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# Specific and Redundant Roles for NFAT Transcription Factors in the Expression of Mast Cell-Derived Cytokines<sup>1</sup>

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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- $\alpha$  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- $\alpha$  and IL-13, whereas NFATc3 is dispensable. Primary murine bone marrow-derived MC from NFATc2<sup>-/-</sup> 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- $\alpha$  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- $\alpha$  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),<sup>3</sup> 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- $\alpha$  (4–6). As a unique feature, MC are able to store preformed TNF- $\alpha$ , 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 Ca<sup>2+</sup>-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<sup>+</sup> T cells to suppression mediated by CD4<sup>+</sup>CD25<sup>+</sup> T 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- $\alpha$ , but not IL-6.

## Materials and Methods

### Mice

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

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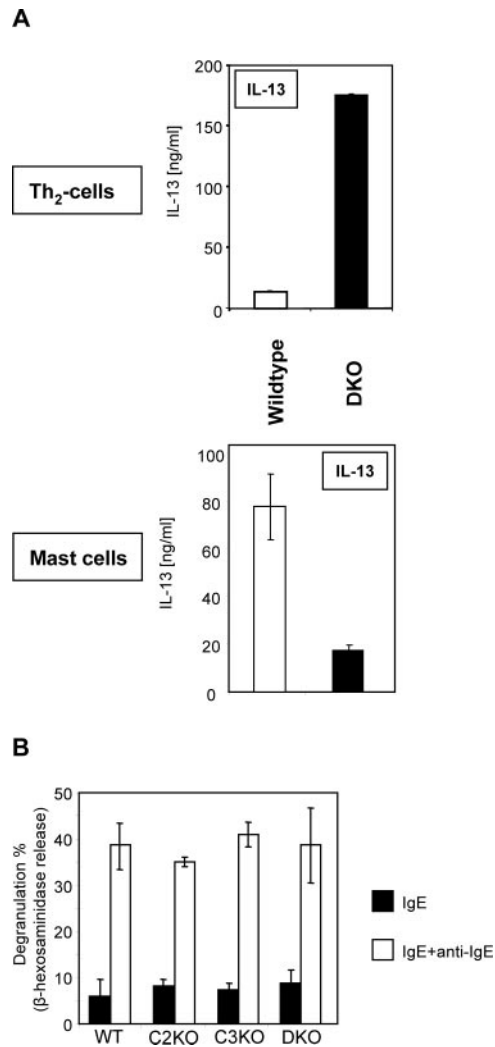
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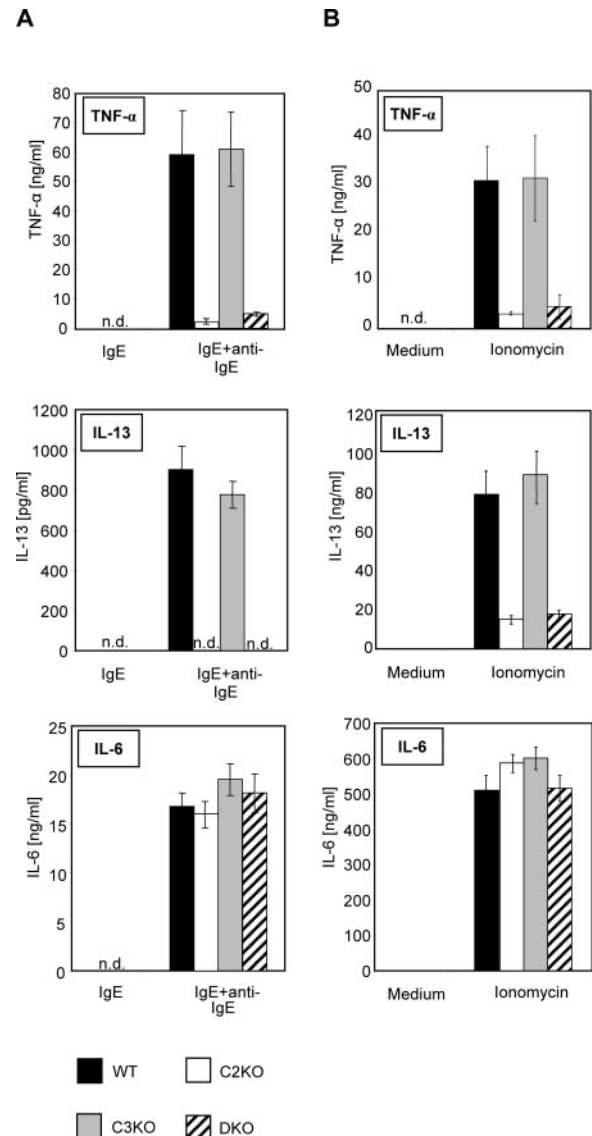
<sup>3</sup> Abbreviations used in this paper: MC, mast cell; SCF, stem cell factor; CsA, cyclosporin A; BMDC, bone marrow-derived MC; qRT-PCR, quantitative RT-PCR; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA.



**FIGURE 1.** 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 BMMC 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; BMMC were activated with ionomycin. After 24 h, cytokine concentrations were determined in the culture supernatants via ELISA. **B**, BMMC 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 ( $\pm$ SD).

