DJ-1 sensitivity and functional role of Na^+/H^+ exchanger 1 (NHE1) activity in T lymphocytes

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ABBREVIATIONS

µ	Micron
µl	Microlitre
µM	MicroMolar
3'-UTR	3'-untranslated region
AE	Ion exchanger
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen presenting cells
B.D.	Becton Dickinson
B6	C57BL/6
bp	Base pairs
Ca	Calcium
CD25	IL-2 receptor α chain
CD62L	L-selectin
cDNA	Complementary deoxy ribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
c-Myc	Cellular- myelocytomatosis viral oncogene
CPM	Counts per minute
CTLA-4	Cytotoxic T lymphocyte-associated protein-4
D	Diversity
D value	Shannon entropy diversity index in percentage
DCs	Dendritic cells
DJ-1/Park7	Parkinson's disease related gene 7
DMSO	Dimethyl sulfoxide
DNA	De-oxy ribonucleic acid
dNTP	Deoxynucleotide triphosphates

DTT	Dithiothreitol; Cleland's reagent
E.coli	Escherichia coli
EDTA	Disodium-ethylenediaminetetraacetic acid
ES	Embryonic stem cells
ETDA	Ethylenediaminetetraacetic acid
F	Forward
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
g/l	Gram per litre
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumour necrosis factor
HBBS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
IELs	Intestinal epithelia cells
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
INS	Insulin
iTregs	Induced peripheral regulatory T cells
J	Junctional
kD	KiloDalton
LACK	Leishmania antigen receptor for c-kinase
mAbs	Monoclonal antibodies
MCT	proton-linked monocarboxylate transporter

MFI	Mean fluorescence intensity
MgCl2	Magnesium chloride
MHC	Major histocompatibility complex
ml	Millilitre
mM	Millimolar
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor κB
Na	Sodium
NBC	Sodium bicarbonate co-transporter
NHE	Sodium hydrogen exchenger
ng	Nanogram
NK cells	Natural killer cells
NP	Nucleoprotein
nTregs	Thymus-derived CD4+CD25+FoxP3+ regulatory T cells
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCD	Programmed cell death
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PLNs	Pancreatic Lymph nodes
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative-real time-polymerase chain reaction
R	Reverse
RAG	Recombinase activating gene
RPMI-1640	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase- Polymerase chain reaction

S.N.	Serial number
SDS	Sodium dodecyl sulphate
Ser	Serine
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta
κ	Kappa

1 SUMMARY

Na^+/H^+ exchanger 1 (NHE1) is fundamentally critical for the maintenance of cytosol pH homeostasis as well as cell volume regulation in a variety of cells including $CD4^+$ T cells. Reactive oxygen species (ROS) formation is paralleled by activation of the NHE1. ROS formation in $CD4^+$ T cells plays a decisive role in regulating inflammatory responses. As a candidate, Parkinson disease related gene DJ-1/Park7 is a redox-sensitive chaperone protein counteracting oxidation and presumably contributing to the control of oxidative stress responses and thus inflammation. DJ-1 gene deletion intensifies the progression of Parkinson's disease presumably by augmenting oxidative stress. In this thesis, studies were carried out to find out whether DJ-1 is expressed in $CD4^+$ T cells and affects ROS production as well as NHE1 in those cells. Furthermore, DJ-1 and NHE1 transcript and protein levels were quantified by qRT-PCR and immunoblotting respectively, intracellular pH (pHi) utilizing bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein (BCECF) fluorescence, NHE activity from recovery after removal sodium followed by a activation by an ammonium pulse, and ROS production utilizing 2',7' – dichlorofluorescin diacetate (DCFDA) fluorescence. Immunoblot data suggests that DJ-1 protein was abundantly found in $CD4^+$ T cells. ROS formation, NHE1 transcript levels, NHE1 protein, and NHE activity were higher in $CD4^+$ T cells from DJ-1 deficient mice than from wild type mice in $CD4^+$ T cells. Antioxidant N-acetyl-cysteine (NAC) and protein tyrosine kinase (PTK) inhibitor staurosporine decreased the NHE activity in DJ-1 deficient $CD4^+$ T cells, and blunted the difference between DJ-$1^{-/-}$ and DJ-$1^{+/+}$ $CD4^+$ T cells, an observation pointing to a role of ROS in the up-regulation of NHE1 in DJ-$1^{-/-}$ $CD4^+$ T cells. Together these results suggest that DJ-1 is a powerful regulator of ROS production as well as NHE1 expression and activity in $CD4^+$ T cells.

Further studies were carried out to identify whether $CD4^+$ T cells subtypes or helper T cells (Th) such as Th1, Th2, Th9, Th17 and iTregs development and functions were also governed by NHE1 or not, we measured the NHE1 activity in these cell types. Th cells subsets were characterized by flow cytometry and intracellular pH measured using 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)- Carboxy--fluorescein-Acetoxymethyl Ester (BCECF-AM) dye. NHE1 activity was estimated by ammonium pulse technique. Surprisingly, Th9 cells had significantly higher intracellular pH and NHE1 activity among other Th cells (Th1/Th2/Th17) and iTregs. NHE1 transcripts and protein expression were significantly higher in Th9 cells than in other Th cells subsets. Inhibition of NHE1 by NHE1-siRNA in Th9 cells downregulated IL-9 production. Functional NHE1 activity as well as development and IL-9 production of Th9 cells was further impaired by pharmacological inhibition of protein kinase Akt1/Akt2. Our findings reveal that Akt1/Akt2 sensitive NHE1 could be an important physiological regulator of Th9 cell differentiation and function. Thus, this thesis highlights the novel role of DJ-1 protein in the regulation of NHE1 in immune helper $CD4^+$ T cells and NHE1 could regulates Th9 cells development and functions.

2 ZUSAMMENFASSUNG

Na^+/H^+ Austauscher 1 (NHE1) sind fundamental entscheidend bei der Erhaltung der zytosolischen pH-Homöostase sowie bei der Regulation des Zellvolumens in einer Reihe von Zellen, einschließlich der $CD4^+$ T-Zellen. Die Bildung von reaktiven Sauerstoffspezies (ROS) wird von einer Aktivierung des NHE1 begleitet. Die ROS-Bildung in $CD4^+$ T-Zellen spielt eine entscheidende Rolle in der Regulierung der inflammatorischen Antwort. Potentiell involviert ist das Parkinson-assoziierte Gen DJ-1/Park7, ein Redox-sensitives Chaperone-Protein, das einer Oxidation entgegenwirkt und vermutlich zur Kontrolle der oxidativen Stressreaktion und dadurch der Inflammation beiträgt. Eine Deletion des DJ-1 Gens verstärkt die Progression der Parkinson-Krankheit vermutlich durch eine Erhöhung des oxidativen Stresses. In dieser Arbeit wurden Studien durchgeführt, um herauszufinden, ob DJ-1 in $CD4^+$ T-Zellen exprimiert wird und die ROS-Produktion sowie NHE1 in diesen Zellen beeinflusst. Weiterhin wurden DJ-1- und NHE1-Transkripte und Proteinlevels durch qRT-PCR und Immunoblotting jeweils quantifiziert, der intrazelluläre pH (pHi) mittels bis-(2-carboxyethyl)-5-(and-6)-Carboxy fluorescein (BCECF)-Floureszenz, die NHE-Aktivität von der Erholung nach Entfernung von Natrium gefolgt von einer Aktivierung durch ein Ammonium-Puls, und die ROS-Produktion mittels 2',7'–Dichloro fluorescindiacetat (DCFDA)-Floureszenz gemessen. Die Immunoblot-Daten deuten darauf hin, dass das reichlich DJ-1-Protein in $CD4^+$ T-Zellen gefunden wurde. Die ROS-Bildung, NHE1 Transkript-Level, NHE1-Protein und NHE-Aktivität waren höher in $CD4^+$ T-Zellen von DJ-1-defizienten Mäusen als in denen von Wildtyp-Mäusen. Das Antioxidans N-Acetylcystein (NAC) und der Protein-Tyrosin-Kinase (PTK)-Inhibitor Staurosporin verringerten die NHE-Aktivität in DJ-1-deficienten $CD4^+$ T-Zellen und dämpften den Unterschied zwischen DJ-$1^{-/-}$ und DJ-$1^{+/+}$ $CD4^+$ T-Zellen, eine Beobachtung, die auf eine Rolle von ROS in der Hochregulation von NHE1 in DJ-1-/- $CD4^+$ T-Zellen hindeutet. Zusammen weisen die Ergebnisse darauf hin, dass DJ-1 ein mächtiger Regulator der ROS-Produktion sowie der NHE1-Expression und -Aktivität in $CD4^+$ T-Zellen ist. Weitere Studien wurden durchgeführt, um die Frage zu klären, ob die Entwicklung und Funktion der $CD4^+$ T-Zell-Subtypen oder T-Helferzellen (Th) wie Th1, Th2, Th9, Th17 auch durch NHE1 geregelt werden oder nicht, wir maßen die NHE1-Aktivität in diesen Zelltypen. Th-Zellen-Subtypen wurden mittels Durchflusszytometrie und intrazellulärem pH mittels BCECF-AM-Färbung charakterisiert. Die NHE1-Aktivität wurde mittels der Ammonium-Puls-Technik abgeschätzt. Überraschenderweise hatten Th9-Zellen ein signifikant höheres intrazelluläres pH und NHE1-Aktivität verglichen zu anderen Th-Zellen (Th1/Th2/Th17) und iTregs.

NHE1-Transkripte und -Proteinexpression waren signifikant höher in Th9-Zells als in anderen Th-Zell-Subtypen. Eine Inhibition von NHE1 durch NHE1-siRNA in Th9-Zellen regulierte die IL-9-Produktion herunter. Die funktionelle NHE1-Aktivität als auch -Entwicklung und IL-9-Produktion von Th9-Zellen wurden darüber hinaus durch eine pharmakologische Inhibition der Proteinkinase Akt1/Akt2 beeinträchtigt. Unsere Ergebnisse zeigen, dass das Akt1/Akt2-sensitive NHE1 ein wichtiger physiologischer Regulator der Th9-Zell-Differenzierung und -Funktion sein könnte. Daher hebt diese Arbeit die neue Rolle des DJ-1-Proteins in der Regulierung von NHE1 in Immun-$CD4^+$-T-Helferzellen hervor, und dass NHE1 die Entwicklung und Funktion der Th9-Zellen regulieren könnte.

3 SCIENTIFIC BACKGROUND

3.1 Adaptive Immunology

Other than the first line of body resistance, which called the innate immune response, there is a substantially more advanced guard line called adaptive immune response (Bonilla and Oettgen 2010). To eliminate pathogens the first line and the second line need to cooperate, and in most cases the innate responses call up the adaptive immune response. The striking difference in-between innate and adaptive response is the specificity among them (Bonilla and Oettgen 2010). The adaptive immune response is very particular to the pathogens that enact them. Alongside the memory effect of some members in the adaptive immune system, the body benefit from the first hit of the pathogens, such as the body recovered from some specific virus infection (measles) will remember the hit and protect from the same infection enduring(Bonilla and Oettgen 2010).

The concept of adaptive immunity can be divided in to cellular immunity and humoral immunity. Cellular immunity, which recognize the non-self-cells, abnormal cells and microbes, and is, ended up to eliminated by the cytotoxic T cells and other Cells (Bonilla and Oettgen 2010). Humoral immunity which correlated with B cells took after by the antigen exposure, antibody secretion from the Plasma cells, and the memory effect for the memory B cells. Both T cells and B cells have their unique receptors, TCRs and BCRs respectively (Germain 2001). During T cells differentiation, TCRs are generated through their developmental pathway by DNA recombination in the thymus. Major histocompatibility complex (MHC) molecules present the antigen followed the formation of processed peptides, which can be recognized by TCRs (Germain 1994). Each TCR and BCR has its conserve and variable region, resulting the combination of a huge diversity of receptors (Germain 2001). Due to huge

diversity, T cells and B cells recognize the diverse array of pathogens, in the mean while; cytotoxic T cells development and antibody production are triggered, as well as the generation of memory cells (Akira 2011). The accompanying figure outlined the procedure in adaptive immunity (Akira 2011). The foundation of adaptive immunity is specificity, plasticity and memory, all coordinated to the construction and homeostasis of functional lymphocyte repertoires (Blattman, Sourdive et al. 2000)

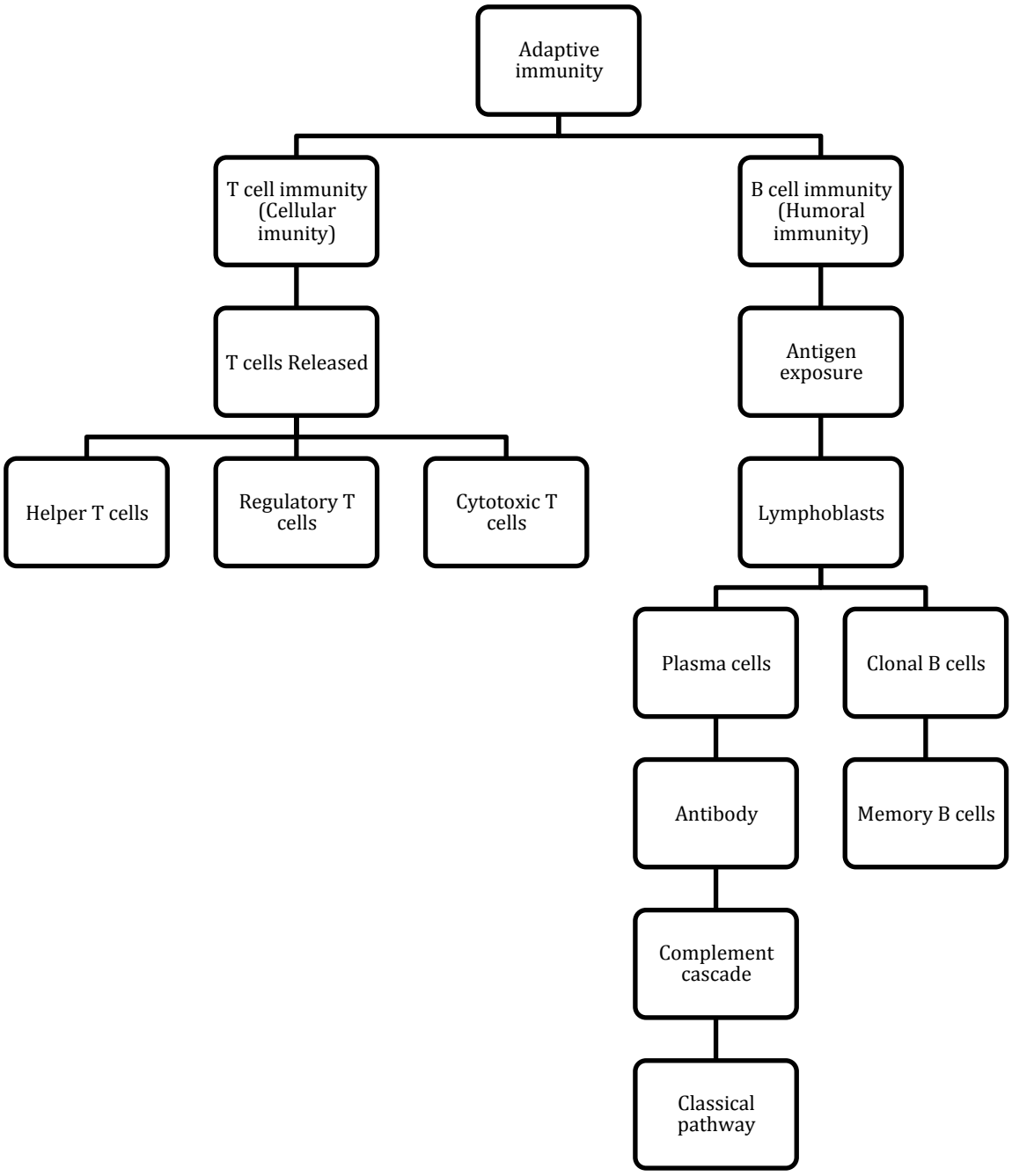


Figure 1 Adaptive Immunity

Outline of the construction of adaptive immune system, the system can be divided into two main brunches, which are T cell immunity and B cell immunity. Followed by T cell releasing, different subtype of T cell accumulated and contribute to the adaptive immunity. On the other hand, in B cell immunity, initiated plasma cells and memory cells built together the humoral immunity.

3.2 $CD4^+$, $CD8^+$ T cells and Dendritic Cells

The CD4 molecule is a glycoprotein, which has been found on the surface of cell membrane, such as T helper (Th), monocytes, macrophages and dendritic cells. The CD4 protein is encoded by the CD4 gene in humans and mice,(Honczarenko, Kulczycki et al. 1977). The major function of $CD4^+$ T cells is to provide help to other immune cells (Zhu and Paul 2008).The $CD4^+$ T cells play a variety of roles by recognizing MHC class II molecules, such as activating the innate immune response, adaptive immune response, Killer T cells, and some non-immune cells, and also act as a regulatory immune response (Zhu and Paul 2008).

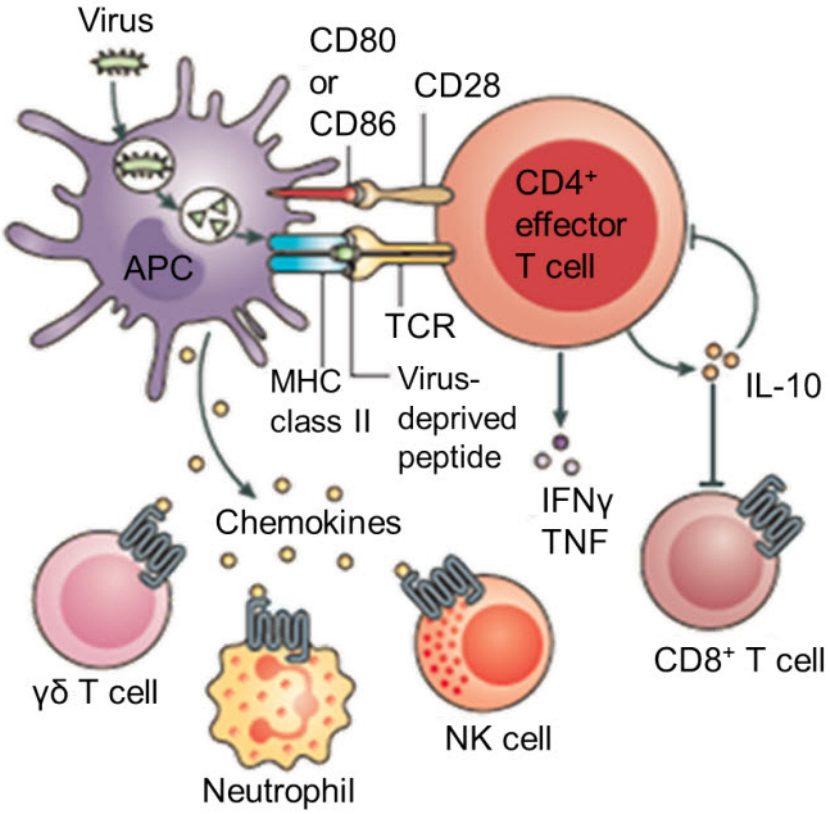


Figure 2 Helper function of $CD4^+$ T cells to APCs

After migrating to sites of infection, effector $CD4^+$ T cells that recognize antigens on antigen-presenting cells (APCs) produce an array of effector cytokines that contribute to the character of the inflammatory responses in the tissue. Some products of highly activated effector $CD4^+$ T cells, such as interleukin-10 (IL-10), dampen inflammation and regulate immunopathology, whereas others, such as interferon-γ (IFNγ), are pro-inflammatory and activate macrophages, which in turn drive further inflammation. The production of IL-10 by effector $CD4^+$ T cells can have a profound impact on the outcome of a viral infection (Swain, McKinstry et al. 2012).

