RESEARCH ARTICLE

Interleukin-7 Modulates Anti-Tumor CD8+ T Cell Responses via Its Action on Host Cells

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Abstract

The adoptive transfer of antigen-specific CD8+ T cells is a promising approach for the treatment of chronic viral and malignant diseases. In order to improve adoptive T cell therapy (ATT) of cancer, recent strategies aim at the antibody-based blockade of immunosuppressive signaling pathways in CD8+ T cells. Alternatively, adjuvant effects of immunostimulatory cytokines might be exploited to improve therapeutic CD8+ T cell responses. For example, Interleukin-7 (IL-7) is a potent growth, activation and survival factor for CD8+ T cells that can be used to improve virus- and tumor-specific CD8+ T cell responses. Although direct IL-7 effects on CD8+ T cells were studied extensively in numerous models, the contribution of IL-7 receptor-competent (IL-7R+) host cells remained unclear. In the current study we provide evidence that CD8+ T cell-mediated tumor rejection in response to recombinant IL-7 (rIL-7) therapy is strictly dependent on IL-7R+ host cells. On the contrary, CD8+ T cell expansion is independent of host IL-7R expression. If, however, rIL-7 therapy and peptide vaccination are combined, host IL-7R signaling is crucial for CD8+ T cell expansion. Unexpectedly, maximum CD8+ T cell expansion relies mainly on IL-7R signaling in non-hematopoietic host cells, similar to the massive accumulation of dendritic cells and granulocytes. In summary, we provide evidence that IL-7R+ host cells are major targets of rIL-7 that modulate therapeutic CD8+ T cell responses and the outcome of rIL-7-assisted ATT. This knowledge may have important implications for the design and optimization of clinical ATT protocols.

Introduction

The size of the peripheral T cell pool is remarkably stable throughout life. Although infections can cause a strong increase in T cell numbers, they usually return to steady-state levels after pathogen clearance. This indicates that self-regulatory mechanisms maintain T cell numbers [1]. A central factor controlling peripheral T cell homeostasis is IL-7. It acts as a growth and survival signal for T cells, which express the IL-7R and constitutively consume IL-7 [2]. Consequently, the size of the peripheral T cell pool becomes self-limiting as soon as IL-7 production

and consumption reach an equilibrium [1]. Due to the lack of IL-7 consumption by T cells, IL-7 availability is increased in lymphopenic humans [3] and mice [4]. Lymphopenia-associated IL-7 overabundance contributes to the activation of naïve T cells, which undergo homeostatic or lymphopenia-induced proliferation (LIP) and convert into memory-like cells, which express high levels of CD44 and IFNγ [5].

The adoptive transfer of antigen-specific T cells is an important therapeutic option for the treatment of viral infections and cancer and has been performed successfully in animal models as well as in the clinic [6,7]. It is well established that the efficacy of adoptive T cell therapy (ATT) can be improved if recipient T cells are depleted by chemotherapy or irradiation prior to T cell transfer [6,8]. This positive effect of lymphodepletion results from the increased availability of T cell growth and survival factors such as IL-7 [9,10].

From our own experiments we know that thymus, lymph nodes, skin and intestine are the major sources of IL-7 in the mouse [11,12]. Nevertheless, steady-state IL-7 production is not sufficient for effective anti-tumor T cell responses under non-lymphopenic conditions. The injection of recombinant IL-7 (rIL-7) circumvents this problem and boosts anti-tumor T cell responses [13,14]. Since IL-7 promotes T cell survival [15,16], activation [17,18], proliferation [19] and memory T cell (T_M) formation [20] its direct action on T cells is supposed to be the major cause for its potent anti-tumor effects [21]. For the effective treatment of viral infections and cancer by ATT high numbers of adoptively transferred CD8+ cells are required *in vivo* [7]. Their longevity and subsequent accumulation can be improved by rIL-7 therapy suggesting that this approach can be used to improve ATT [21]. Importantly, the adjuvant effects of rIL-7 correlate with tumor growth delay rather than complete rejection [13,22,23]. Given that i) regulatory immune cells such as dendritic cells (DCs) and granulocytes expand in response to elevated IL-7 levels [24,25] and ii) non-hematopoietic cells such as fibroblasts and intestinal epithelial cells express functional IL-7 receptors (IL-7R) [12,26], we hypothesized that IL-7R+ host cells might modulate anti-tumor CD8+ T cell responses.

In the current study we asked whether and how host IL-7R signaling affects ATT efficacy. For this purpose we established an ATT model, which enabled us to discriminate between direct and indirect effects of rIL-7 therapy on tumor-specific CD8+ T cells. Our data demonstrate, that LIP of CD8+ T cells and subsequent T_M differentiation are promoted by rIL-7 in a host IL-7R-independent manner. However, tumor rejection strictly requires host IL-7R expression. Furthermore, we show that IL-7R+ non-hematopoietic host cells are crucial for maximum CD8+ T cell expansion and T_M differentiation if rIL-7 therapy is combined with peptide vaccination. Importantly, despite efficient CD8+ T cells expansion, peptide vaccination deteriorates rIL-7-dependent ATT efficacy. In summary, we provide evidence that host IL-7R signaling modulates multiple aspects of CD8+ T cells activation and T_M differentiation and can promote tumor rejection in a context-dependent fashion.

Materials and Methods

Mice

C57BL/6J (WT), B6.PL-Thy1a/Cy (CD90.1+), B6.SJL-*Ptprc*[a] *Pepc*[b]/BoyJ (CD45.1+), B6.129S7-Rag1[tm1Mom]/J (Rag-/-), B6.129S7-Il7r[tm1Imx]/J (IL-7R-/-), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) (expressing a transgenic TCR specific for the chicken ovalbumin (OVA)-derived, H2-K[b]–restricted peptide OVA$_{257-264}$ (SIINFEKL)), IL-7GCDL [11] and ChRluc mice [27] were bred in our animal facilities. All mice were housed under specific pathogen-free conditions. Animal experiments were performed according to institutional guidelines and were approved by the Landesamt für Gesundheit und Soziales Berlin (Permit Number: G0170/08) and Landesverwaltungsamt Sachsen-Anhalt (Permit Number: 2–1155 Uni MD).

Generation of bone marrow (BM) chimeras and bioluminescence detection

BM recipients were anesthetized (Ketamin/Rompun i.p.), irradiated lethally and injected with BM cells i.v. 6–18 hours later. Donor BM was isolated from femur and tibia. BM from one donor was used to reconstitute 3 recipients. BM chimeras received antibiotics via the drinking water for 3–4 weeks and were used for experiments earliest 6 weeks after BM injection. To visualize luciferase activity in live animals, bioluminescence intensities (BLI) were measured using the IVIS Imaging system (Xenogen) as described before [11,27].

Adoptive T cell transfer, peptide vaccination and IL-7 treatment

Naïve CD8$^+$ T cells were purified from spleen and lymph nodes of the respective donor mice using CD8α-specific microbeads and AutoMACS (Miltenyi Biotec). 7 x 10^5–1 x 10^6 CD8$^+$ T cells (purity >97%) were injected i.v. into the tail vein of recipient mice. For peptide vaccination, 50 µg of SIINFEKL were injected i.v. one day after T cell transfer. Control animals were injected with Dulbecco's PBS. Prior to injection, recombinant murine IL-7 (rIL-7) (eBioscience) and anti-IL-7 mAb (M25; BioXCell) were mixed and incubated for 20 min at RT. Unless otherwise stated, 200 µl of 1,5 µg rIL-7 and 10 µg anti-IL-7 mAb in PBS were injected i.p every 3–4 days starting one day prior to T cell transfer.

