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Regulatory Roles of IL-2 and IL-4 in H4/Inducible Costimulator Expression on Activated CD4$^+$ T Cells During Th Cell Development[1]

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We found a tight correlation among the levels of H4/inducible costimulator (ICOS) expression, IL-4 production, and GATA-3 induction, using activated CD4$^+$ T cells obtained from six different murine strains. BALB/c-activated CD4$^+$ T cells expressed ~10-fold more H4/ICOS on their surfaces and produced ~10-fold more IL-4 upon restimulation than C57BL/6-activated CD4$^+$ T cells. BALB/c naive CD4$^+$ T cells were shown to produce much higher amounts of IL-2 and IL-4 upon primary stimulation than C57BL/6 naive CD4$^+$ T cells. Neutralization of IL-4 with mAbs in culture of BALB/c naive CD4$^+$ T cells strongly down-regulated both H4/ICOS expression on activated CD4$^+$ T cells and IL-4 production upon subsequent restimulation. Conversely, exogenous IL-4 added to the culture of BALB/c or C57BL/6 naive CD4$^+$ T cells up-regulated H4/ICOS expression and IL-4 production upon restimulation. In addition, retroviral expression of GATA-3 during the stimulation of naive CD4$^+$ T cells from C57BL/6 or IL-4$^{-/-}$ mice increased H4/ICOS expression on activated CD4$^+$ T cells. A similar effect of IL-2 in the primary culture of BALB/c naive CD4$^+$ T cells appeared to be mediated by IL-4, the production of which was regulated by IL-2. These data suggest that IL-4 induced by IL-2 is critical to the maintenance of high H4/ICOS expression on BALB/c-activated CD4$^+$ T cells. ***The Journal of Immunology,*** **2003, 171: 783–794.**

T cells are not fully activated and can even be rendered anergic or die if their TCRs interact with the antigenic peptide-MHC complex on APCs in the absence of a second, costimulatory signal (1, 2). It is widely accepted that CD28 is a major costimulatory receptor on T cells. The binding of CD28 by its ligands, B7-1 and B7-2, on APCs in addition to TCR binding induces optimal T cell activation (3, 4). In contrast, a molecule structurally related to CD28, CTLA-4, is induced on naive T cells upon activation and suppresses T cell responses by delivering inhibitory signals (5, 6). Recently, a third CD28-related molecule was identified. This molecule is an inducible costimulator (ICOS)[3] and is expressed on activated T cells such as CTLA-4 (7–9). T cell proliferation and various cytokine productions, including those of IL-4, IL-5, IL-10, and IFN-γ, are effectively costimulated by ICOS (7, 8, 10–12). ICOS was shown to regulate predominantly Th2 effector functions in initial studies (13–16); however, Th1 effector functions were later demonstrated as well (17–19).

In some aspects of immune responses, ICOS reveals a preference for Th2 cells over Th1 cells. First, cloned Th2 cell lines and Th2 cells derived from TCR-$\alpha\beta$ transgenic mice predominantly express ICOS as compared with Th1 cells (11, 13). Second, several studies have shown that the engagement of ICOS has a preferential effect on the development of Th2 cells as compared with that of Th1 cells (11, 20). Third, ICOS-deficient mice had profound defects in Th2-mediated immune responses such as class switching to IgE, and IL-4 and IL-13 productions in secondary immune responses (14–16). Questions arise as to how ICOS expression is regulated, resulting in the predominance on Th2 cells, and promotes Th2 development. Concerning the induction of ICOS, recent reports demonstrated up-regulation of ICOS expression on murine CD4$^+$ T cells with CD28 costimulation (11), and reduced ICOS expression in the presence of cyclosporin A during primary stimulation of human CD4$^+$ T cells (21). However, factors regulating ICOS expression are still poorly understood.

In 1996, one of our coauthors (U. Dianzani) and colleagues reported an inducible molecule on murine T cells, H4, which physically associates with TCR and costimulates T cells (22). Later, as inferred by the similarities in function and molecular structure (7, 22), it was indeed shown that H4 and ICOS are identical (23). To elucidate the intracellular signaling events induced by H4/ICOS costimulation, we recently compared the extent of activation of key signaling proteins between CD28 and H4/ICOS engagement. The results indicated that H4/ICOS elicited much stronger activation of serine/threonine protein kinase Akt than CD28, and thus suggested a specific signaling triggered by H4/ICOS (20). Consistent with previous reports showing ICOS expression predominantly on Th2 cells (11, 13), our recent study also found that Th2-skewed BALB/c-activated CD4$^+$ T cells expressed much more H4/ICOS than did Th1-skewed C57BL/6-activated CD4$^+$ T cells (20), suggesting that H4/ICOS expression may be regulated differently depending on the murine strain.

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[3] Abbreviations used in this paper: ICOS, inducible costimulator; GFP, green fluorescent protein; HIgG, hamster IgG; mIgG, mouse IgG; RIgG, rat IgG.

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In the present study, in an attempt to clarify the factors regulating H4/ICOS expression, we investigated the roles of IL-2 and IL-4 in a primary culture of BALB/c and C57BL/6 naive $CD4^+$ T cells in H4/ICOS expression on activated $CD4^+$ T cells. The results show that the amount of IL-4 produced in a primary culture primarily determines the levels of H4/ICOS expression as well as the generation of IL-4-producing cells. Furthermore, IL-2 may have a direct effect on the induction of H4/ICOS expression, and may regulate IL-4 production in primary culture. Based on the results, we discuss in this study an immunoregulatory pathway linking IL-4 to H4/ICOS, which is triggered by IL-2.

Materials and Methods

Animals

BALB/c and C57BL/6 mice were bred in our own colony, and CBF_1 mice were produced by mating C57BL/6 and BALB/c mice at the Department of Microbiology and Immunology, Tokyo Women's Medical University. C3H mice and CBA/J and NC/Nga (24) mice were purchased from Japan SLC (Hamamatsu, Japan) and Charles River Japan (Yokohama, Japan), respectively. IL-$4^{-/-}$ mice (25) were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice, 8–10 wk old, were used in this study.

Abs, cytokines, and reagents

The C398.4A mAb specific for H4/ICOS was produced in Armenian hamsters by immunization with the murine T cell clone D10.G4.1, as described previously (22). mAb to CD28 (37.51) (26) was kindly provided by J. Allison (University of California, Berkeley, CA). mAbs to I-$A^{b,d}$ (28-16-8S), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7 and 83.12.5), CD25 (7D4), and Thy-1.2 (HO13) were described previously (27, 28). The following Abs were used: mAbs to CD44 (IM7) and TCR-$\gamma\delta$ (GL3); FITC anti-CD4 (RM4-5); biotinylated anti-CD28 (37.51) and biotinylated anti-CD44 (IM7); PE anti-IL-4 (BVD4-1D11) and PE anti-IFN-γ (XMG1.2) (BD PharMingen, San Diego, CA); biotinylated anti-CD62L (MEL-14) (Beckman Coulter, Miami, FL); goat anti-hamster IgG (anti-HIgG) (ICN Pharmaceuticals, Aurora, OH); mAbs to NK1.1 (PK136), IL-2 (S4B6), and IL-4 (11B11) (ATCC, Rockville, MD); mAb to GATA-3 (HG3-31); and Ab to actin (I-19) (Santa Cruz Biotechnology, Santa Cruz, CA). Rat IgG (RIgG) and HIgG; anti-IL-2, anti-IL-4, and anti-CD28 mAbs; and anti-H4/ICOS mAb were purified from serum, ascitic fluid, and culture supernatant, respectively, by protein G-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Mouse IgG (mIgG) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-CD3 mAb was used as dialyzed ammonium sulfate precipitates of ascitic fluid. Purified HIgG, anti-H4/ICOS, and anti-CD25 mAbs were conjugated with biotin in our laboratory. PE-streptavidin was purchased from BD PharMingen. The following cytokines were used: human rIL-2 (a gift from Shionogi, Osaka, Japan) and murine rIL-4 (BD PharMingen).

Culture medium

RPMI 1640 supplemented with 100 μg/ml of streptomycin, 100 U/ml of penicillin, 10% FBS, and 5×10^{-5} M 2-ME was used for the culture.

CTLA-4-mIgG Fc fusion protein (CTLA-4-mIg)

The plasmid construct for CTLA-4-Ig fusion protein (pCDM8/CTLA-4-Ig) consisting of extracellular domain of mouse CTLA-4 and human IgG1 Fc portion was previously described (29). The Fc portion was replaced in frame with the corresponding region of mIgG2a (CH2 plus CH3 domain), and whole insert fragment was transferred into *Xba*I site of pEF-BOS vector (30). The resultant construct pEF/CTLA-4-mIg was transiently transfected into 293T cells with calcium phosphate method, and the supernatant was collected, followed by purification with protein G column, and dialyzed against PBS. The protein concentration was measured with Bradford reagent (Bio-Rad, Hercules, CA).

Preparation of cells

Single spleen cell suspensions were prepared in Hanks' solution (Nissui Pharmaceutical, Tokyo, Japan) with 2% FCS. Naive $CD4^+$ T cells from BALB/c, CBF_1, C57BL/6, and IL-$4^{-/-}$ mice were obtained, as described previously (20). $CD4^+$ T cells were obtained by two rounds of treatment of spleen cells with 28-16-8S and 83.12.5, and guinea pig C. Naive $CD4^+$ TCR-$\alpha\beta^+$ T cells were then enriched by treatment with a cocktail of mAbs IM7, PK136, and GL3 and depletion of mAb-coated cells using sheep anti-rat- and sheep anti-mIgG-bound magnetic beads (Dynabeads; Dynal, Lake Success, NY), followed by taking nonadherent cells from goat anti-HIgG (30 μg/ml)-coated dishes (Corning 25020, Corning, NY). PK136 was omitted in the treatment of BALB/c and IL-$4^{-/-}$ spleen cells. Naive $CD4^+$ TCR-$\alpha\beta^+$ T cells from C3H, CBA/J, and Nc/Nga were obtained by treating spleen cells with 53-6.7 and depletion of mAb-coated cells and B cells using sheep anti-rat- and sheep anti-mIgG-bound magnetic beads, and then enriched by treatment with a mixture of mAbs 53-6.7, IM7, and GL3 and depletion of mAb-coated cells using magnetic beads and goat anti-HIgG-coated dishes, as described above. The cells contained <1% $CD44^{high}$ $CD4^+$ T cells. T-depleted spleen cells were obtained by treating spleen cells with HO13 and C, followed by inactivation with mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan), and used as APCs.

