NF-κB-inducing kinase maintains T cell metabolic fitness in antitumor immunity

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Metabolic reprogramming toward aerobic glycolysis is a pivotal mechanism shaping immune responses. Here we show that deficiency in NF-κB-inducing kinase (NIK) impairs glycolysis induction, rendering CD8+ effector T cells hypofunctional in the tumor microenvironment. Conversely, ectopic expression of NIK promotes CD8+ T cell metabolism and effector function, thereby profoundly enhancing antitumor immunity and improving the efficacy of T cell adoptive therapy. NIK regulates T cell metabolism via a NF-κB-independent mechanism that involves stabilization of hexokinase 2 (HK2), a rate-limiting enzyme of the glycolytic pathway. NIK prevents autophagic degradation of HK2 through controlling cellular reactive oxygen species levels, which in turn involves modulation of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that mediates production of the antioxidant NADPH. We show that the G6PD–NADPH redox system is important for HK2 stability and metabolism in activated T cells. These findings establish NIK as a pivotal regulator of T cell metabolism and highlight a post-translational mechanism of metabolic regulation.

The molecular mechanisms connecting T cell signaling and metabolic activities are incompletely understood. One well-defined metabolic pathway in early-phase T cell activation involves activation of phosphatidylinositol 3-kinase and its downstream kinase Akt, which activate the metabolic kinase mammalian target of rapamycin (mTOR) and transcription factors involved in the induction of glycosylation-related genes16–20. How the glycolytic metabolism is maintained in effector T cells is less well understood. Kappa-light-chain-enhancer of activated B cells (NF-κB)-inducing kinase (NIK) is known as a mediator of noncanonical NF-κB activation stimulated by various tumor necrosis factor receptor (TNFR) superfamily members21. Although NIK is known for regulating B cell survival and lymphoid organ development, it is notable that many TNFRs function as costimulatory signals that promote the function of effector T cells22,23. Indeed, recent studies suggest that NIK is crucial for inflammatory CD4+ T cell function and neuroinflammation24. Notably, NIK is dispensable for naive T cell activation but is required for effector T cell function and recall responses25. How NIK regulates T cell responses has been elusive and whether NIK plays a role in regulating antitumor CD8+ T cell responses is also unclear.

In the present study, we identified NIK as a pivotal regulator of T cell metabolism and antitumor immunity. NIK functions post-translationally through mediating the stabilization of HK2, a rate-limiting enzyme of the glycolytic pathway. NIK prevents autophagic degradation of HK2 by controlling cellular ROS levels, which in turn involves NIK-mediated regulation of G6PD and, thereby, the NADPH redox system. These findings establish NIK...
as a pivotal regulator of T cell metabolism and provide a new link between the G6PD–NADPH redox system and glycolysis.

Results

T cell-specific deletion of NIK impairs antitumor immunity.

To examine the role of NIK in regulating antitumor T cell responses, we employed mutant mice harboring T cell-specific deletion of the NIK-encoding gene Map3k14. We challenged Map3k14 T cell conditional knockout (KO) (Map3k14ΔKO) mice and age-matched wild-type (WT) control mice with B16F10 melanoma cells, a poorly immunogenic tumor model. Compared to WT mice, Map3k14ΔKO mice had a drastically increased tumor burden coupled with reduced tumor-infiltrating CD4+ and CD8+ T cells as well as interferon-γ (IFN-γ)-producing effector CD8+ T cells (Fig. 1a–c). The draining lymph nodes of tumor-bearing Map3k14ΔKO mice had a moderately increased frequency of CD4+ and CD8+ T cells, but the percentage of effector CD8+ T cells expressing CD44 and CXCX3 markers was reduced (Fig. 1d,e). A more profound reduction in the frequency and absolute number of CD4+ CXCX3+ CD8+ effector T cells was detected in the tumor (Fig. 1f).

T cell adoptive transfer assays revealed that NIK-deficient OT-I CD8+ T cells were fully functional in tumor infiltration but displayed markedly increased apoptosis and moderately reduced proliferation in the tumor compared to WT CD8+ T cells (Fig. 1g–i). Tumor-infiltrating Map3k14ΔKO CD8+ T cells displayed increased surface expression of PD-1 and Tim3 (Fig. 1j), immune checkpoint molecules associated with T cell exhaustion. On the other hand, NIK deficiency did not increase the frequency of PD-1+ Tim3+ tumor-infiltrating CD8+ T cells and even caused a drastic reduction in their absolute number (Fig. 1k). The latter phenotype was likely due to the overall reduction in tumor-infiltrating CD8+ effector T cells (Fig. 1b) since PD-1 and Tim3 expression is associated with effector T cell generation. These findings suggest that NIK facilitates antitumor immunity by mediating the survival and function of tumor-infiltrating CD8+ effector T cells.

Ectopic NIK expression prevents CD8+ T cell exhaustion and promotes antitumor immunity.

To further investigate the role of NIK in negatively regulating antitumor immunity, we employed a transgenic mouse model, R26StopΔ3Map3k14Δ4, carrying a Map3k14 transgene under the control of a loxp-flanked STOP cassette. By crossing R26StopΔ3Map3k14Δ4 mice with CreER mice, we generated R26StopΔ3Map3k14Δ4CreER mice (hereafter called Map3k14Δ4CreER) and Map3k14Δ4CreER mice, which were then injected with tamoxifen to induce Cre function for producing NIK-induced transgenic (NIKΔG) and WT control mice, respectively (Extended Data Fig. 1a,b). To mimic therapeutic approaches, Map3k14Δ4CreER and Map3k14Δ4CreER mice were preimplanted with tumor cells and then injected with tamoxifen to induce NIK expression (Extended Data Fig. 1c). Ectopic expression of NIK in NIKΔG mice caused a profound suppression of tumor growth and improved survival in both MC38 colon cancer and B16F10 melanoma models (Fig. 2a–c) associated with increased tumor-infiltrating CD4+ and CD8+ T cells as well as IFN-γ-producing CD8+ effector T cells (Fig. 2d–f). Compared to tumor-bearing WT mice, tumor-bearing NIKΔG mice had reduced T cell numbers in draining lymph nodes (Fig. 2h and Extended Data Fig. 1e), probably due to enhanced T cell activation and tumor infiltration induced by NIK. Indeed, the draining lymph nodes and tumors of NIKΔG mice had an increased frequency of effector CD8+ T cells expressing the surface markers CD44 and CXCX3 (Fig. 2i) and Extended Data Fig. 1f). Thus, contrary to NIK deficiency, inducible expression of NIK in adult mice promotes tumor immunity by inducing tumor-infiltrating CD8+ effector T cells.

NIK transgenic expression increased the number of tumor-infiltrating CD8+ T cells with PD-1+ Tim3+ exhaustion markers (Fig. 2k and Extended Data Fig. 1h). However, although WT PD-1+ Tim3+ CD8+ T cells were weak in IFN-γ production, which is characteristic of exhaustion, NIKΔG PD-1+Tim3+ CD8+ T cells were highly competent in IFN-γ production (Fig. 2l and Extended Data Fig. 1i). Ectopic NIK expression did not reduce the level of PD-1 and Tim3 expression (Fig. 2m,n), suggesting that NIK might functionally suppress CD8+ T cell exhaustion.

NIK expression improves the efficacy of adoptive T cell therapy.

To evaluate the therapeutic potential for NIK, we performed adoptive T cell therapy involving transfer of melanoma-specific murine CD8+ effector T cells into B16F10 melanoma-bearing B6.SJL mice. To generate melanoma-specific CD8+ T cells, we employed Pmel1 T cell receptor (TCR) transgenic mice, producing CD8+ T cells recognizing the tumor antigen gp100 expressed in B16F10 cells. We used CD8+ T cells isolated from Map3k14Δ4CreER Pmel1 or Map3k14Δ4CreER Pmel1 mice and treated in vitro with 4-hydroxytamoxifen, along with activation by anti-CD3 plus anti-CD28, to generate NIKΔG Pmel1 and WT Pmel1 CD8+ effector T cells. Tumor-bearing mice transferred with NIKΔG Pmel1 CD8+ T cells displayed a substantially reduced tumor growth rate and drastically increased tumor-infiltrating CD8+ T cells and IFN-γ-producing CD8+ effector T cells, compared to tumor-bearing mice transferred with WT Pmel1 CD8+ T cells (Fig. 2o and Extended Data Fig. 2a,b). NIKΔG Pmel1 CD8+ effector T cells also displayed stronger in vitro cytotoxic activity toward B16F10 tumor cells (Extended Data Fig. 2c). In addition, tumor-infiltrating NIKΔG effector CD8+ T cells had improved survival, although not proliferative, ability compared with WT effector CD8+ T cells (Extended Data Fig. 2d–f). These results suggest that ectopic expression of NIK may be an effective approach to improve the efficacy of adoptive T cell therapy in cancer treatment.

NIK is required for metabolic reprogramming of activated T cells.

