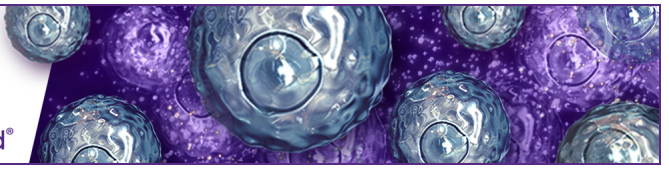

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Direct and Indirect Inhibition of Th1 Development by Progesterone and Glucocorticoids[1]

Hideki Miyaura*† and Makoto Iwata[2]*

Progesterone may contribute to the maternal suppression of immunity to the fetus by modulating the Th1/Th2 balance. To clarify whether progesterone directly or indirectly affects T cell differentiation, we used two experimental systems with isolated T cells in vitro. In one system, isolated $CD4^+CD8^+$ thymocytes differentiated into Th1 and Th2 by two pulse stimulations with defined combinations of ionomycin and PMA followed by the treatment with IL-12, IL-4, and IL-2. In the second system, functional differentiation was induced in purified naive CD4 T cells with cytokines and Abs to CD3 and CD28. In both systems, progesterone added with cytokines suppressed Th1 development at concentrations associated with pregnancy, but enhanced the development of IL-10-producing Th2 cells. Because IL-10 is known to inhibit APC production of IL-12, Th1 development may be also suppressed indirectly by progesterone. However, progesterone failed to enhance IL-10 production in the absence of IL-12. The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 inhibited Th1 development and enhanced Th2 development, as did progesterone, indicating that p38 MAPK and extracellular signal-regulated kinase pathways are involved in Th1 development. However, the progesterone effects may not be simply due to a modulation of MAPK activities, because the inhibitor did not significantly affect the development of IL-10-producing cells in the presence or absence of progesterone. Glucocorticoids exerted effects similar to those of progesterone on Th1/Th2 development even at lower concentrations. These results suggest that progesterone as well as glucocorticoids directly inhibit Th1 development and enhance Th2 development. ***The Journal of Immunology*****, 2002, 168: 1087–1094.**

The fetus bears Ags derived from its paternal chromosomes but is not rejected by the mother during normal pregnancy. To explain how the mother develops immunological tolerance to the fetus, various mechanisms have been proposed, such as 1) regulation of fetal MHC expression and a potential role of the nonclassical MHC class I molecule, HLA-G, on fetal cells (1), 2) suppression of maternal allospecific T cell activation in the decidua by indoleamine 2,3-dioxygenase, an enzyme that catabolizes tryptophan (2), and 3) a maternal shift from Th1-type cell-mediated immunity to Th2-type humoral immunity (3). The shift toward the Th2 responses may be influenced by PGE_2 produced by the placenta (4, 5) or by selective induction of apoptosis in fetal Ag-specific Th1 (6). It has been proposed that progesterone also plays an important role in the maternal shift of the Th1/Th2 balance (7).

Progesterone is known to be essential for the establishment and maintenance of pregnancy including ovulation, uterine, and mammary gland development (8). The major sources of progesterone during pregnancy are the corpus luteum of the ovary and, in many species including humans and rodents, the placenta (9). The serum progesterone concentration increases up to 0.3 μM during pregnancy in mice (10–13). However, in the placenta where the fetal Ags are directly exposed to the mother's immune system, progesterone is synthesized in large quantities, and its concentration reaches much higher levels, e.g., 1–10 μM in humans (9). Progesterone binds to two progesterone receptors (PR),[3] PRA and PRB, which are products of a single gene (14). However, the cellular and molecular mechanisms of progesterone effects on T cell differentiation are vastly unknown. Thus, we first examined whether progesterone directly affects T cell differentiation into Th1 or Th2. We used two experimental systems with isolated T cells in the absence of other cell types in vitro. In one system, we took advantage of our in vitro thymocyte differentiation system (15, 16). Differentiation and survival of isolated $CD4^+CD8^+$ thymocytes to Th1 and Th2 cells were induced by two pulse stimulations with proper combinations of the calcium ionophore ionomycin and the protein kinase C activator PMA, followed by cytokine treatment in the presence or absence of progesterone. In the second system, Th1 or Th2 was induced from isolated naive T cells of normal mice with Abs to CD3 and CD28 together with cytokines in the presence or absence of progesterone. We found that progesterone directly suppressed T cell differentiation into Th1 but enhanced differentiation into IL-10-producing Th2 cells in both systems. Furthermore, we found that glucocorticoids exerted similar effects.

Materials and Methods

Mice and reagents

Male MHC class I and class II double knockout (DKO) mice (C57BL/6 deficient in $A\beta^b$ and β_2-microglobulin) were obtained from Taconic Farms (Immuno-Biological Laboratories, Gunma, Japan). Male C57BL/6 mice (4–6 wk of age) were obtained from Charles River Laboratories (Yokohama, Japan). The mice were kept in our animal facility for at least 1 wk before use. Progesterone, corticosterone, mifepristone (RU-486), and PMA were obtained from Sigma-Aldrich (St. Louis, MO). Dexamethasone was

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[3] Abbreviations used in this paper: PR, progesterone receptor; DKO, double knockout; GR, glucocorticoid receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase.

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obtained from Wako (Osaka, Japan). Ionomycin and SB203580 were obtained from Calbiochem (La Jolla, CA). U0126 was obtained from Promega (Tokyo, Japan). Mouse rIL-2, mouse rIL-4, and mouse rIL-12 were obtained from Genzyme (Cambridge, MA). To express IL-2 and IL-4 activities, U was used by calculating as 1 U/ml = 1 ED_{50} according to the manufacturer's definition of ED_{50}.

