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# AKT/SGK-sensitive phosphorylation of GSK3 in the regulation of L-selectin and perforin expression as well as activation induced cell death of T-lymphocytes

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ABSTRACT

Survival and function of T-lymphocytes critically depends on phosphoinositide (PI) 3 kinase. PI3 kinase signaling includes the PKB/Akt and SGK dependent phosphorylation and thus inhibition of glycogen synthase kinase GSK3α,β. Lithium, a known unspecific GSK3 inhibitor protects against experimental autoimmune encephalomyelitis. The present study explored, whether Akt/SGK-dependent regulation of GSK3 activity is a determinant of T cell survival and function. Experiments were performed in mutant mice in which Akt/SGK-dependent GSK3α,β inhibition was disrupted by replacement of the serine residue in the respective SGK/Akt-phosphorylation consensus sequence by alanine ($gsk3^{KI}$). T cells from $gsk3^{KI}$ mice were compared to T cells from corresponding wild type mice ($gsk3^{WT}$). As a result, in $gsk3^{KI}$ $CD4^+$ cells surface CD62L (L-selectin) was significantly less abundant than in $gsk3^{WT}$ $CD4^+$ cells. Upon activation *in vitro* T cells from $gsk3^{KI}$ mice reacted with enhanced perforin production and reduced activation induced cell death. Cytokine production was rather reduced in $gsk3^{KI}$ T cells, suggesting that GSK3 induces effector function in $CD8^+$ T cells. In conclusion, PKB/Akt and SGK sensitive phosphorylation of GSK3α,β is a potent regulator of perforin expression and activation induced cell death in T lymphocytes.



## 1. Introduction

The glycogen synthase kinase 3 (GSK3α,β) is an ubiquitously expressed serine/threonine kinase, participating in the regulation of a wide variety of functions, including metabolism, cell proliferation, cell differentiation and apoptosis [1–6]. Deranged regulation of GSK3β contributes to the pathophysiology of diabetes, cancer, inflammation, osteoporosis, mood disorders and neurodegenerative disease [7–12]. GSK3 is a key regulator of immune reactions [13–16]. In lymphocytes, coactivation of the T cell receptor and CD28 result in inactivation of GSK3 [17].

GSK3β activity is inhibited by insulin [18,19], an effect mediated by protein kinase B PKB/Akt [20] and the serum and glucocorticoid inducible kinase SGK1 [21]. Both kinases are activated by the phosphoinositide 3-kinase (PI3K) pathway [22].

The present study aimed to define the role of Akt/SGK-dependent regulation of GSK3 in the regulation of T lymphocyte function. To this end, naive T cells were isolated from spleen and cultured from gene-targeted mice in which the serine residues within the respective Akt/SGK phosphorylation sites of GSKα and GSK3β had been replaced by alanin residues ($GSK3\alpha^{21A/21A}$, $GSK3\beta^{9A/9A}$). In those mice ($gsk3^{KI}$), GSKα and GSK3β are resistant against inactivation by AKT/SGK [23]. As shown before, $gsk3^{KI}$ mice are resistant to the effect of insulin on muscle glycogen synthase [23]. Further studies disclosed altered renal function [24,25], gastric acid secretion [26] and behavior [27] of those mice. The $gsk3^{KI}$ mice develop more severe experimental autoimmune encephalitis than wild type mice [28]. The present observations reveal that Akt/SGK-dependent GSK3 phosphorylation participates in the regulation of activation induced cell death and perforin expression in murine cytotoxic T-lymphocytes.

## 2. Materials and methods

### 2.1. Mice

All animal experiments were conducted according to the guidelines of the American Physiological Society. Mice were generated in which the codon encoding Ser9 of GSK3β gene was changed to encode nonphosphorylatable alanine ($GSK3\beta^{9A/9A}$), and simultaneously the codon encoding Ser21 of GSK3α was changed to encode the nonphosphorylatable $GSK3\alpha^{21A/21A}$ thus yielding the $GSK3\alpha/\beta^{21A/21A/9A/9A}$ double knockin mouse ($gsk3^{KI}$) as described previously [23]. The mice were compared to corresponding wild type mice ($gsk3^{WT}$).

