Marked Induction of c-Maf Protein during Th17 Cell Differentiation and Its Implication in Memory Th Cell Development*

Until recently, effector T helper (Th) cells have been classified into two subsets, Th1 and Th2 cells. Since the discovery of Th17 cells, which produce IL-17, much attention has been given to Th17 cells, mainly because they have been implicated in the pathogenesis of various inflammatory diseases. We have performed transcriptome analysis combined with factor analysis and revealed that the expression level of c-Maf, which is considered to be important for Th2 differentiation, increases significantly during the course of Th17 differentiation. The IL-23 receptor (IL-23R), which is important for Th17 cells, is among putative transcriptional targets of c-Maf. Interestingly, the analysis of c-Maf transgenic Th cells revealed that the overexpression of c-Maf did not lead to the acceleration of the early stage of Th17 differentiation but rather to the expansion of memory phenotype cells, particularly with Th1 and Th17 traits. Consistently, mouse wild-type memory Th cells expressed higher mRNA levels of c-Maf, IL-23R, IL-17, and IFN-γ than control cells; in contrast, Maf-/- memory Th cells expressed lower mRNA levels of those molecules. Thus, we propose that c-Maf is important for the development of memory Th cells, particularly memory Th17 cells and Th1 cells.

Acquired immune responses have been divided into two major categories according to the cytokine-production patterns of T helper (Th) cells. Th1 cells produce abundant IFN-γ and play important roles in cellular immune responses. On the other hand, Th2 cells produce various cytokines involved in humoral immunity, such as IL-4. It has been a predominant concept that a skewed balance of Th1/Th2 responses could lead to pathological conditions like autoimmune diseases. Recently, Th17 cells have been discovered as the third type of effector Th cells that produce large amounts of proinflammatory cytokine IL-17A (IL-17) but only minimal amounts of IFN-γ or IL-4 (1, 2). Th17 cells have been shown to play important roles in the pathogenesis of various inflammatory disease models previously considered to be Th1 diseases, such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (3, 4). Thus, Th17 cells have been receiving considerable attention from the viewpoint of the pathological basis of human inflammatory diseases.

Initially, Th17 cells were believed to be differentiated in the presence of IL-23; however, it was thereafter reported that the differentiation factors for Th17 cells are actually TGF-β and IL-6 and that IL-23 is a proliferation factor in mice (5–7). On the other hand, the possibility has been raised that IL-23 is not a mere growth factor for Th17 cells but is important for the differentiation (8–10) and/or proper function (11) of these cells. Recently, TGF-β-independent but IL-23-, IL-6-, and IL-1β-dependent Th17 differentiation has been reported (12). These nonconventional Th17 cells may be more important than conventional TGF-dependent Th17 cells in inflammatory conditions such as experimental autoimmune encephalomyelitis.

In terms of the intracellular mechanisms of Th17 differentiation, Stat3 seems to play an essential role (13, 14). This is not surprising because Stat3 is activated by phosphorylation occurring downstream of IL-6 and IL-23 signaling. In 2006, RAR-related orphan receptor (ROR)γt was reported to be a master regulator transcription factor for Th17 differentiation (15); it is a nuclear receptor the ligand of which is as yet unknown. Another nuclear receptor, RORα, was also implicated to function synergistically with RORγt in Th17 differentiation (16). The entire network of transcription factors in Th17 cells, however, remains to be elucidated. Thus, we first tried to shed light on the network and encountered the transcription factor c-Maf.

* This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Naito Foundation, the Kanae Foundation, and the Promotion of Medical Science, Uehara Memorial Foundation, and the Kato Memorial Bioscience Foundation.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Morohongo 38, Moroyama, Iruma-gun, Saitama 350-0495, Japan. Tel.: 81-49-276-1462; Fax: 81-49-295-4849; Email: satok@saitama-med.ac.jp.

3 The abbreviations used are: ROR, RAR-related orphan receptor; qRT-PCR, quantitative RT-PCR; FC, fold change; cRNA, complementary RNA; IL-23R, IL-23 receptor; luc, luciferase; MARE, Maf recognition element; Tg, transgenic.
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**EXPERIMENTAL PROCEDURES**

Mice—C57BL/6 mice were purchased from CLEA Japan, Inc., and T cell-specific c-Maf Tg mice (under the control of the human CD2 promoter and locus control region) were described previously (17). All the mice were maintained under specific pathogen-free conditions. All animal experiments were performed with the approval of the Animal Study Committee of Saitama Medical University and conformed to relevant guidelines and laws.

