

## Defective Survival of Naive CD8<sup>+</sup> T Lymphocytes in the absence of $\beta 3$ regulatory subunit of Ca<sub>v</sub> channels

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### Abstract

Survival of T lymphocytes requires sustained Ca<sup>2+</sup> influx-dependent gene expression. The molecular mechanism, which governs sustained Ca<sup>2+</sup> influx in naive T lymphocytes, is unknown. Here we report an essential role for the  $\beta 3$  regulatory subunit of Ca<sub>v</sub> channels in the maintenance of naive CD8<sup>+</sup> T cells.  $\beta 3$  deficiency resulted in a profound survival defect of CD8<sup>+</sup> T cells. This defect correlated with depletion of the pore-forming subunit Ca<sub>v</sub>1.4 and attenuation of T cell receptor-mediated global Ca<sup>2+</sup> entry in the absence of  $\beta 3$  in CD8<sup>+</sup> T cells. Ca<sub>v</sub>1.4 and  $\beta 3$  associated with T cell signaling machinery and Ca<sub>v</sub>1.4 localized in lipid rafts. Our data demonstrate a mechanism by which Ca<sup>2+</sup> entry is controlled by a Ca<sub>v</sub>1.4- $\beta 3$  channel complex in T cells.

### Keywords

Calcium; Cacnb3; T lymphocyte; Ca<sub>v</sub> channel; Apoptosis

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Calcium (Ca<sup>2+</sup>) signaling plays a pivotal role in adaptive immune responses. Sustained Ca<sup>2+</sup> influx across the plasma membrane of T cell leads to the activation of NFAT transcription factors. NFATs regulate survival, activation, proliferation and effector functions of T

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Author contributions

M.K.J. conceptualized, designed and did all experiments except calcium imaging and wrote the paper; A.B. performed & analyzed calcium-imaging experiments; A.B., M.M., J.E.M., M.F., and V.F. contributed reagents; R.A.F. provided overall directions, supervised the project and wrote the paper.

lymphocytes<sup>1-4</sup>. Despite the established role of  $\text{Ca}^{2+}$  in T lymphocyte development and functions, the mechanism of sustained  $\text{Ca}^{2+}$  entry in naive T lymphocytes remains elusive to date.

A well-characterized mode of  $\text{Ca}^{2+}$  entry in T cells is the CRAC (Calcium Release Activated  $\text{Ca}^{2+}$ ) pathway, in which, two key players, STIM1 and ORAI1 (also known as CRACM1), have been described<sup>5-7</sup>. STIM1 functions as an intracellular calcium store sensor, which activates the CRAC pore subunit ORAI1 upon release of  $\text{Ca}^{2+}$  from intracellular stores after T cell receptor (TCR) crosslinking<sup>8</sup>. The majority of studies implicated ORAI as being crucial for T cell development and functions<sup>9</sup>. Yet, ORAI deletion had no significant impact on  $\text{Ca}^{2+}$  influx in naive T cells and proliferation of naive T cells upon stimulation<sup>10,11</sup>. These intriguing observations suggest complexity of the  $\text{Ca}^{2+}$  response in primary T cells and indicate a potential role of more than one type of plasma membrane calcium channels.

$\text{Ca}_v$  channels conduct  $\text{Ca}^{2+}$  in a variety of cell types most notably characterized in excitable cells.  $\text{Ca}_v$  channel complexes consist of the pore-forming  $\alpha_1$  subunit, in addition to the  $\alpha_2$ ,  $\delta$ ,  $\gamma$  and  $\beta$  subunits.  $\beta$  subunits are cytoplasmic proteins that strongly regulate  $\text{Ca}_v$  channels through direct interaction with the pore-forming  $\alpha_1$  subunits and are required for the assembly of the channel complex<sup>12</sup>, correct plasma membrane targeting<sup>13-15</sup> and stimulation of the channel activity<sup>16</sup>.  $\text{Ca}_v$  channels are now known to be present in many cells not traditionally considered excitable, such as cells of the immune system<sup>17-20</sup>. We previously showed that  $\text{CD4}^+$  T cells, a major arm of the adaptive immune system, express  $\text{Ca}_v1$  family members and that functional  $\beta_3$  and  $\beta_4$  regulatory subunits are necessary for normal TCR-triggered  $\text{Ca}^{2+}$  responses, NFAT nuclear translocation and cytokine production<sup>19</sup>. Recently we have also shown that deficiency in AHNAK1, a scaffold protein, resulted in reduced interleukin 2 (IL-2) production, reduced proliferation of  $\text{CD4}^+$  T cells<sup>21</sup> and defective cytotoxic T lymphocyte (CTL) responses<sup>22</sup>, which were likely due to the reduced  $\text{Ca}_v1.1$  [<http://www.signaling-gateway.org/molecule/query?afcsid=A000443>] expression and therefore attenuated  $\text{Ca}^{2+}$  signaling. These studies provide convincing evidence for a biological significance of  $\text{Ca}_v$  channels in T cell biology.

In the present study, we investigated the role of  $\text{Ca}_v$  channels in  $\text{Ca}^{2+}$  entry in  $\text{CD8}^+$  T cells. Although CRAC channels have been implicated to play a role in the entry of extracellular  $\text{Ca}^{2+}$  in effector  $\text{CD8}^+$  T cell clones<sup>23</sup>, nothing is known about the mechanism of  $\text{Ca}^{2+}$  entry in TCR-stimulated naive  $\text{CD8}^+$  T cells. Herein, we report an interesting expression pattern of  $\text{Ca}_v1$  family proteins in  $\text{CD8}^+$  T cells and document genetic evidence for a novel role of  $\beta_3$  regulatory protein in the survival of  $\text{CD8}^+$  T cells *in vitro* and *in vivo*.

## Results

### Expression of $\text{Ca}_v$ channel components in $\text{CD8}^+$ T cells

As a first step to analyzing the role of  $\text{Ca}_v$  channels in  $\text{CD8}^+$  T cell functions, we examined the expression pattern of pore-forming  $\alpha_1$  subunits of  $\text{Ca}_v1$  family members ( $\text{Ca}_v1.1$ - $\text{Ca}_v1.4$ ) [<http://www.signaling-gateway.org/molecule/query?afcsid=A000440>; <http://www.signaling-gateway.org/molecule/query?afcsid=A000441>; <http://www.signaling-gateway.org/molecule/query?afcsid=A000442>].

[gateway.org/molecule/query?afcsid=A000442](http://gateway.org/molecule/query?afcsid=A000442)] and their regulatory  $\beta$  subunits ( $\beta 1$ - $\beta 4$ ) in both naive and activated CD8<sup>+</sup> T cells. We designed intron-spanning PCR primer pairs for each gene and obtained the expected size of PCR products. Interestingly, expression of Ca<sub>v</sub>1 family members in naive and effector CD8<sup>+</sup> T cells was temporally regulated (Fig. 1a). *Cacna1s* mRNA was expressed only in effector but not in naive CD8<sup>+</sup> T cells (Fig. 1a). In contrast to *Cacna1s* mRNA abundance, *Cacna1f* mRNA was highly expressed in naive CD8<sup>+</sup> T cells and substantially downregulated in effector CD8<sup>+</sup> T cells (Fig. 1a). We did not observe such temporal regulation of *Cacna1c* and *Cacna1d* mRNA expression (Fig. 1a). We further confirmed the reciprocal expression of Ca<sub>v</sub>1.1 (encoded by *Cacna1s*) and Ca<sub>v</sub>1.4 (encoded by *Cacna1f*) channel protein in wild-type CD8<sup>+</sup> T cells. In agreement with the mRNA data (Fig. 1a), while expression of Ca<sub>v</sub>1.4 was detected only in naive but not activated CD8<sup>+</sup> T cells (Fig. 1b), expression of Ca<sub>v</sub>1.1 was detected only in activated but not in naive CD8<sup>+</sup> T cells (Fig. 1b). Ca<sub>v</sub>1.1 protein expression appeared only on day 4 upon primary stimulation (Supplementary Fig. 1). However, in contrast to the mRNA data, we could not detect Ca<sub>v</sub>1.2 protein (encoded by *Cacna1c*) in naive CD8<sup>+</sup> T cells<sup>22</sup>.

We also examined the expression pattern of  $\beta$  regulatory subunits in CD8<sup>+</sup> T cells. Among all  $\beta$  subunits, only  $\beta 3$  subunit mRNA and protein, encoded by *Cacnb3*, was constitutively and predominantly expressed in naive as well as in effector CD8<sup>+</sup> T cells (Fig. 1c,d). Other  $\beta$  subunits such as  $\beta 1$  (encoded by *Cacnb1*) and  $\beta 2$  (encoded by *Cacnb2*) were either expressed in minimal amounts or undetected such as  $\beta 4$  (encoded by *Cacnb4*) (Fig. 1c). The comparatively high expression of  $\beta 3$  protein in naive and activated CD8<sup>+</sup> T cells led us to hypothesize a potential role for  $\beta 3$  in the CD8<sup>+</sup> T cell biology. To investigate the function(s) of  $\beta 3$  in CD8<sup>+</sup> T cell survival and proliferation, we analyzed *Cacnb3*-deficient mice<sup>24</sup>.

### T cell compartment in *Cacnb3*-deficient mice

We showed previously that T lymphocytes developed normally in the thymus in the absence of the  $\beta 3$  subunit<sup>19</sup>. To identify the physiological roles of  $\beta 3$  in T cells, we analyzed peripheral lymphocytes isolated from *Cacnb3*<sup>+/+</sup> and *Cacnb3*<sup>-/-</sup> mice. *Cacnb3*<sup>-/-</sup> mice exhibited normal number of CD4<sup>+</sup> T cell populations in the periphery (Fig. 2a,b), however, we observed a reduction in the total number of CD8<sup>+</sup> T cells in *Cacnb3*<sup>-/-</sup> mice (Fig. 2b). Although a reduction of the fraction of CD8<sup>+</sup> T cells was not observed consistently (Fig. 2a), the total number of CD8<sup>+</sup> T cells was substantially reduced (Fig. 2b). These observations indicated a perturbation in CD8<sup>+</sup> T cell homeostasis in *Cacnb3*<sup>-/-</sup> mice.

To examine the CD8<sup>+</sup> T cell population in more detail, we determined the activation status of CD8<sup>+</sup> T cells. Compared to control CD8<sup>+</sup> T cells, a severe reduction in both the proportion and absolute numbers of splenic naive (CD44<sup>lo</sup>CD62L<sup>hi</sup>) CD8<sup>+</sup> T cells was observed consistently in *Cacnb3*<sup>-/-</sup> mice (Fig. 2c,d). Similarly, reduction in absolute numbers, but not frequencies, of naive CD8<sup>+</sup> T cells was also observed in peripheral LNs of *Cacnb3*<sup>-/-</sup> mice (Fig. 2c,d). Furthermore, a higher percentage of *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells exhibited a memory like phenotype (CD44<sup>hi</sup>) in spleen (Fig. 2c). On the other hand, the frequency and absolute number of naive CD4<sup>+</sup> T were normal in LNs and spleen of *Cacnb3*<sup>-/-</sup> mice (Fig. 2e and Supplementary Fig. 2). These data indicate a specific defect in the CD8<sup>+</sup> T cells compartment *in vivo* in *Cacnb3*<sup>-/-</sup> mice.

### Ca<sup>2+</sup> influx in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells

The mechanism of Ca<sup>2+</sup> entry in TCR stimulated naive CD8<sup>+</sup> T cells is not known. Since  $\beta$ 3 deficiency resulted in reduced Ca<sup>2+</sup> entry into naive CD4<sup>+</sup> T cells after TCR crosslinking19, we examined if this is also the case in naive *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells. The Ca<sup>2+</sup> response to TCR cross-linking was markedly reduced in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells compared to wild-type cells (Fig. 3a). Both the initial peak and the plateau were lower in the mutant cells as compared to wild-type cells. The ionomycin-induced calcium response in wild-type and *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells was similar, suggesting that the reduced Ca<sup>2+</sup> entry was not due to CRAC deficiency (Fig. 3b).

