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**Immunological and Clinical Long-Term
Effects of Idiotype Vaccination in
Multiple Myeloma Patients**

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بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِیْمِ

وما اوتیتم من العلم الا قليلا

(No matter how much you know, there is still much more to know)

*To those who fought cancer when no
treatment option was offered
and those now living with cancer
when hope for cure may be next door.
We promise to keep knocking that door.*

Table of contents

ABSTRACT	iii
LIST OF ABBREVIATIONS	iv
LIST OF PUBLICATIONS	vi
1 MULTIPLE MYELOMA (MM)	1
1.1 Introduction	1
1.2 History	1
1.3 Epidemiology.....	2
1.4 Etiology	2
1.5 Cytogenetics	3
1.6 Pathogenesis	4
1.7 Clinical manifestations.....	5
1.8 Diagnostic criteria and clinical staging.....	5
1.9 Prognostic factors.....	7
1.10 Current therapy for MM.....	7
1.10.1 Drug therapy and stem cell transplantation.....	7
1.10.2 Donor lymphocyte infusion (DLI)	8
1.10.3 Supportive therapy	8
1.11 Novel therapeutics	9
1.11.1 Thalidomide and its analogues	9
1.11.2 Proteasome inhibitors	10
1.11.3 Arsenic trioxide (As_2O_3).....	11
1.11.4 Other potential new agents	11
2 TUMOR IMMUNOLOGY	14
2.1 Tumor-induced immune responses	14
2.1.1 Tumor antigens	14
2.1.2 Characteristics of tumor-induced immune response.....	14
2.2 Cancer immunosurveillance.....	16
2.3 Cancer immunoediting (immunoselection)	18
2.4 Cancer immunosubversion.....	18
2.4.1 Production of immunosuppressive factors	19
2.4.2 Promotion of T_{reg} cells differentiation.....	19
3 THE IMMUNE SYSTEM AND MM	22
3.1 Origin of the myeloma cell.....	22
3.2 Phenotype of the myeloma cell.....	23
3.3 The immune system in MM.....	24
4 IMMUNOTHERAPY IN MM	27
4.1 General consideration	27
4.2 Idiotype specific immunotherapy in MM	29
4.2.1 Preclinical animal models of Id immunization in MM.....	29
4.2.2 Idiotype specific immunity in human MM.....	30
4.2.3 Idiotype immunization in MM patients	31
5 AIMS OF THE THESIS	37
6 MATERIAL AND METHODS	38
6.1 Patients and healthy donors	38
6.2 Preparation of monoclonal IgG and F(ab') ₂ fragments	38

6.3	Preparation of Id vaccine	38
6.4	Immunization protocol and immune testing times.....	39
6.5	Isolation of peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC).....	39
6.6	Isolation of CD4 and CD8 T cells.....	39
6.7	Proliferation assay.....	40
6.8	Enzyme-linked immunospot assay	40
6.9	Delayed type hypersensitivity (DTH) reaction	40
6.10	Intracellular cytokine (IFN- γ) assessed by flow cytometry (CFC).....	41
6.11	Cell phenotyping by flow cytometry and direct microscopy	41
6.12	Cytometric bead array (CBA)	42
6.13	Total RNA extraction and first strand cDNA synthesis.....	42
6.14	Genomic DNA preparation	42
6.15	Quantitative real-time polymerase chain reaction (QRT-PCR) for cytokines and cytotoxic proteins	42
6.16	Criteria for clinical response, vaccine induced Id-specific T cell response and Th ₁ /Th ₂ immune responses.....	43
6.17	Gene cloning and generation of allele specific oligonucleotide (ASO) probes and primers	44
6.18	Reverse transcription and real-time allele specific oligonucleotide polymerase chain reaction (RT-ASO-PCR and real-time ASO-PCR)	45
6.19	Statistical methods	45
7	RESULTS AND DISCUSSION.....	47
7.1	Background.....	47
7.2	Paper I.....	47
7.3	Paper II.....	48
7.4	Paper III	49
7.5	Paper IV	50
7.6	Paper V	51
7.7	Conclusion.....	51
8	FUTURE PROSPECTS.....	53
9	ACKNOWLEDGMENTS.....	55
10	REFERENCES.....	59

PAPERS I-V

ABSTRACT

In spite of the promising results shown by new anti-myeloma agents, multiple myeloma (MM) remains incurable and additional therapy to overcome the inevitable disease recurrence is greatly needed. Immunotherapy is currently under evaluation as a novel alternative or complementary therapy in many cancer types. The idiotype (Id) protein is a unique myeloma specific antigen that may be targeted in therapeutic vaccination. This thesis presents and discusses results of Id vaccination in early stage MM patients and underscores the immunological and clinical effects of the vaccine.

In the first study we analyzed the time kinetics of cytokine genes expression (IL-2, IL-5, IFN- γ , GM-CSF and TNF- α) and granzyme B in healthy donors to be used as supplementary markers for antigen-specific T lymphocytes in subsequent studies. For most cytokines, the time for maximum accumulation seemed to be obtained after 4 to 8 h of activation. However, a sustained high level could be noticed for up to 24 h. Granzyme B gene expression showed a continuous gradual increase and late maximal accumulation (48-72 h). We concluded that cytokine genes expression would better be measured after 4-8 h of specific stimulation, but also up to 24 h of stimulation is acceptable. Granzyme B may preferentially be measured after 48 h of activation.

In the second study, Id-specific T cell responses were evaluated by multiple read-out systems in 18 patients vaccinated with the Id together with either interleukin (IL)-12 alone or a combination of IL-12 and granulocyte macrophage colony stimulating factor (GM-CSF). IL-12 alone was noted to induce a Th₁ polarized immune response, while the combination of IL-12 and GM-CSF induced a significantly higher frequency of responding patients but with a Th₂ profile.

In the third study Id-specific T cell responses were monitored simultaneously in peripheral blood and bone marrow of 10 patients. Id-specific responses we found to occur at a similar frequency of patients in both compartments. Comparison of the responses during active immunization with those at the late follow-up showed that the responses decreased significantly by time and shifted from a Th₁ to a Th₂ profile.

In the fourth study, 28 patients were immunized as indicated earlier over 110 weeks. Id-specific T cell responses were noted in 33% of patients in the IL-12 group and 85% in the GM-CSF/IL-12 group ($p = 0.003$). Two third of the responsive patients subsequently lost their specific immunity while developing progressive disease. Median time to disease progression (TTP) was found to be significantly longer in immune responders compared to non-responders. Immune non-response was associated with an increase in the numbers of CD4⁺/CD25⁺ cells (T_{reg} cells). Two patients in the IL-12 group had a clinical response ($> 50\%$ and $> 25\%$ reduction of their respective M-component concentrations).

In the last study patients were monitored for an Id-specific T cell response, and the presence of circulating myeloma B cells (CMC) by real time ASO-PCR during a median time of 46 weeks of maintained Id vaccination. Reduction and/or stable levels of CMC were observed in 6/11 patients. Three patients showed progressive increase in the number of CMC and in 2 patients CMC could not be detected. Patients ($n=6$) who showed a reduction and/or a stable CMC level mounted an Id-specific T cell response, while those with increasing numbers of CMC ($n=3$) failed to mount tumor specific T cell immunity ($p < 0.02$).

Taken together, these results indicate that Id immunization in early stage MM patients can induce tumor-specific immune responses that may correlate with reduction of CMC as well as TTP, and clinical responses may also occur. Immune non-response may be associated with increased numbers of T_{reg} cells and progressive disease. Adjuvant cytokines can be a versatile tool for manipulating and directing the anti-tumor immune response.

Key words: Idiotype, vaccination, multiple myeloma, T cell response.

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LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
ASCT	Autologous stem cell transplantation
ASO-PCR	Allele specific oligonucleotide polymerase chain reaction
BM	Bone marrow
BMMC	Bone marrow mononuclear cells
CBA	Cytometric bead array
CD	Cluster of differentiation
CDR	Complementary determining region
CEA	Carcinoembryonic antigen
CFC	Cytokine flow cytometry
CLL	Chronic lymphocytic leukemia
CMC	Circulating myeloma B cells
ConA	Concavalin A
COX2	Cyclooxygenase 2
CR	Complete response
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic lymphocyte antigen 4
D	Diversity
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DTH	Delayed type hypersensitivity
EBV	Epstein Barr virus
EGFR	Epidermal growth factor receptor
ELISPOT	Enzyme-linked immunospot
ELISA	Enzyme-linked immunosorbent assay
EMBT	European group for blood and bone marrow transplantation
EpCAM	Epithelial cell adhesion molecule
F(ab)	Fragment antigen binding
FACS	Fluorescence activated cell sorting
FasL	Fas Ligand
Fc	Fragment crystalizable
FcγR	Fc gamma receptor
FOXP3	Forkhead box P3
FR	Framework region
GM-CSF	Granulocyte macrophage-colony stimulating factor
GvHD	Graft versus host disease

GvM	Graft versus myeloma
HBV	Hepatitis B virus
HD	Hodgkin disease
HDT	High-dose therapy
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
ICAM-1	Intercellular adhesion molecule-1
Id	Idiotype
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgH	Immunoglobulin heavy chain
IL	Interleukin
IMiDs	Immunomodulatory drugs
J	Joining
κ	Kappa
KIR	Killer immunoglobulin-like receptor
KLH	Keyhole limpet hemocyanine
λ	Lambda
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MIP-1 α	Macrophage inflammatory protein-1 alpha
MM	Multiple myeloma
MOPC	Mineral oil-induced plasmacytoma
MP	Melphalan-prednisone
MR	Minor response
MRD	Minimal residual disease
MSC	Myeloid suppressor cells
MUC-1	Mucin-1
NCAM	Neural cell adhesion molecule
NF	Nuclear factor
NHL	Non-Hodgkin's lymphoma
NK	Natural killer cell
NKT	Natural killer T lymphocyte
NSCLC	Non-small cell lung carcinoma

OAF	Osteoclast activating factor
OPG	Osreoprotegerin
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PFS	Progression-free survival
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PI3-k	Phosphatidyl inositol 3-kinase
PPD	Purified protein derivative
QRT-PCR	Quantitative real-time polymerase chain reaction
PR	Partial response
RAG	Recombination activating genes
RANKL	Receptor activator of NF- κ β
RT	Reverse transcription
SCID	Severe combined immunodeficiency
SDF-1 α	Stromal-cell-derived factor-1 alpha
SFU	Spot forming units
SI	Stimulation index
SMM	Smoldering multiple myeloma
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigen
TCR	T cell receptor
TGF- β	Transforming growth factor beta
Th	T helper
TNF- α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TSA	Tumor specific antigen
TTP	Time to progression
V	Variable
VAD	Vincristine-doxorubicin-dexamethasone
VCAM-1	Vascular adhesion molecule-1
VDJ	Variable diversity joining
VEGF	Vascular endothelial growth factor
VH	Variable heavy
VL	Variable light
VLA-4	Very late antigen-4
5TMM	5Tmurine myeloma

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their roman numerals:

- I. **Abdalla AO**, Kiaii S, Hansson L, Rossman ED, Jeddi-Tehrani, Skokri F, Österborg A, Mellstedt H, Rabbani H. Kinetics of cytokine gene expression in human CD4⁺ and CD8⁺ T-lymphocyte subsets using quantitative real-time PCR. *Scand J Immunol.* 2003; 58 (6): 601-6.
- II. **Abdalla AO**, Hansson L, Eriksson I, Näsman-Glaser B, Rossmann ED, Rabbani H, Mellstedt H, Österborg A. Idiotype protein vaccination in combination with the adjuvant cytokines IL-12 and GM-CSF in patients with multiple myeloma - evaluation of a T cell response by different read-out systems. *Haematologica.* 2007; 92 (1): 110-4.
- III. **Abdalla AO**, Hansson L, Eriksson I, Näsman-Glaser B, Mellstedt H, Österborg A. Long-term effects of idiotype vaccination on the specific T cell response in peripheral blood and bone marrow of multiple myeloma patients. (*Submitted*)
- IV. Hansson L, **Abdalla AO**, Moshfegh A, Choudhury A, Rabbani H, Nilsson B, Österborg A, Mellstedt H. Long-term Idiotype Vaccination Combined with IL-12, or IL-12 and GM-CSF, in Early Stage Multiple Myeloma Patients. *Clin Cancer Res.* 2007; 13 (5) pp....
- V. **Abdalla AO**, Kokhaei P, Hansson L, Rabbani H, Österborg A, Mellstedt H. Idiotype vaccination induced reduction/elimination of circulating tumor cells in patients with multiple myeloma. (*Manuscript*)

1 MULTIPLE MYELOMA (MM)

1.1 Introduction

Multiple myeloma (MM) is a B cell neoplasm that is characterized by clonal proliferation of plasma cells and their precursors in the bone marrow (BM) (1). The hallmark of the disease is the presence of a monoclonal immunoglobulin (Ig) in the serum and/or urine (2). In early stages, MM is asymptomatic and often signaled by high erythrocyte sedimentation rate in routine blood analysis. In more advanced disease the most common symptoms are bone pain, fatigue, and recurrent infections. Diagnosis requires the presence of at least 10 percent plasma cells (PCs) in BM and a monoclonal Ig protein in the serum or urine (2).

Despite initial high response rate achieved by high-dose chemotherapy and the promising results shown by newly developed novel anti-myeloma agents the disease remains incurable and there is a great need for additional therapy to overcome the inevitable disease recurrence.

1.2 History

The first described case of MM came to light on Saturday, November 1st 1845 when Dr. William Macintyre, a London leading consultant physician, sent a urine sample along with the following quotation to Dr. Henry Bence Jones, a young chemical pathologist (3);

Dear Dr Jones,

The tube contain urine of very high specific gravity, when boiled it becomes slightly opaque. On the addition of nitric acid, it effervesces, assumes a reddish hue, and becomes quite clear; but as it cools, assumes the consistence and appearance which you see. Heat reliquifies it. What is it? (4).

The patient contributing to the urine sample was Mr. Thomas Alexandar McBean who had a history of progressive pain in the chest, back, and loins along with urinary frequency and fatigue for about one year duration. Dr. Bence Jones tested the urine and identified the heat properties of, what is known today as urinary light chains or Bence Jones protein, as those of an oxide of albumin. He also calculated that the patient could be excreting as much as 67 g of the substance per each day. Mr. McBean died in January 1846 and autopsy material from McBean's bones, revealed soft and brittle ribs, sternum and vertebrae that were filled with a gelatinous matter, a condition at that time designated as mollities ossium (5). Microscopic examination of the gelatinous substance demonstrated that it consisted mainly of oval cells with one

or two nuclei consistent with the appearance of myeloma cells (6). The term multiple myeloma was introduced in 1873 by J. von Rustizky (7), but the classical illustration of MM as a clinical entity was first provided by Kahler in 1889 when he described a case of a fellow physician (8). Wright was the first to point out that MM tumors consisted of plasma cells (9). In 1917, the protein described by Bence Jones was reported to occur concomitantly in both serum and urine of affected patients (10). In 1928 Perlzweig et al reported that serum protein levels in MM patients could actually be elevated (11) and a thorough description of the disease was performed using all reported cases up to that time (12). In 1939, myeloma serum globulins were separated by electrophoresis and the tall narrow (church-spike) peak within the γ -region now designated as the M-component was described (13). Dr. Macintyre question was finally answered when Korngold and Lipari in 1956 demonstrated the antigenic similarity between Bence Jones protein and the light chains prepared from normal as well as MM serum gamma-globulins (14). The two major classes of Bence Jones proteins have been designated kappa (κ) and lambda (λ) as a tribute to Korngold and Lipari.

1.3 Epidemiology

MM is a common neoplasm constituting 1% of all cancers, 10-15% of hematological malignancies and contributing to 2% of all cancer deaths (15). The incidence rates of MM show considerable international variations and increase with advancing age (16). At diagnoses, the median age is approximately 68 years and more than 50% of the patients are over the age of 70 years while only 15% are under 60 years (17). Sweden has one of the highest incidence rates with 60 new cases identified per million of inhabitants each year (18). The disease is rare under the age of 40 years. MM incidence rate is also significantly affected by race and gender. It is more common in the black race, followed by Moaris, Hawaiians, Israeli Jews, northern Europeans, US and Canadian whites, respectively (19). The lowest rates occur in the Middle East, Japan, and China (20). MM is also significantly higher in males than females among both, black and white populations (21).

1.4 Etiology

The cause of MM is not known. Evidences of environmental predisposing factors are murky. The strongest single environmental factor linked to an increased risk of developing MM is ionizing radiation (22). However further studies on nuclear bomb survivors in Japan found no such relation (23, 24). Other factors that have even more mismatching link with increased risk for myeloma disease are smoking (25, 26), exposure to metals, agricultural chemicals, benzene and other petroleum products (22,

27, 28). A direct genetic linkage to the etiology of MM has not yet been established (29). However, the remarkable difference in the incidence rates between different races and the preservation of these incidence patterns regardless of migration to various geographical areas, suggest that susceptibility to MM may be determined by hereditary and genetic rather than environmental factors. A study from the national data base of familial cancer in Sweden has shown that males having fathers with cancer have a relative risk of 3.86 to develop MM (30). Moreover, a substantial familial clustering of MM has been reported in several studies (31-34) as well as a significant association of the HLA-Cw2 allele with occurrence of MM (35).

