T cell immunity against MHC-Ilow tumors in mouse models

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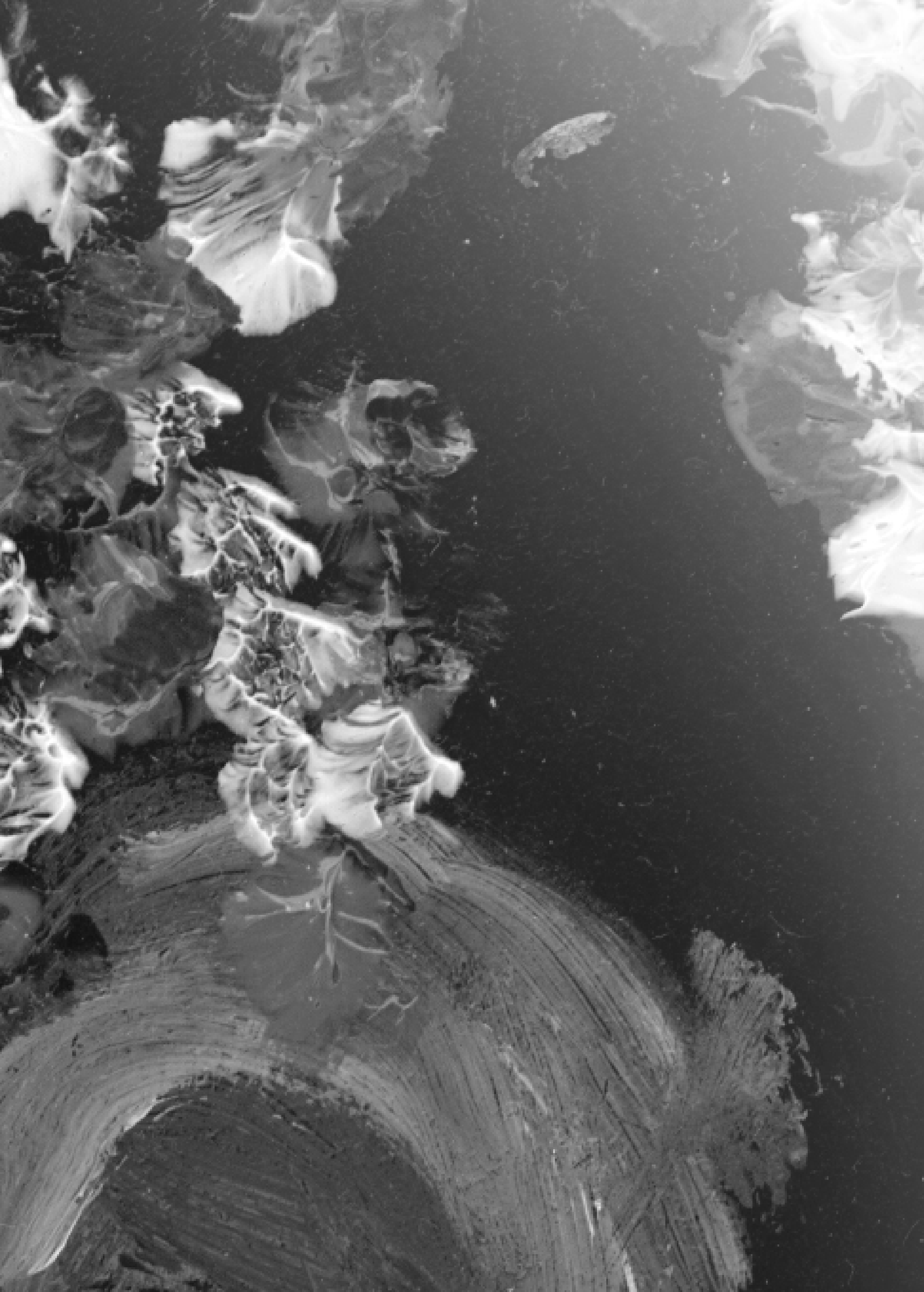

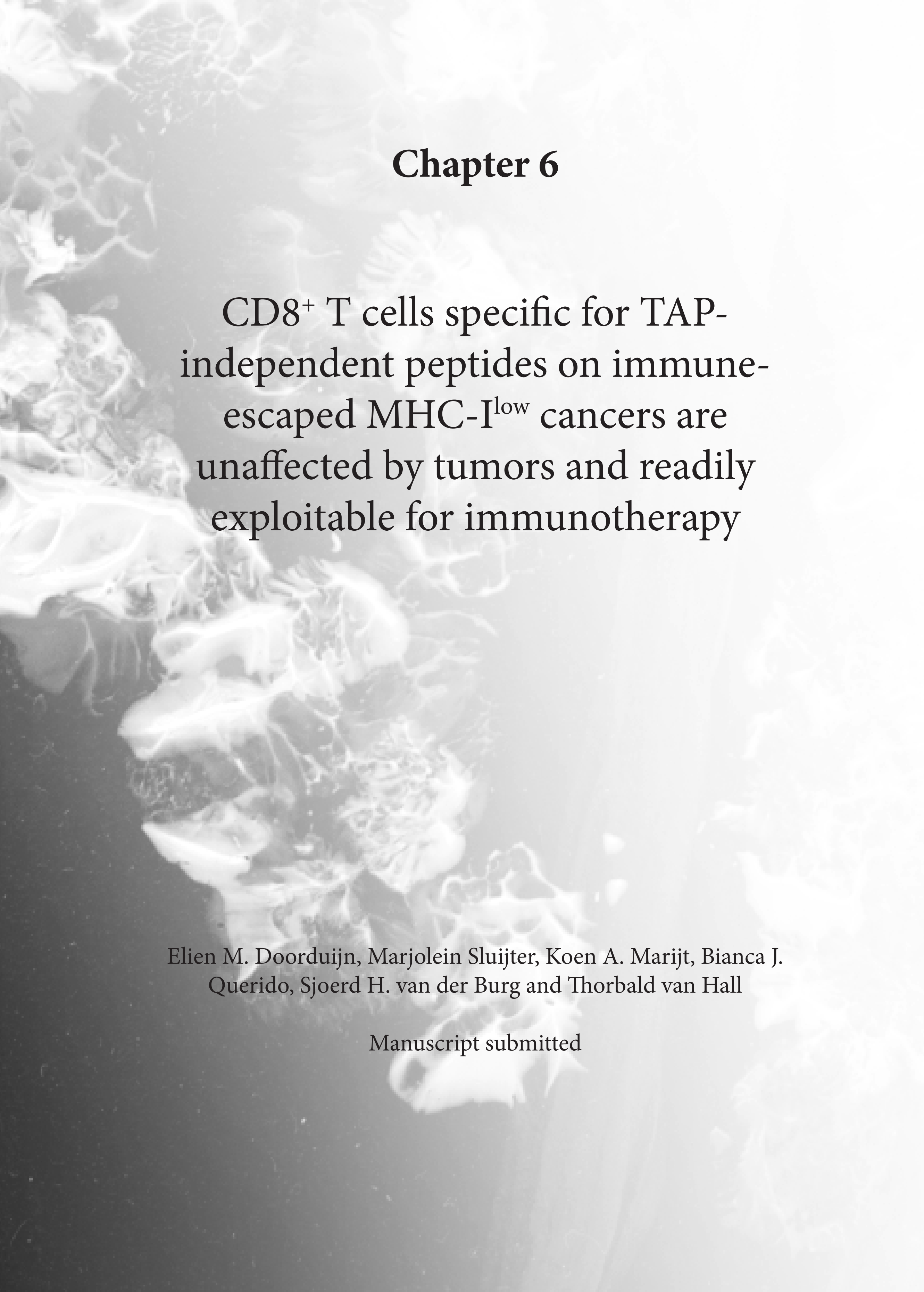

Chapter 6

CD8$^+$ T cells specific for TAP-independent peptides on immune-escaped MHC-I^{low} cancers are unaffected by tumors and readily exploitable for immunotherapy