Thymocyte culture

For induction of in vitro differentiation of thymocytes into CD4 T cells and Th1/Th2 cells, DKO mouse thymocytes were used. T cell differentiation is arrested at the $CD4^+CD8^+$ stage in the thymus of these mice, and most of the thymocytes are $CD4^+CD8^+$ (17). Thymocytes ($3.75–4 \times 10^6$) were suspended in 1 ml of DMEM supplemented with 10% heat-inactivated FCS (Intergen, Purchase, NY), 3 mM L-glutamine, 1 mM sodium pyruvate, 1× MEM nonessential amino acids, 50 μM 2-ME, 20 mM HEPES (pH 7.2), 20 U of penicillin, and 20 μg of streptomycin (complete DMEM) and were cultured with 0.2 μg/ml ionomycin and 0.2 ng/ml PMA for 20 h at 37°C in 24-well tissue culture plates (25820; Corning Glass, Corning, NY) (Fig. 1, step A). Each lot of FCS was selected by its low toxicity to $CD4^+CD8^+$ thymocytes. After culture, the stimulated cells were washed twice with fresh medium and were further cultured in the same volume of complete DMEM without the stimuli to induce $CD4^+CD8^{low/-}$ cells committed to the CD4 T cell lineage (step B) (15). For the secondary stimulation, the cells (5×10^6 cells per ml) were cultured with 0.2 μg/ml ionomycin and 3 ng/ml PMA for 16 h (step C). After culture, the cells were washed twice and further cultured at 1×10^6 cells/ml in complete DMEM containing 40–100 U/ml mouse rIL-2 in the presence of the indicated concentrations of mouse rIL-4 and/or mouse rIL-12 for 3–5 days (step D) (16). For cultures longer than 4 days, cells were expanded 2-fold on the fourth day after the start of cytokine treatment in the continued presence of cytokines. To assess the functional differentiation of the cells to Th1 or Th2, intracellular cytokines or secreted cytokines upon stimulation were detected.

Naive T cells and culture conditions

Splenic CD4 T cells were obtained from C57BL/6 mice by using Dynabeads Mouse CD4 and DetachaBead Mouse CD4 (Dynal Biotech, Oslo, Norway) and were stained with FITC-labeled anti-CD44 mAb (IM7) and PE-labeled anti-CD45RB (16A) (BD PharMingen, San Diego, CA). Naive CD4 T cells ($CD44^{low}CD45RB^{high}$) were isolated by sorting with a FACStar^{Plus} and Consort 30 software program (Nippon Becton Dickinson, Tokyo, Japan). Sorted naive CD4 T cells were plated at a density of 5×10^5 cells/ml in 24-well suspension culture plates (Sumitomo Bakelite, Tokyo, Japan) that had been coated with 3 μg/ml anti-CD3 mAb (145-2C11) (18) and 1 μg/ml anti-CD28 mAb (37.51; BD PharMingen). Cytokines and reagents were added to wells at the initiation of culture as indicated. After 5–8 days, cells were harvested and washed twice and were restimulated for measurement of cytokine production.

Measurement of cytokine production

To assess cytokine secretion into the medium, cells were suspended in fresh medium for 2 days in 24-well suspension culture plates that had been coated with 3 μg/ml anti-CD3 mAb and 3 μg/ml anti-CD28 mAb. The culture supernatants were assessed for cytokine concentrations with OptEIA mouse cytokine kits (BD PharMingen).

To assess intracellular cytokines, the cells were stimulated with 0.4 μg/ml ionomycin and 10 ng/ml PMA for 5 h, and monensin was added for the last 2 h of the culture to inhibit intracellular transport processes. After culture, cells were treated with anti-CD16/32 (FcgIII/IIR) mAb (2.4G2) to block FcR and then washed. The cells were then fixed and permeabilized with a Cytofix/Cytoperm kit (BD PharMingen) and stained with FITC-labeled anti-IFN-γ mAb (XMG1.2), PE-labeled anti-IL-4 mAb (11B11), PE-labeled anti-IL-10 mAb (JES5-16E3), and/or FITC-labeled anti-IL-10 mAb (BD PharMingen). The stained cells were analyzed for fluorescence levels by FACS.

FACS analysis of surface markers

The cells were stained with labeled Abs to PE-conjugated mAb to CD4 (RM4-5) or CD45RB and/or FITC-labeled mAb to CD8 (53-6.7) or CD44 mAb (IM7) (BD PharMingen). Intact cells were gated by using forward and side scatters with a FACScan flow cytometer and FACScan Research Software (BD Biosciences, Lincoln Park, NJ) and were analyzed for marker expression. The gate for viable cells was determined using propidium iodide exclusion and Paint-a-Gate software (Nippon Becton Dickinson).

Results

Progesterone influences the functional differentiation of T cells into Th1 and Th2

To examine the direct effect of progesterone on T cell differentiation, we first used $CD4^+CD8^+$ thymocytes from DKO mice as starting cells. The in vitro differentiation of thymocytes easily provided $>10^8$ T cells per mouse, ready for differentiation into Th1 or Th2 (16). As shown in Fig. 1, suspended thymocytes were transiently stimulated twice with ionomycin/PMA followed by culture with IL-2 and IL-4 and/or IL-12 in the presence of graded concentrations of progesterone (step D) for 3–5 days. IL-4 and IL-12 are required to induce Th2 and Th1 cells, respectively, from naive T cells (19). The cultured cells were washed and restimulated with plate-bound Abs to CD3 and CD28 to assess their ability to produce IFN-γ, IL-4, and IL-10. Progesterone inhibited IFN-γ production dose dependently in the presence or absence of IL-4 (Fig. 2, *A* (▲ and ○) and *D*), but its effect was not significant in the presence of 0.1 ng/ml or higher concentrations of IL-12 (Fig. 2*A*, ■, and data not shown). Progesterone moderately enhanced IL-4 production (Fig. 2*E*), but progesterone at 10 μM in some experiments of 3 days or shorter of step D culture failed to show enhancement (Fig. 2*B*). IL-10 production was dose dependently enhanced by progesterone in the presence of 0.01–1 ng/ml IL-12 with or without IL-4 (80–100 U/ml) (Fig. 2, *C* (■) and *F*; and data not

