Replicating viral vector platform exploits alarmin signals for potent CD8⁺ T cell-mediated tumour immunotherapy

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Viral infections lead to alarmin release and elicit potent cytotoxic effector T lymphocyte (CTL<sup>eff</sup>) responses. Conversely, the induction of protective tumour-specific CTL<sup>eff</sup> and their recruitment into the tumour remain challenging tasks. Here we show that lymphocytic choriomeningitis virus (LCMV) can be engineered to serve as a replication competent, stably-attenuated immunotherapy vector (artLCMV). artLCMV delivers tumour-associated antigens to dendritic cells for efficient CTL priming. Unlike replication-deficient vectors, artLCMV targets also lymphoid tissue stroma cells expressing the alarmin interleukin-33. By triggering interleukin-33 signals, artLCMV elicits CTL<sup>eff</sup> responses of higher magnitude and functionality than those induced by replication-deficient vectors. Superior anti-tumour efficacy of artLCMV immunotherapy depends on interleukin-33 signalling, and a massive CTL<sup>eff</sup> influx triggers an inflammatory conversion of the tumour microenvironment. Our observations suggest that replicating viral delivery systems can release alarmins for improved anti-tumour efficacy. These mechanistic insights may outweigh safety concerns around replicating viral vectors in cancer immunotherapy.
The clinical efficacy of checkpoint blockade, oncolytic viruses and adoptive T-cell therapy heralds success in harnessing the immune system in the combat against cancer1–3. Conversely, active immunization has not yet demonstrated consistent efficacy in clinical Phase III trials4–6, raising an urgent need for improved vaccine formulations that should aim at delivering large numbers of CD8+ cytotoxic effector T lymphocytes (CTLeff)7–9 to the tumour site while simultaneously establishing a pool of self-replenishing memory cells for durable tumour control5–9. The immunological response profile of replicating viral vaccines represents an excellent match to these requirements10. By delivering tumour-associated antigens (TAA) in the context of an acute viral infection, such delivery systems should supply critical alarmin signals, also referred to as damage-associated molecular patterns, as well as pathogen-associated molecular patterns (PAMPs) for optimal CTL induction and differentiation11,12.

Classical tumour vaccination regimens such as peptides-in-adjuvant showed only marginal clinical benefit, despite the induction of sizeable T-cell TA-specific CTL responses13. Major impediments include inefficient tumour infiltration and efficacy of specific CTLeff (refs 14,15). Overcoming these hurdles will critically depend on innate immune activation, which can be achieved by virus-induced inflammation16.

Lymphocytic choriomeningitis virus (LCMV), the prototype member of the arenavirus family, elicits CTLeff responses of high magnitude and cytotoxic capacity, in combination with life-long CTL immunity. These features, together with a low hazard profile for humans, have rendered it a primary workhorse of immunologists since the 1930s (ref. 17). Experimental LCMV infections in humans have documented a systemic inflammatory reaction, accompanied by a lymphoblastic reaction in peripheral blood18, which was reminiscent of the massive CTLeff response in infectious mononucleosis19. Accordingly, studies in accidentally LCMV-infected laboratory workers have confirmed that, analogously to mice, high frequencies of effector memory CTL are maintained for several years after a single acute infection20. These features in combination with low seroprevalence in the human population21,22 raised our interest in LCMV as a model replicating RNA virus for medical application26,27.