The $CD8^+$ (cytotoxic) T cells, like $CD4^+$T cells, are generated in the thymus and express the T cell receptor (Tscharke, Croft et al. 2015). The difference between CD8 and CD4 molecule is laid on that cytotoxic T cells recognize MHC class I molecules which have been found on all nucleated cells (Tscharke, Croft et al. 2015). For the cytosolic pathogen defensing cytotoxic T cells play a crucial role following three major mechanisms: secretion cytotoxic cytokines such as primarily TNF-α and IFN-γ; releasing cytotoxic granules such as perforin, and granzymes; destruction of infected cells via Fas/FasL interactions (Varga, Wissinger et al. 2000).

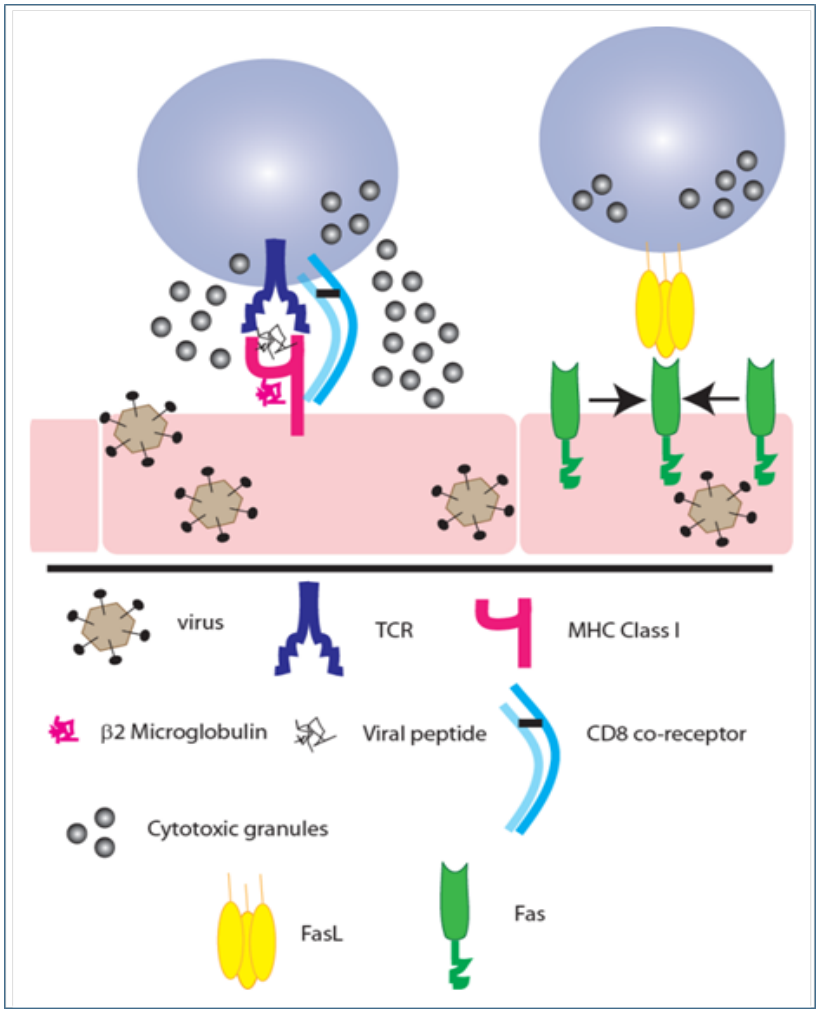


Figure 3 CD8$^+$ T cells Immune response

CD8+ T cells recognize peptides presented by MHC Class I molecules, found on all nucleated cells. The CD8 heterodimer binds to a conserved portion (the α3 region) of MHC Class I during T cell/antigen presenting cell interactions From Erika Wissinger, Imperial College London, UK.

One of the antigen-presenting cells (APCs) called Dendritic cells (DCs), addressed for their formation, dendritic shapes, can trigger the adaptive immune responses and promote the function of the immune system (Lipscomb and Masten 2002). Paul Langerhans first described DCs in human skin in 1868 but thought they were cutaneous nerve cells. DCs are bone marrow derived leukocytes and are contributed as the most potential APC. They can also be propagated in vitro from bone marrow and peripheral cycling system followed by introducing growth factors, such as granulocyte macrophage-colony stimulating factor (GM-CSF).

DCs can specifically capture and present antigens, converting proteins to peptides that are presented on major histocompatibility complex molecules, in most case MHC II which can then be recognized by T cells (Roghanian, Williams et al. 2006, Young, Waller et al. 2008). DC subsets playing differing roles in defining the outcome of an immune response, although clearly some plasticity within defined subsets is possible so that each subset can exert tolerating and polarizing influences on responding T cells (Patterson 2000).

DCs degrade antigens within a MHC class II-rich endosomal compartment (MIIC) yet preserve sufficient peptide structure to be expressed on their cell surface bound to MHC class II molecules (ten Broeke, Wubbolts et al. 2013). DCs take up antigens by phagocytosis, utilizing membrane receptors to trigger uptake, by receptor-mediated pinocytosis in clathrin-coated pits and by fluid-phase pinocytosis (Garrett, Chen et al. 2000). DCs can take up whole cells, including necrotic and apoptotic cells. They can also acquire antigens from live cells for presentation to cytolytic T cells (Harshyne, Watkins et al. 2001)

3.3 $CD4^+$ T cells signaling

Unactivated T cells get activated through the Linker for activation of T cells (LAT) signaling (Keller, Zaidman et al. 2016). When APC present the antigen to T cell, MHC class II get recognized by TCR variety ligation part, SRC superfamily protein tyrosine kinases (PTKs) LCK connected with CD4 and FYN bind with CD3 are activated, leads to phosphorylation of CD3 ζ-chain of the TCR complex and bind activated SYK-family PTKs-ζ-chain-associated protein of 70kD (ZAP70). When ZAP70 get activated, it phosphorylates LAT. LAT then phosphorylates SLP76 (SRC-homology 2 (SH2)-domain-containing leukocyte protein of 76 kD) and recruits SH2-domain-containing proteins growth factor receptor-bound protein 2 (GRB2), GRB2-related adaptor protein (GADS) and phospholipase C-γ1 (PLC-γ1) 4(Abraham and Weiss 2004). The hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate (IP_3) and diacylglycerol (DAG) have been supported by both LAT and SLP76 associates with the SH3 domain of PLC-γ1 activation (Yablonski, Kadlecek et al. 2001). Accumulated IP_3 ended up to increases of cytosolic free Ca^{2+} concentration, as a secondary signal it leads to IL-2 gene expression, cell cycle entry and T cell effector functions. Production of DAG can activate both protein kinase C-θ (PKC-θ) and RAS guanyl nucleotide-releasing protein (RASGRP). Phosphorylated LAT also recruits the SH2 domain of GRB2 (Houtman, Yamaguchi et al. 2006), and therefore, the GRB2-associated RAS guanosine nucleotide-exchange factor (GEF), son-of-sevenless (SOS), as the regulator of RAS indicates the additional possible mechanism of RAS activation through LAT. Tyrosine- phosphorylated SLP76 also associates with the RHO-family GEF, VAV1, and the adaptor protein, NCK (Abraham and Weiss 2004). A threesome complex of SLP76, VAV1 and NCK-associated p21-activated kinase 1 (PAK1) as their

consequential role of SLP76, reveals a potential mechanism for SLP76 regulation of actin cytoskeletal rearrangements following TCR stimulation.

CD28 is a 44-kDa homo-dimeric protein involved in T-cell costimulatory signaling pathways (Rudd, Taylor et al. 2009). One of the main signaling pathways regarding to CD28 on T cell function regulation is CD28-PI3K signaling. PI3K production of PIP2 and PIP3 lipids bind the pleckstrin homology (PH) domains within proteins such as phosphoinositide-dependent protein kinase 1 (PDK1) (Alessi, Deak et al. 1997). PDK1 and PKB can themselves then phosphorylate and regulate multiple pathways linked to protein synthesis, cellular metabolism, and cell survival (Rudd and Schneider 2003).

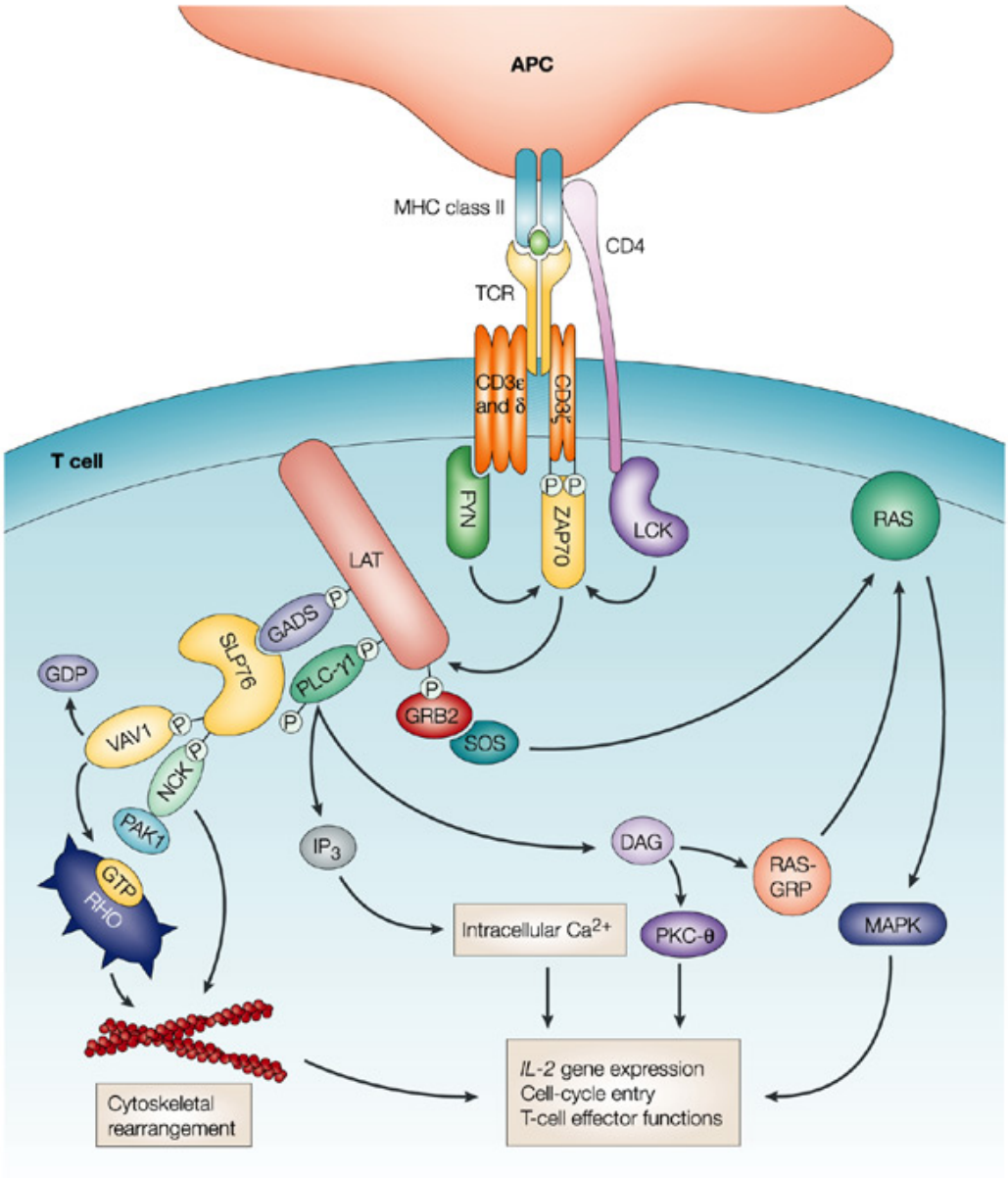


Figure 4 Proximal signalling complexes and downstream responses induced by T-cell receptor (TCR) ligation.

Antigen presenting cells expose the MHC class II to T cell by counteract with TCR, thus triggered T cell signaling pathways contribute to cell cycle entry, IL-2 gene expression and T cell effector functions (Abraham and Weiss 2004).

3.4 T helper Cells

With specific MHC II receptor, $CD4^+$ T cells recognize antigens presented by antigen presenting cells such as dendritic cells by which they get activated and participate in the regulation of the immune response during infections, autoimmunity and cancer. In the importance of adaptive immunity, activated $CD4^+$ T cells can be shaped during their development from conventional T cells. $CD4^+$ T cells participate as a crucial role in the effector adaptive immune system (Zhu and Paul 2010). $CD4^+$ T cells can be differentiated into various subtypes based on their cytokine secretion, such as Th1, Th2, Th9, Th 17 and regulatory T cells.

IFN-γ, TNF-α producing T helper 1 (Th1) cells (Zhou, Chong et al. 2009, Zhu and Paul 2010). Th1 participate in the defense process of preventing intracellular parasites. IL-4 producing Th2 cells (Kudo, Ishigatsubo et al. 2013). In the functional aspect of Th2 cells, controlling extracellular pathogens and parasites as well as play an important role of allergy and anthemia. IL-17 producing Th17 cells (Bettelli, Carrier et al. 2006, Korn, Mitsdoerffer et al. 2008, Awasthi and Kuchroo 2009) defensing against the pathogens, and take part in the autoimmunity, transplant rejection and cancer immunology. IL-9 producing Th9 cells (Dardalhon, Awasthi et al. 2008, Dardalhon, Collins et al. 2015, Kaplan, Hufford et al. 2015), which is a relatively new subtype play an important role in INF-γ dependent antitumor immunology as well as allergic inflammation in lungs and tuberculosis. TGF-β and IL-10 producing suppressive regulatory T cells (Tregs) (Schmitt, Haribhai et al. 2012). Unlike other effect T cells play a suppression role in controlling the balance of T cells subsets in inflammation process and other abnormal abundance of effect T cells. A great deal of experimental efforts attempted to decipher the development and functions of various Th cells subsets. However,

the development and functions of Th9 cells remained incompletely understood.

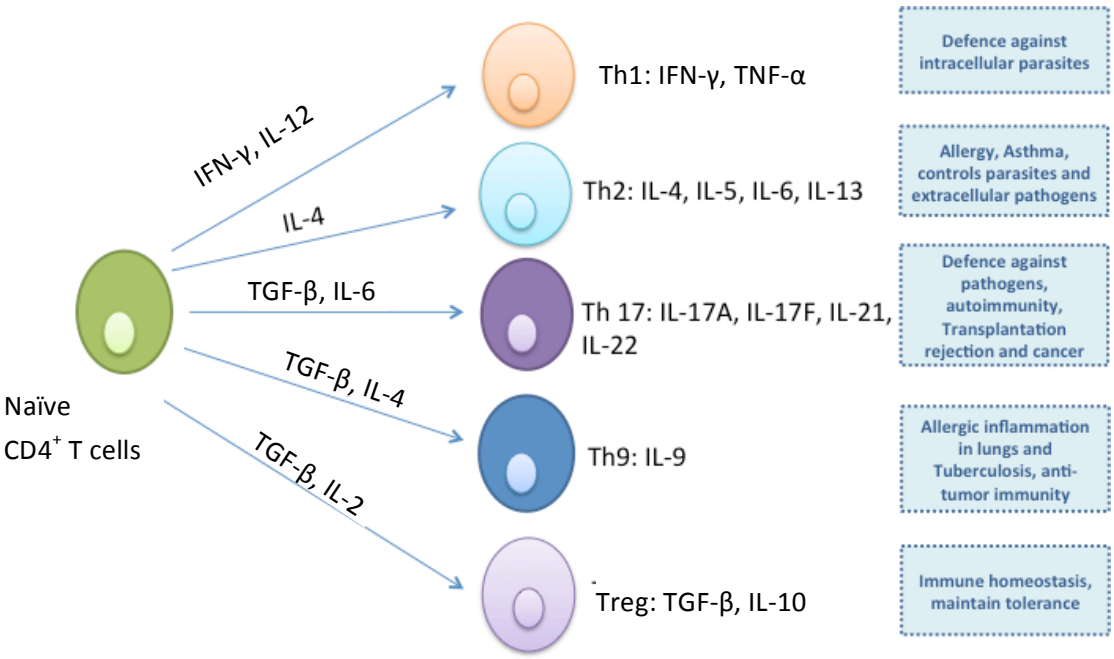


Figure 5 Summary of T helpers development

By introducing cytokines in different combination such as IFN-γ, IL-12, IL-4 TGF-β, IL-6, IL-4 and IL-2, Naïve T cells get differentiated into several subtypes such as Th1, Th2, Th17, Th9 and Treg. Differentiated T cells get involved into regulation of immune system by secretion of varieties of cytokines.

3.5 Intracellular pH

In term of the prototypical mammalian cell, under physiological conditions, the extracellular pH is slightly alkaline (~7.3–7.4) (Casey, Grinstein et al. 2010). In some conditions, such as more alkaline environment-alkaline medium, cell itself will monitor to avoid intensely acidification. The intracellular pH (pHi) will turn into slightly acidification. In the process of cytosol acidification, there are two aspects was majorly discussed to illustrate the phenomenon (Casey, Grinstein et al. 2010). In one hand, the electrical potential across the membrane, which is negative inside, drives the uptake of positively charged protons and the efflux of negatively charged bases, such as HCO3–, through conductive pathways (Casey, Grinstein et al. 2010). In another hand, net acid equivalents are generated by various metabolic reactions (for example, ATP production in the cytoplasm by glycolysis and in mitochondria by oxidative phosphorylation), a situation that is exacerbated during bursts of activity, such as in muscle contraction or on activation of leukocytes by pathogens (Grinstein, Furuya et al. 1986).

