Skewing the T-Cell Repertoire by Combined DNA Vaccination, Host Conditioning, and Adoptive Transfer

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Abstract

Approaches for T-cell–based immunotherapy that have shown substantial effects in clinical trials are generally based on the adoptive transfer of high numbers of antigen-specific cells, and the success of these approaches is thought to rely on the high magnitude of the tumor-specific T-cell responses that are induced. In this study, we aimed to develop strategies that also yield a T-cell repertoire that is highly skewed toward tumor recognition but do not rely on *ex vivo* generation of tumor-specific T cells. To this end, the tumor-specific T-cell repertoire was first expanded by DNA vaccination and then infused into irradiated recipients. Subsequent vaccination of the recipient mice with the same antigen resulted in peak CD8$^+$ T-cell responses of ~50%. These high T-cell responses required the presence of antigen-experienced tumor-specific T cells within the graft because only mice that received cells of previously vaccinated donor mice developed effective responses. Tumor-bearing mice treated with this combined therapy showed a significant delay in tumor outgrowth, compared with mice treated by irradiation or vaccination alone. Furthermore, this antitumor effect was accompanied by an increased accumulation of activated and antigen-specific T cells within the tumor. In summary, the combination of DNA vaccination with host conditioning and adoptive transfer generates a marked, but transient, skewing of the T-cell repertoire toward tumor recognition. This strategy does not require *ex vivo* expansion of cells to generate effective antitumor immunity and may therefore easily be translated to clinical application. [Cancer Res 2008;68(7):2455–62]

Introduction

Virus-induced tumors, such as human papillomavirus (HPV)-induced cervical carcinoma, express foreign antigens that are potential targets for immunotherapy. Unfortunately, clinical trials that assess the efficacy of therapeutic vaccination against HPV oncoproteins E6 and E7 have shown only a limited therapeutic benefit for cervical cancer patients to date (1–5). Furthermore, although vaccine-induced T-cell responses against E6 and E7 are often observed, these responses generally are of limited magnitude, and it may be argued that a substantial enhancement of vaccine-induced T-cell responses is required to improve clinical efficacy.

In line with the notion that the development of marked tumor-specific T-cell responses may be essential, a number of successful T-cell–based immunotherapy trials have been performed that involved the adoptive transfer of high numbers of virus- or tumor-specific T cells. Specifically, infusion of virus-specific T-cell clones has been used for the prophylaxis and treatment of cytomegalovirus- and EBV-associated diseases after hematopoietic stem cell transplantation (6–8). Furthermore, objective cancer regression in patients with metastatic melanoma was accomplished after nonmyeloablative chemotherapy and adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TIL; refs. 9, 10). In these latter studies, T-cell infusion is performed subsequent to the administration of lymphodepleting chemotherapy, and this host conditioning regimen is thought to be essential to allow efficient engraftment of the infused cells. Importantly, the general application of these strategies for the treatment of cancer patients is hampered by the difficulty of expanding sufficient numbers of tumor-reactive T cells *ex vivo*. Our aim in this study was therefore to develop an immunotherapeutic strategy that results in a marked skewing of the T-cell repertoire toward tumor reactivity and that is solely based on a combination of vaccination and host conditioning.

We recently described a vaccination method that uses a high frequency tattoo device to deliver DNA vaccines to the epidermis, a preferred site for the induction of immune responses due to the abundant presence of antigen-presenting cells. In mice, this vaccination strategy generates robust T-cell responses within 2 weeks, eliciting effective immunity toward established HPV-transformed tumors (11). Also, in a nonhuman primate model, DNA tattoo vaccination is highly effective and induces CD8$^+$ T-cell responses that are superior to those obtained upon i.m. DNA vaccination.[1]

To test whether an effective DNA vaccination strategy can be used to achieve a substantial skewing of the T-cell repertoire toward tumor recognition without a requirement for *ex vivo* T-cell expansion, we combined DNA tattoo vaccination with irradiation-induced host conditioning and adoptive transfer. Prior studies that combine host conditioning regimens and tumor cell vaccination have shown that such a combined strategy could protect mice against subsequent tumor challenge and induce regression of established metastases (12, 13). However, as these studies did not assess the effect of host conditioning on vaccine-induced T-cell responses, it is difficult to ascertain whether a substantial skewing of the T-cell repertoire was achieved and if this was responsible for the induction of antitumor immunity.

In the current study, we address this issue and show that the combination of DNA vaccination, host conditioning, and adoptive

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-5254

[1] Verstrepen B, Bins A, Rollier C, Mooij P, Koopman G, Sheppard N, Sattentau Q, Wagner R, Wolf H, Schumacher T, Heeney J, Haanen J. Improved HIV-1 specific T-cell responses by DNA tattooing as compared to intramuscular immunization in non-human primates. Submitted for publication.

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transfer results in a strong skewing of the T cell repertoire toward tumor recognition. This combined therapy resulted in a significant growth delay of established tumors and was associated with a markedly increased accumulation of tumor-specific and IFNγ-producing T cells at the site of the tumor. This strategy may easily be translated to a clinical setting and is therefore an attractive method for the enhancement of vaccine-induced immune responses.

