN-γ in an antigen-independent manner when exposed to IL12/IL18 (Berg et al., 2003; Lertmemongkolchai et al., 2001). Since both STAT4 and T-bet are dispensable for IFN-γ expression in CTLs, it was unclear what transcription factor might be involved in regulating IFN-γ expression in CD8+ T cells. Two years ago, Eomesodermin (Eomes) was identified as the crucial transcription factor for IFN-γ expression and cytolytic effector lineage differentiation in CD8+ T cells (Pearce et al., 2003) (Figure 4). Although expressed in both activated CD4+ and CD8+ effector cells, Eomes mRNA is only significantly induced in CD8+ T cells (Pearce et al., 2003). Eomes, like T-bet, belongs to the T-box transcription factors and was initially described as a key regulator of mesodermal cell fate in vertebrates (Ryan et al., 1996).

Besides the mentioned parameters, other molecular factors and signaling pathways have been associated with regulating IFN-γ production in both CD4+ and CD8+ T cells. Among these are both TCR-dependent and independent factors, such as IL-21, IL-23, IL27 and their respective receptors, members of the SOCS (**s**uppressors **o**f **c**ytokine **s**ignaling) family, IRF (**i**nterferon **r**egulatory **f**actor) family, the GADD45 family, the NFAT family, the MAPK pathway, the JNK (c-**J**un **N**H_2-terminal **k**inase) pathway, the transcription factors CREB1 (**c**yclic AMP-**r**esponsive-**e**lement-**b**inding protein **1**), ATF1/ATF2 (**a**ctivating **t**ranscription **f**actor) ,cJUN and OCT1 (**Oc**tamer binding **t**ranscription factor-**1**) ((Hunter, 2005; Kasaian et al., 2002; Lohoff and Mak, 2005; Murphy et al., 2000; Strengell et al., 2003; Strengell et al., 2002; Yang et al., 2001, Penix, 1996 #393).

3.6. Bicistronic IFN-γ reporter

Various approaches have been employed to determine the role of cytokines in immunity to infection. The depletion of cytokines *in vivo* with antibodies, the use of knockout mice deficient in specific cytokines or cytokine receptors represent just a small selection of successful strategies which lead to a greater appreciation of the importance of cytokines for controlling viral and parasitic infections. However, it is extremely difficult to track cytokine-producing cells *in vivo* due to the rapid secretion of cytokines and the short half-lives of both the proteins and mRNA transcripts. Commonly used methods to identify IFN-γ expression at the single cell level require *in vitro* restimulation thereby altering the *in situ* phenotype and are not able to localize the distribution of cytokine production within populations of cells (Assenmacher et al., 1998; Gessner et al.). Recently, with the development of bicistronic IL-4 and IL-4 dual-reporter mice, novel approaches emerged to overcome these limitations and study cytokine-expressing cells directly in vivo (Mohrs et al., 2005; Mohrs et al., 2001). Applying the same strategy used to create 4get mice (Mohrs et al., 2001), bicistronic IFN-γ reporter mice were generated by targeting a bicistronic reporter cassette, containing an encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) linked to eYFP, into the 3' untranslated region of the endogenous *ifng* locus (Matsuda et al., 2003; Stetson et al., 2003). A genomic fragment of the *ifng* gene, containing exons 2–4 and 2.5 kb of 3' untranslated sequence of the *ifng* gene, was mutated by the addition of an IRES element, eYFP, and a polyadenylation signal, followed by a loxP-flanked neomycin resistance cassette. A herpes simplex derived thymidine kinase was added upstream for counterselection. Cre-mediated recombination of the loxP-flanked neomycin selection cassette in chimeric males resulted in the final mutated *ifng* gene locus.

The novel IFN-γ reporter was designated **Yeti**, an acronym for **y**ellow **e**nhanced **t**ranscript for **I**FN-γ. A schematic map of the murine *ifng* locus, the reporter-targeting construct, and the mutated gene in bicistronic IFN-γ reporter (Yeti) mice is depicted in Figure 5. The insertion of an IRES element into the endogenous *ifng* locus allows for separate translation of IFN-γ and the eYFP reporter. Upon activation, the *ifng* gene, including the bicistronic reporter cassette containing the IRES/eYFP, is being transcribed and processed. The resulting mRNA consists of both the IFN-γ and the eYFP cistron. Due to the IRES element the eYFP protein is translated 5'Cap-independently, which results in

two separate proteins encoded by one mRNA. While the IFN-γ protein is being secreted, the eYFP protein is retained in the IFN-γ expressing cell (Figure 6). The estimated half-life for eYFP within any cell is about 24 to 48 hrs (M. Mohrs unpublished data).

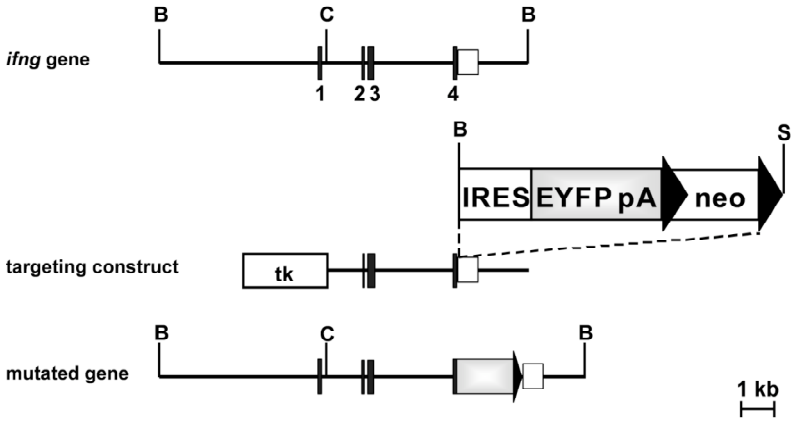


Figure 5:
Schematic map of the murine ifng locus, the targeting construct, and the mutated gene in bicistronic IFN-γ reporter (Yeti) mice

A genomic fragment of the *ifng* gene was mutated by the addition of an IRES (internal ribosomal entry site) element, eYFP, and a polyadenylation signal (pA), followed by a loxP-flanked neomycin resistance (neo) cassette. Thymidine kinase (tk) was added upstream for counterselection. Cre-mediated recombination of the loxP-flanked neomycin cassette in chimeric males resulted in the final mutated locus (bottom). Ifng exons are numbered and depicted as filled boxes. BamHI (B), ClaI (C), and SacI (S) sites are indicated.

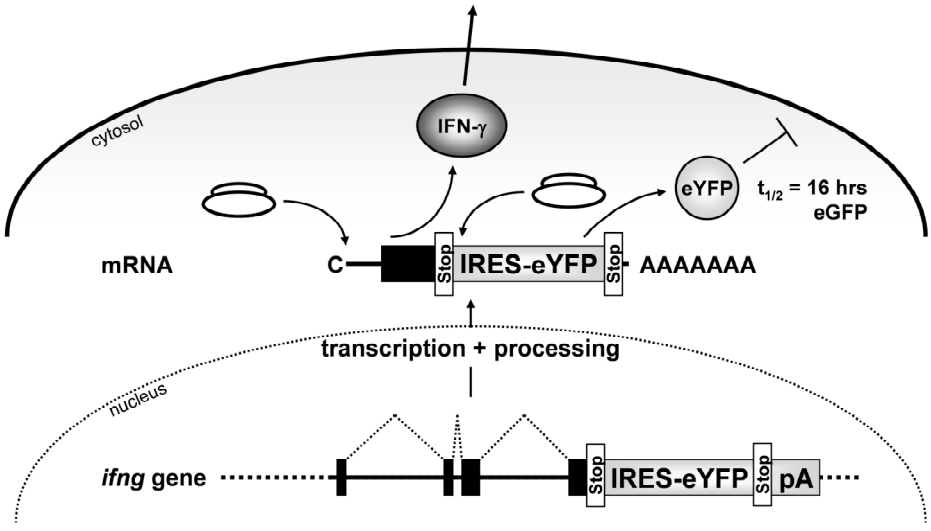


Figure 6:
Function of the IRES-element in Yeti mice

Upon activation, the endogenous *ifng* gene, including the bicistronic reporter cassette containing the IRES element linked to eYFP, is being transcribed and processed. The resulting mRNA consists of both the IFN-γ and the eYFP cistron. The IRES element allows for Cap (c)-independent translation of the eYFP protein, leading to two separate proteins encoded by one mRNA. While the IFN-γ protein is being secreted, the eYFP protein is retained in the IFN-γ expressing cell. IFN-γ expressing cells can then be detected via flow cytometry for example; *Ifng* exons are depicted as filled boxes pA, polyadenylation site

Bicistronic reporter systems are substantially more sensitive for identifying cytokine-expressing cells than currently standardized methods (Mohrs et al., 2001). Additionally, Yeti mice allow non-invasive and unbiased tracking of IFN-γ expressing cells on a single cell level using flow cytometry as well as isolation of live, cytokine-expressing cells (Matsuda et al., 2003; Stetson et al., 2003). Furthermore, both the *ifng* gene regulation and the cytokine production is intact and restimulation of cells is not required to identify cytokine-expressing cells.

3.7. Experimental mouse model of Influenza virus infection

Influenza viruses belong to the taxonomic family *Orthomyxoviridae* and can further be divided into Influenza types A, B and C, based on molecular and serological criteria. Influenza A and B are the two types of influenza viruses that cause epidemic human *Influenza* (also known as *flu*) disease. Besides humans Influenza A viruses infect a variety of mammals, including horses, pigs and whales as well as domestic and wild birds (also known as *avian flu* or *bird flu*). Influenza types B and C are described only in humans. The influenza isolates used in the present study are of the Influenza A subtype. Influenza A viruses are further categorized into subtypes on the basis of two surface antigens: hemagglutinin (H) and neuraminidase (N). Three of the 15 known H subtypes (H1, H2 and H3) and two of the 9 known N subtypes (N1 and N2) are recognized as being capable to cause disease outbreaks in humans (Wright and Webster, 2001). Different strains of Influenza A viruses are described by geographic origin, strain number, year of isolation and the respective H and N subtype. Isolates used in the present thesis are A/HK-x31/H3N2 (referred to as X31) and the heterosubtypic strain A/PR8/34/H1N1 (referred to as PR8). The A/HK-x31/H3N2 virus is a recombinant between A/PR8/34/H1N1 and A/Aichi with the surface H3N2 proteins of A/Aichi and many of the internal components of A/PR8 (Allan et al., 1990; Daly et al., 1995; Kilbourne, 1969). The Influenza A virus particle consists of a segmented single-stranded negative sense RNA genome inside a protein envelope (Figure 7). It presents itself mostly in a spherical shape (80-120 nm in diameter), however it is highly pleomorph. Influenza A virus displays the unique property to undergo extensive antigenic variation in the hemagglutinin and neuraminidase surface

antigens. These two antigens represent the major epitopes for neutralizing and protective antibody responses by the infected host. Frequent development of antigenic variants through antigenic drift is the virologic basis for the seasonal *flu* epidemics, where protective antibody responses are missing. *Antigenic drift* refers to these relatively minor changes in the hemagglutinin and neuraminidase coding RNA segments that occur each year. The World Health Organization (WHO) estimates that in a typical year, 10 to 20 percent of the world's population is infected with influenza, resulting in 3,000,000 to 5,000,000 severe illnesses and 250,000 to 500,000 deaths (Organization, 1999). In contrast, *antigenic shift* refers to major changes in the Influenza A subtype (e.g. from H2N2 to H1N1). This occurs infrequently, only every one to four decades and is the result of genetic reassortment (e.g. introduction of a new RNA segment coding for H and N) (Webster, 2002; Wright and Webster, 2001). Due to the segmented nature of the viral

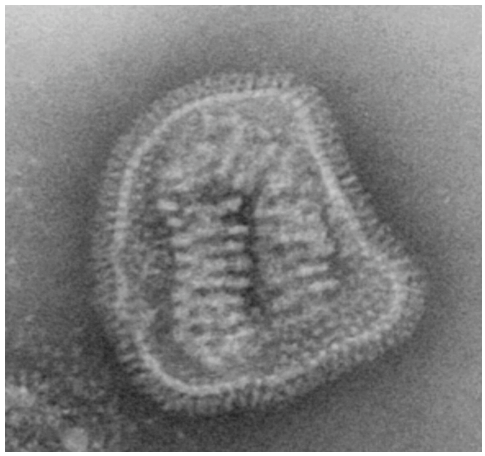


Figure 7:
Negative-stained transmission electron micrograph of Influenza A virus particle.
Ultrastructural details depicted include virus envelope and segmented RNA-genome (content provided by CDC/ Dr. Erskine, L. Pamer; Dr. M.L. Martin)

genome and the existence of a variety of animal hosts, co-infection of cells within a host with human-adapted and animal-adapted strains is likely to occur. This can lead to the emergence of novel strains by genetic reassortment, with the sudden appearance of a new antigenic subtype, and even pandemics. When this reassortment results in a virus with novel surface proteins, it spreads rapidly because most of the population has no protective serum antibody (Webster et al., 1992). Human pandemic viruses have arisen from avian viruses by reassortment (Webster, 2002). In the twentieth century, there were three such

pandemics: the so-called swine flu of 1918–1919, being the most extreme, causing two billion cases and 20 to 40 million deaths worldwide, the “Asian” flu of 1957, and the “Hong Kong” flu of 1969 (Kilbourne, 1975). The latter two pandemics killed an estimated 1 million people each. With the recent H5N1 avian flu outbreaks in Asia, but also in Europe and Africa, the possibility of a new world wide pandemic is strongly debated.

Mice represent the most suitable animal model to study influenza virus infection in mammals (Ada and Jones, 1986). They can be naturally infected with human influenza isolates. After intranasal (i.n.) oculation with influenza virus, mice develop a progressive upper and lower respiratory tract disease with histopathology virtually identical to that seen in human disease (Renegar, 1992; Yetter et al., 1980). Because viral replication is limited to epithelial cells of the upper and lower respiratory tract, influenza infection represents a localized infection (Eichelberger et al., 1991b). Nevertheless, antigen-specific cells can be found in other peripheral lymphoid organs, especially the spleen (Doherty et al., 1996). Much that is known about the details of influenza pathogenesis and host defense was first established in mice and later confirmed in humans (Bender and Small, 1992; Small, 1990). Antibodies to influenza virus can protect against reinfection and when passively transferred antibody can protect naive animals. However, this form of protection is often subtype-specific or even narrowly specific to certain viral surface antigens (Ada and Jones, 1986; de Jong et al., 2000).

In terms of cellular immunity, mediated by effector and memory T cells, the mouse model could establish the following. Effector CD4+ and CD8+ T cells play important roles in clearing influenza virus and protecting against challenge, although they can also cause immunopathology (Doherty et al., 1997b; Wells et al., 1981). Peter Doherty’s and other groups have provided evidence for a beneficial role of class I MHC-restricted CD8+ CTLs in clearing primary influenza virus infection and also in protection against challenge with homologous virus (Doherty et al., 1997b; Lu and Askonas, 1980). A conserved gene product among Influenza A viruses, nucleoprotein (NP) is a major target antigen for CTLs in mice (Yewdell et al., 1985), and MHC class I tetramer staining reagents are available to track NP specific CTLs via flow cytometry (Altman et al., 1996; Crowe et al., 2003). The primary mechanism utilized by CD8+ effector T cells to clear the viral infection is destruction of infected cells mediated by either the perforin or Fas pathways (Topham et al., 1997). Furthermore, adoptive transfer of cloned influenza-specific CTL can prevent death from lethal virus challenge (Lukacher et al., 1984). CD4+ T cells also mediate

protection, although CD8+ T cells are much more efficient in effecting recovery from influenza virus infection (Allan et al., 1990; Bender et al., 1992; Eichelberger et al., 1991a). Both Th1 and Th2 T cells are involved in both the primary and secondary response, though most of the CD4 + T cell clones recovered from influenza virus-infected mice display the Th1 cytokine phenotype (Carding et al., 1993; Graham et al., 1994). MHC class II-restricted cytotoxic activity specific for influenza virus antigens has also been reported (Taylor and Bender, 1995). The most differentiated CD4+ and CD8+ effector cells are present at the site of infection, the lung airways and the lung parenchyma, whereas cells with apparently less differentiated phenotypes can be found in the secondary lymphoid organs, such as the spleen (Homann et al., 2001). Further, immunization with one Influenza A virus subtype can protect animals against challenge with virus of a different subtype. This cross-protection was first demonstrated by Schulman and Kilbourne in 1965 and is referred to as heterosubtypic immunity (Schulman and Kilbourne, 1965). *In vivo* depletion showed that CD4+ and CD8+ T-cells both contribute to control of heterosubtypic virus challenge (Liang et al., 1994). The precise mechanism of more rapid recovery in heterotypic immune mice has not been completely defined but is likely due to augmented anti-influenza pulmonary cellular responses, Th1 cytokines and cross-reactive CTLs from influenza-infected mice recognizing NP (Bennink et al., 1978; Carding et al., 1993; Townsend et al., 1984; Yewdell et al., 1985). This of particular importance for vaccine development, because heterosubtypic T cell memory could potentially provide broad protection against new strains of influenza viruses, displaying altered surface H and N proteins, that are not cross-neutralized by preexisting antibodies.

3.8. Experimental model of Sendai virus infection

Respiratory virus infections, such as those caused by influenza but also parainfluenza viruses (PIVs), are a major cause of morbidity and mortality worldwide. PIVs are medium-sized (150 to 200nm) enveloped viruses that have a non-segmented negative-strand RNA genome and belong to the family *Paramyxoviridae* (Figure 8). Sendai virus, also known as hemagglutinating virus of Japan (HVJ), is a member of the paramyxovirus

family and was classified as the murine counterpart of human parainfluenza virus type 1 (hPIV1), which causes a respiratory infection in children. First identified in the town of Sendai (Japan) in 1952 by Kuroya and colleagues, Sendai virus was initially thought to be a human pathogen, since it was recovered from mice inoculated with an autopsy specimen from an infant with respiratory disease (Kuroya and Ishida, 1953; Kuroya et al., 1953). However, it is recognized now that Sendai virus is indigenous to mice and hardly ever causes disease in humans. Due to its antigenic relationship with hPIV1, Sendai virus is used as a model for human parainfluenza disease, but also respiratory virus infections, such as influenza virus in general (Doherty and Christensen, 2000; Doherty et al., 1997a; Doherty et al., 1996; Doherty et al., 1997b; Swain et al., 2004; Woodland, 2003; Woodland et al., 2001). Similar to Influenza virus infection, intranasal inoculation of mice

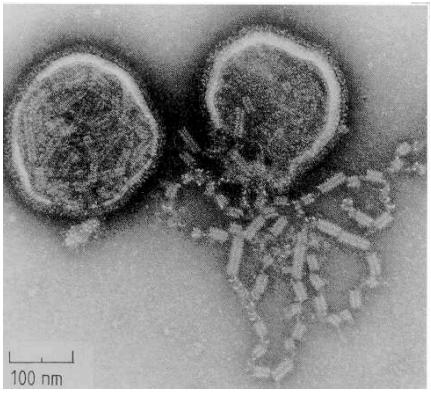


Figure 8.
Transmission electron micrograph of Parainfluenza virus type 1, Sendai strain. An intact virion and a disintegrating particle with free nucleocapsid fragments. (content provided by June Almeida, The Wellcome Research Laboratories, Beckenham, England.)

with Sendai virus results in a localized infection of epithelial cells of the upper and lower respiratory tract of limited duration (Doherty and Christensen, 2000; Flynn et al., 1999; Hou et al., 1992). Both Sendai and Influenza virus are respiratory pathogens, because they require a trypsin-like enzyme, which is anatomically restricted to the respiratory tract, to cleave the surface fusion (Sendai) or hemagglutinin (Influenza) protein, respectively (Horimoto and Kawaoka, 1995; Tashiro et al., 1992; Walker et al., 1992). The immune response against Sendai virus is comparable to that against Influenza virus infection, with similar kinetics of effector T cell recruitment, viral clearance (by 10-12 days) and memory cell formation (Doherty and Christensen, 2000; Doherty et al., 1997a; Doherty et al., 1996; Doherty et al., 1997b; Swain et al., 2004; Woodland, 2003; Woodland et al., 2001). As in the case for many viruses, much of the Sendai virus-specific CD8+ T cell response

is directed against peptides derived from conserved internal components such as the nucleoprotein. The $SenNP_{324-332}/K^b$ epitope (SenNP) is the major immunodominant target of CD8+ T cells, and MHC class I tetramer staining reagents are available to track these CTLs via flow cytometry (Altman et al., 1996; Arnold et al., 2002; Cauley et al., 2002). In fact, the SenNP epitope seems to be immunodominant to an unusual extent by engaging an extremely diverse spectrum of TCR αβ pairs, rendering up to 70% of CD8+ T cells in the lung airways responsive to that one epitope (Cole et al., 1994; Doherty and Christensen, 2000). Importantly, a MHC class II peptide multimer specific for an immunodominant haemagglutinin-neuraminidase epitope $HN_{419-433}/A^b$ (SenHN) is available to track antigen-specific CD4+ T cells (Arnold et al., 2002; Cauley et al., 2002). Finally, Sendai virus attracts increasing attention as an emerging viral vector system for gene transfer approaches and DNA vaccine development. For example, Sendai virus has been employed as a xenogenic vaccine vector for the delivery of respiratory syncitial virus (RSV) antigens and unmodified Sendai virus is currently being studied in clinical trials as a vaccine for the closely related human hPIV1 (Bitzer et al., 2003; Takimoto et al., 2005).