To investigate if reduced Ca<sup>2+</sup> entry in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells affected NFAT translocation and thereby activation, we examined NFATc1 abundance in cytosolic and nuclear extracts prepared from wild-type and *Cacnb3*<sup>-/-</sup> naive CD8<sup>+</sup> T cells stimulated by anti-TCR plus anti-CD28 for 12 h. As expected from the decrease in global Ca<sup>2+</sup> concentrations in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells (Fig. 3a), the nuclear translocation of three isoforms of NFATc1 was reduced in stimulated CD8<sup>+</sup> T cells in the absence of  $\beta$ 3 subunit (Supplementary Fig. 3). Although nuclear translocation of all three isoforms was reduced, NFATc1 activation (as measured by the faster electrophoretic migration by dephosphorylated NFATs) appeared normal in the absence of  $\beta$ 3 (Supplementary Fig. 3). The cytoplasmic concentration of NFATc1 was also remarkably reduced in the stimulated *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells (Supplementary Fig. 3). Therefore, we examined the basal expression of NFATc1 in unstimulated wild-type or *Cacnb3*<sup>-/-</sup> naive CD8<sup>+</sup> T cells. Whole cell lysates were made from equal number of live cells and assessed for the expression of NFATc1 and another survival factor, the kinase Akt. Less NFATc1 protein, but not Akt, was observed in *Cacnb3*<sup>-/-</sup> naive CD8<sup>+</sup> T cells (Fig. 3c). To investigate if reduction in NFATc1 protein was due to altered gene expression, we measured the NFATc1 transcript abundance in wild-type or *Cacnb3*<sup>-/-</sup> naive CD8<sup>+</sup> T cells by real-time assay. *Nfatc1* expression was much less in *Cacnb3*<sup>-/-</sup> naive CD8<sup>+</sup> T cells as compared to wild-type cells (Fig. 3d). These data clearly demonstrate that  $\beta$ 3-dependent Ca<sup>2+</sup> entry is required for the steady-state transcriptional regulation of NFATc1 in naive CD8<sup>+</sup> T cells.

### Physiological significance of $\beta$ 3 in CD8<sup>+</sup> T cells

Given that the Ca<sup>2+</sup>-NFAT pathway is required for the T cell activation and proliferation, we determined whether reduced Ca<sup>2+</sup> entry in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells affected antigen induced proliferation *in vitro*. Sorted naive CD8<sup>+</sup> T cells were labeled with CFSE (carboxyfluorescein succinimidyl ester) dye and cell cycle progression was assessed after 3 days of TCR plus CD28 stimulation in the presence of exogenous murine IL-2. Whereas wild-type cells underwent 4-5 cell divisions, a high number of *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells failed to divide (Fig. 4a). The forward and side scatter pattern of *Cacnb3*<sup>-/-</sup> cells showed remarkably a higher percentage of dead cells on day 3 (Fig. 4b) and cell recovery was also extremely low on d3 (data not shown). These observations led us to determine if *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells underwent activation-induced cell death (AICD).

To quantify AICD, we activated naive CD8<sup>+</sup> T cells from WT and *Cacnb3*<sup>-/-</sup> mice by TCR plus CD28 stimulation for 48 h followed by staining with annexin and propidium iodide

(PI). Annexin-PI staining revealed a very high incidence of AICD in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells compared to wild-type controls (Fig. 4c). *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells in spleen, but not in thymus, exhibited enhanced annexin V positive (Supplementary Fig. 4). We also assessed the kinetics of AICD in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells. Purified naive wild-type and *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells were stained for annexin-PI at 0 h (non-stimulated), or at 12 h or 48 h post-activation. Approximately 40% of naive non-stimulated *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells were already annexin-PI positive compared to 4-5% wild-type cells (Fig. 4d), suggesting that these cells were undergoing spontaneous apoptosis *in vivo* which was further increased upon TCR plus CD28 stimulation *ex vivo*.

To study the longevity of naive T cells *in vivo*, wild-type and *Cacnb3*<sup>-/-</sup> mice were thymectomized just after weaning at 3 weeks of age and the survival of the naive T cell pool was determined. Because thymectomy prevents the addition of new naive T cells to the existing pool, a fixed population of naive T cells was created *in vivo*. We examined spleen and LNs for naive and memory T cells after 6-8 weeks of thymectomy. We observed a substantial reduction in the proportion and absolute numbers of naive CD8<sup>+</sup> T cells in all peripheral lymphoid organs (Fig. 4e,f). On the other hand, although the proportion of central memory CD8<sup>+</sup> T cells (as characterized by CD62L<sup>hi</sup>CD44<sup>hi</sup>) was significantly higher in *Cacnb3*<sup>-/-</sup> mice (Fig. 4e), the total number of memory CD8<sup>+</sup> T cell in spleen was reduced but not LNs (Fig. 4f). The frequency and total numbers of naive CD4<sup>+</sup> T cells were also reduced in spleen and LNs of *Cacnb3*<sup>-/-</sup> mice compared to wild-type mice (Fig. 4e,f). These data clearly establish a critical requirement of  $\beta 3$  in the *in vivo* survival of T cell in general and naive CD8<sup>+</sup> T cells in particular.

### Defective homeostasis of *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells

Since we found that  $\beta 3$  supports the survival of naive CD8<sup>+</sup> T cells, we next investigated the potential reason for the survival defect in the absence of  $\beta 3$ . The survival of mature naive T cells in the peripheral environment requires continuous TCR contact with self-MHC ligands and  $\gamma_c$  family of cytokines such as IL-7 and IL-15 (ref. 25). It has been proposed that T cells constantly compete for the limited amounts of IL-7 available in the periphery and that this competition is influenced by downregulation of expression of the IL-7 receptor (IL-7R) after IL-7 signaling<sup>26</sup>. Therefore we investigated expression of IL-7R and IL-15R in wild-type or *Cacnb3*<sup>-/-</sup> naive CD8<sup>+</sup> T cells. No significant change in expression of either receptor was observed in the absence of  $\beta 3$  in naive CD8<sup>+</sup> T cells (Fig. 5a,b). These data suggested that  $\beta 3$  deficiency in naive CD8<sup>+</sup> T cells did not impair the survival signals generated by  $\gamma_c$  family of cytokines.

To investigate whether defective survival of *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells is cell intrinsic or due to other extrinsic factors, we performed homeostasis experiments. Homeostatic proliferation of naive T cells is most easily studied by transferring small numbers of these cells into T cell-deficient mice such as *Rag1*<sup>-/-</sup>, SCID or nude mice. For these experiments, we backcrossed *Cacnb3*<sup>-/-</sup> mice for eight generations onto congenic C57BL/6 mice and used age- and sex-matched wild-type (CD45.1.1) and *Cacnb3*<sup>-/-</sup> (CD45.2.2) mice for experiments. We transferred a mixture of *Cacnb3*<sup>-/-</sup> and wild-type sorted naive CD8<sup>+</sup> cells into CD45.1.1 *Rag1*<sup>-/-</sup> mice as recipients (Fig. 5c). One week after transfer, whereas wild-

type donor cells survived and expanded their populations extensively to constitute 2-3% of the host cells, we detected 1% *Cacnb3*<sup>-/-</sup>-deficient donor cells (Fig. 5d), even though we transferred more *Cacnb3*<sup>-/-</sup> cells compared to wild-type at the beginning (Fig. 5c). After 4–6 weeks of transfer, we recovered 10–40% of wild-type cells but substantially reduced *Cacnb3*<sup>-/-</sup> cells (Fig. 5d). The disappearance of *Cacnb3*<sup>-/-</sup> cells is thus due to their defective long-term survival.

### Fas-mediated apoptosis of *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells

Numerous studies have established the anti-apoptotic function of NFAT factors in immune system<sup>27-29</sup>. We therefore examined the pro- and anti-apoptotic gene expression in wild-type or *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells. First, we determined Fas expression in *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells. Notably, *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells expressed a very high level of Fas protein compared to that of wild-type (Fig. 6a) and not CD43 or FasL (Supplementary Fig. 5). Similarly, Fas transcript abundance was increased by 2-3 fold in *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells compared to the wild-type control (Fig. 6b), consistent with the earlier report describing the requirement of Ca<sup>2+</sup> in repressing Fas gene expression<sup>30</sup>. Finally, we determined if β3 deficiency affected the expression of anti-apoptotic genes and found expression of Bcl-2, Bcl-x<sub>L</sub> and FLIP mRNAs were down-regulated in *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells (Fig. 6b).

To rescue this loss of CD8<sup>+</sup> T cells *in vivo*, we crossed *Cacnb3*<sup>-/-</sup> mice with *Fas*<sup>-/-</sup> mice. The proportion and numbers of *Cacnb3*<sup>-/-</sup> *Fas*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells was restored to that of wild-type mice (Fig. 6c,d). These data collectively suggest that spontaneous apoptosis occurring in *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells was Fas-mediated.

### Defective CD8<sup>+</sup> T cell responses in *Cacnb3*<sup>-/-</sup> mice

Since the β3 subunit was abundantly expressed in activated CD8<sup>+</sup> T cells (Fig. 1c,d), we analyzed its role in effector CD8<sup>+</sup> T cell functions. Because *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells were unable to proliferate *in vitro* (Fig. 4a), we used a model system previously described to test CD8<sup>+</sup> T cell effector function *in vivo*<sup>31</sup>. For this purpose, wild-type dendritic cells (DCs) primed with SIINFEKL peptide were transferred to both wild-type and *Cacnb3*<sup>-/-</sup> mice. We examined the ability of antigen-specific CD8<sup>+</sup> T cells to produce several effector molecules including interferon-γ (IFN-γ), IL-2, tumor necrosis factor (TNF) and granzyme B on day 7 after immunization. *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells showed a significant deficiency in IFN-γ and IL-2 production (Fig. 7a,b). A similar deficiency was also observed for TNF and granzyme B production by antigen specific *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells (Fig. 7c,d). These data also suggest that the deficiency in effector molecules production by *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells was not due to defective antigen presentation, since we primed *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells with wild-type DCs.

### Ca<sub>v</sub>1.4 is depleted in *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells

β subunits regulate expression of Ca<sub>v</sub>α1 subunits by masking their endoplasmic reticulum retention signals<sup>13</sup>. Since Ca<sub>v</sub>1.4 was found to be expressed in naïve CD8<sup>+</sup> T cells (Fig. 1a&b), we analyzed its expression in naïve CD8<sup>+</sup> T cells purified from wild-type and *Cacnb3*<sup>-/-</sup> mice. A complete loss of Ca<sub>v</sub>1.4 protein (Fig. 8a), but not mRNA (Fig. 8b), was

detected in the *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> cells. Instead, an increase in *Cacna1f* mRNA abundance was observed in the absence of  $\beta 3$  (Fig. 8b), suggesting a compensatory increase in *Cacna1f* mRNA expression. Based on these data, we concluded that  $\beta 3$  was not needed for the upregulation of channel transcription but rather involved in post-transcriptional events.

The  $\beta$ -subunit belongs to the membrane-associated guanylate kinase class of scaffolding proteins (MAGUK) and contains a conserved Src homology 3 (SH3) domain and a conserved guanylate kinase (GK) domain<sup>32</sup> (Supplementary Fig. 6). Interaction between alpha interaction domain (AID) in the  $\alpha 1$  subunit and GK domain is necessary for  $\beta$  subunit-stimulated Ca<sub>v</sub> channel surface expression<sup>33</sup>. Depletion of Ca<sub>v</sub>1.4 in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells (Fig. 8a) indicated a potential interaction between Ca<sub>v</sub>1.4 and  $\beta 3$ . To examine this idea, we co-immunoprecipitated  $\beta 3$ -interacting proteins from wild-type CD8<sup>+</sup> T lymphocytes and immunoblotted with antibody against Ca<sub>v</sub>1.4. We detected Ca<sub>v</sub>1.4 only in those samples immunoprecipitated by antibody against  $\beta 3$  and not in samples immunoprecipitated by control isotype antibody (Fig. 8c). Collectively, these data demonstrate that Ca<sub>v</sub>1.4 and  $\beta 3$  form a stable Ca<sub>v</sub>1.4 calcium channel complex in these T cells and suggests that the absence of  $\beta 3$  destabilizes and leads to degradation of this channel complex without affecting *Cacna1f* gene expression.

