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$CD8^+$ T cells armed with retrovirally transduced IFN-γ

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Abstract

Interferon (IFN)-gamma is considered a key cytokine involved in the preventive and defensive responses of T cells against infectious pathogens and tumors. Therefore, transgenic expression of IFN-γ in specific T cells appears to be an obvious therapeutic possibility. To directly examine whether IFN-γ production can be increased in T cells, we introduced an IFN-γ encoding cDNA into IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ $CD8^+$ effector populations by retroviral transduction. Here we show that CD8 T cells can be equipped with IFN-γ that increases their capacity to secrete the cytokine. Despite constitutive retroviral IFN-γ mRNA transcription, translation and secretion of IFN-γ protein was tightly regulated and only observed in activated T cells. Neither proliferation nor cytolytic activity of CTL were affected by IFN-γ transduction. Importantly, $CD8^+$ T cells retrovirally transduced with IFN-γ exhibit augmented tumor suppressive capacity upon adoptive transfer into IFN-$\gamma^{-/-}$ mice. Thus, T cells can be readily armed with IFN-γ without risking immunopathology by dysregulated production of this highly potent proinflammatory cytokine.

Introduction

Antigen-specific $CD8^+$ T cells are protective against infectious pathogens and tumors by two mechanisms: lysis of infected cells or tumor cells and secretion of cytokines. Among the cytokines produced by activated $CD8^+$ T cells, IFN-γ seems to be of particular importance. Only IFN-γ producing CD8 T cells are able to clear certain viral infections (1-3) and viral clearance is observed before significant cytotoxicity can be demonstrated (1,2). Similarly, in murine experimental tumor systems protective immunity depends on a population of tumor-specific IFN-γ producing CD8 T cells (4-6) and, in tumor patients, $CD8^+$ T cells with tumor rejection potential reside in the fraction of IFN-γ producing $CD8^+$ T lymphocytes (7,8). In addition, IFN-γ deficient mice and IFN-γ receptor deficient mice show impaired T cell dependent immunity against certain infections (3) and tumors (9,10). In the absence of IFN-γ, T cells are not able to clear viral infection (11) and fail to enter the tumor tissue (9). These and other data imply that IFN-γ secretion is a critical effector mechanism of $CD8^+$ T cells *in vivo*. Hence, transgenic expression of IFN-γ in specific T cells appears to be an obvious therapeutic possibility.

Here we show that $CD8^+$ T cells can be readily equipped with IFN-γ by retroviral infection, increasing their IFN-γ secreting capacity and augmenting their therapeutic function.

Material and methods

Mice

Inbred BALB/c mice were obtained from Harlan Olac (Bicester, UK) and BALB/c IFN-γ-knock-out mice were bred and maintained under standard housing conditions in the animal facility of the GBF.

The Clone-4 (CL4) TcR transgenic mouse line expresses an H-2K^d restricted TcR (Vα10, Vβ8.2) against a transmembrane epitope of hemagglutinin (HA) from influenza virus comprising amino acids 512-520 (IYSTVASSL) of the strain A/PR/8/34 (12). The transgenic line has been backcrossed over more than 12 generations onto the BALB/c background and was bred in the GBF animal facility. All mice used in the experiments were sex and age matched.

T cell activation and phenotype differentiation

Splenocytes from CL4TcR-transgenic mice were activated by influenza virus hemagglutinin peptide 512-520 (0,3µg/ml) at 3x10^6 cells/ml in IMDM. Splenocytes from IFN-γ knock-out mice were activated with plate-bound anti-TcR mAb (2C11) and anti-CD28 (37.51) (1 and 4 µg/ml respectively, both Pharmingen) for 48 hrs. Cells were collected, washed and transferred into medium containing 10 U/ml rhIL-2 at a cell density of 2x10^5 cells/ml. To obtain Tc1 and Tc2 cells, T cell cultures were supplemented with IL-12 (10ng/ml) and anti IL-4 mAb (10µg/ml), or IL-4 (20ng /ml), anti-IL-12 (10µg/ml) and anti-IFN-γ (10µg/ml) mAb respectively (cytokines: R&D, antibodies: Pharmingen).

T cells from CL4TcR-transgenic mice were harvested on day 8 after primary activation and purified by cell sorting. At this time point more than 98% of the CD8$^+$ T cells expressed the transgenic T cell receptor as determined by staining with H-2K^d/HA 512-520 tetramers (a kind gift from Dr. D. Bush, LMU, Munich, Germany). For passaging T cells, sorted cells from cultures were stimulated with HA peptide (0,3 µg/ml) at 1,25x10^5 cells/ml with

irradiated BALB/c splenocytes (2,000 rad) as stimulators. Alternatively, sorted cells were expanded by polyclonal activation using anti-CD3-mAb (2C11) and anti-CD28 (37.51) coated plates. Polyclonally activated cells were collected after 24hrs, washed and expanded in fresh medium containing 1ng/ml rhIL-2 and 5ng/ml rmIL-15. For analysis of IL-12/IL-18- induced IFN-γ production, sorted populations were incubated at a density of $2x10^5$ cells per well in a 96 well plate with 10U/ml rmIL-12 and 10ng/ml rmIL-18 (both R&D).

