Supplemental Information

Identification of PGAM5 as a mammalian protein histidine phosphatase that plays a central role to negatively regulate $CD4^+$ T cells

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Supplemental Figures

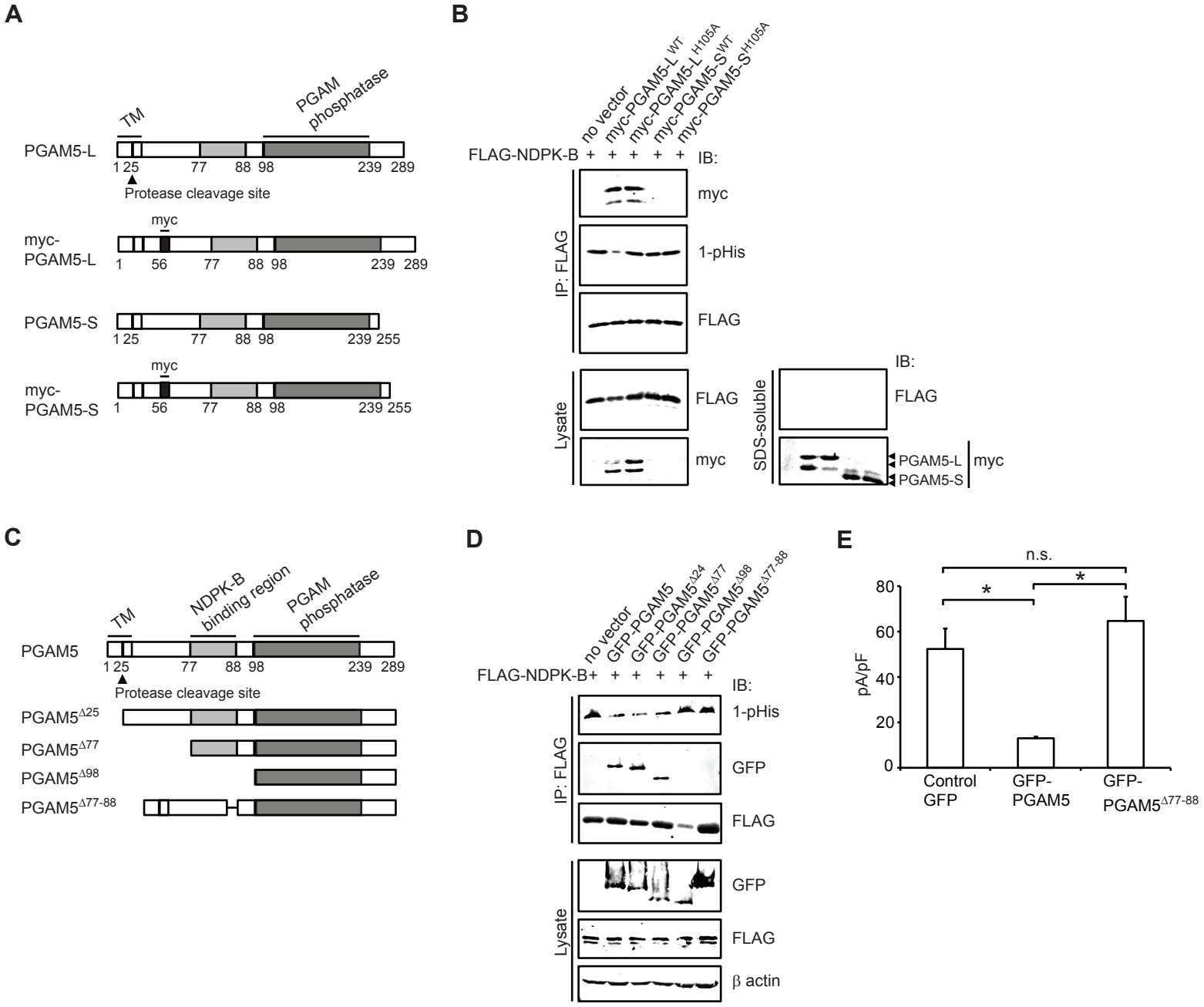

Figure S1, related to Figure 1. ***PGAM5 interaction with NDPK-B is required for NDPK-B de-phosphorylation and inhibition of KCa3.1 channel activity.*** **(A)** Schematic of PGAM5-L and PGAM5-S isoforms showing protease cleavage site and position of myc tag. **(B)** HEK 293T cells were lysed in the lysis buffer to obtain the supernatant (lysate) and insoluble precipitate. The proteins in the insoluble precipitate were extracted using SDS (SDS-soluble fraction), as previously described (Wang et al., 2012). Lysates and SDS-soluble fraction were probed with anti-FLAG and anti-myc antibodies. Lysates were subjected to IP with anti-FLAG and IB with anti-FLAG, anti-myc and anti-1-pHis antibodies (to detect NDPK-B phosophorylation). **(C)** Schematic of PGAM5-L (hereafter referred to as PGAM5) domain structure indicating mitochondrial transmembrane domain; TM, PGAM phosphatase domain and NDPK-B interacting region mapped in Figure S1D. **(D)** Lysates of HEK 293T cells co-expressing FLAG-NDPK-B and the indicated GFP-PGAM5 variants (shown in Figure S1C) were subjected to IP with anti-FLAG antibodies and then IB with