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Haptoglobin Function and Regulation in Autoimmune Diseases

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1. Introduction

Haptoglobin (Hp) is an acute phase protein, primarily synthesized in the liver and secreted into the plasma. Hp is also produced in other tissues including lung, skin, spleen, brain, intestine, arterial vessels and kidney, but to a lesser extent (D'Armiento et al., 1997; Pelletier et al., 1998; Yang et al., 2000). The normal concentration in human plasma ranges from 0.3- 3 mg/ml and increases several fold in the occurrence of local or systemic inflammation. Increased production of Hp is the result of transcriptional activation of the Hp gene (Baumann & Jahreis, 1983; Oliviero et al., 1987) by pro-inflammatory cytokines such as interleukin(IL)-1β, IL-6, and Tumor Necrosis factor (TNF) (Baumann et al., 1989). The result of pro-inflammatory cytokine signalling is the activation of essential transcription factors that are needed for the expression of Hp (STAT, C/EBP, PEA3). Among vertebrate species, the promoter of the Hp gene is conserved and contains three key regulatory elements that include two C/EBPβ recognition sequences that flank a STAT binding site (Wang et al., 2001).

2. Haptoglobin structure and polymorphism

Hp is composed of four chains: 2 α-chains (~9kDa each) and 2 β-chains (~33kDa each). Alpha and beta chains are encoded by a single gene and are synthesized as a single polypeptide chain that is proteolytically cleaved into a short α-chain and a long β-chain that remain connected through a disulfide bond. In addition an α-β unit is linked to another α-β unit also by a disulphide bond (Wejman et al., 1984).
In humans, Hp is characterized by a genetic polymorphism which arises from differences in α-chains while β–chains are identical in all Hp types. The Hp locus is located on chromosome 16 (16q22.1). Two common alleles exist for Hp, Hp1 and Hp2 that give rise to three major phenotypes. Individuals that are homozygous for allele Hp1 express the phenotype 1-1, those homozygous for allele Hp2, express phenotype Hp2-2, and heterozygous individuals express phenotype Hp1-2. The Hp1 allele is organized in 5 exons. The first 4 exons encode for the α-subunit while exon 5 encodes for the β-subunit. The Hp2 allele consists of 7 exons, the first 6 exons encode for a larger form of α–subunit and exon 7 encodes for the β-subunit. The larger form of the Hp α-subunit seems to originate from an

intragenic duplication of exons 3 and 4. As a consequence, the Hp1-1 phenotype consists of homodimers of two α-β units, but Hp1-2 and Hp2-2 consist of polymers, as the cysteine that forms the disulfide bond between α-subunits is duplicated in Hp2. The resultant stoichiometry is for Hp1-1 homodimers of (α1-β)2; for Hp2-1 linear polymers of (α1-β)2 + (α2+β)n (n=0,1,2, etc); and for Hp2-2 cyclic polymers (α2+β)n (n=3,4 etc) (Van et al., 2004). The distribution of Hp1 and Hp2 alleles in the world population differs according to the racial origin. Native populations in South America have the highest frequency of the Hp1 allele (0.7), while Asian populations have the lowest frequency of the Hp1 allele (0.2). The European population has a Hp1 allele frequency of 0.4 with a phenotypic distribution of approximately 15% Hp1-1, 50% Hp 1-2, and 35% Hp2-2 (Carter & Worwood, 2007). Importantly, the three different Hp phenotypes exhibit functional differences that can have clinical and biological consequences.

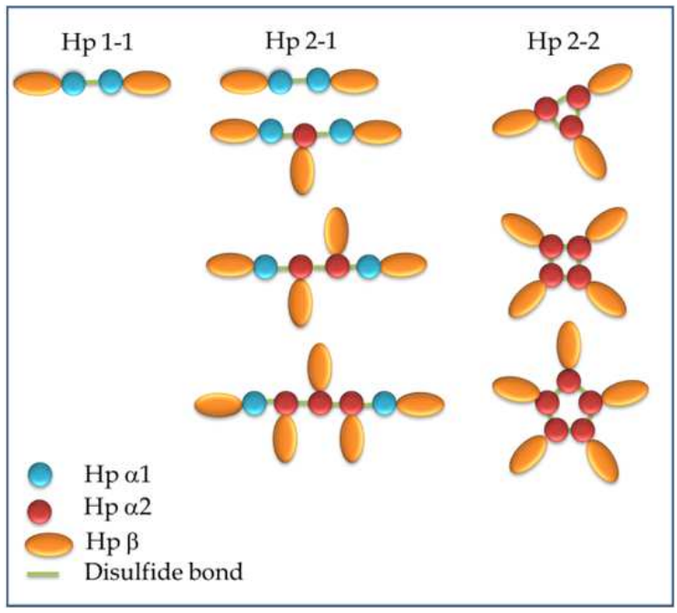

Fig. 1. Schematic representation of the structure of the different haptoglobin polymers determined by phenotype.

