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A Unique Form of Haptoglobin Produced by Murine Hematopoietic Cells Supports B-cell Survival, Differentiation and Immune Response

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Abstract

Haptoglobin (Hp), an acute phase reactant and major hemoglobin-binding protein, has a unique role in host immunity. Previously, we demonstrated that Hp-deficient C57BL/6J mice exhibit stunted development of mature T- and B-cells resulting in markedly lower levels of antigen-specific IgG. The current study identified leukocyte-derived pro-Hp as a relevant mediator of an optimal immune response. Reconstitution of $Hp^{-/-}$ mice with $Hp^{+/+}$ bone marrow restored normal immune response to ovalbumin. Furthermore, transplanting a mixture of bone marrow-derived from B-cell-deficient and Hp-deficient mice into $Rag1^{-/-}/Hp^{+/+}$ recipients resulted in mice with a defective immune response similar to $Hp^{-/-}$ mice. This suggests that Hp generated by the B-cell compartment, rather than by the liver, is functionally contributing to a normal immune response. Leukocytes isolated from the spleen express Hp and release a non-proteolytically processed pro-Hp that uniquely differed from liver-derived Hp by not binding to hemoglobin. While addition of purified plasma Hp to cultured B-cells did not alter responses, pro-Hp isolated from splenocytes enhanced cellular proliferation and production of IgG. Collectively, the comparison of wild-type and Hp-deficient mice suggests a novel regulatory activity for lymphocyte-derived Hp, including Hp produced by B-cells themselves, that supports *in vivo* survival and functional differentiation of the B-cells to ensure an optimal immune response.

Keywords

acute phase response; inflammation; immunoglobin production

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1. INTRODUCTION

Haptoglobin (Hp) restricts oxidative damage at sites of hemolysis[1,2] by sequestering free hemoglobin.[3-5] Additionally, Hp is an IL-6-regulated acute phase plasma protein whose hepatic production is strongly induced during the early stages of inflammation.[6] The overlap in timing of the acute phase induction of Hp with the recruitment of immune cell functions implies a potential relationship between these two events.[7] Supporting this, Hp-deficient mice have stunted development of lymphoid organs.[8] Furthermore, Hp has been shown to contribute to rejection of skin transplants via a TLR/MyD88-dependent mechanism.[9] However, other studies have suggested that Hp also exerts anti-inflammatory[10,11] and immunomodulating effects.[12,13] Plasma Hp binds to monocytes through CD11b/CD18[14,15] and, in complex with hemoglobin, to CD163.[16] It also binds to B-cells through CD22.[17] These interactions may contribute to the pro- and anti-inflammatory functions attributed to Hp.

Hp$^{-/-}$ mice exhibit remarkably reduced production of specific IgG following immunization with antigen.[8] This may be a result of reduced numbers and functions of B- and T-lymphocytes and/or due to a co-activator-like function for Hp on immune cells as suggested by the skin transplant studies.[9] However, treatment of mitogen-stimulated *Hp*$^{-/-}$ T-cells with purified plasma Hp failed to completely restore proliferative responses to the levels of wild-type T-cells.[8] One caveat to these experiments is the assumption that plasma Hp, which is made by the liver, exerts the immune cell-regulating activity. Although non-hepatic sites of Hp expression have been detected,[18-21] Hp released from these sites has been presumed to be functionally equivalent to liver-derived Hp.

To evaluate the regulatory role of Hp in the immune response, we performed bone marrow reconstitution experiments that permitted distinguishing the effects of liver-derived plasma Hp versus hematopoietic-derived Hp. Our results document that Hp produced by splenocytes, including Hp produced by B-cells themselves, contributes to the maturation, differentiation and function of B-cells. Moreover, Hp produced and released by splenocytes is structurally and functionally distinct from plasma Hp. Finally, we demonstrate that interaction with hemoglobin is not an obligatory part of immune cell regulation by Hp.

2. MATERIALS AND METHODS

2.1. Mice

Mice used in this study were all housed under specific pathogen-free conditions and used according to IACUC guidelines. *Hp* knockout mice (*Hp*$^{-/-}$) on a C57BL/6 background were used throughout.[22,23] For bone marrow chimeras, *Hp*$^{-/-}$ host mice were sublethally irradiated with 475-500 RAD and reconstituted with 3×10^6 *Hp*$^{+/+}$ or *Hp*$^{-/-}$ bone marrow cells. For generating mixed bone marrow chimeras, lethally irradiated *Rag1*$^{-/-}$ mice received CD45.2$^+$ *Hp*$^{-/-}$ or *Hp*$^{+/+}$ bone marrow cells mixed 1:1 with bone marrow from B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (which carries the CD45.1 antigen) or *Hp*$^{-/-}$ or *Hp*$^{+/+}$ bone marrow cells mixed 1:1 with bone marrow from a B-cell-deficient strain *Igh-6*^{tm1Cgn} (*muMT*) (Jackson Laboratory, Bar Harbor, ME). Chimeric animals were provided antibiotic-supplemented drinking water for two weeks post-irradiation.

2.2. *Immunization and germinal center analysis*

For immunizations, 0.5 mg chicken ovalbumin (OVA) (Biosearch Technologies, Novato, CA) emulsified in CFA was injected subcutaneously. For recall experiments (49 days later), antigen emulsified in IFA was given. Follicular structure and germinal centers in the spleens were visualized by H&E staining and B220 and PNA immunofluorescence.

2.3. *Blood cell count*

Aliquots of blood were analyzed by a Hemaret 850 Mascat blood cell counter (CDC Technologies, Oxford, CT), which had been calibrated with a standard mixture of mouse leukocytes.

2.4. Flow cytometric analysis (FACS)

Cells from blood and lymphoid organs were stained for surface antigens as previously described.[24] All antibodies were from Becton Dickinson BioSciences (Mountain View, CA) or Biolegend (San Diego, CA) and included: FITC-conjugated anti-CD4, anti-CD21, anti-CD22 and anti-BAFF-receptor; PE-conjugated anti-B220, anti-CD19, anti-CD23, anti-IFNγ, anti-CD5; PE/Cy5-conjugated anti-CD8, anti-CD3; PerCP-conjugated anti-CD8 and APC-conjugated anti-CD45.1 or anti-CD45.2. The cells were analyzed on a FACSCalibur/FACScan flow cytometer using CELLQuest (BD BioSciences, San Jose, CA), WinList 5.0 software (Verity Software House Inc., Topsham, ME) or FlowJo 8.7 software (Ashland, OR).

2.5. Isolation of splenocytes, B-cells, and bone marrow-derived DC

B-cells were purified from spleens using CD43 magnetic microbeads. For sorting of highly purified B-cell subsets by FACS, cells were first enriched using negative selection on CD43 beads and then sorted to isolate $B220^{+}CD21^{+}CD23^{+}$ follicular B-cells and $B220^{+}CD21^{hi}CD23^{lo/neg}$ marginal zone B-cells. Bone marrow plasma cells were enriched with the Miltenyi $CD138^{+}$ Plasma Cell Isolation Kit and sorted by FACS for $B220^{low/neg}CD138^{+}$ cells.

