

Mast cell function is not altered by Coronin-1A deficiency

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ABSTRACT

Coronin-1A is a WD repeat protein family member, highly expressed in all hematopoietic lineages, and acts as a regulator of F-actin dynamics and Ca^{2+} signaling. In *Coro1a*^{Lmb3} mice results in inactivation of the protein and leads to disease resistance in a model of lupus erythematosus. In *Coro1a*^{-/-} and *Coro1a*^{Lmb3} mice, peripheral T cells exhibit impairments in survival, migration, activation, and Ca^{2+} flux. In this study, we show that in vitro-differentiated mast cells from *Coro1a*^{Lmb3} mice are viable, developed normally, and are fully functional in assays of degranulation, cytokine secretion, and chemotactic migration, despite increased F-actin levels. In *Coro1a*^{Lmb3} mast cells, Ca^{2+} flux in response to physiological FcεRI stimulation is unaffected. Finally, *Coro1a*^{Lmb3} mice showed similar in vivo mast cell responses as the WT mice. Coronin-1B and Coronin-1C expression levels were not increased in *Coro1a*^{Lmb3} mast cells but were higher in mast cells than in CD4 T cells or B cells in WT mice. We conclude that Coronin-1A activity is not required for mast cell function. *J. Leukoc. Biol.* 88: 000–000; 2010.

Introduction

Dynamic changes in the actin cytoskeleton regulate many cellular processes, including cell migration, vesicular trafficking, morphogenesis, cell division, and cell survival [1, 2]. The nucleation and subsequent polymerization of branched-chain actin require the activity of the Arp2/3 complex. Members of the Coronin family down-regulate F-actin polymerization through inhibition of the nucleation-promoting Arp2/3 complex [3], displacement of the Arp2/3 from older branch points [1], and activation of the cofilin-actin-interacting pro-

tein 1-mediated F-actin disassembly [4]. The mammalian genome encodes 7 Coronin paralogs (1A, 1B, 1C, 2A, 2B, 6, and 7) [5, 6]. Common structural features include the N-terminal WD40 repeat region that forms a 7-bladed β propeller followed by a variable region that connects to a conserved C-terminal coiled-coil domain, which is absent in Coronin-7 [6, 7].

Coronin-1A is expressed predominantly in hematopoietic cells [5, 6]. In T and B cells, Coronin-1A regulates Ca^{2+} signaling downstream of antigen receptors and is critical for migration and survival of T cells [8–10]. A nonsense mutation in glutamine 262 of Coronin-1A was described recently to lead to lupus resistance (*Coro1a*^{Lmb3}) [8]. The Q262X mutation truncates the β-propeller domain and deletes variable and C-terminal domains, thereby impairing homotrimerization and Arp2/3 binding. *Coro1a*^{Lmb3} T cells, particularly the naïve subset, display elevated levels of F-actin, altered cell surface dynamics, loss of mitochondrial membrane potential, and reduced TCR-mediated Ca^{2+} flux that contributes to profound developmental, migration, and viability defects [8, 9].

Mast cells contribute to protective and pathologic immune responses with a prominent role in allergies and atopic diseases [11]. They express FcεRI, which binds IgE with high affinity to induce mast cell activation [12–14] and secretion of effector molecules, including cytokines, proteases, histamine, PGs, and leukotrienes [15, 16]. However, mast cell activation can be elicited by a variety of stimuli other than FcεRI cross-linking by IgE, such as engagement of complement receptors or TLRs or treatment with endogenous peptides and cytokines [12, 17]. In addition to driving the pathology of allergic diseases, mast cell activation contributes to innate immunity by initiating inflammation and recruiting leukocytes, as well as through secretion of toxins and phagocytosis of pathogens [12–14, 18–22]. In macrophages and neutrophils, Coronin-1A has been proposed to regulate phagocytosis and chemotaxis, at least partially by regulation of Ca^{2+} signaling [23–25]. F-actin levels have been reported to have a negative regulatory role in Ca^{2+} -dependent FcεRI signaling in the basophilic leukemic

Abbreviations: Arp=actin-related protein, Ca^{2+} =calcium, CCK-8=cell counting kit-8, CD62L=CD62 ligand, CFM=calcium-free medium, *Coro1a*^{-/-}=Coronin-1A-KnockOut, *Coro1a*^{Lmb3}=*Lmb3* locus-linked nonsense mutation in Coronin-1A, PCA=passive cutaneous anaphylaxis, PI=propidium iodide, *Ptcd*=peripheral T cell deficiency mutation, qPCR=quantitative PCR, SCF=stem cell factor, TBP=TATA-binding protein

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

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RBL-2H3 cell line [26–28]. However, Coronin-1A function in primary mast cells remains unexplored.

In this study, we detected a high level of Coronin-1A expression in mast cells and set out to determine if mast cell function is altered in the absence of active Coronin-1A. We examined *in vitro* differentiation and Ca^{2+} -dependent degranulation, cytokine secretion, and migration of mast cells derived from the *Coro1a*^{Lmb3} mice [8]. We also examined mast cell function in an *in vivo* assay of PCA. None of the analyzed processes was affected by the lack of Coronin-1A activity. Therefore, we conclude that Coronin-1A function is dispensable for mast cell maturation and activation.

MATERIALS AND METHODS

Animals

C57BL/6J and C57BL/6-*Coro1a*^{Lmb3} mice were bred at The Scripps Research Institute (La Jolla, CA, USA) and housed according to the Scripps Research Institute Institutional Animal Care and Use Guidelines.

