Vav1 regulates MHCII expression in murine resting and activated B cells

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Abstract

Vav1 is a guanine nucleotide exchange factor (GEF) for Rho GTPases, which is exclusively expressed in cells of the hematopoietic system. In addition to its well-documented GEF activity, it was suggested to have other functions due to the presence of multiple domains and nuclear localization signals in its protein structure. Although GEF-dependent and GEF-independent functions of vav have been implicated in T-cell development and T-cell receptor signaling, the role of vav1 in antigen-presenting cells is poorly understood. We found that vav1 is an important regulator of MHCII expression and transport. Microarray analysis of unstimulated bone marrow-derived macrophages revealed a novel role of vav1 in transcriptional regulation of the MHCII locus, possibly by indirect means. Primary immune cells from vav1-deficient mice had a significantly lower constitutive surface expression of MHCII with the strongest impact observed on splenic and peritoneal B cells. Impaired MHCII expression resulted in a diminished capacity for T-cell activation. Using 6-thio-GTP, a specific inhibitor of the GEF function of vav1, we were able to show that the GEF activity is required for MHCII upregulation in B cells after stimulation with LPS. Furthermore, our data show that vav1 not only affects transcription of the MHCII locus but also is an important regulator of MHCII protein transport to the cell surface.

Keywords: antigen presenting cells, guanine nucleotide exchange factor, microarray analysis

Introduction

Rho GTPases are important regulators of cell behavior, linking extracellular stimuli to intracellular signal transduction events. Their activity is controlled by guanine nucleotide exchange factors (GEF) that regulate the exchange from GDP (inactive Rho GTPase) to GTP (active Rho GTPase) (1). One of the best characterized groups of GEF is the vav family that consists of three members. Although vav2 and vav3 are ubiquitously expressed (2, 3), the occurrence of vav1 is restricted to cells of the hematopoietic system (4). The GEF activity of vav proteins is regulated rapidly and transiently in response to various stimuli by direct tyrosine phosphorylation in the DBL homology (DH) domain, which harbors the Rho-GEF activity. Strong genetic and biochemical evidence places vav1 downstream of several immune receptors [e.g. T-cell receptor (TCR), B-cell receptor (BCR) and Fc receptors] whose stimulation leads to intracellular tyrosine phosphorylation (5).

The role of vav1 is well documented during T-cell development and in TCR signaling. Vav1-deficient mice show defects in positive and negative selection in the thymus and therefore have a strongly reduced pool of peripheral CD4+ and CD8+ T cells (6). Vav1 is also required for TCR-induced proliferation and activation, e.g. by regulating IL-2 gene transcription and Ca2+-flux [reviewed in reference (7)]. Vav1 appears to be less important for B-cell functions, which is likely due to a partial redundancy of the three vav family members. Only the generation of the B1-cell population is strongly diminished in vav1−/− mice. Apart from that, conventional B cells proliferate normally after stimulation with LPS or IL-4/anti-CD40, display normal Ca2+-mobilization after BCR activation and show only slight defects in antibody secretion and class switching (8, 9).

In dendritic cells (DC) and macrophages, the clear function of vav1 is poorly understood because most studies examined these cells in vav1/2/3 triple knockout mice (10–13). Bone marrow-derived dendritic cells (BM-DC) generated from vav1−/− mice show normal LPS-induced maturation in vitro and increased rates of migration in vivo (14). Furthermore,
it was proposed by de la Fuente et al. (15) that vav1 might play a role in MHCII clustering on DC. In contrast with the increased rates of migration of BM-DC, BM macrophages from vav1−/− mice show a reduced migration speed (16). Vav1 is also important for the activation of macrophages, as the stimulation of the murine macrophage cell line RAW 264.7 with LPS, rIFNγ or CpG-DNA triggers tyrosine phosphorylation of vav1 (17). Ectopic expression of mutant forms of vav1 lacking GEF activity resulted in an inhibition of tumor necrosis factor-α and inducible nitric oxide synthase production (18).

Vav1 consists of multiple domains that regulate the GEF activity and enable the binding of vav1 to other proteins. Originally, all functions of vav1 were thought to be dependent on its GEF activity, but this notion has been revised in recent years. A prominent example of this changed view is the role of vav1 for TCR signaling. Important downstream events, such as Ca2+-mobilization, have been shown to be vav-dependent but do not require the GEF function (19, 20). GEF-independent functions of vav1 have not been described in DC and macrophages so far.