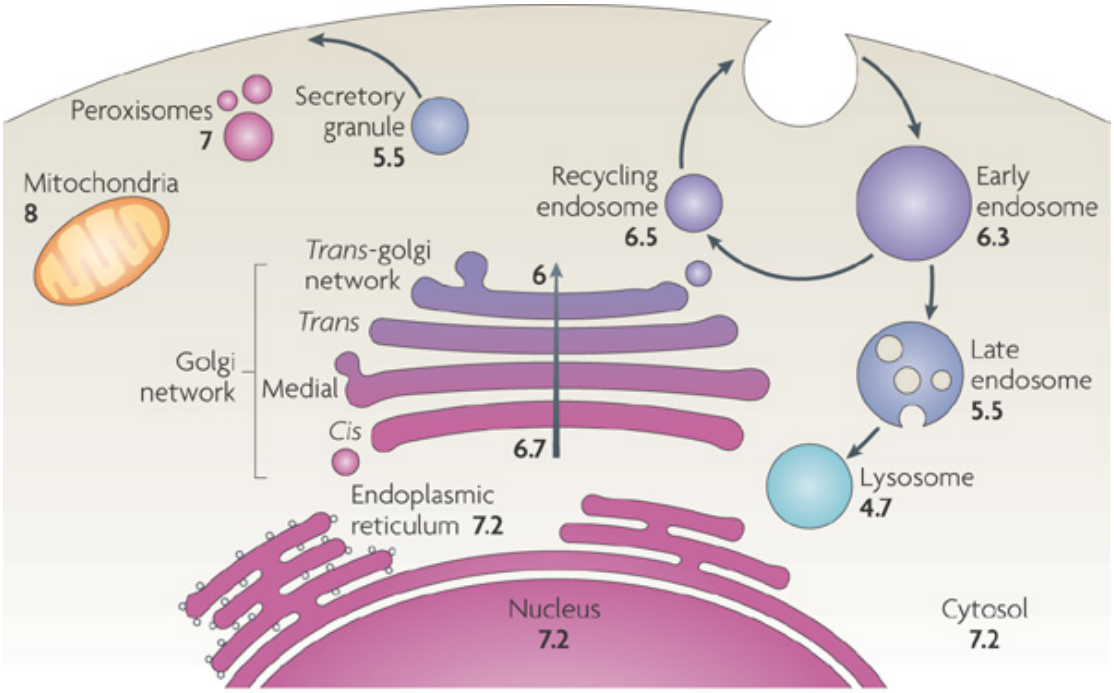


Figure 6 Schematic of cytosolic pH in cell organelles

The pH of individual cellular organelles and compartments in a prototypical mammalian cell. The values were collected from various sources. The mitochondrial pH refers to the matrix, that is, the space contained by the inner mitochondrial membrane. (Casey, Grinstein et al. 2010)

3.6 Ion carriers that regulate cytoplasmic pH

The cytoplasm has a tendency to acidify owing to the activities of various of metabolic pathways, such as the ATP formation in the cytoplasm by glycolysis that generates lactate (the anaerobic segment in the figure) and the oxidative phosphorylation in mitochondria that produces CO2 (the oxygen consuming part appeared in the following figure 7 (Casey, Grinstein et al. 2010). The transcendent pH-administrative transporters that are in charge of alkalization are the plasma membrane sodium hydrogen exchangers (NHEs) and sodium bicarbonate co-transporters (NBCs) (Grinstein, Furuya et al. 1986). A set number of cell sorts additionally alkalinize their cytosol through the activities of sodium dependent chloride bicarbonate exchangers (NDCBEs) (Casey, Grinstein et al. 2010). These transporters utilize the energy stored in the internally coordinated electrochemical Na+ gradient that is built up by sodium potassium ATPase pumps (NKAs) to drive solute transport (Casey, Grinstein et al. 2010). These alkalinizing components are counteracted the activities of plasma membrane chloride bicarbonate or anion exchangers (AEs), which acidify the cell. Plasma membrane Ca^{2+}-ATPases (PMCAs), which exchange cytosolic Ca^{2+} for extracellular H^+, likewise acidify the cytosol in response to boosts that exchange intracellular Ca^{2+}. In tissues experiencing anaerobic metabolism, alkalinization might be favoured by monocarboxylate–H^+ co-transporters (MCTs; for example, in muscle).

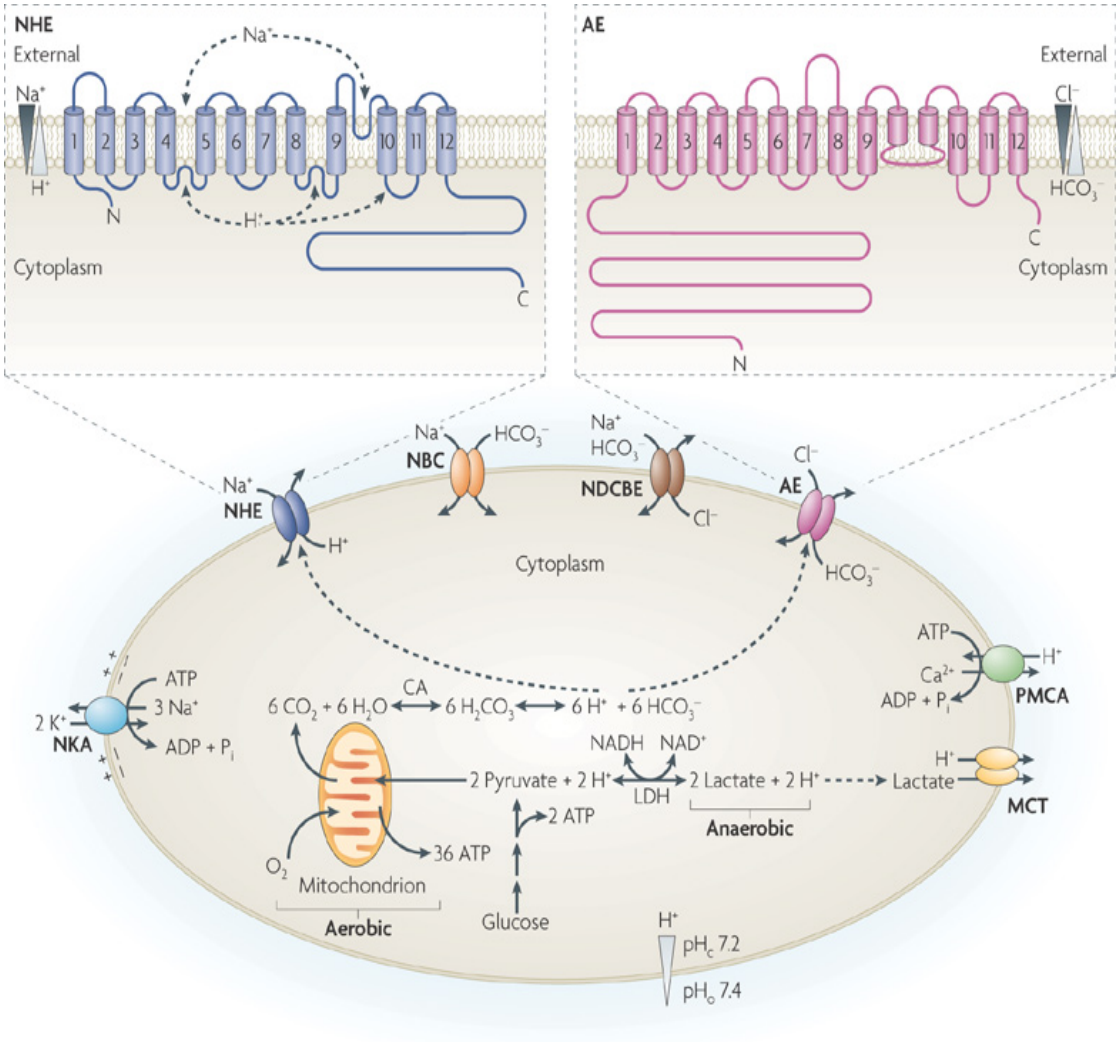


Figure 7 Ion carriers that regulate intracellular pH

The most studied transporters, which take part in the regulation of intracellular pH. Sodium ion, hydrogen, bicarbonate, chloride, ATP and other related elements construct the network in pH regulation. (Casey, Grinstein et al. 2010)

3.7 Sodium hydrogen exchanger

Sodium hydrogen exchanger (NHE) is a group of ATP-independent membrane glycoprotein transporters that are included in the control of pH in cytosol, as well as after hormones and mitogens reaction cell volume and the cellular responses (Valles, Bocanegra et al. 2015). The transporters intervene the concurrent efflux of a hydrogen ion and the influx of a sodium ion transport the plasma membrane (Mahnensmith and Aronson 1985). The internal coordinated electrochemical Na^+ gradient drives this process. There are nine known mammalian NHE isoforms (NHE1-9), all of which contrast in particular biological function as well as subcellular localization (Bobulescu and Moe 2009). Furthermore, except their basic contribution in the media of cytosolic pH and cell volume, the NHE family has additionally been embroiled in diseases including hypertension and organ ischemia (Murphy and Eisner 2009). Whilst the particular system by which NHE activity impacts blood pressure still depending on necessary further studies, it has been recommended to include the inversion of the Na^+/Ca^{2+} exchanger (NCX); this prompts expanded intracellular Ca^{2+} concentration, leads to vascular smooth muscle cell contraction (Iwamoto, Kita et al. 2005).

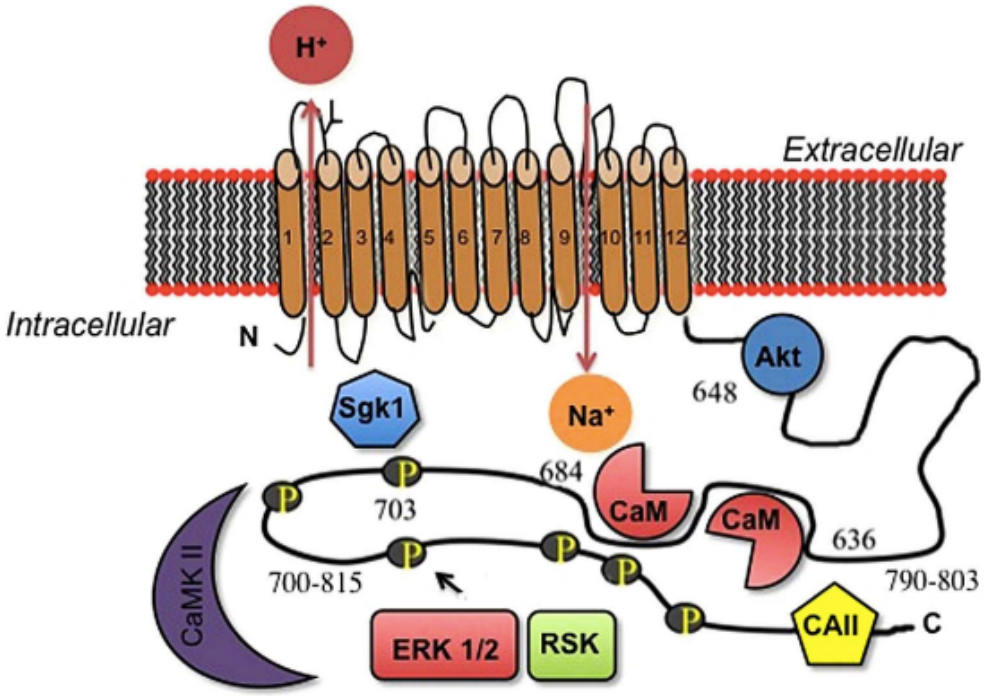


Figure 8 A representative topology model of the NHE1 glycoprotein displaying the extracellular and intracellular cytosolic domains.

A schematic shows the tracing pathways followed by hydrogen exchange, the certain binding point counteract with Sgk1 and Akt then activated the related cell signaling pathways (Mohamed, Iman A et al. 2015).

NHE1 serves as an important alkalinizing mechanism of the cell in defense of H^+ derived from metabolism or electrically-driven H^+-accumulation. In addition, NHE1 constitutes a major pathway for Na+ influx into the cell and when coupled to Cl^- and H2O uptake, it represents a mechanism for restoration of cell volume following cell shrinkage. (Donowitz, Ming Tse et al. 2013). NHE1 is also involved in cell migration (Denker, Huang et al. 2000). NHE2 is involved in some intestinal and renal Na absorption and there is recent evidence that it has a role in repair of epithelial damage (Xue, Aihara et al. 2011). NHE3 is reused between apical membranes and the cytosol compartment of epithelial cells where it has a noteworthy contribution in renal and intestinal Na^+ retention (D'Souza, Garcia-Cabado et al. 1998). Beside their parts in managing intracellular pH and cell volume, Majorly considerable studies are still needed in NHE4, NHE5, NHE6, NHE7, NHE8 and NHE9 (Lemasters, Bond et al. 1996).

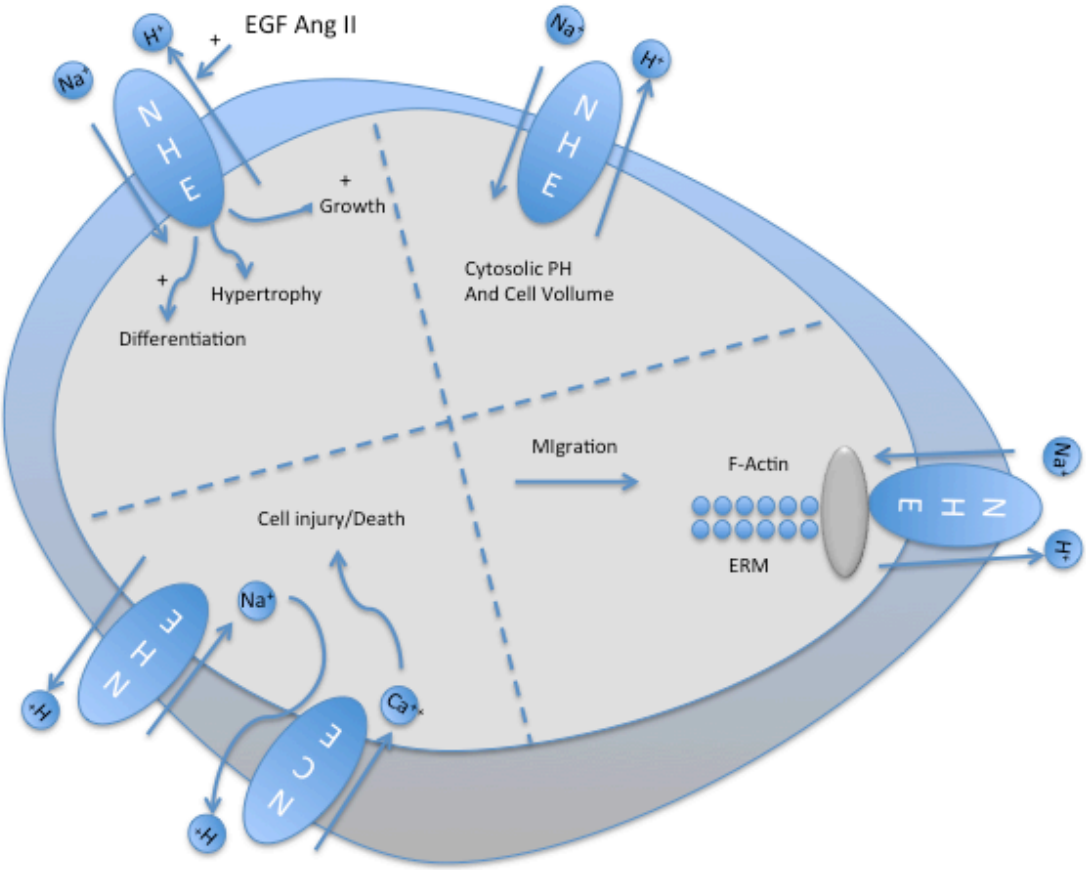


Figure 9 NHE regulates various cellular physiological function

A summary of four major role of NHE in cell function regulation. Beside the role of maintains the pH homeostasis, combine with EGF Ang II, exchange of hydrogen leads to regulate the cell growth, hypertrophy and differentiation. Cross talk with F-Actin NHE regulates the cell migration. The entry of sodium leads to activation of calcium transportation ended up to influence the cell injury or cell death.

3.8 DJ-1

DJ-1, initially distinguished as an oncogene product, a pervasively expressed protein associated in cellular transformation, oxidative stress function, and regulation of transcripts. (Mitsumoto and Nakagawa 2001, Zhong, Kim et al. 2006) DJ-1 assumes an imperative part of cell resistance by dampen p53-Bax-caspase signaling pathway. The defensive impacts of DJ-1 on program cell death are associated with its ability of decreasing Bax expression by dampen p53 transcriptional activity (Bonifati, Rizzu et al. 2003, Fan, Ren et al. 2008).. DJ-1 (PARK7) has been identified as the gene linked to early-onset familial Parkinson's disease. DJ-1 decreases the sensitivity to excytotoxicity and ischemia proved by DJ-1 knockout model, in another word, production of DJ-1 could reverse this sensitivity and indeed enhancing cytoprotection (Aleyasin, Rousseaux et al. 2007). Oxidative stress is caused by the imbalance of the reactive oxygen species production and inhibition. As an oxidation-reduction escort DJ-1 protects neurons against oxidative stress and apoptosis (Bonifati, Rizzu et al. 2003, Billia, Hauck et al. 2013). From tumor hypoxia adaption model, DJ-1 protein has been investigated and revealed the dependent function in cell survival process, through the AKT related signaling pathways, involves in both AKT/mTOR (Vasseur, Afzal et al. 2009) and PTEN/AKT signaling (Billia, Hauck et al. 2013, Klawitter, Klawitter et al. 2013, Dongworth, Mukherjee et al. 2014). DJ-1 counteracts oxidative stress by specifically decreasing reactive oxygen species (ROS) upon oxidative alteration of a conserved cysteine residue or through stabilizing antioxidant transcription factors, for example, erythroid related factor 2 (NRF2) (Clements, McNally et al. 2006, Moscovitz, Ben-Nissan et al. 2015, Shi, Lu et al. 2015).

3.9 ROS and NHE1

Recently study shows that ROS production relies on the activity such as H^+ extrusion by the Na^+/H^+ exchanger NHE1 (Rotte, Pasham et al. 2010, Rotte, Pasham et al. 2011, Yang, Bhandaru et al. 2012, Alvarez and Villa-Abrille 2013, Prasad, Lorenz et al. 2013, Qadri, Su et al. 2014, Park, Lee et al. 2015), which takes part in the control of intracellular pH (pHi) (De Vito 2006) and of several further cellular functions, for example, cell volume, cell proliferation and motility of cells (Putney and Barber 2004, De Vito 2006). NHE1 is ubiquitously expressed (Lacroix, Poet et al. 2004, Putney and Barber 2004, Fliegel 2005, De Vito 2006, Abu Jawdeh, Khan et al. 2011) and the most vital Na^+/H^+ exchanger isoform in the homeostasis modification of intracellular pH (pHi) (De Vito 2006).

Past studies revealed that Na^+/H^+ exchanger activity in $CD4^+$ T cells is sensitive to various stimuli including growth factors (EGF, angiotensin II) and cytokines including IL-2 and IFN-γ (Wakabayashi, Shigekawa et al. 1997, Lacroix, Poet et al. 2004, Chang, Wang et al. 2010). The present study investigated the effect of DJ-1 on ROS production/generation and Na^+/H^+ exchanger activity in $CD4^+$ T cells, which are required for the support of effective immune responses against different intracellular or extracellular pathogens and autoimmune disorders (Bluestone, Mackay et al. 2009, Abou-Jaoude, Monteiro et al. 2014, Bonelli, Shih et al. 2014, Brucklacher-Waldert, Carr et al. 2014, Hale and Ahmed 2015).

But the role of DJ-1 on Na^+/H^+ exchanger activity regulation has not been defined.

4 AIMS OF THE THESIS

- To identify the role of DJ-1 deficiency on NHE1 transcript and protein level as well as Na^+/H^+ exchanger (NHE) activity in activated $CD4^+$ T cells.

- To decipher the regulatory function of intracellular pH and NHE1 in the development of T helper (Th0/Th1/Th2/Th9/Th17 and iTregs) cells.

- To examine the function of the NHE1 in Th9 cells development and functions.

5 MATERIALS AND METHODS

5.1 Mice strains

DJ-1$^{-/-}$ and DJ-1$^{+/+}$ (10-12 week-old) mice on a C57BL/6 background were used for the experiments as described earlier (Billia, Hauck et al. 2013) and kept under standard husbandry conditions. DJ-1$^{+/+}$ and DJ-1$^{-/-}$ mice were obtained by breeding DJ-1$^{+/-}$ heterozygous male and female mice. C57BL/6 mice (8-10 weeks) were used for the Th cells experiments and kept in a conventional specific pathogen free facility. All the animal work was 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 5-6 independent mice were used and data shown in each figure is representative of Means±SEM of independent mice experiment.

