Akt Is a Neutral Amplifier for Th Cell Differentiation*

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Both CD28 and its relative, inducible costimulator (ICOS), have a binding motif for phosphatidylinositol 3-kinase (PI3K) in their cytoplasmic tail, and the binding of PI3K leads to activation of a serine/threonine kinase, Akt. The role of Akt in cytokine production and helper T (Th) cell differentiation remains obscure. In this study, we found that enforced expression of the constitutively active form (E40K) of Akt rendered CD4+ T cells activated. Wild-type of Akt and E40K promoted Th1 cell differentiation in C57BL/6-derived and Th1-polarized BALB/c-derived CD4+ T cells, while both promoted Th2 cell differentiation in BALB/c-derived and Th2-polarized C57BL/6 CD4+ T cells. E40K also facilitated Th1 differentiation in CD4+ T cells from IL-4-deficient mice with the BALB/c background. E40K up-regulated expression of NF-AT and c-Myb, which may be related to the augmentation of cytokine production by E40K. These findings indicate that the mechanism by which Akt augments cytokine production via CD28 and ICOS is Th cell type-specific and reflects the intracellular status affected by the cytokine milieu. We conclude that Akt is a neutral amplifier of T cell activation and Th differentiation.

Naive CD4+ helper T (Th) cells proliferate and differentiate into at least two functionally distinct subsets, Th1 cells, which produce interferon-γ (IFN-γ), IL-2, and tumor necrosis factor-β (TNF-β), and Th2 cells, which produce IL-4, IL-5, IL-6, IL-10, and IL-13 (1). The factors that influence Th cell differentiation include cytokine milieu, transcription factors, antigen dose, nature of antigen, cell cycle, dendritic cell type, genetic background, and costimulation (2). Among them, costimulatory molecules are thought to regulate Th differentiation by altering signal strength, providing Th-specific signals and/or biased expression of themselves on Th cells (2–5). T cell costimulatory molecule CD28 plays a crucial role in the immune system in conjunction with T cell receptor signals, exerting positive effects on proliferation, cytokine production, and an anti-apoptotic effect in T cells, while suppressing anergy induction (2). Current models suggest that greater CD28 signal can achieve Th2 differentiation (6, 7). An inducible costimulator, ICOS, also called H4 (8, 9) or AILIM (10), is a member of the CD28 family and has roles similar to those of CD28 except for IL-2 production (11–13). Accumulating evidence indicates a predominant role of ICOS in Th2-type immune responses, as exemplified by its promoting effect on Th2 cell differentiation, concomitant cytokine secretion, airway hypersensitivity reaction, and Ig-class switching (14–17). However, ICOS has recently been reported to be functionally involved in Th1-type immune responses as well and to regulate the severity of murine experimental autoimmune encephalomyelitis (18).

Both CD28 and ICOS have a YXXM motif for recruitment of phosphatidylinositol 3-kinase (PI3K) in their cytoplasmic tail, whereas a YXXN motif for the Src homology 2 domain of Grb2 family members and a PXXP motif for the Src homology 3 domain of various molecules are present only in CD28. The YXXN motif seems to be responsible for IL-2 production (19). Cross-linking of CD28 or ICOS with TCR/CD3 leads to activation of PI3K (4, 20), and PI3K in turn phosphorylates the D-3 position of the inositol ring of phosphoinositides resulting in production of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, which recruit pleckstrin homology domain-containing molecules including serine/threonine kinases, Akt/protein kinase B, and PDK-1 (21). Akt, upon subsequent phosphorylation at Thr-308 and Ser-473 by PDK-1, becomes activated and plays a critical role in cell survival and cell cycle regulation through a wide variety of downstream molecules (21–24).

