Distinct and Separable Roles of the Complement System in Factor H–Deficient Bone Marrow Chimeric Mice with Immune Complex Disease

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Plasma complement factor H (Cfh) is a potent complement regulator, whereas Cfh on the surface of rodent platelets is responsible for immune complex processing. For dissection between the two, bone marrow chimeras between Cfh-deficient (Cfh−/−) and wild-type C57BL/6 mice were created. Platelet Cfh protein was tracked with the Cfh status of the bone marrow donor, indicating that platelet Cfh is of intrinsic origin. In an active model of immune complex disease, Cfh−/− mice that were reconstituted with wild-type bone marrow had levels of platelet-associated immune complexes comparable to those of wild-type mice and were protected against the excessive glomerular deposition of immune complexes seen in Cfh−/− mice, yet these mice still developed glomerular inflammation. In contrast, wild-type mice with Cfh−/− bone marrow had reduced platelet-associated immune complexes and extensive glomerular deposition of complement-activating immune complexes, but they did not develop glomerular pathology. The large quantities of glomerular C3 in wild-type mice with Cfh−/− bone marrow were in the form of iC3b and C3dg, whereas active C3b remained in Cfh−/− recipients of wild-type bone marrow. These data show that plasma Cfh limits complement activation in the circulation and other accessible sites such as the glomerulus, whereas platelet Cfh is responsible for immune complex processing.


The complement system of proteins identifies pathogens and apoptotic material on which a directed activation can ensue to result in their efficient disposal (1). Besides this important contribution to innate immunity, the complement system is involved in adaptive immunity, such as by providing cues for a directed immune response by B and T cells (2). In addition, complement is crucial for the proper disposal of immune complexes. This multistep process requires complement activation by immune complex Ig that leads to immune complex incorporation of C3b and binding to CR1 on primate erythrocytes, which shuttles and transfers them to CR3 and Fc receptor-bearing cells of the mononuclear phagocyte system (3). Although it is clear that primates use the erythrocyte and its CR1 protein for this function, other species, such as the mouse, rely on platelets to process immune complexes (4). We previously provided evidence that the platelet protein that is responsible for binding C3b in these complexes is complement factor H (Cfh) (5).

Like CR1, Cfh is a member of the regulators of complement activation gene family (6,7). All family members have short consensus repeats (SCR) that are arranged in tandem and that each contain approximately 60 amino acids with a conserved core structure, including four cysteines that form two intra-SCR disulfide bonds. Both CR1 and Cfh have affinity for C3b and can serve as co-factor for the complement factor I (Cfi)-mediated cleavage of C3b to iC3b, which allows transfer of immune complexes to CR3-bearing cells. However, unlike CR1, which is a type I membrane protein with a specific cellular distribution, Cfh exists in the plasma as a soluble protein. Cfh does have the capacity to bind to cells (and other noncellular sites) with specificity, a phenomenon that can lead to protection from complement activation on normal cells, as well as malignant cells and adapted microorganisms (8,9). The inability of Cfh to bind to host tissue sites, particularly because of mutations that affect the final SCR, also may underlie the pathogenesis of atypical hemolytic uremic syndrome (10).

Furthermore, age-related macular degeneration was associated recently with the Tyr402His polymorphism in the seventh SCR, a site for binding C-reactive protein and heparin (11). There also is evidence that cells can produce Cfh and have it remain plasma membrane associated (12,13).

Given these various functions of Cfh, along with our uncertainties of its origin on platelets, here we studied cultured
platelets as well as those that originated in animals with adoptive bone marrow transfers. In the latter, we also investigated an active model of immune complex disease in which immune complex processing and complement activation are key.

Materials and Methods

Animals

Normal wild-type C57BL/6 mice were purchased from Jackson Laboratories. Cfh<sup>−/−</sup> mice (14) were backcrossed >10 generations onto the C57BL/6 strain.

