Nuclear Factor of Activated T Cells 1 (NFAT1) induced permissive chromatin modification facilitates Nuclear Factor-κB (NF-κB) mediated Interleukin-9 (IL-9) transactivation

Arijita Jash§, Anupama Sahoo§§, Gi-Cheon Kim§, Chang-Suk Chae§, Ji-Sun Hwang§, Jung-Eun Kim§ and Sin-Hyeog Im§*

§School of Life Sciences and Immune Synapse Research Center, Gwangju Institute of Science and Technology (GIST), Gwangju 500-712, Republic of Korea
‡Department of Immunology, MD Anderson Cancer Center, Houston, Texas, 77054

Running title: Functional cooperation of NFAT1 and NF-κB in IL-9 expression.

*Address correspondence should be addressed to: Dr. Sin-Hyeog Im, School of Life Sciences and Immune Synapse Research Center, Gwangju Institute of Science and Technology (GIST), 123, Cheomdan-gwagiro, Buk-gu, Gwangju 500-712, Republic of Korea ; Tel: 82-62-715-2503 ; FAX: 82-62-715-2484; Email: imsh@gist.ac.kr

Background: IL-9 is the signature cytokine of Th9 cells.

Results: NFAT1 deficiency or silencing of NF-κB (p65) impair IL-9 production from Th9 cells.

Conclusion: NFAT1 creates an accessible chromatin platform for the recruitment of NF-κB (p65) on to the IL-9 promoter resulting in increased IL-9 expression.

Significance: This is the first report elucidating the molecular mechanism of NFAT1 and NF-κB (p65) mediated IL-9 expression in Th9 cells.

SUMMARY

IL-9 regulates diverse inflammatory immune responses. Although, the functional importance of IL-9 has been investigated in various pathophysiological conditions, molecular mechanisms by which TCR stimulation induced IL-9 gene expression are still unclear. In this study, we investigated the functional importance of the NFAT1 and NF-κB (p65) in IL-9 gene transcription in CD4+ T cells. In vivo binding of NFAT1 and NF-κB (p65) to the IL-9 promoter was observed. NFAT1 binding induced a transcriptionally active chromatin configuration at the IL-9 promoter locus, while NF-κB (p65) binding transactivated the IL-9 promoter. Mouse deficient in NFAT1 shows a significant down regulation of IL-9 expression that resulted from an inaccessible chromatin configuration at the IL-9 promoter. In parallel knock-down of NF-κB (p65) also resulted in reduced IL-9 expression. In this process, NFAT1 plays pivotal role as a core protein that creates an accessible platform for the assembly of transcription activators. Presence of NFAT1 correlates with recruitment of NF-κB (p65), p300 and active histones markers on the IL-9 promoter resulting in a transcriptionally competent promoter. NFAT1 deficiency significantly reduced the recruitment of the above activation complex to the IL-9 promoter. In summary, our data suggest that functional cooperation of NFAT1 and NF-κB synergistically enhances IL-9 transcription in CD4+ T cells.
INTRODUCTION

Naïve CD4+ T cells differentiate into diverse T helper subsets (Th1, Th2, Th9, Th17 and regulatory T cells (Treg)) upon encountering antigens in a unique cytokine microenvironment. While IL-12 and IL-4 induces the differentiation of IFN-γ producing Th1 cells and IL-4, IL-5 and IL-13 secreting Th2 cells, respectively, TGF-β, IL-6, IL-1β and IL-23 stimulate the development of IL-17-producing Th17 (1-4). Adding to this growing list of effector T helper cells are the Th9 cells. This newly discovered Th9 cell subset can be generated under a cytokine milieu enriched in IL-4 and TGF-β. Th9 cells are the major producer of IL-9 among the T helper cell subsets (5,6). The biology and the function of IL-9 are complex and diverse (7). IL-9 functions through the IL-9 receptor (IL-9R) which shares the γ chain with IL-2, IL-4, IL-7, IL-15 and IL-21 cytokine receptors (8,9). Initially, IL-9 was characterized as a growth factor for T cells and mast cells (10,11). IL-9 promotes Th2 specific allergic responses, allergic inflammation and asthmatic symptoms (12-15). IL-9 also promotes the differentiation of Th17 cells and has been implicated in the development and progression of experimental autoimmune encephalomyelitis (EAE) (16-18). On the other hand IL-9 increases the suppressive activity of Treg cells by yet undiscovered mechanisms (16,19,20). Thus, elucidating the underlying mechanism of IL-9 expression will further advance the understanding of the functional variety and diversity of IL-9 producing cells and IL-9 itself.

The activation and expression of specific transcription factors in the T helper cell subsets govern their signature cytokine expression. So far, Pu.1, IRF4 and STAT6 have been reported to play crucial roles in Th9 lineage commitment and regulation of IL-9 expression (21-23). However, these factors do not act alone and require the participation of other transcription factors that may play a pivotal role in inducing IL-9 following TCR stimulation (24). Ubiquitously expressed transcription factors are also known to induce subset specific expression of cytokines. This includes the nuclear factor of activated T cells (NFAT) family members which have diverse roles in T cell subsets (25). The NFAT proteins (NFAT1-4) are regulated by Ca2+ except NFAT5 (TonEBP: toxicity element binding protein or OREBP: osmotic response element binding protein) that responses to osmotic stress (26,27). TCR stimulation coupled with Ca2+ mobilization results in the activation of Ca2+ dependent NFATs via the calcium-calcmodulin phosphatase pathway (28,29). NFATs act in concert with other transcription factors to direct their target gene expression often in cell and tissue specific manner. For example, NFAT1/T-bet interaction directs the Th1 specific IFN-γ expression; NFAT1/IRF4 induces IL-4 and IL-10 expression in Th2 cells, while NFAT2/Smad3 interaction governs Foxp3 expression in Treg cells (30-33). NFATs also interact with active histone modifiers, CBP/p300 to instruct changes in chromatin architecture (34,35) and regulates the inducible gene expression of diverse cytokines in immune response (36). In the context of IL-9, previous reports suggested a potential link of NFAT with IL-9 expression. Treatment of cyclosporin A (CsA) to the mice infected with parasite (Leishmania major) ablated antigen-specific IL-9 production (37). More specifically, umbilical cord blood T cells that express reduced NFAT1 than adult blood T cells produce significantly reduced level of IL-9 (38,39). However no mechanistic investigation on the functional role of NFAT1 in IL-9 regulation has been conducted.

NF-κB family is another well-known ubiquitous transcription factor family that regulates genes involved in diverse biological processes such as growth and survival and also inflammatory immune responses. They bind to specific decameric sequences located within the target gene promoter or other regulatory regions (40-42). This family consists of five members (NF-κB1 (p50/p105), NF-κB2 (p52/p100), c-REL, RELA (p65) and RELB (I-REL)). Their
activity can be regulated by inhibitory protein, IkBs, which sequester NF-κBs in the cytosol (43). This family requires the activity of transcription coactivators to execute their transactivity. CBP/p300 is one of the coactivator with intrinsic histone acetylase (HAT) activity and is required for p65-mediated transactivation (44,45). NF-κB has also been implicated in IL-9 expression in mouse macrophage cells upon LPS stimulation (46). Besides, binding of NF-κB to the human IL-9 promoter in T cells has also been demonstrated (24,47). However, the role of NF-κB in regulating IL-9 expression in mouse Th9 cell is yet unknown.

In this study, we identified the functional synergy of two transcription factors, NFAT1 and NF-κB (p65) in IL-9 expression by Th9 cells. NFAT1 is primarily involved in stabilizing and maintaining a transcriptionally competent chromatin structure, while NF-κB transactivates the IL-9 promoter.

EXPERIMENTAL PROCEDURES

Computational analysis of the IL-9 locus
To identify potential transcription factor binding sites in the IL-9 promoter, comparative genomic analysis was performed. Genomic sequence of the IL-9 gene promoter was analyzed using the web-based alignment software, (http://ecrbrowser.dcode.org/) and VISTA browser 2.0 (48).