A potential role of vav1 in the regulation of transcriptional activation was also discussed (21, 22), e.g. in regulation of IL-2 transcription through the control of Nuclear factor activated T cells (NFAT) activity (23). This type of regulation is mediated by GEF-dependent and GEF-independent functions of vav (22). Additionally, vav1 contains two putative nuclear localization sequences and a C-terminal SH3 domain that has been described to interact with nuclear proteins (24), suggesting a direct or indirect role of vav1 in transcriptional regulation. Indeed, vav1 was reported to be present in the nuclei of immature DC among others (25).

In this study, we describe a new important role of vav1 in various types of antigen-presenting cells (APC), namely B cells, DC and macrophages. Here, we show that vav1 regulates MHCII expression in primary resting APC with the strongest impact on B cells. By using the vav1-GEF inhibitor 6-thio-GTP, we were able to show that the GEF function of the protein is required for upregulation of MHCII expression in B cells following their activation by LPS. Furthermore, we found that vav1 operates on two different functional levels. On one hand, it regulates transcription of histocompatibility genes and on the other hand it mediates the transport of MHCII from the cytoplasm to the cell surface.

Methods

Mice

Balg/c mice were obtained from Charles River (Sulzfeld, Germany). Male and female C57BL/6 wild-type (WT) and vav1−/− mice (23) were bred under specific pathogen-free conditions at the central animal facility of the University of Bonn and were used at 6–12 weeks of age. Mice were sacrificed by cervical dislocation for removal of organs and cells. Animal care and experiments were done in compliance with institutional guidelines and the German law for Welfare of Laboratory Animals.

Preparation and cultivation of primary cells

Spleens were homogenized in PBS and peritoneal cells were harvested by peritoneal lavage with PBS. Both cell preparations were treated with a hypotonic solution (160 mM ammonium chloride in 17 mM Tris) for erythrocyte lysis. For PCR and co-cultivation experiments, B cells from spleen were sorted with CD19 beads (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purity was verified by FACS and was always >92%. For cell culture, splenocytes were incubated in Iscove’s Modified Dulbecco’s Medium (IMDM) (PAA Laboratories, Colbe, Germany) supplemented with 10% FCS (Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamine (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin (PAA Laboratories) and 10 ng/ml streptomycin (PAA Laboratories). Cells were cultured with 1 µg/ml LPS (Sigma-Aldrich) or 400 ng/ml IL-4 (Immunotools, Friesoythe, Germany) together with 2.5 µg/ml anti-CD40 (clone 3/23; BD Pharmingen, Heidelberg, Germany). For inhibitor experiments, cells were pre-treated 1 h before stimulation with 10 µM GTP (Sigma-Aldrich) and 6-thio-GTP (Jena Bioscience, Jena, Germany), respectively.

Generation of BM macrophages

BM macrophages were prepared from the BM of C57BL/6 WT and vav1−/− mice. Cells were filtered through 40 µm pore nylon cell strainers (BD Biosciences, Heidelberg, Germany) and plated in 6 cm petri dishes at 3 × 106 cells in IMDM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 ng/ml macrophage colony-stimulating factor (Peprotech, Hamburg, Germany). The culture medium was half-renewed after 3 days. At day 6, cells were stimulated by adding 200 ng/ml LPS. Efficiency of differentiation was always controlled by counterstaining with anti-CD14.

Surface and intracellular flow cytometry

The following anti-mouse antibodies were obtained from ebioscience (San Diego, CA, USA): anti-B220 (clone RA3-6B2), anti-CD11c (clone N418), anti-CD14 (clone Sa2-8), anti-CD16/32 (clone 93), anti-CD19 (clone eBio1D3) and anti-CD86 (clone H-2D[b] clone 28-8-6) and anti-CD86 (clone 2G9) were purchased from BD Pharmingen. Anti-CD4 (clone GK1.5), anti-CD69 (clone H.12 F3) and anti-I-A/I-E (clone M5/114.15.2) were obtained from BioLegend (San Diego, CA, USA).