### 2.2. Splenic T-lymphocyte culture

T lymphocytes were cultured from the splenic T-cells as described earlier [29]. In brief, for activation of primary-naive T cells,

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spleens from *gsk3*$^{KI}$ mice and their respective wildtype mice *gsk3*$^{WT}$ were disaggregated following red blood cell lysis. Cells were cultured in RPMI-1640 medium containing L-glutamine, 10% heat-inactivated FBS, 50 μM β-mercaptoethanol and 1% penicillin–streptomycin. Single-cell suspensions from splenocytes adjusted to a density of $5 \times 10^6$ cells/ml were stimulated with monoclonal anti-CD3 (5 μg/ml; 145-2C11, R and D Systems) to 'trigger' the TCR. For the generation of mature cytotoxic T-lymphoblasts, mouse T cells grown from spleen preparations cultured for 48 h in the presence of stimulus (monoclonal antibody 145-2C11) were washed and resuspended at a density of $3.5 \times 10^5$ cells per ml with IL-2 (0.02 μg/ml; 360 IU/ml) for 48 h to generate mature T cells blasts. After activation and clonal expansion mature cytolytic T-cell blasts were characterized phenotypically by flow cytometry. As a result, more than 80% of the cells were positive for CD8$^+$. Cells were further maintained in medium containing IL-2 (0.02 μg/ml; 360 IU/ml).

### 2.3. Flow cytometry analysis and phenotyping of the cells

Mature cytotoxic T-cell blasts were analyzed with the standard multicolor flow cytometry settings and commercially available specific fluorescence conjugated antibodies as follows: Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) conjugated anti-CD4, Allophycocyanin (APC) or Cyanine 5.5 (Cy5.5) conjugated anti-CD8, PE conjugated anti-CD25, PE conjugated anti-CD62L, APC conjugated anti-CD25, PE conjugated anti-CD98, PE-anti-CD71 and APC anti-TCRbeta (BD Biosciences, Heidelberg, Germany). A minimum of $2 \times 10^5$ cells were washed and stained for 30 min at 4 °C with saturating concentrations of antibody in RPMI-1640 medium and 0.5% FBS. Cells were washed and resuspended in RPMI-1640 medium and 0.5% FBS before being acquired on a FACS Calibur (BD, Heidelberg, Germany). A minimum of $5 \times 10^4$ relevant events were measured and stored ungated. Live cells (>90% of total acquired events) were gated according to their forward scatter and