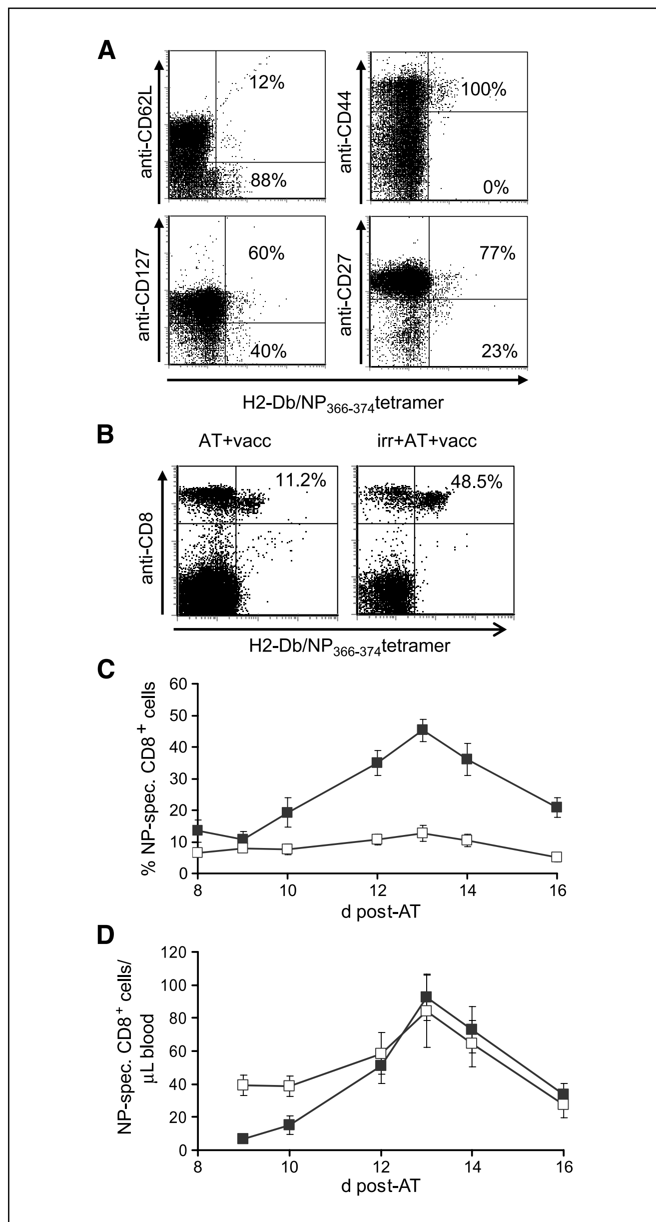

Figure 1. Lymphodepletion strongly enhances vaccine-induced T-cell responses. *A,* donor mice were vaccinated with a DNA vaccine encoding the NP_{366} epitope on day -14, -11, and -8. On day 0, splenocytes were isolated and analyzed by flow cytometry. Numbers in the top and bottom right corners represent the percentage of marker$^+$ cells and marker$^-$ cells of D^b-NP_{366}-tetramer$^+$ $CD8^+$ cells, respectively. *B* to *D,* on day 0, recipient mice were either irradiated or left untreated, and, subsequently, the mice received an adoptive transfer (*AT*) of 3×10^7 splenocytes from previously vaccinated donors. On day 0, 3 and 6, the mice were vaccinated with a DNA vaccine encoding the NP_{366} epitope. *B,* representative flow cytometry plots of peripheral blood samples of nonirradiated (*left*) or irradiated (*right*) mice at day 13 postadoptive transfer. *C* and *D,* peripheral blood of irradiated (■) or nonirradiated (□) mice was collected at different time points after adoptive transfer, and the percentage (*C*) and absolute number (*D*) of D^b-NP_{366}-tetramer$^+$ $CD8^+$ cells were determined by flow cytometry analysis. *Points,* mean ($n = 5$); *bars,* SE. *NP-spec. CD8$^+$ cells,* NP-specific $CD8^+$ cells.

Materials and Methods

Mice and cell lines. Female C57Bl/6 mice were obtained from the experimental animal department of The Netherlands Cancer Institute. Mice were housed under specific pathogen-free conditions and used at ages 6 to 12 wk. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the local Dutch Animal Research Committee.

B16 (H-2^b haplotype) is a spontaneous murine melanoma obtained from the National Cancer Institute tumor repository. The $B16^{NP}$ tumor cell line was obtained by transduction of B16 cells with a retrovirus encoding the influenza A $NP_{366-374}$ epitope as a COOH-terminal fusion with the enhanced green fluorescent gene product (14). The $B16^{NP}$ tumor cell line was maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH), 100 μg/mL streptomycin, and 100 μg/mL penicillin.

DNA vaccination. The luc-NP DNA vaccine was generated as described previously (11). In brief, the influenza A $NP_{366-374}$ epitope was genetically fused to the carboxy terminus of the firefly luciferase gene and cloned into pcDNA3.1. All DNA batches were purified using EndoFree Plasmid kit (Qiagen).

For intradermal DNA vaccination, a droplet of 20 μg DNA in 10 μL endotoxin-free TE buffer (Qiagen) was applied on the shaven hind leg of a mouse. A sterile disposable 11-needle bar (Radical Clean Magnum11; Earl Toupera) mounted on a rotary tattoo device (Cold skin; B&A trading) was then used to administer the vaccine into the skin. Needle depth was adjusted to 0.5 mm, and the needle bar was oscillated at 100 Hz. The DNA vaccine was applied to the skin by a 16s tattoo.

Detection of NP_{366}-specific $CD8^+$ cells in peripheral blood. For analysis of T-cell responses, peripheral blood was drawn at the indicated time points. Erythrocytes were removed by incubation in erylysis buffer [155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA (pH 7.4)] at −20°C for 4 min. Cells were stained with phycoerythrin-conjugated antibody to CD8β (BD PharMingen) plus allophycocyanin-conjugated H-$2D^b/_{NP366-374}$-tetramers for 15 min at room temperature in fluorescence-activated cell sorting buffer (1× PBS, 0.5% bovine serum albumin, and 0.02% sodium azide). Before analysis, propidium iodide (1 μg/mL; Sigma Aldrich) was added to enable selection for propidium iodide–negative (living) cells. Data acquisition and analysis were performed on a FACSCalibur flow cytometer (BD Biosciences) using FCS Express software.

