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The bacterial peptidoglycan sensing molecules NOD1 and NOD2 promote CD8+ thymocyte selection

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Abstract

Nucleotide-binding and oligomerization domain (NOD)-like receptors NOD1 and NOD2 are cytosolic innate immune receptors that recognize microbial peptidoglycans. Whilst studies have addressed the role of NOD proteins in innate immune responses, little attention has been given to their impact on the developing adaptive immune system. We have assessed the roles of NOD1 and NOD2 deficiency on T cell development in mice. Our results demonstrate that NOD1 and NOD2 promote the positive selection/maturation of CD8 SP thymocytes in a thymocyte intrinsic manner. TCR-mediated ERK phosphorylation is significantly reduced in the absence of NOD proteins, but RIP2 is not involved in CD8 SP thymocyte selection or ERK signaling. Commensal bacteria free animals have thymocyte maturation defects and exogenous NOD ligands can enhance thymocyte maturation in culture. These results raise the intriguing possibility that abnormal lymphocyte responses observed in NOD-dependent inflammatory diseases are not driven solely by microbial signals in the gut, but may also involve intrinsic lymphocyte defects resulting from impaired CD8 T cell thymic development.

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Introduction

Nucleotide-binding and oligomerization domain (NOD)1 and NOD2 are intracellular innate immune sensors for the bacterial peptidoglycan derived molecules γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively (1, 2). Their expression is normally associated with myeloid and non-lymphoid cell types. Upon 'sensing' these ligands via leucine rich repeat domains (LRR)(3, 4), dimerization occurs via the oligomerization domain, leading to caspase-recruitment domain (CARD) dependent interaction with the serine threonine kinase RIP2 (Receptor interacting protein 2) (5–7). The peptidoglycan ligand activated NOD: RIP2 complex promotes the potent activation of NFκB, leading to the production of inflammatory mediators and chemokines (8).

NOD1 and NOD2 are important for epithelial and myeloid cell signaling in response to microbial products from a variety of bacteria, including *Mycobacterium* sp, *Salmonella enterica* serovar Typhimurium, *Helicobacter sp*, and *Listeria monocytogenes* (9). In addition to signaling the presence of pathogens, the sensing of commensal microbial products via NOD molecules appears to be important for an increasingly diverse range of biological functions. These include intestinal lymphoid tissue genesis (10), the generation or maintenance of functional Paneth cells in the intestine (11, 12), and systemic neutrophil priming at sites distal to the gut (13).

Polymorphisms in NOD2 are associated with two important human inflammatory diseases, Crohn's disease and Blau syndrome (14–16). Whilst the amino acid changes in the LRR domains of NOD2 that are associated with Crohn's disease reduce MDP ligand driven signaling, Blau syndrome associated sequence changes appear to constitutively activate NFκB, at least when expressed in epithelial cell lines (5, 17). NOD1 and NOD2 can associate with the autophagy related (Atg) molecule ATG16L1 to promote autophagic double membrane restriction of invasive bacteria (18). As hypofunctional polymorphisms of other autophagy genes are also associated with Crohn's Disease (19, 20), it appears that inappropriate control of bacteria by autophagy and inappropriate inflammatory responses driven by NFκB and MAPK are linked to this chronic inflammatory disease (21).

The resulting ileal inflammation in Crohn's disease consists mainly of T lymphocyte infiltrates that are associated with high levels of IFNγ and IL-17 expression (22). During their development in the thymus, all α/β TCR T cells, including α/β TCR intraepithelial lymphocytes (IELs) are subject to positive selection at the $CD4^+CD8^+$ (DP) stage of differentiation (23). The signaling generated by newly rearranged αβ T cell receptors (TCR) interacting with self peptide MHC (pMHC) complexes expressed on cortical thymic epithelial cells (cTEC) promotes positive selection that initiates the differentiation of DPs into MHCII restricted CD4 single positive (CD4 SP) or MHCI restricted CD8 SP thymocytes (see Reviews (24, 25)).

Mature human T lymphocytes express both NOD1 and NOD2 (26, 27) and a CD4 T cell intrinsic function for mouse NOD2 has been reported (28). Although both NOD1 and NOD2 transcripts have been detected in mouse thymic tissue, it is unknown if NOD1 and NOD2 play roles in developing lymphocyte signaling (29, 30). Here we provide evidence that both

NOD1 and NOD2 function intrinsically in thymocytes to promote CD8 SP thymocyte positive selection. Furthermore, we identify a RIP2-independent pathway where NOD proteins are required for efficient TCR-mediated ERK phosphorylation. Finally we show that NOD ligands can promote thymocyte maturation in culture, and that thymocytes from germ free mice show maturation defects.

Materials and Methods

Mice

Wild-type C57BL/6J (B6) and congenic B6.SJL-Ptprc^{a}Pepc^{b} mice were obtained from the Jackson Laboratories. *Ripk2*$^{-/-}$(7), *Nod1*$^{-/-}$(1), *Nod2*$^{-/-}$(12), and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals were obtained from R. Ulevitch. *Ripk2*$^{-/-}$ and all *Nod deficient* animals were backcrossed over ten times to a Scripps C57BL/6 colony originally established from C57BL/6J mice before experimental use. H-Y (31), and OT-I *Tap1*$^{-/-}$ (32) TCR Tg animals were kind gifts from N. Gascoigne. Animals were bred and maintained under specific pathogen-free conditions unless stated. Five weeks old germfree and conventionally housed C57BL/6J animals were from Kew Bioservices, WEHI. Animal procedures were performed as approved by TSRI Institutional Animal Care and Use Committee, the AMREP Animal Ethics Committee, and the WEHI Animal Ethics Committee.

Flow cytometry

Single cell suspensions from spleen, thymus and blood were used for flow cytometry. For surface staining, fluorophore-labeled mAbs specific for CD4, CD8, CD69, CD24 (HSA), CD45.1, CD45.2, H-Y transgenic TCR (T3.70), Vα2, and TCRβ were obtained from BD, Biolegend or eBioscience. For intracellular staining, cells were fixed with 1.5% paraformaldehyde, permeabilized with methanol, and stained for intracellular phospho-Stat5 (clone 47, BD). Samples were acquired on a LSRII flow cytometer and analyzed using FlowJo software (Tree Star).

Quantitative PCR

Total RNA was isolated from sorted thymocyte populations using RNAeasy miniprep kits (Qiagen) and contaminating genomic DNA was removed by DNAse-I digestion. CDNA was generated using Superscript III (Life Technologies) and the PCR reactions were set up using QuantiTect SYBR Green PCR Kits (Qiagen) with the annealing temperature per cycle set to 56°C on a Stratagene MX3005P PCR machine. Quantification of *Nod1* and *Nod2* mRNA was normalized with concomitant amplification of *b-Actin* mRNA in each quadruplicate sample. The following primers were used: NOD1s: 5’-ccaagcctgacaaggtccgaaag, NOD1as: 5’-gaatgagctgggaaggggagaag, NOD2s: 5’-gggtttctgagccagtacgagtg, NOD2as: 5’-ctgacgtgctgtagaaggaaggc, Actins: 5’-cattgctgacaggatgcagaagg, Actinas: 5’-actctggcttgctgatccacat.

Generation of Mixed Bone Marrow Chimeras

T cell depleted bone marrow from non-transgenic or TCR transgenic B6.SJL-CD45.1 and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice were mixed at a 1:1 ratio and injected intravenously into lethally

irradiated (1000 rad) non-transgenic B6.SJL-CD45.1 recipients. Six to 10 weeks after injection, chimeras were analyzed.

Ca^{2+} flux

Ca^{2+} flux measurement was performed as described(33). Single cell thymocyte suspensions from WT mice were loaded for 10 minutes at 37°C with CFSE, washed, then mixed at a 1:1 ratio with unlabeled *Nod1*$^{-/-}$*Nod2*$^{-/-}$ cells. After loading with Indo-1-AM (2mM, Molecular Probes), cells were washed, then stained for 20 minutes on ice with biotinylated antibodies specific for CD3 (145-2C11) and CD4 (RM4.4), and PerCP-Cy5.5-conjugated anti-CD4 (GK1.5), and PE-Cy7-conjugated anti-CD8 (53-6.7, all from Biolegend). Cells were prewarmed to 37°C and streptavidin was added to crosslink biotinylated antibodies. Maximum Ca^{2+}-flux was achieved by the addition of ionomycin (500ng/ml; Calbiochem). The mean fluorescence ratio of Indo-1 violet to Indo-1 blue was calculated using FlowJo software (Tree Star).

OT-I TCR Tg Tap1$^{-/-}$ stimulation assay

To avoid cell signaling triggers by purification, total unpurified 5×10^6 thymocytes from WT or *Nod1*$^{-/-}$*Nod2*$^{-/-}$ OT-I TCR Tg *Tap1*$^{-/-}$ mice were cultured in RPMI 1640 with 10% Fetal Calf Serum, 100U/ml Penicillin, 0.1mg/ml Streptomycin, 55μM 2-mercaptoethanol (GIBCO) 1mM Na pyruvate, 1x MEM Non-Essential Amino Acids (GIBCO). For 15 hours at a set concentration of ionomycin (0.2μg/ml (Sigma), varying concentrations of PMA (ranging from 0.3ng/ml to 0.05ng/ml) (Sigma) and 10μg/ml of MDP or TriDAP (Invivogen) were added to the culture in 24 well plates. After culture with stimuli (+/− NOD ligands) for 15 hours, cells were washed and cultured a further 29 hours at 37°C in growth media alone (total culture time 44 hours). Cells were stained with antibodies specific for CD4, CD8, CD69 and HSA, and live cells (7AAD-) were analyzed using an LSRII flow cytometer (BD).

