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*J Immunol* 2006; 177:6667-6674; doi: 10.4049/jimmunol.177.10.6667
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Specific and Redundant Roles for NFAT Transcription Factors in the Expression of Mast Cell-Derived Cytokines

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By virtue of their ability to express a plethora of biologically highly active mediators, mast cells (MC) are involved in both adaptive and innate immune responses. MC-derived Th2-type cytokines are thought to act as local amplifiers of Th2 reactions, including chronic inflammatory disorders such as allergic asthma, whereas MC-derived TNF-α is a critical initiator of antimicrobial defense. In this study, we demonstrate that the transcription factors NFATc1 and NFATc2 are part of a MC-specific signaling network that regulates the expression of TNF-α and IL-13, whereas NFATc3 is dispensable. Primary murine bone marrow-derived MC from NFATc2−/− mice, activated by either ionomycin or IgE/Ag cross-link, display a strong reduction in the production of these cytokines, compared with bone marrow-derived MC from wild-type mice. Detailed analyses of TNF-α and IL-13 expression using small interfering RNA-mediated knockdown reveals that both NFATc2 and NFATc1 are able to drive the expression of these cytokines, whereas neither degranulation nor the expression of IL-6 depends on NFAT activity. These results support the view that high NFAT activity is necessary for TNF-α and IL-13 promoter induction in MC, irrespective of whether NFATc2 or NFATc1 or a combination of both is present. The Journal of Immunology, 2006, 177: 6667–6674.

It is increasingly being recognized that mast cells (MC), mostly due to their ability to produce a large panel of lipid mediators, biogenic amines, cytokines, and chemokines, are important organizers of a variety of inflammatory reactions. In addition to IgE/Ag-mediated activation, MC are also able to respond to diverse IgE-independent stimuli, thereby participating in both innate and adaptive immune responses (1–3). The arsenal of MC-derived cytokines includes Th2-type cytokines, such as IL-9 and IL-13, which play critical roles in allergic inflammation, as well as the prototypic proinflammatory cytokine TNF-α (4–6). As such unique feature, MC are able to store preformed TNF-α, which can be released on demand within minutes to initiate lifesaving antimicrobial reactions (7–9).

The family of five NFAT transcription factors comprises the four genuine members NFATc1, NFATc2, NFATc3, and NFATc4, as well as the distantly related NFAT5 (TonEBP). With the exception of NFAT5, which is ubiquitously expressed and activated in response to osmotic stress, nuclear translocation and activation of NFAT proteins is induced by the Ca²⁺-calmodulin-dependent phosphatase calcineurin (10–12). Expression of multiple members of the NFAT family in MC was already reported, including NFATc1, NFATc2, and NFATc3 (13–16). Interestingly, targeted disruption of individual Nfat genes in mice leads to the development of quite disparate phenotypes. NFATc1-deficient T lymphocytes display reduced proliferation and IL-4 production, finally leading to impaired Th2 responses (17, 18). In contrast, mice deficient for NFATc2 mount enhanced Th2 responses (19–21), whereas mice bearing an inactivated Nfatc3 gene are phenotypically inconspicuous and express normal cytokine levels (22). Remarkably, double deficiency for both NFATc2 and NFATc3 causes massive lymphadenopathy, splenomegaly, blepharitis, and pneumonitis, which ultimately leads to the death of the affected animals 8–10 wk after birth. At the cellular level, combined NFATc2/NFATc3 deficiency leads to a robust increase in peripheral T cells with an activated phenotype. In addition, T cells from such mice show costimulation-independent TCR-mediated activation, intrinsically differentiate into Th2 cells, and overexpress Th2 cytokines (23, 24). Recently, it was suggested that the fatal phenotype of NFATc2/NFATc3-deficient mice might at least partly be due to the resistance of CD4⁺ T cells to suppression mediated by CD4⁺CD25⁺ regulatory cells (25). Based on these findings, it can be concluded that both NFATc2 and NFATc3 exert overlapping regulatory or inhibitory functions, thereby contributing to lymphoid homeostasis.

The role of distinct NFAT family members in T cell development and function is being studied intensively, whereas comparable information on MC has been sparse. In this study, we report that both NFATc1 and NFATc2 are critically involved in the expression of MC-derived IL-13 and TNF-α, but not IL-6.

