

# Alkaline Cytosolic pH and High Sodium Hydrogen Exchanger 1 (NHE1) Activity in Th9 Cells\*

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CD4<sup>+</sup> T helper 9 (Th9) cells are a newly discovered Th cell subset that produce the pleiotropic cytokine IL-9. Th9 cells can protect against tumors and provide resistance against helminth infections. Given their pivotal role in the adaptive immune system, understanding Th9 cell development and the regulation of IL-9 production could open novel immunotherapeutic opportunities. The Na<sup>+</sup>/H<sup>+</sup> exchanger 1 (NHE1; gene name *Slc9a1*) is critically important for regulating intracellular pH (pH<sub>i</sub>), cell volume, migration, and cell survival. The pH<sub>i</sub> influences cytokine secretion, activities of membrane-associated enzymes, ion transport, and other effector signaling molecules such as ATP and Ca<sup>2+</sup> levels. However, whether NHE1 regulates Th9 cell development or IL-9 secretion has not yet been defined. The present study explored the role of NHE1 in Th9 cell development and function. Th cell subsets were characterized by flow cytometry and pH<sub>i</sub> was measured using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) dye. NHE1 functional activity was estimated from the rate of realkalinization following an ammonium pulse. Surprisingly, in Th9 cells pH<sub>i</sub> and NHE1 activity were significantly higher than in all other Th cell subsets (Th1/Th2/Th17 and induced regulatory T cells (iTregs)). NHE1 transcript levels and protein abundance were significantly higher in Th9 cells than in other Th cell subsets. Inhibition of NHE1 by siRNA-NHE1 or with cariporide in Th9 cells down-regulated IL-9 and ATP production. NHE1 activity, Th9 cell development, and IL-9 production were further blunted by pharmacological inhibition of protein kinase Akt1/Akt2. Our findings reveal that Akt1/Akt2 control of NHE1 could be an important physiological regulator of Th9 cell differentiation, IL-9 secretion, and ATP production.

CD4<sup>+</sup> T cells participate in the regulation of the immune response during infections, autoimmunity, and cancer. CD4<sup>+</sup> T

cells are an important and essential arm of the adaptive immune system (1). CD4<sup>+</sup> T cells can be divided into various subtypes based upon their cytokine secretion: T helper 1 (Th1)<sup>4</sup> cells produce IFN-γ (1, 2), Th2 cells produce IL-4, IL-5, and IL-13 (3), Th17 cells produce IL-17 (4–6), Th9 cells produce IL-9 (7–9), and suppressive regulatory T cells (Tregs) produce TGF-β and IL-10 (10). A great deal of experimental effort has been dedicated to decipher the development and function of various Th cell subsets. However, Th9 cell development and function remain incompletely understood.

Th9 cells are a recently characterized Th cell subset recognized by their potent production of IL-9. Th9 cells play a pivotal role in health and disease. Th9 cells have been shown to exacerbate the airway allergic immune response by recruiting eosinophils and mast cells to the lungs, increasing mucus production, and production of serum IgE (11, 12). Th9 cells further contribute to the host-immune reaction against helminth infections and tumors (7, 13–23). The development and function of Th9 cells are regulated by a constellation of key transcription factors that include Gata3 (7), Pu.1 (13), Stat6 (24), Irf-4 (25), Irf-1 (26), Bcl6 (27), and Batf (28). However, a single faithful transcription factor for Th9 cell development has not yet been described. Recent studies have also implicated glucocorticoid-induced TNFR family-related gene (GITR; gene name *Tnfrsf18*) signaling in Th9 cell development (29, 30). Xiao *et al.* (29) found that GITR signaling controlled chromatin remodeling at the *Foxp3* and *Il9* loci, and consequently was able to convert induced Tregs (iTregs) into Th9 cells. Furthermore, microRNA-15b/16 (miR-15b/16) is involved in fine tuning of iTregs/Th9 cell development thus contributing to the autoinflammation in colitis (31).

Th cells (Th1, Th2, Th17, and iTregs) are different in their energy production and metabolism (32–34). Th cells normally use the aerobic glycolytic pathway to meet their energy demand for cell proliferation and development, with the exception of iTregs, which uses oxidative phosphorylation (OXPHOS) (32–35). All major intracellular processes such as glycolysis-dependent ATP production or protein synthesis require tight regula-

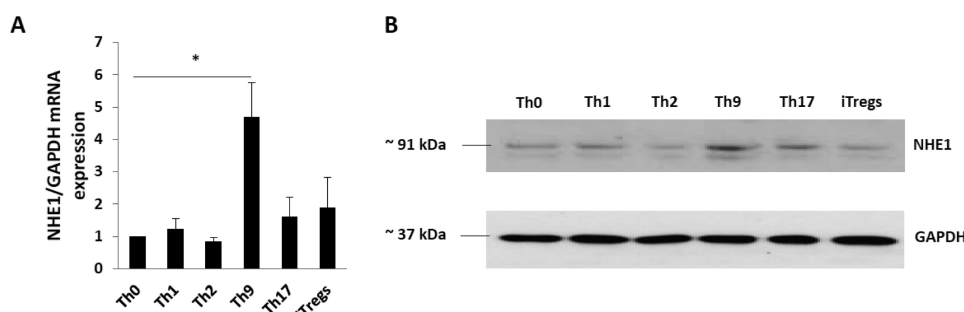
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<sup>4</sup> The abbreviations used are: Th, T helper; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester; Tregs, regulatory T cells; GITR, glucocorticoid-induced TNFR family-related; iTregs, induced Tregs; OXPHOS, oxidative phosphorylation; NHE, Na<sup>+</sup>/H<sup>+</sup> exchanger; q-RT, quantitative RT; TPI, triose-phosphate isomerase; MCT, monocarboxylic acid transporter; mTOR, mechanistic target of rapamycin.



**FIGURE 1. NHE1 expression in Th cell subsets and iTregs at mRNA and protein levels.** *A*, naive T cells were differentiated in Th cell subsets and iTregs as described under "Experimental Procedures" and RNA was isolated for q-RT-PCR to quantify NHE1 transcript levels. Arithmetic means  $\pm$  S.E. ( $n = 5$  independent experiments) of the mRNA levels in Th cell subsets is shown. Th9 cells have significantly higher NHE1 RNA expression than all other Th cell subsets and iTregs. Asterisk (\*) indicates statistically significant difference (\*,  $p < 0.05$ ). *B*, NHE1 protein expression in Th cell subsets and iTregs. Gapdh was used as a loading control.

tion of intracellular pH ( $pH_i$ ) (36). Maintenance of glycolysis and OXPHOS processing enzymes are highly dependent on  $pH_i$ . Accordingly,  $pH_i$  could modify cellular metabolism (37). Understanding regulation of  $pH_i$  and  $Na^+/H^+$  exchanger (NHE) activity could thus open up new avenues to treat autoimmune disorders, allergic inflammation, or cancer by immunotherapeutic manipulation of Th9 cells.