The relevance of the PI3K/Akt pathway to cytokine production remains controversial. Several lines of evidence have demonstrated CD28 to promote IL-2 production independently of its association with PI3K (25, 26). In contrast, PI3K inhibitors have been found to block cytokine production mediated by CD28 in primary cells (27, 28). Kane et al. (29) have demonstrated that Akt can provide the costimulatory signal for activation of the IL-2 promoter, which is indistinguishable from the CD28 costimulatory signal. They further showed that enforced Akt expression in primary CD4+ T cells from CD28-deficient mice restored Th1, but not Th2, cytokine production upon restimulation. Previously, we showed Akt phosphorylation to be enhanced by engagement of CD28 and even more strongly by that of ICOS. Since several reports including ours...

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¶ The abbreviations used are: Th, helper T; ICOS, inducible costimulator; EGFP, enhanced green fluorescent protein; Hlg, hamster IgG; NF-xB, nuclear factor of xB; NF-AT, nuclear factor of activated T cells; IFN, interferon; IL, interleukin; PI3K, phosphatidylinositol 3-kinase; TCR, T cell receptor; Ab, antibody; mAb, monoclonal antibody; PE, phycoerythrin; ELISA, enzyme-linked immunosorbent assay; WT, wild-type; ERK, extracellular signal-regulated kinase; APC, antigen-presenting cell.
have shown that the engagement of ICOS has a preferential effect on Th2 development, Akt may be involved in Th2 development as well under certain circumstances. Furthermore, the question of whether Akt is differentially required for CD28 and ICOS costimulation remains to be answered.

In this report, to address the above questions, we have explored the role of Akt in T cell activation costimulated by CD28 and ICOS, and in Th cell differentiation, by examining the effect of enforced expression of Akt in primary CD4+ T cells under various Th-differentiating conditions.

MATERIALS AND METHODS

Animals—BALB/c and C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). IL-4 knock-out mice with a BALB/c background were purchased from Jackson Laboratories (Bar Harbor, ME) (30). All mice were bred and used in accordance with the guidelines of the Institute of Laboratory Animals, Tokyo Women’s Medical University.

Abs and Reagents—The C938.4A mAb specific for ICOS/H4 has been described previously (8, 9). mAb to CD28 (0.5 μg/ml), polybrene (10 μg/ml) plus syngenic APCs 1 day before were incubated with virus-infected cells to polarize CD4+ T cells (0.5 × 106 CD4+ T cells) that had been preactivated with anti-CD3 mAb (1:200 dilution) and anti-CD28, anti-ICOS, or control Hlg (3 μg/ml), as described previously (13). The IL-2 concentrations in culture supernatants were determined as proliferation of IL-2-dependent CTLL-2 cells in a biosay. IL-4 and IFN-γ in culture supernatants were quantitated by sandwich ELISA according to the manufacturer’s instructions (BD Biosciences). Results are expressed as the mean ± S.D. of triplicate cultures, and the data were analyzed for variance by a one-way analysis of variance followed by Tukey-Kramer’s test for multiple comparisons.

RESULTS

Enforced Expression and Kinase Activity of Akt Constructs—To investigate the role of Akt in CD4+ T cells, we prepared expression constructs for three types of Akt using a bicistronic retrovirus vector and achieved retrovirus-mediated transduction of the Akt constructs into mouse primary CD4+ T cells. The constitutively active form (E40K) and the wild-type (WT) of Akt were highly phosphorylated in BALB/c CD4+ T cells before and after restimulation, although the phosphorylation level was slightly higher in the E40K-infected cells than in WT-infected cells (Fig. 1A). On the other hand, the kinase-inactive form (K179M) of Akt did not show enhanced phosphorylation. These observations indicate the level of phosphorylation to be proportional to the kinase activity of the Akt construct itself. The protein expression levels of Akt and EGFP in WT and E40K were also much higher than those of K179M (Figs. 1 and 2). These differences in expression level do not appear to reflect the difference in virus titer produced but do reflect the kinase activity itself because they were observed even when the infectivity with K179M was higher than that with E40K (data not shown). In addition, as a consequence of the up-regulation of kinase activity, Bcl-XL expression, which is involved in anti-apoptosis through Akt (41), was up-regulated when the infectivity with K179M was higher than that with E40K (data not shown). Thus, the Akt expression levels and kinase activities are well maintained in primary CD4+ T cells infected with the respective retroviral constructs.

