A conformational change of C-reactive protein in burn wounds unmasks its proinflammatory properties

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Abstract

Tissue damage in burn injury leads to a rapid increase of leukocytes and acute phase reactants. Plasma levels of C-reactive protein (CRP) rise within hours after the insult. No deficiency of this protein has been reported in humans, suggesting it plays a pivotal role in innate immunity. CRP in circulation is composed of five identical subunits [pentameric CRP (pCRP)]. Recently, deposits of structurally modified CRP (mCRP) have been found in inflammatory diseases. Little is known about this structural change and how it affects CRP functions. We analyzed CRP deposits in burn wounds and serum by immunohistochemistry, western blot and dot blot analysis. CRP was deposited in necrotic and inflamed tissue, but not in adjacent healthy tissue. Tissue deposited CRP was detected by mCRP-specific antibodies and structurally different from serum pCRP. mCRP but not pCRP induced reactive oxygen species production by monocytes and facilitated uptake of necrotic Jurkat cells by macrophages. In addition, it accelerated migration of keratinocytes in a scratch wound assay. The structural changes that occur in pCRP upon localization to damaged and inflamed tissue in burn wounds result in a functionally altered protein with distinct functions. mCRP exhibits opsonic, proinflammatory and promigratory properties which modulate wound healing.

Keywords: acute phase reactants, burn injury, inflammation, innate immunity, wound healing

Introduction

Thermal injuries can cause a major threat to the body, as large amounts of cells simultaneously undergo apoptosis and necrosis. Cytosolic and nuclear components are released and extracellular proteins are denatured. These danger-associated molecular pattern molecules (DAMPs) initiate a rapid immune response (1). White blood cells and acute phase reactants rise within hours after the injury and closely interact during the inflammatory response (2).

Inflammatory control in burn wounds is critical for wound healing and infection control. Infiltration and activation of leukocytes is necessary to remove cell debris, necrotic and apoptotic cells. In addition, invading bacteria must be cleared, as infection is associated with delayed wound healing, increased scarring and sepsis (3, 4). Still, excessive inflammation in thermal injuries has been shown to also increase burn size and depth (5).

The highly conserved C-reactive protein (CRP) is a pattern recognition molecule that is part of the acute phase response. Plasma levels can increase from undetectable levels in healthy individuals to >500 mg/l after burn trauma (6). Its levels are proportional to the extent of the burn wound and they may further increase if infection is present (7).

Circulating CRP consists of five identical, noncovalently linked subunits, which form a ring-like structure [pentameric CRP (pCRP)]. No deficiency or mutation of this highly conserved protein has been described in humans, suggesting that it plays a pivotal role in innate immunity (8). Deposition of CRP has been reported in acute and chronic inflammatory diseases including myocardial infarction, atherosclerosis and Alzheimer's disease (9–11). Binding of pCRP occurs to cell membranes of necrotic and apoptotic cells, activated...
platelets and β-amyloid plaques, which induce structural modifications in CRP (mCRP). Initially, a partial structural change occurs, producing molecules that express CRP subunit antigenicity, but maintain pentameric symmetry. One of the key neoepitopes that becomes accessible is composed of amino acids 199–206. These amino acids are buried in the intersubunit regions in pCRP, but flip outwards upon membrane binding. The modified variant of CRP has been termed mCRPm and shows altered protein functions, e.g. enhanced complement fixation. Subsequently mCRPm can dissociate into individual CRP subunits. This soluble, monomeric CRP (mCRPm) may detach from the membrane to exert its biological functions (12, 13). As both mCRP variants express the same key epitopes, share similar functions and cannot be distinguished by conformation-specific antibodies, the term mCRP is often used in the literature to describe both isomers.

Whereas circulating serum pCRP is a rather inert molecule (14, 15), mCRP modulates complement activity and exhibits various proinflammatory properties including prolonged survival of neutrophils and enhanced monocyte activation (9, 16). It has therefore been implicated in disease initiation and progression of various inflammatory diseases (17–19).

Despite these observations, the formation and persistence of both mCRP isomers in vivo has been questioned, as pCRP is very stable and in vitro generation of mCRPm is often performed in the presence of harsh denaturing conditions (20). In addition, it is currently not possible to isolate mCRPm, as this variant is only a transient intermediate that occurs during the transition of pCRP to mCRPm. Recent studies, however, showed dissociation of pCRP into its subunits in vitro on cell membranes and liposomes under physiological conditions and in vivo in a rat model with defined tissue inflammation (21). Immunohistochemistry of human tissue confirmed the presence of neoepitope expressing CRP in atherosclerotic plaques, ischemic myocardial tissue and lesions of Alzheimer’s disease (9–11).

