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Carbon Monoxide Produced by Heme Oxygenase-1 Suppresses T Cell Proliferation via Inhibition of IL-2 Production

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Heme oxygenase-1 (HO-1) catalyzes heme into CO, biliverdin, and free iron and serves as a protective enzyme by virtue of its anti-inflammatory, antiapoptotic, and antiproliferative actions. Previously, we have demonstrated that human CD4+ T cells express HO-1 and that HO-1-overexpressing Jurkat T cells tend to display lower proliferative response. The aim of this study is to elucidate the mechanism(s) by which HO-1 can mediate its antiproliferative effect on CD4+ T cells. Among the three HO-1 byproducts, only CO showed suppressive effect on T cell proliferation in response to anti-CD3 plus anti-CD28 Abs, mimicking the antiproliferative action of HO-1. CO blocked the cell cycle entry of T cells, which was independent of the guanylate cyclase/cGMP pathway. CO also suppressed the secretion of IL-2, and this suppressive effect of CO on IL-2 secretion mediated the antiproliferative action of CO. CO selectively inhibited the extracellular signal-regulated kinase pathway, which could explain the suppressive effects of CO on T cell proliferation and IL-2 secretion, possibly via its inhibition of extracellular signal-regulated kinase activation. The Journal of Immunology, 2004, 172: 4744–4751.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the conversion of heme to CO, free iron, and biliverdin, the latter being reduced to bilirubin by biliverdin reductase. HO-1 is induced by a variety of stimuli in many types of cells, including human CD4+ T cells (1). Several studies have demonstrated anti-inflammatory properties of HO-1 (2, 3), the existence of which is further supported by the fact that HO-1-deficient mice develop a progressive chronic inflammatory state (4, 5) and that a human lacking HO-1 enzymatic activity died of an inflammatory syndrome (6).

After the receipt of signals from APCs through TCR and CD28 costimulator, CD4+ T cells are triggered to produce IL-2 and enter the cell cycle. During or after several days of rapid cell division, these cells differentiate into one of two classes of effector CD4+ T cells (Th1 and Th2 cells). It is generally accepted that activation and subsequent proliferation of resting CD4+ T cells are essential processes in the T cell-mediated immune response and that they are associated with increased release of IL-2 (7).

The mitogen-activated protein (MAP) kinase cascade is one of the most ancient and evolutionarily conserved signaling pathways, which is also important for many processes occurring in immune responses. Three major groups of MAP kinases have been described in mammalian cells: they are the extracellular signal-regulated kinase (ERK), the Jun NH2-terminal kinase (JNK), and the p38 kinase (8). ERK, JNK, and p38 pathways are rapidly up-regulated by engagement of the TCR in T cells and play a critical role in the events leading to activation and increased IL-2 secretion (8).

There are two isoforms of ERK, ERK-1 and -2. They can be activated by MAP/ERK kinase (MEK). ERK activation is dependent on p56lck and coupling of the TCR/CD3 complex to p21ras, with subsequent activation of the Raf-1/MEK/ERK kinase cascade (8, 9). JNK activation also requires p21ras and signals generated by the CD28 costimulatory receptor (10). The activation of the Raf-1/MEK/ERK pathway is essential for induction of IL-2 transcription in T cells (11). After phosphorylation of c-jun by JNK, activated c-fos and c-jun combine to form the AP-1 protein required for IL-2 synthesis (12). Interestingly, deficient ERK and JNK activations have been reported to exist in clones that are anergized (13). However, there is also evidence that ERK inhibition alone suppressed T cell proliferation, but did not induce anergy (14).

