

# Hematopoietic progenitor kinase 1 is a critical component of prostaglandin E<sub>2</sub>-mediated suppression of the anti-tumor immune response

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**Abstract** Lung cancer is the leading cause of cancer-related mortality in the world, resulting in over a million deaths each year. Non-small cell lung cancers (NSCLCs) are characterized by a poor immunogenic response, which may be the result of immunosuppressive factors such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) present in the tumor environment. The effect of PGE<sub>2</sub> in the suppression of anti-tumor immunity and its promotion of tumor survival has been established for over three decades, but with limited mechanistic understanding. We have previously reported that PGE<sub>2</sub> activates hematopoietic progenitor kinase 1 (HPK1), a hematopoietic-specific kinase known to negatively regulate T-cell receptor signaling. Here, we report that mice genetically lacking HPK1 resist the growth of PGE<sub>2</sub>-producing Lewis lung carcinoma (LLC). The presence of tumor-infiltrating lymphocytes (TILs) and T-cell transfer into T cell-deficient mice revealed that tumor rejection is T cell mediated. Further analysis demonstrated that this may be significantly due to

the ability of *HPK1*<sup>-/-</sup> T cells to withstand PGE<sub>2</sub>-mediated suppression of T-cell proliferation, IL-2 production, and apoptosis. We conclude that PGE<sub>2</sub> utilizes HPK1 to suppress T cell-mediated anti-tumor responses.

**Keywords** T cell · Tumor immunology · Immunosuppression · Prostaglandin E<sub>2</sub> · Lung cancer

## Introduction

Tumor escape, the last phase of the cancer immunoediting model, is characterized by the emergence of somatic mutants that have acquired sufficient gain/loss of function defects, which allows these cancer cells to resist eradication by the immune system [9]. Of these evasive mechanisms, production of immunosuppressive factors by tumor cells is recognized as one of the most effective means to disarm anti-tumor immunity.

One of the characteristic aberrations found in cancers, such as non-small lung cancers (NSCLCs), is the upregulation of cyclooxygenase 2 (COX-2) [13, 15, 43], the rate-limiting

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enzyme that catalyzes the conversion of arachidonic acid into five classes of prostanoids, of which prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is the predominant by-product [6]. PGE<sub>2</sub> is a multifunctional ligand whose biological actions are determined in part by the type of E prostanoid receptors (EP) it binds [26, 27]. In many transformed cells, PGE<sub>2</sub> engagement initiates signals that block apoptosis, promote angiogenesis and stimulate tumor growth [12, 15, 16, 20, 24, 42]. Paradoxically, the binding of tumor-derived PGE<sub>2</sub> to EP receptors expressed on T cells initiates a cascade of immunoinhibitory signals that block T-cell proliferation, IL-2 production and tumor necrosis factor (TNF) secretion [8, 10, 28, 31, 35]. In addition, PGE<sub>2</sub> can inhibit T-cell functions indirectly by inducing the synthesis of immunosuppressive cytokines such as IL-10 [40]. Therefore, the ability of PGE<sub>2</sub> to promote tumor growth while suppressing the functions of immune cells makes it an ideal mechanism for cancer cells to utilize for their growth and survival.

The pro-growth properties of PGE<sub>2</sub> on epithelial cells and its anti-proliferative effects on hematopoietic cells led us to hypothesize that a hematopoietic cell-restricted negative regulator may contribute to the PGE<sub>2</sub>-mediated immune suppression. Our discovery that PGE<sub>2</sub> stimulation activated hematopoietic progenitor kinase 1 (HPK1) [34] led us to investigate whether this hematopoietic cell-restricted member of the Ste20 family of serine/threonine kinases plays a role in the PGE<sub>2</sub>-mediated suppression of the anti-tumor immune response. HPK1 was considered as a candidate because it is also a known negative regulator of T-cell receptor signaling pathways [17, 19, 38]. In this report, we assess the impact of the loss of HPK1 on PGE<sub>2</sub>-mediated functions. We use a genetic approach to study the role of HPK1 in mounting an effective immune response against a PGE<sub>2</sub>-secreting murine tumor, the Lewis lung carcinoma (LLC).

## Materials and methods

### Animals

*HPK1*<sup>-/-</sup> mice were generated as follows: a 4.7 kb genomic *Bam*HI–*Xho*I fragment covering the first three exons of HPK1 was inserted into a pMC1-neo vector, which served as the long arm of the targeting construct. The second half of exon 1 and the adjacent intron were replaced with a PGK1-neo selection cassette in antisense orientation. A 466 bp fragment starting with exon 2 was generated by PCR amplification and served as the short arm of the targeting construct. The linearized construct was electroporated into E14 embryonic stem cells. Positive clones were identified by PCR with the primers: 5'-GGG AGC CAA GAA ATT TGA GAG CTC-3' (common primer), 5'-CCG GTG GAT GTG GAA TGT GTG-3' (targeted allele) and CCC

TTC TGT CTC CTC CAC CAC (wild-type allele), and injected into C57BL/6 blastocysts. Mice lacking *RAG2* on a C57BL/6 background and wild-type controls were from Taconic (Hudson, NY).

### Antibodies, media and reagents

The following antibodies were used for T-cell stimulation and FACS staining of intracellular and cell surface markers. All were from Becton-Dickinson (BD) Pharmingen (San Jose, CA): anti-murine CD3<sub>ε</sub>, CD28, CD4-FITC, IL-2-PE, and unlabeled and biotinylated anti-murine IL-2. Annexin V-PE, 7-AAD (7-Amino-Actinomycin D), and GolgiStop were also from BD Pharmingen. Anti CD3<sub>ε</sub>, CD4, and CD8 mAbs used for immunohistological studies were purchased from Ventana Medical Systems (Tucson, AZ). Polyclonal rabbit anti-EP receptor antibodies were purchased from Cayman Chemicals (Ann Arbor, MI). Polyclonal rabbit anti-β-actin was purchased from Rockland Immunochemicals (Gilbertsville, PA). RPMI 1640 media (Cellgro, Herndon, VA), supplemented with 10% bovine calf serum (Gemini Bio-products, West Sacramento, CA), β-mercaptoethanol (50 μM) from Gibco (Carlsbad, CA), and L-glutamine (2 mM)/penicillin (100 U/mL)/streptomycin (100 μg/mL) also from Gemini Bio-Products were used as components for complete medium. Quillaja Bark Saponin was from Sigma-Aldrich (St. Louis, MO). Prostaglandin E<sub>2</sub> was from Calbiochem (San Diego, CA).

