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Knockdown of the Antiapoptotic Bcl-2 Family Member A1/Bfl-1 Protects Mice from Anaphylaxis

Eleonora Ottina,*1 Katarina Lyberg,*†,1 Maja Sochalska,* Andreas Villunger,*‡ and Gunnar P. Nilsson†,*2

Many forms of hypersensitivity reactions and allergic responses depend on deregulated mast cell activity. Several reports suggested that the antiapoptotic Bcl-2 family protein Bcl2a1/Bfl-1/A1 plays a critical role in mast cell survival upon activation. However, its in vivo relevance is poorly understood because of quadruplication of the Bcl2a1 gene locus in mice, hindering conventional knockout studies. In this study, we used a mouse model allowing traceable constitutive knockdown of all A1 isoforms expressed in the hematopoietic system by RNA interference. Knockdown of A1 reduced mast cell numbers in the skin and impaired connective tissue–like mast cell survival upon FcεRI-mediated activation in vitro. In contrast, A1 was dispensable for mucosa-like mast cell differentiation and survival. Moreover, knockdown of A1 prevented IgE-mediated passive systemic and cutaneous anaphylaxis in vivo. Our findings demonstrate that A1 is essential for the homeostasis of connective tissue mast cells, identifying A1 as a possible therapeutic target for therapy of certain types of mast cell–driven allergy symptoms. The Journal of Immunology, 2015, 194: 1316–1322.

Abbreviations used in this article: CTLMC, connective tissue–like mast cell; CTMC, connective tissue mast cell; m, murine; MCP, mast cell protease; miRNA, microRNA; mi-shRNA, miR-30–based precursor harboring an shRNA sequence; MLMC, mucosal-like mast cell; MMC, mucosal mast cell; PCA, passive cutaneous anaphylaxis; PCMC, peritoneal cell–derived mast cell, PSA, passive systemic anaphylaxis; qRT-PCR, quantitative RT-PCR; shRNA, short hairpin RNA; TNP, 2,4,6-trinitrophenol; WT, wild-type.

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driven Bax activation in mice (14), human A1/Bfl-1 was suggested to be the major effector in activation-induced survival of mast cells (7, 15). In addition, A1/Bfl-1 has been implicated in mast cell–driven pathologies, as the percentage of Bfl-1+ mast cells was increased significantly in skin biopsies from patients with dermatitis or psoriasis compared with lesion-free skin samples (15).

Despite the high interest in A1/Bfl-1–regulated cell death mechanisms, their role in mast cell biology and pathology is only poorly understood because quadruplication of its gene locus in mice has hindered conventional knockout studies. Indeed, A1 is encoded in three individual genes in the mouse genome (A1-a, A1-b, and A1-d), whereas A1-c is a pseudogene, carrying a premature STOP codon (16). Selective deletion of A1-a in mice by gene targeting uncovered an augmented apoptosis susceptibility of granulocytes, as well as of allergen-sensitized and activated mast cells, in vitro. A1a-knockout animals appeared otherwise normal, most likely as the result of functional redundancies of A1-a with A1-b and A1-d isoforms (7, 17). We recently described a mouse model using RNA interference that allows traceable constitutive ablation of all A1 mRNA species found in the hematopoietic system, demonstrating that A1/Bfl-1 is involved in the normal development, maturation, and survival of lymphocytes and granulocytes (18). We took advantage of this model to characterize the role of A1 in the different mast cell subtypes by analyzing their activation-induced cell survival ex vivo and explored its role in IgE-dependent anaphylaxis models of allergic reaction in vivo.

Materials and Methods

Transgenic mice

The generation of VVA1 mice (strain VVA1.2) and VVFF-transgenic mice was described previously (18). Mice were maintained on a C57BL/6N genetic background. Animal experiments were performed using 6–12-mo-old mice, in accordance with Austrian legislation (BMWF-66.011/0112II/3b/2012). Because of the variegation in transgene expression in...
VVA1 mice, only mice that exhibited >60% Venus+ peripheral blood leukocytes were used for analysis.

**Immunofluorescence analysis, cell staining, cell sorting, and Abs used**

The mAbs used were purchased from either eBioscience or BioLegend. Their specificities are RA3-6B2, anti-B220; RB6-8C5, anti–Gr-1; RMM-1, anti-IgM; 11-26C, anti-IgD; MI/70, anti–Mac-1; 6D5, anti-CD19; 2B8, anti-CD117 (c-Kit); Mar-1, anti-FcεRI; and DX5, anti-CD49b. The analysis was performed using a FACSCalibur or LSR Fortessa cell analyzer (BD). For the morphological characterization of mast cells, May-Grünwald-Giemsa staining was used on cytospins from peritoneal lavage fluid.

