Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation

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Persistence is a hallmark of infection by viruses such as HIV, hepatitis B virus, hepatitis C virus and LCMV. In the case of LCMV, persistence may often be associated with exhaustion of $CD8^+$ T cells. We demonstrate here that persistent antigen suppressed IL-7Rα expression and this correlated with T cell exhaustion and reduced expression of the anti-apoptotic molecule B cell leukemia/lymphoma 2 (Bcl-2). In contrast, exposure to short-lived antigen only temporarily suppressed IL-7Rα expression, failed to induce T cell exhaustion, and primed T cells. Persistent antigen also suppressed IL-7Rα expression on primed T cells and this correlated with exhaustion of a previously stable primed T cell population. These findings suggest that antigen longevity regulates T cell fate.

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Introduction

Infection with viruses that are prone to persist can result in different outcomes. Firstly, in some circumstances complete elimination of the virus is possible, and this is normally associated with increased numbers of stable and "quiescent" memory $CD8^+$ T cells [1]. Secondly, the infection may be largely controlled to low levels, resulting in a state of immune memory involving maintenance of a small number of effector $CD8^+$ T cells [2–4]. This immune surveillance may be stable or, alternatively, fade over-time, allowing re-emergence of the virus [5]. Lastly, infection is initially not controlled,

allowing the systemic persistence of the virus at sufficiently high levels to cause exhaustion of the virus-specific $CD8^+$ T cell population [3, 4, 6]. Exhaustion is preceded by a temporary phase when $CD8^+$ T cells lack lytic mediators such as granzyme B or perforin and exhibit additional functional impairment [7], followed by the eventual physical deletion of virus-specific T cells.

LCMV is a poorly cytopathic RNA-virus which, depending on the strain of the virus and the immune system of the infected host, can model all the possibilities discussed above. Infections by the rapidly replicating LCMV-Docile or LCMV-Clone13 strains usually cause early CTL exhaustion, with those few remaining cells being unable to produce IFN-γ or mediate cytotoxicity [8]. In contrast, LCMV-WE and LCMV-Armstrong, which both have a weaker replication capacity, are rapidly cleared in immune-competent mice largely as a result of efficient $CD8^+$ T cells. LCMV-WE infection of MHC class II deficient mice is initially controlled; however, virus re-emerges after 50–150 days despite a pre-existing initially functional memory $CD8^+$

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T cell population [5]. These observations indicate that the distinct results of CD8^{+} T cell induction and maintenance versus exhaustion closely correlate with virus clearance or persistence. A similar relationship has also been demonstrated for non-viral antigens [9–11].

IL-7 is required for the maintenance of naive and memory CD8^{+} T cells [12], probably as a result of its ability to mediate activation of STAT5 and up-regulation of the anti-apoptotic molecule B cell leukemia/lymphoma 2 (Bcl-2) [13, 14]. A recent study demonstrated that following LCMV infection, a distinct population of CD8^{+} T cells does not express IL-7Rα (CD127) [15]. These cells exhibit effector function but have a short half-life, whereas CD8^{+} T cells expressing high levels of IL-7Rα survive for a long period partly due to high expression of Bcl-2. Here we analyzed the role of IL-7Rα expression on CD8^{+} T cells during persistent or short-term antigen exposure.

Results

Viral persistence suppresses IL-7Rα expression and diminishes the CD8^{+} T cell response

During infection with a high dose (2×10^6 PFU) of LCMV-Docile, the virus cannot be controlled initially and persists [6]. LCMV-specific CD8^{+} T cells are initially primed; however, they lose their function during early infection and have a short lifespan [6, 8]. IL-7 appears to be particularly important for the survival of antigen-experienced CD8^{+} T cells, since expression of the IL-7Rα on the cell surface was found on long-lived "quiescent" or central memory cells but not on short-lived effector CD8^{+} T cells [15, 16].

To investigate the expression of IL-7Rα during the establishment of a persisting viral infection, we infected C57BL/6 mice with 2×10^6 PFU of LCMV-Docile or LCMV-WE. As described earlier, infection with 2×10^6 PFU LCMV-Docile led to virus persistence, whereas infection with 2×10^6 PFU LCMV-WE led to viral clearance (Fig. 1A). After infection with LCMV-WE, T cells specific for the dominant MHC class I-restricted LCMV peptide gp33 (glycoprotein$_{33–41}$; KAVYNFATC) were detected by staining with a tetramer–gp33 (tet-gp33) reagent and contained an IL-7Rα^{hi} population, which is probably responsible for building the memory pool [15]. After infection with 2×10^6 PFU LCMV-Docile, tet-gp33^{+}CD8^{+} T cells were also detectable. However, none of these specific T cells expressed IL-7Rα (Fig. 1B).