### Proliferation assay

Negatively selected, purified T cells were prepared using the Pan T-cell isolation kit from Miltenyi Biotech, Inc. (Auburn, CA). 2 × 10<sup>5</sup> T cells were seeded in a 96-well plate and incubated with various dilutions of anti-CD3<sub>ε</sub> and 0.5 μg/mL of anti-CD28 for 72 h in the presence or absence of 1 nM PGE<sub>2</sub>. Cells were pulsed with 1 μCi/well <sup>3</sup>H-thymidine (MP Biomedicals, Irvine, CA) for 18 h before harvest.

### Enzyme-linked immunosorbent assay (ELISA)

Supernatants from the proliferation assay were collected for ELISA prior to addition of <sup>3</sup>H-thymidine. Supernatants from CTL assay were collected after 18 h of culture in an E:T ratio of 40:1. Protocol used for ELISA followed BD Pharmingen instructions (for IL-2) and R&D instructions (for IFN-γ).

### Intracellular staining, apoptosis, and FACS analysis

RBC-lysed wild-type or *HPK1*<sup>-/-</sup> splenocytes were stimulated with or without 1 nM PGE<sub>2</sub> and 1 μg/mL anti-CD3<sub>ε</sub> plus 0.5 μg/mL anti-CD28 for 72 h in the presence of GolgiStop for the last 5 h of culture. Cells were then fixed

with 2% paraformaldehyde, followed by permeabilization with a buffer containing 0.1% saponin and 0.1% BSA. Cells were then incubated with PE-conjugated anti-IL-2 mAb for 30 min. IL-2 producing cells were determined by flow cytometry using FACSCalibur and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR). For measurement of apoptosis, *HPK1*<sup>-/-</sup> and wild-type T cells were stimulated as above, with or without PGE<sub>2</sub> and then stained with annexin V-PE and 7AAD after 72 h of culture.

#### LLC tumor model

Lewis lung carcinoma tumor cell line (clone 3LL) was obtained from ATCC (Manassas, VA). 3LL cells ( $5 \times 10^5$ ) were injected intravenously into the retro-orbital sinus. Mice were killed 14 days post-cell injection using carbon dioxide asphyxiation, and lung tissue was collected and fixed in 10% buffered formalin for histological analysis. In mice that received drug treatment, 2.5 mg/kg of COX-2 inhibitor II (SC-791) from Calbiochem (San Diego, CA) in PBS was administered to mice intraperitoneally three times per week for the duration of the study. Tumor burden was quantified by both the number of foci in total lung tissue and the total tumor area in the lungs; assessed by microscopic examination with a calibrated grid using Sigma Scan software (Systat Software, San Jose, CA).

#### Histology

Lung tissue was stained with hematoxylin and eosin (H&E), as well as anti-CD3<sub>e</sub>, CD4, and CD8 antibodies for immunohistochemistry analysis. Tumor burden was assessed by microscopic examination of H&E stained sections that show visible foci.

#### Immunohistochemistry

Anti CD3<sub>e</sub>, CD4, and CD8 antibodies require antigen retrieval in 0.01 M citrate of pH 6.0 for 10, 20, and 20 min, respectively. The primary antibodies were detected using biotinylated goat anti-mouse secondary antibody followed by streptavidin–biotin peroxidase conjugate. The complex containing peroxidase was visualized by the addition of diaminobenzidine (DAB). Tissue sections were counter-stained with hematoxylin and samples were mounted using “permount”, a permanent mounting media (Fisher Scientific, Pittsburgh, PA). All other reagents were from Ventana Nexus and the detection was carried out on a Ventana Nexus instrument.

#### CTL and IFN- $\gamma$ assay

3LL cells were irradiated and labeled with 200  $\mu$ Ci/mL <sup>51</sup>Cr. Splenocytes from tumor-bearing animals were

depleted of red blood cells by treating them with RBC lysis buffer (Sigma-Aldrich). Cytotoxicity against 3LL was measured at variable effector to target (E:T) ratios after 6 h of co-culture in V-bottom 96-well plates, in the presence of 10 U/mL of IL-2. Percent lysis was calculated using the following formula: (%cytotoxicity =  $100 \times E - \text{min}/\text{max} - \text{min}$ ) where “min” is the minimum (target cells alone) and “max” is maximum (100%) lysis. For IFN- $\gamma$  analysis, RBC-lysed splenocytes from 3LL-injected mice were stimulated ex vivo at a 40:1 ratio of T cells to irradiated 3LL cells for 18 h, and IFN- $\gamma$  levels were determined by ELISA according to the manufacturer’s instructions. All reagents for IFN- $\gamma$  ELISA were from R&D Systems, Inc. (Minneapolis, MN).

#### T-cell transfer

Cells were purified from the spleen and lymph nodes of *HPK1*<sup>-/-</sup> and wild-type littermates by negative selection as described above. In all experiments, T-cell purity was between 94 and 98% as assessed by anti CD3-FITC staining. Purified T cells ( $20 \times 10^6$ ) were injected intravenously into the retro-orbital sinus of *RAG2*<sup>-/-</sup> mice at the same time as the intravenous injection of 3LL (as above).