Tight regulation of  $pH_i$  by NHE proteins (NHE1, -2, and -3) is vital for survival and function of  $CD4^+$  T cells (38–44). The  $pH_i$  and NHE activity are instrumental for preserving cell viability, cell proliferation, and migration (40, 41, 45, 46).  $pH_i$  is in part affected by modulating signal transduction (40, 41, 45, 47). NHE1 activity is in turn regulated by the protein kinase B (Akt) (48–50). Previous studies in human T cells have suggested that addition of IL-2 to cultured cells could enhance  $pH_i$  and NHE1 protein abundance, thus affecting cell proliferation, cytokine production, and apoptosis (51). It has been further proposed that  $pH_i$  and NHE1 activity are modulated by glucocorticoids in a non-genomic fashion (49, 52–56). However, to which extent  $pH_i$  and NHE activity govern Th9 cell development and function is incompletely understood.

In this study, we explored  $pH_i$  and NHE1 activity in various Th cell subsets. We found that Th9 cells potently up-regulate  $pH_i$  and NHE1 activity compared with other Th cell subsets. Furthermore, we show that regulation of NHE1 activity is dependent on Akt. Finally we reveal that, NHE1 controls the development and function of Th9 cells. In summary our data describe a novel role of NHE1 in the development and IL-9 production of Th9 cells.

## Results

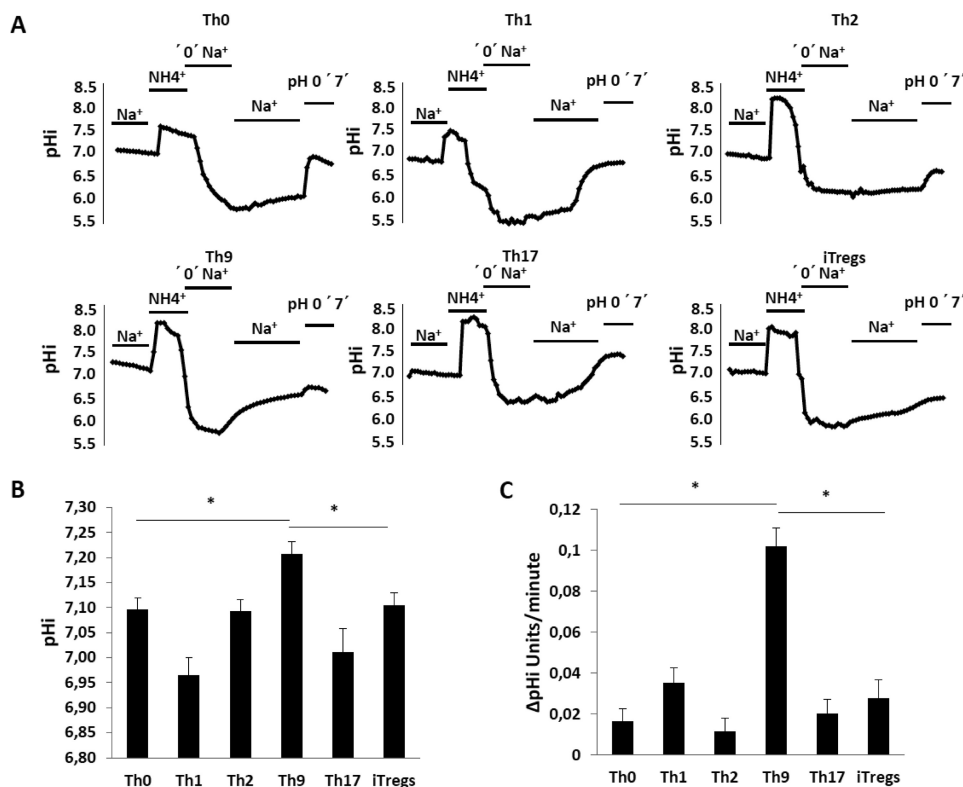
**Characterization of Th Cell Subsets at mRNA and Protein Levels and NHE1 Expression in Th Cell Subsets**—To characterize different Th cell subsets, naive  $CD4^+$  T cells were differentiated into Th1, Th2, Th9, Th17, and iTregs in the presence of defined recombinant cytokine proteins and antibodies as described under "Experimental Procedures." To confirm differentiation, the respective cytokines and transcription factors of particular Th cell subsets and iTregs, were measured by flow cytometry (data not shown). The results were further supported by q-RT-PCR. Th1 cells produce IFN- $\gamma$  transcripts and Th2 cells produce IL-4 transcripts (data not shown). Th9 cells are the major producer of IL-9 transcripts. IL-9 transcripts were

also produced, albeit to a lower extent, by Th2 and iTregs. Th17 cells mainly produced IL-17 transcripts. Induction of Foxp3 transcripts were only associated with iTregs. These results confirmed that our naive T cells were correctly differentiated into various Th cell subsets and iTregs.

Transcript levels of NHE1 were quantified in Th cell subsets and iTregs by q-RT-PCR. NHE1 mRNA transcript levels were significantly higher in Th9 cells than in Th0, other Th cell subsets (Th1, Th2, and Th17), and iTregs (Fig. 1A). Immunoblotting revealed that the differences in NHE1 transcript levels were paralleled by differences in NHE1 protein abundance (Fig. 1B and data not shown). Again, Th9 cells expressed the highest amount NHE1 protein.

**$pH_i$  and  $Na^+/H^+$  Exchanger Activity in Th Cell Subsets and iTregs**—To test whether enhanced NHE1 expression in Th9 cells resulted in enhanced  $Na^+/H^+$  exchanger activity and  $pH_i$ , cytosolic  $pH_i$  was estimated from (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence using fluorescence optics. We found that Th0, Th2, and iTregs had similar  $pH_i$  values (Fig. 2, A and B). To our surprise, Th1 cells had the lowest  $pH_i$  when compared with all other Th cell subsets including iTregs (Fig. 2, A and B). In contrast, Th9 had significantly higher  $pH_i$  than all other Th cell subsets (Fig. 2, A and B).  $Na^+/H^+$  exchanger activity was measured utilizing the ammonium pulse technique. In this method, addition of 20 mM  $NH_4Cl$  (replacing NaCl in the superfusate) was followed by  $NH_3$  entry into the cells with subsequent transient cytosolic alkalinization due to binding of  $H^+$  to  $NH_3$  thus forming  $NH_4^+$ . Subsequent removal of  $NH_4Cl$  was followed by cytosolic acidification due to  $NH_3$  exit with cytosolic dissociation of  $NH_4^+$  and retention of  $H^+$ . In the absence of  $Na^+$ , realkalinization was negligible in all Th cell subsets. Thus, none of the Th cell subsets expressed an appreciable  $Na^+$ -independent  $H^+$  extruding transport system. However, the subsequent addition of  $Na^+$  was followed by rapid cytosolic realkalinization, an observation pointing to  $Na^+/H^+$  exchanger activity (Fig. 2, A and C).  $Na^+/H^+$  exchanger activity was significantly higher in Th9 cells than in any other Th cell subsets (Fig. 2, A and C).  $Na^+/H^+$  exchanger activity in Th9 cells was abrogated in the presence of the NHE1 inhibitor cariporide (data not shown). Thus, these data strongly suggested NHE1 could be involved in development or function of Th9 cells.

