5 Methods

5.1 Molecular biological methods

The general molecular biological methods, *E. coli* cultivation, agarose gel preparation and DNA electrophoresis, DNA extraction and precipitation, measurement of DNA / RNA concentration, DNA digestion were performed according to *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989). DNA plasmid preparations and DNA extraction from agarose gels were performed with kits obtained from *Qiagen*. DNA preparations were ligated in a volume of 10µl using a 3x molar excess of insert and 1 unit T4 ligase (S*tratagene*) in the corresponding ligase buffer at 14° C overnight. DNA sequencing was performed at the sequencing core facility of the institute. Sequences were verified and compared using the web-based program Blast 2 Sequences (http://www.ncbi.nlm.nih.gov/gorf/bl2.html).

5.1.1 *E.coli* heat shock transformation

Competent cells were thawed on ice, 20ng plasmid DNA in H_2O or 1 µl of ligation mix was added and gently mixed with a pipette tip. The suspension was left on ice for 30 min, then heat shocked in a 42°C water bath for 30 seconds, and placed on ice for 2 min. 500µl LB medium was added and cells were incubated at 37°C, 200 rpm for 30-60 min before plating on agarose LB plates.

5.1.2 Electroporation of *E. coli*

A *BioRad* Gene Pulser was used with the following parameters: 0.1cm cuvette, 200 Ω, 1.7 kV, 25µF. Electro-competent cells were thawed on ice, 1 µl ligation mix or 100ng plasmid DNA in H_2O was added and gently transferred into an ice-cold cuvette, left on ice for 10 min, then electroporated. 500µl of pre-warmed LB medium was added and cells were incubated at 37°C, 200 rpm for 30-60 min before plating on LB agarose plates.

5.1.3 Polymerase chain reaction (PCR)

BioTherm Taq polymerase (*Gene Craft*) was used for analytic, Pfu polymerase (*Stratagene*) was used for cloning PCR. Standard PCR conditions were: 5µl of 10x PCR buffer (with $MgCl_2$), 1µl 10mM dNTPs, 1µl template DNA (50-200ng), 0.25µl of each primer (100pmol/µl = 100µM), 0.2 µl (2 U) Taq polymerase, ad 50µl H_2O. 30 cycles, annealing at 55-60°C, denaturation at 95°C. Extension time at 72°C was 0.5-1.0 min/kb for Taq polymerase and 2 min/kb for Pfu polymerase.

5.1.4 Southern Blot

10µg of DNA prepared from tail biopsy was digested with restriction endonucleases in digestion buffer containing 0.1 µg /µl RNAse overnight; separated on a 0.8% agarose gel, denatured in 0.1M NaOH, equilibrated in 1M Tris, 1.5M NaCl, pH 8.0, transferred onto nylon membrane (Hybond N+, *Amersham*), and crosslinked by UV radiation. 500 bp were defined as optimal probe, ^{32}P labeled hybridization probes were prepared with the *Stratagene* "Prime it" kit and purified using "Probe Quant" (*Pharmacia*) micro columns. Sonicated fish sperm DNA was added and probes were denatured for 5 min at 96°C. Hybridization was performed at 68°C for 90 min using "QuickHyb" from *Stratagene*. Blots were washed twice for 15 min at RT with 2x SSC and 0.1% SDS, for a high-stringency wash 30 min at 60°C with 0.1x SSC and 0.1% SDS.

5.1.5 RT-PCR

mRNA was prepared using the Trizol method (*Gibco*) according to the manufacturers instructions followed by DNAse digestion. cDNA was prepared using the Superscript system (*Gibco*) according to the manufacturers instructions. β-actin control templates were routinely diluted 1:200. To exclude contamination with genomic DNA, control PCRs were performed with a twofold amount of non reverse transcribed mRNA.