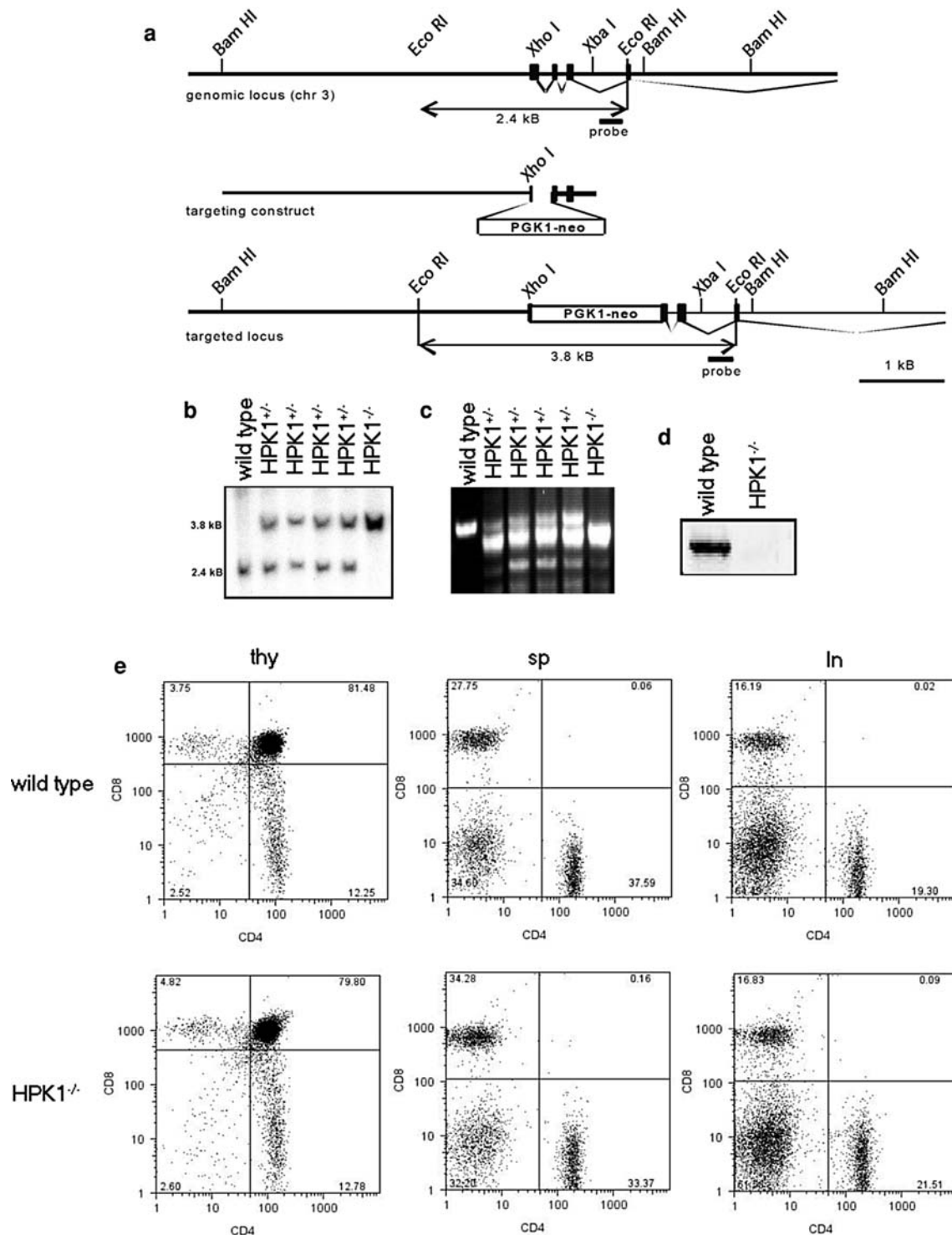
#### Statistical analysis

Error bars represent the standard error of the mean, as indicated in the figure legends. Differences were analyzed using Student’s *t* test. *P* values of <0.05 were regarded as significant.

## Results

#### Generation of HPK1 knockout mice

We have previously demonstrated that PGE<sub>2</sub> activates hematopoietic progenitor kinase 1 (HPK1) [34], a known negative regulator of T-cell receptor signaling [19, 32, 38]. We, therefore, investigated whether HPK1 may be responsible for the PGE<sub>2</sub>-induced suppression of T cell-mediated responses. To address this, we generated mice lacking HPK1 using standard homologous recombination techniques (Fig. 1a–d). *HPK1*<sup>-/-</sup> mice were healthy, reproduced with Mendelian ratios and had a normal life span (data not shown). Analysis revealed that CD4<sup>+</sup> to CD8<sup>+</sup> T-cell ratios in the thymus, lymph nodes, and spleen were normal in *HPK1*<sup>-/-</sup> mice when compared to T cells from wild-type animals (Fig. 1e). These results suggested that the lack of HPK1 does not affect T-cell development, in agreement with a recent report from Tan et al. who independently generated an *HPK1*-deficient mouse [38].



**Fig. 1** Generation of HPK1-deficient mice. **a** Gene-targeting strategy. *Top* a portion of the wild-type murine *HPK1* locus showing relevant restriction sites: *Bam*HI, *Eco*RI, *Xho*I, and *Xba*I. Exons are shown as filled rectangles and the position of the 3' flanking probe is indicated. The structure of the targeting vector (*middle*) and the mutant locus (*bottom*) are also shown. **b** PCR-based genomic analysis for *HPK1*<sup>-/-</sup> mice. Tail genomic DNA was subjected to PCR analysis using primer pairs specific for the wild-type *HPK1* allele and neo-specific primer. A 726-nucleotide fragment is expected for the wild-type allele and a

670-nucleotide fragment for the *HPK1*-disrupted allele. **c** Southern blot analysis of tail DNA isolated from wild-type, heterozygous (*HPK1*<sup>+/-</sup>), and *HPK1*<sup>-/-</sup> mice. Genomic DNA was digested with *Eco*RI and hybridized with the 3' flanking probe. A 2.4-kb fragment is expected for the wild-type allele and a 3.8-kb fragment would indicate the presence of the neomycin cassette. **d** Western blot analysis of protein lysates from spleens of wild-type and knockout mice using antibodies against HPK1. **e** CD4<sup>+</sup> and CD8<sup>+</sup> ratios in the thymus (*thy*), lymph nodes (*ln*) and spleen (*sp*) of wild-type and *HPK1*<sup>-/-</sup> mice

