Analysis of Spontaneous vs. Vaccine-induced Antibody Responses against Antigens MAGE-A3 and MAGE-C2 in Cancer Patients

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List of Abbreviations

AA	Amino acids
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
allo-SCT	Allogeneic stem cell transplantation
ANA	Anti-nuclear antibody
AP	Alkaline phosphatase
APC	Antigen-presenting cell
ASC	Antigen stimulated cells
ASCT	Autologous stem cell transplantation
BCR	B cell receptor
BD	Becton Dickinson
Blimp-1	B-lymphocyte-induced maturation protein-1
BM	Bone marrow
BSA	Bovine serum albumin
BCIP/ NBT	5-bromo-4-chloro-3-indolyl phosphate/nitrobluetetrazolium
Ca	Calcium
CD	Cluster of differentiation
CD40L	CD40 ligand
CEA	Carcinoembryonal antigen
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CR	Complete remission

CTA	Cancer-testis antigen
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dl	Deciliter
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EBMT	European Group for Blood and Marrow Transplantation
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanite
G-CSF	Granulocyte colony-stimulating factor
GSK	GlaxoSmithKline Biologicals
GST	Glutathione S-transferase
GvHD	Graft-versus-host disease
GvM	Graft-versus-myeloma
GvT	Graft-versus-tumor
h	Hour

Hb	Hemoglobin
HCl	Hydrochloric acid
HDT	High-dose chemotherapy
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IC_{50}	Half maximal inhibitory concentration
IF	Immunofixation
IFN	Interferon (e.g., IFN-γ)
Ig	Immunoglobulin
IL	Interleukin (e.g., IL-2)
IMDM	Iscove's Modified Dulbecco's Medium
IMWG	International Myeloma Working Group
IP	Immunoprecipitation
ISC	Immunoglobulin secreting cells
ISS	International staging system
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
L	Liter
LICR	Ludwig Institute for Cancer Research
Lf	Limes flocculation (specific activity of tetanus toxoid)
LPS	Lipopolysaccharides
M	Molar
mAb	Monoclonal antibody
MAGE	Melanoma-associated antigen
mBC	Memory B cell

mg	Milligram
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MHD	MAGE homology domain
min	Minute
ml	Milliliter
mM	Millimolar
MM	Multiple Myeloma
MNC	Mononuclear cells
MP	Melphalan/ Prednisone
MPT	Melphalan/Prednisone/Thalidomide
MPV	Melphalan/Prednisone/Velcade
MRD	Minimal residual disease
mRNA	Messenger Ribonucleic acid
MRI	Magnetic resonance imaging
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
NP	Nucleoprotein (Influenza)
NSCLC	Non Small Cell Lung Cancer
NY-ESO-1	New York Esophagial-1
OD	Optical density
PB	Peripheral blood
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PBS-T	Phosphate-buffered saline-Tween
PC	Plasma cell
PHA	Phytohaemagglutinin
PNPP	Para-Nitrophenylphosphate
PWM	Polyclonal mitogens pokeweed mitogen extract
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SAC	Staphylococcus aureus Cowan
SOX	Sex-determining region Y protein (SRY)-related HMG box
SCT	Stem cell transplantation
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS- Polyacrylamide gel electrophoresis
SMM	Smoldering multiple myeloma
SSX	Synovial sarcoma breakpoint X
TAA	Tumor associated antigen
TCR	T cell receptor for antigen
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TRIS	Tris(hydroxymethyl)aminomethane
TT	Tetanus toxoid
U	Unit

UKE	University Medical Center Hamburg-Eppendorf
VAD	Vincristine/Adriamycin/Dexamethasone
VGPR	Very good partial remission
WB	Western blot
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µL	Microliter
µM	Micromolar

Summary

Background

Cancer-testis (CT) antigens are specifically expressed in bone marrow (BM)-infiltrating plasma cells of patients with multiple myeloma (MM). Certain CT genes of the MAGE family are most commonly detected in MM. In addition, MAGE genes such as MAGE-C1/CT7, MAGE-C2/CT10 and MAGE-A3, seem to independently promote the progression of MM. Therefore, CT antigens (CTAs) such as MAGE-A3 are attractive targets for tumor immunotherapy based on their restricted expression and immunogenicity. However, little is known about the fine specificity of CTA-specific immunity in MM patients, as well as the difference between the spontaneous and vaccine-induced humoral responses.

Objects

We investigated the occurrence of spontaneous humoral responses against the promising target MAGE-A3 and MAGE-C2 for the antigen-specific therapy in MM patients. We further compared it with MAGE-A3 vaccine induced humoral responses with the spontaneous responses. An optimized B cell ELISPOT assay was developed for the evaluation of CT antigen-specific memory B cells.

Methods and Results

We screened a large number of sera (N=1636) consecutively collected from patients with MM (N=333) and monoclonal gammopathy of undetermined significance (MGUS) (N= 10) over six years, as well as sera from healthy blood donors (N=90), for antibody responses against MAGE-A3 and MAGE-C2. We found 4 (1.2%) and 25 (7.5%) out of 333 patients to evidence anti-MAGE-A3 and MAGE-C2 Immunoglobulin G (IgG) antibodies at least on one occasion during the course of their disease. MAGE-A3- and MAGE-C2-specific humoral responses were preferentially induced after allogeneic stem cell transplantation (allo-SCT) and correlated with a survival benefit ($P<0.05$). The antibody against

MAGE-A3 and MAGE -C2 were more often detected in the group of anti-nuclear antibody (ANA) positive patients than in ANA negative patients ($p<0.05$).

Compared with the naturally arising responses, the MAGE-A3 vaccine could rapidly induce high-titered and persisting immunity. An B cell ELISPOT assay was developed and verified to determine the number of antigen-specific memory B cells in the PB of BM and tonsil samples. Using this assay, we observed high frequencies of vaccine-induced MAGE-A3-specific memory B cells in the blood of NSCLC, but not in MM patients with spontaneous responses. Upon repeated antigen exposure, vaccine-induced antibodies underwent affinity maturation. MAGE-A3-specific antibodies consisted of IgG1>IgG3>IgG2>IgG4 subtypes in vaccinated patients, while spontaneous responses were mainly of the IgG2 subtype. We found vaccine-induced antibodies to recognize a much larger number of MAGE-A3 epitopes than spontaneously occurring antibodies. Nevertheless, spontaneous and vaccine-induced responses most frequently recognized a specific MAGE-A3 epitope, namely MAGE-A3$^{51-70}$, which is in accordance with the results calculated with the B cell epitope prediction program, BepiPre.

Conclusions

Cancer-testis antigens of the MAGE family, particularly MAGE-C2/CT10, are capable of inducing a spontaneous humoral response in MM patients. MAGE-A3-, MAGE-C2-specific humoral responses are preferentially induced after allo-SCT in the ANA positive patients, and correlate with a survival benefit. These antigens represent promising targets for antigen-specific immunotherapy in MM patients, and might also be of use as diagnostic and/or prognostic parameters for myeloma. A fundamental difference between spontaneous and vaccine-induced MAGE-A3-specific humoral responses in cancer patients was evidenced. Our findings will contribute to further improving immunotherapies targeting antigens of MAGE family.

CONTENTS

1. Introduction

1.1 Multiple Myeloma

1.1.1 Definition, Epidemiology and Etiology

Multiple myeloma (MM) is the second most frequent malignancy of the blood in the US after non-Hodgkin lymphoma, accounting for approximately 1% of neoplastic diseases and 13% of all hematologic cancers. The annual incidence of multiple myeloma is approximately 6.0/100,000/year in Europe and 4.3/100,000 in the US, with a mortality rate of 4.1/100,000/year [1, 2]. Myeloma and its precursor disease MGUS are slightly more common in males than females, and are twice as high in blacks compared with whites. The median age at diagnosis is between 61 to 70 years old, with only 2% of the patients being younger than 40 years old [3].

Multiple myeloma is a clonal B-cell malignancy characterized by an accumulation of plasma cells in bone marrow, monoclonal protein, osteolytic bone lesions, renal disease and immunodeficiency [1]. The malignant plasma cells phenotypically express CD38, CD56 and CD138, while approximately 20% of malignant plasma cells express CD20. This disease was first well-documented by Solly in 1844 [4]. Over the past decade, there have been major advances in the understanding of the disease biology, as well as in the diagnosis and treatment of myeloma, which in turn has dramatically improved the outcome of patients. However, the etiology of MM remains uncertain. The interaction of MM with its bone marrow (BM) microenvironment is central for the homing pattern, survival and proliferation of malignant plasma cells [5]. Accordingly, chemokines, cytokines and growth factors produced by myeloma cells and stromal cells promote the development and proliferation of MM.

1.1.2 Clinical Manifestations

Bone pain is the most common specific presenting symptom in up to 70% of MM patients. Other common presentations are renal insufficiency, hypercalcemia, anemia, plasmacytoma and susceptibility to infection. Peripheral

neuropathy amyloidosis (AL type) hyperviscocity is also seen in patients with MM.

Bone pain and hypercalcemia are usually secondary to the local osteoclast activation caused by lytic bone lesions. While renal insufficiency is caused by tubular damage by the monoclonal light chain (Bence–Jones protein) excreted by malignant plasma cells, and is related to hypercalcemia and volume depletion. Anemia is related to plasma cell proliferation in bone marrow, renal dysfunction and the inhibition of normal hematopoiesis by excessively produced cytokines such as IL-6. MM patients commonly exhibit a reciprocal decrease in normal immunoglobulin values in the presence of an elevated M protein level. Long-term corticosteroid use and the application of chemotherapies also cause immunologic abnormalities, and patients with myeloma often suffer from repeated infections, with overwhelming infection being a common cause of death in MM [6].

1.1.3 Classification and Diagnosis

Up to the present time, the diagnostic and staging criteria for multiple myeloma and related conditions have not been worldwide standardized. According to the guidelines of the International Myeloma Working Group (IMWG) and European Society for Medical Oncology (ESMO) [2], diagnosis should be based on the presence of monoclonal (M) component detected by serum and urine protein electrophoresis and immunofixation (IF), bone marrow-malignant plasma cell infiltration assessed by bone marrow aspirate and biopsy, and lytic bone lesions screened by skeletal survey and MRI.

To distinguish patients with active or symptomatic MM who require systemic therapy from those with smoldering (asymptomatic) multiple myeloma (SMM) without immediate need of treatment, the IMWG has established the CRAB (C: hypercalcemia; R: renal insufficiency A: anemia; B: bone lesions) criteria in 2009 (Table 1.1) [7]. The major indicator for active therapy is the presence of myeloma-related organ dysfunction or CRAB features based on biopsy and/or other specialized testing.

Table 1.1: Diagnostic criteria for plasma cell disorders

Disorder	Disease definition
Monoclonal gammopathy of undetermined significance (MGUS)	All three criteria must be met: • Serum monoclonal protein <3 g/100 ml • Clonal bone marrow plasma cells <10% and • Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia and bone lesions (CRAB) that can be attributed to the plasma cell proliferative disorder
Smoldering multiple myeloma/ asymptomatic multiple myeloma	Both criteria must be met: • Serum monoclonal protein (IgG or IgA) ≥3 g/100 ml and/or clonal bone marrow plasma cells ≥10% and • Absence of end-organ damage such as lytic bone lesions, anemia, hypercalcemia or renal failure that can be attributed to a plasma cell proliferative disorder
Multiple myeloma	All three criteria must be met except as noted: • Clonal bone marrow plasma cells ≥ 10% • Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma) and • Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically o Hypercalcemia: serum calcium ≥ 11.5 mg/100 ml or o Renal insufficiency: serum creatinine >1.73 mmol/l) o Anemia: normochromic, normocytic with a hemoglobin value of >2 g/100 ml below the lower limit of normal or a hemoglobin value <10 g/100 ml o Bone lesions: lytic lesions, severe osteopenia or pathologic fractures

The prognostic staging system most commonly used in patients with newly diagnosed MM is the Durie-Salmon system (DSS) (Table 1.2) [8, 9] and the International staging system (ISS) (Table 1.3) [10]. Since 1975, myeloma patients were stratified according to the DSS, which is still widely in use until now. However, this system had limitations in the categorization of bone lesions

and a number of prognostically important biological parameters, such as β2-microglobulin, C-reactive protein, lactate dehydrogenase and serum albumin. Combining DSS with serum β2-microglobulin and albumin, Greipp et al developed an International Staging System (ISS) based on data from 11 171 patients [2]. In this system, cytogenetics, as a major prognostic factor, should be performed in all newly diagnosed MM patients as well as subsequently at the time of relapse either by conventional karyotyping or FISH analysis. And del (13q), t (4; 14) and del (17p), are the most relevant abnormalities correlated with a poor outcome. Cytogenetics and/or FISH as patients may develop new chromosomal abnormalities at the time of progression [11].

Table 1.2: Durie-Salmon staging system

Stage	Durie-Salmon Criteria
Stage I	All of the following: ▪ Hemoglobin value >10 g/dL ▪ Serum calcium value normal or =10.5 mg/dL ▪ Bone x-ray, normal bone structure (scale 0), or solitary bone plasmacytoma only ▪ Low M-component production rates: IgG value <5 g/dL IgA value <3 g/dL 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 following: ▪ Hemoglobin value <8.5 g/dL ▪ Serum calcium value >12 mg/dL ▪ Advanced lytic bone lesions (scale 3) ▪ High M-component production rate IgG value >7 g/dL; IgA value >5 g/dL Urine light chain M-component on electrophoresis >12 g/24 h

Durie-Salmon sub classifications (either A or B)

- A: relatively normal renal function (serum creatinine value) <2.0 mg/dL
- B: abnormal renal function (serum creatinine value) >=2.0 mg/dL

Table 1.3: International staging system

Stage	International staging system
Stage I	β2-microglobulin <3.5 mg/L and albumin ≥3.5 g/dL
Stage II	β2-microglobulin <3.5 mg/L and albumin <3.5 g/dL, or β 2-microglobulin 3.5–5.5 mg/L
Stage III	β2-microglobulin >5.5 mg/L

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.

1.1.4 Treatment

The first consideration of treatment is to identify the subset of patients with MGUS and stage I or asymptomatic (smoldering/ indolent) multiple myeloma [12]. For these patients, immediate treatment is not recommended since there is no evidence that the early systemic chemotherapy of patients prolongs survival. Therefore, they could receive supportive care measures alone as a first approach. Meanwhile, clinical trials are ongoing to delay progression with newer agents.

For patients with newly diagnosed advanced stage or symptomatic myeloma (CRAB) (II or III), the treatment aims at the rapid control of the disease, the reversal of disease-related complications where possible, the use of a regimen that is well tolerated with minimal side effects, decreasing the risk of early death and collecting adequate numbers of stem cells for those considering a subsequent autologous stem-cell transplantation (ASCT) [12]. The outcome of conventional chemotherapy is unsatisfactory, while high-dose chemotherapy (HDT) with ASCT was evidenced to extend median survival to 5–7 years [13] in eligible patients. As a result, ASCT is the current standard treatment for patients with good clinical condition, especially in relative younger patients (<65 years old).

The treatment approach of myeloma depends on the eligibility for stem-cell transplantation (SCT) and risk assessment [1]. Although randomized trials have shown equivalent outcomes between an early (immediately after four cycles of induction therapy) or delayed (at the time of relapse as salvage therapy) ASCT [1] [14], an early treatment decision after diagnosis is still quite necessary. The induction therapy before ASCT should be dexamethasone-based and avoid alkylating agents that might cause the induced apoptosis of stem cells. Elderly patients (>65 years old) ineligible for ASCT are treated with standard alkylating agent therapy, with the most commonly used regimens being Melphalan (9 mg/m^2/day for four days), prednisone (30 mg/m^2/day for four days), thalidomide (100 mg/day) (MPT), Melphalan, prednisone, bortezomib (MPV), Melphalan, prednisone, lenalidomide (MPR) [1] [11].

The traditional pretransplantation induction therapy is vincristine and doxorubicin plus an intermittent high-dose dexamethasone (VAD) chemotherapy regimen. In the past decade, novel highly active agents such as thalidomide, bortezomib and lenalidomide have emerged as altering the treatment of myeloma. The most common induction regimens used today are combinations of Thalidomide or bortezomib or lenalidomide plus dexamethasone, which were proved superior to the classical VAD regimen by several large randomized clinical studies [1, 14].

Before each treatment cycle, patients should be evaluated to determine how their disease is responding to therapy. To measure the effect of treatment, the European group for blood and marrow transplant (EBMT) response criteria was widely used from 1998 until 2006. Based on various previously used systems, IMWG developed uniform response criteria in 2006 (Table 1.4) [15]. IMWG added new categories of response, and incorporated the free light chain (FLC) assay for determining the response to therapy, which is reserved for patients with unmeasurable protein in the serum and urine. This response criterion is currently being widely used in clinical trials of myeloma.

Table 1.4: International myeloma working group uniform response criteria for multiple myeloma [15]

Response	IMWG criteria
sCR	CR as defined below plus normal FLC ratio and absence of clonal cells in bone marrow[3] by immunohistochemistry or immunofluorescence[4]
CR	Negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and < 5% plasma cells in bone marrow[3]
VGPR	Serum and urine M-protein detectable by immunofixation but not on electrophoresis or ≥ 90% reduction in serum M-protein plus urine M-protein level < 100 mg/24 hours
PR	≥ 50% reduction of serum M-protein and reduction in 24 hours urinary M-protein by ≥ 90% or to < 200 mg/24 hours If the serum and urine M-protein are unmeasurable,[5] a ≥ 50% decrease in the difference between involved and uninvolved FLC levels is required in place of the M-protein criteria If serum and urine M-protein are not measurable, and serum free light assay is also not measureable, ≥ 50% reduction in plasma cells is required in place of M-protein, provided baseline bone marrow plasma cell percentage was ≥ 30% In addition to the above listed criteria, if present at baseline, a ≥ 50% reduction in the size of soft tissue plasmacytomas is also required
MR	NA
No change/Stable disease	Not meeting criteria for CR, VGPR, PR, or progressive disease
Plateau	NA
Progressive disease[5]	Increase of ≥ 25% from lowest response value in any one or more of the following: • Serum M-component and/or (the absolute increase must be ≥ 0.5 g/dL)[6] • Urine M-component and/or (the absolute increase must be ≥ 200 mg/24 hours) • Only in patients without measurable serum and urine M-protein levels; the difference between involved and uninvolved FLC levels. The absolute increase must be > 10 mg/dL • Bone marrow plasma cell percentage; the absolute percentage must be ≥ 10%[7] • Definite development of new bone lesions or soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas • Development of hypercalcemia (corrected serum calcium > 11.5

	mg/dL or 2.65 mmol/L) that can be attributed solely to the plasma cell proliferative disorder
Relapse	Clinical relapse requires one or more of: Direct indicators of increasing disease and/or end organ dysfunction (CRAB features).[6] It is not used in calculation of time to progression or progression-free survival but is listed here as something that can be reported optionally or for use in clinical practice • Development of new soft tissue plasmacytomas or bone lesions • Definite increase in the size of existing plasmacytomas or bone lesions. A definite increase is defined as a 50% (and at least 1 cm) increase as measured serially by the sum of the products of the cross-diameters of the measurable lesion • Hypercalcemia (> 11.5 mg/dL) [2.65 mmol/L] • Decrease in hemoglobin of ≥ 2 g/dL [1.25 mmol/L] • Rise in serum creatinine by 2 mg/dL or more [177 mmol/L or more]
Relapse from CR (To be used only if the end point studied is DFS) [8]	Any one or more of the following: • Reappearance of serum or urine M-protein by immunofixation or electrophoresis • Development of ≥ 5% plasma cells in the bone marrow[7] • Appearance of any other sign of progression (i.e., new plasmacytoma, lytic bone lesion, or hypercalcemia)

Note: A clarification to IMWG criteria for coding CR and VGPR in patients in whom the only measurable disease is by serum levels: CR in such patients is defined as a normal FLC ratio of 0.26–1.65 in addition to CR criteria listed above. VGPR in such patients is defined as a >90% decrease in the difference between involved and uninvolved free light chain (FLC) levels.

[3] Confirmation with repeat bone marrow biopsy not needed.

[4] Presence/absence of clonal cells is based upon the kappa/lambda ratio. An abnormal kappa/lambda ratio by immunohistochemistry and/or immunofluorescence requires a minimum of 100 plasma cells for analysis. An abnormal ratio reflecting presence of an abnormal clone is kappa/lambda of > 4:1 or < 1:2.

[5] All relapse categories require two consecutive assessments made at anytime before classification as relapse or disease progression and/or the institution of any new therapy. In the IMWG criteria, CR patients must also meet the criteria for progressive disease shown here to be classified as progressive disease for the purposes of calculating time to progression and progression-free survival. The definitions of relapse, clinical relapse and relapse from CR are not to be used in calculation of time to progression or progression-free survival.

[6] For progressive disease, serum M-component increases of $\geq$1 mg/dL are sufficient to define relapse if starting M-component is $\geq$5 g/dL.

[7] Relapse from CR has the 5% cut-off versus 10% for other categories of relapse.

[8] For purposes of calculating time to progression and progression-free survival, CR patients should also be evaluated using criteria listed above for progressive disease.

1.1.5 Prognosis

Although ASCT is the current standard treatment for patients with a good clinical condition, it only sometimes leads to a cure of the myeloma. Despite of remarkable advances in the field of ASCT, the efficacy of ASCT is restricted by a number of challenges, including the occurrence of marrow aplasia after high-dose chemotherapy, mucositis, infections and the relapse of malignancies after ASCT. After high-dose chemotherapy followed by ASCT, the myeloma disappeared completely or partially in about two out of three patients. In roughly half the patients, the myeloma comes back in one to two years, and in approximately 90% of patients, a myeloma relapse occurs in 10 years. The median survival of MM patients has been demonstrated to not have improved substantially. Hence, double ASCT was proposed and developed. However, the superiority of double versus single ASCT to offer the prospect of a cure is currently still controversial [16].

Multiple myeloma is still considered an incurable disease, and a variety of novel approaches are now being investigated to cure myeloma and improve the outcome of this disease. Allogeneic hematopoietic cell transplantation (allo-SCT) in MM is limited by prior reports of a high treatment-related mortality. Although encouraging data with tandem ASCT/reduced intensity conditioning followed by allo-SCT have been published recently, this strategy is currently still being carefully performed in controlled clinical trials. The reason for this is because the transplant-related mortality and the risk of chronic Graft versus host disease (GvHD) remain significant [17].

Residual disease after treatment is an important issue for refractory relapse，but could also be the target of immunologic responses. This immunotherapeutic aspect comprises the major curative effect of allo-SCT [18-20]. After allo SCT, the frequently occurring unspecific graft versus host response could also be accompanied by the graft-versus-myeloma (GvM) effect. The existence of GvM was first indicated by the phenomenon that the clinical remission of patients occurs more frequently following allo-SCT than ASCT [21-23]. The observation of GvM suggests that hematologic malignant cells are susceptible to the killing

effect of graft cytotoxic T lymphocytes [24]. Next, the donor lymphocyte infusion (DLI) alone was demonstrated to lead to long-term remission even after clinical relapse following allo-SCT in patients with MM [25, 26], as well as chronic and acute myeloid leukemia [27, 28]. Correspondingly, the transfusion of T cell-depleted donor lymphocytes [29-31] and immunosuppression [32, 33] was shown to be associated with an increased rate of relapse. Therefore, an active immunotherapy is rationally proposed for the de novo induction and amplification of pre-existing tumor antigen-specific GvM immune responses, without the elicitation of harmful GvHD.

The purpose of vaccination with suitable tumor antigens is to harness the immune system to fight and destroy myeloma cells, which becomes the promising synergetic anti-tumor therapy. Consequently, therapeutic vaccinations become one of the attractive treatment strategies for the attention of researchers.

1.2 Cancer Immunotherapy

Since the middle of the last century, tumor cells were proposed to be recognized and eliminated by the immune system before the manifestation of clinical symptoms [34, 35]. This phenomenon was called the cancer immunosurveillance [34], which was considered to be an important host protection process for inhibiting carcinogenesis and maintaining cellular homeostasis. Many kinds of tumor cells that arise as a result of the onset of cancer are more or less tolerated by the patient's own immune system, as the tumor cells are essentially the patient's own cells. Thus, they differ greatly from pathogens, and are harder to be recognized by the immune system as “foreign.” As a result, they could have the chance to escape the immunosurveillance, growing, dividing and spreading without proper regulatory control.

Cancer immunotherapy is proposed to help the immune system reject cancer, with the main premise being to stimulate the patient's immune system to attack the malignant tumor cells responsible for the disease. The aim of active cancer vaccination is to direct the host’s own immune system against antigens

expressed by tumor cells to help treat existing cancers or prevent the development of cancer in certain high-risk individuals. The components of vaccines usually include tumor antigens, carriers or delivery systems and adjuvants. According to the type of antigens (most frequently peptides, proteins or genetic constructs) and carriers, cancer vaccines can be divided into protein/peptide vaccines, DNA vaccines, DC-based vaccines, tumor-based vaccines, NP-based vaccines and Nanoparticle-Based Vaccines [36].

1.2.1 Strategies for Cancer Immunotherapy

The major strategies applied for cancer immunotherapy were classified into:

- Nonspecific immune activation;

  The nonspecific strategy is used to generically activate the immune response and interfere with the tumor microenvironment, including cytokines, interferons, or Toll-like receptors (TLRs) agonist treatment [36]. The unspecific immune activations work might synergize with the tumor-specific immune stimulation to increase tumor antigen presentation, induce cytotoxic T cell (CTL) activity, guide T-cells to the tumor and down-regulate tumor regulatory T cells or myeloid derived suppressor cells [36].

- Tumor (antigen)-specific immune activation;

  Most immunotherapies in development are primarily focused on a tumor-specific strategy, which could be through:

  - The immunization of the patient to train their own immune system to recognize tumor cells as targets (cancer vaccination);
  - The administration of therapeutic antibodies as drugs to recruit immune systems to destroy;
  - Cell-based immunotherapy, involving immune cells such as the natural killer cells (NK cells), lymphokine activated killer cells (LAK), cytotoxic T lymphocytes (CTLs) and dendritic cells (DC);
  - Radioimmunotherapy.

1.2.2 Tumor-related Antigens

During the development of tumor immunotherapy, the first step is to identify appropriate tumor-specific structures. An ideal target of immunotherapy should be highly restrictedly expressed in tumor tissue, without an inducing of autoimmunity against normal tissue. Among the currently known classes of tumor associated antigens (TAAs).

Cancer cells provide unique gene expression profiles that commonly characterize the respective tumor, as the immunohistochemistry already exploits this phenomenon for diagnostic purposes. The human immune system is also able to recognize structures associated with the malignant cells as indicated by tumor- specific antibody- and T cell responses. During the development of efficient and safe antigen-specific tumor immunotherapies, the first and most crucial step is to identify these structures as appropriate target tumor antigens. The efficacy and safety of tumor-specific immunotherapy will depend on the identification of targeted antigens. The targeted components should ideally be highly restrictedly expressed in tumor tissue, but not in normal cells. If the vaccine component is not specific enough to the cancerous cells, the immune system may not respond at all. Otherwise, it may begin to recognize normal cells as “foreign,” thereby resulting in their destruction by autoimmunity.

