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PROTEASOME INHIBITION IN THE REGULATION OF NATURAL KILLER CELL FUNCTION AND MULTIPLE MYELOMA CELL APOPTOSIS

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To my family!

致我的家人!

ABSTRACT

Multiple myeloma (MM) is a hematologic malignancy mostly occurring in the elderly and characterized by an expansion of monoclonal plasma cells in the bone marrow and increased monoclonal immunoglobulin in plasma. The outcome of this disease has been greatly improved due to introduction of new drugs. The proteasome inhibitor bortezomib (velcade®) is one of the therapeutic drugs showing very pronounced efficacy in the treatment of MM. However, cytotoxic effects of bortezomib on immune-competent cells have also been observed. In the current thesis, we focus on studying regulatory effects of proteasome inhibition on natural killer (NK) cells and MM cell. We found that bortezomb induces apoptosis of NK cells at a clinically relevant dose, and this is mainly due to induction of reactive oxygen species (ROS). Additionally, bortezomib also decreased NK cell activating receptor NKp46 expression, resulting in impaired NKp46-mediated redirected killing activity. Bay 11-7082, a pharmacological inhibitor of NF-κB activation, also reduced NKp46 expression and suppressed redirected cytotoxicity, suggesting NF-κB was involved in the regulation of NKp46 expression. To further study the effects of bortezomib on NK cells, we used human interleukin (IL)-2 activated NK cells. Down regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression at both protein and mRNA level in IL-2 activated NK cells was observed after bortezomib and Bay 11-7082 treatment, suggesting that the proteasome is involved in the regulation of TRAIL expression through modulation of NF-κB activity. Moreover, perforin-independent killing activity of MM cell line RPMI8226 and U266 was also reduced after bortezomib treatment, and blocking cell surface-bound TRAIL impaired NK cell-mediated lysis of the TRAIL-sensitive MM cell line, RPMI8226 cells. Next we studied a novel inhibitor of proteasome deubiquitination, b-AP15. We noted accumulation of polyubiquitinated proteins in RPMI8226 and U266 cells after b-AP15 treatment. Moreover, pro-apoptotic effects of b-AP15 on MM cells were also seen by detecting phosphatidylserine (PS) exposure, processing of pro-caspase-3, and cleavage of poly (ADP-ribose) polymerase (PARP); apoptosis was shown to be caspase-dependent. Additionally, b-AP15 also induced apoptosis in NK cells. However, the pro-apoptotic effect of b-AP15 on NK cells was not as pronounced as the effect of bortezomib, highlighting that b-AP15 may have less adverse effects on the immunosurveillance of NK cells against tumors. Furthermore, we evaluated a multifunctional protein HS-1 associated protein X-1 (HAX-1), which is overexpressed in MM, and its potential role in the regulation of MM. Our data indicated that silencing of HAX-1 expression in the human U266 and RPMI8226 cells can not sensitize cells to bortezomib or b-AP15, nor to NK cell-mediated killing. However, the ability of U266 cells to migration was reduced after HAX-1 knockdown, indicating that HAX-1 could play a role in regulating tumor metastasis. In summary, our studies contributed a better understanding of proteasome inhibitors as anti-cancer drugs and have provided insights into possible adverse effects on immune-competent cells. Our studies also identified HAX-1 as a possible target in MM treatment.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Wang X., Ottosson Wadlund A., Ji C., **Feng X.,** Nordenskjöld M., Henter JI., Fadeel B., Zheng C. Proteasome inhibition induces apoptosis in primary human natural killer cells and suppresses NKp46-mediated cytotoxicity. Haematologica, 2009; 94(4):470-8.

II. **Feng X.,** Yan J., Wang Y., Zierath JR., Nordenskjöld M., Henter JI., Fadeel B., Zheng C. The proteasome inhibitor bortezomib disrupts tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and natural killer cell (NK)-mediated killing of TRAIL receptor-positive multiple myeloma cells. Mol Immunol, 2010 Aug; 47(14): 2388-96.

III. **Feng X.,** Zheng C., Fadeel B. Pro-apoptotic effects of the novel proteasome inhibitor b-AP15 on multiple myeloma cells and natural killer cells. (*submitted*)

IV. **Feng X.,** Ishikawa T., Patarroyo M., Zheng C., Fadeel B. HAX-1 regulates migration of multiple myeloma cells. (*manuscript*)

CONTENTS

LIST OF ABBREVIATIONS

^{51}Cr	Chromium 51
AIF	Apoptosis inducing factor
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
Bcl-2	B lymphoma/leukemia-2
BSA	Bovine serum albumin
CLL	Chronic lymphocytic leukemia
CP	Core particle
Cyt C	Cytochrome C
DCs	Dendritic cells
DD	Death receptor domain
DISC	Death-inducing signaling complex
DUBs	Deubiquitinases
Endo G	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGFR3	Fibroblast growth factor 3
FHL	Familial haemophagocytic lymphohistocytosis
HAX-1	HS-1 associated protein X-1
HDAC	Histone deacetylase
HS-1	Hematopoietic cell-specific Lyn substrate-1
Hsp90	Heat shock protein-90
IAP	Inhibitor of apoptosis protein
IFN-γ	Interferon gamma
IgH	Immunoglobulin heavy chain
IL-2	Interleukin-2
ILK	Integrin-mediated kinase
IMIDs	Immunomodulatory drugs
IST	*In silico* transcriptomics
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
IκB	Inhibitor of nuclear transcriptional factor kappa B
KIR	Killer cell Ig-like receptors
LAK	Lymphokine-activated killer
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMP	Mitochondria membrane potential
MPR	Mannose 6-phosphate receptor
NCRs	Natural cytotoxicity receptors
NF-κB	Nuclear transcriptional factor kappa B
NK	Natural killer

OPG	Osteoprotegerin
P38MAPK	P38 mitogen-activated protein kinase
PARP	Poly (ADP-ribose) polymerase
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PI	Propidium iodide
PS	Phosphatidylserine
RBCs	Autologous red blood cells
rNK	Resting NK cell
ROS	Reactive oxygen species
RP	Regulatory particle
RP	Regulatory particles
SCID	Severe combined immunodeficiency
SCN	Severe congenital neutropenia
SDF-1α	Stromal cell-derived factor 1α
siRNA	Small interfering RNA
Smac	Second mitochondrial activator of caspases
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

1 INTRODUCTION

1.1 MULTIPLE MYELOMA

1.1.1 Clinical presentation

Multiple myeloma (MM) is a hematologic malignancy mostly occurring in the elderly and characterized by an expansion of monoclonal plasma cells in the bone marrow and increased monoclonal immunoglobulin in plasma (Suzuki 2013). It has been reported to cause~12,000 death per year in the United States, which account for~2% of all cancer death and~20% death caused by hematological malignances (Malpas, *et al* 1998). The major clinical presentations of the disease include hypercalcemia, renal failure, anemia and lytic bone destruction (Dolloff and Talamo 2013). Recent studies also indicated that MM is a precursor disease proceeded by the states of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma, which give chance to an early diagnose and prevention of development of MM (Landgren and Waxman 2010). Immunoglubin heavy locus (IgH) is a region contains a gene for the heavy chains of immunoglubin (Cook and Tomlinson 1995). Evidence has shown that MM is a genetic event. Incidence of IgH translocation presents in most MM tumors, and a variety of oncogenes were simultaneously dysregulated including: genes for cyclin D1 and cyclin D3, fibroblast growth factor 3 (FGFR3) combined with the nuclear protein MMSET and the transcription factor c-MAF (Bergsagel and Kuehl 2001, Kuehl and Bergsagel 2002). Chromosomal abnormalities can be found in 90% of MM patients, which further support the vital role of genetic changes in the pathogenesis of MM. (Fonseca, *et al* 2009)

1.1.2 THERAPEUTIC APPROACHES

Clinic outcomes of MM have been greatly improved during the past decade due to introduction of novel therapies. However, MM still remains incurable and most of the patients undergo relapse due to the presence of residual myeloma cells. (Agarwal and Mahadevan 2012, Kumar, *et al* 2008).

Melphalan combined with prednisolone were the first group of therapeutic drugs introduced to the clinic in the 1969s for treatment of MM patients (Alexanian, *et al* 1969). New therapeutic drugs including thalidomide, lenalidomide and bortezomib came to the clinic lately and also showed pronounced improvement for myeloma patients (Kumar, *et al* 2008). Moreover, autologous stem cell transplantation together with one or more novel anti-myeloma agents remains to be standard therapeutic strategy for younger fit MM patients (Al-Farsi 2013). All standard treatment guidelines for MM can be found in The Comprehensive Cancer network (NCCN) guideline (2013).

With the remarkable progression of this disease, there are also some novel agents which are under clinical phase I or II trials, or basic research has found its anti-MM activity. Thalidomide and lenalidomide which belongs to immunomodulatory drugs (IMIDs),

were recently shown to have pronounced effects to human MM cells that currently resistant to the conventional therapy (Hideshima, *et al* 2000). Inhibitors of heat shock protein-90 (hsp90) were found to have anti-MM property by disrupting bone marrow stromal cell-mediated protection to MM cells, and sensitize MM cells to other cytotoxic chemotherapeutic drugs (Mitsiades, *et al* 2006). P38 mitogen-activated protein kinase (p38MAPK) is a downstream target to hsp27, and was reported to enhance the sensitivity to bortezomib-resistant MM cell lines, as well as primary patient MM cells (Hideshima, *et al* 2004). Following the success of bortezomib, more attention has been taken to inhibition of proteolytic pathway, and among them inhibitors for histone deacetylase (HDACs) especially HDAC6 inhibitor was selected as a good candidate (McConkey, *et al* 2012). Moreover, HDAC6 inhibitor alone or in combination with proteasome inhibitor bortezomib triggered apoptosis of MM cells via endoplasmic reticulum (ER) stress and activation of critical caspases, and a further in vivo study in severe combined immunodeficiency (SCID) mice also demonstrated its potential property of inhibition of tumor growth and prolonged life span of these animals (Santo, *et al* 2012). Increased evidence has shown that bone marrow microenvironment plays a crucial role in the pathogenesis of MM (Hideshima, *et al* 2007). MM growth depends on CXCR4 which is expressed on MM cells and stromal cell-derived factor 1α (SDF-1α) which is presented in the bone marrow environment (Aggarwal, *et al* 2006). Thus, therapeutic targets for inhibition of interaction between CXCR4 and SDF-1α and its related signaling pathways were interesting for researchers to evaluate their potential anti-MM function (Alsayed, *et al* 2007, Azab, *et al* 2009). Given these limited observations, other molecular mechanisms such as the role of hypoxia, molecular antibodies against MM survival and other inhibitors which interfere critical pathways for MM survival, could also serve as targets for MM treatment.(Colla, *et al* 2010, Peterson, *et al* 2009, Richardson, *et al* 2011). With the supports of basic science, researchers aim to design more effective and less toxic therapeutic agents for MM patients.