CO, a reaction product of HO-1 activity, has been shown to be highly protective in several rodent disease models (15, 16). It has anti-inflammatory, antiapoptotic, and antiproliferative effects (17), thereby conferring, at least in part, the protective effects of HO-1. Furthermore, the MAP kinase pathway has been shown to mediate the biological effects of CO: the p38 MAP kinase activation mediates the cytoprotective effect of CO on ischemia-reperfusion lung injury (18). CO prevents glucose deprivation-induced cytotoxicity through ERK MAP kinase inactivation in rat hepatocytes (19), and the antiproliferative effect of CO on smooth muscle cells and human airway smooth muscle cells requires p38 MAP kinase activation (20) and ERK MAP kinase inactivation (21), respectively.
We have recently demonstrated that human CD4+ T cells express HO-1 and that HO-1-overexpressing Jurkat T cells tend to display lower proliferative response (1). The aim of this study, therefore, was to elucidate the mechanism(s) by which HO-1 could mediate its antiproliferative effect on CD4+ T cells, and we demonstrated that CO, a reaction product of HO-1, could suppress IL-2 secretion, probably by inhibiting ERK activation, and thereby resulted in suppression of T cell proliferation in response to anti-CD3 plus anti-CD28 Abs.

Materials and Methods

**Reagents, cytokines, and Abs**

RPMA 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Grand Island, NY), and 10% FBS (HyClone Laboratories, Logan, UT) was used as complete medium in all cultures. Anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) Abs were purchased from Immunotech (Westbrook, ME) and BD PharMingen (San Diego, CA), respectively. Phospho (p)-specific rabbit Abs to p-ERK-1/2, p-JNK-1/2, and p-p38 were obtained from Cell Signaling Technology (Beverly, MA), and ERK-1/-2, JNK-1/-2, and p38 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). PE- and FITC-conjugated and HRP-conjugated secondary Abs were purchased from BD PharMingen and Santa Cruz Biotechnology, respectively, and U0126 and cobalt protoporphyrin (CoPP) were from Promega (Madison, WI) and Porpyrin Products (Logan, UT), respectively. SP600125 was purchased from Calbiochem (San Diego, CA). MEK1 (CA-MEK1) were cloned into pcDNA3 (Invitrogen, San Diego, CA). dominant-negative MEK1 (DN-MEK1), and constitutively active (S218A/S222A) mutants of MEK1 were kindly provided by Dr. K. Y. Choi (University of Pittsburgh, Pittsburgh, PA). Recombinant human IL-2, IL-10, IFN-γ, and anti-IL-10 neutralizing Ab were obtained from R&D Systems (Minneapolis, MN). CFSE was purchased from Molecular Probes (Eugene, OR). The other reagents were from Sigma-Aldrich.

**Isolations of resting CD4+ T cells and CD8+ T cells from peripheral blood**

PBMCs were isolated from healthy blood by Ficoll-Paque density gradient centrifugation. After three washes in HBSS, CD4+ T cells were isolated from PBMCs using the MACS negative depletion system (Miltenyi Biotec, Auburn, CA). No contamination with CD8+ T cells, B cells, monocytes, or NK cells was detected. Isolation of CD8+ cells was performed using a negative CD8+ T cell isolation kit (Miltenyi Biotec).

**Cell transfection**

HO-1, dominant-negative MEK1 (DN-MEK1), and constitutively active MEK1 (CA-MEK1) were cloned into pcDNA3 (Invitrogen, San Diego, CA). Jurkat T cells (5 x 10⁶) were transfected with 10 μg of constructs by electroporation at 270V, 950 μl/H9262 from PBMCs using the MACS negative depletion system (Miltenyi Biotec, molecular Probes (Eugene, OR). The other reagents were from Sigma-Aldrich.

**Evaluation of cGMP**

Different pools of CD4+ T cell samples were incubated for 1 h at 37°C in the presence of 20 μM CO or air. To inhibit phosphodiesterase activity, 3′-isobutyl-1-methyl xanthine (10 μM) was added to the cell suspension. The concentration of cGMP was determined by a radioimmunoassay kit using [3H]labeled cGMP (Amersham Pharmacia Biotech). Briefly, after incubation, 500 μl of 10% TCA was added to the cell suspensions. The samples were then centrifuged and TCA was extracted with 0.5 M tri-n-octylamine dissolved in TCA, and the samples were then acetylated with acetic anhydride and the amount of cGMP in the aqueous phase was measured.