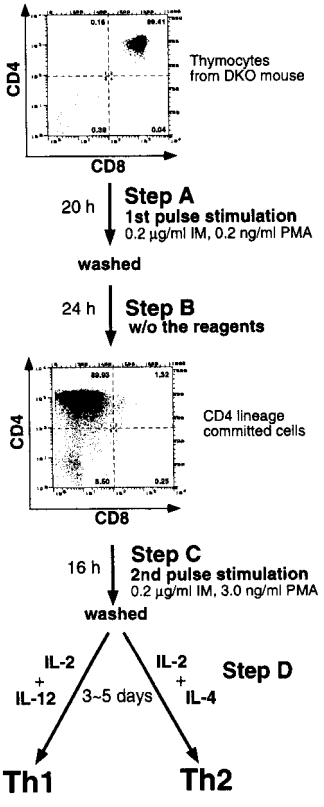

FIGURE 1. A schematic representation of the in vitro thymocyte differentiation system, in which $CD4^+CD8^+$ thymocytes differentiate into $CD4^+CD8^-$ T cells and subsequently into Th1 or Th2 cells. DKO mouse thymocytes were stimulated with 0.2 μg/ml ionomycin (IM) and 0.2 ng/ml PMA for 20 h (step A), washed, and cultured without the reagents for 24 h (step B). The cells became $CD4^+CD8^{low/-}$ and committed to the CD4 T cell lineage. These cells were further stimulated with 0.2 μg/ml ionomycin and 3 ng/ml PMA for 16 h (step C), washed, and incubated for 3–5 days with IL-2 in the presence or absence of IL-12 or IL-4 to induce functional differentiation into Th1 or Th2, respectively (step D). In some experiments, both IL-12 and IL-4 were added to induce both Th1 and Th2.

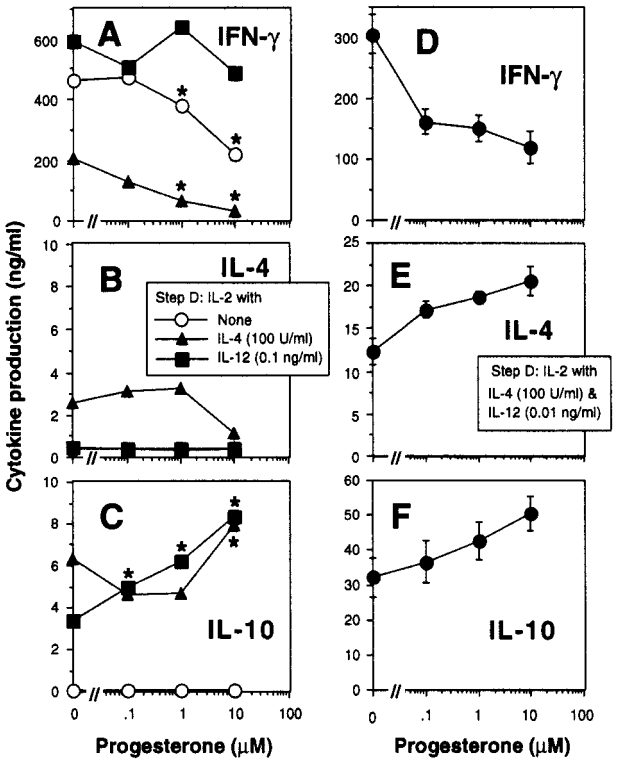

FIGURE 2. Effects of progesterone on the functional differentiation of cultured thymocytes into Th1 and Th2. Differentiation was induced in DKO mouse thymocytes in vitro as shown in Fig. 1, except that graded concentrations of progesterone were added with cytokines to the step D culture for 3 or 4 days. *A–C*, Graded concentrations of progesterone were added to the step D culture in the presence of IL-2 (50 U/ml; ○), IL-2 plus IL-4 (100 U/ml; ▲), or IL-2 plus IL-12 (0.1 ng/ml; ■). *D–F*, Graded concentrations of progesterone were added to the step D culture in the presence of IL-2 (50 U/ml), IL-4 (100 U/ml), and IL-12 (0.01 ng/ml). After 3 (*A–C*) or 4 days (*D–F*) of culture, the cells were collected, washed, and restimulated with plate-bound mAbs to CD3 and CD28. Culture supernatants were collected after 48 h of culture and were assessed for concentrations of IFN-γ, IL-4, and IL-10 by ELISA. The data in *D–F* are expressed as means ± SD of triplicate cultures. The recovery of viable cells after the step D cultures in the presence of 10 μM progesterone was 80.1 ± 13.5% of that in the absence of progesterone. For *A–C*, percentage of control was calculated for each cytokine production in repeated experiments, and statistical analysis was performed with Student's *t* test. Asterisks indicate that the inhibition or enhancement by progesterone is statistically significant ($p < 0.05$) in repeated experiments. A representative result of four (*A–C*) or two (*D–F*) sets of independent experiments is shown.