Mice and Cell lines
C57BL/6 mice 6-8 weeks of age were purchased from Orient Bio (Daejon, Korea). NFAT1 deficient mice (KO) were kindly provided by Dr. A. Rao, Harvard Medical School. Mice were housed in specific pathogen-free barrier facilities and used in accordance with protocols approved by the Animal Care and Ethics Committees of the Gwangju Institute of Science and Technology (GIST).

CD4+ T cell isolation, differentiation and culture
CD4+ T cells were isolated, differentiated and cultured as described previously (30). Briefly, CD4+ T cells were purified from the lymph nodes and spleen of 6-8 weeks-old mice with the use of magnetic beads (Miltenyi, L3T4). For T helper cell differentiation, CD4+ T cells (2-3 × 10^6/ml) were stimulated with 1 μg/ml plate-bound α-CD3 and 2 μg/ml soluble α-CD28 under Th1-skewing (10 ng/ml IL-12, 10 ng/ml IL-4 and 1 ng/ml TGF-β) or left in unpolarized (CD4+ T cell blasts) (6) conditions in RPMI 1640 medium (Welgene, Korea) supplemented with 10% fetal bovine serum, L-glutamine, penicillin–streptomycin, non-essential amino acids, sodium pyruvate, vitamins, HEPES and β-mercaptoethanol. 100 U/ml of recombinant human IL-2 (rhIL-2) was added after 24 hrs. On day 3 cells were shifted to complete medium containing IL-2 and expanded. On day 5, they were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1 μM ionomycin (I) or 1 μg/ml plate-bound α-CD3 and α-CD28 as mentioned in particular cases.

RNA isolation, cDNA synthesis, quantitative real-time PCR (qRT-PCR), conventional PCR and ELISA
Total RNA was extracted from the stimulated or unstimulated cells plus 10 μg/ml α-IL-4), Th2-skewing (10 ng/ml IL-4, 10 μg/ml α-IFN-γ plus 10 μg/ml anti-IL-12) and Th9-skewing (10 using TRIZOL Reagent (Molecular Research Center; Cincinnati, OH) according to the manufacturer’s protocol. For reverse transcription, 1μg of total RNA was used and cDNA was generated using oligo (dT) primer (Promega; Madison, WI) and Improm-II Reverse Transcriptase (Promega; Madison, WI) in a total volume of 20 μl. The mRNA level was determined.
using 1 μl of cDNA by real-time PCR with SYBR green according to the manufacturer's protocol (MJ Research Chromo4). Mouse hypoxanthine-guanine phosphoribosyl transferase (HPRT) primer was used for qRT-PCR to normalize the amount of cDNA used for each condition. The primer sequences used are as follows: HPRT (5′-TTA TGG ACA GGA CTG AAA GAC-3′ (forward) and 5′-GCT TTA ATG TAA TTC AGC AGG T-3′ (reverse)), IL-9 (F-5′-GTG ACA TAC ATC CTT GCC TC -3′ (forward) and R-5′-GTG GTA CAA TCA TCA GTT GGG -3′(reverse)). For ELISA, cell supernatants were collected from unstimulated and 24 hrs PMA/ionomycin or α-CD3/CD28 stimulated cells and cytokines were measured using IL-4, IL-9, and IFN-γ ELISA kits (eBiosciences; San Diego, CA).

**Chromatin immunoprecipitation (ChIP) assay**

ChIP analysis was carried out as described previously with minor modifications (30,49-51). Chromatin prepared from Th9 cells after PMA/ionomycin stimulation were immunoprecipitated using antibodies against RNA Pol II (Santa Cruz; Santa-Cruz, CA), acetyl histone H3 (AcH3), acetyl histone H4 (AcH4), dimethyl lysine histone H3 (H3K4me2) (Upstate; Lake Placid, NY), p300 (Millipore, Billerica, MA), NFAT1 (Santa-cruz; Santa-Cruz, CA), p65 (Abcam; Cambridge, MA) and rabbit IgG (Sigma-Aldrich; St. Louis, MO). After washing, complexes were eluted in elution buffer (1% SDS and 0.1 M NaHCO₃) for the first ChIP. For second immunoprecipitation, complexes were eluted from the primary immunoprecipitation with 10 mM DTT at 37°C for 30 min and diluted 1:50 in buffer (1% Triton X-100, 2mM EDTA, 150 mM NaCl,20 mM Tris-HCl at pH 8.1) followed by re-immunoprecipitation with second antibodies similarly as the first ChIP (52). Following reversal of cross-links, the presence of selected DNA sequences were assessed by qRTPCR using SYBR green PCR mix. As a loading control, PCR was done directly on input DNA purified from chromatin before immunoprecipitation using the primers, IL-9 (5′- CAT TAC CAC CCC TGT AAC TCA C-3′ (forward) and 5′- CTA CCA GGA TCT TCC AGT CTA GC-3′ (reverse)). A pair of primers corresponding to non-relevant region (designated as NR; +1864/+2193) NR-F-ATG TAA CCA GGA ACA AGA TCA CTG CAG and NR-R-5′ GTG GAA GTA CTT ACC TAG ACC TTG GTG TC is used as a control for the time dependent ChIP experiment. Amount of chromatin precipitated by the indicated antibodies as detected by RT-PCR with primers against the target regions relative to that detected in the input (total chromatin) by the same primer pair are represented as fraction of input or as negative images of EtBr stained gels.

**Chromatin accessibility by real time PCR (CHART-PCR) assay**

Th9 cells (5x10⁶ cells /sample) differentiated from wild type (WT) and NFAT1 −/− (KO) CD4⁺ T cells were left unstimulated or restimulated with PMA/ionomycin (PI) for 8 hrs and pelleted by centrifugation at 500xg, washed in ice cold PBS and then re-suspended in 100 μl digestion buffer (10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM CaCl₂) with or without 5U MNase/ml (Roche; Mannheim, Germany) and were incubated at 37°C for 10 min. Reactions were terminated by adding 20 μl Stop solution (100 mM EDTA, 10 mM EGTA- pH 8.1) and 10 ml SDS 10% (w/v). DNA was isolated using the DNA blood genomic prep kit (Intron Biotechnology; Daejon, Korea) and eluted into 100 μl TE. DNA recovered from MNase samples were checked for fragmentation (1000-500bp) in 1% agarose gel. Primers used in the quantitative assays were validated by amplifying serially diluted genomic DNA as templates to create standard curve for each primer set and analyzed using the quantification method. Untreated and MNase treated samples were used in PCR assays to measure the relative abundance of target regions using the primer sets used in ChIP experiments. Actin promoter primers (5′-TTC CGA AAG TTG CCT TTT ATG GCT CGA-3′ (forward)
and 5′-AAG GAG CTG CAA AGA AGC TGT G-3′ (reverse) were used as controls. Chromatin accessibility values were calculated as the ratio of the undigested sample to the digested samples and then the data was plotted as the ratio of accessibility observed in the digested DNA samples.