We analyzed the ability of these T cells to produce IFN-γ after in vitro re-stimulation for 6 h. The gp-33-specific T cells from mice infected with LCMV-WE were able to produce IFN-γ, whereas LCMV-Docile infection led to a functional anergy of specific CD8^{+} T cells. LCMV nucleoprotein (NP) is available longer after LCMV
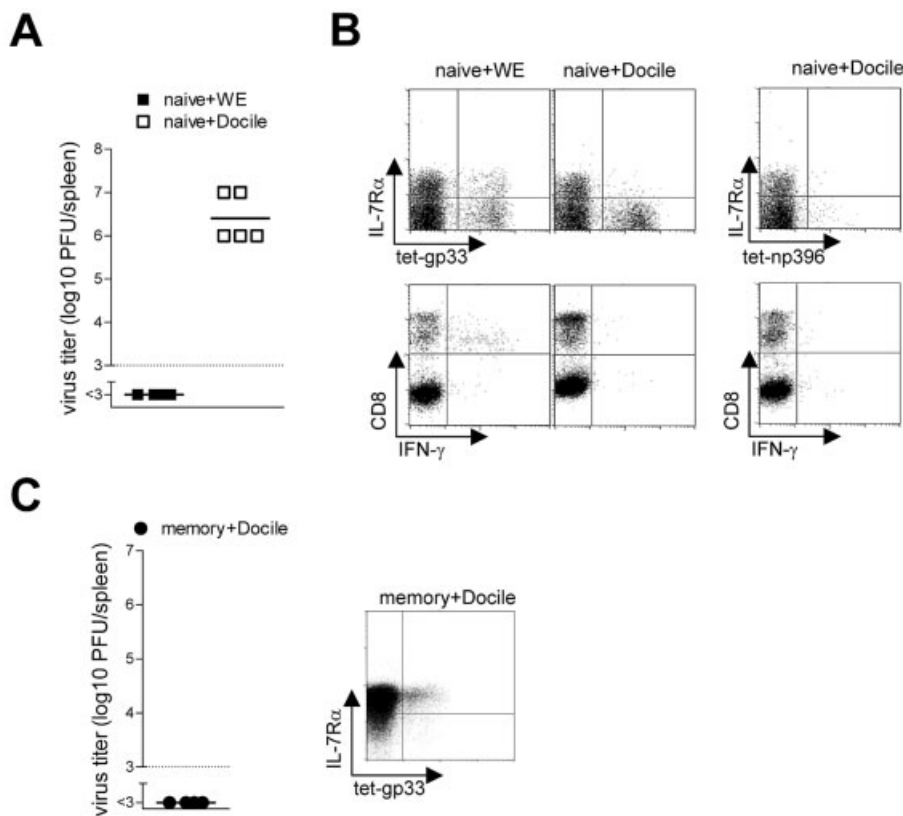
Fig. 1. Viral persistence suppressed IL-7Rα expression and diminished cell survival. Naive mice were infected with 2×10^6 PFU LCMV-WE or -Docile. (A) At day 16 post-infection viral titers were determined in spleens by a plaque assay. (B) At the same time-point CD8^{+} T cells were analyzed for binding to tet-gp33 and tet-np396, and for IL-7Rα expression (gated on CD8^{+} cells, n=4–5). Splenocytes were also re-stimulated in vitro with gp33 (lower left panel) or np396 (lower right panel) and analyzed for the ability to produce IFN-γ. (C) LCMV-memory mice (50 days post-infection with 200 PFU LCMV-WE) were challenged with 2×10^6 PFU LCMV-Docile. Splenocytes were analyzed 16 days later for virus spleen titers and tet-gp33^{+}CD8^{+} T cells as well as IL-7Rα expression.

infection [17] and therefore NP-specific T cells are believed to be more susceptible to T cell exhaustion [17, 18]. As shown in Fig. 1B, tet-np396^{+}CD8^{+} T cells (*i.e.* specific for NP epitope 396–404) were absent after an infection with LCMV-Docile (persistent infection), whereas infection with 200 PFU LCMV-WE (non-persistent infection) led to a high frequency of functional np396-specific CD8^{+} T cells (data not shown and [17]).

