Migration and Homeostasis of Naïve T cells Depends on Coronin 1-mediated Pro-Survival Signals and not on Coronin 1-dependent F-Actin Modulation

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Short title: Coronin 1 signaling controls naive T cell homeostasis

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Abstract

Coronins are WD repeat containing proteins highly conserved in the eukaryotic kingdom implicated in the regulation of F-actin. Mammalian coronin 1, one of the most conserved isoforms expressed in leukocytes, regulates survival of T cells, which has been suggested to be due to its role in preventing F-actin induced apoptosis. We here come to a different conclusion. We show that coronin 1 does not modulate F-actin and that induction of F-actin failed to induce apoptosis. Instead, coronin 1 was required for providing pro-survival signals, in the absence of which T-cells rapidly underwent apoptosis. These results argue against a role for coronin 1 in F-actin-mediated T cell apoptosis and establish coronin 1 as an essential regulator of the balance between pro-survival and pro-apoptotic signals in naive T-cells.

Introduction

Coronin 1 is a member of the conserved WD repeat family of coronin proteins that is exclusively expressed in leukocytes (1). In mice lacking coronin 1, naïve T-cells are deleted from the periphery, (2-6), suggesting that coronin 1 has a specific and essential role in the regulation of cellular homeostasis and the survival of these cells.

Coronin 1 is homologous to *Dictyostelium discoideum* coronin, that was originally isolated as an actin/myosin binding protein (7). Deletion of coronin from *D. discoideum* results in pleiotropic defects such as reduced phagocytosis, macropinocytosis, cell locomotion and cytokinesis (8). In yeast, the single coronin isoform has been suggested to modulate the formation of actin filamentous networks via regulation of the actin-related protein 2/3 (Arp2/3) activity, based on in vitro experiments (9). However, living yeast cells lacking coronin do not show any obvious phenotype (10).

The original isolation of *Dictyostelium* coronin from an actin-myosin affinity matrix has led to the assignment of all coronin protein family members as actin-interacting and regulating proteins (7)**.** However, the evidence for F-actin regulation by mammalian coronin isoforms is largely based on *in vitro* polymerization assays using purified proteins (11, 12), or overexpression of coronin domains in heterologous systems (13). *In vivo*, the only evidence linking coronin 1 to a direct modulation of the F-actin cytoskeleton is based on the observation that coronin 1-deficient leukocytes display a ~2-fold higher phalloidin fluorescence as compared to wild type cells. Phalloidin, due to its ability to specifically bind to filamentous (F-) actin, is widely used to label and quantitate F-actin in cells (14, 15). Consequently, the decrease in cell viability as well as migration of T-cells lacking coronin 1 as observed in coronin 1-deficient mice was attributed to defective functioning

of the F-actin cytoskeleton (3-6). In contrast to linking a role for coronin 1 in T-cell survival to the modulation of F-actin, recent work suggested that coronin 1 promotes T-cell survival by allowing signal transduction downstream of the TCR (2).

We here provide an explanation for this apparent discrepancy. We show that phalloidin cannot be used to quantitate F-actin in cells expressing different coronin 1 levels and furthermore that coronin 1 does not directly modulate F-actin. In cells depleted of coronin 1 by either gene deletion or RNA interference, phalloidin levels increased in direct inverse relation to the amount of coronin 1, without affecting the F/G-actin ratio or cell viability. Conversely, expression of coronin 1 in non-leukocytes resulted in decreased phalloidin fluorescence without affecting F/G actin levels or viability.

Instead of regulating the F-actin cytoskeleton, we describe here that coronin 1 is required for the generation of pro-survival signals upon TCR ligation, in the absence of which naïve T-cells undergo apoptosis and show defective migration. The here described results are therefore crucial for a better understanding of the function of coronin 1 as well as the processes that regulate cellular survival and migration.

Materials and methods

Mice. Mice lacking coronin-1 were generated as described(16). Experiments shown used mice that were backcrossed to C57BL/6 for at least four generations, whereas all experiments were repeated with mice backcrossed for eight generations, using wild-type littermates as a control. Animal experiments were approved by the Kantonales Veterinäramt Basel-Stadt.

Antibodies and reagents. Polyclonal anti-coronin 1 serum has been described (1). Actin was detected using MAB1501 (Chemicon) at 1:1000 dilution. Antibodies to Calcineurin-Aβ were from Santa Cruz. Anti CD95 (Jo-2), anti-CD3 and anti-CD28 were from BD Biosciences, and the secondary mouse anti–hamster IgG was from R&D (clone MAH1.12). Anti-talin antibodies (mouse, monoclonal) were from Sigma). Phalloidin Alexa Fluor-568 and -633 were from Molecular probes. Jasplakinolide was from Calbiochem, Latrunculin B from Sigma, Ionomycin from Calbiochem, Cyclosporine A from Fluka. Annexin V and 7-AAD were from BD Biosciences.

Flow cytometric analysis. Cell counts were determined for single cell suspensions of the indicated organs using a Neubauer chamber. Flow cytometry was carried out by staining the cells with the relevant monoclonal antibodies at saturating concentrations in PBS + 2% FCS and analyzing them on a FACS Calibur (Becton-Dickinson). The following monoclonal antibodies and secondary reagents were obtained from BD Pharmingen: α-CD19 (clone 1D3), α-CD3 (clone 145-2C11), α-CD11b (clone M1/70), α-CD4 (clone RM4-5), α-CD44 (IM7), α-CD69 (H1.2F3), α-CD8a (53-6.7), α-CD62L (clone MEL-14), α-CD24 (M1/69). PE or APC labeled Annexin V and 7-AAD (BD Biosciences) was used according to the manufacturer's protocol.

Phalloidin staining and intracellular antibody labeling for FACS. Where required cells where first labeled with antibodies against extracellular markers such as CD4, washed, fixed in 4% PFA/PBS on ice for 2-4 hours, blocked and permeabilized in PBS, 3% FBS, 0.1% TritonX-100 for phalloidin staining as displayed in Figure 1 or using a commercial cell permeabilization buffer from E-Biosciences according to the manufacturers protocol.

Analysis of F-actin and G-actin. The relative proportions of G-actin and F-actin were analyzed in MACS isolated wild type and coronin 1-deficient T-cells by sedimentation of filamentous actin followed by quantitative analysis of F- and G-actin (17) as described (2, 18). In case of drug treatments, cells were left untreated or incubated with Jasplakinolide (1 μM) or Latrunculin B (4 μM) for 45 min at room temperature (23–26 °C). Alternatively cells were incubated with 250 ng/ml CCL19 for 60 seconds at 37 °C in a water bath, followed by lysis (at room temperature) in F-actin stabilization buffer.

Calcium mobilization measurements. Calcium measurements using Fluo-3 loaded coronin 1-deficient as well as wild type control cells were performed as described previously (2).

T-cell proliferation. Naïve $CD4^+$ splenic T-lymphocytes were isolated from coronin 1-deficient mice and wild-type littermates using magnetic beads according to the manufacturers protocol (MACS, Miltenyi). For stimulation we incubated T-cells with soluble anti-CD3 (0,1 μg/ml) or PMA 20 nM /Ionomycin 100 nM on splenic feeder cells

as described (2). Results were expressed as the average cpm (± s.d.) per well of triplicate cultures.

Preparation of $CD4^+$ SP thymocytes, naïve T-cells and B-cells for survival and functional assays. Naïve T-cells were prepared from erythrocyte depleted splenic cell suspensions using the Pan T-cell isolation kit from Miltenyi supplemented with biotinylated antibodies against CD44 at a final concentration of 5μg/ml. Using this procedure we were able to isolate T-cell populations containing ≥ 95% of naïve T-cells. In order to remove dead or apoptotic cells we passed cell suspensions at room temperature for 20 minutes at 800g over a cushion of HISTOPAQUE-1077 and HISTOPAQUE-1119 (Sigma) mixed at a ratio of 11:3. Using this procedure we were able to recover naïve T-cell populations with ≥ 95% of viable cells (Annexin V, 7-AAD double negative).
For the isolation of $CD4^+$ SP thymocytes we passed thymic single cell suspensions at room temperature for 20 minutes at 800*xg* over a cushion of HISTOPAQUE-1077 and HISTOPAQUE-1119 mixed at a ratio of 11:3, depleted the remaining cell suspensions of CD8 expressing cells using anti-CD8 micro beads from Miltenyi according to the manufacturers protocol. In a next step we positively selected CD8 negative, CD4 positive cells using anti-CD4 micro beads from Miltenyi according to the manufacturers protocol. In order to remove dead or apoptotic cells we passed cell suspensions at room temperature for 20 minutes at 800g over a cushion of HISTOPAQUE-1077 and HISTOPAQUE-1119 mixed at a ratio of 11:3. Using this procedure we were able to recover CD4 single positive thymic cell populations containing approximately 95% of CD4 positive cells which in addition were ≥ 95% viable (Annexin V, 7-AAD double negative).
B-cells were isolated similar to naïve T-cells using anti-CD4, anti-CD8 and anti-CD11b micro beads (Miltenyi) to remove all non B-cells. Using this procedure we were able to

recover CD19 positive splenic cells containing ≥ 95% of CD19 positive cells which in addition were ≥ 95% viable (Annexin V, 7-AAD double negative).

Analysis of talin distribution. In vitro migrated $CD4^+$ single positive naïve T-cells were isolated according to the protocol described in the methods section. Then, the cells were washed 3 times by migration medium (RPMI-1640 supplemented with 0,1% tissue culture grade, lipid free BSA (Sigma) and L-glutamine) to remove the chemo attractants. Afterwards, the cells were seeded on ICAM-1 coated 10-well slides, and incubated at 37 ºC for 1h. The ICAM-1 was dissolved at 6μg/ml in 150 mM Tris buffer (pH9), after which the chambered coverslips were incubated with ICAM 1 solution at 4 ^{O}C overnight for coating), . Then, CCL19 or control medium (migration medium without CCL19) was added and further incubated at room temperature for 5 min. After CCL19 stimulation, the cells were fixed immediately by 4% PFA in RT for 20 min, followed by washing with PBS. Then, the cells were permeabilized in 0.5% PFA at RT for 15 min, followed by a block using 2% BSAat RT for 0.5h. Then, the cells were incubated with talin primary antibodies (1:100 dilution) at 4 ºC overnight, followed with coronin 1 primary antibody incubation (1:1000 dilution) at RT for 1h. After 4 washes, the cells were incubated with corresponding Alexa 488 and Alexa 568 labeled secondary antibodies, respectively. The images were analyzed using a Zeiss confocal laser scanning microscope LSM 510Meta, using 40X objective and 5X zoom.

Videomicroscopy. Migrated $CD4^+$ single positive naïve T-cells (see above), were washed 3 times in migration buffer and seeded on ICAM-1 coated chambered cover slips and incubated at 37 ºC for 1h to let the cells recovery and adhere. Afterwards, the cells were washed 3 times to remove the non-adhered cells. Then, the chambered coverslips were

mounted on an inverted microscope connected to a Cellobserver (Zeiss) and analyzed with a 40X objective and oil immersion, as well as with full temperature and CO_2 incubation during image acquiring. The chemo attractant (CCL19) was directly added to the cell mixtureduring acquisition. The images were taken by 6 frames per min.

Transwell migration assay. For transwell migration cells were washed extensively with migration buffer: RPMI-1640 supplemented with 0,1% tissue culture grade, lipid free BSA (Sigma) and L-glutamine. Cells were then added to the upper chamber of the migration setup in 100 μl of migration medium. 500 μl of migration buffer containing the indicated stimuli at the indicated concentration were added to the lower wells. Migration was monitored after the indicated time span using a FACS Calibur or Neubauer chamber to quantitate migrated cell numbers. In experiments where wild type as well as coronin 1-deficient cells (express GFP) (16) where tested, we used mixtures of both cell types to minimize experimental variations by having exactly the same condition for both wild type as well as coronin 1-deficient cells. GFP was used to distinguish both cell types.

Calcineurin activation. To measure calcineurin activity in naïve coronin 1-deficient or wild type T-cells, untouched cells were isolated using the Pan T-cell isolation kit from Miltenyi supplemented with biotinylated antibodies against CD44 at a final concentration of 5 μg/ml. Cells were either stimulated for 15 minutes using anti-CD3, anti CD28 as well as secondary antibodies as stated for the calcium measurements or using PMA 50 nM/Ionomycin 200 nM. Upon stimulation cell lysis as well as calcineurin activity measurements were performed using a commercial kit (Calbiochem), according to the manufacturers protocol. Protein concentrations measurements were carried out using a commercial BCA protein assay kit (Pierce) according to the manufacturers protocol. In

brief, cells were lysed in the lysis buffer provided with the kit (50 mM Tris, 1 mM DTT, 100 μM EDTA, 100 μM EGTA, 0.2% NP-40, pH 7.5 containing protease inhibitors) by passing them through a 16 gauge needle. To analyze calcineurin activity the lysates was cleared by centrifugation (100,000 g for 1 hr at 4 ^{0}C) and passed through a P6 DG desalting column (provided with the kit) to remove free phosphates from the cell extract. Effective removal of phosphates was qualitatively tested by the addition of the malachite green reagent to the flow through. Calcineurin phosphatase activity (okadaic acid resistant and EGTA sensitive) was detected using equal protein amounts in lysis buffer for the different conditions. The RII phosphopeptide was used as a substrate for calcineurin. Calcineurin-specific phosphate release was measured in the following buffer: 100 mM NaCl, 50 mM Tris, 6 mM MgCl2, 0.5 mM CaCl2, 0.5 mM DTT, 0.025% NP-40 at pH 7.5. Background was determined in the same buffer by excluding the RII phosphopeptide. Plates were incubated at 30 C° for 30 min, and free phosphate released was detected using malachite green by monitoring the absorption at OD 620 nm. Okadaic acid was used at a final concentration of 500 nM. If EGTA was used instead of calcium the 0.5 mM CaCl2 were replaced by 10 mM EGTA. Calcineurin activity was calculated by subtracting the okadaic acid + EGTA sample from the okadaic acid only sample.