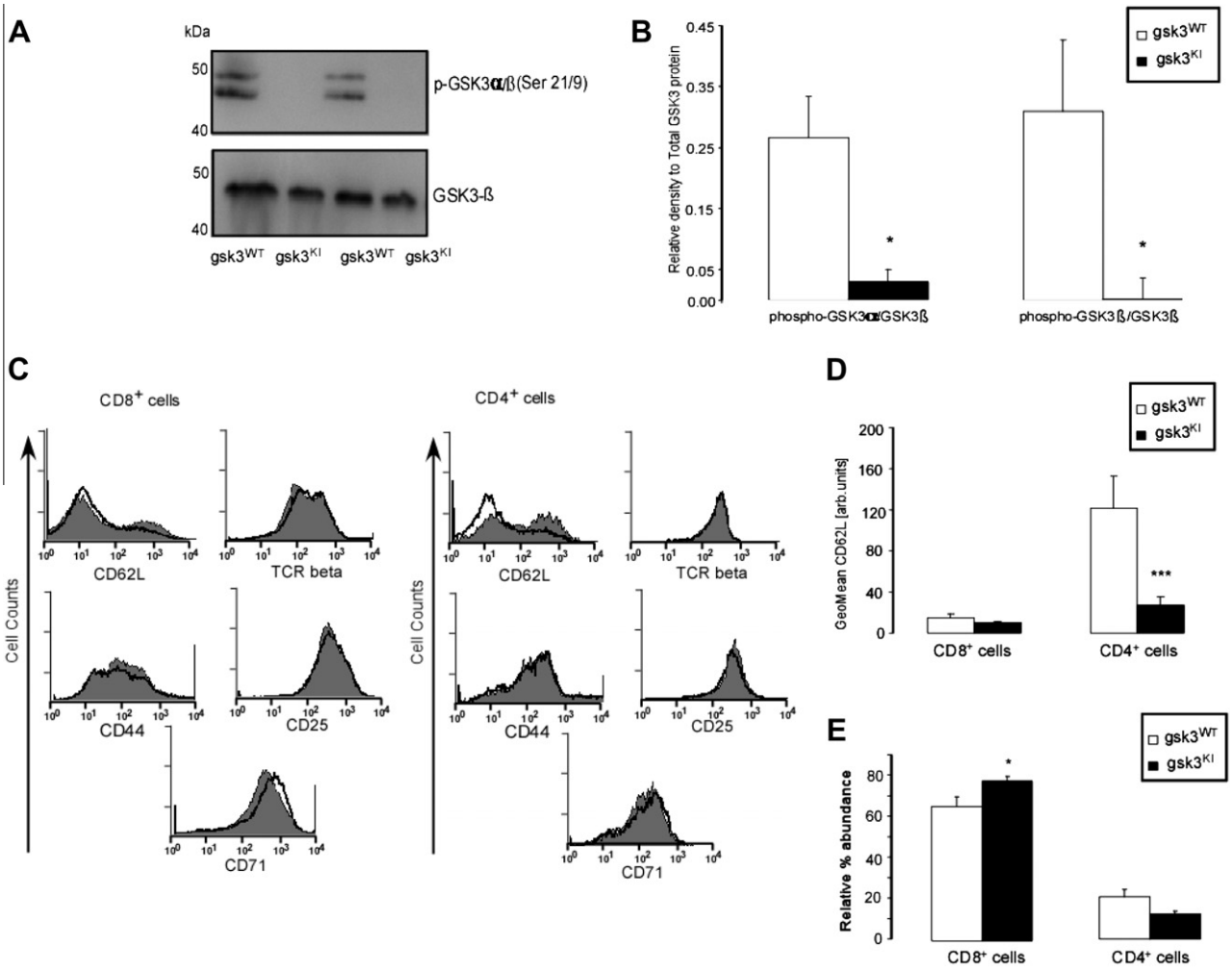


**Fig. 1.** GSK3 phosphorylation and surface markers of cultured CD8$^+$ and CD4$^+$ T cells from *gsk3*$^{WT}$ and *gsk3*$^{KI}$ mice. (A) Original western blot of the expression of total GSK3 protein (lower panel) and of phosphorylated GSK3α/β (upper panel) in T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$) and their corresponding wild type mice (*gsk3*$^{WT}$). (B) Arithmetic means ± SEM ($n = 4$ independent experiments) of phosphorylated over total GSK3 protein abundance in T cells from *gsk3*$^{WT}$ mice (white bar) and *gsk3*$^{KI}$ mice (black bar). *Indicates significant difference ($p < 0.05$) from *gsk3*$^{WT}$ T cells. (C) Original FACS histograms (representative for $n = 5$–7 different cultures) of the surface expression of CD62L, CD71, CD44, CD25 or TCR beta in CD8$^+$ cells (left bars) and CD4$^+$ cells (right bars) derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black line blank histogram) and their corresponding wild type mice (*gsk3*$^{WT}$, grey filled histogram). (D) Arithmetic means ± SEM ($n = 7$ independent experiments) of surface CD62L expression in CD8$^+$ cells (left bars) and CD4$^+$ cells (right bars) derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). ***Indicates significant difference ($p < 0.001$) to *gsk3*$^{WT}$ T cells. (E) Arithmetic means ± SEM ($n = 6$ independent experiments) of the percentage of CD8 (left bars) and CD4 (right bars) cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). *Indicates significant difference ($p < 0.05$) from *gsk3*$^{WT}$ T cells.

