



Heme oxygenase (HO-1) is involved in the negative regulation of contact sensitivity reaction

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Abstract:

Cutaneous contact sensitivity (CS) is a subtype of delayed-type sensitivity and is mediated by either CD4⁺ or CD8⁺ CS-effector T cells. CS can be induced by skin painting with haptens like trinitrophenyl chloride (TNP-Cl). We have previously shown that CS is under the negative regulation of T regulatory cells (Treg) induced by the *iv* injection of a high dose of homologous antigen or *via* epicutaneous application of any protein antigen prior to TNP-Cl painting. In this study, we examined the role of heme oxygenase (HO-1) in the negative regulation of CS in mice.

We found that *ip* injection of heme, an inducer of HO-1, before TNP-Cl sensitization strongly suppresses CS when compared to uninjected controls. Using a transfer out protocol, we showed that suppressor activity can be transferred with lymph node and spleen cells isolated from mice treated with heme for 7 days before TNP-Cl or sham immunization, which suggests a lack of antigen specificity of observed suppression. Negative selection with monoclonal antibodies and complement showed that regulatory cells induced *via* heme injection belong to the population of TCRαβ⁺ lymphocytes. Using CBA/J (H-2^k), SJL (H-2^s), and DBA1 (H-2^q) mice, we showed that the suppression mediated by HO-1 is major histocompatibility complex (MHC) unrestricted. *In vitro* treatment of heme induced Treg cells with tin protoporphyrin IX (SnPPIX), an inhibitor of HO activity, prior to adoptive transfer abolished the suppressor activity.

In summary, injection of heme results in the induction of antigen non-specific and MHC unrestricted TCRαβ⁺ Treg that suppress CS response in mice, possibly in a HO-1-dependent manner.

Key words:

heme oxygenase, T regulatory cells, inflammation, contact sensitivity

Abbreviations: CoPP – cobalt protoporphyrin, CS – contact sensitivity, HO – heme oxygenase, *ip* – intraperitoneal, *iv* – intravenous, LN – lymph node, mAb – monoclonal antibody, MHC – major histocompatibility complex, RC – rabbit complement, SnPPIX – tin protoporphyrin IX, SPL – spleen, TNP-Cl – trinitrophenyl chloride, Treg – T regulatory cells

Introduction

Three isoforms of heme oxygenase (HO) have been reported [14]. Among them, HO-1 is highly inducible by heme itself and several other stimuli including ni-

tric oxide or oxidative stress [4, 12]. On the contrary, HO-2 is constitutively expressed [19]. Moreover, HO-1 and HO-2 are differently regulated and expressed in tissues. HO-1 is ubiquitously induced in mammalian tissues and is localized to the endoplasmic reticulum, caveoli, and mitochondria, whereas HO-2 is expressed in the brain, endothelium, testis, distal nephron segments, and liver [3]. A third isoform, HO-3, was also described [23] but later shown to be a pseudogene [16]. The most widely studied of these proteins is HO-1, which has been reported as an important cytoprotective enzyme modulating tissue response to injury, while HO-2 regulates normal physiological cell activities.

HO-1 catalyzes heme degradation, leading to the local production of equimolar amounts of carbon monoxide (CO), biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase, and free-iron, which induces the expression of heavy chain ferritin, an iron-binding protein [24]. All three metabolites resulting from heme degradation by HO-1 may possess an immune protective effect [25]. Their roles in diabetes, inflammation, heart disease, hypertension, and pulmonary disease are all areas of vigorous research. Studies have focused not only on heme itself but also on its metabolic products as well as endogenous compounds involved in a vast number of genetic and metabolic processes affected when heme metabolism is perturbed. The use of pharmacological agents and genetic probes to manipulate HO has led to new insights into the complex relationship of the heme-HO system with the biological and pathological systems under investigation [1]. It should be noted that although the use of CO and biliverdin/bilirubin as therapeutic agents has been successful, these agents can be toxic to tissue when used at high levels. However, the strategies used to target HO-1 offer promising therapeutic opportunities for the effective treatment of many diseases, including diabetes [17], obesity [2], hypertension [8], heart disease [22], cancer [7], autoimmune diseases [10], and even allograft rejection [27].

