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BRIEF REVIEWS

An Integral Role for Heme Oxygenase-1 and Carbon Monoxide in Maintaining Peripheral Tolerance by $CD4^+CD25^+$ Regulatory T Cells[1]

Todd M. Brusko, Clive H. Wasserfall,* Anupam Agarwal,† Matthias H. Kapturczak,† and Mark A. Atkinson[2]**

Over the past decade, a great deal of interest and attention has been directed toward a population of regulatory T cells (Treg) coexpressing the markers CD4 and CD25. The hallmark phenotype of this cell population resides in its ability to dominantly maintain peripheral tolerance and avert autoimmunity. Despite robust research interest in Treg, their mechanism of action and interaction with other cell populations providing immune regulation remains unclear. In this study, we present a model for Treg activity that implicates carbon monoxide, a by-product of heme oxygenase-1 activity, as an important and underappreciated facet in the suppressive capacity of Treg. Our hypothesis is based on recent evidence supporting a role for heme oxygenase-1 in regulating immune reactivity and posit carbon monoxide to function as a suppressive molecule. Potential roles for indoleamine 2,3-dioxygenase, costimulatory molecules, and cytokines in tolerance induction are also presented. This model, if validated, could act as a catalyst for new investigations into Treg function and ultimately result in novel methods to modulate Treg biology toward therapeutic applications. *The Journal of Immunology,* **2005, 174: 5181–5186.**

Few topics have spurred as much discussion and controversy in the field of immunology as that defining regulatory T cells (Treg).[3] Despite their initial description over 30 years ago by Gershon and Kondo (1), the field of T cell immune regulation offered by "suppressor T cells" was largely abandoned during the 1980s due to a lack of specific characterization. This period of diminished interest reversed with Sakaguchi and colleagues (2) suggested a relatively small population of $CD4^+$ T cells coexpressing CD25 (the α-chain of the IL-2R) was responsible for the prevention of autoimmunity. Since that description, a plethora of studies have, to a large extent, supported the notion that $CD4^+CD25^+$ T cells, often referred to as regulatory T cells (Treg), play a major role in suppressing immune reactivity (reviewed in Refs. 3–7). Although clearly not the only cells providing immunoregulatory activity (i.e., such activities can be afforded by Th3 cells, and subsets of CD8, NK, and NKT cells, among others), CD25 represents an important tool for investigators seeking to identify, isolate, and characterize Treg. Another, more recent finding of significance in this field of immune regulation was that indicating the forkhead box P3 transcription factor (Foxp3) was required for the development and maintenance of the $CD25^+$ Treg cell compartment (reviewed in Ref. 8). The disruption in this gene leads to multiorgan-specific autoimmunity in both mice (scurfy mouse phenotype) and in humans (immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome) (9). Despite advances in our ability to identify and isolate Treg, as well as to appreciate their importance to the maintenance of tolerance, central questions remain regarding the mechanism of action of Treg. This brief review evaluates the existing body of mechanistic studies on Treg and based on that evidence, puts forward a hypothetical model that includes novel and significant roles for molecules not previously subject to significant attention in terms of their potential contribution to the processes underlying immune regulation.

Treg-mediated suppression

Surface interactions by negative costimulatory molecules, suppressive cytokines, and simple competition for IL-2 or APC interactions have all been proposed as contributing to the suppressive phenotype of Treg (outlined in Table I). Despite extensive investigation and the aforementioned enthusiasm for CD25 in studies of immune regulation, little consensus exists with respect to a surface ligand or soluble factor that strictly associates with Treg function. The reasons for discordance between studies varies, but most likely includes differences in experimental conditions and the actual cell populations subject to investigation, among others (7).

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[3] Abbreviations used in this paper: Treg, regulatory T cell; FOXP3, forkhead box P3 transcription factor; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; Teff, effector T cell; HO-1, heme oxygenase-1; CO, carbon monoxide; IDO, indoleamine 2,3-dioxygenase; DC, dendritic cell; HA, 3-hydroxyanthranilic acid; iNOS, inducible NO synthase.

