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**Vav proteins are required for B lymphocyte responses to LPS.**

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Abstract

B Lymphocytes respond to bacterial lipopolysaccharide (LPS) through TLR-4 and CD180 (previously called RP105). We show here that the responses of B lymphocytes to LPS require the function of the Vav family of guanine nucleotide exchange factors. Vav1 mutant mice generate defective humoral IgG responses following administration of low doses of LPS but respond normally to higher doses, while mice lacking both Vav1 and Vav2 manifest defective responses even after a high dose LPS. Vav1/2 mutant B cells fail to divide extensively in vitro in response to LPS or CD180, while deficiency of Vav1 alone impairs CD180 but not LPS driven proliferation. Likewise, activation of Akt and phosphorylation of IκBα in response to CD180 or LPS required Vav1 and Vav2, while Vav1 deficiency led to defective responses to CD180. In addition, activation of ERK required Vav1 and Vav2 in response to CD180 but was Vav1 and vav2 independent in response to LPS. Induction of CD86 and CD25 by anti-CD180 also required Vav function, as did the induction of the anti-apoptotic protein Bcl-XL. These data provide evidence for the function for the Vav proteins in regulating the responses of B cells to LPS.
Introduction

Bacterial lipopolysaccharide (LPS) is a potent B cell mitogen both in vivo and in vitro. Activation of B cells by LPS triggers immunoglobulin (Ig) secretion and Ig class switching. LPS also promotes the ability of B cells to function as antigen presenting cells by increasing expression of MHC-II and co-stimulatory molecules such as CD86. Collectively these responses of B cells contribute to the neutralisation of gram negative bacteria.

B cells express two receptors that function as recognition and signal transducing receptors for LPS. Toll-like receptor (TLR) 4 is a leucine-rich repeat protein with a large cytoplasmic domain which contains a signal transducing Toll/IL-1 receptor (TIR) homology domain. CD180, previously called RP105, similarly contains a leucine-rich extracellular domain but bears an 11 amino acid cytoplasmic domain with no homology to TLR4 or other known proteins. The extracellular domains of TLR4 and CD180 associate with small molecules called MD2 and MD1 respectively. These molecules contribute to recognition of LPS as well as the surface expression and intracellular distribution of TLR4 and CD180. Although B cells express relatively little TLR4 when compared to macrophages, analysis of mutant mice demonstrate TLR4 is essential for B cell responses to LPS. Engineered mutations of CD180 and MD1 have revealed roles for each of these in LPS-mediated activation of B cells and in LPS induced antibody responses. While mutation of TLR4 yields a more severe defect in humoral LPS responses than that of CD180 or
MD1, the genetic experiments indicate a non-redundant requirement for both classes of LPS receptor for the optimal responses of B cells.

TLR4 is ubiquitously expressed and the signal transduction pathways it activates have been well studied. The cytoplasmic domain of TLR4 recruits adapter proteins MyD88, TIRAP (TIR-containing adaptor protein) and TRIF (TIR containing adaptor protein inducing interferon β) which in turn lead to the activation of ERK (extracellular receptor activated kinase), JNK (c-JUN n-terminal kinase) and p38 MAP kinases (mitogen activated kinases) and subsequently to the activation of transcription factors including NF-κB (nuclear factor κB) and IRF3 (interferon response factor 3) (reviewed by Sabroe et. al.11). By contrast, expression of CD180 is restricted to mature B lymphocytes. However the mechanisms by which CD180 signals are less well studied. CD180 crosslinking by antibody leads to activation of ERK, JNK and p3812,13. B cells deficient in MyD88 proliferate normally following CD180 crosslinking14 while B cells deficient in PKCβ or from xid mice lacking normal BTK (Bruton’s tyrosine kinase) function, respond poorly to CD180 crosslinking 13. Recently it has been reported that the B cell co-receptor CD19 is required for optimal proliferative responses to CD180 ligation14. Furthermore it was suggested that Vav1 may participate in CD180 signalling by virtue of its association with CD1914.

