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HPK1 Influences Regulatory T Cell Functions

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ABSTRACT

Hematopoietic progenitor kinase 1 (HPK1) is a negative regulator of TCR-initiated signal transduction. Both the HPK1−/− mice and the genetically engineered mice with a point mutation that disrupts the catalytic activity of HPK1 possess enhanced antitumor immunity, especially when these mice are treated with anti–PD-L1 immune checkpoint Ab. Because CD4+Foxp3+ regulatory T cells (Tregs) play an important role in suppressing tumor immunity, we investigated whether the loss of HPK1 expression could result in the reduction of Treg functions. We found that the number of HPK1−/− Tregs is elevated relative to the number found in wild-type C57/BL6 mice. However, HPK1−/− Tregs lack the ability to carry out effective inhibition of TCR-induced proliferative responses by effector T cells. Furthermore, HPK1−/− Tregs respond to TCR engagement with an elevated and sustained Erk MAPK and p65/RelA NF-κB phosphorylation in comparison with wild-type Tregs. Also, a multiplex cytokine analysis of HPK1−/− Tregs revealed that they demonstrate an aberrant cytokine expression profile when stimulated by anti-CD3e and anti-CD28 crosslinking, including the uncharacteristic expression of IL-2 and antitumor proinflammatory cytokines and chemokines such as IFN-γ, CCL3, and CCL4. The aberrant HPK1−/− phenotype observed in these studies suggests that HPK1 may play an important role in maintaining Treg functions with wider implications for HPK1 as a novel immunotherapeutic target.


INTRODUCTION

Hematopoietic progenitor kinase 1 (HPK1) is a hematopoietic cell–restricted member of the Ste20 family of protein serine/threonine kinases (1, 2). HPK1 becomes catalytically active in response to Ab-mediated TCR crosslinking (3, 4) and functions as a negative regulator of TCR-generated signals (3, 5). Cell-biological and -biochemical studies suggest that HPK1 dampens TCR signal transduction by binding to (6) and phosphorylating serine residue 376 of SLP-76 (7), which in turn induces phosphorylation-dependent binding of SLP-76 by 14-3-3 (7) and the subsequent ubiquitin-induced, proteasome-mediated degradation of SLP-76 (8). Consistent with the known TCR pathways that are regulated by SLP-76 (9), upon TCR engagement, HPK1-deficient T cells had elevated Erk MAPK activation and AP-1– and NFAT-mediated gene transcription (3, 6), which leads to elevated levels of IL-2 and other T helper cytokines (10, 11).

Recent genetic studies revealed that mice lacking HPK1 (HPK1−/−), or those that harbor a catalytically inactivating point mutation (K46E or K46M), are more resistant to tumor growth in four different murine tumor models: the 3LL Lewis lung carcinoma, GL261 glioma, 1956 sarcoma, and the MC38 adenocarcinoma models (11–13). The ability of K46E mice to modestly resist MC38 tumor growth suggests that the kinase activity of HPK1 is at least partially responsible for the enhanced immune response against MC38-mediated tumor growth (12). Interestingly,
this modest resistance could be markedly augmented when the K46E mice were treated an anti–PD-L1 mAb, an immune checkpoint inhibitor (12).

CD4⁢⁺FOX⁢P³ regulatory T cells (Tregs) play an important role in maintaining immune homeostasis by enforcing peripheral tolerance to safeguard the organism from aberrant immune activation, which could lead to autoimmunity, tissue damage, and chronic inflammation (14–16). In the context of cancer immunity, it had been shown in murine tumor models and in cancer patients with a wide variety of tumor types, including melanoma, ovarian cancer, head and neck squamous cell carcinoma, and colorectal cancer, that Tregs suppress antitumor immune responses (17–24). The above studies have shown that the presence of high Tregs to effector T cells (Teffs) ratios (Treg/Teff) in tumor-infiltrating lymphocytes is a prognostic indicator of poor disease outcome in mouse tumor models and in cancer patients. Given the importance of Tregs in dampening the antitumor immunity, we investigated whether the loss of HPK1 might directly impact the presence of Tregs as well as possibly disrupt normal Treg functions.

