Editorial

The Proteasome and Its Implications in Rheumatology

The development of agents against cytokines implicated in the pathogenesis of rheumatic diseases has led to a more rational therapeutic approach to these conditions. Until the present, therapeutic targets have been proteins/cytokines themselves or their synthetic pathways. The recent approval of a drug with antiproteasomal activity (bortezomib) for refractory multiple myeloma[1-3] marks the beginning of a new era: the era of the regulation of protein catabolism. The regulation of the proteasomal complex also has implications and potential benefits for the treatment of rheumatic diseases.

THE PROTEASOME COMPLEX

The proteasome is the central enzyme complex of nonlysosomal protein degradation, an essential component of the ubiquitin-ATP-dependent proteolytic pathway[4,5]. Originally thought of as an indiscriminate protein digestive system, the "waste disposal units of the cell"[6], it is recognized now as a selective, highly complex, temporally controlled, and tightly coordinated regulatory pathway[7-11]. The proteasome is present in the cell nucleus and the cytoplasm of all eukaryotic cells[1,12-14], comprises up to 1% of total cell protein[4,10,15], and targets cytosolic and nuclear proteins as well as membrane-anchored and secretory pathway-compartmentalized proteins[7,8,16].

The sequence of discoveries that led to the current understanding of the proteasome structure and function have been reviewed by Hershko, *et al*[17,18]. Therefore, this review will highlight the essential concepts of the proteasome as they relate to rheumatoid arthritis (RA) and psoriasis. The proteasome consists of different subunits that do not exhibit proteolytic activity when expressed individually[5,19-21]. The 26S proteasome (often called "the proteasome") is a multisubunit protein complex of 2000 kDa that consists of a proteolytic core particle (the 20S proteasome) sandwiched between two 19S "cap" regulatory complexes (19S+20S+19S)[4,7,18]. The 26S proteasome binds ATP and is responsible for the destruction of proteins that have been targeted for degradation via their conjugation with a poly-ubiquitin (Ub) chain (Figure 1)[5,7,8,11,17,22].

The core 20S proteasome has a cylinder shaped structure arranged as 4 axially stacked heptameric rings made up of 2 outer α-rings and 2 inner ß-rings[4,23]. The multiple catalytic sites of this proteolytic complex are exclusively associated with the ß-subunits[16,24,25]. All the catalytic sites face the inner chamber of the cylinder, and the only way for substrates to reach this chamber is through the gated channels in the α-rings, which are completely closed in the free latent 20S proteasomes[4,6,16,20,24]. Such regulation and compartmentalization of proteases prevents indiscriminate digestion of proteins that have not been targeted for elimination[5,11,12,17,26].

When cells are stimulated by inflammatory cytokines [i.e., interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α)], 3 new enzymatic active subunits (immunosubunits) are transcriptionally induced and take the place of their constitutive homologs during proteasome neosynthesis[26-28]. The name "immunoproteasome" has been proposed for the complex containing the specific immunosubunits. Differential regulation of subunit composition serves to control the qualitative properties of proteolytic products by generating peptides that are more appropriate for antigen presentation[6].

The 20S proteasome never functions as an isolated enzyme in cells, but rather function only when bound to regulatory proteins (i.e., 19S, 11S) that mediate proteasome function[4,5,20]. The 19S regulatory complex (PA700), which consists of 2 multi-subunit substructures (a base and a lid), is responsible for: (1) recognition of the proteins to be degraded, (2) energy-dependent unfolding of the peptide chains, (3) recovery of Ub from Ub-protein conjugates by the action of a Ub-isopeptidase, (4) opening of the gated channels in the α-ring, and (5) transfer of the unfolded protein into the inner catalytic chamber, inducing an allosteric activation of catalytic centers of the 20S core[8,12,16,20].