To generate dendritic cells, bone marrow cells were cultured for 7-9 days in media containing 10 ng/ml mouse GM-CSF (eBioscience, San Diego, CA). DC cultures, consisting of ~75% $CD11c^{+}$ cells, were incubated for 24 hours in serum-free media containing 100 ng/ml LPS and 4.5mg/ml glucose.

The mononuclear fraction of splenocytes was isolated on a Histopaque-1083 gradient (Sigma-Aldrich, St. Louis, MO) followed by collection of non-adherent cells after a 2-hour incubation on tissue culture plates. These leukocytes were incubated in serum-free medium containing 4.5 mg/ml glucose for 24 hours.

2.6. Purification of Hp

Plasma-derived Hp was purified from acute phase mouse plasma by affinity chromatography on a column containing hemoglobin bound to Sepharose-6B-CL (Pharmacia, Uppsala, Sweden) as described.[8] To purify hematopoietic Hp, conditioned media from splenocytes, isolated B-cells, or DCs were subjected to ammonium sulfate precipitation and one round of hemoglobin-affinity chromatography. The non-bound and hemoglobin-bound fractions were size separated on a Superose-300 column. Hp proteins eluting at 100-130 kDa were concentrated by dialysis and lyophilization. Proteins were redissolved in pyrogen-free, sterile PBS. The absence of irritant activity in the Hp preparations was tested.

2.7. *RNA analysis*

RNA isolation and RT-PCR detection of Hp mRNA was carried out as described.[8] Primers for the BAFF gene (5′-CCCTGTTCCGATGTATTCAG-3′ and 5′-CACCAAAGAAGGTGTCGTCT-3′), the BAFF receptor gene (5′-AGTGAGTCTGGTGAGCTGGA-3′ and 5′-GGGTTTCTGAGGAGGGTACA-3′) and GAPDH (5′-CATGGCCTTCCGTGTTCCTA-3′ and 5′-

CCTGCTTCACCACCTTCTTGAT-3′) were used. For each amplified segment, the fold enrichment was normalized to GAPDH and calculated using the ΔΔCt method.

2.8. *Protein analysis*

Sample preparation and electrophoresis was carried out as described.[8] Aliquots of cell extract, conditioned media, purified Hp preparations or plasma were separated by SDS-PAGE and immunoblotted using antibodies against Hp, IgG or IgM (Dako, Carpinteria, CA), α_1-acid glycoprotein (AGP) (Springville Laboratories, Springville, NY), BAFF (eBioscience, San Diego, CA), or BLIMP1 (clone 6D3, Santa Cruz Biotechnology). Two-dimensional separation (2D-PAGE) of Hp was carried out using a pH gradient 3.5-10 followed by separation on a 10% polyacrylamide SDS gel. To determine the isoelectric point of desialylated Hp, purified Hp was treated with neuraminidase (Clostridium perfringens, type V; Sigma-Aldrich) and a cocktail of protease inhibitors (Cell Signaling) and incubated for 6 h at 37°.

Quantitative assessment of OVA-specific IgG and IgM in mouse sera was carried out by immunoblotting against OVA.

2.9. *ELISA and ELISPOT*

Polyclonal goat anti-mouse IgG antibodies (to measure total IgG) or ovalbumin (to measure OVA-specific IgG) were used to coat ELISA plates. Mouse serum was diluted and ELISA was performed according to standard procedures. ELISPOT was performed as previously described except that antibody-secreting cells were detected using anti-mouse IgG.[25]

2.10. *B-cell stimulation and thymidine incorporation*

Purified B-cells were cultured in RPMI medium and stimulated for 5 days with 10 μg/ml anti-CD40 and 2 ng/ml mouse IL-4. For quantification of DNA synthesis, [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) was added to cells after 48 hours of growth stimulation and the amount of incorporated tritium was measured 16 hours later.

2.11. Statistics

Significance between control and experimental groups was examined using student's t-test ($p<0.05$ was regarded as significant).

3. RESULTS

3.1. Wild-type bone marrow restores a normal immune response to Hp$^{-/-}$ mice

In an earlier study,[8] two-fold lower numbers of B220$^+$ B-cells were observed in *Hp*$^{-/-}$ mice as compared to *Hp*$^{+/+}$ mice. The reduced B-cell compartment has been tentatively attributed to less efficient B-cell development in the bone marrow.[8] To extend these findings, we analyzed *Hp*$^{+/+}$ and *Hp*$^{-/-}$ mice for the presence of standard B-cell types, including B1a, B1b, and B2 (follicular and marginal zone) cells. Peritoneal lavages showed no statistically significant differences in B1a (29.5% ± 0.1 and 32.2 ± 0.5) or B1b (13.8 ± 2.3 and 15.2 ± 3.4) cells between genotypes (data not shown). However, in the spleen, a significantly lower number of B-cells was detected. Follicular (CD21^{int}CD23$^+$) and especially marginal zone (CD21^{hi}CD23^{lo}) B-cell populations were reduced in *Hp*$^{-/-}$ mice as compared to *Hp*$^{+/+}$ mice ($p=0.01$ and $p=0.006$, respectively; Fig. 1A). CD22, a B cell-restricted protein that can serve as a receptor for Hp, showed a similar mean fluorescent intensity in *Hp*$^{+/+}$ and *Hp*$^{-/-}$ B-cells (Fig. 1B). Although there were fewer B-cells, there was a higher percentage of B220$^{lo/neg}$CD138$^+$ plasma cells in *Hp*$^{-/-}$ mice (0.9% versus 0.1%; Fig. 1C). ELISPOT analysis confirmed an increase in IgM-secreting cells (*Hp*$^{+/+}$ 9000 ± 5000 versus *Hp*$^{-/-}$

31000 ± 6000 cells per 10^6 splenocytes; Fig. 1D), in keeping with the observed elevation of serum IgM in $Hp^{-/-}$ mice (Fig. 1E).

Survival of peripheral B-cells depends on signaling via BAFF ligand binding to the BAFF receptor (BAFF-R) on B-cells.[26] To determine whether this pathway was altered in $Hp^{-/-}$ mice, we assessed expression of BAFF and BAFF-R by quantitative RT-PCR using splenic RNA. Comparable levels of BAFF transcripts and BAFF protein were found in $Hp^{+/+}$ and $Hp^{-/-}$ spleens (Fig. 1F). BAFF-R mRNA levels were reduced in $Hp^{-/-}$ spleens in a manner proportional to the reduced total number of B-cells (data not shown). However, BAFF-R mRNA levels were similar in purified B cells from $Hp^{+/+}$ and $Hp^{-/-}$ spleens (Fig. 1G) and flow cytometry did not indicate a differential expression of BAFF-R protein on a per cell basis on B-cells from $Hp^{+/+}$ and $Hp^{-/-}$ mice (Fig. 1H and I). Thus, it would appear that the lower number of B-cells in $Hp^{-/-}$ mice is not due to a deficiency in expression of BAFF or the BAFF-R.

