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Peripheral deletion of CD8 T cells requires p38 mitogen activated protein kinase in cross-presenting dendritic cells

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Abstract

Peripheral tolerance mechanisms exist to prevent autoimmune destruction by self-reactive T cells that escape thymic deletion. Dominant tolerance imposed by CD4+FoxP3+ T regulatory cells can actively control auto-aggressive T cell responses. Tolerance mechanisms that act endogenous to the T cell also exist. These mechanisms include T cell inactivation (anergy) and deletion. A major difference between anergic T cells and T cells undergoing peripheral deletion is the capacity of the latter to still signal through mitogen activated protein kinases (MAPK) upon TCR stimulation, suggesting these signals may be required for T deletion. In this study, we used several different models of CD8 T cell deletion to investigate the contribution of MAPK activation. Using chemical inhibitors, we established that inhibition of p38, but not ERK or JNK, rescue T cells from undergoing peripheral deletion both *in vitro* and *in vivo*. Using T cell specific murine lines genetically altered in expression of p38α, and mice in which p38α was deleted only in CD11c expressing cells, we surprisingly found that CD8 T cell intrinsic p38α activation was not responsible for increased survival, but rather that inhibition of p38α in the antigen-presenting DCs prevented CD8 T cell deletion.

Introduction

Central tolerance purges the immune system of T cells displaying high affinity T cell receptors specific for self-peptide-MHC complexes. However, this process is not complete and significant numbers of T cells recognizing peripheral self-peptide-MHC molecules emigrate from the thymus (1). Fortunately, peripheral tolerance mechanisms are in place to protect against dangerous auto-reactive T cell responses. These peripheral mechanisms of tolerance are especially important for the control of CD8 T cell immunity, due to widespread expression of MHC class I molecules on all nucleated cells. There are several defined

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mechanisms of peripheral tolerance. Dominant tolerance imposed by CD4+FoxP3+ T regulatory cells can actively prevent or control auto-aggressive T cell responses (2–4). There also exist tolerance mechanisms that act endogenously in self-reactive T cells.

Under non-inflammatory conditions, CD8 T cells undergo tolerance induction in the periphery. This occurs after interacting with cognate peptide-MHC Class I complexes (signal 1) expressed on quiescent APCs that fail to provide sufficiently high levels of co-stimulatory molecules (signal 2) or inflammatory cytokines (signal 3) for proper CD8 T cell activation with development of effector cell functions such as cytotoxicity and cytokine production (5, 6). Defined tolerance mechanisms intrinsic to the T cell include deletion (cell death) and inactivation (anergy) of autoreactive T cells (7–9). T cell anergy is dependent upon the continuous presence of antigen in the absence of inflammation. This is a reversible process, and T cells regain function after a period of rest in the absence of antigen (9–11). During peripheral deletion, T cells proliferate in response to antigen but then die of apoptotic cell death. This has been demonstrated using TCR transgenic CD8+ T cells specific for islet beta cell antigens that are continuously cross-presented by in the pancreatic lymph nodes by DCs. Interaction with the cross-presented antigen in this system abortively activates auto-reactive T cells leading to their deletion (12, 13).

Using *in vivo* models, we and others have shown that differential peripheral T cell tolerance mechanisms can be induced by varying the dose of soluble peptide injected under non-inflammatory conditions. Using several different TCR transgenic models, including CL4 specific for a peptide of HA and OT-1 cells specific for a peptide of OVA, we previously demonstrated that high levels of peptide favour the induction of a form of anergy characterized by the loss of capacity of the cells to signal through MAPKs, including p38 and ERK (14, 15). Low levels of peptides favour deletional tolerance, which has been shown to be BIM-dependent (16, 17). Cells committed toward deletional tolerance are still capable of phosphorylating p38 and JNK after TCR stimulation. The proximal TCR signaling events that result in peripheral deletion rather than anergy of T cells are still poorly defined. Here we tested the hypothesis that MAPK signaling may be required to initiate deletion.

To this end, we established an *in vivo/in vitro* model that mimics peripheral deletion and allowed us to test the effects of MAPK inhibitors on CD8 T cell apoptosis. We established that p38 inhibitors, but not ERK or JNK inhibitors, could partially prevent CD8 T cell deletion. We further confirmed those results *in vivo* using two different protocols to induce peripheral deletion through cross-presenttion of antigen. Surprisingly, we found that CD8 T cell intrinsic p38 activation was not responsible for survival, but rather that inhibition of p38 in the cross-presenting DCs prevented CD8 T cell deletion. Consequently, p38 signalling in DCs appears as a central regulator of peripheral deletion.

Materials and Methods

Mice

Clone 4 (18) TCR transgenic mice express a TCR specific for the $HA_{518–526}$ epitope restricted by MHC class I H-$2K^d$. C57BL/6 Bim−/− mice were kindly provided by Dr. Douglas Green. Bim−/− and Clone 4 TCR RIP-Tag2-HA mice were each backcrossed with

B10.D2 mice for greater than eight generations. P14 T cells express a TCR specific for the $GP_{33–41}$ epitope of LCMV. OT-1 cells express a TCR specific for the SIINFEKL peptide from chicken Ovalbumin. C57BL/6 Gadd45γ−/− and Gadd45β−/− mice were kindly provided by Dr. B. Lu (Department of Immunology, University of Pittsburgh, School of Medicine, Pittsburgh, PA). C57BL/6 p38α and p38β Tyr323-mutated mice were kindly provided by Dr. J. Ashwell (Laboratory of Immune Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD). Permission to use C57BL/6 $Mapk14^{fl/fl}$ ($p38\alpha^{fl/fl}$) mice (19) was kindly given by Dr. K. Otsu (Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan) and were crossed on $CreER^{T2}$ mice obtained from Taconic (USA). To induce the activity of the Cre recombinase mice were administrated 2 mg of tamoxifen by oral gavage for 4 days and used the day after as a recipient or a source of P14 T cells. RIP-OVA mice were obtained from Dr William Heath (University of Melbourne, Australia) and crossed on CD11c-Cre $Mapk14^{fl}$ mice. Animals were housed at The Scripps Research Institute animal facility, and all procedures were performed according to the NIH Guide for Care and Use of Laboratory.

Immunizations

Recombinant vaccinia virus expressing the Ovalbumin gene of chicken ovalbumin was as described previously (20). Mice were infected with 10^6 PFU of recombinant vaccinia virus by i.v. injection. Amounts of the various peptides were injected in PBS. Peptides used in these studies included: the $HA_{518–526}$ (IYSTVASSL) epitope, ILA-HA (ILAIYSTVASSL), which extends the sequence of the nominal peptide by including three amino proximal residues that appear in the natural sequence of the protein, GP33M (KAVYNFATM) and ILA-$OVA_{257–264}$ (ILASIINFEKL). All peptides were synthesized at The Peptide Synthesis Core Facility at The Scripps Research Institute.