Antibodies and reagents

Mouse Coronin-1A-specific antibody was a kind gift from Dr. Andrew Chan (Genentech, Oceanside, CA, USA). Antibody specific for β -actin was from Abcam (Cambridge, MA, USA). Antibody specific for GAPDH was from EMD (Gibbstown, NJ, USA). PE-conjugated anti-Fc ϵ RI antibody, FITC-conjugated anti-CD4 antibody, PerCP-Cy5.5-conjugated anti-CD62L antibody, and unconjugated and biotin-conjugated mouse-specific anti-IL-6 antibodies were from eBioscience (San Diego, CA, USA). Allophycocyanin-conjugated anti-CD44 antibody was from BioLegend (San Diego, CA, USA). Unconjugated and biotin-conjugated antibodies specific for mouse TNF- α were from R&D Systems (Minneapolis, MN, USA). PE-Cy7-conjugated, mouse-specific antibody for c-Kit (CD117), Alexa 488-conjugated Phalloidin, Annexin V-FITC, and TNP-specific IgE C38-2 were from BD Biosciences (San Jose, CA, USA). Mouse-specific anti-CD3 (2C11), anti-CD28 (PV1), and anti-IFN- γ (R4/GA2) antibodies were from Bio Express (West Lebanon, NH, USA). IL-2 was from National Cancer Institute Biological Resources Branch (Frederick, MD, USA). IL-4, IL-12, IL-23, and anti-IL-4 (11B11) antibodies were from eBioscience. IL-6 and TGF- β 1 were from R&D Systems. Quilaja bark saponin, alkaline phosphatase-conjugated avidin, phosphatase substrate tablets, β -ME, sodium deoxycholate, and Evans blue dye were from Sigma-Aldrich (St. Louis, MO, USA). Toluidine blue was from Acros Organics (Geel, Belgium). TNP-BSA was from Biosearch Technologies (Novato, CA, USA). PMA and ionomycin were from EMD. Triton X-100, SDS, 4-nitrophenyl-N-acetyl- β -D-glucosaminide, RPMI 1640, penicillin/streptomycin, L-glutamine, nonessential amino acid solution, and sodium pyruvate were from Fisher (Pittsburg, PA, USA). Indo-1-AM was from Invitrogen (Carlsbad, CA, USA). Hepes was from Mediatech, Inc. (Manassas, VA, USA). FBS was from Atlanta Biologicals (Lawrenceville, GA, USA). PVDF membranes were from Bio-Rad (Hercules, CA, USA). PI was from MP Bio-medicals (Aurora, OH, USA).

BMMC culture

Conditioned media from Wehi-3B cells (American Type Culture Collection, Manassas, VA, USA) was used as the source of murine IL-3. Bone marrow cells from 4- to 6-week-old mouse femurs were grown in RPMI 1640 containing 50% Wehi-3B conditioned media and supplemented with 10% FBS, 0.5% penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, nonessential amino acids, and 50 μ M β -ME (complete media). Media were changed weekly, and the cells were assayed by flow cytometry using fluorescently labeled CD117 and Fc ϵ RI α -specific antibodies on a BD FACSCanto (BD Biosciences). At 4–6 weeks of culture, over 95% of the

cells displayed both markers and were considered to be mature mast cells. BMMC were used for experiments between 4 and 10 weeks of culture.

Th differentiation

Naïve T cells were isolated from mouse spleens and lymph nodes using the CD4⁺CD62L⁺ MACS kit from Mylenyi Biotec (Bergisch Gladbach, Germany) and seeded into plates coated with 1 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 antibodies. Th cell differentiation was induced by the following reagents: for Th1, IL-2 (100 U/ml), IL-12 (10 ng/ml), and anti-IL-4 antibody (10 μ g/ml); for Th2, IL-2 (100 U/ml), IL-4 (10 ng/ml), and anti-IFN- γ antibody (10 μ g/ml); for Th17, IL-6 (20 ng/ml), IL-23 (10 ng/ml), TGF- β 1 (3 ng/ml), anti-IL-4 antibody (10 μ g/ml), and anti-IFN- γ antibody (10 μ g/ml). The cells were analyzed 7 days later. Thymocytes were isolated from mouse thymi using the 70- μ M nylon mesh cell strainers (Fisher). The RBCs were lysed using 1 mM EDTA, 10 mM potassium bicarbonate, and 150 mM ammonium chloride buffer, pH 7.2.

Immunoblotting

BMMC cell extracts were prepared in PBS containing 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100 (RIPA buffer) with 1 mM PMSF, 1 mM sodium orthovanadate, and protease inhibitor tablets. Protein concentration was determined using the bicinchoninic acid assay (Fisher), and equal amounts of protein were subjected to SDS-PAGE and transferred onto PVDF membranes, which were subjected to immunoblotting using antibodies specific for Coronin-1A. As a control for protein load, immunoblotting using antibodies specific for β -actin was performed.

Phalloidin staining for flow cytometry

BMMC were washed once with PBS containing 1% FBS (FACS buffer) and resuspended at 10^6 cells in 100 μ l FACS buffer. The cells were incubated with fluorescently labeled antibodies specific for CD117 and Fc ϵ RI α , followed by fixing in 4% paraformaldehyde and permeabilization with 0.1% saponin in FACS buffer. Permeabilized cells were incubated with Alexa 488-conjugated phalloidin in 0.1% saponin-containing FACS buffer. The cells were washed with FACS buffer and analyzed by flow cytometry. In a separate experiment, BMMC and CD4 T cells were permeabilized and incubated with TRITC-conjugated phalloidin (Sigma-Aldrich) in 0.1% saponin-containing FACS buffer. The cells were washed with FACS buffer and analyzed by flow cytometry. Naïve CD4 T cells were identified as CD4⁺CD62L⁺CD44^{low}.

BMMC proliferation and apoptosis

BMMC proliferation rate was measured using the CCK-8 from Dojindo (Rockville, MD, USA), following the manufacturer's instructions. Briefly, 2×10^5 cells/well were plated in 96-well plates, and cell viability was determined at 0 and 24 h time-points. Cell proliferation after 24 h was measured as a percentage of that observed at 0 h for each cell type. Apoptosis was measured by incubation of cells with PI and FITC-conjugated Annexin V and flow cytometry analysis. Data were plotted as the percentage of Annexin V-positive, PI-negative cells.

BMMC transwell migration

BMMC migration was assayed using 24-well Transwell chambers (Corning, Corning, NY, USA), separated by 8 μ m polycarbonate filters, the lower surface of which had been coated with 20 μ g/ml fibronectin for 16 h at 4°C, followed by blocking with 4% BSA in PBS at 37°C for 1 h. The upper wells contained 10^5 cells/well in 0.2 ml medium consisting of RPMI 1640 supplemented with 1% BSA and 20 mM Hepes, pH 7.4 [29]. Lower wells contained 0.6 ml containing 100 ng/ml SCF or 10 μ g/ml C38-2 IgE. Migration of cells to lower wells containing 0.6 ml media alone was used as control. BMMC migration was quantified after 8 or 24 h by counting cells migrated into lower wells using a hemocytometer and presented as a fold increase compared with migration under control conditions for each cell type.

Cytokine ELISA

BMMC were sensitized with C38-2 IgE as described above, washed, and plated at 2×10^6 cells/ml in a 24-well plate in complete media. The cells were stimulated with 100 ng/ml TNP-BSA for 24 h at 37°C. Cells not previously sensitized with IgE were stimulated with 50 ng/ml PMA and 1 μ M ionomycin. Unstimulated cells were used as controls. Cell supernatants were used in a sandwich ELISA assay to determine the level of IL-6 and TNF- α , following the manufacturer's instructions.