The Vav family of guanine nucleotide exchange factors (GEFs) consists of three members, each of which is phosphorylated in response to BCR (B cell receptor) crosslinking15. There is genetic evidence that all three Vav proteins contribute to signalling by the BCR16. However, there is significant redundancy between the family
members. We and others have shown that B cells from mice deficient in Vav proteins are defective in both thymus dependent and Type II-thymus independent responses\textsuperscript{16-18}. However the responses of Vav-deficient mice to the type I-thymus independent antigen LPS has not been investigated.

In this study we show Vav proteins are required for normal class-switched antibody production following immunisation with a hapten-LPS conjugate. In the absence of normal Vav function LPS and CD180 stimulated signal transduction, gene expression, cell survival and proliferation are defective. The results indicate that Vav protein function is necessary for the response of B cells to LPS.
Study Design

*Mice and immunisation.* Vav1−/−, Vav2−/− and Vav1/2−/− mice have been described previously 17. For this study, all mice were backcrossed to the B10.BR background for five generations as CD180 responses are defective in mice on the 129/Sv background19. Naïve mice were injected i.v. via the tail vein with 20 µg or 3 µg DNP(0.3)-LPS (Biosearch Technologies) in 200 µl PBS, respectively. Sera were collected on days 0, 7 and 14 for analysis for DNP-specific antibodies by ELISA as previously described 17.

*B cell purification and antibody secretion.* B cells were purified from spleens by negative selection to purities between 87% and 97% using previously described methods17. 1.5 x 10⁵ purified B cells/well were cultured with 20 µg/ml LPS (strain 0111:B4, Sigma-Aldrich, Poole, UK) in the presence of 100 U/ml IL-4 (R&D Systems, Oxford, UK) and 1.2 ng/ml IL-5 (Sigma) or without LPS for 5-6 days in 96 well round bottom plates. Supernatants were diluted 1:10 for IgG1 and IgG3 and 1:1000 for IgM and analysed by ELISA using purified rat anti-mouse IgG1, IgG3 and IgM for capture and 2 µg/ml biotinylated Rat anti-mouse IgG1, IgG3 and IgM (Becton Dickenson, Oxford, UK) for detection.

*Cell cycle, survival and proliferation assay.* For cell cycle analysis 2 x 10⁵ purified B cells/well were stimulated either with 5 µg/ml anti-CD180 (Becton Dickenson), 1 µg/ml LPS or cultured without stimulus for 24 and 48 h in 96 well round bottom plates. Cells were harvested, washed and permeabilized in 1 ml ice cold 70% EtOH in PBS by vortexing for 30 sec and incubated on ice for 40 min. Cells were washed in PBS and 100 µg/ml RNaseA were added in 50 µl, incubated at RT for 10 min and
then stained in 50 µg/ml PI in PBS. Samples were analyzed by flow cytometry for linear PI fluorescence intensity gating on single cells. For analysis of proliferation 1 x 10^7 purified B cells/ml were stained in 1µM CFDA-SE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; Vybrant CFDA-SE cell tracer kit, Molecular Probes) in PBS at RT for 10 min. Cells were washed in RPMI containing 10% FCS, seeded into 96 well round bottom plates at 2 x 10^5 cells/well and incubated at 37°C for 30 min. LPS or anti-CD180 were added to a final concentration of 1µg/ml and 5 µg/ml, respectively and cells were cultured for 48h and 72 h. Cells were harvested and stained with 10 nM TO-PRO-3 immediately before acquisition to allow for the identification of dead cells.

**Flow cytometry of splenocytes.** For detection of surface activation markers cultured purified B cells were stained with anti-CD25-PE or anti-CD86-Biotin. Biotinylated antibodies were detected using streptavidin-QuantumRed™ (Sigma). For intracellular Bcl-xL cultured B cells were fixed with Fix/Perm solution (Becton Dickenson) and permeabilized in 0.03% Saponin in PBS/0.5% BSA on ice. They were then stained with anti-Bcl-xL-PE or isotype control (Southern Biotechnology) and analysed using FACS Calibur flow cytometer with Cellquest software for acquisition and Flojo software (Tree Star) for analysis.

