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T_H17 Differentiation is the Default Program for DPP2-deficient T cell Differentiation

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Abstract

DPP2 (dipeptidyl peptidase 2) is an N-terminal dipeptidase, required for maintaining lymphocytes in a resting state. Mutant mice with T cell-specific knock-down (kd) of DPP2 (lck-DPP2 kd) were generated and analyzed for their phenotype. Normal thymocyte development and a modest increase in the proportions of peripheral T cells were observed in these mice compared to littermate controls. Interestingly, the peripheral T cells were hyperactive upon TCR stimulation *in vitro*, although they did not express any activation markers. Furthermore, CD3-crosslinking in the naive $CD4^+$ and $CD8^+$ T cells of lck-DPP2 kd mice resulted mainly in IL-17 production. Similarly, the mutant T cells secreted primarily IL-17 after *in vivo* priming and *in vitro* antigen-specific restimulation. These data suggest that IL-17 production is the default program for T cell differentiation in the absence of DPP2. Thus, DPP2 seems to impose a threshold for quiescent T cells, preventing them from drifting into cell cycle.

DPP2, a member of the serine dipeptidyl peptidase family, is an N-terminal protease that is ubiquitously transcribed in most tissues [1]. It is localized in intracellular vesicles and is also secreted upon cellular activation [2]. The DPP2 expression level is particularly high in quiescent T cells and fibroblasts, but is significantly down-regulated upon activation of these cells [3]. We previously demonstrated that DPP2 inhibition *in vitro* causes apoptosis in quiescent, but not activated, T cells [4] and fibroblasts due to a deregulated entry into the cell cycle [5]. In order to analyze the role of DPP2 in quiescent T lymphocytes *in vivo*, we generated mutant mice where DPP2 is specifically down-regulated in the T cell lineage.

The majority of T cells in the body are in a resting state until encounter with a pathogen. In the presence of exogenous cytokines, TCR-stimulation of naïve $CD4^+$ and $CD8^+$ T cells leads to their maturation into various T_H cell subsets and CTL effector cells. $CD4^+$ cells can differentiate into the classical T_H1 or T_H2 subsets[6] or one of the more recently discovered lineages, T_H17 [7] and inducible T regulatory (iTreg) cells [8]. Differentiation into T_H1 and T_H2 cells depends on exogenous IL-12 and IL-4, respectively. In contrast, T_H17 differentiation can be achieved with TGF-β and IL-6, two cytokines with opposing effects, while TGF-β alone induces iTreg cells [8]. Ghoreschi *et al.* recently demonstrated that IL-1 and /or TGF- β in conjuction with IL-6, IL-21 and IL-23 promote Th17 development [9]. Thus, the cytokine environment determines T_H effector differentiation, a mechanism mediated through

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selective signal transducer and activator of transcription (STAT) proteins [10, 11]. These master regulators include T-bet and Stat4 for T_H1 cells [12-14], GATA-3 and Stat6 for T_H2 cells [10, 11], RORγt and Stat3 for T_H17 cells[15, 16] and Foxp3 and Stat5 for Treg cells [17, 18]. In the absence of exogenous factors, however, CD3-crosslinking in primary T cells results in proliferation without development of effector function, although the activated $CD4^+$ and $CD8^+$ T cells produce IL-2 and IFN-γ, respectively.

T_H1 cells mediate responses against intracellular pathogens and secrete their signature cytokine, IFN-γ. IL-4 is the signature cytokine of T_H2 cells, which are involved in immunity against extracellular parasites, including helminthes. T_H17 cells, as its name implies, secrete IL-17 and are important for immunity against extracellular bacteria and fungi [19]. In addition, these cells have been implicated in various autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) [7] and systemic lupus erythematosus (SLE) [20], although recent reports have described a protective role for IL-17A in inflammatory bowel disease (IBD) [21-23].

Here we report that in the absence of DPP2 T_0 cells respond to CD3 crosslinking by hyper-proliferation and secretion of IL-17, in the absence of any exogenous factors. The same profile was observed after *in vivo* priming and *in vitro* antigen-specific restimulation of the T cells. These data suggest that IL-17 production is the default program for T cell differentiation in the absence of DPP2. Thus, DPP2 seems to prevent quiescent T cells from spontaneously drifting into cell cycle by imposing a threshold.

RESULTS

Generation of constitutive DPP2 kd and lck-DPP2 kd mice

To examine the role of DPP2 *in vivo*, we generated genetically deficient DPP2 mice, using two lentiviral vectors for conditional, Cre-lox-regulated, RNA interference (RNAi) [24]. One vector allows for conditional activation (pSico), whereas the other permits conditional inactivation (pSicoR) of short hairpin RNA (shRNA) expression (**Fig. 1A&B**). Various shRNA sequences designed against mouse DPP2 were cloned into the pSicoR and pSico lentiviral vectors and tested for their effectiveness in reducing DPP2 expression (**Suppl. Fig. 1**). The shRNA sequence with the most significant DPP2 knock-down (kd) was selected and used to infect embryonic stem (ES) cells and ultimately generate chimeric DPP2 kd mice. The constitutive DPP2 kd mouse (**Fig. 1A**), which expresses the shRNA against DPP2 in all tissues, was embryonic lethal, because only three chimeric mice were generated with extremely low chimerism (5-15%), based on coat color and GFP expression. These results were anticipated due to the fact that several previous attempts to generate a traditional DPP2 knock-out (ko) mouse had failed. In contrast, numerous, highly chimeric (90-95%) conditional DPP2 kd mice were generated (**Fig. 1B**). These mice were crossed to lck-Cre transgenic (tg) mice [25], resulting in T cell specific DPP2 kd, originating at the double negative stage in thymocyte development, termed lck-DPP2 kd mice. The GFP^- Cre^+ and GFP^+ Cre^- littermates served as negative controls to ensure that neither the lck-Cre transgene, nor the shDPP2 lentivirus, integration sites had adverse effects.

The lck-DPP kd mice were analyzed for the level and specificity of DPP2 kd. *dpp2* transcript levels were measured, because an antibody against murine DPP2 is currently unavailable. *dpp2* mRNA was reduced by about 50% in whole splenocytes (**Fig.1C**) and by over 90% in isolated peripheral T cells (**Fig.1D**) from lck-DPP2 kd mice compared to littermate controls. Thymic development was indistinguishable in lck-DPP2-kd and control mice, as evidenced by normal absolute numbers (data not shown) and percentages of thymocyte subsets (**Fig.2**). Similarly, the absolute numbers of lymphocytes in the peripheral lymphoid organs were identical to those of littermate controls; however, the proportions of

$CD4^+$ and $CD8^+$ T cells were increased about 40% in the spleen and, to a lesser extent, in the lymph nodes of the lck-DPP kd mice, and the proportion of B cells was decreased (**Fig. 2**). No difference in activation marker expression, $CD4^+CD44^{hi}CD62L$, $CD8^+CD33^{hi}CD122^+$, $CD25^+$ and $CD69^+$, was observed in the peripheral T cells of lck-DPP kd compared to control mice (**Suppl. Fig. 3** and data not shown).

lck-DPP kd T cells are hyper-proliferative when stimulated *in vitro*

DPP2 has been shown to maintain cells in a quiescent state, and its inhibition *in vitro* results in cells drifting into G_1 of the cell cycle [5]. Thus, we reasoned that the loss of DPP2 may cause T cells to proliferate faster than normal cells. To test this hypothesis, splenocytes and lymph node cells from lck-DPP kd mice and littermate controls were stimulated with various concentrations of anti-CD3 alone or in combination with anti-CD28, followed by an 8 h ^{3}H-thymidine pulse at various time points. As shown in **Fig. 3A**, more T cells from lck-DPP kd mice entered S-phase compared to those of control mice. Even after just two days of stimulation, lck-DPP kd T cells incorporated more ^{3}H-thymidine into newly synthesized DNA than control T cells, suggesting that DPP2 inhibition causes T cells to proliferate faster. To analyze the proliferative phenotypes of the individual T cell subpopulations, $CD4^+$ and $CD8^+$ T cells were isolated from the spleen and lymph nodes by negative selection. Similarly to what we had observed in unseparated lymphocytes, both $CD4^+$ and $CD8^+$ T cells from lck-DPP kd mice proliferated more than those of littermate controls (**Fig. 3B&C**), thus, confirming our initial results.