Thymocyte CD69 upregulation assay

Thymocytes from H-Y TCR transgenic WT (CD45.1^{+}), *Ripk2*$^{-/-}$ (Cy5-labeled) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (CD45.2^{+}) were mixed at a 1:1:1 ratio and cultured for 5h with non-transgenic C57BL/6 splenocytes and increasing concentrations of Smcy peptide (KCSRNRQYL, GenScript). Cells were then stained with antibodies specific for CD45.2, CD4, CD8, CD69 and H-Y Tg TCR (T3.70), and were acquired on an LSRII flow cytometer. Gates defining CD69^{+} versus CD69^{-} populations were determined with thymocytes incubated without Smcy peptide.

Immunoblot analysis

Protein lysates from thymocytes were made with Nonident P-40 lysis buffer (50mM Tris, 150mM NaCl, 5mM EDTA, 1% NP-40) supplemented with protease inhibitors (Roche). Soluble fractions were boiled in SDS-containing loading buffer before electrophoresis. Gels were transferred to nitrocellulose membrane and blocked with 0.5% BSA in Tris-buffered saline with Tween 20. Blots were probed with Rabbit anti pErk (Cell Signaling Technologies) and anti rabbit antibodies and signal was detected with the enhanced

chemiluminescence system (Pierce) Blots were then stripped and reprobed with total anti Erk antibody.

Statistics

Statistical differences were calculated with the Student's two-tailed t-test with the assumption of different variances and a confidence level of 95%.

Results

Nod1$^{-/-}$Nod2$^{-/-}$ mice show decreased CD8SP thymocyte cell numbers

Flow cytometry was used to assess any effects the lack of NOD1 and or NOD2 expression might have on the composition of thymocyte subsets. An initial screen of three animals of either *Nod1*$^{-/-}$, *Nod2*$^{-/-}$ or *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals revealed a statistically significant reduction of CD8 single positive (CD8SP) cells in the thymii of *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (Figure 1). Thymocyte subset characterization from thymii where only NOD1 or NOD2 was deficient did not reveal a statistically significant role for either single gene in CD8SP proportion (Figure 1B) or thymocyte numbers (Figure 1C). The findings indicate that both NOD1 and NOD2 expression is required for the generation or maintenance of normal CD8SP thymocyte numbers.

The defect in CD8 SP numbers in Nod1$^{-/-}$Nod2$^{-/-}$ thymocytes is T cell intrinsic

NOD1 and NOD2 are expressed in epithelial and hematopoietic lineages (29, 34, 35), both of which play crucial roles in T cell selection in the thymus. To determine if the reduction in CD8 SP thymocytes observed in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymii was due to a defect intrinsic to thymocytes or altered dendritic or thymic epithelial cell function, mixed bone marrow chimeras were generated (Supplementary Figure 1 A , B). An equal number of T cell depleted bone marrow cells from wild type (WT) (B6.SJL-Ptprc^{a}Pepc^{b} expressing CD45.1) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (expressing the CD45.2 allele) were transplanted into lethally irradiated WT or *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice and thymocyte subsets analyzed nine weeks later. Irrespective of the genotype of the recipient mouse, a statistically significant reduction in the proportion of *Nod1*$^{-/-}$*Nod2*$^{-/-}$ derived CD8 SP thymocytes was observed in these chimeras. Thus WT thymic epithelial and hematopoietically derived cells (including thymocytes and dendritic cells) fail to rescue the defect afflicting *Nod1*$^{-/-}$*Nod2*$^{-/-}$ CD8 SP cell numbers, indicating that the CD8 SP thymocyte defect is intrinsic to *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes. Consistent with an intrinsic thymocyte defect, both *Nod1* and *Nod2* mRNA were detected by quantitative PCR in CD4SP and CD8SP, but not in DP thymocytes (Supplementary Figure 1C,D).

Nod1$^{-/-}$Nod2$^{-/-}$ mice have reduced positive selection and CD8 SP thymocyte maturation—A more thorough analysis of thymic development from a larger cohort of *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice revealed a two fold decrease in number (Fig 2A middle) and proportion (Fig 2A lower) of CD8 single positive (CD8SP) cells in the thymii of *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice. A small but significant decrease (25%) in CD4^{+} CD8^{+} double positive (DP) cell numbers was also noted. In contrast, the proportions and numbers of

double negative (DN) and CD4 single positive (CD4SP) subsets were not significantly different from those in C57BL/6 mice.

After self-antigen/MHC induced positive selection by cTEC, DP thymocytes upregulate CD69 and begin to migrate to the medulla where these cells are then subject to negative selection (reviewed (36)). A subsequent reduction in the expression of Heat Stable Antigen (HSA) and CD69 on SP thymocytes signals the onset of maturation prior to exit from the thymus into the periphery. Analysis of DP CD69 expressing cells from NOD deficient animals revealed a small decrease in the proportion and number of the $CD69^+$ population (Figure 2B). When the proportion (Figure 2C) and number (Figure 2D) of CD4 SP and CD8 SP thymocytes in C57BL/6 and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice that had completed maturation ($CD69^-HSA^{lo}$) were examined, both populations were reduced in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals, although the impact was greater on CD8 SP cells. These findings indicate that the selection/maturation of SP thymocytes in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals is impaired, and that CD8 SP thymocytes are more dependent upon NOD1 and/or NOD2 for efficient completion of maturation after positive selection.

NOD deficiency alters the positive selection of H-Y TCR Tg thymocytes

To confirm that NOD1 and/or NOD2 play roles in the positive selection of MHCI restricted T cells, thymocyte subset development in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals expressing the H-Y T cell receptor transgene (TCR Tg) was examined. The H-Y TCR recognizes a peptide from the protein encoded by the male chromosome–encoded *Smcy* gene presented by $H\text{-}2D^b$ (31). Thymii from female H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals exhibit reduced positive selection of H-Y TCR^+ ($T3.70^+$) CD8 SP thymocytes when compared with H-Y TCR Tg NOD sufficient thymii (Figure 3A). As had been observed in non-transgenic thymic populations, a significant two-fold reduction in the proportion and number of CD8 SP $T3.70^+$ cells was seen in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (Figure 3B). A significant increase in proportion (2.5 fold) and number (4 fold) of $T3.70^+$ CD4SP cells was also observed in the thymii of H-Y TCR *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals (Figure 3B). This may be due to increased endogenous Vα rearrangement.

Both Nod1 and Nod2 contribute to positive and negative selection of H-Y TCR Tg thymocytes

To determine whether *Nod1* or *Nod2* were responsible for the positive selection defects observed in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ animals, we examined H-Y TCR Tg mice that were deficient for either *Nod1* or *Nod2* (Figure 3C). For the H-Y TCR Tg expressing thymocytes, both single deficient *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ female mice showed small decreases in the proportion of CD8 SP cells. As animals deficient for both *Nod* genes (*Nod1*$^{-/-}$*Nod2*$^{-/-}$) displayed greater defects in positive selection (Figure 3C), we concluded that both *Nod1* and *Nod2* promote positive selection signals. Compared to WT H-Y TCR Tg thymocytes, a lower proportion of *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ thymocytes express the H-Y TCR. As there were also increases in CD4 SP thymocytes expressing the H-Y TCR in these mice (Fig 3B, C), it is likely that NOD deficient thymocytes may not efficiently exclude rearrangements of their endogenous TCR loci. Bone marrow chimeras generated using equal proportions of WT and NOD1 or NOD2 deficient H-Y TCR Tg bone marrow revealed that the defects in positive

selection contributed by the loss of NOD1 or NOD2 were thymocyte intrinsic (Supplementary Figure 2A). Collectively, these data indicate that both *Nod1* and *Nod2* play intrinsic roles in promoting positive selection of CD8 T cells expressing the H-Y TCR Tg.

To determine if the absence of NOD proteins also influenced H-Y TCR mediated negative selection, thymocytes were analyzed from male WT, *Nod1*$^{-/-}$, *Nod2*$^{-/-}$, and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ H-Y TCR Tg animals. As expected, thymocytes from WT H-Y TCR Tg male mice showed a depletion of DP cells and reduced thymic cellularity (Figure 3D). While *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ deficient mice both showed an increase in proportion and number of T3.70^{+} DP cells, a feature of impaired negative selection in this model, the greatest defect in negative selection was observed in mice deficient for both *Nod* genes. A proportion of male NOD deficient thymocytes also demonstrated lower levels of H-Y TCR expression, perhaps indicative of ongoing TCR rearrangement, or internalization of the TCR. Importantly, the bone marrow chimera experiments confirmed that the negative selection defect was also intrinsic to NOD deficient thymocytes (Supplementary Figure 2B). We conclude that both *Nod1* and *Nod2* play intrinsic roles that promote thymocyte negative selection in the H-Y TCR transgenic model.