Generation of genetically stable and live-attenuated artLCMV. We aimed at combining tumour antigen vaccination with infection-induced alarmin signals. Hence we sought a strategy how to stably incorporate transgenes into replicating LCMV. The virus’ genome consists of two negative-stranded RNA segments designated L and S, respectively, encoding two viral genes each (LCMVwt, Fig. 1a). 5' and 3' untranslated regions (UTR) and an intergenic region (IGR) flank the ORFs on each segment (Fig. 1a). Reverse genetic tools are available to efficiently tailor the infectious virus’ genome for medical application26,27. As reported28, transgenes of interest can be accommodated in the LCMV genome by segregating the viral glycoprotein (GPC) and nucleoprotein (NP) genes onto artificially duplicated S segments (SNP, SGP in r3LCMV, Fig. 1a). According to the originally published vector design strategy28, both NP and GPC remain under control of their respective regulatory RNA elements (natural positioning; r3LCMV). Genetic and phenotypic stability represent key criteria for manufacturing and clinical translation of live-attenuated viral vector systems29,30. Accordingly, observations on transgene loss in r3LCMV-infected animals (see below) prompted us to search for molecular strategies to stabilize r3LCMV genomes. By placing GPC under 3'UTR control, we generated viruses with an artificial genome organization (artLCMV, Fig. 1a). The reasoning was that, although a rare event in negative strand RNA viruses, inter-segmental recombination32 would reunite NP and GPC on a single S segment, thereby deleting the transgenes (Fig. 1b). The arenavirus promoter consists of an intra-segmental RNA hybrid formed by a highly conserved stretch of 19–21 terminal nucleotides in each segment’s 5'UTR and 3'UTR. Therefore, viral promoter activity requires both, a 5'UTR and a 3'UTR on each RNA template (Fig. 1b)33. Unlike in r3LCMV, a recombination event reuniting the NP and GPC ORFs of artLCMV would occur at the expense of losing the 5'UTR, thus creating an inactive recombination product devoid of a viral promoter (Fig. 1c). Both, r3LCMV and artLCMV were significantly attenuated in cell culture (Fig. 1d). Flow cytometric studies with green (GFP) and red fluorescent protein (tomato) co-expressing viruses (r3LCMV-GFP/tom, artLCMV-GFP/tom) revealed that red/green double-positive virions were outnumbered by a >tenfold excess of bi-segmented replication-deficient particles, carrying either only SGP or SNP in combination with the I segment (Fig. 1e, see Supplementary Fig. 1 for flow cytometry gating strategies). An immunofocus assay (IFF)-based quantification of SNP-only and SGP-only virions yielded analogous results (Supplementary Fig. 2a). Hence, attenuation was, at least in part, due to inefficient co-packaging of SNP and SGP segments. To study the genetic stability of artLCMV and r3LCMV we exploited AGR mice. Owing to targeted deletions of RAG1 (T-cell and B-cell deficiency) as well as of the type I and type II interferon genes, AGR mice readily reveal an attenuated virus’ reversion to virulence34. We measured viral loads in blood by IFF, relying on the detection of the viral structural protein NP (NP-IFF, Fig. 2a). Within the first 20 days after infection, r3LCMV and artLCMV remained at considerably lower titres in blood than LCMVwt, which was in line with published data documenting in vivo attenuation of r3LCMV35. After 30–40 days, however, r3LCMV-infected AGR mice reached NP-IFF titres identical to those infected with LCMVwt. Conversely, artLCMV viremia remained at low levels throughout the observation period of 120 days (Fig. 2a). In artLCMV-infected animals, the assessment of total infectivity by NP-IFF and the quantification of GFP-expressing viruses by GFP-IFF yielded comparable titres on day 5 as well as on day 120 after infection, indicating that the transgene had been retained (Fig. 2b,c). In contrast,
r3LCMV-GFP-infected mice turned GFP-IFF-negative while abundant NP-IFF infectivity persisted, suggesting that the virus had lost its transgene. Indeed, the viral NP was abundant in the liver and spleen of r3LCMV-GFP-infected mice, as expected, while GFP was absent (Fig. 2d, Supplementary Fig. 2b). In contrast, liver and spleen of artLCMV-GFP-infected animals exhibited dense green fluorescence (Fig. 2d, Supplementary Fig. 2b; analogous data for blood monocytes in Supplementary Fig. 2c). To verify whether r3LCMV-GFP had inactivated its transgene by recombining its two S segments, as hypothesized (Fig. 2c), we relied on an RT-PCR with reverse transcription (RT-PCR)-based sequencing strategy (Supplementary Fig. 2d).

Sequence analysis of S_rec segments revealed that individual non-homologous recombination events had occurred in each animal, with breakpoints in the GPC–IGR border region (Fig. 1b, Supplementary Fig. 2e). Genetic tags in the IGR and GPC sequences of r3LCMV-GFP excluded laboratory contaminations as potential confounder in these analyses and assigned genetic elements in S_rec to its parental S_NP or S_GFP segments (Supplementary Fig. 2f,g). We found that a CDNA-derived bi-segmented virus with an exemplary S_rec segment (S_rec#1) grew to LCMVwt titres in cell culture (Fig. 2e). In combination with the inefficient co-packing of S_GFP and S_GPC in tri-segmented particles (Fig. 1d), this finding explained the selective advantage and consistent outgrowth of S_rec segments in r3LCMV-infected AGR mice.

