

JunB Is Required for IgE-Mediated Degranulation and Cytokine Release of Mast Cells¹

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Mast cells are effector cells of IgE-mediated immune responses frequently found at the vicinity of blood vessels, the margins of diverse tumors and at sites of potential infection and inflammation. Upon IgE-mediated stimulation, mast cells produce and secrete a broad spectrum of cytokines and other inflammatory mediators. Recent work identified JunB, a member of the AP-1 transcription factor family, as critical regulator of basal and induced expression of inflammatory mediators in fibroblasts and T cells. To study the impact of JunB on mast cell biology, we analyzed JunB-deficient mast cells. Mast cells lacking JunB display a normal *in vivo* maturation, and JunB-deficient bone marrow cells *in vitro* differentiated to mast cells show no alterations in proliferation or apoptosis. But these cells exhibit impaired IgE-mediated degranulation most likely due to diminished expression of SWAP-70, Synaptotagmin-1, and VAMP-8, and due to impaired influx of extracellular calcium. Moreover, JunB-deficient bone marrow mast cells display an altered cytokine expression profile in response to IgE stimulation. In line with these findings, the contribution of JunB-deficient mast cells to angiogenesis, as analyzed in an *in vitro* tube formation assay on matrigel, is severely impaired due to limiting amounts of synthesized and secreted vascular endothelial growth factor. Thus, JunB is a critical regulator of intrinsic mast cell functions including cross-talk with endothelial cells. *The Journal of Immunology*, 2007, 179: 6873–6880.

Mast cells are tissue-localized effector cells of the immune response that arise from progenitor cells residing in the bone marrow. Mast cells are involved in a variety of inflammatory responses of diverse origin, such as the innate immune response against infection, immune complex-mediated reactions, allergic inflammation, asthma, and delayed hypersensitivity (1–3). In addition, emerging data assign mast cells a relevant role in orchestrating angiogenesis under normal and pathological conditions (4). Exposure of mast cells to an allergen leads to cross-linking of the IgE-loaded surface receptor FcεRI and thereby triggers degranulation and prolonged expression of a variety of cytokines and growth factors (4).

Degranulation leads to the immediate release of various lipid-derived mediators, amines, proteases, proteoglycans, and cytokines into the surrounding tissues (5). Mast cell-derived factors play a critical role in sustaining the inflammatory response and in the recruitment of other immune cells, such as T cells, neutrophils, and macrophages to inflammatory sites (6). FcεR stimulation ini-

tiates signaling cascades including the phospholipase Cγ1, PI3K and MAPK pathways (7) with subsequent calcium raise in the cytosol (8, 9). The increase in intracellular calcium levels, sensed synaptotagmins (SYT)⁴ (10, 11), is a prerequisite for degranulation mediated by SNARE (soluble NSF attachment receptor) complex formation, which is crucially involved in the fusion of vesicle and plasma membrane. SNARE complexes are composed of small membrane-bound proteins, such as VAMPs (vesicle-associated membrane proteins), SNAPs (synaptosome-associated proteins), and STX (syntaxins) (7, 12, 13).

Prolonged production of mast cell-derived factors is governed by a set of proinflammatory transcription factors activated via the FcεR-dependent signaling cascade. A prominent role has particularly been attributed to the transcription factors NFAT (14), NF-κB (15), and AP-1 (15–17). AP-1 is composed of dimeric protein complexes formed by the products of the *jun*, *fos*, and *ATF* gene families and is a major nuclear target of mitogen and stress-induced signal transduction cascades (18, 19). Intensive investigation of AP-1 function in genetically modified cell culture and mouse models led to the well accepted perception that the individual AP-1 subunits may have independent functions as tissue-specific and signal-specific activators of AP-1-dependent genetic programs (18, 19). The AP-1 subunit c-Fos is important for proper mast cell function (16), as c-Fos-deficient bone marrow-derived mast cells (BMMC) display a severe degranulation defect due to a diminished expression of genes implicated in the degranulation process and show altered transcriptional induction of a variety of cytokines (16).

The AP-1 subunit JunB was demonstrated to be crucial for placental development, keratinocyte proliferation, controlling differentiation of myeloid and Th2 cells, and for modulation of the expression and release of inflammatory and angiogenic factors

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⁴ Abbreviations used in this paper: SYT, synaptotagmin; SNARE, soluble NSF attachment receptor; VAMP, vesicle-associated membrane protein; SNAP, synaptosome-associated protein; STX, syntaxin; BMMC, bone marrow-derived mast cell; VEGF, vascular endothelial growth factor.

(20–27). Because mast cells could be shown to be crucial for angiogenic processes in inflammatory and tumorigenic events and global ablation of JunB leads to impaired angiogenesis, we were prompted to study the contribution of JunB for mast cell function in general and in the angiogenic process. In this study, we show that JunB ablation does neither affect mast cell proliferation and differentiation in vitro nor maturation in vivo. However, JunB controls the production and release of various cytokines and growth factors from mast cells upon IgE-mediated FcεR stimulation independently of c-Fos. Consequently, the communication of JunB-deficient BMMC with endothelial cells is severely impaired as monitored by an in vitro model for capillary-like tube formation. This deficiency can be attributed to a failure in vascular endothelial growth factor (VEGF) production, which is strictly JunB dependent.

Materials and Methods

Mice

Collagen1α2-Cre mice were crossed to *junB* +/– mice and their offspring was then mated to floxed *junB* mice (>/>) to obtain mice with conditional *junB* ablation (*junB* –/>>^{Col1α2-cre}) that were generated and kept as previously described (20). The procedures for performing animal experiments were in accordance with the principles of the ATBW and were approved by the Regierungspräsidium (Karlsruhe, Germany).