We next examined the role of NIK in regulating glycolytic metabolism by measuring the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), which are indicators of aerobic glycolysis and oxidative phosphorylation, respectively. As expected, naive CD8+ T cells displayed a low level of OCR and barely detectable level of ECAR, but both ECAR and OCR were profoundly induced on T cell activation (Fig. 3a,b and Extended Data Fig. 3a). NIK deficiency drastically reduced basal and maximum ECAR as well as baseline and maximum OCR (Fig. 3a,b). NIK-deficient CD8+ T cells isolated from B16F10 tumors revealed an even more profound defect in ECAR and OCR, suggesting that NIK deficiency might render effector T cells more vulnerable to metabolic suppression in TME (Fig. 3c,d). The crucial role of NIK in mediating effector T cell metabolism was further demonstrated in an immune response against Listeria monocytogenes infection (Fig. 3e,f).

Contrary to NIK-deficient T cells, NIKΔG CD8+ T cells displayed higher ECAR and OCR than WT control CD8+ T cells under activation conditions (Fig. 3g,h). Since ectopic NIK expression could improve the effector function of CD8+ T cells expressing the exhaustion markers PD-1 and Tim3 (Fig. 2l), we determined whether NIK could prevent PD-1-mediated inhibition of T cell glycolytic metabolism. As expected, PD-1 ligation by its ligand, programmed cell death ligand 1 ligand 1 (PD-L1), suppressed both ECAR and OCR in WT CD8+ T cells (Fig. 3g,h). However, NIKΔG CD8+ T cells were highly resistant to PD-L1-mediated inhibition of ECAR, although they were less effective in preventing PD-L1-mediated OCR inhibition (Fig. 3g,h). Ectopic expression of NIK also prevented PD-L1-mediated inhibition of IFN-γ induction in CD8+ T cells (Fig. 3i). These results establish NIK as a pivotal regulator of CD8+ T cell glycolysis and provide insight into the mechanism by which NIK promotes T cell exhaustion.
NIK prevents autophagic degradation of HK2. NIK deficiency or overexpression did not alter TCR-CD28-stimulated activation of Akt or phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) downstream targets, S6 and 4EBP1 (Fig. 4a,b). NIK deficiency also had little or no effect on TCR-CD28-stimulated expression of a panel of glycolysis-related factors at the messenger RNA or protein level (Fig. 4c,d and Extended Data Fig. 3b,c). Furthermore, NIK was dispensable for glucose uptake in CD8+ T cells stimulated in vitro or isolated from B16F10 tumors (Extended Data Fig. 3d,e). Interestingly, however, NIK-deficient T cells had a drastic reduction in HK2 at the protein, although not mRNA, level (Fig. 4c,e). Conversely, T cells from NIK−/− mice had a markedly higher level of HK2 protein, although not mRNA, than T cells from WT mice (Fig. 4f,g). Consistent with the in vitro experiments, the HK2 level was profoundly reduced in tumor-infiltrating NIK-deficient CD8+ T cells and increased in tumor-infiltrating NIK†/− CD8+ T cells compared to that of WT CD8+ T cells (Fig. 4h–j). The specificity of HK2 flow cytometry was confirmed by using HK2-deficient CD8+ T cells (Extended Data Fig. 3f). The HK2 expression defect in NIK-deficient T cells was not rescued by a proteasome inhibitor but could be efficiently rescued by several lysosomal inhibitors, including bafilomycin A1; a combination of pepstatin A and E64D and chloroquine could rescue HK2 level in NIK-deficient T cells (Fig. 4k–n). Furthermore, NIK deficiency promoted HK2 localization to the lysosome (Extended Data Fig. 3g). These results suggest that NIK prevents lysosomal degradation of HK2.
Since autophagy mediates lysosomal degradation of cellular proteins and damaged organelles\(^3\), we tested the role of autophagy in HK2 degradation by deleting an essential autophagy component, Atg5, in NKI-deficient T cells. While NKI-deficient T cells had severe loss of HK2, this phenotype was no longer detected in T cells derived from Map3k14\(^{loxP}\)/Atg5\(^{loxP}\) (Map3k14\(^{loxP}\)/Atg5\(^{loxP}\)/Pmel1\(^{Cre}\)/Map3k14\(^{loxP}\)/Pmel1\(^{Cre}\)/CD4\(^+\)/Cre) mice (Fig. 4a). Autophagic flux involves lipidation of microtubule-associated protein 1 light chain 3 (LC3) to convert it from the cytosolic form (LC3-I) to a lipaplated form (LC3-II)\(^4\). In addition to nonsclective autophagy, LC3-II also mediates selective autophagy by interacting with specific receptors\(^5\). NKI deficiency did not promote LC3 modification; however, it caused profound loss of p62 (Extended Data Fig. 3h,i), a selective autophagy receptor known to be degraded along with the degradation of cargos\(^6\). Together, these findings suggest the involvement of selective autophagy in mediating HK2 degradation in NKI-deficient T cells.

**HK2 deletion in T cells impairs effector T cell function and antitumor immunity.** Like NKI, HK2 is dispensable for the early stages of T cell activation; however, whether HK2 has an essential role in effector T cell function is elusive\(^7\). We found that HK2 deletion in T cells substantially inhibited glycolysis, as well as oxidative phosphorylation, in activated T cells (Extended Data Fig. 4a–d). Furthermore, T cell–dependent HK2 KO (HK2\(^{KO}\)) mice had a severe defect in mounting antitumor immunity (Fig. 5a–d). As seen with NKI-deficient CD8\(^+\) T cells (Fig. 1i), tumor-infiltrating T cells derived from Map3k14\(^{loxP}\)/Atg5\(^{loxP}\) (Map3k14\(^{loxP}\)/Atg5\(^{loxP}\)/Pmel1\(^{Cre}\)/Map3k14\(^{loxP}\)/Pmel1\(^{Cre}\)/CD4\(^+\)/Cre) mice (Fig. 4o). Autophagic flux involves lipidation of microtubule-associated protein 1 light chain 3 (LC3) to convert it from the cytosolic form (LC3-I) to a lipaplated form (LC3-II)\(^4\). In addition to nonsclective autophagy, LC3-II also mediates selective autophagy by interacting with specific receptors\(^5\). NKI deficiency did not promote LC3 modification; however, it caused profound loss of p62 (Extended Data Fig. 3h,i), a selective autophagy receptor known to be degraded along with the degradation of cargos\(^6\). Together, these findings suggest the involvement of selective autophagy in mediating HK2 degradation in NKI-deficient T cells.

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HK2-deficient CD8+ T cells displayed increased expression levels of PD-1 and Tim3 (Extended Data Fig. 4e). HK2KO mice also had impaired T cell responses to L. monocytogenes infection, displaying reduced IFN-γ-producing effector CD8+ T cells and increased bacterial titer in the spleen (Extended Data Fig. 4f,g). A previous study suggested that HK2 is important for T cell-mediated inflammation in the lung and intestine but is dispensable for T cell responses to acute infection by the Armstrong strain of lymphocytic choriomeningitis virus (LCMV)32. Because of the requirement of HK2 for antitumor immunity, we examined the role of HK2 in...
mediating T cell responses to chronic infections by LCMV clone 13, a well-established model for studying CD8+ effector T cell functions. Interestingly, mice harboring T cell-specific deletion of either HK2 or NIK displayed a defect in CD8+ T cell responses to LCMV clone 13 infection (Extended Data Fig. 4h). Together, these findings suggest an important role for HK2 in regulating the effector function of T cells in immune responses against both tumorigenesis and infections, emphasizing the functional significance of NIK-mediated HK2 stabilization.

To further examine the importance of NIK-mediated HK2 stabilization, we challenged Map3k14loxP/CreER mice with B16F10 melanoma cells along with tamoxifen injection to construct tumor-bearing mice with simultaneous induction of NIK expression and HK2 deletion (NIKloxP/Hk2loxP). While NIKloxP mice displayed profoundly stronger antitumor immunity than WT control mice, these phenotypes were largely reversed in NIKloxP/Hk2loxKO mice (Fig. 5e,f and Extended Data Fig. 5a). HK2 deletion in NIKloxP mice also reduced the frequency and number of tumor-infiltrating PD-1+ Tim3+ CD8+ T cells to the level of WT mice, although it had no obvious effect on the expression level of PD-1 and Tim3 (Extended Data Fig. 5b,c). Furthermore, while NIKloxP PD-1+ Tim3+ CD8+ T cells were competent in IFN-γ production, NIKloxP/Hk2loxKO PD-1+ Tim3+ CD8+ T cells resembled WT PD-1+ Tim3+ CD8+ T cells in the low efficiency of IFN-γ production (Fig. 5g). Collectively, these...
findings suggest that NIK regulates effector T cell function in anti-tumor immunity by stabilizing HK2 and, thereby, facilitating glycolytic metabolism.

**HK2 deletion blocks NIK-mediated autoimmune inflammation.** Mice with T cell-conditional expression of exogenous NIK (Map3k14 expressing a p100 mutant lacking the phosphorylation site and Lym1/Hk2α), hereafter NIKi Tg (iTg) developed lethal autoimmunity, although the underlying mechanism are elusive. Interestingly, while NIKi Tg mice displayed severe pathology, characterized by body weight reduction and organ inflammation, NIKi/Hk2α mice no longer displayed these phenotypes (Fig. 6a–c). NIKi Tg mice also had a severe defect in thymocyte development, causing a notable reduction in the size of the thymus and the number of double-negative, double-positive as well as CD8+ single-positive thymocytes (Fig. 6b,d). Since CD4+-Cre expression occurs at the late stage of double-negative thymocyte development, these data suggest an effect starting from the double-negative stage. Importantly, these abnormalities in thymocyte development were rescued in NIKi/Hk2α mice (Fig. 6b,d). NIKi Tg mice also had a defect in thymic regulatory T (Treg) cell production, which was rescued in NIKi/Hk2α mice (Fig. 6e). In the periphery, NIKi Tg mice had a marked proliferation of Treg cells with CD44hi surface markers, known to be functionally defective; deletion of HK2 also reversed this phenotype (Fig. 6f,g). These findings suggest that deregulated HK2 expression may contribute to the perturbed thymocyte development and autoimmune phenotypes seen in NIKi Tg mice.