1.5 Cytogenetics

The malignant PCs (myeloma cells) carry multiple and complex chromosomal abnormalities that may proportionally increase with disease progression. Microarray analysis have shed more light into the sequential genetic changes from normal to malignant PCs and the multistep transformation of monoclonal gammopathy of undetermined significance (MGUS) to MM (36). Also recent advances in cytogenetic techniques have revealed IgH translocations and aneuploidy state as possible early genetic signatures of MM. The commonly observed numerical cytogenetic anomalies in MM are monosomies which usually involve chromosomes 13, 14, 16, and 22, and trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. These could broadly be categorized as hyperdiploid or non hyperdiploid (hypodiploid and pseudo-hypodiploid) states. Tetraploid features may also occur (37). The most frequently seen structural cytogenetic abnormalities in MM are translocations of IgH genes [t(11;14)(q13;q32), t(4;14), t(14,16), t(6;14)] mediated by VDJ recombination errors. These translocations may increase in frequency with disease progression (38) and result in activation of cyclins D1, D2, and D3 or myeloma Set domain (MMSET) and Fibroblast Growth factor Receptor 3 (FGR3) genes. (37). They may be responsible for the primary and earliest oncogenic event conferring survival and proliferative advantage for the developing malignant plasma cells (37). However, they are not by themselves sufficient for disease progression and a secondary set of translocations and/or other chromosomal anomalies (e.g. involving c-myc, RAS, and p53) may provide the necessary second additional hit for the transformation and expansion of the malignant plasma cell clone (37). Monosomy and interstitial deletions are the most recurrent anomalies of chromosome 13 and have been associated with shorter survival (37). Other abnormalities that have been associated with advanced disease and poor prognosis are deletions in the p53 locus (17p13) and activating RAS mutations (37).

1.6 Pathogenesis

An enormous progression has been made recently in understanding the biology of MM. Myeloma cells interaction with host and BM microenvironment has been shown to have a pivotal role in disease progression and drug resistance. The BM microenvironment is composed of extracellular matrix proteins and BM supportive cells (BM stromal cells, osteoclasts and osteoblasts). The crosstalk and interactions between these elements and myeloma cells determine proliferation, migration and survival of the malignancy as well as its acquisition of drug resistance. Through a plethora of adhesion molecules, myeloma cells adhere to the matrix protein fibronectin inducing cytokine independent drug resistance and inhibiting FAS-mediated apoptosis (39, 40). Myeloma cells also adhere to BM stromal cells mediating resistance to drug-induced apoptosis and enhancing nuclear factor (NF)- κ B-dependent transcription and secretion of interleukin 6 (IL-6) (39-41). IL-6, the major cytokine mediating MM cell growth, proliferation and survival (40), is produced by both myeloma cells and BM stromal cells. It exerts its effects by triggering at least two separate intracellular cascades; the JAK/STAT and the RAS/MAPK signaling pathways (40, 42). Myeloma cells and BM stromal cells also secrete other cytokines in the BM milieu that ultimately support disease progression. The most important of these cytokines are insulin like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF- α), transforming growth factor-beta (TGF- β), IL-10 and stromal-cell-derived factor-1 α (SDF-1 α). This dynamic network of cytokines mediate myeloma cell growth, proliferation, survival, drug resistance and migration (39-41, 43). VEGF together with basic fibroblast growth factor (bFGF) also triggers and mediate angiogenesis, an obligatory event for tumor growth, invasion and progression (42).

The myeloma cells and BM stromal cells also secrete local osteolytic factors collectively known as osteoclast activating factors (OAFs). These include IL-1 β , TNF- α and the central cytokine IL-6. Recently, other factors have come into focus. The receptor activating factor NF- κ B ligand which promote bone resorption and its soluble antagonist osteoprotegerin (RANKL/OPG system) as well as the chemokine macrophage inflammatory protein-1 alpha (MIP- α) are now known to play a pivotal role in myeloma bone disease (44, 45). An imbalance in the bone marrow environment of the RANKL/OPG ratio in favor of RANKL and the direct stimulatory action of MIP- α on osteoclast precursors to differentiate into osteoclasts (46, 47) are important mechanisms for the development of osteolytic bone lesions. Moreover, myeloma cells also produce the dickkopf 1 protein (DKKI) that inhibits osteoblasts differentiation (48). Collectively, these factors enhance myeloma bone disease.

1.7 Clinical manifestations

The clinical symptoms of MM are related to the proliferative capacity and accumulation of myeloma cells in the BM and secretion of paraproteins. Early stage MM is in most cases asymptomatic and detected by the finding of high erythrocyte sedimentation rate upon routine blood testing. In more advanced disease the most common symptom is bone pain affecting around 70% of patients, followed by fatigue and recurrent bacterial infections. The bone lesions of MM are caused by infiltration of BM by myeloma cells leading to imbalanced over activity of osteoclasts that destroy the bone. Susceptibility to bacterial infections, in particular pneumonias and urinary tract infections, is due to mainly a deficiency of normal polyclonal Igs involving IgM, IgG, and the IgA subtypes. Anemia occurs in about 80% of patients, and may contribute to the profound fatigue commonly seen in advanced disease. It is related mainly to inhibition of hematopoiesis by inflammatory cytokines and impaired endogenous erythropoietin production (49). Renal failure occurs in approximately 25% of myeloma patients. Glomerular deposits of amyloid, hyperuricemia, hypercalcemia, tubular damage associated with urinary excretion of light chains and the occasional infiltration of the kidney by myeloma cells all may contribute to renal dysfunction. Symptomatic polyneuropathy is observed in 5-15% of myeloma patients and may be due to the paraneural deposition of amyloid. Subclinical neuropathy is found in about 50% of patients.

1.8 Diagnostic criteria and clinical staging

Diagnostic criteria for MM require the presence of at least 10 percent plasma cells in BM and a monoclonal Ig protein (M-protein) (usually > 30 g/L in the serum) or urine (2). In addition hypercalcemia, renal insufficiency, anemia, and bone lytic lesions may be present.

Overt symptomatic MM must be distinguished from MGUS and asymptomatic MM since the latter two conditions may remain stable for a long time and require no treatment (50-52). MGUS is characterized by the absence of symptoms, M-protein serum level of less than 30 g/L, less than 10% plasma cells in the BM and absence of bone lytic lesions, anemia, hypercalcemia, or renal insufficiency. In asymptomatic MM the M-protein serum level is 30 g/L or greater, the frequency of plasma cells in the BM is 10% or more, and there is no lytic bone lesions, anemia, or hypercalcemia. The proliferative process is of low grade with a very low plasma cell labeling index. In symptomatic MM the frequency of plasma cells in the bone marrow exceeds 10% and in addition to other diagnostic criteria evidence of end organ damage prevails (50, 53). Criteria differentiating symptomatic MM from MGUS or asymptomatic MM are summarized in Table 1.

Since the 70's the most commonly used staging system for MM has been the Durie and Salmon staging criteria (54). However, a newer International Staging System (ISS) that may also reflect the biology of disease and based on two common prognostic features has recently been developed (55) (Table 2).

Table 1. Significant variables differentiating symptomatic MM from MGUS or asymptomatic MM.

Variable	MGUS	Asymptomatic MM	Symptomatic MM
Skeletal destruction or other organ dysfunction*	–	–	++++
Frequency of plasma cells in the bone marrow	< 10 %	> 10 %	++++
M-component concentration	< 30 g/l	> 30 g/l	> 30 g/l
Increasing M-component concentration	–	+	+++
Monoclonal light chains in the urine**	+	+	+++
Subnormal concentrations of normal immunoglobulins (hypogammaglobulinemia)	++	+++	+++

* Anemia, hypercalcemia, renal insufficiency, bone lesions or amyloidosis

** Monoclonal light chains > 1 g/l is often myeloma-related. Occasionally in patients with MGUS the concentrations of urinary light chains may be between 0,5-1 g/l.

Table 2. International Staging System

Stage	Criteria	Median Survival (months)
I	Serum β_2 -microglobulin < 3.5 mg/L Serum albumin \geq 3.5 g/dL	62
II	Not stage I or III*	44
II	Serum β_2 -microglobulin \geq 5.5 g/L	29

*There are two categories for stage II: serum β_2 -microglobulin < 3.5 mg/L but serum albumin < 3.5 g/dL; or serum β_2 -microglobulin 3.5 to < 5.5 mg/L irrespective of the serum albumin level (55).

1.9 Prognostic factors

The individual most powerful prognostic marker is the serum level of β 2-microglobulin, which is a single variable that measures a combination of indices; cell proliferation, cell mass, and renal function. Genetic factors are also important prognostic markers. Favorable prognostic markers include a β 2-microglobulin level < 2.5 mg/L, absence of deletion or monosomy of chromosome 13, and t(11;14). Prognostic markers related to an adverse outcome include increase in plasma cell labeling index, increased levels of serum β 2-microglobulin, and circulating myeloma cells. Complete deletion of chromosome 13 or its long arm, t(4;14) as well as increased density of BM microvessels are also adverse prognostic factors (2).

1.10 Current therapy for MM

1.10.1 Drug therapy and stem cell transplantation

Anti-tumor therapy is currently offered to all patients with advanced or symptomatic disease to reduce tumor cell burden and reverse disease complications such as back pain, renal failure, hypercalcemia, hyperviscosity, and infection. Changes in the level of serum M-protein and/or serum and urinary light chains quantities form the bases of assessing response to therapy and monitoring the progress of disease according to criteria set up by the Myeloma Subcommittee of the European Group for Blood and Marrow Transplantation (EBMT) (56).

With the advent of many newly developed anti-myeloma agents a variety of treatment options are currently being evaluated and geared to optimize therapy for different patient groups and disease stages.

High dose chemotherapy (HDT) supported by autologous stem cell transplantation (ASCT) is still the standard therapy for MM patients below 65 years of age (57). It has substantially increased the frequency of remission and improved progression-free and overall survivals compared to conventional melphalan-prednisone (MP) or vincristine, doxorubicin, dexamethasone (VAD) chemotherapy (39, 57). However, MP-based therapy (possibly in combination with thalidomide) may still be the treatment of choice for elderly patients with symptomatic disease who are not eligible for HDT/ASCT treatment.

Patients eligible for HDT/ASCT should avoid using alkylating agents as these agents may have cumulative toxic effects on the stem cell harvest (58, 59). In the past, patients were commonly treated with a 3 months VAD induction therapy. However, this regimen necessitates the use of indwelling venous catheterization with risk of related infection and thrombo-embolic events and hence a combination of thalidomide and dexamethasone may be a prudent alternative therapy. In Sweden, cyclophosphamide in combination with high dose steroids is commonly used as

induction treatment and, once the stem cell harvest is secured, melphalan 200 mg/m² is the most widely used conditioning regimen (53). Therapy may be continued until the patient reaches a plateau state (stable level of monoclonal protein in serum and/or urine with no other signs of disease progression).

Double and tandem ASCT appears to be superior to single ASCT (60, 61). Allogeneic stem cell transplantation (SCT) was thought to have potential for cure due to the graft versus myeloma (GvM) effect. A high risk of transplant related death due to Graft-versus Host Disease (GvHD) and few eligible patients due to age and HLA type limitations are the main drawback of this treatment option and the reason why it is not recommended as standard therapy (2, 39). However, if given early in the course of the disease, allogeneic SCT may yield molecular remissions (62) and about one third of the patients remain free of disease 6 years later (63). Furthermore, the possibility to obtain new remissions following infusion of donor lymphocytes (DLI) is a major therapeutic advantage (61, 64-66). Transplant related mortality has also been reduced in the more recently performed studies reflecting better patients selection and improved transplant procedures (61). The objective of the forthcoming studies on allogeneic SCT in MM is to reduce transplantation related mortality while still harnessing GvM effect.

The use of reduced intensity conditioning (RIC) non-myeloablative transplantation (mini-allotransplants) is an attractive emerging alternative and may be an option for older patients. Safety and efficacy of this approach are currently under evaluation in clinical trials. Preliminary results are encouraging (67, 68), but longer follow-up is needed.

1.10.2 Donor lymphocyte infusion (DLI)

DLI can induce a response rate of 40-52% in patients with MM (69). It is shown to induce complete and partial remissions in MM patients relapsing after allogeneic BM and SCT transplantations (64, 70, 71). The clinically most relevant treatment related morbidity with DLI is GvHD. Graft failure, infections and immune escape of extramedullary plasmacytoma have also been reported (69). Different clinical strategies to preserve GvM effect while reducing GvHD after DLI include infusion of limited number of donor T lymphocytes, sequential infusion of increasing number of donor T lymphocytes and infusion of selected subsets of donor T cells (72). However, more studies are needed to improve the safety and efficacy for this approach.

1.10.3 Supportive therapy

Bisphosphonates constitute the mainstay for the long-term control of bone disease, a major cause of morbidity and mortality, in MM patients. The use of oral clodronate

and intravenous pamidronate have reduced bone related complications. These agents should be offered to all patients with symptomatic disease for at least 2 years. Osteonecrosis of the jaw may be a risk, especially following zoledronic acid therapy, in particular if extensive dental surgery was performed (73-75). Other supportive treatments include the use of recombinant human erythropoietin (epoetin) (49, 76, 77) to correct anemia, and radiation therapy as palliative treatment for localized lytic lesions and pathologic fractures of long bones, and spinal cord compression.

The median survival is still not more than 4-5 years (40) and almost all patients who initially achieve complete remission eventually relapse and exhibit drug resistance (40, 78).

1.11 Novel therapeutics

1.11.1 Thalidomide and its analogues

The rebirth of thalidomide as a treatment for MM originated from its anti-angiogenic properties. However, extensive research revealed that thalidomide exhibits multiple anti-myeloma activities. It inhibits angiogenesis by blocking VEGF and/or bFGF growth factors. It suppresses TNF- α secretion, inhibits NF- κ B activity, and acts directly on drug resistant MM cells by inducing G1 growth arrest and apoptosis by activation of caspase 8 (39, 40, 43, 79, 80). Thalidomide also induces a Th₁ cellular response and increases natural killer (NK) anti-myeloma cytotoxic activity (40, 81). Moreover, thalidomide interferes with the interactions between MM cells and the BM microenvironment by modulating the expression of cell adhesion molecules and disrupting the cytokine network that orchestrate disease progression (39, 40). Thalidomide is currently an essential component of the standard therapy for relapsed and refractory MM (2). It has been shown to be effective as a single agent and as well as in combination with dexamethasone and/or other alkylating agents (82). The response rate in relapsed myeloma to thalidomide alone is 30%, in combination with steroids about 50%, and around 75% when combined with alkylating agents (83-85). Two randomized controlled clinical trials have recently shown a survival advantage for thalidomide as first line treatment for elderly patients when combined with melphalan and prednisone or used as maintenance therapy (86-88). However, venous thromboembolism has emerged as a rather frequent complication of thalidomide when used together with other agents in newly diagnosed untreated patients (2, 39). Other adverse effects include peripheral neuropathy, sedation, fatigue, and constipation. Side effects usually diminish/disappear upon dose reduction but discontinuation should be considered before neurological damage becomes irreversible.

Structural analogues to thalidomide, immunomodulatory drugs (IMiDs), have also been developed. They are more potent than thalidomide, and seem to have a better safety profile. They might cause reversible, grade 3-4 myelosuppression but no significant sedation, fatigue, constipation and/or peripheral neuropathy (2, 42, 82). IMiDs are so called because of their capacity to expand T cell proliferation with increased secretion of interferon γ (IFN- γ) and interleukin 2 (IL-2). They also augment anti-myeloma natural killer cell activity and antibody-dependent cell-mediated cytotoxicity (ADCC) (42, 82). IMiDs predominantly trigger the caspase-8 mediated apoptotic signaling pathway, enhance MM cell sensitivity to FAS and TRAIL-induced apoptosis, and down-regulate NF- κ B activity (43). Lenalidomide (Revlimid®) is an example of such agents which has undergone rapid clinical development in MM and is entering routine clinical use (recently approved by FDA for use in patients with relapsed disease) (89).

