

CUTTING EDGE

Cutting Edge: Priming of CTL by Transcutaneous Peptide Immunization with Imiquimod¹Gerd Rechtsteiner,^{2*} Tobias Warger,^{2*} Philipp Osterloh,^{*†} Hansjörg Schild,^{3†} and Markus P. Radsak^{*†}

CTL are important in combating cancer and viruses. Therefore, triggering the complete potential of CTL effector functions by new vaccination strategies will not only improve prophylaxis of tumor or virus-related diseases, but also open opportunities for effective therapeutic immunizations. Using transcutaneous immunization, we show that epicutaneous (e.c.)⁴ application of an ointment containing a CTL epitope and the TLR7 ligand imiquimod is highly effective in activating T cells in mice using TCR-transgenic CTL or in wild-type mice. Transcutaneous immunization-activated CTL mount a full-blown immune response against the target epitope characterized by proliferation, cytolytic activity, and the production of IFN- γ that is completely restricted to the epitope used for vaccination. Our results obtained by simple e.c. application of an ointment, without further skin irritating procedures, provide the basis for the development of new, easy to use vaccines against cancer or virus-associated diseases. The Journal of Immunology, 2005, 174: 2476–2480.

Cytotoxic T lymphocytes play a major role in eliminating virus-infected or malignant cells by specifically recognizing antigenic peptides presented on MHC class I molecules (1). To perform their cytotoxic effector functions, CTL need to be activated or primed by professional APC like dendritic cells (DC), which on their own need to acquire an activated phenotype to induce effective priming, otherwise tolerance to the presented Ag is induced (2, 3).

The variety of T cell epitopes for potential therapeutic uses is perpetually expanding and there is so far some success in peptide-based vaccination approaches aiming at the induction of CTL, i.e., using peptide-pulsed DC. To mediate efficient T cell activation, DC have to be in a mature stage characterized by high expression levels of MHC and costimulatory molecules like CD80 or CD86 as well as the release of proinflammatory

cytokines, i.e., IL-6, IL-12, and TNF- α (4, 5). So-called TLR are widely expressed on DC and able to mediate such DC maturation (4, 6–9). Thus, TLR ligands are able to create a proinflammatory milieu required for efficient priming of CTL.

However, DC-based vaccination procedures are expensive and laborious because they involve the purification and in vitro culturing of cells under stringent conditions and require standardized criteria to define various DC preparations. Therefore, targeting CTL responses using a cell-free and nontoxic adjuvant could greatly improve vaccination against viruses and malignant diseases. In this context, transcutaneous immunization (TCI) protocols appear to be particularly promising (10) by gaining direct access to skin-resident DC, so-called Langerhans cells, as highly efficient APC to initiate a CTL response (11). Being easy to use and cost-effective, TCI might be suitable to improve patient compliance as well as feasible for the use in third world countries. In addition, TCI as a noninvasive procedure might increase safety of vaccinations, eliminating the risk of infections related to the recycling and improper disposal of needles (12).

Using the skin as route of delivery for Ags is effective in humans for the induction of Ab responses (10) and in various experimental models for the induction of T cell responses, enhancing the effects of DNA immunization (13) and also in combination with ligands for TLR (14, 15).

The imidazoquinoline derivative imiquimod is a synthetic immunomodulatory ligand for TLR7 (16). In vitro imiquimod has been described to activate DC by inducing DC maturation along with the release of inflammatory cytokines in strict dependence of its intracellular adaptor molecule MyD88 (17). Beyond this, imiquimod also has a proven efficacy in patients with human papillomavirus-associated or malignant skin diseases by topical administration (18–21). The treatment is safe and usually well tolerated with only minor side effects like redness and pruritus at the site of application (21). Concerning the antiviral and antitumor effects, the exact mechanism of action of imiquimod is unknown, but it is proposed that the local activation

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⁴ Abbreviations used in this paper: e.c., epicutaneous; DC, dendritic cell; TCI, transcutaneous immunization; tg, transgenic; BMDC, bone marrow-derived DC; DLN, draining lymph node.

of DC leads to the production of inflammatory cytokines and enhanced Ag presentation of viral or tumor Ags inducing a curative immune response (22).

