JLB

Article

Cooperative immunoregulatory function of the transmembrane adaptor proteins SIT and LAX

Börge Arndt,[*,1] Thomas Kalinski,[†] Dirk Reinhold,[*] Anja Thielitz,[‡] Albert Roessner,[†] Burkhart Schraven,*[,§] and Luca Simeoni*

*Institutes of Molecular and Clinical Immunology and [†]Pathology and [‡]Clinic of Dermatology and Venerology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany; and [§]Department of Immune Control, Helmholtz Centre for Infection Research, Braunschweig, Germany

RECEIVED MARCH 22, 2012; REVISED DECEMBER 17, 2012; ACCEPTED DECEMBER 17, 2012. DOI: 10.1189/jlb.0312152

ABSTRACT

Lymphocyte activation is crucial for the generation of immune responses. In vitro studies have demonstrated that TRAPs are critical regulators of lymphocyte activation. However, more recent in vivo studies have demonstrated that with the exception of LAT, TRAPs, such as SIT, NTAL, and LAX, only minimally affect immune cell functions. Additional studies have suggested that the mild or the apparent lack of a phenotype displayed by most TRAP KO mice may be explained by functional redundancy among this family of adaptors. In fact, it has been shown that the phenotype of NTAL/LAT or SIT/TRIM double-deficient mice is more severe than that of the single KOs. Here, we have evaluated whether SIT and the related transmembrane adaptor LAX have overlapping functions by generating SIT/LAX DKO mice. We show that DKO, in contrast to single KO mice, accumulate large numbers of activated CD4$^+$ T cells in the spleen. Moreover, conventional B cells from DKO mice are hyperproliferative upon CD40 stimulation. Additionally, we found that DKO mice displayed an expansion of the B1 cell pool in the peritoneal cavity, hypergammaglobulinaemia, and an enhanced immune response to the T1-independent antigen, TNP-LPS. Finally, we demonstrate that SIT/LAX double deficiency resulted in a more pronounced breakdown of peripheral tolerance and the development of autoimmunity characterized by ANAs and renal disease (glomerulonephritis and proteinuria). Collectively, our data indicate that SIT and LAX are important negative regulators of immune responses that functionally cooperate.
***J. Leukoc. Biol.* 93: 353–362; 2013.**

Introduction

TRAPs represent a family of molecules that organize membrane-proximal signaling. To date, seven TRAPs have been identified. Among these, LAT, PAG/Cbp, NTAL/linker for activation of B cells and LIME are mainly localized in the lipid rafts, whereas TRIM, SIT, and LAX are excluded from the raft fraction of the plasma membrane (reviewed in ref. [1]).

The common features of all known transmembrane adapters are the presence of a short extracellular domain, a single membrane-spanning region, and multiple TBSMs within the cytoplasmatic tail [1, 2]. These TBSMs become phosphorylated after antigen receptor engagement and allow inducible protein–protein interactions. Therefore, by recruiting other signal-transducing molecules, TRAPs represent important regulators of signaling cascades that are required for the propagation and fine-tuning of the signal from the antigen receptor.

During the last years, the physiological role of TRAPs has been highlighted in studies using cell lines and KO mice. The generation of LAT$^{-/-}$ mice revealed its essential role in T cell development. LAT$^{-/-}$ mice lack peripheral T cells, whereas the generation of B cells and NK cells was not altered [3]. In addition to LAT, other TRAPs are important for proper immune function. However, loss of these adaptors only modestly affects immune functions. For example, NTAL$^{-/-}$ mice developed an autoimmune disease with age [4], whereas LAX$^{-/-}$ mice show abnormally activated lymphocytes, spontaneous germinal center formation in the spleen of unimmunized mice,

Abbreviations: $^{-/-}$=deficient, ANA=antinuclear antibody, Cbp=COOH terminal Src kinase-binding protein, DKO=double-knockout mice, Foxp3=forkhead box p3, Grb2=growth factor receptor-bound protein 2, HEp-2=human epidermoid cancer 2, KLH=keyhole limpet hemocyanin, KO=knockout, L=ligand, LAT=linker for activation of T cells, LAX=linker for activation of X cells, LIME=lymphocyte-specific protein tyrosine kinase-interacting molecule, MAC-1=macrophage 1 antigen, NTAL=non-T cell activation linker, PAG=phosphoprotein associated with genetically engineered mice, PAS=periodic acid-Schiff, SIT=Src homology-containing tyrosine phosphatase-interacting transmembrane adaptor protein, TBSM=tyrosine-based signaling motif, TD=thymus-dependent, TI=thymus-independent, TRAP=transmembrane adaptor protein, Treg=regulatory T cell, TRIM=TCR-interacting molecule, TSAd=T cell-specific adaptor

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence at current address: Medizinische Klinik und Poliklinik IV, Klinikum der LMU, Ziemssenstr. 1, 80336 München, Germany. E-mail: boerge.arndt@med.uni-muenchen.de

hypergammaglobulinemia, and hyperreactive mast cells [5, 6]. Moreover, we have shown that SIT$^{-/-}$ mice displayed an enhanced positive selection in the thymus, hyperactivated peripheral T cells, and an increased homeostatic proliferation [7–9]. Conversely, mice lacking TRIM [10], PAG [11, 12], and LIME [13] have no significant alteration of the immune system.

The observation that TBSMs are highly conserved among transmembrane adaptors and that KO mice have no phenotype or only a mild alteration of the immune system has led to the hypothesis that TRAPs are functionally redundant [1]. Indeed, by generating SIT/TRIM double-deficient mice, we have shown that SIT and TRIM (which share the two TBSMs: YGNL and YASV/L, respectively) modulate T cell development by setting the threshold for thymocyte activation [14]. In addition, the analysis of LAT/NTAL double-deficient mast cells revealed that they display a more severe defect in FcεRI-mediated activation than mast cells lacking LAT alone, thus demonstrating that LAT and NTAL function in a complementary manner [15, 16].

In the present study, we have evaluated whether SIT and LAX cooperate during lymphocyte development and activation by generating SIT/LAX DKO. The rational basis for this approach was given by several structural and functional similarities between the two adaptors: (i) SIT and LAX are both nonraft-associated TRAPs; (ii) they both negatively regulate antigen receptor-mediated signaling; (iii) they share canonical Grb2-binding motifs (YxN) and associate with Grb2 upon antigen receptor stimulation; and finally, (iv) they possess an overlapping tissue distribution in lymphocytes [4, 7–9, 17, 18].

We show that aged DKO mice accumulate larger numbers of activated CD4$^+$ T cells in the spleen. In addition, we demonstrate that despite the fact that SIT and LAX do not overlap during thymic selection and during the development of conventional B cells, they cooperatively regulate the generation of B1a cells and immune functions, such as basal serum Ig levels and TI immune responses. Finally, we found that aged DKO mice develop elevated levels of ANAs in the serum and further develop glomerulonephritis and proteinuria. This lupus-like disease is more severe than in age-matched SIT or LAX single KO mice.

In summary, our studies shed further light on the role of nonraft transmembrane adaptors in lymphocytes and demonstrate that SIT and LAX are important regulators of immune function that partially overlap.

MATERIALS AND METHODS

Animal ethics

All experiments were performed with samples taken from euthanized animals in accordance with the German National Guidelines for the Use of Experimental Animals (Animal Protection Act, Tierschutzgesetz, TierSchG). Animals were handled in accordance with the European Communities Council Directive 86/609/EEC. All possible efforts were made to minimize animal suffering and the number of animals used.

Mice

SIT$^{-/-}$ and LAX$^{-/-}$ mice were described previously [5, 7]. LAX$^{-/-}$ mice were kindly provided by W. Zhang. SIT$^{-/-}$ mice were mated with LAX$^{-/-}$ to generate SIT$^{+/-}$LAX$^{+/-}$ mice, which were then crossed to generate SIT$^{-/-}$LAX$^{-/-}$ double-deficient (DKO) mice. Mice were genotyped for SIT and LAX mutations as described previously [5, 7]. The mice used in this study were between 3 and 12 month of age, as indicated.

