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Generation of reporter mice for detecting the transcriptional activity of nuclear factor of activated T cells

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Abstract: Nuclear factor of activated T cells (NFAT) is a transcription factor essential for immunological and other biological responses. To develop analyzing system for NFAT activity *in vitro* and *in vivo*, we generated reporter mouse lines introduced with NFAT-driven enhanced green fluorescent protein (EGFP) expressing gene construct. Six tandem repeats of −286 to −265 of the human *IL2* gene to which NFAT binds in association with its co-transcription factor, activator protein (AP)-1, was conjunct with thymidine kinase minimum promoter and following EGFP coding sequence. Upon introduction of the resulting reporter cassette into C57BL/6 fertilized eggs, the transgenic mice were obtained. Among 7 transgene-positive mice in 110 mice bone, 2 mice showed the designated reporter mouse character. Thus, the EGFP fluorescence of $CD4^+$ and $CD8^+$ T cells in these mice was enhanced by stimulation through CD3 and CD28. Each of phorbol 12-myristate 13-acetate (PMA) and ionomycin (IOM) stimulation weakly but their combined stimulation strongly enhanced EGFP expression. The stimulation-induced EGFP upregulation was also observed following T cell subset differentiation in a different manner. The EGFP induction by PMA + IOM stimulation was more potent than that by CD3/CD28 stimulation in helper T (Th)1, Th2, Th9, and regulatory T cells, while both stimulation conditions displayed the equivalent EGFP induction in Th17 cells. Our NFAT reporter mouse lines are useful for analyzing stimulation-induced transcriptional activation mediated by NFAT in cooperation with AP-1 in T cells.
Key words: activator protein-1, enhanced green fluorescent protein, helper T cell, ionomycin, phorbol 12-myristate 13-acetate

Introduction

Nuclear factor of activated T cells (NFAT) plays a crucial role in various biological processes by regulating the transcription of functional molecule genes. Particularly in T cells, NFAT augments the expression of numerous cytokines upon binding to *cis*-regulatory elements of their genes in association with or without co-transcription factors [1]. NFAT consists of five members, NFATc1-c4 and NFAT5. Besides activation of NFAT5 by sensing

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an osmotic change, NFATcs are activated upon dephosphorylation by a Ca^{2+}-dependent phosphatase, calcineurin, translocate from the cytoplasm to nuclei, then reach their target gene enhancers [2].

NFAT is involved in the development of several organs and the pathogenesis of various diseases. Calcineurin inhibitors, cyclosporin A and tacrolimus, are used for treating allergic and autoimmune diseases and for preventing allograft rejection [3]. Pharmacological effects of these drugs are principally produced by inhibiting NFAT-dependent transcriptional activity [4].

Since the significance of NFAT on various intracellular events has been indicated and novel drugs for regulating NFAT activity are being developed [2], the establishment of a simplified system for monitoring NFAT activity has been desirable. Recently, fluorescence-based reporter mice by which the activity of another transcription factor, NFκB, can be monitored at a single-cell level were generated [5]. Here we reported the generation of NFAT reporter mice useful for analyzing stimulation-induced NFAT activation in T cells. Through the generation of NFAT/activator protein (AP)-1-combined binding element-driven enhanced green fluorescent protein (EGFP) expressing gene construct transgenic mice, differential regulatory mechanisms of the NFAT/AP-1 site in distinct T cell subsets were suggested.

Materials and Methods

Mice

C57BL/6N mice were obtained from the Jackson Laboratory Japan, Inc. (Yokohama, Japan). All mice were allowed free access to water and food and were kept under specific pathogen-free conditions in an environmentally controlled clean room at 23 ± 2°C and 50 ± 10% humidity with a 12 h light/dark cycle at the animal facility in the Research Institute for Radiation Biology and Medicine, Hiroshima University. The experimental protocols were approved by the Safety Committee for Recombinant DNA Experiments (2022-52) and Animal Use and Care Committees (A22-44) of Hiroshima University.

Generation of NFAT reporter mice

The reporter mouse lines introduced with NFAT-driven EGFP expressing gene construct were generated by transgenesis techniques. Briefly, we subcloned 6 tandem repeats of human *IL2* promoter region containing combined NFAT/AP-1 binding site (−286 to −265: ggaGGAAAAACTGTTTCAtaca) [1], herpes simplex virus (HSV) thymidine kinase minimum (TK-min) promoter, chimeric intron derived from human β-globin gene and immunoglobulin gene heavy chain variable region [6], EGFP, and SV40 early splicing and poly(A) signals into pBlueScript II SK (+) (Addgene, Watertown, MA, USA) (Fig. 1). After the microinjection of a DNA fragment containing the above sequence into the pronuclei, the zygotes were transplanted into the uteri of pseudo-pregnant mice. Following the birth and growth of mice from the zygotes, the transgene was identified by PCR for tail DNA. The optimal primer set, 5′-ACAAGTAAAGCGGCCGTTAACTTGTTTATTGC-3′ plus 5′-CCTTAAGATACATTGATG-3′ were determined from 2 candidate pairs.