Tumor cell challenge

EG7 lymphoma cells produce chicken ovalbumin (OVA) and are targets of CD8$^+$ OT-I T cells. EG7 cells were cultured in RPMI+10% FCS medium with 0.4 mg/ml G418. 1 x 10^6 cells were injected s.c. in the right flank of the indicated mice. Mice with tumors >250 mm^3 were scored as tumor positive. Tumor growth was monitored every 2–3 days. Mice were euthanized by cervical dislocation when tumors reached a diameter of 10–15 mm or when showing the following signs: hunched posture, inactivity, worsening body condition, rough coat, orbital tightening or abnormal breathing. Body weight was not assessed in this study.

Flow cytometry

The following antibodies were used: anti-CD8α (53–6.7; eBioscience/Biolegend/BD), -CD90.1 (OX-7; Biolegend/BD), -CD62L (MEL-14; Biolegend/BD), -CD44 (IM7; eBioscience/Biolegend/BD), -KLRG-1 (2F1; eBioscience), PD-1 (J43; eBioscience), -Ki67 (SolA15; eBioscience), CD127 (A7R34; BD/Biolegend), CD132 (TUGm2; BD), -Bcl-2 (10C4; Biolegend), -IFN-γ (XMG1.2; Biolegend/eBioscience), -TNF-α (MP6-XT22; BD), -CD11c (N418; BD/Biolegend), -CD11b (MI70; BD/Biolegend), Gr-1 (RB6-8-C5; Biolegend). Stimulation of CD8$^+$ OT-I T cells with SIINFEKL and subsequent intracellular cytokine staining was performed as described recently [28]. Samples were measured on FACSCalibur, FACSCanto or LSRII flow cytometers and analyzed by FlowJo software (FlowJo, LLC).

Statistical analysis

Statistical analysis and graphical representations were done using Prism5 software (GraphPad Software). Statistical significance was determined using a non-parametric two-tailed Mann-Whitney, paired Student's t, 1- and 2-way Anova, log-rank or Wilcoxon matched-pairs signed rank test. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

Results

Host IL-7R signaling is crucial for rIL-7-dependent, CD8^{+} T cell-mediated tumor rejection

Naïve CD8^{+} T cells transferred into lymphopenic mice undergo lymphopenia-induced proliferation (LIP), differentiate into CD44^{hi} memory-like T cells [5] and limit tumor growth [8]. Accordingly, naive CD8^{+} TCR-transgenic (tg) OT-I T cells specific for the chicken ovalbumin (OVA)-derived, H2-K[b]-restricted peptide SIINFEKL, expanded and up regulated CD44 in lymphopenic Rag1-deficient (Rag^{-/-}) recipients within 21–27 days after transfer. Expansion and T_M differentiation were not observed in OT-I-reconstituted, non-lymphopenic wildtype (WT) mice (S1 Fig). Whether LIP-associated CD8^{+} T cell activation leads to tumor growth inhibition was tested next. Twenty-two days after reconstitution with naive CD8^{+} OT-I T cells, Rag^{-/-} mice were challenged s.c. with 10^6 OVA-expressing EG7 lymphoma cells. EG7 tumors grew rapidly in untreated Rag^{-/-} mice while adoptive T cell transfer strongly delayed tumor growth (Fig 1A). Nevertheless, only 1 mouse out of 11 remained long-term tumor-free.

In order to investigate whether IL-7 therapy promotes tumor rejection, groups of OT-I-reconstituted Rag^{-/-} mice received rIL-7 every 3–4 days for 18 days starting one day prior to adoptive T cell transfer. To improve its function, rIL-7 was complexed with IL-7-specific antibodies (αIL-7) prior to injection as described before [29]. 7/12 Rag^{-/-} mice receiving OT-I T cells plus IL-7 therapy completely rejected EG7 lymphoma cells demonstrating that IL-7 therapy strongly enhances OT-I-dependent tumor rejection in our experimental system (Fig 1A). Importantly, rIL-7 treatment did not affect primary EG7 growth in either host (data not shown).
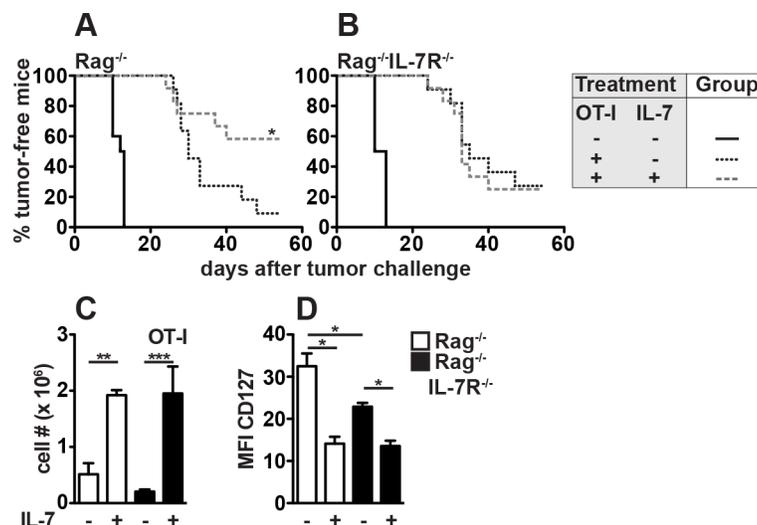

Fig 1. Host IL-7R signaling is required for CD8^{+} T cell-mediated tumor rejection in response to IL-7 treatment. (A-C) Rag^{-/-} and Rag^{-/-}IL-7R^{-/-} mice were reconstituted with 7–10 x 10^5 CD8^{+}CD90.1^{+} OT-I T cells or were left untreated (+/- OT-I). OT-I-reconstituted mice received rIL-7 (+ IL-7) or PBS (- IL-7) every 3–4 days for 18 days starting one day before T cell transfer. (A, B) 22–23 days after T cell transfer, some mice were challenged s.c. with 1 x 10^6 ovalbumin-expressing EG7 lymphoma cells. Mice with tumors >250 mm[3] were scored as tumor positive. Pooled data from 2 independent experiments with a total of 10–12 mice per group are shown. Statistical significance was calculated using the log-rank test. (C, D) Some recipients were not challenged with EG7 but analyzed for OT-I expansion and phenotype. (C) Splenic CD8^{+}CD90.1^{+} OT-I T cells were quantified 21–25 days after adoptive transfer. Pooled data (±SEM) from 2 independent experiments with a total of 7–9 mice/group are shown. (D) CD127 expression of splenic OT-I T cells was determined 5 days after adoptive transfer. Data (±SEM) for 3–4 mice/group are shown.