anti-FLAG, anti-GFP and anti-1-pHis antibodies (to detect NDPK-B phosophorylation). Lysates were also probed with anti-FLAG, anti-GFP and anti-β actin antibodies. **(E)** HEK 293T cells overexpressing KCa3.1 (293-KCa3.1 cells) were transfected with control GFP plasmid or GFP-PGAM5 (full-length or Δ77-88 deletion variant), and KCa3.1 channel activity was determined by whole-cell patch clamp on GFP-positive cells. Bar graph summary of TRAM-34-sensitive currents plotted at +60 mV (n = 15-20 cells each). Data are displayed as mean ±SEM. Data are representative of three independent experiments. Statistical significance was calculated using Student's t test (Figure S1E); *($p<0.05$); **($p<0.01$); not significant (n.s.) ($p>0.05$).

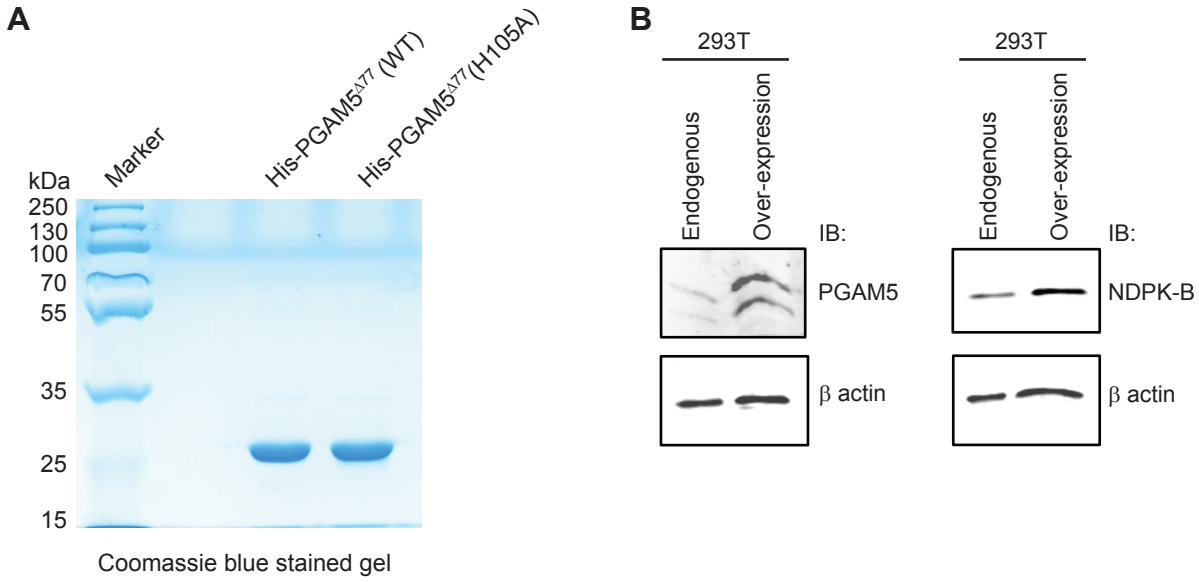

Figure S2, related to Figure 1. Purity of His-*PGAM5*$^{\Delta 77}$ *and comparison of expression levels of endogenous and over-expressed PGAM5 and NDPK-B.* (A) Coomassie blue stained gel to show purity of His-PGAM5$^{\Delta 77}$ (WT and H105A mutant) expressed in *E.coli* and purified by affinity chromatography. **(B)** HEK 293T cells (without or with myc-PGAM5-L or FLAG-NDPK-B over-expression; 5 μg plasmid per 10 cm plate) were lysed in the lysis buffer to obtain the supernatant (lysate). Lysates were probed with anti-PGAM5, anti-NDPK-B and anti-β actin antibodies. Data are representative of three independent experiments.