In our present study, we combined the immunostimulatory capacity of topical activation of DC by imiquimod with specific CTL epitopes to mount a CTL response. We found that TCI with imiquimod is effective using a TCR-transgenic (tg) mouse model. Beyond this, we also demonstrate that immunization by TCI is also suitable for the induction of a primary CTL response in wild-type mice. Taken together, we present a new and straightforward vaccination method to effectively induce potent CTL responses *in vivo*.

Materials and Methods

Mice

C57BL/6 mice at the age of 6–8 wk were used for vaccination experiments or as hosts for adoptive transfer experiments. TCR tg OT-I mice recognizing the H2-K^b-restricted epitope SIINFEKL from chicken OVA (OVA_{257–264}) were generated by Hogquist and coworkers (23) and obtained from Dr. C. K. Kurts (University of Bonn, Bonn, Germany). St35 and St42 mice are tg for a TCR recognizing the peptide SGPSNTTPEI (SGP) from the adenovirus Ad5 E1a protein (aa 234–243) presented on H2-D^b. St35 and St42 mice are on the C57BL/6 background and were generated as described previously (24). They were crossbred with B6.SJL-ptprca/BoCrTac-[KO]Rag2 N10 (RAG2^{-/-}Ly5.1; Taconic Farms) to yield different combinations of CD45.1 or CD90.1, all in the RAG2^{-/-} background. All animal procedures were conducted in accordance with the institutional guidelines.

Adoptive transfer and immunizations

Splenocytes from TCR tg donor mice were prepared and adoptively transferred *i.v.* into the tail vein of recipient mice as described previously (24). TCI was performed by adding the indicated amount of peptide to 50 mg of imiquimod ointment (Aldara; 3M Pharmaceuticals) and applying it to the shaved back of anesthetized mice (~10 cm²). In some experiments, the indicated amount of peptide was injected intracutaneously. For immunizations with bone marrow-derived DC (BMDC), 1 × 10⁵ cells activated by poly(I:C) (50 μg/ml, overnight) were pulsed with SGP (10 nM) and injected *i.p.* Synthetic peptides SGPSNTTPEI derived from Ad5 E1a and SIINFEKL from chicken OVA were kindly provided by Dr. S. Stevanovic (Department of Immunology, Institute for Cell Biology, University of Tübingen, Tübingen, Germany).

Flow cytometric analyses and *in vivo* cytotoxicity assay

The following mAbs were used for analyses by flow cytometry: allophycocyanin-Cy7-conjugated anti-CD8, PE-conjugated anti-CD45.1, PerCP-conjugated CD90.1, allophycocyanin- or FITC-conjugated CD44, PE- or allophycocyanin-conjugated CD62L (all from BD Pharmingen). For detection of peptide-specific CD8⁺ T cells, a SIINFEKL loaded dimeric recombinant H2-K^b-mIgG1 fusion protein (DimerX; BD Pharmingen) was used according to the manufacturer's instructions. Blood samples were collected after tail vein incision and incubated on ice with specific mAbs as indicated after a hypotonic lysis step.

For the assessment of *in vivo* cytolytic activity, C57BL/6 splenocytes were labeled either with low (0.4 μM) or high (4 μM) amounts of CFSE (Molecular Probes) and 3 × 10⁷ cells were adoptively transferred into immunized mice in a 1:1 ratio (CFSE^{low}:CFSE^{high}) after peptide (10 nM SGP or 50 nM SIINFEKL, respectively) labeling of CFSE^{high/low} cells as indicated. After 24 h, the mice were sacrificed and splenocytes from host mice were analyzed by flow cytometry. All analyses were performed with a FACSCanto flow cytometer and FACSDiva software (BD Pharmingen).

Specific lysis was evaluated as follows: specific lysis (percent) = (number of cells nonpeptide pulsed – number of cells pulsed with peptide)/number of cells nonpeptide pulsed.

In vitro cell culture and cytotoxicity assays

BMDC were obtained by 6-day culture of C57BL/6-derived bone marrow cells with GM-CSF as described previously (7). For *in vitro* restimulation of CTL, BMDC were preactivated with poly(I:C) (50 μg/ml; Amersham Bioscience) and loaded with 50 ng/ml SIINFEKL before adding draining lymph node (DLN) cells or splenocytes. CTL activity *in vitro* was assessed by standard ⁵¹Cr release assay as described previously (24) after 5 days of coculture with activated BMDC. Production of IFN-γ was assessed by intracellular staining after restimulation for 6 h with peptide in the presence of brefeldin A (2.5 μg/ml;

Sigma-Aldrich) for the last 4 h of incubations *ex vivo* or alternatively by ELISA (BD Pharmingen) as described previously (24).

