

Extracellular ubiquitin: immune modulator and endogenous opponent of damage-associated molecular pattern molecules

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ABSTRACT

Ubiquitin is a post-translational protein modifier and plays essential roles in all aspects of biology. Although the discovery of ubiquitin introduced this highly conserved protein as a molecule with extracellular actions, the identification of ubiquitin as the ATP-dependent proteolysis factor 1 has focused subsequent research on its important intracellular functions. Little attention has since been paid to its role outside of the cell. During recent years, multiple observations suggest that extracellular ubiquitin can modulate immune responses and that exogenous ubiquitin has therapeutic potential to attenuate exuberant inflammation and organ injury. These observations have not been integrated into a comprehensive assessment of its possible role as an endogenous immune modulator. This review recapitulates the current knowledge about extracellular ubiquitin and discusses an emerging facet of its role in biology during infectious and noninfectious inflammation. The synopsis of these data along with the recent identification of ubiquitin as a CXCR4 agonist suggest that extracellular ubiquitin may have pleiotropic roles in the immune system and functions as an endogenous opponent of DAMPs. Functions of extracellular ubiquitin could constitute an evolutionary conserved control mechanism aimed to balance the immune response and prevent exuberant inflammation. Further characterization of its mechanism of action and cellular signaling pathways is expected to provide novel insights into the regulation of the innate immune response and opportunities for therapeutic interventions. *J. Leukoc. Biol.* 89: 205–219; 2011.

Abbreviations: BALF=bronchoalveolar lavage fluid, Ca²⁺=calcium ion, CK=creatine kinase, CSF=cerebrospinal fluid, DAMP=damage-associated molecular pattern molecule, E1=ubiquitin-activating enzyme, E2=ubiquitin carrier or conjugating enzyme, E3=ubiquitin protein ligase, HCL=hairy cell leukemia, MNSF=monoclonal-nonspecific suppressor factor, MOF=multiple organ failure, RGD=Arg-Gly-Asp, SAB=subarachnoidal hemorrhage, SDF=stromal cell-derived factor, TBI=traumatic brain injury, TL=thymus-leukemia

Introduction

Since antiquity, it has been known that inflammation presents with the unspecific cardinal symptoms of calor, rubor, tumor, and dolor (Aulus Cornelius Celsus, In *De re medicina*, approximately A.D. 30). Similarly, in modern intensive care medicine, the systemic inflammatory response syndrome represents unifying symptoms in critically ill patients [1, 2]. This suggests activation of common pathophysiological mechanisms independent of the nature of the inflammatory stimuli. Today, it is well established that local and systemic symptoms of inflammation are governed by the innate immune system. Various immune cells are activated, and their temporo-spatial production of a vast variety of mediators that induce and determine the magnitude of the local and systemic inflammatory responses is regarded as a key contributing factor for outcome in patients [3, 4].

A significant amount of research has focused on the initial signaling events that trigger immune system activation in infectious and noninfectious inflammation, induce a response that is generally regarded as being proinflammatory, and ultimately, lead to organ damage and failure in critical illnesses. From this research, the current concept of “danger signals” evolved. The importance of microbial PAMPs and their PRRs on immune cells have long been recognized. The significance of potent endogenous immunostimulants (termed “alarmins”), which are released from destroyed tissues and serve as early danger signals to activate innate and adaptive immune systems, emerged more recently [5–10]. This led to the current classification that PAMPs and alarmins form the larger group of DAMPs [11]. The prototypical PAMPs are LPS from Gram-negative bacteria, lipoteichoic acid from Gram-positive bacteria, and bacterial DNA. PAMPs are detected by immune cells through a number of PRRs, such as TLRs, C-type lectin receptors, scavenger receptors, receptor of advanced glycation end products, and complement receptors [9, 10, 12]. Alarmins are endogenous equivalents of PAMPs. The list of alarmins increases continuously and consists of a variety of heterogeneous

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molecules, including high-mobility group proteins, heat shock proteins, defensins, cathelicidins, fibronectin fragments, extracellular ATP, or uric acid [6, 7, 13, 14].

Although numerous review articles have summarized the current concept of danger signals, less attention has been paid to endogenous molecules, which may function to limit immune system activation by DAMPs. Examples for such candidates include adenosine [15, 16], thymosin β 4 [17, 18], apolipoprotein A1 [19, 20], or pulmonary surfactant protein D [21, 22]. During recent years, multiple observations suggest that ubiquitin, a small and highly conserved cellular protein, modulates the immune response to DAMPs when it is released into the extracellular space. Although the discovery of ubiquitin (initially named ubiquitous immunopoietic polypeptide) in 1975 introduced this highly conserved protein as a molecule with extracellular actions [23], the identification of ubiquitin as the ATP-dependent proteolysis factor 1 [24, 25] has focused subsequent research on its important intracellular functions. These functions of the ubiquitin-proteasome system have been discussed in several hundred review articles. In contrast, available information about extracellular ubiquitin has not been integrated into a comprehensive assessment of its possible role as an endogenous immune modulator. It is the goal of this review to recapitulate the current knowledge about extracellular ubiquitin and discuss an emerging facet of its role in biology during infectious and noninfectious inflammation. The synopsis of these data suggests that extracellular ubiquitin may have pleiotropic roles in the immune system and functions as an endogenous opponent of DAMPs. Thus, functions of extracellular ubiquitin could constitute a primordial, anti-inflammatory mechanism that is conserved throughout the phylogenetic system.

INTRACELLULAR UBIQUITIN

Ubiquitin is a small (8.6 kDa) and heat-stable protein in all eukaryotic cells [26, 27]. Its amino acid sequence is highly conserved with little variance in animal species from insects to humans. Only 2–3 aa residues vary between the yeast and plant and the yeast and human protein [26, 28, 29].

It is well established that ubiquitin is a post-translational protein modifier, which plays essential roles in all aspects of biology. The covalent ligation of ubiquitin to intracellular proteins (ubiquitination or ubiquitylation) is catalyzed by ubiquitin protein ligase systems [30, 31]. Traditionally, the ubiquitin protein ligase system is a three-enzyme system, which consists of an E1, an E2, and an E3 [31]. The ubiquitylation reaction is initiated by an E1 enzyme and requires ATP. The E1 enzyme activates the C-terminal glycine of ubiquitin through the generation of an adenylate intermediate, which is followed by the formation of an E1–ubiquitin thiolester and the release of AMP and pyrophosphate. Ubiquitin is next transferred from the E1 enzyme to an E2 enzyme by transesterification. In the last step, which is typically catalyzed by an E3 enzyme, ubiquitin is then linked to the substrate protein in an isopeptide linkage between its C-terminus and an ϵ -amino group of the substrate protein's lysine residues. After binding of ubiquitin to the substrate protein, a polyubiquitin chain can be formed

in which the C-terminus of each ubiquitin molecule is linked to a specific lysine residue of the previous ubiquitin [31, 32]. In the ubiquitin-proteasome pathway of protein degradation, ubiquitylation serves as a signal for degradation of the target protein by the 26S proteasome [31, 32]. Effective degradation of the ubiquitylated protein by the 26S proteasome requires a polyubiquitin tag [33]. In recent years, it became clear that reversible ubiquitylation also occurs and regulates intracellular processes. It is now well accepted that ubiquitylation is more than a proteasomal targeting signal. Today, ubiquitylation is regarded as the second-most common post-translational protein modification following phosphorylation [34, 35].