Combined immunotherapy. Donor mice were vaccinated with NP_{366}-encoding plasmid DNA 2 wk before isolation of splenocytes or at the time points indicated. At day 0, recipient mice were sublethally irradiated with 5 Gy or left untreated, and received an i.v. adoptive transfer of 3×10^7 freshly isolated donor splenocytes 4 to 6 h later. Mice were then vaccinated at day 0, 3, and 6 with the luc-NP DNA vaccine. Peripheral blood samples were collected at the indicated time points by tail-bleed.

In tumor rejection experiments, mice received a s.c. injection of 5×10^4 $B16^{NP}$ tumor cells, 3 d before start of treatment. Tumors were measured with calipers and the products of perpendicular diameters were recorded. In each tumor experiment, tumor-bearing mice were pooled and randomly divided into treatment or control groups.

Analysis of TIL. Tumor-bearing mice were sacrificed at day 14 postadoptive transfer, and s.c. tumors were isolated. To prepare single-cell suspensions, tumors were digested in a mixture of 0.1% collagenase type IV (Worthington) and 0.01% DNase I (Roche) in RPMI. Tumors were then disrupted over a cell strainer, and viable lymphocytes were collected after separation over a Ficoll gradient.

Lymphocytes were stained with H-$2D^b/_{NP366-374}$-tetramers, anti-CD8, anti-CD62L, anti-CD127, anti-CD44, anti-CD27, anti-CD4, or anti-CD25 antibody (BD PharMingen). Alternatively, lymphocytes were incubated for 4 h in the presence of recombinant human interleukin (IL)-2 (40 units/mL;

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Chiron) and Brefeldin A (1 μL/mL; BD Biosciences). Subsequently, cells were stained with anti-CD8 antibody and analyzed for IFNγ production by intracellular cytokine staining with anti-IFNγ antibody (BD PharMingen).

Results

Host conditioning strongly enhances vaccine-induced T-cell responses. To generate high levels of tumor-specific T cells without a need for *ex vivo* expansion, we evaluated the following strategy. First, *in vivo* expansion of antigen-specific precursor cells in donor mice was induced via tattoo vaccination with a DNA vaccine encoding a model viral antigen (the immunodominant epitope of influenza A virus nucleoprotein; $NP_{366-374}$). Using this strategy, an NP-tetramer$^+$ cell population of on average 0.5% to 1% of CD8$^+$ splenocytes was induced 2 weeks postvaccination. Consistent with the previously described phenotype of pathogen- or vaccine-induced CD8+ T cells at the peak of an antigen-induced response, these cells predominantly expressed an effector/effector-memory T-cell phenotype (CD62L$^-$CD27$^+$CD44$^+$ but with heterogeneous CD127 expression; Fig. 1*A*).After this *in vivo* expansion step, splenocytes of donor mice were isolated and transferred to sublethally irradiated recipients or control mice. After transfer, recipient mice were vaccinated with the identical DNA vaccine to assess to what extent the T-cell repertoire could be skewed toward the NP antigens. In nonirradiated recipients, DNA tattoo vaccination induced responses of on average 10% NP-tetramer$^+$ CD8$^+$ cells at day 13. Notably, vaccination-induced responses were strongly enhanced in irradiated mice, amounting to 50% NP-tetramer$^+$ CD8$^+$ cells (Fig. 1*B* and *C*). These data show that a vaccination–conditioning–adoptive cell transfer (ACT)–vaccination (VCAV) protocol can be used to generate T-cell responses that are substantially more pronounced than those induced by vaccination alone.

The use of host conditioning regimens in T-cell–based immunotherapy has largely been based on the observation that T cells display a "homeostatic proliferation" when infused into lymphopenic hosts, and this proliferation is independent of the presence of their cognate antigen. It is however less clear to what extent such homeostatic expansion of antigen-specific T cells still contributes to T-cell accumulation when cognate antigen is offered in parallel. To examine this issue, we determined the absolute number of NP_{366}-specific CD8$^+$ cells that was induced by DNA vaccination of recipients that had or had not been conditioned by irradiation. No significant difference was detected in the absolute number of NP-tetramer$^+$ CD8$^+$ cells in irradiated or nonirradiated recipients (Fig. 1*D*), suggesting that when cognate antigen is offered, homeostatic expansion of antigen-specific cells no longer substantially contributes to the *in vivo* accumulation of transferred T cells. To further test the notion that the expansion of the NP_{366}-specific T-cell pool was unaltered by host conditioning, we specifically analyzed the number of donor-derived NP-tetramer$^+$ CD8$^+$ cells in irradiated or nonirradiated recipients using an Ly5 congenic marker. As expected, the NP_{366}-specific CD8$^+$ cell population in irradiated mice consisted solely of donor cells (Fig. 2*A*). However, also in nonirradiated recipients, 70% to 75% of NP_{366}-specific CD8$^+$ cells were Ly5.1$^+$ and, thus, donor derived (Fig. 2*B*). These data show that the combination of DNA vaccination with host conditioning and adoptive transfer results in a marked skewing of the T-cell repertoire toward the vaccine antigen. Notably, the skewing of the repertoire in irradiated mice is not so much caused by an increased proliferation of the donor-derived NP_{366}-specific CD8$^+$ cells but rather by the fact that a similar proliferative burst now occurs in the absence of other cells.