[³H]Thymidine proliferation assays

Culture medium was IMDM (Invitrogen Life Technologies) supplemented with 2 mM L-glutamine, 5 × 10⁻⁵ M β-ME, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS. Cells from DLN were added to naive splenic T cells into 96-well round-bottom microplates (Costar) in a total volume of 0.2 ml of culture medium. After 48 h, [³H]TdR (0.5 μCi/well; ICN Pharmaceuticals) was added to the cultures and after additional 18 h thymidine uptake was measured by beta scintillation counting.

Results and Discussion

Priming of naive TCR tg CTL by TCI with imiquimod

To assess the potential value of TCI, we adoptively transferred splenocytes from TCR tg St35 RAG2^{-/-} mice into congenic C57/BL6 hosts and subsequently immunized the animals by TCI. The frequency of tg SGP-specific CD8⁺ T cells was monitored in the blood, lymph nodes, and spleen by the congenic marker CD90.1 expressed only in tg cells. As depicted in Fig. 1, already 4 days after TCI with SGP, the TCR tg CD8⁺ T cell population showed a massive (>60-fold) expansion (Fig. 1A) and an activated phenotype (CD44^{high}CD62L^{high/low}) in the lymph nodes, blood, and spleen, whereas CD8⁺ T cells in mice

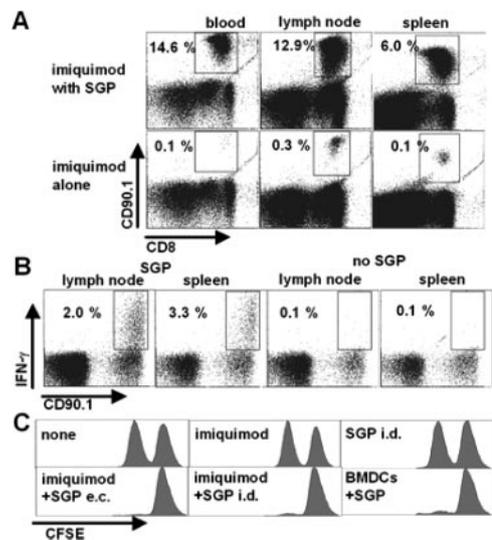


FIGURE 1. Priming of TCR tg CTL by TCI with imiquimod elicits a potent peptide-restricted CTL response. C57BL/6 mice were adoptively transferred *i.v.* with naive TCR tg St42 CTL (1 × 10⁶) and subsequently treated with TCI by applying imiquimod ointment (50 mg) with or without the cognate H2-D^b-restricted peptide SGPSNTTPEI (SGP; 20 μg/treatment) recognized by St42 CTL onto the skin of the shaved back twice on 1 day. After 4 days, the mice were sacrificed and analyzed for the frequency (A) and phenotype (CD44 and CD62L; data not shown) on TCR tg CTL by the congenic marker CD90.1 expressed only on adoptively transferred cells in the blood, lymph nodes, and spleen. A, The depicted numbers are the percentage of CD8⁺CD90.1⁺ lymphocytes in the indicated region. B, Lymph node cells or splenocytes from mice treated with imiquimod + peptide (SGP) were incubated for 6 h *ex vivo* in the presence (upper panel) or absence (lower panel) of SGP, adding brefeldin A for the last 4 h, and analyzed for the production of IFN-γ. The depicted dot plots are gated on CD8⁺ lymphocytes. C, Five days after treatment with either an *e.c.* application of imiquimod, imiquimod + SGP (*e.c.*), intracutaneous (intradermal (*i.d.*)) injection of SGP (20 μg) with or without imiquimod (*e.c.*) or with SGP-pulsed activated BMDC (10⁵ cells; *i.p.*), or no treatment cytotoxicity against splenocytes from C57BL/6 labeled was assessed *in vivo* by *i.v.* transfer of CFSE^{low} (1 × 10⁷ cells; 1 μM SGP) or CFSE^{high} (1 × 10⁷ cells; no peptide)-labeled splenocytes. The results shown are representative of two to four independent experiments using two mice per group.

treated with imiquimod ointment alone did not show any sign of activation by expansion or activation markers (data not shown). Importantly, the expanded CD8⁺ T cell population from TCI-treated mice was able to produce IFN- γ upon peptide restimulation *ex vivo*, indicating they were activated CTL effector cells (Fig. 1B).

