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T cells specific for a TAP-independent self-peptide remain naïve in tumor-bearing mice and are fully exploitable for therapy

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ABSTRACT
Cancers frequently evade immune-recognition by lowering peptide:MHC-I complexes on their cell surface. Limited peptide supply due to TAP-deficiency results in such MHC-Ilow immune-escape variants. Previously, we reported on a category of TAP-independent self-peptides, called TEIPP, with selective presentation by these tumors. Here we demonstrate that in contrast to T cells specific for conventional tumor antigens, TEIPP-directed T cells remain naïve in mice bearing immune-escaped tumors. This unaffected state was caused by low levels of MHC-I on the tumors and the failure to cross-present low levels of antigenic protein by host APCs. Importantly, increased levels of MHC-I, antigen or co-stimulation resulted in potent activation of TEIPP-specific T cells via direct presentation. Genetic knockdown by CRISPR/Cas9 technology of the relevant MHC-I allele in tumor cells indeed abrogated T cell activation. Vaccine-mediated priming of TEIPP-specific T cells induced efficient homing to MHC-Ilow tumors and subsequently protected mice against outgrowth of their MHC-Ilow tumor. Thus, our data open up the search of TEIPP-specific T cells in cancer patients to explore their application against MHC-Ilow tumor cells.

Introduction
Cancer immunotherapy has reached major successes in recent years with the introduction of several new treatment options in the clinic. Especially immune checkpoint therapy with blocking antibodies to PD-1 and PD-L1, which prevent signalling of inhibiting co-receptors on T cells, are FDA- and EMA-approved in an increasing number of oncologic indications. However, the majority of patients still succumb to their disease or relapse after initial successful treatment, highlighting the importance of further delineating critical factors of success. It has become clear that tumors can evade immune recognition by downregulation of MHC-I levels and thereby resist T cell immunity and checkpoint therapy. Two recent studies described that tumors from patients relapsing or not responding to checkpoint inhibitor therapy had mutations in genes encoding the IFNγ pathway, including Jak/STAT signaling. This failure of tumor cells to respond to IFNγ affects levels of MHC-I as cells are impaired to induce expression of the peptide transporter associated with antigen presentation (TAP). Also mutations or epigenetic silencing of components of the antigen-processing machinery, such as TAP, lead to strong reduction of MHC-I on cancer cells. All these alterations result in a general deficiency to present conventional tumor antigens to CD8+ T cells.

We study an alternative CD8+ T cell repertoire that specifically recognizes peptides on cells deficient for the peptide transporter TAP. Due to this TAP deficiency, cells express strongly reduced levels on MHC-I, but in conjunction an alternative peptide repertoire is presented on residual MHC-I molecules, called TEIPP. T cell epitopes associated with impaired peptide processing. TEIPP-specific T cells are therefore a potential candidate to treat immune-escaped, MHC-Ilow tumors. Of note, as TEIPP antigens are presented in MHC-I molecules, tumor cells with a complete loss of MHC-I molecules due to a mutation in β2 m or the heavy chain, will not present TEIPP antigens and are therefore not targetable by TEIPP-specific T cells.

The first identified mouse TEIPP was a C-terminal peptide of Trh4, a ceramide synthase spanning the ER membrane. The protein is ubiquitously expressed in all somatic cells, but its peptide epitope is surprisingly only presented on TAP-deficient cells. Antigen processing and presentation of the epitope is independent of the proteolytic enzyme complex pro tease and the TAP transporter. Instead, release of the epitope depended on intramembrane cleavage by signal peptide peptidase (SPP). Using a T cell receptor-transgenic (TCR tg) mouse based on a Trh4-specific CD8+ T cell clone, we previously demonstrated that these TCR tg T cells (‘LnB5 tg’) undergo normal, efficient thymic selection and are not hampered by central or peripheral tolerance, most likely since the Trh4 self-peptide is only MHC-I presented in TAP-deficient cells. Upon transfer of naïve LnB5T cells in wildtype, tumor-free B6 mice, cells remain naïve as expected. In contrast, transfer of LnB5 T cells to TAP-deficient mice resulted...
in vigorous proliferation and strong activation, especially under inflammatory conditions.\textsuperscript{11}