Lineage diversion of H-Y TCR CD4^{-}CD8^{-} cells in the periphery is impaired by the loss of NOD function

The premature expression of transgenic TCRαβ on CD4^{-}CD8^{-} double negative (DN) thymocytes can alter T cell development, such that it is thymic dependent but MHC independent, a scenario that resembles the γδ T cell developmental pathway. In the periphery, these unusual T cells are identified by expression of the transgenic α/β TCR without CD4 or CD8 co-receptor expression (37, 38). In the case of WT H-Y TCR Tg mice, elegant lineage fate mapping experiments have demonstrated that the majority of T3.70^{+} CD4^{-}CD8^{-} DN T cells in the periphery of either female or male H-Y TCR Tg animals have bypassed the typical TCRαβ RORγt^{+} CD4^{+}CD8^{+} (DP) stage in thymic development (39). An examination of peripheral blood from H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice revealed a stark decrease in the proportion of T3.70^{+} CD4^{-}CD8^{-} DN cells when compared to WT H-Y TCR Tg controls. This decrease coincides with a corresponding increase in the proportion of T3.70^{+} CD8^{+} cells in the blood of H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ female (Figure 4 A) or male mice (Figure 4 B). This finding indicates that NOD1 and/or NOD2 are expressed at DN stages of thymocyte development, and can promote lineage diversion in response to the premature expression of a transgenic TCR.

The CD8 SP thymocyte population is normal in Ripk2$^{-/-}$ mice

RIP2, also known as RICK, is the CARD domain containing kinase that interacts with and activates the NFκB pathway in myeloid cells after NOD1 and NOD2 mediated sensing of bacterially derived ligands (7). However *Ripk2*$^{-/-}$ mice have normal T cell subsets and show no unique role for RIP2 in peripheral T cell survival or effector function (40–42). A limited analysis of thymocyte development in *Ripk2*$^{-/-}$ mice prompted a comparison of thymocyte populations from C57BL/6, *Nod1*$^{-/-}$*Nod2*$^{-/-}$ and *Ripk2*$^{-/-}$ mice to determine if the altered positive selection of CD8 SP thymocytes we identified in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice is mediated by RIP2 (Figure 5A). No significant differences were noted between C57BL/6 and *Ripk2*$^{-/-}$

thymocyte populations indicating that the NOD1 and NOD2 signaling pathway that promotes CD8 SP thymocyte positive selection and maturation is independent of the effector kinase RIP2.

Nod1$^{-/-}$Nod2$^{-/-}$ thymocytes show normal IL-7/STAT5 and Ca^{2+} signaling

To identify which RIP2 independent signaling pathway might be defective in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes, we assessed signaling pathways known to be important for thymocyte positive selection. Although controversial (43), a role for IL-7 cytokine signaling in the selection of CD8 thymocytes has been reported (44). Indeed, conditional deletion of STAT5 in the CD8 compartment leads to a two-fold reduction in CD8 SP thymocytes, a phenotype similar to that observed in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (44). We cultured C57BL/6 and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes with or without IL-7 for 15 minutes and assessed STAT5 phosphorylation by intracellular flow cytometry staining (Supplementary Figure 3A). No differences were detected between C57BL/6 and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes in IL-7 mediated STAT5 phosphorylation, indicating that the proximal IL-7 signaling pathway appears to be intact.

Given calcium signaling plays an important role in regulating positive selection events in the thymus (45, 46), we tested if this pathway was defective in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes (Supplementary Figure 3B). No obvious defects were observed in the magnitude or timing of the Ca^{2+} flux response of *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes after crosslinking of CD3 and CD4. Collectively, we found that IL-7 and Ca^{2+} signaling appear to be intact in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes.

CD69 upregulation after H-Y TCR stimulation requires NOD1 and NOD2

To directly compare the signaling capability of the mutant thymocytes to an agonist ligand, WT (CD45.1^{+}), *Nod1*$^{-/-}$*Nod2*$^{-/-}$ and *Ripk2*$^{-/-}$ H-Y TCR Tg female thymocytes were mixed and co-cultured with C57BL/6 splenocytes that had been pre-incubated with varying concentrations of the Smcy peptide (Figure 5B). The H-Y TCR expressing thymocyte populations were first identified by T3.70 reactivity and the different H-Y TCR Tg thymocytes were distinguished by flow cytometry using the following strategies: The H-Y TCR Tg *Ripk2*$^{-/-}$ thymocytes were labeled with Cy5 prior to culture; WT H-Y TCR Tg cells were identified by lack of expression of CD45.2, while H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes were Cy5^{-} CD45.2^{+}. After stimulation with splenocytes presenting the agonist Smcy peptide, antigen activated thymocytes normally begin to upregulate CD69 in a peptide concentration dependent manner. When compared to H-Y TCR Tg WT and H-Y TCR Tg *Ripk2*$^{-/-}$ thymocytes, the proportion and degree of CD69 induction after antigenic stimulation was significantly reduced on H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes (Figure 5B). The reduction in CD69 upregulation by H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes was observed over a range of agonist peptide concentrations (Figure 5C). At the highest doses of agonist peptide, we observed a small defect in the responding *Ripk2*$^{-/-}$ thymocytes, although this was not as pronounced as the defect in *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocyte responses. These results indicate that the upregulation of CD69 expression after H-Y TCR agonist stimulation is dependent on *Nod1* and/or *Nod2* expression.

NOD1 and NOD2 promote TCR-mediated ERK phosphorylation

CD69 expression is regulated by the transcription factor AP-1, which is downstream of the Ras/ERK pathway (47). To ascertain if the impaired upregulation of CD69 in TCR stimulated NOD-deficient thymocytes was due to a defect in ERK activation, non-transgenic WT and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes were first incubated with biotinylated anti-CD4 and anti-CD3 antibodies on ice, washed, then stimulated with 37°C RPMI media containing streptavidin over a time course of ten minutes. The amounts of phosphorylated ERK1/2 and total ERK1/2 were then determined by western blotting (Figure 6). We consistently found reduced levels of phosphorylated ERK1/2 in TCR activated *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes, summarized here by the ratio of phospho-ERK1/2 to total ERK1/2 protein (Figure 6B). In contrast, no differences in ERK1/2 phosphorylation were detected between C57BL/6 and *Ripk2*$^{-/-}$ stimulated thymocytes (Supplementary Figure 4). We conclude that *Nod1* and *Nod2*, but not *Ripk2* contribute to the TCR induced activation of the ERK pathway in thymocytes.

Lack of NOD1 and NOD2 affects signaling of pre-selection thymocytes—Mouse DP and SP thymocyte subsets have different magnitudes of responses to TCR cross-linking (48, 49). Although unlikely, it is possible that the reduced CD69 up-regulation (as observed on agonist stimulated H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes) and ERK phosphorylation within TCR cross-linked *Nod1*$^{-/-}$*Nod2*$^{-/-}$ thymocytes reflects a reduced number of positively selected mature thymocytes rather than a signaling defect per se. To study a homogenous population of thymocytes that had not undergone any selection dependent signaling, we utilized OT-I TCR Tg *Tap1*$^{-/-}$ mice where the absence of *Tap1* prevents DP thymocytes from receiving a MHCI dependent selection signal (48). Both WT and NOD deficient OT-I TCR Tg *Tap1*$^{-/-}$ thymocytes comprise 90% DP with no CD8SP cells when cultured with media alone (top row Figure 7A). Stimulation of these pre-selection thymocytes with 0.2μg/ml ionomycin and a range of PMA concentrations revealed that WT OT-I TCR Tg *Tap1*$^{-/-}$ thymocytes (left panels) appeared to be more sensitive to stimulation by PMA than their NOD deficient counterparts (as determined by the proportion of CD4+ cells that had down regulated CD8a on their surface). We conclude that the absence of *Nod1* and *Nod2* reduces pre-selection thymocyte responses to signals downstream of the TCR as mimicked by PMA and ionomycin.

NOD2 ligands promote thymocyte maturation

Although NOD deficient thymocytes exhibit signaling deficiencies in the absence of exogenous ligands, we tested whether the addition of NOD ligands could further influence thymocyte selection signals. We added 10μg/ml of NOD1 or NOD2 ligands TriDAP or MDP respectively to the *in vitro* OT-I TCR Tg *Tap1*$^{-/-}$ cultures for the initial 15-hour culture with PMA and ionomycin. After washing and further culture for 29 hours without added ligands we observed that the majority of thymocytes remained immature (HSA^{hi} CD69+) (Figure 7B). However, thymocytes stimulated with PMA at 0.1ng/ml (the concentration that promotes CD8 differentiation in TCR α−/− models (50, 51)) responded to the presence of ligands by down-regulating HSA and CD69, a read out of maturation. MDP, the ligand for NOD2, promoted thymocyte maturation more robustly than TriDAP, and as expected, thymocytes lacking NOD1 and NOD2 did not respond to the presence of either ligand.

Importantly, OT-I TCR Tg *Tap1*$^{-/-}$ thymocytes cultured at the higher concentration of PMA that promotes CD4 differentiation (0.2ng/ml) appeared to be unaffected by the addition of NLR ligands. The capacity of OT-I TCR Tg *Tap1*$^{-/-}$ thymocytes to respond to MDP was restricted to an extremely narrow concentration range of PMA co-stimulation (Figure 7C) and appeared to be linked to conditions that promote CD8 rather than CD4 differentiation (50, 51). We conclude that within narrow signaling thresholds, DP thymocytes can respond to NOD ligands, resulting in the promotion of maturation.