**Apathogenic artLCMV induces potent CTL eff responses.** In vivo attenuation represents a prerequisite for the use of live vaccine delivery platforms in immunocompromised cancer patients.
Infection of wt mice with LCMVwt, but not with artLCMV resulted in viremia (Fig. 3a). Moreover, low level artLCMV infectivity was only transiently detected in spleen and liver on day 4 and was cleared by day 7, whereas LCMVwt persisted for 10 days at high titres (Fig. 3b). LCMVwt can cause choriomeningitis in accidentally infected humans and in mice the virus is invariably lethal at intracranial (i.c.) doses of ≤10 plaque forming units (PFU, Fig. 3c, ref. 36). In contrast, artLCMV failed to cause disease at doses up to 10^8 PFU i.c., and only one of five animals developed terminal disease when given 10^9 PFU i.c. Despite its attenuation, intravenous artLCMV-induced substantial levels of serum type I interferon (IFN-I, Fig. 3d) for at least 48 h. rLCMV triggered a comparably minor and short-lived IFN-I release, whereas neither rAd-based nor replicating vaccinia virus-based vectors induced detectable levels of systemic IFN-I. Analogous results were obtained when rAd vectors were given intramuscularly or upon subcutaneous and intradermal administration of vaccinia virus-based vectors (Supplementary Fig. 3a). Although we used replication-competent vaccinia virus for our study, the lack of systemic IFN-I was expected owing to multiple virally encoded antagonists of the IFN-I response37. Taken together, these results indicated that artLCMV was genetically stable and substantially attenuated, both in vitro and in vivo, but retained the ability to efficiently activate the innate immune system. To assess artLCMV-induced CD8+ T-cell responses to transgenes of choice, we generated an ovalbumin- (OVA-) expressing vector (artLCMV-OVA). It induced OVA epitope-specific CTL numbers, which were substantially higher than those elicited by an OVA-recombinant adenovirus 5-based vector (rAd-OVA) or by a replication-deficient LCMV-based vector (rLCMV-OVA, Fig. 4a). Most notably, artLCMV-OVA-triggered KLRG1+CD127– effector CD8+ T-cell (CTLeff) responses in blood and spleen exceeded those of the other delivery systems by ~tenfold (Fig. 4b,c), while CD127+ CTLmem memory precursor cell numbers were comparable (Fig. 4d). OVA-specific total CTL and CTLeff responses to artLCMV-OVA immunization were also significantly higher than those induced upon intramuscular administration of rAd-OVA and either subcutaneous or intradermal vaccination with rVACC-OVA (Supplementary Fig. 3b,c). Intracellular cytokine staining showed that artLCMV-OVA elicited ten to 20-fold higher numbers of IFN-γ-producing and IFN-γ/TNF co-producing CD8+ T cells than rAd-OVA or rLCMV-OVA, and also IFN-γ/TNF/IL-2 triple producers were significantly more numerous (Fig. 4e). These differences in magnitude and functionality of OVA-specific CTL populations persisted in the memory phase (Fig. 4f). Superior functionality of artLCMV-induced CTLeff responses was also evident in primary ex vivo Gr1+ release assays, with ~tenfold more lytic units in spleen after artLCMV infection than after rLCMV infection, rAd-OVA, or recombinant vaccinia virus (rVACC-OVA) immunization (Fig. 4g). Importantly, artLCMV-based vaccines elicited also robust CD8+ T-cell responses against the tumour self-antigens Her2 and P1A (Fig. 4h,i). Analogously to artLCMV-OVA immunization, P1A-specific CD8+ T cells in blood of artLCMV-P1A-immunized mice were ~tenfold more numerous than in animals receiving rLCMV-P1A (Fig. 4i). LCMVwt evades antibody neutralization by means of its envelope glycan shield38, and even two sequential artLCMV immunizations did not result in viremia (Fig. 3b). LCMVwt persistently infected mice for 10 days at high titres (Fig. 3b). LCMVwt can cause choriomeningitis in accidentally infected humans and in mice the virus is invariably lethal at intracranial (i.c.) doses of ≤10 plaque forming units (PFU, Fig. 3c, ref. 36). Representative liver sections analysed for GFP+ cells on d150 (d, scale bar, 50 μm). (e) Growth kinetics of LCMVwt, r3LCMV-GFP and r2LCMV-Srec#1 on BHK-21 cells (Supplementary Fig. 2e). Symbols represent the mean of three replicates (s.e.m. error bars hidden in the symbol size). N = 2. Data in a (120 days after infection) and e (48 h after infection) were analysed by one-way ANOVA with Bonferroni post hoc test. Data in b were analysed by unpaired two-tailed Student’s t-test. NS, not significant; **P < 0.01 and ***P < 0.001.
such as T-cell immunity to viral backbone epitopes\textsuperscript{39} may compete with and thereby attenuate booster responses to artLCMV-vectorized transgenes.

