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In the absence of its cytosolic domain, the CD28 molecule still contributes to T cell activation.

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Abstract

The CD28 costimulatory receptor has a pivotal role in T cell biology as this molecule amplifies T cell receptor (TCR) signals to provide an efficient immune T cell response. There is a large debate about how CD28 mediates these signals. Here, we designed a CD28 gene targeted knock-in mouse strain lacking the cytoplasmic tail of CD28. As is the case in CD28-deficient (CD28 knock-out) mice, regulatory T cell homeostasis and T cell activation are altered in these CD28 knock-in mice. Unexpectedly, the presence of a CD28 molecule deprived of its cytoplasmic tail could partially induce some early activation events in T cells such as signaling events or expression of early activation markers. These results unravel a new mechanism of T cell costimulation by CD28, independent of its cytoplasmic tail.

Introduction

Costimulatory molecules are essential to achieve complete T cell activation. CD28 is a founding member of the costimulatory receptor family [1]. A large number of experiments have been performed to assess the role of the cytoplasmic tail of this molecule including studies using primary T cells in transgenic (Tg) mice or retroviral reconstitution on the CD28-deficient background [2,3]. In these experiments, CD28 expression is under control of a heterologous promoter, and not under endogenous control.

Several signaling motifs can be identified in the cytoplasmic tail of CD28. One of the strategies used to investigate the importance of the CD28 cosignaling has been to generate tail-less CD28 mutants defective in signaling on a CD28 null background. Such CD28 mutant were expressed using the human CD2 promoter in CD28-deficient mice; cell surface expression of the transgene was detected on splenic T cells at levels higher than those from WT mice [4]. Tail-less CD28 Tg T cells were shown to be functional in terms of cytokine production and proliferation, to be insensitive to CD28 engagement via CD28 mAbs or via B7-transfected CHO cells. These findings have been confirmed by retroviral infection of these tail-less mutants in CD28-deficient T cells [5,6].

However, studies of TCR Tg T cells from CD28-deficient mice expressing a tail-less CD28 mutant showed that the CD28 cytoplasmic tail is important for the recruitment of signaling molecules but not required for CD28 clustering at the T cell surface [7]. This tail-less CD28 receptor clustering has been described to play a regulatory function in the initial steps of T cell activation. In addition to inducing signaling events via its cytoplasmic tail, CD28 surface expression facilitates some TCR-induced signaling events during cell-cell interactions [8]. To study the role of CD28 in adhesion versus signaling in naive T cells where CD28 expression is controlled by endogenous elements, we designed a mouse strain lacking the cytoplasmic tail of CD28, tail-less CD28 gene targeted knock-in mouse (termed CD28 KI). This new

mouse strain allows the analysis of the role of CD28 on T cells allow to exclude specifically, independent of signaling via its cytoplasmic tail. Here, we analyze the influence of the loss of the entire CD28 cytoplasmic tail on T cell activation and the incidence on bacterial infections. In other words, does CD28 ligation induce signal events in the absence of its cytoplasmic tail?

Materials and methods

Animals

CD28-deficient mice (CD28 KO), described previously [9], were bred onto a B6 background and were maintained in our animal facility. C57BL/6 (B6) and BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France) and Janvier Labs SAS (Le Genest St Isle, France). All mice used were cared for in accordance with institutional guidelines. All experimental protocols were in agreement with the French Guidelines for animal handling and were approved by the Inserm ethical committee.

Gene targeting and generation of tail-less CD28 knock-in mice

Tail-less CD28 knock-in (KI) mice (CD28 N163* also termed CD28 KI) were generated in the laboratory (Figure S1A). A targeted mutagenesis in the exon 4 of *cd28* allowed the substitution of an asparagine codon (N163) by a stop codon. A neomycin selection cassette surrounded by loxP sites and a thymidine kinase cassette were introduced. The recombination vector was electroporated into embryonic stem (ES) cells of 129/Sv mice. After dual selection, ES cells were injected into blastocytes of C57BL/6 cells mice by Service d'Expérimentation Animale de Transgénèse et de Recombinaison Homologue (SEAT/UPS 44 CNRS, Villejuif, France). Chimeric males were mated with Cre-transgenic mice, in order to generate heterozygous mice without the neomycin selection cassette. Genotype analyses were performed on genomic DNA from tail biopsies with PCR primers F (5'AAGGCTCCTCAGGGTCATTT3') and R (3'GCTGGTAAGGCTTTCGAGTG5'). Mice heterozygous for the deletion on a mixed 129/Sv by C57BL/6 background were intercrossed to generate homozygous CD28 KI mouse strain. Tail-less CD28 KI mouse strain were backcrossed with C57BL/6 cells mice at least 10 times.

Antibodies

MAbs with the following specificities were used for flow cytometry (FCM): CD3ε (clone: 145-2C11), CD4 (clone: RM4-5), CD8α (clone: 53-6.7), CD25 (clone: PC61.5), CD28 (clone: 37.51), CD44 (clone: IM7), CD69 (clone: H1.2F3), H-2Kb (clone: AF6-88.5.5.3) and FoxP3 (clone: FJK-16s) (all eBioscience, Paris, France), CD127 (clone: A7R34) (Biolegend, San Diego, CA, USA), anti-TCR Vβ6 (clone: RR4-7) and anti-TCR Vβ8.1/8.2 (clone: MR5.2) (BD Biosciences, Le Pont-De-Claix, France). A chimeric antibody CD80-Ig was used to determined CD28 affinity for its ligand CD80 (R&D Systems, Lille, France). Goat polyclonal anti-CD28 biotinylated antibodies were used for immunoblotting (R&D Systems, Lille, France).