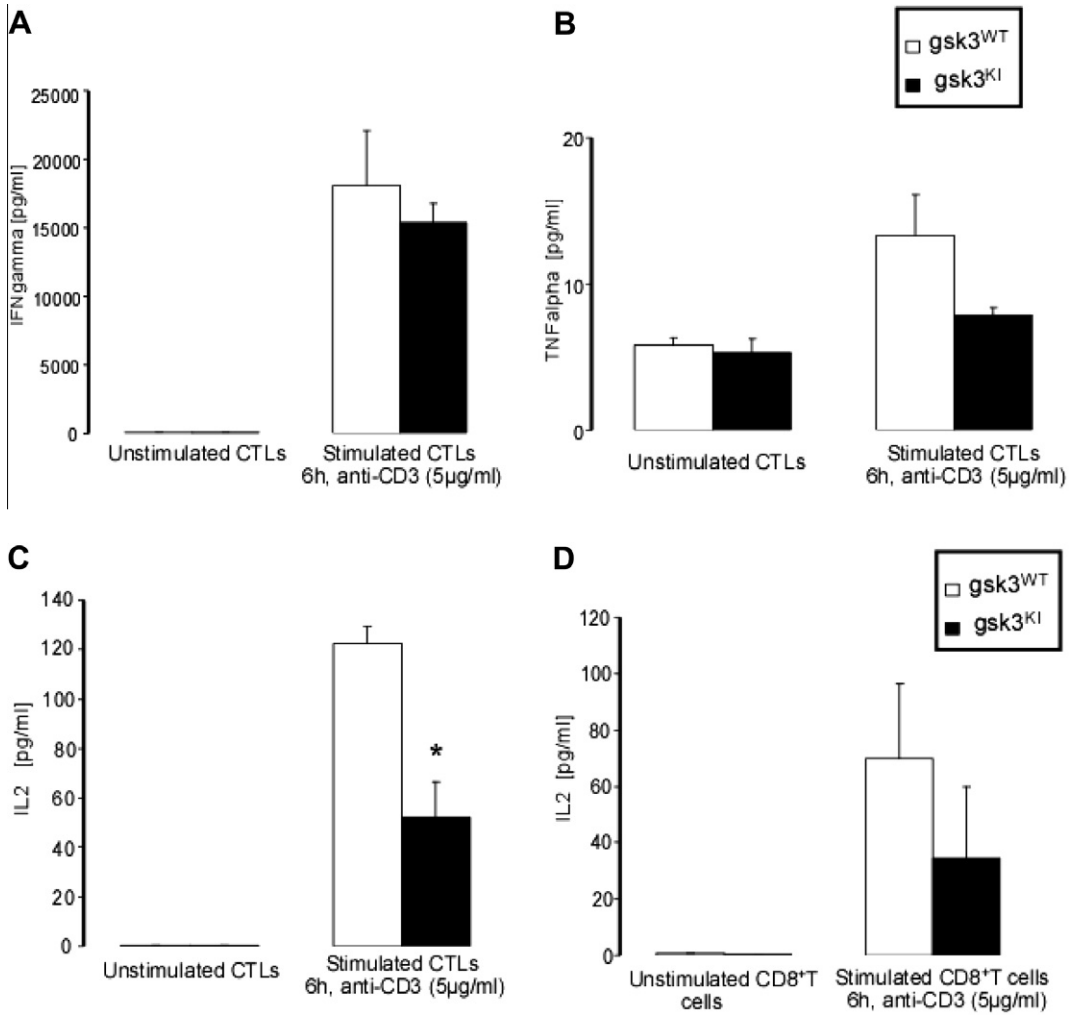


**Fig. 2.** Cytokine secretion of T cells from *gsk3*$^{WT}$ and *gsk3*$^{KI}$ mice with and without re-stimulation with anti-CD3. (A) Arithmetic means ± SEM ($n$ = 6 independent experiments) of IFNγ secretion in CD3-stimulated (with anti CD3) and non-stimulated T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mouse (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). (B) Arithmetic means ± SEM ($n$ = 5 independent experiments) of TNFα secretion in T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). (C) Arithmetic means ± SEM ($n$ = 5 independent experiments) of IL-2 secretion in T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). *Indicates significant difference ($p < 0.05$) from *gsk3*$^{WT}$ T cells. (D) Arithmetic means ± SEM ($n$ = 4 independent experiments) of IL-2 secretion in sorted CD8$^+$ cells from the T cells cultures derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars).

side scatter. Data was analyzed using Cell Quest Pro software (BD Biosciences, Heidelberg, Germany).

### 2.4. MACS sorting of CD8$^+$ cells from total culture

CD8$^+$ cells were sorted from total mixed T cell blast cultures by MACS sorting CD4 beads, as per the instructions of the manufacturers (Miltenyibiotec). The total culture was depleted of CD4 cells. The sorted CD8$^+$ cells were stained with CD8 and CD4 antibodies and acquired on FACS calibur, the CD8$^+$ cells constituted >98–99% of the cells.

### 2.5. Activation induced cell death (AICD)

Mature IL-2 blasts at cell density of $10^6$ cells per ml were restimulated with monoclonal anti-CD3 (5 µg/ml; 145-2C11, R and D Systems), for 24 and 48 h, then acquired on FACS calibur for determination of forward scatter and side scatter yielding the percentage of live and dead cells. Appropriate unstimulated controls were kept in each group for comparison. In addition, annexin binding and Fluo3AM fluorescence were determined to estimate phosphatidylserine exposure and calcium content, respectively. For determination of annexin binding T cells were washed with Ringer solution containing 5 mM $CaCl_2$. The cells were then stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analyzed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For measurement of intracellular $Ca^{2+}$ T cells were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM. The cells were incubated at 37 °C for 20 min and washed in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, $Ca^{2+}$-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

### 2.6. Cytokine secretion

Secretion of IFN-γ, IL-2 and TNF-α was evaluated with or without CD3 stimulation. To this end $5 \times 10^6$ cells from each group (knockin and corresponding control) were incubated in the med-