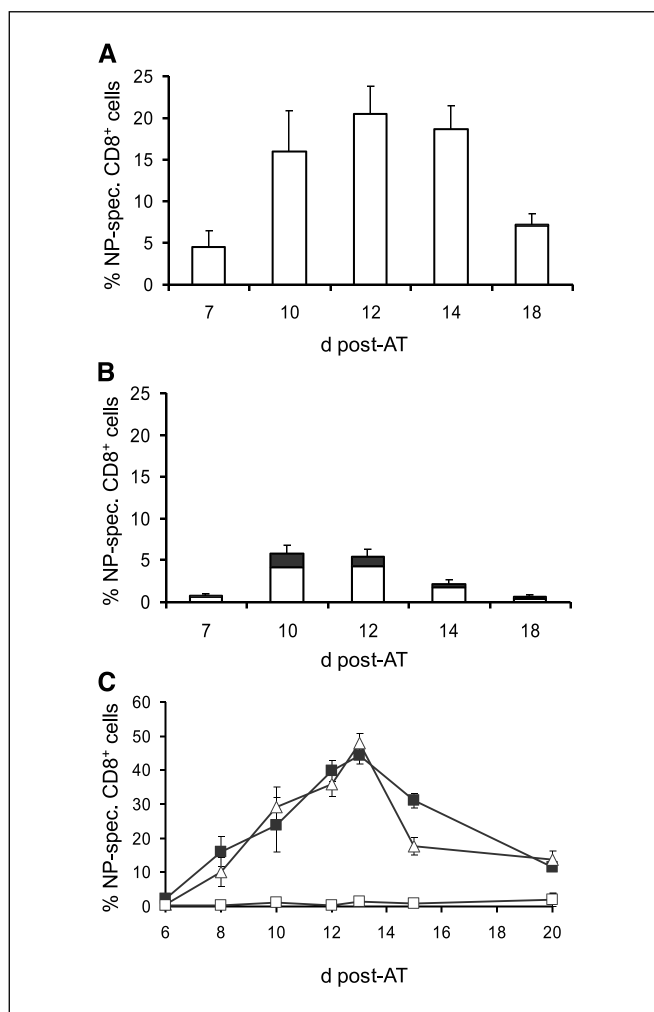

Figure 2. Antigen-specific CD8$^+$ T-cell responses in lymphodepleted mice are mainly donor-derived and require the presence of antigen-experienced T cells within the graft. *A* and *B,* irradiated (*A*) and untreated (*B*) recipients received donor cells from vaccinated Ly5.1$^+$ donors. Peripheral blood was collected at different time points after adoptive transfer and vaccination, and the percentage of Ly5.1$^+$ (□) and Ly5.1$^-$ (■) D^b-NP_{366}-tetramer–binding cells was determined by flow cytometry analysis. *Columns,* mean (n = 5); *bars,* SE. *C,* mice treated by irradiation received an adoptive transfer of 3 × 10^7 cells from naïve donors (□) or from donors that were vaccinated 2 (■) or 4 (△) wk before isolation of splenocytes. Recipient mice were subsequently vaccinated on day 0, 3, and 6. The percentage of D^b-NP_{366}-tetramer$^+$ CD8$^+$ cells in peripheral blood was determined at the indicated time points. *Points,* mean (n = 5); *bars,* SE.

To assess whether the transfer of grafts that contain CD8$^+$ T cells that have prior antigen experience is required for the induction of the profound vaccination-induced skewing in irradiated recipients, recipient mice were given an adoptive transfer of cells from either naive donor mice or from donors that had previously been vaccinated. After adoptive transfer, recipients were vaccinated with the luc-NP DNA vaccine and vaccine-induced T-cell responses were analyzed. Irradiated recipient mice that received donor cells from naive mice did not develop any NP_{366}-specific responses over background (<1.9%). In contrast, recipients that received donor cells from mice that had been vaccinated showed NP_{366}-specific CD8$^+$ responses of 40% to 50% of total CD8$^+$ cells (Fig. 2*C*). These responses were indistinguishable when grafts were prepared 2 or 4 weeks after primary vaccination (the peak of the CD8$^+$ T-cell response and the start of the memory phase, respectively),

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indicating that there is no stringent requirement with respect to the timing of vaccination and ACT (Fig. 2*C*).

VCAV treatment improves immunotherapy of established s.c. tumors. To assess whether skewing of the T-cell repertoire toward tumor recognition could enhance antitumor efficacy, the combined treatment regimen, consisting of DNA vaccination, adoptive transfer in conditioned hosts, and subsequent vaccination was evaluated in a stringent therapeutic tumor setting using the $B16^{NP}$ tumor cell line (14, 15).

Mice were inoculated with tumor cells 3 days before start of immunotherapy, and therapy-induced T-cell responses were monitored. As a control, $CD8^+$ T-cell responses were compared with responses induced by the same regimen in tumor-free mice. Consistent with the data shown above, $CD8^+$ T-cell responses in tumor-free mice were significantly higher when ACT was performed in mice pretreated by irradiation compared with untreated mice (55.4% versus 6.3%, respectively). In tumor-bearing mice, therapy-induced $CD8^+$ cell responses were substantially reduced, consistent with an inhibitory effect of the $B16^{NP}$ tumor, possibly through transforming growth factor (TGF)-β and IL-10 (16–18). Importantly, however, T-cell responses in tumor-bearing mice treated with the combined treatment remained significantly higher than those observed in mice in which host conditioning was omitted (25.5% versus 4.9%). As expected, there were no detectable antigen-specific responses in nontreated mice or mice treated with irradiation and adoptive transfer only (data not shown).

In nontreated mice, tumors grew out rapidly and all mice had to be sacrificed within 18 days. Treatment with irradiation and adoptive transfer only, or vaccination and adoptive transfer only resulted in a slight delay in tumor growth. Combined VCAV treatment resulted in an increased antitumor effect, with a significant difference in tumor size between days 18 and 31 compared with control groups. Combined treatment prolonged mean survival of tumor-bearing mice with 10 days compared with nontreated mice (Fig. 3*B* and *C*). This eventual tumor outgrowth could not be explained by the emergence of antigen-loss variants because analysis of persistent $B16^{NP}$ tumors showed maintainance of NP epitope expression (data not shown).

When tumor-bearing rather than tumor-free mice were used as recipients for the primary vaccination, T-cell responses obtained in secondary recipients were reduced detectably (with peak $CD8^+$ T-cell levels reaching ∼60% of those observed when cell grafts are obtained from tumor-free mice). In line with a reduced potency of cell grafts obtained from tumor-bearing mice, there was a (nonsignificant) trend toward reduced tumor control in this group (Supplementary Fig. S1*A* and *B*; see Discussion).

