GENETIC POLYMORPHISMS AND NATURAL KILLER CELL ACTIVITY IN MULTIPLE MYELOMA

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献给我的母亲, 妻子和儿子

To my mother, wife and son

ABSTRACT

Multiple Myeloma (MM) is a B cell malignancy characterised by an accumulation of clonal neoplastic plasma cells in bone marrow. Its aetiology remains unknown. Remarkable racial differences in the incidence of MM and occurrence of familial MM suggest that genetic factors may play a role in the development of MM. Susceptibility genes for MM, with the exception of MHC class I, have not yet been identified. Cytokines such as IL-10, IL-6, interleukin (IL)-1β and tumour necrosis factor (TNF)-α are involved in normal B cell development and in MM tumour cell growth and/or survival. In addition, IL-10 appears to play an important role in the inhibition of T cell-mediated immune responses mainly through its effects on antigen presenting cells, which could contribute to T cell anergy. Furthermore, increases in the number of T cells producing IL-6 and abnormalities of T cells such as biased expression of T cell receptor variable beta chain in MM patients have been observed. However, potential genetic involvements of cytokine and co-stimulatory molecule genes in the development of MM have not been studied.

The overall aims of this study were to uncover susceptibility genes involved in immunoregulation of MM and to evaluate potential importance of NK cell cytotoxic activity in MM. The specific aims were to investigate a possible association between IL-10, IL-6, IL-1β, TNF-α and CTLA-4 genes and MM, and to analyse NK cell activity in patients with MM in an attempt to uncover a potential role for NK cells in tumour repression. In the current study, our results showed that IL10.G and IL10.R microsatellite polymorphisms in IL-10 promoter region were associated with MM. Moreover, allele 136 was related to a higher IL-10 production as analysed by an *in vitro* assay. The microsatellite polymorphism in the 3′-untranslated region of exon 4 in the CTLA-4 gene was associated with MM and MGUS, and correlated in control samples to the level of CTLA-4 protein expression in $CD4^+$ T cells. However, we did not find an association between the IL-10 (–1082), IL-6 (-174), TNF-α (-308) or IL-1β TaqI polymorphism with MM. These results suggest that the loci in IL-10 and CTLA-4 genes (or alternative genes in linkage disequilibrium) constitute risk factors for the development of MM.

NK cells from MM patients pre-cultured in the presence of rhIL-2 exhibited substantial cytolytic activity against autologous mature DCs that were pre-pulsed with patient's monoclonal immunoglobulin *in vitro*. Furthermore, patient NK cells showed higher cytotoxicity toward autologous DCs as compared to controls. In addition, the activated NK cells also lysed autologous plasma cells, indicating that the NK cell -mediated cytolysis may contribute to tumour regression in MM *in vivo*. On the other hand, the results suggest that NK cell –mediated killing of autologous DCs may influence the outcome of administration of tumour antigen pulsed DCs in clinical trials.

In brief, our results imply that the genetic factors associated with MM may influence cytokine production *in vivo* and control the T cell activation pattern leading to conditions that may favour growth of MM cells and inhibit generation of CTLs. It is likely that cytokine activated NK cells in MM patients are endowed with anti-tumour activities. These results would indicate that there are potential effects of both adoptive and innate immune activities on the development of MM.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals.

I Zheng C, Huang D, Liu L, Wu R, Bergenbrant Glas S, Österborg A, Björkholm M, Holm G, Yi Q, Sundblad A (2001) Interleukin-10 gene promoter polymorphisms in multiple myeloma. *Int J Cancer,* 95 (3): 184-8.

II Zheng C, Huang D, Liu L, Björkholm M, Holm G, Yi Q, Sundblad A. (2001) Cytotoxic T-lymphocyte antigen-4 microsatellite polymorphism is associated with multiple myeloma. *Br J Haematol.,* 112 (1): 216-8.

III Zheng C, Huang DR, Bergenbrant S, Sundblad A, Österborg A, Björkholm M, Holm G, Yi Q. (2000) Interleukin 6, tumour necrosis factor α, interleukin 1β and interleukin 1 receptor antagonist promoter or coding gene polymorphisms in multiple myeloma. *Br J Haematol.,* 109 (1): 39-45.

IV Chengyun Zheng, Margareta Andersson, Masih Ostad, Fredrik Celsing, Göran Holm and Anne Sundblad. Natural cytotoxicity to autologous antigen pulsed dendritic cells in multiple myeloma. *Br J Haematol*. 2002 (in press)

LIST OF ABBREVIATIONS

Ag	antigen
APC	antigen presenting cell
BM	bone marrow
bp	base pair
CD	cluster of differentiation
CI	confidence interval
ConA	concanavalin A
CTLA-4	cytotoxic T lymphocyte antigen-4
CTLs	cytotoxic T lymphocytes
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN	interferon
IL	Interleukin
mAb	monoclonal antibody
imDC	immature dendritic cell
mDC	mature dendritic cell
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
M-Ig	monoclonal immunoglobulin
MM	multiple myeloma
NK	natural killer
OR	odds ratio
P	probability
PCR	polymerase chain reaction
Ra	receptor antagonist
RFLP	restriction fragment length polymorphism
rh	recombinant human
TCR	T cell receptor
TNF	tumour necrosis factor
VNTR	variable number of tandem repeat

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INTRODUCTION

Multiple myeloma (MM) is a clonal B-cell neoplasm characterised by accumulation of malignant plasma cells (myeloma cells) in the bone marrow, where myeloma cells interact with stromal cells for growth and survival. Myeloma cells secrete a monoclonal immunoglobulin (M-Ig) as detected in serum and urine of patients. Based on the evidence that myeloma cells have undergone immunoglobulin gene somatic hypermutation, VDJ recombination and isotype switch recombination (Morgan, 1999), myeloma cells seem to originate from post-germinal centre B cells. The malignant transformation of terminally differentiated plasma cells is considered to be the result of multi-step transformation process (Hallek et al., 1998). However, the aetiology of MM to date has remained obscure.

I. Clinical aspects of MM

In 1850, Dr. William Macintyre was the first to clinically describe MM. Twenty years later Von Rustizky introduced the term multiple myeloma (Gahrton & Durie, 1997). The predominant clinical manifestations of the disease are related to the accumulation of malignant plasma cells and their secretion of M-Ig, mainly consisting of bone pain with pathological fractures, anaemia, symptoms of hypercalcemia, renal insufficiency and immunosuppression related infections.

I-1. Diagnosis criteria

In our study, the diagnosis of MM strictly followed the criteria previously described (Mellstedt et al., 1977; Osterborg et al., 1993). MM diagnosis was established if two of the three criteria below were fulfilled. Briefly, 1. More than 10 percent plasma cells in bone marrow. 2. A monoclonal Ig peak in serum and/or urine, together with subnormal concentrations of at least one non-monoclonal Ig class (IgG, IgM, or IgA). 3. Osteolytic and/or osteoporotic bone lesions compatible with MM.

The diagnosis of monoclonal gammopathy of undetermined significance (MGUS) is based on the following criteria (Pettersson et al., 1981). 1. Less than 10 per cent plasma cells in the bone marrow. 2. A haemoglobin concentration > 120 g/l. 3. Platelet counts > 150 x 10^9 /l. 4. No

osteolytic lesions on bone X-ray examination. 5. A M-Ig peak in serum and/or urine with no rise in concentration during an observation period of more than one year. 6. No clinical symptoms. MGUS is considered to be a pre-malignant lesion, which usually remains stable for years. A recent study of the prognosis in MGUS with a big sample size (1384 cases) demonstrated that the cumulative probability of progression to MM and other related diseases such as macroglobulinemia and primary amyloidosis was 12 percent at 10 years, 25 percent at 20 years, and 30 percent at 25 years, respectively (Kyle et al., 2002).

I-2. Staging

The Durie and Salmon staging system (Durie & Salmon, 1975) was used in the present studies (Table 1).

Table 1. Staging criteria for MM

Stage I:	Haemoglobin > 100 g/l
	Serum calcium < 2.6 mmol/l
	Bone X-ray shows normal bone structure or solitary bone plasmacytoma only
	M-component concentration:
	IgG < 50 g/l
	IgA < 30 g/l
	Urine light chain M-component on electrophoresis < 4 g/24h
Stage II:	Fitting neither stage I nor stage III
Stage III:	One or more of the followings:
	Haemoglobin < 85 g/l
	Serum calcium > 3.0 mmol/l
	Advanced lytic bone lesions
	M-component concentration:
	IgG > 70 g/l
	IgA > 50 g/l
	Urine light chain M-component on electrophoresis > 12 g/24h
Sub-classification (A or B)	
A: Relatively normal renal function (serum creatinine value < 170 μmol/l)	
B: Abnormal renal function (serum creatinine value ≥ 170 μmol/l)	

I-3. Treatment

Most MM patients have a symptomatic disease at diagnosis and require therapy. Without treatment, the median survival of MM patients is less than one year. Treatment approaches consist mainly of chemotherapy (standard and high-dosage), stem cell transplantation, immunotherapies, while other treatments include treatment of myeloma bone disease (Anderson

et al., 2000). Combined chemotherapies with standard-dosage have improved median survival up to 36 months (Crowley et al., 2001). With the application of high dosage chemotherapy and autologous stem cell transplantation, the survival of patients with MM has been further improved to around 5 years (Fermand et al., 1998). However, MM is still an incurable malignancy.

Increasing knowledge about tumour immunology has generated considerable interest in immunotherapy for the disease. Idiotypes, the specific determinants of Ig variable regions, derived from myeloma cells have been considered to represent tumour-specific antigens. Idiotype specific T-cells have been previously observed (Dianzani et al., 1988; Yi et al., 1993; Yi et al., 1995). In addition, CTLs generated *in vitro* from peripheral T cells of MM patients using autologous DCs as APCs demonstrated substantial cytotoxic lysis of autologous primary myeloma cells in a MHC class I-restricted fashion (Li et al., 2000; Wen et al., 2001). It has been proposed that DC based immunotherapy could be an alternative approach to treat myeloma. Clinical trials in patients with MM have revealed that such vaccines can be applied safely without significant side effects and that certain tumour related T cell responses can be induced, but clinical outcome was poor (Liso et al., 2000; Titzer et al., 2000). In one study, DCs generated from adherent cells of PBMCs were administered intravenously to patients with melanoma after pulsing DCs with a melanoma-associated peptide. Unfortunately, this study was ended with a poor clinical outcome (Panelli et al., 2000), suggesting that there are still many problems that need to be solved before DC based vaccines can be routinely used as a therapy approach in the treatment of cancer patients.