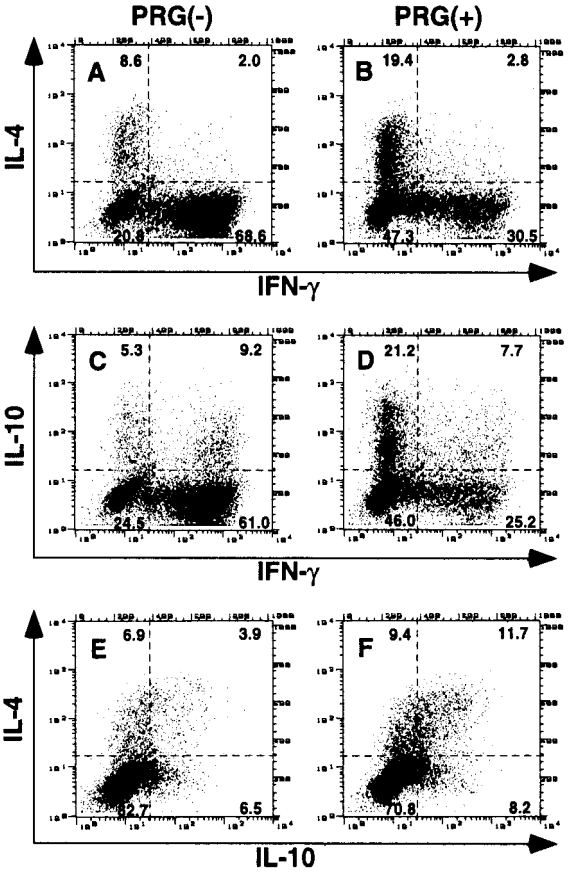

FIGURE 3. Intracellular expression of cytokines in T cells cultured with or without progesterone. Differentiation was induced in DKO mouse thymocytes in vitro as shown in Fig. 1, except that in step D cells were cultured in the presence of IL-2 (50 U/ml), IL-4 (100 U/ml), and IL-12 (0.01 ng/ml) with or without 10 μM progesterone (PRG) for 5 days. The cells were then restimulated with ionomycin/PMA for 5 h, and monensin was added for the last 2 h of culture. The expressions of IFN-γ and IL-4 (*A* and *B*), IFN-γ and IL-10 (*C* and *D*), and IL-10 and IL-4 (*E* and *F*) were determined by FACS analysis after fixing and permeabilizing the cells. A representative result of three experiments is shown.

shown). In the absence of both IL-4 and IL-12, the development of IL-10-producing cells was not induced in the presence or absence of progesterone. However, in the presence of IL-4 without IL-12, progesterone at only 10 μM moderately enhanced IL-10 production (Fig. 2*C*, ▲).

Intracellular cytokine expression in the cells after the step D culture was also assessed after 5 h of restimulation with ionomycin/PMA instead of Abs to CD3 and CD28. Intracellular expression of IL-4 was consistently enhanced by progesterone (Fig. 3). Progesterone also caused a decrease in IFN-γ-expressing cells and an increase in IL-10-expressing cells. Most of the IL-10-expressing cells induced in the presence of progesterone also expressed IL-4 (Fig. 3*F*). These findings indicate that progesterone directly inhibits T cell differentiation into Th1 and enhances T cell differentiation into IL-10-producing Th2. However, in the presence of high concentrations of IL-12, progesterone may not overcome the effect of IL-12 to induce Th1, but it can enhance the development of IL-10-producing cells.

The induction of IL-4- and IL-10-producing cells is significantly enhanced in the late stage of culture with progesterone

Kinetic changes in cytokine productivity of the cultured thymocytes were studied. After two pulse stimulations with ionomycin/PMA, the cells were cultured for graded periods in the presence of both IL-4 (100 U/ml) and IL-12 (0.01 ng/ml) to induce both Th1 and Th2, and the effect of 10 μM progesterone was analyzed. In the absence of progesterone, IFN-γ production assessed by stimulation with Abs to CD3 and CD28 quickly increased during 3 days of culture and thereafter moderately decreased, whereas progesterone suppressed IFN-γ production throughout the culture period (Fig. 4*A*). The IL-4 production increased after 1 days of culture and peaked after 3 days. Thereafter, IL-4 production decreased quickly, but progesterone moderated the decrease (Fig. 4*B*). IL-10 production increased and reached a plateau after 2 days of culture in the absence of progesterone. However, in the presence of progesterone, IL-10 production continued to increase (Fig. 4*C*). Intracellular cytokine staining indicated that the majority of IL-10-expressing cells were also expressing IL-4 after 5 days of culture with progesterone (data not shown).

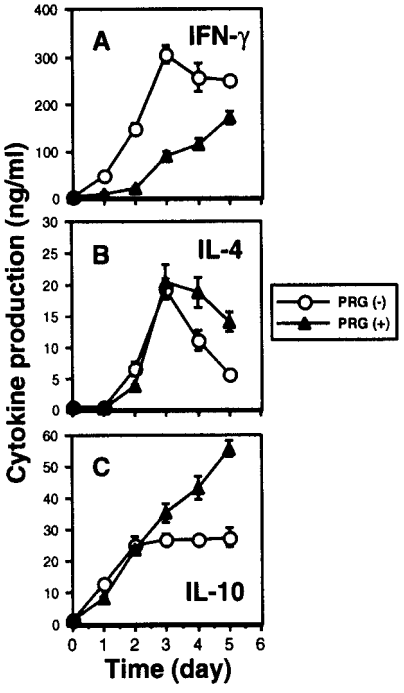

FIGURE 4. Kinetic changes in the cytokine production of the cultured thymocytes. DKO mouse thymocytes were transiently stimulated twice as shown in Fig. 1 and cultured with or without 10 μM progesterone in step D for 0–5 days. The cells were collected, washed, and restimulated for 48 h with plate-bound Abs to CD3 and CD28. Culture supernatants were then assessed for the concentrations of IFN-γ (*A*), IL-4 (*B*), and IL-10 (*C*). Vertical bars in each panel represent SD in triplicate cultures.