MATERIALS AND METHODS

Abs

Anti–phospho-p44/42 MAPK (Erk1/2) (T202/Y204) Ab no. 9101, anti–phospho-SAPK/JNK (Erk1/2) (T183/Y185) (81E11) mAb no. 4668, anti–phospho–NF-κB p65/RelA (S536) (93H1), anti-p44/42 MAPK (Erk1/2), anti-SAPK/JNK no. 9252, anti–NF-κB p65 no. 8242, and anti–HPK1 pAb no. 4472 were purchased from Cell Signaling Technologies. Anti-murine Foxp3 mAb (LS14577382) and HRP-conjugated anti-mouse IgG (LSG21040) were purchased from Invitrogen. The anti-murine CD3ε (145.2C11) and the HRP-conjugated anti-murine β-actin mAb (BA3R) were purchased from Thermo Fisher Scientific. HRP-conjugated anti-rabbit IgG (NA9341ML) was purchased from GE Healthcare Life Sciences.

Cells and mice

Foxp3 locus-driven IRES GFP reporter knock-in mouse on a C57BL/6 background (Foxp3-IRES-GFP) (25) were used as the source for wild-type primary T cells. The Foxp3-IRES-GFP reporter mice were intercrossed with HPKI⁻/⁻ mice (11), creating an HPKI⁻/⁻ bigenic mouse line (HPKI⁻/⁻ Foxp3-IRES-GFP). Exclusive expression of the GFP reporter and Foxp3 by Tregs enabled us to isolate pure CD4⁺ Tregs and Tregs from splenocytes and lymph node cells by FACS sorting (Shared Research Resources, Mount Sinai School of Medicine). To limit the FACS sorting time, CD4⁺ T cells used in all experiments were first isolated by magnetic bead-assisted negative selection (Invitrogen, Carlsbad, CA) of splenocytes and lymph node cells that were prepared from wild-type or HPKI⁻/⁻ Foxp3-IRES-GFP mice. CD4⁺CD25⁻ T effs used in proliferation assays were obtained from freshly isolated splenocytes and lymph node cells by FACS sorting, selecting for CD4⁺ T cells that did not express GFP and CD25.

Proliferation and Treg suppression assay

Wild-type and HPKI⁻/⁻ CD4⁺CD25⁻ T cells were used as responder T cells. Responder T cells were seeded at 5 × 10⁴ cells per well in U-bottomed 96-well plates. Soluble anti-CD3ε mAb (1 μg/ml of 145-2C11) and equal numbers of irradiated syngeneic splenocytes (2500 rad) were added as costimulants to all wells except control wells. Varying amount of either wild-type or HPKI⁻/⁻ Tregs were added as indicated to quadruplicate wells containing Teffs to assess their ability to suppress T cell proliferation. Wells containing the following combinations of cells were included as controls: 1) wild-type and HPKI⁻/⁻ Teffs; 2) wild-type and HPKI⁻/⁻ T effs with irradiated splenocytes; 3) wild-type and HPKI⁻/⁻ splenocytes alone; 4) wild-type and HPKI⁻/⁻ Tregs alone; and 5) wild-type and HPKI⁻/⁻ Tregs with irradiated splenocytes. Cells were stimulated for 72 h, with ³H-thymidine added to the wells in the last 18 h. Suppression of proliferation was measured by ³H-thymidine incorporation by Teffs and depicted as line graphs as normalized ³H-thymidine incorporated by Teffs. Normalization of ³H-thymidine incorporation is achieved by subtracting background values found in wells with Tregs and irradiated splenocytes and soluble anti-CD3ε mAb from values found in wells with Teffs, Tregs, and irradiated splenocytes.

MILLIPLEX cytokine analysis

Supernatants collected from wild-type or HPKI⁻/⁻ C57BL/6 T cells that had been stimulated by anti-CD3 plus anti-CD28 Dynal beads for 48 h were subjected to the LumineX’s MILLIPLEX MAP Mouse TH17 Magnetic Bead Panel (MT17MAG47K-PX25; EMD Millipore). The bead panel can detect the following 25 murine cytokines: CD40L, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28B, IL-31, IL-33, MIP-3α/CCL20, TNF-α, and TNF-β. Cells were seeded in triplicate and the average amount of each cytokine analyte detected in HPKI⁻/⁻ C57BL/6 T cells were compared with the corresponding cytokine analyte produced by wild-type C57BL/6 T cells.