1.1.3 IMMUNITY AGAINST MULTIPLE MYELOMA

The human immune system consists of the innate immune system and adaptive immune system. The innate immune system serves as the first line of immediate defending invaded microbes and pathogens; however, the adaptive immune system composed of B and T cells, serves as the second line by establishing immunologic memory (Schenten and Medzhitov 2011). Moreover, the two components of the immune system are critical players in controlling tumor progression, which was known as immunosurveillance (Dranoff 2004). Transformed cells or tumor cells are first recognized by natural killer (NK) cells, then phagocytes including macrophages and dendritic cells (DCs) uptake the tumor fragments lysed by NK cells, in the end activation of T and B cells lead to a more powerful elimination of tumor cells (Finn 2012). Certainly, a functional immune system is required during cancer treatment (Paget, *et al* 2012).

Multiple myeloma is not an exception, and to understand the function of immune system in MM turns out to be very important. Pessoa de Magalhães et al demonstrated

the fundamental role of immune system in defending tumors. They found that MM patients with long-term disease control had an increased number of cytotoxic CD8(+) T cells and NK cells, bone marrow DCs, as well as tissue microphages (Pessoa de Magalhães, *et al* 2013). Among these immune component cells, the role of NK cells in immunity against MM attracted particular interest due to its powerful anti-tumor activity (Godfrey and Benson 2012). Subsequently, NK cell related immune therapy was introduced into the clinic. Studies have shown that IFN-α together with IL-2 enhanced NK cytotoxicity against MM (Shimazaki, *et al* 1988). Moreover, enhanced NK cell-mediated killing activity was also seen when using other novel anti-myeloma therapies, such as, immunomodulatory agent thalidomide, which could stimulate NK cell function by increasing IL-2 production, while the proteasome inhibitor bortezomib improved NK cell-mediated killing activity via decreasing the major histocompatibility complex (MHC) class I expression of MM cells (Davies, *et al* 2001, Shi, *et al* 2008). NKG2D ligand is expressed in most of multiple myeloma cells, thus, research has targeted NKG2D-expressing T cells as a immunotherapy for MM via NKG2D-mediated killing activity (Barber, *et al* 2008). We believe that understanding effects on the immune system versus MM will lead to the development of improved therapies for this disease.

1.2 APOPTOSIS PATHWAYS

1.2.1 Apoptosis resistance in cancer

The word apoptosis (also known as programmed cell death) derives from a Greek word "falling off", as leaves from the tree. Apoptosis plays a critical role in human tissue development as well as maintenance of homeostasis. Cancer is a genetic disease (Vogelstein and Kinzler 2004), and the development of tumor is thought to be a series of alterations in cell physiology including: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, apoptosis resistance, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis; moreover, another two potential markers: reprogramming of energy metabolism and evading immune destruction were added to this list recently (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Among the eight hallmarks of cancer, we will focus on apoptosis resistance and migration/metastasis in the current thesis.

The p53 gene is consistently believed to play an critical role during cancer development, as half of all human cancers harbor mutations in this tumor-suppressor gene (Lane 1992). The importance of this gene is also due to its regulatory function of other genes such as pro-apoptotic gene BAX (Miyashita and Reed 1995), and BH3-only proteins which triggers the activation of BAX, resulting in the intrinsic apoptosis (which will be discussed below) (Chipuk, *et al* 2004). Of note, a recent study indicated that deletion of p53 gene in patients with extramedullary multiple myeloma result in worse clinic outcome of this disease (Billecke, *et al* 2012). Another candidate gene is survivin, a member of inhibitor of apoptosis protein (IAP), represents one of the most cancer-specific genes in the entire human genome (Reed 2002). The role of survivin was also analyzed in many cancer types. Sarela et al described that poor overall survival of

patients with colon cancer is associated with an increased survivin mRNA expression (Sarela, *et al* 2000). Similar result was also found in patients with multiple myeloma, researchers provided evidence that survivin directly contributed to malignant progression of this disease (Romagnoli, *et al* 2009). Overexpression of anti-apoptotic protein B lymphoma/leukemia-2 (Bcl-2), is common in a variety of hematopoietic malignancies including MM, chronic lymphocytic leukaemia (CLL), acute lymphocytic leukaemia (ALL) and acute myeloid leukaemia (AML). Deregulation of different members of the BCL-2 family was also studied in several cancers, in particular in hematopoietic malignancies (Kitada, *et al* 2002). Taken together, understanding apoptosis resistance in cancer will provide a basis for better anti-cancer treatment.

1.2.2 Extrinsic apoptosis signaling

Apoptosis can be triggered mainly through two pathways, the extrinsic pathway also known as death receptor-mediated pathway and the intrinsic pathway also known as mitochondria-mediated pathway (Fadeel and Orrenius 2005). The extrinsic pathway is initiated by a series of sensors known as death receptors and of them six receptors are described including: TNF receptor 1, Fas, DR3, TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1), TRAIL-R2 and DR6 (Suliman, *et al* 2001). Death receptors bind to their specific ligands (FasR/FasL, TNFR1/TNFα, DR-4/TRAIL, DR5/TRAIL, DR3/Apo-3L/TWEAK). Once activated, the intracellular death receptors domain (DD) bind to adaptor proteins, such as Fas-associated death domain (FADD) to form the death-inducing signaling complex (DISC) with accumulation of pro-caspase 8 (Burz, *et al* 2009). The subsequent pathways are different in different types of cell, one is so called type I cell and another is type II cell. In type I cell, high level of DISC and activation of caspase 8 are sufficient to stimulate downstream effector caspases such as caspase-3 and -7, to initiate apoptosis process. In contrast, type II cells need further amplification via mitochondria in order to cause inevitable apoptosis (Fadeel and Orrenius 2005).

1.2.3 Intrinsic apoptosis signaling

The intrinsic pathway is initiated by toxic stimuli inside the cells, such as DNA damage, oxidative stress, hypoxia and cytotoxic compounds (Danial and Korsmeyer 2004). Mitochondria and associated proteins are major players in this process. There is group of pro-apoptotic proteins located in the mitochondrial intermembrane space which contribute to cell death induction, such as apoptosis inducing factor (AIF), cytochrome c (cyt C), second mitochondrial activator of caspases (Smac/DIABLO), Omi/HtrA2, and Endonuclease G (Endo G). Any change of the inner mitochondria membrane potential (MMP) will cause release of these pro-apoptotic proteins from mitochondria to cytosol. Subsequently, cyt C stimulates apoptosome formation (a complex consist of apoptotic protease-activating factor [Apaf-1], dATP, cyt C and caspase 9) and caspase-9 activation. The "initiator" caspase-9 in turn actives downstream caspase-3 and -7, finally resulting in cell death (Burz, *et al* 2009, Fadeel, *et al* 2008). Smac/DIABLO and Omi/HtrA2 can bind members of the inhibitor of apoptosis protein family (IAP), which

negatively control the process of caspase activation, thus amplifying the process (Cory and Adams 2002).

1.2.4 HS-1-associated protein X-1 (HAX-1)

HS-1 associated protein X-1 (HAX-1) was originally identified as a 35 KD protein that interacts with HS-1 (hematopoietic cell-specific lyn substrate-1), a substrate of Src family tyrosine kinases (Suzuki, *et al* 1997). Moreover, HAX-1 was suggested to have anti-apoptotic function due to its homology with the well-known anti-apoptotic protein Bcl-2 (Suzuki, *et al* 1997). Severe congenital neutropenia (SCN) or Kostmann disease was first described by Rolf Kostmann, a Swedish pediatrician, in his doctoral thesis published in 1965. These patients were characterized by a maturation arrest in the bone marrow and a lack of mature neutrophils in the peripheral blood (Carlsson, *et al* 2008). Subsequently, homozygous mutation of *HAX1* gene was found in patients with kostmann disease, including three patients belonging to the original Swedish kindred described by doctor Kostmann, and HAX-1 was shown to play a role in protecting myeloid stem cells in the bone marrow from apoptosis (Klein, *et al* 2007). Moreover, HAX-1 tissue expression is ubiquitous (Carlsson, *et al* 2008, Suzuki, *et al* 1997) and several different splice variants have been identified (Lees, *et al* 2008).

The localization of HAX-1 may vary in different cell types, and it has been reported to localize in mitochondria, in the cytosol, as well as on the endoplasmic reticulum (Fadeel and Grzybowska 2009). Furthermore, one recent publication indicated that HAX-1 is a nucleocytoplasmatic shutting protein with a possible role of mRNA processing (Grzybowska, *et al* 2013). With its varied localization, HAX-1 has been shown to interact with a number of cellular and viral proteins as well as mRNA, and its involvement in multiple signaling pathway and cellular process has also been discussed and addressed (Fadeel and Grzybowska 2009). Among them, numerous studies have concerned its role in apoptosis or interaction with apoptosis-regulatory proteins. Recent in vivo study supported this notion by showing that, mice that having homozygous deletion of *HAX1* gene fail to eat and drink due to excessive apoptosis of their neurons in the striatum and cerebellum (Chao, *et al* 2008). Another study in *HAX1*-deficient mice also showed that these mice died around that age of 12 week (premature age) accompanied with a severe reduction of lymphocytes in spleen, thymus and bone marrow, further reflecting the role of HAX-1 in protecting certain cell types against apoptosis (Peckl-Schmid, *et al* 2010). Han et al reported that HAX-1 associated with caspase-9. Furthermore, overexpression of HAX-1 in cardiac myocytes protected them from apoptosis (Han, *et al* 2006). In addition, previous study from our laboratory found that HAX-1 was highly expressed in certain hematopoietic malignances, in particular in B lymphoma; they also noted an apparent negative correlation between *HAX-1* and *BCL-2* expression in follicular lymphoma, which might provide some hints to studies its anti-apoptotic role in cancer, especially in hematopoietic malignancies (Kwiecinska, *et al* 2011).

