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 hematological 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 non-myeloablative 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\(^+\) IFN-\(\gamma\)\(^+\) tumor-directed effector cells in secondary lymphoid organs and also for IFN-\(\gamma\)\(^+\) upregulation at the tumor site, which in turn instructed local expression of pro-inflammatory chemokines and intratumoral recruitment of donor-derived T cells for disease regression. Omitting vaccination, transplanting IFN-\(\gamma\) deficient donor T cells or depleting alloreactive T cells all compromised intratumoral IFN-\(\gamma\)-driven inflammation and lymphocyte infiltration, abolishing anti-tumor responses and therapeutic efficacy of the combined approach. Our findings argue that post-transplant tumor-directed vaccination is critical to effectively direct donor T cells to the tumor site in cooperation with allogeneic hematopoietic cell transplantation.
Introduction

Major limitations to efficacious immune-therapy 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), and to the recent approval of Sipuleucel-T by the US Food and Drug Administration (FDA). Sipuleucel-T is the first therapeutic vaccine for the therapy of asymptomatic metastatic castrate-resistant prostate cancer, but it’s impact on overall patient survival remains modest and is still under debate (4, 5). Being among the most successful immunotherapeutic strategies, adoptive cell therapies that leverage on tumor-specific T cells expanded in vitro or generated by TCR gene transfer (6), as well as allogeneic transplantation of hematopoietic stem cells (HSCT) (7) have yielded promising therapeutic responses. The infusion of donor-derived mature T cells (donor lymphocyte infusion; DLI) following HSCT provides further clinical benefit, especially against relapsed hematological malignancies. Therapeutic efficacy is generally due to the allogeneic reaction of donor T cells to host leukemic cells (8) and to transplanted T cells with specificity for host tumor-associated antigens. Expansion of donor T cells is favored under lymphopenic conditions (9).

To date, active and adoptive immunotherapy and HSCT/DLI approaches are still of limited efficacy against most solid tumors, except for melanoma and renal cancer (10), which is possibly due to rather profound mechanisms of peripheral tolerance and the failure of tumor-reactive T cells to properly infiltrate the tumor (8). The combination of HSCT/DLI and tumor-directed vaccination holds the potential to
enhance the graft versus tumor (GVT) response, without favoring the graft versus host (GVH) effect (9). Indeed, post-transplant vaccines have previously been shown to augment the efficacy of HSCT/DLI against hematological 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 post-transplant tumor-directed vaccination, preserved remission from recurrent disease (17) in the transgenic adenocarcinoma of the mouse prostate model (TRAMP; (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 autochthonous prostate cancer found in TRAMP mice, and required the concomitance of tumor-directed T cells. In TRAMP mice, the combination of non-myeloablative irradiation, HSCT and DLI, and post-transplant 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 non-tolerogenic conditions supports tumor regression (17).