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Table I. *Mechanisms proposed to influence suppression mediated by $CD4^+$ $CD25^+$ T cells*[a]

Suppressive Mechanism	Proposed Action	Supporting Evidence	Contradictory Evidence	Refs.
CTLA-4	Binding to B7.1/B7.2; induction of IDO in DC	CTLA-4 knockout mouse exhibits polyautoimmune phenotype	Treg retain suppressive action in APC free cultures	51, 53, 66
LAG-3 (CD223)	Unknown	Blocking Abs abrogate suppression in vitro and in vivo	Lack of overt autoimmunity in LAG-3 knockout mouse	67–69
GITR	Provides T cell costimulatory signal; induces IL-10 production	mAb blockade of GITR in mice abrogates suppression in vitro and in vivo	GITR is also expressed on activated effector cells and blockade may render resistant to suppression by Treg	70–72
IL-2R affinity	Outcompete IL-2 from Teff cells	Neutralization of IL-2 mimics Treg action; addition abrogates suppression mediated by Treg	Stoichiometry of suppression and Treg activation requirements suggests active mechanism	73, 74
Soluble mediators				
TGF-β	Membrane-bound active form exerts suppressive functions	High concentrations of polyclonal Ab abrogate suppression	Transwell experiments, neutralizing Abs, and cytokine knockout mice raise questions about direct effects	18, 21, 75
IL-10	Activation of Th1 reactive cells is controlled	Injection of blocking Ab in vivo abrogates protection in an IBD model	Protective functions of $CD4^+$ $CD25^+$ fraction are independent of IL-10	76, 77

[a] GITR, Glucocorticoid-induced TNFR-regulated gene; IBD, inflammatory bowel disease.

As but one example of such conflicts, the vast majority of published studies involving Treg function have failed to identify a soluble suppressor cytokine (4, 10–15). This notion has most often found support through transwell experiments wherein Treg failed to suppress the proliferation of effector T cells (Teff) cells when incubated independently, thus leading to the conclusion that Treg-mediated suppression requires direct cell-cell contact. Additional experiments indicating a maintenance of the suppressive capabilities by Treg in vitro despite the use of either neutralizing anti-cytokine Abs or cells obtained from mice with targeted deficiency in these molecules (e.g., IL-$10^{-/-}$, IL-$4^{-/-}$) have also supported this notion (10). However, recent findings regarding TGF-β-mediated Treg suppression have rendered the issue of a suppressor cytokine less clear. Specifically, studies in both mice and humans have implicated TGF-β in the induction of Foxp3/FOXP3 expression and a regulatory phenotype in vitro (16, 17). TGF-β has also been reported to be bound to the surface of Treg in an active form and proposed as a possible mechanism by which Treg may mediate suppression (18). High concentrations of polyclonal Ab against TGF-β abrogate the suppressive capacity of Treg (19). Although these represent potentially promising observations, other reports point toward Treg function in the absence of TGF-β, TGF-β signaling elements, or its receptor (20–22). Hence, while the notion for a soluble mediator in Treg function remains unclear, the potential for mechanisms other than contact dependency playing a role in Treg processes do exist.

The anti-inflammatory and immunological role of heme oxygenase-1 (HO-1)