In addition to its role in apoptosis discussed above, there is growing evidence showing that HAX-1 is involved in cell migration. HAX-1 has been shown to interact with

cortactin, a F-actin-associated protein, further silencing HAX-1 expression on a mouse embryonic fibroblast cell line NIH3T3 cells impaired its migration ability (Radhika, *et al* 2004). HAX-1 binds directly to β_6 and regulates clathrin-mediated endocytosis of $\alpha_v\beta_6$ integrin in oral cancer, thereby regulating migration and invasion ability of oral cancer cells (Ramsay, *et al* 2007). Recent studies have shown that HAX-1 regulates integrin-mediated adhesion and motility of neutrophil-like cells (Cavnar, *et al* 2011). Taken together, these data suggest that HAX-1 might be important not only for cell survival/apoptosis but also for cell migration and, hence, for metastatic tumor progression, thus making HAX-1 a potentially important target for therapeutic intervention.

1.3 PROTEASOME INHIBITION

1.3.1 The 26S proteasome

Continuous intracellular protein turnover is critical for the maintenance of cellular homeostasis, and the two major protein degradation systems within cells are: the lysosome system and the ubiquitin-proteasome system (Martinez-Vicente, *et al* 2005). The proteasome, often referred to the 26S proteasome, is a large multicatalytic enzyme complex localized in the cytosol and nucleus of all eukaryotic cells, and responsible for degradation of ubiquinated proteins (Adams 2004). For a protein that further to be recognized by the proteasome, a small peptide called ubiquitin must be first attached as "flags" to the protein, and this process is carried out by a cascade of enzymes (known as E1, E2 and E3) that active free ubiquitin and attach it to the target protein (Pickart 2001, Wilkinson 1999). The 26S proteasome can be further divided into two sub-complexes, a central 20S core particle (CP) associated with one or two 19S regulatory particle (RP) (Zwickl, *et al* 1999) (Figure 1).

The 20S proteasome is the degradation unit and composed of four stacks of heptametrical subunits arranged around a central cavity. It has a barrel-like structure, with two outer rings containing seven different α-subunits that cap two inner rings containing seven different β-subunits (Groll, *et al* 1997). The proteolytic activity is located in the center chamber, and has been named as caspase-like, trypsin-like and chymotrypsin-like sits due to their substrate specificity and activity (Groll, *et al* 2001). Proteins are then degraded by the core particles, and afterword to generate peptides of 3-25 amino acids length (Nussbaum, *et al* 1998). This process that is carried out by proteasome for degrading ubiquitinated proteins is specific and also highly regulated by 19S RP, in order to prevent the erroneous degradation (D'Arcy and Linder 2012). The 19S RP (often referred to as PA700 in mammalian cells) is composed of a lid sub-complex and a base sub-complex. The base subunits of the 19S RP function as the guard of the 20S particle, to unwind the proteins and translocate them into the 20s particle, whereas the lid subunits are required for ubiquitin-dependent degradation (Glickman, *et al* 1998, Lander, *et al* 2012, Rabl, *et al* 2008). Thus, the 19S RP plays an essential role of controlling the degradation of ubiquitin-tagged proteins.

However, protein ubiquitination is a reversible process, in which three human deubiquitinases (DUBs) that are associated with 19s RP are also involved in this process (Fraile, *et al* 2012, Nath and Shadan 2009). Among them, UCHL5/Uch37 and USP14/Ubp16 are two DUBs belonging to cysteine protease, and the third RPN11/POH1 is a Zn^{2+}-dependent protease (Wing 2003). The function of USP14 and UCHL5 is to control and make sure that substrates which contain short or non-degradable ubiquitin chains are released from the proteasome, in order to avoid the accumulation of polyubiquitins (Lam, *et al* 1997). Therefore, functional USP14 and UCHL5 are also essential for proteasome-mediated degradation of proteins, while the lack of DUS activity may impair proteasome activity (Hanna, *et al* 2006).

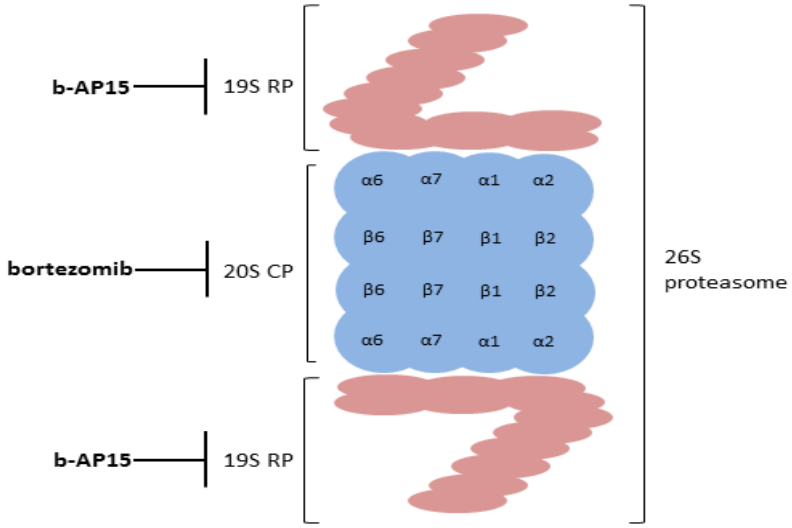

Figure 1. Schematic figure of 26S proteasome and inhibitory actions of proteasome inhibitor bortezomib and b-AP15.

1.3.2 Proteasome as a target for cancer therapy

The idea of targeting proteasome as a cancer therapy is apparent, since the 26S proteasome is involved in numerous signaling pathways including: cell differentiation, proliferation, cell cycling, apoptosis, gene transcription, thus any drug interfering with proteasome function will have pleiotropic effect (D'Arcy and Linder 2012, McCloskey, *et al* 2008). Several intracellular proteins such as p53 and IκB, the inhibitor of nuclear transcriptional factor kappa B (NF-κB), that govern cell growth and survival are regulated by proteasome-mediated pathway (Adams 2004). NF-κB is inactivated in the cytoplasm under normal conditions and will become active once its binding partner, IκB, is degraded by proteasome. However, constitutive NF-κB activity has been observed in a variety of tumors including MM, and this sustained activity of NF-κB

may lead to aberrant expression of target genes that promoting tumor cell proliferation and survival (Gilmore 2007). In addition, major tumor suppressor gene p53 as well as cyclin-dependent kinase inhibitors p21 and p27 can also be stabilized by inhibiting proteasome activity, resulting in p53-dependent apoptosis and growth arrest.(LeBlanc, *et al* 2002, Williams and McConkey 2003). More evidence was shown that the level of several pro-apoptotic proteins such as Bid, Bax were increased after proteasome inhibition, thus lead to augmentation of apoptotic signaling (Zhu, *et al* 2005). Other potential effects related to proteasome inhibition were also testified in numerous studies. ER stress, after proteasome inhibition was first revealed due to the intimate association between the ER and proteasome (Fribley, *et al* 2004). Moreover, rapid production of reactive oxygen species (ROS) was also seen after blocking proteasome degradation pathway (Ling, *et al* 2003). Taken the evidence above, the proteasome emerges as an obvious target for cancer therapy.

The proteasome inhibitor bortezomib (PS-341, NSC 681239, velcade®), a modified dipeptide boronic acid, is a selective 20S proteasome inhibitor (Figure 1). It was approved by the US Food and Drug Administration (FDA) for treatment of refractory MM in 2003, and in 2005 it was introduced to relapse MM due to its prominent anti-cancer function (Richardson and Anderson 2003, Richardson, *et al* 2003). Bortezmib triggers apoptosis of MM by blocking NF-κB pathway, and also an interfering production of IL-6 which is critical for MM growth (Hideshima, *et al* 2005). Bortezomib also induces the process of caspase activity and followed by PARP cleavage (Hideshima, *et al* 2003a). An *in vivo* study also indicated that bortezomib impairs human MM cells growth and angiogenesis associated with the tumor (LeBlanc, *et al* 2002). In addition to its direct cytotoxic effects, bortezomib sensitizes MM cells to DNA-damaging therapeutic drugs, and also inhibit DNA repair (Hideshima, *et al* 2003b, Mitsiades, *et al* 2003). Stromal cells in the bone marrow have been thought to contribute to drug resistance, and bortezomib was shown to interfere with MM and stromal cell interaction by decreasing adhesion molecules of MM cells and also cytokine secretion by stromal cells (Hideshima, *et al* 2001). Another interesting study showed that bortezomib could sensitize tumor cells to NK cell-mediated lysis through TRAIL and/or Fas/Fas ligand pathway (Lundqvist, *et al* 2006).

Besides the success for treatment of MM, bortezomib also became an emerging agent for mantle cell lymphoma (Camara-Clayette, *et al* 2012). In addition, its therapeutic efficacy has been shown in some solid tumors including non-small lung (Aghajanian, *et al* 2002) and androgen-independent prostate carcinoma (Papandreou, *et al* 2004), even though the anti-tumor effects of proteasome inhibitor on solid tumors are less pronounced than MM and mantle cell lymphoma.

Based on the remarkable success of bortezomib, the next generation of proteasome inhibitor has also entered clinical trials, including carfilzomib, which has activity against bortezomib-resistant tumor cells (Kuhn, *et al* 2007), and ONX-0912 which can be administrated orally instead of intravenously (Chauhan, *et al* 2010). b-AP15 (3,5-bis[(4-nitrophenyl)methylidene]-1-prop-2-enoylpiperidin-4-one) was initially identified

as a small molecule that induces p53-independent apoptosis (Erdal, *et al* 2005) with a gene expression pattern characteristic of several known proteasome inhibitors (D'Arcy, *et al* 2011). Further studies showed that b-AP15 blocks the deubiquitinase (DUB) activity of the 19S RP without inhibiting the proteolytic activities of the 20S CP (for an excellent review, see D'Arcy and Linder, 2012) (Figure 1). Interestingly, b-AP15 is capable of induction of apoptosis irrespective of p53 status and overexpression of the anti-apoptotic protein Bcl-2 (D'Arcy, *et al* 2011). Elevated expression of Bcl-2 and lack of p53 contribute to resistance against bortezomib (Ling, *et al* 2010, Paoluzzi, *et al* 2008). DUB inhibitors may thus offer novel therapeutic approaches. Moreover, novel techniques for producing natural proteasome inhibitor, which could serve better therapeutic effects but less side effects are being explored (Stein and Groll 2013).