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Several studies provided evidence that rIL-7 promotes activation, survival, function of CD8$^+$ T cells [15–19,30] and memory T cell (T$_M$) formation [20]. So far, however, these effects were considered to result from direct effects of rIL-7 on CD8$^+$ T cells. Besides CD8$^+$ T cells, however, numerous hematopoietic and non-hematopoietic cells express the IL-7 receptor (IL-7R) [31]. Hence, it remained unclear whether the success of IL-7-assisted adoptive T cell transfers relies on IL-7R signaling in CD8$^+$ T cells and/or host cells. In order to address this question, Rag$^{-/-}$ mice lacking the IL-7Rα chain (Rag$^{-/-}$IL-7R$^{-/-}$) were reconstituted with OT-I T cells, received IL-7 therapy or PBS and were challenged with EG7 lymphoma cells. This approach allowed us to separate direct from indirect effects of rIL-7 on CD8$^+$ T cell-mediated lymphoma rejection. As compared to untreated controls, OT-I cells strongly delayed tumor growth in Rag$^{-/-}$IL-7R$^{-/-}$ recipients (Fig 1B) similar to what we had observed in Rag$^{-/-}$ mice (Fig 1A). This demonstrates that host IL-7R-deficiency does not limit LIP-associated CD8$^+$ T$_M$ differentiation (S1 Fig) and subsequent anti-tumor immunity (Fig 1B). In contrast to Rag$^{-/-}$ mice, rIL-7 treatment of OT-I-reconstituted Rag$^{-/-}$IL-7R$^{-/-}$ mice did not improve anti-tumor CD8$^+$ T cell responses (Fig 1B) indicating that direct effects of rIL-7 on CD8$^+$ T cells are not sufficient for successful tumor rejection. In fact, IL-7R signaling by host cells is crucial for tumor rejection after CD8$^+$ T cell transfer and rIL-7 therapy. Next we studied whether impaired tumor rejection in Rag$^{-/-}$IL-7R$^{-/-}$ mice resulted from reduced LIP. Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice were reconstituted with CD8$^+$ OT-I T cells and spleen cells were analyzed 21–27 days later. CD44 levels (S1A Fig) and recovery rates (S1B Fig) did not differ between OT-I cells from Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice. In conclusion, LIP-driven expansion and T$_M$ formation are independent of host IL-7R signaling.

To analyze the impact of host IL-7R signaling on rIL-7 therapy-related T$_M$ differentiation and expansion, Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice were reconstituted with OT-I T cells and treated with rIL-7 (+IL-7) or PBS (-IL-7) as described above. In accordance with S1B Fig, similar numbers of OT-I cells were recovered from spleens of PBS-treated Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice 21–25 days after adoptive transfer (Fig 1C). IL-7 signaling suppresses IL-7Rα chain (CD127) expression by naive T cells [32]. Accordingly, a strong and host-independent down-modulation of CD127 was observed 5 days after adoptive transfer and rIL-7 treatment (Fig 1D). Importantly, OT-I T cells expressed less CD127 in PBS-treated Rag$^{-/-}$IL-7R$^{-/-}$ than in Rag$^{-/-}$ mice arguing for elevated steady state IL-7 levels in the latter. Nevertheless, this did not affect the long-term abundance (Fig 1C) or early rIL-7 responsiveness of OT-I T cells (Fig 1D).

In conclusion, host IL-7R signaling is dispensable for rIL-7-driven CD8$^+$ T cell expansion (Fig 1C) but not for subsequent tumor rejection. This highlights that high numbers of therapeutic CD8$^+$ T cells do not necessarily correlate with therapeutic success.

Host IL-7R signaling is not required for rIL-7-induced T$_M$ differentiation but for granulocyte and DC expansion

CD8$^+$ T$_M$ express high levels of CD127 and the anti-apoptotic molecule B cell lymphoma protein-2 (Bcl-2) [33], which can be up regulated by IL-7 [34]. In PBS-treated Rag$^{-/-}$ mice CD127^{hi} and Bcl-2^{hi} OT-I T$_M$ were less frequent than in Rag$^{-/-}$IL-7R$^{-/-}$ mice (Fig 2A and 2B). In response to rIL-7 treatment, however, resulting OT-I T$_M$ expressed elevated levels of CD127 and Bcl-2 in both hosts 21–25 days after transfer (Fig 2A and 2B). Hence, despite early CD127 down-regulation (Fig 1D), IL-7 therapy promoted the generation of CD127^{hi}Bcl-2^{hi} OT-I T$_M$ irrespective of the host. IL-7 therapy also promoted the generation of CD62L^{hi} OT-I cells (Fig 2C) expressing low levels of KLRG-1 (Fig 2D). Furthermore, a high percentage of OT-I cells recovered from both hosts rapidly produced IFN-γ after short-term *in vitro* re-stimulation (Fig 2E). Hence, rIL-7 therapy favors the generation of functional CD8$^+$ T$_M$ cells with a CD127^{hi}Bcl-2^{hi}CD62L^{hi}KLRG-1^{lo} phenotype in a host-independent fashion. Interestingly,
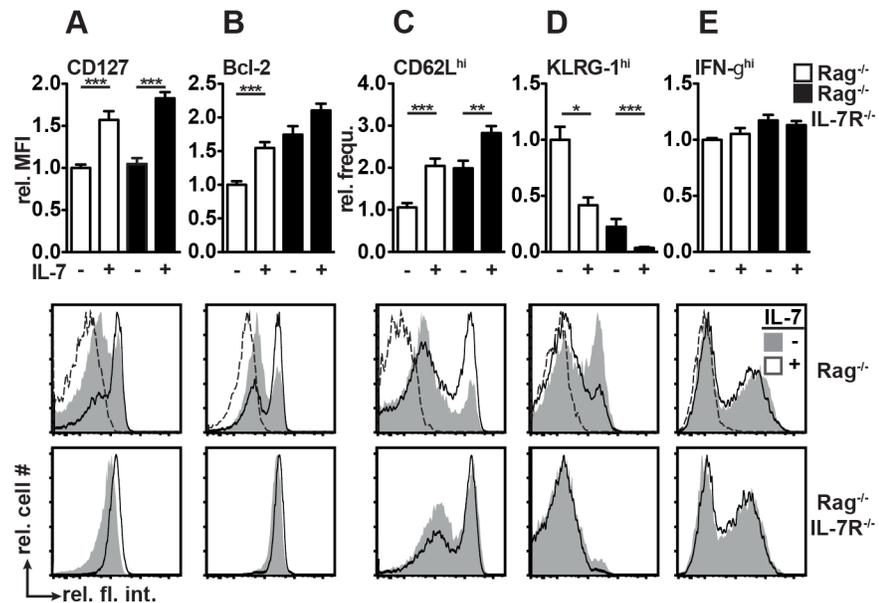
Fig 2. Host IL-7R signaling is not required for rIL-7-induced T_M differentiation. (A-E) Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice were treated with rIL-7 or PBS (+/- IL-7) every 3–4 days starting one day prior to adoptive transfer of 1 x 10^6 CD8$^+$CD90.1$^+$ OT-I T cells. 21–25 days after transfer, splenic OT-I T cells were analyzed by flow cytometry. After gating on CD8$^+$CD90.1$^+$ or CD8$^+$Vα2$^+$ OT-I T cells, mean fluorescence intensities (MFIs) for (A) CD127 and (B) Bcl-2 as well as the frequencies (frequ.) of (C) CD62L^{hi}, (D) KLRG-1^{hi} and (E) IFN-γ^{hi} cells were determined. IFN-γ production was measured after *in vitro* re-stimulation with 1 μM SIINFEKL peptide in the presence of brefeldin A for 6 hours. (A-E) Data shown in bar diagrams were normalized to the mean values determined in PBS-treated Rag$^{-/-}$ mice and are shown as relative (rel.) values. Pooled data (±SEM) from 2–3 independent experiments with a total of 7–13 mice/group are shown. Histogram overlays show relative cell numbers (rel. cell #) and relative fluorescence intensities (rel. fl. int.) for individual mice after gating on OT-I cells. Dashed lines in overlays represent (A-D) FMO control samples or (E) stained cells without prior SIINFEKL stimulation.

despite their high numbers (Fig 1C) and favorable phenotype (Fig 2), rIL-7-induced OT-I T_M cells failed to reject tumors in Rag$^{-/-}$IL-7R$^{-/-}$ mice (Fig 1B). This suggested a contribution of rIL-7-responsive host cells to EG7 elimination.

