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Haptoglobin genotype modulates the balance of Th1/Th2 cytokines produced by macrophages exposed to free hemoglobin

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Abstract

The haptoglobin genotype has been demonstrated to be an independent risk factor for CVD in multiple epidemiological studies. The primary function of haptoglobin is to mitigate the deleterious effects of extracorpuscular hemoglobin. We sought to determine if the protein products of the two haptoglobin alleles differed in their ability to modulate the cytokine profile produced by macrophages in response to hemoglobin. Peripheral blood mononuclear cells were isolated from normal human volunteers and cultured in the presence of complexes formed by the protein products of the two different haptoglobin alleles with hemoglobin. The release of specific cytokines in the conditioned media of these cells was assessed by ELISA. We found that the haptoglobin 1 allele protein product–hemoglobin complex stimulated the secretion of significantly more Il-6 and Il-10 than the haptoglobin 2 allele protein product–hemoglobin complex. We demonstrate that the release of these cytokines is dependent on the liganding of the haptoglobin–hemoglobin complex to the CD163 receptor and the activity of casein kinase II. Haptoglobin genotype modulates the balance of inflammatory (Th1) and anti-inflammatory (Th2) cytokines produced by macrophages exposed to free hemoglobin. This may have implications in understanding inter-individual differences in the inflammatory response to hemorrhage.

Keywords: Haptoglobin; Hemoglobin; Macrophages; Inflammation; Hemorrhage

1. Introduction

Extracorpuscular hemoglobin (Hb), released from the red blood cell, is emerging as an important mediator of vascular disease [1]. The toxicity and inflammatory nature of free Hb is due to its ability to consume nitric oxide (NO) and to serve as a Fenton reagent producing the highly reactive superoxide and hydroxyl radicals. The primary defense mechanism in the body against the deleterious effects of free Hb is provided by the haptoglobin (Hp) protein. Hp binds to Hb, essentially irreversibly, and inhibits in large part the ability of Hb to serve as an oxidant [2]. Hp also promotes the clearance of free Hb. This clearance is important because the Hp–Hb complex is not inert, it can still catalyze formation of reactive oxygen species and consume NO [1]. There exist two pathways in the plasma compartment for clearing the Hp–Hb complex. The first is via a poorly characterized receptor on the liver hepatocyte (90%). The second is via the CD163 receptor expressed on monocytes (10%) [3]. However, in the extravascular compartment the only pathway that exists for the clearance of the Hp–Hb complex is via the CD163 receptor expressed on tissue macrophages. Release of Hb into the extravascular space is a common feature of all hemorrhages and is thought to play a major role in promoting atherosclerotic plaque instability [4] and arterial vasospasm [5]. Due to the vital role of Hp in mitigating the deleterious effects of Hb, genetic variations in the function of Hp would be expected to be of considerable clinical importance.

The Hp locus at chromosome 16q22 is polymorphic in man [6]. There exist two classes of Hp alleles, denoted by 1 and 2. The Hp genotype of an individual can therefore be described as being Hp 1-1 (homozygous for the 1 allele), Hp 2-2 (homozygous for the 2 allele) or Hp 2-1 (heterozygote).

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The Hp polymorphism is an extremely common polymorphism. The frequency of the three genotypes in the western world is 20% Hp 1-1, 50% Hp 2-1 and 30% Hp 2-2 [6]. We and others have demonstrated in multiple longitudinal and cross-sectional studies from diverse geographic and ethnic groups that the Hp genotype is an independent risk factor for the development of vascular disease [7,8]. The ability to recapitulate this association in mice genetically modified at the Hp locus [9] strongly supports the notion that differences in the structure and biological activity of the different Hp allelic protein products can explain the above described clinical–genotype association.

Structural as well as functional differences between the different Hp genotypes have been established. The key structural difference between the different haptoglobin types is the stoichiometry of the haptoglobin polymer [6]. The protein product of the Hp gene is found in serum as a polymer of between 2 and 10 covalently linked monomers. The stoichiometry and therefore the size of the polymer is Hp genotype dependent due to differences in the valencies of the Hp 1 (monovalent) and Hp 2 (bivalent) allelic protein products. The net results of these differences in valency, as confirmed by electron microscopy, is that Hp is found as a dimer in Hp 1-1 individuals, a linear polymer in Hp 2-1 individuals, and a cyclic polymer in Hp 2-2 individuals [6].

