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A role for Ly108 in the induction of PLZF in developing thymocytes¹

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Abstract

The promyelocytic zinc finger transcription factor (PLZF) is required for the development of activated phenotypes in NKT and other innate T lymphocytes. Although strong TCR stimulation has been implicated in the induction of PLZF, the factors regulating PLZF expression are incompletely understood. We show here that costimulation of pre-selection double positive thymocytes through the Signaling lymphocyte activation molecule (SLAM) family receptor Ly108 markedly enhanced PLZF expression compared to that induced by T cell receptor (TCR) stimulation alone. Costimulation with Ly108 increased expression of Egr-2 and binding of Egr-2 to the promoter of *Zbtb16*, which encodes PLZF, and resulted in PLZF levels similar to those seen in NKT cells. In contrast, costimulation with α CD28 failed to enhance Egr-2 binding and *Zbtb16* expression. Moreover, mice lacking Ly108 showed decreased numbers of PLZF expressing CD4⁺ T cells. Together, these results support a potential role for Ly108 in the induction of PLZF.

INTRODUCTION

Innate-like T cells, including natural killer T (NKT) cells differ from their conventional counterparts in that they possess innate, memory-like, rather than naive phenotypes upon maturation, and rapidly secrete effector cytokines upon activation without need for prior differentiation. As such, innate-like T cells play an important role at the interface between the innate and adaptive immune systems (1).

Recent data demonstrate that the Promyelocytic Zinc Finger transcription factor (PLZF) is required for the acquisition of activated phenotypes by iNKT and other innate-like T lymphocytes. While ectopic expression of PLZF is sufficient to impart an activated phenotype to conventional T cells, its absence can impair both the expansion and effector differentiation of iNKT cells (2–6). Therefore, PLZF likely plays a critical role in setting the tone of immediate lymphocyte responses. However, the factors regulating PLZF expression are only minimally understood. While data suggest that strong TCR signals associated with

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high expression of the Ras- and Ca²⁺-dependent Early Growth Response transcription factors Egr-1 and -2 lead to PLZF induction (4, 7), it is not clear that PLZF expression is governed solely by signals from the TCR(8). Unlike conventional $\alpha\beta$ TCR⁺ cells, iNKT and other innate-like T cells undergo a distinct pathway of selection that requires interactions with and selection on MHC and related molecules expressed on other DP thymocytes instead of thymic stroma, suggesting that distinct signaling pathways may be activated during their development (9). Indeed, PLZF can be induced in developing thymocytes by enforced T cell-T cell interactions (10, 11). These data suggest that PLZF expression may be regulated in part by receptor/ligands expressed specifically on DP cells rather than on thymic stroma.

Of interest in this regard are the Signaling Lymphocyte Activation Molecule (SLAM) family receptors, which are expressed at high levels on double-positive (DP) thymocytes, but are absent from the thymic stroma (12). Mutations affecting the SLAM associated protein (SAP), which is required for signaling downstream of SLAM family receptors (13), result in a drastic loss of iNKT and other innate T lymphocytes (14–19). Studies using mixed bone marrow chimeras have further implicated two SLAM members, SLAM and Ly108, in the development of iNKT cells (12). Nonetheless, how these receptors contribute to the development of iNKT and other innate T cell lineages is not well understood.

We have recently found that the homophilic SLAM family receptor Ly108, (CD352, encoded by *Slamf6*), is heavily phosphorylated in the thymus in a contact-dependent manner (20). Given the connection between Ly108 and innate T cells, we examined the effects of Ly108 on the expression of PLZF and other transcription factors required for innate T lymphocyte development. We find that Ly108 engagement amplifies TCR signaling, prolonging phosphorylation of LAT, PLC- γ and ERK and increasing expression of Egr-2. Strikingly, costimulation with Ly108, but not CD28, led to a dramatic induction of PLZF associated with increased Egr-2 binding to the promoter of *Zbtb16*, the gene encoding PLZF. Moreover, Ly108-deficient mice have decreased numbers of PLZF⁺CD4⁺ cells and conversely, a mouse model with enhanced Ly108 signaling (B6.*Sle1b*) showed increased numbers of PLZF⁺CD4⁺ cells. Our results suggest that Ly108 contributes a distinct signal that can potentiate TCR-mediated induction of PLZF.

MATERIALS AND METHODS

Mice

Mice were used in accordance with the Institutional Animal Care and Use Committee, NHGRI, NIH. *Sh2d1a*^{-/-} (21) mice were backcrossed to C57Bl/6J for 10 generations and carry the C57Bl/6J-derived SLAM locus. C57Bl/6J were from Jackson Laboratories. MHC Class I/Class II-deficient (B6.129-*H2-Ab1*^{tm1Gru} *B2m*^{tm1Jae}) (22) and β 2m^{-/-} (*B2m*^{tm1Jae}) (23) mice were from Taconic. *Egr1*^{-/-}*Egr2*^{fl/fl}/*LckCre* mice were previously described (24–26). *Slamf6*^{-/-} mice (Supplemental Fig. S2) were generated by introducing a stop codon into exon 2 and removing part of exons 2 and 3 of *Slamf6* in HGTC-8 C57Bl/6J-derived ES cells (27). B6.*Sle1b* mice have been previously described (28)

Pre-selection DP cell isolation

PS-DP thymocytes were isolated by negative selection (α FITC isolation kit, Miltenyi, CA) using FITC- α CD3, α CD25, and α CD44, which removed post-selection cells (CD3^{hi}), CD25⁺ DN cells, as well as mature (CD44^{hi}) innate T cells (eBiosciences, CA). Post-selection, cells were 98% CD4⁺CD8⁺, and 99% CD69^{lo} and CD44^{lo}. Additionally, over 98% of PLZF⁺ cells were removed by this treatment (Supplemental Fig. S1). In some

experiments, α - $\gamma\delta$ TCR was also included in the negative selection step to deplete $\gamma\delta$ T-cells. Both selection procedures gave comparable results.

Cell culture and staining

5×10^6 PS-DP thymocytes were stimulated in 0.5 mL complete RPMI plus 8% FBS, 1% pen/strep, 2mM L-glutamine and 0.05mM 2-ME in 24 well plates coated with plate-bound α CD3 (2C11, 2 μ g/ml) \pm α CD28 (PC61, 5 μ g/ml) Bio-X-Cell, NH) \pm α Ly108 (5 μ g/ml 13G3-19D, eBiosciences, CA) or an isotype control for 18, 24 or 48h. B cell stimulation experiments were performed by using WT LPS-activated B cells (1 μ g/ml for 72 hrs) that were pre-incubated with α CD3 (2 μ g/ml) for 10 minutes prior to addition of PS-DP thymocytes for 24–48 hrs. Ly108 interactions were blocked by pre-incubating thymocytes with Ly108-Fc fusion protein (Recombinant Mouse NTB-A/SLAMF6 Fc Chimera, R&D systems) for 10 minutes before adding B cells plus α CD3. Cells were harvested, stained for CD4 and CD8, then fixed with FOXP3 buffer (eBiosciences, CA) and stained with α PLZF (clone D-9, Santa Cruz Biotech, CA) followed by PE- α mouse Ig. Controls for staining specificity included staining with α PLZF isotype control (mouse IgG₁) followed by PE- α mouse Ig or staining with PE- α mouse Ig alone. iNKT cells were identified by CD1d-PBS57 tetramer using unloaded tetramer as a control (NIAID tetramer facility, Atlanta, GA). For RNA extraction, cells were harvested and stored at -80°C in Trizol.