#### 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 [<sup>3</sup>H]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 rIL-6 (all from BD Pharmingen). IL-13 was detected by ELISA with MAP413 and BAF413 using known amounts of rIL-13 as reference (R&D Systems). Cyclosporin A (CsA), ionomycin, and *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosamine were purchased from Sigma-Aldrich.

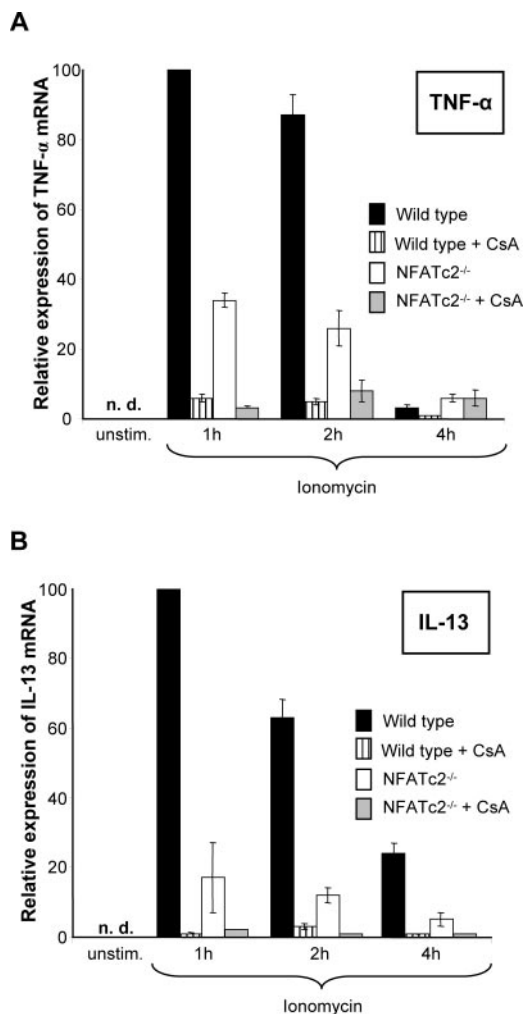


**FIGURE 2.** NFATc2 deficiency impairs expression of MC-derived IL-13 and TNF- $\alpha$ , but does not affect production of IL-6. BMMC from wild-type (■), NFATc2<sup>-/-</sup> (□), NFATc3<sup>-/-</sup> (▣), and NFATc2/c3 double-deficient mice (▨) were activated by IgE receptor cross-link (**A**) or 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 ( $\pm$ SD). n.d., Not detectable.

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-IFN- $\gamma$  mAb XMG1.2 (31). When required, mAbs were purified using protein G-Sepharose (Amersham Biosciences). Proleukin (hrIL-2) was purchased from Chiron (Ratingen).

#### Generation and activation of bone marrow-derived MC (BMMC) and Th2 cells

For the generation of BMMC, 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  $3 \times 10^6$  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 mIL-3, 50 U/ml mIL-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 3.** NFATc2 deficiency diminishes the production of TNF- $\alpha$  and IL-13 mRNA. BMMC derived from either wild-type or NFATc2<sup>-/-</sup> mice were activated with ionomycin in the presence or absence of CsA, and RNA was prepared at the indicated time points. Expression of TNF- $\alpha$  mRNA (A) and IL-13 mRNA (B) was followed by qRT-PCR as detailed in *Materials and Methods*. TNF- $\alpha$  and IL-13 mRNA levels in wild-type BMMC after 1 h of stimulation were arbitrarily set at 100%. Shown are the means ( $\pm$ 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 Fc $\epsilon$ R, MC were incubated with 5  $\mu$ g/ml IgE-anti-DNP Ab A2 (34, 35) for 48–72 h. After washing, cross-linking was performed by 2.5  $\mu$ g/ml anti-IgE mAb EM95.3 (36) in supplemented IMDM ( $10^6$  cells per ml). Alternatively, BMMC were stimulated in the presence of 1  $\mu$ M ionomycin. In some experiments, stimulation was performed in the presence of 300 ng/ml CsA following preincubation with this drug for 30 min.

CD4<sup>+</sup> T cells were isolated from spleens by positive selection using high-gradient magnetic cell separation in combination with MultiSort beads according to the manufacturer's instructions (MACS; Miltenyi Biotec). The CD4 sort was performed twice and resulted in a purity of CD4<sup>+</sup> T cells >99% (37). Cells were skewed toward Th2 by stimulation with plate-bound anti-CD3 mAb 145–2C11 and anti-CD28 mAb 37.51 in IMDM supplemented with 5% FCS (inactivated at 56°C), 2 mM L-glutamine, 1 mM pyruvate,  $5 \times 10^{-5}$  M 2-ME, 100 U/ml penicillin, 100 mg/ml streptomycin, 25 ng/ml rIL-4, and 10  $\mu$ g/ml anti-IFN- $\gamma$  mAb XMG1.2. On day 3, cells were harvested and cultured in the presence of 2.5 ng/ml rIL-4, 100 U/ml proleukin, and 100 U/ml rIL-6 for an additional 3 days. Differentiated Th2 cells ( $10^6$  per ml) were restimulated with a combination of plate-bound anti-CD3 and anti-CD28 Abs for 48 h.