Flow cytometry

Single cell suspensions were prepared from thymus, spleen, LNs, bone marrow, and peritoneal cavity. Subsequently, 1×10^6 cells were stained with different mAb for 15 min at 4°C, washed, and then analyzed on a FACSCalibur using CellQuest software (BD Biosciences, San Diego, CA, USA). Antibodies against CD3ε (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD8 (53-

TABLE 1. Cellularity of Peripheral T Cell Subsets from Young and Aged Mutant and WT Mice

Cells	Age (months)	Absolute cell numbers (1×10^6) DKO	LAX$^{-/-}$	SIT$^{-/-}$	WT	*P* values
Total splenocytes	4	140.0 ± 21.4	115.8 ± 18.3	122.2 ± 18.8	126.7 ± 21.1	ns
CD4$^+$	4	22.4 ± 2.3	18.8 ± 3.1	22.2 ± 5.1	22.1 ± 3.4	ns
CD4$^+$CD44^{hi} CD62L^{lo}	4	9.1 ± 1.2	7.0 ± 1.5	8.2 ± 1.2	8.1 ± 0.6	ns
CD8$^+$	4	12.9 ± 2.2	14.1 ± 1.9	13.1 ± 2.1	14.4 ± 2.5	ns
Tregs (CD4$^+$ Foxp3$^+$)	4	1.09 ± 0.19	1.04 ± 0.13	0.97 ± 0.15	1.19 ± 0.17	ns
Total splenocytes	12	113.3 ± 29.0	109.5 ± 22.0	98.8 ± 26.1	111.3 ± 24.6	ns
CD4$^+$	12	27.3 ± 6.9	20.6 ± 5.6	19.6 ± 4.1	19.6 ± 6.5	DKO versus LAX **0.04** DKO versus SIT 0.01 DKO versus WT 0.01
CD4$^+$CD44^{hi} CD62L^{lo}	12	19.2 ± 5.2	10.1 ± 2.5	11.4 ± 2.0	8.6 ± 3.8	DKO versus LAX **0.0008** DKO versus SIT **0.0034** DKO versus WT **0.0001**
CD8$^+$	12	8.2 ± 2.8	12.1 ± 4.8	8.5 ± 1.8	13.0 ± 3.5	DKO versus LAX 0.11 DKO versus SIT 0.87 DKO versus WT **0.02**
Tregs (CD4$^+$ Foxp3$^+$)	12	1.27 ± 0.25	1.24 ± 0.23	1.44 ± 0.25	1.41 ± 0.24	ns

The absolute numbers of T cell subsets were determined on the basis of the total cell numbers and the percentage of lymphocyte subsets determined by flow cytometric analysis. The absolute cell numbers of the different mouse strains are shown. *P* values were determined by an unpaired two-tailed Student's *t*-test comparing the numbers from each genotype with each other. Data represent means ± SD of the mean. At least six mice/group were analyzed.

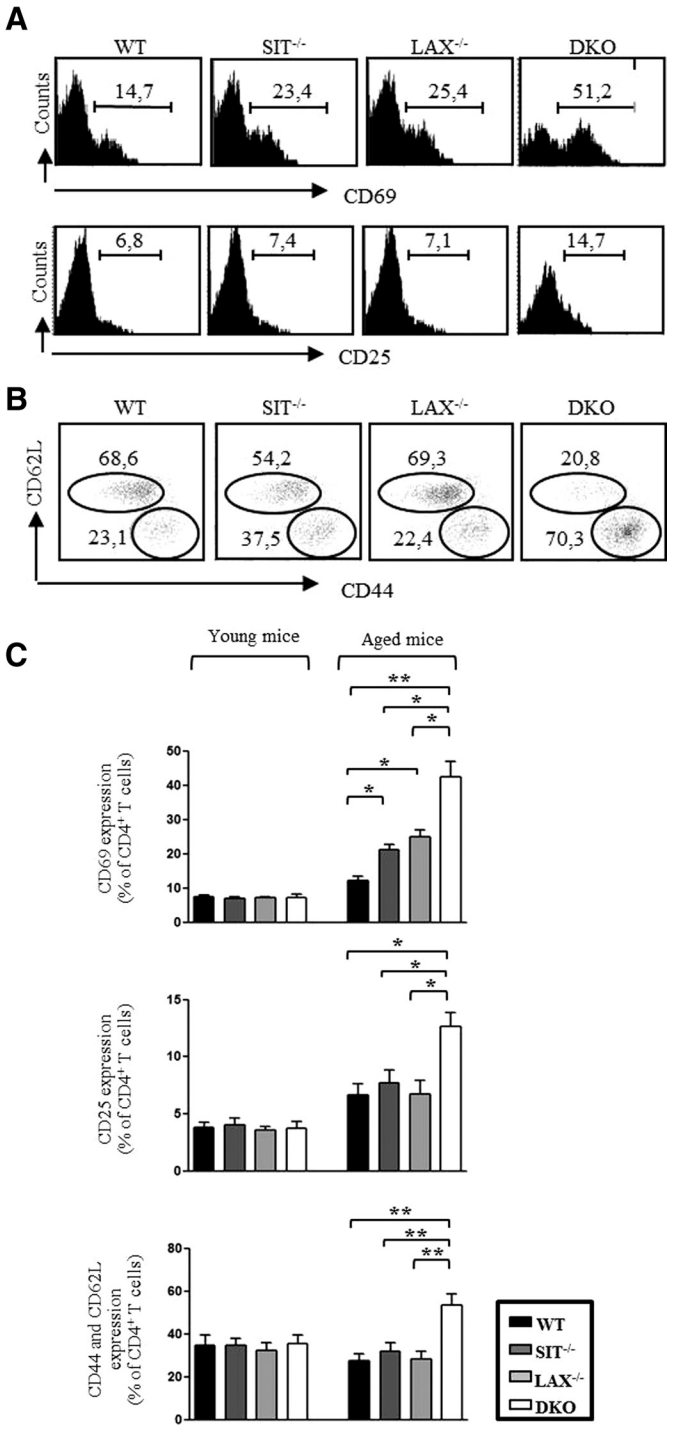

Figure 1. Analysis of T cell activation markers in young and aged mice. (A) Histograms show the expression of the activation marker CD69 or CD25. Numbers within the histograms indicate the percentage of $CD4^+$ T cells that express CD69 or CD25. (B) $CD4^+$ T cells were analyzed for the expression of CD44 and CD62L. Numbers indicate the percentage of cells in the region. (C) Bar graphs show statistical analyses of CD69 (top), CD25 (middle), $CD44^{hi}$ and $CD62L^{lo}$ (bottom) expression and compares the expression pattern of these markers between young (4 months, left) and older mice (12 months, right). Bars represent the mean ± SEM from at least four mice. Statistical significance is indicated (*$P<0.05$; **$P<0.01$).