In the present study, we aimed to understand the behaviour of naïve TEIPP T cells in mice bearing a MHC-I\textsuperscript{low} tumor and, secondly, the molecular requirements for their optimal priming. In contrast to T cells against conventional tumor antigens, we found that naïve TEIPP-specific T cells were not activated by resident tumors and hardly infiltrated MHC-I\textsuperscript{low} tumors, despite the fact that the antigen was presented there. Sufficient TEIPP T cell activation was only reached by tumor cells with high levels of MHC-I as well as cognate antigenic protein. Strikingly, this manipulated activation was operated via direct priming and not via cross-priming of TEIPP antigens and resulted in strong influx in wild type MHC-I\textsuperscript{low} tumors. Importantly, it prevented outgrowth of such immune escape tumors. These results imply that the TEIPP-specific CD8\textsuperscript{T} T cell subset remains naïve even in tumor-bearing mice harboring MHC-I\textsuperscript{low} tumors and indicate that vaccination strategies may optimally exploit these immune cells for immunotherapy.

Results
Activation of TEIPP T cells requires high antigen and MHC-I levels on tumor cells

Previously, we have shown using a TCR transgenic mouse model that TEIPP T cells efficiently egress from the thymus and arrive in the periphery with an antigen-unexperienced phenotype.\textsuperscript{11} To study the potential of MHC-I\textsuperscript{low} tumor cells to activate naïve TEIPP T cells \textit{in vivo}, we applied a model in which congenic naïve TCR-transgenic TEIPP T cells (\textit{LnB}5\textit{tg}) were transferred to recipient mice that were subsequently injected with irradiated MHC-I\textsuperscript{low} RMA-S cells, which display the cognate Trh4/D\textit{b} complex. Previously, we and others showed that RMA-S tumor injection failed to induce TEIPP T cell immunity.\textsuperscript{12,13} Therefore, we overexpressed the Trh4 antigen and/or the co-stimulatory molecule B7.1 (CD80) in RMA-S cells and analysed T cell activation (Fig. 1A). Neither enhanced levels of Trh4, nor B7.1 or the combination of these two resulted in strong expansion of RMA-Trh4 Kb\textsuperscript{+/−} cells, but overexpression of the antigen can also lead to TEIPP presentation in TAP-proficient cells. Together, our data show that high MHC-I antigen presentation and strong expression of the TEIPP antigen are important for the \textit{in vivo} activation of TEIPP T cells.

\textbf{TEIPP T cell activation is mediated by direct priming on tumor cells}

The fact that RMA-Trh4 cells induced a surprisingly strong TEIPP T cell activation \textit{in vivo} prompted us to study how this priming of naïve TEIPP-specific T cells took place. Either via direct interaction with the RMA-Trh4 cells or indirectly via cross-priming a process by which professional antigen-presenting host cells ingest, process and present Trh4 antigen to T cells,\textsuperscript{14,15} To test the capacity of cross-priming, we overexpressed Trh4 in allogeneic P815 cells (Supplementary Figure S2A), a mastocytoma cell line from a DBA/2 mouse on H-2d background, lacking the D\textsuperscript{b}-restricting element for direct presentation to TEIPP T cells. Injection of P815 or P815.Trh4 cells did not elicit accumulation of TEIPP T cells in the blood of mice (Fig. 2A). Some T cell activation was measured in both groups compared to mice that only received T cells, however, these T cells failed to produce IFN\textgamma after a brief \textit{in vitro} stimulation with peptide (Fig. 2A). In contrast, a strong response to MHC-I allo-antigens was detected in these same mice by the endogenous T cell repertoire (Supplementary Figure S2B). So in this setting, injection of allogeneic P815.Trh4 cells did not lead to cross-priming of TEIPP T cells whereas these cells were immunogenic enough to trigger alloreactivity.