To further examine the activation of TCR tg CTL and exclude the potential unspecific activation by the imiquimod ointment or the SGP peptide alone, we adoptively transferred mice with TCR tg St42 CTL and challenged them with either TCI or the components (SGP or imiquimod) separately. For comparison, mice immunized with activated SGP-pulsed BMDC were included as a positive control. After 4 days, the *in vivo* cytotoxicity against peptide-loaded target cells was determined in each group. Only the combination of the peptide with imiquimod led to the specific lysis of target cells that was comparable to the vaccination with peptide-pulsed BMDC (Fig. 1C). Notably, there was no difference whether the peptide was injected intradermally with subsequent *e.c.* application of imiquimod or whether peptide and imiquimod ointment were applied epicutaneously together. This demonstrates that the transcutaneous uptake of the peptide is very efficient. Treatment with either component alone or no treatment did not lead to any detectable killing of peptide-labeled target cells.

Our results clearly demonstrate that priming is required for these TCR tg CTL to perform their effector functions and that neither the peptide nor the imiquimod ointment alone are sufficient for priming. Moreover, this TCI protocol is equipotent compared with DC vaccination and does not require disruption of the skin barrier by tape stripping as reported in a recent study where the application of an antigenic peptide along with CpG oligodeoxynucleotide on tape-stripped skin was effective in inducing CTL responses in mice (14). In this study, we describe a simple, effective, and feasible method to specifically activate CD8⁺ T cell populations that obviates the need for such skin-irritating procedures.

Topically applied peptides are efficiently transported to the DLN

It was shown recently that the topical treatment with imiquimod ointment results in the local activation of skin-resident Langerhans cells followed by the migration to the DLN (25). To provide evidence that locally residing APC are also able to capture and transport peptides from the skin to the DLN where they can present the epitope to CTL, we treated mice (without previous adoptive transfer of TCR tg CD8⁺ T cells) with a single dose of TCI using either SGP or SIINFEKL as target epitopes and harvested the DLN 24 h later. These cells were then used as stimulator cells for SGP-specific St42-derived CD8⁺ T cells or SIINFEKL-specific OT-I CD8⁺ T cells *in vitro*. As shown in Fig. 2, DLN cells from these mice were able to induce only proliferation of TCR tg CD8⁺ T cells that were specific for epitope used for immunization (Fig. 2A) as well as lysis of peptide-loaded target cells *in vitro* (data not shown). These results demonstrate that topically applied peptide Ags are effectively and sufficiently transported from the skin to the DLN where a robust CTL response is initiated. Importantly, the CTL response elicited by TCI is completely restricted to the epitope used for TCI.

Next, we investigated the activation kinetics of TCR tg CTL after immunization by TCI. Mice were adoptively transferred with TCR tg CTL and subsequently immunized by TCI. DLN

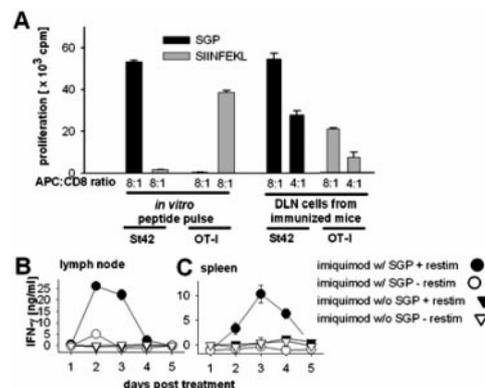


FIGURE 2. Epicutaneous-applied peptides are transported to the DLN and are sufficient for priming of naive CTL. *A*, Mice were treated with a single dose of TCI using either imiquimod + SGP or imiquimod + SIINFEKL and sacrificed 1 day later. Lymph node cells (1×10^6) were irradiated (20 Gy) and used as stimulators for naive TCR tg St42 CTL or OT-I CTL that recognize the H2-K^b-restricted epitope SIINFEKL. Proliferation by [³H]thymidine uptake and cytolytic activity (data not shown) were evaluated after 3 days of *in vitro* culture. *B* and *C*, Mice were adoptively transferred *i.v.* with naive TCR tg St42 CTL (1×10^6) and subsequently treated with TCI by applying imiquimod ointment with or without SGP as described. Lymph node cells (*B*) or splenocytes (*C*) were harvested 1–5 days later and (1×10^6) incubated *in vitro* in the presence (filled symbols) or absence (open symbols) of SGP (10 nM) for 24 h. The release of IFN- γ in the supernatant was analyzed by ELISA. The results shown are representative of 3 independent experiments.