We have also identified functional differences between the Hp genotypes in terms of how well the Hp molecule prevents Hb induced oxidation, with the Hp 1-1 protein being a superior antioxidant to the Hp 2-2 protein [2,10,11]. Amplifying the magnitude of the disparity in antioxidant protection provided by Hp 1-1 and Hp 2-2, we have found that the Hp 1-1–Hb complex is scavenged much more rapidly than the Hp 2-2–Hb complex by the CD163 pathway [10].

In addition to serving as an antioxidant it has been proposed that Hp, as mediated through the CD163 receptor, may also have an immunomodulatory function. Philipidis has recently described the induction of interleukin-10 and heme-oxygenase-1 by the Hp–Hb complex in macrophages [12] but did not investigate whether there may exist differences between Hp 1-1 and Hp 2-2 in this immodulatory function. In this manuscript, we demonstrate that the Hp genotype determines the nature of the cytokine response produced by macrophages in response to hemoglobin. The implications of this finding in modulating the inflammatory response to hemorrhage are discussed.

2. Methods

2.1. Stimulation of human peripheral blood derived mononuclear cells (PBMCs) with Hp–Hb complex

Hp 1-1 and Hp 2-2 were purified by affinity chromatography from human serum [10]. Hb was freshly prepared from lysed red blood cells [10]. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with Histopaque-1077 solution (Sigma) and grown for 18 h in 96-well plates in RPMI-1640 supplemented with 10% FBS and 40 ng/ml dexamethasone. These culture conditions have previously been demonstrated to induce maximal expression of the Hp–Hb receptor CD163 on PBMCs [10]. After 18 h, the cells were incubated with the Hp–Hb complex (1:1 molar ratio) at a defined concentration and for a defined interval as will be described in Section 3.

2.2. Measurement of cytokines in the conditioned media of PBMCs stimulated with Hp–Hb by multi-protein array and ELISA

The relative amount of a battery of cytokines (interleukins-1, 2, 3, 4, 6, 7, 8, 10, 11, 12, 13) in the conditioned media of Hp 1-1–Hb or Hp 2-2–Hb treated PBMCs was determined by a multi-protein array (Ray-Biotech, Atlanta, GA) which permits the simulantaneous assessment of multiple factors from the same conditioned media. The detection system for these arrays is similar to conventional ELISA using high specificity antibodies in a sandwich format coupled to a detection system for the antigen–antibody complex utilizing a chemiluminescent reagent. Using these arrays we assessed the conditioned media at a single time point (24 h) and dose of the Hp–Hb complex (1 mg/ml).

Quantitative assessment of the amount of Il-6, Il-10 or TNFa was assessed by cytokine specific ELISAs (Biosource, USA). Recombinant human Il-10, Il-6 or TNFa was used as a standard.

2.3. Inhibition of cytokine expression with CD163 neutralizing antibodies and kinase inhibitors

PBMCs were stimulated with Hp–Hb in the presence or absence of the following reagents: RM 3/1 CD163 antibody (BMA biomedical, Angst, Switzerland) (20 μg/ml), CD11b antibody (Serotec) (20 μg/ml), genistein (100 μM), bisindolylmaleimide III (Bis III) (5–25 μM), or 5,6-dichloro-1b-D-ribofuranosylbenzimidazole (DRB) (5–100 μM) and the production of cytokines assessed. For these studies the kinase inhibitors were added 30 min prior to the addition of the Hp–Hb complex to the PBMCs.

2.4. Statistical analysis

Data are expressed as the mean ± S.M.E. Differences between groups were assessed by *t*-test.