1.3.3 Adverse effects of proteasome inhibition

As one coin has two sides, the adverse effects of proteasome inhibitor were also pointed out along with its success. The most common side effect of bortezomib reported by hematologists, during clinical treatment, includes gastrointestinal symptoms, fatigue, thrombocytopenia, sensory neuropathy, vomiting, and anoxia (Jagannath, *et al* 2004, Richardson, *et al* 2003, Richardson, *et al* 2005), and among them peripheral neuropathy is the most severe toxicity of bortezomib therapy for advanced MM patients (Cavo 2007). Moreover, basic research also indicated its cytotoxic effects on immune system. Recent studies have demonstrated that bortezomib induces apoptosis in immune-competent cells, such as T cells and DCs, and also adversely affects the biological function of these cells (Berges, *et al* 2008, Blanco, *et al* 2006, Straube, *et al* 2007). One *in vivo* study also indicated that bortezomib affected priming process of T cells, resulting in increased susceptibility of mice to virus infection (Basler, *et al* 2009). Supportively, it has been reported that bortezomib-treated MM patients are sensitive to viral infection due to a toxic effect on NK cells and CD8+ T cells (Kim, *et al* 2008). Taken together, it might be true that MM patients under this treatment could have an impaired immunesurveilince system, which will in the end affect the outcome of this disease. Therefore, more studies are needed to better understand the mechanism of proteasome inhibition, to overcome its side effects and improve its clinical effect.

1.4 NATURAL KILLER CELLS

1.4.1 NK cell function

Natural killer cells (or NK cells) are large granular lymphocytes, principally found in the peripheral blood and lymphoid tissues (Caligiuri 2008). They were named "natural killer" because of the initial notion that unlike T cells, they do not require activation in order to kill cells that are missing "self" markers of MHC class I (Smyth, *et al* 2005). The discovery of NK cells dates back to the early 1970s by doctoral student Rolf Kiessling from Karolinska Institutet (Kiessling, *et al* 1975), in the mouse, and later by Hugh Pross and doctoral student Mikael Jondal, from Karolinska Institutet, in human (Jondal and Pross 1975, Pross and Jondal 1975). NK cells can be defined as a population of CD56(+)CD3(-) cells by flow cytometry. Further studies separated NK

cells into two groups consisting of $CD56^{dim}$ NK cells which with potent cytotoxic effects towards target cells and $CD56^{bright}$ NK cells with the function of cytokine secretion (Caligiuri 2008). Moreover, NK cells as innate lymphoid cells, are critical effector cells of innate immunity and also have a role in tissue modeling (Spits, *et al* 2013).

NK cells serve a very important role in host defense to virus, bacteria, certain parasites and tumors. IL-2-activated NK cells have been used in the clinical trials for treatment of solid primary or metastasized cancer (Rosenberg, *et al* 1993). NK deficiency in humans lead to severe fatal infection during childhood, further supporting this notion (Orange 2006). NK cells mainly use cytolytic granules (perforin/granzyme) and death receptor (eg. Fas, TRAIL)-mediated pathways to process their effector functions (Orange 2006). Furthermore, NK cells can also secret cytokines and chemokines that could influence the host immune response to kill target cells, or interact with adaptive immunity by stimulating DCs and promoting T-cell responses, as well as activate macrophage-mediated killing of obligate intracellular pathogens (Caligiuri 2008, Filipe-Santos, *et al* 2006). Interferon gamma (INF-γ) is considered as the prototypic cytokine secreted by NK cells, which has anti-proliferative effect on virus-infected and transformed cells (Maher, *et al* 2007), as well as the function of shaping Th1 responses (Mocikat, *et al* 2003).

1.4.2 NK cell killing of target cells

The direct killing of cancer or pathogen-infected cells occurs mainly through perforin/granzyme granule-mediated exocytosis or via signaling through death receptor family members (Smyth, *et al* 2005) (Figure 2).

NK cells secret cytotoxic granules upon interaction with target cells, and the cell death mediated by the exocytosis pathway is fast, powerful and multi-faceted, within minutes, therefore limiting pathogen replication and spread (Smyth, *et al* 2005). The cytotoxic granules secreted by NK cells are composed of complex organelles with degradative functions of typical lysosome. Among them, major granular proteins include perforin, a member of disrupting protein, and granzymes, a family of structural similarity with serine protease with biodegradation ability (Smyth, *et al* 2005). Most researchers believe that perforin and granzymes work as partners, with perforin making transmembrane pores on the target cells which facilitates the uptake of granzymes into cells. Further studies about perforin mutation in a severe, inherited human disorder of immue dysregulation known as familial haemophagocytic lymphohistocytosis (FHL), highlighted the importance of perforin in NK cell granule-mediated cytotoxicity (Stepp, *et al* 1999). However, other notions also indicated that the uptake of granzymes does not require perforin and is mediated by receptor-mediated endocytosis, in particular, mannose 6-phosphate receptor (MPR) can act as mediators for granzymes uptake (Motyka, *et al* 2000). Five granzymes (A, B, H, K and M) have been found in human, and among them granzyme B was considered to play a critical role in trigging cell death either directly, or via the activation of cellular caspases (Trapani, *et al* 2000). Once in the cytosol, granzyme B can either directly active caspases and initiate the

caspase cascade to DNA fragmentation and apoptosis, or trigger cell death via activation of mitochondria (intrinsic) pathway, through cleavage of Bid (Lord, *et al* 2003). Thus, granule-mediated exocytosis pathway acts as a main player to initiate cell death of offending cells (Figure 2).

TNF-related apoptosis inducing ligand (TRAIL) or Apol2L belongs to the death receptor family, it has high homology to other members of TNF family and close relation to Fas/Apol-1 ligand (Wiley, *et al* 1995). TRAIL is mostly expressed in T cells and NK cells of immune system, where it plays roles of killing transformed cells (Janssen, *et al* 2005, Smyth, *et al* 2003). TRAIL can band to its cell-bound receptors: two apoptosis-inducing receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), another two receptors are TRAIL-R3 (LIT, DcR1) and TRAIL-R4 (TRUNDD, DcR2), also called decoy receptors which are incapable to transmit apoptotic signaling, as well as the fifth soluble receptor osteoprotegerin (OPG) (Falschlehner, *et al* 2007). Binding of TRAIL to DR1 or DR2 results in apoptosis via extrinsic pathway (Figure 2). Previous studies supported a critical role of TRAIL in NK cell-mediated immunosurveillance against tumors (Falschlehner, *et al* 2009). In addition, normal cells express decoy receptors which could protect them from TRAIL-induced apoptosis (Pan, *et al* 1997), making TRAIL an excellent target for cancer therapy. Studies have reflected that administration of rhTRAIL and agonistic antibodies induce increased apoptosis in many cancer cell lines (Oldenhuis, *et al* 2008). Moreover, the combination of rhTRAIL with various antitumor agents gained more interest, in particular in combination with proteasome inhibitors. Thus, the combination of rhTRAIL and bortezomib has been studied in many cell lines, and also showed a better efficacy than single treatment (Hellwig and Rehm 2012).

Another member of death receptor family is FasL, Fas/FasL-mediated apoptotic signaling pathway also plays essential role during immunosurveillance (Villa-Morales and Fernández-Piqueras 2012) (Figure 2). Moreover, Fas and FasL gene were defined as tumor suppressor genes (Muschen, *et al* 2000). Fas is not constitutively expressed on tumor cells, however, INF-γ secreted by NK cells can up-regulate Fas expression on tumor cells, thus triggers Fas-dependent killing activity (Screpanti, *et al* 2001). Administration of recombinant IL-18 or IL-2 suppresses tumor metastases in mice mainly due to Fas/FasL-mediated killing by NK cells (Hashimoto, *et al* 1999). Collectively, studies have demonstrated the fundamental role of NK cell-mediated killing activity in immune system against tumors.

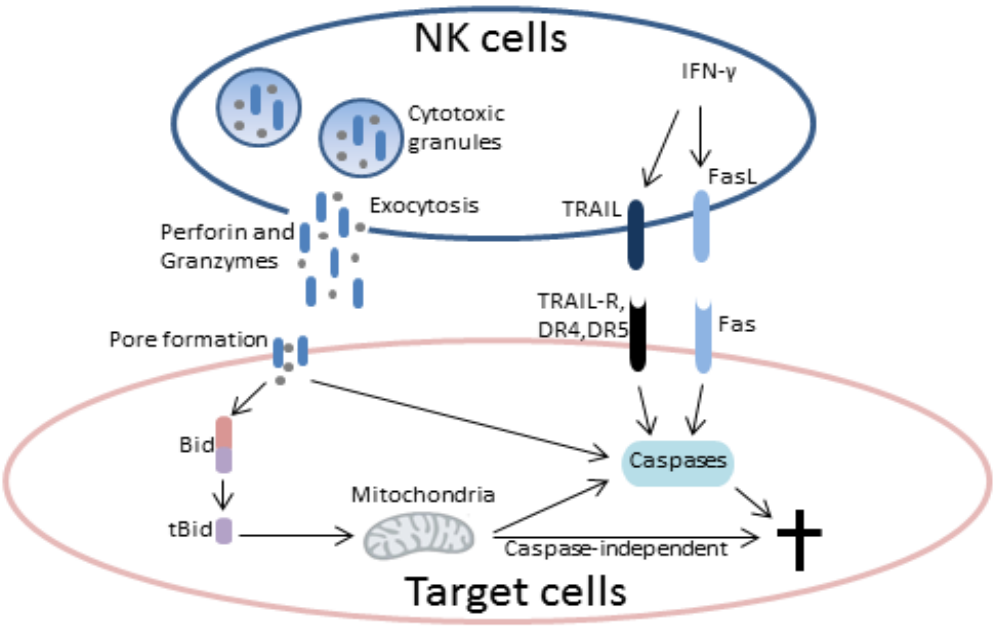

Figure 2. Two pathways of NK cell-mediated killing: granule exocytosis pathway and death receptor mediated pathway.

1.4.3 Inhibitory receptors and activating receptors

The function of NK cells also depends on the balance between inhibitory receptors and activating receptors expressed on them. Three inhibitory receptors have been found and characterized: the killer cell Ig-like receptors (KIR) in humans, the Ly49 lectin-like receptors in mice and CD94/NKG2A lectin-like receptors shared by human and mice, they all signal through intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which located in the cytoplasmic tail of these receptors (Pegram, *et al* 2011, Smyth, *et al* 2005). Target cells that express normal level of MHC class I molecules or MHC class I-related molecules can be protected once NK cell inhibitory receptors bind to these molecules, thus NK cell effector function (cytotoxicity and cytokine production) will be further blocked (Smyth, *et al* 2005). Studies also indicated that inhibitory signal can influence both NK cell-mediated cytotoxicity and adhesion ability of NK cells to target cells (Bryceson, *et al* 2009).