**Plasmid construction, site directed mutagenesis and luciferase reporter assays.**

The promoter containing 5′ region of the murine IL-9 gene (36) encompassing nucleotides -366 to +48 (Fig. 1B) was amplified from T cell genomic DNA using primers F-5′-CAT TAC CAC CCC TGT AAC TCA C and R-5′-GTA TGT CAC CAA CAT GTT GAC (indicated in Fig. 1B) and cloned into pXPG luciferase reporter vector. IL-9 promoter reporter construct that lacks the first NF-κB binding site (designated as ΔNF-κB1-Prom) was amplified using primer F-5′-CCA GAA TTC CTG CTT TTA AAG and cloned into pXPG luciferase reporter vector. The sequences of cloned DNA fragments were confirmed by DNA sequencing. The two NF-κB binding sites at positions -315/-306 (named as NT/κB1) and -48/-38 (named as NT/κB2) were subjected to site directed mutagenesis (NT/κB1) – WT-GGG GAA AAC ACA G to mutant GGG cAc AAC ACA G and (NT/κB2) – WT-GTT TTT CCC GGT to mutant Gac aca CCC GGT). The sequences of cloned DNA fragments were confirmed by DNA sequencing. The two NF-κB binding sites at positions -315/-306 (named as NT/κB1) and -48/-38 (named as NT/κB2) were subjected to site directed mutagenesis (NT/κB1) – WT-GGG GAA AAC ACA G to mutant GGG cAc AAC ACA G and (NT/κB2) – WT-GTT TTT CCC GGT to mutant Gac aca CCC GGT). The sequences of cloned DNA fragments were confirmed by DNA sequencing. HEK-293 and EL-4 cells were transfected using GeneExpresso (Excellgen, Rockville, MD) according to the manufacturer’s protocol and primary Th9 cells were electroporated. Following PMA/ionomycin (PI) stimulation for 8 hrs, cells were harvested and luciferase activity was measured by the dual luciferase assay system (Promega, Madison, WI). Wherever indicated, cyclosporin A (CsA) (Calbiochem, Germany) was added at 0.1 mg/ml, 20 min before PI stimulation. Data were normalized by the activity of Renilla luciferase, which was used as an internal control for transfection.

**DNA affinity purification assay (DAPA)**

DAPA was performed following protocols described previously with minor modifications (53). Briefly, biotinylated complementary oligo nucleotides were annealed in TEN (10mM Tris/HCl ph 8.0, 1mM EDTA, 100mM NaCl) buffer. HEK-293 cells overexpressing NFAT1 and NF-κB (p65) were lysed by sonication in 200 μl of HKMG (10 mM Hepes, pH 7.9, 100 mM KCl, 5 mM MgCl2, 10% glycerol 0.1% NP-40, and 1 mM DTT) buffer containing protease and phosphatase inhibitors (Roche; Mannheim, Germany). The cellular debris was removed by centrifugation. The expression level of NFAT1 and NF-κB (p65) along with that of actin (control) was checked by blotting 30 μg of total cell lysate with α-NFAT1 (Santa-cruz; Santa-Cruz, CA), α-p65 and α-Actin (Abcam; Cambridge, MA) antibodies. The cell extracts (100-500 μg) were precleared with 10 μl M-280 Streptavidin beads (Invitrogen; Carlsbad, CA) for 1 hour at 4°C with gentle agitation. The cleared nuclear extracts were then incubated with 1 μg of biotinylated double-stranded probes and 10 μg of poly (dI-dC) poly (dI-dC) overnight. 10-15 μl of M-280 Streptavidin beads were used to pull down bound proteins for 1 hour at 4°C with gentle agitation. The beads were washed four times with cold HKMG buffer. SDS sample buffer was then added to the beads. The samples were boiled for 5 mins and subjected to SDS-PAGE and western blotting with α-NFAT1 (Santa-cruz; Santa-Cruz, CA), α-p65 (Abcam; Cambridge, MA) antibodies. The nucleotide sequences of oligonucleotides are: NFAT-a and NT/κB-1 (encompassing region -315/-306; 5′-CTA ATG TGG AGG GGA AAA CAC AGA CCT GGG-3′ (forward) and 5′-CCC AGG TCT GTG TTT TTC CCT CCA CAT TAG-3′ (reverse)), NFAT-c (encompassing region -223/-214; 5′-GAT TAA CGA GAA AGT TAA AGA CCT GGG-3′ (forward) and 5′-CTG AAA TAC TAA AGG AAA AGT TAA AGA TC-3′ (reverse)), NFAT-e and NT/κB-2 (encompassing region -48/-38; 5′-GAT GTC AGG GTT TTT CCC GGT TTG-3′ (forward) and 5′-CAA ACC GGG AAA AAC
CCT GAC ATC-3′ (reverse)), mtNT/κB-1 (5′-CTA ATG TGG AGG GcA cAA CAC AGA CCT GG-3′ (forward) and 5′-CCA GGT CTG TGt Ggc CCC CTC CAC ATT AG-3′ (reverse)), mtNT/κB-2 (5′-GAT GTC AGG Gac aca CCC GGT TTG-3′ (forward) and 5′-ATG AAA CAA ATT TTC CTC TTT GGG-3′ (reverse)), Cons (mNF-AT1+2 (murine distal IL-2 NF-AT site); 5′-CCC AAA GAG GAA AAT TTG TTT CAT-3′ (forward) and 5′-ATG GAA ACA AGG GCC TCT TTG GG-3′ (reverse)(54) and NR (5′-CCC AAA GAG GCC TTT GTT TCC AT-3′ (forward) and 5′-ATG GAA ACA AGG GCC TCT TTG GG-3′ (reverse).

NFAT1 reconstitution in NFAT1−/− Th9 cells and siRNA mediated p65 silencing in wild type Th9 cells.

For reconstitution assay, Th9 cells differentiated in vitro for three days from NFAT1−/− mice were transfected with 5 μg of NFAT1 expression construct or empty mock vector (control) into the NFAT1−/− Th9 cells. For knock-down experiment, wild type Th9 cells were transfected with 100 pmole of p65 siRNA (Santa Cruz Biotechnology; Santa-Cruz, CA) or scrambled siRNA (mock control) by using T cell nucleofector kit (Lonza, Cologne, Germany) following manufacturer’s protocol. After 24hrs of transfection cells were re-stimulated with PMA/ionomycin (PI) for 12hrs for RT-PCR and immunoblot analysis and 24 hrs for ELISA, respectively.

Nuclear extract preparation and Immunoblotting

Nuclear extracts were prepared from PMA/ionomycin (PI) stimulated Th9 cells. Briefly, 2×10⁷ cells were washed in ice cold PBS and suspended in 1 ml of lysis buffer (10 mM Tris/HCl, 3 mM CaCl₂, 2 mM MgCl₂) containing protease inhibitor cocktail (Roche, Mannheim, Germany) for 10 min on ice. They were vortexed gently and incubated in 1 ml of NP-40 buffer (10 mM Tris/HCl, 3 mM CaCl₂, 2 mM MgCl₂, 1% NP-40) for 5 min at 4 °C, and centrifuged at 3000 rpm for 10 min at 4 °C. Nuclei were washed with 1 ml of Buffer A (20 mM HEPES-KOH, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) and 100 μl of Buffer C (20 mM HEPES-KOH, 25% Glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 1% Triton X-100) was added to the pellets and they were vortexed vigorously at 4 °C for 10 min. Thirty micrograms of the nuclear extracts were used for SDS-PAGE, and Western blot was carried out with α-NFAT1, α-Pu.1, α-IRF4, α-Lamin B (Santa-cruz; Santa-Cruz, CA) and α-tubulin (Abcam; Cambridge, MA) antibodies.

Statistical analysis

Data are the mean ± SE of at least three independent experiments, unless specified in the text. A Student’s t-test was used to calculate the statistical significance of the experimental data. The level of significance was set at *P<0.05, **P<0.01 and ***P<0.001. Significance was only indicated when appropriate.

RESULTS

Identification of transcription factor binding sites in the IL-9 promoter

To identify the potential transcription factors that are responsible for IL-9 gene expression, the genomic sequences of the human and mouse IL-9 promoter loci (-366/+48) were compared by bioinformatics analysis (Fig. 1A). Along with already identified Pu.1 and IRF4 (Supplemental Fig. S1), five highly conserved (>85%) NFAT binding sites (marked as NFAT-a, -b, -c, -d and NFAT-e, respectively) distributed in the IL-9 promoter were identified (Fig. 1B). Out of the five NFAT binding sites, two of them encompassed the core NF-xB binding sites and marked as NT/κB1 and NT/κB2, respectively (Fig. 1B). The IL-9 promoter showed highly conserved binding sites for other transcription factors such as AP1, STAT proteins, Smad 2 and 3, ETS, Lef1 and Foxp3 (Supplemental Fig. S1). Among the NFAT family members we have mainly investigated the mechanism of NFAT1 mediated IL-9 gene expression since NFAT1 is the predominant NFAT protein in T cells and accounts for 90% of total NFAT.
DNA binding activity in wild type T cells (55).