3. Results

3.1. Cytokine profile of PBMCs stimulated by Hp–Hb

Our initial approach to assess the cytokine profile produced by Hp 1-1 or Hp 2-2 stimulated PBMCs was to analyze the conditioned media using a multi-protein array which

permits the simultaneous semi-quantitative assessment of multiple cytokines from the same conditioned media. We hoped to identify several cytokines that were differentially expressed by Hp 1–Hb and Hp 2–Hb in this approach and then focus more extensively (time course, dose response) on these cytokines using specific ELISAs. While several cytokines were increased with Hp–Hb treatment of the PBMCS, the only reproducible ($n = 3$) difference between Hp 1-1–Hb and Hp 2-2–Hb stimulated PBMCs was in the expression of Il-10 and Il-6 where a several fold increase was found in media conditioned by Hp 1-1 treated macrophages as compared to Hp 2-2 treated macrophages.

3.2. Hp type differences in interleukin-10 production by PBMCs

Subsequent to the findings in the multi-protein array described above we used a quantitative Il-10 specific ELISA to determine the dose dependency and time course of the secretion of Il-10 from PBMCs after stimulation by Hp–Hb (Fig. 1A and B). Consistent with the multi-protein array we found that Hp 1-1–Hb complexes induced significantly more Il-10 than Hp 2-2–Hb complexes. Stimulation of Il-10 production by Hp–Hb was rapid and was found to occur at physiological concentrations of Hp–Hb. The normal concentration of the Hp–Hb complex in blood is 25 nM (5 μg/ml) at which no appreciable stimulation of Il-10 was seen by Hp 1-1–Hb or Hp 2-2–Hb. However, at 150 nM (50 μg/ml) Hp–Hb which could be achieved locally at sites of hemorrhage and hemolysis (50 μg of Hb corresponds to the amount of Hb released from less than 0.5 μl of blood) there was a significant increase in Il-10 release induced by Hp 1-1–Hb complexes as compared to Hp 2-2–Hb. In order to determine if the ability of Hp–Hb to induce Il-10 was mediated via the CD163 receptor we assessed Hp–Hb inducible cytokine production in the presence of the CD163 blocking antibody RM 3/1. We found that RM 3/1 completely blocked the ability of Hp–Hb to induce Il-10 (Fig. 1C). No inhibition of Il-10 production was noted with an antibody to CD11b which does not block the interaction of Hp–Hb with the PBMC (not shown).

3.3. Hp type differences in interleukin-6 production by PBMCs

Analogous to our approach with Il-10 we sought to assess the regulation of Il-6 by Hp–Hb complexes using a quantitative ELISA for Il-6. Both the time course (not shown) and the dose response curve (Fig. 2A) were remarkably similar to that seen for Il-10 induction with Hp 1-1–Hb stimulating significantly greater Il-6 as compared to Hp 2-2–Hb from PBMCs. Furthermore, similar to Il-10 we sought to assess the importance of CD163 in the induction of Il-6 by Hp–Hb and found that RM 3/1 antibody could completely block the induction of Il-6 by Hp–Hb (Fig. 2B).

