PD-1-cis IL-2R agonism yields better effectors from stem-like CD8+ T cells

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Expansion and differentiation of antigen-experienced PD-1+TCF-1+ stem-like CD8+ T cells into effector cells is critical for the success of immunotherapies based on PD-1 blockade1–4. Hashimoto et al. have shown that, in chronic infections, administration of the cytokine interleukin (IL)-2 triggers an alternative differentiation path of stem-like T cells towards a distinct population of ‘better effector’ CD8+ T cells similar to those generated in an acute infection5. IL-2 binding to the IL-2 receptor α-chain (CD25) was essential in triggering this alternative differentiation path and expanding better effectors with distinct transcriptional and epigenetic profiles. However, constitutive expression of CD25 on regulatory T cells and some endothelial cells also contributes to unwanted systemic effects from IL-2 therapy. Therefore, engineered IL-2 receptor β- and γ-chain (IL-2Rβγ)-biased agonists are currently being developed6–10. Here we show that IL-2Rβγ-biased agonists are unable to preferentially expand better effector T cells in cancer models and describe PD1-IL2v, a new immunocytokine that overcomes the need for CD25 binding by docking in cis to PD-1. Cis binding of PD1-IL2v to PD-1 and IL-2Rβγ on the same cell recovers the ability to differentiate stem-like CD8+ T cells into better effectors in the absence of CD25 binding in both chronic infection and cancer models and provides superior efficacy. By contrast, PD-1- or PD-L1-blocking antibodies alone, or their combination with clinically relevant doses of non-PD-1-targeted IL2v, cannot expand this unique subset of better effector T cells and instead lead to the accumulation of terminally differentiated, exhausted T cells. These findings provide the basis for the development of a new generation of PD-1 cis-targeted IL-2R agonists with enhanced therapeutic potential for the treatment of cancer and chronic infections.
of DbGP33- and DbGP276-CD8+ T cells (Extended Data Fig. 1b,c). Conversely, muPD-L1 in combination with muFAP-IL2v was not superior to muPD-L1 monotherapy in increasing the numbers of LCMV-specific CD8+ T cells (Extended Data Fig. 1b,c). In addition to its quantitative advantage over muPD-L1 monotherapy, muPD-L1 in combination with muFAP-IL2wt changed the expression of various phenotypic markers on LCMV-specific CD8+ T cells (Extended Data Fig. 1d). muPD-L1 and muFAP-IL2wt combination therapy elevated the expression levels of CD127, CD218a and CXCR3 on LCMV-specific CD8+ T cells, all of which are critical molecules for functional effector and memory CD8+ T cell differentiation during acute infection. By contrast, expression of the inhibitory receptor TIM-3 was lower on LCMV-specific CD8+ T cells after muPD-L1 and muFAP-IL2wt combination therapy. These phenotypic changes achieved by adding muFAP-IL2wt to muPD-L1 therapy were absent when combining muPD-L1 with muFAP-IL2v (Extended Data Fig. 1d). Expanded LCMV-specific CD8+ T cells obtained after muPD-L1 and muFAP-IL2wt therapy were also more functional in their effector profiles of cytokine production than those obtained from muPD-L1 monotherapy in response to antigenic stimulation, whereas muFAP-IL2v administration had no additive effects to muPD-L1 therapy (Extended Data Fig. 1e,f). Notably, the most effective viral control was observed when combining muPD-L1 with muFAP-IL2wt therapy. By contrast, muFAP-IL2v treatment did not show synergy with muPD-L1 therapy in terms of viral reduction (Extended Data Fig. 1g).

It is important to note that muFAP-IL2v was biologically active in vivo, as muPD-L1 in combination with muFAP-IL2v significantly increased the number of total CD8+ T cells compared with muPD-L1 as monotherapy or in combination with muFAP-IL2wt during chronic LCMV infection (Extended Data Fig. 2a-c). However, when we characterized the increased number of CD8+ T cells, we found that combination of muPD-L1 with muFAP-IL2v mainly expanded non-LCMV-specific PD-1+CD8+ T cells during chronic infection (Extended Data Fig. 2d,e). This was in marked contrast to the muPD-L1 and muFAP-IL2wt combination, which preferentially expanded PD-1-CD8+ T cells that included LCMV-specific CD8+ T cells (Extended Data Fig. 2d,e). Expansion of non-LCMV-specific CD8+ T cells by muPD-L1 and muFAP-IL2v combination therapy implied a requirement for targeted delivery of IL-2v to PD-1-expressing LCMV-specific CD8+ T cells to achieve desirable biological outcomes.

**PD1-IL2v mediates cis delivery of IL-2v to PD-1+ T cells**

PD-1 is expressed on the surface of chronically activated antigen-specific T cells, including virus- and tumour-reactive T cells, and is a bona fide marker to identify antigen-specific T cells14-16. We designed PD1-IL2v to provide IL-2R agonism preferentially to PD-1 tumour-reactive T cells by binding and blocking the PD-1 inhibitory pathway while agonizing IL-2R signalling on the same cell. To measure the potency of PD1-IL2v versus FAP-IL2v, used here as IL-2v not targeted to T cells, we briefly incubated in vitro-activated PD-1-expressing polyclonal human CD4+ T cells with increasing amounts of either PD1-IL2v or FAP-IL2v before measuring IL-2R signalling through the levels of phosphorylated STAT5 (STAT5-P). In this assay, PD1-IL2v was found to be approximately 40-fold more potent than FAP-IL2v in delivering IL-2R agonism to PD-1+ T cells (Fig. 1a). To verify that PD-1 targeting mediated the observed difference in potency between the two compounds, we included a ‘versus’ assay in which PD1-IL2v was internalized with bound PD-1 receptors

**PD1-IL2v is internalized with bound PD-1 receptors**

Given that, following binding to IL-2, T cells internalize IL-2R within minutes18, we assessed the internalization of fluorescently labelled PD1-IL2v and of FAP-IL2v as a control using in vitro-activated PD-1 expressing polyclonal human CD4+ T cells. Additionally, we tracked the fate of simultaneously bound PD-1 receptors using a fluorescently labelled, non-competing anti-PD-1 antibody. We observed that, while FAP-IL2v was internalized within 1 h at 37 °C (Extended Data Fig. 3a,b), PD1-IL2v was internalized with slower kinetics (Extended Data Fig. 3a,c). Pretreatment of PD-1+ T cells with a competing anti-PD-1 antibody prevented binding of PD1-IL2v to PD-1, therefore inducing PD1-IL2v internalization through the IL-2R at rates similar to those induced by binding and blocking the PD-1 inhibitory pathway while agonizing IL-2R signalling on the same cell. In agreement with this finding, we observed an approximately twofold-higher number of PD-1 receptors per cell on PD1-IL2v, as compared to PD-1 receptors in PD1-IL2v treated T cells (Fig. 1d).
by FAP-IL2v, while leaving the PD-1 receptors on the T cell surface (Extended Data Fig. 3c). Pretreatment with the anti-PD-1 antibody did not affect FAP-IL2v internalization or surface expression of PD-1 receptors (Extended Data Fig. 3b).

These data suggest an unexpected additional mechanism of action of PD1-IL2v at a cellular level, where longer interaction of IL-2v with IL-2R could result in continuous signalling followed by internalization and removal of bound PD-1 receptors from the T cell surface.

**PD1-IL2v potently drives T cell effector functions**

IL-2 has been shown to induce secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) by T cells\(^2,3\), which is important for dendritic cell activation and maturation, in addition to enhancing T cell cytotoxic effector functions\(^2,3\). For this reason, we tested whether PD1-IL2v could also elicit GM-CSF secretion, in addition to granzyme B, from PD-1-expressing polyclonal human CD4\(^+\) T cells activated in vitro for 5 d. As expected, PD1-IL2v induced GM-CSF and granzyme B secretion by activated T cells in a dose-dependent fashion for dendritic cell activation and maturation, in addition to enhancing T cell cytotoxic effector functions\(^2,3\). For this reason, we tested whether PD1-IL2v could also elicit GM-CSF secretion, in addition to granzyme B, from PD-1-expressing polyclonal human CD4\(^+\) T cells activated in vitro for 5 d. As expected, PD1-IL2v induced GM-CSF and granzyme B secretion by activated T cells in a dose-dependent fashion and was roughly 30-fold more potent than untargeted FAP-IL2v, while PD-1 blockade alone did not induce any significant change in effector functions. Interestingly, PD1-IL2v was as potent as wild-type IL-2 with intact CD25 binding (aldesleukin) in eliciting T cell effector functions, in line with the hypothesis that PD-1-mediated cis delivery of IL-2v acts as a surrogate of CD25 for cis binding on the T cell surface (Fig. 1f and Extended Data Fig. 4a).
**Comparison of PD1-IL2v to IL-2Rb-biased IL-2 mutant**

An alternative approach to engineering IL-2 for systemic therapy is to increase its affinity for IL-2Rβ, with the aim of making signalling less dependent on cis anchoring through CD25. One such engineered IL-2 has been termed ‘superkine’ (ref. 24). This is a different approach as compared with targeting cytokines to specific immune cells by fusion to immune receptor-targeting antibodies such as in PD1-IL2v or other recently reported fusion proteins25,26. We therefore produced a FAP-IL2 superkine analogue with increased binding affinity for IL-2Rβ and compared it with PD1-IL2v for potency and cis targeting in the cis–trans STAT5-P assay in activated T cells. As a control, we used FAP-IL2v, which binds to IL-2Rβγ with an affinity comparable to that of wild-type IL-2 in the absence of IL-2Rα. We observed that the FAP-IL2 superkine analogue was tenfold more potent than FAP-IL2v in delivering IL-2R agonism to T cells. However, both the FAP-IL2 superkine analogue and FAP-IL2v were as active on PD-1− T cells as on PD-1+ T cells, regardless of PD-1 expression (Extended Data Fig. 4b). Conversely, PD1-IL2v was roughly 40-fold more potent on PD-1− T cells than on PD-1+ T cells, and on PD-1− T cells PD1-IL2v was fivefold more potent than the FAP-IL2 superkine analogue (Extended Data Fig. 4b).

We then extended our observations to more physiologically relevant conditions, by exposing peripheral blood mononuclear cells (PBMCs) from healthy donors to a non-saturating concentration (630 pM) of PD1-IL2v, FAP-IL2 superkine analogue or FAP-IL2v for 30 min before staining with a phycoerythrin (PE)-conjugated anti-PGLALA antibody to detect the bound molecule and with a panel of antibodies to phenotypically characterize the T cell subsets through flow cytometry. PD1-IL2v significantly bound to both PD-1− TCF1+ stem-like CD8+ T cells and PD1− TCF1− CD8+ T cells when compared with naive CD8+ T cells, Treg cells and natural killer (NK) cells (Extended Data Fig. 4c). By contrast, the FAP-IL2 superkine analogue and FAP-IL2v bound modestly to PD-1− TCF1+ CD8+ T cells and did not bind to PD-1− TCF1− stem-like CD8+ T cells, potentially because of their lower IL-2Rβ expression levels. In addition, FAP-IL2 superkine analogue was the only fusion protein to strongly bind to NK cells (Extended Data Fig. 4c).

This finding suggests a fundamental functional difference between targeted delivery of a mutated IL-2 devoid of CD25 binding to antigen-experienced cells that are PD-1+ and just engineering IL-2 for increased affinity for its receptor on all cells. The former approach provides increased selectivity for the IL-2R agonism on specific T cell populations, whereas the latter increases the potency of signalling on many cells irrespective of their antigen experience and is only regulated by the expression profile of IL-2R by overall lymphocytes.

**Targeted delivery of IL-2v to PD-1+CD8+ T cells**

Given the effective cis delivery of IL-2v to PD-1-expressing T cells by PD1-IL2v in vitro, we wondered whether muPD1-IL2v (Extended Data Fig. 4d) could efficiently deliver IL-2v to LCMM-specific CD8+ T cells in vivo during chronic infection. It is worth noting that PD-1 expression was highest on LCMM-specific CD8+ T cells compared with other T cell populations during chronic infection (Extended Data Fig. 5a). We compared the therapeutic efficacy of muPD-L1, muPD-L1 in combination with muFAP-IL2v, and muPD-L1 in combination with muPD1-IL2v during chronic infection and performed quantitative and qualitative analyses of LCMM-specific CD8+ T cells (Extended Data Fig. 5b). As previously shown, muFAP-IL2v therapy did not have additive effects in comparison to muPD-L1 therapy in terms of enhancing LCMM-specific CD8+ T cell responses (Extended Data Fig. 5c–e). Interestingly, combination of muPD-L1 with muPD1-IL2v was significantly superior to muPD-L1 monotherapy in increasing the numbers of LCMM-specific CD8+ T cells in all tissues analysed (Extended Data Fig. 5d). Moreover, muPD-L1 in combination with muPD1-IL2v induced qualitative changes in LCMM-specific CD8+ T cells, as exemplified by the polyfunctional signature (IFN-γ ‘TNF-α’ and IFN-γ ‘IL-2’) (Extended Data Fig. 5e), and altered the expression profiles of several phenotypic markers such as TIM-3, CD127, CD218α and CXC-R3 (Extended Data Fig. 5f).

To gain further insights into the qualitative attributes of LCMM-specific CD8+ T cells after muPD-L1 and muPD1-IL2v combination therapy, we performed a transcriptional analysis by RNA sequencing (RNA-seq) of LCMM-specific CD8+ T cells after the treatments. Principal-component analysis (PCA) showed that the transcriptional signature of LCMM-specific CD8+ T cells after muPD-L1 monotherapy was very similar to that of the untreated group (Extended Data Fig. 5g). Notably, adding muPD1-IL2v to muPD-L1 therapy changed the transcriptional signature of LCMM-specific CD8+ T cells, indicating that combination of muPD-L1 with muPD1-IL2v generated LCMM-specific CD8+ T cells that were distinct from those in the untreated or muPD-L1 single-treatment group (Extended Data Fig. 5g). The heatmap of differentially expressed genes across the treatment groups highlights the therapeutic potential of muPD1-IL2v therapy resulting from modulation of the differentiation status of LCMM-specific CD8+ T cells during chronic infection (Extended Data Fig. 5h). For example, muPD-L1 in combination with muPD1-IL2v elevated the expression levels of Cd28, an essential co-stimulatory molecule for improved CD8+ T cell responses to anti-PD-1 therapy27,28. Upregulated cytokine receptors included Il2ra, Il7r, Il18r1, Ifng1 and Il18rap, suggesting that LCMM-specific CD8+ T cells generated by muPD-L1 and muPD1-IL2v combination therapy are more responsive to inflammatory cytokines (IL-2, IL-18 and interferon-γ (IFNγ)) and the homeostatic cytokine IL-7, the latter of which is an important cytokine for survival and maintenance of naive and memory CD8+ T cells39–41, muPD-L1 and muPD1-IL2v also increased the abundance of molecules regulating T cell migration (Ccr2, Cx3cr1 and Cxcr3)42,43, adhesion (Ly6c2 and Cd44) and egress from lymphoid tissues (Sip1r1 and Klf2)44,45. All of these features are essential components for functional effector CD8+ T cells to respond to various co-stimulatory signals, cytokines and chemokines, followed by their migration to major sites of infection to exert effector functions. Indeed, Tbx21, a crucial transcription factor for effector CD8+ T cell differentiation46,47, was also upregulated by co-administration of muPD-L1 and muPD1-IL2v. Conversely, genes downregulated by muPD-L1 and muPD1-IL2v therapy included Tox and Pdcd1, which are two major regulators of T cell exhaustion48–50. Other inhibitory receptors (Lag3, Cd244a and Hvaccr2) and transcriptional factors (Tox2, Nra4a2, Nra4a1, Prdm1 and Egr2) associated with exhausted CD8+ T cells were also downregulated by muPD-L1 and muPD1-IL2v combination therapy (Extended Data Fig. 5h). Overall, LCMM-specific CD8+ T cells generated by combining muPD-L1 and muPD1-IL2v possessed increased expression of molecules critical for functional effector cells and decreased expression of major transcription factors and inhibitory receptors related to exhausted CD8+ T cells, in line with induction of antigen-specific CD8+ T cell states with better effector potential and skewed away from T cell exhaustion. Most notably, these quantitative and qualitative changes in LCMM-specific CD8+ T cells accomplished by co-treatment with muPD-L1 and muPD1-IL2v were linked to improved biological outcome, and muPD-L1 and muPD1-IL2v therapy resulted in the best viral control across the treatment groups (Extended Data Fig. 5i).