After rIL-7 treatment, spleen cell numbers increased dramatically in Rag$^{-/-}$ mice (Fig 3A). In agreement with previous studies [24,25], CD11b$^+$Gr-1$^+$ granulocytes and CD11c$^+$MHCII$^+$ DCs expanded most efficiently in response to rIL-7 (Fig 3B and 3C). Among the latter, CD8$^+$ lymphoid and CD8$^-$ myeloid DCs responded similarly well (Fig 3D and 3E). In Rag$^{-/-}$IL-7R$^{-/-}$ mice rIL-7 therapy failed to induce the expansion of splenocytes, granulocytes and DCs (Fig 3A–3E) excluding IL-7R-independent side effects of our treatment regimen. In summary, host IL-7R signaling is crucial for rIL-7-induced, CD8$^+$ T cell-mediated tumor rejection (Fig 1A and 1B), is not essential for CD8$^+$ T_M differentiation (Fig 2) but promotes the expansion of granulocytes and DCs.

Host IL-7R signaling promotes CD8$^+$ T cell expansion and modulates T_M differentiation in response to peptide vaccination and IL-7 therapy

IL-7 administration improves vaccination-induced T cell responses [13,22]. To test whether this also requires host IL-7R signaling, Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice were reconstituted with OT-I cells, immunized with SIINFEKL and treated with rIL-7. Peptide-vaccinated mice receiving PBS served as controls. As shown in Fig 4A rIL-7 treatment strongly increased spleen cell numbers in Rag$^{-/-}$ but not Rag$^{-/-}$IL-7R$^{-/-}$ mice. Furthermore, rIL-7 treatment promoted OT-I
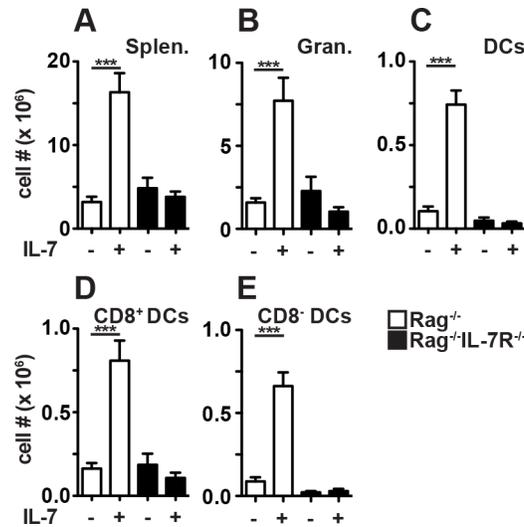
Fig 3. Host IL-7R signaling is required for granulocyte and DC expansion in response to rIL-7. Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice were treated with rIL-7 or PBS (+/- IL-7) every 3–4 days and spleens were analyzed by flow cytometry after 10–24 days. (A-E) Shown are numbers of (A) splenocytes (Splen.), (B) CD11b$^+$Gr1$^+$ granulocytes (Gran.), (C) CD11c$^+$MHC-II$^+$ dendritic cells (DCs), (D) CD8$^+$ and (E) CD8$^-$ DCs. Shown are pooled data (±SEM) from 2 independent experiments with a total of 7–8 mice/group.

cell expansion in Rag$^{-/-}$ mice ([Fig 4B](#)). Although to a much lesser extent, this was also observed in rIL-7-treated Rag$^{-/-}$IL-7R$^{-/-}$ mice ([Fig 4B](#)). Thus, IL-7R signaling in CD8$^+$ T cells promotes some degree of rIL-7-induced CD8$^+$ T cell expansion although the full-blown response requires host IL-7R expression.

To test whether IL-7R$^+$ host cells also modulate T$_M$ differentiation the phenotype of peripheral blood OT-I cells was determined by flow cytometry. In Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice, frequencies of CD62L^{hi} OT-I cells were similar after vaccination ([Fig 4C](#)). This was further increased by rIL-7 and was independent of host IL-7R expression ([Fig 4C](#)). OT-I cells expressed similar levels of CD127 after vaccination of Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice. To our surprise, rIL-7 therapy further promoted CD127 expression by OT-I cells only in Rag$^{-/-}$ mice ([Fig 4D](#)). KLRG-1^{hi} OT-I cells were more frequent in vaccinated Rag$^{-/-}$IL-7R$^{-/-}$ than in Rag$^{-/-}$ mice. In both hosts their abundance decreased in response to rIL-7, though to a lesser extent in Rag$^{-/-}$IL-7R$^{-/-}$ mice ([Fig 4E](#)). After vaccination, OT-I proliferation was lower in Rag$^{-/-}$ mice as shown by low numbers of Ki67^{hi} cells ([Fig 4F](#)). Bcl-2 expression was similar in vaccinated Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice and was up regulated by rIL-7 only in the latter ([Fig 4G](#)). In summary, the rIL-7-induced up-regulation of CD62L by OT-I cells was largely independent of IL-7R$^+$ host cells. In contrast, host cells promoted the expansion of OT-I cells ([Fig 4B](#)) and modulated their expression of CD127, KLRG-1 and Bcl-2 in response to rIL-7 therapy ([Fig 4D, 4E and 4G](#)).

After vaccination, OT-I cells isolated from Rag$^{-/-}$ mice produced high levels of IFN-γ ([Fig 4H](#)). This response was less pronounced in Rag$^{-/-}$IL-7R$^{-/-}$ mice but could be improved by rIL-7 treatment ([Fig 4H](#)). Importantly, rIL-7 treatment could not further promote the generation of IFN-γ^{hi} OT-I cells in Rag$^{-/-}$ mice ([Fig 4H](#)). OT-I cells producing high levels of TNF-α were similarly frequent in Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice and further expanded in both hosts in response to rIL-7 treatment ([Fig 4I](#)). The expression of PD-1, a marker for dysfunctional T cells, was comparable for OT-I cells recovered from mice of both strains. In agreement with a previous study [35], rIL-7 administration reduced PD-1 expression on OT-I cells in Rag$^{-/-}$ mice ([Fig 4J](#)). Surprisingly, this was not the case in Rag$^{-/-}$IL-7R$^{-/-}$ mice ([Fig 4J](#)) indicating that IL-7R$^+$ host
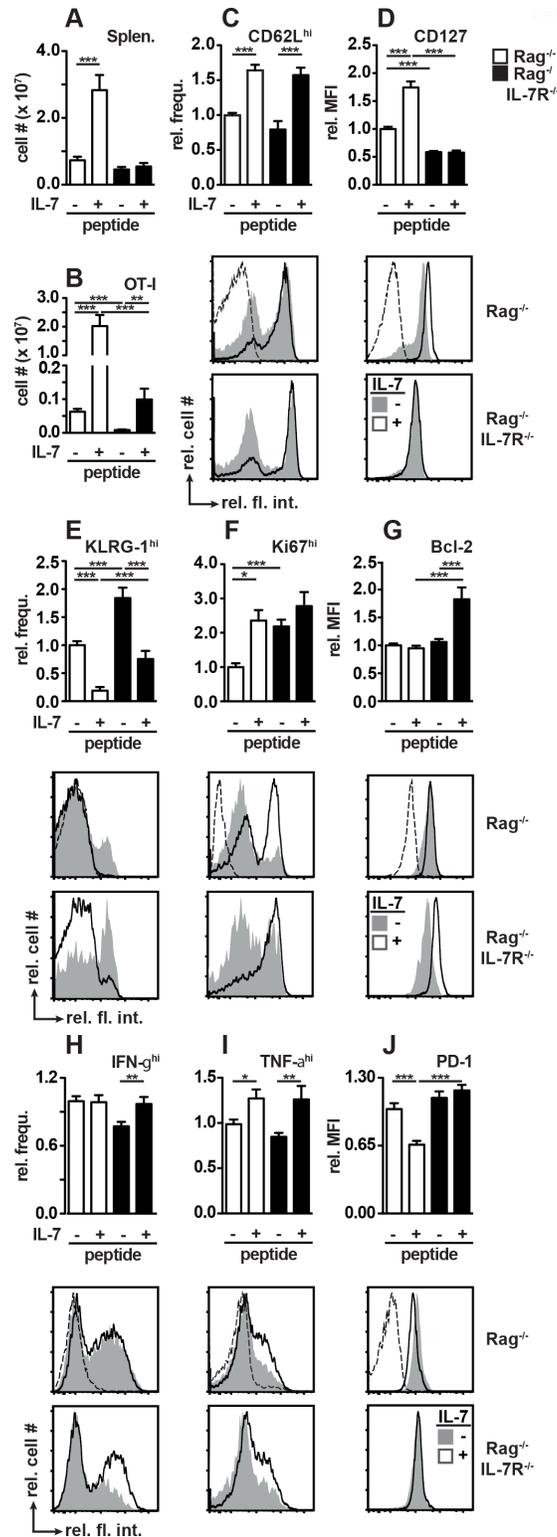
Fig 4. Host IL-7R signaling modulates CD8$^+$ T cell expansion and differentiation in response to peptide vaccination and IL-7 therapy. (A-J) Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice were reconstituted with CD8$^+$CD90.1$^+$ OT-I T cells and treated with SIINFEKL +/- rIL-7 as described in Fig 4. Three weeks after T cell transfer (A, B) spleens and (C-J) peripheral blood were analyzed by flow cytometry. Shown are numbers of (A) splenocytes and (B) splenic CD8$^+$CD90.1$^+$ OT-I T cells. After gating on OT-I T cells, relative frequencies