Another regulatory protein that can be bound to 20S is the 11S activator (PA28)[4,6]. *In vitro,* the complex formation with PA28 is ATP-independent[20]. The PA28 + 20S + PA28 complex has been implicated in the immune response, as it can be induced by IFN-γ[12,29]. Its function is to trim large peptides generated by the 26S complex into smaller antigenic peptides for the purposes of presentation to T cells in the context of the MHC class I complex[7,16]. The 20S proteasome can also simultaneously bind to PA700 and PA28 activators (19S/20S/11S), forming the hybrid proteasome complex that has the potential to carry out, in a consecutive manner, initial proteolysis of large peptides and final trimming to the antigenic peptides[30].

The primary function of the proteasome is to degrade polyubiquitinated proteins into small peptides[4]. Included among these proteins are rate-limiting enzymes that control events as fundamental as cell-cycle regulation and division, apoptosis, gene transcription, DNA repair, oncogenesis, development, growth and atrophy of developed tissues, cellular responses to stress and to extracellular effectors, morphogenesis of neuronal networks, flux of substrates through metabolic pathways, antigen processing and modulation of cell-surface receptors, viral replication, and signal transduc-

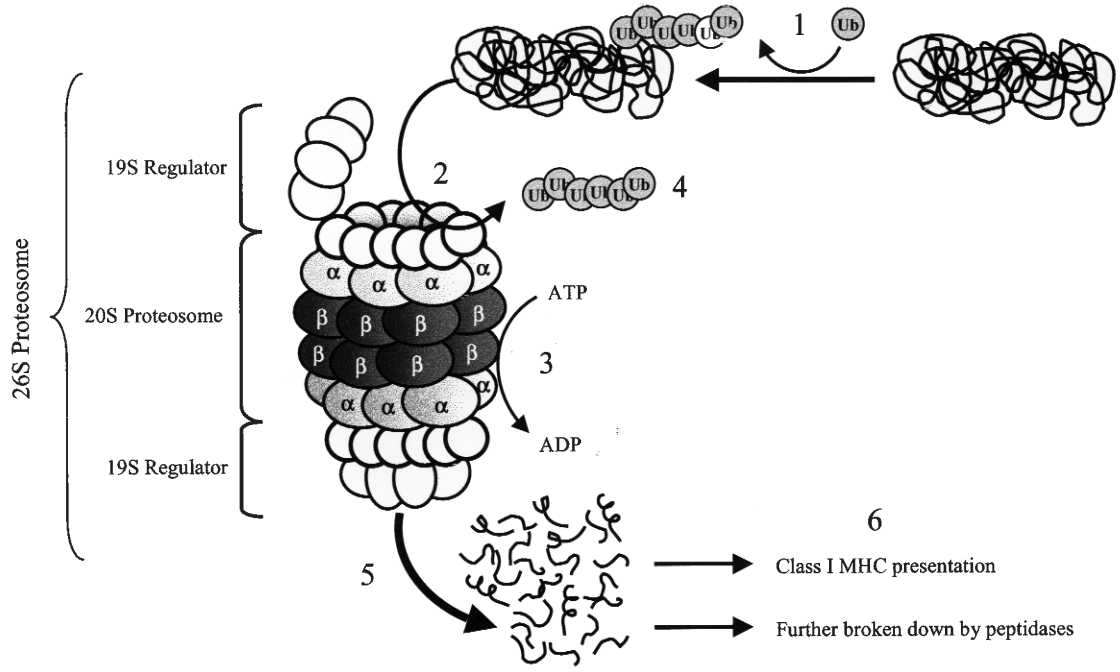

Figure 1. The proteasome complex. Degradation of a protein via the Ub-proteasome pathway involves 2 successive steps. First, multiple Ub molecules are covalently attached to the substrate (1). Second, the tagged protein is degraded by the 26S proteasome (2) in an ATP-dependent manner (3). Ub is recycled via the activity of Ub-C-terminal hydrolases (4). Following protein degradation, short peptides are generated (5) that can be either hydrolyzed by cytoplasmic exopeptidases or delivered to the cell surface for MHC class I antigen presentation (6).

tion[1,7,8,17,20]. Transcriptional-specific regulatory proteins and abnormal proteins that arise via mutation or by post-translational damage are also processed by the proteasome[5,16,31]. Proteasomes degrade these proteins to short peptides[12], which are then rapidly hydrolyzed by cytoplasmic exopeptidases. However, in higher vertebrates, some of the peptides are delivered to the cell surface for MHC class I antigen presentation[4,6,11,14,16,32].