**HK2 degradation in NIK-deficient T cells involves aberrant ROS accumulation.** To examine the potential involvement of noncanonical NF-kB in HK2 regulation, we employed a mouse strain, Nfkbi2Δm1, carrying a point mutation in the Nfkbi2 gene and expressing a p100 mutant lacking the phosphorylation site and defective in NIK-induced processing. We used the Lym1 heterozygous mice, Nfkbi2Δm1 since they display strong phenotype in p100 processing and biological functions. Despite the impaired noncanonical NF-kB activation (p100 processing to generate p52), Nfkbi2Δm1 mice had normal levels of HK2 expression (Extended Data Fig. 6a). Consistently, Nfkbi2Δm1 T cells were also normal in TCR-CD28-stimulated glycolysis and oxidative phosphorylation (Extended Data Fig. 6b,c), suggesting an NF-kB-independent function of NIK in the regulation of HK2 stability and T cell metabolism.

NIK and HK2 did not physically interact, suggesting an indirect mechanism of HK2 regulation by NIK (Extended Data Fig. 6d). NIK-deficient T cells had a markedly higher level of ROS compared to WT T cells (Fig. 7a). NIK deficiency also made CD8+ T cells aberrantly produce ROS in immune responses against L. monocytogenes infection and in the TME (Fig. 7b,c). Conversely, NIK+ CD8+ T cells had reduced ROS levels (Fig. 7d,e). Since ROS has been implicated as an intracellular trigger for autophagy, we sought to determine the potential connection of ROS with HK2 degradation. Incubation of NIK-deficient T cells with nicotinamide (NAM), a well-defined ROS inhibitor, largely rescued the expression of HK2 in NIK-deficient T cells, which was coupled with reduced ROS levels (Fig. 7f and Extended Data Fig. 7a). Incubation of NIK-deficient T cells with several other antioxidants, such as glutathione (GSH), vitamin C and N-acetylcycteine (NAC), also restored the level of HK2 (Fig. 7g and Extended Data Fig. 7b). Conversely, CD8+ T cell activation in the presence of ROS inducers, such as hydrogen peroxide, paraquat and 1-chloro-2,4-dinitrobenzene, caused HK2 degradation (Extended Data Fig. 7c,d), which was inhibited in Atg5-deficient T cells (Extended Data Fig. 7d). Furthermore, the lysosomal localization of HK2 was inhibited by NAC.
NIK regulates the G6PD–NADPH redox system. Cellular ROS levels are maintained by a balance between ROS production and antioxidant mechanisms; a major antioxidant mechanism is provided by the NADPH redox system. NIK deficiency did not seem to affect signal-induced ROS production since both WT and NIK-deficient T cells increased ROS production on activation by the TCR-CD28 signals (Fig. 7a). NIK deficiency also had no obvious effect on the mass and membrane potential of mitochondria (Extended Data Fig. 7f). NIK-deficient CD8 T cells had profoundly decreased activity of the antioxidant NADPH when activated in vitro by TCR-CD28 stimuli or in vivo by L. monocytogenes infection (Fig. 7h). Conversely, NADPH activity was increased in NIK-deficient CD8 T cells (Fig. 7i). An important function of NADPH is to act as a cofactor of GSH reductase for converting oxidized GSH, GSH disulphide (GSSG), to a reduced form of GSH. In line with the role of NIK in NADPH regulation, the GSH/GSSG ratio was substantially reduced in NIK-deficient (Map3k14<sup>−/−</sup>) CD8 T cells and increased in NIK-overexpressing (NIK<sup>Tg</sup>) CD8<sup>+</sup> T cells (Extended Data Fig. 7g).

NADPH is produced by the pentose phosphate pathway, which is dependent on a rate-limiting enzyme, G6PD<sup>1,11</sup>. By maintaining the concentration of NADPH, G6PD prevents aberrant ROS accumulation and oxidative stress<sup>1,13</sup>. Interestingly, G6PD expression was induced along with T cell activation, which was moderately reduced at the protein level and not affected at the mRNA level by NIK deficiency (Fig. 7i). However, the G6PD enzymatic activity was substantially reduced in NIK-deficient OT-I CD8 T cells that were activated in vitro by anti-CD3 plus anti-CD28 or in vivo by L. monocytogenes infection (Fig. 7k). Conversely, NIK<sup>Tg</sup> T cells displayed increased G6PD activity than WT T cells (Fig. 7l). Functionally, overexpression of exogenous G6PD could restore HK2 expression in NIK-deficient T cells (Fig. 7m), suggesting the involvement of G6PD in NIK-mediated HK2 stabilization.

G6PD is required for HK2 stable expression and T cell functions. We further studied G6PD function using a G6pd<sup>−/−</sup> mouse strain with impaired G6PD expression due to a point mutation in the 3’ end of the untranslated exon 1 of the G6pd
genotype. G6pdmut T cells had markedly reduced concentration of NADPH, which was associated with a lower level of HK2 protein although not HK2 mRNA, under activated conditions (Fig. 8a–c). The HK2 protein level in G6pdmut T cells was restored on incubation with the ROS inhibitor NAM (Fig. 8d). Reconstitution of G6pdmut T cells with exogenous G6PD also efficiently restored HK2 expression (Fig. 8e). To confirm the T cell-intrinsic function of G6PD, we generated bone marrow chimeric mice by adoptively transferring WT or G6pdmut bone marrow cells into Rag2 KO recipient mice. T cells derived from G6pdmut chimeric mice had markedly reduced HK2 expression and increased ROS levels compared to WT control T cells (Fig. 8f,g). These findings demonstrate a crucial role for G6PD in regulating the fate of HK2, which involves control of ROS production via the G6PD–NADPH redox system.

G6pd mutation did not alter the frequency of peripheral T cells but impaired T cell function, as shown by substantially reduced cytokine production and glycolytic metabolism (Fig. 8h,i and Extended Data Fig. 8a). Regarding the mechanism of G6PD regulation by NIK, noncanonical NF-xB activation was dispensable since Nfkb2Δ31/31 and WT T cells had comparable levels of G6PD (Extended Data Fig. 8b). Nfkb2Δ31/31 T cells also did not display aberrant ROS concentration (Extended Data Fig. 8c). Interestingly, NIK physically interacted with G6PD in both OT-I CD8+ T cells and transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfected 293 cells, and NIK phosphorylated G6PD transiently transfe...
G6PD is required for HK2 stable expression and T cell functions. a–c, Whole-cell NADPH concentration (a), immunoblot (b) (right panel in b is a summary graph of densitometric quantification of three independent experiments) and qRT-PCR (c) assays using total T cells isolated from WT (G6pdwt) or G6pdmut mice in vitro-activated with anti-CD3 plus anti-CD28 for 72 h (a,b) or as indicated (c) (n = 2 per genotype; c = n = 4 per genotype). d, Immunoblot analysis using lysates of G6pdwt or G6pdmut T cells, stimulated with anti-CD3 plus anti-CD28 for 48 h in the presence (+) or absence (−) of NAM. e, Summary graph of densitometric quantification of two independent experiments (n = 4 per genotype). f, Immunoblot analysis using lysates of G6pdmut CD8+ T cells transduced with either a control vector or Flag-G6PD. g, Immunoblot (f) and flow cytometry analysis of ROS levels (g) using total T cells isolated from chimeric mice transplanted with G6pdwt or G6pdmut bone marrow, in vitro-activated with anti-CD3 plus anti-CD28 for 48 h. h, Flow cytometry analysis of CD4+ and CD8+ T cells in the spleens of G6pdwt and G6pdmut mice. i, Enzyme-linked immunosorbent assay of IFN-γ and IL-2 in the supernatant of G6pdwt and G6pdmut CD8+ T cell cultures stimulated with anti-CD3 plus anti-CD28 for 66 h. j, Co-immunoprecipitation analysis of endogenous NIK-G6PD interaction using whole-cell lysates of CD8+ T cells isolated from NIK−/− mice and activated for 48 h with anti-CD3 plus anti-CD28 in the presence of 4-hydroxytamoxifen. MG132 and BV6 were added during the last 4 h to block NIK degradation. An immunoprecipitation with IgG was included as a negative control. k, l, Summary graph of G6PD activity and NADPH concentration (k) and flow cytometry analysis of ROS levels (l) in splenic CD8+ T cells of chimeric mice adoptively transferred with G6pdmut bone marrow cells transduced with an empty vector or expression vectors encoding G6PD WT or mutants. m–p, Tumor growth curves (m,o) and flow cytometry analysis of tumor-infiltrating CD8+ T cells producing IFN-γ (n,p) in chimeric mice adoptively transferred with G6pdmut bone marrow cells transduced with the indicated expression vectors (m,n, vector: n = 7, 1 survived, WT: n = 4, 3 survived, S40D: n = 5, 5 survived; o,p, WT: n = 4, 3 survived, S40A: n = 5, 2 survived). q, Immunoblot using T cells of chimeric mice adoptively transferred with G6pdmut bone marrow cells that had been transduced with an empty vector or HK2. r, Flow cytometry analysis of IFN-γ-producing CD8+ T cells derived from chimeric mice of vector-or HK2-transduced G6pdmut bone marrow cells (described in q), in vitro-stimulated for 5 h with phorbol 12-myristate 13-acetate plus ionomycin in the presence of monensin (n = 3 per genotype). Data are representative of one (k–r), two (a,d,e,i) or three (b,c,j) independent experiments. Summary data are shown as the mean ± s.e.m. with P values determined by two-tailed Student’s t-test.