Bone Marrow Chimeric Mice

Wild-type or Cfh<sup>−/−</sup> C57BL/6 mice at 4 to 6 wk of age received 1050 cGy (irradiation). Bone marrow cells were isolated by standard techniques from femurs of wild-type or Cfh<sup>−/−</sup> C57BL/6 mice, and CD-117 (c-kit)-positive progenitor cells were isolated from bone marrow cells using a mAb magnetic positive selection technique (Miltenyi CD117 MicroBeads, Auburn, CA). One day after irradiation, mice received 1 × 10<sup>6</sup> CD-117–positive progenitor cells intravenously. In pilot studies, this protocol rescued animals from lethality and led to full hematopoietic reconstitution within 3 wk.

Serum Sickness

Immune complex disease was induced by actively immunizing chimeric mice 6 wk after bone marrow transfer with a daily intraperitoneal dose of 4 mg of horse spleen apoferritin (Calzyme Laboratories, San Luis Obispo, CA) (15–18). Controls included wild-type mice into which wild-type bone marrow was transferred and wild-type and Cfh<sup>−/−</sup> mice that were not subjected to bone marrow transfer yet were immunized with the same schedule of apoferritin or saline vehicle alone. After 5 wk, mice were killed and renal disease was characterized.

Western Blots

Glomeruli were isolated from 4-μm frozen sections by laser capture microdissection using a Leica AS LMD microdissection microscope. A total of 450 glomerular sections in 150 mM sodium chloride, 1% NP-40, 50 mM Tris (pH 8.0), and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) were separated by SDS-PAGE (10%), transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and incubated with horseradish peroxidase-anti-mouse C3 (Cappel), MP Biomedicals, Irvine, CA) or rabbit anti-human C3d (Dako, Carpinteria, CA) followed by horseradish peroxidase-anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO). Bound antibodies were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).

As controls for mouse C3 cleavage products, C3 was purified from normal mouse plasma using previously described techniques (19) and incubated in trypsin (1% wt/wt) at 37°C for various times, followed by neutralization with soybean trypsin inhibitor. The resultant C3b and further cleavage fragments were subjected to Western blotting with the anti-C3 and anti-C3d antibodies that were used for glomerular immunoblotting. As in our previous studies with these same antibodies (19), each was reactive with the 110-kD C3b α chain, attributable to non-overlapping reactivities of anti-C3 with α<sub>43</sub> and anti-C3d with α<sub>43</sub>.

Platelet Culture

Published protocols were used with minor modifications (20,21). CD-117–positive progenitor cells were isolated and cultured in Iscove’s Modified Dulbecco’s Medium supplemented with 1% Nutridoma (Roche Applied Science) and 3 ng/ml murine thrombopoietin (Cell Sciences, Canton, MA) for 3 d. Megakaryocytes were isolated on a discontinuous BSA density gradient and cultured again in the same medium.

Flow Cytometry

Platelets were isolated ex vivo (5,19) or from culture supernatants and stained either directly with FITC-goat anti-mouse IgG (Cappel) or indirectly with sheep anti-platelet factor H or goat anti-mouse thrombocyte antibodies (Accurate Scientific, Westbury, NY) followed by FITC-conjugated antibodies to sheep or goat IgG (Cappel) (5). Samples were analyzed using the Becton Dickinson FACSCalibur and CellQuest software (BD Biosciences, Bedford, MA).

Immunofluorescence Microscopy

Four-micrometer sections from frozen mouse kidneys were fixed in ethanol:ether (1:1) for 10 min followed by 95% ethanol for 20 min and washed with PBS and stained with FITC-conjugated goat antibodies to mouse IgG or C3 (Cappel). Slides were viewed with an Olympus BX-60 IF microscope (Melville, NY) and scored in a masked manner from 0 to 4. Representative photomicrographs were taken at identical settings.

Renal Pathology

Tissues were fixed in 10% buffered formalin and embedded in paraffin, from which 4-μm-thick sections were cut and stained with periodic acid-Schiff. Each slide was scored in a blinded manner by a renal pathologist (M.H.) for the extent of glomerulonephritis (GN) on a scale of 0 to 4 (in increments of 0.5) according to the schema of Passwell et al. (22) as described previously (23). In addition, the fraction of glomeruli with segmental sclerosis and/or hyalinosis was determined; no crescents were seen in any of the specimens.