**NFAT1 binds directly to the IL-9 promoter in Th9 cells**

We utilized *in vitro* differentiated murine Th9 cells as our model considering the fact that they are the major IL-9 producing T helper cell subset identified (5). To compare IL-9 expression among the T helper cell subsets, CD4+ T cells were cultured *in vitro* under Th1, Th2 and Th9 differentiation conditions by following the previously reported studies (6,51). Expression level of IL-9 was measured among the various *in vitro* differentiated T helper subsets. Indeed, Th9 cells showed the highest IL-9 expression upon restimulation (Supplemental Fig. S2 A-F). During *in vitro* Th9 cell differentiation, IL-9 expression was detectable in day 3 of culture and peaked at day 5 upon restimulation and the highest expression of IL-9 was found between 8 to 12 hrs of stimulation and maintained until 24hrs (Supplemental Fig. S3 A-B) (56,57). Next we performed chromatin immunoprecipitation (ChIP) assay to detect a physical binding of NFAT1 to the predicted NFAT binding sites on the IL-9 promoter. NFAT1 enrichment was much higher in the IL-9 promoter of Th9 cells compared with Th1 cells, which well correlates with the IL-9 expression profiles between them (Fig. 2A). To further confirm the binding of NFAT1 to the IL-9 promoter, DNA affinity purification assay was performed. Out of the five NFAT binding sites, three probes were designed based on their highest matrix similarity (0.8) such as NFAT-a (-315/-306), NFAT-c (-223/-214) and NFAT-e (-48/-38). Biotinylated NFAT-e (-48/-38) probe efficiently pulled down NFAT1 protein from HEK-293 cell lysate (Fig. 2B). This result confirmed that NFAT1 directly binds to the IL-9 promoter. NFAT1 binding at the IL-9 promoter could enhance IL-9 transcription by performing IL-9 promoter reporter analysis. IL-9 promoter driven luciferase activity was measured upon NFAT1 over expression in HEK-293 and EL-4 T lymphoma cell lines. As a positive control for NFAT1 driven transactivation, CNS-9 containing IL-10 promoter was employed. Interestingly, overexpression of NFAT1 failed to transactivate the IL-9 promoter in both HEK-293 (Fig. 2C) and EL-4 T cells (Fig. 2D) while NFAT1 significantly increased the transactivity of CNS-9 containing IL-10 promoter in EL-4 cells. Reduction of CNS-9 containing IL-10 promoter activity in the presence of CsA further confirms the NFAT1 mediated transactivity of this CNS element (Fig. 2D) (30). These results suggest that although NFAT1 binds to the IL-9 promoter it does not transactivate IL-9 promoter.

**NFAT1 dependent IL-9 expression in Th9 cells**

To elucidate the functional importance of NFAT1 we tested the effect of NFAT1 deficiency on IL-9 expression in Th9 cells. CD4+ T cells isolated from wild type (WT) and NFAT1−/− (KO) mice were differentiated *in vitro* for 5 days into Th9 cells and were restimulated with PMA/ionomycin, or α-CD3/α-CD28. The expression level of IL-9 transcript and protein between the groups were analyzed by qRT-PCR and ELISA respectively. Compared with WT, the expression level of IL-9 in Th9 cells from NFAT1−/− mice was significantly decreased under all the time points and stimulation conditions analyzed (Fig. 3A and B). Down regulated IL-9 mRNA level in NFAT1−/− Th9 cells is well correlated with the decreased level of IL-9 protein in the cell culture supernatants (Fig. 3C). To further validate the functional role of NFAT1 in IL-9 expression we tested whether a reconstitution of NFAT1 into NFAT1−/− Th9 cells can restore IL-9 expression. NFAT1 expression plasmid was nucleofected in the Th9 cells derived from NFAT1−/− mice on day 3 of culture. After 48 hrs of nucleofection
cells were stimulated with PMA/ ionomycin for 12hrs. Reconstitution of NFAT1 into NFAT deficient Th9 cells was confirmed by checking NFAT1 protein expression by western blotting (Fig. 3D). Upon reconstitution (RC) of NFAT1, down-regulated IL-9 expression in NFAT1 deficient cells (KO) was successfully restored to the comparable levels of wild type (WT) Th9 cells both in the mRNA (Fig. 3E) and protein levels (Fig. 3F). These results indicate the pivotal role of NFAT1 in IL-9 expression by Th9 cells.

Reduced chromatin accessibility of the IL-9 promoter in NFAT1⁻⁻ Th9 cells

Previous studies showed that transcription factors, IRF4, Pu.1 and STAT6 play decisive roles in Th9 cells lineage commitment. We tested whether a defect in IL-9 expression in NFAT1⁻⁻ Th9 cells was due to any changes in the expression levels of these transcription factors. In vitro differentiated Th9 cells from WT and NFAT1⁻⁻ (KO) mice were left unstimulated or restimulated with PMA/ionomycin for the indicated time and nuclear extracts were used to check the nuclear level of NFAT1, Pu.1, IRF4, Lamin B (nuclear control) and tubulin (cytosolic control) by western blotting. However, no significant change was observed in the nuclear levels of IRF4 and Pu.1 between the wild type (WT) and NFAT1⁻⁻ Th9 cells (Fig. 4A). We also confirmed that down-regulated IL-9 expression in NFAT1⁻⁻ condition is not mediated by alteration of Th9 development program since IL-9 expression was also significantly lower in the NFAT1⁻⁻ CD4⁺ T cell blasts compared with wild type counterparts (Supplemental Fig. S4A and B). Next, we questioned how the absence of NFAT1 could affect IL-9 gene expression without inducing any defect in Th9 differentiation or enhancing IL-9 promoter activation? We tested the possibility that NFAT1 is involved in the epigenetic modifications of the IL-9 promoter locus. One of the possible causes of reduced gene expression is the alteration of the chromatin architecture to a condensed and inaccessible form. This can be detected by analyzing accessibility of chromatin to micrococcal nuclease (MNase) based PCR assay (58,59). Thus we analyzed the differential chromatin architecture of IL-9 promoter region between the WT and NFAT1⁻⁻ Th9 cells by measuring relative MNase accessibility. Indeed, IL-9 promoter region in NFAT1⁻⁻ Th9 cells was more resistant to MNase digestion compared to that of the WT cells (Fig. 4B). The accessibility of the IL-9 promoter from NFAT1⁻⁻ Th9 cells was restricted regardless of stimulation while wild type Th9 cells showed a significant increase of accessibility upon stimulation (Fig. 4B). The actin promoter region was employed as a control for constitutively active housekeeping gene and showed a similar accessibility regardless of NFAT1 deficiency (Fig. 4B).

In general, enrichment of acetyl histone H3 (AcH3), acetyl histone H4 (AcH4) and histone H3 lysine 4 dimethylation (H3K4Me2) to promoters well correlates with their transcriptionally active status (30,50,51). Thus, to further confirm the differential chromatin structure of the IL-9 promoter between WT and NFAT1⁻⁻ Th9 cells, relative amounts of recruited AcH3, AcH4 and H3K4Me2 levels were analyzed by ChIP assay. Indeed, the enrichment levels of all the tested active histone markers on the IL-9 promoter were significantly lowered in NFAT1⁻⁻ Th9 cells (Fig. 4C). We also measured RNA polymerase II (Pol II) binding to the IL-9 promoter since RNA Pol II enrichment marks actively transcribing promoter (34,35). Correlating with the physiologically decreased binding of active histone markers, significantly lowered binding of Pol II was observed in NFAT1⁻⁻ Th9 cells (Fig. 4D). These results suggest that NFAT1 deficiency results in a transcriptionally inactive chromatin configuration at the IL-9 promoter.