(frequ.) of (C) CD62L^{hi}, (E) KLRG-1^{hi}, (F) Ki67^{hi}, (H) IFN-γ^{hi}, (I) TNF-α^{hi} OT-I cells and relative MFIs for (D) CD127, (Γ) Bcl-2 and (J) PD-1 were determined. (H, I) Cytokine production was measured after *in vitro* stimulation of PBMCs with 1 μM SIINFEKL peptide in the presence of Rag$^{-/-}$ splenocytes, brefeldin A and monensin for 6 hours. Relative (rel.) values were calculated as described in Fig 2. Bar diagrams show pooled data (±SEM) from 3 independent experiments with a total of 15–18 mice/group. Histogram overlays show representative results for individual mice. Dashed lines in overlays represent (C-G and J) FMO control samples or (H, I) stained cells without prior SIINFEKL stimulation.

cells determine the levels of PD-1 expression by CD8$^+$ T cells. Hence, immunomodulation by rIL-7 relies on complex interactions between IL-7R$^+$ host and therapeutic CD8$^+$ T cells.

IL-7R$^+$ non-hematopoietic cells are crucial for CD8$^+$ T cell expansion in response to vaccination and rIL-7 therapy

Prolonged exposure to elevated levels of IL-7 down modulates *il-7* gene activity in non-BM-derived cells [4,12], which are the major source of IL-7 *in vivo* [20]. To determine whether non-hematopoietic cells respond to rIL-7 therapy, IL-7 reporter mice expressing luciferase under the control of the *il-7* promoter [11] were treated with rIL-7. As a readout for *il-7* gene activity, bioluminescence intensities (BLI) were determined before and after rIL-7 treatment. As shown in S2 Fig, rIL-7 administration reduced *il-7* promoter activity significantly demonstrating that IL-7-producing non-BM-derived cells indeed respond to rIL-7 therapy.

In order to study the relative contribution of IL-7R signaling in hematopoietic versus non-hematopoietic host cells to rIL-7-induced CD8$^+$ T cell responses, we generated bone marrow (BM) chimeric mice. Lethally irradiated CD45.1$^+$ Rag$^{-/-}$ mice received BM from CD45.1$^+$ Rag$^{-/-}$ (R → R chimeras) or CD45.2$^+$ Rag$^{-/-}$IL-7R$^{-/-}$ mice (RR → R chimeras). Furthermore, lethally irradiated CD45.2$^+$ Rag$^{-/-}$IL-7R$^{-/-}$ mice received BM from CD45.2$^+$ Rag$^{-/-}$IL-7R$^{-/-}$ (RR → RR chimeras) or CD45.1$^+$ Rag$^{-/-}$ mice (R → RR chimeras). Flow cytometric analysis of CD45.1/.2-disparate RR → R and R → RR chimeras revealed that donor BM contributed to the generation of around 97% of splenic CD11b$^+$ cells (data not shown). BM-reconstituted mice received OT-I cells, vaccination and rIL-7 therapy as described above. Three weeks after adoptive T cell transfer, we first quantified splenic CD11b$^+$ cells and DCs. To our surprise, IL-7R expression by BM-derived cells was dispensable for CD11b$^+$ cell expansion if non-BM-derived cells expressed the IL-7R (Fig 5A; R → R vs RR → R chimeras). If, however, BM- and non-BM-derived cells lacked the IL-7R, CD11b$^+$ cell expansion was strongly impaired (RR → RR). Only a partial recovery was observed if BM-derived cells produced the IL-7R (Fig 5A; R → RR) suggesting that IL-7R signaling in BM- and non-BM-derived cells synergize to promote therapy-induced CD11b$^+$ cell expansion. Similarly, the expansion of IL-7R-competent and -deficient DCs was comparable in Rag$^{-/-}$ BM recipients (R → R vs RR → R). On the contrary, DC expansion was strongly impaired in RR → RR chimeras and only partially recovered in R → RR chimeras (Fig 5B). Hence, IL-7R signaling in non-BM cells is crucial for therapy-associated DC expansion. However, this appeared to be more important for CD8$^+$ DCs than for CD8$^-$ DCs (Fig 5C and 5D).

Similar to DCs, OT-I accumulation in the spleen was independent of IL-7R expression by BM derived cells if non-BM-derived cells expressed the IL-7R (Fig 5E; R → R vs RR → R). In accordance with Fig 4B, OT-I responses were least efficient in chimeras lacking the IL-7R on BM-derived and non-BM-derived cells (Fig 5E; RR → RR). Importantly, IL-7R expression by BM-derived cells alone was insufficient to fully recover OT-I accumulation (Fig 5E; R → RR).