5.2 Naïve T cells isolation

To perform the Th differentiation or iTreg induction experiments, naïve CD4^{+}CD62L^{high+}CD25^{-} T cells were isolated using magnetic bead selection from spleen and lymph nodes as described earlier (Singh, Dyson et al. 2011, Singh, Chen et al. 2015). To isolate the CD4^{+} T cells, spleen and lymph nodes (inguinal, axillary, brachial, mediastinal, superficial cervical, mesenteric) were collected from the mice and macerated using syringe plunger. Cell suspension was centrifuged at 600x*g* at 4^{0}C for 5 minutes and cells pellet treated with RBC lysis buffer for 1 minute and washed three times. After washing cells were kept on roller at 4^{0}C (cold room) for 30 minutes in the presence of 40µl/mouse antibody mix containing anti-CD8, anti-MHC II, anti-CD11b, anti-CD16/32,

anti-CD45R, and Ter-119 (Dynabeads® Untouched™ Mouse CD4 cells kit, Invitrogen). Cells were washed after antibody incubation and Dynabead was added and incubated at 4^0C (cold room) for 30 minutes on roller to deplete the $CD8^+$ T cells, B cells, NK cells, monocytes/macrophages, dendritic cells, erythrocytes and granulocytes and isolated $CD4^+$ T cells. Further, to isolate $CD4^+CD25^+$ T cells, purified $CD4^+$ T cells were incubated with 2 µl/mouse biotinylated-anti-CD25 (7D4 clone; BD Biosciences, UK) for 30 minutes in cold room on roller, washed and kept for 15 minutes with 20µl/mouse streptavidin-Microbeads (Miltenyi Biotech, Germany) for the indirect magnetic labelling of $CD25^+$ T cells. Using MACS separation columns $CD4^+CD25^+$ T cells were positively selected and remaining cells were $CD4^+CD25^-$ T cells (Niedbala, Besnard et al. 2014). To enrich naïve $CD4^+CD62L^{high+}CD25^-$ T cells from $CD4^+CD25^-$ T cells, these cells were again incubated with 10 µl/mouse biotinylated-anti-CD62L (clone MEL-14; BD Biosciences) for 30 minutes in cold room on roller, washed and kept for 15 minutes with 20µl/mouse streptavidin-Microbeads for the indirect magnetic labelling of $CD62L^+$ T cells. Using MACS separation columns naïve $CD4^+CD62L^{high+}CD25^-$ T cells were positively selected. Remaining cells were $CD4^+CD62L^-CD44^+CD25^-$ T cells. Purity of these cells was checked by flow cytometry. As a result more than 90% cells were positive for naïve T cells.

Purified $CD4^+$ T cells ($1x10^6$ cells) from DJ-$1^{+/+}$ and DJ-$1^{-/-}$ mice were activated in the presence of anti-CD3:anti-CD28 (1:2) (eBioscience, Frankfurt, Germany) for 1-3 days in R10 medium at 37° C in a 5% CO_2 incubator. The cultured cells were used for pH measurements, immunoblotting, and q-RT-PCR. Further inhibitors used were cariporide (10 µM) (Sigma, Munich, Germany), antioxidant N-acetyl-cysteine (NAC; 10 µM) (Sigma) and ROS scavenging enzyme catalase (10 µM) (Sigma).

5.3 Th cells differentiation

Naïve $CD4^+CD62L^{high+}CD25^-$ T cells were activated in the presence of plate-bound anti-CD3/anti-CD28 antibodies (eBiosciences, Frankfurt, Germany) with a ratio of 1:2::anti-CD3:anti-CD28 (1ug/ml anti-CD3: 2 µg/ml anti-CD28) for Th1, Th2 and iTregs and 1:10::anti-CD3:anti-CD28 (1 µg/ml anti-CD3: 10 µg/ml anti-CD28) for Th9 and Th17. Briefly, T naïve cells were differentiated into Th1 using 20 ng/ml recombinant-IL-12 (eBiosciences, Frankfurt, Germany), anti-IL-4 (5 µg/ml; eBiosciences, Frankfurt, Germany), Th2 using 20 ng/ml recombinant-IL-4 (eBiosciences, Frankfurt, Germany), anti-IFN-γ (5 µg/ml; eBiosciences, Frankfurt, Germany), Th9 using 2.5 ng/ml recombinant-TGF-β, 40 ng/ml recombinant-IL-4, anti-IFN-γ (10 µg/ml), Th17 using 2.5 ng/ml recombinant-TGF-β, 50 ng/ml recombinant-IL-6 (eBiosciences, Frankfurt, Germany), anti-IFN-γ (5ug/ml), anti-IL-4 (5 µg/ml) and anti-IL-2 (5 µg/ml; eBiosciences, Frankfurt, Germany) and iTregs using 2.5 ng/ml recombinant-TGF-β, 5 ng/ml recombinant-IL-2 (eBiosciences, Frankfurt, Germany), cultured for 3-4 days (Zhou, Chong et al. 2009, Perumal and Kaplan 2011, Kudo, Ishigatsubo et al. 2013, Chatterjee, Schmidt et al. 2014, Singh, Garden et al. 2015). Cells were harvested at day 3 and used for intracellular staining for characterising the Th cells using flow cytometry, q-RT-PCR, pHi, NHE1 activity and immunoblotting experiments.

5.4 Intracellular pH (pHi) measurement

Equipment for pHi imaging

Names	Manufacturer and country of origin
Discofix® Stopcock for Infusion Therapy	B.Braun, Melsungen, Germany
Centrifuge RotiFix 32	Hettich Zentrifugen, Tuttlingen, Germany
Camera Proxitronic	Proxitronic, Bensheim, Germany
Eppendorf pipettes 1000 µl, 100 µl, 10 µl	Eppendorf AG, Hamburg, Germany
Eppendorf cups 1.5, 2 ml	Eppendorf AG, Hamburg, Germany
Filter Set for BCECF-AM	AHF Analysentechnik AG, Tübingen, Germany
Filter tips 10, 100, 1000µl	Biozym Scientific, Hess. Oldendorf, Germany
Filter wheel	Sutter Instrument Company, Novato, USA
Heraeus Incubator	Thermo Electron Corporation, Dreieich, Germany
Lamp XBO 75	Leistungselektronik Jena GmbH, Jena, Germany
Metafluor software	Universal Imaging, Downingtown, USA
Microscope Axiovert 100	Zeiss, Oberkochen, Germany
Microscope cover glasses round, 30mm diameter, 0.13-0.16 mm	Karl Hecht KG, Sondheim, Germany
Multiwell™ 6 well	Becton Dickinson Labware, Franklin Lakes, USA
Needles BD Microlance ™3,	Becton Dickinson Labware, Franklin

1.2X40mm	Lakes, USA
Objective neo fluar 40x/1.3 oil	Carl Zeiss, Oberkochen, Germany
PP-Test Tubes 15, 50 ml	Greiner bio-one, Frickenhausen, Germany
Syringde BD 10ml, Leur-Lok™ Tip	Becton Dickinson Labware, Franklin Lakes, USA
Syringde BD, Perfusion™ 50ml	Becton Dickinson Labware, Franklin Lakes, USA
Tissue Culture Dishes 35x10 mm	Becton Dickinson Labware, Franklin Lakes, USA

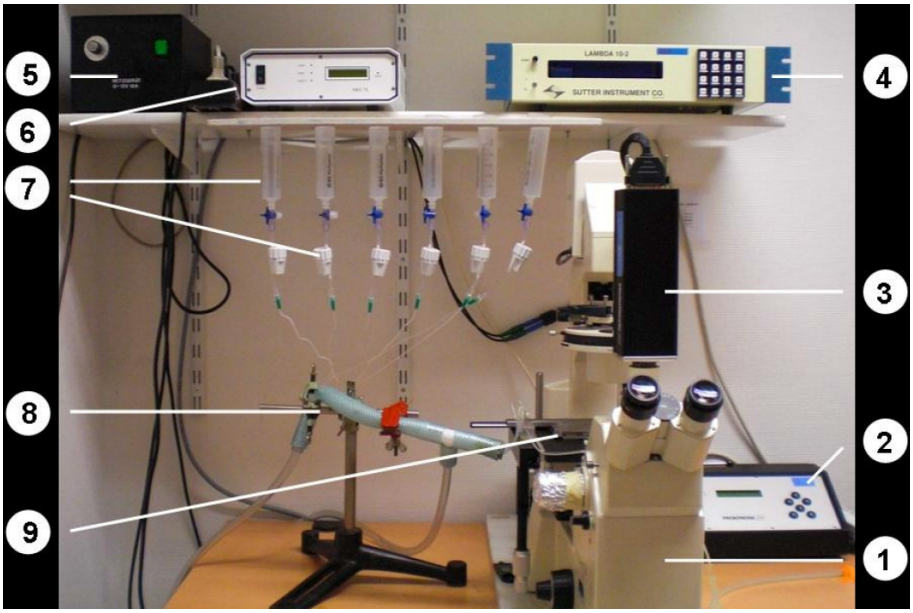


Figure 10 Intracellular pHi imaging setup.

1-Micrsocope, 2-Camera control panel, 3-Camera, 4-Shutter, 5-Light source, 6-Xenon lamp control panel, 7-Perfusion system, 8-Flow heating system, 9-Measuring chamber

pH measurement chemicals

Substances	Manufacturer and country of origin
Ampuwa	Fresenius KABI, Bad Homburg,

	Germany
$CaCl_2$ x 2 H_2O	Carl Roth, Karlsruhe, Germany
CXCL12	Peprotech, Germany
Ethylene **g**lycol **t**etraacetic **a**cid (EGTA)	Sigma, Taufkirchen, Germany
BCECF-AM	Invitrogen, Karlsruhe, Germany
Glucose	Carl Roth, Karlsruhe, Germany
HEPES	Sigma, Taufkirchen, Germany
Immersol 518F	Carl Zeiss, Göttingen, Germany
KCl	Carl Roth, Karlsruhe, Germany
LPS	Sigma, Taufkirchen, Germany
$MgSO_4$ x 7 H_2O	Sigma, Taufkirchen, Germany
Na_2HPO_4 x 2 H_2O	Sigma, Taufkirchen, Germany
N-**M**ethyl-**D**-**g**lucamin (NMDG)	Sigma, Taufkirchen, Germany
NaCl	Sigma, Taufkirchen, Germany
Poly-L-Lysine	Sigma, Taufkirchen, Germany
Phosphate **b**uffered **s**aline (PBS)	GIBCO, Carlsbad, Germany
Silicone	Grease Carl Roth, Karlsruhe, Germany
Trypan blue solution 0,4%	Sigma, Taufkirchen, Germany

pH measurement buffer composition

Table I Standard HEPES solution

Substance	[mM/L]
NaCl	115
KCl	5
$MgSO_4$*7H_2O	1.2

HEPES	32.2
$Na_2HPO_4*2H_2O$	2
$CaCl_2*2H_2O$	1
Glucose	10

pH 7.4 (NaOH); H_2O (at the 37 ^{0}C)

Table II Na^+ free solution

Substance	[mM/L]
NMDG	132.8
KCl	3
$CaCl_2*2H_2O$	1
$MgSO_4*7H_2O$	1.2
KH_2PO_4	2
HEPES	32.2
Mannitol	10
Glucose	10
HCL approx.	109

pH 7.4 (HCL); H_2O
(at the 37 ^{0}C)

Table III Standard NH_4Cl

Substance	[mM/L]
NMDG	122.8
KCl	3
$CaCl_2*2H_2O$	1
$MgCl_2*6H_2O$	1.2
KH_2PO_4	2
HEPES	32.2

Glucose	10
HCL approx.	109
NH_4Cl approx.	20

pH 7.4 (HCL or NMDG); H_2O (at the 37 ^{0}C)

Table IV High K+ for Calibration

Substance	**[mM/L]**
NMDG	32.8
KCl	105
$CaCl_2*2H_2O$	1
$MgSO_4*7H_2O$	1.2
HEPES	32.2
Mannitol	5

pH 7.0 (HCl); H_2O (at the 37 ^{0}C)

Cytosolic pH (pH_i) was measured in Th cells as described previously (Salker, Zhou et al. 2015) using pH sensitive BCECF, AM dye (Life Technologies, USA). Naïve T cells were differentiated into Th cells and after 3 days of differentiation of various subsets of Th cells were subjected to measurement of pHi with and without treatment. To measure the pHi and NHE1 activity of Th cells subsets, 300 µl of cells were collected and were fixed on a coverslip coated by Poly-L-lysine (Sigma, Germany), which was then placed in a chamber. Th cells were co-incubated with 10 µM BCECF, AM (Life Technologies, USA) for 15 min at 37^{0}C. Once the incubation was finished, chamber was then placed on the stage of an inverted microscope (Zeiss Axiovert 135) with epifluorescence mode 40x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 nm 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, AM fluorescence. The results from each cell were averaged and used for final data analysis.

High-K^+/nigericin calibration technique was applied for converting intensity ratio (490/440) data into pH_i values. The cells were perfused at the end of each experiment for 5 minutes with standard high-K^+/nigericin (10 µg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5) (Rotte, Pasham et al. 2010).

For acid loading, Th cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of pH_i due to entry of NH_3 and binding of H^+ to form NH_4^+. The acidification of pHi upon removal of ammonia allowed calculating the mean intrinsic buffering power (ß) of the cell. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$ß = \Delta [NH_4^+]_i / \Delta pH_i$,

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta [NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is equal to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 $[20/(1+10^{pHo-pK})]$. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from:

$[NH_4]_i = 19.37 \cdot 10^{pHo-pHi}$.

The calculation of the buffer capacity required that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence

of Na^+, suggesting that there was no relevant further exit of NH_4^+.

To calculate the Δ pH/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 pHi and NHE1 measurements were composed of (in mM) as described earlier (Zhou, Pasham et al. 2015): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG Cl, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 5 µM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 using HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37^0C.

5.5 Surface and intracellular antibodies staining

Th cells were characterised by using surface and intracellular staining with relevant antibodies. In brief, Th cells were stimulated with anti-CD3/anti-CD28 or 1 mg/ml PMA (Sigma, Germany) and 1 mg/ml Ionomycin (Sigma, Germany) for 4 hours and after 2 hours of PMA+Ionomycin treatment 1 µg/ml Brefeldin A (eBiosciences, Frankfurt, Germany) was added to the cultured cells for 2 hours. After 4 hours, cells were collected and used for surface staining for various antibodies dependent on the experiment (anti-Foxp3-APC and anti-IFN-γ -FITC; eBiosciences, Frankfurt, Germany, anti-IL-4-PE anti-IL-17A-PE; BD Bioscience, UK, anti-IL-9-PE; Biolegends, USA), and washed with PBS. Cells were fixed with Foxp3 fixation/permeabilization buffer (eBiosciences, Frankfurt, Germany) for intracellular staining and incubated for 30 minutes. After incubation, cells were washed with 1x permeabilization buffer (eBiosciences,

Frankfurt, Germany) and intracellular monoclonal antibodies were added and incubated for additional 30 minutes. Cells were washed again with permeabilization buffer and PBS was added to acquire the cells on flow cytometry (FACSCalibre™, BD Bioscience, Heidelberg, Germany).

5.6 q-RT-PCR

Total mRNA was isolated from different Th cells (Th0, Th1, Th2, Th9, and Th17) and iTregs using mRNAeasy isolation kit (QIAGEN, Germany) as described by the manufacturer. 1 µg mRNA was converted into cDNA using Superscript III cDNA synthesis kit (Life Technologies, Germany). Briefly, in 10 µl reactions, 10 ng cDNA, 2X SYBR green mastermix (Peqlab, Germany) and 250 nM primers (Sigma, Germany) were used for q-RT-PCR reactions. q-RT-PCR run and data analysis was performed as described previously (Soroosh and Doherty 2009) for NHE-1 (F primer: 5'-TCGCCCAGATGACCACAATTT-3' and R primer: 5'-GGGGATCACATGGAAACCTATCT-3') and GAPDH (F primer: 5'-CGTCCCGTAGACAAAATGGT-3' and R primer: 5'- TTG ATG GCA ACA ATC TCC AC-3') using universal cycling conditions (95^0C for 3 minutes, 95^0C for 10 seconds and 60^0C for 1 minute for 40 cycles followed by melting curve analysis).

5.7 Semi-quantitative-RT-PCR for NHE1 isoforms

Total mRNA was isolated from 3 days activated $CD4^+$ T cells using the mRNAeasy isolation kit (QIAGEN, Germany) as described by the manufacturer. 1.0 µg mRNA was converted into cDNA using the Superscript III cDNA synthesis kit (Invitrogen, Germany). Briefly, in 10 µl reactions, 10 ng cDNA, 2x Taq polymerase Master-mix (Peqlab, Germany) and 250 nM primers

were used for RT-PCR reactions (Singh, Ferreira et al. 2010). NHE isoforms primers used in reactions are described in a table below with their product size. PCR was run using standard PCR conditions (95^0C) for 3 minutes, 95^0C for 15 seconds, 60^0C for 30 seconds and 72^0C for 30 second for 40 cycles followed by final extension at 72^0C for 10 minutes. All primers were purchased from Sigma.

S.N.	Primers name	Primer sequences	Product size
1	NHE1 F	TCGCCCAGATGACCACAATTT	172bp
2	NHE1 R	GGGGATCACATGGAAACCTATCT	
3	NHE2 F	TGGCCAAGATTGGTTTTCATCTC	197bp
4	NHE2 R	TGGCATGAAGTAGCCAGCAT	
5	NHE3 F	TCAGTGGCTTGATGGGTGAA	71bp
6	NHE3 R	AGCTGCAATGAGGCTACCAA	
7	NHE4 F	AACCCACAGATGATGATGGCA	117bp
8	NHE4 R	TCTTCATAGGGATCACTGCCTG	
9	NHE5 F	ATGGTGTGGCTAATCCCGAA	73bp
10	NHE5 R	ACTGCAGCTGTGTCCTGAAA	
11	NHE6 F	TGTTTGCTGGTCTTCGTGGT	70bp
12	NHE6 R	TGTCGTGCATAAGTGGCAGT	
13	NHE7 F	TCTCGAAGTCGAAGCAAGCA	144bp
14	NHE7 R	GATGGCAACAAAAGCACCGA	
15	NHE8 F	TTGAAGTCGCATTGGTGGAG	95bp
16	NHE8 R	TGCAGATGGCTTCTTTGAAACAG	
17	NHE9 F	TGTTTTCAGGTTTGCGAGGTGT	70bp

18	NHE9 R	TGTTTGGGCTGAGATTCCG	
19	SLC4a1 F	GGCACCTACACACAGAAACTCT	128bp
20	SLC4a1 R	ACGGAGGCAAACATCATCCA	
21	SLC4a2 F	TAAAGCACAGCCACCCAAGT	188bp
22	SLC4a2 R	TGGTAGCTCACGCTCTCTAT	
23	SLC4a4 F	TTTAGCAACCCTGATAATGGCAG	160bp
24	SLC4aF R	CCCCAACAAGCACATTGGAA	

5.8 Immunoblotting

Naïve T cells were differentiated into Th0, Th1, Th2, Th9, Th17 and iTregs from WT mice. After 48 hours of incubation, Th cell and iTregs were washed once with PBS and equal amount of H_2O and 2X Lammelli's Buffer added for cell lysis. Proteins were denatured at 95^0C for 5 minutes and stored at -20^0C. Sample proteins were loaded on 10% SDS-PAGE gel and run for 80-120 V for 120 minutes. Proteins were electrotransferred onto membranes. Membranes were probed with the indicated primary antibodies for NHE1 (1:1000 NHE1 rabbit antibody from Genetex®, USA), followed by HRP-conjugated secondary antibodies (1:2000 Cell Signalling, Germany). Membranes were washed and visualized with enhanced chemiluminescence detection system (ECL; Life Technologies, Germany) and data were analysed using Image J software.