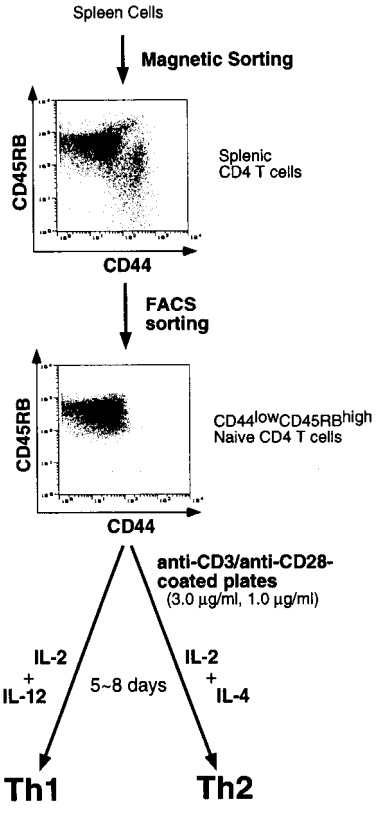

FIGURE 5. A schematic representation of the in vitro naive T cell differentiation system. CD4 T cells were purified from normal mouse spleens by magnetic sorting, followed by removal of anti-CD4 mAb from the cells with an anti-idiotypic Ab to the anti-CD4. Naive T cells were collected from the CD4 T cells by FACS sorting of $CD44^{low}CD45RB^{high}$ cells and were stimulated with plate-bound Abs to CD3 and CD28 in the presence of IL-2 and IL-12 and/or IL-4 to induce functional differentiation of the cells into Th1 and/or Th2.

Based on these results, we examined whether progesterone exerts similar effects on the functional development of normal naive T cells into Th1 and Th2. Naive T cells were obtained from normal mouse spleens and were stimulated to induce functional differentiation as shown in Fig. 5. Progesterone dose dependently inhibited naive T cells from differentiating into IFN-γ-producing cells and promoted their differentiation into IL-4-producing cells (Fig. 6). Progesterone slightly enhanced IL-10 production by the cells after 5 days of culture (Fig. 6*C*), but kinetic studies indicated that the enhancement of IL-10 production became significant after 8 days of culture in this condition (Fig. 6*F* and data not shown).

IL-10 is known to inhibit APCs, including macrophages and dendritic cells, from producing IL-12, and thus it inhibits Th1 differentiation in vivo (20, 21). Because we used isolated T cells essentially free from APCs or other cell types in the two experimental systems, it is unlikely that the inhibition of Th1 development was dependent on IL-10 production. Indeed, the addition of anti-IL-10 mAb into the culture failed to affect the progesterone-induced changes in cytokine productivity assessed by intracellular and secreted levels of IFN-γ, IL-4, and IL-10 (data not shown).

We also examined the effect of progesterone on Th1 or Th2 development in the skewed condition to induce either one of them in the presence of IL-12 or IL-4. Progesterone significantly suppressed IFN-γ production at 0.3 and 10 μM in both culture conditions (Fig. 6, *G* and *J*; $p < 0.02$ or 0.001), although percent inhibition in the presence of 0.1 ng/ml IL-12 was lower than that in the absence of IL-12. Progesterone at 10 μM but not 0.3 μM enhanced both IL-4 and IL-10 production when the cells were cultured with IL-12 ($p < 0.05$) but not IL-4 (Fig. 6, *H*, *I*, *K*, and *L*).

These results collectively suggest that progesterone directly suppresses T cell differentiation into Th1 and that it directly enhances T cell differentiation into IL-10/IL-4-producing Th2 at a relatively late stage. IL-10 productivity appears to be affected with the concentrations of IL-4 and IL-12.

Corticosterone exerts effects similar to those of progesterone on T cell differentiation

Because progesterone is known to cross-react significantly with glucocorticoid receptors (GR) (22, 23), we examined whether glucocorticoids might exert similar effects on T cell differentiation. As shown in Fig. 7, the major glucocorticoid in mice, corticosterone, dose dependently suppressed cultured thymocyte differentiation into IFN-γ-producing cells and enhanced their differentiation into IL-4- and IL-10-producing cells. The induction of cells expressing IL-4 but not IL-10 was suppressed, but that of the cells expressing both IL-4 and IL-10 was significantly enhanced (Fig. 7*B*). The proportion of the cells expressing IL-10 but not IL-4 was small; however, induction of these cells was also enhanced by corticosterone. Similar results were obtained with the synthetic glucocorticoid dexamethasone at 0.01 μM (data not shown). Corticosterone was 10–100 times more effective than progesterone (Fig. 7 and data not shown). IL-4 production was significantly increased in the cells treated with 1 μM corticosterone (Fig. 7*C*), but the intracellular IL-4-expressing cells were decreased (Fig. 7*A*), indicating that the IL-4 production per IL-4-expressing cell increased. In naive T cells, similar results were obtained, although corticosterone at concentrations of 0.01–0.1 μM somewhat suppressed IL-10 production (Fig. 7*D*). Nonetheless, the results collectively suggest