**Cell sorting and real time PCR.** Splenocytes were stained with anti-CD23 and anti-CD21 antibodies. Follicular and marginal zone B cells were identified as CD21+ and CD23+ or CD21high and CD23low, respectively and sorted on an Aria cell sorter (Becton Dickinson). Re-analysis of sorted cells revealed >97% purity. For gene expression analysis RNA was extracted using TRIzol reagent (Invitrogen, Paisley,
and cDNA was prepared using high capacity cDNA Archive kit (Applied Biosystems, Oxford UK) according to manufacturer’s instructions. Real time PCR reactions used Taq-Man Universal Master Mix (Applied Biosystems) and an ABI Prism 7700 Sequence Detection System. TLR4 RNA concentrations were calculated relative to HPRT expression. Primers and FAM labeled probes for TLR4 and HPRT were obtained from Applied Biosystems (assays on demand).

For western blots 10^7 purified B cells/condition were stimulated in RPMI with 10% FCS, cells were spun down, lysed by adding 50 mM TRIS pH 7.4, 80 mM KCl 10 mM EDTA, 1% NP-40, phosphatase and protease inhibitors: 1mM Na_3VO_4, 5 mM NaF, 10 μM leupeptin, 2 μg/ml antipain, 6 μg/ml chymostatin, 1 μM pepstatin A, 1 μg/ml AEBSF) and incubated for 5 min on ice. Detergent insoluble fractions were removed by centrifugation and supernatants were analysed by western blot. For JNK assay, cells were lysed for 20 min on ice in 150 μl kinase assay lysis buffer (25mM HEPES pH 7.6, 0.3 M NaCl, 1.5mM MgCl_2, 0.2 mM EDTA, 0.1% Triton X-100, phosphatase and protease inhibitors), diluted with 450 μl ice cold dilution buffer (25 mM HEPES pH 7.6, 2.5 mM MgCl_2, 0.05 mM EDTA, 0.025% Triton X-100, phosphatase and protease inhibitors) per sample and incubated with 15 μl of GST-Jun(aa1-91) conjugated to glutathione beads for 1 h. Beads were washed with ice cold wash buffer (20 mM HEPES pH 76, 50mM NaCl, 2.5 mM MgCl_2 0.1 mM EDTA, 0.05% Triton X-100, phosphatase and protease inhibitors) and incubated for 30 min at 30°C in kinase reaction buffer (20 mM HEPES pH 7.6, 20 mM MgCl_2, 2 μM DTT, 20 μM β-glycerophosphate, phosphatase inhibitors, 25 μM ATP), beads were spun down and analysed for phosphorylated c-Jun by western blot. Antibodies used were phospho-
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serine-473 Akt, phospho-IκBα, phospho-Jun and phospho-ERK (Rabbit, Cell Signaling Technologies, Hitchin, UK), IκBα and ERK (Rabbit, Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase conjugated Goat anti-Rabbit (Dako, Ely, UK).
Results and Discussion

*LPS stimulated IgG production in mice requires Vav function.*

To further investigate the involvement of Vav proteins in B cell responses we analyzed the thymus-independent type I (TI-I) antibody responses of mice with single or combined mutations in Vav1 and Vav2. To this end, groups of wild-type (WT), Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav1/2<sup>-/-</sup> mice were injected with two different doses of DNP-LPS and sera were analyzed for DNP-specific antibodies of the IgM, IgG2a, IgG2b and IgG3 subclasses. At the high dose of DNP-LPS Vav1/2<sup>-/-</sup> mice produced levels of antigen-specific IgM which were similar to WT at day 7 but significantly higher than WT on day 14 (Figure 1A).
By contrast, there were significantly lower levels of antigen specific IgG2a, IgG2b and IgG3 in sera collected on days 7 and 14 from Vav1/2−/− mice (Figure 1A). When injected with a lower dose of DNP-LPS Vav1/2−/− mice produced normal levels of
antigen-specific IgM and reduced levels of antigen specific IgG2a, IgG2b and IgG3 (Figure 1B). Additionally, Vav1−/− mice, which responded in a similar manner to WT mice when administered the high dose of DNP-LPS, produced normal levels of DNP-specific IgM but significantly decreased levels of antigen-specific IgG2b and IgG3 after challenge with the low dose of antigen (Figure 1B). Antigen specific antibody levels in immunized Vav2−/− mice resembled those of WT mice at both doses of DNP-LPS. These experiments demonstrate a clear defect in the class switched TI-I response of mice lacking Vav1 or both Vav1 and 2. This defect is manifested in Vav1/2−/− mice at both high and low doses of antigen and in Vav1−/− mice at low doses of antigen. To investigate if this defect was due to decreased responsiveness of Vav-deficient B cells to LPS, we purified splenic B cells from the four genotypes and tested their antibody secretion in response to LPS in vitro. Purified splenic B cells (purities of > 90%) were incubated in the presence of LPS and culture supernatants analyzed for IgM, IgG1 and IgG3. B cells lacking Vav1 or Vav2 produced lower levels of IgM than WT and this was further reduced in B cells lacking both Vav1 and Vav2 (Figure 1C). The reduced IgM secretion following addition of LPS to the in vitro cultures of B cells contrasts with our in vivo data that showed antigen-specific IgM production was unimpaired in Vav1−/−, Vav2−/− and Vav1/2−/− mice. This may reflect the involvement of other cell types, absent from the in vitro assay, for the normal IgM responses of B cells lacking Vav proteins. Following in vitro LPS stimulation Vav1−/− and Vav1/2−/− B cells generated reduced levels of IgG1 and IgG3 while Vav2−/− B cells yielded IgG levels similar to WT B cells (Figure 1C). Collectively, these results demonstrate that Vav proteins are necessary for optimal production of class switched antibodies in response to LPS. The data suggest that
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there may be some redundancy between Vav1 and Vav2 when LPS concentrations are high but that Vav1 is required when LPS concentrations are low.