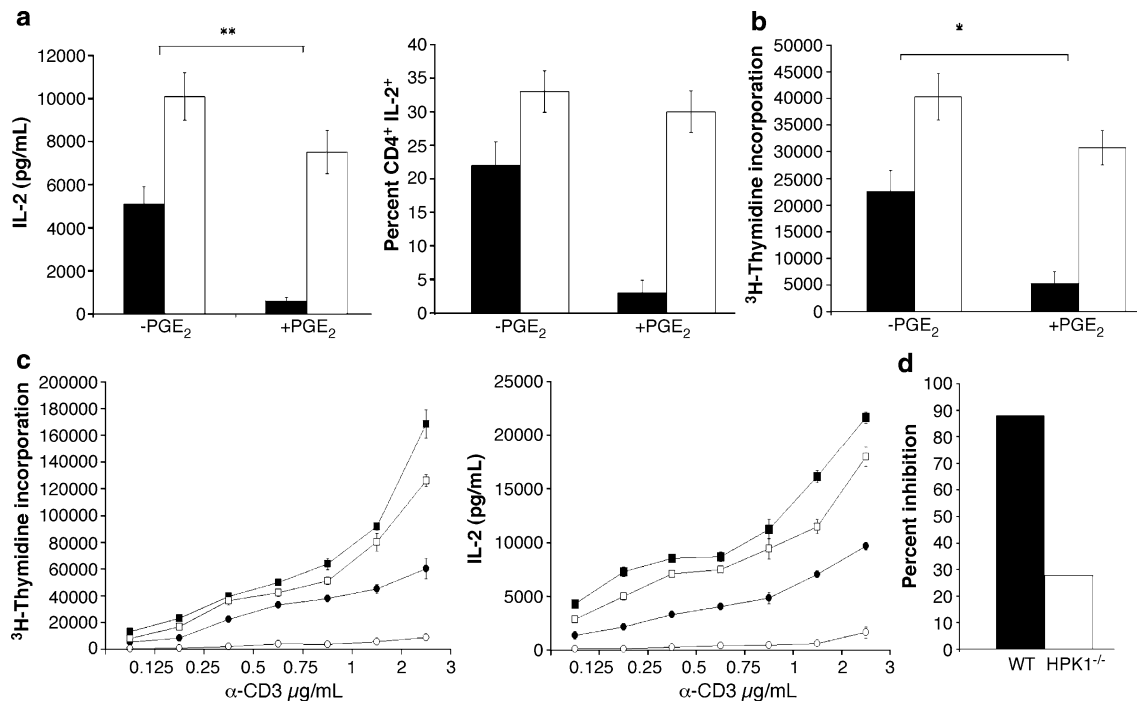
### *HPK1*<sup>-/-</sup> T cells are resistant to PGE<sub>2</sub>-mediated suppression of IL-2 production

The best-characterized effects that PGE<sub>2</sub> exerts on T cells are its negative impact on TCR-induced IL-2 production and proliferation [8, 22, 28]. Therefore, we investigated whether the absence of HPK1, a known negative regulator of TCR-induced IL-2 production [19], would alter the ability of PGE<sub>2</sub> to inhibit IL-2 production. T cells from the spleens of *HPK1*<sup>-/-</sup> or wild-type mice were co-stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs (CD3 + CD28) for 72 h, in the presence or absence of PGE<sub>2</sub>. The amount of IL-2 in the supernatants of these stimulated T cells was determined by ELISA (Fig. 2a, left panel). *HPK1*<sup>-/-</sup> T cells exposed to PGE<sub>2</sub> were markedly resistant to the inhibitory effect of PGE<sub>2</sub> on IL-2 production when compared to identically treated wild-type T cells. Specifically, *HPK1*<sup>-/-</sup> T cells stimulated with PGE<sub>2</sub> produced 26% less IL-2 than those left untreated, whereas an 88% reduction was observed in identically stimulated wild-type T cells (Fig. 2a, left panel). Anti-IL-2 intracellular staining confirmed that *HPK1*<sup>-/-</sup> T cells were more resistant to PGE<sub>2</sub>-mediated inhibition of IL-2 production

(Fig. 2a, right panel), as indicated by the 9% reduction of IL-2<sup>+</sup> CD4<sup>+</sup> *HPK1*<sup>-/-</sup> T cells, compared to a 75% reduction of IL-2<sup>+</sup> CD4<sup>+</sup> T cells from wild-type mice. Because the resistance to PGE<sub>2</sub>-mediated suppression could have been caused by the diminished expression of the prostaglandin E2 receptors (EP1–EP4) in *HPK1*<sup>-/-</sup> T cells, we performed Western blot analyses on lymphocyte whole cell lysates prepared from wild-type and *HPK1*<sup>-/-</sup> lymph nodes, using antibodies that recognize specific EP receptors. Analysis of the EP Western blot data revealed that the absence of HPK1 did not reduce the expression of the EP receptors, when the EP expression levels were compared to the amounts of proteins in the loading control lanes (see Supplemental Fig. 1). These findings support the conclusion that the lack of HPK1 renders T cells significantly resistant to PGE<sub>2</sub>-mediated inhibition of IL-2 production.