Cell culture

Isolated bone marrow cells were in vitro differentiated to BMMC as previously described (28). The amount of fully differentiated mast cells was determined by flow cytometric analysis using FITC-conjugated α-FcεR1α Ab (Acris Abs) and a PE-conjugated α-CD117 Ab (BD Biosciences). Primary lung endothelial cells were isolated and cultured as previously described (24). BMMC were retrovirally transduced as described previously (24).

Quantification of tissue mast cells

To determine the peritoneal mast cell populations control and *junB* –/>>^{Col1α2-cre} mice were sacrificed, peritoneal lavage was performed with 5 ml of Hank's buffer solution (Sigma-Aldrich) and the number of cells was determined. 2×10^5 cells were transferred on glass slides, fixed in methanol and subsequently stained by the Enerbac protocol (29).

Mast cells residing in the ear of control and *junB* –/>>^{Col1α2-cre} mice were determined as previously described (30). The average number of mast cells in peritoneum and ear was determined on cytospin slides and tissue sections, respectively, by counting mast cells in ten randomly chosen optical fields.

Degranulation assay

Degranulation efficiency was determined as described (16).

RNA isolation and quantitative RT-PCR analysis

RNA of resting BMMC and mast cells activated with 1 μg/ml IgE and stimulated with 100 ng/ml DNP in medium for 6 h was isolated as described previously (31). mRNA transcript levels were measured using real-time quantitative RT-PCR as described (20). Oligonucleotide sequences are available upon request.

Measurement of intracellular calcium concentrations

BMMC were sensitized with 1 μg/ml IgE (α-DNP, SPE-7, Sigma Aldrich) for 6 h and incubated with the calcium-sensitive dyes Fluo-4 (Invitrogen Life Technologies) and FuraRed (Invitrogen Life Technologies) for 30 min at 37°C. Cells were washed twice and resuspended in 1 ml of BMMC medium without FCS. During flow cytometric-based calcium measurement (FACSCalibur; BD Bioscience), mast cells were stimulated by addition of either 100 ng/ml DNP or 0.5 μM ionomycin (Invitrogen Life Technologies). To inhibit influx of extracellular calcium, 50 mM EGTA was added for 90 s before IgE/DNP activation. The ratio of calcium flux was determined by using FCSpress and FlowJo (Treestar) software.

Quantification of protein

BMMC were activated as described above. Amount of cytokines and growth factors released into the supernatant was determined by CBA tech-

nology (BD Biosciences) using a FACSarray (BD Biosciences) for IL-6 and IL-10 and by ELISA (R&D Systems) for VEGF, respectively. Analysis was performed according to manufacturers' instructions.

Tube formation assay

BMMC were sensitized as described above, resuspended in 1 ml of BMMC medium without IL-3 and with 1% FCS and subsequently activated with 100 ng/ml DNP for 6 h at 37°C. The supernatant was applied to the capillary-like tube formation assay. In brief, 4×10^5 wild-type endothelial cells were incubated in 150 μl of supernatant from IgE-activated BMMC on a matrigel (BD Biosciences). Complexity of the tubule network was determined 24 h later by counting the number of interendothelial spaces in randomly chosen optical fields (24). For VEGF inhibition 0.15 μg/ml α-VEGF Ab (AF-493-NA; R&D Systems) was applied to supernatant of wild-type BMMC.

Results

Mast cell differentiation, proliferation, and maturation is JunB independent

To investigate the role of JunB in mast cells, we made use of a conditional mouse mutant (*junB* –/>>^{Col1α2-cre}) (20), which is viable in contrast to the complete *junB* knockout (23), and shows *junB* deletion in various cell types and tissues (20). To prove loss of JunB in mast cells derived from these mice, bone marrow cells were isolated and differentiated for 3 wk in the presence of IL-3. Subsequently, genomic DNA was isolated and complete deletion of the floxed *junB* allele was confirmed by PCR using specific primers for the respective *junB* alleles (20, 23) (Fig. 1A). In vitro differentiated control (*junB* –/>>) and JunB-deficient (*junB* –/Δ) bone marrow cells consisted of a mast cell population of high purity as monitored by *c-kit* (CD117) and FcεRI staining. Differentiated double-positive mast cells could be detected for 97 and 96% of control and *junB* –/Δ mast cell cultures, respectively (Fig. 1B). Thus, JunB has no obvious impact on mast cell differentiation in vitro and BMMC from *junB* –/>>^{Col1α2-cre} mice are suitable to generate *junB* –/Δ mast cells for further investigations.

Because loss of JunB affects the cell cycle profile of mouse embryonic fibroblasts, myeloid cells, and osteoblasts (27, 32–35), we first measured cell cycle phases of in vitro differentiated, logarithmically growing BMMC by propidium iodide staining (Fig. 1C) or by BrdU incorporation (data not shown) and subsequent FACS analysis. Both analyses revealed no significant differences in the cell cycle profile of control and *junB* –/Δ BMMC.

In the absence of the survival factor IL-3, in vitro differentiated BMMC stop proliferation, enter apoptosis and die within 2 to 4 days (36). To study the impact of JunB deficiency on induced cell death, we determined the number of apoptotic cells 24 and 48 h post IL-3 withdrawal by annexin V staining and subsequent FACS analysis (Fig. 1D), but the percentage of annexin V-positive *junB* –/Δ BMMC was not significantly different from control cells.

In vivo mast cell progenitor cells leave the bone marrow, enter the circulation as undifferentiated cells and then colonize peripheral tissues such as lung, bowel, and skin, to differentiate into mature mast cells (37). In line with the normal mast cell differentiation in vitro, maturation and distribution of *junB* –/Δ mast cells was not affected in vivo as determined by counting the number of mast cells in the peritoneum (Fig. 1E) and in the ears (Fig. 1F) of control and *junB* –/Δ mice.