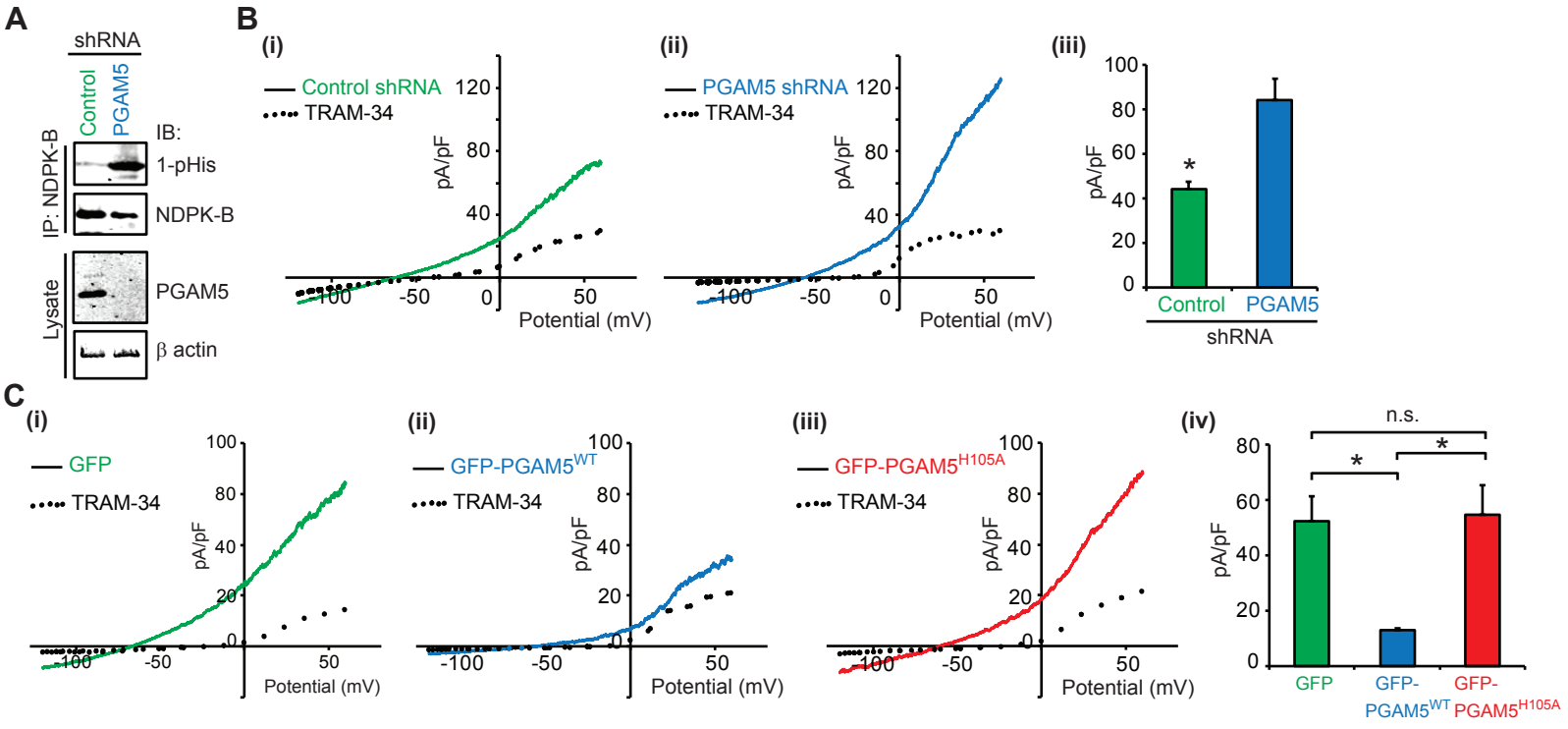

Figure S3, related to Figure 2. ***PGAM5 negatively regulates KCa3.1 channel activity.*** **(A)** HEK 293T cells overexpressing KCa3.1 (293-KCa3.1 cells) were stably transfected with control or PGAM5 shRNA. NDPK-B was IP from control and PGAM5 shRNA transfected cell lysates and IB with anti-NDPK-B or anti-1-pHis antibodies. Lysates were blotted with anti-PGAM5 (to demonstrate knockdown of PGAM5) and anti-β actin (as a loading control) antibodies. **(B)** KCa3.1 channel activity was determined by whole-cell patch clamp of 293-KCa3.1 cells shown in Figure S3A. Shown are I/V plots of 293-KCa3.1 cells treated with: (i) control shRNA and (ii) PGAM5 shRNA. (iii) Bar graph summary of TRAM-34-sensitive currents plotted at +60 mV (n = 15-20 cells each). **(C)** 293-KCa3.1 cells were transfected with control GFP or GFP-PGAM5 (WT or H105A mutant), and KCa3.1 channel activity was determined by whole-cell patch clamp of GFP-positive cells. Shown are I/V plots of 293-KCa3.1 cells: (i) control, (ii) overexpressing GFP-PGAM5^{WT}, and (iii) overexpressing GFP-PGAM5^{H105A}. (iv) Bar graph summary of TRAM-34-sensitive currents plotted at +60 mV (n = 15-20 cells each). Data are representative of three independent experiments. Statistical significance was calculated using Student's t test (Figures S2B and S2C); *($p<0.05$); **($p<0.01$); n.s. ($p>0.05$).