IL-7 treatment is known to alter homing patterns of CD8$^+$ T cells [36]. To exclude migration-related differences in splenic OT-I cell numbers, BM chimeras were reconstituted with renilla luciferase-transgenic (ChRluc^{tg}) CD8$^+$ OT-I T cells [27], vaccinated and treated with
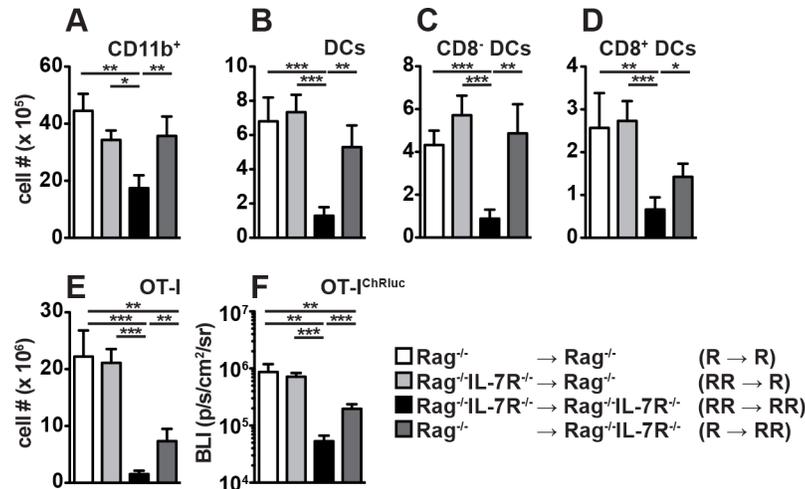
Fig 5. IL-7R signaling in non-hematopoietic host cells contributes to granulocyte, DC and CD8$^+$ T cell expansion in response to IL-7 therapy. Lethally irradiated CD45.1$^+$ Rag$^{-/-}$ and CD45.2$^+$ Rag$^{-/-}$IL-7R$^{-/-}$ mice were reconstituted with the indicated bone marrow (BM) cells (donor→recipient). After successful BM reconstitution, BM chimeras received 1 x 10^6 CD8$^+$ OT-I T cells. One day later, mice were vaccinated with 50 µg SIINFEKL. IL-7 treatment was done as described above. (A-E) Three weeks after T cell transfer, recipient spleens were analyzed by flow cytometry and the numbers of the indicated cell types were determined. Shown are pooled data (±SEM) of 3 independent experiments with a total of 10–17 mice/group. (F) BM chimeras received 1 x 10^6 renilla luciferase-transgenic (ChRluc^{tg}) CD8$^+$ OT-I T cells (OT-I^{ChRluc}) and were treated with SIINFEKL and rIL-7 as described above. Six days after T cell transfer, mice were treated with 100 µg colenterazine to determine bioluminescence intensities (BLI) as a readout for OT-I abundance and distribution. Shown are pooled results (±SEM) of 2 independent experiments with a total of 5–10 mice/group.

rIL-7. Six days later, whole-body BLI levels were determined to quantify relative OT-I cell numbers in an unbiased, tissue-independent fashion. As shown in Fig 5F, ChRluc^{tg} OT-I were similarly abundant in R → R and RR → R chimeras. On the contrary, their frequency was strongly impaired in RR → RR chimeras and only partially recovered in R → RR chimeras. Hence, these results confirm Fig 5E and further emphasize the importance of IL-7R$^+$ non-BM cells for rIL-7-induced CD8$^+$ T cell expansion.

IL-7R$^+$ non-BM cells modulate CD8$^+$ T$_M$ differentiation in response to vaccination and rIL-7 therapy

Having shown that non-BM cells promote the expansion of CD8$^+$ T cells, we analyzed their phenotype. Three weeks after adoptive transfer, OT-I cells isolated from the spleens of BM chimeras were analyzed by flow cytometry. In agreement with Fig 4E, KLRG-1^{hi} OT-I cells were more frequent in RR → RR than in R → R chimeras (Fig 6A) further emphasizing that T$_M$ differentiation into KLRG-1^{hi} cells is modulated by IL-7R$^+$ host cells. These cells appear to be of BM and non-BM origin as shown by the fact that R → RR chimeras contained most KLRG-1^{hi} OT-I cells. However, their numbers were reduced in RR → RR chimeras and lowest in R → R and RR → R chimeras (Fig 6A).

The frequencies of CD62L^{hi} OT-I cells were identical in R → R and RR → RR chimeras suggesting their host IL-7R-independence (Fig 6B). However, CD62L^{hi} OT-I cells were less abundant in RR → R and R → RR (Fig 6B). This indicates that IL-7R$^+$ BM- and non-BM-derived cells are part of a complex network exerting opposing functions on CD62L^{hi} T$_M$ differentiation.

Highest levels of CD127 were found on OT-I cells from R → R and RR → R chimeras and lowest on those from RR → RR chimeras (Fig 6C) demonstrating that IL-7R expression by
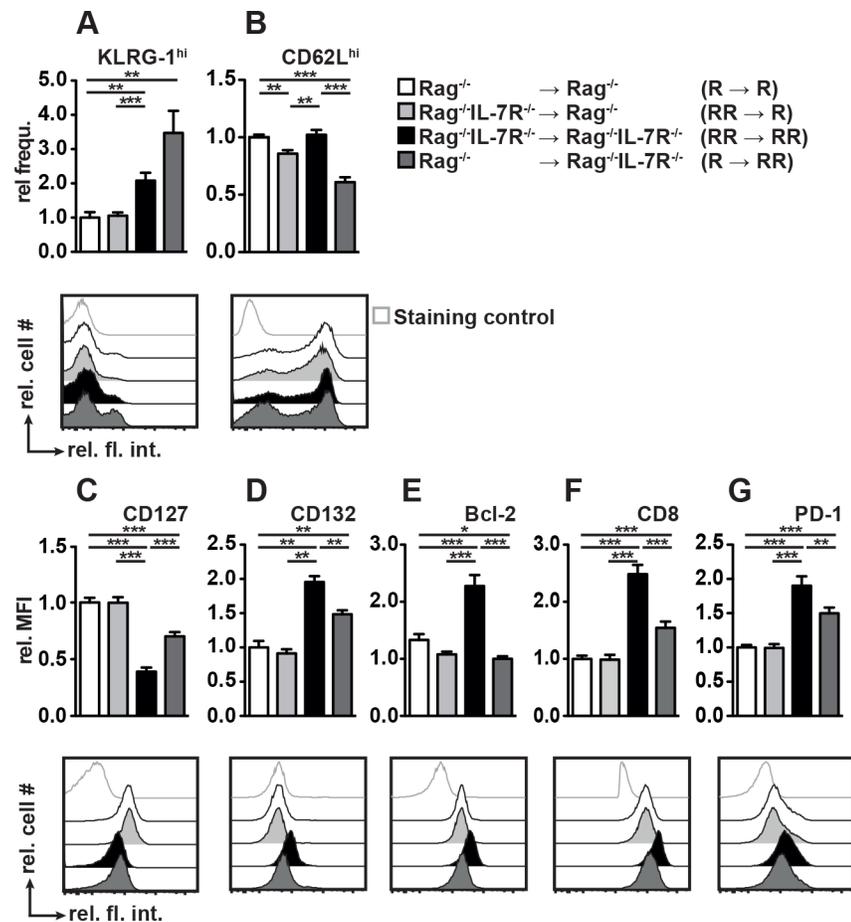
Fig 6. IL-7R signaling in non-hematopoietic cells regulates CD8⁺ T cell differentiation in response to peptide vaccination and IL-7 therapy. (A-G) The indicated BM chimeras received CD8$^+$ OT-I T cells and were treated with SIINFEKL and rIL-7 as described above. Three weeks after T cell transfer, splenic CD8$^+$ OT-I T cells were analyzed by flow cytometry. Bar diagrams show pooled results (±SEM) of 2–3 independent experiments with a total of (A, B, C, E, G) 10–17 or (D) 4–10 mice/group. Histogram overlays show representative results for individual mice. Grey-lined histograms represent FMO staining controls.