NF-κB (p65) transactivates the IL-9 promoter

Generally, NFAT1 positively regulates transcription of a large number of inducible cytokine genes by directly binding to their promoters. ChIP and DAPA analysis showed the physiological binding of NFAT1
to the IL-9 promoter (Fig. 2A and B). The results that NFAT1 deficiency resulted in an inactive chromatin configuration (Fig. 4B-D) and over-expression of NFAT1 failed to transactivate the IL-9 promoter (Fig. 2C-D), suggest that NFAT1 may play a role in inducing or stabilizing the active chromatin status of the IL-9 promoter rather than activating it. So we questioned if NFAT1 is not involved in the activation of IL-9 promoter then which factor can drive IL-9 production? To identify candidate proteins that are subsequently recruited to the active IL-9 promoter and trigger the maximal expression of IL-9 upon stimulation, IL-9 promoter driven luciferase reporter analysis was performed in the presence of the predicted transcription factors (Supplemental Fig. S1) in HEK-293 cells. Among the tested transcription factors, only NF-κB (p65) significantly enhanced the IL-9 promoter activity (Fig. 5A). Significant increase of IL-9 promoter activity was also observed in EL4 T cells upon over-expression of NF-κB (p65) (Fig. 5B). To further confirm the NF-κB (p65)-dependent transactivation of IL-9 promoter, deletion or mutations were introduced in the NF-κB binding sites corresponding to the NT/κB1 (-315/-307) and NT/κB2 (-48/-38) (Fig. 1B and Supplemental Fig. S1). A deletion or mutation of the predicted NF-κB binding sites (NT/κB1 and NT/κB2 or both (NT/κB1,2)) completely abolished NF-κB mediated transactivation of the IL-9 promoter in HEK-293 cells (Fig. 5C) and significantly reduced the activity of the IL-9 promoter in primary Th9 cells as well (Fig. 5D). Next we tested whether NF-κB (p65)-driven IL-9 enhancement is directly associated with physiological binding of NF-κB mediated transactivation of the IL-9 promoter in HEK-293 cells (Fig. 5C) and significantly reduced the activity of the IL-9 promoter in primary Th9 cells as well (Fig. 5D). Next we tested whether NF-κB (p65) antibody was used to precipitate the chromatin prepared from in vitro differentiated and restimulated Th9 cells from wild type (WT) and NFAT1−/− (KO) mice. Indeed, significant enrichment of NF-κB (p65) was observed at the IL-9 promoter in the Th9 cells compared with that of Th1 cells that produce negligible amount of IL-9 (Fig. 6A). By performing DNA affinity purification assay we further confirmed the binding of NF-κB to the two predicted NF-κB (p65) binding sites at the IL-9 promoter. Efficient binding of NF-κB (p65) was detected when biotinylated NT/κB1 (-315/-306) probe was used to pull down NF-κB from HEK-293 cells that were transfected with NF-κB (p65) expression plasmid. Nonbiotinylated competitor probes significantly reduced NF-κB binding (lanes 4 and 5) whereas mutant competitors (lanes 6 and 7) failed to do (Fig. 6B). To validate the functional involvement of NF-κB, the effect of p65 siRNA on IL-9 gene expression was tested. Th9 cells differentiated from wild type (WT) mice were transfected with mock or p65 siRNA as described in Materials and Methods section. Transfection of p65 siRNA diminished the expression level of p65 compared with the cells transfected with control siRNA (mock) as confirmed by western blotting (Fig. 7A). Indeed, knockdown of p65 expression significantly reduced IL-9 expression both in the mRNA and protein levels compared to scrambled siRNA transfected cells (mock) (Fig. 7B and C). These results suggest that the presence and physiological binding of NF-κB (p65) to the IL-9 promoter are essential for IL-9 expression in Th9 cells.

**NFAT1-mediated recruitment of transcriptional activation complex enhances IL-9 expression.**

To further delineate the mechanism of functional synergy between the transcription factors in driving IL-9 expression, we tested the effect of over-expression of each transcription factors or their combinations on IL-9 promoter activity. Over-expression of NFAT1 alone or NFAT1 together with NF-κB failed to further enhance the NF-κB induced IL-9 promoter activity (Fig. 8A). Interestingly the histone acetyl transferase p300 has been reported to interact with both NFAT and NF-κB and is essential for NF-κB mediated transactivation (34,44,45). Therefore, we tested the functional synergism between the factors p300, NF-κB (p65) and NFAT1 by performing IL-9 reporter assay. Indeed, co-expression of p300, NF-κB (p65) and NFAT1 significantly increased NF-κB (p65) mediated IL-9 promoter activity (lane 9 in
Fig. 8A). However, the promoter construct with mutations in both the NF-κB binding sites (mt-IL-9) abolished the enhancement of promoter activity in presence of these three factors (lane 10 in Fig. 8A). This result indicates that coactivator protein p300 acts as a bridge between the two transcription factors leading to a functional synergy between them. This was further proved by the reduced binding of p300 to the IL-9 promoter in NFAT1−/− Th9 cells (Fig. 8B). Next, we tested the effect of NFAT1 deficiency on the recruitment of NF-κB (p65) to the IL-9 promoter. Upon stimulation, significant enrichment of NF-κB (p65) to the IL-9 promoter was observed in WT Th9 cells. However NFAT1 deficient cells did not show such enrichment (Fig. 8C). These results suggest that NFAT1 is mainly involved in remodeling of IL-9 promoter with transcriptional active status where recruitment of p300 and NF-κB (p65) transactivates IL-9 promoter activity. We further tested the effect of NFAT1 deficiency on co-recruitment of p300 and NF-κB (p65) to the IL-9 promoter in Th9 cells by performing ChIP-re-ChIP experiment. Chromatins were prepared from stimulated WT or NFAT1−/− Th9 cells and ChIP assay was performed with anti-NFAT1 (Fig. 8D; left panel), anti-p65 (Fig. 8D; middle panel) and anti-p300 (Fig. 8D; right panel) antibodies. The precipitated chromatin from each group was subjected to second round of ChIP assay using indicated antibodies. Specific enrichment of the indicated factors was compared with control IgG. Next, to further characterize the functional role of NFAT1 as active chromatin modifier and NF-κB (p65) as transactivator respectively, we tested the recruitment kinetics of activation complex (NFAT1, p300, and NF-κB (p65)) and its effect on IL-9 expression. Th9 cells were stimulated for indicated time period and the expression level of IL-9 (Fig. 8E, upper panel) and relative recruitment of NFAT1 and other factors to the IL-9 promoter (Pro) or non-relevant region (NR) were analyzed (Fig. 8E, lower panel). Compared with other proteins, earlier binding of NFAT1 was observed within 30 mins of stimulation and its level was maintained till later period of stimulation (Fig. 8E, lower panel). The binding levels of p300, AcH3 and NF-κB (p65) to the IL-9 promoter were increased with time and the maximal binding of NF-κB p65 was detected at 8 hrs of stimulation (Fig. 8E, lower panel). This result well correlated with the time dependent increase of IL-9 expression that reaches peak at later hours of stimulation (Fig. 8E, upper panel and Supplemental Fig. S3B) (56,57). A non-relevant control region (NR) however failed to show enrichment with the tested proteins compared with control IgG antibody (Fig. 8E, lower panel). Collectively, these results suggest that the activation complex assembled on the IL-9 promoter constitutes of NFAT1 (as a chromatin activator), p300 (as an active chromatin modifier) and NF-κB (p65) (as a transactivator). A functional synergy between these factors may potentiate high levels of IL-9 expression in Th9 cells.

