Vaccine-Instructed Intratumoral IFN-γ Enables Regression of Autochthonous Mouse Prostate Cancer in Allogeneic T-Cell Transplantation

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Abstract
Vaccination can synergize with transplantation of allogeneic hematopoietic stem cells to cure hematologic malignancies, but the basis for this synergy is not understood to the degree where such approaches could be effective for treating solid tumors. We investigated this issue in a transgenic mouse model of prostate cancer treated by transplantation of a nonmyeloablative MHC-matched, single Y chromosome–encoded, or multiple minor histocompatibility antigen-mismatched hematopoietic cell preparation. Here, we report that tumor-directed vaccination after allogeneic hematopoietic stem cell transplantation and donor lymphocyte infusion is essential for acute graft versus tumor responses, tumor regression, and prolonged survival. Vaccination proved essential for generation of CD8+ Tumor-directed effector cells in secondary lymphoid organs and also for IFN-γ+ upregulation at the tumor site, which in turn instructed local expression of proinflammatory chemokines and intratumoral recruitment of donor-derived T cells for disease regression. Omitting vaccination, transplanting IFN-γ-deficient donor T cells, or depleting alloreactive T cells all compromised intratumoral IFN-γ-driven inflammation and lymphocyte infiltration, abolishing antitumor responses and therapeutic efficacy of the combined approach. Our findings argue that posttransplant-tumor-directed vaccination is critical to effectively direct donor T cells to the tumor site in cooperation with allogeneic hematopoietic cell transplantation. Cancer Res 2013;73:4641-52. ©2013 AACR.

Introduction
Major limitations to efficacious immunotherapy are mechanisms of central and peripheral T-cell tolerance, which weaken tumor immunogenicity (1). Several strategies have been attempted to break such tolerance with variable degrees of success in preclinical mouse models and in clinical trials (2). Active immunotherapy in the form of vaccines targeted to tumor-associated antigens have led to promising results (3). Molecular Medicine and Institute for Immunology, Charité, Berlin, Germany

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previously been shown to augment the efficacy of HSCT/DLI against hematologic cancers (11–15) and in some instances also against transplantable solid tumors (16).

We recently reported that HSCT and DLI from HY-mismatched female donors, presensitized against male antigens, when combined with posttransplant tumor-directed vaccination, preserved remission from recurrent disease (17) in the transgenic adenocarcinoma of the mouse prostate model (TRAMP; ref. 18). Of note, while minor H antigen–directed T-cell responses have been reported sufficient to promote the regression of transplantable tumors (19–21), they proved inefficacious in clearing the autologous prostate cancer found in TRAMP mice, and required the concomitance of tumor-directed T cells. In TRAMP mice, the combination of nonmyeloablative irradiation, HSCT and DLI, and posttransplant vaccination proved capable of overcoming the profound central and peripheral mechanisms of tolerance to the tumor-driving SV40 large T antigen (Tag). In brief, providing a fresh repertoire of cells capable of responding to the vaccination under nontolerogenic conditions supports tumor regression (17).

Whether the combined strategy might be efficacious also in the case of more clinically relevant settings, that is, in mice transplanted with HSCT/DLI from naive, nonsensitized donors, mismatched to the recipient for defined sets of multiple minor H antigens remains to be defined. Here, we addressed this issue and also investigated the mechanism of action of posttransplant tumor-directed vaccination in TRAMP mice transplanted with HSCT/DLI from naive donors, mismatched to the TRAMP for either Y chromosome–encoded or multiple minor H antigens. We also show that in the case of nonsensitized donors, HSCT/DLI can be of therapeutic efficacy, can initiate tumor regression and significantly prolong survival of mice with autologous prostate cancer, but only when combined with postransplant tumor-directed vaccination. This is best explained by the vaccine’s ability to promote the generation of optimal numbers of tumor-reactive CTL, the intratumoral expression of IFN-γ, and the development of a local inflammatory response critical for GVT reactions.

Materials and Methods
Mice, cell lines, and reagents
Congenic C57BL/6 CD45.2 (B6.CD45.2), C57BL/6 CD45.1 (B6.CD45.1), and BALB.B mice were obtained from Charles River Laboratories. CD45.2+ C57BL/6 TRAMP heterozygous mice were genotyped as described previously (22) and housed, bred along with CD45.1− or CD45.1−CD45.2+ wild-type (WT) mice and OT-II TCR transgenic mice (kindly provided by Prof. Roncarolo, San Raffaele Institute, Milan, Italy) in a specific pathogen-free animal facility in accordance with European Union guidelines and with the approval of the Institutional Ethical Committee (Institutional Animal Care and Use Committee # 388). IFN-γ−deficient mice on the C57BL/6 genetic background were obtained from The Jackson Laboratory and bred at the animal facility of the Max-Delbrück Center (MDC, Berlin, Germany). Unless specified, all chemical reagents were obtained from Sigma-Aldrich, and monoclonal antibodies were purchased from BD Pharmingen. Peptides (Espikem) used in the study were derived from the large T [Tag404–411, i.e., Tag-IV: YYDFLKC; Tag362–384, i.e., T-helper cell (T_h) Tag: TNRFNFDLDMFDFGSTGSDAI], the ovalbumin (OVA252–264 ISQAVHAAHA1EAGR), the Leishmania receptor for activated C Kinase (LACK: FSPSLEHIPIVYSGSWD), or the Y-encoded Uty (WMHHNMDLI) and Dby (NAGFNSNRANSSRSS) antigens.