Statistical Analyses

Numerical data are presented in the text or Table 1 as medians with ranges in parentheses. Graphical data are presented from individual mice with different experiments depicted with different symbols or as means ± SEM. Statistical significance between groups was determined by Mann-Whitney testing (Minitab v.12) or by ANOVA followed by Fisher pairwise tests. Potential correlation among variables was determined by calculating the Pearson product moment correlation coefficient.

<table>
<thead>
<tr>
<th>BM → Host</th>
<th>Anti-Apoferitin IgG (U)</th>
<th>Circulating Immune Complexes (U)</th>
<th>Platelet Cfh (MCF)</th>
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<tbody>
<tr>
<td>Cfh&lt;sup&gt;−/−&lt;/sup&gt; → wt</td>
<td>0.80 (0.72 to 0.99)</td>
<td>0.35 (0.32 to 0.67)</td>
<td>204 (16 to 794)</td>
</tr>
<tr>
<td>wt → Cfh&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1.19 (0.67 to 1.41)</td>
<td>0.50 (0.31 to 1.20)</td>
<td>3934 (220 to 4829)&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Data are medians (ranges); n = 5 per group. Cfh, complement factor H; MCF, mean channel fluorescence.

<sup>b</sup>Mean channel fluorescence.

<sup>bp = 0.02 versus Cfh<sup>−/−</sup> → wt.</sup>
Results

Platelet Cfh Is of Intrinsic Origin

Although Cfh is an abundant plasma protein, our previous studies demonstrated that it was specifically expressed on the platelet and not other blood cells, such as the erythrocyte, despite their continuous exposure to plasma proteins (5). By analogy to erythrocyte CR1, we considered it possible that platelets are endowed with Cfh upon exit from the bone marrow. To investigate this, we cultured megakaryocytes from normal C57BL/6 mice. These had the expected arborizing processes that released into the culture supernatant a continuous supply of platelets with features that were comparable to those of blood platelets (20,21). Megakaryocytes and platelets had mRNA for Cfh (Figure 1A), and cultured platelets bore Cfh protein on their surface (Figure 1B). These results provide proof that platelets and their precursors have the intrinsic capacity to produce Cfh as a plasma membrane protein.

To investigate this in vivo, we performed adoptive bone marrow transfers between mice with targeted deficiency of Cfh (14) (N10, C57BL/6) and wild-type C57BL/6 mice. For elimination of any contribution from native bone marrow cells, mice were lethally irradiated 1 d before transfer of bone marrow cells. Platelets from Cfh−/− recipients of wild-type bone marrow had quantities of Cfh that were comparable to those from un manipulated mice, whereas platelets in wild-type recipients of Cfh−/− bone marrow had a marked reduction in Cfh (Figure 2). These results are consistent with data from cultured platelets and indicate that platelet-bound Cfh is largely of intrinsic (i.e., megakaryocyte) origin. Because in these studies Cfh-deficient platelets were bathed in a Cfh-rich plasma, it is likely that what platelet-associated Cfh was identified in these studies could be attributable to its absorption from plasma onto the platelet surface.

Platelet Cfh Binds Immune Complexes In Vivo

To investigate the role of platelet Cfh in immune complex metabolism, we studied chronic serum sickness in bone marrow chimeric mice. Mice immunized with apoferritin generated an appropriate anti-apoferritin IgG immune response. After 5 wk of daily immunization with apoferritin, there were no differences in antibody titers between wild-type recipients of Cfh−/− bone marrow and Cfh−/− recipients of wild-type bone marrow (Table 1). Similar to animals without serum sickness, platelets from wild-type recipients of Cfh−/− bone marrow had markedly decreased platelet-associated Cfh, whereas the transfer of wild-type bone marrow to Cfh−/− hosts was capable of reconstituting platelet Cfh (Table 1).