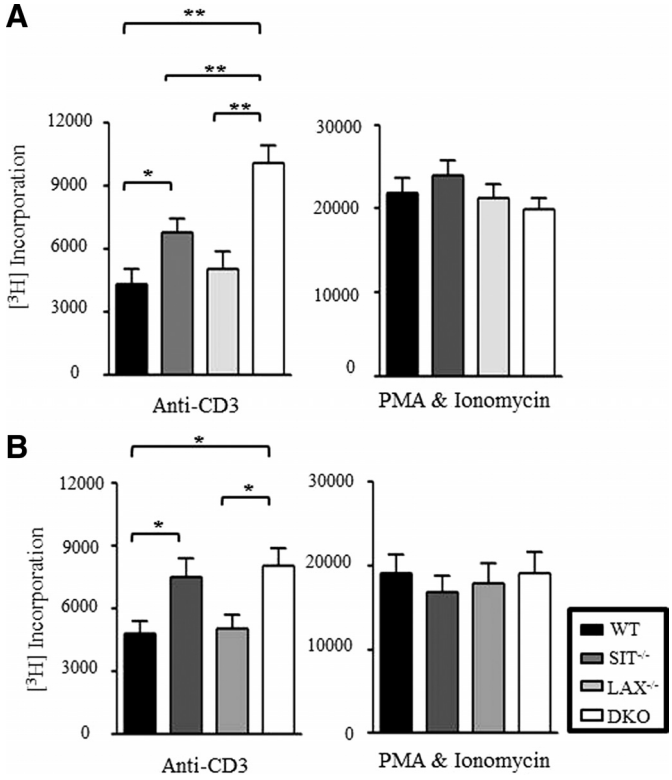

Figure 2. $CD4^+$ T cells from DKO mice are hyper-responsive to antigen receptor stimulation. Purified splenic $CD4^+$ T cells from aged (12-month-old) mutant and control mice (A) or from young mutant and WT mice (B) were stimulated in a 96-well plate with 1 μg/ml coated CD3 or PMA plus ionomycin (2 ng/ml PMA and 0.5 μg/ml ionomycin) for 72 h. Cells were pulsed with [^{3}H]-thymidine and processed for standard scintillation counting. Data represent mean counts/min ± SEM from five mice. *$P < 0.05$; **$P < 0.01$.

6.7), CD21 (7G6), CD23 (B3B4), CD25 (7D4), CD40 (3/23), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD86 (GL1), β-TCR (H57-597), B220 (RA3-6B2), IgM (11/41), IgD (11-26c.2a), and MAC-1 (M1/70) were purchased from BD Biosciences. CD40L (MR1) and FoxP3 were purchased from eBioscience (San Diego, CA, USA).

Proliferation assay

Splenic B lymphocytes or $CD4^+$ T cells were purified using a B cell isolation kit or a $CD4^+$ isolation kit (Miltenyi Biotec, Germany) on an AutoMACS magnetic separation system. The purity that we obtained during the isolation process was usually > 94% and was confirmed by flow cytometry. Purified B cells (5×10^4 cells/well) and purified $CD4^+$ cells (2×10^5 cells/well) were cultured in RPMI medium (supplemented with 10% FCS, antibiotics, and β-ME) and left unstimulated or activated with the following mitogens: anti-IgM F(ab)′2 fragment, CD40, LPS, CD3, and PMA and ionomycin. Anti-IgM F(ab)′2 was added in solution, whereas CD40 and CD3 were coated to the plate. LPS was used at 1 μg/ml. Cells were cultured for 72 h at 37°C with 5% CO_2. During the last 8 h, cells were pulsed with 0.5 μCi/well [^{3}H]-thymidine. Subsequently, cells were harvested, and [^{3}H]-thymidine incorporation was measured by using the microplate liquid scintillation counter Wallac MicroBeta TriLux from Perkin Elmer (Waltham, MA, USA).

Mouse immunization and ELISA

For TI immune responses, mice were immunized i.p. with 50 μg TNP-LPS (Sigma, St. Louis, MO, USA). Sera were collected before immunization and

at Day 10 after immunization. Ig levels were determined as described previously [19]. Basal IgE levels were determined by using a Mouse IgE ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX, USA).

Cell stimulation and immunoblotting

Total B lymphocytes were left unstimulated or stimulated with 10 μg/ml anti-IgM antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 37°C. $CD4^+$ T cells were stimulated with biotinylated anti-CD3ε mAb (145-2C11; BD Biosciences), followed by cross-linking with 25 μg/ml streptavidin. Cells were lysed in lysis buffer containing 1% Nonidet P-40, 1% laurylmaltoside (*N*-dodecyl-β-D-maltoside), 50 mM Tris (pH 7.5), 140 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 mM PMSF, and 1 mM Na_3VO_4. Cell lysates were subjected to SDS-PAGE, blotted onto PVDF membranes, and probed with the following antibodies: antiphosphotyrosine (4G10), antiphospho-ERK1/2 (T202/Y204), antiphospho-JNK (T183/Y185), antiphospho-p38 (T180/Y182), and antiphospho-AKT (S473; all Cell Signaling Technology, EMD Millipore, Billerica, MA, USA).

ANA production

The presence of ANAs was analyzed by using a 12-well microscope slide (Innogenetics, Belgium) covered with human HEp-2 cells, which were blocked and then incubated with the indicated mouse serum dilutions for 30 min. Successively, the slides were washed and incubated for an additional 30 min with a FITC-labeled goat anti-mouse IgM antibody (Sigma) or donkey anti-mouse IgG antibody (Dianova, Switzerland). Slides were analyzed by fluorescence microscopy.

Histology

Kidneys from 12-month-old C57BL/6 WT and mutant mice were fixed in formalin and embedded in paraffin for histological studies. Sections were stained with H&E and additionally with PAS. Slides were analyzed by light microscopy in a blinded fashion.

Immunofluorescence

For immunohistochemistry, kidneys were quickly frozen in liquid nitrogen. Sections were stained with FITC-conjugated goat anti-mouse IgG and analyzed by fluorescence microscopy.

Proteinuria

Proteinuria was measured by using urine analysis sticks (Bayer, Germany).

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). *P* values were determined by an unpaired two-tailed Student's *t*-test.

RESULTS

Accumulation of activated $CD4^+$ T cells in DKO mice

Recently, it has been shown that SIT and LAX are negative regulators of lymphocyte activation [5, 7, 9]. However, as $SIT^{-/-}$ and $LAX^{-/-}$ mice display only mild alterations in immune functions, we investigated whether the loss of SIT and LAX would result in a more severe phenotype than the single KOs. To assess this issue, we generated $SIT^{-/-}LAX^{-/-}$ DKO mice. We have shown previously that loss of SIT altered thymic selection. Conversely, LAX is dispensable for lymphocyte development [5]. To assess whether the concomitant ablation of LAX aggravates the defective thymic development of $SIT^{-/-}$ mice, we analyzed primary and secondary lymphoid organs from $SIT^{-/-}$, $LAX^{-/-}$, and DKO mice. The numbers, as well as the CD4/CD8 profiles of thymocytes from DKO, were comparable with those of $SIT^{-/-}$ mice (data not shown). Moreover, a detailed analysis of positive and negative selection in TCR transgenic models also revealed no difference between $SIT^{-/-}$ and DKO mice (data not shown). Thus, it appears that SIT and LAX do not functionally overlap during T cell development. Also, the size of the spleen and LNs was comparable between mutant and WT mice, thus indicating that DKO, similar to single mutant mice, do not develop spontaneous lymphopenia or lymphoproliferative disease (**Table 1**, and data not shown). However, a detailed analysis of lymphocyte subsets revealed that aged, but not young, DKO mice have clearly elevated numbers of $CD4^+$ T cells in the spleen (Table 1). The expansion of $CD4^+$ T cells was not detected in LAX or SIT single KO mice. Next, we examined whether $CD4^+$ T cells from aged DKO mice showed additional abnormalities. FACS analyses revealed that $CD4^+$ T cells from aged DKO mice express significantly higher levels of CD69 compared with the single mutant mice (**Fig. 1A** and **C**). In contrast to $CD4^+$ T

TABLE 2. Cellularity of Peripheral B Cell Subsets from Young and Aged Mutant and WT Mice