Real time PCR

RNA was isolated with Trizol and the RNeasy® mini kit (Qiagen). cDNA was generated by Taqman reverse transcription reagents (Applied Biosystems, CA). cDNA was amplified with Taqman probe primer mixes (Applied Biosystems, CA).

Chromatin Immunoprecipitations (ChIPs)

PS-DP thymocytes were harvested 18 h post-stimulation and ChIPs performed using Magna ChIP-A kit (Millipore, MA). DNA was sheared in 500 μ l using a Misonix 3000 water bath sonicator (Output 5.5, 6 min 30 sec On, 30 sec Off) and 50 μ l (1×10^6 cell equivalent) sheared DNA was immunoprecipitated and sequences amplified by SYBR green qPCR with primers: Egr-2 F: 5' ATCCGGAGCAACAGTTCCCC 3', Egr-2 R 5' TGGGCGGTGCGAGTCCTTC 3'; CREB F 5' AGCACTAAAGATGGAGAGGCG 3', CREB R 5' TCGCAGTACCCGCTCTCG 3'; NFAT F 5' TTCTGACTCTGAGTTTGGGGATAAATGAC 3', NFAT R 5' TGGGTTTGCTAGTTATTAGTCAGAGATG 3'. ChIPs were normalized to input DNA and expressed as fold-enrichment relative to isotype control from non-stimulated (NS) thymocytes.

Stimulations and Protein analyses

Thymocytes were incubated with biotinylated α CD3 ϵ , α CD4 (BD Biosciences, NJ) \pm α Ly108 (eBiosciences, CA), washed, stimulated with streptavidin and stopped with 2X SDS loading buffer. Immunoblots were probed with 4G10, LAT and PLC γ -1 (Millipore, MA); pLAT Y191 and pPLC γ -1 Y783 (Biosource-Invitrogen, NY); pPKC θ Thr 538, pERK and ERK (Cell Signaling, MA), and PKC θ (Santa Cruz, CA) followed by TrueBlot® HRP-secondary Ab. For analysis of Ly108 phosphorylation in intact B6 and B6.*Slc1b* thymi, freshly harvested intact thymi were crushed in RIPA lysis buffer and Ly108 immunoprecipitated from cleared lysates for 2hrs at 4°C using α Ly108 monoclonal (13G3-19D clone, eBiosciences) and Protein A agarose beads (Santa Cruz, CA). Immunoprecipitates were resolved on 10% Tris Glycine gels and were probed for phosphotyrosine using 4G10 and for total Ly108 protein using polyclonal α Ly108 rabbit antibody (20). Western blots were developed using enhanced chemiluminescence detection

reagents (GE Healthcare). Films were scanned and intensities quantitated using imageJ software (NIH, <http://rsb.info.nih.gov/ij>).

Ca²⁺ Flux

PS-DP thymocytes were incubated with Fluo-3 and Fura-red (Molecular Probes/Invitrogen) and incubated with biotinylated α CD3e and α CD4 \pm α Ly108 for 30s, at 37°C, followed by crosslinking with streptavidin. Data were collected for 10 min (FACS Calibur, BD Biosciences, NJ), analyzed using FlowJo (Tree Star, Inc., CA), and plotted as the ratio of Fluo-3/Fura-red.

Statistical analysis

A two-tailed, paired Student's *t*-test was used to calculate *P* values.

RESULTS

Ly108 costimulation enhances *Zbtb16* induction in pre-selection DP thymocytes

To evaluate factors that regulate PLZF expression, we first isolated pre-selection DP (PS-DP) thymocytes by negative selection using antibodies to CD3, CD25, and CD44. (Supplemental Fig. S1A). This selection enriches for pre-selection thymocytes that have not upregulated their TCR and also depletes CD25⁺ DN cells as well as innate lymphocytes, which are CD44^{hi}. After purification, the isolated cells were over 98% DP and greater than 99% CD69^{lo}, CD44^{lo}. Over 98% of pre-existing PLZF⁺ cells were removed by this treatment (Supplemental Fig. S1A–E). As seen for developing $\gamma\delta$ cells (4), stimulation of PS-DP thymocytes with plate-bound α CD3, led to an induction of *Zbtb16* (Fig. 1A). However, costimulation with both α CD3 and an α Ly108 antibody capable of inducing Ly108 phosphorylation (Supplemental Fig. S1F, (20)), dramatically increased *Zbtb16* expression above stimulation with α CD3 alone (Fig. 1B). This enhancement of *Zbtb16* message required stimulation of both CD3 and Ly108, as α Ly108 alone was unable to induce *Zbtb16* expression above background.

To confirm that the induction in *Zbtb16* did not result from a specific outgrowth or survival advantage of pre-existing *Zbtb16*-expressing population, we evaluated pre-selection thymocytes that were isolated from β 2m-deficient mice, which lack most classical iNKT cells (29–31), and which were further negatively selected with α TCR $\gamma\delta$ to minimize contribution from PLZF expressing $\gamma\delta$ T cells. A similar induction of *Zbtb16* was also seen in pre-selection thymocytes from these mice (Fig. 1C), consistent with the fact that we did not detect appreciable PLZF expression in freshly isolated WT PS-DP thymocytes prior to stimulation (Fig. S1D and S2A). Furthermore, Ly108 costimulation did not induce any appreciable proliferation of PS-DP thymocytes in plate-bound stimulations (Fig. S2B). Thus, the induction of *Zbtb16* by Ly108 costimulation is unlikely to result from the expansion of *Zbtb16* expressing cells that may have escaped the purification of pre-selection DP cells. Although α CD3 stimulation of DP thymocytes does induce cell death, particularly by 48h, Ly108 costimulation did not appear to further diminish cell viability (Fig. S2C), also suggesting that we were not enriching for a *Zbtb16*⁺ population upon Ly108 costimulation.

To determine whether the plate-bound Ly108 antibody stimulation assay could be recapitulated using the endogenous self-ligand, and to evaluate the effects of homotypic Ly108 interactions similar to those observed *in vivo*, we next stimulated thymocytes with LPS-activated B cells, which express high levels of Ly108 when activated (32), and induced TCR signaling with α CD3. Since Ly108 is a self-ligand, this stimulation allowed engagement of Ly108 on thymocytes. Costimulation by Ly108 presented by B cells induced *Zbtb16* expression in WT PS-DP thymocytes, but not in those from mice deficient in SAP,

which is required for Ly108 activation (Fig. 1D). Moreover, pre-incubation of thymocytes with a blocking Ly108-Fc fusion protein (33) prevented this induction of *Zbtb16*, supporting the idea that Ly108 engagement contributes to *Zbtb16* expression.