Whether the combined strategy might be efficacious also in the case of more clinically relevant settings, i.e. in mice transplanted with HSCT/DLI from naïve, non-sensitized donors, mismatched to the recipient for defined sets of multiple minor H antigens remained to be defined. Here we addressed this issue and also investigated the mechanism of action of post-transplant tumor-directed vaccination in TRAMP mice transplanted with HSCT/DLI from naïve donors, mismatched to the TRAMP for either Y chromosome-encoded or multiple minor H antigens. We show that also in the case of non-sensitized donors, HSCT/DLI can be of therapeutic efficacy, initiates
tumor regression and significantly prolongs survival of mice with autochthonous prostate cancer, but only when combined with post-transplant tumor-directed vaccination. This is best explained by the vaccine’s ability to promote the generation of optimal numbers of tumor-reactive cytotoxic T lymphocytes (CTL), the intratumoral expression of IFN-γ, and the development of a local inflammatory response critical for graft versus tumor 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. CD45.2+C57BL/6 TRAMP heterozygous mice were genotyped as described (22) and housed, bred along with CD45.1+ or CD45.1+ CD45.2+ wild type mice and OT-II TCR transgenic mice (kindly provided by Prof. Roncarolo, San Raffaele Institute) in a specific pathogen-free animal facility in accordance with EU guidelines and with the approval of the Institutional Ethical Committee (IACUC # 388). IFN-γ deficient mice on the C57BL/6 genetic background were obtained from the Jackson Laboratories and bred at the animal facility of the MDC, Berlin. Unless specified, all chemical reagents were obtained from Sigma-Aldrich, and monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). Peptides (Espikem, Italy) used in the study were derived from the large T (Tag404-411, i.e. Tag-IV: VYDFLKC; Tag362-384, i.e. Th Tag: TNRFNDLLDRMDIMFGSTGSADI), the ovalbumin (OVA323-339: ISQAVHAAHAEINEAGR), the Leishmania receptor for Activated C Kinase (LACK: FSPSLEHPIVVSGSWD), or the Y-encoded Uty (WMHHNMDLI) and Dby (NAGFNSRNANSSRSS) antigens.

Hematopoietic cell transplantation and tumor-directed vaccination. Mice were subjected to non-myeloablative HSCT as previously described (17). Briefly, recipient mice were sub-lethally irradiated (600 rad) and transplanted intravenously (i.v.) with 1x10⁷ viable bone marrow (BM) cells on consecutive days. A DLI consisting of 6x10⁷ splenocytes was i.v. injected after two additional weeks. The infusion of donor lymphocytes was followed by a tumor-directed vaccination administered the
following day. For post-transplant vaccination, bone marrow-derived dendritic cells (DC) were LPS matured, pulsed for 1h at 37°C with 2μM Tag-IV peptide (VYDFLKC) and injected subcutaneously (s.c.) (4×10^5 DC/mouse), as previously described (22). Mice were vaccinated one day after DLI, boosted three 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 DC were simultaneously pulsed with the class I Tag-IV peptide and the class II Tag362-384 peptide (both at 2μM). Mice were vaccinated one day after DLI, boosted three 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 re-stimulated *in vitro* with 2μM 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 performed 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 H&E were scored by a pathologist as previously described (17). Briefly, a score of 0 was given to prostates showing complete tumor 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.
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(PR). Prostate tissue sections (5μm thick) were stained with anti-CD3 antibody (Serotec, Space Import, Milan) 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 performed 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 normalized against the housekeeping gene TATA-binding protein (TBP) and were expressed as ∆CT. Statistical analysis was done by two-tailed Mann-Whitney-Wilcoxon test.

**Antigen presentation competition assay.**
LPS-matured DC (5x10^4 cells per well) were co-cultured with 10^5 OT-II TCR transgenic CD4^+ T cells (expressing a TCR specific for the ovalbumin peptide 323-229 presented by I^A^b), obtained from pooled lymph nodes and spleen, and 1μM OVA323-339 peptide (ISQAVHAAHAEINEAGR) in the absence or the presence of various concentrations of competitor peptides (1-100μM; Espikem, Italy). The Th Tag-derived peptide (TNRFNDLLDRMDIMFGSTGSADI) was compared to the I^A^d-restricted LACK (FSPSLEHPIVVSGSWD) and of I^A^b-restricted Dby (NAGFNSRNANSSRSS) peptides, used as negative and positive controls, respectively. 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 24h of culture. Proliferation was determined by pulsing parallel wells 1Ci of $[^3]H$Thymidine on day two and the incorporation of labeled nucleotides was determined after another 18h.