T-cell transfer: Fresh or migrated CD4 SP thymocytes were isolated as described elsewhere in methods, labeled with Cell Tracker Orange and injected into the tail vain of recipient wild type mice. In order to minimize experimental variations thymi from wild type and coronin 1-deficient mice were pooled after isolation of the thymi, prior to preparing single cell suspensions. Coronin 1-deficient CD4 SP thymocytes were distinguished from the wild type cells via the GFP they express instead of coronin 1. Mice were sacrificed after 20h and analyzed for distribution of Cell Tracker Orange positive

cells and the ratio of wild type and coronin 1-deficient CD4 positive cells. For the ratio calculation the input material was analyzed by FACS and the ratio of coronin 1-deficient to wild type cells normalized to 1. Further we took into account the ratio of CD62L high (mature) coronin 1-deficient versus wild type cells, as measured for input material using FACS, when calculating the ratio of cells homed to peripheral as well as mesenteric lymph nodes. This was done since CD62L is a crucial homing molecule for lymph node homing of T cells. As only part of the total CD4 SP thymocytes do express CD62L and are as such homing competent, the ratio of these cells in our input material was compared with the ratio of T-cells that had homed to lymph nodes after 20h. For spleen, the ratio of total CD4 SP thymocytes was used.

Results

Phalloidin Fluorescence, F-actin and Apoptosis

Coronin 1 has been proposed to prevent apoptosis via modulation of the F-actin cytoskeleton. This conclusion was based on the observed ~2-fold increase in phalloidin fluorescence intensity in coronin 1-deficient versus wild type T-cells (3-6). To analyze the correlation between phalloidin fluorescence and apoptosis, the different leukocyte populations as indicated in Fig. 1 and Supp. Fig. 1 were isolated from wild type or coronin 1-deficient mice or differentiated *ex vivo* in case of bone marrow derived macrophages, and analyzed for coronin 1 expression, phalloidin fluorescence and annexin V staining. Staining of all leukocyte populations with fluorescent phalloidin resulted in a ~2-fold increase of fluorescence in coronin 1-deficient cells versus wild type cells (Fig. 1A and B). However, with the exception of $CD4^+$ single positive thymocytes as well as T-cells, none of the leukocyte populations lacking coronin 1 showed an increase in apoptosis, as judged by Annexin V labeling (Fig. 1C). Notably, the heterozygous $CD4^+$ single positive thymocytes as well as T-cells showed intermediate levels of phalloidin staining but no elevated Annexin V staining.

However, as the thymocytes and T-cells analyzed in Figure 1 were freshly isolated from mice, the results might not fully reflect the extend of *in vivo* cell death, as dead and dying cells are rapidly removed *in vivo* by phagocytic cells. To circumvent the problem of i*n vivo* loss of apoptotic and dead cells due to clearance, we have further performed an *ex vivo* analysis of the different subsets of coronin 1-deficient and wild type leukocytes. To this end, wild type and coronin 1-deficient T cell subsets as well as B-cells and thymic subsets were analyzed *ex vivo* for spontaneous apoptosis. Naïve $CD4^+$ T-cells and B-cell

preparations (Fig. 2), freshly isolated from wild type or coronin 1-deficient mice, were depleted of dead cells, left in medium for the indicated time and stained with Annexin V and 7-AAD to assess the number of viable cells. Double negative cells were scored as viable. Our results demonstrate that freshly isolated coronin 1-deficient naïve T-cells show elevated levels of spontaneous apoptosis, while in B-cells, which in the absence of coronin 1 show elevated levels of phalloidin fluorescence similar to that observed in T-cells (see Fig. 1), cellular viability is not affected by the loss of coronin 1.
Using the same experimental setup we also analyzed double positive thymocytes, $CD4^+$ single positive thymocytes and memory/effector T-cells for spontaneous apoptosis (Fig. 2). We found that coronin 1-deficient single positive thymocytes are affected to a similar extend as naïve T-cells with respect to spontaneous apoptosis, but that coronin 1-deficient double positive thymocytes as well as memory/effector T-cells showed only a mild increase in apoptosis when compared to the wild type control cells.

These results indicate that the increased phalloidin fluorescence upon depletion of coronin 1 does not correlate with induction of apoptosis in the analyzed leukocyte populations. The phalloidin signal was increased in all cellular subsets analyzed lacking coronin 1, when compared to wild type cells. However, only the single positive thymic and naïve T-cell subsets showed an increase in apoptosis. Moreover, since the cellular numbers in the thymus are only modestly decreased, a developmental defect is unlikely to be the reason for the decreased naïve T cell numbers.

Possibly, the observed increase in phalloidin fluorescence is a result of coronin 1 depletion rather than of increased F-actin. To directly address this possibility, the F/G-actin ratio in wild type or coronin 1-deficient cells was analyzed under conditions where

the F-actin cytoskeleton was perturbed by the addition of F-actin interfering drugs Jasplakinolide (to induce polymerization of F-actin (19)) or Latrunculin B (to depolymerize F-actin (20)). As expected, the addition of Jasplakinolide resulted in F-actin polymerization while the presence of Latrunculin B induced depolymerization of F-actin in both wild type as well as coronin 1-deficient cells to the same extent (Fig. 3A). In order to demonstrate the sensitivity as well as the reproducibility of the biochemical F versus G-actin assay, untreated wild type and coronin 1-deficient T-cells were compared with wild type cells exposed to CCL19, a member of the chemokines that result in the induction of F-actin polymerization (21). The amount of CCL19 was titrated to yield a similar elevation of phalloidin staining as observed in the untreated coronin 1-deficient T-cells (Fig. 3B). While CCL19 induced the same increase in phalloidin fluorescence as coronin 1 deletion (Fig. 3B), only CCL19 triggering resulted in an elevation of the F/G-actin ratio as measured biochemically (Fig. 3C, D). Finally, to analyze whether F-actin modulation results in elevated spontaneous apoptosis, as suggested earlier (3), wild type $CD4^+$ single positive thymocytes were incubated with the F-actin modulating reagents as indicated in panel E and the viability of the cells was analyzed by Annexin V and 7-AAD staining. As shown in Fig. 3, Panel E, neither depolymerization nor polymerization of the F-actin cytoskeleton in wild type cells resulted in an altered viability while untreated coronin 1-deficient cells showed a severe defect in survival.

To further analyze the correlation between coronin 1 expression, F-actin accumulation and Annexin V labeling two additional experiments were performed. First, coronin 1 was depleted in the human T-cell line Jurkat using siRNA specific for mouse (control) or human coronin 1. While coronin 1 expression was effectively knocked down in Jurkat cells expressing the human, but not the mouse siRNA (Supp. Fig. 2A), the ratio of F/G

actin as measured biochemically was similar in wild type and coronin 1 depleted cells (Supp Fig. 2B). However, phalloidin fluorescence increased ~2-fold upon coronin 1 depletion (Supp Fig. 2C). Analysis of Annexin V labeling revealed no differences (Supp Fig. 2D). Also, incubation of Jurkat cells expressing the murine or human specific siRNA with the apoptosis inducing agent tumor necrosis factor-α (TNF-α) revealed no differences in either the time course or degree of apoptosis induction (Supp Fig. 2D, insert). Conversely, while in the non-leukocyte cell line Mel JuSo transfected with cDNA encoding coronin 1 (1), expression of coronin 1 resulted in a decrease of phalloidin fluorescence, as observed in T-cells, no differences were found in the F/G actin ratio or Annexin V labeling of control versus coronin 1 expressing cells (Supp Fig. 2E-H). In addition, incubation of control or coronin 1 expressing Mel JuSo cells with the apoptosis inducing agent staurosporin revealed no differences in either the time course or degree of apoptosis induction (Supp Fig. 2H, insert). In accordance with these findings, staurosporin induced the same degree of apoptosis in both wild type and coronin 1-deficient B-cells (Supp. Fig. 3).

These results therefore corroborate the conclusion that expression or deletion of coronin 1 interferes with phalloidin fluorescence, but does not affect F-actin levels nor has a direct effect on or protects cells from apoptosis.

Chemokine induced migration of wild type and coronin 1-deficient T-cells

An important question to address was why, in the absence of an F-actin phenotype, coronin 1-deficient T-cells as opposed to B-cells (5), macrophages (16, 22) and neutrophils (23), show a significantly lower migratory capacity in addition to a survival defect (Figure 1 and 2), when compared to wild type cells.

To assess the differences in migratory capacity between different subsets of coronin 1-deficient and wild type leukocytes, wild type and coronin 1-deficient T cell subsets as well as B-cells and thymic subsets were analyzed for their ability to migrate in a transwell migration assay. Naïve $CD4^+$ T-cells and B-cells (Fig. 4), isolated from wild type or coronin 1-deficient mice, were analyzed for their ability to migrate in a transwell migration assay towards the indicated concentrations of chemo-attractants. When compared with the data presented in Figure 2, our results demonstrate that freshly isolated coronin 1-deficient naïve T-cells show elevated levels of spontaneous apoptosis and that at the same time the number of migration competent naïve T-cells is far lower in case of coronin 1-deficient cells as compared to the wild type control. However, in B-cells, which in the absence of coronin 1 show elevated levels of phalloidin fluorescence similar to that observed in T-cells (see Fig. 1), neither the cellular viability nor their capacity to migrate is affected.

Using the same experimental setup we have also analyzed double positive thymocytes, $CD4^+$ single positive thymocytes and memory/effector T-cells for their ability to migrate in a transwell migration assay (Fig. 4). We found that coronin 1-deficient single positive thymocytes are affected to a similar extend as naïve T-cells regarding spontaneous apoptosis (Figure 2) as well as transwell migration (Fig. 4), but that coronin 1-deficient double positive thymocytes as well as memory/effector T-cells only show a mild increase in apoptosis as well as a minor reduction in transwell migration when compared to the wild type control.

This prompted us to hypothesize that the two observed defects are connected, due to the same molecular defect and are naïve T-cell specific as well as coronin 1-dependent.

A feature which distinguishes T-cells from other leukocytes is their TCR. Downstream signaling of the TCR is essential for naïve T-cell survival (24) as well as defective in coronin 1-deficient naïve T-cells (2). To analyze whether the migration defect is a cell intrinsic property of coronin 1-deficient cells, related to a defect in F-action regulation, or secondary to another defect, such as defective TCR signaling (2), the following experiment was designed (Fig. 5A): Wild type and coronin 1-deficient single positive thymocytes as well as naïve T-cells ($CD62L^{high}$, $CD44^{low}$) were analyzed for phalloidin staining, spontaneous apoptosis as well as their ability to migrate in a transwell migration assay. After this first transwell migration assay, the migrated wild type and coronin 1-deficient cells were washed extensively and further recovered in fresh medium for 1 hour at 37°C. The recovered cells were used for the same analysis as described above for a second time, i.e. phalloidin staining, spontaneous apoptosis and transwell migration. If reduced viability and migration were cell intrinsic properties of coronin 1-deficient naïve T cells the previously migrated coronin 1-deficient cells should display the same defects when reanalyzed after recovery using the same assays and conditions. As can be seen in Fig. 5 B and C, freshly isolated coronin 1-deficient $CD4^+$ single positive thymocytes as well as naïve T-cells displayed defects in cellular viability and migration as well as yielded a ~2 fold higher phalloidin fluorescence when compared to the wild type control. However, when coronin 1-deficient single positive thymocytes as well as naïve T-cells that had migrated in the first assay, were allowed to recover and were subjected to the second round of assays, neither higher levels of spontaneous apoptosis nor a defect in cell migration were observed when compared to the wild type control (Fig. 5D, E).

In order to exclude that CCL19 by itself was responsible for the observed effects and not selection by migration, the same assay as depicted in Fig. 5 was performed without selection for migrated cells (Supp. Fig. 4). Survival and migration was analyzed for cells

that had been left in migration medium for 3h, had been incubated with the same amount of CCL19 used for transwell migration for 3h or had migrated for 3h as described above. As can be seen from our data CCL19 by itself had no effect on migration or survival of coronin 1-deficient cells in any of the conditions tested.

Noteworthy is also the fact that migrated and recovered coronin 1-deficient cells still showed the same elevated phalloidin fluorescence as observed for freshly isolated cells.

The data presented thus far demonstrate a clear correlation between the reduced viability of coronin 1-deficient T-cells and their inability to migrate in a transwell assay. To directly address the importance of cellular viability in T-cell migration, the ability of wild type naïve T-cells in which apoptosis had been induced using agents such as Etoposide, C6 Ceramide, Dexamethasone and anti-CD3/CD95/cyclosporine A was analyzed (data not shown). As expected, the manipulated cells were no longer capable of migrating to the same extent as the untreated control cells.

Conversively, to analyze the result of increasing the viability of coronin 1-deficient naïve T-cells on migration as well as subsequent survival, cells were incubated for 20h with the anti-apoptotic cytokine interleukin-7 (IL-7) (25, 26) and the same assays as described above (Fig. 5) were performed, namely phalloidin staining, transwell migration and survival with or without added IL-7. As shown in Fig. 6, IL-7 restored the viability of coronin 1-deficient naïve T-cells and also restored the migratory capacity to wild type levels. Together these data therefore establish that reduction of T-cell viability severely affects their ability to migrate, and conversively, that increasing the viability of coronin 1-deficient naïve T-cells restores their ability to migrate and survive.

Talin distribution, video microscopy and in vivo migration of wild type and coronin 1-deficient T-cells

To further analyze a role for coronin 1 in cytoskeletal reorganization, the distribution of talin was analyzed. Talin is a uropod-associated protein that is involved in cytoskeletal organization of migrating cells, the localization of which was found to be deregulated in coronin 1-deficient T cells (3). However, when recovered wild type or coronin 1-deficient T cells were stimulated with CCL19, no difference was observed either in talin distribution or uropod formation (Fig. 7A and B). In addition, video microscopy of a mixed culture of wild type and coronin 1-deficient T cells showed qualitatively and quantitatively similar migratory behavior (Supp movies 1 and 2 and Fig. 7C, D). Finally, to analyze T cell migration *in vivo*, cell tracker orange labeled, mixed wild type and coronin 1-deficient (expressing GFP) CD4 single positive thymocytes were transferred into recipient wild type mice and homing to lymphoid organs was analyzed after 20h (Fig 7E). The results show that freshly isolated coronin 1-deficient cells fail to migrate to the same extend as the wild type control, whereas cells selected using a transwell assay show no *in vivo* migration defect.