Hp is primarily expressed by the liver, but can also be produced by non-hepatic tissues. Hence, a key question is whether the impaired B-cell differentiation of $Hp^{-/-}$ mice is due to the absence of liver-derived Hp or Hp from other sources. To address this question, we transplanted $Hp^{+/+}$ bone marrow into $Hp^{-/-}$ mice. Host animals were sub-lethally irradiated to provide a niche for the donor bone marrow. After 8 weeks of reconstitution, the peripheral blood of chimeric mice exhibited a composition of 56.7% ± 2.7% B-cells and 22.7% ± 1.5% T-cells, similar to wild-type mice (not shown). $Hp^{-/-}$ chimeric mice reconstituted with wild-type bone marrow showed a basal level of IgM and IgG in their blood that was very similar to that found in non-irradiated, non-reconstituted $Hp^{+/+}$ controls (Fig. 2A).

After an additional 9 days, chimeric mice were immunized with ovalbumin (OVA). OVA-specific IgM and IgG levels were similar in bone marrow reconstituted mice and in wild-type controls as measured by both Western blotting and ELISA assays (Fig. 2 B and C). In $Hp^{-/-}$ mice reconstituted with $Hp^{+/+}$ bone marrow cells, only bone marrow-derived cells produce Hp. Hence Hp from hematopoietic cells is sufficient to support a normal immune response and abundant liver-derived Hp is not essential. Notably, none of the immunized $Hp^{-/-}$ chimeras showed a detectable level of Hp in their blood (Fig. 2A&B), suggesting that Hp produced by hematopoietic cells does not contribute to circulating Hp.

To further characterize the immune response, we quantitated the number of OVA-specific IgG-producing plasma cells in the spleens of immunized non-chimeric $Hp^{+/+}$ and $Hp^{-/-}$ mice (Fig. 2D). $Hp^{-/-}$ mice had 6-7 fold fewer anti-OVA IgG-secreting cells than $Hp^{+/+}$ mice. However, the fact that $Hp^{-/-}$ mice have a ~2-fold lower number of B-cells in their spleen should be taken into account (Fig. 1B).[8] B-cells from immunized $Hp^{+/+}$ and $Hp^{-/-}$ mice expressed Blimp1, a master regulator of plasma cell differentiation,[27] but its level was lower in $Hp^{-/-}$ cells (Fig. 2E). This is consistent with the fact that $Hp^{-/-}$ spleens contained fewer IgG-secreting plasma cells detectable by ELISPOT.

3.2. Hp expression in B-cells contributes to immune response

To test whether the effects of Hp on the immune response were B-cell autonomous, we generated mixed bone marrow chimeras in Rag1-deficient hosts. $Rag1^{-/-}$ mice lack mature T- and B-cells, but express liver-derived Hp. First, to show that $Hp^{-/-}$ bone marrow does not have a competitive disadvantage in mixed chimeras, we reconstituted $Rag1^{-/-}$ mice with a 1:1 mixture of CD45.1 $Hp^{+/+}$ marrow and CD45.2 $Hp^{-/-}$ marrow. Bone marrow of either *Hp* genotype yielded comparable engraftments (Fig. 3A).

We also generated mixed bone marrow chimeras in which all B-cells were derived from $Hp^{+/+}$ or $Hp^{-/-}$ cells to test the role of B-cell-derived Hp. This was accomplished by

transplanting a 1:1 mixture of marrow from B-cell-deficient *muMT* mice and marrow from either $Hp^{-/-}$ mice or $Hp^{+/+}$ mice (Fig. 4A). Although the contribution of each bone marrow type in these chimeras cannot be easily distinguished as both donors are $CD45.2^{+}$, we anticipate that the resulting chimeras will contain ~50% $Hp^{-/-}$ cells and 50% muMT cells ($Hp^{+/+}$), since $Hp^{-/-}$ bone marrow does not have a competitive disadvantage. In the resulting mixed bone marrow chimeras, all B-cells are derived from either $Hp^{-/-}$ or $Hp^{+/+}$ donor cells, whereas T-cells and other immune cells can arise from either donor (and ~50% should express Hp). The $Rag1^{-/-}$ host also provides liver-derived Hp. Hence, we anticipate sufficient levels of circulating Hp and Hp produced by T-cells and/or myeloid cell types will be available in these chimeras.

Eight weeks post-transplant, flow cytometry of blood confirmed presence of B- and T-cells in host $Rag1^{-/-}$ mice (Fig. 3B). Interestingly, while the relative number of T-cells was comparable between the two groups, mice that had received $Hp^{-/-}$ bone marrow had fewer B-cells in the circulation than mice that received $Hp^{+/+}$ bone marrow. The lower number of B-cells found in $Hp^{-/-}$ chimeras may possibly account in part for the lower spleen weight and fewer splenocytes in these chimeras (Fig. 3B). These data suggest that deficiencies in development and differentiation of B-cells in $Hp^{-/-}$ mice are largely correlated with absence of Hp in these B-cells and independent of Hp produced by the liver, T-cells, DCs and myeloid cells. Sections of the spleen isolated from these mice demonstrated the development of lymphoid follicles, which are absent in the host $Rag1^{-/-}$ mice (data not shown, see also Fig. 4B below). At 8 weeks (day 56) post reconstitution, serum IgM and IgG antibodies were also detected in reconstituted chimeric mice, unlike in the host $Rag1^{-/-}$ mice (Fig. 3C & D). The basal concentration of IgM was higher in mice receiving $Hp^{-/-}$ bone marrow compared to those receiving $Hp^{+/+}$ marrow, whereas total IgG showed the inverse relationship. Thus, B-cell-derived Hp appears effective in regulating basal immunoglobulin levels.

The reconstituted mice were immunized with OVA (Fig. 4A) and analyzed nine days later for restoration of B-cell follicles (Fig. 4B), IgG-producing splenocytes (Fig. 4C) and production of anti-OVA antibodies (Fig. 4D and F). A separate cohort of mice received both primary immunization as well as a booster immunization 40 days after the primary immunization (Fig. 4E and F). Although the overall size of the B cell follicles was smaller in $Rag1^{-/-}$ mice reconstituted with either $Hp^{-/-}$ bone marrow due to the lower number of B cells, the number and the size of the germinal centers in the spleen was comparable between $Rag1^{-/-}$ mice reconstituted with either $Hp^{-/-}$ or $Hp^{+/+}$ bone marrow (Fig. 4B). To assess the contribution of B-cell-derived Hp to the anti-OVA immune response, we measured IgM and IgG anti-OVA antibodies. $Hp^{-/-}$ mixed bone marrow chimeras produced less OVA-specific IgG and IgM, as well as less total IgG, nine days post-immunization (Fig. 4D) and at day 112, following ovalbumin re-challenge, as compared to the $Hp^{+/+}$ mixed bone marrow chimeras (Fig. 4E). Western blot data were confirmed by ELISA (Fig. 4F).