Thymic CD8SP maturation is reduced in germ free mice

The ability of NOD ligands to influence thymocyte development in culture suggested that endogenous commensal organism derived ligands might also influence thymic development. Commensal bacteria and their NLR specific ligands influence neutrophils and the formation of gut lymphoid structures, so thymocyte development from germ free C57BL/6 animals was compared to that from conventionally housed SPF C57BL/6 mice (Figure 8). Consistent with the notion that endogenous bacterial ligands can influence thymocyte development, a decrease in DP cell number and proportion and a 1.5 fold decrease in CD8SP thymocyte numbers was detected when animals were raised in a germ free environment. Analysis of the maturation of the thymocytes from germ free mice revealed a similar pattern to that identified for *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice. In germ free animals, an increase in proportion of semi-mature SP thymocytes (HSA^{+}CD69^{+}) is observed for both CD4 and CD8 lineages, correlating with a decrease in maturing SP thymocytes (HSA^{lo}CD69^{-}) (Fig 8B and C). Importantly, the final maturation step defined by the down-regulation of HSA and CD69 seemed most affected by the lack of commensal organisms, and again seemed to affect the CD8SP thymocyte numbers more so than for CD4SP thymocytes. We conclude that the presence of commensal organisms can promote thymocyte maturation.

Discussion

In this study we reveal an unexpected role for the innate sensing molecules NOD1 and NOD2 in CD8 SP thymocyte development. Although there is increasing evidence that pattern recognition receptors play intrinsic roles in augmenting peripheral T cell responses (28, 52, 53), their contribution in the early development of lymphoid cells has not been studied. Our results indicate that both NOD1 and NOD2 promote the positive and negative selection/maturation of CD8 SP thymocytes. Analysis of H-Y TCR transgenic animals where the thymocyte TCR repertoire is artificially restricted revealed non-redundant roles for each NOD molecule in thymocyte selection. In the H-Y TCR Tg experimental model, NOD2 appears to be slightly more important than NOD1 in regulating positive and negative selection. These developmental roles appeared to be additive, in that deficiency of both *Nod* genes revealed the greatest defects in both positive and negative selection of the HY+ subsets. However roles for either NOD molecule alone in thymic subset development of non-transgenic thymocytes was not observed, perhaps indicating redundancy or other compensatory mechanisms. The TCR signals generated in selecting thymocytes are influenced by interactions with pMHC expressed on antigen presenting cells of hematopoietic and epithelial origin in the cortex and medulla. Despite the expression of NOD1 and NOD2 in epithelial and myeloid cell lineages, data from our bone marrow

chimeras point to functional defects in thymic selection when NOD1 and NOD2 are only absent in thymocytes.

Importantly, we did not find a role for RIP2 in the NOD mediated CD8 SP thymocyte developmental pathway, complementing other studies where RIP2 was not required for mature T cell function (40, 42). In a *Toxoplasma gondii* infection model, a RIP2 independent pathway operating in mature T cells has been reported to involve the NFκB transcription factor c-Rel interacting with NOD2 to promote optimal T cell responses (28). However, as c-Rel plays no role in either positive or negative thymocyte selection in the H-Y TCR Tg model (54) and we found no obvious defects in nuclear NFκB activity in NOD deficient thymocytes (not shown), our study highlights a novel RIP2-independent NOD signal transduction pathway operating intrinsically in selecting CD8 SP thymocytes.

Although it is well established that ERK signaling is important in thymocyte positive selection (55–57), our work demonstrates for the first time that NOD1 and NOD2 promote ERK activation via TCR signaling in thymocytes. In the absence of a link with RIP2, the focus turns to other molecules known to associate with NOD proteins, which include AAMP, Grim-19, ACAP1 and Erbin (58–61).

CD4 thymocytes appear to be more dependent upon ERK signal transduction than CD8 thymocytes (55), yet our data indicates a greater role for NOD1 and NOD2 in CD8 thymocyte development. In TCRα$^{-/-}$ thymocyte pre-selection models, doses of PMA between 0.1- 0.11ng/ml drive differentiation towards a CD8+ lineage, while doses of PMA at 0.2 – 0.22ng/ml drive DP cells towards a CD4SP fate (51). Our results using similar cultures indicate that thymocytes receiving “weaker” CD8 lineage promoting signals are more responsive to the presence of NOD ligands, perhaps explaining the genetic sensitivity of CD8SP thymocytes to *Nod1* and *Nod2* ablation. However, in addition to the expected lack of response to NLR ligands, NOD deficient thymocytes also exhibit reduced responses to signals generated through the TCR, or mimicked by PMA and Ionomycin. How NOD1 and NOD2 influence the transduction of these signals and if ligand sensing is important in this regard is an exciting question.

While some microbes, including *Mycobacteria* can infect the thymus, systemic bacterial infections often lead to atrophy of the thymic cortex (62, 63), a process presumably designed to prevent central tolerization of developing thymocytes in the presence of microbial antigens. Whether ligand sensing via LRR domains is regulating NOD1 and NOD2 signaling downstream of the TCR in thymocytes is unknown. Certainly the presence of systemic peptidoglycan ligands derived from commensal bacteria can play a role in neutrophil function and priming via NOD1 (13). Recent work also indicates that in thymic epithelial cells, the expression level of the transcription factor Aire is regulated by commensal bacteria and NOD1 expression (64). Whether peptidoglycan products found in the serum are able to cross the thymus/blood barrier and influence thymocyte maturation is unknown but analysis of germ free mice suggests this to be true. The SLC15A family of peptidyl transporters is important in the transport of NOD peptidylglycan ligands across cellular membranes. In myeloid and dendritic cells, SLC15A3 and SLC15A4 are highly expressed and are important for the transport of endocytosed NOD ligands from endosomes

and lysosomes into the cytosol (65, 66). However neither SLC15A3 or SLC15A4 mRNA appear to be expressed at high levels in thymocytes. Intestinal epithelial cells, but not macrophages, use SLC15A1 to transport NOD ligands across the cell membrane (67–69). Interestingly, SP thymocytes express ten fold higher levels of SLC15A1 mRNA than either DP thymocytes or peripheral T cell subsets, suggesting that SP thymocytes could selectively transport NOD1 and NOD2 ligands across the membrane (70).

In summary, our study clearly identifies a novel role for the innate immune sensing molecules NOD1 and NOD2 in promoting positive selection of CD8SP thymocytes. Noncaseating granulomas are one of the diagnostic markers of Crohn's disease and an increase in proportion of CD8+ T cells is associated with Crohn's granuloma but not lymphoid aggregates or lymphoid follicles (71). As the peripheral T cell repertoire is influenced by the strength of TCR signals developing cells encounter in the thymus, it is possible that abnormal lymphocyte responses in inflammatory diseases like Crohn's disease and Blau syndrome may not be solely driven by microbial signals, but may also include contributions of abnormal lymphocyte signaling and repertoire selection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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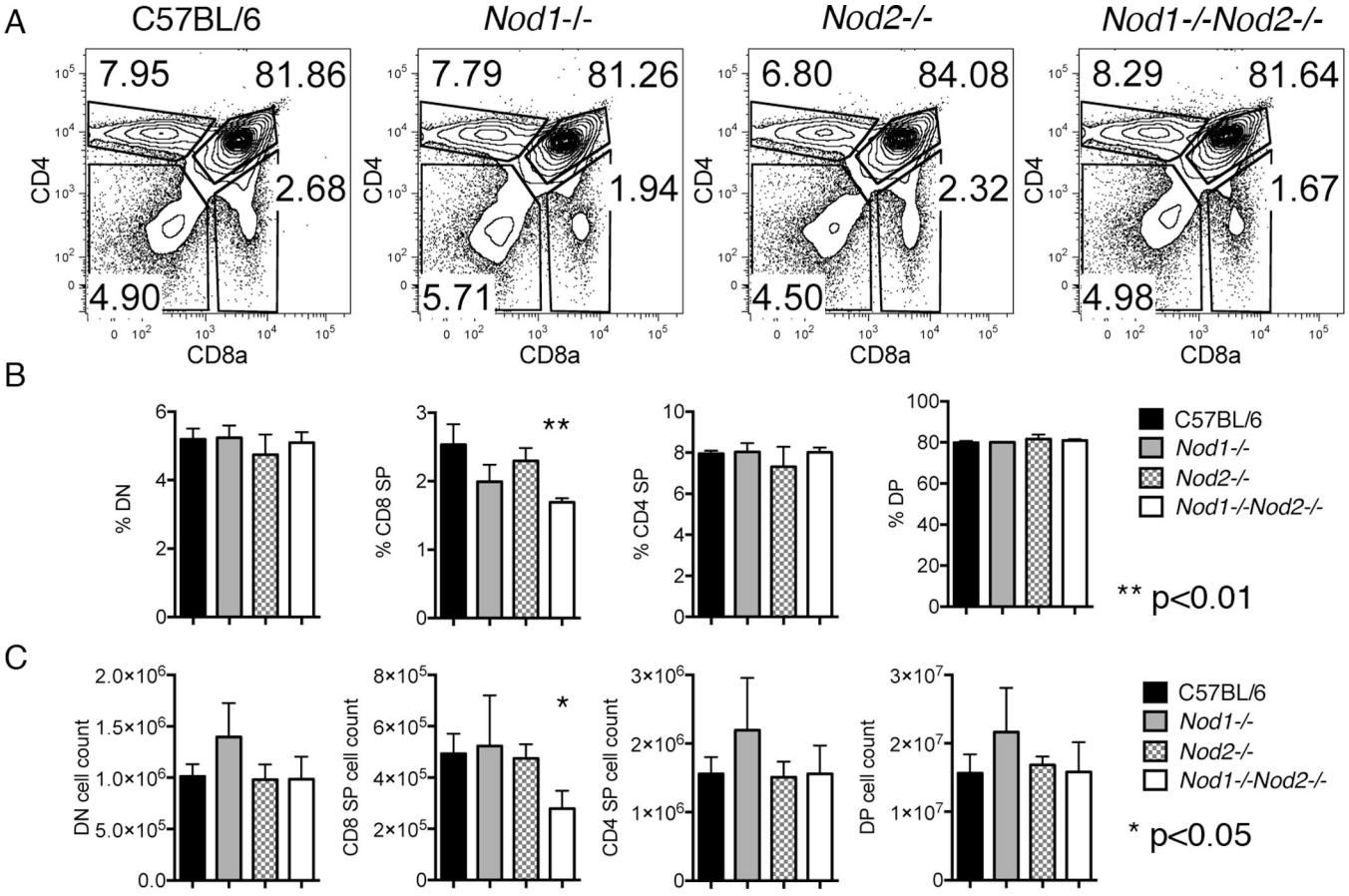