Materials and Methods

Mice

NFATc2−/− mice were created in our laboratory (26) or provided by L. Glimcher (Harvard University, Boston, MA), along with NFATc3−/− mice, all on a BALB/c genetic background. NFATc2−/−c3−/− (DKO) mice were obtained by intercrossing these lines accordingly. All mice were used at the age of 6–8 wk.

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Received for publication December 19, 2005. Accepted for publication August 29, 2006.

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1 This work was supported by Deutsche Forschungsgemeinschaft Grant SCHM1001/4/2 (to E.S. and M.S.) and Sonderforschungsbereich Grant 548/10 (to M.S.).

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3 Abbreviations used in this paper: MC, mast cell; SCF, stem cell factor; CsA, cyclosporin A; BMMMC, bone marrow-derived MC; qRT-PCR, quantitative RT-PCR; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA.

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Cytokines, cytokine assays, and mAbs

Murine IL-3 was isolated from supernatants of myelomonocytic WEHI-3B cells using DEAE chromatography. The cDNA of his-tagged murine stem cell factor (SCF) provided by G. W. Bornkamm (GSF-National Research Center for Environment and Health, Institute of Clinical Molecular Biology and Tumor Genetics, Munich, Germany) (27) was expressed in Escherichia coli, and SCF was affinity purified using the QIAexpress system, according to the manufacturer’s directions (Qiagen). The biological activity of SCF was verified using a proliferation assay measuring \[^{3}\text{H}]\text{thymidine incorporation by MC. Murine rIL-4 was a gift from Dr. W. Müller (German Research Center for Biotechnology, Department of Experimental Immunology, Braunschweig, Germany). For the detection of IL-6 via ELISA, we used purified rat anti-mouse IL-6 and biotinylated anti-mouse IL-6. Standard curves were obtained using known amounts of rmIL-6 (all from BD Pharmingen). IL-13 was detected by ELISA with MAP413 and BAF413 using known amounts of rmIL-13 as reference (R&D Systems). Cyclosporin A (CsA), ionomycin, and p-nitrophenyl-N-acetyl-\(\beta\)-\(\alpha\)-glucosamine were purchased from Sigma-Aldrich.

Biological activity of TNF-\(\alpha\) was measured using WEHI-164 target cells as described previously (28). In addition, the following Abs were used: anti-CD3 mAb 145-2C11 (29), anti-CD28 mAb 37.51 (30), and anti-INF-\(\gamma\) mAb XMG1.2 (31). When required, mAbs were purified using protein G-Sepharose (Amersham Biosciences). Proleukin (hrIL-2) was purchased from Chiron (Ratigen).

Cytokines, \(\beta\)-deficient mice, and Th2 cells

For the generation of BMCC, mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and BM cells were harvested by repeat flushing with MEM (32). Cell cultures were established at a density of \(5 \times 10^6\) cells/ml in IMDM supplemented with 10% FCS (inactivated at 56°C), 2 mM l-glutamine, 1 mM pyruvate, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 20 U/ml IL-3, 50 U/ml IL-4, and 200 ng/ml SCF. Non-adherent cells were transferred to fresh culture plates every 2–3 days for a total of at least 21 days to remove adherent macrophages and fibroblasts.

![FIGURE 1.](http://www.jimmunol.org/)

Th2 cells from NFATc2/c3 double-deficient (DKO) mice display an increased production of IL-13, whereas expression of MC-derived IL-13 is severely impaired. A, Th2 cells and BMCC were prepared from DKO and wild-type mice as described in Materials and Methods. Th2 cells were restimulated with a combination of plate-bound anti-CD3 and anti-CD28 Abs; BMCC were activated with ionomycin. After 24 h, cytokine concentrations were determined in the culture supernatants via ELISA. B, BMCC derived from NFATc2-deficient (C2KO), NFATc3-deficient (C3KO), or NFATc2/c3 double-deficient (DKO) animals were sensitized with IgE, and degranulation was induced by the addition of anti-IgE. Shown are representatives of at least three independent experiments, each performed in triplicate (±SD).