Statistical analysis

Error bars represented the deviation from the mean numbers. Differences were analyzed using Student t test. Results were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0. The p values <0.05 were regarded as significant.

RESULTS

HPKI⁻/⁻ mice possess elevated levels of Tregs in secondary lymphoid organs

We and others have previously reported that genetic disruption of the HPKI alleles has no impact on CD4 and CD8 T cell development, as evident by the comparable ratio of CD4⁺ and CD8⁺ T cells in HPKI⁻/⁻ mice (10, 11). Not only is the CD4⁺/CD8⁺ ratio comparable between wild-type and in HPKI⁻/⁻ spleen, no significant difference in the number of CD4⁺ T cells present in

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the spleens of wild-type and HPK1−/− C57BL/6 mice were found ($p < 0.8$) (Supplemental Fig. 1). This observation is consistent with the report that no significant difference in the number of CD4+ cells found in the spleens of wild-type C57/BL6 and the catalytically inactive K46E point mutant HPK1 mouse strain (13). Given the critical role Tregs play in thwarting effective antitumor immunity, we assessed the impact of HPK1 deletion on the number of Tregs present in HPK1−/− mice. Freshly isolated primary T cells were prepared from spleen and inguinal lymph nodes of wild-type and HPK1−/− C57BL/6 mice. CD3+CD4+ T cells were probed intracellularly with anti-Foxp3 Ab to determine the number of Foxp3+ Tregs among 10^6 CD4+ T cells sampled by FACS analysis from in these organs. Analysis revealed that a higher number of Tregs was found in HPK1−/− mice relative to their wild-type counterpart (Fig. 1A). The mean number of Tregs among CD4+ HPK1−/−–splenic T cells (8767 ± 0.7691%, n = 10) was significantly higher than the number of Tregs in wild-type CD4+ HPK1−/−–splenic T cells (4252 ± 0.2344%, n = 10, $p < 0.0001$). Similarly, the mean number of Tregs in 10^6 CD4+ HPK1−/−–splenic T cells sampled by FACS from the inguinal lymph nodes (7.637 ± 0.6631%, n = 10) was also significantly higher than the percentage of Tregs in wild-type CD4+ inguinal lymph node T cells (4.35 ± 0.2954%, n = 10, $p < 0.00003$) (Fig. 1A). However, Student t-tests analysis revealed that there were no statistical differences in the mean fluorescence intensity (MFI) of Foxp3 in the samples examined ($p < 0.2$) (Fig. 1B). These data suggest that HPK1−/− mice could inhibit the growth of a variety of murine tumors despite possessing elevated numbers of Tregs in the periphery (11). These observations raised the question whether Tregs lacking HPKI retain normal inhibitory activity.