Persistence of antigen or, alternatively, an LCMV-Docile-specific trait could potentially be responsible for the observed down-regulation of IL-7Rα. Therefore, mice that had been infected 50 days previously with LCMV-WE (200 PFU) were then challenged with 2×10^6 PFU LCMV-Docile (memory-CTL challenge). These mice cleared LCMV from spleen by day 16 post-challenge (Fig. 1C) and most tet-gp33^{+}CD8^{+} T cells expressed IL-7Rα (Fig. 1C). This indicated that antigen persistence itself regulated IL-7Rα expression on specific CD8^{+} T cells.

Prolonged down-regulation of the IL-7Rα on responding CD8^{+} T cells by persistent antigen

Despite antigen persistence, many other factors could lead to suppression of IL-7Rα expression during infection with LCMV. Cytokine profiles differ significantly after infection with different virus strains (K. S. Lang, unpublished) and they have been demonstrated to influence IL-7Rα expression. To avoid such differences and to directly evaluate the impact of antigen persistence, we treated mice with peptides via the i.p. route using 1 mg gp33 delivered in PBS or IFA. Peptide in PBS was functionally short lived in vivo (see Supporting Information, Supplementary Fig. A). In contrast, peptide in IFA functionally persisted for at least one week in vivo (Supplementary Fig. A), which is consistent with published observations [9].

We transferred splenocytes from LCMV-gp33–H-2D[b]-specific TCR-transgenic mice (P14) [19] into naive C57BL/6 recipients and treated them with 1 mg gp33 in PBS, or with various doses (1.0 and 0.1 mg) of gp33 in IFA. Mice receiving gp33 in PBS (days 0 and 3) exhibited elevated frequencies of tet-gp33^{+}CD8^{+} T cells in the spleen (Fig. 2A). When gp33 was given in IFA (days 0 and 3), tet-gp33^{+} cells expanded until day 3 (similar to tet-gp33^{+} cells, treated with gp33 in PBS; Fig. 2A and Supplementary Fig. B); however, by day 12 tet-gp33^{+} cells could no longer be detected (Fig. 2A), and recipient mice were no longer protected from a challenge LCMV infection (Fig. 2D).