3. Haptoglobin: Biological functions

3.1 Haptoglobin prevents oxidative stress

Hp scavenges free hemoglobin (Hb) in the event of intravascular and/or extravascular hemolysis or during normal red blood cell turnover. In plasma, free Hb instantly binds Hp with extremely high affinity (KD ~10^{-15} M) but low dissociation speed, in a ratio of 1Hp:1Hb, to form the Hp-Hb complexes. These complexes are rapidly cleared and degraded by macrophages via the CD163 cell-surface receptor. CD163 receptor is expressed in monocytes and mature tissue macrophages. Hb-Hp complexes once internalized by tissue macrophages are degraded in lysosomes. The heme fraction is converted by heme oxygenase into bilirubin, carbon monoxide, and iron (Buehler et al., 2009). By doing so, Hp prevents the oxidative damage caused by free Hb. Free Hb is highly toxic due to its content of iron-heme that in the presence of H_2O_2 can generate reactive hydroxyl radicals (Fenton reaction) that in

turn cause damage to lipids, proteins and DNA (Lim et al., 2001). Additionally, free Hb acts as a potent scavenger of nitric oxide (NO), an endogenous antioxidant and a regulator of vascular homeostasis, via deoxygenation. NO depletion by Hb has severe consequences such as blood vessel constriction and pulmonary and systemic hypertension, thrombosis, platelet activation, and smooth muscle responses (Alayash, 2011). NO is also important as a toxic defence molecule against infectious organisms and regulates the functional activity, growth and death of many immune and inflammatory cell types that results in an anti-inflammatory effect. The consumption of nitric oxide by Hb reduces the anti-inflammatory properties of NO (Tripathi, 2007).

Independently of its binding capacity to Hb, Hp is an effective antioxidant molecule of Cu2+ and AAPH-induced LDL oxidation. Hp also plays a role in cellular resistance to oxidative stress since its expression renders cells more resistant to damage by oxidative stress induced by hydrogen peroxide (Tseng et al., 2004).

Its capacity of Hp to prevent oxidative stress is directly related to its phenotype. Hp1-1 has been demonstrated to provide superior protection against Hb-iron driven peroxidation than Hp2-2. The superior antioxidant capacity of Hp1-1 seems not to be related with a dissimilar affinity with Hb but the functional differences may be explained by the restricted distribution of Hp2-2 in extravascular fluids as a consequence of its high molecular mass (Melamed-Frank et al., 2001).

The lessened antioxidant function of Hp2-2 is translated into clinical consequences. For instance, low levels of Hp (hypo-haptoglobinemia) and the Hp2-2 phenotype are associated with idiopathic familial and posttraumatic epilepsy. The mechanism behind this association can be the defective inhibition of Hb-driven brain-lipid peroxidation after micro-haemorrhage events within the central nervous system (CNS) caused by the null or low concentration of Hp in this tissue as a result of the low diffusion of Hp2-2 high molecular mass-polymers into the interstitial compartment of the CNS (Panter et al., 1985; Sadrzadeh et al., 2004). In subarachnoid haemorrhage (SAH) and intracerebral haemorrhage (ICH), Hp is important for protecting brain from damage caused by free Hb. In experimental SAH Hp1-1 showed higher protection of the brain tissue from vasospasm, inflammation and improved survival compared to Hp2-2 (Levy et al., 2007). In the ICH model, lack of Hp promoted axonal and oligodendroglia damage (Zhao et al., 2009).

3.2 Haptoglobin beyond its antioxidative role

Besides its anti-oxidative capacity, Hp has been demonstrated to exert direct angiogenic properties and to be involved in arterial restructuring. Hp has been shown to stimulate angiogenesis in both *in vitro* and *in vivo* models. Hp is implicated in arterial restructuring through the formation of a temporary gelatin-based matrix that enhances cell migration (De Kleijn et al., 2002). Additionally, Hp has been reported to function as an extracellular chaperone as it inhibits the heat and stress-induced precipitation of a wide variety of purified proteins by forming soluble high molecular weight complexes with missfolded proteins. All three phenotypes of Hp have efficient chaperone function, however Hp1-1 has the highest efficacy (Yerbury et al., 2005). Moreover, Hp can inhibit formation of amyloid fibrils from diverse proteins by interacting with prefibrillar species to maintain the solubility of amylodogenic proteins, avoiding the incorrect accumulation of proteins in extracellular spaces (Yerbury et al., 2009).

Haptoglobin forms part of the innate unspecific defence against pathogenic bacteria. Hp has a bacteriostatic role by binding Hb and preventing the utilization of iron by pathogenic

bacteria that require iron for their growth (Eaton et al., 1982). Hp2-2 can bind the T4 antigen on the surface of streptoccocus bacterium and agglutinates this pathogen, thus inhibiting its growth (Delanghe, J. et al., 1998). However, Hp can also have a detrimental function in parasite infection. Hp-Hb complexes bind a receptor expressed on *Trypanosoma brucei* surface. Parasites seem to acquire heme from Hp-Hb complexes in order to increase their growth rate and resistance to the oxidative response of the host to eliminate parasites. On the other hand, a highly similar protein to Hp called "haptoglobin related protein" (Hpr) binds Hb and also associates with trypanosome lytic factor (TLF). TLF is a deadly toxic molecule for parasites. The penetration of TLF into the parasite is mediated by Hpr-Hb complexes. Hpr-Hb complexes are recognized by the same trypanosomal receptor that binds Hp-Hb complexes (Pays & Vanhollebeke, 2009).