EXTRACELLULAR UBIQUITIN

Ubiquitin levels in health and disease

Ubiquitin is also a natural plasma protein. The normal ubiquitin concentration in human plasma and serum lies in the low nanomolar range (<10 nM; <100 ng/mL) [36–48]. Previously reported concentrations of ubiquitin in normal plasma and serum differ considerably among individual studies, and average ubiquitin concentrations range from 9 ng/mL to more than 100 ng/mL. In these studies, ubiquitin was quantified with ELISAs, radioimmunoassays, and fluorooimmunoprecipitation assays, using various mono- and polyclonal ubiquitin antibodies. Thus, differences in the cross-reactivity of the ubiquitin antibodies with circulating ubiquitin chains and ubiquitin protein conjugates may account for this variability [39, 46, 47, 49, 50]. In addition, differences in blood storage and processing conditions are likely to contribute to the wide range of normal concentrations reported previously, as ubiquitin is released from damaged erythrocytes during prolonged blood storage [39, 51, 52]. This is reflected by the findings that ubiquitin concentrations did not exceed 50 ng/mL when normal serum was isolated immediately after the blood draw [38] and that normal ubiquitin serum concentrations averaged 32.7 ± 13.8 ng/mL and 126 ± 24 ng/mL (means $\pm$ SD) when sera were prepared within 3 h of storage at 4°C and within 6 h of blood storage at room temperature, respectively [39]. Except for an inverse correlation of plasma ubiquitin concentrations with increasing body mass index [44], no other associations of circulating ubiquitin levels with epidemiological variables, such as age, gender, or ethnicity, have been reported.

Besides its presence in the bloodstream, ubiquitin is also detectable in CSF, BALF, seminal plasma, and urine. Normal CSF ubiquitin concentrations are three- to fivefold lower than its circulating concentrations [46, 48, 51]. Ubiquitin concentrations in normal urine are slightly lower than in serum [46, 53]. Multiple studies demonstrated the presence of ubiquitin in BALF. However, as a result of methodological limitations intrinsic to the procedure of the BALF collection, the actual ubiquitin concentration in lung epithelial-lining fluid is currently unknown [54–58]. As compared with all other extracellular fluids, large amounts of ubiquitin have been detected in seminal plasma [59]. It has been shown that an extracellular ubiquitin-proteasome system plays a role in fertilization and gametogenesis [60, 61], and this nontraditional role of the

ubiquitin proteasome pathway has been reviewed recently elsewhere [62, 63].

Multiple diseases are known to be associated with significantly increased concentrations of ubiquitin in the systemic circulation and in other extracellular fluids (see **Table 1**). This list includes diseases as diverse as HCL, allergic, autoimmune, and neurodegenerative diseases, kidney failure, infections, or trauma. In the majority of these studies, extracellular ubiquitin was considered as a disease biomarker without specific function. Increased ubiquitin concentrations were thought to resemble tissue ubiquitin expression and cell damage associated with the disease processes. Only two of the studies (see Table 1) evaluated a possible functional role of extracellular ubiquitin [43, 46], which will be discussed in the forthcoming sections.

Release and clearance of ubiquitin

The exact cellular origin and release mechanism of extracellular ubiquitin remain to be determined. Nevertheless, one obvious source is its passive release from cells undergoing physiological turnover in normal baseline conditions and from damaged cells and tissues in pathological conditions. The latter would explain the heterogeneity of diseases that is associated with increased ubiquitin levels. Clinical observations are consistent with this assumption, as initial ubiquitin serum concentra-

tions on hospital admission correlated with the severity of injury and the extent of damaged tissue in blunt trauma and burn patients [47], and ubiquitin serum levels paralleled the release of CK, CK-myocardial band, and lactate dehydrogenase within 3–17 h after acute myocardial infarction [45]. Similarly, consistent with its release from damaged cells are the observations that CSF ubiquitin levels increased continuously during progression to irreversible brain damage and brain death in patients with TBIs [51] and correlated with impairment of neurological function after global brain ischemia [64]. Besides its origin from damaged tissues after physical, thermal, or ischemic cell damage, release of ubiquitin from lysed erythrocytes was identified as another significant source [51, 52]. In fact, 25% of CSF ubiquitin could be attributed to erythrolysis in patients with TBIs, and lysis of only 150 μ L blood could explain the CSF ubiquitin concentrations after TBI and nontraumatic SAB in patients [51]. Interestingly, in previous studies, CSF and serum ubiquitin levels were not associated with each other [48, 51, 65], and exogenous ubiquitin does not cross the blood brain barrier under normal conditions (unpublished results). This suggests that ubiquitin in the bloodstream and in CSF originates from distinct cellular sources.

In addition to passive release from damaged cells and tissues, active cellular secretion of ubiquitin likely constitutes another release mechanism. This possibility was inferred from in vitro studies in which ubiquitin was detectable in the secretome of the plant *Arabidopsis thaliana* [66], in supernatants from the cultured water mold *Phytophthora infestans* [67], and from cultured *Spodoptera frugiperda* Sf9 insect cells [68], leptomeningeal cells [69], HCL cells [70], and rat ventricular myocytes [71]. Furthermore, eight- to tenfold increased ubiquitin concentrations were detectable in supernatants from the murine pro-B cell line Ba/F3 and the human kidney cell line 293T after transfection with a ubiquitin expression vector [43]. The notion that ubiquitin can be secreted was corroborated further by the observations that it is cosecreted from human parathormone-producing transformants of the yeast *Pichia pastoris* [72] and that ubiquitin is present in various exosomes derived from multiple mammalian cell types [53, 73, 74] and by the detection of ubiquitin in secretory vesicles from adrenal neuroendocrine chromaffin cells [75, 76]. As ubiquitin was secreted from cultured chromaffin cells together with catecholamines only after direct depolarization by nicotine, it appears not to be secreted constitutively [75]. As sympathetic, splanchnic nerves activate nicotinic acetylcholine receptors on chromaffin cells and stimulate release of catecholamines into the bloodstream in preparation for the fight and flight reactions [77], extracellular ubiquitin could be a component of the neuroendocrine system that controls physiological functions in health and disease. The recent observation that direct β -adrenergic receptor stimulation of cultured ventricular myocytes also increased ubiquitin secretion into conditioned media further supports the idea of an interaction between extracellular ubiquitin and the neuroendocrine system [71].

After i.v. administration of exogenous ubiquitin, a systemic half-life of ~60 min has been determined [78–80]. Ubiquitin

TABLE 1. Diseases Associated with Significantly Increased Concentrations of Extracellular Ubiquitin

	Increase (n-fold)	Reference(s)
Serum/plasma		
HCL	5	[43]
Chronic renal failure/hemodialysis	2–10	[37, 39, 42]
Alcoholic liver cirrhosis	2	[40]
Nonalcoholic steatohepatitis	2	[85]
Diabetes mellitus type 2	2	[41]
Lupus erythematosus	2	[38]
Hymenoptera venom hypersensitivity during desensitization	5	[38]
Acute myocardial infarction	3	[45]
Schistosomiasis	10	[38]
Sepsis	6	[46]
Trauma	3–6	[46, 47]
Burns	3–8	[47]
CSF		
Creutzfeld-Jakob	3–30	[65, 86, 87]
Alzheimer's disease	1.3–2	[48, 88–90]
Vascular dementia	1.3	[48]
TBI	4–30	[51]
Nontraumatic SAB	5	[51]
Anoxic-ischemic encephalopathy	10–100	[64]
BALF		
Idiopathic pulmonary fibrosis	>10	[54, 56]
Hypersensitivity pneumonitis	>10	[56]
Blunt chest trauma ^a	10	[57]
Urine		
Sepsis	4–5	[46]

^aAnimal study.

plasma concentrations are not associated with kidney function [36], and only 10% of i.v.-administered ubiquitin could be recovered in the urine after five serum half-lives [80]. These data indicate that most of ubiquitin is cleared from the systemic circulation through extrarenal mechanisms. In CSF after experimental brain injuries, ubiquitin concentrations peaked within 30–60 min after injury and declined with a half-life of 1–2 h [51, 80]. The similarity of ubiquitin's systemic and intrathecal half-life also makes it unlikely that the lung or liver determines its systemic clearance.