Taken together, these data suggest that the observed migratory defect in coronin 1-deficient T-cells is a secondary defect which is directly linked with T-cell survival and not a result of disturbed F-actin dynamics.

The molecular basis of the observed defects in coronin 1-deficient naïve T-cells

Maintenance of naïve T-cell homeostasis depends on signals that are predominantly delivered via the TCR (24, 27, 28). In the absence of coronin 1, naïve T-cells show a specific defect in TCR dependent signaling and in particular calcium signaling (see Supp Fig. 5A and (2)). The specificity of this defect is demonstrated by the fact that in the presence of a TCR-specific stimulation, proliferation was strongly reduced in coronin 1-deficient naïve T-cells, while stimulation with PMA/Ionomycin, which bypasses the TCR thus directly acting on downstream signaling pathways (29), was comparable between coronin 1-deficient cells and the wild type control (Supp Fig. 5B).

A direct consequence of TCR ligation and subsequent elevation of the cytosolic calcium concentration is activation of the phosphatase calcineurin which results in nuclear translocation of nuclear factor of activated T-cells (NFAT) and subsequent transcriptional activation of target genes such as IL-2 (30, 31). Calcineurin is required for naïve T cell survival (32, 33), and interestingly has been previously reported to depend on coronin 1 for its activation during mycobacterial infection in macrophages (16). Therefore, the levels and activity of calcineurin was measured in wild type or coronin 1-deficient naïve T-cells. While the total levels of calcineurin Aβ, which is the predominantly expressed isoforms in T lymphocytes (33), were identical in wild type and coronin 1-deficient cells (Supp Fig. 5C), calcineurin phosphatase activity was severely reduced upon TCR ligation in the absence of coronin 1 (Supp Fig. 5D). However, when coronin 1-deficient as well as wild type naïve T-cells were stimulated with PMA and Ionomycin, no significant differences in calcineurin activation were detected (Supp Fig. 5E), consistent with the proliferation data presented above.

These data therefore demonstrate that the defect in coronin 1-deficient naïve T-cells is TCR specific as both coronin 1-deficient as well as wild type cells showed similar

responses upon stimulation with the TCR bypassing reagents PMA and Ionomycin, whereas a TCR specific stimulus resulted in defective calcium signaling, calcineurin activation as well as proliferation in coronin 1-deficient naïve T-cells. Coronin 1 exerts its function in TCR signaling only at the stage of phospholipase Cγ1 activation and endoplasmic reticulum calcium release as early signaling events such as CD3 zeta phosphorylation are not affected (Supp. Fig. 6 and (2)). We conclude that coronin 1 by regulating calcium/calcineurin signaling upon TCR stimulation is a key regulator of naïve T-cell survival.

To independently show that calcineurin activation is the critical step in the signaling regulated by coronin 1, migrated wild type and coronin 1-deficient naïve T-cells (as used in Figure 5D and E), were incubated with or without the specific calcineurin inhibitor cyclosporine A (CsA) in the presence or absence of either α-CD3/CD28 coated dynal-beads or plate bound α-CD3/CD28 (Fig. 8A,B). Measurement of cell viability after 20h revealed that α-CD3/CD28 treatment increased the rate of apoptosis in coronin 1-deficient naïve T-cells as compared to the untreated control. Importantly, the coronin 1-deficient phenotype is phenocopied by incubating wild type cells with CsA. Also, the beneficial effect of IL-7 on cell survival was ablated by α-CD3/CD28 treatment in the absence of coronin 1, indicating that naïve T-cells are susceptible to activation induced cell death upon α-CD3/CD28 stimulation in the absence of coronin 1 or upon inhibition of calcineurin via CsA. One expected consequence of defective calcineurin activation in T-cells is the down-regulation of the anti-apoptotic molecule Bcl-2 (33). Bcl-2 levels were significantly lower in coronin 1-deficient cells as compared to the wild type control (Fig. 8C). This effect could be mimicked by incubating wild type cells with the specific

calcineurin inhibitors CsA or FK506 (Fig. 8D), again showing that blocking calcineurin in wild type cells phenocopies the coronin 1 deletion.

Furthermore, while in freshly isolated cells Bcl-2 levels were significantly lower in the absence of coronin 1, migrated coronin 1-deficient cells showed levels similar to the wild type control (Fig. 8E).

Finally, given the recent report that that inhibition of calcineurin in the presence of TCR stimulation and co-stimulation via CD28 leads to a super-induction of caspase 3, rendering cells susceptible to apoptosis (34) the levels of caspase 3 in wild type and coronin 1-deficient naïve T cells were analyzed (Fig. 8F). While in freshly isolated cells, caspase 3 levels were significantly elevated in the absence of coronin 1, migrated coronin 1-deficient cells showed levels similar to the wild type control.

These findings explain the elevated levels of apoptosis observed in freshly isolated coronin 1-deficient naïve T-cells in molecular terms, namely that the lack of coronin 1 causes a defect in calcineurin activation upon TCR stimulation which in turn leads to a massive induction of caspase 3 and reduced expression of Bcl-2.

Discussion

Coronin 1, also known as P57 or TACO (for tryptophan aspartate containing coat protein (1)), is a leukocyte specific molecule that is crucial for the survival and migration of naïve T-cells in the periphery (2-6). Based on the ~25% homology of coronin 1 with *Dictyostelium* coronin, a molecule that co-purifies with an actin/myosin complex, as well as *in vitro* actin binding assays, it has been widely suggested that coronin 1 allows cellular survival and migration by preventing F-actin formation. Since coronin 1 deletion results in a ~2-fold increase in phalloidin staining, absence of coronin 1 was concluded to lead to excessive F-actin accumulation thereby preventing cell migration and inducing apoptosis (3). In this paper, we show that coronin 1 does not modulate the F-actin cytoskeleton in leukocytes and non-leukocytes, and furthermore, that F-actin accumulation does not correlate with apoptosis in naïve T-cells as proposed previously (3). Instead, we find that both survival as well as migration defects occurred as a result of defective signaling and calcineurin activation in coronin 1-deficient naïve T-cells. We further demonstrate that cellular viability and the ability to migrate are linked, providing an explanation for the observed migration defect. We conclude that instead of regulating the F-actin cytoskeleton, coronin 1 functions in balancing pro- and anti-apoptotic signals by regulating Ca^{2+} fluxes and calcineurin activation downstream of the TCR.

Coronin 1 and F-actin

The evidence linking coronin 1 to the modulation of the F-actin cytoskeleton is predominantly based on the observed two-fold difference in phalloidin fluorescence between wild type and coronin 1-deficient cells (3-5). Several lines of evidence however argue against the differential phalloidin fluorescence being an appropriate measure for altered F-actin dynamics in cells expressing different levels of coronin 1. First, a sensitive

biochemical assay, that accurately measured the influence of chemoattractant-induced F-actin accumulation on the F/G-actin ratio, failed to show any difference in the F/G actin ratio between coronin 1 negative and coronin 1 expressing cells. This is not only the case for T-cells, but also for macrophages deficient for coronin 1 (16) or depleted for coronin 1 using siRNA (22), Jurkat cells depleted for coronin 1 using siRNA and Mel JuSo cells over expressing coronin 1 (this manuscript). Second, depletion of coronin 1 in macrophages, B cells and neutrophils does not affect any of the F-actin dependent functions, such as phagocytosis, macropinocytosis and migration (16, 22, 23, 35) and this manuscript). Third, rather than being an indication of reduced F-actin formation in coronin 1 expressing cells, the reduced phalloidin fluorescence may be a result of a reduction in phalloidin binding to F-actin because of the presence of large amounts of coronin 1 that sterically hinder phalloidin binding to F-actin at the cell cortex. A similar observation has been reported previously, where F-actin cables could not be stained using phalloidin due to accessory F-actin binding factors but could be labeled using α-actin antibodies (36). These findings are also consistent with the observation that despite a 2-fold difference in phalloidin fluorescence we were unable to detect any differences regarding cortical F-actin levels using α-actin antibodies in coronin 1-deficient versus wild type cells (2).

While *in vitro*, coronin 1 has been shown to co-precipitate with F-actin (37, 38), and modulate F-actin filament dynamics (39-41), a survey of all available literature on coronin 1 reveals that no data exist showing that coronin 1 directly participates in modulating F-actin dynamics *in vivo*. Most of the previous studies linking coronin 1 activity to F-actin dynamics rely either on (i) *in vitro* co-sedimentation of coronin 1 fusion proteins with F-actin, (ii) expression of coronin 1 domains in cell lines or (iii) a difference in phalloidin

fluorescence. However, it is important to note that the here described results relate to the activity of mammalian coronin 1. In contrast to the single coronin gene in *Dictyostelium*, mammals express seven coronin isoforms. It is well possible that other mammalian coronin isoforms have maintained an F- actin regulatory function, and that this function has been lost in mammalian coronin 1.

F-actin and Apoptosis

One important argument put forward to implicate coronin 1 in preventing F-actin induced apoptosis is based on the observed increase in cell death upon forced F-actin formation in yeast (42) as well as mammalian cell lines (43). However, when the actin cytoskeleton of primary murine naïve T-cells was polymerized or depolymerized using actin modulating drugs, no differences in apoptosis were apparent (this study). Further, none of the other leukocyte populations such as B-cells and macrophages, which have been found to be sensitive to F-actin accumulation (44), display a survival defect despite showing elevated phalloidin staining similar to T-cells. Moreover, induction of apoptosis in a variety of coronin 1-expressing and –deficient cell types by TNF-α or staurosporin demonstrated that coronin 1 does not protect cells from apoptosis. It is noteworthy that at least staurosporin-induced apoptosis involves F-actin and that proteins which prevent F-actin formation such as human gelsoline, unlike coronin 1, are able to prevent the onset of apoptosis upon staurosporin treatment (45).

We therefore conclude that F-actin modulation cannot account for the elevated levels of apoptosis in coronin 1-deficient cells and that coronin 1 does not protect cells from apoptosis.

Coronin 1 and Calcineurin Activation

Although early signaling events such as CD3 zeta chain phosphorylation as well as PLCγ1 phosphorylation and activation are normal in the absence of coronin 1 ((2) and Supp. Fig. 6), in coronin 1-deficient cells calcineurin activation following TCR ligation cannot occur because of defective calcium mobilization. Interestingly, there is a striking resemblance between a recently described calcineurin Aβ-deficient mouse model and mice lacking coronin 1 (32, 33). Both calcineurin Aβ- and coronin 1-deficient single positive thymocytes as well as naïve T-cells are specifically depleted *in vivo* and show elevated spontaneous apoptosis *in vitro* while double positive thymocytes as well as memory/effector T-cells and B-cells show none or only mild defects. Experiments with calcineurin Aβ deficient mice have demonstrated that defective activation of the phosphatase leads to susceptibility of T-cells to apoptosis induction due to an imbalance between pro- and anti-apoptotic stimuli, leading to reduced naïve T-cell viability and finally cell death. Consistent with these results, we found that inhibition of calcineurin in naïve wild type T cells phenocopies the coronin 1-deficient T cells, demonstrating that calcineurin activation is the critical step in signaling regulated by coronin 1.

The data presented in this manuscript not only highlight the importance of coronin 1 in calcineurin activation but also provide a rational for the observed migration and survival defects in the absence of coronin 1. As we demonstrate, Bcl-2 levels are reduced and caspase 3 is induced at high level in coronin 1-deficient naïve T-cells as a consequence of defective calcineurin activation. Furthermore, the data described here provide, on the molecular level, an explanation for the diverse phenotypes such as migration and survival defects described for naïve T-cells from coronin 1-deficient mice.

These data may further explain the dependence of T-cells on coronin 1, in relation to their differentiation status. On the one hand, it has been shown that Bcl-x, another anti-apoptotic molecule of the Bcl-2 family, is selectively expressed in double positive thymocytes and activated T-cells, which are largely unaffected by the loss of coronin 1 (46, 47). On the other hand, the loss of Bcl-2 has only little effect on the thymic development of T-cells but severely effects naïve T-cell survival in the periphery (48). A possible explanation for the predominant loss of naïve T-cells as observed upon coronin 1 deletion could thus be, that during thymic development as well as once T cells have developed beyond the naïve state, expression of Bcl-x can, at least in part, compensate for the loss of coronin 1.

The here presented results highlight the importance of coronin 1 dependent calcium and calcineurin signaling for cellular survival and provide a direct link between coronin 1, TCR mediated calcineurin activation and calcineurin-dependent cellular viability. It should be noted, that the experiments described in Figure 8 and Supplementary Figure 5/6 were not meant to mimic tonic TCR signals, which are fundamental for naïve T-cell homeostasis in vivo (49-52), but to delineate the underlying molecular defect and to show that calcineurin activation is the crucial step in the signaling cascade regulated by coronin 1. Of note, calcineurin Aβ deficient naïve T-cells still demonstrate a partial proliferative and cytokine responsiveness upon CD3 receptor cross-linking, as do coronin 1-deficient naïve T-cells, indicating that other, calcineurin Aβ independent and probably also coronin 1 independent, signaling pathways influence T-cell activation and peripheral survival (53), providing an explanation as to why there are some non apoptotic naïve T-cells left in these mice. As some TCR downstream signals are annihilated or at least attenuated in coronin 1-deficient mice (2) (in contrast to calcineurin Aβ deficient T-cells, calcineurin is

fully functional in the absence of coronin 1 as demonstrated by PMA/ionomycin stimulation, Supplementary Figure 5) the hampered downstream signal delivery, upon TCR triggering, is likely to increase the signaling/activation threshold that has to be reached in order to keep naïve, peripheral T-cells alive. Consequently, less naïve T-cells will receive sufficient survival signals, resulting in the observed loss of these cells in the absence of coronin 1. Through the analysis of healthy, non-apoptotic coronin 1-deficient naïve T-cells from an otherwise heterogeneous population, these data also allow to compare healthy, perfectly viable coronin 1-deficient naïve T-cells with wild type cells, explaining most if not all of the discrepancies with previous studies. Furthermore, these data offer an explanation of why the observed defects are specific for single positive thymocytes as well as naïve T-cells as these cells are heavily dependent on calcineurin Aβ for their survival (31).