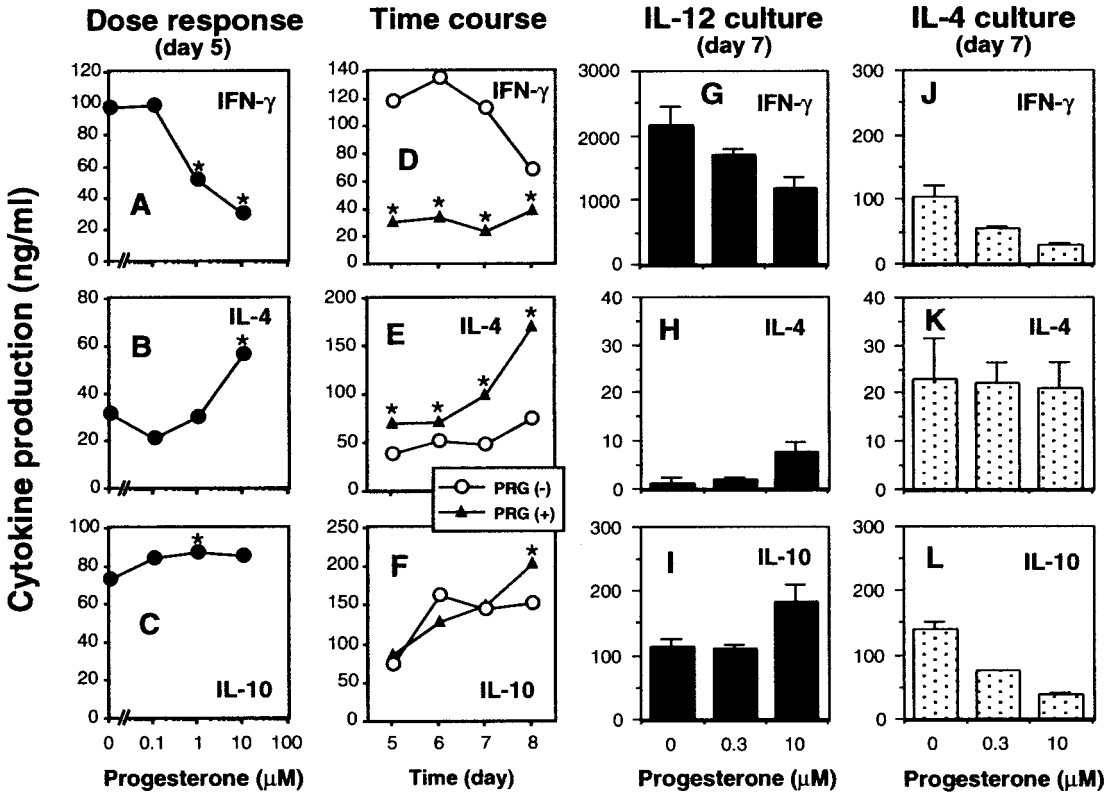

FIGURE 6. Progesterone directly inhibits naive T cell differentiation into IFN-γ-producing cells but enhances differentiation into IL-4- and IL-10-producing cells. *A–F*, Naive T cells were purified and stimulated as shown in Fig. 5, except that graded concentrations of progesterone were included in the culture for 5 days in the presence of IL-12 (0.01 ng/ml), IL-4 (100 U/ml), and IL-2 (50 U/ml) (*A–C*) or that 10 μM progesterone was added to the culture for 5–8 days in the presence of the same combination of cytokines (*D–F*). A representative result of three experiments is shown. Asterisks indicate that the inhibition or enhancement by progesterone is statistically significant ($p < 0.05$) in repeated experiments. *G–L*, Naive T cells were cultured as shown in Fig. 5, but with IL-12 (0.1 ng/ml) and IL-2 (50 U/ml) (IL-12 culture; *G–I*) or with IL-4 (100 ng/ml) and IL-2 (50 U/ml) (IL-4 culture; *J–L*) for 7 days in the presence or absence of 0.3 or 10 μM progesterone. The cells were then restimulated with plate-bound Abs to CD3/CD28 for analyzing cytokine productivity. Vertical bars in each panel represent SD in triplicate cultures.

that corticosterone exerts effects similar to those of progesterone on T cell differentiation, but more strongly.

The effects of progesterone on T cell differentiation are not simply due to direct modulation of p38 MAPK or ERK

Involvement of mitogen-activated protein kinases (MAPKs) has been suggested in Th1 and Th2 differentiation (24–26). Thus, we compared the effects of MAPK inhibitors on T cell differentiation with those of progesterone. The p38 MAPK inhibitor SB203580 (27) suppressed cell differentiation into IFN-γ-producing cells and enhanced differentiation into IL-4-producing cells (Fig. 8). In contrast, the MAPK kinase (MEK) inhibitor U0126 (28) suppressed the cell differentiation into IL-4-producing cells and enhanced differentiation into IFN-γ-producing cells (Fig. 8). The findings indicate that p38 MAPK and extracellular signal-regulated kinase (ERK) pathways are indeed involved in Th1 and Th2 development, respectively, and that the effects of SB203580 on the development of IFN-γ-producing cells and IL-4-producing cells resemble those of progesterone. Progesterone synergized with SB203580 to suppress the development of IFN-γ-producing cells but suppressed the SB203580 enhancement of development of IL-4-producing cells (Fig. 8). Furthermore, SB203580 neither affected the development of IL-10-producing cells by itself nor synergized with progesterone to enhance the development of IL-10-producing cells (Fig. 8*C*). Thus, it is unlikely that the progesterone effects are simply due to inhibition of p38 MAPK activity.

U0126 also failed to significantly affect the development of IL-10-producing cells in the presence or absence of progesterone (Fig. 8*C*). The MEK1 inhibitor PD 98059 exerted effects similar to, but weaker than, those of U0126 (data not shown). The results suggest that the effects of progesterone on T cell differentiation were not simply due to direct modulation of p38 MAPK or ERK activity.