Figure 1. Thymic subset characterization of thymii from *Nod1*$^{-/-}$ *Nod2*$^{-/-}$ or *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice

(A) Representative flow cytometry plots from the thymus of C57BL/6 , *Nod1*$^{-/-}$, *Nod2*$^{-/-}$, and *Nod1*$^{-/-}$*Nod2*$^{-/-}$mice (labeled above) stained for CD4 and CD8a. Numbers in plots represent percentages of depicted populations. Total proportions (panel B) and numbers (panel C) of thymocyte subpopulations of C57BL/6 (black), *Nod1*$^{-/-}$(grey), *Nod2*$^{-/-}$(grey hatched), and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (white) are shown. Bars represent mean +/− SD. Statistical analysis with paired t tests was performed. Data represent three animals per group.

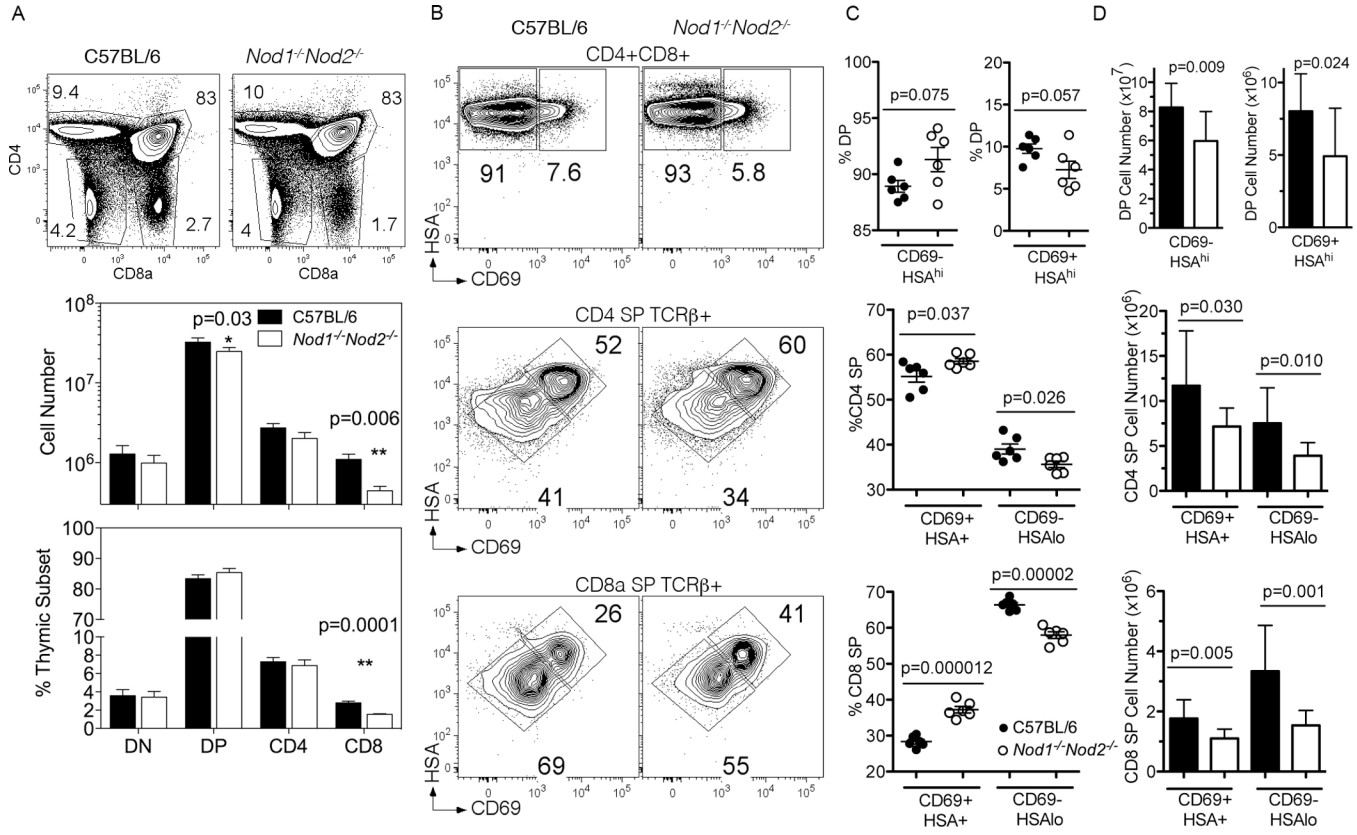

Figure 2. Thymic maturation of CD8 SP thymocytes is less efficient in the absence of *Nod1* and *Nod2*

(A) Representative flow cytometry plots from the thymus of C57BL/6 (left column) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$mice (right column) stained for CD4 and CD8a. Numbers in plots represent percentages of depicted populations. Total numbers (middle panel) and proportions (lower panel) of thymocyte subpopulations of C57BL/6 (black) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (white) are shown. Bars represent mean +/− SD. Statistical analysis with paired t tests was performed. Data represent pooled results from three independent experiments with a total of 13 mice per group analyzed. (B) Representative flow cytometry plots from gated thymocyte subsets of CD4^{+}CD8^{+} (DP) (top row), CD4 SP TCRβ^{+} (middle row) and CD8 SP TCRβ^{+} thymocytes (bottom row) from C57BL/6 (left column) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$mice (right column). Total thymocytes were stained for CD4 and CD8a, and gated thymocyte populations were stained for HSA and CD69. Numbers in plot represent percentages of depicted population. Proportions (C) and numbers (D) of thymocyte subpopulations of C57BL/6 (black) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice (white). Bars represent the mean +/− SD. Statistical analysis with two-tailed unpaired t tests was performed. (C) Data representative of one of two independent experiments with 6 mice per group. (D) Data represent pooled results from two independent experiments with a total of 11 mice per group.