Proteasomes form a new class of proteolytic enzymes called threonine proteases[5,11,12], in that, unlike other proteases, all the proteolytic sites in proteasomes utilize N-terminal threonines of ß-subunits as the active site nucleophiles[13,24,25]. The active site can also be targeted by pharmacophores linked to short peptides[1,5]. Although the proteasome has multiple active sites, inhibition of all of them is not required to significantly reduce protein breakdown. Inhibition of the chymotrypsin-like site or its inactivation by mutation alone causes a large reduction in the rate of protein breakdown; thus most synthetic and natural inhibitors of the proteasome act predominantly on this chymotrypsin-like activity[5,32]. The antiproteasomal agents most frequently evaluated in research and clinical trials are: the nonpeptide inhibitors lactacystin (a naturally-occurring proteasome inhibitor)[33] and PS-519, the peptide aldehydes (i.e., MG132), and the peptide boronates. Belonging to the latter group, the dipeptidyl boronic acid bortezomib (PS-341) is an extremely potent, stable, reversible, and selective inhibitor of chymotryptic threonine protease activity[1,2,5,13,34,35].

THE UBIQUITIN-PROTEASOME SYSTEM: IMPLICATIONS IN THE IMMUNE RESPONSE

The rationale of antiproteasomal therapy for autoimmune conditions is based on the fact that several steps of the immune response are regulated by the proteasome, as follows.

1. T and B cell activation

PA28 expression is upregulated during T cell activation[36]. This correlates to the augmentation of the proteasome activity seen in activated lymphocytes and might reflect the need to degrade or process regulatory proteins in a timely fashion[9]. In contrast, the inhibition of the proteasome activity (i.e., by lactacystin) represses mitogen-induced T cell proliferation[9,37]. Many of the transcription factors and signaling molecules that play positive regulatory roles in activation of T and B cells, as well as monocytes/macrophages and dendritic cells, are degraded via the proteasome pathway[26,28]. Of major importance is the regulation of nuclear factor-κB (NF-κB; p50-RelA)[5], which is activated in response to proinflammatory cytokines (interleukin 1 and TNF-α), T and B cell mitogens, bacterial lipopolysaccharide, viral proteins, double-stranded RNA, and physical and chemical stresses[28,38]. NF-κB regulates the transcription of a large number of genes, encoding stress-response enzymes, cell-adhesion molecules, proinflammatory cytokines, and anti-apoptotic proteins[39]. Genes regulated by NF-κB that are involved in immune inflammatory responses include T cell receptor-ß chain, interleukin 1 (IL-1), IL-2, IL-6, IL-8, gran-

ulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, TNF-α, IFN-ß, CD25, CD54, CD62E, CD62L, inducible nitric oxide synthase, MHC class I α-chain, and $ß_2$-microglobulin[14,26].

The regulation of NF-κB by the proteasome is complex. NF-κB consists of 2 subunits, p50 and p65[38]. The p50 subunit is generated by novel cotranslational biogenesis requiring the 26S proteasome[26]. The proteasome is also important in the activation of NF-κB[17]. Bound to an inhibitory protein (IκB), NF-κB is retained in the cytoplasm. When IκB undergoes regulated serine phosphorylation, it is ubiquitinated and then degraded by the proteasome. The released NF-κB moves to the nucleus, where it induces the transcription of numerous genes[4,16,38]. Inhibition of IκB degradation by proteasome inhibitors keeps NF-κB in the cytoplasm, thereby preventing it from acting on nuclear DNA and regulating immune-specific genes[5,38].