3.9. Experimental mouse model of *Toxoplasma gondii* infection

Toxoplasma gondii (*T. gondii*) is an Apicomplexa obligate intracellular protozoan parasite that commonly infects mammals and birds throughout the world. Infections can be categorized into an acute phase, in which the asexual invasive parasite form (tachyzoite) rapidly proliferates and invades host cells, and a chronic phase, which is characterized by disappearance of tachyzoites and formation of tissue cysts (Figure 9). *T. gondii* is a food born pathogen and the natural reservoir are cats. It exists in three stages throughout its life cycle in various hosts: the tachyzoite, the tissue cyst (containing bradyzoites) and the oocyst (containing sporozoites), which is produced during the sexual cycle in the intestine of cats (Figure 9) (Dubey et al., 1998). Following ingestion of tissue cysts or oocysts, either bradyzoites or sporozoites respectively, invade cells of the small intestine and transform into the tachyzoite form, a stage that is highly motile and can invade and replicate in any nucleated cell type eventually leading to lysis of the cell and further dissemination throughout the body via the blood and lymphatics (Dubey et al.,

1998). Tachyzoites are present in tissues during the acute phase or after reactivation of the chronic infection. Formation of tissue cysts, approximately 10-14 days after infection, is accompanied with disappearance of the tachyzoite stage and appears to be associated with development of immunity and acquisition of resistance against rechallenge with the parasite (Aliberti, 2005; Dubey et al., 1998; Filisetti and Candolfi, 2004). Thus, the parasite successfully avoids elimination from the host by forming tissue cysts in multiple organs. Immunocompromised hosts, such as HIV infected individuals, suffer from widespread dissemination with pneumonitis, myocarditis and encephalitis, eventually leading to death (Martinez et al., 1995). Toxoplasmosis has emerged as one of the most common opportunistic infections in AIDS patients. Maternal-fetal transmission in

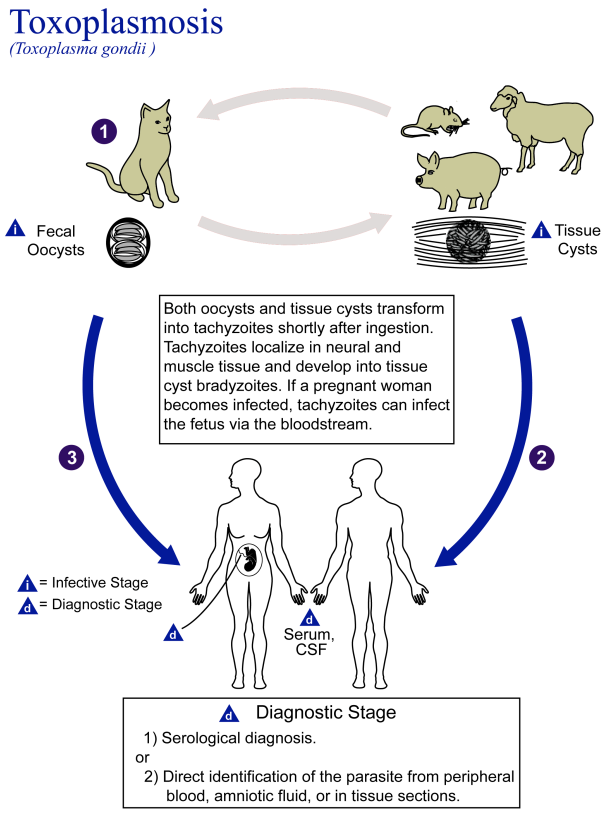


Figure 9:
Life cycle of *T. gondii.* Details are discussed in the text.(content provided by CDC)

immune-competent females continues to be an important cause of congenital defects (Swisher et al., 1994; Wong and Remington, 1994), although *T. gondii* infection is frequently regarded as asymptomatic. However, there is a growing recognition that even in

immune-competent individuals this parasite is a cause of severe morbidity, ocular disease and even death (Swisher et al., 1994; Wong and Remington, 1994). Most of what is known today about the immune response against *T. gondii* was established in the mouse model of Toxoplasmosis. Cell-mediated immunity plays a central role in resistance against *T. gondii*, with both CD4+ and CD8+ effector T cells involved in conferring protection (Araujo, 1991; Gazzinelli et al., 1991; Suzuki and Remington, 1988). *T. gondii* induces an extremely potent type I immune response, characterized by a strong IFN-γ production of both parasite-specific CD4+ and CD8+ T cells. T cells confer long term protection, at least in part by secretion of IFN-γ, and these mechanisms are most effective when working in combination (Gazzinelli et al., 1991; Suzuki et al., 1989; Suzuki et al., 1988; Suzuki and Remington, 1988). IFN-γ is of extreme importance for resolution of acute parasite burdens and IFN-γ deficient mice succumb rapidly to infection (Scharton-Kersten et al., 1996). From a historical perspective, the study of protective immunity against *T. gondii* was focused on adaptive responses, because T cells were considered the major source for IFN-γ. It is now recognized that *T. gondii* stimulates also a strong innate response, with IL-12 release by accessory cells which stimulates NK cell production of IFN-γ (Gazzinelli et al., 1994; Hunter et al., 1994; Johnson, 1992; Khan et al., 1994). Thus, both T cells and NK cells are important sources of IFN-γ during infection. One of the major anti-microbial functions of IFN-γ is (in combination with TNF-α) to activate macrophages to produce NO, which inhibits the growth of *T. gondii* (Chao et al., 1993; Sibley et al., 1991). While there is evidence that tryptophan-starvation can also be involved in the inhibition of parasite replication, it is clear that there is an iNOS independent pathway for the control of *T. gondii* that is dependent on a family of IFN-γ-induced GTPases, including LRG-47 and IGTP (Collazo et al., 2002; Collazo et al., 2001; Pfefferkorn, 1984; Taylor et al., 2004). Infection also induces an early (d3) peak in systemic levels of Type I IFNs, but while the cellular source of these factors is unclear, they have been implicated in activating NK cells and mediating anti-parasite effector function (Hunter et al., 1994; Johnson, 1992; Khan et al., 1994). Cytotoxic T cells are generated during infection (Subauste et al., 1991), but it appears that lysis of infected cells plays a secondary role in protection (Denkers and Gazzinelli, 1998; Denkers et al., 1997). Finally, humoral immunity does not appear to be crucial for protection against *T. gondii* (Blackwell et al., 1993).

The outcome of *T. gondii* infection in mice varies widely depending on the genetic background, the virulence of the parasite strain and the route of infection. All experiments

in the present study were conducted with the cystogenic ME49 strain of *T. gondii* by oral infection of mice on the susceptible C57BL/6 background.

4. AIMS OF THIS THESIS

IFN-γ is the signature cytokine of type I immune responses against invading pathogens, such as intracellular bacteria, parasites and viruses. Understanding the dynamic regulation of its expression is of importance, not only for the comprehension of cell mediated type I immune responses per se, but also for optimizing vaccine strategies against type I associated pathogens. The goal of the present thesis is to define the expression patterns of IFN-γ by the two major T cell sources for this cytokine, CD4+ and CD8+ T cells, after infection. Although IFN-γ is one of the most intensively studied cytokines, there are still many unaddressed questions: 1) Do IFN-γ expressing T cells represent a homogeneous population? 2) Is IFN-γ expression tied to a specific surface phenotype or anatomical location? 3) Are viral and protozoan infections similar in their IFN-γ response? 4) What are the differences in the IFN-γ expression patterns between CD4+ and CD8+ T cells? 5) What is the role of IFN-γ receptor mediated functions for the expression of IFN-γ? To address these and related questions, the immune response against various infectious agents is studied, using bicistronic IFN-γ reporter mice and MHC class I and II multimer technology to assess antigen-specific CD4+ and CD8+ T cell responses.

5. MATERIALS AND METHODS

5.1. Mice

Bicistronic IFN-γ reporter (Yeti WT) mice (Stetson et al., 2003) were backcrossed for a minimum of 10 generations to C57BL/6 obtained from the Animal Breeding Facility (ABF) at the Trudeau Institute (Saranac Lake, USA). Yeti mice were crossed to IFN-γR1KO mice (B6.129S7-*Ifngr1*^{tm1Agt}/J) and the F1 generation was subsequently backcrossed to the IFN-γR1KO background to generate Yeti mice homozygous for the receptor knockout (Yeti IFN-γR-/-). Additionally Yeti WT mice were crossed to C57BL/6 mice congenic for CD90.1 (B6.PL-*Thy1*^{a}/CyJ) and CD45.1 (B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ). B6.129S7-*Ifngr1*^{tm1Agt}/J, B6.PL-*Thy1*^{a}/CyJ, B6.SJL-*Ptprc*^{a}*Pep3*^{b}/Boy, B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J (TCRβδKO) and B6.129S7-*Ifng*^{tm1Ts}/J (GKO) mice were purchased from the ABF at the Trudeau Institute. Yeti WT, Yeti x CD90.1, Yeti x CD45.1 and Yeti IFN-γR-/- were heterozygous for the bicistronic reporter knockin and wild-type (B6 WT) littermates were used as controls. Experimental animals were between 8 and 12 weeks of age at the onset of experiments and were kept under specific pathogen-free conditions in filter top cages at the animal facility of Trudeau Institute. The Institutional Animal Care and Use Committee (IACUC) at Trudeau Institute approved all experimental procedures involving mice.

5.2. Viral Infections

Mice at 8-12 weeks of age were anesthetized with 2,2,2-tribromoethanol (also known as Avertin) intraperitoneal (i.p.) and infected intranasally (i.n.) with either 300 50% egg infectious doses (EID_{50}) Influenza virus A/HK-x31 (H3N2; referred to as X31) in 30 μl of phosphate buffered saline (PBS) (Crowe et al., 2003) or 250 EID_{50} of Sendai virus (Enders) in 30 μl of PBS (Cauley et al., 2002; Hou et al., 1992). Experimental procedures

involving Sendai virus and Sendai virus-infected mice were carried out in a designated Biosafety Level 3 facility at the Trudeau Institute.

5.3. *T. gondii* Infections

Cysts of the *T. gondii* strain ME49 were obtained from brains of chronically infected C57BL/6 mice and infections were initiated by oral gavage of 10 cysts in 0.1 ml of diluted brain suspension using a 19-gauge gavage needle (Johnson et al., 2003). Sham-infected mice received similarly diluted brain suspensions from uninfected mice.

5.4. Tissue Sampling and Preparation

Peripheral blood was collected into heparin before pleural exudates cells (PLC) were isolated by lavage through the diaphragm. Next, bronchoalveolar lavage (BAL) cells were collected by 5 consecutive washes with 1 ml PBS each. Mice were perfused through the heart after the portal vein was cut for drainage. Perfused lung and liver tissues were either mechanically cut into small pieces or dispersed by passage through a 70 μm cell strainer and subsequently digested for 45 min at 37°C with collagenase IV (100 U/ml, Sigma-Aldrich) and DNase I (10 U/ml, Sigma-Aldrich). Hepatocytes were then sedimented at 30 x *g* for 3 min and non-hepatocytes collected from the supernatant. Subsequently lymphocytes were enriched in the interphase of a discontinuous 60%/40% Percoll (Amersham Biosciences) gradient spun at 1200 x *g* for 20 min at room temperature. Single cell suspensions were prepared from spleen, mediastinal lymph nodes (medLN) and bone marrow (BM) by mechanical dispersion through a 70 μm cell strainer. Erythrocytes were removed from blood, spleen and BM by ammonium chloride lysis. Adherent cells from BAL and PLC were depleted by incubation in complete RPMI (cRPMI; supplemented with 10% heat-inactivated fetal calf serum , 100 U/ml penicillin/streptomycin, 50 μM 2-mercaptoethanol and 2 mM L-glutamine; Fisher/Cellgro) medium, for 2 h in tissue culture dishes at 37°C, 5% CO_2. Panning on goat anti-mouse IgG H+L-coated (Jackson

ImmunoResearch Laboratories Inc.) Primaria flasks (BD Falcon) for 1 hr at 37°C, 5% CO_2, depleted B cells and adherent cells from the spleen.

5.5. Flow Cytometric Analysis

Surface antigens were analyzed by flow cytometric analysis using monoclonal antibodies that were purchased from Caltag Laboratories, eBioscience or BD Biosciences if not stated otherwise and clone designations are given in parentheses. Single cell suspensions were kept on ice at all times and stained in 1% bovine serum albumine (BSA), 0.1% azide in PBS (FACS buffer). All samples were first incubated with anti-CD16/32 (2.4G2) to block non-specific binding of antibodies to Fc III/II receptors. Monoclonal antibodies were directly conjugated to phycoerythrin (PE), Peridinin chlorophyll protein (PerCP), allophycocyanin and Biotin or tandem conjugates of PE-Texas Red, PE-Cy7 and APC. Where necessary Streptavidin (SA)-PE, SA-PerCP, SA-allophycocyanin or SA-allophycocyanin-Cy7 were used to detect biotin labeled antibodies. The following monoclonal antibodies were used: CD3ε (145-2C11), CD4 (RM4-5), CD8α (CT-CD8α), CD11a (M17/4), CD11c (HL3), CD19 (1D3), CD25 (PC61 5.3), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD90.1 (HIS51 and OX-7), CD90.2 (53-2.1), CD122 (TM-γ1) and NK1.1 (PK136), CD27 (LG.3A10), H-2D^b (KH95), I-A^b (AF6-120.1), ICOS (C398.4A). MHC class I peptide tetramers specific for the Influenza nucleoprotein (NP) epitope $NP_{366-374}/D^b$, the Influenza acid polymerase (PA) protein epitope $PA_{224-233}/D^b$ and the Sendai nucleoprotein epitope $NP_{324-332}/K^b$ (SenNP) as well as the MHC class II peptide multimer specific for the Sendai hemagluttinin-neuraminidase epitope (HN) $HN_{419-433}/A^b$ (SenHN) were generated according to described methods (Altman et al., 1996; Arnold et al., 2002; Cauley et al., 2002) and purchased from the Molecular Biology Core facility (MBCF) at the Trudeau Institute. Tetramer and multimer staining was performed for 1 h at room temperature prior to staining of surface antigens. Sendai virus infected samples were then fixed in 1% paraformaldehyde and subsequently analyzed by flow cytometry. Dead cells were identified in unfixed samples by addition of DAPI (4',6-Diamidino-2-phenylindole, 0.1

μg/ml; Sigma-Aldrich) or PI (propidium iodide, 3 μg/ml, Sigma-Aldrich) and excluded from the analyses.

Four-color samples were acquired on a FACSCalibur™ (Beckton Dickinson) cytometer with CellQuest software, or a FACScan™ (Beckton Dickinson) cytometer upgraded to 5 colors (Cytek) equipped with Rainbow software (Cytek Development) and CellQuest software. 5-9 color samples and samples stained with tandem conjugate fluorochromes other than allophycocyanin-Cy7 were acquired on a CyAn (DAKO Cytomation) flow cytometer equipped with Summit 3.3 software.

Data were analyzed using FlowJo (Tree Star) software. Electronic compensation matrices for data acquired on the CyAn cytometer were calculated and verified using the FlowJo compensation platform based on proper single stain controls. All analyses of unfixed samples were gated on lymphocytes within a live (PI- or DAPI-) gate.

5.6. IFN-γ Secretion Assay

Splenocytes from day 9 influenza infected mice were cultured at 5 x 10^6/ml in the presence or absence of PMA (50 ng/ml, Sigma) and ionomycin (500 ng/ml; Sigma) in cRPMI for 4 h at 37°C, 5% CO_2. The IFN-γ Secretion Assay is a flow cytometry-based assay for measuring cytokine secretion by individual T lymphocytes (Brosterhus et al., 1999). The assay is designed for the detection, isolation, and analysis of T cells responding by IFN-γ secretion to brief (approximately 3- to 16-h) *in vitro* stimulation with a either antigen or PMA and ionomycin. The secreted IFN-γ is captured on the cell surface of the secreting cell, using an affinity matrix for the secreted cytokine (catch reagent) which consists of a bispecific antibody able to capture IFN-γ conjugated to a cell-surface specific antibody (Brosterhus et al., 1999). Captured IFN-γ is then detected by a PE-labeled second antibody specific for a distinct IFN-γ epitope (detection antibody). The subsequent analysis by flow cytometry allows for assessment of lymphocytes secreting IFN-γ, on a single cell level. The IFN-γ Secretion Assay was performed according to the manufacturer's instructions (Miltenyi Biotec). A "High Control" (Hu-Li et al., 2001) was

included by the addition of recombinant murine IFN-γ (100 ng/ml, eBioscience) and the staining specificity was confirmed by using a PE-labeled rat IgG1 isotype control (R3-34).

5.7. Intracellular Cytokine Staining

For immunofluorescent staining of intracytoplasmic cytokines, cells were stimulated with either PMA (50 ng/ml, Sigma) and ionomycin (500 ng/ml; Sigma) or peptide ($SenNP_{324-332}/K^b$ 10 μg/ml; $SenHN_{419-433}/A^b$ 10 μg/ml; soluble toxoplasma antigen, STAg 10 μg/ml) in cRPMI for 4 hrs at 37°C, 5% CO_2. Brefeldin A (10 μg/ml; Sigma) was added for the last 2 hrs of stimulation to allow for accumulation of cytokines within the cells. 2 x 10^6 cells were stained for surface markers prior to fixation and permeabilization with BD Cytofix/Cytoperm solution (Cytofix/Cytoperm™ Kit; BD Biosciences, 20 min 4°C). Subsequently cells were stained with α-IFN-γ (XMG1.2; BD Biosciences), α-IL-4 (11B11, BD Biosciences), α-IL-17 (TC11-18H10; BD Biosciences) or rat IgG1 (R3-34; Calteg) in 1x BD Perm/Wash solution (Cytofix/Cytoperm™ Kit; BD Biosciences; 30 min 4°C in darkness) and analyzed by flow cytometry.

5.8. SNARF®-1 labeling of cells

SNARF®-1 carboxylic acid, acetate, succinimidyl ester (SNARF-1; Molecular Probes) is a vital red fluorescent dye, similar to CFSE, for monitoring cytokinesis (Lyons et al., 2001). Up to 10^7 cells/ml were labeled with 5 μM SNARF-1 in PBS for 7 min. at 37°C in a waterbath. Cell division was monitored by Flow cytometry in the FL2 channel.

5.9. Cell Sorting

For in vitro priming, single cell suspensions from the lymph nodes and spleens of naïve Yeti WT or Yeti IFN-γR-/- were depleted of B cells and adherent cells (see 3.4)prior to purification of CD8+ or CD4+ T cells. CD8+ and CD4+ T cells were then purified using either negative selection with magnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec) or were stained with CD4-PE, CD8α-PE-Cy5 and CD62L-APC or CD44-APC, using buffers without azide. The cells were sorted into eYFPneg/CD44hi or eYFPneg/CD62Llow CD4+ and CD8+ populations using a FACSVantage^{TM} (Becton Dickinson) cell sorter with DiVa enhancement software. In some experiments the CD8+ and CD4+ T cell cultures were resorted according to their eYFP expression into eYFP negative (eYFPneg), eYFP intermediate (eYFPint) and eYFP high (eYFPhi) populations on day 1 and day 5 of culture, respectively.

On day 9 after influenza infection T cells from BAL, lung and medLN from 12-15 Yeti WT mice were prepared as described using buffers without azide. Samples were then stained with CD4-PE, CD8α-PE-Cy5 and CD62L-APC and subsequently sorted. MedLN samples were sorted into eYFPneg and eYFPint populations within a CD62Llow, CD4+ or CD8α+ lymphocyte gate. Pooled BAL/lung samples were first sorted into total CD8+ cells and CD62Llow/CD4+ cells with an eYFPneg, eYFPint and eYFPhi phenotype. CD8+ cells were subsequently resorted and separated according to eYFP fluorescence within a CD62Llow gate.

5.10. *In Vitro* T Cell Priming and Cultures

Purified CD4+ or CD8+ T cells (1 x 10^6/ml) in cRPMI media were stimulated in 48-well plates in the presence of anti-CD3ε mAb (145-2C11, 2 μg/ml, BD Pharmingen) and anti-CD28 mAb (37.51, 5 μg/ml, eBioscience) and recombinant murine IL-2 (5 ng/ml, BD Pharmingen) and irradiated (3000 rad from a ^{137}Cs source) B6 WT or GKO splenocytes as APCs (5 x 10^6/ml) (neutral priming conditions). The following cytokines and antibodies were added to the cultures as indicated: recombinant murine IL-4 (50 ng/ml, R&D Systems), recombinant murine IL-12 (5 ng/ml, Peprotech), recombinant murine IL-18

(30ng/ml, R&D Systems), recombinant murine IFN-γ (30 ng/ml, eBioscience), anti-IL-4 (11B11, 20 μg/ml, MBCF Trudeau Institute), anti-IL-12 (C17.8, 20 μg/ml, MBCF Trudeau Institute) and anti-IFN-γ (XMG1.2, 20 μg/ml, MBCF Trudeau Institute). Under type 1 priming conditions, IL-12 and anti-IL-4 mAb, and, under type 2 priming conditions, IL-4 and anti-IFN-γ, were added to the respective cultures. Some cells were labeled with the vital red fluorescent dye SNARF-1 (Molecular Probes) to monitor cell division.

T cells sorted from Influenza virus-infected animals were cultured at 5 x 10^5/ml in flat-bottom 96-well plates for 24 h in cRPMI at 37˚C, 5% CO_2. Cells were activated by plate-bound anti-CD3ε (10 μg/ml) where indicated.

5.11. Cytokine and Chemokine Quantification using RT-PCR

For RT-PCR, RNA was extracted using the RNAqueous-4PCR kit (Ambion) and reverse transcribed with the Superscript II RNase H^- kit (Invitrogen) using oligo$(dT)_{18}$ priming. To analyze mRNA expression of cytokines and chemokines, quantitative real-time RT-PCR was performed using gene-specific primers and probes (Johnson et al., 2003, Overbergh, 1999 #361), purchased from the MBCF at the Trudeau Institute, and the ABI Prism 7700 Sequence BioDetector (PE Biosystems) according to the manufacturer's instructions (TaqMan, Perkin Elmer). Ct (cycle threshold) values were normalized to levels of the housekeeping gene GAPDH (routinely between 15 and 18 cycles) and normalization to the housekeeping gene ß-2-microglobulin gave similar results.