Retroviral construct and retroviral transduction.

The IFN-γ coding sequence was amplified by RT-PCR from the transfectant X63/BCMG NEO IFNγ (kindly provided by Dr. Karasuyama, Tokyo, Japan) using the forward primer GCGCGCACTAGTGCCACCATGAACGCTACACACTGC and the reverse primer ATAAGAGCGGCCGCTCAGCAGCGACTCCTTTTCCGC and cloned into the pBluescript via *Spe*I/*Not*I resulting in the plasmid pBS-IFNγ. The IFN-γ coding fragment was excised from pBS-IFNγ using *Bam*HI and *Not*I. This fragment was cloned into a *Xho*I digested, blunt ended and subsequently *Bgl*II digested GFP-RV retroviral vector containing an IRES element for bi-cistronic expression (a kind gift from Dr. Kenneth Murphy, Howard Hughes Medical Institute, MD, USA) resulting in IFN/GFP-RV. Thus, the final vector contains the coding sequence for IFN-γ without untranslated regions of the gene followed by an IRES and the GFP coding sequence. Integrity and function were confirmed by enzymatic digestion of the vector and analyzing supernatants from plasmid-transfected 3T3 fibroblasts by IFN-γ ELISA.

Phoenix-Eco packaging cells (Nolan/Stanford CA, purchased through ATCC) were transfected according to Dr. Nolan's protocol. Primary T cells were activated as described above and infected after 48 hrs using 1 volume of viral supernatant and polybrene (6µg/ml, Sigma), centrifuged at 1,800 rpm for 45 min at room temperature and incubated at 37°C for 48 hrs, before being supplied with fresh media and expanded until day 8 after primary activation.

RT-PCR

Transcription of the retroviral DNA was analyzed using RT-PCR. Retrovirally infected, $CD8^+/GFP^+$-sorted CL4 TcR Tc1 cells were either stimulated for 24 hrs with plate-bound anti-CD3 antibodies or left unstimulated. RNA extraction and reverse transcription was carried out using standard procedures. Primers used for PCR were:

GAPDH: ATCTTCTTGTGCAGTGCCAGG (forward), ACTCCACGACATACTCAGCACC (reverse); total IFN-γ: AGTGGCATAGATGTGGAAGAAAAGAGTCTCTTCTTG forward), GGCGCTGGACCTGTGGGTTG (reverse); retroviral IFN-γ: CGTCATTGAATCACACCTG (forward), GAACTTCAGGGTCAGCTT (reverse, binding to GFP); endogenous IFN-γ: AGTTCTGGGCTTCTCCTCCT (forward), GTCACCATCCTTTTGCCAGT (reverse).

Intracellular IFN-γ staining

For intracellular staining of IFN-γ, cells were fixed for 20 minutes at room temperature in PBS 1% paraformaldehyde, followed by incubation in 0,1% NP40 in PBS for 3 minutes on ice. After washing, cells were incubated with PE-conjugated anti-IFN-γ mAb or the appropriate PE-conjugated isotype control (both Pharmingen) for 30 minutes on ice. Cells were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Cytotoxicity assay

The cytotoxic activity of CL4TcR CTL was determined using the JAM assay (13). Briefly, 5,000 [^{3}H]-thymidine-labeled P815 were incubated in 96-well U-bottom plates with or without 1µg/ml HA peptide 512-520 for 1h. CL4TcR effector T cells were added at different ratios and the plates were incubated for a further 4hrs. Percent cytotoxicity was calculated from the mean of triplicate wells.

HA tumors and tumor cell injections

Full-length HA cDNA from the Mt. Sinai strain of the PR8 influenza virus was subcloned into the pCDNA3 expression vector. The BALB/c colon carcinoma CT26 was transfected with

this vector using standard methodology. CT26-HA were selected and grown in complete medium supplemented with G418 (500µg/ml). The level of HA expression on transfected cells was determined by flow cytometry analysis, using the FITC-conjugated HA-specific monoclonal antibody H36-4-5 (14).

BALB/c IFN-γ knock-out (KO) mice, 8-10 weeks of age, were inoculated s.c. with 1 x 10^5 CT26-HA transfectants with high HA-expression and monitored for tumor growth every day. Tumor size was measured in two dimensions and given as the mean diameter. Explants of CT26-HA, obtained after 20 days of tumor progression, demonstrated that expression of HA was maintained *in vivo* as determined by staining with the antibody H36-4-5 against HA (data not shown).

Results

$CD8^+$ T cells can be retrovirally equipped with IFN-γ

To test whether IFN-γ production can be increased in T cells by ectopic expression of the IFN-γ gene, we retrovirally introduced an IFN-γ encoding cDNA into $CD8^+$ T cells. A bi-cistronic retroviral vector was used to co-express murine IFN-γ together with GFP under the control of the MSCV LTR (IFN/GFP-RV; Fig. 1A). GFP-RV (Fig. 1A) served as negative control for these experiments. Using GFP as a reporter gene, transduced $CD8^+$ T cells from transgenic CL4 mice were routinely enriched by cell sorting to purities greater than 95% (Fig. 1B and C).