**Flow cytometry**

Cells were suspended in HBSS containing 5% FBS, fixed by the drop-wise addition of ice-cold 70% ethanol to a final 50% concentration, and held on ice for 1 h. After extensive washing, the cells were suspended in HBSS containing 50 μg/ml propidium iodide (Sigma-Aldrich) and 50 μg/ml RNase A (Boehringer Mannheim, Indianapolis, IN) and were incubated for 1 h at room temperature. In some experiments, the states of ERK activation and HO-1 expression were determined by intracellular staining with either anti-p-ERK or anti-HO-1 Abs labeling either FITC- or PE-conjugated secondary Ab, as described previously (23). Stained cells were analyzed by flow cytometry on a FACSVantage with CellQuest software (BD Biosciences, Franklin Lakes, New Jersey). G/G, S, and G/M populations were quantified using the ModFIT program (BD Biosciences).

**CFSE dilution assay**

Naïve CD4+ T cells were labeled with 1.5 μM CFSE for 10 min at 37°C in serum-free RPMI 1640. Cells were washed twice in RPMI 1640 containing 10% FBS and were stimulated as indicated. Cell division was assessed at 4 days by determining the pattern of CFSE dilution using flow cytometry.

**Statistics**

Data were expressed as mean ± SEM of the individual titer. Levels of significant differences between groups were determined by the Student t test. Values of p < 0.01 were considered statistically significant.

**Results**

**HO-1 expression in T cells is antiproliferative**

We found that pharmacological expression of HO-1 by the HO-1 inducer CoPP in PBMCs containing monocytes and lymphocytes was antiproliferative in response to anti-CD3 plus anti-CD28 Abs (Fig. 1A). This suggested that HO-1 expression in monocytes and/or lymphocytes could suppress T cell proliferation. Therefore, we examined whether CoPP could induce HO-1 expression in human CD4+ T cells purified from PBMCs and also whether HO-1 expression by CoPP could be antiproliferative. Preincubation of
FIGURE 1. Effects of HO-1 expression on T cell proliferation. PBMCs (A) and CD4⁺ T cells (B) purified from PBMCs were incubated for 12 h with CoPP, and Jurkat T cells (C) were transfected with human HO-1 gene (Jurkat/HO-1) or empty vector (Jurkat/pcDNA). HO-1 expressions (top panels) were confirmed by Western blot analysis after 12-h incubation of either PBMCs or CD4⁺ T cells with CoPP (20 μM) or after stable transfection of Jurkat T cells with either HO-1 gene or empty vector. One of three experiments is shown. T cell proliferation (bottom panels) was determined after 4-day stimulation of T cells with anti-CD3 plus anti-CD28 Abs. Values are the mean ± SEM of six triplicate experiments. *, p < 0.01.

CD4⁺ T cells with CoPP for 12 h resulted in an apparent increase in HO-1 expression and a significant decrease in T cell proliferation (Fig. 1B). CoPP preincubation also suppressed the proliferative response of CD8⁺ T cells (data not shown). To further investigate the contribution of HO-1 expression to T cell proliferation, we transfected the HO-1 gene into human Jurkat T cells (Fig. 1C). In agreement with the CoPP data, overexpression of HO-1 suppressed T cell proliferation (Fig. 1C). These data demonstrate that HO-1 expression in T cells is antiproliferative.

Exogenous and endogenous CO can suppress human T cell proliferation, mimicking antiproliferative action of HO-1

We next determined which of the HO-1 metabolites could mediate the antiproliferative effect of HO-1 on human CD4⁺ T cells. Thus, we preincubated CD4⁺ T cells with CO gas, bilirubin, or free iron (Fe²⁺) for 1 h before stimulation of the T cells with anti-CD3 plus anti-CD28 Abs. Only CO gas had an antiproliferative effect on CD4⁺ T cells (Fig. 2A). Similarly, the CO-releasing compound RuCO suppressed the proliferative response of CD4⁺ T cells in a dose-dependent manner (IC₅₀ = 20 μM), but not in the presence of the CO scavenger Hb (Fig. 2B). In addition, a 12-h preincubation of CD4⁺ T cells with CoPP significantly suppressed the proliferative response, whereas a 12-h preincubation of the cells with CoPP in the presence of Hb showed no suppressive effect (Fig. 2C). Similarly, CoPP in the presence of Hb did not suppress the proliferation of either CD8⁺ T cells or Jurkat T cells (data not shown). CO gas, RuCO, CoPP, or Hb at concentrations used in these experiments showed no effect on T cell viability (data not shown). These results suggest that exogenous and endogenous CO can mimic the antiproliferative action of HO-1.