$CD4^+$ T cells from DJ-$1^{+/+}$ and DJ-$1^{-/-}$ mice were activated in the presence of anti-CD3:anti-CD28 (1:2) (eBioscience) for collecting the samples for immunoblotting. Activated T cells were washed once with PBS and equal amounts of H_2O and 2 x Lamelli buffer added for cell lysis. Proteins were denatured at 95^0 C for 5 minutes followed by storage at -20^0 C. Sample proteins were loaded on 10% SDS-PAGE gel run for 120 V per gel for 100-120

minutes. Proteins were electro-transferred onto PVDF membranes (GE healthcare, Freiburg, Germany). Membranes were probed with the indicated primary antibodies (DJ-1 (1:1000; #5933), p-AKT(S473) (1:1000; #9271); GAPDH (1:1000; #2118), (Cell Signaling Technology, Leiden, The Netherlands) and NHE1 (1:1000; #GTX85046); GeneTex, Irvine, USA) followed by HRP-conjugated secondary antibodies (1:2000; Cell Signalling). Membranes were washed and visualized with enhanced chemiluminescence detection system (ECL; peqlab, Erlangen, Germany). Blot image quantification was done by ImageJ software.

5.9 ROS production

To determine ROS production in 3 days activated $CD4^+$ T cells from both DJ-$1^{+/+}$ and DJ-$1^{-/-}$, approximately 2×10^5 cells were co-incubated with 10 µM 2',7'–dichlorofluorescin diacetate (DCFDA) (Sigma) for 30 min in the dark at room temperature. Cells were then washed with 100 µl PBS and then resuspended into 200 µl of fresh PBS. Furthermore, ROS in T cells were detected by Flow cytometry using the FACS Calibur (BD Biosciences, Heidelberg, Germany). DCFDA fluorescence intensity was measured in FL-1 with as excitation wavelength of λ 488 nm and an emission λ wavelength of 530 nm. Geometric mean of the FL-1 signal intensity was used to show the amount of ROS production. Cell volume was reflected by the geometric mean of forward scatter. Data were analysed with flow FlowJo (Treestar,USA) software.

5.10 siRNA transfection of T cells

Naïve T cells were transfected with siRNA-control and siRNA-NHE1 using DharmaFECT3 transfection reagent (Dharmacon, USA) as recommended by

manufacture's guidelines. Briefly, Naïve T cells were washed 3x with PBS to remove any residual serum and antibiotics from the cells and 0.75 - 1x10^6 cells per well cultured in the presence of antibiotic free media in 24 well plate coated with anti-CD3::anti-CD28. Final concentration of 200nM of 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.

5.11 Statistical analysis

Prism software (GraphPad software) was used for statistical analyses. Student's t-test was used for significance. Flow cytometry data were analysed by Flowjo (Treestar, USA). Figures were made in Excel and GraphPad prism software. ImageJ was used for WB data analysis. P values of equal or less than 0.05 were considered significant.

6 RESULTS AND DISCUSSION

Part1: Role of DJ-1 protein in NHE1 activity:

In this chapter, I have described how DJ-1 protein is involved in the regulation of NHE1 activity and the mechanisms how DJ-1 is possibly controlling the NHE1 activity and ROS production

RESULTS:

6.1 Enhanced Na^+/H^+ exchanger activity in DJ-1 deficient $CD4^+$ T cells

To find out the impact of DJ-1/Park-7 on the activated $CD4^+$ T cells, a ratio matric imaging system was utilized. As the nature of BCECF-AM followed by duplicate emission florescence 490/440. The impact of DJ-1 deficiency on Na^+/H^+ exchanger activity and intracellular pH (pHi) was detected at day 2 of activated $CD4^+$ T cells. The initial pHi was not significantly different between DJ-$1^{-/-}$ $CD4^+$ T cells and DJ-$1^{+/+}$ $CD4^+$ T cells. Na^+/H^+ activity of $CD4^+$ T cells was subsequently measured using the ammonium pulse technique as described in method section. In following Fig.11 A and B, the addition of NH_4Cl to the extracellular bath was followed by a sharp cytosolic alkalinization due to NH_3 entry into the cells followed by binding of cytosolic H^+ to form NH_4^+. The subsequent removal of NH_4Cl was followed by sharp cytosolic acidification due to NH_3 exit and cellular H^+ retention in a totally non-sodium environment. As long as Na^+ was absent, neither in DJ-$1^{+/+}$ $CD4^+$ T cells nor in DJ-$1^{-/-}$ $CD4^+$ T cells, acidification was followed by significant realkalinization. Thus, the cells did not express appreciable Na^+-independent H^+ extrusion irrespective of absence or presence of DJ-1. Addition of Na^+ was followed by a recovering process since cell has to rescue its self into the initial level of pH_i, this part was considered to reveal Na^+/H^+ exchanger activity. After 2 days of $CD4^+$ T cell activation, the pH_i recovery following addition of Na^+ was significantly more rapid in DJ-$1^{-/-}$ $CD4^+$ T cells than in DJ-$1^{+/+}$ $CD4^+$ T cells, illustrating higher Na^+/H^+ exchanger activity in DJ-$1^{-/-}$ $CD4^+$ T cells. No significant difference of buffer capacity was observed between DJ-$1^{+/+}$ $CD4^+$ T cells and DJ-$1^{-/-}$ $CD4^+$ T cells. Thus, following activation, DJ-1 deficiency enhanced Na^+ dependent realkalinization reflecting enhanced Na^+/H^+ exchanger activity.

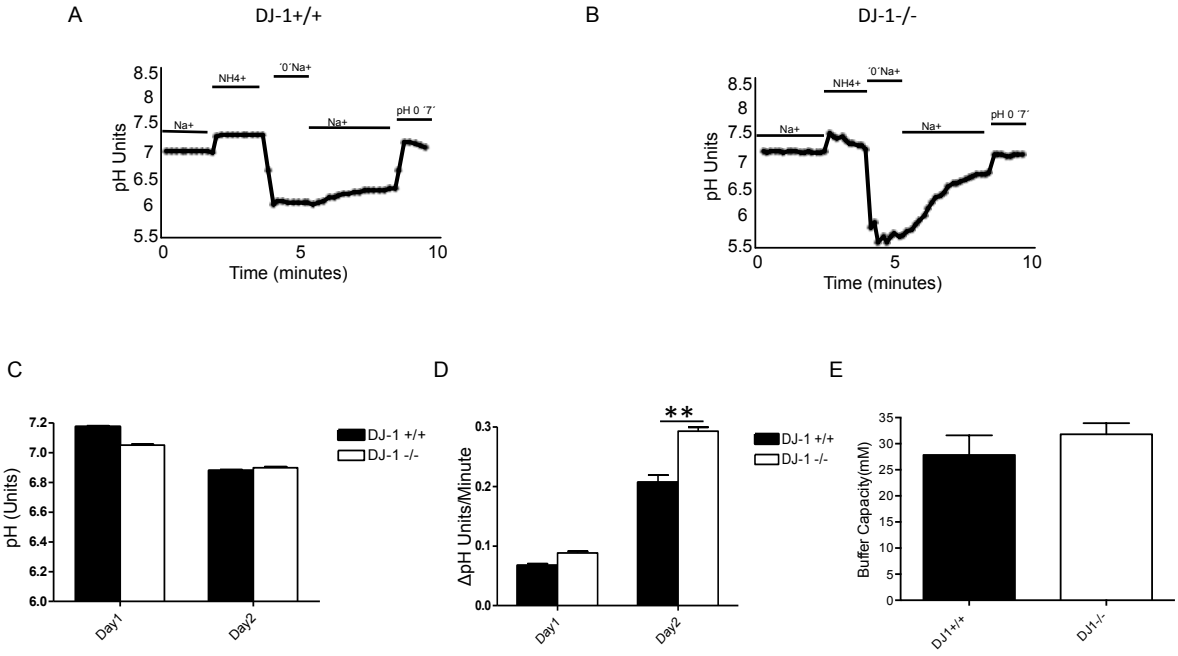


Figure 11 DJ-1 deficiency enhances Na^+/H^+ activity in $CD4^+$ T cells.

A,B. Original tracings reflecting alterations of cytosolic pH (pH_i) following an ammonium pulse in $CD4^+$ T cells isolated after 2 days of activation from DJ-$1^{+/+}$ (**A**) and DJ-$1^{-/-}$ (**B**) mice. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG) in a first step, NH_4Cl removed in a second step, Na^+ added in a third step and nigericin (pH_o 7.0) applied in a fourth step to calibrate each individual experiment.

C-E. Arithmetic means ± SEM (n = 3 - 5 independent experiments) of cytosolic pH (pH_i) prior to the ammonium pulse (**C**) Na^+-dependent recovery of cytosolic pH (ΔpH/min) following an ammonium pulse (**D**) and buffer capacity (**E**) in 1 or 2 days activated *DJ-$1^{+/+}$* $CD4^+$ T cells (black bars) or DJ-$1^{-/-}$ $CD4^+$ T cells (white bars). ** indicates statistically significant difference ($p<0.005$) between DJ-$1^{+/+}$ and DJ-$1^{-/-}$ $CD4^+$ T cells.

6.2 DJ-1 deficiency enhances the mRNA and protein expression of NHE1 in $CD4^+$ T cells

Functional NHE1 was upregulated in DJ-1 deficient T cells but how DJ-1 deficiency is affecting NHE1 at transcript or protein level is not known. Thus, NHE1 transcription and/or translation, quantitative RT-PCR and immunoblotting, respectively, were performed. As illustrated in the following figure, the abundance of mRNA (Fig.12 A) and protein (Fig.12 B, C) was significantly higher in DJ-$1^{-/-}$ $CD4^+$ T cells than DJ-$1^{+/+}$ $CD4^+$ T cells. Western blotting of NHE1 yielded two bands. The top band was used for quantification (Fig.12 B, C). We also verified the presence of DJ-1 protein expression in $CD4^+$ T cells in both mice strains by immunoblotting (Fig.12 B). As expected, DJ-$1^{-/-}$ $CD4^+$ T cells did not express DJ-1 protein whereas DJ-1 was abundantly present in DJ-$1^{+/+}$ $CD4^+$ T cells. To find out whether NHE1 was expressed in the cell membrane and in mitochondria, we stained the cells with NHE1 antibody and visualized NHE1 in both DJ-$1^{-/-}$ and DJ-$1^{+/+}$ $CD4^+$ T cells using the confocal microscopy. As a result expression of NHE1 was higher in DJ-$1^{-/-}$ $CD4^+$ T cells than in DJ-$1^{+/+}$ $CD4^+$ T cells, whereby NHE1 was mainly localized in the cell membrane (Fig.12 D). Additional experiments identified different NHE isoforms (Fliegel 2005) in $CD4^+$ T cells. All NHE isoforms were present except NHE3 in $CD4^+$ T cells (Fig.12 D). Thus, in summary $CD4^+$ T cells express various NHE isoforms and NHE1 expression is significantly higher in DJ-$1^{-/-}$ deficient $CD4^+$ T cells than in DJ-$1^{+/+}$ $CD4^+$ T cells.

§ WB data were kindly provided by Xiaolong Shi, Tubingen University.

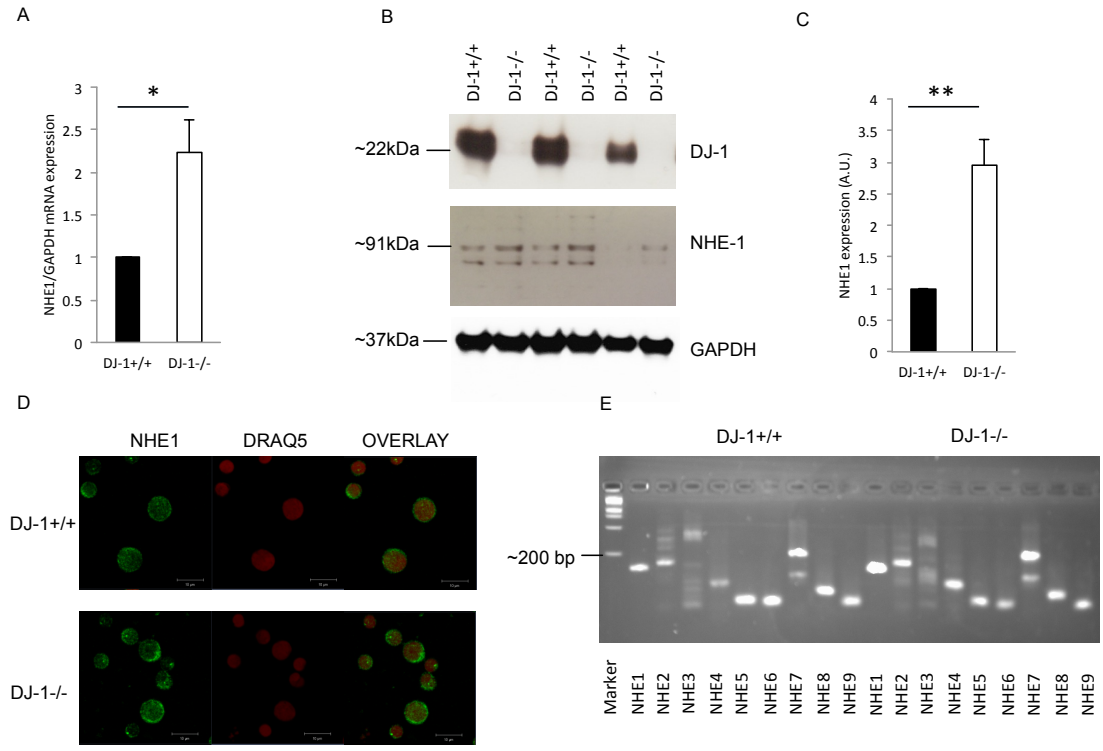


Figure 12 DJ-1 deficiency enhances NHE1 mRNA transcript and protein levels in CD4$^+$ T cells

A. Arithmetic means ± SEM (n = 3 - 5) of NHE1 mRNA levels in DJ-1$^{+/+}$ (black bar) and DJ-1$^{-/-}$ (white bar) CD4$^+$ T cells activated in the presence of anti-CD3 and anti-CD28 for 3 days.

B. Original Western blots showing DJ-1, NHE1 and GAPDH protein abundance in DJ-1$^{+/+}$ and DJ-1$^{-/-}$ CD4$^+$ T cells.

C. Arithmetic means ± SEM (n = 3 - 5) of NHE1 mRNA levels in DJ-1$^{+/+}$ (black bar) and DJ-1$^{-/-}$ (white bar) CD4$^+$ T cells activated in the presence of anti-CD3 and anti-CD28 for 3 days.

D. Characterisation of NHE1 in DJ-1$^{+/+}$ and DJ-1$^{-/-}$ CD4$^+$ T cells by confocal microscopy. NHE1 expression (green) and nuclear staining (DRAQ5) is shown in upper and lower panel for DJ-1$^{+/+}$ and DJ-1$^{-/-}$ CD4$^+$ T cells. NHE1 appears to be express mostly on cell the membrane.

E. Expression of different NHE isoform (1-9) from DJ-1$^{+/+}$ and DJ-1$^{-/-}$ CD4$^+$ T cells activated in the presence of anti-CD3 and anti-CD28 for 3 days by RT-PCR. CD4$^+$ T cells express most of the NHE isoforms expect NHE3.

*($p<0.01$), **($p<0.008$) indicate statistically significant difference between DJ-1$^{+/+}$ and DJ-1$^{-/-}$ CD4$^+$ T cells.

§ WB data were kindly provided by Xiaolong Shi, Tubingen University.

6.3 ROS in $CD4^+$ T cells is upregulated in the absence of DJ-1

Recently study reveals DJ-1 deficiency leads to oxidative stress (Shi, Lu et al. 2015). Oxidative stress may in turn stimulate expression of NHE1 (De Vito 2006). To uncover the link between ROS and DJ-1 in $CD4^+$ T cells, we measured ROS abundance using Flow cytometry utilizing 2',7' – dichlorofluorescin diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell later deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). DCF is a highly fluorescent compound, which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively. As illustrated in Fig.13 A, B, By detecting FL-1 fluorescence, ROS level was indeed significantly enhanced in DJ-$1^{-/-}$ $CD4^+$ T cells as compared to DJ-$1^{+/+}$ $CD4^+$ T cells. According to the forward scatter, cell volume was similar in both DJ-$1^{-/-}$ $CD4^+$ T cells and DJ-$1^{+/+}$ $CD4^+$ T cells (Fig.13 C,D).