Preparation and adoptive transfer of naive TCR transgenic T cells

CD8+ T cells were isolated from the lymph nodes of CL4, P14 or OT-1 TCR mice (6–8 wk of age) by negative selection using the MACS CD8+ T cell isolation kit (Miltenyi Biotec). T cell purity was >85% with no contaminating CD4+ cells. For adoptive transfer experiments, the indicated number of cells was injected *i.v.* in a volume of 100 μl of PBS.

Cell culture condition

For *in vitro* culture after in vivo peptide injections, 5.10^6 total splenocytes were cultured for 3 additional days in 10% FCS complete RPMI, without addition of any peptide. For flow cytometry experiments, stimulation were performed with peptides at 10^{-6} M or PMA (10 ng/ml) Ionomycine (200 ng/ml).

In vitro and in vivo kinase inhibition

SKF86002 (10 μM), SB220025 10 uM, SP600125 (JNK inhibitor −10 μM), JNK Inhibitor II (CAS 129-56-6 - 5 μM), PD98059 (5 μM), U0126 5 (μM) (Sigma Aldrich) were used at the indicated concentration *in vitro*. For in vivo inhibition mice received twice daily SB220025 (0.6 mg per injection), SB203580 (1 mg per injection) or vehicle.

Flow cytometry

Antibodies were ordered from BD Biosciences (CD8, Thy1.1, Thy1.2, CD25, CD44). Cells were analyzed on a FACSCalibur or a FACSLSRII cytometer (BD Biosciences). Data were analyzed using FlowJo (Treestar Inc., CA) or Diva (BD Biosciences) software. For phospho antibody labeling, after ex-vivo restimulation cells were fixed in ice-cold 1.6% paraformaldehyde for 15 min. Cells were permeabilized with 90% methanol and stored at −20°C. Cells were labeled with anti-CD8, anti-Thy1.1, and anti-phospho antibodies specific for Erk and p38 (BD Biosciences) for 1 h in FACS buffer (HBSS, 1% FCS, 0.1 mM EDTA).

Loading of β2M KO spleen cells with ILA-peptide

ILA Ag-loaded spleen cells were prepared by osmotic shock as previously described (21). Briefly, Splenocytes from β2M KO mice were washed in RPMI 1640 and resuspended in hypertonic medium (0.5 M sucrose, 10% w/v polyethylene glycol 1000, and 10 mM HEPES in RPMI 1640 (pH 7.2)). Pre-warmed hypotonic medium (40% H2O, 60% RPMI 1640) was added. Cells were pelleted by centrifugation and washed twice with ice-cold HBSS, and 30.10^6 in 0.2 ml of ILA-loaded splenocytes was injected i.v. into each recipient mouse.

Statistical test

Data are expressed as mean + SD for each group. Unless mentioned, statistical differences between groups were evaluated with a non parametric Mann-Whitney t test using GraphPad Prism 5.0b software; $p>0.05$ was considered statistically significant ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).

Results

CL4 T cells undergoing peripheral deletion are partially anergized

Using a previously reported model of CD8 tolerance (8), we wished to compare the capacity of anergized T cells and T cells undergoing peripheral deletion to activate MAPK signaling upon restimulation. B10.D2 recipients that previously received purified TCR transgenic CL4 CD8 T cells (CL4 cells) were injected i.v. daily for 3 days with 1μg (low dose antigen (LDA)) or 100μg (high dose antigen (HDA)) of cognate K^dHA peptide to induce peripheral deletion or anergy, respectively (15). We monitored the activation status and numbers of CL4 cells in the spleen between days 3 and 6 following peptide injection. Flow cytometry analysis revealed comparable expression levels of the activation markers CD44, while CD25 was not expressed (Fig. 1A). Cell numbers peaked on day 3 (Fig. 1B). As anticipated, there was a rapid loss in the numbers of CL4 cells thereafter in mice treated with LDA (Fig. 1B). The rapid decrease in cell number was not due to an absence of peptide between days 3 and 6, as CL4 T cell numbers contract with the same kinetics if 1 μg peptide is administered daily throughout the duration of the experiment to day 6 (Fig. S1A). Treatment with HDA was not associated with a decrease in CL4 T cell number after day 3, as similar numbers of CL4 cells were detected in the spleens on days 3 to 6 (Fig. 1B). The number of CL4 cells in mice treated with PBS did not change significantly throughout the duration of the experiment (Fig. 1B).

Anergy is associated with an inability to signal through the TCR after re-stimulation (9). We measured by flow cytometry the ability of CL4 cells to phosphorylate the mitogen activated protein kinases (MAPK) Erk and p38 after K^dHA peptide re-stimulation *in vitro*. HDA treatment prevented Erk phosphorylation as early as 24 hours after the first peptide injection (5% of pERK+ CL4 cells; Fig. 1C,D). CL4 cells from mice treated with a low amount of peptide are partially anergized, but can still phosphorylate Erk to some extent (from 29% of pERK+ CL4 cells at 24 hours to 10.5% at 72 hours; Fig. 1C,D). The results are similar for p38 phosphorylation at 72 hours post-treatment (Fig. 1E, F). 41 % of naïve CL4 cells are phospho-p38 positive, versus 28% and 14% of CL4 cells after low dose or high dose of antigenic peptide injection, respectively (Fig. 1D). Thus, HDA treatment induces almost complete blockade of signaling through Erk or p38 MAP kinases, while this inhibition is only partial after LDA treatment of CL4.

The p38 MAPK pathway is involved in peripheral deletion

As the capacity to activate the MAPK pathway is an early difference between T cells undergoing peripheral deletion or anergy, we assessed the role of MAPK signaling pathways in the regulation of peripheral deletion. JNK and p38 MAPK pathways have been shown to regulate cell death in various cell types, including T cells (22–25). We analyzed the effects of chemical inhibitors of MAP kinases in an *in vivo/in vitro* model of deletion. CL4 cells were transferred into B10D2 mice, and the host mice treated with LDA or HDA for 3 days as previously described. One hour after the last antigen injection, splenocytes were isolated and cultured *in vitro* for 72 hours (Fig. 2A). As a consequence, both antigen presenting cells and T cells are present in the culture well. Numbers of non-apoptotic (annexinV negative) CL4 cells at the beginning and the end of the culture period were compared. Most of the CL4 cells from untreated animals remained annexinV negative throughout the experiment. Only 16% and 54% of the initial pool of CL4 cells from mice treated with LDA or HDA respectively remained annexinV negative at the end of the culture (Fig. 2B). Cell death in this system was BIM-dependent, as LDA-treated bim−/− CL4 cells did not undergo apoptosis as previously described (Fig. 2B and (16)). To assess the importance of MAPK signaling pathways in apoptosis induction in this system we added small molecule inhibitors of ERK, p38 and JNK during the *in vitro* culture phase (Fig. 2C, D). Neither JNK nor ERK inhibition increased cell survival. Inhibitors for p38 were associated with a more than 2-fold increase in survival rate of CL4 cells compared to control.