Over the decades, several categories of antigens were found to fulfill this requirement，which have been historically classified as tumor-specific antigen (TSA) or tumor-associated antigen (TAA) [37].

Tumor-specific antigens are likely to be expressed endogenously only by tumor cells resulting from somatic mutations, internal deletions, chromosomal translocations or normal immunoglobulin (Ig)- or T cell receptor (TCR) recombination events peculiar to leukemic B- or T-cell idiotypes [38]. They are usually classified as proteins, carbohydrates, glycoproteins or glycopeptides, gangliosides or gene (DNA or RNA) encoding cancer-associated antigens.

The natural advantage of TSA as immunogens and as targets of an effective immune response is clear. Their selective expression on tumor-initiating cells

conferred by the mutations could avoid the risk of autoimmunity from T cells targeting normal tissue. On the other hand, many cancers only have a relatively small number of consensus coding mutations. Thus, the T cell determinants generated are unique to a specific patient's tumor, which makes TSA-specific therapy individual and costly. Furthermore, some mutated proteins and tumor-specific posttranslational modified proteins could not be presented by MHC molecules, or the region is masked by modifications such as glycosylation [36]. Therefore, they cannot be good targets for immunotherapies.

Tumor-associated antigens are often aberrantly expressed endogenous proteins which present in limited normal tissues as a result of epigenetic control [38]. Compared with TSA, a much broader array of potential TAAs is available for any given cancer. They are classed as differentiation antigens (e.g. Melan-A in melanoma), cancer-testis antigens (CTAs, e.g. MAGE-A3, NY-ESO-1), those which arise from changes in protein structure as a result of point mutations (e.g. ras), over-expressed antigens (e.g. p53) and viral antigens (e.g. HPV-derived E6) [39]. The available naturally arising spontaneous immune responses against TAA antigens are likely to be of a lower avidity or against poorly cross-presented determinants. Immunotherapies that selectively enhance the recognition of specific TAAs by delivering high concentrations of synthetic antigen might overcome such hurdles [38].

1.3 Cancer-testis Antigen

1.3.1 Tumor-restricted Expression

Among the currently known classes of TAAs，cancer-testis antigens (CTAs) represent one of the most relevant for the development of cancer vaccines and are of the most interest to us. CTAs are a class of proteins particularly suited as targets for immunotherapeutic approaches, and include a family of more than 100 protein antigens with the expression normally restricted to immune-privileged tissues such as developing germ cells and the placenta. The expression of CTAs has also been detected, as a result of an aberrant activation, heterogeneously in a broad range of human cancers, including

melanoma, ovarian-, lung-, renal-, colon-, pancreatic cancers and hematopoietic malignancies [40-42]. CTAs could be classified by normal tissue restriction: I expression in normal tissues restricted to testis; II predominant expression in testis and brain; III expression in other tissues, but a strong expression in testis (testis-selective) [43]. Most of the CTAs, including NY-ESO-1, MAGE-A, CT7/MAGE-C1, CT10/MAGE-C2, GAGE, CT47, SAGE1 and NXF2, etc., belong to the first group.

The melanoma antigen (MAGE) family proteins are well-known CTAs comprising more than 60 genes. MAGE-1 was identified as the first tumor antigen recognized by host cytotoxic T-lymphocytes (CTL) in 1991 [44]. And most members of the MAGE family have been initially detected during screenings of T cell responses against autologous tumor cells from melanoma patients [45-48]. The MAGE antigens are characterized by a large conserved domain termed MAGE homology domain (MHD), a region of ~170 amino acids (AA) that comprises approximately 70% of each MAGE protein. MAGE proteins are normally expressed only in developing germs, trophoblasts and placentas. Unlike tumor cells, they lack a protein essential to present the antigen to T cells. The nearly tumor-specific expression patterns of MAGE proteins make them ideal therapeutic targets [41, 49]. Several clinical trials underway investigate full-length recombinant MAGE protein or partial MAGE peptides as anti-tumor vaccines in melanoma and other solid cancers [50, 51].

1.3.2 Biological Function

Antigens of the MAGE family, in addition to other CTAs, were frequently expressed in various solid tumors such as lung cancers and melanomas, as well as in hematological malignancies such as MM [52-57]. Clinical evidence is accumulating that a higher frequent expression of CTAs, including of MAGE antigens. It is also often correlated with progressive tumor stage, tumor metastasis and a worse outcome [52, 57-62]. In multiple myeloma, MAGE-A3 MAGE-C1,and MAGE-C2 were repeatedly demonstrated to be frequently expressed [1] (in more than 50% of patients). This expression is correlated with

an early relapse and reduced survival of the patients [52, 63-65]. In prostate cancer, MAGE-C2/CT10 expression correlates with the degree of malignancy and indicates a higher risk for biochemical recurrence after radical prostatectomy [58].

In vitro data further evidenced the correlation between the expression of these antigens and the survival and progress of myeloma cells [66, 67]. The myeloma-specific expression and its correlation with myeloma progress made MAGE antigens a prime target for immunotherapy.

However, the precise biological function of CTAs in both normal tissues and tumors remains as not yet clearly understood. It was indicated that MAGE expression may accelerate cell cycle progression and the rate of migration and invasion *in vitro*, as well as increases in lung metastases in an orthotopic mouse model of human thyroid cancer [57, 68, 69]. Recent studies have proven that full-length MAGE-A3, full-length MAGE-C2 or corresponding MAGE homology domain constructs, bind to and form complexes with full-length KAP1 and RBCC-KAP1, a known co-repressor of p53 [70, 71]. The suppression of these MAGE genes by siRNA induces apoptosis and causes an increased p53 expression *in vitro*, suggesting that MAGE-A3 might be oncogenic [71].

1.3.3 CTAs as Target for Cancer Immunotherapy

The expression of MAGE proteins in normal tissues is restricted to germ cells and placental trophoblasts. The missing expression of histocompatibility complex (MHC) molecules on these normal cells prevents them from being targeted by anti-MAGE-3 immunity. In contrast, the immunogenicity of CTAs was evidenced in various tumors by corresponding humoral responses and cytotoxic T lymphocytes [70]. The tumor-restricted expression and their capability to evoke natural immunity [72] make CTAs a promising target for immunotherapies.

1.3.4 Clinical Trials

Multiple clinical trials have been conducted using Melanoma-associated antigen-A3 (MAGE-A3) or NY-ESO-1 on patients with non-small-cell lung cancers (NSCLC) and melanomas, the most devastating diseases associated with a high rate of recurrence. A clinical benefit by postoperative vaccines with MAGE A3 in NSCLC and in Stage IV melanomas have been demonstrated by recent phase II trials [73, 74]. Both trials with MAGE-A3 or NYESO-1 have resulted in tumor regression in melanoma patients, and immunological responses were also detected in both patients (Figure 1.1) [50, 75-78].

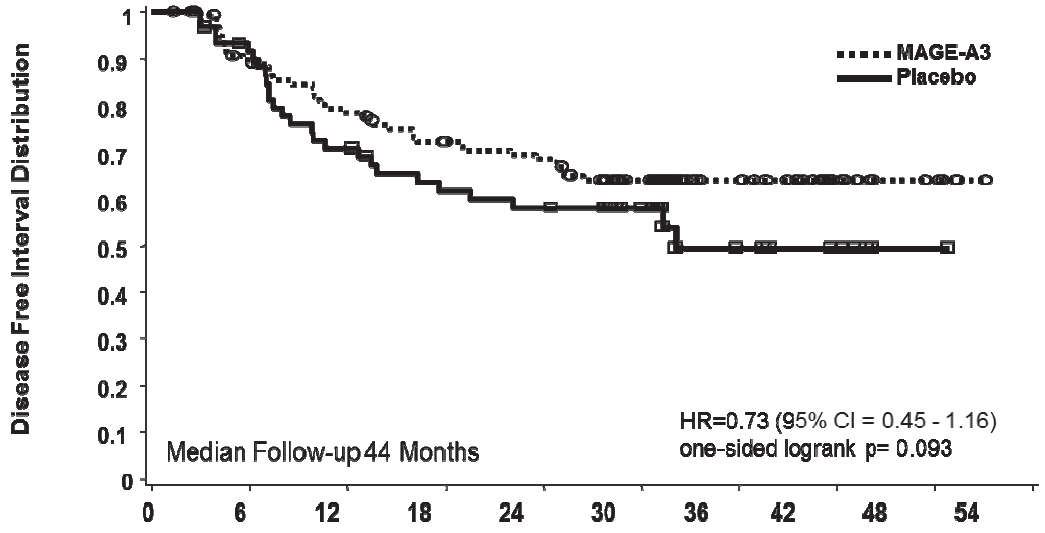

Figure 1.1: Kaplan-Meier curve for disease-free survival - 182 patients (122 Stage IB, 60 Stage II) with completely resected MAGE-A3 (+) NSCLC were randomly assigned to postoperative MAGE-A3 or placebo (2:1), with five administrations at three-week intervals, followed by eight administrations every three months. Group comparisons of disease-free intervals (DFI), disease-free survival (DFS) and overall survival (OS) gave a hazard ratio (HR) of 0.74 (95% CI 0.44-1.20, p=0.107), 0.73 (95% CI 0.45-1.16) and 0.66 (95% CI 0.36-1.20), respectively, in favor of the MAGE-A3 group. The final analysis of this randomized Phase II study shows a positive trend for the activity of MAGE-A3 treatment in NSCLC with a relative improvement of DFI and DFS of 27% [74].

Based on these promising results, large Phase III trials [79] have been initiated in NSCLC and melanomas patients investigating the efficacy of MAGE-A3 protein vaccination adjuvant and metastasized settings [50, 79]. The field of antigen-specific cancer vaccines is so far still in its early stages, thus it is anticipated that it will be used to treat a wider scope of malignant diseases in the future.

1.3.5 Side Effect

Cancer vaccines can be powerful therapeutic methods for cancer therapy. However, since cancer vaccines stimulate specific immune responses and direct them against the targets, some potential disadvantages should be noted. As vaccines, they could induce flu-like symptoms, including fever, chills, dizziness, nausea and vomiting, as well as inflammation, including pain, swelling, itchiness and rash. In addition, more serious symptoms such as asthma, autoimmune disease and severe hypersensitivity have also been reported in a few cases [39]. Furthermore, for protein and DNA vaccines, viral-based vaccines, NP-based vaccines and anti-liposome or PEG , we should pay more attention to side effects such as cell transformation, the risk of genetic recombination, pre-existing immunity and antibody responses against the drug delivery system, respectively [39].

1.4 CTAs and Hematologic Malignancies

1.4.1 CTA's Expression in Hematologic Malignancies and Multiple Myelomas

During the last decade, an abundance of CTAs have been identified in hematologic malignancies that may be used for tumor immunotherapy (Table 1.5) [80], with CTA expression in multiple myeloma being the most characterized of all the hematologic malignancies. Multiple myeloma is the second most common hematological malignancy, with an incidence of approximately 30,000 new cases per year in the European Union alone. Despite the introduction of novel agents and treatments over the last decade, and even

after the application of targeted strategies most patients will eventually relapse and succumb to the disease [81]. MM is essentially incurable and the prognosis of MM patients remains poor with a median survival of only 4-5 years [1].

Table 1.5: CTAs identified in hematologic malignancies [80]

CTA	Chromosomal location	Diseases and frequency of expression (%)
SSX	Xp11.23-p11.22	Lymphoma (36%)
Sp17	11q24.2	MM (26%); DLCL (54.5%)
Ropporin 1	3q21.1	CLL (43%); AML (18%); MM (37.5%)
CT45	Xq26.3	HD (58%); DLCL (22%)
BAGE	21p11.1	MM (14%)
GAGE 1	Xp11.4-p11.2	MM (41%)
MAGE-A	xq28	MM (Stage III, 100%; Stage I/II, 33%)
MAGE C1	Xq26-q27.2	MM (82%)
NY-ESO-1	Xq28	MM (60%)
SLLP 1	17q11.2	AML (22%); CML (29%); CLL (29%); MM (35%)
SPAN-Xb	Xq27.1	MM (20%); CML (60%); CLL (33%); AML (50%)
SCP1	1p13-p12	MM (10%); CML (23%); AML (5.7%)
SEMG 1	20q12-q13.2	CML (62%); CLL (42%); CLL (42%); MM (7%)
HAGE	6q12-q13	CML (57%)
PRAME	22q11.22	MM (48%); AML (30%); ALL (17%); CML (34%); CLL (28%)
PASD 1	Xq28	AML (33%); CML (17%)

1.4.2 Expression, Prognostic Value and Immunogenicity in Multiple Myeloma

In multiple myeloma, CTAs, particularly MAGE-A3, MAGE-C1 and MAGE-C2, were repeatedly demonstrated to be frequently expressed (in more than 50% of patients) [1]. The frequent expression of CTAs correlated with a reduced survival and early relapses of the patients [52, 63-65]. In vitro data further evidenced the correlation between the expression of these antigens and the survival and progress of myeloma cells [66, 67]. The myeloma-specific expression and its correlation with myeloma progress make MAGE antigens an attractive target for immunotherapy. The immunogenicity of CTAs was also verified by spontaneous antibodies and T immune responses identified in patients with MM, AML or CML [82], even in patients' post allogeneic hematopoietic stem cell transplantation (allo-HSCT). These data indicated the potential feasibility of using TAA (especially CTAs)-specific immunotherapy in MM patients.

1.4.3 Therapeutics Cancer Vaccines for MM Patients

The development of safe and effective vaccine myeloma vaccines could conceivably lengthen progression-free survival with minimal side effects, and could ultimately be used as an alternative maintenance option for myeloma patients. However, although therapeutic cancer vaccines have been intensely researched for more than a decade, only one has thus far been approved for use in the United States. Provenge (sipuleucel-T) received FDA approval in April 2010 for the treatment of certain patients with advanced prostate cancer. However, there are still numerous hurdles to be overcome, such as finding the right targets to generate the vaccine as well as making the vaccine elicit a strong enough immune response.

Vaccine therapy for multiple myeloma is an active area of research, with currently more than a dozen ongoing clinical trials for myeloma vaccine therapy. Because approximately 55% of myelomas express the MAGE-A3 antigen [82], MAGE-treatment may be a tumor-specific, well tolerated and effective adjuvant therapy. MAGE-A3-specific vaccine has also been studied in MM patients. One

healthy donor was reported to be immunized with MAGE-A3 protein formulated in an adjuvant system AS02B. The induced immunity was evidenced to be transferred to the identical twin, who was diagnosed with MAGE-A3-positive MM. The results showed that immunization of the healthy donor induced immune responses could be transferred and expanded in the recipient post SCT. The immunization with cancer-testis antigen MAGE-A3 may be a useful adjunctive therapeutic option to high-dose Melphalan-based PB SCT for high-risk MM [83].

Most recently, the phase I clinical trial of “MAGE-A3 Protein + AS15 as Consolidation for Multiple Myeloma Patients Undergoing Autologous Stem Cell Transplantation” is actively recruiting participants. The patients should have completed induction therapy with at least a very good partial response, making them eligible for high dose chemotherapy with auto-SCT. This study seeks to evaluate the safety and immunogenicity of recombinant MAGE-A3 protein plus AS15 adjuvant in patients with symptomatic multiple myeloma. The estimated completion date of this open label, single-arm pilot study will be in May 2014.

2. Objectives of Work and Study Design

The overall aim of this study is to investigate the immunogenicity of MAGE-A3 and MAGE-C2 in MM patients, and to discuss the the possibility of using them as therapeutic vaccine for MM patients.

To achieve these goals, first of all, we began with developing an improved method for the enumeration of antigen-specific memory B cells (mBCs) in various compartments of the human body. We planned to incubate ehe mBCs with different combinations of stimuli, and to analyze the proliferation-dependent terminal differentiations of plasma cells. We would apply the optimized method later to quantification of mBCs specific for microbial antigens and CT antigens in patients' samples.

Next, we set out to carry out a systematic investigation of B cell-mediated spontaneous immunity against MAGE-A3 and MAGE-C2 in a larger number of patients with multiple myeloma and MGUS. The repertoire of naturally developed humoral responses was to be characterized. Correlating MAGE-A3 and MAGE-C2-specific immunity and the clinical characteristics and treatment of patients, we aimed at answering the question whether MAGE-A3 and MAGE-C2 indeed represent the natural target for spontaneous anti-myeloma immune responses, at which time-points during course of the disease and under which clinical conditions these humoral responses occurred, and at whether the presence of allo-immune responses and/or graft-versus-host disease (GVHD) might further amplify the frequency and strength of MAGE-specific immune responses.

At last, we planned to compare MAGE-A3 vaccine-induced humoral responses and spontaneous responses with respect to the frequency, titer, persistence, affinity maturation, IgG subtypes, targeted epitope and MAGE-A3-specific memory B cells. Through this comparison, we would demonstrate whether there is substantial difference between the spontaneous and vaccine-induced antibody responses, and seek to contribute to further improving immunotherapies targeting MAGE-A3.

3. Materials and Methods

3.1 Sample Resource

3.1.1 Patients and Healthy Donors

Patients (N=343) with multiple myeloma or monoclonal gammopathy of undetermined significance were enrolled at University Medical Center Hamburg-Eppendorf. The Durie-Salmon system was used for prognostic staging in patients with newly diagnosed MM (Table 2) [8, 9]. All patients provided written informed consent for sample collection, and the study protocol was approved by the local ethics committee (decision number OB-038/06). Peripheral blood (PB) mononuclear cells and plasma were also collected from patients with written informed consent to donate blood for immunological monitoring in accordance with the revised version of the Declaration of Helsinki. The study protocol had received approval by the local ethics committee (OB-038/06). A total of 1,636 PB samples from MM patients were obtained during routine diagnostic procedures performed from January 2004 to October 2010.

3.1.2 MAGE-A3 Vaccination Study

Patients with non-small cell lung cancer (NSCLC) (N=4) without evidence of disease after surgical resection of Stage I or II NSCLC were enrolled into a phase II trial (LUD99–010) initiated by the Ludwig Institute for Cancer Research and Weill Medical College of Cornell University. The vaccine consisted of 300 µg of a full-length MAGE-A3/influenza protein D fusion protein (GlaxoSmithKline, GSK), and was administered to patients with MAGE-A3-expressing tumors once every three weeks for eight consecutive intramuscular injections, respectively. The vaccine was given in combination with adjuvants AS02B (monophosphoryl lipid A, MPL, and QS21; GSK) and AS15 (CpG 7909, MPL, and QS21; GSK). All patients were provided written informed consent to participate in the experimental vaccination study and to donate blood for immunological monitoring in accordance with the revised version of the Declaration of Helsinki. The vaccination protocol had been approved by the IRBs of the respective

institutions, and PB samples were collected from patients before each vaccination, as well as three weeks after the last injection.

3.1.3 Criterion for remission status

The therapy response level was evaluated for serologically positive, previously treated MM patients. The remission status was determined based on both the EBMT response criteria [84, 85] and the IMWG uniform response criteria (Table 4) [15]. In this retrospective assessment, the criteria were modified to account for the limited data available for each sample [52].

Complete remission (CR) was defined as < 10% plasma cells (PC) in BM, negative IF in both serum and urine, physiological levels of the patients' respective paraprotein, and absence of progressive bone or kidney pathologies.

Partial remission (PR) was defined as < 10% PC in BM, absence of progressive bone or kidney pathologies, and one of the following criteria: positive or ambivalent serum immunofixation, or moderately elevated patient's respective paraprotein (≤ 150% of physiological levels).

Progressive disease was defined as one of the following criteria: ≥ 10% PC in BM, highly elevated respective paraprotein (>150% of physiologic levels), or progressive bone or kidney pathologies.

Relapse was defined as one of the direct indicators of increasing disease and/or end organ dysfunction (CRAB features).

Overall survival was defined as the time from the date ofallo-SCT until death immediately related to MM as determined by the attending physician. Cases were censored when a death was not directly related to MM.

3.1.4 Patient Samples

Tonsils and PB were obtained from adult patients with a non-malignant disease undergoing tonsillectomy.

Peripheral blood mononuclear cells (PBMCs) and plasma samples were separated after collection and were frozen immediately. All PBMCs were cryopreserved and plasma was stored in −80°C freezer until use.

3.1.5 Cell Lines

All Myeloma cell lines (EJM, OPM-2, RPMI-8226, KMS-12-BM, U-266), lung cancer cell line A-427, as well as the african green monkey kidney fibroblast-like cell line COS-7, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The SK-MEL-37 was kindly provided by the New York branch of the Ludwig Institute for Cancer Research (LICR). Lines were maintained in RPMI 1640, IMDM or DMEM with 10-20% fetal calf serum (FCS), 2 mM L-glutamine and 50 units/ml penicillin/streptomycin. The cultivation of cell lines was performed in incubators at 37 °C in a water-saturated atmosphere with 5% CO_2. Once the cells were confluent to 90%, they were passaged. First, the medium was aspirated and the adherent cells were washed with PBS without Ca^{2+} and Mg^{2+}. By incubation with trypsin/EDTA, the cells were dissolved from the bottom of the culture flask, and the respective pre-warmed complete medium was added to the detached cells. Next, the suspension of cells was centrifuged for 3 min at 1,200 rpm. The cells were then resuspended in 5 to 10 ml culture medium and seeded at a density of 10-20%, and the cultured cells were subjected to a mycoplasma test at regular intervals.

List of cell lines used in this study:

A-427 (lung carcinoma, human)	DSMZ	Culture medium 2 - DMEM
COS-7 (kidney fibroblast-like cell line, African green monkey)	DSMZ	Culture medium 2 - DMEM
EJM (MM, human)	DSMZ	Culture medium 3 - IMDM
KMS-12-BM (MM, human)	DSMZ	Culture medium 1B - RPMI
OPM-2 (MM, human)	DSMZ	Culture medium 1A - RPMI

RPMI-8226 (MM, human)	DSMZ	Culture medium 1A - RPMI
SK-MEL-37 (MM, human)	LICR	Culture medium 2 - DMEM
U-266 (MM, human)	DSMZ	Culture medium 1A - RPMI

3.2 Cell Separation

3.2.1 Chemicals and Equipments

Centrifuge Megafuge 1.0R	Heraeus	Hanau, Germany
Ficoll Paque	Biochrom AG	Berlin, Germany
Microscope Telaval 31	Zeiss	Jena, Germany
PBS	Gibco	Paisley, GB
Tryptan blue solution 0,4%	Sigma-Aldrich	St. Louis, MO, USA

3.2.2 Medium

Erythrolysis buffer

NH_4CL	8,29 g
KHCO3	1 g
EDTA	45,2 mg
Distilled water	1 L

Freezing medium

RPMI-1640	70% (w/v)
FCS	20% (w/v)
DMSO	10% (w/v)

3.2.3 Cell Separation by Centrifugation

Buffy coat cells were obtained from University Medical Center Hamburg-Eppendorf. Plasma was collected and stored at either -80^{o}C or -20^{o}C depending on its later use. Buffy coats and heparinized whole-blood samples were diluted 1:1 with PBS at room temperature (RT). PBMCs were further isolated by density gradient centrifugation using the lymphocyte separation medium, Ficoll-Paque. Due to its high specific weight, Ficoll-Paque allows for the separation of low-density MNCs from erythrocytes and granulocytes. To retrieve a layer of mononuclear cells, diluted samples were slowly layered on top of Ficoll-Paque, and underwent density-gradient centrifugation at 2,000 rpm for 20 minutes (at 4ºC, without a break). The mononuclear cell layer, containing both lymphocytes ($CD4^{+}$/$CD8^{+}$ T-cells, natural killer T-cells, B-cells) and macrophages, was extracted via gentle aspiration with a Pasteur pipette. After the separation and lysis of the remaining erythrocytes, MNCs were washed twice with PBS. PBMCs were either suspended in an appropriate volume for immediate use or frozen in a freeze mix.

3.2.4 Cell Counting

The cell number was determined by use of trypan blue staining, with the dying process only taking place in dead cells. In contrast to living cells, dead cells no longer have an intact cell membrane. Hence, by diffusion, the dye enters the dead cell and binds to proteins. To determine the number of live cells, a 10 µL cell suspension was mixed with the same volume of trypan blue solution. The mixture was then placed in a Neubauer hemocytometer and the trypan blue stained cells within the four major quadrants were counted under a microscope with transmitted light. Since each quadrant corresponds to a volume of 0.1 mm^3 (10^{-4} ml), the cell concentration will be the average count per square x 2 x 10^4 per ml.

3.2.5 Separation of MNCs from Tissue Using BD Medimachine System Methods

The BD Medimachine System is a standardized sample preparation system for the automated, mechanical disaggregation of solid animal or plant tissues for flow cytometric analysis, cell culture or DNA amplification techniques. The system consists of three components: the Medimachine, the Medicon and the Filcon. According to product literature, the compact Medimachine unit is the heart of the system. It works together with the Medicons, and operates at a constant speed of approximately 80 rpm. Tonsils were dissected with two sterile scalpels into 96 mm glass Petri dishes containing an ice-cold separation medium (PBS), and cut into 1-2 mm^3 pieces after removal of the fat and connective tissue. The Medicon was opened and moistened with PBS, and together with 1.0 mL of ice-cold PBS, four to five of these pieces, were immediately moved into the disposable disaggregator Medicon with a 50 µm separator mesh. The Medicon was then inserted into the Medimachine and the machine was run for three minutes. The Medicon is a disposable polyethylene chamber containing a rotating mincing device and an immobile stainless steel screen with approximately 100 hexagonal holes. Around each hole, there are six microblades designed for efficient cutting of hard and soft tissues. The tissue is brought to each hole by a metal rotor inside the Medicon chamber and disaggregated by passing over the sharpened holes and through the metal screen, while a micropump under the screen supplies liquid and flushes out the holes. As the tissue is disaggregated, the cells pass through the screen into the suspension liquid in the bottom of the Medicon. After the disaggregation of the tissue, the Medicon could be removed from the Medimachine. The cell suspension is transferred from the Medicon unit with a 1000 µl pipette with the tip end cut off. The cell suspension is subsequently filtered through a 50-µm Filcon (a disposable filter device constructed of nylon mesh with a plastic housing) and placed on ice. Depending upon the chosen Filcon pore size, cells or cell aggregates pass through, while larger particles are trapped. The Medicon is refilled and rinsed with PBS four times, and the process is repeated to ensure

maximum cell recovery [86]. The MNCs are then separated by density centrifugation on a Ficoll-Hypaque.