Despite the presence of systemic Hp, Hp-expressing non-B-cells and germinal centers, ELISPOT showed a 3-fold lower number of IgG-secreting cells for $Hp^{-/-}$ bone marrow chimeras compared to $Hp^{+/+}$ bone marrow chimeras (Fig. 4C). The difference was less prominent than the 6-7-fold lower number noted for the donor mice (Fig. 2D), suggesting a supporting influence of systemic Hp and/or the presence of wild-type immune cells (except for B-cells). The failure to fully reconstitute the immune response in $Hp^{-/-}$ mixed bone marrow chimeras indicates that Hp is partly required in B-cells for a normal immune response. This represents the first evidence that defects in Hp-deficient mice can be linked in part to a B-cell-autonomous role for Hp.

3.3. Hematopoietic cells synthesize distinct Hp forms

The fact that B-cell-derived Hp contributes to the immune response and abundant liver-derived Hp is not sufficient to support immune responses, suggest that there may be a difference between the Hp forms. To determine whether B-cells produce a unique form of Hp, we first analyzed Hp transcripts from immune cells. Northern blot and RT-PCR for sequences corresponding to the 5 exons of the *Hp* gene did not indicate the presence of alternatively spliced Hp mRNA in any cell types and tissues tested (data not shown).[8] The amount of Hp expressed by immune cells was minor (<5%) compared to the amount produced by the liver during an acute phase response. Due to the low-level of expression, as well as absence of intracellular retention, the detection of Hp protein in lymphocytes was technically challenging (Fig. 5A). Among all the bone marrow-derived cells, only Gr1-positive granulocytes proved to contain large amounts of Hp (Fig. 5A, lanes 9 & 10) due to their ability to store Hp intracellularly.[21]

We characterized Hp secreted by *in vitro* cultured splenocytes and purified cell types and compared these to plasma Hp. We assessed: (a) molecular size; (b) binding to hemoglobin; and (c) posttranslational modifications (Fig. 5B & C). Hp in mouse plasma represents proteolytically processed α/β subunits that are assembled into a disulfide-linked complex of ~110 kDa that, under reducing conditions, dissociates into α (~10 kDa) and β (~45 kDa) subunits.[3] Hp forms were identified with polyclonal antibodies that recognize epitopes in the β subunit (Fig. 5B). Pro-Hp (Hp not proteolytically cleaved into α/β subunits) was undetectable in plasma under the conditions used for analysis. All plasma Hp bound hemoglobin and, thus, the identical patterns of Hp isoforms could be purified by hemoglobin-affinity chromatography as detected in non-fractionated plasma (Fig. 5C).[8,28]

In contrast to liver, splenocytes secreted Hp that migrated on SDS-PAGE with an apparent molecular size of ~120 kDa in the non-reduced state and 50-55 kDa after reduction (Fig. 5B). Splenocyte-derived Hp corresponds to pro-Hp based on the ~10 kDa larger size for the charge-heterogeneous monomer compared to the β-subunit of plasma Hp (Fig. 5C & D). Desialylation of splenic Hp converted it into more cationic isoforms of which the most basic product has a pI ~6.4, which is predicted for mouse pro-Hp (Fig. 5D). This form was distinguishable from the desialylated β subunit of plasma Hp of which the most basic form has a pI of ~6.9. The more basic pI of plasma β Hp is due to the removal of the acidic α subunit.[28]

Two-dimensional separation indicated that splenic pro-Hp displayed a similar charge heterogeneity as plasma Hp, but with a larger size range of the individual components (Fig. 5C & D). The size heterogeneity was tentatively been attributed to variations in the glycan structures. Only a minor fraction (~5%) of splenocyte-derived Hp was recovered by hemoglobin-affinity chromatography (Fig. 5B). While this hemoglobin-bound Hp migrated on 1D-PAGE with same size as the plasma Hp (Fig. 5B), 2D-PAGE revealed that it too was a pro-Hp form that was incompletely glycosylated/sialylated (Fig. 5C). Splenocyte-derived pro-Hp primarily represented lymphocyte-derived protein, since conditioned medium from purified B-cells contained the same Hp forms (Fig. 5A). This pro-Hp differed structurally from those produced by other cells, such as bone marrow-derived myeloid DCs (Fig. 5B), $Gr1^+$ neutrophils (Fig. 5A) or recombinant mouse Hp expressed by transfected MCF7 cells (Fig. 5B). Moreover, all Hp forms from these non-lymphocytic sources bound hemoglobin, whereas the majority of splenocyte-derived Hp did not.

Due to the relatively low amount of Hp-derived from spleen cell preparations, we restricted the functional analysis to two key forms, the major splenocyte-derived non-hemoglobin-bound Hp (termed form I) and the minor splenocyte-derived hemoglobin-bound Hp (form

II), which were both isolated from conditioned media of mass cultures of untreated splenocytes.

3.4. Effects of Hp on B-cells

B-cells were isolated from untreated $Hp^{-/-}$ and $Hp^{+/+}$ mice and cultured with anti-CD40 and IL-4 to activate the cells and induce differentiation. While cells from both genotypes proliferated and underwent homotypic association (Fig. 6A), B-cells from $Hp^{-/-}$ mice displayed consistently smaller, less-structured cell clumps containing more dead cells and debris. Staining with 7-AAD showed 1.4 to 2-fold more dead cells in $Hp^{-/-}$ B-cell cultures (Fig. 6B). Measurements of thymidine incorporation indicated a two-fold lower DNA synthesis for $Hp^{-/-}$ B-cells (Fig. 6C).

Unlike $CD4^+$ and $CD8^+$ T-cells,[8] neither $Hp^{+/+}$ nor $Hp^{-/-}$ B-cells proliferated more in response to purified plasma Hp (Fig. 6C). However, the prevalent, non-hemoglobin-binding splenic pro-Hp form was able to stimulate proliferation of $Hp^{-/-}$ B-cells to almost the level of $Hp^{+/+}$ B-cells. This stimulatory activity was neutralized by polyclonal anti-Hp antibody (Fig. 6C).

The production of IgG followed the pattern of proliferation. ELISA indicated that $Hp^{-/-}$ B-cell cultures had a 2-fold lower IgG output per live cell unit as compared to $Hp^{+/+}$ B-cell cultures (Fig. 6D). While treatment with plasma-derived Hp was ineffective in increasing IgG production, splenic non-hemoglobin-binding Hp enhanced it by two-fold (Fig. 6D). This finding suggested that pro-Hp functions as an autocrine or paracrine coactivator/survival factor for B-cells.

4. DISCUSSION

Hp is a prominent protein produced at high levels mainly by the liver in inflammatory conditions. Hp participates in the acute phase response and has known anti-inflammatory properties based on its ability to bind free hemoglobin and inhibit the production of free radicals. Non-hepatic cells, including immune cells, can also produce Hp, albeit to a much lower level than the production by acute phase liver.