In contrast, NK cell activating receptors currently refer to the receptors that induce release of cytolytic granules and trigger cytokine production. Characterized activating receptors on NK cells include the natural cytotoxicity receptors (NCRs) (e.g. NKp30, NKp44 and NKp46), NKG2D, NKRP1, CD16 and activating KIRs, moreover, other receptors such as LFA-1, DNAM and members of the CD2 family are also involved in trigging NK cell activity. (Kirwan and Burshtyn 2007).

NCRs consist of NKp30, NKp44 and NKp46 as described above. Among them, the NKp30 and NKp46 receptors are expressed on both resting and activated NK cells,

whereas NKp44 receptor is expressed on activated NK cells only (Bottino, *et al* 2000). Activation of all NCRs results in direct killing of target cells. The NKp46, studied in the current thesis, is considered as a major player for NK cell-mediated killing function (Mandelboim and Porgador 2001). NKp46 is a member of immunoglobulin superfamily, characterized by two extracellular C2-type Ig-like domains. The function of NKp46 was shown to bind putative ligands of tumor cells, then lyse the target cells, conversely, block of NKp46-mediated pathway using NKp46 mAb prevents the killing of large panel of tumor cells (Sivori, *et al* 1999). It was also reported that NKp46 interacts with virus hemagglutinins (HAs), therefore leading to the lysis of virus-infected cells (Mandelboim and Porgador 2001). Other activating receptors, such as NKG2D, which is expressed on NK cells, $\gamma\delta^+$ T cells, $CD8^+$ $\alpha\beta$ T cells and activated macrophages, also play a fundamental role of defending infected and transformed cells in both innate and adaptive immune responds (Diefenbach, *et al* 2000, Jamieson, *et al* 2002). The NKG2D molecule recognizes several different ligand, and has been reported to have a role of stimulating immunity against tumors, as well as the induction of CTL, Th1 and Th2 responses (Diefenbach, *et al* 2001, Westwood, *et al* 2004).

Taken together, a functional immunity is considered to be a powerful barrier against cancer development, and it is very important to maintain its function during cancer therapy.

2 AIM OF THE STUDY

The overall aim of this Ph.D. project is to uncover potential roles of proteasome inhibition in the regulation of natural killer (NK) cell function and multiple myeloma (MM) cell apoptosis, We hope that these studies will enable the design of an optimized proteasome inhibition-based treatment protocol for multiple myeloma patients. The specific aims are:

I, To investigate a possible role of proteasome inhibition by inhibitors such as bortezomib in the regulation of NK cell survival and the underlying mechanisms related to treatment in vitro.

II, To explore potential effects of proteasome inhibition by bortezomib on death receptor-mediated functions, including TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL)-associated cytotoxicity by NK cells.

III, To analyze the potential pro-apoptotic effects of the novel proteasome inhibitor b-AP15 on human MM cells and NK cells.

IV, To study the putative regulatory function of HS1-associated X1 (HAX-1) on MM cells, including sensitization of MM cells to apoptosis induced by NK cells and proteasome inhibitors, as well as regulation of cell migration.

3 MATERIALS AND METHODS

Detailed description of the techniques, materials and methods used in our studies can be found in the publications and manuscripts included in the current thesis. The sections below provide an overview of the methods.

3.1 CELL CULTURE

3.1.1 Cancer cell lines

K562 cells

K562 cell line is the first human immortalized myelogenous leukemia line to be established, the line was derived from a 53 year old female CML patient in blast crisis (Lozzio and Lozzio 1975). K562 cell line was obtained from the American Tissue Culture Collection (ATCC) and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) plus penicillin (10 U/mL) and streptomycin (10 U/mL), in a humidified incubator at 37^{o}C and 5% $CO_{2.}$ Cells were kept in a logarithmic growth phase for all experiments (paper I and II).

P815 cells

FcγR^{+}P815 is a murine mastocytoma cell line provided by Dr Yenan Bryceson, Karolinska Institutet. Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) plus penicillin (10 U/mL) and streptomycin (10 U/mL), in a humidified incubator at 37^{o}C and 5% $CO_{2.}$ Cells were kept in a logarithmic growth phase for all experiments. FcγR+P815 cells were pre-incubated with NKp46 antibody (2.5µg/mL) for 30 min, before applying them to redirected cytotoxicity assay (paper I).

RPMI8226 cells and U266 cells

RPMI8226 cells were derived from the peripheral blood of a 61 years old male with multiple myeloma (Matsuoka, *et al* 1967). U266 cells were derived from peripheral blood of a 53 years old male with myeloma (Nilsson, *et al* 1970). Both cell lines were obtained from the ATCC and maintained in RPMI-1640 medium supplemented with 15% (U266) or 10% (RPMI8226) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) plus penicillin (10 U/mL) and streptomycin (10 U/mL). Cells were kept in a logarithmic growth phase for all experiments (paper II, III and IV).

Jurkat cells

The human Jurkat cells were established from the peripheral blood of 14 years old boy with T cell leukemia (Schneider, *et al* 1977). This cell line was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) plus penicillin (10 U/mL) and streptomycin (10 U/mL), in a humidified incubator at 37^{o}C and 5% $CO_{2.}$ Cells were kept in a logarithmic growth phase for all experiments (paper IV).

3.1.2 LAK and NK cells

Human peripheral blood mononuclear cells (PBMCs) from adult blood donors (Karolinska University Hospital, Stockholm, Sweden) were separated by Ficoll-Paque gradient centrifugation (Amersham Biosciences AB, Uppsala, Sweden). Peripheral blood lymphocytes (PBLs) were generated by culturing PBMCs (5.0×10^6/mL) for 45 min, followed by collection of non-adherent cells (PBLs) and re-suspension in cell culture medium at 1.0×10^6/mL. To generate lymphokine-activated killer (LAK) cells, PBLs were cultured in presence of 400 U/ml of IL-2 (R&D Sys- tems, Abingdon, UK) for 48 h (paper II).

We applied two kinds of methods to isolate NK cells from PBMCs. In paper I, II and IV, NK cells were isolated from PBMCs using the RosetteSep NK cell purification kit (Stemcell Technologies, Vancouver, Canada) and following the method described previously (Marcenaro, *et al* 2006), with minor modifications. Briefly, 20×10^6 PBMCs were mixed with autologous red blood cells (RBCs) with an RBC/PBMC ratio of 40:1, suspended in 1 ml complete RPMI-1640 medium and incubated with 50 µL RosetteSep cocktail for 20 min at RT. Then, the sample was diluted 2 times with complete medium before loading on Ficoll-Hypaque for separation. After centrifugation, NK cells were recovered from the interface. In paper III, NK cells were isolated from NK Cell Isolation Kit (Miltenyi Biotec Norden AB, Lund, Sweden), PBMCs were suspended in PBS buffer containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. Then, NK Cell Biotin-Antibody Cocktail was added, and samples were refrigerated for 10 min followed by centrifugation at 300xg for 10 min, whereupon the cell pellet was re-suspended in NK Cell Microbead Cocktail, and refrigerated for 15 min. Cells were re-suspended in buffer and centrifuged at 300xg for 10 min, then re-suspended again in buffer and applied onto the magnetic column, to enrich for NK cells. Purity of NK cells ($CD56^+$ $CD3^-$) from the two methods was approx. 90%, as determined by staining with CD56 and CD3 mAbs.

3.1.3 Co-culture system

Standard (4 h) chromium 51 (^{51}Cr)-release assay (Paper I, II and IV) or prolonged (12 h) ^{51}Cr-release assay (Paper II) were performed using protocol described previously (Zheng, *et al* 2002), to analyze cytotoxicity of resting NK (rNK) cells (paper I) , LAK cells (paper II) and PBLs (paper IV)to target cells. Briefly, effector cells were incubated with ^{51}Cr (Amersham Biosciences, Uppsala, Sweden) labeled target cells (K562 and $Fc\gamma R^+$ P815 for paper I), (K562, RPMI8226 and U266 for paper II) and (RPMI8226 and U266 for paper IV) in triplicates at various effector to target (E/T) ratios. After 4 h or 12 h incubation at 37^{o}C, 100 µl of cell-free supernatants were collected and the amount of ^{51}Cr released into the supernatants was measured using an automatic gamma counter (Wallac, Upplands Väsby, Sweden). Specific lysis was calculated by the method described previously (Zheng, *et al* 2002).

CD107a surface translocation assay was performed to quantify NK cell degranulation activity following the method published previously with minor modifications

(Bryceson, *et al* 2007). LAK (effector) cells and K562 (target) cells were seeded at an effector to target (E:T) ratio of 1:1 in 200µL complete medium in a 96-well plate in duplicate, and then the cells were centrifuged at a speed of 300 rpm for 3 min, FITC-conjugated CD107a (BD Pharmingen) was added into each well, and cells were incubated at 37^{o}C for 2 h. Then, cells were harvested, washed with cold PBS and stained with PE-conjugated anti-human CD3 and PECy5-conjugated anti-human CD56 mAbs (BD Pharmingen) as described above. $CD107a^{+}CD56^{+}CD3^{-}$ cells were defined as degranulated NK cells and quantified by using the CELLQUEST software (Becton Dick- inson) (paper II).

To deliver endogenous granzyme B to RPMI8226 cells, purified NK cells were isolated from PBMCs using the RosetteSep NK cell purification kit (Stemcell Tech-nologies, Vancouver, Canada) as described above, NK cells from healthy donor were seeded with RPMI8226 cells at effector to target (E:T) ratio of 2:1, after incubation for 4 h, cells were collected and prepared for western blot analysis (paper IV).

3.2 FLOW CYTOMETRY

3.2.1 Phosphatidylserine exposure

Plasma membrane exposure of phosphatidylserine (PS) was quantified by dual labeling with Annexin V-Fluos (AV) (Roche Diagnostics GmbH, Mannheim, Germany) and propidium iodide (PI) (Sigma Aldrich) according to the protocol provided by the manufacturer. Cells were then collected and washed twice with cold PBS, re-suspended in 100 µL of binding buffer containing AV and PI, followed by incubation for 15 min at RT. Then, 400 µL of incubation buffer was added, and cells were analyzed by flow cytometry within 30 min on a FACS Calibur (Becton Dickinson, San Jose, CA). $AV^{+}PI^{+}$ cells and $AV^{+}PI^{-}$ cells were defined as apoptotic cells (paper I, II, III and IV).