In summary, we show that the decreased viability of naïve coronin 1-deficient T-cells is not a result of deregulated F-actin levels and that F-actin increase by itself was not sufficient to induce apoptosis in these cells. Furthermore, we demonstrate that the reduced migratory capacity of coronin 1-deficient T-cells is not an intrinsic property of cells lacking coronin 1, but due to reduced viability and could be restored by providing pro-survival signals; vice versa, when wild type cells were incubated with apoptosis inducing agents these cells showed a reduced ability to migrate.

We further show that upon deletion of coronin 1, the balance between bcl-2 and caspase 3 is disturbed as a result of deficient TCR mediated calcineurin activation, that is known to be coronin 1-dependent in leukocytes (16), thus providing an explanation for the increased susceptibility of coronin 1-deficient naïve T-cells to undergo apoptosis. Finally, the compromised viability of coronin 1-deficient naïve T-cells, as a result of defective

TCR signaling leads to a reduced migratory capacity of coronin 1-deficient cells and consequently hampers naïve T-cell homing in these animals.

The definition of coronin 1 as an essential regulator of pro-survival signals in naïve T-cells and consequently their ability to survive and migrate may allow a better understanding of leukocyte activation, recruitment and homeostasis.

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I think JI does not like financial statements here!!!

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Figure Legends

Figure 1: Coronin 1 expression, phalloidin fluorescence and apoptosis in wild type, heterozygous and coronin 1-deficient cells. Indicated cell populations from wild type, heterozygous and coronin 1-deficient mice were stained for coronin 1 using an AlexaFluor633 conjugated polyclonal α-coronin 1 antibody (A, *left column*) as well as Alexa Fluor 568 or 633 conjugated phalloidin (B, *middle column*) and PE or APC-conjugated Annexin V (C, *right column*). In order to achieve the best possible separation (phalloidin staining), we used different voltage settings for macrophages (lowest voltage), T-cells and B-cells (same, medium voltage) as well as double and single positive thymocytes (same, highest voltage). Within a figure the settings were the same. Depicted are mean +/– SD of three mice in each group. Stainings were repeated at least two times and analyzed using FACS.

Figure 2 Ex vivo detection of spontaneous apoptosis in splenic T-cell and B-cell as well as Thymocyte Subsets in the presence and absence of coronin 1. Spontaneous apoptosis of freshly isolated splenic T-cell and B-cell as well as thymocyte subsets, depleted of apoptotic and dead cells upon isolation (0h), was measured after 20h in culture as described in methods. Depicted are means +/– SD of duplicates. The experiments were repeated at least 3 times.

Figure 3: F/G-actin ratio, spontaneous apoptosis and F-actin induced apoptosis in wild type and coronin 1-deficient T-cells. (A) Wild type (+/+) or coronin 1-deficient (–/–) splenic T-cells were left untreated or treated with either Jasplakinolide (1 μM) or Latrunculin B (4 μM) for 45 min at room temperature followed by cell lysis. Proteins from supernatants (S) and pellets (P) prepared as described in methods were separated by

SDS-PAGE and immuno-blotted using α-actin antibodies. (B) Wild type T-cells were treated with CCL19 (250 ng/ml for 60 seconds) to result in the same phalloidin staining as coronin 1-deficient T-cells, shown is a representative histogram of untreated and CCL19 wild type T-cells as well as untreated coronin 1-deficient T-cells stained with phalloidin-633. (C) Cells treated as in B were analyzed for F/G actin as described in methods. (D) Quantitation of a representative experiment out of three as described under B and C. Shown are mean +/– SD of three independent samples. (E) Wild type $CD4^+$ single positive thymocytes were incubated for 4 and 20h with DMSO (carrier), Latrunculin B (LatB, 4 μM) or Jasplakinolide (JP, 1 μM) at 37°C, 5% CO_2 as indicated and stained with Annexin V and 7-AAD at the indicated time points. Coronin 1-deficient cells treated with DMSO were used as an internal control. Cells negative for both Annexin V and 7-AAD were considered as viable cells. Experiments were performed at least three times in triplicates.

Figure 4: In vitro Migration of splenic T-cell and B-cell as well as Thymocyte Subsets in the presence and absence of coronin 1 Transwell migration of freshly isolated splenic T-cell and B-cell as well as thymocyte subsets, using the indicated chemo-attractants. Depicted are means +/– SD of duplicates. The experiments were repeated at least 3 times.

Figure 5: Phalloidin fluorescence, survival and migration of migrated wild type and coronin 1-deficient single positive thymocytes and naive T-cells following recovery. (A) Schematic outline of the experiment as described under B-E. (B) Freshly isolated $CD4^+$ single positive thymocytes from which all dead and apoptotic cells had been removed (see method) were stained for phalloidin and assayed for spontaneous apoptosis

as well as transwell migration against 300 ng/ml of CCL19 in the lower chamber. (C) Assay as described under B performed with naive splenic T-cells. (D and E) Cells as used in B and C were subjected to a transwell migration assay against 300 ng/ml of CCL19 in the lower chamber. Migrated cells were washed to remove all CCL19 and left to recover in complete medium for 1h at 37°C/5% CO_2. After this period, cells were again subjected to phalloidin staining and assayed for spontaneous apoptosis as well as transwell migration as done under B and C. Depicted are means +/– SD of duplicates. The experiments were repeated at least 3 times.

Figure 6: Phalloidin fluorescence, survival and migration of wild type and coronin 1-deficient naive T-cells following IL-7 treatment.

(A) Freshly isolated naive splenic T-cells from which all dead and apoptotic cells had been removed (see method) were stained for phalloidin and assayed for spontaneous apoptosis as well as transwell migration against 300 ng/ml of CCL19 in the lower chamber. (B) Cells as used in A were treated with interleukin-7 (IL-7, 20ng/ml, mouse recombinant from R&D). After this period, cells were analyzed using Annexin V/7-AAD staining to determine the number of viable cells. (C) Dead cells were removed from the IL-7 treated population as described in methods, viable cells were subjected to phalloidin staining, assayed for spontaneous apoptosis (with or without the addition of 20ng/ml recombinant IL-7) and transwell migration as done under A. Depicted are means +/– SD of duplicates. The experiments were performed 3 times.

Figure 7: Talin redistribution and in vivo and vitro migration of transwell selected coronin 1-deficient T-cells. (A and B) Mixed wild type and coronin 1-deficient cells, selected by transwell migration, were seeded on ICAM-1 coated coverslips and incubated

for 1h at 37 degree to allow the cells to attach. Cells were left untreated or were stimulated by adding CCL19 to a final concentration of 250 ng/ml for 5 min. After this, cells were immediately fixed using 4% PFA and subject to immunofluorescence staining of talin and coronin 1. The experiments were repeated at least 3 times. (C, D) Mixed wild type and coronin 1-deficient cells, selected by transwell migration, were seeded on ICAM-1 coated coverslips and incubated for 1h at 37 degree to allow the cells to attach. Cells were stimulated by adding CCL19 to a final concentration of 250 ng/ml. Time lapse movies were recorded (40x magnification, 1.25 N.A) monitoring phase contrast and the GFP channel simultaneously with a time interval of 10s.The migrated cells were tracked by Image-J using a manual tracking plug-in. Average speed (C) and speed distribution (D) were calculated from 100 wild type and 100 coronin 1-deficient cells using data from 15 different movies obtained from 5 independent experiments. See also Supp. Movies 1 and 2. (E) Cell tracker orange labeled, mixed wild type and coronin 1-deficient (expressing GFP) CD4 single positive thymocytes, freshly isolated or selected for migration in a transwell assay, were injected into the tail vain of recipient wild type mice and analyzed for their homing to lymphoid organs after 20h. Shown are averages of 7 mice for each condition from 4 independent experiments +/– SD.

Figure 8: TCR signaling in wild type and coronin 1-deficient naive T-cells in the presence and absence of IL-7 and calcineurin inhibitors. Migrated wild type and coronin 1-deficient naive T-cells were analyzed for their survival using the indicated reagents and (A) α-CD3/28 coated dynal beads (T-cell expander, Dynal) or (B) plate bound α-CD3/28 (coated with 5/10 μg/ml of the indicated antibodies in PBS at 4°C o/n). Cyclosporine A was used at 5μM and IL-7 at 20ng/ml. Depicted are means +/– SD of duplicates. The experiments were repeated 3 times.. (C) Bcl-2 levels were detected in

lysates of freshly isolated naive T-cells from wild type or coronin 1-deficient mice using hamster α-mouse Bcl-2 from BD. (D) Bcl-2 levels were detected in lysates of freshly isolated wild type naive T-cells or the same cells incubated o/n on α-CD3/28 plates (coated with 5/10 μg/ml of the indicated antibodies in PBS at 4°C o/n) with or without the addition of the calcineurin inhibitors cyclosporine A or FK506 (5μM and 10μM respectively). The experiments were repeated 2-4 times. (E) Bcl-2 levels were detected in lysates of freshly isolated or migrated naive T-cells from wild type or coronin 1-deficient mice. (F) Caspase-3 levels were detected in lysates of freshly isolated or migrated naive T-cells from wild type or coronin 1-deficient mice The experiments were repeated 2 times.

Figure 1

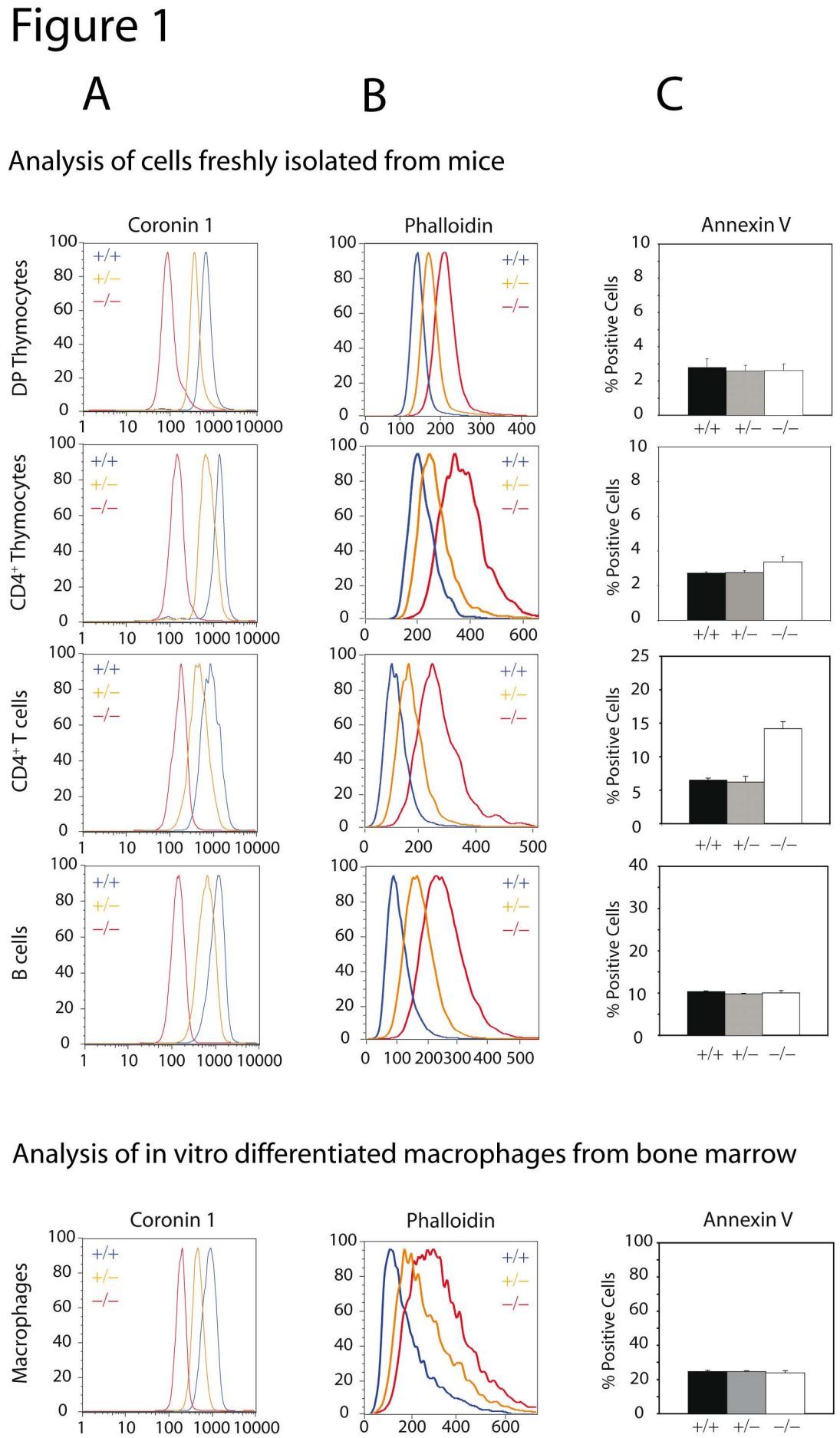

A
B
C
Analysis of cells freshly isolated from mice
Coronin 1
Phalloidin
Annexin V
DP Thymocytes
CD4+ Thymocytes
CD4+ T cells
B cells
+/+
+/–
–/–
% Positive Cells
Analysis of in vitro differentiated macrophages from bone marrow
Macrophages