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Abstract

Tumors can evade immune-recognition by lowering peptide:MHC-I complexes on the cell surface due to deficiencies in the peptide transporter TAP. We previously reported on a novel CD8$^+$ T cell category specifically targeting such MHC-I^{low} tumors by recognition of TAP-independent peptides, called TEIPP. In the present study, we show that TEIPP-specific T cells do not become activated nor traffic to tumors in mice bearing MHC-I^{low} tumors, in contrast to T cells specific for tumor-specific or differentiation antigens. Using a panel of irradiated tumor variants, we revealed that this unaffected state was caused by low levels of MHC-I and antigen on the tumors. Surprisingly, artificial increase of both these factors or co-stimulation resulted in potent activation of TEIPP T cells via direct interaction with tumor cells but not via cross-presentation. Complete removal of MHC-I on these artificial tumors by CRISPR/CAS9 technology abrogated T cell activation and, moreover, allogeneic cells expressing the TEIPP antigen also failed to induce a T cell response. Our data demonstrate the unaffected state of TEIPP T cells in MHC-I^{low}-tumor-bearing mice, suggesting they were not affected by tumor-induced tolerance mechanisms. Therefore, the TEIPP T cell repertoire is ready to be exploited for immunotherapeutic strategies.

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[1,2]. However, the majority of patients still succumb to their disease, highlighting the importance of studying why some patients fail to respond to therapy or relapse after initial successful treatment. 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 signalling[3,4]. This failure of tumor cells to respond to IFNγ affects levels of MHC-I as cells are impaired to induce TAP expression. Also mutations or epigenetic silencing of components of the antigen-processing machinery, such as the peptide transporter TAP lead to strong reduction of MHC-I on cancer cells[5,6]. All these alterations result in a general deficiency to present conventional tumor antigens to $CD8^+$ T cells. We study an alternative $CD8^+$ T cell category that specifically recognize 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[7,8]. TEIPP-specific T cells are therefore a potential candidate to treat immune-escaped, MHC-I^{low} tumors. The first identified mouse TEIPP was a C-terminal peptide of Trh4, a ceramide synthase spanning the ER membrane[7,9]. The protein is ubiquitously expressed in all somatic cells, but its peptide epitope is surprisingly only presented on TAP-deficient cells[8]. Antigen processing and presentation of the epitope is independent of the proteolytic enzyme complex proteasome and the TAP transporter, instead, release of the epitope depended on intramembrane cleavage by signal peptide peptidase (SPP)[9]. 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[10], most likely since the Trh4 self-peptide is only MHC-I presented in TAP-deficient cells. Upon transfer of naïve LnB5 T 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[10].

In the present study, we aimed to understand the conditions under which MHC-I^{low} tumors spontaneously can stimulate a TEIPP T cell response. We found that transferred naïve TEIPP-specific T cells hardly infiltrated MHC-I^{low} solid 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 Trh4 protein. Strikingly, this activation was operated via direct priming and not via cross-priming of TEIPP antigens. These results imply that the TEIPP-specific $CD8^+$ T cell subset remains naïve even in tumor-bearing mice harboring MHC-I^{low} tumors and that vaccination strategies might optimally exploit these immune cells for immunotherapy.

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[7,8]. B16 and B16.OVA cells were also described before[11]. P815.Trh4 cells were generated by retroviral transduction of P815 cells with the long Trh4 transcript as previously performed[8]. 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[7,9]. All cells were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Invitrogen) at 37° C in humidified air with 5% CO_2. C57BL/6 mice were purchased from Charles River (L'Arbresle, France). OT-I TCR transgenic mice, transgenic for the $OVA_{257-264}/K^b$-restricted T cell receptor were derived from Jackson's Laboratory (stock no. 003831). The pmel 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[10]. 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^b knock-out cells using CRISPR/Cas9 system

CRISPR/CAS9 sgRNA's targeting both D^b and K^b were designed using online CRISPR Design software (http://crispr.mit.edu). The sgRNA sequence (5'- AGATGTACCGGGGCTCCTCG-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^b deficient cell populations, in line with homology between the genes. From these transfected cells, D^b or K^b –deficient cells were FACS-sorted and used for further experiments.

Tumoinoculation and adoptive T cell transfer

For tumor cell inoculation, 0.1x10^6 (B16 and B16.OVA), or 2x10^6 cells (RMA-S , RMA-S.B7 and RMA.S-Trh4) were injected in 200 µl 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 3x10^6 cells were injected in 200 µl PBS intravenously in recipient mice. For tumor homing experiments, cells were labeled with 5 µM 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 ug anti-NK1.1 antibody (PK136), intraperitoneally in 200 µl PBS, every 3-4 days.

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), CD62L (MEL-14), H2-D^b (28-14-8), H2-K^b (AF6-88.5), eBioscience specific for NK1.1 (PK136) and CD90.1 (HIS51). 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 fixation buffer on ice, washed twice in 1x 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 RNAeasy kit (Qiagen), according to manufactures 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'-GTGCTGAGTATGTCGTGGAGTCTAC-3', rev: 5'GGCGGAGATGATGACCCTTTTGG -3'. For the Trh4 transcript, the common forward primer was used: 5'-GCAGACCCCTTACTGGAAGCTGCC-3' and reverse: 5'- CGGTCATCCTTAGACACATGCAAAGG-3'. For the splice variant, lacking an exon and therefore not encoding for the C-terminal TEIPP epitope, the reverse primer used was 5'-CTGCGGTCATCCTTAGACACCTTTCC -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[10].

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.