3.3 Cell Culture

3.3.1 Chemicals and Materials

DMEM	Gibco BRL,	Eggenstein,Germany
CD40L	GenWay Biotech	San Diego, CA
CpG 2006	DNA Technology	Risskov, Denmark
FCS	Invitrogen	Carlsbad, CA
IL-2	Roche	Roche, Basel, Switzerland
IL-7	R&D Systems	Minneapolis, MN
IL-10	Immuno Tools	Friesoythe, Germany
IL-21	PeproTech	Hamburg, Germany
IMDM	Gibco, Life Technologies	Paisley, United Kingdom
L-Glutamine	Gibco BRL	Eggenstein,Germany
PHA-HA15	Murex Diagnostics	Dartford, United Kingdom
PWM	Sigma-Aldrich	St Louis, MO
RPMI 1640	Life Technologies	Paisley, United Kingdom
SAC	Sigma-Aldrich	St Louis, MO
Streptomycin /Penicillin	Gibco BRL	Eggenstein,Germany
Tissue culture flasks	Sarstedt	Nümbrecht, Germany
Trypsin/ EDTA (25%)	Gibco BRL	Eggenstein,Germany
X-VIVO -15	BioWhittaker	Walkersville, MD

3.3.2 Culture Medium and Trypsin-EDTA- medium

Culture medium 1A- RPMI

RPMI - 1640	90% (v/v)
FCS	10% (v/v)
L-Glutamine	2 mM
Nonessential amino acids	1%
Penicillin	100 U/ml
Streptomycin	100 U/ml

Culture medium 1B - RPMI

RPMI - 1640	80% (v/v)
FCS	20% (v/v)
L-Glutamine	2 mM
Nonessential amino acids	1%
Penicillin	100 U/ml
Streptomycin	100 U/ml

Culture medium 2- DMEM

DMEM	90% (v/v)
FCS	10% (v/v)
L-Glutamine	2 mM
Nonessential amino acids	1%
Penicillin	100 U/ml
Streptomycin	100 U/ml

Culture medium 3- IMDM

IMDM	90% (v/v)
FCS	10% (v/v)
L-Glutamine	2 mM
Nonessential amino acids	1%
Penicillin	100 U/ml
Streptomycin	100 U/ml

Culture medium 4- RPMI

RPMI - 1640	90% (v/v)
FCS	10% (v/v)
L-Glutamine	2 mM
Nonessential amino acids	1%
Penicillin	100 U/ml
Streptomycin	100 U/ml
CD40L	500 ng/ml
IL-21	50 ng/ml
CpG 2006	6 µg/ml

Culture medium 5- RPMI

RPMI - 1640	90% (v/v)
Human AB serum	10% (v/v)
L-Glutamine	2 mM
Nonessential amino acids	1%
Penicillin	100 U/ml
Streptomycin	100 U/ml
IL-2	10U/ml
IL-7	20 ng/ml

Trypsin-EDTA- medium (1x)

Trypsin	0.25% (w/v)
EDTA	1 mM
PBS	without Mg2+ und Ca2

3.4 Cryopreservation of Eukaryotic Cells

Eukaryotic cells can be preserved by cooling to low sub-zero temperatures with cryopreservation technology in liquid nitrogen (-196 ° C) for a long time. The addition of the highly hygroscopic cryoprotectant dimethyl sulfoxide (DMSO) increases viscosity at sub-zero temperatures, thereby decreasing the mobility of water molecules. The constrained water molecules are prevented from forming ice crystal nuclei. Thus, the cryoprotectant solutions can protect the cells from being damaged due to freezing or thawing during the approach to low temperatures or warming to room temperature.

The viable cells suspension was centrifuged at 1,000 rpm for five minutes at RT and the supernatant was discarded. $2x10^6$ to $2x10^7$ cells were resuspended in an 1 ml freezing medium containing 20% (v/v) FCS and 10% (v/v) DMSO. Cell suspensions were then stored in 2 ml cryovials (Nunc, Wiesbaden, Germany), which were placed in a plastic holder, a Mr. Frosty Control Freeze container (Nalgene, Roskilde, Denmark) containing a propane-1, 2,-diol bath. The container was immediately transferred to a -80°C freezer overnight. Then cryovials were transferred to liquid nitrogen for long-term storage.

3.5 Defrosting Cells

Cells were taken out of the liquid nitrogen, and quickly thawed by hand in a 37 ° C water bath for 1-2 minutes, and washed once by centrifugation in a wash solution at 1,200 rpm for 5 minutes. Pelleted cells were resuspended with the appropriate medium and removed to a culture flask. The cells were incubated for 24 hours before the medium was refreshed.

3.6 ELISA

3.6.1 Materials

Recombinant MAGE-A3 protein produced in the baculovirus system and E. coli-derived protein D, a surface protein of Haemophilus influenzae B (PD), were kindly provided by GSK. A total number of 11 CTAs produced as glutathione S-transferase (GST)-tagged proteins in the wheat germ system were obtained from Abnova (Taipei, Taiwan). E. coli-derived cancer-testis antigens SSX-2 and NY-ESO-1 were kindly provided by the New York branch of the LICR. Carcinoembryonic antigen (CEA) produced in E. coli was obtained from CellSystems (Troisdorf, Germany), and E.coli-derived SSX-4 protein was purchased from ProSci (Poway, CA). Tetanus toxoid (TT) was kindly provided by Chiron Behring (Marburg, Germany), and GST protein expressed in E. coli (CellSystems) or wheat germ (Abnova) was used as the irrelevant control.

A series of 20mer peptides (n = 31) overlapping by 10 AAs, spanning the complete sequence of MAGE-A3 and consisting of 314 AAs, were obtained from Multiple Peptide Systems (San Diego, CA).

A series of 20mer peptides (n = 37) overlapping by 10 AAs, spanning the complete MAGE-C2 sequence consisting of 373 AAs, were obtained from IRIS Biotech (Marktredwitz, Germany).

Antibody (Ab) responses were screened by ELISA fusing various recombinant full-length protein antigens: MAGE-A3 (produced in baculovirus; GSK), influenza protein D (produced in E. coli; GSK), MAGE-C2 (produced as a GST tagged protein in wheat germ; Abnova, Taiwan) and TT. The protein glutathione S-transferase (GST) expressed in E. coli (CellSystems) or wheat germ (Abnova) was used as the irrelevant control. All related information is shown below.

Recombinant proteins:

Protein	expression system	company	
CEA	E. coli	CellSystems	Troisdorf, Germany
GST	E. coli	CellSystems	Troisdorf, Germany
GST	wheat germ	Abnova	Taipei, Taiwan
MAGE 3	baculovirus	GSK Biologicals	Rixensart, Belgium
MAGE A1	wheat germ	Abnova	Taipei, Taiwan
Mage A11	wheat germ	Abnova	Taipei, Taiwan
Mage A12	wheat germ	Abnova	Taipei, Taiwan
MAGE A2	wheat germ	Abnova	Taipei, Taiwan
MAGE A8	wheat germ	Abnova	Taipei, Taiwan
MAGE A9	wheat germ	Abnova	Taipei, Taiwan
Mage B1	wheat germ	Abnova	Taipei, Taiwan
MAGE-C2	wheat germ	Abnova	Taipei, Taiwan
Mage H1	wheat germ	Abnova	Taipei, Taiwan
NYESO-1	E. coli	LICR	New York, USA
P53	E. coli	Abnova	Taipei, Taiwan
PRAME	wheat germ	Abnova	Taipei, Taiwan
Protein D	E. coli	GSK Biologicals	Rixensart, Belgium
SOX-2	wheat germ	Abnova	Taipei, Taiwan
SSX2	E. coli	LICR	New York, USA
SSX4	E. coli	ProSci	Poway, CA
TT	Clostridium tetani	Chiron Behring	Marburg, Germany

MAGE-A3 20-mer peptides:

number	location	sequence
# 1	1 - 20	M P L E Q R S Q H C K P E E G L E A R G
# 2	11 - 30	K P E E G L E A R G E A L G L V G A Q A
# 3	21 - 40	E A L G L V G A Q A P A T E E Q E A A S
# 4	31 - 50	P A T E E Q E A A S S S S T L V E V T L
# 5	41 - 60	S S S T L V E V T L G E V P A A E S P D
# 6	51 - 70	G E V P A A E S P D P P Q S P Q G A S S
# 7	61 - 80	P P Q S P Q G A S S L P T T M N Y P L W
# 8	71 - 90	L P T T M N Y P L W S Q S Y E D S S N Q
# 9	81 - 100	S Q S Y E D S S N Q E E E G P S T F P D
# 10	91 - 110	E E E G P S T F P D L E S E F Q A A L S
# 11	101 - 120	L E S E F Q A A L S R K V A E L V H F L
# 12	111 - 130	R K V A E L V H F L L L K Y R A R E P V
# 13	121 - 140	L L K Y R A R E P V T K A E M L G S V V
# 14	131 - 150	T K A E M L G S V V G N W Q Y F F P V I
# 15	141 - 160	G N W Q Y F F P V I F S K A S S S L Q L
# 16	151 - 170	F S K A S S S L Q L V F G I E L M E V D
# 17	161 - 180	V F G I E L M E V D P I G H L Y I F A T
# 18	171 - 190	P I G H L Y I F A T C L G L S Y D G L L
# 19	181 - 200	C L G L S Y D G L L G D N Q I M P K A G
# 20	191 - 210	G D N Q I M P K A G L L I I V L A I I A
# 21	201 - 220	L L I I V L A I I A R E G D C A P E E K
# 22	211 - 230	R E G D C A P E E K I W E E L S V L E V
# 23	221 - 240	I W E E L S V L E V F E G R E D S I L G
# 24	231 - 250	F E G R E D S I L G D P K K L L T Q H F
# 25	241 - 260	D P K K L L T Q H F V Q E N Y L E Y R Q
# 26	251 - 270	V Q E N Y L E Y R Q V P G S D P A C Y E
# 27	261 - 280	V P G S D P A C Y E F L W G P R A L V E
# 28	271 - 290	F L W G P R A L V E T S Y V K V L H H M
# 29	281 - 300	T S Y V K V L H H M V K I S G G P H I S
# 30	291 - 310	V K I S G G P H I S Y P P L H E W V L R
# 31	300 - 314	Y P P L H E W V L R E G E E

MAGE-C2 20-mer peptides:

number	location	sequence
# 1	1 - 20	M P P V P G V P F R N V D N D S P T S V
# 2	11 - 30	N V D N D S P T S V E L E D W V D A Q H
# 3	21 - 40	E L E D W V D A Q H P T D E E E E E A S
# 4	31 - 50	P T D E E E E E A S S A S S T L Y L V F
# 5	41 - 60	S A S S T L Y L V F S P S S F S T S S S
# 6	51 - 70	S P S S F S T S S S L I L G G P E E E E
# 7	61 - 80	L I L G G P E E E E V P S G V I P N L T
# 8	71 - 90	V P S G V I P N L T E S I P S S P P Q G
# 9	81 - 100	E S I P S S P P Q G P P Q G P S Q S P L
# 10	91 - 110	P P Q G P S Q S P L S S C C S S F S W S
# 11	101 - 120	S S C C S S F S W S S F S E E S S S Q K
# 12	111 - 130	S F S E E S S S Q K G E D T G T C Q G L
# 13	121 - 140	G E D T G T C Q G L P D S E S S F T Y T
# 14	131 - 150	P D S E S S F T Y T L D E K V A E L V E
# 15	141 - 160	L D E K V A E L V E F L L L K Y E A E E
# 16	151 - 170	F L L L K Y E A E E P V T E A E M L M I
# 17	161 - 180	P V T E A E M L M I V I K Y K D Y F P V
# 18	171 - 190	V I K Y K D Y F P V I L K R A R E F M E
# 19	181 - 200	I L K R A R E F M E L L F G L A L I E V
# 20	191 - 210	L L F G L A L I E V G P D H F C V F A N
# 21	201 - 220	G P D H F C V F A N T V G L T D E G S D
# 22	211 - 230	T V G L T D E G S D D E G M P E N S L L
# 23	221 - 240	D E G M P E N S L L I I I L S V I F I K
# 24	231 - 250	I I I L S V I F I K G N C A S E E V I W
# 25	241 - 260	G N C A S E E V I W E V L N A V G V Y A
# 26	251 - 270	E V L N A V G V Y A G R E H F V Y G E P
# 27	261 - 280	G R E H F V Y G E P R E L L T K V W V Q
# 28	271 - 290	R E L L T K V W V Q G H Y L E Y R E V P
# 29	281 - 300	G H Y L E Y R E V P H S S P P Y Y E F L
# 30	291 - 310	H S S P P Y Y E F L W G P R A H S E S I
# 31	301 - 320	W G P R A H S E S I K K K V L E F L A K
# 32	311 - 330	K K K V L E F L A K L N N T V P S S F P
# 33	321 - 340	L N N T V P S S F P S W Y K D A L K D V
# 34	331 - 350	S W Y K D A L K D V E E R V Q A T I D T
# 35	341 - 360	E E R V Q A T I D T A D D A T V M A S E
# 36	351 - 370	A D D A T V M A S E S L S V M S S N V S
# 37	361 - 374	S L S V M S S N V S F S E

Chemicals:

Diethanolamine	Sigma-Aldrich®,	Hamburg, Germany
D-PBS (10x)	Invitrogen	Karlsruhe, Germany
D-PBS (1x)	Invitrogen	Karlsruhe, Germany
Goat-Anti-human-IgA-AP	Southern Biotech	Birmingham, USA
Goat-Anti-human-IgG-AP	Southern Biotech	Birmingham, USA
Goat-Anti-human-IgG1-AP	Southern Biotech	Birmingham, USA
Goat-Anti-human-IgG2-AP	Southern Biotech	Birmingham, USA
Goat-Anti-human-IgG3-AP	Southern Biotech	Birmingham, USA
Goat-Anti-human-IgG4-AP	Southern Biotech	Birmingham, USA
Goat-Anti-human-IgM-AP	Southern Biotech	Birmingham, USA
Goat-Anti-mouse-IgG-HRP	R&D Systems	Minneapolis, MN, USA
Magnesium chloride ($MgCl_2$)	Fluka	Basel, Switzerland
Sodium hydroxide (NaOH)	Sigma-Aldrich	Hamburg, Germany
Nonfat dry milk	Spinnrad	Bad Segeberg, Germany
Para-Nitrophenylphosphate (PNPP)	Southern Biotech	Birmingham, USA
Hydrochloric acid (HCl)	Roth	Karlsruhe, Germany
TMB substrate solution	Sigma -Aldrich	Munich, Germany
Tween 20	Sigma-Aldrich	Hamburg, Germany

Laboratory equipments/ instruments:

ELISA-Reader	Sunrise, Tecan	Männedorf, Switzerland
Microplate Washer	Columbus Pro, Tecan	Männedorf, Switzerland
pH-Meter	CG 811, Schott	Mainz, Germany
Shakers	IKA-Vibrax-VXR Typ VX7, Janke & Kunkel	Staufen, Germany
Vortex	Lab Dancer S40, VWR	Darmstadt, Germany
Scale	PB 300 AT 261 Delta Range, Mettler	Bergisch-Gladbach, Germany

Laboratory consumable:

Multi-channel pipette	10 – 100 µl	Eppendorf	Hamburg, Germany
Parafilm	PM-996	Brand	Wertheim, Germany
Pipettes	0,5 – 10 µl 10 – 100 µl 100 – 1000 µl	Eppendorf	Hamburg, Germany
Pipettes for pipetting	Falcon 5 ml, 25 ml	Becton Dickinson	Franklin Lakes, USA
Pipette tips	20 µl, 200 µl,	Sarstedt	Nümbrecht, Germany
Pipetting	Pipetus	Hirschmann	Eberstadt, Germany
PP-test tubes, Cellstar	15 ml 50 ml	Greiner Bio-One	Frickenhausen, Germany
Reagent reservoir	4873	Costar	Cambridge, USA

PNPP substrate buffer

Double distilled water	400 mL
MgCl2.6H20	24.5 mg
Diethanolamine	48 mL

Adjust pH to 9.8 with 5N HCl and make up to 500 mL with distilled water.

3.6.2 Methods

Antigen-specific Immunoglobulin A (IgA), IgG and IgM antibody responses were determined in plasma and culture supernatants by ELISA as previously described [82]. Briefly, half-area, 96-well microtiter plates (Corning Costar, Lowell, MA) were coated overnight at 4°C with recombinant protein, peptides or goat anti-human IgG-Fab (Bethyl Laboratories Inc., Montgomery, TX), diluted in PBS at a final concentration of 1 µg/ml if not otherwise specified. After washed twice with PBS containing 0.05% Tween 20 and once with PBS, ELISA plates were blocked with PBS containing 5% non-fat dry milk for two hours at RT. Plasma diluted at 1:100 in a blocking buffer or undiluted supernatants from cell cultures were added to the plates at 30 µl/well, then were incubated at RT for two hours after washing. For the detection of antigen-specific antibody responses, alkaline phosphatase (AP)-conjugated second antibody goat antihuman IgG goat anti-human pan IgG and IgG1, 2, 3, 4 antibodies (Southernbiotech, Birmingham, AL) diluted 1:3000 in block buffer were applied at 30 µl/well after washing and were incubated for one hour at RT. After another washing, plates were developed with the detection reagent para-nitrophenyl phosphate (PNPP, Southern Biotech) 100 µl/well RT for 30 minutes, followed by reaction arrest with 3N NaOH. Specific absorption was measured using an ELISA reader (Tecan, Männedorf, Switzerland) at a wavelength of 405 nm with a reference wavelength of 620 nm.

In the screening part of the study, a sample was considered positive if the OD > Mean ($OD_{healthy\ donors}$) + $2SD_{healthy\ donors}$ or OD> 2×Mean ($OD_{healthy\ donors}$) and the OD>150% of the background against GST control protein.

Titration

In the titration part of the study, serial serum dilutions were performed for antibody-positive samples, and titers obtained with GST protein were used as reference values. For the calculation of titers, regression analyses were performed for the linear part of the serum titration curves of the patient sample,

and pooled the sera of five representative healthy donors. Titers were defined mathematically as the dilution at the intersection of both regression lines.

For antibody isotyping, AP-labeled secondary anti-IgG1 anti-IgG2, anti-IgG3 and anti-gG4 antibodies (Southern biotech) were used at a dilution of 1:3000.

Competition ELISA

The competition ELISA was performed as previously described [87] [88] [89]. Briefly, half-area, 96-well microtiter plates were coated with 1 µg/ml MAGE-A3 protein and 1µg/ml control protein GST, respectively. Serially diluted plasma samples were incubated with increasing concentrations of recombinant MAGE-A3 protein at dilutions of 3M, 2M, 1M and 0.5M, respectively, for two hours at room temperature. The samples were subsequently added to the coated ELISA plates. Free MAGE-A3-specific antibodies were determined as described above.

Antibody Absorption

Half-area, 96-well microtiter plates were coated with two-fold serial dilutions of recombinant protein GST or MAGE-A3 or MAGE-A3 peptide 51-70. Plasma diluted at 1:100 in blocking buffer was added to the plates, and the antibody absorption lasted two hours at room temperature. The samples were then removed to the coated ELISA plates for anti-MAGE-A3 antibody testing as mentioned above.

3.7 Immunoprecipitation and Western Blot

3.7.1 Materials

Chemicals

Amersham ECL Detection reagents	GE Healthcare	Buckinghamshire, UK
DTT	Sigma-Aldrich	Poole, UK
HRP-conjugated anti-mouse IgG	R&D Systems	Minneapolis, MN, USA
HRP-conjugated anti-human IgG	Sigma Aldrich	Buchs, Switzerland
Methanol	J.T.Baker	Deventer, Netherland
Mouse anti-ACTB antibody	Santa Cruz Biotechnology	Santa Cruz, CA, USA
Mouse anti-MAGE-A3 antibody	GSK Biologicals	Rixensart, Belgium
Mouse anti -MAGE-A3 antibody	LICR	NY, USA
Mouse anti -MAGE-C2 antibody	LICR	NY, USA
NuPage 4-12% Bis-Tris SDS-PAGE gel	Invitrogen	Karlsruhe, Germany
NuPage running buffer,	Invitrogen	Karlsruhe, Germany
NuPage transfer buffer	Invitrogen	Karlsruhe, Germany
NuPage LDS Sample Buffer(4x)	Invitrogen	Karlsruhe, Germany
NuPage Sample Reducing Agent (10x)	Invitrogen	Karlsruhe, Germany
NuPage Antioxidant	Invitrogen	Karlsruhe, Germany
Protease inhibitor	Sigma Aldrich	Buchs, Switzerland
RIPA Lysis Buffer	Sigma Aldrich,	Buchs, Switzerland
Sodium chloride	J.T.Baker	Deventer, Niederlande
Tris base	Sigma-Aldrich	Steinheim, Germany
Tween 20	Sigma-Aldrich	Steinheim, Germany
Vibrax VXR, JK	IKA Werke	Staufen, Germany

Instruments

Amersham Hyperfilm MP	GE Healthcare	Buckinghamshire, UK
Amersham Hyperprocessor	GE Healthcare	Buckinghamshire, UK
Consort EV202 power supply	Sigma-Aldrich	Steinheim, Germany
Developing chamber 18x24cm	Rego	Augsburg, Germany
E-Cups/Eppendorf tubes 1,5ml	Eppendorf	Hamburg, Germany
Falcon centrifuge tube	Greiner	Kremsmünster, Austria
Gel blotting paper	Whatman	Dassel, Germany
Nitrocellulose membrane	Whatman	Dassel, Germany
NuPage transfer buffer	Invitrogen	Karlsruhe, Germany
Sponge Pad for XCell II Blotting	Invitrogen	Karlsruhe, Germany
Vibrax VXR, JK	IKA Werke	Staufen, Germany
Water bath	WalterF.C.Ebel	Hamburg, Germany
Xcell II blot chamber	Invitrogen	Karlsruhe, Germany
Xcell SureLock electrophoresis cell	Invitrogen	Karlsruhe, Germany

TBS 10x (concentrated Tris-buffered saline) 1 liter

Tris base	24.2 g
NaCl	88 g

Dissolve in 900 ml distilled water

PH to 7.6 with 12N HCl

Add distilled water to a final volume of 1 liter

IP lysis buffer

NaCl	140 mM
$MgCl_2$	5 mM

Tris/HCl pH 7,6	20mM
NP40	1%
Complete Protease Inhibitor	

IP Wash buffer B

NaCl	150mM
Tris/HCl pH 7.6	10mM
EDTA	2mM
NP40	0,2%

IP Wash buffer C

NaCl	500mM
Tris/HCl, pH 7.6	10 mM
EDTA	2mM
NP40	0.2%

IP Wash buffer D

Tris/HCl, pH 8.0	10mM

3.7.2 Methods

The western blot, or protein immunoblot, is an investigation technique used to evidence specific proteins in an appropriate sample. With the aid of gel electrophoresis, the proteins are separated according to length (denatured proteins) or its tertiary structure (native/non-denatured proteins). This separation is followed by the transfer of the proteins to a suitable membrane (e.g. nitrocellulose), blocking, hence the incubation with the target protein-specific antibodies and further the reagent conjugated secondary anti-antibody to optically display the results.

Cell Lysate Preparation

A protein lysis buffer containing a protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) for western blot was stored at -80°C until needed. Immunoprecipitation (IP) and western blot were performed using the cell lines MAGE-A3-positive cell line A-427 and MAGE-A3-negative line COS-7 as protein sources. Exponentially growing cells lines were washed twice in phosphate buffered saline (PBS) (Gibco) by centrifugation. The supernatant was discarded and the concentrated cell mass was mixed with standard lysis buffer containing a protease inhibitor cocktail in a 15mL Falcon tube. The tube was shaken at 4 °C for 40 minutes to lead to the dissolution of the cell structure of the particular cell walls and the release of the cytoplasm including the intracellular proteins. After being transferred into a 1.5 mL Eppendorf tube, the mixture was centrifuged at 13,000/minute for 30 minutes (4 °C). The prepared protein lysates could then be directly used or be stored at -80 °C.

Protein Quantification

The amount of protein in cell lysates was quantified to ensure equal loading in western blot gels. The Bio-Rad laboratories protein assay was applied, and known bovine serum albumin (BSA, Promega Corporation, Southampton, UK) concentrations (0-8 µg/ml) were used as reference values. The standard samples were prepared with an 1x protein assay solution in sterile water as a 1:10 dilution of 10 mg/ml stock solutions of BSA. The 5x dye reagent concentrate was diluted 1 in 5 with deionized water.

Immunoprecipitation

Immunoprecipitation was performed using cell lines such as MAGE-A3-positive cell line A-427 and MAGE-A3-negative line COS-7 as protein sources. Protein lysates were prepared using a standard lysis buffer containing a protease inhibitor cocktail. Protein G sepharose 60 µl were pre-linked over night with 10 µg of a monoclonal antibody (mAb) against MAGE-A3 (GSK) or an IgG1 isotype

antibody (Miltenyi Biotech, Cologne, Germany) at 4°C. The mAb-protein G sepharose mixture was then incubated with 600 µl of cell lysate on a rotating shaker at 4°C for 4 hours. Subsequent to a series of washing steps, precipitated protein was resolved in lysis buffer/LDS-Loading buffer/DTT, and boiled for 5 minutes at 95°C. The precipitated protein was collected for loading on the SDS-PAGE.

Western Blot Analysis

The samples were prepared as follows: 13 µL protein sample (containing Recombinant protein 20 ng or 30 µg total protein of cell lysates) were mixed with 5uL NuPAGE LDS sample buffer (4x) and 2µL NuPAGE sample reducing agent (10X). After vortex and centrifuge, the mixture was denatured in a 70°C water bath for 10 minutes. Thus, the tertiary structures of the proteins were dissolved, and different lengths of polypeptide chains were available.

In the meantime, the XCell SureLock Mini-Cell and NuPAGE 4–12% Bis–Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA) were prepared for electrophoresis. A buffer made from NuPAGE MES SDS running buffer was prepared and added. After centrifuge, 15 µL denatured protein was loaded into the corresponding gel pockets. The electrophoresis started at a constant voltage of 195 votage (v) for one hour (Power Supply Model 250/2, Biorad), and the necessary cooling of the electrophoresis was provided by the water bath surrounding the experimental setup. Next, the gel was removed from the panel, cut and then placed on the activated Amersham Hybond-ECL Nitrocellulose membrane between (order from outside to inside) each sponge pad and moist commercial cardboard. This blot “sandwich” was slotted into the XCell II Blot Module Kit, which was then inserted into the XCell SureLock Mini-Cell. The Mini-Cell was filled with transfer buffer, and the outer chamber was filled with conventional water. The transfer of the previously separated proteins from the electrophoresis gel to the nitrocellulose membrane was carried out at a constant voltage of 25v for one hour. The cooling was performed using an ice water bath. The nitrocellulose membrane was then incubated in a 25mL 3% nonfat dry milk

buffer at 4°C for at least 12 hours. This so-called "blocking" is intended to block nonspecific binding sites on the nitrocellulose membrane and proteins in order to avoid the false positive binding reaction of the specific antibodies. The blocking solution was discarded and blocking buffer with primary antibody was added. Here, we either used 6 µg/10ml of monoclonal primary antibody against MAGE-A3 or MAGE-C2 (kindly provided by the New York branch of the LICR) or anti-beta-actin mAb (Santa Cruz Biotechnology, Santa Cruz, CA) or the patient's plasma at a dilution ranging between 1:200 and 1:1000. The blotted membrane was shaken in the antibody solution for 3-4 hours at RT in the dark. The antibody solution was then discarded and the membrane was washed four times for 10 minutes each in a 25 mL TBS-Tween buffer (washing buffer) to avoid nonspecifically binding to primary antibodies.