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**FIGURE 2.  $pH_i$  and  $Na^+/H^+$  exchanger activity in Th cell subsets and iTregs.** A, alterations of cytosolic pH ( $pH_i$ ) following an ammonium pulse in Th cell subsets and iTregs. Cells were loaded with  $H^+$  by treating with 20 mM  $NH_4Cl$ .  $Na^+$  was then removed by replacement with NMDG.  $NH_4Cl$  was then removed in a second step and  $Na^+$  was added with nigericin ( $pH_o$  7.0) to calibrate each individual experiment. Original tracings of typical experiments are shown for  $pH_i$  measurement and  $Na^+/H^+$  exchanger activity in Th cell subsets. B, arithmetic means  $\pm$  S.E. ( $n = 3-5$  independent experiments) of cytosolic pH prior to the ammonium pulse ( $pH_i$ ) in Th cell subsets and iTregs. Asterisk (\*) indicates a statistically significant difference (\*,  $p < 0.05$ ). C, arithmetic means  $\pm$  S.E. ( $n = 3-5$  independent experiments) of  $Na^+$ -dependent recovery of cytosolic pH ( $\Delta pHi/min$ ) in Th cell subsets. Asterisk (\*) indicates a statistically significant difference (\*,  $p < 0.05$ ).

*Glycolytic Rate Is Higher in Th9 Cells Than Other Th Cell Subsets and iTregs*—Elevated  $pH_i$  is implicated in neoplastic transformation in response to overexpression of proton transporters and different oncogenes. Oncogenes take advantage of NHE-1-induced cellular alkalinization to produce a unique cancer-specific regulation (36). High  $pH_i$  leads to increased glycolytic flux in cancer cells reducing the requirement of oxidative phosphorylation (36). Previous studies also suggested that Th cell subsets have different energy requirements (32–35). Th cells primarily use aerobic glycolytic pathways to meet their energy demand, whereas Tregs use OXPHOS for their energy supply (32–35). NHE1 activation can lead to enhanced glycolysis. Glucose consumption is dependent on a series of reactions catalyzed by a chain of multiple enzymes. Catalysis of one molecule of glucose yields two molecules of ATP and lactic acid (a by-product of this process). We postulated that NHE1 activity would therefore affect glycolysis in Th9 cells. We thus performed q-RT-PCR on key regulatory genes in the glycolytic pathway. We found that Th9 cells have the higher expression of glucose transporter 1 (*Glut1*), hexokinase 2 (*Hk2*), triose-phosphate isomerase (*Tpi*), lactate dehydrogenase 2 $\alpha$  (*Ldh2 $\alpha$* ), and monocarboxylic acid transporter members 1 and 4 (*Mct1* and *Mct4*) transcript levels than Th0 cells or iTregs (Fig. 3, A and B).

*NHE1 Impacts on Regulation of Cell Metabolism, IL-9, and ATP Production*—To uncover the functional importance of NHE1 in Th9 cells, we knocked down NHE1 expression in Th9

cells using siRNA. The transfection efficiency of siRNAs was tested using control siRNA, which was labeled with FAM dye. FAM fluorescence was measured by flow cytometry and more than 90% of  $CD4^+$  T cells were positive for the FAM dye (data not shown). After NHE1 knockdown,  $pH_i$  and NHE1 activity were measured using BCECF, which demonstrated a drastically reduced activity of the  $Na^+/H^+$  exchanger (Fig. 4, A and C) and a decreased  $pH_i$  (Fig. 4, A and B). NHE1 knockdown further reduced intracellular IL-9 cytokine expression (Fig. 4D) and importantly, the ability of Th9 cells to convert into iTregs (Fig. 4E).

We next explored whether genes in the glycolytic pathway decreased due to the knockdown of NHE1 in Th9 cells. Several genes related to glycolysis such as *Glut1*, *Tpi*, and *Mct1* were significantly down-regulated (Fig. 5A). Interestingly, siRNA knockdown of NHE1 expression affected *Foxp3* mRNA and reduced IL-9 transcript levels. To explore the impact of NHE1 activity on the intracellular energy status of Th9 cells, we measured ATP production in Th9 cells in the presence and absence of the NHE1 inhibitor cariporide. In keeping with the q-RT-PCR data, treatment with cariporide significantly decreased ATP levels in Th9 cells (Fig. 5B).

*$Na^+/H^+$  Exchanger Activity Is Dependent on Akt Signaling in Th9 Cells*—Akt signaling is critically involved in NHE1 regulation (48, 49, 57). Akt/Rictor/mTOR (mechanistic target of rapamycin) signaling is also involved in the development of Th

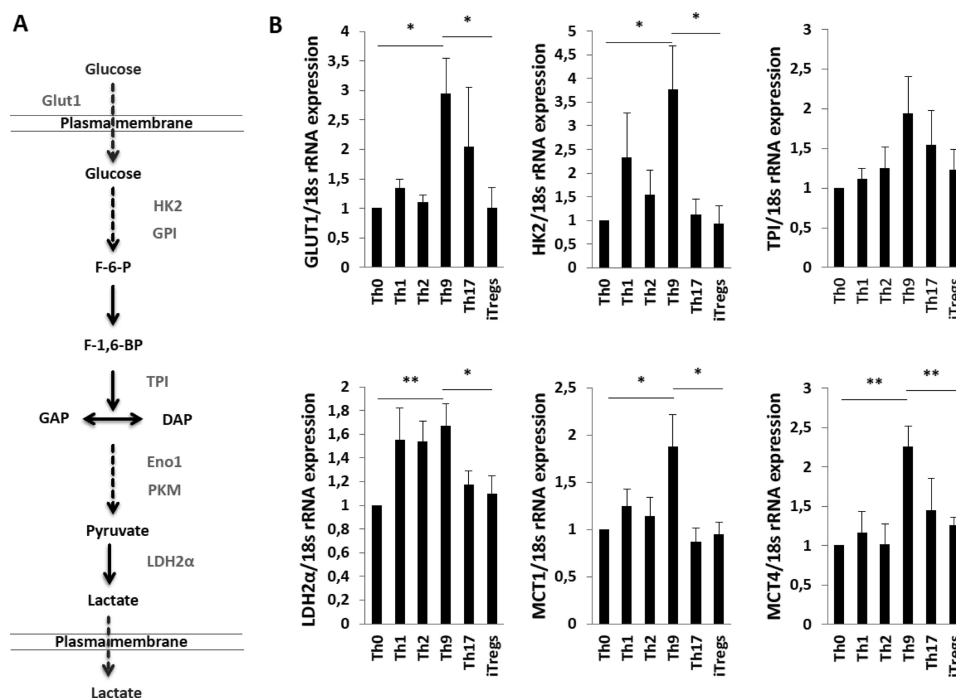


FIGURE 3. Th9 cells augmented the glycolysis compared with Th cell subsets and iTregs. A, a diagram is shown displaying the enzymes involved in the glycolysis pathway. B, arithmetic means  $\pm$  S.E. ( $n = 5-6$  independent experiments) of mRNA transcripts for glycolytic pathways enzymes genes (*Glut1*, *Hk2*, *Tpi*, *Ldh2α*, *Mct1*, and *Mct4*) from Th cell subsets and iTregs. Asterisk (\*) indicates a statistically significant difference (\*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ).

