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Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies

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Identifying key factors that enhance immune responses is crucial for manipulating immunity to tumors. We show that after a vaccine-induced immune response, adjuvant interleukin-7 (IL-7) improves antitumor responses and survival in an animal model. The improved immune response is associated with increased IL-6 production and augmented T helper type 17 cell differentiation. Furthermore, IL-7 modulates the expression of two ubiquitin ligases: Casitas B-lineage lymphoma b (Cbl-b), a negative regulator of T cell activation, is repressed, and SMAD-specific E3 ubiquitin protein ligase-2 (Smurf2) is enhanced, which antagonizes transforming growth factor-β signaling. Notably, we show that although short term IL-7 therapy potently enhances vaccine-mediated immunity, in the absence of vaccination it is inefficient in promoting antitumor immune responses, despite inducing homeostatic proliferation of T cells. The ability of adjuvant IL-7 to antagonize inhibitory networks at the cellular and molecular level has major implications for immunotherapy in the treatment of tumors.

Tremendous efforts have been made to induce antitumor immunity with various vaccine strategies. Despite much enthusiasm and optimism, numerous trials have shown minimal efficacy[1]. This highlights the need for new approaches to augment antitumor immunity *in vivo*. To this end, we have studied a mouse model in which an antitumor response can be elicited through viral vaccination. RIP-TAG2 transgenic mice expressing the SV40 large T antigen (TAG) under the control of the rat insulin promoter (RIP) develop pancreatic β-islet cell tumors[2]. As the tumors grow, more insulin is produced, and blood glucose readings directly reflect the tumor burden[2]. These mice have been crossed with transgenic mice expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) under the control of the RIP[3] to produce RIP(GP × TAG2) mice. Although the tumors expressed GP, they were not cleared by the immune system[4]. Additionally, if these mice were infected with LCMV, mimicking a live viral antitumor vaccine, a limited increase in survival was observed, but the antitumor response was not maintained[4].

Studies have shown that an immune response can be sustained if immune effector cells lack the proapoptotic molecule Bim[5,6]. *In vitro*, this improved survival can be mimicked by addition of IL-7 (ref. 5), and *in vivo* adjuvant IL-7 together with immunization improved survival of effector and memory T cells[7,8]. IL-7 is required for the development of the immune system, and studies have shown that exogenous IL-7 increases T cell and B cell numbers in mice[9]. A similar T cell effect was reported in a clinical trial in which IL-7 was administered subcutaneously to patients[10]. The limited toxicity associated with IL-7 treatment, as opposed to other cytokines such as IL-2, highlights the potential of this cytokine as a therapeutic agent. Several studies have examined the therapeutic potential of IL-7 in diverse models of disease[11–14]. Our limited understanding of how this cytokine functions beyond its role in development, homeostasis, survival and proliferation and, indeed, whether vaccination is a prerequisite for IL-7 immune treatments, has limited its translation to human therapy.

Here we show that a vaccine-induced antitumor response can be augmented and sustained by providing exogenous IL-7. The enhanced response can be harnessed to directly target spontaneously arising tumors, and the resulting immune-mediated antitumor effect profoundly improves survival. These studies have also identified new ways through which IL-7 can enhance tumor specific immunity *in vivo*.

RESULTS

IL-7 boosts vaccine-induced antitumor immunity and survival

To investigate the effectiveness of exogenous recombinant human IL-7 in sustaining a primary *in vivo* response, we treated LCMV-vaccinated tumor-bearing RIP(GP × TAG2) mice with IL-7 or PBS for 14 d. We initiated treatment 8 d after LCMV infection, coinciding with the peak

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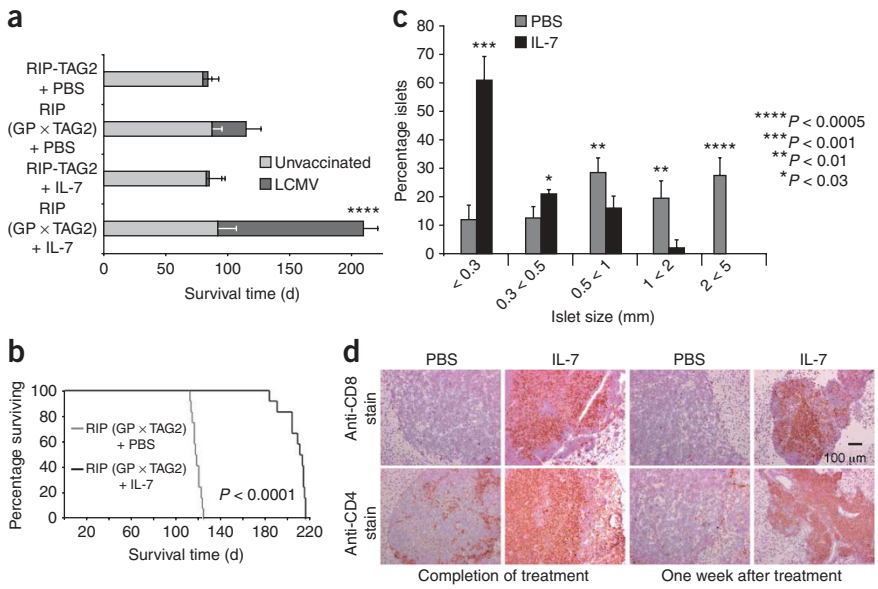

Figure 1 IL-7 prolongs survival, decreases tumor burden and enhances tumor T cell infiltration in vaccinated mice. (**a**) Survival of tumor-bearing mice after birth. Survival times for mice of the indicated genotypes that were either unvaccinated or infected with LCMV and then treated with IL-7 or PBS. Error bars represent mean ± s.d. obtained from 12 mice of each genotype and in each treatment arm. Differences in survival between naive or LCMV-vaccinated RIP(GP × TAG2) and RIP-TAG2 mice treated with IL-7 or PBS. Statistically significant differences are indicated (*$P \leq 0.0001$). (**b**) Survival curves of treatment groups. Time from birth until death is indicated in the survival curves for the two indicated treatment arms ($n = 12$ in each group). (**c**) Tumor sizes in treated mice. RIP(GP × TAG2) mice were vaccinated and treated with PBS or IL-7 and islet tumor sizes were measured on histological sections 1 week after completion of treatment. The bar graphs represent the average proportion of islets, from 4 mice in each group, with the indicated diameters. Error bars represent means ± s.d. (**d**) Pancreas histology. LCMV-vaccinated mice were killed at the completion of IL-7 or PBS treatment or 1 week after the last dose of IL-7 or PBS. Sections were stained for CD4 and CD8 with specific antibodies (anti-CD4 and anti-CD8).

of the cytotoxic T lymphocyte (CTL) response, viral elimination and the onset of the contraction phase. Mice receiving IL-7 tended to have a faster onset and more pronounced level of euglycemia or mild hyperglycemia than PBS-treated mice; this was most obvious at late time points, suggesting an enhanced antitumor response (**Supplementary Fig. 1** online). Survival of IL-7–treated mice was prolonged by over 100 d compared to control mice (**Fig. 1a**,**b**). This profound increase in survival was dependent on efficient vaccination and the expression of GP on the tumor (**Fig. 1a**).