After washing, a secondary horseradish peroxidase (HRP)-conjugated anti-mouse monoclonal antibody (R&D Systems, Minneapolis, MN) or anti-human IgG-Fcγ antibody (Sigma Aldrich) was applied at a dilution of 1:4000 and incubated with the membrane for 1 hour at RT.

The second antibody solution was discarded and the membrane was again washed four times as mentioned above. At the last step, the membrane was reacted in a 6 ml Hybond-ECL western blotting analysis system for 1 minute with shaking. The membrane was then slightly dried using a filter paper and transferred into an X-ray film cassette. The specific binding was visually presented on Amersham Hyperfilm MP (18 × 24 cm) by developer in the darkroom.

3.8 ELISPOT

3.8.1 Chemicals and Equipments

Anti-IFN-γ mAb (7-B6-1-biotin)	Mabtech	Stockholm, Sweden
B Cell Isolation Kit II, human	Miltenyi Biotech Biotech	Cologne, Germany
BCIP/ NBT	Sigma-Aldrich	St. Louis, MO
BSA	Biomol	Hamburg,Germany
Carbonate-Bicarbonate Buffer capsule	Sigma-Aldrich	St. Louis, MO
CD4 Positive Isolation Kit	Dynabeads; Dynal,	Oslo, Norway
CD8 Positive Isolation Kit	Dynabeads; Dynal,	Oslo, Norway
ELISPOT reader	AutoimmuneDiagnostika	Strassberg, Germany
EliSpot software version 3.2.3	AutoimmuneDiagnostika	Strassberg,Germany
Goat anti-human IgG-Fab Ab	Bethyl Laboratories	Montgomery, USA
Goat anti-human IgG antibody-AP	Mabtech	Stockholm, Sweden
IFN-γ mAb	Mabtech	Stockholm, Sweden
LD Columns	Miltenyi Biotech Biotech	Cologne, Germany
MidiMACS Separator	Miltenyi Biotech Biotech	Cologne, Germany
MiniMACS Separator	Miltenyi Biotech Biotech	Cologne, Germany
MS Columns	Miltenyi Biotech Biotech	Cologne, Germany
Multi Screen-HA	Millipore	Bedford, USA
Streptavidin-AP	Mabtech	Stockholm, Sweden
X-VIVO-15	BioWhittaker	Walkersville, MD

3.8.2 Monitoring of CD4+ and CD8+ T Cell Responses

In Vitro Presensitization

The IFN-γ-producing $CD4^+$ and $CD8^+$ T cells specific for MAGE-A3 were monitored by ELISPOT after a single *in vitro* presensitization as described [90]. $CD4^+$ and $CD8^+$ T lymphocytes were purified from PBMCs using magnetic beads coated with anti-human CD4 or CD8 Ab (Dynabeads; Dynal, Oslo, Norway). The isolated T cells were seeded into round-bottom 96-well plates (Corning, NY) at 5×10^5cells/well in an RPMI 1640 medium with 10% human AB serum (culture medium 5 without IL-2 and IL-7). The remaining $CD4^-/CD8^-$ PBMCs were used as Ag-stimulating cells (ASC) for the presensitization. They were pulsed with 10 μM overlapping 20-mer peptides pools covering the entire MAGE-A3 sequence (overlapping by 10 AAs; NeoMPS, San Diego, CA). Influenza nucleoprotein peptide 206-229 and irrelevant NY-CO-58 peptide 151-180 were used as controls. The ASCs were stimulated in 500 μl of serum-free medium X-VIVO-15 (BioWhittaker, Walkersville, MD) overnight at 37°C. Pulsed $CD4^-/CD8^-$ ASCs were then washed, irradiated and added to plates containing $CD4^+$ or $CD8^+$ T cells at a concentration of 1×10^6 ASCs/well. After 20 hours, IL-2 (10 U/ml; Roche Molecular Biochemicals, Indianapolis, IN) and IL-7 (20 ng/ml; R&D Systems, Minneapolis, MN) were added. Subsequently, half of the medium was replaced by fresh culture medium 5 containing IL-2 (20 U/ml) and IL-7 (40 ng/ml) twice a week. Before the ELISPOT assay, the effector T cells were washed twice to remove serum and were resuspended in a plain RPMI medium for testing.

Generation and Culture of Target Cells

$CD4^+$ T cells from the initial separation were resuspended at a concentration of 1×10^6 cells/ml in a complete culture medium 5 supplemented with 10 μg/ml PHA (PHA HA15; Murex Diagnostics, Dartford, U.K.). The T cell suspension was then seeded at 1 ml/well into 24-well plates (Corning Glass, Corning, NY), fed and expanded twice a week with a complete medium 5. After 10–20 days, the activated T cell antigen presenting cells (T-APC) were harvested and used

as target cells. Before the ELISPOT assay, the T- APCs were washed twice with an X-VIVO-15 medium to remove serum and were resuspended in an appropriate medium for testing [90, 91].

Enumeration of IFN-specific Antibody-secreting Cells by ELISPOT

The Ag-specific effector $CD8^+$ T cells were determined by use of an enzyme-linked immunospot technique (ELISPOT) on the 10^{th} day of presensitizing culture [90, 91]. The ELISPOT assay for the determination of Ag-specific $CD4^+$ T cells was performed on the 20^{th} day. A flat-bottom, 96-well nitrocellulose plate (MultiScreen-HA; Millipore, Bedford, MA) was coated with 2-5 μg/ml anti-human IFN-γ mAb (1-D1K; Mabtech, Stockholm, Sweden) in a carbonate-bicarbonate coating buffer (Sigma-Aldrich, St. Louis, MO). The plate was incubated overnight at 4°C, and was washed three times with RPMI-1640. The plate was then blocked for 2 hours at 37°C using RPMI-1640 with 10% human AB-type serum. Before being used, the coated plate was washed three times with plain RPMI-1640 again.

$5x10^5$ target T cells (the T-APCs) were pulsed with 10 μM peptides in 500 μl of serum-free medium X-VIVO and were transferred into a 1.0 ml Nunc tuber. The target T cells were incubated at 37°C overnight, and were counted, washed twice and resuspended at a $1x10^6$/ml RPMI 1640 medium without serum. The target T cells were then seeded at $5x10^4$ per well in the coated ELISPOT plate.

$CD8^+$ and $CD4^+$ T cells were harvested and incubated with autologous PHA-activated T cells (T-APCs) pulsed with cognate or control peptides. A total of 5×10^4 or 1×10^4 presensitized $CD4^+$ or $CD8^+$ T effector cells were added to each well and incubated for 20 hours. The plate was then washed six times with water containing 0.05% Tween 20, and was rinsed 20 times with normal water. Biotin-conjugated anti human IFN-γ mAb (0.2 μg/ml, 7-B6-1-biotin; Mabtech) was added at 100 μl per well. The plate was incubated at 37°C for 2 hours, and was then washed and developed with 1 μg/ml streptavidin-alkaline phosphatase (Mabtech) for 1 hour at RT. After washing, the substrate buffer with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT;

Sigma-Aldrich, St. Louis, MO) was added and incubated for 5 to 10 minutes. The development was stopped with water and the plate was dried. The dark violet spots displayed on the plate membranes represented the respective IFN-γ -producing effector T cells. Finally, the plate was scanned with an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany), and the spots were counted using AID EliSpot software. The average from duplicates was calculated, and a positive response was determined as >20 spots and >two times the number of spots for irrelevant control target. The results were representative of at least two repeated experiments.

3.8.3 Memory B Cell Assay

Purification of B Cells

PBMCs were prepared by density centrifugation on Ficoll-Hypaque. Untouched B cells and non-B cells were separated using the B cell Isolation kit II (Miltenyi Biotech), which contained a cocktail of avidin-conjugated antibodies against a variety of non-B cells and biotin-conjugated microscopic magnetic beads.

Induction of Immunoglobulin Secreting Cells (ISCs) from PBMCs

Briefly, 3×10^5 PB-derived B cells mixed with 5.7×10^6 non-B cells separated from autologous PBMCs were re-suspended in a 2 ml RPMI 1640 medium (Life Technologies, Paisley, United Kingdom), and supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA), penicillin, streptomycin and glutamine. Cells were seeded into 6-well plates and were incubated with combinations of 6 μg/ml 24-mer DNA oligonucleotide containing a phosphorothioated optimized human CpG motif (CpG 2006: 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′; DNA Technology, Risskov, Denmark), 500 ng/ml human CD40 Ligand (CD40L; GenWay Biotech, San Diego, CA), 50 ng/ml Interleukin (IL)-21 (PeproTech, Hamburg, Germany), 100 U/ml IL-2 (Roche, Mannheim, Germany) and 20 ng/ml IL-10 (ImmunoTools, Friesoythe, Germany). Cells were cultured for 3–6

days at 37 °C with 5% CO_2. For the enumeration of CTA-specific memory B cells, cells were incubated with culture medium 4 containing 6 µg/ml CpG 2006, 500 ng/ml human CD40L and 50 ng/ml IL-21 for six days at 37 °C with 5% CO_2.

Enumeration of Antibody-Secreting Cells by ELISPOT

To enumerate antigen-specific immune globulin-secreting memory B cells (mBCs), an B cell ELISPOT was used. Briefly, flat-bottomed 96-well nitrocellulose plates (Multi Screen-HA; Millipore, Bedford, USA) were coated overnight at 4 °C with 30 µl of the following Ags or monoclonal antibodies: 30 Lf/ml tetanus toxoid (TT; Chiron Behring, Marburg, Germany), 2 µg/ml Glutathione-S-Transferase (GST; Abnova, Taipei, Taiwan), 2 µg/ml MAGE-A3 and 2 µg/ml goat anti-human IgG-Fab antibody (Bethyl Laboratories, Montgomery, USA) in a coating buffer made of a carbonate–bicarbonate buffer (Sigma-Aldrich). Free protein-binding sites were blocked by incubation with 3% albumin bovine (BSA; Biomol) at 37 °C for 2 hours. Stimulated B cells and PBMCs ($2.5–5\times10^5$/well) were incubated in RPMI 1640 (Gibco, Paisley, United Kingdom) at 37 °C for 20 hours. The plates were then washed thoroughly with water containing 0.05% Tween 20 (Sigma-Aldrich), followed by incubation with 1 µg/ml Alkaline phosphatase (ALP)-conjugated goat anti-human IgG antibody (Mabtech, Stockholm, Sweden) for 2 hours at 37 °C. After washing, substrate BCIP/NBT was added, and plates were developed at room temperature for 15 minutes. Spots were counted using an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

3.9 Flow Cytometry Analysis

3.9.1 Chemicals and Equipments

Anti-human CD3- APC	ImmunoTools	Friesoythe, Germany
Anti-human CD14 -PerCP	BD	Heidelberg, Germany
Anti-human CD19-PerCP	BD	Heidelberg, Germany
Anti-human CD19-PE	BD	Heidelberg, Germany
Anti-human CD27-APC	BD	Heidelberg, Germany
Anti-human CD38-FITC	Caltag	Hamburg, Germany
Anti-human CD38-PE	Immunotech	Marseille, France
Anti-human CD138-PC5	Beckmann Coulter	Krefeld, Germany
Anti-human IgD-PE	BD	Heidelberg, Germany
BD Cell QuestTM Pro (Version 5.2.1)	BD	NJ, USA
CellTrace CFSE Cell Proliferation Kit	Invitrogen,	Carlsbad, CA, USA
FACSCalibur Flow Cytometer	BD	NJ, USA
FlowJo Version 7.2.5	Tree Star Inc	OR, USA

3.9.2 CFSE Labeling and Flow Cytometry Test

Purified B cells or MNCs were suspended at 1 x 10^7 cells/ml in a plain PBS with 0.5 µmol/l carboxyfluorescein succinimidyl ester (CFSE; Invitrogen/Molecular Probes, Eugene, OR), and incubated at 37 °C for 10 minutes. The staining was stopped by immediately adding 1.5 ml cold RPMI with 10% FCS and washed. The labeled cells were cultured with CD40L, CpG and IL21 for 3-6 days. In vitro-activated cells were harvested, counted and centrifuged at 1,200rpm for 5 minutes, and then re-suspended in PBS at 1x10^6 cells/ tube. The cell suspension was added to polystyrene round bottom tubes (BD Falcon) at 50µl/tube. A combination of fluorochrome-conjugated anti-CD19, -CD27, -CD38,

-CD138, or -IgD mAb was applied to the appropriate FACS tube. Samples incubated with an isotype antibody or without an antibody were used as a negative control. Nonspecific binding sites were blocked by pre-incubating with normal mouse IgG, and the cells were then incubated at 4°C for 20 minutes. After washing, 300μl PBS was added and the cells were analyzed on FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences).

4 Statistical Analyses

All statistical analyses were performed using SPSS software (SPSS Inc., IL, USA). Since most of the data were not distributed normally, a Mann-Whitney rank sum test was performed to compare the values between groups. A Spearman's rank correlation coefficient was calculated to analyze correlations between groups such as clinicopathological parameters and CT antigen-specific humoral responses. Cox regression analyses were performed for the evaluation of survival and relapse in MM patients. For all statistical analyses, results were considered statistically significant if the p values were less than 0.05. Figures in the following chapters were created using SPSS, Microsoft Excel 2003 (Microsoft Deutschland GmbH, Unterschleißheim, Germany).

5. Results

5.1 An Optimized Assay for Enumeration of Antigen-specific Memory B Cells in Different Compartments of Human Body

In the framework of humoral immunity, mBCs have been suggested to persist for a lifetime and be essential for maintaining long-term antibody-mediated immunity [92-94]. The enumeration of antigen-specific mBCs in human subjects is critical for the analysis of humoral immune responses. However, it has remained technically challenging due to the relatively low frequencies of these cells in PB. In our current study, we began with developing an improved method for the enumeration of antigen-specific mBCs in various compartments of the human body. This part of work was performed by Yanran Cao with Maja Gordic. This method was later used for CTA-specific mBCs evaluation in patients'samples.

5.1.1 CD40L, CpG, and IL-21 Represent the Optimal Combination of Stimuli to Induce TT-specific IgG-secreting B cells

Due to the relatively low frequencies of Ag-specific mBCs, it is necessary to use polyclonal stimuli to amplify Ag-specific mBCs before enumerating them in a read-out assay. For a first step, we investigated which combination of stimuli would be optimal for an efficient *in vitro* induction of ISCs from PBMCs. To the end, we stimulated whole PBMCs from healthy donors separately with PWM, CpG 2006, IL-2, IL-10, IL-21, CD40L and SAC. After six days of *in vitro* incubation with different stimuli, the number of TT-specific ISCs was determined in an ELISPOT read-out assay. In addition, TT-specific antibodies produced during the *in vitro* culture were assessed by ELISA. We found that PWM represented the most potent inducer of TT-specific ISCs from precursor B cells compared with other stimuli when used as single agents (Figure 5.1A). CpG 2006 and IL-21 as single agents were also able to amplify significant numbers of ISCs from TT-specific mBCs, though with a lower efficiency. In contrast, no TT-specific ISC was induced by IL-2, IL-10, CD40L and SAC when used as single agents (Figure 5.1A). Importantly, these results corroborated by the

ELISA, were showing that IL-21, CpG, and PWM led to elevated levels of TT-specific IgG produced by ISCs during the in vitro stimulation (Figure 5.1 B).

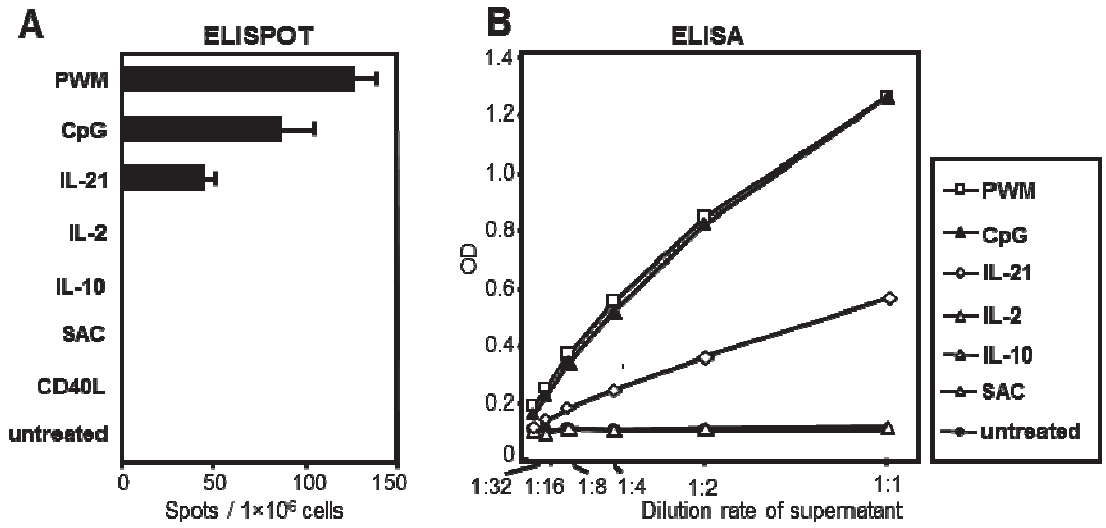

Figure 5.1: Single stimulus for the amplification of antibody-secreting B cells from TT-specific mBCs - PBMCs were stimulated with single agents PWM, CpG, IL-21, CD40L and SAC for six days. TT-specific mBCs precursors were detected by ELISPOT and ELISA. The bars represents the mean (+SEM) count of TT-specific ISCs per 1×10^6 cells by ELISPOT (A). The OD values represent the level of TT-specific antibodies measured in ELISA (B).

Next, we aimed at determining the most effective combination of stimuli for the induction of TT-specific ISCs from mBCs precursors. Incubating PBMCs with combinations of 2-5 stimuli for six days, we observed that the combination of CD40L, CpG 2006 and IL-21 could most efficiently induce ISC from TT-specific mBCs (Figure. 5.2A), but not IgA or IgM secreting B cells (data not shown). Although PWM had shown the strongest capacity for the induction of TT-specific ISC when used as a single stimulus, the addition of PWM to a combination of stimuli did not further increase the yield of TT-specific ISC (Figure 5.2A). These findings were largely corroborated by observations made in the ELISA analysis of culture supernatants after in vitro stimulation of whole PBMCs (Figure 5.2B).

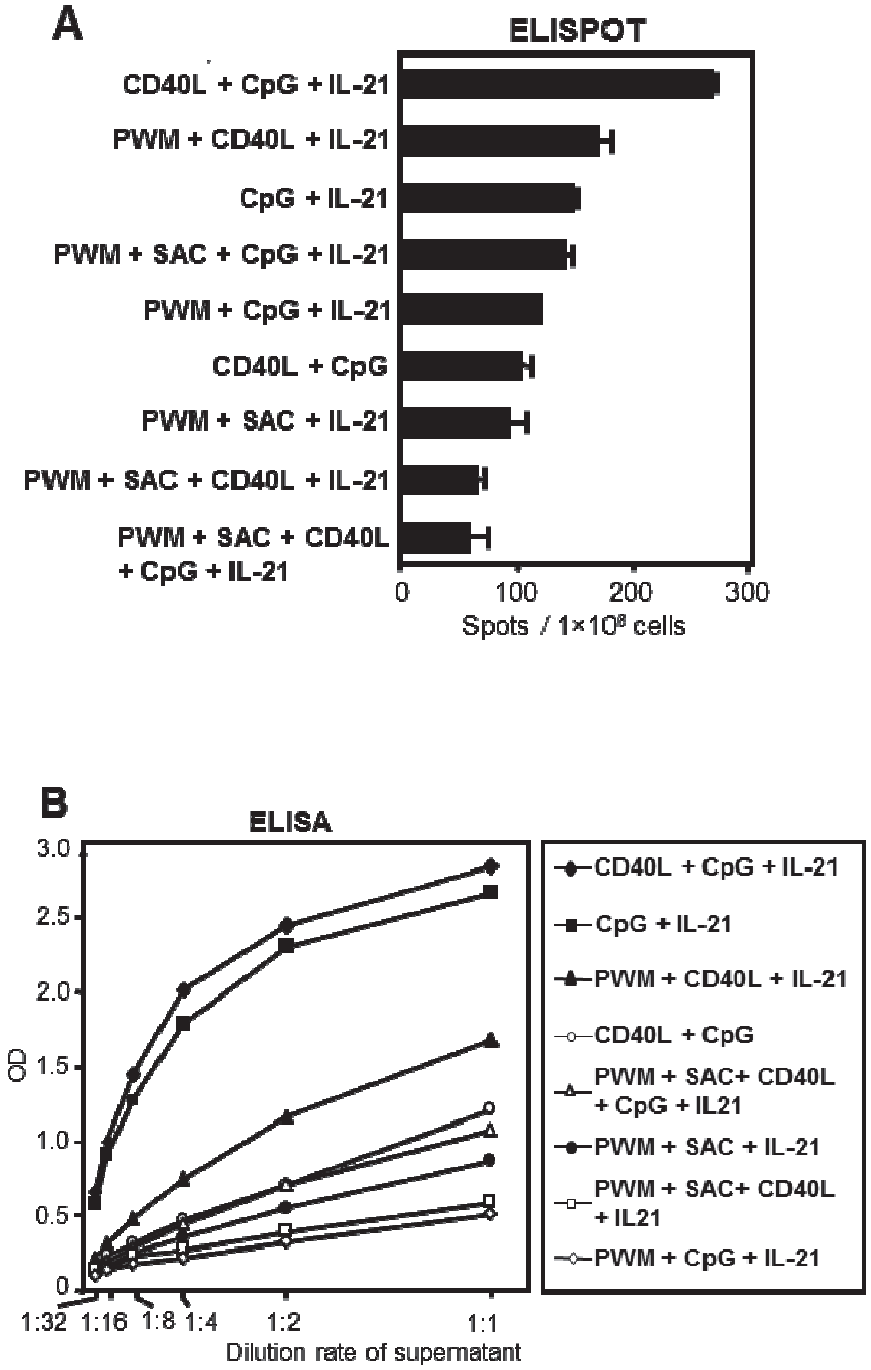

Figure 5.2: A Cocktail of CD40L, CpG and IL-21 represents the ideal stimulus for the amplification of antibody-secreting B cells from mBCs - PBMCs were stimulated with combinations of 2-5 stimulants (PWM, CpG, IL-21, CD40L, and SAC) for six days. TT-specific mBCs precursors were detected by ELISPOT and ELISA. The bars represent the mean (+SEM) count of TT-specific ISCs per 1×10^6 cells by ELISPOT (A). OD value responses represent the level of TT-specific antibodies measured in ELISA (B).

Of all the cytokines, IL-2 and IL-10 have previously been the most commonly used in memory B cell assays. IL-10 can induce the differentiation of memory B cells into ISCs, while IL-2 enhances the response of CD40L-stimulated B cells [95-97]. However, stimulation of a combination of CD40L/CpG 2006, with cytokine IL-21, which was identified as a very potent stimulant in our current study, led to higher numbers of TT-specific ISCs compared to a combination with IL-2 or IL-10 (Figure 5. 3A, B). Furthermore, the addition of IL-10 and/or IL-2 to our cocktail of stimulants consisting of CD40L/CpG 2006/IL-21 did not further increase Ig secretion (data not shown).

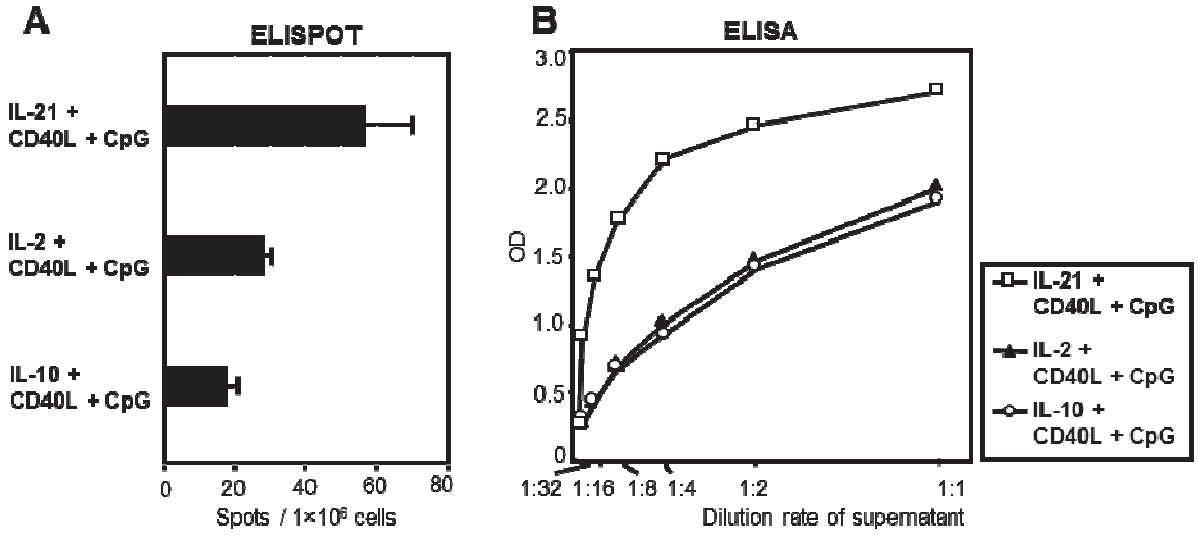

Figure 5.3: A comparison of IL-2, IL-10 and IL-21 for the amplification of antibody-secreting B cells from mBCs - PBMCs were stimulated with CD40L and CpG, and combined with either IL-2, IL-10 or IL-21 for six days. TT-specific mBCs precursors were detected by ELISPOT and ELISA. The bars represent the mean (+SEM) count of TT-specific ISCs per 1×10^6 cells by ELISPOT (A). OD value responses represent the level of TT-specific antibodies measured in ELISA (B).

5.1.2 Memory B Cells Proliferate and Differentiate into ISCs upon Stimulation with CD40L/ CpG 2006/IL-21

It has been long known that polyclonal stimulation is necessary to induce antigen-specific ISCs from memory B cells present among PBMCs. However, it

is unclear by which mechanisms exactly such stimuli amplify B cell signals. We used CFSE staining in order to monitor the proliferation of different B cells upon an unspecific stimulation. We found that after three days, the unspecific stimulation had induced the division of $CD19^+$ B cells. In addition, the number of $CD138^+$ plasma cells presented in the culture had significantly increased (Figure 5.4). After six days, $CD138^+$ plasma cells had all divided multiple times, while the proportion of cells that had undergone cell division was much lower among $CD19^+$ conventional B cells. Importantly, other cell types such as T cells and monocytes had not undergone any cellular proliferation (Figure 5.4).