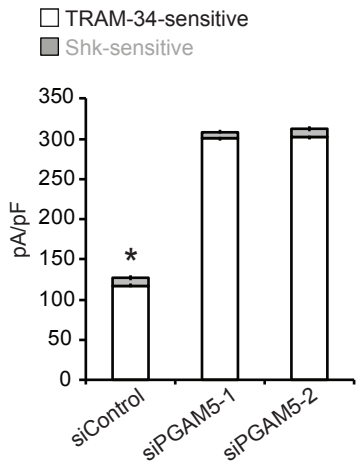

Figure S4, related to Figure 4. ***PGAM5 negatively regulates KCa3.1 in human CD4$^+$ T cells.*** Primary human naïve CD4$^+$ T cells were transfected with a control siRNA or individual PGAM5 siRNAs, rested overnight, and then activated with anti-CD3 and anti-CD28 antibodies for 48h (to generate Th0 CD4$^+$ T cells). Whole cell patch clamp of activated cells (transfected with control or two single PGAM5 siRNAs) as described in Figure 4B. Shown is a bar graph summary of TRAM-34-sensitive and Shk-sensitive currents plotted at +60 mV (n = 15 to 20 cells each). Data are representative of three independent experiments. Data are shown as mean±SEM. Statistical significance was calculated using Student's t test; *($p<0.05$); **($p<0.01$); n.s. ($p>0.05$).

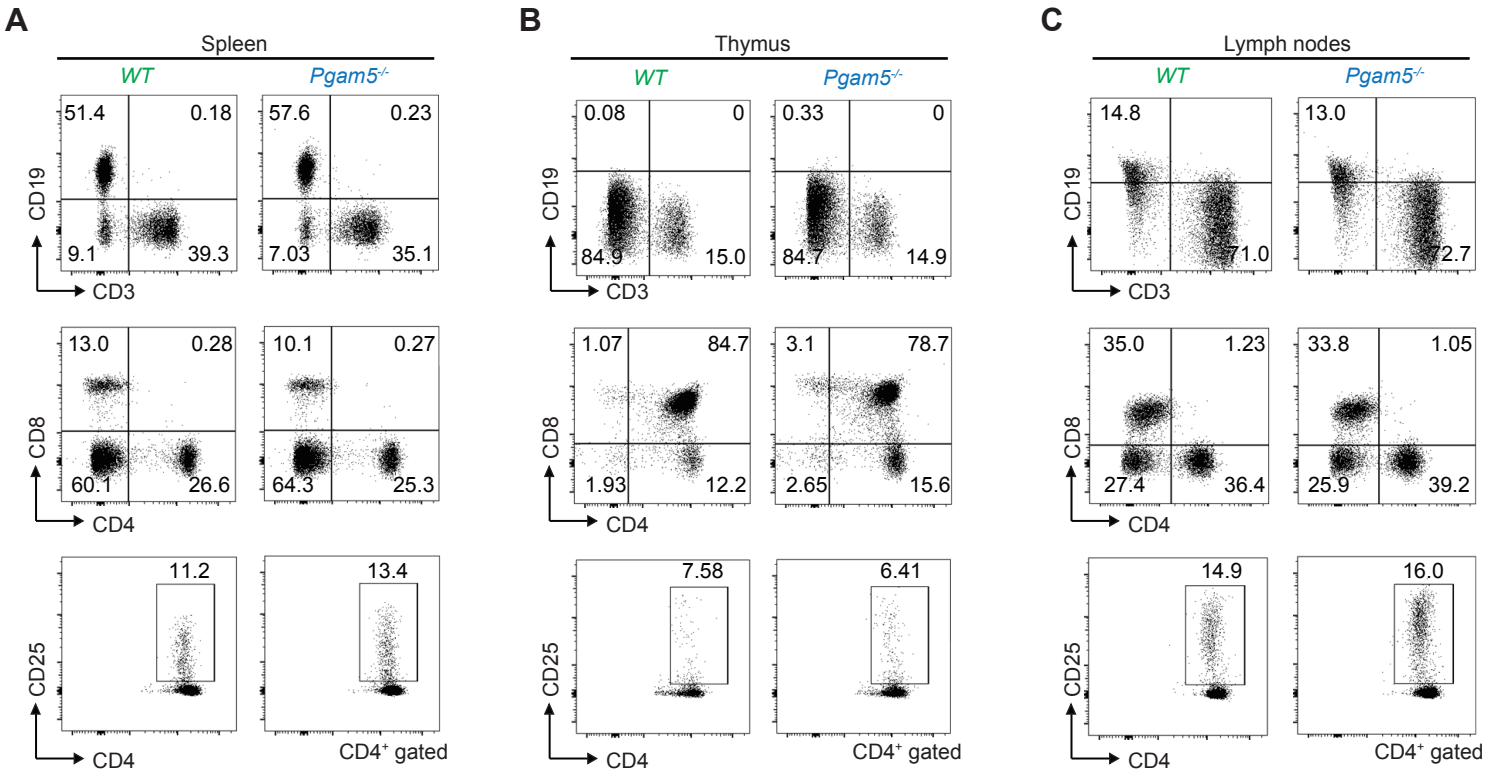

Figure S5, related to Figure 6. ***Normal lymphocyte development in Pgam5$^{-/-}$ mice.*** **(A-C)** Cells were isolated from spleen **(A)**, thymus **(B)** and lymph nodes **(C)** from WT and *Pgam5$^{-/-}$* mice, stained with antibodies to CD3, CD19, CD4, CD8 and CD25, analyzed by flow cytometry (n=3 mice per group per experiment). Data are representative of three independent experiments.