### $\beta$ -Hexosaminidase release

Degranulation of MC was quantified by assaying the activity of the enzyme  $\beta$ -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  $\mu$ l 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- $\beta$ -D-glucosamine 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  $\mu$ l of 0.2 M glycine (pH 10.7). Hydrolysis of the substrate was measured at 405 nm.  $\beta$ -Hexosaminidase activity in supernatant and lysate were added and defined as the total enzyme content. The results are expressed as percentage of  $\beta$ -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), GTT GGA TAC AGG CCA GAC TTT GTT G; HGPRT (reverse), GAG GGT AGG CTG GCC TAT AGG CT; TNF- $\alpha$  (forward), GGT CGA ATC TGG CTG GAG CGG, TNF- $\alpha$  (reverse), GGT AGG TGC AGG ACC GGT GGG; IL-6 (forward), GCC AGA GTC CTT CAG AGA GAT AC, IL-6 (reverse), CCC AAC GAT TCA TAT TGT CAG; IL-13 (forward), GGA GCT GAG CAA CAT CAC ACA, IL-13 (reverse), GGT CCT GTA GAT GGC ATT GCA; NFATc1 (forward), TGC CCT TGA CTG GCA GCT CC; and NFATc1 (reverse), AAT GAA CAG CTG TAG CGT GAG. For detection of NFATc2 mRNA, the QuantiTect primer assay Mm\_NFATc2\_SG (Qiagen) was used.

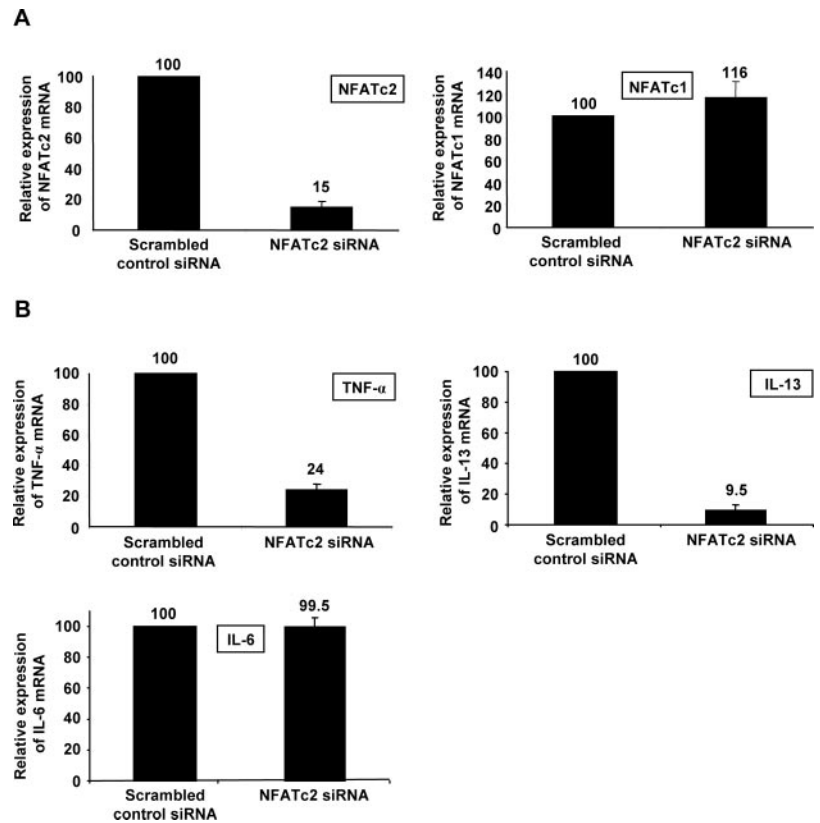
PCR was performed on an iCycler (Bio-Rad) using ABabsolute SYBR Green fluorescein (ABgene). Data were normalized according to the expression of HGPRT, and relative mRNA expression levels were calculated.

### Transfection of BMMC

NFATc1/A cDNA was cloned into the expression vector pLGP3. BMMC ( $2\text{--}3 \times 10^6$  cells in 0.2 ml of serum-free IMDM) were transfected with either 2  $\mu$ g 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  $\mu$ F. 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\_Nfatc2.2\_HP siRNA and Mm\_NFATc1.6\_HP siRNA purchased from Qiagen). siRNAs (1  $\mu$ M) 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 ( $2 \times 10^7$ ) 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 lysis buffer (25 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40, and protease inhibitors) and incubated for 15 min on ice. Nuclei were pelleted and resuspended in sonication buffer (50 mM HEPES (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and protease inhibitors). The suspension was sonicated 15–18 times for 10 s with 1 min cooling on ice in-between using a Bandelin SonoPlus UW2070 device set at 20% power. The samples were precleared with protein A-Sepharose blocked with salmon sperm DNA (Upstate Biotechnology). An aliquot of chromatin, referred to as input control, was saved after this preclearing step. One hundred micrograms of chromatin in a total volume of 300  $\mu$ l was immunoprecipitated with 2  $\mu$ g of polyclonal rabbit Abs raised against human NFATc2 (AB1-209; ImmunoGlobe), NFATc1 (AB1-205; ImmunoGlobe), anti-acetyl-histone H3 (no. 06-599; Upstate Biotechnology), and anti-GST (sc-459; Santa Cruz Biotechnology), respectively, and the immune complexes were precipitated with protein A-Sepharose. The precipitates were washed twice each with sonication buffer, high salt buffer (50 mM HEPES (pH 7.9), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS), LiCl buffer (20 mM Tris (pH 8), 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) and TE (20 mM Tris (pH 8), 1 mM EDTA). The complexes were eluted with 1% SDS in TE at 65°C,