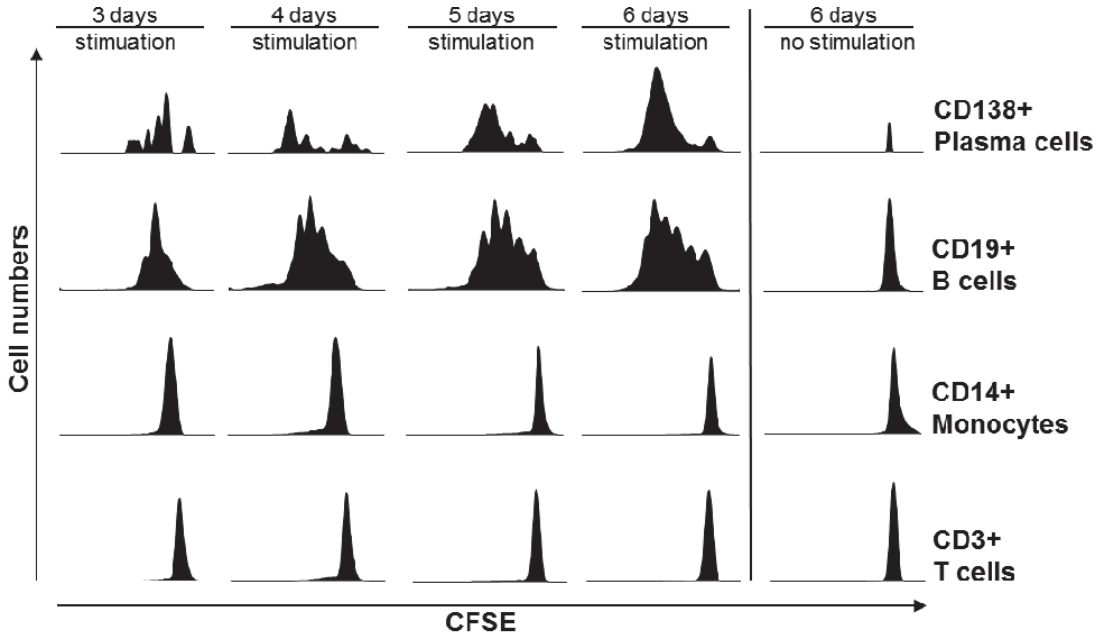

Figure 5.4: Selective proliferations of human memory B lymphocyte subsets in response to CD40L, CpG 2006, and IL-21 - CFSE-labeled PBMCs were cultured both with and without CD40L, CpG 2006 and IL-21 in a human B cell medium. After 3, 4, 5 and 6 days, cells were collected. The proliferation of total B cells, plasma cells, monocytes and T cells were determined.

Further dissecting the B cell-restricted differentiation induced by stimuli CD40L/CpG 2006/IL-21, we found that after six days of stimulation B cells could be divided into two distinct groups, $CD19^{high}$ and $CD19^{intermediate}$ cells. Most of

the $CD19^{high}$ B cells were negative for CD27, which is a marker for memory B cells. These $CD19^{high}$ B cells had proliferated less frequently and exhibited almost no differentiation into CD38+ or CD138+ plasma cells (Figure. 5.5). In contrast, the population of $CD19^{intermediate}$ B cells evidenced a strong expression of the memory B cell marker CD27 had undergone several cycles of division, and had developed into $CD38^{+}CD138^{+}$ terminally differentiated plasma cells. Overall, these combined data suggested that the amplification of the ISC signal caused by our mixture of stimuli was most likely based on the induction of an B cell-restricted proliferation and differentiation.

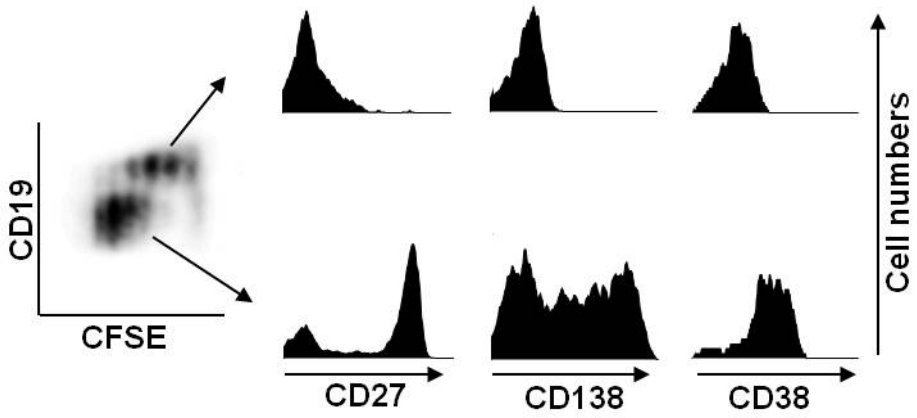

Figure 5.5: Selective differentiation of human memory B cells to plasma cells in response to CD40L, CpG 2006, and IL-21 - CFSE-labeled memory B cells were stimulated for six days with CD40L, CpG 2006 and IL-21. The expression of CD19, CD27, CD38 and CD138 were measured as a function of cell activation and differentiation. The cell division was evaluated in variant B cell subgroups.

5.1.3 IgG Antibody Responses Observed in an ELISPOT Assay Generated by TT-specific Memory B Cells but Depend on Help from Non-B Cells

To further confirm that the TT-specific IgG responses observed after the stimulation of whole PBMCs were indeed generated by mBCs, but not by other cell types present in the culture, we isolated B cells from the stimulating culture after six days of incubation. When we applied these cells to our standard ELISPOT assay, we observed enriched TT-specific ISCs with reduced

background signal against control protein GST (Figure 5.6). These results demonstrated that stimulated memory B cells were indeed the source of the TT-specific responses observed in our *in vitro* assays.

We also stimulated isolated B cells with CD40L, CpG 2006 and IL-21 for six days, and applied these stimulated B cells to our ELISPOT read-out assay. We observed that both numbers of detectable TT-specific B cells, as well as the total numbers of IgG-secreting cells, were much lower than those produced by stimulated whole PBMCs (Figure 5.6). These observations suggested that B cells present among total PBMCs needed the help of non-B cells in order to proliferate and/or fully differentiate into competent antibody-secreting cells upon unspecific stimulation. As expected, non-B cells themselves could not be transformed into antibody-secreting cells by stimulation (data not shown).

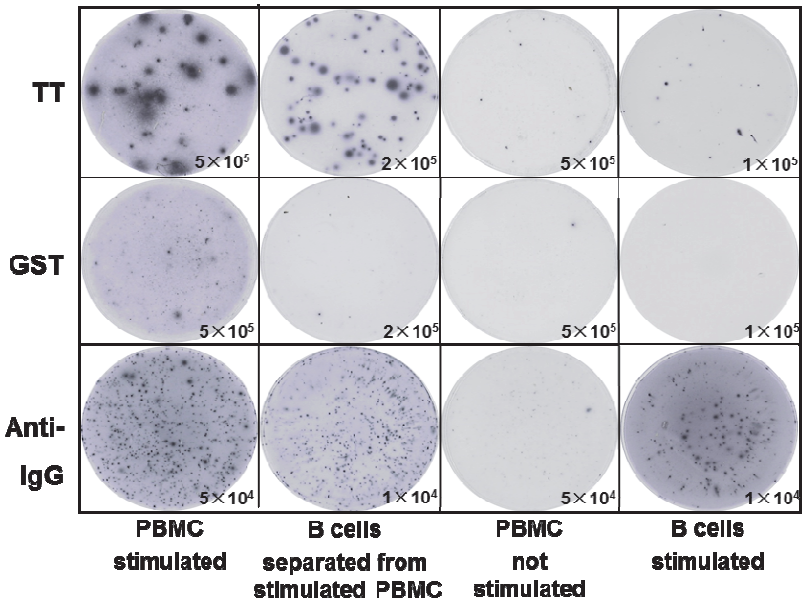

Figure 5.6: Antigen-specific responses determined by ELISPOT six days after polyclonal stimulation - For the analysis of TT-specific responses, PBMCs were cultured without stimulation, or were stimulated with CD40L/CpG 2006/IL-21. After six days, cells were collected and applied to an ELISPOT assay. ELISPOT plates were coated with TT and anti-human IgG antibody to enumerate antigen-specific ISCs and total IgG-secreting B cells, respectively, while GST protein was used as a negative control. In some experiments, B cells were isolated before or after polyclonal stimulation. Total B cells were also isolated before stimulation and cultured with CD40L, CpG, and IL-21 for six days and were followed by an ELISPOT assay.

5.1.4 Antibody-secreting B Cells with Specificities Other than TT Induced Using a CD40L/CpG/IL-21 Cocktail of Stimuli

Having established an optimized assay for the enumeration of antigen-specific mBCs, we next aimed at answering whether the same assay was also suitable for the analysis of B cell responses against Ag other than TT. Therefore, we applied our method to viral Ag such as CMV pp28 and influenza virus A nucleoprotein. Stimulating PBMCs from healthy donors with the aforementioned combination of CD40L, CpG 2006 and IL-21 for six days, we clearly detected an ISC specific for both antigens by ELISPOT (Figure 5.7). Corresponding IgG antibodies secreted during *in vitro* stimulation were observed in the ELISA (data not shown).

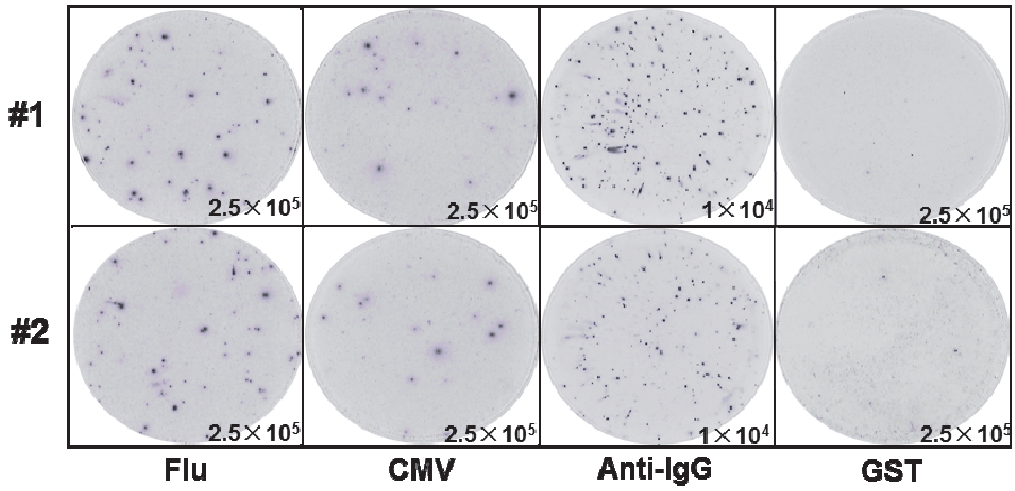

Figure 5.7: Influenza NP- and CMV-specific mBCs could also be identified by ELISPOT - Based on the analysis of TT-specific B cells, we further determined the number of influenza NP- and CMV-specific B cells among human PBMCs. Experiments were performed in duplicate wells (#1 and #2). The antigen-specific ISCs were shown as spots. Total ISCs were shown as positive control by coating with anti-human IgG antibodies. GST was used here as a negative control.

5.2 Analysis of Naturally Occurring Humoral Responses against MAGE-A3 and MAGE-C2

5.2.1 Autoantibodies against MAGE-A3, MAGE-C2 Arose Spontaneously in Patients with Myeloma

Firstly, we screened a large number of sera (N=1636) consecutively collected from patients of MM (N=333) and MGUS (N= 10), as well as sera from healthy blood donors (N=90), for antibody responses against MAGE-A3 and MAGE-C2. The antibody response against MAGE-C2 was screened by Miss Katrin Bartels. The antibody response against MAGE-A3 in samples before February 2008 was screened by Marina Ristic. The antibody response against MAGE-A3 in samples between January 2005 and June 2006, and samples between February 2008 and October 2010, was screened by Yanran Cao. The patients' characteristics are shown in Table 5.1. We found that four and 25 out of 333 patients (1.2% and 7.5%, respectively) evidenced anti-MAGE-A3 and anti-MAGE-C2 IgG antibodies at least on one occasion during the course of the disease. In contrast, none of the 90 analyzed healthy donors evidenced MAGE-A3- or MAGE-C2-specific IgG antibodies.

The specificity of the antibody against MAGE-C2 in sera from serologically positive MM patients was further analyzed by western blot assay, and the same recognition of recombinant human MAGE-C2 protein by the patient's plasma was verified (Figure 5.8 A). Next, we asked whether the patient-derived antibody against recombinant protein was also able to recognize the naturally produced antigen. We performed an immunoprecipitation (IP) assay with a lysate of MAGE-C2 expressing melanoma cell line SK-MEL-37 [98] and MAGE-C2-specific mouse mAb. We observed that the plasma from MM patients, as well as the mouse antibodies, could really recognize the blotted immunoprecipitated product (Figure 5.8 B). We further incubated the blotted lysates of MAGE-C2 expressing MM cell line EJM and OPM-2 with diluted seropositive patients' plasma before exposure to MAGE-C2-specific mouse mAbs. We observed an inhibition of binding between MAGE-C2 and the mouse mAb (Figure 5.8 C). This blocking effect confirmed that the antibodies in the

plasma of seropositive patients could definitely recognize and bind to the target antigen MAGE-C2.

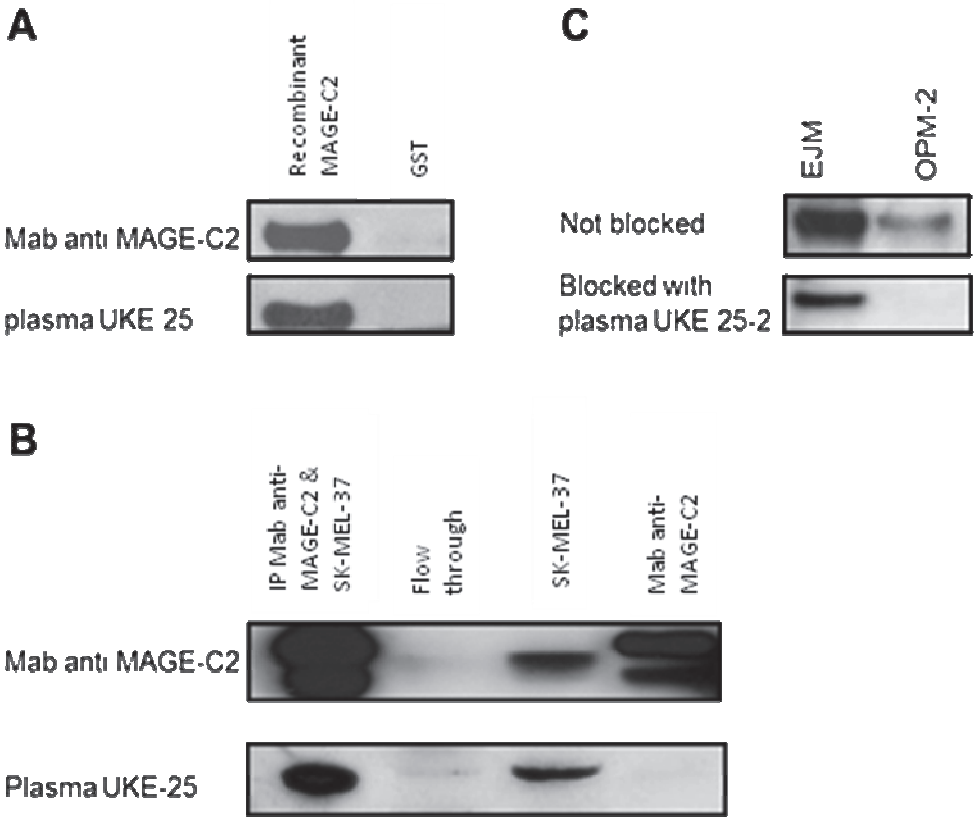

Figure 5.8: Analysis of the specificity of the antibody responses for MAGE-C2 by western blot - Antibodies from patients' plasma and mAb against MAGE-C2 bound to recombinant MAGE-C2 in Western blot assay. Recombinant protein GST was used here as a control (A). MAGE-C2 expressed by myeloma cell lines SK-MEL-37, as well as the respective immunoprecipitated product using anti-MAGE-C2 mAb, could be recognized by plasma from seropositive patients (B). The binding of anti-MAGE-C2 mAb to MAGE-C2 expressed in the myeloma cell line EJM and OPM-2 could be blocked or partially blocked by diluted plasma from serologically positive patients (C).

We further analyzed the sera of MM patients for the distribution of anti-MAGE-C2, MAGE-A3 immunoglobulin isotypes. We observed a mixed profile of MAGE-C2 antibodies. Variant isotypes were induced, with IgG1, 2, 3, 4 in three, four, three and one out of eight patients and IgM in one patient (Figure 5.9

upper). By contrast, the MAGE-A3-specific IgG mainly consisted of IgG2 and IgG3 in three and one out of four patients. None of the patients had IgM, IgG1, or IgG4 MAGE-A3-specific antibodies (Figure 5.9 middle). As a control, we evaluated Ig isotypes of antibodies targeting microbial antigen TT in myeloma patients. Differently from MAGE-specific humoral responses, we found that the TT-specific antibodies primarily consisted of IgG2 (8 out of 10 patients), IgG1 and IgG4 (7 from 10 respectively). TT-specific IgG3 was observed in just one patient, and no IgM antibody against TT was detected (Figure 5.9 lower).

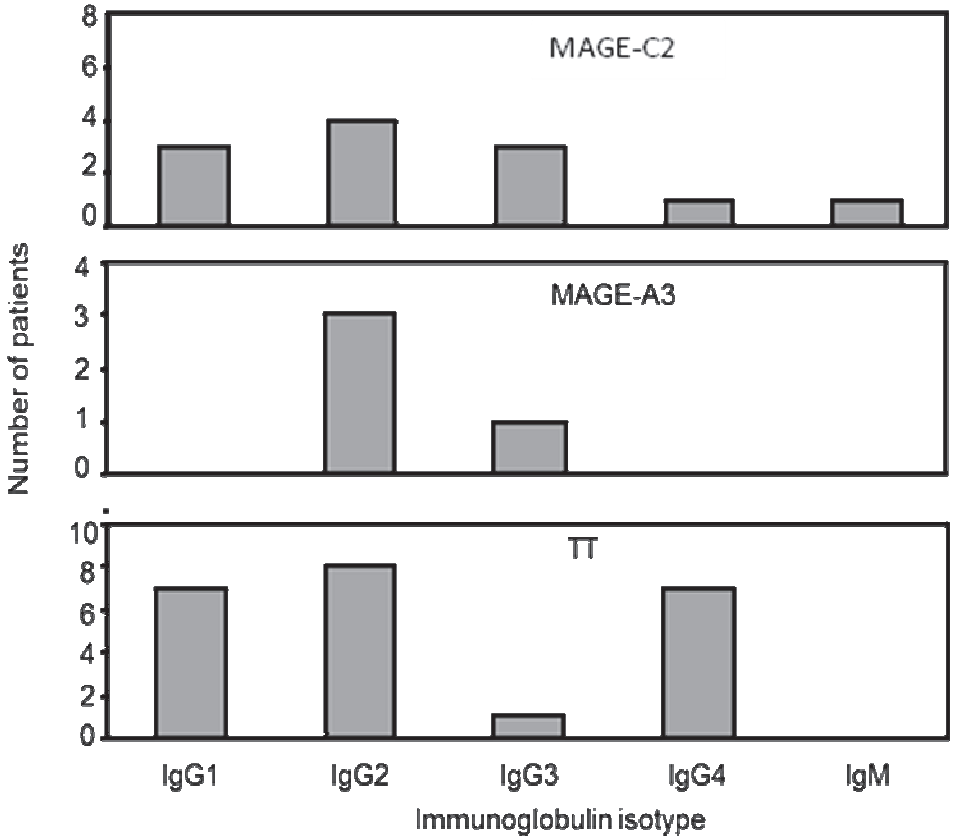

Figure 5.9: Distribution of immunoglobulin isotypes of antibodies against MAGE-C2, MAGE-A3 and TT - Immunoglobulin isotypes of MAGE-C2 (upper), MAGE-A3 (middle) and TT-specific antibodies (lower) in plasma from MM patients were identified by ELISA. Bars represent numbers of individuals who evidenced MAGE-C2-, MAGE-A3- or TT-specific IgG subtype or IgM, respectively.

Table 5.1: Clinicopathological characteristics of the patients

Data are shown for all patients and for the subgroup of MAGE-A3- or MAGE-C2-seropositive patients.

Parameter	Total	MAGE-A3 seropositive	MAGE-C2 seropositive
Sex		Total 4	Total 25
Male	217	1	14
Female	126	3	11
Age			
> 60	160	0	7
≤ 60	183	4	18
Karyotype			
normal	141	2	12
Complex	31	1	0
del13q14	59	0	6
del17p13	12	0	0
t(4;14)	9	1	1
Not tested	91	0	6
LC isotype			
Light lambda	113	2	10
Light kappa	187	2	14
HC isotype			
IgG	230	3	15
IgA	68	1	9
IgM	9	0	0
Maximum			
MGUS	10	0	0
Untreated	33	0	0
Chemotherapy	97	0	3
autoSCT	54	0	1
alloSCT	149	4	21
Stage			
I	57		3
II	63	1	4
III	177	3	17

Abbreviations: LC = light chain, HC = heavy chain

5.2.2 MAGE-A3-, MAGE-C2-specific Humoral Responses Preferentially Induced after Allogeneic Stem Cell Transplantation and Correlated with a Survival Benefit

Since we have revealed that MAGE-A3 and MAGE-C2 are naturally immunogenic in MM patients, we next proposed the question, under what circumstance these humoral responses could arise in MM. Firstly, we evaluated the relevance between a number of known clinicopathological attributes of these patients and the presence or absence of such serological responses. An obvious male predominance was noted among all 343 patients enrolled in our study, while no discrepancy of serological response was found between males and females. To the contrary, a pronounced deficiency was manifested in patients over the age of 60 years old compared with younger patients (P=0.01). The remaining clinicopathological parameters, such as the karyotype, the paraprotein isotype and the stage of the disease, lacked an association with the occurrence of a humoral response against MAGE-A3 and MAGE-C2 (Table 5.1).

Secondly, we pursued an investigation into the effect of a myeloma-specific therapeutic intervention. We divided all the patients into five groups with regard to the disease and maximum therapies they have received respectively: MGUS, MM untreated, MM post chemotherapy, MM post autologous SCT and MM post allo-SCT (Figure 5.10). A remarkable enhancement of serological response against MAGE-A3 and MAGE-C2 was evidenced in patients post allo-SCT compared with the patients who had just undergone conventional chemotherapy or auto-SCT maximally (P<0.01) (Figure 5.11). All four cases with antibodies against MAGE-C2 belong to the group post allo-SCT. In sharp contrast, this humoral response was completely absent in untreated patients with MGUS and MM (Figure 5.11).

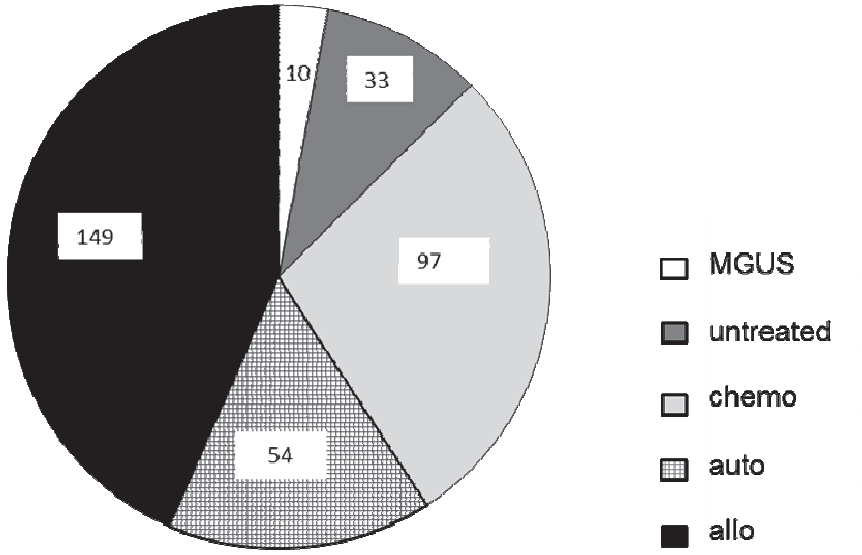

Figure 5.10: Study population - All 373 patients were divided into five groups according to the disease and maximal therapies they received: MM patients post allo-SCT (black), post ASCT (small grid), post chemotherapy (light grey), untreated MM patients (dark grey) and MGUS patients (white).

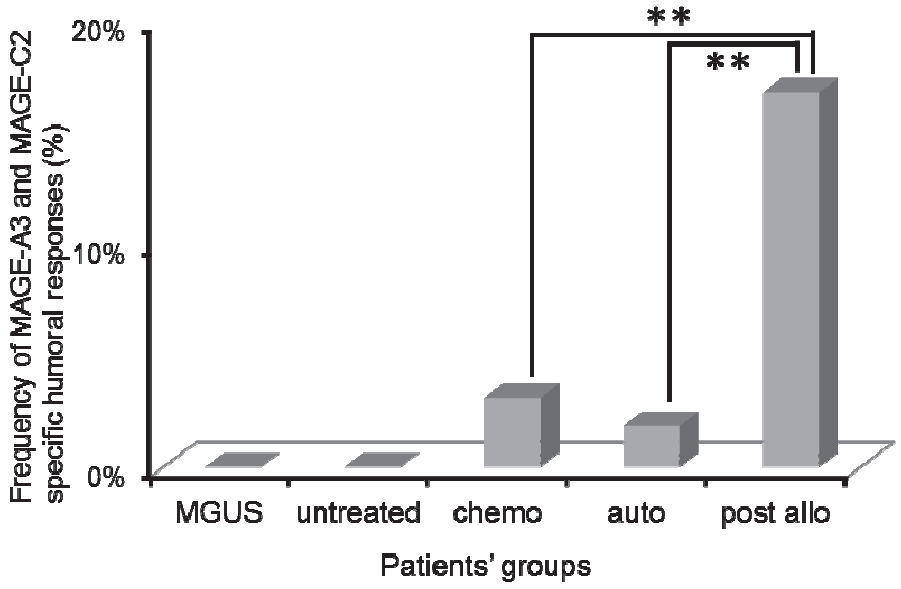

Figure 5.11: MAGE-A3 and MAGE-C2 positive patients in all groups - The MAGE-A3- or MAGE-C2-specific Ab response was detected in 17% of patients post allo-SCT (25 out of 149), 2% of patients post ASCT (1 out of 54) and 3% of patients post chemotherapy (3 out of 97), while the MAGE-A3- or MAGE-C2-specific Ab response was absent in untreated patients with MM and MGUS. (** indicates $p < 0.01$).