and spleen cells from these animals were harvested 1–5 days later and analyzed for the production of IFN- γ *in vitro*. As depicted in Fig. 2, there were large amounts of IFN- γ present at 2 and 3 days after immunization in the DLN (Fig. 2B) and on days 3 and 4 in the spleen (Fig. 2C). Notably, on day 2 there was some IFN- γ detectable even without peptide restimulation *in vitro*, although this was not sufficient for a sustained response. On day 3, peptide-specific IFN- γ production became detectable in the spleen.

As recently demonstrated by Nair and coworkers (26), the injection of immature DC into imiquimod-treated skin induces DC maturation and activation *in vivo*. Beyond this, we now demonstrate that creating an inflammatory milieu by activating skin-resident cells, most likely Langerhans cells, by TLR ligands in the presence of a T cell epitope is sufficient for CTL priming. This procedure has the potential to replace the *ex vivo* generation and injection of peptide-pulsed DC.

Effective priming of naive CTL by TCI in wild-type mice

Next, we explored whether vaccination by TCI is also suitable to induce a primary CTL response in a non-tg system. We therefore immunized wild-type C57BL/6 mice by TCI with the SIINFEKL peptide. DLN and spleens were collected 16–120 h after TCI and restimulated for 5 days *in vitro* using SIINFEKL-loaded activated BMDC. As depicted in Fig. 3, at 16 or 30 h after immunization, a cytolytic response was detectable in DLN, but not the spleen, whereas after 120 h we found CTL activity in the spleen as well as DLN. These results are complementary to our previous adoptive transfer experiments and clearly demonstrate that after TCI naive CTL first become activated in the DLN and later in the spleen.

To investigate whether the observed *in vitro* cytotoxic activity is also relevant *in vivo*, we treated mice as above with TCI

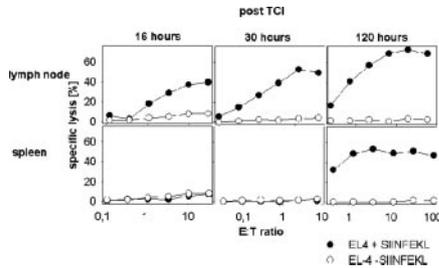


FIGURE 3. Cytolytic activity in vitro after TCI treatment of wild-type mice. C57BL/6 mice were treated with TCI by applying imiquimod ointment (50 mg) with SIINFEKL (150 μ g) twice daily on 2 consecutive days and were sacrificed after 16, 30, or 120 h after treatment. Lymph node cells or splenocytes were collected and 1×10^6 cells were incubated with SIINFEKL (50 nM) pulsed, activated BMDC (ratio 5:1) for 5 days in vitro. Cytolytic activity against SIINFEKL-loaded (200 nM) EL-4 cells was analyzed by a standard 4-h ^{51}Cr release assay.

using the SIINFEKL epitope as Ag peptide and additionally included mice that were treated with the peptide or ointment separately as controls. After 8 days, in vivo cytotoxicity was evaluated with SIINFEKL-labeled target cells. As shown in Fig. 4, this treatment led to the specific lysis of target cells in vivo (Fig.