Cytokine profile of *in vitro* activated naïve T cells from lck-DPP kd mice

The hyper-proliferative phenotype of the activated T cells from lck-DPP kd mice prompted the analysis of the cytokines secreted by these cells. Whole splenocytes and lymph node cells or isolated $CD4^+$ and $CD8^+$ T cells were simulated with anti-CD3 plus anti-CD28, and supernatants were collected 24, 48 and 72 h later and tested by ELISA for the level of IL-2, IFN-γ, IL-4 and IL-17 cytokines. Very little IL-2 was observed in the supernatant of unseparated lymphocytes (**Fig. 4A**), probably due to the rapid use of this cytokine by the activated $CD8^+$ T cells. This was verified in isolated $CD4^+$ T cells from normal mice, which produced significant levels of IL-2. Surprisingly however, the $CD4^+$ T cells from lck-DPP kd mice secreted virtually no IL-2 (**Fig. 4A**). As expected, the activation of isolated $CD8^+$ T cells resulted in accumulation of only small amounts IL-2, and no difference between mutant and wild type cells was observed (**Fig. 4A**). IFN-γ is secreted mainly by activated $CD8^+$ cells and differentiated T_H1 cells. DPP2 kd $CD8^+$ T cells produced slightly more IFN-γ than controls at 24 h of stimulation, but no significant difference was observed at the other time points tested (**Fig. 4B**). Of special significance is the observation that IL-17 production, a cytokine secreted exclusively by differentiated T_H17 cells, was upregulated in unseparated lymphocytes, as well as in isolated $CD4^+$ and $CD8^+$ T cells from lck-DPP-kd mice, most notably at 72 h (**Fig. 4C**). Lck-DPP2-kd mice have a higher level of intracellular staining of IL-17A compared to littermate controls (Suppl. Fig. 4) which supports this data. IL-4, the signature T_H2 cytokine, was produced at extremely low levels by both DPP2 kd and control cells, and no difference was discernable (data not shown).

To determine whether $CD4^+$ T cells from lck-DPP2 kd mice indeed produced less IL-2, as opposed to increased usage of this cytokine by the highly proliferating DPP2 kd T cells, *il-2* transcripts were quantified by qRT-PCR. As shown in **Fig. 5A** left panel, *il-2* steady state mRNA levels were significantly decreased in activated $CD4^+$ T cells from lck-DPP kd versus control mice, suggesting that DPP2 kd $CD4^+$ T cells indeed have a defect in IL-2 production. In parallel, *ifn-γ* mRNA levels were measured by qRT-PCR in activated $CD8^+$ T cells and were found to be significantly lower in the lck-DPP2 kd versus control cells (**Fig. 5A**, right panel). On the other hand, *il-17* transcript levels were significantly

upregulated in both $CD4^+$ and $CD8^+$ T cells from lck-DPP2 kd compared to control mice (**Fig. 5B**). RORγt is a transcription factor required for Th17 cell differentiation. Stimulated T cells from lck-DPP kd mice were analyzed for RORγt transcript levels by qRT-PCR, they were upregulated in $CD4^+$, but not $CD8^+$ (data not shown), (**Fig. 5C**).

Immunization of lck-DPP2 kd mice leads to Th17 memory cells that are hyper-proliferative when restimulated *in vitro*

Mice were immunized with OVA in CFA intradermally (i.d.) and boosted with OVA in IFA i.d. two weeks later. Ten days after boosting, the draining lymph nodes were harvested, restimulated *in vitro* with OVA and pulsed for 8 h with ^{3}H-thymidine at various time points (**Fig. 6A**). Consistent with the anti-CD3 plus anti-CD28 stimulation results obtained with naïve T cells, OVA-immune DPP2 kd T cells were hyper-proliferative and responded to lower doses of OVA compared to those from littermate controls. These data demonstrate that immune T cells from lck-DPP2 kd mice have a lower threshold of activation, when restimulated *in vitro* with specific antigen. To examine whether these T cells had a similar cytokine profile as those of naïve mice, the respective supernatants were tested for the various cytokines by ELISA. We found that IL-2, IL-4 and IFN-γ levels were extremely low in both DPP2 kd and control mice (data not shown). This is most likely due to the low percentage of OVA-specific T cells responding to antigen restimulation *in vitro*. In contrast, the level of IL-17 was significantly increased in DPP2 kd lymphocytes (**Fig. 6B**). Thus, in the absence of DPP2, the *in vivo* immunization led to the generation of T_H17 memory cells, although the adjuvants CFA and IFA had presumably induced the full set of exogenous cytokines, necessary for T_H1 and T_H2 differentiation *in vivo*. Consistent with the higher level of IL-17 production, DPP2 kd T cells also upregulated *il-17a* (**Fig. 6C**) and *rorγt* (**Fig. 6D**) transcript levels.

lck-DPP2 kd mice have elevated autoantibodies

T_H17 cells are potent inducers of autoimmunity. Since activation of T cells from lck-DPP kd mice leads to differentiate into Th17 cells, these mice were examined for signs of autoimmunity. Interestingly, we observed that the level of circulating anti-nuclear antibodies (ANA) was increased in 6 months old lck-DPP2 kd compared to control littermates (**Fig.7**). ANA were detected on HEp-2 cells at serum dilutions of 1:50 and 1:100, but not 1:300, indicating that DPP2 kd mice have relatively low titers of circulating autoantibodies. The localization of the ANA to the nucleolar of the HEp-2 cells suggests the presence of anti-RNA, rather than anti-DNA, autoantibodies. Total Ig and IgM serum levels were quantified by ELISA, but no differences were observed between DPP2 kd and control mice (**Suppl. Fig. 2**). Furthermore, pathological studies performed on these mice revealed no inflammation, lesions or cellular infiltrates. It is possibly, therefore, that the full development of autoimmunity takes 12-15 months.

DISCUSSION

Our data indicate that DPP2 is a quiescence factor that is required for the maintenance of T cells in G_0 *in vivo*. In the presence of this dipeptidase, T cell differentiation into effector cells depends on TCR signals, as well as exogenous factors. In lck-DPP2 kd mice, however, the threshold of TCR-mediated activation is lowered, resulting in increased proliferation and differentiation into IL-17 secreting cells, independently of exogenous cytokines. Thus, IL-17 production seems to be the default pathway for T cell differentiation, a process that is actively prevented by DPP2, providing a new model for the control of T cell activation and differentiation.

In our previous work we observed that *in vitro* inhibition of DPP2 enzyme activity or down-regulation of its expression in quiescent T cells and fibroblasts leads to deregulated entry into the cell cycle, resulting in apoptosis of these cells [3-5]. To further elucidate the function of DPP2, development of an *in vivo* model was essential. For this purpose, we constructed two lentiviral vectors for conditional, Cre-lox-regulated, RNAi [23], infected ES cells and generated chimeric mice.

The constitutive DPP2 kd approach, where the DPP2-specific shRNA is expressed in all tissues, appeared to be embryonic lethal. This was surmised from the fact that only three chimeric mice were obtained which had extremely low chimerism (5-15%), based on coat color and GFP expression. These results were anticipated due to the earlier observation that the traditional DPP2 ko mouse was embryonic lethal (Huber lab, unpublished observation), suggesting that DPP2 plays an essential role during development. Further experiments are required to determine the stage of embryonic lethality and the defects associated with loss of DPP2.