**Th1/2/17 Cell Differentiation in Vitro**—Mouse naive Th cells were purified from mouse spleens using a magnetic sorter and microbeads (AutoMACS system and CD4+CD62L- T Cell Isolation Kit II, Miltenyi Biotec). They were cultured in RPMI 1640 medium containing 10% FCS (culture medium) and stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (1 µg/ml each) for 3 days. Th1 cells were cultured with 10 ng/ml IL-12 and 10 µg/ml anti-IL-4 mAb; Th2 cells with 10 ng/ml IL-4 and 10 µg/ml anti-IFN-γ mAb; and Th17 cells with 10 ng/ml each of IL-6 and IL-23, 3 ng/ml TGF-β and 10 µg/ml each of anti-IFN-γ and anti-IL-4 mAbs. All of the antibodies were purchased from BD Biosciences, and the cytokines were purchased from R&D Systems.

**GeneChip Analysis**—Total RNA was used for cDNA synthesis by reverse transcription followed by the synthesis of biotinylated cRNA through in vitro transcription. After cRNA fragmentation, we performed hybridization with a mouse A430 GeneChip (Affymetrix). The raw data were analyzed using Affymetrix Microarray Suite (version 5.0) and normalized.

**qRT-PCR and ELISA**—We performed qRT-PCR analysis using an ABI PRISM 7000 Sequence Detection System with TaqMan Gene Expression Assay probes. The GAPDH expression level was used as the internal control. As to the analysis of cytokine production of Th cells, the cells were stimulated with phorbol 12-myristate 13-acetate (40 ng/ml) and ionomycin (1 µg/ml) for 5 h in the culture medium. Then, RNA was extracted from the cells, and the supernatant was subjected to ELISA. The mouse IL-17 ELISA kit was from R&D Systems.

**Intracellular Staining of Transcription Factors and Cytokines**—Mouse Th cells differentiated in vitro were preincubated with an anti-mouse CD16/CD32 (Fcy receptor) mAb for 15 min on ice to block nonspecific staining. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Bioscience) and stained with the primary Abs (anti-c-Maf Ab, M-153; anti-GATA-3 Ab, HG3-31, Santa Cruz Biotechnology). They were then stained with appropriate secondary Abs conjugated with Alexa Fluor 488 (Invitrogen). For intracellular cytokine staining, Th cells were cultured in the culture medium in the presence of phorbol 12-myristate 13-acetate (40 ng/ml) and ionomycin (1 µg/ml) for 5 h. In the last 1 h, monensin (Golgistop) was added. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm and intracellularly stained with anti-IFN-γ-FITC plus anti-IL-17-PE, or anti-IFN-γ-FITC plus anti-IL-4-PE Abs (all reagents were from BD Biosciences). Stained cells were analyzed by FACScan or FACSCanto (BD Biosciences).

**Factor Analysis**—Transcription factors whose expression levels (normalized signals) were higher than 100 under at least one of the Th cell differentiation conditions were selected. Forty-three probes (including overlapping probes for the same genes) were subjected to factor analysis using SPSS software (version 15.0). By the unweighted least-squares method, two principal factors were extracted, and, after rotation by the varimax method with Kaiser normalization, each gene was positioned on a plane defined by Factors 1 and 2 according to its factor loadings.

**Luciferase Assay**—To construct the reporter plasmid pTA-Il23r-luc, the mouse Il23r promoter region (-1440 to +110) was ligated in the Nhel and XhoI gap of the pTA-luc plasmid (Clontech). The following primers were used for PCR: 5′-GCT AGC TGG AGG CAT TTC CTC AGC TG-3′ (sense) and 5′-CTC GAG TTC AGG AAT TAG GGT CTC CT-3′ (antisense). A deletion mutant of pTA-Il23r-luc, which lacks a putative Maf binding site (MARE-like element) was constructed as described previously (18). DNA transfection and luciferase assays were performed as described previously (19, 20). Briefly, the reporter plasmid was transfected along with a c-Maf expression vector (21), a GATA-3 expression vector (22), and/or the control vector pcDNA3.1 into HEK293T cells using FuGENE 6 (Roche Applied Science). After 24 h, the cells were harvested, and luciferase activity was measured.

**Statistical Analyses**—Error bars indicate S.D. Student’s t test was used for statistical analyses (*, p < 0.05 and **, p < 0.01).