The secretion of Hp is enhanced in inflammatory states, and at the same time, Hp has important anti-inflammatory properties. As a consequence of macrophage activation, a variety of inflammatory mediators are released, including prostaglandins, leukotrienes, and platelet-activating factor, which are products of the metabolism of the membrane polyunsaturated fatty acid arachidonic acid (AA) by 5-lipoxygenase (5-LO), cyclooxygenase (COX) or cytochrome P450 epoxygenase activity. Leukotrienes have chemotactic, chemokinetic, vasoactive and immunomodulatory properties. Hp possesses significant inhibitory activity on the biosynthesis of prostaglandins via COX and on 12-hydroxyeicosatetrenoate (12-HETE) via lipoxygenase (Saeed et al., 2007). Consequently, during the initial events of inflammation, Hp participates actively in reducing tissue damage.

During the inflammatory process and tissue injury, proteases such as cathepsin B are released. Cathepsin B is an abundantly expressed cysteine peptidase involved in many physiological processes, such as remodelling of the extracellular matrix (wound healing), apoptosis, and activation of thyroxin and rennin. Cathepsin B importantly participates in many pathological processes, such as inflammation, parasite infection and cancer, where it is highly up-regulated. Hp specifically inhibits cathepsin B activity (Kalsheker et al., 1981; Pagano et al., 1980; Snellman & Sylven, 1967). Therefore, Hp might have a regulatory role in tissue proteolysis associated with the inflammatory reaction.

Neutrophils are recruited at sites of inflammation within minutes after injury or infection. In this context Hp suppresses neutrophil function. Hp is synthesized in myelocytes/metamyelocytes during granulocyte differentiation, stored in specific granules of fully differentiated neutrophils and exocytosed immediately in response to activation. Hp binding to activated neutrophils inhibits not only the activity of both 5-LO and COX but also calcium influx and subsequent generation of reactive oxygen species. Hp inhibits respiratory burst activity in neutrophils stimulated with fMLP, arachidonic acid, and opsonized zymosan (Oh et al., 1990). Hp also inhibits the chemotactic response of human granulocytes and differentiated HL-60 cells to fMLP, but does not affect chemotaxis by IL-8. Hp inhibits phagocytosis and reduces intracellular bactericidal activities of granulocytes (Rossbacher et al., 1999; Theilgaard-Monch et al., 2006). Thus, Hp secreted at sites of injury or inflammation mitigates potential tissue damage locally.

4. Haptoglobin expression, polymorphism and disease

Expression of Hp has been associated with diverse inflammatory autoimmune diseases and as a marker of disease activity. Also, genetic polymorphism of Hp has been shown to influence the course of several inflammatory pathologies.

Infection

Patients infected with HIV-1 and with Hp2-2 phenotype have a worse prognosis, related to a more rapid rate of viral replication, than HIV-1 infected patients with the Hp1-1 phenotype. Hp2-2 patients also show higher iron levels and oxidize more vitamin C. It is possible that the less efficient protection against heme-driven oxidative stress by Hp2-2 may stimulate the viral replication (Delanghe, J. R. et al., 1998).

Cancer

High amounts of Hp in plasma and locally in tumoral tissue have been observed in diverse types of malignancies including, lung, bladder, breast cancer, leukemia, glioblastoma, malignant lymphoma, and ovarian cancer (Abdullah et al., 2009; Carter & Worwood, 2007; Kumar et al., 2010; Smeets et al., 2003). The suggested functions of Hp in cancer are as a biomarker of malignancy, as a regulator of the immune response against tumor cells, and as a facilitator of metastasis, since Hp seems to participate in cell migration and angiogenesis (Cid et al., 1993; De Kleijn et al., 2002).

Atherosclerosis

In cross sectional studies, the Hp2-2 phenotype is also associated with an increased risk of atherosclerotic vascular disease and acute myocardial infarction. The lower antioxidant capacity of Hp2-2 together with a lower capacity of Hp2-2 in stimulating macrophages to secrete anti-inflammatory cytokines after binding CD163, might be the reason why individuals with Hp2-2 phenotype are more susceptible for cardiovascular diseases (Levy et al., 2007).

Myasthenia gravis

High serum levels of Hp have been found in patients with myasthenia gravis. Hp serum level is directly correlated with the severity of the disease. This correlation could be explained by the presence of high levels of pro-inflammatory cytokines during active disease. To determine the Hp serum levels in patients with myasthenia can be useful to monitor the severity of the disease (Oliveira et al., 2008).

Arthritis

In patients with active rheumatoid arthritis, high serum levels of Hp have been found. Levels of Hp correlate with clinical disease activity (Cylwik et al., 2010). In juvenile idiopathic arthritis (JIA), a heterogeneous group of inflammatory diseases, Hp was found in inflamed joints. Hp was locally produced in synovial fluid of patients with JIA. Moreover, Hp was expressed differentially between JIA subtypes. Hp expression was increased in systemic JIA. The presence of Hp in the inflamed tissue suggests that Hp plays a role in the progression and pathology of the disease and can also be used as a biomarker of disease activity (Rosenkranz et al., 2010).

Psoriasis

In psoriasis, a structure modification of Hp was described that might impair the Hb binding function and also the activity of the lecithin-cholesterol acyltransferase (LCTA) enzyme (Cigliano et al., 2008). The structural modification of Hp in psoriasis patients is an abundance or structure change of specific glycans that differ or do not exist in Hp from healthy donors. These changes are associated with altered function of that might have an impact on the disease activity (Maresca et al., 2010). Interestingly, it was found that there is no higher prevalence of any of the three phenotypes of Hp in psoriasis.

