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No antigen presentation defect in *Unc93b1*^{3d/3d} (3d) mice

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To the editor:

Unc93b1 is a critical regulator of Toll-Like Receptor (TLR) signaling¹. The original description of mice with an inactivating mutation in Unc93b1 (H412R, also termed 3d) reported that these mice have defective TLR3, TLR7 and TLR9 signaling as well as major histocompatibility complex (MHC) class II presentation and cross-presentation defects¹. Mutations in Unc93b1 have also been identified as a genetic etiological factor in herpes simplex virus-1 encephalitis, where susceptibility is associated with defects in TLR signaling². Interestingly, *in vitro* studies of human cells have not found defects in MHC class II presentation after Unc93b1 knock-down³, raising the possibility that the role of Unc93b1 in antigen presentation, at least for MHC class II, is limited to mice. Further studies have shown that Unc93b1 controls TLR signaling by acting as a trafficking chaperone, associating with TLRs (such as TLR3, 7, 8, 9, 11 and 13) and delivering them from the endoplasmic reticulum to endosomes^{4, 5, 6}. On the other hand, despite ample studies of *Unc93b1*^{3d/3d} mice, a mechanistic understanding for the role of Unc93b1 in antigen presentation is still lacking.

Because of the discrepancies between human and mouse studies and the lack of any mechanistic basis for an antigen presentation defect in *Unc93b1*^{3d/3d} mice, we sought to confirm the antigen presentation defect reported in Tabeta *et al.*'s original description of *Unc93b1*^{3d/3d} mice¹. First, we measured proliferation of ovalbumin (OVA)-specific OT-II T cells transferred into either wild-type or *Unc93b1*^{3d/3d} recipients, after injection of soluble OVA. In contrast to the findings reported by Tabeta *et al.*, cells transferred into an *Unc93b1*^{3d/3d} host proliferated to a similar extent as in a wild-type host, both in terms of frequency of proliferating cells and average number of divisions (Fig.1a,b). Of note, the activation state of cells, followed by CD44 upregulation, was also identical in both wild-type and Unc93b1-deficient hosts (not shown).

Next, we tested the ability of *Unc93b1*^{3d/3d} hosts to cross-present antigens to CD8⁺ T cells, using Act-OVA cells as an antigen source, as described in the initial characterization of *Unc93b1*^{3d/3d} mice¹. To ensure that we only measured cross-presentation, we injected OVA-expressing *H-2*^{Kbm1} cells in wild-type and *Unc93b1*^{3d/3d} mice. As *H-2*^{Kbm1} cells cannot directly present peptides derived from OVA on MHC class I molecules⁷, CD8⁺ T cell activation must be the result of cross-presentation by host cells. In this setting, we also failed to detect any significant differences between the proliferation profiles of OVA-specific OT-II T cells transferred in wild-type or Unc93b1-deficient hosts (Fig.1c,d). Moreover, the activation status of CD8⁺ T cells also appeared similar in both strains. Indeed, upregulation of CD44 and production of interferon- γ (IFN γ) after restimulation with either peptide or PMA-ionomycin (not shown) were not affected by the genotype of the host (Fig.1e, f). We

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also studied CD4⁺ T cell proliferation in this setting and found similar proliferation 7 days after activation in wild-type and *Unc93b1*^{3d/3d} mice (not shown).

Taken together, our data do not support a direct role for Unc93b1 in antigen presentation – either MHC class II presentation or cross-presentation. We do observe defective TLR3, TLR7, and TLR9 function as originally reported for *Unc93b1*^{3d/3d} mice⁵. Given the well-recognized role of TLRs in activating antigen-presenting cells to enhance T cell priming⁸, it is certainly likely that Unc93b1 plays an indirect role in T cell activation in some models through its control of TLR trafficking. It is possible that differences in the level of contaminating TLR ligands may account for the discrepancy between our findings and those published by Tabeta *et al.* Regardless of the explanation, it is important to establish that there is no evidence to support a function for Unc93b1 beyond control of TLR localization. This clarification is particularly relevant to studies that employ *Unc93b1*^{3d/3d} mice as a model of multiple TLR deficiencies⁹ because any additional functions for Unc93b1 would complicate interpretation of such studies. Thus, *Unc93b1*^{3d/3d} mice, especially when crossed with other TLR-deficient strains, may offer a more straightforward model of multiple TLR deficiencies than the commonly used *Myd88*^{-/-}*Trif*^{-/-} mice, which also lack signaling downstream of the Interleukin-1 receptor family.