First, we determined whether IFN-γ can be expressed in $CD8^+$ T cells via retroviral transduction. Therefore, we infected $CD8^+$ T cells from IFN-γ knock-out mice after anti-CD3 stimulation. Sorted GFP^+ $CD8^+$ T cells infected with IFN/GFP-RV (Fig.2A), but not control-vector-infected $CD8^+$ T cells, secreted IFN-γ after restimulation with anti-CD3. When comparing transduction efficiency under Tc1 or Tc2 culture conditions, IFN-γ secretion was found to occur under both priming conditions, although TcR-activated Tc2 cells secreted less recombinant IFN-γ per cell than Tc1 cells did(Fig. 2A). Interestingly, no IFN-γ production could be observed in resting T cells (Fig. 2A), although the IFN-γ encoding cDNA was under the control of the viral LTR and the IFN/GFP-RV retrovirus did not contain any upstream regulatory elements of the IFN-γ gene (15,16). Pertinent to this observation, fibroblasts constitutively secreted the cytokine upon infection with IFN/GFP-RV virus (Fig. 2B).

When IFN-γ competent $CD8^+$ T cells were transduced with IFN/GFP-RV, an increase in TcR-induced IFN-γ secretion was observed compared to the levels produced by GFP-RV infected control $CD8^+$ T cells (Fig. 2C) or GFP^- cells from IFN/GFP-RV-infected cultures (not shown). Similar data were obtained when IFN/GFP-RV-infected $CD8^+$ T cells were

stimulated with stimulator cells and peptide instead of anti-CD3 (Fig. 2D). Together these data demonstrate that IFN-γ can be enhanced in $CD8^+$ T cells by retroviral transduction. However, despite retroviral LTR control of the cDNA, IFN-γ secretion depends on activation of the T cells.

Constitutive transcription of retroviral IFN-γ in resting T cells transduced with IFN/GFP-RV

It was unexpected that the secretion of retrovirally derived IFN-γ driven by the viral LTR was dependent on activation of the transduced T cells. This regulation could be either on a transcriptional or translational level. Since only the IFN-γ coding sequence was introduced into the vector and the reporter protein GFP, co-encoded by bi-cistronic mRNA, was found to be expressed, it was unlikely that regulation was on the transcriptional level. This was confirmed by RT-PCR on resting transduced T cells. Using primers that distinguished the virally derived IFN-γ specific mRNA from the endogenous transcript, we could show that significant amounts of IFN-γ encoding mRNA were present in resting T cells transduced with IFN/GFP-RV (Fig. 3A). This mRNA could not be detected in T cells transduced with the GFP-RV control vector (Fig. 3B). The amount of virally derived IFN-γ mRNA did not change upon stimulation of the T cells, although mRNA encoding endogenous IFN-γ became readily detectable under these conditions.

Despite the presence of functional mRNA no IFN-γ protein could be detected by intracellular staining in resting T cells transfected with IFN/GFP-RV (Fig. 3C). In contrast, when such T cells were stimulated with plate-bound anti-CD3, strong IFN-γ production could be detected intracellularly in most of the GFP^+ T cells. Thus, the absence of production of recombinant IFN-γ by resting T cells is not simply a block in secretion. Rather, our results suggest a T cell specific regulation of IFN-γ production at the post-transcriptional or post-translational level.

Influence of Tc1 and Tc2 stimulating conditions on retroviral IFN-γ expression

To analyze the influence of cytokines on IFN-γ production by IFN-γ transduced $CD8^+$ T cells, we infected CL4-TcR-transgenic T cells with IFN/GFP-RV under Tc1 or Tc2 inducing conditions. IFN-γ and IL-4-secretion of sorted GFP^-CD8^+ and GFP^+CD8^+ T cells was analyzed subsequent to antigenic expansion of the sorted populations. Transduction of IFN-γ highly augmented TcR-induced IFN-γ secretion in Tc1, and significantly in Tc2 CL4-TcR $CD8^+$ T cells, after 8 and 16 days of expansion, respectively (Fig. 4). Under Tc2 conditions, IFN-γ transduction partially repressed IL-4 production in IFN/GFP-RV transduced cells (Fig. 4). This is likely due to a feedback loop of IFN-γ acting on the IL-4 promotor via IRF-1 and IRF-2 as previously described for human $CD4^+$ T cells (17). Uninfected cells from the same culture (GFP^-) were not affected, suggesting that a short exposure to IFN-γ present in the viral supernatant during infection does not alter IL-4 production by $CD8^+$ Tc2 cells. As observed by others (18,19), Tc2 cells remain capable of secreting IFN-γ upon stimulation with antibodies against the T cell receptor. Together these results demonstrate that IFN-γ transduction enforces IFN-γ production in $CD8^+$ T cells even under Tc2 conditions.