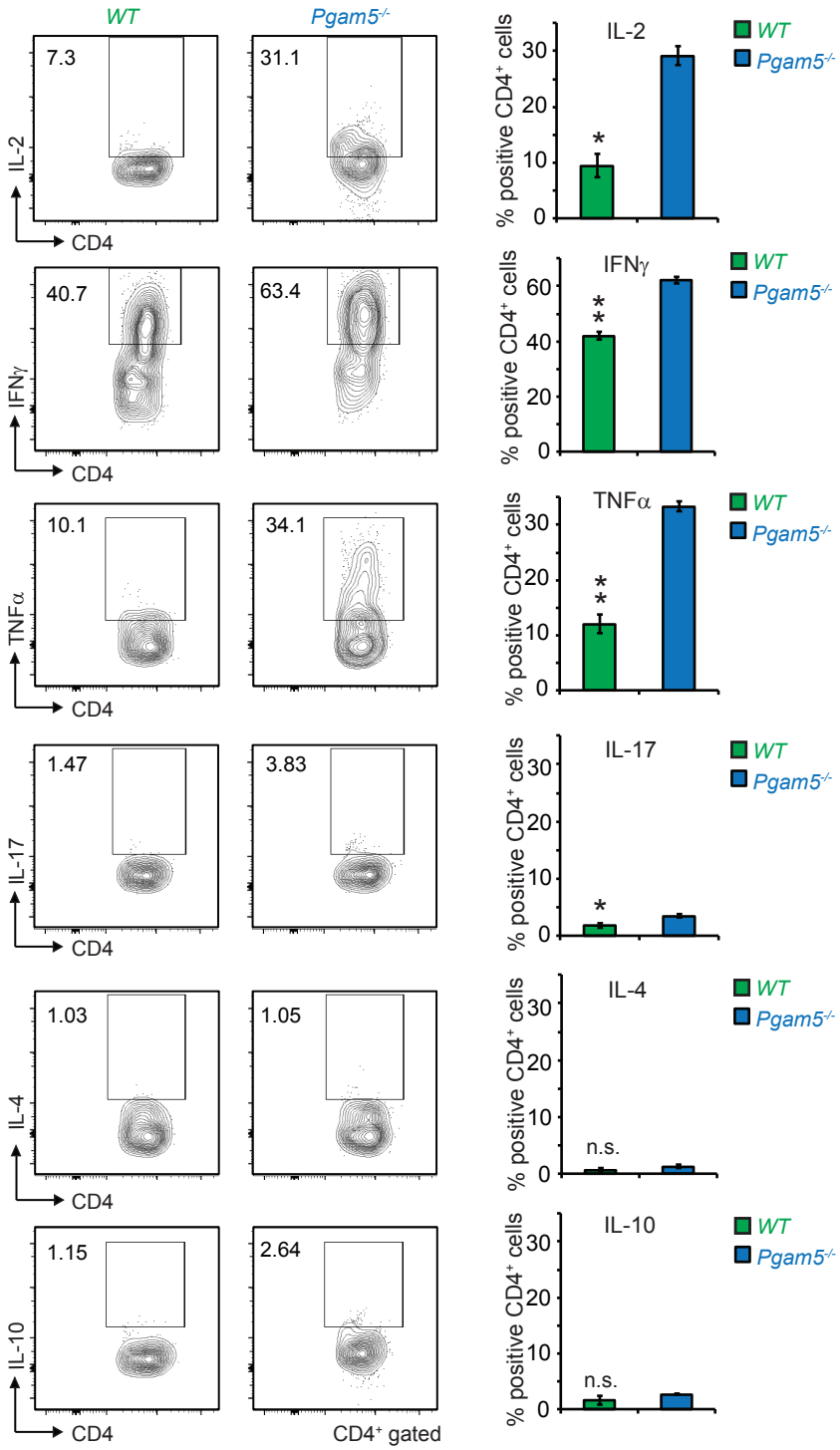

Figure S6, related to Figure 7. ***Allogeneic Pgam5$^{-/-}$ donor CD4^{+} T cells express more pro-inflammatory cytokines during GvHD.*** Representative intracellular flow cytometry detecting cytokine expression of splenic CD4^{+} donor T cells at day 6 post HCT (left). Total spleen cells were re-stimulated for 6h with PMA/ionomycin. Quantification of data from three mice per group per experiment (right). Data are representative of two independent experiments. Data are shown as mean±SEM. Statistical significance was calculated using Student's t test; *($p<0.05$); **($p<0.01$); n.s. ($p>0.05$).