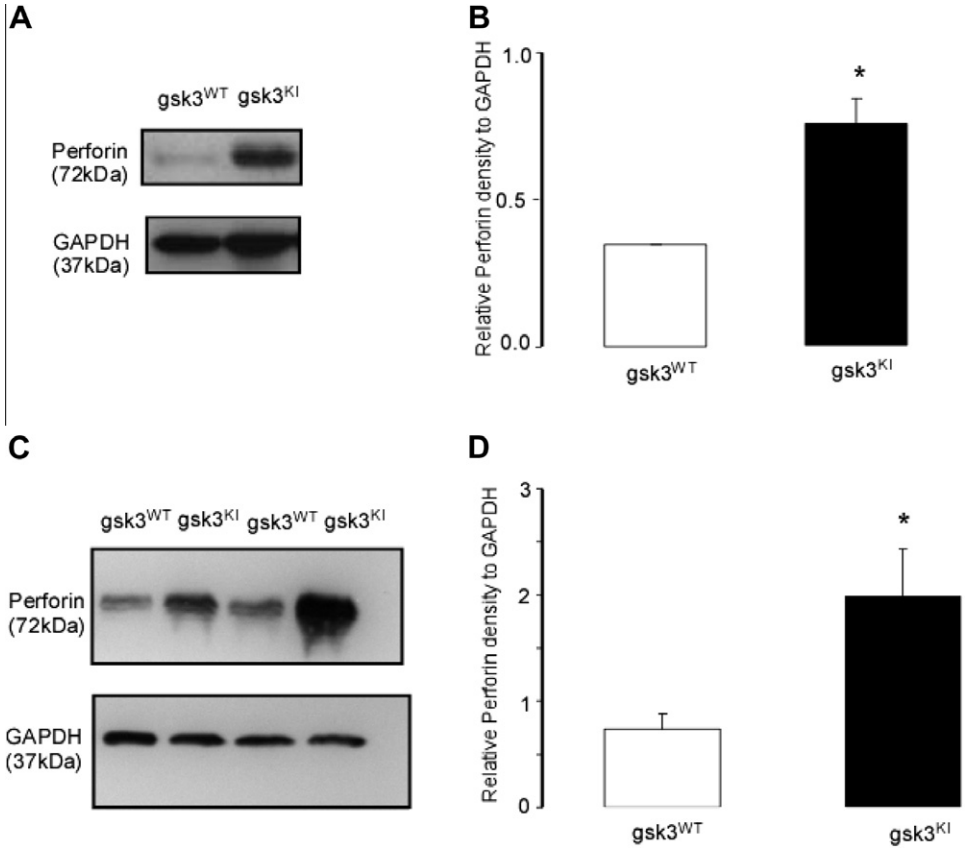


**Fig. 3.** Perforin abundance in T cells and sorted CD8$^+$ T cells from *gsk3$^{KI}$* and *gsk3$^{WT}$* mice. (A) Original western blot of the expression of perforin in T cells derived from spleen of GSK3$\alpha$/$\beta^{21A/21A/9A/9A}$ double knockin mice (*gsk3$^{KI}$*) and their corresponding wild type mice (*gsk3$^{WT}$*). (B) Arithmetic means ± SEM ($n$ = 3 independent experiments) of perforin protein abundance in T cell cultures from *gsk3$^{WT}$* mice (white bar) and *gsk3$^{KI}$* mice (black bar). *Indicates significant difference ($p < 0.05$) from *gsk3$^{WT}$* T cells. (C) Original western blot of the expression of perforin in CD8$^+$ sorted T cells from cytotoxic T cell (CTL) cultures derived from spleen of GSK3$\alpha$/$\beta^{21A/21A/9A/9A}$ double knockin mouse (*gsk3$^{KI}$*) and their corresponding wild type mice (*gsk3$^{WT}$*). (D) Arithmetic means ± SEM ($n$ = 4 independent experiments) of perforin protein abundance in CD8$^+$ sorted T cells from CTL cultures from *gsk3$^{WT}$* mice (white bar) and *gsk3$^{KI}$* mice (black bar). *Indicates significant difference ($p < 0.05$) from *gsk3$^{WT}$* CTL.

ium with (stimulated) or without (non-stimulated) addition of monoclonal anti-CD3 (5 μg/ml; 145-2C11) for 6 h. After the incubation period cells were separated by centrifugation and the supernatant stored in aliquotes in −20 °C. The secreted cytokines in the medium were detected using commercially available ELISA kits from BD Biosciences (Becton Dickinson, Heidelberg, Germany).