### **$\beta 3$ and Ca<sub>v</sub>1.4 interact with T cell signaling proteins**

Many downstream TCR signaling proteins, such as Lck, Vav, Zap-70, Grb2, and Nck contain SH2 and SH3 motifs that are known to facilitate the assembly of multi-protein signaling complex. Similarly, SH3 and GK domains in  $\beta$  subunits highlight a multiplicity of potential protein partners. To investigate whether  $\beta 3$  interacts with such proteins and participate in multi-protein T cell signaling complex,  $\beta 3$ -interacting proteins were immunoprecipitated from wild-type or *Cacnb3*<sup>-/-</sup> primary CD8<sup>+</sup> T lymphocytes using antibody against  $\beta 3$  and immunoblotted with antibodies against known key T cell signaling kinases and adaptors. Interestingly,  $\beta 3$  antibody recovered Lck, Vav, Zap70, Nck, and Grb2 only in wild-type but not in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells (Fig. 9a).

We performed coimmunoprecipitation experiments to determine whether Ca<sub>v</sub>1.4 channel is directly present in the TCR signaling complex. Ca<sub>v</sub>1.4-interacting proteins were immunoprecipitated from wild-type CD8<sup>+</sup> T lymphocytes and immunoblotted with antibodies against known key T cell signaling kinases and adaptors. Lck and Vav were co-immunoprecipitated by a monoclonal antibody against Ca<sub>v</sub>1.4 (Fig. 9b). Overall, these data indicate a possible synergy of crosstalk between Ca<sub>v</sub> channels and the T cell signaling machinery in a cellular context where interaction of these molecules is required for rapid Ca<sup>2+</sup> influx once TCR is stimulated either in an antigen-dependent or -independent manner.

### **Ca<sub>v</sub>1.4 localizes in lipid rafts of CD8<sup>+</sup> T cells**

Recently, Ca<sub>v</sub>1.4 channel protein has been shown to interact with filamin proteins in immune cells<sup>34</sup>. Filamin is expressed in T lymphocytes and participates in T cell activation<sup>35</sup>. Interestingly, CD28 associated with filamin which is required for CD28-induced lipid raft recruitment into the immunological synapse<sup>36</sup>. These lipid rafts concentrate glycosphosphatidylinositol-linked proteins, glycosphingolipids, as well as several

molecules involved in signal transduction such as Lck, LAT, Ras, and G proteins and thereby lipid rafts provide a signaling platform<sup>37</sup>.

Our finding that Ca<sub>v</sub>1.4 was in a complex with raft resident proteins, Lck and Vav (Fig. 9b) coupled with the reported interaction of Ca<sub>v</sub>1.4 with filamins<sup>34</sup> led us to speculate that Ca<sub>v</sub>1.4 might be localized in lipid rafts to facilitate the TCR-mediated sustained calcium influx. To investigate this possibility, lipid rafts were purified from CD8<sup>+</sup> T cells of wild-type mice and were analyzed by immunoblot for the presence of Ca<sub>v</sub>1.4 and known raft resident signaling proteins such as Lck and Vav. Interestingly, Ca<sub>v</sub>1.4 along with Lck and Vav were found in lipid raft fractions (Fig. 9c). As a control for the purity of lipid raft fractions, we analyzed the presence of ganglioside GM1, which is a commonly used raft marker. GM1 staining was only positive in detergent-resistant lipid raft fractions (3–5) and not in detergent-soluble cytosolic fractions (8–11) suggesting the clear separation of raft from non-raft fractions (Fig. 9c). These data identify Ca<sub>v</sub>1.4 as a potential calcium channel in lipid microdomains of T cells.

## Discussion

In this study, we have examined the biological significance of Ca<sub>v</sub>1 channels in CD8<sup>+</sup> T cells. Surprisingly, we found Ca<sub>v</sub>1.4, but not Ca<sub>v</sub>1.1, is highly expressed in naïve CD8<sup>+</sup> T cells whereas Ca<sub>v</sub>1.1, not Ca<sub>v</sub>1.4 (or Ca<sub>v</sub>1.2), is highly expressed in activated CD8<sup>+</sup> T cells. We also observed a significant and robust expression of only β3 subunit at all developmental stages of peripheral CD8<sup>+</sup> T cells. *Cacnb3*<sup>-/-</sup> mice have a highly reduced pool of naïve CD8<sup>+</sup> T cells due to spontaneous apoptosis *in vivo*. In the absence of thymic output, survival of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells was affected *in vivo*. Increased expression of Fas caused spontaneous apoptosis since *Fas* deficiency rescued loss of CD8<sup>+</sup> T cells in *Cacnb3*<sup>-/-</sup> mice. This observation supports an elegant prior study which showed that CD8<sup>+</sup> T cells die via a Fas-dependent pathway in the absence of a survival signal provided by engagement of host MHC-self peptide complexes<sup>38</sup>.

Compared to wild-type, *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells have attenuated Ca<sup>2+</sup> entry upon TCR crosslinking. Although our data lack a measurement of basal Ca<sup>2+</sup> levels in unstimulated cells due to technical limitations, we found a severe reduction in basal NFATc1 levels in naïve *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells (in the absence of any TCR stimulation). This suggested a reduction in NFAT-dependent *NFATc1* gene expression since the *NFATc1* gene has functional binding sites for NFATs and an autoregulatory role of NFAT factors has been described in murine T cells<sup>39</sup>.

We found that β3 associates with Ca<sub>v</sub>1.4 subunit and β3 deficiency completely depletes the cellular levels of pore forming Ca<sub>v</sub>1.4 subunit, suggesting that β3 is required for a functional and stable Ca<sub>v</sub>1.4 channel in T cells probably by protecting Ca<sub>v</sub>1.4 from degradation. Interestingly, we found that β3 and Ca<sub>v</sub>1.4 are associated with a T cell signaling complex in primary CD8<sup>+</sup> T cells, which was not dependent on TCR-stimulation suggesting that a preformed complex of these proteins exists in naïve T cells. Furthermore, we identified a fraction of Ca<sub>v</sub>1.4 as a lipid raft resident calcium channel protein. Combining the previously reported interaction of Ca<sub>v</sub>1.4 with filamins in spleen cells<sup>34</sup> with our finding of its



association with Lck and Vav highlight a  $Ca_v$  channel dependent molecular architecture of signaling complex in specialized microdomains of T cells. These observations further gain significance in light of a widely accepted model that the specificity, reliability and accurate execution of many of these processes depend on tightly regulated spatiotemporal  $Ca^{2+}$  signals restricted to precise microdomains that contain  $Ca^{2+}$ -permeable channels and their modulators<sup>40,41</sup>.

It has been suggested that  $Ca_v1.4$  is a unique channel which supports only minute amounts of  $Ca^{2+}$  entry<sup>42</sup>. Low intensity of calcium influx has been shown to regulate survival of naïve T cells in the absence of antigen<sup>43</sup>. Based on these observations we believe that a  $Ca_v1.4$ - $\beta3$  complex in naïve  $CD8^+$  T cells is involved in antigen-independent, MHC-triggered  $Ca^{2+}$  response which generates tonic signaling for the survival of these cells. Tonic signaling might be required to express a threshold level of important transcription factors like NFATs which, in turn, repress the expression of pro-apoptotic genes such as Fas and also maintain a threshold expression levels of anti-apoptotic genes such as Bcl-2/ $x_L$  and cFLIP. Our observation that the  $Ca_v1.4/\beta3$  complex regulates  $Ca^{2+}$  influx required for the survival of naïve  $CD8^+$  T lymphocytes has fundamental importance in understanding the regulatory mechanisms of calcium signaling in primary T cells. Since  $Ca_v$  channels are clinically well-established targets in many pathological conditions, this study identifies a  $\beta3/Ca_v1.4$  dependent pathway in  $CD8^+$  T cells, which may be used to design therapeutic agents to inhibit aggressive  $CD8^+$  T cells in autoimmune and lymphoproliferative diseases.

## Methods

### Mice

*Cacnb3*<sup>-/-</sup> mice were as previously described<sup>24</sup>. Mice within experiments were age (5–6 weeks in all experiments) and sex-matched. Bulk of experiments was done either on 129svj or the mixed background (2 generations backcrossed with C57BL/6) mice. Mice were kept in accordance with institutional animal care and used according to committee-approved protocols at the Yale University School of Medicine animal facility.

### T cell purification, culture and flow cytometry

Lymphocytes were isolated from 5–6- week-old mice. Peripheral  $CD8^+$  T cells were purified with CD8-coupled beads (Miltenyi Biotec) and were sorted for naïve T cell population (gated on CD8, CD62L<sup>hi</sup> and CD44<sup>lo</sup>) on a VantageSE station (Becton Dickinson). Unless otherwise stated, all experiments were done on sorted naïve  $CD8^+$  T cells. T cells were cultured at 37°C with 5% CO<sub>2</sub> in Bruff's medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin and 25 U/ml of IL-2.

### RNA isolation and semiquantitative RT-PCR

Total RNA from cells was isolated with Trizol reagent and subjected to reverse transcriptase with Superscript II (Invitrogen) according to the manufacturer's protocol. PCR was done with sense and antisense primers: *Cacnb1* (NM\_031173), 5'-GAAAGGGCGGTTCAAAGGTC-3' (sense) and 5'-TTGGGCTCAAAGGTGATGGC-3' (antisense); *Cacnb2* (NM\_023116.3), 5'-CGGACCAATGTCAGATACAGCG-3' (sense)

and 5'-TGGAAGTGGGCACTATGTCACC-3' (antisense); *Cacnb3* (NM\_001044741), 5'-AACCTGTGGCATTGCTGTGAG-3' (sense) and 5'-CCTGCTTTTGCTTCTGCTTGG-3' (antisense). RT-PCR primers for *Hprt* (hypoxanthine phosphoribosyltransferase), *Cacnb4* and  $Ca_v\alpha1$  subunits were described previously<sup>19</sup>. Real-time PCR analysis of *Cacna1f*, *NFATc1*, *Fas*, *Bcl-2*, *Bcl-xL* was performed by using commercially available primers and probe from Applied Biosystems. Cycling conditions were 2 min at 94°C followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. *Hprt* was included as an internal control for all samples and quantified by  $C_t$  (change in cycle threshold) method.

### Flow cytometry

Cells were stained with fluorochrome-conjugated Abs in 1% FBS in Bruffs medium and subsequently fixed in 2% paraformaldehyde. The following Abs were used: CD8 $\alpha$  (53-6.7), TCR $\beta$  (H57-597), CD44 (clone IM7), CD62L (clone MEL-14), Fas (Jo2), and CD4 (RM4-5) (all Abs from BD Pharmingen). Cells were analyzed with a FACS Calibur (BD Biosciences) and data were analyzed by FlowJo v. 6.1 (TreeStar, Inc.). Staining with annexin V and propidium iodide (BD Pharmingen) or CFSE (Molecular Probes) were done according to the manufacturer's protocol.

### Antibodies

Following commercial antibodies were used: mouse monoclonal Ab to NFATc1 (Affinity Bioreagents), anti-actin goat polyclonal Ab, anti- $Ca_v1.1$  (Santa Cruz Biotechnology), anti-AKT (Cell signaling # 4685), pan-Cadherin (abcam # ab6528), anti-Grb2 (BD transduction #610111), anti-Nck (BD Transduction # 610099), anti-Vav (Upstate # 05-219), anti-Lck (Cell Signaling # 610097) and anti-Zap70 (Sigma # Z0627). The Ab to  $\beta3$  (Ab 828) was provided by V.F.44 and polyclonal anti- $Ca_v1.4$  was provided by J.M.45 and monoclonal anti- $Ca_v1.4$  was from Sigma.

### Protein extracts and immunoblot analysis

For immunoblot assays, equal number ( $3-5 \times 10^6$ ) of WT and KO cells were directly lysed in the LDS buffer (Invitrogen) and sonicated for 30 sec. Cell lysates were electrophoresed on 3-8% Bis-Tris (neutral pH) or 4-12% MOPS (neutral pH) NuPAGE gradient gel (Invitrogen), transferred to polyvinylidene difluoride membrane (Millipore), and immunoblotted with appropriate Abs.