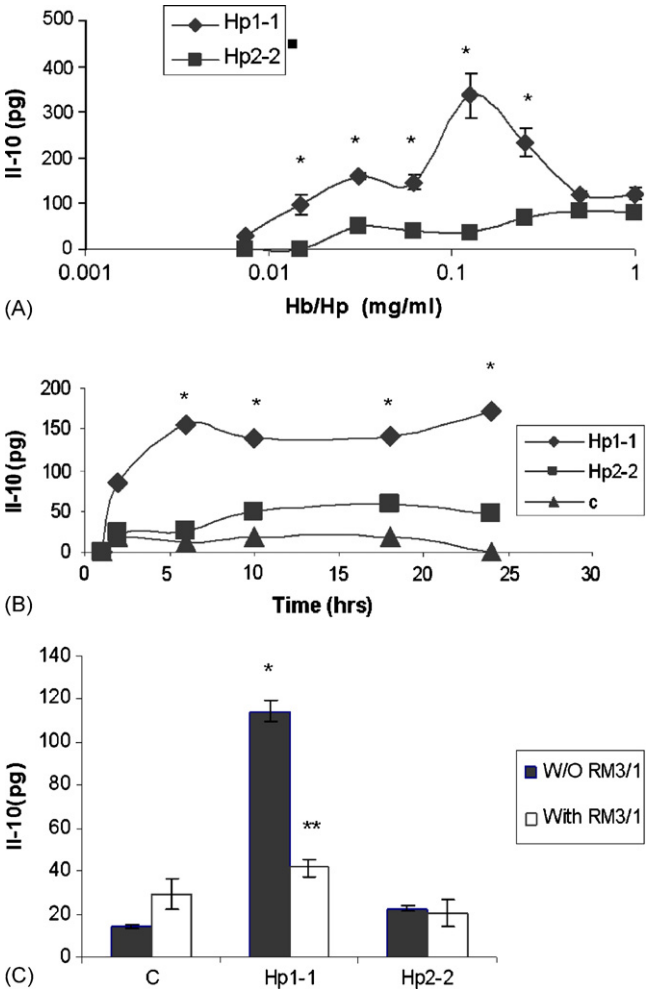

Fig. 1. Regulation of Il-10 by Hp–Hb in PBMCs. (A) Dose reponse curve. Hp 1-1–Hb or Hp 2-2–Hb were incubated with PBMCs for 24 h and the amount of Il-10 (in 50 μl of media) determined by ELISA. Data are composite of 10 independent experiments. Asterisk (*) indicates a significant difference in Il-10 production between Hp 1-1 and Hp 2-2 ($p < 0.01$). (B) Time course. Hp 1-1–Hb or Hp 2-2–Hb at a concentration of 0.250 mg/ml were incubated with PBMCs and the amount of Il-10 assessed by ELISA after 2, 5, 10, 18 or 24 h. Data are the composite of six independent experiments. Asterisk (*) indicates a significant difference in Il-10 production between Hp 1-1 and Hp 2-2 ($p < 0.01$). (C) Inhibition of the induction of Il-10 by anti-CD163 antibody. PBMCs were incubated with 0.1 mg/ml Hp–Hb in the presence or absence of antibody RM 3/1 (20 μg/ml) and the amount of Il-10 assessed after 24 h by ELISA. Asterisk (*) indicates a significant increase in Il-10 production with Hp 1-1–Hb compared to control cells or to Hp 2-2–Hb ($p < 0.01$). Asterisks (**) indicate a significant reduction in Hp 1-1–Hb stimulated Il-10 production by RM 3/1 ($p < 0.01$). Data are the composite of three independent experiments.

3.4. Hp–Hb induction of TNFa in PBMCs is CD163 independent

The production of the pro-inflammatory cytokine TNFa is also increased in PBMCs when they are stimulated by Hp–Hb, albeit to a significantly less degree than Il-6 or Il-10 (TNFa is increased 2–3-fold by Hp–Hb as opposed to Il-6 or Il-10 which are increased 100- or 20-fold, respectively, by Hp–Hb). We sought to determine if the induction of TNFa by