**Statistical analyses.** Statistical analyses were performed 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 pre-sensitized to male antigens and tumor-directed vaccination promoted long-term survival of tumor-prone TRAMP mice. Since donor sensitization against host antigens might be ethically questionable in clinical settings, we evaluated the therapeutic potential of HSCT/DLI from naïve, non-sensitized 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. While 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 eighteen 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 T antigen (Tag) (22-24), were preconditioned through non-myeloablative total body irradiation (TBI, 600 rad) and transplanted the following day with 1x10⁷ bone marrow cells from (B6.CD45.1+ x B6.CD45.2+) F1 congenic donors (HSCT) (a schematic representation of the transplant scheme is provided in Figure 1A). Two weeks later, mice received a donor lymphocyte infusion (DLI) of 6x10⁷ splenocytes (syngenic to the HSCT). To understand the need for vaccination, one day after DLI a fraction of
transplanted mice was vaccinated with sex-matched donor-derived DC pulsed with the tumor-associated Tag-IV peptide (vax) (Figure 1A), and in some cases boosted after two additional weeks. Likewise, to understand the contribution of DLI, a set of mice received fHSCT and vaccination, but no DLI. Mice were sacrificed either one week after vaccination (week 1 after DLI), or one week after boosting (week 4 after DLI). In mice sacrificed 4 weeks after DLI, tumor regression can be evaluated within the urogenital apparatus (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. Based on 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 (NR). In contrast, the histological 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 fDLI/vax mice was 1.13 ± 1.64. All mice that underwent the treatment had evidence of a clinical response (Figure 1B) and 5 out of 8 mice showed morphological characteristics of a complete regression (CR), such as well-lined normal epithelial cells and expanded thick-walled tubules with hyalinosis of the fibromuscular wall (indicative of past disease occurrence) (representative H&E pictures are depicted in (17)). When vaccination was however omitted, the majority of fDLI 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 post-transplant
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vaccination was provided (fBALB.BDLI/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 (Figure 1B). Thus, in this setting, the infusion of donor-derived mature T cells (DLI) containing precursors for both tumor and minor H antigen (fDLI) followed by tumor-directed vaccination is critical for acute tumor remission.

We next evaluated whether this combined treatment would prolong overall survival. While control mice had a median survival time of 300 days, up to 33% of fDLI/vax mice (Figure 2A) and 25% of fBALB.BDLI/vax mice (Figure 2B, p=0.0013) survived until day 430 and day 476, respectively, when the studies were concluded to allow for immunological and histological 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 Figure 2C, 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 post-transplant tumor-directed vaccination (in the form of DC 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 cytotoxic T cell activity (17) in the spleen of TRAMP mice, transplanted as described in Figure 1A.

While IFN-\(\gamma\) CD8\(^+\) Tag-IV-specific T cells readily accumulated in frequency (Figure 3A and B) and total number (Figure 3C) in the spleens of mice transplanted with congenic (mDLI) or allogeneic (fDLI) cells and subjected to post-transplant DC-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 f\(\text{BALB.B}\) DLI, 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, post-transplant 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 DC-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 pro-inflammatory responses. This was revealed by analyzing snap frozen prostates one 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
intrapathological expression of Th-1-like pro-inflammatory 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 (Figure 4) within the tumor of mDLI and fDLI recipients. Among Th-2-like cytokines, both IL-4 and IL-13 appeared to be increased, of which the latter to levels of statistical significance. Expression of other factors including CXCL12, IL-5 (Figure 4) and TGF-β (not shown) appeared instead rather insensitive to the treatment. Elevated expression of pro-inflammatory cytokines and chemokines was found to comparable levels in recipients of male and female derived DLI, and could not be detected when DLI (TBI/HSCT/vax only; not shown) or vaccination was omitted (Figure 4). Thus, vaccination following HSCT and DLI is critical to instruct an inflammatory response within the tumor mass, likely due to the infiltration of vaccine-primed tumor-specific donor T cells.