Contact dermatitis is defined as an inflammatory response that may occur as a result of contact with external factors. Contact dermatitis can be caused by exposure to an irritant substance (irritant contact dermatitis) or an allergen in sensitized individuals (allergic contact dermatitis) [11]. Contact allergies affect nearly 15–20% of the general population [33], whereas occu-

pational contact dermatitis comprises up to 30% of all occupational diseases [11].

The treatment modalities for contact dermatitis are limited and accompanied by many side effects [15]. Thus, numerous efforts have been undertaken to develop a treatment able to control inflammatory reaction observed in contact dermatitis. Research on the pathogenesis of contact dermatitis, and development and testing of new drugs is possible thanks to animal models that imitate human diseases. Contact sensitivity (CS) in mice has been used as an experimental model of T cell mediated allergic contact dermatitis [5, 6, 28, 30]. The local inflammation caused by T cell recruitment and extravascular activation on antigen presenting cells often peaks at 24 h and is a model for Th1-mediated inflammation in diseases such as autoimmunity [21], allergies, immune resistance responses to some microbes and tumors [29], and immune response in graft rejection [20].

In the current study we determined the role of HO-1 in the negative regulation of inflammatory response observed during CS reaction. Our data suggest that activation of HO-1 *via* heme injection results in the induction of antigen non-specific and major histocompatibility complex (MHC) unrestricted TCR $\alpha\beta$ + T regulatory cells (Treg) that suppress the CS response in mice.

Materials and Methods

Animals

Six to eight-week-old male CBA/J (H-2^k), SJL/J (H-2^s), and DBA1 (H-2^d) mice were obtained from the breeding unit of the Department of Human Developmental Biology, Jagiellonian University, College of Medicine. Mice were fed autoclaved food and water. All experiments were conducted according to guidelines of the Jagiellonian University College of Medicine.

Reagents

Trinitrophenyl chloride (TNP-Cl) (Chemica Alta, Edmonton, Canada), heme (heme chloride), and tin protoporphyrin IX (SnPPIX) were purchased from Alexis Biochemicals (USA). Protein A was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, and Se-

pharose 4 Fast Flow was obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

Low-tox rabbit complement (RC) was obtained from Pel-Freeze Biologicals, Brown Deer, WI, USA.

Monoclonal antibodies and hybridomas

The following hybridomas were used: anti-TCR β (clone H57-597) from Dr. R. Kubo, Cytel Inc. (La Jolla, CA) and anti-TCR δ (clone UC7-13D5) from Dr. J. Bluestone, University of California, San Francisco, CA. Culture supernatants were purified with Protein A as described previously [32].

Active sensitization and measurement of contact sensitivity (CS) *in vivo*

Mice were actively sensitized by topical application of 0.15 ml of 5% TNP-Cl in an acetone-ethanol mixture (1:3) to the shaved abdomen and hind feet. Control mice were shaved and painted with the acetone-ethanol mixture alone as a sham sensitization. Four days later, mice were challenged on both sides of the ears with 10 μ l of 0.4% TNP-Cl in an olive oil-acetone mixture (1:1) [34].

The subsequent increase in ear thickness was measured 24 h later with an engineer's micrometer (Mitutoyo, Tokyo, Japan) and expressed in units of 10^{-2} mm \pm SE [9]. The background increase in ear thickness (\pm 2 units at 24 h) of similarly challenged non-sensitized littermates was subtracted from each experimental group to yield the net ear swelling, which was expressed in units of 10^{-2} mm \pm SE. Each experimental and control group consisted of 5–6 mice.

Treatment with heme and inhibition of HO-1 with SnPPIX

Mice were injected *ip* with 20 mg of heme per kg of body weight daily for seven days. In a model of actively induced CS, mice were sensitized with TNP-Cl and tested for CS four days later.

To test if treatment with heme induced regulatory cells, mice were *ip* injected with heme for one week as described above and auxiliary and inguinal lymph node (LN) were then isolated and used as a source of regulatory cells.