5.12. Cytokine and Chemokine Quantification in Supernatants

Cytokines and chemokines in culture supernatants were quantified using traditional ELISA (**e**nzyme-**l**inked **i**mmunosorbent **a**ssay) or a multiplex flow cytometric suspension microbead array (Carson and Vignali, 1999). Plates and reagents were purchased from Fisher Scientifc if not otherwise stated. Cytokine quantification by ELISA was performed

using paired antibodies for IFN-γ (R4-6A2; XMG1.2; both eBioscience). Briefly, Nunc™ MaxiSorp™ 96-well immunoplates were coated overnight at 4°C with unconjugated rat anti-mouse IFN-γ (R4-6A2, 2μg/ml) in PBS containing 0.05% NaN_3, washed with PBS containing 0.05% Tween 20 (PBS-Tween) and subsequently blocked with PBS containing 0.05% NaN_3, 1% BSA and 0.5% sucrose (Amresco) for 2 hours at room temperature (RT). After washing, samples and recombinant mouse IFN-γ (eBioscience) as standard were serially diluted in PBS containing 0.05% NaN_3 and 1% BSA and applied for 2 hours at RT. Cytokine-antibody binding was detected using unconjugated mAb against mouse IFN-γ (XMG1.2, 0.5μg/ml in PBS 0.05% NaN_3, 1% BSA, 1 hour RT) and visualized with a conjugate of biotinylated alkaline phosphatase/streptavidin (Caltag, 45 min RT) and p-nitrophenyl phosphate as substrate (Sigma, in alkaline buffer). If necessary, reactions were stopped adding 0.5M NaOH. Absorbance was read at 405 nm with a Spectramax™ 190 ELISA microplate reader (Molecular Devices) equipped with SOFTmax™ Pro Version 4.0 (Molecular Devices) software.

Multiplex flow cytometric suspension microbead arrays are based on 5.6 micron polystyrene microbeads that are internally dyed with fluorochromes. Using different intensities of the dyes for different batches of microbeads results in unique spectral signatures determined by its mixtures. Thus, it is possible to distinguish up to 100 different parameters via flow cytometry in one sample. The surface chemistry of the microbeads allows coupling of capture reagents such as antibodies, oligonucleotides, peptides or even receptors. In our case, monoclonal antibodies specific for a cytokine or chemokine are covalently linked to a fluorescent bead set, which captures the cytokine. A complementary biotinylated monoclonal cytokine/chemokine antibody then completes the immunological sandwich and the reaction is detected with SA-PE. Multiplex flow cytometric microbead array analysis was performed according to the manufacturer's instructions (Beadlyte® Multi-Cytokine/Chemokine Flex-Kit; Upstate USA). Briefly, 50 μl of sample or recombinant mouse cytokine/chemokine standards were diluted in Beadlyte® assay buffer, mixed with 25 μl of relevant mouse cytokine or chemokine capture beads, and incubated for 2 h at RT in a microplate shaker. Unbound material was removed from the bead mixture by placing the filter plate over a vacuum manifold (Millipore Inc.). Beads were washed extensively in Beadlyte® assay buffer, resuspended in 75 μl of assay buffer and mixed with 25 μl of biotinylated secondary antibodies. The

mixture was then incubated for 1.5 h at 25°C in a microplate shaker. Next, 25 µl of SA-PE was added, incubated for 30 min, washed and samples were finally resuspended in 125 µl of assay buffer. The following mouse Beadmates™ were combined in a multiplex analysis: IL-2, IFN-γ, GM-CSF, TNF-α, RANTES (CCL5), SDF (CXCL12) and MIP-1β (CCL4). The median fluorescence intensity (MFI) was measured from at least 50 beads per set using the Luminex *x*MAP™ system (Luminex). The concentration of cytokines and chemokines was determined using a 5 parameter curve-fit generated from the MFI's of the respective mouse cytokine and chemokine standards.

5.13. Dual Adoptive Transfer System

For dual transfer experiments single cell suspensions from the lymph nodes and spleens of naïve Yeti WT (CD45.1 or CD90.1) and Yeti IFN-γR-/- (CD45.2, CD90.2) were depleted of B and adherent cells as described above. An aliquot of the cells was analyzed prior to transfer by FACS analysis in order to determine the percentage of CD4+ and CD8+ T cells and subsequently combined such that the total number of CD4+ and CD8+ cells in each donor population was equal. The combined donor cells were then transferred (1.7 x 10^7 total cells) in 200 µl into naive TCRβδKO recipient mice via tail vein injection of 200 µl. 1 d later, recipient mice were challenged with Sendai virus or *T. gondii* as described above, and T cell responses were analyzed on day 9 or day 7 post infection, respectively.

5.14. Bone marrow Chimeras

Bone marrow (BM) chimeras were generated by reconstituting lethally irradiated (2x 475 rads from a ^{137}Cs source) TCRβδKO recipient mice with a total of 1 x 10^7 donor-derived whole BM cells (Lee et al., 2003). Erythrocytes were removed from donor BM by ammonium chloride lysis. Donor BM cells from naïve Yeti WT (CD45.1 or CD90.1) and Yeti IFN-γR-/- (CD45.2, CD90.2) donor mice were transferred at equal numbers via tail

vein injection in a volume of 200 µl. Mice were put on an antibiotic diet for at least 3 weeks after transfer and allowed to reconstitute for 6-8 weeks prior to Sendai virus or *T. gondii* infection.

5.15. Statistical Analysis

Statistical analysis was performed using Prism 3.0c (Graphpad Software). Asterisks indicate statistical differences with p values <0.05 in a Student's t test.

6. RESULTS

6.1. Assessing IFN-γ expression by CD4+ and CD8+ effector T cells

6.1.1. IFN-γ expression by CD4+ and CD8+ T cells is heterogeneous *in vitro*

Yeti mice represent a unique tool to directly visualize IFN-γ expressing cells (Stetson et al., 2003). Briefly, in these mice eYFP is linked to the endogenous *ifng* gene and activation and transcription of the endogenous *ifng* gene locus results in one mRNA with two cistrons. Due to the IRES element, eYFP and IFN-γ are translated separately. While IFN-γ is secreted, eYFP is retained in the cell, thereby marking the IFN-γ expressing cell. Yeti mice allow therefore non-invasive and unbiased tracking of any IFN-γ expressing cell using flow cytometry. Thus, to analyze the expression of IFN-γ during the activation of T cells, CD4+ and CD8+ T cells were purified from naïve Yeti mice or B6 WT littermates and stimulated with anti-CD3ε and anti-CD28 in the presence of irradiated APCs. The cultures were supplemented with IL-12 plus α-IL-4 (Th1/Tc1 conditions) or IL-4 plus α-IFN-γ (Th2/Tc2 conditions) as indicated. To assess cell division, some cells were labeled with the vital red-fluorescent dye SNARF-1 (Lyons et al., 2001) and cultured under the same conditions. As shown in Figure 1A, CD8+ T cells expressed high levels of eYFP within 24 h of priming although they had not divided yet. The eYFP fluorescence was broadly heterogeneous and Tc1 conditions clearly increased IFN-γ expression while Tc2 conditions reduced the frequency and brightness. Both effects were more apparent after 3 days of culture. In contrast to CD8+ T cells, CD4+ T cells required 2 days of priming to become eYFP fluorescent and were only induced to do so under Th1 conditions (Figure 10B). Although at least one cell division was apparent, even undivided cells were eYFP+ indicating that the time in culture rather than cytokinesis is the limiting factor for IFN-γ expression. Heterogeneous eYFP expression was maintained in Th1 cells over the 5-day culture period. To confirm that eYFP fluorescence correlates with the expression of IFN-γ we sorted CD8+ and CD4+ T cells into eYFP negative (eYFPneg), eYFP intermediate

(eYFPint) and eYFP high (eYFPhi) populations and determined the abundance of IFN-γ transcript by real time RT-PCR. The eYFP brightness of both CD8+ (Figure 10C) and CD4+ (Figure 10D) T cells correlated positively with the abundance of IFN-γ transcripts of the respective population. Together, these data show that IFN-γ expression by CD4+ and CD8+ is broadly heterogeneous and can be directly quantified by the brightness of the bicistronic eYFP reporter.

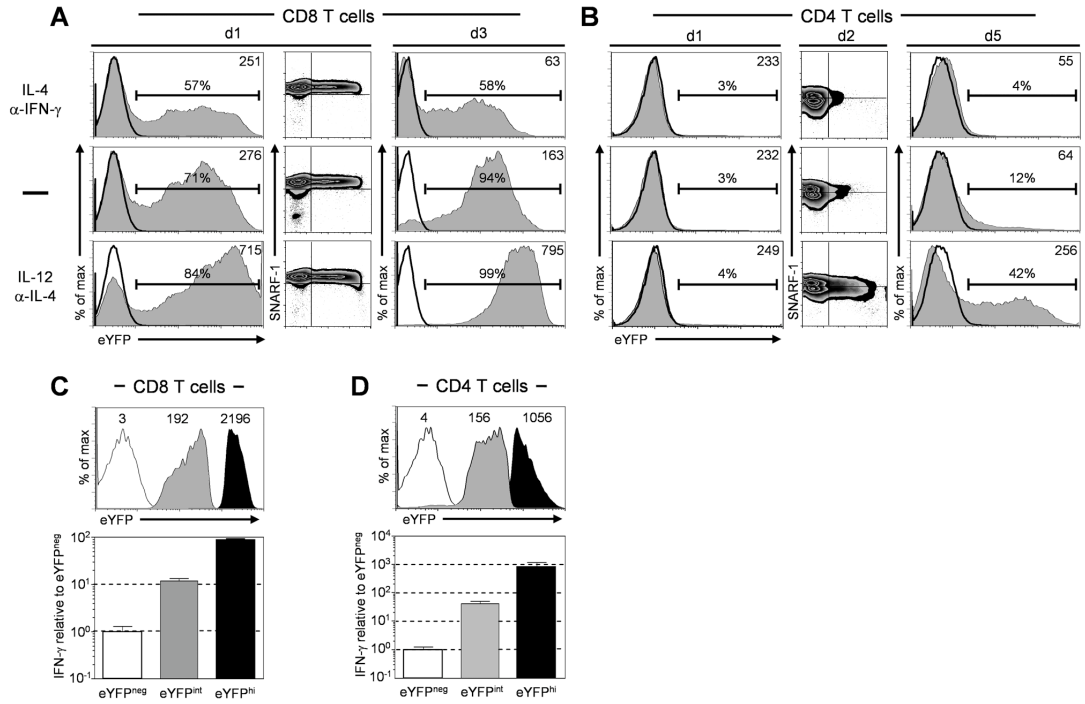


Figure 10:
Heterogeneous fluorescence of the bicistronic IFN-γ-eYFP reporter correlates with IFN-γ expression.

CD8+ **(A)** or CD4+ **(B)** T cells were purified from the lymph nodes of naïve Yeti mice (gray histograms) or wt littermate controls (bold line) and stimulated with anti-CD3ε and anti-CD28 in the absence or presence of polarizing cytokine conditions and APCs. Some cells were labeled prior to culture with the vital red-fluorescent dye SNARF-1. On the indicated days, the cells were analyzed by FACS for eYFP expression and SNARF-1 fluorescence. Cultures of CD8+ **(C)** T cells cultured under neutral conditions and CD4+ **(D)** T cells cultured under Th1 conditions were sorted on day 1 and day 5, respectively, into eYFP^{neg} (open histogram and bar), eYFP^{int} (gray histogram and bar) and eYFP^{hi} (black histogram and bar) populations (top panels). The abundance of IFN-γ transcripts in the sorted populations was determined by real-time RT-PCR and is depicted relative to the eYFP^{neg} population. The frequency and MFI of the eYFP positive cells are noted in the histograms. All data are representative of two or more independent experiments.

6.1.2. Expression of IFN-γ in respiratory virus-infected Yeti mice is broadly heterogeneous and highly fluorescent cells are restricted to the infected lung

To investigate the expression of IFN-γ during infection, we analyzed the response to intranasal challenge with Influenza or Sendai virus, respectively. Yeti mice and B6 WT littermates were infected i.n. with Influenza virus or Sendai virus and analyzed 9 days later, at the peak of the cellular response, by FACS for eYFP expression. Consistent with published data, non-infected Yeti controls revealed only a background of eYFP fluorescence in all examined tissues, (Figure 11 and 12) (Matsuda et al., 2003; Stetson et al., 2003). The background fluorescence intensity was low and the number of eYFP+ cells in the BAL and lung was prior to infection very small (Figure 11A and 12). Upon infection with Influenza or Sendai virus the number and frequency of eYFP+ cells

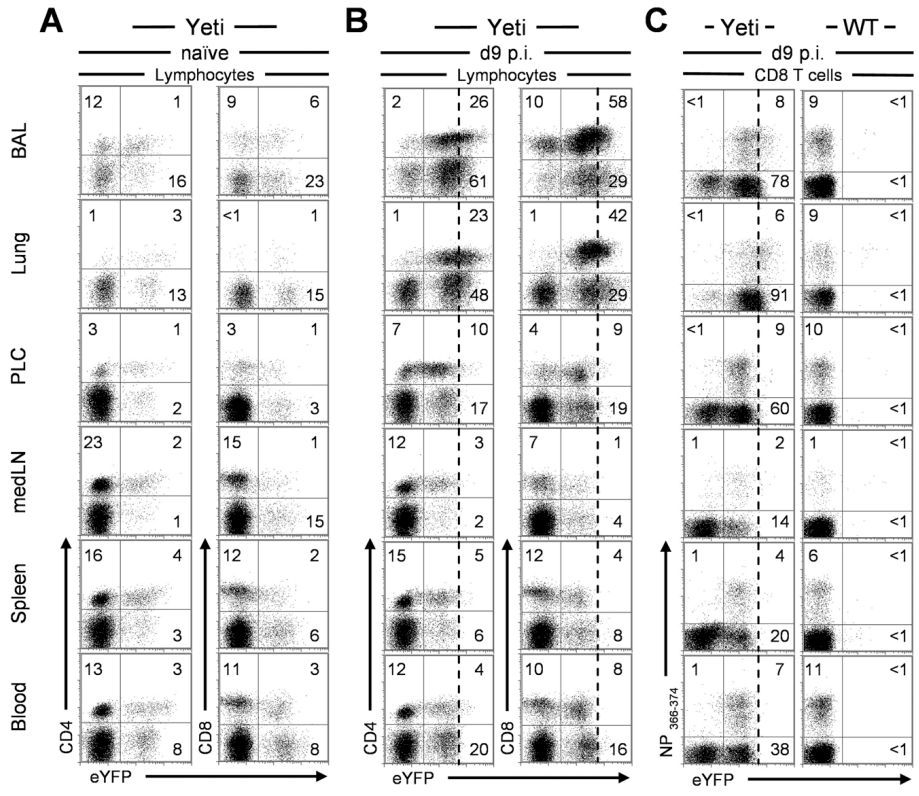


Figure 11:
Expression of the bicistronic IFN-γ- eYFP reporter in naïve and Influenza-infected Yeti mice.

A Indicated organs from naive Yeti mice were analyzed by FACS for eYFP fluorescence of $CD4^+$ and $CD8^+$ T cells. **B** Yeti mice were infected i.n. with Influenza virus and FACS was performed 9 days later together with the analysis of naïve mice in (A). **C** Yeti mice and wt littermate controls were infected as in (B) and $CD8a^+$-gated T cells from the indicated organs were analyzed by flow cytometry for the expression of eYFP and $NP_{366-374}$ tetramer staining. The vertical dashed line demarcates $eYFP^{int}$ and $eYFP^{hi}$ cells. Data are representative of multiple independent experiments. In some of these experiments five individual mice per cohort were analyzed with comparable results.

increased substantially, mainly in the BAL (>100-fold), the lung (>6-fold) and the pleural cavity (>7-fold) (Figure 11B, 12A, 13B and data not shown). Essentially all eYFP+ cells were in all organs contained within a FSC/SSC lymphocyte gate and were predominantly identified as CD4+ or CD8+ T cells (60-95%) with a minor contribution of NK and NK T cells (Figure 11B, 12B and data not shown). T lymphocytes in the lung parenchyma and airways were almost exclusively eYFP positive (Figure 11B and 13B) although only one *ifng* allele was marked by the bicistronic reporter. In contrast, the frequency of eYFP positive T lymphocytes remained low in all secondary lymphoid organs, including the draining medLN and in other peripheral tissues besides the lung (Figure 11B, 13B and data not shown). The eYFP fluorescence intensity of eYFP+ CD4+ and CD8+ T cells was remarkably heterogeneous in all examined tissues and highly fluorescent cells (eYFPhi) were present only in the lung airways (BAL) and parenchyma of infected mice (Figure 11B, 13B). Even the pleural cavity, which harbors the infected lung and had a similar increase and frequency of eYFP+ cell as the lung, did not contain cells of comparable brightness (Figure 11B, 11C and data not shown).

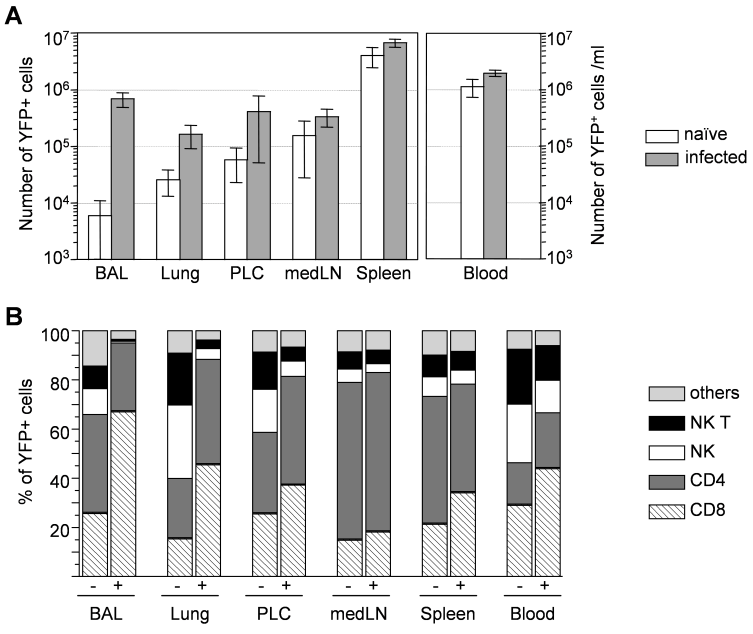


Figure 12:
Total numbers and identity of eYFP+ cells in naïve and Influenza-infected Yeti mice.

Naïve and day 9 Influenza virus infected Yeti mice were analyzed by flow cytometry for eYFP expression.

A Viable cells were counted by trypan blue exclusion using a hemacytometer and the total number of eYFP+ cells from naïve (white bars) and day 9 influenza-infected (grey bars) Yeti mice was calculated according to the flow cytometric analysis within a negative PI or DAPI gate. Depicted are the mean and standard deviation obtained from three mice per group. These data are representative of six independent experiments with similar result.

B The relative contribution of cellular subsets to the eYFP+ population of naïve (-) and day 9 influenza-infected (+) Yeti mice was determined by flow cytometry using mAb to CD4, CD8α, NK1.1 and CD3ε. The following classifications were made and indicated in the legend: CD4 (CD4+, CD8α-; dark grey bars), CD8 (CD4-, CD8α+, hatched bars), NK (NK-1.1+, CD3ε-, white bars), NK T (NK-1.1+, CD3ε+, black bars) and uncharacterized others (light grey bars). Depicted is the mean from three mice per group. These data are representative of three independent experiments with similar result.

We next used MHC class I tetramers to analyze IFN-γ expression in antigen-specific CD8+ T cells after Influenza or Sendai virus infection (Belz et al., 2000; Cauley et al., 2002; Flynn et al., 1998). Both Influenza $NP_{366-374}$- and $PA_{224-233}$-specific and Sendai $NP_{324-332}$ (SenNP) specific CD8+ T cells disseminated into all examined tissues. These cells were almost exclusively eYFP positive but nonetheless highly heterogeneous in their fluorescence intensity (Figure 2C, 4C and data not shown). Moreover, highly fluorescent CD8+ antigen-specific cells were also only present in the lung airways and parenchyma after infection with both Influenza and Sendai virus. The frequency of Influenza $NP_{366-374}$- or $PA_{224-233}$-specific cells was comparable between Yeti and B6 WT littermate controls in all organs, demonstrating that the insertion of the bicistronic reporter did not affect the cellular response to infection with the Influenza virus (Figure 11C and data not shown).

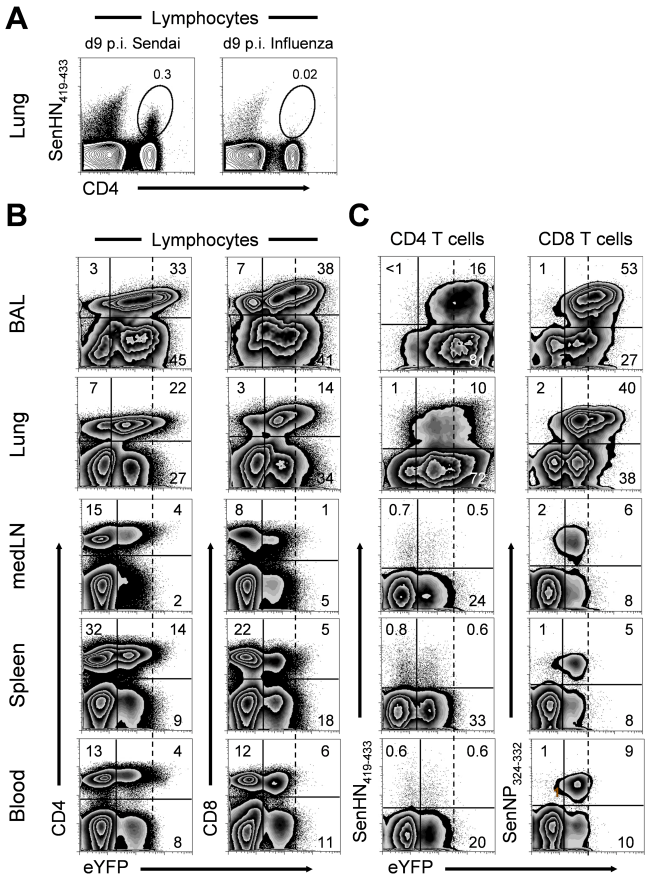


Figure 13:
Expression of the bicistronic IFN-γ- eYFP reporter in Sendai-infected Yeti mice.