Materials and methods

Mice and in vivo T cell activation

C57BL/6, C57BL/6.SJL (CD45.1), B6.C-H2-*K^{bm1}* and B6.C-H2-*K^{bm1}* Act-OVA mice were purchased from Jackson and bred in our colony. OT-I and OT-II mice were originally from Jackson and were bred on a C57BL/6.SJL background in our colony. *Unc93b1*^{3d/3d} mice were obtained through the MMRRC (UC Davis). All animal experiments were performed in accordance with University of California Animal Care and Use Committee guidelines. Sex-matched animals of 6-10 weeks of age were used for experiments. As all results were analyzed by flow cytometry, no specific blinding method was used.

For *in vivo* experiments, splenic CD45.1⁺ OT-I and OT-II T cells were purified using the appropriate Dynabeads negative selection kit (Invitrogen). Purities were over 90% in all experiments as tested by flow cytometry. Cells were labeled with 2.5μM or 5μM CFSE (Invivogen) for analysis at day 3 or day 7 after activation, respectively, and 5×10⁶ cells were injected i.v. in wild-type or *Unc93b1*^{3d/3d} mice. The next day, mice were injected with either 0.25mg soluble OVA (Sigma) or saline as a control, or 10⁷ irradiated *H2-K^{bm1}* Act-OVA cells or *H2-K^{bm1}* cells as a control.

Flow cytometry

At the indicated time points, spleens and peripheral lymph nodes (inguinal, brachial, axillary) were harvested and dissociated on a cell strainer (70μm, BD). For restimulation, cells were cultivated for 4 hours in the presence of 10⁻⁷M OVA₃₃₂₋₃₃₉ peptide or PMA (50ng/mL) + ionomycin (1μg/mL) and brefeldin A.

Fc receptors were blocked with anti-CD16/32 (24G2, UCSF Antibody Core) before staining with the following antibodies (all from eBiosciences): anti-CD4-PerCP-Cy5.5 (RM4-5), CD45.1-Pacific Blue (A20), CD62L-APC (MEL-14), CD44-Alexa700 (IM7), CD8α-APCAlexa780 (53-6.7). For intracellular staining, cells were fixed with Cytofix/Cytoperm (BD) according to manufacturer's instructions before staining with anti-IFNγ-PE-Cy7 (XMG1.2, BD).

Acquisition was performed on a LSR Fortessa and data were analyzed using FlowJo (Treestar, v9.6).

Statistical analysis

Based on Tabeta et al.'s original study, we estimated that >4-fold differences would be observed. Assuming typical standard deviations, a sample size of 5 should lead to a statistical power of 100% (DSS Research's calculator for a two-tail test). Data were pooled from at least two individual experiments for statistical analysis by one-way ANOVA followed by a Tukey-Kramer post-test using Prism (GraphPad, v5). Of note, direct comparison of samples injected with OVA by unpaired two tailed t-tests generated similar results. All test samples (n=5-6) respected the assumption of normality as tested by Kolmogorov-Smirnov's test. P-values lower than 0.05 were considered significant and denoted by a *. ** and *** denote p-values lower than 0.01 and 0.001, respectively. Error bars represent SEM.

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J.D. and G.M.B. designed research and analyzed data, B.L.L. and Z.R.N. performed preliminary studies, J.D. performed the experiments appearing in the manuscript, and J.D. and G.M.B. wrote the manuscript.

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Figure 1. Antigen presentation is unaffected in *Unc93b1*^{3d/3d} mice

a. Representative histograms of CFSE dilution in CD45.1⁺ CD4⁺ T cells harvested from the spleens of wild-type (WT) or *Unc93b1*^{3d/3d} (*Unc*) hosts, 3 days after injection of soluble OVA (black line, 5 mice/group) or saline as a control (solid grey, 2 mice/group). **b.** Percentage of divided cells (left) and the average number of divisions (right) in the CD45.1⁺ CD4⁺ T cell population harvested from the spleen or peripheral lymph nodes (pLN) of mice described in (a). **c.** Representative histograms of CFSE dilution in CD45.1⁺ CD8⁺ T cells harvested from the spleens of WT or *Unc93b1*^{3d/3d} hosts, 7 days after injection of *H-2Kbm1* Act-OVA cells (black line, 6 mice/group) or *H-2Kbm1* cells as a control (solid grey, 2 mice/group). **d-e.** Percentage of divided cells (**d**, left), the average number of divisions (**d**, right) and percentage of CD44⁺ cells (**e**) in the CD45.1⁺ CD8⁺ T cell population from the mice described in (c). **f.** Representative histograms (left) and the percentage (right) of IFN- γ ⁺ cells among CD45.1⁺ CD8⁺ T cells from the peripheral lymph nodes of mice described in (c), after restimulation with OVA₃₃₂₋₃₃₉ peptide. P-values lower than 0.05 were considered significant and denoted by a *. ** and *** denote p-values lower than 0.01 and 0.001, respectively. Error bars represent SEM.