Ly108 is required for full induction of PLF *in vitro*

To confirm that these effects were specific for Ly108, we evaluated thymocytes from mice deficient in Ly108, (*Slamf6*^{-/-} mice), which we generated on a pure C57BL/6 background to eliminate potential effects of *Slam* gene family polymorphisms. These mice showed no expression of Ly108 as detected by immunoblotting or flow cytometry (Fig. S2D). As previously reported in a mixed genetic background, *Slamf6*^{-/-} mice showed no gross defects in conventional CD4⁺ and CD8⁺ T cell development (Fig. 2A and Table 1). However, the effects of Ly108 on *Zbtb16* expression were abrogated in Ly108-deficient thymocytes, as well as in thymocytes from mice deficient in SAP or Fyn, which are required for Ly108 phosphorylation (20, 34) (Fig. 2B and data not shown). Moreover, evaluation of PLZF protein, by intracellular staining, confirmed that Ly108 costimulation increased PLZF protein levels over that induced by CD3 stimulation alone in nearly the entire population of WT PS-DP cells (Fig. 2C–D and Supplemental Fig. S2E), reaching levels similar to those seen in iNKT cells (Fig. 2E). Thus, Ly108 costimulation strongly enhanced α CD3-induced PLZF expression, to levels observed in innate T cells.

Ly108-deficient mice have fewer PLZF-expressing cells

To evaluate whether Ly108 contributes to PLZF expression in CD4⁺ T cells *in vivo*, we evaluated PLZF expressing thymocytes and thymic iNKT cell numbers from Ly108-deficient (*Slamf6*^{-/-}) mice. As previously reported, we found that thymic iNKT cells were reduced by approximately 50% in *Slamf6*^{-/-} mice (12). However, both the frequency and absolute numbers of CD4⁺PLZF⁺ thymocytes were even more compromised in *Slamf6*^{-/-} mice (Fig. 3A–B and Table 1). Despite the reduction of PLZF positive thymocytes in *Slamf6*^{-/-} mice, the intensity of PLZF in the remaining iNKT cells was similar between WT and *Slamf6*^{-/-} thymocytes (Fig. 3C–D), suggesting compensating roles for other SLAM family members or other receptors in PLZF expression.

Increased PLZF expressing cells in a mouse model of enhanced Ly108 signaling

B6.*Slle1b* congenic mice carry the SLAM gene locus of lupus-prone NZM2410 mice introgressed into C57BL/6 background, and develop high levels of autoantibodies with age (28). This phenotype has been linked to higher expression of the Ly108-1 isoform, which is more heavily phosphorylated upon engagement (20, 28, 34). Consistent with these observations, we have found that lysates from intact thymi from B6.*Slle1b* mice show increased phosphorylation of Ly108 (Fig. 4A). Using plate-bound α CD3 \pm α Ly108 stimulation, we further found that B6.*Slle1b* PS-DP thymocytes showed higher levels of *Zbtb16* induction upon Ly108 costimulation compared to C57BL/6 controls (Fig. 4B). Additionally, *ex vivo* staining of B6.*Slle1b* thymocytes also revealed increased percentages and numbers of PLZF positive cells compared to C57BL/6 controls (Fig. 4C–D and Table 1). Taken together, our results suggest that Ly108 signaling is a potent positive regulator of PLZF expressing cells.

Ly108 costimulation potentiates TCR signaling

The observation that the effect of Ly108 on PLZF expression requires stimulation with α CD3 suggests that Ly108 functions as a costimulatory molecule to potentiate TCR signaling. To evaluate whether Ly108 affects TCR signaling, we compared tyrosine-phosphorylated proteins from thymocytes stimulated with α CD3 (or α CD3+ α CD4) \pm α Ly108. Ly108 stimulation alone elicited minor changes in tyrosine phosphorylation.

However, α Ly108 plus α CD3 (or α CD3/CD4), prolonged the tyrosine phosphorylation of multiple proteins, including an approximately 37 kDa protein corresponding to the molecular weight of LAT, in WT, but not *Slamf6*^{-/-} thymocytes, compared to α CD3 (or α CD3/CD4) stimulation alone (Fig. 5A, Supplementary Fig. S3A). Although subtle, these effects were confirmed by probing for specific phospho-proteins, including LAT (Y191), PLC γ -1 (Y783), PKC θ (Thr538), and ERK (Fig. 5B and Supplementary Fig. S3B). To confirm that this increased signaling occurred in pre-selection thymocytes, we also evaluated the effects of Ly108 costimulation on thymocytes from MHC-class I/class II-deficient mice that lack mature conventional SP thymocytes. We observed a similar prolongation of phosphorylation of signaling proteins downstream of TCR upon Ly108 costimulation (Supplementary Fig. S3C). Evidence of prolonged signaling was also observed as a stronger resurgence of ERK phosphorylation after an hour of Ly108 costimulation (Fig. 5C).

In line with increased PLC γ -1 phosphorylation, Ly108 costimulation also slightly increased the prolonged phase of Ca²⁺ mobilization in WT, but not *Slamf6*^{-/-} PS-DP thymocytes. In contrast, Ca²⁺ flux was minimal with α Ly108 stimulation alone (Fig. 5D). Thus, although these effects are subtle, Ly108 costimulation enhanced signaling downstream of the TCR.

Egr-2 binds to PLZF promoter sequences and links Ly108 signaling to PLZF expression

The transcription factor Egr-2 is induced by TCR stimulation in a Ca²⁺ and NFAT-dependent manner and is important for both NKT cell development and expression of PLZF (7, 25, 35). Sequence analyses (NCBI; Mulan - <http://mulan.dcode.org>) revealed two conserved overlapping binding sites for Egr-2 in the *Zbtb16* promoter (Fig. 6A). These sites were also found in a recent study using ChIP-Seq to evaluate potential Egr-2 binding sites, supporting a role for Egr-2 in regulating PLZF expression (7). Notably, we found that Ly108 costimulation enhanced CD3-induced *Egr-2* expression in WT but not Ly108-deficient thymocytes (Fig. 6B). This effect was detectable as early as 6h and peaked by 12h (Fig. 6C), preceding the increase in *Zbtb16* expression (Fig. 6D). Using Chromatin Immunoprecipitations (ChIPs), we further found that Ly108 costimulation enhanced NFATc2 binding at a conserved site upstream of *Egr-2*, suggesting that Ly108 costimulation increases *Egr-2* expression via effects on NFATc2 (Fig. 6E).

Consistent with these observations, ChIP analyses revealed that Ly108 costimulation strongly enhanced Egr-2 binding to the conserved sites within the *Zbtb16* promoter in WT but not *Slamf6*^{-/-} thymocytes (Fig. 6F). In contrast, CREB, a TCR-activated transcription factor (36) that also has conserved binding sites on the *Zbtb16* promoter (Fig 6A), failed to show increased *Zbtb16* binding upon Ly108 costimulation (Fig. 6G). Thus, Ly108 costimulation enhanced Egr-2 expression and binding of Egr-2 to the PLZF promoter. Consistent with a critical role for Egr proteins in the regulation of PLZF, stimulation of PS-DP thymocytes from T cell-specific *Egr1*^{-/-}*Egr2*^{-/-} conditional mice showed only minimal CD3-mediated induction of PLZF, which did not increase upon Ly108 costimulation (Fig. 6H). Conversely, stimulation of PS-DP thymocytes from B6.*Slc1b* mice that have enhanced Ly108 phosphorylation also showed increased Ly108-induced prolonged Egr-2 expression when compared to C57Bl/6 controls (Fig.6I). Taken together our results support the hypothesis that Ly108 costimulation can increase and sustain Egr-2 expression and binding to the *Zbtb16* promoter in developing thymocytes.