Enhanced IFN-γ production in IFN-γ transduced $CD8^+$ T cells is stable

To examine whether or not enhanced IFN-γ production in IFN/GFP-RV transduced $CD8^+$ T cells is transient or stable, transduced $CD8^+$ T cells were expanded *in vitro* by restimulation on stimulator cells plus peptide under Tc1 inducing conditions. Already after the first round of restimulation, such T cells produced IFN-γ and T cells transduced with IFN/GFP-RV exhibited increased production of IFN-γ (Fig. 5). This property was maintained over several rounds of restimulation. No influence on the viability and growth were observed throughout a 3-4 week period of cell culture (not shown). Thus, IFN/GFP-RV transduction stably augments TcR-regulated IFN-γ secretion in IFN-γ sufficient Tc1 polarized cells during expansion and

differentiation.

Retroviral IFN-γ transduction increases IL-12/IL-18-induced IFN-γ secretion

At least two receptor-mediated pathways can induce IFN-γ secretion in T cells. Besides a TcR-dependent pathway, an alternative antigen-independent pathway for IFN-γ secretion is implemented by upregulation of both IL-12 receptor and IL-18 receptor expression (20). Triggering via the two cytokine receptors also results in IFN-γ production. We therefore analyzed IFN-γ production of transgenic $CD8^+$ T cells transduced with GFP-RV or IFN/GFP-RV in the presence of IL-12 and IL-18. As shown in Fig. 6, stimulation with these cytokines resulted in a strong increase in IFN-γ secretion by IFN/GFP-RV infected Tc1 cells as compared to GFP^- cells from the same culture (uninfected internal control) or GFP^+ cells from GFP-RV infected T cells (vector control). Thus, retroviral IFN-γ expression enhanced the antigen independent IFN-γ secretion in $CD8^+$ T cells.

Retroviral IFN-γ transduction does not alter specific cytolytic activity

An influence of IFN-γ on the homeostasis of T cells has been shown before (21), although mature $CD8^+$ T cells seem to be insensitive to IFN-γ due to reduced IFN-γR2 chain expression (22). No significant difference in cytolytic activity could be discerned between GFP and IFN/GFP-RV transduced CL4 T cells. When the cytolytic activity of T cells sorted for $GFP^+/CD8^+$ was compared at day 8 or day 16 after retroviral infection, IFN/GFP-RV infected cells exhibited only a slightly higher unspecific cytolytic activity than vector controls (Fig. 7A). Similar results were obtained with GFP and IFN/GFP-RV transduced CL4 T cells additionally expanded for ten days in culture (data not shown). Thus, IFN/GFP-RV transduction does not significantly affect the induction of a fully competent cytolytic response.

Tumor protective potential of CTL retrovirally transduced with IFN-γ

Adoptive transfer of IFN/GFP-RV transduced CL4-Tc1 cells into syngeneic mice that have received a challenge with HA expressing CT26 tumor cells, enabled us to evaluate the anti-tumor potential of such T cells *in vivo*. We compared two $CD8^+$ T cell populations that only differed in their capacity to produce IFN-γ. Control mice received no T cells at all, or CL4 Tc1 cells that had been infected with the GFP-RV control vector. Adoptive transfers were carried out in mice deficient for IFN-γ production to exclude contribution of IFN-γ by host cells.

Under these circumstances, IFN/GFP-RV transduced CL4 T cells exhibited improved therapeutic potency compared to GFP-RV-infected Tc1 cells (Fig. 7B). Thus, under limiting conditions, IFN-γ production driven by retrovirus transduction is able to improve the therapeutic function of $CD8^+$ T cells.

Discussion

This study makes several noteworthy observations. First, we show that antigen specific $CD8^+$ T cells can readily be armed with IFN-γ by retroviral transduction. Interestingly, a tight regulation of IFN-γ production in $CD8^+$ T cell was observed and only activated T cells were able to secrete the cytokine. In contrast, fibroblasts constitutively produced the cytokine upon infection with the same retroviral vector, demonstrating that regulation of IFN-γ production is intrinsic for the T cell.

Down regulation of IFN-γ was not due to the inactivation of transcription when the T cells went into quiescence as has been observed by others (23, 24). Virally derived mRNA encoding IFN-γ was unambiguously demonstrated in resting T cells by RT-PCR, and the amount of virally derived specific mRNA was not further enhanced upon T cell stimulation. The controversy might be due to a different retroviral construct used in our work or to our selection of GFP expressing T cells by cell sorting. Thus, we would have selected for cells that constitutively express the LTR driven genes. In favor of this argument is that IFN-γ secretion is resumed upon T cell stimulation but virally derived mRNA is not increased.

This suggests that although on/off cycling of IFN-γ production by T cells is thought to be transcriptionally regulated (25), post-transcriptional mechanisms contribute to the control of IFN-γ production in $CD8^+$ T cells. In agreement with our finding, it was recently shown that primed T helper cells can regulate cytokine secretion by controlling ribosomal loading of cytokine mRNA (26). Since only the coding sequence of IFN-γ was introduced into the retroviral vector, appropriate regulatory motifs should be restricted to this region.