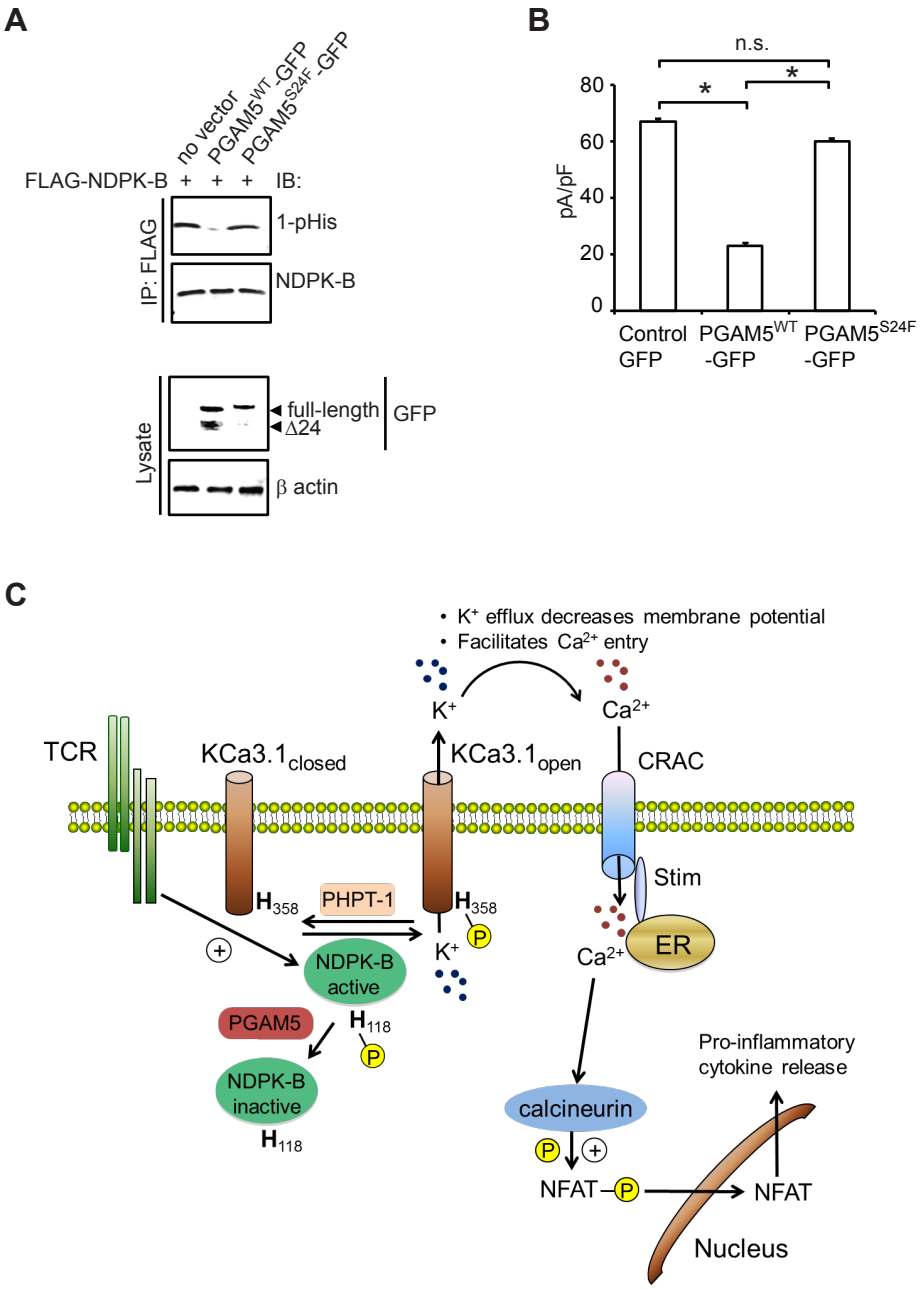

Figure S7, related to Figure 1. Cytosolic portion of *PGAM5-L dephosphorylates NDPK-B and inhibits KCa3.1 channel activity.* (A) HEK 293T cells were transfected with PGAM5 (WT or S24F protease-resistant mutant) with a C-terminal GFP tag together with FLAG-NDPK-B. Lysates were probed with anti-GFP and anti-β actin antibodies. The cells expressing PGAM5^{WT}-GFP showed 2 bands corresponding to full-length and Δ24 fragment of PGAM5, whereas those expressing PGAM5^{S24F}-GFP showed only the full-length form of PGAM5 in the GFP blot. Lysates were then subjected to IP with anti-FLAG antibodies and then IB with anti-FLAG and anti-1-pHis antibodies (to detect NDPK-B phosophorylation). **(B)** HEK 293T cells overexpressing KCa3.1 (293-KCa3.1 cells) were transfected with control GFP or PGAM5-GFP (WT or S24F mutant), and KCa3.1 channel activity was determined by whole-cell patch clamp on GFP-positive cells. Bar graph summary of TRAM-34-sensitive currents plotted at +60 mV (n = 15-20 cells each). Data are displayed as mean ±SEM. Data are representative of three independent experiments. Statistical significance was calculated using Student's t test (Figure S6B); *($p<0.05$); **($p<0.01$); not significant (n.s.) ($p>0.05$). **(C)** Schematic showing PGAM5 as a mammalian histidine phosphatase that negatively regulates CD4$^+$ T cell activation. Activation of the TCR activates the class II PI3K-C2β to generate PI3P, which is required for NDPK-B to transfer the autophosphorylated phosphate on H118 from NDPK-B to H358 on KCa3.1, leading to KCa3.1 activation. By mediating the efflux of K^+, KCa3.1 maintains a negative membrane potential required to maintain the electrical gradient that facilitates Ca^{2+} entry via Ca^{2+}- release-activated channels (CRAC). Ca^{2+} influx then activates NFAT-mediated transcription of various cytokines. Here, we show that PGAM5 functions as histidine phosphatase that inhibits TCR signaling by specifically dephosphorylating H118 on NPDK-B, leading to inhibition of NDPK-B-mediated activation of KCa3.1.