Next, we examined direct priming by tumor cells using the CRISPR/CAS9 technology to knock-out the H2-D\textsuperscript{b} gene in RMA.Trh4 cells. As control, we knocked-out the irrelevant H2-K\textsuperscript{b} gene. Gene knock-out phenotypes were verified at the protein level by flow cytometry and cells were sorted twice to obtain pure populations (Fig. 2B). Indeed, RMA.Trh4.D\textsuperscript{b−/−} cells failed to present the Trh4 epitope to a Trh4-specific T cell clone \textit{in vitro}, whereas strong T cell recognition was observed when wildtype RMA.Trh4 cells and RMA.Trh4 K\textsuperscript{b−/−} cells were tested (Fig. 2C). Importantly, when irradiated RMA.Trh4 D\textsuperscript{b−/−} or K\textsuperscript{b−/−} cells were injected in mice to study the effect on priming of naïve TEIPP T cells \textit{in vivo}, the lack of D\textsuperscript{b} molecules caused a complete loss of activation capacity, while removal of the K\textsuperscript{b} molecule did not result in decreased priming efficiency (Fig. 2D). \textit{Ex vivo} analysis of IFN\textgamma release by \textit{in vivo} activated TEIPP T cells corroborated these results (Fig. 2D). Of note, RMA.Trh4.D\textsuperscript{b−/−} and RMA.Trh4.K\textsuperscript{b−/−} cells overexpressed the
Trh4 transcript to comparable degree (Supplementary Figure S2C). Moreover, the applied in vivo model required two injections with tumor cells for clear results, but heterologous prime-boost schedules with the RMA-Trh4 panel demonstrated that the first injection was responsible for the priming event of TEIPP T cells (Supplementary Figure S2D). Together, these data show a critical role for direct priming by tumor cells of TEIPP T cells and elucidate why MHC-I low RMA-S tumor cells fail to activate this T cell specificity, leaving this subset ‘untouched’.

**TEIPP-T cells are not activated in tumor-bearing mice**

Since the thus far applied model with irradiated tumor cells does not precisely reflect the situation of tumor-bearing mice, we subcutaneously inoculated mice with progressively growing RMA-S tumors, with or without overexpression of the Trh4 antigen or the co-stimulatory molecule B7.1, and transferred naive, congenic and CFSE-labelled LnB5 T cells at the time tumors were palpable (Fig. 3A). Seven days after T cell transfer, tumor-draining lymph nodes (dLN), non-draining lymph nodes (nDLN) and tumors were removed, dispersed and analysed for the presence and activation status of the transferred TEIPP T cells. Although TEIPP T cells were still detectable in dLN and nDLN and comprised 2–4% of the CD8+ T cell population, hardly any cells infiltrated the RMA-S tumors (Fig. 3A). Moreover, we did not observe any division of the T cells nor loss of the CD62 L marker, in these mice. Since RMA-S cells are optimal targets for in vitro pre-activated LnB5 T cells, the lack of TEIPP T cell activation in RMA-S-tumor bearing mice underlined our earlier conclusion on the failure of T cell priming by this MHC-I low tumor, despite presentation of the cognate peptide-epitope.

To compare these data on LnB5tg T cells to other TCR tg CD8+ T cells in tumor models, we examined priming efficiency of pmel-1 T cells (specific for the gp100 melanocyte differentiation self-antigen) and OT-I T cells (specific for the OVA foreign antigen). Pmel-1 T cells were transferred in mice harbouring B16F10 melanomas and OT-I T cells were tested in B16F10 tumors with transgenic ovalbumin. In tumor-draining LN, a significantly increased frequency of transgenic pmel-1 T cells was observed compared with contralateral non-draining

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**Figure 1. High antigen and MHC-I levels are required for TEIPP T cell priming.** CFSE labelled TCR-transgenic TEIPP T cells were transferred to recipient mice and T cell activation and proliferation were measured after challenge with indicated irradiated tumor cells. (A) Blood samples were analysed for the presence and activation status of T cells by flow cytometry five days after the second injection of MHC-Ilow RMA-S cell lines. ‘Trh4’ indicates cells transfected with full length cDNA of the cognate antigen and ‘B7’ indicated RMA-S cells transfected with the mouse CD80 gene. IFNγ production by TEIPP T cells was measured in blood after the second injection, upon overnight stimulation with short Trh4 peptide. (B) Blood samples from mice challenged with MHC-Ihigh RMA cell lines. Means and SEM are plotted from one (RMA-S.B7, RMA-S.Trh4.B7), two (RMA and RMA-S.Trh4) or five experiments (RMA.Trh4). Student T-test compared to T cells only: *P < 0.05, **P < 0.01, ***P < 0.001.
cells were primed in the presence of a tumor, whereas the RMA-S tumor did not induce priming of naïve TEIPP T cells.