A. Lung lymphocytes from Sendai (left plot) and Influenza (right plot) infected mice were analyzed 9 days after infection for specificity of the $SenHN_{419-433}$ multimer staining. **B.** Yeti mice were infected i.n. with Sendai virus and FACS was performed 9 days later. Indicated organs were analyzed by FACS for eYFP fluorescence of CD4+ and CD8+ T cells. **C** CD4+ and CD8a+-gated T cells from the indicated organs were analyzed by flow cytometry for expression of eYFP and $SenHN_{419-433}$ multimer or $SenNP_{324-332}$ tetramer staining, respectively. The vertical dashed line demarcates $eYFP^{int}$ and $eYFP^{hi}$ cells. Data are representative of multiple independent experiments. In some of these experiments five individual mice per cohort were analyzed with comparable results.

To investigate the expression of IFN-γ in antigen-specific CD4+ T cells after Sendai virus infection we used a MHC class II multimer, recognizing CD4+ T cells specific for the hemagluttinin-neuraminidase $HN_{419-433}$ epitope (SenHN) in the context of I-A^b (Arnold et al., 2002; Cauley et al., 2002). In a control experiment, the SenHN MHC class II multimer did not stain in mice infected 9 days earlier with Influenza virus, demonstrating the specificity of the reagent (Figure 13A). SenHN-specific CD4+ T cells also disseminated into all examined tissues but were only in the lung airways and lung parenchyma exclusively eYFP positive (Figure 13C). Antigen-specific CD4+ T cells were also highly heterogeneous in their fluorescence intensity (Figure 13C). Moreover, eYFPhi SenHN-specific CD4+ T cells were only present in the lung airways and parenchyma after Sendai virus infection.

In summary, the heterogeneity in eYFP fluorescence and the anatomical restriction of eYFPhi cells was observed in Influenza $NP_{366-374}$, Influenza $PA_{224-233}$-specific and SenNP-specific CD8+ T cells as well as SenHN-specific CD4+ T cells and is therefore not due to differences in T cell lineage, antigen-specificity or the nature of the respiratory virus.

6.1.3. eYFP fluorescence correlates directly with the expression of acute activation markers

Next we investigated whether heterogeneous eYFP fluorescence intensity correlates with the expression of surface activation markers. Conventional methods to identify cytokine-producing cells at the single-cell level require *in vitro* restimulation (Assenmacher et al., 1998; Openshaw et al., 1995) which alters the expression patterns of surface antigens and prevents their assessment directly ex vivo (Taylor-Fishwick and Siegel, 1995; Ziegler et al., 1994). In contrast, bicistronic cytokine reporter mice allow the direct phenotypic analysis of cytokine-expressing cells *ex vivo* (Mohrs et al., 2005; Mohrs et al., 2001; Shinkai et al., 2002; Stetson et al., 2002; Stetson et al., 2003). Yeti mice were infected with Influenza virus and analyzed 9 days later via flow cytometry. T lymphocytes with a naïve phenotype (CD62Lhi, CD44low, CD45RBhi, CD11alow) were eYFP negative (Figure 14A and data not shown). Conversely, eYFP positive CD4+ and CD8+ T cells were CD62Llow, CD44hi, CD45RBlow and CD11ahi consistent with an

activated/memory phenotype. The activated/memory phenotype of eYFP positive cells was displayed on lymphocytes derived either from secondary lymphoid tissues or peripheral sites and was independent of eYFP brightness. In contrast, the surface expression of acute activation markers such as CD69, CD25, and CD122 on eYFP positive CD4+ and CD8+ T cells was clearly heterogeneous (Figure 14B and data not shown). The expression of these markers on CD4+ and CD8+ T cells correlated positively with eYFP fluorescence. For example, eYFPhi cells in the lung expressed higher levels of CD69 on the surface than eYFPint or eYFPneg cells suggesting a higher level of acute activation.

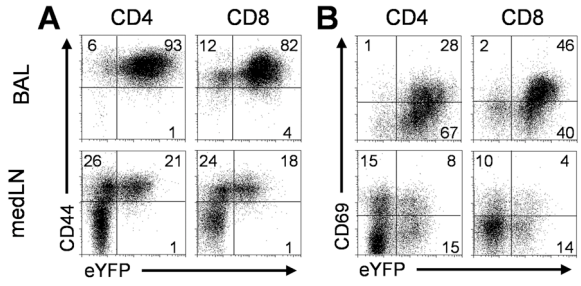


Figure 14:
Surface phenotype of T cells in correlation with eYFP fluorescence.
Yeti mice were infected i.n. with influenza virus. BAL and medLN were analyzed by flow cytometry 9 days later. Depicted dot plots were gated on $CD4^+$ or $CD8a^+$ cells. Shown are eYFP fluorescence versus CD44 **(A)** or CD69 **(B)**. Depicted plots are representative of three individual mice. Similar results were obtained in three independent experiments.

6.1.4. IFN-γ production correlates with eYFP fluorescence but is only induced after stimulation

As described above, we observed remarkable fluorescence heterogeneity in eYFP positive CD4+ and CD8+ T cells after infection with Influenza or Sendai virus (Figures 11,13,14). We next asked whether the brightness of reporter expression correlated with the secretion of IFN-γ protein. Yeti mice and B6 WT controls were infected with Influenza virus and the production of IFN-γ by splenocytes was analyzed 9 days later by cytokine secretion assay (Hu-Li et al., 2001). As shown in figure 6A, the vast majority of eYFP positive but not eYFP negative cells secreted IFN-γ upon short-term stimulation. The secretion of IFN-γ by CD4+, total CD8+ and antigen-specific CD8+ T cells correlated directly with the eYFP fluorescence. In fact, eYFPhi cells secreted the maximum amount

of IFN-γ that can be measured by this assay as determined by the addition of recombinant IFN-γ as a High Control (Hu-Li et al., 2001) (Figure 15A). However, the frequency of IFN--secreting cells was very low when the same cells were cultured in the absence of stimulation with plate-bound anti-CD3ε despite robust eYFP fluorescence. Similar results were obtained by intracellular cytokine staining and antigen-specific stimulation with the Influenza $NP_{366-374}$ peptide (data not shown). Although the cytokine secretion assay is ideal to analyze the secretion of IFN-γ at the single cell level, it is limited to the detection of one cytokine at a time and it is unclear whether the brightness of staining reflects the secreted amount. To directly corroborate the positive correlation between eYFP fluorescence and IFN-γ production and to assay for additional cytokines and chemokines, we measured the accumulation of effector molecules in culture supernatants of cells that were isolated based on different levels of eYFP fluorescence. Yeti mice were infected with Influenza virus and CD4+ and CD8+ T lymphocytes were sorted from the pooled BAL/lung and the medLN into eYFPneg, eYFPint and eYFPhi cells (Figure 15B). We gated on CD62Llow cells since the vast majority of eYFP negative cells in the lymph node have a naïve phenotype (CD44low, CD62Lhi, CD45RBhi), whereas essentially all lymphocytes in the periphery display an effector/memory phenotype (CD44hi, CD62Llow, CD45RBlow). As mentioned earlier, eYFPhi cells were present only in the pooled BAL and lung, while eYFP negative cells could not be obtained in sufficient numbers from these sites (Figure 11). The sorted cells were then cultured in the absence or presence of plate-bound anti-CD3ε for 24 h and the supernatants were analyzed for IFN-γ using a multiplexed cytokine bead array. As shown in figure 15B, IFN-γ secretion by CD4+ and CD8+ cells isolated from either the peripheral effector tissue or the draining medLN correlated positively with the eYFP fluorescence intensity of the sorted population. CD4+ T lymphocytes isolated from the effector site generally secreted more IFN-γ than cells with similar reporter expression isolated from the lymph node. Surprisingly, IFN-γ was almost undetectable in cultures without further *in vitro* restimulation despite the robust eYFP fluorescence of the sorted cell populations at the time of isolation. Upon *in vitro* restimulation with plate bound α-CD3ε (Figure 15B) or PMA + ionomycin (data not shown), the IFN-γ secretion was more than 1000-fold increased. Similar results were obtained when the culture supernatants were harvested after 4 h or 8 h (data not shown). These observations suggest that the potential of CD4+

and CD8+ T cells to secret IFN-γ correlates directly with the expression of the bicistronic reporter; however, cytokine secretion is largely dependent on stimulation

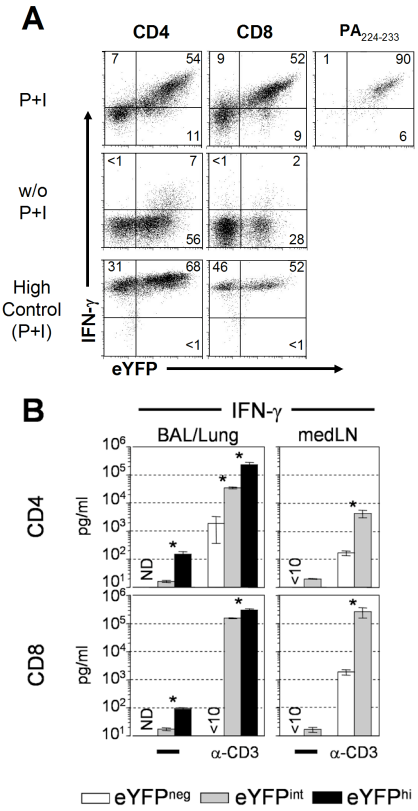


Figure 15:
***Ex vivo* IFN-g production by T cells in correlation with eYFP fluorescence**.

Yeti and wt mice were infected i.n. with Influenza virus and analyzed 9 days later. **(A)** Splenocytes were cultured for 4 h in the presence or absence of PMA+ ionomycin and analyzed by IFN-g secretion assay. Depicted dot plots were gated on $CD4^+$, total $CD8a^+$ or $PA_{224-233}$-specific T cells. The spleens from three mice per group were pooled. Similar results were obtained in two independent experiments. **(B)** Single cell suspensions were prepared from lungs (BAL+lung) and medLN. $CD4^+$ and $CD8a^+$ T lymphocytes with a $CD62L^{lo}$ phenotype were separated by cell sorting according to fluorescence intensity into $eYFP^{neg}$, $eYFP^{int}$ and $eYFP^{hi}$ populations. Purified populations were cultured in the absence (-) or presence (a-CD3) of plate-bound anti-CD3e. Culture supernatants were analyzed for IFN-g using a cytokine bead array. Depicted are mean ± SD. Asterisks indicate statistical differences with p values <0.05 in a Student's t test. Certain cell populations were not obtained in sufficient numbers and IFN-g production could not be determined = ND. Similar results were obtained in two independent experiments.

6.1.5. eYFP fluorescence correlates directly with the production of additional effector cytokines and chemokines

We speculated further that increased reporter fluorescence might correlate not only with the secretion of IFN-γ but also with the enhanced secretion of additional effector cytokines and chemokines. To test this hypothesis we analyzed the culture supernatants from cells sorted by different levels of eYFP fluorescence as described in figure 15B for additional effector cytokines and chemokines. Indeed, the secretion of the type 1 effector cytokines TNF-α and GM-CSF (Figure 16A) and the pro-inflammatory chemokines CCL5 (RANTES) and CCL4 (MIP-1β) (Figure 16B) was largely restricted to eYFP positive cells

and correlated positively with the eYFP brightness of CD4+ and CD8+ from the medLN or the BAL/lung. In contrast, the secretion of IL-2 (Figure 16A) and the chemokine CXCL12 (SDF-1) (Figure 16B) was not restricted to eYFP positive cells and did not correlate with eYFP fluorescence intensity. As seen for IFN-γ, tissue-derived CD4+ T lymphocytes secreted larger amounts of these soluble effector molecules than cells from the lymph node. In fact, among the CD4+ T population, GM-CSF was only detectable in the supernatants of stimulated cells derived from BAL/lung. As observed for the production of IFN-γ, the secretion of these cytokines and chemokines was likewise largely dependent on activation of the CD3 complex. These data show that the brightness of the bicistronic eYFP reporter correlates positively with the potential to coordinately secret a select set of effector cytokines and chemokines in addition to IFN-γ. The production of

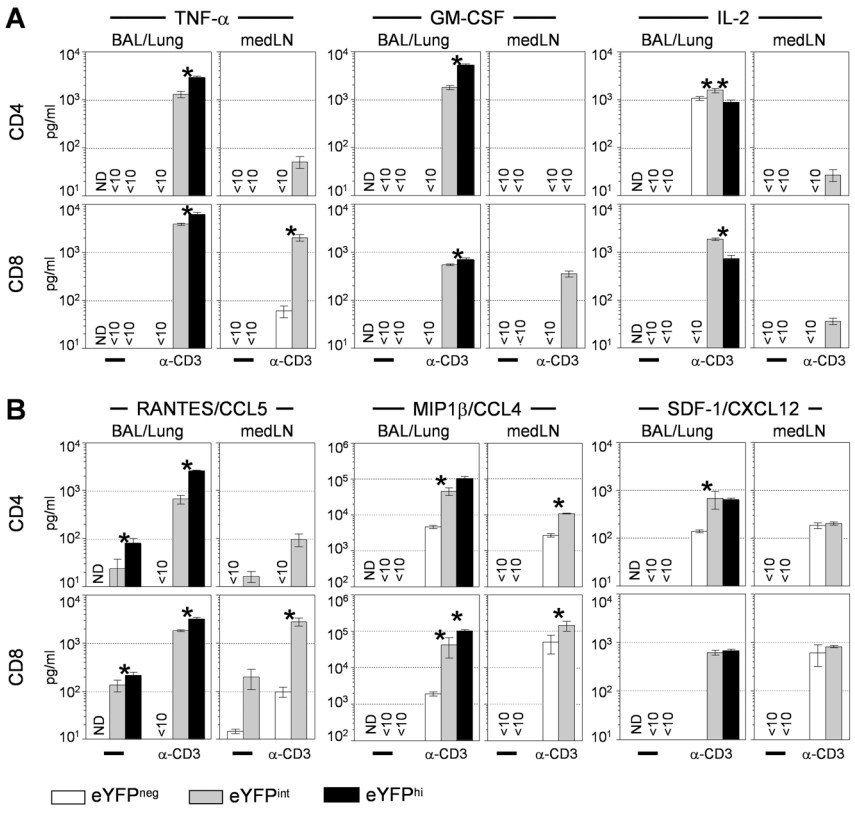


Figure 16:
***Ex vivo* cytokine and chemokine production by T lymphocytes.**

Yeti mice were infected with influenza virus and the indicated populations were isolated 9 days later as described in Fig. 4. Subsequently eYFP^{neg}, eYFP^{int} and eYFP^{hi} populations were cultured in the absence (-) or presence (α-CD3) of plate-bound anti-CD3ε. Culture supernatants were analyzed for **(A)** the cytokines TNF-α, GM-CSF, IL-2, and **(B)** the chemokines RANTES/CCL5, MIP-1β/CCL4 and SDF-1/CXCL12 using a cytokine bead array. Depicted are mean ± SD. Detection limits for the cytokines or chemokines are indicated in the respective graphs. Asterisks indicate statistical differences with p values <0.05 in a Student's t test. Certain cell populations were not obtained in sufficient numbers and protein production could not be determined = ND. Similar results were obtained in two independent experiments.

these cytokines and chemokines by CD4+ and CD8+ T cells *ex vivo* is largely restricted to eYFP positive cells and is dramatically increased upon stimulation. These data suggest that increased eYFP fluorescence reflects the progressive effector differentiation of activated CD4+ and CD8+ T cells and that highly differentiated cells are restricted to the site of infection.

6.1.6. The anatomical restriction of eYFPhi cells, but not the heterogeneity of eYFP, depends on the pathogen

Finally we wanted to study whether the observed heterogeneity of eYFP fluorescence or the anatomical restriction of eYFPhi cells after respiratory virus infection are specific for a given type of pathogen. To this end, Yeti mice were infected orally with the protozoan parasite *T. gondii*, which induces a vigorous type I response, and various organs were analyzed 1 week later by FACS (Figure 17). *T. gondii*-infected mice revealed a similar heterogeneity of eYFP fluorescence of CD4+ and CD8+ T cells as Influenza- and Sendai-infected animals. However, in contrast to Influenza or Sendai-infected mice, eYFPhi cells were restricted to other organs such as the mesLN, liver, lung or the blood

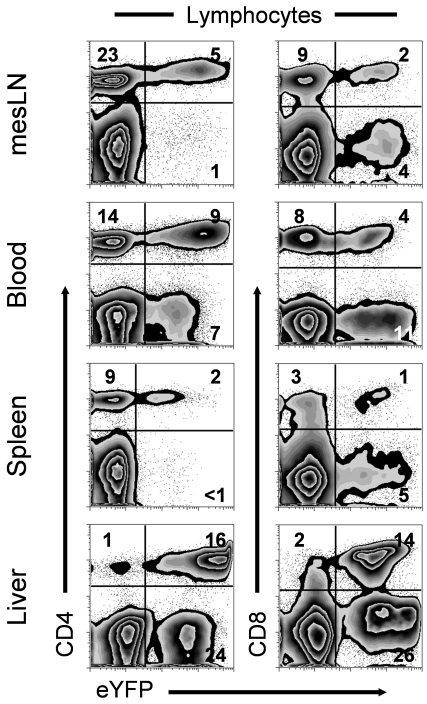


Figure 17:
Expression of the bicistronic IFN-g-eYFP reporter in T. gondii-infected Yeti mice.
Yeti mice were infected orally with *T. gondii* and $CD4^+$ and $CD8^+$ T cells in the indicated organs were analyzed after 1 week for their eYFP fluorescence. The vertical dashed line demarcates $eYFP^{int}$ and $eYFP^{hi}$ cells. Data are representative of two independent experiments with a minimum of 3 individual mice or 3 pooled mice per group.

(Figure 17 and data not shown). In fact, blood-borne CD4+ T cells from *T. gondii*-infected animals had substantially higher MFI (eYFP) values than cells in any organ of Influenza or Sendai virus-infected animals (Figure 11, 13 and 17). Consistent with this observation, *T. gondii*-infected mice have high serum IFN-γ concentrations during acute infection ((Denkers and Gazzinelli, 1998; Johnson et al., 2003) and data not shown). Thus the heterogeneous expression of IFN-γ by CD4+ and CD8+ T cells and the selective accumulation of eYFPhi cells in certain tissues are not limited to localized viral infections. In contrast, in which tissue eYFPhi cells accumulate is dependent on the pathogen, despite the wide dissemination of eYFP positive cells.

6.2 The Role of IFN-γ receptor mediated signals for IFN-γ expression by CD4+ and CD8+ T cells

6.2.1. Differential requirement for IFN-γ receptor mediated signals for IFN-γ expression by CD4 and CD8 T cells *in vitro*

To investigate the requirement of IFN-γ receptor mediated signals for the subsequent expression of IFN-γ in T cells, naïve CD4+ and CD8+ T cells were purified from CD45.1+ Yeti WT and Yeti IFN-γR1-deficient (Yeti IFN-γR-/-) mice by sorting for an eYFP negative/CD44low phenotype. Purified cells were stimulated with α-CD3ε, α-CD28 and IL-2 (neutral conditions) in the presence of irradiated IFN-γ deficient (GKO) APCs (Dalton et al., 1993; Huang et al., 1993; Stetson et al., 2003). The splenic APCs were obtained from Thy1.1+ GKO mice to exclude APCs as a potential source of IFN-γ and to omit live non-Yeti effectors based on their Thy1 disparity from the T cell analysis (Dalton et al., 1993). CD4+ and CD8+ T cells were cultured with IL-12 plus α-IL-4 (Th1/Tc1 conditions) or IL-4 plus α-IFN-γ (Th2/Tc2 conditions) where indicated and analyzed on d5 or d3 post priming, respectively (Figure 18). Consistent with data shown in paragraph 4.1.1, neither Yeti WT nor Yeti IFN-γR-/- CD4+ T cells induced the eYFP reporter under neutral or Th2 conditions, while Th1 polarizing conditions lead to robust eYFP expression by Yeti WT CD4+ T cells (Figure 18A). In contrast, the frequency of eYFP positive Yeti IFN-γR-/- CD4+ T cells was substantially decreased under Th1 polarizing condition, despite similar cell recovery (Figure 18A and data not shown), suggesting a critical role of IFN-γ mediated signals for optimal IFN-γ expression by CD4+ effector T cells. And, consistent with previous data (paragraph 4.1.1), robust eYFP expression by Yeti WT CD8+ T cells could be detected in all priming conditions and the addition of IL-12 resulted in the highest frequency and brightness (Figure 18B). Yeti IFN-γR-/- CD8+ T cells also induced IFN-γ under all conditions, however, their frequency and brightness was substantially lower under neutral and Tc2 polarizing conditions when compared to Yeti WT cultures (Figure 18B). Interestingly, the addition of IL-12 to Yeti IFN-γR-/- cultures restored the defect in IFN-γ expression, revealing that IL-12 is able to compensate for the

lack of IFN-γR signals in CD8+ T cells. Thus, activated CD8+ T cells express IFN-γ independently of IFN-γR mediated signals but IFN-γ mediated functions are required for optimal IFN-γ expression in the absence of IL-12.