Discussion

A normal pregnancy involves a shift in immunity toward Th2-type responses. In fact, pregnancy impairs mouse resistance to *Leishmania major* infection by down-regulation of Ag-specific Th1 responses and up-regulation of Th2 cytokine expression (3). Pregnancy improves the symptoms of rheumatoid arthritis, a disease driven primarily by Th1 immune responses, whereas systemic lupus erythematosus, a disease linked to excess Th2 cytokine production, tends to flare during pregnancy (29, 30). Furthermore, estradiol and estriol at concentrations typical of pregnancy can cause an increase in IL-10 production by human CD4 T cells (31). However, the adaptive immune responses during normal pregnancy might not be always skewed toward Th2-type responses. Maternal T cells acquire a transient state of tolerance mainly specific for paternal alloantigens (32, 33). The Th1 to Th2 shift is likely to occur upon T cell Ag recognition at the maternal-fetal interface. Progesterone may be especially important for the Th1 to Th2 shift at the interface during pregnancy, because the free concentration of progesterone in the placenta can be much higher than

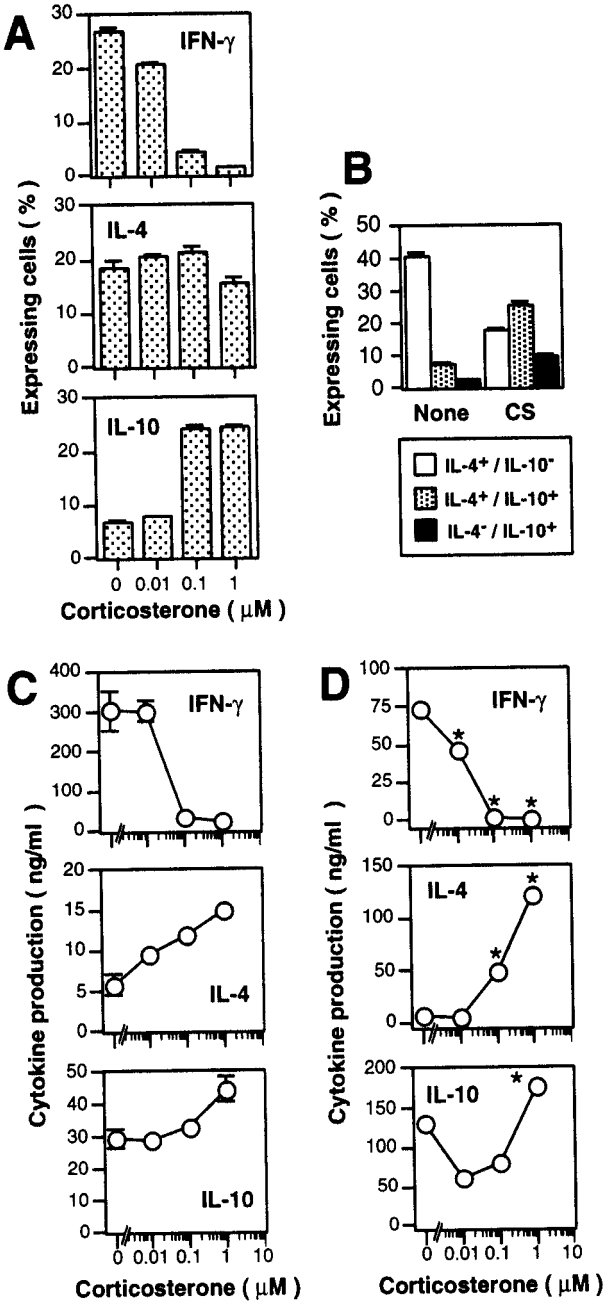

FIGURE 7. The glucocorticoid corticosterone inhibits T cell differentiation into IFN-γ-producing cells and enhances differentiation into IL-4/IL-10-producing cells. Functional differentiation was induced in DKO mouse thymocytes (*A–C*) or in naive T cells (*D*). DKO thymocytes were transiently stimulated twice with ionomycin/PMA as shown Fig. 1, except that graded concentrations of corticosterone (CS) were added into the step D culture in the presence of IL-2 (50 U/ml), IL-12 (0.01 ng/ml), and IL-4 (80 U/ml) for 3 days. The cells were washed and restimulated with ionomycin/PMA or plate-bound Abs to CD3 and CD28 for analyzing intracellular expression (*A* and *B*) or production (*C*) of IFN-γ, IL-4, and IL-10. *B*, Proportions of IL-4-expressing but not IL-10-expressing cells (open bars), IL-4- and IL-10-expressing cells (hatched bars), or IL-10-expressing but not IL-4-expressing cells (filled bars) were calculated. Vertical bars in each panel represent SD in triplicate cultures. *D*, Naive T cells were stimulated as shown Fig. 5, except that graded concentrations of corticosterone were included in the culture in the presence of IL-2 (50 U/ml), IL-12 (0.01 ng/ml), and IL-4 (100 U/ml) for 5 days. The cells were then restimulated with plate-bound Abs to CD3/CD28 for analyzing cytokine productivity. Asterisks indicate that the inhibition or enhancement by progesterone is statistically significant ($p < 0.05$) in repeated experiments. A representative result of three experiments is shown.

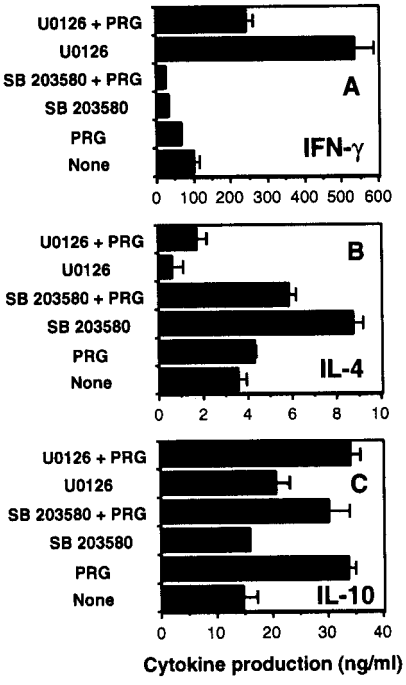

FIGURE 8. The p38 MAPK inhibitor SB203580 suppresses T cell differentiation into Th1 and enhances differentiation into Th2, whereas the MEK inhibitor U0126 enhances differentiation into Th1 and suppresses differentiation into Th2. DKO mouse thymocytes were stimulated twice as shown in Fig. 1, except that 5 μM SB203580 or 5 μM U0126 was added to step D culture with or without 10 μM progesterone in the presence of IL-12 (0.01 ng/ml), IL-4 (100 U/ml), and IL-2 (100 U/ml) for 3 days. The cells were collected, washed, and restimulated for 48 h with plate-bound Abs to CD3 and CD28. Culture supernatants were then assessed for the concentrations of IFN-γ (*A*), IL-4 (*B*), and IL-10 (*C*). The data are expressed as means ± SD of triplicate cultures. A representative result of four experiments is shown.