Flow cytometry (FCM) analysis

Cells were washed twice in FCM buffer (PBS, 2% FCS, 1mM EDTA, 0.02% NaN_3). Cell number was determined by Trypan blue staining. From 2.5 x 10^5 to 10^6 cells were then incubated with conjugated-antibodies for 30 min at 4°C, washed twice with FCM buffer and fixed with 4% paraformaldehyde. For intracellular staining, cells were permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences, Le Pont-De-Claix, France), incubated with conjugated antibodies for 30 min at 4°C and washed twice with Permwash buffer (BD Biosciences, Le Pont-De-Claix, France). All data were acquired on an FACS LSR II SORP 4 lasers flow cytometer (Becton Dickinson, Le Pont-De-Claix, France) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

T cell activation

For CD3/CD28 stimulation, 2.5 x 10^5 $CD4^+$ T cells isolated from the spleen were mixed (ratio 1:1) with Dynabeads Mouse CD3/CD28 T Cell Expander (Life Technologies, Cergy-

Pontoise, France) in 96-well round bottom plates (200 µL) and incubated for 3 days (37°C, 5% CO_2) with recombinant IL-2 (60 U/mL). For staphyloccoccal enterotoxin B (SEB; Sigma Aldrich Chemie S.a.r.l., L'isle d'Abeau Chesnes, France) stimulation, SEB was dissolved in sterile phosphate buffered saline (PBS) at 1 mg/ml and further dilutions were done in RPMI-1640 media supplemented with 10 % FCS. 5 x 10^5 splenocytes were incubated for 24 hrs with different concentraions of SEB in 96-well round bottom plates. For allogeneic stimulation, BALB/c mouse splenocytes were pre-activated for 24 hrs with LPS (10 µg/mL). Then, 2.5 x 10^5 $CD4^+$ T cells were mixed (ratio 1:3) with 7.5 x 10^5 BALB/c mouse splenocytes irradiated with 50Gy as antigen presenting cells (APCs) and incubated for 3 or 4 days with recombinant IL-2 (60 U/mL). For PMA/ionomycin stimulation, 2.5 x 10^5 $CD4^+$ T cells were incubated for 3 to 4 days with 10 ng/mL phorbol myristate acetate and 1 µg/mL ionomycin. Activation was assessed by FACS analysis for activation markers expression at the cell surface and by ELISA (eBioscience, Paris, France) for IFN-γ production.

Regulatory T cell stimulation

$CD4^+$ cells were obtained by antibody-mediated magnetic bead depletion from lymph nodes or spleen (Miltenyi Biotec, Paris, France). Purity was checked by FCM and was always more than 97%. For regulatory T cell stimulation, 10^5 splenocytes were mixed (ratio 1:2) with Dynabeads Mouse CD3/CD28 T Cell Expander and were incubated for 72h (37°C, 5% CO_2) with recombinant IL-2 (1 µg/mL corresponding to 10^5 units/mL) and TGF-β (1 ng/mL) purchased from R&D Systems (Lille, France). Regulatory T cell stimulation was assessed by FACS analysis.

Phosphoflow by FACS analysis

Cells were stimulated 5-30 min with Dynabeads Mouse CD3/CD28 T Cell Expander and recombinant IL-2 (10ng/mL). Phosphoflow analysis was then performed by cytometry as previously described [10]. Briefly, cells were fixed and permeabilized, incubated with anti-phospho-Akt S473 or anti-phospho-ERK-1/2 T202/Y204 mAbs (Cell Signaling Technology, Danvers, MA, USA) and appropriate biotinylated secondary antibodies. Finally, revelation was performed using Streptavidin-phycoerythrin solution (Beckman Coulter, Roissy, France). All data were acquired by FACS analysis.

Bacterial preparation, mice inoculation and antibodies response analysis

Coxiella burnetii RSA493 Nine Mille strain was cultured in L929 cell line as previously reported [11]. Monolayers of L929 cells were infected for 7 to 10 days and bacteria were harvested, sonicated and quantified by Gimenez stain. Bacterial viability was assessed using the LIVE/DEAD BacLight kit (Molecular Probes) as previously reported [11]. Wild type (WT), CD28 KO and CD28 KI mice housed in biosafety level 3 laboratory were inoculated with $5x10^5$ bacteria per mouse using intra-peritoneal route (i.p.). Whole blood specimens were sampled by retro-orbital puncture at days 14 and 21 post-inoculation (pi) (n = 5 for WT and KO mice and n = 4 for KI mice) for serology experiments (see below). Serum was obtained by centrifugation of blood from infected mice at 700g for 10 min. Obtained serum was stored at –80°C until analysis for the presence of specific antibodies anti- *C. burnetii.* Smears of *C. burnetii* antigens methanol-fixed were incubated with serial dilutions of serum for 30 min at 37°C. After washing, the presence of immunoglobulins G (IgG) against *C. burnetii* was revealed using fluorescein-conjugated goat antibodies (Abs) directed against mouse IgG diluted at a 1:400. The samples were titered by the end point.

Statistical analysis

Results shown represent means ± SD. Statistical analysis was performed with Student and ANOVA tests using Prism software (La Jolla, CA, USA).