Supplemental Experimental Procedures

Detailed method for mass spectrometry:

The eluted samples were reduced with DTT at 57℃ for 1 hour (2 µl of 0.2 M in 100mM ammonium bicarbonate) and next alkylated with iodoacetamide at room temperature in the dark for 45 minutes (2 µl of 0.5 M in 100mM ammonium bicarbonate). Immediately after alkylation, the samples were loaded onto a NuPAGE® 4-12% Bis-Tris Gel 1.0 mm (Life Technologies Corporation) and run for approximately 15 minutes at 200 V. The gel was stained using GelCode Blue Stain Reagent (Thermo Scientific, Rockford, IL) following manufactures instruction.

The short gel lane was cut into approximately $1mm^3$ pieces. The gel pieces were destained in 1:1 v/v solution of methanol and 100 mM ammonium bicarbonate at 4°C with agitation. The destain solution was changed every 15 minutes at least 5 times and until the gel pieces had no visibly blue stain left. Gel pieces were partially dehydrated with an acetonitrile rinse and further dried in a SpeedVac concentrator for 20 minutes. 300 ng of sequencing grade-modified trypsin (Promega) was added to the dried gel pieces. After the trypsin was absorbed, 200µl of 100 mM ammonium bicarbonate was added to cover the gel pieces and digestion proceeded overnight on a shaker at room temperature. Peptide extraction was performed, as described[1], by adding a slurry of R2 20 µm Poros beads (Life Technologies Corporation) in 5% formic acid; 0.2% trifluoroacetic acid (TFA) to each sample at a volume equal to that of the ammonium bicarbonate. Samples were incubated with agitation at 4℃ for 4 hours. The beads were loaded onto equilibrated C18 ziptips (Millipore) using a microcentrifuge for 30 sec at 6000 RPM. Gel pieces were rinsed three times with 0.1% TFA and each rinse was added to the corresponding ziptip followed by microcentrifugation. Extracted poros beads were further washed with 0.5% acetic acid. Peptides were eluted off the beads by addition of 40% acetonitrile in 0.5% acetic acid followed by the addition of 80% acetonitrile in 0.5% acetic acid. The organic solvent was removed using a SpeedVac concentrator and the samples were reconstituted in 0.5% acetic acid.

An aliquot of each sample was loaded onto the EASY spray 50 cm C18 analytical HPLC column with <2µm bead size using the auto sampler of an EASY-nLC 1000 HPLC (ThermoFisher) in solvent A (2% acetonitrile, 0.5% acetic acid). The peptides were gradient eluted directly into an Orbitrap Elite (Thermo Scientific) mass spectrometer using a 90 minute gradient from 2% to 31% solvent B (95% acetonitrile, 0.5% acetic acid), followed by 15 minutes from 31% to 100% solvent B. The Orbitrap Elite mass spectrometer acquired high resolution full MS spectra with a resolution of 60,000, an AGC target of 1e6, with a maximum ion time of 200 ms, and scan range of 300 to 1500 m/z. Following each full MS twenty data-dependent high resolution

HCD MS/MS spectra were acquired using the following instrument parameters: resolution of 15000, AGC target of 5e5, maximum ion time of 500 ms, one microscan, 2 m/z isolation window, fixed first mass of 150 m/z, and NCE of 30, and a dynamic exclusion of 30 seconds. The MS/MS spectra were searched against the Uniprot homo sapiens database using Sequest within Proteome Discoverer (ThermoFisher). The results were filtered using a 1% False Discovery Rate (FDR) searched against a decoy database and excluding proteins with less than two unique peptides. Proteins identified in the control were subtracted from the proteins identified in the NDBK-B affinity purification and a shortened list interrogated for potential NDBK-B interacting proteins.