To determine if HO-1 is involved in heme induced suppression, 5×10^7 heme-induced regulatory cells were preincubated with 3×10^{-4} mM of SnPPIX for

60 min at 37°C before incubation with 7×10^7 of the CS-effector immune cells from TNP-Cl contact sensitized donors. After incubation, the cell mixture was washed and transferred *iv* into naive recipients. Mice were ear challenged with TNP-Cl within 30 min of cell transfer and tested for CS at 24 h, as described above.

Adoptive cell transfer of CS and cell mixing assay to evaluate suppression ("transfer out" protocol)

Donors of CS-immune effector cells were contact sensitized with 5% TNP-Cl. Auxiliary and inguinal LN and spleens were harvested on day "+4" and 7×10^7 immune cells were incubated for 30 min at 37°C in RPMI 1640 medium alone, washed, and then injected *iv* into normal syngeneic recipients (positive transfer). For the cell mixing assay, 7×10^7 of the CS-effector immune cells from TNP-Cl contact sensitized donors were incubated for 30 min at 37°C with 5×10^7 auxiliary and inguinal LN cells from mice that were *ip* injected with heme for 7 days (20 mg/kg). After incubation, the cell mixture was washed and transferred *iv* into naive recipients. Mice were ear challenged with TNP-Cl within 30 min after cell transfer and tested for CS at 24 h, as described above.

Phenotype of regulatory cells

To determine the phenotype of regulatory cells induced by heme treatment *in vivo*, LN cells isolated from mice *ip* injected with heme were incubated in PBS on ice with purified anti-TCR β or anti-TCR δ mAbs (1 μ g Ab/ 10^6 cells), or with PBS alone for 45 min. The cells were then washed and incubated with a predetermined 1:25 dilution of RC for 60 min at 37°C, and then washed and resuspended in PBS. After that, 4 day TNP-Cl immune CS-effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control), with 5×10^7 heme-induced regulatory cells treated with RC alone (suppressor control), or with 5×10^7 cell aliquots of regulatory cells treated with each appropriate monoclonal antibody (mAb) and RC. Cells were then washed and finally transferred *iv* into naive recipients that were challenged with TNP-Cl and their CS reactions were measured 24 h later. Positive controls and suppression controls were prepared as described in the previous paragraph.

Statistics

The paired two-tailed Student's *t*-test was used with $p < 0.05$ taken as the level of significance.

Results

In vivo induction of HO-1 inhibits contact sensitivity to hapten TNP-Cl

To determine if HO-1 is involved in the immunoregulation of inflammatory response in the skin we studied the contact sensitivity response to reactive hapten TNP-Cl.

CBA/J mice were *ip* injected with heme (Group B) or vehicle alone (Group C) for 7 days before sensitization with TNP-Cl. The positive control was mice that were TNP-Cl painted and did not receive any other treatment (Group A).

Data presented in Figure 1 show that treatment with heme strongly suppressed the CS response (Group B vs. A) whereas treatment with vehicle alone did not affect CS response (Group C vs. A). These data suggest that HO-1 is involved in the negative regulation of inflammatory response in the skin.

Treatment with heme induces regulatory cells that suppress CS T effector cells

The previous experiment showed that injection with heme strongly inhibited CS response. This could be a result of many different mechanisms. Among them

is induction of regulatory cells that could inhibit the action of CS T effector cells.

To find out whether suppression of CS observed after treatment with heme is a result of the action of heme-induced regulatory cells, we employed the transfer out model of CS. Data presented in Figure 2 show that LN cells isolated from either 7 day heme treated and sham immunized mice (Group B) or mice treated with heme and immunized with TNP-Cl (Group C) suppressed the adoptive transfer of CS when compared to the positive control (Group A). LN cells from mice injected with vehicle alone and co-transferred with CS-effector cells did not affect the CS reaction (Group D) when compared to the positive control (Group A). These data indicate that treatment with heme induces antigen non-specific regulatory cells that suppress CS response.

TCR $\alpha\beta$ but not TCR $\gamma\delta$ lymphocytes are involved in heme induced suppression of CS response

Transfer out experiments showed that suppression of the CS response caused by treatment with heme is a result of the action of regulatory cells. Therefore, in the next step we determined which type of cell is responsible for heme-induced suppression of the CS response.