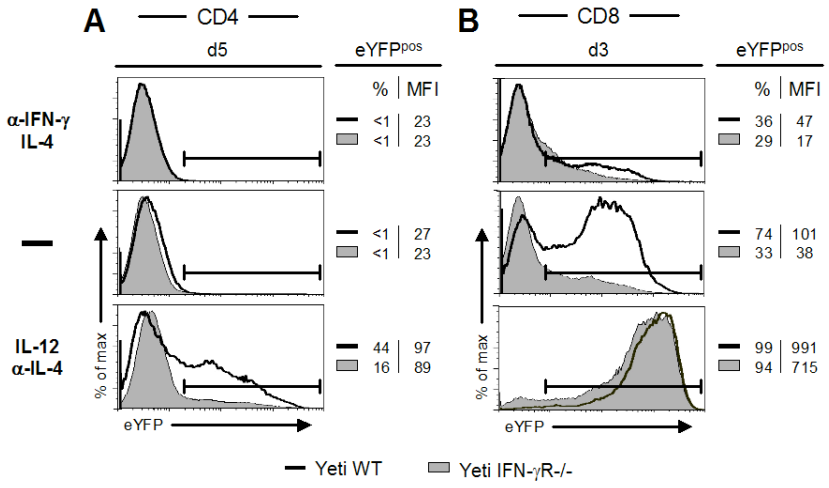


Figure 18:
Expression of the bicistronic IFN-γ-eYFP reporter in IFN-γR-/- Yeti mice after in vitro polarization.

CD44low/eYFP negative CD4+ **(A)** or CD8+ T cells **(B)** were purified from the lymph nodes of naïve Yeti mice (Yeti WT, bold line) or naïve IFN-γ R-/- Yeti mice (Yeti IFN-γ R-/-, gray histograms) and stimulated with anti-CD3ε, anti-CD28 and IL-2 (━, neutral conditions) in the absence or presence of polarizing cytokine conditions (as indicated) and GKO APCs. The cells were analyzed by FACS for eYFP expression on d5 and d3, respectively. The frequency and median fluorescence intensity (MFI) of the eYFP^{pos} cells are noted next to the histograms. All data are representative of three or more independent experiments.

6.2.2. IFN-γ by itself is not sufficient to induce IFN-γ expression by CD4+ or CD8+ T cells

As reviewed in the introduction, IFN-γ is thought to play a critical role in inducing its own expression, via T-bet-activation by the IFN-γR complex and STAT1, respectively (Figure 12 and 13). However, the sequential roles of IFN-γ vs. IL-12 in the induction of IFN-γ expression remain controversial (Murphy and Reiner, 2002b; Robinson and O'Garra, 2002). We therefore asked whether IFN-γ by itself is capable of inducing substantial IFN-γ expression by CD4+ and CD8+ T cells. To answer this question we analyzed the eYFP expression of Yeti WT and Yeti IFN-γR-/- CD4+ and CD8+ T cells cultured *in vitro*, as described in figure 18, after addition of exogenous rIFN-γ and

concurrent neutralization of IL-12 and IL-4 (Figure 19). Recombinant IFN-γ alone was insufficient to induce IFN-γ expression, as its presence did not result in significant eYFP expression by Yeti WT CD4+ T cells (Figure 19A). In fact, comparable eYFP expression was achieved by CD4+ under neutral or cytokine neutralizing conditions without addition of IFN-γ (Figure 18A and 19A). Likewise, the addition of rIFN-γ did not increase the frequency of eYFP+ Yeti WT CD8+ T cells when compared to conditions without rIFN-γ but neutralization of IL-12 and IL-4 (Figure 19B).

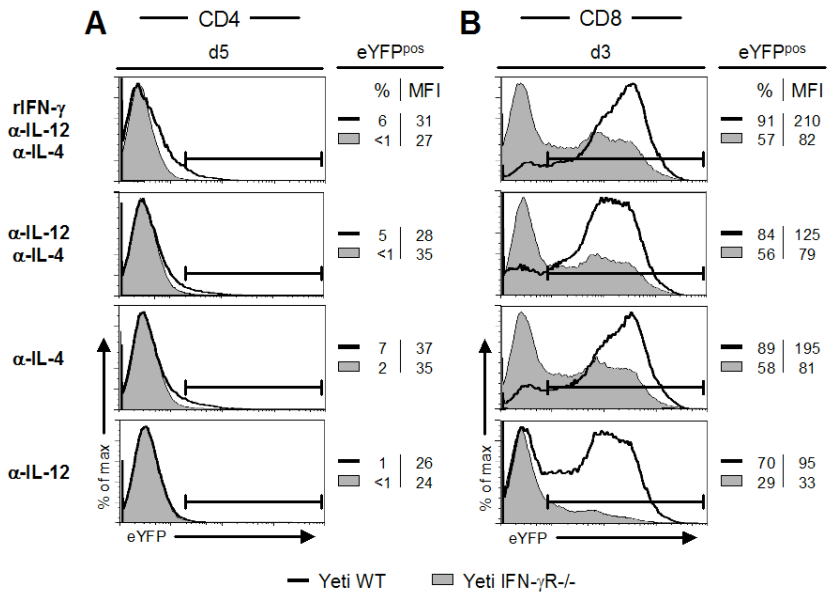


Figure 19:
Expression of the bicistronic IFN-γ-eYFP reporter in IFN-γR-/- Yeti mice after in vitro polarization with recombinant IFN-γ

CD44low/eYFP negative CD4+ **(A)** or CD8+ T cells **(B)** were purified from the lymph nodes of naïve Yeti mice (Yeti WT, bold line) or naïve IFN-γ R-/- Yeti mice (Yeti IFN-γ R-/-, gray histograms) and stimulated with anti-CD3ε, anti-CD28 and IL-2 in the presence of rIFN-γ and neutralizing Ab against IL-12 and IL-4 (as indicated) and GKO APCs. The cells were analyzed on d5 or d3 by FACS for eYFP expression. The frequency and median fluorescence intensity (MFI) of the eYFP^{pos} cells are noted next to the histograms. All data are representative of three or more independent experiments.

Moreover, the absence of IL-4, in cultures where neutralizing Ab against IL4 was added, increased the frequency of IFN-γ expressing CD4+ and CD8+ T cells. This observation is in concordance with earlier studies demonstrating the dominance of IL-4 over IL-12 in T cell differentiation cultures (Hsieh et al., 1993). Overall, IFN-γ alone is not sufficient for the induction of IFN-γ during priming of naïve CD4+ and CD8+ T cells *in vitro*.

6.2.3. Impaired Th1 response after Sendai-virus infection of IFN-γ receptor deficient mice

Our previous studies revealed that IFN-γ receptor-mediated signals are required for an optimal IFN-γ response by both CD4+ and CD8+ effector T cells after *in vitro* priming. To examine the role of the IFN-γR complex for IFN-γ expression by antigen-specific CD4+ and CD8+ T cells *in vivo*, B6 WT, Yeti WT and Yeti IFN-γR-/- mice were infected intranasally with the respiratory Sendai virus (Cauley et al., 2002). The IFN-γ response was analyzed 9 days later in various organs (Figure 20). B6 WT mice were included as controls to discriminate autofluorescence from low eYFP fluorescence. IFN-γR-/- antigen-specific CD4+ and CD8+ T cells displayed heterogeneous IFN-γ expression after Sendai infection as assessed by eYFP fluorescence. Thus, the observed heterogeneity in eYFP expression in CD4+ and CD8+ T cells after infection is not the result of IFN-γ mediated events. Also consistent with our previous *in vivo* data, the presence of highly eYFP fluorescent cells was restricted to the infected lung airways and parenchyma in antigen-specific IFN-γR-/- CD4+ and CD8+ T cells (Figure 20C and D). In agreement with our *in vitro* observation, both the frequency and brightness of eYFP positive cells within the CD4+ SenHN-specific population were reduced in all examined organs of Yeti IFN-γR-/- mice when compared to Yeti WT mice (Figure 20C). In contrast, the antigen-specific CD8+ T cell response was normal in terms of frequency and brightness of the IFN-γ reporter positive cells (Figure 20D). Moreover, while the overall frequency of the SenNP-specific CD8+ response was similar between Yeti IFN-γR-/- and Yeti WT, the SenHN-specific CD4+ T cell response was substantially reduced in all examined organs (Figures 20A, 20B and 21A, 21B). Therefore, IFN-γ receptor-mediated functions are critical for both the magnitude of the SenHN-specific CD4+ T cell response and their IFN-γ expression, while eYFP expression was not compromised in SenNP-specific CD8+ T cells. While our experiments clearly demonstrated a critical role of IFN-γ receptor-mediated functions for the T helper cell response and IFN-γ expression upon infection with Sendai virus, it remains unclear whether the observed defects in the SenHN-specific CD4+ T cell compartment are T cell-intrinsic or due to indirect effects, such as impaired antigen presentation. Although the overall frequency of recently activated antigen-specific T cells, as assessed by CD69 expression, was similar between Yeti IFN-γR-/- and Yeti

WT controls in the lung parenchyma, there was a substantial eYFPnegative CD69hi population present within SenHN-specific CD4+ T cell which was absent in Yeti WT controls (Figure22). As described in the introduction, IFN-γ is critically involved in the up-regulation of MHC class II expression by APCs. Therefore, IFN-γ might be required for optimal activation and differentiation of naïve CD4+ T cells in order to acquire an IFN-γ expressing phenotype. Indeed, Sendai-infected Yeti IFN-γR-/- displayed substantially lower levels of I-A^b expression when compared to the Yeti WT controls (data not shown). Thus, it remains possible that IFN-γ receptor signals directly or indirectly regulate the IFN-γ expression by T cells in mice that entirely lack the IFN-γ receptor.

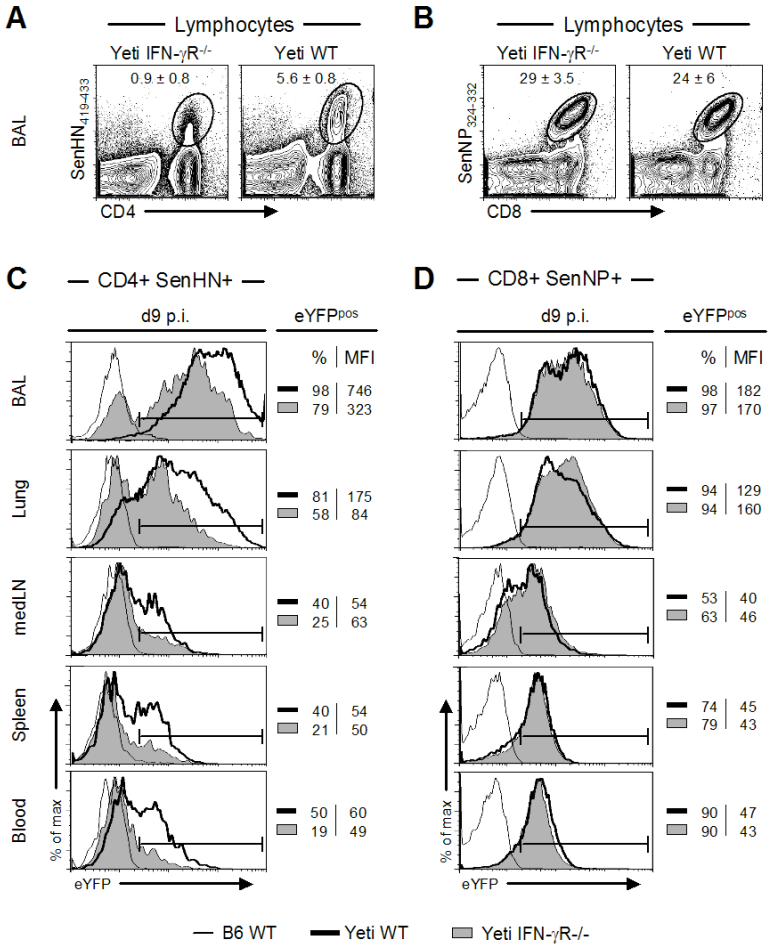


Figure 20:
Expression of the bicistronic IFN-g-eYFP reporter in Sendai-infected IFN-γR-/-Yeti mice.

IFN-γR-/- Yeti mice (Yeti IFN-γR-/-, gray histograms), Yeti mice (Yeti WT, bold line) and WT littermate controls (B6 WT, thin line) were infected i.n. with 250 EID_{50} Sendai virus and FACS was performed 9 days later. **(A)** Antigen-specific CD4+ T cells in the BAL were determined by $HN_{419-433}/A^b$ multimer staining. **(B)** Antigen-specific CD8 T cells in the BAL were determined by Sendai $NP_{324-332}/K^b$ tetramer staining. (**C**) $CD4^+HN_{419-433}/A^{b+}$-gated T cells (CD4+SenHN+) from the indicated organs were analyzed for the expression of eYFP. **(D)** $CD8a^+NP_{324-332}/K^{b+}$-gated T cells (CD8+SenNP+) from the indicated organs were analyzed for the expression of eYFP. The frequency and MFI of the eYFPpos cells are noted next to the histograms. Data are representative of four independent experiments. A minimum of three individual mice per cohort were analyzed with comparable results.

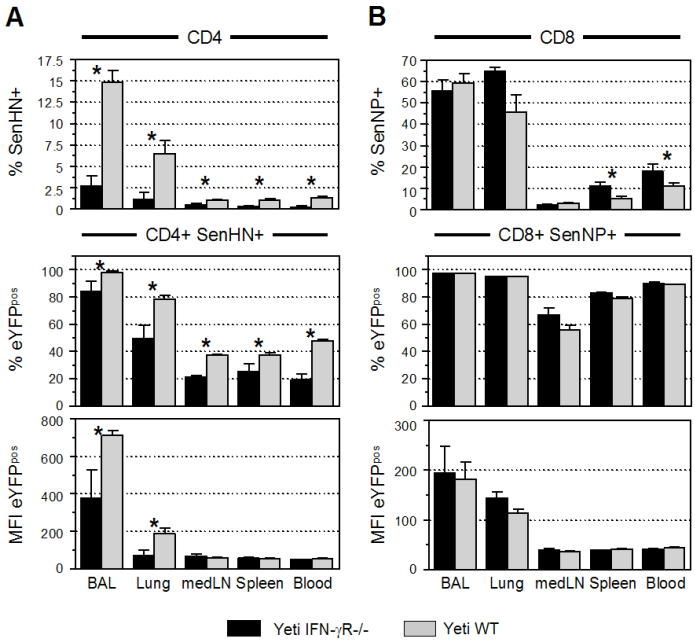


Figure 21:
Defective response of antigen-specific IFN-γR-/- CD4+ T cells after Sendai virus infection.

Yeti IFN-γR-/- (black bars) mice and Yeti WT (gray bars) were infected with Sendai virus and analyzed 9 days later. **(A)** Frequency of $SenHN_{419-433}$+ T cells within the CD4+ T cell population and frequency and MFI of eYFP positive cells within $SenHN_{419-433}$+ (CD4+SenHN+) cells **(B)** Frequency of $SenNP_{324-332}$+ T cells within the CD8+ T cell population and frequency and median fluorescence intensity (MFI) of eYFP positive cells within $SenNP_{324-332}$+ (CD8+SenNP+) cells. Depicted are the mean and SD (n=5). Data are representative of four independent experiments. A minimum of three individual mice per cohort were analyzed with comparable results. Asterisks indicate statistical differences of at least $p<0.05$ as determined by Student's t-test.

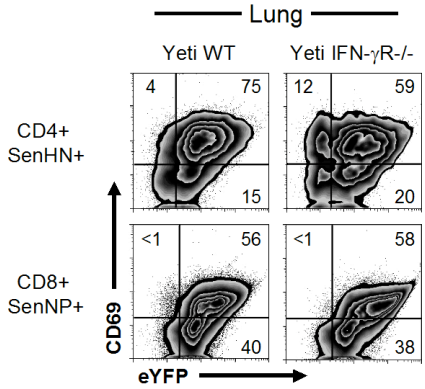


Figure 22:
Expression of the acute activation marker CD69 on IFN-γR-/- antigen-specific T cells after Sendai virus infection.

Yeti IFN-γR-/- mice and Yeti WT mice were infected i.n. with 250 EID_{50} Sendai virus and FACS was performed 9 days later to assess CD69 expression the lung parenchyma. Data are representative of three independent experiments. A minimum of three individual mice per cohort were analyzed with comparable results.

6.2.4. IFN-γ acts directly on antigen-specific CD4+ T cells after Sendai-virus infection to induce optimal IFN-γ expression

To test whether the observed CD4+ T cell defects in IFN-γ expression were due to functions mediated by IFN-γ directly onto the T cells or indirectly onto other cell compartments, we wanted to examine IFN-γR-/- T cells in an IFN-γR sufficient environment. Moreover, to compare IFN-γ expression of IFN-γR deficient and WT T cells side by side in the same animal, we generated dual BM chimeric mice (Lee et al., 2003). We used TCRβδKO hosts to exclude potential contamination by radio-resistant T cells eYFP negative cells. TCRβδKO hosts were lethally irradiated and reconstituted with 50% CD45.1- Yeti IFN-γR-/- BM and 50% CD45.1+ Yeti WT BM. The use of CD45.1 as a congenic marker allows to discriminate respective populations. Because hematopoietic cells in these chimeras will be derived from both donors the non-T cell compartment is IFN-γR sufficient. Thus, indirect defects that might result from a lack of IFN-γR 1 expression in the non-T cell compartment are avoided by the presence of WT cells. Moreover, the presence of WT T cells functions as an internal control in individual experimental animals. The irradiated BM chimeric mice were allowed to reconstitute for 6-8 weeks and reconstitution was assessed by flow cytometric analysis of blood samples prior to infection with Sendai virus. IFN-γ expression was analyzed 9 days in various organs. As shown in Figure 23B and 24B, eYFP expression by SenNP-specific IFN-γR-/- CD8+ was unimpaired when compared to WT cells. In contrast, the frequency of eYFP positive cells within the SenHN-specific IFN-γR-/- CD4+ T cells was significantly reduced in all examined organs (Figure 23A and 24A). The greatest defect in IFN-γ expression by SenHN-specific IFN-γR-/- cells was observed in the lymphoid organs such as the spleen and the draining medLN. In contrast the relative defect at the site of infection was less pronounced in terms of frequency of eYFP expression (Figure 23A and 24A). In concordance with our previous data, the presence of eYFP bright SenHN-specific IFN-γR-/- CD4+ T cells was restricted to the lung airways and parenchyma. Furthermore, the reporter fluorescence intensity of SenHN-specific IFN-γR-/- CD4+ T cells was unimpaired in these organs, but defective in the spleen and the medLN. Thus, T cell intrinsic IFN-γR-/- signals are required for optimal IFN-γ expression in CD4+ but not CD8+ T cells.

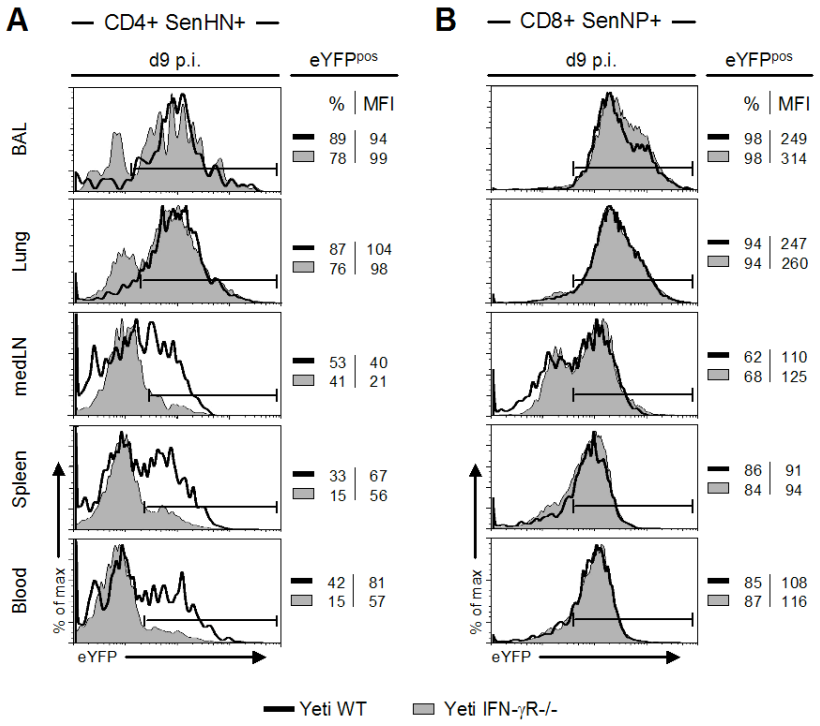


Figure 23:
Expression of the bicistronic IFN-γ-eYFP reporter in Sendai-infected dual BM chimeric mice.

TCRβδKO hosts were lethally irradiated and reconstituted with 50% CD45.1- Yeti IFN-γR-/- BM and 50% CD45.1+ Yeti WT BM. Chimeric mice were allowed to reconstitute for 6-8 weeks and were subsequently infected i.n. with 250 EID_{50} Sendai virus and FACS was performed 9 days later to assess eYFP expression in the indicated organs. Yeti IFN-γR-/- (gray histograms) and Yeti WT (bold line) T cells were identified on the basis of their CD45.1 expression. **(A)** $CD4^{+}HN_{419-433}/A^{b+}$-gated T cells (CD4+SenHN+) and **(B)** $CD8\alpha^{+}NP_{324-332}/K^{b+}$-gated T cells (CD8+SenNP+) from the indicated organs were analyzed for the expression of eYFP. The frequency and MFI of the eYFPpos cells are noted next to the histograms. Data are representative of three independent experiments. A minimum of five individual mice per cohort were analyzed with comparable results.