**RESULTS**

**Transcriptome Analysis of Th1/2/17 Cells during Course of Their Differentiation**—First, we obtained the basic profiles of Th1/2/17 cell differentiation. Mouse naive Th cells were sorted and cultured under Th1, Th2, and Th17 cell differentiation conditions, and harvested on days 1 and 3. We also harvested Th0 cells on day 1. RNA extracted from the cells was subjected to transcriptome analysis using GeneChip. The expression levels of genes encoding IFN-γ, IL-4, and IL-17, which are “signature” cytokines produced by Th1, Th2, and Th17 cells, respectively, were up-regulated significantly in each subset, indicating that the differentiation conditions were appropriate (Fig. 1A).

**Factor Analysis of Transcription Factors**—To focus on transcription factors that are specifically up-regulated during the course of Th1/2/17 differentiation, we selected transcription factor genes whose expression levels (signals) were >100 in at least one of the Th conditions that we examined. Then, we performed factor analysis using the seven sets of data (days 1 and 3 for Th1/2/17 cells and day 1 for Th0 cells). Two principal “Factors” were extracted, and each gene is plotted in the twodimensional space defined by Factors 1 and 2, according to its factor loading (Fig. 1B). As expected, dots representing RORγ (one dot) and RORα (four dots, because four different probes are attributed to RORα in the chip we used) were closely located in this diagram and both were high in Factor 2 but not in Factor 1. Interestingly, all the dots representing c-Maf were plotted nearest to the dot representing Rorc, suggesting that c-Maf is even more closely related to RORγ than RORα is to RORγ. Histograms of the GeneChip data of Maf, Rorc, and Rora are shown in Fig. 1C. Consistent with Fig. 1B, Maf and Rorc demonstrated a more Th17-specific expression pattern than Rora.
Expression of c-Maf but Not GATA-3 in Th17 Cells—The above data were rather unexpected in that c-Maf was reported to play important roles in Th2 differentiation (23). If c-Maf is highly expressed in Th17 cells, the question arises as to why they do not become Th2 cells. Indeed, similar levels of c-Maf protein expression were observed in the cells cultured under the Th2 and Th17 conditions (Fig. 2A, left panel). Interestingly, there was a large difference in GATA-3 expression level between the two subsets, whereas GATA-3 was barely detected in Th17 cells (Fig. 2A, right panel). This finding may explain why Th17 cells do not become Th2 cells. Indeed, IL-6 induces c-Maf by activating Stat3, which directly binds to the Maf promoter (24). GATA-3 is induced by IL-6, as well, but more indirect mechanisms are implicated (24). On the other hand, TGF-β, another important cytokine for the differentiation of mouse Th17 cells, is a potent inhibitor of Th2 development and has been shown to down-regulate GATA-3 (25). We added these cytokines to naive CD4⁺ T cells separately or in combination and quantified the expression levels of two established markers of Th17 cells, IL-17 and IL-23R, in the screening for putative transcriptional targets of c-Maf. IL-6 alone induced Il23r but...
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Promoter Analysis of Putative c-Maf Target Genes—To obtain further insight, we performed luciferase promoter analysis using the promoters of the two genes. c-Maf over-expression induced the promoter activity of Il23r. c-Maf binds to a sequence called the Maf recognition element (MARE) (26). Indeed, the promoter region of Il23r contained a MARE-like sequence, and the deletion mutant luciferase vector that lacks the element lost responsiveness to c-Maf (Fig. 2C). Thus, c-Maf likely regulates Th17 proliferation via IL-23R induction. On the other hand, another 5'-flanking region of Il17a responded to neither c-Maf nor GATA-3, suggesting that IL-17 is not a direct target of these factors (data not shown).

Ex Vivo Analysis of c-Maf Transgenic Mice—To confirm the hypothesis that c-Maf plays important role(s) in Th17 differentiation, we employed T cell-specific c-Maf Tg mice (17). As expected, the expression of IL-23R and IL-17 at the mRNA level was up-regulated in Tg Th cells. The expression of IFN-γ was also significantly up-regulated in Tg Th cells, but that of IL-4 was not (Fig. 3A). Flow cytometric analysis confirmed that the numbers of both IFN-γ-positive cells and IL-17-positive cells, but not that of IL-4-positive cells, were increased among Tg Th cells (Fig. 3B). To our surprise, although c-Maf Tg Th cells produced significantly more IL-17 than control cells in vitro, this difference disappeared when naive Th cells were sorted and stimulated under the same condition (Fig. 3C). This unexpected finding seems to be derived from the fact that most of the c-Maf Tg Th cells from the spleen demonstrated an effector-memory phenotype (CD62Llow CD44high, Fig. 4A). Similar to the results in Fig. 3, these cells also expressed significantly higher mRNA levels of Ifng and Il23r (Fig. 4B). This was also consistent with the data, flow cytometric analysis revealed more IFN-γ-positive and IL-17-positive cells than WT naive cells (Fig. 4D and data not shown). Interestingly, an IFN-γ/IL-17 double-positive population was evident (IL-17 single-positive cells and IFN-γ/IL-17 double-positive cells were present at similar levels). On the other hand, IL-4-positive cells were scarcely observed.