In vitro differentiation and activation of T cells

$CD4^+$ and $CD8^+$ T cells were isolated from splenocytes of the transgenic mouse lines by magnetic cell sorting with EasySep Mouse CD4 and CD8 T Cell Isolation Kits (Veritas, Santa Clara, CA, USA), respectively, at >97% purity. The $CD4^+$ cells were cultured in OpTmizer CTS medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal calf serum (Hyclone Laboratories, San Angelo, TX, USA). At the start of culturing, pre-washed Dynabeads Mouse T-Activator CD3/CD28 (2 μl/10^5 cells, Thermo Fisher Scientific) and recombinant IL-2 (10 U/ml, PeproTech, Inc., Cranbury, NJ, USA) were added. To promote differentiation into each subset, cells were cultured for 7 to 10 days in the presence of appropriate cytokines and anti-cytokine antibodies as follows [7–12]. Th1: 10 U/ml IL-12 (PeproTech) and 10 μg/ml anti-IL-4 monoclonal antibody (mAb) (BD Biosciences, San Jose, CA, USA), Th2: 10 U/ml IL-4 (PeproTech) and 10 μg/ml anti-IFN-γ mAb (R4-6A2, eBioscience, San Diego, CA, USA), Th9: 10 U/ml IL-4, 5 ng/ml TGF-β (BioLegend, San Diego, CA, USA), and 10 μg/ml anti-IFN-γ mAb, Th17: 10 ng/ml IL-1β (Peprot-

Fig. 1. Schematic illustration of the injection fragment to generate transgenic mice. Six-tandem repeats of human *IL2* promoter region containing combined nuclear factor of activated T cells (NFAT)/activator protein (AP)-1 binding site [4] and enhanced green fluorescent protein (EGFP) coding sequence are shown as blue and green boxes, respectively. Herpes simplex virus (HSV) thymidine kinase minimum (TK-min) promoter, chimeric intron derived from human β-globin gene and immunoglobulin gene heavy chain variable region [6], and SV40 early splicing and poly(A) signals are shown in gray boxes. Positions of genotyping PCR primer pair were indicated by red bars.

ech), 20 ng/ml IL-6 (Peprotech), 1 ng/ml TGF-β, 10 ng/ml IL-23 (R & D Systems, Minneapolis, MN, USA), 40 μg/ml anti-IL-4 mAb, and 40 μg/ml anti-IFN-γ mAb, Treg: 5 ng/ml TGF-β, 2 nM trans-Retinoic Acid (Calbiochem, San Diego, CA, USA), 40 μg/ml anti-IL-4 mAb, and 40 μg/ml anti-IFN-γ mAb. Freshly isolated $CD4^+$ and $CD8^+$ T cells and differentiated T cell subsets were stimulated through CD3/CD28 as described above or stimulated by 20 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Saint Louis, MO, USA), a conventional protein kinase C (PKC) activator, 1 μM ionomycin (IOM, Sigma-Aldrich), a $Ca2^+$ ionophore, or their combination in bulk-culture for 24 h, then the fluorescence of EGFP was determined by flow cytometry.

Statistics

All experimental data are presented as means ± SD. Statistical analyses were performed using Student's *t*-test or one-way analysis of variance with Tukey's multiple comparison test. Statistical significance was set at $P<0.05$.

Results

Generation of NFAT reporter mice

Upon screening through tail DNA PCR, we identified seven transgene-positive mice in 110 mice bone. From these mice, we selected two mouse lines based on the reporter activity as described below, named them as C57BL/6-Tg(NFAT/AP-1-GFP)75Osmk (#75) and C57BL/6-Tg(NFAT/AP-1-GFP)97Osmk (#97), and maintained them through mating with C57BL/6N mice. Offspring of both lines was bone and grew healthy and did not show any apparent abnormalities. The introduction of at least one copy of the full-length DNA injection fragment into those mouse genomes was confirmed by sequencing.