Virally derived IFN-γ mRNA was apparently not translated in resting IFN/GFP-RV transduced $CD8^+$ T cells, although these cells remained GFP^+. Possibly, translation of the

complete bi-cistronic mRNA might be down-regulated and cells might remain GFP^+ due to the longevity of the GFP protein. More likely, however, is that the IRES element sufficiently uncouples the translation of both cistrons to allow independent GFP expression.

Whereas retroviral IFN-γ is down-regulated in resting IFN/GFP-RV $CD8^+$ T cells, it contributes to $CD8^+$ T cell IFN-γ production under T cell stimulating conditions. This is most evident in IFN/GFP-RV transduced $CD8^+$ T cells from IFN-γ deficient mice. In IFN-γ competent $CD8^+$ T cells, IFN/GFP-RV transduction resulted in increased T cell receptor driven, as well as IL-12/IL-18-induced, IFN-γ secretion as compared to GFP-RV transduced T cells. Thus, the antigen dependent, as well as the antigen independent, activation pathway induces translation and/or secretion of recombinant IFN-γ in $CD8^+$ T cells.

Retrovirally derived IFN-γ can be secreted by $CD8^+$ T cells under different polarizing conditions. In such cases, secretion of retrovirally derived IFN-γ can be modulated in a similar way as endogenous IFN-γ is. Thus, Tc1 cells exhibit stronger secretion of recombinant IFN-γ than Tc2 cells, an observation most obvious at day 16 after stimulation. On the other hand, retroviral IFN-γ expression participates in the polarization of $CD8^+$ T cells, as illustrated by a decrease in IL-4 production by transduced Tc2 cells.

IFN-γ plays a key role in the homeostatic control of $CD8^+$ T cells (21). Thus, expression of this cytokine by retroviral transduction might impair T cell function. However, in our hands, infected Tc1 cells that over-expressed IFN-γ did not exhibit a significantly diminished viability. Growth and cytotoxic function of these T cells were also not altered throughout a 3-4 week restimulation period *in vitro*, in spite of stably increased IFN-γ levels. Thus, the control of IFN-γ secretion by physiological signals apparently permits normal T cell functions.

It has been shown that IFN-γ can directly inhibit tumor cell growth (27,27), enhance tumor immunogenicity by increasing tumor antigen presentation (29) and recruit and activate innate anti-tumor responses (30,31). Furthermore, IFN-γ suppresses tumor angiogenesis by inducing angiogenesis-inhibitory chemokines (22) and, most notably, invasion of anti-tumor T cells into the tumor stroma seems to depend on IFN-γ signaling (9). Comparison of IFN-γ deficient $CD8^+$ T cells and/or Tc1 and Tc2 populations (18,19) in different experimental tumor systems suggested that the capacity to secrete large amounts of IFN-γ is the most critical anti-tumor effector mechanism mediated by adoptively transferred $CD8^+$ T cells *in vivo*. In the present study, we show that retrovirally armed $CD8^+$ T cells producing increased amounts of IFN-γ delay the outgrowth of an experimental tumor when transferred into IFN-γ deficient mice. This observation suggest that $CD8^+$ T cells can be readily equipped with IFN-γ to augment their therapeutic function.

One other group has previously transduced murine tumor specific $CD8^+$ T lymphocytes with the IFN-γ gene (30,31). In these studies, a tumor-specific T cell line that did not produce IFN-γ was transduced with a retrovirus encoding a mono-cistronic IFN-γ cDNA. One T cell clone obtained from the IFN-γ transduced cell line was shown to constitutively secrete IFN-γ *in vitro* and displayed enhanced cytolytic activity against a tumor *in vivo*. In contrast, $CD8^+$ T cells transduced with the IFN-γ gene still depended on physiological signals to secrete the cytokine in our experimental setting. This result corroborates the general observation that IFN-γ secretion and cytotoxicity are regulated independently in $CD8^+$ T cells (32). Thus, our study establishes compellingly that $CD8^+$ T cells can be selectively equipped with interferon γ without the risk of immunopathology by deregulated production of this pro-inflammatory cytokine.

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Figure Legends

Figure 1: IFN-γ and GFP expressing retroviral vectors.

A) Retroviral constructs. The retroviral vector containing only GFP (GFP-RV) was used in all experiments to control for the effects of retroviral infection. The IFN-γ expressing retrovirus (IFN/GFP-RV) contains murine IFN-γ placed downstream of the murine stem cell virus (MSCV) long terminal repeat (LTR) and upstream of the internal ribosomal entry site (IRES) from encephalomyocarditis virus. **B**) Infection of CL4 transgenic T lymphocytes. CL4 transgenic T cells were infected on day 2 after primary activation by either control (GFP-RV) or IFN-γ-expressing (IFN/GFP-RV) retrovirus, harvested on day 8, stained with anti-CD8-PE and analyzed by two color flow cytometry. GFP expressing $CD8^+$ T cells were purified by FACSorting. **C**) GFP and CD8 positive T cells that were sorted from the populations described in (B). Data are given as dot blot of live-gated events.