II. Incidence of MM

MM accounted for 14.1% of new haematological malignancies and 18.5% of deaths from haematological malignancies in the United States in 1996 (Parker et al., 1996). In Malmö, Sweden, the average annual incidence rate per 100 000 inhabitants was 3.4, 4.9 for males and 3.7 for females between 1950 and 1979 (Turesson et al., 1984). In America, the average annual incidence rates per 100 000 for Caucasian inhabitants in the 1978-1990 period, were 4.1 for both sexes, 5.4 for males and 2.8 for females (Kyle et al., 1994). The incidence of MGUS was 1.4% and 2.6% in populations aged 50 to 69 and 70 to 89 years, respectively (Axelsson et al., 1966). Age and race are two main factors related to the incidence of MM.

II-1. Age

The occurrence rate of MM is closely related to age. The data from the Turesson et al study showed that the incidence increased progressively with age in Malmö, Sweden between 1970 and 1979 (Turesson et al., 1984). In this investigation, myeloma was not found in the population who were under age 40. In contrast, the incidence of MM reached 64.5 per 100 000 in males and 36.6 per 100 000 in females over the age of 80 years. The median age of MM in North America was 62 years (Belch et al., 1988) and 72 years in Sweden (Hjorth et al., 1992). Fewer than 3% of patients are younger than 40 year old (Blade et al., 1996). The mechanism behind such age-associated phenomenon is not known.

II-2. Race

There are striking differences in the occurrence of MM cases among races. The age-adjusted incidence of MM from selected population-based cancer registries around the world ranges from $0.5/10^5$ in Hawaiian Japanese males to $8.2/10^5$ in Bay area African American males, USA (Riedel & Pottern, 1992). The average annual age-adjusted (1970 US standard) incidence rates for African Americans in America were $10.8/10^5$ for males and $7.2/10^5$ for females (Bergsagel, 1995). Lower incidences of MM ($1.4/10^5$ or less) in Chinese and Japanese were consistently observed in Shanghai of China and Singapore and in the Japanese of Osaka and Hawaii (Bergsagel, 1995). This racial difference in the incidence of MM is even observed among population living in the same regions. The incidence of MM was $2.3/10^5$ in male Chinese, $1.7/10^5$ in Japanese and $4.6/10^5$ in Caucasian in the same regions of USA (Bergsagel, 1995).

III. Aetiology

Development of cancers including MM seems to be associated with genetic factors or a combination of potential susceptibility factors and certain environmental exposures. However, causes of myeloma are largely unknown. Certain environmental factors related to MM development have been suggested. So far, none of them has been proved to contribute to the occurrence of MM. Furthermore, little is known about the genetic background for developing MM.

III-1. Environmental factors

Smoking

A prospective cohort study showed that smoking was related to an increased risk of developing MM (Mills et al., 1990). In contrast, another cohort study with a larger population in Sweden did not show such an effect (Adami et al., 1998).

Radiation

An excess frequency of MM was observed in American radiologists (Matanoski et al., 1975). However, studies of the incidence of MM in the atom bomb survivors in Japan did not show an association of the radiation exposure with MM (Neriishi et al., 1993; Preston et al., 1994).

Benzene

Benzene was thought to be related to an increased frequency of MM (Aksoy et al., 1984). However, several case-control studies did not support a causal relationship between exposure to benzene and the risk of developing MM (Bergsagel et al., 1999).

Autoimmune disease

Increased frequency of MM in patients with rheumatoid arthritis has been previously reported (Isomaki et al., 1978). In addition, an increased number of cases of rheumatoid arthritis has been detected in MM patients (Eriksson, 1993). These would suggest that there might exist a connection between the B cell malignancy and autoimmune diseases.

Others

Human herpesvirus-8 (HHV-8) has been considered to play a role in the pathogenesis of MM (Rettig et al., 1997), but subsequent studies have questioned the existence of an association between MM and HHV-8 infection (Cannon et al., 2000; Dominici et al., 2000). Agriculture work is the occupation most frequently associated with MM (Nanni et al., 1998).

III-2. Genetic factors

Racial difference in the incidence

As mentioned earlier, there are remarkable differences in the incidence of MM among different races, lowest in Japanese/Chinese, intermediate in Caucasian and highest in African Americans. The low incidence of MM in Japanese and Chinese populations in Asia is kept when they migrate to America, indicating that the difference in the incidence of the disease between different racial populations is mainly determined by genetic rather than environmental factors.

Familial MM

Substantial familial clustering of MM has been reported (Shoenfeld et al., 1982; Grosbois et al., 1999; Lynch et al., 2001; Roddie et al., 1998). Moreover, several affected family members were found to have inherited identical HLA haplotypes (Bizzaro & Pasini, 1990; Grosbois et al., 1999). In familial MM, the mean age at diagnosis in successive generations was younger for children than for parents (Deshpande et al., 1998; Grosbois et al., 1999). National database studies of familial cancer in Sweden showed that the risk for a son to develop MM was elevated with a relative risk of 3.9 when his father had cancer (Hemminki & Vaittinen, 1998). Consistently, individuals who had first-degree relatives with MM had a significant higher risk for developing MM than those without a first-degree relative with MM (Brown et al., 1999; Eriksson & Hallberg, 1992). These findings suggest that the predisposition to MM may be inherited.

Association of HLA loci with MM

Pottern et al investigated human leukocyte antigens (HLA) class I allele distributions by serological typing in a population-based case control study including 46 African America patients and 88 ethnic controls, and 85 Caucasian patients and 122 Caucasian controls (Pottern et al., 1992). Their results demonstrated that there was a significant association of the HLA-Cw2 with MM among both African American and Caucasian males. In addition, the frequency of the HLA-Cw2 allele in African Americans was similar to that in Caucasian male population.

They concluded that the Cw2 allele, or a gene close to the C loci, might confer susceptibility to the development of MM for both African Americans and Caucasians (Pottern et al., 1992).

IV. Cytokines in MM

Cytokines are a group of low-molecular weight regulatory proteins secreted by white blood cells and a variety of other cells including stromal cells in the bone marrow. Cytokines generally exert their effects locally and bind to their receptors on the membrane of target cells with high affinity, therefore, picomolar concentrations of cytokines can mediate a biological effect. Cytokines play an important role in finely regulating the immune system. Under certain circumstances, certain cytokines may be directly and/or indirectly involved in the development of tumours such as MM.

Myeloma cells appear to be long-lived plasma cells localised in the bone marrow (BM) (Hallek et al., 1998). In BM, myeloma cells closely interact with the stromal cells which support myeloma cell growth and survival mainly by secreting cytokines (Hallek et al., 1998). In particular, the pathogenesis of MM depends upon the presence of some of these cytokines such as interleukin (IL)-6, TNF-α, IL-1β and IL-10. These cytokines are involved not only in MM pathogenesis but also in normal B cell development.

IV-1. IL-6, TNF-α and IL-1β

IL-6, TNF-α and IL-1β are important factors for B cell growth, differentiation and antibody production (Chiplunkar et al., 1986; Brieva et al., 1990; Rieckmann et al., 1991; Burdin et al., 1995), and are implicated in the development of MM. IL-6 supports the proliferation and survival of MM cells (Hallek et al., 1998) via autocrine and paracrine pathways (Anderson & Lust, 1999). Myeloma cells per se can produce IL-6, mediating an autocrine tumour growth. Studies on CD40 signalling in MM cells from Urashima et al revealed that triggering MM cells via CD40 induced tumour cell growth mediated by autocrine IL-6 (Urashima et al., 1995), indicating that $CD4^+$ T cells may regulate MM cell growth in the BM microenvironment via their cytokine productions induced by the CD40L-CD40 signal pathway. TNF-α can be secreted by myeloma cells (Sati et al., 1999) and induces IL-6 production by BM stromal cells (Carter et al., 1990). Producers for IL-1β in BM appear to be non-myeloma cells (Sati et al., 1999). IL-6, TNF-α and IL-1 are considered to be important osteoclastic stimulators

contributing to the development of osteolytic bone disease (Bataille et al., 1997). Increased serum level of these cytokines, especially IL-6, have been reported (Kyrstsonis et al., 1996) and the higher levels of IL-6 and TNF-α were correlated to a progressive disease (Filella et al., 1996; Wierzbowska et al., 1999).

IV-2. Interleukin-10

Interleukin-10 (IL-10) was first described as a cytokine synthesis inhibitory factor by Mosmann and colleagues in 1989 (Fiorentino et al., 1989). During the last 13 years, IL-10 has been extensively studied and found to play essential roles in regulating immune response and tumour growth, especially B cell malignancies.

B cells

IL-10 has been shown to induce proliferation and differentiation of B-cells into plasma cells and to enhance survival of normal human B cells (Moore et al., 2001). Moreover, IL-10 could inhibit apoptosis of germinal center B cells by up-regulating bcl-2 expression (Levy & Brouet, 1994) and could function as a switch factor for IgG1 and IgG3 (Briere et al, 1994). Telomerase is a ribonucleoprotein polymerase consisting of a telomerase RNA subunit, the catalytic protein component/telomerase reverse transcriptase (hTERT) and telomerase-associated protein, which seems to be involved in oncogenesis (Nicol Keith et al., 2001). Telomerase activity is observed in germline cells and most tumours and is absent in the majority of somatic cells (Keith et al., 2001). IL-10 induces hTERT expression and telomerase activity in human B cells independently of cellular proliferation (Hu & Insel, 1999), suggesting that IL-10 may be involved in B cell immortalization and/or in B cell malignancy transformation.