Hematopoietic cell transplantation and tumor-directed vaccination
Mice were subjected to nonmyeloablative HSCT as previously described (17). Briefly, recipient mice were sublethally irradiated (600 rad) and transplanted intravenously with 1 × 107 viable bone marrow cells on consecutive days. A DLI consisting of 6 × 107 splenocytes was intravenously injected after 2 additional weeks. The infusion of donor lymphocytes was followed by a tumor-directed vaccination administered the following day. For posttransplant vaccination, bone marrow–derived dendritic cells (DC) were lipopolysaccharide (LPS) matured, pulsed for 1 hour at 37°C with 2 μmol/L Tag-IV peptide (VVYDFLKC) and injected subcutaneously (4 × 106 DC/mouse), as previously described (22). Mice were vaccinated 1 day after DLI, boosted 3 weeks later, and sacrificed after an additional week, or boosted once a month for survival studies. Mice with an early onset of highly aggressive, poorly differentiated neuroendocrine tumors were excluded from the survival analysis (generally observed in 10%–15% of mice). In selected experiments, LPS-matured dendritic cells were simultaneously pulsed with the class I Tag-IV peptide and the class II Tag362–384 peptide (both at 2 μmol/L). Mice were vaccinated 1 day after DLI, boosted 3 weeks later, and sacrificed after an additional week for disease scoring.

Flow cytometry, histology, and immunohistochemistry
Single-cell suspensions of spleen and peripheral lymph nodes were either stained with the appropriate fluorochrome-labeled monoclonal antibodies ex vivo or restimulated in vitro with 2 μmol/L Tag-IV, Uty (WMHHNMDLI), or Dby (NAGFNSNRANSSRSS) peptide and were subsequently incubated for 4 hours, of which the last two were in the presence of brefeldin A. Thereafter, cells were surface-stained, fixed, and permeabilized, and intracellular cytokine measurement was conducted as previously described (17).

The urogenital apparatus (UGA) was embedded in paraffin and processed for histology and immunohistochemistry. Coded slides of UGA sections stained by hematoxylin and eosin (H&E) were scored by a pathologist as previously described (17). Briefly, a score of 0 was given to prostates showing complete regression (CR) and a score of 5 was given to invasive adenocarcinoma or metastases. Prostates with areas of CR scattered among acini affected by adenocarcinoma were defined as being of partial regression (PR). Prostate tissue sections (5-μm thick) were stained with anti-CDS antibody (Serotec, Space Import) according to the manufacturer’s instructions, were digitally scanned (ScanScope, Aperio), and then analyzed with Spectrum Plus software (Aperio).
Real-time PCR

Prostates were dissected from the UGA and snap-frozen in liquid nitrogen. Frozen organs were homogenized and RNA was extracted with phenol/chloroform. Reverse-transcription was conducted with oligo-(dT)12–18 primers and M-MLV Reverse Transcriptase (Invitrogen). Real-time amplification was carried out with commercially available TaqMan probes and primers on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). Obtained mRNA expression levels were cultured with 10^5 OT-II TCR transgenic CD4^+ T cells (expressing a TCR-specific for the OVA peptide 323–229 presented by I^A^*), obtained from pooled lymph nodes and spleen, and 1 μmol/L OVA323–339 peptide (ISQAVHAAHAEINEAGR) in the absence or the presence of various concentrations of competitor peptides (1–100 μmol/L; Espikem). The T_H Tag-derived peptide (TNRFDNLDRLDIMFPTGSAD) was compared with the IA^A^-restricted LACK (FPSLEHPIVVSGLSWD) and of IA^B^-restricted Dby (NAGFNSRNASSRSS) peptides, used as negative and positive controls, respectively. Interleukin (IL)-2 secretion and T-cell proliferation were measured as indicative of OVA peptide-driven responses. IL-2 was measured by capture ELISA in culture supernatants after 24 hours of culture. Proliferation was determined by pulsing parallel wells 1 Ci of [^3^H] Thymidine on day 2 and the incorporation of labeled nucleotides was determined after another 18 hours.