Degranulation of JunB-deficient BMMC is strongly impaired

As *junB* is induced upon IgE-mediated FcεR stimulation (38), it was obvious to investigate the impact of JunB loss on FcεR-dependent mast cell degranulation, one key feature of activated mast cells and the decisive event for proper mast cell function. For this purpose, in vitro differentiated control and *junB* –/Δ BMMC were

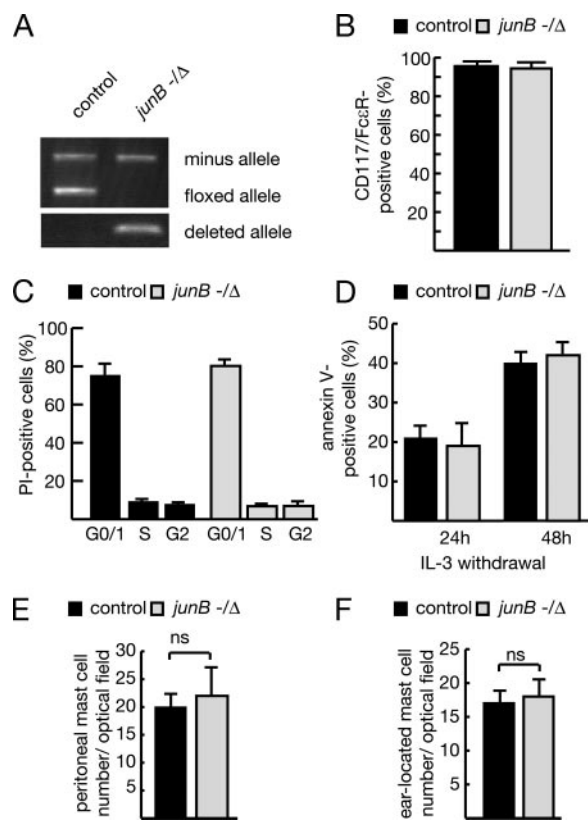


FIGURE 1. JunB-deficient BMMC: in vitro differentiation, characterization and in vivo localization. *A*, PCR analysis demonstrating complete recombination of the floxed *junB* allele in BMMC generated from *junB*^{-/-}>*Col1α2-cre* mice (*junB*^{-/-}) in contrast to cre-negative BMMC (*control*). *B*, Quantification of CD117/FcεRI double-positive cells. Cell numbers (means ± SEM) of at least three independent experiments are given. *C*, Percentage of propidium iodide (PI) positive control and *junB*^{-/-} BMMC residing in G_{0/1}, S, and G₂ as detected by FACS analysis. The result represents the means ± SEM of three independent experiments. *D*, Apoptosis of control and *junB*^{-/-} BMMC was determined at the indicated time points post IL-3 withdrawal by measuring the percentage of annexin V-positive cells by FACS. Rate of spontaneous apoptosis was deduced. Means ± SEM of three independent experiments are reported. *E* and *F*, Peripheral tissue mast cells. Numbers of peritoneal (*E*) and ear-located (*F*) mast cells from control and *junB*^{-/-}>*Col1α2-cre* mice were determined after staining with the Enerbac protocol (29) or with 0.1% toluidine blue (30), respectively. The results represent the means ± SEM of positively stained cells counted on cytosin slides in ten randomly chosen optical fields. ns = not significant.

sensitized with IgE raised against α-DNP and stimulated by addition of increasing concentrations of the Ag human serum albumin conjugated to α-DNP (abbreviated throughout the text as DNP) (Fig. 2A). The degranulation efficiency was measured by the release of the indicator enzyme β-hexosaminidase, an abundant enzyme stored in mast cell secretory granules. Although degranulation of control cells was strictly dependent on DNP concentration and reached a maximum at 100 ng/ml DNP, degranulation of *junB*^{-/-} mast cells resulted in a different, more flattened dose-response curve displaying an up to 5-fold reduced percentage of degranulation efficiency (Fig. 2A). An influence of JunB loss on β-hexosaminidase expression and granule loading could be excluded as both quantitative RT-PCR analysis for mRNA levels and determination of β-hexosaminidase release upon ionophore treatment did not reveal any difference compared with control cells (data not shown). Thus, JunB is required for FcεR-mediated degranulation of mast cells.

The observed phenotype is not due to deregulation of c-Fos, which was previously reported to play an important role for mast cell degranulation (16), because *c-fos* levels were not impaired in *junB*^{-/-} BMMC but rather marginally elevated (data not shown).

Degranulation involves the regulated fusion of granule membrane with plasma membrane. This process requires increased cytosolic calcium levels. Subsequently, calcium sensors are activated and initiate membrane fusion, which is achieved by the SNARE complex (13). To study the influence of JunB loss on this machinery, we determined mRNA levels of SWAP-70, a protein required for calcium release (39) and FcεRI-mediated degranulation (40), of the calcium sensing SYT-1 and of the SNARE complex components SNAP-23 and VAMP-3, -4, -8, and -7, of which the latter is c-Fos dependently regulated (16). Quantitative RT-PCR revealed strongly reduced mRNA levels for *SWAP-70*, *SYT-1*, and *VAMP-8* in *junB*^{-/-} BMMC, whereas the transcript levels of the SNARE proteins *SNAP-23* and *VAMP-3*, -4, and -7 were not altered (Fig. 2B). Thus, JunB regulates the expression of a whole set of components of the degranulation machinery, which may account for the observed degranulation defect of *junB*^{-/-} BMMC.

To determine whether SWAP-70 is functionally implicated in the degranulation process, we attempted to rescue the phenotype. By retroviral gene transfer SWAP-70 or empty control vector were reintroduced into *junB*^{-/-} BMMC. A transduction efficiency of >92% was measured by FACS analysis for the coexpressed IRES-GFP reporter (data not shown). Immunoblot analysis confirmed a robust re-expression of SWAP-70 in transduced *junB*^{-/-} mast cells as compared with strongly diminished SWAP-70 protein in *junB*^{-/-} cells or cells transduced with empty vector (Fig. 2C). Degranulation levels upon re-expression of SWAP-70 in *junB*^{-/-} BMMC reach >50% of those observed for control cells confirming a direct effect of JunB-dependent SWAP-70 on mast cell degranulation (Fig. 2D).