Although inflammation has been shown to contribute to various stages of coronary artery disease (CAD), there is an ongoing debate on whether CRP is causally involved or merely a marker of increased systemic inflammation (22–24). Despite these controversies regarding its proinflammatory potential in CAD, there is compelling evidence that high CRP serum concentrations contribute to the severity and outcome of ischemic tissue injury (18, 25).

Still, little is known about the physiological function of CRP in regulating acute inflammatory responses during the acute phase response. We therefore investigated the physiological functions of CRP in burns as a disease model of defined tissue damage in respect to its immune modulatory and wound healing properties.

Methods

Antibodies and CRP preparations

pCRP was purchased from Calbiochem (Nottingham, UK) and thoroughly dialyzed against Dulbecco’s phosphate buffered saline (D-PBS) supplemented with 2 mM CaCl2. mCRPm was recombinantly expressed in Escherichia coli, purified by affinity purification and subsequently tested for LPS contamination with a Limulus assay (Sigma-Aldrich, Taukirchen, Germany). Recombinant mCRPm preparations and conformation-specific anti-mCRP antibodies 9C9 and anti-pCRP antibodies 8D8 were a gift from Dr Potempa (College of Pharmacy, Roosevelt University, Chicago, IL, USA). Anti-CRP polyclonal serum, anti-CRP antibody clone-8 and anti-rabbit CF™ 633 antibodies were purchased from Sigma-Aldrich. Anti-CD 68 clone KP1 and HRP-labeled anti-mouse antibodies were purchased from Dako (Glostrup, Denmark) and anti CD14-PE from Miltenyi Biotec (Bergisch Gladbach, Germany).

Sample collection

Collection of blood and burned skin occurred from patients (n = 3, Supplementary Table S1, available at International Immunology Online) with deep second degree to full thickness burn wounds who needed surgical excision on day 4–8 following injury. Skin samples were taken from the center and margins of burn lesions that were completely excised and primarily closed. Burn depth was judged by at least two experienced plastic surgeons. Serum was collected on the day of surgery. Healthy control skin was taken from excess material of abdominoplasty procedures (n = 2) and blood from healthy volunteers (n = 3) who had not taken any medication within the past 2 weeks. Written informed consent was obtained from all patients. Sample collection was in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Freiburg, Germany. Tissue specimens were directly frozen in liquid nitrogen and stored at −80°C. Blood samples were centrifuged for 15 min at 1500 × g after clotting. The serum fraction was collected, frozen in liquid nitrogen and stored at −80°C.

Histological staining of human skin

Histological staining of human skin was performed as described previously with the following modifications (9). After incubation with the primary antibody for 1 h at room temperature, slides were incubated with an HRP-labeled anti-mouse antibody for 30 min. Reaction products were stained with HistoGreen substrate kit for peroxidase from Linaris (Dossenheim, Germany) resulting in a green reaction product.

Skin tissue lysates

Frozen skin was cut into small cubes and incubated in lysis buffer (D-PBS supplemented with 2 mM CaCl2, 0.5% Triton X-100, 1 mM orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Tissue was subsequently homogenized with an UltraTurrax disperser from IKA (Staufen, Germany) and centrifuged at 17,000 × g for 10 min at 4°C. Protein concentrations were determined with a BCA protein assay kit from Pierce (Rockford, IL, USA) and adjusted with lysis buffer if necessary.

SDS–PAGE, western blot and dot blot analysis

Tissue lysates were precipitated with an equal volume 10% trichloroacetic acid on ice. Protein pellets were denatured at 95°C for 5 min in SDS loading dye and separated on 15% SDS–polyacrylamide gels followed by western blotting on polyvinylidene fluoride membranes. After blocking in 5% milk
powder in Tris-buffered saline–Tween 20 (TBS-T), membranes were incubated with anti-CRP clone-8 antibody (1:500) followed by an HRP-conjugated anti-mouse antibody (1:5000) in 1% BSA TBS-T. ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) were used to visualize protein bands in a Fusion Fx7 chamber (Peqlab, Erlangen, Germany). Band intensity was determined with ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA) and CRP concentrations were calculated with the aid of a standard curve obtained from purified CRP. Serum CRP levels were determined by an immunoturbidimetric assay (C-Reactive Protein Gen.3, Roche Diagnostics, Mannheim, Germany) according to the manufacturers protocol. For dot blot analysis, native samples containing 0.2 µg CRP were directly spotted on nitrocellulose membranes and dried for 20 min at room temperature. Proteins were visualized as described for western blots.