**FIGURE 4.** siRNA-mediated knockdown of NFATc2 impairs the expression of TNF- $\alpha$  and IL-13 mRNAs. *A*, BMDC derived from wild-type mice were transfected with NFATc2-specific or nonspecific siRNA (scrambled control). Cells were activated with ionomycin for 4 h, and expression of mRNAs encoding NFATc2 and NFATc1 was assayed by qRT-PCR to confirm efficiency and specificity of the knockdown. *B*, MC were transfected as described in *A*, and mRNA species were measured by qRT-PCR at 1 h (TNF- $\alpha$  mRNA) and 4 h (IL-13 and IL-6 mRNA) poststimulation with ionomycin. mRNA levels in cells transfected with irrelevant siRNA were arbitrarily set at 100%. Each figure shows the means  $\pm$  SD of three independent experiments.

adjusted to 200 mM NaCl, and incubated at 65°C overnight to reverse the formaldehyde cross-links. After treatment with proteinase K and RNaseA at 55°C, the samples were extracted with phenol/chloroform and precipitated with ethanol in the presence of 5  $\mu$ g of linear acrylamide. One-tenth of the immunoprecipitated DNA and input DNA were analyzed by PCR using the following oligonucleotides: TNF- $\alpha$  promoter (forward), TTT CAG TTC TCA GGG TCC TAT ACA A; TNF- $\alpha$  promoter (reverse), TGT GCA GAC GGC CGC CTT TAT AG (322-bp product); IL-13 promoter (forward), ACC AAA GTG ATG ACG CCT CA; IL-13 promoter (reverse), CCT GCC CAA AGG GTG ACA (300-bp product); IL-6 promoter (forward), GTA TGT GTG TGT CGT CTG TCA; and IL-6 promoter (reverse), GAC TCA TGG GAA AAT CCC ACA (327-bp product). Amplifications were performed in the presence of 2  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]dCTP. After separation by PAGE, amplification products were visualized by autoradiography.

## 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 Th2-type cytokines. To this end, we generated both BMDC 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 BMDC 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 Ca $^{2+}$ . Elevation of intracellular Ca $^{2+}$  concentration is also

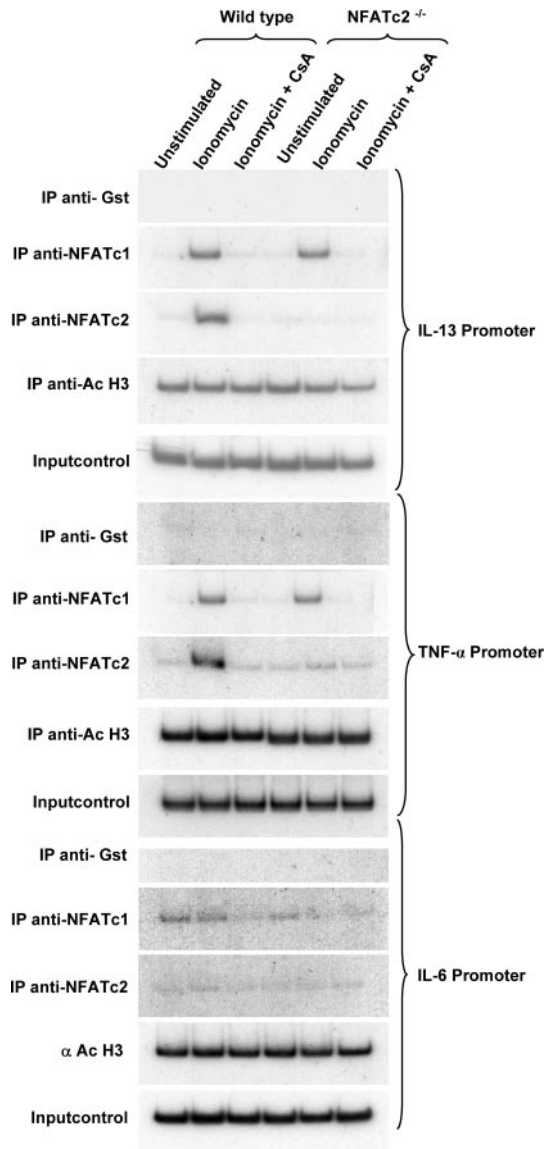
necessary for rapid degranulation of MC, but early signaling events leading from the high-affinity IgE receptor to the exocytosis of stored granules do not rely on protein de novo synthesis (39). In line with this, we did not observe any impairment in degranulation of MC derived from DKO animals or mice 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- $\alpha$*

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 BMDC derived from NFATc2 $^{-/-}$ , NFATc3 $^{-/-}$ , DKO, and wild-type mice. NFATc2 deficiency severely impaired production of IL-13 and TNF- $\alpha$  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 BMDC. Irrespective of the stimulation method used, the expression of IL-6 remained unimpaired in all kinds of NFAT-deficient BMDC. Therefore, NFATc2, but not NFATc3, is an essential transcription factor for the expression of both IL-13 and TNF- $\alpha$  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 BMDC 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- $\alpha$*