We then tested mice with RMA-S tumors that overexpressed Trh4 or B7.1. Overexpression of Trh4 in RMA-S did not enhance the frequencies or the percentage of dividing lymph node resident TEIPP T cells when compared to RMA-S tumor-bearing mice (Fig. 3D). However, the frequency of intratumoral TEIPP T cells was slightly increased when compared to RMA-S tumors. Additionally, the majority of tumor-infiltrating TEIPP T cells had proliferated and displayed an CD62Llow activated phenotype (Fig. 3D). Thus, overexpression of the Trh4 antigen mildly improved the activation and number of TEIPP T cells in tumors. Of note, MHC-I surface levels of RMA-S and RMA-S. Trh4 tumor cells were comparably low, suggesting that higher Trh4 expression can result in modestly improved priming (Supplementary Figure S1A and 1B).

Transfer of naïve TEIPP T cells in RMA-S.B7 tumor-bearing mice resulted in a slightly increased frequency of TEIPP T cells in the dLN when compared to ndLN, albeit that the percentage of dividing cells was low in both cases (Fig. 3E). Tumor-infiltration of RMA-S.B7 was heterogeneous with only half of the tumors displaying high numbers of dividing TEIPP T cells (Fig. 3E).

We concluded that TEIPP-specific T cells are not primed by MHC-Ilow RMA-S tumors and therefore fail to infiltrate these lesions. Consequently, the TEIPP T cell repertoire remains ‘untouched’ in tumor-bearing mice and might be optimally primed by immunotherapeutic strategies.

**Successful MHC-Ilow tumor-infiltration and prevention of outgrowth by RMA.Trh4-induced priming**

To study if activated TEIPP T cells could migrate to MHC-Ilow tumors, naïve mice received LnB5tg T cells and were injected twice with irradiated RMA.Trh4 to allow T cells to become activated. Mice were inoculated with RMA-S cells after the first injection of RMA.Trh4 cells (Fig. 4A). Activated TEIPP T cells strongly infiltrated MHC-Ilow RMA-S tumors, in that more than 50% of the intratumoral CD8+ T cell population represented LnB5tg cells in the majority of mice (Fig. 4B). This was in contrast to the very few tumor-infiltrating TEIPP T cells in mice receiving T cells only. Of the infiltrating TEIPP T cells in the RMA.Trh4-injected mice, all had an activated phenotype as measured by CD62 L downregulation (Fig. 4B). These results show that TEIPP T cells are capable to infiltrate MHC-Ilow tumors once they are properly activated.

Finally, we examined the efficacy of these activated TEIPP T cells to control outgrowth of tumors. Therapeutic setup of this experiment was not successful, since RMA-S tumor growth was too fast to allow for full activation of TEIPP T cells using a prime-boost scheme (Supplementary Figure S3). Therefore, a prophylactic setting was chosen in which mice with activated TEIPP T cells were challenged with RMA-S tumors. The combination of T cell transfer and *in vivo* activation by RMA.Trh4 injections resulted in a strong prevention of tumor outgrowth (Fig. 4D and Fig. 4E). More than seventy percent of the challenged mice were still alive at day 65 after tumor challenge, whereas...
naive mice or mice only receiving T cells or only RMA. Trh4 injections succumbed to tumor outgrowth (Fig. 4E).

Overall, this study showed that TEIPP-specific T cells remain naive in the presence of MHC-I\textsuperscript{low} tumors, but can efficiently be activated by cells expressing high levels of the D\textsuperscript{b}/Trh4 complex. Once activated, TEIPP T cells strongly infiltrate MHC-I\textsuperscript{low} tumors and control further outgrowth of the malignant lesions. Our data highlight the potential of TEIPP antigens and TEIPP-specific T cells to target immune-escaped tumors.