Histology revealed that tumors from IL-7–treated mice were heavily infiltrated with both $CD4^+$ and $CD8^+$ T cells compared to PBS-treated mice, which consistently had larger tumors (**Fig. 1c**,**d**). We quantified the absolute number of lymphocytes, granulocytes and macrophages in the pancreatic draining lymph nodes (PDLNs), nondraining inguinal lymph nodes (ILNs), spleen and pancreas of mice after 2 weeks of IL-7 or PBS treatment. IL-7–treated mice with tumors had a 3.5- to 10-fold increase in both $CD4^+$ and $CD8^+$ T cell numbers compared to PBS control mice (**Supplementary Fig. 2a** online and data not shown). B cell numbers were also higher in IL-7–treated mice, but we did not observe differences in the number of granulocytes or macrophages (**Supplementary Fig. 2a**). Similarly, the number of $CD4^+$ T cells with an activated phenotype was higher in IL-7–treated mice (**Supplementary Fig. 2a**). Tetramers, incorporating peptides representing the viral epitope sequences of LCMV glycoprotein (GP33–41 and GP276–286) and nucleoprotein (NP396-404), were used to stain cells. The numbers of GP33-, GP276- and NP396-specific $CD8^+$ T cells were at least 16 times higher in the PDLN of IL-7–treated mice compared to PBS controls (**Supplementary Fig. 2a**). Although less pronounced, there was also a significantly ($P < 0.005$) higher number of GP33- and GP276-specific $CD8^+$ T cells in the spleen (**Supplementary Fig. 2a**) and ILN (data not shown), perhaps reflecting preferential expansion in nodes draining the tumors. Most notably, we isolated pancreatic infiltrating leukocytes (PILs) from tumor-bearing RIP(GP × TAG2) mice that had been infected with LCMV and treated for 7 d with PBS or IL-7. We observed an approximately three times higher number of GP61-specific $CD4^+$ T cells and GP33-, GP276- and NP396-specific $CD8^+$ T cells in IL-7–treated mice (**Supplementary Fig. 2c**). Therefore, the enhanced tumor immune response directly correlated with a significant accumulation and persistence of GP-specific T cells at the tumor site.

IL-7 enhances survival of activated T cells

Despite the downregulation of the IL-7 receptor subsequent to antigen recognition, the higher number of LCMV-specific cells in IL-7–treated mice could be due to enhanced survival and or proliferation of activated cells (**Supplementary Fig. 3** online). IL-7 could also be improving antigen-presenting cell (APC) function and thereby enhancing T cell priming[15]. To investigate the mechanism causing the large T cell expansion, we isolated virus-activated, GP-specific $CD8^+$ or $CD4^+$ T cells from LCMV-infected mice expressing a T cell receptor (TCR) specific for the major histocompatibilty complex (MHC) class I–restricted ($H\text{-}2D^b$) LCMV-GP33 epitope or the MHC class II ($I\text{-}A^b$)-restricted LCMV-GP61 epitope, known as P14 or Smarta mice, respectively[16,17]. We isolated splenic T cells just before the contraction of the immune response. In the absence of IL-7, *in vivo*–primed LCMV-specific T cells died rapidly in culture, a finding consistent with previous *in vitro* studies[18] (**Fig. 2a**).

We also transferred *in vivo*–primed LCMV-specific $CD8^+$ carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T cells into mice that had been infected with LCMV 8 d earlier. We found that 6 d of IL-7 treatment enhanced both survival and expansion of cells (**Fig. 2b**). Consistent with these data, we found that mice treated with IL-7 for 5 d, commencing on day 8 after infection, had a smaller proportion of LCMV-specific apoptotic T cells in the spleen compared to control mice (**Supplementary Fig. 4** online). Collectively, these data show that the high numbers of LCMV-specific T cells in IL-7–treated mice are due to enhanced survival of $CD8^+$ T cells, which can continue to proliferate in conditions when antigen and other cytokines are limiting.

IL-7 treatment enhances cytolytic activity *in vivo*

We next investigated whether IL-7 is able to maintain and promote the cytotoxic activity of LCMV-specific T cells[19]. Notably, although we observed three times more GP33-specific T cells in the pancreas

of vaccinated IL-7–treated tumor-bearing mice compared to controls (**Supplementary Fig. 2c**), we observed eight to ten times higher *ex vivo* PIL-mediated GP33-specific cytotoxic activity (**Fig. 2c**).

On a per cell basis, we observed a threefold higher GP33-specific cytotoxic activity (**Supplementary Fig. 5** online). Of note, we found that GP33-specific CD8$^+$ T cells isolated from vaccinated IL-7–treated tumor-bearing mice showed a tenfold increase in granzyme B abundance and more rapid and robust kinetics and capacity to degranulate (**Fig. 2d,e**). This enhanced activity could be a direct effect of IL-7 on CTLs, or, alternatively, IL-7 may indirectly create a milieu that is more conducive to efficient effector activity.

IL-7 augments and promotes multiple effector cell responses

Recently, the T helper type 17 (T_H17) subset of CD4$^+$ T cells has been shown to be crucial in promoting inflammation and pathology in animal models[20–22]. We characterized the CD4$^+$ T cells in the PIL population of PBS– or IL-7–treated, LCMV-infected tumor-bearing mice. In addition to a sevenfold higher proportion of cells that produce IL-2, there was an 18-fold higher number of IL-17–producing CD4$^+$ T cells in IL-7–treated mice compared to PBS-treated mice (**Fig. 2f,g**).

A similar increase was also seen in the number of natural killer (NK) and NKT cells infiltrating the pancreas of IL-7–treated, LCMV-vaccinated tumor-bearing mice (**Fig. 2h**). These increases in