Data presented in Figure 3 show that LN cells from 7 day heme treated mice depleted of TCR $\alpha\beta$ (Group C) lose their suppressor activity when compared to the suppression control (LN cells treated with RC alone, Group B). However, depletion of TCR $\gamma\delta$ cells did not affect suppressor activity of Treg cells (Group D).

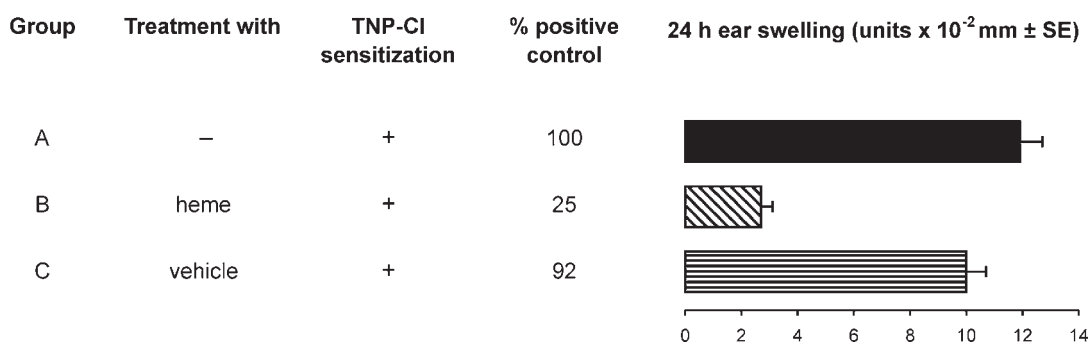


Fig. 1. *In vivo* induction of HO-1 inhibits contact sensitivity to hapten trinitrophenyl chloride (TNP-Cl). CBA/J mice were *ip* injected with 20 mg/kg of heme (Group B) or vehicle alone (Group C) for seven days. Animals were then actively immunized with TNP-Cl and tested for contact sensitivity (CS) four days later. In a positive control mice were sensitized with TNP-Cl only (Group A). Results were expressed in units of 10⁻² mm \pm SE. Each experimental group consisted of 5 or 6 mice. Statistical significance: Group B vs. A $p < 0.001$ and Group C vs. A $p = \text{NS}$