DISCUSSION

The principle aim of our study is to elucidate the molecular mechanism of IL-9 gene transcription in Th9 cells. We demonstrated that functional cooperation of NFAT1 and NF-κB (p65) synergistically enhances IL-9 expression in Th9 cells. Binding of NFAT1 to the IL-9 promoter results in a transcriptionally competent IL-9 promoter by modifying it with active histone marks. This active chromatin configuration thereby favors the recruitment NF-κB (p65) to enhance IL-9 expression in Th9 cells. Consequently, NFAT1 deficiency or knock-down of NF-κB (p65) results in a significant down-regulation of IL-9 expression by affecting both the chromatin architecture and the formation of activation complex, respectively, at the IL-9 promoter.

Recent studies have identified the transcription factors involved in regulation of Th9 cell differentiation and IL-9 transcription. The ETS family and TGF-β induced transcription factor Pu.1, has been implied in controlling IL-9 expression and high level of Pu.1 is essential for Th9 cell differentiation program (21,23,24). Mouse with T cell specific deletion of Pu.1 and the knock-down of Pu.1 in human T cells
displays a significantly reduced IL-9 expression (21). The Pu.1 interacting and IL-4 upregulated protein IRF4 is another transcription factor proven to be indispensable for Th9 cell development. Absence of IRF4 also prevented the Th9 cell generation (22). STAT6 is yet another newly found transcription factor acting downstream of IL-4 signalling that affects Th9 lineage commitment by inducing IRF4 expression and repressing Tbet and FoxP3 expression in Th9 cells (23). However the role of TCR induced transcription factors responsible for acute IL-9 induction from Th9 cells is still unclear.

In this study, we have mainly identified two important TCR induced transcription factors, NFAT1 and NF-κB that functionally synergize to activate IL-9 gene transcription. NFAT1−/− Th9 cells display significantly reduced IL-9 expression (Fig. 3A–C), while reconstitution of NFAT1 into NFAT1−/− Th9 cells restored IL-9 expression (Fig. 3 D–F). Based on these finding we have investigated the underlying mechanism of NFAT1 mediated IL-9 expression in Th9 cells.

Firstly, we tested whether NFAT1 deficiency could alter the Th9 differentiation program, thereby down-regulating IL-9 expression. However, we did not find any changes in the nuclear levels of reported Th9 lineage determinants such as Pu.1 and IRF4 between the WT and NFAT1−/− Th9 cells (Fig. 4A). In addition, NFAT1 deficiency also decreased IL-9 expression in CD4+ T cell blasts that were not polarized into any specific T helper cell types (Supplemental Fig. S4A and B). This result thereby rules out the possibility that absence of NFAT1 results in dysregulated Th9 differentiation at least in the context of Pu.1 and IRF4 expression. Another possibility of NFAT1 deficiency mediated IL-9 reduction could be the differential expression of the cytokines necessary for IL-9 production. IFN-γ has been shown to be a negative regulator of IL-9 expression in human Th9 cells (60). The absence of Th9 lineage factor IRF4, results in huge increase in IFN-γ expression and hence could be another potential mechanism by which IL-9 expression is reduced in IRF4−/− mice (22). In this study, to rule out the possibility of IFN-γ involvement in dysregulated IL-9 expression in NFAT1−/− Th9 cells, Th9 cells were differentiated with IL-4 and TGF-β in the presence and absence of α-IFN-γ. Expression of IFN-γ was reduced upon neutralization of IFN-γ and IL-9 expression was further increased both in the wild type and NFAT1−/− Th9 cells. Thus, IFN-γ signaling may not interfere with IL-9 expression in NFAT1−/− Th9 cells (Supplemental Fig. S5A and B). In addition to IFN-γ, IL-21 has been shown to be a positive regulator of IL-9 expression in human Th9 cells (60). IL-21 is itself regulated by NFAT1 (33) and thus this axis may also account for decreased IL-9 expression in NFAT1−/− cells.

Secondly, we tested the role of NFAT1 as a transcription factor that potentiates IL-9 promoter activity by directly binding to the IL-9 promoter. Indeed, NFAT1 binds to the NFAT1 responsive elements at the IL-9 promoter, but it failed to enhance its activity (Fig. 2). So we tested the possibility whether NFAT1 could act as a chromatin remodeling factor. Previous studies have demonstrated that the N-terminal of NFATs interact physically with intrinsic histone acetylase (HATs) CBP/p300, which enables NFAT to act as a chromatin remodeling factor (34,35). For example, in the case of GM-CSF enhancer, NFAT is responsible for the formation of a permissive chromatin architecture, thereby providing an access to AP1 that is obligatory for transactivating the GM-CSF enhancer (61). A recent report demonstrated that NFAT binding to the c-Myc promoter resulted in an increased acetylation of the c-Myc promoter, via recruitment of p300 coactivator. This acetylated c-Myc promoter subsequently binds to the ETS family transcription factor ELK-1 and triggers the maximal expression of c-Myc in pancreatic cancer cell line (35). In our present study, we also found that NFAT1 deficiency reduced chromatin accessibility and recruitment of active histone markers and Pol II to the IL-9 promoter (Fig. 4B–D).

The immediate follow-up question is what are the other factors that can
potentiate IL-9 expression while acting in concert with NFAT1 in Th9 cells? In this context, we identified NF-κB (p65) as another TCR induced transcription factor that works in functional synergy with NFAT1 and drives IL-9 expression. NF-κB serves as a transcriptional activator to enhance IL-9 gene expression in Th9 cells. Significant enrichment of NF-κB (p65) at the IL-9 promoter potentiated its activity while siRNA mediated knock-down of p65 in Th9 cells significantly decreased IL-9 expression (Fig. 5, 6 and 7). In addition, we also elucidated the mechanism by which a functional synergy is achieved between NFAT1 and NF-κB in the context of IL-9 expression. NFATs often influence gene transcription acting synergistically with several other transcription factors by assembling enhanceosomes together with coactivators CBP and p300 (25,62-64). In addition to histone acetyl transferase activity, CBP/p300 has well known functions as scaffolding proteins, which leads to the formation of enhanceosomes onto gene promoter and regulatory elements (65). Incidentally, NF-κB (p65) also physically interacts with the p300/CBP and this is essential and crucial for NF-κB (p65) mediated transactivation (44,45,66,67).

Interestingly, in our study we found that in the absence of NFAT1, there is a significant reduction of p300 recruitment to the IL-9 promoter (Fig. 8). This accounts for the creation of a hypo-acetylated chromatin environment at the IL-9 locus and also manifests in the impaired and unstable binding of NF-κB (p65) to the IL-9 promoter (Fig. 8B and C). However, the IL-9 promoter reporter plasmid showed similar activity in the NFAT1- Th9 cells as that in the wild type (Supplemental Fig. S6). The fold activation of IL-9 reporter construct with respect to the mock transfected (empty vector) samples were ~2.6 and 2.9 in case of WT and KO cells, respectively (Supplemental Fig. S6). The activity of the mutant promoter construct also decreased as in the case of transfection in WT cells (Supplemental Fig. S6). This could be due to the following facts: (i) Abundant amount of p65 protein was present both in the WT and NFAT1- Th9 cells (Supplemental Fig. S6; inset). (ii) As p65 and NFAT1 share the same binding sites (two out of the five NFAT biding sites) in the IL-9 promoter (Fig 1A, 2B and 6B), p65 can successfully maintain the promoter activity in the absence of NFAT1. Overlapping NFAT and NF-κB binding sites have been identified in several genes such as in the HIV-1 LTR, IL-8, IL-13, GMCSF, IFN-γ and TNF-α etc. (36,68-71). This is due to the presence of similar DNA binding domain (RHR domain) in the NFAT and NF-κB/Rel family of proteins (72). In the context of IL-9 promoter, we have found that NFAT1 mediated chromatin activation is crucial for p65 binding to the IL-9 promoter, thereby indicating that IL-9 promoter is responsive to both NFAT1 and NF-κB. The differential in vivo binding of p65 to the IL-9 promoter in the WT and NFAT1 deficient (KO) Th9 cells (Fig. 8C & 8D) and decrease in IL-9 expression upon p65 knock-down (Fig. 7) clearly establishes the fact that both NFAT1 and p65 are indispensable for IL-9 expression. (iii) Additionally, the IL-9 reporter plasmid is an artificial in vitro designed construct used in transient transfection assay. Thus, in this situation, its reporter activity is not susceptible to chromatin configuration changes unless it integrates into the genome as a transgene which is possible only in stable transfection system and not in transient transfection assay (73,74).

