Peptide Vaccination after T-Cell Transfer Causes Massive Clonal Expansion, Tumor Eradication, and Manageable Cytokine Storm

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Abstract
Adoptive T-cell transfer (ACT) is successfully applied as a cancer treatment that is based on the activation and effector functions of tumor-specific T cells. Here, we present results from a mouse model in which ACT is combined with a long peptide–based vaccine comprising gp100 T-cell epitopes. Transferred CD8+ T cells expanded up to 1,000-fold after peptide vaccination, leading to a 3-fold increase in white blood cell count and a very high frequency in the generation of antigen-specific memory T cells, the generation of which tended to correlate with effective antitumor responses. An enormous pool of effector T cells spread widely to different tissues, including the skin and the immune-privileged eye, where they mediate tumor eradication. Importantly, these striking T-cell dynamics occurred in immunocompetent mice without prior hematologic conditioning. Continued activation of the specific T-cell pool by vaccination led to strong T-cell–mediated cytokine storm and lethality due to multi-organ failure. However, this immunopathology could be prevented by controlling the rapid biodistribution of the peptide or by using a weakly agonistic peptide. Together, these results identify a peptide vaccination strategy that can potently accentuate effective ACT in non-lymphodepleted hosts.

Introduction
Adoptive cell transfer (ACT) with tumor-specific T cells is successfully applied in patients with post-transplant lymphoproliferative disease, leukemia, and melanoma (1–3). Adoptively transferred T lymphocytes are frequently obtained from stem cell donors in the case of hematopoietic tumors, but also autologous T cells have the capacity to control the outgrowth of malignant cells. In melanoma, transfer of tumor-infiltrating lymphocytes that have been activated and expanded in vitro is capable of inducing the regression of large metastasized lesions and results in long-term survival in approximately half of the stage IV melanoma patients (3). The function and maintenance of the transferred melanoma-reactive T cells were greatly improved when the recipients were pretreated with a lymphodepleting or even myeloablative regimen (3–6). In the absence of host conditioning, the transferred T cells were hardly detectable after a few days, despite the fact that high doses of the T-cell growth factor interleukin (IL)-2 were provided. The “empty” host apparently promotes the proliferation and survival of the transferred T cells, leading to a more efficient tumor regression. Furthermore, lymphodepletion may help to overcome immunosuppressive mechanisms orchestrated by tumors (6–8).

These clinical findings on melanoma nicely illustrate that the patients’ own T-cell repertoire does comprise functional tumor-specific lymphocytes, although their contribution to the natural tumor resistance seems to be limited. Therapeutic peptide vaccination aims at the recruitment of such endogenous tumor-specific T cells, but its clinical success has thus far been limited. Recently, we have observed much more robust immune and clinical responses following therapeutic vaccination when using longer peptides comprising T-cell epitopes (9, 10). The efficient activation of tumor-specific T cells that was observed in these patients did not depend on any kind of hematologic conditioning of the host, in contrast to the findings for ACT.

We here report on the combination treatment of ACT and peptide vaccination. Long peptides comprising the melanoma gp10025–33 epitope were applied to activate adoptively transferred T cells in vivo. Extreme clonal expansions up to 1,000-fold and effective antitumor responses were observed in the absence of lymphodepletion. Our results imply that the field of T-cell–based immunotherapy reaches an era in which, next to clinical efficacy, also adverse immunopathology needs to be carefully evaluated.

Materials and Methods
Mice
C57BL/6jico mice, 8 weeks old, were obtained from Charles River (France). T-cell receptor (TCR) transgenic mice containing...
gp100 20-39 the H-2Dβ specific receptors (designated as pmel ref. 11) were a kind gift of Dr. N.P. Restifo (Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD) and were bred to express the congenic marker CD90.1 (Thy-1.1). All animals were housed under specific pathogen-free conditions and cared for in accordance with the guidelines of the local University Committee for the Care of Laboratory animals (DEC), NIH guidelines on laboratory animal welfare, and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Adoptive cell transfer and immunization of mice