Fc receptors on cells were blocked by preincubation with unconjugated anti-CD16/32 before staining. All surface stainings were performed for 20 min at 4°C. For intracellular staining, cells were fixed overnight with 2% PFA at 4°C. Cells were washed and subsequently incubated in permeabilization buffer (PBS with 0.5% BSA, 0.1% saponin and 0.1% sodium azide) for 10 min at 4°C. Staining was performed in permeabilization buffer for 30 min at 4°C. Cells were measured with a FACSCanto II flow cytometer (Becton Dickinson) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

MHCII internalization and re-emergence

To evaluate internalization rates of MHCII, splenocytes from WT and vav1−/− mice were incubated in the presence of 5 µg/ml Brefeldin A (Sigma-Aldrich) for 1–6 h. The transport of MHCII from the cytoplasm to the cell surface was analyzed by re-emergence. For this purpose, MHCII on the cell surface of
splenocytes was blocked by incubation with an unconjugated anti-I-A/II-E (clone M5/114.15.2; BioLegend) for 20 min at 4°C. After removal of the unconjugated antibody, cells were incubated for the indicated time points. Reactions were stopped by adding NaN3 (final concentration 1%) and immediate storage on ice. Cells were stained with a fluorochrome-conjugated anti-I-A/II-E (clone M5/114.15.2; BioLegend) and anti-CD19 and analyzed by FACS. Relative internalization was calculated by the mean fluorescent intensity (MFI) of the remaining MHCII in comparison with the MFI of untreated cells that was set to 100%. Re-emergence was determined by increased MFI; cells stored on ice to inhibit transport processes served as controls.

Mixed lymphocyte reaction

T cells from Balb/c mice (haplotype H2-d) were isolated from lymph nodes and spleen and purified with the CD4+ T cell isolation kit II from Miltenyi Biotech. CD4+ T cells (2 × 10^5) were co-cultivated with different numbers of purified B cells isolated from spleen of C57BL/6 WT and vav1–/– mice (haplotype H2-b). T-cell activation was evaluated by IL-2 ELISA (ebioscience) after 48 h and expression of the activation markers CD25 and CD69 was analyzed by FACS after 72 h of co-cultivation.

Microarray analysis

RNA from unstimulated BM macrophages from WT and vav1–/– mice was extracted with TRIzol (Invitrogen) and purified by standard methods. Preparation of cRNA, labeling and hybridization on Mouse WG-6v2.0 arrays (Illumina, San Diego, CA, USA) were done according to the manufacturer’s instructions. For cDNA synthesis, 1 µ of each primer, 1 µl of final volume, containing 10 mM of each primer, 1 µl of cDNA and RNase-free water. Amplification conditions were 40 cycles of 3 s at 95°C for denaturation, 30 s at 58°C for primer annealing, 10 s at 72°C for elongation and 2 s at 72°C for fluorescence detection. Expression levels were calculated proportionately to the expression of the housekeeping gene rps6. Primer sequences—\textit{ciita} forward: acacctgtgacctggtctcc, \textit{ciita} reverse: gtgacaaagggacacagac; \textit{cd74} forward: tccatggtactacctctct, \textit{cd74} reverse: gggagtcttctgtctcta; \textit{h2-α} forward: agacctgtgtgaggtaaga, \textit{h2-α} reverse: gtgacaggtggtaatgta; \textit{h2-β} forward: gtgacaggtggtaatgta; \textit{rps6} forward: attccgtgctacacagac, \textit{rps6} reverse: gcgttcttctgtctgtc. Primers were obtained from MWG Operon (Cologne, Germany).

Western blot analysis

Cell pellets from WT and vav1–/– B cells were re-suspended in Igepal-lysis buffer, containing 10 mM Hepes, pH 7.5, 10 mM KCl, 10 mM MgCl2, 150 mM NaCl, 1% Igepal and protease inhibitors. Protein concentrations were measured by standard BCA assay. Equal amounts of proteins were loaded onto 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking with Odyssey™ blocking buffer (LI-COR Biosciences GmbH, Lincoln, NE, USA), membranes were incubated with primary antibodies for 3–20 h at room temperature (anti-vav1, anti-vav2 and anti-vav3 were obtained from Santa Cruz, Biotechnology, Inc., Santa Cruz, CA, USA; anti-β-actin was purchased from Sigma-Aldrich). The appropriate secondary antibodies were purchased from LI-COR Biosciences GmbH. Signals were detected with the Odyssey infrared imaging system from LI-COR Biosciences GmbH.

Statistical analysis

Student’s t-test was calculated with GraphPad Prism software (GraphPad, San Diego, CA, USA) and used to assess statistical significance of data. Values of \( P < 0.05 \) were considered as significant.