As a measure of immune complexes bound to platelets, the quantities of IgG that were associated with platelets were determined. To explore this in more detail, we also examined platelets from wild-type and Cfh−/− mice (i.e., without bone marrow transplant) that were immunized with apoferritin or saline as controls. As shown in Figure 3, low levels of platelet-associated IgG were found in both wild-type and Cfh−/− mice that were immunized with saline. In wild-type mice that were immunized with apoferritin, there was a significant increase in platelet-associated IgG, which was not seen in Cfh−/− mice. As with platelet-associated Cfh, IgG on platelets was reduced in wild-type recipients of Cfh−/− bone marrow, whereas Cfh−/− recipients of wild-type bone marrow had amounts of platelet-associated IgG that were comparable to apoferritin-immunized wild-type animals. These data are consistent with the hypothesis that intrinsically produced platelet Cfh binds immune complexes. Further support for this is the significant correlation between platelet-associated Cfh and IgG levels (r = 0.68, P = 0.03). There was not total elimination of IgG from Cfh-deficient platelets could reflect that acquired from plasma was some platelet Cfh that was capable of binding immune complexes.
and/or binding occurred in a Cfh-independent manner, such as to Fc receptor on the platelet surface (24).

Platelet Cfh Limits Deposition of Immune Complexes, whereas Plasma Cfh Restricts Complement Activation in Glomeruli

Glomerular manifestations of serum sickness in the chimeric mice were investigated next by killing of mice after 5 wk of apoferritin immunization and examining glomeruli for immune reactants. Cfh<sup>−/−</sup> mice with wild-type bone marrow had moderate glomerular deposition of IgG (Figure 4B) and C3 (Figure 4F), primarily in the mesangium, findings that are comparable to that seen in wild-type mice (but not Cfh<sup>−/−</sup> mice) with serum sickness (18). Similar findings were seen in control studies in which serum sickness was induced in wild-type mice that had received wild-type bone marrow (Figure 4, C and G), thereby excluding an effect of the bone marrow transfer procedure itself on immune complex processing and complement activation in glomeruli of C57BL/6 mice. In contrast, wild-type mice with Cfh<sup>−/−</sup> bone marrow had significantly greater deposition of IgG (Figure 4, A and D) and C3 (Figure 4, E and H) in glomeruli, with these being distributed throughout the glomerular capillary wall, a pattern that otherwise was observed only in Cfh<sup>−/−</sup> mice with serum sickness and also in the case of IgG and C3 in unmanipulated Cfh<sup>−/−</sup> mice as a spontaneous manifestation (14,18). These data indicate that the platelet Cfh limits glomerular accumulation of immune complexes in situations in which they are formed in excess, such as in serum sickness. Furthermore, it seems that the presence of immune complexes in glomeruli and not the deficiency of fluid-phase Cfh is responsible for the activation of complement in glomeruli in these settings; presumably, this is initiated through the classical pathway that is not appreciably affected by Cfh.

Glomerular Inflammation Occurs When C3 Is Not Inactivated by Cfh

Despite the presence of abundant IgG-containing immune complexes and complement activation products, glomeruli of wild-type mice with Cfh<sup>−/−</sup> bone marrow were completely

Figure 3. Platelet-associated IgG in mice with serum sickness. Platelets from wild-type and Cfh<sup>−/−</sup> mice and bone marrow chimeric mice that had been immunized with apoferritin to induce chronic serum sickness were studied, as were control mice that were immunized with saline. Platelet-associated IgG was increased significantly in wild-type mice and Cfh<sup>−/−</sup> mice with wild-type bone marrow in which serum sickness was induced (*P < 0.01 versus all other groups). The number of mice studied in each group is given in the bars.

Figure 4. Immunohistologic manifestations in glomeruli from Cfh<sup>−/−</sup> bone marrow chimeric mice with serum sickness. Shown are representative photomicrographs for glomerular IgG (A through C) and C3 (E through G) staining in wild-type recipients of Cfh<sup>−/−</sup> bone marrow (A and E), Cfh<sup>−/−</sup> recipients of wild-type bone marrow (B and F), and wild-type recipients of wild-type bone marrow (C and G). The staining intensity scores for IgG (D) and C3 (H) from individual mice are shown.
normal when examined histopathologically (Figure 5A). In contrast and despite having markedly less IgG and C3 evident in glomeruli, every Cfh−/− mouse with wild-type bone marrow developed significant GN, characterized by increased cellularity as well as the accumulation of matrix material (Figure 5B and quantified in all animals in Figure 5, D and E). This is a pattern with similarities to the membranoproliferative GN (MPGN) that can occur spontaneously in Cfh−/− mice on 129/Sv × C57BL/6 mixed backgrounds as well as that actively produced by serum sickness in C57BL/6 Cfh−/− mice (14,18).