Analysis of WT Memory Phenotype Th Cells—The above findings on Tg Th cells prompted us to analyze WT memory Th cells. Under specific pathogen-free conditions, nearly 80% of the Th cells from the spleens of WT mice demonstrated the naive phenotype (CD62L<sup>high</sup> CD44<sup>low</sup>, Fig. 4A, left panel). To facilitate the analysis of memory phenotype WT Th cells, we utilized the system of homeostatic expansion; when naive T cells are transferred into lymphopenic mice, they proliferate vigorously and acquire the memory phenotype (27). Thus, we transferred WT Th cells into Rag-2-deficient mice. In 4 weeks, most of the splenic Th cells from the recipient mice acquired an effector-memory phenotype (CD62L<sup>low</sup> CD44<sup>high</sup>, Fig. 4A, right panel). When compared with WT naive Th cells, these cells expressed significantly higher mRNA levels of Maf and Il23r (Fig. 4B). Similar to the results in Fig. 3, these cells also expressed higher levels of Il17a and Ifng mRNAs, but not Il4 mRNA (Fig. 4C). Consistent with the data, flow cytometric analysis revealed more IFN-γ-positive and IL-17-positive cells than WT naive cells (Fig. 4D and data not shown).

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Ex Vivo Analysis of c-Maf-deficient Th Cells—As c-Maf-deficient mice are embryonic lethal, we generated bone marrow chimeras by injecting \( \text{Maf}^{+/+} \) fetal liver cells containing hematopoietic stem cells into irradiated Rag-2-deficient mice. Six weeks later, the donor mice were sacrificed, and flow cytometric analysis of splenocytes was performed. The ratio of effector-
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memory phenotype Th cells (CD62L<sub>low</sub> CD44<sub>high</sub>) was not significantly different from that of WT Th cells (data not shown). As Maf<sup>−/−</sup>-naive Th cells were shown to have the capacity to differentiate into Th17 cells in vitro (28), we decided to analyze the memory-phenotype c-Maf-deficient Th cells by transferring sorted splenic Th cells to Rag-2-deficient mice as in the experiment whose results are shown in Fig. 4 (Fig. 5A). In 4 weeks, most of the transferred Th cells acquired an effector-memory phenotype, and these cells were analyzed by qRT-PCR for the expressions of cytokines and Il23r. As expected, Il23r, Il17a, and Ifng expression levels were significantly lower in these cells than in the control WT cells. The expression level of Il21, which was reported to be a transcriptional target of c-Maf, was also lower (Fig. 5B).

**DISCUSSION**

In this study, we performed transcriptome analysis during the course of Th differentiation and unexpectedly found that c-Maf, considered to be a Th2-type transcription factor, is more prominently induced in Th17 cells than in Th2 cell. Moreover, factor analysis using the data of Th1-, Th2- and Th17-conditioned cells on days 1 and 3 demonstrated that Maf is more closely related to Rorc than Rora is to Rorc, at least in terms of the expression profiles in Th cells. As both RORγ and RORα are considered to play essential roles in Th17 differentiation (16), it occurred to us that c-Maf may also play an important role in Th17 cells. Bauquet et al. reported that inducible T cell costimulator (ICOS) is important for the expression of c-Maf in the development of follicular T helper cells (Tfh cells) and Th17 cells (28). In our culture system, we did not use ICOS-ligand for Th-cell stimulation. Thus, it is obvious that c-Maf can be induced in an ICOS-independent manner, probably through Stat3 activation by IL-6 (24).

As shown in the factor analysis results (Fig. 1B), Maf, Rorc and Rora were all high in Factor 2 but low in Factor 1. In contrast, Gata3, which encodes the master regulator transcription factor of Th2 cells, was very high in Factor 1 but low in Factor 2, and so was Stat5a, which is implicated in Th2 differentiation (29, 30). From these data, we may safely call Factor 1 “a Th2-related factor” and Factor 2 “a Th17-related factor”. Indeed, Tbx21, which encodes the master regulator of Th1 cells, T-bet, was not high in either Factor 1 or Factor 2 and was positioned near the coordinate origin. Thus, these transcription factor groups specific to Th1, Th2 and Th17 cells are separately placed on the two-dimensional space defined by Factors 1 and 2.