S40D (Extended Data Fig. 8h). CD8+ T cells derived from G6PD WT and S40D, but not S40A, bone marrow chimeric mice displayed strong G6PD activity (Fig. 8k). Reconstitution of G6pdmut T cells with G6PD WT or S40D, but not S40A, also rescued NADPH production (Fig. 8k), prevented aberrant ROS production (Fig. 8l) and increased HK2 expression (Extended Data Fig. 8l). Furthermore, chimeric mice expressing G6PD S40D displayed stronger antitumor immunity, while those expressing S40A displayed reduced antitumor immunity, compared to chimeric mice expressing WT G6PD (Fig. 8m–p). Chimeric mice were also generated using G6pdmut bone marrow cells transduced with either a vector control or HK2 (Fig. 8q). HK2 overexpression efficiently rescued the defect of
G6pd<sup>−/−</sup> T cell IFN-γ production (Fig. 8r). Together, these results suggest that NIK-mediated G6PD regulation plays an important role in regulating the level of HK2 and, thereby, the function of CD8<sup>+</sup> effector T cells.

**Discussion**

The results presented in this article establish NIK as a new regulator of T cell metabolism and antitumor immunity. In contrast to the phosphatidylinositol 3-kinase–Akt–mTORC1 signaling axis, which triggers the initiation of glycolysis by promoting glucose uptake and transcriptional induction of glycolytic enzymes, NIK functions through a post-translational mechanism involving stabilization of HK2. NIK controls cellular ROS levels by maintaining the G6PD–NADPH redox system. These findings not only identified NIK as a pivotal regulator of T cell metabolism but also suggested a new link between the redox system and metabolic pathways.

We have previously shown that NIK is dispensable for naïve T cell activation but plays a crucial role in mediating effector T cell function and recall responses<sup>2,3</sup>. Our present study provides a mechanistic insight into NIK function since competent glycolysis is essential for effector T cell functions. Our data suggest that NIK is crucial for CD8<sup>+</sup> T cell responses against tumorigenesis. Interestingly, transgenic expression of NIK using an inducible system strongly promoted antitumor immunity, which is associated with increased glycolytic metabolism and effector function of CD8<sup>+</sup> T cells. Using a preclinical model of cancer therapy, we further demonstrated that ectopic NIK expression in CD8<sup>+</sup> effector T cells substantially improved the efficacy of adoptive T cell therapy. We found that ectopic NIK expression rendered PD-1<sup>+</sup> Tumor-infiltrating CD8<sup>+</sup> T cells competent in IFN-γ production, a result reminiscent of the recent finding that immune checkpoint inhibitor therapy reinvigorates exhausted CD8<sup>+</sup> T cells without reducing checkpoint molecule expression<sup>1,2</sup>.

Our work revealed an NF-κB-independent mechanism of NIK function in regulating the fate of HK2, a rate-limiting enzyme catalyzing the first step of glycolysis. Previous studies suggested a dispensable role for HK2 in mediating T cell responses to acute infections by herpes simplex virus<sup>1</sup> and the Armstrong strain of LCMV<sup>2,11</sup>. Interestingly, our present study demonstrates an important role for HK2 in regulating CD8<sup>+</sup> T cell function in antitumor immunity. The discrepancies between this study and previous ones are likely due to the different experimental models used: previous studies employed acute viral infection models, whereas this study used tumor immunity models that mimic chronic viral infections. In further support of this explanation, we found that HK2 deficiency impairs CD8<sup>+</sup> T cell responses to chronic infections by LCMV clone 13. This study also led to the identification of a T cell-intrinsic role for HK2 in regulating thymocyte development and mediating the lethal autoimmune caused by constitutive overexpression of NIK in T cells. Together, these findings suggest that HK2 plays a crucial role in regulating specific aspects of T cell functions, particularly CD8<sup>+</sup> T cell responses to tumorigenesis and chronic viral infections.

Our data suggest that NIK deficiency causes HK2 degradation by selective autophagy due to aberrant ROS accumulation. NIK facilitates production of NADPH, a major redox system controlling ROS levels, by phosphorylating and promoting the activity of G6PD, an enzyme catalyzing NADPH generation in the pentose oxidation pathway. We obtained genetic evidence that G6PD was crucial for controlling ROS concentrations, preventing HK2 degradation and supporting T cell metabolism. Collectively, these findings demonstrate a new mechanism of metabolic regulation involving post-translational regulation of HK2 by NIK and establish NIK as an important regulator of effector T cell function in antitumor immunity. These findings also suggest that NIK regulates T cell function via both noncanonical NF-κB-dependent and independent mechanisms.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-00829-6.

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Methods

Mice. Map3k14 fox mice (C57BL/6 background), provided by Genentech, were crossed with Cd4-Cre transgenic mice (B6.Cg-Tg(Cd4-cre)1Cwbl/Bluj, Jackson Laboratory) to produce age-matched Map3k14+/−Cd4-Cre (WT) and Map3k14+/-CreER (mutant) mice. The NIK transgenic mice R26StopFL Map3k14+/− (Map3k14; conditional knock-out or NIK+/-; mice). The NIK transgenic mice R26StopFL Map3k14+/−, carrying a Map3k14 transgene encoding WT NIK under the control of a loxP-flanked STOP cassette (C57BL/6-G(Rosa)26StopFL(CreERT2); the Jackson Laboratory), were crossed with ROSA26-CreERT (B6.129-G(Rosa26Sor); Map3k14+/−R26StopFL/+ (for WT cells) or 1 mM of carboxyfluorescein succinimidyl ester (CFSE) (for NIK+/-mice) were further purified by fluorescence-activated cell sorting (FACS) based on the tumor-infiltrating OT-I CD8+ T cells isolated from WT OT-I or Map3k14+/−OT-I mice. The gating strategy used is shown in Supplementary Fig. 1. in the presence of monensin (eBioscience). Single-cell suspensions of the cells were subjected to flow cytometry analysis. FACS data were analyzed with FlowJo v.10.5.3 (FlowJo LLC). The gating strategy used is shown in Supplementary Fig. 1.

To isolate tumor-infiltrating T cells, tumors were weighed, cut into pieces and digested for 30 min at 37 °C in an 70–100 μm cell strainer and cells were collected by centrifugation at 1,500 r.p.m. for 5 min at room temperature. After incubation in a red blood cell lysis buffer, cell suspensions were passed through a 70-μm cell strainer and cells were selected for flow cytometry analysis of tumor-infiltrating T cell frequencies. For T cell restimulation and cytokine production analyses, T cells were enriched using Percoll gradient and further purified with a Pan T Cell Isolation Kit (Thermo Fisher Scientific). In some experiments, cells from two or three tumor samples were combined to obtain sufficient T cell numbers for the analyses.

Adaptive cell therapy was performed using Pmel1 TCR transgenic mice that produce CD8+ T cells recognizing the B16 melanoma antigen gp100.T lymphom. Map3k14+/−CreER and Map3k14+/CreER mice were crossed with Pmel1 mice to generate Map3k14+/−CreER-Pmel1 and Map3k14+/CreER-Pmel1 mice. Splenocytes were isolated from these mice and treated with 0.2 μg ml−1 of 4-hydroxytamoxifen in vitro along with activation with plate-coated anti-CD3 (1 μg ml−1) and soluble anti-CD28 (1 μg ml−1) to produce activated WT and NIK+/- mice. The culture was provided with murine interleukin-2 (IL-2; 10 ng ml−1) on day 2 and CD8+ T cells were purified from the culture on day 5 and used for the adoptive transfer experiments. To generate tumor-bearing mice, WT B6.SJL mice (expressing the CD45.1 congenital marker) were injected subcutaneously with 105 B16F10 melanoma cells; after 4 d, tumor-bearing mice were subjected to whole-body irradiation (300 rad, 137Cs irradiator) to induce lymphodepletion. One day after the irradiation, mice were injected with in vitro-activated WT Pmel1 or NIK+/- Pmel1. Control mice were not irradiated and injected with Pmel1 T cells. Tumor size was measured every other day for the indicated time.