### *HPK1*<sup>-/-</sup> T cells are resistant to PGE<sub>2</sub>-mediated suppression of proliferation

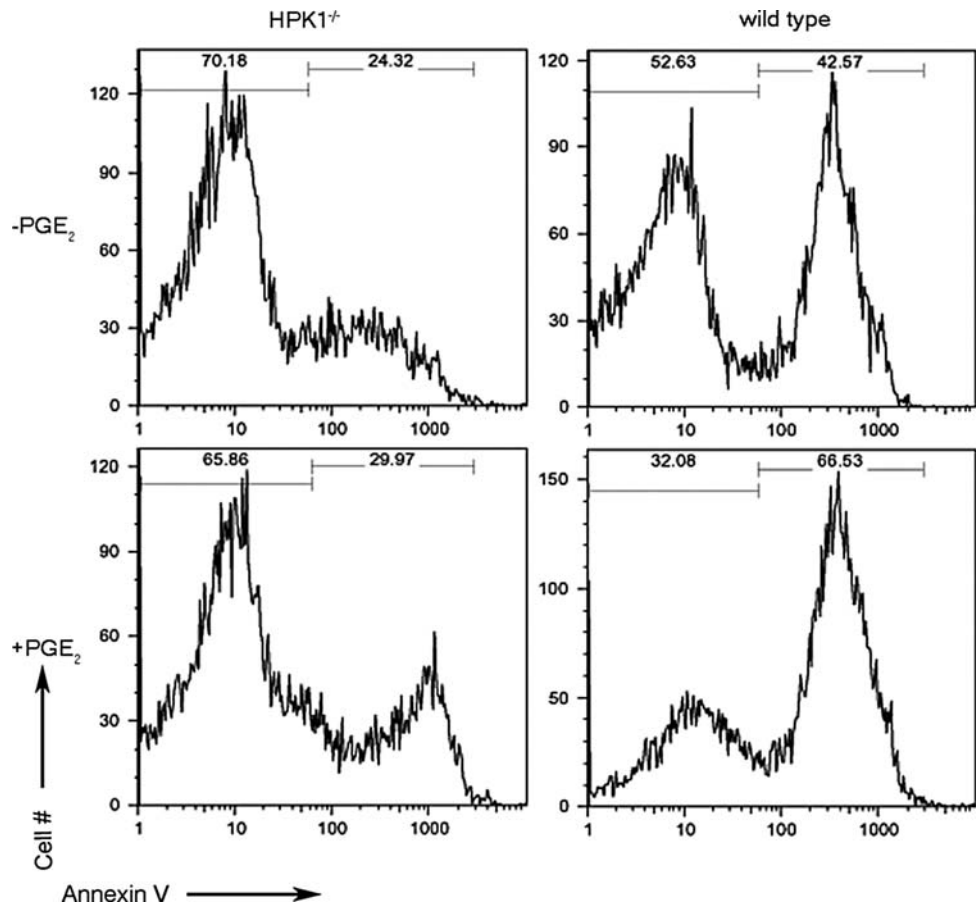
Given that PGE<sub>2</sub> stimulation also inhibits TCR-induced T-cell proliferation [22], we investigated whether the loss of HPK1 would have an impact on the anti-proliferative effect



**Fig. 2** Resistance of *HPK1*<sup>-/-</sup> T cells to PGE<sub>2</sub> inhibition of IL-2 production and proliferation. T cells stimulated with 1 μg/mL anti-CD3 and 0.5 μg/mL anti-CD28 in the presence or absence of 1 nM PGE<sub>2</sub>. **a** IL-2 levels of supernatants measured by ELISA (left panel) or intracellular staining (right panel). **b** T-cell proliferation by <sup>3</sup>H thymidine incorporation. <sup>3</sup>H thymidine was added in the last 18 h of stimulation. **c** Line graphs representing proliferation (left panel) or IL-2 levels (right panel) at varying anti-CD3 concentrations, as shown on the X-axis. Cells were stimulated in 96-well plates for 3 days with the

varying anti-CD3 concentrations, and a fixed amount of CD28 (0.5 μg/mL) in the presence or absence of 1nM PGE<sub>2</sub>. As shown, filled squares are *HPK1*<sup>-/-</sup> and filled circles are wild-type T cells. Solid symbols indicate conditions without PGE<sub>2</sub> and open symbols are with PGE<sub>2</sub>. **d** The degree of inhibition of proliferation on the addition of PGE<sub>2</sub> was compared when *HPK1*<sup>-/-</sup> or wild-type T cells produced comparable levels of IL-2 at 0.25 or 2 μg/mL of anti-CD3 used for stimulation, respectively. Error bars represent the standard error of the mean of an experiment done in triplicate (\**P* < 0.05)

**Fig. 3** Resistance to apoptosis by *HPK1*<sup>-/-</sup> T cells. *HPK1*<sup>-/-</sup> or wild-type T cells, in the absence or presence of PGE<sub>2</sub>, were stained with 7AAD and Annexin V. The 7AAD negative population, which represents living cells, was assessed for Annexin V staining



induced by PGE<sub>2</sub>. We compared the levels of <sup>3</sup>H-thymidine incorporation by wild-type and *HPK1*<sup>-/-</sup> T cells that were stimulated with CD3 + CD28 for 72 h and found that proliferation of *HPK1*<sup>-/-</sup> T cells was only inhibited by 24% in the presence of PGE<sub>2</sub> (Fig. 2b). This degree of inhibition of *HPK1*<sup>-/-</sup> T cells by PGE<sub>2</sub> was in contrast to the 77% reduction in the proliferation of wild-type T cells treated with PGE<sub>2</sub>. Since we observed an increase in the levels of proliferation and IL-2 production from *HPK1*<sup>-/-</sup> when compared to wild-type conditions in the absence of PGE<sub>2</sub>, the amount of anti-CD3 used for stimulation was titrated to address whether the resistance to PGE<sub>2</sub>-mediated inhibition may be dispensable at a lower threshold levels of stimulation. At all degrees of IL-2 production (Fig. 2c, right panel) and proliferation (Fig. 2c left panel) examined, there remained a significant resistance to the addition of PGE<sub>2</sub> in the absence of HPK1 (Fig. 2c). Furthermore, we recognized that the amount of IL-2 produced might affect the degree of proliferation. Therefore, we compared two points at which *HPK1*<sup>-/-</sup> and wild-type T cells produced similar amounts of IL-2, but at different anti-CD3 concentrations. The proliferation of *HPK1*<sup>-/-</sup> T cells at 0.25 μg/mL of anti-CD3 used for stimulation was inhibited by 28% when compared to the 88% inhibition observed by wild-type T cells that produced similar amounts of IL-2 at 2 μg/mL of anti-CD3 (Fig. 2d). Taken

together, our results confirm that the absence of HPK1 confers T cells with a significant resistance to PGE<sub>2</sub>-mediated inhibition of both proliferation and IL-2 production.

*HPK1*<sup>-/-</sup> T cells are resistant to PGE<sub>2</sub>-mediated induction of apoptosis

In addition to suppressing T-cell functions, PGE<sub>2</sub> stimulation has been shown to promote apoptosis in thymocytes and mature peripheral T cells [25, 29, 41]. Since HPK1 is known to act as a pro-apoptotic molecule in T cells [36], it is possible that *HPK1*<sup>-/-</sup> T cells are less susceptible to apoptosis-inducing stimuli, such as PGE<sub>2</sub>. Indeed, in the absence of HPK1, T cells stimulated by CD3 + CD28 for 72 h underwent less apoptosis as evidenced by Annexin V staining when compared to identically treated wild-type T cells (Fig. 3). Not only are *HPK1*<sup>-/-</sup> T cells resistant to TCR-induced apoptosis, but they are also resistant to apoptosis triggered by PGE<sub>2</sub>.