IL-23R, which is required for the expansion of Th17 cells, is among the candidates for the transcriptional targets of c-Maf. Indeed, the promoter analysis revealed that MARE located in the 5′-prime lesion of Il23r is important for the luciferase activity induced by c-Maf overexpression, indicating that IL-23R is a direct target of c-Maf (Fig. 2). IL-21 (28) and IL-10 (31) have also recently been reported to be targets of c-Maf in the context of Th17 differentiation. The analysis of c-Maf Tg mice, however, demonstrated that the overexpression of c-Maf did not seem to accelerate the early stage of Th17 differentiation (Fig. 3). This finding is consistent with that of Bauquet et al., in which c-Maf-deficient Th cells were capable of producing IL-17, although at a lower level (28). Instead, the significant
deviation of Th cells toward the memory phenotype was observed in the Tg mice, suggesting that c-Maf may play a role in the development and/or maintenance of memory Th cells. The fact that WT mouse memory Th cells express higher mRNA levels of not only \( \text{Maf}, \text{Il23r} \) and \( \text{Il17a} \) but also \( \text{Ifng} \) than non-memory cells suggests that c-Maf indeed plays a role in memory Th17 and Th1 cells (Fig. 4B and C). In the earliest studies of Th cells and IL-17, it was memory Th cell that was shown to mainly produce the cytokine (32) particularly in response to IL-23 (33). It was not until the discovery of Th17 cells that these IL-17-producing cells were considered to be distinct from Th1 cells. Recently, however, Th cells that produce both IFN-\( \gamma \) and IL-17 have been gathering attention particularly in the context of inflammation, making the difference between Th1 and Th17 subsets less clear again (34–36). Although the relationship between memory Th cells and Th17 cells differentiated in vitro seems to be close in that both subsets express IL-23R, we have yet to determine whether Th17 cells differentiated in vitro are indeed the precursors of memory Th cells in vivo. If that is the case, c-Maf, which is expressed highly in both de novo Th17 cells and memory Th cell, may be a transcription factor that mediates the differentiation of the former into the latter.

In regard to this point, it is interesting that an IFN-\( \gamma \)/IL-17 double-positive population was evident in memory phenotype Th cells (Fig. 4D), which was not so apparent in Maf Tg cells. It is likely that the forced expression of c-Maf is sufficient for the expression of surface markers of memory Th cells, but not sufficient for the differentiation of IFN-\( \gamma \)/IL-17 double-positive Th cells.

The homeostatic expansion and induction of memory phenotype Th cells are important in a variety of clinical situations, such as during immunosuppressive therapy or chemotherapy. There is little doubt that the expansion of memory phenotype Th cells plays an important role in the defense against numerous pathogens, for example, those residing in the gut. At the same time, such expansion also bears the risk of autoinflammation, causing damage to self tissues. It has been shown that adoptive transfer of naive Th cells into lymphopenic hosts induces inflammatory bowel disease, which can be prevented by cotransfer of regulatory T (Treg) cells (37). Th1 response is implicated in the pathogenesis, whereas the role Th17 cells play in this disease model is still controversial (38). In either case, Treg cells seem to be essential for controlling the excessive response of homeostatically expanding Th cells.

It was unexpected that c-Maf Tg Th cells did not produce more IL-4 than WT cells (Fig. 3A and B), although c-Maf was reported to play an important role in IL-4 production (39). Consistently, the expression of \( \text{Il4} \) was not reduced in Maf\(^{-/-}\) memory Th cells and control cells. Data represent three independent experiments. PMA+ iono, phorbol 12-myristate 13-acetate + ionomycin. **, \( p < 0.01 \).
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FIGURE 6. Schematic diagram of Th cell differentiation. IL-6 induces both c-Maf and GATA-3 via STAT3 phosphorylation and activation. GATA-3 expression can lead to Th2 differentiation, but this route is blocked in the presence of TGF-β, which inhibits GATA-3 expression. c-Maf induces IL-23R, and then IL-23 signaling activates STAT3, constituting a positive feedback loop (boldface lines). Although this loop does not seem to be essential to early Th17 differentiation, it may play an important role in the development and/or maintenance of memory Th cells, particularly memory Th17 cells.

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doi: 10.1074/jbc.M111.218867 originally published online March 14, 2011

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