General introduction and introduction to the chapters
RHEUMATOID ARTHRITIS AND IMMUNE EFFECTOR CELLS INVOLVED

Rheumatoid Arthritis (RA) is a chronic, progressive autoimmune disease which affects the adult population of developed regions with an estimated prevalence of ~1%.

The cause of this disease is still unclear, but likely involves a combination of genetic factors, environmental triggers and chance. Chronic inflammation of the synovial joints is accompanied by hyperplasia, autoantibody production, cartilage and bone destruction of which the underlying cause lies in immune regulatory factors such as the loss of tolerance. How these processes are linked to a localized onset of inflammation in the joint is still unclear but it involves immune effector cells including macrophages and osteoclasts, myeloid and plasmacytoid dendritic cells (DCs), B cells and T cells.

Figure 1 depicts a few key players and processes in the pathophysiology of RA: 1) B cells are involved in the regulation of immune responses through cytokine production and the production of antibodies such as rheumatoid factor (RF) and autoantibodies against citrullinated proteins (anti-CCP) and carbamylated proteins and also in antigen presentation to T cells.

2) Migrating monocytes from the blood home to tissues where they develop into M1-like macrophages releasing pro-inflammatory cytokines, e.g. TNFα, IL-18, IL-12 and IL-23. 3) Specialized bone-resident macrophages called osteoclasts become involved in bone remodeling and triggering bone loss via receptor activator of nuclear factor-kB (RANK) activation.

4) Crucial for the function of T lymphocytes are T-cell receptor (TCR) engagement, co-stimulation and distinct cytokine receptor ligation which in concert promote their activation and/or differentiation into specialized subsets. In particular, a CD4+ T-helper (Th) subset called Th17, which produces IL-17 and IL-21, in combination with regulatory T cells (Tregs), dominate the infiltrating T cell population in RA.

5) As the most powerful professional antigen presenting cells (APCs), dendritic cells (DCs) control other players and processes of the immune system through the release of regulatory or stimulatory cytokines, depending on their developmental stage and activation state. DCs, for instance, control peripheral tolerance by either inducing anergy or depletion of autoreactive T cells.
**CURRENT THERAPY OPTIONS IN RA AND A WINDOW FOR INNOVATIVE THERAPEUTICS**

Traditionally, early RA symptoms were relieved with nonsteroidal anti-inflammatory drugs (NSAIDs), e.g. ibuprofen, diclofenac, naproxen, but nowadays RA therapy is initiated early and aggressively with disease-modifying anti-rheumatic drugs (DMARDs) and/or biological agents. Table 1 gives an overview of several currently applied treatment options with either single agent DMARDs, combinations of DMARDs, and refined schedules of these, such as the recently reported COBRA-light schedule. In general, DMARDs have limited long-term efficacy and considerable toxicity at higher (effective) doses, which calls for switching to (expensive) biological agents when failing on DMARD therapy.
The list of biological agents includes several antibodies with neutralizing capacities to the pro-inflammatory cytokines tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and interleukin 6 (IL-6). Other biological agents which interfere with the underlying immune/inflammatory events include antibodies directed against the B cell specific surface molecule CD20 (to achieve B cell depletion) and antibodies against CTLA-4 and CD80/86 on antigen presenting cells (which inhibit co-stimulation of T cells). However, these agents are also restricted in their long term efficacy, for instance due to the onset of immunogenicity. Large clinical studies with long-term follow up demonstrated that the use of combinations of conventional DMARDs, particularly methotrexate (MTX), with biological agents was highly effective in achieving clinical remission and preventing radiological deterioration in approximately 50% of RA-patients, but the remaining 50% of patients still experienced insufficient disease activity reduction or sustained active disease. As such, in RA treatment there is still room for investigational drugs with novel mechanisms of action, such as for instance antibody- and small molecule-mediated targeting of specific cell types (T/B cells, macrophages, synoviocytes), cytokines and their receptors, and intracellular (signaling) pathways. Janus kinase (JAK) inhibitors and Spleen kinase (Syk) inhibitors represent examples of these latter drugs, displaying promising pre-clinical potential, but safety/toxicity issues as with biological agents also apply.

Beyond signaling pathways, the protein degradation pathway has also been recognized for potential therapeutic interventions. Figure 2 depicts the role of proteasomes and aminopeptidases as the key elements of protein degradation and peptide processing for either antigen presentation or complete hydrolysis to amino acids for reutilization in protein synthesis. Detailed background information on proteasomes, aminopeptidases and their therapeutic targeting is provided in two later chapters in this thesis. Briefly, from an anti-inflammatory/autoimmune disease perspective, proteasome inhibitors (PIs), with bortezomib (BTZ) as its main representative, represent another category of promising investigational drugs related to their ability to (a) inhibit the activation of NF-κB and transcriptional regulation of pro-inflammatory cytokine release, and/or (b) induce apoptosis of activated immune cells. Aminopeptidase inhibitors
Table 1. Overview of current and experimental therapeutics in RA