CD28 costimulation does not enhance PLZF expression

To test whether the enhancement of PLZF expression was specific for Ly108, we examined the effects of CD28, which also potentiates TCR signaling. To minimize CD28 costimulation-induced thymocyte death (37–39), analyses were performed after 18–24 h of

stimulation (Supplementary Fig. S2C). While α CD28 costimulation slightly increased *Egr-2* expression compared to α CD3 alone, it failed to increase *Egr-2* expression to levels observed with Ly108 costimulation (Fig. 7A), nor did CD28 costimulation increase *Egr-2* binding to the PLZF promoter (Fig. 7B). Notably, CD28 costimulation also failed to induce PLZF expression in PS-DP thymocytes (Fig. 7C). These data suggest that Ly108 provides a distinct signal that enhances PLZF expression in developing thymocytes.

DISCUSSION

Although SLAM family members have been implicated in iNKT and other innate T lymphocyte development, the functions of these receptors in this process have not been clear. In this paper, we demonstrate that Ly108 can potentiate CD3-induced expression of *Egr-2* and PLZF, a master regulator of innate T lymphocyte phenotypes. Using either plate-bound antibody or the endogenous ligand, we found that costimulation with Ly108 in vitro potently enhanced TCR-induced PLZF expression in PS-DP thymocytes, reaching levels observed in iNKT cells. Although we cannot absolutely rule out outgrowth of a previously PLZF⁺ population, we have rigorously purified cells to minimize PLZF⁺ (and iNKT) cells in our starting cultures. Moreover, as far as we can detect, Ly108 stimulation did not induce proliferation, nor did it further enhance α CD3-induced cell death, suggesting that we were not enriching for a specific PLZF-expression population in our stimulations. Indeed, the PLZF⁺ cells in these cultures did not react with the CD-1d tetramer that recognizes iNKT cells.

Ly108 costimulation also increased expression of *Egr-2*, which is highly expressed in iNKT cells and has recently been shown to be critical for both iNKT development and PLZF expression (7, 25, 35). Supporting an important role for Ly108 in this pathway, we observed that Ly108 strongly increased *Egr-2* binding to the *Zbtb16* promoter and that Ly108 could not induce PLZF expression in thymocytes from mice deficient in *Egr-1* and -2. Thus while high levels of *Egr-2* in iNKT cells have been interpreted as indicative of these cells having received strong TCR signals (7), our data suggest that Ly108 is an important contributor to the induction of *Egr-2*, which is critical for PLZF expression. These findings were further supported by observations in B6.*Sle1b* mice, a model of enhanced Ly108 signaling, which show increased *Egr-2* expression and increased numbers of PLZF expressing CD4⁺ cells.

While the observations that the iNKT cells that do develop in Ly108-deficient mice express normal levels of PLZF could be used to argue against a role for Ly108 in regulating PLZF expression, an alternative interpretation is that cells that successfully upregulate PLZF have a selective advantage and preferentially develop. This idea is consistent with data showing decreased iNKT cells in *Zbtb16*^{-/-} animals (12). Alternatively, given that bone-marrow chimeras that generate a functional double-deficiency in both Ly108 and SLAM have a more complete block in the development of PLZF expressing iNKT cells (12), other SLAM family members (or other receptors) may compensate for the absence of Ly108. Consistent with this idea, we have seen that an α -SLAM (CD150) antibody can modestly increase expression of *Egr-2* and PLZF in similar plate-bound stimulation assays, although not nearly to the level seen with α -Ly108 (data not shown). Whether this is the result of functional differences between SLAM and Ly108 or the relative efficiency of the particular stimulating antibodies requires further evaluation.

It has been argued that SLAM/SAP-mediated signaling does not play a major role in the regulation of PLZF in part because a) the defect in iNKT cell development occurs earlier in SAP-deficient than that in PLZF-deficient mice (5, 12); b) some PLZF induction is still seen in SAP-deficient stage 0 iNKT cells (3) and c) restoring PLZF expression by transgenic expression does not restore iNKT development in SAP-deficient mice (5). However, each of

these arguments does not rule out a role for SLAM family members in the regulation of PLZF. Indeed, our results are consistent with the idea that SLAM family members affect multiple factors and stages in NKT cell differentiation, and with recent data showing that the absence of SAP leads to strong inhibitory signals that may differ from the lack of SLAM family signaling (40, 41)

Our results further suggest that the expression of PLZF can be induced in most (pre-selection) thymocytes that receive the appropriate signals. We have recently found that Ly108 is heavily phosphorylated in the thymus, but that phosphorylation of Ly108 is lost within minutes of dispersion of thymocytes into a single cell preparation. In contrast, α -Ly108-mediated induction of Ly108 tyrosine phosphorylation is relatively slow compared to the global patterns of tyrosine phosphorylation induced by α CD3 (20). These results suggest that there is dynamic contact-mediated regulation of Ly108 phosphorylation in the thymus. Given the dramatic effects of Ly108 on PLZF induction, the rapid dephosphorylation (with relatively slow induction of phosphorylation) suggests that Ly108 activity is tightly controlled, perhaps preventing the full induction of PLZF unless contact is maintained between developing DP cells, as may occur during the selection of iNKT and other hematopoietically-selected T cells. This rapid dephosphorylation could provide a safety mechanism that allows only certain cells to develop and maintain high PLZF expression, and thereby limits the number of CD4⁺ T cells that develop innate-like characteristics.

However, whether Ly108 acts solely by potentiating TCR signaling and Egr-2 expression is not clear. While the effects of Ly108 co-stimulation observed at the level of tyrosine phosphorylation and Ca²⁺ mobilization are subtle, these may affect duration of signals, which could lead to distinct outcomes in terms of gene expression and cell fate. However, the contrast with CD28 costimulation for the induction of PLZF expression suggests that the effects of Ly108 may go beyond that of boosting TCR signaling. The identification of distinct Ly108 signaling pathway(s) that contribute to PLZF expression therefore remains an important question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DP	double positive
DN	double negative
SP	single positive
NKT	Nature Killer T
SLAM	Signaling Lymphocyte Activation Molecule
SAP	SLAM associated protein
XLP	X linked lymphoproliferative disease
PSDP	Pre-selection Double Positive
PLZF	Promyelocytic Zinc Finger transcription factor

Egr	Early growth response protein
ChIP	Chromatin immunoprecipitation
LAT	Linker of activated T cells
PLC γ-1	Phospholipase C gamma -1
PKCθ	Protein kinase C θ