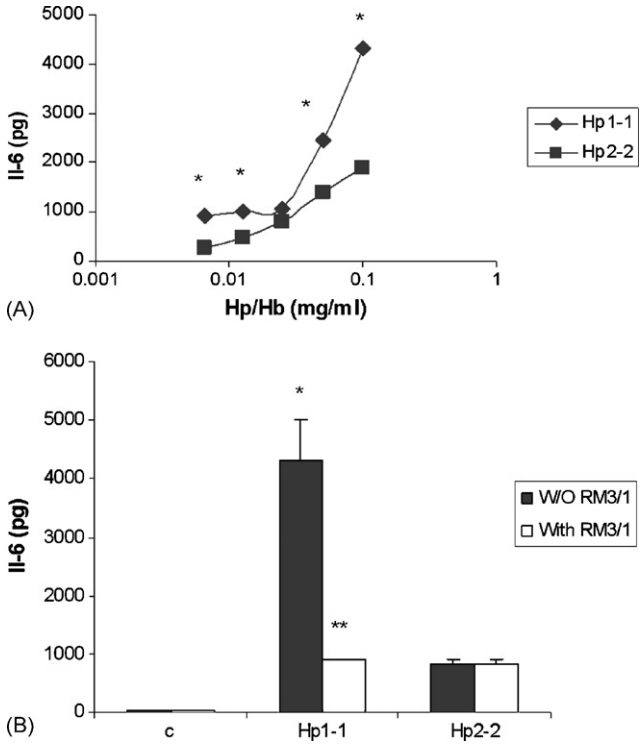

Fig. 2. Regulation of Il-6 by Hp–Hb in PBMCs. (A) Dose reponse curve. Hp 1-1–Hb or Hp 2-2–Hb were incubated with PBMCs for 24 h and the amount of Il-6 (per 50 μl of media) determined by ELISA. Data are composite of six independent experiments. Asterisk (*) indicates a significant difference in Il-6 production between Hp 1-1–Hb and Hp 2-2–Hb complexes ($p < 0.01$). (B) Inhibition of the induction of Il-6 by anti-CD163 antibody. PBMCs were incubated with 0.1 mg/ml Hp–Hb in the presence or absence of antibody RM 3/1 (20 μg/ml) and the amount of Il-10 assessed after 24 h by ELISA. Asterisk (*) indicates a significant increase in Il-6 production with Hp 1-1–Hb compared to control cells or to Hp 2-2–Hb ($p < 0.01$). Asterisks (**) indicate a significant reduction in Hp 1-1–Hb stimulated Il-6 production by RM 3/1 ($p < 0.01$). Data are the composite of three independent experiments.

Hp–Hb was CD163 dependent using the RM 3/1 antibody. Using the same conditioned media in which we were able to demonstrate an inhibition of Il-6 and Il-10 production we failed to see any effect of blocking the CD163 receptor on the regulation of TNFa suggesting that the induction of TNFa in PBMCs by Hp–Hb is CD163 independent (Fig. 3).

3.5. Hp–Hb induction of Il-6 and Il-10 in PBMCs is absolutely dependent on casein kinase II (CKII)

Prior to the identification of Hp–Hb as the physiological ligand for CD163 it was demonstrated that numerous cytokines could be induced by cross-linking CD163 with a monoclonal antibody and that this cytokine induction could be blocked using tyrosine kinase, protein kinase C and CKII inhibitors [13]. We investigated the ability of these protein kinase inhibitors for their ability to block the Hp–Hb induced induction of Il-6 and Il-10. We found that tyrosine kinase inhibition had no effect ($10 \pm 5\%$, $p = \text{NS}$) and protein kinase C inhibition had only a small affect on

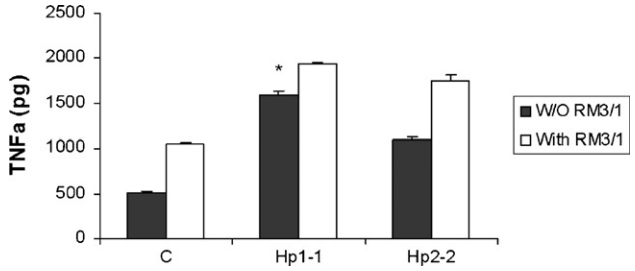

Fig. 3. The induction of TNFa by Hp–Hb is not dependent on the CD163 receptor. PBMCs were incubated with 0.1 mg/ml Hp–Hb in the presence or absence of antibody RM 3/1 (20 μg/ml) and the amount of TNFa assessed after 24 h by ELISA. Asterisk (*) indicates a significant increase in TNFa production with Hp–Hb compared to control cells ($p < 0.01$). Hb alone also resulted in a significant increase in TNFa (not shown). RM 3/1 antibody did not block the Hp–Hb induced induction of TNFa but rather resulted in a non-significant increase in TNFa production by the PBMCs. Data are the composite of three independent experiments.

the induction of these cytokines by Hp–Hb (inhibition of $25 \pm 10\%$, $p = 0.05$). However, the casein kinase II inhibitor DRB was found to completely inhibit the induction of Il-6 and Il-10 by Hp–Hb (Fig. 4). Furthermore, a structurally distinct CKII inhibitor, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), was similarly capable of completely blocking the induction of Il-6 and Il-10 by Hp–Hb (not shown). Notably, treatment of PBMCs with casein kinase II inhibitors did not block Hp–Hb induced induction of TNFa (Fig. 4). On the contrary, TNFa production was increased after treatment with casein kinase II inhibition, most likely due to inhibition of the negative feedback mechanism that Il-6 and Il-10 have on TNFa production [14,15].