BMMC degranulation assay

BMMC were plated at 3×10^5 cells/ml and sensitized with 500 ng/ml C38-2 IgE for 16 h at 37°C. The cells were washed once with Tyrodes buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaH_2PO_4 , 0.1 mM MgCl_2 , 1.3 mM CaCl_2 , 0.1% glucose, 0.1% gelatine, and 10 mM Hepes, pH 7.3), and 5×10^5 cells were plated in 100 μ l/well of a 96-well plate. BMMC degranulation was stimulated by 50 ng/ml PMA and 1 μ M ionomycin or a range of TNP-BSA concentrations (0–100 ng/ml). Unstimulated cells were used as controls. After 1 h at 37°C, the cells were pelleted by centrifugation and cell extracts prepared in PBS containing 1% Triton X-100. The amount of mast cell enzyme β -hexosaminidase activity in cell supernatants and extracts was measured using the substrate 4-nitrophenyl-N-acetyl- β -D-glucosaminide. Degranulation was plotted as percent of β -hexosaminidase activity detected in the cell supernatants compared with total activity present in the supernatants and cell extracts.

BMMC Ca^{2+} flux

BMMC were sensitized with C38-2 IgE as described above, washed, and resuspended at 2×10^6 cells/ml in complete media. The cells were loaded with 0.25 μ M Indo-1-AM for 30 min at 37°C. BMMC were washed twice with complete media and once with CFM (containing HBSS supplemented with 10% FBS, 10 mM Hepes, 1 mM MgCl_2 , and 1 mM EGTA) and resuspended at 2×10^6 cells/ml in CFM. Changes in violet and blue Indo-1 fluorescence with time were determined by flow cytometry after the addition of 50 ng/ml TNP-BSA, 10 mM CaCl_2 , and 1 μ g/ml ionomycin at the indicated time-points. The violet:blue ratio was plotted over time as relative Ca^{2+} flux.

PCA

Cohorts of C57BL/6 ($n=8$) and C57BL/6-*Coro1a*^{Lmb3} ($n=7$) were anesthetized using isoflurane. TNP-specific C38-2 IgE (50 ng) was injected i.d. into the left ear pinna in 20 μ l PBS. Contralateral ear was injected with vehicle only. After 24 h, the mice were subjected to i.v. injection with 100 μ g TNP-BSA in 200 μ l 1% Evans blue dye in PBS. After 90 min, the mice were killed, and both ears were removed and minced into 2×2 mm pieces. Dye was extracted by incubation in 1 ml formamide for 16 h at 55°C. Hair and tissue fragments were eliminated by filtration through a 70- μ m nylon cell strainer (BD Biosciences), and absorbance was read at 620 nm. Data were plotted as a ratio between the ear injected with IgE and the vehicle-injected ear.

Coronin-1 expression analysis by qPCR

CD4 T cells and B cells were purified from the spleen using the CD4⁺ T cell or the B cell MACS kit from Miltenyi Biotec. Total RNA was prepared from BMMC, CD4 T cells, and B cells using the RNeasy Plus mini kit from Qiagen (Hilden, Germany) following the manufacturer's instructions. After DNase digestion (Invitrogen), cDNA was prepared using the iScript cDNA synthesis kit from Bio-Rad. QuantiTect SYBR Green PCR kits (Qiagen) were used to detect expression of Coronin-1A, Coronin-1B, Coronin-1C, and the housekeeping gene TBP by qPCR using the 7900HT Fast System real-time PCR instrument from Applied Biosystems (Foster City, CA, USA).

Statistics

Statistical significance was determined by the Student's *t* test using GraphPad Prism 5 software. *P* values of <0.05 were considered statistically significant.

Online Supplemental material

CD4 T cells were purified from the spleen using the CD4⁺ T cell MACS kit from Miltenyi Biotec. BMMC and CD4 T cell extracts were prepared in RIPA buffer containing protease inhibitors. Equal amounts of protein were subjected to SDS-PAGE and transferred onto PVDF membranes, which were subjected to immunoblotting using antibodies specific for Coronin-1A. As a control for protein load, immunoblotting using antibodies specific for GAPDH was performed.

RESULTS

Mast cell characterization and F-actin levels in the absence of Coronin-1A

To determine whether Coronin-1A is expressed in mast cells, we prepared lysates from WT BMMC, thymocytes, CD4 T cells isolated from the spleen, and in vitro-differentiated Th cells Th1, Th2, and Th17. SDS-PAGE-separated lysates were immunoblotted with a Coronin-1A-specific antibody. We found that Coronin-1A is expressed at comparable levels in mast cells and T cells (Fig. 1 and Supplemental Fig. 1). Equal protein loading was determined by immunoblotting with β -actin and GAPDH antibodies. The specificity of the Coronin-1A antibody was confirmed using BMMC lysates derived from *Coro1a*^{Lmb3} mice homozygous for the Coronin-1A Q262X mutation (referred to as Q262X BMMC throughout this manuscript; Fig. 1B). This mutation within Coronin-1A yields a nonfunctional, truncated protein that is undetectable [8].

To examine if the maturation of *Coro1a*^{Lmb3} BMMC is altered by the loss of Coronin-1A, BMMC were incubated with fluorescently labeled antibodies specific for mast cell markers, the SCF receptor c-Kit, and the high-affinity IgE receptor Fc ϵ RI. After 4–6 weeks of culture, BMMC from the WT or *Coro1a*^{Lmb3} mice were over 95% Fc ϵ RI⁺c-Kit⁺ (Fig. 1C). We observed no difference in the total cell numbers in BMMC cultures from the WT or *Coro1a*^{Lmb3} mice. In addition, we examined cellular dehydrogenase activity as a measure of proliferation. We did not detect any proliferation defect in *Coro1a*^{Lmb3} BMMC in response to the mast cell mitogen SCF (Fig. 1D) [30]. Further, BMMC from the WT and *Coro1a*^{Lmb3} mice contained comparable metachromatic granularity when stained with toluidine blue (Fig. 1E). These results indicate that a functional Coronin-1A protein is dispensable for mast cell differentiation, proliferation, and maturation.

Coronin-1A is an inhibitor of Arp-2/3-mediated F-actin polymerization and promotes branched F-actin degradation [1, 3, 4]. Indeed, *Coro1a*^{Lmb3} CD4⁺ T cells, CD8⁺ T cells, and B cells have increased levels of cellular F-actin [8]. Therefore, we examined F-actin levels by flow cytometry using fluorescently labeled phalloidin and observed increased cellular F-actin levels in mast cells derived from *Coro1a*^{Lmb3} mice (Fig. 2A). Although the overall F-actin level of increase in *Coro1a*^{Lmb3} mast cells ($114.1 \pm 5.2\%$) was lower than that observed in *Coro1a*^{Lmb3} naive CD4 T cells ($151.8 \pm 4.6\%$), F-actin was increased significantly in comparison with mast cells from WT mice (Fig. 2B). Thus, we concluded that the absence of a functional *Coro1a* allele affects F-actin dynamics in mast cells.