Lymphocytes from the spleen and lymph nodes of naïve CD90.1-positive pmel mice were isolated and enriched for T lymphocytes by nylon wool. Enriched spleen cells (3 x 10^6) T lymphocytes were adoptively transferred via injection into the tail vein. Mice were immunized 1 day later by shaving the left flank and s.c. injecting 150 μg of homologous human gp100 20-39 KVP (AVGALKVPRNQDWLGVPRQL) or mouse gp100 20-39 EGS (AVGALEGSRNQDWLGVPRQL) peptide solved in PBS. Toll-like receptor (TLR) ligands were simultaneously applied: 60 μg of Aldara cream containing 5% imiquimod on the skin at the injection site or 25 μg of CpG 1826 from the Leiden Institute of Chemistry. Immunizations were repeated on day 7 and then combined with two s.c. injections of 600,000 IU human recombinant IL-2 (Novartis) on the day of the second vaccination and one on the next day. To achieve slow release of gp100 peptide in vivo, 150-μg peptides were emulsified in Montanide-ISA-51 with PBS. Adoptive transfer of OT-1 cells and vaccination with long OVA peptide were described previously (12).

Melanoma treatment

The B16F10 melanoma cell line was derived from the American Type Culture Collection and fresh batches were thawed every year. No further authentication was used, but the expression of melanoma antigens and MHC-I molecules was tested during the experiments. Cells were cultured in the expression of melanoma antigens and MHC-I molecules thawed every year. No further authentication was used, but melanoma treatment described previously (12).

Peripheral blood lymphocyte (PBL) samples were collected from tail veins and red blood cells were lysed for 10 minutes on ice. Cells were stained with the monoclonal antibodies anti-CD62L—phycoerythrin, anti-CD8—peridinin chlorophyll protein, and anti-90.1—allophycocyanin (BD Pharmingen); acquired on a Caliber flow cytometer (BD Biosciences); and analyzed with CellQuest Pro software (BD Biosciences). For determination of pmel T cells in the eyes and lymph nodes, mice were euthanized by cervical dislocation. After removal, eyes were incubated for 45 minutes at 37°C in a 5% CO2 atmosphere in IMDM and 8% FCS supplemented with 250 units/mL collagenase IV (Sigma-Aldrich) and 50 μg/mL DNase (Sigma-Aldrich). Eyes were then pressed over a cell strainer to generate single-cell suspensions. Lymph nodes and spleen were directly pressed through a cell strainer. For intracellular cytokine staining of IFN-γ, PBL were incubated overnight with mouse gp100 peptide EGSRNQDWL (1 μg/mL) stimulation at 37°C in a 5% CO2 atmosphere and stained as described before (14). The capacity of ACT-treated and immunized mice to eradicate peptide-pulsed target cells was analyzed as previously described (14).

Histology and immunohistochemistry

Enucleated eyes and organs were embedded in Tissue-Tek optimum cutting temperature (Sakura Fine-Tek) for cryosections or fixed in 4% buffered neutralized formalin for paraffin sections. Four-micrometer-thick serial sections were mounted on a slide; routine staining was performed with H&E on paraffin sections. Staining with anti-CD90.1 was performed on cryosections with a 1:1,000 dilution of biotin-labeled mouse anti-mouse antibody (clone HIS51, eBioscience). Paraffin sections were stained for CD3 with a polyclonal rabbit anti-human/mouse CD3 diluted 1:500 (clone A0452, Dako). Subsequently, biotinylated swine anti-rabbit IgG antibody (clone E0431, Dako) was used at 1:100 as secondary antibody. Stainings were visualized with alkaline phosphatase-streptavidin, diluted 1:100 (clone no. K0391, Dako), and developed with Fast Red (Scytek) in naphthol-phosphate buffer (Scytek) with 50 mmol/L levamisole. The slides were counterstained with Mayer’s hematoxylin. Control sections were incubated with secondary antibodies alone.