On the third step, we pursued the relevance to the prognosis parameter of the patients post allo-SCT. Surprisingly, a notably prolonged overall survival was observed in seropositive patients than in negative patients (P<0.05) (Figure 5.12).

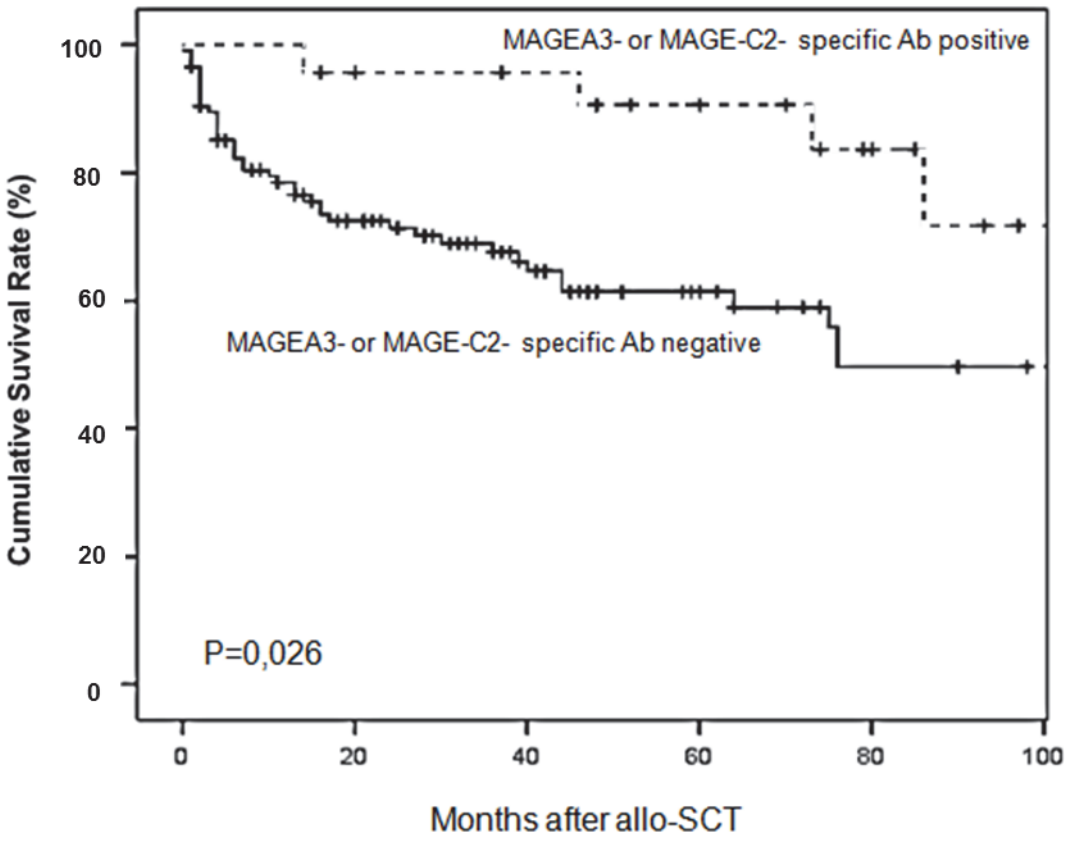

Figure 5.12: Survival of MM patients post allo-SCT with or without antibody responses - 52 MM Patients, who received allo-SCT with completed follow-up data available, were analyzed for overall survival. The patients were divided into two groups according to the existence of antibody response against MAGE-A3 and MAGE-C2 detected by ELISA. Curves represent Kaplan-Meier estimates of the percentages of patients evidenced in a disease-related death during the time of observation.

5.2.3 Presence of Autoimmune Antibodies ANAs Correlated with the MAGE-A3, MAGE-C2-specific Antibody Responses in MM Patients Post Allo-SCT

To further address the impact of allo-SCT on MAGE-A3, MAGE-C2-specific immunity, we measured the presence of the auto-reactive anti-nuclear antibodies (ANAs) and looked for the correlation with the MAGE-A3 or MAGE-C2- specific antibody responses in MM patients post allo-SCT. The ANAs level was measured in Professor Dr. Friedrich Haag's diagnostic lab with an indirect immunofluorescence assay of HEp-2 cells. We found seven out of 46 MM patients, who were negative for anti- MAGE-A3 or anti-MAGE-C2 response, evidenced ANAs positive. On the contrary, 14 out of 21 anti-MAGE-A3 or anti-MAGE-C2 seropositive patients evidenced ANAs positive. In the group of anti-MAGE-A3 or anti-MAGE-C2 positive patients, the ANAs were detected more frequently than in the seronegative patients ($p<0.05$).

We further evaluated the patients' disease states during the period when the anti-MAGE-A3 or anti-MAGE-C2 antibody was initially developed (Table 5. 2). We identified 14 post allo-SCT patients who used to be detected as serologically negative after the transplantation. They became serologically positive two months to six years after allo-SCT (median 18 months). Nine of these patients had received a donor lymphocyte infusion at least once. At the time point when they were initially identified seropositive, two patients were in disease relapse or progress, six patients showed a partial remission and six patients evidenced a complete BM cytological remission and Immunofixation (IF) negative, including four who were detected with an abnormally elevated serum FLC (Table 5.2).

We next asked whether the occurrence of MAGE-specific antibodies might be influenced by an elevated paraprotein level or general humoral response. We surveyed the plasma level of paraprotein, pathologic light chain, total IgG and IgG anti-bodies against microbial antigen TT and NP both before and after the induction of the MAGE-specific antibodies. The represented results are shown in Figure 5.13 and 5.14. We observed that there was neither an enhanced total

IgG level nor an increase of paraprotein or pathological light chain, during the arising of MAGE antibodies in the 14 patients. The immunization directed against microbial antigens post allo-SCT had no impact on the development of MAGE antibodies, either (Figure 5.13, Figure 5.14).

Table 5.2: The individual clinical characteristics of patients when the first antibody response was detected

	subtype	Progress /relapse	PR	CR	IF	MRD mPC	LK	mPC	DLI	post-allo (months)
UKE-96	λ	+			+	NA	-	-	+	2
UKE-975	λ	+			NA	NA	NA	NA	NA	6
UKE-43	λ		+		+	NA	+	+	+	72
UKE-245	κ		+		+	NA	+	+	+	6
UKE-300	κ		+		+	+	+	+	+	5
UKE-16	IgA κ			+	-	-	+	+	+	36
UKE-53	κ			+	-	NA	+	+	+	24
UKE-54	κ			+	+	+	+	+	+	72
UKE-64	κ			+	-	-	-	-	+	36
UKE-65	κ			+	+	NA	+	+	-	30
UKE-139	κ			+	-	-	+	+	+	18
UKE-164	κ			+	+	+/-	+	+	+	8
UKE-396	λ			+	-	NA	+	+	NA	2
UKE-945	λ			+	-	-	-	-	-	12

Abbreviations:

PR: BM cytological partial remission

CR: BM cytological complete remission

IF: Immunofixation

MRD: Minimal residual disease

mPC: malignant plasma cells/ myeloma plasma cells detected by flow cytometry assay[170]

FLC: abnormal serum free light chain level

DLI: donor lymphocyte infusion

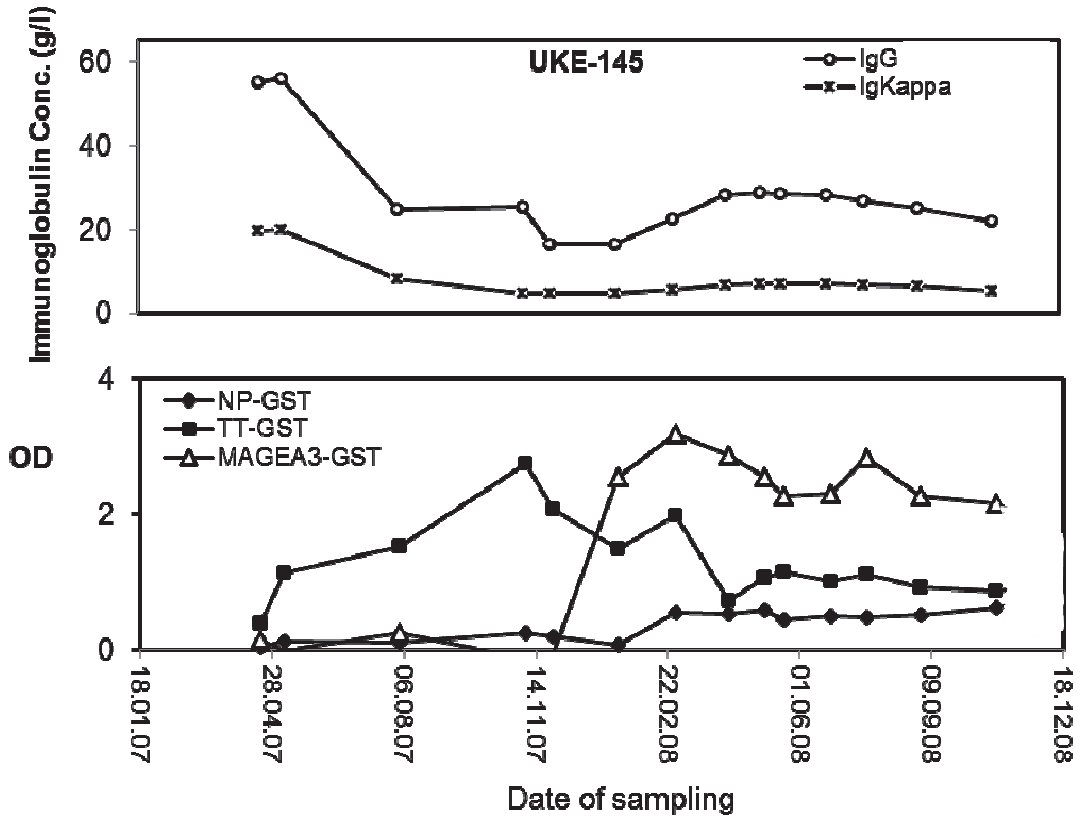

Figure 5.13: Pathogenic and general immune globulin level during the induction of MAGE-A3-specific Ab - Post allo-SCT, the level of paraprotein, as well as total IgG and Ab against TT and NP were evaluated in patients with MAGE-A3 Ab during the courses of their diseases. In representing seropositive patients UKE-145, the total IgG and pathogenic light chain level were shown as IgG concentration (upper). Ab against TT, NP and MAGE-A3 were shown as OD value by standard ELISA assay (lower).

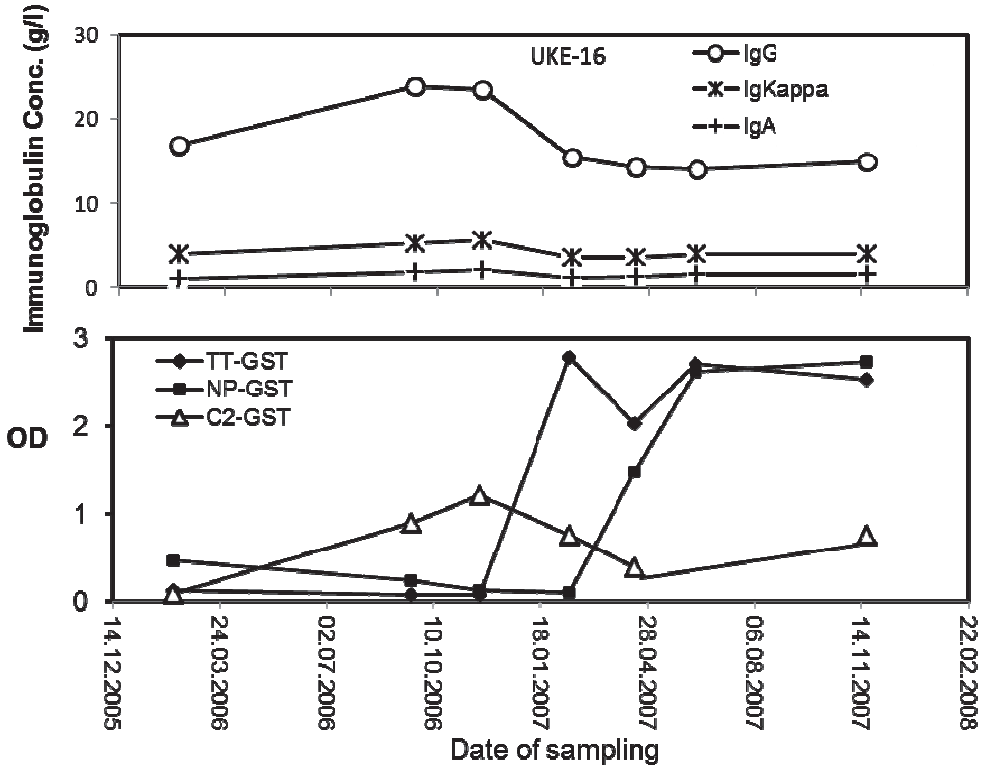

Figure 5.14: Pathogenic and general immune globulin level during the induction of MAGE-C2-specific Ab - Post allo-SCT, the level of paraprotein, as well as total IgG Ab against TT and NP, were evaluated in patients with MAGE-C2 Ab, during the course of their disease. In representing seropositive patients UKE-16, the total IgG and pathogenic light chain level were shown as IgG concentration (upper). Ab against TT, NP, and MAGE-A3 were shown as OD value by standard ELISA assay (lower).

5.2.4 Two Major Areas throughout Full Sequence of MAGE-C2 Frequently Recognized by MM Patients

At the last step, we aimed at defining the fine profile of MAGE-C2-specific antibodies from MM patients. To help identify the linear epitopes recognized by the MAGE-C2-specific antibodies, we designed a series of 20mer MAGE-C2 peptides. These peptides overlapped each other by 10 amino acids, covering the complete sequence of the antigen (373 amino acids long). Using these peptides, we were able to define certain regions of MAGE-C2, which were preferentially targeted by the antibodies in 13 seropositive MM patients.

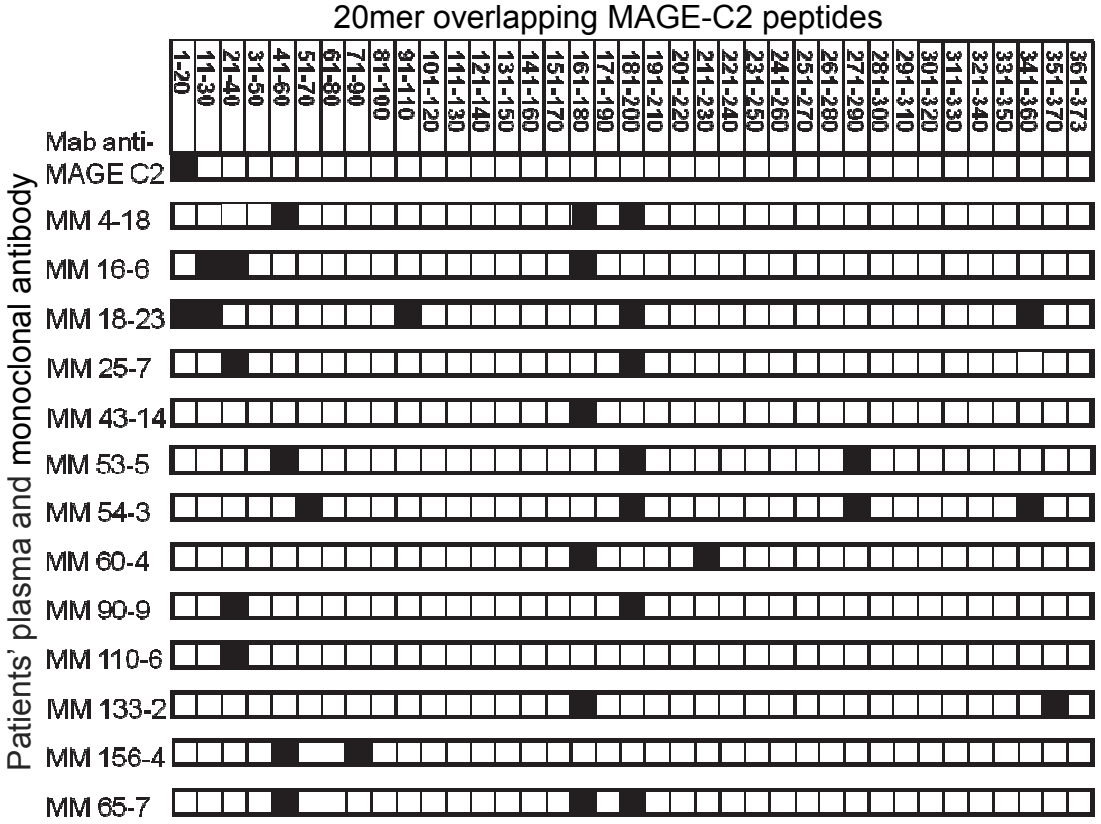

Figure 5.15: MAGE-C2 epitopes recognized by plasma of MM patients - The fine specificity of the antibody responses was examined by ELISA using overlapping 20mer peptides spanning the whole sequence of MAGE-C2. The represented linear epitopes targeted by B cells from seropositive MM patients were indicated as black blocks. The linear epitope recognized by the mouse monoclonal antibody against MAGE-C2 was shown on the top.

The localization of the reactive peptides in the MAGE-C2 sequence for each patient is shown in Figure 5.15, in which the black block indicates the peptide recognized by a patient-derived antibody. Firstly, a MAGE-C2-specific mAb targeted linear epitope was identified in the area of AA 1-20 in the N-terminal of MAGE-C2 protein. The most interesting regions targeted by patients' plasma were located on the N-terminal and in the middle of the MAGE-C2 protein. The linear sequences of AA 160-180 and 180-190 in the middle of the MAGE-C2 full-length protein were most commonly recognized by six and seven patients. And the linear sequence of AA 20-40 and 40-60 in the N-terminal was recognized by four and five patients. Additionally, the epitopes located on AA

10-30, 270-290 and 340-360 were recognized respectively by plasma from two patients, (Figure 5.15).

5.2.5 MAGE-A3-, MAGE-C2-specific B Cells Rarely Observed in Peripheral Blood of MM Patients

Using an improved memory B cell assay, we enumerated antigen-specific immune globulin-secreting B cells in the PB of MM patients.

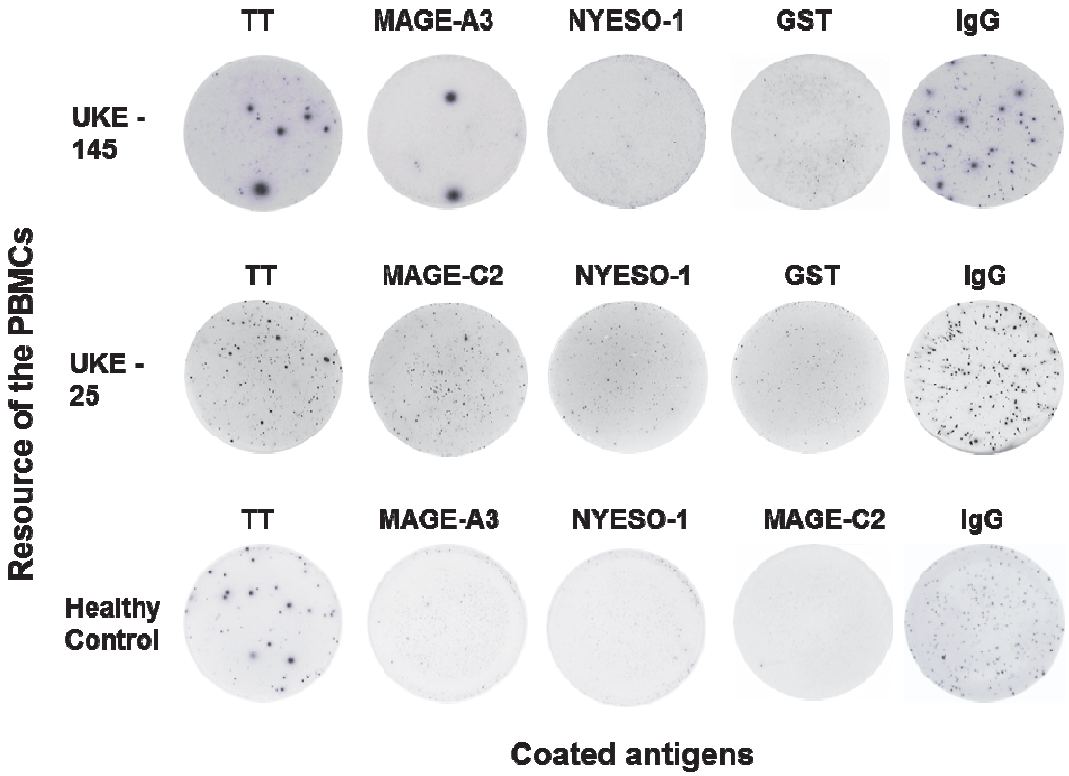

Figure 5.16: MAGE-C2-specific memory B cells in peripheral blood - The Ag-specific memory B cells in PB were evaluated by a B cell ELISPOT Assay. Spots indicate the total numbers of immunoglobulin secreting cells (ISCs) as well as Ag-specific B cells. ELISPOT result of representing MAGE-C2 seropositive patients UKE-25 was shown (middle). TT, MAGE-A3, NY-ESO-1, GST and IgG were used here as controls. A PBMC sample from UKE-145 was used as a positive control for MAGE-A3-specific ISC (upper). A PBMC from a normal donor was used here as a negative control for MAGE-A3 and MAGE-C2 (lower). TT-specific ISC and total IgG were used as positive controls. NY-ESO-1 and GST were used here as negative controls.

The PBMCs of vaccinated patients, serologically positive MM patients and normal donors were firstly stimulated with a combination of CD40L, CpG and IL21 for six days, and then applied for the quantitative analysis of MAGE-A3-specific B cells by ELISPOT and ELISA assay. Using our ELISPOT-based technique, we detected TT-specific B cells and total ISCs equally in both MM patients and normal individuals. In the PB of MAGE-A3 serologically positive patients, we observed a low level of antigen-specific ISCs (0-8/1×10^6 MNCs), while the MAGE-C2-specific ISC was almost not detectable in the PB from corresponding seropositive patients (Figure 5.16).

5.3 Comparison of Spontaneous and Vaccine-induced Humoral Responses against MAGE-A3

5.3.1 Anti-MAGE-3 Antibodies Efficiently Induced by MAGE-3 Protein in Combination with Adjuvant AS02B

Firstly, sera from NSLC patient vac-1, vac-2, vac-3, and vac-4 before and after a cycle of vaccination were screened by ELISA for antibody responses against recombinant human MAGE-A3 protein. Unfortunately, pre-vaccination sera from patient vac-3 and vac-4 were not available and could not be analyzed in this study. Using stringent criteria for the definition of seropositivity, we observed significant IgG antibodies responses against MAGE-3 protein in all four patients who had been vaccinated with MAGE-3 protein in combination with adjuvant AS02B (Figure 5.17). Strong humoral responses were detected in three weeks after the patients had received the vaccine for the second time. The serum levels were kept until the end of the observation period after the last vaccine injections.

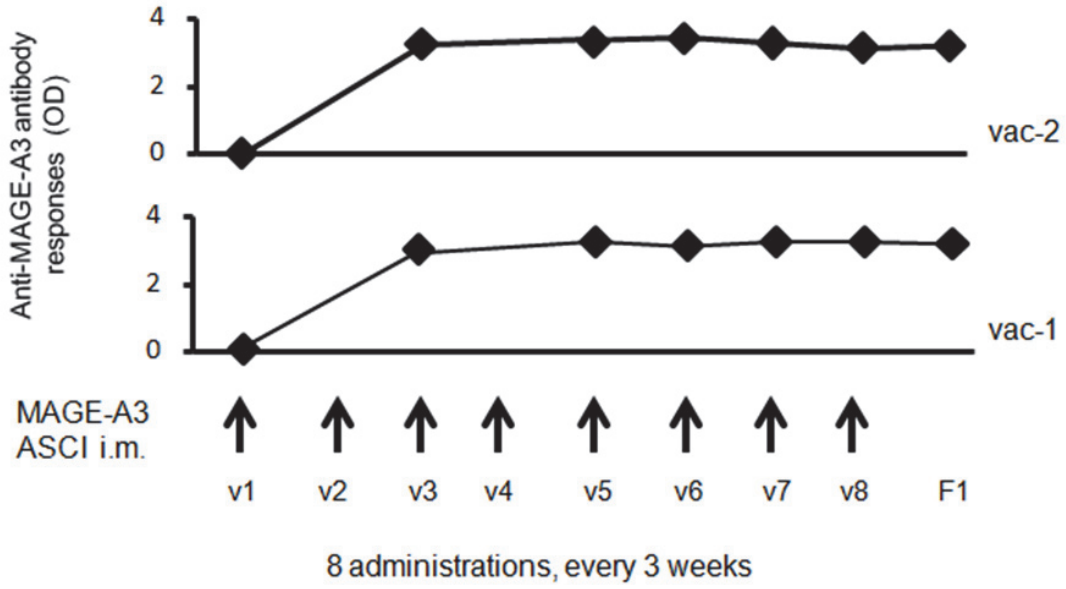

Figure 5.17: MAGE-A3-specific IgG antibodies induced by vaccination with full-length recombinant MAGE-A3 protein (ELISA) - Sera from all vaccinated NSLC patients vac-1, vac-2, vac-3, and vac-4 were screened for MAGE-A3- and protein D-specific antibodies by ELISA both before and after a cycle of vaccine administration. To help facilitate a comparison between patients, all sample OD values at a 1:100 serum dilution were normalized according to positive and negative controls. MAGE-A3-specific antibodies from representative NSCLC patient vac-1 and vac-2, who have received MAGE-A3 vaccine, were shown for each time point from before the study (pre) to every three weeks postvaccination. The black arrow indicates each administration of MAGE-A3 vaccine (v1-v8; F1 indicates the first follow up five weeks after v8).

Western Blot Analysis

Next, the specificity of antibodies against MAGE-A3 in sera from patients vaccinated with MAGE-A3, in addition to from serologically positive MM patients, was further analyzed by western blot. We confirmed that the MAGE-A3-specific IgG antibodies presented in the PB of both groups of patients, as well as the monoclonal MAGE-A3-specific antibody (M3H67), could bind to the MAGE-A3 protein at 42 kDa, but not to the recombinant protein, NY-ESO-1 protein. We further proved the antibodies from both groups could also bind to natural

MAGE-A3 protein in lysates of MAGE-A3-expressing myeloma cell line OPM-2, RPMI-8226, KMS-12-BM and U-266, and melanoma cell line SK-MEL-37 at 42 kDa (Figure 5.18A and B). In contrast, both anti-MAGE-A3 antibodies did not bind to the lysate of the kidney cell line from the African green monkey cos-7, which does not express MAGE-A3 at 42 kDa. The antibody derived from the patients' sera was also shown to bind to the immunoprecipitated product of MAGE-A3-expressing lung cancer cell line (Figure 5.18 C).