Figure 2

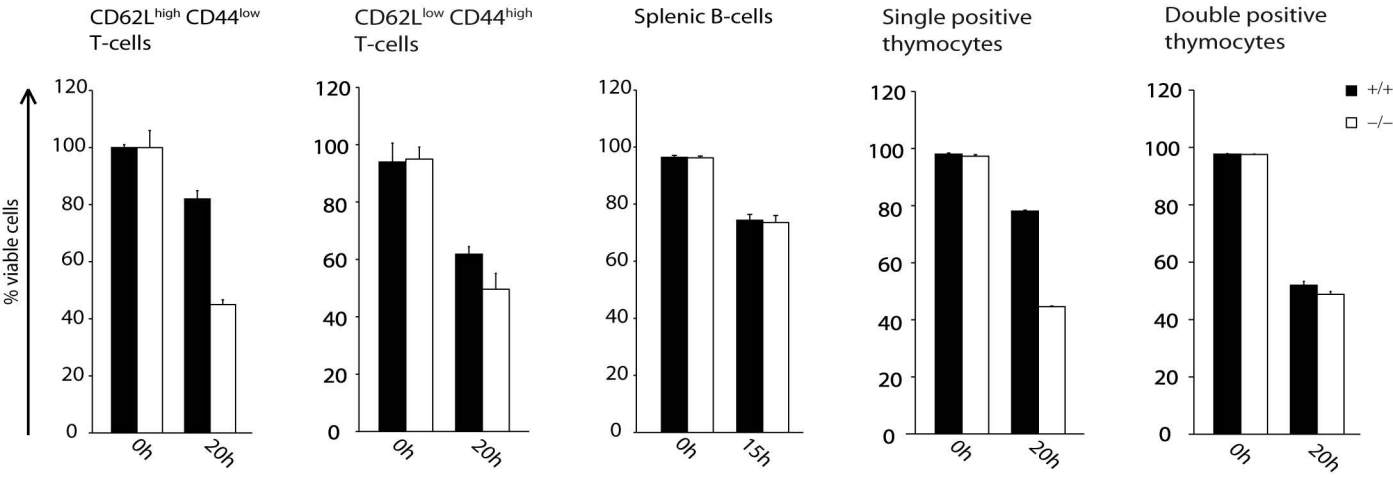

CD62L^high CD44^low T-cells
CD62L^low CD44^high T-cells
Splenic B-cells
Single positive thymocytes
Double positive thymocytes
+/+
−/−
% viable cells
120
100
80
60
40
20
0
0h
20h
15h

Figure 3

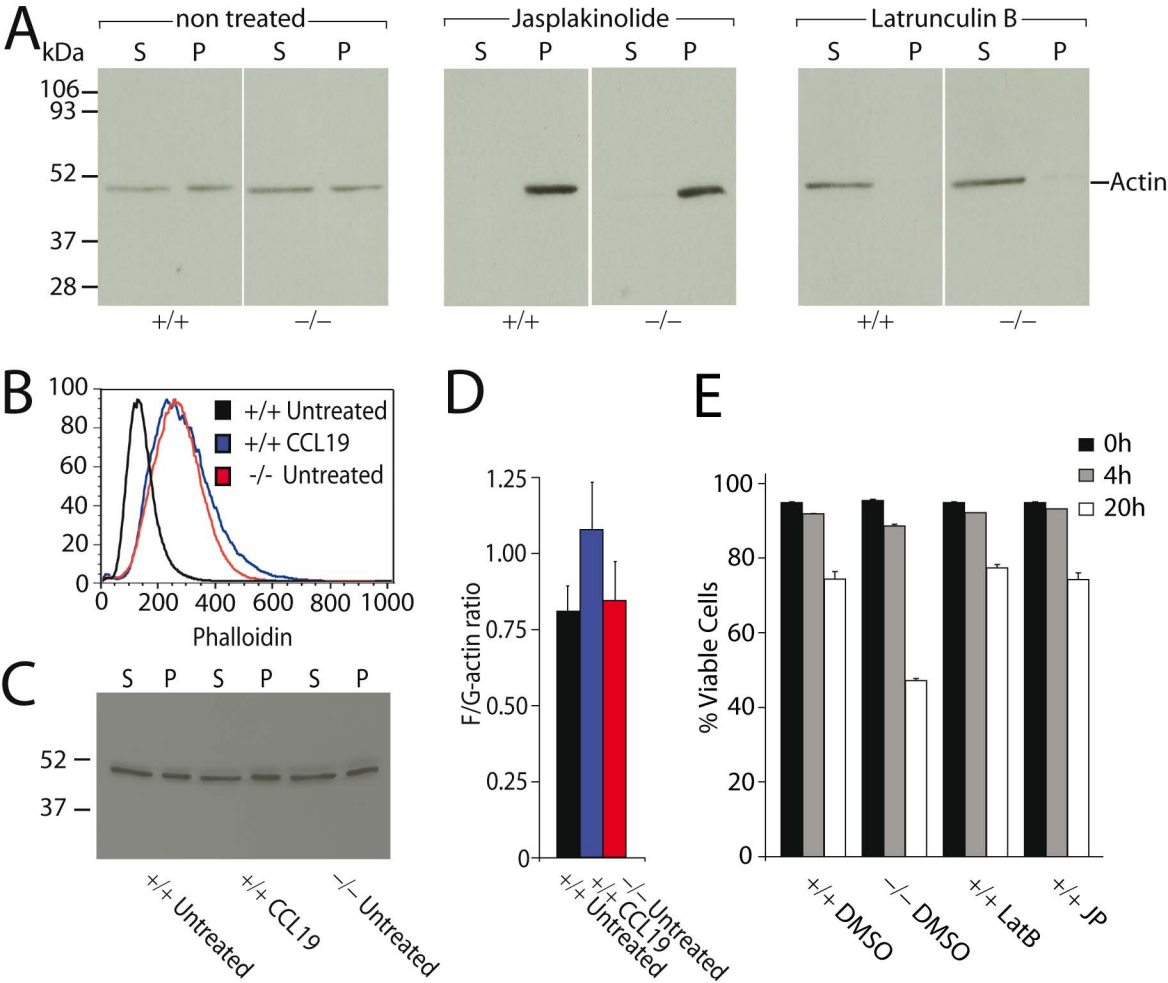
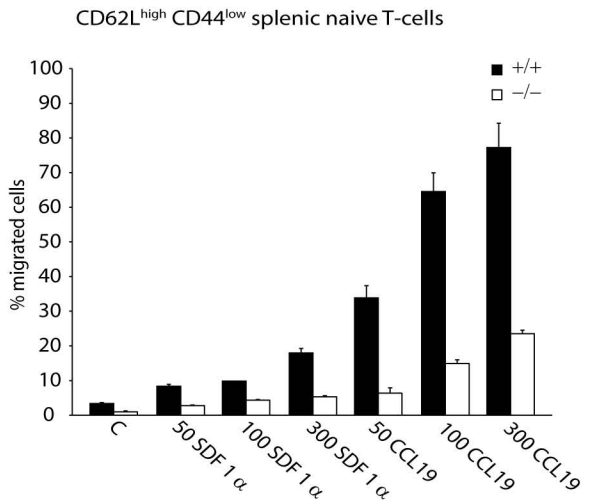
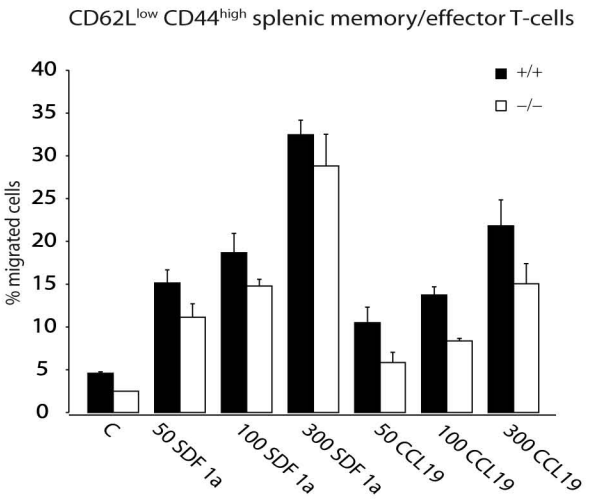
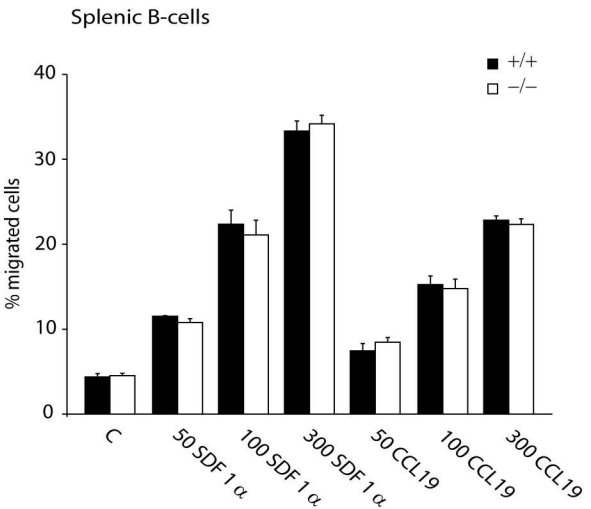
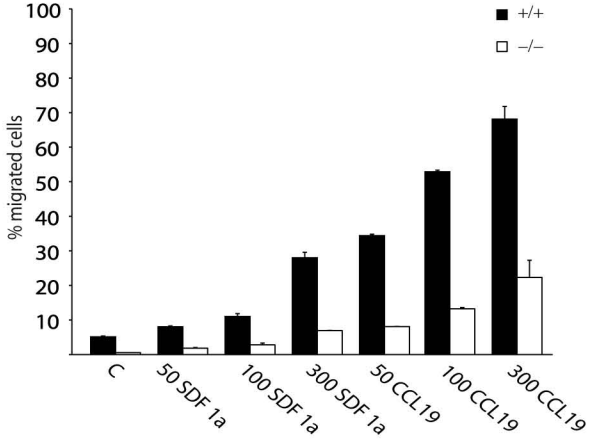

A
non treated
Jasplakinolide
Latrunculin B
kDa
S P S P
106
93
52
37
28
+/+ −/−
Actin
B
100
80
60
40
20
0
0 200 400 600 800 1000
Phalloidin
+/+ Untreated
+/+ CCL19
-/- Untreated
C
S P S P S P
52
37
+/+ Untreated
+/+ CCL19
−/− Untreated
D
F/G-actin ratio
1.25
1.00
0.75
0.50
0.25
0
+/+ Untreated
+/+ CCL19
−/− Untreated
E
% Viable Cells
100
80
60
40
20
0
0h
4h
20h
+/+ DMSO
−/− DMSO
+/+ LatB
+/+ JP

Figure 4


CD62L^high CD44^low splenic naive T-cells
% migrated cells
+/+
–/–
C
50 SDF 1α
100 SDF 1α
300 SDF 1α
50 CCL19
100 CCL19
300 CCL19


CD62L^low CD44^high splenic memory/effector T-cells
% migrated cells
+/+
–/–
C
50 SDF 1a
100 SDF 1a
300 SDF 1a
50 CCL19
100 CCL19
300 CCL19


Splenic B-cells
% migrated cells
+/+
–/–
C
50 SDF 1α
100 SDF 1α
300 SDF 1α
50 CCL19
100 CCL19
300 CCL19


Single positive thymocytes
% migrated cells
+/+
–/–
C
50 SDF 1a
100 SDF 1a
300 SDF 1a
50 CCL19
100 CCL19
300 CCL19

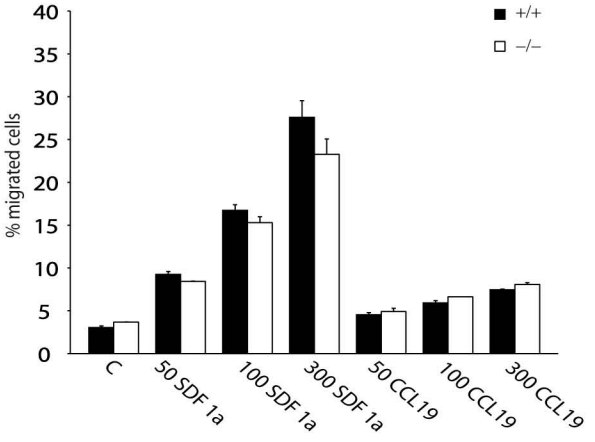

Double positive thymocytes
% migrated cells
+/+
–/–
C
50 SDF 1a
100 SDF 1a
300 SDF 1a
50 CCL19
100 CCL19
300 CCL19