T cell transfer to measure tumor infiltration and intratumoral survival. CD8+ T cells isolated from WT OT-I or Map3k14+/−OT-I mice were stimulated in vitro using plate-bound anti-CD3 (1 μg ml−1) plus soluble anti-CD28 (1 μg ml−1) antibodies, provided with murine IL-2 (10 ng ml−1) on day 2 and cultured for an additional day. For the tumor infiltration analysis, the activated OT-I T cells were labeled with 1 μM of chloromethyl tetramethylrhodamine (CMTRM) (Biotium, San Carlos, CA) or 1 μM of tetramethylrhodamine isothiocyanate (CMTCRM; Invitrogen, Carlsbad, CA), mixed at a 1:1 ratio (1.1 millions of each) and adoptively transferred into B16-OVA-bearing B6 mice (17 d after tumor cell injection). After 24 h of T cell transfer, tumor-infiltrating WT and Map3k14+/− OT-I CD8+ T cells were quantified by flow cytometry based on CMTRM (red) and CFSE (green), respectively. For the intratumoral survival and proliferation assays, activated OT-I CD8+ T cells were labeled with CFSE and adoptively transferred into day 14 B16-OVA-tumor-implemented B6.SJL mice. After 7d, apoptosis and proliferation of the tumor-infiltrating OT-I CD8+ T cells was analyzed by flow cytometry based on caspase-3 cleavage and CFSE dilution, respectively.

T cell isolation, treatment and flow cytometry analysis. Total and CD8+ T cells were isolated from spleens and lymph node cells with anti-CD90.2- and anti-CD8-conjugated magnetic beads (Miltenyi Biotec), respectively. Naïve T cells were further purified by fluorescence-activated cell sorting (FACS) based on CD44+ CD62L+ surface markers. For in vitro generation of NIK+/- T cells, T cells isolated from Map3k14+/−CreER and Map3k14+/−CreER in a specifically pathogen-free facility of the University of Texas MD Anderson Cancer Center and all animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Antibodies and reagents. Antibodies used for the western blot were: G6PD (clone DS52, catalog no. 122635; Cell Signaling Technology); HK2 (clone C64G5, catalog no. 2867; Cell Signaling Technology); NIK (clone A12, catalog no. sc-8417; Santa Cruz Biotechnology); H2 (clone 14F6, catalog no. 11962-1-AP, ProteinTech); Glut1 (clone EPR3915, catalog no. ab115730; Abcam); Pim2 (clone D78A4, catalog no. 4053; Cell Signaling Technology); Akt (clone C67E7, catalog no. 4691; Cell Signaling Technology); p-Akt (Ser473) (clone D9E, catalog no. 4060; Cell Signaling Technology); p-Akt (Thr308) (clone D25E6, catalog no. 13038, Cell Signaling Technology); c-Myc (clone D814C2, catalog no. 5605; Cell Signaling Technology); phospho-S6 ribosomal protein (Ser235/236) (clone D357.2,2E, catalog no. 4858; Cell Signaling Technology); PpKα/β M2A (ERK2/1) (clone 1375S, catalog no. 4695; Cell Signaling Technology); IκBα (clone J5A5, catalog no. 4814; Cell Signaling Technology); NF-κB p100/p52 (catalog no. 4882; Cell Signaling Technology); SQSTM1/p62 (clone D6M5X, catalog no. 23214, Cell Signaling Technology); LC3 (catalog no. PM056; MBL International); Atg6 (clone D535U, catalog no. 12994; Cell Signaling Technology); LAMP1 (clone C54H11, catalog no. 3245; Cell Signaling Technology); LAMP-2A (clone AMC52, catalog no. M21-200; Thermo Fisher Scientific); HSP70 (REF 4872; Cell Signaling Technology); GAPDH (clone no. 10494-1-AP, ProteinTech); β-actin (clone C4, catalog no. sc-47778; Santa Cruz Biotechnology); HA-peroxidase (clone 3F10, catalog no. 1201381901; Roche); FLA-G-PE (clone G10L33, catalog no. SAB4305520; Sigma-Aldrich); γ-H2AX (clone 6C1, catalog no. ab245777; Abcam); Proteohistone H2B (clone EPR3887, catalog no. ab209847, Abcam); goat-anti-rabbit IgG H&L (Alexa Fluor 488) (catalog no. ab110077; Abcam). Pepsatin A, E64d, chloroquine, NAM, GSH, L-ascorbic acid (vitamin C), NAC, paraquat dichloride, 1-chloro-2,4-dinitrobenzene, sodium taurocholate, and monensin were obtained from Sigma-Aldrich. The CellROX Deep Red Reagent was obtained from Invitrogen.

Tumor models. Map3k14+/− or H2a+/- mice and their age- and sex-matched WT controls (6–8 weeks old) were injected subcutaneously with B16F10 murine melanoma cells (ATCC) or with 5 × 10^5 MC38 colon cancer cells (ATCC) and monitored for tumor growth and survival. Mice with tumor size reaching 22 mm were considered lethal and euthanized according to protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. At the indicated time point, all mice were euthanized for flow cytometry analysis of immune cells from both the draining lymph nodes and tumors to test the therapeutic effect of ectopic NIK expression in tumor-bearing mice. Age- and sex-matched Map3k14+/−CreER and Map3k14+/CreER mice were injected subcutaneously with B16F10 or MC38 tumor cells and tumor-bearing mice were then injected with tamoxifen intraperitoneally to induce NIK expression as indicated in the experiments, producing tumor-bearing WT and NIK+/- mice.

Quantitative PCR with reverse transcription (qRT–PCR) assays. RNA was isolated using the TRIzol reagent and subjected to qRT–PCR assays using the SYBR reagent (Bio-Rad Laboratories) and the following primers for mouse genes: HK2 (forward: 5′-GATGGCGCCTTTAGGGACAGA-3′; reverse: 5′-GGTCACTGCTGTTAGGCTTC-3′); G6PD (forward: 5′-GAATTCTGGTGTGCTGCTC-3′; reverse: 5′-CCAGTACCCAGACCCACCC-3′); p-CD28 (forward: 5′-CCGAAAAGATGGACCCAGATCA-3′; reverse: 5′-CAGCCTGGTGAAGTGCAGT-3′); Pdcd1 (forward: 5′-GGGACCTTGGCTCCTCCTAC-3′; reverse: 5′-ATATCCCCCCTTTCCTCCCT-3′); Haver2 (forward: 5′-AGGTATTGCAAGGAAATGTTAGTGTGGAAG-3′; reverse: 5′-CAGTAGGGCCTAGGATGACCC-3′).

L. monocytogenes infection. Age- and sex-matched wild-type and Map3k14+/− or wild-type and H2a+/- mice (6–8 weeks old) were infected intravenously with 1 × 10^5 c.f.u. of recombinant L. monocytogenes expressing chicken ovalbumin (OVA) (provided by H. Shen, University of Pennsylvania). For analysis of bacterial burden in spleen or liver, the mice were killed four days after infection. For analysis of
OVA-specific CD8+ effector T cells in the spleen, the mice were killed seven days after infection. Then, splenocytes were stimulated overnight with 10 μg ml−1 of the OVA257–264 peptide (SHINEKEL, Genemed Synthesis), with a protein transport inhibitor, monensin, added during the last hour of culture. The cells were then subjected to intracellular IFN-γ staining and flow cytometry analysis.

LCMV infection. Age- and sex-matched WT and Map3k14mut/− or WT and HK2mut/− mice (6–8 weeks old) were infected intravenously with 4×106 plaque-forming units of LCMV clone 13 as provided by E. John Wherry, University of Pennsylvania. Eighty-four days after infection, mice were euthanized for the analysis of antigen-specific CD8+ T cells in the spleen. In brief, splenocytes were stimulated with 3 μg ml−1 of LCMV GP33–41 peptide (KAYVYNMAT; AnaSpec) for 14 h with monensin added during the last hour. Cells were then subjected to intracellular IFN-γ staining and flow cytometry analysis.

Metabolic assays. ECAR and OCR were measured with an XF96 extracellular flux analyzer (Seahorse Bioscience) as described previously47. In brief, naive CD8+ T cells isolated from the spleen of the indicated mice were either not treated (defined as naive) or activated for 24 h in vitro with anti-CD3 plus anti-CD28 (defined as activated) and then subjected to metabolic assays. For the metabolic assay of tumor-infiltrating lymphocytes (TILs), Map3k14mut/− and age- and sex-matched WT control mice (6–8 weeks old) were injected subcutaneously with 2×106 B16F10 murine melanoma cells. TILs isolated from 5 pairs, day 16 tumor was mixed and further sorted with CD90.2+ MACS beads (Milteny Biotec) followed by activation with anti-CD3 and anti-CD28 for 24 h. The activated TILs were subjected to ECAR and OCR analysis. In some experiments, OT-I CD8+ T cells were isolated from the day 7 spleen of L. monocytogenes–infected mice directly subjected to metabolic analysis. ECAR was measured with an XF96 extracellular flux analyzer in a glycolysis stress test medium under basal (injection of glucose) and maximum or stressed (injection of oligomycin) conditions. The OCR measured using a Mito Stress kit (Seahorse Biosciences) under baseline (no treatment) and maximum or stressed (injection of FCCP) conditions, as described previously38. NADPH measurements were performed with an NADP/NADPH Quantitation Colorimetric Kit (BioVision) and G6PD activity was determined with the Glucose-6-Phosphate Dehydrogenase Activity Assay Kit (Sigma-Aldrich). The GSH/GSSG ratio was detected with the GSH/GSSG Ratio Detection Assay Kit (Abcam).