**HPK1−/− Tregs are unable to effectively suppress TCR-induced cellular proliferation**

We crossed HPK1−/− mice with the Foxp3-ires-GFP mice (25), allowing us to use GFP as reporter signal to isolate Foxp3+ Tregs. FACS analysis of GFP reporter signal revealed the identical trend of higher percentages of GFP+CD4+ Tregs in spleen and lymph nodes of HPK1−/− mice that mirror to the expression level of Foxp3 observed in Fig. 1A (data not shown). Tregs were prepared from wild-type Foxp3-ires-GFP reporter mice (25) and the HPK−/− Foxp3-ires-GFP via FACS sorting of GFP-marked Tregs. We used the Treg in vitro suppression assay to assess the ability of wild-type and HPK1−/− Tregs to suppress the TCR-induced cellular proliferation of wild-type Teffs. Analysis of the mean 3H-thymidine incorporation number revealed that there was a statistically significant loss in the ability to suppress TCR-induced Teff proliferation by HPK1−/− Tregs (Fig. 2A, triangles) when compared with the suppressive activity exhibited by wild-type Teffs (Fig. 2A, squares), $F(4, 30) = 405.4$, ****$p < 0.0001$. At a 1:1 Teff/Treg ratio, wild-type Tregs were able to suppress a TCR-induced proliferative response by 85% (Fig. 2A, squares), whereas HPK1−/− Tregs were able to suppress Teff proliferation by only 26% (Fig. 2A, triangles). Comparison of the wild-type Treg-induced or the HPK1−/− Treg-induced suppression of the proliferative response revealed that HPK1−/− Tregs were unable to suppress the proliferative response of wild-type Teffs at Teff/Treg ratios of 0.05:1 ($p < 0.05$), 0.1:1 (**$p < 0.0005$), 0.2:1 (***$p < 0.00002$), and 1:1 (****$p < 0.00002$) (Fig. 2A). When HPK1−/−
Teffs were used as target cells in the suppression assay (Fig. 2B), statistically significant loss of suppressive activity was also observed in HPK1<sup>−/−</sup> Tregs (Fig. 2B, triangles) when compared with the suppressive activity exhibited by wild-type Tregs (Fig. 2B, squares), \( F(4, 30) = 97.44, ****p < 0.0001 \), revealing that the loss of HPK1 does not confer Teffs with resistance to suppressive activity of wild-type Tregs. Comparison of the wild-type Treg–induced or the HPK1<sup>−/−</sup> Treg–induced suppression of the proliferative response revealed that HPK1<sup>−/−</sup> Tregs were unable to suppress the proliferative response of HPK1<sup>−/−</sup> Tregs at Treg/Teff ratios of 0:1 (\( *p < 0.0001 \)), 0.2:1 (\( **p < 0.0002 \)), and 1:1 (\( ***p < 0.0003 \)) (Fig. 2A). These results clearly implicate HPK1 as an important molecule needed by Tregs to perform effective suppressive function.

Elevated and sustained phosphorylation of Erk and p65/RelA NF-\( \kappa \)B in HPK1<sup>−/−</sup> Tregs in response to TCR engagement

Detailed biochemical characterization of Tregs that were undergoing TCR-induced signal transduction had been previously reported (26). The study revealed that all Treg signal transduction cascades examined were found to be markedly inhibited relative to the levels found in Teffs. Given that the lack of HPK1 expression in murine Teffs results in hyperresponsive activation of the Erk MAPK pathway (10), we determined whether the lack of suppressive function observed in HPK1<sup>−/−</sup> Tregs would correlate with increased signal transduction in the Erk MAPK pathway. Using phosphospecific Abs for Erk MAPK, Western blot analysis of FACS-sorted GFP<sup>+</sup> Treg lysates (cells were lysed with Mammalian Protein Extraction Reagent and sonicated six times with Bioruptor Plus; Diagenode, Denville, NJ) revealed that Erk MAPK has sustained phosphorylation at 40 and 60 min post-TCR engagement in HPK1<sup>−/−</sup> Tregs, compared with wild-type Treg responses, \( *p < 0.03 \) and \( **p < 0.03 \), respectively (Fig. 3A, upper panel). Reprobing of the Western blot membrane with Abs against equal number of irradiated syngeneic splenocytes. GFP<sup>+</sup> Tregs from spleens and lymph nodes of wild-type mice were FACS sorted, and these wild-type Tregs were added to quadruplicate wells to stimulated T cells at varying Treg to Teff ratios, as indicated on the x-axis. After 3 d of stimulation, \( ^{3}\text{H-} \)thyidine was added to each well (1 \( \mu \text{Ci} \) per well during the last 18 h of stimulation. The following symbols represent the different genotypes of Treg and Teff mixtures in the suppression assay: wild-type Tregs and wild-type Teffs, squares; HPK1<sup>−/−</sup> Tregs and wild-type Teffs, triangles. \( *p < 0.05, **p < 0.00005, ***p < 0.00002, ****p < 0.00002 \), \( *****p < 0.00001 \). (B) HPK1<sup>−/−</sup> CD4<sup>+</sup> CD25<sup>−</sup> Teffs were used instead of the wild-type Teffs in an identical experimental set up as in (A). The following symbols represent the different genotypes of Treg and Teff mixtures in the suppression assay: wild-type Tregs and HPK1<sup>−/−</sup> Teffs, squares; HPK1<sup>−/−</sup> Tregs and HPK1<sup>−/−</sup> Teffs, triangles. Error bars represent the SEM from experiments done in quadruplicates in both (A and B). The data shown is from one experiment that is representative of three independently performed experiments. \( *p < 0.0001, **p < 0.00002, ***p < 0.00003, ****p < 0.00001 \).
Figure 3. Elevated and sustained phosphorylation of Erk and p65/RelA NF-κB in HPK1−/− Tregs in response to TCR engagement. Foxp3+ Tregs were separated from CD4+ Teffs based on the expression of the GFP+ reporter signal as described in the legend for Fig. 1. (A, C, E, and G) Wild-type and HPK1−/−GFP+ Tregs (1 × 10⁶) were stimulated by mAb-mediated TCR crosslinking for the indicated time at 37°C, and the total cell lysates were resolved by SDS-PAGE and probed and reprobed with the indicated Abs. For (A), (C), and (I), numbers under the upper panels are normalized densitometric values relative to the levels of corresponding nonphosphorylated protein levels. For (E) and (G), the densitometric numbers were normalized with their corresponding densitometric numbers for actin. (B and D) Mean fold increase from two replicate experiments at the indicated time poststimulation. *p < 0.03, **p < 0.03. (F and H) Densitometric numbers for HPK1 and Foxp3 expression were normalized to densitometric numbers for actin. The normalized numbers for HPK1 and Foxp3 collected from two replicate experiments were averaged and plotted. (I) Wild-type and HPK1−/−GFP+ Tregs (1 × 10⁶) were stimulated by soluble anti-CD3ε and anti-CD28 mAb-mediated crosslinking for the indicated time at 37°C. Lysates were probed with anti-p65/RelA NF-κB Ab. Numbers under the upper panels are normalized densitometric values relative to the levels of corresponding nonphosphorylated p65/RelA NF-κB. The black vertical line indicated where the image was cropped so the lanes could be presented adjacent with one another. (J) Mean fold increase from two replicate experiments at the indicated time poststimulation. Error bars represent the SEM. *p < 0.002, **p < 0.003.
FIGURE 4. *HPK1*−/− Tregs produce increased levels of IFN-γ, IL-2, and other inflammatory cytokines in response to TCR engagement.