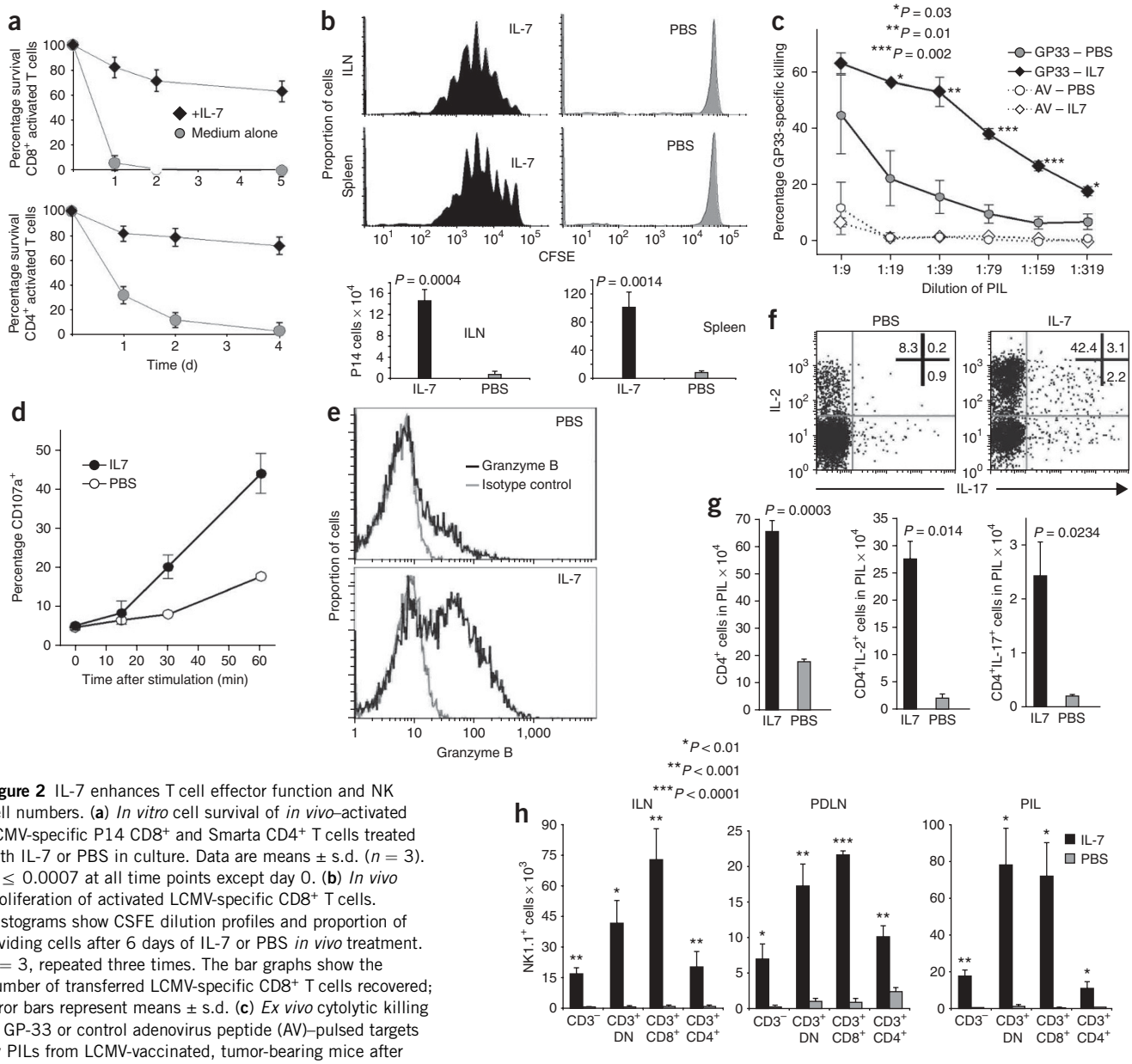

Figure 2 IL-7 enhances T cell effector function and NK cell numbers. (**a**) *In vitro* cell survival of *in vivo*–activated LCMV-specific P14 CD8$^+$ and Smarta CD4$^+$ T cells treated with IL-7 or PBS in culture. Data are means ± s.d. ($n = 3$). $P \leq 0.0007$ at all time points except day 0. (**b**) *In vivo* proliferation of activated LCMV-specific CD8$^+$ T cells. Histograms show CSFE dilution profiles and proportion of dividing cells after 6 days of IL-7 or PBS *in vivo* treatment. $n = 3$, repeated three times. The bar graphs show the number of transferred LCMV-specific CD8$^+$ T cells recovered; error bars represent means ± s.d. (**c**) *Ex vivo* cytolytic killing of GP-33 or control adenovirus peptide (AV)–pulsed targets by PILs from LCMV-vaccinated, tumor-bearing mice after 7 d of IL-7 or PBS treatment. $n = 3$ mice. Error bars represent means ± s.e.m.; the experiment was repeated three times. (**d**) *Ex vivo* degranulation of GP33-specific CD8$^+$ PILs cultured with EL4 GP33 peptide–pulsed cells. The proportion of CD107a$^+$ GP33-specific CD8$^+$ T cells is shown. $n = 3$; error bars represent means ± s.d.; the experiment was repeated three times. (**e**) Granzyme B expression in PILs; the experiments were repeated three times (12 mice). (**f,g**) Dot plot profiles (**f**) and number (**g**) of *in vitro*–re-stimulated CD4$^+$ PILs producing IL-2 and IL-17 (gated on CD4). (**h**) Number and subsets of NK1.1$^+$ cells in ILNs, PDLNs and pancreas (PIL) of vaccinated tumor-bearing mice after 1 week of IL-7 or PBS treatment. $n = 3$; error bars represent means ± s.d.

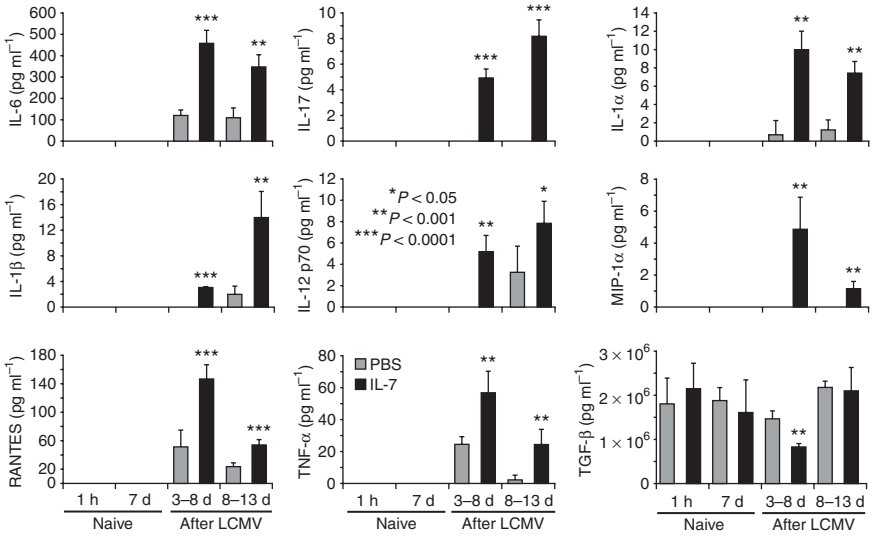

Figure 3 Serum cytokine levels in naive and LCMV-infected mice treated with PBS or IL-7. Naive mice were given 20 μg IL-7 or PBS intravenously and killed 1 h later for serum collection (naive 1 h). Alternatively, naive mice were injected subcutaneously once daily for 7 d with 10 μg IL-7 or PBS (naive 7 d). LCMV-infected mice received 10 μg of IL-7 or PBS subcutaneously once daily for 5 d commencing 3 d after infection or 8 d after infection (3–8 d and 8–13 d, respectively). Error bars represent means ± s.d., $n = 4$ for each group. The experiment was reproduced twice. MIP-1α, macrophage inflammatory protein-1α.

IL-2–producing cells, NK cells and T_H17 cells in tumor-bearing mice treated with IL-7 may create a pathogenic milieu that overcomes tumor-associated inhibitory factors.

IL-7 enhances cytokine production after infection

In view of the enhanced T cell effector function and increase in T_H17 cell numbers in IL-7–treated, vaccinated, tumor-bearing mice, we evaluated the serum cytokine concentrations in naive or LCMV-infected mice treated with IL-7 or PBS. Neither a single nor a repeated daily dosing of IL-7 over 7 d affected serum cytokine concentrations (**Fig. 3**). However, we observed a marked three- to tenfold increase in serum concentrations of IL-6, IL-1α, IL-1β, IL-12, tumor necrosis factor-α, C-C chemokine ligand-5 (RANTES), macrophage inflammatory protein-1α and other cytokines when we administered IL-7 daily for 5 d starting 3 or 8 d after LCMV infection (**Fig. 3**).