Cytokine and blood chemistry analysis

A cytometric bead array (BD Biosciences) was performed on inflammatory cytokines [IL-12p70, IL-6, IL-10, tumor necrosis factor-α (TNF-α), IFN-γ, and MCP-1] in serum obtained by heart puncture. Blood samples were centrifuged at 10,000 rpm for 10 minutes and were diluted 1:4 for CBA analysis, according to the manufacturer’s instruction, and analyzed on a Calibur machine with CellQuest software (BD). Standard curves were included for each cytokine to determine blood concentrations. Samples of 150 μL of heparin blood from cardiac punctures were analyzed for functions of renal (creatinine) and liver aspartate aminotransferase.
(AST), alanine aminotransferase (ALT), and total bilirubin with a fully automated Modular P 800 system (Roche). All coefficient variations were below 3%.

**Statistical analysis**

Statistical analyses were performed using GraphPad software. The precise statistical method that was applied is indicated in the figure legends.

**Results and Discussion**

**In vivo T-cell activation by long peptide causes massive T-cell expansion and persistence**

Immunization with a 20-amino-acid long peptide comprising the human version of the gp10025–33 CTL epitope activated melanocyte-specific T cells from the endogenous T-cell repertoire but failed to control the outgrowth of B16 melanomas (14). We then analyzed the effects of peptide vaccination after adoptive transfer of gp10025–33-specific TCR-transgenic pmel T cells. Strong expansions of T cells were observed after s.c.

immunization with peptide in PBS and topically applied imiquimod, a TLR7 ligand (TLR7L; Fig. 1A). Five days after the second immunization, around 50% of all CD8+ T cells in the blood were pmel cells, as determined with the CD90.1 congenic marker on the transferred pmel T cells. Addition of low-dose IL-2 even enhanced this frequency up to 90% (Fig. 1A and B), showing that the CD8+ T-cell pool was heavily skewed toward the transferred T cells. Comparable high frequencies were observed in the spleen and lymph nodes, indicating that the in vivo activated pmel T cells spread systemically (Supplementary Fig. S1). Peptide and imiquimod were both essential for the massive clonal expansions of pmel T cells in vivo (Supplementary Fig. S2A), as replacement of imiquimod with TLR9L CpG DNA resulted in much lower frequencies (Fig. 1B).

Measurement of the pmel T-cell frequencies over time revealed a strong increase of pmel T-cell frequencies up to the week after the last immunization and then a slow contraction of this population (Fig. 1C). Notably, even 120 days after the last immunization, the peripheral CD8+ T-cell compartment...
of mice treated with ACT, peptide, imiquimod, and IL-2 still consisted of more than 50% pmel cells, pointing at a very high abundance of memory T cells. These memory T cells produced IFN-γ on brief in vitro stimulation (Supplementary Fig. S2B) and stained positive for the lymph node–homing molecules CD62L and CD27 (Supplementary Fig. S2C), showing a normal memory phenotype and function (15). Similar dynamics were found with 10 times less transferred T cells (Supplementary Fig. S2A) or in the context of the independent OT-1 TCR-transgenic T cells and OVA peptide model (Supplementary Fig. S2D), indicating that these striking expansions and high memory frequencies were commonly found in ACT combined with this vaccine consisting of long peptide, imiquimod, and IL-2.

Phenotypic analysis of the blood of these mice seemed to suggest that the numbers of endogenous hematopoietic cells of other lineages were strongly decreased after vaccination (CD90.1+CD8- cells in Fig. 1A). However, this was only a relative decrease because the total white blood cell count tripled owing to the extreme pmel T-cell expansions (Fig. 1D). The absolute numbers of the endogenous hematopoietic cells, including red blood cell counts, were hardly affected (Fig. 1D). Our data implied that peptide vaccination with imiquimod and IL-2 induces a 1,000-fold expansion of adoptively transferred T cells. Previous findings with this model also showed that transferred pmel T cells need in vivo activation or otherwise remain ignorant to the endogenous gp100-expressing melanocytes. However, immunization with antigen-encoding DNA, short peptides, or recombinant viruses, as previously tested, did not yield the extreme expansions that we observed here (4, 11, 16–19). Apparently, the combination of peptide in PBS, a topical overlay of imiquimod, and a low dose of IL-2 is optimally stimulating to CD8+ T cells. The same dynamics were observed in the OVA-specific OT-1 model, indicating that the effect of this vaccine mixture is not limited to the pmel system. The importance of imiquimod is further underlined by the finding that replacement with TLR9L CpG resulted in much less T-cell expansions. It is known that topically applied imiquimod leads to strong skin infiltration of dendritic cells (20). We would like to emphasize that these striking CD8+ T-cell dynamics were obtained in immunocompetent hosts in the absence of lymphodepletion or other hematologic conditioning.