(A) GFP-sorted wild-type and *HPK1*−/− Tregs were seeded on anti-CD3ε-coated 96-well plate at 5 × 10^4 cells per well in triplicates in the presence of soluble anti-CD28 costimulation (2 μg/ml). Supernatants collected 24 h poststimulation were subjected to multiplex cytokine/chemokine analysis and the quantity of cytokines and chemokines produced by wild-type and *HPK1*−/− Tregs are displayed as histograms. The data shown is from one experiment that is representative of two independently performed experiments. (B) Stimulated wild-type and *HPK1*−/− regs were subjected to intracellular staining using anti–IFN-γ Ab, (C) and anti–IL-2 Ab as probes. (D) Mean percentage of wild-type and *HPK1*−/− (Continued)
the nonphosphorylated form of Erk MAPK revealed that the lanes were loaded with similar amounts of Erk (Fig. 3A, lower panel). Numbers representing the densitometric values of the strength of the phosphor-Erk signal relative to the amount of Erk loaded are shown below each lane (Fig. 3A, upper panel). Fig. 3B shows the mean fold increase of phosphor-Erk signals over nonstimulated control lanes. Analysis of multiple Student t tests of fold increase values at each time point revealed that HPK1−/− Tregs sustain statistically elevated Erk phosphorylation at 40 and 60 min post-TCR engagement, with significance of p < 0.03 and **p < 0.02, respectively (Fig. 3B). These data suggest that the Erk MAPK pathway in HPK1−/− Tregs is able to sustain elevated Erk MAPK activation in response to TCR-induced activation signals better than their wild-type Treg counterpart.

The membrane was stripped and reprobed with anti–phospho-JNK Ab to assess the status of JNK MAPK activation. Western blot revealed that JNK MAPK is basally phosphorylated even in the absence of TCR activation in Treg of both genotypes, with HPK1−/− Tregs having a higher phospho-JNK at every time point measured (Fig. 3C, upper panel). When the actin-normalized data were analyzed by multiple Student t tests for difference in fold increase values at each time point, no statistical significance was found (Fig. 3D). The membrane was also stripped and reprobed for HPK1 and it confirmed the absence of HPK1 in all HPK1−/− Treg lanes (Fig. 3E). Fig. 3F shows the mean of actin-normalized HPK1 levels from two experiments (Fig. 3F). Similarly, the reprobing of the membrane to determine the level of Foxp3 was performed and revealed the presence of Foxp3 in all lanes (Fig. 3G). Although the mean of actin-normalized Foxp3 levels from two experiments showed that Foxp3 appeared to be slightly elevated in HPK1−/− Tregs, multiple Student t test indicated that there was no statistically significant difference found between wild-type and HPK1−/− Tregs (Fig. 3H).