Cellular Phenootypes Induced by the Constitutively Active Form of Akt—Following Akt infection, we gated EGFP+ cells to monitor infected cells and examined the phenotypes of cells with the active or the inactive form of Akt. In the cells transfected with E40K, but not K179M, increases in the number and size of cell aggregates were evident (Fig. 2A), suggesting up-regulation/activation of integrins. E40K up-regulated several activation/adhesion molecules, CD69, LFA-1, and to a lesser extent, CD44, as well as increasing cell size (forward scatter (FS)) of both BALB/c and C57BL/6 CD4+ T cells, although...
These changes were more marked in BALB/c cells (Fig. 2, B and C, and data not shown). CD69 expression in E40K-transfected activated CD4+ T cells was consistently higher than in K179M- and empty vector-transfected cells, and a high level of CD69 expression was sustained in rested CD4+ T cells (Fig. 2C). In contrast, the expression levels of CD3 and CD4 did not differ between these transfectants. Consequently, these findings indicate that the Akt signal is involved in T cell activation and may account for the costimulation mediated by CD28 and ICOS.

Akt Promotes Th1 Cell Differentiation of C57BL/6 CD4+ T Cells—In the next experiments, we explored the effects of Akt on cytokine production by CD4+ T cells prepared from C57BL/6 mice. CD4+ T cells infected with Akt constructs were enriched by cell sorting and assessed for cytokine production upon restimulation. In accordance with a previous report by another group (29), transfection with WT and E40K markedly enhanced IFN-γ production in response to anti-CD3 plus anti-CD28 and anti-CD3 plus anti-ICOS, as compared with an empty control vector, whereas K179M had no effect (Fig. 3A). Furthermore, E40K significantly augmented IFN-γ production upon restimulation even with anti-CD3 Ab alone, corroborating that the effects of costimulation could be achieved by Akt alone in the absence of anti-CD28 (29). IL-2 was also increased in these cells, showing the same pattern as IFN-γ. However, the effect on IL-4 production was very small or negligible.

There are two possible interpretations of the results. One is that Akt simply transactivated Th1 cytokine production, and the other is that Akt promoted Th1 differentiation resulting in elevated IFN-γ production upon restimulation. To test these possibilities, we conducted intracellular cytokine staining after anti-CD3 plus anti-CD28 stimulation. In WT- and E40K-infected CD4+ T cells, the percentage of IFN-γ-producing cells was elevated, and the ratio of IFN-γ to IL-4+ cells was much higher as compared with the control vector (Fig. 3, B and C, top). These changes were more marked in the EGFP+ (retrovirus-infected) cells than in the EGFP- cells (Fig. 3C, bottom). The EGFP- cells also appeared to be affected to a lesser extent by transfected Akt through the environmental cytokines produced in the same wells in a paracrine manner. In contrast, K179M-infected cells did not show any change in numbers of IFN-γ-producing cells. The difference in the percentage of IL-4-producing cells was essentially negligible in all transfectants. In addition, ICOS and CD28 surface expression levels were each consistent among transfectants (data not shown). Consequently, these results indicate that Akt activity facilitates Th1 cell differentiation of C57BL/6 CD4+ T cells.