DCs and T cells

In vitro generation and maturation of monocyte-derived DCs can be inhibited by exogenous IL-10. In the absence of GM-CSF and IL-4, IL-10 can induce differentiation of imDCs into macrophage-like cells (Allavena et al., 1998; Fortsch et al., 2000). In addition, IL-10 down-regulates MHC class II and co-stimulatory molecule expression on DCs (Kawamura & Furue, 1995). Inhibitory effects of IL-10 on T cell cytokine production (Th1 type) and activation seem

to occur mainly via its effects on APC function (Fiorentino et al., 1991). It has been shown that IL-10 directly affects T cell function, and inhibits IL-2 production (Taga et al., 1993). *In vitro*, about 50% of proliferation of a tetanus toxiod-specific T cell clone induced by anti-CD3 mAb was inhibited by IL-10 (Taga et al., 1993). One animal experiment showed that T cell-derived IL-10 favors tumour cell (Lewis lung carcinoma) growth *in vivo* (Sharma et al., 1999). In addition, APCs from IL-10 transgenic mice were found to have significantly decreased ability to induce allo-genetic T cell response as observed in the mixed lymphocyte reaction, IL-12 production and CTL cytolytic activity (Sharma et al., 1999). Moreover, IL-10 plays a crucial role in generating regulatory T (Tr) cells (Groux et al., 1997). Tr cell inhibitory effects on the antigen specific responses seems to be mediated by cell-cell contact and their production of IL-10 together with TGF-β (Doetze et al., 2000; Seo et al., 1999). Deletion of Tr cells from the tumour lesions augmented tumour-specific CTL as well as NK cell activities and led to a resultant regression or growth inhibition of the tumours in an animal model (Seo et al., 1999). These results strongly suggest an inhibitory role for IL-10 in regulation of specific immune activity against tumours.

Myeloma cells

More recently, one study demonstrated that U266, RPMI-8226 and 6 other myeloma cell lines expressed IL-10 receptor mRNA and most of the cell lines also expressed IL-10 mRNA (Otsuki et al., 2000), suggesting that IL-10 may support MM cell growth via an autocrine pathway. Furthermore, IL-10 was found to stimulate the proliferation of MM cell lines and of freshly explanted MM cells in short-term BM cultures (Lu et al., 1995) by induction of an oncostatin M autocrine loop (Gu et al., 1996). In addition, IL-10 induces IL-11 receptor expression thereby enhancing stimulatory effect of IL-11 on myeloma cell growth (Lu et al., 1995). Increased serum IL-10 levels in patients with MM are observed to be associated with progressive disease (Urbanska-Rys et al., 2000). In view of effects of IL-10 on the immune system and myeloma cells, it seems very likely that IL-10 is involved in the development of MM.

IV-3. Genetic polymorphisms of the cytokines.

IL-6, TNF-α, IL-1β and IL-1RA

A number of genetic polymorphisms within the genes of IL-6, TNF-α, IL-1β, IL-10 and IL-1 receptor antagonist (Ra) have been previously defined (Olomolaiye et al., 1998; Pociot et al., 1992; Tarlow et al., 1993; Wilson et al., 1992). Position –174 in the promoter region of IL-6 gene consists of a G/C transversion polymorphism (Olomolaiye et al., 1998). The allele C in this locus is associated with low levels of plasma IL-6 in healthy subjects (Fishman et al., 1998). The promoter region of TNF-α gene includes a bi-allelic polymorphic site at position -308 (Wilson et al., 1992). The presence of adenine (allele 2) instead of a guanine (allele 1) at position -308 is associated with high TNF-α production by peripheral blood mononuclear cells (PBMC) in healthy individuals (Louis et al., 1998). There is a C (allele 2) to T (allele 1) transition polymorphism in exon 5 of the IL-1β gene and the allele 2 is associated with high IL-1β production by lipopolysaccharides (LPS) -activated PBMCs in healthy individuals (Pociot et al., 1992). IL-1Ra binds to the IL-1 receptor and inhibits the biological effects of IL-1 (Dinarello, 1996). IL-1Ra levels in human plasma were found to correlate to the polymorphism resulting from an 86 bp variable number of identical tandem repeats (VNTR) in intron 2 of the gene (Hurme & Santtila, 1998).

Interleukin-10

In humans, the gene encoding IL-10 has been mapped to chromosome 1 (Kim JM & Jenkins NA, 1992) between 1q31 and 1q32 (Eskdale J, 1997). The promoter of the gene spans a region of 5kb upstream of the transcription-starting site, and is known to contain several polymorphic loci. There are two (AC)n repeat microsatellites at approximately 1.1 and 4 kb upstream, which were termed IL10.G and IL10.R respectively (Eskdale & Gallagher, 1995; Eskdale et al., 1996). In addition, three single nucleotide polymorphisms, at positions -1082, -819 and –592 (Kube et al., 1995; Lazarus et al., 1997), were defined. IL-10 production was correlated to the bi-allelic polymorphism at position -1082, constituted by a guanine (G) to adenine (A) substitution, where G was associated with a higher IL-10 production by concanavalin A (ConA) stimulated PBMCs from normal individuals (Turner et al., 1997). In addition, the two

microsatellite polymorphisms (IL10.G and IL10.R) in promoter region are also described to be related to IL-10 production (Eskdale et al., 1998). IL-10 polymorphisms have been previously implicated in non-malignant B cell related autoimmune disorders (Eskdale et al., 1997; Huang et al., 1999). Considering the involvement of these cytokines in the development of MM (Fig 1.), one hypothesis is raised that the polymorphisms in the loci of the cytokine genes may constitute risk factors for developing MM.

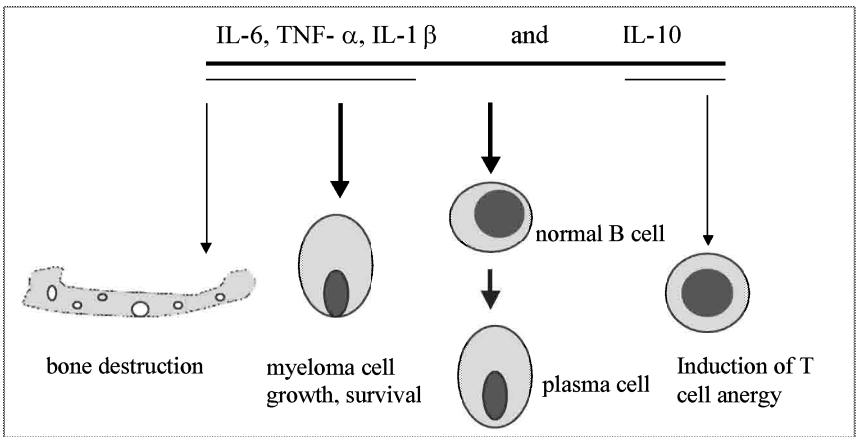


Fig 1. Involvement of IL-6, TNF-α, IL-1β and IL-10 in immune regulation and disease development.

V. T cells, Co-stimulatory molecules and DCs and NK cells

V-1. T cells

T cells are major components of specific immunity, which includes two well-defined subpopulations, T helper ($CD4^+$) and T cytotoxic ($CD8^+$) cells. T cell receptor (TCR) expression on T cells allows recognition of antigens, which are bound to major histocompatibility complex (MHC) molecules on antigen presenting cells (DCs, B cells and macrophages). $CD4^+$ T cells can recognise and interact with antigen/MHC class II molecules. Based on the cytokine production pattern, $CD4^+$ T cells are sub-grouped into Th1 and Th2 cells. Th1 cells secrete IL-2 and IFN-γ thereby playing an important role in cellular immunity against cancer, virus infections and intracellular parasites. Th2 cells secrete IL-4, IL-10 and IL-6 helping B cells to proliferate, differentiate and consequently playing a major role in humoral

immunity (Constant & Bottomly, 1997). $CD8^+$ T cells recognise and interact with antigen-MHC class I molecule complexes and differentiate into an effector cells called cytotoxic T lymphocyte (CTL) under the influence of Th1 type cytokines particularly IL-2. CTLs play an important role in immune surveillance, eliminating tumour cells, virus infected cells and foreign tissue graft.

For complete T cell activation, an antigen specific signal via the TCR complex and co-stimulatory signals are required. In the absence of co-stimulation, engagement of the T cell receptor fails to induce T cell proliferation and results in anergy (Greenfield et al., 1998).
In MM patients, T cell alterations such as an abnormal ratio of $CD4^+$ and $CD8^+$ T cells (Mills & Cawley, 1983; San Miguel et al., 1985) and biased expression of the T cell receptor variable beta chain (Janson CH, 1991; Kay et al., 1999) have been previously described. In a murine model, T cells were implicated in the development of plasmacytoma (Hilbert et al., 1995). Furthermore, a recent report demonstrated that the incidence of B-cell lymphoproliferative disease (BLPD) induced by inoculation of PBMCs from Epstein-Barr virus seropositive blood donors was substantially reduced after deletion of $CD4^+$ T cells (Johannessen et al., 2000). These findings indicate that T cells, especially $CD4^+$ cells, might play an important role *in vivo* in the B cell tumourigenesis.