Results

Characterization of CD28 tail-less KI mice

To address the role of the CD28 cytoplasmic tail in T cell activation, we generated CD28 tail-less KI (CD28 KI) mice. This truncated CD28 protein, CD28 tail-less (CD28 TL) contains the first 162 residues and lacks the intracellular domains involved in the binding to signaling molecules (Figure 1A). CD28 surface expression in CD28 KI thymocytes is similar to WT thymocytes (Figure S1B). By western blot, we assessed the molecular weight of CD28 TL, which is lower than that of CD28 WT (Figure S1C). The CD28 extracytoplasmic Ig domain binds to the CD80 and CD86 ligands. CD80-Fc (Figure S1D) and CD86-Fc (data not shown) fusion proteins bind similarly to the surface of thymocytes from CD28 KI or WT mice. As reported for CD28 KO mice [9], analysis of CD4/CD8, CD25/CD44 and H-2Kb/CD69 fractions in thymus showed no major difference in T cell development between CD28 KI and WT mice (Figure S1E-G). Moreover, CD28 KI splenocytes were analyzed for their surface expression of CD28 (Figure 1A), showing no difference compared to WT cells. Thus, the truncated CD28 receptor is normally expressed at the surface of the CD28 KI T cells and is recognized by its natural ligands B7.

CD28 tail-less (CD28 TL) engagement can amplify T cell signals during T cell costimulation

In the context of TCR crosslinking, CD28 ligation induces an optimal activation of signaling pathways such as PKB/Akt and ERK-1/2 activation [12]. Using phosphoflow analysis, we analyzed the phosphorylation of Akt (Ser-473) and ERK-1/2 (Thr-202 / Tyr-204) from $CD4^+$ T cells both in $CD25^-$ or $CD25^{high}$ subtypes. Cells were stimulated with CD3/CD28 beads and phosphorylation was measured as fold induction compared to untreated control. As expected, Akt and ERK-1/2 phosphorylation was higher in $CD25^{high}$ cells than $CD25^-$ cells (Figure 1B). Phosphorylation rates in CD28 KI cells were intermediate between CD28 KO and WT cells:

respectively for Akt 1.74 ± 0.05, 1.15 ± 0.10 and 1.86 ± 0.06, and for ERK-1/2 1.85 ± 0.14, 1.30 ± 0.21 and 2.32 ± 0.16 ($CD25^{high}$ cells with 30 min CD3/CD28 stimulation). These results show that CD28 lacking its cytoplasmic tail, is able to significantly increase the TCR activation signal by activation of PI3K-Akt and MAPK pathways.

Late T cell activation events are less impaired in CD28 KI than in CD28 KO T cells

$CD4^+$ T cells isolated from the three different mouse strains were stimulated with CD3/CD28 beads for 3 days. The percentage of activated T cells corresponding to $CD69^{POS}$ or $CD44^{POS}$ cells (Figure 2A & 2B, left panels) was measured and production of IFN-γ was evaluated (Figure 2C). Similar results were obtained in CD28 KO and CD28 KI conditions for CD69 expression (Figure 2A), however the percentage of $CD44^{POS}$ cells in CD28 KI is higher than CD28 KO T cells (Figure 2B). The detection of another activation marker, CD25 shows similar results in that CD28 KI appears at an intermediate range between CD28 KO and WT conditions (Figure S2B). This difference is mainly due to a difference in the percentage of cells with a low level of CD25 expression (Figure S2C). The percentage of cells with a high level of CD25 expression (Figure S2D) is very low both in CD28 KI and CD28 KO T cells. As this high $CD25^+$ population could be due to a endogenous IL-2 stimulation, this suggests that both CD28 KI and CD28 KO T cells are defective for the production of this cytokine. Therefore, upon CD3/CD28 beads stimulation, IFN-γ production is reduced both in CD28 KI and CD28 KO T cells (Figure 2C). By phospho-Akt flow analysis, a PI3K/Akt signaling induction is detected in $CD4^+$ T cells from CD28 KI mice (Figure 1B). The loss of IL-7Rα (CD127) expression was reported as a hallmark of PI3K activation in effector $CD4^+$ T cells [13]. Therefore, the percentage of $CD127^{POS}$ cells was measured in $CD4^+$ T cells from lymph nodes of the different mouse strains (Figure S3). Upon CD3/CD28 T cell stimulation, the

percentage of CD127^{POS} cells is lower in WT than in CD28 KO mice and CD28 KI condition has an intermediate phenotype.

Using a bacterial superantigen such as Staphylococcal enterotoxin B (SEB) to activate T cells, Similar results were obtained in CD28 KO and CD28 KI conditions for CD69 expression (Figure S4). Furthermore, CD4$^+$ T cells were also stimulated by irradiated BALB/c splenocytes as antigen presenting cells (APC) for 3 or 4 days, then activation marker expression (CD69 and CD44) were measured by flow cytometry (Figure 2A & 2B). No significant increase of CD69 and CD44 expression level was detected at day 3 either in CD28 KI or in CD28 KO T cells (Figure 2A & 2B, left panels). However, at day 4 post-allostimulation, CD28 KI T cells showed an intermediate induction phenotype compared to CD28 KO and WT cells (Figure 2A & 2B, right panels). These results show that the cytoplasmic tail of CD28 is required to induce functional costimulatory events in primary T cells. However, in some cases, signaling events induced by CD28 engagement occur in absence of its cytoplasmic tail.