Results

Poor tumor infiltration of TEIPP-specific T cells

Previously, we showed that TEIPP-specific T cells were not activated upon transfer in naïve syngeneic mice, but strongly proliferated upon transfer in $TAP^{-/-}$ hosts[10]. To study if these $CD8^+$ T cells also become stimulated in syngeneic mice bearing a $TAP^{-/-}$ tumor, we subcutaneously inoculated the TAP2-deficient tumor RMA-S, which displays the cognate Trh4/Db complex, and transferred naïve, congenic and CFSE-labeled LnB5 TCR transgenic cells at the time that palpable tumors were present. Seven days after T cell transfer, tumor-draining lymph nodes (dLN), non-draining lymphnodes (ndLN) and tumors were removed, dispersed and analysed for the presence and activation status of the transferred T cells. TEIPP T cells were detectable in both dLN and ndLN and comprised 2-4% of the $CD8^+$ T cell population (Fig 1A). In contrast, hardly any TEIPP T cells infiltrated the tumors as less than 1% of the total intratumoral $CD8^+$ T cell population was TEIPP specific (Fig 1A). In the lymph nodes, the majority of the cells had not divided and still displayed a naïve phenotype as reflected by the high expression of CD62L. We previously showed that *in vitro* pre-activated LnB5 tg T cells do recognize RMA-S cells[10], suggesting that the lack of TEIPP T cell infiltration of RMA-S tumors was due to the fact that there was no TEIPP T cell activation in these tumor-bearing mice. In line, very few TEIPP T cells in the tumor had proliferated or displayed decreased levels of CD62L. The poor infiltration of TEIPP T cells in the tumor as well as their naïve phenotype in the dLN suggested that naïve TEIPP T cells were not spontaneously activated by a $MHC\text{-}I^{low}$ tumor that does express the cognate TEIPP antigen and can be recognized by activated LnB5 TCR tg T cells. As control TCR tg $CD8^+$ T cells, pmel-1 cells which are specific for the melanocyte differentiation self-antigen gp100 and OT-I cells responding to the foreign antigen ovalbumin were used[12,13]. Pmel-1 cells were transferred in mice harbouring B16F10 melanomas. In this model a basal frequency of around 2% naïve T cells was detected in ndLN. In the dLN this frequency was significantly higher (Fig 1B) and this coincided with a modest increase of divided T cells (Fig 1B). This suggests that pmel-1 cells were activated by the gp100 melanoma antigen in the dLN. This notion is in line with a recent study, showing that intratumoral $CD103^+$ dendritic cells (DC) take up tumor antigens in melanomas and can activate specific T cells in dLN[14]. Despite this modest priming efficiency of pmel-1 cells, we found that infiltration of activated pmel-1 cells in B16F10 tumors was very poor (Fig 1B). These data indicate that, in contrast to TEIPP antigens, melanoma differentiation antigens can lead to some priming of T cells. We expected more clear effects from foreign antigen-driven T cell activation and turned to B16.F10 tumors expressing the OVA protein. After transfer of naïve OT-I cells in B16F10. OVA tumor-bearing mice, comparable frequencies of OT-I T cells were present in LN as found in the TEIPP and gp100 models (Fig 1C). However, a much higher proportion of OT-I cells in lymph nodes had proliferated and a strikingly high proportion of intratumoral $CD8^+$ T cells of up to 70% constituted OT-I cells (Fig 1C). Moreover, the majority of intratumoral OT-I cells displayed an activated phenotype as illustrated by low levels of CD62L and had underwent multiple divisions, confirming that the foreign OVA protein from the tumor was capable to efficiently induce T cell priming and homing to the tumor. Comparable results were observed previously in RIP-OVA mice, where the OVA protein was expressed under the control of the rat insulin promoter[15]. In conclusion, compared to both the melanoma self-antigen as well as the foreign OVA antigen, the TEIPP antigen failed to stimulate CD8⁺ T cells and cause tumor infiltration. The difference in nature of the antigen, as well as the antigen and MHC-I levels on tumor cells might affect tumor-specific T cell activation.

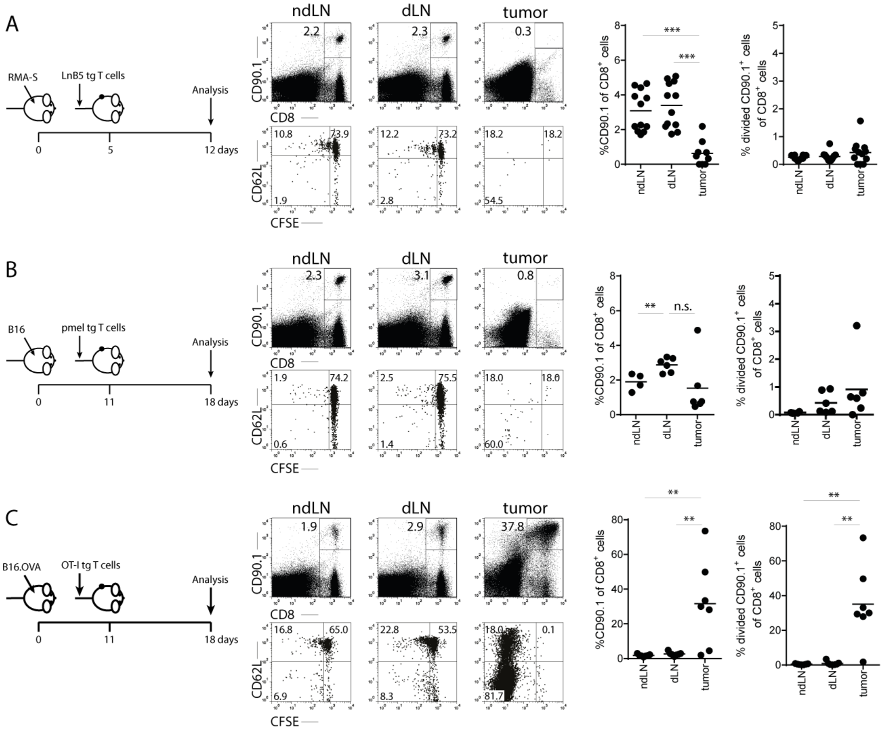


Figure 1: Tumor infiltration by tumor-specific TCR-transgenic T cells

TEIPP-specific LnB5 (A), melanoma-specific pmel (B) or OVA-specific OT-I (C) transgenic T cells (CD90.1⁺) were labelled with CFSE and transferred to (CD90.1⁻)mice bearing palpable RMA-S (A), B16F10 (B) or B16F10.OVA (C) tumors. After seven days, tumor-draining and non-draining lymph nodes (dLN and ndLN) and tumors were harvested, dispersed and analysed for the presence and phenotype of transferred T cells by flow cytometry. Representative dot plots are shown and data from 2 or 3 independent experiments with 3 mice per group were pooled. Student T-test: **P<0.01, ***P<0.001.