Most notably, IL-17 was undetectable in PBS-treated mice, but elevated concentrations were detected in IL-7–treated, LCMV-infected mice (**Fig. 3**). These findings emphasize that, in its homeostatic role, IL-7 alone is insufficient to initiate cytokine production. Instead, IL-7 potently augments serum cytokine concentrations after LCMV infection.

IL-7 promotes T cell refractoriness to inhibitory networks

A further explanation for the increased functional antitumor activity in IL-7–treated mice could involve suppression of the negative regulators of lymphocyte function. One report suggests that IL-7 may inhibit the ability of T regulatory (T_{reg}) cells to suppress effector cells *in vitro*[23]. We found that IL-7–treated RIP(GP × TAG2) tumor-bearing mice had a lower percentage of T_{reg} cells amongst total T cells in the ILN and PDLN (**Supplementary Fig. 6** online). However, we found a relative increase in the proportion of T_{reg} cells amongst the T cell population in the pancreas of IL-7–treated mice (**Supplementary Fig. 6**). Nonetheless, in IL-7–treated mice we observed an increase in GP33-specific *ex vivo* cytolytic activity (**Fig. 2c**) and a corresponding decrease in insulinoma size (**Fig. 1c**) compared to PBS–treated controls. Therefore the T_{reg} cells do not completely block CTL activity or prevent tumor destruction *in vivo*.

Alternatively, IL-7 may render effector cells refractory to T_{reg} cell–mediated inhibition. To examine this possibility, we performed a suppression assay with $CD4^+CD25^-$ T cells or T_{reg} cells from mice treated for 3 d with IL-7 or PBS. We found that $CD4^+$ T cells pretreated *in vivo* with IL-7 were refractory to T_{reg} cell inhibition (**Fig. 4a**), whereas T_{reg} cells pretreated *in vivo* with IL-7 were still able to suppress proliferation of $CD4^+$ T cells from PBS-treated mice (data not shown). Collectively, these data indicate that IL-7 increases $CD4^+$ LCMV-specific T effector cell survival, expansion and function directly and also makes them refractory to T_{reg} cell inhibitory pathways.

Other inhibitory factors such as TGF-β, IL-10, programmed death ligand-1 (PDL-1) and PDL-2 can antagonize antitumor and antiviral responses[24–28]. We found that tumors in uninfected RIP-TAG2 mice produce TGF-β and express PDL-1 but not PDL-2 (**Supplementary Fig. 6b**), and this was not affected by 7 d of IL-7 treatment (data not shown). We investigated whether IL-7 could render effector cells refractory to the effects of some of these inhibitory molecules. Notably, we found that PD-1 expression on effector T cells was marginally lower in IL-7–treated mice compared to control mice, which may reflect clearance of antigen or a direct or indirect effect of IL-7 on PD-1 expression (**Supplementary Fig. 6c**). It has been suggested that IL-7 may interfere with TGF-β signaling in fibroblasts[29]. We examined the effect of IL-7 on TGF-β signaling in T cells and found that TGF-β–induced inhibition of $CD8^+$ T cell proliferation was abrogated by the addition of IL-7 (**Fig. 4b**). The refractory state of IL-7–treated $CD4^+$ T cells to T_{reg} cells and the refractoriness of $CD8^+$ T cells to TGF-β effects may contribute to the enhanced capacity of these cells to degranulate and kill targets, respectively. Indeed, TGF-β has been shown to inhibit degranulation and thus cytolytic efficiency of tumor-specific $CD8^+$ T cells[30].

IL-7 regulates Smurf2 and TGF-β signaling *in vivo*

To elucidate the molecular mechanisms underlying the refractory phenotype of T effector cells and their indifference toward TGF-β and T_{reg} cells, we administered IL-7 intravenously or subcutaneously in naive or LCMV-infected mice and isolated T cells at various time points. TGF-β signaling involves receptor-mediated phosphorylation and activation of a set of proteins called Smads, which function as the core transcription factors in this pathway. We examined downstream TGF-β receptor signaling events and found that the abundance of phosphorylated Smad2 and Smad3 was lower in $CD8^+$ T cells from LCMV-infected mice treated for 5 d with IL-7 compared to PBS-treated mice (**Fig. 4c**). Total Smad3 expression was not altered by IL-7 (**Fig. 4c**). Smad3 has been implicated in promoting T cell tolerance by induction of the cyclin dependent kinase inhibitor protein $p27^{Kip1}$ (ref. 31). This potential tolerogenic pathway is antagonized by IL-7, given the marked reduction in $p27^{Kip1}$ levels observed in T cells from IL-7–treated mice (**Fig. 4d**),