To analyze the influence of NFATc2 on the expression of MC-derived cytokines in more detail, we first focused on the regulation of TNF- $\alpha$  and IL-13 production at the mRNA level. As depicted in



**FIGURE 5.** ChIP reveals the binding of both NFATc2 and NFATc1 to the TNF- $\alpha$  and IL-13 promoters in MC. BMMC derived from NFATc2<sup>-/-</sup> and wild-type mice were either left unstimulated or stimulated for 4 h with ionomycin  $\pm$  CsA. Chromatin fragments were prepared and immunoprecipitated with Abs against NFATc2, NFATc1, Gst (negative control), and acetyl-histone H3 (positive control). The immunoprecipitated and input DNAs were analyzed by PCR using specific promoter primers for IL-13, TNF- $\alpha$ , and IL-6 as a negative control. Shown are representatives of at least three independent experiments.

Fig. 3, both mRNA species were rapidly induced upon stimulation of wild-type BMMC and mRNA expression peaks  $\sim$ 1 h after stimulation. The potency of MC to initiate lifesaving inflammatory responses in murine models of acute bacterial inflammation has been ascribed mainly to their unique ability of storing preformed TNF- $\alpha$  within their secretory granules, which can be released within minutes upon stimulation. However, in our experiments, unstimulated BMMC contained virtually no TNF- $\alpha$  and did not constitutively express the respective mRNA. This is in accordance with previous observations that TNF- $\alpha$  is not constitutively produced and stored in BMMC or most MC lines (7, 28, 41). The data shown in Fig. 3 also demonstrate that production of TNF- $\alpha$  and IL-13 mRNA in MC from wild-type mice can be effectively repressed in the presence of CsA, a specific inhibitor of calcineurin

and, hence, of NFAT activation. In line with this, induction of both cytokine mRNAs in BMMC derived from NFATc2<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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 unimpaired. 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).

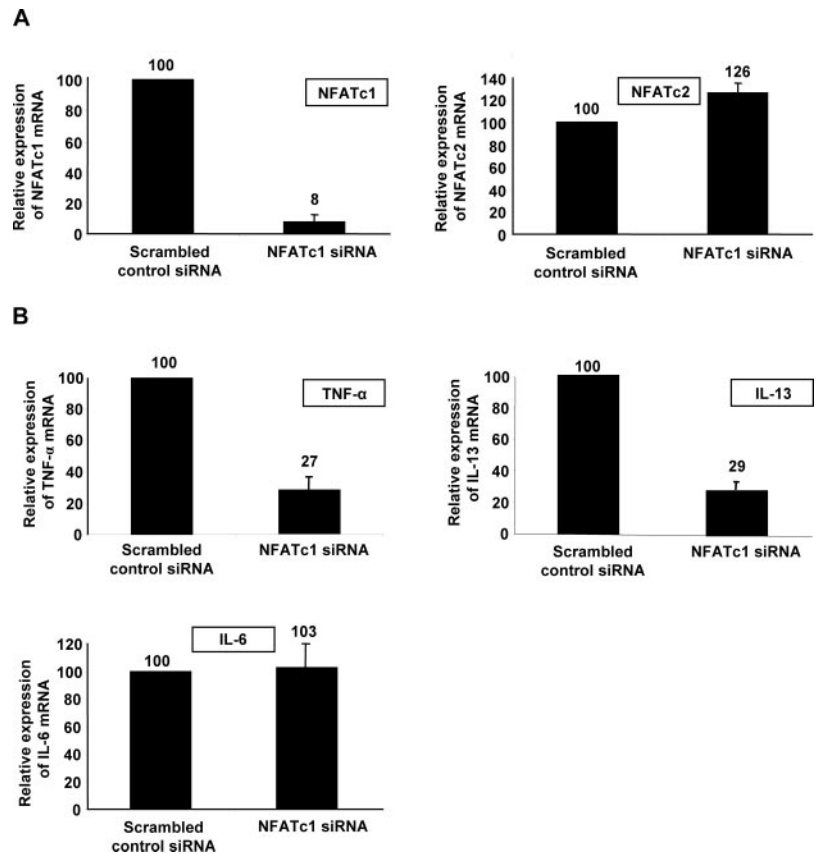
To examine whether NFATc2 binds directly to the promoter regions of the *Il-13* and *Tnf- $\alpha$*  genes, we performed ChIP analyses. As depicted in Fig. 5, inducible binding of NFATc2 to the promoter regions of both genes is detectable upon stimulation of BMMC and can be completely abrogated in the presence of CsA. Using chromatin derived from NFATc2<sup>-/-</sup> 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<sup>-/-</sup> mice (Fig. 5). Binding of NFATc1 might account for CsA sensitivity in NFATc2<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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+c3-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



**FIGURE 6.** NFATc1 positively contributes to the production of TNF- $\alpha$  and IL-13 mRNA. **A**, BMMC derived from wild-type mice were transfected with NFATc1-specific or nonspecific siRNA (scrambled control). Cells were activated with ionomycin, and the expression of NFATc1 and NFATc2 mRNAs was measured 4 h after stimulation by qRT-PCR. **B**, BMMC were transfected as detailed in **A**, and mRNAs were measured by qRT-PCR at 1 h (TNF- $\alpha$ ) or 4 h (IL-13, IL-6) postactivation of cells with ionomycin. mRNA levels expressed in cells transfected with irrelevant siRNA were arbitrarily set at 100%. Each figure shows the means  $\pm$  SD of three independent experiments.