Lupus

It has been shown that in systemic lupus erythematosus (SLE) patients, Hp plasma levels correlate with severity of the disease and that the Hp2-2 phenotype is over-represented in SLE patients (Pavon et al., 2006; Rantapaa et al., 1988). The association of the Hp2-2 phenotype with SLE can have several implications. SLE is an autoimmune disease mediated by B cells that secrete self-reactive pathogenic antibodies. Individuals with the Hp2-2 phenotype seem to have a higher number of CD22 binding sites compared to other Hp phenotypes. Additionally, it is known that cardiovascular disease is a common complication of SLE. The finding that Hp2-2 has lower anti-oxidant capacity (Van et al., 2004) might be an explication for this.

Celiac disease

The frequency of the Hp1-2 phenotype in celiac disease patients has been reported to be higher than in the general population. However, Hp2-2 phenotype was associated with a more severe clinical course of the disease (Papp et al., 2008). The structural differences and the functional differences between Hp phenotypes may account for phenotype association of Hp to the more severe form of celiac disease. Hp2-2-Hb complexes upon binding CD163 induce lower expression of IL-10 compared to Hp1-1-Hb complexes. Hp2-2 is as well associated to a stronger immune response (Guetta et al., 2007).

Diabetes type I

In patients with a long duration of type I diabetes, an increased risk of cardiovascular disease was observed in patients with the Hp2-2 phenotype. Again, as Hp2-2 has low anti-oxidant capacity and a low efficiency in preventing heme release, this can contribute to the higher cardiovascular risk in type I diabetes (Costacou et al., 2008). Also, it was shown that the Hp phenotype may be an independent determinant of early renal function decline and progression to end-stage renal disease (Costacou et al., 2009).

Inflammatory bowel diseases (IBD)

Inflammatory bowel disease (IBD) is a chronic, relapsing intestinal inflammatory condition that is classified into two major forms, Crohn's disease (CD) and ulcerative colitis (UC). The etiology is unknown, but the pathogenesis is likely dependent on the interaction between local immune reaction and environmental factors in susceptible individuals. To explore a possible role of Hp in IBD patients we recently studied polymorphisms in the Hp locus in a cohort of CD and UC patients. It was found that the Hp2 locus was overrepresented in CD and UC patients compared to healthy individuals (Marquez, et al. in press). These results indicate that Hp phenotype can be a risk factor for IBD. Since Hp phenotypes differ in their function, further research is necessary to understand how Hp genotype modulates IBD pathogenesis.

5. Haptoglobin and the immune response

Disease associations mentioned above are strongly suggestive for a modulatory effect of Hp on immune responses. Several experimental data support this concept.

5.1 Receptors for Hp on immune cells

The Hb-Hp complex is removed from the circulation by binding to CD163 expressed on the monocyte-macrophage system. CD163 is a member of the SRCR family class B and is

expressed on most subpopulations of mature tissue macrophages. Triggering CD163 by ligand binding (Hb-Hp) results in a protein tyrosine kinase-dependent signal and secretion of IL-6 and IL-10. Moreover, IL-6 and IL-10 are known to up-regulate CD163 and this might function as a positive feedback mechanism for CD163 induction. In fact, CD163 has an important immunomodulatory function. The Hp genotype modulates the balance of Th1 and Th2 cytokines produced by macrophages exposed to free Hb via a CD163 dependent mechanism. The Hp1-1–Hb complex stimulated the secretion of significantly more IL-6 and IL-10 than the Hp2-2–Hb complex (Guetta et al., 2007). Moreover, CD163 also exerts an immunomodulatory role by degradation of heme which results in the production of metabolites with suggested anti-inflammatory effects (Moestrup & Moller, 2004; Nielsen et al., 2007).

Hp also binds to the integrin adhesion receptor, Mac-1 (Macrophage-1 antigen, integrin alphaMbeta2, CD11b/CD18) (El Ghmati et al., 1996) which is expressed on dendritic cells, neutrophils, monocytes, macrophages, NK cells, and a small subset of T cells. Mac-1 is involved in maturation, phagocytosis and adhesion of monocytes and is required for adhesion and transmigration of monocytes into tissues. Engagement of Mac-1 by its ligands initiates an intracellular signalling cascade that results in activation and cytokine secretion (Shi et al., 2004; Shi & Simon, 2006).

Hp is a ligand of CD22 on B cells and CD22 is implicated in B cell activation and survival (Langlois et al., 1997). By binding CD22, Hp can inhibit the interaction with other CD22 ligands and as a consequence may negatively modulate the B cell function (Hanasaki et al., 1995).

Hp has also been demonstrated to bind a not-yet-identified receptor on mast cells interfering with their spontaneous proliferation (El-Ghmati et al., 2002), and perhaps with other functions that have not been explored.

5.2 Immunomodulatory effects of Hp

Hp participates actively in several processes of the immune response, from activation of the innate and adaptive immune response to tissue repair and regeneration. Hp released locally and systemically modulates numerous cellular activities, including prostaglandin synthesis, leukocyte activation, recruitment and migration, modulation of cytokine patterns, and tissue repair (Wang et al., 2001). Hp rises during inflammatory process, including those caused by autoimmunity, and the general picture is that Hp has an anti-inflammatory effect (Arredouani et al., 2005; Quaye, 2008; Wang et al., 2001).