Interestingly, muPD1-IL2v monotherapy was sufficient to elicit the expansion of LCMM-specific CD8+ T cells in different organs (Fig. 2a and Extended Data Fig. 6a). However, combination with muPD-L1 was even more effective at increasing the numbers of polyfunctional LCMM-specific CD8+ T cells and imprinting marked phenotypic and transcriptional changes that were induced by muPD1-IL2v therapy (Fig. 2b–d and Extended Data Fig. 6b), resulting in significantly improved viral control in comparison to muPD1-IL2v monotherapy (Fig. 2e).

Finally, we assessed the responsiveness to IL-12 and IL-18 of the LCMM-specific CD8+ T cells generated following muPD-L1 and muPD1-IL2v co-treatment and the respective monotherapies. Splenocytes from in vivo-treated mice were briefly stimulated ex vivo with both cytokines...
before measuring the secretion of IFNγ by D\textsuperscript{b}GP33\textsuperscript{+} CD8\textsuperscript{+} T cells (Extended Data Fig. 6c). Interestingly, among the LCMV-specific CD8\textsuperscript{+} T cells obtained from mice treated with muPD1-IL2v monotherapy or muPD1-IL2v in combination with muPD-L1, a subset of T cells expressing the receptor for IL-18 rapidly secreted IFNγ after being exposed to IL-12 and IL-18 (Extended Data Fig. 6d,e).

These results together illustrate that targeted delivery of IL-2v to PD-1\textsuperscript{+} CD8\textsuperscript{+} T cells by muPD1-IL2v therapy was highly effective in enhancing LCMV-specific CD8\textsuperscript{+} T cell responses with a transcriptional signature of better effectors. In addition, combination of muPD1-IL2v with muPD-L1 further improved some effector attributes such as polyfunctionality as compared with muPD1-IL2v monotherapy and was particularly effective at viral control during chronic infection in this model.

**muPD1-IL2v acts on PD-1\textsuperscript{+} TCF-1\textsuperscript{+} stem-like CD8\textsuperscript{+} T cells**

During chronic infection, PD-1\textsuperscript{+} LCMV-specific CD8\textsuperscript{+} T cells are a heterogeneous cell population with distinct biological features, and the
stem-like (TIM-3 TCF-1') and terminally differentiated (exhausted; TIM-3 TCF-1') subsets are two major components. Stem-like CD8+ T cells act as resource cells to maintain the pools of LCMV-specific CD8+ T cells by self-renewal as well as by providing terminally differentiated (exhausted) CD8+ T cells to peripheral tissues of major sites of infection. It is also the stem-like subset that provides the proliferative burst of PD-1+ LCMV-specific CD8+ T cells during anti-PD-L1 therapy in chronic infection.

To elucidate which CD8+ T cell subset is targeted by muPD1-IL2v, we performed adoptive transfer experiments. Two PD-1+ CD8+ T cell subsets, stem-like (PD-1+ CXCR5 TIM-3) and terminally differentiated (exhausted; PD-1+ CXCR5 TIM-3), were sorted from the pooled splenocytes of chronically LCMV-infected mice (CD45.2+), and each CD8+ T cell subset was transferred into infection-matched mice (CD45.1+), followed by muPD1-IL2v therapy. After 2 weeks of treatment, congenically marked CD45.2+ donor cells were checked in recipient CD45.1+ mice (Fig. 2f). Notably, we found that the proliferative burst came exclusively from the stem-like CD8+ T cell subset after muPD1-IL2v therapy, whereas the exhausted CD8+ T cell subset did not expand in multiple tissues (Fig. 2g and Extended Data Fig. 6f).

Two weeks after transfer in untreated recipient mice, stem-like donor CD45.2+ cells maintained a TIM-3+ population, but they also converted to TIM-3+ cells, indicating their self-renewal and differentiation potential (Fig. 2h and Extended Data Fig. 6g). Both of these TIM-3+ and TIM-3− compartments expressed minimal levels of CD218a, suggesting that during chronic infection the transferred stem-like T cells went through a conventional differentiation pathway from stem-like to terminally differentiated (exhausted) CD8+ T cells (Fig. 2h and Extended Data Fig. 6g). By contrast, muPD1-IL2v therapy altered this differentiation process and transferred stem-like T cells underwent optimal effector differentiation, represented by marked upregulation of CD218a with low to intermediate expression of TIM-3 (Fig. 2h and Extended Data Fig. 6g). These results together demonstrate that muPD1-IL2v therapy acts on stem-like CD8+ T cells, enhancing their proliferation and effector differentiation.

muPD1-IL2v eradicates mouse pancreatic tumours

We then assessed muPD1-IL2v in an in vivo efficacy study in C57BL/6 mice implanted orthotopically with the pancreatic adenocarcinoma syngeneic cell line Panc02-H7-Fluc. Mice were treated once a week for 4 weeks with muPD1-IL2v (0.5 and 1 mg kg−1), muPD-1 antibody (10 mg kg−1), muFAP-IL2v (2.5 mg kg−1) or combinations thereof. muPD1-IL2v eradicated tumours in treated animals and provided long-term survival benefit in four of seven and seven of seven treated mice at doses of 0.5 and 1 mg kg−1, respectively (Fig. 3a). Only one mouse from the group treated with parental muPD-1 antibody in combination with muFAP-IL2v survived until the end of the experiment. All mice from the vehicle-treated control group and those receiving muPD-1 antibody or muFAP-IL2v as monotherapy died within 100 d (Fig. 3a).

Immunohistochemical analysis for the expression of PD-1 and granzyme B by tumours obtained from mice across the different treatments showed that muPD1-IL2v induced a significantly higher number of PD-1+ (Fig. 3b) and granzyme B+ (Fig. 3c) tumour-infiltrating lymphocytes (TILs) than the other treatments.

muPD1-IL2v favours CD8+ over CD4+ TILs

To better characterize the phenotype and function of TILs generated by the muPD1-IL2v treatment, Panc02-H7-Fluc tumour cells were implanted subcutaneously in syngeneic mice. Once tumours reached a size of 200 mm3, the mice were treated with muPD1-IL2v, muFAP-IL2v and muPD-1, using the above doses, once a week for 2 weeks and monitored for tumour growth. Treatment with muPD1-IL2v resulted in control of tumour growth and led to tumour eradication in three of six mice, while the other treatments failed to do so, both as monotherapies and in combination (Fig. 3d). Phenotypic characterization of TILs across the different treatment groups showed a significant and preferential -20-fold expansion of CD8+ over CD4+ T cells in tumours from mice treated with muPD1-IL2v, compared with the control and other treatment groups (Fig. 3e). Notably, by contrast, the ratio of CD8+ to CD4+ T cells in blood was -seven- to eightfold increased and comparable in mice receiving either muPD1-IL2v or muFAP-IL2v, the latter either as monotherapy or in combination with muPD-1 antibody (Fig. 3e). This observation is consistent with the notion of higher PD-1 expression on TILs than peripheral blood T cells.

Further characterization of CD8+ TILs across the various treatment groups highlighted dissimilarities in their differentiation stage. While the anti-PD-1 therapy enriched terminally differentiated TILs, muPD1-IL2v generated and expanded effector memory TILs (Fig. 3f). Conversely, muFAP-IL2v expanded naive TILs, and its combination with muPD-1 retained the features of both molecules by encompassing both naive and terminally differentiated TILs. CD8+ TILs induced by muPD1-IL2v were multifunctional and co-expressed significantly higher levels of granzyme B, IFNγ and tumour necrosis factor-α (TNFα) than CD8+ TILs isolated from mice from the other treatment groups (Extended Data Fig. 7a,b). In blood, the effect of muPD1-IL2v treatment was comparable to that of muFAP-IL2v (Fig. 3f), highlighting the importance of higher PD-1 expression, such as in TILs versus peripheral blood T cells, for the differentiated effects of muPD1-IL2v treatment over muFAP-IL2v.

To verify that CD8+ T cells are critical for the efficacy associated with muPD1-IL2v therapy, we depleted CD8+ cells 1 week before administering either muPD1-IL2v or muFAP-IL2v and monitored the number of CD8+ T cells in the blood over time. The effect of CD8+ T cell depletion in the muFAP-IL2v-treated group was not appreciable owing to the lack of efficacy of muFAP-IL2v in this tumour model (Extended Data Fig. 7c). However, depletion of CD8+ T cells prevented muPD1-IL2v from achieving tumour growth inhibition when compared with muPD1-IL2v-treated mice that were not depleted of CD8+ T cells (Extended Data Fig. 7c,d), demonstrating that CD8+ T cells are indeed required for the efficacy observed under muPD1-IL2v therapy.

CD8+ TILs are preferentially targeted by PD1-IL2v

To better understand the tumour tropism of PD1-IL2v, we isolated leukocytes from the blood and tumours of human PD-1-transgenic mice bearing subcutaneous Panc02-H7-Fluc tumours. We then measured, ex vivo, the frequencies of T cells expressing PD-1 and IL-2Rβ and quantified on these cells the numbers of both receptors per T cell. While the frequencies of T cells expressing PD-1 and IL-2Rβ on peripheral blood and tumours (Extended Data Fig. 7e,f), we found in tumours an effector memory population of CD8+ T cells expressing much higher levels of PD-1, approximately 15,000 PD-1 receptors per T cell (Fig. 3g). Interestingly, in blood, the corresponding T cell subset expressed ~700 PD-1 receptors per T cell, relatively comparable in peripheral blood and tumours (Extended Data Fig. 7e,f). We then treated human PD-1-transgenic mice, implanted subcutaneously with Panc02-H7-Fluc tumours, with either 0.5 mg kg−1 PD1-IL2v, comprising the anti-human PD-1 antibody binder fused to IL-2v, or high-dose IL-2 (aldesleukin) as single agents or in combination with pembrolizumab. By the end of the experiment, 7 of 12 mice receiving either muPD1-IL2v or muFAP-IL2v had a tumour smaller than 100 mm3, while 11 of 12 had a tumour smaller than 500 mm3. By contrast, only one of 11 mice receiving either muPD1-IL2v or muFAP-IL2v had a tumour smaller than 100 mm3.
Fig. 3 | muPD1-IL2v favours CD8+ versus CD4+ T cell in the tumour microenvironment and expands less differentiated TILs, which provide tumour eradication and survival benefit to treated mice. In vivo efficacy study in syngeneic or human PD-1 transgenic mice bearing orthotopic or subcutaneous Panc02-H7-Fluc tumours treated for 4 or 2 weeks, respectively, with the indicated treatment options. 

a. Survival curve, in days, of control syngeneic mice and mice treated with the indicated therapies (n = 4 mice per treatment group; mean ± s.e.m.). 

b. Percentage of surviving mice (n = 7 mice per treatment group).

c. Frequency of granzyme B+ cells (n = 3 mice per treatment group; mean ± s.e.m.). 

d. Tumour growth curves of subcutaneous tumours in syngeneic control mice and mice treated with the indicated therapies (n = 6 mice per treatment group; mean ± s.e.m.).

e. Tumour growth curves of subcutaneous Panc02-H7-Fluc tumours treated for 4 or 2 weeks, respectively, in syngeneic control mice and mice treated with the indicated therapies (n = 4 mice per treatment group).

f. Granzyme B expression 4 d after first therapy.

g. Percentage of surviving mice (n = 9 mice, respectively, from more than two independent experiments). 

h. Quantification of PD-1 receptors per cell on the surface of T cells isolated from the tumours and blood of untreated human PD-1 transgenic mice (n = 4 and n = 9 mice, respectively, from more than two independent experiments; box plots represent the median, minimum/maximum and individual points).

i. Number of PD-1+ cells (n = 3 mice per treatment group; mean ± s.e.m.).

j. Tumour growth curves of subcutaneous tumours in syngeneic mice treated with the indicated therapies (n = 7–12 mice per treatment group; mean ± s.e.m.). 

k. Tumour growth curves of subcutaneous tumours in human PD-1 transgenic mice receiving the respective therapies (n = 7–12 mice per treatment group; mean ± s.e.m.). 

l. Tumour growth curves of subcutaneous tumours in human PD-1 transgenic mice receiving the respective therapies (n = 3 independent experiments). 

m. To test for significant differences in tumour growth inhibition between groups means for multiple comparisons, standard ANOVA (one-way ANOVA) was used with Dunnett’s post hoc test in the Panc02 mouse tumour model. Wilcoxon’s test was used for survival analysis of the orthotopic Panc02 mouse tumour model. Statistical comparisons among multiple immuno-pharmacodynamic groups were performed using one-way ANOVA with Tukey’s multiple-comparisons test.

(Fig. 3h). Although the PD-1:IL-2 binding domain in PD1-IL2v competes for PD-1 binding with pembrolizumab, combination of PD1-IL2v with pembrolizumab did not impair PD1-IL2v efficacy. This can be explained by the superior functional affinity of PD1-IL2v, resulting from an approximately fourfold-higher monovalent PD-1 affinity and from simultaneously binding in cis to IL-2R, allowing PD1-IL2v to displace pembrolizumab even at saturating concentrations of the latter (Extended Data Fig. 7i). In line with this, combination of PD1-IL2v with pembrolizumab did not provide any additional benefit in comparison to PD1-IL2v monotherapy, as 8 of 12 treated animals (66%) had a tumour smaller than 100 mm3 at experiment termination (Fig. 3h). These data confirm that PD1-IL2v as monotherapy is more efficacious than the combination of pembrolizumab with high-dose aldesleukin, that PD1-IL2v does not require additional PD-1 blockade to increase its efficacy in this tumour model at the tested doses and that CD8+ TILs express roughly 20-fold more PD-1 receptors per T cell than CD8+ T cells in the blood, supporting the rationale for a tumour-preferential effect of PD1-IL2v.

muPD1-IL2v yields better effector CD8+ TILs

Immuno-pharmacodynamic analysis of subcutaneous Panc02-H7-Fluc tumours from syngeneic mice showed a progressive and large expansion of CD8+ T cells within the tumours of animals treated with muPD1-IL2v and a beneficial CD8+ T cell/Treg ratio after two single doses...
Fig. 4 | muPD1-IL2v expands and differentiates PD-1+TCF-1+ stem-like resource CD8+ TILs into a new population of better effector CD8+ TILs. Immuno-pharmacodynamic study on the effect of the different therapies, according to subset (g) and specific treatment effect (h). Average relative expression of selected genes (RNA and/or protein level) across the distinct T cell subsets within the CD8+ TILs depicted in g and h. j. Expression of selected markers, signature scores and TCR clonal expansion among CD8+ TILs using a 2D UMAP visualization as in g and h. log(cp10k), natural logarithm of counts per 10,000; log(clone size), natural logarithm of clone size. k. Percentage of better effectors and exhausted CD8+ T cells relative to all CD8+ T cells across the different treatments and average signature enrichment scores among effector CD8+ T cells per treatment group and individual animal (3–4 mice per group; box plots represent the median, minimum/maximum and individual points).

of muPD1-IL2v (Fig. 4a,b). This observation is consistent with the in vitro findings of preferential targeting and activity of PDL-1+IL2v on effector T cells rather than Treg cells. 

On the basis of PD-1 and TCF-1 expression, as reported by Hashimoto et al48 and by previous publications32,46, we identified antigen-experienced stem-like T cells as PD-1+TCF-1+ whereas their progeny were identified as PD-1+TCF-1low (Fig. 4c–f). We then further discriminated the functionality and degree of exhaustion of more mature PD-1+CD8+ T cells on the basis of their expression levels of granzyme B and TIM-3 (Fig. 4d). Interestingly, muPD1-IL2v drove the expansion of stem-like CD8+ TILs (Fig. 4c) and significantly increased the frequency of a granzyme B+TIM-3+ population within PD-1+TCF-1low CD8+ TILs (Fig. 4d,e). Here named ‘better effectors’, to underscore their highly functional effector profile and lower degree of exhaustion. Conversely, muPD-1...
monotherapy and combination of muPD-1 with muFAP-IL2v significantly increased the frequency of granzyme B ‘TIM-3’ cells within PD-1+ TCF-1+CD8+ TILs (Fig. 4d,f), showing low functionality and a higher degree of exhaustion.