**Histology**

Tissue specimens were fixed with 2% paraformaldehyde and embedded in paraffin. Four-micrometer sections were stained with 0.1% toluidine blue and fast green for histological examination and enumeration of mast cells.

**FIGURE 2.** Loss of CTMCs in A1-knockdown mice. Enumeration of mast cells/mm² in skin, tongue, and ear pinna (connective tissue) (A) and in the glandular stomach wall and duodenum (mucosal tissues) (B) was performed using ImageJ (n = 5–15 animals/genotype). **p < 0.01, ***p < 0.001, versus WT and VVFF mice, Student t test. (C) Percentage of mast cells (FcεRI+ CD117+) in the peritoneal cavity. *p < 0.05, versus WT mice, Student t test. (D) Proliferation of PCMCs in culture was monitored over 4 wk. **p < 0.01, ***p < 0.001, versus WT and VVFF cells, two-way ANOVA with Bonferroni correction for multiple comparisons. (E) Percentage of basophils (FcεRI+ CD49b+) in the peritoneal cavity. n.s., not significant.

**FIGURE 3.** A1 knockdown does not affect MLMCs. (A) Representative dot plots confirming expression of CD117 and FcεRI in MLMCs after 4 wk in culture. (B) Number of MLMCs in culture over time. Cells were sensitized with 1 µg/ml of IgE-anti-TNP for 90 min and left treated or subsequently challenged with 100 ng/ml TNP to induce FcεRI cross-linking (FcεRI-XL) or activated with the ionophores ionomycin (0.5 µg/ml) or A23187 (0.5 µM). As a control, the cells were kept in media without cytokines. Release of β-hexosaminidase (C) and change in cellularity (D) were measured 24 h posttreatment. Symbols represent means ± SEM of six MLMC cultures from individual mice/genotype.
Cell culture

Connective tissue–like mast cells (CTLMCs) were derived from bone marrow by cultivation of the cells in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME, 50 ng/ml SCF, and 1 ng/ml murine (m)IL-4 (PeproTech). Macosal-like mast cells (MLMCs) were generated by culturing bone marrow cells in DMEM 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μM penicillin, 50 μg/ml streptomycin, 50 ng/ml SCF, 1 ng/ml human TGF-β1, 5 μg/ml IL-9 and 1 ng/ml IL-3 (PeproTech), as previously described (19). Peritoneal cell–derived mast cells (PCMCs) were generated by cultivating peritoneal cells in Opti-MEM (Life Technologies) supplemented with 10% FBS, 2 mM l-glutamine, 50 μg/ml penicillin, and 4% SCF containing hybridoma supernatant harvested from CHO-KL cell cultures or 50 ng/ml recombinant mSCF (PeproTech), as previously described (20). Maturity and purity of CTLMCs, MLMCs, and PCMCs were examined by toluidine blue staining or flow cytometric analysis using anti-CD117 and anti-FcεRI Abs.

Mast cell activation ex vivo and β-hexosaminidase release

CTLMCs and MLMCs were sensitized with IgE by incubation with 15% hybridoma supernatant containing 1 μg/ml monoclonal mouse anti–2,4,6-trinitrophenol (TNP) IgE Ab (IgE-b4; American Type Culture Collection) for 90 min. The cells were washed and resuspended in PBS with 0.2% BSA or normal media without cytokines before the addition of 100 ng/ml TNP-BSA (coupling ratio of 15:1; Sigma-Aldrich). Alternatively, mast cells were stimulated with 0.5 μg/ml ionomycin or 0.5 μM A23187 (both from Sigma-Aldrich) for 24 h, as previously described (21). The activation was measured by a β-hexosaminidase-release assay (19). In experiments in which activation-induced survival after FcεRI cross-linking was measured, mast cells were deprived of cytokines postactivation for 24 h and analyzed by enumeration using trypan blue and a Countess automatic cell counter (Invitrogen).