Extracellular ubiquitin can be taken up by various eukaryotic cells, including plants [81], the fission yeast *Schizosaccharomyces pombe* [82], adult rat ventricular myocytes [71], human monocytic cell lines [83, 84], human T cell lines [43], and freshly isolated human PBMCs [83]. Therefore, and besides its possible extracellular degradation, cellular uptake and metabolism of ubiquitin by a large variety of cells are attractive mechanisms, which could explain its clearance from the systemic circulation and other body compartments.

Associations with clinical disease parameters

Most of the studies listed in **Table 1** reported ubiquitin concentrations at a single time-point and/or in small patient populations. Accordingly, information about the correlation of extracellular ubiquitin with disease characteristics, progression, and outcomes is sparse. Besides a correlation of serum ubiquitin with increased injury severity after trauma and burns [47] and of CSF ubiquitin with impairment of neurological function after global brain ischemia [64], higher ubiquitin serum and plasma levels have been associated with impairment of the function of peripheral nerves in diabetes mellitus type 2 and dialysis patients [36, 41]. In patients with Creutzfeldt-Jakob disease, significantly elevated CSF ubiquitin decreased during transition into the end-stage of the disease in which patients progressed into a vegetative neurological state and died [86]. In patients with acute viral hepatitis, increased ubiquitin serum levels correlated with levels of C-reactive protein [39]. Interestingly, an inverse correlation between serum ubiquitin levels and sepsis/MOF development has been established recently in burn patients [47]. Ubiquitin serum levels in burn patients who developed sepsis/MOF and in those who died were significantly lower than in patients with comparable injury severity who recovered uneventfully or survived [47]. Furthermore, ubiquitin serum levels showed an inverse correlation with sequential organ failure assessment scores and correlated independently negative with sepsis/MOF development and survival in partial correlation analyses. This suggests that lower ubiquitin levels are associated with a higher degree of organ dysfunction or failure after burns [47]. These data imply that patients who develop sepsis/MOF after burns have a relative ubiquitin deficiency and provide initial clinical evidence for a possible protective role of ubiquitin during the systemic inflammatory response to burn injury.

Properties and mechanism

Several lines of evidence suggest that extracellular ubiquitin plays pleiotropic roles in the immune system and partici-

pates in the regulation of host defense mechanisms during microbial and sterile inflammation. Properties of ubiquitin include antimicrobial activities, regulation of hematopoiesis, and effects on leukocytes, platelets, and nonhematological cells (**Table 2**).

Antimicrobial activity. In the lysosomal compartment of macrophages, peptides generated by the hydrolysis of ubiquitin are known to contribute to the killing of intracellular bacterial pathogens [108–110]. Similarly, several findings suggest that ubiquitin and ubiquitin-derived peptides are also involved in the defense against microbial invasion in the systemic circulation and on biological surfaces. During isolation of natural antimicrobial molecules, the N-terminal sequence 1–25 (2.8 kDa) of ubiquitin was detected in the rat bladder [91], and a 4.3-kDa peptide of the N-terminal sequence of ubiquitin was identified as a potent antimicrobial peptide with activity against a broad spectrum of pathogens in human amniotic fluid [92]. Furthermore, ubiquitin was identified as a naturally occurring protein with antimicrobial activity in the midgut of the American dog tick *Dermacentor variabilis* [93] and in human vernix caseosa [94]. Although the native ubiquitin molecule (Ub_{1–76}) appears to have limited antimicrobial activity [75, 108], the C-terminal peptide Ub_{65–76} displays potent antimicrobial activity against several gram-positive and gram-negative bacteria, filamentous fungi, and yeast, and the N-terminal peptide Ub_{1–34} synergistically enhances its antifungal activity [75]. At concentrations comparable with its antifungal minimal inhibitory concentrations, Ub_{65–76} interacts with the fungal cell wall, penetrates and accumulates in the microorganisms, and inhibits calcineurin [75], which is important for cellular proliferation of *Neurospora crassa* [111]. Thus, besides destabilization of the fungal cell wall, the antifungal activities of Ub_{65–76} may also be related to its inhibitory effects on essential microbial enzymes. The amount of ubiquitin that is proteolytically processed in the systemic circulation is currently unknown. Nevertheless, microbial enzymes, such as *Staphylococcus aureus* endoprotease V8 or cathepsins released from activated leukocytes could generate ubiquitin peptides with potent antimicrobial activity during local and systemic infections [108, 112] and thus, enhance microbial defense at the site of inflammation.

Hematopoiesis. Ubiquitin was discovered during purification of polypeptide hormones from bovine thymus and initially ascribed a role in lymphocyte differentiation [23]. At concentrations comparable with its physiological plasma concentrations (1–100 ng/mL), purified ubiquitin induced T cell and B cell differentiation in induction assays with splenic mouse lymphoid precursor cells in vitro [23]. In this initial study, T cell differentiation was assessed with the Komuro-Boyse assay, in which expression of the thymic differentiation antigen TL is detected in a cytotoxicity assay using TL antiserum and complement, and the acquisition of complement receptors was used to assess B cell differentiation [23, 113, 114]. As the inductive properties of ubiquitin could be blocked with propanolol, a cAMP-dependent mechanism was implied [23]. Indeed, nanomolar concentrations of extracellular ubiquitin increased cellular cAMP in BALB/c LN cells and in sarcoma 180 cells in later studies [115, 116]. However, it should be noted

TABLE 2. Reported Properties of Extracellular Ubiquitin

	Reference(s)
Antimicrobial activities^a	[75, 91–94]
Hematopoiesis	
Induction of cell surface markers on myeloid/lymphoid precursors	[23, 95–97]
Induction of the capacity to phagocytose in immature granulocytes	
Homing of bone marrow cells into the spleen	[98]
Inhibition of hematopoietic progenitor cell colony formation	[99]
Growth regulation in leukemic cell lines	[43, 99]
Induction of apoptosis in KT-3 and HL-60 cells	[43]
Leukocytes	
Enhances chemotaxis in bone marrow-derived granulocytes	[97]
Attracts lymphocytes into the tissue	[100]
Regulation of cytokine production in response to danger signals	[46, 52, 78, 101, 102]
Inhibition of mixed leukocyte reaction	[103]
Reduction of the number of antibody-forming cells ^a	[104]
Inhibition of IgG production after LPS activation	[105]
Inhibition of plasma membrane-associated exoproteolysis in DCs	[106]
Platelets	
Inhibition of cytotoxicity	[107]
Inhibition of IgE-dependent production of oxygen metabolites	[107]
Enhancement of aggregation induced by suboptimal doses of ADP, collagen, and thrombin	[107]
Nonhematological cells	
Inhibition of growth via delayed cell-cycle progression in fission yeast and myeloblast C2C12 cells	[82, 100]
Inhibition of apoptosis in cardiomyocytes after β -adrenergic receptor stimulation	[71]

^aFunctions reported for ubiquitin and ubiquitin-derived peptides.

that the observed effects of ubiquitin on T and B cell surface marker expression and cAMP levels showed bell-shaped dose-response curves [115, 117, 118]. This indicates that its effects are probably not mediated by a simple interaction with a single cell surface receptor. Nevertheless, daily i.p. injections of 0.2 μ g ubiquitin for 14 days also induced T cell differentiation in the spleen and LNs of athymic nu/nu mice [95], which confirms the effects of ubiquitin, detected earlier in induction assays in vitro. Whether ubiquitin treatment also affected T or B cell function in these studies remains unknown.