Recent studies have also highlighted the important biological significance of reactive products of the HO-1 enzymatic reaction in models of inflammation and transplant rejection (reviewed in Refs. 23–25). HO-1 catalyzes the rate-limiting step in the degradation of heme, resulting in the liberation of equimolar amounts of iron, carbon monoxide (CO), and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. We and others have observed that HO-1-deficient mice develop chronic inflammation characterized by hepatosplenomegaly, lymphadenopathy, leukocytosis, hepatic periportal inflammation, and occasionally glomerulonephritis (26, 27). Our work analyzing splenocytes from HO-1 knockout mice also demonstrated a marked Th1 response following T cell stimulation, suggesting that genetic absence of HO-1 correlated with abnormal T cell function (27). Interestingly, a patient with HO-1 deficiency was reported with several phenotypic similarities with the HO-1 knockout mouse, including growth failure, anemia, increased iron binding capacity and ferritin, tissue iron deposition, lymphadenopathy, leukocytosis, and increased sensitivity to oxidant injury (28). Modulation of HO-1 expression by pharmacological or genetic approaches significantly influences graft survival in organ transplantation (29, 30). Collectively, the induction of HO-1 tends to prolong graft survival, while inhibition worsens outcome (see Ref. 31 for a review of HO-1 actions in transplantation). Although HO-1 clearly demonstrates nonspecific cytoprotective functions against oxidative damage and inflammation, evidence is now mounting that HO-1 acts more prominently to inhibit allogeneic immune responses following transplantation. Indeed, viral gene transfer of HO-1 promoted long-term allograft survival in an acute cardiac allograft rejection model (32). Another recent study suggests HO-1 action specifically modulates T cell responses by promoting activation-induced cell death of alloreactive T cells (33). Taken collectively, HO-1 clearly represents a molecule of immunologic significance. However, these findings also raise the question of how this molecule could impact specific aspects of immune regulation including that of Treg function.

Evidence for HO-1 in Treg function

To that question, recent (and unconfirmed) work by Pae et al. (34) has demonstrated that HO-1 is differentially expressed between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations in a manner analogous to the aforementioned findings for human FOXP3 expression (35). Follow-up experiments by that same group observed that upon anti-CD3/anti-CD28 costimulation, human $CD4^+CD25^-$ T cells could also be induced to express

HO-1; a finding that once again paralleled the expression pattern of human FOXP3 (36). In those same studies, the investigators demonstrated that stable transfection of HO-1 into Jurkat T cells was capable of inhibiting cellular proliferation. This latter observation, when applied to natural Treg, could explain the anergic and refractory phenotype of this cell population.

In light of the observation that HO-1 and Foxp3 have similar expression profiles in T cells, the question then arises of whether one of these genes influences the expression of the other. To address this issue, we would note the studies of Choi et al. (37) involving transfection of Foxp3 into Jurkat T cells; a process that subsequently induced expression of HO-1. HO-1 gene expression in these "de novo regulatory cells" suppressed proliferation and cytokine production of nontransfected cells in a cell-cell contact-dependent manner. Furthermore, the suppressive capacity of Foxp3-transfected Jurkat T cells and primary human $CD4^+CD25^+$ T cells was blocked in the presence of an HO-1 inhibitor (37). Although subject to the need for confirmation by other investigators, these reports do provide enticing evidence that the transfer of Foxp3 does indeed lead to the induction of HO-1 expression and a Treg phenotype in vitro. Still needed are more thorough investigations into the other transcriptional targets of Foxp3 and their contribution to the phenotype of Treg.

A model for HO-1 in Treg function

Based on a collection of the aforementioned evidence, we would propose a model wherein the phenotype of Treg (i.e., the suppression of proliferation and effector cytokine production) results, in part, from a biochemical reaction involving the production of CO via HO-1 in activated Treg (Fig. 1). The mechanism outlined herein indicates that suppression elicited by Treg is an active process that must first be "primed" by the actions of tolerogenic APC. This hypothetical model provides a framework by which repeated exposures to Ags (including self Ags) in the thymus or periphery, or even environmental agents, may lead T cells to a refractory and anergic phenotype.