As in native wild-type mice, serum sickness in wild-type mice that had received wild-type bone marrow failed to induce histologic glomerular disease (Figure 5C), thereby excluding an effect of the bone marrow transfer procedure itself on this disease in C57BL/6 mice. Therefore, deficiency of fluid-phase Cfh seems to be the key aspect accounting for the susceptibility of Cfh−/− mice to develop glomerular histopathology. In contrast, deficiency of platelet Cfh, with the attendant marked deposition of IgG and complement activation products in glomeruli, is not sufficient for GN to develop.

As shown above, the presence of C3 tracked closely with the extent and distribution of IgG in glomeruli. Because it was surprising that the Cfh−/− mice with wild-type bone marrow had much less C3 deposition in glomeruli but developed GN, we examined the form of C3 in glomeruli by Western blotting.

To examine specifically glomerular C3 proteins (rather than renal cortex with its significant plasma contribution), we isolated glomeruli by laser capture microdissection and subjected them to immunoblotting with anti-C3 antibodies. Consistent with the immunohistological data, wild-type recipients of Cfh−/− bone marrow had significantly greater amounts of C3 activation products in the kidney compared with Cfh−/− recipients of wild-type bone marrow (Figure 6A). However, despite the marked increase in C3 quantity in the former group, there also was evidence of efficient cleavage of the C3b 110-kD α′-chain to iC3b (containing α′67, α′43, and α′40 chains) and C3dg (Figure 6, B and C), which presumably occurred because the Cfi co-factor function of plasma Cfh was intact in these animals. In contrast, Cfh−/− recipients of wild-type bone marrow had intact α′110- chain, as shown with antibodies to C3 (Figure 6A) and C3d (Figure 6B), and therefore seemed to have impaired inactivation of C3b to iC3b as a result of the deficiency of plasma Cfh.

**Discussion**

Besides its physiologic roles, activation of the complement system can contribute to pathology. In the case of a number of immunologic diseases that affect the renal glomerulus, immune complexes and complement activation products are found in affected glomeruli, supporting a pathogenic role for immune complex injury.

![Figure 5](image_url) **Figure 5.** Glomerular pathologic features in Cfh−/− bone marrow chimeric mice with serum sickness. Representative photomicrographs are shown from a wild-type recipient of Cfh−/− bone marrow (A), a Cfh−/− recipient of wild-type bone marrow (B), and a wild-type recipient of wild-type bone marrow (C). Scores for the extent of glomerulonephritis (D) and glomerular sclerosis/hyalnosis (E) from individual animals are shown graphically. The arrow in B points to an area of segmental hyalinosis in a glomerulus from a Cfh−/− mouse with wild-type bone marrow.
complex-directed complement activation (25). Further evidence for the role of complement activation in glomerular diseases also has been provided by years of study in rodent models of disease (26,27). For example, in glomeruli of Cfh-/- mice on a mixed 129/Sv/H11002 background, there is unrestricted alternative complement activation and accumulation of immune complexes (14). At a relatively advanced age, these animals can spontaneously develop glomerular disease that has pathologic similarities to human MPGN.

Serum sickness and the resultant GN have been studied in various animal species, using a variety of immunization protocols. Rats of several strains have been used to generate serum sickness nephropathy (28–34). The resultant glomerular lesions seem to depend in large part on the amount of free antigen administered and range from deposition of immune deposits in the mesangium without cellular proliferation to a severe exudative GN (29,30). GN is mediated in part by C5a recruitment and activation of inflammatory cells and/or by C5b-9-mediated glomerular cell injury with resultant proliferative events (e.g., in the anti-thymocyte mesangial proliferative GN model in rats [39]). Because disease was not eliminated completely in C5-deficient mice, proximal complement components, such as the C3a and C3b cleavage products of C3, or noncomplement mediators of disease also must be playing a role in this model of GN (17). The finding of C5-independent yet C3-dependent GN has been observed in another murine model of immune complex GN, in which mice were immunized with cationized BSA (40).