Apoptosis of *Coro1a^{Lmb3}* BMMC

An increase in F-actin can induce apoptosis by altering mitochondrial membrane potential [9]. However, despite elevated F-actin in *Coro1a^{Lmb3}* T cells and B cells, only *Coro1a^{Lmb3}* T cells display an increase in apoptosis [8]. Therefore, we examined

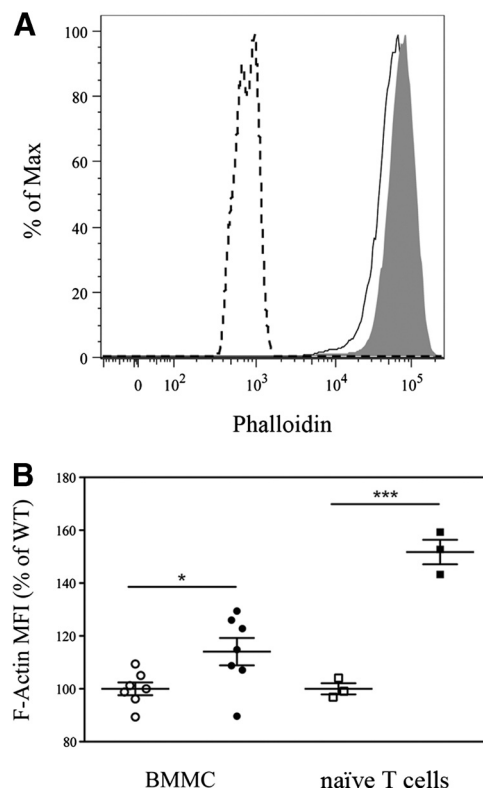
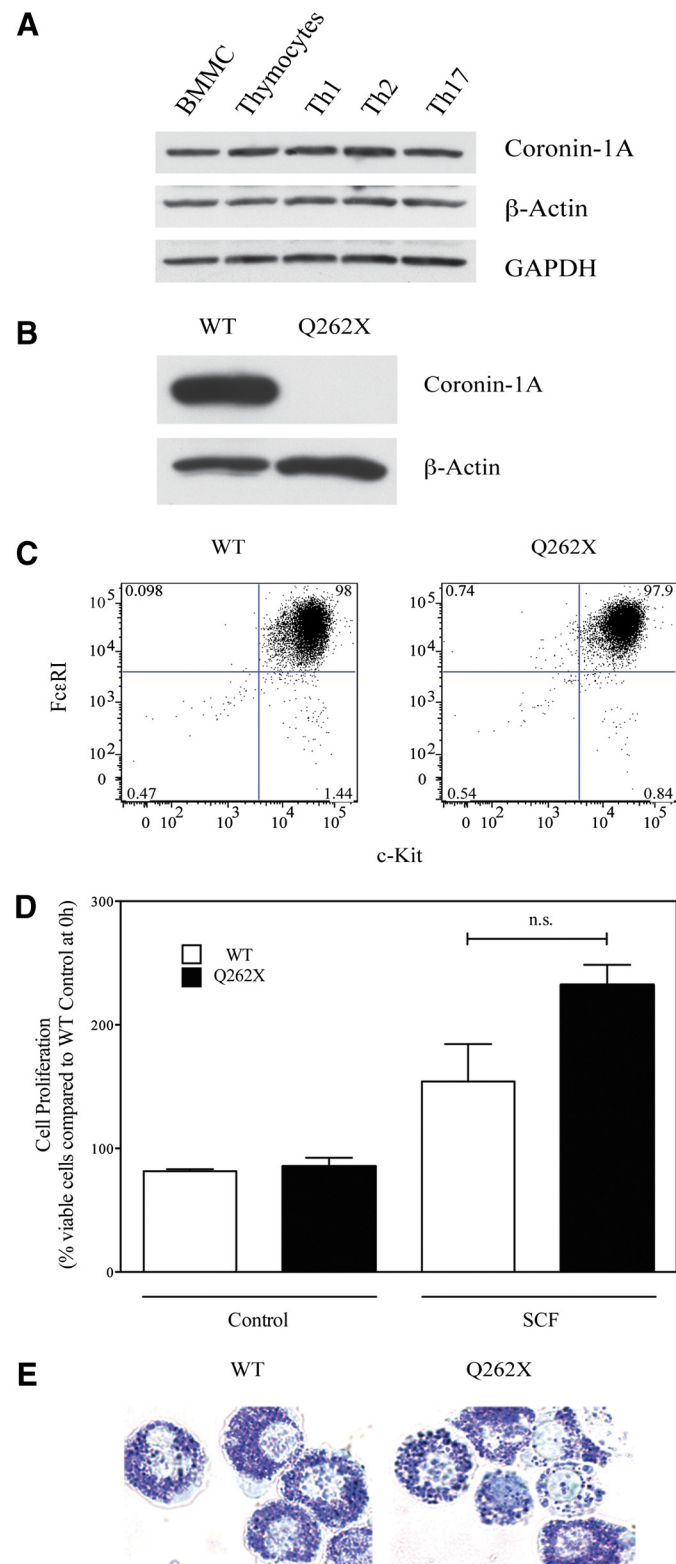


Figure 2. Increased levels of F-actin in *Coro1a^{Lmb3}* BMMC. (A) WT (solid black line) and *Coro1a^{Lmb3}* (filled gray area) BMMC were incubated with Alexa 488-labeled phalloidin and subjected to flow cytometry analysis in comparison with unstained cells (dashed black line). A representative of 3 independent experiments is shown. (B) BMMC (circles; $n=7$) and $CD4^+CD62L^+CD44^{low}$ (naïve) T cells (squares; $n=3$) from the WT (open symbols) and *Coro1a^{Lmb3}* mice (closed symbols) after incubation with TRITC-labeled phalloidin and flow cytometry analysis. Results are presented as percent of WT median fluorescence intensity (MFI) for each cell type. * $P \leq 0.05$; ** $P \leq 0.001$.

mast cell apoptosis by measuring the percentage of annexin V-positive cells. Treatment of BMMC with PMA and ionomycin leads to a significant increase in the percentage of apoptotic cells, and the rate of apoptosis is comparable between WT and

Figure 1. *Coro1a^{Lmb3}* BMMC characterization. (A) Cell extracts were prepared from the BMMC, thymocytes, or in vitro-differentiated Th1, Th2, or Th17 cells and subjected to SDS-PAGE and immunoblot analysis using the antibody specific for Coronin-1A. Cell extracts were immunoblotted for β-actin or GAPDH as a measure of protein load. (B) Cell extracts from the WT or *Coro1a^{Lmb3}* (Q262X) BMMC were prepared and immunoblotted as in A. (C) BMMC were stained with c-Kit and FcεRI and subjected to flow cytometry analysis. Percentage of stained cells is shown in a representative of 7 experiments. (D) WT and *Coro1a^{Lmb3}* BMMC (Q262X) were left unstimulated (Control) or were stimulated with the SCF for 24 h at 37°C. Cell viability was determined at 0 h and 24 h of culture by adding the CCK-8 reagent, as described in Materials and Methods. Percentage of cell proliferation was measured by comparing cell viability at 24 h with that observed at 0 h for each cell type. A representative of 2 independent experiments is shown; $P > 0.05$ (n.s.). (E) WT and Q262X BMMC were incubated with Toluidine blue, washed, cytospun onto slides, and examined by microscopy.