IgE-dependent passive systemic anaphylaxis

Mice were sensitized passively with IgE by i.p. injection of 10 μg DNP-specific IgE Ab (clone SPE-7; Sigma-Aldrich) in 500 μl PBS and then challenged i.p. 24 h later with 200 μg DNP (Sigma-Aldrich) in 500 μl PBS. Body temperature was measured immediately before and at 5-min intervals after Ag challenge for up to 1 h (Physitemp model BAT-12).

IgE-dependent passive cutaneous anaphylaxis

IgE-dependent passive cutaneous anaphylaxis (PCA) was induced in the ear pinna as described previously (22). Briefly, mice under anesthesia and analgesia were sensitized passively with IgE by intradermal injection of 20 ng DNP-specific IgE Ab (clone SPE-7; Sigma-Aldrich) in 20 μl PBS and were challenged 24 h later by i.v. injection with 200 μg DNP (Sigma-Aldrich) in 100 μl PBS. Ear swelling was measured immediately before and at 30-min intervals after Ag challenge for 6 h using a micrometer (Mitutoyo Digimatic Micrometer 0–25 mm).

Analysis of histamine, mast cell protease-1, and mast cell protease-2 release

Blood was collected from the submandibular vein 10 min after DNP injection. Serum was isolated by centrifugation using microvette 500 Z-Gel columns (Sarstedt). Levels of histamine were quantified by histamine-release assay (RefLab, Tagensieve, Denmark). Mouse mast cell protease (MCP)-1 (eBioscience) or MCP-2 (R&D Systems) serum level was quantified by ELISA using a 1:10 dilution of sera.

qRT-PCR analysis of mRNA expression levels

Quantitative RT-PCR (qRT-PCR) on mRNA was performed with an Omniscript RT Kit (QIAGEN) using 1 μg total RNA pretreated with 1 U/μl RQ1 DNase (Promega). PCR conditions were 95˚C for 7 min and 40 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s. qRT-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG reagent (Invitrogen). Primers used were A1 fwd 5’-CATCACCATCTTCCAGGAGCG-3’; A1 rev 5’-GATAACCATTCTCGTGGGAG-3’; GAPDH fwd 5’-GTCTTCTTGCTGAACTCAGTG-3’; and GAPDH rev 5’-GATGTA-3’. Reactions were performed in triplicates. Relative quantification was performed using the ΔΔCT method, using the
To explore the role of A1 in CTMCs versus MMCs, we compared the in vitro development and phenotype of CTLMCs and MLMCs derived from bone marrow. Mast cells from VVA1-derived bone marrow cultivated in the presence of SCF/IL-3/IL-9/TGF-β1, conditions that promote development into MLMCs, proliferated at rates similar to cells derived from WT and VVFF mice and showed normal CD117 and FcεRI expression levels and morphology (Fig. 3A, 3B, data not shown). In addition, MLMCs were activated by IgE cross-linking or the calcium ionophores, ionomycin or A23187, to the same extent as were the relevant controls and did not show differences in survival (Fig. 3C, 3D). These findings are in line with published data suggesting that A1 plays only a minor role in the survival of MLMCs (19). In contrast, VVA1 mast cells cultured in SCF plus IL-4, promoting differentiation into CTLMCs, showed normal differentiation and activation (Fig. 4A–C) but impaired activation-induced survival after FcεRI cross-

A1 knockdown impairs CTMC homeostasis
Metachromatic staining of skin or tongue sections revealed an 
~50% reduction in the CTMC number in VVA1 mice compared 
with WT or VVFF control mice (Fig. 2A, Supplemental Fig. 2). 
Ear pinna sections showed a trend toward lower mast cell 
numbers in VVA1 mice, but a statistically significant difference 
was not achieved (Fig. 2A). Interestingly, MMC numbers in the 
glandular stomach wall, as well as in the duodenum, were not 
reduced in A1-knockdown mice (Fig. 2B, Supplemental Fig. 2B). 
These results suggest that A1 may be a more crucial survival 
factor for CTMCs than for MMCs or that the knockdown of 
A1 in these cells is inefficient in the latter. Consistent with 
a reduction in the number of CD117⁺ (c-Kit) cells in the peritoneal 
cavity of VVA1 mice (Fig. 2C), PCMCs from A1-

Statistics
All data are presented as the mean ± SEM, unless otherwise stated. The 
differences among different groups were determined by two-way ANOVA 
with the Bonferroni correction for multiple comparisons or the Student 
t test. The p values < 0.05 were considered significant.