5.2 Cell culture and transfection

58$\alpha^-\beta^-$ cells, EL 4 cells, 54ζ17 cells and all stable transfectants were grown in RPMI supplemented with 10% FCS, 0.2mM L-glutamine, 10 U/ml penicillin and streptomycin. Cells were cultured in cell culture plastic flasks and diluted 1 to 50 every 3-4 days. Phoenix cells were cultured in 10 cm culture dishes in DMEM supplemented with 10% FCS, 0.2mM L-glutamine, 10 units/ml penicillin and streptomycin. 70-80% confluent plates were diluted 1:4 or 1:5 every 2-3 days. All media and supplements were obtained from *Biochrom*. The hsp60-specific CD8+ T cell clone UZ3/4 was maintained as described (Steinhoff et al., 1999).

5.2.1 Electroporation of cultured cells

Electroporation of 54ζ17 and 58$\alpha^-\beta^-$ cells was performed using a *BioRad* Gene Pulser with the following parameters: 0.4cm cuvette, 960µF, 275V and 1x10^7cells in 250 µl PBS. 10µg of each linearized TCR vector DNA + 1µg linearized pCMV/Zeo vector was mixed with the cells and placed into cuvette on ice for 1 min, electroporated, and left on ice for another 10 min on ice. Cells were then cultured in 10ml non selective medium in 10 cm culture dishes. After 1-2 days, zeocin selection was started. Dishes were placed in the 4°C fridge for two hours to enhance killing and selection, according to the manufacturers manual. A zeocin concentration of 300µg/ml had been determined as the optimal concentration for selection (**Figure 6**).

Figure 6. Various cell lines were cultured in duplicates with the indicated zeocin concentrations to determine the optimal concentration for selection. Line 54CD8 was not used in this work.

5.2.2 Retroviral Transduction

Complete cDNA of the TCR Vβ8, Vα7 and Vα8 chains were cloned from pP14α2AR and pP142β2AR TCR-expression plasmids into the vector pMSCV-2.2-IRES-GFP using the unique endonuclease restriction sites ClaI and Xho1. Phoenix packaging cells (Kinsella and Nolan, 1996) were transiently transfected via calcium phosphate precipitation with pMSCV-2.2-IRES-GFP containing the TCR Vβ8, Vα7 and Vα8 chains (**Figure 7**). Briefly, $3x10^6$ cells were plated 10h prior to transfection in 10 cm tissue culture dishes. 15 min prior to transfection, medium was replaced by 10 ml fresh growth medium containing 25µM chloroquine to inhibit lysosomal DNAses. DNA Ca_2PO_4 precipitates were prepared as follows: 10µg of each plasmid DNA was dissolved in 438 µl H_2O. 61 µl 2M $CaC1_2$ was added and mixed thoroughly with finger tapping. 500µl of 2x Hepes buffer (50mM Hepes, pH 7.05, 10mM KCl, 12mM dextrose, 280mM NaCl, 1.5mM NaH_2PO_4) was added dropwise while bubbling air into the solution with pasteur-pipette using a pipette aid. Precipitation mix was added to Phoenix cells immediately. After 48h, supernatants were harvested and 2ml were used for transduction of 10^5 54ζ17 cells. Two days after transduction, GFP positive transfectants were sorted by FACS in the cell sorting facility of the Max-Planck-Institute for Infection Biology. GFP expression levels remained stable for at least 60 days. For double viral transduction, cells were first transduced with the α chain constructs, sorted for GFP expression and subsequently supertransduced with the β chain construct, vice versa, or with both the α and β constructs at a time.

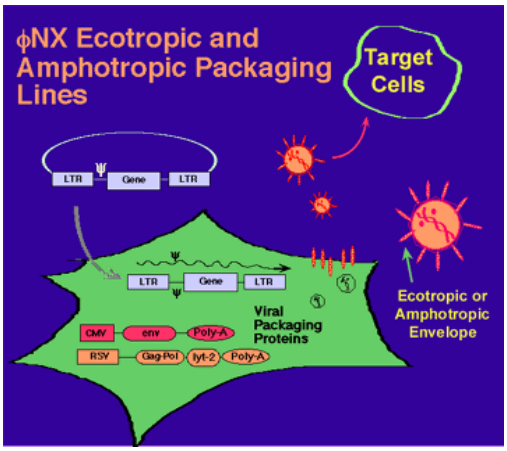

Figure 7. Phoenix packaging cells contain stable integrations of viral *env* and gag-pol. Retroviral vector is produced upon CaPO4 transfection with pMSCV-2.2-IRES-GFP containing the gene of interest. Figure 7 taken from (Nolan 2002)