Analysis of IL-7Rα expression revealed that stimulation with either persistent or non-persistent antigen (given only on day 0) resulted initially in the rapid down-regulation of IL-7Rα on all antigen-specific CD8^{+}
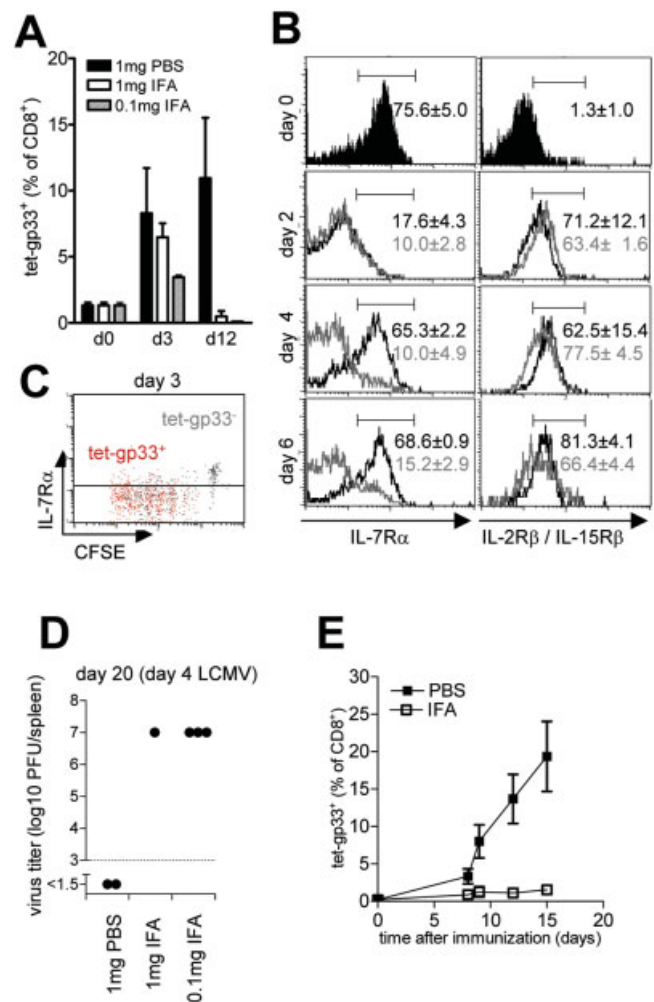
Fig. 2. Permanent down-regulation of the IL-7Rα on responding CD8^{+} T cells stimulated by persistent antigen. (A–D) P14 splenocytes were injected into naive C57BL/6 mice at day –1 (10^7 splenocytes per mouse with or without CFSE labeling). (A) On day 0 and day 3 mice were treated with gp33 (1 mg or 0.1 mg) in IFA or 1 mg in PBS and splenocytes were analyzed for tetramer binding on days 0, 3 and 12 (n=3–6). (B) Mice were immunized with 1 mg gp33 in PBS (black lines, black numbers) or IFA (grey lines, grey numbers) on day 0 and expression of IL-7Rα and IL-2Rβ/IL-15Rβ was analyzed on days 0, 2, 4, and 6. Histogram plots show splenocytes gated on tet-gp33^{+}CD8^{+} T cells (n=3–5; IL-7Rα expression on day 4 and day 6: p<0.05). FACS analysis of naive tet-gp33^{+}CD8^{+} T cells (day 0) is shown as black histogram plots (n=2–3). (C) Mice were treated with 0.1 mg gp33 in IFA and on day 3 splenocytes were analyzed for IL-7Rα and CFSE content (grey dots gated on tet-gp33^{−}CD8^{+} T cells, red dots gated on tet-gp33^{+}CD8^{+} T cells, n=3). (D) Mice were immunized as described in (A) and challenged with 200 PFU of LCMV-WE on day 16. Virus titers in spleens were analyzed 4 days later. (E) Naive C57BL/6 mice were treated with 50 μg of cytidine guanosine dinucleotide (CpG) together with 1 mg gp33 in PBS (black squares) or together with 1 mg gp33 in IFA (white squares) given i.p. on days 0, 2 and 5, then boosted with peptide on day 9. The numbers of tet-gp33^{+} cells present in the blood were determined by tetramer staining (n=6–7).

T cells (Fig. 2B, day 2) following the first rounds of cellular divisions (Fig. 2C). All CD8^{+} T cells responding to non-persistent antigen (gp33 in PBS) regained expression of IL-7Rα (Fig. 2B, days 4–6) at a time-point that correlated with the disappearance of antigen (Supplementary Fig. A). In contrast, the large majority of cells stimulated with persistent antigen failed to re-express IL-7Rα (gp33 in IFA, Fig. 2B, days 4–6). Peptide was still present at these time-points (Supplementary Fig. A); therefore, it was likely that a constant stimulation of the TCR was responsible for the continued down-regulation of the IL-7Rα. Tet-gp33^{+}CD8^{+} T cells stimulated by either non-persistent or persistent antigen up-regulated the β-chain of the receptor for IL-2 and IL-15 (IL-2Rβ/IL-15β, CD122), and this increased expression was maintained for the investigated period of 6 days (Fig. 2B). Thus, persistent antigen reduced CD8^{+} T cell survival (also in a non-transgenic system, Fig. 2E), which closely correlated with reduced expression of the functional receptor for the survival cytokine IL-7.

Enhanced apoptosis of CD8^{+} T cells with down-regulated IL-7Rα

One mechanism by which IL-7Rα expression could influence apoptosis and cellular survival would be through regulation of the anti-apoptotic molecule Bcl-2 [20–22]. IL-7Rα-expressing cells are known to signal through STAT5 and express high levels of Bcl-2 [15, 23], an anti-apoptotic molecule demonstrated to play a crucial role in cell survival [14]. Indeed, stimulation of P14 splenocytes with gp33 in either IFA or PBS led to down-regulation of Bcl-2 on day 3 (Fig. 3A), paralleled by IL-7Rα down-regulation. By day 5, Bcl-2 was re-expressed on tet-gp33^{+}CD8^{+} T cells in mice treated with gp33 in PBS, whereas cells from mice treated with gp33 in IFA continued to express reduced levels Bcl-2 (Fig. 3A), correlating with limited IL-7Rα expression. To determine whether IL-7Rα re-expression was indeed crucial for cell survival, we stained splenocytes for Annexin-V at day 5 after injection of mice with gp33 in IFA. Tet-gp33[+] splenocytes contained an increased number of apoptotic cells within the fraction expressing low levels of IL-7Rα (IL-7Rα[lo]) as compared with the few remaining cells expressing high levels of IL-7Rα (IL-7Rα[hi], Fig. 3B).