To obtain $CD4^+$ T cell blasts, indicated numbers of naive $CD4^+$ T cells were cultured with an indicated concentration of anti-CD3 mAbs in the presence of an equal number of syngenic APCs in 24-well culture plates (3047 Falcon; BD Labware, Franklin Lakes, NJ) or 48-well culture plates (3078 Falcon; BD Labware), or cultured in a 48-well culture plate coated with anti-CD3 mAbs (30 μg/ml) (the plate was precoated with anti-HIgG Abs (10 μg/ml)). After 40 h of culture, blasts were collected by applying the cell suspension to a Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient (density, 1.075), and expanded with 100 U/ml of human rIL-2 for an additional 2 days. The blasts obtained were >95% $CD4^+$, <0.8% $NK1.1^+$, and <0.2% TCR-$\gamma\delta^+$ after expansion with IL-2, and >90% $CD4^+$, <1.0% $NK1.1^+$, and <0.2% TCR-$\gamma\delta^+$ before expansion with IL-2.

Assay of naive and activated $CD4^+$ T cell responses

For the primary proliferative response, 2×10^5 naive $CD4^+$ T cells were cultured for 40 h or 2.5 days with titrated amounts of anti-CD3 mAbs and 2×10^5 syngenic APCs in a 96-well flat-bottom culture plate (3072 Falcon; BD Labware). Cells were pulsed with 0.5 μCi of [^{3}H]thymidine for the last 16 h, and the amount of [^{3}H]thymidine incorporated was measured. Results were expressed as the mean cpm ± SD of triplicate cultures, or as the mean inhibition (%) ± SD of triplicate cultures, as described in the legend for Fig. 3*A*. For primary cytokine production, naive $CD4^+$ T cells were cultured, as described above, for 43 h, and supernatants were collected to determine cytokine concentrations. For secondary cytokine production, activated $CD4^+$ T cells after expansion with IL-2 were stimulated, as described previously (20). In brief, 2.5×10^5 $CD4^+$ T cell blasts were cultured in a 48-well culture plate serially coated with anti-CD3 mAbs (0.1 μg/ml) and anti-CD28 mAbs (3 μg/ml). The plate was precoated with anti-HIgG Abs (10 μg/ml). Culture supernatants were collected at 24 h of culture to determine cytokine concentrations.

Flow cytometric analysis

The entire procedure was conducted on ice. To detect expressions of surface molecules on activated $CD4^+$ T cells before or after expansion with IL-2, blasts were stained by incubation with various biotinylated mAbs, followed by a cocktail of FITC anti-CD4 mAb and PE-streptavidin. Samples of 10,000 viable cells were analyzed by an Epics XL flow cytometer (Beckman Coulter). After gating for $CD4^+$ T cells, expression of surface molecules on activated $CD4^+$ T cells was calculated by subtracting mean log fluorescence intensity with background biotinylated HIgG staining from mean log fluorescence intensity with the corresponding biotinylated mAb staining. One sample for each molecule or culture condition was examined in independent experiments. In the figure indicated, results are presented as mean expression ± SD of at least three samples from separate experiments, and otherwise are representative of separate experiments.

Determination of cytokine concentrations

The IL-2 concentrations in culture supernatants were determined in a bioassay as the proliferation of IL-2-dependent CTLL-2 cells, as described previously (31), in the presence of anti-IL-4 mAbs (3 μg/ml). IL-4 and IFN-γ in culture supernatants were quantified by sandwich ELISA, according to the manufacturer's instructions (BD PharMingen). Results are presented as the mean concentration ± SD of triplicate cultures, or in the figure indicated, as mean concentration ± SD of at least three mean concentrations of triplicate cultures from separate experiments.

Assay for the induction of GATA-3

For analysis of the induction of GATA-3 in activated $CD4^+$ T cells, blast cells before or after expansion with IL-2 were lysed by incubation for 20 min on ice with TNE lysis buffer (10 mM Tris, pH 7.5, 0.15 M NaCl, 2 mM EDTA, 10% glycerol) containing protease inhibitors. The lysates were cleared by centrifugation to remove insoluble materials and stored at −80°C until needed. The lysates from 7×10^5 cells were applied to SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with 1–3%

skim milk in TBST (10 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween) for 1 h to reduce the background. The membranes were incubated overnight with anti-GATA-3 and anti-actin Abs of appropriate concentration in the TBST containing 1% milk. After being washed four times with TBST, they were incubated with HRP-conjugated secondary Abs for 1 h and developed on film using an ECL substrate (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's instructions.

Retrovirus-mediated gene transfer

The culture supernatant containing recombinant retrovirus was collected from Plat-E packaging cells (32) at 2 or 3 days after transfection with control enhanced green fluorescent protein (GFP)-retrovirus vector (pMX-IRES-EGFP) (a generous gift from Y. Kamogawa (University of Tokyo, Tokyo, Japan)) or GATA-3 cDNA-inserted pMX-IRES-EGFP (GATA-3 cDNA (33), a generous gift from J. Engel, Northwestern University, Evanston, IL) using the calcium phosphate method. Splenic $CD4^+$ T cells prepared as above were cultured with 2 μg/ml of anti-CD3 mAbs in the presence of an equal number of syngenic APCs in a 24-well culture plate. After 1 day, for infection with the recombinant virus, culture medium was replaced with supernatant from Plat-E cells supplemented with anti-CD3 mAbs (2 μg/ml) and polybrene (10 μg/ml) (Sigma-Aldrich), and the plate was centrifuged for 1 h at 1900 rpm at 30°C and incubated for 3–4 h at 37°C. Then, an equal volume of fresh DMEM (Invitrogen, Carlsbad, CA) containing 10% FCS was added to the well, followed by incubation overnight. The following day, the cells were reinfected as above. The infected cells were expanded and/or rested in the presence of 100 U/ml of human rIL-2 for an additional 2 days. The cells were collected and subjected to surface molecule staining by incubation with biotinylated mAbs, followed by PE-streptavidin. Remaining cells were stimulated with immobilized anti-CD3 and anti-CD28 mAbs, as described above, for 20 h, and with PMA (Sigma-Aldrich) (10 ng/ml) and A23187 (Sigma-Aldrich) (0.4 μM) in the presence of brefeldin A (Sigma) (10 μg/ml) for the last 4 h. Intracellular cytokine staining was then performed using IntraPrep permeabilization reagent (Immunotech, Marseille, France), according to the manufacturer's instructions. Briefly, the cells were fixed for 15 min, washed once, and permeabilized for up to 30 min while staining each cytokine with PE anti-IL-4 or PE anti-IFN-γ.

Statistical analysis

Results were analyzed for variance using a one- or two-way ANOVA, as appropriate, followed by Tukey-Kramer's test for multiple comparisons. Pearson's correlation coefficient was calculated between H4/ICOS expression and IL-4, IFN-γ production, or GATA-3 expression, and between IL-4 production and GATA-3 expression. Normalized amounts of GATA-3 protein (relative to the actin controls, as determined by densitometry) were used for statistical analysis. Results were considered significant if $p < 0.05$.

Results

Correlation between the level of H4/ICOS expression on activated $CD4^+$ T cells and the amount of IL-4 produced

Being influenced by the genetic background, the immune responses of BALB/c mice are Th2 prone, whereas those of C57BL/6 mice are Th1 prone (34). Using these mice, we previously reported that BALB/c-activated $CD4^+$ T cells exhibited much higher H4/ICOS expression and more Th2-derived cytokine production than C57BL/6-activated $CD4^+$ T cells (20). A representative profile of H4/ICOS expression on activated $CD4^+$ T cells from two murine strains is shown in Fig. 1*A*. To clarify that the above association is a general phenomenon, further study including four additional murine strains, C3H, CBA/J, CBF_1, and NC/Nga, was conducted. The NC/Nga mouse is known as a model of atopic dermatitis (24), thus being expected to show high H4/ICOS expression. Naive $CD4^+$ T cells obtained from spleen cells were stimulated with anti-CD3 mAbs in the presence of syngenic APCs. After expansion with IL-2, activated $CD4^+$ T cells were examined