We next assessed the *in vivo* role of p38 in LDA-induced deletional tolerance by injecting soluble p38 inhibitors (SB220025 or SB203580) in mice harboring adoptively transferred CL4 T cells (Fig. 3A). Data from 2 independent experiments using each inhibitor showed that p38 inhibition partially protected CL4 cells from deletion during LDA treatment, with a 4- to 7-fold increase in survival (Fig. 3A, B).

To confirm that the p38 pathway was not specific to LDA induced deletion, we analyzed its role in another model of deletional tolerance, in which cell death is induced after activation by cross-presented antigen *in vivo*. We had previously shown that CL4 cells were deleted after activation by a ILA-extended HA antigen variant that could not directly bind H-$2K^d$, but must be cross-presented by DCs in order to stimulate CL4 cells (21). Treatment with the

cross-presented antigen resulted in efficient CL4 deletion that could be inhibited by the p38 inhibitor SB203580 *in vivo* (Fig. 3C). These data demonstrate that inhibition of the p38 pathway rescues CL4 cells from deletion after LDA treatment with nominal or a cross-presented antigen.

Deficiency of CD8+ T cell intrinsic p38 signaling does not inhibit LDA-mediated peripheral deletion

Two pathways of p38 signaling have been described for T cells, i) the conventional pathway involving Gadd45 molecules, and ii) the alternative pathway initiated after signaling through the TCR, leading to the autophosphorylation of p38 (26). To determine if p38 signaling within the CD8+ T cell was critically involved in LDA-induced deletional tolerance, mice deficient for key signaling molecules involved in p38 signaling pathways were obtained. Since these various KO/KI mice were only available on the C57BL/6 background, we used the C57BL/6 CD8 P14 TCR transgenic model in these experiments. The P14 TCR recognizes the LCMV derived epitope gp33m in the context of H2-K^b. P14 T cells undergo a similar kinetic of peripheral deletion upon treatment with LDA compared to CL4 cells, with a peak of proliferation at day 3 and a gradual decrease in cell number until day 6 where most of the cells are gone (Fig. S1B). However, the dose of peptide required for deletion is lower than the one used for CL4 cells (0.2 μg vs 1 μg). We confirmed that most of the cells have been deleted by infecting the mice with LCMV 30 days after LDA treatment (Fig. S1C). This process is BIM dependent, as *bim*-KO P14 T cells do not undergo LDA induced deletion (Fig. 4A; S1B). This was further confirmed by using the same *in vivo/in vitro* model used in Fig 2 and observed that the p38 inhibitor SB220025 rescues P14 T cells from undergoing deletion (Fig. S1D).

Gadd45β and Gadd45γ have been demonstrated to be important molecules in the potentiation of MAPK p38 signaling in T cells after exposure to inflammatory mediators (27). In the absence of Gadd45β and Gadd45γ, p38 signaling is attenuated in T cells. We investigated the effect such deficiency had on the induction of deletional tolerance. In the absence of Gadd45β and Gadd45γ, P14 T cells are still efficiently deleted (Fig. 4B), indicating that the conventional pathway of activation of p38 in T cells was not required for deletion.

To assess whether the alternative pathway was critical for LDA-induced deletion, we obtained mice that carried a mutation in the p38α at position 323 that prevents TCR-mediated p38α activation (26). P14 T cells carrying this mutation were adoptively transferred into B6 mice, and the hosts treated with LDA. P14 T cells lacking the alternative p38α activation pathway were efficiently deleted (Fig. 4C). Although p38α is the major isoform expressed in T cells, p38β has been shown to possess some activity in T cells (28). To rule out a compensatory role for p38β we used P14 cells containing Tyr323 mutations on both p38 α and β. These cells were also efficiently deleted after LDA treatment (Fig. 4C).

Finally, to fully exclude a critical role for p38α signaling intrinsic T cell deletion, we obtained ERT2-Cre *Mapk14*$^{fl/fl}$ P14 cells (p38α^{del}). After tamoxifen treatment, p38 activation is completely abrogated upon TCR stimulation in these T cells (Fig. S1E). P38α^{del} cells were efficiently deleted following LDA-treatment (Fig. 4D). Taken together,

these results indicate that p38 signaling intrinsic to CD8 T cells is not involved in peripheral deletion.

Role for T cell extrinsic p38 signaling in CD8+ T cell deletional tolerance

The fact that CD8 T cell deficiency in p38 had no significant effect on deletion was surprising, as p38 inhibition using small molecule inhibitors saved CD8+ T cells from undergoing deletion after LDA treatment (Fig.2C). We next considered whether p38 signaling in a non-T cell population was required for deletion. WT P14 cells were transferred into p38α-deficient hosts. P14 cells were deleted significantly less efficiently after receiving LDA treatment in a p38α-deficient host compared to WT host (Fig. 5A). Furthermore, upon challenge with LCMV virus 30 days after LDA treatment, a robust recall response is observed 7 days post-infection in the p38α-deficient host compared to a negligible response in the WT host (Fig. 5B). There was no significant difference in the deletion efficiency between p38α-deficient and WT P14 cells in a p38α-deficient host (Fig. 5 A–D), further suggesting intrinsic p38 MAPK signaling is not essential in the T cell population undergoing deletional tolerance. We noticed a 10-fold decrease in the amplification of virus-specific T cells when the host is p38α-deficient. Systemic p38 inhibition has been shown to inhibit viral replication and viral protein syntheis (29). This could explain those results.

Role of p38 in DCs to induce peripheral T cell deletion

It was previously established that cross-presenting $CD8\alpha^{+}CD103^{+}$ DCs are required to induce T cell peripheral deletion (12, 13). To determine whether deficiency of p38α in DCs affects T cell peripheral deletion, we used CD11c-Cre *Mapk14*$^{fl/fl}$ mice with a specific inactivation of the p38α gene in CD11c expressing cells ($p38^{del\text{-}CD11c}$ mice). We tested two models in which peripheral deletion is induced by antigen cross-presented by DCs. We previously showed that when the cognate peptide recognized by OT-1 cells is extended at the N-terminus 3 residues (ILA) it must undergo TAP dependent processing and cross-presentation by DCs before it can be recognized by CD8 cells, and that such activation results in T cell delection (22). As a second model of deletion we used RIP-OVA^{hi} mice, which express ovalbumin under the rat insulin promoter in the beta cells of the pancreas (30). OT-1 cells injected in to these mice are deleted through recognition of cross-presented antigen in the pancreatic lymph nodes (31). In order to use this model of endogenous deletion, it was necessary to employ a third TCR transgenic model, i.e OT-1 T cells.