Infiltration of TEIPP T cells in MHC-I^{low} tumors is enhanced by increased antigen levels or co-stimulation

The poor infiltration of TEIPP-specific T cells in RMA-S tumors could be related to the low levels of Trh4 protein in these cells[8]. Although we previously showed that overexpression of Trh4 in RMA-S cells (RMA-S.Trh4 cells) only slightly enhanced T cell recognition[8], it may enhance cross-presentation and subsequent T-cell activation *in situ*. Therefore, mice were inoculated with RMA-S.Trh4 cells and naïve TEIPP-specific T cells were again tracked with a congenic marker and CFSE. The 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 2A). In contrast, the frequency of intratumoral TEIPP T cells was increased when compared to RMA-S tumors (Fig 2A versus 1A). Additionally, the majority of tumor-infiltrating TEIPP T cells had proliferated and displayed an activated phenotype (Fig 2A). Thus, overexpression of the Trh4 antigen improves the activation and number of TEIPP T cells in tumors. Importantly, RMA-S and RMA-S.Trh4 tumor cells expressed comparable low levels of MHC-I, excluding an effect of MHC-I levels as such to explain the increased TEIPP T cell infiltration (Suppl Fig 1A and 1B). Previously, we showed that TEIPP T cell recognition of RMA-S cells *in vitro* could also be improved by expression of the co-stimulatory molecule B7.1 (RMA-S.B7) [16]. Transfer of TEIPP T cells in RMA-S.B7 tumor-bearing mice resulted in an 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 2B). Tumor-infiltration of RMA-S.B7 was heterogeneous with only half of the tumors displaying high numbers of dividing TEIPP T cells (Fig 2B). Apparently, a yet unknown factor may tip the balance towards efficient T cell priming in this model.

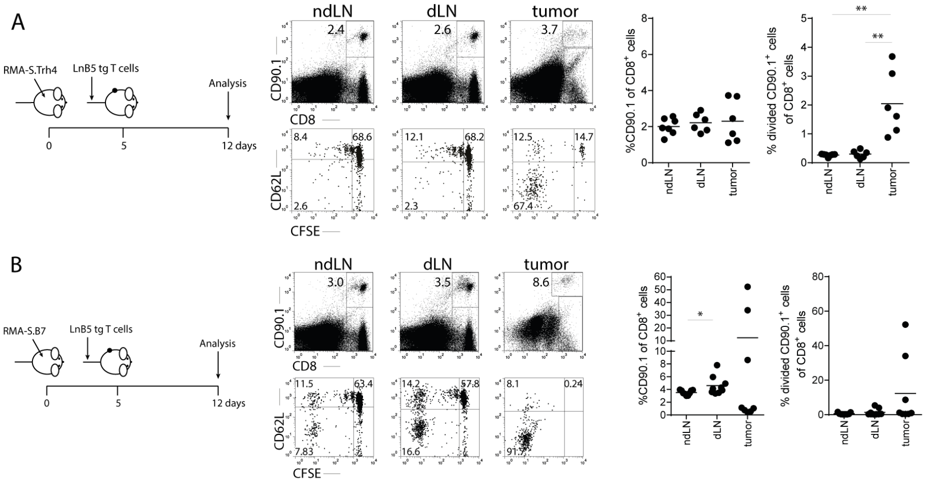


Figure 2: Addition of antigen or co-stimulation results in some TEIPP T cell activation

TEIPP-specific LnB5 transgenic T cells (CD90.1^{+}) were labelled with CFSE and transferred to mice bearing palpable RMA-S.Trh4 (A) or RMA-S.B7 (B) tumors. After seven days, tumor-draining and non-draining lymph nodes (dLN and ndLN) and tumors were harvested, dispersed and analysed for the presence and phenotype of transferred T cells by flow cytometry. Representative dot plots are shown and data from 2 or 3 independent experiments with 3 mice per group were pooled. Student T-test: *P<0.05, **P<0.01.

These findings are in line with the important co-stimulatory function of B7.1 in the activation of T cells, maybe even in the effector phase. We concluded that TEIPP-specific T cells hardly infiltrate the parental MHC-I^{low} tumors, but that their activation and intratumoral infiltration can be promoted by overexpression of the cognate antigen or a co-stimulatory molecule.

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

The previous results suggested that antigen density levels and the expression of co-stimulatory molecules are important factors for TEIPP T cell activation and expansion. To study the potential of the MHC-I^{low} tumor cells to stimulate the activation and expansion of TEIPP T cells in vivo,, we adopted a model in which non-tumor-bearing mice received naïve TEIPP T cells and were subsequently injected with irradiated tumor cells, enabling a bigger time window to follow T cell activation in blood of mice. Two injections of RMA-S.Trh4 cells or RMA-S.B7 cells were needed to convincingly increase the percentage of circulating TEIPP T cells in this system (Fig 3A and B). Since RMA-S.B7 cells are also potent targets for NK-cell mediated killing and thereby result in fast tumor cell clearance, we depleted host NK cells during tumor cell injections, but this did not overtly improve the priming efficiency (Fig 3A and B). After the second injection, there was increased TEIPP T cell activation upon challenge with tumor cells overexpressing the Trh4 antigen or the co-stimulatory molecule B7, as measured by CD62L expression or by the production of IFNγ after short *in vitro* stimulation with the Trh4 peptide (Fig 3B and C). However, the heterogeneity in responses between mice indicated that the activation of TEIPP T cells still was not optimal under these conditions.

We recently showed that vaccination with long peptides containing TEIPP antigens potently activated T cells *in vivo* by dendritic cells, suggesting that high levels of antigen and MHC-I are necessary for efficient priming[10]. The fact that RMA-S cells were generally poor in activating TEIPP T cells *in vivo* could be related to the low MHC-I levels on these cells, leading to poor TCR:MHC-I interactions that are crucial for proper T cell activation. To test if the level of MHC-I on tumor cells plays a role in TEIPP T cell activation, we made advantage of the TAP-proficient RMA.Trh4 cells, in which the Trh4 antigen was overexpressed to similar levels as in RMA-S.Trh4, but clearly express higher levels of MHC -I (Suppl Fig1). Notably, we have shown before that overexpression of the Trh4 antigen overcomes the TAP barrier for the presentation of the Trh4 epitope in MHC-I at the cell surface of normal cells[8]. Strikingly, the majority of mice displayed a strong activation and expansion of TEIPP T cells when injected with RMA.Trh4 cells (Fig 3D and E). On average, the frequency of activated TEIPP T cells in these mice was 80% out of all CD8^{+} T cells in the blood as shown by their CD62L^{low} phenotype. , showing that injection with RMA.Trh4 was very efficient in the priming of TEIPP T cells. In addition, a strong increase in the percentage of TEIPP T cells producing IFNγ after *in vitro* challenge with Trh4 peptide was observed (Fig 3F). The more homogeneous activation of TEIPP T cells by RMA.Thr4 is in sharp contrast to the the heterogenous activation found when RMA-S.Trh4 was injected and highlights the importance of the level of MHC-I Thr4 since both cell lines strongly overexpress the Trh4 antigen but differ in surface display of MHC-I (Suppl Fig 1). Notably, parental RMA cells only expressed low endogenous levels of Trh4 (Suppl Fig 1) and failed to prime TEIPP T cells (Fig 3D and E). Together, our data show that the level of MHC-I and antigen expressed at the tumor cell surface is important for the activation of TEIPP T cells.