CO blocks the cell cycle entry of human T cells, independent of the guanylate cyclase/cGMP pathway

We observed that addition of CO to CD4⁺ T cells 24 h after stimulation with anti-CD3 plus anti-CD28 Abs had no effect on proliferation (Fig. 3A). This suggested that CO was blocking early events leading to T cell activation. Therefore, we examined the effect of RuCO on the cell cycle and could show that CD4⁺ T cells did not progress past G₂/M phase, if the cells were stimulated after preincubation with RuCO for 1 h (Fig. 3B, lower left panel). We could also show that CO was not inhibitory to cell cycle progression, if the activation was already progressed beyond TCR signaling (Fig. 3B, lower right panel). Furthermore, a kinetic CFSE analysis with intracellular HO-1 staining revealed that CO was not inhibitory to T cell activation.

FIGURE 2. Suppressive effects of CO on the proliferation of CD4⁺ T cells. A, CD4⁺ T cells were preincubated for 1 h with CO gas (200 ppm), bilirubin (20 μM), or Fe²⁺ (20 μM) before stimulation. B, CD4⁺ T cells were preincubated for 1 h without (control) or with indicated concentrations of RuCO in the absence or presence of Hb (80 μM) before stimulation. C, CD4⁺ T cells were preincubated for 12 h with CoPP (20 μM) in the absence or presence of Hb (80 μM). The CD4⁺ T cells were then stimulated for 4 days with anti-CD3 plus anti-CD28 Abs. Proliferation was measured, as described in Materials and Methods. Data (B) are expressed as percent of control. Values are the mean ± SEM of six triplicate experiments. *, p < 0.01.
FIGURE 3. Blocking effects of CO on the cell cycle progression of human T cells. A, CD4$^+$ T cells labeled with CFSE were cultured for 1 h with medium (upper right panel) or were stimulated for 4 days with anti-CD3 plus anti-CD28 Abs (upper left panel). CD4$^+$ T cells labeled with CFSE were preincubated for 1 h with RuCO (20 μM) and stimulated for 4 days with anti-CD3 plus anti-CD28 Abs (lower left panel). RuCO (20 μM) was added after 24-h stimulation of CD4$^+$ T cells with anti-CD3 plus anti-CD28 Abs (upper right panel), and the T cells were further cultured for 3 days. CFSE fluorescence was measured by flow cytometry. B, CD4$^+$ T cells were cultured for 48 h with medium (upper left panel) or were stimulated with anti-CD3 plus anti-CD28 Abs (upper right panel). CD4$^+$ T cells were preincubated for 1 h with RuCO (20 μM) and were stimulated for 48 h with anti-CD3 plus anti-CD28 Abs (lower left panel). RuCO (20 μM) was added after 24-h stimulation of CD4$^+$ T cells with anti-CD3 plus anti-CD28 Abs (lower right panel), and the T cells were further cultured for 24 h. Cell cycle analysis was performed, as described in Materials and Methods. Cells with sub-2N amounts of DNA were not gated and comprised from 10 to 25% of the population. C, CD4$^+$ T cells labeled with CFSE were stimulated for 4 days with anti-CD3 plus anti-CD28 Abs. Cells were harvested and stained with anti-HO-1-PE. Dotted box in one of the representative dot plots of HO-1 expression vs CFSE represents the population of HO-1-positive cells. D, CD4$^+$ T cells were preincubated for 1 h with medium or RuCO (20 μM) in the presence or absence of ODQ (30 μM) and were stimulated for 48 h with anti-CD3 plus anti-CD28 Abs. Each bar represents the percentage of cells belonging to each cell cycle in flow cytometric analysis. Values are the mean ± SEM of six triplicate experiments.

cell cycle progression after a certain stage of activation because HO-1/CO was induced during T cell activation (Fig. 3C).