Single-cell RNA-seq (scRNA-seq) and feature barcoding of CD8+ TILs obtained from the same in vivo experiment showed a unique gene expression signature following administration of muPD1-IL2v versus muPD-1 as monotherapy and in combination with muFAP-IL2v (Fig. 4g,h and Extended Data Fig. 5a–d). muPD1-IL2v drove the enrichment of a new CD8+ T cell population of better effectors (clusters 3, 4, 12, 14, 16 and 17), which was missing or under-represented in the other treatment groups (Fig. 4g,h and Extended Data Fig. 5a–d). muPD-1 therapy, and muPD-1n in combination with muFAP-IL2v even more so, drove the expansion of terminally differentiated/exhausted TILs (clusters 1 and 11) (Fig. 4g,h and Extended Data Fig. 5a–d). Similarly, the findings previously reported for chronic LCMV settings, we observed that muPD1-IL2v induced in the population of better effectors gene expression of receptors for pro-inflammatory cytokines, such as Il2rg, Il18r1, Il18rap and Ifngr, as well as those for homeostatic proliferation and memory formation, including Il7r (Fig. 4i,j) and Extended Data Fig. 8d). In addition, this population of CD8+ TILs also expressed high levels of transcripts for Pdcd1 (PD-1) and intermediate levels of Lag3 while expressing low levels of Havcr2 (TIM-3), Tigit and Tox, in line with a non-exhausted profile (Fig. 4i,j) and Extended Data Fig. 8d). The presence of expression of Ifitm1 and Tbx21 together with the polyfunctional effector signature of Tnf and Ifng, as well as the cytotoxic effector signature of the Gzm family gene and Lamp1, supports the finding that muPD1-IL2v is able to promote durable, productive and protective immune memory. Conversely, terminally differentiated CD8+ TILs (clusters 1 and 11) generated following treatment with muPD-1 as monotherapy or in combination with muFAP-IL2v, expressed high levels of Havcr2, Lag3, Tigit, Tox and Il10, typical of exhausted T cells (Fig. 4i,j) and Extended Data Fig. 8d).

These differences were both qualitative and quantitative, as illustrated by the significantly higher frequency of better effectors in response to muPD1-IL2v treatment, in contrast to the significantly higher frequency of exhausted CD8+ TILs elicited by muPD-1 alone and in combination with muFAP-IL2v (Fig. 4k). The CD8+ TILs generated by muPD1-IL2v possessed significantly higher stem-like and migration signature scores, indicating that they retain some of the transcriptional characteristics of stem-like PD-1+ TCF-1+ CD8+ TILs (Fig. 4k). By contrast, the immune-checkpoint and exhaustion signature scores were significantly higher in CD8+ TILs generated by the combination treatment of muPD-1 and muFAP-IL2v (Fig. 4k).

Single-cell TCR-seq showed that both better effectors, generated following muPD-1-IL2v treatment, and the exhausted T cells that arise following treatment with muPD-1 as monotherapy or in combination with muFAP-IL2v consisted of clonally expanded CD8+ TILs (Fig. 4j), a bona fide indicator of tumour specificity and productive immune response.40,41 We then assessed the total number of clones present in the stem-like CD8+ TILs and their progeny of effector cells across the different treatment groups, regardless of their functional phenotype. In the muPD1-IL2v-treated group, we found a high number of clones among the effector cells (768 clones), 97 of which were shared with stem-like CD8+ TILs (Extended Data Fig. 8e, top). We also observed the highest number of cells with highly (>10) expanded clones in the effector cells generated by muPD1-IL2v treatment, corresponding to a more than twofold difference compared with muPD-1 as monotherapy or in combination with muFAP-IL2v (Extended Data Fig. 8e, middle).

In addition, the muPD1-IL2v-treated group had the highest fraction of clones shared between the effector progeny and stem-like CD8+ TILs (46.4%) when compared with muPD-1 as monotherapy (17.1%) or in combination with muFAP-IL2v (22.1%). Of the 46.4% of shared clones, 16.4% were highly expanded clonotypes, as opposed to only 5.9% and 8.1% of the shared clones with muPD-1 as monotherapy and in combination with muFAP-IL2v, respectively (Extended Data Fig. 8e, bottom).

These results when taken together demonstrate that muPD1-IL2v therapy acts on stem-like CD8+ TILs and leads to the expansion of a unique CD8+ T cell population of better effectors with a transcriptional signature containing the hallmark of productive and protective immune memory. In addition, better effector CD8+ TILs have a high overlap in clonotypes with stem-like CD8+ TILs, indicating their developmental path, and the highest number of expanded clones, suggestive of their tumour specificity.

**Better effector CD8+ TILs provide survival benefit**

We then assessed muPD1-IL2v in an in vivo efficacy study in C57BL/6 mice implanted subcutaneously with the B16-F10-OVA syngeneic cell line. Mice were treated once a week for 2 weeks with muPD1-IL2v (0.5 mg kg⁻¹) or muFAP-IL2v (1.5 or 3 mg kg⁻¹) as monotherapy or in combination with muPD-1 (10 mg kg⁻¹). muPD1-IL2v provided longer survival benefit to 50% of the treated animals, and the tumours were eradicated in 20% of the total mice (Fig. 5a and Extended Data Fig. 9a). None of the mice receiving muFAP-IL2v as monotherapy or in combination with muPD-1 survived until the end of the experiment or showed controlled tumour growth (Fig. 5a and Extended Data Fig. 9b,c). muPD1-IL2v significantly increased the total number of intratumoural CD8+ T cells (Fig. 5b) but, more notably, significantly expanded the frequency and total count of ovalbumin (OVA)-specific CD8+ TILs when compared with muFAP-IL2v as monotherapy or in combination with muPD-1 (Fig. 5c,d, top). Interestingly, the combination of muPD-1 with muFAP-IL2v significantly expanded the frequency and total count of OVA-specific CD8+ T cells in the blood but not in the tumour (Fig. 5c,d, bottom).

In addition, muPD1-IL2v significantly expanded intratumoural OVA-specific PD-1+ TCF-1+ stem-like T cells in comparison to the other treatments (Fig. 5e).

Phenotypic and functional characterization of the PD-1+ TCF-1+OVA-specific CD8+ TILs showed that muPD1-IL2v induced a significant expansion of the frequencies of granzyme B ‘TIM-3’ and granzyne ‘TIM-3’ populations, whereas muFAP-IL2v and its combination with muPD-1 increased the frequency of the granzyne B ‘TIM-3’ population (Fig. 5f,g). The observed differences in the TIM-3 expression profile following treatment with muPD1-IL2v in the B16-F10-OVA mouse model and the subcutaneous Panc02-H7-Fluc mouse model might reflect the different immunogenicity of the two types of tumours and the relative difference in avidity of the T cell receptors (TCRs) for tumour antigens.40 CD8+ TILs isolated from mice treated with muPD1-IL2v showed the ability to mount a fast antigen-specific effector response when re-stimulated for 5 h with an OVA peptide (Fig. 5h).

We further explored two additional mouse models for responsiveness towards muPD1-IL2v: MCA-205 sarcoma, which is partially sensitive to PD-1 blockade, and RIP-Tag5, a spontaneous pancreatic neuroendocrine tumour model that is unresponsive to anti-PD-1 therapy. In the MCA-205 tumour model, muPD1-IL2v provided superior tumour growth inhibition to the treated mice in comparison to muPD-1 and muFAP-IL2v as monotherapy and in combination (Fig. 5i). Similarly, treatment of RIP-Tag5 mice with muPD1-IL2v resulted in increased survival benefit compared with the combination therapy of muPD-1 plus untargeted muL-2v (Fig. 5j). Of interest, two complete responders from the RIP-Tag5 study had to be killed during the study because of hyperglycaemia as a consequence of the potent anti-tumour immune response elicited by muPD1-IL2v that evidently resulted in organ-specific autoimmunity.

**Discussion**

Stem-like, antigen-experienced PD-1+ TCF-1+CD8+ T cells, or ‘stem-like T cells’, have emerged as important determinants of the immune response in chronic infections and cancer, with the size of their tumour-associated pool critical to the success of cancer immunotherapies.
In vivo efficacy study and immunopharmacodynamic study on the effect of the different therapies, given twice, on the number, phenotype and effector function of intratumoural and peripheral CD8+ T cells in syngeneic mice bearing subcutaneous B16-F10-OVA tumours. 

**Fig. 5** | **muPD1-IL2v** provides survival benefit and control of tumour growth in mice with subcutaneous B16-F10-OVA tumours by expanding cytotoxic OVA-specific better effector CD8+ TILs. 

- **a**, **d**: Survival (a), counts of total CD8+ T cells (b), and frequency (c) and count (d) of OVA-specific CD8+ T cells in the tumour and blood of syngeneic mice bearing subcutaneous B16-F10-OVA tumours receiving the indicated treatment (n = 5–8; box plots represent the median, minimum/maximum and individual points).

- **b**, **c,** **e**: Effect on frequencies of granzyme B+TIM-3− (left), granzyme B+TIM-3+ (middle) and granzyme B+TIM-3+CD8+ T cells (right) in tumour-infiltrating OVA-specific CD8+ T cells (n = 5–8; box plots represent the median, minimum/maximum and individual points).

- **f**: Representative contour plots depicting PD-1+OVA-Dextramer+CD8+ T cells (left); PD-1+OVA-Dextramer+CD8+ T cells with expression of TCF-1+ (middle); and PD-1+OVA-Dextramer+CD8+ T cells with expression of TCF-1+ and CD107a+IFNγ (right).

- **g**: Frequency of intratumoral OVA-specific PD-1+TCF-1−GrzB–TIM-3–CD8+ T cells.

- **h**: Fold increase in the frequency of CD107a+IFNγ+ CD8+ T cells from the different treatment groups following re-stimulation for 5 h with either SINGFLKL or gp100 peptide (n = 3; box plots represent the median, minimum/maximum and individual points). Tumour growth inhibition in the MCA 205 sarcoma model in syngeneic mice (n = 9 mice per treatment group; mean ± s.e.m.). Survival graph of tumour-bearing RIP-Tag5 mice either left untreated or subjected to treatment as indicated. Tumour progression was monitored by ultrasound imaging for 16 weeks. Two mice in the muPD1-IL2v group developed hyperglycaemia due to complete islet tumour regression and had to be killed before the predefined study end of 16 weeks. These mice were still counted as complete responders. Numbers of mice were as follows: untreated, n = 4; muPD1-untargeted muIL-2v, n = 5; muPD1-IL2v, n = 10. Statistical analysis was performed by log-rank Mantel–Cox test: muPD1-IL2v versus muPD1-untargeted muIL-2v, P < 0.0001. In **a–h**, n = 5–8 mice per treatment group, 2 independent experiments; statistical comparisons were performed using one- or two-way ANOVA with Dunnett’s multiple-comparisons test.

Blocking PD-1 or PD-L1 (refs. 1–3), we show that PD-1 inhibition alone acts on stem-like T cells to expand a population of transitory effector cells but eventually leads to the accumulation of exhausted T cells. By contrast, adding IL-2 triggers an alternative differentiation path from stem-like cells to a distinct subset of highly proliferative and cytotoxic CD8+ T cells, or ‘better
effects. We found that IL-2 binding to the non-signalling component of its receptor, CD25, is required for this process. However, CD25 binding can also contribute to unwanted effects of systemic IL-2 therapy, as occurs with high-dose adalimumab therapy, and this has led to the development of various IL-2 receptors targeted by agonists with reduced or abolished CD25 binding, currently in clinical trials. To address the challenge of systemic IL-2 therapy without losing the beneficial properties of IL-2 on stem-like T cells, we substituted binding to CD25 by targeting PD-1 with a blocking, high-affinity anti-PD-1 antibody fused to an IL-2 variant devoid of CD25 binding. This allowed specific delivery of enhanced cis IL-2:R agonist to PD-1 antigen-experienced T cells, such as virus-specific and tumour-reactive T cells. We found that binding in cis of PD-1 IL-2v to PD-1 and IL-2R by on the cell surface of the same T cell allows IL-2v to differentiate stem-like CD8+T cells into better effectors in the absence of CD25 binding in both chronic infection and cancer models. In the chronic LCMV infection model, we showed that these better effectors generated by PD1-IL2v from stem-like T cells have a transcriptional profile closely resembling that of the effector CD8+ T cells described in the accompanying article, generated following treatment with a combination of PD-1 inhibition and IL-2, with normal CD25 binding. They share characteristics of effectors generated during an acute infection, having lower levels of inhibitory receptors (for example, TIM-3) and transcription factors associated with T cell exhaustion (for example, Tim3) and higher levels of IFNy and IL-2 production. Better effectors also have higher levels of effector molecules (for example, granzyme family members) and immunological cytokine receptors (for example, Cxcr3). In addition, expression of genes encoding factors associated with memory (for example, IL-7R) and migration (for example, Cxcr3) was enhanced in this effector subset. Expansion of these highly proliferative and cytotoxic CD8+ T cells with a distinct transcriptional profile was associated with superior anti-viral and anti-tumour responses. By contrast, antibodies blocking PD-1 and PD-1 alone, or in combination with clinically relevant doses of IL-2 molecules devoid of CD25 binding and not targeted to PD-1, could not expand better effectors and instead induced exhausted T cells, leading to inferior treatment efficacy. In the last decade, immune-checkpoint inhibitors targeting the PD-1–PD-L1 pathway have revolutionized the standard of care for several types of tumours by acting on stem-like T cells and expanding tumour-specific transitory effector T cells. The findings described here provide a basis for the development of a new generation of PD-1-cis-targeted IL-2R agonists, preferentially targeting antigen-specific stem-like T cells but expanding an alternative population of better effector cells with enhanced therapeutic potential for the treatment of cancer and chronic infections.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-022-05192-0.
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Methods

Human PBMC isolation
Blood samples from healthy volunteers were obtained through a blood donation centre (Zurich, Switzerland) with approval of the Cantonal Ethics Committee (Zurich). PBMCs were isolated from the blood of different healthy donors using density gradient centrifugation with Histopaque-1077 (Sigma). All cells were cultured in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), GlutaMAX (Gibco) and 1% penicillin-streptomycin (100×; Gibco).

Human and mouse CD4+ T cell isolation and in vitro activation
Human CD4+ T cells were sorted by using a CD4+ selection Miltenyi bead system following the manufacturer's instructions. Thereafter, the cells were labelled with CFSE to track the proliferation of both populations. Tconv cells were labelled with CFSE and Treg cells fraction from selection of CD25+ cells (Miltenyi) followed by enrichment of soluble anti-CD28 antibody (1 μg ml−1; clone CD28.2, BioLegend). Cells were cultured for 3 d to induce activation and upregulation of the PD-1 receptor on the surface of CD4+ T cells.

Splenes of C57BL/6 mice were homogenized to a single-cell suspension by mashing the spleen through a 100-μm cell strainer, and erythrocytes were lysed with ACK (ammonium chloride – potassium) lysis buffer for 5 min at 4 °C. CD4+ T cells were sorted with a CD4 negative selection Miltenyi bead system following the manufacturer’s instructions. CD4+ T cells were seeded into a plate precoated with anti-CD3/anti-CD28 antibody (1 μg ml−1; clone OKT3, BioLegend; overnight, 4 °C) with addition of soluble anti-CD28 antibody (1 μg ml−1; clone CD28.2, BioLegend). Cells were cultured for 3 d to induce activation and upregulation of the PD-1 receptor on the surface of CD4+ T cells.

CD4+ T cells were seeded into a plate precoated with anti-CD3 antibody (1 μg ml−1; clone OKT3, BioLegend; overnight, 4 °C) with addition of soluble anti-CD28 antibody (1 μg ml−1; clone CD28.2, BioLegend). Cells were cultured for 3 d to induce activation and upregulation of the PD-1 receptor on the surface of CD4+ T cells.

IL-2R signalling (STAT5-P) in PD-1+ and PD-1-blocked CD4+ T cells
After 3 d of in vitro activation, cells were collected and washed multiple times to remove endogenous IL-2. A portion of the CFSE-labelled T cells were exposed to 10 μg ml−1 of parental anti-PD-1 antibody to block the PD-1 epitope for 30 min at room temperature and, thereafter, unbound antibody was washed away.

To assess IL-2R signalling (STAT5-P) on human T cells following treatment, both anti-PD-1-pre-treated and untreated cells were exposed to increasing concentrations of PD1-IL2v, pembrolizumab or non-blocking PD1-IL2v for 30 min at 4 °C. After a washing step, cells were incubated for an additional 30 min at 4 °C with saturating concentrations (10 μg ml−1) of a parental anti-PD-1-antibody conjugated directly to AF647 to generate PD1-IL2v. Cells were fixed with CellFIX (BD) after an additional wash.