Figure 2: Retroviral expression of IFN-γ in IFN-γ sufficient and deficient $CD8^+$ T cells.

A) Retroviral expression of IFN-γ in IFN-$\gamma^{-/-}$ $CD8^+$ T cells. $CD8^+$ T cells were prepared from BALB/c IFN-$\gamma^{-/-}$ spleen cells and stimulated with plate-bound anti-CD3 and anti-CD28 mAb for 48 hrs under either Tc1 or Tc2 conditions as indicated. At 48 hrs cells were infected with retroviral GFP-RV or IFN/GFP-RV supernatants containing Tc1 or Tc2 cytokines and appropriate mAb in addition to 10 ng/ml rhIL-2. On day 8 cells were harvested, washed and stimulated at $1x10^5$ cells per well in a 96 well plate with 1 µg/ml plate bound anti-CD3 mAb. IFN-γ production was measured by ELISA from supernatants taken after 24 hrs of activation. Dotted line indicates the detection limit of the ELISA. **B**) Constitutive secretion of IFN-γ by transduced fibroblasts. 3T3 fibroblast were infected with GFP-RV or IFN/GFP-RV respectively. $1x10^5$ cells were seeded into wells of a 24-well plate and supernatants taken 24hrs later.

C) Retroviral expression of IFN-γ in $CD8^+$ T cells from CL4 transgenic mice sufficient for IFN-γ. CL4 transgenic primary T cells were activated under Tc1 conditions and infected with GFP-RV or IFN/GFP-RV on day 2. $GFP^+/CD8^+$ T cells were purified by cell sorting on day 8 (as in figure 1) and restimulated at a density of $1x10^5$ cells per well in a 96 well plate using 1 µg/ml plate bound anti-CD3 mAb. IFN-γ production was measured by ELISA from supernatants taken after 24 hrs of activation. **D)** Same cells as under C) were restimulated with irradiated feeder cells and 1µg/ml peptide. Data are representative for at least three independent experiments. Note that the scales are different in the three panels.

Figure 3: Constitutive expression of retroviral IFN-γ mRNA in resting T cells.

CL4 transgenic primary T cells were activated under Tc1 conditions and infected with GFP-RV or IFN/GFP-RV on day 2. $GFP^+/CD8^+$ T cells were purified by cell sorting on day 8 and mRNA was prepared from $2x10^6$ $GFP^+/CD8^+$ T cells either after stimulation for 24hrs with plate bound anti-CD3 antibody or from cells that were left unstimulated. IFN-γ mRNA was analyzed by semiquantitative RT-PCR. To differentiate the origin of the IFN-γ message, primers were used that bind either to the 5'-UTR of the mRNA of endogenous IFN-γ or the message derived from the viral vector. Total IFN-γ was analyzed using primers located inside the IFN-γ encoding region of the mRNA. Data are representative of three independent experiments. Amplification of GAPDH encoding mRNA was taken to control the amount of cDNA employed.

A) resting or activated $CD8^+$ T cells transduced with IFN/GFP-RV, **B**) resting or activated $CD8^+$ T cells transduced with GFP-RV control vector**. C)** Intracellular staining of transduced T cells for IFN-γ. No IFN-γ could be revealed in resting T cells whereas roughly 70% of such GFP^+ T cells became positive for IFN-γ after stimulation with plate bound anti-CD3

Figure 4: IFN-γ and IL-4 production by transduced Tc1 and Tc2 cells.

CL4 transgenic primary T cells were activated under Tc1 or Tc2 conditions and infected by IFN/GFP-RV on day 2. $GFP^-/CD8^+$ T cells (open bars) and $GFP^+/CD8^+$ T cells (filled bars) were purified by cell sorting on day 8, and a fraction of the sorted populations was restimulated at a density of $2x10^5$ cells per well in a 96 well plate with 1 µg/ml plate bound anti-CD3 mAb. Supernatants were harvested after 24 hrs. The remaining population of sorted cells were stimulated at $5x10^5$ per well in a 24 well plate with 1µg/ml HA-peptide and irradiated BALB/c splenocytes as APCs. 8 days later $2x10^5$ $CD8^+$ T cells from the expanded populations were stimulated with anti-CD3 mAb and supernatants collected after 24 hrs as above. IFN-γ and IL-4 content of the supernatants was determined by ELISA. (n.d. = not detectable). Dotted lines indicate the detection limit of the ELISA. Data are representative for at least three independent experiments.

Figure 5: IFN/GFP-RV transduction results in stably elevated levels of IFN-γ.