### Analysis of intracellular calcium concentration

Concentrations of intracellular calcium were measured using the ratiometric calcium-binding dye Fura-2/AM as previously described<sup>19</sup>.

### Thymectomy

3-weeks-old (just after weaning) mice were anesthetized with a mixture of 100 mg/ml ketamine (Fort Dodge Laboratories) and 20 mg/ml xylazine (Abbott Laboratories) by IP (0.1 ml per 10 g body weigh just before surgery). Thymectomy was performed by aspiration of the thymus through an incision in the skin and muscle over the thymus in the suprasternal notch. The skin and body wall were sutured using soluble sutures and Vetbond. Successful

extraction of the thymus was verified upon sacrifice of the animals at the time of experiment.

### ***In vivo* priming of CD8<sup>+</sup> T cells**

BMDCs were generated *in vitro* in GM-CSF containing complete Bruffs medium for 7 days according to the method published previously<sup>31</sup>. On day 7, cells were washed and incubated overnight with H-2K<sup>b</sup> binding SIINFEKL peptide (OVA<sub>257-264</sub>) (10 µg/ml) and LPS (200 ng/ml). Next day, cells were washed three times with sterile PBS and  $1 \times 10^6$  cells were injected i.p. into WT and *Cacnb3*<sup>-/-</sup> mice. After 7 days spleens were harvested and splenocytes were *in vitro* stimulated with SIINFEKL peptide (10 µg/ml) for 6 h in the presence of Golgi stop (BD Biosciences, Cat # 554715). Cells were stained with CD8<sup>+</sup> then fixed and permeabilized for intracellular staining of IFN- $\gamma$ , IL-2, Granzyme B and TNF. Stained cells were analyzed by FACS calibur.

### **Coimmunoprecipitation**

CD8<sup>+</sup> T cells were resuspended in 500–1000 µl of cell lysis buffer (Cell signaling #9803) or RIPA buffer (Cell signaling #9806) containing protease- and phosphatase inhibitors (PMSF, leupeptin, NEM and cocktail tablets, Roche Diagnostics). Resulting extracts were pre-cleared by rocking with 1 ml of a 50% slurry of equilibrated Protein A/G beads (Santa Cruz Biotechnology) for 2 h at 4°C and removal of the precipitate. The resulting supernatant was then subjected to immunoprecipitation for overnight at 4°C followed by immunoblot assays.

### **Lipid raft isolation and characterization**

Lipid rafts were isolated from CD8<sup>+</sup> T cells as previously described<sup>46</sup>. Cells were lysed in 1 ml of 25 mM HEPES (pH 6.5), 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin (all from Sigma) and Roche protease inhibitor cocktail. The homogenate was overlaid with 5 - 40% step gradient of sucrose solution and subjected to ultracentrifugation at 200,000g for 20 h at 4°C. The floating opaque band corresponding to the detergent-resistant membrane fraction was found at the interface between the 30% and 5% sucrose gradients. Fractions were characterized for the raft purity by blotting 1 µl of each fraction onto Duralon-UV membranes (Stratagene), followed by blocking and immunoblotting with cholera toxin B subunit from *Vibrio cholerae* - peroxidase (Sigma # C3741).

### **Statistical analysis**

Statistical analyses were performed using Prism<sup>TM</sup>. *P* values were calculated with Student's *t*-test. *P* values of less than 0.05 were considered significant. Unless otherwise stated, numbers shown are mean±standard deviation.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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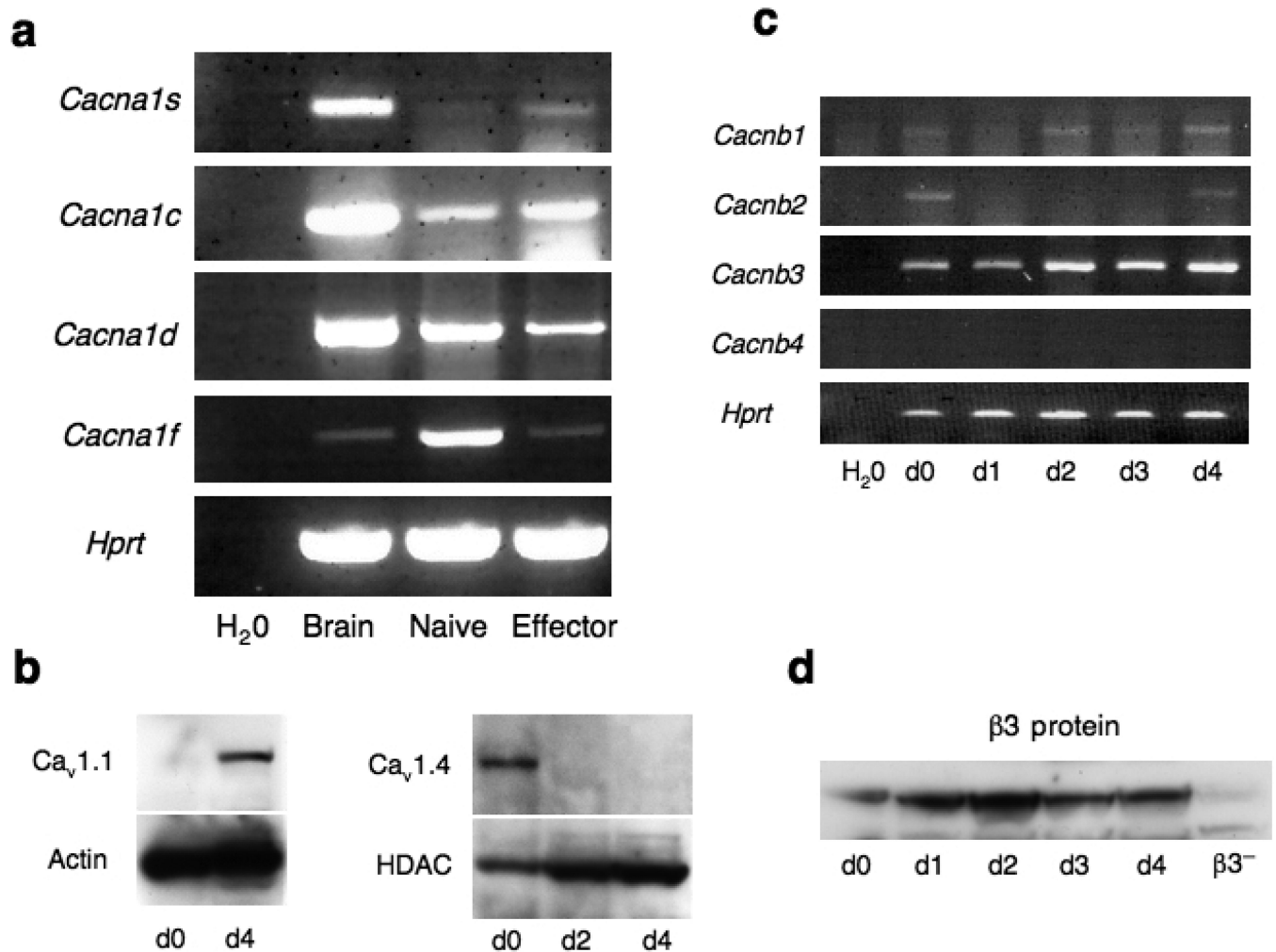
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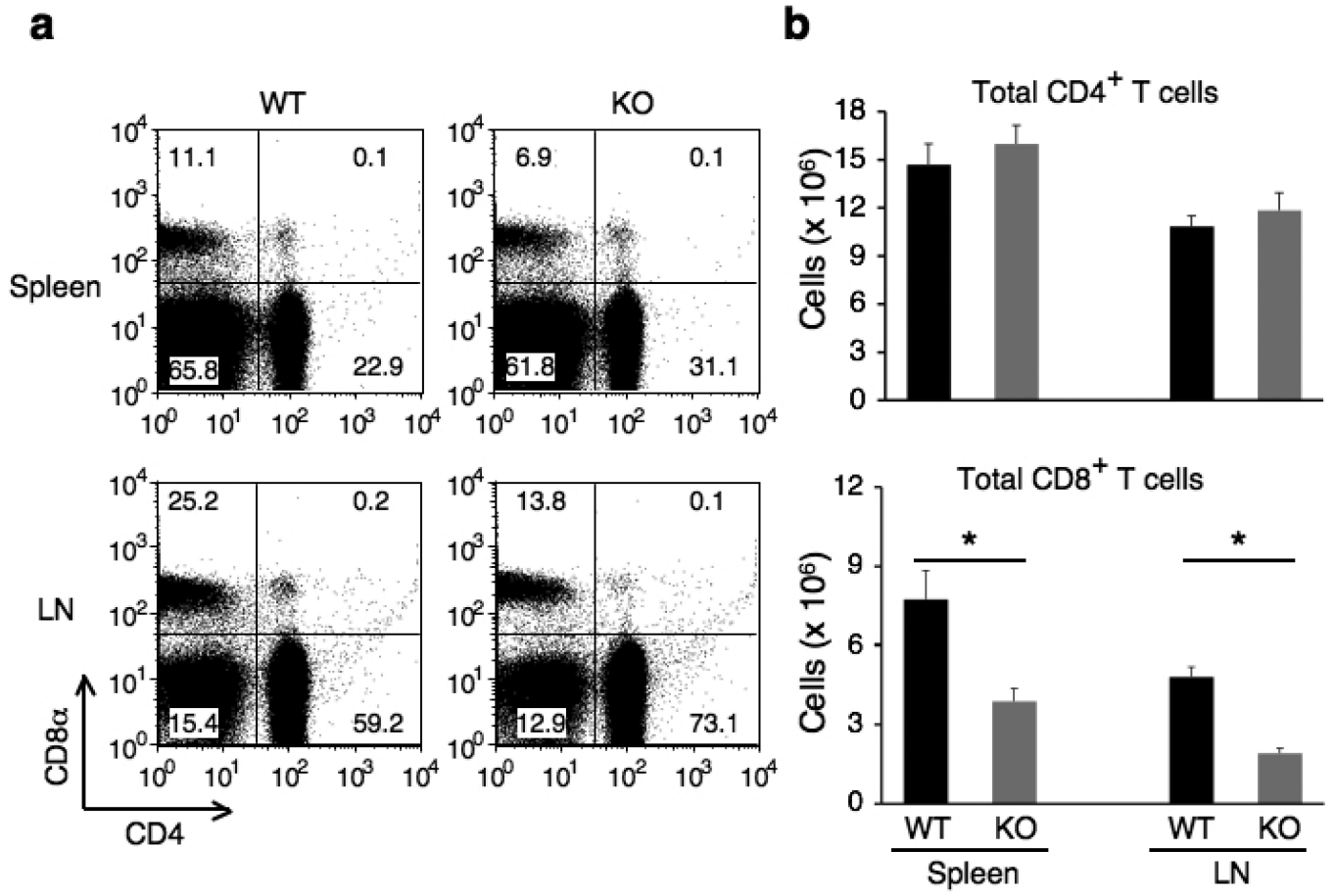
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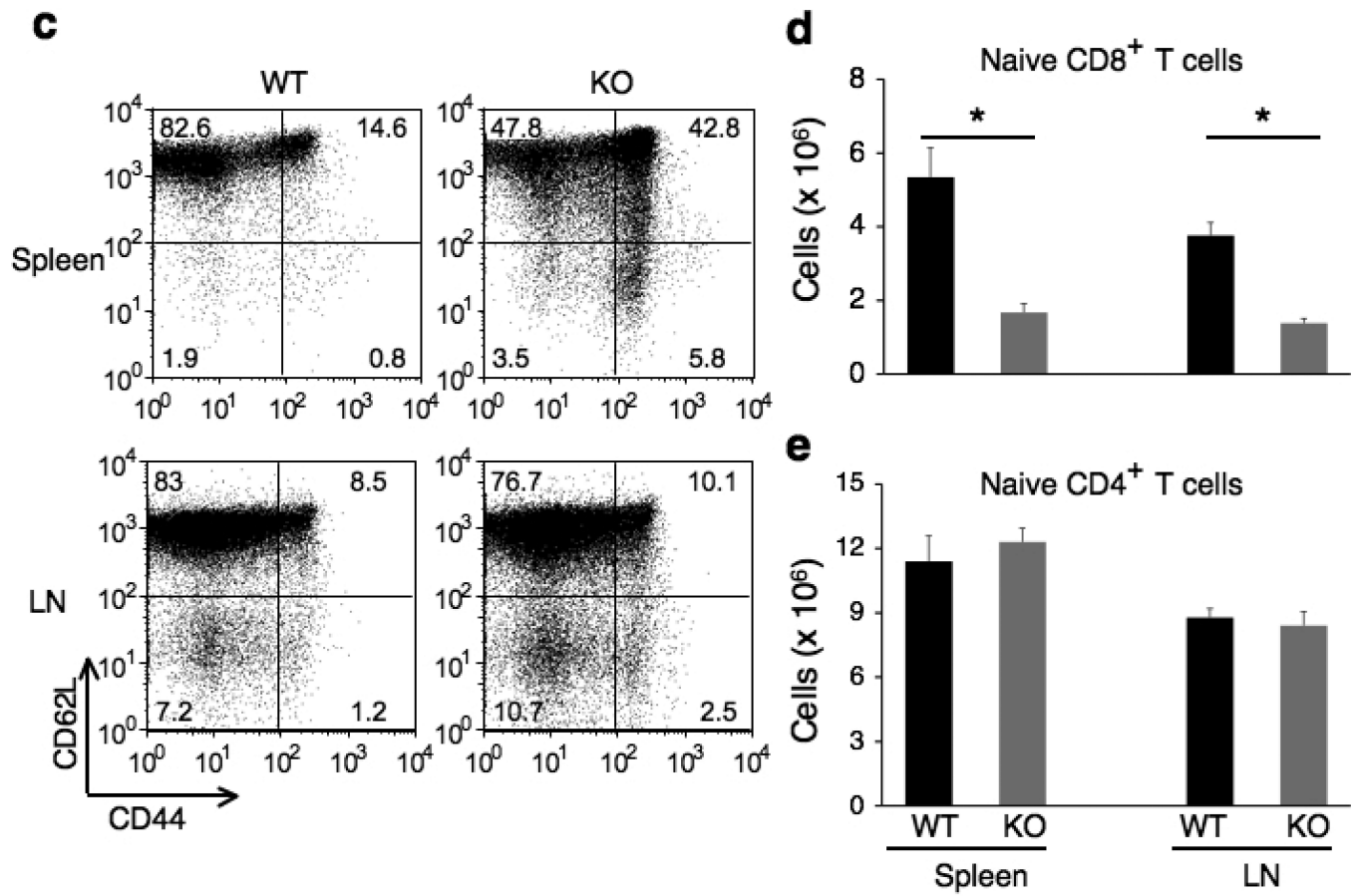