**artLCMV triggers IL-33-driven CTL by targeting stromal cells.** We investigated the mechanisms underlying exceptionally potent CTL\textsubscript{eff} responses upon artLCMV immunization. First we studied its tropism for antigen-presenting cells in vivo as a basis for potent CTL induction. While both, rLCMV and artLCMV, efficiently targeted plasmacytoid DCs (pDCs), artLCMV infected a significantly higher number of conventional dendritic cells and macrophages than its replication-deficient counterpart rLCMV (Fig. 5a). In addition we hypothesized that artLCMV, analogously to LCMVwt, triggered the IL-33–ST2 alarmin pathway, thereby potentiating CTL responses and CTI\textsubscript{eff} responses in particular\textsuperscript{11}. When immunized with artLCMV-OVA, wt mice mounted ~tenfold higher OVA-specific CTL responses than animals lacking the IL-33 receptor ST2 (Illr1 \textsuperscript{+/−} ) in both spleen and blood (Fig. 5b,c). These differences were particularly pronounced in the KLRG1 \textsuperscript{+}CD127 \textsuperscript{−} CTL\textsubscript{eff} subset (Fig. 5b,c). Conversely, the responses to rAd-OVA, rVACC-OVA and rLCMV-OVA were unaffected by ST2 deficiency, suggesting that artLCMV-OVA immunization but neither replication-deficient rLCMV- nor rAd- or vaccinia-vectorized vaccination triggered the IL-33–ST2 axis.

The capacity to replicate in vivo differentiates artLCMV and rLCMV and was apparently required to activate this pathway. Bioactive IL-33 is released from non-haematopoietic stromal cells\textsuperscript{40}, which are a target of replicating LCMV infection\textsuperscript{40}. To test the hypothesis that artLCMV triggered ST2 signalling by infecting IL-33-expressing stromal cells inside secondary lymphoid organs, we developed and validated a green fluorescent IL-33 reporter IL-33-expressing stromal cells inside secondary lymphoid organs, the hypothesis that artLCMV triggered ST2 signalling by infecting bioactive IL-33 was released from dying artLCMV-infected lymphoid tissue stromal cells, offering a mechanism whereby replicating viral delivery systems supply IL-33 to the ensuing CTL response inside secondary lymphoid organs.

**IL-33-driven tumour control upon artLCMV immunotherapy.** To compare the efficacy of several viral vector platforms in cancer immunotherapy, we first exploited a transplanta\textsuperscript{e}l OVA-expressing tumour model (EG7-OVA). Unlike rLCMV-OVA and rAd-OVA, which showed partial or no clinical benefit, respectively, when administered to mice with an established solid tumour, treatment with artLCMV-OVA afforded substantial tumour control and prolonged the animals’ survival in a CD8 \textsuperscript{+} T-cell-dependent manner (Fig. 6a,b, Supplementary Fig. 5a,b). Importantly, an irrelevant artLCMV vector (artLCMV-GFP)
failed to prevent tumour progression, indicating that tumour antigen-specific T-cell induction was essential for clinical efficacy (Fig. 6a,b). Moreover, artLCMV-OVA-based tumour immunotherapy of EG7-OVA tumours was only effective in ST2-sufficient wt animals but failed in Il1rl1−/− mice (Fig. 6c–f). These differences correlated with OVA-specific CTLs and CTLeff, which were >tenfold higher in the blood of artLCMV-OVA-treated wt mice than in analogously treated Il1rl1−/− mice (Supplementary Fig. 5c), altogether attesting to the critical function of the IL-33–ST2 axis in artLCMV-based cancer immunotherapy. To assess the potency of artLCMV-based immunotherapy in a mouse tumour model without artificially introduced non-self antigens, we exploited the P815 mastocytoma model. Immunotherapy with artLCMV expressing the cancer testis (self) antigen P1A (artLCMV-P1A) significantly delayed the growth of established subcutaneous tumours and prolonged the animals’ survival, whereas replication-deficient rLCMV-P1A and irrelevant artLCMV-GFP were ineffective (Fig. 6g,h). Of note, P815 tumours were refractory to anti-PD1 checkpoint inhibition but responded to artLCMV-P1A (Fig. 6i,j). Anti-PD1 unresponsiveness of P815 tumours has been previously documented and may be due to several mechanisms including insufficient expression of PD-1 ligands, as reported41. Accordingly, the combination of artLCMV-P1A and anti-PD-1 checkpoint blockade was not superior to artLCMV-P1A monotherapy (Supplementary Fig. 5h).