Besides NF-κB, other transcription factors that play a role in immune inflammatory responses are regulated via the proteasome pathway: AP-1 (Jun and Fos subunits rely on the Ub-proteasome pathway for their elimination), c-Myc, c-Myb, OBF-1, STAT3, STAT4, STAT5b, HIF-1α, Smad1 and Smad2, and IRF-1. The proteasome is also responsible for the removal of several nonreceptor kinases that are pivotal in T and B cell signaling (Lyn, Srs and Fyn, Syk and Zap-70, ERK3, Raf-1, and the p21-activated protein kinase family member γ-PAK)[26].

2. Cell-cycle control

The eukaryotic cell cycle is coordinated by the interaction of families of cyclins with cyclin-dependent kinases (CDK)[40,41]. The proteasome, by the action of Ub-protein-ligase complexes[42], carries out the selective degradation of cell-cycle regulators, such as mitotic cyclins (e.g., cyclin E), G_1 cyclins, some inhibitors of cyclin-dependent kinases (p27 and p21), and proteins whose degradation is required for the onset of anaphase[11,18]. The complexity of this process has been extensively reviewed[39-41].

Regarding mitogen-stimulated T cells, entry into the S phase depends on proteasome activity, specifically via activation of CDK2 and most likely the cyclin E-associated CDK2 during the G_1 phase[9]. Further, identification of NF-κB binding sites in the promoter region of the cyclin D1 gene has provided direct evidence of the involvement of NF-κB in cell-cycle regulation[39]. Treatment of T cells with the proteasome inhibitor lactacystin induces apoptosis in the cycling but not in the resting T cells[9]. Similarly, in replicating cells *in vitro,* bortezomib leads to an increase in intracellular concentrations of the cyclin kinase inhibitor p21[34] and causes cell-cycle arrest at the G_2–M transition, resulting in apoptosis[2,34,43].

3. Cell adhesion and migration

The Ub-proteasome pathway is required for expression of adhesion molecules [CD54, CD11a, CD62E, and vascular cell adhesion molecule-1 (VCAM-1)]. Proteasome inhibitors suppress the transcription of these adhesion molecules in immune as well as endothelial cells. In some cases they act at a posttranscriptional level, decreasing the expression or affinity of these molecules[26,44]. Similarly, T cell chemotactic activity induced by IL-16 and RANTES is proteasome-dependent. Thus, proteasome inhibitors can repress antigen presentation, costimulation, chemotaxis, homing, and cytotoxic activities of lymphocytes by suppressing cell-cell interaction and cell migration[26].

4. Apoptosis of T and B lymphocytes and monocytes

The proteasome degrades pro-apoptotic factors (Smac/Diablo, Omi/HtrA2, Bid, Bax, Nix, Id1, Id2, and Id3) in some cell types, but in others it degrades anti-apoptotic factors (Bcl-2 TC3, IAP, and XIAP)[22]. Thus, proteasome inhibition by disturbance of the ratio of anti-apoptotic to pro-apoptotic signals within a cell can be pro- or anti-apoptotic for particular cell types at a particular stage[9,45]. In mature and activated lymphocytes, however, the proteasome inhibitor lactacystin induces DNA fragmentation and apoptosis in a dose-dependent manner[37], suggesting that in these cells, the proteasome normally promotes anti-apoptotic signals.

5. Antigen presentation

The proteasome is the main machinery for the production of antigenic peptides (derived from endogenously expressed intracellular proteins) with a high affinity for the MHC class I-binding domain that can be recognized by cytotoxic T cells[11,14,28,32]. The peptides are produced by the immunoproteasome and are delivered to nascent MHC class I molecules in the lumen of the endoplasmic reticulum by specialized transporters that are associated with antigen-processing proteins[40]. Aberrations in processing of these proteins may lead to the presentation of differently cleaved self-peptides that will be recognized as non-self, potentially inducing autoimmune diseases[16]. In addition, an *in vivo* study of proteasome inhibitors found that blocking the proteasome reduces the generation of peptides for MHC class I antigen presentation[32].