To test the biological effects of Hp, mice lacking Hp were previously derived.[8,22] Interestingly, in the basal state, these $Hp^{-/-}$ mice demonstrate reduced numbers of peripheral B cells (particularly of the marginal zone type), but increased numbers of IgM secreting plasma cells and higher than normal titers of serum IgM. The effects of Hp on the basal differentiation of B cells is at least in part B cell-intrinsic as mixed bone marrow chimeras in which all B cells are $Hp^{-/-}$ showed a diminished number of peripheral B cells, similar to that found in non-chimeric $Hp^{-/-}$ mice.

Although basal numbers of IgM secreting plasma cells are higher in $Hp^{-/-}$ mice, antigen-specific IgM and IgG antibody responses are several-fold lower in immunized $Hp^{-/-}$ mice than in immunized $Hp^{+/+}$ mice, suggesting defects in the immune response. Antibody responses to the T-dependent antigen OVA arise mainly from germinal center B cells, while basal IgM typically arises from non-germinal center derived, short-lived plasmablasts. Since germinal center numbers and size appeared similar in mixed bone marrow chimeric mice with $Hp^{-/-}$ B cells, it is plausible that the main effect of Hp on B cell responses to immunization might be an ability to control germinal center B cell differentiation into plasma cells. Thus, there appears to be a differential requirement for Hp in antibody-production by germinal center-derived and non-germinal center-derived antibody-secreting cells.

Bone marrow transplantation experiments led to the unexpected finding that Hp produced by hematopoietic cells, not plasma Hp produced by the liver, supports the immune response. Furthermore, mixed bone marrow chimeras indicated that Hp produced by B-cells is important to maximal antibody responses. Bone marrow chimera results were verified by treating $Hp^{-/-}$ B-cells with purified splenic Hp *in vitro*, which stimulated their proliferation and antibody secretion.

In addition to its function in mature B-cells, Hp likely also has an supporting role in earlier stages of B-cell development given that $Hp^{-/-}$ mice have fewer $B220^{+}IgM^{-}$ and $B220^{+}IgM^{+}$ cells in the bone marrow[8]. In this manuscript, we show that splenic Hp can stimulate the proliferation of mature, peripheral B cells. Splenic or liver-derived Hp might also be able to stimulate proliferation of B cell precursors in the bone marrow and thereby its absence would result in fewer B cell precursors.

4.1. Extrahepatic source of Hp as a potential biological effector

Most of the current information on structure and function of Hp is derived from the plasma form and primarily concerns its role as hemoglobin-binding protein,[4,5] antioxidant,[29,30] and chaperone.[31] Extrahepatic Hp expression has been found in epithelial cells, [32-34] neutrophils,[21] adipocytes,[35] DCs and macrophages.[8] Analysis of Hp isolated from neutrophils[21] revealed that it includes both Hp sequestered from plasma[36,37] as well as neutrophil-synthesized Hp. The larger size of the β-subunit of neutrophil-synthesized Hp was attributed to differential glycosylation.[21] Despite the differences in posttranslational modifications, all Hp forms from neutrophils bind hemoglobin and function as antioxidants at the site of neutrophil activation.[36]

Splenocyte Hp differs from plasma Hp by posttranslational modifications. The characteristic charge and size heterogeneity of splenic Hp indicates the synthesis of cell-type specific carbohydrate structures. Unexpectedly, the major splenic Hp isoform (form I) was unable to bind hemoglobin. Hp from splenocytes is not proteolytically cleaved into α/β subunits suggesting that mouse splenocytes lack the C1r-like protease reported to process pro-Hp.[38] However, the absence of proteolysis alone is not sufficient to render pro-Hp unable to bind hemoglobin. The pro-Hp made by liver cells in culture,[28] or other cells such as DCs or epithelial cells, has hemoglobin binding activity. We presume that lymphocyte-specific modifications of the protein (potentially specific glycosylation events) prevent its interaction with hemoglobin. In support of this is the fact that the minor splenic Hp isoform with less carbohydrate processing (form II) binds hemoglobin.

The level of Hp expression in hematopoietic cells, including lymphocytes, is low compared to that expressed by the liver during an acute phase reaction. However, in non-inflamed mice kept in a pathogen-free environment, hepatic Hp expression is also quite low and comparable to that found at extrahepatic sites.[32,35] Even under such conditions, no proteins corresponding to the splenic or other extrahepatic Hp forms were identifiable in the blood. Similarly, $Hp^{-/-}$ mice with engrafted $Hp^{+/+}$ bone marrow did not show immunodetectable Hp in the plasma. Two processes may account for this: non-hepatic Hp is either rapidly cleared from the circulation or does not appreciably exit from sites of production.

4.2. Hp action on lymphocytes

Lack of Hp attenuates the immune response to immunization. Recently, Hp has been identified as a pro-inflammatory mediator in skin transplantation experiments and in co-stimulation of DCs.[9] In contrast to these pro-inflammatory and pro-immunogenic roles, Hp has also been proposed to limit immune cell activation and serve as an antioxidant.[10] The

precise role of Hp in a particular experimental system may rely on the particular immune cell types involved and the microenvironment of these cells.

While the previous characterization of $Hp^{-/-}$ mice uncovered the link between Hp and immune cell regulation[8] and the regulation of inflammation,[9-11] it did not permit identification of the cellular origin of the biologically relevant Hp. We[8] and others[11,39,40] have demonstrated that plasma Hp mediates *in vitro* responses that partly explain *in vivo* processes. However, plasma-derived Hp could only partially restore T-cell activity and had no effect on B-cells, the two lymphocyte populations most prominently affected by Hp deficiency. Thus, plasma Hp is not fully responsible for the phenotype of $Hp^{-/-}$ mice. Mixed bone marrow chimeras where all B-cells are Hp-deficient demonstrated that B-cell-derived Hp exerts a cell-autonomous action. However, the fact that exogenously added splenic Hp was effective in co-stimulating B-cells *in vitro* suggests the possibility of paracrine action of Hp as well.

Although we have identified lymphocyte-derived pro-Hp as a regulatory component for B-cell function, the identity of the receptor for this Hp form is still unclear. The previously proposed Hp receptors, in particular CD22 for B-cells,[41] may not be involved as their function had been defined with plasma-derived Hp, the form that was ineffective in our B-cell assay system. We have begun some preliminary experiments to determine the mechanism of action of Hp on B-cells. However, this remains technically challenging, since the relevant receptor and its downstream signaling effectors remain unknown. Future analyses will be needed to clarify issues regarding binding specificity of plasma versus splenic Hp to potential Hp receptors and to identify the signals transduced from these receptors.

Taken together, our study indicates that hematopoietic Hp contributes to the homeostasis and function of the lymphocyte compartment and promotes execution of an optimal immune response. This adds Hp to the list of plasma proteins that, by cell-specific modification, assumes a distinct regulatory role not previously recognized. Potentially, supplementation of vaccine regimens with the splenic form of Hp could stimulate antibody responses to the immunization. Additional studies would be needed to determine if this is feasible.