Cells	Age (months)	Absolute cell numbers (1×10^6) DKO	$LAX^{-/-}$	$SIT^{-/-}$	WT	*P* values
Total splenocytes	4	140.0 ± 21.4	115.8 ± 18.3	122.2 ± 18.8	126.7 ± 21.1	ns
$B220^+$	4	75.1 ± 10.5	65.6 ± 13.4	66.7 ± 8.9	69.6 ± 12.0	ns
IgM^+CD23^{hi} $CD21^{int}$ (FO)	4	60.7 ± 8.9	51.6 ± 10.6	52.9 ± 6.6	55.5 ± 9.4	ns
IgM^+CD21^{hi} $CD23^{int}$ (MZ)	4	2.5 ± 0.4	2.1 ± 0.5	2.1 ± 0.5	2.1 ± 0.5	ns
Total splenocytes	12	113.3 ± 29.0	109.5 ± 22.0	98.8 ± 26.1	111.3 ± 24.6	ns
$B220^+$	12	58.6 ± 10.1	55.1 ± 8.8	50.3 ± 8.6	57.2 ± 10.1	ns
IgM^+CD23^{hi} $CD21^{int}$ (FO)	12	47.2 ± 8.1	44.1 ± 7.4	39.5 ± 7.5	45.8 ± 8.2	ns
IgM^+CD21^{hi} $CD21^{in}$ (MZ)	12	2.1 ± 0.4	2.2 ± 0.4	1.9 ± 0.4	2.3 ± 0.5	ns

The absolute cell numbers of B cell subsets were determined on the basis of the total cell numbers and the percentage of lymphocyte subsets determined by flow cytometric analysis. The absolute cell numbers of the different mouse strains are shown. *P* values were determined by an unpaired two-tailed Student's *t*-test comparing the numbers from each genotype with each other. Data represent means ± SD of the mean. At least five mice/group were analyzed. FO, Follicular; MZ, marginal zone.

cells from aged LAX$^{-/-}$ and aged SIT$^{-/-}$ mice, those from aged DKO also displayed elevated expression levels of the IL-2Rα chain CD25 (Fig. 1A and C). Moreover, aged DKO mice, but not single mutant mice, displayed a striking increase in the numbers of CD4$^+$ T cells expressing high levels of CD44 and low levels of CD62L corresponding to activated/effector T cells (Fig. 1B and C and Table 1). Contrary to aged mutant mice, the expression patterns of CD25 and CD69 on T cells from younger DKO, SIT$^{-/-}$, and LAX$^{-/-}$ mice were comparable with WT controls (Table 1 and Fig. 1C).

Given the fact that T cells from aged DKO and SIT$^{-/-}$ appear to be expanded in vivo, we investigated their proliferative capability in vitro. As shown in **Fig. 2** and in agreement with our previously published data [7], loss of SIT enhanced CD3-mediated proliferation. Moreover, the data presented in Fig. 2A show that CD4$^+$ T cells obtained from 12-month-old DKO mice proliferate stronger in response to the applied stimuli in comparison with cells from single KO mice or WT controls. However, when we analyzed young mice (4 month), we found that proliferation was enhanced in SIT$^{-/-}$ and DKO mice in a comparable manner (Fig. 2B). Taken together, it appears that SIT and LAX synergize to regulate the activation status of peripheral CD4$^+$ T cells in aged mice.

Normal development of conventional B cells but increased numbers of B1 B cells in SIT/LAX double-deficient mice

We next evaluated the role of SIT and LAX in the B cell compartment. We found that the development of conventional B cells is not affected by single or double deletion of the two transmembrane adaptors (data not shown). In agreement with this observation, we found normal B cell numbers in all mutant mice (**Table 2**). To assess whether other B cell subsets, such as B1 cells that appear to have a distinct origin from conventional B cells [20], were affected by the loss of SIT and LAX, B1 cells were prepared from the spleen and peritoneal lavages, stained with IgM, Mac-1, CD43, and CD5 mAb, and analyzed by flow cytometry. As shown in **Fig. 3A** and **B**, DKO mice possess a twofold higher proportion of B1a cells (IgM$^+$Mac1$^+$CD43$^+$CD5$^+$) compared with WT mice in the peritoneal cavity and the spleen. It is important to note that the proportion of B1a cells is normal in SIT and LAX single KO mice. In contrast to B1a cells, the number of B1b cells that do not express CD5 was comparable in all mutant mice (Fig. 3C). Thus, our data reveal that although SIT and LAX do not possess a redundant function during the development of conventional B lymphocytes, they collaborate in regulating the size of the peripheral B1a cell pool.

B cells from DKO mice are hyperproliferative upon CD40 stimulation

We next investigated the function of the peripheral B cell pool in 4-month- and 12-month-old mice. We found that splenic B cells from all mutant mice displayed normal expression of the activation markers CD69 and CD86 (**Fig. 4A**

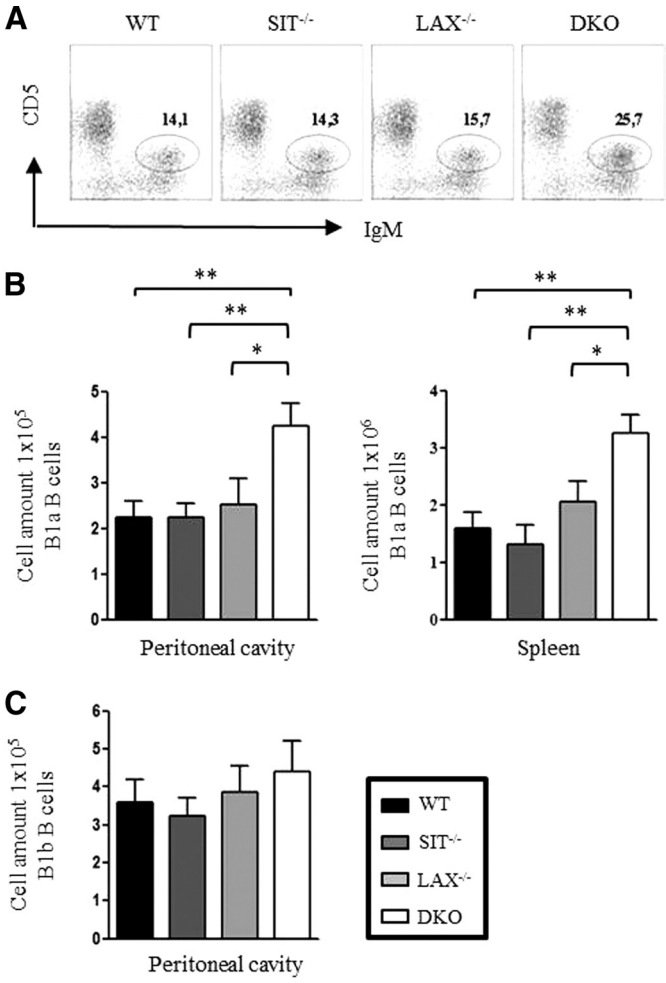

Figure 3. Increased numbers of B1a cells in DKO mice. (A) Peritoneal cells were stained with mAb against CD5, IgM, CD43, and MAC-1. B1a cells were defined as IgM^{hi}, MAC-1$^+$, CD43$^+$, and CD5$^+$. Numbers indicate the percentages of living cells that reside in the corresponding gate. One representative mouse out of 10/each group is shown. (B) Bar graphs show the absolute numbers of B1a cells in the peritoneal cavity (left) and spleen (right). (C) Bar graphs show the absolute number of B1b cells (defined as IgM^{hi}, MAC-1$^+$, and CD5$^-$) in the peritoneal cavity. *$P < 0.05$; **$P < 0.01$.

and **B** and Supplemental Fig. 1A and B) in young and aged mice.