Results

\textit{Vav1 regulates expression of genes associated with MHC biology}

The role of vav1 in the immune system has extensively been investigated in T cells, and increasing evidence suggests a role for vav1 in transcriptional regulation in a GEF-dependent and GEF-independent manner \( (7, 21, 22) \). Nevertheless, there is only very limited knowledge on the role of vav1 in myeloid cells and APC. To obtain comprehensive information on the genes that are differentially expressed in WT versus vav1–/– BM macrophages, we performed a microarray-based mRNA expression analysis. RNA samples from unstimulated BM macrophages derived from WT or vav1–/– mice were analyzed using beadchip arrays. A limited set of differentially expressed genes was identified by this approach \( [57 \text{ genes, fold change (FC)} > 2, P < 0.05] \). Forty-five genes were expressed significantly more strongly in WT BM macrophages, whereas 12 genes showed higher expression in vav1–/– cells. Gene Ontology analysis using the most significantly downregulated annotated genes (\( n = 126 \)) as sample input and the MGI database as background revealed 7 of the 15 most significant GO terms to be directly associated with antigen processing and presentation (\textit{Supplementary Table 1}, available in \textit{International Immunology} Online). This indicated that genes associated with MHC biology were clearly overrepresented. \textit{Table 1} shows the expression of vav1 and differentially regulated genes (FC \( > 2, P < 0.05 \)) that are relevant for MHCII function. The expression of MHCII

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components (h2-α, h2-αβ1 and h2-εβ1) and of the invariant chain (CD74) necessary for MHCII transport were all found to be significantly diminished in vav1–/– macrophages.

We confirmed the microarray results by semi-quantitative PCR of unstimulated and stimulated BM macrophages from WT and vav1–/– mice (Fig. 1A–D). h2-α was significantly reduced in vav1–/– BM macrophages (4.5-fold compared with WT cells, Fig. 1A). The influence of vav1 on h2-αβ1 was much more pronounced because h2-αβ1 was hardly detectable in vav1-deficient cells (Fig. 1B). The differential expression of both histocompatibility genes was maintained after LPS stimulation (Fig. 1A and B). We included the expression profile of ciita, the best-known transcriptional regulator of MHCII (27), which was slightly (1.2-fold) but significantly (P = 0.01) reduced on the array in vav1–/– cells. This marginal change was proved by PCR analysis, where ciita was only weakly

Table 1. Differentially expressed genes related to MHCII in vav1–/– BM macrophages

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</table>

*Gene names listed by alphabetic order. *Every gene has a distinct identification number on the array. *Mean value from three independent preparations of unstimulated BM macrophages from vav1–/– mice. *Mean value from four independent preparations of unstimulated BM macrophages from WT mice.

Fig. 1. Diminished expression of genes related to MHCII in vav1–/– BM macrophages. BM macrophages were generated as described in Methods and were stimulated for 24h with 200 ng/ml LPS or left unstimulated (us). Relative expression levels of genes were measured by real-time PCR from WT (white bars) and vav1–/– (black bars) cells against the housekeeping gene rps6. Given is the mean of at least five independent experiments with SD (*P < 0.05, **P < 0.01, ***P < 0.001).
reduced in vav1−/− cells in comparison with WT cells (1.4-fold; Fig. 1C). Because the differential expression of h2-<i>α</i> and h2-<i>αβ</i>1 was much more pronounced than that of <i>ciita</i>, we conclude that vav1 regulates MHCII transcription independently of alterations in <i>ciita</i> transcription.

**Basal MHCII expression levels depend on vav1 expression in splenic and peritoneal APC**

To validate the observed effects on gene expression levels we compared MHCII protein expression on splenic APC from WT, vav1 heterozygous and vav1 knockout mice by FACS. All three cell types analyzed namely B cells, DC and macrophages showed a diminished MHCII expression (MFI) on vav1−/− cells (Fig. 2A and B). On splenic B cells isolated from vav1−/− mice, MHCII expression was reduced by 50%, and B cells isolated from vav1−/− mice expressed ~25% of MHCII surface levels found on WT cells. In DC and macrophages, the differences in MHCII expression between WT and vav1−/− cells were not significant (reduction to 83 and 79%, respectively), but there was a clear and significant reduction of MHCII surface expression on vav1−/− cells by 50% on both cell types compared with WT cells. Figure 2C displays the percentage of B cells, DC and macrophages in the spleen of WT, vav1 heterozygous and vav1 knockout mice. The high proportion of B cells in spleens from vav1−/− mice results from the strongly reduced T-cell numbers observed in these mice (6).