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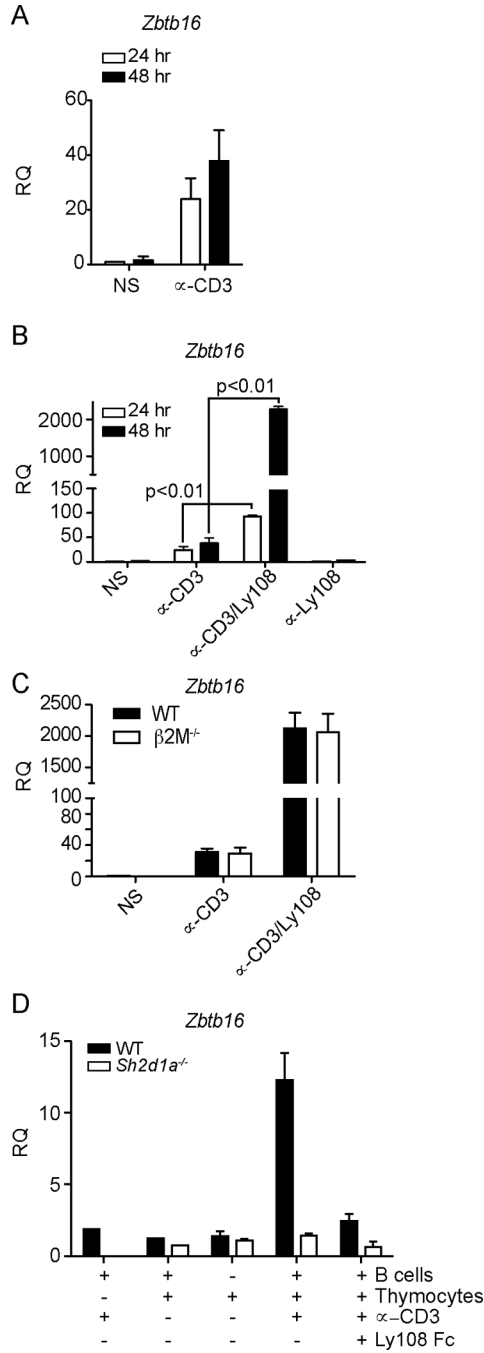


Figure 1. Ly108 costimulation enhances PLZF expression in developing PS-DP thymocytes
 (A) Expression of *Zbtb16* (encoding PLZF) evaluated by qRT-PCR of WT PS-DP thymocytes stimulated with media alone (NS; non-stimulated) or plate-bound α CD3 for 24 or 48h. (B) *Zbtb16* expression of WT PS-DP thymocytes stimulated with media alone (NS), α CD3 \pm α Ly108 or α Ly108 alone. RQ is relative quantitation. Values in A–B were normalized to NS WT samples. Similar results were obtained using freshly isolated PS-DP cells as normalization controls (Supplemental Fig. S2A). Data are the means \pm SEM of 5 independent experiments, using cells from 3 mice each. (C) *Zbtb16* expression in WT and $\beta 2m$ -deficient PS-DP thymocytes stimulated with plate-bound α CD3 \pm α Ly108. Data are from two independent experiments. Cells in these experiments were also negatively selected

to remove TCR $\gamma\delta^+$ cells. (D) *Zbtb16* expression in WT and SAP-deficient (*Sh2d1a^{-/-}*) PS-DP thymocytes in the presence or absence of Ly108Fc fusion protein were stimulated with LPS-activated B cells (which express high surface levels of Ly108) plus α CD3. Data are the means \pm SEM of 3 independent experiments, using cells from 3 mice each.

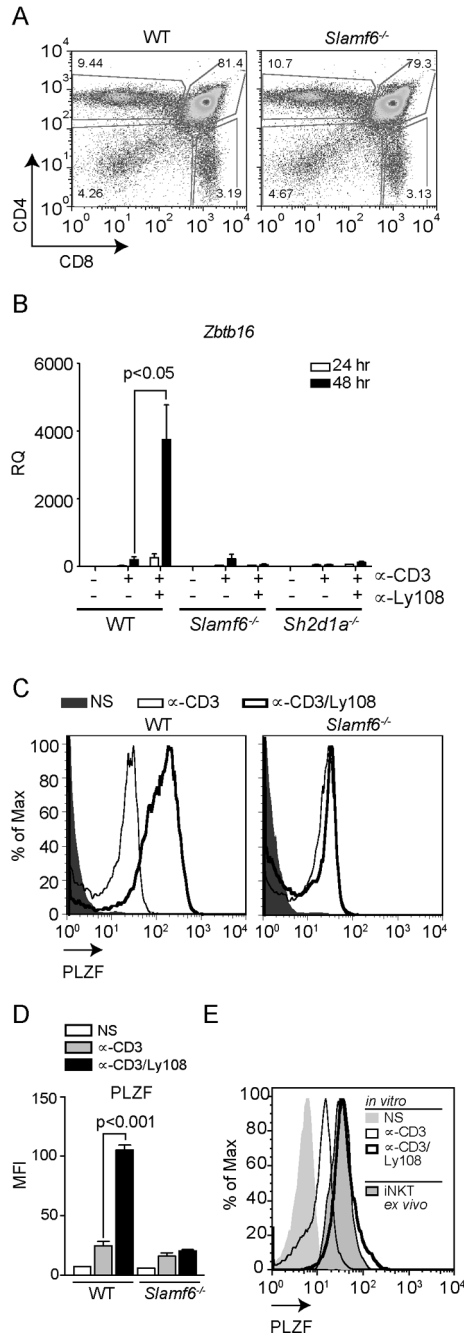


Figure 2. Ly108 is required for full induction of PLZF *in vitro*

(A) Characterization of Ly108-deficient (*Slamf6*^{-/-}) mice. Representative flow plots of thymocyte populations from WT and *Slamf6*^{-/-} mice stained for CD4 and CD8. Frequencies and absolute numbers of thymocyte populations (N=6) stained for DP, DN CD4⁺ and CD8⁺ cells are depicted in Table 1. (B) *Zbtb16* expression in WT, *Slamf6*^{-/-} and SAP-deficient (*Sh2d1a*^{-/-}) thymocytes stimulated as in Fig.1. Values were normalized to NS WT samples. Similar results were obtained using freshly isolated PS-DP cells as controls (Supplemental Fig. S2A). Data are the means ± SEM of 3 independent experiments, using cells from 3 mice each. (C) Representative histograms of intracellular PLZF staining in PS-DP thymocytes from WT and *Slamf6*^{-/-} mice, NS (solid histogram) or stimulated with plate-

bound α CD3 \pm α Ly108 for 48 h. Staining controls are shown in Supplemental Fig. S2E. (D) MFI \pm SD of PLZF from 3 mice per genotype. (E) Intracellular PLZF expression in PS-DP thymocytes stimulated as in (C) compared to PLZF⁺CD1d-PBS57 tetramer⁺ iNKT cells. Histograms are representative of triplicates for each condition. Data in (E) were generated with a different antibody lot than in (C).