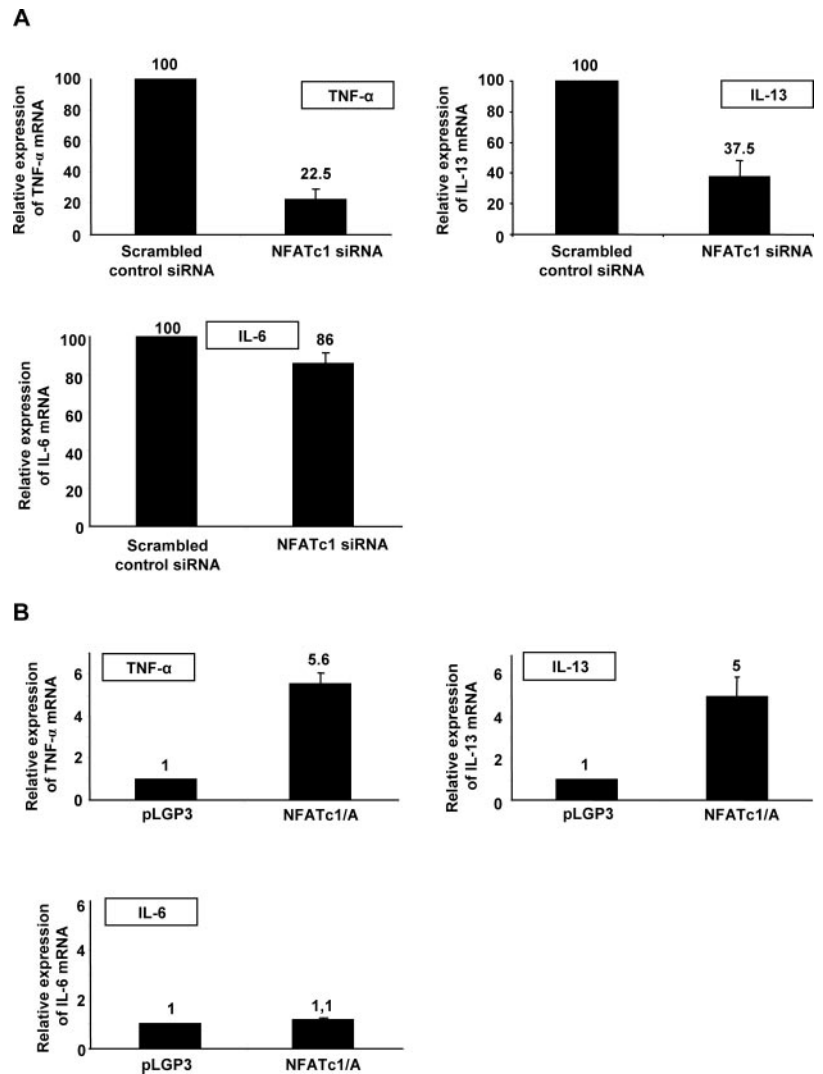
about Th2 cytokine expression. In transient transfection studies, NFATc2 has often been shown to be a strong transactivator of IL-4, IL-5 and of other Th2-type lymphokine genes (44), and in primary stimulations of NFATc2<sup>-/-</sup> T cells IL-4 RNA synthesis appeared to be impaired instead of enhanced (45). The strong enhancement of IL-4 synthesis by NFATc2<sup>-/-</sup> 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, NFATc2-deficient mice were shown to mount increased Th2 responses both upon infection with the nematode *Nippostrongylus brasiliensis* (20) and in a model of allergic pleurisy (21). However, as host immunity to *N. brasiliensis* is MC-independent (46), impaired MC responses to this parasite in NFATc2<sup>-/-</sup> mice would not come to attention. To our knowledge, the role of MC in allergic pleurisy has not been investigated, but the strong sensitization protocol used in this study, using OVA in complete Freund's adjuvant (21), suggests a MC-independent mechanism (47, 48). However,

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- $\alpha$  expression in murine MC, while NFATc3 is dispensable. As in T cells, NFATc1 is expressed in murine MC, binds to the TNF- $\alpha$  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



**FIGURE 7.** Residual expression of TNF- $\alpha$  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- $\alpha$  mRNA (1 h after treatment with ionomycin) and IL-13/IL-6 (after 4 h) was performed. *B*, BMMC from NFATc2<sup>-/-</sup> 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- $\alpha$  mRNA (after 1 h) and IL-13/IL-6 (after 4 h) was performed. Each figure shows the means  $\pm$  SD of three independent experiments.

of one NFAT factor, i.e., either NFATc2 or NFATc1, severely impairs promoter activity.

## Disclosures

The authors have no financial conflict of interest.