**In vivo effector functions of transferred T cells**

The heavily expanded pmel T cells were examined for effector functions in vivo. First, fluorescence-labeled and peptide-loaded spleen cells were injected as target cells. These targets were efficiently killed in vaccinated mice that received pmel T cells, whereas target cells loaded with a control peptide were still detectable (Supplementary Fig. S3A and B). Interestingly, the low pmel frequencies in the TLR9L group were sufficient to eliminate all gp100-loaded targets, pointing at the sensitivity of this assay. Second, we scored the degree of skin depigmentation, which is a result of on-target pathology due to elimination of gp100-positive skin-residing melanocytes. All animals receiving pmel T cells and peptide vaccination showed depigmentation after 2 to 3 months, indicating that the activated T cells were capable of skin infiltration and eradication of healthy gp100-expressing melanocytes (Supplementary Fig. S3C and D). Depigmentation was most obvious at the site of imiquimod application on the flank, but in mice receiving additional low-dose IL-2 injections, the whiskers and eyelids turned white as well. Interestingly, intentional skin injury in these mice by a short tattoo treatment resulted in focused white spots in the fur at the site of tattooing (data not shown), suggesting that local on-target damage to healthy skin melanocytes depends on tissue responses evoked by imiquimod- or tattooing-mediated inflammatory signals.

Third, we tested the capacity of pmel T cells to eradicate subcutaneous B16 melanomas. Treatment of established melanomas was successful in half of the mice and tumor growth was delayed in the other mice (Fig. 2A). Finally, to challenge the tumor-protective capacity of our treatment modality, we investigated the tumor growth of melanomas in the anterior chamber of the eye. The eye is defined as an immune-privileged site harboring distinct immunosuppressive mechanisms (21). We inoculated B16 melanoma cells in the anterior chamber of the eye using microinjections, reflecting uveal melanoma in humans. Progressive and fast melanoma outgrowth was observed, and mice were sacrificed when the tumor completely filled this site, which was already achieved in most mice after 2 weeks (Fig. 2B). In vivo activated pmel T cells homed to both eyes regardless of the presence of B16 melanoma (Fig. 2C). Interestingly, homing to the eyes was scarcely accompanied with entry of other CD8+ T cells, and animals receiving only transferred pmel cells hardly contained CD8+ T cells at all (Fig. 2C). Addition of low-dose IL-2 to the protocol resulted in the highest level of pmel T-cell infiltrate. We stained tissue sections of the B16 tumors with CD3- and CD90.1-specific antibodies and confirmed that the pmel T cells were located within the tumor beds in close contact with tumor cells (Fig. 2B and data not shown). Furthermore, large numbers of T cells were found in the gp100-expressing choroidal layer of the uvea, even in control eyes (Fig. 2B). This structure of the eye was clearly thickened compared with the mice receiving pmel T cells alone. These findings are similar to recently published data on uveitis in a mouse model and a cutaneous melanoma patient who was treated with immunotherapy (22, 23). Importantly, treatment with ACT and peptide vaccination caused a significant delay in B16 melanoma outgrowth in the eye compared with naïve mice (Fig. 2D). This delay was even more pronounced when low-dose IL-2 was added to the treatment regimen. The limited treatment efficacy might be due to the immunosuppressive environment of the anterior chamber of the eye. In conclusion, in vivo activation of adoptively transferred T cells by peptide vaccination endows these T cells with the capacity to home to the skin and eyes and mediate tumor control at these sites.