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non-BM cells is a prerequisite for maximum CD127 expression by CD8$^+$ T cells. If only BM-derived cells expressed the IL-7R (R → RR) CD127 levels were significantly higher than in RR → RR chimeras but still below those found on OT-I cells primed in RR → R chimeras ([Fig 6C](#)). Thus, IL-7R$^+$ non-BM cells are major regulators of CD127 expression by OT-I cells.

The genes encoding Bcl-2 and CD8 are direct targets of IL-7 and other cytokines utilizing the IL-2Rγ_c (CD132) for signal transduction [37]. CD127^{lo} OT-I cells from RR → RR chimeras expressed highest levels of CD132, Bcl-2 and CD8 ([Fig 6D–6F](#)) suggesting that other CD132-dependent cytokines than IL-7 caused the differentiation of CD127^{lo}CD132^{hi}Bcl-2^{hi}CD8^{hi} T$_M$ cells in RR → RR chimeras. In contrast, R → R and RR → R chimeras rather contained CD127^{hi}CD132^{lo}Bcl-2^{lo}CD8^{lo} OT-I cells ([Fig 6C–6F](#)). However, if only BM-derived cells expressed the IL-7R (R → RR) we observed an intermediate OT-I phenotype ([Fig 6C–6G](#) and [6F](#)).

In response to rIL-7 treatment PD-1 was downregulated only on OT-I cells primed in Rag$^{-/-}$ mice but not on those primed in Rag$^{-/-}$IL-7R$^{-/-}$ mice ([Fig 4J](#)). In agreement with this, PD-1 expression was most pronounced in RR → RR chimeras ([Fig 6G](#)). PD-1 levels were significantly lower in both groups of chimeras expressing IL-7R on non-BM-derived cells and (R →

R and RR → R). In contrast, OT-I cells from R → RR chimeras showed an intermediate phenotype (Fig 6G) indicating that IL-7R$^+$ non-BM-derived cells are major regulators of PD-1 expression by CD8$^+$ T cells.

In summary, IL-7R$^+$ host cells promoted the expansion of CD8$^+$ T$_M$ cells in response to rIL-7 therapy (Fig 4B–4J). Surprisingly, regulatory host cells were mainly of non-BM origin (Figs 5E, 5F and 6).

The combination of rIL-7 therapy and peptide vaccination impairs T cell-dependent tumor rejection in Rag$^{-/-}$ mice

CD8$^+$ CD62L^{hi}KLRG-1^{lo}IFN-γ^{hi}PD-1^{lo} T$_M$ cells are well suited to provide long-term protection against chronic infections and tumors [7,38,39]. Peptide vaccination and rIL-7 therapy induced the generation of such T$_M$ cells in Rag$^{-/-}$ mice (Fig 4B–4J), a process that was mainly controlled by IL-7R$^+$ non-hematopoietic cells (Figs 5E, 5F and 6). However, their therapeutic potential remained unclear. To test this, Rag$^{-/-}$ mice were reconstituted with CD8$^+$ OT-I T cells and vaccinated with SIINFEKL one day later. Additionally, mice received rIL-7 or PBS according to the scheme described above. To ensure appropriate OT-I expansion and differentiation, mice were challenged with EG7 cells 21 days after adoptive T cell transfer. In untreated Rag$^{-/-}$ control mice, EG7 tumors grew rapidly (Fig 7A). In contrast, 6/12 peptide-vaccinated Rag$^{-/-}$ mice remained tumor free (Fig 7A). Surprisingly, however, only 2/12 Rag$^{-/-}$ mice rejected EG7 lymphomas after rIL-7 therapy (Fig 7A). Importantly, this was not due to impaired DC expansion in rIL-7-treated Rag$^{-/-}$ mice (Fig 7C). Hence, the protective effect of peptide vaccination was blunted by rIL-7.

Correlating with their comparably low frequency (Fig 4B) and KLRG-1^{hi}IFN-γ^{lo} phenotype (Fig 4E and 4H), SIINFEKL-induced T$_M$ cells rejected EG7 tumors only in 2/12 Rag$^{-/-}$IL-7R$^{-/-}$ mice (Fig 7B). After rIL-7 therapy only 1/12 Rag$^{-/-}$IL-7R$^{-/-}$ mice remained tumor free (Fig 7B). In summary, the protective effect of peptide vaccination relied on host IL-7R signaling and was blunted by rIL-7 therapy.

Discussion

The major goal of our study was to clarify whether and how host IL-7R signaling contributes to rIL-7-driven anti-tumor CD8$^+$ T cell responses. For this purpose, SIINFEKL-specific CD8$^+$
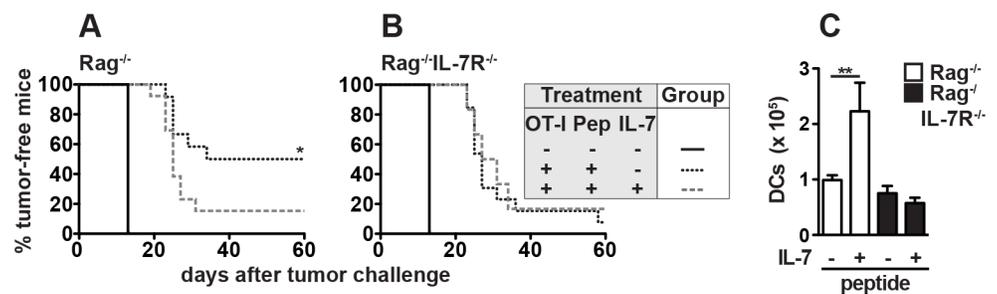

Fig 7. The combination of rIL-7 therapy and peptide vaccination impairs T cell-dependent tumor rejection in Rag$^{-/-}$ mice. (A) Rag$^{-/-}$ and (B) Rag$^{-/-}$IL-7R$^{-/-}$ mice were reconstituted with 1 x 10^6 CD8$^+$CD90.1$^+$ OT-I T cells or were left untreated (+/- OT-I). One day later, OT-I-reconstituted mice were either vaccinated with 50 μg SIINFEKL or received PBS (+/- Pep). rIL-7 or PBS (+/- IL-7) were injected every 3–4 days for 19 days starting one day before T cell transfer. Mice were challenged s.c. with 1 x 10^6 EG7 tumor cells three weeks after T cell transfer. Mice with tumors larger than 250 mm^3 were scored as tumor positive. Shown are pooled data from 2 independent experiments with a total of 12–13 T cell reconstituted mice. Primary tumor growth was analyzed in untreated Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice (n = 3). Statistical significance was calculated using the log-rank test. (C) The numbers of splenic DCs were determined in tumor-bearing mice 28–37 days after tumor challenge. Pooled data (±SEM) from 2 independent experiments with a total of 6–11 mice/group are shown. Statistical significance was calculated using the Mann-Whitney test.

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OT-I cells were transferred into Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice, which were then treated with rIL-7. As shown in Fig 1A, OT-I-reconstituted Rag$^{-/-}$ mice receiving IL-7 therapy rejected SIINFEKL-expressing EG7 lymphoma cells. This was not the case for Rag$^{-/-}$IL-7R$^{-/-}$ mice demonstrating that the success of rIL-7 therapy is dependent on host IL-7R expression in our experimental system. However, CD8$^+$ T cell expansion and differentiation were largely independent of host IL-7R signaling. Irrespective of host IL-7R expression nearly identical numbers of OT-I cells were recovered from spleens after rIL-7 treatment. Similarly, the differentiation of CD8$^+$ T$_M$ with a CD127^{hi}Bcl-2^{hi}CD62L^{hi}KLRG-1^{lo}IFN-γ^{hi} phenotype was induced in rIL-7-treated Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice. Hence, our data show that rIL-7-induced CD8$^+$ T cell expansion and subsequent T$_M$ differentiation are not affected by host IL-7R expression and related differences in IL-7 availability (Fig 1D). In agreement with previous studies [21], the beneficial effects of rIL-7 on CD8$^+$ T cell function, differentiation and survival appear to result mainly from IL-7R signaling in CD8$^+$ T cells. Nevertheless, it is important to stress that effective CD8$^+$ T cell expansion and differentiation do not necessarily correlate with tumor rejection. This conclusion is supported by the fact that rIL-7-induced OT-I expansion and subsequent T$_M$ differentiation occurred efficiently in Rag$^{-/-}$IL-7R$^{-/-}$ mice while tumor rejection failed. This finding emphasizes the importance of IL-7-responsive host cells for rIL-7-assisted ATT in our model system.