Binding of CRP to apoptotic cells
Whole blood from healthy volunteers was incubated with 5 µg/ml staurosporine (Sigma-Aldrich, Taukirchen, Germany) in the presence or absence of 100 µg/ml pCRP for 5 h. Red blood cells were lysed with BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) and the remaining white blood cells were stained with polyclonal anti-CRP serum and anti-rabbit CF 633 antibodies. CRP binding was analyzed on a LSR Fortessa FACS analyzer from Becton Dickinson (Franklin Lakes, NJ, USA) and further processed by Flowjo (TriStar Inc., Ashland, OR, USA). Displayed values show the difference in fluorescence in the presence and absence of CRP.

Binding of CRP to liposomes
Large multilamellar liposomes were prepared as previously described (26). Indicated concentrations of pCRP were incubated with 0.2 mg/ml liposomes at 37°C for 30 min in D-PBS supplemented with 2 mM CaCl2 and 1 mg/ml albumin. EDTA (10 mM) was added to inhibit Ca2+-dependent CRP binding. Liposomes with bound CRP were subsequently pelleted for 60 min at 20000 × g. The input control and the resuspended pellets were precipitated with an equal volume of 10% trichloroacetic acid and subsequently separated on a 15% SDS–PAGE followed by western blot and detection with anti-CRP clone-8 antibodies. Quantification was performed as described above.

Macrophage phagocytosis
Engulfment of necrotic Jurkat cells by human macrophages was analyzed by a modified flow cytometry assay that has been described previously (27). Human macrocytes were isolated from peripheral blood of healthy human volunteers by Ficoll density gradient centrifugation and cultured on six-well plates for 4 days. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FCS and labeled with CMFDA (5-Chloromethylfluorescein Diacetate, Life Technologies, Darmstadt, Germany) according to the manufacturer’s instructions. Necrosis of labeled cells was induced in serum-free medium by heating for 30 min at 60°C. Cell death was verified by trypan blue staining with generally more than 90% trypan blue positive cells. Labeled cells (1 × 106) were then incubated with 0.3 × 106 adherent macrophages for 2 h at 37°C in the presence of 10% autologous human serum (AHS), 25 µg/ml pCRP or 25 µg/ml mCRP. After washing, cells were released, stained with anti-CD14-PE and analyzed by two-color flow cytometry. Macrophages were gated by forward scatter, side scatter and CD14 positivity to exclude gating of unbound target cells. Unlabeled necrotic Jurkat cells served as negative controls. The percentage of macrophages that bound or ingested labeled Jurkat cells was calculated. In addition, engulfment of target cells was verified by confocal fluorescence microscopy (Leica TCS SP2 AOBS, Leica Microsystems, Wetzlar, Germany) after further fixation with 2% formaldehyde and staining with 1 µg/ml 4′,6-diamidino-2-phenylindole.

Reactive oxygen species production by monocytes
Rat mononuclear cells were isolated from whole blood by Ficoll density gradient centrifugation and incubated with 50 µg/ml LPS (E. coli; Sigma-Aldrich), pCRP or mCRP. Reactive oxygen species (ROS) formation was determined by electron spin resonance (ESR) spectroscopy using CMH (1 mM, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; Noxygen, Elzach, Germany) as the spin label and was prepared as previously described (28). The cell concentration was determined prior to the experiments by flow cytometry. Subsequently, 500 µl of cell suspension was incubated at 37°C for 30 min with 1 mM CMH. The oxidation of CMH by ROS generated stable 3-methoxy carbonyl-aryl radicals (CM*) that were quantified by the ESR spectroscopy referring to standard CM* solutions. Measurements were performed using a MiniScope MS 200 ESR Spectrometer (Magnettech, Berlin, Germany) with the following instrument settings: center field, 3340 G; sweep wide, 60 G; sweep time, 5 ms over 10 scans; modulation amplitude, 2.4 G; microwave power, 10 mW. Results were normalized against the cell concentration and expressed as a percentage of values of the control group.