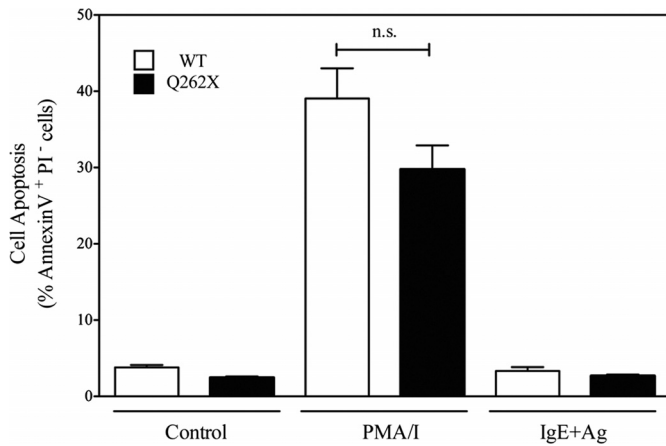


Figure 3. Apoptosis in *Coro1a*^{Lmb3} BMMC. WT or *Coro1a*^{Lmb3} BMMC (Q262X) were left unstimulated (Control) or were stimulated with PMA and ionomycin (PMA/I) or with IgE followed by antigen TNP-BSA (IgE+Ag) for 24 h at 37°C. Cell death by apoptosis was measured after 24 h by staining of cells with FITC-labeled Annexin V and PI, and flow cytometry analysis. The average percentage of Annexin V-positive, PI-negative cells in 2 independent experiments is shown; $P > 0.05$ (n.s.).

Coro1a^{Lmb3} BMMC (Fig. 3). FcεRI stimulation did not lead to significant apoptosis in either genotype (Fig. 3). Therefore, despite increased F-actin levels in *Coro1a*^{Lmb3} BMMC, Coronin-1A does not contribute to apoptosis resistance in mast cells.

Cell migration of *Coro1a*^{Lmb3} BMMC

F-actin polymerization regulates many cellular processes, including phagocytosis, cell division, endocytosis, exocytosis, and migration [31]. The Coronin requirement for cell motility was first described in *Dictyostelium discoideum* [5]. Further, evolutionary conservation of this function is underscored by the migration defects of *Coro1a*^{-/-} or mutant T cells [8–10]. Mast cells accumulate in inflammatory mucosal tissues in which IgE is present [32–37]. In addition, IgE acts as a mast cell chemoattractant in vitro [29]. Furthermore, certain growth factors, including the SCF, induce chemotactic mast cell migration in vitro [38]. Using the Transwell migration assay, we examined BMMC chemotaxis toward SCF or IgE [29]. SCF induced robust and equivalent migration of WT and *Coro1a*^{Lmb3} BMMC (Fig. 4A). The migration response to IgE was modest, and we detected no difference between WT and *Coro1a*^{Lmb3} cells (Fig. 4B). These data suggest that Coronin-1A is not required for mast cell chemotactic migration.

Cytokine secretion in *Coro1a*^{Lmb3} BMMC

Coronin-1A is required for CD4⁺ T cell activation and IL-2 secretion [8, 39]. Mast cell activation results in secretion of preformed and newly synthesized cytokines [15, 16]. Thus, we examined BMMC IL-6 and TNF-α cytokine secretion following FcεRI or PMA and ionomycin stimulation. BMMC produced abundant amounts of IL-6 (Fig. 5A) and TNF-α (Fig. 5B) in response to either treatment. However, no significant differ-

ence in cytokine secretion was observed between WT and *Coro1a*^{Lmb3} cells.

Degranulation of *Coro1a*^{Lmb3} BMMC

Bcl10-Malt1 activation downstream of the FcεRI drives NF-κB-dependent TNF-α and IL-6 production [40]. In parallel, FcεRI stimulation induces PKC and Ca²⁺ mobilization pathways, uncoupling processes such as degranulation from NF-κB-driven cytokine secretion [40, 41]. Mast cell degranulation requires Ca²⁺ mobilization and extensive cytoskeletal rearrangements. Therefore, we investigated whether the absence of a functional Coronin-1A protein and the resulting alterations in F-actin levels (Fig. 2) would lead to defects in mast cell degranulation. Surprisingly, FcεRI-mediated degranulation, as measured by β-hexosaminidase release into cell supernatants, remained intact in *Coro1a*^{Lmb3} BMMC in comparison with WT BMMC (Fig. 6A). Furthermore, treatment with PMA and the Ca²⁺ ionophore ionomycin also resulted in equivalent degranulation between the WT and *Coro1a*^{Lmb3} BMMC, indicating that Coronin-1A is dispensable for degranulation.