**IFN-γ competent 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 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 fDLI and post-transplant DC-Tag-IV vaccination, neither intratumoral pro-inflammatory cyto- and chemokines, nor tumor remission could be detected one week after priming (Figure 4), or one week after boosting (Figure 5A), respectively. Loss of therapeutic efficacy was not due to the failure of IFN-γ deficient T cells to exert effector function, since vaccination elicited comparable numbers of Tag-IV-specific TNF-α+ CD8+ T cells (Figure 5B), and mediated lethal GVHD when infused into irradiated BALB.B mice (Figures 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 post-transplant vaccination.

**Alloreactive T cells are required to promote therapeutic graft versus tumor responses.** Data depicted so far indicate that following HSCT and DLI, tumor-directed vaccination is critical to initiate a pro-inflammatory reaction within the tumor as early as one week after DLI. The contribution of alloreactive T cells was however not yet apparent (Figure 4 shows no statistically significant differences comparing mDLI/vax and fDLI/vax). Results depicted in Figure 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 (Fig. S1), in fDLI/vax recipients to clearly exceed those in mDLI/vax and fDLI recipients (Fig. S2A), and to correlate with tumor debulking (Fig. S2B). Even though a further characterization of the infiltrate (Th/CTL/Treg 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 (26)), and to promote a local graft versus tumor 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 (Figure 6A). In spite of comparable expansion of Tag-IV-specific CD8+ T cells in secondary...
lymphoid organs (Figure 6B), tumor infiltration by CD3\(^+\) cells (Figure 6C) was significantly lower in TRAMP mice subjected to CD4-depleted fDLI/vax, when compared to recipients of fDLI/vax. Mice that had been injected with a CD4-deprived fDLI showed suboptimal tumor remission, at best revealing signs of partial regression (Figure 6D). Replenishing the CD4-depleted fDLI with male-derived CD4\(^+\) cells could also not restore optimal tumor infiltration (Figure 6C) or tumor regression (Figure 6D), supporting a critical role for alloreactive CD4\(^+\) T cell help.

In previous studies in TRAMP mice, it was shown that tumor-specific Th cells were sensitive to tolerance (29, 30), and that provision of tumor (Tag)-specific TCR transgenic T helper cells 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 Th epitope (Tag\(_{362-384}\)) (32) in the vaccination regimen. Although this peptide was identified as recognized by a Tag-reactive IA\(^k\)-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\(^b\) (even though with low affinity). Furthermore, when tested in a competition for antigen presentation assay, employing the high affinity OVA\(_{323-339}\) peptide and TCR transgenic OT-II T cells (see methods), it proved able to inhibit OVA-induced IL-2 secretion and proliferation of OT-II CD4 T cells (Figure 7A, B). Thus, we simultaneously pulsed LPS-matured DC with the Tag-IV CTL epitope and the Th Tag\(_{362-384}\) peptide and used them to vaccinate and boost preconditioned TRAMP mice previously transplanted with male-derived HSTC and DLI (as outlined in Figure 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 (Figure 1B). Results indicate that copulsing LPS-matured DC with both the
CTL and the Th epitopes evoked a more potent Tag-IV-directed CD8 T cell responses when compared to a vaccine only containing the CTL epitope (Figure 7C), but yet revealed suboptimal therapeutic potential (Figure 7D). Indeed, the optimized vaccine formulation following syngenic transplantation failed to reproduce tumor eradication induced by the combination of an allogenic DLI and a vaccine containing only the Tag-IV CTL epitope (Figure 1B). Thus, an allogenic DLI appears 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 non-myeloablative HSCT and DLI from minor H antigen mismatched donors, followed by post-transplant 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 pro-inflammatory 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.

While we had previously demonstrated the efficacy of the combination of HSCT and DLI from pre-sensitized female donors and post-transplant vaccination, our recent work provides two important additional advancements. First, we demonstrate that therapeutic efficacy is retained also when exploiting non-sensitized donors, mismatched for defined sets of multiple minor H antigens, which better reflects the clinical situation where multiple disparities are inevitable. And second, we now provide for mechanistic proof regarding the need for post-transplant tumor-directed vaccination.