Glucose uptake assay for T cells. Sorted CD8+ T cells activated with anti-CD3 and anti-CD28 for the indicated time or CD8+ TILs prepared as described above were washed with PBS at room temperature and then incubated in glucose-free RPMI 1640 medium containing 100 μM of 2-NBDG (Cayman Chemical). For negative controls, the cells were incubated in glucose-free RPMI 1640 medium without 2-NBDG. After 1 h incubation in a 37 °C water bath, cells were washed with ice-cold PBS and immediately subjected to flow cytometry analysis of 2-NBDG uptake.

Immunoblot and co-immunoprecipitation. For the immunoblot analysis, T cells or 29T cells were lysed in radioimmunoprecipitation assay buffer (50 mM of Tris-HCl pH 7.4, 150 mM of NaCl, 1% (vol/vol) Nonidet P-40, 0.5% (vol/vol) sodium deoxycholate and 1 mM of EDTA). Cytosolic and lysosomal fractions were prepared with the Lysosome Purification Kit from Biovision according to the manufacturer’s instructions. Co-immunoprecipitation and western blotting were performed essentially as described in Xiao et al.46. Primary OT-I CD8+ T cells treated with anti-CD3 plus anti-CD28 for the indicated times along with the proteasome inhibitor MG132 and the c-IAP inhibitor BV6. MG132 and BV6 were added to stabilize NIK. Treated OT-I CD8+ T cells or transiently transduced 29T cells were lysed in radioimmunoprecipitation assay buffer and subjected to immunoprecipitation using the indicated antibodies followed by immunoblot analysis of the co-immunoprecipitated proteins.

Mapping sites of G6PD phosphorylation induced by NIK. G6PD was phosphorylated by NIK in an in vitro kinase assay performed as described previously45. In brief, the reaction was carried out in 50 μl of reaction mix containing 50 mM of Tris-HCl (pH 7.5), 2 mM of ATP, 5 mM of MgCl2, 0.1 μM OA (okinon), 500 ng of recombinant human G6PD (Novus Biologicals) as the substrate and different doses (200 ng and 400 ng) of recombinant human NIK protein (Thermo Fisher Scientific). The mixture was incubated at 30 °C for 30 min and analyzed by immunoblot analysis with Phos-tag SDS–PAGE from Wako Chemicals. The product from the in vitro kinase assay was subjected to LC–MS/MS analysis at the Proteomics Core Facility of the University of Texas MD Anderson Cancer Center.

In vitro cytotoxic T cell assays. CD8+ T cells isolated from Map3k14mut/−CreER or Map3k14mut/−CreER Pmel1 mice were activated in vitro for 1 d with anti-CD3 plus anti-CD28 in the presence of the CreER inducer 4-hydroxymethylbenzen and then cultured in IL-2–supplemented medium. Map3k14mut/−CreER Pmel1 effector CD8+ T cells were labeled with CFSE and cocultured for 3 h with B16F10 tumor cells (1×105) at the indicated ratios in a U-bottom 96-well plate. Cells in all wells were assayed with 0.25% trypsin–EDTA and CFSE− tumor cells were subjected to flow cytometry to measure cleaved caspase-3 to assess the cytotoxicity of the effector CD8+ T cells.

Retroviral transduction. Human HK2 complementary DNA was cloned into the pMIGR1-green fluorescent protein (GFP) retroviral vector and murine G6pd cDNA was cloned into the pPRich-mCherry retroviral vector. To produce retroviral particles, HEK293 cells were transfected with the retroviral expression vectors along with the packaging vector pC-L. Eco. To transduce primary T cells, T cells were cultured in 48-well plates (1×105 per well) for 24 h with plate-bound anti-CD3 (1 μg ml−1) plus anti-CD28 (1 μg ml−1) in the presence of 10 ng ml−1 of IL-15 and 5 ng ml−1 of IL-2, then infected twice (at 48 and 72 h) with retroviruses. For bone marrow cell infection, bone marrow cells were cultured in 48-well plates (1×106 per well) for 24 h with 100 ng ml−1 stem cell factor, 10 ng ml−1 IL-6 and 10 ng ml−1 IL-3 and then infected twice (at 48 and 72 h) with retroviruses. Twenty-four hours after the second retroviral transduction, transduced cells were enriched by flow cytometry cell sorting on the basis of GFP (for pMIGR1-based vectors) or mCherry (for pPRich-based vectors) expression.

Bone marrow adoptive transfer. Bone marrow cells were isolated from G6pdmut/− and WT control mice under sterile conditions. After removing red blood cells in a red blood cell lysis buffer (Sigma-Aldrich), bone marrow cells were counted and resuspended in ice-cold sterile PBS and 2×106 bone marrow cells were transferred into lethally irradiated (1,000 rad) Rag1 KO (where indicated, Rag2 KO) mice. After 6 weeks, bone marrow chimeric mice were used for the indicated experiments. In some experiments, G6pdmut/− bone marrow cells were transduced with the indicated expression vectors and enriched by flow cytometry sorting as described above.

RNA sequencing (RNA-seq) analysis. Naive CD8+ T cells were isolated from the spleen of young adult (6–8-week-old) WT OT-I or Map3k14mut/− OT-I mice and were either immediately lysed for RNA preparation or activated for 24 h with plate-bound anti-CD3 (1 μg ml−1) and anti-CD28 (1 μg ml−1). Total RNA was isolated with TRIzol (Invitrogen) and subjected to RNA-seq analysis using an Illumina sequencer in the sequencing and microarray facility of the University of Texas MD Anderson Cancer Center as described previously47.

Statistical analysis. Statistical analysis was performed with Prism 8 (GraphPad Software). For tumor growth curves, differences between groups were evaluated by two-way analysis of variance (ANOVA) with Bonferroni correction. For survival, differences between groups were evaluated by two-sided log-rank test. Other statistical analyses were performed by two-tailed unpaired t-test. All statistical values were considered as appropriate and data met the assumptions of the tests. Variance was similar between the groups being statistically compared. All data are shown as the mean±s.e.m. Each experiment was repeated independently with similar results. The number of animals, number of independent experiments and methods of the statistical tests used are indicated for each experiment in the figure legends.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNA-seq datasets have been deposited in the Gene Expression Omnibus under accession no. GSE155576. The mass spectrometry data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with dataset identifier PXD020943 and https://doi.org/10.6019/PXD020943. Other datasets generated during the current study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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**Author contributions**

M.G. designed and performed the research, prepared the figures and wrote part of the manuscript. X. Zhou designed and performed the research and prepared the figures. L.Z., Z.J., J.-Y.Y., X.X., J.Y., Y.S. and X.C. contributed to the experiments. X. Zheng and J.W. performed the RNA-seq data analysis. J.H.S. and J.B.K. contributed the G6pd<sup>−/−</sup> mouse materials. H.D.B. contributed the Map3k14 flox mice, and S.-C.S. supervised the work and wrote the manuscript.

**Competing interests**

J.Y. is an employee of Precision for Medicine. H.D.B. is an employee of Genentech. The other authors declare no competing interests.

**Additional information**

Extended data is available for this paper at [https://doi.org/10.1038/s41590-020-00829-6](https://doi.org/10.1038/s41590-020-00829-6).

Supplementary information is available for this paper at [https://doi.org/10.1038/s41590-020-00829-6](https://doi.org/10.1038/s41590-020-00829-6).