In human bone marrow-derived lymphocyte precursor cells, ubiquitin also induced several cell surface markers, such as surface IgM expression or the ability of spontaneous rosette formation with sheep erythrocytes [96]. In contrast to its effects on splenic lymphocyte precursor cells, ubiquitin did not induce complement receptor expression on immature bone marrow-derived lymphocytes [23, 96]. The latter effect, along with enhancement of the capacity to phagocytose, was detectable in differentiation assays with predominantly immature bone marrow-derived human granulocytes [97]. Unlike effects of ubiquitin on surface marker expression of human and murine lymphoid precursor cells, which showed bell-shaped dose responses [97, 115, 117, 118], ubiquitin's property to induce the capacity to phagocytose in immature granulocytes appeared to be saturable in dose-response experiments [97].

After these initial observations about functions of ubiquitin had been published, the involvement of intracellular ubiquitin in ATP-dependent protein degradation was discovered [24, 25]. Subsequently, this initial line of investigation ceased, and it took 15 years until further and independent evidence for a role of extracellular ubiquitin in hematopoiesis was reported

[98]. Incubation of murine hematopoietic progenitor cells with ubiquitin significantly reduced their homing into the spleen of lethally irradiated, syngeneic mice [98] when tested in the Till and McCulloch spleen colony assay [119]. Subsequently, ubiquitin was found to inhibit colony formation of human hematopoietic progenitor cells (CFU-GM, CFU-erythroid) and of the G-CSF-dependent human myeloblastic leukemic cell line OCI/AML/1 α [99]. In addition, high concentrations of ubiquitin inhibited the growth of the cell lines KT3 (T lymphoblast, >50 μ g/mL), K562 (erythroleukemia, 20 μ g/mL), Daudi (Burkitt's lymphoma, 20 μ g/mL), HL-60 (promyelocytic leukemia, >50 μ g/mL), U937 (monocytic leukemia, 100 μ g/mL), MT-4, YTC-3, and MOLT-4 (human T cell lines, 100 μ g/mL) and of PHA-stimulated normal human PBMCs (100 μ g/mL) [43, 99]. However, it should be noted that lower concentrations of ubiquitin (20 μ g/mL) stimulated growth of Jurkat and HL-60 cells and had no effect on U937 cells [99]. Nevertheless, inhibitory effects of ubiquitin on the growth of HL-60 and KT-3 cells could be attributed to the induction of apoptosis via a proteasome-dependent mechanism [43]. As patients with HCL have elevated ubiquitin plasma concentrations, and HCL cells release ubiquitin, the proapoptotic and inhibitory effects on clonogenic growths of CFU-GM and GFU-E of ubiquitin were interpreted to correspond to pancytopenia, which develops frequently in HCL patients [43, 70, 99, 120]. Taken together, the reported effects of ubiquitin on the differentiation and homing of normal hematopoietic precursor cells and on growth regulation of leukemic cell lines suggest a possible role during normal and pathological hematopoiesis.

Leukocytes. Initial evidence that ubiquitin is able to influence leukocyte function was obtained from in vitro experiments with normal human bone marrow cells. Preincubation of a bone marrow-derived granulocyte preparation (band forms > segmented > metamyelocytes) with ubiquitin enhanced their chemotactic response significantly toward a casein-concentration gradient [97]. In contrast, ubiquitin did not enhance chemotactic movements toward fMLP when tested with peripheral blood PMN leukocytes from healthy blood donors [121]. As for its differential effects on the differentiation of lymphocyte precursor cells from the bone marrow and the spleen [23, 96], this may again suggest that the effects of ubiquitin depend on the grade of cellular maturation or the cellular compartment. Although it is currently unknown whether ubiquitin itself produces a chemotactic response, the observation that injection of ubiquitin into skeletal muscle led to an accumulation of lymphocytes in the absence of cytotoxicity may indicate chemotactic activity [100]. The latter observation is surprising, as ubiquitin does not elicit antibody production when administered in the absence of a covalently linked immunogen and thus, is commonly believed to lack immunogenicity as a result of its small size and highly conserved structure [122, 123].

Recent data further indicate that ubiquitin is able to suppress immune responses. In murine splenocyte cultures, ubiquitin dose-dependently inhibited the number of antibody-producing cells upon antigen re-exposure [104], IgG production after LPS activation [105], and one-way mixed leukocyte reaction [103]. In addition, a decapeptide corresponding to the ubiquitin sequence Ub_{50–59}, which is located on the external loop of the molecule, showed strong, suppressive activities on humoral and cellular immune responses, comparable with cyclosporine [124]. Thus, ubiquitin appears to suppress B and T cell functions. Consistent with immune-suppressive properties of ubiquitin are the findings that it inhibited LPS-stimulated TNF- α and IL-8 production of normal whole blood and isolated PBMCs in vitro [46, 52] and also reduced LPS-stimulated TNF- α production of whole blood after administration in vivo [78]. Inhibitory effects of ubiquitin on LPS-stimulated TNF- α production were detectable in multiple species, could be demonstrated for exogenous and endogenous extracellular ubiquitin, and occurred in a dose-dependent manner [46]. Interestingly, maximal inhibitory activity of ubiquitin was detectable at concentrations consistent with its plasma levels after severe injuries and sepsis. As hyporesponsiveness to LPS is a well-described parameter of impaired immune function after trauma and during sepsis in patients [4, 125–130], this observation led to further studies about its possible physiological role during the systemic inflammatory response. In severely injured patients, circulating ubiquitin concentrations correlated inversely with the capacity of whole blood to produce TNF- α in response to LPS during a 14-day period. Neutralization of endogenous extracellular ubiquitin with anti-ubiquitin normalized the reduced whole blood responsiveness to LPS in trauma and sepsis patients. These findings, along with the inhibitory activity of endogenous and exogenous ubiquitin on LPS-induced TNF- α pro-

duction of normal blood, established a causal relationship in vitro and identified ubiquitin as an endogenous immune modulator with anti-inflammatory properties [46]. Subsequent studies demonstrated that ubiquitin is not only able to suppress the production of proinflammatory cytokines in response to LPS but also enhances the production of the classical Th2 cytokines IL-4, IL-10, and IL-13 during sterile inflammation in the lung in vivo [101]. This further supports the notion that ubiquitin is a molecule with anti-inflammatory actions. However, it should be noted that conflicting results about the effects of ubiquitin on LPS-stimulated TNF- α production have also been reported. In the mouse macrophage cell line RAW264.7, a supraphysiological dose (1 μ M, 8.6 μ g/mL) of ubiquitin augmented LPS-stimulated TNF- α mRNA expression and secretion [102]. Besides the limitation that a significant twofold effect on TNF- α production was detectable only at a single concentration and a single time-point after LPS stimulation [102], clonal line differences may explain this observation, as neither stimulating nor inhibitory effects of ubiquitin were detectable in the murine macrophage cell line J774 [46]. As evidence was provided that chemical modification of extracellular ubiquitin can lead to a more acidic isoelectric point of the protein and results in a molecule that irreversibly inhibits chemotaxis of peripheral blood PMN leukocytes [121], such a mechanism can currently not be excluded for its divergent effects on LPS-induced TNF- α production in freshly isolated blood cell and cell line cultures.