Statistical analyses

Statistical analyses were conducted using two-tailed Student t test or two-tailed Mann–Whitney–Wilcoxon test as detailed in figure legends. Statistical significance: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Tumor-directed vaccination is critical for therapeutic efficacy of hematopoietic cell transplantation against autochthonous prostate cancer in TRAMP mice

In our previous study, we found that combining transplantation of HSCT and DLI from female mice presensitized to male antigens and tumor-directed vaccination promoted long-term survival of tumor-prone TRAMP mice. Because donor sensitization against host antigens might be ethically questionable in clinical settings, we evaluated the therapeutic potential of HSCT/DLI from naïve, nonsensitized donors, mismatched to the recipient for defined sets of multiple minor H antigens, and the need for tumor-directed post-transplant vaccination.

As donors of HSCT and DLI, we adopted congenic male B6 mice, allogeneic female B6 mice, and allogeneic female BALB.B mice. Although male B6 donors are matched to the TRAMP recipients in MHC as well as minor H antigens, female B6 donors are mismatched for Y-encoded minor H antigens (HY). Third in line, female BALB.B donors are mismatched to TRAMP recipients in multiple minor H antigens, including HY. This last mismatch setting best reflects the scenario found in patients. Seventeen- to 18-week-old male TRAMP mice (CD45.2^+), an age at which mice show well-developed prostate cancer and are fully tolerant to the tumor-associated large Tag (22–24), were preconditioned through nonmyeloablative total body irradiation (TBI; 600 rad) and transplanted the following day with 1 × 10^7 bone marrow cells (HSCT; a schematic representation of the transplant scheme is provided in Fig. 1A). Where appropriate, TRAMP mice were transplanted with congenic CD45.1^+ HSCT and CD45.1^+ CD45.2^+ DLI. Two weeks later, mice received a DLI of 6 × 10^7 splenocytes (syngeneic to the HSCT). To understand the need for vaccination, 1 day after DLI a fraction of transplanted mice was vaccinated with sex-matched donor-derived dendritic cells pulsed with the tumor-associated Tag-IV peptide (vax; Fig. 1A), and in some cases boosted after 2 additional weeks. Likewise, to understand the contribution of DLI a set of mice received HSCT and vaccination, but no DLI. Mice were sacrificed either 1 week after vaccination (week 1 after DLI) or 1 week after boosting (week 4 after DLI). In mice sacrificed 4 weeks after DLI, tumor regression can be evaluated within the UGA by H&E analyses (17), and either a disease score (reflecting the state of tumor burden) or a rejection score (reflecting the efficacy of the therapy) was assigned to each individual animal. On the basis of the analysis of coded H&E-stained slides, the UGA of age-matched and vaccinated control TRAMP mice revealed enlarged prostate tubules, due to the presence of the adenocarcinoma. Such control mice were assigned an average disease score of 4.05 ± 0.52 (17) and their prostates showed no signs of disease regression (no regression).

In contrast, the histologic appearance of the UGA in the majority of TRAMP mice transplanted with allogeneic female cells and subjected to tumor-directed vaccination (fDLI/vax) resembled that of healthy mice. The mean disease score of DLI/vax mice was 1.13 ± 1.64. All mice that underwent the treatment had evidence of a clinical response (Fig. 1B) and 5 of 8 mice showed morphologic characteristics of a CR, such as well-lined normal epithelial cells and expanded thick-walled tubules with hyalination of the fibromuscular wall (indicative of past disease occurrence; representative H&E pictures are depicted in ref. 17). When vaccination was, however, omitted, the majority of DLI-transplanted mice failed to reject the tumor (disease score of 3.13 ± 1.25) and did not reveal signs of tumor regression. Also TRAMP mice transplanted with DLI from multiple minor H antigen–mismatched female BALB.B donors were found to be free of disease by week 4 after DLI when posttransplant vaccination was provided (fBALB.BD LI/vax). Again, mice failed to reject the tumor when vaccination was omitted (fBALB.BDLI). When mice were transplanted with male-derived HSCT/DLI and vaccinated (mDLI/vax), or when mice received fHSCT and the vaccination, but not the DLI (vax, noDLI), the majority of transplanted TRAMP mice failed to reject the tumor and did not reveal signs of tumor regression (Fig. 1B). Thus, in this setting the infusion of donor-derived mature T cells (DLI) containing precursors for both tumor and minor H antigen (DLI) followed by tumor-directed vaccination is critical for acute tumor remission.
We next evaluated whether this combined treatment would prolong overall survival. Although control mice had a median survival time of 300 days, up to 33% of fDLI/vax mice (Fig. 2A) and 25% of fBALB.BDLI/vax mice (Fig. 2B; \( P = 0.0013 \)) survived until day 430 and day 476, respectively, when the studies were concluded to allow for immunologic and histologic analysis. At the time of sacrifice, all transplanted and vaccinated mice provided sizeable populations of Tag-specific IFN-γ+ CD8+ T cells, which were exclusively of donor origin, as identified by the expression of congenic markers CD45.1, respectively, or Ly9.1 (representative dot plots and quantifications for fBALB.BDLI/vax are depicted in Fig. 2C and D), likely contributing to long-term protective immunity. Taken together, these data indicate that HSCT and DLI from HY or multiple minor H antigen–mismatched donors per se are rather ineffective in promoting tumor regression. However, combining them with posttransplant tumor-directed vaccination (in the form of dendritic cells pulsed with the tumor-associated Tag-IV peptide) promotes acute tumor regression in the majority of mice and prolongs survival of transplanted animals.