Next, we wanted to investigate whether SIT and LAX regulate the proliferative capability of B cells in vitro. Freshly purified splenic B cells were cultured and stimulated with different concentrations of anti-IgM F(ab)′2, CD40, and LPS. B cells from aged DKO mice displayed an equal proliferation potential compared with single KO mice upon anti-IgM F(ab)′2 stimulation (**Fig. 5A**). In contrast, upon stimulation with anti-CD40, B cells from aged but not young DKO mice proliferated stronger than those from SIT$^{-/-}$, LAX$^{-/-}$, or WT mice (Fig. 5B and C). Taken together, these results demonstrate that SIT and LAX alone do not regulate the proliferative B cell response in vitro upon antigen receptor stimulation in B cells. In contrast, both adaptors together appear

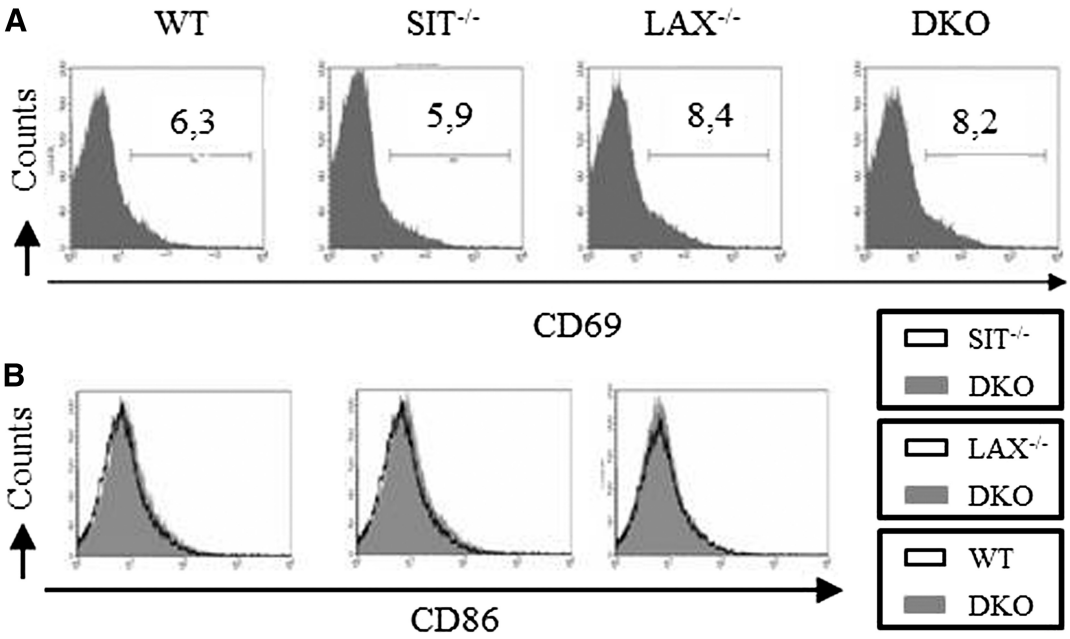

Figure 4. Analysis of B cell activation markers in aged mice. (A) Histograms display the expression of the activation marker CD69 on $B220^+$-gated cells from aged DKO, $SIT^{-/-}$, or $LAX^{-/-}$ and WT mice. Numbers indicate the percentage of living cells that express CD69. (B) $B220^+$-gated splenic cells were analyzed for the expression of CD86. One representative mouse out of four is shown.

to regulate proliferation upon CD40 stimulation in B cells in a redundant manner.

SIT/LAX double-deficient mice display hypergammaglobulinemia and enhanced TI immune responses

Having demonstrated that SIT and LAX cooperatively regulate the activation status of $CD4^+$ T cells and the number of B1 B cells, we wanted to evaluate whether the absence of SIT and LAX also affects the immune response in vivo. We have shown previously that the absence of SIT does not alter the basal serum Ig levels. Moreover, TD and TI responses were also normal in $SIT^{-/-}$ mice [7]. Conversely to $SIT^{-/-}$ mice, $LAX^{-/-}$ mice have been reported to possess elevated levels of basal Igs [5]. In agreement with these data, we found that $SIT^{-/-}$ mice possess normal levels of serum Ig, whereas $LAX^{-/-}$ mice display higher levels of IgG1, IgG3, IgM, and IgE (**Fig. 6A**). Importantly, when we investigated the Ig levels in the sera of DKO mice, we observed that the production of IgG3, IgE, and particularly IgM is enhanced further in the DKOs compared with $LAX^{-/-}$ mice (Fig. 6A). As $SIT^{-/-}$ mice possess normal levels of Ig, the augmented concentration of IgG3, IgE, and IgM in the sera of DKO mice appears to indicate that SIT and LAX function in a cooperative manner in regulating Ig production in vivo.

The augmented basal Ig levels observed in DKO mice prompted us to examine the humoral immune responses in these mice. To address this point, mice were immunized with the TD antigen DNP-KLH and the TI antigen TNP-LPS. The concentration of antihapten antibodies after primary and secondary immunization with the DNP-KLH antigen was indistinguishable among $SIT^{-/-}$, $LAX^{-/-}$, SIT/LAX double-deficient, and WT mice (not shown). These observations confirm previous data and demonstrate that SIT and LAX are dispensable for TD immune responses [5, 7]. However, when mice were immunized with the TI-1 antigen TNP-LPS, DKO mice showed a slight but significant enhancement of the antibody titer (Fig. 6B). Conversely, single mutant mice generated a normal antibody response against the TI-1 antigen TNP-LPS (Fig. 6B). Collectively, these data suggest that SIT and LAX cooperatively regulate TI antibody production.

SIT and LAX cooperate to limit autoimmunity

The data presented above indicate that T and B cells from DKO have an altered function. We next analyzed whether our mutant mice spontaneously develop autoimmune diseases with age. We initially determined the constitutive production of autoantibodies by analyzing sera from 12-month-old $SIT^{-/-}$, $LAX^{-/-}$, and DKO mice and WT littermates for the presence of ANAs. HEp-2 epithelial cells were permeabilized and incubated with sera from mutant and control mice. The immunofluorescence data presented in **Fig. 7A** show that sera collected from 12-month-old $SIT^{-/-}$ and $LAX^{-/-}$ mice display a homogeneous antinuclear staining similar to what is observed in human systemic lupus erythematosus [21]. We next determined the ANA titer in $SIT^{-/-}$ and $LAX^{-/-}$ mice, through serial dilution of the serum, and found a titer of 247.3 ± 53.3 in $SIT^{-/-}$ and 221.8 ± 55.9 in $LAX^{-/-}$ mice, whereas the mean of ANA titer in control mice was only 16.7 ± 9.1 (Fig. 7B). DKO mice also showed a homogeneous antinuclear staining (Fig. 7A) but displayed an antibody titer that was approximately twofold higher than in either single KO (480 ± 97; Fig. 7B). Kidneys from DKO mice were markedly abnormal and displayed a more severe glomerulonephritis and an augmented IgG deposition compared with single KO mice (Fig. 7C). Moreover, glomeruli, showing pathological signs, were more frequent in DKO mice (data not shown). Additionally, DKO mice displayed significantly enhanced proteinuria compared with single mutant mice (Fig. 7D). Thus, it appears that DKO mice suffer from a more severe disease than the single