4. Discussion

We have provided evidence in this study for a plausible mechanism contributing to the increased vascular protection found in Hp 1-1 individuals as compared to Hp 2-2 individuals by altering the pro-inflammatory (Th1) and anti-inflammatory (Th2) balance of cytokine production by

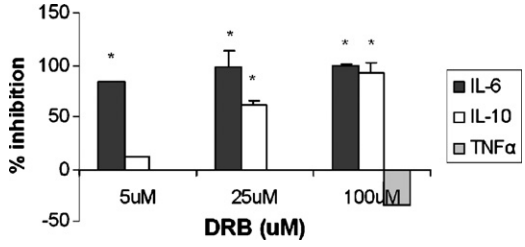

Fig. 4. Regulation of Hp–Hb induced cytokine expression by the casein kinase II inhibitor DRB. DRB was added at the concentrations indicated to PBMCs 30 min prior to the addition of the Hp 1-1–Hb complex (0.100 mg/ml). Il-6, Il-10 and TNFa were assessed by ELISA at 24 h. Data are expressed as the percent inhibition of cytokine production induced by Hp 1-1–Hb in the absence of DRB. DRB inhibited the release of Il-6 and Il-10 induced by Hp–Hb ($^{*}p < 0.01$) but failed to inhibit the induction of TNFa. Data are the composite of six independent experiments.

macrophages. We have shown for the first time that Hp–Hb 1-1 complexes induce a significantly greater amount of two important anti-inflammatory cytokines, Il-6 and Il-10, as compared to Hp–Hb 2-2 complexes in macrophages via a CD163 dependent mechanism.

There exists considerable data supporting an anti-inflammatory role for both Il-10 and Il-6 as well as CD163 [14–23]. Il-10 has been proposed to play a major role in protection against atherosclerosis and ischemia-reperfusion injury [14,16–18]. Il-10 has been shown to decrease TNFa and Il-12 production, inhibit NF-κB activation, matrix metalloproteinase synthesis, tissue factor expression and cell death. In vivo, Il-10 has been shown to prevent exaggerated atherosclerosis development in ApoE knockout mice and to protect the reperfused myocardium from injury by the suppression of neutrophil recruitment. Il-6 was initially suggested to be a pro-inflammatory cytokine perhaps due to the ability of Il-6 to stimulate the production of acute phase proteins which were thought to amplify the inflammatory response [15,19–23]. However, recent findings have shown that Il-6 has many anti-inflammatory and immunosuppressive effects [15,19–29]. First, in vitro Il-6 stimulates the production of tissue inhibitor of matrix metalloproteinase (TIMP) [23], stimulates the production of the soluble receptors for Il-1 and TNFa (thereby blocking the pro-inflammatory activity of Il-1 and TNFa) [15] and decreases the production of TNFa by macrophages [15]. Second, Il-6 knockout mice display increased atherosclerotic lesion size [19] and a more exaggerated inflammatory and TNF response to a variety of stimuli [20]. Third, the anti-inflammatory effects of both Il-10 and Il-6 are associated with the expression of members of the SOCS which serve as negative regulators of cytokine signaling by downregulating transcription and release of pro-inflammatory cytokines such as TNFa that depend on the JAK/STAT pathway [27,28]. Finally, many of the acute phase proteins which are induced by Il-6 are themselves anti-inflammatory (haptoglobin, anti-trypsin, macroglobulin) as they serve to put the brakes on the inflammatory reaction [15]. The distribution and temporal expression of CD163 has suggested that CD163 is part of the anti-inflammatory response. Specifically, peak high levels of CD163 expression are generally seen in the resolving phase of an injury and in the margins of the wound where healing is occurring [30].