Proliferation in response to LPS and anti-CD180 requires Vav function.

We next tested the ability of purified B cells to progress through the cell cycle and to proliferate in response to LPS and CD180 crosslinking. In response to LPS WT, Vav1<sup>+/+</sup>, Vav2<sup>+/+</sup> and Vav1/2<sup>+/+</sup> B cells all progressed to S/G2 (Figure 2A). In response to anti-CD180 there was reduced DNA synthesis in Vav1<sup>+/+</sup> B cells. However, there was virtually no detectable DNA replication in Vav1/2<sup>+/+</sup> B cells (Figure 2A). By staining cells with CFDA-SE measured the proportion of cells that divided and how many divisions individual cells underwent. In response to CD180 stimulation, proliferation of Vav1<sup>+/+</sup> B cells was reduced and there was virtually no proliferation of Vav1/2<sup>+/+</sup> B cells. Only a very small proportion of WT, Vav1<sup>+/+</sup> and Vav2<sup>+/+</sup> B cells failed to proliferate in response to LPS (Figure 2A). By contrast, a large proportion of Vav1/2<sup>+/+</sup> B cells treated with LPS did not divide at all. However, a minority population of Vav1/2<sup>+/+</sup> B cells underwent a similar number of cell divisions as did the majority of WT B cells. These results are consistent with the reduced thymidine incorporation in Vav1/2<sup>+/+</sup> B cells in response to LPS (18 and E.V. unpublished data). Vav1/2<sup>+/+</sup> B cells survived less well when compared to WT after culture in media alone or in media with LPS (Figure 2B&C). However, the failure of Vav1/2<sup>+/+</sup> B cells to proliferate was not only due to cell death as LPS still rescued a large fraction of Vav1/2<sup>+/+</sup> B cells from apoptosis (Figure 2C). Even though Vav1/2<sup>+/+</sup> B cells are able to progress through the cell cycle in response to LPS they do not divide as efficiently. This might be the basis for the reduced antibody secretion since proliferation has been implicated as a prerequisite of switched antibody production20. Our results indicate
that Vav proteins are required for both the proliferative and survival responses of B cells following exposure to LPS.
To confirm that the failure to respond to anti-CD180 or LPS treatment was not due to reduced CD180 or TLR4 expression we analyzed CD180 levels by flow cytometry and TLR4 levels by real-time PCR. Marginal zone (MZ) and follicular (FO) B cells can be distinguished through differential expression of CD21 and CD23 and the spleens of WT and Vav1/2−/− mice contain similar percentages of these two subsets (Figure 3A). The expression of CD180 is about 4.5 times higher on MZ when compared to FO B cells of WT or Vav1/2−/− mice but CD180 expression does not differ between WT and Vav1/2−/− B cells (Figure 3A). To examine the expression of TLR4 we used real-time PCR (Figure 3C). TLR4 mRNA was expressed equivalently between FO and MZ B cell subsets from WT and Vav1/2−/− mice. We conclude that the defects Vav1/2−/− B cells display in response to CD180 or LPS are unlikely to result from reduced CD180 or TLR4 expression. We further conclude that the failure of Vav1/2−/− mice to respond to LPS is not a consequence of the absence of MZ B cells which have been shown to be more responsive to LPS than FO B cells. Moreover, the elevated expression of CD180 on MZ B cells may contribute to the heightened LPS responsiveness of this population.
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CD180 and LPS signal transduction requires Vav proteins.