An anti-proliferative capacity of Hp on lymphocytes has been demonstrated in a variety of conditions. Purified Hp or Hp present in sera and ascites fluid of cancer patients has been shown to inhibit the polyclonal proliferation of mitogen-stimulated T cells (Arredouani et al., 2003; Oh, S. K. et al., 1987b). Hp modifies the T helper Th1/Th2 balance (Arredouani et al., 2003; Oh, S. K. et al., 1987a; Oh, S. K. et al., 1987b). Furthermore, Hp inhibits or enhances proliferation of B-cells in response to bacterial endotoxins, depending on its concentration (Quaye, 2008; Wang et al., 2001).

Dendritic cells (DC) are central regulators of immune responses and the bridge between innate and adaptive immune response. It has been demonstrated that Hp prevents epidermal Langerhans cells (LC) from spontaneous functional maturation in the skin. Hp is stored in the epidermal LC cytoplasm, but LC do not produce Hp. Though, Hp is produced in keratinocytes (KC). KC play an important role in regulating the function of LC and T cells

in the skin by producing cytokines and possibly by expressing Hp (Li et al., 2005; Wang et al., 2005; Xie et al., 2000).

5.3 Haptoglobin has a negative regulatory role in a CNS-autoimmune response

Experimental autoimmune encephalomyelitis (EAE) is a model for organ specific autoimmunity. The immunopathological events of EAE encompass the initial T-cell priming in the secondary lymphoid organs, followed by the recruitment of primed T-cells in the CNS and the subsequent effector phase. Systemic immunisation with myelin antigens in CFA is sufficient to prime myelin-reactive T cells. Neuroantigen-reactive T cells recognize their cognate antigen presented by professional antigen-presenting cells (APCs) within secondary lymphoid organs where those cells are activated and expanded (Becher et al., 2006). Once myelin-reactive T cells are activated in the periphery, they migrate to the CNS traversing the blood brain barrier (BBB). Activated T cells in the CNS recruit other lymphocytes, monocytes, and granulocytes by secreting inflammatory mediators. All inflammatory immune cells orchestrate damage to myelin that covers axons, a process called demyelination. In turn, demyelination leads to a variety of neurological symptoms including paralysis as a final result.

Until recently there were no studies yet that specifically analyzed the effect of Hp on development and course of CNS autoimmune inflammatory disease. We aimed to characterize the role of Hp in EAE induced by immunisation with MOG35-55 peptide in C57BL/6 mice. We immunised wild-type (WT) and Hp-/- C57BL/6 mice with MOG35-55 in complete Freunds adjuvans (CFA) and showed that Hp influences the severity of EAE, as the lack of Hp results in clinically and in pathologically exacerbated EAE (Galicia et al., 2009). Our further results indicated that Hp has an important modulatory effect on the infiltration of mononuclear cells into the CNS and on the production of Th1 cytokines and IL-17A by auto-reactive T cells. Exacerbated disease in Hp-/- mice was related to an increased expression of IFN–γ, IL-6 and IL-17A in the CNS of these animals. Furthermore, the number of IL-17+ cells in the CNS was increased (Galicia et al., 2009). Neutralization of IL-17A with anti-IL-17A monoclonal antibodies (mAbs) in Hp-/- mice significantly reduced the severity of the disease but did not completely block it as it did in WT animals (Fig2) (unpublished data).

These results strongly suggest that Hp has a protective role in reducing the severity of an autoimmune inflammatory process, and this protection is related with the suppression of auto-aggressive cells that produce inflammatory cytokines.

5.4 Haptoglobin has a regulatory role in experimental colitis

Several animal models of inflammatory bowel disease have been developed, in which colitis can be induced using chemical compounds such as dextran sulphate sodium (DSS) or oxazolone in susceptible strains of mice. The resulting inflammation is mediated by polymorphonuclear cells, macrophages, and lymphocytes. Interestingly, Hp is expressed by intestinal epithelial cells (Pelletier et al., 1998).

To study whether Hp has an effect on the course of inflammatory colitis, we induced colitis with DSS and oxazolone in wild-type and in Hp deficient mice. We found that in both forms of colitis, the severity of the disease was exacerbated in Hp-/- mice compared to WT. The more severe inflammation in Hp-/- mice was related to high mRNA expression of IL-17, IFN-γ, TNF and IL-6 in colonic tissue in Hp-/- DSS colitis mice, while there was a slight

increase in IL-13 mRNA in Hp-/- mice with oxazolone-induced colitis. In order to determine the immunological mechanisms for the protective role of Hp in these models, draining lymph nodes from DSS or oxazolone colitis mice were cultured in the presence of IL-23, a cytokine that contributes to the survival and effector functions of IL-17-producing cells (Th17). Cells obtained from DSS and oxazolone colitis Hp-/- mice produced high amounts of IL-17 compared with WT mice, suggesting the presence of increased numbers of Th17 cells in Hp-/- mice. However, we found that *in vitro* Hp does not interfere with Th17 differentiation but that it rather suppresses IL-17 production (Marquez, et al. in press). Thus again, Hp exerts n immunomodulatory function by reducing the activity of pro-inflammatory lymphocytes.