Because HPK1 had been shown to play a role in regulating TCR-induced NF-κB activation in T cells through phosphorylation of CARMA1 (27), we assessed the impact of HPK1 deletion on the phosphorylation status of the p65/RelA NF-κB at serine residue 536 in response to TCR engagement. The normalized densitometric values indicate that HPK1−/− Tregs are able to sustain a higher phosphorylation level of the p65/RelA NF-κB at 20 min post-TCR engagement (Fig. 3I). Student t test analysis of two replicate experiments revealed that the levels of NF-κB phosphorylation status found in HPK1−/− Tregs at 10 and 20 min post-TCR engagement is significantly higher than the levels found in wild-type Tregs (Fig. 3J, *p < 0.02 and **p < 0.03, respectively).

**HPK1−/− Tregs express an aberrant cytokine expression profile in response to TCR engagement**

We and others have reported that HPK1−/− T cells produce elevated levels of IL-2, IFN-γ, and TNF-α in response to crosslinking by anti-CD3ε and anti-CD28 mAb (10, 11). In this study, we examined whether the loss of HPK1 would alter the TCR-induced cytokine and chemokine expression profiles of Foxp3+ HPK1−/− Tregs. Multiplex cytokine/chemokine analysis of supernatants collected from FACS-sorted GFP+ wild-type and HPK1−/− Tregs that had been stimulated by anti-CD3ε plus anti-CD28 mAb-mediated crosslinking revealed that HPK1−/− Tregs express several cytokines and chemokines at much higher levels than those found in wild-type Tregs. Immunomodulatory and proinflammatory cytokines that possess antitumor activity such as IL-2, IFN-γ, TNF-α, and CCL3 and CCL4 are found to be elevated in HPK1−/− Tregs (Fig. 4A). Although the elevated IFN-γ and IL-2 levels had been reported as one of the phenotypic hallmarks of HPK1 deletion in Teffs (10, 11), our multiplex survey of cytokines/chemokines produced by HPK1−/− Tregs identified CCL3 and CCL4 as previously unappreciated members of cytokine/chemokine family whose expression levels in Tregs are positively affected by the loss of HPK1. In addition to the elevated levels of proinflammatory, antitumor cytokines/chemokines, we also observed that the level of IL-10, an anti-inflammatory cytokine with a known role in suppressing antitumor immunity, was also elevated in HPK1−/− Tregs but by a smaller margin (Fig. 4A). The altered cytokine/chemokine levels identified by the multiplex analysis have been confirmed by ELISA (data not shown).

To ensure that the elevated cytokine levels observed were produced by the actual Tregs and not by cross-contaminated HPK1−/− Teffs, we used FACS analysis to confirm the elevated expression of two of these cytokines, IFN-γ and IL-2, in GFP-expressing Foxp3+ Tregs (Fig. 4B, 4C). Intracellular staining of wild-type and HPK1−/− Tregs revealed that 5.91% of HPK1−/− Tregs produce IFN-γ with an MFI of 4116, whereas only 1.02% of wild-type Tregs produce IFN-γ, an MFI of 1872 (Fig. 4B). Student t test analysis of two replicate experiments revealed that the mean percentage of IFN-γ detected in HPK1−/− Tregs is significantly higher than the percentage found in wild-type Treg (Fig. 4D, gray bars, *p < 0.004), but no significant difference is detected in the MFI of IFN-γ between the two Treg genotypes (Fig. 4E, gray bars). Similarly, a higher percentage of HPK1−/− Tregs also produce IL-2 compared with the wild-type Tregs. Analysis of IL-2 expression by intracellular staining revealed that 2.78% of HPK1−/− Tregs produce IL-2, with an MFI of 8728, as compared with 0.13% of wild-type Tregs that possess an MFI of 631 (Fig. 4C). Student t test analysis of two replicate experiments revealed that the mean percentage of IL-2 detected in HPK1−/− Tregs was significantly higher than the percentage found in wild-type Treg (Fig. 4D, white bars, **p < 0.04). The mean MFI of IL-2 detected in HPK1−/− Tregs is also significantly higher than the MFI found in wild-type Treg (Fig. 4E, white bars, *p < 0.0005).
DISCUSSION