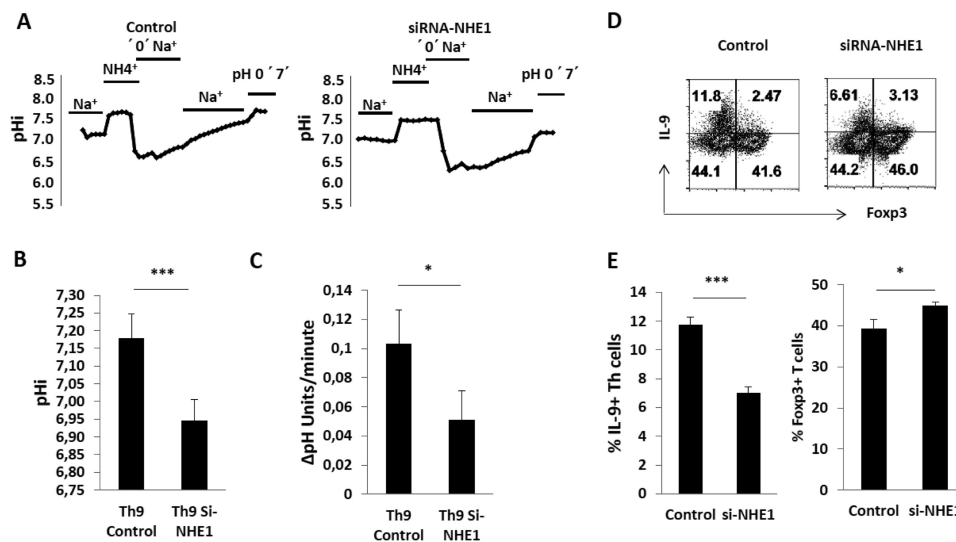
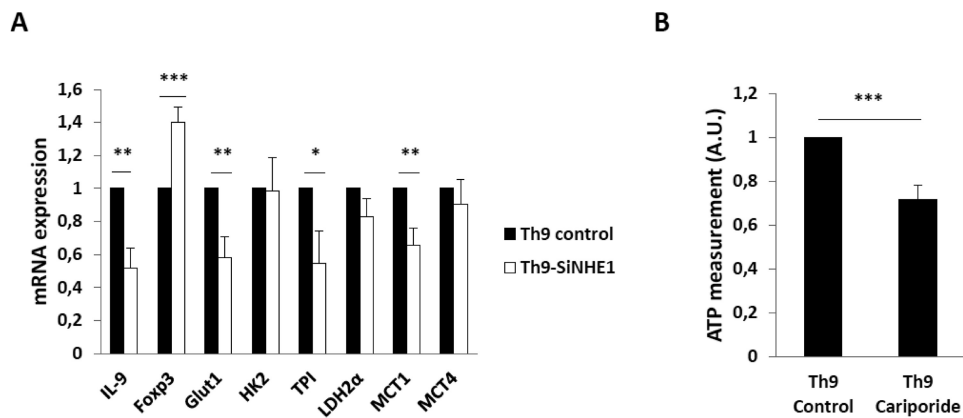


FIGURE 4. Regulation of Th9 cells and iTregs development by NHE1. A, alterations of cytosolic pH (pHi) following an ammonium pulse in transfected Th9 cells with control and NHE1 siRNAs. Cells were loaded with  $H^+$  as described in the legend to Fig. 2A. Original tracings of typical experiments are shown for pH<sub>i</sub> measurement and  $Na^+/H^+$  exchanger activity in Th cell subsets. B, arithmetic means  $\pm$  S.E. ( $n = 3-5$  independent experiments) of cytosolic pH prior to the ammonium pulse (pHi) in siRNA control and siRNA NHE1 Th9 cells. Asterisk (\*) indicates a statistically significant difference (\*,  $p < 0.05$ ). C, arithmetic means  $\pm$  S.E. ( $n = 3-5$  independent experiments) of  $Na^+$ -dependent recovery of cytosolic pH ( $\Delta pHi$ /min) in siRNA control and siRNA NHE1 Th9 cells. Asterisk (\*) indicates a statistically significant difference (\*,  $p < 0.05$ ). D, differentiation of Th9 cells transfected with control and NHE1 siRNAs. At day 4, Th9 cells were activated with PMA, ionomycin, and brefeldin A for 4 h and stained for IL-9 and Foxp3. Representative FACS plots show the Th9 differentiation after knock-down of NHE1. E, arithmetic means  $\pm$  S.E. ( $n = 3-5$  independent experiments) of IL-9 and Foxp3 expression in siRNA control and siRNA NHE1 Th9 cells. Asterisk (\*) indicates a statistically significant difference (\*\*\*,  $p < 0.001$ ; \*,  $p < 0.05$ ).