TEIPP T cell activation is mediated by direct interaction with tumor cells

The fact that RMA.Trh4 cells, with a similar overexpression of antigen but higher levels of cell surface expressed MHC-I, displayed a surprising strong TEIPP T cell activation capacity *in vivo* prompted us to study how the TEIPP-specific T cells activation took place. TEIPP T cells may be activated via direct interaction with the RMA.Trh4 cells or indirectly via cross-priming, in which professional antigen-presenting cells ingest, process and present Trh4 antigen to naïve CD8$^+$ T cells[17,18]. To test this, we overexpressed Trh4 in P815 cells (Suppl Fig 2A), a mastocytoma cell line from a DBA/2 mouse on H-2^d background, lacking the D^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 4A and B). After the second injection, some T cell activation was measured in both groups compared to mice that only received T cells (Fig 4B). However, these CD62L^{low} T cells failed to produce IFNγ after a brief *in vitro* stimulation with peptide (Fig 4C), whereas a strong response to allogeneic MHC-I of the endogenous T cell repertoire was detected in these mice (Suppl Fig 2B). So in this setting, P815.Trh4 cells did not induce TEIPP T cell activation and apparently did not provide cross-presentation of the Trh4 epitope to TEIPP T cells.

Next, we used CRISPR/CAS9 technology to knock-out the H2-D^b molecule in the genome of RMA.Trh4 cells to analyze direct priming. As a control, we knocked-out the irrelevant H2-K^b gene. Gene knock-out phenotypes were verified at the protein level

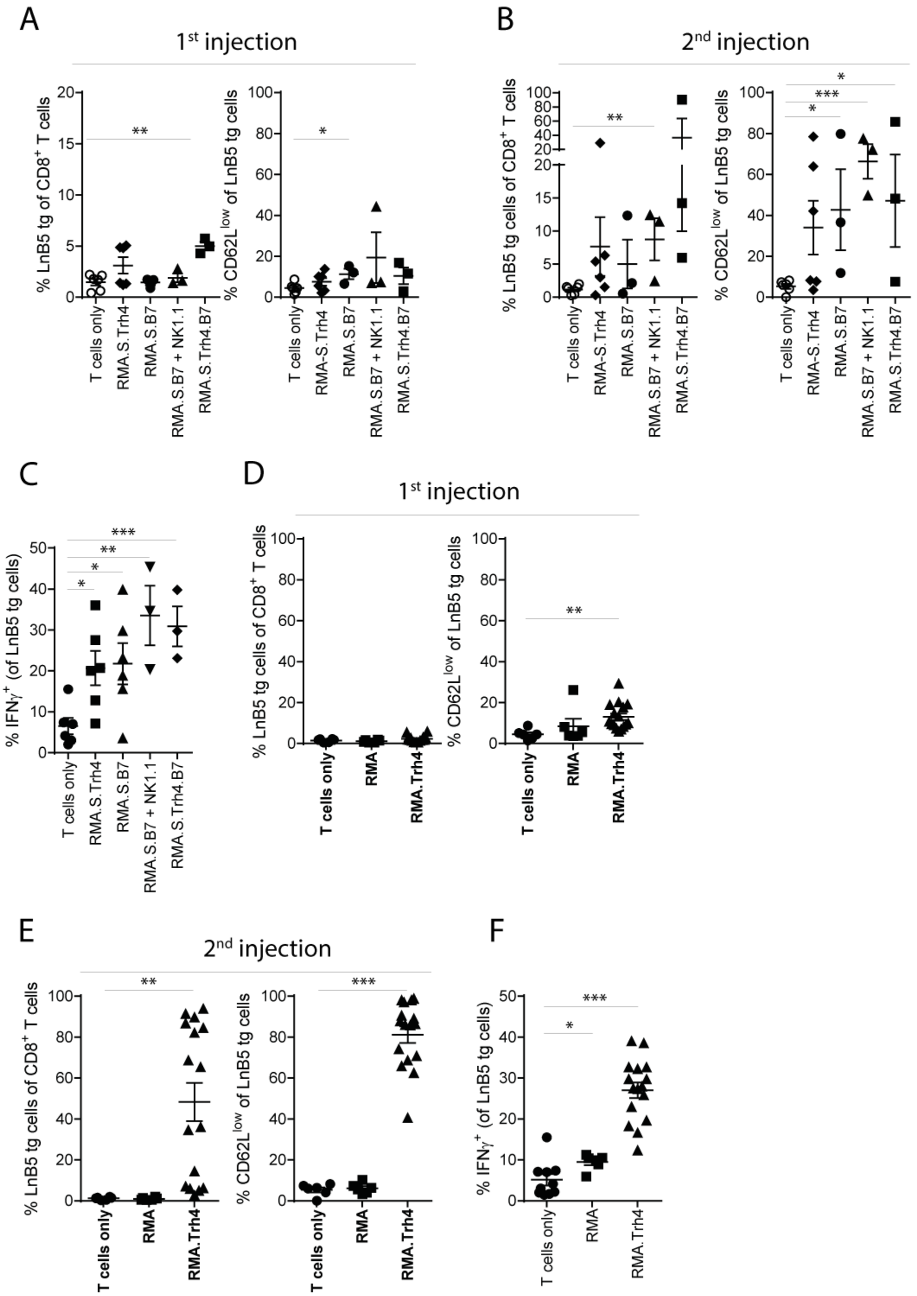


Figure 3: High antigen and MHC-I levels are required for TEIPP T cell priming
CFSE labelled TCR-transgenic TEIPP T cells were transferred to mice and activation and proliferation were measured in mice after challenge with indicated irradiated tumor cells. Blood samples were analysed by flow cytometry five days after the first (panel A and D) or second injection (panel B and E) for the presence and activation status of T cells. C and F) IFNγ production by TEIPP T cells in blood after the second injection, upon overnight stimulation with short Trh4 peptide. Data is pooled from one (RMA-S.B7, RMA-S.B7+αNK1.1, RMA-S.Trh4.B7), two (RMA and RMA-S.Trh4) or five experiments (RMA.Trh4). Student T-test: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

by flow cytometry and cells were sorted twice to obtain pure populations (Fig 4D). To validate this RMA.Trh4 cell panel, the cell variants were co-cultured with the Trh4-specific T cell clone *in vitro* and indeed only strong T cell recognition was observed for wildtype RMA.Trh4 cells and RMA.Trh4 K$^{b-/-}$ cells, whereas this recognition was completely abrogated with RMA.Trh4 D$^{b-/-}$ cells (Fig 4E). Next, irradiated RMA.Trh4 D$^{b-/-}$ or K$^{b-/-}$ cells were injected in mice to study the effect on activation of naïve TEIPP T cells *in vivo*. Strikingly, the lack of D^{b} molecules on RMA.Trh4 cells caused a complete loss of TEIPP T cell activation, while removal of the K^{b} molecule did not result in decreased priming (Fig 4F and G). *Ex vivo* analysis of IFNγ release by *in vivo* activated TEIPP T cells corroborated these results, highlighting the absolute requirement for H2-D^{b} on target cells to induce TEIPP T cell activation (Fig 4H). Of note, RMA.Trh4.D$^{b-/-}$ and RMA.Trh4.K$^{b-/-}$ cells overexpressed the Trh4 transcript to comparable degree (Suppl Fig 2C).