Although the functions of ectoenzymes on the cell surface of APCs are incompletely understood, they have been associated with various cellular functions, such as antigen processing, inactivation of soluble mediators, or cell adhesion and migration [106]. The finding that extracellular ubiquitin inhibits CD13 or CD13-like exoproteolytic activity associated with the cell membrane of human DCs could therefore also correspond to its anti-inflammatory and immune-suppressive properties [106].

Platelets. In line with immune-suppressive and anti-inflammatory effects on leukocyte functions, ubiquitin inhibited IgE- and IFN- γ -induced platelet cytotoxicity when assayed with schistosomula as targets and inhibited the generation of oxygen metabolites by platelets when stimulated by the IgE-anti-IgE reaction [107]. The finding that inhibition of platelet cytotoxicity by ubiquitin was also detectable in assays with platelets from patients with hymenoptera venom hypersensitivity and aspirin-sensitive asthma after exposure to the relevant antigen indicated that this effect could have pathophysiological relevance [107]. Similar to some of its effects on leukocytes described above, these inhibitory effects on platelet functions showed bell-shaped dose dependencies in dose-response experiments. The maximal inhibitory activities were detectable at concentrations between 0.1 and 1 ng/mL and disappeared when concentrations in the normal and pathological range were tested (10–1000 ng/mL) [107]. As platelet functions were tested in assay reactions with platelet-rich plasma and plasma components, which contain ubiquitin, the absolute concentrations are difficult to interpret and probably underestimate the required amounts of ubiquitin. Nonetheless, addi-

tion of ubiquitin to platelet-rich plasma enhanced platelet aggregation induced by suboptimal doses of ADP, thrombin, or collagen, suggesting that ubiquitin-induced effects are not suppressive in general but depend on the specific cell function [107].

Nonhematological cells. Besides regulatory effects on the growth of various leukemia cell lines [43, 99], high concentrations (10–100 $\mu\text{g/mL}$) of ubiquitin have been shown to inhibit growth of the fission yeast *S. pombe* [82] and the mouse myoblast cell line C2C12 [100]. Although the mechanism of these effects is largely unknown, growth retardation has been attributed to a delayed progression through the cell cycle in *S. pombe* and C2C12 cells, with significantly fewer C2C12 cells in the S-phase after ubiquitin treatment [82, 100]. In contrast to its proapoptotic effects in human leukemia cell lines, ubiquitin did not induce apoptosis in C2C12 cells [100], and antiapoptotic effects of ubiquitin have been detected in adult rat ventricular myocytes [71]. Although small concentrations (0.01–1 $\mu\text{g/mL}$) of ubiquitin partially inhibited apoptosis, high concentrations (100 $\mu\text{g/mL}$) almost completely abrogated cardiomyocyte apoptosis induced by β -adrenergic receptor stimulation [71]. This antiapoptotic effect was attributed to activation of PI3K and inhibition of glycogen synthase kinase-3 β /c-Jun N-terminal kinase and mitochondrial pathways by ubiquitin [71]. Thus, these data suggest that extracellular ubiquitin may serve as a survival factor in nonhematological cells and tissues.

Mechanism of action. Despite the multitude of effects of ubiquitin that have been reported in previous studies, little is known about its mechanism of action. The existence of a cell surface receptor for ubiquitin has been hypothesized with its initial description [23]. As ubiquitin inhibited homing of murine hematopoietic progenitor cells, and bone marrow cells with morphological resemblance to lymphocytes stained positive for biotin after incubation with biotinylated ubiquitin, a specific ubiquitin-binding site on a homing receptor was postulated [98]. Initial biochemical evidence for a ubiquitin cell surface receptor was provided when ubiquitin was used as a control protein in binding studies with MNSF- β , a lymphokine that consists of a ubiquitin-like protein (36% identity with ubiquitin) fused to the ribosomal protein S30. Specific MNSF- β (20 nM) and ubiquitin (100 nM) binding was detectable in the Con A-stimulated murine T cell line D.10.G4.1 [131]. However, ubiquitin was unable to replace MNSF- β from its binding site in competition experiments [131], suggesting a separate binding site for ubiquitin. In addition, quantification of ubiquitin uptake into the human monocytic cell line Mono-Mac 6 revealed a saturable process with a K_d in the nanomolar range, which further supported the idea that its uptake is mediated by a cell surface receptor [83]. Consequently, it was shown that ubiquitin binds to various human (THP1, U937, HL-60) and murine (Wehi-3, P388.D1, J774) monocyte/macrophage cell lines and to freshly isolated human monocytes at 4°C [84]. Ubiquitin binding fulfilled all classical receptor-binding characteristics with a K_d of ~ 100 nM in THP1 cells, promoted intracellular Ca^{2+} flux within seconds, and reduced cellular cAMP levels within minutes. The ubiquitin-induced intracellular Ca^{2+} flux was PTX-sensitive, suggesting involve-

ment of GPCRs [132], and LPS treatment reduced ubiquitin-binding sites. In combination with the reported expression profile of GPCRs on murine macrophages [133], these data enabled identification of a limited number of GPCR candidates. Overexpression of the candidate receptors and gene knockdown studies, as well as pharmacological characterization of its binding properties, identified ubiquitin as a natural agonist of the chemokine and HIV coreceptor CXCR4 (CD184, fusin) [84]. Similar to ubiquitin, CXCR4 and its cognate ligand, the chemokine SDF-1 α (CXCL12), are also highly conserved throughout evolution, with $>90\%$ aa sequence identity among different species [134, 135]. Consistent with effects of ubiquitin in various cell types, CXCR4 is abundantly expressed in leukocytes, platelets, and most human tissues [136, 137]. The SDF-1 α /CXCR4 axis is known to be important for normal development and hematopoiesis, plays pleiotropic roles in the immune system, and is involved in cancer metastasis, leukemia, and HIV infection [132, 138–145]. Reported functions of ubiquitin and SDF-1 α are equally consistent with CXCR4 as their common receptor. For example, both ligands promoted platelet aggregation [106, 137], reduced the production of TNF- α , enhanced the production of IL-10 in inflammatory conditions [46, 101, 146], and decreased apoptosis in a PI3K-dependent mechanism [71, 147]. Similar to observed effects of ubiquitin, biological properties of SDF-1 α are also known to depend on the cell type and to result in opposite effects depending on the concentration [148–151].