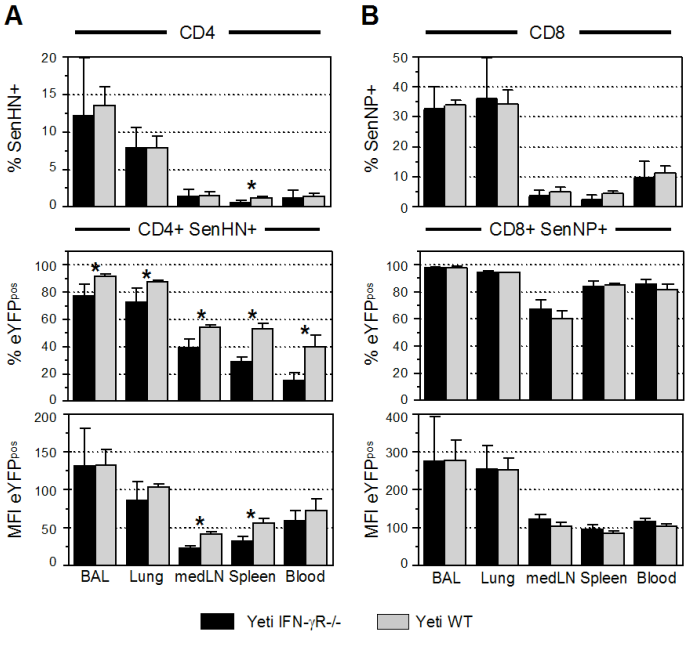


Figure 24:
Impaired response of antigen-specific CD4+ T cells lacking the IFN-γR1 in Sendai-infected BM chimeric mice.

TCRβδKO hosts were lethally irradiated and reconstituted with 50% CD45.1- Yeti IFN-γR-/- BM and 50% CD45.1+ Yeti WT BM Chimeric mice were allowed to reconstitute for 6-8 weeks and were subsequently infected with Sendai virus and FACS was performed 9 days later. Yeti IFN-γR-/- (black bars) and Yeti WT (gray bars) T cells were identified on the basis of their CD45.1 expression. **(A)** Frequency of $SenHN_{419-433}$+ T cells within the CD4+ T cell population and frequency and MFI of eYFP positive cells within $SenHN_{419-433}$+ (CD4+SenHN+) **(B)** Frequency of $SenNP_{324-332}$+ T cells within the CD8+ T cell population and frequency and median fluorescence intensity (MFI) of eYFP positive cells within $SenNP_{324-332}$+ (CD8+SenNP+). Depicted are the mean and SD (n=5). Data are representative of three independent experiments. A minimum of five individual mice per cohort were analyzed with comparable results. Asterisks indicate statistical differences of at least $p<0.05$ as determined by Student's t-test.

6.2.5. Repeated *in vitro* stimulation enhances IFN-γ expression in IFN-γR-/- CD4+ T cells

Our studies using BM chimeric mice revealed that the eYFP brightness of IFN-γR-/- CD4+ T cells in the infected lung airways and parenchyma is unimpaired. Moreover, the relative decrease in YFP expression was less pronounced at these sites when compared to WT CD4+ T cells. Therefore, it is conceivable that the highly inflammatory environment in the infected lung enhances IFN-γ expression, potentially synergizing with repeated antigenic stimulation (Cerwenka et al., 1999; Ely et al., 2003; Wiley et al., 2001). Indeed, the frequency of cells presenting MHC class II-restricted antigenic peptides is the highest in the infected lung (S. R. Crowe and D. L. Woodland, unpublished observations; (Crowe et al., 2003; Hamilton-Easton and Eichelberger, 1995; Usherwood et al., 1999)). As elucidated in the introduction, IFN-γ production in differentiated T cells can be induced via TCR-dependent and independent stimulation, such as IL-12 + IL-18. We wished to examine whether the observed defect in IFN-γ expression by IFN-γR-/- CD4+ T cells in non-infected sites was solely the result of sub-optimal priming and whether this defect could be overcome by repeated stimulation *in vitro* via TCR engagement or IL-12/IL-18 cytokines, respectively. To this end, we primed CD4+ T cells under Th1 polarizing conditions (Figure 25, top panel) as described in figure 9 and re-stimulated the cultures 7 days later in the presence of IL-12 and IL-18, α-CD3ε and α-IL-4 or under Th1 polarizing conditions as indicated in figure 17 (bottom row) and analyzed eYFP expression 5 days later. TCR stimulation in the absence of IL-12 did not increase the frequency or brightness of eYFPpositive IFN-γR-/- CD4+ T cells (Figure 25, bottom row, left). In contrast, repeated stimulation under Th1 polarizing conditions increased both frequency and brightness. A second TCR independent IFN-γ stimulation via the IL-12/IL-18 pathway also increased the proportion of IFN-γ expressing IFN-γR-/- CD4+ T cells (Figure 25). However, the fact that IL-12 or IL-18 alone did not increase eYFP expression (data not shown), asserted the synergistic effect of IL-12 and IL-18 for induction of IFN-γ expression in differentiated Th1 cells (Nakanishi et al., 2001; Szabo et al., 2003). Thus, the defect in IFN-γ expression by IFN-γR-/- CD4+ T cells can at least partially be overcome by repeated TCR stimulation and cytokines.

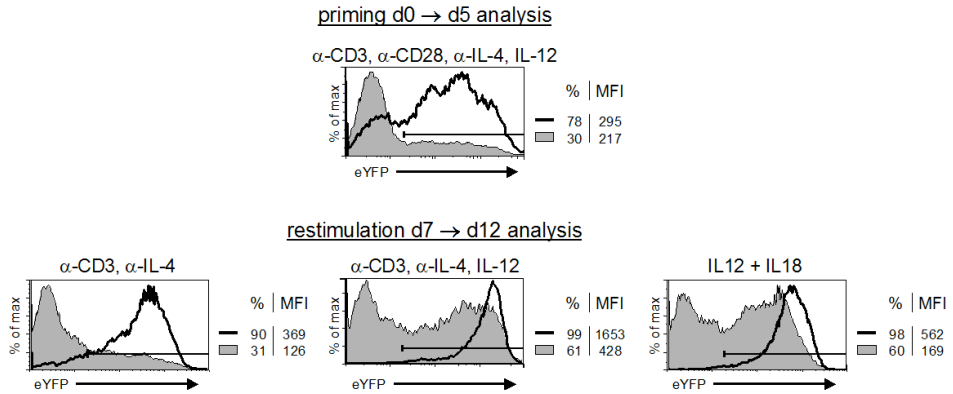


Figure25:
YFP- expression of IFN-gR-/- CD4+ T cells after repeated *in vitro* stimulationCD4+ T cells from lymph nodes of naïve Yeti mice (Yeti WT, bold line) or naïve IFN-gR-/- Yeti mice (Yeti IFN-gR-/-, gray histograms) were sorted for a naïve (CD44low/eYFPneg) phenotype and primed (d0) with anti-CD3ε and anti-CD28 in the presence of Th1 polarizing cytokine conditions (top panel). On d7 cells were restimulated either with anti-CD3ε plus anti-IL-4 (bottom row, left), Th1 polarizing cytokines (bottom row, middle) or in the presence of IL-12 + IL-18 (bottom row, right). Cells were analyzed 5 days after the respective stimulation by FACS for eYFP expression. The frequency and median fluorescence intensity (MFI) of eYFP^{pos} cells are noted next to the histograms. All data are representative of two or more independent experiments.

6.2.6. Increased IFNγ expression of IFN-γ receptor deficient mice after *T.gondii* infection

Since we were able to ameliorate the defect in IFNγ expression by IFN-γR-/- CD4+ T cells via Th1-associated inflammatory signals *in vitro*, we speculated that IFNγ expression by IFN-γR-/- CD4+ T cells might be differentially regulated in different disease models. We further speculated that in a model, which induces a vigorous type I response, IFN-γR-/- CD4+ T cells would display little or no defect in IFNγ expression. To test this hypothesis we infected Yeti IFN-γR-/- and Yeti WT mice orally with the protozoan parasite *T. gondii*, a strong inducer of type I-associated inflammation, and various organs were analyzed 1 week later by FACS (Figure 26). To exclude contamination by naïve eYFP negative CD4 or CD8+ T cells and to assess reporter expression within a defined activated population, eYFP fluorescence was analyzed within CD44hi T cells. *T. gondii*-infected Yeti IFN-γR-/- mice revealed, like their WT counterparts, heterogeneous eYFP fluorescence by CD4+ and CD8+ T cells. However, *T. gondii*-infected Yeti IFN-γR-/- mice revealed a significantly higher frequency and brightness of eYFP positive CD44hi CD4+ T cells when compared to Yeti WT cells (Figure 26A).

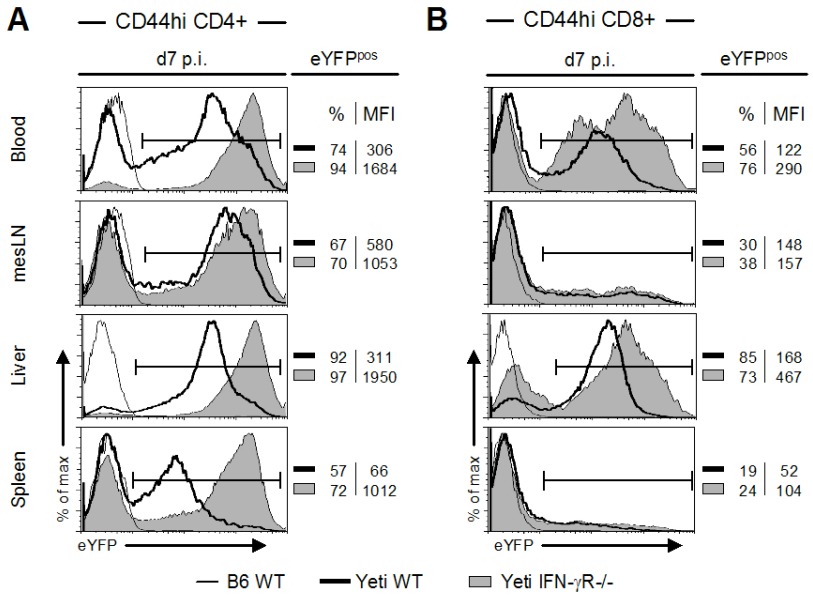
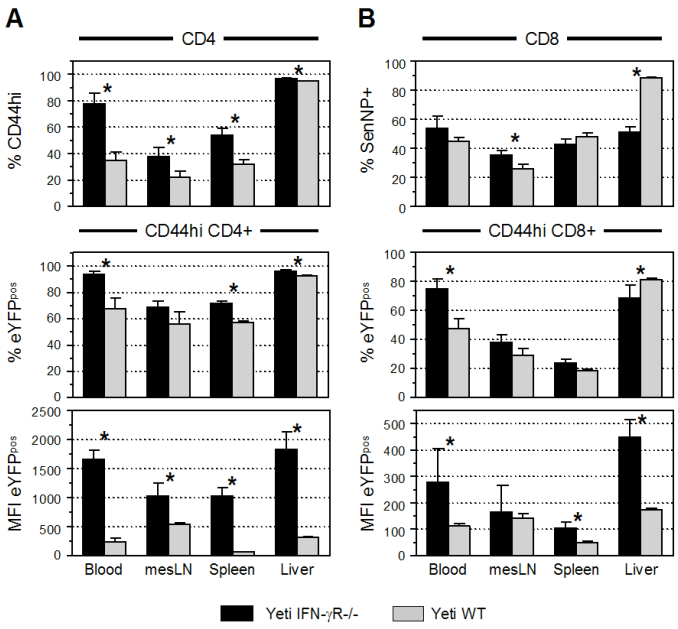


Figure 26:
Expression of the bicistronic IFN-γ-eYFP reporter in *T.gondii*-infected IFN-γR-/-Yeti mice.
IFN-γR-/- Yeti mice (Yeti IFN-γR-/-, gray histograms), Yeti mice (Yeti WT, bold line) and WT littermate controls (B6 WT, thin line) were infected orally with *T. gondii* and CD44hiCD4+ gated T cells **(A)** and CD44hiCD8+ gated T cells **(B)** in the indicated organs were analyzed after 1 week for their eYFP fluorescence. The frequency and MFI of the eYFPpositive (eYFPpos) cells are noted next to the histograms. Data are representative of four independent experiments. A minimum of three individual mice per cohort were analyzed with comparable results.

Figure 27:
Increased response of CD44hi CD4+ T in T.gondii-infected Yeti IFN-γR-/- mice.
Both Yeti IFN-γR-/- (black bars) mice and Yeti WT (gray bars) were infected with *T.gondii* and analyzed 7 days later. (A) Frequency of CD44hi cells within the CD4+ T cell population and frequency and median fluorescence intensity (MFI) of eYFP positive (eYFPpos) cells within CD44hi CD4+ T cells (B Frequency of CD44hi cells within the CD8+ T cell population and frequency and median MFI of eYFP positive cells within CD44hi CD8+ T cells. Depicted are the mean and SD (n=5). Data are representative of three independent experiments. A minimum of three individual mice per cohort were analyzed with comparable results. Asterisks indicate statistical differences of at least $p<0.05$ as determined by Student's t-test.



This data suggest that under strong inflammatory conditions IFN-γ expression by IFN-γR-/- CD4+ T cells can be restored. In fact, the MFI of eYFP positive CD44hi CD4+ T cells was significantly higher in Yeti IFN-γR-/- mice than in Yeti WT mice (Figure 26A and 27A). Additionally, the frequency of activated CD4+ T cells, as assessed by high CD44 expression, was higher in all examined organs of *T. gondii*-infected Yeti IFN-γR-/- mice (Figure 27 A). In contrast, the CD44hi CD8+ response was only increased in the mesenteric LN (Figure 27B). Our previous *in vivo* studies demonstrated that IFN-γ expression can be regulated independently of IFN-γR mediated functions in CD8+ T cells. Here, we observed a significant increase in eYFP-reporter fluorescence after *T. gondii* infection in the absence of IFN-γR expression by CD44hi CD8+ T cells in both the liver and the blood (Figures 26 B and 27B). Overall these data show that IFN-γ receptor signals are not required for maximal IFN-γ expression by CD4+ and CD8+ T cells after infection with *T. gondii*. However, the caveat remains in Yeti IFN-γR-/- mice, that the observed increase in eYFP brightness could be mediated by indirect effects, such as increased parasite burden. Indeed, Yeti IFN-γR-/- mice displayed a 10-fold higher parasitic burden than Yeti WT mice (Figure 28).

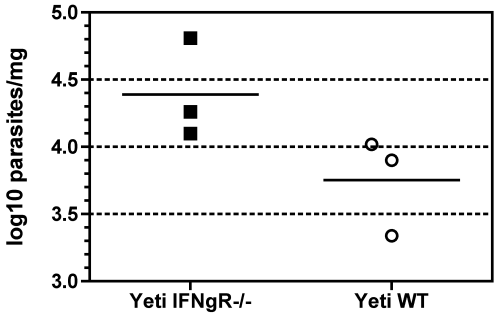


Figure 28:
Increased parasite burden in the lungs of *T.gondii*-infected Yeti IFN-γR-/-mice

Yeti IFNγR-/- (black squares) mice and Yeti WT (open circles) were infected with ***T.gondii*** and analyzed 7 days later for ***T.gondii*** parasite levels, via quantitative RT-PCR of infected lungs. Depicted are the results of three individual mice. Data are representative of two independent experiments.

6.2.7. T cell intrinsic IFN-γR signals are required for optimal IFN-γ expression after *T. gondii* infection

To distinguish between T cell-intrinsic and indirect IFN-γR functions and directly compare IFN-γ expression by Yeti IFN-γR-/- and Yeti WT CD4+ T cells side by side in the same animal after acute *T.gondii* infection, we generated dual BM chimeric mice as explained in chapter 3.2.3. The irradiated dual BM chimeric mice were allowed to reconstitute for 6-8 weeks and reconstitution was assessed by flow cytometric analysis of blood samples prior to oral infection with *T.gondii* cysts. Various organs were analyzed 7 days later for IFN-γ expression (Figure 29 and 30). In contrast to IFN-γR-/- mice, the frequency of eYFP positive cells within CD44hi CD4+ and CD8+ IFN-γR-/- T cells was reduced in lymphoid organs and in the blood, while the frequency not reduced in the liver (Figures 29 and 30). Furthermore, while the frequency was reduced in the lymphoid

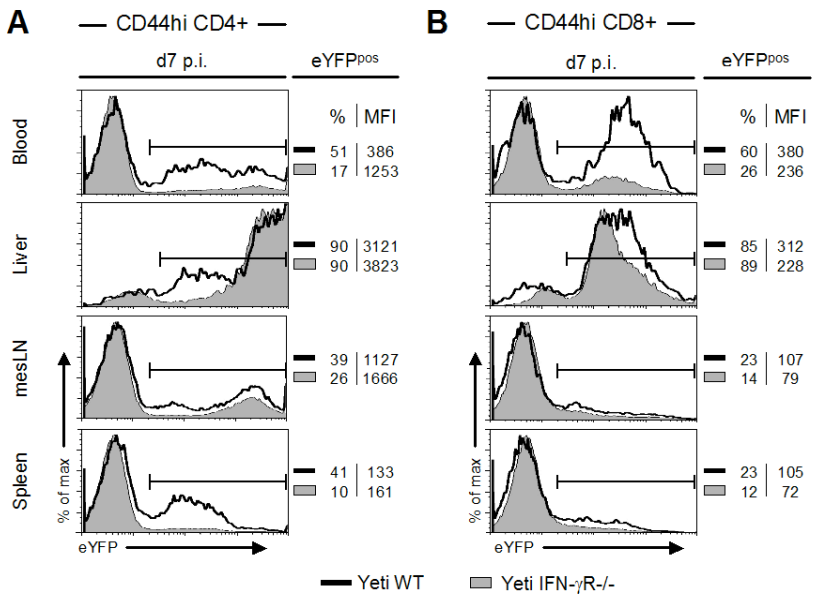


Figure 29:
Expression of the bicistronic IFN-γ-eYFP reporter in *T.gondii* -infected BM chimeric mice.
TCRβδKO hosts were lethally irradiated and reconstituted with 50% CD45.1- Yeti IFN-γR-/- BM and 50% CD45.1+ Yeti WT BM. Chimeric mice were allowed to reconstitute for 6-8 weeks and were subsequently infected orally. with 10 ***T.gondii*** cysts and FACS was performed 7 days later to assess eYFP expression in the indicated organs. Yeti IFN-γR-/- (gray histograms) and Yeti WT (bold line) T cells were identified on the basis of their CD45.1 expression. **(A)** CD44hi CD4+ T cells from the indicated organs were analyzed for the expression of eYFP. **(B)** CD44hi CD8+ from the indicated organs were analyzed for the expression of eYFP. The frequency and MFI of the eYFPpos cells are noted next to the histograms. Data are representative of three independent experiments. A minimum of five individual mice per cohort were analyzed with comparable results.

organs and the blood, the fluorescence intensity of eYFP positive CD44hi CD4+ IFN-γR-/- T cells was increased in dual BM chimeric mice (Figures 29A and 30A). eYFP fluorescence was not increased in eYFP positive IFN-γR-/- CD44hi CD8+ T cells. Contrary to infection of IFN-γR-/- mice, the proportion of CD44hi cells within the IFN-γR-/- CD4+ and CD8+ cell populations was unaltered or even decreased in blood-derived and splenic CD4+ T cells when compared to WT cells in dual BM chimeras (Figure 30). In the liver and the lung no differences in eYFP-expression were observed in both CD4+ and CD8+ IFN-γR-/- T cells in terms of frequency or brightness (Figures 29, 30 and data not shown). Thus, infection of dual BM chimeric mice with *T.gondii*, revealed that in lymphoid organs T cell intrinsic IFN-γR signals are required for optimal IFN-γ induction. However, at sites known for high parasitic burden, such as the liver, IFN-γ-expression can be regulated independently of IFN-γR mediated signals.

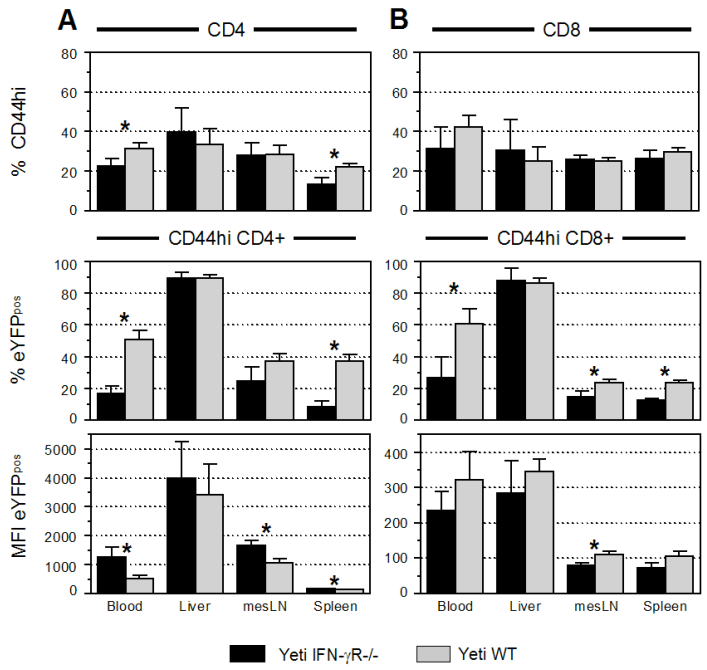


Figure 30.
Response of CD44hi CD4+ T cells lacking the IFN-γR1 in *T.gondii* infected dual BM chimeric mice.

TCRβδKO hosts were lethally irradiated and reconstituted with 50% CD45.1- Yeti IFN-γR-/- BM and 50% CD45.1+ Yeti WT BM. Chimeric mice were allowed to reconstitute for 6-8 weeks and were subsequently infected with *T.gondii* and FACS was performed 7 days later. Yeti IFN-γR-/- (black bars) and Yeti WT (gray bars) T cells were identified on the basis of their CD45.1 expression. **(A)** Frequency of CD44hi cells within the CD4+ T cell population and frequency and median fluorescence intensity (MFI) of eYFP positive (eYFPpos) cells within CD44hi CD4+ T cells **(B)** Frequency of CD44hi cells within the CD8+ T cell population and frequency and median MFI of eYFP positive cells within CD44hi CD8+ T cells. Depicted are the mean and SD (n=5). Data are representative of three independent experiments. A minimum of five individual mice per cohort were analyzed with comparable results. Asterisks indicate statistical differences of at least $p<0.05$ as determined by Student's t-test.