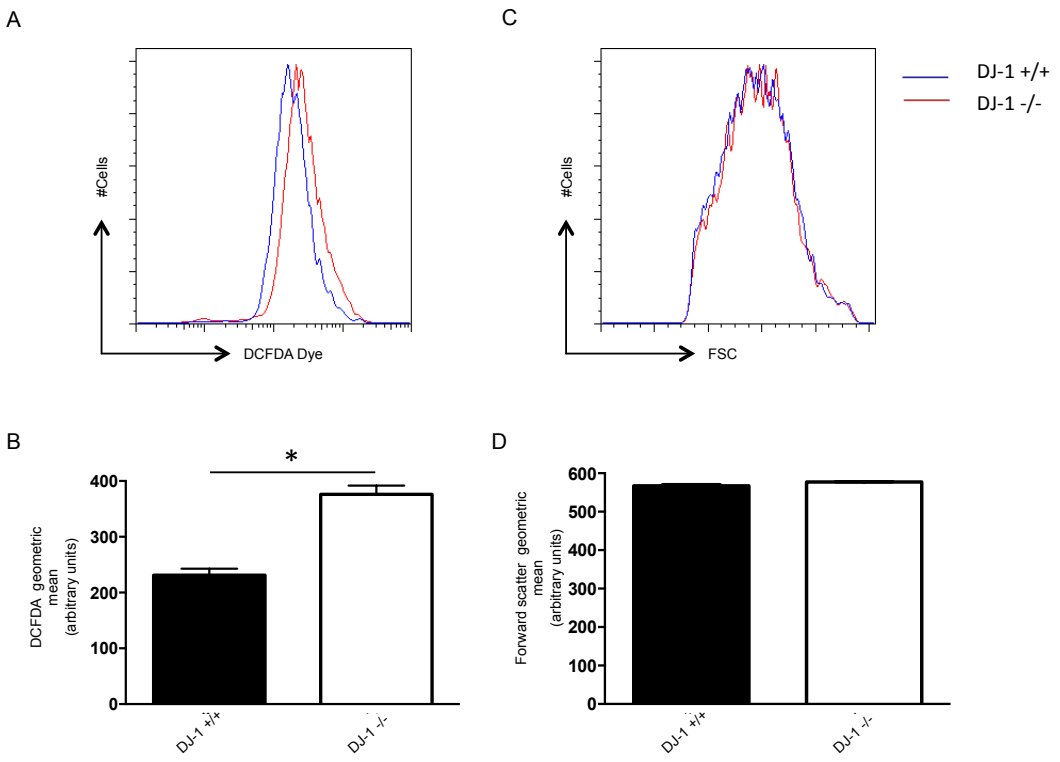


Figure 13 Enhanced ROS production but normal forward scatter in DJ-1$^{-/-}$ CD4^{+} T cells

A. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in CD4^{+} T from DJ-1$^{-/-}$ knockout mice (*DJ-1$^{-/-}$*, red) and their wild type littermates (*DJ-1$^{+/+}$*, blue) following 3 days of activation in the presence of anti-CD3 and anti-CD28 (upper panel).
B. Arithmetic means ± SEM (n = 3-5 independent experiments) of the ROS Geometric Mean Fluorescence (MFI) intensity in *DJ-1$^{-/-}$* (white bars) and *DJ-1$^{+/+}$* (black bars) CD4^{+} T cells following 3 days of activation.
C. Representative FACS histograms depicting forward scatter of CD4^{+} T from DJ-1$^{-/-}$ knockout mice (*DJ-1$^{-/-}$*, red) and their wild type littermates (*DJ-1$^{+/+}$*, blue) following 3 days of activation in the presence of anti-CD3 and anti-CD28 (upper panel).
D. Arithmetic means ± SEM (n = 3-5 independent experiments) of forward scatter of *DJ-1$^{-/-}$* (white bars) and *DJ-1$^{+/+}$* (black bars) CD4^{+} T cells following 3 days of activation.*($p<0.05$) indicates statistically significant difference

6.4 Na^+/H^+ exchanger activity is decreased by NHE1 inhibitor cariporide, antioxidant NAC and catalase

In order to further elucidate the mechanisms achieving and regulating Na^+ dependent realkalinization, experiments were performed in DJ-$1^{-/-}$ $CD4^+$ T cells in the absence and presence of NHE1 inhibitor cariporide (10 μM), of antioxidant N-acetyl-cysteine (NAC; 10 μM) and of ROS scavenging enzyme catalase (10 μM). The respective original tracings are illustrated in Fig.14 A-C. As shown in Fig.14 D, cariporide treatment (Fig.14 D), but not NAC treatment (Fig.14 E) or catalase treatment (Fig.14 F) significantly reduced the pHi of DJ-$1^{-/-}$ $CD4^+$ T cells. Na^+-dependent cytosolic alkalinisation was significantly blunted by each, cariporide (Fig. G), NAC (Fig.14 H) and catalase (Fig.14 I).

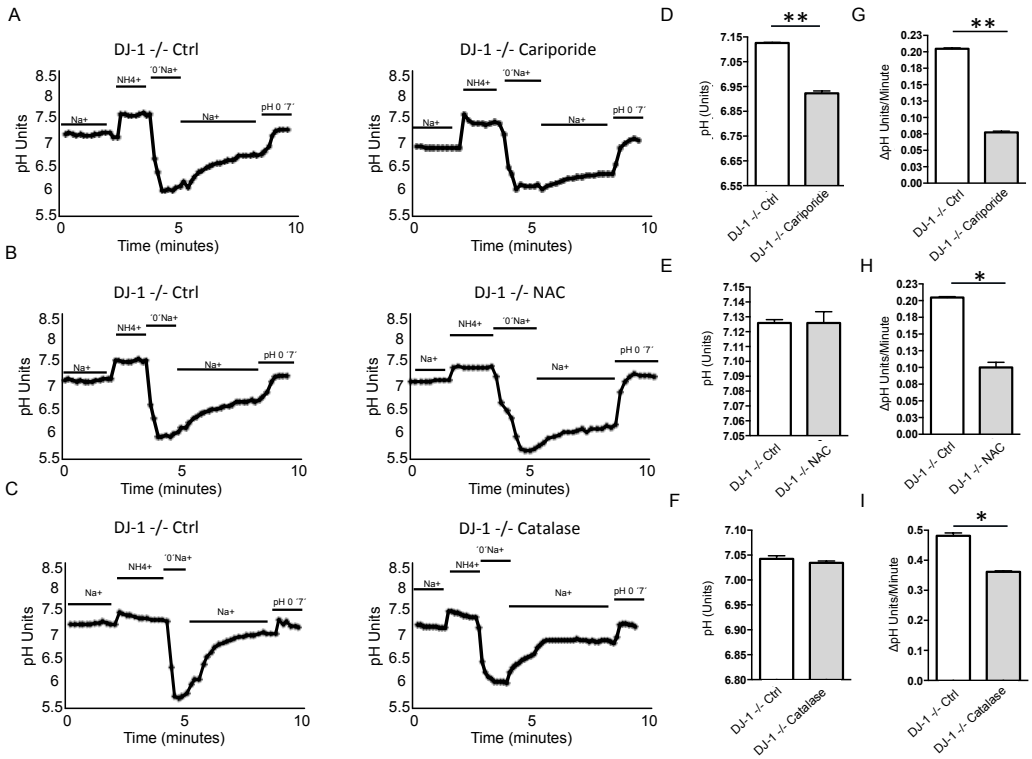


Figure 14 Attenuation of NHE1 activity by cariporide, NAC and catalase in DJ-1$^{-/-}$ CD4^{+} T cells

A-C. Original tracings of typical experiments as described in Fig.1 for determination of pH_i and Na^+/H^+ exchanger activity in CD4$^+$ T cells from DJ-1$^{-/-}$ mice without (left panels) and with (right panels) treatment with Cariporide (10 μM), (**A**, 15 minutes), NAC (10 μM), (**B**, 4 hours) and catalase (10 μM), (**C**, 4 hours) after 3 days of activation.

D-F. Arithmetic means ± SEM (n = 3 - 5 independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in *DJ-1$^{-/-}$* CD4$^+$ T cells without (white bars) and with (grey bars) treatment with cariporide (**D**), NAC (**E**) and catalase (**F**).

G-I. Arithmetic means ± SEM (n = 3 - 5 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in *DJ-1$^{-/-}$* CD4$^+$ T cells without (white bars) and with (grey bars) treatment with cariporide (10 μM) (**G**), NAC (**H**) and catalase (**I**). *($p<0.05$), **($p<0.01$) indicate statistically significant difference.

6.5 Cariporide, NAC and catalase reduce the ROS production

As illustrated in Fig.15 A-F, treatment with cariporide (10 μM), NAC (10 μM), and catalase (10 μM), each significantly decreased ROS production in DJ-$1^{-/-}$ $CD4^+$ T cells. As shown in Fig.15 G-M, cariporide and NAC treatment, but not catalase treatment, significantly decreased cell volume.

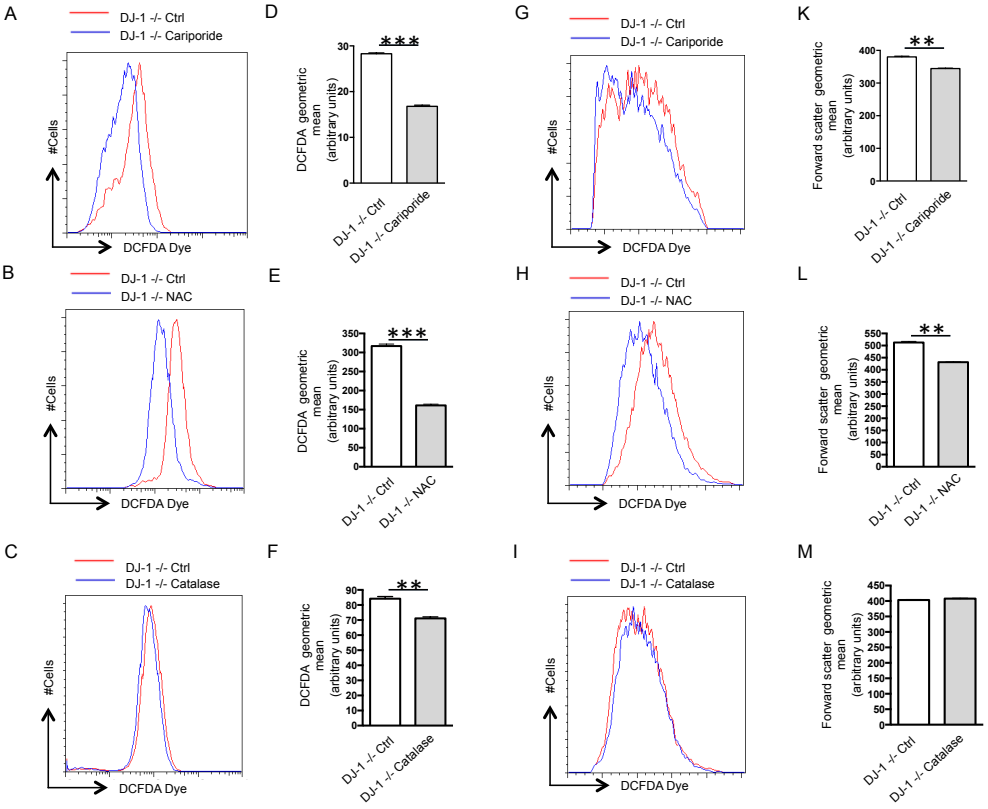


Figure 15 Impact of cariporide, NAC and catalase on ROS abundance and forward scatter in DJ-1$^{-/-}$ CD4^{+} T cells

A-C. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in CD4^{+} T from DJ-1$^{-/-}$ knockout mice (*DJ-1$^{-/-}$*) without (red lines) and with (blue lines) treatment with Cariporide (10 μM), (**A**, 15 minutes), NAC (10 μM), (**B**, 4 hours) and catalase (10 μM), (**C**, 4 hours) after 3 days of activation.

D-F. Arithmetic means ± SEM (n = 3 - 5 independent experiments) of ROS-dependent DCFDA fluorescence in *DJ-1$^{-/-}$* CD4^{+} T cells without (white bars) and with (grey bars) treatment with cariporide (**D**), NAC (**E**) and catalase (**F**).

G-I. Representative FACS histograms depicting forward scatter of CD4^{+} T from DJ-1$^{-/-}$ knockout mice (*DJ-1$^{-/-}$*) without (blue lines) and with (red lines) treatment with cariporide (**G**, 15 minutes), NAC (**H**, 4 hours) and catalase (**I**, 4 hours) after 3 days of activation.

K-M. Arithmetic means ± SEM (n = 3 - 5 independent experiments) of forward scatter of *DJ-1$^{-/-}$* CD4^{+} T cells without (white bars) and with (grey bars) treatment with cariporide (**K**), NAC (**L**) and catalase (**M**). **(p<0.01),

***(p<0.001) indicate statistically significant difference.

6.6 Protein tyrosine kinase (PTK) inhibitor significantly reduces the NHE1 and ROS production

DJ-1 redox-sensitive chaperone protein may play a role in the redox regulation of T cell receptor (TCR) signaling. Therefore, we studied whether protein tyrosine kinase (PTK) activation is involved in ROS production and NHE activation. As a result PTK inhibitor staurosporine (100 nM; for 4 hours treatment after 3 days of activation) significantly decreased the NHE1 activity in DJ-$1^{-/-}$ $CD4^+$ T cells (Fig.16 A). Further, ROS production was also significantly reduced after application of PTK inhibitor staurosporine to DJ-$1^{-/-}$ $CD4^+$ T cells (Fig.16 B). In theory, the link of ROS production and NHE1 activity may have resulted from Ca^{2+} influx in activated T cells. We thus performed the experiments in the presence and absence of a Ca^{2+} buffer. As a result, the buffer did not appreciably alter pHi and NHE1 activity (Fig.16 C).

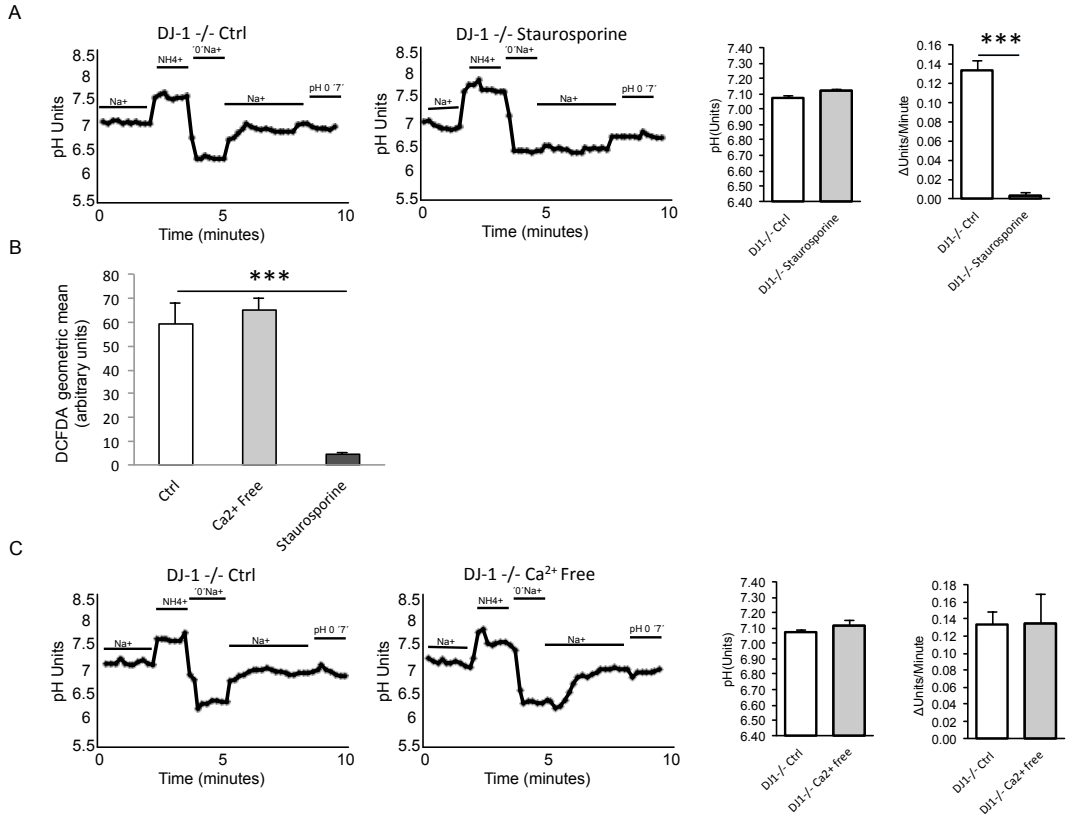


Figure 16 Effect of protein tyrosine kinase (PTK) inhibitor staurosporine and extracellular free Ca^{2+} for pHi and NHE1 regulation and ROS production in DJ-$1^{-/-}$ $CD4^+$ T cells

A. Original tracings of typical experiments as described in Fig.1 for determination of pH_i and Na^+/H^+ exchanger activity in $CD4^+$ T cells from DJ-$1^{-/-}$ mice without (left panels) and with (right panels) treatment with Staurosporine (100 nM) (4 hours), after 3 days of activation. Arithmetic means ± SEM (n = 3 independent experiments) of pHi and ∆pHi/minutes (NHE activity) of *DJ-$1^{-/-}$* $CD4^+$ T cells without (white bars) and with (grey bars) treatment with Staurosporine. ***(p<0.001) indicate statistically significant difference.

B. Arithmetic means ± SEM (n = 3 independent experiments) of ROS-dependent DCFDA fluorescence in *DJ-$1^{-/-}$* $CD4^+$ T cells without (white bars), Ca^{2+} free buffer (grey bars) and treatment with Staurosporine (dark grey bars). ***(p<0.001) indicate statistically significant difference.

C. Original tracings of typical experiments as described in Fig.1 for determination of pH_i and Na^+/H^+ exchanger activity in $CD4^+$ T cells from DJ-$1^{-/-}$ mice with (left panels) and without (right panels) Ca^{2+}, after 3 days of activation. Arithmetic means ± SEM (n = 3 independent experiments) of pHi and ∆

pHi/minutes (NHE activity) of *DJ-1*$^{-/-}$ $CD4^+$ T cells with (white bars) and without (grey bars) Ca^{2+}.

6.7 HCO_3^- has no effect on NHE1 production

Besides NHE, other exchanger or transporters such as Cl^-/HCO_3^- or Na^+-HCO_3^- may be involved in pHi regulation in $CD4^+$ T cells. Abnormalities in pHi regulation by these mechanisms could lead to immune dysfunctions. As there is an evidence that the Ae2a gene or Slc4a4 mRNA (an acid loader with electroneutral and Na^+ independent Cl^-/HCO_3^- anion exchanger) in $CD8^+$ T cells is involved in pHi regulation by exchanging bicarbonate for chloride across the plasma membrane in an electroneutral fashion (Concepcion, Salas et al. 2014). We measured the mRNA expression level of SLC4a1, SLC4a2 and SLC4a4 by q-RT-PCR for DJ-$1^{+/+}$ and DJ-$1^{-/-}$ $CD4^+$ T cells and found no significant difference (Fig.17 A). Further, we performed twin NH4Cl pre-pulse protocol for DJ-$1^{-/-}$ and DJ-$1^{+/+}$ $CD4^+$ T cells showing the time scale of the experiments in both HEPES and HCO_3^-, however, we did not observe any significant difference for pHi in HEPES and HCO_3^- in 2 pulse chase experiments (Fig.17 B).

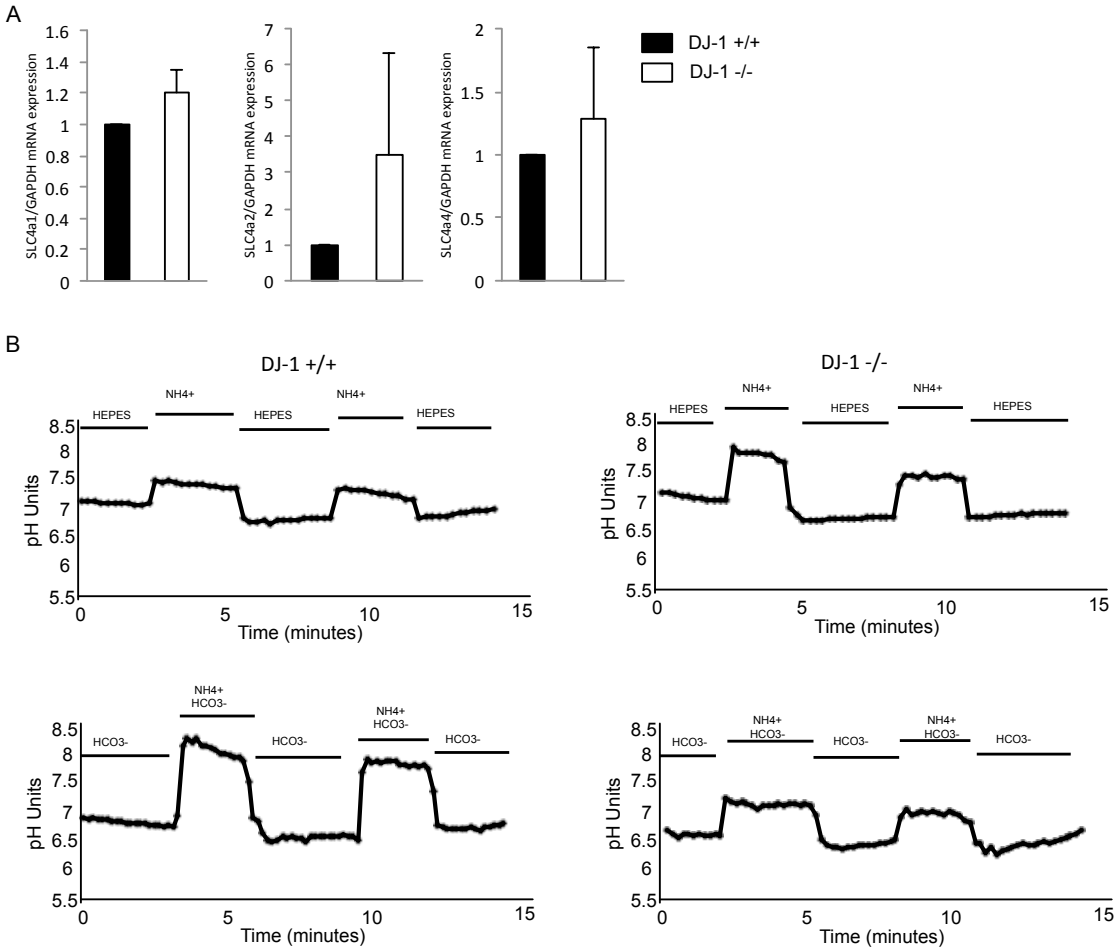


Figure 17 HCO_3^- has no impact on pHi and NHE1 regulation in DJ-$1^{-/-}$ $CD4^+$ T cells

A. mRNA expression of SLC4a1, SLC4a2 and SLC4a4 by q-RT-PCR for DJ-$1^{+/+}$ and DJ-$1^{-/-}$ $CD4^+$ T cells (n = 3 independent experiments).