that in the maternal or fetal plasma (9). In previous reports, the effect of progesterone on Th1/Th2 differentiation has been studied in vivo or in vitro with a mixture of T cells and other cell types including APCs, and thus it remained to be determined whether the effect is exerted through a direct or indirect mechanism. We have shown here that the local progesterone levels found at the human placenta could directly affect T cell differentiation in the absence of other types of cells and that Th1 development was significantly suppressed by progesterone at concentrations observed in sera during pregnancy.

The reagent RU-486 is known to be an antagonist of PR and GR (34). However, RU-486 itself at 1 and 10 μM exerted similar effects to progesterone and corticosterone in our experimental systems (data not shown). Because RU-486 has predominantly glucocorticoid agonist actions in several tissues (35, 36), RU-486 might exert an agonist effect on T cell differentiation via GR or PR. PR expression appears to be low in T cells (23, 37, 38) but increased upon exposure to progesterone and probably with TCR-mediated stimulation (39). Indirect effects of progesterone on T cells through binding to PR in other cell types may also be possible, as reported in other situations. The indirect mechanism is involved in the hormonally controlled accumulation and survival of uterine mucosal NK cells, because neither PR nor estrogen receptors were detected in these NK cells (37). PR in thymus is localized in reticuloepithelial cells (38) and is required for normal fertility, probably through a PR-dependent paracrine mechanism that blocks T cell lymphopoiesis during pregnancy (40). Progesterone has one-third the affinity exhibited by corticosterone for GR

(22), whereas corticosterone has a very high affinity for its own receptor ($K_d \approx 10^{-9}$ M) (22, 41). In contrast, corticosterone has a relative affinity 25-fold lower than progesterone for PR in rat placenta and in human mononuclear leukocytes (23, 42). GR is expressed in immature and mature T cells (43, 44). Thus, physiological concentrations of progesterone during pregnancy are sufficient to bind to GR as well. We have shown in this study that the glucocorticoid corticosterone can directly affect T cells to give a skewed differentiation into Th2 cells. Progesterone may exert its effect on the functional differentiation of T cells partly through GR. During the second half of pregnancy in mice, plasma corticosterone levels reach a peak of 4 μM on day 16, ~60 times the nonpregnant resting level (45). Most of the plasma corticosterone is of adrenal origin, and the remaining portion of the corticosterone is feto-placental in origin (45). It has been previously suggested that glucocorticoids indirectly affect T cell differentiation and skew it toward the Th2 type, because glucocorticoids reduce the secretion of the Th1-promoting factor IL-12 in LPS-stimulated dendritic cells (46) or because IL-4-producing NK1.1$^+$ T cells are more resistant to glucocorticoid-induced apoptosis than conventional T cells in vivo (47). These and present results collectively suggest that both progesterone and glucocorticoids directly and indirectly contribute to inducing a shift in differentiation of T cells into the Th2 type during pregnancy, particularly at the maternal-fetal interface. The placental levels of progesterone in mice may be lower than in humans (13) but may be supplemented by glucocorticoids in mice.

Induction of IL-10-producing T cells was also directly enhanced by progesterone and glucocorticoids. IL-10 is known to inhibit the production of IL-12 and the expression of MHC class II Ags or costimulatory molecules by macrophages, monocytes, or various types of dendritic cells (48). Furthermore, IL-10 treatment of dendritic cells contributes to a state of anergy in alloantigen-activated T cells (49, 50). Thus, progesterone and glucocorticoids are likely to inhibit Th1 differentiation also through up-regulating IL-10 production in vivo and may contribute to a state of anergy in paternal Ag-activated T cells. Most of the IL-10-expressing cells induced in the presence of progesterone or corticosterone in vitro were also expressing IL-4 (Figs. 3 and 7), indicating that these cells were not Tr1, which produce high levels of IL-10, normal levels of IL-5, and low levels of IL-2 and IL-4 (51), at least within the culture period in this study. The enhanced induction of IL-10-producing cells from naive T cells appeared to be influenced by several conditions. The progesterone concentration was necessary to be 1–10 μM, and the presence of IL-12 (0.01–0.1 ng/ml) was required (Fig. 6).

The molecular mechanism of the direct effect of progesterone and glucocorticoids on T cell differentiation remains unclear. Inhibition of IL-12-induced Stat4 phosphorylation may contribute to the effect of glucocorticoids (52). In the presence of progesterone, activated human lymphocytes, especially $\gamma\delta$ T cells, synthesize a 34-kDa molecule (progesterone-induced blocking factor) that inhibits NK activity and exerts an antiabortive effect in vivo (53). Progesterone-induced blocking factor may be responsible in part for the progesterone effect on T cells (53). Progesterone also plays a role in the induction of leukemia inhibitory factor, essential for embryo implantation, in the presence of IL-4, and the production of leukemia inhibitory factor and/or Th2 cytokines by decidual T cells contributes to the maintenance of pregnancy (54).

Our findings indicate that progesterone and glucocorticoids directly suppress T cell differentiation into Th1 and enhance IL-10-producing Th2. However, in the presence of high concentrations of the Th1-promoting factor IL-12, progesterone cannot suppress or less efficiently suppresses the Th1 differentiation that may cause abortive responses, but can enhance the induction of IL-10-producing cells. IL-10 may then indirectly inhibit Th1 differentiation.

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