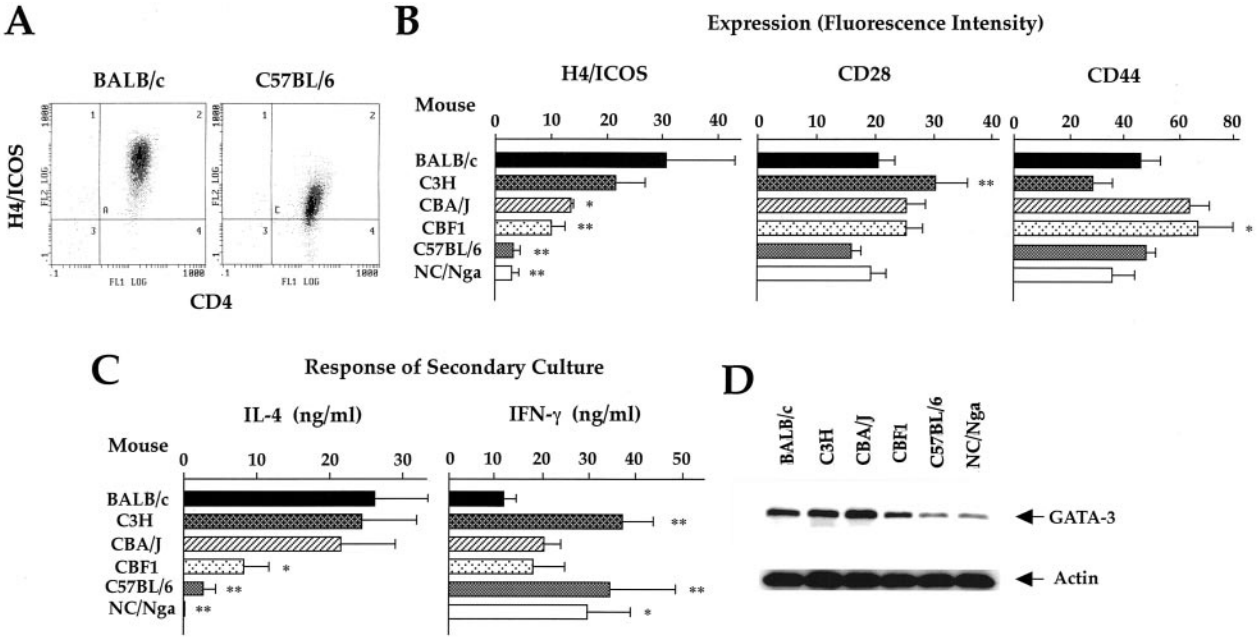

FIGURE 1. Activated $CD4^+$ T cells from different murine strains show a correlation between their H4/ICOS expression and IL-4 production. *A*, 1.5×10^6 BALB/c or C57BL/6 naive $CD4^+$ T cells were stimulated with anti-CD3 mAbs (2 μg/ml) in the presence of 1.5×10^6 syngenic APCs in a 24-well plate. After 40 h of culture, blasts were collected and expanded with 100 U/ml of human rIL-2 for 2 more days. $CD4^+$ T cell blasts were examined for expression of H4/ICOS and CD4 by flow cytometry. *B*, Activated $CD4^+$ T cells from different murine strains were obtained and expanded, as in *A*, and examined for expression of H4/ICOS, CD28, and CD44 by flow cytometry, as described in *Materials and Methods*. *C*, A total of 2.5×10^5 activated $CD4^+$ T cells obtained and expanded in *B* were stimulated in a 48-well plate serially coated with anti-CD3 mAbs (0.1 μg/ml) and anti-CD28 mAbs (3 μg/ml). The plate was precoated with anti-HIgG Abs (10 μg/ml). Culture supernatants were collected at 24 h of culture, and concentrations of IL-4 and IFN-γ were determined by sandwich ELISA. The level of IL-4 and IFN-γ production from each type of activated $CD4^+$ T cells without stimulation was below the detection level, <7.8 and <31.3 pg/ml, respectively. *D*, The cell lysate from 7×10^5 activated $CD4^+$ T cells obtained and expanded in *B* was subjected to SDS-PAGE, followed by blotting with anti-GATA-3 and anti-actin Abs. Results are shown as mean expression or concentration ± SD from at least three separate experiments for each murine strain. *, $p < 0.05$; **, $p < 0.01$ as compared with BALB/c.

for expressions of H4/ICOS, CD28, and CD44 and the pattern of cytokine production upon restimulation with immobilized anti-CD3 and anti-CD28 mAbs. There was a broad range of H4/ICOS expression levels on activated $CD4^+$ T cells among the murine strains analyzed (Fig. 1*B*). BALB/c showed the highest expression, and C3H, CBA/J, and CBF_1 followed in that order. C57BL/6 and, unexpectedly, NC/Nga expressed the lowest levels, which were ~10-fold lower than those of BALB/c. This pattern of variation among strains was not observed in CD28 and CD44 expressions (Fig. 1*B*). The amount of IL-4 produced from each type of activated $CD4^+$ T cell showed a similar variation, and correlated well with the level of H4/ICOS expression (Fig. 1*C*) ($r = 0.794$, $p < 0.0001$). A weak negative correlation appeared to exist between the levels of H4/ICOS expression and IFN-γ production (Fig. 1*C*) ($r = -0.582$, $p = 0.0018$). In addition, the induction of a Th2-associated transcription factor, GATA-3 (35), in each type of activated $CD4^+$ T cell also varied widely, and was consistent with the level of IL-4 production (Fig. 1*D*) ($r = 0.975$, $p = 0.0002$). Collectively, these results indicate H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production in these cells to be tightly correlated and regulated differently among murine strains. To clarify factors regulating H4/ICOS expression, we thought that comparative studies of immune responses between high and low H4/ICOS-expressing strains would be informative, and elucidate a cause for the different level of H4/ICOS expression in different murine strains. Therefore, we selected BALB/c and C57BL/6 mice and used them in the present study.

The degree of down-regulation of molecules strongly affects the level of H4/ICOS expression on activated $CD4^+$ T cells

It is well known that the dose of Ag and strength of the signal through TCR affect Th cell differentiation (36). Therefore, the above correlation may be due to a difference in the degree of activation of $CD4^+$ T cells among murine strains. To clarify this, we stimulated naive $CD4^+$ T cells from BALB/c and C57BL/6 with different amounts of anti-CD3 mAbs, and compared their proliferations, H4/ICOS expression, and various activation/memory markers on activated $CD4^+$ T cells before and after expansion with IL-2. As shown in Fig. 2*A*, before expansion with IL-2, BALB/c-activated $CD4^+$ T cells exhibited ~3- and ~2-fold higher expression of H4/ICOS than C57BL/6-activated $CD4^+$ T cells at 0.2 and 2 μg/ml of anti-CD3, respectively. The highest amount of anti-CD3 (20 μg/ml) gave rise to very similar expressions in the two murine strains. Among the molecules examined, CD25 showed a similar pattern of difference in expression between BALB/c- and C57BL/6-activated $CD4^+$ T cells. CD44 and CD69 expressions, the number of blast cell recovered, and proliferation indicated a similar level of cell activation in the two corresponding blasts, thus ruling out the above possibility, although a slight difference was observed in down-regulation of CD62L.

After expansion with IL-2, some of the molecules analyzed exhibited substantial down-regulations (Fig. 2*A*). H4/ICOS expression on BALB/c-activated $CD4^+$ T cells, however, was maintained at a slightly lower level than that before expansion with IL-2, whereas H4/ICOS on C57BL/6-activated $CD4^+$ T cells showed strong down-regulation, resulting in 6-, 10-, and 5-fold differences at 0.2, 2, and 20 μg/ml of anti-CD3, respectively, between two murine strains (Fig. 2*A*). Thus, higher induction followed by weaker down-regulation caused remarkably higher expression of H4/ICOS on BALB/c-activated $CD4^+$ T cells than on C57BL/6-activated $CD4^+$ T cells.

Much higher production of IL-2 and IL-4 in primary culture of BALB/c naive $CD4^+$ T cells than in that of C57BL/6 naive $CD4^+$ T cells

Taking the fact that IL-2 secreted from T cells increases and prolongs CD25 expression (37) into account, the difference in CD25 expression in BALB/c and C57BL/c suggests that the amount of IL-2 produced in the primary culture may be different. In addition, IL-4 may be a key to H4/ICOS expression, as indicated by its effect on the generation of IL-4-producing cells (38–40). To assess these possibilities, the production of cytokines was examined after stimulation of naive $CD4^+$ T cells from BALB/c and C57BL/6 mice with different amounts of anti-CD3 mAbs. The results showed BALB/c naive $CD4^+$ T cells to be stimulated to produce substantial amounts of IL-2 and IL-4, whereas C57BL/6 naive $CD4^+$ T cells produced ~3- and ~10-fold lower amounts of IL-2 and IL-4, respectively, as shown by dose-response curves (Fig. 2*B*). IFN-γ was produced at a similar level by these two naive $CD4^+$ T cells. Production of IL-2 and IL-4, but not IFN-γ, at different times after stimulation with 2 μg/ml of anti-CD3 mAbs also revealed a remarkable difference in BALB/c and C57BL/6 mice (data not shown). Thus, the amounts of IL-2 and IL-4 produced during activation of naive $CD4^+$ T cells appear to be regulated differently among murine strains, and may thereby affect the expression of H4/ICOS.

Role of CD28 costimulation in the difference in H4/ICOS expression

It has been shown that IL-2 production (41) and ICOS expression (11) are up-regulated with CD28 costimulation. Thus, the differences in H4/ICOS expression may be due to differences in the level of CD28 costimulation that the different murine strains receive. To test this, we next examined the effect of blocking of CD28 costimulation on the difference in H4/ICOS expression between BALB/c and C57BL/6 mice. CTLA-4-mIgs (160 μg/ml, at which the maximal blocking of CD28 costimulation was achieved (Fig. 3*A*)) were added in the same culture system as above, or purified naive $CD4^+$ T cells were stimulated with immobilized anti-CD3 mAbs in the presence of 160 μg/ml of CTLA-4-mIgs. After expansion of blasts with IL-2, H4/ICOS expression on BALB/c- and C57BL/6-activated $CD4^+$ T cells was compared. As shown in Fig. 3*B*, BALB/c-activated $CD4^+$ T cells exhibited ~6-fold higher H4/ICOS expression than C57BL/6-activated $CD4^+$ T cells in the presence of control mIgG. CTLA-4-mIgs strongly reduced H4/ICOS expression on BALB/c-activated $CD4^+$ T cells, resulting in ~2-fold difference in H4/ICOS expression between two murine strains. Thus, CD28 costimulation exerts a strong effect on the difference in H4/ICOS expression. In addition, stimulation of naive $CD4^+$ T cells with anti-CD3 mAbs alone gave rise to ~3-fold difference. Taken together with the above ~2-fold difference, other factors than CD28 costimulation may also affect the level of H4/ICOS expression in BALB/c and C57BL/6.