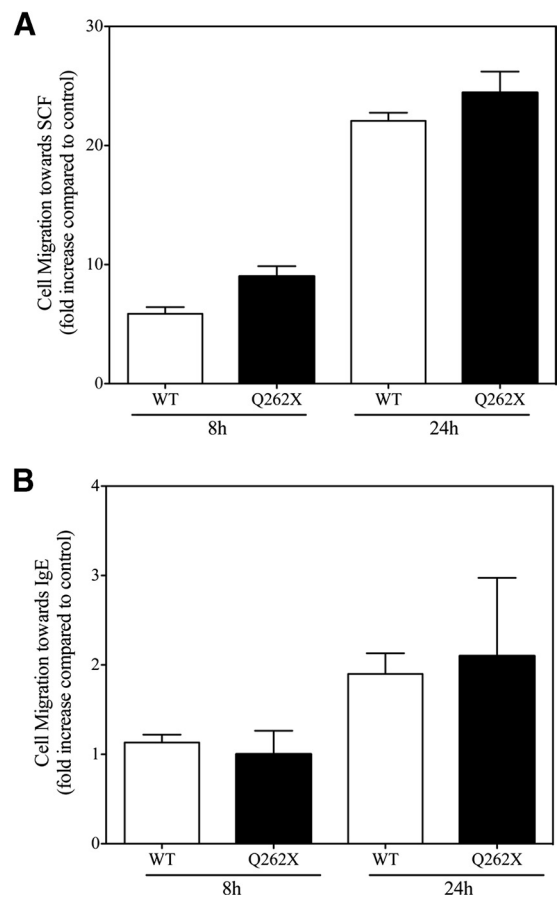


Figure 4. In vitro migration of *Coro1a*^{Lmb3} BMMC. We analyzed migration of WT, and *Coro1a*^{Lmb3} (Q262X) BMMC toward 100 ng/ml SCF (A) or 10 μg/ml IgE (B) for 8 or 24 h at 37°C, as described in Materials and Methods. Chemotactic cell migration is shown as the fold-increase in migration compared with cell migration toward wells containing media only (control) for each cell type. A representative of 2 experiments is shown.

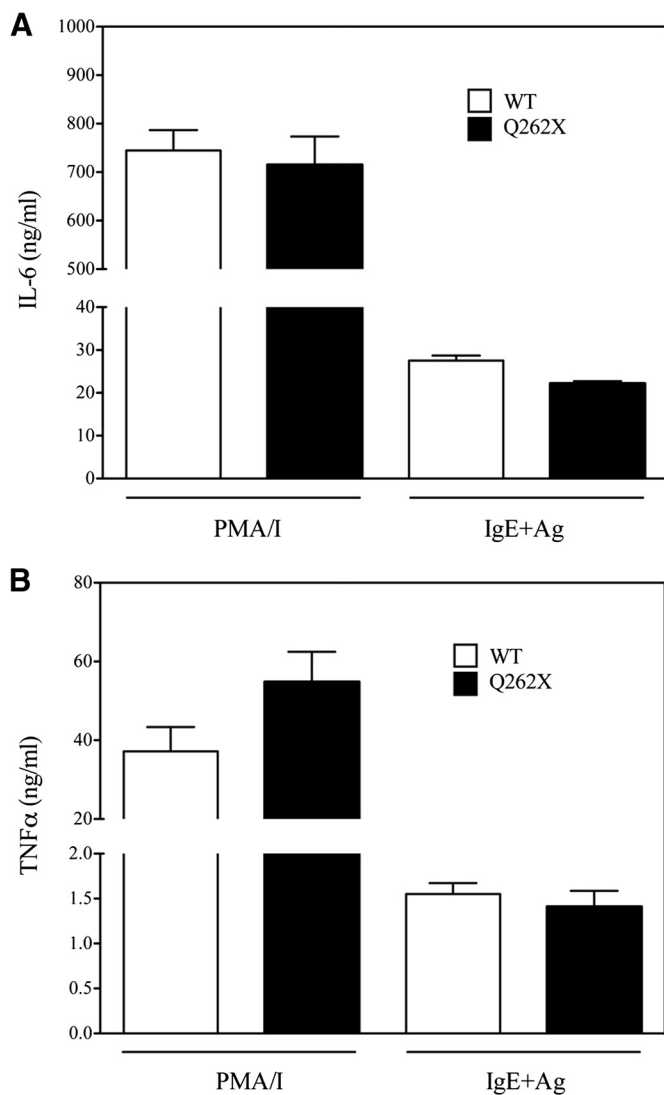


Figure 5. Cytokine secretion by *Coro1a^{Lmb3}* BMMC. WT or *Coro1a^{Lmb3}* (Q262X) BMMC were stimulated with PMA and ionomycin or IgE followed by antigen TNP-BSA, (IgE+Ag) and cytokine levels in the supernatants were determined by ELISA. (A) IL-6. (B) TNF- α . A representative of 3 independent experiments is shown.

Ca²⁺ flux in *Coro1a^{Lmb3}* BMMC

As Coronin-1A regulates cytosolic Ca²⁺ mobilization upon antigen-induced leukocyte stimulation and may be required for Ca²⁺-dependent signaling [8–10, 25, 39, 42], we examined induction of the Ca²⁺ flux in WT and *Coro1a^{Lmb3}* BMMC. TNP-specific, IgE-sensitized cells loaded with Ca²⁺ indicator Indo-1-AM were transferred into Ca²⁺-free medium. The initial Ca²⁺ release from the ER was measured following the addition of TNP-BSA (Fig. 6B). Administration of exogenous Ca²⁺ enabled measurement of extracellular Ca²⁺ influx. Finally, ionomycin was added to induce maximal Ca²⁺ flux. We detected no difference in either phase of Ca²⁺ mobilization between the WT and *Coro1a^{Lmb3}* BMMC (Fig. 6B). In contrast to T cells, B cells, and macrophages, functional Coronin-1A is not required for Ca²⁺ mobilization in mast cells.

PCA in *Coro1a^{Lmb3}* mice

Finally, to establish whether the Coronin-1A mutation impairs the in vivo function of mast cells, WT and *Coro1a^{Lmb3}* mice were subjected to PCA, a model of vascular leakage and edema, dependent on mast cell recruitment and degranulation [43]. The PCA response developed equally well in the *Coro1a^{Lmb3}* mice as in the WT mice (Fig. 7). Thus, the absence of Coronin-1A function does not impair mast cell responses in vivo.