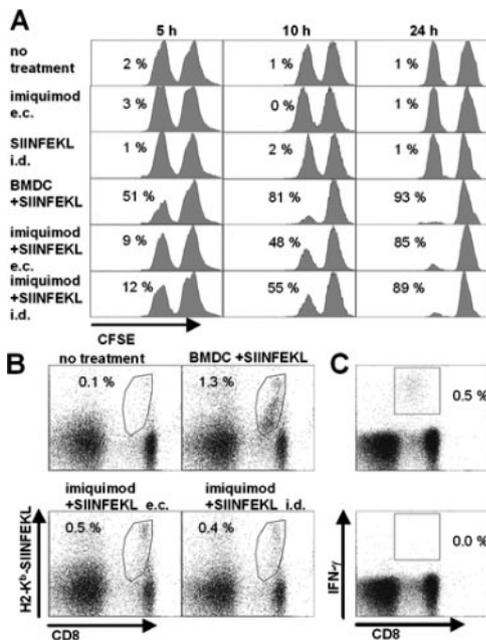


FIGURE 4. Transcutaneous peptide immunization of wild-type mice with imiquimod elicits a potent CTL response in vivo. *A*, C57BL/6 mice were treated with TCI using imiquimod ointment (50 mg) or peptide (SIINFEKL, 150 μ g) separately or in combination twice daily on 2 consecutive days. After 8 days in vivo, cytolytic activity against SIINFEKL (1 μ M)-loaded CFSE^{low} or non-peptide-loaded CFSE^{high} syngenic splenocytes was evaluated after 5–24 h. The depicted numbers correspond to the percentage of specific lysis. *B*, Splenocytes from treated mice were stained for peptide-specific CD8⁺ T cells using a SIINFEKL-loaded recombinant dimeric H2-K^b-mIgG1 fusion protein to detect peptide-specific CD8⁺ T cells. The depicted numbers represent the percentage of cells in the indicated region. *C*, Splenocytes from imiquimod + SIINFEKL e.c.-treated mice were stimulated for 6 h in the presence (*upper panel*) or absence (*lower panel*) of SIINFEKL (50 nM), adding brefeldin A for the last 4 h of incubation. The production of IFN- γ ex vivo was then analyzed by intracellular staining for IFN- γ . The numbers shown indicate the percentage of cells in the indicated region. All depicted results are representative of three independent experiments using two mice per group.

4*A*), expansion of peptide-specific CD8⁺ T cells (Fig. 4*B*), and ex vivo production of IFN- γ (Fig. 4*C*), indicating that TCI also induces a primary CD8⁺ T cell response in wild-type mice. Notably, immunization using peptide-pulsed BMDC led to a slightly enhanced cytolytic activity at early time points (Fig. 4*A*, *left and middle panels*) and frequency of peptide-specific CD8⁺ T cells, although there was no detectable difference in target cell lysis at 24 h (Fig. 4*A*, *right panel*). These results demonstrate that the amount of antigenic peptide and costimulation provided by transcutaneous delivery is adequate to induce a primary CTL response in wild-type mice. Whether the observed delay in cytolytic activity after TCI compared to immunization with activated BMDC is functionally relevant in terms of protection against tumors or memory formation is currently under investigation.

One major obstacle in therapeutic vaccination against viruses and tumors is the current inability to target Ags for effective presentation on professional APC without concomitantly creating significant toxicity due to unspecific adjuvant effects. Using topical vaccination protocols might help to manage some of the related problems: applying a vaccine directly onto the skin might deliver Ags to local DC, circumventing the laborious need for ex vivo generation and injection of DC. In addition, making vaccination a facile and noninvasive procedure might potentially overcome some of the side effects created by injections and thus increase patient compliance as well as needle-associated morbidity (12). In DNA vaccination, Thomsen et al. (13) found an enhancing effect of topical imiquimod treatment before immunization that leads to an improved activation of both CD4⁺ and CD8⁺ T cells. Whether the potent primary CTL response induced by our new TCI protocol also confers long-term protection by the induction of memory CTL is yet to be determined and is under current investigation. However, TLR-mediated signals as provided by imiquimod in our approach might help to overcome the need for a CD4 helper T cell response for memory formation as suggested by Melief and co-workers (4, 27). Furthermore, the question whether this vaccination approach also allows the direct peptide-specific activation of CD4⁺ T cells has to be addressed and is currently under investigation.

In conclusion, we describe a novel simple and feasible method for vaccination to mount an efficient CTL response in vivo. Further studies evaluating and adopting this technique for use in humans are currently underway. Given that our TCI protocol is also effective in humans, this vaccination method might help to improve prophylactic and therapeutic vaccination approaches against any defined CTL epitope, providing new treatment options in persistent virus infections such as CMV or human papillomavirus and malignancies.

Disclosures

The authors have no financial conflict of interest.

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