Figure 5

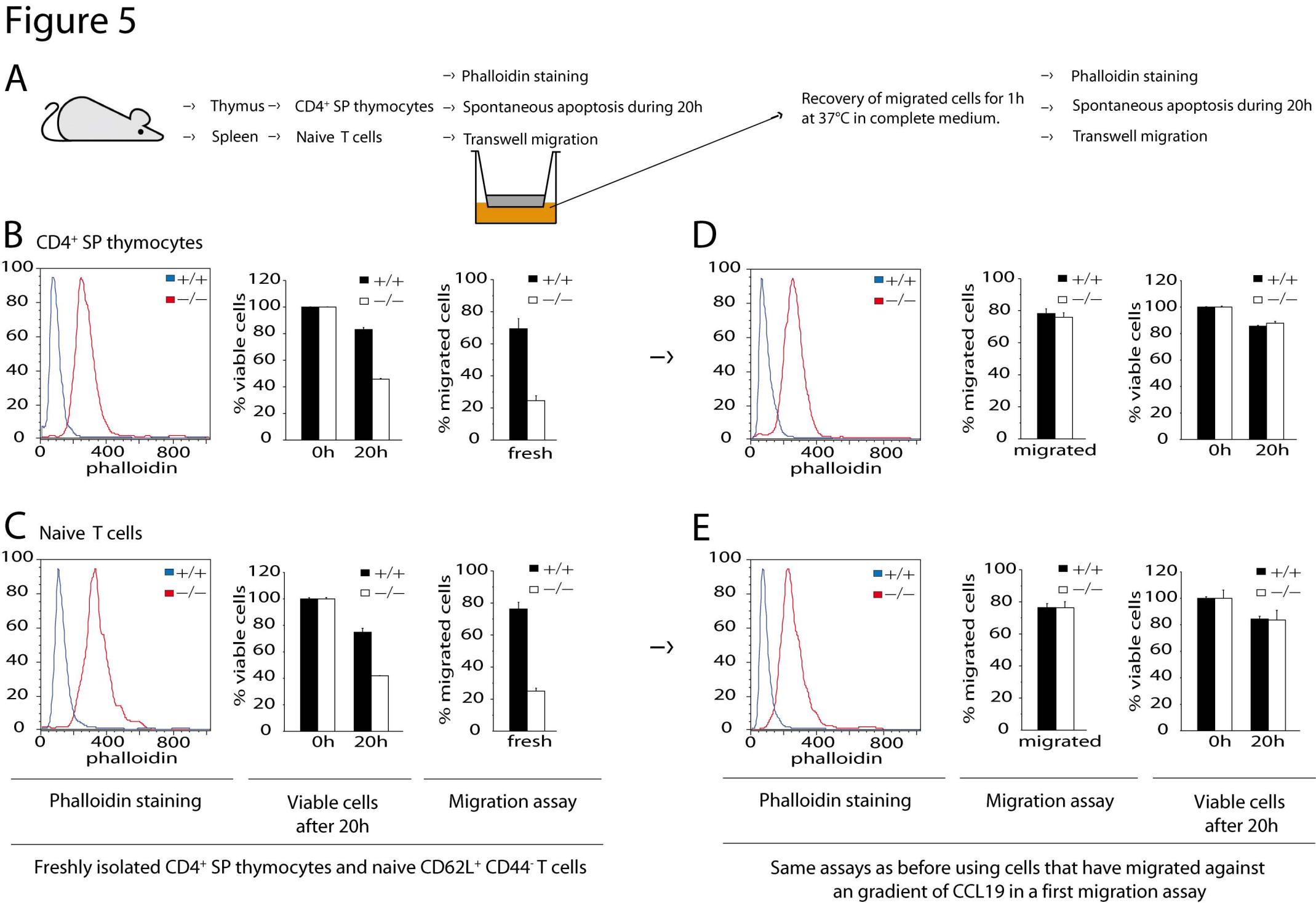

A
→ Thymus → CD4+ SP thymocytes
→ Spleen → Naive T cells
→ Phalloidin staining
→ Spontaneous apoptosis during 20h
→ Transwell migration
Recovery of migrated cells for 1h at 37°C in complete medium.
→ Phalloidin staining
→ Spontaneous apoptosis during 20h
→ Transwell migration
B CD4+ SP thymocytes
C Naive T cells
D
E
+/+
−/−
phalloidin
% viable cells
% migrated cells
0h
20h
fresh
migrated
Phalloidin staining
Viable cells after 20h
Migration assay
Freshly isolated CD4+ SP thymocytes and naive CD62L+ CD44- T cells
Same assays as before using cells that have migrated against an gradient of CCL19 in a first migration assay

Figure 6

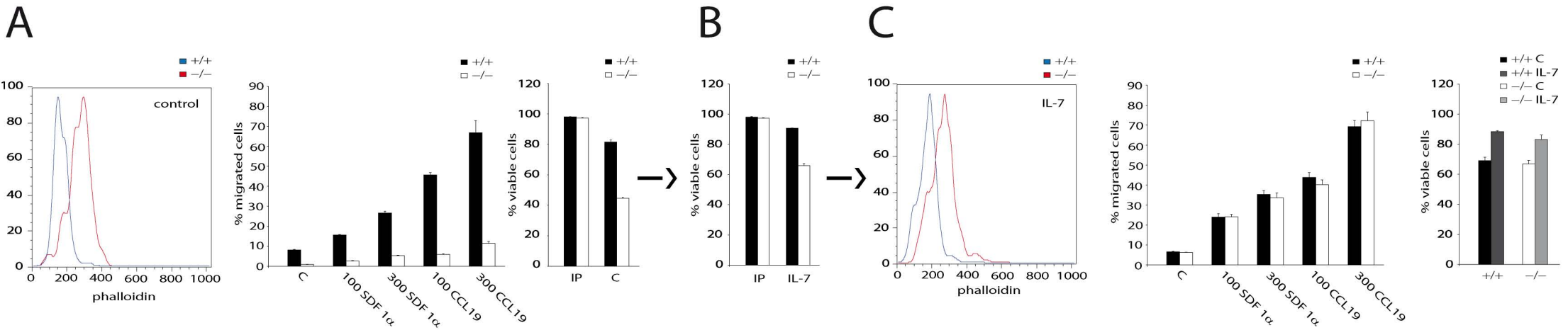

A
B
C
+/+
−/−
control
phalloidin
% migrated cells
% viable cells
C
100 SDF 1α
300 SDF 1α
100 CCL19
300 CCL19
IP
IL-7
+/+ C
+/+ IL-7
−/− C
−/− IL-7
Phalloidin staining
Migration assay
Untreated cells after 20h
Freshly isolated naive T-cells
Cells after 20h drug treatment
Same assays as before using drug treated cells after drug removal, recovery and dead/apoptotic cell removal

Figure 7

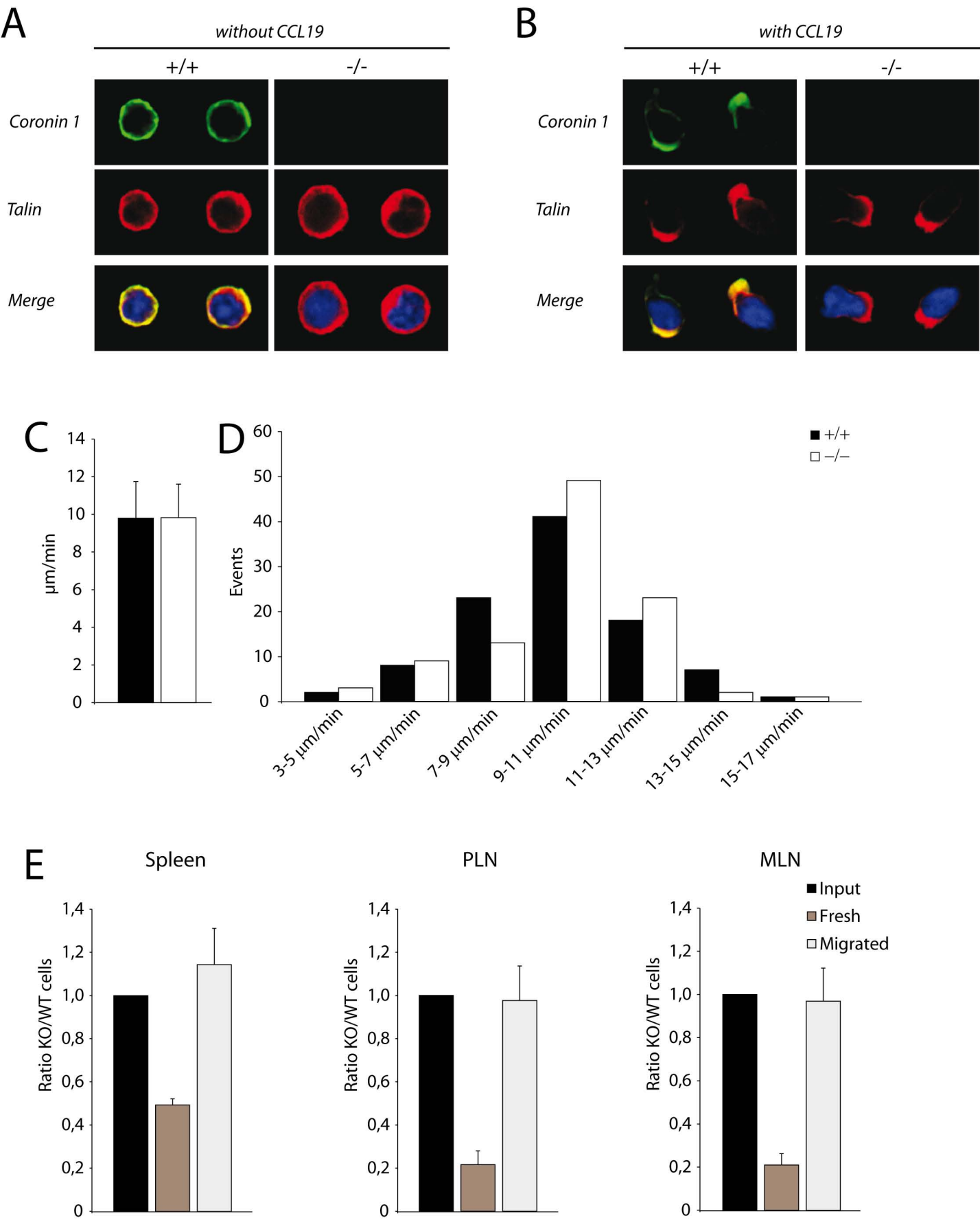

A
without CCL19
+/+
-/-
Coronin 1
Talin
Merge
B
with CCL19
+/+
-/-
Coronin 1
Talin
Merge
C
µm/min
0
2
4
6
8
10
12
14
D
Events
0
10
20
30
40
50
60
+/+
–/–
3-5 µm/min
5-7 µm/min
7-9 µm/min
9-11 µm/min
11-13 µm/min
13-15 µm/min
15-17 µm/min
E
Spleen
PLN
MLN
Input
Fresh
Migrated
Ratio KO/WT cells
0
0,2
0,4
0,6
0,8
1,0
1,2
1,4

Figure 8

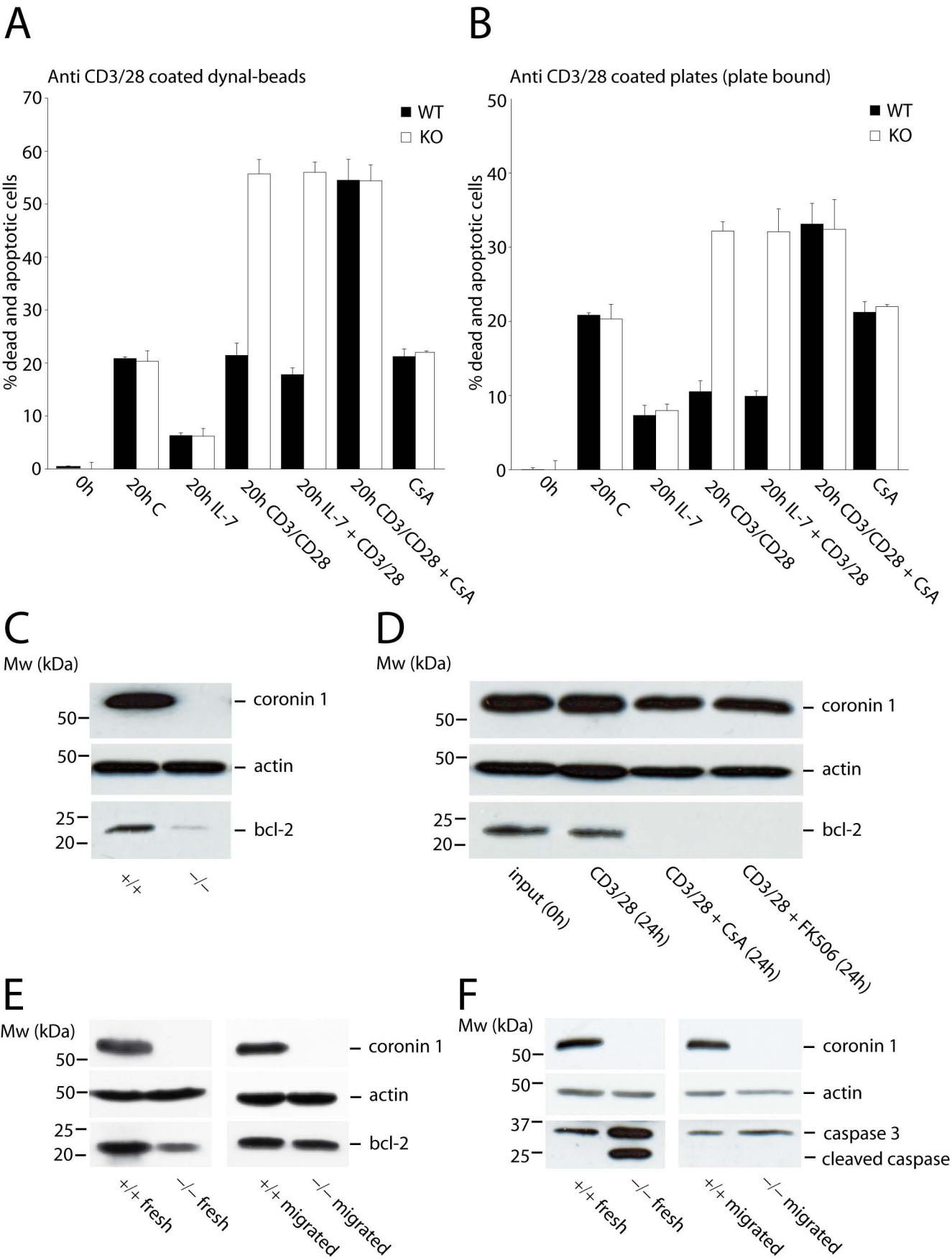

A
Anti CD3/28 coated dynal-beads
% dead and apoptotic cells
WT
KO
0h
20h C
20h IL-7
20h CD3/CD28
20h IL-7 + CD3/28
20h CD3/CD28 + CsA
CsA
B
Anti CD3/28 coated plates (plate bound)
% dead and apoptotic cells
WT
KO
0h
20h C
20h IL-7
20h CD3/CD28
20h IL-7 + CD3/28
20h CD3/CD28 + CsA
CsA
C
Mw (kDa)
coronin 1
actin
bcl-2
+/+
−/−
D
Mw (kDa)
coronin 1
actin
bcl-2
input (0h)
CD3/28 (24h)
CD3/28 + CsA (24h)
CD3/28 + FK506 (24h)
E
Mw (kDa)
coronin 1
actin
bcl-2
+/+ fresh
−/− fresh
+/+ migrated
−/− migrated
F
Mw (kDa)
coronin 1
actin
caspase 3
cleaved caspase 3
+/+ fresh
−/− fresh
+/+ migrated
−/− migrated