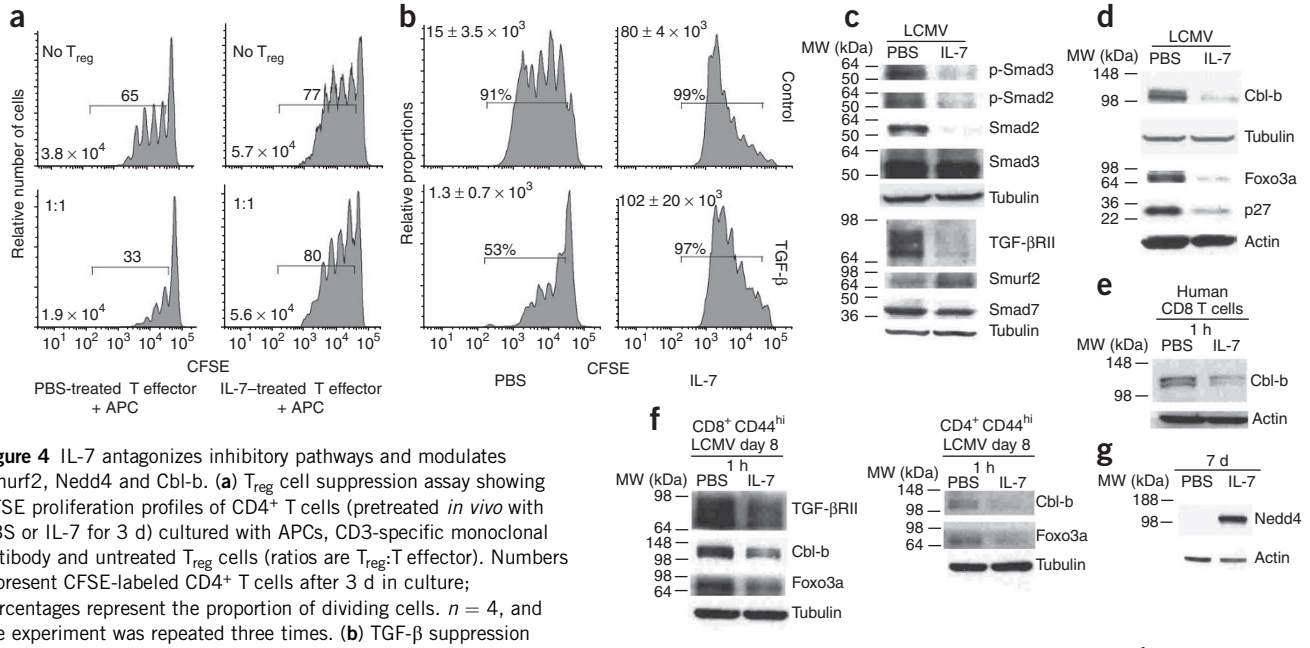

Figure 4 IL-7 antagonizes inhibitory pathways and modulates Smurf2, Nedd4 and Cbl-b. (**a**) T_{reg} cell suppression assay showing CFSE proliferation profiles of $CD4^+$ T cells (pretreated *in vivo* with PBS or IL-7 for 3 d) cultured with APCs, CD3-specific monoclonal antibody and untreated T_{reg} cells (ratios are T_{reg}:T effector). Numbers represent CFSE-labeled $CD4^+$ T cells after 3 d in culture; percentages represent the proportion of dividing cells. $n = 4$, and the experiment was repeated three times. (**b**) TGF-β suppression assay showing CFSE proliferation profiles of $CD8^+$ T cells cultured with APCs, CD3-specific monoclonal antibody and TGF-β (10 ng ml^{-1}) with IL-7 (500 ng ml^{-1}) or PBS. Numbers represent cells that have undergone at least one division after 3 d in culture (means ± s.d.); percentages represent the proportion of dividing cells. $n = 6$ mice, and the experiment was repeated twice. (**c**,**d**) Western blot analysis of purified $CD8^+$ T cells from LCMV-infected mice treated with PBS or IL-7 between days 3 and 8. TGF-β signaling components (**c**) and Cbl-b and Foxo3a proteins (**d**). Experiments were repeated 4 times. p-Smad3 and p-Smad2 indicate the phosphorylated proteins. TGF-βRII, TGF-β receptor II; MW, molecular weight. (**e**) Western blot analysis of human purified $CD8^+$ T cells treated with 500 ng ml^{-1} IL-7 for 1 h. (**f**) Western blot analysis of purified $CD8^+/CD44^{hi}$ T cells or $CD4^+/CD44^{hi}$ T cells isolated from LCMV-infected mice 8 d after infection. One hour before isolation, the mice had received 20 μg IL-7 or PBS intravenously. (**g**) Direct *ex vivo* western blot analysis of Nedd4 in splenic $CD4^+$ and $CD8^+$ T cells from naive mice treated for 7 d with IL-7 or PBS.

consistent with the pronounced drop in forkhead box O3 expression (**Fig. 4d**).The expression of total Smad2 was also downregulated by IL-7 (**Fig. 4c**).

We also observed a decrease in TGF-β receptor II expression in IL-7–treated mice (**Fig. 4c**). Smurf2, a ubiquitin ligase, and Smad7 have been shown to target Smad2 and/or the TGF-β receptor for degradation[32,33]. We found that Smurf2, but not Smad7, was upregulated in $CD8^+$ T cells treated *in vivo* with IL-7 (**Fig. 4c**). Consistent with the refractory nature of T cells treated with IL-7 toward TGF-β (**Fig. 4b**), our results indicate that IL-7 may have a role in modulating inhibitory signaling by downregulating TGF-β receptor II and Smad2 via Smurf2 activity.

Cbl-b expression in T cells is negatively regulated by IL-7 *in vivo*

Cbl-b–deficient T cells are resistant to TGF-β and T_{reg} cell suppression, much like IL-7–treated cells[34,35]. We next investigated whether IL-7 could be regulating Cbl-b expression in T cells. Indeed, *in vivo* administration of IL-7 lowered Cbl-b expression in $CD8^+$ T cells from LCMV-infected mice (**Fig. 4d**). These effects were also observed when human T cells were treated *in vitro* with IL-7 (**Fig. 4e**). Furthermore, all effects in mouse T cells were evident even after 1 h of *in vivo* IL-7 treatment, comparing cells expressing the same set of activation markers (**Fig. 4f**).

We next tested whether IL-7 administration could recapitulate the effects of Cbl-b deficiency in an *in vivo* model. We compared the outcome of an immune response in mice transgenically expressing the LCMV GP on the surface of β-islet cells (RIP-GP) after infection with recombinant vaccinia virus expressing the same GP (vacc-GP). Normally in RIP-GP mice, vacc-GP infection does not cause substantial β-islet cell destruction, and mice do not develop diabetes (**Supplementary Fig. 7a** online). However, diabetes was induced in vacc-GP–infected, Cbl-b–deficient RIP-GP mice and vacc-GP–infected, wild-type RIP-GP mice treated with once-daily subcutaneous injections of IL-7 (**Supplementary Fig. 7a**). Indeed, the parallels between IL-7 treatment and Cbl-b deficiency became more apparent when we examined the ability of PILs to produce cytokines in response to *ex vivo* peptide re-stimulation. Both IL-7–treated and Cbl-b–deficient RIP-GP mice, infected with vacc-GP 9 d earlier, showed the same two to three times higher numbers of IFN-γ– or IL-2–producing cells amongst the PIL population compared to control mice (**Supplementary Fig. 7b**,**c**). Collectively, our data show that IL-7 stimulation of T cells can lead to the downregulation of Cbl-b, and, accordingly, IL-7 treatment of wild-type mice generates similar phenotypes as $Cblb^{-/-}$ mice.

Recent work has shown that the E3 ubiquitin ligase Nedd4 augments the adaptive immune response by promoting ubiquitin-mediated degradation of Cbl-b in activated T cells[36]. Evidence suggests that Nedd4 promotes the conversion of naive T cells into activated T cells through this mechanism *in vivo*[36]. As IL-7 is a negative regulator of Cbl-b expression (**Fig. 4d**,**e**), we investigated whether this repression may, in part, be due to Nedd4 upregulation. Naive T cells isolated from mice treated with IL-7 for 7 d showed a marked increase in Nedd4 abundance (**Fig. 4g**). Therefore, it is likely that IL-7 leads to the degradation of Cbl-b by upregulating Nedd4.