Although the therapy-induced T-cell responses are marked in this regimen, high levels of antigen-specific cells are only maintained for a short period; vaccine-induced responses peak around day 14 but then rapidly decline to baseline level. Because the persistence of tumor-specific $CD8^+$ T cells is associated with tumor regression (19), the rapid contraction of the population of tumor-specific $CD8^+$ cells seen here seems a plausible causal factor in the subsequent outgrowth of $B16^{NP}$ tumors. To test whether prolonged presence of the antigen could maintain high levels of antigen-specific $CD8^+$ T cells for a longer period of time, recipient mice were treated with a continuous vaccination regimen after ACT and host conditioning.

When adoptive transfer of donor cells in irradiated recipient mice was followed by continuous vaccination, a peak $CD8^+$ T-cell response was observed that was comparable with that observed in recipients that received the standard vaccination regimen. However, responses declined to baseline levels more slowly upon continuous vaccination, resulting in a significant higher level of antigen-specific cells on day 20 (Fig. 4*A*). Furthermore, absolute numbers of NP-specific $CD8^+$ cells were slightly enhanced in mice treated with continuous vaccination (Fig. 4*B*). Tumor growth in mice treated with continuous vaccination was delayed compared with mice treated with the

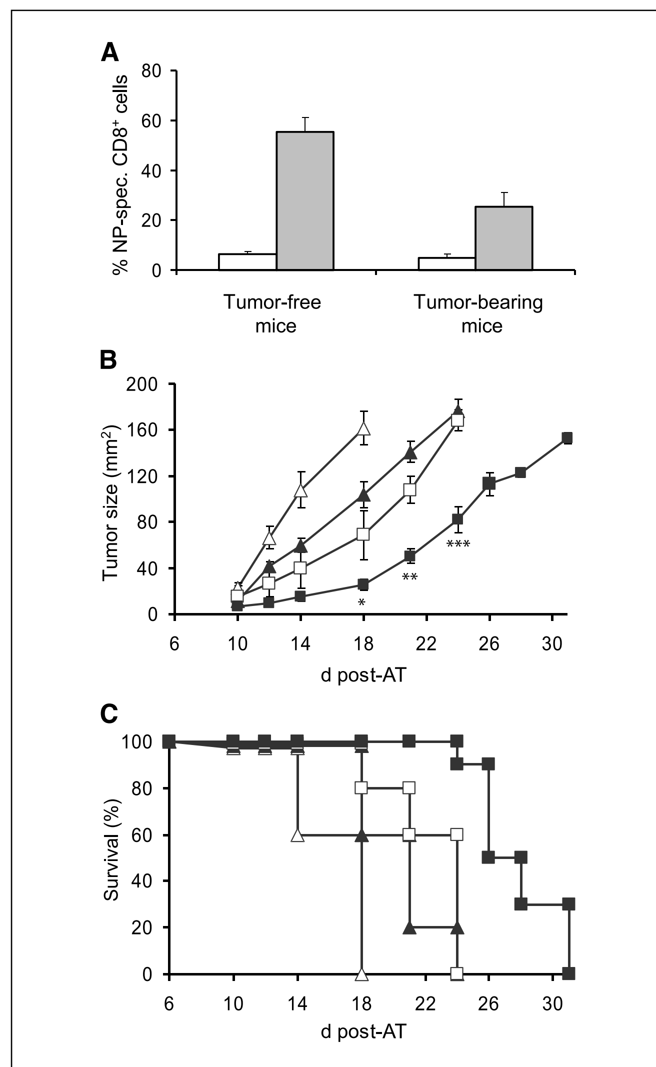

Figure 3. VCAV treatment improves immunotherapy of established s.c. tumors. *A,* mice were inoculated with 5×10^4 $B16^{NP}$ cells s.c. in the right flank on day 3 (*right*) or were left tumor free (*left*). On day 0, mice were either irradiated (▨) or not irradiated (□). On the same day, all mice received an adoptive transfer of 3×10^7 cells from previously vaccinated donors and were subsequently vaccinated on day 0, 3, and 6. The percentage of D^b-NP_{366}-tetramer$^+$ $CD8^+$ cells at the peak of the response is depicted. *Columns,* mean (n = 4); *bars,* SE. *B* and *C,* mice were inoculated with 5×10^4 B16-NP cells s.c in the right flank on day 3. Subsequently, mice were left untreated (△); treated by irradiation and adoptive transfer (▲); adoptive transfer and vaccination (□); or by the combination of irradiation, adoptive transfer, and vaccination (■). *B,* analysis of tumor development. Tumor size was measured thrice per week. *Points,* mean (n = 5); *bars,* SE. Student's t tests based on a one-tailed distribution were performed to determine differences between the VCAV treatment group and the adoptive transfer and vaccination control group; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. *C,* Kaplan-Meier survival plot. Mice were sacrificed when the longest diameter was >15 mm.

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standard vaccination regimen, although the effect on survival was marginal (Fig. 4*C* and *D*).

These data show that a slight increase in the persistence of vaccine-induced $CD8^+$ cells led to a small enhancement of the antitumor effect of this treatment strategy. This suggests that it may be worthwhile to analyze strategies that can result in a more pronounced enhancement of T-cell persistence in ACT protocols (see Discussion).

Functional characterization of TIL. Delayed tumor growth in mice treated with the combined VCAV treatment is associated with a marked presence of tumor-specific $CD8^+$ cells in peripheral blood. However, peripheral blood T-cell responses do not necessarily reflect the quantity or quality of T cells present at the site of the tumor. To characterize $CD8^+$ cell infiltrates at the effector site, TIL were isolated from $B16^{NP}$ tumors at day 14 postadoptive transfer, at which time peripheral blood T-cell responses are maximal. Infiltrating lymphocytes were analyzed for CD8 expression, NP-tetramer binding, and spontaneous IFNγ production, as well as for several phenotypic markers such as CD62L and CD127.