**Figure 1. Expression analysis of pore forming  $\alpha$  subunits and regulatory  $\beta$  subunits of  $\text{Ca}_v$  channels**

(a) Naive  $\text{CD8}^+$  T cells ( $\text{CD62L}^{\text{high}}$  and  $\text{CD44}^{\text{low}}$ ) were left unstimulated (naive) or were stimulated with plate-bound anti-CD3 plus anti-CD28 Abs for 4 days (effector). Total RNA was extracted from each time point and subjected to RT-PCR analysis of the expression of mRNA encoding pore forming  $\alpha$ 1 subunits of  $\text{Ca}_v$ 1 family:  $\text{Ca}_v$ 1.1 (*Cacna1s*),  $\text{Ca}_v$ 1.2 (*Cacna1c*),  $\text{Ca}_v$ 1.3 (*Cacna1d*) and  $\text{Ca}_v$ 1.4 (*Cacna1f*) (b) Purified naïve or *in vitro* generated effector  $\text{CD8}^+$  T cells were lysed and expression of  $\text{Ca}_v$ 1.1 and  $\text{Ca}_v$ 1.4 was examined by immunoblot analysis using anti- $\text{Ca}_v$ 1.1 and anti- $\text{Ca}_v$ 1.4 antibodies. (c) Total RNA from cells, generated as described in panel (a) and stimulated as indicated, was subjected to RT-PCR analysis of the expression of mRNA encoding regulatory  $\beta$  subunits of  $\text{Ca}_v$  channels:  $\beta$ 1 (*Cacnb1*),  $\beta$ 2 (*Cacnb2*),  $\beta$ 3 (*Cacnb3*), and  $\beta$ 4 (*Cacnb4*), (d) Cells, as prepared in panel (c), were lysed and  $\beta$ 3 expression level was detected by immunoblot analysis. Results of all panels are representative of two independent experiments.

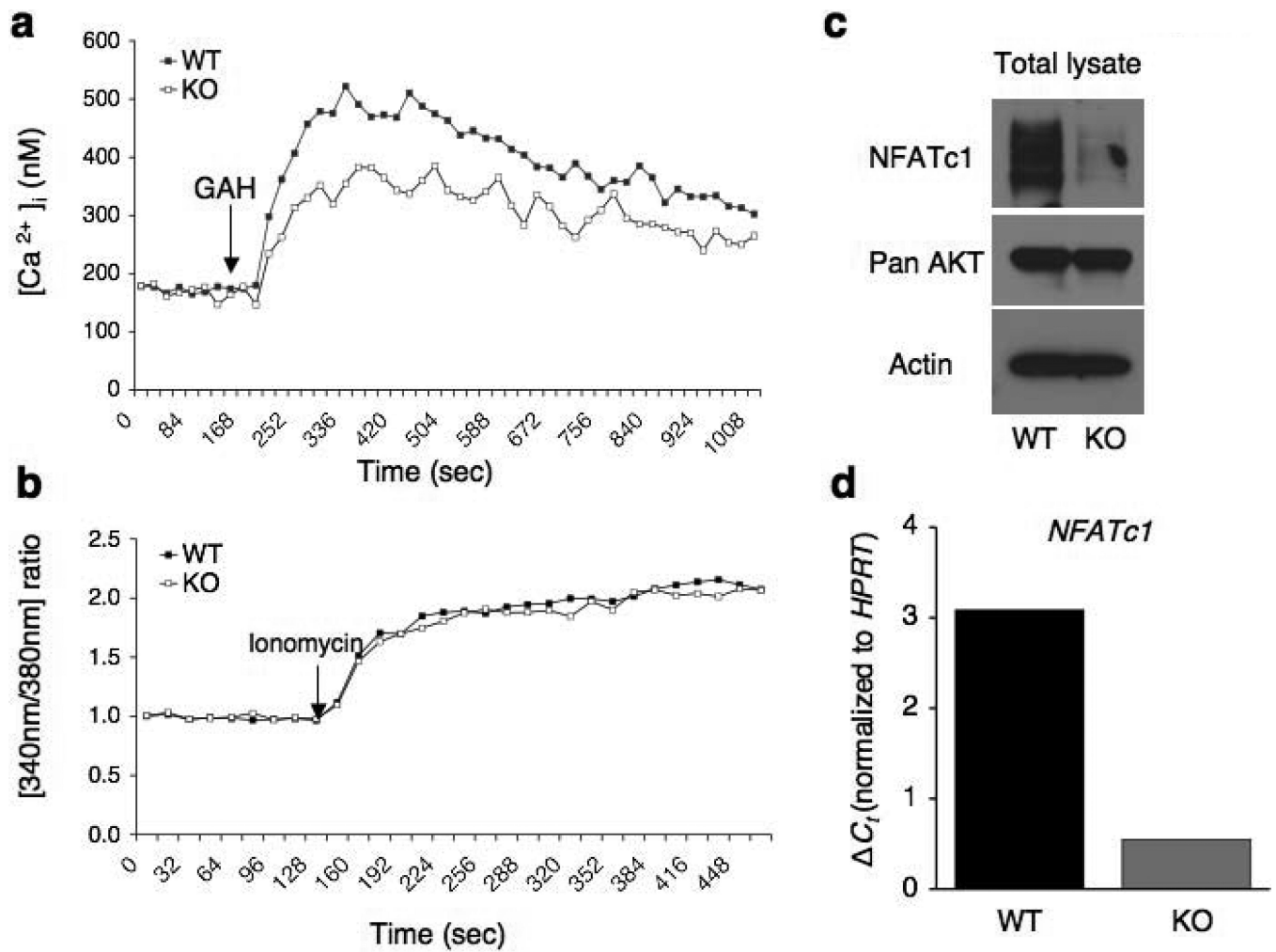






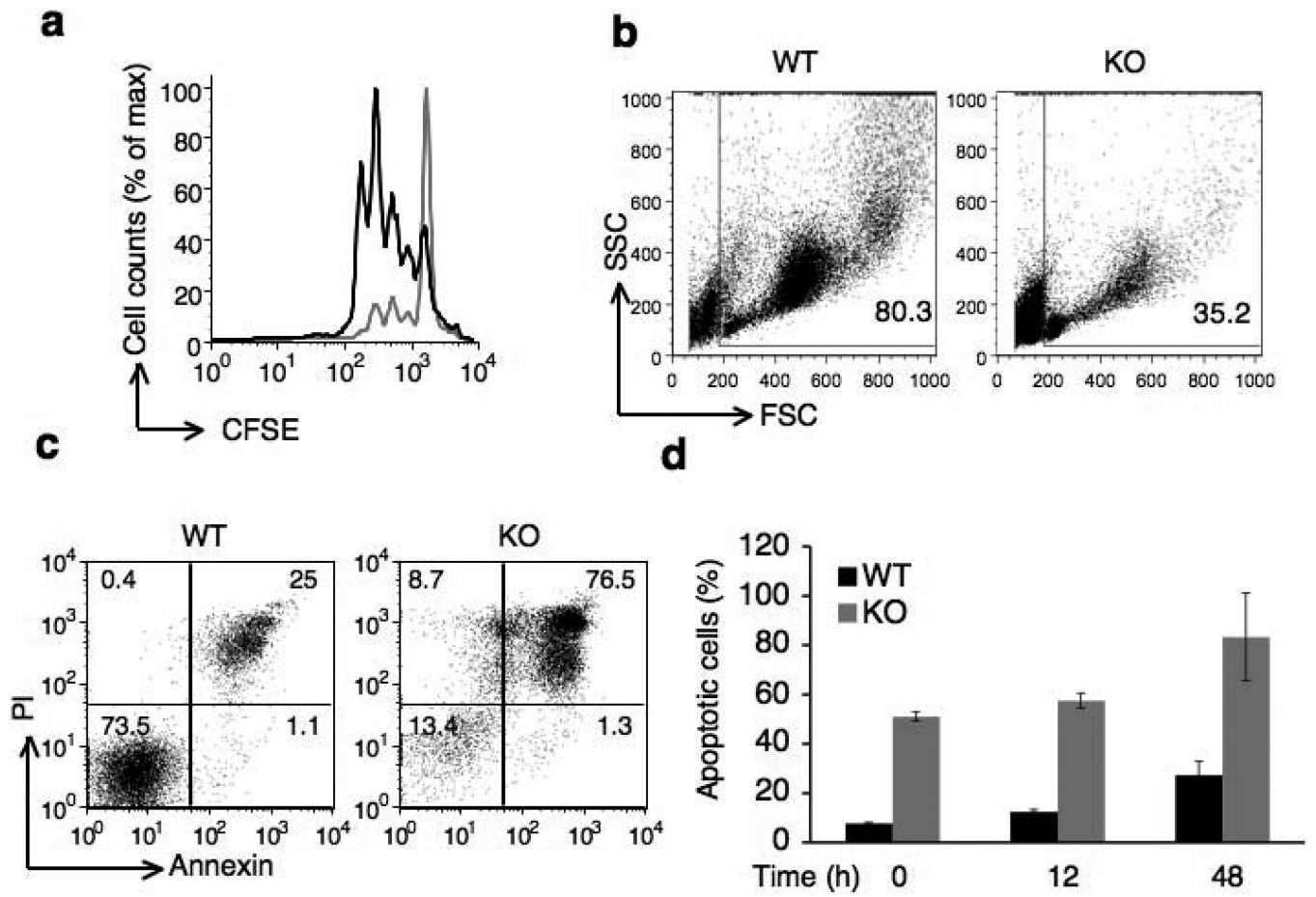
**Figure 2. Characterization of T cells in *Cacnb3*<sup>-/-</sup> mice**

(a) Splenocytes and LNs from 5 weeks old wild type and *Cacnb3*<sup>-/-</sup> male mice were analyzed by flow cytometry for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (b) Absolute numbers of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in splenocytes and LNs. (c) Flow cytometry analysis of surface markers CD44 and CD62L expressed on CD8<sup>+</sup> T cells of spleen and LNs. (d, e) Absolute numbers of naïve CD8<sup>+</sup> or naïve CD4<sup>+</sup> T cells in splenocytes and LNs. Results in panel a, b, c, d, and e are representative of two independent experiments (n=5 per experiment). Error bars in panel b, d and e show s.d. of 5 mice. \**P* < 0.05 (*t*-test).