![FIGURE 2.](http://www.jimmunol.org/)

NFATc2 deficiency impairs expression of MC-derived IL-13 and TNF-\(\alpha\), but does not affect production of IL-6. BMCC from wild-type (\(\bullet\)), NFATc2\(-/-\) (\(\square\)), NFATc3\(-/-\) (\(\bigcirc\)), and NFATc2/c3 double-deficient mice (\(\bigcirc\)) were activated by IgE receptor cross-link (A) or stimulated with ionomycin (B). IL-13 and IL-6 concentrations were determined 24 h poststimulation in the supernatants via ELISA. TNF-\(\alpha\) was assessed 4 h after activation using a WEHI-164 bioassay. Shown is one representative of at least three independent experiments, each performed in triplicates (±SD). n.d., Not detectable.
NFATc2 deficiency diminishes the production of TNF-α and IL-13 mRNA. BMMC derived from either wild-type or NFATc2−/− mice were activated with ionomycin in the presence or absence of CsA, and RNA was prepared at the indicated time points. Expression of TNF-α mRNA (A) and IL-13 mRNA (B) was followed by qRT-PCR as detailed in Materials and Methods. TNF-α and IL-13 mRNA levels in wild-type BMMC after 1 h of stimulation were arbitrarily set at 100%. Shown are the means (±SD) from three experiments.

FACS analyses using an anti-CD13 Ab (R3-242; BD Pharmingen) (33) and IgE plus anti-IgE mAb (34, 35) as well as May-Grünwald-Giemsa and toluidine blue staining revealed that the resulting cell population consisted of ~99% BMMC (data not shown). For stimulation of BMMC via their FceR, MC were incubated with 5 μg/ml IgE-anti-DNP Ab A2 (34, 35) for 48–72 h. After washing, cross-linking was performed by 2.5 μg/ml IgE anti-DNP Ab A2 using 3 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate, (pH 4.5) was added, and the plates were incubated for 90 min at 37°C. The reaction was stopped by the addition of 150 μl of 0.2 M glycine (pH 10.7). Hydrolysis of the substrate was measured at 405 nm. β-Hexosaminidase activity in supernatant and lysate were added and defined as the total enzyme content. The results are expressed as percentage of β-hexosaminidase activity released into the medium.

Quantitative RT-PCR (qRT-PCR)

RNA was isolated using TRIzol (Invitrogen Life Technologies), and cDNA was synthesized with RevertAid M-murine leukemia virus reverse transcriptase following the recommendations of the supplier (MBI Fermentas). qRT-PCR was performed using the following oligonucleotides: HGPRT (forward), GGT GTA TAC AGG CCA GAC TTT GTT G; HGPRT (reverse), GAG GGT AGG CTG GCC TAT AGG CT; TNF-α (forward), GGT CGA ATC TGG CTG CAG GGG; TNF-α (reverse), GGT CGA TGC AGG ACC GGT GGG; IL-13 (forward), GCC AGA GTC CTG AGA GAT AC; IL-13 (reverse), GGT CCT GTA GAT GGC ATT GCA; NFATc1 (forward), TGC CCT TGA CTC GCA CCT CC; and NFATc1 (reverse), AAT GAA CAG CTG NTAG GTG GAG. For detection of NFATc2 mRNA, the QuantiTect primer assay Mm_Nfatc2_cSG (Qiagen) was used. PCR was performed on an iCycler (Bio-Rad) using ABssolute SYBR Green fluorescent (ABgene). Data were normalized according to the expression of HGPRT, and relative mRNA expression levels were calculated.

Transfection of BMMC

NFATc1/A cDNA was cloned into the expression vector pLP.G3. BMMC (2–3 × 106 cells in 0.2 ml of serum-free IMDM) were transfected with either 2 μg of NFATc1/A or empty vector by electroporation in 0.4-cm cuvettes at room temperature using a Bio-Rad Gene Pulser II set at 290 V and 600 μF. Cells were allowed to recover in medium for 16–24 h, harvested, washed with IMDM, and stimulated as outlined in the figure legends. RNA knockdown experiments were performed using chemically synthesized and annealed small interfering RNAs (siRNAs) specific for NFATc1 and NFATc2 (Mm_Nfatc2_c.SiRNA purchased from Qiagen). siRNAs (1 μM) were delivered into BMMC by electroporation as described above. As a negative control, negative control siRNA 1 was used (Silencer; Ambion). In pilot experiments, transfection efficiency of fluorescently labeled siRNA was shown to be >70% (data not shown).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was conducted essentially as described previously (38). BMMC (2 × 106) were fixed for 7 min at room temperature with 1% formaldehyde. After incubation, glycine was added to a final concentration of 125 mM for 5 min. Cells were rinsed with cold PBS and resuspended in 0.2 M glycine (pH 10.7). Hydrolysis of the substrate was measured at 405 nm. β-Hexosaminidase release