Therapeutic efficacy was achieved when allogenic HSCT and DLI were combined and followed by post-transplant 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 (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), while those of bone marrow origin remained negligible both after priming (data not shown) and boosting (Figure 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 DC-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 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 non-myeloablative total body irradiation, 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 pre-sensitized 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 pre-sensitized donors. These, by virtue of their memory phenotype, may more readily infiltrate the tumor than their naïve counterparts, and may then target the transformed epithelium and components of the
tumor-associated stroma, favoring antigen 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 graft versus tumor response. Indeed, only transplanted mice subjected to vaccination revealed sizeable expression of pro-inflammatory 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 DC 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 Th cells) mediate the local secretion of IL-2, IFN-γ, TNF-α, IL-13 and other cytokines. The finding that both Th-1 and Th-2-like cytokines were upregulated within the tumor could be indicative of a tumor-directed CD4⁺ Th reaction, which however might be sensitive to tolerance induction and suboptimal in the absence of a CD4-directed vaccination ((30), and see below). Intratumoral secretion of pro-inflammatory cytokines likely activates stromal components, provoking the local release of CCL2 and CXCL9/10, favoring the up-regulation of MHC class I and class II molecules on various cell types (IFN-γ and TNF-α) and also possibly instructing remodeling of the tumor microenvironment (IFN-γ and IL-13, (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 (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+ 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 allogenic 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 graft versus tumor 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 the groups of H. Schreiber (28) and L. Sherman (27) demonstrating 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 tumoricidal activity (27). Of note, in our study alloreactive CD4+ T cells could not be substituted by reconstituting CD4-depleted fDLI with male-derived CD4+ cells (likely containing tumor-reactive
CD4^+ helper T cells). Likewise, optimizing post-transplant vaccination by pulsing LPS-matured DC with the immunodominant Tag-derived CTL epitope and the only available Th 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 IAb^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 to 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
allotransplantation against solid tumors in humans, which at present suffers of scarce therapeutic efficacy. Towards clinical translation of this combined strategy for prostate cancer patients, 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 reflects 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. While we could not detect a skewed Th repertoire in 20-25 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 non-sensitized donors (shown here) when compared to 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 pre-sensitized 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). While we could not directly enumerate HY-specific T cells in the current manuscript, we did visualize TCR transgenic HY-specific naïve T cells in our previous study, and found these T cells able to expand in peripheral lymphoid organs and to infiltrate the tumor (17), but incapable to persist (unpublished data).
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 fDLI (17), when compared to those receiving non sensitized fDLI (shown here).

Having found that vaccine-induced T cell priming and graft versus tumor responses can be observed over the course of graft versus host disease [(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, 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. Based on 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.
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Abbreviations: HSCT: hematopoietic stem cell transplantation; DLI: donor lymphocyte infusion; mDLI: DLI of male origin; fDLI: DLI of female origin; H: histocompatibility; B6: C57Bl/6; NR: no regression; PR: partial regression; CR: complete regression; GVHD: graft versus host disease; GVT: graft versus tumor.
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Figure legends

Figure 1. Tumor-directed vaccination is required to instruct regression of autochthonous prostate cancer following minor H antigen mismatched HSCT/DLI. (A) Schematic representation of the transplant setting. TRAMP and WT controls were transplanted with CD45.1+ CD45.2+ F1 HSCT and CD45.1+ DLI from female B6 (fDLI) or BALB.B (fBALB.BDLI) donors and were vaccinated or not with syngeneic DC pulsed with the Tag-IV peptide (vax) as described in the text. TRAMP mice subjected to vaccination only (vax), to male HSCT/DLI and vax (mDLI/vax) or to fHSCT and vax (vax, no DLI) were also analyzed. B) To determine therapeutic efficacy, the UGA was recovered one week after boosting and embedded in paraffin. Coded samples were scored by H&E analyses and defined as being of complete tumor regression (CR; black area), partial tumor regression (PR; gray area) or no response (NR; white area), as described in the text.