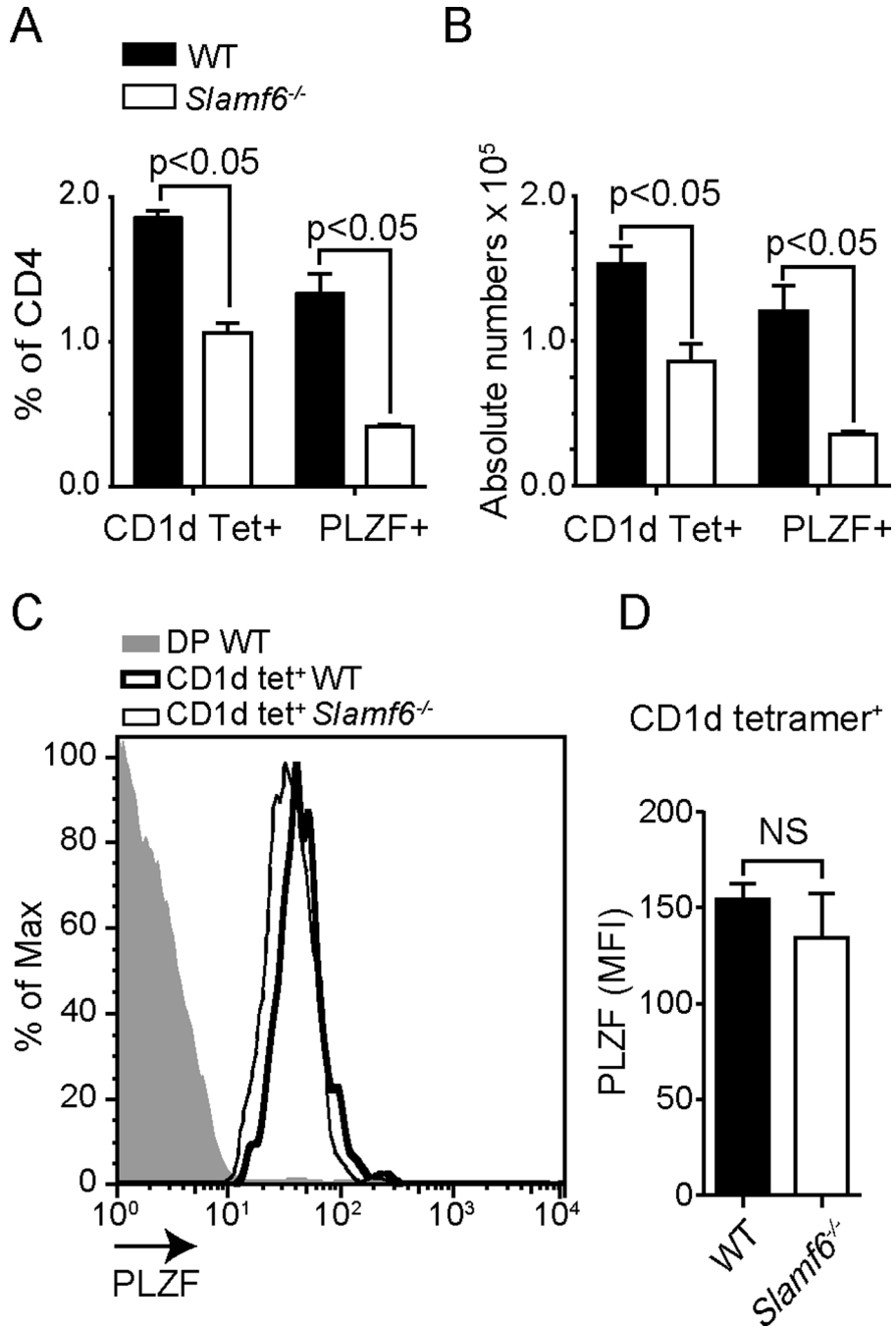


Figure 3. Ly108-deficient mice have fewer PLZF⁺CD4⁺ thymocytes
 Percentages (A) and absolute numbers (B) of CD1d-PBS57 Tetramer⁺ and PLZF⁺, CD4⁺ thymocytes in WT and *Slamf6*^{-/-} mice. (C–D) PLZF levels in CD1d-PBS57 Tetramer⁺ iNKT cells from WT and *Slamf6*^{-/-} mice. Data are the mean ± SD of 3 mice per group.

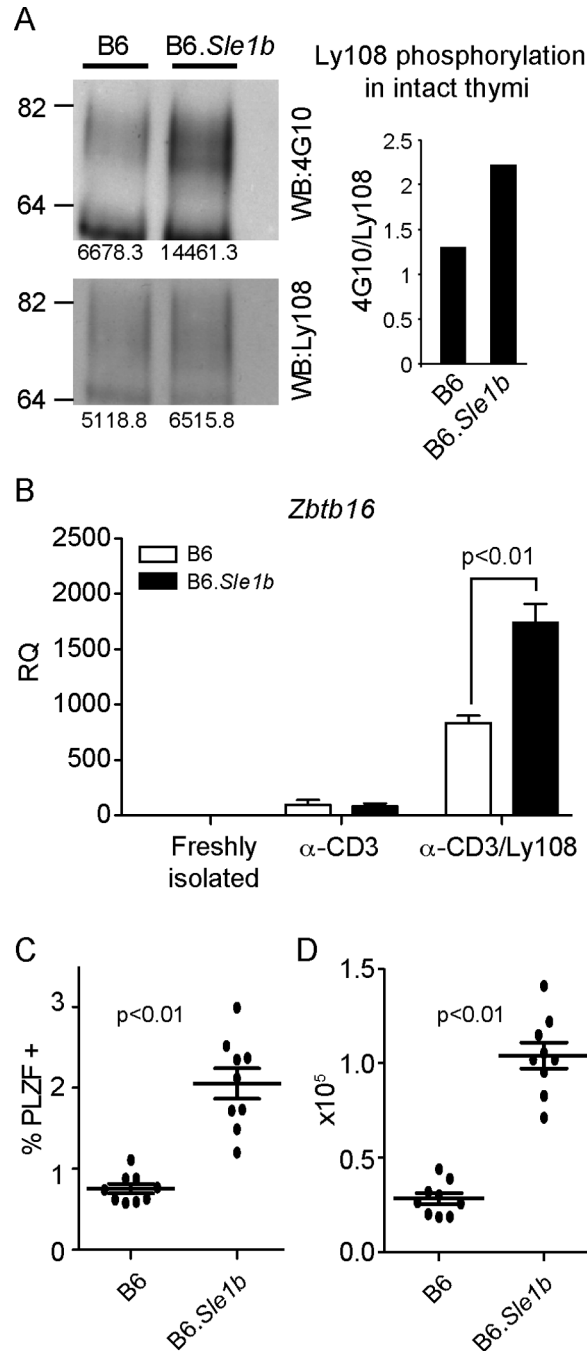


Figure 4. Increased PLZF expressing cells observed in a mouse model with enhanced Ly108 signaling

(A) Enhanced phosphorylation of Ly108 in intact thymi of B6.Sle1b mice. Ly108 was immunoprecipitated from lysates of intact thymi of C57Bl/6J (B6) and B6.Sle1b mice and probed for phosphotyrosine using 4G10 antibody (top) and for total Ly108 protein (bottom). Quantitation of relative tyrosine phosphorylation is shown on the right. (B) *Zbtb16* expression in C57Bl/6J (B6) and B6.Sle1b PS-DP thymocytes stimulated for 48 hrs with plate-bound α CD3 \pm α Ly108 as in Figure 1. Data are representative of three independent experiments. Percentages (C) and absolute numbers (D) of PLZF⁺ CD4⁺ thymocytes in C57Bl/6J (B6) and B6.Sle1b mice.