The CD28 cytoplasmic tail is required for regulatory T cells homeostasis

CD28 costimulation by its ligands CD80/CD86 is essential for regulatory T cells (Tregs) homeostasis and Treg subset is strongly decreased in CD28 KO mice [14]. The presence of CD25$^+$/FoxP3$^+$ Tregs was measured in splenocytes from the three different mouse strains (Figure 3A, left panel). As was observed in CD28 KO, CD28 KI mice showed a clear reduction of CD25$^+$/FoxP3$^+$ cells compared to WT mice. We used a Treg stimulation assay to induced Treg differentiation and proliferation. By focusing on regulatory CD25$^+$/FoxP3$^+$ subset, we obtained a strong decrease in CD28 KI similar to CD28 KO, compared to WT cells (respectively 4.52 ± 0.53, 6.44 ± 0.62 and 15.05 ± 1.45) (Figure 3A, right panel). However, in the same assay, the total CD25$^+$ T cell population corresponding mainly to activated effector

T cells is decreased in CD28 KO, but not in CD28 KI conditions (Figure 3B). The results suggest that the cytoplasmic tail of CD28 is mandatory for Treg homeostasis and activation, but not for effector T cell activation.

The cytoplasmic tail of CD28 is required to drive an optimal IgG production during *Coxiella burnetii* infection

Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate intracellular microorganism that replicates in myeloid cells. The development of a protective immune response against *C. burnetii* requires an adaptive immune response as shown by Honstettre *et al.* [15]. Despite the fact that the CD28 gene inactivation in mice leads to a decrease of IgG production, it also leads to a decrease of *C. burnetii* burden in tissues [16]. We analyzed the CD28 KI mice immune response to infection by *C. burnetii*. There were no significant differences in *C. burnetii* burden in both blood and spleen compared to CD28 KO mice, but we found an increased burden in liver (Figure 4A). IgG production was assessed and we found a large decrease of Igs both in CD28 KI and CD28 KO mice compared to B6 (WT) mice. Ig titers are slightly but significantly higher in CD28 KI than in CD28 KO mice at 14 days, but then this decrease is comparable in CD28 KO and in CD28 KI mice, 21 days after infection by *C. burnetii* (Figure 4B). These results prove that the cytoplasmic tail of CD28 is required to drive an optimal IgG production during *C. burnetii* infection.

Discussion

The CD28 receptor has been identified as a costimulatory molecule that can amplify TCR-driven signals to induce functional events in T cells. Several partners have been identified to be associated to the cytoplasmic tail of CD28 [2,17], and we contributed to identify some of them such as the phosphatidylinositol 3'-kinase (PI3K) and some protein tyrosine kinases [18,19]. In order to precisely define the relative importance of each individual signaling motif in the CD28 sequence, some CD28 gene targeted KI mouse strains have been generated [20,21]. Despite the description of these elegant mouse models, in 2010, J. Boomer and J. Green entitled their review article in the Cold Spring Harbor Perspectives in Biology, "*An enigmatic tail of CD28 signaling*" [2]. Previous data assessing the function of tail-less version of CD28 have been performed using retroviral transfer into previously activated cells (which cannot achieve T cell development and activation of naïve T cells) or using transgenic CD28 which is generally overexpressed compared to the endogenous CD28 expression levels. In this context, we generated a mouse strain lacking the entire cytoplasmic tail of CD28, tail-less CD28 gene targeted KI mouse. In contrast to the previously described CD28 tail-less models, CD28 engagement delivers some co-signals in CD28 tail-less KI T cells, suggesting that CD28 allows partial amplification of TCR signals in the absence of its cytosolic tail. How an adhesion molecule expressed at the T cell surface can participate to the T cell costimulatio is still a matter of debate [22].

Expression of the CD28 receptor and its ability to recognize B7 ligands are similar in C57BL/6 mice and CD28 KI animals. T cell homeostasis is well described in CD28 KO mice, where the numbers of effector T cells are normal but associated to a dramatic decrease of Tregs [9,14]. The overall T cell phenotype of the CD28 KI mouse strain is similar to the CD28 KO model, showing that the cytoplasmic tail of CD28 is mandatory for Treg development. Moreover, in both CD28 KO and CD28 KI mice, T cell responses are impaired

for activation marker expression, IFN-γ secretion or IgG production during bacterial infection. Unexpectedly, some differences appear between CD28 KO and CD28 KI mice, when T cell activation in effector T cells is assessed. In the context of TCR+CD28 costimulation, the magnitude of signaling events induced in CD28 KI T cells is always lower than WT T cells, but significantly higher than in CD28 KO T cells where by definition the CD28 molecule is not expressed. These results suggest that the engagement of the CD28 molecule is able to amplify TCR signaling at least partially via the presence of the CD28 costimulatory receptor at the plasma membrane.

The lipid kinase, PI3K that directly binds to the cytoplasmic tail of CD28 [18], can be triggered via CD28 in the absence of a direct binding to the YxxM CD28 binding motif [23]. However, these observations do not exclude an indirect binding to the cytoplasmic tail. Here, we showed that in the context of TCR engagement, stimulation of CD28 can promote PI3K/Akt signaling in absence of its entire cytoplasmic tail. Bacterial superantigens can trigger T cell activation; these potent T cell activation inducers can create a bridge between TCR and CD28 receptor at the T cell surface to facilitate T cell signaling [24]. CD28 triggering can enhance the PI3K/Akt signaling pathway in T cells when this receptor is located in specialized plasma membrane structures called raft nanodomains [25]. This specific membrane localization of CD28 followed by a receptor aggregation induced via its engagement, is able to promote the TCR cell signaling. Before the engagement of signaling proteins with the cytoplasmic tail, receptor oligomerization and plasma membrane sublocalization could represent the initial step of the costimulation events induced by CD28. It is well established that aggregation of receptors at the T cell surface is a potent mechanism to induce cell signaling events [26]. Among the signaling proteins associated with CD28 signaling [2], the Src-family protein tyrosine kinase member Lck is anchored to the inner phase of the plasma membrane and is a resident of the lipid rafts. The CD28 molecule could