We next sought to determine the signalling pathways that Vav proteins were mediating for LPS and anti-CD180. CD180 stimulation induced activation of ERK and JNK that was defective in Vav1/2−/− B cells (Figure 4A). By contrast, ERK activation by LPS was readily apparent in B cells lacking both Vav1 and Vav2 (Figure 4B). JNK activation was not consistently observed in WT B cells activated by LPS (data not shown) thus we have not been able to establish a requirement for Vav proteins in LPS mediated JNK activation. As Vav proteins have been shown to regulate PI3K activation in response to diverse stimuli in several cell types15 we used Akt phosphorylation on serine 473 as a surrogate for PI3K activation. The phosphorylation of Akt in response to CD180 stimulation was much reduced in the absence of Vav1 or Vav1/2 (Figure 4C). Akt phosphorylation in response to LPS was unaffected by the lack of Vav1 but was reduced in Vav1/2−/− B cells (Figure 4D). These data indicate Vav proteins contribute to PI3-K activation by LPS in B cells. As Vav proteins have previously been shown to be dispensable for PI3-K activation following surface immunoglobulin crosslinking22 the data presented here further indicate that the requirement for Vav proteins in PI3-K activation is stimulus specific. Activation of NF-κB by LPS and CD180 is necessary for the B cell response to these mitogens23,24. We measured phosphorylation of IκBα as a marker for the induction of this class of transcription factor. Following CD180 stimulation phosphorylation of IκBα was defective in the absence of Vav1 alone or both Vav1 and Vav2 (Figure 4C). By contrast, LPS stimulated IκBα phosphorylation was unaffected by the lack of Vav1 (Figure 4D). Instead, IκBα phosphorylation induced by LPS was reduced in B
cells lacking Vav1 and Vav2 (Figure 4D). PMA stimulated IκBα phosphorylation was normal in B cells of all genotypes suggesting the IKK complex was functional (Figure 4C). Taken together these results suggest the signalling pathways activated by LPS and CD180 require Vav function. CD180 requires Vav1 while LPS activation of some signalling pathways in B cells can bypass the loss of Vav1 presumably by utilizing Vav2.
Figure 4

Vav function is required for activation of gene expression. Increased expression of CD25 and CD86 requires NF-κB activation and is a hallmark of B cell stimulation.
enabling functional interaction with other cells or cytokines. We therefore measured CD25 and CD86 expression on B cells from WT and Vav1/2°/° mice after stimulation with anti-CD180. Unlike WT B cells, Vav1/2°/° B cells failed to significantly increase expression of CD25 or CD86 (Figure 5A) indicating a role for Vav proteins in this process.
We also analyzed Bcl-xL expression following anti-CD180 or LPS stimulation of B cells as Bcl-xL is an NFκB responsive anti-apoptotic protein. WT B cells showed increased Bcl-xL expression in response to anti-CD180 or LPS, however there was no increase of Bcl-xL expression in Vav1/2−/− B cells in response to anti-CD180 (Figure 5B). Furthermore the induction of Bcl-xL was significantly decreased in response to LPS. The failure of Vav1/2−/− B cells to express normal levels of Bcl-xL following LPS stimulation could be one of the factors contributing to the reduced survival and proliferation.