Degranulation of MC was quantified by assaying the activity of the enzyme β-hexosaminidase as described previously (32). Briefly, BMMC were activated for 30 min at 37°C. The cells were spun down and lysed in 0.5% Triton X-100. Two aliquots of 20 μl from each supernatant and the corresponding lysate were transferred to separate plates. Fifty microliters of substrate solution (1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate, (pH 4.5)) was added, and the plates were incubated for 90 min at 37°C. The reaction was stopped by the addition of 150 μl of 0.2 M glycine (pH 10.7). Hydrolysis of the substrate was measured at 405 nm. β-Hexosaminidase activity in supernatant and lysate were added and defined as the total enzyme content. The results are expressed as percentage of β-hexosaminidase activity released into the medium.
**Results**

The effects of NFATc2/NFATc3 double deficiency in Th2 cells and MC are diametrically opposed with respect to the production of IL-13

Mice double deficient for NFATc2 and NFATc3 (DKO) mount increased Th2 responses, evidenced by overexpression of IL-4, IL-5, IL-6, and IL-10 (23, 24). This prompted us to investigate whether deficiency for both NFATc2 and NFATc3 also affects the production of MC-derived cytokines. To this end, we generated both BMMC and Th2 cells from DKO mice and assayed their IL-13 production upon stimulation (Fig. 1A). IL-13 production by DKO Th2 cells was found to be enhanced at least 10-fold, compared with their wild-type counterparts. In sharp contrast with this, production of IL-13 was severely impaired in BMMC derived from DKO mice (Fig. 1A). This finding led us to conclude that NFATc2/NFATc3 also negatively regulate the expression of IL-13 by Th2 cells but positively influence the production of MC-derived cytokines.

Activation of NFAT critically depends on an increase in intracellular Ca²⁺. Elevation of intracellular Ca²⁺ concentration is also necessary for rapid degranulation of MC, but early signaling events leading from the high-affinity IgE receptor to the exocytosis of stored granules do not rely on protein de novo synthesis (39). In line with this, we did not observe any impairment in degranulation of MC derived from DKO animals or mice deficient for either NFATc2 or NFATc3, compared with wild type (Fig. 1B).

NFATc3 is dispensable, but NFATc2 is critical for the production of MC-derived IL-13 and TNF-α

To investigate whether the presence of NFATc2, NFATc3, or a combination of both factors is required to ensure proper expression of MC-derived cytokines, we compared BMMC derived from NFATc2−/−, NFATc3−/−, DKO, and wild-type mice. NFATc2 deficiency severely impaired production of IL-13 and TNF-α by MC activated with either IgE cross-link (Fig. 2A) or calcium ionophor (Fig. 2B). In contrast, cytokine production by MC derived from NFATc3−/− mice was hardly affected, compared with wild-type BMMC. Irrespective of the stimulation method used, the expression of IL-6 remained unimpaired in all kinds of NFAT-deficient BMMC. Therefore, NFATc2, but not NFATc3, is an essential transcription factor for the expression of both IL-13 and TNF-α by MC. With respect to the NFATc2-mediated production of IL-13 by MC, our results corroborate a finding published previously on the positive effect of NFATc2 on the expression of IL-13 (40). It should also be noted that, in accordance with a previous publication, we did not observe an altered expression of NFATc1 or NFATc3 in BMMC derived from NFATc2-deficient mice (Ref. 14 and data not shown).