Nevertheless, it appears unlikely that nature developed and conserved two ligands with identical properties on the same receptor throughout evolution. Furthermore, chemokine receptors and their ligands are considered to be highly promiscuous, being able to bind multiple receptors/ligands [132, 152]. Although SDF-1 α has been shown to bind to CXCR4 and CXCR7, endogenous CXCR4 ligands are SDF-1 α , macrophage migration inhibitory factor, and ubiquitin [84, 132, 152–154]. Thus, it is possible that ubiquitin also binds to other as-yet unidentified receptors. This assumption could correspond to the bell-shaped dose-response curves that have been described for various ubiquitin-induced effects and may correspond to inhibitory effects on one receptor and activating properties at different concentrations on another. Alternatively, the physicochemical interaction of ubiquitin with CXCR4 could be distinct from SDF-1 α and induce a different set of biological effects. Such a behavior could be explained by biased agonism or functional selectivity of the CXCR4 interaction with its agonists. Biased agonism has been reported for other GPCRs [155, 156] and is suggested for CXCR4 by divergent biological actions of the monomeric and dimeric form of SDF-1 α [157] and by findings that suggest that alternative agonist-binding sites are present on CXCR4 [158]. Although the structural requirements of ubiquitin for CXCR4 binding and signaling remain to be determined, the external loop 50–59 of the ubiquitin molecule has been suggested as a possible receptor-interaction site [104, 124]. Interestingly, this loop contains a retro-RGD sequence and has been described to correspond partially to the $\beta 164$ –172 loop of HLA-DQ. The latter contains the RGD sequence, which is thought to be important in mediating cell adhesion interactions [104, 124].

As described before, ubiquitin is taken up by a variety of eukaryotic cells. Recent data suggest that cellular uptake of extracellular ubiquitin is followed by its covalent conjugation to intracellular proteins of the target cell [43, 83]. This implies that it can be reused in the target cell and is introduced into the ubiquitin proteasome pathway. Interestingly, it has been shown that the tight regulation of cellular ubiquitin pools is significantly disturbed during sepsis in human PBMCs with reduced total amounts of ubiquitin, which were caused by a reduction in ubiquitin protein conjugates [159]. Quantification of ubiquitin uptake into LPS-stimulated MonoMac 6 cells showed that its uptake could significantly increase the available pool of free intracellular ubiquitin [83]. Thus, in addition to its receptor-mediated cell signals, uptake of extracellular ubiquitin could be used to replenish the depleted pools of ubiquitin and to maintain the essential functions of the ubiquitin proteasome pathway in the immune system [34, 35, 160–162].

SDF-1 α has been shown to activate STAT3 within a few minutes [163]. Furthermore, previous observations suggest that extracellular ubiquitin can be conjugated to STAT3, leading to its proteasomal degradation at later time-points (>30 min) [43]. Therefore, it is an attractive alternative hypothesis that post-translational modification of intracellular proteins with extracellular ubiquitin produces a secondary cell signal, which could function as an autoregulatory mechanism to terminate its receptor-mediated biological signals on the level of downstream effector molecules.

Therapeutic potential

As for SDF-1 α and CXCR4, lack of ubiquitin is associated with embryonic lethality [138, 164, 165]. At present, neutralization or removal of endogenous extracellular ubiquitin *in vivo* has not been possible. Therefore, information about the physiological and pathophysiological role of endogenous extracellular ubiquitin *in vivo* is not available. Nevertheless, exogenous ubiquitin has been administered to normal animals and to animals in multiple disease models (Table 3). Besides the effects

described above regarding ubiquitin on T cell differentiation in mice and on *ex vivo* LPS-stimulated blood TNF- α production in pigs, no physiological consequences have been observed in these studies when ubiquitin was administered in normal conditions [78, 95]. In contrast, administration of exogenous ubiquitin produced notable effects in various disease models, including autoimmune diseases, transplantation, and infectious and sterile inflammation.

L. erythematosus. In well-accepted mouse models of spontaneous immunopathology resembling human *L. erythematosus*, *i.p.* ubiquitin treatment (5 days/week; 1 or 10 μ g/injection), starting at 4 weeks of age, delayed the appearance of Coombs' antibodies by 4 months and reduced splenomegaly significantly at 6 months of age, as compared with animals injected with vehicle [166]. This observation matches well with *in vitro* effects of ubiquitin on antibody production that have been described by other investigators in later studies [104, 105]. When ubiquitin treatment was started at 6 months of age, at a time-point when Coombs' antibodies had appeared already, disease progression could not be influenced [166]. This may suggest that ubiquitin is able to suppress or delay initiation of pathological immune activation but does not reverse immunopathology once the disease process has been established.

Transplantation. The effects of ubiquitin on allograft survival were evaluated in a murine model of skin transplantation in fully mismatched strain combinations (C3H/HEJ; DBA2) [103]. Ubiquitin (25 μ g/h) or albumin was delivered *i.p.* via mini-osmotic pumps for 14 days. *i.p.* ubiquitin administration was well-tolerated and dose-dependently increased ubiquitin serum concentrations. The obtained serum concentrations compared well with the average serum concentration produced after *i.v.* bolus injection in other studies. One day after pump implantation, recipient DBA2 mice simultaneously received syngeneic and allogeneic skin grafts on full-thickness skin excisions. Syngeneic skin-graft survival was >90% in both groups. At the end of the ubiquitin administration period (Day 14), 61% of the allogeneic skin grafts survived with ubiq-

TABLE 3. Therapeutic Effects of Exogenous Ubiquitin

Disease model	Effect	Reference(s)
<i>L. erythematosus</i>	Delays appearance of Coombs' antibodies	[166]
	Reduces splenomegaly	
<i>Skin transplantation</i>	Prolongs allograft survival	[103]
<i>Endotoxic shock</i>	Reduces systemic fluid requirements	[78]
	Reduces lung injury	
	Reduces edema and erythema formation	
	Reduces mortality	
<i>Polymicrobial sepsis</i>	Reduces lung edema formation	[167]
<i>Extremity trauma plus hemorrhage</i>	Reduces systemic fluid requirements	[79]
<i>TBI plus hemorrhage</i>	Reduces intracranial pressure	[80]
	Reduces systemic fluid requirements	
	Reduces lung injury	
<i>Mild/moderate TBI</i>	Reduces cortical contusion volume	[168]
<i>Cerebral ischemia</i>	Improves spontaneous motor activity	[169]
	Reduces neuronal death	
<i>Lung ischemia-reperfusion injury</i>	Reduces lung edema formation	[101]
	Improves oxygenation	

ubiquitin and 9% with albumin. Median allograft survival was significantly longer in the ubiquitin group (median: 17 days) than in the albumin group (median: 10 days; survival ratio: 1.7; 95% confidence interval of ratio: 1.266–2.134), which confirms its immune-suppressive effects that have been detected in vitro [46, 52, 103–105, 107]. In subsequent pilot experiments, ubiquitin administration was extended to 4 weeks but did not result in a longer allograft survival (unpublished results). As for effects of ubiquitin in a mouse lupus model [166], these observations imply that ubiquitin exerts its actions during the initiation of the immune response but does not produce long-term immune suppression or induce transplant tolerance. Ubiquitin was identified recently as an intracellular immunophilin that binds IL-2-suppressing drugs, such as tacrolimus and sirolimus, and mediates their suppressive effects on T cells [170, 171]. As extracellular ubiquitin can be taken up into the cell, synergistic effects of a coadministration of ubiquitin with tacrolimus or sirolimus appear likely and may be useful to reduce adverse side-effects associated with commonly used immune-suppressive treatment protocols after organ transplantation.

Infectious inflammation. Based on the inhibitory effects of ubiquitin on LPS-induced responses [46, 52, 78, 105], its actions were studied in a lethal endotoxic shock model [78]. Fully instrumented, anesthetized, and mechanically ventilated pigs received a single i.v. dose of 1.3 mg/kg ubiquitin 15 min before or 45 min after the start of a continuous endotoxin infusion for 180 min. Within the experimental procedures, the only intervention was i.v. fluid resuscitation when the mean arterial blood pressure reached 69 mmHg, and fluid resuscitation was continued until a mean arterial blood pressure of 70 mmHg was achieved. All other experimental settings remained unchanged.