NP-specific TIL of both the VCAV treatment group and the adoptive transfer plus vaccination group displayed an effector phenotype of $CD62L^-$, $CD127^+$, $CD44^+$, and $CD27^+$ cells (Fig. 5*A*). However, whereas absolute numbers of NP-specific $CD8^+$ T cells in peripheral blood were not increased upon combined treatment, both the percentage (31%) and absolute number (200,000 cells) of tumor-infiltrating $CD8^+$ T cells were enhanced in mice that received VCAV treatment, compared with control mice (20% and 60,000 cells in mice treated with adoptive transfer and vaccination only). Furthermore, a higher percentage (24%) and a higher absolute number (60,000 cells) of $CD8^+$ cells from mice treated with the combined treatment bound NP-tetramers, compared with mice treated with adoptive transfer and vaccination (12% and 7,000 cells, respectively). Thus, inclusion of the conditioning regimen led to a >8-fold increase in the number of tumor resident NP-specific $CD8^+$ cells (Fig. 5*C* and *D*). More importantly, combined treatment resulted in a marked increase in TIL that displayed intratumoral effector activity, as assessed by direct *ex vivo* INFγ production in the absence of further antigenic stimulation (45.3% versus 6.5% of $CD8^+$ cells, corresponding to a more than 10-fold increase in absolute numbers of IFNγ-producing cells; Fig. 5*B*, *C*, and *D*). These results suggest that the improved antitumor effect of a combined vaccination-irradiation-adoptive transfer-vaccination strategy is associated with a strongly enhanced accumulation of tumor-specific $CD8^+$ cells at the site of the tumor, and that these cells display an enhanced capacity for effector function at this site.

Although recent data have suggested that the ratio of intratumoral cytotoxic T cells to regulatory T cells predicts capacity for immune control (20–22), the percentage of $CD4+CD25^{high}$ T cells was not affected by combined VCAV treatment, and neither was the ratio of $CD4+CD25^{high}$ T cells versus total $CD8^+$ or antigen-specific $CD8^+$ T cells (Supplementary Fig. S2*A* and *B*). These data suggest that the enhanced antitumor reactivity of TIL in VCAV-treated mice may not be accounted for by an effect of VCAV treatment on regulatory T cells.

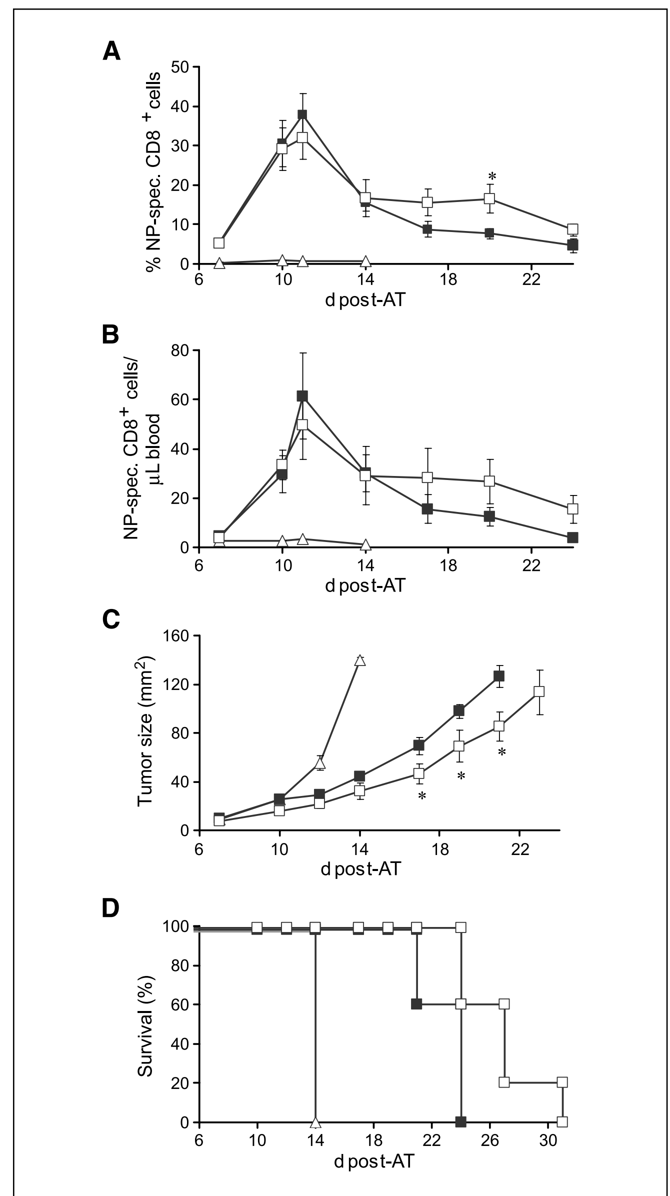

Figure 4. Effect of continued vaccination. *A* and *B*, mice were inoculated with 5×10^4 $B16^{NP}$ cells s.c. in the right flank on day 3. On day 0, mice were irradiated and subsequently received an adoptive transfer of 3×10^7 donor cells. Vaccination was performed on day 0, 3, and 6 (■), or every 3 d up to day 21 (□). Control mice were left untreated (△). Peripheral blood was collected at the indicated time points after adoptive transfer, and the percentage (*A*) and absolute number (*B*) of D^b-NP_{366}-tetramer+ $CD8^+$ cells were determined by flow cytometry analysis. *Points,* mean ($n = 5$); *bars,* SE. *C,* analysis of tumor development. Tumor size of mice in the treatment groups as indicated in *A* and *B* was measured thrice per week. *Points,* mean ($n = 5$); *bars,* SE. Student's *t* tests based on a one-tailed distribution were performed to determine differences between the standard and continuous vaccination groups; *, $P < 0.05$. *D,* Kaplan-Meier survival plot. Mice were sacrificed when the longest diameter was >15 mm.