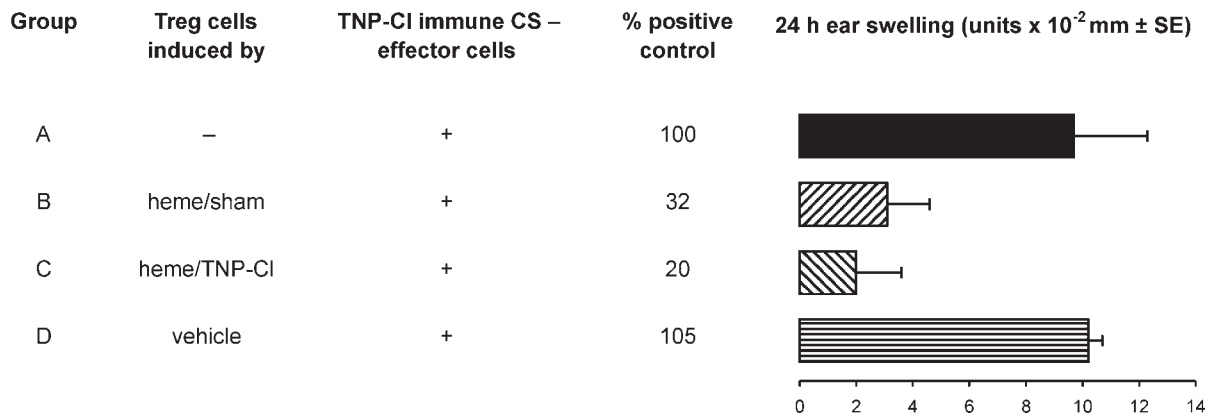


Fig. 2. Treatment with heme induces regulatory cells that suppress contact sensitivity (CS) T effector cells. 7×10^7 of 4 day trinitrophenyl chloride (TNP-CI) immune lymph node (LN) and spleen (SPL) cells were incubated for 30 min at 37°C in RPMI 1640 medium alone, washed and then injected *iv* into normal syngeneic recipients (positive transfer) (Group A). For the cell mixing assay, 7×10^7 of the contact sensitivity (CS)-effector immune cells from TNP-CI contact sensitized donors were incubated for 30 min at 37°C with 5×10^7 auxiliary and inguinal LN cells from mice *ip* injected with heme and sham sensitized (Group B) or heme injected and sensitized with TNP-CI (Group C) or injected with vehicle alone (Group D). After incubation, the cell mixtures were transferred *iv* into naive recipients that were tested for CS. Results were expressed in units of 10^{-2} mm ± SE. Each experimental group consisted of 5 or 6 mice. Statistical significance: Groups C and B vs. A $p < 0.001$ and Group D vs. A $p = NS$

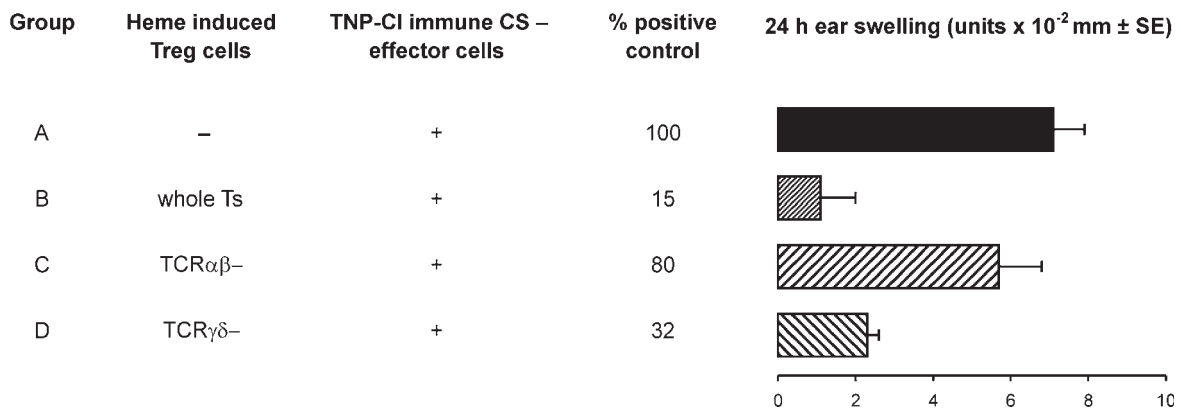


Fig. 3. TCR $\alpha\beta$ but not TCR $\gamma\delta$ lymphocytes are involved in heme induced suppression of contact sensitivity (CS) response. Lymph node cells isolated from mice *ip* injected with heme were incubated in PBS on ice with purified anti-TCR β mAb, anti-TCR δ mAb, or with PBS alone for 45 min. The cells were then washed and incubated with rabbit complement (RC) for 60 min at 37°C and then washed and resuspended in PBS. After that, 4 day trinitrophenyl chloride (TNP-CI) immune CS-effector cells (7×10^7) were incubated for 30 min at 37°C with medium alone (positive control) (Group A), or with 5×10^7 heme-induced regulatory cells treated with RC alone (whole Ts, suppressor control) (Group B), or with 5×10^7 cell aliquots of regulatory cells treated with either anti-TCR β or anti-TCR δ mAbs (Groups C and D respectively). The cells were then washed and transferred *iv* into naive recipients that were tested for CS. Results are expressed in units of 10^{-2} mm ± SE. Each experimental group consisted of 5 or 6 mice. Statistical significance: Groups B and D vs. A $p < 0.001$ and Group C vs. A $p = NS$

These data clearly show that treatment with heme induced Treg that belong to the population of TCR $\alpha\beta$ + lymphocytes.

Heme-induced TCR $\alpha\beta$ regulatory cells are MHC unrestricted

To determine whether heme-induced Treg are MHC restricted we performed a transfer out experiment

where CS-effector cells from CBA/J (H-2^k) donors were treated with heme-induced regulatory cells isolated from syngeneic CBA/J (H-2^k) mice (Group B) or allogeneic SJL/J (H-2^s) or DBA1 (H-2^q) mice (Groups C and D, respectively) before adoptive transfer into CBA/J (H-2^k) recipients.