In addition, we separated P14 splenocytes exposed to persistent antigen in vivo (1 mg gp33 in IFA for 4 days) into IL-7Rα^{lo} and IL-7Rα^{hi} populations (to 95% and 86% purity of CD8^{+} T cells, respectively; Fig. 3C), labeled each population with CFSE, then injected the labeled cells into separate naive C57BL/6 hosts. The survival of the tet-gp33[+] population contained within the transferred population was then monitored by FACS. Shortly
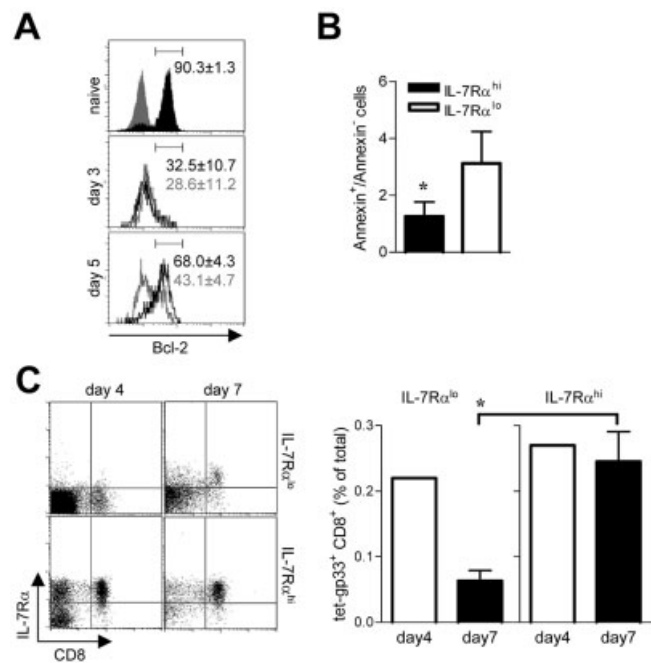
Fig. 3. Enhanced apoptosis by CD8^{+} T cells with down-regulated IL-7Rα. P14 splenocytes were injected into naive C57BL/6-mice at day –1 (10[7] splenocytes per mouse) and at day 0 mice were immunized with 1 mg gp33 in PBS or IFA. (A) On days 3 and 5 splenocytes were stained with anti-Bcl-2 antibody or with an isotype control. Splenocytes from mice treated with 1 mg of gp33 in PBS (black lines, black numbers) or in IFA (grey lines, grey numbers) were gated on tet-gp33^{+}CD8^{+} expression and for large cell size (as blasts showed the biggest difference in Bcl-2 expression), then analyzed for expression of Bcl-2 (n=4–5, Bcl-2 expression on day 5; p<0.05). Naive tet-gp33^{–}CD8^{+} T cells were stained with anti-Bcl-2 (black histogram, naive) and with the isotype control (grey histogram, naive). (B) On day 5 splenocytes from mice treated with 1 mg of gp33 in IFA were stained for IL-7Rα and Annexin-V. The degree of apoptosis of IL-7Rα^{hi} and IL-7Rα^{lo} is shown (ratio of Annexin^{+}/Annexin^{–} cells) for splenocytes gated on tet-gp33^{+}CD8^{+} T cells (n=4, *p<0.05). (C) On day 4 splenocytes pooled from three mice treated with 1 mg of gp33 in IFA were sorted into IL-7Rα^{hi} and IL-7Rα^{lo} populations by MACS and labeled with CFSE. Each fraction was injected into a separate group of naive C57BL/6 mice (n=3). Dot plots show CD8 and IL-7Rα expression on CFSE^{+} transferred cells (day 4, shortly before transfer and day 7, 3 days after transfer). The bar charts indicate tet-gp33^{+}CD8^{+} T cells as a % of total transferred splenocytes (p<0.05 between IL-7R^{hi} and IL-7R^{lo} on day 7). One of two experiments is shown.