V-2. Co-stimulatory molecules

Co-stimulatory molecules and T cell activation

A number of co-stimulatory signal pathways have been described, such as CD28/B7, CD152 (CTLA-4)/B7 and the inducible co-stimulatory molecule (ICOS)/B7h. Among them, the interaction of CD28 with B7 molecules (CD86 and CD80) is relatively well defined. CD28-mediated co-stimulation induces cytokine production, primarily IL-2, following activation of naïve T cells (Lenschow et al., 1996). IL-2 functions as a T cell growth factor thereby supporting T cell expansion and differentiation of the antigen-specific T cells. Cytotoxic T lymphocyte antigen-4 (CTLA-4), a homologue of CD28, binds to B7 molecules with much higher affinity than CD28 (van der Merwe et al., 1997). Inhibition of T cells by CTLA-4/B7 signalling has been demonstrated *in vitro* and *in vivo* (Chambers et al., 2001). Cross-linking of CTLA-4 on normal murine T-cells has been shown to reduce the production of IL-2 and

prevent the up-regulation of IL-2 receptors (Walunas et al., 1996). Evidence that CTLA-4 deficient mice exhibit a massive lymphoproliferative disorder (Tivol et al., 1995) strongly suggests that CTLA-4 acts as a crucial negative regulator of T cell activation (Chambers et al., 2001). CTLA-4 can interfere with T cell activation by two mechanisms: through competition with CD28 for binding to B7, or via inhibition of TCR- and CD28-mediated signal transduction (Chambers et al., 2001) by recruitment of phosphatase SHP-2 and PP2A that may oppose the action of kinases downstream of CD3 and CD28 (Frauwirth & Thompson, 2002). It appears that CD28/CTLA-4 plays an essential role in the differentiation of Th2 cells. Oosterwegel et al demonstrated that in CTLA-$4^{-/-}$ mice, $CD4^{+}$ T cells developed into Th2 cells after priming and re-stimulation *in vitro* by specific peptide Ag, however, wild type T cells developed into Th1 cells. In addition, administration of an anti-CD28 Ab *in vivo* induced IL-4 production in CTLA-$4^{-/-}$ mice but not in wild type mice. A newly identified ICOS shares approximately 30 to 40% sequence similarity with CD28 and CTLA-4, binds to other B7 family molecules such as B7h and B7RP-1 to enhance T cell proliferation and increase the secretion of IL-4, IL-5, IL-10 and IFN-γ but not IL-2 (Chambers et al., 2001). $ICOS^{-/-}$ T cells display a substantial defect in the ability to secret Th2 type cytokines (Tafuri et al., 2001), suggesting that ICOS may play an important role in regulating Th2-like cell development. CTLA-4 engagement could prevent ICOS co-stimulated T cells from producing IL-4 and IL-10 *in vitro* (Riley et al., 2001). These results indicate that CTLA-4 is a potent and critical inhibitor of Th2 cell differentiation via antagonism of CD28/B7 and ICOS/B7h mediated co-stimulation.

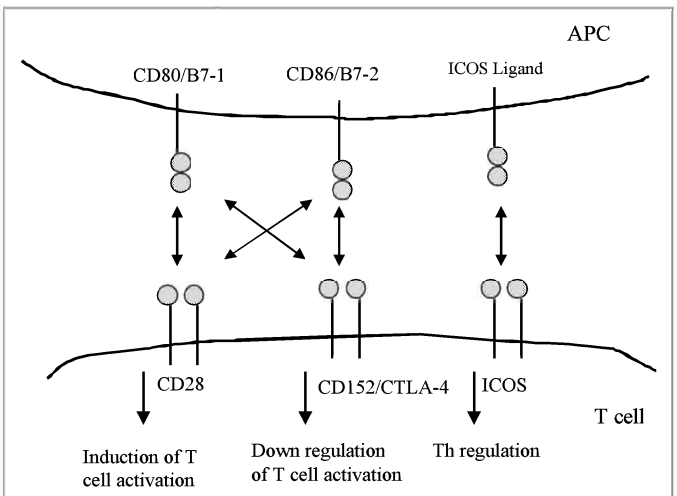


Fig 2. Co-stimulation signalling and T cell response.

Briefly, CD28/B7 and ICOS/B7h deliver co-stimulatory signals for T cell activation, while CTLA-4/B7 down-regulates T cell activation (Fig 2.)

CTLA-4 gene and genetic polymorphisms

The human CTLA-4 gene is located on chromosome 2q33 (Harper et al., 1991). Three polymorphic loci in the gene have been defined (Fig 3.).

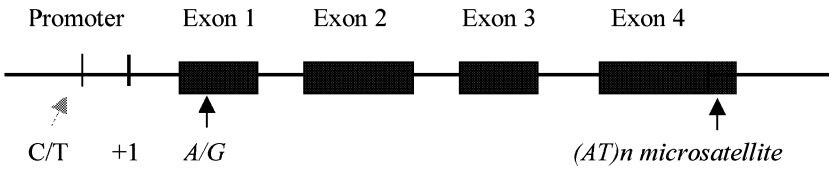


Fig 3. Locations of the three polymorphic loci in the CTLA-4 gene.

The CTLA-4 (AT) repeat microsatellite polymorphism in the 3′-untranslated region of exon 4 (Ling et al., 1999) was initially defined as exon 3 in the CTLA-4 gene (Dariavach et al., 1988). The polymorphism at position 49 in exon 1 was shown to affect T cell proliferation (Kouki et al., 2000) and CTLA-4 expression on T cells (Ligers et al., 2001) *in vitro*.

Because B cells have close interactions with T cells *in vivo,* particularly in germinal centres, different T cell behaviour or activity regulated by genetic factors may influence B cells including "myeloma progenitor cells" development thereby affecting disease progression. Indeed, a crucial role of T cells in the formation of plasmacytoma in an animal model has been demonstrated (Hilbert et al., 1995). However, there is a shortage of knowledge concerning potential genetic roles of the CTLA-4 in MM.

V-3. Dendritic cells

DCs were first described by Steinman and Chon about 30 years ago (Steinman & Cohn, 1973). DCs include Langerhans cells in epithelium (skin, mucosa, lung), veiled DCs in the afferent lymphatic and thoracic ducts, interstitial DCs in the connective tissue of solid organs such as heart and kidney, blood DCs, interdigitating DCs of lymphoid organs, and follicular DCs in germinal centre. The origin of DCs is thought to be diverse. Most types of DCs are considered to be myeloid such as Langerhans. In addition, there is another type of DC, which can be generated from human thymic precursors ($CD34^{+}CD1a^{-}$) *in vitro* in the presence of GM-CSF and IL-3. These thymic precursors can give rise to NK cells under proper culture conditions but not granulocytes or macrophages (Kelly et al., 2001). This evidence suggests the existence of a

lymphoid DC origin in humans. Recently, del Hoyo et al defined a DC-committed precursor population in mice (del Hoyo et al., 2002). In blood $CD11c^{+}MHC\text{-}II^{-}$ cells have the capacity to generate all the DC sub-populations present in lymphoid organs including $CD8\alpha^{-}$, $CD8\alpha^{+}$ and $B220^{+}$ DC subsets (del Hoyo et al., 2002).

DC development is proposed to have three stages. Firstly, DC precursors patrol through blood and lymphatic, and enter into tissues. In most tissues, DCs are present in a so-called "immature" state. They are well equipped to capture antigens through their endocytic and phagocytotic capacity mediated by certain receptors such as the mannose receptor and c-type lectin receptor. Secondly, imDCs capture antigens in tissues; Antigens are able to induce full maturation and mobilisation of DC. Upon activation, DCs migrate to lymphoid tissues such as the spleen and lymph nodes. The chemokine receptor CCR-7 is considered to be an important factor for DC homing to lymphoid tissues (Kellermann et al., 1999). Mature DCs acquire high capacity to activate T cells. Finally, DCs reside temporarily within lymphoid organs, where they present antigens and select rare antigen-specific T cells including $CD4^{+}$ and $CD8^{+}$ T cells thereby initiating an immune response (Banchereau & Steinman, 1998). In addition to their effect on T cells, DCs enhance NK cell cytolytic activity and IFN-γ production as well (Fernandez et al., 1999), which further suggest that DCs are involved in the interaction between innate and adaptive immune response.

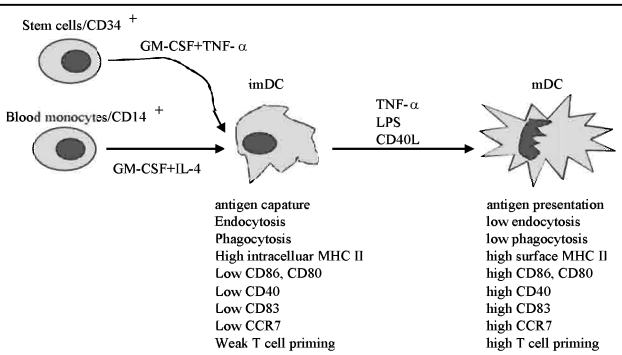


Fig 4. Generation of DCs from CD34 and CD14 positive cells and characteristics of imDCs and mDCs.

There are two main ways to generate human DCs (Langerhans DC-like) *in vitro*, either from monocytes or $CD34^{+}$ precursors. Peripheral blood monocytes or BM stem cells can be induced to develop imDCs *in vitro* by GM-CSF and IL-4 or TNF-α (Caux et al., 1992; Inaba et al., 1992). Pathogens (i.e. LPS), cytokines (e.g. TNF-α) and CD40L can induce the maturation of imDCs into mDCs (Banchereau & Steinman, 1998). Figure 4 briefly displays the steps of generation of DCs *in vitro* and the main characteristics of imDCs and mDCs.

V-4. Natural killer (NK) cells

NK cells are bone marrow derived granular lymphocytes and account for 5-15% of circulating lymphocytes, which were first termed in mice by Kiessling et al (Kiessling et al., 1975). Most NK cells are larger than resting lymphocytes, and their expanded cytoplasma contains granules used in cytotoxicity. Since NK cells, unlike B and T cells, do not require prior sensitisation (previous stimulation) and immediately perform their cytolytic activity, they are considered to be an important component in the innate immune system and provide a first line of defence against infections and malignancy.

Useful markers of human NK cells

In humans, NK cells lack T cell receptors and half of them express CD8. CD56 and CD16 are characteristic markers for human NK cells (Seaman, 2000), although other types of cells such as granulocytes also express CD16. NKp46, NKp30 and NKp44 are considered to be NK cell specific markers and are only expressed on resting and/or activated NK cells (Pende et al., 1999; Sivori et al., 1997; Vitale et al., 1998). Functionally, these receptors are involved in triggering NK cell mediated natural cytotoxicity towards different tumours and have therefore been named natural cytotoxicity receptors (NCR) (Moretta et al., 2000).

NK cell receptors for recognition of target cells

The fact that NK cells preferentially kill target cells lacking surface expression of MHC class I molecules (Moretta et al., 1997) implies that NK cells recognise MHC class I molecules and that MHC class I molecules provide a protective effect for target cells. The discovery of inhibitory receptors for MHC class I and activating receptors allows a better understanding of how NK cells interact with target cells through surface molecules. Table 1 is a brief summary of inhibitory receptors, activating receptors and corresponding ligands (Moretta et al., 2000; Vales-Gomez et al., 2000; Miller, 2001).