![Fig. 2. Constitutive MHCII surface expression depends on vav1 expression. (A–C) Splenocytes were isolated from WT, vav1 heterozygous and vav1−/− mice. Cells from at least three mice were analyzed by FACS after staining for B220 (B cells), CD11c (DC), CD14 (macrophages) and MHCII. Representative histograms are shown in (A) (WT, black curves vav1 heterozygous, dotted grey curves vav1−/− filled, grey curves). (B) Shown is the average MFI from at least three WT (white bars), vav1 heterozygous (striped bars) and vav1−/− (black bars) mice with standard deviation (SD) (*<i>P</i> < 0.05, **<i>P</i> < 0.01, ***<i>P</i> < 0.001). (C) The percentage of B cells, DC and macrophages from at least three WT (white bars), vav1 heterozygous (striped bars) and vav1−/− (black bars) mice is shown with SD. (D–F) Cells taken from peritoneal cavity from WT and vav1−/− mice were stained for B220, CD11c, CD14 and MHCII and analyzed by FACS. (D) The shown histograms are representative for at least three independent experiments from WT (black curves) and vav1−/− (filled grey curves) mice. (E) Given is the average MFI from at least three WT (white bars) and vav1−/− (black bars) mice with SD (**<i>P</i> < 0.001). (F) Shows the percentage of B cells, DC and macrophages from at least three WT (white bars) and vav1−/− (black bars) mice with SD. (G) Splenocytes were isolated from WT and vav1−/− mice and stained for CD19, CD11c, CD14, CD3 and MHC. MHC expression levels of each cell type were calculated in comparison with WT cells set to 100% (n = 4).
To confirm these results, we also investigated MHCII expression on APC isolated from peritoneal exudates of WT and vav1–/– mice. Figure 2D shows representative histograms of MHCII expression on B cells, DC and macrophages. The MFI of MHCII on WT B cells was about five times higher than on vav1–/– B cells (average MFI 21 733 versus 4221; P < 0.001). As in splenic DC, MHCII expression was reduced by 50% on vav1–/– peritoneal DC (P < 0.001), whereas there was no significant difference in the MHCII levels of peritoneal macrophages from WT and vav1–/– mice (average mean of three to four individual mice is shown in Fig. 2E). The percentages of the different cell types in the peritoneal cavity were independent of the genotype (Fig. 2F).

Together, these data show that vav1 is an important regulator of basal MHCII expression on primary APC, with the strongest impact observed on B cells.

We also analyzed expression of MHCI on splenic cells and found reduced expression levels on vav1–/– cells in comparison with WT cells as well (Fig. 2G). In comparison with MHCII, the differences between WT and vav1–/– cells with respect to MHCI expression were much weaker. Whereas MHCII expression on vav1–/– cells was reduced by 50% on DC and macrophages and 75% on B cells (Fig. 2B), MHCI levels were only reduced by 10–40%.

Induction of MHCII on activated B cells depends on vav1 expression

Because MHCII expression on B cells was affected most by vav1 deficiency, we analyzed whether the transcription of histocompatibility complex genes was altered in this cell type. We therefore performed semi-quantitative PCR analysis with mRNA from purified splenic B cells and found that, similar to the situation in BM macrophages, expression levels of h2-αα and h2-αβ were significantly reduced in vav1–/– B cells compared with WT B cells (Fig. 3A and B). By performing a western blot analysis of WT or vav1–/– B cells, respectively, we were able to exclude that the absence of vav1 has an influence on the expression of either vav2 or vav3, respectively (Fig. 3C). Whereas vav1 was clearly absent in vav1–/– cells, vav2 and vav3 were equally expressed in splenic B cells independently of the vav1 genotype. Notably, vav3 shows a much weaker expression in splenic B cells compared with vav1 or vav2.

To analyze whether enhanced expression of MHCII during B-cell activation was also regulated by vav1, splenic B cells from WT and vav1–/– mice were stimulated with IL-4/anti-CD40 (Fig. 4A). As opposed to WT B cells (delta MFI = 5423), MHCII expression levels on vav1–/– B cells were only marginally enhanced after stimulation (delta MFI = 2524). Reduced upregulation of MHCII on vav1–/– cells was not due to a general maturation defect because CD86 expression was strongly enhanced both in WT and vav1–/– B cells following IL-4/anti-CD40 stimulation (Fig. 4B). These results confirm that vav1 is important for basal MHCII expression, but also plays a role in MHCII upregulation after stimulation.