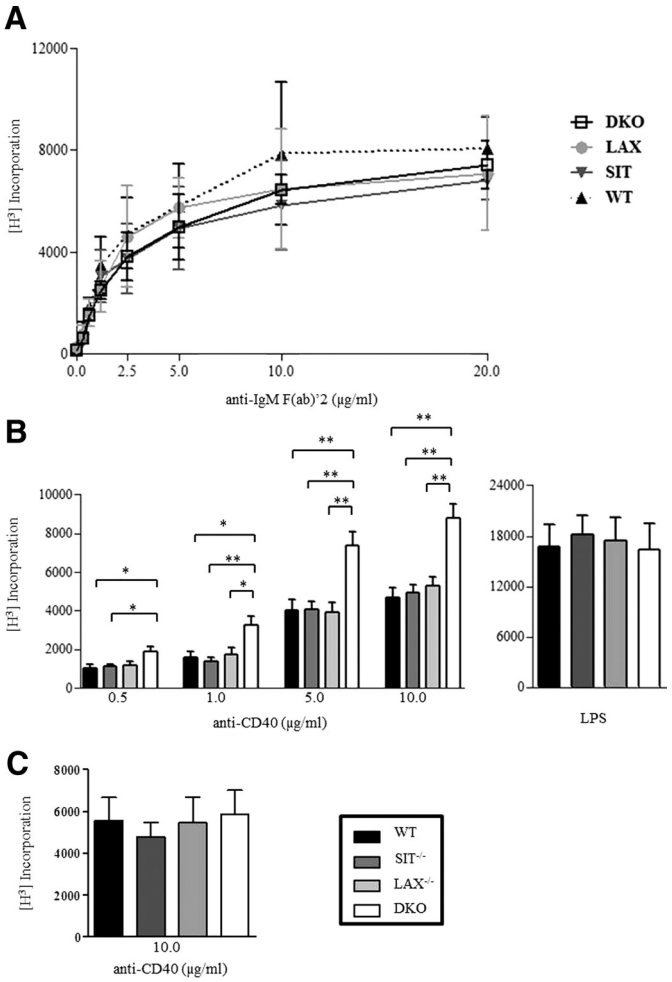

Figure 5. Hyperproliferation of B cells from aged DKO mice upon CD40 stimulation. Purified splenic B cells from aged mutant and control mice were stimulated with the indicated concentrations of soluble anti-IgM F(ab)′2 (A) or plate-bound CD40 mAb or LPS (B) in a 96-well plate for 72 h. (C) Purified splenic B cells from 4-month-old mice were stimulated with the indicated concentration of plate-bound CD40 mAb. Proliferation was assessed by [H^3]-thymidine incorporation for the last 6–8 h. Data represent mean counts/min ± SEM from triplicate cultures of four mice/each group. Statistical significance is indicated (*P<0.05; **P<0.01).

KO mice, as they display higher ANA titers and a more severe kidney disease. However, this seems to be an additive effect of the two phenotypes observed in single KO mice. Conversely to aged (12-month-old) mice, young (4-month-old) mice do not display ANAs and also have normal kidney architecture (data not shown).

To explore the possibility that reduced numbers of $CD4^+Foxp3^+$ Tregs in mutant mice contribute to the development of autoimmunity, splenic $CD4^+$ T cells were tested for the expression of Foxp3 in young as well as aged mice. As shown in Table 1, we did not detect any difference in the number of $CD4^+Foxp3^+$ Tregs between mutant and WT mice, thus excluding the possibility that a reduction in the number of Tregs causes autoimmunity.

DISCUSSION

SIT and LAX possess similar structure, function, tissue distribution, and membrane localization. Here, we have investigated whether these two TRAPs also share overlapping functions in lymphocytes. The principal finding of our study is that SIT and LAX negatively regulate several aspects of the immune system in a cooperative manner. More importantly, SIT and LAX also appear to cooperate to limit autoimmunity. The clinical features of the lupus-like disease in our mice are similar to those seen in KO mice for other adaptor molecules, such as NTAL and TSAd [4, 22], as the disease is late in onset and does not affect mortality/morbidity. In $NTAL^{-/-}$ and $TSAd^{-/-}$ mice, the disease appears to be triggered by an abnormal T cell function. In the case of $SIT^{-/-}$, $LAX^{-/-}$, and DKO mice, it is not clear whether T cells play a major role in the development of the lupus-like disease. The observation that $SIT^{-/-}$ and $LAX^{-/-}$ T cells are hyperactive indicates that these cells may play a role in the development of the disease [5, 7, 9]. Here, we show that T cells from aged mutant mice display enhanced CD69 and CD25 expression. Furthermore, and as shown previously [9], we found that naïve $CD4^+$ T cells from all mutant mice express slightly more CD40L on the surface (data not shown). Interestingly, it has been shown that increased expression of CD40L was found on $CD4^+$ T cells from lupus patients [23, 24]. Moreover, CD40L appears to play a role also in murine lupus models [25, 26]. Additionally, we display here that $CD4^+$ T cells from aged DKO mice are hyperproliferative upon CD3 stimulation when we used a low concentration of CD3 mAb (1 μg/ml). Titration experiments revealed that by using higher concentrations of plate-bound CD3 mAb, the differences in the proliferation capability becomes smaller (data not shown). Thus, it may be that this abnormal $CD4^+$ cell function in $SIT^{-/-}$, $LAX^{-/-}$, and DKO mice contributes to the autoimmune syndrome, e.g., by supporting the production of high-affinity IgG autoantibodies. Interestingly, DKO mice, but not single mutant mice, display an increase in effector $CD4^+$ T cells, which may also contribute to the development of the disease.

Moreover, B cells from aged DKO but not from single mutant mice are hyperproliferating upon CD40 stimulation and display a more pronounced hypergammaglobulinemia than single mutant mice. Thus, it is tempting to speculate that in addition to T cells, B cells may contribute to the disease in DKO mice. Nevertheless, additional experiments are required to asses the contribution of T and B cells to the development of the lupus-like disease in these mice.

Our data suggest that SIT and LAX synergistically regulate the development of B1a cells, which express different surface markers and also have different functions compared with conventional B2 cells [27]. In fact, B1a cells are resistant to anti-IgM-induced proliferation and apoptosis, produce low-affinity antibodies with a broad specificity, and mediate mainly T-independent immune responses [27]. The importance of B1 cells is emphasized by the discovery that this lymphocyte subpopulation is implicated in the pathogenesis of many human diseases, such as autoimmunity and malignant transformation [20, 28]. The origin of B1 cells is still under debate, but sev-

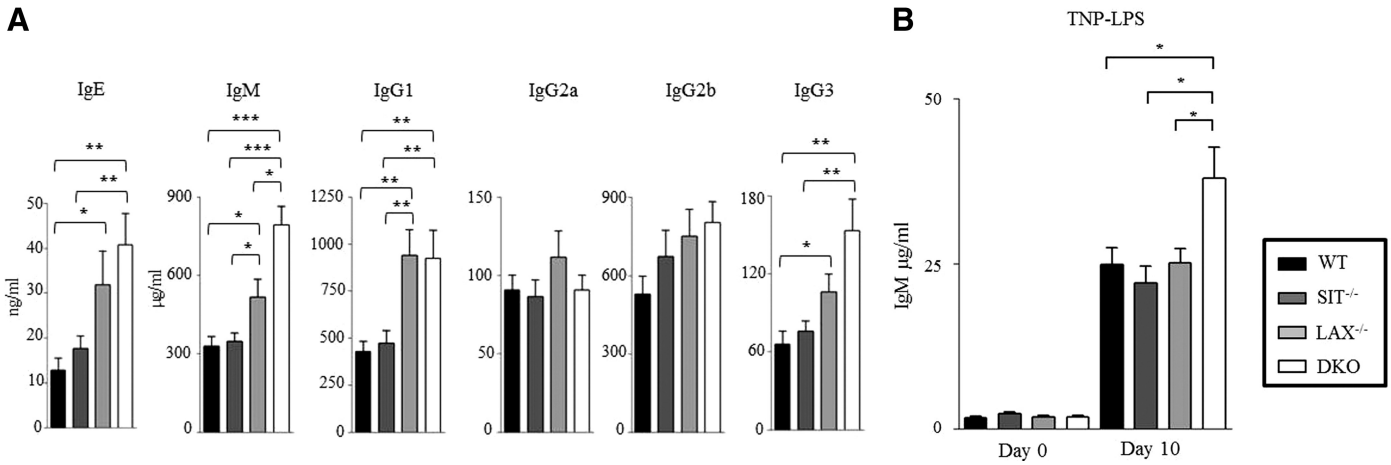

Figure 6. Hypergammaglobulinemia in DKO mice. (A) Basal Ig levels in the sera from 10 DKO, SIT$^{-/-}$, LAX$^{-/-}$, and WT mice were determined by ELISA. (B) TI immune response. Mice were immunized with 50 μg TNP-LPS, and sera were collected before (Day 0) and after (Day 10) immunization and analyzed for TNP-specific antibodies. A total of 11 mice/group was analyzed. Asterisks indicate the statistical significance (*P<0.05; **P<0.01; ***P<0.0001).