### 2.7. Western blotting

The expression levels of each protein were analyzed by Western blotting. In brief, 2–4 × $10^7$ mature T cell blasts or sorted CD8$^+$ cells from *gsk3$^{KI}$* and *gsk3$^{WT}$* mice were washed twice with ice cold phosphate-buffered saline (PBS) and cells were lysed for 10 min with cell lysis buffer (20 mM tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM $Na_3VO_4$ , 1 μg/ml leupeptin and 1 mM PMSF, added immediately prior to use). The extracts were centrifuged at 14,000*g* for 10 min at 4 °C to remove insoluble material. The protein concentration of the supernatant was determined and 1:5 Laemmli sample buffer added. Total protein (50–100 μg) was subjected to 7.5–10% SDS–PAGE (1:30, bis: acrylamide). Proteins were transferred to a nitrocellulose or polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.), and the membranes were then blocked for 2 h at room temperature with 5% non-fat dried milk in tris-buffered saline (NFDM/TBS) containing 0.1% Tween 20. Immunoblotting was carried out with overnight incubation at 4 °C with either 5% BSA/TBS- or 3% NFDM/TBS-containing rabbit Perforin antibody (1:1000) (Cell Signaling, Boston, MA, USA), goat Granzyme B antibody (1:1000, R and D Systems, Minneapolis, MN, USA) rabbit GSK3β antibody, and/or phospho-GSK3α/β (Ser 21/9) antibody (Cell signaling Technology, Boston, MA, USA). A rabbit GAPDH antibody (1:1000) (Cell signaling Technology, Boston, MA, USA) was used as a loading control. Specific protein bands were visualized after subsequent incubation with a 1:2000 dilution of anti-rabbit IgG or 1:5000 dilution of anti-goat IgG conjugated to horseradish peroxidase and a super signal enhanced chemiluminescence detection procedure (GE Healthcare, UK). Specific bands were quantified by densitometry with the Quantity one software (Bio rad gel doc system, Chemidoc XRS). Levels of each protein were expressed as the ratio of signal intensity for the target protein relative to that of GAPDH or target phospho-protein relative to the total protein.

### 2.8. Statistics

Data are provided as means ± SEM, $n$ represents the number of independent experiments. All data were tested for significance using paired or unpaired Student $t$-test or ANOVA by GraphPad InStat (GraphPad Software, San Diego, USA). Only results with $p < 0.05$ were considered statistically significant.

## 3. Results

The present study analyzed cultured T cells from knockin mice expressing Akt/SGK resistant GSK3α and GSK3β (*gsk3$^{KI}$*) and from