On the other hand, numerous, highly chimeric conditional DPP2 kd founder mice were generated. These mice were crossed to lck-Cre tg mice [25] to produce lck-DPP2 kd mice, where DPP2 kd is restricted to the T cell lineage, beginning at the double negative stage in thymocyte development. T lymphocytes were chosen for this *in vivo* analysis, because DPP2 was initially discovered in T cells and the majority of *in vitro* data had been performed in T cells. Upon further breeding, we observed expected ratios and normal maturation of lck-DPP2 kd mice. Contrary to our expectations from the *in vitro* data however, thymocyte development was normal in the mutant mice in terms of overall cellularity and proportions of specific subsets. Furthermore, the peripheral T cell pool was increased by about 40% in these mice, and no apoptosis was observed. Thus, in the absence of DPP2 *in vivo*, the T cells appeared to be rescued from cell death.

It is possible that the increased peripheral T cell number in lck-DPP2 kd mice is a result of defective homeostatic proliferation. In the absence of DPP2, T cells would drift into early G_1 and enter the cell cycle, as observed *in vitro* [5]. However, these cells could be rescued from apoptosis due to environmental signals provided by stromal cells, which secrete numerous cytokines and chemokines. These factors are not present in *in vitro* cultures and could account for the discrepancy in the *in vitro* and *in vivo* results obtained by down-regulation of DPP2. One such factor is IL-7, which is required for the development of peripheral T cells [26-29] and is produced by many cell types, including stromal cells, B-cells, monocytes/macrophages, follicular dendritic cells, keratinocytes, and gut epithelial cells [26]. IL-7 promotes survival in part through expression of target genes, such as pro-survival *bcl-2* [30] and the stabilization of $p27^{kip1}$[30]. The importance of TCR-MHC interactions has also been established as a key factor in T cell survival *in vivo* [31, 32]. Brocker *et al.* demonstrated that continued survival of mature T lymphocytes is dependent on MHC class II-expressing dendritic cells [33].

When tested *in vitro* by TCR activation, the T cells of the lck-DPP2 kd mice demonstrated a lower activation threshold and higher proliferation than those of the control littermates. Similarly, T cells from *in vivo* immunized mice responded to a lower antigen dose and increased proliferation when restimulated *in vitro*. This may possibly be due to a shortened G_1 phase caused by DPP2 kd, an observation that we made in DPP2 kd fibroblasts, which proliferate faster than wild type cells in the presence of serum (unpublished result). Similarly, it has been reported that T cells lacking transactivator of ErbB2 (TOB1) have a reduced threshold of activation [34]. Furthermore, loss of Lung-Kruppel-like factor 2 (KLF2) leads to a loss of quiescence defined by proliferation, increased metabolism and altered expression of activation markers [35]. Interestingly, we previously demonstrated that DPP2 is

transcriptionally activated by KLF2 and TOB1, linking them in a program that maintains lymphocyte quiescence, which is regulated by quiescence-specific transcription [3]. Collectively, these data support the role of DPP2 in preventing proliferation and promoting quiescence.

Of particular interest is the finding that naïve T cells from lck-DPP2 kd mice mainly produce IL-17, the signature cytokine of T_H17 cells [36], upon TCR-mediated activation *in vitro.* In addition, these cells significantly upregulate *ror*γt mRNA, the master regulator of T_H17 differentiation [15]. In agreement with this observation, we found that IL-2 and IFN-γ production was down-regulated in the activated mutant T cells. $CD4^+$ and $CD8^+$ T cells, respectively, produce these cytokines after TCR activation in the absence of exogenous factors. Furthermore, IL-2 has been shown to induce Foxp3 expression and inhibit T_H17 cell differentiation [37]. Collectively, our data support the notion that loss of DPP2 causes T cells to differentiate into T_H17 cells and IL-17 producing $CD8^+$ T cells upon TCR stimulation.

It can be surmised from these results that the production of the inflammatory cytokine IL-17 is the default pathway in T cell differentiation and is actively suppressed by DPP2. Such control may be important to prevent expansion of autoreactive T cells. In agreement with this hypothesis, we observed increased levels of ANA in the lck-DPP2 kd mice, indicative of augmented autoantibody production in these mice. T_H17 cells have been implicated in numerous human diseases, such as psoriasis, rheumatoid arthritis, multiple sclerosis, asthma and some bacterial and fungal infections [38]. A recent report on the effects of the loss of early growth response gene-2 (Egr-2) in T cells suggests that autoimmune disorders can result from a loss of effector T cell expansion and inflammatory activation [39]. This is consistent with the observations made in lck-DPP2 kd mice, where T cells are hyper-proliferative and differentiate into IL-17-producing cells. Several other reports have also shown examples of proteins that act to prevent abnormal T cell proliferation and autoimmunity associated with the production of IL-17. Similar to the role of DPP2 demonstrated in this report, proprotein convertase, furin, which regulates proteolytic maturation of proproteins, has been implicated in central tolerance, and break of tolerance leads to autoimmunity and IBD [40]. However, recent reports have described a protective role of IL-17A in IBD [21-23]. In this regard, it is of interest that the lck-DPP2 kd mice showed no signs of IBD (results not shown).

In summary, the data presented here on the activation phenotype of T cells from lck-DPP2 kd mice point to a model in which DPP2 lifts the threshold of T cell activation, preventing spontaneous cell division. Upon kd of DPP2, cells may drift into early G_1 of the cell cycle and may proliferate faster upon stimulation, because they have an advantage by being poised to enter S phase sooner. This would provide an explanation for the hyper-proliferative behavior of DPP2 kd T cells upon stimulation. Activated DPP2 kd $CD4^+$ cells differentiate into T_H17 cells through a default pathway bypassing the required cytokines, IL-6, IL-1 and/or TGF-β, for Th17 cell differentiation. Interestingly, DPP2 kd $CD8^+$ T cells also generate increased amounts of IL-17A, suggesting that IL-17 production is the default pathway for all T_0 cells. In the presence of DPP2, exogenous factors are required to overcome this threshold of activation, allowing differentiation into effector cells. Collectively, these results imply that DPP2 is an essential protease that is intricately involved in the G_0/G_1 transition in T cells, preventing their differentiation into IL-17-producing effector cells.

METHODS

shRNAs

The shRNAs against mouse DPP2 were generated using the pSicoOligomaker1.5 which can be found at http://web.mit.edu/jackslab/protocols/pSico.html and were verified on the Dharmacon website http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx.

The selected oligos were cloned in pSicoR and pSico vectors [24] according to the protocol described on the Tyler Jacks Website. Double-stranded RNA was synthesized by Dharmacon (Lafayette, CO). All DNA sequencing was done at the Tufts University Core Facility. shRNA sequences that had the most significant knock down of mouse DPP2 measured by qRT-PCR was selected to infect 129/SVEV ES cells (#CMT1-1, Chemicon). The empty lentiviral vector was used as a control.

Sense strand against mouse DPP2 (shDPP2): 5'-TGG TTC CTA GTG TCA GAT AA-3

Virus Generation and Infection

Lentiviruses were generated essentially as described [41]. Briefly, 10 μg of lentiviral vector and 4 μg of each packaging vector were cotransfected in 293T cells by using the calcium phosphate method (Current Protocols in Molecular Biology). Supernatants were collected 36–40 h after transfection, filtered through a 0.45-μm filter, followed by centrifugation of the viral supernatant at 25,000rpm in a Bechman SW28 rotor for 1.5 h to concentrate the virus. The viral pellet was resuspended in 200ul ES cell media and added to 10,000-20,000 ES cells that were plated on a feeder layer of irradiated MEFs and incubated for 6 h at 37°C. Sequabrene (Sigma) was added (8 μg/μl) to the ES cell media during leniviral infection to increase infection efficiency. GFP positive colonies were isolated 3–4 days after infection. On average, 15–30% of ES colonies were GFP positive.