3.2.2 Cell surface and intracellular protein detection

For surface staining, cells were first incubated with 10% FCS-PBS buffer for 15 min at RT to block non-specific binding of antibodies to Fc receptors. The cells were then stained with fluorescence labeled antibodies in 10% FBS-PBS buffer for 30 min at 4°C (paper I, II, III, and IV). Detection of intracellular protein expression was performed by using the Intrastain Kit (DAKO, Glostrup, Denmark) according to instructions provided by the manufacturer. Briefly, NK cell surface marker (CD56) was stained first as described above and cells were then fixed using reagent A for 15 min at RT. After washing, cells were re-suspended in reagent B for cell membrane permeabilization, and specific antibodies or corresponding isotype control mAb was added and cells were incubated for 15 min at RT (paper I and II). Finally, cells were washed with PBS and re-suspended in 400 µl PBS prior to analysis by flow cytometry. The collected data were analyzed using CELLQUEST software (Becton Dickinson).

3.3 CASPASE-3 ACTIVITY

Caspase activation was detected as previously described (Fadeel, *et al* 1998). Briefly, cell lysates were combined with the fluorogenic substrate, DEVD-AMC (Sigma) in a reaction buffer (20 mM HEPES, pH=7.5, 10% glycerol, and 10mM dithiothreitol). Real-time measurements of enzyme-catalyzed release of AMC were obtained using a Tecan Infinite F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). Fluorescence values were converted to pmol and the maximum rate of AMC release (pmol/min) was estimated for each sample. To assess the role of caspase activation, zVAD-fmk (Sigma) was applied as indicated (paper I and III).

3.4 MITOCHONDRIAL MEMBRANE POTENTIAL

Functional mitochondria were labeled by Mitotracker Red CMXRos (Mitotracker Red) (Molecular Probes, Eugene, OR) as described previously (Jayaraman 2005), with minor modifications. In brief, a total of $1.0x10^6$ cells were pelleted and suspended in 1 ml of pre-warmed complete medium with a final concentration of 125 nM Mitotracker Red for 35 min at 37^{o}C. Excess dye was removed with two washes in pre-warmed complete medium at 37^{o}C, and finally the labeled cells were re-suspended in PBS for mitochondrial membrane potential (MMP) measurement. Mitotracker Red fluorescence was detected using a FACS Calibur (Becton Dickinson) (paper I).

3.5 REAL-TIME POLYMERASE CHAIN REACTION

For real-time polymerase chain reaction (PCR), RNA was extracted from cells using RNase Mini Kit (Qiagen, Hilden, Germany). One microgram of RNA was then treated with DNase I using a DNA-free kit (Ambion, Austin, TX) according to the manufacturer's protocol, and cDNA was synthesized using the SuperScript first strand synthesis system (Invitrogen, Carlsbad, CA) with random hexamers. cDNA quantity was measured using real-time PCR with the ABI PRISM 7000 sequence detector system and fluorescence-based SYBR-green technology (Applied Biosystems, Warrington, UK). PCR was performed in a final volume of 25 µL, consisting of diluted cDNA sample plus 1×SYBR-green PCR Master Mix (Applied Biosystems). Primer sequences were designed according to a previous publication (Morissette, *et al* 2008). All samples were analyzed in duplicate (two independent experiments). The ratio of mRNA expression/18S rRNA expression for each sample was determined to represent the mRNA expression level (paper II).

3.6 SMALL INTERFERING RNA (SIRNA) DELIVERY

We applied the AMAXA Nucleofector System (Lonza Cologne AG, Germany) to transfect cells with targeted small interfering RNA (siRNA) and a matched control siRNA. Cells were re-suspended in fresh medium in a concentration of $0.5x10^6$/mL the day before use. After determining cell density, we centrifuged the required number of cells at 90xg for 10 min, then removed the supernatant completely and re-suspended the cell pellet gently with 100 µl Nucleofector reagent per sample. Cells were

electrophoresed using Nucleofector Program X-005 per million cells, and cells were incubated for 24 hours to have efficient knockdown (paper IV).

3.7 WESTERN BLOT

To obtain cell lysates, cells were lysed on ice for 30 min in RIPA buffer (50 mM Tris, PH8.0, 150 mM Nacl, 10% glycerol, 1% NP-40, 0.5% deoxycholate) supplemented with protease inhibitors. Protein concentration was measured using BCA™ Protein Assay (Pierce Biotechnology Inc., Rockford, IL). Fifty µg of protein were loaded into a Nu-PAGE 4-12% Bis-Tris Gel (Invitrogen) and then transferred onto PVDF membranes. Following probing with the indicated primary antibodies the relevant secondary antibody (DAKOCytomation, Glostrup), the protein bands were visualized by enhanced chemiluminescence (BioRad Laboratories). The films were scanned using the Epson Perfection 4490 Scanner (EPSON Europe B.V, Sollentuna, Sweden) (paper III and IV).

3.8 DELIVERY OF RECOMBINANT GRANZYME B

To delivery recombinant granzyme B, cells ($2x10^6$) were permeabilized using 100 µl permeabilization buffer (containing 150 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-x-100) with 10 µl 0.01% digitonin for 5 min, then 2 µl recombinant granzyme B (stock solution 0.2 µg/µl) were added and cells were incubated for 1 h (paper IV).

3.9 CELL MIGRATION ASSAY

To monitor the *in vitro* migration of cells, we utilized a 24-well Transwell system with polycarbonate membranes of 8 mm pore size (Corning Costar, Cambridge, MA). The membranes were pre-coated with 20 µg/ml fibronectin (R&D System) at 37°C for 1h, followed by blocking of the membranes with 0.1% BSA PBS at 37°C for another 30 min. Cells were re-suspended at $2.5x10^6$/ml in serum-free complete RPMI1640 medium, and were seeded onto the upper-side chamber (100 µl/well), 0.6ml of the same medium supplied with specific chemoattractant was added to the lower chamber. The cells were allowed to migrate overnight, and cells that migrated into the lower chamber were collected and counted using a Countess® Automated Cell Counter (Invitrogen) (paper IV).

3.10 *IN SILICO* ANALYSIS OF MICROARRAY DATA

Global gene expression profiling has become a powerful tool for exploring the underlying pathogenesis and biology of human cancer for many years (Kwiecinska, *et al* 2011). Expression of indicated mRNA in different cancer types was assessed using the *In Silico* Transcriptomics (IST) database, an online resource that contains data from 9.783 Affymetrix gene expression analyses of 43 normal tissues, 68 cancer types and 64 other diseases (Kilpinen, *et al* 2008) (paper IV).

4 RESULTS

4.1 PAPER I: PROTEASOME INHIBITION INDUCES APOPTOSIS IN PRIMARY HUMAN NK CELLS AND SUPRESSES NKP46-MEDIATED CYTOTOXICITY

In this study, we investigated the effects of proteasome inhibitor bortezomib on natural killer (NK) cell survival and function.

Cytotoxic effects of bortezomib on NK cells were evaluated in highly purified resting NK (rNK) in the presence of bortezomib for 12 and 24 h. We found that bortezomib markedly induced apoptosis in rNK cells in a dose- and time-dependent manner. Furthermore, we asked the underlying mechanism of apoptosis induced by bortezomib. Our results demonstrated that GSH, a potent ROS scavenger, markedly abolished apoptosis induced by bortezomib at 4.7 ng/ml for 24 h. However, blocking caspase activation by zVAD-fmk, a pan-caspase inhibitor, did not effectively block total apoptosis in rNK cells induced by bortezomib, indicating that ROS generation contributes to apoptosis in rNK cells induced by bortezomib at the doses tested. To obtain more information on the potential mechanisms involved in bortezomib-induced apoptosis of rNK cells, we evaluated the role of mitochondria, which plays pivotal roles in the process of apoptosis, in the bortezomib-induced apoptosis in rNK cells. Mitochondrial membrane potential (MMP) of rNK cells cultured with bortezomib for 12 or 24 h was measured. Our data has shown that bortezomib markedly induced a loss of Mitotracker Red fluorescence intensity in rNK cells in a dose-dependent manner, and GSH partially and significantly prevented the loss of MMP induced by bortezomib, further indicated bortezomib induced apoptosis of rNK cells is ROS-dependent.

Next, we examined putative effects of proteasome inhibition on NK cell activation receptors (NKp30, NKp46, NKG2D and DNAM-1). Particular NKp46 expression in rNK cells was significantly reduced upon treatment with bortezomib in a time- and dose-dependent manner, but not expression of DNAM-1, NKG2D and NKp30, or perforin expression after bortezomib treatment. Since NF-κB is one of the main targets of proteasome inhibition (Adams 2004), we further tested NKp46 expression on rNK cells after blocking of NF-κB activity by a NF-κB blocker Bay 11-7082, and found that blocking of NF-κB activity induced significant loss of NKp46 expression on rNK cells, indicating that the bortezomib-induced decrease in NKp46 expression may be, at least in part, attributable to the known inhibitory effects of this agent on NF-κB function.

To investigate the functional consequence of decreased NKp46 expression on rNK cells following bortezomib treatment, we employed a redirected NK cell cytotoxicity assay, using $Fc\gamma R^+$ P815 as target cells, in presence of anti-NKp46 antibody. We found that the lysis of $Fc\gamma R^+$ P815 cells by the rNK cells exposed to a low dose of bortezomib (4.7 ng/mL) for 12 h was significantly decreased. Interestingly, blocking of NF-κB

activation by Bay 11-7082 for 12 h substantially inhibited rNK cell-mediated lysis of $Fc\gamma R^+$ P815 cells in the presence of anti-NKp46 antibody. These results further support the role of NF-κB in the regulation of bortezomib-induced decrease of NKp46 expression.

Our findings demonstrated that bortezomib induces rNK cell apoptosis in a time- and dose-dependent manner, and reduces NKp46 receptor expression as well as NK cell cytotoxicity mediated through the NKp46 activation pathway. These in vitro results caution that bortezomib may disrupt NK cell-dependent immunosurveillance in patients treated with this drug.

4.2 PAPER II: THE PROTEASOME INHIBITOR BORTEZOMIB DISRUPTS TRAIL EXPRESSION AND NK CELL KILLING OF TRAIL RECEPTOR-POSITIVE MULTIPLE MYELOMA CELLS

Based on the findings explored in paper I, we want to further evaluate the regulatory role of bortezomib in TNF-related apoptosis inducing ligand (TRAIL) and FasL of NK cells.

The dosages and time point of bortezomib treatment applied in this study were nontoxic to multiple myeloma cell line RPMI8226 and U266. However, we found that TRAIL expression (both protein level and mRNA level) of IL-2 activated NK cells was decreased in a dose-dependent manner, as determined by flow cytometry, but no increase in apoptosis was detected in IL-2 activated NK cells under these conditions. NF-κB was shown previously to be involved in the regulation of TRAIL expression (Wajant 2004). We further demonstrated that Bay 11-7082 substantially decreased both TRAIL protein and mRNA expression, indicating the role of NF-κB involved in bortezomib-mediated down regulation of TRAIL expression. In contrast, we also confirmed that bortezomib treatment did not affect intracellular perforin expression in IL-2-activated NK cells nor did the bortezomib treatment affect FasL cell surface expression in these cells. Therefore, bortezomib appears to selectively reduce TRAIL expression in activated human NK cells.