The simplified figure (Fig 5.) illustrates that the fate of target cells depends upon the balance between inhibiting and activating signals. Ligation of activating receptors with their ligands transmits signals which initiate NK cell cytotoxicity and lead to a target cell lysis. In contrast,

inhibitory receptors bind to a corresponding MHC class I molecule on the target cell surface which counter-activate signals and block the induction of cytotoxicity and prevent the lysis of

Table 1. Activation, inhibitory receptors and their ligands

Activation/inhibitory Receptor (NK cell)	Ligand (Target cell)	Induction of cytotoxicity
Natural cytotoxicity receptor		
NKp30, NKp40, NKp46	unknown	+
NKp80	unknown	+
2B4	CD48	+
Inhibitory receptors		
Killer cell lectin-like receptors (KLRs)		
CD94/NKG2A/B	HLA-E	-
CD94/NKG2C/E	HLA-E	+
NKG2D	MICA/B*	+
killer Ig-like receptors (KIRs)		
KIR2Ds	HLA-C	+
KIR2DL	HLA-C	-
KIR3D	HLA-A,B	-

*MICA/B are stress-induced molecules, which are mostly expressed on epithelial tumour cells such as breast and lung cancers (Bauer et al., 1999; Groh et al., 1999).

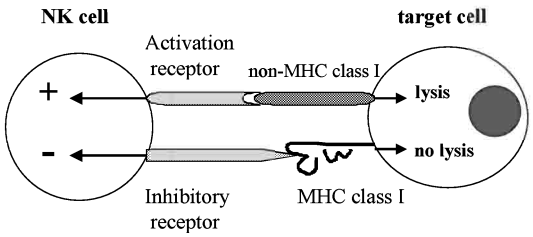


Fig 5. NK cell cytotoxicity mediated by activating (+) and inhibitory (-) signallings

target cells. Main function of HLA-class I specific inhibitory receptors including KIRs and KLRs is considered to check the integrity of cells and to prevent normal cells from damage by NK cells (Moretta et al., 2001).

Mechanisms of cytotoxicity

To lyse targets, NK cells use two main mechanisms. One is perforin-mediated cytotoxicity. NK cells, like CTLs, lyse targets primarily through a calcium dependent release of granules containing perforin, a protein forming pores on target cell membrane (Podack, 1995).

Granzyme A and B release from granules and enter target cells where they initiate DNA fragmentation and apoptosis (Russell & Ley, 2002). The perforin-mediated pathway accounts for most of NK cell killing in the experimental systems. Another cytotoxic pathway involves Fas (CD95) which is a member of the tumour necrosis factor superfamily and belongs to the subgroup of the apoptosis-inducing "death receptors" characterised by a death domain in the cytoplasmic region which is essential for triggering apoptosis (Wallach et al., 1999). Activated NK cells kill Fas positive target cells in a pathway independently of perforin (Zamai et al., 1998). Resting NK cells express TNF related apoptosis-inducing ligand (TRAIL) thereby activating apoptosis in target cells that express receptors for TRAIL (Zamai et al., 1998).

Cytokine production

In addition to their cytotoxicity, NK cells can secrete a number of cytokines such as IFN-γ, TNF-α and GM-CSF (Perussia, 1996). The cytokine production pattern is dependent upon the stimuli. IFN-γ production can be induced by stimulation with IL-2, IL-12, IL-15 and IL-18 when used alone or in combination with each other (Fehniger et al., 1999; Perussia, 1996). As discussed earlier, NK cells kill targets through exocytosis and Fas/FasL pathways. It is claimed that these pathways are, to a certain extent, influenced by NK cell–derived cytokines (Sayers et al., 1998). For example, IFN-γ can up-regulate TNF family ligands on NK cells and TNF family receptors on tumour cells (Kashii et al., 1999) thereby enhancing apoptosis of target cells mediated by NK cells. Through NK-derived cytokines such as IFN-γ, NK cells also play a role in the regulation of T cell mediated immune response. IFN-γ plays an essential role in inducing differentiation of naïve T cells into Th1- type cells after acute infection (Scharton & Scott, 1993). Reciprocally, Th1 produced cytokines such as IL-2 can influence NK cell activity. Moreover, NK cell may be indirectly involved in DC development via NK cell secreted GM-CSF. NK cells could thus provide an important link between innate and adaptive immune mechanisms in a reciprocal fashion.

NK cells in MM patients and their anti-tumour activity

It has previously been reported that NK cell numbers in the peripheral blood and/or BM of MM patients are increased, and that NK cell cytolytic activity against tumour cells is consistently enhanced (Garcia-Sanz et al., 1996; Gonzalez et al., 1992; Osterborg et al., 1990; Uchida et al.,

1984). A significant increase of CD57 expression, an activation marker, in bone marrow NK cells was observed especially in patients at early stages (I+II) (Garcia-Sanz et al., 1996). Furthermore, bone marrow mononuclear cells from myeloma patients expressed substantial levels of cytotoxicity against K562 cells in an *in vitro* assay, which was comparable to that of blood lymphocytes. In contrast, such NK-cell activity was markedly low or absent in BM of normal donors (Garcia-Sanz et al., 1996). The activity and number of NK cells were found to be related to disease activity with a higher number and activity of NK cells found in patients with a low tumour burden and low values in patients with advanced disease (Osterborg et al., 1990). When RPMI 8226, a myeloma cell line, was used as a target, peripheral blood lymphocytes from MM patients showed significantly higher spontaneous cytotoxicity than that from controls (Peest et al., 1995b). The recombinant IL-2 treatment protocol has been applied in an attempt to induce an anti-tumour cytotoxicity in MM. An increased NK cell cytolytic activity *in vitro* and NK cell number *in vivo* have been observed (Peest et al., 1995a). In this study, 18 patients with advanced disease were investigated, and 6 of 17 cases showed clinical response (Peest et al., 1995a). The results from this study highlights that induction of natural cytotoxicity in addition to enhancement of a specific immune response by cytokines (i.e., IL-2) could be a promising approach to treat MM patients. In *in vitro* and animal experiments, IL-12 and IL-18 showed significant effectivity in inducing NK cell cytotoxicity against tumours and in producing IFN-γ especially when used together with IL-2 (Arai et al., 2000; DeBlaker-Hohe et al., 1995). Therefore, application of combined cytokine therapy (i.e., IL-2 with IL-18) to the patient would be expected to improve the clinical outcome in comparison with using IL-2 alone.

AIMS OF THE STUDY

The overall aims of this study were to uncover susceptibility genes involved in immunoregulation of MM and to evaluate potential importance of NK cell cytotoxic activity in MM. The specific aims were as follows:

* To investigate whether the IL-10 gene polymorphisms are associated with multiple myeloma.

* To study if there is a correlation between the CTLA-4 gene polymorphisms and MM, and if the genetic polymorphisms influence the CTLA-4 protein expression.

* To identify possible associations between the cytokine genes IL-6, TNF-α and IL-1β as well as IL-1Ra with MM.

* To analyse NK cell activity in patients with MM using autologous DCs and primary tumour cells as targets to uncover a potential role of NK cells in tumour repression.

METHODOLOGICAL ASPECTS

This section presents the methods used in this thesis. Information of the samples from patients and controls were provided in detail in the respective papers. The ethics committee at Karolinska hospital approved all the studies.

I. DNA extraction from blood

Genomic DNA was extracted from EDTA treated peripheral blood using a standard proteinase K digestion and phenol/chloroform method. DNA was dissolved in sterile distilled water.

II. Polymorphism screening

Polymerase chain reaction (PCR) was used for all screenings. PCR reaction was run in thin-wall PCR tubes containing 100 ng of genomic DNA, 200 μM of dNTPs, 0.4 μM of each primer, 1 x buffer with $MgCl_2$ and 0.5 u Taq gold DNA polymerase. Sequences of primers or the source of the primers used in these studies are shown in the papers (I to IV).

II-1. Genotyping IL-10-1082 and IL-1Ra variable number of identical tandem repeats loci (Paper I, III)

PCR products were loaded onto 2% agarose gels (NuSieve GTG, FMC BioProducts, Rockland, Maine USA) containing 0.1% ethidium bromide. After electrophoresis for 30 minutes, the bands of the PCR products were visualised under ultraviolet light.

II-2. Genotyping of the restriction fragment length polymorphism (RFLP) (Paper III)

IL-6 (-174) RFLP, TNF-α (-308) RFLP and IL-1β TaqI RFLP were investigated. The PCR products were digested by restriction enzymes, Hsp92II for IL-6, NcoI for TNF-α and TaqI for IL-1β following the instructions provided by the company (Promega, USA) respectively. To make sure that the digestion system works properly, one DNA sample with known genotype was included in each experiment as a positive control. In addition, the amplified fragment of the IL-6 constitutively contains a Hsp92II enzyme digestion, which was used as an internal control for the digestion.

II-3. Microsatellite typing of the IL-10 and CTLA-4 genes (Paper I, II)

In the promoter region of the IL-10 gene there are two microsatellite loci, referred to as IL10.G and IL10.R (Eskdale & Gallagher, 1995; Eskdale et al., 1996). The polymorphic (AT)n repeat locus is located in the 3´-untranslated region of exon 4 (see Fig 3), which was previously called exon 3, of the human CTLA-4 gene. The primers used for genotyping IL10.G, IL10.R and CTLA-4 were designed as previously described (Eskdale & Gallagher, 1995; Eskdale et al., 1996) (Polymeropoulos MH, 1991). The 5' -end of each forward primer was labelled with fluorescent HEX with an approximated wavelength of 557 nm (Pharamacia-Biotech, Uppsala, Sweden).

A mixture of PCR products and an internal size standard (Gene Scan 350-TAMRA, Perkin-Elmer) were loaded onto 5% denaturing polyacrylamide gels in the ABI 377 sequencer (Perkin-Elmer) after denaturation. Data was analysed with the GeneScan 672 (version 1.0) and the Genotyper software (version 2.1, Perkin-Elmer). PCR fragment size was determined according to the standard and alleles were designated accordingly.

III. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a quantitative technique for measurement of a soluble protein in a cell-free sample (serum, plasma, supernatant and other fluids). This assay was used in the present studies for detection of IL-10 in the supernatants from LPS stimulated PBMCs of MM patients. (Paper I)

IV. Cell purification

Negative selection kits were used to purify NK and T cells according to the instructions provided by the company to avoid possible cross-linking induced activation of NK cells or T cells. (Paper IV)

V. Immunofluorescence staining and flow cytometry

Immunofluorescence staining and FACS Calibure flow cytometer with CELLQuest software were used to analyse surface markers on DC, NK and T cell surface (paper IV). To reveal whether there is a possible correlation between the length of the alleles of CTLA-4

microsatellite polymorphism and its protein expression, differences in the CTLA-4 surface and intracellular expressions were compared between longer and shorter allele carriers in healthy controls. Total (surface plus intracellular) CTLA-4 expression on $CD4^+$ or $CD8^+$ T cells, which were previously stimulated by anti-CD3 mAb or ConA for 72 hours respectively, was assessed by intracellular immunofluoresence staining technique. The procedure of staining has been previously described (Alegre et al., 1996). Briefly, PBMCs prior to and after stimulation were stained with FITC labelled anti-CD4 or anti–CD8 mAb for 30 min, cells were fixed by 2% paraformaldehyde, permeabilized by 0.3% saponin and stained with CD152 (CTLA-4) mAb or isotype control mAb (Becton Dickinson, USA) for 30 min at 6 ^{o}C. The cells were washed and kept in PBS before running flow cytometry.

VI. Cytotoxicity assays (paper IV)

In the current study, a standard 4h ^{51}Cr release assay was employed to measure the cytotoxicity of IL-2 activated NK cells or T cells against autologous, allogenetic DCs or primary autologous plasma cells.

VII. Statistical analysis

In paper I-III, genotype and allele frequencies were compared between the study groups by Chi-square test with Yates correction or Fisher's exact test when necessary. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. A two-sided p or pc (corrected p value) < 0.05 was considered to be significant. In paper I and IV, Mann-Whitney U-test was used for comparisons made between groups. A two-sided p value less than 0.05 was considered to be significant.

RESULTS AND DISCUSSIONS

I. IL-10 genetic polymorphisms (paper I)

In the present study, three polymorphisms in the promoter region of the IL-10 gene were analysed in 73 patients with MM, 27 patients with MUGS and 109 unrelated healthy controls. As figure 6 demonstrates, homozygosity for allele 136 was significantly increased in MM but not in MGUS groups when compared with controls by Fisher´s exact text (*** $p<0.0001$, $pc<0.003$, OR = 6.93, # $p>0.1$). We further analysed IL10.R and found the frequencies of the genotype 112/114 and 114/116 to be significantly increased and decreased in the MM group in comparison with those in controls, respectively (Fig 7). (*$p<0.002$, $pc<0.05$, OR=3.11, **$p<0.0001$, $pc<0.001$, OR=0.20). The genotype 114/116, which accounted for 34.6% in the control group, was not present in any of the MGUS patients ($pc<0.001$, OR=0.03). Our results did not show an association between the IL-10-1082 locus and MM or MGUS.

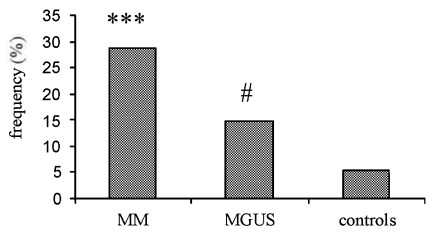


Fig 6. Distribution of IL10.G 136/136 in patients and controls

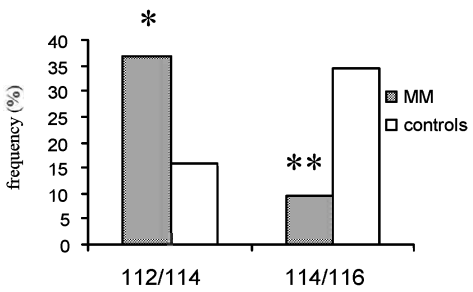


Fig 7. IL10.R genotype 112/114 and 114/116 in MM and controls

Our findings suggest that IL10.G 136/136 and IL10.R 112/114 may be risk factors and that IL10.R 114/116 may be a protective factor for developing MM. Likewise, the IL10.R 114/116 could be protective from the development of MGUS. The finding that the IL10.R 114/116 was less frequent in both MM and MGUS might suggest that there would be a common genetic element for the development of both MM and MGUS. This raises the question of what

possible mechanism could explain this genetic association with the diseases. One study demonstrated striking differences between individuals in their ability to produce IL-10 following LPS stimulation in whole blood cultures *in vitro* from first-degree family members (Westendorp et al., 1997), indicating that differences in IL-10 production contain a considerable hereditary component. Analysis of IL-10 production in monozygotic or dizygotic twins and unrelated individuals indicated that variability in IL-10 production has a heredity of about 74% (Westendorp et al., 1997). Based on the these findings, IL-10 genetic polymorphisms may be involved in disease development by influencing IL-10 production *in vivo* by corresponding cells. Therefore, we asked the question whether the MM associated alleles or genotypes in the IL10.G and IL10.R loci could influence the IL-10 production. To answer this, PBMCs from 20 patients were stimulated by LPS for 24 hours and IL-10 concentration in the supernatants was measured using ELISA. Our results demonstrated that the median IL-10 protein concentration in supernatants of activated PBMCs from the IL10.G allele 136 carriers (n=14) was significantly higher than that from the non-carriers (n=6) ($p<0.005$, by Mann-Whitney Test). Moreover, a wide-range in variation in IL-10 production by PBMCs from the allele 136 carriers (data not shown) was observed, indicating that other alleles in the locus and/or other nearby loci could also be involved in IL-10 production. Eskdale et al investigated the IL-10 production by whole blood stimulated by LPS among different IL10.R/G haplotypes and found that the IL-10 gene haplotypes were associated with differential production of IL-10, i.e., haplotype R2/G14 gave the highest IL-10 secretion overall (Eskdale J & TW, 1998). This suggests that there is a functional cross linkage between these two loci and the ability to produce IL-10 may vary in individuals based on the genetic composition of the IL-10 gene. Since we used a different technique for the genotyping, we can not precisely correlate our nomenclature for the IL10.G and IL10.R with the previous reports (Eskdale & Gallagher, 1995; Eskdale et al., 1996). The nomenclatures of the alleles need to be unified in the future so as to allow for comparison of data from different sources. Unlike IL10.G, the IL10.R and IL-10 –1082 loci were not shown to be associated with IL-10 production in our current study (data not shown). The latter locus was previously found to be related to IL-10 production by PBMCs following ConA stimulation *in vitro* (Turner et al., 1997). The reason for such a difference may be due to the different types of IL-10 producing cells investigated in the two assays. Obviously, LPS stimulation mainly affects monocytes and

B cells, whereas ConA affects T cells. Therefore, the results from LPS activated PBMCs are particularly relevant to genetic differences in monocyte and B cell IL-10 production, suggesting that genetic elements may govern differences in IL-10 production between cell types (Eskdale J & TW, 1998) and that additional potential factors regulating IL-10 production may exist as well.

Such putative alterations of IL-10 protein production as a disease-promoting factor in MM could work at different levels by different or combined mechanisms. First, IL-10 may act as a co-factor for the initiation of MM. We have previously discussed IL-10 as a normal B cell growth, differentiation and isotype switch factor. Additionally, IL-10 could up-regulate bcl-2 expression (Levy & Brouet, 1994) and telomerase activity in B cells (Hu & Insel, 1999), which would suggest that IL-10 might be involved in tumourigenesis machinery. Furthermore, IL-10 could be implicated in MM by inducing tumour-associated anergy, as discussed in the introduction, allowing for tumour cells to evade a T cell-mediated immune response. In line with this notion, it has been shown that melanoma cells are protected from antigen specific cytotoxic T cell lysis via an IL-10-dependent reduction of tumour cell MHC class I expression (Matsuda et al., 1994) or by an IL-10-mediated induction of specific anergy in $CD8^+$ T cells (Steinbrink et al., 1999). In addition, IL-10 has been described to function as a growth factor for a population of regulatory T cells, which could suppress antigen-specific immune responses (Groux H & MG, 1998). The inhibitory effect of IL-10 on immune response may hamper the immune surveillance or clearance of malignant transformed cells at an early stage of the disease. Finally, IL-10 has a direct effect on MM cells by supporting their growth, which suggests a role for IL-10 in the progression of the disease.

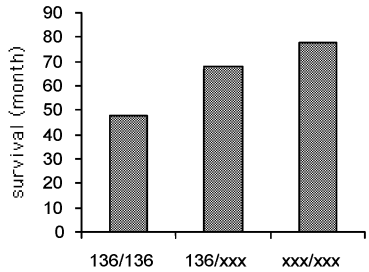


Fig 8. The correlation between the IL10.G allele136 carriage status and survival

To investigate whether the IL10.G polymorphic status has impact upon survival of patients, we analysed the mean values of survival among individuals with different allele 136 status. The IL10.G 136/136 carriers (n=7) showed a shorter mean value of survival than heterozygous

(136/xxx) (n=10) and non-allele 136 carriers (xxx/xxx) (n=12) (unpublished data). These results suggest that the allele 136 may be related to a poor prognosis (Fig 8). However, the difference among them was not significant after statistical analysis, due to the small sample size. This work is currently under investigation.

II. CTLA-4 genetic polymorphism (Paper II)

Allele 86 was the most frequent allele in the MM and control groups. Significant decreases in the frequencies of the genotype 86/86 and allele 86 were observed in the MM group (21.9% vs. 37%, $p<0.05$, OR=0.48; 36% vs. 51%, $p<0.01$, $x^2=6.8$, OR=0.55, respectively) when compared with controls. Likewise, the genotype 86/86 distribution and allele 86 frequency in the MGUS group deviated from that in controls (7.4 % vs. 37%, $p<0.003$, OR=0.14; 32% vs. 51%, $p<0.02$, $x^2=5.7$, OR=0.44, respectively). Accordingly, longer allele carriers were more frequently observed in the MM group than in the control group (data not shown). The genotype 86/86 was significantly less frequent in the λ light chain expression group than in control group (5% vs. 37%, $p<0.005$, OR = 0.09). Likewise, the allele 86 frequency in the λ expressing group deviated from the control group (20% vs. 51%, $p<0.0005$, OR=0.24, by Fisher's exact test). Compared with the κ group, this genotype was significantly less frequent in the λ group (5% vs. 29%, $p<0.05$, OR=0.13), suggesting that the longer alleles may preferentially increase the risk of developing the λ chain type of MM.