Most of the biological effects of CO are attributed to its abilities to modulate the activity of guanylate cyclase and to increase the cellular cGMP levels (17). Therefore, we examined whether the blockage of cell cycle progression by RuCO could be due to elevation of cGMP and found that ODQ, a potent inhibitor of the soluble form of guanylyl cyclase, had no effect on RuCO-induced blockage of the cell cycle (Fig. 3D). However, ODQ significantly blocked RuCO-induced cGMP synthesis in T cells (data not shown).

**CO inhibits IL-2 secretion by activated T cells, contributing antiproliferative effect of CO**

IL-2 is an important regulator of T cell proliferation and is released by activated T cells (8–14). We observed that, among three HO-1 reaction products, only CO gas inhibited IL-2 secretion by activated CD4$^+$ T cells (Fig. 4A). Similarly to CO gas, RuCO also inhibited the secretion of IL-2 by CD4$^+$ T cells in a dose-dependent manner (IC$_{50}$ = 20 μM), but not in the presence of Hb (Fig. 4B). Additionally, RuCO at higher concentrations reduced the secretion levels of IFN-γ and IL-10 (Table I). Moreover, preincubation of CD4$^+$ T cells with CoPP inhibited IL-2 secretion, whereas preincubation with CoPP in the presence of Hb showed no inhibitory effect (Figs. 4C).

To test whether the observed CO-induced suppression of T cell proliferation could be due to reduction of secretion levels of IL-2, we added recombinant human IL-2 to the cultures of T cells stimulated with anti-CD3 plus anti-CD28 Abs in the presence of RuCO. The exogenous IL-2 effectively reversed the antiproliferative effects of CO in T cell culture (Fig. 4D). Unlike IL-2, neither IFN-γ nor IL-10 reversed the antiproliferative effects of CO (Fig. 4D). These data indicate that the suppression of T cell proliferation by CO could be due to its inhibition of IL-2 secretion.

**CO inhibits ERK phosphorylation in activated T cells, probably mediating the inhibitory effect of CO on IL-2 secretion**

The ERK MAP kinases, which have been implicated as an intracellular target to contribute to certain CO-induced biological actions (19, 21), also play critical roles in T cell proliferation and IL-2 secretion (13, 14). Therefore, we examined the effects of CO gas and RuCO on ERK-1/ERK-2 activation in CD4$^+$ T cells. CO
gas and RuCO significantly inhibited ERK phosphorylation in the activated CD4+ T cells (Figs. 5, A and B). In contrast, RuCO had no effect on either p38 phosphorylation or JNK phosphorylation (Fig. 5B). In Jurkat T cells, RuCO also inhibited ERK phosphorylation in a dose-dependent manner (data not shown). Next, we were interested in determining the effects of blocking the ERK, p38, and JNK pathways on T cell proliferation. U0126, a selective inhibitor of ERK pathway, SB20358, a selective inhibitor of p38 pathway, and SP600125, a selective inhibitor of JNK pathway, were used to block each pathway. U0126 significantly suppressed T cell proliferation, but SB20358 and SP600125 did not (Fig. 5C). Suppression of T cell proliferation by U0126 was most likely due to reduction of IL-2 levels, because U0126-treated CD4+ T cells could proliferate when IL-2 was exogenously added to the cultures (Fig. 5C). In addition, RuCO at 20 μM reduced proliferative response even when p38 and JNK pathways were blocked by SB20358 and SP600125, respectively (Fig. 5C). However, RuCO did not further reduce T cell proliferative response when ERK pathway was effectively blocked by U0126 (Fig. 5C). These results further suggest that the ERK pathway might be involved in the suppressive effects of CO on T cell proliferation.

CO may block upstream signaling cascades of MEK1 in activated T cells, thereby resulting in ERK inactivation

Several studies have brought evidence that the intracellular signals that mediate activation of transcription factors regulating IL-2 gene transcription in human T cells involve p21ras-mediated signaling pathways (24–27). These studies obtained with T cell lines collectively suggest that IL-2 gene transcription might require the p21ras/Raf-1/MEK/ERK phosphorylation cascade.