1.11.2 Proteasome inhibitors

The 26S proteasome is a multicatalytic enzyme complex present in the cytoplasm and nucleus of all eukaryotic cells. It is responsible for the synchronised degradation of intracellular proteins, including those regulating cell cycle and cell survival, a fundamental metabolic process essential for cellular homeostasis (57, 78). Cancer cells were found to be more susceptible to proteasome inhibitors due to dysregulated cell cycle control (78). Bortezomib (Velcade®), the prototype of proteasome inhibitors, is a potent reversible inhibitor with a high affinity and specificity for the catalytic activity of the proteasome (39, 78, 90). The antimyeloma effects of bortezomib involve a combination of effects on pro-apoptotic and anti-apoptotic pathways (78). Mitsiades et al. (80) demonstrated that bortezomib targets MM cells by up-regulating pro-apoptotic cascades (e.g., mitochondrial/caspase 9 and JNK/FAS/caspase 8) and down-regulating the transcription of molecules promoting cell growth and survival. Further studies have confirmed that bortezomib induces apoptosis in drug resistant MM cells, down-regulates the expression of adhesion molecules, inhibits angiogenesis, and blocks constitutive and MM cell adhesion-induced NF- κ B-dependent cytokine secretion in BM stromal cells (40). The adverse effects of bortezomib are mild and well tolerated (57). Drug related gastrointestinal toxicities and fatigue are manageable. Thrombocytopenia and neuropathy occur mainly in patients in whom these conditions are pre-existent (39, 40). Recent clinical studies demonstrated that bortezomib had remarkable anti-tumor activity in refractory and relapsed MM (39, 78, 91). Bortezomib is also under evaluation in phase III trials for the treatment of patients with newly diagnosed MM and as maintenance therapy.

1.11.3 Arsenic trioxide (As₂O₃)

Arsenic trioxide has shown remarkable clinical effects in patients with acute promyelocytic leukemia and shown to affect pathways involved in the pathogenesis of MM (57). It has been shown to overcome the anti-apoptotic effects of IL-6 and could induce apoptosis in drug resistant MM cell lines and fresh myeloma cells by activation of caspase 9 (40, 41). It also inhibits the binding of MM cells to BM stromal cells and blocks NF-κB activation (40, 41) Furthermore arsenic trioxide augments the killing of MM cells by lymphokine activated killer cells (40, 41) suggesting an immunomodulatory mechanism of action as well. Arsenic trioxide mediated cytotoxicity of MM cells may be enhanced by ascorbic acid and dexamethasone (40). Results of clinical trials of As₂O₃ in patients with refractory MM are encouraging and indicate an acceptable safety profile (92-95).

1.11.4 Other potential new agents

2-Methoxytradiol (2ME2) inhibits angiogenesis by blocking VEGF and IL-6 secretion in the BM microenvironment (39). It induces apoptosis of drug resistant MM cells, overcomes the protective effects of IL-6 and IGF-1, and enhances dexamethasone induced apoptosis (40). The apoptotic effect of 2ME2 is mediated by mitochondrial release of Smac and cytochrome C proteins followed by the activation of the caspase cascade (39, 40).

Lysophosphatidic acid (LPA) acyltransferase-β inhibitors were shown to have potent cytotoxic activity against MM cell lines and fresh myeloma cells. This group of compounds mediate apoptosis through activation of caspases and cleavage of poly (ADP-ribose) polymerase in MM cells (39, 40).

NF-κB inhibitor (PS-1145) blocks NFκB activation in both MM cells and BM stromal cells.

P38 MAPK inhibitor (VX-745) inhibits IL-6 and VEGF secretion from BM stromal cells, as well as IL-6 secretion triggered by adherence of MM cells to BM stromal cells (40).

To my knowledge the 4 above compounds have not yet entered clinical trials.

Inhibitors of angiogenesis. Neovastat (AE-941) is a pleiotropic inhibitor of angiogenesis. It inhibits matrix metalloproteinases that are involved in angiogenesis and play a role in tumor progression and metastasis (42). It suppresses the production of VEGF and bFGF (key stimulators of angiogenesis) by MM cells and indirectly

increases the production of angiostatin, an endogenous inhibitor of angiogenesis (42). PTK787 (ZK222584) and SU5416 are specific inhibitors of VEGF-RII which are under clinical evaluation. A phase II clinical study showed that SU5416 exhibited a biological activity that reduced plasma VEGF levels, but no objective responses have been shown in that small trial.

Franesyltransferase inhibitors and rapamycin are agents that inhibit franesylation of proteins. They principally target RAS/MAPK and P13K/AKT pathways as well as various cytokine dependent MM cell proliferation. Results of preclinical and early clinical reports on these agents are encouraging (96).

Histone deacetylase inhibitors. A histone deacetylase inhibitor known as suberoylanilide hydroxamic acid (SAHA) was found to induce growth arrest and apoptosis in drug resistant MM cells (40, 43). LAQ824 is another histone deacetylase inhibitor that induces caspase dependent MM cell apoptosis and inhibits both proteasome activity and constitutive activation of NF- κ B in MM cells. More recently two other histone deacetylase inhibitors, depsipeptide (FR901228) (97) and valproic acid (98) were shown to induce apoptosis in myeloma cell lines and human myeloma cells.

Other novel agents with significant anti-myeloma activity are the heat shock protein-90 inhibitors which target multiple pathways promoting survival and growth of myeloma cell (99-101), and IGF-1 receptor inhibitors (102). Inhibition of IGF-1 and other growth factors signaling cascades in MM cells and BM microenvironments when combined with conventional anti-myeloma therapy may enhance cytotoxicity and drug susceptibility of myeloma cells (103).

The principle mechanisms of action of the main biologically based novel therapeutics are summarized in Fig 1.

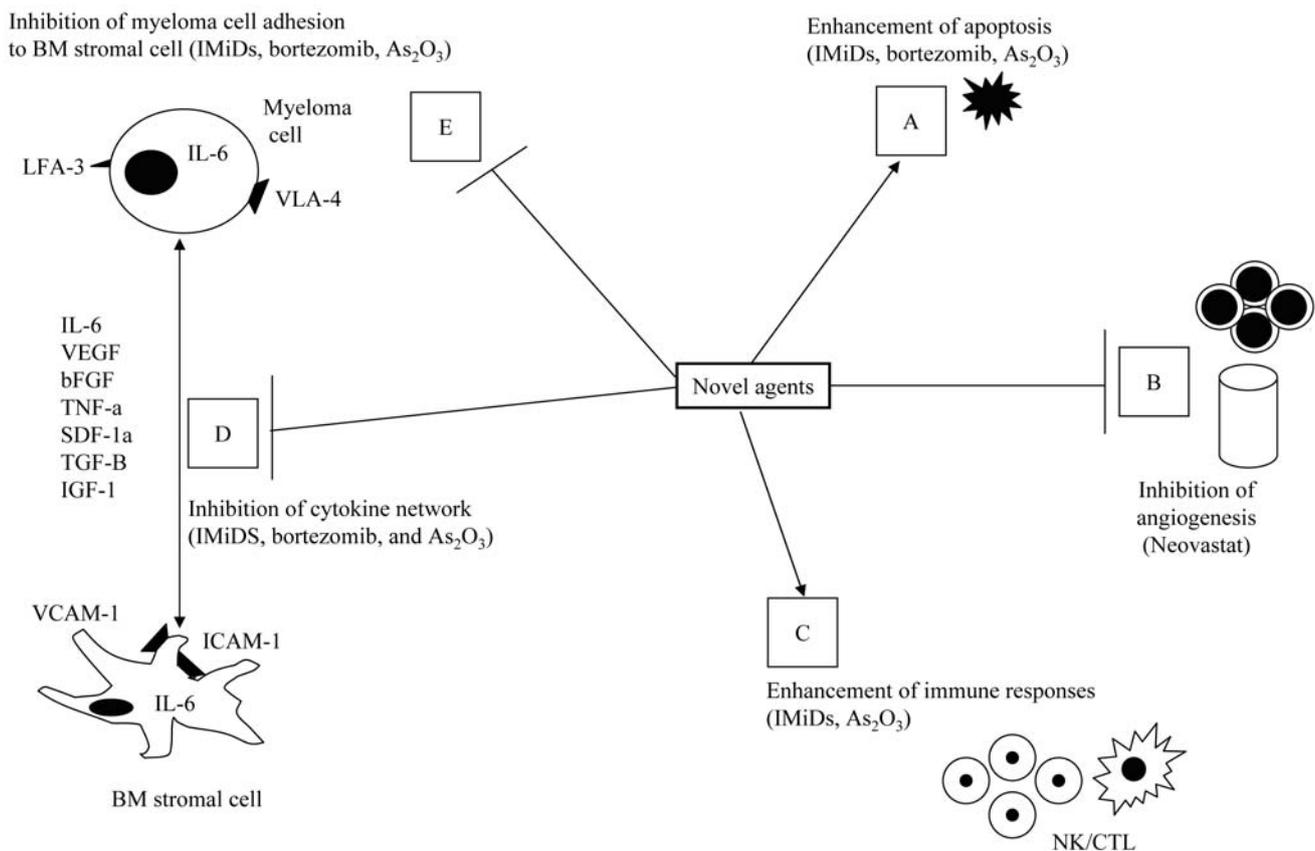


Figure 1. Principle mechanisms of action of main novel therapeutic agents (A-E) in multiple myeloma.

BM = bone marrow. NK = natural killer cell. CTL = cytotoxic T lymphocytes.
 IL-6 = interleukin 6. VEGF = vascular endothelial growth factor.
 bFGF = basic fibroblast growth factor. TNF- α = tumor necrosis factor alpha.
 SDF-1 α = stromal-cell-derived factor-1 alpha. TGF- β = transforming growth factor beta.
 IGF-1 = insulin like growth factor-1. LFA-3 = leukocyte function associated antigen-3.
 VLA-4 = very late antigen-4. VCAM-1 = vascular cell adhesion molecule-1.
 ICAM-1 = intercellular adhesion molecule-1. —→ = Enhancement. —| = Inhibition.

Adapted from Hideshima et al (40).

2 TUMOR IMMUNOLOGY

2.1 Tumor-induced immune responses

2.1.1 Tumor antigens

Tumor cells contain antigens that may induce spontaneous humoral and cell mediated immune responses and may be targeted for therapeutic immunotherapy. A large number of such antigens have been identified and it seems that an increasing number of, perhaps, more relevant tumor antigens may be characterized in the forthcoming future. Currently tumor antigens are categorized into two main groups, tumor associated antigens (TAAs) and tumor specific antigens (TSAs). TAAs represent the majority of tumor antigen and are over-expressed in tumor cells and may also be present in normal cells (104). They are typically oncofetal antigens that are expressed on normal cells during fetal development and down-regulated after birth. Reactivation of the genes encoding these antigens during oncogenesis results in their expression on the fully differentiated tumor cells. Typical examples of TAAs are the cancer testis antigens (MAGE-1 and MAGE-2), melanocyte differentiation antigens, carcinoembryonic antigen (CEA), alfafetoprotein, and prostate specific antigen (PSA). On the other hand, TSAs are unique to tumor cells and not expressed by normal cells of the body (105). They are rare but highly desirable and constitute an elegant target for immunotherapy. They typically arise as a result of oncogenic transformation, but may also be the product of genetic mutations in the tumor cells that generate altered cellular proteins. Examples of TSAs are the clonal Ig idiotype (Id) expressed on the surface of B cell malignancies, bcr-abl fusion product in chronic myeloid leukemia, and the TCR on T cell lymphoma. Cytosolic processing of these antigenic proteins would give rise to peptides that may be presented with MHC class I molecules inducing tumor-specific cytotoxic T lymphocyte (CTLs) mediated immune response (106).

Lastly, oncogenic viruses may produce tumors that integrate pro-viral genetic material in their genomes and express viral genome-encoded proteins. These tumor associated viral antigens may be classified as TSAs. Examples of oncogenic viruses are the Epstein-Barr and human papilloma viruses. The former is associated with endemic Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma, nasal T cell lymphoma, lymphomas in immunosuppressed patients, and Hodgkin's disease (107) and the latter predisposes to cervical cell carcinoma (108, 109).

2.1.2 Characteristics of tumor-induced immune response

From the current scientific literature, it is possible to identify several cell types and a range of effector molecules that are involved in anti-tumor immunity and cancer

immunosurveillance. The key effector immune cells that can directly engage tumor cells are the CD8⁺ T cells, Th₁ CD4⁺ cells and NK cells. These cells mediate their effector function in cooperation with a complex and highly organized network of other immune cells and effector molecules that synchronize the immune response either in favor or against tumor killing, depending on preference of specific circuits in a vast array of signaling commands. The final outcome greatly depends on various intrinsic-autonomous and extrinsic immunogenic characteristics of cancer cells as well as the immune competence of patients (110).

Tumor antigens are typically presented to T cell receptor (TCR) in the context of major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs). The professional APC is the dendritic cell (DC), but macrophages and B lymphocytes can also present antigens to the T cells. Exogenous tumor antigens, released by tumors cells, are usually presented to CD4⁺ T cells after being processed through the MHC class II pathway while endogenous antigens are processed through the MHC class I pathway to CD8⁺ T cells (classical presentation). However APCs (DC and macrophages) can also present exogenous tumor antigen in the context of MHC class I molecule by a process known as cross-presentation and prime CD8⁺ T cells (CTLs) (cross-priming) (111). In addition to malignant cells, cross-presentation is involved in responses to viral infections and transplanted organs (112).

Activated CD4⁺ T cells mediate many anti-tumor responses (113). They can recognize tumor infiltrating macrophages and convert IL-10 producing macrophages (M1) into IFN- γ producing macrophages (M2) (110). They also provide signals to activate CTLs and stimulate eosinophils and macrophages to produce toxic molecules (114). Primed CTLs secrete IFN- γ and kill tumor cells in a perforin-dependent manner (110). Th₁ CD4⁺ T cells can release IFN- γ which has a direct cytolytic anti-tumor activity and may inhibit angiogenesis (110) They may also exert a perforin mediated cytotoxic activity (115). Th₂ CD4⁺ cells can activate B cells promoting the secretion of tumor specific antibodies and may also block angiogenesis indirectly through an effect on stromal fibroblast (110).

NK cells can release perforin and granzyme B from their granules when they encounter target cells (116) and may induce Fas mediated cytotoxicity (117). Granzyme B mediates apoptosis while perforin disrupt endosomal trafficking (118, 119). NK cells may also exhibit anti-tumor activity when activated by DCs, either by direct cell-cell contact or in an NKG2D (NK group 2, member D)-dependent manner (110). Other immune cells that mediate anti-tumor activities include macrophages and macrophage-activated NKT cells which can secrete IFN- γ . NKT cells may also lyse tumor cells in a tumor-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and perforin-dependent manners (110).

These numerous anti-tumor immune responses, as we shall see later, usually end short of the desired goal, and ultimately fail to shrink or eliminate tumors. Such

incomplete anti-tumor immune responses might benefit the tumor but not the host and promote carcinogenesis by the resultant chronic inflammation due to long standing antigenic activation (110). An example of such effect is the contribution of CD4⁺ T cells to squamous cell carcinogenesis induced by human papillomavirus antigens (120). Also vascular leukocyte cells (VLCs) and plasmacytoid DCs (pDCs) may be recruited to tumor beds by pro-inflammatory mediators and contribute to angiogenesis which sustain tumor growth (121, 122).

2.2 Cancer immunosurveillance

The original immunosurveillance theory claims that cancer cells frequently arise in the body, recognized as abnormal or foreign, and eliminated by the immune system (123). It was also suggested that cell mediated immunity has evolved to patrol the body and eliminate cancer cells (124). Both notions were proved to be either largely incorrect or over-speculative. Cancer may develop, in most instances, regardless of immune malfunction. In fact it is now generally accepted that the most defined six hallmarks of cancer are all due to autonomous intrinsic-cellular phenomena (125). Cancer cells characteristically provide their own growth signals, ignore growth-inhibitory signals, avoid programmed cell death (apoptosis), replicate without limits, sustain angiogenesis, and invade tissues through basement membranes and capillary walls (125). However, avoidance of immunosurveillance might be a cell-extrinsic seventh hallmark of cancer (126, 127). Both, cell-extrinsic (immune-mediated) and cell-intrinsic mechanisms need to be subverted for cancer to develop and both mechanisms might be amenable to manipulation for cancer therapeutic purposes.