Besides NFATc2, NFATc1 also positively contributes to the expression of MC-derived IL-13 and TNF-α

To analyze the influence of NFATc2 on the expression of MC-derived cytokines in more detail, we first focused on the regulation of TNF-α and IL-13 production at the mRNA level. As depicted in...
and, hence, of NFAT activation. In line with this, induction of both cytokine mRNAs in BMMC derived from NFATc2$^{-/-}$ mice was strongly diminished, indicating that NFATc2 influences the expression of the Tnf-$\alpha$ and Il-13 genes at the level of transcription. Interestingly, residual production of TNF-$\alpha$ and IL-13 mRNA in NFATc2$^{-/-}$ BMMC was still sensitive to CsA, which suggested that other NFAT factors, i.e., NFATc1, also might positively contribute to the expression of TNF-$\alpha$ mRNA.

To rule out the possibility that the observed reduction of cytokine production by NFATc2$^{-/-}$ BMMC represents an epiphenomenon due to abnormal development of BMMC, we performed knockdown experiments using NFATc2-specific siRNA in BMMC derived from wild-type mice. As shown in Fig. 4A, delivery of specific siRNA into BMMC strongly reduced expression of NFATc2 mRNA, whereas the level of NFATc1 mRNA was unchanged. Inducible expression of TNF-$\alpha$ mRNA and IL-13 mRNA could be efficiently reduced by targeting NFATc2 using siRNA, whereas the expression of IL-6 mRNA was unaffected under these conditions (Fig. 4B).

To examine whether NFATc2 binds directly to the promoter regions of the Il-13 and Tnf-$\alpha$ genes, we performed ChIP analyses. As depicted in Fig. 5, inducible binding of NFATc2 to the promoter regions of both genes is detectable upon stimulation of BMMC and can be completely abrogated in the presence of CsA. Using chromatin derived from NFATc2$^{-/-}$ BMMC as additional control, all signal intensities fade into the background, as expected. Therefore, we conclude that NFATc2 acts in cis-on the expression of the Tnf-$\alpha$ and Il-13 genes.

Interestingly, ChIP analyses of TNF-$\alpha$ and IL-13 promoters also revealed the inducible and CsA-sensitive binding of NFATc1 in both BMMC derived from either wild-type or NFATc2$^{-/-}$ mice (Fig. 5). Binding of NFATc1 might account for CsA sensitivity in NFATc2$^{-/-}$ BMMC (see Fig. 3).

RNA interference technology was again used to study the effects of NFATc1-specific knockdown in BMMC from wild-type mice (Fig. 6). Fig. 6A shows that NFATc1 mRNA was reduced by $>$90%, whereas expression of NFATc2 mRNA was not inhibited. Furthermore, siRNA-mediated knockdown of NFATc1 severely impaired production of TNF-$\alpha$ and IL-13 mRNA, but not of IL-6 (Fig. 6B), clearly indicating that NFATc1 also plays a role for the expression of MC-derived TNF-$\alpha$ and IL-13. In addition, residual TNF-$\alpha$ and IL-13 mRNA production by NFATc2$^{-/-}$ BMMC was further reduced by knockdown of NFATc1 (Fig. 7A). Finally, expression of both TNF-$\alpha$ mRNA and IL-13 mRNA was efficiently up-regulated by overexpressing NFATc1 in BMMC derived from NFATc2$^{-/-}$ mice (Fig. 7B). Thus, we conclude that NFATc1 and NFATc2 are functionally equivalent with respect to the induction of TNF-$\alpha$ and IL-13 production, and that both are necessary to reach full expression levels. The expression of IL-6 mRNA remained unaffected under all conditions shown in Fig. 7B.