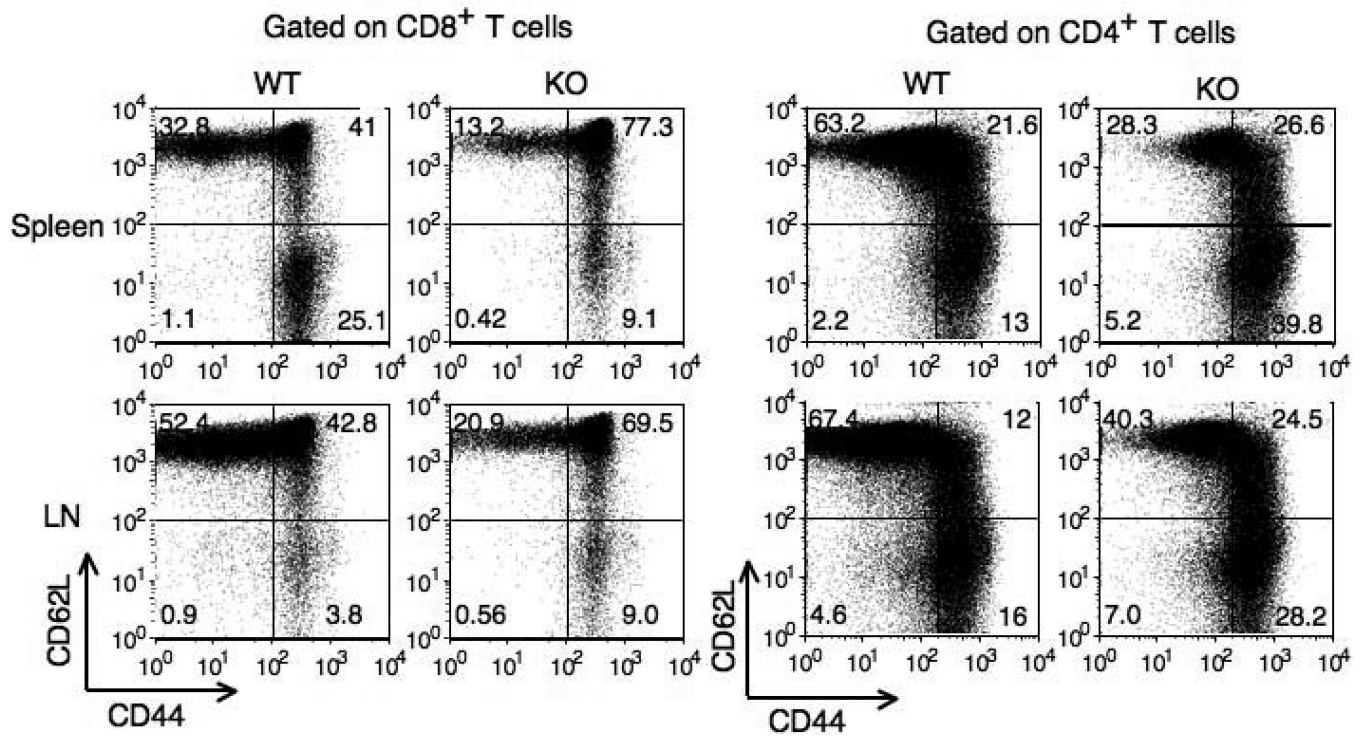


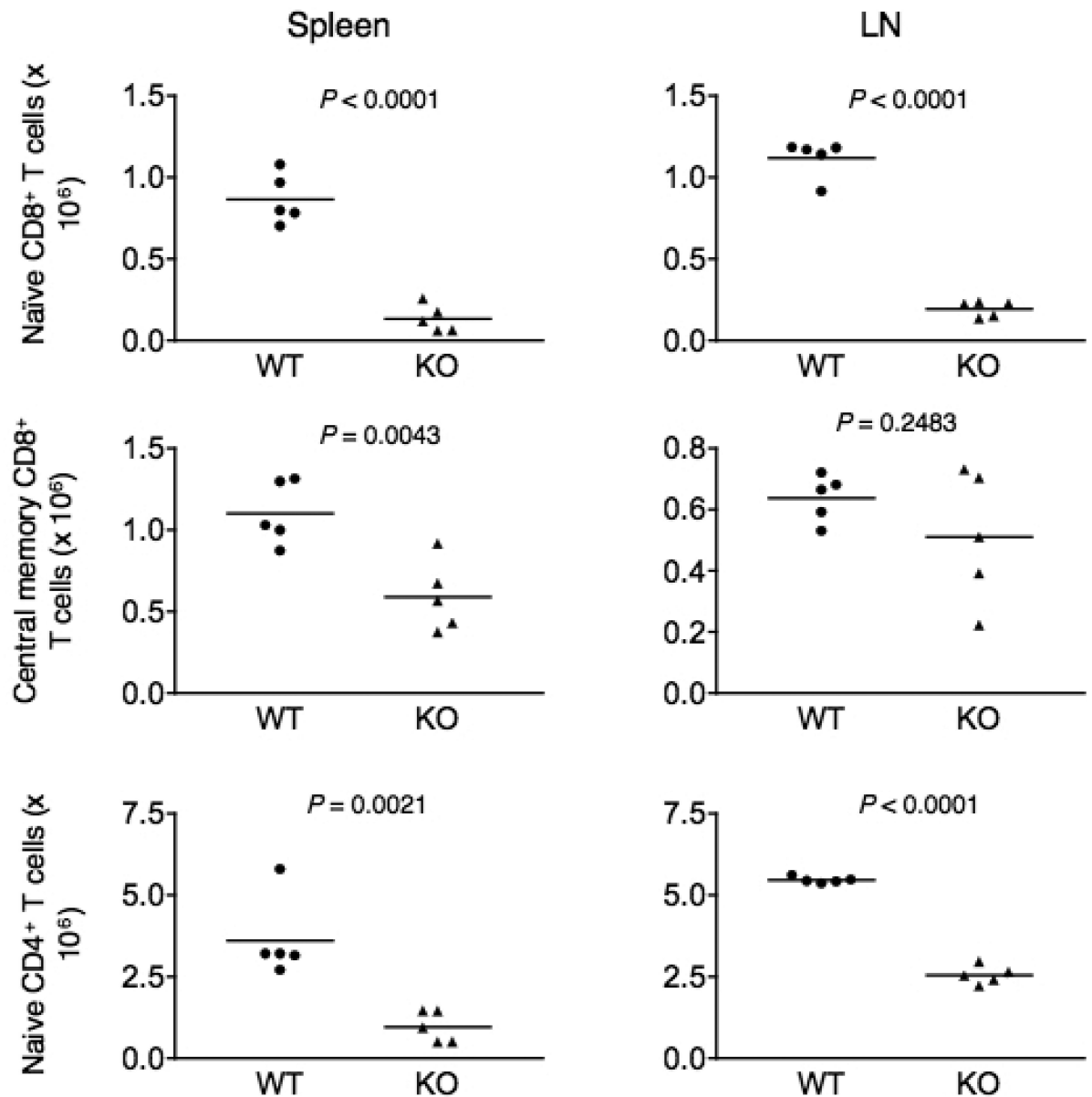
**Figure 3. Calcium/NFAT pathway is impaired in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells**

(a, b) The calcium response of one million sorted naïve CD8<sup>+</sup> T cells (CD62L<sup>high</sup> and CD44<sup>low</sup>) from *Cacnb3*<sup>-/-</sup> mice and WT mice was evaluated by using 2C11 anti-CD3 Ab and goat anti-hamster (GAH) in a cross-linking system and upon ionomycin treatment. (c) Western blot assay was performed on whole cell lysates prepared from naïve CD8<sup>+</sup> T cells (CD62L<sup>high</sup> and CD44<sup>low</sup>) of *Cacnb3*<sup>-/-</sup> and control mice. Actin was used as an internal control. (d) Total RNAs prepared from nonstimulated WT and *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells were analyzed for the levels of *NFATc1* transcripts using commercially available probe (ABI) by real-time PCR. Results are representative of three (a and b), and two (c and d) independent experiments.