Acknowledgments

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Abbreviations

AGP	α_1-acid glycoprotein
BAFF	B-cell activation factor of the TNF family
BAFF-R	BAFF receptor
BLIMP-1	B lymphocyte-induced maturation protein-1
DC	dendritic cell
ECL	enhanced chemiluminescence

Hp	haptoglobin
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
OVA	ovalbumin

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Highlights

- A novel form of haptoglobin which does not bind hemoglobin is produced by hematopoietic cells
- Posttranslational modifications distinguish hepatic derived and extrahepatic forms of haptoglobin
- B-cell-derived haptoglobin facilitates proper B-cell function and drives B-cell dependent immune responses

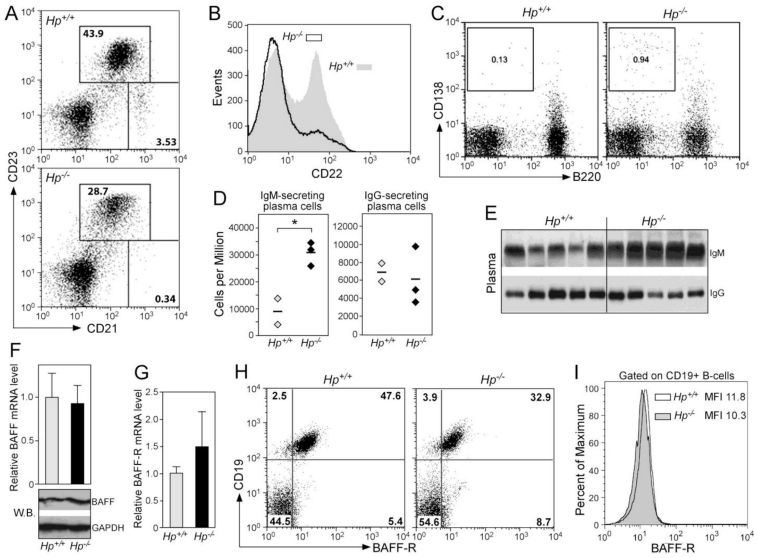

FIGURE 1.

Maturation of B-cells in $Hp^{+/+}$ and $Hp^{-/-}$ mice. **A,** A representative flow cytometric analysis of follicular ($CD21^{int}CD23^{+}$) and marginal-zone ($CD21^{hi}CD23^{lo}$) B-cell types from the spleen of $Hp^{+/+}$ (top panel) and $Hp^{-/-}$ mice (bottom panel). Numbers within the boxed regions represent the percent of each boxed population in the spleen. **B**, Expression of the B-cell marker CD22 in splenocytes of $Hp^{+/+}$ and $Hp^{-/-}$ mice. Note that fewer $CD22^{+}$ B-cells are present in $Hp^{-/-}$ spleens, but that the mean fluorescent intensity (MFI) of CD22 is similar between $Hp^{+/+}$ and $Hp^{-/-}$ B-cells. **C & D**, Increased IgM-secreting plasma cells in the spleens of $Hp^{-/-}$ mice detected by flowcytometry of $B220^{low/neg}CD138^{hi}$ plasma cells (C) and by ELISPOT analysis of IgM- and IgG-secreting plasma cells in the spleens of unimmunized, 8 week-old $Hp^{+/+}$ and $Hp^{-/-}$ mice (D). **E**, Level of IgM and IgG in equivalent aliquots of plasma from 5 individual, 8 week-old $Hp^{+/+}$ and $Hp^{-/-}$ mice detectable by immunoblotting for the corresponding heavy chains. **F**, mRNA and protein analyses indicate normal expression of BAFF by *Hp−/−* spleen. Upper panel: qRT-PCR with primers to the mouse BAFF gene using cDNA prepared from splenocytes (N=3, mean ± SD). Lower panel: Western blot analysis of BAFF protein in whole spleen extracts from wild-type and *Hp−/−* mice. **G**, Relative level of BAFF-R mRNA in B-cells purigied from $Hp^{+/+}$ and $Hp^{-/-}$ spleens as determined by qRT-PCR. **H**, Expression of BAFF-R protein by $CD19^{+}$ B-cells in the spleens of $Hp^{+/+}$ and $Hp^{-/-}$ mice determined by flow cytometry. **I**, Histogram and MFI of BAFF-R expression by CD19+ cells as determined in H.

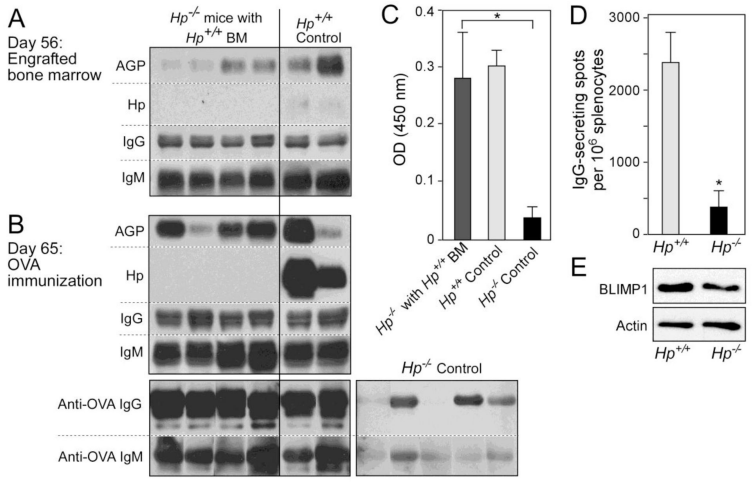

FIGURE 2.

$Hp^{+/+}$ bone marrow reconstitutes a wild-type immune response to OVA in $Hp^{-/-}$ mice. Groups of 4 age-matched $Hp^{-/-}$ mice received $Hp^{+/+}$ bone marrow. After 8 weeks of reconstitution (56 days), these animals, together with two $Hp^{+/+}$ (controls) and 5 non-reconstituted $Hp^{-/-}$ mice, were immunized with OVA. Nine days later (day 65), the level of anti-OVA antibodies was evaluated by Western blotting and ELISA. **A,** Western blots to detect basal levels of serum antibodies and acute phase proteins in chimeric mice versus wild-type controls. The acute phase protein α_1-acid glycoprotein (AGP), which is produced by the liver and induced during immunization, was measured as an internal control. Also measured were the levels of serum Hp, total serum IgM and IgG. **B,** After immunization, anti-OVA IgM and anti-OVA-IgG antibodies were measured. Anti-OVA antibodies were also measured in immunized non-chimeric $Hp^{-/-}$ mice (side panel). **C**, ELISA of OVA-specific IgG in serum of immunized reconstituted $Hp^{-/-}$ mice as well as immunized $Hp^{+/+}$ and $Hp^{-/-}$ controls (N=4-5; mean ± SD; p=0.03). **D**, Quantification of IgG-secreting plasma cells from $Hp^{+/+}$ and $Hp^{-/-}$ mice 7 days after immunization with OVA using ELISPOT (N=5; mean ± SD; p = 0.01). **E**, Immunoblot to assess BLIMP-1 expression in lysates of B-cells isolated from $Hp^{+/+}$ and $Hp^{-/-}$spleens.