The current concept of cancer immunosurveillance predicts that the immune system can recognize precursors of cancer and often destroy these precursors before they become clinically apparent. In clinical practice, it is well known that immune suppressed patients such as organ transplanted patients or patients with AIDS have a high risk of developing malignant tumors e.g. lymphomas. Ample experimental evidence has also recently accumulated supporting this concept. Experimental models of mice that lack essential component of the innate or adaptive immune system are more susceptible to the development of spontaneous or chemically induced tumors (110). Experiments using antibodies to deplete natural killer (NK) cells and NKT cells or to neutralize TRAIL or the activating receptor NKG2D have revealed similar results (128). Furthermore, immunostimulatory regimen designed to augment the number of NK cells and NKT cells reduced the development of malignant tumors in mouse models (129). Further evidences supporting the importance of immunosurveillance in tumor suppression emerged from the observation that patients with early stage cancerous lesions and pre-malignant conditions, in whom the immune function is largely preserved, can mount vigorous anti-tumor immune

responses. For example, patients with MGUS, which is pre-malignant, mount strong T cell responses to autologous pre-malignant B cells, whereas such responses are absent or less pronounced in patients with MM, which is malignant (130). Also the bone marrow of patients with early operable breast cancer contains tumor specific CD8⁺ T cells that can mediate the regression of autologous human tumors when transplanted into immunodeficient mice (131). Similar observations have been reported for patients with pancreatic cancer (132). These examples and others indicate that cancer can still develop despite recognition of cancer cells by the immune system and the initial vigorous anti-tumor response. It seems that the multistep cancer development is accompanied by a proportional progressive immune dysfunction. Cancer cells escape immunosurveillance (innate and adaptive anti tumor immune responses) by immunoediting (immunoselection), which is the selection of non-immunogenic tumor cell variants or by immunosubversion which is the active suppression of the immune response (133). The central concept of multistep carcinogenesis resulting from crosstalk of cancer cell intrinsic factors and host immune system (cell extrinsic) effects is illustrated in Figure 2.

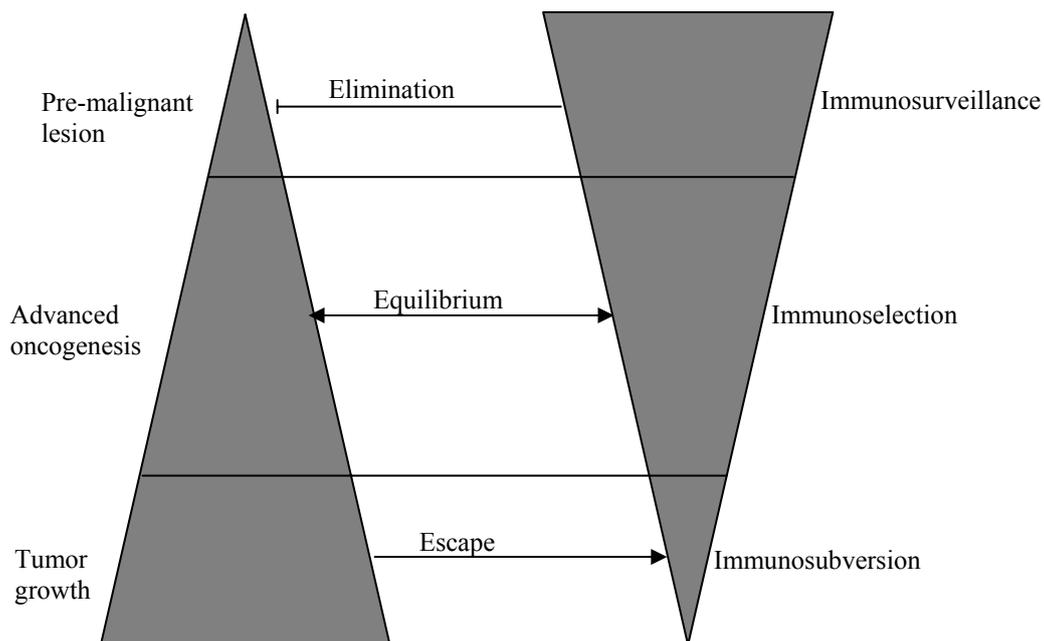


Figure 2. Relationship between cell intrinsic and cell extrinsic aspect of tumor progression.

Adapted from Zitvogel, L et al (110).

2.3 Cancer immunoediting (immunoselection)

Immunoselection comprises a series of strategies developed by tumors to evade immunosurveillance in response to the selective pressure exerted by the immune system. These strategies are designed to result in the development of less or weakly immunogenic tumor cell variants.

A common strategy is to down-regulate or lose the expression of HLA class I molecules and IFN- γ receptors to elude T cell mediated immune responses (110). Loss of HLA class I expression is especially common in lung cancer (134). Other molecules involved in antigen processing and presentation through the HLA class I pathway and are also often down-regulated by tumor cells. These include, transporter associated with antigen processing 1 (TAP1), low-molecular mass protein 2 (LMP2), LMP7, and tapasin. The expression of these molecules is progressively lost during the development of colorectal carcinoma (135). Another strategy adopted by tumors to evade immunosurveillance is to develop various mechanisms by which they can avoid killing by CTLs. Tumors may over-express the serine-protease inhibitor P19 that can efficiently block the granzyme B-perforin pathway (136). They may exhibit down-regulation or mutation of the genes encoding death receptors as reported for Fas in MM (137), non-Hodgkin's lymphoma (138), and melanoma (139). Methylation or mutation of the gene encoding caspase-8, and over-expression of FLIP [FLICE (caspase-8)-like inhibitory protein] or decoy receptors for TRAIL may also occur leading to resistance to CTLs mediated killing of tumor cells (140).

Tumors may also down-regulate or lose their specific antigen to avoid immunosurveillance as has been reported for the melanoma-melanocyte differentiation antigens in progressive melanoma (141) and the clonal Id in patients with B cell lymphomas receiving anti-Id therapy (142). Alternatively, tumor cells might down-regulate or lose costimulatory molecules leading to immune tolerance or ignorance of the immune system to tumor antigens (143-145).

Ultimately immunoselection produces tumor variants that have lost their antigen processing machinery and specific tumor antigens, as well as their sensitivity to immune effectors.

2.4 Cancer immunosubversion

Immunosubversion denotes the active destruction and suppression of the immune responses. Induction of immune tolerance might indeed be a prerequisite for the initial steps of tumorigenesis (146). Malignant tumors may actively suppress the immune system by producing various factors and molecules that are dispensable of cell-intrinsic cancer-cell characteristics (110) as well as by favoring the induction and differentiation of regulatory T cells (T_{reg} cells) (147).

2.4.1 Production of immunosuppressive factors

Tumors might overproduce nitric oxide and increase their arginase 1 activity to inhibit T cell function (148). More importantly human tumors constitutively express indoleamine 2,3-dioxygenase (IDO) that degrades tryptophan and consequently promotes resistance to immune-mediated rejection of tumor cells (149). Locally tumor-produced IDO can block the proliferation of CTLs at the tumor site and promote apoptosis of CD4⁺ T cells (150). Interdigitating DCs may also over express IDO and exert more resistance to the elimination of cancer cells by the immune system (149). Tumors may also express CD95L and induces death of CD95-expressing tumor specific T cells (151). In murine systems advanced cancer invariably subverts immune function. Typically CTLs show progressive loss of cytolytic function (152) and tumor specific CD4⁺ T cells progressively lose their anti-tumor activity (153). On the other hand the number of T_{reg} cells increases (146). In addition to IDO, nitric oxide and arginase 1, tumor beds contain various immunosuppressive factors (VEGF, IL-6, IL-10, TGFβ, M-CSF, NOS2, PGE2, COX2 and gangliosides) produced by the tumors and supportive stromal cells. These factors mediate multiple immunosuppressive pathways including inhibition of maturation and function of DCs (154). Consequently, local immature DCs will mediate immunosuppressive, rather than immunostimulatory, effects leading to defective T cell priming and promotion of T_{reg} cells differentiation (155). Also tumor-associated macrophages mostly belong to the M1 class of macrophages, which produce arginase 1, IL-10, TGFβ, and PGE2 and favor Th₂ cell responses (156). Moreover, a tumor-infiltrating variant of NKT cells (CD4⁺ NKT cells) produces IL-13, an immunosuppressive factor which can directly suppress CTLs-mediated tumor rejection or activate myeloid suppressor cells (MSC) to produce TGFβ that also suppress CTL activity (157). Furthermore, immunostimulatory tumor characteristics at early stage disease or small tumors can become immunosuppressive in advanced disease or large tumors. For example the expression of NKG2D ligands by tumor cells, which stimulate anti-tumor immune responses at early stage disease may play an immunosuppressive role as the increasing number of ligands in the growing tumor and soluble ligands shed from tumor cells down-regulate NKG2D receptor expression by CTLs and NK cells (158). Similarly, it could be argued that large number of tumor antigens in large tumors may induce high dose tolerance to tumor antigen and down-regulate both tumor specific and general T cell responses. The mechanisms of tumor induced immune suppression are summarized in Figure 3.

2.4.2 Promotion of T_{reg} cells differentiation

T_{reg} cells constitute a heterogeneous group of immunoregulatory cells that are under continuous evaluation and scientists are looking for more specific markers to

characterize the various phenotypes of the T_{reg} cells. Currently it is generally accepted that antigen experienced T_{reg} cells should minimally express CD4 and CD25 markers as well as the transcription factor FOXP3. Characteristics of $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells are their anergic state, their ability to actively inhibit $CD4^+CD25^-$ T cells, $CD8^+$ T cells, DCs, NK cells, NKT cells, and B cells in a cell to cell contact and dose-dependent manner (147). Additionally, IL-10 and TGF- β might have functional importance for T_{reg} cells immunosuppression in vivo (159, 160). Other prominent cell-surface markers associated with T_{reg} cell phenotype and function, are cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced TNFR-related protein (GITR) (161, 162).

Recent research findings clearly suggest that T_{reg} cells are major regulators of anti-tumor immune responses (163) in addition to their general role in immunologic self-tolerance and negative control of immune responses. Naturally occurring T_{reg} cells protect the host from autoimmune disease by suppressing self-reactive immune cells (164, 165). Antigen-induced T_{reg} cells also coexist and contribute to immune suppression in various conditions (164, 166-168).

Tumor antigens are mostly self-derived and consequently T_{reg} cells might also block the immune responses directed against these antigens and promote tumor development and growth. In mice solid tumor models, highly immunosuppressive tumor-induced T_{reg} cells have been detected early during tumor development (169). Suppression of immune response by T_{reg} cells occurs predominantly at the tumor site (170, 171). However, increased frequencies of T_{reg} cells and associated production of suppressive cytokines that correlated with tumor progression were also observed in peripheral blood (172). In human, increased frequencies of T_{reg} cells both, locally at the tumor microenvironment and systemically in peripheral blood have been shown in many solid tumors. These include invasive breast or pancreatic cancers (173), malignant melanoma (174-176), gastrointestinal malignancies (177, 178), ovarian cancer (179), head and neck cancer (180) and hepatocellular carcinoma (181). Similarly, high frequencies of T_{reg} cells have been shown to occur locally at the tumor site as well as in peripheral blood in patients with hematological malignancies. These included patients with Hodgkin lymphoma (HL) (182), B cell non-Hodgkin lymphomas (B-NHLs) (183), B cell chronic lymphocytic leukemia (B-CLL) (184), acute myeloid leukemia (AML) (185), and recently also those with MGUS and MM (186). In many of these cancer types higher frequencies of T_{reg} cells seemed to be stage-dependent and correlate with poor prognosis and decreased survival rates underlying the close correlation of tumor growth and T_{reg} cell frequencies (147).

Although the increase of T_{reg} cells seems to be a common theme and a characteristic feature in most tumors, little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated levels of these cells in cancer patients. A possible mechanism for expansion of T_{reg} cells in cancer

patients was recently suggested by Wolf et al who demonstrated that the increased frequencies of T_{reg} cells in the peripheral blood of cancer patients are due to active proliferation rather than redistribution from other compartments (187). Tumor derived PGE₂ were also shown to increase T_{reg} cells activity and FOXP3 expression, while in vivo COX2 inhibition reduced T_{reg} cell activity and FOXP3 expression as well as decreased tumor burden (147). CCL22 produced by tumor cells and surrounding macrophages can also mediate trafficking of T_{reg} cells to tumor sites via CCR4 (147). However, more elaborative research is needed to identify the principle key mechanisms that drive and maintain the high level of T_{reg} cells in cancer patients and pave the way to manipulate the kinetics of these cells for therapeutic purposes. One key question to be addressed is whether the increase of T_{reg} cell frequencies is an early event at the onset of disease that might contribute to disease progression or more likely a response of the immune system during disease progression. It would also be interesting to see how does the current cancer therapeutic regimens influence T_{reg} cells number.

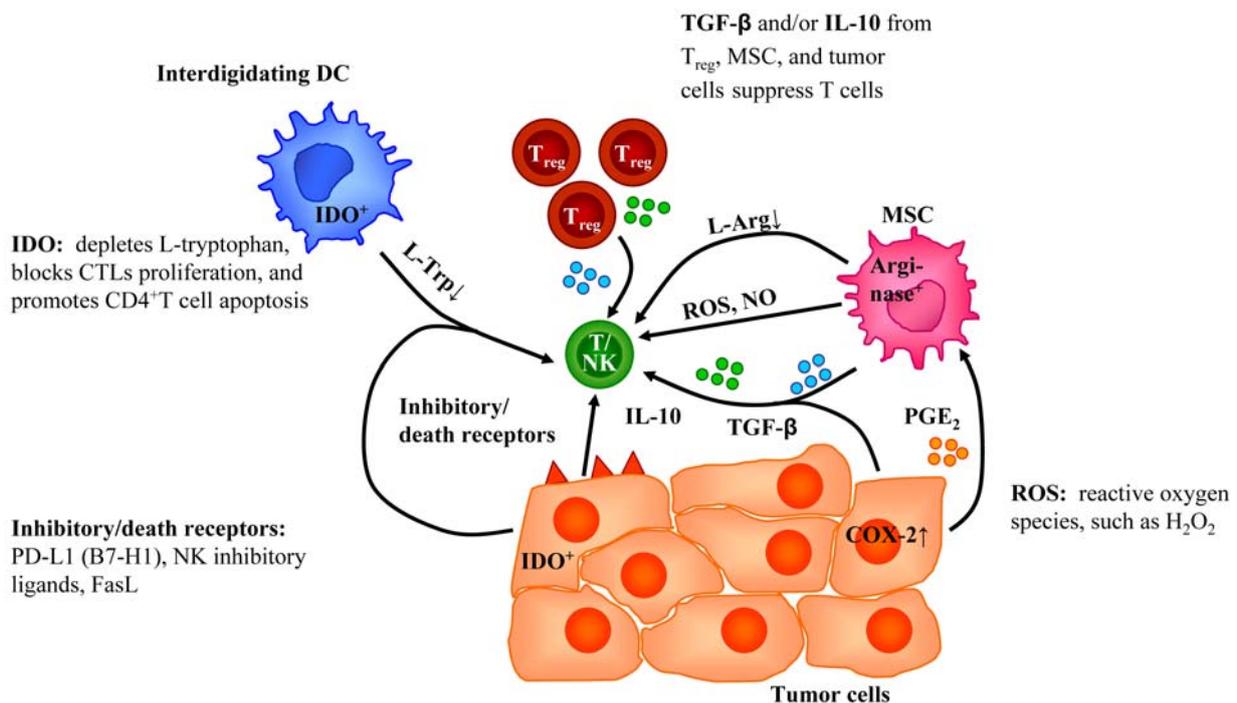


Figure 3. Tumor induced immune suppression.

IDO = indoleamine 2,3-dioxygenase. L-Arg = L-arginase. ROS = reactive oxygen species. No = nitric oxide. PEG2 = prostaglandin E2. MSC = myeloid suppressor cells. Cox2 = cyclooxygenase 2.

This figure was kindly provided by Dr Helena Laven.

3 THE IMMUNE SYSTEM AND MM

3.1 Origin of the myeloma cell

During the entire course of MM the malignant PCs (myeloma cells) reside almost exclusively in the BM. However, the exact origin of the myeloma cells is not known (188). The identity and biological characteristics of the cells that belong to the malignant clone in MM is a subject for an ongoing research since the early 70s (189). A B cell with stem cell characteristics and ability to replicate and subsequently differentiate into malignant plasma cell has been postulated (190). Gene analysis the variable region of the heavy chain of the Ig (IgH) revealed that myeloma cells share a unique variable (V) diversity (D) joining (J) regions of the IgH (VDJ rearrangement) (191, 192). The myeloma VDJ rearrangement is characterized by the occurrence of isotype switching from C μ to one of the down-stream post-switch constant regions C α or C γ . Earlier stage B cells with clonotypic VDJ rearrangements identical to myeloma cells have also been detected and thought to be precursors of myeloma cells (193). The presence of B lymphocytes as part of the tumor clone was suggested also by flow cytometry studies in which circulating B lymphocytes bearing the same Id surface structures and light chain isotypes as those of the serum M-component and cytoplasmic Ig of myeloma cells were identified (189, 194, 195). Also cloning and sequencing of the genes encoding the variable regions of the IgH (VH genes) from patients revealed that the myeloma cells have already passed through the phase of somatic hypermutation and antigen selection and, importantly for immunotherapy, they do not undergo any further somatic mutation during the course of disease (196, 197). Strict intraclonal homogeneity for myeloma cells and the B cells precursors are also revealed by analysis of clonotypic VDJ sequences for the isotype (193, 196, 197) and clonal stability throughout the course of the myeloma disease has been demonstrated (193). These findings strongly imply that the malignant clone in MM evolves from a cell late in B cell development and the presence of pre-switch antigen-selected post-germinal centre clonal B cell was proposed as an origin for the myeloma cell clone (198, 199). However, a study on the 5T murine model of myeloma (5TMM) showed that isotype variants of myeloma may arise from rare secondary isotype switching or trans-switching events in the terminally differentiated post-switch plasma cells (200) challenging the idea of clonotypic pre-switch cells as an origin.