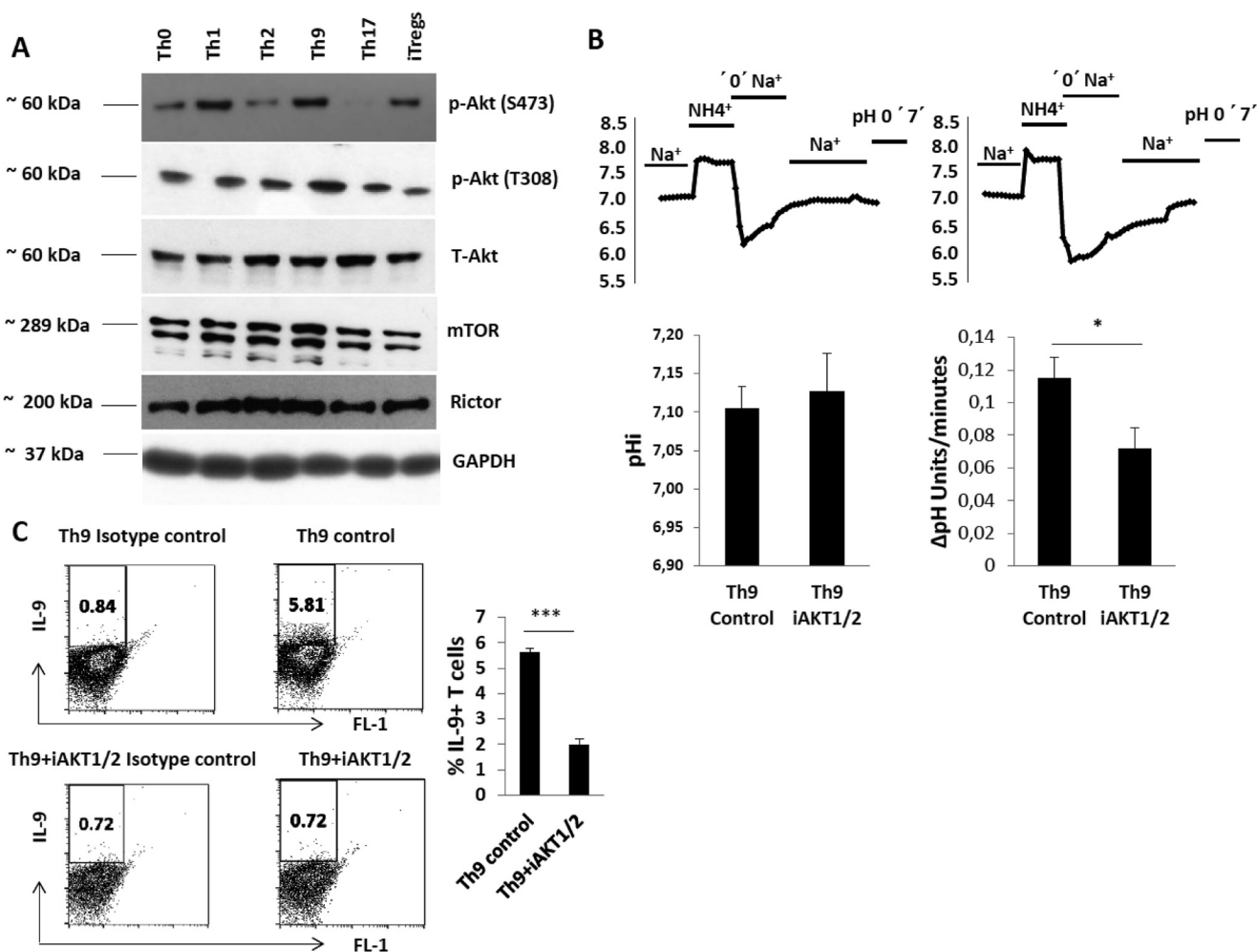
cell subsets (58–61). However, the role of Akt/Rictor/mTOR signaling has not been defined in Th9 cell development. To identify the possible role of Akt and NHE1 in Th9 cell development, we examined Akt/mTOR proteins and their enzymatic activity. We found that the level of Akt phosphorylation at serine 473 (Ser-473) is highest in Th9 and Th1 cells (Fig. 6A and data not shown). In contrast, Akt phosphorylation at threonine

308 (Thr-308) was higher in Th9 than in any other Th cell subsets (Fig. 6A). As Akt Ser-473 is phosphorylated by Rictor, we also measured Rictor expression and found that Th1, Th2, and Th9 cells have higher amounts of Rictor than Th0 and Th17 cells, or iTregs (Fig. 6). However, mTOR expression was higher in Th9 cells than in any other Th cell subsets (Fig. 6A). Therefore, we reasoned that the increased phosphorylation level of

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**FIGURE 5. NHE1 inhibition leads to decreased glycolysis and ATP production.** *A*, arithmetic means  $\pm$  S.E. ( $n = 5-6$  independent experiments) of mRNA transcripts for glycolytic pathway enzymes genes (*Glut1*, *Hk2*, *Tpi*, *Ldh2α*, *Mct1*, and *Mct4*) from Th9 cells with and without inhibition of NHE1 by siRNAs. Asterisk (\*) indicates a statistically significant difference (\*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ). *B*, arithmetic means  $\pm$  S.E. ( $n = 4$  independent experiments performed in duplicate) of intracellular ATP levels. Naive T cells were differentiated into Th9 cells with or without NHE1 inhibitor cariporide ( $10 \mu\text{M}$ ) and intracellular ATP was measured using luciferase assay kit. Asterisk (\*) indicates a statistically significant difference (\*\*\*,  $p < 0.001$ ).



**FIGURE 6. Regulation of  $\text{Na}^+/\text{H}^+$  exchanger activity and IL-9 production by Akt in Th9 cells.** *A*, levels of total and phosphorylated Akt at Ser-473 and Thr-308 plus levels of Rictor and mTOR in Th cell subsets. Data are representative of Western blot analysis of 4 independent experiments. *B*, NHE1 activity and  $\text{pH}_i$  of Th9 cells with or without the Akt1/2 inhibitor ( $0.3 \mu\text{M}$ ). Original tracings of typical experiments for  $\text{pH}_i$  measurement and  $\text{Na}^+/\text{H}^+$  exchanger activity are shown. Arithmetic means  $\pm$  S.E. ( $n = 3-4$  independent experiments) of cytosolic  $\text{pH}_i$  prior to the ammonium pulse ( $\text{pH}_i$ ) and  $\text{Na}^+$ -dependent recovery of cytosolic  $\text{pH}$  ( $\Delta\text{pH}/\text{min}$ ) in Th9 cells with and without Akt1/2 inhibitor are shown. Asterisk (\*) indicates a statistically significant difference (\*,  $p < 0.05$ ). *C*, a representative FACS plot showing differentiated Th9 cells with and without Akt1/2 inhibitor ( $0.3 \mu\text{M}$ ). Data are representative of  $n = 3$  experiments. Asterisk (\*) indicates a statistically significant difference (\*\*\*,  $p < 0.0005$ ).

Akt at Ser-473/Thr-308 could lead to enhanced expression of NHE1 in Th9 cells. To explore whether Akt influences NHE1 expression, we differentiated naive T cells into Th9 cells in the presence and absence of Akt1/2 inhibitor and measured  $\text{Na}^+/\text{H}^+$  exchanger activity. As shown in Fig. 6B, Akt inhibition significantly decreased  $\text{Na}^+/\text{H}^+$  exchanger activity. Moreover, inhibition of Akt reduced the expression of IL-9 (Fig. 6C). These data reveal that Akt is crucial for the up-regulation of  $\text{Na}^+/\text{H}^+$  exchanger activity and IL-9 production in Th9 cells.

## Discussion

The present study uncovered a novel role of cytosolic pH regulation and  $\text{Na}^+/\text{H}^+$  exchanger activity in development and function of Th cell subsets, in particular Th9 and iTregs.

Previously, it was suggested that IL-2 signaling could induce NHE1 activity in human T cells (51) as well as in murine T cells (52, 53). In extension to these previous findings, our study revealed that each Th cell subset differs in  $\text{pH}_i$ , which is governed by  $\text{Na}^+/\text{H}^+$  exchanger activity.  $\text{Na}^+/\text{H}^+$  exchanger activity is highly sensitive to cytosolic  $\text{pH}_i$  and is switched off upon cytosolic alkalization (62). NHE1 mRNA and protein levels were highest in Th9 cells than in any other Th cell subsets or iTregs. Moreover, Th9 cells had the highest and Th2 cells had the lowest  $\text{Na}^+/\text{H}^+$  exchanger activity when compared with all other Th cell subsets (Th0, Th1, and Th17, or iTregs). Up-regulation of  $\text{Na}^+/\text{H}^+$  exchanger activity in Th9 cells enhances the extrusion of  $\text{H}^+$  thus contributing to the maintenance of alkaline  $\text{pH}_i$ .