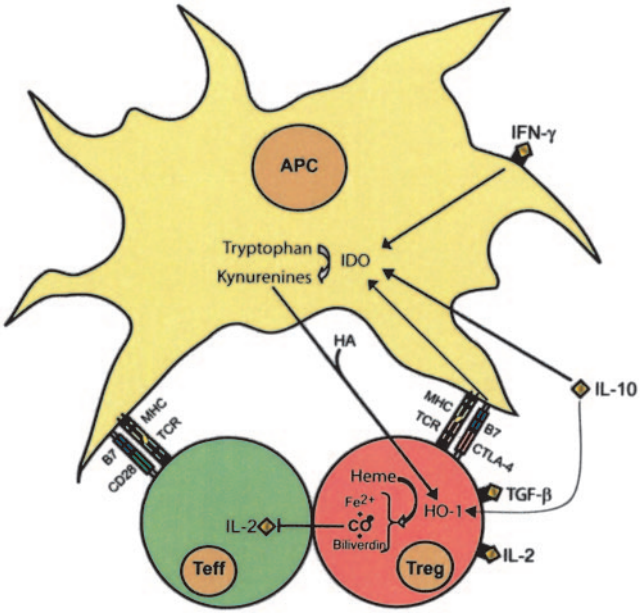

FIGURE 1. Cooperative mechanism of immune regulation at the junction of Treg, Teff, and APC. CTLA-4 expression on primed Treg induces tryptophan metabolism in APC via IDO following ligation of B7 and IFN-γ signaling. The metabolic by-product HA suppresses iNOS and NO production by up-regulating HO-1 expression. CO, a catabolic product of heme via HO-1, functions in an intimate manner to suppress Teff proliferation and cytokine production. Cytokines play a key role in maintaining suppression by reinforcing expression of FOXP3, HO-1, and IDO.

Why would this model place an emphasis on CO? Of the three HO-1 reaction products, CO has been shown to exert broad antiproliferative effects in both immune and nonimmune cells (24). Generally, CO is thought to exert those antiproliferative actions by modulating the activity of guanylate cyclase to increase the levels of cellular cGMP as well as through the MAPK pathway. The mechanism by which CO elicits its antiproliferative effects appears to differ somewhat with respect to the target cell type. In vascular smooth muscle cells, CO exerts its antiproliferative effects via the p38 and MAPK pathway (38). In T cells, a mechanism of action for CO has been proposed which involves inhibition of ERK activation (39).

Also central to the hypothesis of how CO would mediate suppression by Treg are the findings indicating that CO produced by HO-1, and not the other reaction products (Fe^{2+} or billirubin), are capable of mediating the antiproliferative effects of HO-1 in human $CD4^+$ T cells (39). Specifically, those investigations indicate that both endogenously and exogenously produced CO is capable of inhibiting T cell proliferation. More specifically, CO was shown to block production of IL-2, a principal cytokine responsible for T cell proliferation (39). This observation is in line with what has previously been reported as the suppressive mechanism of Treg, without delineating the exact effector molecule (11). These studies also highlight that CO was unable to inhibit cellular proliferation if a T cell had progressed beyond TCR signaling (39). This represents an especially interesting finding, which may help explain the ability to overcome the anergic threshold of Treg with the addition of strong TCR signaling and costimulatory signals. CO functioning as a nonspecific suppressor also fits with the observation that Treg cells are capable of suppressing proliferation of various immune cell types without MHC restriction and in a non-Ag-specific manner (40). Furthermore, CO production via HO-1 in Treg alone explains the capacity of these cells to suppress Teff cells in systems void of APC as well as the direct effects of metabolites of indoleamine 2,3-dioxygenase (IDO). Although Treg cells clearly suppress effector T cell proliferation in vitro, questions exist as to whether this phenomenon reflects the natural in vivo phenotype of Treg. To this end, additional evidence exists which indicates that CO exposure is also capable of suppressing inflammatory cytokine production (41).

The observed in vitro phenotype of Treg would require that CO act in an autocrine fashion and by intimate diffusion across membranes into Teff cells. There is a precedent for CO functioning in an analogous manner. In the brain, CO produced by the heme oxygenase-2 isoform (HO-2) functions as a retrograde neurotransmitter by diffusing across the postsynaptic membrane to the presynaptic membrane. This process, termed long-term potentiation, may play a critical role in the generation or consolidation of memories (42). Hence, HO-1, through actions of CO, could provide one mechanism by which the regulatory capacity of Treg is generated.