Migration of human keratinocytes
Migration of human keratinocytes was observed in a scratch wound assay according to Liang et al. (29). Briefly, HaCaT cells were cultured in DMEM medium supplemented with 2 mM l-glutamine and 10% fetal calf serum. Confluent monolayers were scraped in a straight line with a p200 tip and washed to remove debris. Subsequently, 25 µg/ml mCRP and pCRP were added in cell culture media and closure of the gap monitored over 24 h. Images were captured at indicated time points and the area of the remaining defect determined [ImageJ software (NIH)]. The relative defect size was calculated by comparing the defect area at indicated time points to the defect size at t = 0.

Statistical analysis
Statistical analysis was performed with GraphPad prism (GraphPad Software, La Jolla, CA, USA). A paired t-test was used to compare two groups, and one-way ANOVA was used to compare multiple groups.
CRP aggravates inflammation in burn injury

Results

CRP is deposited in burned but not in healthy skin

We analyzed deposition and distribution of CRP in mid to deep dermal skin burns of serial tissue sections taken from the center of burn wounds 4–8 days post injury. We observed staining by the monoclonal anti-CRP antibody clone-8 in burned skin, but not in skin from healthy controls (Fig. 1A). Staining was restricted to the superficial to mid-dermal layer and thus coincided with the predicted burn depth. CRP appeared to be diffusely distributed in the interstitial space but also in part bound to cell membranes. We also observed excessive infiltration of burn wounds by CD68-positive macrophages in areas of CRP deposits (Fig. 1B). We did not observe any background staining in control sections, where the primary anti-CRP antibody was omitted (Fig. 1C).

Deposition of CRP at the site of tissue damage was further verified by immunohistochemical staining of the transition zone between the burn wound and adjacent healthy skin. We observed massive CRP deposits in damaged tissue with a gradual decrease towards uninjured skin (Fig. 1D). To exclude cross-reactivity of anti-CRP antibodies with unknown epitopes that are expressed in burned but not in healthy skin, CRP deposition was further verified by western blotting of tissue lysates. CRP antibodies specifically recognized a band of ~23 kDa in lysates from burned skin, which could not be observed in healthy skin lysates. This size is in line with the predicted molecular mass of a CRP monomer (23 kDa) and was also observed with control CRP. A weak band migrating on top of the CRP band was also observed in control skin lysates and is probably due to unspecific antibody binding to common skin epitopes (Fig. 1E).

CRP is deposited at sites of tissue injury

Previous reports noticed binding of CRP to apoptotic but not to non-injured Jurkat cells (12). As we observed CRP deposition in areas of tissue damage and inflammation but not in healthy skin, we further analyzed binding of CRP to apoptotic cell membranes. Apoptosis of leukocytes was induced in whole peripheral blood with staurosporine in the presence and absence of CRP. CRP binding was subsequently quantified by FACS analysis. CRP was found to bind only to staurosporine-treated cells but not to untreated cells, which could explain deposition of CRP in the burn wounds (Fig. 2A and B).

To further explore the possibility that CRP is selectively enriched on damaged cell membranes in burn wounds, we determined CRP binding to lysophosphatidylcholine (LPC)-containing liposomes. These liposomes mimic damaged cells, because in both damaged cells and liposomes, the phospholipid headgroups are accessible and allow Ca^{2+}-dependent CRP attachment (12, 30).

We observed binding of CRP to large multilamellar liposomes (Fig. 2C) that could be inhibited by EDTA, which suggests a specific, Ca^{2+}-dependent interaction. Strikingly, ~80% of the input material could be recovered from the liposomal pellet at CRP concentrations of 10 µg/ml and ~40% at 1 µg/ml (Fig. 2D). We additionally tested whether changes in the LPC or cholesterol content of our liposomes affected CRP binding. Similar to previous reports, the addition of LPC to liposomes was necessary to increase CRP binding. However, increasing the LPC content from 10 to 20% did not further enhance CRP binding (Fig. 2E). These in vitro findings suggest that large amounts of CRP might be deposited in damaged tissue. This hypothesis was verified in vivo by determining CRP concentrations in burn wounds by quantitative western blots. Considerably, higher CRP concentrations were present in burn wounds when compared to CRP serum levels at the day of surgery (Fig. 2F).