deliver a *trans*-signal to the TCR in naive primary T cells to prime TCR signaling. In this case, the CD28 molecule in the absence of its cytosolic tail could participate to TCR-mediated signals in a quantitative-manner by increasing TCR clustering and/or lipid raft coalescence [27]. One hypothesis in favour of a qualitative costimulatory model would be that the transmembrane domain of CD28 could be associated with unknown protein binding partners involved in cell signaling and/or cytoskeleton reorganization.

Taken together, our results show that the cytoplasmic tail of CD28 is mandatory for Treg development and is required to promote a full activation of effector T cells. However, in the absence of its cytoplasmic tail, CD28 can also induce some signaling events. These initial steps of cosignaling at the plasma menbrane should be further investigated. It is important to understand the behaviour of these cosignaling molecules when there are recognized by a ligand as a monomer or an oligomer, as therapeutic strategies using monoclonal antibodies have been designed to target cosignaling molecules [17].

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Figure legends

Figure 1. Analysis of signaling events induced by CD28 triggering in CD28 tail-less (CD28 KI) T cells. (A) Peptide sequence corresponding to the deleted CD28 cytoplasmic region in CD28 KI mouse strain. Expression of CD28 measured by flow cytometry in $CD4^+$ T cells from spleen of WT, CD28 KO and CD28 KI mice. (B) Phosphorylation induction of signaling key molecules, Akt (S473) and ERK-1/2 (T202/Y204) measured by phosphoflow in $CD4^+/CD25^-$ and $CD4^+/CD25^-$ cell subsets after CD3/CD28 beads stimulation of splenocytes from WT, CD28 KO and CD28 KI mice. Representative data from 3 independent experiments (n= 8-9 mice each genotype/experiment, mean ± SD), * $P < 0.05$.

Figure 2. *Ex vivo* stimulation of CD28 tail-less (CD28 KI) $CD4^+$ T cells. Isolated $CD4^+$ T cells from spleen of WT, CD28 KO and CD28 KI mice were stimulated for 3 or 4 days.(A) Expression of CD69 activation marker measured by flow cytometry upon 3 days (left panel) or 4 days (right panel) of stimulation. (B) Expression of CD44 activation marker measured by flow cytometry upon 3 days (left panel) or 4 days (right panel) of stimulation. (C) IFN-γ accumulation is measured by ELISA following the 3 days of CD3/CD28 beads stimulation. Representative data from 2 independent experiments (n= 6 - 7 mice each genotype/experiment, mean ± SD), *** $P < 0.001$; ** $P < 0.01$ and * $P < 0.05$.

Figure 3. (A) $CD25^+/FoxP3^+$ regulatory T cells subsets were measured by flow cytometry in total $CD4^+$ splenocytes from WT, CD28 KO and CD28 KI mice expression upon Treg stimulation conditions (CD3/CD28 beads + IL-2 + TGF-β). (B) Under same stimulation conditions, $CD25^+$ effector T cells were measured by flow cytometry in $CD4^+$ splenocytes from WT, CD28 KO and CD28 KI mice expression upon Treg stimulation conditions (* $P<$ 0.05 compared to KO).

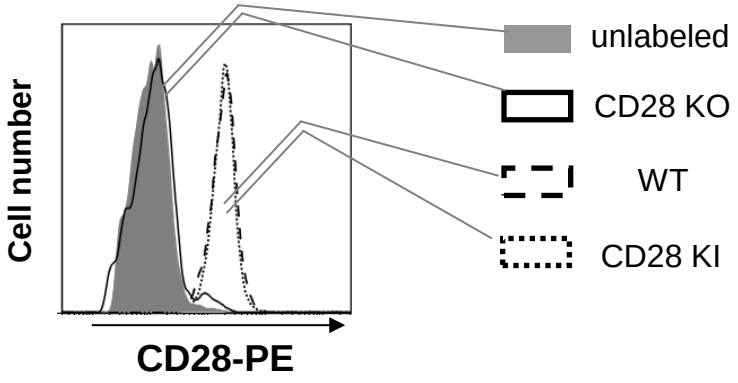
Figure 4. The response of mice to *Coxiella burnetii* infection. (A) Mice were infected with $5x10^5$ *C. burnetii* via i.p. route and blood (200 µl), liver (25 mg) and spleen (10 mg) from WT, CD28 KO and CD28 KI mice were collected at day 14 post-infection. DNA was extracted in a 100µl final volume and the presence of *C. burnetii* was determined by qPCR using a 5 µl DNA extract. Results represented the number of DNA copies for 5 µl DNA extract and were expressed as mean ± SD, n = 5 for each group of mice. *, *P* value < 0.05. (B) IgG response in WT, CD28 KO and CD28 KI mice. Whole blood specimens were sampled by retro-orbital puncture where serum was collected at 14 and 21 days post-inoculation. The presence of IgG directed against *C. burnetii* was assessed by immunofluorescence. The results as titers are expressed as mean ± SD (n = 5 for each group of mice and per time point). * $P < 0.05$.