CL4 transgenic primary T cells were activated under Tc1 conditions and infected with GFP-RV or IFN/GFP-RV on day 2. $GFP^+/CD8^+$ T cells were purified by cell sorting on day 8 as described in Figure 1 and cells were restimulated with stimulator cells and peptide once per week for two consecutive rounds. **A**) GFP expression of parallel GFP-RV and IFN/GFP-RV infected CL4 $CD8^+$ T cells at day 25 of culture. T cells were stained with PE-conjugated anti-Vβ 8.1/8.2 antibody to identify cells that express the transgenic Vβ 8.2 TcR β-chain of CL4 T cells and analyzed by flow cytometry. **B**) Intracellular IFN-γ staining of the same T cells described in A). Fractions of the cultures were stimulated with stimulator cells and peptide for 4 hrs in presence of 2 µg/ml Brefeldin A. Subsequently, cells were stained for intracellular IFN-γ and analyzed by flow cytometry gating on GFP-expressing cells. **C**) IFN-γ production

of the cultures was measured by specific ELISA. By restimulating $2x10^5$ cells from the cultures described in (A) or (B) with plate-bound anti-CD3 mAb.

Figure 6: Retroviral expression of IFN-γ enhances IL-12/IL-18-induced IFN-γ production by $CD8^+$ T cells.

CL4 transgenic primary T cells were activated under Tc1 conditions and infected by GFP-RV or IFN/GFP-RV on day 2. $GFP^+/CD8^+$ T cells from the GFP-RV infected culture as well as $GFP^-/CD8^+$ T cells and $GFP^+/CD8^+$ T cells from the IFN/GFP-RV infected culture were purified by cell sorting on day 8 and a fraction of the sorted populations was restimulated at a density of $2x10^5$ cells per well in a 96 well plate with 10 U/ml IL-12 and 10 ng/ml IL-18. Supernatants were harvested after 24 hrs and IFN-γ content of the supernatants was determined by ELISA. Addition of either cytokine alone was not sufficient to induce secretion of IFN-γ (data not shown). The dotted line indicates the detection limit of the ELISA. Data are representative for at least three independent experiments.

Figure 7: IFN-γ transduction does not affect specific cytolytic activity but enhances tumor immunity upon adoptive transfer.

A) CL4TcR-transgenic primary T cells were activated under Tc1 and infected by GFP-RV (squares) or IFN/GFP-RV (circles) on day 2. $GFP^+/CD8^+$ T cells were purified by cell sorting on day 8 as described in Figure 1 and incubated with 5,000 [^{3}H]-thymidine-labeled P815 cells in 96-well U-bottom plates with (circles) or without (squares) 1µg/ml HA peptide 512-520 for

1h. Sorted CL4 effector T cells were added at different ratios and plates incubated for 4 hrs. Percent cytotoxicity was calculated from the mean of triplicate wells as described.

B) IFN-γ deficient mice were injected s.c. with 1 x 10^5 CT26-HA cells. On the same day mice received $3x10^6$ GFP-RV infected (●-●), IFN/GFP-RV-infected (○-○), CL4 Tc1 cells i.v. or were left untreated (■-■). Shown is the mean tumor diameter after tumor cell injection.

p-values (**Student's t-test**) are shown for mice that received GFP-RV infected T cells versus IFN/GFP-RV-infected T cells. P-values: ✱ < 0.05, ✱✱ < 0.005.

References

1 **Guidotti, L. G., P. Borrow, A. Brown, H. McClary, R. Koch, and F. V. Chisari. 1999. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. *J. Exp. Med.* 189:1555.**

2. **Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari. 1999. Viral clearance without destruction of infected cells during acute HBV infection. *Science* 284:825.**

3 **Bartholdy, C., J. P. Christensen, D. Wodarz, and A. R. Thomsen. 2000. Persistent virus infection despite chronic cytotoxic T-lymphocyte activation in gamma interferon-deficient mice infected with lymphocytic choriomeningitis virus. *J. Virol.* 74:10304.**

4 **Barth RJ Jr, Mule JJ, Spiess PJ, Rosenberg SA.1991. Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8+ tumor-infiltrating lymphocytes. *J Exp Med* 173:647**

5 **Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD.1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A* 95:7556.**

6 **Qin, Z., J. Schwartzkopff, F. Pradera, T. Kammertoens, B. Seliger, H. Pircher, and T. Blankenstein. 2003. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res.* 63:4095.**

7 **Schwartzentruber DJ, Topalian SL, Mancini M, Rosenberg SA. 1991. Specific release of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-alpha, and IFN-gamma by human tumor-infiltrating lymphocytes after autologous tumor stimulation. *J Immunol.* 146:3674.**

8 **Becker C, Pohla H, Frankenberger B, Schuler T, Assenmacher M, Schendel DJ, Blankenstein T. 2001. Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. *Nat Med.* 7:1159.**

9 **Nakajima C, Uekusa Y, Iwasaki M, Yamaguchi N, Mukai T, Gao P, Tomura M, Ono S, Tsujimura T, Fujiwara H, Hamaoka T.2001. A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. *Cancer Res* 61:3399.**

10 **Wilcox RA, Flies DB, Wang H, Tamada K, Johnson AJ, Pease LR, Rodriguez M, Guo Y, Chen. L.2002. Impaired infiltration of tumor-specific cytolytic T cells in the absence of interferon-gamma despite their normal maturation in lymphoid organs during CD137 monoclonal antibody therapy. *Cancer Res* 62:4413.**

11 Von Herrath, M. G., B. Coon, and M. B. Oldstone. 1997. Low-affinity cytotoxic T-lymphocytes require IFN-gamma to clear an acute viral infection. *Virology* 229:349.