**Tumor-directed vaccination of hematopoietic cell transplant recipients is vital for the generation of tumor-reactive effector CD8+ T cells**

To understand the need for tumor-directed vaccination, we first enumerated tumor (Tag-IV)–specific CD8+ T-cell responses, which we previously found to well correlate with CTL activity (17) in the spleen of TRAMP mice, transplanted as described in Fig. 1A. Although IFN-γ+ CD8+ Tag-IV–specific T cells readily accumulated in frequency (Fig. 3A and B) and total number (Fig. 3C) in the spleens of mice transplanted with congenic (mDLI) or allogeneic (fDLI) cells and subjected to postransplant dendritic cell–mediated vaccination, the number of Tag-IV–specific CTLs remained within background levels, if mDLI- or fDLI-treated TRAMP mice were not vaccinated. Also, after multiple mismatched allogeneic fBALB.BDLI, Tag-IV–specific T cells were enumerable only if the DLI was combined with a DC-Tag-IV vaccine (not shown). Thus, while preconditioning and minor H antigen–mismatched HSCT and DLI are insufficient to allow for the accumulation of tumor-directed CTL, postransplant vaccination allows for optimal priming and differentiation of donor-derived CTL in secondary lymphoid organs. Of note, TRAMP recipients of male and female DLI responded equally well to the dendritic cell vaccine. This supports the notion that Tag-IV–specific T-cell priming was not influenced by concurrent alloreactivity, as we previously showed to be the case under tumor-free condition (25).

Vaccine-induced T-cell responses in secondary lymphoid organs correlated with the onset of intratumoral proinflammatory responses. This was revealed by analyzing snap-frozen prostates 1 week after DLI by real-time PCR to measure expression of immune-related genes. Results show that cytokine and chemokine gene expression was low in control TRAMP mice, but it was readily upregulated following HSCT/DLI and tumor-directed vaccination. Indeed, DC-vax instructed the intratumoral expression of TGF-β-like proinflammatory cytokines, such as IL-2, IFN-γ, and TNF-α, of chemokines such as CCL2, CXCL9, and CXCL10, and of the cytotoxic molecule granzyme B (Fig. 4) within the tumor of mDLI and fDLI recipients. Among TGF-β-like cytokines, both IL-4 and IL-13...
for tumor-homing of IFN-γ–competent Tag-IV–specific T cells, we next directly addressed the role of IFN-γ in tumor aggression by transplanting IFN-γ–deficient female donor lymphocytes. Following HSCT combined with an IFN-γ–deficient DLI and posttransplant DC-Tag-IV vaccination, neither intratumoral proinflammatory cyto- and chemokines, nor tumor remission could be detected 1 week after priming (Fig. 4), or 1 week after boosting (Fig. 5A), respectively. Loss of therapeutic efficacy was not due to the failure of IFN-γ–deficient T cells to exert effector function, as vaccination elicited comparable numbers of Tag-IV–specific TNF-α+ CD8+ T cells (Fig. 5B), and mediated lethal GVHD when infused into irradiated BALB.B mice (Fig. 5C and D).

We conclude that IFN-γ sufficiency of donor T cells is critical to promote an intratumoral inflammatory response, and to favor disease remission following posttransplant vaccination.