5.3 Biochemical methods

5.3.1 Western Blot

Cells ($1x10^8$) were washed twice in PBS and lysed in Lysis Buffer (0.5% Triton X-100, 300mM NaCl, 50mM TrisCl, pH 7.6, protease inhibitors 0.5 mM PEFA-block, 1mM leupeptin, 1mM pepstatin A) on ice. Nuclei and cell debris were removed by centrifugation of the lysate for 10 min at 10.000 x *g*. 50µg of protein was separated on a discontinuous SDS-PAGE (4-12%) and blotted onto nitrocellulose (*BioRad*). The constant region of mouse TCR α chain was detected with mAb H-28 710, followed by incubation with a peroxidase-conjugated goat anti Armenian hamster IgG (*Jackson Immuno Research*). Blots were developed with the enhanced chemiluminescence Western blotting kit (Renaissance*, NEN*).

5.3.2 Pulse-chase metabolic labeling and immunoprecipitation.

Thymi from various mice were excised and single-cell suspensions were prepared using an iron mash sieve and cells were washed twice with a 1:1 mixture of complete RPMI and PBS. $3x10^7$ cells were incubated at 37°C in 10 ml L-cysteine and L-methionine free DMEM (*ICN*) for 1h, pulsed with 500µCi [^{35}S]-methionine / cysteine (TRAN^{35}S-LABEL; *ICN*) for 5 min and chased in 10 volumes of medium containing 10% fetal calf serum and 4mM L-methionine and 4mM L-cysteine (*Sigma*) for various time periods at 37°C. Cells were washed twice in PBS and solubilized in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 50mM sodium borate pH 8.0, 150mM NaCl, 1 µg/ml of aprotinin, 1 µg/ml of leupeptin, 100 µg/ml of PMSF, all obtained from *Roche*) on ice for 30 min. Lysates were clarified by 10 min centrifugation at 12,000 x g to remove nuclei and insoluble material and precleared with 50µl protein-A-agarose suspension (*Roche*) at 4°C for 1h. Immunoprecipitation was performed by addition of 10µg of anti TCR β mAb (H57-597) at 4°C for 1h and precipitation with 50µl protein-A-agarose suspension overnight. Precipitates were washed five times for 20 min at 4°C: Twice in 50mM Tris, 150mM NaCl, 0.1% Nonidet P-40, twice in 50mM Tris, 500mM NaCl, 0.1% Nonidet P-40 and finally in 10mM Tris, denatured in 4 x LDS sample buffer (*NOVEX*) under reducing conditions and separated by discontinuous SDS-PAGE

using a NuPAGE pre-cast 4-12% gel (*NOVEX*). Gels were dried and subjected to autoradiography and densitometric analysis using a Fujifilm FLA-2000 IMAGER and the software *image gauge*. Nonlinear regression curves were determined using GraphPad Prism software and half-lives were calculated as ln 0.5 / -k.

5.4 Generation of transgenic mice

5.4.1 Cloning strategies for TCR expression constructs

The rearranged TCR Vα8.2-J42 and TCR Vβ8.1–Dβ1–Jβ1.1 chains were amplified from cDNA prepared from the clone UZ3/4 using the primer-pairs Valpha8-EcoRI-FW and Calpha-250-REV as well as Vbeta8-Xho1-Ban-FW and Cbeta-762-REV, respectively. Using these primers, additional 5' restriction sites were introduced, and the glycine 2 codon of the TCR β chain was silent mutated from GGC to GGG to delete an unwanted BanII restriction site. PCR products were inserted into the pCR2.1 vector and sequenced. The TCR α chain was cut at the introduced EcoRI and a natural EcoRV site in the constant region, the resulting fragment was ligated into the pP14αAR vector to yield a plasmid containing full length TCR α8 cDNA, p14x–α8. Accordingly, the TCR β chain was cut at the introduced Xho1 and a natural BanII site in the constant region, the resulting fragment was placed into the pP142βAR vector to yield the full length TCR β8 cDNA plasmid p14x–β8. These plasmids were digested with Xho1 and BamH1, the cDNAs were subcloned into SalI / BamH1 cut pHSE3' TCR expression vector thereby deleting the Xho1 restriction site.