To further evaluate the role of the ERK pathway in CO-induced inhibition of IL-2 secretion, we transfected DN-MEK1 and CA-MEK1 genes into Jurkat T cells to selectively inhibit or activate the ERK1 pathway. DN-MEK1 expression suppressed ERK1 activation and IL-2 secretion by the simultaneous ligation of CD3 and CD28 Abs (Fig. 6), which was similar to RuCO effects on IL-2 secretion. Conversely, CA-MEK1 expression enhanced ERK1 activation and IL-2 secretion by the same stimuli (Fig. 6). No significant inhibition of IL-2 secretion by RuCO was observed in DN-MEK1- or CA-MEK1-transfected cells (Fig. 6). These results suggest that CO might block or inactivate upstream signaling cascades of MEK1, thereby inhibiting ERK pathway.

IL-10 may be involved in HO-1 expression in activated CD4+ T cells

It has been reported that IL-10 is able to induce HO-1 expression and to exert its anti-inflammatory effects via the HO-1-dependent pathway in monocytes (28). In human CD4+ T cells, IL-10 secretion (see Table I) as well as HO-1 expression (see Fig. 3C) was induced by the simultaneous ligation of CD3 and CD28 Abs. Thus, one may ask about whether IL-10 could be involved in HO-1 expression in CD4+ T cells. Although IL-10 itself did not induce HO-1 expression in naive CD4+ T cells, this cytokine further enhanced HO-1 expression in CD4+ T cells stimulated with CD3 plus CD28 Abs (Fig. 7). Moreover, anti-IL-10 neutralizing Ab reduced the level of HO-1 expression in activated CD4+ T cells (Fig. 7). It is most likely that there are many complex signals, including an IL-10 signal, to sufficiently induce HO-1 expression in human CD4+ T cells.

Discussion

The antiproliferative and anti-inflammatory HO-1 may play important roles in regulating T cell responses. It has been reported that HO-1-deficient mice contract a progressive chronic inflammatory disease, demonstrated by enlarged spleen and lymph nodes, high peripheral white blood cell counts, and high splenic and
lymph node CD4+/CD8+ T cell ratios with numerous activated CD4+ T cells (4, 5). It has also been reported that splenocytes isolated from HO-1-overexpressing mice tend to display lower proliferation indices against allogeneic stimulation in both CD4+ and CD8+ subsets and markedly increased allotransplant survival by inhibiting infiltrations of inflammatory cells and CD4+ T cells (29). The administration of a HO-1 inducer to normal mice results in suppressions of T cell-mediated cytotoxicity and Th1-mediated cytokine production and decreases in the lymphoproliferative alloresponse and differentiation of CTLs (30). HO-1 inducer also prevents the induction of T cell-mediated experimental autoimmune encephalomyelitis in rats (31). In accord with these observations, we recently demonstrated that human CD4+ T cells express HO-1 and that HO-1-overexpressing Jurkat T cells tend to display lower proliferative response. In the present study, we explored the mechanism(s) by which HO-1 mediated its antiproliferative effect on CD4+ T cells and found that the HO-1/CO system was an important regulator of T cell responses. CO suppressed proliferation and IL-2 secretion of CD4+ T cells, most likely by inhibiting ERK activation.

The exact mechanisms of HO-1 expression in activated T cells are currently unknown. However, the antiproliferative and anti-inflammatory IL-10 produced by activated CD4+ T cells was involved at least in part in HO-1 expression in T cells (4, 5). It has also been reported that splenocytes isolated from HO-1-overexpressing mice tend to display lower proliferation indices against allogeneic stimulation in both CD4+ and CD8+ subsets and markedly increased allotransplant survival by inhibiting infiltrations of inflammatory cells and CD4+ T cells (29). The administration of a HO-1 inducer to normal mice results in suppressions of T cell-mediated cytotoxicity and Th1-mediated cytokine production and decreases in the lymphoproliferative alloresponse and differentiation of CTLs (30). HO-1 inducer also prevents the induction of T cell-mediated experimental autoimmune encephalomyelitis in rats (31). In accord with these observations, we recently demonstrated that human CD4+ T cells express HO-1 and that HO-1-overexpressing Jurkat T cells tend to display lower proliferative response. In the present study, we explored the mechanism(s) by which HO-1 mediated its antiproliferative effect on CD4+ T cells and found that the HO-1/CO system was an important regulator of T cell responses. CO suppressed proliferation and IL-2 secretion of CD4+ T cells, most likely by inhibiting ERK activation.