artLCMV-induced CTL drive inflammatory conversion of tumours. CTL infiltrates predict survival in many human cancers7,9 and the recruitment of circulating specific CTLs into the tumour...
Figure 5 | artLCMV infects DCs and IL-33-expressing stromal cells to trigger IL-33-driven CTL expansion. (a) We infected C57BL/6 mice with artLCMV-tom, rLCMV-tom or LCMVwt and quantified virus-infected tomato-reporting conventional dendritic cells (lineage−CD11c(hi)B220−), plasmacytoid DCs (pDCs; lineage−CD11c(int)B220+) and macrophages (lineage−CD11b+Ly6G−) in spleen on d4. Representative FACS plots (left) and quantifications (right) are shown. Bars represent the mean ± s.e.m. of four mice per group. N = 2. (b,c) Wt and ST2-deficient (Il1rl1−/−) mice were infected with artLCMV-OVA, rLCMV-OVA, rAd-OVA or rVACC-OVA. Spleens (b) were analysed on d9, peripheral blood (c) on d8. OVA-tetramer-binding total CD8+ T cells (b, left), as well as OVA-tetramer-binding CTLeff (Klrg1+CD127−) in spleen and blood were enumerated by FACS (b right, c). Bars represent the mean ± s.e.m. of four mice per group. N = 2. (d) FACS analysis of splenic fibroblastic reticular cells (FRC) and blood endothelial cells (BEC) (gated as outlined in Supplementary Figs 1 and 4c) from hemizygous IL-33 reporter mice (IL-33gfp/wt) on d3 and d7 after infection with artLCMV-tom, rLCMV-tom or LCMVwt (control). Representative FACS plots are shown. Quadrant statistics and quantifications of IL-33-expressing (GFP+) viral vector-infected (tom+) cells (bar graphs) are shown as mean ± s.e.m. of four mice per group. N = 2. Horizontal dashed lines show technical backgrounds of uninfected controls. Data in a–c were analysed by unpaired two-tailed Student’s t-test ((b,c) with Bonferroni correction) and data in d were analysed by one-way ANOVA with Bonferroni post hoc test. NS, not significant; *P < 0.05, **P < 0.001.
Figure 6 | artLCMV-based immunotherapy affords antigen-specific and ST2-dependent tumour control. (a,b) We implanted EG7-OVA tumour cells subcutaneously into the flank of C57BL/6 mice. On day 7, when tumours became palpable, we treated them with artLCMV-OVA, artLCMV-GFP, rLCMV-OVA, α-Ad-OVA or left them untreated. Tumour growth over time (a, terminated when the first animal was lost from follow-up owing to humane endpoint) and survival curves (b) are shown. Symbols represent the mean ± s.e.m. of nine mice per group. N = 3. (c–f) We implanted EG7-OVA tumour cells subcutaneously into the flank of C57BL/6 (wt, d,e) and ST2-deficient (Il1rl1−/−, f) mice. When tumour masses became palpable on d7, we treated them with artLCMV-OVA (red lines) or left them untreated (black lines). Tumour growth (c,e, lines depict individual mice) and Kaplan–Meier survival curves (d,f, n = 9 (wt untreated), n = 10 (other groups)) are shown. N = 2. (g,h) We implanted P815 tumour cells subcutaneously into the flank of DBA/2 mice. When tumour masses became palpable on d9, we treated them with artLCMV-P1A, artLCMV-GFP, rLCMV-P1A or left them untreated. Tumour volumes (g, mean ± s.e.m.) and Kaplan–Meier survival curves based on humane endpoints (h) show combined data of nine (rLCMV-P1A), 12 (untreated, artLCMV-GFP) and 14 mice per group (artLCMV-P1A) from two independent experiments. N = 2. (i,j) DBA/2 mice bearing P815 tumours as in g,h were treated on d9 with artLCMV-P1A or 12.5 mg kg−1 anti-PD1 antibody on d15 (earliest possible onset of the artLCMV-P1A-induced CTL response), d18, d22 and d25. Tumor volumes (i, mean ± s.e.m.) and survival rates (j) of eight (artLCMV-P1A) or nine mice per group (untreated, α-PD1) are shown. We analysed tumour growth curves in a,g,i by comparing the area under the curve (AUC) using one-way ANOVA with Bonferroni post hoc test. Survival data in b,d,f,h,j were analysed by log-rank tests with Bonferroni correction in b,h,j. NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001.
represents an important goal of cancer immunotherapy. artLCMV-OVA immunotherapy yielded higher numbers of circulating OVA-specific CTLs and CTL\(^{\text{eff}}\) in blood of EG7-OVA tumour-bearing mice than did artLCMV-GFP, rAd-OVA or rLCMV-OVA (Fig. 7a). Flow cytometry and immunohistochemistry documented also that artLCMV-OVA immunotherapy yielded significantly higher densities of tumour-infiltrating CTLs (TILs), OVA-specific TILs and tumour-infiltrating OVA-specific CTL\(^{\text{eff}}\) (Fig. 7b,c). Analogously, artLCMV-P1A immunotherapy augmented P1A-specific CTLs and CTL\(^{\text{eff}}\) in blood of tumour-bearing mice and in tumour tissue (Supplementary Fig. 5d–f). The administration of irrelevant artLCMV-GFP resulted in only modestly elevated TIL numbers and was clinically ineffective, corroborating that artLCMV immunotherapy operated in a largely antigen-specific manner (Fig. 7b,c). Accordingly, immunohistochemical analysis did not reveal artLCMV antigen in the tumour, while viral antigen was clearly detected in spleen (Supplementary Fig. 5g), arguing against an oncolytic effect of artLCMV. Inflammatory gene expression profiling of EG7-OVA tumours on day 9 after artLCMV-OVA immunotherapy identified 30 genes, which were significantly different from tumours of untreated control mice. Thereof 20 were IFN-inducible (Fig. 7d). Validation by TaqMan RT-PCR confirmed that artLCMV-OVA immunotherapy induced several inflammatory mediators namely including the chemokines Ccl5, Cxcl9, Ccl4 and Cxcl10, which are predictive of prolonged survival in human cancers (Fig. 7e)\(^{42–44}\). Of note, expression of CCL5 was significantly higher in tumours of artLCMV-OVA-treated animals than in those receiving rLCMV-OVA or rAd-OVA (Fig. 7e, similar trend for Cxcl9). Importantly, however, only tumours of artLCMV-OVA immunized but not of artLCMV-GFP control-treated animals exhibited substantial chemokine induction. This indicated that OVA-specific CTL\(^{\text{eff}}\) infiltration was essential for