As exocytosis of perforin-containing granules is a major mechanism for NK cell-dependent cytotoxicity. To explore a potential role of bortezomib in the regulation of the granule-exocytosis pathway, the CD107a surface translocation assay was applied (Bryceson, *et al* 2007). Our studies showed that the percentage of degranulated NK cells treated by bortezomib was not significantly different from the control (untreated) group. Supportively, bortezomib treatment did not affect LAK-mediated killing of target cells, as evidenced using the standard 4 h ^{51}Cr release assay. CMA has been shown to reduce perforin content and to block perforin-mediated cytotoxicity in NK cells (Saudemont, *et al* 2005). Moreover, for comparison, inactivation of perforin-

mediated cytotoxicity by CMA treatment of LAK substantially blocked the killing of target cells in this assay.

Additionally, a modified 12 h ^{51}Cr release assay was employed to evaluate perforin-independent killing activity. Using this assay, we observed that bortezomib treatment of LAK cells significantly reduced the percentage of lysis of RPMI8226 and U266 cells as compared to untreated LAK cells. To further dissect the potential mechanism underlying the killing defect, and to determine the role of TRAIL-dependent killing in this assay, we employed a specific anti-TRAIL antibody to block TRAIL-mediated killing pathway. Interestingly, LAK cell-mediated killing activity to RPMI8226 cells was impaired, but did not affect the killing of U266 cells. Moreover, RPMI8226 cells expressed both TRAIL receptors while U266 cell line expressed DR4 and DR5 at low level, indicating that TRAIL is involved in the LAK cell-mediated killing of RPMI8226 cells.

To conclude, proteasome inhibition by bortezomib disrupts TRAIL expression in human NK cells but does not affect Fas ligand or perforin expression. This may potentially hamper NK-dependent immunosurveillance against tumors in patients treated by this drug.

4.3 PAPER III: PRO-APOPTOTIC EFFECTS OF THE NOVEL PROTEASOME INHIBITOR B-AP15 ON MULTIPLE MYELOMA CELLS AND NK CELLS

Recent studies have shown that the small molecule b-AP15, a novel inhibitor of proteasome deubiquitination, displays anti-tumor activity in several pre-clinical solid tumor models (D'Arcy, *et al* 2011). Herein, we wanted to study the potential regulatory effects of b-AP15 on multiple myeloma cells, as well as NK cells.

We first demonstrated that b-AP15 induces time- and dose-dependent apoptosis in MM cell lines. Moreover, accumulation of ubiquitin-conjugated proteins was evaluated by western blot, indicating that b-AP15-induced apoptosis in MM cells is associated with proteasome inhibition. Caspase-3 is a key protease in the apoptosis pathway (Andón and Fadeel 2012), we further monitored activation of pro-caspase-3, as well as cleavage of the nuclear protein, PARP, a downstream target protein of caspase-3, our results revealed the underlying mechanism of b-AP15-triggered apoptosis in MM cells. Additionally, b-AP15-induced caspase-3 activation was also quantified by DEVD-AMC cleavage. As noted above, caspase activation seems to be involved in b-AP15-induced apoptosis in MM cells. Interestingly, b-AP15-triggered apoptosis of MM cells was significantly reduced after pre-incubation with zVAD-fmk (a pan-caspase inhibitor), further suggesting the importance of caspase activation in b-AP15-induced apoptosis.

Next, we examined whether b-AP15 has any pro-apoptotic effects on NK cells. Highly Purified NK cells were then exposed to b-AP15 at indicated does- and time-point. Our data demonstrated that, besides its cytotoxic effects on MM cells, b-AP15 also triggers apoptosis in NK cells in a dose- and time-dependent manner. We also applied zVAD-fmk to study the role of caspase in b-AP15 induced apoptosis of NK cell, our finding suggested that both caspase-dependent and -independent pathways are engaged in this process. For comparison, we employed bortezomib in this system. Importantly, however, the pro-apoptotic effect of b-AP15 on NK cells is much lower than that of bortezomib, implying that b-AP15 may have less side effects on the immune system than bortezomib. This suggests that b-AP15 or similar drugs might have advantages to bortezomib in treatment of cancer patients.

Taken together, we demonstrate herein that the novel proteasome inhibitor, b-AP15 induces caspase-dependent apoptosis of human MM cells. b-AP15 also has toxic effects on primary human NK cells but the effect is less pronounced when compared to bortezomib. Therefore, b-AP15 may provide a useful approach for the treatment of MM with less adverse effects on immune-competent cells.

4.4 PAPER IV: HAX-1 REGULATES MIGRATION OF MULTIPLE MYELOMA CELLS

Recent studies from our laboratory have shown that HAX-1 is overexpressed in certain hematopoietic malignancies including B lymphoma (Kwiecinska, *et al* 2011). We wanted to elucidate the potential role of HAX-1 in multiple myeloma.

By studying the publically available IST database, which contains microarray data from close to 10.000 Affymetrix analyses of normal and disease tissues (Kilpinen, *et al* 2008), we found *HAX1* mRNA level was elevated in multiple myeloma samples. Additionally, high level of HAX-1 protein expression in the human MM cell lines U266 and RPMI8226 was also observed. Based on our knowledge of HAX-1 in the regulation of apoptosis (Jitkaew, *et al* 2009, Li, *et al* 2009), we wanted to assess the putative role of HAX-1 for regulation of apoptosis in MM cells. First, we efficiently silenced HAX-1 expression in MM cells, then, control siRNA and HAX-1 siRNA transfected U266 cells were exposed to either bortezomib or b-AP15 at indicated dose- and time-points. We found that silencing of HAX-1 expression in U266 cells did not affect the sensitivity of these cells to these stimuli. However, a down-regulation of HAX-1 expression was seen after bortezomib and b-AP15 treatment, as well as Bay11-7082 (a NF-κB inhibitor) treatment, indicating that the expression of HAX-1 is subject to NF-κB-dependent regulation in MM cells, in line with our previous experiments using normal EBV-transformed B cell lines (Jitkaew, *et al* 2009). However, we did not observe the up-regulation of Bcl-2 expression after HAX-1 knockdown, as shown in follicular lymphoma (Kwiecinska, *et al* 2011).

In line with the previous finding that granzyme B cleaves HAX-1 (Han, *et al* 2010), we observed HAX-1 cleavage in the RPMI8226 cell line in our study upon incubation with recombinant granzyme B. We also found for the first time that HAX-1 is cleaved in target cells co-cultured with NK cells. To further assess the possible role of HAX-1 in the protection of MM cells against NK cell-mediated killing, we applied human primary PBL-mediated killing to HAX-1 siRNA or control siRNA transfected U266 or RPMI8226 cells. Our results showed that silencing of HAX-1 in MM cells fails to modulate NK cell-mediated killing of these cells.

HAX-1 is a multi-functional protein (Fadeel and Grzybowska 2009), and recent publications also revealed its role in the regulation of cell migration (Mekkawy, *et al* 2012, Ramsay, *et al* 2007). We used the Transwell assay to study a possible role of HAX-1 for the migration activity of MM cells. Our data demonstrated that inhibition of HAX-1 expression in the U266 cell line using specific siRNA significantly decreased migration ability. However, the underlying mechanisms need to be further explored in future studies.

5 DISCUSSION

Multiple myeloma (MM) still remains incurable, even though the clinic outcomes of this disease have been greatly improved due to introduction of novel therapies (Suzuki 2013). Among these, proteasome inhibitor-based treatment has shown success; however, the clinical management of this novel agent needs to be further studied, in order to design a more effective proteasome inhibitor-based treatment. Moreover, we are also aiming to explore other potential target for the treatment of myeloma.

Recent studies have revealed cytotoxic effects of proteasome inhibition on immune-component cells, including T cells and DCs (Berges, *et al* 2008, Straube, *et al* 2007). The studies presented in the current thesis are aimed at elucidating the potential role of proteasome inhibition in the regulation of NK cells. For this purpose we investigated the effects of this agent on NK cell survival and function. In the first paper our studies showed for the first time that primary human rNK cells undergo apoptosis in response to proteasome inhibition by bortezomib, a novel anti-cancer agent. Importantly, the dose selected in this study (4.7 ng/ml and 18.8 ng/ml) are below the dose range mostly applied in patients. As known, the most commonly used dose of bortezomib in the clinic is 1.3 mg/m^2 on day 1, 4, 8 and 11 of every 21 days defined as one cycle, and as a result the concentration of bortezomib in plasma ranges from 60 to 120 ng/ml (Utecht and Kolesar 2008). It is clear from others studies that both generation of ROS and caspase activation triggered by proteasome inhibition play a critical role in bortezomib-induced tumor cell apoptosis (Perez-Galan, *et al* 2006). Interestingly, our findings suggest that ROS generation rather than caspase activation is a key triggering factor for bortezomib-induced apoptosis in rNK cells. Consistently, our results showed that GSH almost completely prevented the dissipation of MMP, as well as caspase activation induced by bortezomib. Similar effects of bortezomib on malignant NK cells have been reported (Shen, *et al* 2007). Indeed, our data indicated that bortezomib may act via similar mechanism(s) to trigger apoptosis toward primary resting NK cells.

Another question we might ask is whether NK cell-mediated cytotoxicity is also influenced by proteasome inhibition. NK cell function is finely regulated by a balance between activating and inhibiting signaling with corresponding ligands expressed on the target cells (Bryceson, *et al* 2006).We found that bortezomib selectively down-regulated the expression of NKp46 but not other NK receptors tested (NKp30, NKG2D and DNAM-1) and this effect was not attributable to the apoptosis inducing-property of bortezomib. Consequently, NK cell cytotoxicity to FcγR$^+$ P815 cells mediated by the NKp46 activation pathway was impaired, and these results were in line with the decreased NKp46 expression observed after administration of bortezomib. Moreover, NF-κB was shown to play an important role in regulating the expression of NK surface molecules in mice (Pascal, *et al* 2007), as well as a main target for proteasome inhibition (Ciechanover 1994). Our data indeed showed the blocking effects of NF-κB mediated by bortezomib may be involved in the selective down-regulation of NKp46

expression. In addition, another novel discovery by Gur et al makes our study more interesting. These authors found that NKp46 recognize its ligands on human and mouse pancreatic beta cells, thus mediated the damage that caused by this activation and contributed to the development of type 1 diabetes (Gur, *et al* 2010). This might suggest a clinical potential of bortezomib in prevention of NK cell-mediated killing of pancreatic beta cells in type 1 diabetes patients.