Figure 2. Minor H antigen mismatched HSCT/DLI followed by tumor-directed vaccination prolongs TRAMP mice survival. Seventeen week-old CD45.2+ TRAMP mice underwent HSCT and fDLI as depicted in Figure 1A. Mice were boosted monthly and monitored for survival. Untransplanted, vaccinated TRAMP mice are depicted as controls. A, B) The depicted Kaplan-Maier plots report the survival curves of vax (n=13), fDLI/vax (n=10, A); fBALB.B/vax (n=12, B) TRAMP mice. C, D) At day 430 and 476, remaining fDLI/vax (n=3) and fBALB.B/vax (n=3) animals were sacrificed to perform immunological analysis. Splenocytes were recovered from long-term survivors and analyzed ex vivo by flow cytometry for intracellular secretion of IFN-γ following control (unpulsed) or Tag-IV stimulation. In
C, representative dot plots of CD8+ T cells of host or donor (HSCT+DLI)-origin in TRAMP mice transplanted with fBALB.B HSCT and DLI are depicted. In D, cumulative data of Tag-IV-specific IFN-γ release from host and donor (HSCT+DLI)-CD8+ T cells of independent mice is shown).

Figure 3. Post-transplant tumor-directed vaccination is required for IFNγ+ CD8+ T cell priming. Seventeen week-old CD45.2+ TRAMP mice underwent mDLI or fDLI as depicted in Figure 1A. Following DLI, mice were randomized and subjected or not to vaccination. One week after DLI mice were sacrificed and splenocytes were analyzed ex vivo by flow cytometry for intracellular secretion of IFN-γ following 4h control (Nil) or Tag-IV stimulation. A) Representative dot plots are shown after gating on viable CD8+-DLI-derived cells. The frequency (B) and total number (C) of Tag-specific DLI-derived CD8+ IFN-γ+ cells (subtracted of background) of individual mice analyzed in independent experiments are shown. Statistical significance was evaluated by two-tailed Mann-Whitney-Wilcoxon test.

Figure 4. Post-transplant 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 Figure 1A. Prostates were recovered one 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 ∆CT and differences in gene expression were evaluated by employing two-tailed Mann-Whitney-Wilcoxon test, comparing the following treatment groups: TRAMP vs. mDLI/vax, TRAMP vs. fDLI/vax, mDLI/vax vs fDLI/vax, fDLI/vax vs. fDLI/no vax and fDLI/vax vs fDLI IFNγ ko/vax.
**Figure 5. IFN-γ-competent donor lymphocytes are required to support prostate tumor infiltration and regression.** Splenocytes (DLI) were derived from control or IFN-γ deficient female B6 mice and infused into seventeen week-old CD45.2⁺ male TRAMP mice. DLI recipients had been pre-conditioned, transplanted with T cell depleted HSCT and vaccinated according to the protocol described in Figure 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 Figure 4). A) Remaining mice were boosted three weeks after DLI and sacrificed after an additional week. The UGA were then recovered and processed for disease scoring. B) Depicted are vaccine-induced CD8⁺ T cell responses in the spleen of differentially treated animals, as measured by TNF-α release one week after DLI. C) IFN-γ deficient splenocytes of B6 origin were transplanted into BALB.B mice to evaluate occurrence of GVHD. The Kaplan-Maier plot visualizes survival of mice.