Mice received 1×10^{4} purified $CD8^{+}$ Thy1.1 OT-1 cells, followed by injection of apoptotic splenocytes from B6 mice loaded with the long ILA-SIINFEKL peptide. This peptide requires cross-presentation to induce deletion of OT-1 cells, as previously described (21). At day 4 after injection, OT-1 cells have undergone proliferation in both WT and $p38^{del\text{-}CD11c}$ hosts (Fig. S2). Mice were infected 4 weeks post treatment to expand the surviving OT-1 T cells for easier detection by flow cytometry. In PBS treated mice, OT-1 T cells represented approximately 1% of CD8 T cells after infection induced proliferation (Fig. 6A; S2E). In mice treated with ILA-SIINFEKL this percentage was significantly reduced. Numbers of OT-1 detected in CD11c-Cre *Mapk14*$^{fl/fl}$ mice were similar to the numbers observed without treatment, strongly suggesting that peripheral deletion is dependent on p38α expression in

CD11c+ cells (Fig. 6A; S2E). To further confirm those results, CD11c-Cre p38α$^{fl/fl}$ were crossed onto RIP-OVA mice, which express ovalbumin under the rat insulin promoter in the beta cells of the pancreas. It has been demonstrated in this model that deletion of OT-1 cells requires cross-presentation of OVA by CD11c DCs (12). To measure the efficiency of peripheral deletion mice were infected with vaccinia-SIINFEKL 4 weeks after OT-1 transfer in order to expand the numbers of OT-1 cells for detection. Very few OT-1 cells were recovered from inguinal LNs, pancreatic LNs or spleens from WT RIP-OVA mice (Fig. 6B; S2F). In contrast, a significantly greater percentage of OT-1 cells were still present in those organs from CD11c-Cre *Mapk14*$^{fl/fl}$ RIP-OVA mice. Taken together, these results demonstrate that p38α expression in DCs is necessary for the induction of peripheral deletion of CD8 T cells.

Discussion

It is well established that the strength of TCR signal as determined by the amount of antigen (7–9) and the affinity for antigen (15) are critical factors to direct a CD8 T cell toward anergy or peripheral deletion. The proximal events that translate those differences into various cellular signaling pathways and their consequences on the fate of the CD8 T cells are still poorly characterized. We hypothesized that MAPK signaling could affect T cell survival. Using an *in vitro*/*in vivo* model for T cell tolerance, we established that p38, but not ERK or JNK signaling, was involved in peripheral deletion. We also showed that p38 inhibition could rescue CD8 T cells from Bim-dependent apoptosis. Activation of the MAPK kinase p38 signaling pathway had previously been reported to selectively induce apoptosis in CD8 T cells *in vivo* (23). Furthermore, T cell endogenous p38 has been reported to be required for Fas induced death in CD8 T cells (22). However, through the use of various mouse strains deficient in components of the p38 signaling pathways, we found that the role of p38 during T cell peripheral deletion is not intrinsic to T cells, but rather an effect mediated by DCs. To our knowledge, this is the first report implicating p38-signaling extrinsic to the T cell in deletional tolerance. Our current hypothesis is that activation of the p38 pathway supports peripheral deletion in an antigen presenting cell population, possibly by sustaining a tolerogenic profile in the DC.

The role of p38 in DCs has been studied in different contexts. During immune tolerance it was shown that p38α is constitutively activated in cross-presenting DCs from the mesenteric LNs, leading to the generation of induced Tregs and inhibition of Th1 T cells through a TGFβ dependent mechanism (32). The loss of p38α signaling in DCs was reported to result in impaired induction of oral tolerance and generation of Ag specific iTregs *in vivo*. Another study showed that the loss of p38α protected mice from development of autoimmune encephalomyelitis, and demonstrated that p38α acts in DCs to drive Th17 differentiation and inflammation, by reciprocal regulation of IL-6 and IL-27 (33). A very recent paper reported that p38α has a role in Ag processing by conventional CD8$^+$ DCs, whereas p38α has no function in antigen uptake by these DCs or their co-stimulation for T cells. This study also showed that p38α inactivation led to reduced production of IL-12p40 and IL-12p70 by conventional DCs (34).

In cancer-associated studies, it was established that tumor-derived suppressive factors inhibit the differentiation and function of DCs by upregulating p38 MAPK activity in DC precursors in both murine tumor models and cancer patients (35, 36). DCs from melanoma-bearing mice secreted significantly more IL-10 and less IL-12p70, and showed a decreased capacity to activate T cells compared with DCs from tumor-free animals. This cytokine profile was linked to the strong activation of p38 MAPK in those DCs (37). A more recent study shows that the inhibition of p38 MAPK resulted in decreased PPARγ expression in DCs, thus reducing the functional inhibition of p50 transcriptional activities by PPARγ. The activation of p50 upregulated surface expression of OX40L on DCs, which increases immunostimulatory potency of these DCs to activate antigen-specific effector T cells and to inhibit Treg conversion and function, thus facilitating tumor rejection (38). Altogether, p38 deletion can have multiple effects on DCs. One of these parameters, or a combination of them could be responsible for the rescue in deletional tolerance observed in our models. Considering this complexity, further investigations are required to discriminate between these various mechanisms in DCs, and their effects on T cell deletional tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

DC	Dendritic Cell
LDA	Low Dose Antigen
HDA	High Dose antigen
ILA	isoleucine, leucine, alanine
HA	hemagglutinin
KO	knockout
CL4	Clone 4
RIP	rat insulin promoter
LCMV	lymphocytic choriomeningitis virus.