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Extended Data Fig. 1 | NIK regulates T cell exhaustion and antitumor immunity. a, Genotyping PCR of R26Stop\(^{\text{fl}}\)Map3k14\(^{\text{Tg}}\), Map3k14\(^{+/+}\)CreER, and R26Stop\(^{\text{fl}}\)Map3k14-CreER (Map3k14\(^{+/+}\)CreER) mice, showing the PCR products of NIK-GFP in Map3k14\(^{+/+}\) allele and CreER. b, Immunoblot analysis of NIK expression in tamoxifen-treated Map3k14\(^{+/+}\)CreER (WT) and Map3k14\(^{Tg\text{CreeR}}\) (iTg) mice. c, Schematic of experimental design for producing B16F10 tumor-bearing NIK\(^{\text{tum}}\) and WT control mice. Each mouse was injected s.c. with 5 \times 10^5 B16F10 cells (WT = 9, iTg = 7). d–e, Flow cytometric analysis of the frequency and absolute cell number of CD4 and CD8 T cells in the tumor (d) or draining lymph node (e) of day 18 B16F10 tumor-implanted NIK-iTg and WT mice (d, WT: n = 4; iTg: n = 5; e, n = 6 per genotype). f–g, Flow cytometric analysis of the frequency and absolute number of CD44\(^{+}\)CXCR3\(^{+}\)CD8 effector T cells in the draining lymph node (f) or tumor (g) of day 18 B16F10 tumor-implanted NIK-iTg and wildtype mice (f, n = 6 per genotype; g, n = 4 per genotype). h–i, Flow cytometric analysis of the frequency and absolute number of PD1\(^{+}\)Tim3\(^{+}\)CD8\(^{+}\) T cells (h) or IFN-\(\gamma\)-producing PD1\(^{+}\)Tim3\(^{+}\)CD8\(^{+}\) T cells (i) in the tumor of day 18 B16F10-implanted NIK\(^{\text{tum}}\) and wildtype control mice (h, n = 4 per genotype). Data are representative of three independent experiments. Summary data are shown as mean \(\pm\) s.e.m. with P values determined by two-tailed Student’s t test.
Extended Data Fig. 2 | Ectopic expression of NIK improves CD8 T cell function in adoptive T cell therapy. a,b, Flow cytometric analysis of the frequency of total donor CD8 T cells (a) and IFNγ-producing donor CD8 T cells (b) in the tumor of B16F10 melanoma-bearing B6.SJL recipient mice adoptively transferred with wildtype or NIKi Tg Pmel1 CD8 T cells as described in Fig. 2o (n = 4 per genotype). c, In vitro cytotoxicity assay of wildtype (WT) and NIKi Tg Pmel1 CD8 effector T cells towards B16F10 tumor cells at the indicated effector to tumor (E:T) ratios. d-f, Schematic of experimental design (d) and flow cytometric analysis of apoptosis based on caspase 3 cleavage (e) or proliferation based on CFSE dilution (f) of tumor-infiltrating wildtype (WT) and NIKi OT-I CD8 T cells in B16-OVA-tumor bearing B6.SJL mice adoptively transferred with in vitro activated and CFSE-labeled WT or NIKi CD8 T cells for 7 days (e, n = 3 per genotype). Data are representative of two independent experiments. Summary data are shown as mean ± s.e.m. with P values determined by two-tailed Student’s t test.
Extended Data Fig. 3 | NIK deficiency has no effect on mRNA expression of glycolysis-regulatory genes and glucose uptake but promotes lysosomal localization of HK2. **a**, Seahorse analysis of OCR under baseline (no treatment) and maximum or stressed (injection of FCCP) conditions in untreated Map3k14^tKO (tKO) or wildtype (WT) naïve CD8 T cells. Data are shown as a representative plot (upper) and summary graphs (lower, each circle represents a well). **b**, Volcano plot of RNA sequencing analysis of differentially expressed genes in Map3k14^tKO OT-I CD8 T cells relative to WT OT-I CD8 T cells, activated with anti-CD3 plus anti-CD28 for 24 hr. **c**, Immunoblot analysis of the indicated proteins in WT or Map3k14^tKO OT-I CD8 T cells stimulated with anti-CD3 plus anti-CD28 for the indicated time periods. **d,e**, Flow cytometric analysis of glucose uptake using in vitro activated (d) or B16F10 tumor-infiltrating (e) CD8 T cells. **f**, Flow cytometry analysis of HK2 expression in tumor-infiltrating CD8 T cells of B16F10-bearing wildtype (WT) or Hk2^ko mice, showing the specificity of the HK2 staining. **g**, Immunoblot analysis of HK2 and the indicated control proteins in the cytoplasmic (Cy) and lysosomal (Ly) fractions of wildtype (WT) and Map3k14^tKO CD8 T cells. **h**, Immunoblot analysis of the indicated proteins in whole cell lysates of wildtype or Map3k14^tKO CD8 T cells treated for the indicated time points in the presence (+) or absence (−) of lysosomal inhibitors, E64D plus pepstatin A. **i**, Summary of **h** based on densitometric quantification of three independent experiments. Data are representative of two (**b,f**) or three (**a,c-e,g,h**) independent experiments. Summary data are shown as mean ± s.e.m. with P values determined by two-tailed Student’s t test.
Extended Data Fig. 4 | T cell-specific deletion of HK2 impairs T cell metabolism and immune responses against tumorigenesis and bacterial infection.

**a, b.** Genotyping PCR (a) and immunoblot (b) analysis of the Hk2\(^{tKO}\) (tKO) and wildtype (WT) control mice.

**c, d.** Seahorse analysis of basal ECAR (measured after glucose injection, Glc) and maximum ECAR (measured after oligomycin injection, Oligo) (c) and Seahorse analysis of baseline OCR (no treatment) and maximum OCR (FCCP injection) (d) in Hk2\(^{tKO}\) or wildtype OT-I CD8 T cells either naïve or activated with anti-CD3 and anti-CD28 for 24 h.

**e, f, g.** Seahorse analysis of the expression level of PD1 and Tim3 in the CD8 T cells isolated from day-20 tumor of the B16F10-implanted Hk2\(^{tKO}\) and wildtype control mice (n = 4 per genotype).

**h, i.** Flow cytometric analysis of the frequency and absolute number of IFN\(\gamma\)-producing CD8 effector T cells in splenocytes (f) and bacterial burden in the spleen (g) of Hk2\(^{tKO}\) or wildtype mice infected i.v. with L. monocytogenes (1 \(\times\) 10\(^5\) CFU/mouse) for 7 (f) or 4 (g) days (f, n = 4 per genotype; g, n = 6 per genotype).

**j, k.** Flow cytometric analysis of IFN\(\gamma\)-producing CD8 T cells in the spleen of the indicated mouse strains infected for 84 days with LCMV clone 13 (4 \(\times\) 10\(^6\) PFU/mouse), restimulated in vitro with 3 \(\mu\)g/ml LCMV gp33-41 peptide for 14 h with monensin added during the last hour; WT (n = 6), Map3k14\(^{tKO}\) (n = 6) and Hk2\(^{tKO}\) (n = 3). Data are representative of two (f-h) or three (a-e) independent experiments. Summary data are shown as mean ± s.e.m. with P values determined by two-tailed Student’s t test.
Extended Data Fig. 5 | NIK-mediated stimulation of antitumor T cell responses requires HK2. Flow cytometric analysis of the frequency and absolute cell number of CD4 and CD8 T cells (a), PD1+Tim3+ CD8 T cells (b) and flow cytometric analysis of the expression level (MFI) of PD1 and Tim3 in CD8 T cells (c) in day-27 tumor of wildtype (WT), NIKi Tg (iTg), NIKi Tg Hk2iKO (iTg-iKO) mice injected s.c. with 5 × 10^5/mouse MC38 colon cancer cells (a, b, WT: n = 4; iTg: n = 4; iTg-iKO: n = 3). Data are representative of two independent experiments. Summary data are shown as mean ± s.e.m. with P values were determined by two-tailed Student’s t test.
Extended Data Fig. 6 | Noncanonical NF-κB activation is not required for HK2 stabilization or T cell metabolism. 

a, Immunoblot analysis of HK2 expression and p100 processing (as a measure of noncanonical NF-κB activation) in WT or Nfkb2−/− (Lym1+/+) OT-I CD8 T cells stimulated with anti-CD3 plus anti-CD28 for the indicated time periods. 

b, Seahorse analysis of basal ECAR (after glucose injection) and maximum ECAR (after oligomycin injection) and Seahorse analysis of baseline OCR (no treatment) and maximum OCR (FCCP injection) of WT or Nfkb2−/− (Lym1+/+) OT-I CD8 T cells, either naïve or activated with anti-CD3 and anti-CD28 for 24 h, under baseline and stressed conditions. 

c, Coimmunoprecipitation analysis of NIK-HK2 interaction using whole-cell lysates of 293 cells transfected with (+) or without (-) the indicated expression vectors. Data are representative of three independent experiments. Summary data are shown as mean ± s.e.m. based on multiple wells (each circle represents a well) with P values determined by two-tailed Student’s t test.
Extended Data Fig. 7 | ROS is involved in HK2 degradation in NIK-deficient CD8 T cells. a, Flow cytometric analysis of ROS levels in Map3k14\(^{\text{tKO}}\) OT-I CD8 T cells activated with anti-CD3 plus anti-CD28 for 48 h in the presence of NAM or solvent control DMSO. b, c, Immunoblot analysis of HK2 expression in Map3k14\(^{\text{tKO}}\) (tKO) or wildtype (WT) OT-I CD8 T cells activated with anti-CD3 plus anti-CD28 for 48 h in the presence of the antioxidant N-acetylcysteine (NAC) or medium control (b) or the indicated ROS inducers and DMSO control (c). d, Immunoblot analysis of HK2 in wildtype (WT) or Atg5\(^{-}\)tKO CD8 T cells activated with anti-CD3 plus anti-CD28 for 48 h in the presence of the indicated ROS inducers or medium control. e, Immunoblot analysis of HK2 and the indicated loading controls in lysosomal (Ly) and cytoplasmic (Cy) fractions of wildtype (WT) and Map3k14\(^{\text{tKO}}\) CD8 T cells activated with anti-CD3 plus anti-CD28 for 48 h in the presence of NAC or medium control. f, Flow cytometry analysis of the mass and membrane potential of mitochondria in wildtype (WT) and Map3k14\(^{\text{tKO}}\) (tKO) CD8 T cells stimulated with anti-CD3 plus anti-CD28 for 24 h. g, Ratio of reduced (GSH) and oxidized (GSSG) forms of glutathione in Map3k14\(^{\text{tKO}}\) (upper), NIK\(^{\text{Tg}}\) (lower), or wildtype (WT) control OT-I CD8 T cells activated in vitro with anti-CD3 plus anti-CD28 for 48 h. Data are representative of three independent experiments. Summary data are shown as mean ± s.e.m. with P values determined by two-tailed Student’s t test.
Extended Data Fig. 8 | G6PD phosphorylation by NIK and role in regulating HK2 expression and T cell metabolism.