Flow cytometry staining for cytokine detection and receptor quantification
Cells were stained in PBS with antibodies to cell-surface markers for 30 min at 4 °C and for live/dead status (with either Aqua Dead Cell Stain eFluor 780 (eBioscience) or CTV (5 μM, 5 min at room temperature; Thermo Scientific) to measure cell proliferation.

Binding competition on Treg and Tconv cells and Treg suppression assays
CD4+CD25+CD127low Treg cells were isolated from human peripheral blood with the two-step Regulatory T Cell Isolation kit (Miltenyi). In parallel, CD4+CD25+ Tconv cells were isolated by collecting the negative fraction from selection of CD25+ cells (Miltenyi) followed by enrichment of CD4+ cells (Miltenyi). Tconv cells were labelled with CFSE and Treg cells were labelled with CTV to track the proliferation of both populations.

For PD-1 and IL-2Rβ receptor quantification and PD1-IL2v binding competition, Treg and Tconv cells were co-cultured at a 1:1 ratio in a plate precoated with anti-CD3 antibody (1 μg ml−1; clone OKT3, BioLegend) with soluble anti-CD28 antibody (1 μg ml−1; clone CD28.2, BioLegend). After 3 d of stimulation, a competitive binding assay was conducted with 1 μg ml−1 (6.3 nM) of either parental anti-PD-1 antibody or PD1-IL2v, which were both directly labelled with AF647. Cells were incubated with the directly coupled antibodies for 30 min at 4 °C and fixed with CellFIX (BD).

In the Treg suppression assay, the rescue of Tconv granzyme B production following treatment with PD1-IL2v was measured after co-culturing Tconv cells together with Treg cells at a 2:1 ratio for 5 d, in the presence or absence of treatment. Irradiated (40 Gy) feeders from an unrelated donor were used to elicit allospecific stimulation. Suppression by Treg cells was calculated with the following formula:

\[
\text{% Cytokine suppression} = 100 - \left( \frac{\text{cytokine}_{\text{Tconv+Treg,therapy}} - \text{cytokine}_{\text{Tconv+Treg,untreated}}}{\text{cytokine}_{\text{Tconv+Treg,untreated}}} \right) \times 100
\]

where % cytokine_{Tconv+Treg,therapy} is the level of cytokine secreted by Tconv cells in the presence of Treg cells ± treatment and % cytokine_{Tconv,untreated} is the level of cytokine secreted by Tconv cells in the absence of Treg cells and without treatment.

GM-CSF, granzyme B and IFNγ secretion by CD4+ T cells
Sorted and CTV labelled human polygonal CD4+ T cells were activated with soluble anti-CD3 antibody (1 μg ml−1) in the presence of irradiated (40 Gy) feeder cells from the same donor at a 1:1 ratio and increasing concentrations of treatment antibodies or aldesleukin (Proleukin, Novartis). After 5 d, GM-CSF secretion was measured by ELISA (BioLegend) following the manufacturer’s instructions. For intracellular flow cytometry staining, accumulation of cytokines in the Golgi complex was induced by re-stimulating cells with ionomycin (500 ng ml−1) and phorbol 12-myrystate 13-acetate (PMA; 50 ng ml−1) together with protein transport inhibitors (1 μl GolgiPlug and GolgiStop, BD) for 5 h before staining.

Binding competition
CD4+ T cells activated for 3 d were exposed to increasing equimolar concentrations of PD1-IL2v, pembrolizumab or non-blocking PD1-IL2v for 30 min at 4 °C. After a washing step, cells were incubated for an additional 30 min at 4 °C with saturating concentrations (10 μg ml−1) of a parental anti-PD-1-antibody conjugated directly to AF647 to generate PD1-IL2v. Cells were fixed with CellFIX (BD) after an additional wash.
Mice, virus and infection model
Six- to 8-week-old female C57BL/6J and CD45.1 congenic mice were purchased from the Jackson Laboratory. The following housing conditions for the mice were used: 12-h light-cycle (7:00 am on, 7:00 pm off), temperature between 68–74 °F, humidity between 30–70 g m⁻³. Mice were maintained under specific-pathogen-free conditions with daily cycles of 12 h light/12 h dark-ness according to guidelines (GV-SOLAS, FELASA) and food and water were provided ad libitum. Continuous health monitoring was carried out, and the experimental study protocol was reviewed and approved by the Veterinary Department of Canton Zurich. Tumour volume was measured using a calliper. Tumour volume was calculated with the formula:

\[ \text{Tumour volume} = \text{length} \times \text{width} \times \text{depth} \times \frac{4}{3} \pi \]

Therapy was started when tumour volume reached 150–200 mm³. For the CD8⁺ depletion study, all mice bearing Panc02-H7-Fluc subcutaneous tumours were administered intravenously with anti-mouse CD8 antibody (In VivoPlus Anti-Mouse CD8α, clone 2.43, BioXCell), 5 mg kg⁻¹ three times a week, 1 week before the first administration of therapy. Depletion of mouse CD8⁺ cells was evaluated in blood by flow cytometry before the start of therapy.

B16F10-OVA. A subcutaneous melanoma syngeneic model was used to assess the in vivo efficacy of muPD1-IL2v compared with the single agents muPD1 and muAP-IL2v or their combination in C57BL/6J mice. Tumour growth inhibition and survival rate were the readouts for this subcutaneous model. In brief, 6- to 8-week-old female C57BL/6J mice (Charles River) were inoculated subcutaneously with 2 × 10⁶ B16F10-OVA cells from a B16 cell line overexpressing the OVA protein. As described previously for the Panc02-H7-Fluc model, mice were maintained under specific-pathogen-free conditions with daily cycles of 12 h light/12 h dark-ness according to guidelines (GV-SOLAS, FELASA) and food and water were provided ad libitum. Continuous health monitoring was carried out, and the experimental study protocol was reviewed and approved by the Veterinary Department of Canton Zurich. Tumour volume was measured using a calliper. Tumour volume was calculated with the formula:

\[ \text{Tumour volume} = \text{length} \times \text{width} \times \text{depth} \times \frac{4}{3} \pi \]

Mice were randomized, 10 d after tumour inoculation, into different treatment groups on the basis of tumour size. Therapy started 11 d after tumour inoculation. All treatments were administered subcutaneously once a week, with the following doses investigated: muPD1-IL2v at 0.5 mg kg⁻¹, muAP-IL2v at 1.5 and 3 mg kg⁻¹, combination of muPD1 at 10 mg kg⁻¹ and FAP-IL2v at 1.5 mg kg⁻¹. For survival rate curves, the termination criterion to kill animals was either tumour size or tumour ulceration. Therapy was considered to begin when tumour volume reached 150–200 mm³.

MCA-205 sarcoma. A subcutaneous fibrosarcoma syngeneic model was also used to assess the in vivo efficacy of muPD1-IL2v compared
with the single agents muPD1 and muFAP-IL2v or their combination in C57BL/6j mice at the CRO Antineo (Lyon, France). Tumour growth inhibition was the readout for this subcutaneous model. In brief, 6- to 8-week-old female C57BL/6j mice (Charles River) were inoculated with 5 × 10^5 MCA-205 cells injected subcutaneously. Mice were maintained under specific-pathogen-free conditions with continuous health monitoring according to guidelines (Animalerie Commune Scar Rockefeller, Lyon, France).

Mice were randomized into different treatment groups, and therapy started when tumours reached an average volume of 100 mm^3 as measured by calliper in the subcutaneous model. All treatments were administered intravenously, with the following doses investigated: muPD1-IL2v at 1 and 2 mg kg^-1, muFAP-IL2v at 2 mg kg^-1 and muPD1 at 3 mg kg^-1. Tumour volume was measured using a calliper and calculated with the formula:

\[
\text{Tumour volume} = \text{length} \times \text{width} \times \text{depth} \times \frac{4}{3\pi}
\]

Tumour growth inhibition was used as the readout; to test for significant differences in group means for multiple comparisons, standard ANOVA (one-way ANOVA) was used with Dunnett’s method. The JMP statistical software program was used for analyses.

**RIP-Tag5 transgenic mouse model of PanNET.** The generation of RIP-Tag5 mice has previously been described. The RIP-Tag5 mice in this study were on a C57BL/6/N background (Charles River) and were males aged 21 to 31 weeks. Animal experiments were conducted according to protocols approved by the Veterinary Authorities of the Canton of Vaud and Swiss law.

To enrol RIP-Tag5 mice into the trial, mice were from 22 weeks of age displaying blood glucose levels below 7 mmol L^-1 for 2 weeks and the average starting age was 26 weeks and the average starting glucose level was 5.5 mM. Tumours were monitored by ultrasound imaging every 2 weeks, or every 4 weeks for complete responders, for a maximum of 16 weeks following the start of treatment. Blood glucose levels were monitored weekly using an Accu-Chek glucometer (Roche). The criteria for the endpoint were defined according to tumour burden (>50 mm^2 or 2- to 4-fold increase on progression for relapsing tumours), hypoglycaemia (blood glucose levels at or below 3 mM) and health status.

Therapies were administered by intraperitoneal injection with the following amounts per mouse: muPD1, 250 μg once a week; DP47-muIL2v (untargeted muIL2v), 25 μg once a week; PD1-IL2v, 25 μg once a week for a duration of 8 weeks.

**Histology**

For histological analysis, tissue samples were collected, fixed in 10% formalin (Sigma) and later processed for FFPE (Leica, 1020). Four-micrometre paraffin sections were subsequently cut in a microtome (Leica, RM2235). Haematoxylin and eosin staining was performed in an automated Leica system following the manufacturer’s instructions. Mouse PD-1 immunohistochemistry was performed with anti-mouse PD-1 (1:250; clone AF1021, R&D Systems) while mouse granzyme B staining was performed with anti-mouse GZMB (1:250; clone ab4059, Abcam) on CD3^+ (1:100; clone SP7, Diagnostic Biosystems) and CD8^+ (1:300; clone 4S1M15, eBiosciences) T cells. Staining was performed in the Leica autostainer (Leica, STS010) following the manufacturer’s protocols. Sections were counterstained with haematoxylin (Sigma-Aldrich), and slides were scanned using the Olympus VS120-L100 Virtual Slide Microscope scanner. Quantification of positive cells from scan images was performed with Definiens software. For this, whole scans were uploaded in the tissue developer module and necrotic areas were excluded with segmentation analysis. Second, a threshold was set to recognize the brown staining of targeted cells, and the algorithm for cell quantification or percentage positive area was subsequently automatically run. The output data were then transferred to GraphPad Prism (v8) for analysis of significance by standard ANOVA (one-way ANOVA) with Dunnett’s correction.

**Cell lines**

Vero E6 cells were obtained from the American Type Culture Collection, mouse pancreatic cancer cell line Panc02-H7-Fluc was generated at Roche Glycart and the B16-OVA cell line was purchased from ProQinase. The MCA-205 mouse fibrosarcoma cell line was purchased from Sigma-Aldrich and was derived from 3-methylcholanthrene-induced fibrosarcoma in C57BL/6 mice. Tumours were maintained in vivo by serial subcutaneous transplantation in syngeneic mice, and single-cell suspensions were prepared from solid tumours by enzymatic digestion. From these cells, the MCA-205 cell line was established and maintained in vitro. Vero E6 cells were not authenticated, while MCA-205, B16-OVA and Panc02-H7-Fluc cells were authenticated through morphology and PCR assays with species-specific primers. MCA-205 cells tested negative for infectious diseases using a Mouse Essential CLEAR panel by Charles River Animal Diagnostic Services and were negative for mycoplasma contamination. Batches of the Panc02-H7-Fluc and B16-OVA cell lines are routinely tested in house for mycoplasma and are negative.

**Lymphocyte isolation**

Chronic infection experiments. Lymphocytes were isolated from the blood, spleen and lung as described previously. In brief, spleens were dissociated by passing them through a 70-μm cell strainer (Corning). Lungs were treated with 1.3 mM EDTA in HBSS for 30 min at 37 °C, with shaking at 200 r.p.m., followed by treatment with 150 U ml^-1 collagenase (Thermo Fisher Scientific) in RPMI-1640 containing 5% FBS, 1 mM MgCl_2 and 1 mM CaCl_2, for 60 min at 37 °C with shaking at 200 r.p.m. Collagenase-treated lung tissues were homogenized and filtered through a 70-μm cell strainer. Lymphocytes from lungs were purified using a 44–67% Percoll gradient (800 g at 20 °C for 20 min).

Cancer model experiments. Mice were killed according to animal welfare guidelines; tumour tissue and blood were isolated in the animal facility. Tumour tissue was transferred to PBS and was disrupted using manual scissors and the Miltenyi Gentle MACS machine. Subsequently, it was digested in an enzyme mix consisting of RPMI with 10 mg ml^-1 DNase (Sigma-Aldrich) and 0.25 mg ml^-1 Liberase (Sigma-Aldrich). After 30 min of digestion at 37 °C, the tissue mix was filtered through a 70-μm filter and resuspended as a single-cell suspension with an appropriate volume for subsequent staining with fluorescently labelled antibodies. Blood was transferred to heparin tubes, and red blood cells were lysed with erythrocyte lysis buffer. After red blood cell lysis, cells were resuspended as a single-cell suspension with an appropriate volume for subsequent staining with fluorescently labelled antibodies. Lymphocytes were mechanically isolated from draining lymph nodes with a pestle, filtered through a 70-μm filter and resuspended as a single-cell suspension with an appropriate volume for subsequent staining with fluorescently labelled antibodies.

**Reagents, flow cytometry and in vitro stimulation**

**Chronic infection experiments.** All antibodies for flow cytometry were purchased from BD Biosciences, BioLegend, Thermo Fisher Scientific, Cell Signaling Technology and R&D Systems. D^4^GP33–41 and D^4^GP276–286 tetramers were prepared in house and were used to detect LCMV-specific CD8^+ T cells. Streptavidin-PE or streptavidin-APC was purchased from Thermo Fisher Scientific. Dead cells were excluded by using the Live/Dead...
Fixable Near-IR or Yellow Dead Cell Stain kit (Thermo Fisher Scientific). For cell-surface staining, antibodies were added to cells at dilutions of 1:20 to 1:500 in PBS supplemented with 2% FBS and 0.1% sodium azide for 30 min on ice. Cells were washed three times and fixed with 2% paraformaldehyde. To detect cytokine production, 1 × 10^6 splenocytes were stimulated with a pool of nine LCMV-specific peptides (200 ng/ml each of GP33–41, GP70–77, GP92–101, GP115–125, GP276–286, NP166–175, NP205–212, NP235–249 and NP396–404) in a 96-well round-bottom plate for 5 h at 37 °C in a CO2 incubator in the presence of GolgiPlug (BD Biosciences). Samples were acquired on a Canto II, LSR II or FACSymphony A3 instrument (BD Biosciences) with FACSDiva (v9.1; BD Biosciences), and data were analysed by using FlowJo (v9.9.6 or v10.8.1; BD Biosciences).