before transfer, the IL-7Rα^{lo}tet-gp33^{+}CD8^{+} fraction represented 0.22% of all transferred splenocytes, whereas 0.27% of total splenocytes were IL-7Rα^{hi}tet-gp33^{+}CD8^{+}. Three days later, the IL-7Rα^{lo}tet-gp33^{+}CD8^{+} fraction represented only 0.06±0.02% of the total transferred population. In contrast, the IL-7Rα^{hi}tet-gp33^{+}CD8^{+} fraction survived, now representing 0.25±0.05% of the total transferred cells (Fig. 3C). Interestingly, those few CD8^{+} T cells that were initially IL-7Rα^{lo} but that did survive following transfer were

found to have re-expressed higher levels of IL-7Rα (Fig. 3C). These data suggested that IL-7Rα expression was both directly and causally linked to the survival of antigen-experienced CD8^{+} T cells.

Loss of IL-7Rα expression and continuously diminished cell survival on memory CD8^{+} T cells exposed to persistent antigen *in vivo*

To analyze the expression of IL-7Rα on memory CD8^{+} T cells after antigen challenge we compared tet-gp33^{+}CD8^{+} T cells in LCMV-immune mice (200 PFU, day 300 post-infection), or LCMV-immune mice which had received 1 mg gp33 in PBS 2 days previously. As observed for naive CD8^{+} T cells (Fig. 2B), peptide stimulation of memory CD8^{+} T cells resulted in the rapid down-regulation of IL-7Rα expression (Fig. 4A). Those T cells that had down-regulated IL-7Rα exhibited an effector phenotype in that they also expressed granzyme B, plus increased levels of CD27, CD43 and CD44 (Fig. 4A) [24], and a slight down-regulation of CD45RB. Furthermore, peptide-treated splenocytes were able to effectively lyse gp33-labeled target cells directly *ex vivo* within 7 h (Fig. 4A).

These data indicate that administration of antigen resulted in the rapid activation of memory CD8^{+} T cells to exhibit full effector function but low IL-7Rα expression. We hypothesized that exposure of memory CD8^{+} T cells to persistent antigen may result in continued IL-7Rα down-regulation, eventually resulting in the loss of these cells. To address this question we treated LCMV-immune mice (day 30 after LCMV-WE infection) with gp33 in IFA, or as a control with IFA alone, and then examined IL-7Rα expression, cellular survival and CD8^{+} T cell function. Persistent antigen resulted in the permanent down-regulation of IL-7Rα on memory T cells, and the numbers of tet-gp33^{+} cells continuously declined over time when compared with IFA-treated controls (Fig. 4B).