Akt Promotes Th2 Cell Differentiation of BALB/c CD4+ T Cells—We further examined whether the above findings obtained with Th1-prone C57BL/6 mice were applicable to Th2-prone BALB/c mice. Unexpectedly, infection of BALB/c CD4+ T cells with WT and E40K Akt constructs enhanced IL-4 production, as compared with the control, in response to anti-CD3 plus anti-CD28 (Fig. 4A). To a lesser extent, WT and E40K increased IL-4 production in response to anti-CD3 plus anti-ICOS. E40K resulted in costimulation of IL-4 production upon restimulation with anti-CD3 Ab alone. In contrast, IFN-γ production in WT and E40K cells was reduced by about half in response to both types of stimulation. IL-2 production was also reduced in WT to the same extent as IFN-γ. K179M had no effect on cytokine production. Intracellular cytokine staining yielded essentially the same results in which the percentages of IL-4-producing cells were increased and the ratios of IFN-γ to IL-4+ cells were much lower in EGFP+ WT- and E40K-transfected cells than in EGFP- K179M-transfected cells, although variation was observed (Fig. 4, B and C). These changes in EGFP- cells were less marked than those in EGFP+ cells (Fig. 4C, bottom). In addition, ICOS expression of E40K was much higher than that of K179M, although CD28 expression was slightly decreased (Fig. 4D). Moreover, expressions of the Th2-specific transcription factors GATA-3 (42) and c-Maf (43) were induced in WT and E40K cells and correlated well with the amount of IL-4 produced by each transfectant (Fig. 4E). Taken together, these findings indicate enforced expression of Akt in BALB/c CD4+ T cells to direct their Th2 differentiation.

Akt-mediated Th Differentiation Is Modified by the Cytokine Milieu—APCs, such as dendritic cells, have recently been reported to regulate Th cell polarization (44), and thus it was conceivable that the above results were attributable to APCs derived from a particular strain of mice and not to T cells. Alternatively, a small number of already activated (polarized) T cells remaining in the cell preparations above may have been the determinant of the above results. To address these questions, we purified C57BL/6 (B6) and BALB/c naive CD62L+cd44low CD4+ T cells by cell sorting (Fig. 5A, top) and stimulated them in the presence of C57BL/6 or BALB/c APCs (irradiated T-depleted splenocytes) during Akt infection. The results again showed that E40K up-regulated IFN-γ in C57BL/6 naive CD4+ T cells and IL-4 in BALB/c naive CD4+ T cells as compared with the empty vector control (Vec), irrespective of the APC type (Fig. 5A, bottom). Thus, the results ruled out the above possibilities and indicated that enforced expression of Akt in naive CD4+ T cells promoted Th differentiation. Since the effects of Akt on cytokine production and Th differentiation were opposite in C57BL/6 and
BALB/c mice, the question arose as to whether the dependence of Akt on the genetic background was absolute or could be modified by extracellular cytokines. Thus, we conducted in vitro polarization experiments during Akt infection to answer this question. Under Th1-polarizing conditions in Th2-prone BALB/c CD4⁺ T cells, E40K markedly up-regulated IFN-γ-producing cells as compared with the control vector, whereas GATA-3 consistently increased IL-4 producing cells (Fig. 5B, top). Contrary to this, under Th2-polarizing conditions in Th1-prone C57BL/6 CD4⁺ T cells, E40K up-regulated IL-4-producing cells as compared with K179M and vector alone, while T-bet, a Th1-specific transcription factor (36), reversed the deviation and consistently up-regulated IFN-γ-producing cells (Fig. 5B, bottom). Furthermore, to test the cytokine requirement for Akt-mediated differentiation, we introduced Akt into CD4⁺ T cells from IL-4-deficient mice with the BALB/c genetic background. We found that IFN-γ-producing cells increased among WT and E40K cells as compared with K179M cells, whereas IL-5 (another Th2 cytokine)-producing cells were unchanged (Fig. 5C). In contrast, WT and E40K in normal BALB/c mice up-regulated IL-5-producing cells, which was consistent with IL-4 production (Figs. 4 and 5C). We therefore concluded that Akt per se is essentially neutral in regard to Th1/Th2 balance and that its promoting activity on Th skewness. Akt appears to indirectly drive T cells to further Th polarization, especially when concomitant costimulation is provided.