**Alloreactive T cells are required to promote therapeutic GVT responses**

Data depicted so far indicate that following HSCT and DLI, tumor-directed vaccination is critical to initiate a proinflammatory reaction within the tumor as early as 1 week after DLI. The contribution of alloreactive T cells was, however, not yet apparent (Fig. 4 shows no statistically significant differences comparing mDLI/vax and fDLI/vax). Results depicted in Fig. 3 indicate that tumor-specific CTL accumulate to similar extents in the spleens of mDLI and fDLI TRAMP recipients, supporting the notion that alloreactive T cells do not contribute to Tag-specific T-cell priming (25). Analysis of UGA sections revealed the number of tumor-infiltrating CD3+ cells, including both CD4+ and CD8+ T cells (Supplementary Fig. S1), in fDLI/vax recipients to clearly exceed those in mDLI/vax and DLI recipients (Supplementary Fig. S2A), and to correlate with tumor debulking (Supplementary Fig. S2B). Even though a further characterization of the infiltrate (Treg/CTL/Th17 subsets and relative functions) is needed before reaching definitive conclusions, this supports the notion that alloreactive T cells are critical for promoting intratumoral infiltration, a critical issue in solid tumors (reviewed in ref. 26), and to promote a local GVT response. This is consistent with our previous data showing that depletion of alloreactive CD8+ cells from DLI of male-sensitized female origin hinders transplant-mediated tumor remission (17). As CD4+ T cells can orchestrate tumor infiltration (27, 28), we also addressed the contribution of allo (HY)-specific CD4+ T cells in our treatment setting. To this aim, fDLI were depleted of CD4+ cells and repleted or not with male-derived CD4+ cells (Fig. 6A). Despite comparable expansion of Tag-IV–specific CD8+ T cells in secondary lymphoid organs (Fig. 6B), tumor infiltration by CD3+ cells (Fig. 6C) was significantly lower in TRAMP mice subjected to CD4-depleted fDLI/vax, when compared with recipients of fDLI/vax. Mice that had been injected with a CD4-deprived DLI showed suboptimal tumor remission, at best revealing signs of PR (Fig. 6D). Replenishing the CD4-depleted fDLI with male-derived CD4+ cells could also not restore optimal tumor infiltration (Fig. 6C) or tumor regression (Fig. 6D), supporting a critical role for alloreactive CD4+ T-cell help.

**IFN-γ-compotent T cells are required for tumor infiltration and regression**

Having found tumor-directed vaccination following HSCT and DLI to be critical for the priming, and potentially also
In previous studies on TRAMP mice, it was shown that tumor-specific T<sub>H</sub> cells were sensitive to tolerance (29, 30), and that provision of tumor (Tag)-specific TCR transgenic T<sub>H</sub> prolonged responsiveness of transgenic CTL and slowed the progression of the tumor (31). To test whether tumor-specific T-cell help might overcome the need for allogenic DLI, we incorporated the previously described Tag-derived T<sub>H</sub> epitope (Tag<sub>662–384</sub>) in the vaccination regimen. Although this peptide was identified as recognized by a Tag-reactive IA<sup>k</sup>-restricted T-cell clone obtained from C3H/HeN mice (32), according to the IEDB prediction method (33), it is predicted to also bind to IA<sup>B</sup> (even though with low affinity). Furthermore, when tested in a competition for antigen presentation assay, using the high-affinity OVA<sub>323–339</sub> peptide and TCR transgenic OT-II T cells (see Materials and Methods), it proved able to inhibit OVA-induced IL-2 secretion and proliferation of OT-II CD4<sup>T</sup> T cells (Fig. 7A and B). Thus, we simultaneously pulsed LPS-matured dendritic cells with the Tag-IV CTL epitope and the T<sub>H</sub> Tag<sub>662–384</sub> peptide and used them to vaccinate and boost preconditioned TRAMP mice previously transplanted with male-derived HSTC and DLI (as outlined in Fig. 1A). We opted for this setting, as it mostly fails to reject prostate tumors when provided with a vaccine containing only the Tag-IV CTL epitope (Fig. 1B). Results indicate that copulsing LPS-matured dendritic cells with both the CTL and the T<sub>H</sub> epitopes evoked a more potent Tag-IV–directed CD8<sup>T</sup> T-cell responses when compared with a vaccine only containing the CTL epitope (Fig. 7C), but yet revealed suboptimal therapeutic potential (Fig. 7D). Indeed, the optimized vaccine formulation following syngeneic transplantation failed to reproduce tumor eradication induced by the combination of an allogenic DLI and a vaccine containing only the Tag-IV CTL epitope (Fig. 1B). Thus, an allogenic DLI seems to mediate a more powerful vaccine helper function. These results, together with our previous study (17), support a unique role for alloreactive T cells in propagating vaccine-initiated T-cell responses within the tumor mass.

Discussion

For an ideal immune response to tumors, several components should be in place: a functional repertoire of T cells capable of responding, an appropriate immunization strategy, the elimination/inhibition of potentially suppressive mechanisms, and a favorable tumor microenvironment. We report here that nonmyeloablative HSCT and DLI from minor H antigen–mismatched donors, followed by posttransplant tumor-directed vaccination, provides for this unique combination. The combined treatment grants for a fresh repertoire of lymphocytes and a powerful vaccination strategy, jointly enabling the generation of sufficiently high numbers of functional tumor-reactive CTL and instructing intratumoral expression of proinflammatory factors. Obeying the laws of inflammation, bulk leucocytes and among them alloreactive lymphocytes are recruited into the now inflamed prostate and ultimately cause the regression of advanced stage autochthonous prostate cancer.