Multiple IL-7 effects can enhance antitumor immunity

Evidence suggests that IL-7 coordinates a multifaceted response to augment immunity by favoring T_H17 differentiation, repressing Cbl-b, inhibiting TGF-β–associated pathways and rendering the immune system refractory to the inhibitory effects of T_{reg} cells. To dissect the relevance of each of these parameters, we examined their individual contribution in augmenting an antitumor immune

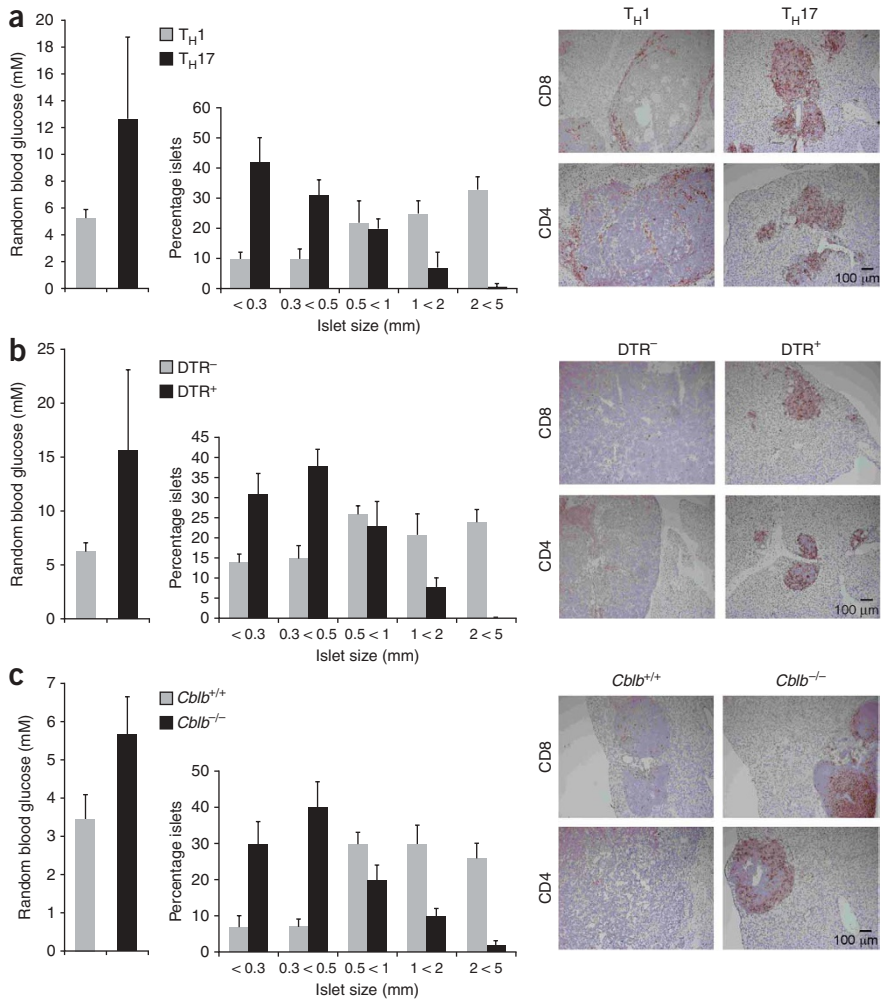

Figure 5 The presence of T_H17 cells, the depletion of T_{reg} cells or the absence of Cbl-b in T cells promotes an antitumor immune response. (**a**) Effect of adoptively transferred, *in vitro*–skewed tumor-specific Smarta T_H17 cells or T_H1 cells in RIP(GP × TAG2) LCMV vaccinated mice. Blood glucose levels 2 weeks after treatment (left), tumor sizes (middle) and tumor T cell infiltration (right). (**b**) Effect of T_{reg} cell depletion in RIP(GP × TAG2) LCMV-vaccinated mice. T_{reg} cells were depleted 6 d after LCMV infection and antitumor immune responses were measured (as in **a**) after 2 weeks of depletion. (**c**) Effect of adoptively transferred, *in vivo* activated, Cbl-b–deficient T cells in RIP(GP × TAG2) LCMV vaccinated mice. Bar graphs represent means ± s.d. of data obtained from three mice in each group. These experiments were repeated twice.

response. We examined the role of T_H17 cells by adoptively transferring *in vitro*–activated and skewed primary Smarta $CD4^+$ T cells into RIP(GP × TAG2) mice that had been immunized with LCMV 8 d earlier. We found that adoptive transfer of T_H17-differentiated Smarta cells was far superior to transfer of T_H1-differentiated Smarta cells in eliciting antitumor immunity (**Fig. 5a**). Two weeks after transfer, mice receiving T_H17 cells showed an improved antitumor response, as measured by blood glucose concentration, reduction in tumor burden and promotion of both $CD4^+$ and $CD8^+$ T cell tumor infiltration (**Fig. 5a**). Similar antitumor responses were reported in a mouse model using transplantable melanoma[37]. The effectiveness of T_H17 cells in promoting an antitumor response supports the likelihood that the increased numbers of T_H17 cells observed in the IL-7–treated mice contributes to the antitumor effect seen with IL-7 therapy.

To examine the role of T_{reg} cells in antitumor immunity, we crossed RIP(GP × TAG2) mice with bacterial artificial chromosome–transgenic Dereg mice, which express a diphtheria toxin receptor–GFP fusion protein under the control of the *Foxp3* gene locus (encoding forkhead box P3)[38]. This allowed us to follow and selectively deplete $Foxp3^+$ T_{reg} cells by diphtheria toxin injection in tumor-bearing mice. Depletion of T_{reg} cells in RIP(GP × TAG2) × DEREG mice 6 d after LCMV infection resulted in an enhanced antitumor response (**Fig. 5b**). Our data indicate that T_{reg} cells suppress antitumor immune responses. Furthermore, IL-7's ability to antagonize T_{reg} inhibitory networks may contribute to the effect of IL-7 in mediating antitumor immunity.

Studies have shown that Cbl-b deficiency results in enhanced immune-mediated rejection of transplantable or induced tumors[39,40]. To confirm that Cbl-b would also have a role in mediating tumor destruction of spontaneously arising insulinomas, we transferred Cbl-b–deficient or Cbl-b–sufficient T cells from mice infected with LCMV into RIP(GP × TAG2) mice also infected with LCMV. Again, we observed an enhanced antitumor response in mice receiving Cbl-b–deficient T cells compared to mice receiving wild-type T cells (**Fig. 5c**). These results indicate that Cbl-b also antagonizes T cell–mediated antitumor immune responses in this model. As IL-7 represses Cbl-b expression in T cells, this effect may contribute to IL-7's ability to promote antitumor immune responses. Collectively, our data suggest a role for each of these IL-7–modulated pathways in promoting an antitumor immune response resulting in tumor regression.

Adjuvant IL-7 promotes efficacy of multiple immunotherapies

We next investigated whether the effects of IL-7 in our LCMV vaccination model are applicable to other vaccines and clinically relevant approaches. We found that adjuvant IL-7 potently enhanced antitumor immune responses initiated by dendritic cell (DC) vaccination or vaccinia virus immunization in mice with spontaneous pancreatic tumors (**Fig. 6a,b**). *In vitro*–differentiated and matured DCs pulsed with GP33, GP276 and GP61 were transferred into RIP(GP × TAG2) tumor-bearing mice. IL-7 was administered at the time of transfer and continued daily for 2 weeks, at which time mice were analyzed for antitumor immune responses. Mice receiving DC vaccination together with IL-7 showed an enhanced antitumor response (**Fig. 6a**).