**Transcription Factors Induced by the Akt Signal**—To identify the transcription factors that are located downstream of Akt and are responsible for the above phenomena, Akt-infected CD4⁺ T cells were restimulated with plate-bound anti-CD3 Ab, and nuclear extracts were assessed for induction of the tran-
Fig. 3. Akt up-regulates IFN-γ production by C57BL/6 CD4⁺ T cells. A, C57BL/6 CD4⁺ T cells were infected with three types of Akt and enriched by cell sorting as in Fig. 1. The cells were restimulated for 24 h with anti-CD3 and anti-CD28, anti-ICOS, or control Ig, as indicated, and cytokine production was estimated by ELISA. *, p < 0.01 as compared with an empty vector. B, Akt retrovirus-infected CD4⁺ T cells without cell sorting were restimulated with anti-CD3 plus anti-CD28, followed by intracellular cytokine staining. Representative results of the flowcytometric analysis are shown. The percentages positive for cytokine-producing cells among EGFP⁺ cells are shown above the upper right quadrant of the figures. C, the percentages positive for cytokine-producing cells among EGFP⁺ cells (top) and the ratio of IFN-γ⁺ to IL-4⁺ cells among EGFP⁺ cells and EGFP⁻ cells (bottom) from four independent experiments (exp. 1–4) are shown as graphs.

scription factors. Expression of the NF-κB component c-Rel in E40K-transfected cells was slightly higher than in K179M cells (Fig. 6). c-Myb expression, which has been reported to be up-regulated by Akt (45), was augmented in E40K transfectants, especially in BALB/c, as compared with K179M cells. NF-ATc1 (NF-AT2), which has been found to be involved in Th2 differ-
FIG. 4. Akt up-regulates IL-4 production by BALB/c CD4+ T cells. A, Akt-infected BALB/c CD4+ T cells enriched by sorting were restimulated and examined for cytokine production as in Fig. 3A. *, p < 0.01 as compared with an empty vector. B, the infected cells without cell sorting were restimulated and subjected to intracellular cytokine staining as in Fig. 3B. Representative results of the flow cytometric analysis are shown. The percentages positive for cytokine-producing cells among EGFP+ cells are shown above the upper right quadrant of the figures. C, the percentages positive for cytokine-producing cells among EGFP+ cells (top) and the ratio of IFN-γ to IL-4+ cells among EGFP+ cells and EGFP− cells (bottom) from four independent experiments (exp. 1–4) are shown as graphs. D, the cells infected with E40K (shaded histogram) or K179M (open histogram) were examined for CD28 and ICOS expression. E, lysates from Akt-infected CD4+ T cells without cell sorting were blotted with the specific Abs indicated. These figures are representative of three independent experiments with similar results (D and E).
NF-ATc2 (NF-AT1) was augmented in E40K cells of both C57BL/6 and BALB/c mice, although it is noteworthy that nuclear NF-ATc2 expression, especially of the hypophosphorylated (lower molecular weight) form, was reduced following TCR stimulation. Collectively, these findings indicate that Akt directly and/or indirectly up-regulates the expression of multiple transcription factors, some of which may be responsible for Akt-mediated T cell activation, cytokine production, and Th cell differentiation.

**DISCUSSION**

This study was conducted to clarify the role of PI3K/Akt signaling in T cell activation and Th cell differentiation. The results showed that enforced expression of Akt activated T and purified by cell-sorting. The purity of naive T cells obtained was >99%. These naive CD4+ T cells were primed with anti-CD3 Ab in the presence of APCs (irradiated T-depleted splenocytes) derived from C57BL/6 (B6) or BALB/c mice during Akt infection. The infected T cells were restimulated with anti-CD3 (0.1 μg/ml) plus anti-CD28 (1 μg/ml) and assessed for cytokine production by ELISA. *, p < 0.01 as compared with an empty vector. B, BALB/c-derived CD4+ T cells were infected with the retrovirus for Akt or GATA-3 in the absence or presence of supplements promoting Th1 polarization, then restimulated with anti-CD3 plus anti-CD28 and subjected to intracellular cytokine staining. The percentages positive for cytokine-producing cells among EGFP+ cells are presented as graphs (top). C57BL/6-derived CD4+ T cells were infected with the retrovirus for Akt or T-bet with or without supplements promoting Th2 polarization. The infected CD4+ T cells were subjected to intracellular cytokine staining. The results are presented as graphs (bottom). The above results are presented as graphs (bottom). These figures are representative of two independent experiments with similar results.
Role of Akt for Cytokine Production