**a.** Seahorse analysis of basal ECAR (measured after glucose injection, Glc) and maximum ECAR (measured after oligomycin injection, Oligo) and Seahorse analysis of baseline OCR (no treatment) and maximum OCR (FCCP injection) in G6PDmut or wildtype (WT) control T cells activated by anti-CD3 plus anti-CD28 for 48 h.

**b, c.** Immunoblot analysis of the indicated proteins (b) and ROS detection (c) in wildtype or Nfkb2myl/+(Lym1/+) CD8 T cells, which were either not treated (NT) or stimulated with anti-CD3 plus anti-CD28 for the indicated time periods.

**d.** CoIP analysis of NIK-G6PD interaction (upper) and immunoblot analysis of HA-NIK and Flag-G6PD expression in whole-cell lysates (WCL) of 293 cells transfected with (+) or without (−) Flag-G6PD and HA-NIK.

**e.** Phos-tag SDS PAGE and Immunoblot analysis of phosphorylated (p-) and total G6PD as well as GST-NIK in an in vitro kinase assay mix containing 500 ng recombinant His-G6PD and the indicated amounts of recombinant GST-NIK.

**f.** NIK-induced G6PD phosphorylation sites identified by mass spectrometry analysis of G6PD phosphorylated in vitro by NIK.

**g.** G6PD activity in 293 T cells transiently transfected with Flag-tagged wildtype G6PD or the indicated G6PD mutants along with either an empty vector or HA-NIK.

**h, i.** Schematic of experimental design (h) and immunoblot analysis of G6PD expression in T cells isolated from bone marrow chimeric mice constructed using G6PDmut bone marrow cells reconstituted with either an empty vector (Vector) or the indicated G6PD expression vectors (i). For a and g, data are shown as representative plots, each circle represents a well. Data are representative of one (f) or three (a–e, g–i) independent experiments. Summary data are shown as mean ± s.e.m. with P values determined by two-tailed Student’s t test.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- The Flow cytometric data: FACS fortesa and FACSArria [BD Biosciences]
- The RNA seq data: HiSeq 4000 [illumina, USA]
- The qRT-PCR data: Bio-Rad CFX384
- the seahorse data: Seahorse XFe96

Data analysis
- Statistical analysis: GraphPad Prism (Ver 8)
- Flow cytometric analysis: Flowjo (ver. 10.5.3)
- RNA seq data analysis: TopHat2 RNASeq alignment software, Multiplet in Genepattern from Broad institute, Genesis
- qRT-PCR analysis: Bio-Rad CFX manager (version 3.1 for Windows)

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: Sample sizes were chosen based on prior experience with same experimental design according to the former publication of the group (PMCID: PMC658407), without prior power analysis. We usually used more than 3-5 mice per group to ensure the statistically significant difference could be obtained from unpaired two-tailed Student’s t-test, ANOVA analysis with Bonferroni’s post-test or Log-Rank test. We described the exact numbers of animals/samples for each experiments in the figure legends.

Data exclusions: No data were excluded from the analysis

Replication: The experimental findings were reliably reproduced. The replication numbers were described in the corresponding figure legends

Randomization: Age- and sex- matched mice were assigned randomly to experimental and control groups. Other experimental allocation is random.

Blinding: The assessment of tumor sizes was performed in a blinded fashion. Experimental analyses of mouse samples were obtained by automated methods (flow cytometry, qRT-PCR machine, et al.). Other experimental techniques were not necessary blinded since mice were grouped by genotype and/or treatment rather than subjective allocation, however, data analyzed were blinded.

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| Antibodies                       | ChiP seq |
| Eukaryotic cell lines            | Flow cytometry |
| Palaeontology                    | MRI-based neuroimaging |
| Animals and other organisms      |         |
| Human research participants      |         |
| Clinical data                    |         |

Antibodies

For flow cytometry antibodies used were:
- CD4 clone GKL1.5, REF 25-0041-82, ebioscience, 1:100
- CD8 clone 53-6.7, Cat 100725, Biolegend, 1:200
- CD44 clone 1M7, REF 11-0441-85, ebioscience, 1:100
- CD45.1 clone A20, REF 11-0453-85, ebioscience, 1:100
- CD45.2 clone 104, REF 25-0454-82, ebioscience, 1:100
- PD-1 clone J43, REF 11-9985-82, ebioscience, 1:100
- TIM-3 clone RMT3-23, Cat 119703, Biolegend, 1:200
- IFN-γ clone XVIG1.2, Cat 505810, Biolegend, 1:100
- CXCR3 clone CXCR3-173, REF 17-1831-82, ebioscience, 1:100
- H2K clone EPR20839, REF ab209847, abcam, 1:50
- Goat anti-Rabbit IgG, H&L (Alexa Fluor® 488) (REF ab150077, abcam, 1:2000)

For western blot antibodies used were:
- GSPD clone DS02, REF 12263, CST, 1:1000
- HK2 clone C64G5, REF 2867, CST, 1:1000
- NIK clone A12, REF sc-8417, SantaCruz, 1:250
- HK1 REF 19662-1-AP, Proteintech, 1:1000
- Glut1 clone EPR3915, REF ab115730, abcam, 1:1000
- PKM2 clone D7/8A4, REF 4053, CST, 1:1000
- AKT clone C66E7, REF 4691, CST, 1:1000
- p-AKT (Ser 473) clone D9E, REF 4060, CST, 1:1000
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) HEK293T, B16F10, B16OVA and MC38 were purchased from American Type Culture Collection.

Authentication None of the cell line have been authenticated.
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All cell lines were tested negative for mycoplasma contamination.

Commonly misidentified lines

(See ITAC register)

No commonly misidentified lines were used.

Animals and other organisms

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Laboratory animals

Map3k14 flox mice, provided by Genentech 37, were crossed with Cd4-Cre transgenic mice (Jackson Laboratory) to produce age-matched Map3k14fl/+ Cd4-Cre (named WT) and Map3k14fl/fl Cd4-Cre (named Map3k14 T cell-conditional knockout or Map3k14 KO) mice. The NIK transgenic mice R26StopFlMap3k14, carrying a Map3k14 transgene encoding wildtype NIK under the control of a loxP-flanked STOP cassette1E (Jackson Laboratories), were crossed with Rosa26-CreER (Jackson Laboratory) to generate Map3k14fl/+ CreER and R26StopFlMap3k14 CreER (hereafter called Map3k14tg CreER) mice, which were injected intraperitoneally with tamoxifen (1 mg/mouse) in corn oil daily for five consecutive days to induce cre function for generation of wildtype and NIK-induced transgenic (NIKtg) mice, respectively. The R26StopFlMap3k14 mice were also crossed with Cd4-Cre to generate Map3k14fl/+ Cd4-Cre (called WT) and R26StopFlMap3k14 Cd4-Cre (called NIK T cell-conditional transgenic or NIKtg) mice. H2b flox mice were purchased from the European Mouse Mutant Archive (EMMA) and described previously. H2b flox mice were crossed with Cd4-Cre transgenic mice to produce age-matched H2f1fl+/+ Cd4-Cre (named WT) and H2f1fl/fl Cd4-Cre (named T cell-conditional H2 knockout or H2kKO) mice. Atg5 flox mice were purchased from Jackson Laboratory. Atg5 mice were crossed with Cd4-Cre transgenic mice to produce age-matched Atg51fl+/+Cd4-Cre (named WT) and Atg5fl/fl Cd4-Cre (named T cell-conditional Atg5 knockout or Atg5tKO) mice. Atg5 mice were crossed with Map3k14fl/fl Cd4-Cre to produce age-matched Atg5fl/fl Map3k14 fl/fl Map3k14 fl/fl Cd4-Cre (named Map3k14 fl/fl Cd4-Cre) mice. OT-I and Pmell TCR-transgenic mice, C57Bl/6, Ragl and Rag2 KO were from Jackson Laboratory. Experiments were performed with young adult (6 to 8-week-old) female and male mice except where indicated otherwise. Mice were maintained in a specific-pathogen-free facility of the University of Texas MD Anderson Cancer Center, mice were housed in a 12hr/12hr light/dark cycle at an ambient temperature of 18-24 degree Celsius and a humidity range of 30-70%.

Wild animals

This study did not involve the use of wild animals.

Field-collected samples

No field-collected samples were used in this study.

Ethics oversight

All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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Methodology

Sample preparation

Spleen, peripheral draining lymph nodes, and tumor sample were gently ground under nylon mesh using the flat end of syringes. Red blood cells were removed by 1X RBC lysing buffer, following by washing cell with isolation buffer. Cell were then filtered, pelleted and staining for FACS or sorting. Cells were blocked with Fc blocker (CD16/32), and stained for specific surface markers. For intracellular staining, cells were fixed and permeabilized and stained for intracellular cytokines by fixation/permeabilization kit (BD bioscience).

Instrument

Flow cytometry data were collected by FACSFortessa and FACSaria.

Software

Flow cytometry data were analyzed by FlowJo software (TreeStar, Ashland, OR).

Cell population abundance

The purities of the sorted T cells were more than 99%.

Gating strategy

For immune cells, first we gated the lymphocytes based on the FSC-A and SSC-A. Singlets were gated according to the pattern of FSC-H versus FSC-A. The specific cell population was gated on the indicated surface markers as described in the manuscript.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.