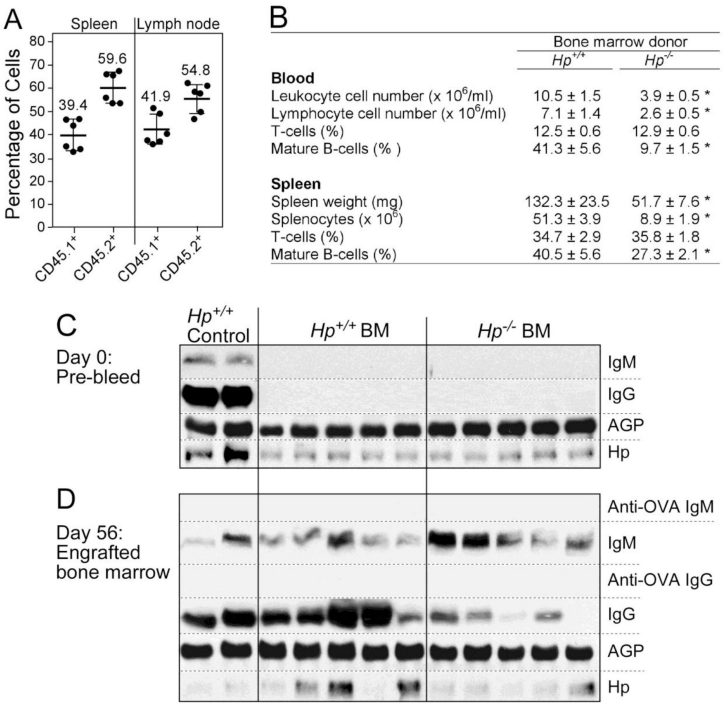

FIGURE 3.

Generation of mixed bone marrow chimeras. **A**, $Hp^{+/+}$ and $Hp^{-/-}$ bone marrow cells engraft with similar efficiencies in host mice. $Rag1^{-/-}$ mice received a 1:1 mixture of $CD45.1^+$ $Hp^{+/+}$ marrow and $CD45.2^+$ $Hp^{-/-}$ marrow. After 8 weeks, the relative number of $CD45.1^+$ and $CD45.2^+$ cells in spleen and lymph nodes was determined, which showed that $Hp^{-/-}$ bone marrow was as efficient as $Hp^{+/+}$ bone marrow in engrafting. **B,** $Rag1^{-/-}$ mice received a transplantation of a 1:1 mixture of bone marrow from *muMT* B-cell deficient mice along with bone marrow from $Hp^{+/+}$ or $Hp^{-/-}$ mice. The reconstitution of the immune system was assessed after 8 weeks (56 days) by determining the cellular composition of spleen and blood from $Hp^{+/+}$ and $Hp^{-/-}$ bone marrow chimera mice (5 mice per group, means ± SD, *$p<0.05$). **C & D**, Serum aliquots, collected prior to or after reconstitution of $Rag1^{-/-}$ mice with mixed bone marrow containing either $Hp^{+/+}$ or $Hp^{-/-}$ cells, were analyzed by immunoblotting for IgM, IgG, Hp and AGP as an internal control. Also measured were serum anti-OVA IgM and IgG antibodies, which were undetectable in these unimmunized mice. Two control $Hp^{+/+}$ mice served as reference.

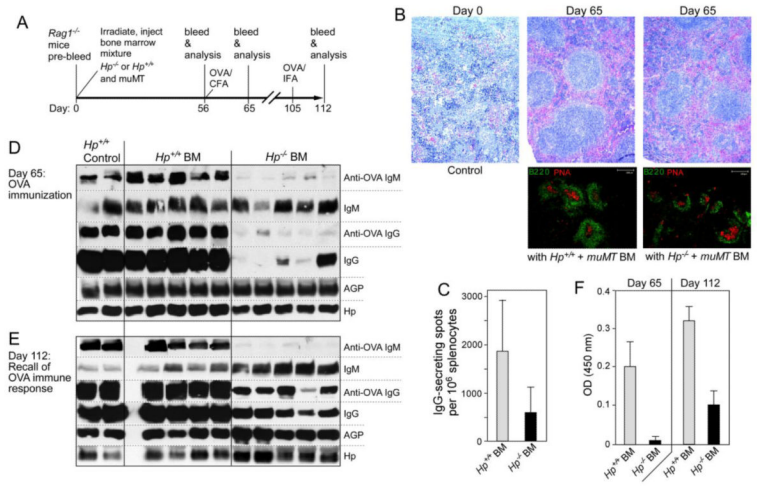

FIGURE 4.

Hp-deficiency at the level of B-cells prevents a full immune response. **A,** Time course of the experiments to analyze responses of bone marrow chimeric mice to immunization. Groups of 10 *Rag1*$^{-/-}$ mice received a transplantation of a 1:1 mixture of bone marrow from *muMT* B-cell deficient mice along with bone marrow from *Hp*$^{+/+}$ or *Hp*$^{-/-}$ mice (as described in Figure 3). **B, upper panels,** Microphotographs of H&E stained sections (100X magnification) of the spleens of *Rag1*$^{-/-}$ mice without (Control, day 0) and with (day 65) bone marrow from *Hp*$^{+/+}$ or *Hp*$^{-/-}$ in combination with *muMT*. Note the presence of lymphoid follicles in reconstituted mice. **B, lower panels**, Spleen sections from immunized mixed bone marrow chimeric mice stained with B220 (green) to identify B-cell follicles and with peanut agglutinin (PNA, red) to identify germinal centers. **C,** The relative number of IgG-secreting cells in spleens day 65 were quantified by ELISPOT assay (N=5, p= 0.05). **D & E,** Serum aliquots, collected at the days indicated from *Rag1*$^{-/-}$ mice containing marrow from either *Hp*$^{+/+}$ or *Hp*$^{-/-}$ mice, were analyzed by immunoblotting for the proteins indicated at the right. AGP immunoblotting served as an internal control. Two control *Hp*$^{+/+}$ mice served as reference. Animal #1 with *Hp*$^{+/+}$ bone marrow died during the week of the recall reaction and is not shown in panel E. **F**, The relative amounts of anti-OVA IgG in the serum samples taken after initial immunization (day 65; N=5, p<0.01) and after recall (day 112; N=4-5, p=0.03) were determined by ELISA. The OD values for the samples at 1/80 dilution are shown.