IL-4 in the primary culture is required for maintenance of H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production by these T cells

To assess the role of IL-4 more directly, the effect of neutralization of IL-4 on H4/ICOS expression was examined by adding anti-IL-4 mAbs in the primary stimulation of BALB/c naive $CD4^+$ T cells. As shown in Fig. 4*A*, in the presence of titrated amounts of control RIgG in the primary culture, the expression of H4/ICOS on

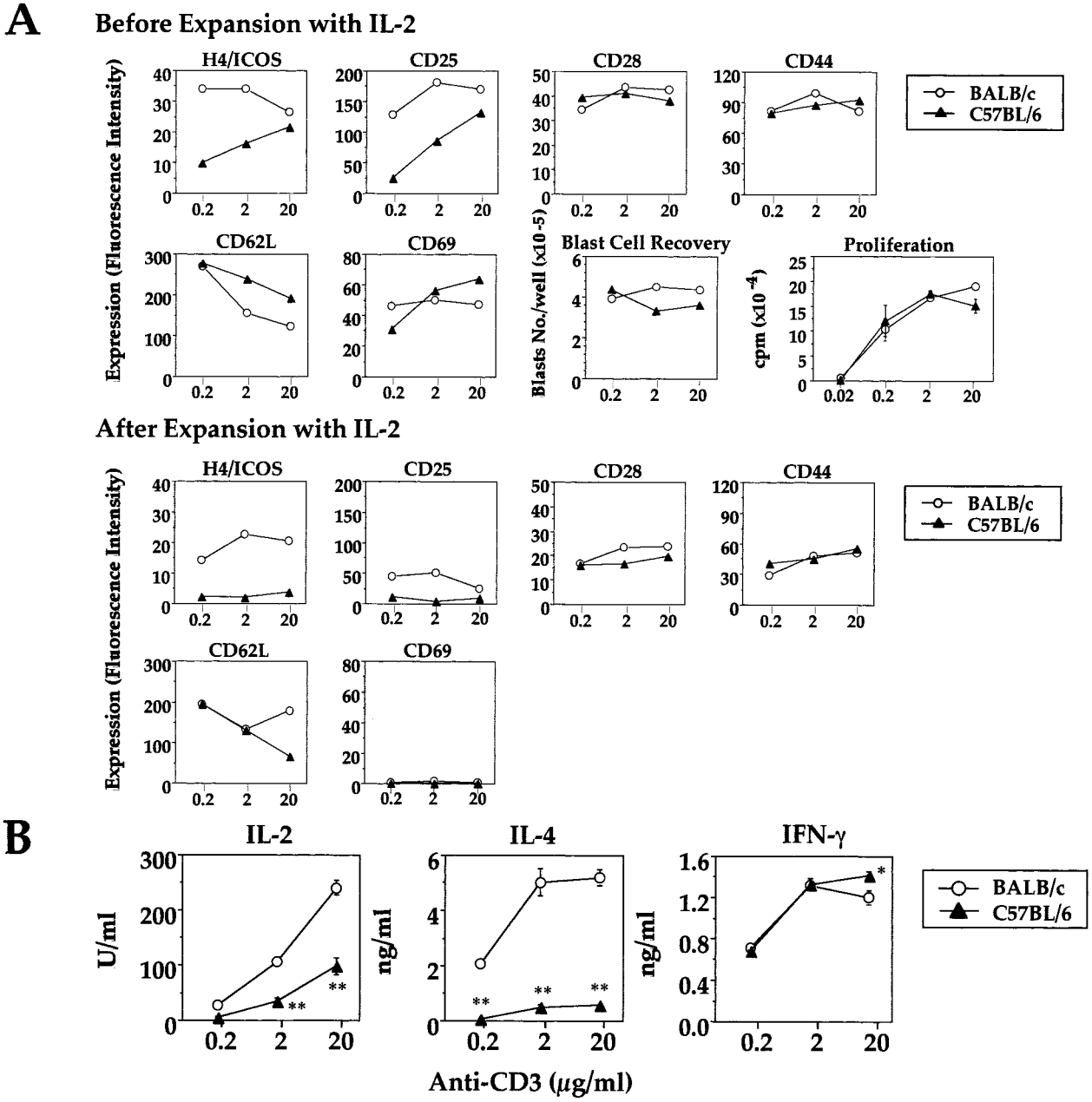

FIGURE 2. Comparison of the levels of activation and cytokine production upon primary stimulation of BALB/c and C57BL/6 naive $CD4^+$ T cells. *A*, A total of 5×10^5 BALB/c or C57BL/6 naive $CD4^+$ T cells were stimulated with titrated amounts of anti-CD3 mAbs in the presence of 5×10^5 syngenic APCs in a 48-well plate. After 40 h of culture, blasts were examined for expression of various activation/memory markers on activated $CD4^+$ T cells by flow cytometry, and for the number of blast cells recovered. At the same time, 2×10^5 naive $CD4^+$ T cells were stimulated for 40 h with titrated amounts of anti-CD3 mAbs in the presence of 2×10^5 syngenic APCs in a 96-well plate, and uptakes of $[^3H]$thymidine were examined. After expansion of blasts obtained in the above culture with IL-2 for 2 more days, expressions of molecules on activated $CD4^+$ T cells were examined. *B*, A total of 2×10^5 naive $CD4^+$ T cells from BALB/c and C57BL/6 mice were stimulated with titrated amounts of anti-CD3 mAbs in the presence of 2×10^5 syngenic APCs in a 96-well plate. After 43 h of culture, culture supernatants were collected, and the IL-2 concentration was determined in a bioassay using CTLL-2 cells, and those of IL-4 and IFN-γ were determined by sandwich ELISA. These figures are representative of three independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$ as compared with BALB/c.

activated $CD4^+$ T cells after expansion with IL-2 was maintained at a slightly lower level than that before expansion with IL-2. The addition of anti-IL-4 mAbs reduced H4/ICOS expression on activated $CD4^+$ T cells only partially (~30% at 30 μg/ml of mAbs) and strongly (~6-fold at 30 μg/ml of mAbs) before and after expansion with IL-2, respectively (Fig. 4*A*), indicating the down-regulatory effect of IL-4 neutralization on H4/ICOS expression. Because the addition of anti-IL-4 mAbs did not affect the level of IL-2 production in the primary culture (Fig. 4*B*) or the number of blast cells recovered (data not shown), it is unlikely that the reduced H4/ICOS expression was due to insufficiency of cell activation. After expansion with IL-2, activated $CD4^+$ T cells obtained from the primary culture with 3 μg/ml of anti-IL-4 mAbs secreted much less IL-4, but a similar amount of IFN-γ upon restimulation as compared with the control cells (Fig. 4*C*). GATA-3 induction in these cells was consistently lower, and a similar reduction of GATA-3 was also observed before expansion with IL-2 (Fig. 4*D*). Thus, these results indicate a critical role for IL-4 in the

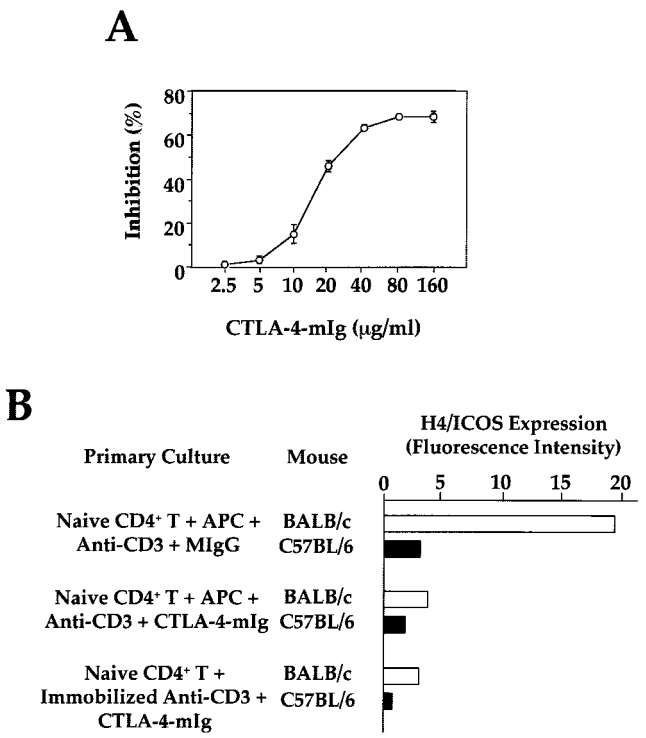

FIGURE 3. Effect of blocking of CD28 costimulation on the difference in H4/ICOS expression between BALB/c- and C57BL/6-activated $CD4^+$ T cells. *A*, A total of 2×10^5 BALB/c naive $CD4^+$ T cells were cultured for 2.5 days with anti-CD3 mAbs (2 μg/ml) and 2×10^5 syngenic APCs in the presence of titrated amounts of control mIgG or CTLA-4-mIgs in a 96-well plate, and uptakes of [^{3}H]thymidine were examined. Mean cpm was calculated from triplicate cultures at each concentration of mIgG. Results are expressed as the mean percentage of inhibition compared with control ± SD of triplicate cultures in the presence of CTLA-4-mIgs. *B*, A total of 5×10^5 BALB/c or C57BL/6 naive $CD4^+$ T cells were cultured for 40 h with anti-CD3 mAbs (2 μg/ml) and 5×10^5 syngenic APCs in the presence of control mIgG or CTLA-4-mIgs (160 μg/ml) in a 48-well plate. At the same time, 5×10^5 BALB/c or C57BL/6 naive $CD4^+$ T cells were stimulated in a 48-well plate coated with anti-CD3 mAbs (30 μg/ml) in the presence of CTLA-4-mIgs (160 μg/ml). The plate was precoated with anti-HIgG Abs (10 μg/ml). Blast cells were obtained, expanded with IL-2, and examined for expression of H4/ICOS by flow cytometry. The results are representative of three independent experiments with similar results.

maintenance of H4/ICOS expression on activated $CD4^+$ T cells and in the induction of IL-4 production upon restimulation.