Results
The Vav gene promoter drives mi-short hairpin RNA-A1 
expression in mast cells
VVA1 mice (18) express a microRNA (miRNA)-30–based pre-

sequence that is processed into a small interfering RNA that 
that targets all A1 isoforms. This sequence is embedded in the 3’ untranslated region of the cDNA encoding the fluorescent protein 
Venus that allows detection and isolation of cells expressing the 
mi-shRNA, under control of the VavP promoter (23), limiting A1 
knockdown to hematopoietic cells (Fig. 1A). As a control, we used 
mice expressing a mi-shRNA targeting firefly luciferase (VVFF 
mice). To examine the extent of mi-shRNA expression, we mon-
tored the levels of the Venus reporter in FcεRI⁺CD117⁺ mast cells 
and FcεRI⁺CD49b⁺ basophils in the peritoneal lavage fluid from 
these mice by flow cytometric analysis (Fig. 1B, 1C). Notably, 
although we detected high expression levels of Venus in VVFF 
mice, VVA1 mice displayed a fraction of mast cells and basophils 
that no longer expressed the reporter gene, suggesting variedagated 
transgene expression (Fig. 1B, 1C; see Supplemental Fig. 1 for 
gating strategies), a phenomenon that we noted previously when 
using the VavP promoter to drive Venus expression alone (24). 
Peritoneal mast cells from aged VVA1 mice, as well as VVFF 
mice, presented normal morphology, with abundant cytoplasmic 
secretory granules (Fig. 1D). We also examined the knockdown 
efficiency by quantifying A1 mRNA levels by qRT-PCR, because 
reliable commercial Abs are still lacking, in PCMCs from wild-type 
(WT), VVFF, and VVA1 mice. PCMCs were enriched by culti-
vating peritoneal cells in the presence of SCF for 1 wk until >80% 
of the cells stained positive for CD117 and FcεRI (Fig. 1E). Cons-
istent with our previously published data on splenocytes (18), we 
observed an ~80% reduction in total A1 mRNA expression in 
VVA1-derived mast cells compared with WT and VVFF controls 
(Fig. 1F, 1G).

FIGURE 5. IgE-dependent systemic PSA is markedly reduced in A1-

knockdown mice. (A) WT mice (n = 12), VVFF mice (n = 8), and VVA1 
mice (n = 8) were sensitized passively by i.p. injection of 10 ng of DNP-
specific IgE (SPE-7) and then challenged by i.p. injection the next day with 
200 μg of DNP. Body temperature was measured immediately before and 
in 5-min intervals after Ag challenge as a read-out of the allergic response. 
Symbols represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, 
versus WT and VVFF mice. Two-way ANOVA with Bonferroni correction 
for multiple comparisons. The data shown were pooled from four inde-
pendent experiments performed, each of which gave similar results. Serum 
histamine (B) and MCP-1 (C) and MCP-2 (D) concentrations were eval-

ulated in blood samples drawn 10 min post-DNP injection. **p < 0.01, 
versus WT and VVFF mice. Student t test.
linking or ionophore-mediated activation (Fig. 4D), again in line with our previous data reporting increased survival of CTLMCs by induction of A1 expression (19, 25). Collectively, our data indicate that A1 knockdown mainly affects CTMC survival upon activation ex vivo, consistent with previous findings that A1 mRNA is significantly induced upon activation in CTLMCs but not in MLMCs (19).

**A1 knockdown impairs passive systemic and cutaneous anaphylaxis**

To determine whether the noted reduction in CTMCs in VVA1 mice also resulted in decreased systemic responses in vivo, we performed an experiment of mast cell–driven IgE-dependent passive systemic anaphylaxis (PSA). Mice were sensitized with Ag-specific IgE and subsequently challenged with DNP-Ag. This induces the cross-linking of IgE bound to FcεRI on mast cells, triggering systemic and acute activation resulting in marked hypothermia. Consistently, we noted a median drop in body temperature of 3.5˚C in WT and VVFF mice. In contrast, IgE-sensitized VVA1 mice were significantly less responsive, with a median drop in body temperature of only 1.5˚C (Fig. 5A). Assessment of serum histamine levels 10 min postactivation revealed strongly reduced plasma histamine levels in VVA1 mice compared with WT and VVFF controls (Fig. 5B). To investigate whether the impaired response of VVA1 mice to PSA was due to the reduction in CTMCs or impaired activation of MMCs in vivo, we also measured the levels of MCP-1 and MCP-2, proteases found exclusively in MMCs (26). In line with a minor role for A1 in MMC survival (Fig. 3), the levels of MCP-1 and MCP-2 in the sera from VVA1 mice were comparable to the ones observed in WT and VVFF mice (Fig. 5C, 5D), indicating that the reduced severity of anaphylaxis in VVA1 mice was caused by the reduction in CTMCs and impaired survival upon activation, matching our ex vivo data.