Prevention of Lewis lung carcinoma development by the ablation of HPK1

The ability of *HPK1*<sup>-/-</sup> T cells to resist the immunosuppressive and pro-apoptotic action of PGE<sub>2</sub> suggests that

*HPK1*<sup>-/-</sup> tumor-infiltrating lymphocytes (TILs) may continue to carry out their effector function in a PGE<sub>2</sub>-rich tumor microenvironment. The PGE<sub>2</sub>-producing Lewis lung carcinoma cell line possesses immunosuppressive properties and is widely used as a murine model of lung cancer with a strong T-cell involvement [23, 37, 39]. To test whether the loss of HPK1 would result in a resistance to the growth of 3LL, we injected 5 × 10<sup>5</sup> 3LL cells intravenously into wild-type or *HPK1*<sup>-/-</sup> mice. The presence of tumor foci in the lungs was assessed 14 days after 3LL injection by hematoxylin and eosin (H&E) staining. No foci were detected in 75% of the *HPK1*<sup>-/-</sup> mice examined, and the remaining 25% only developed one or two tumor foci compared to an average of 22 foci in wild-type mice (Fig. 4a, b). Furthermore, when compared to wild-type controls, the size of individual foci in the lungs of *HPK1*<sup>-/-</sup> mice was significantly smaller. Immunohistochemistry revealed that CD3<sup>+</sup> lymphocytic infiltrates were present in those solitary tumor foci, whereas none was detected in the foci of wild-type mice (Fig. 5a). Further analysis revealed both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltrates. When PGE<sub>2</sub> production was blocked in wild-type mice by intraperitoneal administration of a COX-2 inhibitor (SC-791), the number of foci was markedly reduced compared to that from untreated wild-type mice (Fig. 4a). Furthermore, these foci were now infiltrated by lymphocytes (Fig. 5a), in agreement with prior reports [39]. COX-2 inhibitors reduced the number of lung foci, but not as much as the reduction in foci seen in the *HPK1*<sup>-/-</sup> mice. These results suggest that besides resistance to PGE<sub>2</sub>, removing HPK1 results in an enhanced anti-tumor response by these T cells. This interpretation would be consistent with the enhanced T-cell response seen in the *HPK1*<sup>-/-</sup> mice, reported by Tan et al. Taken together, our results indicate that *HPK1*<sup>-/-</sup> mice have a robust anti-tumor response against PGE<sub>2</sub> producing 3LL that may consist of an enhanced T-cell response to the tumor, as well as a resistance to PGE<sub>2</sub> suppression.

#### T cell-dependent resistance of *HPK1*<sup>-/-</sup> mice to tumor growth

T-cell infiltration in tumors that developed in HPK1-deficient mice implicates T cells in their resistance to the growth of Lewis lung carcinoma. To compare the anti-tumor T-cell response of wild-type and *HPK1*<sup>-/-</sup> mice, splenocytes were isolated from mice that received 3LL cells 14 days earlier, and their ability to lyse 3LL cells was determined ex vivo by a cytotoxicity assay. When *HPK1*<sup>-/-</sup> splenocytes were co-cultured with irradiated 3LL cells at varying effectors to targets (E:T) ratios, they were five times more effective in killing 3LL cells than wild-type splenocytes (Fig. 5b). Furthermore, when supernatants from this assay were collected and analyzed for the production of

IFN- $\gamma$  by ELISA, significantly more IFN- $\gamma$  was detected in the supernatants collected from *HPK1*<sup>-/-</sup> T-cell co-cultures versus those from wild-type T cells (Fig. 5b).

To verify the role of *HPK1*<sup>-/-</sup> T cells in the rejection of 3LL cells, we purified and intravenously transferred wild-type or *HPK1*<sup>-/-</sup> T cells along with 3LL cells into T cell-deficient, *RAG2*<sup>-/-</sup> hosts. After 14 days, there was a profound reduction in the number and size of tumor foci in the lungs of mice that received *HPK1*<sup>-/-</sup> T cells when compared to the number and size of tumors from mice that received wild-type T cells or to controls that received no T cells (Fig. 5c). These findings confirm that the resistance to 3LL tumor development in *HPK1*<sup>-/-</sup> mice is T cell-dependent.