**Discussion**

There is an urgent need to target tumors with low MHC-I expression which are not responsive to conventional T-cell based immunotherapies. Natural killer cells are well known to target MHC-I\textsuperscript{low} cells,\textsuperscript{19} and NK cell transfer in cancer patients has proven to be feasible and show promising results,\textsuperscript{20,21} but have had little success in clinical trials yet. Here, we show that CD8\textsuperscript{+} TEIPP T cells, specific for TAP-independently processed, non-mutated self-antigens, can be effectively exploited.
for the treatment of these aggressive tumors. Naïve TEIPP T cells remain ‘untouched’ in tumor-bearing mice, and as a consequence do not infiltrate these tumors. Potent activation of TEIPP T cells resulted in a strong influx in these non-immunogenic tumors and, consequently, efficiently protected mice against a tumor outgrowth. Importantly, since TEIPP T cells only recognize TAP-deficient cells and remain naïve in wild-type mice, there is no risk for autoimmunity.

TEIPP antigens are unusual in their intracellular processing mechanism as they are MHC-I presented independent of the peptide transporter TAP and have to compete with TAP-mediated peptides for their loading on MHC-I in the endoplasmic reticulum. We previously described that the here studied Trh4-derived peptide-epitope is intramembraneously cleaved by the enzyme Signal Peptide Peptidase (SPP) at the C-terminus and does not require the proteasome. These unusual features of TEIPP antigens might impact the priming of the cognate CD8+ T cell repertoire in tumor-bearing mice.

Using an artificial model in which irradiated tumor cells were used to study the requirements for TEIPP T cell activation, we showed that high levels of both MHC-I and Trh4 by tumor cells were needed to induce potent T cell priming. Interestingly, this activation did not depend on the most common pathway of cross-priming via host dendritic cells but in fact required direct priming by tumor cells engineered to present high MHC-I and antigen levels (Fig. 2).

Cross-priming has been described by many studies to induce an anti-tumor T cell response, in which tumor-antigens are taken up by dendritic cells (DCs) and ‘crossed’ in the endogenous MHC-I pathway to be presented in the context of MHC-I to CD8+ T cells. Indeed, the importance of cross-priming has been described in several tumor models, including a recent study showing that CD103+ DCs in lymphnodes of mice bearing a TAP-deficient melanoma, overexpressing OVA (B78. OVA), could induce proliferation of both pmel and OT-I transgenic T cells. The lack of cross-priming as a pathway for CD8+ T cell induction in the TEIPP model could be related to the nature of the peptide. An elegant study demonstrated that signal peptides, which are small peptides liberated by the SPP enzyme, are less efficiently presented through cross-presentation by host APC, whereas efficient priming is induced through direct presentation. Such small peptide intermediates might not be suitable to picked up by dendritic cells. Notably, the effective TEIPP T cell priming could also be mediated via a process called cross-dressing, in which peptide:MHC complexes are transferred from the surface of tumor cells to professional APCs in lymphnodes, thereby inducing T cell activation. Interestingly, we showed before that TEIPP T cells can be efficiently activated in vivo upon vaccination with a
long synthetic peptide containing the Trh4 epitope, most likely via cross-priming by host DCs, suggesting that large quantities of the peptide-epitope are able to reach host DC in the animals and be loaded in the MHC-I processing pathway.\(^{11}\)

One of the important implications of the poor priming capacity of MHC-I\(^{-}\)low tumors, due to their low general levels of MHC-I and lack of co-stimulatory ligands, is the naive status of the TEIPP T cell repertoire even in tumor-bearing mice. Obviously, the T cells fail to home to infiltrate tumors and therefore not experience tumor-induced tolerance or exhaustion.\(^{28,29}\) As infiltration of T cells in the tumor is clearly one of the requirements for a good protective anti-tumor response,\(^{20}\) TEIPP T cells need to be primed and activated for optimal exploitation in immunotherapeutic strategies. The expectation is that simply blocking inhibitory receptors will not suffice for this T cell subset. Also the blocking TAP function in DCs or tumor cells by for example an oncolytic virus, might not induce a potent TEIPP T cell response due to lack of high antigen:MHC-I complexes. As mentioned, vaccination with long synthetic peptides is effective in inducing potent TEIPP T cell responses, resulting in a subsequent influx in MHC-I\(^{-}\)low tumors, which are hardly immunogenic. The surprising data in our study that MHC-I\(^{\text{high}}\) RMA.Trh4 tumor cells could efficiently prime TEIPP T cells, whereas RMA-S.Trh4 cells failed, even though Trh4 peptide:MHC-I surface levels were similar between these two cell lines,\(^{9}\) might be explained by essential non-cognate peptide/MHC interactions with the TCR.\(^{31}\) Once TEIPP T cells were efficiently activated by synthetic long peptide or engineered tumor cells, mice were capable to control MHC-I\(^{-}\)low tumors, the majority of which remaining tumor-free for more than two months (Fig. 4). However, peptide vaccination represents a much better controllable platform compared to engineered tumor cells that need to be fine-tuned and genetically expressed for each antigen.