Data presented in Figure 4 show that either syngeneic (Group B) or allogeneic LN cells (Groups C and D) were able to suppress the activity of CS-effector cells.

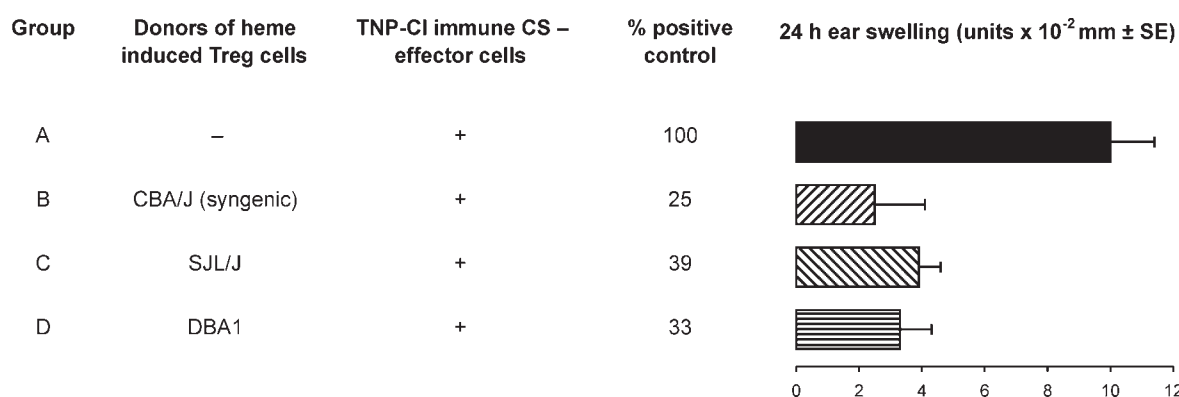


Fig. 4. Heme-induced TCR $\alpha\beta$ regulatory cells are major histocompatibility complex (MHC) unrestricted. To determine whether heme-induced T regulatory cells (Treg) are MHC restricted, transfer out experiments were carried out where 7×10^7 of contact sensitivity (CS)-effector cells from CBA/J (H-2^k) donors were incubated with 5×10^7 of heme-induced regulatory cells isolated from syngeneic CBA/J (H-2^k) mice (Group B) or allogeneic SJL/J (H-2^s) or DBA1 (H-2^d) mice (Groups C and D respectively) before adoptive transfer into CBA/J (H-2^k) recipients. The recipient mice were then challenged with TNP-CI and tested for CS. Results are expressed in units of 10^{-2} mm \pm SE. Each experimental group consisted of 5 or 6 mice. Statistical significance: Groups D, C and B vs. A $p < 0.001$

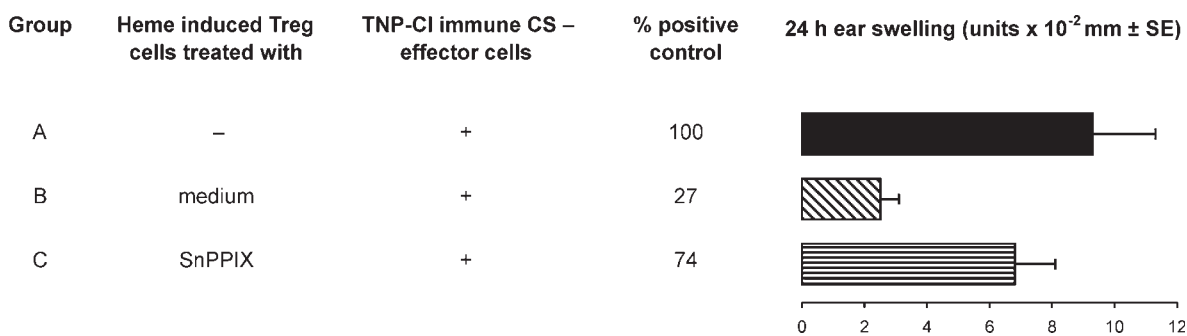


Fig. 5. Blocking of HO-1 with tin protoporphyrin IX (SnPPIX) reverses heme induced suppression of contact sensitivity (CS) response. To determine if HO-1 is indeed involved in heme induced suppression, 5×10^7 of LN cells isolated from mice treated for 7 days with heme were preincubated with SnPPIX (Group C) or with medium alone (Group B) for 60 min at 37°C before incubation with 7×10^7 of CS-effector immune cells and subsequent adoptive transfer into naive syngeneic recipients. Recipients in a positive control received CS-effector immune cells only (Group A). Statistical significance: Group B vs. A $p < 0.001$ and Group C vs. B $p < 0.001$