The exact mechanisms of HO-1 expression in activated T cells are currently unknown. However, the antiproliferative and anti-inflammatory IL-10 produced by activated CD4+ T cells was involved at least in part in HO-1 expression in T cells (Fig. 7), raising an interesting question of whether HO-1 expression could be involved in modulating T cell responses. Pharmacological induction or gene transfer of HO-1 in human T cells was antiproliferative (Fig. 1). Among the three HO-1 byproducts, only both exogenously added and endogenously generated CO-suppressed T cell proliferation (Fig. 2), mimicking the antiproliferative action of
CO suppresses T cell proliferation via IL-2 inhibition

Neither bilirubin nor free iron showed any effect on proliferative response under our experimental conditions. CO blocked the cell cycle entry of T cells, which was independent of guanylate cyclase/cGMP pathway (Fig. 3). However, it was of great interest to observe that CO was not inhibitory to cell cycle progression if the T cell activation already progressed beyond TCR signaling (Fig. 3, A–C). This suggests that CO blocks early events in T cell activation. CO also inhibited IL-2 secretion in CD4+ T cells stimulated with anti-CD3 plus anti-CD28 Abs (Fig. 4). This inhibitory effect of CO on the IL-2 secretion appears to be responsible for the antiproliferative action of CO, because T cell proliferation occurred when IL-2 was exogenously added to the culture (Fig. 4D). At high doses, CO also inhibited both IFN-γ and IL-10 secretions (Table I), but the additional inhibitions of these cytokines by CO were not associated with the antiproliferative effect of CO (Fig. 4D). MAP kinase plays an important role in IL-2 secretion, and there exists a strong correlation between decreased IL-2 secretion and the inhibition of ERK activation in T cells (13, 14). In support of these findings, our data clearly showed that CO selectively inhibited the ERK pathway in the activated T cells (Fig. 5). Furthermore, U0126, a selective inhibitor of ERK activation, suppressed T cell proliferative response, but not in the presence of exogenously added IL-2 (Fig. 5C). Similarly, an expression of DN-MEK1 inhibited IL-2 secretion (Fig. 6). These findings led us to suggest that CO might be able to inhibit the ERK activation, which leads to inhibition of IL-2 secretion, eventually resulting in suppression of T cell proliferation in response to the simultaneous ligation of CD3 and CD28 Abs.

Naive CD4+ T cells could be potentially exposed to endogenous CO produced by CD4+CD25+ regulatory T cells, which constitutively express HO-1 (1), as well as activated CD4+CD25− responder T cells, which can express HO-1 after stimulation. Thus, we speculate that CO may directly and/or indirectly affect T cell responses in vivo. It is of interest that CO shows cGMP-independent suppressive effects on T cell responses and it can inhibit ERK activation in activated CD4+ T cells. CO could indirectly inhibit ERK phosphorylation, probably by blocking upstream signals of MEK (Fig. 6). It could be possible that CO activates the small G protein, Rap1, which has been shown to inhibit ERK activation by blocking Ras-dependent activation of the MAP kinase kinase, Raf-1 (32). Further studies are in progress to explore potential mechanisms of CO effects on ERK signaling pathways.

In summary, our findings suggest that HO-1/CO induces suppressive effects on T cell proliferation and IL-2 secretion, possibly via its inhibition of the ERK MAP kinase pathway, which is currently believed to be an important signaling pathway for mediating T cell activation. Our findings may contribute not only to our deeper understanding of the basic roles of HO-1 in the immune system, but also to our search for novel targets for new therapeutic approaches to modulate T cell-mediated immune responses.

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