Low MHCII expression on vav1–/– B cells constrains their antigen-presentation capacity toward T cells

To evaluate if the reduced MHCII expression on vav1–/– cells is of functional relevance, we performed a mixed lymphocyte reaction and measured the capacity of WT and vav1–/– B cells to activate allogeneic T cells. The activating stimulus in this assay is the foreign histocompatibility antigen. To this end, 2 × 10^5 Balb/c T cells (H2-d) were co-cultivated with different numbers of purified B cells isolated from WT or vav1-deficient C57BL/6 mice (H2-b). After 48 h of co-cultivation, we analyzed the supernatant for IL-2 by ELISA, at 72 h we additionally analyzed the expression of the activation markers CD25 and CD69 on T cells. As shown in Figure 5, vav1–/– B cells had a significantly decreased capacity to stimulate T cells in comparison with WT B cells. Notably, about 5-fold more vav1–/– than WT B cells per T cell were required to stimulate the production of similar IL-2 levels (Fig. 5A), which resembles the difference in MHCII expression observed between WT and vav1–/– B cells (Fig. 2B). Furthermore, at all B-cell/T-cell ratios used in this experiment we found a significantly reduced percentage of activated T cells when co-cultivated with vav1–/– cells as shown by the expression of CD25 and CD69 (Fig. 5B). These results demonstrate that the impaired expression of MHCII is of importance for an efficient T-cell activation.

In B cells, vav-mediated MHCII upregulation after stimulation with LPS is GEF dependent

To elucidate whether the GEF activity of vav1 is required for MHCII expression, we used the vav1-GEF inhibitor 6-thio-GTP that binds to small GTPases, and which specifically inhibits the
GDP/GTP exchange mediated by vav1 (28). Splenocytes from WT and vav1–/– mice were pre-incubated with 10 µM 6-thio-GTP or with GTP as a control. After 1 h, cells were stimulated with LPS (Fig. 6A), IL-4/anti-CD40 (Fig. 6B) or were left unstimulated. Twenty-four hours later, cells were analyzed for MHCII expression levels and upregulation in comparison with unstimulated GTP-treated WT cells, normalized to 100%. Treatment of unstimulated cells with 6-thio-GTP had no influence on MHCII expression compared with control cells treated with GTP either in WT or in vav1–/– B cells (compare open and closed circles in Fig. 6A and B). As depicted in Fig. 6A, WT cells stimulated with LPS showed a strong upregulation of MHCII if pre-treated with 10 µM GTP. In contrast, LPS-stimulated WT cells pre-treated with 10 µM 6-thio-GTP showed only a weak MHCII induction. Compared with LPS-stimulated GTP-treated WT cells, the MHCII induction on 6-thio-GTP-treated cells was significantly lower (P = 0.0064). No differential MHCII induction on LPS-stimulated vav1–/– cells treated with GTP or 6-thio-GTP was detectable. Surprisingly, 6-thio-GTP treatment of IL-4/anti-CD40-stimulated B cells led to a significantly weaker induction of MHCII on WT as well as on vav1–/– B cells (Fig. 6B).

We thus conclude that the requirement of the GEF activity of vav1 for MHCII induction is stimulus specific.

**MHCII transport in B cells depends on vav1 expression**

The microarray and PCR results suggest that MHCII transport regulated by invariant chain (CD74) is affected in vav1–/– cells (See Table 1 and Fig. 1D). To validate this finding, splenocytes from WT and vav1–/– mice were analyzed with regard to their intracellular MHCII storage. To this end, cells were treated with saponin for intracellular staining while separating a fraction that was surface-labeled for comparison. Figure 7A shows representative histograms of surface and intracellular staining from WT and vav1–/– splenocytes. In Fig. 7B, black bars depict the proportion of cells expressing MHCII on their cell surface, whereas white bars indicate the increase of MHCII-positive cells after intracellular staining. Nearly all APC (90.5%) in WT spleen express the entire MHCII pool on the cell surface. Immunostaining of permeabilized cells revealed a minor increase of MHCII-positive cells (from 51.6 to 57.1% of all cells analyzed). However, within the population...
of vav1−/− splenocytes, 57.1% of the cells expressed MHCII on their cell surface and this was strongly enhanced by intracellular staining (82.6%). This means that >30% of all MHCII-bearing cells in the spleen of vav1−/− mice store this molecule intracellularly. The high frequency of MHCII-expressing cells in the spleen of vav1−/− mice in comparison with WT spleen results from the strongly reduced T-cell numbers observed in vav1−/− mice (6) (see also Fig. 2C). Notably, total MHCII expression levels of vav1−/− cells never reach the levels observed in WT cells (Fig. 7C), confirming the PCR data (Fig. 3A and B).