![Figure 7](image_url)

Figure 7 | artLCMV immunotherapy leads to CTL infiltration and inflammatory conversion of the tumour. (a–e) We implanted EG7-OVA tumour cells subcutaneously into the flank of C57BL/6 mice. On day 7, when tumours became palpable, we treated them with artLCMV-OVA, artLCMV-GFP, rLCMV-OVA, rAd-OVA or left them untreated. Peripheral blood (a) and tumour tissue (b–e) were analysed on d8 and d9 after treatment, respectively. We enumerated OVA-tetramer-binding total CTLs and CTL\(^{\text{eff}}\) (Klrg1\(^{+}\)) in blood (a) and tumour (b) as indicated. CTLs were gated as live CD8\(^{+}\) CD4\(^{−}\) CD3\(^{−}\) B220\(^{−}\) lymphocytes. For normalization to EG7 tumour cells the latter were differentiated from infiltrating inflammatory cells by size and granularity. CTL\(^{\text{eff}}\) were identified as Klrg1\(^{+}\). Bars represent the mean ± s.e.m. of five (artLCMV-OVA) or four mice (all other groups). N = 2. (c) Tumours from mice as in a–b were analysed for infiltrating CD8\(^{+}\) cells by immunohistochemistry. Representative pictures from four to five mice as in b are shown. Scale bars 10 µm (inset) and 100 µm (overview). N = 2. (d) Gene expression profiles of inflammation-associated genes from tumour tissue of untreated or artLCMV-OVA-treated animals as in a–c by Nanostring technology. Differentially expressed genes (fold change ≥2, adjusted P value < 0.05) are displayed. Each lane represents a tumour from an individual mouse. Interferon-regulated genes are indicated according to www.interferome.org. (e) TaqMan RT-PCR validation of select genes identified in d. Bars represent the mean ± s.e.m. of 4–5 mice as in b. Data in a,b and e were analysed by one-way ANOVA with Dunnett’s post hoc test. NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001.
inflammatory activation of the tumour microenvironment in artLCMV-OVA immunotherapy whereas the accompanying non-specific inflammation, which was also induced by artLCMV-GFP, was insufficient to mediate these effects in full.

Discussion
These results identify virally delivered alarmin signals as key drivers of protective CTL responses in vectored cancer immunotherapy. The function of such damage-associated molecular patterns appears non-redundant with PAMPs, which can also be provided by replication-deficient viral delivery systems. As schematically outlined in Supplementary Fig. 6, the ability of artLCMV to trigger IL-33/ST2-dependent CTL induction correlated with the vectors’ spread into IL-33-expressing lymphoid stroma cells. Subsequent death of these cells, possibly as a consequence of CTL attack, offers a likely mechanism for IL-33 release to neighbouring T cells. Activated CTLs inside secondary lymphoid organs express ST2, and IL-33 sensing is known to potentiate their expansion, effector differentiation and survival. The present observations suggest that upon expansion to the tumour bed, these alarmin-implicated CTLs contribute essentially to tumour microenvironment changes and to tumour control, thus providing a mechanistic rationale for the exploitation of replicating delivery platforms in the fight against cancer. Besides renewed interest in the tumour immunotherapy field, replicating live vaccine approaches currently experience a revival in indications such as Ebola hemorrhagic fever, tuberculosis and immunodeficiency virus infection. These infectious diseases and cancer have in common that potency is rate-limiting, outweighing potential concerns related to the safety profile of replicating vector systems or to the release of genetically modified organisms into the environment. Accordingly, the safety profile of genetically engineered live viruses has become acceptable for oncolytic virus therapy, with a licensed product already on the market.