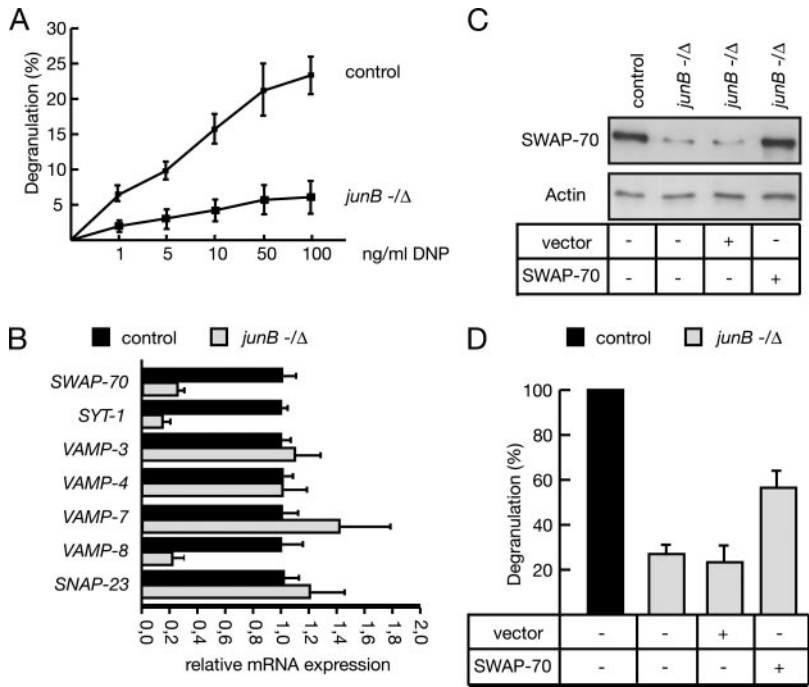
As reported recently (39), SWAP-70-deficient BMMC are strongly impaired in calcium efflux upon *c-kit* stimulation. The reduced SWAP-70 expression in *junB*^{-/-} BMMC prompted us to analyze calcium levels in the cytosol of control and mutant mast cells upon activation by IgE cross-linking. To distinguish between calcium recruitment from intracellular stores and extracellular calcium influx cells were either pretreated with the calcium chelator EGTA or left untreated before DNP activation and subsequent calcium measurement. Whereas in control BMMC the concentration of cytoplasmic calcium rapidly rose by a factor of 3 upon activation, in *junB*^{-/-} BMMC only a very marginal increase (1.8-fold) of cytosolic calcium was measured. However, calcium release from intracellular stores was not altered in *junB*^{-/-} BMMC when compared with controls as demonstrated by pretreatment of BMMC with EGTA (Fig. 3). The degranulation phenotype cannot be explained by differences in calcium loading of the endoplasmic reticulum because we observed for both genotypes similar calcium levels released in BMMC upon stimulation with thapsigargin or ionomycin (data not shown).

Taken together, our data indicate that upon IgE-dependent stimulation JunB is essential for the efficient degranulation of mast cells and JunB loss interferes on different levels with the process of exocytosis including proper calcium triggering and expression of key molecules of the vesicle fusion machinery.

Cytokine expression upon IgE/DNP activation is altered in JunB-deficient BMMC

As a consequence of FcεR stimulation, the expression of mast cell-derived cytokines and growth factors is induced. JunB has already been reported to be crucial for the regulation of cytokine and growth factor expression in keratinocytes and fibroblasts (20,

FIGURE 2. Impaired degranulation of JunB-deficient BMMC. *A*, Percentage of IgE-dependent degranulation of control and *junB* $^{-/-}$ BMMC upon treatment with increasing concentrations of DNP as indicated. The degranulation efficiency was calculated by measuring the relative release of β -hexosaminidase. The data represent the means $\pm$ SEM of three independent experiments. *B*, mRNA levels of *SWAP-70*, *SYT-1*, *VAMP-3*, *-4*, *-7*, and *-8* and *SNAP-23* in unstimulated control and *junB* $^{-/-}$ BMMC were assessed by quantitative RT-PCR analysis. The expression levels were normalized to the housekeeping gene β -actin. Data were obtained from three independent analyses. *C*, Re-expression of *SWAP-70* upon retroviral gene transfer. Immunoblotting shows reduced levels of *SWAP-70* in *junB* $^{-/-}$ BMMC that can be restored to levels in control cells by retroviral transduction of *SWAP-70* but not of empty control vector. *D*, Degranulation phenotype can be partly restored by reintroduction of *SWAP-70* in *junB* $^{-/-}$ BMMC. IgE-sensitized control, *junB* $^{-/-}$ and retrovirally transduced BMMC (empty vector or *SWAP-70*) BMMC were activated with 100 ng/ml DNP. The degranulation efficiency was calculated by measuring the relative release of β -hexosaminidase. The data represent the means $\pm$ SEM of three independent experiments.



21). It was therefore evident to ask whether loss of JunB does also affect the cytokine expression of mast cells. Thus, we analyzed a variety of cytokines and growth factors for their transcriptional induction upon IgE/DNP activation in control and *junB* $^{-/-}$ BMMC by quantitative RT-PCR (Fig. 4A) using primers specific for *IL-3*, *IL-4*, *IL-5*, *IL-6*, *IL-10*, *IL-13*, *GM-CSF*, *MIP-1 α* , and *TNF- α* . Except for *GM-CSF*, whose mRNA levels were already increased in unchallenged *junB* $^{-/-}$ mast cells, the basal expression of all cytokines and growth factors analyzed did not differ in control and *junB* $^{-/-}$ BMMC (data not shown). But, upon IgE-mediated stimulation we found a strong impact of JunB ablation on transcriptional induction of all cytokines and growth factors examined with the exception of *IL-5* and *IL-13*. The induction of *IL-6* and *GM-CSF* mRNA was strongly increased while expression of *IL-3*, *IL-4*, *IL-10*, *MIP-1 α* and *TNF- α* was diminished in *junB* $^{-/-}$

BMMC (Fig. 4A). By contrast, induction of other mast cell-specific transcripts such as β -hexosaminidase, carboxypeptidase A and the microphthalmia transcription factor MITF were not altered in *junB* $^{-/-}$ mast cells (data not shown).