We further revealed the critical influence of Akt on  $\text{Na}^+/\text{H}^+$  exchanger activity in Th9 cells. Akt is known to govern the signaling for other Th1, Th2, and Th17 cell subsets and iTregs. However, the role of Akt has not been defined in Th9 cells. We found that Th9 cells had the highest level of Akt phosphorylation. This could be due to high mTOR/Rictor activity in Th9 cells compared with iTregs. This higher activity of Akt could affect the  $\text{Na}^+/\text{H}^+$  exchanger activity in Th9 cells as previous studies have suggested a role of Akt in governing the  $\text{Na}^+/\text{H}^+$  exchanger activity (48–50). Furthermore, Akt could also be required for up-regulation of IL-9 cytokine expression, as Akt inhibition decreased the expression of IL-9 in Th9 cells. It appears that Akt is vitally important for regulating development and function of Th9 cells. Phosphorylation of Akt is also up-regulated in Th1 cells; however, they do not have an appreciable  $\text{Na}^+/\text{H}^+$  exchanger activity.

A recent study also suggested that Th9 cells have increased expression of Hif-2 $\alpha$  (31). Hif-2 $\alpha$  is upstream of Rictor and it is also up-regulated in Th9 cells. Thus it could contribute to or even account for the enhanced glycolysis. Previous studies have revealed that mTOR is a metabolic sensor in various immune T cells (32, 34). Theoretically, mTOR could regulate glycolysis within Th9 cells. In keeping with a recently published finding (63), the glycolytic rate is higher in Th9 cells than in any other Th cell subsets and iTregs. In contrast to Th9 cells, iTregs depend on OXPHOS for their metabolic energy production. Following NHE1 inhibition, Th9 cells lose glycolytic potential and convert into iTregs. Our data thus confirm that NHE1 is a controller of glycolytic flux in Th9 cells. However, other pathways cannot be ruled out.

The Th9/iTregs axis in cancer is further affected by GPCR signaling (29, 30). Our studies suggest that NHE1 could also be instrumental for the Th9/iTregs balance. In view of our siRNA knock-down data, it is tempting to speculate that NHE1 is essential for the Th9/iTregs axis in cancer patients. Tumor cells generate lactate thus leading to a highly acidic environment (64). An adequate immune response against tumor cells requires survival of the respective immune cells in the acidic environment. Augmented  $\text{Na}^+/\text{H}^+$  exchanger activity may indeed confer some protection against an acidic environment. On the other hand, cytosolic alkalization is known to stimulate glycolysis (65–67), which may support energy supply to Th9 cells. It is tempting to further speculate that the enhanced glycolysis in Th9 cells contributes to the deprivation of glucose for neighboring cancer cells. Clearly, additional experimental effort is required to define the exact role of  $\text{Na}^+/\text{H}^+$  exchanger activity for Th9 cells survival and function.

In summary, we have shown that NHE1 is required for the development of Th9 cells. These data will help to understand the physiological function of Th9 cells and the metabolic reprogramming of Th9 cells.

## Experimental Procedures

**Mice Strains**—C57BL/6 mice (8–12 weeks) were used for the experiments and kept in a conventional specific pathogen-free facility. Animal experiments were performed according to the EU Animals Scientific Procedures Act and the German law for the welfare of animals. The procedures were approved by the authorities of the state of Baden-Württemberg and the research has been reviewed and approved by an Institutional Animal Care and Use Committee. For each experiment 3–6 mice were used and data shown in each figure is representative of arithmetic means  $\pm$  S.E. and numbers ( $n$ ) of independent experiments.

**Naive T Cells Isolation**—To perform the Th differentiation or iTreg induction experiments, naive  $\text{CD4}^+\text{CD62L}^{\text{high}}\text{CD25}^-$  T cells were isolated using magnetic bead selection from spleen and lymph nodes as described earlier (68, 69). To isolate the  $\text{CD4}^+$  T cells, the spleen and lymph nodes (inguinal, axillary, brachial, mediastinal, superficial cervical, mesenteric) were collected from the mice and macerated using a syringe plunger. The cell suspension was centrifuged at  $600 \times g$  at  $4^\circ\text{C}$  for 5 min and the cell pellet was treated with RBC lysis buffer for 1 min and washed three times. After washing, the cells were kept on a roller at  $4^\circ\text{C}$  (cold room) for 30 min in the presence of 40  $\mu\text{l}$ /mouse antibody mixture containing anti-CD8, anti-MHC II, anti-CD11b, anti-CD16/32, anti-CD45R, and Ter-119 (Dyna-beads<sup>®</sup> Untouched<sup>™</sup> Mouse CD4 cells kit, Invitrogen, Karlsruhe, Germany). Cells were then washed and Dynabeads<sup>®</sup> were added. The cells were incubated at  $4^\circ\text{C}$  (cold room) for 30 min on a roller to deplete  $\text{CD8}^+$  T cells, B cells, NK cells, monocytes/macrophages, dendritic cells, erythrocytes, and granulocytes and to isolate  $\text{CD4}^+$  T cells. Furthermore, to isolate  $\text{CD4}^+\text{CD25}^+$  T cells, purified  $\text{CD4}^+$  T cells were incubated with 2  $\mu\text{l}$ /mouse of biotinylated anti-CD25 (7D4 clone; BD Biosciences, UK) for 30 min on a roller ( $4^\circ\text{C}$ ), washed, and kept for 15 min with 20  $\mu\text{l}$ /mouse of streptavidin microbeads (Miltenyi Biotech, Germany) for the indirect magnetic labeling of  $\text{CD25}^+$

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T cells. Using MACS separation columns CD4<sup>+</sup>CD25<sup>+</sup> T cells were positively selected and the remaining cells were CD4<sup>+</sup>CD25<sup>-</sup> T cells (18). To enrich naive CD4<sup>+</sup>CD62L<sup>high+</sup>CD25<sup>-</sup> T cells from CD4<sup>+</sup>CD25<sup>-</sup> T cells, these cells were again incubated with 10  $\mu$ l/mouse of biotinylated anti-CD62L (clone MEL-14; BD Biosciences) for 30 min on a roller (4 °C), washed, and kept for 15 min with 20  $\mu$ l/mouse of streptavidin microbeads for the indirect magnetic labeling of CD62L<sup>+</sup> T cells. Using MACS separation columns naive CD4<sup>+</sup>CD62L<sup>high+</sup>CD25<sup>-</sup> T cells were positively selected. Remaining cells were CD4<sup>+</sup>CD62L<sup>-</sup>CD44<sup>+</sup>CD25<sup>-</sup> T cells. Purity of these cells was confirmed by flow cytometry. As a result, more than 90% cells were positive for naive T cells.