eral studies indicate that the development of B1 cells appears to be critically dependent on BCR signal strength. Disruption of genes that decrease BCR-mediated signaling results in a reduction of B1 cells, whereas mutations that enhance BCR-mediated signal strength result in an expansion of B1 cells [29]. Thus, mice lacking or carrying mutations in negative regulators of BCR-mediated signaling, such as CD22, Lyn, programmed death-1, and Src homology region 2 domain-containing tyrosine phosphatase-1, show increased numbers of B1 cells. As SIT and LAX also represent negative regulators of antigen receptor-mediated signaling, the observation that B1 cell numbers are increased in DKO mice is in line with the idea that signals emanating from the BCR critically regulate B1 cell numbers. However, when we analyzed BCR-mediated

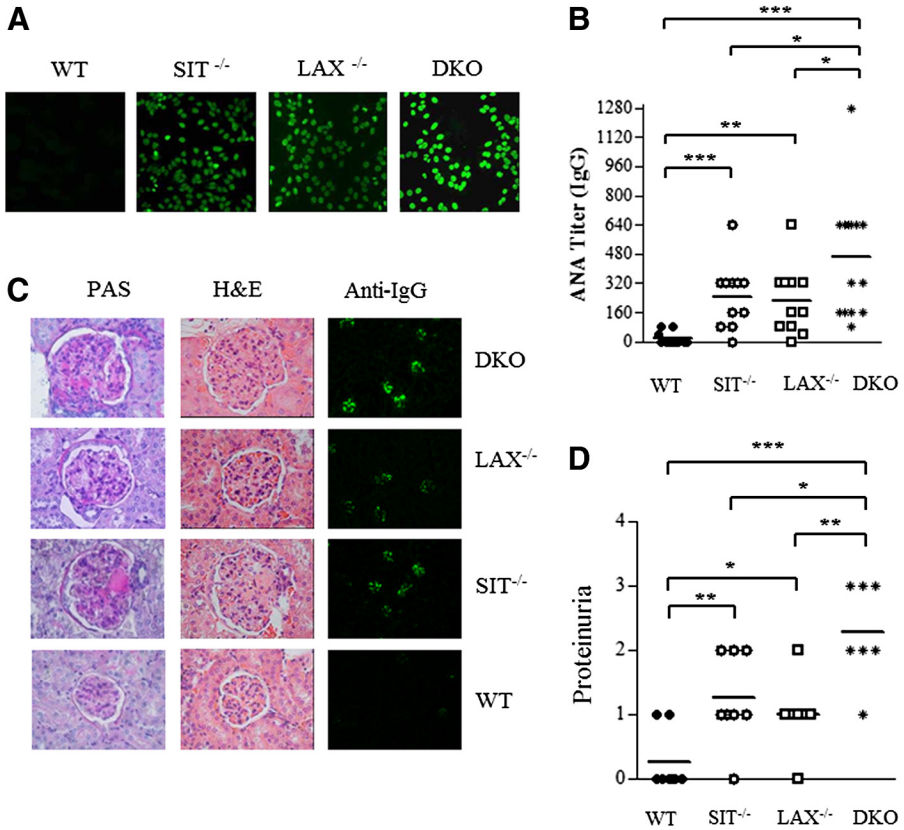

Figure 7. Development of ANAs and glomerulonephritis in DKO, SIT$^{-/-}$, and LAX$^{-/-}$ mice. (A) The presence of autoantibodies (ANAs) in sera from aged mutant and WT mice was determined by immunofluorescent staining of HEp-2 epithelial cells. The pattern of nuclear staining of HEp-2 cells using 1:80 dilution is shown. ANAs were detected with FITC-conjugated secondary antibody against mouse IgG. (B) Statistical analysis of ANA titer in mutant and WT mice. The bars represent the mean values of ANA titers. (C) Kidney sections from 1-year-old mutant and WT mice were stained with PAS, H&E, or with FITC-conjugated goat anti-mouse IgG. Sections were analyzed in a blinded fashion by light microscopy and scored for mesangial cellularity, increased mesangial matrix, infiltration of inflammatory cells, and thickness of basal membranes or capillary walls. Representative glomeruli from control and mutant mice are shown (1×400). Kidney cryosections were also stained with FITC-conjugated goat anti-mouse IgG, and the presence of immune complexes was visualized by fluorescence microscopy. (D) Statistical analysis of proteinuria in mutant and WT mice. The bars represent the mean values of proteinuria, which was measured by using urine analysis sticks and was graded, according to the following scale: negative, 0 mg/dl; 1, <30 mg/dl; 2, <100 mg/dl; and 3, >100 mg/dl. Asterisks in B and D indicate statistical significance (*P<0.05; **P<0.01; ***P<0.0001).

signaling using strong BCR cross-linking (i.e., anti-IgM), we did not find any difference between DKO and WT mice (Supplemental Fig. 2). Therefore, we propose that these adaptors may regulate homeostatic signals, which are induced by low-affinity self-ligands rather those that are induced by high-avidity foreign antigens. This hypothesis is supported by our previous observations showing that SIT negatively regulates T cell homeostasis and inhibits TCR-mediated signaling induced by weak but not strong stimuli [8, 9]

How SIT and LAX together regulate TCR-mediated signaling remains still elusive. A major obstacle in the analysis of the biochemical events that are regulated by SIT and LAX is the development of sensory adaptation upon loss of SIT. This means that the activation threshold of $SIT^{-/-}$ T cells is elevated, leading to reduced responsiveness upon TCR engagement. In fact, we have shown previously that $SIT^{-/-}$ T lymphocytes up-regulate CD5, a molecule required to reprogram the activation threshold in response to an altered signaling strength, and negatively regulate TCR-mediated signals, down-regulate the coreceptor CD8, and display impaired ZAP-70 activation and TCRζ-chain phosphorylation [8]. It appears that compensatory mechanisms are set in motion also in double mutant mice, as similarly to $SIT^{-/-}$ T cells, T cells from DKO mice also up-regulate CD5 and are hyporesponsive to strong TCR stimulation (data not shown and Supplemental Fig. 3). In line with that idea, hyperproliferation of $CD4^+$ T cells from $SIT^{-/-}$ and DKO mice was more obvious when low concentrations of CD3 mAb were used. Therefore, additional mouse models, such as $SIT^{-/-}/LAX^{-/-}/CD5^{-/-}$ triple KOs will be required to address how SIT and LAX regulated TCR-mediated signaling in T cells.

Collectively, our data suggest that lymphocytes are endowed with a set of TRAPs that exert subtle effects in lymphocytes. Thus, the question arises why lymphocytes need to express several TRAPs. Our data suggest that it is not the action of a single TRAP but rather, the coordinated action of multiple TRAPs that finely tunes the immune response. It is tempting to speculate that functional redundancy might apply to all TRAPs. In fact, the structural similarities between LAX and TRIM (note that both molecules are capable of binding PI3K and are strongly expressed in $CD4^+$ T cells) or between NTAL and LAX (which both carry several Grb2-binding sites and are expressed in B cells and mast cells) are suggestive of the existence of sophisticated and redundant mechanisms that regulate signaling. In addition to antigen receptors, it is possible that TRAPs control signals that are transduced through other surface receptors and thereby, modulate the general activation threshold of lymphocytes. The generation of additional DKO or even triple KO mice will help to assess how TRAPs control the intricate signal transduction network within immune cells.

Nevertheless, it is striking to note that NTAL, SIT, TRIM, and LAX all appear to primarily exert negative regulatory signaling functions within the immune system. Similarly, although $PAG/Cbp^{-/-}$ mice do not show an obvious phenotype [11, 12], small interference RNA studies demonstrate that PAG is an important negative regulatory protein in T cells [30]. Thus, it appears as if the strong, positive regulatory function of LAT is balanced by a plethora of negative regulatory TRAPs that help to maintain homeostasis within the immune system.

AUTHORSHIP

B.A. was the principle investigator and corresponding author. L.S. was the coprinciple investigator. Coinvestigators include T.K., D.R., A.T., A.R., and B.S.

ACKNOWLEDGMENTS

This work was supported by grants from the German Research Foundation (DFG), FOR-521 (SI861/1), GRK-1167 (TP12), and SFB-854 (TP19). We are grateful to Dr. Jonathan Lindquist and Tilo Beyer for critically reading the manuscript and helpful discussion and to Ines Meinert, Marita Lotzing, and Britta Bogel for excellent technical assistance. We thank Dr. Weiguo Zhang for providing $LAX^{-/-}$ mice and the employees of the animal facility for maintenance of the animals.