The rearranged Vα7.2 Jα18 region was amplified by PCR from cDNA of the CD8+ T cell clone UZ3/4 using the primer pair UZ-α-7.2 FW and UZ-α-7.2 REV adding a 5' Xho1 and a 3' Not1 site. The PCR product was put into the pCR2.1 vector and sequenced. Xho1 and partial Not1digestion gave a fragment containing the TCR Vα7.2-Jα18 region flanked by splice acceptor and donor sites which was introduced into the genomic TCR α chain shuttle vector pSHVα.

5.4.2 Preparation of transgenic vector samples

To separate the plasmid-backbone from the construct, the pHSE3' TCR plasmids were cut with Xho1 and the 6.2 kb fragments were eluted from an ethidium bromide free preparative gel. Analog, the pSHVα plasmid was cut with the restriction enzymes Cla1 and Sal1 to yield a 12.7 kb fragment. Linearized constructs were eluted in 2 mM Tris pH 7.5 using the QiaQuick protocol. For injection, the DNA was diluted in injection buffer (10 mM Tris/0.1 mM EDTA, pH 7.5) to a final concentration of 3-5 ng/µl.

5.4.3 Pronucleus injection of transgenic vector samples into fertilized eggs

To obtain a large quantity of more than 100 fertilized eggs for injection, C57BL/6 x CBA F1 females or C57BL/6 females (5-6 weeks of age) were superovulated by using consecutive PMS and HCG hormone injections (PMS: pregnant mere serum, 5 IU in 100µl PBS given i.p. at day 3 before microinjection, 14:00-16:00h; HCG: Human chorionic gonadotropin, 5 IU in 100µl PBS given i.p. at day 1 before microinjection). Females were mated to C57BL/6 stud males immediately following the HCG injection. The next day, mated females were checked for vaginal plugs. Eggs were harvested from the ampulla of the oviduct at 0.5 days post conception. Egg-cumuli were treated with hyaluronidase to remove nurse cells, and were then washed through several dishes of M2 (Hogan et al., 1986) media. Fertilized eggs were stored in M16 medium (Hogan et al., 1986) at 37°C and in 5% CO2 until injection. For injection, 20-30 eggs were removed from the incubator at a time. Each egg was individually injected into the male pronucleus (**Figure 8**) with a few hundred copies of the DNA fragment by constant pressure under high magnification according to (Brinster et al., 1981; Hogan et al., 1986). When each egg had been injected, they were returned to the incubator overnight. The next day, eggs which had survived injection and had proceeded into the two cell stage were implanted in groups of 10-15 bilaterally into the oviduct of pseudopregnant CD1 females (i.e. females which have been mated to vasectomized males). The animals were allowed to recover from anesthesia under a warming lamp, and then returned to the animal room.

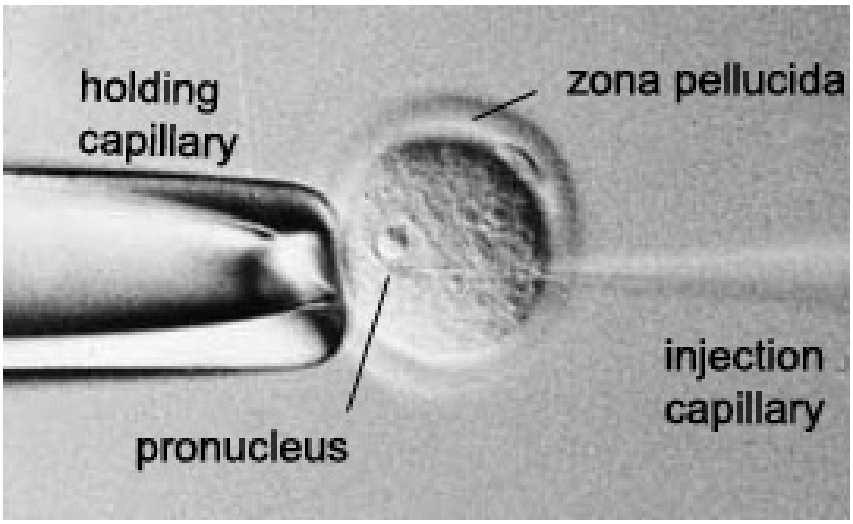

Figure 8. Injection capillary delivering vector DNA into the male pronucleus of a fertilized mouse egg. Pronucleus is swollen to 1.5x-2x of original volume. Figure 8 modified from http://www.hsc.wvu.edu/neurosci/faculty/michalkiew