**Cancer model experiments.** Single-cell suspensions from tumours and blood were stained with the following antibodies: Fixable Viability Dye eFluor 453UV (1:500), AF700 anti-CD45 (1:300; clone 30-F11, Biologend), PerCP-Cy5.5 anti-TCRβ (1:200; clone H57-597, Biologend), APC–Cy7 anti-CD8 (1:200; clone 53-6.7, Biologend), PE-Cy7 anti-CD4 (1:200; clone GK1.5, Biologend), FITC anti-CD62L (1:200; clone MEL-14, Biologend), PE anti-CD127 (1:100; clone A7R34, Biologend), BV421 anti-CD4 (1:200; clone GK1.5, Biologend), AF647 anti-granzyme B (1:100; clone GB11, Biologend), BV786 anti-IFNγ (1:100; clone XMGL2, Biologend), PE-Cy7 anti-TNFα (1:100; clone M6P-XT22, Biologend), BV421 anti-FoxP3 (1:100; clone MF-14, Biologend), AF647 anti-CD39 (1:200; clone Du49a5, Biologend), AF700 anti-granzyme B (1:100; clone QA16a02, Biologend), PE–Cy7 anti-Ki67 (1:300; clone IA68, Biologend), PE–Cy7 anti-CD11c (1:200; clone RMPI30-30, Biologend), BV711 anti-CD25 (1:200; clone RMT3-23, Biologend), PE–Dazzle594 anti-TiG1T (1:100; clone iG9, Biologend), BV605 anti-IFNγ (1:100; clone XMGL2, Biologend), BV421 anti-TNFα (1:100; clone M6P-XT22, Biologend), AF488 anti-CD107a (1:100; clone 1D4B, Biologend), BV510 anti-CD44 (1:200; clone IM7, BD Biosciences), BV605 anti-CD45 (1:100; clone 30-F11, Biologend), BV786 anti-TCRβ (1:100; clone H57-597, Biologend), BV496 anti-CD6 (1:100; clone RM6-4, BD Biosciences), BV395 anti-CD8 (1:100; clone 53-6.7, BD Biosciences), BV737 anti-PD-1 (1:100; clone RMPI30-30, Biologend), PE–CF594 anti-CD25 (1:100; clone PC61, BD Biosciences), BV650 anti-CD30-L (1:100; clone SD12, BD Biosciences), PE anti-TCF–1 (1:100; clone S33-966, BD Biosciences), BV650 anti-LAG3 (1:100; clone C9B7W, BD Biosciences), BV510 anti-SLAMF6 (1:150; clone 1G9, BD Biosciences), and FITC anti-CD218a (1:50; clone REA947, Milteny).

Detection of OVA-specific CD8+ T cells was performed by using APC-labelled Dextramer H-2Kb (SIINFEKL) from Immudex (1:100). Staining with Dextramer was performed by using 0.1% BSA in PBS. For intracellular staining, cells were fixed and permeabilized using the FOXP3 Transcription Factor Staining Buffer Set from ebioscience or the Transcription Buffer Set from BD.

For detection of cytokines, tumour cell suspensions were re-stimulated with 6.25 ng/ml PMA (Sigma-Aldrich) and 1.87 μg/ml ionomycin (Sigma-Aldrich) for 5 h at 37 °C. After 1 h of re-stimulation, GolgiPlug (BD) and GolgiStop (BD) were added to the cell suspension. For antigen re-stimulation, tumour cell suspensions were re-stimulated with 0.1 μg/ml 1 gpi100 or SIINFEKL peptide, for 5 h at 37 °C. Anti-CD107a antibody was added together with the peptides for 5 h at 37 °C. As before, after 1 h of re-stimulation, GolgiPlug (BD) and GolgiStop (BD) were added to the cell suspension.

Discrimination of living cells from dead cells was performed using DAPI (Sigma-Aldrich), Fixable Viability Dye eFluor 780 (ebioscience) or Live/Dead APC-Cy7 (ebioscience). Samples were acquired with a BD LSRII Fortessa and a BD FACSymphony A5 instrument by using FACSDiva (v9.1; BD Biosciences). Data obtained were analysed by using FlowJo (v10.8.1; BD Biosciences).

**Cell sorting**

**Chronic infection experiments.** Cell sorting was performed on a FACSAria II (BD Biosciences). For adoptive transfer experiments, two PD-1-expressing CD8+ T cell subsets (PD-1+CXCR5+ TIM-3- and PD-1+CXCR5+ TIM-3+) were sorted from the pooled spleens (n = 40–60) of chronically LCMV-infected mice. For RNA-seq analysis of LCMV-specific CD8+ T cells after muPD-L1, muPD1-IL2v and muPD-L1 + muPD1-IL2v therapy, chronically LCMV-infected mice (more than 40 after infection; n = 1–18) were left untreated or treated for 2 weeks, and ΔG33+CD8+ T cells were sorted from pooled spleens to obtain at least 2 × 10^4 cells. Naive (CD44low) CD8+ T cells were sorted from the pooled spleens of uninjected mice (n = 2–3). All samples had purities of greater than 95%.

**Cancer model experiments.** Single-cell tumour suspensions were kept on ice during the staining and sorting procedure. Cell suspensions from 3–5 tumours of the same treatment group were stained with the following antibodies: AF700 anti-CD45 (1:100; clone 30-F11, Biologend), BV711 anti-CD8 (1:100; clone 53-6.7, Biologend) and bi-channel BV605 anti-CD4 (1:100; clone GK1.5, Biologend), and BV605 anti-CD11c (1:100; clone N418, Biologend). Discrimination of living cells from dead cells was performed using Live/Dead APC-Cy7 (ebioscience, 65-0865-14: 1:500 for non-fixed samples and cells with incubation for 20 min). Cells were washed twice, filtered through a 40-μm cell strainer, sorted on a FACSAria III instrument and acquired with FACSDiva (to enrich viable single CD45+CD8+CD11c-CD4- cells).

**Mouse PD-L1 blockade and treatment with muFAP-IL2wt, muFAP-IL2v and muPD1-IL2v in vivo**

For PD-L1 blockade, 200 μg of mouse PD-L1 antibody with the DAPG mutation (Roche) was administered intraperitoneally into chronically LCMV-infected mice every 3 d for 2 weeks. Appropriate isotype control (mouse IgG1 isotype control (MOPC-21, BioXcell)) was administered in untreated mice. For muFAP-IL2 therapy, 1 mg kg⁻¹ muFAP-IL2wt or muFAP-IL2v was administered intraperitoneally into chronically LCMV-infected mice twice weekly for 2 weeks. For muPD1-IL2V therapy, 1 mg kg⁻¹ muPD1-IL2v was administered intraperitoneally into chronically LCMV-infected mice twice weekly for 2 weeks.

**Adoptive transfer of two CD8+ T cell subsets**

Cells from two CD8+ T cell subsets (4–8 × 10^4 cells; PD-1+CXCR5+TIM-3+ and PD-1+CXCR5+ TIM-3-) isolated from chronically LCMV-infected mice (CD45.2+) were transferred into infection-matched recipient mice (CD45.1+), followed by muPD1-IL2v treatment for 2 weeks.

**RNA isolation and RNA-seq**

**Chronic infection experiments.** Total RNA was extracted by using the RNeasy Micro kit (Qiagen) or Direct-zol RNA Microprep kit (Zymo Research) according to the manufacturer’s protocols at the Emory Integrated Genomics Core or in house. Preparation of a standard RNA-seq library was performed at Hudson Alpha or the Emory Yerkes Nonhuman Primate Research Center (NPRC) Genomics Core. In brief, RNA amplification was performed using the Nugen Ovation RNAseq v2 kit or Clontech SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio). Amplified cDNA was fragmented, and samples were prepared using the KAPAHyper Prep kit or Nextera XT DNA Library Preparation kit (Illumina). Pooled libraries were sequenced on an Illumina NovaSeq 6000 with 100-bp paired-end reads.

**Cancer model experiments.** Tumours and lymph nodes were digested as previously described, and 1–10 Mio cells were stored in liquid nitrogen in Iibi freezing medium. The samples were randomized and processed in four different batches with ten samples each (tumours and lymph nodes were processed separately). After thawing a batch of samples, the cells were stained with a mix of flow cytometry and oligonucleotide-labelled antibodies (Table I) and sorted for CD8+ T cells before performing scRNA-seq. In brief, cells were washed once with PBS before evaluation of both cell number and viability using a Nexcelom Cellometer Auto 2000. Approximately one Mio cell per sample was
resuspended in 50 μl PBS and incubated with 5 μl of Mouse TruStain FcX Fc Blocking reagent (BioLegend). The mix of flow cytometry and oligonucleotide-labelled antibodies was added to the cells in a volume of 50 μl (final volume, 10 μl). After incubation for 30 min at 4 °C, the cells were washed three times with PBS and resuspended in 500 μl PBS to obtain a concentration of approximately 1 × 10^6 cells per ml. Cells were filtered through a 40-μm cell strainer and sorted on the BD FACSAria III system. The cell number and viability of the sorted cells were determined using a Nexcelom Cellometer Auto 2000, and a total of 10,000 viable cells per sample were loaded into the 10x Genomics Chromium controller. CDNA and library preparation were performed according to the manufacturer’s indications (scRNA-seq v5 v2 kit with TCR and feature barcoding), and the resulting libraries were sequenced on an Illumina NovaSeq 6000 sequencer according to 10x Genomics recommendations (R1 = 26, I = 10, I5 = 10, R2 = 90) to a depth of approximately 20,000 reads per cell for the GEX library and 5,000 reads per cell for both the TCR and feature barcoding libraries.

**Analysis of RNA-seq data for virus-specific CD8+ T cells during chronic infection**

Reads were mapped to the GRCm38/mm10 genome with HISAT2 (v2.1.0)\(^{54}\). Gene expression was quantified with scRNA-seq v5 v2 kit with TCR and feature barcoding. DESeq2 (ref.\(^{57}\); v1.24.0) was used to normalize for library size and calculate differential expression across groups. A gene was considered differentially expressed between two groups with an adjusted P value of <0.05 and an average expression of >20 normalized counts across all samples. PCA was performed on all detected genes using the regularized log transformation from DESeq2. RNA-seq data were visualized by using Prism software (v9.3.1; GraphPad) and the ComplexHeatmap R package (v2.2.0)\(^{58}\).

**Single-cell RNA, protein and TCR sequencing analyses of TILs**

Fastaq files were aligned to the mouse transcriptome (mm10-2020-A) using CellRanger (count and vdj) v6.0.0 with the parameter ‘--expect-cells=6000’. All cells showing >200 counts were further merged across all samples and processed with scany\(^{55}\) and the besca\(^{40}\) standard workflow. Filtering was performed with the parameters min_genes = 500, min_cells = 20, min_counts = 1000, n_genes = 6000, percent_mito = 0.08, max_counts = 40000. In addition, cells with no antibody counts were removed. Two samples were excluded because of low overall quality and very low cell number; all other samples were included in the analysis. In brief, RNA counts were normalized per 10,000, the top most highly variable genes were selected, total gene and mitochondrial reads were regressed out, PCA was performed and the first 50 principal components were used for nearest-neighbour calculations and Leiden clustering, as well as for UMAP-based visualization. Protein counts were central log ratio (CLR) transformed. Annotation was performed using besca’s sig-annot module, and more detailed CDS* subpopulations were attributed on the basis of RNA and protein marker expression and signature enrichment (scany.tl.score_genes).

Only clusters containing CDS* T cell populations were retained in the analysis; clusters 21 (non-immune), 18 (non-T cell), 7 and 19 (myeloid T cell doublet), 22 (T helper 17 cell) and 20 (Treg cell) were excluded as likely contaminants. Gene signatures used in Fig. 4 are provided in Table 2. TCR analysis was performed in Python with the toolkit scirty\(^{41}\), and clonotypes were determined on the basis of CDR3 sequence identity, with the parameters receptor_arms = “all”, dual_ir = “primary_only”. Jupyter notebooks are available for data preprocessing, clustering and visualization, and cell annotation, as well as for TCR analysis, at https://github.com/bedapub/PD1-IL2v_in-vivo_TILs_Panc02_publication.

**Internalization assays**

**Imaging.** Human CD4+ T cells freshly purified from PBMCs were activated on anti-CD3/CD28-coated plates. Three days after activation, CD4+ T cells were collected and stained with 10 μM CellTracker Blue CMAC Dye (Invitrogen, C2110) for 15 min at 37 °C. 150,000 cells were seeded on RetroNectin-coated imaging slides and allowed to adhere for 30 min at 37 °C. For RetroNectin coating, imaging slides were treated with 1 μg μl−1 RetroNectin (Takara, T100B) for 40 min at room temperature. Subsequently, T cells were treated for 1 h or 3 h at 4 °C or 37 °C with 630 pM (0.1 μg ml−1) of in-house-produced PD1-IL2v AF647 or 630 pM (0.1 μg ml−1) of in-house-produced PD1-IL2v AF647. Where indicated, cells were pretreated with 10 μg ml−1 anti-CD-PD1 to saturate all PD-1 binding sites. Afterwards, samples were fixed and permeabilized (BD Cytofix/Cytoperm, 554714) for 20 min at 4 °C and then stained with a non-competing unconjugated anti-PD-1 antibody (1:100; D4W2), Cell Signaling Technology) for 45 min at 4 °C, followed by staining with goat anti-rabbit IgG (H+L), F(ab’), Fragment AF488 (Cell Signaling Technology, 4412, lot 18; 2 μg ml−1; 1:1,000). All samples were then imaged with an inverted confocal microscope (Leica Sp8), adopting a ×40 objective. For each image, one optical section was acquired with a resolution of 1,024 × 1,024 at a pixel size of 0.379 μm.

| Application | Marker | Clone | Oligonucleotide tag | Source | Concentration |
|------------|--------|-------|---------------------|--------|---------------|
| Feature barcoding | CD28 | 37.51 | ATTAAGACCGCTTG | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD44 | IM7 | TGCTTCAGTCCTTA | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD62L (L-selectin) | MEL-14 | TGGGCCTAATGACATC | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD279 (PD-1) | RMP1-30 | GAAATGCAAACAGCT | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD366 (TIM-3) | RTM1-23 | ATTCGACCACAGT | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD223 (LAG-3) | C987W | ATTCCGCTTCTTAAGG | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD183 (CXCR3) | CXCR3-173 | GTTCACGCCGTGTTA | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD185 (CXCR5) | L138D7 | ACCTGATCACCTAGT | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD127 (IL-7Rα) | A7R34 | GTGGAGGCACCTTT | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD185 (IL-7Rα) | PC61 | ACCATGAGACACAGT | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | Ly108 (SLAM-F6) | 330-AJ | CGATTCTTTCGCGT | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | CD137 (4-1BB) | 1785 | GCCCGTATAGTATA | TotalSeq-C, BioLegend | 1 μg ml−1 |
| Feature barcoding | IL-21R | 4A9 | GATCCGACAGTAGA | TotalSeq-C, BioLegend | 1 μg ml−1 |
**Table 2** | Gene signatures used for Fig. 4 and Extended Data Fig. 8.

| Migration | Immune checkpoint | Stem-like CD8+ T cells (cluster 6, vehicle) | Exhausted CD8+ T cells | Cytotoxicity |
|-----------|-------------------|--------------------------------------------|-------------------------|-------------|
| Ccr2      | Cd160             | Pag1                                       | Pdcd1                   | Gzma        |
| Cxcr3     | Lag3              | Sloc3a1                                    | Haver2                  | Gzmb        |
| Cxcr4     | Ccl244a           | Ifi1b1                                     | Lag3                    | GzmC        |
| Cx3cr1    | Btla              | Itgae                                      | Entpd1                  | Gzmf        |
| Stpr1     | Pdcd1             | Baiap3                                     | Cd38                    |             |
| Itga1     | Hcav2             | Ripor2                                     | Tax                     |             |
| Itga4     | Tigit             | Rasa3                                      |                         |             |
| Itgae     | Cd101             | Ly6a                                       |                         |             |
| Itgb1     | Oas3              |                                           |                         |             |
| Itgb7     | Samhd1            |                                           |                         |             |
| Ccd4      | Gm45552           |                                           |                         |             |
| Ly6c2     | Cxcr3            |                                           |                         |             |
| Cxcr5     | Acss2            |                                           |                         |             |
| Gpr55     | Ifti208           |                                           |                         |             |
| Aht4c     | Ifi213            |                                           |                         |             |
| Ccr2      | 5830432E09Rik     |                                           |                         |             |
| Ly9       | Rtp4              |                                           |                         |             |
| Nod1      | Dtx1              |                                           |                         |             |
| Slf1n     |                   |                                           |                         |             |

**Data analysis.** Images were analysed using Imaris 9.5.1 (Bitplane), MATLAB 2020a (Mathworks) and GraphPad Prism (v8; GraphPad Software). In Imaris, images were opened and the cytoplasm was segmented on the basis of the CellTracker Blue CMAC Dye channel (surface grain size, 0.758). Subsequently, the images were transformed to 32 bits; then, using the Imaris Xtenion ‘Distance Transformation’, the distance to the ‘cytoplasm isosurface’ was calculated and saved as a separate channel. The next segmentation, to approximate the membrane position, was based on this ‘distance to cytoplasm’ channel (surface grain size, 0.758; thresholds, 0–0.759 μm for distance from cytoplasm). Any touching segmentation objects were split, such that for every segmented cytoplasm a segmented membrane was present. Membrane and cytoplasm statistics were exported for further analysis.