ES Cell Manipulation and Generation of Chimeras

129/SVEV ES cells were cultivated on irradiated MEFs in DMEM containing 15% FCS, leukemia-inhibiting factor, penicillin/streptomycin, l-glutamine, and nonessential amino acids. As described above, ES cells were infected with pSico or pSicoR, GFP^+ clones were isolated and tested for kd DPP2 by qRT-PCR. The clone that suppressed DPP2 expression by 90% was selected to inject into the blastocysts of pregnant mice. Only two pSicoR chimeric mice were obtained with extremely low chimerism (5%-15%). Fourteen male pSico chimeric mice were obtained that differed in GFP expression. The two male mice with highest GFP expression were chosen to mate with transgenic mice that express Cre in tissue-restricted manner. lck-Cre mice (C57BL/6, cat#004197) [25] were purchased from Taconic Farms, Inc. (Hudson, NY).

Flow Cytometry

Lymphocytes from thymus, spleen and LN were stained with αCD4-APC and αCD8-PEcy5 (BD Biosciences) in PBS for 15min at room temperature followed by FACS calibur (BD Biosciences) analysis to determine the percentage of T cell populations in these tissues.

qRT-PCR

qRT-PCR were performed on total RNA isolated from cells (RNeasy mini kit, Qiagen) using mouse *Dpp2* (primer pair: GGAGGCCCTGCTTGTCTTT and CACCGAACGGAAGCGATTTC; TaqMan MGB probe: 6-FAM-CTGAGCACCGGTACTATG-NFQMGB) and RT-PCR reagents (#4304971) (Applied Biosystems), were run and analyzed on ABI 7200 sequence detection system. The probe for 18S RNA (#4308329, Applied Biosystems) was used to normalize individual samples. The calculation is based on the relative differences ddC(t) method as described.[3] Transcript levels were similarly quantitated using the murine IL-17A (Mm004369619), IFN-γ (Mm00801788), RORγt, and IL-2 ABI probes.

T cell separation and stimulation

Lymphocyte single cell suspensions were generated from thymus, spleen or lymph nodes of sacrificed mice using mesh filters. $CD4^+$ or $CD8^+$ cells were isolated from splenocytes and LN cell populations using negative selection magnetic beads CD8 enrichment and CD4 enrichment sets (#558131 and #558131, BD Biosciences) according to the manufacturer's protocol. Cells were cultured in RPMI-1640 (Gibco, Grand Island, NY), supplemented with Hepes pH7.4, Penicillin-Streptomycin, L-glutamine, beta-Mercaptoethanol (all Gibco) and 10% fetal calf serum (Atlanta Biologicals, Norcross, GA). Lymphocytes were stimulated with plate bound αCD3 alone or αCD3 and αCD28 antibody (#553238, BD Biosciences). 96-well round bottom plates were coated with protein A for 1h at 37°C, washed 2Xs with 1XPBS, followed by addition of αCD3 alone or αCD3 and αCD28 antibody.

Intracellular Staining

CD4+ T cells were isolated and stimulated with αCD3 and αCD28 for 3 days followed by stimulation with PMA/Ionomycin in the presence of GolgiPlug (BD Biosciences) for 5h. Cells were fixed and stained with αIL-17A-PE according to the manufacturer's protocol (#555028 BD Biosciences) and analyzed on the FACS calibur.

T cell Proliferation Assay

Forty hours and sixty-four hours poststimulation, 1 μCi of [^{3}H]thymidine (ICN Biochemicals) was added to each well containing 50,000 of unseparated splenocytes and LN cells, for $CD4^+$ and $CD8^+$ cells 25,000 cells were used, followed by additional 8h incubation. Plates were harvested with the TOMTEC cell harvester and ^{3}H incorporation was measured by TRILUX Microbeta counter (PerkinElmer Life Science). Data were obtained from triplicate samples for each treatment.

ELISAs

Flat bottom Immulon 2HB plates (Fisher Scientific) were coated overnight with 3 ug/ml of capture anti-mouse IL-17 antibody (R&D Systems, Minneapolis, MN) in 1× phosphate buffered saline (PBS). Plates were blocked with 2% bovine serum albumin and 5% sucrose in 1× PBS at room temperature for 1 hour. Recombinant mouse IL-17 (standard curve) and the supernatant from the *in vitro* stimulation were diluted 1:2 then added in duplicate to the ELISA plates and incubated for 2 hrs at room temperature. Plates were washed and incubated with biotinylated anti-mouse IL-17 (R&D Systems) for 1 hour at 37°C, followed by additional washes and incubation with neutravidin-alkaline phosphatase for 30 minutes at room temperature. Plates were then developed with the AP substrate, *para*-nitrophenyl phosphate (Pierce), in 0.2% diethanolamine substrate buffer (Pierce) and were read at 405 nm in a SpectraMax spectrophotometer (Molecular Devices). Similar procedures were used for IFNγ, IL-2 and IL-4 ELISAs according to the manufacturer's protocol.

Immunizations

lck-DPP2 kd and littermate controls were immunized with 100ug of OVA and complete Freud's adjuvant (Sigma) by subcutaneous injection. Ten to fourteen days later mice were boosted with 100ug of OVA and incomplete Freud's adjuvant (Sigma) by subcutaneous injection. Ten to fourteen days after boosting the mice were sacrificed and the draining lymph nodes were harvested for *in vitro* stimulation with OVA.

Immunofluorescence Staining of Hep-2 Cells

Fixed human HEp-2 cells (Antibodies, Inc.) were stained with mouse serum according to the manufacturer's instructions, except the secondary antibody was FITC-conjugated F(ab)2 goat antimouse IgG (Jackson Immunoresearch). The slides were mounted with ProLong

Gold antifade reagent (Invitrogen) and digitally photographed with a Nikon E400 fluorescence microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Underwood R, et al. Sequence, purification, and cloning of an intracellular serine protease, quiescent cell proline dipeptidase. J Biol Chem. 1999; 274:34053–34058. [PubMed: 10567372]
2. Chiravuri M, Agarraberes F, Mathieu SL, Lee H, Huber BT. Vesicular localization and characterization of a novel post-proline-cleaving aminodipeptidase, quiescent cell proline dipeptidase. J Immunol. 2000; 165:5695–5702. [PubMed: 11067927]
3. Bista P, Mele DA, Baez DV, Huber BT. Lymphocyte quiescence factor Dpp2 is transcriptionally activated by KLF2 and TOB1. Mol Immunol. 2008; 45:3618–3623. [PubMed: 18555530]
4. Chiravuri M, et al. A novel apoptotic pathway in quiescent lymphocytes identified by inhibition of a post-proline cleaving aminodipeptidase: a candidate target protease, quiescent cell proline dipeptidase. J Immunol. 1999; 163:3092–3099. [PubMed: 10477574]
5. Mele DA, Bista P, Velez-Baez D, Huber BT. Dipeptidyl peptidase 2 is an essental survival factor in the regulation of cell quiescence. Cell Cycle. 2009; 8 e-pub Aug.
6. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989; 7:145–173. [PubMed: 2523712]
7. Langrish CL, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005; 201:233–240. [PubMed: 15657292]
8. Chen W, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF- β induction of transcription factor Foxp3. J Exp Med. 2003; 198:1875–1886. [PubMed: 14676299]
9. Ghoreschi K, et al. Generation of pathogenic T_H17 cells in the absence of TGF-β signaling. Nature. 2010; 467:967–971. [PubMed: 20962846]
10. Dong C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. Nat Rev Immunol. 2006; 6:329–333. [PubMed: 16557264]
11. Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev. 2000; 14:1693–1711. [PubMed: 10898785]
12. Szabo SJ, et al. A novel transcription factor, T-bet, directs T_H1 lineage commitment. Cell. 2000; 100:655–669. [PubMed: 10761931]
13. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of T_H2 cells in Stat4-deficient mice. Nature. 1996; 382:174–177. [PubMed: 8700209]
14. Thierfelder WE, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature. 1996; 382:171–174. [PubMed: 8700208]
15. Ivanov II, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006; 126:1121–1133. [PubMed: 16990136]
16. Kleinschek MA, et al. IL-25 regulates T_H17 function in autoimmune inflammation. J Exp Med. 2007; 204:161–170. [PubMed: 17200411]
17. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299:1057–1061. [PubMed: 12522256]
18. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4:330–336. [PubMed: 12612578]

19. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity. 2006; 24:677–688. [PubMed: 16782025]
20. Doreau A, et al. Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol. 2009; 10:778–785. [PubMed: 19483719]
21. O'Connor W Jr. et al. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. Nat Immunol. 2009; 10:603–609. [PubMed: 19448631]
22. Yang XO, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity. 2008; 28:29–39. [PubMed: 18164222]
23. Ogawa A, Andoh A, Araki Y, Bamba T, Fujiyama Y. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. Clin Immunol. 2004; 110:55–62. [PubMed: 14962796]
24. Ventura A, et al. Cre-lox-regulated conditional RNA interference from transgenes. Proc Natl Acad Sci U S A. 2004; 101:10380–10385. [PubMed: 15240889]
25. Lee PP, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity. 2001; 15:763–774. [PubMed: 11728338]
26. Maeurer MJ, Lotze MT. Interleukin-7 (IL-7) knockout mice. Implications for lymphopoiesis and organ-specific immunity. Int Rev Immunol. 1998; 16:309–322. [PubMed: 9505193]
27. Freitas AA, Rocha B. Peripheral T cell survival. Curr Opin Immunol. 1999; 11:152–156. [PubMed: 10322146]
28. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol. 2000; 1:426–432. [PubMed: 11062503]
29. Tan JT, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A. 2001; 98:8732–8737. [PubMed: 11447288]
30. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell. 1997; 89:1033–1041. [PubMed: 9215626]
31. Barata JT, Cardoso AA, Nadler LM, Boussiotis VA. Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). Blood. 2001; 98:1524–1531. [PubMed: 11520803]
32. Marrack P, Kappler J. Control of T cell viability. Annu Rev Immunol. 2004; 22:765–787. [PubMed: 15032596]
33. Brocker T. Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. J Exp Med. 1997; 186:1223–1232. [PubMed: 9334361]
34. Tzachanis D, et al. Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. Nat Immunol. 2001; 2:1174–1182. [PubMed: 11694881]
35. Buckley AF, Kuo CT, Leiden JM. Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc--dependent pathway. Nat Immunol. 2001; 2:698–704. [PubMed: 11477405]
36. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004; 21:467–476. [PubMed: 15485625]
37. Laurence A, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007; 26:371–381. [PubMed: 17363300]
38. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009
39. Zhu B, et al. Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease. J Exp Med. 2008; 205:2295–2307. [PubMed: 18779345]
40. Pesu M, et al. T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. Nature. 2008; 455:246–250. [PubMed: 18701887]
41. Rubinson DA, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet. 2003; 33:401–406. [PubMed: 12590264]

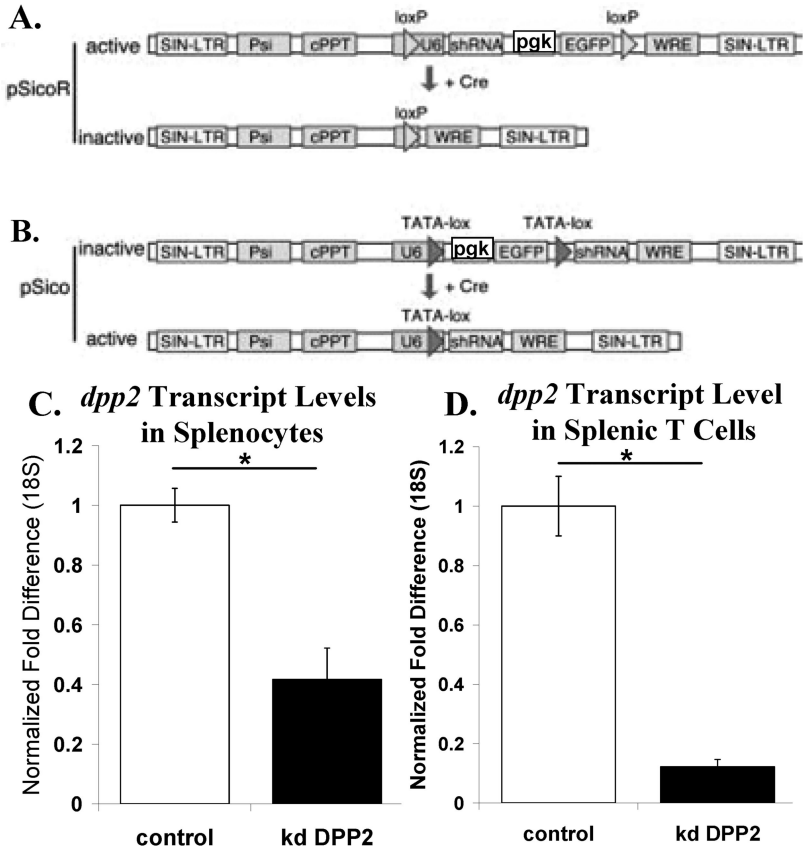

Figure 1. Schematic of shRNA expressing lentiviral vectors

A. Schematic of the generation of constitutive lck-DPP2 kd mice before and after Cre-mediated recombination. B. Schematic generation of conditional lck-DPP2 kd mice before and after Cre-mediated recombination. SIN-LTR, self-inactivating long terminal repeats; Psi, required for viral RNA packaging; cPPT, central polypurine tract; EGFP: enhanced GFP; WRE, woodchuck regulatory element (Adapted from [23]). C. Splenocytes were harvested from lck-DPP2 kd mice and control littermates, followed by RNA isolation; qRT-PCR was used to quantify DPP2 transcript levels. D. T cells were sorted for CD8 and CD4 expression from splenocytes of lck-DPP2 kd mice and control littermates, followed by RNA isolation, and qRT-PCR was used to determine DPP2 transcript levels. Student's two-tailed t test was used to determine the statistical differences in C. and D. *, p=0.02.