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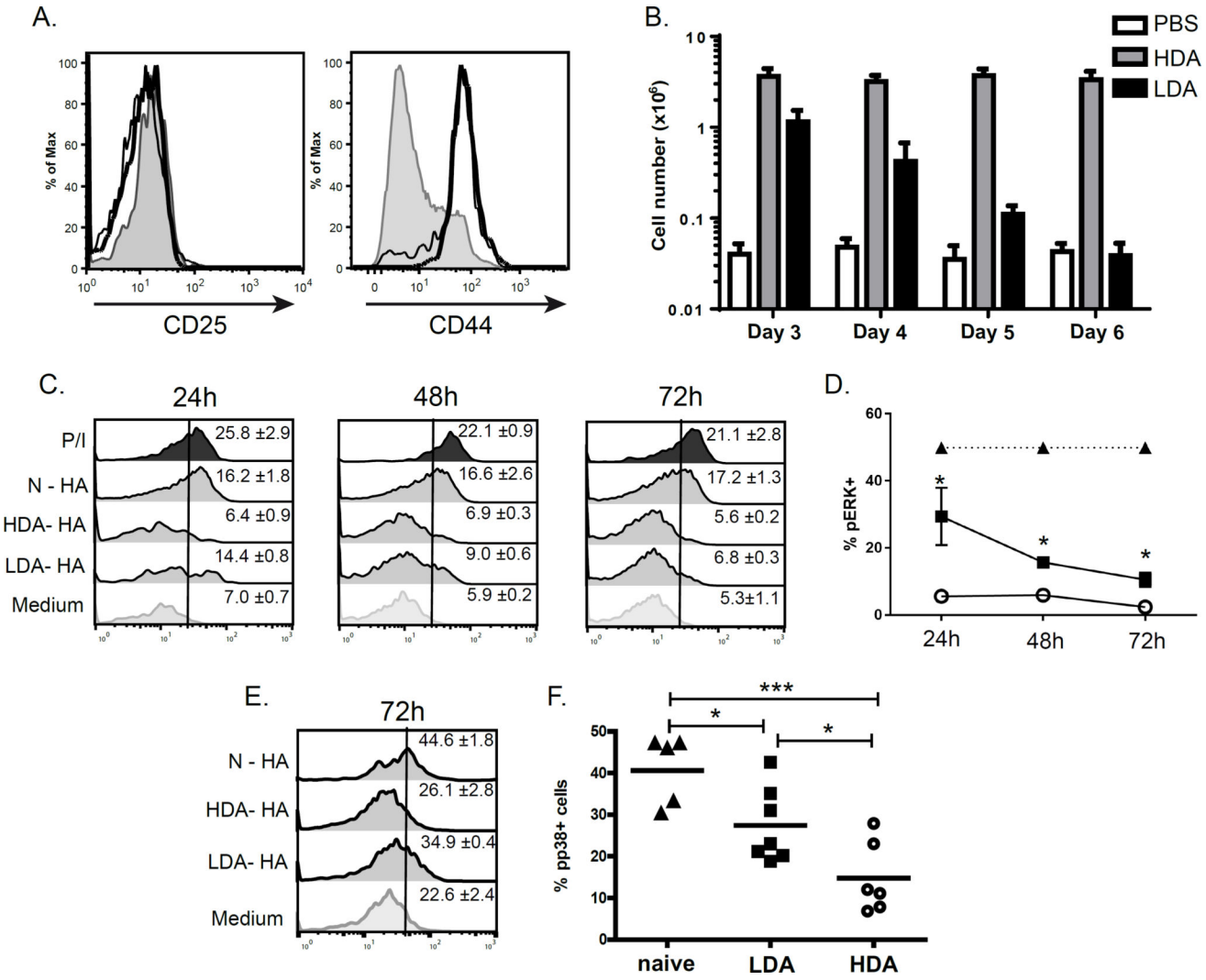

Figure 1. Injection of low or high dose of soluble antigen induces deletion or anergy of CD8 T cells

CL4 Thy1.1+ CD8 T cells (1.10^6) were adoptively transferred into B10D2 mice. Mice received daily injections of low (1 μg) or high (100 μg) dose of antigenic HA peptide for 3 days. (A) Levels of expression of CD25 and CD44 at day 3 on naïve CL4 cells (N - tinted) or CL4 cells coming from mice injected with low (LDA - thin line) or high dose (HDA - thick line) of antigenic peptide are shown. Results are representative of at least 3 independent experiments. (B) CL4 cell numbers in splenocytes from mice that received low (black), high (grey) dose of antigenic peptide or PBS (white) were determined at indicated days after the first peptide injection. (C–F) Mice were sacrificed 24, 48 or 72 hours after the first injection of antigenic peptide. Splenocytes from mice treated with PBS (black triangle), LDA (black square) or HDA (empty circle) of antigenic HA peptide were stimulated for 10 min with medium alone, with antigenic peptide (HA) or PMA and ionomycine (PI), fixed and labeled for FACS analysis. (C) Representative histograms of phosphor-ERK+ CL4 cells from the various conditions and Median Fluorescence Intensity ± SD (average of 4 mice from one representative experiment per condition) for pERK labelling are shown. (D)

Average of 4 mice per condition/per time point of one representative experiment out of two for pERK positive population is shown. (E) Representative histograms of phosphor-P38+ CL4 cells from the various conditions and Median Fluorescence Intensity ± SD (average of 3 mice per condition from one representative experiment) are shown. (F) Pooled percentages of phosphor-P38+ CL4 cells from two independent experiments are shown.