5.4.4 Screening of potential transgenic founders

At the age of 3-4 weeks, mice were weaned and genders were separated. At the same time, they were ear marked, and a 3mm tail biopsy was cut off to prepare a sample of genomic DNA from each potential founder. To do so, DNA was precipitated from proteinase K digested tails (shake overnight at 56 °C in 500µl of tail buffer: 100mM Tris pH 8.5, 5mM EDTA, 200mM NaCl, 0.2% SDS, +10 µl Proteinase K 10mg/ml). Transgene-carrying mice were identified by PCR of tail biopsy DNA and transgene integration was confirmed by Southern blotting. While taking the tail biopsy, a 20µl drop of blood was taken and used to check transgenic TCR expression of potential TCR α8 and TCR β8 transgenic mice. Transgene-expression of TCR α 7 transgenic mice was confirmed by RT-PCR.

5.5 Animal procedures

5.5.1 BCG infection

M. bovis BCG (Copenhagen) organisms were grown in Middlebrook 7H9 broth (*Difco*) supplemented with albumin-dextrose complex containing 0.05% Tween 80 and were stored in aliquots at -70°C. Mice were infected intravenously (i.v.) in the lateral tail vein with 5 x 10^6 colony forming units (cfu) of BCG.

5.5.2 Peptide immunization

Mice were immunized subcutaneously (s.c.) weekly for the indicated times (1x, 2x, and 3x) with 20µg or 40µg of synthetic $hsp60_{499\text{-}508}$ peptide dissolved in 100µl PBS mixed with 100µl of IFA. Control mice were immunized s.c. with 200µl of the same PBS / IFA emulsion without peptide. Frequencies of $hsp60_{499\text{-}508}$ peptide-specific CD8+ cells were analyzed 3 days after the last immunization.

5.5.3 Heterozygous breeding

To compare TCR α 7.2 transgenic and nontransgenic α -/- mice as well as control animals from the same litter, breeding pairs heterozygous both for the TCR α null mutation and for the TCR α 7.2 transgene were employed. Offspring were typed by PCR and by FACS of peripheral blood cells (PBC). TCR α 7.2 transgenic mice showing severe signs of IBD were sacrificed.

5.6 Immunological methods

5.6.1 Flow Cytometry and Cell Sorting

Cells were incubated in 200µl PBS with conjugated antibodies (Table 1) for 20 min on ice, washed in PBS and resuspended in 300µl PBS containing 0.1% BSA. Counting was performed with a FACS Calibur (*Becton Dickinson*) and the software FCS-express and Cell Quest was used to analyze the data. FACS assisted cell sorting was performed in the flow cytometry core facility of Max Planck Institute for Infection Biology using a FACS-VANTAGE cell sorter (*Becton Dickinson*).