Discussion

MC-derived cytokines supposedly play decisive roles in shaping and maintaining chronic inflammatory disorders, including allergic asthma, a common illness in Western countries (4, 42). A detailed understanding of molecular mechanisms driving the expression of these cytokines might promote the development of novel therapeutic strategies. The family of NFAT transcription factors displays a complex mode of action on the regulation of cytokine gene expression. Studies using NFATc2$^{+/-}$c+-double-deficient mice strongly suggested that both transcription factors suppress the expression of Th2-type cytokines, as their combined absence leads ultimately to an enhancement of Th2 phenotype in vivo (23, 43). However, it is currently unclear how this negative effect is brought...
about Th2 cytokine expression. In transient transfection studies, NFATc2 has often been shown to be a strong transactivator of IL-4, IL-5 and of other Th2-type lymphokine genes (44), and in primary stimulations of NFATc2/H11002/H11002/H11002 T cells IL-4 RNA synthesis appeared to be impaired instead of enhanced (45). The strong enhancement of IL-4 synthesis by NFATc2/H11002/H11002/H11002 T cells was detected in long-term cultures or elder mice (19, 26), suggesting that, in these situations, NFATc2 and NFATc3 control Th2-type lymphokine expression through an NFATc2/NFATc3-dependent suppressor. This suppressor does not affect IL-13 synthesis in MC, because their lymphokine synthesis was found to be strongly reduced, and not enhanced, in the absence of NFATc2. Previous results also provide evidence that, in T cells, the absence of NFATc2 might be compensated for by NFATc3, implying that both proteins perform at least partly overlapping functions.

Th2-type cytokines are produced by both Th2 cells and MC, and both cell types were shown to be involved in the pathogenesis of allergic asthma, but the relative contribution of each cell type, especially at later stages of the disease, remains obscure. Interestingly, increased MC numbers can be observed in NFAT DKO mice, which can be hypothesized to support the development of an allergic phenotype (23). However, our results favor the interpretation that this increase in MC might be rather a secondary effect due to increased cytokine production by T cells. Furthermore, reconstitution experiments of MC-deficient mice with BMMC from NFATc2-deficient mice might be a valuable tool to study the role of MC-derived cytokines in the development of allergic asthma using MC-dependent models for the disease.

The experimental results of our study indicate that NFATc1 and NFATc2 play an essential role in controlling IL-13 and TNF-α expression in murine MC, while NFATc3 is dispensable. As in T cells, NFATc1 is expressed in murine MC, binds to the TNF-α and IL-13 promoters (see our ChIP assays in Fig. 5), and was shown to be transcriptionally active (49). Our results contradict those reported by Monticelli et al. (40), who reported that only NFATc2, but not NFATc1, is involved in IL-13 expression in MC. They conclude this from the fact that 1) ES cell-derived MC from NFATc1-deficient mice produced normal levels of IL-13, and that 2) a constitutively active mutant of NFATc1, introduced via retroviral transduction into BM cells used to generate BMMC, did not influence the expression of IL-13. Although NFATc1 is expressed in MC, the authors provide evidence that the difference between NFATc2 and NFATc1 might be due to a preferential synergistic interaction of NFATc2 with GATA proteins at the IL-13 promoter.

Individual members of transcription factor families often have overlapping functions, which might prevent the loss of important unique regulatory genes. NFATc1 and NFATc2 are the most prominent NFAT factors in peripheral T cells, and both contribute to activity of IL-2 promoter and of numerous other lymphokine promoters. Although inactivation of nfatc1 or nfatc2 gene alone does not affect IL-2 promoter activity, the inactivation of both genes abolished any IL-2 promoter activity in T cells (50). This indicates that a very low NFAT threshold level is necessary for IL-2 promoter induction in T cells. This might be different for cytokine induction in MC, where high NFAT threshold levels seem to be necessary for promoter induction. This means that loss...
of one NFAT factor, i.e., either NFATc2 or NFATc1, severely impairs promoter activity.

Disclosures

The authors have no financial conflict of interest.

References


FIGURE 7. Residual expression of TNF-α and IL-13 mRNAs in NFATc2-deficient BMMC is mostly mediated by NFATc1. A. MC from NFATc2-deficient mice were transfected with NFATc1-specific siRNA or scrambled control siRNA. qRT-PCR for TNF-α mRNA (1 h after treatment with ionomycin) and IL-13 mRNA (after 4 h) was performed. B. BMMC from NFATc2 −/− mice were transfected with either an expression vector encoding NFATc1/A or with the empty parental plasmid. At 24 h posttransfection, cells were stimulated with ionomycin and qRT-PCR for TNF-α mRNA (after 1 h) and IL-13 mRNA (after 4 h) was performed. Each figure shows the means ± SD of three independent experiments.
NFATc1/NFATc2 DRIVE THE EXPRESSION OF MC-DERIVED CYTOKINES


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