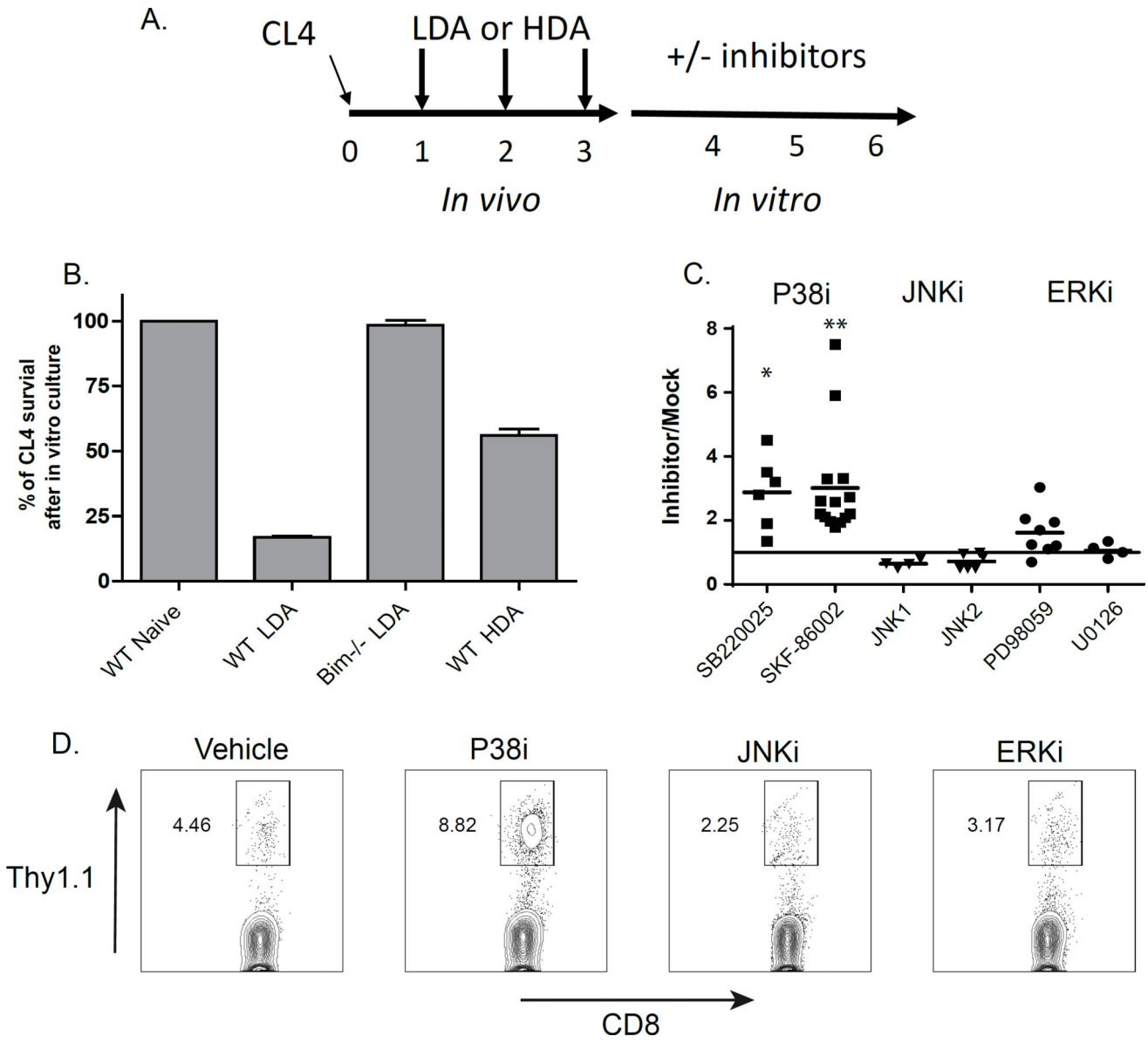

Figure 2. p38 inhibition rescues LDA induced deletion in vitro

(A–B) 10^6 WT or bim−/− CL4 Thy1.1+ CD8 T cells were adoptively transferred into B10D2 mice. For 3 days, mice received daily injections of PBS, 1 μg (LDA) or 100 μg (HDA) of HA peptide. One hour after the last injection of peptide, mice were sacrificed and 5.10^6 total splenocytes were cultured for 3 additional days in the absence or presence of the indicated MAPK inhibitor. (B) Percent of survival by comparing the final number of Annexin V negative CL4 cells with the initial number at the beginning of the culture is shown (3 mice per condition from one representative experiment out of three). (C) Ratio of CL4 cell number between each inhibitor (p38 inhibitors (p38i), JNK inhibitors (JNKi) and ERK inhibitors (ERKi)) and mock condition at the end of the culture after LDA treatment is shown. Pooled results from 2 to 6 independent experiments are shown. Each dot represent data from one mouse. Anova using Kruskal-Wallis non-parametric test with multiple

comparison was used. (D) A representative FACs plot from experiment in (C) with the indicated markers is shown (Gated on CD8+, Topro3-, lymphocytic cells using FSC/SSC).

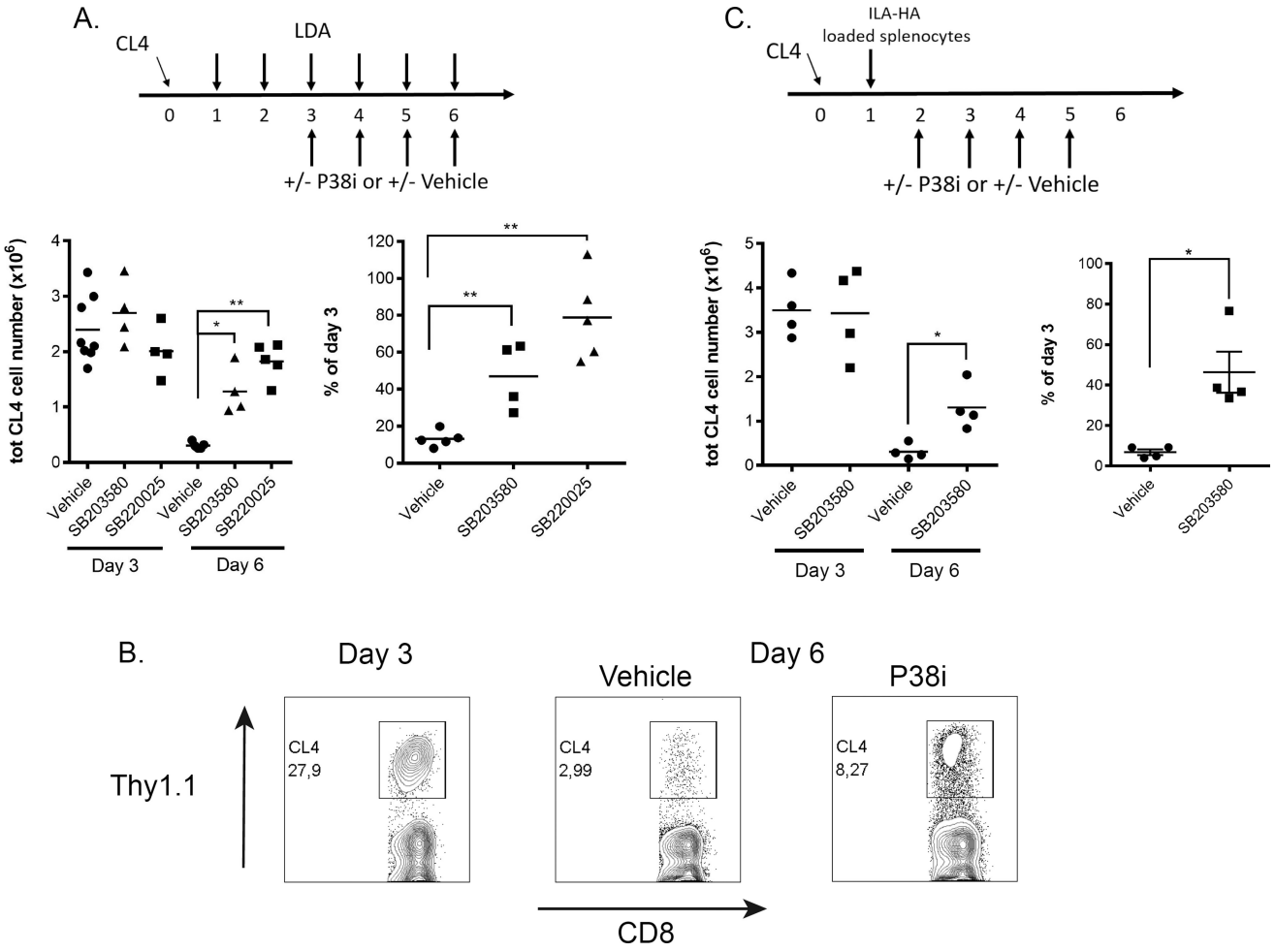

Figure 3. p38 inhibition rescue LDA induced deletion in vivo

(A, B) Mice harboring CL4 T cells received daily i.v. injections of 1 μg of HA peptide and were injected twice a day with 600 μg of SB220025, 1 mg of SB203580 or vehicle alone at the indicated time points. CL4 cell numbers from spleen were determined 3 and 6 days after the first HA peptide injection (left hand graph) and data at day 6 were normalized to data from day 3 (right hand graph). Pooled results from 2 independent experiments for each inhibitor are shown. (B) Representative dot plots at day 3 and 6 are shown. (C) Mice harboring CL4 T cells received 30.10^6 apoptotic splenocytes loaded with ILA-HA peptide and were treated twice a day with 1 mg of SB203580 or vehicle alone as indicated. CL4 cell numbers at days 3 and 6 (left hand graph) and data from day 6 normalized to data from day 3 (right hand graph) are shown. Pooled results from 2 independent experiments are shown.