5.6.2 Generation of MHC class I tetramers and staining of cells with tetramers

Modified forms of the full-length cDNA of H-2K^d and human β2m were kindly provided by Drs. E. Pamer and D. Busch. Tetrameric H-2Kd/peptide complexes were generated as described by Busch et al. (Busch et al., 1998). Briefly, human β2m and the extracellular domains of H-2K^d fused with a peptide containing a specific biotinylation site were expressed as recombinant proteins in *E. coli*. Proteins were purified, dissolved in 8 M urea, and diluted in a refolding buffer containing the mycobacterial hsp60$_{499\text{-}508}$ peptide to generate monomeric soluble MHC class I-peptide complexes. These complexes were purified by gel filtration and enzymatically biotinylated using the biotin protein ligase BirA (Avidity, Denver, CO). Free biotin was removed and MHC-peptide complexes were purified by gel filtration. To generate tetrameric MHC-peptide complexes, PE-conjugated streptavidin (*Molecular Probes*) was added to the monomers at a molar ratio of 4:1. Tetramers were purified by gel filtration and stored at 4°C. For flow cytometry analysis, 1 x 10^6 cells were incubated for 15 min at 4°C with polyclonal rat Abs, anti-CD16/CD32 mAb, and streptavidin (*Molecular Probes*) in PBS containing 0.5% BSA and 0.01% sodium azide to block unspecific binding of mAb and tetramer. After incubation, cells were stained for 60 min at 4°C with Cy5-conjugated anti-CD8α mAb, FITC-conjugated anti-CD62L mAb, and PE-conjugated MHC class I-hsp60$_{499\text{-}508}$ tetramers. Subsequently, cells were washed with PBS containing 0.5% BSA and 0.01% sodium azide, and resuspended in PBS. Propidium iodide was added before 4-color flow cytometry analysis. The optimal amount of PE-conjugated MHC class I- hsp60$_{499\text{-}508}$ tetramers to stain 10^6 cells was titrated using the CD8+ T cell clone UZ3/4 (**Figure 9**).

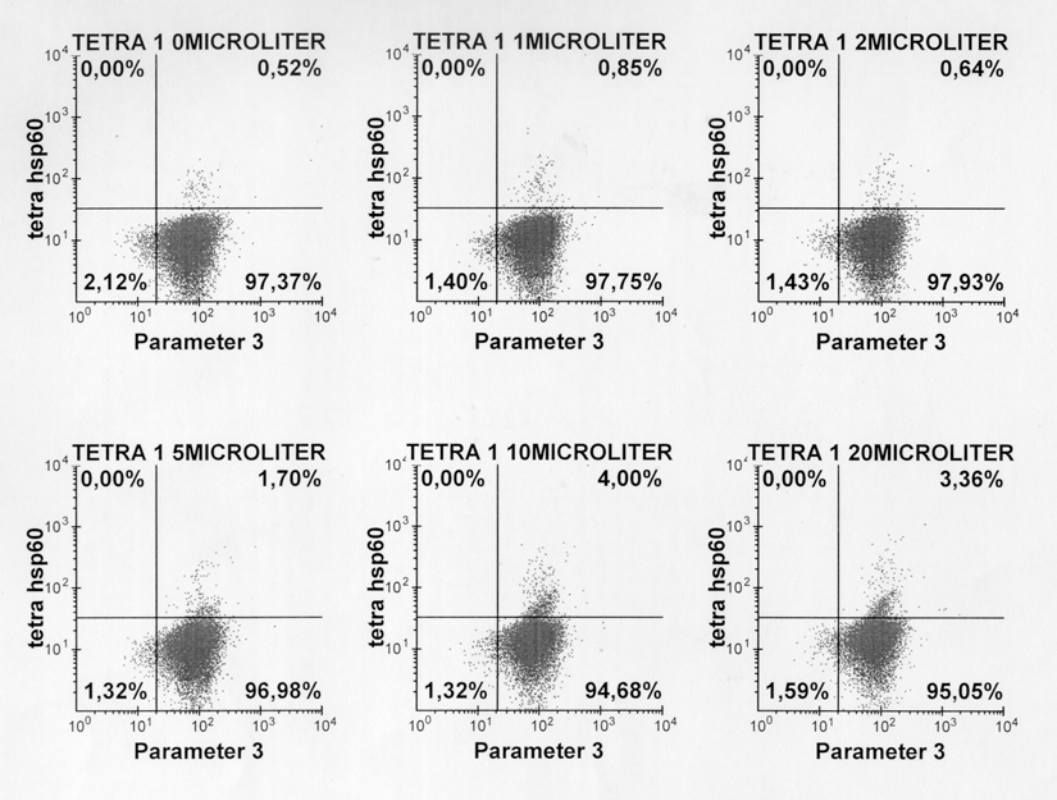

Figure 9. Titration of PE-conjugated MHC class I- hsp60$_{499\text{-}508}$ tetramers with the clone UZ3/4. The percentage of tetramer positive cells is given in the upper right quadrant. Best results were obtained with 10µl of tetramer solution per sample. Staining with unspecific control tetramers gave no positive cells (not shown).