In this model, ubiquitin treatment reduced inflammation significantly when administered before and after the start of the endotoxin infusion. It reduced i.v. fluid requirements to maintain systemic hemodynamics by 90% and 70% with pretreatment and post-treatment, respectively. Ubiquitin preserved oxygenation and prevented a continuous increase in peak inspiratory pressure and development of pulmonary failure that was detectable in the control group. Edema and erythema development were visibly attenuated with ubiquitin. Although mortality was 50% in the control group, ubiquitin pre- and post-treatment reduced mortality to 0%. The antimicrobial activities of ubiquitin, in combination with its beneficial effects during endotoxemia [78] and the observation that ubiquitin treatment reduced lung edema formation during the early period after cecal ligation and puncture in mice [167], suggest a therapeutic potential for the treatment of infections and sepsis.

Sterile inflammation. Exogenous ubiquitin has been administered i.v. in several models of trauma and ischemia-reperfusion injury. In a clinically relevant extremity trauma and hemorrhage model in pigs, ubiquitin (1.5 mg/kg i.v.) was administered at the end of a 40-min shock period as initial bolus injection prior to standard fluid resuscitation [79]. Similar to its effects in endotoxic shock, ubiquitin treatment reduced fluid requirements significantly to maintain systemic hemody-

namics by at least 60%. As hemodynamic parameters, hematocrit, and hemoglobin levels were similar in the treatment and control groups, these data indicate that ubiquitin reduced fluid shifts as a measure of diffuse capillary leak. Furthermore, the therapeutic potential of ubiquitin was explored in a clinically relevant porcine fluid-percussion brain injury and hemorrhage model. Ubiquitin (1.5 mg/kg i.v.), administered at the end of a 35-min shock period prior to standard fluid resuscitation, reduced systemic fluid requirements significantly to maintain mean arterial and cerebral perfusion pressures [80]. Consistent with reduction of fluid shifts into tissues during the early systemic inflammatory response [78, 79], ubiquitin treatment reduced intracranial pressure significantly as a parameter of brain edema formation. In addition, ubiquitin treatment reduced peak inspiratory pressure during mechanical ventilation as a parameter of lung edema formation and improved oxygenation, suggesting protective effects against lung injury [80].

Ubiquitin's profound effects on intracranial pressure after fluid-percussion brain injury provided indirect evidence that it reduced brain edema formation and possesses neuroprotective properties [80]. Direct evidence for neuroprotective properties of ubiquitin after brain injury could be obtained in a well-described, controlled cortical impact model in rats [168]. The same dose of ubiquitin administered i.v. 5 min after brain injury reduced the cortical contusion volume significantly by 25%, as assessed 1 week after the injury [168]. Consistent with the assumption that exogenous ubiquitin confers neuroprotection is the finding that ubiquitin treatment with the identical dosing regimen improved spontaneous motor activity significantly after transient cerebral ischemia in gerbils [169]. Besides this functionally relevant treatment effect, ubiquitin reduced neuronal death and reactive gliosis significantly in the hippocampus CA1 region [169], which provides additional morphological evidence for direct neuroprotective effects of ubiquitin [168]. Remarkably, a recent study demonstrated a transient but almost complete loss of SDF-1 expression in microvessels of the hippocampus, whereas CXCR4 expression was selectively increased in the subgranular layer after transient global cerebral ischemia in rats [172]. Thus, it is tempting to speculate that loss of SDF-1 α up-regulates CXCR4 in a negative-feedback loop and that neuroprotective effects of ubiquitin are caused through the maintenance of adequate CXCR4 signaling.

Although ubiquitin treatment showed therapeutically relevant effects on lung mechanics and function during resuscitation in endotoxic and traumatic shock [78, 80], these studies were performed using fluid resuscitation protocols with strict hemodynamic targets. The latter may have resulted in iatrogenic fluid overloading and aggravation of lung edema formation. Nevertheless, when the same dose of ubiquitin was administered immediately before reperfusion of the lung after a 90-min period of ischemia, treatment significantly reduced lung edema formation and improved oxygenation, independent of rigid resuscitation protocols [101]. The beneficial actions of ubiquitin during lung ischemia-reperfusion injury confirm its protective effects against lung injury that have been observed during traumatic and

endotoxic shock. As ubiquitin reduced lung edema formation and improved oxygenation independent of fluid resuscitation protocols [101], these findings strengthen the hypothesis that its actions are of physiological and therapeutic relevance.

Collectively, previous studies about the therapeutic potential of ubiquitin during sterile inflammation suggest that exogenous ubiquitin limits physiological sequelae, which occur during the systemic inflammatory response to trauma and ischemia-reperfusion injury. Third spacing of fluids into tissues is one of the earliest physiological consequences that occur during the infectious and sterile systemic inflammatory response. The observations that ubiquitin reduced fluid shifts in models of infectious and sterile inflammation support the assumption that it dampens activation of the immune system in response to PAMPs and alarmins.

A concept for a role of extracellular ubiquitin during inflammation

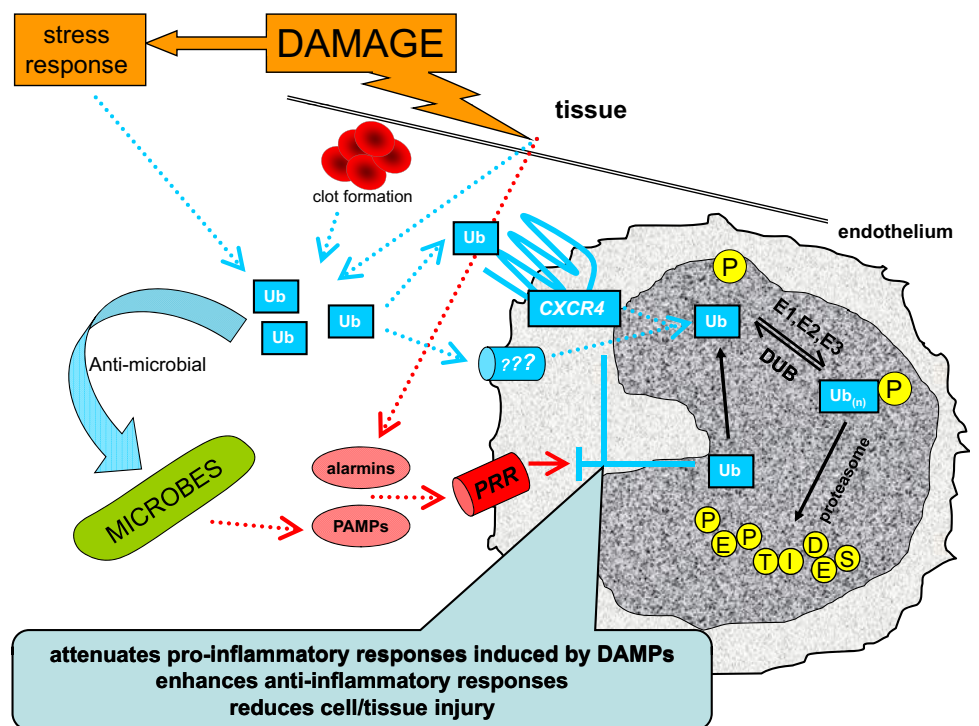
Unlike proinflammatory cytokines, which are also found in lower vertebrates, anti-inflammatory and immunosuppressive cytokines appear to occur later during vertebrate evolution [173, 174]. Thus, it stands to reason that other molecules exist that balance the immune response to microbial invasion or tissue injury and prevent detrimental consequences of exuberant immune system activation. Based on the highly conserved structure of ubiquitin in all eukaryotes, the available information about its release mechanisms and biological actions outside of the cell can be integrated into the

working concept that functions of extracellular ubiquitin constitute an evolutionary conserved control mechanism that is aimed to balance the immune response to DAMPs and prevent exuberant inflammation (Fig. 1). In this model, erythrocytes could serve as universally and immediately available ubiquitin carriers. Although mature erythrocytes contain high amounts of ubiquitin, they have lost substantial amounts of enzymes of the ubiquitin-proteasome pathway [39, 175]. As a consequence, it is generally believed that erythrocyte ubiquitin is a remnant of the cellular remodeling that occurs during maturation of reticulocytes to erythrocytes. Alternatively, ubiquitin release from clotted erythrocytes may constitute a strikingly simple mechanism to deliver it specifically and at high, local concentrations at the site of cellular injury and inflammation.