Although we had previously shown the efficacy of the combination of HSCT and DLI from presensitized female donors and posttransplant vaccination, our recent work provides two important additional advancements. First, we show that therapeutic efficacy is also retained when exploiting nonsensitized donors, mismatched for defined sets of multiple minor H antigens, which better reflects the clinical situation in...
which multiple disparities are inevitable. And second, we now provide for mechanistic proof about the need for posttransplant tumor-directed vaccination.

Therapeutic efficacy was achieved when allogenic HSCT and DLI were combined and followed by posttransplant tumor-directed vaccination. This possibly mimics the clinical setting of non-T cell–depleted or T cell–repleted allotransplantation, now commonly used even in HLA-haploidentical clinical setting to favor engraftment, prevent opportunistic infections, and avoid disease recurrence (reviewed in ref. 34). The need for mature T cells stemming from the graft (DLI) as well as vaccination was best explained by the facts that: (i) tumor-directed IFN-γ+ Tag-IV–specific T cells were of DLI-origin (independently of a syn- or allogenic transplantation), whereas those of bone marrow origin remained negligible both after priming (data not shown) and boosting (Fig. 6), and (ii) priming of tumor-directed CTL and the initiation of intratumoral inflammation relied on vaccination. Indeed, while IFN-γ+ Tag-specific CTL remained below the level of detection in HSCT/DLI–transplanted mice not subjected to the vaccination, they were readily enumerable a few days after dendritic cell–mediated vaccination, and persisted in long-term survivors. Omitting tumor-directed vaccination compromised the accumulation of tumor-directed CTLs and failed to mediate tumor regression or prolong the survival of TRAMP mice subjected to HSCT and DLI from minor H antigens mismatched donors. The need for tumor-directed vaccination likely underlines inefficient tumor antigen cross-presentation in secondary lymphoid organs, even in mice treated with nonmyeloablative TBI, which is known to promote tumor-antigen shedding and activation of antigen-presenting cells (35). Of note, in a previous study, we found that vaccination was dispensable for the initial expansion of tumor (Tag)-reactive CTL, and acute tumor aggression, if TRAMP mice were transplanted with lymphocytes from female donors presensitized to recipient male antigens (17). We speculate that these differences may best be explained by the presence of minor H antigen-specific memory cells within DLI of presensitized donors. These, by virtue of their memory phenotype, may more readily infiltrate the tumor than their naive counterparts, and may then target the transformed epithelium and components of the tumor-associated stroma, favoring antigen

Figure 4. Posttransplant tumor-directed vaccination instructs an inflammatory response within the tumor mass. Seventeen-week-old CD45.2+ TRAMP mice underwent mDLI/vax or fDLI/vax as depicted in Fig. 1A. Prostates were recovered 1 week after DLI and vaccination, and were processed for retrotranscription and real-time PCR analysis. Gene-specific mRNA expression was normalized against the housekeeping gene TBP. Data are depicted as ΔCt and differences in gene expression were evaluated by using two-tailed Mann–Whitney–Wilcoxon test, comparing the following treatment groups: TRAMP versus mDLI/vax, TRAMP versus fDLI/vax, mDLI/vax versus fDLI/vax, fDLI/vax versus fDLI/no vac, and fDLI/vax versus fDLI IFN-γ ko/vax.
shedding and cross-presentation in the lymphoid organs. In support of this hypothesis, we found that TRAMP mice receiving a DLI from a male-sensitized female donor experienced accelerated CD3+ cell infiltration of the prostatic tissue (unpublished data). Thus, it is likely that the use of primed T cells, or of other strategies favoring tumor infiltration (36) and/or tumor antigen cross-presentation, might mimic the effect of tumor-directed vaccination.