The exact role of the clonal B cells in the disease process is not yet fully understood (193, 198, 201-203). The presence of clonal B cells in the blood of patients with MM at the time of diagnosis might suggest a biologically aggressive disease with a poor prognosis (204), and in patients with MGUS indicates subsequent malignant course (205). There is also a growing evidence indicating that these

circulating precursors might be relatively resistant to conventional therapy and partially responsible for disease recurrence (206-209).

These findings indicate that MM may be regarded as an ongoing differentiating B cell malignancy, which consists of a large population of slowly proliferating plasma cells and encompasses small compartment of differentiating clonal B cells that may display proliferative capacity as well as MDR (multi-drug resistance) phenotype. However, it remains unclear whether these precursor B cells have a fully malignant phenotype or not.

3.2 Phenotype of the myeloma cell

Myeloma cells are heterogeneous in different patients as well as different disease stages in the same patient, reflecting the ongoing differentiation process that characterizes myeloma cells development. During development, myeloma cells change and modulate their surface and adhesions molecules with disease progression, a process that enable them to home to bone marrow and enhance their proliferative and invasive capacity (210, 211). Like normal plasma cells, myeloma cells express high level of CD138 and CD38, but lower CD19, CD20 and CD45 compared to their normal counterparts. CD28 expression is associated with more aggressive disease, while immature myeloma cells may express CD45 and IL-6 receptors. The expression of some of the surface and adhesion molecules in myeloma cells and normal plasma cells is shown in Table 3.

Table 3. Surface and adhesion molecules in myeloma versus normal plasma cells.

Surface/adhesion molecule	Myeloma cell	Normal plasma cell
CD138	+++	+++
CD38	+++	+++
CD19	+ -	+
CD20	+ -	+
CD27	+ -	+
CD28	+	-
CD40	+	+
CD45	- ^a	+
CD44	+	+
CD54	+	+
CD56	+++	-
CD58	+	-
LFA-1 ^b	+ -	-
VLA-4 ^c	+	+
Syndecan-1	+	+

^a On immature myeloma cells. ^b Lymphocyte function associated antigen-1. ^c Very late antigen-4. + = up-regulation of molecules and - = down-regulation of molecules.

Adapted from Bataille et al (210) and Munshi et al (212).

3.3 The immune system in MM

The immune system in MM exhibits several defects that may be common to most cancer types or specific to myeloma disease. Tumor antigens, which are in most cancer types self-proteins that inherently show weak immunogenicity (213, 214) may fail to send danger signals (215) and induce immune tolerance (216). This may hold true for many myeloma antigens. An example is the Id protein, which is a myeloma specific antigen, when used alone as a vaccine induced a transient weak anti-idiotype T cell response (217). These anti-Id T cell responses could be augmented only after addition of various adjuvants (218-220) and few clinical effects could be noted.

A selective reduction in the number of CD4⁺ T cells is observed in MM patients (221) as well as phenotypic and functional abnormalities of both CD4⁺ and CD8⁺ T cells. The biologic bases for these abnormalities are still unclear and need further

investigations. T cells expanded from MM patients are difficult to prime due to down-regulation of the expression of CD28 costimulatory molecules (143). In both untreated and conventionally treated MM patients a significant reduction in frequency and proliferative capacity of CD8⁺ cells against Epstein-Barr and influenza A viruses is observed (222). CD4⁺ and CD8⁺ T cells in MM patients are also shown to exhibit multiple intracellular defects in T cell receptor (TCR) signaling pathways associated with a reduced capacity to produce cytokines (IL-2, IFN- γ , and IL-4) (223). Not surprisingly, these defects increase with disease progression (223). Furthermore, there is evidence that a reversible defect in NK cell function is associated with progression from MGUS to MM (224). DCs of patients with MM are also functionally inhibited by TGF β and IL-10 at the tumor bed and may fail to up-regulate the costimulatory molecule CD80 (B7-1) upon stimulation (225). Moreover, circulating myeloid DCs in MM patients are reported to be significantly reduced in number and also have impaired up-regulations of CD80 and the adhesion molecule CD54 when stimulated *ex vivo* with GM-CSF and IL-4 (226).

The cytokine network in MM patients is deregulated and patients with active disease characteristically show defective Th₁ immune response and accelerated Th₂ polarization with disease progression (227). Such a cytokine milieu may be conducive to disease progression as effective and functional Th₁ immune responses are crucial for tumor rejection (228) while a bias towards a Th₂ response is said to favor tumorigenesis and an indicator for poor prognoses (229). The central cytokine that drives myeloma disease seems to be IL-6 (227, 230). However a myriad of immunosuppressive cytokines in the tumor microenvironment orchestrates the maintenance and progression of the disease. These include the well-known immunosuppressive factors TGF β and IL-10 as well as other cytokines and factors that sustain angiogenesis and promote myeloma bone disease (see pathogenesis of MM).

An increasingly recognized factor in cancer related immune suppression is the existence of elevated numbers of T_{reg} cells. Functional FOXP3⁺ T_{reg} cells of naïve, central and effector memory phenotypes are significantly expanded in MM patients (186). The existence of these cells, which exert strong suppression after TCR stimulation (231-233), may be a response to the malignant transformation since they are numerically increased and fully functional in MGUS as well as early untreated or late stage treated MM patients (186). A recent report has suggested reduction in the number and function of FOXP3⁺ T_{reg} cells in both MGUS and MM, but argued that such a reduction may account for a nonspecific increase of CD4⁺CD25⁺ T cells and dysfunctional T cell responses (234).

Despite these immunological dysfunctions in MM, as we shall see later, the situation is not direly bleak since many reports support the existence of naturally occurring cytotoxic tumor specific T cells in both, peripheral blood and bone marrow

of MGUS and MM (130, 235-237). In addition autologous T cells isolated from bone marrow exhibit preferential lysis of myeloma cells and their precursors after nonspecific stimulation through CD3 and CD28 molecules (238). The existence of such tumor reactive T cells gives hope that some tumor specific antigens (e.g. Id) may have the potentials of breaking immune tolerance and inducing effective anti-tumor immunity if used with optimal conditions for immunotherapy.

4 IMMUNOTHERAPY IN MM

4.1 General consideration

Knowledge about the molecular identities of many tumor-associated antigens has provided a major stimulus for the development of new immunotherapeutic approaches for the treatment of cancer patients. An essential prerequisite for immunotherapy is relative immune competence in the recipient and hence it is generally accepted that patients with early stage cancer disease are the best candidates. Currently two major forms of cancer immunotherapy are in practice; nonspecific and specific immunotherapies, each could be induced either passively or actively (see Table 4). In this section we shall discuss specific active immunotherapy in MM patients with greater emphases on immunization with the idiotypic protein, the myeloma specific antigen.

Table 4. Current approaches to cancer immunotherapy.

Category	Example
Nonspecific passive	1) Cytokines; IL-2, Interferons (IFN- α , β , γ) and TNF 2) Local application of BCG in patient with superficial bladder carcinoma
Nonspecific active	1) Nucleic acid with CPG motifs 2) Adoptive immunotherapy; allogeneic bone marrow transplantation, transfer of lymphokine activated killer cells (LAK)
Specific passive	Monoclonal antibodies
Specific active	Cancer vaccines; peptide or protein, idioype, recombinant viral vector (vaccinia, adenoviruses, etc) based vaccines. DNA vaccines, modified autologous tumor cells or tumor cell lysate vaccines. DC based vaccines (pulsing DCs with peptides, idiotypes, or loading DCs with tumor cell lysate, tumor cells apoptotic bodies, tumor RNA or tumor DNA, fusing DCs with autologous or allogeneic tumor cells) and many others.

Specific active immunotherapy principally involves vaccines directed against tumor antigens. Most cancer vaccines aim at stimulating the adaptive immune system of the patient (CD4 and CD8 as well as antibody responses) to attack and kill the tumor cells. Tumor vaccines can be used on prophylactic bases such as hepatitis B vaccine against liver cancer or human papilloma virus vaccine against cervical cancer. However most of the cancer vaccines are designed for the therapeutic setting aiming

at reducing tumor burden or treating minimal residual disease (MRD), or for the adjuvant setting as a complementary treatment.

Since tumors cells of B cell malignancies, including MM, express various potential target antigens, active immunotherapy is being investigated as a novel treatment modality in these tumors. The idiotypic structure is by far the most exploited tumor specific antigen for therapeutic vaccination in these malignancies, particularly in NHL and MM. Vaccination with the Id coupled with KLH \pm GM-CSF induced tumor regression and clearance of t(14;18)⁺ cells in patients with low grade NHL (239, 240). This and other promising results from phase II trials (241) stimulated the initiation of phase III studies comparing Id-KLH plus GM-CSF with either KLH or placebo (sponsored by Genitope and the National Cancer Institute). In MM, many TAAs antigens apart from the Id may be targeted for immunotherapy. These include mucin-1 (MUC-1) (242-244) and the cancer/testis (C/T) (245, 246) antigens. Immunization trials with some of these antigens are ongoing (see www.clinicaltrials.gov and www.kimt.de).

Tumor antigens may be delivered as peptides or protein vaccines in various adjuvant settings (protein plus cytokine adjuvants and peptide or protein pulsed DCs). TSAs may also be administered as DNA vaccines. An example is the Id antigen encoded by the variable regions genes, V_H and V_L. The V genes may be assembled in a single chain Fv (scFv) format and fused into the C fragment of tetanus toxin to make DNA scFv-FrC vaccines. One patient has been vaccinated so far and a strong durable anti-Id T cell response combined with a slow fall of the paraprotein was noted (247). An alternative principle to vaccination of MM patients against defined tumor antigen is cell-based myeloma vaccines that aim at stimulating the immune system with the entirety of myeloma cells antigens. This approach has regained new momentum after earlier disappointing results. Current approaches for cell-based myeloma vaccination use tumor cell lysates or apoptotic tumor cells as a source of antigen. T cells from the tumor microenvironment of patients with progressive myeloma were shown to generate strong tumor-specific cytolytic immune responses against autologous DCs loaded with the myeloma cells (235). Antigen presentation from myeloma cells by the DCs in this approach is greatly enhanced by coating the myeloma cells with specific antibody such as antiCD138 (248). Similar results were obtained when DCs were loaded with autologous myeloma cells lysed by repeated freeze-thaw cycles (249). In a direct comparison irradiated apoptotic tumor cells appeared to be a superior source of tumor antigen compared to tumor lysates for dendritic cell-mediated T cell stimulation (250). Other models for cell-based myeloma vaccines are fusion of myeloma cells with DCs (251) and infection of myeloma cells with cytokine expressing adenovirus e.g. IL-2 (252). Although these cell-based myeloma vaccination experiments seem promising many issues such as

tumor cell purity and specifications need to be solved before large scale clinical testing.

In general, successful induction of anti-myeloma immunity must overcome the marked immunodeficiency of myeloma patients mentioned earlier and should aim at generating sufficient number of functional immune cells that can recognize the tumor cells with high avidity as well as breaking tolerance and the immune suppression produced by the tumor or the immune system itself (253).

4.2 Idiotype specific immunotherapy in MM

4.2.1 Preclinical animal models of Id immunization in MM

In vitro studies using various human MM cell lines may be relatively easy (254, 255). However, most human MM cell lines are derived from refractory cells and by no means represent *in vivo* MM cells. *In vivo* animal models that overcome extrapolation problems of *in vitro* systems and enable meaningful preclinical studies are needed. Id vaccination in human has evolved from animal models that are still in use and being improved to explore and develop more efficient and reproducible models for Id vaccination. Several *in vivo* myeloma models have been described.

The pristane mineral-oil-induced plasmacytoma (MOPC)-315 mouse model raised in BALB/c mice is one of the most widely used model and has provided considerable data on plasmacytomagenesis (256, 257). However, in the MOPC-315 model the plasma cell tumors are not spontaneous and are primarily localized in the peritoneal environment and not in the BM. These plasmacytoma models mainly produce IgA and lack typical features of human myeloma disease (258). A more generalized myeloma disease could be reproduced in severe combined immunodeficiency (SCID) mice models (SCID xenograft and SCID-hu) (259, 260). Nevertheless, the immune system of SCID mice is different from that of MM patients and results from these models may need further validation. The most analogous to human myeloma is the 5TMM model that originated from spontaneously developed MM in elderly mice of the C57BL/kalwRij inbred strain (261) and has since been propagated by intravenous injection of BM cells from MM bearing elderly mice into young naïve syngenic recipients (262). Like human MM, the clinical characteristics of the 5TMM model include the selective localization of MM cells in the BM, presence of serum M-component, induction of osteolytic bone lesions, and increased angiogenesis in BM. This model has been used to study BM homing, osteolytic bone disease, DNA vaccination, and cytogenetics in MM.

The earliest Id immunizations were performed in syngenic BALB/c mice with inoculation of myeloma Id purified from MOPC tumors and coupled with complete Freund's adjuvant. The vaccinated mice developed a prolonged humoral immune

response that was specific for the individual Ids used for immunization (263-265). Specificity of anti-tumor immunity was further confirmed by showing that these mice are resistant to subsequent tumor challenge of the parental neoplasm but not to other MOPC tumors. Years later, immunization with MOPC-315 plasmacytoma derived light chains alone was shown to be sufficient to protect against subsequent tumor challenge (266). Preclinical DNA vaccination with expression plasmids encoding the myeloma idiotype in an scFv format has yielded a greatly enhanced anti-myeloma immune response when the scFv gene was genetically fused with a cDNA encoding fragment C on the tetanus toxin (267). Induction of Id-specific protective anti-tumor immunity by Id immunization has been further supported by successful experiments in lymphoma (268-271). The anti-tumor immunity seen after Id immunization is unlikely to be mediated by anti-Id antibodies (272) and Id-specific immune cells are more likely to be responsible since Id-specific T cells were detected in mice after Id immunization (273, 274). An important observation in an experimental animal model is the progressive deletion of Id-specific T cells in the presence of high concentration of myeloma protein (275) which may imply that patients with advanced MM may be less prone to respond to Id vaccination and the vaccine should preferentially be offered to patients with low tumor burden.

4.2.2 Idiotype specific immunity in human MM

Naturally occurring cytotoxic T cells against the autologous plasma cells have been described in patients with MGUS and MM (276). Also clonal expansion of both, CD4⁺ and CD8⁺ T cells have been demonstrated in peripheral blood of MM patients (277). CD8⁺ clonal T cells were more frequently seen in patients with low tumor burden as compared to those with advanced disease (278). It has been suggested that the occurrence of clonal T cells in MM may be associated with favorable prognoses. Activated T cells that recognize myeloma cells and produce IL-2 and IFN- γ have also been demonstrated in the bone marrow of MM patients. (279). However, T cells of MM patients are more susceptible to apoptosis due to deregulated Fas and Bcl-2 expression (280) as well as increased frequency of CD8⁺ CD57⁺ suppressor cells (281, 282).

The existence of naturally occurring T cells reactive with the Id of the autologous monoclonal Ig has been reported (236, 237). Th₁ T cells were detected in patients with indolent MM (236), while Th₂ cells were observed in those with advanced disease (283). The myeloma cells themselves may present the Id antigen (284) and may be recognized and lysed by the Id reactive T cells (249, 285, 286).

T cells, specifically recognizing peptides corresponding to the hypervariable stretches within the variable part of the heavy chain of the tumor derived Ig (Ig-V_H), known as complementary determining regions (CDRs) I-III were identified for

CDRIII in B-CLL and B cell lymphoma patients (287, 288). Moreover, these T cells were shown to secrete IFN- γ as well as lyse autologous PBMCs loaded with the relevant peptide in the lymphoma patients (288). Peptides derived from the framework regions (FRs) may also express cytotoxic T cell epitopes (289).

In MM, specificity of Id-reactive T cells was mapped using peptides corresponding to the complete CDRI-III regions of both Ig-V_H and Ig-V light chain (Ig-V_L) as well as MHC restricted sequences of the CDRII and III regions of Ig-V_H. T cells reactive CDRIII peptides were found in the Ig-V_H (290). However, peptides corresponding to the CDRs of the Ig-V_L or other unrelated Ig fragments were found to be not efficient in eliciting an Id-specific T cell response (290-292). A more comprehensive search for T cell epitopes within the Id sequences of myeloma patients by HLA binding prediction algorithms confirmed these findings and demonstrated that most epitopes are located in the CDR II-FR III-CDR III region (293). These findings convincingly support the existence of MHC restricted Id-specific T cells that may target immunogenic CDR peptides in monoclonal gammopathies. Anti-Id antibodies have been detected in both, MGUS and MM patients, but such antibodies are most likely to exist in vivo as immune complexes with the tremendous excess of freely circulating monoclonal Ig and may not be important for anti-tumor immunity. Moreover, the myeloma cells characteristically lack surface Ig expression, which make them unsusceptible to active or passive antibody mediated immunity (294).