**e**

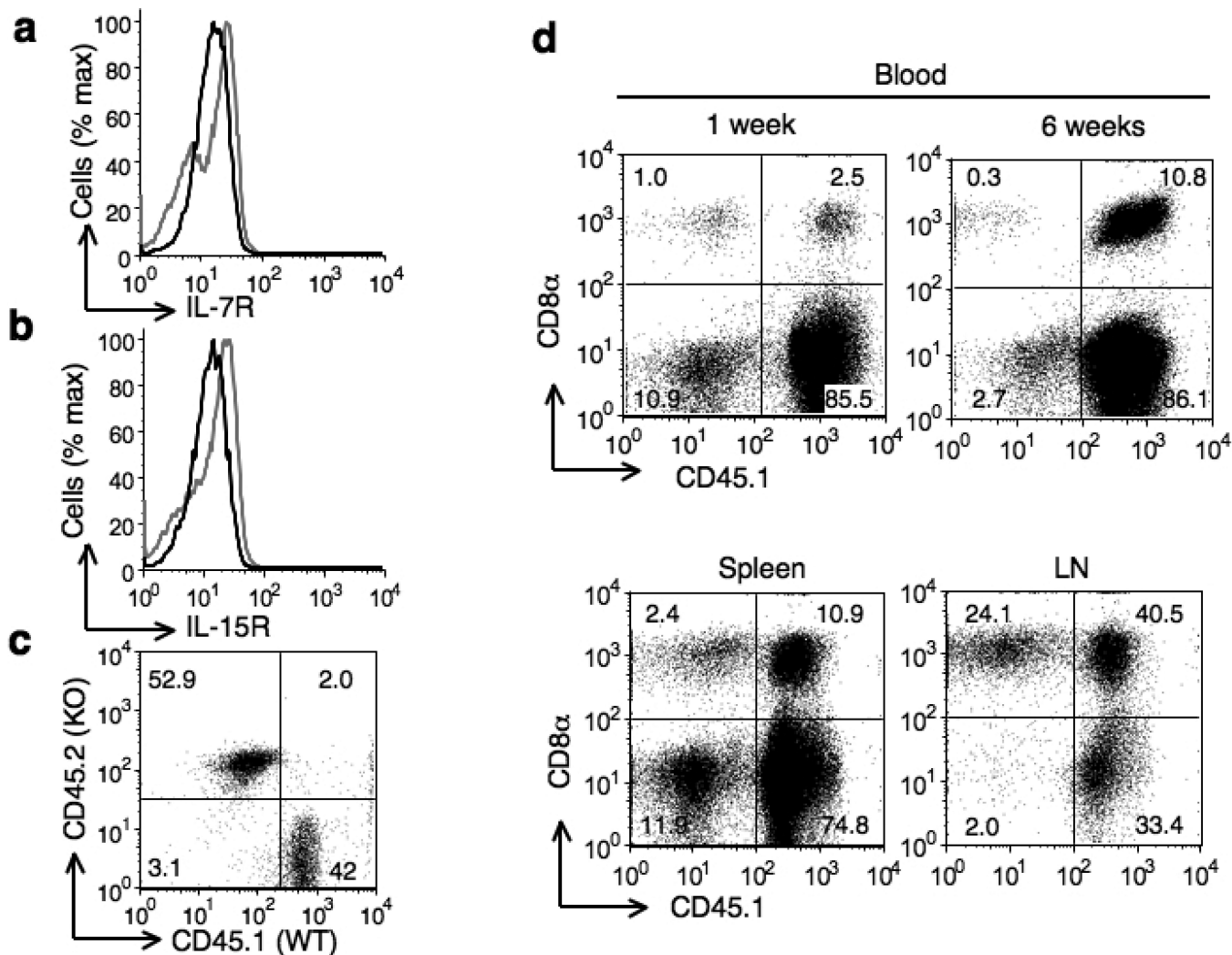


**f**

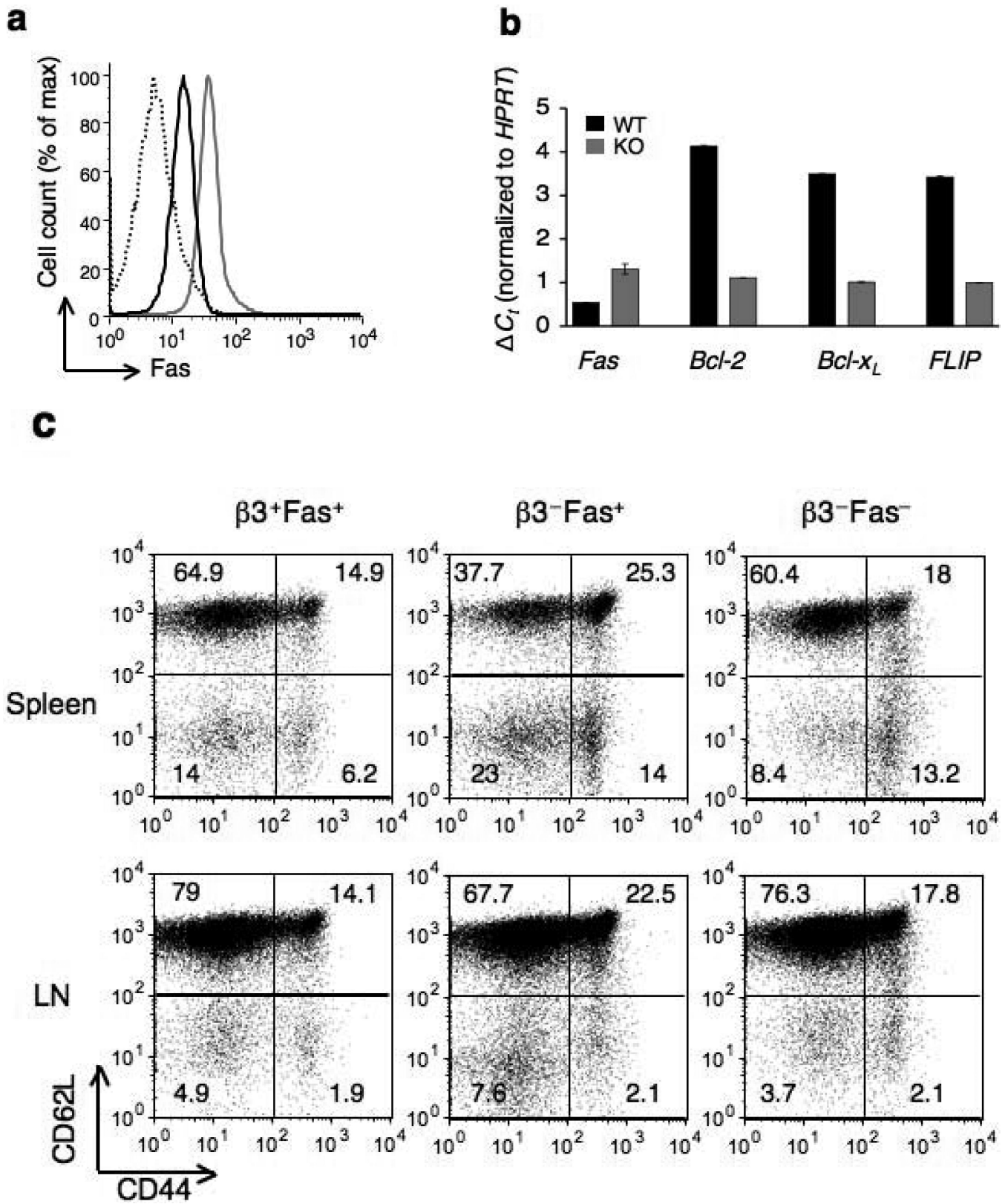
**Figure 4. Survival of naïve CD8<sup>+</sup> T cells requires  $\beta 3$  subunit**

(a) Proliferation of *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells stimulated by plate-bound antibodies to CD3 and soluble CD28 assessed after 72 hours by CFSE. (b) Forward and side scatter pattern of *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells after 72 hrs as stimulated in panel (a). (c) *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells were stimulated as in panel (a), stained with annexin V and PI after 48 hrs of stimulation and analyzed. (d) *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells were stimulated for indicated time, stained with annexin V and PI and analyzed in flow cytometry. (e and f) 21 day old mice were thymectomized and analysis of T cell populations in spleen and LN was done six to eight weeks post thymectomy. Frequency and numbers of all T cell populations were shown from each individual mouse (n=5) of one experiment (P value - unpaired, two-tailed Student's t-test). Results in a, b, c, and d are representative of three independent experiments (error bars (d), s.d.) and results in

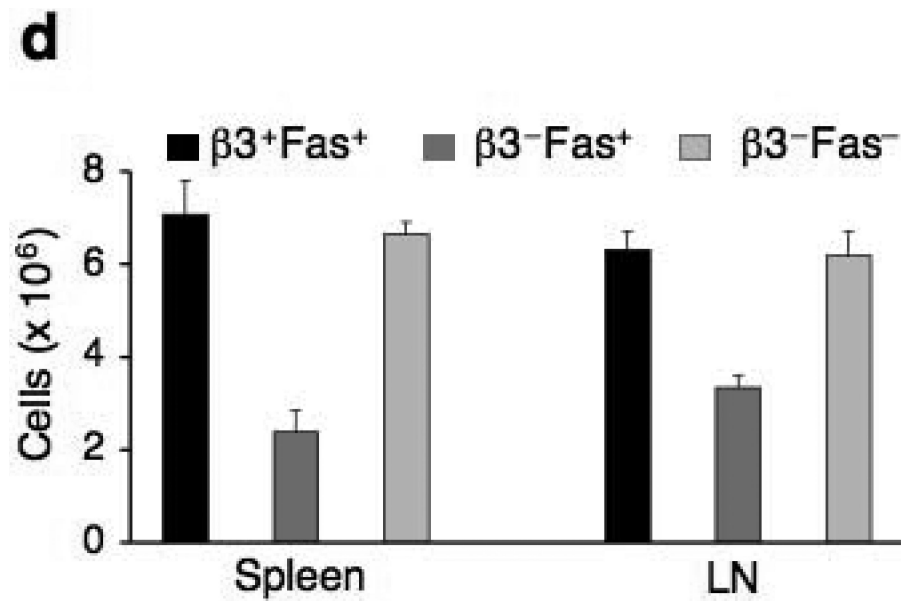
(e) and (f) are representative of four independent experiments with 2-5 mice per group in each experiment.



**Figure 5. Homeostatic maintenance of naïve CD8<sup>+</sup> T cells is critically dependent on  $\beta 3$  subunit** (a and b) IL-7R and IL-15R protein expression were analyzed in non-stimulated *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells (CD62L<sup>high</sup> and CD44<sup>low</sup>). (c) Frequency of mixture of sorted naïve CD8<sup>+</sup> T cells (CD62L<sup>high</sup> and CD44<sup>low</sup>) from WT (CD45.1.1<sup>+</sup>) mice and *Cacnb3*<sup>-/-</sup> (CD45.2.2<sup>+</sup>) mice before transfer. (d) Mixed cells as prepared in panel (c) was transferred into *Rag1*<sup>-/-</sup>CD45.1.1<sup>+</sup> recipient mice, followed by analysis at 1 and 6 weeks after transfer in peripheral blood (*upper*) or at 6 weeks in spleen and LN (*lower*). Numbers inside boxed areas indicate percent recovered CD8<sup>+</sup> T cells of *Cacnb3*<sup>-/-</sup>CD45.2.2<sup>+</sup> cells (left top) or *Cacnb3*<sup>+/+</sup>CD45.1.1<sup>+</sup> cells (right top). Results are representative of three (a & b) and two (c & d) independent experiments.