The accuracy of quantitative western blots for determining CRP tissue concentrations was verified by comparing it to a commercial immunoturbidimetric assay, which was used to measure CRP serum levels. CRP levels in two patient sera with similar CRP concentrations to burn patient 3 were analyzed. Both assays measured similar CRP concentrations (Supplementary Figure S1, available at International Immunology Online).

Tissue-deposited CRP expresses subunit antigenicity

Recent results suggest that at least two different CRP isoforms exist in vivo. Whereas pCRP is predominant in human serum, deposits of neoepti-le-expressing CRP were found in atherosclerotic plaques, necrotic myocardium and on circulating microparticles. Conformation-specific antibodies that recognize epitopes that are not accessible in pCRP are commonly used to distinguish mCRP from pCRP. These antibodies detect both mCRP isoforms, as they express the same key antigens (9, 10, 31).

Immunohistochemistry of burned skin with the mCRP-specific antibody 9C9 again revealed diffuse as well as cell associated staining in the superficial dermis. In contrast, we detected only minimal staining with the pCRP specific antibody 8D8 (Fig. 3A).

In addition, nearly exclusive cellular staining of mCRP was found in areas where new granulation tissue was forming. These areas were also characterized by accumulation of CD68 macrophages adjacent to newly forming vessels (Fig. 3B). As fixation of tissue sections by acetone might lead to neoepitope expression of CRP, we also omitted this step during the sample preparation. We did not observe differences in the staining patterns by 9C9 and 8D8 antibodies in unfixed sections, compared to acetone-treated tissue (Fig. 3C).

Dot blot analysis confirmed that serum CRP in burn victims is structurally different from tissue-deposited CRP. The anti-pCRP antibody 8D8 strongly recognized control pCRP and serum CRP from burn victims, whereas burn tissue lysates, recombinant control mCRP, and control serum showed only minimal reactivity (Fig. 3D).

The presence of CRP in burn patient serum and burn tissue lysate was confirmed by SDS–PAGE and western blot of denatured samples (Fig. 3E), which induces pCRP dissociation. This demonstrates that tissues and serum of burn victims contain distinguishable CRP variants.

Engulfment of necrotic cells by human macrophages is accelerated in the presence of mCRP

We assessed the influence of pCRP and mCRP, on removal of necrotic cells in a phagocytosis assay. Labeled Jurkat cells were heated to mimic burn-induced cell necrosis and their engulfment by human macrophages was then determined.
CRP is deposited in burn wounds. Serial tissue sections of a mid to deep dermal burn wound (patient 1, 6 days post injury) and healthy control skin were stained with α-CRP clone-8 (recognizes pCRP and mCRP) and α-CD68 antibodies. Subsequent detection was conducted with HistoGreen, yielding a green reaction product. (A) CRP (green) can be detected in the superficial to mid-dermal layer in burned skin. It is in part diffusely distributed within the interstitial space and in part bound to cells. (B) Burned skin additionally shows excessive infiltration of CD68-positive macrophages, and no unspecific staining was observed, if the primary antibody was omitted (C). (D) Immunohistochemical staining of the transition zone between the burn wound and adjacent healthy skin (patient 2, 4 days post injury). CRP gradually decreases towards healthy tissue. Note the changes in the epithelium between damaged and healthy skin. (E) Western blot of tissue lysates from a burn wound and healthy skin. CRP was detected with α-CRP clone-8 antibodies in burn wounds but not in healthy skin and migrated at the same size as control CRP.
in the presence of AHS, pCRP or mCRP, and analyzed by flow cytometry and fluorescence microscopy. AHS increased uptake of labeled bait cells, which is most likely due to opsonizing complement and natural IgM antibodies (32). mCRP showed a more pronounced effect that could not be enhanced by the addition of AHS. pCRP had no significant effect on the uptake of necrotic cells, neither alone nor in the presence of AHS (Fig. 4A–C). Although recombinant mCRP preparations were free of endotoxin contamination as indicated by the Limulus assay, LPS amounts below the detection limit of the assay might still be present. We therefore determined phagocytosis of necrotic cells in the presence of 20ng/ml and 1 µg/ml LPS (Supplementary Figure S2, available at International Immunology Online). Both concentrations showed only minimal effects on phagocytosis and thus confirm that mCRP specifically enhances phagocytosis of necrotic cells and facilitates their removal.