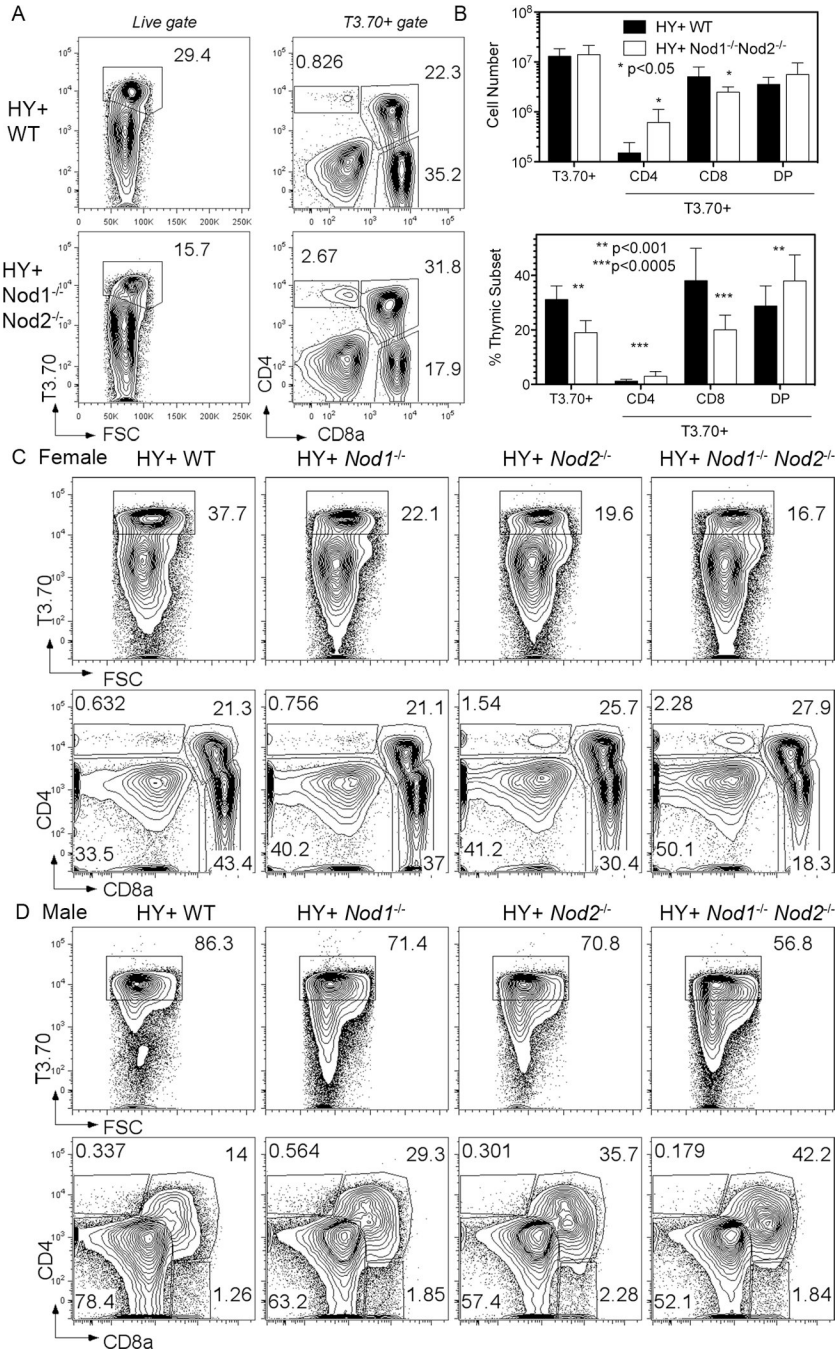

Figure 3. Both NOD genes contribute to positive and negative selection of H-Y TCR Tg thymocytes

(A) Representative flow cytometry plots from the thymus of H-Y TCR Tg WT (top row) and H-Y TCR Tg *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (bottom row) female mice. The left column shows the percentage of thymocytes expressing the H-Y TCR (T3.70^{+} cells), whilst the right shows the percentages of T3.70^{+} thymocyte subpopulations after staining for CD4 and CD8a.

(B) Total numbers (upper graph) and proportions (lower graph) of T3.70^{+} cells and T3.70^{+} subpopulations in the thymus of H-Y TCR Tg WT (black bars) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (white bars) female mice. Bars represent the mean +/− SD. Statistical analysis with unpaired t tests

was performed. Data represent pooled results from three independent experiments with a total of 9 mice per group analyzed.

(C and D) Representative flow cytometry plots from the thymus of H-Y TCR Tg WT (first column), *Nod1*$^{-/-}$ (second column), *Nod2*$^{-/-}$ (third column) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (fourth column) female (C) and male (D) mice. Upper row shows the percentage of T3.70^{+} thymocytes, whilst the lower row shows the percentages of T3.70^{+} thymocyte subpopulations after staining for CD4 and CD8a. Data are representative results of three mice per group analyzed.

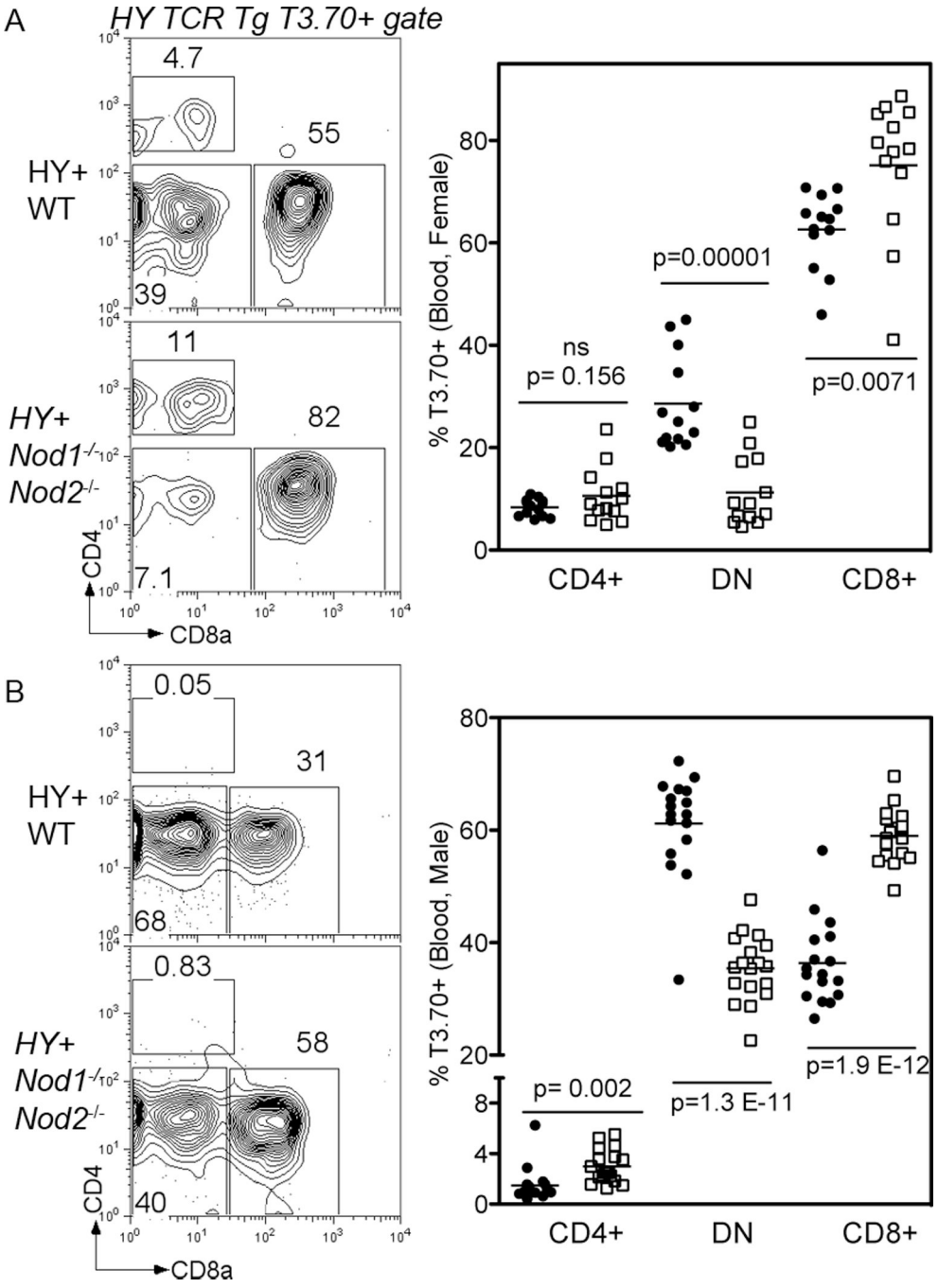

Figure 4. NOD deficiency alters lineage diversion in the H-Y TCR Tg model

(A and B) Representative flow cytometry plots (left) from peripheral blood of H-Y TCR Tg WT (upper row and filled circles) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (bottom row and white squares) female (A) and male (B) mice. Right panels show the proportions of T3.70^{+} CD4 SP, CD4- CD8- (DN) and CD8 SP T cells in the blood. Data is pooled from multiple experiments. A total of 13 female and 17 male mice of each group were analyzed by the unpaired t test.