Filling in the gaps: the regulatory loop between Treg and APC

As previously indicated, the direct mechanism of suppression by Treg appears to be APC-independent, yet Treg must first be

primed to exert their suppressive effects. Evidence in several mouse models suggests that multiple costimulatory interactions between Treg and APC are required for the development and function of Treg (43–45). A poignant example of this resides in the observation that Treg fail to develop in mice which lack CD28/B7 interactions (46). Evidence also suggests that Treg require IL-2 and must be activated through the TCR for suppressive functions to remain operative (47). In addition, one of the characteristic markers of Treg is their constitutive expression of the negative costimulatory molecule CTLA-4 (48, 49). It has been postulated that this expression may contribute to the Treg cell phenotype by inhibiting T cell signaling and by outcompeting CD28-B7 costimulatory interactions on APC needed to prompt Teff proliferation (50). However, an additional mechanism of action for CTLA-4 expression on Treg has been proposed by Fallarino et al. (51) that involves CTLA-4 signaling through B7 engagement on dendritic cells (DC). Specifically, the interaction of CTLA-4 on Treg with B7 leads to the induction of the immunomodulatory enzyme IDO in DC. Recent findings indicate that another transcription factor of the forkhead box class (FOXO3a) may be involved in this signaling interaction leading to the full activation of tryptophan metabolism (52). The induction of IDO is also regulated at the transcriptional level by IFN-γ, which appears to signal in an autocrine fashion (51).

Another potential gap: a role for IDO in modifying Treg function

IDO is an enzyme expressed primarily in monocytes, macrophages, and DCs and is associated with broad immunoregulatory activities (reviewed in Ref. 53). IDO is responsible for the conversion of tryptophan to kynurenine and the by-products 3-hydroxyanthranilic acid (HA), picolinic acid, and quinolinic acid (54). A key yet unanswered question is how IDO expression could modify a broad array of immune responses. At least two non-mutually exclusive processes have previously been proposed. First, a reduced proliferation by T cells may result from the depletion of the essential amino acid tryptophan in the local microenvironment. The alternative hypothesis suggests that the by-products of tryptophan metabolism are proapoptotic and/or antiproliferative to T cells (53, 54). Strong arguments can be made for both cases. For our model, we questioned whether HO-1 expression by Treg is affected by IDO metabolites or if HO-1 serves as a survival mechanism in the "cytotoxic" environment of IDO expressing APC.

Although preliminary at this time, a recent publication by Oh et al. (55) demonstrated that the IDO metabolite HA may influence the expression of HO-1. The authors showed that HA was capable of blocking inducible NO synthase (iNOS) expression and NO production by up-regulating HO-1 expression. More specifically, the gaseous molecule CO, but not Fe^{2+} or bilirubin, was responsible for the inhibition of iNOS. Additionally, CO was observed to feedback on IDO leading to an up-regulation of its expression. These findings link the production of HA via IDO with the induction of the cytoprotective and immunomodulatory enzyme HO-1. However, it should be noted that these experiments were conducted in RAW 264.7 macrophages stimulated with LPS and IFN-γ and it remains to be shown whether the production of HA functions to up-regulate HO-1 expression in Treg. This may be a significant point following the observation that NO donors such as sodium nitroprusside also tend to up-regulate HO-1 expression in Jurkat T cells (56). These apparent contradictions may represent some of the differences in the cellular distribution and control of HO-1 and iNOS between APC and T cells, which need to be addressed. Finally, progression through an immune response must now be considered in the context of both APC and T cell maturation state. Major signals provided by activated T cells induce IDO expression including CTLA-4, CD40L, and IFN-γ signaling (51, 57). This would suggest that interactions between activated T cells or Treg and IDO expressing APC may constitute an important negative feedback loop for the resolution of immune responses or the maintenance of peripheral tolerance. One could also speculate that conditions associated with lymphopenia could perturb the homeostatic balance between APC and T cells, resulting in a shift toward an effector rather than a regulated response (58, 59).