POTENTIAL THERAPEUTIC USES OF PROTEASOME INHIBITORS

Dysregulation of the Ub-proteasome pathway has been implicated in the pathogenesis of inherited and acquired diseases such as Alzheimer's disease, amyotropic lateral sclerosis, multiple sclerosis, asthma, cancer, autoimmune thyroid disease, type I diabetes, ischemia-reperfusion injury, cachexia, graft rejection, hepatitis B, inflammatory bowel disease, and sepsis (reviewed in[5,7,8,11,13,14,16,28,35]). Thus, the Ub-proteasome pathway could be a reasonable target for the treatment of numerous diseases. A main concern in con-

sidering the proteasome as a therapeutic target, however, is the broad spectrum of basic cellular processes that this complex modulates, which may be nonspecifically affected by proteasome inhibitors[26,34]. Nevertheless, a proof of concept comes from clinical trials that led to the accelerated US Food and Drug Administration approval of bortezomib for the treatment of advanced multiple myeloma. Bortezomib phase I and II studies, with particular reference to safety issues, are detailed in Table 1[2,3,13,46]. Moreover, promising studies of bortezomib as a therapeutic agent in patients with solid and hematologic tumors are also evidence for the potential use of these agents[16,35,40]. Among the rheumatic conditions, the use of proteasome inhibitors may have implications in the treatment of RA and psoriatic arthritis.

ANTIPROTEASOMAL AGENTS IN RA

The constitutive activation of the NF-κB pathway is associated with inflammatory diseases such as RA. Immunohistochemical analysis of synovial samples from patients with RA detected nuclear localization of NF-κB in type A synoviocytes, macrophages, and vascular endothelium, indicative of its activation[47-50]. The involvement of NF-κB in RA is also confirmed in the NF-κB-deficient mouse model, which is refractory to the induction of both acute and chronic destructive arthritis[51]. Furthermore, antibodies that bind to the NF-κB subunit sites, normally hidden by IκB, have also been used and functionally help to support its role in synovial samples *in vivo*[52]. The role of constitutive NF-κB expression in RA pathogenesis is critical, as it leads to (1) transcription of the main proinflammatory mediators (IL-6, IL-8, TNF-α, IL-1ß) essential to the development of a Th1-type response[45,48]; (2) induction of adhesion molecules on endothelial cells [VCAM-1, E-selectin, intercellular adhesion molecule-1 (ICAM-1)] with recruitment of inflammatory cells to extravascular sites; (3) tissue remodeling and increased vascular permeability through the expression of metalloproteinases, inducible nitric oxide synthase, and cyclooxygenase-2; and (4) inhibition of TNF-α- and Fas-L-mediated apoptosis, which promote synovial hyperplasia[50,53,54].

Different approaches to "suppress" NF-κB in arthritis have been developed, including the modulation of IκB kinases by gene therapy[55], the use of IκB super-repressors[52], and the use of genetic constructs that overexpress IκB[56]. The latter made use of an adenoviral vector-encoding IκB. Expression of IκB *in trans* effectively inhibited NF-κB, resulting in suppression of TNF-α production in both RA synovial cells and macrophages[45]. It has also been shown that by inhibiting NF-κB activity, salicylates exert antiinflammatory effects[57]. Another approach to regulate NF-κB expression includes the use of NF-κB decoy oligonucleotides that bind the transcription factor, block the activation of proinflammatory cytokine genes and thus suppress the severity of joint destruction[58]. RNA interference tech-

Table 1. Clinical studies that supported FDA approval of bortezomib: safety issues.