In the following study, we aimed at evaluating potential effects of proteasome inhibition by bortezomib on death receptor-mediated functions. We chose two MM cell lines as target cells, since bortezomib was first approved for the treatment of patients with MM (Richardson and Anderson 2003). FasL/Fas and TRAIL/TRAIL-R mainly contribute to the death receptor-mediated pathways by NK cells (Falschlehner, *et al* 2009). Herein, we demonstrated that bortezomib significantly downregulates TRAIL expression in IL-2-activated NK cells, and suppresses perforin-independent killing activity of activated NK cells against human MM cells. Moreover, the inhibitory effect by bortezomib seems to be selective as the expression of FasL as well as perforin was not impaired at the same condition. TRAIL expression level on resting NK cells in blood is low or undetectable, but it is inducible upon stimulation of Th1 type cytokines including IL-2, interferon (IFN)-γ, and IL-15 (Smyth, *et al* 2003), to this notion, we used IL-2 activated NK cells in this study. Several studies have shown that TRAIL induces apoptosis in MM cells in vitro (Balsas, *et al* 2009, Lincz, *et al* 2001). Moreover, recombinant soluble TRAIL induced MM cell apoptosis and protected against MM cell-induced lytic bone destruction in a mouse model (Labrinidis, *et al* 2009). These studies indicated that a disruption of TRAIL expression may impair its cytotoxicity against MM.

The NF-κB pathway seems to be involved in the regulation of NKp46 expression after bortezomib treatment (paper I). Consistently, our data showed that Bay11-7082 significantly reduced the expression of TRAIL both at the mRNA and protein level, indicated that proteasome inhibition regulates TRAIL expression at transcriptional level, and the NF-κB pathway plays a key role in the regulation of TRAIL expression in activated human NK cells. NK cell cytotoxicity applied in paper I is classical short-term (4 h) lysis assay. However, in paper II we utilized a long-term (12 h) ^{51}Cr release assay, plus CMA pretreatment to exclude perforin-dependent pathway (Phillips and Lanier 1986). Using this modified assay, we could demonstrate that bortezomib treatment significantly suppressed LAK or NK cell-mediated killing of human MM cell lines. Moreover, the lysis of RPMI8226 cells, which expressed high level of DR4 and DR5, were partially, but significantly blocked by anti-TRAIL antibody, indicating that TRAIL is involved in the NK cell-mediated killing of RPMI8226 cells. Further studies are warranted to clarify the underlying mechanisms by which bortezomib disrupts NK cell cytotoxicity towards RPMI8226 cells besides impairing TRAIL-mediated pathway.

In paper III, we studied a small molecule b-AP15, which is an inhibitor of proteasome deubiquitination, and its pro-apoptotic effect on MM cells and human primary NK cells. Bortezomib is used as a first-line therapy for multiple myeloma patients (Goldberg

2012); however, relapse often occurs in patients who initially responded to bortezomib. Moreover, the cytotoxic effect of this drug to immune-component cells, including its side effect on NK cells (paper I and paper II), makes the use of bortezomib a complicated issue (Buac, *et al* 2012). Thus, basic research needs to explore novel proteasome inhibitors, which could provide better therapeutic effects but with less side effects. One recent publication gave an opportunity to study the novel proteasome inhibitor b-AP15, which differs from bortezomib insofar as it acts through inhibition of the 19S regulatory-particle associated deubiquitinases (DUBs) (D'Arcy, *et al* 2011). We observed dose- and time-dependent apoptosis of MM cells induced by b-AP15, in line with the accumulation of polyubiquitin, as well as activation of caspase-3 and cleavage of its downstream substrate PARP, indicating that caspase-dependent pathways might be involved in this process. In fact, our studies showed that b-AP15 triggered caspase-dependent apoptosis in MM cells.

In paper I, we demonstrated that proteasome inhibitor bortezomib influence NK cell survival at the dose below clinically used doses in myeloma patients. In paper III, we noted that b-AP15 induces apoptosis of purified human NK cells at doses equal to or lower to the doses that killed MM cells, further confirming our previous observation in paper I that NK cells are sensitive to proteasome inhibition. However, more interestingly, we found that b-AP15 was less toxic to NK cells than bortezomib. It will be of interest to determine whether this is a common feature of DUB inhibitors and whether this translates into less adverse effects on immune cells *in vivo*. Of course, it remains to be investigated whether NK cell-mediated killing activity is also impaired after exposing NK cells to b-AP15.

In the last paper, we studied the regulatory effect of multi-functional protein HAX-1 on MM cells. The reason to study the function of HAX-1 in MM is due to our finding that *HAX1* mRNA expression is high in multiple myeloma, even higher than the previous reported high expression in B lymphomas (Kwiecinska, *et al* 2011). HAX-1 was initially believed to be an anti-apoptotic protein. A very important evidence of this notion is the discovery of patients with genetic defects of *HAX1* gene, which is associated with severe congenital neutropenia (also known as Kostmann disease), in which HAX-1 was shown to display a role of preventing myeloid stem cell from apoptosis in the bone marrow (Carlsson, *et al* 2004). Moreover, the HAX-1-interacting protein HS-1 also plays an important role in B cell receptor (BCR)-mediated apoptosis and proliferative responses (Taniuchi, *et al* 1995). Therefore, knocking down of HAX-1 expression on MM cell lines with siRNA as well as a control siRNA was applied to achieve the aims of studying its putative anti-apoptotic function. However, we noted that silencing of HAX-1 expression in MM cell lines does not affect sensitivity to the apoptotic stimuli, bortezomib or b-AP15 applied in this study.

Han *et al*. (2010) reported granzyme B-mediated cleavage of HAX-1 and identified HAX-1 cleavage as a novel mechanism for granzyme B-mediated mitochondrial depolarization (Han, *et al* 2010). We corroborated the observation that recombinant granzyme B is able to cleave HAX-1 using MM cells and we reported for the first time

that NK cells can mediate granzyme B-mediated cleavage of HAX-1 in target cells. However, we could not demonstrate a role for HAX-1 in the regulation of target cell susceptibility to NK cell killing using the standard 4 h ^{51}Cr release assay. Since HAX-1 has been shown to interact with several viral proteins (Kawaguchi, *et al* 2000, Sharp, *et al* 2002, Yedavalli, *et al* 2005), its cleavage could serve to prevent such interactions, thereby affecting virus function(s), this remains to be studied.

HAX-1 is a multi-functional protein (Fadeel and Grzybowska 2009) and recent publications also revealed its role in the regulation of cell migration (Mekkawy, *et al* 2012, Ramsay, *et al* 2007). We applied fibronectin coated Transwell assay to study a possible role of HAX-1 in the regulation of migration activity of MM cells. Our data demonstrated that inhibition of HAX-1 expression in the U266 cell line significantly decreased its migration ability to specific chemoattractant, SDF-1α. Of note, silencing of HAX-1 in MM cells did not trigger apoptosis, indicating that the reduced migration was not due to an increase in cell death. It was known that expression of CXCR4, the receptor for SDF-1α, is decreased in B cells from HAX-1-deficient mice (Peckl-Schmid, *et al* 2010). Moreover, Dobreva et al described the physical interaction of HAX-1 and integrin-linked kinase (ILK), a kinase known to participate in integrin signaling, including regulation of integrin-mediated cell migration (Dobreva, *et al* 2008). We detected surface expression of CXCR4, and integrin receptors, α_4, α_5 and β_1, using specific antibodies and flow cytometry based analysis. However, we could not detect a reduction of surface expression of CXCR4 on U266 cells after silencing of HAX-1, nor did we detect a decrease in expression of integrin receptors, α_4, α_5 and β_1. Ramsay et al pointed out in their study that HAX-1 regulates carcinoma cell migration and invasion via clathrin-mediated endocytosis of integrin alphavbeta6 (Ramsay, *et al* 2007). However, our experiments only provided the surface expression of these molecules after HAX-1 deletion. One could further study the endocytosis of these receptors after HAX-1 knockdown. We also want to assess HAX-1 protein expression in MM patient biopsies using immunohistochemical techniques and it will be of interest to determine if HAX-1 expression correlates with the biological/clinical behavior of the tumor in patients.

We believe that the results generated in this thesis will improve our knowledge of proteasome inhibition in the regulation of NK cell function. We aim to provide important knowledge for designing a more effective proteasome inhibitor-based treatment for patients with multiple myeloma, with the goal to maintain proteasome inhibition-associated apoptosis induction activity in tumor cells, with reduction of the toxic effects of proteasome inhibition on immune cells including NK cells. Moreover, we have observed that HAX-1 is overexpressed in MM and played a role in the regulation of myeloma cell migration, which might point to HAX-1 as a potential target in the treatment of myeloma. Overall, our studies should serve to improve the clinical management of patients with MM, and may also aid in the understanding of the utility of proteasome inhibition in the treatment of other types of cancer.

6 CONCLUSIONS

In summary, the main results generated from the studies suggest the following:

Paper I:

- Bortezomib induces resting NK cell apoptosis in a time- and dose-dependent manner.

- Glutathione, a reactive oxygen species scavenger, protects against bortezomib-induced apoptosis in resting natural killer cells.

- Bortezomib reduces activating receptor NKp46 expression as well as NK cell cytotoxicity mediated through the NKp46 activation pathway.

- Bay 11-7082, a pharmacological inhibitor of NF-κB activation, also reduces NKp46 expression and suppressed redirected cytotoxicity.

Paper II:

- Bortezomib selectively reduces the surface expression of TRAIL in activated human NK cells.

- Perforin-independent killing of MM cell lines is impaired by bortezomib at doses that does not induce apoptosis of NK cells or MM cells.

- The anti-TRAIL antibody blocks the lysis of TRAIL sensitive RPMI8226 cells.

Paper III:

- The novel proteasome inhibitor, b-AP15 induces caspase-dependent apoptosis of human MM cells.

- b-AP15 induces apoptosis in primary human NK cells.

- The pro-apoptotic effect of b-AP15 on NK cells is not as pronounced as the effect of bortezomib.

Paper IV:

- HAX-1 is highly expressed in MM cells.

- HAX-1 does not seem to play a role in regulation of apoptosis in MM cells.

- HAX-1 is shown here to play a role in the regulation of myeloma cell migration.

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