**Lethal cytokine storm after continued peptide vaccination**

When mice were vaccinated with long peptide and imiquimod for a third time, they presented with fever chills, ataxia,
and ruffled fur. These symptoms were apparent after 5 hours and resulted in death in 25% of the animals within 12 hours. Addition of low-dose IL-2 increased the percentage of death to 90% (Table 1). We observed that pmel T-cell frequencies in the blood were correlated to these side effects, as mice with more than 85% pmel T cells before the third vaccination all succumbed to the peptide vaccination protocol, whereas mice with lower pmel frequencies showed symptoms but did not die (Table 1). The pathophysiologic cause underlying these unexpected side effects was investigated by blood analyses and autopsies. Blood chemistry of sera taken from the mice at 10 hours after the last vaccination revealed strongly elevated levels of the liver enzymes AST, ALT, and creatinine, indicating liver and kidney damage (Supplementary Fig. S4A), whereas total bilirubin was not different between the treatment and control groups (data not shown). Again, the addition of low-dose IL-2 led to more pronounced toxicity. H&E staining of lungs, spleens, livers, and kidneys of mice before and after the last vaccination protocol. Both treatment groups were significantly different from the control group (log-rank test). Figure shows compiled data from three independent experiments that all displayed significant differences between the groups.
peptide vaccination showed tremendous differences in tissue structure (Fig. 3A). The splenic architecture was disrupted, as no clear white pulp zones were visible anymore. The lungs were massively infiltrated with neutrophil granulocytes and T cells, and also the livers and kidneys showed clear signs of immunopathology (Fig. 3A). Together, these data suggested multiorgan failure as cause of death.

Blood samples at 5 and 10 hours after the third vaccination were also analyzed for six different cytokines: IL-12, IFN-γ, IL-10, MCP-1, TNF-α, and IL-6 (Fig. 3B). The pro-inflammatory cytokine IL-12 was not elevated in the diseased animals, whereas all the other cytokines were strikingly high. In line with the degree of mortality, vaccination without IL-2 resulted in a peak at 5 hours and a subsequent decrease. Most of these mice spontaneously recovered from cytokine storm symptoms. However, cytokine levels remained high in the group with inclusion of IL-2. Of note, these extreme cytokine levels were not found after two vaccinations with peptide and strictly correlated with clinical symptoms (Table 1).

We reasoned that the multiorgan failure was caused by Table 1. Symptoms of systemic inflammatory response syndrome after treatment

<table>
<thead>
<tr>
<th>Treatment modality</th>
<th>No. of ill mice (%)</th>
<th>Adverse effects</th>
<th>No. of dead mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>0/10 (0)</td>
<td>−</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>ACT</td>
<td>0/10 (0)</td>
<td>−</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>ACT + peptide + TLR7L</td>
<td>20/20 (100)</td>
<td>+</td>
<td>5/20 (25)</td>
</tr>
<tr>
<td>ACT + peptide + TLR7L + IL-2</td>
<td>20/20 (100)</td>
<td>+++</td>
<td>18/20 (90)</td>
</tr>
</tbody>
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*Symptoms were fever, chills, ataxia, and gathering.
†Twenty-four hours after the last vaccination.
‡Means of pmel frequency of CD8+ T cells in peripheral blood.

Figure 3. A third vaccination with peptide results in multiorgan failure and cytokine storm. Mice treated with ACT of pmel T cells and vaccination with gp100 peptide, TLR7L, and low-dose IL-2 presented with fever chills and ataxia and died within 24 h. A, histologic analysis of the lung, spleen, liver, and kidney showing immunopathology. Tissues were taken just before (left) and 10 h after (right) the third vaccination. A summary of toxicity is shown in Table 1. B, the serum levels of cytokines in animals from different treatment groups were determined. Columns represent different treatment groups: naïve mice (light gray); ACT of pmel only (medium gray); ACT, peptide vaccination, and TLR7L (dark gray); and treatment including IL-2 (black). Sera were taken on different time points: before, 5 h after, and 10 h after the third vaccination. Each group contained five mice. Columns, mean; bars, SD. Comparable data were obtained in an independent experiment.
cytokine storm and resembled an aseptic systemic inflammatory response syndrome (SIRS), an immune-mediated constitution found in severe graft-versus-host disease; antithymocyte globulin injection for prevention of transplant rejection; and acute respiratory distress syndrome, in which the lungs are injured intrinsically or by trauma (24–26). However, the closest resemblance is found in the recently published clinical studies of agonistic anti-CD40 and anti-CD28 administration (27, 28). This latter study, which was conducted in healthy young volunteers, was not successful because massive cytokine release by T cells was accompanied with serious multiorgan failure; fortunately, the lives of all subjects were saved with the use of corticosteroids and in vivo capture of IL-2 (27).