These data indicate that heme-induced Treg are MHC unrestricted.

Blocking of HO-1 with protoporphyrin reverses heme-induced suppression of the CS response

To determine if HO-1 is indeed involved in heme-induced suppression, heme-induced Treg were preincubated with SnPPIX for 60 min at 37°C before incubation with CS-effector immune cells and subsequent adoptive transfer into naive syngeneic recipients (Group C).

Data presented in Figure 5 show that heme-induced Treg lose their suppressor activity when pretreated

with the HO-1 inhibitor SnPPIX (Group C) compared to untreated Treg (Group B).

Discussion

The goal of our current work was to determine the role of HO-1 in the negative regulation of inflammatory response observed during the contact sensitivity reaction. Whereas the general anti-inflammatory activity of HO-1 is well studied [10, 27, 35, 36], its role in cutaneous inflammation and the impact of pharma-

cological inhibition or induction of HO-1 on T cell dependent skin reactions have not been described.

Our work using a model of CS shows that 7 days of treatment with heme before sensitization with TNP-CI results in strong inhibition of the inflammatory response localized in hapten challenged skin. Our observation is in line with findings made by Listopad et al., who showed that cutaneous inflammation was abrogated by treatment with the HO-1 inducer cobalt protoporphyrin (CoPP) [18].

The adoptive cell transfer experiments showed that LN cells isolated from mice injected with heme for 7 days suppress the ability of CS effector cells to transfer the CS reaction. These data suggest that treatment with heme induces cells that can suppress the activity of CS effector cells. Moreover, suppressor activity possess LN cells isolated from donors who were either TNP-CI sensitized or sham immunized after heme injection. These data indicate that heme-induced regulatory cells are antigen non-specific. Our finding is similar to observations made in other systems, like animal model of asthma [36] and graft rejection [27], where authors showed lack of antigen specificity of HO-1 induced suppression.

Additionally, both syngeneic and allogeneic heme-induced regulatory cells could suppress adoptive transfer of the T cell mediated CS response. This experiment clearly shows that heme-induced regulatory cells are MHC unrestricted. Our previous work on immunoregulation showed that T cells with regulatory activity may lack MHC restriction [26, 31].

Negative selection of heme induced regulatory cells showed that TCR $\alpha\beta$ but not TCR $\gamma\delta$ lymphocytes are involved in the negative regulation of CS response. Similarly, other researchers using various experimental models found that TCR $\alpha\beta$ T lymphocytes are involved in HO-1 induced immunosuppression [13, 24]. It was shown that HO-1 exerts its protective effect on asthma through a mechanism mediated by foxp3+CD4+CD25+ Treg, interleulin (IL)-10, and membrane-bound transforming growth factor (TGF)- β [35]. Other studies showed that HO-1 inhibits T cell-dependent skin inflammation by affecting antigen presenting cells [18]. CoPP induced HO-1 inhibited antigen-presenting cells and depressed secretion of tumor necrosis factor- α and IL-12, whereas it increased synthesis of IL-10.

At present the mechanism of heme induced suppression in the CS reaction to TNP is not clear. We have shown that treatment with heme induces regula-

tory cells that belong to the population of TCR $\alpha\beta$ T lymphocytes and inhibit the activity of TNP specific CS effector cells. Additionally, the heme induced suppression was reversed by the HO-1 inhibitor SnPPiX, confirming the involvement of HO-1 in heme induced suppression of TNP specific CS response.

Further work is required to better understand the role of HO-1 in the negative regulation of the inflammatory reaction observed in the CS response, which can have an important impact on therapy of inflammatory diseases of the skin.

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