In addition to this microsatellite locus, there is an A/G transition polymorphism at position 49 in exon 1 of the gene (exon 1 polymorphism). To investigate if the CTLA-4 exon 1 polymorphism is another susceptibility locus for MM, we investigated the prevalence of exon 1 polymorphism in the patients and controls. However, our data did not show any association between the exon 1 polymorphism and MM (unpublished data).

The functional implication of CTLA-4 genetic polymorphism is not well known. To uncover whether there is a potential relationship between the variants in the loci and CTLA-4 protein expression, we analysed the CTLA-4 expression in T cells by flow cytometry technique in relation to the genotypes within the loci of the CTLA-4 gene (unpublished data). PBMCs from 19 healthy donors were cultured in the presence of anti-CD3 mAb or ConA for 72 hours. A positive correlation between the allele 86 carriage status of the microsatellite and the percentage

of CD152 positive cells in $CD4^+$ but not $CD8^+$ T cells was observed. As Figure 9 demonstrates, the percentage of $CD152^+$ cells in the $CD4^+$ T cell population from samples who were genotype 86/86 carriers (homozygous) (n=7) was significantly higher than that from non-allele 86 carriers (xx/xx) (n=8) by Mann-Whitney U test ($p<0.005$) when PBMCs were stimulated by anti-CD3 mAb. There was a tendency toward increased CD152 expression in the group of heterozygous for allele 86 (n=4) compared to the non-allele 86 carrier group in $CD4^+$ T cells (83% vs. 71%, $p=0.11$). The low power of the p value may be due to the limited number of cases analysed particularly of the heterozygous group. After Con A stimulation, the number of CD152 expressing cells in $CD4^+$ T cells from the homozygous group was higher than that from the heterozygous and non-allele 86 carrier groups (83%, 80% and 73% respectively). The difference between the homozygous and non-allele carrier groups was nearly significant by Mann-Whitney U test ($p=0.059$). Ligers et al consistently found a correlation between the number of CTLA-4 positive T cells and the microsatellite polymorphism in healthy controls (Ligers et al., 2001). Moreover, $CD4^+$ T cells from the homozygous samples expressed higher CTLA-4 protein (surface plus intracellular) compared to heterozygous and non-allele 86 carrier groups (124, 116 and 80, respectively) at single cell level in terms of mean fluorescence intensity (MFI) after CD3 stimulation. The difference in the CTLA-4 expression between the homozygous and non-allele carrier groups was toward significance ($p=0.07$). This phenomenon of genotype dependent CTLA-4 expression needs to be further confirmed by enlarging the sample size. Nevertheless, our data from the functional study would suggest that the CTLA-4 microsatellite could possibly be involved in regulation of $CD4^+$ T cell response *in vivo* by influencing both the number of CTLA-4 expressing cells and quantitative CTLA-4 expression per cell. In agreement with this notion, one study demonstrated that the length of the (AT)n repeats in the microsatellite locus was positively correlated to the

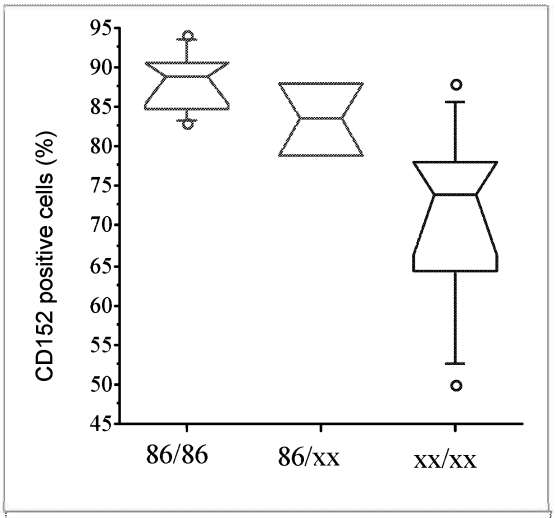


Fig 9. CD152 expression in $CD4^+$ T cells is correlated with allele 86 carriage status.

level of soluble IL-2 receptor in serum from patients with myasthenia gravis (Huang et al., 2000), implying that this microsatellite polymorphism in the CTLA-4 is involved in regulating T cell activity *in vivo*. Based on our results and the role of CTLA-4 in the immune system, we may thus speculate that the CTLA-4 microsatellite polymorphism could be involved in the development of the diseases through regulating T cell activation, especially Th cell differentiation.

IL-10 and IL-6, Th2 -type cytokines, are considered to be growth factors for myeloma cells (Hallek et al., 1998) and for normal B cell differentiation (Burdin et al., 1995). It has been claimed that Th1 or Th2 $CD4^+$ T cell development is controlled at least partly if not completely by CTLA-4-B7 and CD28–B7 signalling. In mice, CTLA-4 co-stimulation was shown to suppress Th2 type cytokine production upon priming of naïve $CD4^+$ T cells with anti-CD3 in the presence of spleen adherent cells. On the other hand, the naïve $CD4^+$ T cells were polarised toward the Th2 subset when the CTLA-4 co-stimulation was blocked by anti-CTLA-4 (Kato & Nariuchi, 2000). Therefore, it is very likely that CD28 signalling promotes Th2 cell differentiation and CTLA-4 signalling limits their differentiation (Oosterwegel et al., 1999). Furthermore, it has been shown that a Th2 cell line expresses much higher cell surface CTLA-4 protein than a Th1 cell line (Alegre et al., 1996), which suggests that CTLA-4 may preferentially regulate Th2 cells rather than Th1 cell differentiation. In addition to its antagonism of CD28, CTLA-4 also inhibits ICOS induced IL-10 and other, mainly Th2-type, cytokine productions as discussed in the introduction. Considering the lower frequency of the shortest allele in MM and MGUS patients and the fact that allele 86 is responsible for a higher CTLA-4 expression in $CD4^+$ T cells, individuals carrying longer alleles would be expected to acquire a lower CTLA-4 expression in $CD4^+$ T cells. Theoretically, in this condition, more Th2 $CD4^+$ T cells might be polarised due to a predominant CD28-B7 signalling thereby producing more Th2-type cytokines favourable for MM developments through multiple effects on B, T, APC and myeloma cells. Selective expansions of TCR Vβ expression in CD4 subsets were demonstrated in the patients at diagnosis (Kay et al., 1999). Are these expanded $CD4^+$ T cells Th1 or Th2 -type? Our previous study has demonstrated that Th2-type cytokine (IL-4) producing cells were frequently detected in patients with advanced disease, when PBMCs from these patients were stimulated by the F(ab´)2 fragments of M-Ig (Yi et al., 1995). Frassanito et al analysed cytokine producing cells in peripheral blood of patients with MM using an

intracellular immunofluoresence staining technique (Frassanito et al., 2001). They found that $CD3^+$ T cells producing IL-6 but not IL-2 or IFN-γ in patients with active disease were significantly increased as compared with age-matched healthy controls, when PBMCs were stimulated *in vitro* by phorbol myristate acetate/ionomycin (Frassanito et al., 2001). This would indicate that preferentially expanded Th2 like cells may exist in the peripheral blood of patients with advanced disease. This phenomenon may be driven under genetic control to a certain extent.

Taken together, CTLA-4 microsatellite but not exon 1 polymorphism may contribute to an increased risk for developing MM, particularly subgroups of MM, via the regulation of CTLA-4 expression in T cells thereby influencing T cell immunity *in vivo*.

III. IL-6, TNF-α, IL-1β and IL-1Ra genetic polymorphisms (paper III)

The IL-6 (-174), TNF-α (-308) and IL-1β TaqI RFLPs as well as IL-1Ra VNTR polymorphism were analysed in 73 patients with MM, 27 with MGUS and 129 healthy individuals. None of them were associated with MM or MGUS in the current study. Although these cytokines, IL-6, TNF-α and IL-1β, are considered to be important in the MM development at the protein level (Hallek et al., 1998), it is unlikely that their genetic polymorphisms confer susceptibility to the development of MM and MGUS. However, this does not exclude the possibility that one of the cytokine genes may be associated to the disease by linkage with other genes. In fact, it has been shown that there was no association between TNF-α (-308) and MM when this locus was analysed alone. However, a significant correlation was observed when individuals were defined as haplotypes based on the alleles of TNF-α (-308) and lymphotoxin alpha (LTa) polymorphism at position +252 (Davies et al., 2000). In the current study, combined genotype distributions of the IL-6 (-174)/TNF-α (-308) / IL-1βTaqI were further analysed in the patients and controls, but no biased combined genotype distribution was found (unpublished data).

IV. MM NK cell cytotoxicity against autologous mature DCs and primary myeloma cells (Paper IV)

To analyse the potential importance of autologous lymphocyte cytolytic activity for cell based immunotherapy in MM, *in vitro* generated DCs loaded with patient specific M-Ig and purified

primary plasma cells from patients served as autologous target cells in cytotoxicity assays. Purified NK cells ($CD56^+$, $CD3^-$) and T cells ($CD3^+$) from the peripheral blood of patients with clinically stable disease or of healthy controls were employed as effectors.

Our results demonstrated that purified NK cells from patients pre-cultured in 400 units/ml of rhIL-2 exhibited a substantial cytolytic activity against autologous mDCs that were pre-pulsed with patients M-Ig or un-pulsed. This phenomenon was observed in all the experiments from 5 different patients. The data from five independent experiments were presented as mean±SEM in Figure 10. Consistent with previous reports that MM NK cells acquired higher cytotoxicity

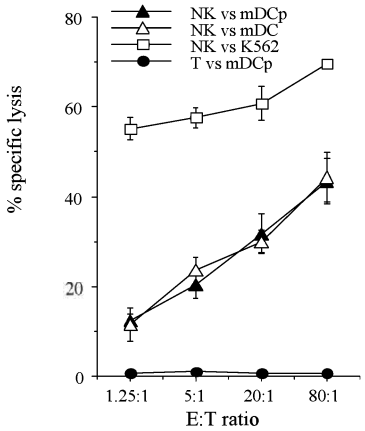


Fig 10. MM NK cell cytotoxicity against autologous DCs.