A custom MATLAB script was used to match the values for ‘drug average intensity’ and ‘PD-1 average intensity’ of membrane and cytoplasm objects, on the basis of the closest distance for segmented object centroids. Subsequently, the ratio was calculated and exported as a .csv file. From the exported .csv files, values were copied into GraphPad Prism for plotting and statistical analysis.

**Statistical analysis**

Pram software (v8 and v9.3.1; GraphPad) was used for statistical analysis. Differences among the experimental groups were assessed by using an unpaired test or a Mann–Whitney test for comparing two groups. One-way or two-way ANOVA with Dunnett’s or Tukey’s post hoc test or a Kruskal–Wallis test was used for comparison of more than two groups. To test for significant differences in tumour growth inhibition between groups means for multiple comparisons, standard ANOVA (one-way ANOVA) was used with Dunnett’s post hoc test in the Panc02 mouse tumour model. Log-rank Mantel–Cox tests were used to compare muPDI-IL2v versus muPDI + untargeted muIL-2v survival in the RIP-Tag5 mouse tumour model; Wilcoxon’s test was used for comparison of survival in the orthotopic Panc02 mouse tumour model.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The scRNA-seq, TCR-seq and CITE-seq data discussed in this publication have been deposited in ArrayExpress with accession number E-MTAB-11773. RNA-seq data from chronic infection experiments are available in the NCBI Gene Expression Omnibus database under accession number GSE208556. Source data are provided with this paper.

**Code availability**

For scRNA-seq, TCR-seq and CITE-seq data analysis, we used CellRanger (v6.0.0), scanypy (v1.6.0), besca (v2.4.1) and scirpy (v0.7.1); all additional custom workbooks are available at https://github.com/bedapub/PD1-IL2v_in-vivo_TILs_Panc02_publication. Custom code for RNA-seq analysis in the chronic infection model is available from the corresponding authors on reasonable request.

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L.C.D., V.N., M.H., R.A., L.L., M.K., M.R., E.B., J.S., S.W. and M.T. analysed the in vivo and in vitro experiments. M.H., L.L., M.K., X.G., S.L., A.F.-G., S.S., V. Teichgräber, C.K. and P.U. designed experiments. L.C.D., V.N., M.H., R.A., L.L., M.K., R.A., C.K. and P.U. wrote the manuscript, with M.B., S.H., S.C., D.H., S.L., V. Teichgräber and A.F.-G. contributing by providing feedback and their expertise. P.C.S., L.L. and E.M.V. contributed equally.

Competing interests Patent application number 15/943,237, with relevance to this work, has been filed by Roche. L.C.D., C.K., L.L., V.N., M.H., L.L., M.K., X.G., S.L. and P.U. are named inventors on this patent family. R.A. holds patents related to the PD-1 pathway. K.H., M.H. and R.A. declare no additional financial interests. L.C.D., V.N., M.H., R.A., L.L., M.K., R.A., C.K. and P.U. are employees of Roche and declare ownership of Roche stock. Work performed at Emory for this manuscript was supported by the Roche pRED ROADS program.

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Extended Data Fig. 1 | MuFAP-IL2v fails to synergize with muPD-L1 therapy during chronic LCMV infection. **a**, Chronically LCMV-infected mice (> day 40 post-infection) were left untreated or treated with muPD-L1, muPD-L1 + muFAP-IL2wt, and muPD-L1 + muFAP-IL2v therapy for 2 weeks and then CD8+ T-cell responses and viral titer were examined. **b**, Representative FACS plots for D\(^{\text{GP33}}\) CD8+ T cells in spleen. **c**, Numbers of D\(^{\text{GP33}}\) and D\(^{\text{GP276}}\) CD8+ T cells. **d**, Phenotypic marker expression on D\(^{\text{GP33}}\) CD8+ T cells. **e**, Representative FACS plots for IFN-γ+ and INF-γ+TNF-α+ LCMV-specific CD8+ T cells. **f**, Numbers of IFN-γ+ and INF-γ+TNF-α+ LCMV-specific CD8+ T cells. **g**, Viral titer in spleen. Results were pooled from 2-3 experiments with n = 2–5 mice per group in each experiment. Data are presented as geometric mean and 95% CI (**c, f**) or mean and SD (**d, g**) with p values. Statistical comparisons were performed using one-way ANOVA with Tukey’s multiple comparison test. Untx, untreated; AF, Alexa Fluor.
Extended Data Fig. 2 | MuFAP-IL2v is biologically active in vivo but non-specifically expands CD8 T cells. a, Chronically LCMV-infected mice (> day 40 post-infection) were left untreated or treated with muPD-L1, muPD-L1 + muFAP-IL2wt, and muPD-L1 + muFAP-IL2v for 2 weeks and then analyzed for expansion of CD8 T cells. b, Representative FACS plots for CD8+ T cells in PBMCs. c, Numbers of CD8+ T cells per 10^6 PBMCs. d, Representative FACS plots for CD44 and PD-1 expressions on CD8+ T cells in PBMCs. e, Numbers of CD44+PD-1- and CD44+PD-1+ CD8+ T cells per 10^6 PBMCs. Results were pooled from 3-4 experiments with n = 2-5 mice per group in each experiment. Data are presented as geometric mean and 95% CI with p values. Statistical comparisons were performed using one-way ANOVA with Tukey’s multiple comparison test. Untx, untreated.
Extended Data Fig. 3 | PD1-IL2v is internalized upon binding to PD-1 and IL-2Rβγ and drives the internalization of the bound PD-1 receptors.  

**a.** Representative confocal images of IL2v and PD-1 receptor internalization kinetics at 37 °C in in vitro activated polyclonal PD-1+ CD4 T cells upon incubation with 630 pM of PD1-IL2v or FAP-IL2v for 1 or 3 h, with or without anti-PD1 pre-treatment to prevent PD-1 binding by PD1-IL2v. PD1-IL2v and FAP-IL2v are in pink, PD-1 surface staining in yellow, and the cytoplasm is in cyan.

**b, c.** Quantification of average drug intensity in membrane/cytoplasm (Log₂). Log₂ = 0: Equal amount of drug at the membrane and in the cytoplasm (dotted line). Log₂ > 0: More drug is on the membrane. Log₂ < 0: Drug localizes in the cytoplasm. Each dot represents quantification from a single CD4 T cell; clear and dark dots indicate T cells derived from two different donors, from 2 independent experiments. Mean and SD are shown. One-way ANOVA with a post hoc Tukey multiple comparison test.
Extended Data Fig. 4 | In vitro CD4 T cell activation and cytokine release by PD1-IL2v and in vivo expansion of antigen-specific polyfunctional CD8 T cells by muPD1-IL2v. 

a. Dose dependent increase in frequencies of GM-CSF+ and IFN-γ+ human polyclonal CD4 T cells upon 5 days of in vitro stimulation with increasing concentrations of either PD1-IL2v, Aldesleukin, FAP-IL2v or PD-1 antibody (n = 4 healthy donors, 2 independent experiments, mean ± SEM).

b. Frequency of in vitro activated, polyclonal human STAT5-P+ CD4 T cells upon exposure for 12 min to increasing concentrations of either PD1-IL2v, FAP-IL2v or FAP-IL2 superkine-analogue. As additional control, part of the PD-1+ T cells were pre-treated with PD-1 antibody to prevent PD-1-mediated targeting of PD1-IL2v (dotted line) (n = 2 donors from 2 independent experiments, mean ± SEM). c. Targeting of several T cell subsets and NK cells from fresh PBMCs by PD1-IL2v, FAP-IL2v and FAP-IL2 superkine-analogue (n = 8 healthy donors from 4 independent experiments, box plots representing median, minimum/maximum and individual points). Statistical comparisons were performed using two-way ANOVA with Tukey’s multiple comparison test.

d. Frequency of in vitro activated, STAT5-P+ murine CD4 T cells upon exposure for 12 min to increasing concentrations of either muPD1-IL2v or muFAP-IL2v in vitro (n = 2 mice from 2 independent experiments, mean ± SEM).
**Extended Data Fig. 5** See next page for caption.
Extended Data Fig. 5 | Comparative analysis of muPD1-IL2v versus muFAP-IL2v in combination therapy with muPD-L1 during chronic LCMV infection.

a, Representative histogram for expression of PD-1 by D^bGP33^ CD8^+^ T cells, Foxp3^+^ CD4^+^ T cells (Tregs), conventional (Foxp3^−^) CD4^+^ T cells, and naive (CD44^lo^) CD8^+^ T cells. All T cells except naïve CD8^+^ T cells were isolated from spleens of chronically LCMV-infected mice (> day 40 post-infection). naïve CD8^+^ T cells were isolated from uninfected C57BL/6J mice. The results are representative of two experiments (n = 6 for chronically LCMV-infected mice and n = 2 for uninfected mice).
b, Chronically LCMV-infected mice (> day 40 post-infection) were treated with muPD-L1, muPD-L1 + muFAP-IL2v, and muPD-L1 + muPD1-IL2v for 2 weeks and then analyzed for CD8 T-cell responses and viral titer.
c, Representative FACS plots for D^bGP33^ CD8^+^ T cells in spleen.
d, Numbers of D^bGP33^ CD8^+^ T cells in the indicated tissues.
e, Numbers of IFN-γ^+, INF-γ^+^TNF-α^+, and INF-γ^+^IL-2^+^ LCMV-specific CD8^+^ T cells in spleen.
f, Phenotypic marker expression on D^bGP33^ CD8^+^ T cells in spleen.
g, PCA plot of RNA-seq for naïve CD8^+^ T cells from uninfected mice and D^bGP33^ CD8^+^ T cells from chronically LCMV-infected mice after the indicated treatments.
h, Heat map showing mean relative expressions of all differentially expressed genes (n = 1954) across treatment groups.
i, Viral titer in the indicated tissues. Results were pooled from 3–5 experiments with n = 2–5 mice per group in each experiment (c–f, i). RNA-seq data for groups of naive, Untx, and muPD-L1 were obtained from GSE206722. RNA-seq data for muPD-L1 + muPD1-IL2v group were generated from biological triplicates with n = 2 mice per replicate (g, h).

Data are presented as geometric mean and 95% CI (d, e) or mean and SD (f, i) with p values. Statistical comparisons were performed using Kruskal-Wallis test with Dunn’s multiple comparison test (d, e), one-way ANOVA with Tukey’s multiple comparison test (f, i). Untx, untreated.
Extended Data Fig. 6 | Immunotherapy of chronically LCMV-infected mice with muPD-L1 and muPD1-IL2v. Chronically LCMV-infected mice (> day 40 post-infection) were left untreated or treated with muPD-L1, muPD1-IL2v, and muPD-L1 + muPD1-IL2v for 2 weeks and then analyzed for CD8 T-cell responses. a, Numbers of D\textsuperscript{o}GP33\textsuperscript{+} CD8\textsuperscript{+} T cells per 1 x 10\textsuperscript{6} PBMCs. b, CD127 expression on D\textsuperscript{o}GP33\textsuperscript{+} CD8\textsuperscript{+} T cells in spleen. c, Spleen cells were isolated from chronically LCMV-infected mice after the indicated treatments for 2 weeks. One million cells were cultured with recombinant mouse IL-12 and IL-18 (20 ng/ml each) for 5 h, then GolgiPlug was added, followed by culturing for 1 h. Cells were stained with surface markers including D\textsuperscript{o}GP33-specific tetramer, fixed, and followed by intracellular staining for IFN-γ. d, Representative FACS plots for co-staining of CD218a and IFN-γ gated on D\textsuperscript{o}GP33\textsuperscript{+} CD8\textsuperscript{+} T cells. e, Frequency of IFN-γ\textsuperscript{+} cells among D\textsuperscript{o}GP33\textsuperscript{+} CD8\textsuperscript{+} T cells in response to stimulation with IL-12 + IL-18. f, g, Sorted stem-like (PD-1\textsuperscript{+}CXCR5\textsuperscript{−}TIM-3\textsuperscript{−}) and exhausted (PD-1\textsuperscript{+}CXCR5\textsuperscript{−}TIM-3\textsuperscript{+}) CD8\textsuperscript{+} T cells isolated from CD45.2\textsuperscript{+} chronically LCMV-infected mice (> 40 days post-infection) were adoptively transferred into infection-matched CD45.1\textsuperscript{+} recipient mice, followed by muPD1-IL2v therapy for 2 weeks. Representative FACS plots for the frequency of donor CD45.2\textsuperscript{+} cells (f) and TIM-3 and CD218a expression on transferred donor stem-like CD45.2\textsuperscript{+} CD8\textsuperscript{+} T cells in spleen of recipients after 2 weeks of the treatments (g). Results were pooled from 2–6 experiments with n = 2–4 mice per group in each experiment. Data are presented as geometric mean and 95% CI (a) or mean and SD (b–e) with p values. Statistical comparisons were performed using Kruskal-Wallis test with Dunn’s multiple comparison test (a) or one-way ANOVA with Tukey’s multiple comparison test (b–e). Untx, untreated; AF, Alexa Fluor.
Extended Data Fig. 7. CD8 T cells acquire a polyfunctional effector profile upon muPD1-IL2v and are critical for its efficacy. Frequency and amount of PD-1 and IL-2Rβ per T cell in the tumor and blood of huPD1-transgenic mice. a, b. Left, representative FACS contour plot of PD-1+ CD8 T cells secreting granzyme B, IFN-γ and TNF-α across different treatment groups; right, frequency of PD-1+ granzyme B+ and IFN-γ+ TNF-α+ CD8 TILs (n = 4 mice per group per experiment from 3 independent experiments, box plots representing median, minimum/maximum and individual points). Statistical comparisons were performed using one-way ANOVA with Tukey’s multiple comparison test. c. Tumor growth inhibition and d. CD8 T cell count in blood of syngeneic mice bearing subcutaneous Panc02-H7-Fluc tumors with or without CD8 depletion before the start of the indicated treatments (n = 11 mice per treatment group, mean ± SEM). e–h. Frequencies of receptor positive T cells and quantification of PD-1 receptors and IL-2Rβ on T cells isolated from tumors and blood of untreated human PD-1 transgenic mice bearing Panc02-H7-Fluc (n = 4 and n = 9 mice respectively, box plots representing median, minimum/maximum and individual points). i. (Top) Percentage of directly conjugated Alexa Fluor-647 parental anti-PD-1 bound to 3 days activated CD4 T cells previously exposed to increasing concentrations of either PD1-IL2v, pembrolizumab or non-blocking PD1-IL2v; (bottom) percentage of PD-1 receptors occupied by either PD1-IL2v, pembrolizumab or non-blocking PD1-IL2v and therefore unavailable for binding of the directly conjugated Alexa Fluor-647 parental anti-PD1 (n = 2 healthy donors from 2 independent experiments, mean ± SEM).
Extended Data Fig. 8 | MuPD1-IL2v expands PD-1+ TCF-1+ stem-like resource CD8+ TILs and their progeny and enhances their cytotoxicity. a. Joint 2D UMAP visualization of all cells across all treatments and individual mice colored according to Leiden clusters. b. Selected relative average marker expression at RNA and protein (in capitals) level identifies the majority of cells as CD8 T cells as expected (21, 18, 19 exception, 7 myeloid/T cell doublet, 22 Th17, 20 regulatory T cell specific expression within CD8+) (left). Among CD8 T cell clusters, 5 shows naïve specific, 6 stem-like, 11 and 1 exhausted-like, 3, 4, 12, 14, 16, 17 better effector and the rest more broadly memory/effector-like (right). c. 2D UMAP visualization of the CD8 T cell distribution in the vehicle (control) group. d. Relative marker expression stratified per cell type and treatment for 3 main treatment groups: muPD1-IL2v, muPD-1 monotherapy and combination with FAP-IL2v. e. (Top) Number of distinct TCR clones present within stem-like T cells, within their progeny (all CD8 effector/exhausted subsets) and the ones shared between the two per treatment group; (middle) number of cells (normalized to 10,000) with a high clonotype expansion (≥10), per treatment group; (bottom) percentages of TCR clones in the stem-like T cells that are shared with their progeny with low clonotype (<10) or high clonotype expansion (≥10). (a–e). n = 3–4 mice per treatment group.
Extended Data Fig. 9 | Tumor growth inhibition and survival curves in B16-F10-OVA tumor mouse model. a. Tumor growth kinetics upon muPD1-IL2v, b. muFAP-IL2v and c. muPD-1 in combination with muFAP-IL2v. (a–c) n = 5–8 mice per treatment group, 2 independent experiments.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a  Confirmed
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficients) and variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

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For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.
Data

Policy information about availability of data.
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy.