We have also found that TNFa is induced by the Hp–Hb complex and that this induction appears to be slightly greater for Hp 1-1–Hb than for Hp 2-2–Hb. However, there are several fundamental differences between the induction of TNFa, an inflammatory cytokine, and Il-6/Il-10 anti-inflammatory cytokines by Hp–Hb. First, Il-6 and Il-10 are induced over 100- and 20-fold, respectively, while TNFa is only induced 2–3-fold by Hp–Hb. Second, for Il-6/Il-10 there is a 5–10-fold difference in the amount of cytokine produced by Hp 1-1 versus Hp 2-2–Hb complexes while for TNFa there is only a 50% difference between them. Third, Hb alone can induce the production of TNFa while it has no effect on the induction of Il-6/Il-10. Finally, TNFa is stimulated by a CD163 casein kinase independent pathway unlike Il-10 and Il-6.

Therefore, we propose that the Hp genotype plays a critical role after hemorrhage by specifying the nature and intensity of the macrophage response to extracorpuscular and extravascular Hb (Fig. 5). The Hp 1 and Hp 2 allelic protein products appear to play opposite roles in modulating macrophage function after hemorrhage. After hemorrhage in individuals with the Hp 1 genotype, a redox inactive Hp–Hb 1 complex is generated that binds to the CD163 receptor and induces the secretion of anti-inflammatory cytokines. Conversely,

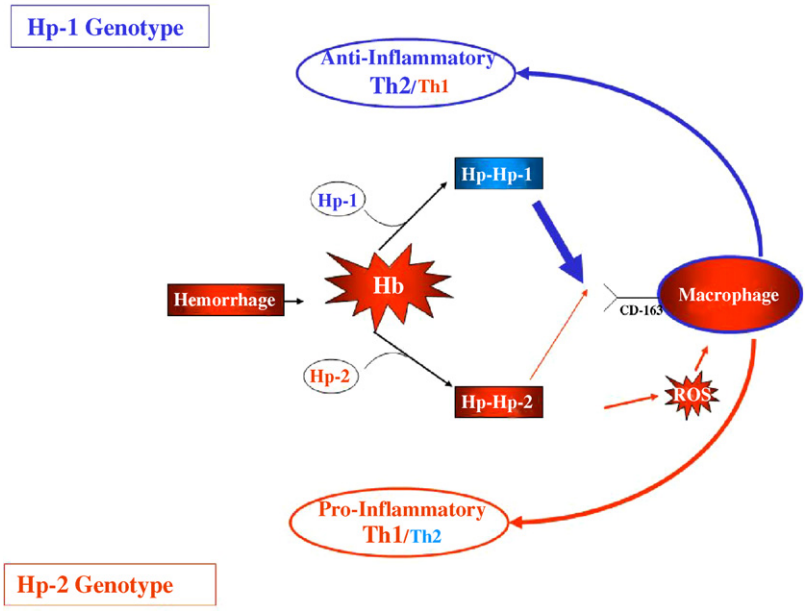

Fig. 5. Role of the Hp genotype in determining the response of the macrophage to hemorrhage. Th1, inflammatory cytokines; Th2, anti-inflammatory cytokines.

after hemorrhage in individuals with the Hp 2 genotype, a redox active Hp–Hb 2 complex is generated that produces reactive oxygen species (ROS) [11] which may mediate vascular damage and inflammation. Notably the Hp–Hb 2 complex is a poor inducer of the anti-inflammatory CD163 pathway.

The differences we have observed between Hp 1-1 and Hp 2-2 in CD163 dependent signaling cannot be explained by differences between the Hp types in their affinity for CD163 [10,11]. We have shown here that there is an absolute dependence of CD163 signaling on casein kinase II. There exists a direct association between casein kinase II and the CD163 receptor as demonstrated using the yeast two hybrid approach [13]. Future studies will establish if the nature of the association between casein kinase II or the activity of casein kinase II are differentially modulated by the binding of the different Hp–Hb complexes to CD163.

Acknowledgments

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