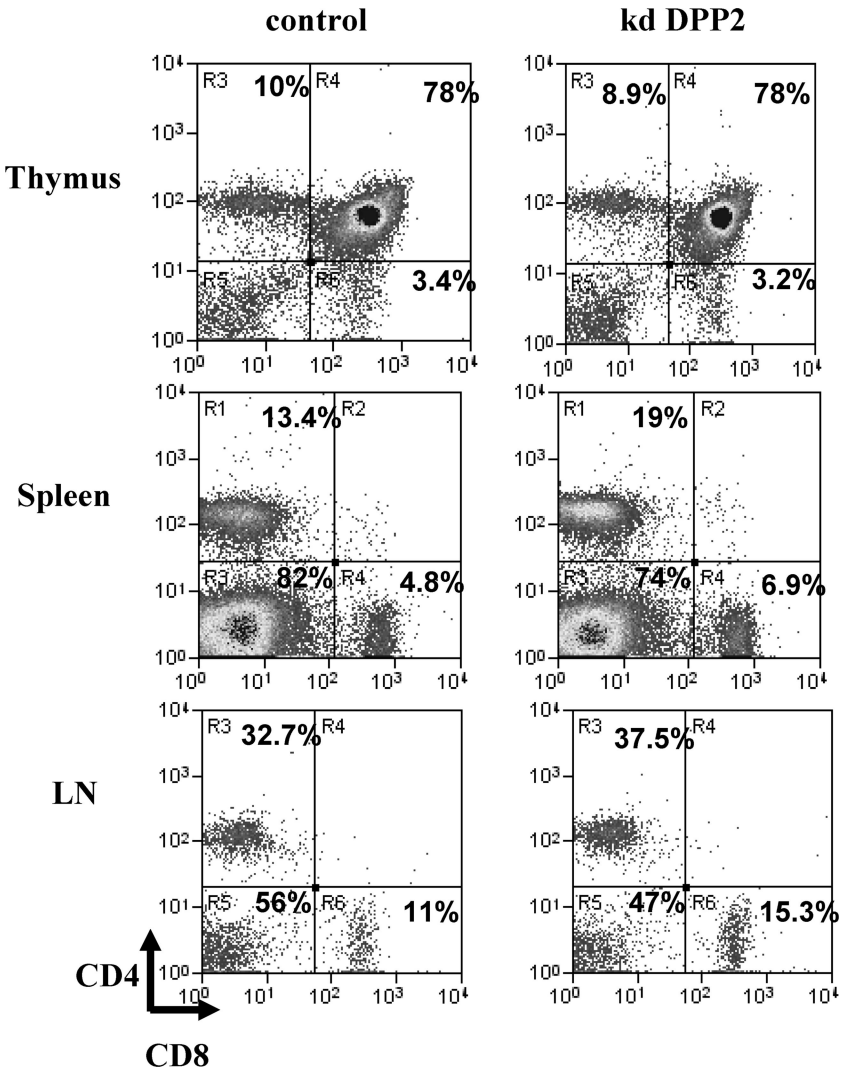

Figure 2. Lymphocyte profile of lck-DPP2 kd mice

Thymocytes, splenocytes and lymph node cells were stained for CD4 and CD8, followed by FACS analysis. The presented data are representative of the analysis of at least 4 mice per group.

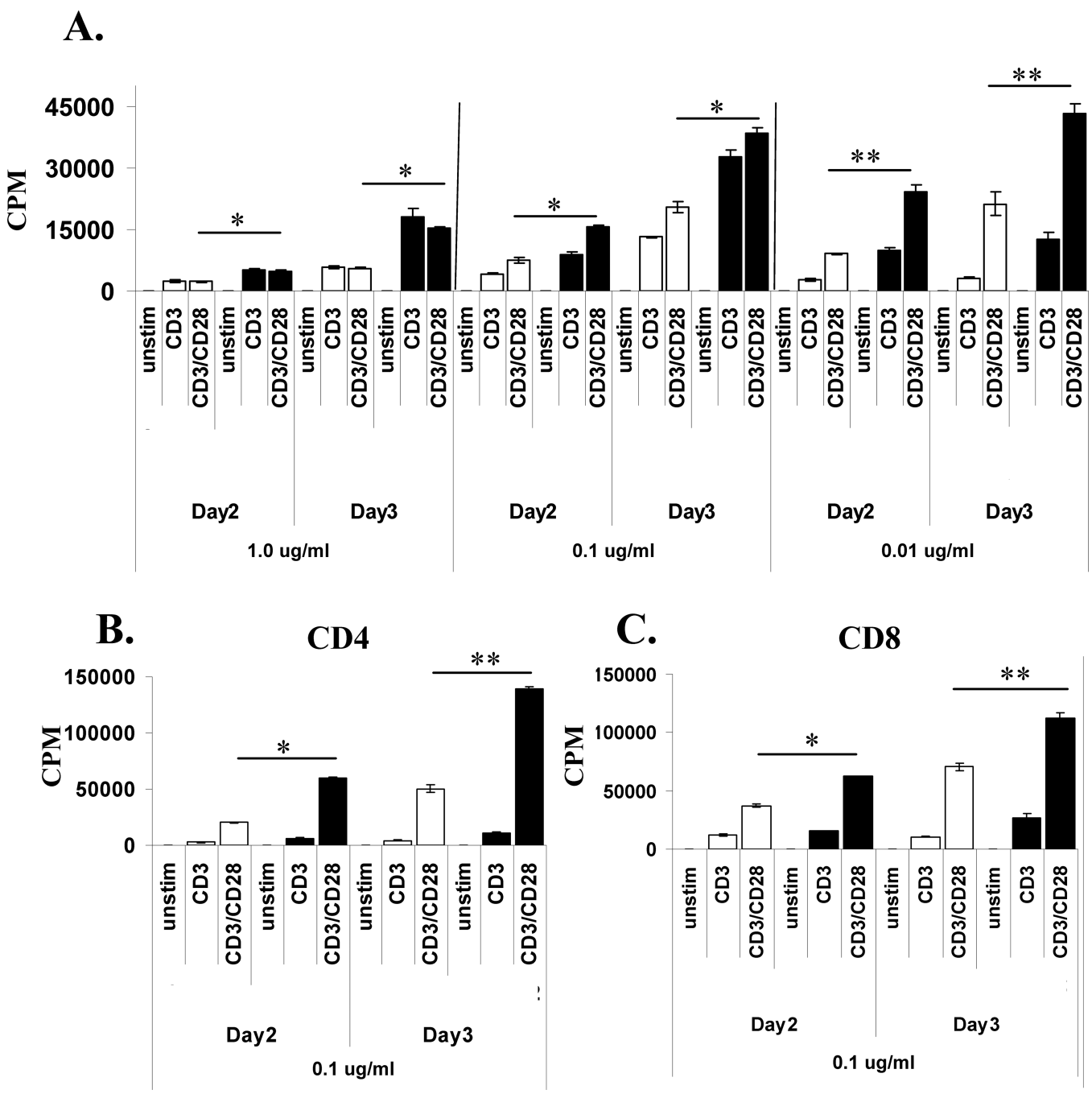

Figure 3. lck-DPP2 kd T cells are hyper-proliferative

A. Unseparated splenocytes and lymph node cells from lck-DPP2 kd mice (black bars) and control littermates (white bars) were cultured with various concentrations (1.0 μg/ml, 0.1μg/ml, 0.01μg/ml) of plate-bound anti-CD3 alone or anti-CD3 & anti-CD28 or were left unstimulated. Lymphocytes were pulsed with ^{3}H-thymidine for 8 h at the various time points and harvested. *, $p<0.007$; **, $p<0.014$. B. CD4^{+} T cells were isolated by negative selection from lymph node cells and splenocytes from lck-DPP2 kd (black bars) and control mice (white bars) and were cultured as in A. *, $p=0.001$; **, $p=0.001$. C. CD8^{+} T cells were isolated and cultured, following the same procedure as B. *, $p=0.004$; **, $p=0.005$. Student's two-tailed t test was used to determine the statistical significance.