Figure 1

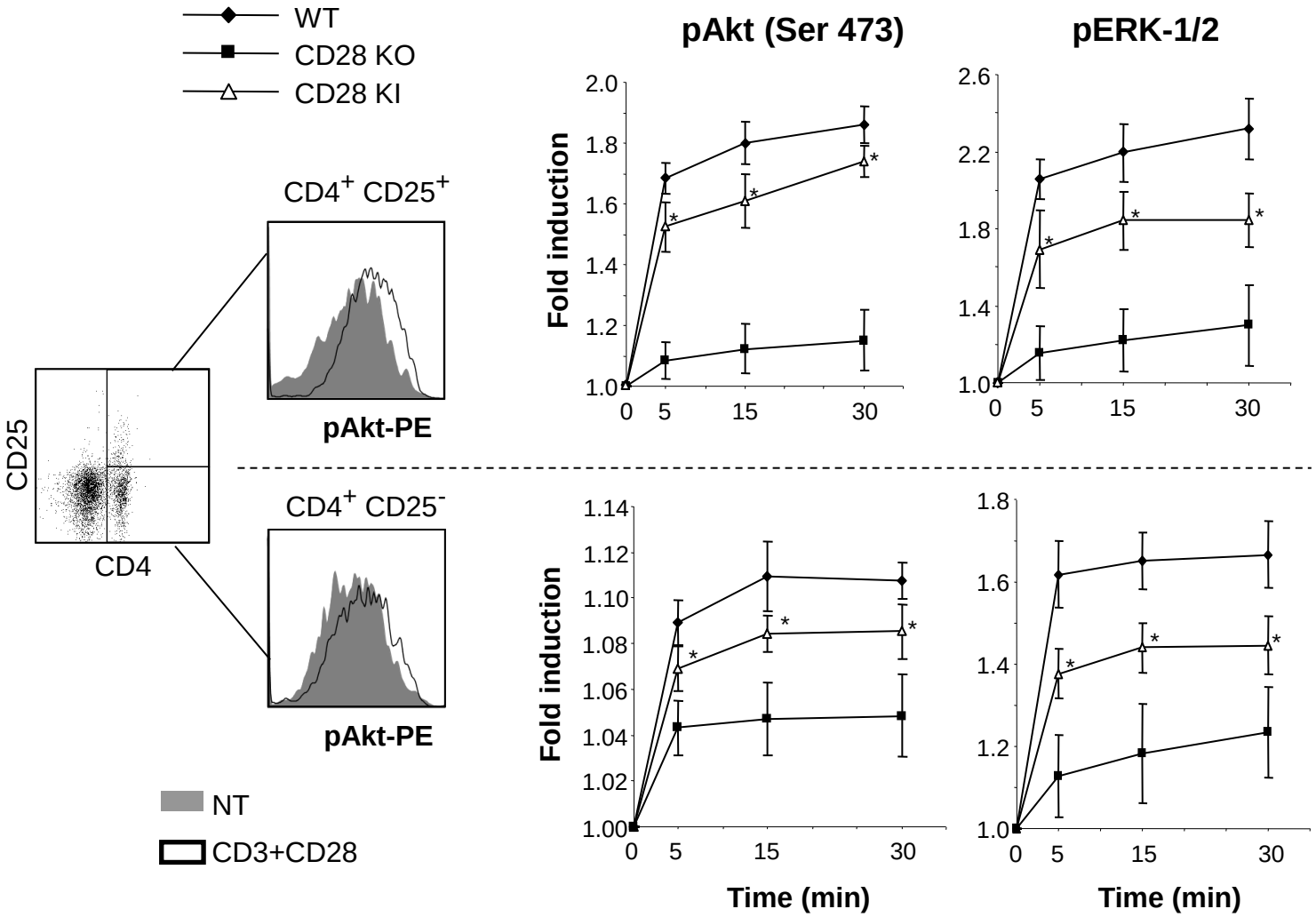
A

CD28 WT … SRRNRLLQSDYMNMTPRPGLTRKPYQPYAPARDFAAYRP$_{198}$

CD28 tail-less KI … SRR$_{162}$



B



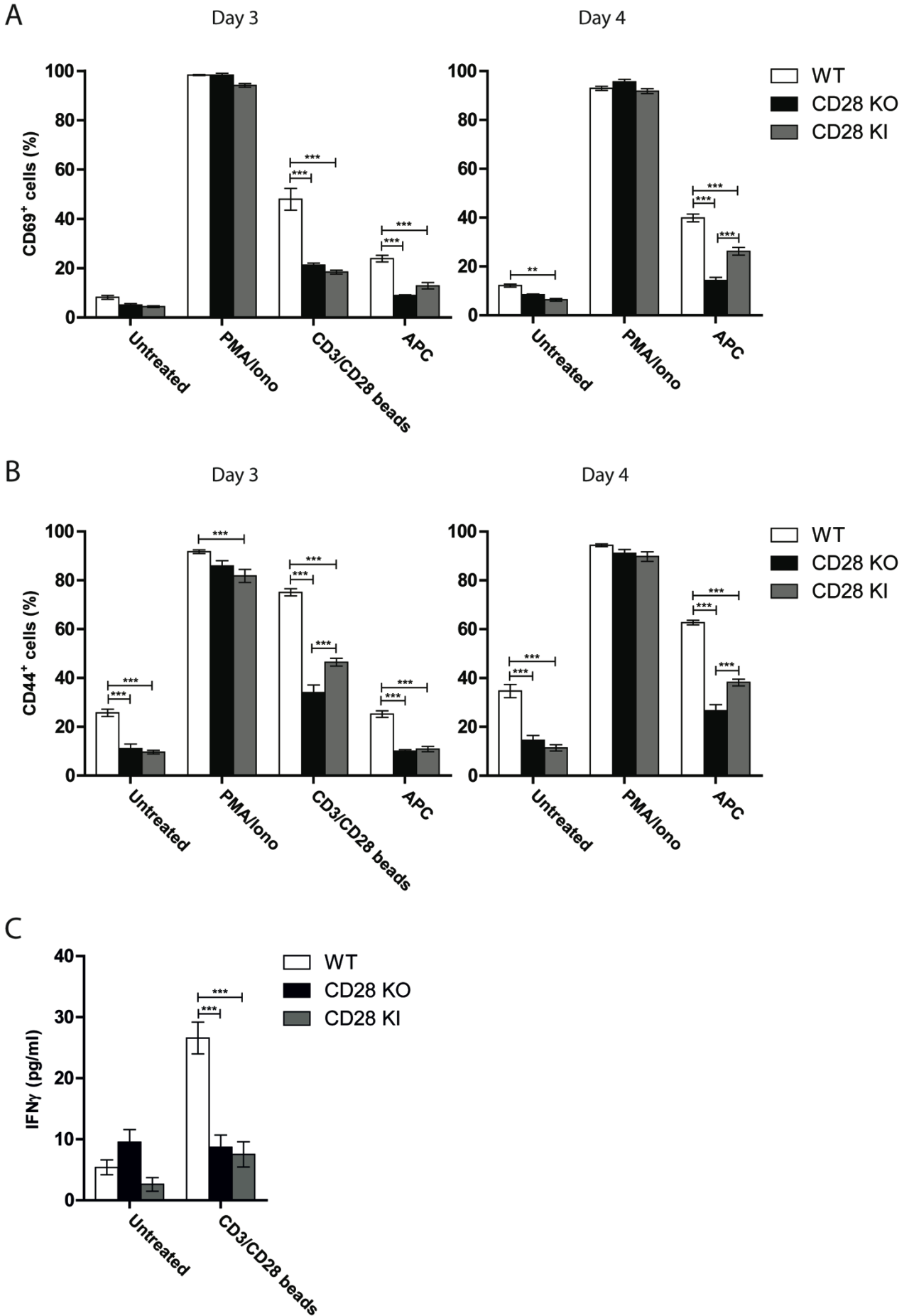

A
Day 3
Day 4
CD69+ cells (%)
WT
CD28 KO
CD28 KI
Untreated
PMA/Iono
CD3/CD28 beads
APC
B
Day 3
Day 4
CD44+ cells (%)
WT
CD28 KO
CD28 KI
Untreated
PMA/Iono
CD3/CD28 beads
APC
C
IFNγ (pg/ml)
WT
CD28 KO
CD28 KI
Untreated
CD3/CD28 beads