Studies using syngeneic murine tumor models have implicated HPKI as an important negative regulator of antitumor immunity (11–13). The elevated granzyme B, perforin, and IFN-γ levels in CD8+ tumor-infiltrating lymphocytes suggest that this T cell subset is responsible for the enhanced antitumor immunity exhibited by HPKI−/− mice (12). Given the role Tregs play in suppressing antitumor immunity (17–24), we assessed whether the loss of HPKI might also impact the Treg subset either in terms of altering the number of Tregs present in HPKI−/− mice or by interfering with the ability of HPKI−/− Tregs to carry out their normal role as immune suppressor cells. The observation that HPKI−/− mice have more Tregs than wild-type mice is an unexpected finding (Fig. 1), given the enhanced antitumor immunity of HPKI−/− mice (11). Because we found that HPKI−/− Tregs possess diminished suppressive activity in vitro (Fig. 2), we speculate that a similar reduction in suppressive activity may also be occurring in vivo. This reduced homeostatic suppressive activity, which is necessary to maintain peripheral tolerance, may have triggered a compensatory positive feedback mechanism that causes an increase in Tregs in the secondary lymphoid organs of HPKI−/− mice. We are currently examining the cell-autonomous impact of HPKI deletion in Tregs using genetically engineered mice in which the floxed HPKI alleles are conditionally deleted by Foxp3 promoter-driven YFP-Cre chimera in Tregs (28). Such studies should allow us to elucidate the definitive role HPKI plays in Treg development and its contribution to antitumor immunity.

Sustained TCR-induced Erk MAPK activation might offer a plausible mechanism as to why HPKI−/− Tregs exhibit aberrant Th1-like functional profiles. It had been shown that a strong and prolonged Erk signal would give rise to the development of Th1 cells (29). Conversely, inhibition of Erk activation promotes TGF-β–induced Foxp3 expression and Treg development (30). In a study where the TCR-induced signal transduction pathways were compared between Tregs and Teffs, the finding confirmed that Erk phosphorylation level in Tregs is much less robust than the levels found in their CD4+ Teff counterpart (26). Thus, it is plausible to propose that the strong and sustained Erk phosphorylation we observed in HPKI−/− Tregs in response to TCR engagement (Fig. 3A) may contribute toward destabilization of the Treg phenotype. It is important to note that the Erk pathway is thought to impact Treg functions through its control of the Foxp3 gene transcription (30). However, as we have found from our FACS and Western blot analyses (Figs. 1B, 3G), the loss of HPKI does not significantly alter the expression level of Foxp3 in HPKI−/− Tregs, suggesting that other Erk-regulated mechanisms that are independent of its transcriptional control of Foxp3 may exist, and that such mechanisms might be disrupted by the absence of HPKI.

Enhanced Erk MAPK response is not the only TCR-generated pathway positively impacted by the loss of HPKI. We observed that the NF-κB pathway is also hyperresponsive to TCR signals in the absence of HPKI, as evidenced by the elevated phosphorylation levels of p65/RelA at serine residue 536 in HPKI−/− Treg (Fig. 3I). Although HPKI had been proposed as the critical molecule that couples TCR-generated signals to the CARMA-BCL-10-MALT1 (CBM) complex to activate NF-κB (27), our results reveal that TCR signal transduction to NF-κB in HPKI−/− Tregs is not negatively impacted by the absence of HPKI, but it becomes hyperresponsive to TCR engagement (Fig. 3I). Given that NF-κB activation is an essential component of the transcriptional control of the IL-2 gene in response to TCR engagement (31, 32), the elevated NF-κB activation is consistent with the observed increase in TCR-induced IL-2 production by HPKI−/− and K46M and K46E HPKI mutant T cells (10–13). In addition to CARMA being an HPKI kinase substrate (27), it could also be phosphorylated by PKC-β, IKKβ, AKT, and CaMKII (33, 34). Therefore, it is possible that, in the absence of HPKI, one or more of these kinases could compensate for the loss of HPKI-mediated CARMA activation, resulting in competent TCR-induced NF-κB activation.