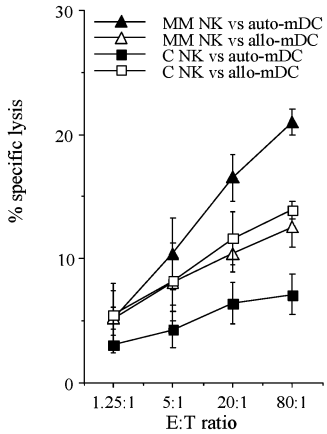


Fig 11. Lysis of autologous DCs by NK cells in low dosage of IL-2.

against tumour cells as discussed in the introduction, our results demonstrated that the lysis rate of autologous DCs by MM NK cells was higher than that of control NK cells, particularly after culturing NK cells in low IL-2 concentrations (10 IU/ml) (Fig 11). The NK cell activity of control donors showed an expected pattern of higher allogenetic versus autologous killing efficiency (Fig 11).

Potential mechanism behind the MM associated effects observed is not clear. CD80 (Chambers et al., 1996) or CD40 (Martin-Fontecha et al., 1999) mediated NK cell activation or

MHC class I mediated protection could not explain this phenomenon since DCs from patients and healthy controls expressed identical levels of these molecules in the current study. Certain ligands such as MIC A/B are able to provide activation signals to NK cells (Table 1). It will be of importance to know whether there is a difference in the expressions of such ligands on the DCs from MM patients and controls.

As discussed in the introduction, both inhibiting and activating signaling pathways by distinct receptors are implicated in the NK–target cell interactions. NK cell -mediated lysis of DCs indicates that activation signals are apparently dominant during the interaction of the NK cells with DCs. NK cell -mediated cytotoxicity triggered by CD80 signaling could override the protection mediated by MHC class I molecules (Chambers et al., 1996). Our results, however, do not exclude a MHC-dependent negative regulation of the NK-mediated cytotoxicity of DCs from MM patients since blocking of MHC-class I enhanced the lysis of autologous DC targets. In addition to the CD80 triggering effect, cytokines derived from DCs such as IL-12 and IL-18 are able to activate NK cells as well thereby regulating DC-NK cell interactions.

DCs from MM patients have been shown to be able to induce tumour-specific CTLs *in vitro*. Therefore, our observation that MM NK cells mediated lysis of autologous DCs would imply DCs as a target of activated NK cells *in vivo*. Accordingly, activated NK cells may also interfere with the effects of DC-based vaccines for initiating or enhancing anti-tumour T-cell responses (Esche et al., 1999). Increased serum IL-2 in MM patients has been previously observed (Cimino et al., 1990). Moreover, NK cells from MM patients stimulated by low concentration of IL-2 corresponding to that previously measured in the circulation of most MM patients (Cimino et al., 1990), considerably lysed autologous DCs as observed in the current study. Consistently, increased NK cell numbers and cytolytic activities have also been documented (Garcia-Sanz et al., 1996; Osterborg et al., 1990). These findings, together with our current results, point to *in vivo* conditions permissive for NK cell activation which could subsequently lyse DCs in MM. Thus, NK cell-mediated lysis of DCs may be an important reason for the limited clinical response reported so far in trials utilizing tumour antigen loaded DCs for treating patients with cancers.

To test the activated NK cell activity against primary myeloma cells, purified myeloma cells from the bone marrow of patients were used as targets in the ^{51}Cr release assay. The results showed that the IL-2 activated NK cells induced a considerable myeloma cell lysis at higher E:

T ratios. NK cell-mediated lysis of tumour cells has been implicated in tumour regression in MM (Peest et al., 1995b). When myeloma cells are in contact with NK cells, are the myeloma cells able to induce NK cell natural cytolytic activity? It may be possible, since myeloma cells express a certain level of the co-stimulatory molecules CD80, CD86 and CD40 (Yi et al., 1997) that are involved in induction of NK cell mediated lysis of tumour target cells both in mice and humans (Martin-Fontecha et al., 1999; Wilson et al., 1999; Chambers et al., 2001). It will be of importance to explore how to induce NK cell cytotoxicity against tumour cells in an efficient way by regulation of triggering molecule expressions on target or NK cells.

The T cells in the current study did not show any cytolysis of autologous M-Ig pulsed DCs and primary myeloma cells after short-term co-culture with autologous DCs pre-pulsed with M-Ig. This may reflect the low frequency of tumour specific T cells in the peripheral blood. Moreover, we found that a high level of IL-10 and undetectable levels of IL-2 and IFN-γ in the supernatants from cultures of PBMCs from MM patients after *in vitro* stimulation by autologous M-Ig pulsed DCs, indicating that a Th2 cell but not Th1 or CD8 cell expansion was induced (manuscript in preparation). This would suggest that the generation of CTLs from patients with MM might be hampered *in vivo*. In agreement with this notion, one recent report described that DCs purified from the peripheral blood of MM patients displayed a functional defect characterised by a reduced ability to up-regulate CD80 expression mediated by CD40 ligand (Brown et al., 2001). Since the expression of CD80 is considered to be essential for induction of antitumour immunity (Martin-Fontecha et al., 1999), DCs with this functional defect *in vivo* may lead to T cell anergy instead of activation.

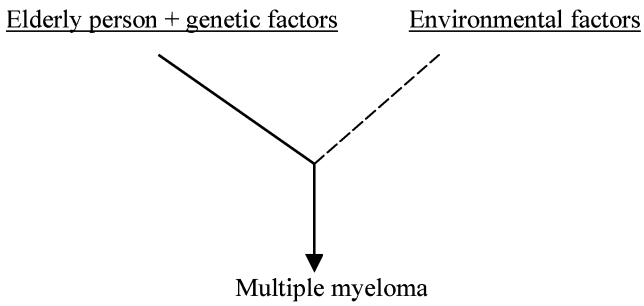
It has been recently described that DCs are able to enhance NK cell cytotoxicity against tumour cells and to induce NK cell proliferation (Ferlazzo et al., 2002). Consistently, our unpublished data demonstrated that mature autologous DCs induced resting peripheral NK cells from MM patients and healthy individuals to proliferate after co-culture for 5 to 14 days *in vitro* as determined by a CFSC labelling assay. Our results further demonstrated that such DC induced NK cell proliferation was significantly impaired after separating NK cells and DCs in culture by transwell membranes, indicating that cell-cell interaction rather than the DC released cytokines is essential for such DC-mediated effect on NK cell proliferation. Like mDCs, imDCs also have such function (Ferlazzo et al., 2002). The DC induced NK cell proliferation and cytotoxicity was found mainly through engagement of the NKp30 but not the NKp46 or NKp44

receptor (Ferlazzo et al., 2002). Regarding the application of DC based vaccines, DCs may trigger non-specific anti-tumour activities in vivo. The observations that DCs promote NK cell-dependent anti-tumour immunity in mice (Fernandez et al., 1999), and that DCs derived from human cord blood (Yu et al., 2001) or DCs from blood of MM patients (unpublished data) enhance NK cell cytotoxicity towards K562 tumour cells, are consistent with this notion.

Taken together, the present observations that NK cell-mediated lysis of autologous DCs and myeloma cells suggest the existence of competent and inducible polyclonal NK cells in the circulation of MM patients. Since we analysed patients who were in long-term remission, the observed NK-DC activity possibly reflects that NK cells contribute to tumour regression *in vivo*. This also points to the possibility that NK cells would exert anti-tumour effects more efficiently by involvement of activation triggering signals mediated by DCs and cytokines such as IL-2, IL-12 and IL-18.

SUMMARY AND CONCLUSIONS

MM is an age dependent malignancy. Reduction of immune function or immunosenesence, mainly involving specific immunity, concomitantly occurs with ageing (Ginaldi et al., 1999; Pawelec et al., 2001). Here, we propose a model that the development of MM in an individual may be related to potential internal factors including the carrying of risk alleles and age related defects in the immune system, plus external factors or environmental factors such as viral infections as identified in some patients.



In this study, two IL-10 microsatellite and CTLA-4 microsatellite loci were found to be associated with MM and MGUS. The IL10.G genotype 136/136 and IL10.R 112/114 were significantly increased in MM and IL10.R 114/116 and the CTLA-4 86/86 were decreased in MM and MGUS patients as compared to those in controls, respectively, suggesting that they may act as risk and protective factors, respectively, for the development of MM and MGUS. Moreover, such associations between the IL10.R and CTLA-4 microsatellite polymorphisms with MM and MGUS indicate the existence of a common genetic background for the development of both diseases even though MGUS is considered to be a benign disease. Since our preliminary results showed that the IL10.G allele 136 was related to a higher IL-10 production and that the CTLA-4 allele 86 was related to a higher CTLA-4 expression in $CD4^+$ T cells, it is likely that the IL-10 and CTLA-4 genetic polymorphisms may contribute to the

disease development by regulation of their protein expression thereby influencing immune responses and tumour cell growth and survival.

Although IL-6, TNF-α and IL-1β seem to be involved in the pathogenesis of MM, our results in the present study do not support a role for these genes in conferring susceptibility to MM and MGUS.

In the current study NK cells mediated autologous DC lysis. This, together with previous findings of increased NK cell numbers and cytotoxicity against tumour cells, indicates the existence of competent and inducible polyclonal NK cells in the circulation of patients with MM. This suggests an alternative pathway of tumour targeting therapy for further exploration in MM. In addition, such NK-mediated lysis of DCs may contribute to the poor clinical outcome of DC vaccines, especially when DCs are administered intravenously. This message may be an important factor when designing a DC vaccine approach.

Taken together, in view of the roles of cytokines, T, NK cells and DCs in the immune system, myeloma development seems to be regulated by specific and non-specific immune responses at the gene and protein levels.

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