## References

- Stassen, M., L. Hultner, and E. Schmitt. 2002. Classical and alternative pathways of mast cell activation. *Crit. Rev. Immunol.* 22: 115–140.
- Stassen, M., L. Hultner, C. Muller, and E. Schmitt. 2002. Mast cells and inflammation. *Arch. Immunol. Ther. Exp.* 50: 179–185.
- Galli, S. J., S. Nakae, and M. Tsai. 2005. Mast cells in the development of adaptive immune responses. *Nat. Immunol.* 6: 135–142.
- Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 54: 577–589.
- Hauber, H. P., C. Bergeron, and Q. Hamid. 2004. IL-9 in allergic inflammation. *Int. Arch. Allergy Immunol.* 134: 79–87.
- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
- Gordon, J. R., and S. J. Galli. 1990. Mast cells as a source of both preformed and immunologically inducible TNF- $\alpha$ /cachectin. *Nature* 346: 274–276.
- Echtenacher, B., D. N. Mannel, and L. Hultner. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* 381: 75–77.
- Malaviya, R., T. Ikeda, E. Ross, and S. N. Abraham. 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF- $\alpha$ . *Nature* 381: 77–80.
- Serfling, E., F. Berberich-Siebelt, S. Chuvpilo, E. Jankevics, S. Klein-Hessling, T. Twardzik, and A. Avots. 2000. The role of NF-AT transcription factors in T cell activation and differentiation. *Biochim. Biophys. Acta* 1498: 1–18.
- Serfling, E., F. Berberich-Siebelt, A. Avots, S. Chuvpilo, S. Klein-Hessling, M. K. Jha, E. Kondo, P. Pagel, J. Schulze-Luehrmann, and A. Palmethofer. 2004. NFAT and NF- $\kappa$ B factors—the distant relatives. *Int. J. Biochem. Cell Biol.* 36: 1166–1170.
- Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* 5: 472–484.
- Hock, M. B., and M. A. Brown. 2003. Nuclear factor of activated T cells 2 transactivation in mast cells: a novel isoform-specific transactivation domain confers unique Fc $\epsilon$ RI responsiveness. *J. Biol. Chem.* 278: 26695–26703.
- Solymar, D. C., S. Agarwal, C. H. Bassing, F. W. Alt, and A. Rao. 2002. A 3' enhancer in the IL-4 gene regulates cytokine production by Th2 cells and mast cells. *Immunity* 17: 41–50.
- Turner, H., M. Gomez, E. McKenzie, A. Kirchem, A. Lennard, and D. A. Cantrell. 1998. Rac-1 regulates nuclear factor of activated T cells (NFAT) C1 nuclear translocation in response to Fc $\epsilon$  receptor type 1 stimulation of mast cells. *J. Exp. Med.* 188: 527–537.
- Hutchinson, L. E., and M. A. McCloskey. 1995. Fc $\epsilon$ RI-mediated induction of nuclear factor of activated T-cells. *J. Biol. Chem.* 270: 16333–16338.
- Yoshida, H., H. Nishina, H. Takimoto, L. E. Marengere, A. C. Wakeham, D. Bouchard, Y. Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity* 8: 115–124.
- Ranger, A. M., M. R. Hodge, E. M. Gravalles, M. Oukka, L. Davidson, F. W. Alt, F. C. de la Brousse, T. Hoey, M. Grusby, and L. H. Glimcher. 1998. Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. *Immunity* 8: 125–134.
- Xanthoudakis, S., J. P. Viola, K. T. Shaw, C. Luo, J. D. Wallace, P. T. Bozza, D. C. Luk, T. Curran, and A. Rao. 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. *Science* 272: 892–895.
- Erb, K. J., T. Twardzik, A. Palmethofer, G. Wohlleben, U. Tatsch, and E. Serfling. 2003. Mice deficient in nuclear factor of activated T-cell transcription factor c2 mount increased Th2 responses after infection with *Nippostrongylus brasiliensis* and decreased Th1 responses after mycobacterial infection. *Infect. Immun.* 71: 6641–6647.