Figure S1

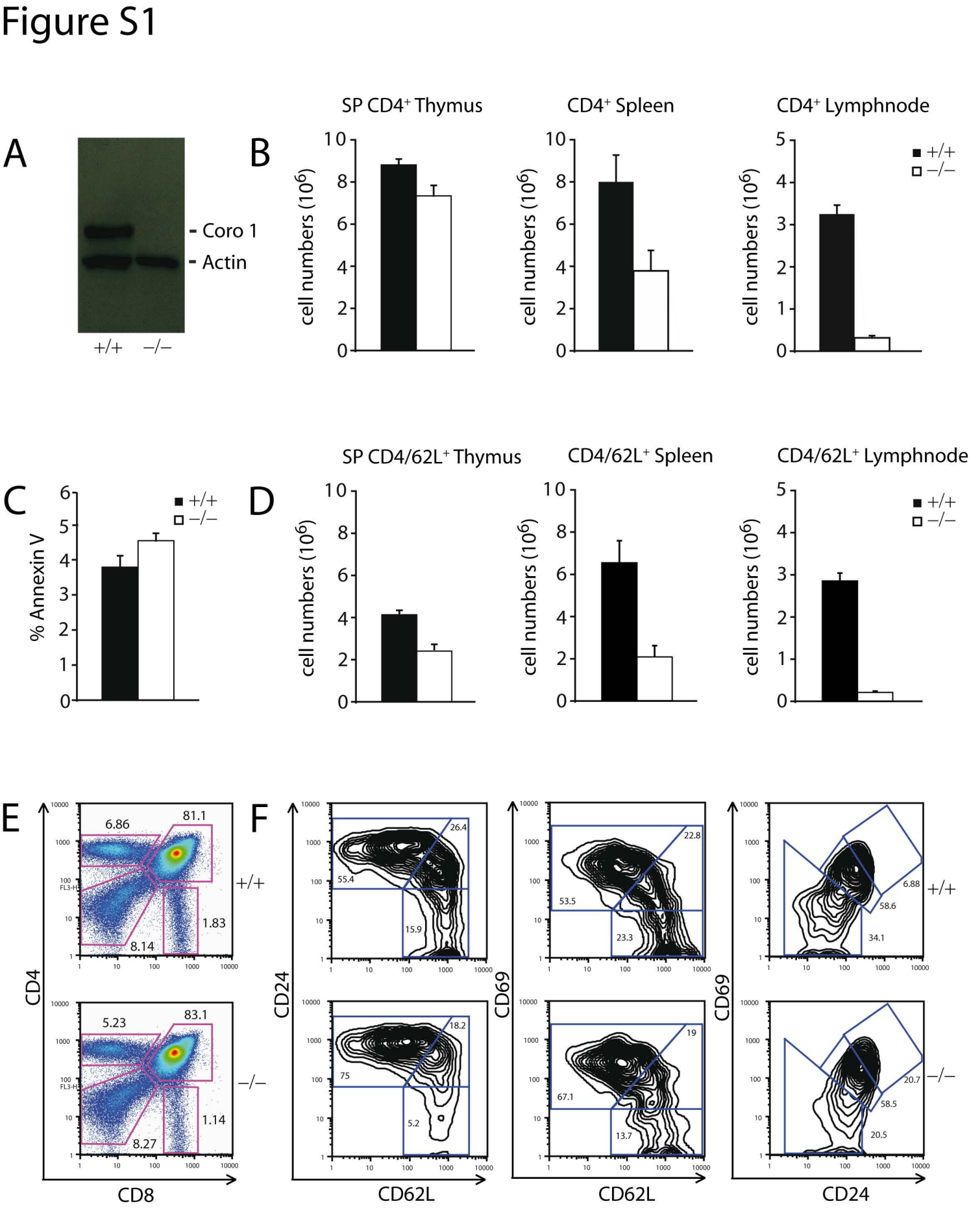
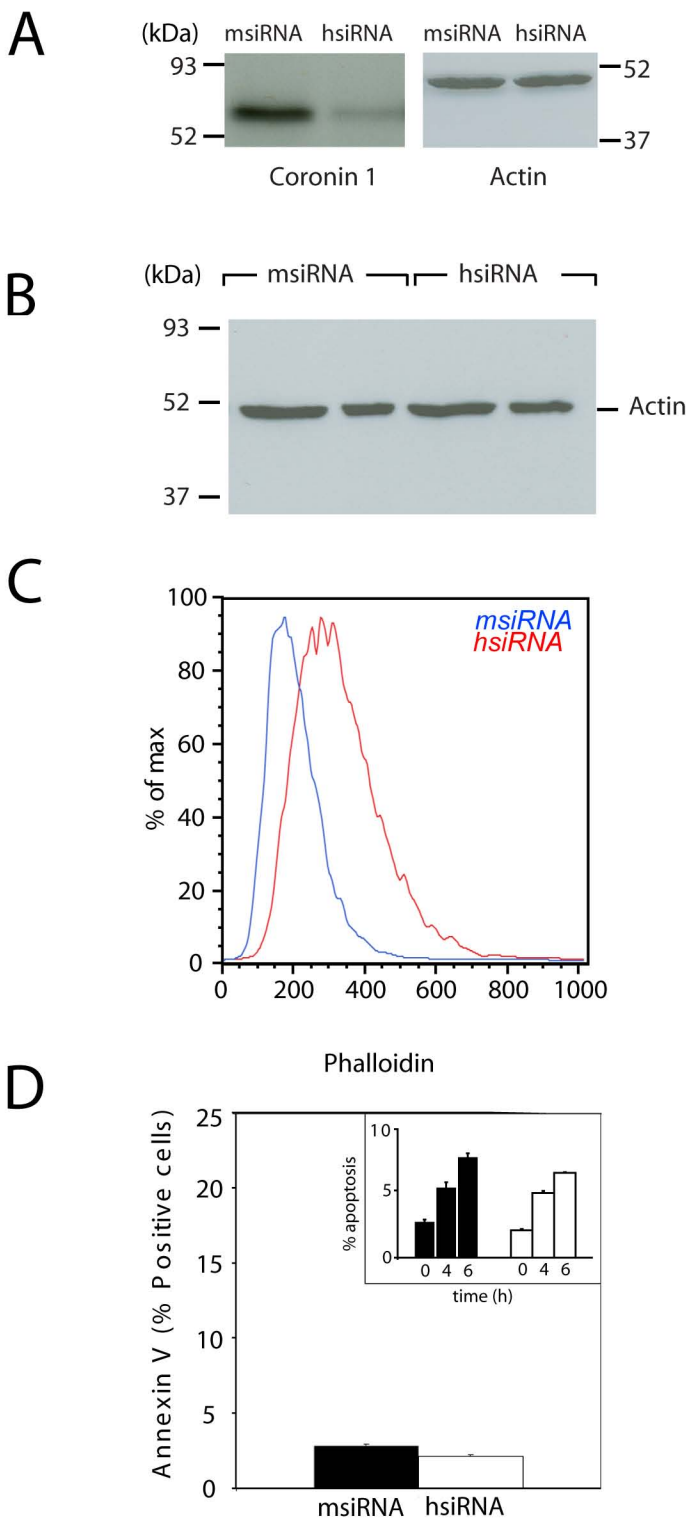

A
- Coro 1
- Actin
+/+
−/−
B
SP CD4+ Thymus
CD4+ Spleen
CD4+ Lymphnode
cell numbers (10^6)
+/+
−/−
C
% Annexin V
D
SP CD4/62L+ Thymus
CD4/62L+ Spleen
CD4/62L+ Lymphnode
E
6.86
81.1
1.83
8.14
5.23
83.1
1.14
8.27
CD4
CD8
F
26.4
55.4
15.9
22.8
53.5
23.3
6.88
58.6
34.1
18.2
75
5.2
19
67.1
13.7
20.7
58.5
20.5
CD24
CD62L
CD69

Figure S2

Jurkat T cells



Mel JuSo

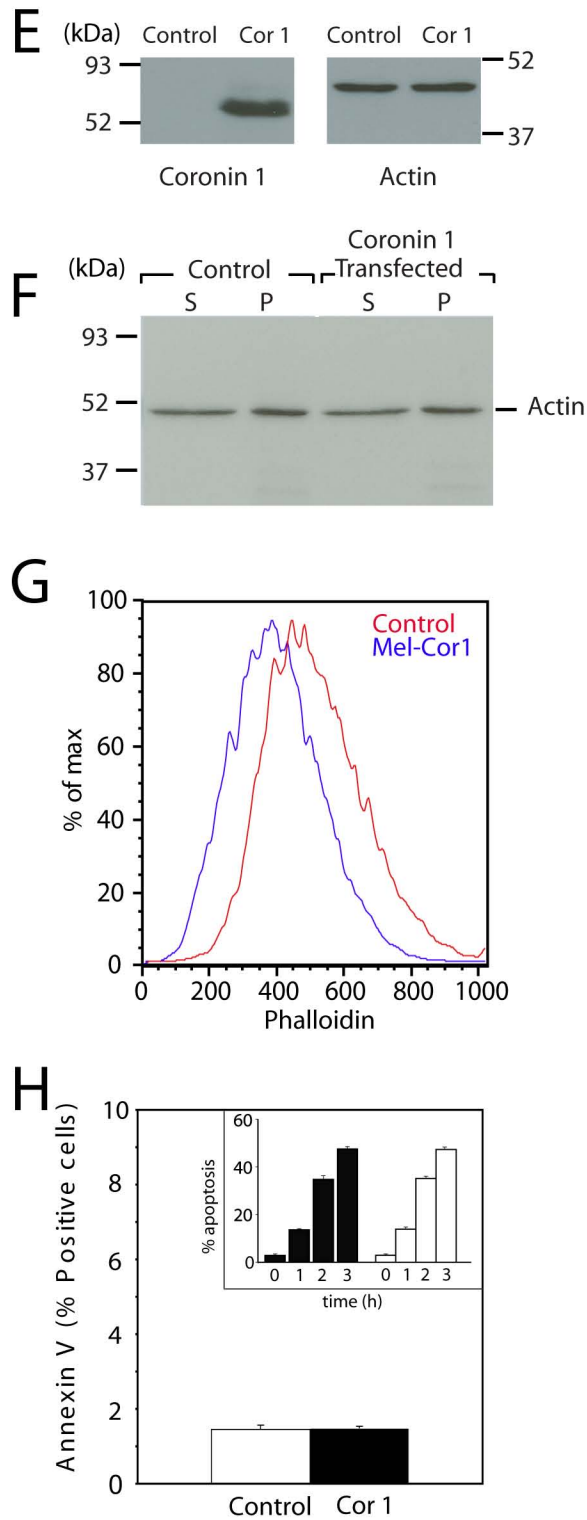


Figure S3

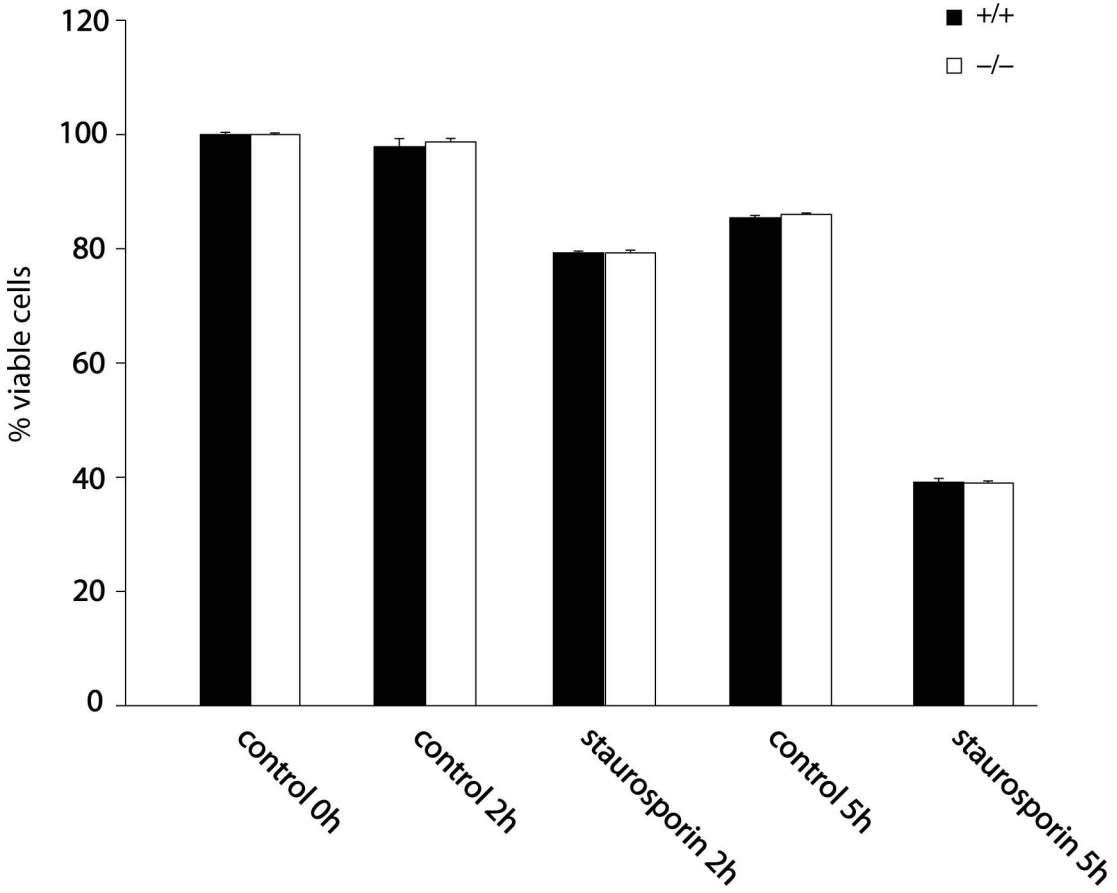
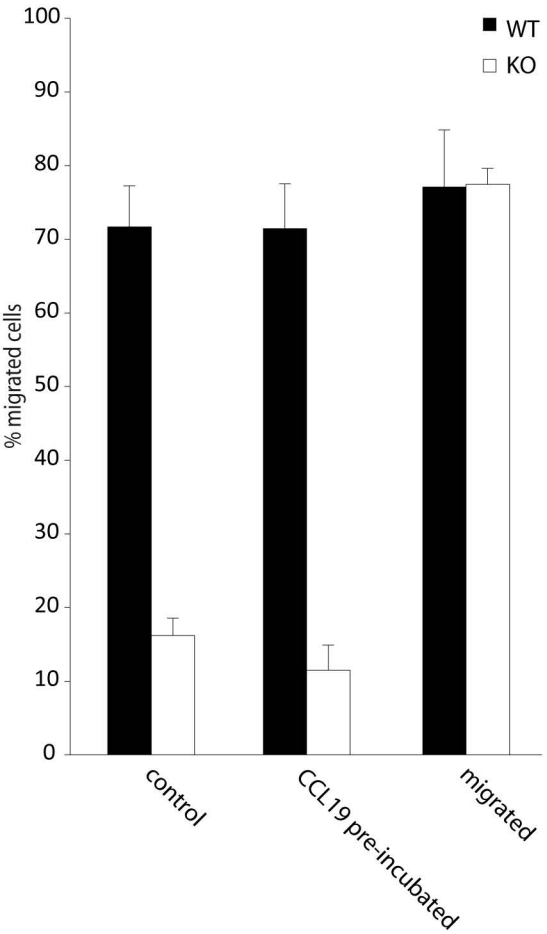