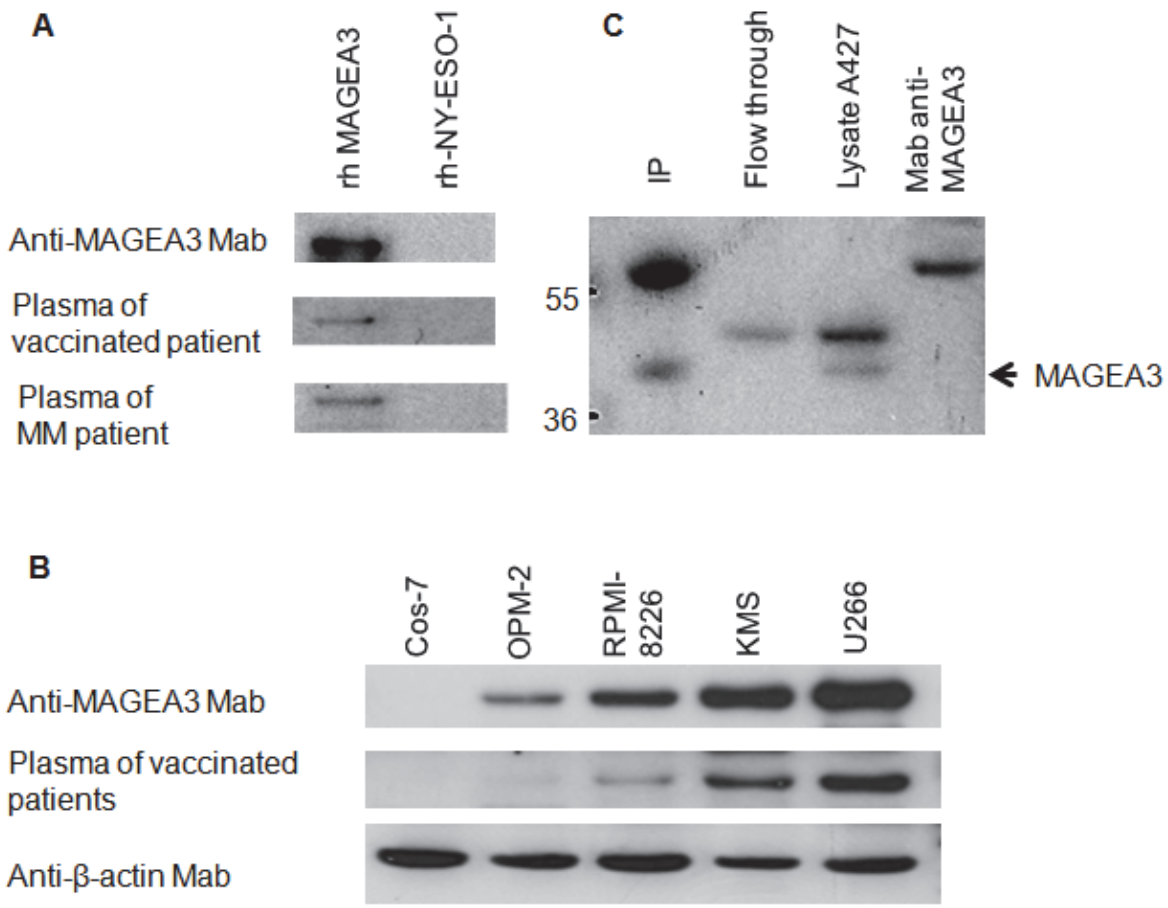

Figure 5.18: Analysis of the specificity of the antibody responses for MAGE-A3 with Western blot- Antibodies from patients' plasma-bound recombinant MAGE-A3 (A), MAGE-A3 expressed in myeloma cell lines (B), as well as immunoprecipitated (IP) MAGE-A3 from melanoma cell line SK-MEL-37 (C). Cell lysate of COS-7 was used as a negative control. Anti-actin beta (ACTB) antibody was used as a positive control. Results of the representative experiment are shown.

5.3.2 Antibody Response against 17 Tumor Antigens in MAGE-A3-vaccinated Patients

We analyzed antibody responses against MAGE-A3, MAGE-A3-related antigen MAGE-A1, MAGE-A2, MAGE-A8, MAGE-A9, MAGE-B1, MAGE-A11, MAGE-A12, CT10/MAGE-C2, MAGE-H1, other CTA NY-ESO-1,SSX2 and SSX4, SOX2, PRAME, and other tumor antigens CEA, p53 in all NSLC patients vac-1, vac-2, vac-3, and vac-4 both before and after a cycle of MAGE-A3 vaccine treatment (Figure 5.19) (Pre-vaccination sera from patient vac-3 and vac-4 were not available and could not be analyzed in this study.).

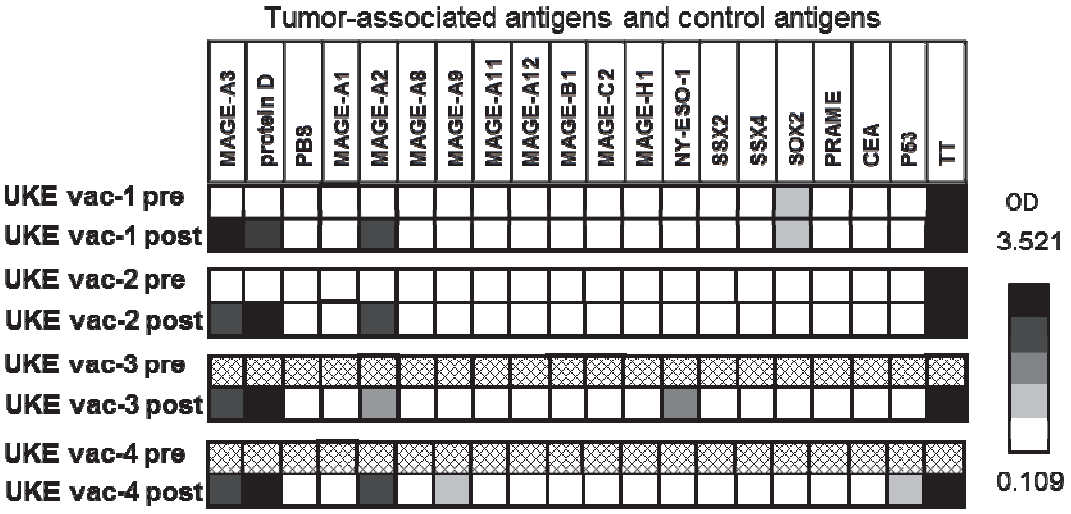

Figure 5.19: A measurement for humoral responses against 17 tumor-associated antigens in NSCLC patients before and after MAGE-A3 vaccine administration - Plasma samples from eight NSCLC patients before (pre) and after (post) a cycle of treatment were tested by ELISA for the antibodies against MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A8, MAGE-A9, MAGE-B1, MAGE-A11, MAGE-A12, CT10/MAGE-C2, MAGE-H1, NY-ESO-1, SSX2 and SSX4, SOX2, PRAME, p53 and CEA. Pre-vaccination sera from patient vac-3 and vac-4 were not available and could not be analyzed in this study (shown with small grid). Patient vac-1, vac-2, vac -3, and vac-4 were treated with MAGE-A3/influenza protein D fusion protein. Antibodies against protein D and TT were tested as relevant and irrelevant controls. The darkness of the blocks represented the intensity of the response detected by the ELISA assay as the OD value.

Before vaccination, antibody against SOX2 was detected in patient vac-1, whereas no antibody response against MAGE-A3 and/or other CTAs was observed. Additionally, antibody response against p53 was observed in patient vac-4. After vaccination, the antibody responses against MAGE-A3 and MAGE-A2 were induced in all patients. Antibody against SOX2 in patient vac-1, as well as response against p53 in patient vac-4 was not changed. A new response against SOX2 and p53 was not induced in the rest of the patients. Humoral responses against other CTAs antigens and CEA were not induced. Furthermore, no variation of antibody response against microbial antigen TT was observed after MAGE-A3 vaccination.

5.3.3 MAGE-A3-specific Antibodies Undergo Antigen–driven Affinity Maturation during the Course of Vaccination

Once challenged with an antigen, these antigen-specific B cells will undergo somatic hypermutations, thereby enhancing the antibody's affinity for its specific target [99][100]. The most specific B cells will receive the strongest survival signal, whereas less specific B cells will vanish. A repeated antigen challenge will trigger Ig affinity through somatic hypermutations [100]. In this study, the MAGE-A3-specific antibodies consisted of a mixture of antibodies targeting various epitopes. Here, the affinity of the antibodies was presented as the avidity of the antisera. Using an competitive ELISA assay, we tested the avidity of MAGE-A3-specific antibodies of patients [101]. As we know, the avidity and concentration of the inhomogeneous Abs in the patients' plasma were varied at different time points. To help make the half maximal inhibitory concentration (IC_{50}) value comparable, we first calibrated the concentration of the antibodies with a serial dilution of the plasma.

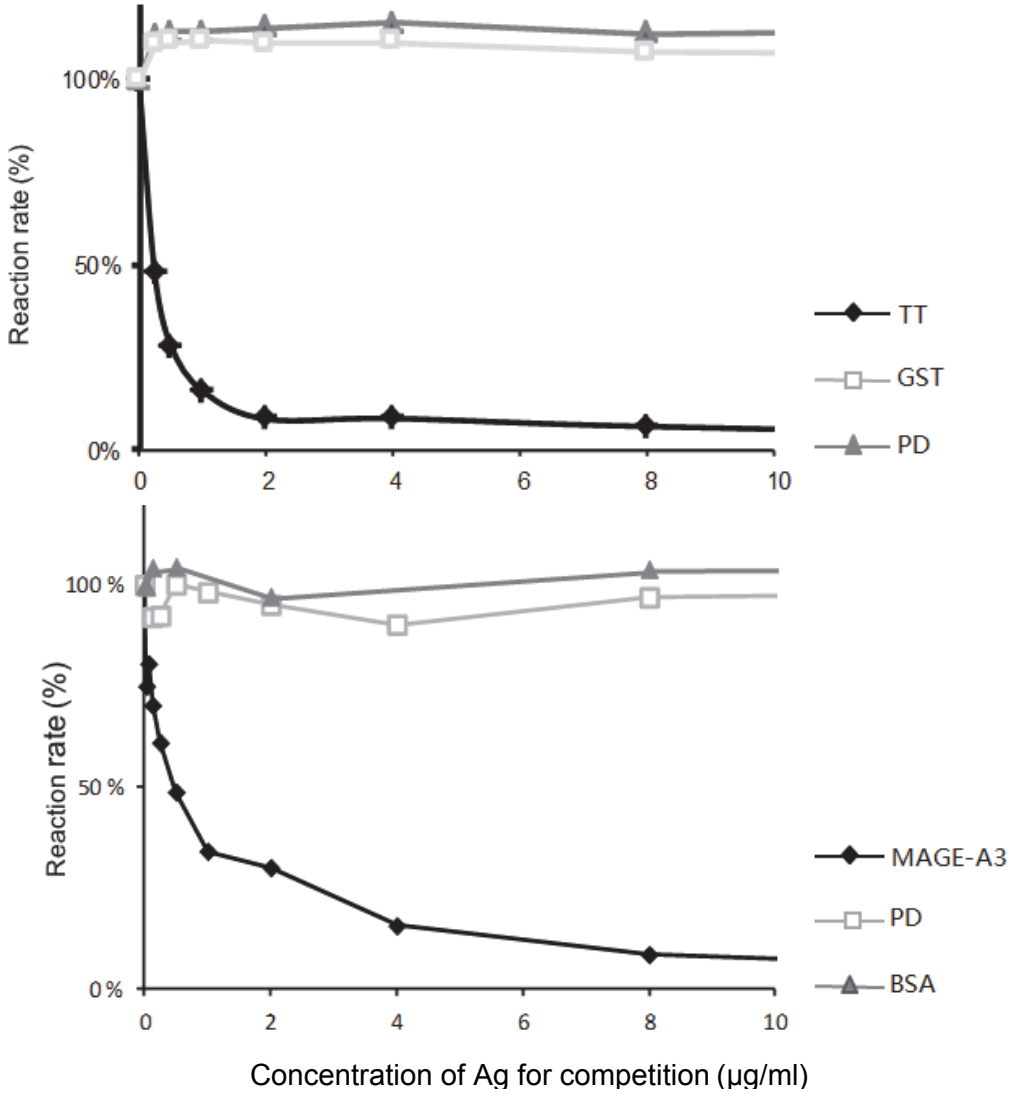

Figure 5.20: Competition ELISA assay at a plasma dilution of 1:100 - The plates for the completion of ELISA were coated with TT (upper) or recombinant protein MAGE-A3 (lower).The competition was performed by incubating the diluted plasma samples with antigens at serial increasing dilutions before being added to the coated ELISA plates. The inhibition of the combination between coated antigen (TT or MAGE-A3) and the antibodies in the plasma was increased with the elevated concentration of the respective competition antigen. In contrast, the irrelevant protein GST and protein D (PD) (upper), or PD and BSA (lower), could not inhibit the reaction. The IC_{50} value was determined as the concentration of the competitive antigen, which inhibited 50% of the reaction rate in the ELISA assay. In the figure, GST, PD, MAGE-A3,BSA are in µg/ml, while TT is presented in multiples of coating concentration.

At certain dilutions of the plasma sample, a series of competitive ELISA calibration curves were performed with increasing concentrations of antigen for pre-incubation, namely competition (Figure 5.20). In each single curve, the higher ELISA signal was observed at lower concentrations of corresponding competitive antigen. This means the reaction rate in ELISA is inversely proportional to the concentration of corresponding competitive antigen. The IC_{50} could be considered a measure (inverse) of the avidity of the antibodies for a given antigen, and was determined at each dilution of the plasma sample. No reaction inhibition was observed when an irrelevant protein was used for competition.

The IC_{50} value of plasma was then plotted against corresponding OD values at each dilution, and a calibration curve was made for the plasma of each time point (Figure 5.21A). The avidities of antibodies from variant time points were compared at a certain defined OD value. To obtain a certain OD value in ELISA, a fixed amount of coated antigens must be recognized and bound by corresponding antibodies. The lower that the avidity of the Abs is, the more that free Abs is required, and even more Ags are expected to be consumed to achieve a 50% reduction of the reaction in ELISA. In other words, as an inverse indicator of the antibody avidity, the IC_{50}, could be compared at a certain OD value.

With the extension of the duration post vaccination, the IC_{50} curve of MAGE-A3 vaccinated patient returns to baseline (Figure 5.21B and C), which indicated less and less amount of MAGE-A3 protein were needed to half reduce the equal signal in ELISA. Therefore a trend for an increasing affinity for MAGE-A3 over the total cycle of dosage was shown. In Figure 5.21C, the reduction of $IC_{50,}$ indicating an increasing affinity, demonstrated the similar tendency of an antigen–driven affinity maturation of the MAGE-A3 antibodies, as is usually seen in a vaccination setting [102]. The same change of antibody affinity over time was not seen in the serologically positive MM patients, which might have completed the antigen–driven affinity maturation before the earliest positive sample was available.

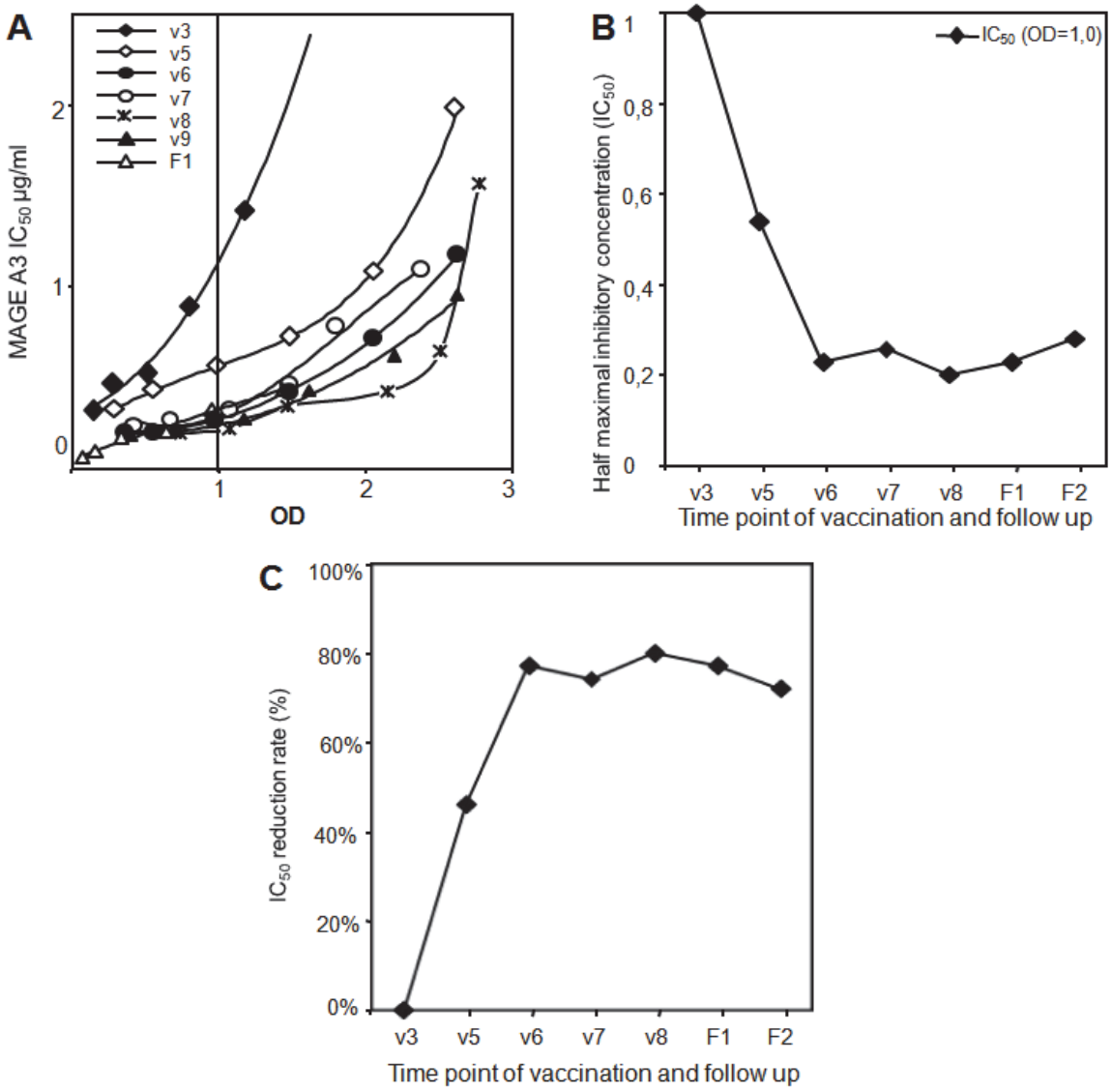

Figure 5.21: MAGE-A3-specific antibodies underwent affinity maturation during the cycle of vaccine - The relative avidity of MAGE-A3-specific antibodies of representative patient vac-2 was indicated by the half maximal inhibitory concentration (IC_{50}) based on a competitive ELISA assay. v3, v5, v6, v7, and v8 represent the time point of 6, 12, 15, 18, 21, 24 weeks after the first dosage of vaccine; F1 and F2 represent the time point of five and ten weeks after the last dosage of vaccine, respectively. Against the corresponding OD value (at a certain dilution of the plasma), the IC_{50} value was then plotted for plasma from all time points (A). At the dilutions that produced equal signals in ELISA (such as OD=1.0), the IC_{50} of the sample from all the time points was compared with the fist antibody response IC_{50} (v3) (B). The reduction rate of IC_{50} was calculated as (IC_{50} (v3)-IC_{50} (actual))/ IC_{50} (v3) × 100% and was shown in figure(C).

5.3.4 MAGE-A3 Vaccine-induced Humoral Responses with High-titered Antibodies and Frequent Corresponding B cells in Peripheral Blood

We next compared the mean titer of MAGE-A3-specific antibodies and the frequency of memory B cells in the PB of vaccinated patients and MM patients' spontaneous responses. We demonstrated that the average reciprocal titer of MAGE-A3-specific antibodies induced by vaccine was much higher than that of the spontaneous response ($p<0.05$) (Figure 5.22A).

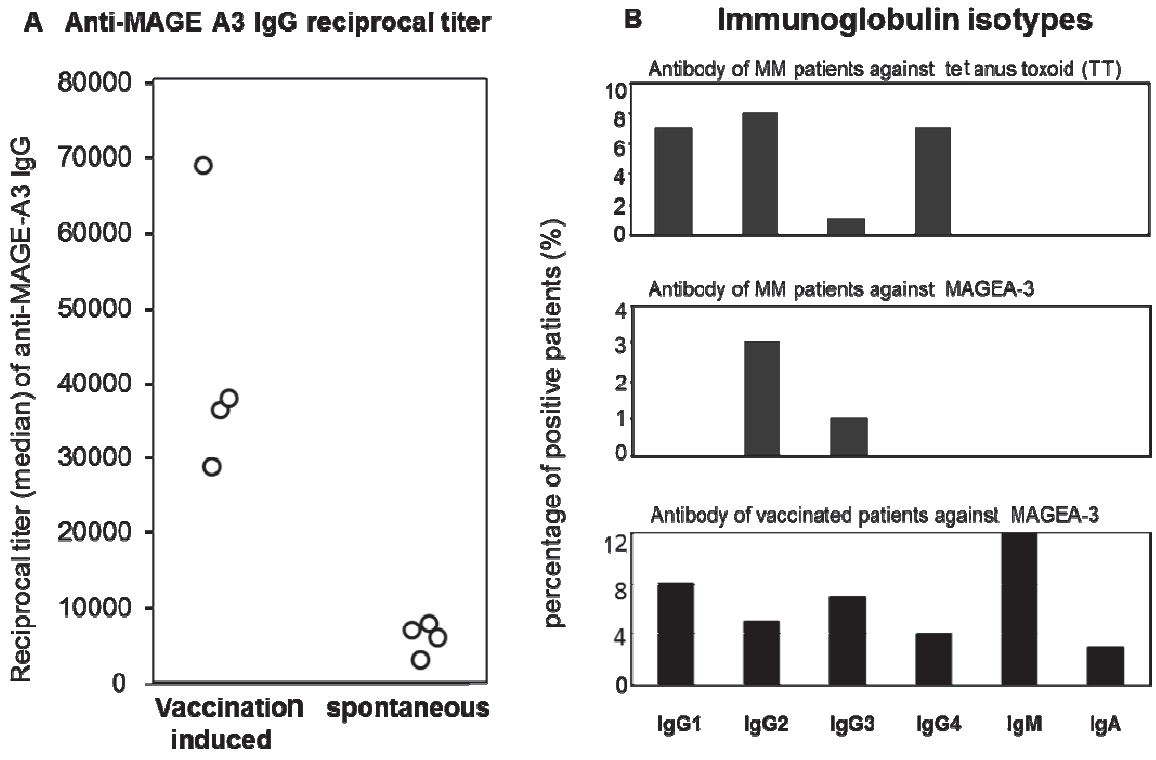

Figure 5.22: MAGE-A3 recombinant protein induced antibodies with much higher titer and more distribution of immunoglobulin isotypes - The averaged reciprocal titer of the anti-MAGE-A3 antibodies was calculated and compared between MAGE-A3 vaccinated patients and non-vaccinated seropositive patients (A); Comparison of immunoglobulin isotypes of MAGE-A3-specific antibodies induced by recombinant MAGE-A3 protein and spontaneous; Immunoglobulin isotypes of TT-specific antibodies in plasma was assessed in 10 MM patients (B).

We further compared the sera of the patients with spontaneous and vaccine-induced MAGE-A3-specific antibodies for the distribution of anti-MAGE-A3 immunoglobulin isotypes. For this test, we introduced plasma from eight additional patients who experienced MAGE-A3 vaccine treatment. We found that antibodies of variant isotypes were induced, with IgG and IgM in all 12 patients and IgA in three out of 12 patients. The MAGE-A3-specific IgG mainly consisted of IgG1 and IgG3 in eight and seven out of 12 patients, and also IgG2 in five and IgG4 in four patients, respectively (Figure 5.22B lower). By contrast, the spontaneous humoral responses primarily consisted of IgG2 antibodies in three out of four patients. None of the four MM patients has shown IgA, IgM, IgG1 or IgG4 MAGE-A3-specific antibodies. However, one patient evidenced an anti-MAGE-A3 response of the IgG3 subtype (Figure 5.22B middle). These results suggested that the antibody response against MAGE-A3 in MM patients might mainly be IgG1-driven or IgG3-driven. As a control, we evaluated Ig isotypes of antibodies targeting microbial antigen TT in myeloma patients. Differently from the MAGE-A3-specific humoral responses, we found that the TT-specific antibodies mainly consisted of IgG2 (eight out of 10 patients), IgG1 and IgG4 (seven out of 10, respectively). TT-specific IgG3 was observed in just one patient. Moreover, no IgM and IgA antibodies against TT were detectable (Figure 5.22B upper).

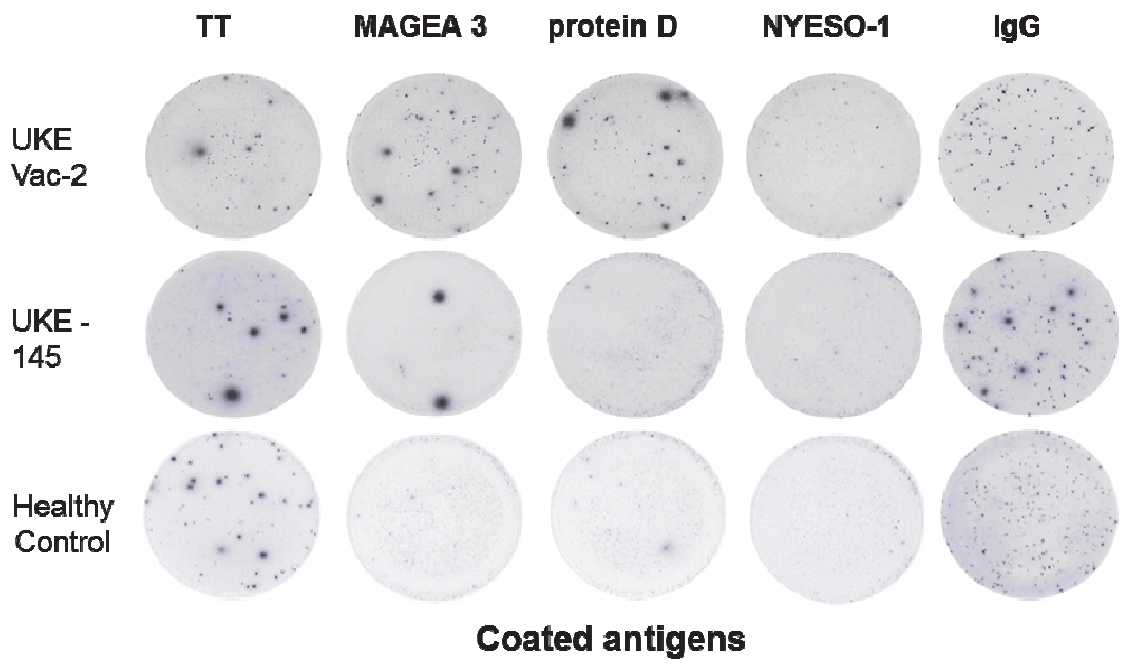

Figure 5.23: Comparison of MAGE-A3-specific memory B cells in peripheral blood - The MAGE-A3-specific memory B cells in PB were evaluated by a B cell ELISPOT Assay. The spots indicate the total number of ISCs, as well as the number of tetanus toxoid (TT)-, MAGE-A3-, protein D-, NY-ESO-1- and GST-specific B cells. High frequencies of vaccine-induced, MAGE-A3-specific memory B cells were evidenced in the PB of NSCLC patients postvaccination, while they remained barely detected in most seropositive myeloma patients.