Discussion

The more successful approaches for T-cell–based immunotherapy of cancer used to date have required *ex vivo* expansion of tumor-specific T cells to generate sufficiently high numbers for subsequent adoptive transfer. As the immune system has an intrinsic capacity to support massive expansion of antigen-specific T-cell populations, it seems plausible that a similar skewing of the T-cell repertoire may, in theory, also be achieved *in vivo*. Here, we describe our first efforts toward this goal. A strategy was developed that combines *in vivo* T-cell expansion with host conditioning and adoptive transfer, thereby circumventing the difficulties associated with current adoptive cell therapy.

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This combination of vaccination, host conditioning, and ACT resulted in a marked skewing of the T-cell repertoire toward the vaccine antigen: T-cell responses in mice that were treated with this strategy reached maximal levels of close to 50%. Furthermore, when analyzing TIL during the peak of the T-cell response, an increased accumulation of both MHC-tetramer$^+$ CD8$^+$ cells and CD8$^+$ cells capable of spontaneous IFNγ production was apparent. These data are in agreement with other studies showing increased accumulation (23) or improved effector function (24) of T cells at the tumor site of irradiated mice receiving *in vitro* activated or T-cell antigen receptor (TCR) transgenic cells. Notably, irradiation led to a specific increase in the number (and activity) of tumor-specific T cells at the tumor site but not in peripheral blood. This indicates that the beneficial effect of conditioning regimens may not always be reflected by a change in the level of tumor-specific immunity detected in blood samples.

The higher levels of activated, IFNγ-producing cells may indicate a more effective differentiation of the transferred cells upon host conditioning, for instance due to the removal of endogenous cells competing for cytokines, such as IL-7 and IL-15 (24–26), or by a direct effect of host conditioning on the local tumor environment, inducing tumor necrosis and apoptosis, and thereby enhancing the presentation of tumor antigens (27–29). Although host conditioning is also known to remove regulatory T cells (30, 31), the combined treatment regimen in this study did not affect the frequency of regulatory T cells within TIL. Regardless of the mechanism, the 10-fold increase in TIL that display *in vivo* effector activity is striking.

When donor T cells were derived from tumor-bearing hosts, both vaccine-induced T-cell responses and antitumor efficacy seemed to be impaired to some extent (Supplementary Fig. S1*A* and *B*). It is somewhat difficult to generalize this finding, as the effect of tumor growth on immune status will likely be variable. However, the data do suggest that at least for some tumor types, the ability of VCAV to induce strong tumor-specific T-cell responses may be reduced through the action of tumor-derived factors such as TGF-β. It will therefore be interesting to determine whether the immunosuppressive effect of established tumors can be counteracted *in vivo*, for example, via *in vivo* blockade of TGF-β (32).

Based on prior data by other groups (33, 34), it seems likely that only those T cells in the primary T-cell graft that retain the capacity to migrate to secondary lymphoid organs contribute to the vaccine-induced T-cell response in secondary recipients. In line with this, T-cell responses in secondary recipients were identical when T-cell grafts were obtained from primary recipients at the peak of the T-cell response or when the majority of effector T-cells had disappeared. Conceivably, the magnitude of secondary T-cell responses could therefore be further enhanced with primary vaccines that would selectively induce antigen-specific T-cell populations with a central memory phenotype. Although such selective induction of T_{cm} cells by manipulation of culture conditions is feasible *in vitro* (35), it is still unclear whether robust