**Prevention of SIRS with peptides in a slow-release depot or weak agonistic peptides**

Long peptides were administered in a physiologic salt solution in this study because this formulation is known to mediate excellent in vivo T-cell priming when a strong adjuvant is provided (10, 12). The observed fast and massive cytokine release by pmel T cells after the third vaccination (Fig. 3) suggested that the 20-mer long peptides used in the current study quickly diffused from the s.c. injection site and thereby systemically stimulated pmel T cells. Our previous studies on long peptides, however, indicated that long peptides are only drained to the local lymph node (10, 12). Indeed, the biodistribution of the 20-mer long gp100 peptide was largely restricted to the local draining lymph node, in contrast to the minimal 9-mer peptide epitope, which was also detected at the distant contralateral side (Fig. 4A). Higher quantities of the long peptide resulted in systemic spread.4 We concluded that a local pulse of peptide presentation was sufficient for fast and vigorous stimulation of pmel T cells leading to fatal cytokine storm and multiorgan failure.

To prevent cytokine storm, we replaced the PBS in the third vaccination with the mineral oil Montanide-ISA-51 to restrain the release of the peptide. This completely prevented the symptoms of SIRS, despite high pmel T-cell frequencies. The levels of liver enzymes and creatinine and the concentrations of cytokines confirmed the absence of morbidity and massive pmel T-cell activation (Supplementary Fig. S4B; Fig. 4B). Alternatively, vaccination with a long peptide comprising the wild-type sequence of mouse gp100 (EGSRNQDWL) also prevented SIRS (Fig. 4B). This peptide epitope binds with less affinity to the presenting MHC class I molecule and is a weaker agonist than the widely used altered peptide (KVPRNQDWL; ref. 14). These results showed that the strong immunopathology of SIRS could be prevented either by applying weak agonistic peptides or by formulating the peptide in a slow-release depot, such as mineral oil. Thus, the adverse effect of the massive T-cell activation by peptide vaccination can be readily prevented.

In clinical studies of adoptive cell therapy, hematologic conditioning has been considered necessary for optimal T-cell activation and antitumor efficacy (5, 7, 29, 30). The mechanisms involved are likely quite diverse: creating physical space for the introduced cells, removal of cytokine "sinks" and suppressor cells, and activation of antigen-presenting cells through the intestinal flora (7). Here, we show that a 1,000-fold expansion of T cells and antitumor efficacy can also be observed in immunocompetent, nonconditioned hosts. This sheds new light on the previously defined requirements and might open up possibilities of reaching clinical tumor responses without severe pretreatment conditioning.

4 Unpublished data.
of the patient. The combination of ACT with peptide vaccine-mediated activation in vivo and TLR ligands, as applied in our study, is certainly an avenue for further exploitation in cancer therapy. However, the power of this combination also warrants precautions concerning overstimulation of tumor-specific CD8+ T cells when the levels of circulating specific T cells are pushed to the limit. Harnessing this power is imperative and must include careful peptide formulations that prevent rapid spread of the peptide, for example, by using slow-release adjuvant or optimal peptide design.

**References**

4. Kohlmeyer J, Cron M, Landsberg J, et al. Complete regression of tumors: a consideration for the role of chronic antigenic stress in the destruction of tumor-specific CD8+ T cells when the levels of circulating specific T cells are pushed to the limit. Harnessing this power is imperative and must include careful peptide formulations that prevent rapid spread of the peptide, for example, by using slow-release adjuvant or optimal peptide design.

**Disclosure of Potential Conflicts of Interest**

S.H. van der Burg is advisor and C.J.M. Melief is employee of the biotech company ISA Pharmaceuticals, but the company did not financially support this study.

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