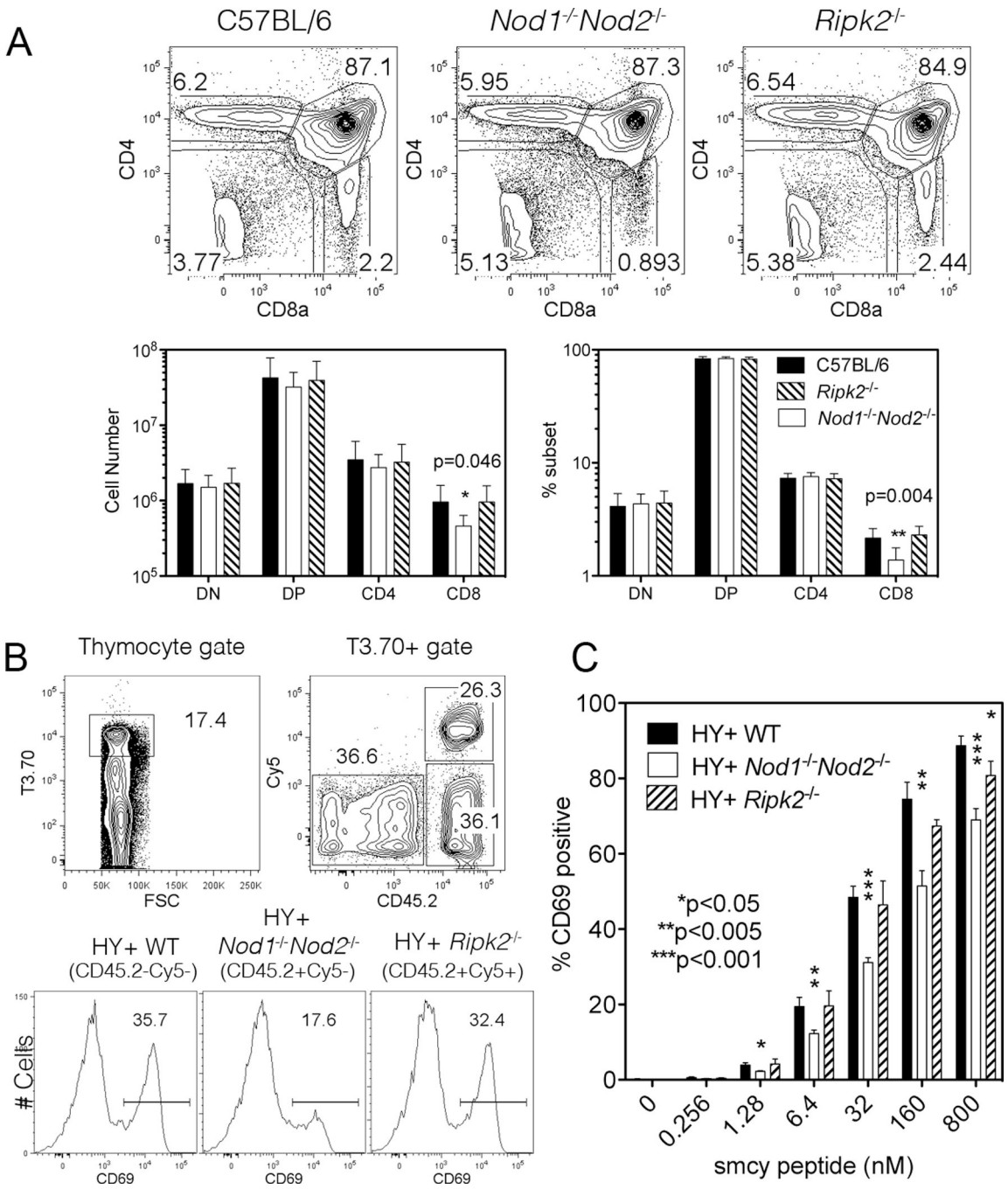

Figure 5. *Ripk2*$^{-/-}$ thymocytes show normal CD8 frequencies and activation

(A) Representative flow cytometry plots from the thymus of C57BL/6 (left plot), *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (middle plot) and *Ripk2*$^{-/-}$ (right plot) mice stained for CD4 and CD8a. Numbers in plots represent percentages of depicted populations. Total numbers (lower left) and percentages (lower right) of thymocyte subpopulations of C57BL/6 (black), *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (white) and *Ripk2*$^{-/-}$ mice (striped) are shown. Bars represent the mean +/− SD. Statistical analysis with unpaired t tests was performed. A representative of two independent experiments is shown (n=3 mice per group/ experiment analyzed).

(B and C) H-Y TCR Tg thymocytes from WT ($CD45.2^{-}Cy5^{-}$), *Ripk2*$^{-/-}$ ($CD45.2^{+}Cy5^{+}$) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ ($CD45.2^{+}Cy5^{-}$) female mice were cultured with female C57BL/6 stimulator spleen cells pulsed with different concentrations of the H-Y peptide Smcy. (B) Representative flow cytometry plots where cells were gated on $T3.70^{+}$ cells (left plot), followed by identification of the three different thymocyte populations according to their CD45.2 vs. Cy5 staining (right plot). Individual thymocyte populations were stained for CD69 upregulation. Histogram plots show the percentage of $CD69^{+}$ H-Y TCR Tg WT (left), *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (centre), and *Ripk2*$^{-/-}$ (right) thymocytes after 5h-culture with 32nM of the H-Y peptide Smcy. (C) CD69 upregulation in H-Y TCR Tg WT (black), *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (white) and *Ripk2*$^{-/-}$ (striped) $T3.70^{+}$ $CD8^{+}$ thymocytes stimulated with increasing concentrations of Smcy peptide. Data are representative of two independent experiments with 3 mice per group analyzed. Bars represent the mean +/− SD. *Refers to statistical significance between H-Y TCR Tg WT and mutant thymocytes using the unpaired t test.

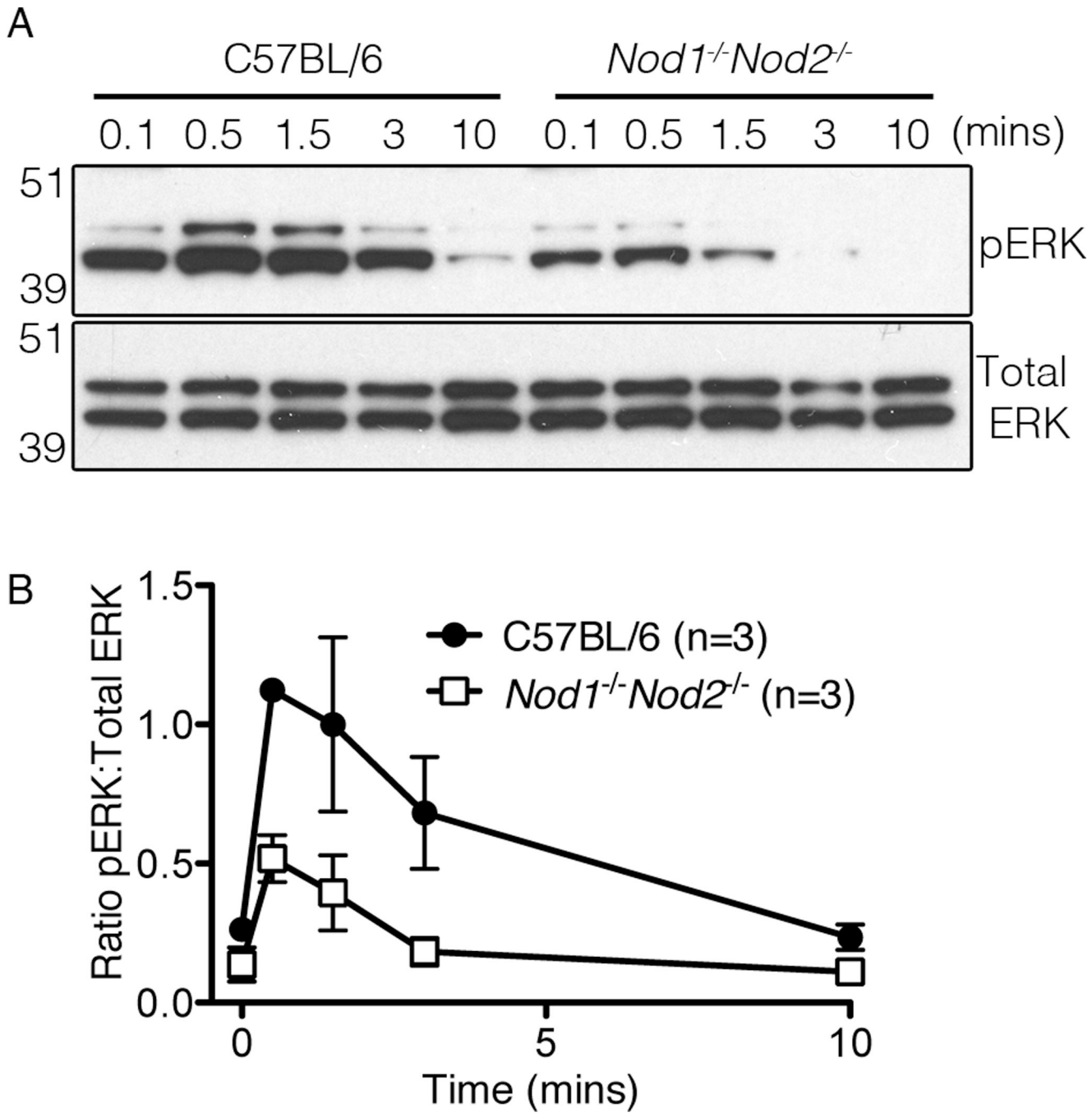

Figure 6. NOD deficiency reduces TCR-mediated ERK activation

(A) Immunoblot analysis of phosphorylated (pERK) and total ERK1/2 in lysates of TCR-stimulated C57BL/6 (left) and *Nod1*$^{-/-}$*Nod2*$^{-/-}$ (right) thymocytes. Thymocytes were bound with biotinylated anti-CD3 and anti-CD4 followed by stimulation with streptavidin over time. After pERK was detected, the membranes were stripped and re-probed with an antibody detecting total ERK1/2. Molecular weight standards are indicated on the left. Data are representative of three independent experiments. (B) Densitometry graph of the ratio of pERK to total ERK levels after stimulation. After measuring the densitometry of pERK to

total ERK expression in the same lane/ time point over three experiments, the graph plots mean values +/− SD.