DISCLOSURES

We declare that we, the authors, have no conflicting interest.

REFERENCES

1. Horejsi, V., Zhang, W., Schraven, B. (2004) Transmembrane adaptor proteins: organizers of immunoreceptor signalling. *Nat. Rev. Immunol* **4,** 603–616.
2. Simeoni, L., Kliche, S., Lindquist, J., Schraven, B. (2004) Adaptors and linkers in T and B cells. *Curr. Opin. Immunol.* **16,** 304–313.
3. Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E., Samelson, L. E. (1999) Essential role of LAT in T cell development. *Immunity* **10,** 323–332.
4. Zhu, M., Koonpaew, S., Liu, Y., Shen, S., Denning, T., Dzhagalov, I., Rhee, I., Zhang, W. (2006) Negative regulation of T cell activation and autoimmunity by the transmembrane adaptor protein LAB. *Immunity* **25,** 757–768.
5. Zhu, M., Granillo, O., Wen, R., Yang, K., Dai, X., Wang, D., Zhang, W. (2005) Negative regulation of lymphocyte activation by the adaptor protein LAX. *J. Immunol.* **174,** 5612–5619.
6. Zhu, M., Rhee, I., Liu, Y., Zhang, W. (2006) Negative regulation of Fc εRI-mediated signaling and mast cell function by the adaptor protein LAX. *J. Biol. Chem.* **281,** 18408–18413.
7. Simeoni, L., Posevitz, V., Kolsch, U., Meinert, I., Bruyns, E., Pfeffer, K., Reinhold, D., Schraven, B. (2005) The transmembrane adapter protein SIT regulates thymic development and peripheral T-cell functions. *Mol. Cell. Biol.* **25,** 7557–7568.
8. Posevitz, V., Arndt, B., Krieger, T., Warnecke, N., Schraven, B., Simeoni, L. (2008) Regulation of T cell homeostasis by the transmembrane adaptor protein SIT. *J. Immunol.* **180,** 1634–1642.
9. Arndt, B., Krieger, T., Kalinski, T., Thielitz, A., Reinhold, D., Roessner, A., Schraven, B., Simeoni, L. (2011) The transmembrane adaptor protein SIT inhibits TCR-mediated signaling. *PLoS One* **6,** e23761.
10. Kolsch, U., Arndt, B., Reinhold, D., Lindquist, J. A., Juling, N., Kliche, S., Pfeffer, K., Bruyns, E., Schraven, B., Simeoni, L. (2006) Normal T-cell development and immune functions in TRIM-deficient mice. *Mol. Cell. Biol.* **26,** 3639–3648.
11. Xu, S., Huo, J., Tan, J. E., Lam, K. P. (2005) Cbp deficiency alters Csk localization in lipid rafts but does not affect T-cell development. *Mol. Cell. Biol.* **25,** 8486–8495.
12. Dobenecker, M.W., Schmedt, C., Okada, M., Tarakhovsky, A. (2005) The ubiquitously expressed Csk adaptor protein Cbp is dispensable for embryogenesis and T-cell development and function. *Mol. Cell. Biol.* **25,** 10533–10542.
13. Gregoire, C., Simova, S., Wang, Y., Sansoni, A., Richelme, S., Schmidt-Giese, A., Simeoni, L., Angelisova, P., Reinhold, D., Schraven, B., Horejsi, V., Malissen, B., Malissen, M. (2007) Deletion of the LIME adaptor protein minimally affects T and B cell development and function. *Eur. J. Immunol.* **37,** 3259–3269.
14. Koelsch, U., Schraven, B., Simeoni, L. (2008) SIT and TRIM determine T cell fate in the thymus. *J. Immunol.* **181,** 5930–5939.

15. Zhu, M., Liu, Y., Koonpaew, S., Granillo, O., Zhang, W. (2004) Positive and negative regulation of FcεRI-mediated signaling by the adaptor protein LAB/NTAL. *J. Exp. Med.* **200,** 991–1000.
16. Volna, P., Lebduska, P., Draberova, L., Simova, S., Heneberg, P., Boubelik, M., Bugajev, V., Malissen, B., Wilson, B.S., Horejsi, V., Malissen, M., Draber, P. (2004) Negative regulation of mast cell signaling and function by the adaptor LAB/NTAL. *J. Exp. Med.* **200,** 1001–1013.
17. Pfrepper, K. I., Marie-Cardine, A., Simeoni, L., Kuramitsu, Y., Leo, A., Spicka, J., Hilgert, I., Scherer, J., Schraven, B. (2001) Structural and functional dissection of the cytoplasmic domain of the transmembrane adaptor protein SIT (SHP2-interacting transmembrane adaptor protein). *Eur. J. Immunol.* **31,** 1825–1836.
18. Zhu, M., Janssen, E., Leung, K., Zhang, W. (2002) Molecular cloning of a novel gene encoding a membrane-associated adaptor protein (LAX) in lymphocyte signaling. *J. Biol. Chem.* **277,** 46151–46158.
19. Togni, M., Swanson, K. D., Reimann, S., Kliche, S., Pearce, A. C., Simeoni, L., Reinhold, D., Wienands, J., Neel, B. G., Schraven, B., Gerber, A. (2005) Regulation of in vitro and in vivo immune functions by the cytosolic adaptor protein SKAP-HOM. *Mol. Cell. Biol.* **25,** 8052–8063.
20. Kantor, A. B., Herzenberg, L. A. (1993) Origin of murine B cell lineages. *Annu. Rev. Immunol.* **11,** 501–538.
21. Tan, E. M., Chan, E. K., Sullivan, K. F., Rubin, R. L. (1988) Antinuclear antibodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. *Clin. Immunol. Immunopathol.* **47,** 121–141.
22. Drappa, J., Kamen, L. A., Chan, E., Georgiev, M., Ashany, D., Marti, F., King, P. D. (2003) Impaired T cell death and lupus-like autoimmunity in T cell-specific adapter protein-deficient mice. *J. Exp. Med.* **198,** 809–821.
23. Yellin, M. J., Thienel, U. (2000) T cells in the pathogenesis of systemic lupus erythematosus: potential roles of CD154-CD40 interactions and costimulatory molecules. *Curr. Rheumatol. Rep.* **2,** 24–31.
24. Grammer, A. C., Slota, R., Fischer, R., Gur, H., Girschick, H., Yarboro, C., Illei, G. G., Lipsky, P. E. (2003) Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions. *J. Clin. Invest.* **112,** 1506–1520.
25. Ma, J., Xu, J., Madaio, M. P., Peng, Q., Zhang, J., Grewal, I. S., Flavell, R. A., Craft, J. (1996) Autoimmune lpr/lpr mice deficient in CD40 ligand: spontaneous Ig class switching with dichotomy of autoantibody responses. *J. Immunol.* **157,** 417–426.
26. Mohan, C., Shi, Y., Laman, J. D., Datta, S. K. (1995) Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. *J. Immunol.* **154,** 1470–1480.
27. Rothstein, T. L. (2002) Cutting edge commentary: two B-1 or not to be one. *J. Immunol.* **168,** 4257–4261.
28. Kasaian, M. T., Casali, P. (1993) Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. *Autoimmunity* **15,** 315–329.
29. Hardy, R. R., Hayakawa, K. (2001) B cell development pathways. *Annu. Rev. Immunol.* **19,** 595–621.
30. Smida, M., Posevitz-Fejfar, A., Horejsi, V., Schraven, B., Lindquist, J. A. (2007) A novel negative regulatory function of the phosphoprotein associated with glycosphingolipid-enriched microdomains: blocking Ras activation. *Blood* **110,** 596–615.

KEY WORDS:
molecules · T cells · B-1 cells · autoimmunity · cell activation · transgenic/knockout mice