Next, we asked whether the enhanced intracellular pool of MHCII in vav1−/− cells is caused by an enhanced internalization or reduced transport from the cytoplasm to the cell surface of this molecule. To examine internalization, splenocytes were treated with Brefeldin A to inhibit MHCII transport to the outside. Surface MHCII levels were determined after different time points on B cells (Fig. 7D). Because the decreases in MHCII levels were comparable in WT and vav1−/− cells over time, we conclude that internalization of MHCII is independent of vav1 expression. To evaluate transport of MHCII to the surface, we first saturated these molecules by incubation with an unconjugated anti-I-A/I-E antibody and chased re-emerged MHCII molecules by staining with a fluorochrome-conjugated anti-I-A/I-E antibody and compared this with the unstimulated WT cells (Fig. 7E). In WT B cells, the re-emergence on the cell surface occurred significantly faster than in vav1−/− B cells at all tested time points.

Taken together, these results show that, vav1−/− B cells are characterized by an impaired transport of MHCII molecules to the cell surface.

Discussion

Since the discovery of vav1 in 1989 by Katzav et al. (4), its role has mainly been investigated in T-cell development and TCR signaling (7). In contrast, the function of vav1 in APC is only poorly understood. In the present work, we analyzed APC, namely B cells, DC and macrophages from vav1−/− mice and report for the first time that vav1 is required for both basal and inducible MHCII surface expression. We observed the strongest effects of vav1 deficiency on splenic and peritoneal B cells. In the past, substantial efforts have been directed at the generation of mice that are genetically deficient for two or three vav family members. Nevertheless, some examples illustrate that the double vav1/2, vav1/3 and triple vav1/2/3 knockout mice do not necessarily enhance or even display a phenotype obtained with vav1−/− cells (12, 18, 29). MHCII expression on single-knockout vav1−/− B cells in comparison with WT B cells has not been reported yet. One study analyzed MHCII expression on B cells by investigating double-vav1/3-deficient mice (30). In that study, no difference in MHCII upregulation after BCR stimulation was observed and potential differences in basal MHCII expression levels of these cells were not addressed. Here, we describe a significantly reduced basal and inducible MHCII expression on single vav1−/− B cells in comparison with WT B cells. Graham et al. used triple vav1/2/3−/− BM-DC to depict a role for the vav family in recycling of peptide-loaded MHCII in which vav acts as a linker protein of the ITAM adapter molecules DAP12 and FcγR. Although they gave conclusive data for enhanced internalization and degradation of MHCII in vav1/2/3−/− BM-DC, surprisingly no difference in basal MHCII expression was observed (11). Thus, we present here the unambiguous role of vav1 in these processes and found that in vav1−/− B cells the transport of MHCII to the cell surface is severely impaired. Interestingly, we could not determine an enhanced internalization of MHCII as described by Graham et al., and therefore internalization might selectively depend on vav2 or vav3 expression. However, we cannot exclude cell type specific differences between primary splenic B cells and BM-DC. We also found a diminished MHCII expression on splenic cells. This effect was less pronounced compared with MHCII levels. Because MHCII and MHCII have similar pathways for endocytosis and recycling (31), this result was expected and confirms the previous findings.

![Fig. 6](https://academic.oup.com/intimm/article-abstract/25/5/307/667865/figure-6)
We further show that vav1 regulates surface expression of MHCII on two different functional levels. On the one hand, vav1 regulates the transport of MHCII molecules from the cytoplasm to the cell surface. Vav1 is well known for its important role in cytoskeletal re-arrangements through the regulation of Rho GTPases (32) that are also involved in the trafficking of endosomal vesicles (33). It is hence conceivable that vav1 regulates the transport of MHCII from the endoplasmic reticulum.