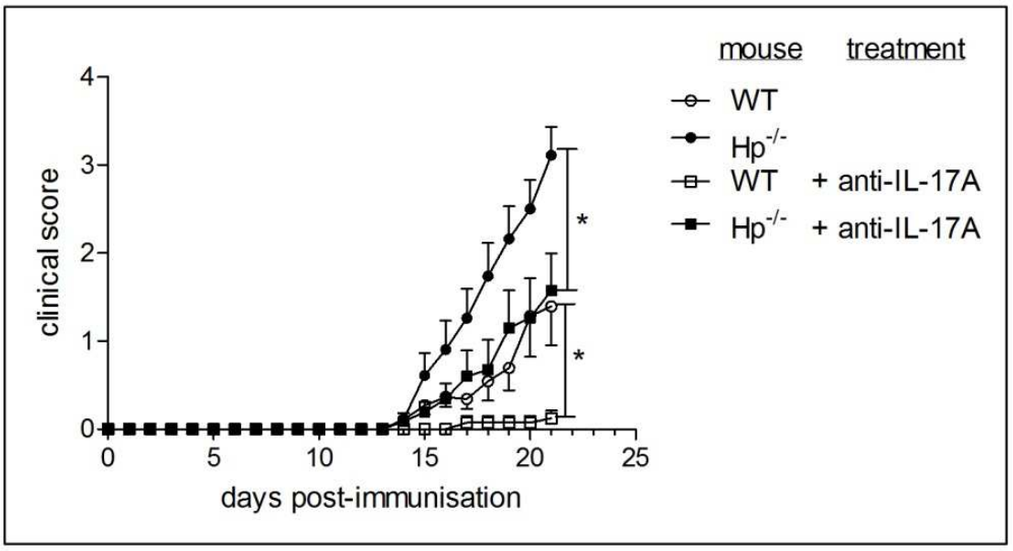

Fig. 2. Blockade of IL-17A reduces the severity of EAE in WT and Hp-/- mice. Clinical scores of WT and Hp-/- mice immunised with MOG35-55/CFA untreated or treated with murine anti-mouse IL-17A mAbs. Anti-IL-17A mAb was administered intra peritoneally two times per week (0.2 mg/mouse) from day 7 until day 21 after immunisation. Data shown are from two different experiments (n=10). *p≤ 0.05. (Galicia, et al. unpublished data)

5.5 Hp is required to induce mucosal tolerance

The immune response serves primarily to protect the organism from foreign invading pathogens and tumor cells. Reduced or absent responsiveness of the immune system against self-antigens is necessary to allow the immune system to mount an effective response to eliminate infectious invaders while leaving host tissues intact. This condition is known as immune tolerance. Self-tolerance is maintained by central (thymus-dependent) and by peripheral mechanisms. The mechanism of central tolerance is the deletion of self-reactive T cells in the thymus during T cell maturation. During ontogeny auto-reactive cells encounter self-antigens in the medullary-cortical junction in the thymus, and those cells that react with high affinity to the antigen are eliminated in a process called negative selection (Kyewski & Klein, 2006). Nevertheless, some self-reactive T cells can escape from deletion in the thymus. These auto-reactive cells must be kept in a state of reduced or absent responsiveness to avoid pathogenic immune reactivity. Peripheral tolerance provides a second line of

protection against autoimmune responses by regulating potentially pathogenic autoreactive lymphocytes, a process in which regulatory T cells have a major role (Li & Boussiotis, 2006). When self-tolerance is broken, the immune reaction against self-antigens can lead to autoimmune disease.

Mucosal tolerance induction is a naturally occurring immunological phenomenon that originates from mucosal contact with inhaled or ingested proteins. The largest areas of the body exposed to the external environment are the mucosal surfaces. Regular contact of antigens with mucosal surfaces prevents harmful inflammatory responses to non-dangerous proteins, such as food components, harmless environmental inhaled antigens and symbiotic microorganisms. The lymphoid tissue associated to the mucosa of the gastrointestinal tract and to the airway system mucosa therefore plays an important role in tolerance maintenance (Dubois et al., 2005). Administration of antigens by either oral or nasal route, in the absence of costimulatory signals, leads to specific suppression of systemic immune responses against these antigens in mice. The induction of oral or nasal tolerance to antigens is an active process that results in anergy, generation of antigen-specific suppressive T lymphocytes, production of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β, and deletion of antigen reactive T cells (Dubois et al., 2005; Faria & Weiner, 2006). On the contrary, mucosal tolerance induction is abrogated by applying the antigen in the presence of an adjuvant, such as LPS or cholera toxin (Kraal et al., 2006).

Oral and nasal tolerance has been used successfully to prevent a number of experimental autoimmune diseases including, arthritis, diabetes, uveitis, and EAE (Faria & Weiner, 2006). Particularly, administration of MOG35-55 peptide by the nasal route is highly efficient in suppressing EAE (Shi et al., 1998; Xu et al., 2000). Shortly after intranasal antigen instillation of a soluble, harmless antigen, it can be detected in the nose-draining lymph nodes. The majority of inhaled antigen detected in lymph nodes is associated with a variety of dendritic cells (DC), such as CD8αlow CD205+, plasmacytoid DCs and CD8αhigh DC (Wikstrom & Stumbles, 2007). These DC play a pivotal role in induction of tolerance through the differential expression of surface molecules and cytokines that make them able of inducing T-regulatory cells (Coombes et al., 2007). After antigen presentation under non-inflammatory conditions to antigen-specific naïve CD4+ T cells, those T cells proliferate transiently and become tolerant. The CD4+ T cell population that arises after harmless antigen administration in the nose is able to transfer tolerance and to suppress specific immune responses in naïve animals (Unger et al., 2003). The draining lymph nodes of the nose which are the internal jugular (IJLN) and superficial cervical lymph (CLN) nodes are essential for tolerance induction towards inhaled antigens. Removing these lymph nodes abrogates the capacity of nasal tolerance induction and their function cannot be restored by peripheral lymph nodes when transplanted to this site (Kraal et al., 2006; Wolvers et al., 1999). Importantly, Hp expression is increased in cervical lymph nodes shortly after nasal protein antigen instillation without adjuvant (Boots et al., 2004). Thus, Hp might have an important function in nasal tolerance induction.