7. DISCUSSION

IFN-γ is a hallmark cytokine of type I immune responses against a diversity of pathogens. Bicistronic cytokine reporter mice have been instrumental to visualize, quantify and functionally characterize cytokine-expressing cells *in vivo* (Mohrs et al., 2005; Mohrs et al., 2001; Stetson et al., 2003). Using bicistronic IFN-γ reporter (Yeti) mice (Stetson et al., 2003), we show here that the expression of the IFN-γ-eYFP reporter by CD4+ and CD8+ T cells is broadly heterogeneous, *in vitro* and *in vivo* upon infection. Increased expression of the reporter correlated positively with the abundance of IFN-γ transcripts and with the amount of secreted IFN-γ protein upon stimulation. Therefore, heterogeneous eYFP expression reflects important functional differences because it correlates with both the abundance of IFN-γ transcripts and the potential for IFN-γ secretion upon stimulation. Increased expression of the reporter also correlated with enhanced secretion of additional proinflammatory cytokines and chemokines after stimulation. Highly eYFP fluorescent CD4+ and CD8+ T cells were found only in select tissues and their restriction was dependent on the infectious agent. Applying multimer technology after respiratory virus infection, we found that antigen-specific CD4+ T cells are exclusively eYFP positive in the lung airways and parenchyma, while all antigen-specific CD8+ T cells were eYFP positive in all examined organs. Furthermore, we show that T cell-intrinsic IFN-γR signals are not necessary to induce IFN-γ expression but greatly enhance the proportion of IFN-γ expressing cells within CD4+ T cells *in vitro* and *in vivo*. In contrast, the abundance of IFN-γ transcripts in either eYFP fluorescent CD4+ or CD8+ T cells was regulated independently of IFN-γR mediated functions. Finally, IFNγ expression by CD8+ T cells was less dependent on IFN-γR mediated functions than CD4+ T cells

Heterogeneous reporter expression and post-transcriptional regulation of IFN-γ

In vitro, the expression of IFN-γ was broadly heterogeneous in both CD4+ and CD8+ T cells and occurred prior to cell division (Figure 10A and B). Increased expression of the reporter correlated positively with the abundance of IFN-γ transcripts, as assessed by

quantitative RT-PCR (Figure 10C and D). In contrast to CD4+ T cells, which expressed IFN-γ only under Th1 polarizing conditions, CD8+ T cells activated the *ifng* gene irrespective of the polarizing cytokine milieu. However, eYFP expression occurred more rapidly and the expression of IFN-γ in CD8+ T cells was substantially regulated by exogenous factors (Figure 10B). This mechanism might be important for the local modulation of CD8+ effector functions *in vivo*. Indeed, a similar heterogeneity of IFN-γ expression by both CD4+ and CD8+ T cells was observed upon infection with Influenza virus, Sendai virus, or the obligate intracellular protozoan parasite *T. gondii* (Figures 11, 13 and 17). When T cells were isolated from Influenza virus infected mice, according to their eYFP brightness and subsequently cultured in the presence or absence of plate-bound α-CD3, eYFP expression correlated positively with IFN-γ protein production upon stimulation (Figure 15). Most interestingly, despite robust expression of the reporter at the time of isolation, IFN-γ production was more than 1000-fold increased upon stimulation. Thus, the bicistronic IFN-γ reporter marks cells competent for rapid cytokine production, while the secretion of this effector cytokine is largely dependent on antigenic simulation. In fact, the rapid on/off cycling of IFN-γ production by CD4+ and CD8+ T cells has been demonstrated (Corbin and Harty, 2005; Slifka et al., 1999; Slifka and Whitton, 2000). Both IFN-γ transcript levels and the amount of IFN-γ that is secreted upon stimulation correlated directly with the expression of the bicistronic eYFP reporter (Figures 10C, 10D and 15). The accessible *ifng* gene locus and the constitutive presence of transcripts may allow accelerated cytokine production (Ansel et al., 2003), while the requirement for antigen-specific stimulation could limit collateral damage. The greatly enhanced secretion of cytokines upon TCR-mediated stimulation might additionally be important to focus antigen-specific T helper functions to MHC class II-bearing cells (Ingulli et al., 1997; Itano and Jenkins, 2003; Jenkins et al., 2001). The translational silencing of IFN-γ has recently been demonstrated in NK and NK T cells derived from wild-type and Yeti mice (Matsuda et al., 2003; Stetson et al., 2003). Moreover, CD8+ memory T cells have been shown to contain high levels of IFN-γ and RANTES /CCL5 transcripts even in the absence of detectable protein production (Bachmann et al., 1999; Grayson et al., 2001; Swanson et al., 2002). In fact, we observed that antigen-specific CD8+ T cells remain eYFP positive for at least one year after primary Influenza virus infection (K.D. Mayer and M. Mohrs manuscript in preparation). Thus, the disconnect

between the presence of IFN-γ transcripts and the actual secretion of IFN-γ protein, suggests that IFN-γ is being post-transcriptionally regulated. Additionally, our *in vivo* studies with Yeti mice imply that this mechanism of post-transcriptional IFN-γ regulation is also operational in conventional CD4+ and CD8+ effector T cells in a situation of acute infection.

In conclusion, the presence of eYFP fluorescence in Yeti mice reflects the potential for IFN-γ production in a qualitative fashion, while the eYFP brightness quantitatively reflects the potential for IFN-γ production. The heterogeneity in eYFP expression reflects both the abundance of IFN-γ transcripts and the potential for IFN-γ secretion upon stimulation. Thus, it has important functional consequences. Furthermore, heterogeneous IFN-γ expression represents a distinct functional adaptation of CD4+ and CD8+ T cells because innate IFN-γ producers such as NK and NK T cells, which are constitutively eYFP positive in Yeti mice (Matsuda et al., 2003; Stetson et al., 2003) do not reveal a similar heterogeneity in their reporter fluorescence in naïve or infected animals (K.D. Mayer and M. Mohrs data not shown).

High eYFP fluorescence reflects a more differentiated effector status

eYFP fluorescence of CD4+ and CD8+ T cells also correlated positively with the enhanced production of a select set of cytokines and chemokines after Influenza virus infection. In addition to IFN-γ, eYFP high cells also produced the highest amount of TNF-α, GM-CSF, MIP-1β/CCL4, RANTES/CCL5, IP-10/CXCL10 and SDF-1/CXCL12 (Figure 16). Similar to IFN-γ, these cytokines and chemokines were 100-1000 fold induced upon stimulation *ex vivo*. The positive correlation between the secretion of these cytokines and chemokines with eYFP brightness, implies that eYFPhi cells reflect a generally more differentiated effector status. The association of IFN-γ and TNF-α with the immune response to viral infections is well established (Harty et al., 2000; Wong and Goeddel, 1986; Wong and Pamer, 2003). The synergistic function of GM-CSF and TNF-α is supported by data showing that the combined exposure of antigen presenting cells in the lung to both cytokines is required for optimal antigen presentation and Th1 priming (Holt et al., 1993; Holt and Stumbles, 2000; Nelson and Holt, 1995). The chemokine receptors for the inflammatory chemokines RANTES/CCL5 and MIP-1β/CCL4 (CCR1, 3, 5) are expressed on a variety of cell types including effector and memory T cells and

macrophages (Mackay, 2001; Moser and Loetscher, 2001; Zlotnik and Yoshie, 2000). Thus, eYFPhi cell efficiently recruit other effector cells into the lung. The increased secretion of soluble effector molecules was selective and therefore not simply due to a generally enhanced secretory capacity. For example, the production of IL-2 was not limited to eYFP positive cells and the secreted amounts did not correlate positively with eYFP fluorescence intensity (Figure 16A). In fact, eYFPhi CD4+ and CD8+ cells produced less IL-2 than eYFPint cells, and reduced IL-2 production has been associated with progressive Th1 effector polarization (Seder, 1994; Szabo et al., 2000; Wu et al., 2002). Our notion, that high eYFP fluorescence reflects a generally more differentiated effector status, is further substantiated by the fact that the surface expression of acute activation markers, such as CD25, CD69 and CD122, correlated positively with eYFP fluorescence (Figure 14). These markers indicate recent antigenic stimulation (Ortega et al., 1984; Sancho et al., 2005; Taniguchi and Minami, 1993; Waldmann, 1991; Ziegler et al., 1994).

Anatomical restriction of highly eYFP fluorescent cells

T cells with heterogeneous eYFP fluorescence disseminated systemically after both respiratory virus and parasitic infection, but eYFPhi cells were restricted to certain tissue sites (Figures 11, 13 and 17). In the case of respiratory virus-infection, the frequency of cells presenting MHC class I- and class II-restricted antigenic peptides is highest in the infected lung (S.R. Crowe and D.L. Woodland unpublished data and (Crowe et al., 2003; Hamilton-Easton and Eichelberger, 1995; Usherwood et al., 1999)). Therefore, it is conceivable that increased antigenic stimulation is potentially synergizing with the highly inflammatory environment in the infected lung to enhance IFN-γ expression in T cells (Cerwenka et al., 1999; Ely et al., 2003; Wiley et al., 2001). In fact, eYFPhi cells were only present in the lung airways and parenchyma after respiratory virus infection. Employing secondary infections with viral mutants of Influenza virus, lacking either the $NP_{366-374}$- or $PA_{224-233}$-epitope, inflammation alone, without the context of antigen-presence, did not result in conversion of eYFPint to eYFPhi cells (data not shown, K.D. Mayer and M. Mohrs manuscript in preparation). Similarly, recruitment into tertiary sites alone did not result in high levels of eYFP expression, because eYFPhi cells were not present in the lung-harboring pleural cavity, despite similar recruitment and frequency of antigen-specific cells as the infected lung (Figure 11C). Moreover, identical patterns of restriction

were observed after Sendai or Influenza virus infection, in Influenza-specific CD8+ T cells recognizing $NP_{366-374}$- or $PA_{224-233}$-epitopes and Sendai-specific CD8+ and CD4+ T cells (Figures 11C, 13C and data not shown). Therefore, the fluorescence heterogeneity and anatomical restriction of eYFPhi cells after respiratory virus infection is not linked to a particular virus species, antigen-specificity or T cell lineage. Additionally, oral infection with the protozoan parasite *T. gondii* also resulted in functional heterogeneity, and highly fluorescent cells were similarly restricted to certain tissues despite the widespread dissemination of the parasite. eYFPhi T cells were present within the CD44hi T cell population of the mesLN, the blood, the liver and the lung parenchyma (Figure 17 and data not shown). However, the highest proportion of eYFPhi cells was found in the liver and the lung parenchyma (Figure 17 and data not shown). Indeed, the lung and the liver are among the organs with the highest parasitic burden after acute *T.gondii* infection (L.L. Johnson unpublished data and (Kobayashi et al., 1999; Luo et al., 1997)). Again, it is conceivable that in these organs increased antigen presentation in conjunction with inflammation induces high expression of IFN-γ in CD4+ and CD8+ effector T cells. Thus, fluorescence heterogeneity and anatomical restriction of eYFPhi cells represent general immunological mechanisms that are not limited to viral infections of the lung. However, in which tissue eYFPhi cells accumulate is dependent on the pathogen.

Biallelic expression of IFN-γ

The potential for IFN-γ secretion was tightly linked to clonal expansion of antigen-specific CD8+ lymphocytes because all $NP_{366-374}$- or $PA_{224-233}$-specific T cells were eYFP+ in all tissues (Figure 11C and data not shown). Because we used heterozygous reporter knockin mice throughout all experiments, this observation implies that both *ifng* alleles are frequently expressed, because otherwise a substantial fraction of the cells would be eYFP negative. Expression of the *ifng* gene in CD4+ T cells was also biallelic, because after Influenza virus infection >95% of these cells in the lung airways and parenchyma were eYFP+ and after Sendai virus infection SenHN-specific CD4+ cells in these organs were almost exclusively eYFP positive (Figure 11B and 13C). Thus, IFN-γ expression by both CD4+ and CD8+ T cells occurred biallelically. This finding stands in contrast to previous reports which suggest monoallelic expression of IFN-γ by CD4+ T cells (Hsieh et al., 2000; Mullen et al., 2001).

IFN-γR signals are not necessary for IFN-γ expression by CD4+ T cells

Expression of IFN-γ by T cells is the result of a sequential and complex combination of different mechanisms, including the activation of crucial transcription factors and up-regulation of pivotal cytokine receptors (Grogan and Locksley, 2002; Murphy and Reiner, 2002b). In the case of Th1 differentiation, it is well established that IL-12 and its receptor, T-bet, STAT4 and IFN-γ are crucial in inducing Th1 development (Magram et al., 1996; Szabo et al., 2002; Wurster et al., 2000; Zhang et al., 2001), but the sequence of events leading to Th1 differentiation is unclear. IFN-γ, probably produced by NK cells, is thought to be among the cytokines initiating the differentiation program (Bradley et al., 1996; Lohoff and Mak, 2005; Murphy and Reiner, 2002b). However, the sequential role of IFN-γ vs. IL-12 in the induction of both IFN-γ expression and Th1 differentiation by CD4+ T cells remains controversial with existing models in favor of either IFN-γ or IL-12 (Murphy and Reiner, 2002b; Robinson and O'Garra, 2002). We are able to show here, that IFN-γ by itself is a very poor inducer of IFN-γ expression by CD4+ and CD8+ T cells *in vitro* (Figure 19). Therefore, in our hands, IFN-γ is not able to efficiently drive IFN-γ expression or Th1 differentiation, when IL-12 is neutralized. Our finding stands in contrast to a previous report from Bradley and colleagues, which showed that autocrine IFN-γ, independently of IL-12, is able to drive Th1 differentiation and IFN-γ production (Bradley et al., 1996). However, most studies confirm that IFN-γ alone is insufficient for the development of Th1 cells and that it is more likely to enhance IL-12 responsiveness in naive T cells and to stabilize the Th1 phenotype (Seder et al., 1993; Swihart et al., 1995; Wenner et al., 1996; Zhang et al., 2001). Furthermore, IFN-γR expression by CD4+ T cells was not a prerequisite for the induction of IFN-γ or Th1 differentiation per se, because both occurred in the absence of autocrine IFN-γR signaling *in vitro* (Figure 18). Since our current understanding of the role of IFN-γ responsiveness for its own expression is extensively extrapolated from *in vitro* culture systems, we employed IFN-γR1-/- Yeti mice to revisit the role of IFN-γ for its own expression *in vivo*. After infection with the respiratory Sendai virus or with the protozoan parasite *T. gondii*, IFN-γR-/- CD4+ T cells were able to acquire an IFN-γ expressing Th1 phenotype (Figures 20, 23, 26 and 29). It has been reported that IFN-γR1 colocalizes with the TCR, during, but not before, naïve CD4+ T cells become activated (Maldonado et al., 2004). Thus, IFN-γR1 recruitment into the immunological synapse could be a prerequisite for the generation of Th1 cells. Our

data clearly indicate that this is not the case. Indeed, recent reports confirm that antigen-specific Th1 cells were generated in the absence of the IFN-γR, after infection with *L. monocytogenes* or LCMV, respectively (Haring et al., 2005a; Seder et al., 1993). In contrast to our experiments, these studies used TCR transgenic T cell populations and IFN-γ secretion was assessed by intracellular cytokine staining after restimulation *ex vivo*. Using two different infectious models our data clearly demonstrate that IFN-γ responsiveness is not required to initiate IFN-γ expression or Th1 differentiation of CD4+ T cells.

IFN-γR signals enhance the induction of IFN-γ in CD4+ T cells

Yeti mice uniquely allow to visualize differences in both quality (eYFP positive cells) and quantity (eYFP brightness) of IFN-γ expression in T cells. For example, we found that the proportion of IFN-γ expressing IFN-γR-/- CD4+ cells under Th1 priming conditions *in vitro* was reduced, despite similar cell recovery (Figure 18A). These data reveal a critical role of IFN-γR mediated functions for maximal IFN-γ induction by CD4+ T cells *in vitro*. Similarly, *in vivo,* after Sendai virus infection of IFN-γR-/- Yeti mice, the proportion of IFN-γ expressing SenHN-specific CD4+ T cells was also reduced (Figure 20C). Of note, the reduced frequency of eYFPpositive cells within the SenHN-specific CD4+ T cell response cannot be explained by monoallelic expression of the *ifng* locus, because homozygous eYFP-reporter IFN-γR-/- mice displayed the same defect (K.D. Mayer and M. Mohrs unpublished data). Additionally, the overall magnitude of the SenHN–specific CD4+ T cell response was drastically reduced in IFN-γR-/- Yeti mice, irrespective of IFN-γ expression (Figure 21A and C). Therefore, IFN-γ receptor-mediated functions are critical for both the magnitude of the SenHN-specific CD4+ T cell response and the development of Th1 cells within this population. IFN-γ is critically involved in the up-regulation of MHC class II expression on APCs. Thus, IFN-γ might be required for optimal activation and differentiation of naïve CD4+ T cells in order to acquire an IFN-γ expressing phenotype. Indeed, Sendai-infected Yeti IFN-γR-/- expressed substantially lower levels of I-A^b when compared to Yeti WT controls (data not shown). Because none of the cells in these knockout mice express the receptor, it remains possible that the effect of IFN-γ on T cells is indirect. Nevertheless, these and other indirect effects did not result in decreased IFN-γ expression by CD4+ T cells after infection of IFN-γR-/- mice with

T.gondii. In contrast, the proportion of IFN-γ expressing CD44hi CD4+ T cell was substantially increased after *T.gondii* infection (Figures 26A and 27A). It is conceivable that these indirect effects could also cause an increase in IFN-γ expression by Th1 cells. Of note, *T.gondii* infected IFN-γR-/- mice displayed a consistently higher parasitic burden (Figure 28). This is due to impaired activation of macrophages by IFN-γ. Macrophages are crucial for the elimination of the parasite, by inducing tryptophan degradation in host cells and NO production (Beaman et al., 1994; Chao et al., 1993; Deckert-Schluter et al., 1996; James, 1995; Nathan et al., 1983; Pfefferkorn, 1984; Scharton-Kersten et al., 1996; Sibley et al., 1991). Moreover, proinflammatory cytokines such as IL-12 and IFN-α have been shown to induce high levels of IFN-γ (Wenner et al., 1996). Indeed, it is known that *T. gondii* induces systemic levels of type I IFNs early after infection (Hunter et al., 1994; Johnson, 1992; Khan et al., 1994). Similarly, a pathway for IFN-γ induction by gram-negative bacteria has been reported, which is based on IL-18 signaling and STAT4 activation by type I IFNs (Freudenberg et al., 2002). Thus, it might be possible that a strong inflammatory environment, caused by high parasite numbers, favors the induction of IFN-γ, independently of its receptor. Even more so, cytokine dependent IFN-γ induction together with increased antigen-presentation could be sufficient to overcome the observed defect in IFN-γ induction by IFN-γR-/- CD4+ T cells. In fact, we could demonstrate that repeated TCR and cytokine stimulation *in vitro* is able to greatly enhance IFN-γ expression by IFN-γR-/- CD4+ T cells (Figure 25).

CD4+ T cell intrinsic IFN-γR functions enhance the induction of IFN-γ

We wished to determine whether the observed CD4+ T cell defect in IFN-γ expression was due to functions mediated by IFN-γ directly on the T cell compartment. To compare IFN-γ expression of IFN-γR-/-and WT T cells side by side in the same animal, we generated dual BM chimeric mice (Lee et al., 2003). Infection of these mice with Sendai virus or *T. gondii* resulted in a reduced proportion of IFN-γ expressing cells within the IFN-γR-/- CD4+ population (Figures 23A, 24A, 26A and 27A). This defect was also apparent in IFN-γ production after *in vitro* stimulation with specific antigen (SenHN peptide or STAg) (data not shown). Moreover, the proportion of antigen-specific eYFP negative IFN-γR-/- CD4+ cells was not due to the generation of Th2 cells, because IL-4 producing CD4+ T cells could not be detected (data not shown). In conclusion, our studies

in two different infectious disease models reveal a CD4+ T cell-intrinsic requirement for IFN-γ responsiveness for maximal IFN-γ induction.

Regulation of reporter brightness occurs independently of IFN-γR functions

As mentioned above, bicistronic IFN-γ reporter mice uniquely allow to visualize differences in IFN-γ expression not only in terms of quality (eYFP positive cells) but also quantity (eYFP brightness). The median fluorescence intensity (MFI) of eYFP expression allows to assess the abundance of IFN-γ transcripts in individual cells and for cell populations (Figure 10C and D). In all examined organs, infection of Yeti IFN-γR-/- mice with Sendai virus resulted in decreased MFIs of eYFP fluorescence within the eYFP positive Th1 population (Figure 20A). As discussed above, indirect effects in these mice, such as sub-optimal priming, could similarly be responsible for the observed defect in eYFP brightness of IFN-γR-/- Th1 cells. Accordingly, repeated *in vitro* stimulation with TCR dependent and independent stimuli could restore the eYFP fluorescence intensity in knockout CD4+ T cells (Figure 25). After infection of IFN-γR-/- mice with *T. gondii*, a inducer of a vigorous type I immune response, the generated Th1 cells displayed no defect in eYFP fluorescence intensity (Figures 26A and 27A). Indeed, the potent inflammatory environment and/or increased antigen load in IFN-γR-/- mice resulted in a strong increase in eYFP brightness (up to 10 fold higher MFIs, Figure27). Therefore, the up-regulation of IFN-γ transcripts within a cell can occur in the absence of IFN-γR-/- mediated functions. After infection of dual BM chimeric mice with Sendai virus or *T.gondii*, eYFP brightness within eYFP positive IFN-γR-/- CD4+ cells was unaltered in non-lymphoid organs (Figure 24A and 30A and data not shown). Thus, IFN-γ transcript levels were regulated in a IFN-γR independent manner, while the induction of IFN-γ was enhanced when IFN-γR is present on CD4+ T cells

IFNγ expression by CD8+ T cells is less dependent on IFN-γR mediated functions than CD4+ T cells

As discussed in the introduction, various mechanisms govern IFN-γ induction in CD8+ and CD4+. IFN-γ induces T-bet expression in both cell types, but only CD8+ T cells employ additionally the transcription factor Eomes for the expression of IFN-γ (Afkarian et al., 2002; Lighvani et al., 2001; Pearce et al., 2003; Szabo et al., 2002; Yin et al., 2002).