Figure 3

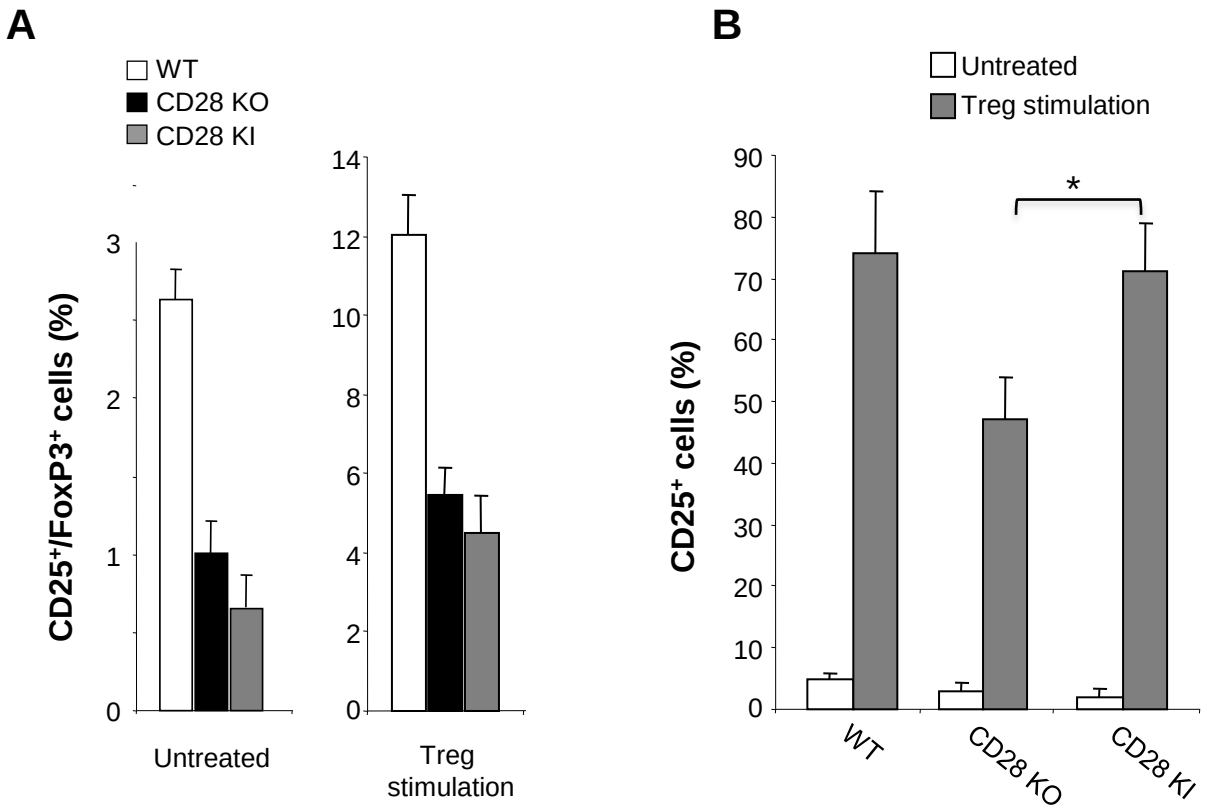
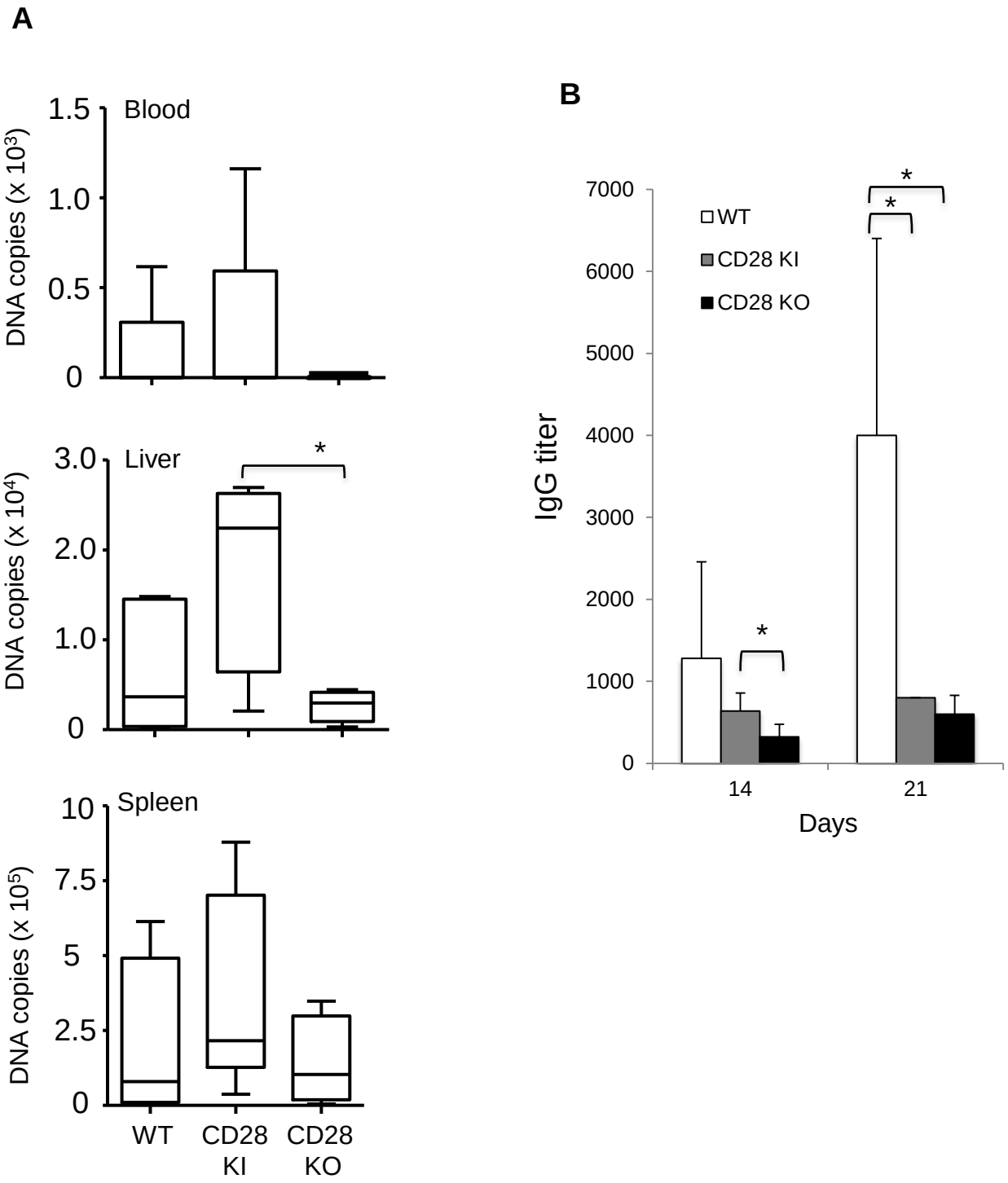

A
WT
CD28 KO
CD28 KI
CD25+/FoxP3+ cells (%)
0
1
2
3
0
2
4
6
8
10
12
14
Untreated
Treg stimulation
B
Untreated
Treg stimulation
CD25+ cells (%)
0
10
20
30
40
50
60
70
80
90
*
WT
CD28 KO
CD28 KI

Figure 4


A
Blood
DNA copies (x 10³)
1.5
1.0
0.5
0
Liver
DNA copies (x 10⁴)
3.0
2.0
1.0
0
*
Spleen
DNA copies (x 10⁵)
10
7.5
5
2.5
0
WT
CD28 KI
CD28 KO
B
IgG titer
7000
6000
5000
4000
3000
2000
1000
0
WT
CD28 KI
CD28 KO
*
*
*
14
21
Days

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