The importance to target epitopes on MHC-I\(^{-}\)low tumors to counteract immune evasion was recently highlighted by a study in IFN\(\gamma\)-unresponsive tumors.\(^{32}\) Here, they showed that T cells specific for an IFN\(\gamma\)-independently processed epitope were potent in eradicating MHC-I\(^{-}\)low, IFN\(\gamma\)-unresponsive tumors in mice, whereas T cells targeting a conventional epitope of the same antigen requiring IFN\(\gamma\) for its presentation failed to do so.\(^{32}\) As TEIPP antigens are selectively presented on TAP-deficient cells and do not depend on IFN\(\gamma\) signalling for their presentation, TEIPPs and their cognate T cell receptors might effectively be exploited for immunotherapy of MHC-I\(^{-}\)low tumors which have escaped from conventional immunotherapies.

Materials and methods

Cell lines and mice

The tumor cell lines RMA, RMA-S (TAP2-deficient), RMA-S.B7.1 (RMA-S transfected with mouse CD80 gene), RMA-S.Trh4 and RMA.Trh4 cell lines have been described before.\(^{8,9}\) B16 and B16.OVA cells were also described before.\(^{33}\)

and RMA cells were originally derived from Klas Kärre (Karolinska Institutet, Sweden) and B16 cells were bought from ATCC (Manassas, Virginia, USA). All cells were cultured no longer than one month and regularly tested by flow cytometry for MHC class I expression. Mycoplasma testing for all cell lines was performed every 2 months by PCR. P815.Trh4 cells were generated by retroviral transduction of P815 cells with the long Trh4 transcript as previously performed.\(^{9}\) The generation and culture of TEIPP T cell clone ‘LnB5’ specific for the Trh4 derived peptide MCLRMTAVM in the context of H2-D\(^{b}\) (hereafter named D\(^{b}\)) has been previously described.\(^{8,10}\) All cells were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS (Gibco), 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin and 2 mM L-glutamine (Invitrogen) at 37\(^\circ\)C in humidified air with 5% CO\(_2\). C57 BL/6 mice were purchased from Charles River (L’Arbresle, France). OT-I TCR transgenic mice, transgenic for the OVA\(_{257–264}\)/K\(^{\beta}\)-restricted T cell receptor were derived from Jackson’s Laboratory (stock no. 003831). The pmel-1 TCR transgenic mice, containing gp100\(_{25–33}\)/D\(^{b}\)-specific T cells, were a gift from Dr. N.P. Restifo (National Cancer Institute, Bethesda, Maryland). Generation and phenotype of the LnB5 TCR transgenic mouse model has been described before.\(^{11}\) Mice were housed in individually ventilated cages and used at 6 to 12 weeks of age. All animal experiments were approved by the Central Committee Animal Experiments of the Netherlands (AVD116002015271).

Generation of RMA.Trh4 D\(^{b}\) or K\(^{\beta}\) knock-out cells using CRISPR/Cas9 system

CRISPR/CAS9 sgRNA’s targeting both D\(^{b}\) and K\(^{\beta}\) were designed using online CRISPR Design software (http://crispr.mit.edu). The sgRNA sequence (5’- AGATGTAACGGGCTCGCTCTCG-3’) was cloned into a sgRNA expression vector (Addgene 41824) using a Gibson In-fusion kit. RMA-Trh4 cells were transfected with the vector containing the sgRNA and a plasmid containing Cas9 WT (Addgene 41815), using lipofectamine 2000. Flow cytometry analysis of cells transfected with the sgRNA/CAS9WT plasmids generated both D\(^{b}\) and K\(^{\beta}\) deficient cell populations, in line with homology between the genes. From these transfected cells, D\(^{b}\) or K\(^{\beta}\)-deficient cells were FACSorted and used for further experiments.