Unresolved issues and directions for future investigations

In this brief review, we have proposed that CO produced by HO-1 in Treg may function as a suppressive molecule. It is important to note facets that currently lack evidence or are at conflict to the model presented; both of which will require further investigation (Table II) to bring clarity and validity. Among these is the need to understand the role, if any, of HO-1 expression and CO production by non-Treg (i.e., activated Teff). This will be achieved by evaluating T cell subpopulations in the presence and absence of HO-1 using cells derived from HO-1 knockout and wild-type mice. This model would also allow for testing the effects of CO and other products in the HO-1 reaction (e.g., biliverdin, bilirubin) on mediating specific effects on Treg and Teff functions.

Also of question is the source of substrate for HO-1 in T cells. Heme proteins are fairly ubiquitous and critical for a multitude of cellular functions. Heme proteins include not only hemoglobin and myoglobin, but several intracellular proteins such as cytochromes, respiratory burst enzymes, catalase, NO synthases, peroxidases, and pyrrolases (60). It has been postulated that these heme-containing proteins may liberate their heme prosthetic group in the course of tissue injury creating a pro-oxidant environment (61, 62). The free heme moiety can damage several cellular targets including lipid bilayers, cytoskeleton components, mitochondria, and the nucleus. Tissue damage, and the physiological responses to it, produce various molecules that induce HO-1 expression including heme, growth factors, and cytokines (63, 64). Thus, HO-1 protection is 2-fold, by reducing the effects of pro-oxidant heme and by producing anti-inflammatory by-products. In this context, the HO-1 system

Table II. *Future directions for testing the hypothesis that HO-1 contributes to the suppressive activities of Treg*[a]

Future Directions
Assessment of Treg function in HO-$1^{-/-}$ mice.
Induce and/or overexpress HO-1 in Treg/Teff; monitor for enhancement or induction of suppressive capacity.
Overexpression of HO-1 in T cells from Scurfy (Foxp3 mutant) mice as well as human subjects with IPEX; determine rescue of suppressive activity or in vivo attenuation of autoimmunity.
In vivo enhancement of EAE model of autoimmunity in HO-$1^{-/-}$ mice vs wild type.
Determine immunological memory (i.e., recall) to antigenic challenge in HO-$1^{-/-}$ mice; assess Ag-specific regulation and role of TCR.

[a] EAE, Experimental autoimmune encephalomyelitis.

may represent a progressive pathway by which tissue damage could elicit cytoprotection by activation of HO-1. In fact, a recent review has suggested that HO-1 may be a "therapeutic funnel" that mediates the action of several molecules including IL-10, rapamycin, ROS, and growth factors, via one of more of its reaction products, CO, biliverdin, or bilirubin (25).

At the same time, we would also note it remains unlikely that this proposed mechanism acts independently in vivo to elicit the collective suppressive effects of Treg. A more likely scenario involves CO acting in concert with the network of surface receptors and soluble factors associated with Treg. Undoubtedly, these complex interactions, along with the heterogeneity of T cells associated with regulatory functions, have contributed in the past to the discordant findings in regard to Treg function. Nonetheless, the mechanistic pathways outlined herein link the functions of tolerogenic APC and Treg through the biochemical pathways of heme and, potentially, tryptophan metabolism. Understanding these pathways will undoubtedly lead to novel insights into the pathogenesis of a variety of disorders associated with aberrant immunoregulation. The ability to manipulate Treg function should also facilitate the translation of Ag-specific immune therapies directed toward autoimmunity, allergy, and transplantation; applications of which are outlined in Ref. 65. Clearly, a greater understanding of Treg-mediated suppression is required to facilitate this progress.

Disclosures

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