To determine whether the deficiency in CTMCs in A1-knockdown mice and their decreased activation in vivo also prevented cutaneous allergic reaction, we performed an experiment of IgE-dependent PCA. Mice were sensitized with Ag-specific IgE intradermally in the ear pinna and subsequently challenged by i.v. injection of DNP-Ag. This induces activation of the dermal mast cells and a robust local inflammatory response, with leukocyte infiltration and tissue swelling. Inflammation peaked between 60 and 90 min after Ag challenge in control mice but was markedly reduced in VVA1 mice (Fig. 6A). Interestingly, VVA1 mice also exhibited a significant reduction in mast cell numbers in ear pinnae at sites of PCA reactions (Fig. 6B, 6C), suggesting that cutaneous tissue mast cells suffer from impaired activation-induced survival in vivo, as well as ex vivo. Collectively, these data indicate that the reduced mast cell number and their impaired survival upon activation, due to A1 knockdown, also inhibit cutaneous allergic reactions.

**Discussion**

Recently, A1/Bfl-1 was reported to be an important regulator of leukocyte survival and differentiation in mice (18). A1/Bfl-1 also was suggested to be the major effector in activation-induced survival of mast cells in mice and humans (7, 15, 27). Human mast cells activated by IgER cross-linking show increased viability that is diminished by inhibition of A1/Bfl-1 (15). In line with these findings, A1/Bfl-1 seems to be an important player in some forms of hypersensitivity reactions in humans. A1/Bfl-1 expression was increased significantly in challenged skin of birch pollen–allergic patients, as well as in skin lesion biopsies from patients with atopic dermatitis or psoriasis, compared with nonlesion skin (15). In this article, we present data that indicate that A1 is indeed required for the normal development and survival of CTMCs. This is supported by histological analysis that shows reduced mast cell numbers in the skin and tongue from A1-knockdown mice, whereas the numbers of mast cells present in the mucosa of the gastrointestinal system or in the duodenum were not altered significantly (Fig. 2). Furthermore, in vitro bone marrow–derived CTLMCs from VVA1 mice showed impaired activation-induced survival when stimulated by IgER cross-linking (Fig. 4), which is comparable to what was shown using mast cells from A1-a–knockout mice (7). Our results are in accordance with previous findings that showed that A1 is differentially expressed in in vitro–derived MLMCs and CTLMCs. Indeed, A1 mRNA appears to be

![FIGURE 6](http://www.jimmunol.org/ by guest on June 5, 2017)
present in both mast cell types, but only a minor upregulation was observed in MLMCs after FceRI cross-linking. In contrast, FceRI cross-linking caused a profound upregulation of A1 in CTLMCs (19). The molecular basis for this difference is unknown. However, consistent is the observation that MLMCs do not respond with increased survival after activation, whereas CTLMCs activated by either Ca++ ionophores or FceRI cross-linking Abs showed impaired survival (Figs. 3, 4) (19). Although it remains possible that insufficient knockdown in MLMCs accounts for a lack of phenotype, we propose that the repeated activation of CTMCs in vivo causes their gradual depletion over time.

In line with the marked reduction in the number of CTMCs (Fig. 2), A1-knockdown mice also exhibited a significant reduction in the severity of features of IgE-dependent PSA (Fig. 5). The impaired response to the allergic reaction correlated with reduced levels of histamine in the sera, but not of MCP-1 or MCP-2, which are secreted selectively by MLMCs (26). Accordingly, A1-knockdown mice exhibit a strong reduction in mast cell numbers in ear pinnae at sites of PCA reactions, indicating that CTMCs in vivo causes their gradual depletion over time.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplementary Figure 1: Representative dot-plots and gating strategies used to assess the Venus expression in peritoneal mast cells (CD117+FcεRI+) and basophils (CD49b+FcεRI+) described in Figure 1.
Supplementary Figure 2: Representative Toluidine blue staining of mast cells in 4-µm-thick paraffin sections of belly skin, glandular stomach wall, tongue and duodenum isolated from wild-type, VVFF and VVA1 mice.