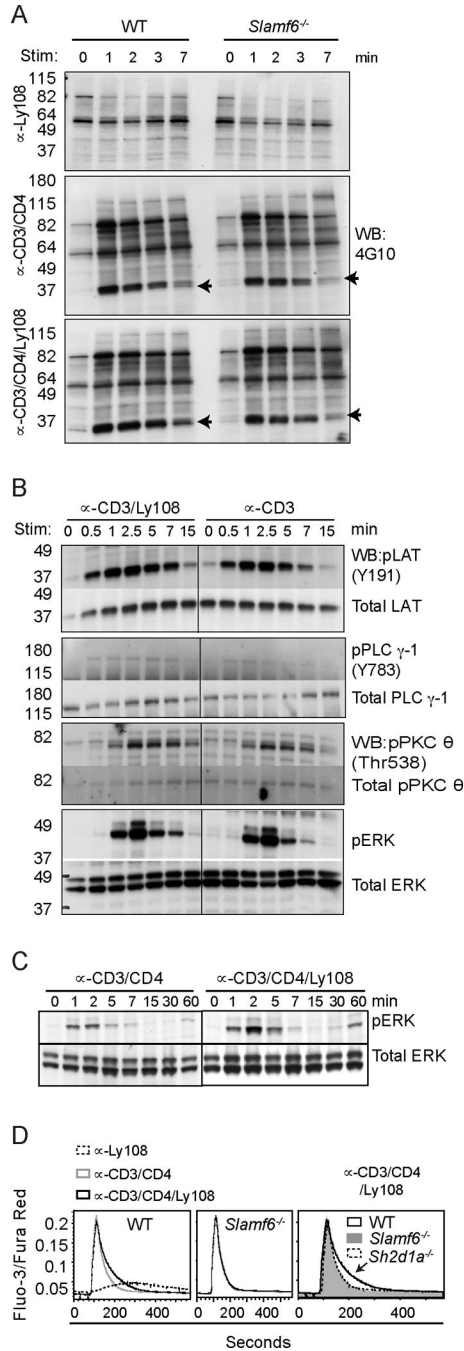


Figure 5. Ly108 costimulation prolongs TCR signaling

(A) Rested WT and *Slamf6*^{-/-} thymocytes, pre-incubated with biotinylated α Ly108 (top), α CD3+ α CD4 (middle), and α CD3+ α CD4+ α Ly108 (bottom), were stimulated with streptavidin for indicated times and lysates immunoblotted for α -phosphotyrosine (4G10). Arrows indicate predicted migration of LAT (left). Quantitation of 37 kDa band corresponding to LAT in (A) is shown in supplemental Fig. S3A (B) WT thymocytes were stimulated with α CD3+ α Ly108 as in (A) and lysates probed for pLAT (Y191), LAT; pPLC γ -1 (Y783), PLC γ -1; pPKC θ (Thr 538), PKC θ ; pERK and ERK. Quantitation of band intensities are shown in supplemental Fig. S3B. (C) WT thymocytes were stimulated with α CD3+ α CD4+ α Ly108 for extended times and probed for phospho and total Erk proteins.

(D) Ca^{2+} flux of WT and *Slamf6*^{-/-} PS-DP thymocytes stimulated with biotin-conjugated $\alpha\text{CD3/CD4}\pm\alpha\text{Ly108}$ followed by streptavidin, as indicated by the ratio of Fluo-3/Fura-Red. Arrow indicates enhanced intracellular Ca^{2+} levels seen in WT, but not Ly108- or SAP-deficient thymocytes, upon $\alpha\text{CD3/CD4/Ly108}$ stimulation. A–D are representative of 3 independent experiments.

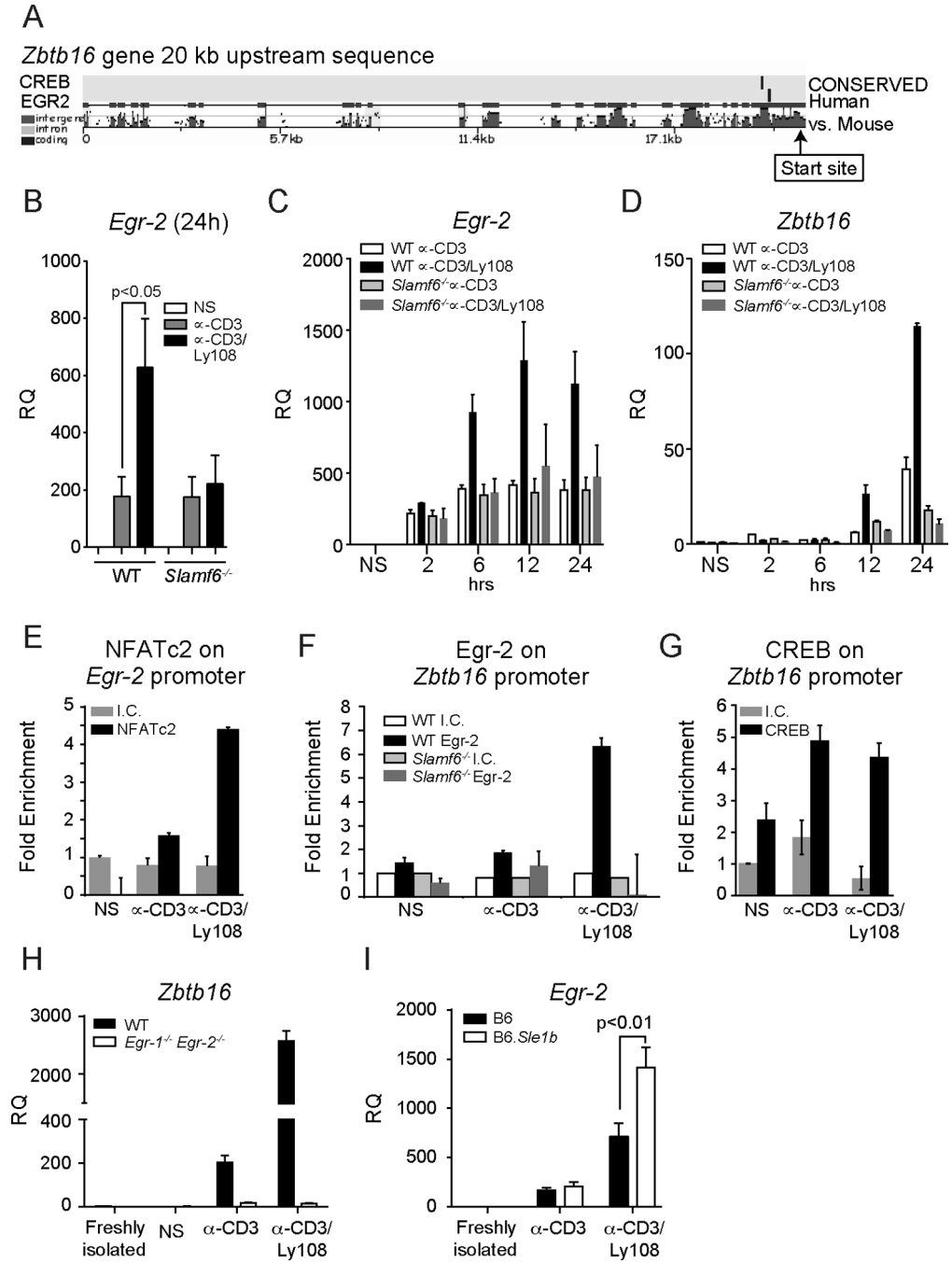


Figure 6. Ly108 induces *Egr-2* expression and binding to the *Zbtb16* promoter

(A) Mouse and human *Zbtb16* sequences, 20 kb upstream of first exon, were aligned and analyzed for conserved binding sites for Egr-2 and CREB. (B) *Egr-2* expression in WT or *Slamf6*^{-/-} PS-DP thymocytes stimulated as in 1B for 24 h, normalized to NS controls. Data are the mean ± SEM from 6 independent experiments. *Egr-2* (C) or *Zbtb16* (D) expression by qRT-PCR from WT and *Slamf6*^{-/-} PS-DP thymocytes stimulated for 2, 6, 12 or 24 h. Time courses are representative of 4 independent experiments. (E–G) Chromatin immunoprecipitations (ChIPs) from thymocytes stimulated as in 1B. (E) NFATc2 binding on the *Egr-2* promoter. (F) Egr-2 binding on the *Zbtb16* promoter. (G) CREB binding on the *Zbtb16* promoter. ChIPs show average of 2 replicates ± SD from one of 2 or more

independent experiments. (H) *Zbtb16* expression in *Egr-1^{-/-}Egr-2^{fl/fl} Lck Cre* or littermate control PS-DP thymocytes stimulated as in Fig. 1B for 48 hours and normalized to NS controls. (I) *Egr-2* expression in C57Bl/6J (B6) and B6.*Sle1b* PS-DP thymocytes stimulated with plate-bound α CD3 \pm α Ly108 for 48 hrs. Data are representative of three independent experiments.