Our study also suggests a functional importance of crosstalk between the transcription factors and their co-activators. Physical association of NFAT1, NF-κB (p65) and p300 with the IL-9 promoter was confirmed by ChIP- re-ChIP experiment (Fig. 8D). NFAT1 deficiency significantly reduced the recruitment of NFAT interacting activation complex such as NF-κB and p300 to the IL-9 promoter while co-over expression of NFAT1, NF-κB (p65) and p300 significantly enhanced IL-9 promoter activity (Fig. 8D and 8A). A recent paper suggested a two-step process in the assembly of transcription factor complex on to the IL-2 promoter. NFAT1, Jun and Fos are the early and Oct2, Rel A (p65), c-Rel and NFAT2 are the late binding factors, respectively (75). Thus sequential binding
of transcription factors and coactivators are probably essential and general series of events for the TCR induced IL-9 expression also. Indeed, time dependent ChIP assay revealed that binding of NFAT1 to the IL-9 promoter is an initial event compared with other factors such as p300, AcH3 and NF-κB (p65) (Fig. 8E). NFAT1 binding to the IL-9 promoter region thus induces a net histone acetylation by co-recruitment of histone acetyl transferase p300, which results in the formation of a hyperacetylated, transcriptionally competent IL-9 promoter. This active promoter thereby recruits NF-κB (p65) and ensures maximal expression of IL-9. Therefore we suggest that IL-9 transcription in Th9 cells is driven by a transcription activator complex consisting of NFAT1, p300 and NF-κB (p65) and in this process p300 serves as a bridge which not only acetylates the promoter but also stabilizes the binding of NF-κB (p65). In this study, we have identified the functional importance of NFAT1 and NF-κB in enhancing IL-9 expression. However we certainly cannot exclude the role of other transcription factors involved. The expression level of IL-9 is further enhanced under Th9 differentiation condition compared with CD4+ T cell blasts, which suggests a possibility that NFAT1 may cooperate with Th9 lineage determining transcription factors as well. IL-9 has also newly been described as a Th17 type cytokine and subsequent roles of IL-9 in Th17 mediated inflammatory responses have also been implicated (18,76). Interestingly, the HTLV-transformed cell line Hut-102 has an IL-9 producing Th17 phenotype. It is shown that in this cell, specific knock down of NF-κB protein c-Rel results in reduced IL-9 production (77). We have also found that NFAT1 deficient Th17 cells have defect in IL-9 expression (data not shown). Thus, the ubiquitous expression pattern of NFAT1 and NF-κB, yet a differential effect in the T helper cell subsets, can be exploited to elucidate their roles in regulating IL-9 expression in association with other transcription factors partners.

In summary, our study constitutes the first report showing that TCR induced transcription factors NFAT1 and NF-κB (p65) play a crucial role in the regulation of IL-9 expression in Th9 cells. NFAT1 essentially creates an accessible platform for the assembly of transcriptional coactivators on to the IL-9 promoter while NF-κB (p65) potentiates maximal expression of IL-9. Thus, our study delineates an important role of NFAT1 and NF-κB (p65) in IL-9 expression by T lymphocytes.

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SUPPLEMENTAL DATA
Supplemental material is available at Journal of Biological Chemistry online.

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Conflict of interest:
The authors have no financial conflict of interest

FIGURE LEGENDS

**Figure 1.** Comparative bioinformatic analysis of the mouse and human *IL-9* gene loci. *A,* rVISTA 2.0 analysis depicting % conservation between mouse (as a base) and human IL-9 loci and the boxed region indicates the IL-9 promoter. *B,* The nucleotide sequence comparison between mouse (-366/+48) and human IL-9 promoter region. The predicted TATA site is boxed and +1 denotes the TSS (transcription start site). The predicted NFAT sites with a matrix similarity of 0.8 are marked as following; NFAT-a (-315/-306) NFAT-b (-290/-281), NFAT-c (-223/-214), NFAT-d (-165/-155) and NFAT-e (-48/-38), respectively. The composite sites for NFAT and NF-κB sites are marked as NT/κB1 (-315/-306) and NT/κB1 (-48/-38), respectively.

**Figure 2.** Physical association of NFAT1 with the IL-9 promoter. *A,* ChIP assay was performed with PMA/ionomycin-stimulated Th1 and Th9 cells using control IgG and NFAT1
antibody. The amounts of precipitated DNA were measured by quantitative PCR with primers specific for the IL-9 and IFN-γ promoter regions and represented relative to their amount in total chromatin (input) as fraction of input. B, Left panel, antibodies against NFAT1 and actin (control) were used to perform western blot for detecting NFAT1 expression in untransfected and NFAT1 transfected HEK cells. Right Panel, Biotin conjugated probe corresponding to NFAT binding site (at -48/-38 in Fig. 1A; NFAT-e) were incubated with NFAT1 overexpressing HEK-293 cell lysate in the presence of indicated non-biotinylated competitor probes. Cons or mt-Cons indicate the conserved NFAT binding or NFAT nonbinding mutant probes, respectively. The protein DNA complexes were precipitated with streptavidin (Strep) and analyzed by immunoblotting with α-NFAT1 antibody. First Lane indicates input which is 2.5% of the total cell extract (CE) used for pull-down. The data are representative of three independent experiments. C,D, HEK-293 (C) or EL-4 cells (D), respectively, were transfected with empty control (mock) or luciferase reporter constructs containing IL-9 promoter (IL-9 Pro: encompassing -366/+48 region as shown in Fig. 1A) or enhancer containing IL-10 promoter (IL-10 CNS-9; (30)) in the presence of NFAT1 expression vector. Cells were stimulated with PMA/ionomycin for 8 hrs or with cyclosporine A (CsA) 20 min prior to PMA/ionomycin stimulation and luciferase assay was conducted. The luciferase activity was calculated relative to the activity of Renilla luciferase and represented as Relative Luciferase Unit (RLU) as a fold difference relative to the control (Mock/empty plasmid) value. The data shown are expressed as mean ± SEM, n = 3 and ** P<0.01, ***P<0.001.

**Figure 3.** NFAT1 reconstitution restores IL-9 expression in the NFAT1 deficient Th9 cells. Th9 cells differentiated from wild type (WT) and NFAT1−/− (KO) were stimulated by A, PMA/ionomycin (PI) or B, α-CD3/CD28 for the indicated time and IL-9 expression was measured by qRT-PCR by normalizing with the level of housekeeping gene HPRT. C, The IL-9 protein levels in the culture supernatant of unstimulated (W/O) or upon 24 hrs by PI or α-CD3/CD28 restimulation were quantified by ELISA. The data shown are expressed as mean ± SEM, n = 3 and *P<0.05, ** P<0.01, ***P<0.001. D, Relative level of NFAT1 was analyzed in wild type (WT), KO or NFAT1 reconstituted NFAT1−/−Th9 (RC) cells by western blotting with α-NFAT1 antibody and α-Lamin B (control). E-F, The relative amount of IL-9 expression in the wild type (WT), NFAT1−/− (KO) and NFAT1 reconstituted NFAT1−/− Th9 (RC) cells was measured by qRT-PCR and ELISA, respectively. The data shown are expressed as mean ± SEM, n = 3 and ** P<0.01.