Consequently, this feature of CD8+ T cells could allow for robust IFN-γ expression in the absence of IFN-γR mediated functions, such as the activation of T-bet. Indeed, CD8+ T cells were less dependent on IFN-γR mediated functions for the induction of IFN-γ than CD4+ T cells. For example, *in vitro* under culture conditions where IL-12 was absent, the relative defect in IFN-γ expression by IFN-γR-/- CD8+ T cells was less pronounced, when compared to the relative defect in IFN-γR-/- CD4+ T cells (Figure 18). Moreover, in the presence of IL-12, optimal IFN-γ expression by IFN-γR-/- CD8+ T cells was completely restored (Figure 18B). Thus, *in vitro,* IL-12 is able to compensate for the lack of IFN-γR signals in CD8+ T cells but not CD4+ T cells. Additionally, after infection of knockout mice with Sendai virus, both IFN-γ expression and eYFP brightness were unaltered in IFN-γR-/- CD8+ T cells (Figures 20B and 21B). After infection with *T. gondii,* the proportion and brightness of eYFP expressing IFN-γR-/- CD8+ T cells were increased in the liver, the spleen and the blood (Figures 26B and 27B). However, the relative increase was little when compared to WT CD8+ T cells or IFN-γR-/- CD4+ T cells. IFN-γR-/- CD4+ T cells consistently displayed 5 to 10 fold higher eYFP MFIs than IFN-γR-/- CD8+ T cells (Figure 27B). As discussed above, the indirect effects in these knockout mice are very divers, yet they affect CD4+ T cells to a greater extent than CD8+ T cells. Moreover, in dual BM chimeric mice the IFN-γ expression of IFN-γR-/- CD8+ T cells was unaltered after Sendai virus infection (Figure 24B). After *T. gondii* infection, IFN-γR-/- CD8+ T cells the frequency of eYFP expression was reduced in the blood, but not other examined organs (Figure 30B). Taken together these data suggest IFN-R independent mechanisms are in place for optimal IFN-γ induction in CD8+ but not CD4+ T cells.

Proposed model for IFN-γ expression by CD4+ and CD8+ T cells

Bicistronic IFN-γ reporter mice provide valuable insights into the IFN-γ expression of defined cell populations and individual cells. The visualization of IFN-γ expression in terms of quality (eYFP positive cells) and quantity (eYFP brightness) are unique attributes of bicistronic cytokine reporter mice (Matsuda et al., 2003; Stetson et al., 2003). Qualitative statements can be made for defined cell populations (frequency of eYFP), while quantitative statements (MFI) refer additionally to individual cells. Thus, based on our data obtained from Yeti mice *in vitro* and *in vivo*, the following distinctive features of IFN-γ expression by CD4+ and CD8+ T cells could be identified.

1) eYFP-reporter brightness (MFI) faithfully reflects the abundance of IFN-γ transcripts.
2) The abundance of IFN-γ transcripts is highly regulated within individual cells (heterogeneous eYFP fluorescence).
3) Upon stimulation, eYFP-reporter brightness (MFI) correlates with the amount of secreted IFN-γ protein.
4) The dependence of robust IFN-γ protein secretion/production on antigenic stimulation, despite heterogeneous IFN-γ transcript levels, implies post-transcriptional regulation of IFN-γ.
5) High eYFP fluorescence reflects a more differentiated effector status.
6) IFN-γ expression occurs biallelically.

Based on these observations, we propose a model for IFN-γ expression by CD4+ and CD8+ T cells (Figure 31). Naïve CD4+ and CD8+ T cells are non-competent for IFN-γ production while at the same time they are able to respond to IFN-γ (Gajewski and Fitch, 1988; Groux et al., 1997; Pernis et al., 1995). They have little or no IFN-γ transcript and are negative for the IFN-γ-eYFP reporter. According to the above-mentioned features, we were able to dissect two distinct steps in the regulation of IFN-γ expression. During priming and differentiation, naïve cells become activated and given the appropriate stimuli, are rendered competent for IFN-γ production (i.e. eYFP positive). Thus, the acquisition of IFN-γ competence, as assessed by the presence of eYFP fluorescence, represents the first step in the regulation of IFN-γ expression by both CD4+ and CD8+ T cells (Step 1 in Figure 31). IFN-γR expression on T cells is not necessary for this step. However, T cell-intrinsic expression of IFN-γR augments the proportion of IFN-γ competent cells within CD4+ and CD8+ T cells. In CD8+ T cells *in vitro*, this partial requirement for the IFN-γR can be overcome by IL-12. Besides the IFN-γR, various other parameters, involved in both T cell priming and Th1 differentiation, are able to influence the acquisition of IFN-γ competence. Factors that influence Th differentiation and priming include the cytokine microenvironment (especially IL-12 and IL-4), the signal strength and duration of the APC-T cell interaction, the type of APC and its activation status, co-stimulatory molecules and cell cycle (Amsen et al., 2004; Bird et al., 1998; Constant and Bottomly, 1997; Kuchroo et al., 1995; Reiner and Seder, 1995; Seder, 1994). Furthermore,

the acquisition of IFN-γ competence includes inter- and intrachromosomal interactions, epigenetic modifications, initiation of crucial transcription factors and up-regulation of pivotal cytokine receptors (Grogan and Locksley, 2002; Murphy and Reiner, 2002b; Spilianakis et al., 2005).

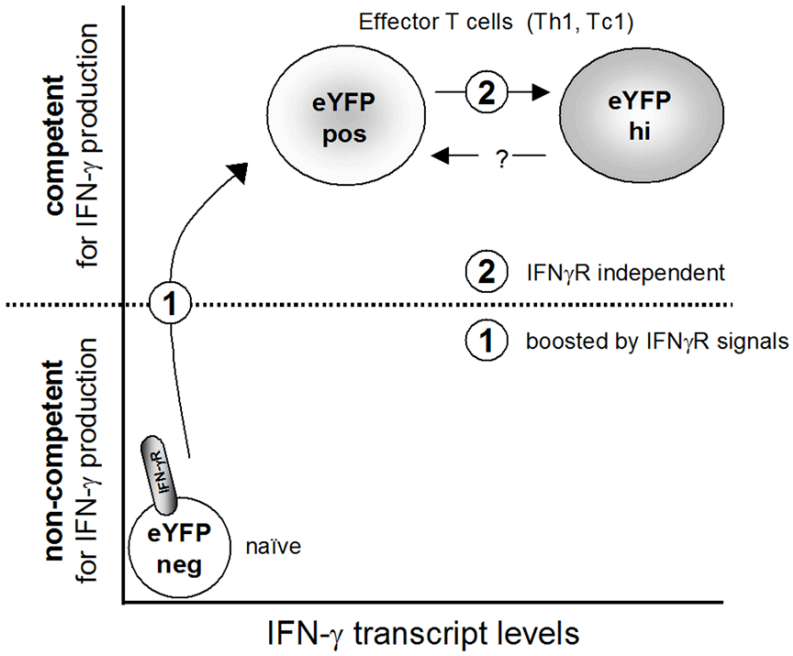


Figure 31:
Proposed model for IFN-γ expression by CD4+ and CD8+ T cells in context of IFN-γR requirements. Details are described in the text.

Once rendered IFN-γ competent, CD4+ and CD8+ T cells are able to modulate the abundance of IFN-γ transcripts (Step 2 in Figure 31), as detected by heterogeneous reporter expression. The ability to up-regulate IFN-γ transcripts represents the second distinct step in the regulation of IFN-γ expression. The modulation of IFN-γ transcript levels occurs largely independent of IFN-γR mediated functions in both CD4+ and CD8+ T cells. This is supported by the fact that differentiated Th1, but also Tc1 cells, become unresponsive to IFN-γ (Gajewski and Fitch, 1988; Groux et al., 1997; Haring et al., 2005b; Pernis et al., 1995; Rigamonti et al., 2000; Whitmire et al., 2005). Furthermore, the modulation of transcript abundance within a cell reflects the observed functional

heterogeneity in IFN-γ expression. Highly up-regulated IFN-γ transcript levels approximate a more differentiated effector phenotype, and high eYFP fluorescence could mark IFN-γ secreting/ producing cells *in situ*. The anatomical restriction of eYFP hi cells mainly to tertiary sites and/or sites of pathogen replication, as in the case for respiratory virus infections, could support this concept. Moreover, while secretion of IFN-γ was largely dependent upon antigenic-stimulation, eYFPhi cells produced detectable amounts even without stimulation. Most interestingly, the dependence of robust IFN-γ protein secretion/production on antigenic stimulation implies post-transcriptional regulation of IFN-γ. Thus, the abundance of IFN-γ transcripts within a cell reflects its potential to produce a corresponding amount of IFN-γ protein upon stimulation. In differentiated Th1 and Tc1 cells, which are IFN-γ competent by nature, various stimuli can up-regulate IFN-γ transcripts. Once rendered IFN-γ competent (Step 1 in Figure 31), both TCR-dependent and independent stimuli, such as IL-12/IL-18 exposure, can increase IFN-γ expression (Step 2 in Figure 31) (Ahn et al., 1997; Campbell et al., 1996; Nakahira et al., 2002; Nakanishi et al., 2001; Robinson et al., 1997; Sica et al., 1997; Sweetser et al., 1998; Szabo et al., 2003). In contrast, the acquisition of IFN-γ competence by naïve T cells (Step 1 in Figure 31) is strictly TCR dependent.

Recently, a two-step process for IL-4 cytokine production could be identified by using IL-4 dual-reporter mice (Mohrs et al., 2005). In these mice, insertions of two different reporter genes into both copies of the endogenous *Il4* locus allow for simultaneous analysis of IL-4 transcripts and IL-4 protein secretion within the same cell. According to this two-step model of cytokine production, the first step generates IL-4 competent CD4+ cells that disseminate systemically, while during the second step the cytokine is rapidly produced in response to local stimulation. Thus, the acquisition of competence for cytokine production and the functional separation between cytokine transcript levels (competence) and actual cytokine secretion upon stimulation, are likely to reflect general regulatory mechanisms of cytokine expression.

Understanding the regulation of cytokine expression *in situ* is of great importance for vaccine development and identifying novel therapeutic approaches for cancer and autoimmunity. For example, enhancing IFN-γ transcripts could lead to more potent effectors. Similarly, down-regulating IFN-γ could ameliorate autoimmune diseases or decrease pathology. The functional separation between IFN-γ transcript levels and actual

IFN-γ secretion could be exploited for controlling IFN-γ production. The molecular basis for the anatomical restriction of eYFPhi cells could aid in targeting the most differentiated effectors to desired organs, when developing vaccines. Future investigations might allow to integrate these concepts of cytokine regulation and develop means for *in vivo* adaptation to control IFN-γ expression in humans.

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9. APPENDIX

9.1. Table I

A selection of IFN-γ regulated genes according to their association with IFN-γ effector functions (modified after (1, 2))

Antigen Processing and Presentation (genes up-regulated by IFN-γ)

Gene	Function	References
α1, α2, β1, β2 MHC II chains	Constituents of the heterodimeric MHC II. MHC II displays foreign and self-peptides on the cell surface for immune surveillance by CD4+ T cells. The MHC II α and β chains are encoded by the class II MHC locus.	(3, 4)
Cathepsins B, H, L	Lysosomal proteases implicated in peptide production for class II MHC loading.	(5, 6)
Class I MHC heavy chain	The heavy chain associates with ß2-microglobulin to form the MHC class I complex (MHC I). MHC I displays foreign and self-peptides on the cell surface for immune surveillance by cytotoxic T cells.	(7, 8)
PAα28, PA28β	Proteasome activator (PA)28α:PA28β dimer is a nonenzymatic proteasome subunit, which alters the specificity of peptides generated to increase efficiency of class I MHC peptide delivery.	(9, 10)
ß2-microglobulin	Light chain that associates with the class I MHC heavy chain to form the MHC I. MHC I displays foreign and self-peptides on the cell surface for immune surveillance by cytotoxic T cells. The ß2-microglobulin light chain is not MHC-encoded.	(11, 12)
TAP-1, TAP-2	The transporter associated with antigen processing (TAP) is a heterodimer consisting of TAP-1 and TAP-2 subunits. TAP functions as a transmembrane pump to transfer peptides from the proteasome into the endoplasmic reticulum (ER) lumen. It also aids in peptide delivery to class I MHC. TAP-1 and TAP-2 map to class II MHC.	(13, 14)
Tapasin	Tapasin is a chaperone that aids in the retention of empty MHC I in the ER and peptide loading into MHC I peptide-binding cleft.	(15)

Antiproliferative Effect (genes up-regulated by IFN-γ)

Gene	Function	References
p202	p202 is a strong cell cycle repressor that can bind to E2F and inactivate its DNA-binding activity, thereby preventing transcription of E2F-dependent genes required for S phase.	(16, 17)
p21, p27	p21 and p27 are cyclin-dependent kinase (CDK) inhibitors of the Cip/Kip family. p21 and p27 inhibit the activity of CDK2 and CDK4, respectively, causing cell cycle arrest at the G1/S checkpoint.	(18-20)
PKR	PKR is an antiviral enzyme, which functions as a serine/threonine kinase when activated by dsRNA. PKR inhibits cellular proliferation, thereby halting protein synthesis. May also suppress c-myc function.	(21, 22)

Apoptotic Effect (genes up-regulated by IFN-γ)

Gene	Function	References
Caspase 1	Caspase-1 is a cysteine protease involved in the generation of bioactive IL-1β and IL-18 and implicated in mediating macrophage apoptosis.	(23)
Fas/Fas ligand	IFN-γ may increase cellular sensitivity to apoptosis by up-regulating expression of Fas and Fas ligand.	(24, 25)
IRF-1	interferon regulatory factor 1 (IRF-1) is a tumor-suppressor gene required for the induction of apoptosis by signals such as DNA damage.	(26-28)
TNF-α receptor	IFN-γ may promote cellular sensitivity to the proapoptotic effects of tumor necrosis factor-α (TNF-α) by promoting surface expression of a TNF-α receptor on tumor cells.	(29)

Table I. (continued)

Antimicrobial Effect (genes up-regulated by IFN-γ)

Gene	Function	References
C2, C4, Factor B	Complement proteins are secreted by macrophages and fibroblasts in response to IFN-γ. Complement functions to opsonize extracellular pathogen for receptor-mediated phagocytosis by mononuclear phagocytes.	(30)
FcRγI	Expression of the high-affinity Fc receptor (FcRγI) is increased in myeloid cells by IFN-γ stimulation. FcRγI binds extracellular pathogens via IgG in the adaptive phase of the immune response.	(31)
GBPs	The guanylate-binding proteins (GBP) are GTPases with antimicrobial properties that function as regulators of immunity to intracellular pathogens	(32)
gp67^{phox}, gp91^{phox}	subunits of the NADPH oxidase, associate with gp22^{phox} and gp47^{phox} to form the active complex capable of the generation of ROS during the respiratory burst.	(33-35)
iNOS/NOS2	The NOS enzymes (NOS1, iNOS, NOS3) catalyze the reduced nictonimide adenine dinucleotide phosphate (NADPH)-dependent conversion of L-arginine to L-citrulline, forming NO as a by-product. Of these, iNOS is the only isoform inducible by cytokine and/or microbial stimulus.	(36, 37)
Mac-1	Complement receptors CR3 of mononuclear phagocytes, up-regulated by IFN-γ to promote receptor-mediated phagocytosis of opsonized extracellular pathogens.	(38)

Antiviral Effect (genes up-regulated by IFN-γ)

Gene	Function	References
ADAR	The dsRNA-specific adenosine deaminase (ADAR) catalyzes the deamination of adenosine to form inosine on dsRNA substrates and thus may be responsible for the generation of "edited" viral mRNA. The cellular translational machinery treats inosine as guanosine, and thus, A → I editing of viral mRNA may cause mistranslation into nonfunctional viral proteins to inhibit viral replication	(39)
GBP1, GBP2	The guanylate-binding proteins (GBP) are GTPases with antiviral properties	(32, 40)

Immunomodulation, T helper development, and leukocyte trafficking
(genes up-regulated by IFN-γ)

Gene	Function	References
B7-1/B7-2	Surface molecules on APCs that provide costimulus for antigen-specific T cell activation.	(41)
B7/BB-1	B cell activation antigen, Type I membrane protein, natural ligand for the T cell antigen CD28	(42)
CCL2 (MCP-1), CCL7 (MCP3), CCL8 (MCP-2),	Monocyte chemoattractant protein (MCP), MCP-1, MCP-2 and MCP-3 are chemoattractants for monocytes, macrophages, basophils, eosinophils and activated T cells and NK cells	(43-45)
CCL3, CCL4	(MIP-1α, MIP-1β) chemoattractants for CD4+, CD8+, and memory T cells.	(46)
CCL5 (RANTES)	regulated on activation, normal T expressed and secreted (RANTES) is a chemoattractant for memory CD4+ T cells and monocyte/macrophages.	(47-49)
CD11a, CD18 (LFA-1)	Lymphocyte function- associated antigen-1(LFA-1), interacts with ICAM-1 and ICAM-2, activation marker for T cells	(50-52)
CD40	Member of the TNF-α superfamily expressed on APCs, fibroblast and endothelial cells, interacts with CD40 Ligand on T cells to induce B cell activation	(53, 54)
CD44	CD44 is a surface molecule that mediates cell-cell and cell-matrix adhesion, Highly expressed on activated/memory T cells	(55)
CD62L	L-selectin (CD62L) is a cell surface adhesion protein that mediates the adherence of leukocytes to high endothelial venules, highly expressed on naïve T cells	(56)
CXCL10 (IP-10)	IFN-inducible protein (IP-10) is a chemoattractant for monocytes and T cells.	(57, 58)

Table I. (continued)

Immunomodulation, T helper development, and leukocyte trafficking

(genes up-regulated by IFN-γ) ***continued***

Gene	Function	References
ICAM-1	Adhesion molecule-binding to lymphocyte function-associated antigen-1 and Mac1.	(59)
IFN-γ	Autostimulatory loop	(60-62)
IgG2a, IgG3	Up-regulation of immunoglobulin heavy chain gene class switch transcripts	(63-65)
IL-12	NK cell activator and differentiation factor driving CD4+ cell development to a Th1 phenotype.	(66, 67)
IL-4 receptor	IL-4 is the hallmark cytokine of type 2 immune responses. In mouse cells IL-4 receptor is up-regulated by IFN-γ, whereas it is down-regulated in human cells	(68, 69)
Hyaluronic acid (HA)	Hyaluronic acid (HA) is a component of the extracellular matrix and is a ligand for CD44	(70)
PNAd	Peripheral lymph node addressin (PNAd, MECA-79 antigen), ligand for L-selectin; required for lymphocyte homing	(50)
SCF	(stem cell factor) c-kit ligand	(71)
T-bet	T-box transcription factor that is key regulator in Th1 differentiation	(72, 73)
VCAM-1	Adhesion molecule-binding to very late antigen-4.	(74-76)

Immunomodulation, T helper development, and leukocyte trafficking
(genes down-regulated by IFN-γ)

Gene	Function	References
CD15	Ligand for L-selectin, inhibits neutrophil phagocytosis and bacterial activity	(77, 78)
CD31 (PECAM-1)	Platelet endothelial cell adhesion molecule 1 (PECAM-1) acts as an cell adhesion molecule expressed on platelets and at endothelial intercellular junctions	(79, 80)
CD34	Ligand for L-selectin on endothelial cells	(81)
CD35	Low affinity IgE receptor	(82, 83)
CD147	interaction between CD147 (Basigin, OX-47) and cyclophilin A is important for the infectivity of HIV	(84, 85)
Fibronectin	Involved in cell adhesion, cell motility, opsonization, wound healing and maintenance of cell shape	(86, 87)
IgG1, IgE	Down-regulation of immunoglobulin heavy chain gene class switch transcripts	(63, 88, 89)
VLA-4, VLA-5	Very late antigen (VLA) 4 and 5 interact with fibronectin and VCAM-1	(90-92)

9.2. Table I references

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Publications:

Trujillo-Vargas CM, **Mayer KD**, Bickert T, Palmetshofer A, Grunewald S, Ramirez-Pineda JR, Polte T, Hansen G, Wohlleben G, Erb KJ.
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Mayer KD, Mohrs K, Cookenham T, Roberts A, Woodland DL, Mohrs M.
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(manuscript in preparation)

Mayer KD, Woodland DL, Mohrs M.
The competence for IFN-γ production is imprinted in antigen-specific T cells and modulated over the course of infection.
(manuscript in preparation)

Oral presentations:

2004 12th International Congress of Immunology, Montréal, Canada

2005 University of Pennsylvania, Department of Pathobiology, Philadelphia, USA (invited by Dr. C.A. Hunter)

2005 National Institute of Allergies and Infectious Diseases, Department of Parasitic Diseases, Bethesda, USA (invited by Dr. A. Sher)

2006 University of Connecticut, Health Center, Farmington, USA (invited by Dr. L. Lefrancois)

2006 10th Woods Hole ImmunoParasitology meeting (WHIP), Marine Biology Institute Woods Hole, USA

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Eidesstattliche Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation in allen Teilen selbständig angefertigt und keine anderen als die genannten Quellen und Hilfsmittel verwendet habe.

Weiterhin versichere ich, dass ich bisher keinen Promotionsversuch unternommen oder die vorliegende Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt habe.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften an der Bayerischen Julius-Maximilians-Universität Würzburg.

Saranac Lake, April 2006

Katrin Doris Mayer