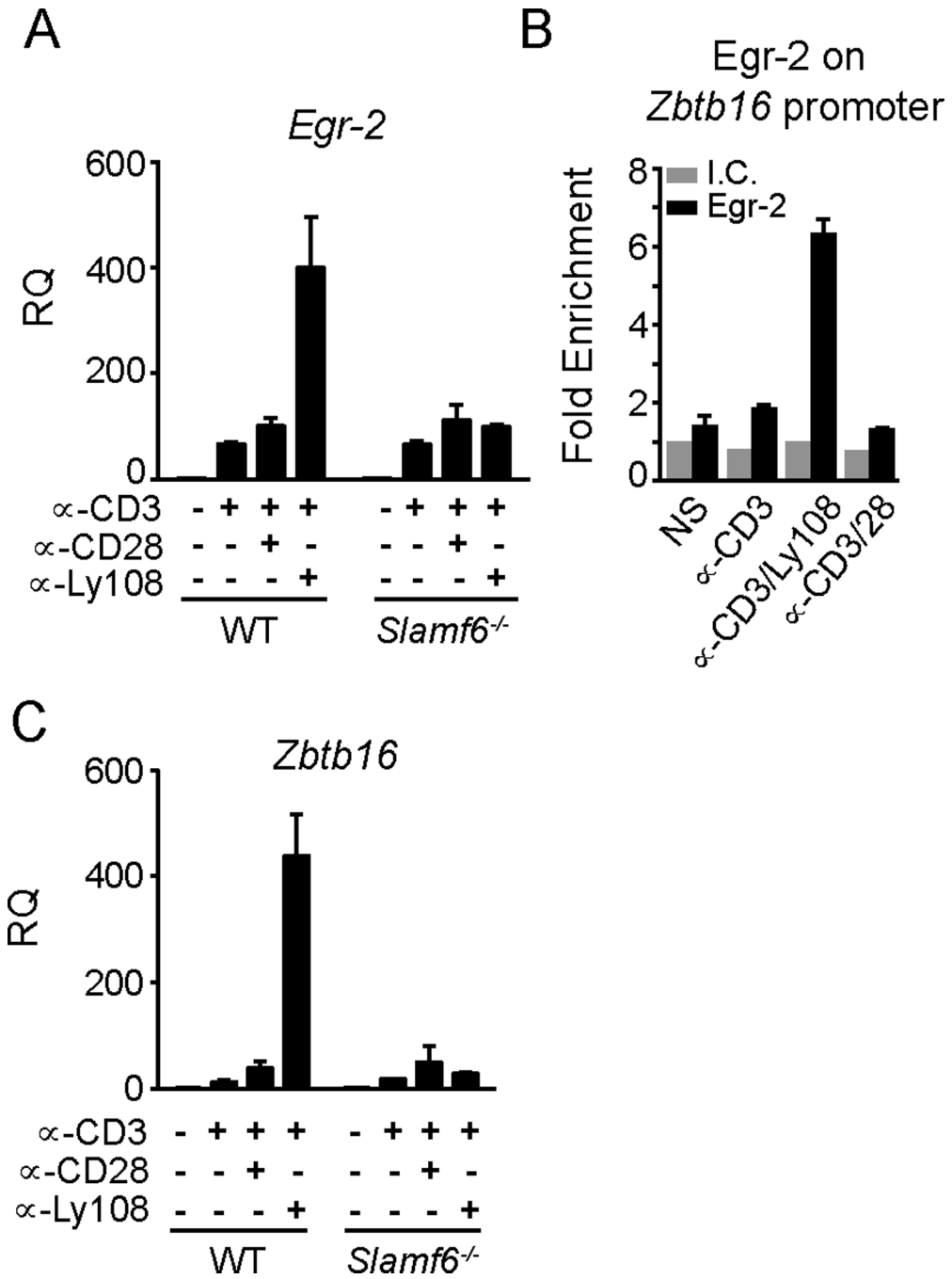


Figure 7. CD28 costimulation does not enhance PLZF expression
 (A–C) PS-DP thymocytes were stimulated with αCD3±αCD28 or ±αLy108 and evaluated for *Egr-2* expression by qRT-PCR at 18h (A), *Egr-2* binding at the *Zbtb16* promoter by ChIP at 18h (B), or *Zbtb16* expression at 24h (C). Data show the mean of 2 replicates ± SD from one of two or more independent experiments.

Table 1

Conventional and PLZF⁺ T Cell populations in *Slamf6*^{-/-} and *B6.Sle1b* mice

	DN % ± SEM (# × 10 ⁶)	DP % ± SEM (# × 10 ⁶)	CD8 SP % ± SEM (# × 10 ⁶)	CD4 SP % ± SEM (# × 10 ⁶)	PLZF ⁺ % ± SEM (# × 10 ⁵)*	CD1d tet ⁺ % ± SEM (# × 10 ⁵)*
WT C57BL/6 N=6 (95.9×10 ⁶ ±3.85)	4.99±0.47 (4.79±0.57)	80.9±0.29 (77.6±2.17)	2.97±0.11 (2.84±0.16)	9.47±0.23 (9.08±0.42)	1.33±0.14 (1.21±0.18)	1.85±0.05 (1.53±0.12)
<i>Slamf6</i> ^{-/-} N=6 (96.5×10 ⁶ ±6.6)	4.98±0.47 (4.78±0.48)	81.8±1.14 (79.1±2.49)	2.64±0.37 (2.55±0.33)	8.91±0.84 (8.6±0.8)	0.42±0.01 (0.36±0.02)	1.06±0.07 (0.91±0.08)
WT C57BL/6# N=9 (71.7×10 ⁶ ±6.31)	3.08±0.26 (2.21±0.26)	87.2±1.38 (62.5±3.2)	1.46±0.18 (1.05±0.15)	5.23±0.79 (3.75±0.63)	0.76±0.18 (0.29±0.09)	0.9±0.11 (0.34±0.06)
<i>B6.Sle1b</i> N=9 (99.5×10 ⁶ ±5.44)	3.63±0.66 (3.61±0.64)	84.9±3.7 (84.5±6.61)	2.43±0.59 (2.42±0.57)	5.53±0.48 (5.5±0.58)	1.91±0.24 (1.11±0.08)	1.44±0.17 (0.79±0.15)

* Percentages are of gated CD4 single positive cells.

Controls for *B6.Sle1b* experiments (performed at a different time)