Finally, we wanted to exclude the possibility that the first injection with tumor cells would induce TEIPP T cell activation via cross-presentation and that the D^{b} molecule was only required for directly boosting this response. To this end, mice were first injected with RMA.Trh4.D$^{b-/-}$ cells and then challenged with the wildtype RMA.Trh4 cells. These experiments revealed that the priming of TEIPP T cells during the first injection depended on D^{b} expression by the tumor cells (Suppl Fig 2D and E).

These results firmly demonstrated that priming of TEIPP-specific T cells required direct interaction with peptide/MHC-I complexes on target cells and that direct priming, but not cross-priming, is the major pathway of T cell activation for this TEIPP response. Importantly, the fact that immune escaped cancers usually display low peptide/MHC-I complexes at their cell surface further underlines our previous notion that TEIPP T cells will mostly remain naïve and unaffected in tumor-bearing hosts.

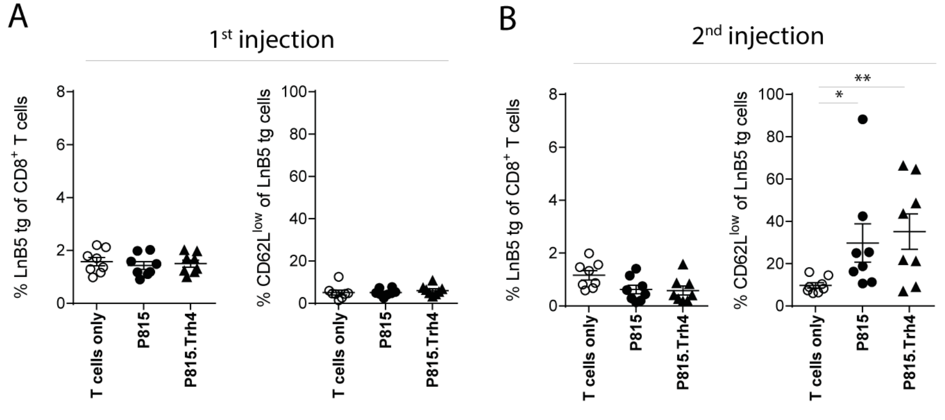
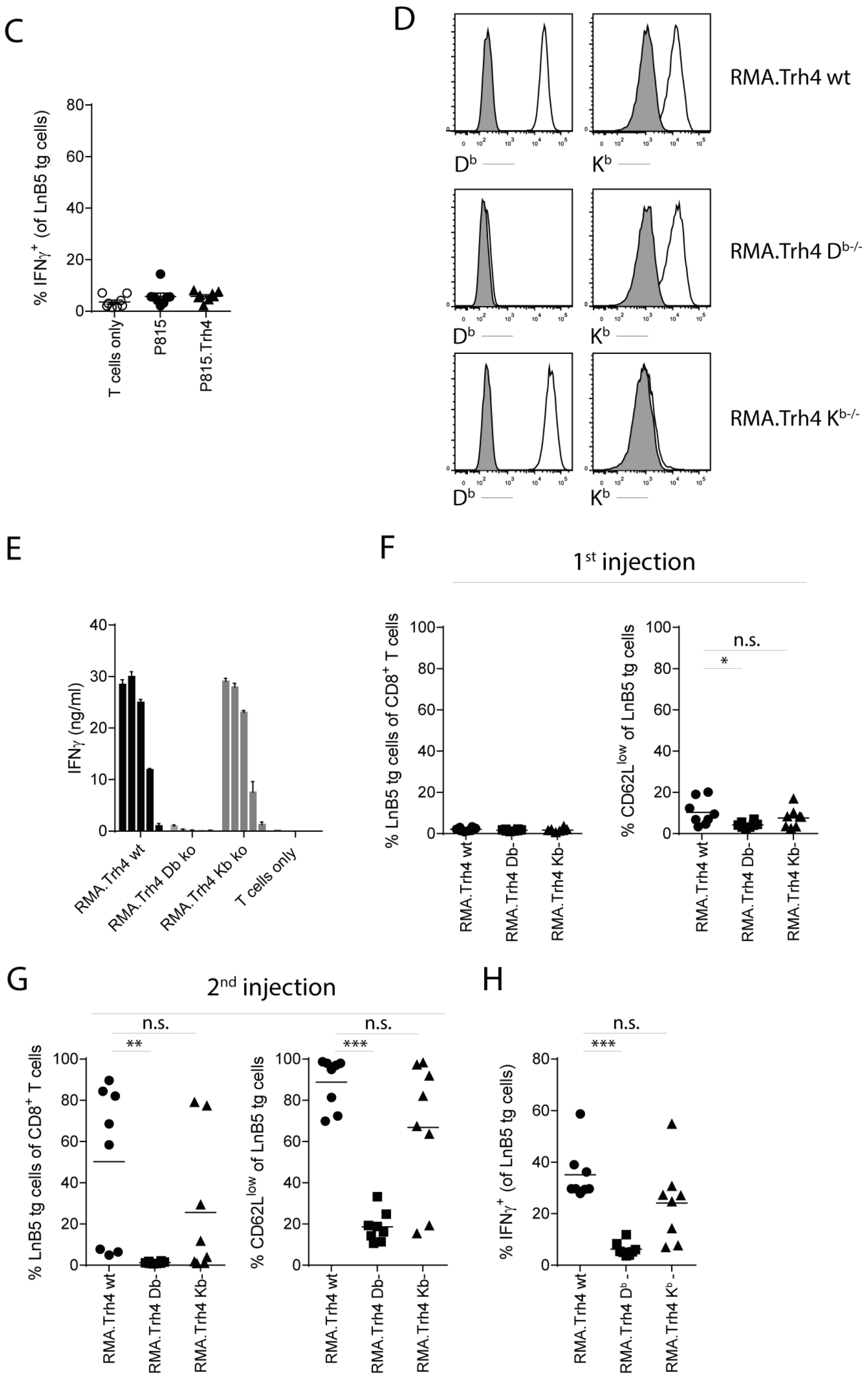