21. Viola, J. P., A. Kiani, P. T. Bozza, and A. Rao. 1998. Regulation of allergic inflammation and eosinophil recruitment in mice lacking the transcription factor NFAT1: role of interleukin-4 (IL-4) and IL-5. *Blood* 91: 2223–2230.
22. Oukka, M., I. C. Ho, F. C. de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1998. The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* 9: 295–304.
23. Ranger, A. M., M. Oukka, J. Rengarajan, and L. H. Glimcher. 1998. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity* 9: 627–635.
24. Rengarajan, J., B. Tang, and L. H. Glimcher. 2002. NFATc2 and NFATc3 regulate T(H)2 differentiation and modulate TCR-responsiveness of naive T(H) cells. *Nat. Immunol.* 3: 48–54.
25. Bopp, T., A. Palmethofer, E. Serfling, V. Heib, S. Schmitt, C. Richter, M. Klein, H. Schild, E. Schmitt, and M. Stassen. 2005. NFATc2 and NFATc3 transcription factors play a crucial role in suppression of CD4<sup>+</sup> T lymphocytes by CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. *J. Exp. Med.* 201: 181–187.
26. Schuh, K., B. Kneitz, J. Heyer, U. Bommhardt, E. Jankevics, F. Berberich-Siebelt, K. Pfeffer, H. K. Muller-Hermelink, A. Schimpl, and E. Serfling. 1998. Retarded thymic involution and massive germinal center formation in NF-ATp-deficient mice. *Eur. J. Immunol.* 28: 2456–2466.
27. Reisbach, G., I. Bartke, B. Kempkes, G. Kostka, J. Ellwart, A. Birner, K. Thalmeier, R. Mailhammer, G. W. Bornkamm, and A. Ullrich. 1993. Characterization of hemopoietic cell populations from human cord blood expressing *c-kit*. *Exp. Hematol.* 21: 74–79.
28. Stassen, M., C. Muller, C. Richter, C. Neudorfl, L. Hultner, S. Bhakdi, I. Walev, and E. Schmitt. 2003. The streptococcal exotoxin streptolysin O activates mast cells to produce tumor necrosis factor- $\alpha$  by p38 mitogen-activated protein kinase- and protein kinase C-dependent pathways. *Infect. Immun.* 71: 6171–6177.
29. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* 84: 1374–1378.
30. Gross, J. A., E. Callas, and J. P. Allison. 1992. Identification and distribution of the costimulatory receptor CD28 in the mouse. *J. Immunol.* 149: 380–388.
31. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166: 1229–1244.
32. Stassen, M., A. Valeva, I. Walev, and E. Schmitt. 2006. Activation of mast cells by streptolysin O and lipopolysaccharide. *Methods Mol. Biol.* 315: 393–403.
33. Hansen, A. S., O. Noren, H. Sjoström, and O. Werdelin. 1993. A mouse aminopeptidase N is a marker for antigen-presenting cells and appears to be coexpressed with major histocompatibility complex class II molecules. *Eur. J. Immunol.* 23: 2358–2364.
34. Mazurek, N., H. Schindler, T. Schurholz, and I. Pecht. 1984. The cromolyn binding protein constitutes the Ca<sup>2+</sup> channel of basophils opening upon immunological stimulus. *Proc. Natl. Acad. Sci. USA* 81: 6841–6845.
35. Eshhar, Z., M. Ofarim, and T. Waks. 1980. Generation of hybridomas secreting murine reagenic antibodies of anti-DNP specificity. *J. Immunol.* 124: 775–780.
36. Baniyash, M., and Z. Eshhar. 1984. Inhibition of IgE binding to mast cells and basophils by monoclonal antibodies to murine IgE. *Eur. J. Immunol.* 14: 799–807.
37. Stassen, M., H. Jonuleit, C. Muller, M. Klein, C. Richter, T. Bopp, S. Schmitt, and E. Schmitt. 2004. Differential regulatory capacity of CD25<sup>+</sup> T regulatory cells and preactivated CD25<sup>+</sup> T regulatory cells on development, functional activation, and proliferation of Th2 cells. *J. Immunol.* 173: 267–274.
38. Soutoglou, E., and I. Talianidis. 2002. Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. *Science* 295: 1901–1904.
39. Blank, U., and J. Rivera. 2004. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 25: 266–273.
40. Monticelli, S., D. C. Solymar, and A. Rao. 2004. Role of NFAT proteins in IL13 gene transcription in mast cells. *J. Biol. Chem.* 279: 36210–36218.
41. Gordon, J. R., and S. J. Galli. 1991. Release of both preformed and newly synthesized tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )/cachectin by mouse mast cells stimulated via the Fc $\epsilon$ RI: A mechanism for the sustained action of mast cell-derived TNF- $\alpha$  during IgE-dependent biological responses. *J. Exp. Med.* 174: 103–107.
42. Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255–281.
43. Hodge, M. R., A. M. Ranger, D. L. B. Charles, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity* 4: 397–405.
44. Monticelli, S., and A. Rao. 2002. NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription. *Eur. J. Immunol.* 32: 2971–2978.
45. Schuh, K., B. Kneitz, J. Heyer, F. Siebelt, C. Fischer, E. Jankevics, E. Rude, E. Schmitt, A. Schimpl, and E. Serfling. 1997. NF-ATp plays a prominent role in the transcriptional induction of Th2-type lymphokines. *Immunol. Lett.* 57: 171–175.
46. Urban, J. F., Jr., L. Schopf, S. C. Morris, T. Orekhova, K. B. Madden, C. J. Betts, H. R. Gamble, C. Byrd, D. Donaldson, K. Else, and F. D. Finkelman. 2000. Stat6 signaling promotes protective immunity against *Trichinella spiralis* through a mast cell- and T cell-dependent mechanism. *J. Immunol.* 164: 2046–2052.
47. Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192: 455–462.
48. Taube, C., X. Wei, C. H. Swasey, A. Joetham, S. Zarini, T. Lively, K. Takeda, J. Loader, N. Miyahara, T. Kodama, et al. 2004. Mast cells, Fc $\epsilon$ RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. *J. Immunol.* 172: 6398–6406.
49. Sherman, M. A., D. R. Powell, D. L. Weiss, and M. A. Brown. 1999. NF-ATc isoforms are differentially expressed and regulated in murine T and mast cells. *J. Immunol.* 162: 2820–2828.
50. Peng, S. L., A. J. Gerth, A. M. Ranger, and L. H. Glimcher. 2001. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. *Immunity* 14: 13–20.