4.2.3 Idiotypic immunization in MM patients

As previously stated, the Id, which harbors the antigenic determinant of the immunoglobulin (Figure 4) is a myeloma specific antigen and as such could be targeted in therapeutic vaccination for MM patients. In animal models Id immunization induced resistance to tumor cell challenge as well as both humoral and cellular anti-Id immune responses. However, in human no solid firm evidence of improved clinical outcome has been reported with Id vaccines. We have recently reported longer time to disease progression (TTP) in patients with positive anti-Id immune response compared to immune non-responders (see paper IV).

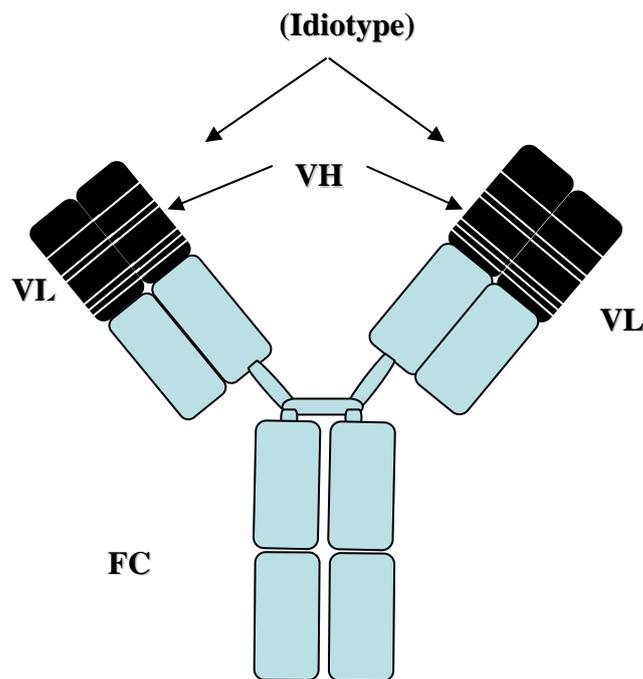


Figure 4. Schematic presentation of the idiotype structure within the immunoglobulin molecule.

VH = variable regions of the immunoglobulin heavy chains. VL = variable regions of the immunoglobulin light chains. The white lines denote the hypervariable CDR I, II and III regions. FC = constant fragment of the immunoglobulin.

Although autologous Id vaccines are patient-specific and have to be prepared individually for each patient, they are feasible and relatively easy to prepare since native Id protein can usually be prepared from the serum of MM patients through various straightforward purification steps. In this thesis, similar to clinical trials in other B cell malignancies where immunological and clinical responses have been reported (240, 295), we used the Id protein as a tumor antigen with two adjuvant cytokines (IL-12 and GM-CSF). Addition of cytokine adjuvants and coupling of the Id protein to exogenous carriers such as keyhole limpet hemocyanine (KLH) or aluminium phosphate (alum) are imperative if adequate immune response is to be obtained from a weakly immunogenic self derived antigen like the idiotype. DCs may also be used as adjuvants onto which the idiotype can be loaded and administered as a vaccine. However DCs based vaccines are more laborious and expensive compared the protein-cytokine adjuvant approach. The principle of Id protein vaccination is summarized in figure 5. A necessary condition for tumor control was the uptake and presentation of the Id by DCs (296). Activation of infiltrating macrophages through

IFN- γ also appeared to be indispensable for generating T cell mediated tumor protection (113).

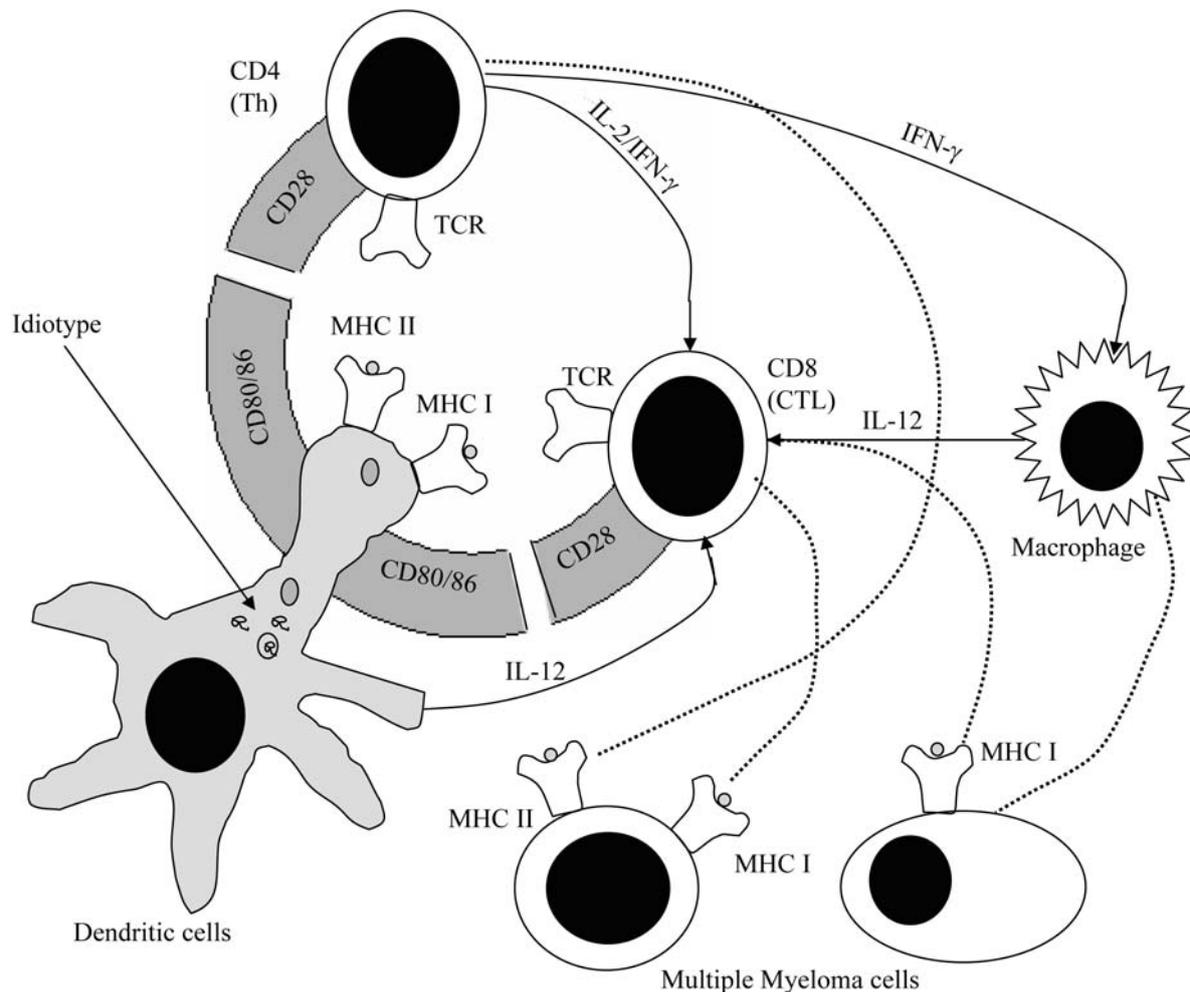


Figure 5. Schematic presentation of the principles of Id vaccination.

The Id is brought by various means to be taken up *in vivo* or *ex vivo* by DCs. It is then presented on MHC class I and II molecules of DCs. Specific CD4 T cells interact with MHC class II and produce cytokines e.g. IL-2 and IFN- γ . These cytokines and others (IL-12 from macrophages and DCs) activate CD8 T cells that have specifically recognized Id on MHC class I molecules on DCs. Id-specific CD4 and CD8 T cells can then specifically react with myeloma cells expressing the Id within their MHC class II and I respectively and kill myeloma cells (classic pathway). Activated CD4 T cells (IFN- γ) also activate macrophages that inhibit myeloma cell growth.

In one of the earliest Id vaccination trials in MM patients the Id protein was given alone (precipitated in alum) to 5 MM patients with stage I-IIA disease. A transient anti-Id T cell response that appeared to be insufficient to generate sustained anti-multiple myeloma immunity was observed in 3 patients (217). When the Id is administered in combination with granulocyte macrophage-colony stimulating factor (GM-CSF) to other 5 patients with stage IIA disease in the following study an increase in the number of IFN- γ and IL-2 secreting T cells was noted in all patients and in one patient partial remission ($> 50\%$ reduction of M-component serum level) was observed (219). In another trial, 15 MM patients were vaccinated with KLH-coupled Id and GM-CSF while they were in MRD in the first remission after HDT and ASCT. Delayed-type hypersensitivity (DTH) reactions to the vaccine were induced in 85% of the patients, but in vitro testing provided little evidence for specific T cell responses and no clinical effects were seen (297, 298). The first clinical study where DCs were used as an adjuvant to augment the Id-specific T cell response was published in 1998 where a MM patient vaccinated with DCs pulsed with autologous Id protein developed Id-specific cellular and humoral responses and showed a transient fall in M-component level (299). In a study by Lim and coworkers 6 MM patients were treated with Id-pulsed DCs. A minor clinical response (MR) was observed in one patient (300). Two out of 12 MM patients vaccinated with Id-pulsed DCs after ASCT developed Id-specific T cell proliferative response (301). Liso et al reported an Id-specific T cell response in 4/26 MM patients who were vaccinated with Id-pulsed DCs while in PR or CR after HDT (302). Seventeen patients were also vaccinated with Id-pulsed DCs after HDT. Three patients entered CR and 2 PR (303). It was also reported that all 6 MM patients in PR after ASCT, vaccinated with Id or Id (VDJ)-derived HLA class I restricted peptides coupled to KLH-pulsed DCs, developed an Id-specific T cell proliferative response and 4 of them showed circulating IFN- γ secreting T cells by ELISPOT. One of these patients also developed CR (304). When additional GM-CSF was given together with Id-pulsed DCs, 3 and 4 out of 10 MM patients with advanced disease developed humoral and cellular Id-specific responses respectively (305). Also vaccination of 12 patients with serum-free generated Id-pulsed DCs combined with GM-CSF, after HDT/ASCT, showed Id-specific T cell proliferative response in 2 patients and a low level of Id-specific cytotoxic T cells in one patient (306). Subcutaneous administration of Id-pulsed DCs together with IL-2 to 5 patients in PR following HDT yielded Id-specific T cell response in 4 patients and PR in one patient (220).

Patients with advanced refractory MM have also been vaccinated with Id-pulsed DCs. Two such patients received Id-pulsed DCs combined with GM-CSF. Anti-Id T cell proliferative response as well as Id-specific T cell cytokine release was observed in both patients (307). Finally we have recently reported that vaccination with the Id protein coupled with alum together with IL-12 or a combination of IL-12 and GM-

CSF induced Id-specific T cell responses in patients with early stage MM associated with a reduction in the circulating myeloma B cells and a prolongation of TTP.

Comparison between these phase I-II trials might be difficult due to differences in both, patient characteristics and vaccines particularities. Nevertheless, they have collectively shown that the induction of tumors specific cellular immune responses is possible in the setting of minimal disease burden after ASCT. However, unlike the results of Id vaccination in NHL mentioned earlier, no firm evidence could be obtained from these trials that the natural course of the disease has been altered by Id vaccination and continuous efforts to improve the efficacy of Id vaccination are ongoing.

Among the newer strategies to improve the results of vaccination in MM is to combine immunotherapy with HDT and ASCT in high risk myeloma patients by harvesting and expanding primed anti-myeloma T cells and reinfusing them to patients after HDT. This principle has been done with DC-based whole myeloma cells vaccines so far with inconclusive results (308, 309). Another special approach to improve active Id immunotherapy in MM is the induction of Id-specific immunity in donors of hematopoietic stem cell for MM patients by Id immunization followed by adoptive transfer of the specific immune cells into the transplanted patient. This approach may render allogeneic stem cell transplantation (SCT) into a specific form of tumor immunotherapy. Few formal clinical trials of donor Id immunization were recently reported. In a study performed by Neelapu and coworkers, 5 MM patients and their related donors were immunized with the Id coupled to KLH plus GM-CSF prior to allogeneic SCT (310). All donors developed cellular and humoral anti-Id immune responses. Three patients received 3 booster vaccinations with KLH-coupled Id and GM-CSF after BM transplantation. All the 3 patients survived without evidence for disease recurrence for 5.5 to more than 8 years and all had evidence of Id-specific immunity after allogeneic SCT. Alternatively, to avoid immunization of healthy donors, donors T cells were stimulated *in vitro* with monocyte-derived Id presenting DC (311). Table 5 summarizes the clinical outcome of patients so far vaccinated with various Id vaccines.

Table 5. Recent Id-vaccine clinical trials in MM.

Treatment	Disease stage	Immune responses	Clinical results	References
Id-alum	I-IIA	3/5 Id-specific T-cell cytokine release 3/5 Id-specific antibodies	0/5	(217)
Id-alum + GM-CSF	IIA	1/5 Id-specific T-cell proliferation 5/5 Id-specific T-cell cytokine release 5/5 Id-specific antibodies	1/5 PR	(219)
Id-alum + IL-12 ± GM-CSF	I-II	15/28 Id-specific T-cell response 4/6 decrease/disappearance of blood circulating tumor cells	1/28 PR 1/28 MR	(218, 312)
Id-KLH + GM-CSF or IL-2	1 st remission after HDCT and PBSCT	13/15 Id-specific positive skin test	0/17	(297, 298)
Id-KLH-pulsed DCs	Advanced patient	Id-specific T-cell proliferation Id-specific T-cell cytokine release Id-specific CTL Id-specific antibodies	1/1 MR	(299)
Id-KLH-pulsed DCs	I	5/6 Id-specific T-cell proliferation 2/6 Id-specific T-cell cytokine release 3/6 Id-specific antibodies	1/6 MR	(300)
Id- or Id-KLH-pulsed DCs → Id-KLH	Remission after HDCT and PBSCT	4/26 Id-specific T-cell proliferation	2/21 CR 8/21 MR	(302)
Id-pulsed DCs → Id + GM-CSF	Advanced patients	3/10 Id-specific antibodies 4/10 Id-specific T-cell cytokine release	1/10 MR	(305)
Id-pulsed DCs	Remission after HDCT and PBSCT	Id-specific T-cell proliferation in responding patients	3/17 CR 2/17 PR	(303)
Id-pulsed DCs + IL-2	Remission after HDCT and PBSCT	2/5 Id-specific T-cell proliferation 4/5 Id-specific T-cell cytokine release 5/5 Id-specific antibodies	1/5 MR	(220)
Id-pulsed DCs → Id-KLH + GM-CSF	Remission after HDCT and PBSCT	2/10 Id-specific T-cell proliferation 1/10 Id-specific CTL	Not stated	(306)
Id/Peptide-KLH pulsed DCs	Remission after HDCT and PBSCT	6/6 Id-specific T-cell proliferation 5/7 Id-specific T-cell cytokine release	1/10 CR	(304)
Id-KLH-pulsed DCs + GM-CSF	Advanced patients	2/2 Id-specific T-cell proliferation 2/2 Id-specific T-cell cytokine release 1/2 Id-specific antibodies	0/2	(307)

5 AIMS OF THE THESIS

1. To characterize the Id-specific cellular immune responses in MM patients vaccinated with the Id protein and adjuvant cytokines.
2. To analyze the kinetics of circulating myeloma B cells during maintained Id vaccination in MM patients.
3. To evaluate the long-term effects of repeated Id vaccination on Id-specific T cells and the clinical effects in MM patients.

6 MATERIAL AND METHODS

6.1 Patients and healthy donors

Six healthy donors provided buffy coats for the first study (paper I). Their median age was 39 (range 35-63).

Patients included in papers II, IV, and V had stage I-II IgG MM stable disease and were either in stable response plateau phase following radio- or chemotherapy or asymptomatic and had received no treatment. Their median age was 65 years (range 46-82). Paper III included 4 patients with stage I-II stable non-progressive disease and 6 patients with stage II-III symptomatic progressive disease requiring therapy. Their median age was 71 years (range 50-83). Ten age matched healthy donors were used as controls.

The studies were approved by the regional Ethics Committee and written informed consent was obtained from each patient.

6.2 Preparation of monoclonal IgG and F(ab')₂ fragments

The procedure was performed as described (313). Briefly, patient sera were fractionated using a sterile MabTrapG column (Pharmacia, Uppsala, Sweden). IgG was eluted with 0.1 M glycine-HCl (pH 2.7). Isoelectric focusing (Pharmacia Phast system) indicated that more than 90% of the IgG was monoclonal. The monoclonal IgG was dialyzed against sterile NaCl followed by filtration through a Millipore filter (0.20 µm). F(ab')₂ fragments were obtained following pepsin digestion of the IgG. F(ab')₂ fragments of different myeloma patients prepared in the same way as the autologous F(ab')₂ were used as controls in the immunological assays.