B. Original tracings of typical experiments for determination of pH_i in $CD4^+$ T cells from DJ-$1^{+/+}$ and DJ-$1^{-/-}$ mice with HEPES (Na^+ free) and HCO_3^- solutions (n = 3 independent experiments).

DISCUSSION 1

This part of the thesis described a novel functional role of the DJ-1 protein, i.e. the regulation of NHE1 activity and ROS production in $CD4^+$ T cells. These findings are the first to uncover enhanced Na^+/H^+ exchanger 1 activity in DJ-1 deficient $CD4^+$ T cells, which is evident with no treatment with cytokines (IL-2, IFN-γ), growth factors (EGF, angiotensin II) and hormones (glucocorticoids) (Chang, Wang et al. 2010, Voelkl, Pasham et al. 2013, Chatterjee, Schmidt et al. 2014, Qadri, Su et al. 2014). The impact of DJ-1 on NHE1 expression is somehow evidence to the effect of DJ-1 on NHE1 activity. It is proved follow higher abundance of NHE1 mRNA levels and NHE1 protein abundance. NHE1 as a transmenberane protein is exist in the plasma membrane of most mammalian cells and described as the housekeeping isoform of NHE family (Fliegel 2005, De Vito 2006). Other NHE isoforms such as NHE6 and NHE7 are exclusively found in intracellular organelles such as mitochondria and trans-Golgi (De Vito 2006). In $CD4^+$ T cells, the NHE1 protein is mostly localized in the plasma membrane, as apparent from confocal microscopy. Localization to other structures cannot be discounted, nonetheless.

T-cell receptor initiation by lectin, antibodies and cytokines leads to the activation of the amiloride-sensitive NHE activity (Lacroix, Poet et al. 2004, Fliegel 2005, De Vito 2006, Chang, Wang et al. 2010). To our best knowledge, the expression of the NHE1 in T cells was transcendent compared to other NHE isoforms, at least compare with NHE2 and NHE3. In our study we also characterized all NHE isoforms (1-9) by RT-PCR and we also discovered comparative results as beforehand depicted (Wakabayashi, Shigekawa et al. 1997, Lacroix, Poet et al. 2004, Fliegel 2005, De Vito 2006, Chien, Liao et al. 2007, Chang, Wang et al. 2010, Odunewu and Fliegel 2013). NHE5, NHE6, NHE8 and NHE9 were also expressed in wild type $CD4^+$ T cells. However,

DJ-1$^{-/-}$ CD4^{+} T cells predominantly expressed NHE1, which is transparently evidenced by the PCR gel imaging. Previous findings suggested that the DJ-1 protein is associated in the control of ROS production (Billia, Hauck et al. 2013, Shi, Lu et al. 2015). Thus, the higher NHE1 mRNA and protein as well as Na^{+}/H^{+} exchanger activity could have been due to enhanced ROS production in DJ-1$^{-/-}$ CD4^{+} T cells. The impact of ROS production on Na^{+}/H^{+} exchanger activity is apparent from the sharp decrease of Na^{+}/H^{+} exchanger activity following treatment of the cells with antioxidant NAC, and in the presence of the ROS scavenging enzyme catalase. DJ-1 redox-sensitive chaperone protein may play a role in the redox regulation of TCR signaling. Our data suggested that PTK is involved in regulation of NHE1 production as PTK inhibitor staurosporine blocks the NHE1 activity as well as ROS production in DJ-1$^{-/-}$ CD4^{+} T cells. This observation suggests that DJ-1 plays a role in the regulation of TCR signaling. Removal of extracellular Ca^{2+} had, however, no effect on pHi and NHE1 activity in DJ-1$^{-/-}$ CD4^{+} T cells.

Beyond its role in the regulation of cytosolic pHi, Na^{+}/H^{+} exchanger activity also participates in the regulation of cell volume, which involves parallel activity of Na^{+}/H^{+} and Cl^{-}/HCO_3^{-} exchanger (Boyarsky, Ganz et al. 1988, Watts, George et al. 2013, Lee, Mele et al. 2014). The carriers mediate the entry of NaCl in exchange for H^{+} and HCO_3^{-}, which are replenished from CO_2 and are thus not osmotically relevant (Lang, Busch et al. 1998, Hoffmann, Lambert et al. 2009). Increase of cell volume in a timely fashion is a prerequisite for cell proliferation and stimulation of cell proliferation is paralleled by a shift of the Na^{+}/H^{+} exchanger cell volume regulatory set point to larger volumes (Lang, Busch et al. 1998, Allantaz, Cheng et al. 2012, Feske, Skolnik et al. 2012, Chatterjee, Schmidt et al. 2014). However, we did not observe significant differences of cell volume between DJ-1$^{+/+}$ and DJ-1$^{-/-}$ CD4^{+} T cells. Since the formation of ROS is paralleled by activation of NHE1, it is necessary to study

whether the link of DJ-1 to responses on ROS production and NHE1 activity increase are dependent on intracellular calcium elevation or Ca^{2+} influx in activated T cells. Furthermore, there is a possibility that other acid or base transporting carriers or exchangers are expressed to control the pHi in DJ-$1^{-/-}$ $CD4^+$ T cells such as Cl^-/HCO_3^- or Na^+-HCO_3^-. However, we did not observe any significant difference in pHi after the twin NH4Cl pre-pulse experiments performed in the presence of either HEPES or HCO_3^-.

According to previous studies, progesterone induces cytosolic acidification in human T cells (Chien, Liao et al. 2007, Chang, Wang et al. 2010). These observations lead to the conclusion that inhibition of NHE1 activity by progesterone contributes to acidification in human T cells. Further, the expression of NHE1 mRNA was also increased in human lymphocytes during metabolic acidosis (Lacroix, Poet et al. 2004, Odunewu and Fliegel 2013). It is well known that cytosolic pH modifies the ROS production, which may regulate the NHE1 activity or conversely ROS is activating the NHE1 activity (Meima, Webb et al. 2009, De Giusti, Caldiz et al. 2013, Salker, Zhou et al. 2015, Zhou, Pasham et al. 2015). A recently published study also suggested that regulatory T cells also have enhanced production of ROS in DJ-$1^{-/-}$ mice (Singh, Chen et al. 2015). The enhanced ROS production in DJ-1 deficient $CD4^+$ T cells strengthens the idea that a relationship occurs between DJ-1/ROS/NHE1 activities. According to observations of others (Lim and Zhang 2013, Parsanejad, Bourquard et al. 2014, Shi, Lu et al. 2015) and us (Singh, Chen et al. 2015), we suggest that the DJ1-ROS-NHE1 relationship appears to be general mechanism in other cell types, such as neurons, muscles etc. Possibly DJ1 dysfunction can be rescued by down-regulating NHE1 using pharmacological inhibitors. It is tempting to speculate that NHE1 inhibitors could be used for the treatment of Parkinson's disease.

These data suggest that DJ-1 deficient $CD4^+$ T cells are prone to

upregulate NHE1 due to ROS production during the activation of T cells with anti-CD3 and anti-CD28 antibodies. The significance of Na^+/H^+ exchanger activity for ROS production is illustrated by the observation that ROS production is inhibited by NHE1 blocker cariporide. In conclusion, this study discloses a novel impact of DJ-1 on ROS production, NHE1 expression, and NHE1 activity in activated $CD4^+$ T cells.

In the future, it is worthy to investigate a role of various of ion channels like NHE, AE, NBC, NCX, and MCT in the universal physiological network by alteration of DJ-1 in nervous immune cells like Neuroglia utilizing ratio-matric imaging system which may contribute to understand more about the role of physiological factors in DJ-1 related Parkinson diagnose and treatment.

Part2: Potential role of NHE1 activity in T helper 9 (Th9) cells:

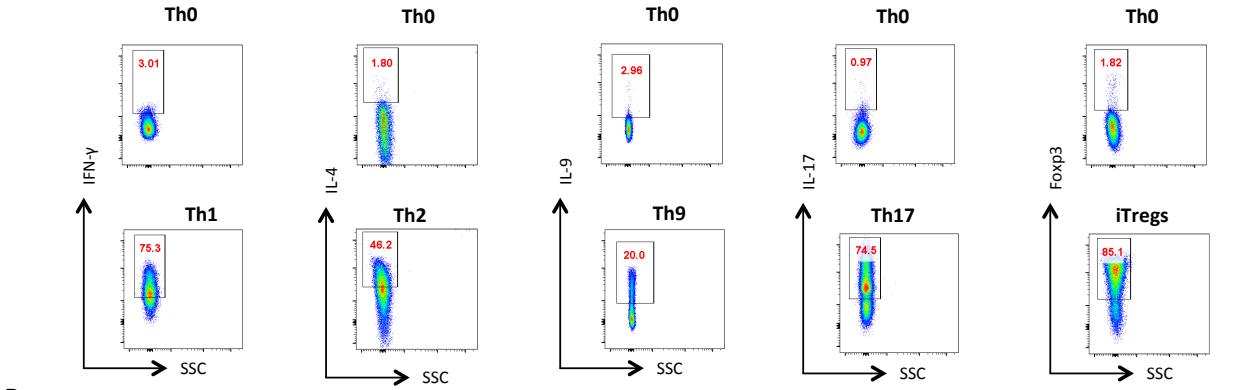
In this chapter, I have described how pH and NHE1 activity are upregulated in various Th cells subsets and how NHE-1 could be affecting the development and functions of Th9 cells in vitro.

RESULTS:

6.8 Characterisation of Th cells subsets at mRNA and protein level

In this part of thesis, I intend to characterize Th cells subsets and iTregs, naïve $CD4^+$ T cells were differentiated into Th1, Th2, Th9, Th17 and iTregs in presence of various cytokines recombinant proteins and antibodies described in methods. As shown in the following Fig.18 A, we measured the respective cytokines and transcription factors of particular Th cells subsets and iTregs, respectively, by flow cytometry to identify whether cells are differentiated appropriately into particular Th cells lineage. Further, we performed q-RT-PCR for Th cells subsets to explore whether Th cells subsets are truly unique (Fig.18 B). IFN-γ is produced by the majority of Th1 cells, but not by other Th cells subsets, while IL-4 is produced by Th2 cells but not by other Th cells. IL-9 is largely produced by Th9 cells. However, a little amount of IL-9 is also produced by iTregs and Th2 cells. Th17 cells produce mainly IL-17 (Fig.18 B). These results suggested that our naïve T cells were correctly differentiated into various Th cells subsets and iTregs.

A



B

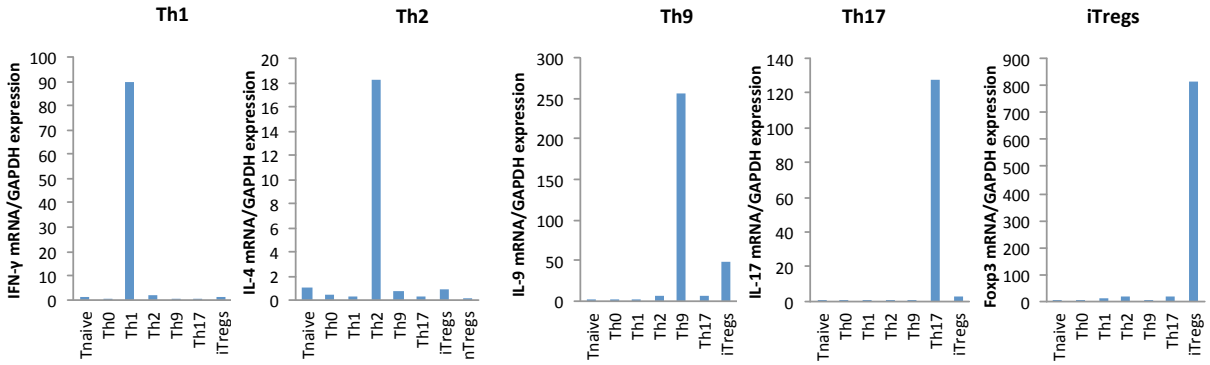


Figure 18 Differentiation of Th cells and iTregs

A. Naïve T cells were differentiated into Th cells and iTregs as described in methods and stained for corresponding cytokines. Each Th cells subsets is specific for respective cytokines and transcription factor (Th1; IFN-γ, Th2; IL-4, Th9; IL-9, Th17; IL-17A and iTregs; Foxp3) expression at protein level. FACS plot shown here is representative for 5-6 independent experiments.

B. Naïve T cells were differentiated in Th cells and iTregs as described in methods sections and RNA was isolated to perform q-RT-PCR. Each Th cells subsets is specific for respective cytokines and transcription factor (Th1; IFN-γ, Th2; IL-4, Th9; IL-9, Th17; IL-17A and iTregs; Foxp3) expression at transcript level.

§ mRNA and Protein data were kindly provided by Dr. Yogesh Singh, Tubingen University.

6.9 NHE1 expression in Th cells subsets

Transcript levels of NHE1 were quantified in various Th cells subsets and iTregs by q-RT PCR. NHE1 mRNA transcript levels were significantly higher in Th9 cells than in Th0, other Th cells subsets (Th1, Th2 and Th17) and iTregs (Fig.19 A). To confirm this finding, we measured the NHE1 protein by immunoblotting and found very similar results as those of transcript levels (Fig.19 B). Similar to transcript levels, NHE1 protein levels were higher in Th9 cells than in any other Th cells subsets and iTregs.

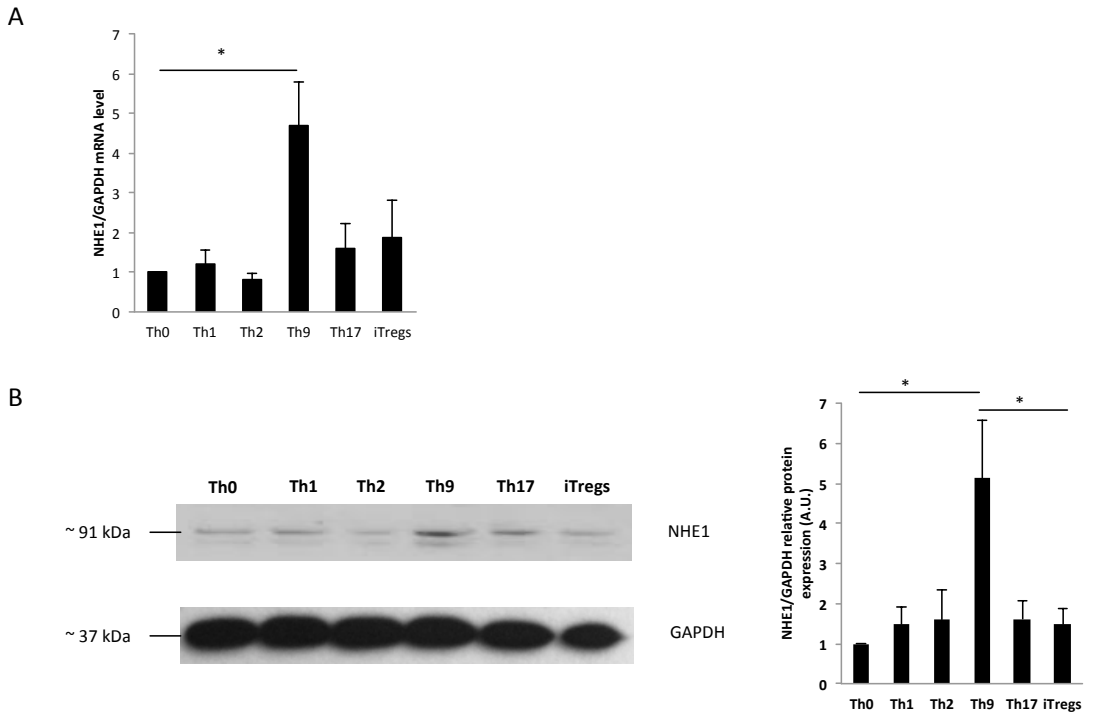


Figure 19 NHE1 expression in Th cells subsets and iTregs at mRNA and protein level

A. Naïve T cells were differentiated in Th cells and iTregs as described in methods sections and RNA was isolated for q-RT-PCR quantifying NHE1 transcript levels. Th9 cells have significantly higher NHE1 RNA expression than other Th cells subsets and iTregs. Arithmetic means ± SEM (n = 5 independent experiments) of the Th cells mRNA level. *indicates statistically significant difference (*p<0.05).

B. NHE1 protein expression in Th cells subsets and iTregs. NHE1 expression was statistically significantly higher in Th9 than in other Th cells subsets and iTregs. Arithmetic means ± SEM (n = 5 independent experiments) of the NHE1 protein level in Th cells. *indicates statistically significant difference (*p<0.05).

6.10 pHi and NHE1 activity in Th cells

In order to test, whether enhanced NHE1 expression in Th9 cells resulted in enhanced Na^+/H^+ activity and alkaline cytosolic pH (pHi), pHi was estimated from BCECF fluorescence using Fluorescence Microscopy. We found that Th0, Th2 and iTregs had similar pHi values (Fig.20 A,B). To our surprise, Th1 cells had the lowest pHi compared with all Th cells subsets including iTregs (Fig.20 A,B). In contrary, Th9 had significantly higher pHi than any of the other Th cells subsets including iTregs (Fig.20 A,B). Na^+/H^+ 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 alkalinisation 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 cells subsets as well as iTregs. Thus, none of the Th cells subsets and iTregs 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 NHE activity (Fig.20 A,C). NHE activity was significantly higher in Th9 cells than in other Th cells and iTregs (Fig.20 A,C). Thus, these data strongly suggested NHE1 could be involved in Th9 cells development or functions.