The overall production of mast cell-derived cytokines and growth factors is a result of different processes over time such as the basal expression of factors loaded in the granules, degranulation efficiency and the prolonged production and release of these factors after Fc ϵ RI stimulation. To prove our hypothesis that the cytokine and growth factor profile of *junB* $^{-/-}$ mast cells is severely affected, we measured the amount of cytokine produced, stored and released in control and *junB* $^{-/-}$ mast cells post Fc ϵ RI stimulation (Fig. 4B).

In agreement with unaltered β -hexosaminidase granule loading, we detected by ionophore treatment in *junB* $^{-/-}$ BMMC normal granule loading for IL-6 and IL-10 (data not shown) as examples for cytokines that are transcriptionally up- or down-regulated, respectively. However, degranulation of these two cytokines was impaired in *junB* $^{-/-}$ BMMC (data not shown and Fig. 4B, SN at 1 h).

To determine in the late phase of BMMC activation the amounts of newly synthesized IL-6 and IL-10, we replaced the growth medium 1 h 45 min after activation with fresh medium. 6 h 45 min later (8 h post induction) we measured the amount of released IL-6 (Fig. 4B, SN at 8 h) and IL-10 (data not shown). Another 45 min later the remaining vesicle-stored IL-6 (Fig. 4B, cells plus ionomycin at 8 h 45 min) and IL-10 (data not shown) quantities were analyzed upon ionomycin-triggered retrieval. For IL-10 we could approve the diminished transcription on the protein level (data not shown), whereas for IL-6, at first glance, the robust increase of IL-6 transcripts present in *junB* $^{-/-}$ BMMC was not reflected in the secreted protein levels present in the supernatant. However, IL-6 protein levels retrieved from the vesicles were 8-fold increased when compared with control cells. Altogether, the total amount of IL-6 protein (secreted plus vesicle-stored; Fig. 4B, right panels) is 2.5-fold higher in *junB* $^{-/-}$ BMMC and, thus, reflects the increase in IL-6 mRNA levels caused by loss of JunB. In summary, these data demonstrate an important role of JunB in proper

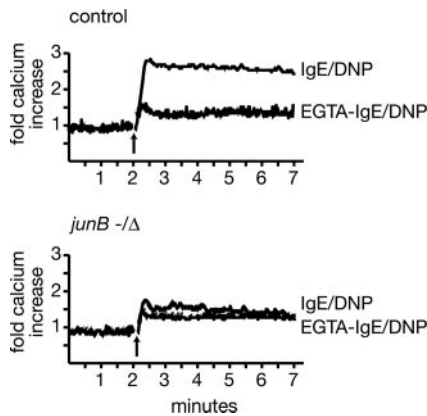


FIGURE 3. Impaired calcium influx in *junB* $^{-/-}$ BMMC. Calcium levels were assessed by FACS analysis using the calcium-indicative dyes fluo-4 and FuraRed. One representative measurement of calcium release after Fc ϵ RI-mediated activation of control and *junB* $^{-/-}$ BMMC upon treatment of 100 ng/ml DNP is shown (IgE/DNP). Intracellular calcium levels were determined upon treatment of BMMC with 5mM EGTA prior to activation with 100 ng/ml DNP (EGTA-IgE/DNP). The arrows indicate the time when the stimulus was added.