The development of artLCMV represents an innovative addition to a limited quiver of replicating viral vaccine delivery systems. Several mechanistic features suggest it holds promise for tumour immunotherapy. Efficient DC targeting results in potent CTL priming. In vivo spread and infection of IL-33-expressing lymphoid stromal cells unleashes the IL-33–ST2 alarmin pathway, thereby augmenting CTL function and effector differentiation. Low CMV seroprevalence in the human population predicts high response rates. Inefficient vector-neutralizing antibody induction facilitates repeated vector re-administration. The intravenous administration of LCMV is safe in humans and non-human primates.

In recent years, checkpoint inhibitors such as anti-PD-1/PD-L1 or anti-CTLA-4 antibodies had groundbreaking success in a variety of malignancies. However, immune checkpoint blockade merely disinhibits ongoing T-cell responses and, by consequence, tends to fail in tumours with a paucity of pre-existing tumour-infiltrating CTLs. Such ‘cold’ tumours can be the result of immunoeediting and T-cell escape. Alternatively, tumours can exhibit immunogenic determinants but fail to induce clinically significant CTL responses, which is referred to as immune exclusion or ignorance. As a powerful tool for active immunization artLCMV immunotherapy delivered significant numbers of tumour-reactive CTL eff to the tumour bed, resulting in an inflammatory conversion of the tumour microenvironment (Fig. 7, Supplementary Fig. 5). This transition is thus predicted to render the corresponding tumours more responsive to immunomodulatory therapy. Beyond the commonly benign nature of human LCMV infection and a lack of horizontal transmission in humans, inefficient co-packaging of the three artLCMV genome segments represents a molecularly defined mechanism of attenuation. The >1,000-fold increase in i.c. mouse LD50 exceeds the ~100-fold safety margin of the clinically used, live-attenuated Junin arenavirus vaccine Candida® (ref. 55). Last but not least, genetic stability (Fig. 2a–d) should enable industrial exploitation. During scale-up in batch production, but also upon administration to vaccinees, rearranged genomes with a fitness gain are readily selected, compromising product safety as well as efficacy. Accordingly, stable transgene expression and attenuation of artLCMV, together with the simplicity and rapidity of vector generation, represent critical assets for clinical translation and the vector’s exploitation in personalized medicine approaches.

Taken together, our study identifies alarmin signals as crucial for the induction of protective anti-tumour CTL responses in vectored immunotherapy. By demonstrating mechanistically that alarmin release depends on viral in vivo spread, our work suggests that live viruses deserve their place in the rapidly evolving array of cancer treatment modalities. artLCMV may represent the prototype of a novel class of live microbial delivery systems, which leverage not only PAMPs but also alarmin release for CTL eff differentiation and enhanced anti-tumour efficacy.

Methods
Cells
BHK-21 cells were obtained from ECACC (Clane 13, Cat #85011433), MC37 cells (CRL-2295), EL-4 cells (TIB-39), EG7 thymoma cells (EL-4 cells expressing OVA, CRL-2113) and P815 mastocytoma cells (TIB-64) were obtained from ATCC. Stably transfected BHK-21 cells expressing the LCMV-NP and -GP proteins, respectively (BHK-NP, BHK-GP), and expressing 293T cells have previously been described. All cell lines were tested mycoplasma negative.

Vaccines
artLCMV and r3LCMV were routinely given at a dose of 105 PFU i.v., except for virus tracing experiments in C57BL/6 mice (106 PFU) and experiments in AGRAG mice (104 PFU). rLCMV vectors were used at 105 PFU for standard immunization and for Cr51 release experiments. Except for prime-boost immunizations in the experiments to Supplementary Fig. 2, artLCMV and r3LCMV were routinely given at a dose of 105 PFU i.v., except for virus tracing experiments in C57BL/6 mice (106 PFU) and experiments in AGRAG mice (104 PFU). rLCMV vectors were used at 105 PFU for standard immunization experiments or at 106 PFU for immunization with tumour self-antigens, for tumour immunotherapy and for GTPC experiments. LCMV was used for immunization experiments in Fig. 4j,k. Animals were given only a single shot of viral vector or virus, respectively. LCMVwt was administered i.v. at the same dose.
as artLCMV was used. Intracranial LCMV challenge was performed through the skull and animals developing terminal disease were euthanized by CO₂ inhalation in accordance with the Swiss law. PD-1-blocking antibody (clone RMP1-14, from BioXcell) was administrated at a dose of 12.5 mg kg⁻¹ intraperitoneally. CD₈⁺ T cells were depleted by injecting 200 μg anti-CD8 antibody (YTS169, from BioXcell) intraperitoneally. Animal experiments were performed at the Universities of Geneva and Basel in accordance with the Swiss law for animal protection. Permission was granted by the Direction de l’alimentation de la santé, Doctors Committee of the Experimentation animals of the Canton of Geneva and by the Veterinärämter Basel-Stadt, respectively. Experimental groups were sex- and age-matched. Animals in tumour therapy experiments were assigned to groups in a manner to assure even distribution of tumour volumes between groups at the time of tumour therapy. The groups were neither randomized, nor were experiments conducted in a blinded manner. The Swiss law for animal protection requires that mice with wounds on the tumour or exhibiting signs of distress (evident mainly in lethargy, hunchback, piloerection, emaciation and agonal breathing) be euthanized by CO₂ inhalation irrespective of tumour size and diameter. Accordingly, animals not reaching humane endpoints of tumour volume or diameter were excluded from survival curves.