Phase I studies (n=3)	• Analysis of 123 patients with advanced malignancies • Weekly/twice-weekly dosing schedules	Adverse reactions with doses > 1.6 mg/m^2: diarrhea, sensory neurotoxicity, hypotension, tachycardia, syncope
Phase II studies (n=2)	SUMMIT study: **S**tudy of **U**ncontrolled **M**ultiple**M**yeloma managed with proteasome **I**nhibition **T**herapy • 202 patients • Doses: 1.3 mg/m^2 IV bolus days 1, 4, 8, and 11 in a 21-day cycle. Maximum 8 cycles	Analysis of 256 patients. Adverse reactions: Frequency > 50%: fatigue, malaise, weakness, nausea, diarrhea Frequency 30-50%: anorexia, constipation, thrombocytopenia, peripheral neuropathy, pyrexia, vomiting, anemia Frequency < 30%: headaches, arthralgia, edema, neutropenia Severity grade 3 or 4 adverse events: thrombocytopenia (31%), peripheral neuropathy (12%), neutropenia (14%), anemia (8%), diarrhea, nausea, vomiting (each 7%)
	CREST study: **C**linical **R**esponse and **E**fficacy **S**tudy of bortezomib in the **T**reatment of relapsing multiple myeloma • 54 patients • Doses: 1.3 mg/m^2 vs 1 mg/m^2	

nology to modulate the expression of genes that participate in the NF-κB pathway has also been examined[59]. Finally, NF-κB modulation could also be achieved through the inhibition of proteasome-dependent degradation of IκB.

Proteasome inhibition may theoretically benefit patients with RA via modulation of 3 different mechanisms: Th1 response, apoptosis, and angiogenesis[52]. *In vitro*, PS-341 can enter mammalian cells and inhibit NF-κB activation and NF-κB-dependent gene expression. PS-341 also inhibits TNF-α-induced expression of the cell-surface adhesion molecules E-selectin, ICAM-1, and VCAM-1 on primary human umbilical vein endothelial cells. This inhibition is at the level of gene expression and the concentration of PS-341 that completely suppresses adhesion molecule expression is ~10-fold lower than that needed to inhibit NF-κB DNA binding[44,60]. In a rat model of streptococcal cell wall-induced polyarthritis, which is clinically and histologically similar to RA[61], PS-341 attenuated the neutrophil-predominant acute phase and markedly inhibited the progression of the T cell-dependent chronic phase of the inflammatory response[60]. As a consequence, there was a marked reduction in the subchondral bone erosions. In relation to apoptosis, *in vivo,* the proteasome inhibitor MG132 increases the frequency of apoptosis in the synovium of rats with streptococcal cell wall-induced arthritis[53].

Angiogenesis is a fundamental factor of disease progression in RA. In joint synovial studies from RA patients, exuberant angiogenesis is present and precedes all other pathological features of the disease[62]. Lactacystin inhibited angiogenesis in a dose-dependent manner in an *in vivo* model of neovascularization (the developing chick embryo chorioallantoic membrane), causing an avascular zone through (1) inhibition of plasminogen activator secretion and (2) antiproliferative activity on endothelial cells[63].

ANTIPROTEASOMAL AGENTS IN PSORIASIS AND PSORIATIC ARTHRITIS (PsA)

Psoriasis is a T cell-mediated autoimmune disease[64] (the most prevalent T cell-mediated inflammatory disease in humans)[65] in which there is increasing evidence for the pathogenic role of bacterial superantigens in genetically predisposed patients[14,65]. The clinical heterogeneity of psoriasis and the apparent multigenic pattern of inheritance suggest that a combination of variables are involved in psoriasis development[64].

In psoriasis, activated T cells (CD4+ and CD8+) infiltrate the skin. CD8+ T cells predominate in the epidermis and are responsible for the persistence of the psoriatic lesions, but CD4+ T cells, which predominate in the dermis, may help initiate the skin lesions[65]. Activated T cells also induce the expression of their skin-homing receptor [cutaneous lymphocyte-associated antigen (CLA)] and produce other mediators, predominantly inflammatory cytokines (IFN-γ and TNF-α). After binding to their receptors, these cytokines activate several cellular signaling pathways, including the NF-κB pathway[66]. NF-κB-mediated inflammation in skin appears to be a final common pathway for the translation of environmental insults into inflammation, and is a crucial element of innate immunity. Among the many genes regulated by NF-κB in skin cells, those that are central to the initiation of cutaneous inflammation include the genes that encode for E-selectin, chemokines and cytokines, defensins (antibacterial peptides), ICAM-1, and VCAM-1. As in RA, angiogenesis and NF-κB activation are central to the pathogenesis of PsA[49,67].