The scRNA-seq, TCRseq, and CITeseq, data on tumor infiltrating lymphocytes discussed in this publication have been deposited in ArrayExpress with the accession number E-MTAB-1773.
RNA-seq data from chronic infection experiments are available in the NCBI Gene Expression Omnibus (GEO) database under the accession number GSE208556.
Reads were mapped to the GRCh38/mm10 genomeS3 with HISAT2 version 2.1.0.s4. Gene expression was quantified by featureCounts55 (v.1.5.2). DESeq2 (Love et al., 2014, v.1.24.0) was used to normalized for library size and calculate differential expression across groups.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

| Reporting on sex and gender | Sex: 39 females and 11 males |
| Population characteristics | Healthy donors, born between 1949-2001 |
| Recruitment | Healthy donors that donate blood on volunteer basis at the blood donation center (Blutspende Zürich, Switzerland, https://www.blutspendezurich.ch/) randomly allocated to Roche |
| Ethics oversight | Approval of the Cantonal Ethics Committee (Zürich) |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For the chronic infection experiments: No statistical methods were used to predetermined sample size. Sample sizes were chosen based on previous experiences (West E. E. et al, PD-L1 blockade synergizes with l-2 therapy in reinvigorating exhausted T cells. J Clin Invest 123, 2604-2615, doi:10.1172/JCI67008 (2013)), balancing statistical robustness and animal welfare. For the cancer model experiments: group size is determine statistically using JMP statistic software program to allow significant difference with minimum amount of mice per group to comply with country animal welfare guidelines. For in-vitro and ex-vivo experiments 3 to 6 different donors from 3 to 6 independent experiments were used based on inter-donor variability, data homogeneity and statistical significance. For imaging studies at least 12 cells were analyzed from the selected optical section in order to have statistical significance. |
| Data exclusions | For scRNaseq on TILs: two samples were excluded because of low overall quality and very low cell number, respectively, all other samples were included in the analysis. |
| Replication | All data were reliably reproduced. The number of repeats and sample sizes are provided in each figure legend. |
| Randomization | For chronic infection model: LCMV chronically infected mice were randomly assigned to experimental groups. For mouse tumor models: Randomization is performed with the use of an automated software in the POMES platform. Group size is determine statistically to allow significant difference with minimum amount of mice per group to comply with country animal welfare guidelines. For in vitro studies all used donors were either left untreated (negative control) or were exposed in parallel to equimolar concentrations of all the indicated therapies. |
| Blinding | For the chronic infection model: investigators were not blinded to group allocation during experimental setup, data collection, and analysis. No blinding was performed since we did not have the personnel resources to consistently perform blinding. For the mouse tumor models: investigators were blinded to group allocation during data collection and analysis by means of assigning letters to the treatment groups by people involved in preparing the drug dilutions that need to be injected. Technical involved personnel are |
Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

- [ ] n/a Involved in the study
- [ ] Antibodies
- [ ] Eukaryotic cell lines
- [ ] Palaeontology and archaeology
- [ ] Animals and other organisms
- [ ] Clinical data
- [ ] Dual use research of concern

### Methods

- [ ] n/a Involved in the study
- [ ] ChIP-seq
- [ ] Flow cytometry
- [ ] MRI-based neuroimaging

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**Antibodies**

| Antibodies used | The complete antibody list is provided as supplementary information. Ex-vivo binding on human PBMCs Marker Fluorophore Clone #CAT Company Dilution/Final Concentration Live/Dead APC-Cy7 66-0865-14 eBioscience 1:500 CD45 AF700 H350 56-9459-42 eBioscience 1:100 CD3 BV605 OKT3 317322 Biologend 1:100 CD4 BV496 OKT4 612936 BD Biosciences 1:100 CD8 BV395 RPA-TB 563795 BD Biosciences 1:100 CD366 (TIM-3) BV711 F38-212 345024 Biologend 1:20 LAG3 (CD223) PerCP-Cy5.5 305223H 46-2239-42 eBioscience 1:20 TIGIT BV786 741182 747388 BD Biosciences 1:20 CD218a (IL-18Ra) FITC H44 313810 Biologend 1:100 CD56 BV5480 NCAM16.2 566124 BD Biosciences 1:20 TCR g/d PE-Cy7 B1 331272 Biologend 1:50 PG-LALA PE NA Roche 1:650 (2.5µg/ml) TCF-1/TCF-7 AF647 C6309 67095 Cell Signaling Technology 1:100 PD-1 IC D8WJ 861635 Cell Signaling Technology 1:100 Goat anti-Rabbit secondary ab BV421 Polyclonal 565014 BD 1:100 FOXP3 PE-C594 2060 320126 Biologend 1:50 In-vitro experiment on human CD4 T cells (incl. Tcon and Tregs) Marker Fluorophore Clone #CAT Company Dilution/Final Concentration Stat5 PE BV994 47/Stats (pY694) AF647 47/Stats (pY694) 562076 BD 1:20 PD-1 PE EH12.2H7 329906 Biologend 2.5µg/ml IL2Rb PE TU27 339006 Biologend 2.5µg/ml isotype PE MOPC-21 400112 Biologend 2.5µg/ml Parental PD-1 AF647 0376 NA Roche 1µg/ml PD1-L2v AF647 0376-L2v fused NA Roche 1µg/ml CD4 AF700 RPA-T4 56-0049-42 eBioscience 1:50 live/dead Aqua Dead Cell Stain - L34966 Invitrogen 1:1000 Granzyme B AF647 615199 BD Biosciences 1:100 GM-CSF PE BV02-21C11 502306 Biologend 1:100 IFNγ PE-Cy7 45.B3 25-7319-82 eBioscience 1:200 Ex-vivo receptor quantification on mouse TILs and T cells from blood Marker Fluorophore Clone #CAT Company Dilution/Final Concentration PD-1 PE 29F-1412 135206 Biologend 2.5µg/ml PD-1 PE EH12.2H7 329906 Biologend 2.5µg/ml IL2Rb PE SH4 105906 Biologend 2.5µg/ml TCRR BV21 H57-597 109230 Biologend 1:200 CD8 BV395 53.6 7 565958 BD Biosciences 1:200 CD4 AF488 GK1.5 100406 Biologend 1:100 CD62L BV711 MEL-14 104445 Biologend 1:200 CD44 BV480 IM7 566116 BD Biosciences 1:200 FOXP3 AF647 1500 320014 Biologend 1:100 Antibodies used for the LCMV-chronic infection Marker Fluorophore Clone #CAT Company Dilution CD4 BV563 RM4-5 741217 BD Biosciences 1:500 CD4 FITC RM4-5 553046 BD Biosciences 1:500 |
CD4  V505  RM4-5  S60782  Biolegend 1:500
CD4  BV6505  RM4-5  100548  Biolegend 1:500
CD4  BV711  RM4-5  100557  Biolegend 1:500
CD4  PE-Cy7  RM4-5  25-0042-82  Thermo Fischer Scientific 1:500
CD4  APC-eFluor  780  RM4-5  47-0042-82  Thermo Fischer Scientific 1:500
CD8a  BV496  53-6.7  563786  BD Biosciences 1:100
CD8a  BV421  53-6.7  100753  Biolegend 1:150
CD8a  BV605  53-6.7  100744  Biolegend 1:100
CD8a  PerCP-Cy5.5  56-553036  BD Biosciences 1:100
CD19  BV563  13D  749028  BD Biosciences 1:150
CD19  BV510  13D  115546  Biolegend 1:150
CD19  BV605  103  115540  Biolegend 1:150
CD19  PE-Cy7  103  25-0193-82  Thermo Fischer Scientific 1:150
CD19  APC-eFluor  780  103  47-0193-82  Thermo Fischer Scientific 1:150
CD44  BV805  IM7  741921  BD Biosciences 1:500
CD44  FITC-M7  561859  BD Biosciences 1:500
CD44  AF700  IM7  56-0441-82  Thermo Fischer Scientific 1:100
CD45.2  APC  104  109814  Biolegend 1:100
CD127  BV737G  58/199  612841  BD Biosciences 1:100
CD127  PE-A7R34  12-1271-83  Thermo Fischer Scientific 1:100
CD218a  PE  P3TUNYA  12-5183-82  Thermo Fischer Scientific 1:100
CD218a  PE-Cy7  P3TUNYA  25-5183-82  Thermo Fischer Scientific 1:100
CXCR3  PE-Cy7  CXCR3-173  25-1831-82  Thermo Fischer Scientific 1:100
CXCR5  BV421  L138D7  145512  Biolegend 1:50
CX3CRI  BV785  SA011F1  149031  Biolegend 1:500
Foxp3  PE-Cy7  FJK-16s  25-5773-82  Thermo Fischer Scientific 1:250
IL-2  PE-JES6  5H4  554428  BD Biosciences 1:100
IFN-γ  BV421  XM1.2  505830  Biolegend 1:100
IFN-γ  APCXM1G1.2  554413  BD Biosciences 1:100
PD-1  PE  RMP1-30  109104  Biolegend 1:100
PD-1  APC  RMP1-30  109112  Biolegend 1:100
TCF-1  PE  S33-966  564217  BD Biosciences 1:100
Tim-3  BV395  SD12  747620  BD Biosciences 1:100
Tim-3  AF488  215008  FAB1529G R&D systems 1:20
TNF-α  BV421  MP6-XT22  506328  Biolegend 1:100
TNF-α  PE  MP6-XT22  554419  BD Biosciences 1:100

Name #Catalogue
anti-mouse PD-L1 with DAPG mutation  NA NA Roche 200 μg/mouse/injection
mouse IgG1 isotype control  MOPC-21 BE0083 BioXcell 200 μg/mouse/injection
anti-mouse CD4  GK1.5 BE0003-1 BioXcell 300 μg/mouse/injection

Antibodies used for TILs characterization in mouse tumor-model
Marker Fluorochrome clone Cat number Provider Dilution
Fixable Viability Dye  eFluor™ 455 555UV 65-0868-14 1:500
CD45  AF700  30-F11  103128  Biolegend 1:300
TCRβ  PerCP-Cy5.5  H57-597  109228  Biolegend 1:200
CD6  APC-Cy7  53-6.7  100714  Biolegend 1:200
CD4  PE-Cy7  GX1.5  100422  Biolegend 1:200
CD62L  FITC-MEL-14  104406  Biolegend 1:200
CD127  PE  A7R34  135010  Biolegend 1:100
TCRβ  PerCP-Cy5.5  H57-597  109228  Biolegend 1:200
CD4  BV421  GK1.5  100438  Biolegend 1:200
Granzyme B  AF647  G811  515406  Biolegend 1:100
IFN-γ  BV786  XM11.2  505838  Biolegend 1:100
TNF-α  PE-Cy7  MP6-XT22  506306  Biolegend 1:100
Foxp3  BV421  MF-14  126419  Biolegend 1:100
CD39  AF647  Daub9  143808  Biolegend 1:200
Granzyme B  AF700  QA16A02  372222  Biolegend 1:100
k67  PE-Cy7  16A8  652426  Biolegend 1:300
PD1  PE-Cy7  RMP1-30  109110  Biolegend 1:200
CD25  BV711  RMT3-23  102049  Biolegend 1:200
TGIT  PE-Dazzle594  109  142110  Biolegend 1:100
IFN-γ  BV605  XM11.2  505840  Biolegend 1:100
TNF-α  BV421  MP6-XT22  506328  Biolegend 1:100
CD107a  AF488  1D4B  121608  Biolegend 1:100
CD44  BV510M7  563316  BD Biosciences 1:200
CD45  BV805  30-F11  608748370  BD Biosciences 1:100
TCRβ  BV786  H57-597  742484  BD Biosciences 1:100
CD4  BV496  RM4-5  612952  BD Biosciences 1:100
CD8  BV395  53-6.7  563786  BD Biosciences 1:100
PD-1  BV737  RMP1-30  749306  BD Biosciences 1:100
CD25  PE-CF594  PCG1  562694  BD Biosciences 1:100
Tim-3  BV550  SD12  747623  BD Biosciences 1:100
TCF-1  PE  S33-966  564217  BD Biosciences 1:100
LAG3  BV650  C9B7W  740560  BD Biosciences 1:100
SLAMF6  BV510  `13G3 745073  BD Biosciences 1:50
CD218a  FITC REA947 130-115-703 Miltenyi 1:50
OVA-dextramer H-2 Kb (SIINFEKL) APC JD2163 Immudex 1:100
Rat anti-mouse CD8a  InVivoPlus  2.43 BP0061 BioXcell 100 µg/mouse/injection

**Feature barcoding**

| Marker | Oligo Tag Clone Source Concentration (µg/ml) |
|--------|---------------------------------------------|
| CD28 | ATTAATGACGCTCTGGTGGG 37:1 TotalSeq-C, Biolegend 1 |
| CD44 | TGGCTTCAGTCCTGA 1M7 TotalSeq-C, Biolegend 1 |
| CD62L (L-selectin) | TGGGCTCAATGCATC ME-14 TotalSeq-C, Biolegend 1 |
| CD39 | CGCTTTAATCCGGGT Duha59 TotalSeq-C, Biolegend 1 |
| CD279 (PD-1) | GAAAGTCGAACGACT RPM1-30 TotalSeq-C, Biolegend 1 |
| CD366 (Tim-3) | ATGGGACCTCAGTATG RMT-23 TotalSeq-C, Biolegend 1 |
| CD223 (LAG-3) | ATTTGCTCCATAGG C9B7W TotalSeq-C, Biolegend 1 |
| CD183 (CXC3) | GTACGACGGCTGG GTA CXC3-173 TotalSeq-C, Biolegend 1 |
| CD185 (CXCR5) | AGTGAAGCTCACTG T138D7 TotalSeq-C, Biolegend 1 |
| CD127 (IL-7R) | GGTGTCGACCTC TCTAF34 TotalSeq-C, Biolegend 1 |
| TIGIT (Vst3M) | GAAAGTGCGACCAAG C6G TotalSeq-C, Biolegend 1 |
| CD205 (ACCATAGCAAGCAG PC61 TotalSeq-C, Biolegend 1 |
| Ly108 (SLAM-F6) | CGATCTTTCGGCAGG 330-AJ TotalSeq-C, Biolegend 1 |
| CD137 (4-18B) | TCCCTGTATAGTG A1785 TotalSeq-C, Biolegend 1 |
| IL-2R | GATTCCGAGTACAG A49 TotalSeq-C, Biolegend 1 |

**Histology**

| Marker Fluorochrome clone Cat number Provider Dilution |
|-------------------------------------------------------|
| CD3 | SP7 RMA8005 Diagnostic Biosystems 1:100 |
| CD8 | 4S15 14-0808-82 ebioscience 1:300 |
| PD-1 | Polyclonal AF1021 R&D Systems 1:250 |
| Granzyme B | Polyclonal Ab4095 Abcam 1:250 |

**Confocal imaging**

| Marker Fluorochrome clone Cat number Provider Dilution |
|-------------------------------------------------------|
| PD-1 IC D4W2J 861635 Cell Signaling Technology 1:100 |
| Goat anti-rabbit IgG (H+L), F(ab)2 Fragment AF488 Polyclonal 4412S Cell Signaling Technology 1:1000 |
| Parental PD-1 Pure 0376 NA Roche 10 µg/ml |
| PD1-IL2v AF647 0376-IL2v fused NA Roche 1 µg/ml |
| FAP-IL2v AF647 489-IL2v fused NA Roche 1 µg/ml |

**Cell sorting**

| Marker Fluorochrome clone Cat number Provider Dilution |
|-------------------------------------------------------|
| CD45 AF700 30-F11 103128 Biolegend 1:100 |
| CD8 BV711 53-6.7 100748 Biolegend 1:100 |
| CD4 BV605 (B cell channel) GK1.5 100451 Biolegend 1:100 |
| CD11c BV605 (B cell channel) N481 117334 Biolegend 1:100 |
| Live/Dead APC Cy7 65-0805-14 ebiosciences 1:500 |

**Validation**

For FACS and CITE-seq, the antibodies have been titrated (2 fold serial dilutions of the recommended concentration) on human healthy donor PBMCs or mouse splenocytes for meaningful biological patterns against common cell line markers (CD45, CD3, CD4, CD8, CD56, FOXP-3, gd T cells) and differentiation state (CD62L, CD44 and CD127) by monitoring the frequencies of positive cells (known from previous experiments and the available literature) and the background signal by using also FMO or an isotype control staining; for markers associated with exhaustion or activation (PD-1, TIM-3, LAG-3, TIGIT, CD218a, CD25) cytokine secretion (GM-CSF, Granzyme B, IFN-g and TNF-a) and IL-2R signalling (STAT-SP) manufacturers’ recommendations have been followed and the staining assessed on either 3 days polyclonally activated human T cells or murine splenocytes.