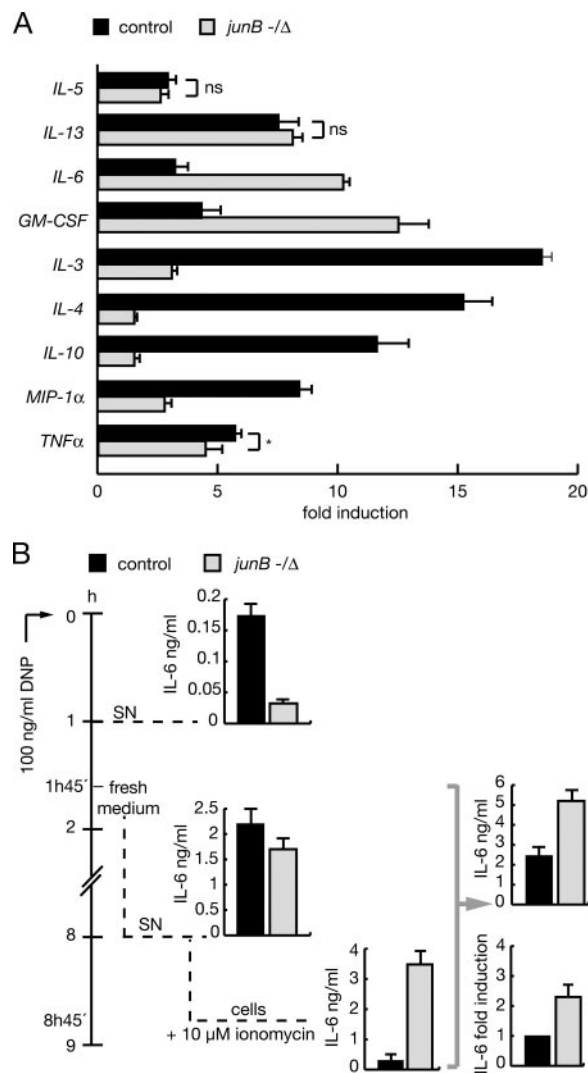


FIGURE 4. FcεRI-mediated induction of cytokine mRNAs in BMMC is JunB-dependent. *A*, Transcriptional induction of various cytokines in control and *junB*^{-/-Δ} BMMC was investigated 6 h post activation with 100 ng/ml DNP by quantitative RT-PCR analysis. Expression levels of cytokines were normalized to the housekeeping gene β -actin. The graph shows the fold-increase in expression. Data were obtained from three independent experiments that were each performed in triplicates. *B*, Determination of IL-6 protein secreted over time and stored in vesicles upon BMMC activation by cytometric bead array (CBA) analysis. *Left panels*, levels of secreted IL-6 were measured in supernatants (SN) of control and *junB*^{-/-Δ} BMMC at 1 h and subsequent medium change (at 1 h 45 min) at 8 h post IgE-mediated activation (*middle panel*), to determine the amount of vesicle stored IL-6, BMMC were harvested 8 h 45 min post stimulation and resuspended in medium containing 10 μ M ionomycin to retrieve IL-6 from all secretory granules. *Right panels*, the amount of newly synthesized IL-6 as sum of protein measured in SN plus IL-6 retained within cytoplasmic granules is depicted as absolute numbers but also as fold induction over expression in control cells which was set to 1 (*right panel, top*). The data represent the means $\pm$ SEM of three independent experiments. (*, $p < 0.01$, ns = not significant).

synthesis and secretion of various cytokines, which are essential for adequate mast cell function.

The communication of mast cells with endothelial cells is JunB dependent

Since mast cells are a source of various mediators with angiogenic properties, we wondered whether hampered protein

production and secretion caused by JunB deficiency causes impairment in angiogenesis induction. For this purpose, we studied the communication of BMMC with endothelial cells in a capillary-like tube formation assay. Previously it has been shown that the supernatant of activated mast cells is sufficient to induce capillary-like tube formation of endothelial cells on matrigel in vitro (41, 42). Therefore, we cultured wild-type primary endothelial cells on matrigel in the presence of supernatant medium from control or *junB*^{-/-Δ} BMMC, 6 h post IgE stimulation and analyzed the formed capillary-like structures 24 h after addition of the mast cell supernatant (Fig. 5*A, left panel*). Counting interendothelial spaces revealed that induction of endothelial tube formation by *junB*^{-/-Δ} BMMC was reduced by >50% compared with the control (Fig. 5*A, right panel*).

VEGF-A and angiopoietin-1 may represent candidate factors responsible for the induction of capillary-like tube formation in this assay as both factors are major proangiogenic molecules and can be secreted by mast cells (42, 43). Thus, we determined for both factors the mRNA levels in control and *junB*^{-/-Δ} BMMC 6 h post FcεRI stimulation by quantitative RT-PCR. VEGF mRNA induction in *junB*^{-/-Δ} BMMC (3.5-fold) was >6-fold reduced compared with activated control BMMC (21.6-fold induction; Fig. 5*B*). In contrast, FcεRI-mediated induction of angiopoietin-1 mRNA did not significantly differ between control and *junB*^{-/-Δ} BMMC. These data are underscored by the diminished amount of VEGF protein, which is produced and secreted by IgE-activated *junB*^{-/-Δ} BMMC when compared with control cells. Although control BMMC secreted >935 pg/ml (± 34 pg/ml) VEGF 6 h post stimulation, VEGF concentration in the supernatant of FcεRI stimulated *junB*^{-/-Δ} mast cells was only 84 pg/ml (± 17 pg/ml) (Fig. 5*C*) and did not increase 24 h post activation (data not shown). In line with these findings, supernatant from control BMMC treated with a neutralizing Ab against VEGF failed to induce capillary-like tube formation of endothelial cells similar to supernatant of *junB*^{-/-Δ} BMMC (Fig. 5*D*). Thus, our data demonstrate that the diminished VEGF levels due to loss of JunB are not sufficient for normal tube formation process and are responsible for the phenotype caused by *junB*^{-/-Δ} BMMC in the in vitro angiogenesis assay.

Taken together, these data demonstrate that JunB is required for the IgE-dependent production and release of mast cell derived factors necessary for the communication of mast cells with endothelial cells.

Discussion

In the present study, we describe the phenotype of in vitro differentiated *junB*^{-/-Δ} BMMC. The general characterization of in vitro differentiated *junB*^{-/-Δ} BMMC revealed no change in CD117 and FcεRI surface expression demonstrating that loss of JunB has neither impact on the development of mature mast cells nor on proper trafficking of mast cell membrane proteins from the endoplasmic reticulum to the cell membrane. In addition, the cell cycle profile in *junB*^{-/-Δ} mast cells was unchanged and apoptosis induction upon IL-3 withdrawal was similar to control BMMC. In contrast, key features of mast cell function, such as degranulation upon IgE-mediated activation, and overall production of mast cell-derived cytokines and growth factors was severely affected. The observed degranulation phenotype cannot be explained by impaired loading of intracellular calcium stores as *junB*^{-/-Δ} BMMC pretreated with EGTA and stimulated with ionophore (data not shown) did not exhibit a difference in intracellular calcium raise when compared with control cells. As a consequence of the deregulated cytokine and growth factor profile, the fine tuned communication of BMMC with endothelial cells is strongly impaired