Sixty days after the first peptide administration a subset of mice were sacrificed and their splenocytes analyzed for CD8^{+} T cell effector function. Memory splenocytes from mice treated with gp33 in IFA did not exhibit significant lytic capacity (Fig. 4B). Splenocytes from mice treated with IFA alone were able to effectively lyse targets after a 12- or 20-h incubation (Fig. 4B) [25]. We infected the remaining mice with a recombinant vaccinia virus expressing the gp33 peptide (VV-gp33) [17]. As expected, tet-gp33^{+}CD8^{+} T cells from mice treated with IFA alone expanded in both the spleen and the peritoneum, whereas tet-gp33^{+}CD8^{+} T cells of gp33-IFA treated mice only showed minimal expansion (Fig. 4B). Thus, persistent antigen exposure resulted in continuous exhaustion/deletion even of "quiescent" or central memory T cells.
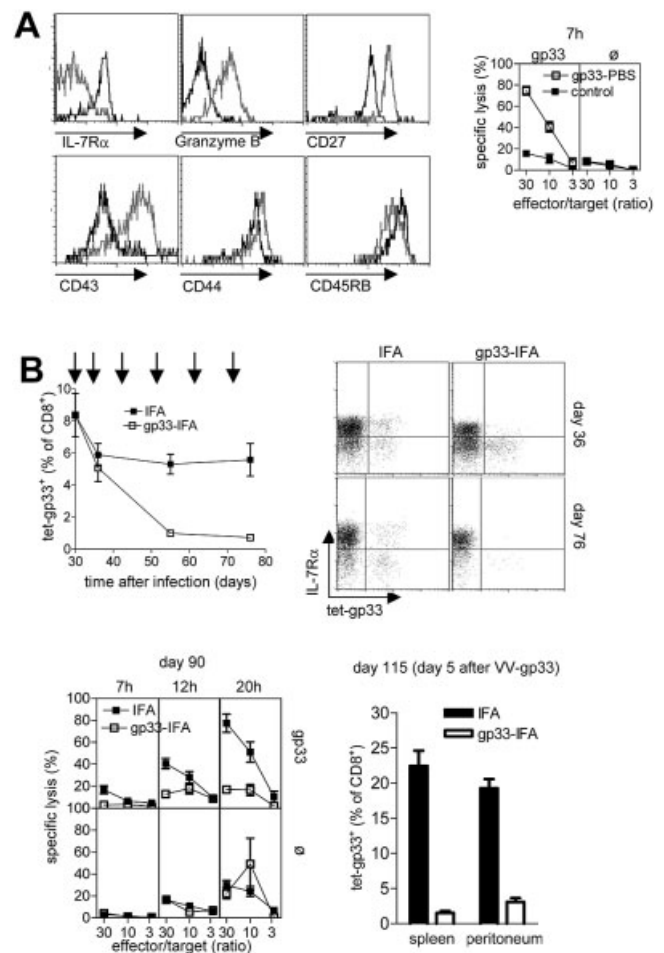
Fig. 4. Loss of IL-7Rα expression and diminished cell survival of memory CD8^{+} T cells induced by persistent antigen. (A) Naive C57BL/6 mice were infected with LCMV-WE. Mice were injected 300 days later with 1 mg gp33 in PBS (grey line, *n*=3), or left untreated (black line, *n*=2). Two days after peptide injection, tet-gp33^{+}CD8^{+} splenocytes were analyzed for expression of IL-7Rα, intracellular granzyme B, CD27, CD43, CD44 and CD45RB. Splenocytes were tested for the ability to lyse gp33-labeled target cells directly *ex vivo* in a 7-h assay (*n*=2–3, right panel). (B) C57BL/6 mice were infected with LCMV-WE, and 30 days after infection mice were injected with IFA alone or 0.1 mg gp33 in IFA at weekly intervals (see arrows). Numbers of tet-gp33^{+}CD8^{+} T cells were analyzed in blood samples (*n*=6–7, upper left). On days 36 and 76 CD8^{+} T cells were analyzed for expression of tet-gp33 and IL-7Rα (*n*=6–7) as indicated. At day 90 the ability of splenocytes to lyse gp33-labeled target cells directly *ex vivo* was determined by cytotoxicity assays (*n*=3). On day 110, mice were challenged with VV-gp33 and 5 days later mice were analyzed for numbers of tet-gp33^{+}CD8^{+} T cells present in the spleen and peritoneum.

Loss of IL-7Rα expression on memory CD8^{+} T cells preceded and predicted virus re-emergence

Our data implied that viral antigen that persists despite a specific CD8^{+} T cell response may continuously delete the memory T cell population. Eventually this could lead

to a loss of CD8$^+$ T cell memory below a certain threshold that is required to successfully control residual virus replication. The result would be virus re-emergence, a hallmark of chronic virus infections. In the LCMV model, a situation as described above is observed in LCMV-infected MHC class II deficient mice (MHC II$^{-/-}$), where after an initial phase of CD8$^+$ T cell mediated immune control, re-emergence of viremia is observed by day 50–150. We therefore followed IL-7Rα expression over time in LCMV-infected MHC class II deficient mice or C57BL/6 controls (Fig. 5). Whereas IL-7Rα expression on tet-gp33$^+$CD8$^+$ T cells remained high after infection of C57BL/6 mice (control), we observed a continuous loss of IL-7Rα expression after infection of MHC class II deficient mice, directly correlating with and accurately predicting virus re-appearance.
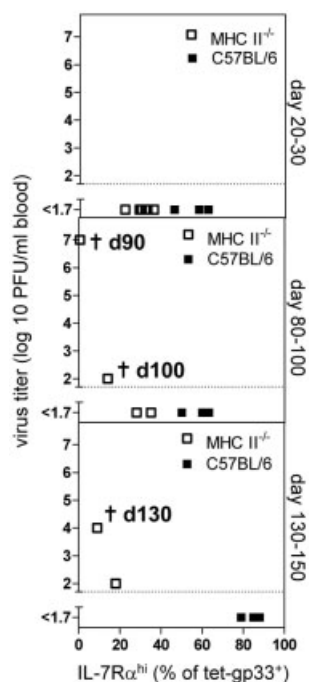
Discussion

In summary, TCR stimulation by specific antigen resulted in a rapid down-regulation of the α-chain of the IL-7R in a comparable way on both naive and memory CD8$^+$ T cells. When antigen-induced TCR stimulation was only available for a short period, responsive T cells were able to re-express IL-7R. These cells were apparently able to survive for extended periods of time and to differentiate into "quiescent" or central memory CD8$^+$ T cells. In contrast, persistent antigen prolonged IL-7Rα down-regulation and resulted in deletion of antigen-specific naive or memory T cells.