The reporter activity of T cells in NFAT reporter mice

The reporter activity of peripheral T cells in the established mouse lines was analyzed. As shown in Fig. 2, the fluorescence of EGFP was equivalently upregulated in both $CD4^+$ and $CD8^+$ T cells upon stimulation through CD3/CD28 or with PMA. The relatively weak enhancement of EGFP expression was induced by IOM, though combined PMA + IOM stimulation induced the strongest

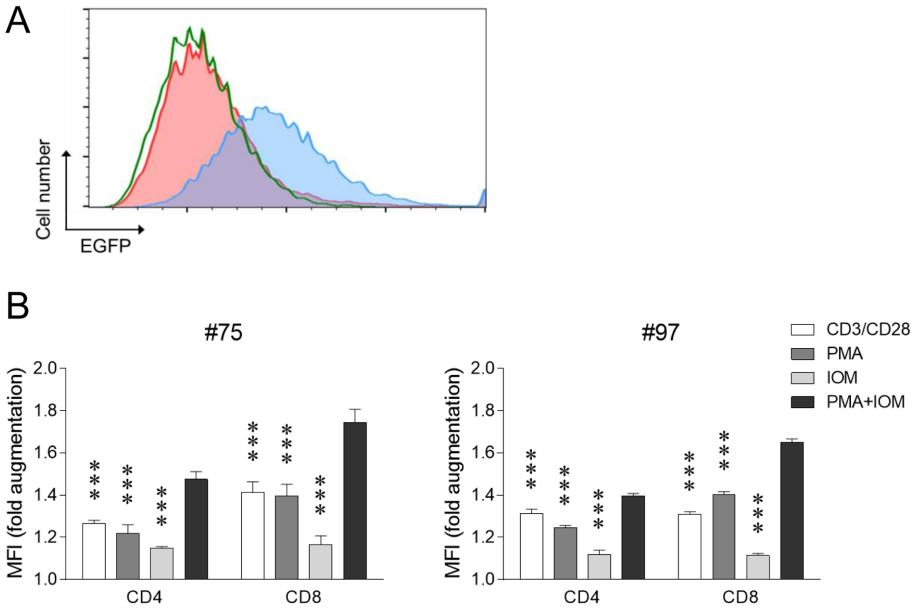

Fig. 2. Expression of enhanced green fluorescent protein (EGFP) in nuclear factor of activated T cells (NFAT) reporter mouse T cells. Peripheral $CD4^+$ and $CD8^+$ T cells of C57BL/6-Tg (NFAT/activator protein (AP)-1-GFP) 75Osmk (#75) and C57BL/6-Tg(NFAT/AP-1-GFP)97Osmk (#97) were left unstimulated or stimulated with anti-CD3/CD28 antibodies, phorbol 12-myristate 13-acetate (PMA), ionomycin (IOM), or PMA + IOM for 24 h. Then the fluorescence of EGFP was determined by flow cytometry. (A) Representative histogram data for #75 $CD4^+$ T cells with (blue) and without (red) PMA + IOM stimulation and unstimulated wild-type mouse $CD4^+$ T cells (green) were shown. (B) Data are expressed as means ± SD of fold augmentation of mean fluorescence intensity (MFI) compared with unstimulated control in quadruplicate cultures. *** $P<0.001$, compared with PMA + IOM-stimulated cells (Tukey's test).

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EGPF expression. Essentially the same responses were observed in both the #75 and #97 lines, though the stimulation-induced upregulation of EGFP fluorescence was hardly detectable by microscopic analysis.

The difference in the reporter activity of established NFAT reporter mouse lines was further evaluated in differentiated helper T (Th)1, Th2, Th9, Th17, and regulatory T (Treg) cells. Th1 cells differentiated by our procedure preferentially express IFN-γ and CXCR3, Th2 express IL-4, IL-5, IL-13, and CCR4, Th9 express IL-9, Th17 express IL-17A, IL-22, and RORγt, and Treg express TGF-β1, IL-10, CTLA-4, CD25, and FoxP3 as described previously [7–12]. As shown in Fig. 3, The expression of EGFP was upregulated by CD3/CD28 and PMA + IOM in all subsets. Among them, the weakest enhancement by each stimulation was observed in Th1 cells. Th17 and Treg cells displayed relatively higher EGPF augmentation by CD3/CD28 stimulation rather than other subsets. The enhancement of EGFP expression by PMA + IOM stimulation was more potent than that by CD3/CD28 stimulation in almost all the subsets, though both stimulations almost equivalently upregulated EGFP in Th17 cells.

Discussion

The present findings suggest that the NFAT reporter mouse lines we generated in this study are useful for analyzing the transcriptional activity mediated by the combined NFAT/AP-1 binding element at least in T cells. To evaluate NFAT-dependent transcriptional activity, the reporter system has widely been used. The transient transfection of NFAT binding site-driven reporter plasmids enables quantifying the NFAT activity in various cell types [13]. Based on this procedure, the difference in the activity of promoter/enhance elements to which NFAT binds in cooperation with or without other transcription factors, such as AP-1, can be examined [13, 14]. Several types of corresponding reporter plasmids and their stably transfected cells are commercially available. Reporter mice expressing luciferase directed by a hybrid promoter composed of a minimal mouse alpha myosin heavy chain cardiac promoter with upstream copies of the NFAT binding site sequence are also available [15]. At the NFAT site derived from the mouse *Il4* promoter, NFAT binding without AP-1 was demonstrated [16]. We further expanded the possibility of the NFAT/AP-1-driven reporter system in further physiological cells and conditions.