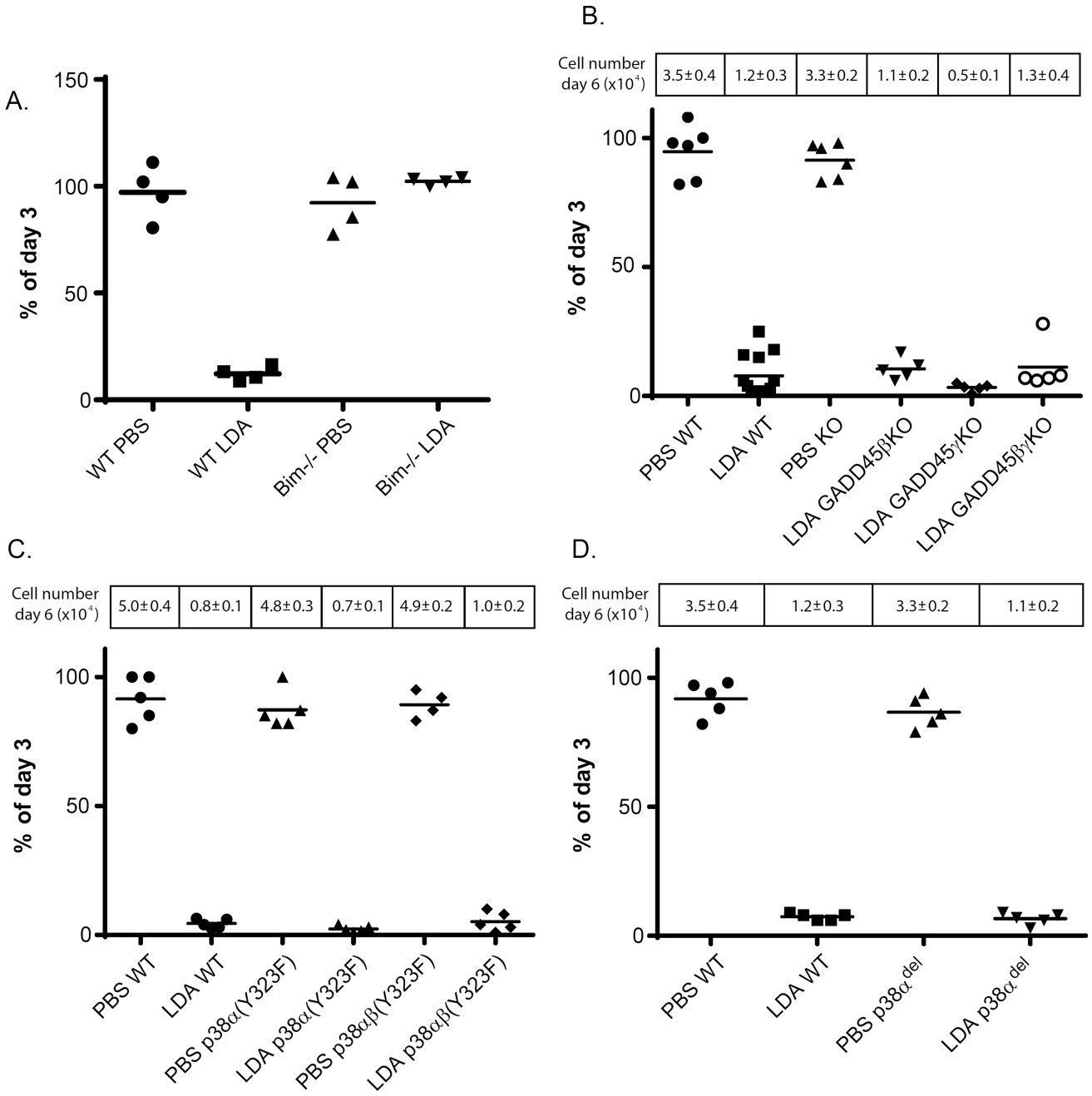

Figure 4. CD8 T cell intrinsic p38 deficiency does not rescue them from peripheral deletion

C57/BL6 mice were adoptively transferred with 1.10^6 WT (A–D) and bim−/− (A), GADD45β−/−, GADD45γ−/− and GADD45βγ−/− (B), p38α(Y323F) and p38αβ(Y323F) (C), p38αflox (D) P14 Thy1.1+ CD8 T cells. For 3 days, host mice received daily injections of PBS or 0.2 μg (LDA) of GP33m peptide. Mice were sacrificed on days 3 and 6, and P14 T cell numbers in the spleen were determined by flow cytometry. Survival of P14 T cells was determined by comparing day 6 cell numbers to day 3 cell numbers (% of day 3). Cell numbers at day 6 are indicated above the histograms. Pooled results from 2 to 3 independent

experiments are shown. Each dot represent data from one mouse. (B) PBS KO stands for a pool of the 3 KO mice (2 per KO).