Figure 4: TEIPP T cell activation is mediated by direct interaction with tumor cells
Naïve mice received TEIPP T cells and were challenged with H-2^{d} allogeneic P815 or P815.Trh4 cells. Blood samples were analysed for the presence of transferred TEIPP T cells by flow cytometry after the first (A) or second (B, C) injection. D) Expression of H2-D^{b} and H2-K^{b} molecules on RMA.Trh4 cell panel generated by Crispr/CAS9 technology: wildtype (wt), D$^{b-/-}$ or K$^{b-/-}$ cells. E) IFNγ release by LnB5 T cell clone upon *in vitro* co-culture with the different RMA.Trh4 cells. E) Naïve TEIPP T cells were injected together with irradiated RMA.Trh4, RMA.Trh4 D$^{b-/-}$ or RMA.Trh4 K$^{b-/-}$ cells and TEIPP T cell activation was measured in blood of mice after the first (E) or second (F and G) injection. Representative data are shown from at least 2 experiments with comparable results. Student T-test: *P<0.05, **P<0.01, ***P<0.001.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% IFNγ+ (of LnB5 tg cells)
80
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20
0
RMA.Trh4 wt
RMA.Trh4 Db-
RMA.Trh4 Kb-
H
% IFNγ+ (of LnB5 tg cells)
n.s.
***
80
60
40
20
0
RMA.Trh4 wt
RMA.Trh4 Db -
RMA.Trh4 Kb -

Discussion

Here we show that naïve TEIPP T cells are poorly activated by a growing MHC-I^{low} tumor in mice and as a consequence hardly infiltrate MHC-I^{low} tumors. Overexpression of the Trh4 antigen to increase signalling via the MHC-I or expression of B7.1 to improve co-stimulation slightly enhanced the activation of TEIPP T cells. TEIPP antigens are unusual in their intracellular processing mechanism as they are independent of the peptide transporter TAP and might even have to compete with TAP-mediated peptides for their loading on MHC-I in the endoplasmic reticulum[8]. In fact, 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[9]. These unusual features of TEIPP antigens might impact the activation of the cognate CD8$^+$ T cell repertoire.

Using an artificial model in which irradiated tumor cells were used to study the activation requirements for TEIPP T cell activation, we showed that high levels of both MHC-I and Trh4 by tumor cells was required to induce potent T cell activation. Interestingly, this activation did not depend on the most common pathway of cross-priming via host dendritic cells priming but in fact requires direct priming. The absolute requirement of Trh4 presentation in MHC-I expressed by the tumor cells was shown using a cell panel generated via Crispr/CAS9 technology in which we deleted the Trh4-presenting D^b or the irrelevant K^b molecules. Additionally, TEIPP T cells did not become activated upon encounter with allogeneic P815 cells overexpressing the Trh4 transcript.

There has been quite some debate whether or not tumors can directly prime tumor-specific CD8$^+$ T cells or whether this occurs via cross-priming[19-21]. Activation of naïve T cells requires both T cell receptor signalling (signal 1) and co-stimulation (signal 2) and as tumor cells do not express co-stimulatory molecules, they are poor in direct priming of naïve T cells. In line with that, cross-priming has been described by many studies to induce an anti-tumor 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[17,18,22]. The importance of cross-priming has been described in several tumor models, including tumors transformed with the early region 1A of the human adenovirus (E1A)[23,24]. Importantly, a recent study showed CD103$^+$ DCs in lymph nodes of mice bearing TAP-deficient melanoma, overexpressing OVA (B78.OVA) could induce proliferation of both pmel and OT-I transgenic T cells[14]. Also the use of long peptides requires cross-presentation of antigens by dendritic cells to tumor-specific CD8$^+$ T cells and induce robust CD8$^+$ T cell-mediated tumor control[25,26]. Only when cells overexpress co-stimulatory molecules, direct priming of CD8$^+$ T cells can be induced, as shown in a study using an engineered antigen-presenting cell (APC), overexpressing co-stimulatory molecules, direct priming of CD8$^+$ T cells was possible and induced tumor protection[27]. This is in line with our results using mice bearing a RMA-S.B7 tumor, in which some TEIPP T cells were primed and infiltrated the tumor.

On the other hand, the importance of direct priming has been demonstrated in other tumor and virus models. Using tumor cells overexpressing the glycoprotein of the LCMV virus, inability of tumor cells to drain to the lymph node failed to induce an antigen-specific CD8$^+$ T cell response, suggesting APC as third party are required for CD8$^+$ T cell induction[28,29]. However, when H-2^b x H-2^d mice were challenged with irradiated H-2^b tumor cells, only CD8$^+$ T cell responses restricted against H-2^b antigens could be measure and not against H-2^d restricted antigens, arguing that in that model, there was no role for cross-priming[28,29]. The lack of cross-priming as a pathway for CD8$^+$ T cell induction in this 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[30]. The authors speculated that the position of the epitope in a signal sequence could result in rapid degradation of these peptides after synthesis. Alternatively, transfer of ER-located small peptides from tumor cells to DC might not be optimally facilitated. In our study, the lack of cross-priming of the Trh4 antigen could be related to the fact that TEIPP peptides are under normal conditions outcompeted by TAP-dependently processed antigens, as well as to the location of the studied peptide-epitope and the alternative proteolytic liberation from its protein context. The importance of location of an immunogenic epitope was shown in a parasite model, where the immunodominance of an epitope in generating a CD8$^+$ T cell response was correlated with its position at the C-terminal part of the protein[31]. Parasites expressing the immunodominant epitope internal, instead of at the C-terminus, failed to induce a potent CD8$^+$ T cell response. The Trh4 epitope is located at the C-terminus, but does not induce potent T cell activation unless the antigen is highly overexpressed together with high levels of MHC-I. This suggest that in our model, the levels of MHC-I are the determining factor whether or not a T cell responses is initiated, rather than the location of the peptide.

So, although the current dogma in the field of cancer immunology is that most tumor-specific CD8$^+$ T cell are primed via cross-priming of tumor antigens, we found strong evidence that TEIPP T cell priming can be mediated via direct recognition of tumor cells. Importantly, in mice bearing a MHC-I^{low} tumor, TEIPP-specific T cells had a naïve phenotype, due to the absence of sufficient MHC-I and antigen levels. These immune-escaped tumors have very low levels of MHC-I and antigen presentation and, moreover, do not express co-stimulatory ligands, and therefore will not induce TEIPP T cell activation. As TEIPP T cells remain their naïve phenotype in these tumor-bearing mice, they are a potent candidates to be used for immunotherapeutic strategies.

Infiltration of T cells in the tumor is one of the requirements for a good anti-tumor response, as shown by many studies in which the presence of intratumoral tumor-specific T cells correlates with good clinical outcome in different human tumor types[21]. Checkpoint inhibitor therapy is potent in inducing anti-tumor responses in T cell inflamed tumors[3,32]. However, since TEIPP T cells are still naïve in tumor-bearing mice, checkpoint blockade as a single modality will not work. TEIPP T cells first need to be activated and recruited to the tumor by vaccines or alternatively infused in recipients in order to induce a tumor-rejection response.