Mechanistically, in our model, tumor-directed vaccination proved critical to initiate a local GVT response. Indeed, only transplanted mice subjected to vaccination revealed sizeable expression of proinflammatory cytokines within the tumor. This was likely initiated by vaccine-primed tumor-directed T cells of DLI origin, as cytokine gene upregulation was comparable in vaccinated TRAMP mice transplanted with syngenic (male) or allogenic DLI (and was absent in TRAMP mice subjected to HSCT/vax; not shown). We thus envisage peptide-pulsed dendritic cells favoring the generation of sufficiently high numbers of tumor-directed CTL, which relocate to the tumor and upon local antigen recognition (possibly in conjunction with tumor-directed T\(\text{H}\) cells) mediate the local secretion of IL-2, IFN-\(\gamma\), TNF-\(\alpha\), IL-13, and other cytokines. The finding that both T\(\text{H}1\)- and T\(\text{H}2\)-like cytokines were upregulated within the tumor could be indicative of a tumor-directed CD4+ T\(\text{H}\) reaction, which however might be sensitive to tolerance induction and suboptimal in the absence of a CD4-directed vaccination (ref. 30; and see later). Intratumoral secretion of proinflammatory cytokines likely activates stromal components, provoking the local release of CCL2 and CXCL9/10, favoring the upregulation of MHC class I and class II molecules on various cell types (IFN-\(\gamma\) and TNF-\(\alpha\)), and also possibly instructing remodeling of the tumor microenvironment (IFN-\(\gamma\) and IL-13; ref. 37) to facilitate the entry of newly recruited T cells (38). Although a further characterization of the CD3+ infiltrate, allowing for instance for the measurements of effector to FoxP3+ regulatory T-cell ratio or of the subsets/TCR repertoire representation, would be needed to clearly define the role of infiltrating cells, our data indicate CD3+ cell numbers to directly correlate with the clinical response, and to be optimal in recipients of allogenic DLI (fDLI/vax and fBALB. BDLI/vax). This is a critical issue, given the notion that T-cell infiltration within solid tumors is generally suboptimal (reviewed in ref. 26), and the need to reach defined CD8+ T-cell frequencies within tumors for effective clearance (39) and more favorable prognosis (40). We speculate that in response to vaccine-instructed intratumoral inflammation, minor H antigen–specific T cells accumulate within the prostate (41) and contribute to tumor regression by recognizing HY+

Figure 5. IFN-\(\gamma\)-competent donor lymphocytes are required to support prostate tumor infiltration and regression. Splenocytes (DLI) were derived from control or IFN-\(\gamma\)-deficient female B6 mice and infused into 17-week-old CD45.2+ male TRAMP mice. DLI recipients had been preconditioned, transplanted with T cell-depleted HSCT, and vaccinated according to the protocol described in Fig. 1A. One week after DLI, a group of mice was sacrificed and prostates were snap-frozen for real-time PCR analysis (data are depicted in Fig. 4). A, remaining mice were boosted 3 weeks after DLI and sacrificed after an additional week. The UGA response in vaccinated TRAMP mice transplanted with syngenic (male) or allogenic DLI (and was absent in TRAMP mice subjected to HSCT, and vaccinated according to the protocol described in Fig. 1A. One week after DLI, a group of mice was sacrificed and prostates were snap-frozen for real-time PCR analysis (data are depicted in Fig. 4). A, remaining mice were boosted 3 weeks after DLI and sacrificed after an additional week. The UGA survival of mice (D). The Kaplan-Maier plot visualizes survival of mice (D).
stromal or tumor components. In line with this model, T cells failed to accumulate within the prostate when vaccination was omitted or when mice were either transplanted with syngenic male DLI or allogeneic IFN-γ-deficient DLI. In these animals, tumor regression could no longer be observed.

Several evidences support alloreactive CD4\(^+\) and CD8\(^+\) T cells to play a unique role in promoting local GVT responses, in transplanted TRAMP mice. Depleting female DLI of CD4\(^+\) cells did not impact on tumor-directed CTL priming in the periphery, and yet it abrogated CD3\(^+\) cell intratumoral infiltration and tumor regression. These findings are in line with results generated by Schietinger and colleagues (28) and Bos and Sherman (27) showing that optimal targeting of the tumor and its associated stroma necessitates the cooperation of CD4\(^+\) and CD8\(^+\) T cells. In these studies, CD4\(^+\) T cells were critical to mobilize CD8\(^+\) effector T cells to peripheral sites (28), and to obtain local CTL-mediated tumorcidal activity (27). Of note, in our study, alloreactive CD4\(^+\) T cells could not be substituted by reconstituting CD4-depleted DLI with male-derived CD4\(^+\) cells (likely containing tumor-reactive CD4\(^+\) T\(_{il}\)). Likewise, optimizing posttransplant vaccination by pulsing LPS-matured dendritic cells with the immunodominant Tag-derived CTL epitope and the only available T\(_{il}\) Tag-derived epitope could not enable tumor eradication following syngenic HSCT/DLI. Although at present we cannot exclude that alternative vaccination strategies (i.e., mediated by CpG or poli I:C injection) or vaccination with more immunogenic IA\(^B\)-restricted tumor-derived peptides may achieve superior tumor-specific T-cell help, our data suggest that a DLI of an allogenic nature might provide a more powerful vaccine helper function that tumor-directed T-cell help for therapeutic GVT in TRAMP mice. This might be due to the polyclonal nature of CD4 T cells within the DLI, and/or to better antigen-driven reactivation within the tumor. Indeed, the ubiquitous expression of several minor H antigens (such as HY) might enable better reactivation of alloreactive CD4 T cells when compared with tumor-restricted CD4 T cells. Alloreactive CD4\(^+\) cells might also exert a wider local cytotoxic activity against both transformed epithelial cells, and/or components of the tumor-associated stroma and by that contribute to stromal remodeling (42). This could in turn further promote CD8\(^+\) effector T-cell homing to the tumor site (as found in the case of transplantable tumors (28) and infections (43)), and favor antigen spreading, initiated by tumor-directed vaccination (44), ultimately leading to potent tumor aggression. Ongoing experiments are aimed at defining the target(s) of minor H antigen–directed T cells within the tumor to provide additional data for a novel mechanism, by which tumor-specific T cells best induced in vaccinated mice, synergize with allo-specific CD4\(^+\) and CD8\(^+\) T cells in promoting infiltration and eradication of established autochthonous tumors.