120
100
80
60
40
20
0
% viable cells
+/+
–/–
control 0h
control 2h
staurosporin 2h
control 5h
staurosporin 5h

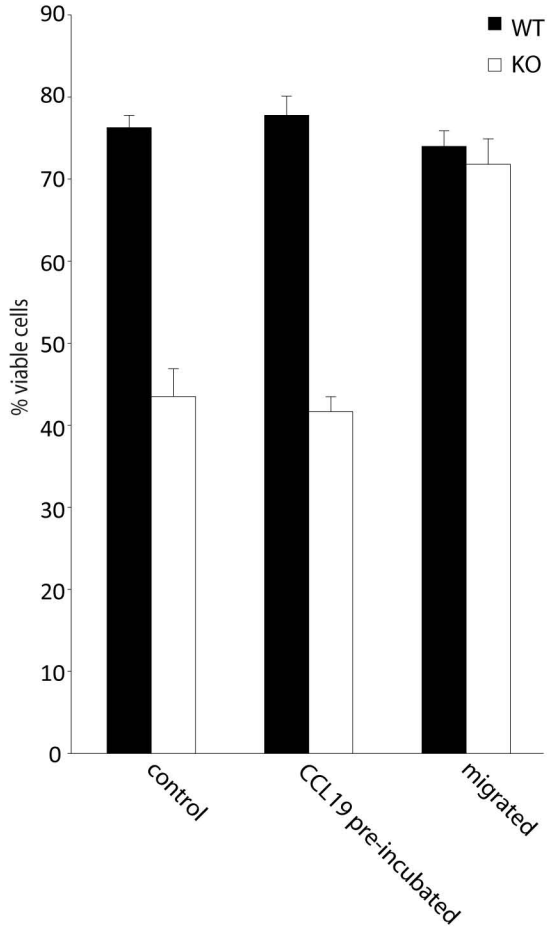
Figure S4

CD62L^{high} CD44^{low} splenic naive T-cells

A

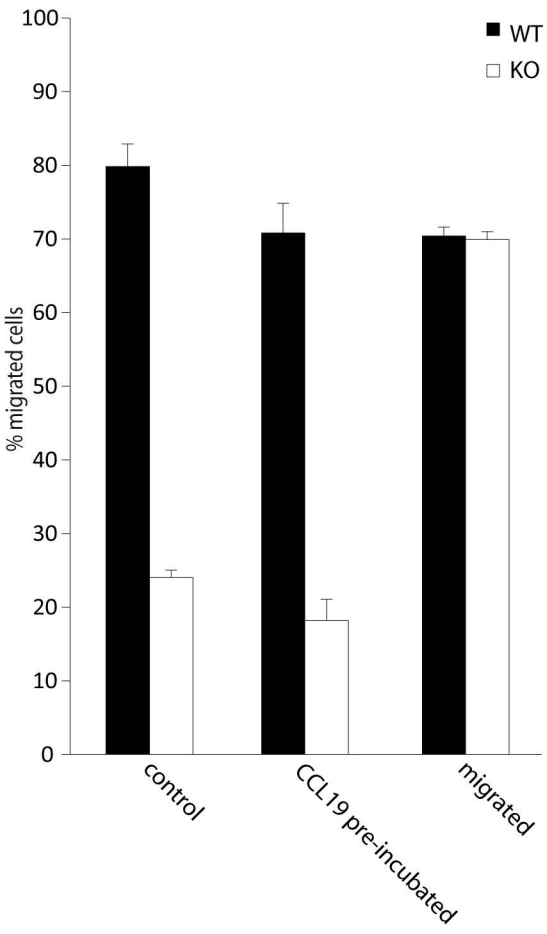


B



Single positive thymocytes

C



D

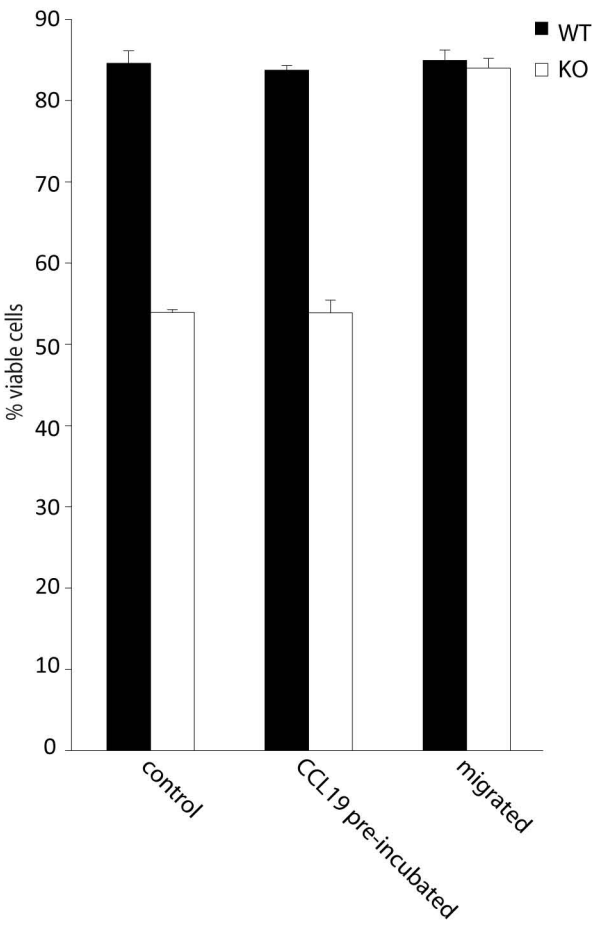


Figure S5

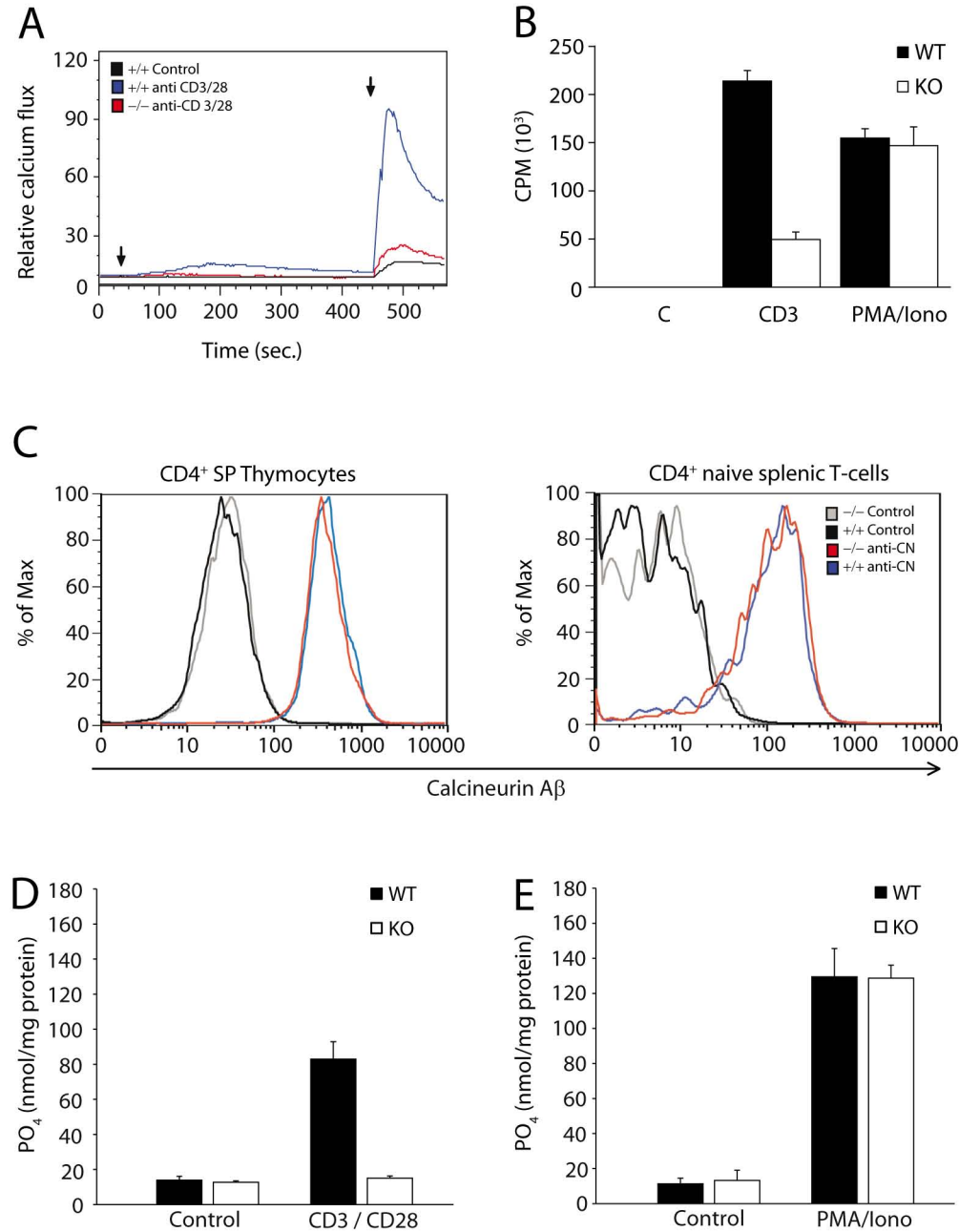
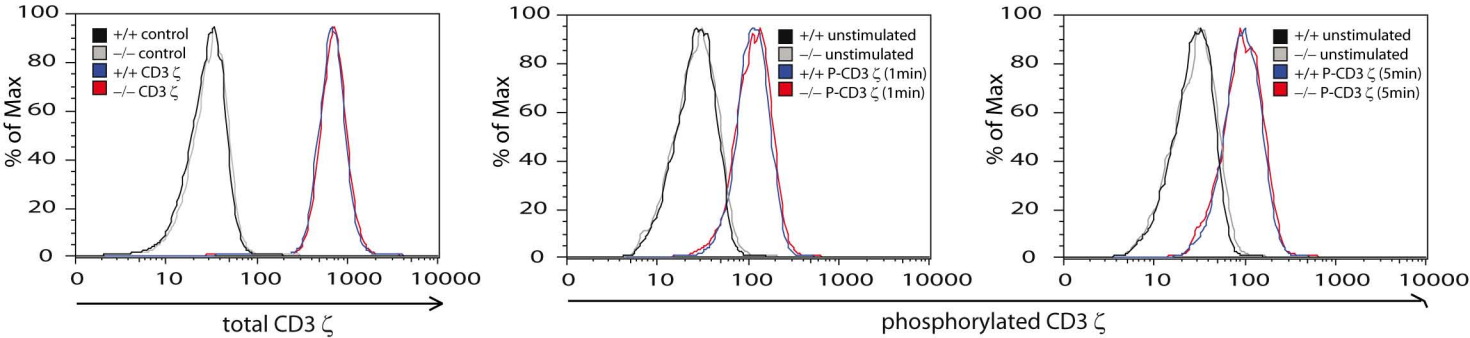

A
Relative calcium flux
+/+ Control
+/+ anti CD3/28
–/– anti-CD 3/28
Time (sec.)
B
CPM (10³)
WT
KO
C
CD3
PMA/Iono
C
CD4⁺ SP Thymocytes
CD4⁺ naive splenic T-cells
% of Max
–/– Control
+/+ Control
–/– anti-CN
+/+ anti-CN
Calcineurin Aβ
D
PO4 (nmol/mg protein)
Control
CD3 / CD28
E
PMA/Iono

Figure S6


100
80
60
40
20
0
% of Max
+/+ control
–/– control
+/+ CD3 ζ
–/– CD3 ζ
0
10
100
1000
10000
total CD3 ζ
+/+ unstimulated
–/– unstimulated
+/+ P-CD3 ζ (1min)
–/– P-CD3 ζ (1min)
+/+ P-CD3 ζ (5min)
–/– P-CD3 ζ (5min)
phosphorylated CD3 ζ