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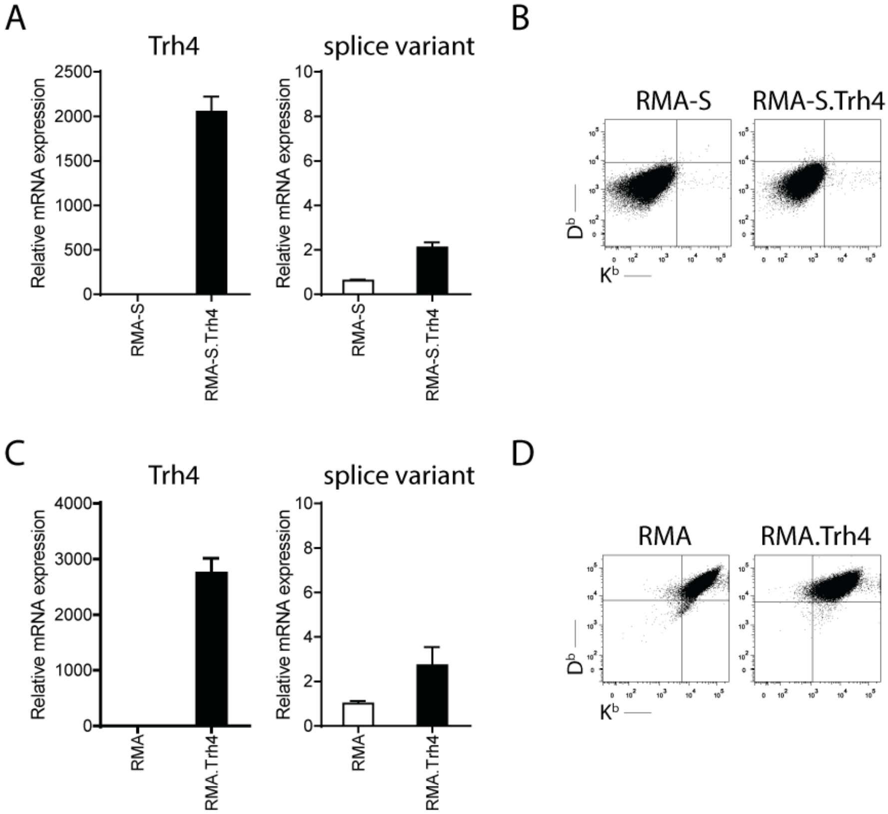
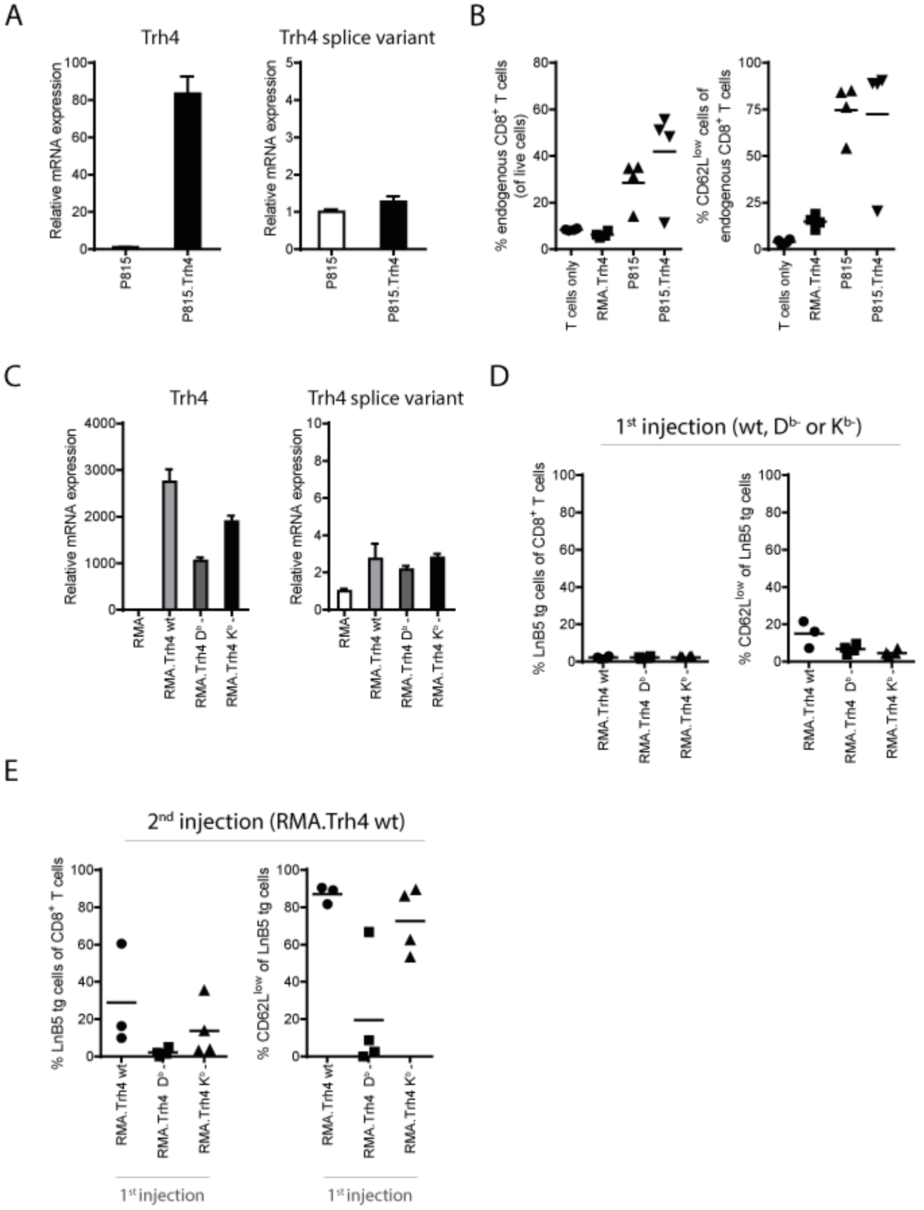
Supplemental Figure 1: Phenotype of RMA-S.Trh4 and RMA.Trh4 cells

Overexpression of the Trh4 transcript in RMA-S (A) or RMA (C) cells was analysed by RT-PCR. The splice variant lacks an exon an therefore does not encode for the C-terminal TEIPP epitope. mRNA levels are corrected to the housekeeping gene GAPDH and relative to expression levels to RMA-S (A) or RMA (C) cells. Shown is one of two experiments with comparable results, and is shown as mean with standard deviation. B and D) MHC-I expression levels was measured by flow cytometry on tumor cells. Dot plots are representative of at least two independent experiments.



Supplemental Figure 2

Expression levels of the Trh4 transcript or its splice variant in different cell lines. Expression levels are corrected for the housekeeping gene GAPDH and relative to expression in P815 (A) or RMA (B) cells. Data is shown from one of two independent experiments with comparable results and shown is mean with standard deviation. C and D) Naïve TEIPP T cells were transferred and mice were challenged with RMA.Trh4 wt, RMA.Trh4 $D^{b-/-}$ or RMA. Trh4 $K^{b-/-}$ irradiated tumor cells and as a second injection all mice were injected with RMA.Trh4 wt cells. Data from one experiment, shown as mean.



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