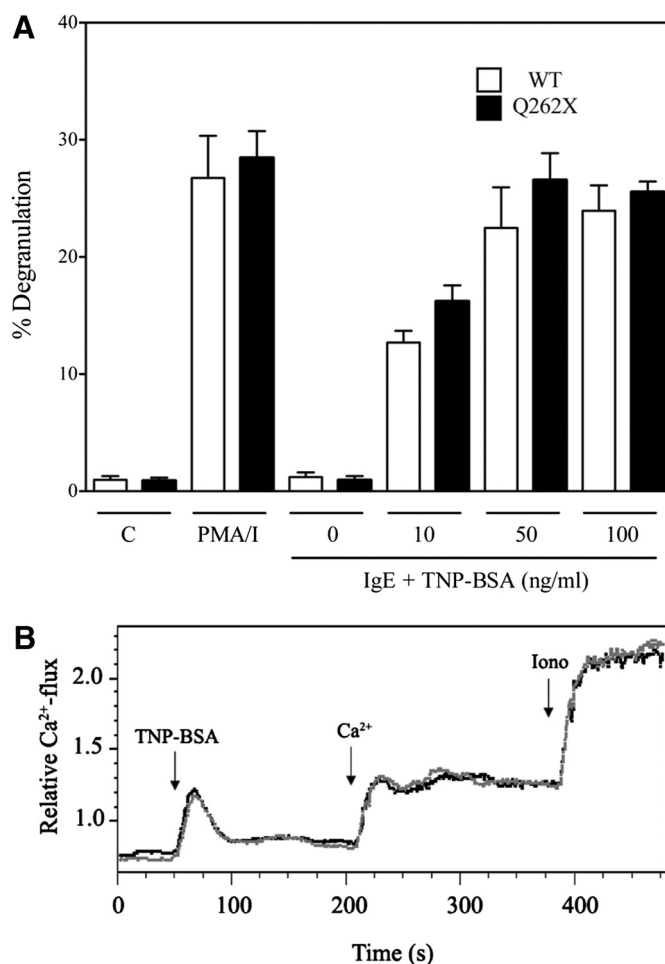


Figure 6. *Coro1a^{Lmb3}* BMMC degranulation and Ca²⁺ flux. (A) WT and *Coro1a^{Lmb3}* (Q262X) BMMC were left unstimulated (C) or were stimulated with PMA and ionomycin or IgE followed by the indicated concentration of antigen TNP-BSA for 1 h at 37°C. Percent of β -hexosaminidase activity in the cell supernatants was measured in comparison with total β -hexosaminidase levels in the supernatants and lysates. The average of 3 independent experiments is shown. (B) WT (black trace) and *Coro1a^{Lmb3}* (gray trace) BMMC were sensitized with TNP-specific IgE (0.5 g/ml) for 16 h at 37°C. The cells were washed, loaded with Indo-1-AM, and kept in Ca²⁺-free medium. Intracellular Ca²⁺ levels over time were measured as the following stimuli were added at the indicated time-points: 50 ng/ml TNP-BSA, 10 mM CaCl₂ (Ca²⁺), and 1 g/ml ionomycin (Iono), indicated by the arrows. A representative of 3 independent experiments is shown.

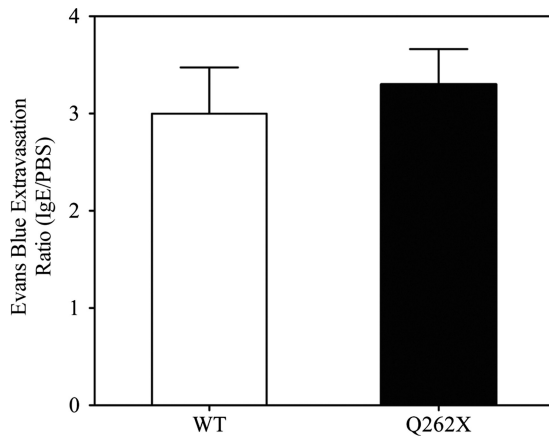


Figure 7. PCA in *Coro1a*^{Lmb3} mice. WT (n=8) or *Coro1a*^{Lmb3} (Q262X; n=7) mice were subjected to the PCA assay, as described in Materials and Methods.

Coronin-1 expression in cells from *Coro1a*^{Lmb3} mice

Coronin-1A deficiency leads to profound defects in CD4 T cell function [8–10]. Our data suggest that mast cells, similarly to B cells, do not require Coronin-1A in vivo [8, 42]. We examined expression of Coronin family members in mast cells, CD4 T cells, and B cells from the WT and *Coro1a*^{Lmb3} mice to determine if other members of the Coronin family compensate for the lack of Coronin. As Coronin-2A and Coronin-2B expression is restricted to the brain and reproductive tissues, and Coronin-7 is expressed at low levels in comparison with other Coronin family members [6], we focused on Coronin-1B and Coronin-1C. We observed decreased expression of Coronin-1A in *Coro1a*^{Lmb3} mast cells, CD4 T cells, and B cells in comparison with their WT counterparts (Fig. 8A). Coronin-1B and Coronin-1C were expressed at a higher level in mast cells than in CD4 T cells or B cells from the WT mice (Fig. 8B and C). Surprisingly, neither Coronin-1B nor Coronin-1C was up-regulated significantly in cells from the *Coro1a*^{Lmb3} mice in comparison with the WT cells. We concluded that Coronin-1A deficiency in mast cells, CD4 T cells, or B cells in *Coro1a*^{Lmb3} mice is not compensated by an increase in Coronin-1B or Coronin-1C expression. However, the higher levels of Coronin-1B and Coronin-1C expression in mast cells may be sufficient for maintaining normal mast cell function in the absence of a functional Coronin-1A allele.

DISCUSSION

Mast cells contain stores of preformed mediators within cytosolic granules. Ligation of FcεRI triggers Ca²⁺-dependent signaling processes, resulting in new gene expression and the release of cytosolic granules via cytoskeletal rearrangements [44]. Indeed, F-actin disassembly is critical for mast cell degranulation [27, 45]. Coronin-1A is a leukocyte-specific actin-binding protein that is involved in Ca²⁺-dependent signaling processes in macrophages and T and B cells [9, 25, 42]. The

murine *Coro1a*^{Lmb3} mutation leads to lupus resistance due to the inactivation of Coronin-1A function in T cells [8]. Here, we examined whether Coronin-1A activity is required for mast cell function. We found that the Coronin-1A protein is expressed abundantly in BMMC, at levels comparable with those observed in T cells (Fig. 1 and Supplemental Fig. 1). As predicted from the lack of Coronin-1A activity, *Coro1a*^{Lmb3} BMMC had increased levels of F-actin (Fig. 2). Coronin-1A is not essential for mast cell growth or differentiation. Furthermore, degranulation, viability, cytokine secretion, migration, and Ca²⁺ flux of *Coro1a*^{Lmb3} BMMC were comparable with their WT counterparts. We conclude that despite dysregulated F-actin levels, surprisingly, mast cells do not require a functional Coronin-1A molecule to control F-actin or Ca²⁺-mediated processes.