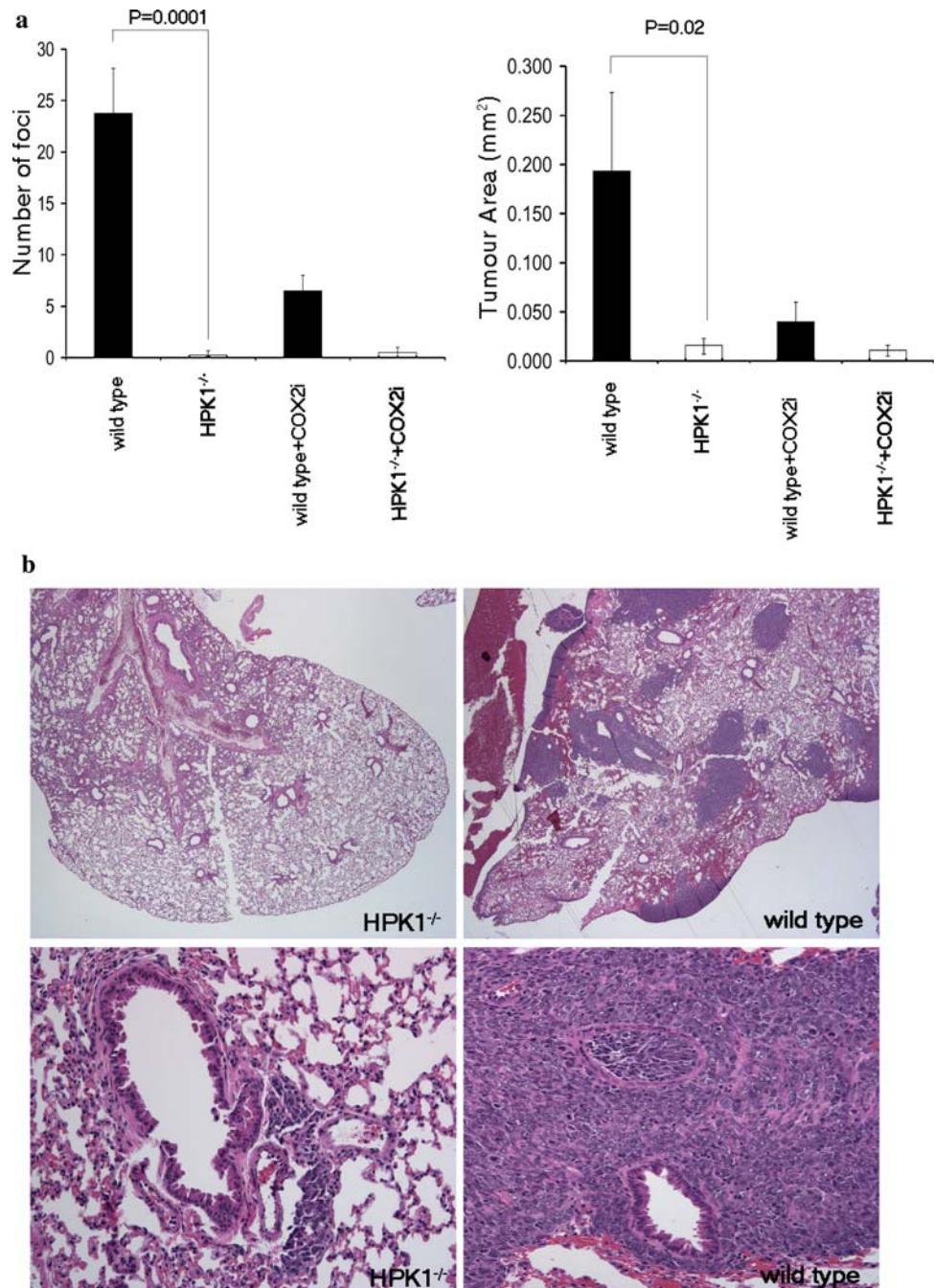
## Discussion

The primary objective of cancer immunotherapy is to manipulate a non-responsive immune system to effectively participate in tumor rejection. Our finding that the lack of *HPK1* resulted in an efficient rejection of Lewis lung carcinoma suggests that HPK1 is involved in the control of anti-tumor immune responses. The involvement of HPK1 in the control of anti-tumor activity is consistent with its role as a negative regulator of TCR-induced signals [19, 38]. Similar enhanced anti-tumor responses in mice that are deficient in known negative regulators have been documented. For instance, the loss of the ubiquitin ligase activity of Cbl-b correlates with an enhanced ability of *Cbl-b*<sup>-/-</sup> mice to reject transplanted, as well as spontaneously arising, tumors [7, 21]. Administering neutralizing mAbs capable of blocking negative signaling pathways downstream of CTLA-4 and PD-1 also resulted in enhanced anti-tumor activity [2, 11, 14, 18, 30]. Because HPK1 and the aforementioned negative regulators may utilize unique signaling mechanisms to inhibit T-cell activation, they represent possible targets for the development of cancer therapies.

Our initial observation that the kinase activity of HPK1 is responsive to PGE<sub>2</sub> stimulation led us to propose that HPK1 plays a functional role downstream of PGE<sub>2</sub>-induced signals. Through genetic ablation of HPK1, we have generated mice whose hematopoietic cells are resistant to immunosuppression mediated by PGE<sub>2</sub> stimulation, but whose T cells also demonstrate enhanced functions, such as proliferation, IL-2 production, and cytotoxicity. In aggregate, the resistance to PGE<sub>2</sub>, in conjunction with the resistance to apoptosis and the increased T-cell responsiveness to TCR-generated signal, may contribute toward the enhanced anti-tumor activity observed in *HPK1*<sup>-/-</sup> mice.

Lewis lung carcinoma is a PGE<sub>2</sub>-dependent murine model of lung cancer. The importance of PGE<sub>2</sub> in this model system has been demonstrated through the use of COX-2 inhibitors and anti-PGE<sub>2</sub> neutralizing antibodies,

**Fig. 4** *HPK1*<sup>-/-</sup> mice are resistant to the growth of Lewis lung carcinoma. 3LL cells were injected intravenously into *HPK1*<sup>-/-</sup> or wild-type mice, in the presence or absence of a COX-2 inhibitor (COX2i), and tumor burden in the lungs was assessed after 14 days. **a** Total number of lung foci and total tumor area in the lungs with H&E staining. White and black bars represent *HPK1*<sup>-/-</sup> and wild-type conditions, respectively. **b** With H&E staining, a 20× magnification (top panels) of a single lobe from the lungs of *HPK1*<sup>-/-</sup> or wild-type mice, respectively. A single tumor focus of 60× magnification from *HPK1*<sup>-/-</sup> or wild-type lungs (bottom panels). *P* values shown represent the standard error of the mean of an experiment (*n* = 6)