Similarly, we found that RIP(GP × TAG2) tumor-bearing mice immunized with recombinant vaccinia virus expressing LCMV GP and treated concurrently with IL-7 for 2 weeks had a pronounced

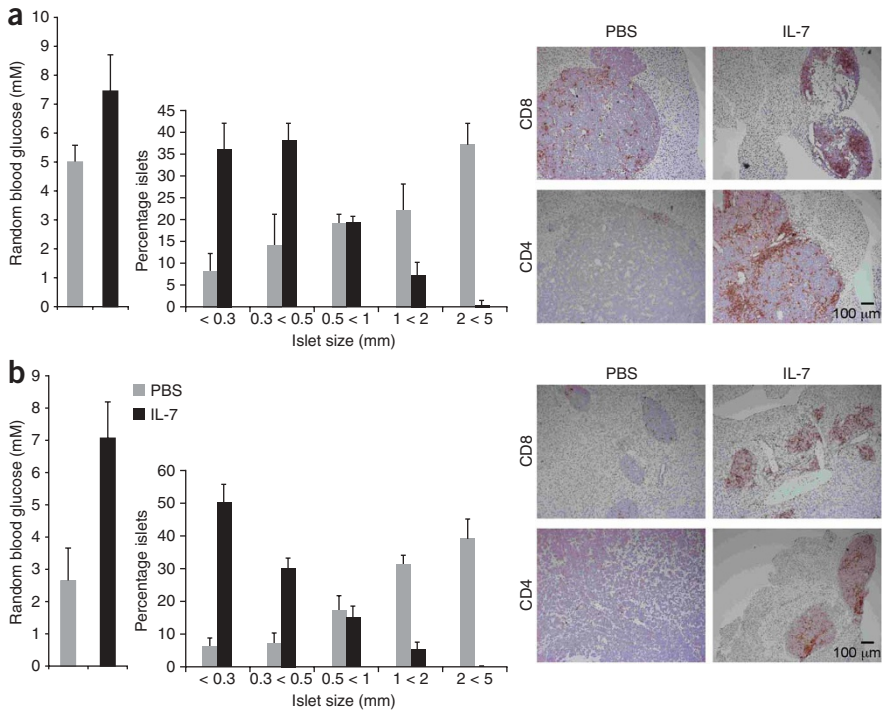

Figure 6 IL-7 therapy enhances antitumor immune responses in DC and vaccinia vaccination models. (**a**) Quantification of blood glucose (left), tumor size (middle) and T cell tumor infiltration (right) in DC vaccinated RIP(GP × TAG2) mice after 2 weeks of IL-7 or PBS treatment. (**b**) Blood glucose, tumor size and T cell tumor infiltration in vacc-GP immunized RIP(GP × TAG2) mice after 2 weeks of IL-7 or PBS treatment. Bar graphs represent means ± s.d. of data obtained from three mice in each group. These experiments were repeated twice.

antitumor response compared to PBS-treated mice (**Fig. 6b**). These data underscore the potential clinical relevance and impact of IL-7 therapy in promoting efficacy using various protocols.

DISCUSSION

Previous studies have defined a role for IL-7 in homeostasis and have elucidated some of the potential intracellular mechanisms at play[7,13]. To rationally use IL-7 as a therapeutic tool and maximize its efficacy, a thorough understanding of the cellular and molecular networks affected by this cytokine in the relevant pathological settings is essential. We show that IL-7 combined with vaccination has functions beyond its simple survival- and proliferation-promoting roles. Effector cells isolated from IL-7–treated mice showed enhanced functional activity at the single-cell level. We found that after IL-7 treatment, CTLs acquire a more pathogenic phenotype with higher expression of granzyme B, faster and amplified degranulation kinetics, and more effective cytolytic activity. Recent *in vitro* studies suggest that these IL-7–mediated effects may be further improved by the addition of IL-21, which may act to prevent downregulation of IL-7 receptor[41].

IL-7 also affected $CD4^+$ T cells in our spontaneous tumor model, causing an 18-fold increase in the number of IL-17–secreting $CD4^+$ effector cells, indicating a strong skewing toward T_H17 differentiation. Current models predict that the relative proportions of TGF-β and IL-6 determine whether T_{reg} or T_H17 differentiation is favored[42]. The serum concentrations of IL-6 were greatly enhanced in vaccinated mice receiving IL-7. Recently, an IL-17–triggered positive feedback loop promoting IL-6 expression has been described[43]. IL-6, in turn, promotes further T_H17 differentiation, perpetuating the loop. Our data suggest that IL-7 may synergize in promoting this positive feedback loop.

We have characterized some of the molecular pathways to provide a deeper understanding of the mechanisms by which IL-7 promotes antitumor responses (**Supplementary Fig. 8** online). Several ubiquitin ligases have been implicated in negatively regulating T cell activity and modulating TGF-β signaling pathways[44–48].We show that IL-7 upregulates Smurf2, which downregulates TGF-β signaling. IL-7 also decreases expression of Cbl-b, which has been associated with multiple alterations in T cell function. Reports have shown that the absence of Cbl-b can render T cells refractory to T_{reg} cell suppression[34,35]. However, others studies have shown that Cbl-b may increase the threshold for T cell activation[49,50], and the ability of IL-7 to modulate Cbl-b expression may explain the observation that IL-7 has been shown to expand T cells with low-affinity TCRs[7]. Accordingly, the absence of Cbl-b has also been shown to augment immune responses to low-affinity antigens[51]. We have shown that IL-7 regulates expression of Nedd4, another E3 ubiquitin ligase, which converts naive T cells into activated effector cells through the targeted degradation of Cbl-b[36].

Notably, we found that the effect of IL-7 on antitumor immune responses was dependent on efficient vaccination, a major issue that stresses the need for optimal vaccination strategies to translate IL-7–based therapy to human cancers and chronic infections. This may explain the lack of a clinically favorable outcome in a recent pilot study in individuals with human melanoma[10] and suggests that, although limited spontaneous immune surveillance may occur, it is unlikely that these responses are sufficiently improved by IL-7 treatment alone.

Another immunotherapeutic approach has focused on adoptive transfer of tumor-specific T cells[52]. A key component of successful adoptive immune therapy protocols is preconditioning recipients with nonmyeloablative regimens to establish a lymphopenic state. Lymphopenia is associated with an increased availability of cytokines, such as IL-7 and IL-15, and is used as a preconditioning strategy to augment the efficacy of adoptive immunotherapy[13,53]. Our results provide alternative mechanistic insights underlying the improved efficacy. Modified protocols could provide exogenous IL-7 beyond the transient lymphopenic state to maintain high levels of this cytokine as endogenous IL-7 availability wanes. A more practical approach could be to promote *in vivo* priming and expansion of tumor-specific cells by administering IL-7 concurrently with targeted chemotherapy or other vaccination strategies[54].

In conclusion, our findings show that the addition of IL-7 along with vaccination can substantially increase antitumor immunity. The pleiotropic effects associated with IL-7 may act together to antagonize inhibitory networks. The relative importance, redundancy or crucial nature of any one effect remains to be explored. Further studies extending the length of IL-7 therapy, combining IL-7 with other strategies or using alternative repeated vaccination protocols will help elucidate the most effective use of this cytokine.

METHODS

Mice and tumor monitoring. Mice are described in detail in the **Supplementary Methods** online. We depleted T_{reg} cells from Dereg mice by administering 40 ng per g body weight diphtheria toxin (Merck) intraperitoneally every 3 d. We confirmed depletion by the absence of GFP^+ cells in peripheral blood using flow cytometry. We isolated Cbl-b–deficient[49] or Cbl-b–sufficient T cells from spleens of mice infected 8 d earlier with LCMV by using a pan–T cell enrichment kit (Miltenyi Biotec). We injected a total of 4×10^7 cells into the tail veins of host mice that had been infected 8 d earlier with LCMV. We determined blood glucose concentrations as previously described[55]. Mice were maintained and mouse experiments were performed at the Ontario Cancer Institute animal facility according to institutional guidelines and with approval of the Ontario Cancer Institute Animal Ethics Committee.