The enhanced expression of EGFP in NFAT reporter mice in response to PMA is convincing. Because AP-1, a heterodimer of Jun and Fos, is activated in the downstreaming cascade of PKC [17]. The relative weak upregulation of EGFP by IOM, regardless of the dependency of NFAT nuclear translocation to Ca^{2+}-dependent calcineurin, is supported by our previous reports, indicating the substantial nuclear localization of NFATc2 but not NFATc1 in the nuclei of unstimulated T cells [18]. The cooperation of Ca^{2+}-dependent NFAT and PKC-regulated AP-1 in their combined binding site, as described in previous studies [19], was confirmed in our reporter mouse T cells.

Our findings suggested the differential regulatory mechanisms of the NFAT/AP-1 site in distinct Th cell subsets. The dependency on NFAT and its co-transcription factors are different among T cell subset-specific cytokines. In contrast to the apparent cooperation of NFAT and AP-1 in IL-2, IL-4, and IL-17 promoters [20–22], NFAT was reported to associate with NFκB and Jun/activating transcription factor (ATF)-2 for activating

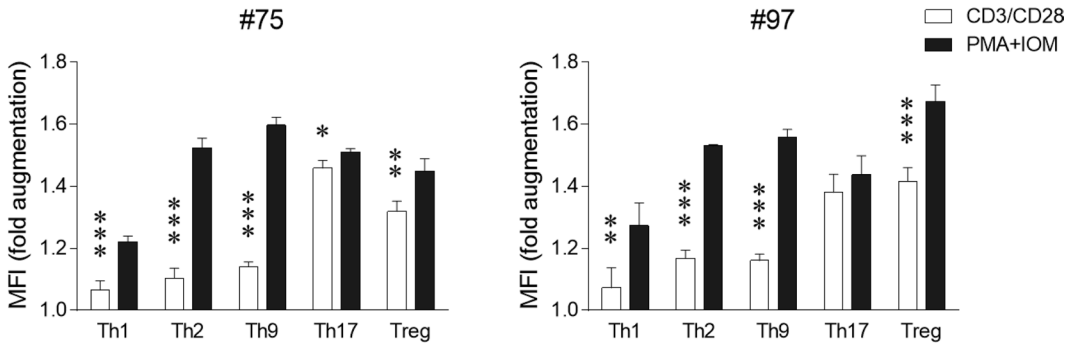

Fig. 3. Expression of enhanced green fluorescent protein (EGFP) in T cell subsets derived from nuclear factor of activated T cells (NFAT) reporter mice. Upon differentiation of $CD4^+$ T cells from #75 and #97 mouse lines into helper T (Th)1, Th2, Th9, Th17, and regulatory T (Treg) cells, they were left unstimulated or stimulated with anti-CD3/CD28 antibodies or PMA + IOM for 24 h. Then the fluorescence of EGFP was determined by flow cytometry. Data are expressed as means ± SD of fold augmentation of mean fluorescence intensity (MFI) compared with unstimulated control in quadruplicate cultures. *P<0.05, **P<0.01, ***P<0.001, compared with PMA + IOM-stimulated cells (Student's test).

the promoter of IFN-γ and TNF-α, respectively [23, 24]. Other than cytokines, the expression of forkhead box P3, a Treg-specific transcription factor, is transactivated by the combined binding of NFAT/AP-1 to its promoter [25]. Several NFAT binding sites independently of other co-transcription factors were also identified [14]. Furthermore, cytokine gene transactivating property is different among NFAT isoforms. For example, probably due to the difference in cooperated co-transcription factors, NFATc2 but not NFATc1 efficiently transactivates the *TNFα* gene, in contrast to the equivalent activation of *IL2* gene promoter by NFATc1 and NFATc2 [26]. Detailed elucidation of the complexed NFAT-dependent regulatory mechanisms in T cells and other cells/tissues also *in vivo* including various models of immunological diseases deserves further examination with employing our NFAT reporter mice.

In conclusion, we generated fluorescence-based reporter mice that enable us to analyze NFAT-dependent transcriptional activity at least in peripheral T cells. They are promisingly useful for elucidating the mechanisms of immunological and other biological events in various physiological and disease conditions.

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Declaration of Interests

The authors declare that they have no conflicts of interest.

Author Contributions

O.K., M.G., and N.K. designed the study. N.Y., K.M., S.O., S.M., A.U., Y.S., and M.T. performed the experiment. N.Y., K.M., and O.K. wrote the manuscript. N.H. revised the manuscript. All authors reviewed the manuscript.

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