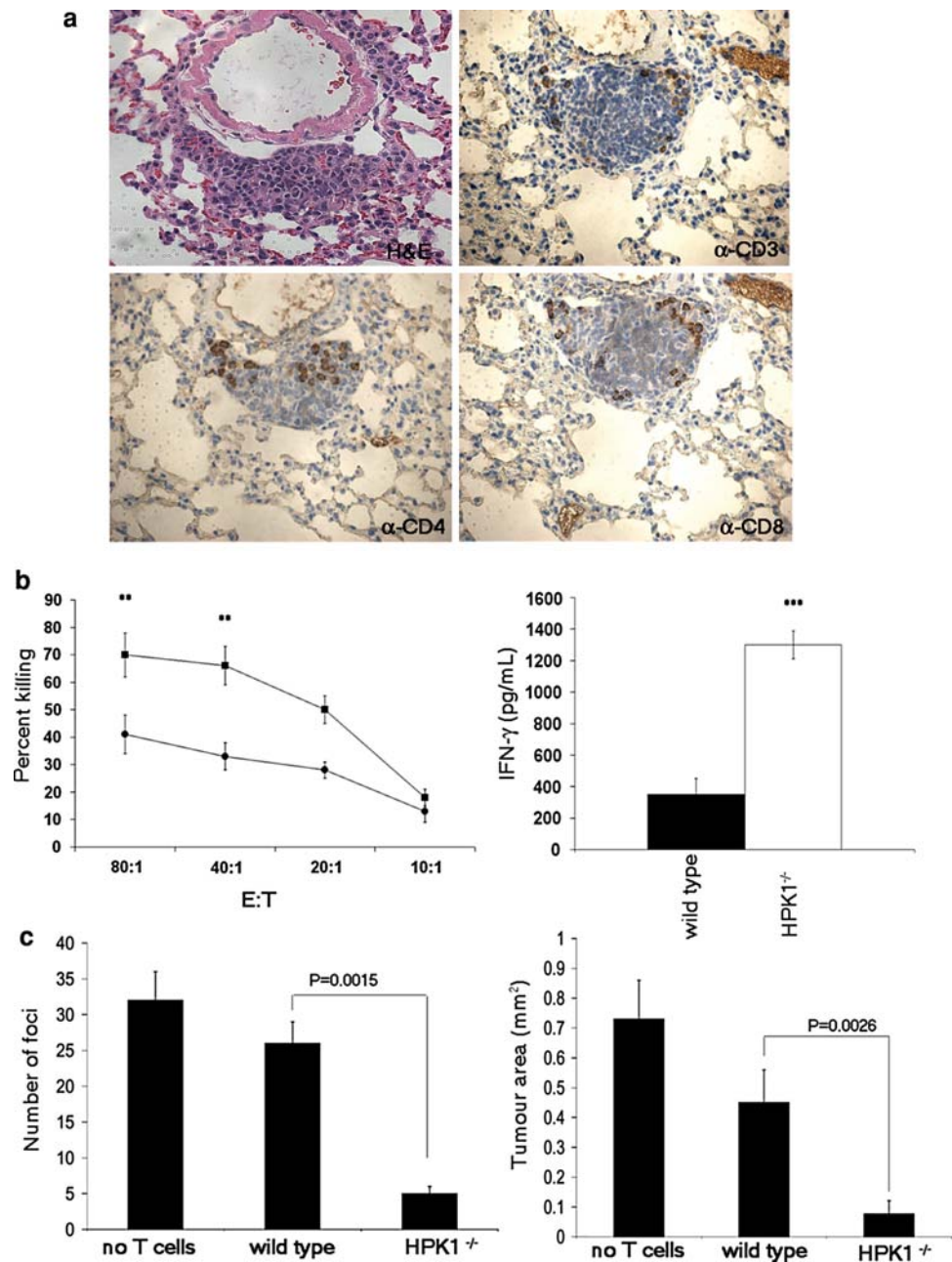
resulting in an increased anti-tumor activity and subsequent tumor rejection by PGE<sub>2</sub>-manipulated mice [39]. The inability of COX-2 inhibitor-treated wild-type mice to eradicate 3LL tumors as efficiently as *HPK1*<sup>-/-</sup> mice does suggest that an HPK1-regulated, PGE<sub>2</sub>-independent anti-tumor mechanism(s) may also exist. Our results here demonstrate that *HPK1*<sup>-/-</sup> T cells are also more resistant to PGE<sub>2</sub>-induced apoptosis, a finding that is consistent with the existing data that implicates HPK1 as a pro-apoptotic molecule in TCR signaling pathways [3–5]. Not only did we

observe resistance to PGE<sub>2</sub>-induced apoptosis in *HPK1*<sup>-/-</sup> T cells, but fewer Annexin V<sup>+</sup> T cells were found in CD3 + CD28 expanded *HPK1*<sup>-/-</sup> cultures in the absence of PGE<sub>2</sub>. These findings strengthened the conclusion that HPK1 functions as a pro-apoptotic molecule downstream of both the TCR and the EP receptors.

The concept that HPK1 function is regulated by both PGE<sub>2</sub> and the TCR pathways is consistent with our finding that TCR and PGE<sub>2</sub> stimulation utilize distinct signal transduction mechanisms to activate HPK1 [33]. Protein



**Fig. 5** Enhanced anti-tumor T-cell response in *HPK1*<sup>-/-</sup> mice. **a** 40× magnification of H&E staining with superimposed IHC of anti-CD3, CD4, and CD8 staining, respectively, of a tumor focus from *HPK1*<sup>-/-</sup> lungs. **b** Varying ratios of splenocytes, from 3LL-injected *HPK1*<sup>-/-</sup> (filled squares) or wild-type (filled circles) mice were co-cultured with irradiated, <sup>51</sup>Cr-labeled 3LL cells. Irradiated 3LL cells were co-cultured with splenocytes from tumor-bearing *HPK1*<sup>-/-</sup> (white bar) or wild-type (black bar) mice, and supernatants were collected for IFN- $\gamma$  ELISA ( $n = 3$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). **c** Purified T cells from wild-type or *HPK1*<sup>-/-</sup> mice were injected intravenously with 3LL cells into *RAG2*<sup>-/-</sup> mice. The number of tumor lung foci and the tumor area was assessed as in Fig. 4 ( $n = 5$ ). Error bars in all experiments represent the standard error of the mean of an experiment



tyrosine kinase-dependent HPK1 activation via TCR engagement also requires the interaction between HPK1 and Src homology domain-containing adapter proteins as well as PKD activation [1]. This signaling mechanism contrasts dramatically with the G-protein-coupled EP receptor signaling pathway used by PGE<sub>2</sub> stimulation to activate HPK1. PGE<sub>2</sub>-induced HPK1 activity requires PKA activation and does not need protein tyrosine kinase activation or adapter protein interactions with HPK1 [33]. Taken together, these data suggest that both TCR and EP receptors may activate HPK1 via different signaling

mechanisms to achieve the same goal. Therefore, the role of HPK1 in T-cell-mediated signaling events in response to PGE<sub>2</sub>-producing tumors can be clearly differentiated from its role as a negative regulator of TCR-mediated events.

In conclusion, understanding the mechanism whereby HPK1 is regulated by, and, in turn, regulates PGE<sub>2</sub> signal transduction pathways could provide a novel approach that will facilitate the development of HPK1-based immunotherapy for cancer and perhaps inflammatory diseases that also utilize PGE<sub>2</sub>.

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