There is a consensus between our study and that of Liu et al. (13) that HPKI plays a role in either regulating the Treg suppressive activity or their number. Although Shui et al. (10), Hernandez et al. (12), and Liu et al. (13) did not assess the suppressive function of Tregs in their HPKI−/− mice or their kinase-inactive K46E and K46M HPKI point mutant mice, Liu et al. (13) evaluated the 1956 sarcoma tumors grown in K46M HPKI mice for the presence of Tregs and found that the number of CD4+CD25hiFoxp3+ present in the K46M HPKI mice are at a significantly lower level than those found in the 1956 sarcoma tumors grown in wild-type mice (15). The authors proposed that the resistance of the K46M T cells to PGE2-mediated immune suppression might also have an analogous impact on the ability of PGE2 to promote Treg development. Although this is a plausible model that could explain why there are fewer Tregs in tumors from the K46M mice and why the K46M mice possess superior antitumor immunity, it does not rule out the possibility that the K46M Tregs found in these tumors are not also functionally impaired because the authors did not assess the suppressive activity of their K46M Tregs. We did not find a decrease in Treg numbers in spleen and lymph node of our HPKI−/− mice (Fig. 1A), which is consistent with the observation of Liu et al. in that they did not find a significant difference in Treg numbers in draining lymph nodes of wild-type and K46M mice. Because their study did not assess the suppressive ability of the K46M Treg population, further studies using K46M mice to study Treg numbers and their suppressive activity are needed. Comparing HPKI−/− and K46M HPKI mutant mice in terms of their Treg numbers and the ability of their Tregs to suppress Teffs may reveal whether these aberrant Treg phenotypes observed in HPKI−/− Tregs could also be found in the K46M HPKI mutant Tregs, implicating a kinase activity–dependent process. Should the aberrant Treg phenotypes be found only in HPKI−/− Tregs, this would implicate the noncatalytic functions of HPKI as the cause of the Th1-like Treg phenotype we observed.

Our observation that HPKI−/− Tregs produce IFN-γ and IL-2 (Fig. 4A–C) in the presence of stable levels of Foxp3 expression (Fig 1A) is most consistent with the proposed “fragile Treg” model in which Tregs produce IFN-γ and lose their ability to suppress...
Teffs without the corresponding reduction in Foxp3 level (35, 36). The mechanism of IFN-γ-mediated Treg fragility is not necessarily cell intrinsic, because IFN-γ-expressing Tregs could also cause a reduction in suppressive activity by non–IFN-γ–producing bystander Tregs (35). This phenomenon may help explain how HPKI−/− Tregs could lose their ability to suppress Teffs when only ~6% of the cells produces IFN-γ (Fig. 4B). Certainly, it is possible that the aberrant expression of IFN-γ and the other proinflammatory cytokines/chemokines by HPKI−/− Tregs (Fig. 4A) may contribute directly to superior antitumor immunity through their direct cytolytic activity against tumor cells.

In conclusion, we have identified HPKI as a regulatory molecule capable of influencing Treg functions. In the absence of HPKI, several Foxp3-meditated Treg phenotypes, such as the ability to inhibit TCR-induced T cell proliferation and the ability to suppress IFN-γ, IL-2, CCL3, and CCL4 production, are adversely affected. Interestingly, the Foxp3-driven expression of CD25 was not affected by the absence of HPKI (data not shown). These findings suggest that selective Foxp3-dependent functions, namely Teff suppression and the production of certain proinflammatory cytokines/chemokines, may be influenced by HPKI. Our findings suggest that HPKI might be an interesting therapeutic target to consider for the modification of Treg suppressive functions.

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Supplemental Figure 1. Number of CD4$^+$ T cells in wild type and HPK1$^{-/-}$ spleens. CD4$^+$ T cells isolated by magnetic bead-assisted negative selection from wild type (n=6) and HPK1$^{-/-}$ splenocytes (n=6) were counted and the number of CD4$^+$ T cells present in each spleen are depicted. Error bars represent the standard error of the mean.