mCRP locally aggravates inflammation by activating monocytes

Monocytes were isolated from peripheral blood of healthy Wistar rats and incubated with LPS, pCRP and mCRP. ROS production was used as a surrogate marker for monocyte

Fig. 2. CRP binds to altered cell membranes. CRP binds to apoptotic cells. (A) Whole blood was treated with staurosporine (stauro) to induce apoptosis. Binding of CRP to live and apoptotic human leukocytes was subsequently determined by flow cytometry with polyclonal α-CRP antibodies. * P < 0.05 compared to live cells, ** P < 0.01 compared to live cells. Shown are the mean values and error bars indicate SEM of at least three independent experiments. (B) Representative fluorogram of live (control, left panel) and apoptotic (stauro, right panel) granulocytes in the presence (tiled curve) and absence (blank curve) of CRP. CRP attaches to liposomes. (C) Indicated concentrations of CRP were incubated with liposomes [40% phosphatidylcholine (PC), 10% LPC, 50% cholesterol (CHO)] in buffer containing 2mM Ca++. Bound CRP was isolated by centrifugation, separated on SDS–PAGE and detected with α-CRP clone-8 antibodies after western transfer. (D) Relative amounts of CRP that were recovered from the liposomal pellet fraction. Displayed are the mean values of four independent experiments. ** P < 0.01 and *** P < 0.001 compared to EDTA. (E) Binding of CRP (10 µg/ml) to liposomes with different lipid compositions. Bars indicate the mean values of CRP that were recovered from the pellet fraction. *** P < 0.001 compared to PC or PC/CHO. Error bars indicate SEM. (F) Quantification of CRP in serum and burned tissue from a representative patient (patient 3, 8 days post injury). Shown are mean CRP values and error bars indicate SEM.
Tissue-deposited CRP expresses subunit antigenicity. **(A)** Detection of CRP isoforms in burned skin (patient 2) by α-mCRP 9C9 antibody and α-pCRP 8D8 antibody. mCRP antibodies show bright staining, whereas pCRP antibodies show only minimal reactivity. Close-up sections reveal diffuse interstitial as well as cell-bound CRP deposits. **(B)** Some areas, which are rich in CD68-positive macrophages, exhibit exclusive cellular CRP staining. **(C)** Burn wound tissue sections were stained as in (A), however, acetone fixation was omitted. Again, α-mCRP 9C9 antibodies recognized CRP, whereas α-pCRP 8D8 antibodies showed only minimal reactivity. **(D)** Control pCRP and serum from a burn patient react with α-pCRP 8D8 antibodies in a dot blot experiment, whereas CRP in the burn wound lysate and control mCRP are not recognized. **(E)** Detection of CRP in serum and burn wound lysate by western blot. To obtain uniform detection by α-CRP clone-8 antibodies in all samples, samples were boiled in SDS loading dye prior to SDS-PAGE. Samples were from the same patient as in (D).
Fig. 4. Effects of mCRP on burn wound healing. mCRP accelerates engulfment of necrotic cells by human macrophages. CMFDA-labeled necrotic Jurkat cells were incubated with human macrophages in the presence of autologous human sera (AHS), pCRP or mCRP, when indicated. Macrophages were released, stained with CD14-PE and analyzed by flow cytometry. (A) Percentage of macrophages that ingested or bound target cells. Displayed are mean values of three independent experiments. Bars indicate SEM. *P < 0.05 compared to untreated control, **P < 0.01 compared to pCRP + AHS, ***P < 0.001 compared to pCRP. (B) Representative fluorograms of CD14-PE-positive macrophages after engulfment of necrotic CMFDA-labeled Jurkat cells. Unlabeled cells: blank curves, CMFDA-labeled cells: tiled curves. (C) Engulfment of necrotic cells was verified by fluorescence microscopy. Representative image of CMFDA-labeled necrotic cell (green) that is ingested by a macrophage. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Brightfield image displays the dimensions of the macrophage. (D) mCRP activates monocytes. Rat peripheral blood monocytes were incubated with LPS, pCRP and mCRP (50 µg/ml) for 30 min. ESR spectrosopy was performed to determine ROS production. Values are mean of 7–10 independent experiments and bars indicate SEM. *P < 0.05 compared to control group, *P < 0.05 compared to pCRP group. (E) mCRP enhances keratinocyte migration. Keratinocyte migration was observed in a scratch wound assay over 24 h in the presence of mCRP, and pCRP. After scratching the surface, closure of the gap was monitored over 24 h. Displayed are the mean values of at least three independent experiments and error bars indicate the SEM. **P < 0.01 and ***P < 0.001 compared to pCRP and control group.
activation. LPS- and mCRP-treated monocytes showed increased production of ROS, whereas pCRP evoked no significant changes when compared to untreated controls (Fig. 4D).