Dendritic cells promote CD8$^+$ T cell responses under lymphopenic conditions [40], cross-present tumor-derived antigens [41] and expand in response to rIL-7 treatment [24], which promotes T-DC interactions [42]. Additionally, rIL-7 stimulates myelopoiesis and the subsequent accumulation of CD11b$^+$ cells [25], which can cross-present tumor antigens and promote CD8$^+$ T cell-mediated tumor rejection [43]. After rIL-7 treatment, DCs and granulocytes accumulated only in IL-7R-competent mice. However, this accumulation was not required for expansion and functional maturation of OT-I cells but correlated positively with tumor rejection. This suggests that rIL-7-expanded DCs and granulocytes support CD8$^+$ T cell responses in the late effector phase rather than in the early phase after adoptive transfer.

IL-7R signaling in combination with TCR stimulation boosts CD8$^+$ T cell responses in multiple experimental systems. TCR signal strength and the timing of IL-7R signaling appear to be important to achieve optimal IL-7 effects [44]. To generate maximum CD8$^+$ T cell responses, we reconstituted Rag$^{-/-}$ and Rag$^{-/-}$IL-7R$^{-/-}$ mice with OT-I cells and vaccinated them with SIINFEKL. Unlike after rIL-7 treatment alone, host IL-7R signaling was crucial for maximum OT-I expansion in response to vaccination and rIL-7. Additionally, OT-I cells up-regulated CD127 and down-modulated PD-1 in a host-IL-7R-dependent fashion. This was not the case for all other markers tested. The host-dependent modulation of CD8$^+$ T cell differentiation was confirmed in BM chimeras. Surprisingly, we identified IL-7R$^+$ radio-resistant host cells as major regulators of CD8$^+$ T cell expansion and differentiation. For maximum levels of CD127 expression and restriction of KLRG-1, CD132, Bcl-2, CD8, and PD-1, IL-7R expression by radio-resistant host cells was sufficient. CD127 expression is modulated by multiple intracellular signaling events in T cells. Whether non-BM-derived host cells affect these or other signaling pathways remains open.

Besides OT-I expansion and differentiation, IL-7R$^+$ non-BM-derived cells also controlled granulocyte and DC expansion. Even if donor BM was devoid of the IL-7R, its expression by radio-resistant cells supported the expansion of CD11b$^+$ cells and DCs. However, IL-7R expression by BM-derived cells was largely sufficient to rescue the expansion of CD11b$^+$ cells and CD8$^-$ DCs. Surprisingly, this was not the case for CD8$^+$ DCs. Their accumulation depended more on IL-7R expression by non-BM- than by BM-derived cells. Together, we demonstrate that IL-7R signaling in BM- and non-BM-derived cells contributes to rIL-7-driven DC expansion. This might help to reconcile apparently conflicting results regarding the relative

importance of cell autonomous IL-7R signaling for DC generation obtained in different experimental systems [24,45].

Fibroblastic reticular cells (FRCs) and lymphoid endothelial cells (LECs) are major sources of IL-7 in secondary lymphoid organs [46]. Furthermore, other cells of non-BM origin such as intestinal epithelial cells (IECs) [47], keratinocytes [48], hepatocytes [49] and fibroblasts [50] were shown to produce IL-7 *in vivo*. The maintenance of CD8^{+} T cell homeostasis requires IL-7R signaling in CD8^{+} T cells which is triggered by IL-7 from non-BM derived cells [20]. However, only little is known about the consequences of IL-7R signaling in non-BM-derived cells and subsequent immune modulation [51]. As we have shown in S2 Fig, the application of rIL-7 leads to the systemic down-modulation of *il-7* gene activity supporting previous reports demonstrating that *il-7* gene activity is regulated in an IL-7-mediated negative feedback loop [4,12]. Given that IL-7R signaling modulates gene expression profiles in multiple non-BM-derived cell types [4,12,26], long-term rIL-7 therapy would not only affect immune cell but also non-immune cell homeostasis. For instance, prolonged IL-7 overabundance promotes IEC expansion, survival and subsequent alterations in intestinal physiology [12]. Since many cell types of non-BM-derived origin can express the IL-7R [31], the local down regulation of endogenous IL-7 production and alterations in tissue homeostasis might be as yet underestimated side effects of rIL-7 therapy. Whether i) non-BM-derived cells located in the tumor stroma and/or other tissues are the main targets of rIL-7, and ii) whether rIL-7 signaling in such cells promotes or suppresses rIL-7-assisted ATT remains to be shown.

IL-7R^{+} host cells appear to promote antigen-dependent CD8^{+} T cell function also in an IL-7-independent fashion. For example, successful peptide vaccination and subsequent tumor rejection, in the absence of rIL-7 treatment, strictly required host IL-7R expression (Fig 7A and 7B). Importantly, however, peptide vaccination blocked the therapeutic effect of rIL-7 (Figs 1A and 7A) in Rag^{-/-} mice although IL-7R-dependent DC expansion was normal (Fig 7C). It has been reported that TCR signaling can interfere with the beneficial effects of IL-7 on T cells [52]. Hence, impaired tumor rejection in rIL-7-treated, peptide vaccinated Rag^{-/-} mice might have been a result of altered T cell rather than host cell function.

In summary, our data provide evidence for the complex interplay between IL-7R^{+} host and CD8^{+} T cells in the course of anti-tumor CD8^{+} T cell responses. While productive host-CD8^{+} T cell interactions can be promoted by rIL-7 therapy, the inappropriate combination with other immune stimuli can cause adverse effects.

Supporting Information

S1 Fig. Lymphopenia-induced T cell proliferation (LIP) is independent of host IL-7R signaling. (A, B) Rag^{-/-}, Rag^{-/-}IL-7R^{-/-} and WT mice were reconstituted with 1 x 10^6 CD8[+-]CD90.1^{+} OT-I T cells. Spleens were analyzed by flow cytometry 21–27 days later. Shown are (A) relative fluorescence intensities for CD44 on CD8^{+}CD90.1^{+} OT-I T cells. (B) Cell numbers were normalized to the mean values determined in treated Rag^{-/-} mice and are shown as relative (rel.) values. Data (±SEM) from 3 independent experiments with a total of 9–19 mice/group are shown.
(EPS)

S2 Fig. IL-7 administration decreases host *il-7* expression. (A, B) IL- representative mouse before (0h) and 24 hours (24h) after IL-7 treatment are shown. (B) BLI levels measured at 24h were calculated in relation to those determined before treatment and relative BLI (rel. BLI) values were calculated. Shown are pooled data (± SEM) of 2 experiments with a total of 6 mice. Statistical analysis was performed using Wilcoxon matched-pairs signed rank test.
(EPS)

Acknowledgments

Author Contributions

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