Fig. 7. MHCII transport from the cytoplasm to the cell surface depends on vav1 expression. Splenocytes were fixed with 2% PFA. Cells were split into two groups and stained either for surface MHCII or intracellular MHCII after permeabilization with saponin. (A) Shown is a representative histogram of splenocytes with the surface MHCII expression (black dotted curve) and intracellular MHCII (grey curve), (dotted grey line, unstained control). In (B), the percentage of MHCII-positive cells after surface staining (black bars) and the additional MHCII-positive cells after intracellular staining (white bars) is shown. Given is the average of three independent splenocyte preparations with SD. Significances refer to the ratio of surface MHCII to total MHCII (\( P < 0.05 \)). In (C), total MHCII levels after intracellular staining are given for WT (white bar) and vav1–/– cells (black bar) (\( n = 3, * p < 0.05 \)). (D) Splenocytes from WT and vav1–/– mice were incubated in the presence of Brefeldin A or left untreated as controls (set to 100%). After the indicated time points, cells were harvested and remaining MHCII on the surface was analyzed on CD19+ B cells by FACS (\( n = 3 \) with SD). (E) After blocking surface MHCII splenocytes from WT and vav1–/– mice were incubated for the indicated time points and stained with an anti-I-A/I-E antibody recognizing the same epitope. Re-emergence of MHCII on the cell surface of B cells was analyzed by FACS (\( n = 3 \) with SD).
through the Golgi apparatus to the cell surface. Furthermore, we found a weaker mRNA expression of invariant chain (CD74) in vav1−/− macrophages. On the other hand, vav1 appears to have a direct or indirect effect on the expression of genes in the histocompatibility gene locus. Whereas the expression of the major transcriptional regulator of MHCII expression ciita (27) is only marginally reduced in vav1−/− macrophages in comparison with WT cells, h2-aβ1 was nearly undetectable in vav1−/− cells. Also, expression levels of h2-α and h2-εβ1 were diminished as shown by PCR and/or microarray analysis. Our data, therefore, suggest that vav1 regulates MHCII expression at the level of both transcription and endocytosis/recycling. We cannot exclude, however, that the intracellular accumulation of MHCII in vav1−/− cells induces a negative feedback mechanism resulting in a reduced transcription of MHCII.

A possible role for vav1 in transcriptional activation has been addressed before (22, 24, 34). Vav1 contains two putative nuclear localization sequences and a C-terminal SH3 domain that regulates its cellular localization (24). In several systems vav1 was detected in the nucleus, e.g. in stimulated mast cells, Jurkat T cells (24) and immature DC (25), which suggests a function for it in the control of transcription. Furthermore, vav associates with nuclear proteins such as Ku70 (35) or the heterogeneous nuclear ribonucleoprotein (36). A convincing argument for a direct regulatory role of vav1 in transcription is given by Brugnoli et al. (37), who described that vav1 and PU.1 are recruited to the CD11b promoter. Moreover, there is a well-described indirect role for vav1 in regulation of transcription, e.g. of IL-2 through the control of NFAT activity (23) that is mediated by GEF-dependent and GEF-independent functions (22). The discrimination between GEF-dependent and GEF-independent functions of vav1 has not been fully resolved and has thus far only been addressed in T cells (19, 20).

This study demonstrates for the first time the importance of vav1 in MHCII expression on B cells and extends previous publications that demonstrated the role of small GTPases and GEF proteins in regulating MHCII expression. Specifically, it was shown that the ablation of the GEF protein SWAP70 (38) and the knockdown of GEFH1 (39) both have suppressive effects on MHCII surface expression after stimulation.

The GEF activity of vav1 strictly depends on tyrosine phosphorylation that takes place after stimulation (5). In this study, we observed significant differences in basal MHCII expression in unstimulated cells. Because the GEF function of vav1 is usually inactive in unstimulated cells, one might speculate that regulation of basal MHCII levels is independent of its GEF activity. Employment of the vav1-GEF-inhibitor 6-thio-GTP, which specifically inhibits the GDP/GTP exchange mediated by vav1 (28), yielded data that support this assumption. Pre-treatment of cells with 6-thio-GTP affected only inducible MHCII expression, but had no effect on basal MHCII levels in unstimulated cells. In contrast to LPS where 6-thio-GTP had no effect on stimulated vav1−/− cells, MHCII induction on IL-4/anti-CD40-stimulated vav1−/− B cells was affected. These data are in line with previous findings describing that, e.g. proliferation of B cells induced by IL4/anti-CD40 or LPS has a differential requirement for vav proteins (8).

As a metabolite of the prodrug azathioprine, which is widely used in organ transplantation, autoimmunity and chronic inflammatory diseases, 6-thio-GTP partially mediates the immunosuppressive effects of this substance (40). Its effects on the immune system pertaining to vav1 functions had only been described in T cells (28, 41, 42). Our findings that a diminished MHCII induction following treatment of APC with 6-thio-GTP expands this knowledge and indicates that inhibition of vav1 might be useful for the treatment of inflammatory diseases.

Supplementary data

Supplementary data are available at International Immunology Online.

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