**mCRP enhances keratinocyte migration**

We assayed keratinocyte migration in a scratch wound assay in the presence of pCRP and mCRP. After scratching the surface, closure of the gap was monitored over 24 h. mCRP, at a concentration of 25 µg/ml significantly increased migration of cultured keratinocytes (P < 0.01 at 8 h and P < 0.001 at 24 h) when compared to control and pCRP. pCRP showed only a small increase in keratinocyte migration when compared to control cells, which was not statistically significant (Fig. 4E).

**Discussion**

Local inflammation in burn wounds is fundamental for wound healing, as cell debris has to be removed and the wound needs to be protected from invading bacteria. Subsequently, fibroplasia and granulation tissue formation in conjunction with epithelialization restore the protective layer of the skin (33). Still, excessive inflammation has also been implicated in promoting further tissue destruction and excessive scarring. Therefore, inflammation needs to be tightly regulated and restricted to the site of tissue injury. Little is known about how cellular and soluble factors of the innate immune system balance these pro- and anti-inflammatoryary processes.

**Deposition of CRP in damaged tissue**

Elevated CRP serum levels have been studied extensively in burn patients and have been linked to the extent and depth of burn wounds (7). Recently, deposition of CRP in conjunction with C3d and leukocytes was shown in burn tissue by immunohistochemistry (34). This was confirmed in the current study, which demonstrates that CRP is deposited in burn wounds but not in healthy skin. Remarkably, deposition occurs exclusively in areas of damaged tissue and acute inflammation, as the CRP deposits correlate with the predicted burn depth and colocalize with CD68-positive cells. These findings are in line with previous reports where CRP deposits were found in infarcted myocardium but not in adjacent healthy muscle tissue (10). This suggests that CRP plays a fundamental role in innate inflammation in general and that its mechanism of action is universally conserved in acute inflammatory processes.

The phosphorylcholine head groups of membrane phospholipids are the major cell surface ligands for CRP (12, 30, 35). In healthy cells, this ligand is camouflaged but becomes accessible if cells undergo apoptosis and necrosis (12). As heat-damaged necrotic and apoptotic cells as well as apoptotic leukocytes are abundantly found in burn wounds, they provide the basis of CRP recruitment. We could show binding of CRP to apoptotic leukocytes and our binding assay with liposomes confirms that structurally altered cell membranes provide the basis for CRP deposition in damaged tissue. As efficient binding could already be observed at low CRP concentrations of 1 µg/ml and 10 µg/ml, CRP is able to localize to damaged tissue already during the early acute phase response. These findings are supported by in vivo data, which show that CRP concentrations in burned skin are higher than in serum. As we did only record CRP serum concentrations on the day of surgery, high CRP levels in the burn wound might also result from a preoperative CRP serum spike that was deposited. Thus, although the in vivo data confirm deposition of large CRP amounts in damaged tissue, it remains unclear, whether this is a result of CRP accumulation at low serum levels or of deposition during CRP serum spikes.

Although these data might suggest that CRP is exclusively cell bound, we still observed diffuse interstitial CRP deposits in addition to cell bound CRP. Diffuse staining is probably due to shedding of small apoptotic bodies and microparticles by dying and stimulated cells, which bind CRP (31, 36). These particles rapidly diffuse within the extracellular matrix because of their small size and are able to subsequently transfer CRP to other cells (31).