IL-2 in the primary culture appears to regulate the induction of H4/ICOS expression, and has an effect on H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production from these cells similar to that of IL-4

We next asked whether IL-2 regulates H4/ICOS expression on activated $CD4^+$ T cells in similar neutralization experiments. Neutralizing anti-IL-2 mAbs were titrated into the primary culture of BALB/c naive $CD4^+$ T cells, and H4/ICOS expression on activated $CD4^+$ T cells was examined before expansion with IL-2. In contrast to the slight inhibitory effect of anti-IL-4 mAbs (Fig. 4*A*), anti-IL-2 mAbs strongly and dose dependently reduced H4/ICOS expression on activated $CD4^+$ T cells (~4-fold at 30 μg/ml of mAbs) as compared with control RIgG (Fig. 5*A*). This result suggests that IL-2 is a factor regulating the induction of H4/ICOS expression on BALB/c-activated $CD4^+$ T cells. Although inhibition of the proliferative response was barely detectable (Fig. 5*A*), anti-IL-2 mAbs inhibited the production of IL-4 in the primary culture (Fig. 5*A*). The inhibition was dose dependent, and was almost complete at >3 μg/ml of anti-IL-2 mAbs.

After expansion with IL-2, activated $CD4^+$ T cells obtained from the primary culture with 30 μg/ml of anti-IL-2 mAbs showed the level of H4/ICOS to be ~10-fold lower than that seen with none or RIgG with a level of CD28 similar to that of the control (Fig. 5*B*). Thus, IL-2 neutralization in the primary culture promoted the down-regulation of H4/ICOS expression during expansion with IL-2. Upon restimulation, activated $CD4^+$ T cells from the primary culture with anti-IL-2 mAbs secreted >50-fold less IL-4, but a similar amount of IFN-γ as compared with the control levels (Fig. 5*B*). Consistent with this, GATA-3 expression in these cells was strongly suppressed as compared with that in control cells (Fig. 5*C*). The effect of anti-IL-2 mAbs in the primary culture was dose dependent, such that the suppression of H4/ICOS expression and IL-4 production with 3 μg/ml of anti-IL-2 mAbs was about one-half of that with 30 μg/ml (data not shown). Taken collectively, these observations suggest the amount of IL-2 in the primary culture of naive BALB/c $CD4^+$ T cells to affect the level of H4/ICOS expression and IL-4 production from activated $CD4^+$ T cells.

The effect of IL-2 is likely to be indirect and mediated by IL-4

As IL-2 regulated IL-4 production (Fig. 5*A*), it is possible that the above inhibitory effect of IL-2 neutralization was secondary to the reduction of IL-4. To test this possibility, we examined whether exogenous IL-4 cancels the effect of anti-IL-2 mAbs during primary stimulation of BALB/c naive $CD4^+$ T cells. As shown in Fig. 6*A*, 30 μg/ml of anti-IL-2 mAbs in the primary culture, which was capable of almost completely inhibiting the production of endogenous IL-4 (Fig. 5*A*), greatly inhibited the expression of H4/ICOS on activated $CD4^+$ T cells before and after expansion with IL-2, and the production of IL-4 upon restimulation. Addition of IL-4 (2 ng/ml) to this culture restored both H4/ICOS expression on activated $CD4^+$ T cells after expansion with IL-2 and IL-4 production by these cells to the levels seen in the control culture with RIgG alone. H4/ICOS expression on activated $CD4^+$ T cells before expansion with IL-2 was still reduced by ~35% in the presence of anti-IL-2 and IL-4, and this may support the regulatory effect of IL-2 on the induction of H4/ICOS. In our experimental system, we consistently observed the addition of IL-4 to promote not only IL-4, but also IFN-γ production. GATA-3 expression in each activated $CD4^+$ T cell preparation was consistent with the amount of IL-4 produced upon restimulation (Fig. 6*B*). Taken collectively, these results indicate the effect of IL-2 on the H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production upon restimulation to be mediated mainly by IL-4, the production of which is regulated by IL-2.

H4/ICOS expression on C57BL/6-activated $CD4^+$ T cells and IL-4 production by these cells are increased by exogenous IL-4 and retroviral expression of GATA-3

If the above regulation of IL-2 and IL-4 in BALB/c mice occurs in C57BL/6 mice as well, an increase in the amount of IL-2 or IL-4 in the primary culture of C57BL/6 naive $CD4^+$ T cells would be likely to increase H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production by these cells. To evaluate this, exogenous IL-2 or IL-4 was added to the primary stimulation of C57BL/6 or BALB/c naive $CD4^+$ T cells, and H4/ICOS expression on activated $CD4^+$ T cells after expansion with IL-2 and cytokine production by these T cells were examined. As shown in Fig. 7*A*, the addition of IL-4 increased the level of H4/ICOS expression on C57BL/6-activated $CD4^+$ T cells ~4-fold, and induced a polarized Th2 response with a ~5-fold increase in IL-4 production and

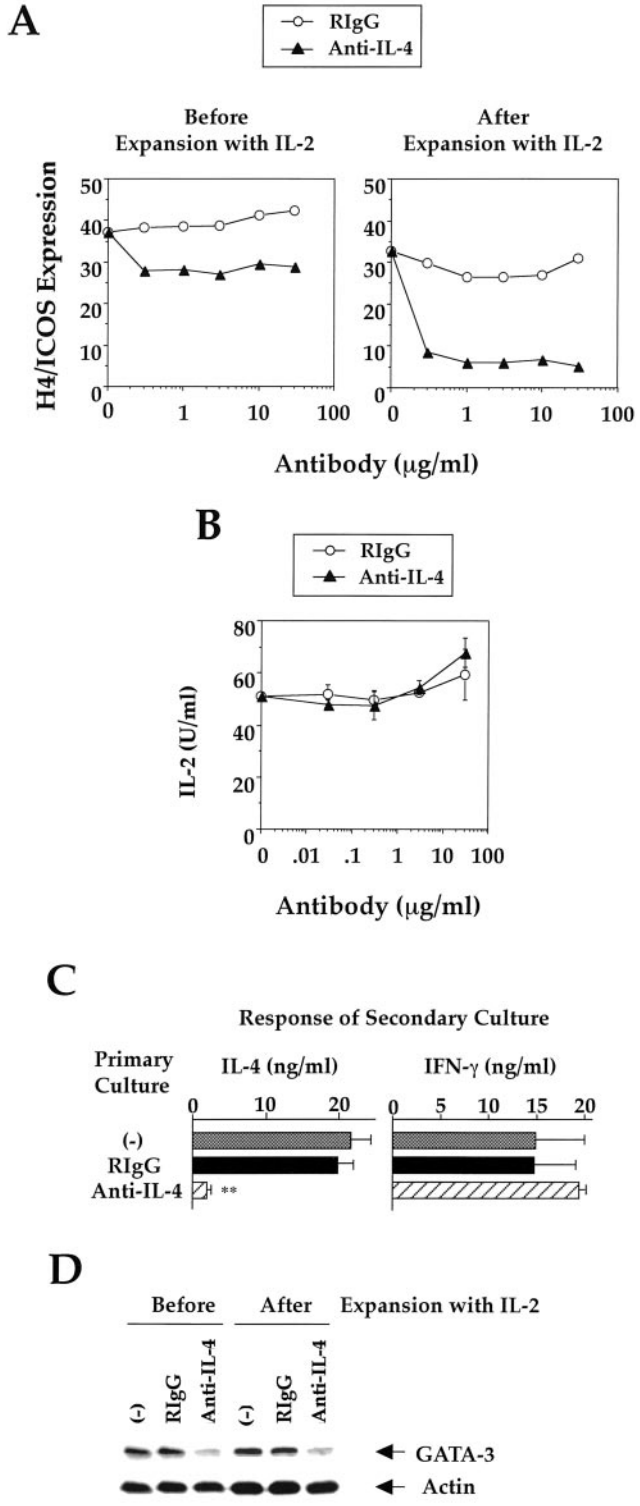

FIGURE 4. Neutralization of IL-4 in the primary culture of naive $CD4^+$ T cells leads to down-regulation of H4/ICOS expression on activated $CD4^+$ T cells and of IL-4 production by these cells. *A*, A total of 5×10^5 BALB/c naive $CD4^+$ T cells were stimulated with anti-CD3 mAbs (2 μg/ml) and 5×10^5 syngenic APCs in the absence or presence of titrated amounts of RIgG or anti-IL-4 mAbs in a 48-well plate. After 40 h of culture, blasts were collected and examined immediately or 2 days after expansion with IL-2 for expression of H4/ICOS on activated $CD4^+$ T cells by flow cytometry. *B*, A total of 2×10^5 BALB/c naive $CD4^+$ T cells were stimulated for 43 h with anti-CD3 mAbs (2 μg/ml) and 2×10^5 syngenic APCs in the absence or presence of titrated amounts of RIgG or anti-IL-4 mAbs in a 96-well plate, and IL-2 concentrations in culture supernatants were determined. *C* and *D*, BALB/c-activated $CD4^+$ T cells obtained in the absence or presence of RIgG or anti-IL-4 (3 μg/ml) were examined for the production of IL-4 and IFN-γ after expansion with IL-2 (*C*) and for GATA-3 and actin expressions before and after expansion with IL-2 (*D*), as in Fig. 1. These figures are representative of three independent experiments with similar results. **, $p < 0.01$ as compared with controls in the absence and presence of RIgG.

a ~4-fold decrease in IFN-γ production, as compared with control cells. However, unexpectedly, the addition of IL-2, at an amount similar to that produced in the primary culture of BALB/c naive $CD4^+$ T cells, did not increase H4/ICOS expression on C57BL/6-activated $CD4^+$ T cells or IL-4 production by these T cells. In contrast, the addition of IL-2 slightly increased H4/ICOS expression on BALB/c-activated $CD4^+$ T cells in a dose-dependent manner, and promoted production of both IL-4 and IFN-γ as did the addition of IL-4 (Fig. 7*B*). The effect of IL-2 became clearer when endogeous IL-2 produced in the primary culture was neutralized with mAbs (Fig. 7*C*).