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Abbreviations: MTX: Methotrexate, SSZ: Sulfasalazine, HCHQ: Hydroxychloroquine, BTZ: bortezomib
Figure 2. (A) Cycle of protein synthesis and protein degradation: role of proteasomes and aminopeptidases. After initial synthesis, proteins at the end of their life-span, or damaged/misfolded, are subject to degradation after conjugating with a ubiquitin (Ub) tag. Recognition by the proteasome initiates protein degradation to smaller peptides which are further processed by aminopeptidases, depicted in (B) Complete hydrolysis of intracellular proteins into free amino acids that are reutilized for protein synthesis and to peptides presented to MHC class I molecules. Central to this process are the proteasome and aminopeptidases. (C) Mechanism of blockage of NFκB activation by bortezomib which prevents degradation of IkB, the nuclear localization of p50/p65 and thereby the transcription of pro-inflammatory cytokines/molecules. Abbreviations; aa: amino acid; TOP: thimet oligopeptidases; MHC: major histocompatibility complex. Modified from Saric et al. 2004.49

are less well studied in an autoimmune diseases setting but rational design and medical chemistry has come up with a new generation of aminopeptidase inhibitors that warrant further exploration.
Like for established therapeutics, a recurrent theme for experimental therapeutics will be the assessment of their long term efficacy following chronic administration and/or development of drug resistance related phenomena. In this thesis, these issues will be investigated at the level of immune-effector cells for a class of drugs that interferes in protein degradation, i.e. proteasome inhibitors and aminopeptidases. Since proteasome inhibitors and aminopeptidase inhibitors are also emerging drugs in cancer therapy, study outcomes and implications will be viewed not only from an autoimmune disease but also from a cancer perspective in terms of possible treatment applications.

**Aim and Outline of the Thesis**

The search for more efficacious, less toxic and less expensive drugs for the treatment of autoimmune diseases (such as RA) and common types of cancer is an ongoing challenging mission, in which investigational drugs with novel mechanisms of action are attractive candidates for detailed examination. Although treatment objectives differ in autoimmunity treatment (aimed at dampening the immune system to avoid reactions to self antigens) vs. cancer immunotherapies (aimed at boosting the immune system and eliminating malignant cells), there are drug targets and efficacy issues that can be of mutual interest. Indeed, as cancer immunotherapy in many ways represents the flip side of the immunology coin, aiming at the actual induction of a kind of autoimmunity (i.e. directed against tumor-related autoantigens), it holds high relevance, with many lessons to be drawn, for the treatment of autoimmunity.

In this thesis a central theme is to define molecular mechanisms that determine long-term efficacy or loss of efficacy to experimental drugs interfering with the protein degradation pathway, specifically proteasome inhibitors and aminopeptidase inhibitors. The next chapter provides an extensive overview of the available clinical and experimental drugs within this class of therapeutics and their respective mechanisms of action. The topic of drug efficacy and drug resistance was investigated in immune effector cells and human model systems of immune effector cells by assessing the impact of chronic drug administration on specialized functions of these immune effector cells and unraveling molecular mechanisms of drug resistance.
INTRODUCTION TO THE CHAPTERS

Chapter 2 covers a review on the diversity and relevance of proteasome subtypes in immune-competent cells in relation to autoimmune diseases. The review also provides an up-to-date overview of the several classes of proteasome inhibitors (PIs) designed and exploited for therapeutic interventions. Pre-clinical data related to the impact of the first clinically applied and registered PI Bortezomib (BTZ) and next generation PIs on functional properties of immune-effector cells (T-cells, B-cells, dendritic cells, macrophages, osteoclasts) are presented along with factors influencing long-term efficacy of the PIs. The current (pre)clinical status of PIs and future perspectives for their use as anti-inflammatory and anti-arthritis agents are also discussed.

In Chapter 3 we assessed the effects of long-term BTZ exposure in a cell line model system of human dendritic cell development (MUTZ-3). Investigations focused on the impact on DC differentiation and maturation and the assessment of alterations in signalling pathways implicated in DC development and function.

In Chapter 4 we examined the effects of long-term exposure to BTZ on the human B lymphoblastoid cell line JY. We unravelled the molecular basis of acquired resistance to BTZ in these cells and explored strategies to overcome resistance by next-generation PIs or by anti-CD20/rituximab-mediated complement-dependent cytotoxicity (CDC).

In Chapter 5 we investigated whether next-generation irreversible PIs, including carfilzomib, ONX 0912 and ONX 0914, might overcome two established BTZ resistance mechanisms in the myeloid THP-1 cell line, one involving point mutations in the PSMB5 gene encoding the proteasome β5 subunit to which BTZ binds, and another mechanism involving cellular extrusion by multidrug resistance efflux transporters of the family of ATP-Binding cassette transporters. The role of drug efflux transporters in relation to efficacy of proteasome inhibition was also examined on mononuclear blood cells from RA patients.
Chapter 6 reports on short-term effects of proteasome inhibition by BTZ and by the next-generation PI ONX 0914 on differentiation and maturation of inflammatory DCs generated from monocytes in the presence of IFN-α, which have previously been implicated in several autoimmune diseases.

Aminopeptidases operate downstream of the proteasome in the cellular process of protein degradation. Chapter 7 covers an overview of various types of aminopeptidase and their roles in immune-effector cells and malignant cells. The positioning of aminopeptidase inhibitors in cancer and autoimmune disease therapy schedules is discussed.

In Chapter 8 we investigated the molecular mechanism underlying acquired resistance to the aminopeptidase inhibitor prodrug CHR2863 in human U937 myelomonocytic cells. Multifactorial and novel mechanisms contributing to CHR2863 resistance are discussed.

In Chapter 9 a general discussion is provided on the data resulting from the studies presented in this thesis. Short- and long-term effects of (immuno)proteasome and aminopeptidase inhibitors on immune effector cell function in relation to therapeutic efficacy in autoimmune and malignant disease treatment is discussed as well as future perspectives for these types of drugs.

Chapter 10 will cover an English summary of the work presented in this thesis, while Chapter 11 include Dutch and Papiamentu summaries.