The results presented here demonstrate that the Vav proteins are required for B cells to respond efficiently to LPS. The data support the hypothesis that Vav proteins are involved in signaling pathways downstream of CD180 and TLR4 in B cells. CD180 signaling, which is B cell specific, was very sensitive to loss of Vav1. As the CD180 pathway additionally involves CD19 and Btk, it might provide B cells with a mechanism for integrating LPS responses with signals from the antigen receptor and CD19 complex.
References

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Legends

Figure 1. Defective TI-1 responses. 8-wk-old WT (open columns), Vav1-/- (splitted columns), Vav2-/- (hatched columns) and Vav1/2-/- (black columns) mice were injected intravenously with 20 µg (A) or 3 µg (B) of DNP-LPS. Sera were collected at the times shown and analysed by ELISA for DNP specific antibodies of the isotypes indicated. * P<0.05, ** P<0.005 by ANOVA; n = 5. Bar graphs represent the mean of the OD values for 5 mice per group and error bars correspond to the standard error of the mean. (C) Purified B cells of WT and Vav deficient mice
(genotypes as described above) were cultured for 5 days in presence of 20 µg/ml LPS and secreted antibodies were analyzed by ELISA. Error bars indicate Standard Deviation of six wells; * P<0.05, ** P<0.005 by ANOVA.

**Figure 2. Requirement for Vav proteins in cell cycle progression.** (A) B cells of the indicated genotypes were stained with CFDA-SE and cultured in the presence of 1 µg/ml of LPS (left panels) or 5 µg/ml anti-CD180 (right panels). The first and third panels show analyses of cells gated for lymphocytes by forward and side scatter of CFDA-SE fluorescence after 72 h of culture, numbers of divisions are indicated. The second and fourth panels show cell cycle analyses of PI staining after 48 h of culture, percentage of cells in G1 and G2/S/M phases are given. (B) B cells from WT or Vav1/2-/- mice were loaded with CFDA-SE and cultured with or without 1 µg/ml of LPS. After 48 hours, cells were harvested, stained with ToPRO3 and analyzed by FACS. Results from a representative mouse of each genotype are displayed. The boxes indicate live cells identified as To-PRO3 negatives. (C) The graph shows the percentage of live cells from three WT (open circles) or three Vav1/2-/- mice (closed circles) after culture with or without LPS for 48 hours. Statistical significance was determined using the Student’s t Test.

**Figure 3. Expression of CD180 and TLR-4 in WT and Vav1/2-/- mice.** (A) 5% probability Contour Plots showing CD21 and CD23 surface staining of B220+ gated splenocytes from WT and Vav1/2-/- mice. MZ B cells fall within the CD21High CD23Low gate and the percentage of MZ and FO B cells are indicated. (B) Expression of CD180 on FO and MZ B cells from WT and Vav1/2-/- mice using the gating from (A). (C) Marginal zone and follicular B cells from WT or Vav1/2-/- mice were
FACS sorted according to CD21 and CD23 levels as described in (A). Expression of TLR-4 was measured by real time PCR on cDNA from the indicated populations, open bars correspond to WT mice and closed bars to Vav1/2-/- mice. No significant differences were observed between the groups.

**Figure 4.** Vav deficient mice show defects in MAP kinase, Akt and IκB activation in response to LPS or anti CD180 stimulation. B cells were stimulated with (A) Anti-CD180 (5 µg/ml for 15 mins) then analysed for activation of JNK and ERK. Upper panel: JNK activity assessed by measuring c-Jun phosphorylation. Middle panel: ERK activity assessed by blotting with anti-phospho ERK. Lower panel: total ERK levels assessed by blotting with anti-ERK 1 and 2 antibodies. (B) LPS (20µg/ml for 20 minutes) then analysed for activation of ERK activation using anti phospho ERK. In this experiment anti ERK-2 was used as the loading control. (C) 5µg/ml anti-CD180 or 50ng/ml PMA for the indicated times or (D) 10µg/ml LPS for 20 minutes. Akt and IκBα phosphorylation were assessed by western blot using phosphospecific antibodies. Subsequently, blots were stripped and re-probed with AKT or IκBα antibodies, respectively, to confirm equivalent protein loading.

**Figure 5.** Vav deficient B cells show defects in expression of activation markers following anti-CD180 and LPS stimulation. Purified splenic B cells from WT or Vav1/2-/- mice were cultured for 26 h and analyzed for the expression of (A) activation markers CD86 and CD25 or (B) intracellular levels of Bcl-xL. Open columns: media-only cultured cells, closed columns: CD180 stimulated cells, grey columns: LPS stimulated cells. Error bars represent Standard Deviation, (n=3). Difference in increase: * < 0.05; ** < 0.01; *** < 0.001 by Student’s t test.
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