GATA-3 is induced by signaling through IL-4Rs (42). Therefore, we next tested whether GATA-3-dependent transcription regulates H4/ICOS expression. GATA-3-GFP expression constructs were introduced into C57BL/6 $CD4^+$ T cells during primary stimulation of naive $CD4^+$ T cells using retroviral infection. Retroviral expression of GATA-3 promoted Th2 development, such that GFP-positive cells in activated $CD4^+$ T cells infected by GATA-3-GFP virus showed ~7-fold more IL-4-producing cells and ~2-fold fewer IFN-γ-producing cells after secondary stimulation than GFP-positive cells among activated $CD4^+$ T cells infected by control virus (Fig. 8*A*). The amount of H4/ICOS on GFP-positive activated $CD4^+$ T cells infected by GATA-3-GFP virus was ~3-fold higher than that on GFP-positive activated $CD4^+$ T cells infected by control virus, while retroviral GATA-3 expression did not affect the level of CD3 expression or resulted in a ~3-fold decrease in CD28 expression (Fig. 8*B*). It is, however, possible that retroviral expression of GATA-3 increased IL-4 production, and that IL-4 up-regulated H4/ICOS expression through a GATA-3-independent pathway. To examine this possibility, similar experiments were performed using IL-$4^{-/-}$ naive $CD4^+$ T cells. Retroviral expression of GATA-3 in IL-$4^{-/-}$-activated $CD4^+$ T cells increased H4/ICOS expression ~4-fold, with similar and ~3-fold lower levels of CD3 and CD28 (Fig. 8*B*). Western blot analysis confirmed the increased expression of GATA-3 by retroviral infection (Fig. 8*C*). Thus, these results suggest that GATA-3 at least partially up-regulates H4/ICOS expression in activated $CD4^+$ T cells.

Discussion

In the present study, activated $CD4^+$ T cells capable of producing more IL-4 consistently expressed more H4/ICOS on their surfaces among different murine strains (Fig. 1, *B* and *C*). In keeping with this finding, the degree of induction of the Th2-associated transcription factor GATA-3 in activated $CD4^+$ T cells also correlated with the level of H4/ICOS expression in these cells (Fig. 1*D*) ($r = 0.848$, $p = 0.0305$). Several observations in the present study support the notion that IL-4 produced in the primary stimulation of naive $CD4^+$ T cells is a key factor determining the level of H4/ICOS expression on activated $CD4^+$ T cells. First, there was a correlation between the amount of IL-4 produced in the primary stimulation of naive $CD4^+$ T cells and the level of H4/ICOS on activated $CD4^+$ T cells. A much higher amount of IL-4 was produced by BALB/c naive $CD4^+$ T cells than C57BL/6 naive $CD4^+$ T cells (Fig. 2*B*). The amount of IL-4 produced by C3H naive

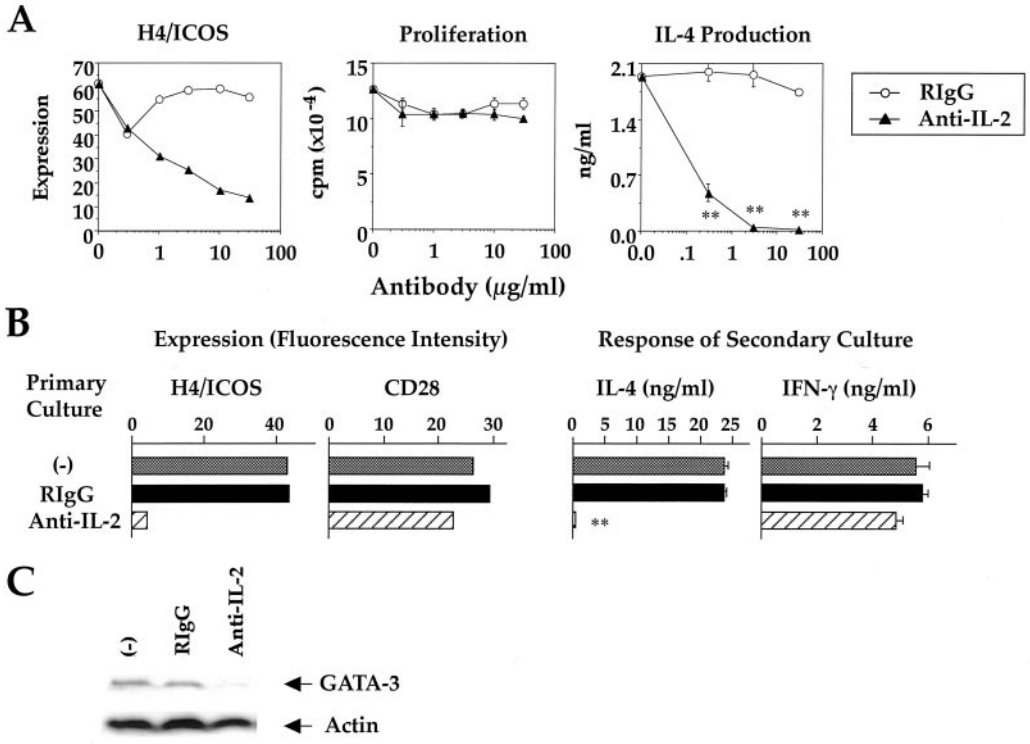

FIGURE 5. Neutralization of IL-2 in the primary culture of naive $CD4^+$ T cells decreases the induction of H4/ICOS expression and the levels of H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production by these cells. *A*, A total of 5×10^5 naive $CD4^+$ T cells from BALB/c mice were stimulated with anti-CD3 mAbs (2 μg/ml) and 5×10^5 syngenic APCs in the absence or presence of titrated amounts of RIgG or anti-IL-2 mAbs in a 48-well plate. After 40 h of culture, blasts were examined for expression of H4/ICOS on activated $CD4^+$ T cells by flow cytometry. A total of 2×10^5 BALB/c naive $CD4^+$ T cells was stimulated for 40 h with anti-CD3 mAbs (2 μg/ml) and 2×10^5 syngenic APCs in the absence or presence of titrated amounts of RIgG or anti-IL-2 mAbs in a 96-well plate, and uptakes of [^{3}H]thymidine were examined. A total of 2×10^5 BALB/c naive $CD4^+$ T cells was stimulated, as described above, for 43 h, and concentrations of IL-4 in culture supernatants were determined by sandwich ELISA. *B* and *C*, A total of 5×10^5 BALB/c naive $CD4^+$ T cells were stimulated for 40 h with anti-CD3 mAbs (2 μg/ml) and 5×10^5 syngenic APCs in the absence or presence of RIgG or anti-IL-2 mAbs (30 μg/ml) in a 48-well plate. Blast cells were obtained, expanded with IL-2, and examined for expression of H4/ICOS and CD28, and production of IL-4 and IFN-γ (*B*), and GATA-3 and actin expressions in cell lysates (*C*), as in Fig. 1. These figures are representative of three independent experiments with similar results. **, $p < 0.01$ as compared with control culture in the presence of RIgG (*A*) or control cultures in the absence and presence of RIgG (*B*).

$CD4^+$ T cells was intermediate between those of BALB/c and C57BL/6, and that by NC/Nga naive $CD4^+$ T cells was lower than that of C57BL/6 naive $CD4^+$ T cells (data not shown). NC/Nga mice are known as an atopic dermatitis model, because they develop atopic dermatitis-like lesions when kept under conventional conditions (24). However, the results of the present study clearly indicated NC/Nga naive $CD4^+$ T cells to be prone to differentiate into Th1 cells, which express low levels of H4/ICOS, when kept under specific pathogen-free conditions. Second, culture of BALB/c naive $CD4^+$ T cells with neutralizing anti-IL-4 mAbs led to a strong down-regulation of H4/ICOS expression on activated $CD4^+$ T cells during expansion with IL-2, and the suppression of GATA-3 induction (Fig. 4, *A* and *D*), as seen in C57BL/6-activated $CD4^+$ T cells (Figs. 1*D* and 2*A*). Third, exogenous IL-4 or retroviral expression of GATA-3 in the primary stimulation of C57BL/6 or IL-$4^{-/-}$ naive $CD4^+$ T cells conversely up-regulated the expression of H4/ICOS on activated $CD4^+$ T cells (Figs. 7*A* and 8*B*). We consistently found retroviral expression of GATA-3 to suppress the expression of CD28. We do not yet have any data concerning the effect of retroviral expression of GATA-3 on the turnover or recycling of CD28, but it would be interesting to clarify how CD28 expression is specifically down-regulated. Taken collectively, our findings indicate IL-4 produced during the differentiation of Th cells, the effect of which is at least partially mediated by GATA-3, to be critical for maintaining the level of H4/ICOS induced on activated $CD4^+$ T cells. Hence, Th2 cells appear to sustain a high level of H4/ICOS expression on their surfaces, whereas Th1 cells down-regulate H4/ICOS expression. Then it is possible that factors affecting IL-4 and/or GATA-3 expression also regulate H4/ICOS expression on activated $CD4^+$ T cells. Supporting this, the addition of IFN-γ (43) (9 ng/ml) in the primary stimulation of naive $CD4^+$ T cells reduced H4/ICOS expression on BALB/c- and C57BL/6-activated $CD4^+$ T cells after expansion with IL-2 by ~40 and ~30%, respectively, and the addition of anti-IFN-γ mAbs (10 μg/ml) conversely increased the level of H4/ICOS expression by ~30 and ~80%, respectively (results are the means of three independent experiments) (our unpublished observations).