Tumor inoculation and adoptive T cell transfer

For tumor cell inoculation, \(0.1 \times 10^6\) (B16 and B16.OVA), or \(2 \times 10^6\) cells (RMA-S, RMA-S.B7 and RMA-S.Trh4) were injected in 200 \(\mu\)l 0.1% BSA/PBS subcutaneously. After 5 days (RMA-S, RMA-S.B7 or RMA-S.Trh4) or 11 days (B16 or B16.OVA), when a palpable tumor was present, CFSE labeled T cells were injected intravenously. For T cell transfers, lymph nodes and spleen were isolated from the TCR transgenic mice and mechanically disrupted. Cells were passed through nylon wool to enrich for T cells and 3 \(\times\) \(10^6\) cells were injected in 200 \(\mu\)l PBS intravenously in recipient mice. For tumor homing experiments, cells were labeled with 5 \(\mu\)M CFSE (Invitrogen) prior to transfer. Injection of irradiated tumor cells was performed at day one and day eight after T cell transfer. These
tumor cell were harvested, washed twice with PBS and irradiated at 60 Gy. Five million irradiated cells were injected i.p. per mouse. At day eight and nine after T cell transfer, mice received 600,000 IU recombinant human IL-2 (proleukin, Novartis) intraperitoneally in 100 μl PBS. To deplete NK cells, mice were given 100 μg anti-NK1.1 antibody (PK136), intraperitoneally in 200 μl PBS, every 3–4 days. Blood was taken from mice five days after the second injection and analysed for the frequency and phenotype of transgenic T cells.

**Flow cytometry analyses**

For flow cytometry analysis, tumor-draining lymphnode (dLN) and non-draining (mesenteric) lymphnode (ndLN) were isolated and mechanically disrupted. The tumor was cut in small pieces and treated with liberase (Roche) for 15 minutes at 37°C and then put over a cell strainer. Single cell suspensions were stained in 0.1% BSA/PBS with antibodies from Biolegend specific for CD4 (clone RM4–5), CD8 (53.6–7), CD3 (145–2C11), CD62 L (MEL–14), H2-D^b (28–14–8), H2-K^b (AF6–88.5), ebioscience specific for NK1.1 (PK136) and CD90.1 (HI5). Intracellular cytokine staining was performed using the ICS kit from BioLegend according to manufactures protocol. In short, cells were permeabilized for 20 min with the permeabilization/washing buffer and thereafter stained for IFNγ (XMG1.2, Biolegend). Cells were analyzed on a FACS Calibur or Fortessa (BD) and all analysis was performed using FlowJo (Treestar).

**qPCR analysis**

Cell pellets were washed twice with PBS and snapfrozen in liquid nitrogen. RNA was isolated using the RNeasy kit (Qiagen), according to manufacturers protocol. cDNA was synthesized using the High capacity RNA-to-cDNA kit (Applied Biosystems). qPCR analysis were performed using the SybrGreen supermix (Bio-Rad) and Ct values were normalized to the expression levels of housekeeping gene GAPDH (fw primer: 5'-GTGCCTAGATGTCGTGGACTAC-3', rev: 5'GGCCGAGATGTAGACCTTTTGG -3'). For the Trh4 transcript, the common forward primer was used: 5'- GCCAAGCCCTTACTGGAAAGCTGCC-3' and reverse: 5'- CGGTGATCCCTAGACACATGCAAAGG-3'. For the splice variant, lacking an exon and therefore not encoding for the C-terminal TEIPP epitope, the reverse primer used was 5'-CTGCCGTCGATCCCTAGACACCTTTCC -3'. Data was analyzed using Bio-Rad CFX software.

**In vitro stimulations**

To verify the recognition of the RMA.Trh4 knock-out variants, 3000 cells of the LnB5 T cell clone were co-cultured overnight with the RMA.Trh4 knock-out variants, at different cell concentrations. The next day, supernatant was harvested and IFNγ was measured by ELISA as described before.11

**Statistics**

Statistical analysis was done in GraphPad Prism (version 6). The specific test is indicated in the Figure legends. P values below 0.05 were considered statistically significant.

**Author contributions**

EMD, KAM, SHVDB, and TVH developed the concept and designed experiments. EMD, MS, KAM, BJQ conducted experiments and analyzed data. EMD, SHVDB, and TVH interpreted results. EMD and TVH wrote the manuscript.

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The authors declare no potential conflicts of interest.

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