**Structural changes in CRP upon localization to burn wounds**

CRP can occur in different isoforms: pCRP, which is the major species in circulation, and mCRP, which is predominantly found in inflamed tissue (37). Cell plasma membranes of necrotic and apoptotic cells can generate mCRP from pCRP. This mechanism seems to be universally conserved as it can be observed on necrotic cardiomyocytes, necrotic retinal pigment epithelial cells, apoptotic Jurkat cells, human platelets and microparticles (10, 13, 17, 31, 38). We showed by immunohistochemistry and dot blot analysis that tissue deposits of CRP in burn wounds are nearly exclusively composed of mCRP whereas corresponding serum CRP exhibits pCRP reactivity. mCRP detection was not an artifact due to acetone fixation, as mCRP reactivity was also observed in the absence of acetone. Although conformation-specific antibodies reliably distinguish pCRP from mCRP, they are not able to differentiate between mCRP and mCRP, as both isoforms express the same key epitopes (13). Due to these restrictions, most published studies, which showed mCRP deposits in human tissue, could not distinguish between the two mCRP isoforms. The exact composition of mCRP and mCRP in inflamed tissues thus remains to be determined.

**Physiological effects of modified CRP and complement**

The two modified CRP isoforms appear to be the active conformations of CRP, as they possess several proinflammatory properties that are not observed for pCRP (14, 15). As mCRP is only as a transient intermediate that occurs during the transition of pCRP to mCRP, it is currently not possible to isolate stable mCRP for in vitro use. In contrast, mCRP can either be recombinantly expressed in E. coli or generated from pCRP. As expression of neoepitopes is the key feature that distinguishes both mCRP variants from pCRP, the two isoforms are likely to have overlapping functions (10, 13, 39). mCRP is thus commonly used to study effects of modified CRP.

We consistently used recombinant mCRP, as it is composed of neoepitope-expressing, monomeric CRP only. Although mCRP generation from pCRP by chemical treatment...
is reported to be complete within minutes, we often observed incomplete dissociation (9, 40). In contrast to recombinant mCRP, these preparations might thus contain two different CRP species, what might confound results.

Binding of mCRP, but not pCRP to human monocytes causes cell activation as evidenced by increased ROS formation. As large amounts of mCRP are present in the burn wound but not in serum, activation of monocytes is restricted to the site of tissue injury.

In addition, mCRP, opsonizes necrotic cells and accelerates their uptake by macrophages. Interestingly, we observed this effect even in the absence of serum, which contains opsonizing complement and natural IgM antibodies. mCRP might additionally control complement-mediated phagocytosis on necrotic cells as it initiates the complement cascade by binding complement factor C1q, but also prevents cell lysis by recruiting complement inhibitors such as C4bp and factor H (10). Complement components have been implicated in promoting tissue destruction in burn patients, as complement C1-inhibitors as well as complement factor C4 knockout mice showed reduced scarring and contracture (41–43). It is thus likely that elevated CRP levels, beyond the inflammatory phase, might also exert adverse effects and promote scar formation and wound contraction (34, 44).

A similar CRP- and complement-dependent mechanism was proposed to aggravate tissue destruction in an animal model of myocardial infarction (18). Inhibitors that target CRP are currently under investigation (25) and it might thus be possible to reduce potential adverse effects of CRP in burn patients, which result from increased inflammation. Still, timing and concentration of this treatment is probably critical, as it may imply an increased susceptibility to bacterial infections and delay epithelialization of the burn wound.

Interestingly, mCRP, also promotes migration of keratinocytes and may thus accelerate wound epithelialization in burn patients. This effect was observable at physiological CRP concentrations of 25 µg/ml. Similar to monocyte activation, it also required neoepitope expression of the CRP molecule, as pCRP showed only minimal promigratory effects that were not statistically significant. The observed small increase in keratinocyte migration in the presence of pCRP might result from partial conversion of pCRP to mCRP on cell membranes and hydrophobic surfaces of the cell culture plates which has already been observed in previous in vitro experiments (45).

Monocyte chemotactic activity of mCRP has been reported previously and is most likely mediated by specific receptors, in particular Fc-γ receptors (9, 16). Enhanced keratinocyte migration in the presence of mCRP is likely to be mediated by a similar mechanism, as they also express Fc-γ receptors (46, 47).

Taken together, heat-damaged tissue catalyzes a molecular switch in CRP, which unmasks its proinflammatory properties. Our data suggest that structurally modified CRP facilitates efficient removal of cellular debris, activates monocytes and promotes the establishment of a protective skin layer by accelerating wound epithelialization (Fig. 5). As generation of mCRP is restricted to sites of tissue damage, uninjured adjacent tissue is not affected by its proinflammatory properties.

Supplementary data
Supplementary data are available at International Immunology Online.

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