The selected titration for murine antibodies were the then further validated on mouse TILs isolated from tumors of untreated mice. Detailed information and references for validation and QC are shown in https://www.biolegend.com/en-us/quality/quality-control/https://www.bdblismatics.com/en-ch/products/reagents/flow-cytometry-reagents/research-reagents/quality-and-reproducibility/.

Antibodies for IHC are set up and validated using negative and positive mouse tissue and cell pellets from in-vitro 3 days activated murine T cells, isotype control antibodies and omission of primary antibody during the staining. 3 serial dilutions are tested to address the dynamic range of the assay.

For Imaging staining the intracellular PD-1 staining antibody was used at concentrations suggested by Cell Signaling Technologies (https://www.cellsignaling.com/products/primary-antibodies/pd-1-d4w2j-xp-rabbit-mab/85183)

For in-vivo depletion of CD8 T cells with Rat anti-mouse CD8a InVivoPlus clone 2.43 from BioXcell we referred to Lin J-S, Szaba FM, Kummer LW, Chromy BA, and Smiley ST. 2011. J. Immunol. 187: 897-904. Wozniak KJ, Young ML, and Wormley FL. 2011. Clin. Vaccine Immunol. 18(5):717-723 and Hufford MM, Kim TS, Sun J, and Braciale TJ. 2011. J. Exp. Med. 208: 167-180.

For in-vitro experiments immunocytokines (PD1-IL2v, FAP-IL2v, nBP01-IL2v) were used at EC50 concentration (630 pM) obtained from the dose response IL-2R signaling curves on 3 days polyclonally activated CD4 T cells.

Anti-mouse PD-L1 antibody with DAPG mutation was validated by Roche and the previous study (Klein, C. et al. Cergutuzumab amunaleukin (CEA-IL2v), a CEA-targeted IL-2 variant-based immunocytokine for combination cancer immunotherapy: Overcoming limitations of aldesleukin and conventional IL-2-based immunocytokines. Oncoimmunology 6, e1277306,
Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

| Vero E6 cells (ATCC) |
|----------------------|
| Panc02-H7-Fluc cell line was generated at Roche Glycart. |
| B16-OVA cell line was purchased from ProQinase. |
| SCC173 Sigma-Aldrich Mouse Fibrosarcoma Cell Line: MCA-205 was derived from 3-methylcholanthrene-induced fibrosarcoma in C57Bl/6 mice. Tumors were maintained in vivo by serial subcutaneous transplantation in syngeneic mice and single-cell suspensions were prepared from solid tumors by enzymatic digestion. From these cells the MCA-205 cell line was established and maintained in vitro. |

Authentication

| Vero E6 cells were not authenticated. |
| MCA-205, B16-OVA and Panc02-H7-Fluc were authenticated through morphology and PCR assays with species specific primers. |

Mycoplasma contamination

| MCA-205: Cells are tested negative for infectious diseases by a Mouse Essential CLEAR panel by Charles River Animal Diagnostic Services: cells are negative for mycoplasma contamination. |
| Panc02-H7-Fluc cell line and B16-OVA cell line batches are routinely tested for mycoplasma and are negative. |

Commonly misidentified lines

(See CIAC register)

| No commonly misidentified cell lines were used in this study |

Animals and other research organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals

| Six to 8-week-old female C57Bl/6j and CD45.1 congenic mice were purchased from the Jackson Laboratory for chronic infection model and Charles Rivers, Lyon, France for the mouse tumor models except the RIP-Tag mouse tumor model performed on males aged from 21 to 31 weeks. |
| For the chronic infection model: |
| All animal experiments were performed in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines. |
| The following housing conditions for the mice are used. |
| For the chronic infection experiments: |
| - Light Cycle is 7:00 am ON, 7:00 pm OFF |
| - Temperature is between 68-74 degrees Fahrenheit |
| - Humidity is between 30-70 g/m³ |
| For the mouse tumor model: |
| Mice were maintained under specific-pathogen-free conditions with daily cycles of 12 hours light/darkness according to guidelines (temperature of 22°C, dark/light cycle of 12h, and humidity of 50%, GV-SOLAS, FELASA) and food and water were provided ad libitum. Continuous health monitoring was carried out and the experimental study protocol was reviewed and approved by the Veterinary Department of Canton Zürich. |

Wild animals

| No wild animals were used |

Reporting on sex

| Female mice were used in the chronic infection and tumor models |

Field-collected samples

| No field collection was performed |

Ethics oversight

| For the chronic infection: All animal experiments were performed in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines. |
| For mouse tumor models: experimental study protocol was reviewed and approved by the Veterinary Department of Canton Zürich. |
| For the RIP-Tag mouse tumor model: Animal experiments were conducted according to protocols approved by the Veterinary Authorities of the Canton of Vaud and the Swiss Law. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g., CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Human PBMC isolation

Blood samples from healthy volunteers were obtained via the blood donation center (Zürich, Switzerland) with approval of the Cantonal Ethics Committee (Zürich). PBMC were isolated from the blood of different healthy donors using density gradient centrifugation with Histopaque-1077 (Sigma). All cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), GlutaMAX (Gibco), and 1% penicillin-streptomycin 100× (Gibco).

Human and murine CD4 T cell isolation and in vitro activation

Human CD4T cells were sorted by using a CD4-positive selection Miltenyi beads system following manufacturer instructions. Thereafter the cells were labelled with CFSE (5 μM, 5 min at RT, eBioscience) or CTV (5 μM, 5 min at room temperature, Thermo Scientific) to measure cell proliferation.

CD4 T cells were seeded into an αCD3 pre-coated plate (1 μg/ml, clone OKT3, BioLegend overnight, 4°C) with addition of soluble αCD28 (1 μg/ml, clone CD28.2, BioLegend). The cells were cultured for 3 days to induce activation and upregulation of the CD-1 receptor on the surface of the CD4 T cells.

Spleens of C57BL/6 mice were homogenized to a single cells suspension by mashing the spleen through a 100 μm cell strainer and the erythrocytes were lysed with ACK (ammonium-chloride-potassium) lysis buffer for 5 min at 4°C. CD4 T cells were sorted with a CD4-negative selection Miltenyi beads system following manufacturer instructions. CD4 T cells were seeded into an αCD3/αCD28 pre-coated plate (5 μg/ml, clone 145-2C11, BioLegend and 5 μg/ml, clone 37.51 Biologicd) and activated for 3 days.

Binding competition on Treg and Tconv and Treg suppression assay

CD4+ CD25+ CD127dim regulatory T cells (Tregs) were isolated from human peripheral blood with the two-step regulatory T cell isolation kit (Miltenyi). In parallel the CD4+ CD25− conventional T cells (Tconv) were isolated by collecting the negative fraction of a CD25 positive selection (Miltenyi) followed by CD4+ enrichment (Miltenyi). Tconv were labelled with CFSE and the Tregs were labelled with Cell Trace Violet to track the proliferation of both populations. For the PD-1 and IL2Rb receptor quantification and the PD-1-IL2v binding competition, Tregs and Tconvs were cocultured at a 1:1 ratio into a αCD3 pre-coated plate (1 μg/ml, clone OKT3, BioLegend) with soluble αCD28 (1 μg/ml, clone CD28.2, BioLegend).

In the Treg suppression assay, the rescue of Tconv granzyme B production upon PD1-IL2v treatment was measured upon coculturing Tconv together with Treg at 2:1 ratio for 5 days, in presence or absence of treatment. Irradiated (40 Gy) feeders from an unrelated donor where used to elicit an allospecific stimulation.

GM-CSF, granzyme-B and IFN-γ secretion by CD4 T cells

Sorted and CTV labelled human polyclonal CD4 T cells were activated with soluble αCD3 (1 μg/ml) in presence of irradiated (40 Gy) feeder cells from the same donor at 1:1 ratio and increasing concentrations of treatment antibodies or aldesleukin (Proleukin, Novartis). After 5 days, GM-CSF secretion was measured with ELISA (Biolegend) following manufacturer instructions. For intracellular FACs staining, the accumulation of cytokines in the Golgi complex was induced by re-stimulating the cells with ionomycin (500 ng/ml) and PMA (50 ng/ml) together with protein transport inhibitors (GolgiPlug and GolgiStop, BD) for 5 hours prior to the FACs staining.

Flow cytometry staining for cytokine detection and receptor quantification

The cells were stained in PBS with surface antibodies for 30 min at 4°C for being live/dead (either Aqua Dead Cell Stain, Invitrogen, during the last 10 min of incubation, or Fixable Viability Dye eFluor 780, eBioscience, for 30 min, 4°C). For intracellular staining, cells were permeabilized with FACs permeabilization buffer (fixation/permeabilization, BD Biosciences or Foxp3 Transcription Factor Fixation Kit, eBioscience) and then incubated with antibodies specific for cytokines for 60 min at 4°C. The following antibodies mixes were used:

1) Human: PD1 (clone EH12.2H7 Biologicd, IL2Rb (clone TU27, BioLegend), Isotype control (clone MOPC-21, BioLegend), CD4 (clone RPA-T4, eBioscience), GM-CSF (clone BV2D-2C11, BioLegend), GrzB (GB11, BD Biosciences), IFNγ (clone 4S.83, eBioscience)

2) mouse: PD1 (clone 29F.1A12 Biologicd, IL2R (clone SH4, Biologicd), Isotype control (clone RKT2758, Biologicd), TCRβ (clone HS7.597, Biologie), CD3 (clone 145-2C11, Biologicd), CD8 (clone 53-6.7, BD), CD4 (clone GK1.5, Biologicd), CD45 (clone 30-F11, Biologicd), C602L (clone Mel 14, Biologicd), CD44 (clone IM7, BD), FoxP3 (clone 150D, Biologicd). The number of PD-1 and IL-2Rβ receptors were quantified on the cell surface of PBMC and TIL of huPD1 transgenic mice bearing Panc02-H7-Fluc tumors and on human activated Tregs and Tconvs with the PE Phycoerythrin Fluorescence
Quantitation Kit (BD) following manufacturer’s instructions. 2.5 µg/ml of PE-labeled monoclonal antibodies were used to quantify the receptor of interest on gated populations of interest. The cells and the PE Quantibrite beads were fixed following the same protocol and fluorescence data acquired while using the same settings. The number of receptors was quantified following the kit instructions.

Ex vivo binding of PD1-IL-2v, FAP-IL-2v and FAP-Superkine analogue was performed by incubating 630 µM of the constructs for 30 min on healthy donors PBMCs. After a washing step the cells were incubated for an additional 30 min at 4°C with a PE-labeled antibody recognizing the FGLAIA mutation in the Fc portion of the primary antibodies together with a panel of antibodies to characterize the phenotype of the immune cytokine targeted cells: CD3 (clone OKT3), CD4 (clone OKT4), CD8 (clone RPA T8), TIM 3 (clone F38.2E2), CD218a (clone H44), CD56 (clone NCAM16.2), TCF 1 (C6309) FOXP3 (clone 206D) and PD-1 (clone D4W2).

Lymphocyte isolation
1. For chronic infection experiments
Lymphocytes were isolated from the blood, spleen and lung as described previously. Briefly, spleens were dissociated by passing them through a 70 µm cell strainer (Corning). Lungs were treated with 1.3 mM EDTA in HBSS for 30 min at 37°C, shaking at 200 rpm, followed by treatment with 150 U/ml collagenase (Thermo Fisher Scientific) in RPMI 1640 medium containing 5 % FBS, 1 mM MgCl2, and 1mM CaCl2 for 60 min at 37°C shaking at 200 rpm. Collagenase treated lung tissues were homogenized and filtered through a 70 µm cell strainer. Lymphocytes from lungs were purified by a 44-67% Percoll gradient (800 g at 20°C for 20 min).

2. For cancer model experiments
Tumor tissue and blood were isolated in the animal facility. The tumor tissue was transferred into PBS and was disrupted using manual scissors and the Miltenyi Gentle MACS machine. Subsequently, it was digested in an enzyme mix containing RPMI with 10 mg/ml DNase (Sigma Aldrich) and 0.25 mg/ml latherase (Sigma Aldrich). Upon 30 min of digestion at 37°C, the tissue was filtered through a 70 µm filter and resuspended to a single-cell suspension with an appropriate dilution for subsequent fluorescently labeled antibody staining. The blood was transferred in heparin tubes and was lysed with erythrocyte lysis buffer. Upon red blood lysis, cells were resuspended with an appropriate dilution for subsequent fluorescently labeled antibody staining. Lymphocytes were mechanically isolated from draining lymph nodes with a pestle, filtered through a 70 µm filter and resuspended to a single-cell suspension with an appropriate dilution for subsequent fluorescently labeled antibody staining.

For detection of cytokines, tumor cell suspensions were restimulated with 6.25 ng/ml of PMA (Sigma Aldrich) and 1.87 µg/ml of ionomycin (Sigma Aldrich) for 5h at 37°C. Upon 1 hour of restimulation, Golgiplug (BD) and Golgistop (BD) were added in the cell suspensions. For antigen restimulation, tumor cell suspensions were restimulated with 0.1 µg/ml of gp100 or SIINFEKL peptide, for 5 hours at 37°C. Anti-CD107a antibody was added together with the peptides for 5h at 37°C. As before, upon 1 hour of restimulation, Golgiplug (BD) and Golgistop (BD) were added in the cell suspensions.

Discrimination of living cells versus dead cells was performed using DAPI (Sigma Aldrich) for the non-fixed samples and Fixable Viability Dye eFluor™ 780 (eBioscience) for the fixed ones. Samples were acquired with a BD LSRII Fortessa and a BD FACSYMPHONY A5. Data obtained were analyzed by using FlowJo (v10.8.1, BD Biosciences).

Cell sorting
1. For chronic infection experiments
Cell sorting was performed by a FACSAria II (BD Biosciences). For adoptive transfer experiments, two PD-1 expressing CD8+ T cell subsets (CD4+CXCR5+Tim3+ and PD-1+CXCR5+Tim3+) were sorted from pooled spleens (n=40-60) of chronically CMV infected mice. For RNA-seq analysis of CLMV-specific CD8+ T cells after muPD-1-L1, muPD1-IL2v, and muPD-1-L1 + PD1-IL2v therapy, chronically CMV infected mice (> day 40 post-infection n=3-18) were untreated or treated for 2 weeks, and DCGP32+ CD8+ T cells were sorted from pooled spleens for obtaining at least 2x104 cells. Naïve (CD44lo) CD8+ T cells were sorted from pooled spleens of uninfected mice (n=2-3). All samples had purities of greater than 95%.

2. For cancer model experiments
Single cell tumor suspensions were kept on ice during the staining and sorting procedure. Cell suspensions from 3-5 tumors of the same treatment group were pooled and incubated with Fc receptor block (BD, #101320) for 10 minutes, before staining with the following antibodies: Alexa Fluor 700 anti-CD45 (30-F11), BV711 anti-CD8 (53-6.7), BV605 anti-CD4 (GK.1.5), BV605 anti-CD11c (N418). Discrimination of living cells versus dead cells was performed using DAPI (Sigma Aldrich) for the non-fixed samples and cells incubated for 20 minutes. Cells were washed twice, filtered through a 40µm cell strainer and sorted on FACS AriaII to enrich the viable, single, CD45+ CD8+ CD11c- CD4- population.

Detailed isolation and sample handling are described in the Method section.

Instrument
Cell sorting was performed by FACSAria II or Aria III (BD Biosciences).

FACS data was collected on BD Canto II, LSR II Fortessa, FACS Symphony A3 or A5.

Software
FACSDiva v8.0.1 or 9.1, Flowsio v. 9.9.6 or 10.8.1 (BD Biosciences)

Cell population abundance
The purities of the sorted cells were more than 95%.

Gating strategy
Gate boundaries were set accordingly to control samples [FMO- fluorescence minus one or isotype controls] or based on density distribution. Gating examples are provided as supplementary information.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.