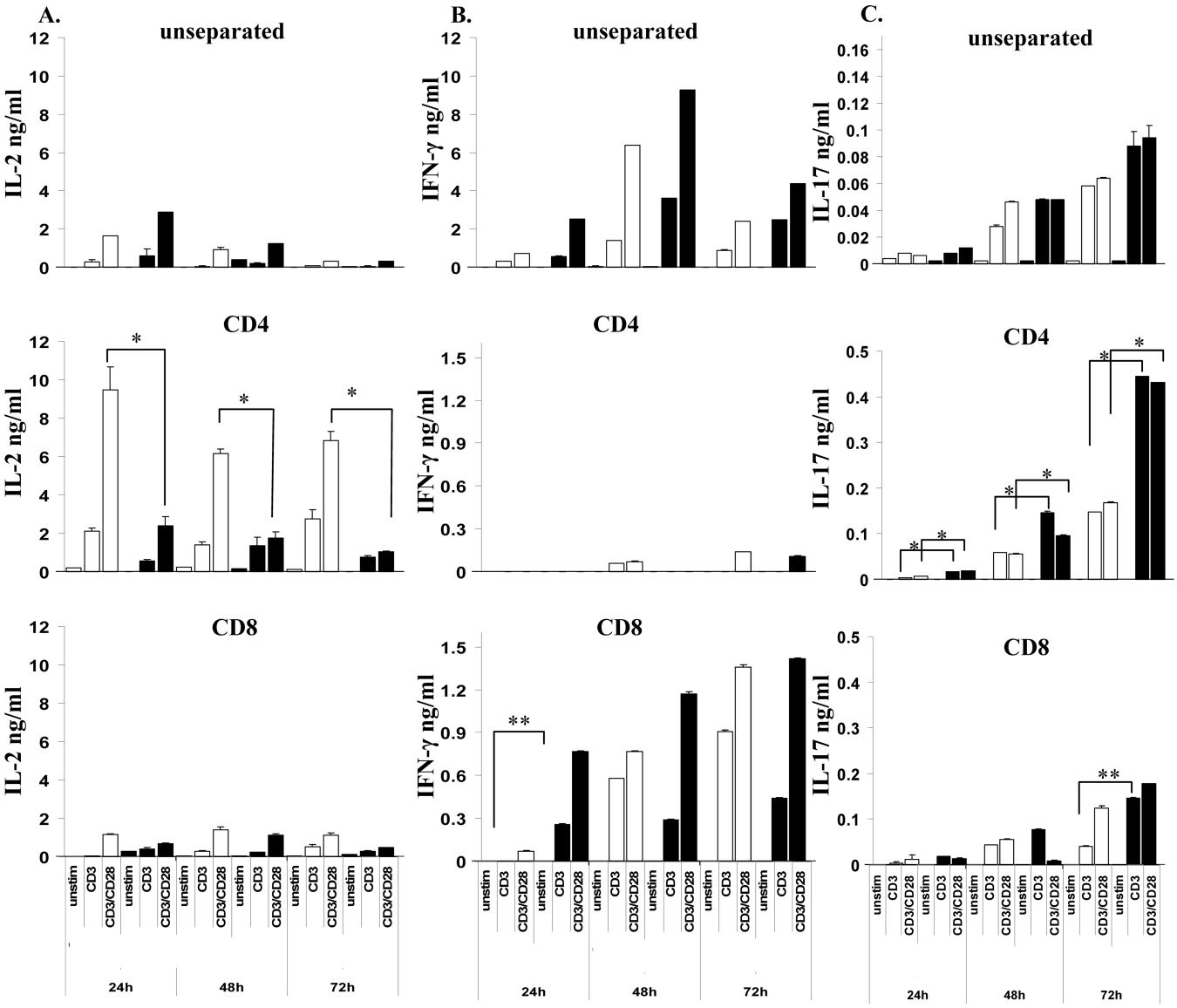

Figure 4. Cytokine profile of lck-DPP2 kd T cells

Unseparated lymphocytes, or either $CD4^+$ or $CD8^+$ T cells from lck-DPP2 kd (black bars) and control mice (white bars) were stimulated with 0.1 μg/ml of plate-bound anti-CD3, anti-CD3 and anti-CD28 or left unstimulated. Cell supernatants were analyzed by ELISA at 24, 48 and 72 h for IL-2 (A.), IFN-γ (B.), IL-17 (C.) The presented data are representative of four independent experiments. Student's two-tailed t test was used to determine the statistical significance in A. *, $p<0.05$; B. **, $p=0.04$, $p=$ C. *, $p<0.05$; **, $p=0.03$.

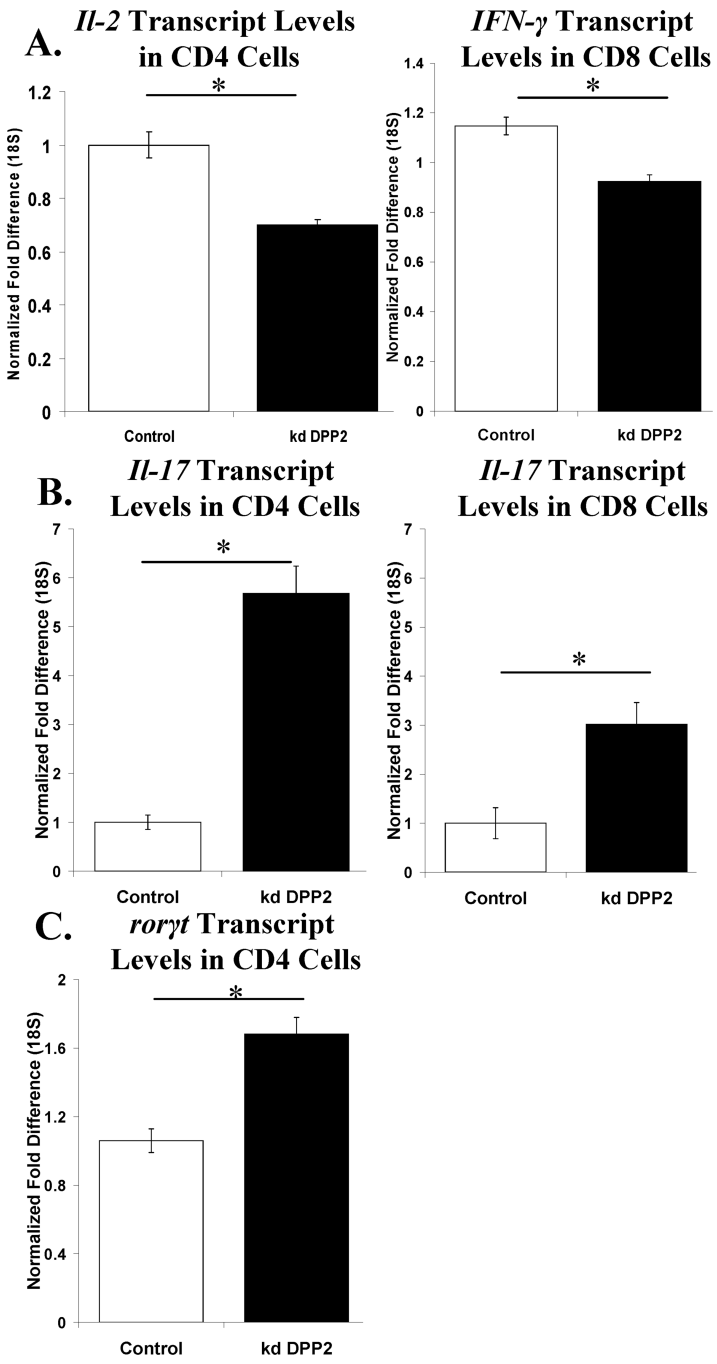

Figure 5. Cytokine and transcription factor mRNA levels in lck-DPP2 kd mice

Isolated $CD4^+$ or $CD8^+$ T cells from lck-DPP2 kd and control mice were stimulated with 0.1 μg/ml of plate-bound anti-CD3 & anti-CD28, as described in Fig. 4, and RNA was isolated 2 d later. A. *il-2* and *ifn-γ* transcript levels were quantified by qRT-PCR and normalized to 18S in isolated CD4 and CD8 T cells, respectively. B. *il-17* transcript levels were quantified by qRT-PCR and normalized to 18S in isolated CD4 and CD8 T cells. C. *rorγt* transcript levels were compared in isolated $CD4^+$ cells by qRT-PCR and normalized to 18S. Student's two-tailed t test was used to determine the statistical differences in A. *, p=0.03; B. *, p=0.003 and C. *, p=0.0015