**Figure 6. Alloreactive CD4⁺ T cells sustain vaccine-instructed graft versus tumor reactions.** Seventeen week-old CD45.2⁺ TRAMP mice underwent HSCT and fDLI as depicted in Figure 1A. In some cases, fDLI were depleted of CD4⁺ T cells (fDLICD4⁻) and were occasionally repleted with highly purified CD4⁺ cells from male donors (fDLICD4⁻/mCD4). A) Schematic representation of the experiment. B) Splenocytes were analyzed one week after boosting by flow cytometry for intracellular secretion of IFN-γ following a 4h Tag-IV stimulation. Total numbers of host and DLI-derived Tag-IV-specific CD8⁺ IFN-γ⁺ cells (subtracted of background) are shown. C) Paraffin embedded sections of UGA were analyzed by immunohistochemistry with anti-CD3 Ab and H&E staining (not depicted). Sections were digitally scanned and the number
of CD3+ cells/mm² was electronically quantified. C) Clinical responses were assessed on the basis of coded samples. Symbols reflect complete tumor regression (CR; black area), partial tumor regression (PR; gray area) or no response (NR; white area).

**Figure 7.** Optimized DC vaccines including Tag-derived Th and CTL epitopes do not overcome the need for allogenic DLI. A, B) Antigen presentation competition assays were performed by incubating LPS-matured DC (5x10⁴ cells per well) with 1μM OVA peptide in the presence of various concentrations of the Th Tag, LACK (IA𝑏) or Dby (IA𝑏) peptides (1-100μM). IL-2 secretion (A) and [3H]Thymidine incorporation (B) were evaluated as described in the methods section. Data is depicted after subtraction of background measures. Similar results were obtained in unfractionated splenocyte cultures. C, D) Seventeen week-old CD45.2+ TRAMP mice underwent male-derived HSCT/DLI and were subjected to vaccination with DC pulsed with the Tag-derived CTL epitope only, or co-pulsed with the Tag-derived Th epitope, and boosted accordingly. Mice were sacrificed after an additional week. c) Splenocytes were analyzed by intracellular secretion of IFN-γ following a 4h Tag-IV stimulation. Total numbers of host and DLI-derived Tag-specific CD8+ IFN-γ+ cells (subtracted of background) are shown. D) Coded samples of paraffin embedded and sectioned UGA were scored for clinical responses. Symbols reflect complete tumor regression (CR; black area), partial tumor regression (PR; gray area) or no response (NR; white area).
A

Time (days) 0 1 14 15 21 35 42

TBI  HSCT  DLI  vax  vax  vax  vax

Survival

Immunology

Histology and immunology

B

vax  n=22

fDLI  n=8

fDLI/vax  n=8

fBALB.BDLI  n=4

fBALB.BDLI/vax  n=7

mDLI/vax  n=7

vax, no DLI  n=4

CR

PR

NR

Hess Michelini-Manzo, Figure 1
Hess Michelini-Manzo, Figure 3
**Figure 5**

A. Pie charts showing the distribution of responses to fDLI/vax and IFN-γ−/− fDLI/vax. n=6 for IFN-γ−/− fDLI/vax, n=8 for fDLI/vax.

B. Bar graph showing % TNF-α+CD8+ cells in fDLI, fDLI/vax, and IFN-γ−/− fDLI/vax.

C. Graph showing changes in body weight over days after HSCT for B6 WT fDLI→mB6, B6 WT fDLI→mBALB.B, IFN-γ−/− fDLI→mBALB.B.

D. Graph showing survival rates over days after HSCT for B6 WT fDLI→mB6, B6 WT fDLI→mBALB.B, IFN-γ−/− fDLI→mBALB.B.
**Figure 7**

A. Graph showing IL-2 (ng/ml) levels against competitor (μM) concentration.

B. Graph showing 3HThymidine (c.p.m.) levels against competitor (μM) concentration.

C. Bar graph showing the number of CD8+ IFN-γ (x10^3) cells.

D. Pie chart showing mDLI/vax (4+8) outcomes with n=4.

Legend:
- LACK
- DBY
- Tag

h DLI mDLI/vax (CTL) h DLI mDLI/vax (Th/CTL)

CRPRNR

Hess Michelini-Manzo, Figure 7
Vaccine-instructed intratumoral IFN-γ enables regression of autochthonous mouse prostate cancer in allogeneic T cell transplantation

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