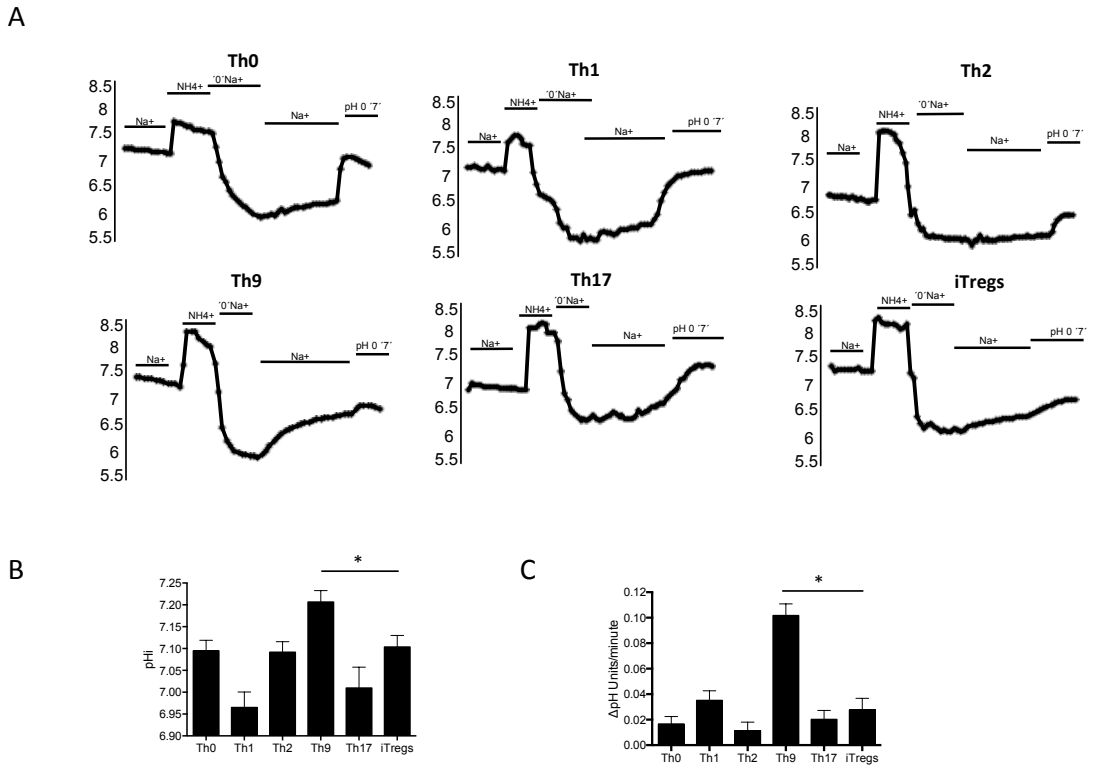


Figure 20 pHi and NHE1 activity in Th cells subsets and iTregs

A. Alterations of cytosolic pH (pHi) following an ammonium pulse in Th cells subsets and iTregs. To load the cells with H^+, 20 mM NH4Cl was added and Na^+ removed (replaced by NMDG) in a first step, NH_4Cl removed in a second step, Na^+ added in a third stem and nigericin (pH_0 7.0) applied in a fourth step to calibrate each individual experiment. Original tracing of typical experiments for pHi measurement and Na^+/H^+ exchanger activity in Th cells subsets and iTregs

B. Arithmetic means ± SEM (n = 3-5 independent experiments) of cytosolic pH prior to the ammonium pulse (pHi) in Th cells subsets and iTregs. *indicates statistically significant difference (*p<0.05).

C. Arithmetic means ± SEM (n = 3-5 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in Th cells subsets and iTregs. *indicates statistically significant difference (*p<0.05).

6.11 NHE1 activity is dependent on Akt signalling in Th9 cells

Previous studies have suggested that AKT signalling is involved in NHE1 regulation (Wu, Khan et al. 2004, Meima, Webb et al. 2009, Abu Jawdeh, Khan et al. 2011). Akt/mTOR signalling events are further involved in the development of Th cells subsets (Pierau, Engelmann et al. 2009, Lee, Gudapati et al. 2010, Kim, Sklarz et al. 2013) and iTregs (Haxhinasto, Mathis et al. 2008). However, the role of Akt/mTOR signalling has not been defined in Th9 cells development. To identify the possible role of Akt and NHE1 in Th9 cells development, we explored the phosphorylation of Akt at S473 site and found that Th9 cells have significantly higher rates of Akt phosphorylation than the other Th cells subsets and iTregs (Fig.21 A). Therefore, we reasoned that increased phosphorylation level of Akt leads to enhanced expression of NHE1 in Th9 cells compared with other Th cells subsets including iTregs. To explore whether Akt influenced NHE1 expression, we differentiated naïve T cells into Th9 cells in presence and absence of Akt1/2 inhibitor and measured NHE1 activity. As shown in Fig, B, Akt inhibition significantly decreased NHE1 activity. Moreover, inhibition of Akt reduced the expression of IL-9 (Fig.21 C). Those data reveal that AKT is crucial for the up-regulation of NHE1 activity and IL-9 production in Th9 cells.

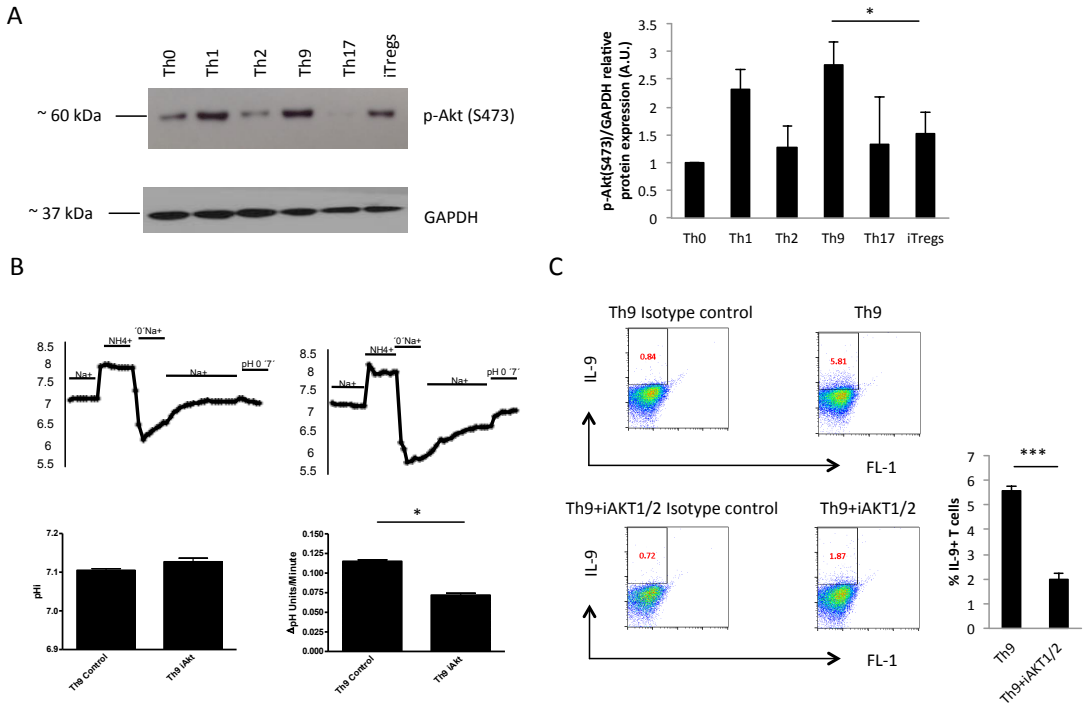


Figure 21 Regulation of NHE1 activity and IL-9 production by Akt in Th9 cells

A. Characterisation of Akt in Th cells subsets and iTregs. Phosphorylation of Akt at Ser 473 is significantly higher in Th9 cells compared to iTregs. Arithmetic means ± SEM (n = 5 independent experiments) of the Th cells protein level. *indicates statistically significant difference (*p<0.05).

B. Naïve T cells were differentiated into Th9 cells with and without Akt1/2 inhibitor (0.3µM) and pHi as well as NHE1 activity measured. Original tracing of typical experiment for pHi measurement and Na^+/H^+ exchanger activity in Th9 cells and after treatment of Akt1/2 inhibitor. Arithmetic means ± SEM (n = 3-4 independent experiments) of cytosolic pH prior to the ammonium pulse (pHi) and Na^+-dependent recovery of cytosolic pH (Δ pH/min) in Th9 cells with and without Akt1/2 inhibitor. *indicates statistically significant difference (*p<0.05).

C. A representative FACS plot showing differentiated Th9 cells with and without Akt1/2 inhibitor (0.3 µM). Data shown here are representative of n=3 experiments.*indicates statistically significant difference (***p<0.0005).

6.12 NHE1 is directly involved in regulation of IL-9 production

To uncover the importance of NHE1 in Th9 cells functions and development, we used NHE1-siRNA to knock-down NHE1 in Th9 cells. The transfection efficiency of siRNAs was tested using control siRNA which was labelled with FAM dye. FAM fluorescence was measured by flow cytometry. As a result more than 90% $CD4^+$ T cells were positive for FAM dye (green colour) (data not shown). After siRNA treatment, pH and NHE1 activity were measured using BCECF. As a result knock-down of NHE1 drastically reduced the activity of the Na^+/H^+ exchanger (Fig. 22 A, C) and decreased intracellular pH (Fig 22 A, B). NHE1 knock-down further reduced IL-9 production (Fig.22 D) and the ability of Th9 cells becoming Tregs (Fig.22 E). Thus, NHE1 is apparently an essential component of Th9 cells development.

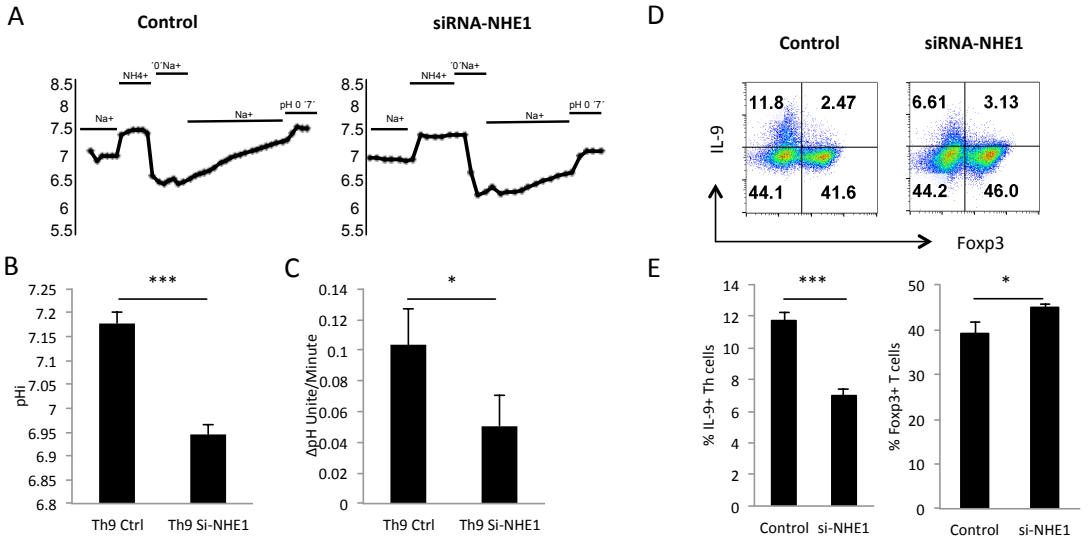


Figure 22 Regulation of Th9 cells and iTregs development by NHE1

A. Alterations of cytosolic pH (pHi) following an ammonium pulse in transfected Th9 cells with control and NHE1 siRNAs. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG) in a first step, NH_4Cl removed in a second step, Na^+ added in a third step and nigericin (pH_0 7.0) applied in a fourth step to calibrate each individual experiment. Original tracings of typical experiments for pHi measurement and Na^+/H^+ exchanger activity in Th cells subsets and iTregs

B. Arithmetic means ± SEM (n = 3-5 independent experiments) of cytosolic pH prior to the ammonium pulse (pHi) in siRNA control and siRNA NHE1 Th9 cells. *indicates statistically significant difference (*p<0.05).

C. Arithmetic means ± SEM (n = 3-5 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in siRNA control and siRNA NHE1 Th9 cells. *indicates statistically significant difference (*p<0.05).

D. Differentiation of Th9 cells transfected with control and NHE1 siRNAs. At day 4 day, Th9 cells were activated with PMA, Ionomcyin and Brefeldin for 2 hours and stained for IL-9 and Foxp3. Representative FACS plots show the Th9 differentiation after knock-down of NHE1.

E. Arithmetic means ± SEM (n = 3-5 independent experiments) of IL-9 and Foxp3 expression in siRNA control and siRNA NHE1 Th9 cells. *indicates statistically significant difference (***p<0.001, *p<0.05).

DISCUSSION2

In this part of thesis, to understand more about how NHE function in the Th cells pro-activation process, the characteristics of NHE from several Th cells subsets (Th0, Th1, Th9 and Th17) and suppressive T cell were further tested. The present study reveals a totally novel importance of intracellular pH regulation and Na^+/H^+ exchanger activity in development and functions of Th cells subsets, in particular of Th9 and iTregs.

Past reports shows that IL-2 signalling can prompt the NHE1 activity in human T cells (GORDON B. MILLS 1986). In our study, we also got comparable results as described earlier in murine T cells (Chien, Liao et al. 2007, Chang, Wang et al. 2010). Further experiments suggested that among Th cells subsets, variety of intracellular pH, which is governed by Na^+/H^+ exchanger activity of specific Th cells. Na^+/H^+ exchanger activity is highly sensitive to intracellular pH and is switched off upon cytosolic alkalinisation (Wakabayashi, Shigekawa et al. 1997). Th9 cells had the highest and Th2 cells the lowest Na^+/H^+ exchanger activity compared to other in vitro induced T helpers (Th0, Th1 and Th17) and suppressive T cell. Up-regulation of Na^+/H^+ exchanger activity in Th9 cells enhances the extrusion of H^+ thus contributing to the maintenance of alkaline pHi. Moreover, NHE1 mRNA and protein levels were higher in Th9 cells than in any other Th cells and iTregs.

In addition, this study uncovered the important regulation role of Akt on NHE1 activity in Th9 cells. Akt is known to govern the signalling for other Th1, Th2, Th17 cells 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 Rictor activity in Th9 cells compared with iTregs. This higher activity of Akt could affect the NHE1 activity in Th9 cells as previous studies suggested a role of Akt in regulating the NHE1

activity (Wu, Khan et al. 2004, Meima, Webb et al. 2009, Zhou, Pasham et al. 2015). Akt could also be required for up-regulation of IL-9 cytokine expression, as Akt inhibition decreased the production of IL-9 in Th9 cells. It appears that Akt is critically important for regulating function and development of Th9.

GITR signalling has been shown as a tool of regulating the Th9/iTregs balance in cancer (Kim, Kim et al. 2015, Xiao, Shi et al. 2015). Our studies suggest that NHE1 may have the instrumental effect for the Th9/iTregs axis associated with GITR signalling. From the analysis of our siRNA knock-down data it is tempting to speculate that NHE1 is essential for Th9/iTregs axis in cancer patients. Tumor cells generate lactate thus leading to a highly acidic environment (Cairns 2015). An adequate immune response against tumor cells requires survival of the respective immune cells in the acidic environment. Enhanced NHE1 activity may indeed confer some protection against an acidic environment. On the other hand, cytosolic alkalinisation is known to stimulate glycolysis (Boiteux and Hess 1981, Heinrich, Melendez-Hevia et al. 1999, Tennant, Duran et al. 2010), which may support energy supply to Th9 cells. It can be explained by the enhanced glycolysis in Th9 cells contributes to deprivation of neighbouring cancer cells from glucose. Clearly, Unmistakably, extra trial exertion is required to characterize the role of NHE1 activity for Th9 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. Thus it is tempting to speculate that NHE1 could contribute to the cancer metabolism checkpoint therapy.

In the future, it would be very interesting to investigate especially how the high NHE activity is involved in the regulation of Th9 cell functions in vivo. For example, as a key member of antitumor immune system, it is worthy to test the role of high NHE activity in assistance to Th9 function in the tumour niche.

SUMMARY

To sum up both the result chapters, this thesis described that DJ-1 protein is very crucial to maintain the ROS production and NHE1 activity. In addition to this, further NHE1 is also involved in the development and functions of Th9 cells. This thesis, elucidate the novel role of NHE1 in $CD4^+$ helper T cells and potential implications of NHE1 in Parkinson's disease as well as in cancer pathogenesis.

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PUBLICATIONS

A. Published Papers (* Co-First Authorship)

1. **Zhou Y**, Pasham V, Chatterjee S, Rotte A, Yang W, Bhandaru M, Singh Y, Lang F.Regulation of Na+/H+ Exchanger in Dendritic Cells by Akt1. *Cell Physiol Biochem* 2015;36:1237-1249
2. **Zhou Y***, Salker MS*, Singh Y, Brosens J, Lang F. LeftyA sensitive cytosolic pH regulation and glycolytic flux in Ishikawa human endometrial cancer cells. *Biochem Biophys Res Commun.* 2015 May 8;460(3):845-9.(Joint-first authorship)
3. Singh Y, Chen H, **Zhou Y**, Foller M, Mak TW, Salker MS, Lang F. Differential effect of DJ-1/Park-7 on development of natural and induced regulatory T cells. *Sci Rep.*2015 Dec 4;5:17723.
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B. Publication under revision or in prep

1. **Zhou Y**, Shi X, Chen H, Salker MS, Föller M,Mak TW, Singh Y, Lang F. DJ-1/Park-7 sensitive pH and sodium hydrogen exchanger 1 (NHE1) activity in $CD4^+$ T cells. *J Cell Physiol* (Under revison)
2. **Zhou Y***, Singh Y*, Shi X, Umbach AT, Salker MS, Lang KS, Cobb BS, Lang F. T helper-9 (Th-9) cells are distinct from other Th cells subsets and induced regulatory T cells (iTregs) in pH and sodium hydrogen exchanger 1 (NHE1) regulation. (*J Biol Chem*) (Under revison) (Joint-first authorship)
3. **Zhou Y**, Salker MS, Singh Y, Lang F. Differential effect of ASM on development of induced regulatory T cells (submitted)
4. **Zhou Y**, Salker MS, Singh Y, Lang F. The regulation effect on regulatory T cell development by Nicotinamide (in prep)
5. Singh Y, Munzer P, Shi X, **Zhou Y**, Schimidt S, Umbach AT, Salker MS, Lang F A differential Ca2+ influx activity distinguishes the IL-9 producing T helper-9 (Th-9) cells from other Th cells subsets and iTregs (in prep)
6. Yogesh Singh, Shaqiu Zhang, Kübra Vurat, Christopher Schipp, Bernart Alvira, Abul Fajol, **Yuetao Zhou**, Hong Chen, Anja T Umbach, Madhuri S Salker,

Lionel Apetoh, Graham Pawelec, Roland H Wenger, Florian Lang. Role of HIF-2α in the development of $CD4^+$ T helper 9 (Th9) cells and induced regulatory T cells (in prep)

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C. Published abstracts:

1. **Y Zhou**, MS Salker, Y Singh, JJ Brosens, F Lang. Lefty A sensitive cytosolic pH regulation and glycolytic flux in Ishikawa cells. *Acta Physiologica* 213, 82-82 (Conference Abstract)
2. Y Singh*, **Y Zhou***, AT Umbach, MS Salker, F Lang.IL-9 producing T helper-9 (Th-9) cells are distinct from-Th1-/-Th2/Th17 cells subsets and iTregs in sodium hydrogen exchanger 1 (NHE1) activity and pH regulation. *Acta Physiologica* 213, 52-52 (Conference Abstract)

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