Mass spectrometry results for PGAM5 identification as an interaction partner in NDPK-B IP (not present in control vector IP):

The affinity purified samples were digested with trypsin and analyzed on an Orbitrap Elite using high resolution and HCD for fragmentation. The MS/MS spectra were searched against the Uniprot human database using Sequest within Proteome Discoverer (Thermo Fisher). The results were filtered using a 1% False Discovery Rate (FDR) searched against a decoy database and proteins with less than two unique peptides were excluded. Proteins identified in the control were subtracted from the proteins identified in the NDBK-B affinity purification and this shortened candidate list interrogated for potential NDBK-B interacting proteins. Using this approach, PGAM5 was our top hit.

- Accession number: Q96HS1
- Description: Serine/threonine-protein phosphatase PGAM5, mitochondrial OS=Homo sapiens GN=PGAM5 PE=1 SV=2 - [PGAM5_HUMAN]
- Search algorithm: Sequest
- Score: 41.35 (sum of the scores of the individual peptides)
- Coverage: 40.14% (the percentage of the protein sequence identified in this analysis)
- # Peptides: 10 (the number of distinct peptide sequences identified per protein)
- #PSMs: 11 (Peptide Spectrum Matches: the total number of identified peptide sequences for the protein, including those redundantly identified)
- Confidence: confidence level of peptide identification (we included only high confidence peptides based on a better than 1% FDR searched against a decoy database)
- Mass accuracy: better than 5 ppm for parent and fragment ions

Antibodies, reagents and plasmids

Anti-NDPK-B (Abgent, Cat# ALS16689); anti-KCa3.1 (Alomone labs, Cat# ALM-051); anti-PGAM5 (Abcam, Cat# ab126534); anti-FLAG (Sigma, Cat# F3165); anti-β actin (Sigma, Cat# A5316); anti-c-myc (Santa Cruz, Cat# sc-40) and anti-GFP (Life technologies, Cat# A6455) were purchased. Rabbit monoclonal anti-1-pHis (clone SC1-1) and anti-3-pHis (clone SC56-2) antibodies have been previously described (Fuhs et al., 2015). For FLAG-tag immuno-precipitation, anti-FLAG M2 beads (Cat# A2220) and elution buffer (3x FLAG peptide, Cat# F4799) were purchased from Sigma. For Streptavidin-tag immuno-precipitation, Strep-Tactin Sepharose beads (Cat# 2-1201-010) and Desthiobiotin elution buffer (Cat# 2-1000-002) were purchased from IBA-Life Sciences. For measuring serum IL-2, TNFα and IFNγ, Ready-Set-Go! ELISA kits (eBioscience) were used according to manufacturer's instructions. NDPK-A, -B or -C cDNA were sub-cloned into a FLAG-mcherry vector. Myc-PGAM5 (WT and H105A mutant) and PGAM5 shRNA plasmids were kindly provided by Dr. Zhigao Wang (UT Southwestern)(Wang et al., 2012). The myc tag is inserted after AA 56 and hence anti-myc antibodies detected both full-length and Δ24 forms of PGAM5-L and PGAM5-S, as previously reported(Wang et al., 2012). PGAM5-L cDNA (full-length, Δ24, Δ77, Δ98, Δ77-88) was sub-cloned into pEGFP-C1 vector. PGAM5-L cDNA (full-length or S24F mutant) was also sub-cloned into pEGFP-N1 vector. For the purpose of *in vitro* kinase/phosphatase assay, NDPK-B cDNA was sub-cloned into pGEX-4T-1 and PGAM5 (AA 76-289) was sub-cloned into His-tag bacterial expression vectors.

Flow cytometry

Cells isolated from spleens and lymph nodes of mice were passed through 70 μm cell strainer and washed once in FACS buffer (PBS containing 1mM EDTA and 0.1% BSA) before blocking with anti-FcγRII/FcγRIII (2.4G2, BD Pharmingen). Staining of cell surface molecules was performed in FACS buffer on ice for 30 min in dark using flurophore-conjugated antibodies to: CD3 (17A2), CD19 (6D5), CD4 (RM4-5), CD8α (53-6.7) and CD25 (PC61). For intracellular cytokine staining, cells were re-stimulated with PMA (10 ng/ml, Sigma)

and ionomycin (5 nM, Merck) for 6 h in the presence of brefeldin-A (1 μg/ml, Sigma). Intracellular staining was performed using the Fixation/Permeabilization kit (BD Biosciences, Cat # 554714) and antibodies to: IL-2 (JES6-5H4), IFNγ (XMG1.2), TNFα (MP6-XT22), IL-4 (11B11), IL-10 (JES5-16E3) and IL-17 (eBio17B7), according to manufacturer's instructions. For primary human $CD4^+$ T cell staining, cells were stained with fluorophore-conjugated antibodies to CD4 (RPA-T4), followed by intracellular staining for IL-2 (MQ1-17H12), IFNγ (4S.B3), TNFα (MAb11) and IL-4 (MP4-25D2) using the Fixation/Permeabilization kit (BD Biosciences, Cat # 554714). All antibodies were from eBioscience or BioLegend. Cells were analyzed on a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).