6.3 Preparation of Id vaccine

Equal volumes of sterile filtered monoclonal IgG and alum solution (0.5% aluminium phosphate) (SBL Vaccine AB, Stockholm, Sweden) were mixed under aseptic conditions and adjusted with sterile 0.9% NaCl to a final IgG concentration of 1 mg/mL as described (217, 219). Tests for sterility, pyrogens, and viruses were performed and shown to be negative.

6.4 Immunization protocol and immune testing times

Patients received 0.5 mg of the autologous Id on day 1 intradermally (i.d.) in the left arm. One group of patients (n=15) also received 2 µg of the adjuvant cytokine IL-12 (Genetics Institute, Inc./Wyeth-Ayerst Research, Cambridge, MA, USA) subcutaneously (s.c.) in the contra lateral arm (IL-12 group). Another group of patients (n=13) were given the autologous Id and IL-12 as described above with the addition of 75 µg of GM-CSF (Schering-Plough, Kenilworth, NJ, USA) i.d. at the Id vaccine site daily, days 1 to 4 (GM-CSF/IL-12 group). The complete vaccination procedure was repeated after 2, 4, 6, 8, 14 weeks (induction phase) and continued at weeks 30, 46, 62, 78, 94 and 110 (maintenance phase). One patient included in papers III and V received the autologous Id vaccine plus 75 µg of GM-CSF (Schering-Plough) s.c. at the vaccine site daily, days 1 to 4 and the vaccination was repeated after 2, 4, 6, 8, 14 weeks (induction phase). This patient did not receive maintenance immunization. Patients were tested for cellular immune responses before vaccination and at weeks 4, 8, 10, 14, 16, 30, 32, 46, 48, 62, 64, 78, 80, 94, 96, 110, 112, 126, 142 and 158 or until progressive disease.

6.5 Isolation of peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC)

PBMC and BMMC were isolated from blood and bone marrow of patients using Ficoll/Hypaque (Amersham-Bioscience, Uppsala, Sweden) density gradient centrifugation as previously described (314). Isolation of PBMC was performed in all papers and isolation of BMMC was done in papers III and V only.

6.6 Isolation of CD4 and CD8 T cells

Fresh unstimulated cells and cells activated with PHA and PPD (see below) for 0.5, 4, 8, 24, 48, 72, and 96 h were subjected to magnetic bead separation using anti-CD4 and anti-CD8 monoclonal antibody coated Dynabeads (Dynabeads[®] CD8⁺&CD4⁺, Dynal A/S, Oslo, Norway) according to the manufacturer's instructions. The purity of separated CD4 and CD8 T cells was always > 95%. The procedure was performed in paper I.

6.7 Proliferation assay

The method has been described in detail (236) and was applied in papers II, III, IV and V. Briefly, PBMC were stimulated with purified F(ab')₂ fragments of the autologous idiotype IgG and allogeneic monoclonal isotype-matched IgG F(ab')₂ fragments (purified from other myeloma patients) as control. Unstimulated cells and cells stimulated with purified protein derivative of tuberculin (PPD) (Statens Seruminstitut, Copenhagen, Denmark) and phytohemagglutinin (PHA) (Sigma-Aldrich, Stockholm, Sweden) were also used as controls. Cells were cultured for 6 days and [³H]-thymidine (Amersham, Life Sciences, Amersham, UK) was added during the last 18 h of culture. Tests were run in triplicates. Mean thymidine incorporation was calculated for each triplicate. Stimulation index (SI) was calculated by dividing mean thymidine incorporation of antigen-stimulated cells with that of unstimulated cells. An SI cut-off level of ≥ 3.00 was used to indicate the presence of an idiootype specific cellular response as previously reported (236).

6.8 Enzyme-linked immunospot assay

The ELISPOT assay for identification of IFN- γ secreting cells was performed as described (236) and was applied in papers II, III, IV and V. Briefly, PBMC were incubated with F(ab')₂ fragments of the autologous idiootype or the isotypic (control) monoclonal IgG for 48 h in humidified air with 5% CO₂ at 37 °C. PBMC were also stimulated with medium alone, PPD (Statens Seruminstitut), and (PHA) (Sigma) as controls. Spots forming units (SFU) corresponding to cells secreting IFN- γ were quantified using an automated computer assisted video imaging analysis system (Axioplan2) (Carl Zeiss Vision, Jena, Germany). The number of SFU was obtained by subtracting the number of spots in cells incubated with medium alone from that of stimulated cultures. Results are expressed as number of SFU/10⁶ PBMC. A cut-off level of ≥ 70 SFU/10⁶ PBMC was used to indicate the presence of an idiootype specific cellular response as previously reported (218).

6.9 Delayed type hypersensitivity (DTH) reaction

This procedure was performed in paper IV at week 10. Sterile autologous monoclonal IgG (0.1 mg) and control human pooled polyclonal IgG (0.1 mg) were injected intracutaneously. Induration and erythema were measured after 24, 48, and 72 hours. A positive reaction was defined as an induration with a diameter ≥ 10 mm.

6.10 Intracellular cytokine (IFN- γ) assessed by flow cytometry (CFC)

The method was performed as described (223) and applied in paper II. PBMC were incubated with purified F(ab')₂ fragments of the autologous or isotype (control) monoclonal IgG (10 mg/ml) for 20 h. Brefeldin A (GolgiPlug, BD Biosciences Immunocytometry Systems, San Jose, CA) was added during the last 4 h. Cells were fixed with 2% paraformaldehyde (on ice) for 15 min, washed with PBS and stained with monoclonal antibodies specific for CD3, CD4, and CD8 (on ice) (BD Biosciences, Stockholm, Sweden) for 30 min in dark. Cells were washed again with PBS, permeabilised with FACS permeabilising solution (BD) and incubated with anti IFN- γ monoclonal antibodies (BD) for 30 min at room temperature in dark. Cells were finally washed, suspended in PBS and analyzed using a FACSCalibur flow cytometer (BD). The data were processed by the CellQuest software program (BD). A minimum of 5×10^4 lymphocyte-gated events were acquired.

The frequency of IFN- γ producing cells was presented as percent of CD4 or CD8 T cells. Mean + 2 SEM of the ratio between the frequency of IFN- γ producing cells induced by the isotypic IgG F(ab')₂ fragments and unstimulated cells was 1.29 (n = 66). First, an idiotype specific IFN- γ response was considered to be present when the ratio comparing the frequency of IFN- γ producing cells induced by the autologous idiotypic IgG F(ab')₂ fragments and the isotypic control IgG F(ab')₂ fragments was ≥ 1.3 . Then, the frequency of idiotype specific IFN- γ producing cells, as shown in the result section, was calculated by subtracting the frequency of IFN- γ producing cells of isotype-matched IgG F(ab')₂ stimulated cells from that of autologous IgG F(ab')₂ stimulated cells.

6.11 Cell phenotyping by flow cytometry and direct microscopy

The method has described in detail (315, 316) and was applied in paper III. Briefly, PBMC or BMMC (1×10^6) were incubated with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against CD3, CD4, CD8, CD56, and CD19 (Becton-Dickenson, Mountain View, CA, USA) (BD) and isotype-matched mouse IgG (BD) as control for 30 min (on ice) in the dark. Cells were then washed twice with FACS buffer (PBS, 1% BSA, and 0.1% NaN₃) and processed by a FACSCalibur flow cytometer (BD). Data were analyzed by the CellQuest software program (BD). A minimum of 5×10^4 lymphocyte-gated events was acquired. Plasma cells were visualized by direct microscopy of Giemsa stained smears prepared from peripheral blood and bone marrow aspirates. To determine the frequency of plasma cells a total of 300 to 400 cells were counted.

6.12 Cytometric bead array (CBA)

The BD™ CBA Human Th₁/Th₂ Cytokine kit was used according to manufacturer's instruction and was applied in papers II and III. The sensitivity of the kit is considered to be comparable to conventional ELISA. The lowest detection level using BD™ CBA Human Th₁/Th₂ Cytokine kit is 20 pg/ml and the highest is 5000 pg/ml (317). Briefly, supernatants of PBMC or BMMC (0.5x10⁶/ml) stimulated with the autologous idiotype or the isotype control monoclonal IgG F(ab')₂ fragments as well as of unstimulated cells (medium alone) were collected after 5 days of culture and incubated in duplicates with the cytokine-capture bead array for 3 h. Standards and samples were processed by FACSCalibur flow cytometer (BD) and data analyzed by the CBA software program (BD). The concentrations of the cytokines were calculated using standard curves. The presence of Id-specific cytokine secretion was considered when the concentration of a cytokine in the supernatant of autologous Id stimulated cells subtracted by that of unstimulated cells exceeded that of isotype-matched stimulated cells.

6.13 Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from BMMC and/or PBMC by the guanidium thiocyanate-phenol-chloroform extraction technique (318) using RNazol B (AMS Biotechnology Europe, Stockholm, Sweden). First strand cDNA synthesis was performed according to standard protocols (314) with minor modifications using the First-strand cDNA Synthesis Kit (Amersham-Pharmacia Biotech, Uppsala, Sweden). The procedure was performed in papers I, II, III and V.

6.14 Genomic DNA preparation

Genomic DNA was extracted from PBMC using the QRAprep Spin Miniprep kit (Qiagen® Helden, Germany) according to manufacturer's instruction. The procedure was performed in paper V.

6.15 Quantitative real-time polymerase chain reaction (QRT-PCR) for cytokines and cytotoxic proteins

The method was performed as described (319) and was applied in papers I, II, III and V. Briefly, PBMC were incubated with F(ab')₂ fragments of the autologous idiotype or the isotype-matched (control) monoclonal IgG for 8-18 h and 48 h in humidified air with 5% CO₂ at 37 °C. Cells were also incubated with medium alone and PHA (Sigma) as controls. Cells incubated for 8-18 h were used for cytokine (IFN-γ, TNF-

α , IL-4, IL-5, IL-10) analyses, while those stimulated for 48 h were used for granzyme B and perforin analyses. MgCl₂ concentrations were optimized for the individual primers to obtain maximum efficiency. The efficiencies of probes and primers used in the assays were in the range of 90-100% that corresponds to slopes between 3.6 and 3.1. PCRs and quantification of cytokine mRNA were performed as previously described (319). An Id-specific cytokine response was considered when the relative fold increase of a gene induced by the autologous Id was higher than the isotypic control i.e. fold increase ratio >1.

6.16 Criteria for clinical response, vaccine induced Id-specific T cell response and Th₁/Th₂ immune responses

Partial clinical response (PR) was defined according to the Bladé criteria for evaluating disease progression and response (56) as $\geq 50\%$ reduction of the M-component and minor response (MR) as 25-49% reduction of the M-component. Progressive disease (PD) was attained when one or more of the following criteria were fulfilled: a) an increase in the serum M-component concentration by at least 25% of pretreatment or response value; b) >25% increase in 24-h urinary light chain excretion; c) >25% increase in plasma cells in bone marrow; d) development of hypercalcemia and; e) development of new bone lesions or progression of osteolytic lesions.

In papers II, III and V, a vaccine induced idiotypic specific T-cell response was considered to be present when all of the following three criteria were met: 1) an idiotypic specific SI, SFU, or mRNA cytokine gene expression ratio \geq the corresponding cut-off levels (3, 70, and 1 respectively); 2) the idiotypic specific SI, SFU, or mRNA cytokine expression ratio values had to be \geq twice the respective pre-vaccination baseline value and; 3) an idiotypic specific response in at least two of these three tests detected at different testing times after start of vaccination. In paper IV a patient was considered to have developed a vaccine induced Id-specific T cell response if the following three criteria were fulfilled; 1) an Id induced SI and/or SFU value \geq the respective cut-off level (see above); 2) an Id induced SI and/or SFU \geq twice the baseline value; and 3) a positive test (proliferation, ELISPOT or DTH) at a minimum of two different time points. The CFC and/or CBA in paper II and III were used to detect the frequency Id-specific responses regardless of whether they were or not induced by vaccination.

The cellular immune response was defined as a Th₁ response when only Th₁ cytokine genes (IFN- γ and/or TNF- α) were expressed and as a Th₂ response when only the Th₂ cytokine genes (IL-4, IL-5 and /or IL-10) were expressed. When both types of cytokine genes were expressed the immune response was considered to be a mixed Th₁/Th₂ with a predominance of Th₁ cells when Th₁ cytokine genes fold

increase ratios were higher or expressed at more testing times than the Th₂ cytokine genes. The immune response was considered as mixed Th₁/Th₂ with a predominance of Th₂ cells when the Th₂ cytokine genes fold increase ratio was higher or expressed at more testing times than Th₁ cytokine genes.

6.17 Gene cloning and generation of allele specific oligonucleotide (ASO) probes and primers

The procedure was performed in Paper V. The gene encoding the variable heavy chain (V_H) for each patient was determined by reverse transcription-PCR (RT-PCR) using consensus V_H family primers (320) and a consensus gamma constant (C_γ) region primer (198). RT-PCR was performed in serial cDNA dilutions (1:10, 1:20, 1:50, 1:100, 1:500) prepared from patient's BMMC. The dominant V_H gene family (tumor clone) was identified as the clone amplified at the lowest dilution. The V_H PCR products were visualized on 1.5% ethidium-stained agarose gel and the V_H amplicons representing the myeloma clone were excised from the gel and purified with a QIAquick Gel Extraction Kit© (Qiagen® Helden, Germany). The purified V_H amplicons were ligated into a pGEM-T Easy Vector© (V_H plasmid) and then transformed into competent E.coli (JM 109©, Promega® Madison, USA) and cultivated in LB-Ampicillin agar plates (100 g/ml) pre-treated with IPTG and X-Gal. Five V_H plasmid-positive colonies were re-cultivated over night and plasmids were extracted with QIAprep Spin Miniprep kit© (Qia-gen®), and sequenced using ABI PRISM 310© (Applied Biosystem®) with T7 and Sp6 primers and a Big Dye© reagent (321). The sequences were confirmed by aligning to the closest published germline genes using the V-BASE directory (Medical Research Council, Centre for Protein Engineering, UK), and the IgBlast and Blast (National Center for Biotechnology Information, USA) databases (322). The β actin gene was also similarly cloned and sequenced.

The Complementary Determining Regions (CDR) II and III sequences were identified for all patients. For the cDNA assay, patient specific ASO sense primers corresponding to the CDRIII regions were designed for each patient to obtain maximum specificity (Table 1). A C_γ anti-sense primer (GGAAGTAGTCCTTGACCA) and probe (CCTCCACCAAGGGCCCATCG) were used for all patients. For the genomic DNA assay, a patient specific sense primer corresponding to the V_H 3 or 1 region, ASO (CDRIII) anti-sense primer and ASO (CDRII) probe were used (Table 1 B). The V_H 3/1, CDRIII and CDRII primers were tested on a panel of DNA from different patients to ensure that the primer amplified only the V_H gene from the patient of interest.

6.18 Reverse transcription and real-time allele specific oligonucleotide polymerase chain reaction (RT-ASO-PCR and real-time ASO-PCR)

The procedure was performed in Paper V. Patients were first tested for the presence of the malignant clone by RT-ASO-PCR using patients specific ASO primers. All positive patients were then subjected to quantitative real time ASO-PCR analyses performed as previously described (323) with minor modifications. Briefly, PCRs were performed in a 25 μ l reaction volume and contained 1xTaqMan Buffer A (Perkin Elmer), $MgCl_2$ (optimized for each ASO assay) and 3 mM $MgCl_2$ for the β actin assay (Perkin Elmer), 0.5 mM dNTPs (Perkin Elmer), 5.0 pmol forward (CDRIII) ASO primer (Cybergene AB, Stockholm, Sweden), 5.0 pmol reverse (C γ) primer (Cybergene), 2.5 pmol (C γ) TaqMan probe (Cybergene), 0.5 units Ampli-Taq Gold and 0.1 units uracil-N-glycosylase (UNG) (Perkin Elmer). Two μ l of patient's cDNA were included in each reaction volume except for the negative controls. In 2 patients, the assay was additionally performed in genomic DNA for comparison. For the generation of standard curves serial dilutions of known concentrations of patient specific V_H and β actin plasmid DNA were used. For each sample at least 4 or 6 aliquots (when the material allowed) were subjected to analysis, 2-3 aliquots for quantitation of clonal B cells and 3 aliquots for β actin. Aliquots were amplified for an initial period of 2 min at 50 °C and 10 min at 95 °C followed by 40 concurrent cycles involving denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 1 min. ABI PRISM 7700 Sequence Detection System (Perkin Elmer), was used for online quantification.