Lymphocytic choriomeningitis virus and interleukin-7 treatment. We immunized mice by intravenous infusion of LCMV Armstrong strain (3,000 plaque-forming units (PFU), except for P14 mice, which received 2×10^5 PFU). We infected tumor-bearing mice after two consecutive hypoglycemic readings ($\leq$5 mM, one week apart). Eight days after infection, we injected mice subcutaneously once daily with 10 μg of recombinant human IL-7 (Cytheris) or PBS for 2 weeks. We performed and repeated experiments with both recombinant *Escherichia coli*–derived and fully glycosylated Chinese hamster ovary cell–derived human IL-7 with similar results.

Histology, pancreatic infiltrating leukocyte isolation and flow cytometry. Immunohistological staining and analysis has been previously described[55]. We measured tumor sizes on histological sections from H&E-stained pancreas using vernier gradations on a light microscope. We randomly cut six sections from each organ and determined the sizes for all islets in the sections. The specific monoclonal antibodies used are detailed in the **Supplementary Methods**. All flow cytometry data was acquired on a FACSCalibur or FACSCanto (BD) and analyzed with FlowJo software (Tree Star). Tetramer production has been detailed previously[55]. We isolated PILs as previously described[55]. We stained for Foxp3 with a mouse T_{reg} cell staining kit (eBioscience). To quantify $CD4^+$ antigen-specific cells, we used γ-irradiated (30 Gy) T cell–depleted splenocytes, obtained with IMag cell separation system (BD Pharmingen), to re-stimulate antigen-specific cells. We plated T cell–depleted preparations at a density of 3×10^6 cells per well in a 24-well plate and added 1 μg ml^{-1} of GP61 peptide (GLNGPDIYKGVQFKSVEFD, New England Peptide) or an irrelevant OVA323 control peptide (ISQAVHAAHAEINEAGR) to the cultures. We added 4×10^5 PILs to each well in the presence of the intracellular protein transport inhibitor monensin, according to the manufacturer's instructions (BD Bioscience). We collected cells from the PIL cultures after 4 h and stained them for cytokine production as previously described[55]. To quantify the number of IL-17– and IL-2–secreting cells, we cultured PILs in the presence of phorbol myristate acetate (100 ng ml^{-1}), ionomycin (100 ng ml^{-1}) and 1 μl ml^{-1} monensin (BD Bioscience) for 4 h and then stained them as described above with IL-17– and IL-2–specific antibodies (BD Bioscience clones TC11-18H10 and JES6-5H4, respectively). To assess granzyme B expression, we collected PILs and labeled them with a monoclonal antibody specific for CD8 (BD Bioscience clone 53-6.7), fixed them, permeabilized them and stained them directly *ex vivo* with monoclonal antibody specific for granzyme B (eBiosciences clone GB11).

Degranulation assay. We assessed the ability of PILs to degranulate in the presence of GP33-pulsed (1×10^{-8} M) EL-4 mouse lymphoma cells (American Type Culture Collection TIB-39) as detailed in the **Supplementary Methods**.

Survival assays and adoptive transfer. We assayed *in vitro* and *in vivo* survival by propidium iodide (P.I.) staining and flow cytometry as detailed in the **Supplementary Methods**.

Cytotoxic T lymphocyte assays. We incubated EL-4 target cells with 200 μCi ^{51}Cr and 1×10^{-8} M GP33 peptide or nonstimulatory adenovirus peptide (SGPSNTPPEI) for 1 h at 37 °C. We then used these peptide-pulsed targets in an *ex vivo* killing assay as described in the **Supplementary Methods**.

T regulatory cell and transforming growth factor-β suppression assays. We cultured CFSE-labeled $CD4^+$ T cells treated *in vivo* with IL-7 or PBS in the presence of T_{reg} cells or TGF-β for 3 d and assessed proliferation by flow cytometry as detailed in the **Supplementary Methods**.

Serum cytokine assays. We assayed serum cytokine abundance with the SearchLight Array Service (Pierce Biotechnology).

Western blots. We performed all treatments with PBS or IL-7 *in vivo* for mouse cells. Direct *ex vivo* western blot analysis is detailed in the **Supplementary Methods**. We isolated human peripheral blood mononuclear cells from the peripheral blood of healthy donors after informed consent was obtained in accordance with the Ontario Cancer Institute.

T helper type 1 and T helper type 17 skewing and adoptive transfer. We isolated Smarta $CD4^+$ splenic T cells from transgenic mice. We skewed the cells *in vitro* and adoptively transferred them as described in the **Supplementary Methods**.

Vacc-GP infection. We infused vacc-GP (2×10^6 PFU) intravenously in mice and commenced IL-7 or PBS treatement on the day of infection. We isolated PILs from mice on day 9 after infection for *ex vivo* cytokine production.

Dendritic cell vaccination. We produced bone marrow–derived DCs as previously described[55] and matured them and peptide-pulsed them for vaccination as detailed in the **Supplementary Methods**.

Statistical analyses. We used unpaired two-tailed Student's *t*-tests for all comparisons except for data with multiple groups, for which we used analysis of variance. We used a log-rank test on survival data.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.P. and T.C. directed the project, designed and performed experiments, interpreted data and wrote the manuscript. P.S.O. and T.W.M. directed the project, assisted with experimental design, interpreted data and contributed to the preparation of the manuscript. A.R.E., A.S., A.E.L., D.D., S.D., L.T.N. and M.A.G. assisted with experiments. M.M., B.A., K.L. and T.S. provided reagents and contributed conceptually to the project.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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Erratum: Copy number analysis indicates monoclonal origin of lethal metastatic prostate cancer

Wennuan Liu, Sari Laitinen, Sofia Khan, Mauno Vihinen, Jeanne Kowalski, Guoqiang Yu, Li Chen, Charles M Ewing, Mario A Eisenberger, Michael A Carducci, William G Nelson, Srinivasan Yegnasubramanian, Jun Luo, Yue Wang, Jianfeng Xu, William B Isaacs, Tapio Visakorpi & G Steven Bova

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In the version of this article initially published, some of the sample identifiers were missing from Figure 1e. The error has been corrected in the HTML and PDF versions of the article.

Corrigendum: Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies

Marc Pellegrini, Thomas Calzascia, Alisha R Elford, Arda Shahinian, Amy E Lin, Dilan Dissanayake, Salim Dhanji, Linh T Nguyen, Matthew A Gronski, Michel Morre, Brigitte Assouline, Katharina Lahl, Tim Sparwasser, Pamela S Ohashi & Tak W Mak

Nat. Med. **15, 528–536 (2009); published online 26 April 2009; corrected after print 7 July 2009**

In the version of this article initially published, the two right histological sections in Figure 5a were duplicated in Figure 5b. The error has been corrected in the HTML and PDF versions of the article.