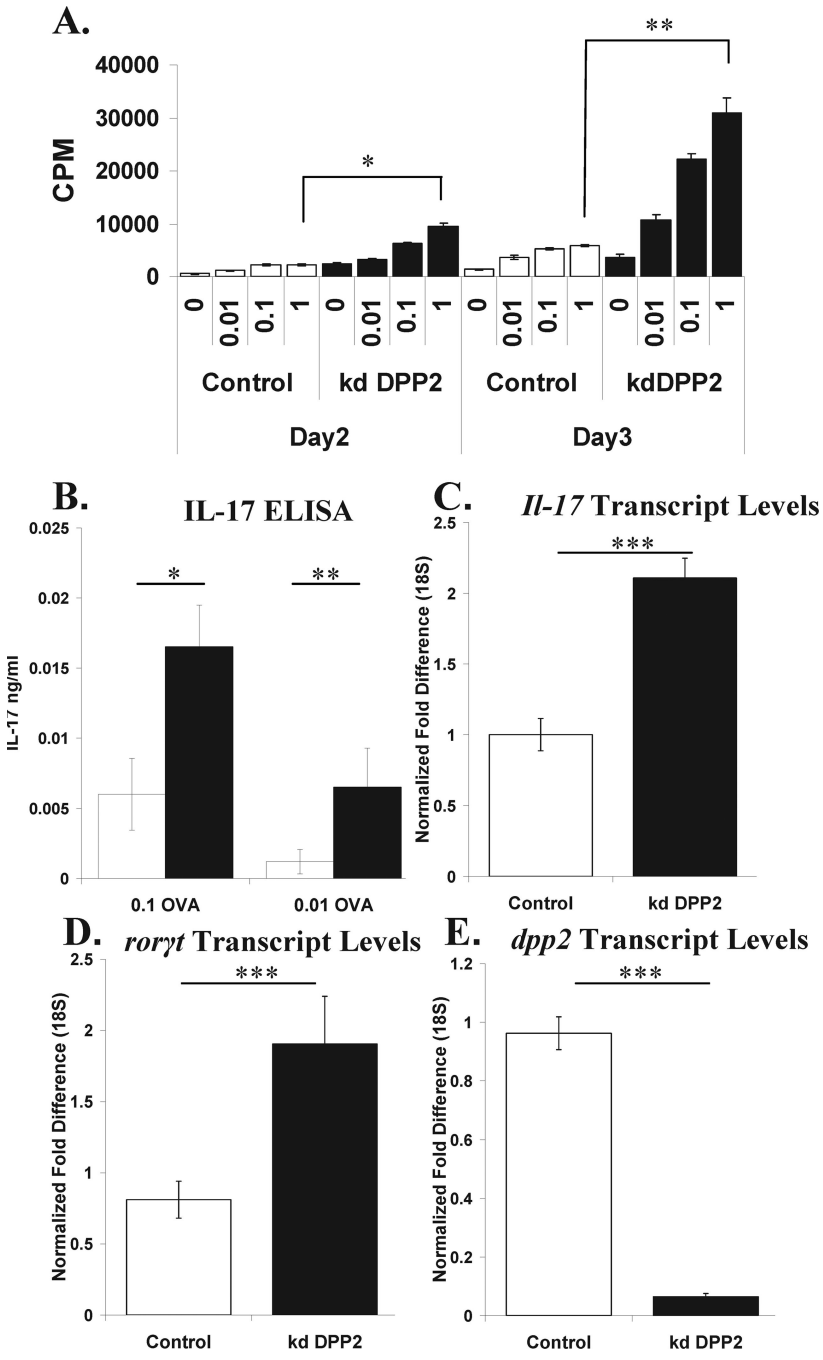

Figure 6. OVA- immune T cells from lck-DPP2 kd mice are hyper-proliferative and differentiate into Th17 cells when restimulated *in vitro*

The draining lymph nodes were harvested from OVA-immunized lck-DPP2 kd and control littermates, single cell suspensions were prepared and cultured *in vitro* with 0.01μg/ml, 0.1μg/ml, 1.0 μg/ml of OVA. A. Cultures were then pulsed with ^{3}H-thymidine for 8h at various time points after stimulation and harvested. These data are representative of 4 independent experiments. Paired student's t test was used to determine the statistical differences *, p=0.005; **, p=0.009. B. The supernatant from these cells was analyzed for IL-17 by ELISA on day 3 of stimulation. These data represent the average of 6 mice per group. Non-paired t test was used to determine the statistical differences *,p<0.05; **,

$p<0.065$. C., D., E. RNA was prepared from these cells on day 3 of stimulation and tested by qRT-PCR for the following transcript levels and normalized to 18S: *il-17* (C.), *rorγt* (D.) and *dpp2* (E.) Paired student's two-tailed t test was used to determine the statistical differences, ***, $p=0.005$.

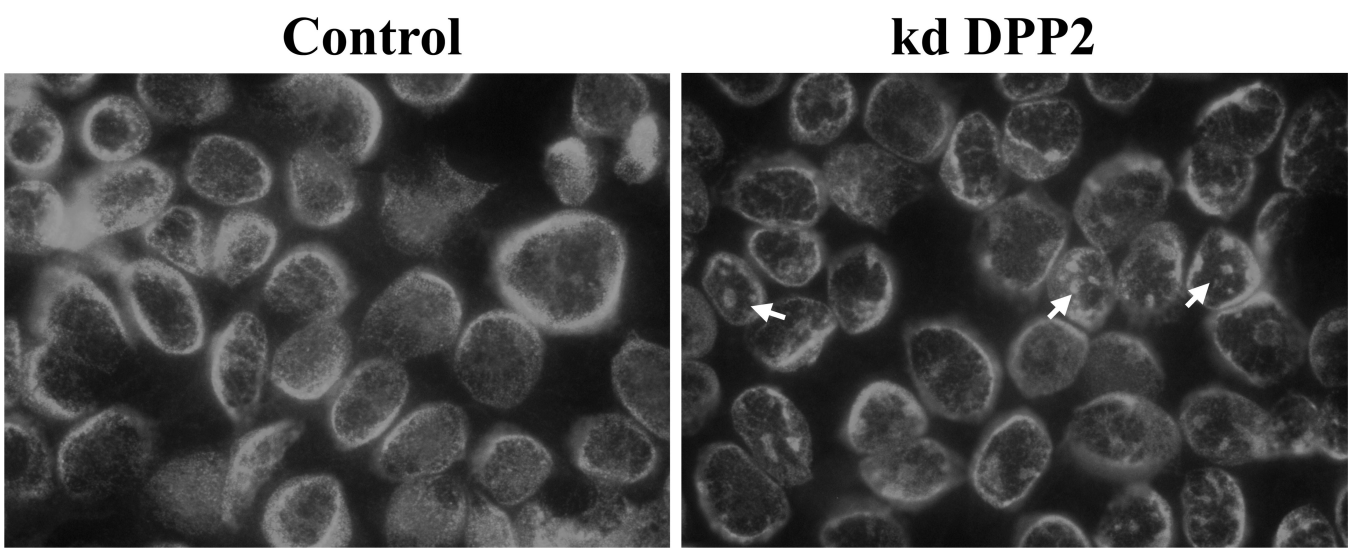

Figure 7. lck-DPP2 kd mice have elevated ANA autoantibodies

Sera (diluted 1:50) from naïve control littermates and lck-DPP2 kd mice were used to stain fixed HEp-2 cells. The nucleolar staining of the HEp-2 cells by the serum of lck-DPP2 kd, but not control mice, indicates that these Abs recognize RNA, rather than DNA. Results are representative of 3 independent experiments.