Inflammation induced by infection, trauma, or autoimmune processes is always associated with cell damage and subsequent initiation of the complement and coagulation cascades. Ubiquitin is released into the extracellular space from damaged tissues and clotted erythrocytes at the site of inflammation. In situations in which inflammation produces a systemic inflammatory response syndrome, local ubiquitin release is augmented through its release along with catecholamines from the adrenal gland during the stress response. In the extracellular space, ubiquitin and ubiquitin-derived peptides, which may be generated by microbial proteases or proteases released by activated leukocytes, enhance antimicrobial defense. Ubiquitin binds to and signals through CXCR4 and possibly to other cell surface receptors

Figure 1. Concept for a role of extracellular ubiquitin during inflammation.

Microbial invasion and cell and tissue damage lead to the liberation of PAMPs and alarmins, which activate the immune system and produce a proinflammatory response. Similarly, ubiquitin is released from damaged tissues and also from clotted erythrocytes. In situations in which inflammation produces a systemic inflammatory response syndrome, local ubiquitin release is augmented through its release from the adrenal gland during the stress response. Besides direct antimicrobial actions, ubiquitin binds to and signals through CXCR4 and possibly other receptors. It is then taken up into the cell through receptor-mediated endocytosis. Ubiquitin is, at least partially, recycled and reused in the ubiquitin-proteasome system of the target cell. Its CXCR4/receptor-mediated effects and possibly its secondary effects through post-translational modification of target proteins attenuate proinflammatory responses induced by DAMPs, enhance anti-inflammatory responses, and reduce cell and tissue injury. Ub, Ubiquitin; Ub_(n), ubiquitin/ubiquitin chains covalently linked to intracellular proteins; P, intracellular proteins; CXCR4, CD184, fusin; DUB, de-ubiquitylating enzymes.



expressed in leukocytes, platelets, and tissues. Its receptor-mediated actions down-regulate the release of proinflammatory cytokines in response to DAMPs from activated leukocytes and enhance the production of anti-inflammatory cytokines, promote cell survival through inhibition of apoptosis in tissues, and may induce apoptosis in leukocytes to terminate uncontrolled perpetuation of immune system activation.

During inflammation, activated neutrophils release cathepsin G and neutrophil elastase, both of which are able to cleave the N-terminus of SDF-1 α , leading to a truncated form that is unable to interact with CXCR4 [132, 176]. Although the exact proportion of SDF-1 α that is inactivated during inflammation is unknown, increased levels of ubiquitin may substitute for its inactivation and maintain appropriate CXCR4 signaling.

Following its receptor interactions, ubiquitin is taken up into the cell through receptor-mediated endocytosis and at least partially, recycled and used by the ubiquitin proteasome system of the target cell. In the target cell, ubiquitin augments the available pool of free ubiquitin to maintain the multiple functions of the ubiquitin-proteasome system in the immune system and other tissues. Alternatively, conjugation of extracellular ubiquitin to intracellular target proteins may produce a secondary cell signal, which terminates its receptor-induced, initial cell signals and thus, may constitute an autoregulatory feedback loop.

This working model is an agreement with clinical observations in patients with severe injuries, burns, and sepsis, with the affinity of the interaction between ubiquitin and CXCR4, and with the observation that supraphysiological doses of exogenous ubiquitin and SDF-1 α have beneficial and therapeutically relevant effects during inflammation. As outlined before, the normal ubiquitin plasma concentration is below 10 nM and increases during infectious and noninfectious inflammation. Remarkably, ubiquitin plasma levels during inflammation approach its K_d for CXCR4, its EC_{50} for the reduction of cAMP levels and induction of Ca²⁺ fluxes, and its effective concentration to reduce LPS-stimulated TNF- α production [46, 84]. Treatment of animals with 1.3–1.5 mg ubiquitin/kg i.v. produced peak concentrations of >1 μ M [79, 80]. This is more than 1 order of magnitude higher than its K_d for CXCR4, suggesting complete receptor engagement.

Although the affinity of SDF-1 α for CXCR4 (K_d : 1.5–54 nM; refs. [153, 177–181]) is higher than the affinity of ubiquitin, its systemic concentrations in physiologic baseline conditions are 20- to 200-fold lower [182–184]. Systemic SDF-1 α levels also increase in patients after burns and during sepsis, but ubiquitin levels are even higher in these conditions and exceed SDF-1 α levels 200- to 700-fold [183, 184]. In agreement with the therapeutic potential of exogenous ubiquitin, SDF-1 α treatment also showed anti-inflammatory, neuro- and tissue-protective effects in various models of sterile inflammation [146, 157, 185, 186]. This further supports the concept that endogenous and exogenous ubiquitin mediate many of its functions through CXCR4.

CONCLUSIONS

Multiple lines of evidence obtained throughout the past decades indicate that ubiquitin is an endogenous immune modulator when it is present outside of the cell. Observations in patients, reported in vitro and in vivo functions, and its recent identification as a CXCR4 agonist support the notion that ubiquitin is involved in the regulation of innate and adaptive immune responses. Although it is possible that its actions are not mediated exclusively via CXCR4, its CXCR4 agonist properties shed a new light on the possible role of extracellular ubiquitin in leukocyte differentiation and justify to revisit its functions during normal and pathological hematopoiesis. Furthermore, these data imply a novel role of CXCR4 during the initiation of the innate immune response. As ubiquitin is one of the most highly conserved proteins in all eukaryotes, its actions may represent a phylogenetically early anti-inflammatory pathway that has been available already in lower vertebrates, in which a broad repertoire of specialized immune-suppressive and anti-inflammatory cytokines has not evolved. Erythrocytes appear to serve as universally available ubiquitin carriers, which deliver it specifically to the site of inflammation during clot formation through the activation of the complement and coagulation cascades. Ubiquitin appears to function as an endogenous opponent of DAMPs, which may prevent inadequate immune system activation and physiological symptoms of inflammation during subclinical infections and after minor tissue injury. Although the release of DAMPs dominates during clinically relevant infections and after severe tissue damage and induces a proinflammatory response that leads to the clinically relevant symptoms of inflammation and to organ system failure in critical illness, ubiquitin treatment has the potential to attenuate these symptoms and to reduce organ injury. Further characterization of its mechanism of action and cellular signaling pathways is expected to provide novel insights into the regulation of the innate immune response and opportunities for therapeutic interventions.

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DISCLOSURE

The therapeutic use of ubiquitin has been patented (U.S. Patent #7,262,162). The author is an inventor. The author has not received any income related to the patent.

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KEY WORDS:

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