Given the tight association between the amount of H4/ICOS expression on activated $CD4^+$ T cells and the level of IL-4 induction by these cells, an obvious question is whether the increased expression of H4/ICOS results in greater generation of IL-4-producing cells. Previous studies using BALB/c background mice found the development of Th2 cells to be inhibited by the presence of ICOS-IgG fusion proteins (11), and promoted by the presence of anti-H4/ICOS mAbs coated on the surface of culture wells (20), indicating that the strength of H4/ICOS-dependent signaling affects Th2 development. Consistent with this notion, we recently observed that H4/ICOS binding dominantly elicited the serine/threonine protein kinase Akt pathway, in contrast to CD28 (20),

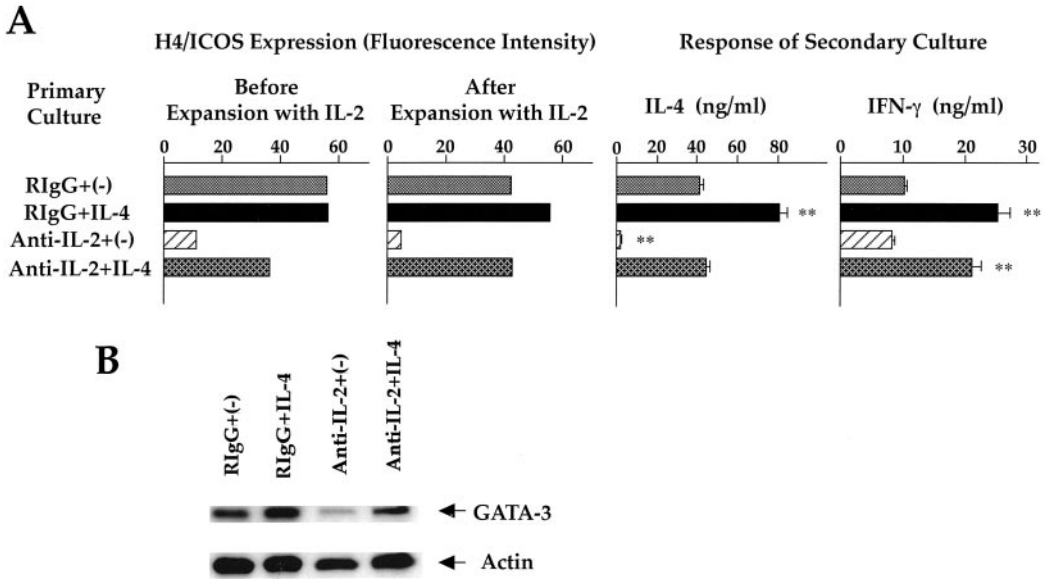

FIGURE 6. The effect of anti-IL-2 mAbs is blocked by the addition of IL-4. *A*, 5×10^5 BALB/c naive $CD4^+$ T cells were stimulated with anti-CD3 mAbs (2 μg/ml) and 5×10^5 syngenic APCs in the presence of RIgG or anti-IL-2 (30 μg/ml) with or without exogenous IL-4 (2 ng/ml) in a 48-well plate. After 40 h of culture, blasts were collected and examined for expression of H4/ICOS by flow cytometry immediately or 2 days after expansion with IL-2. Blast cells after expansion with IL-2 were also examined for the production of IL-4 and IFN-γ, as in Fig. 1*C*. *B*, GATA-3 and actin expressions in the blasts obtained and expanded in *A* were examined, as in Fig. 1*D*. These figures are representative of three independent experiments with similar results. **, $p < 0.01$ as compared with control culture in the presence of RIgG alone.

and that retroviral expression of the activated form of Akt during primary stimulation of BALB/c naive $CD4^+$ T cells promoted Th2 cell differentiation.[4] Furthermore, higher expression of H4/ICOS appears to induce stronger H4/ICOS-dependent signaling, as seen in our recent study in which Akt was far more strongly activated upon H4/ICOS costimulation in BALB/c-activated $CD4^+$ T cells, in which H4/ICOS expression is 10-fold higher, than in C57BL/6-activated $CD4^+$ T cells (20). Thus, when bound by the ligand, higher expression of H4/ICOS on the cell surface in BALB/c mice most likely promotes the generation of Th2 cells through stronger H4/ICOS-dependent signaling.

ICOS also plays a critical role in Th1 responses, as shown recently in in vivo infectious Th1 models (44, 45). The ability of H4/ICOS to costimulate T cells in IFN-γ secretion most likely contributes to resistance to certain infections. In addition, it may be possible that H4/ICOS costimulation also promotes Th1 differentiation. Our recent study found that the activated form of Akt promoted Th1, but not Th2 cell differentiation in C57BL/6 naive $CD4^+$ T cells.[4] Furthermore, under Th1-polarizing conditions with

[4] Y. Arimura, J. Yagi, F. Shiroki, S. Kuwahara, H. Kato, U. Dianzani, and T. Uchiyama. Akt is a neutral amplifier for Th cell differentiation. *Submitted for publication.*

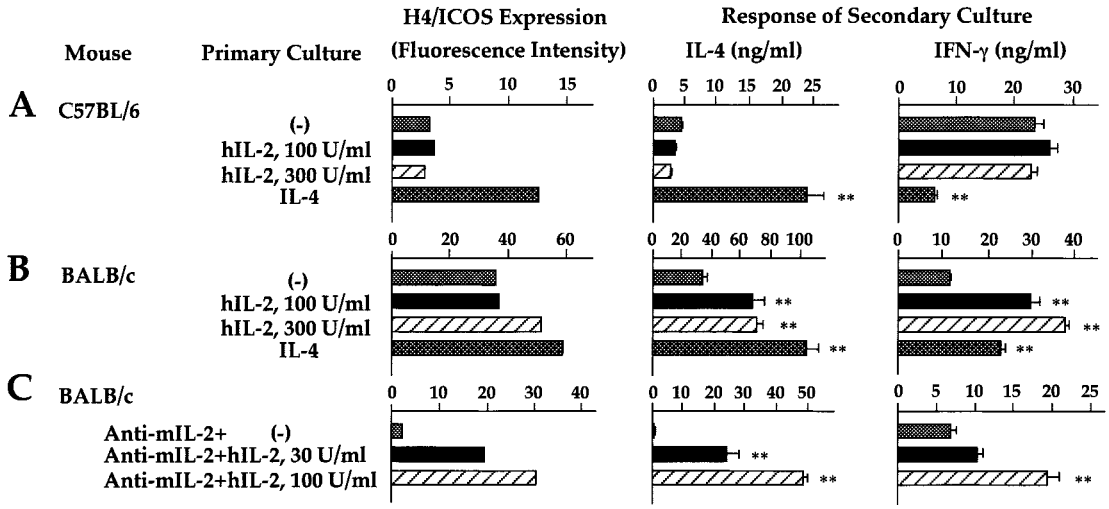

FIGURE 7. The effect of adding IL-2 or IL-4 to the primary culture of naive $CD4^+$ T cells on the expression of H4/ICOS on activated $CD4^+$ T cells and their cytokine production. *A* and *B*, A total of 5×10^5 C57BL/6 (*A*) or BALB/c (*B*) naive $CD4^+$ T cells were stimulated with anti-CD3 mAbs (2 μg/ml) and 5×10^5 syngenic APCs in the absence or presence of human rIL-2 (hIL-2) (100 or 300 U/ml) or IL-4 (2 ng/ml) in a 48-well plate. *C*, BALB/c naive $CD4^+$ T cells were stimulated, as in *B*, with anti-mouse IL-2 (anti-mIL-2) mAbs (30 μg/ml) in the absence or presence of hIL-2 (30 or 100 U/ml). After 40 h of culture, blasts were collected, expanded with IL-2, and examined for expression of H4/ICOS and productions of IL-4 and IFN-γ, as in Fig. 1. These figures are representative of three independent experiments with similar results. **, $p < 0.01$ as compared with control culture without addition of lymphokines (*A* and *B*) or control culture in the presence of anti-IL-2 alone (*C*).