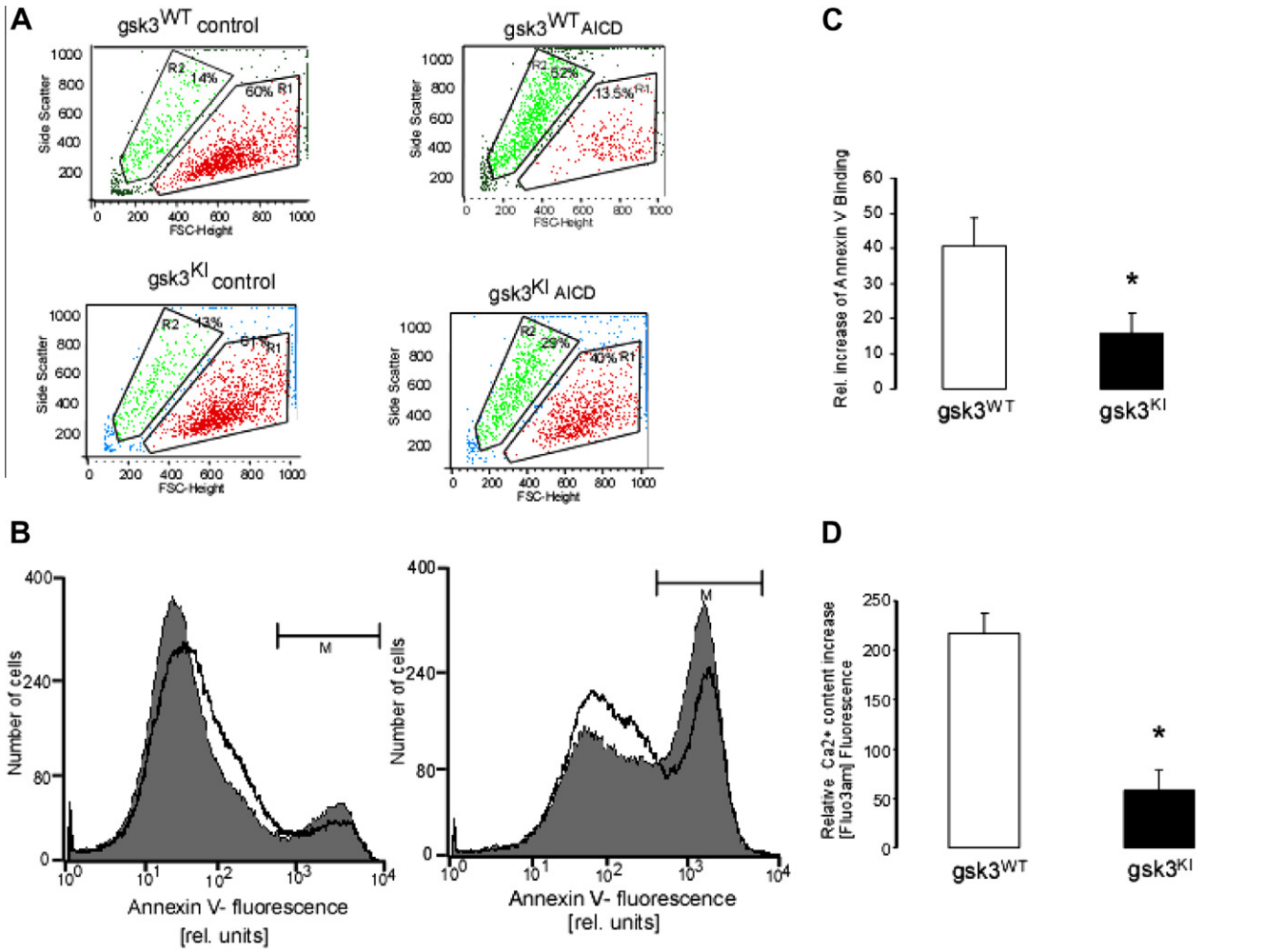


**Fig. 4.** Activation induced cell death (48 h) of T cells from *gsk3*$^{WT}$ and *gsk3*$^{KI}$ mice. (A) Original dot blots demonstrating changes in forward scatter (FSC) reflecting cell size in a representative experiment of T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, right panel) and their corresponding wild type mice (*gsk3*$^{WT}$, left panel). R1 represents live cell gating, and R2 represents the region for gating of dead cells. (B) Histogram of annexin V-binding reflecting phosphatidylserine exposure in a representative experiment of T cells from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black) and their corresponding wild type mice (*gsk3*$^{WT}$, grey). M indicates annexin V-positive cells. (C) Arithmetic means ± SEM ($n = 3$ independent experiments) of annexin V-binding following activation (48 h) in T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). *Indicates significant difference ($p < 0.05$) from *gsk3*$^{WT}$ T cells. (D) Arithmetic means ± SEM ($n = 3$ independent experiments) of Fluo3AM fluorescence, reflecting intracellular calcium content, following activation (48 h) of T cells derived from spleen of GSK3α/β$^{21A/21A/9A/9A}$ double knockin mice (*gsk3*$^{KI}$, black bars) and their corresponding wild type mice (*gsk3*$^{WT}$, white bars). *Indicates significant difference ($p < 0.05$) from *gsk3*$^{WT}$ T cells.

corresponding wild type mice (*gsk3*$^{WT}$). According to Fig. 1A and B, phosphorylated GSK3 was detectable in *gsk3*$^{WT}$ T cells but completely absent in *gsk3*$^{KI}$ T cells.

According to FACS analysis, *gsk3*$^{KI}$ cultures had a slightly but significantly higher percentage of CD8$^+$ cells as compared to *gsk3*$^{WT}$ cultures and tended to have a lower percentage of CD4 cells, a difference, however, not statistically significant (Fig. 1E). The surface expression of CD71, CD44, CD25 or TCRbeta in CD8$^+$ and CD4$^+$ cells was similar in *gsk3*$^{KI}$ and *gsk3*$^{WT}$ mice (Fig. 1C). In contrast, CD62L (L-selectin) expression tended to be lower in CD8$^+$ cells and was significantly lower in *gsk3*$^{KI}$ than in *gsk3*$^{WT}$ CD4$^+$ cells (Fig. 1C and D).

In order to possibly disclose functional differences between T cells from both genotypes cytokine secretion upon re-stimulation with anti-CD3 was determined. The secretion of TNFα and IFNγ was again similar in *gsk3*$^{KI}$ and *gsk3*$^{WT}$ T cells, while *gsk3*$^{KI}$ T cells secreted significantly less IL-2 than *gsk3*$^{WT}$ T cells upon re-stimulation with anti-CD3 antibody (Fig. 2).