12 Morgan DJ, Liblau R, Scott B, Fleck S, McDevitt HO, Sarvetnick N, Lo D, Sherman LA. 1996. CD8(+) T cell-mediated spontaneous diabetes in neonatal mice. *J Immunol.* 157:978.

13 Matzinger, P. 1991. The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods* 145:185.

14 Staudt LM, Gerhard W. 1983. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. I. Significant variation in repertoire expression between individual mice. *J Exp Med.* 157:687.

15 Young, H. A. 1996. Regulation of interferon-gamma gene expression. *J. Interferon Cytokine Res.* 16:563.

16 Ben-Asouli, Y., Y. Banai, Y. Pel-Or, A. Shir, and R. Kaempfer. 2002. Human interferon-gamma mRNA autoregulates its translation through a pseudoknot that activates the interferon-inducible protein kinase PKR. *Cell* 108:221.

17 Elser B, Lohoff M, Kock S, Giaisi M, Kirchhoff S, Krammer PH, Li-Weber M.2002. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. *Immunity.* 17:703.

18 Dobrzanski MJ, Reome JB, Dutton RW. 1999. Therapeutic effects of tumor-reactive type 1 and type 2 CD8+ T cell subpopulations in established pulmonary metastases. *J Immunol.* 162:6671.

19 Kemp RA, Ronchese F. 2001. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. *J Immunol.* 167:6497.

20 Yang J, Zhu H, Murphy TL, Ouyang W, Murphy KM. 2001. IL-18-stimulated GADD45 beta required in cytokine-induced, but not TCR-induced, IFN-gamma production. *Nat Immunol.* 2:157.

21 Badovinac, V.P., A.R. Tvinnereim, and J.T. Harty. 2000. Regulation of antigen-specific $CD8^+$ T cell homeostasis by perforin and interferon-gamma. *Science.* 290:1354–1358

22 Tau GZ, Cowan SN, Weisburg J, Braunstein NS, Rothman PB. 2001. Regulation of IFN-gamma signaling is essential for the cytotoxic activity of CD8(+) T cells. *J Immunol.* 167:5574.

23 Bestor TH. 2000. Gene silencing as a threat to the success of gene therapy. *J Clin Invest.* 105:409

24 Cooper, IJ, Topp, MS, Pinzon, C, Plavec, I, Jensen, MC, Riddell, SR, Greenberg, PD. 2004. Enhanced transgene expression in quiescent and activated human CD8+ T cells.*Hum Gene Ther.* 7:648.

25 Slifka MK, Rodriguez F, Whitton JL. 1999. Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. *Nature.* 401:76.

26 Scheu, S., Stetson, D.B., Reinhardt, R.L., Leber, J.H., Mohrs, M., Locksley, R.M. 2006. Activation of the integrated stress response during T helper cell differentiation. *Nat Immunol.* 7:644.

27 Arenberg DA, Kunkel SL, Polverini PJ, Morris SB, Burdick MD, Glass MC, Taub DT, Iannettoni MD, Whyte RI, Strieter RM. 1996. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J Exp Med* 184:981.

28 Krasagakis K, Garbe C, Zouboulis CC, Orfanos CE. 1995. Growth control of melanoma cells and melanocytes by cytokines. *Recent Results Cancer Res.* 139:169.

29 Bohm W, Thoma S, Leithauser F, Moller P, Schirmbeck R, Reimann J. 1998. T cell-mediated, IFN-gamma-facilitated rejection of murine B16 melanomas. *J Immunol.*161:897.

30 Nishihara K, Miyatake S, Sakata T, Yamashita J, Kikuchi H, Kawade Y, Zu Y, Namba Y, Hanaoka M, Watanabe Y.1988. Augmentation of tumor targeting in a line of glioma-specific mouse cytotoxic T-lymphocytes by retroviral expression of mouse gamma-interferon complementary DNA. *Cancer Res* 48:4730.

31 Miyatake S, Nishihara K, Kikuchi H, Yamashita J, Namba Y, Hanaoka M, Watanabe Y.1990. Efficient tumor suppression by glioma-specific murine cytotoxic T lymphocytes transfected with interferon-gamma gene. *J Natl Cancer Inst* 82:217.

32 Snyder, JE, Bowers, WJ, Livingstone, AM, Lee, FE, Federoff, HJ, Mosmann, TR. 2003. Measuring the frequency of mouse and human cytotoxic T cells by the Lysispot assay: independent regulation of cytokine secretion and short-term killing. *Nat Med.* 9:231.