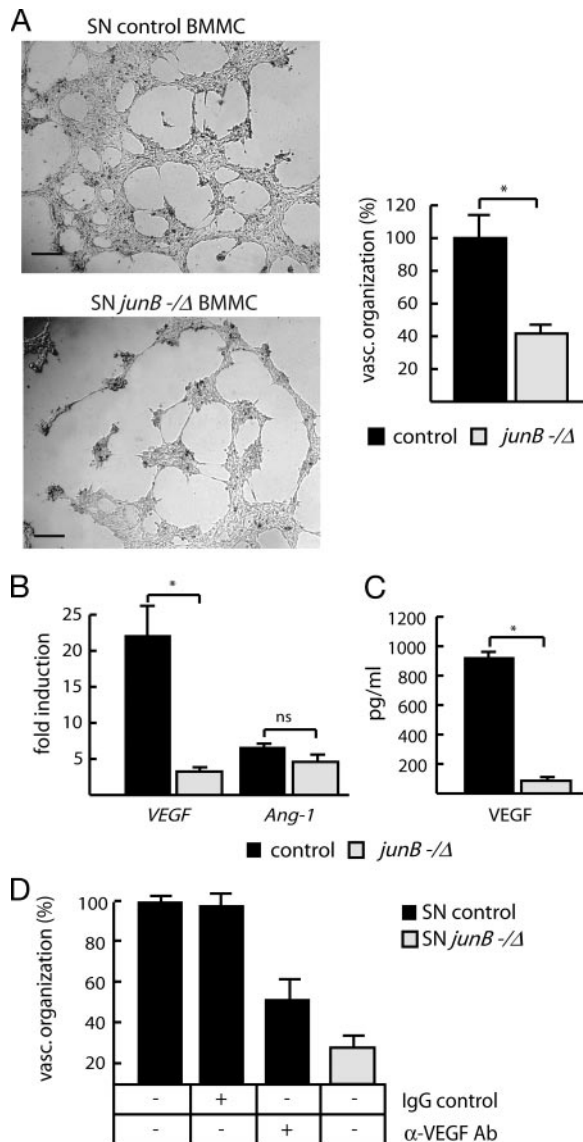


FIGURE 5. Mast cell-mediated angiogenesis requires JunB. **A**, Capillary-like tube formation of lung endothelial cells cultivated in the presence of the supernatant from activated control or *junB* Δ/Δ BMMC. BMMC were sensitized by 1 μ g/ml IgE-DNP over night and activated with 100 ng/ml DNP for 6 h. Supernatants of control and *junB* Δ/Δ BMMC were collected and used as incubation medium for endothelial cells (left panel). Quantification of tube formation assays (right panel): 24 h after addition of BMMC supernatant capillary-like structures formed by wild-type endothelial cells were counted in 6 randomly chosen optical fields. Data of one representative experiment carried out in triplicates are given. Experiment was reproduced twice. **B**, Induction of *VEGF* and *angiopoietin-1* mRNA in control and *junB* Δ/Δ BMMC upon IgE/DNP-activation with 100 ng/ml DNP for 6 h was determined by quantitative RT-PCR. The data were obtained from two independent experiments that were carried out in triplicates. Significantly impaired induction of *VEGF* but not of *angiopoietin-1* is detected in *junB* Δ/Δ BMMC. **C**, Quantitative measurement of secreted VEGF protein of 2×10^6 IgE-sensitized control and *junB* Δ/Δ BMMC activated with 100 ng/ml DNP for 6 h. Supernatant was harvested at the indicated time points. The release of VEGF was detected by ELISA analysis of supernatants and revealed significant impaired VEGF protein levels in *junB* Δ/Δ BMMC. (*, $p < 0.01$, ns = not significant). **D**, Quantification of three independent capillary-like tube formation assays of wild-type endothelial cells as described in **A** cultivated with supernatant from activated control BMMC (black bars) in the absence or presence of control (IgG control) or neutralizing antibody against VEGF (α -VEGF Ab). For comparison, wild-type endothelial cells were incubated with *junB* Δ/Δ BMMC (grey bars).

when JunB is ablated as shown by the disturbed tube formation capability of endothelial cells in the presence of *junB* Δ/Δ BMMC supplemented medium.

The process of degranulation is tightly controlled and dependent on factors controlling calcium triggering (8, 44), such as SWAP-70 (39), proteins sensing the calcium concentration in the cytosol such as SYTs (45), and proteins forming the SNARE complex such as VAMPs and SNAPs (46). SYT-1 mediates regulated exocytosis by acting as a calcium sensor triggering membrane fusion upon calcium influx into the cytosol (45). SWAP-70 localizes to membrane ruffles, the site of membrane rearrangements, and plays an important role in mast cell development and *c-kit* (CD117)-mediated signaling (39, 40, 47). SWAP-70 knockout mice have reduced numbers of mast cells pointing to an important role of this signaling molecule in mast cell development (40). Additionally, SWAP-70 deficient BMMC are strongly impaired in calcium efflux upon *c-kit* stimulation and in Fc ϵ RI-mediated degranulation (39, 40). We found strongly reduced SWAP-70 mRNA and protein levels in *junB* Δ/Δ BMMC. However, mutant mast cells differentiate normally and mast cell numbers in vivo do not differ from control mice. We, therefore, assume that the residual SWAP-70 protein found in *junB* Δ/Δ mast cells is sufficient to overcome the maturation defect described for the complete SWAP-70 knock out (40).

Reduced SWAP-70 levels in *junB* Δ/Δ BMMC may explain the observed diminished rise in intracellular calcium levels upon IgE stimulation and concomitantly the impaired degranulation capability. Our detailed analysis with calcium chelator pretreatment as well as ionophore-triggered exocytosis show that JunB-dependent SWAP-70 is required to enable influx of extracellular calcium into the cytosol. These findings are in line with previously reported normal ionomycin-triggered degranulation of SWAP-70 $-/-$ BMMC (39). The involvement of SWAP-70 in the JunB-dependent degranulation defect is evidenced by a 50% restoration upon SWAP-70 re-expression. A complete rescue upon SWAP-70 re-expression could not be achieved, most likely due to reduced SYT-1 and VAMP-8 expression hampering calcium sensing and SNARE complex formation, respectively. In addition, other components of the vesicle fusion machinery might be deregulated and have to be investigated in detail in future studies. However, remaining SYT-1 and VAMP-8 is sufficient to induce calcium-triggered exocytosis when the cell is flooded by calcium due to ionophore treatment. Altogether, the defects in SWAP-70 and SYT-1 expression may account for the diminished degranulation capability due to JunB ablation, albeit, at the current state, we cannot rule out defects in other pathways involved in the degranulation process (48) caused by the loss of JunB.