The notion that tumor-directed vaccination bears the potential to ameliorate GVT after allogenic HSCT is under discussion (9), and might rejuvenate the enthusiasm for allo-transplantation against solid tumors in humans, which at present suffers from scarce therapeutic efficacy. Toward
clinical translation of this combined strategy for patients with prostate cancer, it would be critical to address efficacy following androgen-deprivation, which has been reported to impact on T-cell responsiveness (29), and in the context of castration-resistant disease, which would better reflect the clinical state of patients eligible for this experimental treatment. In such a setting, the importance of a skewed tumor microenvironment will also need to, once again, be addressed. Although we could not detect a skewed TH repertoire in 20- to 25-week-old TRAMP mice, we cannot preclude predominant TH2/Treg milieus in mice at later stages of the disease (45, 46), which may then possibly impact on donor T-cell function within the tumor. Thus, efficacy of tumor-directed vaccination following transplantation remains to be proved in androgen-independent disease, as it is the possibility to adopt improved vaccine formulations to ameliorate the fraction of allo HSCT/DLI TRAMP mice with long-term survival.

It is worth mentioning that a higher fraction of TRAMP mice relapsed after transplants from nonsensitized donors (shown here) when compared with host-sensitized donors (17). We speculate that this might be attributed to the nature of the minor H antigen-specific T-cell response, represented by naïve T cells in the current study and by memory T cells in the case of DLI from presensitized donors. Alloreactive memory T cells might simply outperform naïve T cells by being more resistant to clonal adaptation/exhaustion, which is frequently induced by ubiquitously expressed antigens (47). Although we could not directly enumerate HY-specific T cells in the current article, we did visualize TCR transgenic HY-specific T cells in our previous study, and found that these T cells are able to expand in peripheral lymphoid organs and to infiltrate the tumor (17), but are incapable of persisting (unpublished data). In contrast, HY-reactive T cells within DLI from male-sensitized female donors persisted for longer times. Thus, faster and more prolonged responses to host minor H antigens might enable acute tumor debulking and dampen intratumoral immunosuppression, known to hinder T-cell responses in TRAMP mice (31, 48) and explain the lower rate of tumor relapse in TRAMP mice receiving male-sensitized IDLI (17), when compared with those receiving nonsensitized IDLI (shown here). Having found that vaccine-induced T-cell priming and GVT responses can be observed over the course of GVHD (ref. 25 and Manzo, in preparation), several strategies might be envisaged to favor minor H antigen T-cell function/persistence. For instance, in vitro
strategies able to sensitize donor cells to host minor H antigen might be well worth considering. Host-restricted minor H antigen–specific donor T cells could then be selected and engineered with safety switches (i.e., suicide genes) to avoid graft-related toxicity. Alternatively, autologous T cells could be cultured under controlled in vitro conditions to generate T-cell subsets with given phenotypes (i.e., effector, effector memory, and central memory cells) and may be equipped with tumor and minor H antigen–specific TCRs and safety genes (49, 50) to create therapeutic and yet safe lymphocyte combinations. On the basis of our data and considering state of the art technologies, we propose to take advantage of tumor and minor H antigen–directed T-cell responses to treat solid tumors with combined adoptive and active immunotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Hess Micheliné, T. Manzo, M. Bellone, A. Mondino Development of methodology: R. Hess Micheliné, T. Manzo, T. Sturmheit Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Hess Micheliné, T. Manzo, T. Sturmheit, V. Bassò, M. Rocchi, J. Listopad, T. Blankenstein, A. Mondino Writing, review, and/or revision of the manuscript: R. Hess Micheliné, T. Manzo, T. Sturmheit, J. Listopad, T. Blankenstein, M. Bellone, A. Mondino Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Bassò, M. Rocchi, M. Bellone Study supervision: M. Bellone, A. Mondino

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