It is important to note that active immunization of experimental animals with cationized antigens, as originally described by Border et al. (41) and also applied to the apoferritin model by Iskandar et al. (42), is considered to result in their being “planted” in the negatively charged glomerular capillary wall, leading to in situ formation of immune complexes (43–45). In contrast, chronic immunization with the highly anionic unmodified apoferritin (46) results in the formation of circulating immune complexes that subsequently deposit in glomeruli, as we have shown in this and previous studies using this model (18,47). In either circumstance, complement depletion or its absence leads to decreased glomerular clearance of immune complexes (34,44,47–49), highlighting the relevance and complexities of the complement system in immune complex processing in the glomerulus (3,45).

Unlike some strains, C57BL/6 mice are resistant to developing glomerular inflammation in apoferritin-induced chronic serum sickness (16–18). This is despite the development of a strong humoral immune response to the immunogen and the deposition of immune complexes and complement activation products in glomeruli. However, in our previous studies, all C57BL/6 Cfh-/- mice with serum sickness did develop GN (18). Thus, Cfh deficiency converts the C57BL/6 strain from one that is resistant to GN into one that is susceptible to GN. Arguably, the most important function for Cfh is its ability to limit complement activation in plasma and in sites that are not served by other complement regulators, such as the glomerular capillary wall and choroidal capillaries (10). However, it is a versatile protein with a number of other functions, such as serving as the platelet protein that is responsible for immune complex processing in rodents (5). As such, it could not be stated for certainty which of these predominated in the spontaneous glomerular disease that occurred in mixed background Cfh-/- mice or in the disease that was induced by serum sickness in C57BL/6 Cfh-/- mice.

The data presented in this study suggest that in a disease such as serum sickness, immune complex deposition and complement activation occur in glomeruli but the C3b (generated through the classical pathway but with the likely contribution by Falk and Jennette in their studies that compared the C5-deficient B.10.D2.OSN strain with congenic C5-sufficient B.10.D2.NSN mice (17). GN was reduced significantly in C5-deficient mice; however, there still was detectable disease in some of the C5-deficient B.10.D2.OSN mice in association with glomerular deposition of immune complexes and C3. These results indicated that GN is mediated in part by C5a recruitment and activation of inflammatory cells and/or by C5b-9-mediated glomerular cell injury with resultant proliferative events (e.g., in the anti-thymocyte mesangial proliferative GN model in rats [39]). Because disease was not eliminated completely in C5-deficient mice, proximal complement components, such as the C3a and C3b cleavage products of C3, or noncomplement mediators of disease also must be playing a role in this model of GN (17). The finding of C5-independent yet C3-dependent GN has been observed in another murine model of immune complex GN, in which mice were immunized with cationized BSA (40).

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The data presented in this study suggest that in a disease such as serum sickness, immune complex deposition and complement activation occur in glomeruli but the C3b (generated through the classical pathway but with the likely contribution
of alternative pathway amplification) is efficiently inactivated to iC3b when Cfh is present in plasma to serve as C6 co-factor (50). Cfh is an intrinsically derived platelet protein in mice that is responsible for the processing of immune complexes in a manner that is analogous to erythrocyte CR1 in primates. Therefore, when absent, excessive immune complex accumulation occurs over time in glomeruli, which is accelerated in a condition such as serum sickness. However, in these circumstances, the presence of plasma Cfh remains sufficient to limit the proinflammatory effects of complement activation. In the absence of Cfh in the plasma and glomerular capillaries, alternative pathway complement activation in glomeruli occurs spontaneously over time in Cfh−/− mice (14) and is accelerated through the classical pathway in serum sickness (18), such as also occurs in the prototypical immune complex disease systemic lupus erythematosus (51,52). This unrestricted alternative complement pathway activation converts the normally resistant C57BL/6 strain into one that is susceptible to glomerular complement pathway activation converts the normally resistant C57BL/6 strain into one that is susceptible to glomerular disease, through the proinflammatory actions of C3b and other downstream products of complement activation, such as C3a, C5a, and C5b-9 (25). These data contribute to our growing appreciation for the potency of alternative complement pathway activation (53) and the importance of Cfh as its physiologic regulator.

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