As a consequence of Fc ϵ RI stimulation, expression of mast cell derived inflammatory (5, 17, 49) and angiogenic factors (50) is induced. These factors are synthesized in the course of a prolonged IgE-mediated mast cell response and are important for sustained mast cell-dependent physiological responses, such as innate immune response (51), allergy-associated inflammation (2, 52), inflammatory arthritis (1), angiogenesis in allergic disease (53) and tumor angiogenesis (54–56). Our data revealed a severely deregulated transcriptional cytokine expression profile of stimulated *junB* Δ/Δ BMMC. Although some interleukins, such as *IL-5* and *IL-13*, are normally induced upon IgE/DNP-activation, strong JunB-dependent transcriptional regulation could be demonstrated for *IL-3*, *IL-4*, *IL-10*, *MIP-1 α* , and *TNF- α* , being reduced in mutant cells and for *IL-6* and *GM-CSF*, which exhibit superinduction in IgE-activated *junB* Δ/Δ mast cells.

The observed deregulation in the cytokine pattern is in line with previous studies on JunB ablation in other cell types. Enhanced

transcriptional induction of *GM-CSF* upon loss of JunB has been described for JunB-deficient fibroblasts studied in a skin equivalent model in vitro (21). In addition, cutaneous wound healing experiments performed with *junB* $-/\Delta^{Col1\alpha2-cre}$ mice demonstrated enhanced *GM-CSF* transcripts levels in unchallenged skin as well as enhanced induction upon wounding (20). Increased *GM-CSF* and *IL-6* induction can be explained by the antagonistic function of JunB on c-Jun/c-Fos dimers that have been shown to positively regulate these two genes (57–60). Up-regulated *IL-4* transcription and normal *IL-13* induction observed in the present studies in mast cells reflects the cytokine pattern of JunB-deficient CD4⁺ T cells in vitro polarized to the Th2 direction (22). However, for mast cells we did not detect the diminished *IL-5* expression observed in JunB-ablated Th2 cells (22). This difference in *IL-5* regulation in mast cells and CD4⁺ cells might be explained by the well known cell type specificity of JunB action and the diversity of AP-1 transcription factor complex composition (19).

In both, JunB- and c-Fos-deficient mast cells, induction of *IL-10* upon IgE-dependent stimulation was decreased on transcriptional level (16), indicating that it is commonly activated by JunB and c-Fos. However, except for *IL-10*, all other cytokines examined as well as *MITF* and *VAMP-7* are differently regulated in JunB- and c-Fos-mutant mast cells (16) implying that at least for these AP-1 dependent genes there is no concerted action of JunB and c-Fos. We could also rule out a direct impact of JunB on Fos levels and NFAT activity (data not shown).

The late response of FcεRI stimulation, namely synthesis and release of various cytokines is severely affected in *junB* $-/\Delta$ BMMC. Reduction in *IL-10* protein levels reflects the impaired transcriptional induction found in stimulated *junB* $-/\Delta$ mast cells.

Although IL-6 is transcriptionally superinduced, IL-6 protein is only poorly secreted from *junB* $-/\Delta$ BMMC. However, the vast amount of IL-6 protein can be retrieved from secretory vesicles by ionophore treatment. Thus, the total amount composed of secreted plus vesicle-retained IL-6 reflects the transcriptional superinduction on protein level. Presumably, the poor IL-6 secretion is due to a failure in exocytosis. This process involves tethering of transport vesicles to the membrane and their subsequent docking mediated by Rab GTPases and the SNARE proteins, respectively (61, 62). Analysis of different components of the SNARE complex revealed a severe impairment in VAMP-8 expression. Based on its presence on mediator containing secretory granules, a participation of VAMP-8 in mast cell degranulation has been suggested (63). VAMP-8 also couples with SYT proteins and thus, appears to be involved in the terminal step of cytokinesis in mammalian cells (64).

In recent years it became clear that mast cells are important players for angiogenesis during inflammation and allergic diseases (65), wound healing (66), rheumatoid arthritis (67) and tumorigenesis (4, 50, 53). Moreover, mast cell-deficient mice exhibit a decreased rate of tumor-associated angiogenesis (55) and are much less susceptible to the development of experimentally induced intestinal tumors (56). In this context, mast cells communicate with endothelial cells by releasing proangiogenic factors such as VEGF, basic fibroblast growth factor, angiopoietin-1, and matrix metalloproteinases (41–43, 68–70). We hypothesized that proper cell-cell communication is disturbed in JunB-ablated mast cells as a consequence of the deregulated production of mast cell-mediated protein factors. In support with this hypothesis, we could demonstrate that *junB* $-/\Delta$ BMMC are unable to induce tube formation in endothelial cells and that this is most probably due to the attenuated VEGF production (68, 69, 71). This is in line with previous data demonstrating that JunB is required for basal, hypoxia and hypoglycemia-induced VEGF expression in endothelial cells, fibroblasts and embryonic stem cells (24–26). However, the tran-

scriptional induction of another angiogenic factor such as *angiopoietin-1* is not affected mast cells lacking JunB. This could explain the residual capability of *junB* $-/\Delta$ BMMC to induce at least some branching in the wild-type endothelial cells in the tube formation assay.

Taken together, aside from JunB's role as a negative regulator for previously described AP-1-dependent genes, we have identified novel target genes in mast cells, which are commonly activated by JunB and c-Fos, such as *SWAP-70* and *SYT-1*. Furthermore, we could assign JunB important functions in mast cell biology and cellular communication. The inability of *junB* $-/\Delta$ BMMC to degranulate and the deregulated cytokine profile predicts severe consequences for mast cell-dependent physiological and pathophysiological processes such as immune responses and tumor progression. Application of appropriate mouse models in the future will unravel the role of JunB in paracrine mast cell signaling in the organism.

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Disclosures

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