**Th Cell Subsets Differentiation**—Naive CD4<sup>+</sup>CD62L<sup>high+</sup>CD25<sup>-</sup> T cells were activated in the presence of plate-bound anti-CD3/anti-CD28 antibodies (eBioscience, Frankfurt, Germany) with a ratio of 1:2 anti-CD3:anti-CD28 (1:2  $\mu$ g/ml, anti-CD3:anti-CD28) for Th1, Th2, and iTregs and 1:10 anti-CD3:anti-CD28 (1  $\mu$ g/ml of anti-CD3: 10  $\mu$ g/ml of anti-CD28) for Th9 and Th17. Briefly, T naive cells were differentiated into Th1 using 20 ng/ml of recombinant IL-12 (eBioscience), anti-IL-4 (5  $\mu$ g/ml; eBioscience), Th2 using 20 ng/ml of recombinant-IL-4 (eBioscience), anti-IFN- $\gamma$  (5  $\mu$ g/ml; eBioscience), Th9 using 2.5 ng/ml of recombinant-TGF- $\beta$ , 40 ng/ml of recombinant-IL-4, anti-IFN- $\gamma$  (10  $\mu$ g/ml), Th17 using 2.5 ng/ml of recombinant-TGF- $\beta$ , 50 ng/ml of recombinant-IL-6 (eBioscience), anti-IFN- $\gamma$  (5  $\mu$ g/ml), anti-IL-4 (5  $\mu$ g/ml), and anti-IL-2 (5  $\mu$ g/ml; eBioscience) and iTregs using 2.5 ng/ml of recombinant-TGF- $\beta$ , 5 ng/ml of recombinant-IL-2 (eBioscience), and cultured for 3–4 days (2, 3, 19, 47, 70). Cells were harvested at day 3 and used for intracellular staining for characterizing the Th cells using flow cytometry, q-RT-PCR, pH<sub>i</sub>, NHE1 activity, and immunoblotting experiments.

**Intracellular pH (pH<sub>i</sub>) Measurement**—Cytosolic pH (pH<sub>i</sub>) was measured in Th cell subsets as described previously (71) using pH-sensitive BCECF-AM dye (Invitrogen). Naive T cells were differentiated into Th cell subsets and after 3 days of differentiation various Th cell subsets were subjected to measurement of pH<sub>i</sub> with and without treatment. To measure the pH<sub>i</sub> and NHE1 activity of Th cells subsets, 300  $\mu$ l of cells were collected and were fixed on a coverslip coated with poly-L-lysine (Sigma, Darmstadt, Germany), which was then placed in a chamber. Th cell subsets were co-incubated with 10  $\mu$ M BCECF-AM for 15 min at 37 °C. Once the incubation was finished, the chamber was placed on the stage of an inverted phase-contrast microscope with the incident-light fluorescence illumination system (Axiovert 135, Zeiss, Göttingen, Germany) with epifluorescence mode  $\times$ 40 oil immersion objective (Zeiss). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Approximately 30–40 cells were outlined and monitored during the course of the measurements of BCECF fluorescence. The results from each cell were averaged and used for final data analysis.

High-K<sup>+</sup>/nigericin calibration technique was applied for converting intensity ratio (490/440) data into pH<sub>i</sub> values. The

cells were perfused at the end of each experiment for 5 min with standard high-K<sup>+</sup>/nigericin (10  $\mu$ g/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the  $r_{\max}$ ,  $r_{\min}$ , and pK<sub>a</sub> values previously generated from calibration experiments to establish a standard nonlinear curve (pH range 5 to 8.5) (72).

For acid loading, Th cell subsets were transiently exposed to a solution containing 20 mM NH<sub>4</sub>Cl leading to initial alkalinization of pH<sub>i</sub> due to entry of NH<sub>3</sub> and binding of H<sup>+</sup> to form NH<sub>4</sub><sup>+</sup>. The acidification of pH<sub>i</sub> upon removal of ammonia allowed calculating the mean intrinsic buffering power ( $\beta$ ) of the cell. Assuming that NH<sub>4</sub><sup>+</sup> and NH<sub>3</sub> are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH<sub>3</sub>,

$$\beta = \Delta[\text{NH}_4^+]_i / \Delta\text{pH}_i \quad (\text{Eq. 1})$$

where  $\Delta\text{pH}_i$  is the decrease of cytosolic pH (pH<sub>i</sub>) following ammonia removal and  $\Delta[\text{NH}_4^+]_i$  is the decrease of cytosolic NH<sub>4</sub><sup>+</sup> concentration, which is equal to  $[\text{NH}_4^+]_i$  immediately before the removal of ammonia. The pK for NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub> is 8.9 and at an extracellular pH (pH<sub>o</sub>) of 7.4 the NH<sub>4</sub><sup>+</sup> concentration in extracellular fluid ( $[\text{NH}_4^+]_o$ ) is 19.37 [20/(1 + 10<sup>pH<sub>o</sub>-pK</sup>)]. The intracellular NH<sub>4</sub><sup>+</sup> concentration ( $[\text{NH}_4^+]_i$ ) was calculated from Equation 2.

$$[\text{NH}_4^+]_i = 19.37 \times 10^{\text{pH}_o - \text{pH}_i} \quad (\text{Eq. 2})$$

The calculation of the buffer capacity required that NH<sub>4</sub><sup>+</sup> exits completely. After the initial decline, pH<sub>i</sub> indeed showed little further change in the absence of Na<sup>+</sup>, suggesting that there was no relevant further exit of NH<sub>4</sub><sup>+</sup>. To calculate the  $\Delta\text{pH}/\text{min}$  during re-alkalinization, a manual linear fit was placed over a narrow pH range with time which could be applied to all measured cells.

The solutions used in the pH<sub>i</sub> and NHE1 measurements were composed of (in mM) as described earlier (50): standard Hepes, 115 NaCl, 5 KCl, 1 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 2 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 32.2 Hepes; sodium-free Hepes, 132.8 NMDG-Cl, 3 KCl, 1 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 2 KH<sub>2</sub>PO<sub>4</sub>, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium-free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH<sub>4</sub>Cl); high K<sup>+</sup> for calibration, 105 KCl, 1 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 32.2 Hepes, 10 mannitol, 5  $\mu$ M nigericin. The pH of the solutions was titrated to 7.4 or 7.0 using HCl/NaOH, HCl/NMDG, and HCl/KOH, respectively, at 37 °C. In some cases, NHE1 inhibitor cariporide (10  $\mu$ M; sc-337619A, Santa Cruz Biochemical, Santa Cruz, CA) and AKT1/2 (0.3  $\mu$ M; A6730, Sigma) were used.