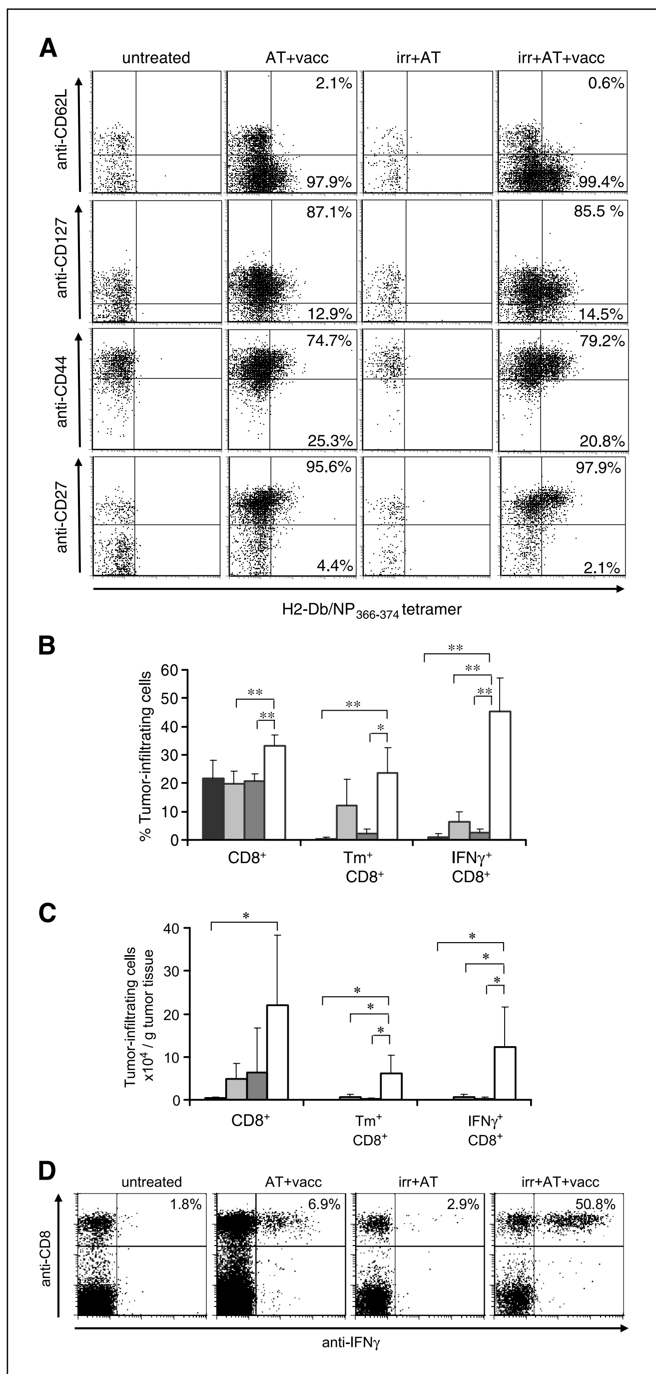

Figure 5. Functional characterization of tumor-infiltrating lymphocytes. Mice were inoculated with 5×10^4 B16^{NP} cells s.c. in the right flank on day 3. On day 0, mice were irradiated and subsequently received an adoptive transfer of 3×10^7 donor cells. Vaccination was performed on day 0, 3, and 6. Control mice were left untreated, were treated by irradiation and adoptive transfer only, or by adoptive transfer and vaccination only. At day 14 after adoptive transfer, TIL were isolated from the tumors and analyzed by flow cytometry. *A,* phenotypic analysis of TIL. Flow cytometry plots are gated on CD8$^+$ cells and show D^b-NP$_{366}$-tetramer staining in combination with anti-CD62L (*first row*), anti-CD127 (*second row*), anti-CD44 (*third row*), and anti-CD27 staining (*fourth row*). Numbers in the top and bottom right corners represent the percentage of marker$^+$ cells and marker$^-$ cells of D^b-NP$_{366}$-tetramer$^+$ CD8$^+$ cells, respectively. *B* and *C,* analysis of TIL from untreated mice (*black bars*), mice treated by irradiation and adoptive transfer (*light gray bars*), adoptive transfer and vaccination (*dark gray bars*), or VCAV combined treatment (*white bars*). The percentage (*B*) and absolute number (*C*) of total CD8$^+$ cells, D^b-NP$_{366}$-tetramer$^+$ CD8$^+$ cells, and spontaneous IFNγ-producing CD8$^+$ cells were determined by flow cytometry analysis. *Columns,* mean ($n = 4$); *bars,* SD. Student's *t* tests based on a one-tailed distribution were performed to determine differences between the VCAV treatment group and control groups; *, $P < 0.05$; **, $P < 0.005$. *D,* spontaneous IFNγ release by TIL. Flow cytometry shows anti-CD8 staining in combination with intracellular anti-IFNγ staining. Plots are representative for four mice per group, one of three experiments.

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central memory T-cell responses can be induced *in vivo* by manipulation of vaccination conditions.

In addition to potential changes in the way by which primary T-cell responses are induced, we consider it likely that substantial further improvements can also be made downstream. Specifically, therapy-induced T-cell responses in secondary recipients were relatively transient with a peak around day 14 followed by a rapid decrease to levels below 10%. The kinetics of these T-cell responses are reminiscent of those seen during classic pathogen-induced T-cell responses, in which the majority of T-cell output is predestined to die by apoptosis (36, 37). In line with the notion that the drop in tumor-specific T-cell frequency at later time points is caused by contraction rather than an increased abundance of other T-cell specificities, absolute numbers of antigen-specific $CD8^+$ T cells also go down. As a possible correlate of the transient nature of the skewing of the T-cell repertoire toward tumor recognition upon VCAV, the antitumor effect of combined therapy was also temporary, with tumors growing out in all mice eventually. Notably, when the presence of antigen is prolonged, by continuous vaccination, this results in only a somewhat slower decline in levels of antigen-specific cells.

These data suggest that vaccination may form a suboptimal way to steer the T-cell repertoire after ACT when long-term persistence is required. In the current setting, vaccination post-ACT was required to boost tumor-specific T-cell frequencies from the low frequencies present in the original graft (0.5–1% of total donor splenocytes). It therefore seems attractive to prepare grafts that are already highly enriched for tumor reactivity, thereby potentially obviating the need for subsequent vaccination. Based on prior data using TCR transgenic T cells, it seems plausible that the high level of tumor reactivity present in such selective cell grafts would be maintained during homeostatic expansion, thereby resulting in a long-term dominance of the tumor reactive T-cell repertoire. Selective T-cell grafts may be prepared by MHC multimer–based sorting, using either classic MHC tetramers (38) or reversible MHC tetramers that may result in a higher viability of the resulting cell product (39). Furthermore, the production of clinical-grade MHC multimers for a large collection of T-cell epitopes seems a realistic option with the development of more efficient production methods (40).

It should be relatively straightforward to translate the strategy described here, or further modifications that use selective T-cell grafts, to a clinical setting. As is the case for all immunotherapeutic strategies that are based on a mobilization of the endogenous T-cell repertoire, VCAV may in particular be suitable for tumor types for which a high avidity tumor-specific T-cell repertoire is present. For instance, patients with cervix carcinoma may be vaccinated with vaccines encoding the HPV E6 and E7 oncoproteins. Such a vaccination may either involve the type of DNA vaccines used here or one of the approaches that have previously been shown to yield $CD8^+$ T-cell reactivity against HPV E6 and E7 in clinical trials (3–5). After isolation of peripheral blood lymphocytes and administration of lymphodepleting chemotherapy, reinfusion of the autologous cells followed by a second round of vaccination could be used to induce skewing of the T-cell repertoire toward the HPV oncogenes. This type of study would be valuable to determine whether this approach for skewing the antigen-specific T-cell repertoire can be effective in a clinical setting.

Acknowledgments

Received 9/11/2007; revised 12/31/2007; accepted 1/29/2008.

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