**Virus sequencing and gene expression profiling.** Viral RNA was extracted from cell culture supernatant or serum of infected mice using the QIAamp Viral RNA Mini Kit (QIAGEN, cat. no. 52906). Reverse-transcription was performed with ThermoScript RT-PCR System (Invitrogen) and an LCMV-NP-specific primer (5’-GGGCGCCAGITTAAACCTGTGCAAC-3’). A GPC specific primer together with a GPC–specific primer (5’-GGTGCTGGTCTGCTAATGGCTC-3’) Amplified products were purified for Sanger sequencing (Microsynth). Whole-cell RNA was extracted from tumour tissue using QIAzol (QIAGEN).

**Flow cytometry.** Antibodies against CD4 (RM4-5 or GK1.5), CD8 (53-6.7), CD45R/B220 (RA3-6B2), CD45.2 (104), Ter-119 (TER-119), CD11b (M1/70), CD19 (6D5), NK1.1 (PK136), CD90.2 (30-H12), GR-1 (Podoplanin; 8.1.1), CD3 (17A2), Klrg1 (2F1), CD127 (A7R34), CD11c (N418), CD45R/B220 (RA3-6B2), CD45.2 (104), Ter-119 (TER-119), CD31 (390), gp38 (Podoplanin; 8.1.1), CD3 (17A2), Klrg1 (2F1), CD127 (A7R34), CD11b (M1/70), CD19 (6D5), NK1.1 (PK136), CD90.2 (30-H12), GR-1 (RB6-8C5), IFN-γ (XMGL2), TNF (MIP-6/IX22) and IL-2 (IL-2E6/SH) were provided by Biologeon, Pharmingen and ebioScience. All fluorescently labelled monoclonal antibodies were used at a 1:100 dilution, except for gp38 (Podoplanin) and Ter-119, which were diluted 1:1,000, and 1:1,0 respectively. Dead cells were excluded with Zombie UV Fixable Viability Kit (Biologeon, cat. no. 423108). OVA- SIFNEFKL peptide was used at established procedures from dose–response curves69.

**Statistical analysis.** For statistical analysis, GraphPad Prism software (Version 6.0, GraphPad Software) was used. Differences between two groups were assessed by unpaired two-tailed Student’s t-tests. Single values of multiple groups were compared by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test or Dunnett’s post hoc test when comparing against a reference group. A Bonferroni correction was made when comparing multiple parameters. Two-way ANOVA with Bonferroni post hoc test was used to compare multiple groups with multiple measurements. Survival curves were analysed by log-rank tests with Bonferroni correction. For the comparison of tumour growth curves over the under the curve was compared70. The analysis ended when the first animals reached human endpoints.

Viral load data were log–converted to obtain a near-normal distribution prior to statistical analysis. Variations within different groups in a given experiment were similar. In accordance with current standard practice in the field of viral immunology, only very substantial differences were reported, obviating the need for variance testing.

P values of P < 0.05 were considered significant (*), P < 0.01 (**) and P < 0.001 (***) as highly significant.

**Data availability.** Nanostar data that support the findings of this study have been deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO) with the accession code GSE84039. The nucleotide sequences of recombinant and recombined LCMV segments have been deposited in GenBank with the accession codes KX462116-KX462128. All relevant data are available from the authors upon request.

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Author contributions

S.M.K., S.D., W.V.B., M. Kreutzfeldt, N.P., P.M., M. Kreuzaler, F.K., M.L., S.A.L., A.Z., D.M. and D.D.P. conceived and designed the experiments. S.M.K., S.D., W.V.B., M. Kreutzfeldt, N.P., P.M., M. Kreuzaler, M.L. and S.F. performed the experiments. S.M.K., S.D., W.V.B., M. Kreutzfeldt, P.M., A.Z., D.M. and D.D.P. analysed the data. S.M.K., A.Z., D.M. and D.D.P. wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: D.D.P. is a shareholder and also a consultant to Hookipa Biotech AG commercializing arenavirus-based vector technology. S.M.K., S.D., MarK, N.P., D.M. and D.D.P. are listed as inventors on a patent entitled ‘Tri-segmented arenaviruses as vaccine vectors’ (application number PCT/EP2015/076458) describing artLCMV-based vector technology. The remaining authors declare no competing financial interests.

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