5.6.3 ^{51} Cr-release assay

Cytolytic activities of hsp60-specific T cells were measured in a ^{51}Cr release assay using EL 4 cells as targets as previously described (Zugel et al., 1995). Cloned T cells were incubated with 2x10^3 ^{51}Cr-labeled EL4 cells in the presence or absence of synthetic peptides of hsp60 for 4 hr at 37°C in 5% CO_2 at various effector to target ratios. After 4hrs, 100µl of the supernatant were removed and measured in a gamma counter. Percent specific lysis was calculated as follows: (Experimental ^{51}Cr-release – spontaneous ^{51}Cr-release) x 100 / (Maximum ^{51}Cr-release – spontaneous ^{51}Cr-release). TCR blocking experiments were performed in the presence of mAb (Table1) directed against TCR-Vα8 (B21.14) and TCR-Vβ8 (M5-2R) at a final concentration of 5µg/ml.

5.6.4 Cell isolation from different tissues

Blood samples were collected in PBS supplemented with 2% FCS, 0.2% NaN_3, 20mM EDTA, and treated with Tris-buffered ammonium chloride to lyse red blood cells. Prior to staining, cells were washed twice with PBS. Spleens and lymph nodes were removed and single-cell suspensions were prepared using an iron mash sieve. In spleen preparations red blood cells were lysed and cells were washed twice with a 1:1 mixture of complete RPMI and PBS. Intraepithelial lymphocytes (IEL) from small and large intestine were isolated as previously described (Steinhoff et al., 1999) with some modifications. Briefly, after excision of the Peyer's patches, large intestine and small intestine of mice were cut open and washed twice in PBS, 1% BSA. Intestines were stirred at 37°C for 20 min in complete RPMI medium, and then washed twice by shaking in complete RPMI medium for 0.5 min. Supernatants were filtered through a 70 µm nylon sieve and centrifuged to pellet the cells. Cells were resuspended and centrifuged through a 40%/70% Percoll gradient (*Biochrom*) for 30 min at 600 x g. Liver lymphocytes were obtained by perfusion of the liver with PBS through the vena hepatica, removed and homogenized using an iron mash sieve. Cell suspensions were washed with PBS, centrifuged for 1 min at 50 x g and the supernatants were collected. This step was repeated 4 times. Cells from pooled supernatants were further purified by a 40%/70% Percoll gradient. IEL and liver lymphocytes were collected at the interface of the gradient and washed in complete RPMI medium.

5.6.5 CD69 upregulation assay

TCR Vα8/ Vβ8 transduced 54ζ17 cells ($1x10^5$) were sorted for TCR expression and cocultured with spleen cells ($1x10^6$) from C57BL/6 mice in the presence or absence of mycobacterial hsp60 peptides (SALQNAASIA, $AA_{499-508}$) or murine hsp60 (KDIGNIISDA, $AA_{162-171}$) at concentrations ranging from 2 to 500µM. After 20h, the fraction of GFP expressing CD69 positive cells was determined by FACS analysis using PE-conjugated anti-CD69 antibody (Table 1).

5.7 Histology.

Organs were snapfrozen in liquid nitrogen and subsequently kept at -70°C. Cryostat frozen sections were cut at 5 µm, air dried, fixed in acetone for 10 min and stained with hematoxylin and eosin. Immunohistology was performed as follows: Cryostat sections were preincubated with normal goat serum and then incubated with the primary mAb for 60 min. For primary incubation, anti-CD4 and anti-TCRγ/δ mAb (Table 1) were applied. Secondary Ab incubation was performed with goat anti rat Ab and goat anti Armenian hamster Ab for 40 min, respectively. Alkaline peroxidase (AP) conjugated donkey anti goat was added for 30 min (secondary and tertiary Ab from *Dianova*). Then sections were stained for 10 min with magenta III (*Merck*). Tris-buffered saline (TBS) was used for washing steps between incubations. Finally, sections were counterstained with hematoxylin. Samples incubated with isotype-matched control mAb and without primary mAb served as negative controls.