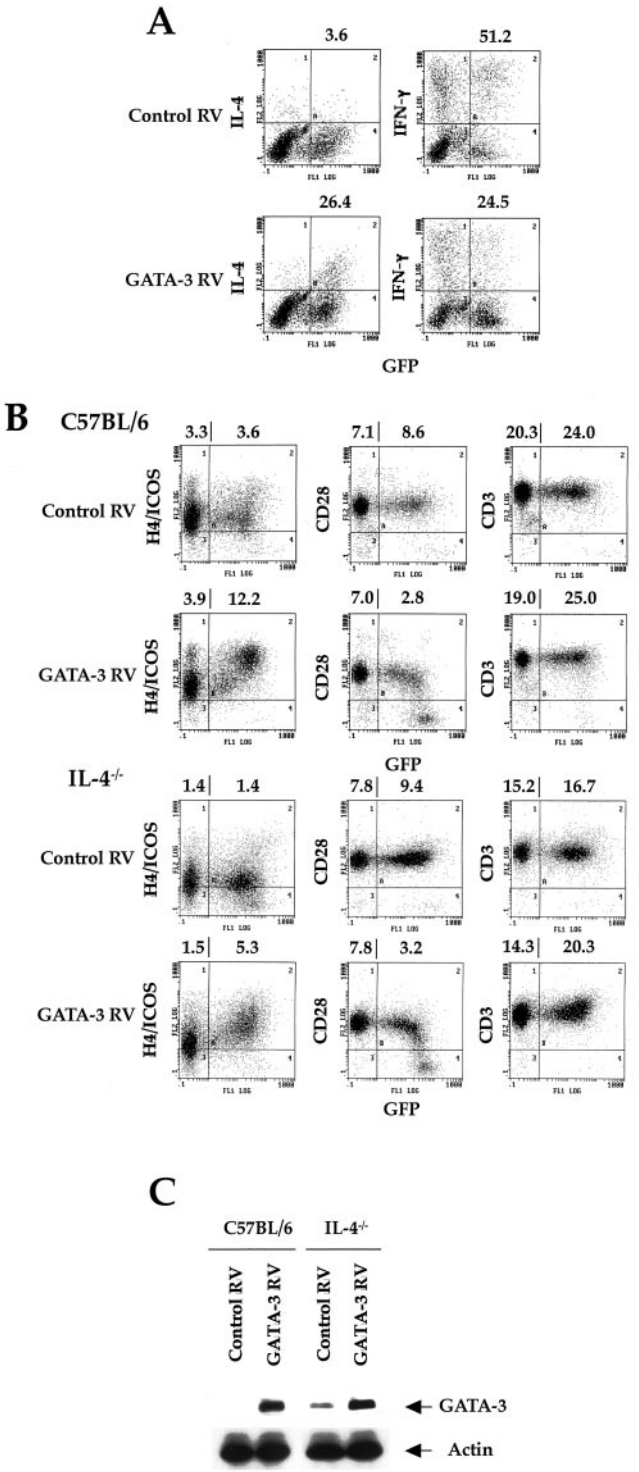

FIGURE 8. Retroviral expression of GATA-3 during primary stimulation of naive $CD4^+$ T cells increases H4/ICOS expression on activated $CD4^+$ T cells. A control empty construct (control RV) or a GATA-3-GFP expression construct (GATA-3 RV) was introduced into C57BL/6- or IL-$4^{-/-}$-activated $CD4^+$ T cells during primary culture with anti-CD3 mAbs (2 μg/ml). Blast cells were collected, expanded with IL-2, and examined for IL-4- and IFN-γ-producing cells among C57BL/6 blasts by intracellular staining upon restimulation (*A*), and H4/ICOS, CD28, and CD3 expressions on both blasts by flow cytometry (*B*). Figures in *A* and *B* indicate the percentages of IL-4- or IFN-γ-producing cells among GFP^+ cells and mean fluorescence intensities of GFP^- or GFP^+ cells corrected for background stainings, respectively. *C*, GATA-3 and actin expressions in the cells after expansion with IL-2 were examined, as in Fig. 1*D*. These figures are representative of two independent experiments with similar results.

the addition of IL-12 and anti-IL-4 mAbs, Akt promoted Th1 differentiation even in BALB/c naive $CD4^+$ T cells.[4] From these findings, we assume that H4/ICOS-dependent signaling by itself is not specific for Th2 differentiation, and facilitates Th differentiation depending on intracellular status, which is primarily based on the murine genetic background, but can be affected by the extracellular cytokine milieu. As shown in the present study, IL-4 production is much higher in BALB/c naive $CD4^+$ T cells than C57BL/6 naive $CD4^+$ T cells, and IL-4 up-regulates H4/ICOS expression. It is thus conceivable that IL-4 links to H4/ICOS, and that this pathway is important for effectively promoting Th differentiation into Th2 cells in Th2-prone BALB/c mice. In contrast, in Th1-prone C57BL/6 mice, H4/ICOS, although not highly expressed, may promote Th1 differentiation. H4/ICOS appears to play a critical role in both Th1 and Th2 responses by enhancing cytokine productions from effector Th cells.

Neutralization of IL-2, or addition of IL-2, to the primary culture of BALB/c naive $CD4^+$ T cells had a profound inhibitory, or promoting effect on the H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production by these cells (Figs. 5*B* and 7, *B* and *C*). As shown in the present and previous studies, anti-IL-2 mAbs inhibited IL-4 production in a primary culture of BALB/c naive $CD4^+$ T cells (Fig. 5*A*) (46), while conversely, the addition of IL-2 increased it (data not shown). These results suggest a regulatory role for IL-2 in IL-4 production in primary culture, although the mechanism underlying the IL-2-regulated IL-4 production remains to be elucidated. Thus, IL-2 may be crucial for triggering the pathway linking IL-4 to H4/ICOS in BALB/c mice, thereby exhibiting an effect on H4/ICOS expression on activated $CD4^+$ T cells and IL-4 production by these T cells similar to that of IL-4. Consistent with this scenario, the results of the present study suggest that the effect of IL-2 is mediated by IL-4 produced in the primary culture of BALB/c naive $CD4^+$ T cells. First, the inhibitory effect of anti-IL-2 mAbs on H4/ICOS expression and IL-4 production in activated $CD4^+$ T cells was completely blocked by the addition of IL-4 to the primary culture (Fig. 6*A*). Second, neutralization of IL-4 in the primary culture suppressed most of the H4/ICOS expression on activated $CD4^+$ T cells after expansion with IL-2 and IL-4 production by these cells upon restimulation, regardless of whether IL-2 production in the primary culture was intact (Fig. 4, *A*, *B*, and *C*). Several previous studies documented IL-2 to play a role in up-regulating the generation of IL-4-producing cells (47–49). Our results in the present study most likely rule out a direct effect of IL-2 in such regulation, rather supporting the possibility that the effect of IL-2 is mediated secondarily by IL-4.

Strength of signaling through TCR appears to be an important factor in the induction of H4/ICOS expression, because a higher anti-CD3 concentration in the primary stimulation of C57BL/6 naive $CD4^+$ T cells gave rise to greater H4/ICOS expression (Fig. 2*A*). IL-2 production in the primary culture of C57BL/6 naive $CD4^+$ T cells was increased in a dose of anti-CD3 mAb-dependent manner, while IL-4 production was not substantially increased even at the highest concentration of anti-CD3 mAb (Fig. 2*B*). Taken together with the inhibitory effect of anti-IL-2 mAbs on the induction of H4/ICOS expression (Fig. 5*A*), which was much greater than that of anti-IL-4 mAbs (Fig. 4*A*), this up-regulation of H4/ICOS expression on C57BL/6-activated $CD4^+$ T cells was most likely induced through the IL-2 production increase. During the expansion of C57BL/6-activated $CD4^+$ T cells with IL-2, the level of H4/ICOS expression was markedly down-regulated (Fig. 2*A*). Thus, it is conceivable that IL-2 may be important in the induction of H4/ICOS expression on activated $CD4^+$ T cells, but is not effective in maintaining the induced level of H4/ICOS expression.

CD28 costimulation has a strong effect on the difference in H4/ICOS expression between BALB/c and C57BL/6 (Fig. 3*B*). However, when CD28 costimulation was blocked with CTLA-4-mIgs or immobilized anti-CD3 mAbs alone were used in the stimulation of naive $CD4^+$ T cells, the differences in the level of H4/ICOS between BALB/c- and C57BL/6-activated $CD4^+$ T cells were still observed (Fig. 3*B*), suggesting that other mechanisms may also be involved in the difference in H4/ICOS expression. The addition of IL-2 in the primary stimulation of BALB/c naive $CD4^+$ T cells increased IL-4 and IFN-γ productions from activated $CD4^+$ T cells and their H4/ICOS expressions, whereas the addition of IL-2 in C57BL/6 naive $CD4^+$ T cells did not affect the levels of cytokine production and H4/ICOS expression in activated $CD4^+$ T cells (Fig. 7). Several lines of evidence indicate that uncommitted precursor cells that secrete IL-2 differentiate into Th1 or Th2 cells (50, 51). One possibility would be that IL-2 secreted from such cells in the early phase of the immune response induces IL-4 production, and triggers a pathway linking IL-4 to H4/ICOS, as described above. C57BL/6 mice may be less efficient, compared with BALB/c mice, in terms of IL-2-induced IL-4 production during primary stimulation of naive $CD4^+$ T cells, resulting in the Th1 skewness and down-regulation of H4/ICOS expression. Thus, the efficiency in IL-2-induced IL-4 production in addition to the level of CD28 costimulation may be crucial for determining the difference in H4/ICOS expression on activated $CD4^+$ T cells between these two strains. Further study is now in progress to clarify this possibility. Finally, because the amount of IL-2 and IL-4 in the primary culture of OVA-specific, DO11.10 TCR-$\alpha\beta$-transgenic naive $CD4^+$ T cells with OVA peptides and APCs also affected H4/ICOS expression on activated $CD4^+$ T cells (data not shown), the above regulatory pathway appears to function in Ag-specific T cell responses as well.

In conclusion, the present study showed IL-4 to be a key factor in the regulation of H4/ICOS expression because it maintains the level of expression on activated $CD4^+$ T cells. Assuming that increased expression of H4/ICOS on IL-4-producing cells is not only a consequence, but most likely promotes further generation of IL-4-producing cells, a pathway linking IL-4 to H4/ICOS appears to have an important role in Th2 development. IL-2 may be crucial for triggering this pathway. This scenario suggests that manipulation of H4/ICOS expression may be a therapeutic approach to alleviating Th2-mediated diseases such as asthma and atopy. To achieve this end, further studies must be performed to elucidate the roles of cytokines in the regulation of H4/ICOS expression.

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