The mean gene copy number of patient's specific V_H was divided by that of the corresponding β actin copy number for normalization and the value obtained from samples taken before vaccination was set to 100%. The values at the different testing times during follow-up are presented as percent of the initial pre-vaccination value. Where genomic DNA was also analyzed the absolute gene copy number was plotted against the different testing times during follow-up and compared to the corresponding gene copy number in the cDNA real time ASO-PCR assay.

6.19 Statistical methods

In paper I, the Mann-Whitney test was applied to compare time of maximum accumulation of cytokines between activated CD4⁺ and CD8⁺ T cells. In paper II, Chi-square test was used to compare differences between groups and Spearman's Rank correlation test to estimate correlation between assays. In paper III, Wilcoxon Signed Rank test was used to compare immune responses of PBMC and BMMC as well as immune responses during the early active phase of immunization and the later

phase of the disease. The Chi-Square test was used to assess a shift from a Th₁ to a Th₂ response. The Mann-Whitney test was applied to compare the proliferative and IFN- γ (ELISPOT) responses, respectively in PBMC of patients and healthy donors. In paper IV, the Chi-Square test was applied to compare differences between the groups and differences in immune response between patients with a pre-existing immunity and non-pre-existing immunity. The Wilcoxon (Gehan) statistics was used to test differences in TTP between vaccination groups and between immune overall responders/non-responders respectively. The Mann Whitney test was used to test differences in levels of T_{reg} cells following immunization compared to baseline (before start of vaccination) in responding and non-responding patients. In paper V, Fisher's exact test was used to compare the kinetics of CMC between immune responders and non-responders.

7 RESULTS AND DISCUSSION

7.1 Background

Cancer immunotherapy is currently under rigorous evaluation in a plethora of cancer vaccine trials as a novel alternative or complementary therapy in many cancer types. The Id is a unique myeloma specific antigen that is expressed by the entire myeloma clone (myeloma cells and their precursors) and as such constitutes an attractive target for immunotherapy. Preclinical studies in murine myeloma models have clearly shown that the induction of anti-Id immunity confers protective and therapeutic immunity against tumor cell challenges. In human MM, however, it seems that the anti-Id cellular immunity induced, so far, by various vaccination strategies stands short of achieving solid objective clinical responses. The aim of this thesis is to have a close short range look at the detailed characteristics of the Id-specific cellular immune responses in a classical Id vaccination setting (Id protein combined with adjuvant cytokines) and study the long-term effects of the vaccine in the clinical outcome as well as the immune system of MM patients. Such a careful look might open gates to newer more effective Id based vaccination approaches.

7.2 Paper I

Kinetics of cytokine gene expression in human CD4⁺ and CD8⁺ T-lymphocyte subsets using quantitative real-time PCR (*Scand J Immunol.* 2003; 58 (6): 601-6)

Cytokines are critical modulators of innate and adaptive immunity, and may execute the final effector stages of T cell responses. This study was designed to examine the time for maximal accumulation of multiple cytokines produced by activated T cell subsets to be used as supplementary markers for antigen-specific T lymphocytes in subsequent studies. We analyzed the time kinetics of multiple cytokine genes (IL-2, IL-5, IFN- γ , GM-CSF and TNF- α) and granzyme B in purified CD4⁺ and CD8⁺ T cells of healthy donors following *in vitro* stimulation with PHA and PPD using real-time quantitative PCR. Although a wide inter-individual variability in the cytokine gene expression pattern was demonstrated, for most cytokines the time to induction was within 30 min of activation, and maximum accumulation seemed to be obtained after 4 to 8 h of activation. However, a sustained high level could be noticed for up to 24 h. Granzyme B gene expression showed a continuous gradual increase and late maximal accumulation (48-72 h). Minor variations were seen in the kinetics of various cytokines depending on the activator used and the T cell subset. We concluded that cytokine gene expression would better be measured after 4-8 h of specific stimulation, but also up to 24 h of stimulation is acceptable. Granzyme B gene expression may preferentially be measured after 48-72 h of activation.

7.3 Paper II

Idiotype protein vaccination in combination with the adjuvant cytokines IL-12 and GM-CSF in patients with multiple myeloma - evaluation of a T cell response by different read-out systems (*Haematologica*. 2007; 92 (1): 110-4)

Immune monitoring is one of the corner stones for evaluating the efficacy of anti-tumor vaccines. Due to scarcity of clinical responses in cancer vaccine trials, it has been difficult to validate any of the available immune assays as a useful surrogate for clinical response. Many workshops have been held for immune monitoring in cancer vaccine trials, but the debate on which assay would optimally evaluate tumor-specific immune responses is ongoing and the golden rule is to use multiple read-out systems. The main objective of this study was to characterize the Id-specific cellular immune response during Id vaccination (Id protein with IL-12 ± GM-CSF) in early stage disease. GM-CSF was chosen for its known capacity to increase the magnitude of both cellular and humoral tumor-specific responses and IL-12 was added to promote Th₁ immunity and augment the frequency of memory T cells. Results of 3 different assays: Id-specific T cell proliferative activity (³H-thymidine incorporation), ELISPOT (IFN-γ), and multiple cytokine gene expression (QRT-PCR) were evaluated in 18 patients and in 5 of these patients additional two assays (CFC and CBA) were included. This set of assays was selected as it might have a great possibility to detect a response against a weak self-antigen and to characterize the immune response in detail.

In the IL-12/GM-CSF group 78% of patients developed Id-specific immunity vs 22% in the IL-12 group ($p < 0.05$) as evaluated by proliferation/ELISPOT assays. By including QRT-PCR the frequency of responding patients increased from 50% to 78%. Predominantly a Th₁ response was noted in the IL-12 group while in the IL-12/GM-CSF group a Th₂ response predominated ($p = 0.053$). Vaccine induced immunity was more frequently seen in patients with a pre-existing Id-specific immunity than those without ($p < 0.02$). A correlation was noted between IFN-γ gene (QRT-PCR) and intracellular IFN-γ protein (CFC) expression ($r = 0.79$, $P < 0.0001$).

In conclusion, IL-12 alone seemed to induce a Th₁ polarized immune response, while the combination of IL-12 and GM-CSF induced a higher frequency of responding patients but with a Th₂ profile. Patients with naturally occurring tumor-specific immunity are more likely to benefit from vaccination than those lacking such immunity. It also seems that, until an assay that firmly and consistently correlates with clinical responses is validated, the use of multiple read-out systems may be necessary for the evaluation of immune responses of patients in vaccine trials.

7.4 Paper III

Long-term effects of idiotype vaccination on the specific T cell response in peripheral blood and bone marrow of multiple myeloma patients (*Submitted*)

This study was undertaken to evaluate the long-term effects of Id vaccination on tumor-specific T cells of MM patients and compare the frequency of Id-specific T cell responses in peripheral blood and bone marrow. Id-specific T cell responses of PBMC were compared with those of BMMC (tumor site) in 10 MM patients vaccinated with the Id protein after a median time of 41 months since last immunization. By then 6 of the patients had progressive disease. The responses of PBMC were also compared to those of PBMC during active immunization when all 10 patients had stable disease. T cell responses were measured by multiple read-out systems (proliferation assay, ELISPOT (IFN- γ), CBA for secreted cytokines and QRT-PCR for cytokine gene expression).

At the late testing time an overall Id-specific T cell response was detected in 5/10 patients in PBMC and in 4/10 patients in BMMC. A T cell response in both compartments was noted in 3 patients and in either compartment in 2 patients. The cytokine gene profile was consistent with the predominance of Th₂ cells (IL-4, IL-5, IL-10) both, in PBMC and BMMC. The concentrations (CBA) and fold increase ratios (QRT-PCR) of cytokines were generally higher in PBMC as compared to BMMC.

Comparison of the Id-specific T cell responses of PBMC during active immunization with those at the late follow-up, showed that the frequency and magnitude of the Id-specific immune responses had decreased significantly by time (proliferation/ELISPOT) ($p < 0.02$) and shifted at the gene level from a Th₁ (IFN- γ , TNF- α) to a Th₂ profile ($p < 0.05$).

This study shows that an Id-specific T cell response may be found at a similar frequency of patients in peripheral blood and bone marrow of MM patients indicating no preference for either site. The low proportion of the T cells in the bone marrow as compared to blood might dilute a detectable response in BMMC. Following Id vaccination, and during un-maintained immune therapy, there was a decrease by time, towards disease progression, in the Id-specific T cell response and a shift from a Th₁ to Th₂ immunity. Such a decrease in the tumor-specific immunity may be comparable to the progressive deletion of Id-specific T cells in the presence of high concentration of myeloma protein in the myeloma murine system and may necessitate the adoption of more frequent immune boosting strategies.

7.5 Paper IV

Long-term idiotype vaccination combined with IL-12, or IL-12 and GM-CSF, in early stage multiple myeloma patients (*Clin Cancer Res, in press*)

This study describes the general outlines of a phase II Id vaccination trial and gives a summary of the most important end points of the trial. The number of patients for the trial was set according to Gehan's design which states that the frequency of clinical responses in the first 14 patients determines the number of additional patients to be recruited. This enables the estimation of the response frequency with a SE of $\pm 10\%$. The maximum number of patients to be included in each group was set to 25. However, due to discontinued production of IL-12 as well as GM-CSF only twenty-eight patients were included in the study. Fifteen patients were allocated to the IL-12 group and 13 patients to the IL-12/GM-CSF group on an alternating basis. All patients received at least 3 vaccinations (≥ 4 weeks). Two patients did not complete the induction vaccination schedule (14 weeks) due to PD. Thirteen patients were withdrawn from the study at various time points during the maintenance immunization phase; twelve due to PD (6 in the IL-12 and 6 in the IL-12/GM-CSF group) and 1 patient due to a stroke not related to vaccination (IL-12/GM-CSF group). As indicated earlier, patients were generally immunized over 110 weeks and tested for cellular immune responses before vaccination and at regular intervals up to 158 weeks or until progressive disease.

Id-specific immune responses were noted in 5/15 patients (33%) in the IL-12 group and 11/13 patients (85%) in the IL-12/GM-CSF group ($p = 0.003$). Immune response were seen only in patients with M-component concentration < 50 g/L. Two third of the responsive patients subsequently lost their specific immunity and developed progressive disease suggesting the onset of T cell tolerance. Immune non-response was associated with an increase in the numbers of CD4⁺/CD25⁺ cells (T_{reg} cells). Median time to progression (TTP) for immune responders ($n=16$) was 108 weeks compared to 26 weeks for non-responders ($n=12$) ($p=0.03$). Two of the patients in the IL-12 group had a clinical response ($> 50\%$ and $> 25\%$ reduction of their respective M-component concentrations).

This study was the first trial that combined IL-12 and GM-CSF as adjuvant cytokines with the Id protein in human MM. It is also one of the largest trials in Id based vaccination in MM patients. An important finding in this study, excluding the two clinical responses, is the significant prolongation of TTP observed in immune responders compared to non-responders. The results of this study also support our observation in paper III concerning loss of Id-specific T cell responses with disease progression and the general notion that T_{reg} cells play an important role in immune suppression in cancer patients. Of special interest is the late occurrence of the two clinical responses and both being in the IL-12 group.

7.6 Paper V

Idiotype vaccination induced reduction/elimination of circulating tumor cells in patients with multiple myeloma (*Manuscript*)

Blood circulating tumor cells exist as part of MRD in many cancer types. In follicular lymphoma Id vaccination induced elimination of circulating lymphoma cells shown by a PCR assay based on detecting t(14;18) translocations on bcl-2-rearranged lymphoma cells (240, 324). We have previously reported, in a small pilot study, that the frequency of circulating myeloma B cells (CMC) exhibiting the same immunoglobulin heavy chain (IgH) gene rearrangement as the myeloma plasma cells, may be reduced following Id vaccination. These cells could be a source for relapse and may contribute to the aggressiveness of the disease. This study is designed to confirm our previous observations and to see whether CMC susceptibility to Id vaccination is durable and may result in clinical benefit.

Eleven MM patients were enrolled in this study and immunized with the autologous Id protein together with GM-CSF, IL-12, or a combination of the two cytokines. CMC were detected by real time ASO-PCR and the Id-specific T cell response was monitored by multiple read-out systems (proliferation assay, ELISPOT (IFN- γ), and QRT-PCR for cytokines (IFN- γ , TNF- α , IL-4, IL-5) and cytotoxic proteins (granzyme B and perforin).

Reduction of CMC was observed in 4/11 patients and in 2 of them a molecular remission of CMC in blood was observed. Two patients had stable levels of CMC while 3 patients showed progressive increase. In the remaining 2 patients, CMC were below the quantification level. All patients (n=6) who showed a reduction and/or a stable frequency of CMC mounted an Id-specific T cell immune response, while those with increasing numbers of CMC (n=3) failed to mount a tumor specific T cell immunity ($p < 0.02$).

The results of this study confirm and extend our previous observations and indicate that long-term idiotype vaccination might be associated with elimination/reduction of circulating myeloma B cells in patients with early stage MM. Similar to the observations in follicular lymphoma (240), the study also suggests that vaccine induced anti-idiotype immunity may be associated with long-lasting molecular remission in blood.

7.7 Conclusion

Id-immunization in early stage MM patients induces anti-Id immune responses that may correlate with reduction and/or elimination of blood circulating myeloma cells (CMC) as well as time to disease progression (TTP). Objective clinical responses may occur. Immune non-response may be associated with increased numbers of T_{reg}

cells. Adjuvant cytokines can be used to alter the anti-tumor immune response and need to be optimized with regard to dose, time of administration and appropriate combinations. Frequent boosting of immunity seemed to be necessary, but should be carefully gauged and balanced to avoid immune suppression.

The thesis argues that the Id remains an attractive target for immunotherapy, albeit it is, like other auto-antigens, weakly immunogenic. Efforts and resources may be justifiable to improve the immunogenicity and prolong the anti-tumor immunological memory of the vaccine as well as to identify an optimal strategy for delivery. Due to the various and complex deficiencies in the immune system of patients with MM, a multi-prong approach may be adopted e.g. a combination of active and adoptive transfer therapies as well as treatment modalities that correct the imbalance in T_{reg} cells.

8 FUTURE PROSPECTS

The Id remains to be an attractive antigen for immunotherapy in MM since shared tumor antigens in MM such as the cancer testis antigens (MAGE/NY-ESO-1) and MUC-1 may induce immune tolerance and be associated with autoimmune diseases due to their co-expression as self antigens in other normal tissues (325, 326). If Id-vaccination is to produce objective clinical benefits for MM patients, the Id specific cellular mediated immunity must be augmented. The vaccine must be able to generate, *in vivo*, sufficient number of tumor-specific T cells that can recognize the Id antigen with high avidity and traffic to the bone marrow to eradicate tumor cells (253). This is of utmost importance since anti-Id antibodies are virtually blocked from reaching tumor cells by the large quantities of soluble monoclonal Ig secreted by the tumor cells and may play very little role, if any, in tumor elimination. Furthermore, even if these antibodies escape peripheral blockade and reach the tumor site they are unlikely to be effective since the myeloma cells express little or no surface Ig (327). Both, CD8⁺ and CD4⁺ T cells must be generated since the Id antigen is presented by the tumor cells as well as by APC (DCs) in the context of MHC class I molecules to CD8 T cells and by APC to CD4 T cells in the context of MHC class II molecules.

An effective way of securing sufficient number of Id- specific T cells may be to harvest and expand autologous primed anti-myeloma T cells *in vitro* and re-infuse them to patients after HDT. Alternatively allogeneic Id-reactive T cells from healthy donors may be expanded *in vitro* and adoptively transferred to MM patients following *in vitro* priming or Id-vaccination of healthy donors. It is also equally important to select suitable patient candidates for Id vaccination i.e. patients with low tumor burden and early stage asymptomatic disease or adopt methods that can reverse T cell suppression in patients with advanced disease prior to Id vaccination e.g. reduction of serum myeloma protein levels as in patients with prolonged remission (MRD) after HDT. In study IV, Id-specific T cell responses were observed only in patients with serum M-component concentrations of < 50 g/L. A similar observation was also reported in Id-specific TCR-transgenic mouse model (327).

Other known myeloma associated immune dysfunctions must also be addressed. For example murine dysfunctional DCs were reported to regain functionality if generated *ex vivo* and treated with specific inhibitors of p38 mitogen activated protein kinase (MARK) (328), and Id vaccines incorporating allogeneic donor derived fully functional DCs are currently under evaluation (329). The complete remission induced by immunomodulatory drugs such as thalidomide in the MPT regimen or other regimens including lenalidomide or bortezomib is being investigated as a treatment modality that may preserve immune competence in MM patients and allow Id vaccination as a complementary treatment.

Lastly, various methods of delivery of the Id, still under investigation (see above), and a careful tuning of adjuvant cytokine combinations and balancing of the level of T_{reg} cells might all be an integral part of an optimal vaccination strategy.

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