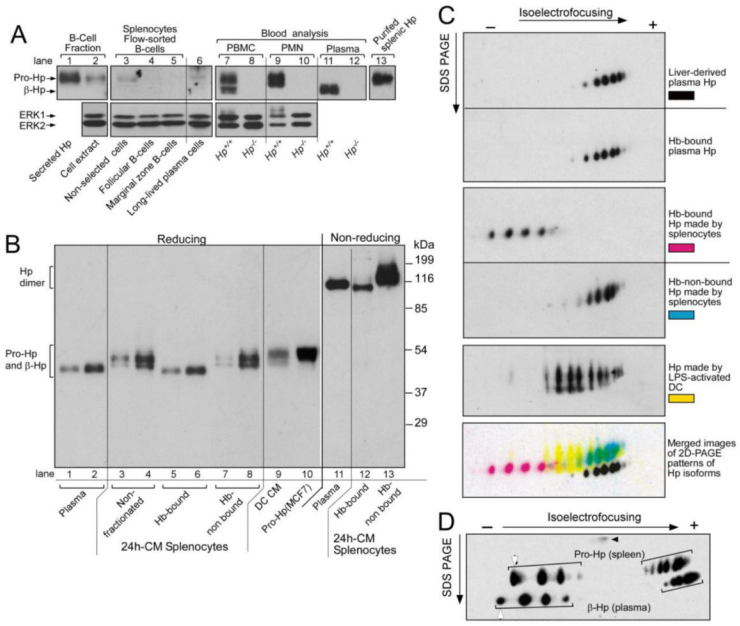

FIGURE 5.

Identification of a unique form of Hp produced by hematopoietic cells. **A**, Western blot of Hp expression in various subsets of B-cells and other leukocytes purified as described in the Methods. Shown is the secreted and intracellular forms of Hp from bulk B-cells isolated from the spleen (first two lanes) as well as intracellular Hp from highly-purified flow cytometry sorted B-cell subsets (lanes 3-6), peripheral blood mononuclear cells (lanes 7-8), Gr1+ neutrophils (lanes 9-10), blood plasma (lanes 11-12) and purified splenic Hp (lane 13). Erk serves as marker for sample loading in cases of cell extracts. **B**, Electrophoretic analysis of variants of Hp made by hematopoietic cells. Hp preparations were separated on one-dimensional SDS PAGE under reducing (lanes 1-10) and non-reducing condition (lanes 11-13) and visualized by immunoblotting. For better quantification, two-fold different amounts of samples were loaded into the first 4 lane pairs (lanes 1-8). The higher amount was also separated under non-reducing conditions (lanes 11-13). The samples represent: Hp in acute phase mouse plasma (lanes 1, 2 & 11); conditioned medium of *in vitro* cultured splenocytes before purification (lanes 3 & 4), Hp eluted from hemoglobin-containing columns (*Hb-bound*, lanes 5, 6 & 12), and Hp found in the flow through of hemoglobin-containing columns (*Hb-non-bound*, lanes 7, 8 & 13). For comparison, Hp in conditioned medium of LPS-activated mouse DCs (DC CM, lane 9) and MCF7 cells transduced with an mouse Hp expression vector (MCF7, lane 10) were co-analyzed. **C**, 2D-PAGE-immunoblots represent: Hp in unfractionated plasma and after hemoglobin-affinity and size chromatography, Hp isolated from conditioned medium of splenocytes, and Hp in unfractionated conditioned medium of LPS-activated DC. The separations were carried out under identical conditions. The electrophoretic mobilities of the various Hp forms were compared by overlaying colorized images of each pattern. **D**, Identification of isoelectric points of desialylated forms of pro-Hp and β-Hp. Hp isolated from splenocytes (same as in lane 7/8 in B) and plasma was digested with neuraminidase. Aliquots of the samples before and after desialylation were separated by 2D-PAGE. The immunoblot patterns (non-digested Hp forms at the right; desialylated Hp form at the left) were graphically merged to indicate the relative positions of the glycosylated forms of the splenic (upper row of protein spots) and plasma Hp (lower row of protein spots). The positions of the most basic form are marked by open arrowhead. Internal reference protein spot for positioning of the patterns is indicated by the closed arrowhead.

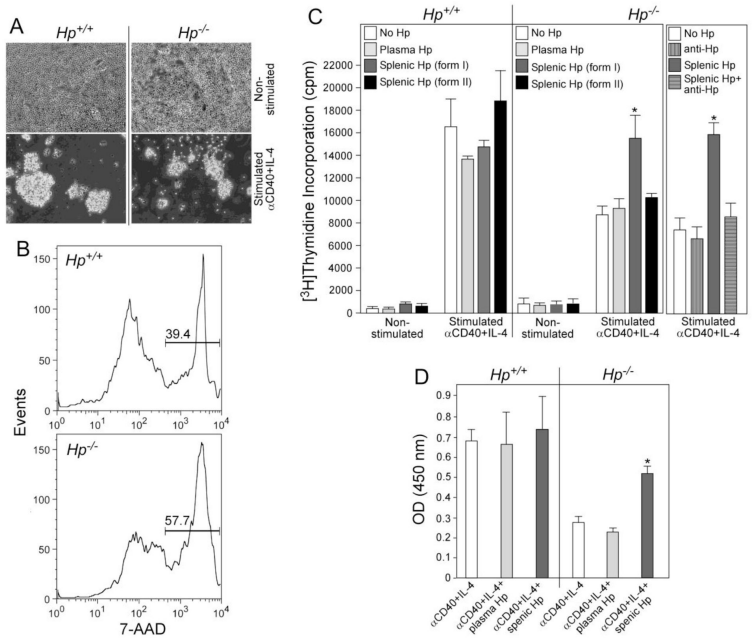

FIGURE 6.

Response of isolated B-cells to purified Hp preparations. **A**, Photomicrographs (40X) of purified B-cell cultures from spleens of $Hp^{+/+}$ and $Hp^{-/-}$ mice (day 5) either unstimulated or stimulated with αCD40 and IL-4. **B**, Relative number of dead cells in B-cell cultures maintained for 5 days in presence of anti-CD40 and IL-4 was determined by 7-AAD staining and flow cytometry. **C**, Left panel: Thymidine incorporation for 16 hours after 2 days of treatment in either control medium or growth stimulatory medium containing anti-CD40 and IL-4. The media included also Hp from plasma (hemoglobin binding, 300 ng/ml) or from splenocytes, form I (does not bind hemoglobin, 300 ng/ml) or form II (binds hemoglobin, 50 ng/ml). Right panel: Replicate cultures of a separate B-cell preparation from $Hp^{-/-}$ mice were incubated in growth stimulatory medium containing anti-CD40 and IL-4 alone, or in addition anti-haptoglobin immunoglobulins (100 μg/ml) and splenic form I Hp (300 ng/ml) as indicated. (N=3-5, ** $p<0.01$). **D**, IgG detectable by ELISA in the supernatant of stimulated B-cells from $Hp^{+/+}$ and $Hp^{-/-}$mice (adjusted based on viable cell number at day 5). The OD values of the culture media at 1/5000 dilution are shown (N=3, $p<0.01$).