According to western blotting, the expression of perforin protein was significantly higher in *gsk3*$^{KI}$ T cell cultures than in *gsk3*$^{WT}$ T cell cultures (Fig.4). Similar observations were made in isolated CD8$^+$ T cells. In CD8$^+$ T cell preparations, *gsk3*$^{KI}$ CD8$^+$ T cells again expressed significantly higher levels of perforin than *gsk3*$^{WT}$ CD8$^+$ T cells (Fig. 4).

Moreover, *gsk3*$^{KI}$ T cells were more resistant than *gsk3*$^{WT}$ T cells to activation induced cell death (AICD) when restimulated with monoclonal anti-CD3 antibody for 24 h (data not shown) and for 48 h (Fig. 3). The increase in annexin V binding following 48 h AICD was significantly lower in *gsk3*$^{KI}$ T cells then in *gsk3*$^{WT}$ T cells (Fig. 3). Also, the increase in intracellular calcium content following AICD was significantly lower in *gsk3*$^{KI}$ T cells than in *gsk3*$^{WT}$ T cells (Fig. 3).

## 4. Discussion

The present study reveals that disruption of AKT/SGK dependent regulation of GSK3α,β impacts on activation induced cell death (AICD). Accordingly, AICD is blunted in T cells isolated from spleen of knockin mouse expressing AKT/SGK resistant GSK3α and

GSK3β ($gsk3^{KI}$) as compared to AICD in T cells derived from corresponding wild type mice ($gsk3^{WT}$). Thus, AICD is apparently inhibited by AKT/SGK resistant GSK3α and GSK3β. Presumably; at restrained GSK3 activity, T cells once activated undergo AICD. If GSK3 activity is enhanced, AICD is suppressed and cell survives at the stage of short life effector cells. AICD involves the Fas receptor CD95 [30–33]. GSK3 is known to inhibit via negative regulation of Nuclear Factor of Activated T Cells NFATc the production of the proapoptotic Fas ligand [34]. As shown in cardiac tissue, Fas receptor triggering leads in turn to GSK3 inhibition [1]. Thus, it is feasible that AKT/SGK resistant GSK3 interferes with the upregulation of Fas dependent cell death. The present study did not address the mechanisms involved. GSK3 could influence gene expression by phosphorylation of NFAT [35–37], β-catenin [8,38] and PSF (PTB [polypyrimidine tract-binding protein]-associated splicing factor) [16].

In addition to differences in AICD, $gsk3^{KI}$ T cells express significantly higher levels of perforin than $gsk3^{WT}$ T cells, suggesting that the effector function is maintained by GSK3. According to earlier studies GSK3 stimulates the release of TNFα [39], IL-1 [39], IL-2 [36], IL-6 [3,39] and IL-12 [40] and inhibits the release of IL-10 [15,17]. To the best of our knowledge, GSK3 sensitive regulation of perforin expression and release has never been shown. In line with our observation of enhanced perforin expression we found that in $gsk3^{KI}$ CD4 cells, CD62L expression was significantly less pronounced than in $gsk3^{WT}$ CD4 cells. During activation CD62L is downregulated on T cells. As CD62L low cells are considered short life effector T cells, this observation provides further evidence that enhanced GSK3 activity maintains effector function in T cells [41].

The decreased AICD and the enhanced perforin expression could both contribute to the stimulation of inflammation in autoimmune disorders, such as experimental autoimmune encephalitis, which is ameliorated by lithium treatment and takes a more severe clinical course in $gsk3^{KI}$ mice than in $gsk3^{WT}$ mice [28]. Lithium has been shown to suppress the differentiation of reactive effective T lymphocytes and the proliferation of mononuclear cells [28]. Along those lines, inhibition of GSK3 in cutaneous T-cell lymphomas (CTCL) increases the cytotoxicity of enzastaurin [42]. Thus, AKT/SGK dependent regulation of GSK3 may impact on the survival and function of several cell types participating in the inflammatory response. Those earlier studies did not define the cellular mechanisms involved in altered lymphocyte survival and function.

In conclusion, in cytotoxic T lymphocytes AKT/SGK dependent inhibition of GSK3α,β fosters the activation induced lymphocyte death and decreases perforin expression, effects prone to counteract inflammation and autoimmune response.

## 5. Disclosure

The authors of this manuscript state that they have neither financial nor any other conflicts of interests.

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