The proteasome is preferentially involved in the initiation and perpetuation of autoimmune cytotoxic T cell response, thus proteasomes theoretically could be a target for psoriasis therapy. For example, the immunosuppressive drug cyclosporin A, which acts as a noncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome *in vitro*[68], has been used as therapy for PsA. Thus the proposed mechanisms of antiproteasome therapy that could be of benefit for the treatment of psoriasis include the following:

1. Interference with the superantigen-mediated activation of T cells, namely with the expression of CLA[13]. Since the CLA gene is under the control of NF-κB, its expression would be expected to be reduced in the presence of proteasome inhibitors[66].
2. Modulation of cutaneous inflammation and epidermal hyperproliferation through the suppression of NF-κB activation, thereby decreasing the transcription of genes encoding proinflammatory proteins[53].
3. The anti-angiogenic effect[63].

The selective proteasome inhibitor PS-519 can significantly inhibit numerous parameters in the process of superantigen-mediated T cell activation[14,69]. *In vitro,* PS-519 has a profound inhibitory effect on the formation of NF-κB DNA complexes in activated T cells. Also, it significantly reduces the superantigen-mediated T cell proliferation (in a dose-dependent manner) and blocks the expression of T cell activation markers and CLA. As a result, there is a profound reduction in the ability of T cells to bind to the endothelial cell adhesion molecule E-selectin[13,69].

In vivo studies in a SCID-hu model for psoriasis (xenogeneic transplantation model) showed that the effects of PS-519 were equivalent to those obtained with dexamethasone without adverse effects (infection or wasting)[13,69]. The antiproteasomal therapy was characterized by:

1. Reduced superantigen-mediated T cell activation *in vitro* and *in vivo* within the psoriatic lesion. The expression of very early (CD69), early (CD25), and late (HLA-DR) T cell activation molecules was also reduced.
2. The inhibition of T cell proliferation and T cell expression of adhesion molecules (i.e., the expression of E-selectin ligands relevant for T cell homing to the skin).
3. Inhibition of neutrophils, macrophages, and keratinocytes within the psoriatic lesion[14].

A study of bortezomib (Velcade, formerly PS-341) in acute graft-versus-host disease (another CLA-positive T cell-mediated inflammatory disease)[70] confirmed *in vitro* a potent dose-dependent inhibitory effect of this agent on mixed lymphocyte responses and a selective induction of apoptosis in proliferating alloreactive T cells. Similarly, early administration of bortezomib is capable of preventing the occurrence of lethal graft-versus-host disease after allogenic bone marrow transplant in a murine model. This effect is associated with an initial reduction of donor T cell engraftment, increased alloreactive T cell apoptosis, and reductions in systemic TNF-α concentrations[70].

PROSPECTUS

The vast expansion of Ub-proteasome research over the past decade, along with the award of the 2004 Nobel Prize in Chemistry to A. Ciechanover, A. Hershko, and E. Rose, pioneers in the study of this system, is a sight to behold[17]. The recent approval of bortezomib for patients with advanced myeloma is likely to accelerate the focus in developmental therapeutics that target the Ub-proteasome system and NF-κB modulation[39]. The current knowledge of the Ub-proteasomal system, its relation to the pathogenesis of rheumatic diseases, and the evidence derived from clinical trials of the safety and efficacy of proteasome inhibitors in other diseases all point toward the possibility that these agents or future ones can target more specific elements of the system to become an option for the treatment of rheumatic conditions. Continuing studies should provide more evidence on the risk/benefits ratio of the regulation of protein catabolism in the treatment of rheumatic diseases.

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