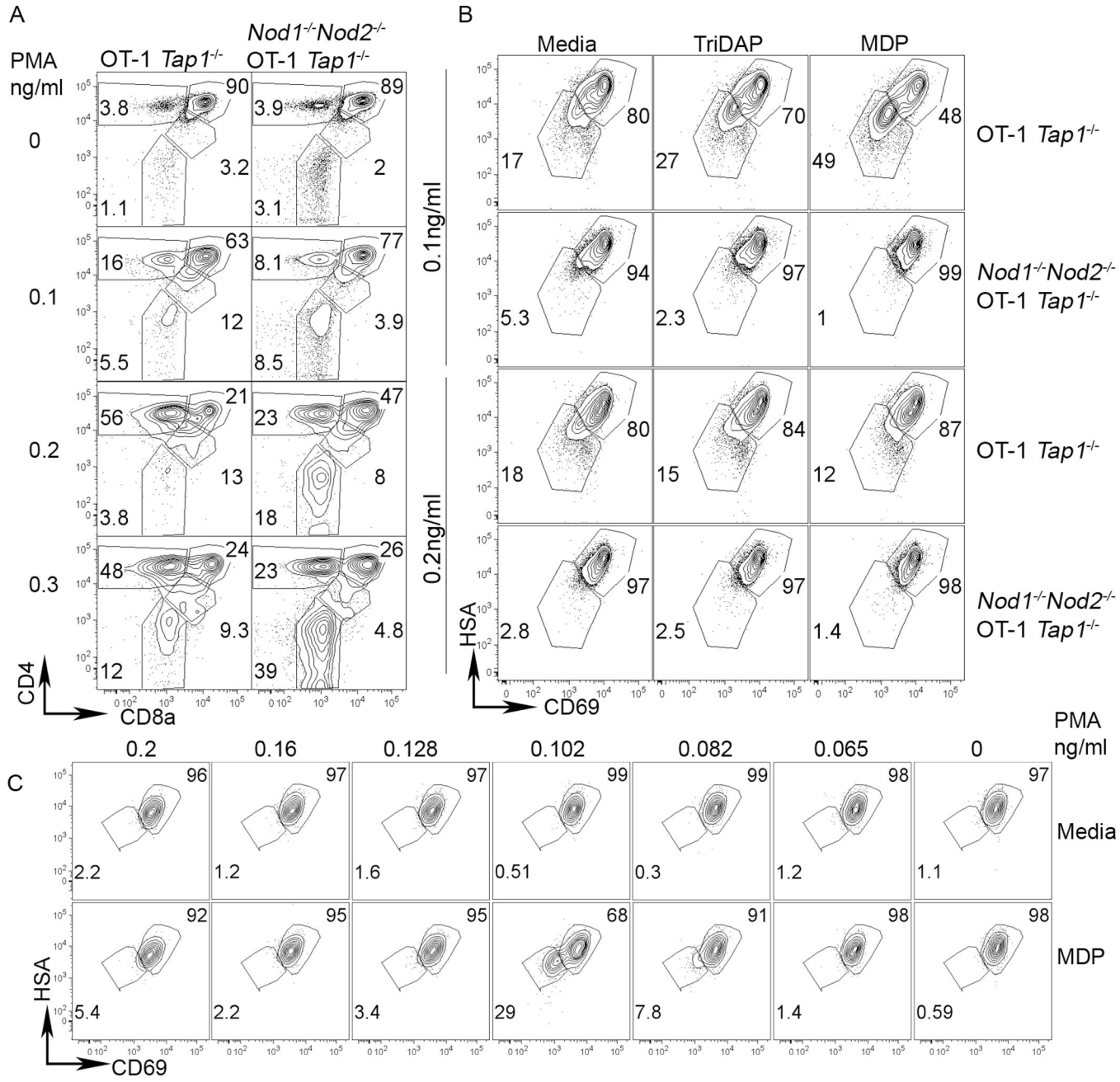

Figure 7. NOD proteins modify signal thresholds in pre-selection thymocytes

(A) Representative flow cytometry plots from the thymus of WT OT-I TCR Tg *Tap1*$^{-/-}$ (left plots), *Nod1*$^{-/-}$*Nod2*$^{-/-}$ OT-I TCR Tg *Tap1*$^{-/-}$ (right plots) mice stained for 7AAD, CD4 and CD8a (top row). For 15 hours, cells were stimulated with 0.2ug/ml ionomycin and a range of PMA concentrations (shown on left). After washing, cells were cultured without stimuli for a further 29 hours before analysis by flow cytometry. Numbers in plots represent percentages of depicted populations after gating on live cells (7AAD- gate). A representative of four independent experiments is shown.

NOD ligands promote maturation of thymocytes receiving selection signals. (B) Thymocytes from WT OT-I TCR Tg *Tap1*$^{-/-}$ or *Nod1*$^{-/-}$*Nod2*$^{-/-}$ OT-I TCR Tg *Tap1*$^{-/-}$ mice were cultured as in (A) with 0.1ng/ml PMA (upper two rows) or with 0.2ng/ml PMA (lower two rows) with no ligand (left panels), 10ug/ml Tri-DAP (middle panels) or with 10ug/ml MDP (right panels). Live cells (7AAD-) were stained for HSA and CD69. (C) Titration of PMA dose (shown above) without (upper row) or with MDP (bottom row) reveals CD69 and HSA down regulation occurs when cells are cultured with the NOD2 ligand MDP specifically at 0.1ng/ml PMA.

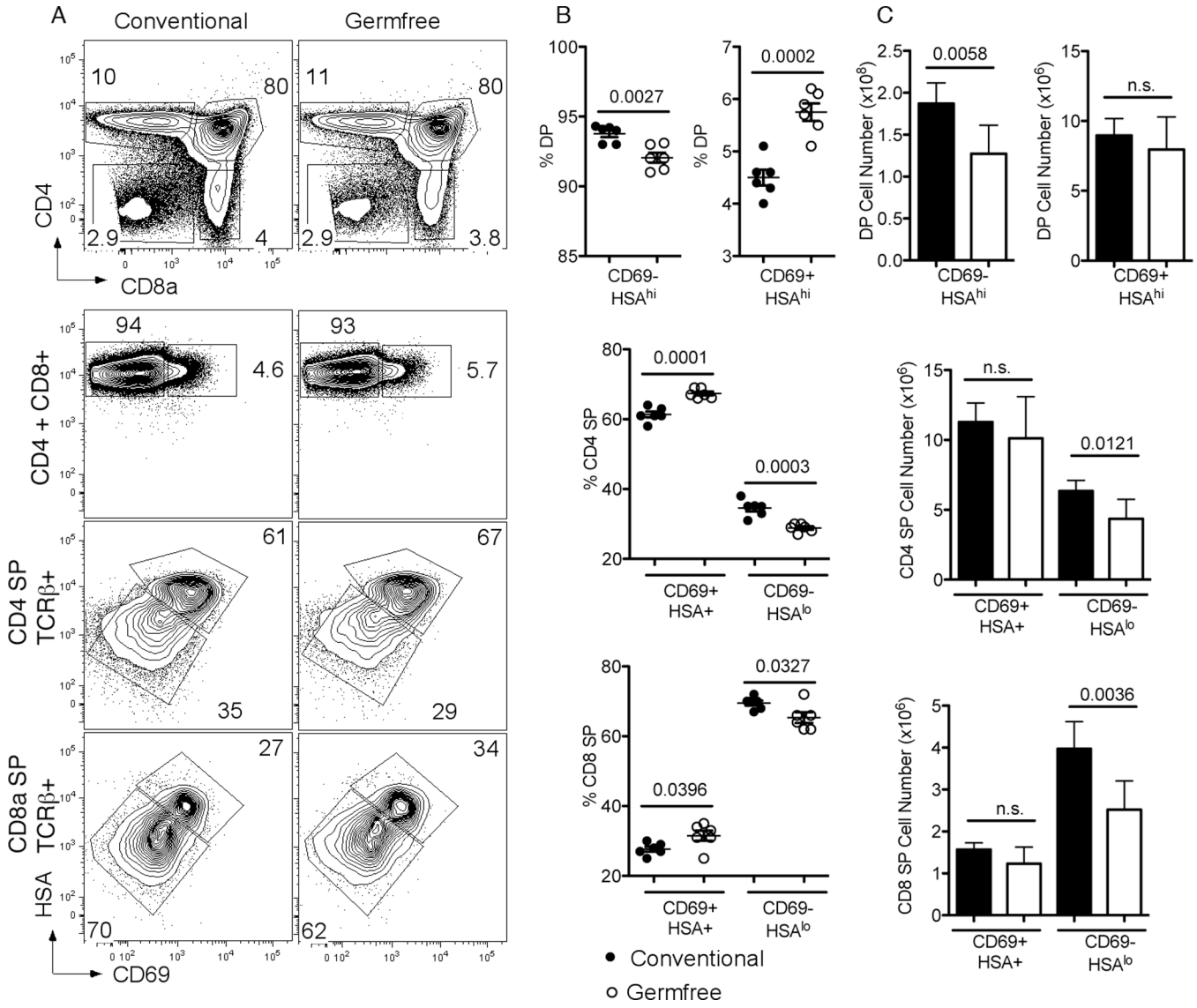

Figure 8. Absence of commensal bacteria affects thymocyte maturation

(A) Representative flow cytometry plots from the thymus of conventionally housed C57BL/6 (left column) and germ-free mice (right column) stained for CD4 and CD8a (top row). Numbers in plots represent percentages of depicted populations. Representative flow cytometry plots of thymocytes stained for HSA and CD69 (bottom three rows). Shown are gated thymocyte subsets of $CD4^+CD8^+$ (DP) (top row), CD4SP $TCRb^+$ (middle row), and CD8SP $TCRb^+$ thymocytes (bottom row) from conventionally housed C57BL/6 (left column) and germ-free C57BL/6 mice (right column). Numbers in plot represent percentages of depicted population. Proportions (B) and numbers (C) of thymocyte subpopulations of conventionally housed C57BL/6 (black) and germ-free C57BL/6 mice (white) are shown. Bars represent the mean 6 SD. Statistical analysis with two-tailed unpaired t tests was performed. In (B) and (C), data represent results from six mice per group analyzed.