**Figure 4.** NFAT1 regulates chromatin architecture at the IL-9 promoter. A, Nuclear extracts were prepared from Th9 cells differentiated from wild type (WT) and NFAT1−/− (KO) mice and stimulated with PMA/ionomycin for indicated time. The nuclear levels of Pu.1, IRF4, Lamin B (nuclear control) and β-tubulin (cytosolic control) were analyzed by immunoblotting with the respective antibodies. B, Nuclei isolated from either unstimulated (W/O) or PMA/ionomycin-stimulated (PI) wild type (WT) and NFAT1−/− (KO) Th9 cells were left untreated or subjected to MNase digestion. Relative chromatin accessibility at the promoters of the IL-9 and actin promoter (as a control for accessible region) was measured by qRT-PCR using specific primers. The results are represented as ratio of PCR product obtained from digested samples normalized to the PCR products from undigested samples and mean ± SEM, n = 3 and * P<0.05. C-D, PMA/ionomycin stimulated Th9 cells from wild type (WT) and NFAT1−/− (KO) mice were used for ChIP assay with antibodies against acetylated histones (AcH3, AcH4, H3K4Me2), RNA-Pol II or control IgG. qRT-PCR with primer spanning the IL-9 promoter locus (-366/+48) was used to detect the precipitated DNA and represented as negative images of EtBR stained gels (C) or relative to the amount in total chromatin (input) as fraction of input (D). All data are representative of at least three independent experiments.
**Figure 5. NF-κB (p65) transactivates the IL-9 promoter.**
A, HEK-293 or EL-4 cells. B, were transfected with the empty vector (Mock) or IL-9 promoter (-366/+48) (IL-9 Pro) containing luciferase reporter constructs in the presence of indicated expression vectors. C, HEK-293 cells were transfected with empty vector (Mock), IL-9 promoter or mutant IL-9 promoter reporter constructs in the presence of NF-κB (p65) expression vector. D, Th9 cells were transfected with the empty vector (Mock), IL-9 promoter reporter construct or mutated IL-9 promoter reporter construct that has mutation in both NF-κB binding sites (mt-IL-9 Pro; NT/κB1 and NT/κB2). The luciferase activity was calculated relative to the activity of Renilla luciferase and represented as Relative Luciferase Unit (RLU) as a fold difference relative to the control (Mock/empty plasmid) value. The data is representative of at least 3 independent experiments. The data shown are expressed as mean ± SEM, n = 3 and *P<0.05 ** P<0.01, ***P<0.001.

**Figure 6. In vivo and in vitro binding of NF-κB (p65) to the IL-9 promoter.**
A, ChIP assay was performed with in vitro differentiated and PMA/ionomycin-stimulated Th1 and Th9 cells using control IgG and NF-κB (p65) antibodies. The amounts of precipitated DNA were measured by qRT-PCR with primers specific for the IL-9 and IL-2 promoter regions. Relative NF-κB (p65) enrichment in the precipitated samples compared with total chromatin (input) is shown as a fraction of input. B Left panel, antibodies against NF-κB (p65) and actin (control) were used to perform western blot for detecting NF-κB (p65) expression in untransfected and NF-κB (p65) transfected HEK cells. Right Panel, Biotin conjugated probe corresponding to NF-κB binding site 1 (NT/κB-1; -315/-306 in Fig. 1B) were incubated with NF-κB (p65) overexpressing HEK-293 cell lysate in the absence or presence of indicated non-biotinylated competitor probes. The protein DNA complexes were precipitated with streptavidin (Strep) and analyzed by immunoblotting with α-NF-κB antibody. First Lane indicates input which is 2.5% of the total cell extract (CE) used for pull-down. The data are representative of three independent experiments.

**Figure 7. Knockdown of NF-κB (p65) reduces IL-9 expression.**
In vitro differentiated primary Th9 cells were transfected with siRNA against NF-κB (p65) or non-relevant siRNA (mock). A, The knockdown efficiency of p65 was confirmed by immunoblotting the respective cell lysates with antibodies against p65 and Lamin B (control). B, Relative level of IL-9 transcript in the mock and p65 siRNA transfected cells were analyzed by qRT-PCR and represented relative to the expression level of house-keeping gene HPRT. C, p65 siRNA or mock transfected Th9 cells were stimulated for 24 hrs with PMA/ionomycin and the IL-9 protein levels in the culture supernatant were measured by ELISA. The data shown are expressed as mean ± SEM, n = 3 and *P<0.05, ** P<0.01.

**Figure 8. NFAT1 mediates the recruitment of activation complex to enhance IL-9 promoter activity.** A, HEK-293 cells were transfected with empty control vector (Mock), IL-9 promoter (IL-9 Pro) or mutant IL-9 promoter (mt-IL-9 Pro (mutations in both NT/κB1 and NT/κB1 sites) reporter constructs in the presence of indicated expression vectors. Cells were stimulated with PMA/ionomycin for 8 hrs and harvested for luciferase assay. The luciferase activity was calculated relative to the activity of Renilla luciferase and represented as Relative Luciferase Unit (RLU) as a fold difference relative to the control (Mock/empty plasmid) value. The data represent mean ± SEM, n = 3 and * P<0.05, ***P<0.001. B-C, ChIP assay was performed with Th9 cells differentiated from wild type (WT) and NFAT1−/− (KO) mice using control IgG, p300 B and p65 antibodies C. The amounts of precipitated DNA were measured by qRT-PCR with primers specific for the IL-9 promoter region and represented relative to the amount in total chromatin (input) as a fraction of input. The data represents at least three independent experiments. The data represent mean ± SEM, n = 3 and *P<0.05, ** P<0.01. D, ChIP-re-ChIP experiment. Th9 cells from wild type (WT) and NFAT1−/− (KO)
mice were stimulated with PMA/ionomycin and subjected to ChIP with either α-NFAT1, α-p65, α-p300 or control IgG antibodies (first ChIP) and the immunoprecipitates were eluted either with elution buffer or 10mM DTT and proceeded for second ChIP with indicated antibodies. Amount of precipitated DNA was then detected by qRT-PCR with the primer spanning the IL-9 promoter locus and presented as negative images of EtBr stained gel. Data are representative of at least three independent experiments. E, Th9 cells were restimulated with PMA/ionomycin for indicated time periods and IL-9 levels were measured by qRT-PCR (upper panel). ChIP assay was performed with indicated antibodies and relative amount of precipitated IL-9 promoter DNA was then detected by qRT-PCR and presented as negative images of EtBr stained gel (lower panel). Data are representative of at least three independent experiments.
FIGURE 2.

A. Graph showing the fraction of input IFN-γ promoter compared to the IL-9 promoter.

B. Table summarizing pull down results with various conditions.

C. Bar graph illustrating RLU values for different treatments.

D. Graph depicting RLU values with different conditions and treatments.
FIGURE 3

A. IL-9/HPRT levels in WT and KO cells with or without PI treatment.

B. IL-9/HPRT levels in WT and KO cells stimulated with α-CD3/CD28 for different hours.

C. IL-9 levels in WT and KO cells with or without α-CD3/CD28 treatment.

D. Western blot analysis of NFAT1 and Lamin B in WT, KO, and RC cells.

E. IL-9/HPRT levels in WT, KO, and RC cells.

F. IL-9 levels in WT, KO, and RC cells.
FIGURE 4

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B

Relative accessibility

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<tr>
<td>IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>input</td>
<td></td>
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</tbody>
</table>

D

Fraction of input

IgG
α-Pol II
FIGURE. 6.

Graph A shows the fraction of input IL-2 promoter for different treatments: α-p65 and IgG.

Graph B illustrates the pull down of various treatments with p65 and β-Actin as controls.
A

B

C

FIGURE 7.
FIGURE 8.
Nuclear factor of activated T cells 1 (NFAT1) induced permissive chromatin modification facilitates nuclear Factor-κB (NF-κB) mediated interleukin-9 (IL-9) transactivation

Arijita Jash, Anupama Sahoo, Gi-Cheon Kim, Chang-Suk Chae, JI-Sun Hwang, Jung-Eun Kim and Sin-Hyeog Im

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