Several murine models of autoimmune diseases can be prevented by nasally administering the self-antigens prior to disease induction with the same antigen, including EAE (Li et al., 1998; Shi et al., 1998). To study the participation of Hp in nasal tolerance induction, we administrated 100μg of MOG35-55 to Hp-/- and WT sex-age matched mice by nasal instillation at day 7, 5, and 3 before EAE induction by immuniszation with MOG in CFA. The control group was given PBS as vehicle control. We found that Hp-/- mice were partly resistant to the development of nasal tolerance induction, as Hp-/- mice that received

nasally MOG peptide in the nose still developed severe EAE, while the WT mice were protected. These data suggest that Hp is a critical modulator of mucosal tolerance induction (Fig3) (Galicia, et al. unpublished data). Lack of immune tolerance in Hp-/- mice was associated with deficient suppression of antigen-dependent IL-17A production by T cells (unpublished data).

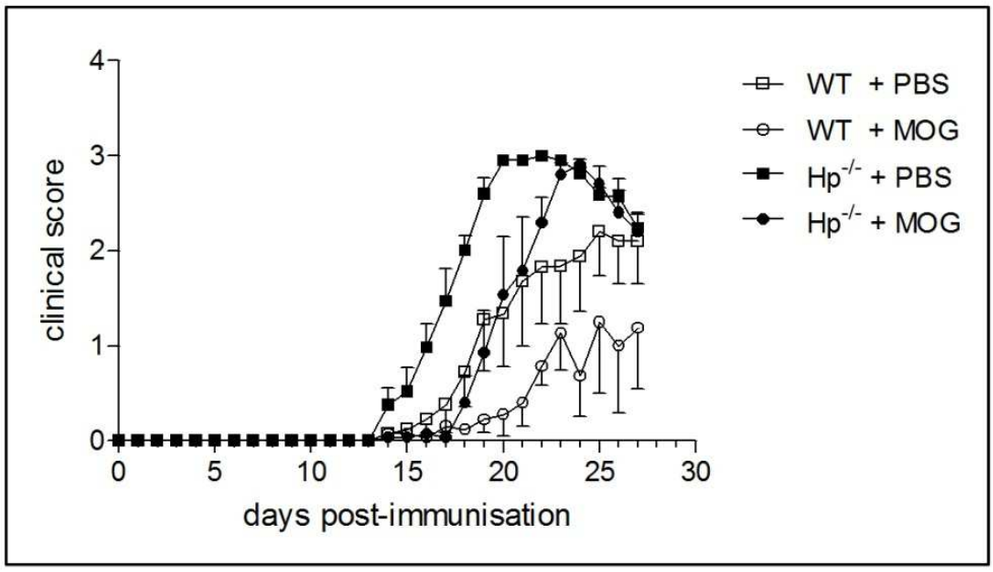

Fig. 3. Lack of Hp hampers induction of nasal tolerance. Mice received 100µg MOG35-55 or PBS by nasal instillation, on day 7, 5 and 3 before MOG-immunisation. EAE was induced by injection of MOG35-55 in CFA in the footpads. Clinical score was assessed daily. A representative experiment out of three independent experiments with similar results is shown. Each experimental group consisted of 5 mice. WT+PBS vs WT+MOG: p=0.0066; WT+PBS vs Hp-/- +PBS: p=0.048. Hp-/-+PBS vs Hp-/-+MOG: p=0.06.

6. Conclusion

Apart from the Hb scavenger function of Hp, several recent studies have revealed new functions of Hp on immune responses and in autoimmunity. Furthermore, Hp polymorphism seems to be related with the outcome of various autoimmune diseases. The differential capacity to prevent oxidative stress, modulation of immune responses, and control of inflammation by the three major Hp phenotypes (Van et al., 2004) can account for differences in susceptibility to, or severity of the autoimmune inflammation. Previously, our laboratory demonstrated that Hp was able to modulate not only the function of T cells, by regulating *in vivo* and *in vitro* the Th1/Th2 response, but also macrophage cytokine secretion. Whereas in some cells the Hp binding receptor is known (Mac-1 on macrophages, dendritic cells; CD22 on B cells), additional receptors might exist. However, the evidence that Hp acts directly on immune cells points out that Hp plays an important negative regulatory role in immune response and likely in autoimmunity. To explore this hypothesis we used models of organ-specific autoimmunity mediated by T cells. We showed in two different models of autoimmune inflammation that Hp has a crucial role in controlling inflammation mediated by Th1 and Th17 cells. Though the mechanism behind exacerbated

Th1/Th17 responses is still not clear, we propose that Hp may regulate differentiation and/or activity of T cells indirectly through the negative regulation of dendritic cell and macrophage functions.