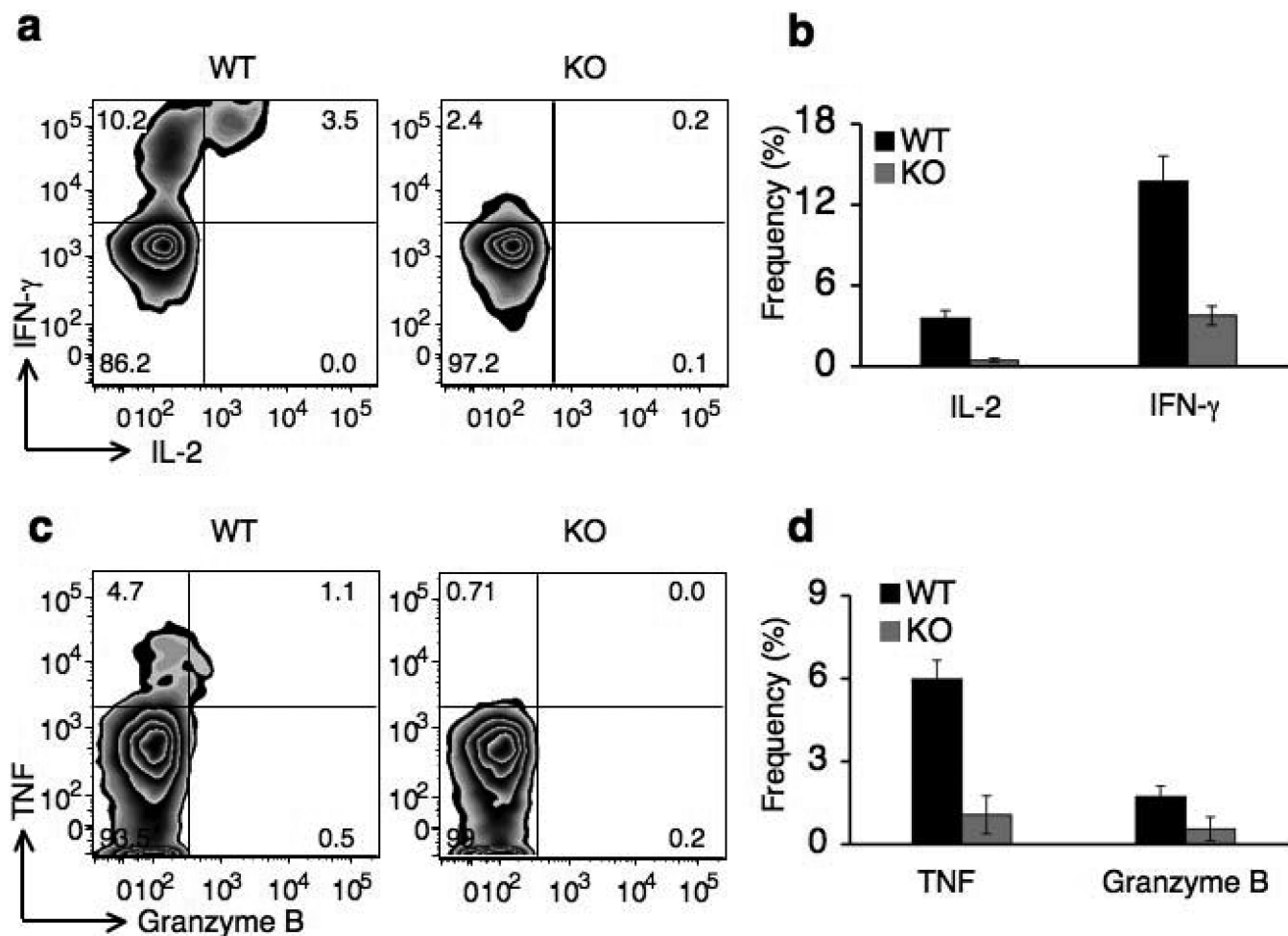




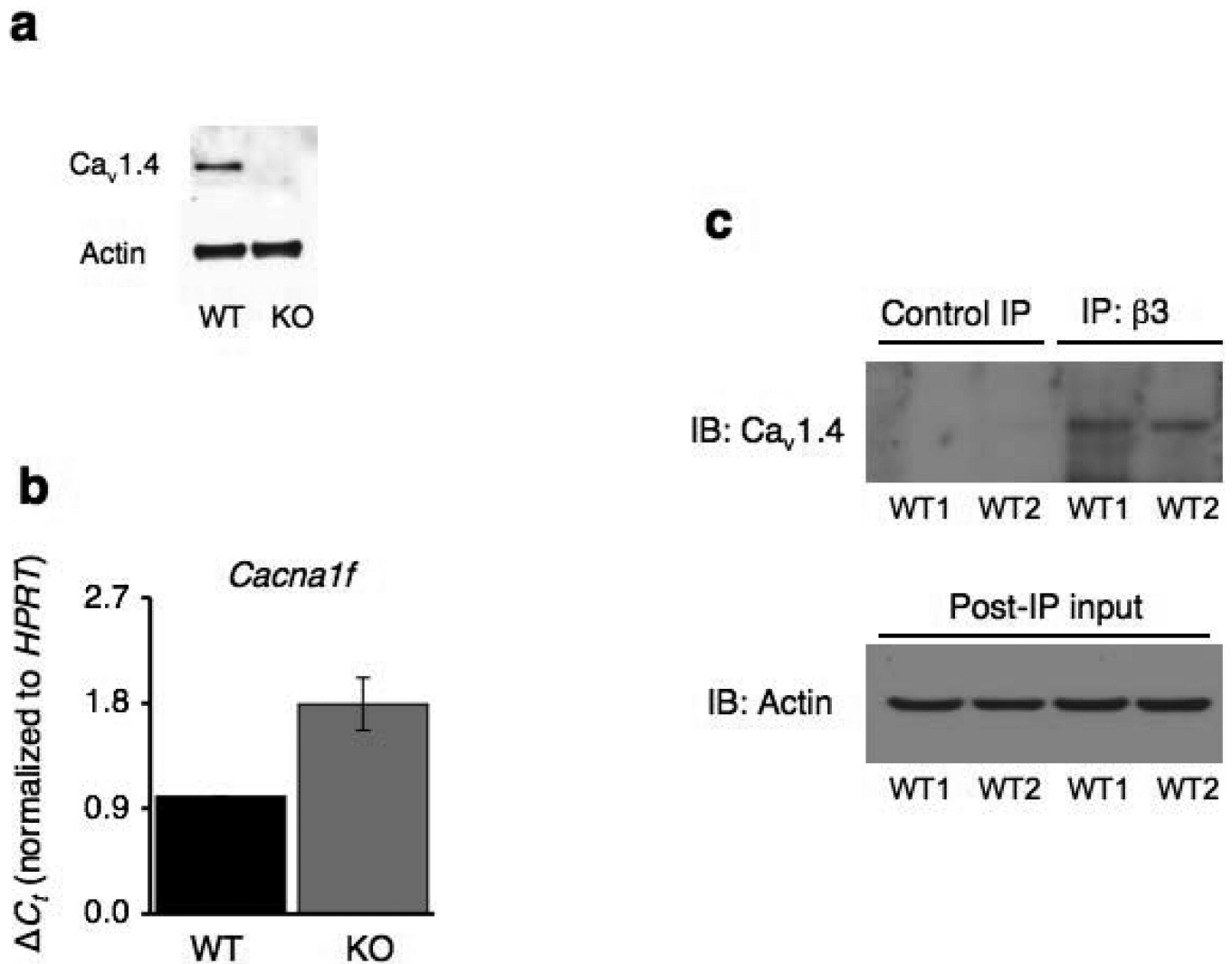


**Figure 6. Upregulation of Fas and downregulation of anti-apoptotic gene expression in *Cacnb3*<sup>-/-</sup> CD8<sup>+</sup> T cells**

**(a)** Fas protein expression in non-stimulated *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells (CD62L<sup>high</sup> and CD44<sup>low</sup>). **(b)** Analysis of *Fas*, *Bcl-2*, *Bcl-xl* and *FLIP* mRNA expression levels in nonstimulated *Cacnb3*<sup>-/-</sup> and WT naïve CD8<sup>+</sup> T cells using real-time quantitative PCR. **(c)** Flow cytometry analysis of surface markers CD44 and CD62L expressed on CD8<sup>+</sup> T cells of spleen and LNs. **(d)** Absolute numbers of naïve CD8<sup>+</sup> T cells in splenocytes and LNs. Results are representative of three (a) and two (b) independent experiments (error bars (b), s.d. of triplicate samples). Histograms in panel (c) represent 3 individual mice and panel (d) show s.d. of 3 mice.

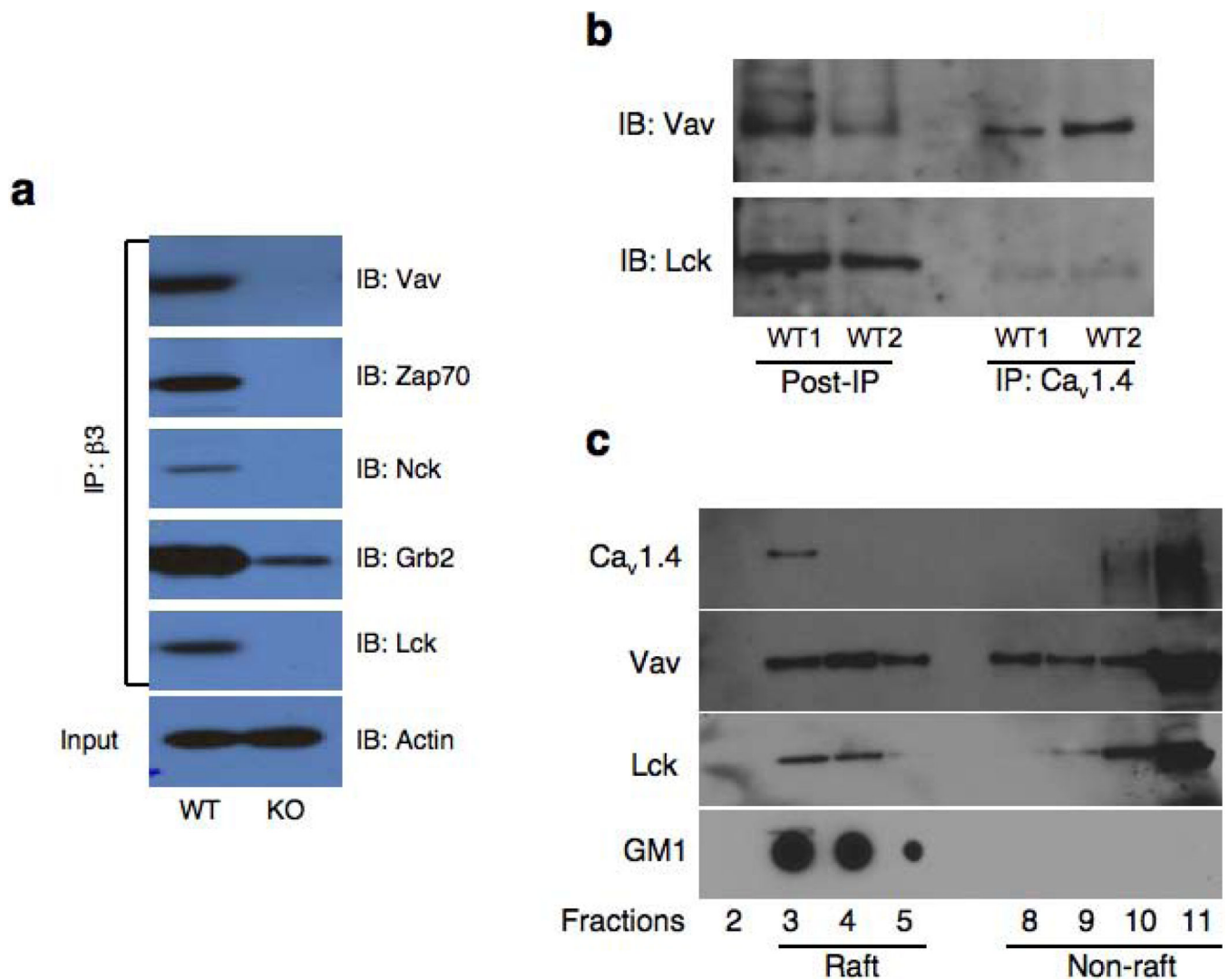


**Figure 7. Defective CD8<sup>+</sup> T cell dependent *in vivo* effector response in *Cacnb3*<sup>-/-</sup> mice**  
 Wild-type DCs primed with H-2K<sup>b</sup> binding-SIINFEKL peptide (OVA) were transferred into WT and *Cacnb3*<sup>-/-</sup> mice. Seven days later, splenocytes were purified and restimulated with SIINFEKL peptide for 6 hrs followed by analysis of IFN $\gamma$  and IL-2 (a) or Granzyme B and TNF (c) expression in CD8<sup>+</sup> T cells by intracellular staining. Each histogram in panels (a) and (c) for both staining represents 5 wild-type and *Cacnb3*<sup>-/-</sup> mice. Results shown in panels (b) & (d) are mean  $\pm$  SD for 5 mice per group. Unstimulated cells and CD4<sup>+</sup> T cells serve as negative control in setting gates. Another independent experiment yielded similar results.



**Figure 8. Loss of Ca<sub>v</sub>1.4 pore forming subunits in *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells**

(a) Nonstimulated WT and *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells (CD62L<sup>high</sup> and CD44<sup>low</sup>) were lysed and Ca<sub>v</sub>1.4 expression was detected by immunoblot analysis using polyclonal antibody provided by JEM. (b) RNAs prepared from nonstimulated WT and *Cacnb3*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells were analyzed for the abundance of *Cacna1f* transcripts by real-time PCR. (c) Whole cell lysates were prepared from unstimulated CD8<sup>+</sup> T cells purified from pLNs of approximately 20-25 WT mice per group. 1 mg protein extracts were taken for co-immunoprecipitation experiment and incubated with anti-β3 antibody (clone 828) overnight at 4°C followed by immunoblotting and detection by anti-Ca<sub>v</sub>1.4 antibody. Results are representative of three (a & b) and two (c) independent experiments.



**Figure 9. Association of  $Ca_v1.4$  and  $\beta 3$  with T cell signaling components and localization of  $Ca_v1.4$  in lipid rafts**

(a) Whole cell lysates were prepared from unstimulated  $CD8^+$  T cells purified from pLNs of approximately 20-25 WT mice per group. 1 mg protein extracts were taken for co-immunoprecipitation experiment and incubated with anti- $\beta 3$  antibody (clone 828) overnight at  $4^\circ C$  followed by immunoblotting and detection by respective antibodies. (b) 1 mg protein extracts as prepared in panel (a) were taken for co-immunoprecipitation experiment and incubated with monoclonal anti- $Ca_v1.4$  antibody (Sigma) overnight at  $4^\circ C$  followed by immunoblotting and detection by anti-Lck and anti-Vav antibodies. (c) Total  $CD8^+$  T cells, purified from spleen and LNs of approximately 8 - 10 wild-type mice, were subjected to lipid raft isolation protocol. 1 to 11 fractions were collected. Fractions # 3, 4, 5 were raft fractions as per GM1 staining and fractions # 8, 9, 10, 11 were non-raft fractions. Usually fraction # 1, 2, 6 and 7 were very low in protein concentrations and therefore not loaded. These fractions were analyzed for the presence of different proteins by immunoblotting.

GM1 staining was done separately on these fractions. Results in all panels are representative of two independent experiments.