Increased F-actin levels in *Coro1a*^{-/-} or *Coro1a*^{Lmb3} T cells have been linked to enhanced apoptosis [8, 9, 39]. The cataract Shinogi mouse strain was isolated initially as a result of

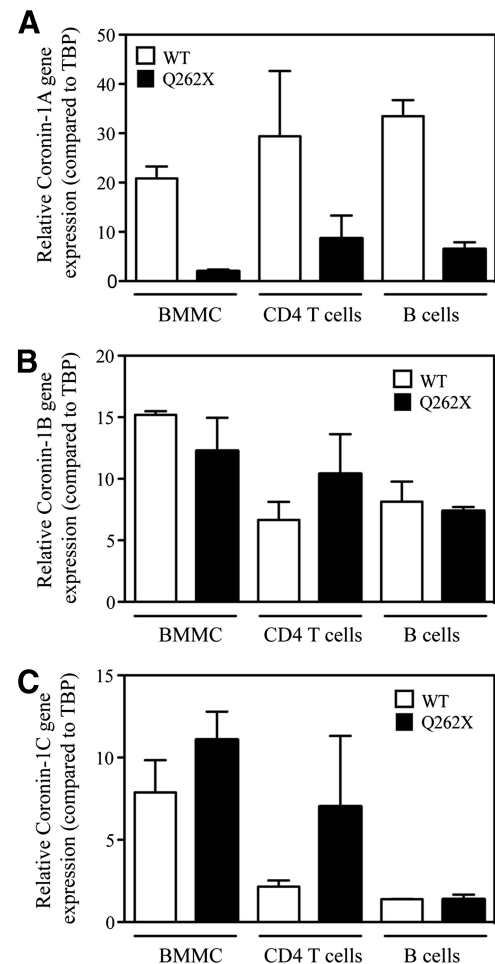


Figure 8. Coronin-1 expression in mast cells, CD4 T cells, and B cells from *Coro1a*^{Lmb3} mice. Total RNA was extracted and cDNA prepared from the WT (white bars) and *Coro1a*^{Lmb3} (Q262X; black bars) BMMC, CD4 T cells, and B cells. qPCR was performed as described in Materials and Methods to detect expression of (A) Coronin-1A, (B) Coronin-1B, or (C) Coronin-1C. A representative of 3 experiments is shown.

cataracts and microphthalmia but was reported later to have a severe peripheral T cell deficiency [10], and the *Ptcd* locus contains function-impairing E26K substitution within Coronin-1A. *Ptcd* thymocytes display elevated F-actin levels as a result of increased binding and inhibition of the F-actin depolymerizing Arp2/3 complex [10]. Survival of *Ptcd* thymocytes is unaffected, paralleling our observation in *Coro1a^{Lmb3}* mast cells, where we find no decrease in viability, despite increased F-actin levels. Further, B cell viability is comparable between *Coro1a^{-/-}* or *Coro1a^{Lmb3}* mice and their WT counterparts, despite a similar increase in F-actin levels [8, 42]. Therefore, an increase in F-actin levels does not necessarily lead to impaired survival.

Cellular migration requires actin-dependent cytoskeleton rearrangements. *Coro1a^{-/-}* or mutant T cells are impaired in migration toward chemokines, including CCL19, CXCL12, CCL25, CCL21, and S1P [9, 10]. Thus, reduced thymus egress likely contributes to the reduced number of peripheral T cells seen in Coronin-1A deficiency [8–10]. Similarly, TAT-mediated transduction of a dominant-negative Coronin-1A protein inhibited fMLP-induced chemotaxis of human neutrophils [24]. In contrast, migration of murine *Coro1a^{-/-}* neutrophils was reported not to be impaired [46]. Mast cells migrate readily toward IgE, growth factors, and chemokines [47]. Functional Coronin-1A was not required for mast cell migration, as WT and *Coro1a^{Lmb3}* BMMC migrated equivalently toward the SCF or IgE.

Coronin-1A expression is limited to hematopoietic cells and to a lesser extent, neuronal tissues [48]. Mammals express 7 Coronin family members [6]. Thus, the lack of an obvious phenotype in *Coro1a^{Lmb3}* BMMC may be the result of redundancy in Coronin activity. Coronin-1B and Coronin-1C, which are expressed ubiquitously, could play a role in regulating actin dynamics [6]. Interestingly, expression of Coronin-1B and Coronin-1C is not increased in *Coro1a^{Lmb3}* mast cells in comparison with the WT cells (Fig. 8). However, we found higher expression levels of Coronin-1B and Coronin-1C in mast cells than in CD4 T cells or B cells (Fig. 8B and C). These data suggest that Coronin-1B and Coronin-1C may be expressed at high enough levels in mast cells to compensate a deficiency in Coronin-1A function without requiring further up-regulation. Although the Q262X mutation could possibly result in the synthesis of an undetectable, truncated protein, we found that the overall expression level of Coronin-1A is decreased greatly in *Coro1a^{Lmb3}* mast cells (Fig. 8A). Additionally, such a protein would lack the C-terminal domains necessary for Coronin-1A trimerization and activity [3, 4, 49]. Further, when overexpressed in Jurkat cells, Q262X Coronin-1A fails to localize to the cell membrane during the immune synapse formation, suggestive of improper function [8]. Importantly, lack of Coronin-1A activity in *Coro1a^{Lmb3}* mice is supported further by the observed deficiencies in *Coro1a^{Lmb3}* T cell function, resembling the phenotype of *Coro1a^{-/-}* T cells [8, 9]. Finally, increased levels of F-actin observed in *Coro1a^{Lmb3}* BMMC (Fig. 2) indicate defective regulation of actin polymerization. As the increase in F-actin levels was more profound in naïve CD4 T cells than in mast cells (Fig. 2B), we speculate that the lack of Coronin-1A activity can be overcome as a result of higher lev-

els of Coronin-1B and Coronin-1C expression in mast cells, when compared with CD4 T cells. However, the comparable levels of Coronin-1B and Coronin-1C expression in T cells and B cells cannot account for the lack of an in vivo phenotype in Coronin-1A deficiency B cells, and further studies are needed to address this question.

Antigen receptor stimulation induces Ca^{2+} flux generation, initially through the inositol triphosphate-mediated release of Ca^{2+} from intracellular stores, followed by the influx of extracellular Ca^{2+} through Ca^{2+} release-activated Ca^{2+} channels [50]. Coronin-1A regulates cytosolic Ca^{2+} levels downstream of antigen receptors in B and T cells [8–10, 42]. In addition, macrophages from *Coro1a^{-/-}* or Coronin-1A mutant mice exhibit defects in the activation-induced influx of extracellular Ca^{2+} [8–10, 25, 42]. Surprisingly, *Coro1a^{Lmb3}* mast cells stimulated through the Fc ϵ RI were not defective in cytosolic Ca^{2+} increases from intracellular store release or from extracellular entry. Precisely how Coronin-1A regulates cytosolic Ca^{2+} levels is unknown. The discrepancy in Coronin-1A dependence for Ca^{2+} flux in mast cells versus T cells, B cells, and macrophages suggests that mast cells possess a unique Ca^{2+} mobilization machinery.

AUTHORSHIP

S.A. designed and executed experiments and interpreted data. D.W., S.H., S.S.L., and D.H.K. participated in the design of experiments and interpretation of the data. K.A.M. designed the experiments, interpreted data, and together with S.A., wrote the manuscript. All authors contributed to the editing of the final draft.

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