**Surface and Intracellular Antibodies Staining**—Th cell subsets were characterized by using surface and intracellular staining with relevant antibodies. In brief, Th cell subsets were stimulated with anti-CD3/anti-CD28 or 1 mg/ml of PMA (Sigma) and 1 mg/ml of ionomycin (Sigma) for 4 h and after 2 h of PMA + ionomycin treatment 1  $\mu$ g/ml of brefeldin A (eBioscience) was added to the cultured cells for 2 h. After 4 h, cells were collected and used for surface and intracellular staining for various antibodies dependent on the experiment (anti-CD4-PerCP, anti-Foxp3-APC, and anti-IFN- $\gamma$ -FITC; eBioscience, anti-IL-4-PE anti-IL-17A-PE; BD Bioscience, Heidelberg, Ger-

many, anti-IL-9-PE; BioLegend®, London, UK), and washed with PBS. Cells were fixed with Foxp3 fixation/permeabilization buffer (eBioscience) for intracellular staining and incubated for 30 min. After incubation, cells were washed with 1× permeabilization buffer (eBioscience) and intracellular monoclonal antibodies were added and incubated for an additional 30 min. Cells were washed again with permeabilization buffer and PBS was added to acquire the cells on flow cytometry (FACSCalibur™, BD Bioscience).

**q-RT-PCR**—Total mRNA was isolated from different Th cell subsets (Th0, Th1, Th2, Th9, and Th17) and iTregs using miRNeasy isolation kit (Qiagen, Hilden, Germany) as described by the manufacturer. 1 µg of mRNA was converted into cDNA using SuperScript III cDNA synthesis kit (Invitrogen). Briefly, in 10-µl reactions, 10 ng of cDNA, 2× SYBR Green mastermix (Peqlab, Erlangen, Germany) and 250 nM primers (Sigma) were used for q-RT-PCR. q-RT-PCR run and data analysis was performed as described previously (21) for NHE-1 (*Slc9α1*) (F primer, 5'-TCGCCAGATGACCACAATTT-3' and R primer, 5'-GGGGATCACATGGAAACCTATCT-3'), *Glut-1* (F primer, 5'-ATGGATCCAGCAGCAAGAA-3' and R primer, 5'-ACTCCTCAATAACCTTCTGGGG-3'), *Hk2* (F primer, 5'-TGATCGCTGCTTATTACGG-3' and R primer, 5'-AACCGCTAGAAATCTCCAGA-3'), *Tpi* (F primer, 5'-CCAGGAAGTTCTTCGTTGGGG-3' and R primer, 5'-CAAAGTCGATGTAAGCGGTGG-3'), *Ldh2α* (F primer, 5'-AACTTGGCGCTCTACTTGCT-3' and R primer, 5'-GGACTTGAATCTTTGAGACCTTG-3'), *Mct1* (F primer, 5'-AAAATGCCACCTGCGATTGG-3' and R primer, 5'-TCACTGGTTCGTTGCACTGAA-3'), *Mct4* (F primer, 5'-CTGTTTGGCAGCACACCATT-3' and R primer, 5'-GGCTGCTTTACCAAGAAGACT-3'), *Il-9* (F primer, 5'-CTCTCCGTC-CCAAGTATGATT-3' and R primer, 5'-AAAGGACGGACACGTGATGT-3'), *Foxp3* (F primer, 5'-GGTACACCCAGGAAGACAG-3' and R primer, 5'-ATCCAGGAGATGATCTGCTTG-3'), *18S* rRNA (F primer, 5'-GTAACCCGTTGAACCC-ATT-3' and R primer, 5'-CCATCCAATCGGTAGTAGCG-3'), and *Gapdh* (F primer, 5'-CGTCCCGTAGACAAAATGGT-3' and R primer, 5'-TTGATGGCAACAATCTCCAC-3') using universal cycling conditions (95 °C for 3 min, 95 °C for 10 s, and 60 °C for 1 min for 40 cycles followed by melting curve analysis).

**Immunoblotting**—Naive T cells were differentiated into Th0, Th1, Th2, Th9, Th17, and iTregs from WT mice. After 48 h of incubation, Th cell subsets and iTregs were washed once with PBS and equal amounts of H<sub>2</sub>O and 2× Laemmli's buffer added for cell lysis. Proteins were denatured at 95 °C for 5 min and stored at -20 °C. Sample proteins were loaded on 10% SDS-PAGE gel and run for 80–120 V for 120 min. Proteins were electrotransferred onto membranes. Membranes were probed with the indicated primary antibodies for NHE1 (1:1000 NHE1 rabbit antibody number GTX85047 from Genetex®, Irvine, CA), Akt (number 4691), AktS473 (number 4060), AktT308 (number 13038), Rictor (number 2114), and mTOR (number 2983) (1:1000 dilution and all from Cell Signaling Technology, Leiden, The Netherlands), followed by HRP-conjugated secondary antibodies (1:2000, Cell Signaling Technology). Membranes were washed and visualized with enhanced chemiluminescence detection system (WesternBright ECL; Biozym®

Scientific GmbH, Hessisch Oldendorf, Germany) and data were analyzed using ImageJ software.

**siRNA Transfection of T Cells**—Naive T cells were transfected with siRNA-control and siRNA-NHE1 using DharmaFECT3 transfection reagent (GE Dharmacon, Lafayette, LA) as recommended by the manufacturer's guidelines. Briefly, naive T cells were washed 3 times with PBS to remove any residual serum and antibiotics from the cells and 0.75–1 × 10<sup>6</sup> cells/well cultured in the presence of antibiotic free media in 24-well plate coated with anti-CD3:anti-CD28. Final concentration of 200 nM non-targeting siRNA-control and siRNA-NHE1 was added to 500 µl of media and cells were incubated with Th9 differentiating conditions as described earlier. Cells were further incubated for 4 days and stained for IL-9/Foxp3 antibodies.

**ATP Measurement**—Naive CD4<sup>+</sup> T cells were differentiated into Th9 cells and also incubated with cariporide (10 µM). Intracellular ATP measurement by luciferase-based assay (ATP Bioluminescence Assay kit CLS II, Roche Diagnostics and Sigma) was performed as described earlier (73). Data were normalized with Th0 cells, performed in triplicate, and relative ATP levels estimated.

**Statistical Analysis**—Prism software (GraphPad software, La Jolla, CA) was used for statistical analysis. Student's *t* test was used for determination of significance. Flow cytometry data were analyzed by Flowjo (Treestar). Figures were made in Excel and GraphPad Prism software. ImageJ was used for Western blot data analysis. *p* values of equal or less than 0.05 were considered significant.

**Author Contributions**—Y. S., K. S. L., and F. L. conceived and coordinated the study and wrote the paper. Y. S. designed, performed, and analyzed the experiments shown in Figs. 1 and 3–6. Y. Z. performed and analyzed the experiments shown in Figs. 2 and 4–6. X. S., S. Z., and M. S. S. performed and analyzed the experiments shown in Figs. 1 and 3–6. A. T. U. provided technical assistance and contributed to the preparation of figures. All authors reviewed the results and approved the final version of the manuscript.

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