Transgenic expression of Bcl-2 restored T cell numbers in mice that lacked IL-7 signaling, proving that IL-7 signaling mediated protection from apoptosis [22]. In addition to expression of anti-apoptotic bcl-2, serine-protease inhibitors are critical determinants of CD8$^+$ T cell survival [26]. Over-expression of serine-protease inhibitor 2A in CD8$^+$ T cells led to an impaired CD8$^+$ T cell contraction phase following LCMV infection, whereas a lack of these enzymes was followed by enhanced contraction. Lack of CD8$^+$ T cell contraction was similarly observed by analyzing virus-infected mice which were deficient for the pro-apoptotic molecule Bim [27]. A recent report demonstrated that loss of Bim increased T cell production and function in IL-7R-deficient mice [28].

It is noteworthy that additional factors than antigen persistence may influence the fate of virus-specific CD8$^+$ T cells. In particular, T helper cells have been shown to rescue memory CD8$^+$ T cells from their ongoing depletion [29]. In these experiments, persisting viral antigen was experimentally excluded. Thus, the presence of T help represents another effective means to keep CD8$^+$ T cell memory stable. On the other hand, persistent antigen in our model deleted CD8$^+$ memory T cells even in the presence of CD4$^+$ T cells, demonstrating that here bystander help might be inefficient in sustaining IL-7Rα expression [29]. In addition, our data identified loss of IL-7Rα expression on specific CD8$^+$ T cells as a marker that highlights memory T cell exhaustion and virus re-appearance. Thus, IL-7R expression on virus-specific T cells may serve as a clinical marker to follow T cell responses in chronic viral diseases such as HIV or hepatitis C virus in man.

Materials and methods

Mice and viruses

LCMV strains were used as described previously [17]. VV-gp33 (2×10^6 PFU) [17] was injected i.v. P14 mice [19], MHC II$^{-/-}$ mice and C57BL/6-mice were bred locally and kept under specific pathogen free conditions. Animal experiments were performed in accordance with the Zürich, Switzerland, Swiss animal protection law.

Fig. 5. Loss of IL-7Rα expression on memory CD8$^+$ T cells precedes and predicts virus re-emergence. MHC II$^{-/-}$ and C57BL/6 mice were infected with 200 PFU LCMV-WE. At the indicated time-points, mice were analyzed for viral blood titer and expression of IL-7Rα on tet-gp33$^+$CD8$^+$ T cells.

Peptides, adoptive transfer and virus titers

Peptide gp33 was dissolved in DMSO (10 mg/ml) and then diluted in PBS. It was administered i.p. in PBS in a volume of 300 µl or in IFA (diluted 1:1 with IFA and administered i.p. in

300-μl volumes). For adoptive transfer experiments 10^7 P14 splenocytes were injected i.v. into naive C57BL/6 mice, which were then immunized with peptide in PBS or IFA as described above. Virus titers were measured by using a focus-forming assay [17].

FACS analysis, cell sorting and cytotoxicity assays

Extracellular and intracellular stainings were performed as described previously [17] or by following the manufacturers' instructions. Antibodies used included anti-IL-7R antibody (eBioscience), anti-CD8, anti-Bcl-2, anti-IFN-γ and Annexin-V (Pharmingen). Tetramers were made in-house as described previously [17]. For labeling, P14 splenocytes were incubated for 10 min with 5 μM CFSE (Molecular Probes). For cell sorting, splenocytes were stained with allophycocyanin (APC)-conjugated anti-IL-7Rα mAb, washed, and further labeled with anti-APC microbeads (Miltenyi Biotec). IL-7Rα^{hi} and IL-7Rα^{lo} cell fractions were separated as described previously [30]. For cytotoxicity assays, ^{51}Cr-release assays were performed as described previously [17].

Statistical analysis

Data are expressed as the mean±SEM; *n* = number of different animals used for an experiment; * denotes a statistically significant difference in a Student *t*-test ($p<0.05$).

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