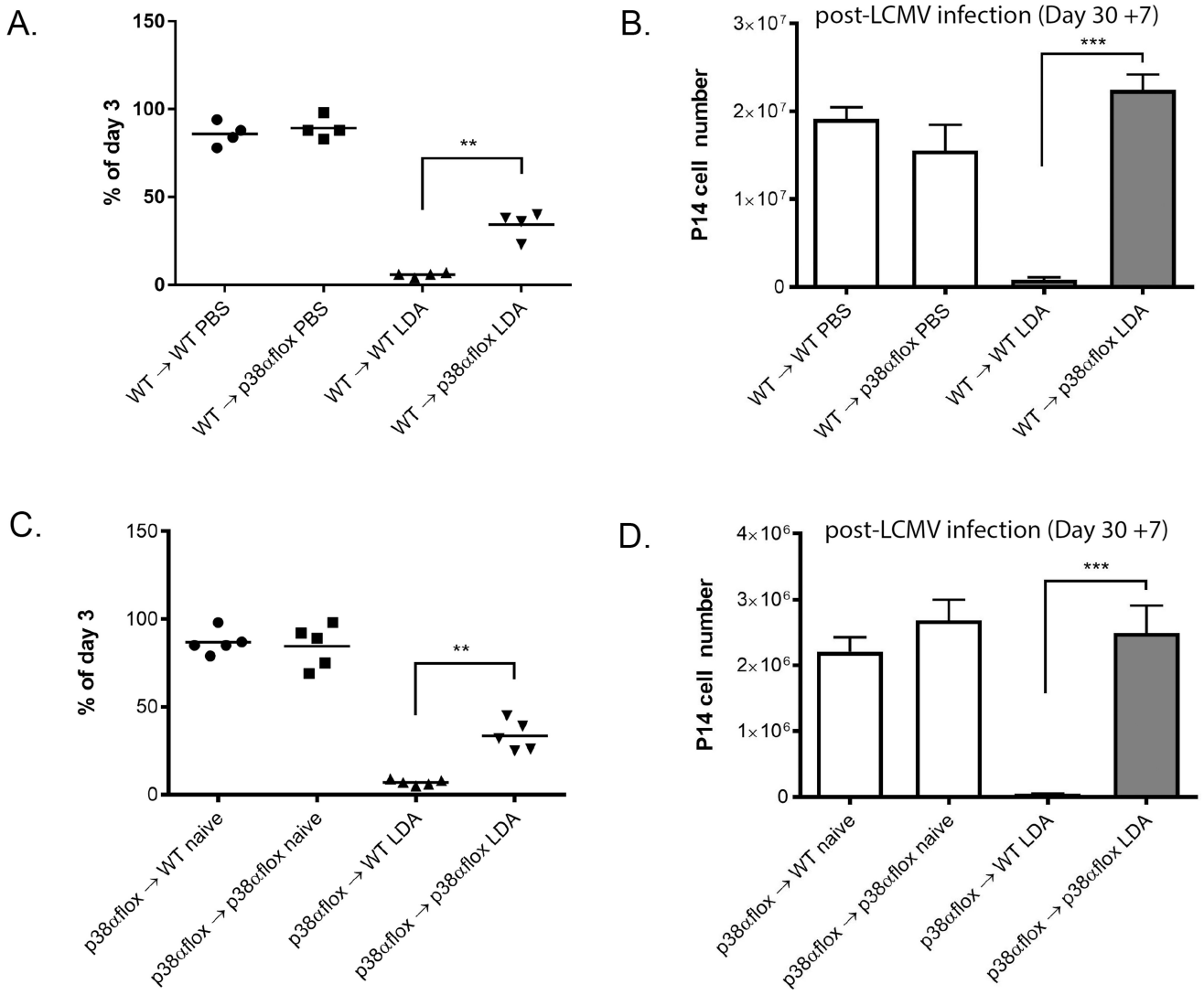

Figure 5. Host p38 deficiency rescues CD8+ T cells from LDA-induced deletion

WT (A–B) or p38α^{del} (C–D) P14 Thy1.1 CD8 T cells (10^6) were adoptively transferred into B6 WT or p38α^{del} hosts. For 3 days, host mice received daily i.v. injections of PBS or 0.2 μg (LDA) of Gp33m peptide. (A & C) Mice were sacrificed on days 3 and 6, and P14 T cell numbers in the spleen were determined by flow cytometry. (B & D) On day 30 mice were challenged with LCMV-Armstrong i.p., and 6 days later mice were sacrificed and P14 T cell numbers in the spleen were determined by flow cytometry. Pooled results for 3 independent experiments are shown (A–D).

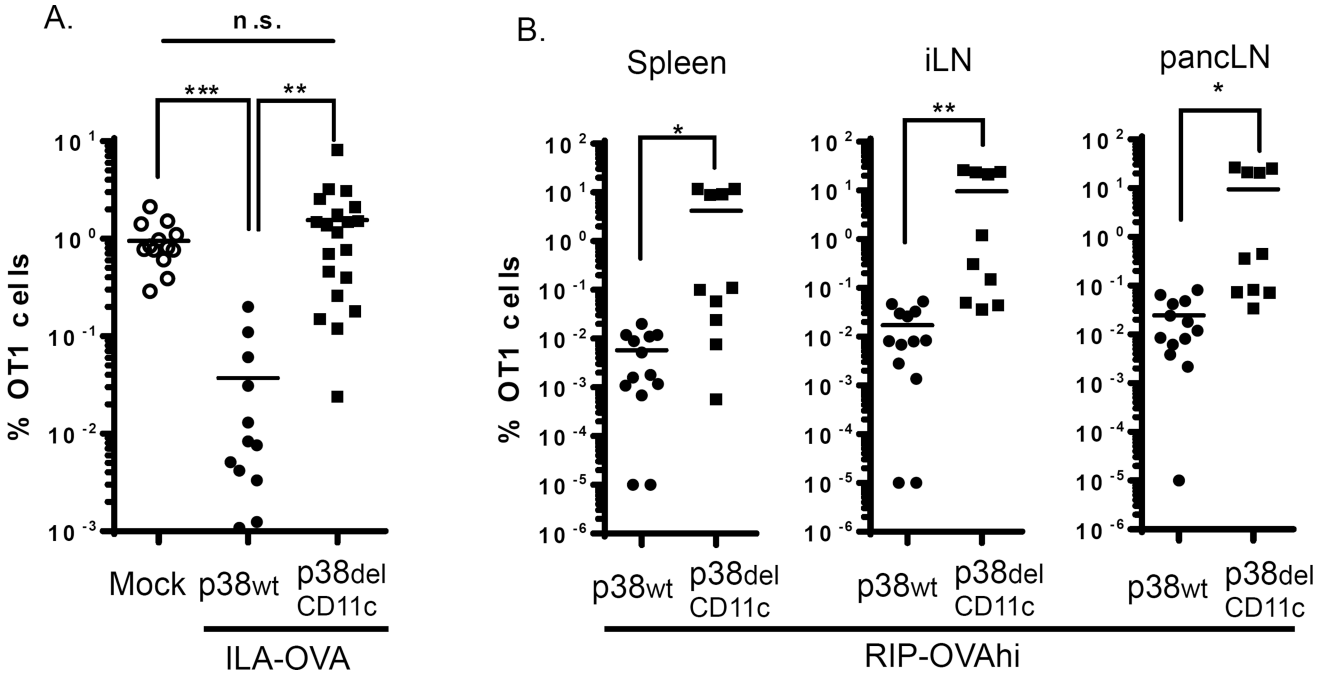

Figure 6. P38 deficiency in DCs rescues CD8+ T cells from peripheral deletion

(A) OT-1 Thy1.1 CD8 T cells (10^4) were adoptively transferred into B6 WT or CD11c-Cre *Mapk14*$^{flox/flox}$ hosts. The day after mice were injected with ILA-SIINFEKL (5μg) loaded splenocytes (30×10^6). Mice were infected 4 weeks later with vaccinia-OVA and spleens were analyzed 7 days post-infection. Pooled data from 3 experiments are shown. (B) OT-1 Thy1.1 CD8 T cells (3×10^6) were adoptively transferred into RIP-OVA^{high} × CD11c-Cre *Mapk14*$^{flox/flox}$ mice or control littermates. After 4 weeks the indicated organs (pLN: pancreatic Lymph Nodes; iLN: inguinal Lymph nodes) were analyzed by flow cytometry. Pooled data from 2 independent experiments are shown.