Dendritic cells are the most efficient cells in driving the activation and differentiation of naïve T cells (Ueno et al., 2007). Hp has been described as an alternative low affinity ligand for the CD11b/CD18 (Mac-1) integrin (El Ghmati et al., 1996). Moreover, Mac-1 has been demonstrated to play an important role in the function of Mac-1 expressing cells and in migration and phagocytosis. Thus, it is likely that Hp, by binding Mac-1, may negatively regulate the function of dendritic cells, macrophages, and any cell that express Mac-1. Consequently, we propose that the lack of Hp allows for a stronger activation of DC, and therefore more potent activation and differentiation of auto-aggressive T cells with a pro-inflammatory cytokine profile (Fig4).

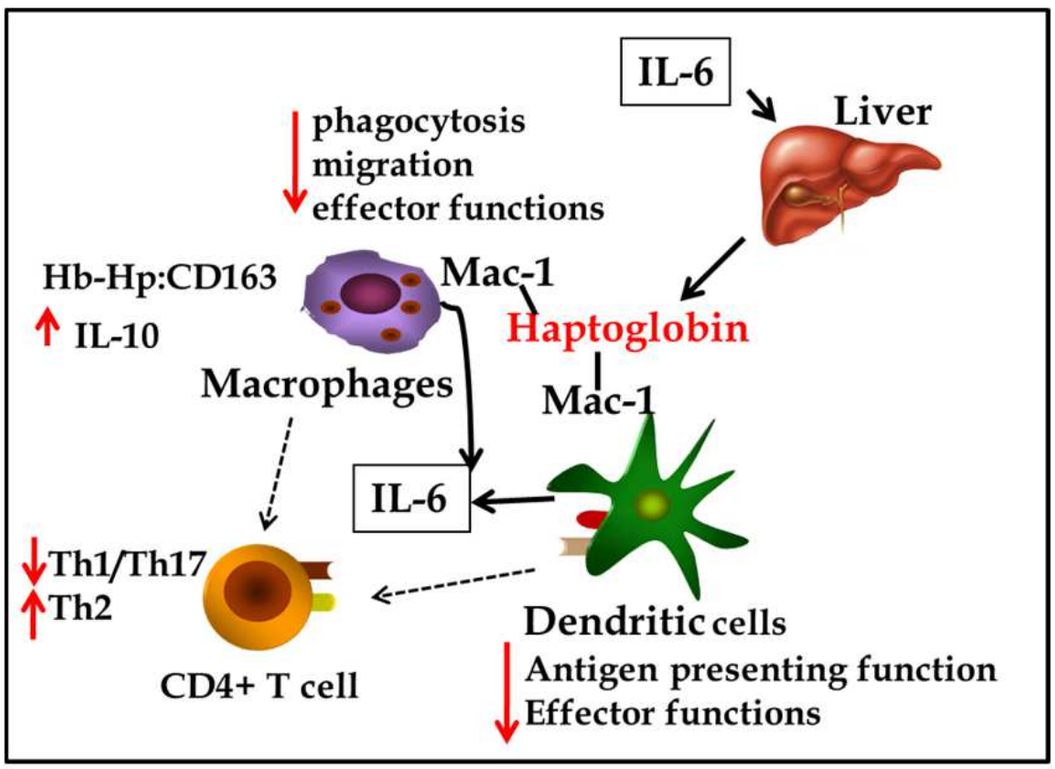

Fig. 4. Diagrammatic scheme of the potential mechanism of Hp regulation of immune cell functions. Hp is proposed to act through binding Mac-1 on dendritic cells and macrophages, thus reducing their activity and IL-6 secretion. Dotted arrows indicate an indirect effect of Hp on T cells through function modulation of macrophages and dendritic cells, resulting in decreased Th1 and Th17 activity.

Our results imply that Hp can be considered as a highly relevant player in controlling immunity and inflammation. Additionally, we showed that Hp polymorphism is associated with inflammatory bowel disease. Finally, we also demonstrated that Hp is an essential component for the mucosal tolerance induction. Further studies are needed to obtain more insight into the mechanism through which Hp restrain the Th1/Th17 response and how Hp is relevant in creating a proper environment in the lymph nodes to induce tolerance.

7. Acknowledgments

This work was supported by the Fund for scientific research Vlaanderen and by the SCAP foundation for allergy research.

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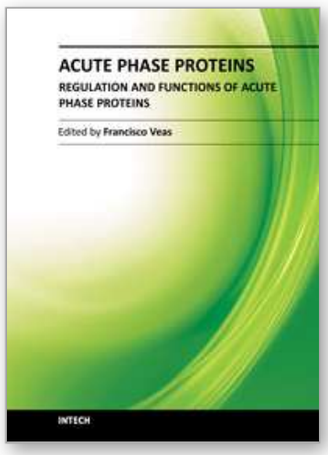

Acute Phase Proteins - Regulation and Functions of Acute Phase Proteins

Edited by Prof. Francisco Veas

ISBN 978-953-307-252-4
Hard cover, 368 pages
Publisher InTech
Published online 03, October, 2011
Published in print edition October, 2011

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How to reference

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Georgina Galicia and Jan L. Ceuppens (2011). Haptoglobin Function and Regulation in Autoimmune Diseases, Acute Phase Proteins - Regulation and Functions of Acute Phase Proteins, Prof. Francisco Veas (Ed.), ISBN: 978-953-307-252-4, InTech, Available from: http://www.intechopen.com/books/acute-phase-proteins-regulation-and-functions-of-acute-phase-proteins/haptoglobin-function-and-regulation-in-autoimmune-diseases

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