Originally, ICOS was reported to be more intimately involved in Th2-type immunity than in Th1 species. This preferential involvement of ICOS could be explained in two ways. The first involves the much higher expression of ICOS in Th2 cells than in Th1 cells (4, 13). The mechanism is likely to be a direct effect of elevated expression of GATA-3 in Th2 cells, which is a critical transcription factor for Th2 cytokines, since enforced expression of GATA-3 in CD4+ T cells from C57BL/6 and IL-4-deficient mice increased ICOS expression (48). The second involves signaling via ICOS mediated by cross-linking with specific Abs or by blockade with ICOS-Ig fusion protein during priming, which can directly control the appearance of Th2 cells (13, 49). In Th2-prone BALB/c-activated CD4+ T cells, Akt is more strongly elicited via ICOS than in Th1-prone C57BL/6-activated CD4+ T cells (13). In this study, GATA-3 and c-Maf expressions, which are induced by IL-4, and expression of NF-ATc1, which is involved in Th2 differentiation (3, 46), were markedly elevated in WT- and E40K-transfected cells from BALB/c mice (Figs. 4D and 6). Taken together, these observations raise the possibility that once CD4+ T cells start to differentiate into Th2 cells, ICOS expression is gradually up-regulated, thereby providing stronger activation of Akt than in Th1 cells and in turn leading to an increase in IL-4 production. Consequently, Akt could be a driving factor toward further Th2 cell maturation, through ICOS engagement, the expression of which was elevated in this lineage, in a positive feedback manner, although Akt per se is a neutral player in Th differentiation.

Absence or blockade of CD28 can lead to global immunosuppression and/or a selective limitation in the generation of normal Th responses. CD28-deficient mice show reduced basal levels of IgG1 and IgG2b, increased IgG2a, and inefficient help to B cells (50, 51). In addition, IL-4 production and, to a lesser extent, IFN-γ production have been shown to be regulated by CD28-mediated signals (52, 53). Thus, CD28 costimulation was predicted to be required for maturation of Th2 cells. However, the relevance of CD28 to Th differentiation is limited, and CD28 is not essential (54). The CD28 requirement for Th differentiation varies according to the genetic background and the nature of the immunogen (54). Linsley's group (55) recently reported that TCR and CD28 signals up-regulated a similar set of transcripts, suggesting the major consequence of CD28 costimulation to be quantitative rather than qualitative. PI3K/Akt activation is, in fact, elicited by TCR as well as CD28, and its activity is most potent when cross-linked with both receptors, thereby indicating that PI3K/Akt is one of convergence points for two signaling pathways. In the current study, the effect of Akt was found to be influenced by genetic background as well as the cytokine milieu. Consequently, Akt is not a critical determinant of Th cell development and concomitant cytokine production. Akt thus appears to provide quantitative, but not qualitative, effects on T cell function.

What are the downstream targets for Akt, via which it activates T cells and up-regulates cytokine secretion at the initial step in each Th cell type? A large number of molecules have been reported for substrates of Akt, in which consensus sequences for phosphorylation are RXRXXXS/T (21, 22). In this study, multiple transcription factors were found to be up-regulated by E40K of Akt. Among them, NF-AT members are well known to be crucial to the production of various cytokines (46, 56), thus suggesting the possibility that Akt-mediated cytokine production and Th differentiation are achieved through these transcription factors. NF-ATc1 plays an important role in Th2 cytokine expression (46). On the other hand, NF-ATc2 was originally reported to down-regulate Th2 cytokines (57); however, a recent report has shown that NF-ATc2 is a positive
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and c-Myb expression remains unknown and is now under investigation. Screening of the other target molecules of Akt is also in progress.

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