Next, we enumerated antigen-specific immune globulin-secreting B cells using the recently improved memory B cell assay. PBMCs, with 5% B cells from vaccinated patients, serologically positive MM patients and normal donors, were first stimulated with a combination of CD40L, CpG and IL21 for six days, and then applied for the quantitative analysis of MAGE-A3-specific B cells by ELISPOT and ELISA assay. Using the ELISPOT-based technique, we detected significant numbers of MAGE-A3-specific IgG antigen-specific ISC spots, as well as protein D-specific IgG ISC, in PBMCs of vaccinated NSCLC patients. While in the PBMCs of spontaneously seropositive MM patients, both MAGE-A3-specific IgG ISC and protein D-specific IgG ISC spots were hardly observed. Furthermore, the MAGE-A3-specific B cell was totally undetectable in the

PBMCs of normal individuals. In contrast, the total IgG antibody secreting cells were equally detected in the PBMCs of these three groups, as well as in the TT-specific B cell in the serologic plasma of all the groups (Figure 5.23).

5.3.5 Peptide ELISA

In a last step, we aimed at defining linear epitopes recognized by the MAGE-A3-specific antibodies from two groups of patients. We designed a series of 20mer MAGE-A3 peptides overlapping each other by 10 amino acids and covering the complete sequence of the antigen (313 amino acids long). Using these peptides, we were able to define certain regions of MAGE-A3 preferentially targeted by the antibodies. In this assay, values were considered positive when they were at least twice as higher than those obtained in control wells. The localization of the reactive peptides in the MAGE-A3 sequence recognized by each sample is shown as black blocks (Figure 5.24). Firstly, a MAGE-A3-specific mAb targeted linear epitope was identified on a sequence of AA 261-280 on MAGE-A3 protein (Figure 5.24 top). Sera from all serologically positive patients recognized at least one peptide-based linear sequence. Throughout the full length of the MAGE-A3 protein, the linear epitopes targeted by antibodies in plasma from all 12 postvaccination patients mainly focused on the N terminal and the region of amino acid 211 to 230. In particular, the regions located on amino acid 51 to 80 and 221 to 230 were recognized by 11 and all 12 vaccinated patients.

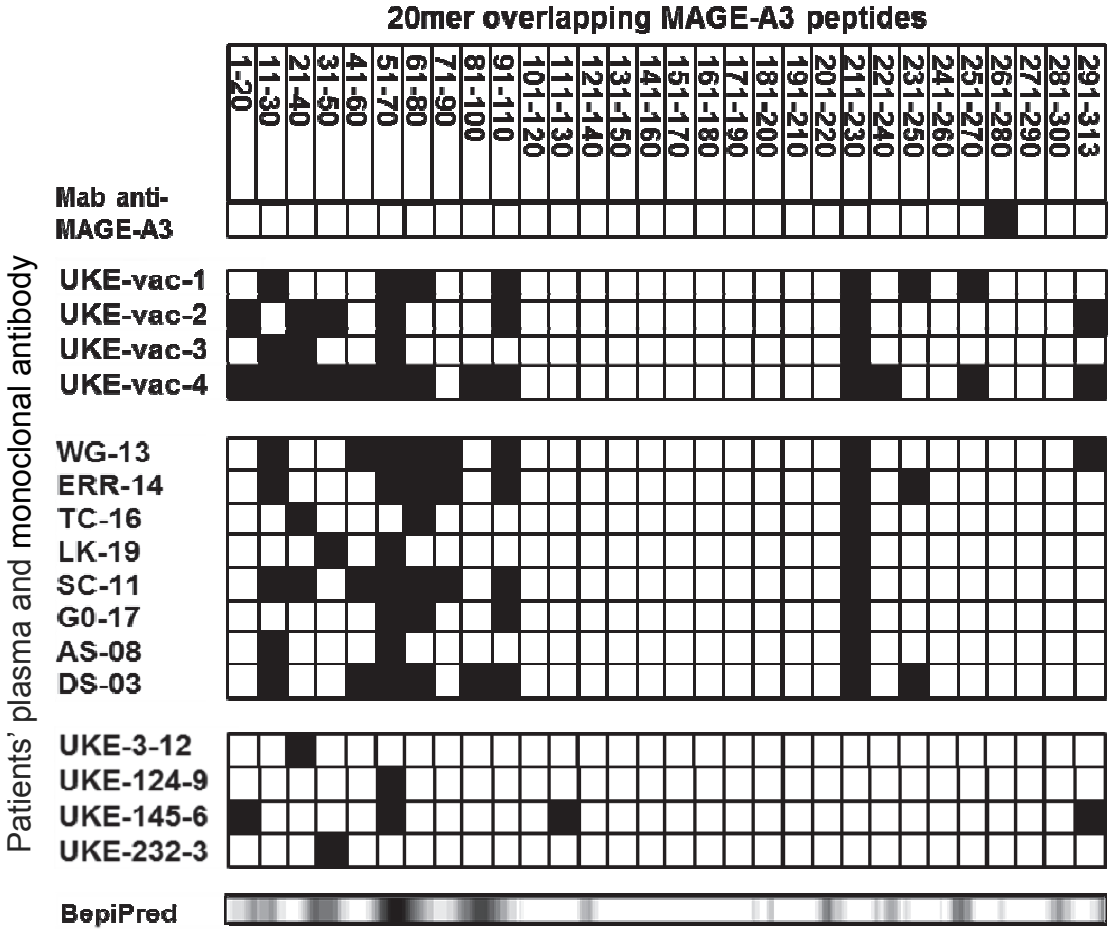

Figure 5.24: MAGE-A3 epitopes recognized by plasma of NSCLC patients and MM patients – The fine specificity of the antibody responses was examined by ELISA using overlapping 20mer peptides spanning the whole sequence of MAGE-A3. The linear epitope recognized by the mouse monoclonal antibody against MAGE-A3 is shown (top). The plasma samples from 12 MAGE-A3 vaccinated patients and four non-vaccinated seropositive MM patients were measured. Using a B cell epitope prediction program BepiPre [103], we calculated the location of linear B-cell epitopes. The darkness represents the possibility as a linear epitope targeted by B cells. The epitopes recognized by patients' plasma were compared with that expected by the B cell epitope prediction software BepiPred (bottom).

On the contrary, the antibodies from the MM patients recognized much less peptides. Interestingly, the epitope located on amino acid 51-70 was targeted by

both two spontaneous serologically positive MM patients and 11 vaccinated patients. Using a B cell epitope prediction program BepiPre based on a combination of a hidden Markov model and a propensity scale method [103], we calculated the location of linear B-cell epitopes. The most predicted humoral immunogenic linear area was also located on amino acid 51-70 (Figure 5.24).

The interaction between the linear epitope and the MAGE-A3-specific antibodies was verified by an absorption ELISA assay (Figure 5.25). In this assay, the MAGE-A3-specific antibodies of the plasma from representative seropositive patient UKE-124 could be 100% absorbed by pre-incubation with a MAGE-A3 coated plate. The pre-incubation with peptide 51-70 could also absorb most of the MAGE-A3-specific antibodies in the same plasma, which diminished 90% of the reaction in ELISA. This result confirmed the antibody against peptide 51-70, which was detected by the peptide ELISA, comprised the majority of the anti-MAGE A3 antibodies in this plasma. The absorption result also indicated there could be certain amount of antibodies recognizing those other than the linear epitopes, such as the structural epitopes, which could not be detected by the current assay.

We further compared the linear epitopes recognized by the MAGE-A3-specific antibodies and the epitopes recognized by cellular responses. MAGE-A3 epitopes recognized by both naturally derived- and vaccine-induced humoral responses were all collected from this study (Figure 5.26 upper). The MAGE-A3 epitopes for T cell responses were summarized based on the results of our previously vaccinated patients and previous descriptions [90] (Figure 5.26 middle, lower). Comparing both targeted epitopes, we noticed that the most common B cell linear epitopes were located on AA 51-80 and 211-230, while the T cell epitopes were located on AA 111-130, 140-180, 241-290. No common MAGE-A3 linear for both humoral and cellular responses was indicated in this comparison.

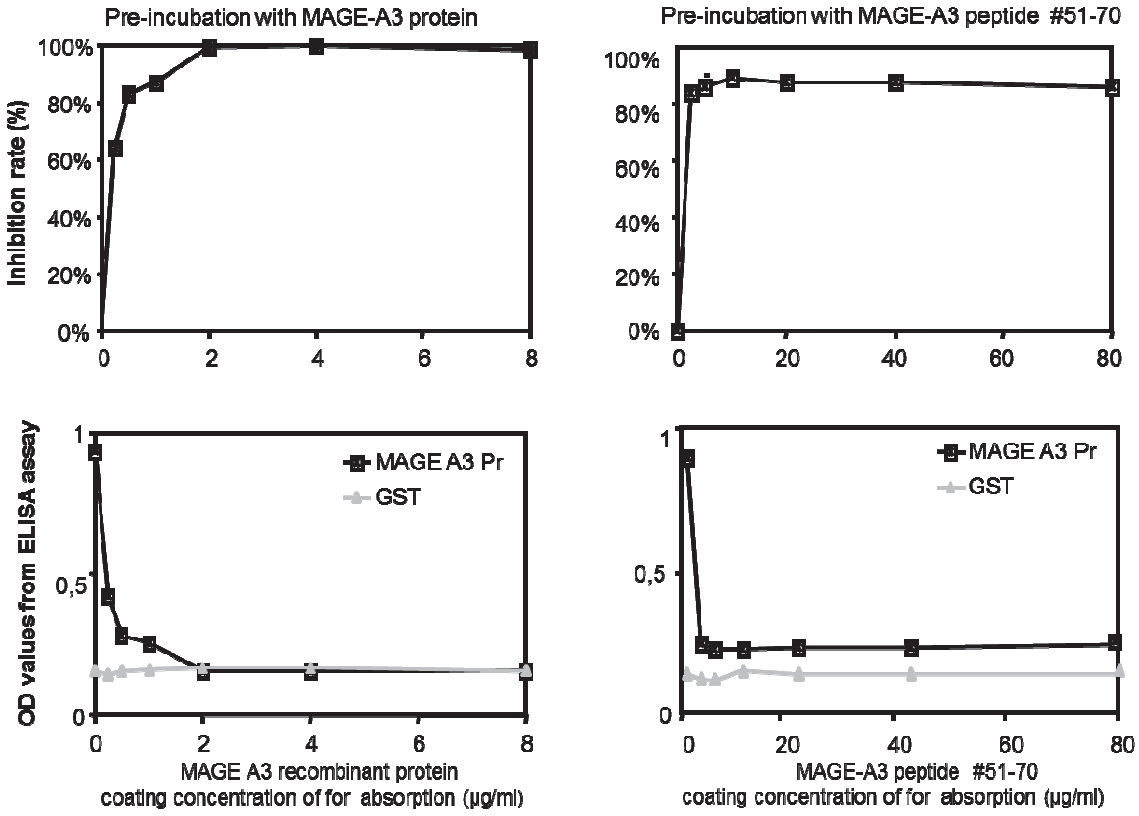

Figure 5.25: Absorption of MAGE-A3-specific antibodies - In the antibody absorption assay, diluted plasma from representative seropositive patient UKE-124 was pre incubated with half-area, 96-well microtiter plates coated with two-fold serial dilutions of MAGE-A3 (dark line, left), peptide 51-70(dark line, right) or control protein GST (grey line) at RT for 2 hours. The post absorption samples were then transferred to coated ELISA plates for anti-MAGE-A3 antibody testing. The results were shown as both the inhibition rates of the MAGE-A3-specific reactions (upper) and the OD values (lower).

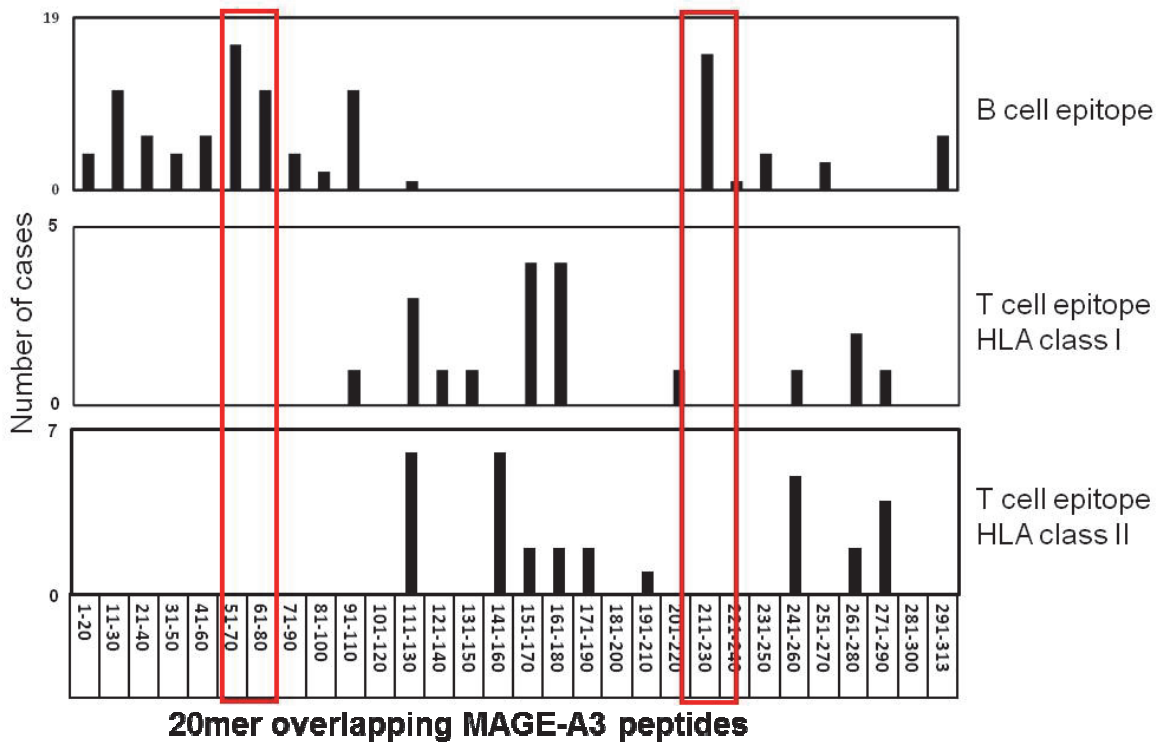

Figure 5.26: Comparison of MAGE-A3 linear epitopes recognized by humoral immune responses and epitopes recognized by cellular responses - The bar graphs (upper) represent the numbers of patients who evidenced antibody responses against certain linear epitopes of MAGE-A3. T cell epitopes scaled along the amino acid sequence of full-length MAGE-A3 protein are shown by ELISPOT. The epitopes of MAGE-A3 found in our vaccinated patients and previously described were summarized. A selection of potential HLA restriction alleles is indicated [90]. The number of cases that evidenced HLA class I (middle) or class II (lower) restricted epitopes of MAGE-A3 are shown as bar graphs.

Next, we applied this peptide ELISA assay to the supernatant of B cell stimulation culture. We demonstrated that the antibodies secreted from B cells upon multiclonal stimulation recognized the similar epitopes as antibodies in the sera of the same patients (Figure 5.27).

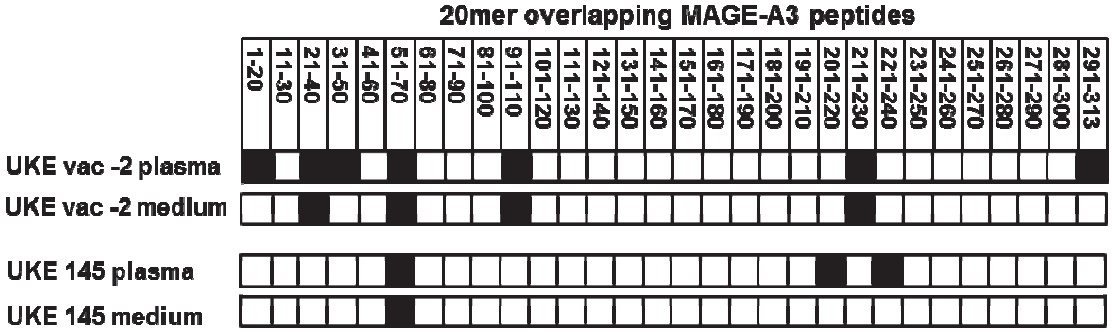

Figure 5.27: MAGE-A3 epitopes recognized by antibodies derived from the memory B cell culture - Representative MAGE-A3 epitopes recognized by antibodies derived from the supernatant of memory B cell cultures (medium) were estimated and identified comparable with those recognized by the corresponding plasma (plasma). Representative results of vaccinated patient UKE vac-2 (upper) and seropositive MM patient UKE 145 (lower) are displayed.

6. Discussions

6.1 Stimulation and Enumeration of Antigen-Specific Memory B cells

The enumeration of antigen-specific mBCs in human subjects is critical for the analysis of humoral immune responses following vaccination or infection, but has remained technically challenging due to the relatively low frequencies of these cells in PB. In the framework of this study, we developed an improved method for the quantification of antigen-specific mBCs in samples derived from different body compartments.

An effective amplification of the B cell signal before application to the read-out assay is critical for increasing the sensitivity of any given method for the quantification of Ag-specific mBCs. Mitogens such as PWM, as well as microbial products, have previously been used to amplify mBCs signals with varying degrees of success [104]. Out of all microbial products tested in our current study, we found CpG 2006 to be the most effective for the induction of ISC from memory B cells when added to a cocktail of stimuli. CpG 2006 contributed significantly to the proliferation and maturation of mBCs, probably by acting through Toll-like receptor 9 (TLR9), which is expressed on human memory B lymphocytes [105, 106].

The binding of CD40 to CD40L (CD154) expressed on activated T lymphocytes plays a central role in B lymphocyte activation [107, 108]. Accordingly, T cells activated by a third-party antigen have been used for stimulating B cells in a non-cognate fashion via CD40L and cytokine production [107, 109]. Alternatively, CD40L-expressing cell lines [110-115], soluble recombinant CD40L or anti-CD40 antibodies have been used for B cell stimulation [116-120]. We show here that in an assay for the enumeration of Ag-specific mBCs, recombinant CD40L represents a critical component for the amplification of mBCs signals.

Finally, cytokines such as IL-2, IL-4 and IL-10, which influence B cell development and homeostasis by regulating their proliferation and survival, have also been indicated to be useful for the induction of ISC from mBCs [121]. We show here for the first time that IL-21 is an important component of any

mixture of stimulants used for the induction of antigen-specific ISC from mBCs. Importantly, the capacity of IL-21 to induce ISC from mBCs greatly exceeded that of other cytokines such as IL-2 and IL-10. The high efficiency of IL-21 in amplifying B cell signals may be related to the fact that it seems be a surrogate for TLR signaling inducing the proliferation and differentiation of human mBCs into Ig-secreting plasma cells in the presence of B cell receptor- and CD40 signals [118, 122-125]. Most recently, CD40L and IL-21 have been applied for converting PB memory B cells into highly proliferating, immunoglobulin-secreting B cells [126]. We believe that the introduction of this cytokine into B cell monitoring approaches will significantly facilitate the investigation of antigen-specific humoral immunity.

Previous studies have indicated that an efficient proliferation of human B cells and their subsequent differentiation into antibody-secreting cells requires the combination of B cell receptor triggering, cognate T cell help and TLR stimulation [127]. We show here that cellular stimuli in the form of “help” from blood leukocytes are indispensable for the generation of ISC from mBCs precursors. In addition to this cellular signal, the most efficient combination of soluble stimuli to induce TT-specific ISC consists of CD40L, CpG 2006 and IL-21. Importantly, we are able to show that a CD40L/CpG 2006/IL-21-induced proliferation of memory B cells and their differentiation into antibody-secreting plasma cells is most likely the basis of the amplified ISC signal.

Using our optimized method for the enumeration of Ag-specific B cells, we are also able to quantify mBCs with specificities other than TT. Hence, we could demonstrate significant numbers of mBCs specific to viral antigens such as CMV and the influenza virus in the peripheral blood of healthy donors. Therefore, we suggest that our methodology is applicable to a wide variety of Ag, probably even Ag inducing specific mBCs at a lower frequency, such as tumor antigens. Currently, this approach has already been applied in our studies on monitoring immune responses against CTAs.

In addition, this optimized method could be used for the identification of antigen-specific memory B cells in different body compartments. To exclude a possible

influence of different microenvironments containing different subsets of non-B cells, we standardized the composition of the indispensable “helper” cells by mixing a total of 3×10^5 B cells separated from PBMCs, BMMCs or tonsil MNCs with 5.7×10^6 B cell-depleted PBMCs from the same donor. Consequently, B cells comprised approximately 5% of all cells used in the culture, a proportion comparable to that usually seen among the PBMCs of healthy subjects. This mixture of cells was treated for six days with our optimized cocktail of stimuli consisting of CD40L, CpG 2006 and IL-21. Applying the stimulated cells to our ELISPOT read-out assay, we enumerated the antigen-specific memory B cells in mononucleocytes from PB, BM and tonsils (data not shown).

In summary, we have developed an optimized methodology for the enumeration of antigen-specific mBCs in various compartments of the human body. We have demonstrated that the combination of CD40L, CpG2006 and IL-21 is optimal for the amplification of the ISC signal causing an expansion and differentiation of mBCs. Applying this methodology, we could also assess the antigen-specific mBCs residing in human tonsils and BM. We suggest that this approach can be used for the enumeration of mBCs specific for a wide variety of Ag (microbial, tumor-related, auto-antigens) in different human tissues, which will lead to significant improvements regarding our knowledge about the biology of humoral immunity.

6.2 Spontaneous Humoral Responses against MAGE-A3 and MAGE-C2 in Patients with Myeloma

Elucidation of the immune responses in patients with cancer has been the research topic of many investigations, and numerous TAAs have been identified up to now [128]. The cancer-testis antigens represent a family of TAAs potential targets for immunotherapy. Although CTA expression has been frequently detected, the spontaneous immune responses against CTAs, especially against MAGE antigens, have not been reported so often. In our previous study, we noticed that most of the immune responses against cancer-testis antigen NY-ESO-1 or SOX2 were remarkably identified in patients with malignant hematologic disease post allo-SCT. However, the sample bios could not be completely excluded, as the study group was limited to patients post allo-SCT. In this study, we recruited more patients before allo-SCT to explore the impact of clinical remedies, particularly allo-SCT, on the development of immune responses against CTA MAGE-A3 and MAGE-C2.

In patients post allo-SCT, we have observed a remarkable increase of cases with a MAGE-A3- and MAGE-C2-specific serological response than in patients only treated with maximally conventional chemotherapy or auto-SCT, while this humoral response was completely absent in untreated patients with MGUS and MM. This observation is in accordance with our previous hypothesis that immunological responses induced by allo-SCT may be capable of breaking the tolerance towards TAAs.

Allo-SCT is currently a potential curative therapy for patients with malignant hematological disorders, and can be applied to patients with various hematological diseases [129]. After HSCT, the immune system is substantially disturbed [130]. During the ensuing complex process of immune reconstitution in recipients, autoantigens and tumor-associated antigens may become immunogenic and initiate graft versus host responses [130, 131]. The expected graft-versus-tumor/ leukemia (GvT/GvL) effect is capable of killing malignant cells and is central for the therapeutic potential of allo-SCT [132]. Whereas the graft versus host response may affect nearly all organ systems and resemble

many clinical and laboratory features of a variety of classical autoimmune disorders such as lupus, Sjögren's syndrome and systemic sclerosis [131, 133-136]. GvHD is a major cause of morbidity and mortality after allo-SCT [137]. The development of autoantibodies has long been recognized in GvHD. The most common antibody was ANA, the occurrence of which was found to be significantly associated with chronic GvHD [138, 139]. The homeostatic expansion after transplantation-induced lymphopenia is thought to be a trigger for a loss of self-tolerance and the proliferation of auto reactive lymphocytes in these patients [131]. The patients with autoantibodies evidenced a better survival post allo-SCT [138, 140]. Thus, autoantibodies themselves were proposed to have some role in GvL effects [141].

With the aim of addressing the inducing factor of the CTA-specific response in patients after allo-SCT, we further analyzed their possible association with the occurrence of autoantibodies, in addition to the variation of the disease load and immune recovery after allo-SCT.

We noticed that the development of the MAGE-A3- and MAGE-C2-specific antibody has no special correlation with the variation of the general humoral response level, which was indicated by the concentration of immunoglobulin and antibody response against microbial antigen TT and NP. When the patients were identified as being serologically positive for the first time, most of them manifested a sign of residual myeloma disease. The development of the MAGE antigen-specific responses seemed not to be related with the variation of tumor load, as represented by the fluctuation of paraprotein level and the remission of disease. Interestingly, the occurrence of the MAGE-A3- and MAGE-C2-specific response was correlated positively with the expression of ANAs. This observation suggests the initiation of immune responses against TAAs, as well as the autoantigens, benefited from the failure of peripheral tolerance after allo-SCT; whereas they seemed to not be the direct consequence of an elevated general immune response or the variation of tumor load. In addition, this result also indicates the potential synergistic effect of a non-TAA-specific immune stimulating treatment on TAA-targeting therapies.

Tumor cells by themselves are commonly poor at initiating host-protective immunity. Correspondingly, the naturally arising humoral responses have been suggested to be ineffective. We also showed that MAGE-A3 and MAGE-C2 expression was frequently detected, and correlated with the progress of myeloma [52, 63, 67, 82, 142]. The frequency of naturally accruing immune responses in MM patients is quite low, which is in accordance with previous studies on anti-CTA response in other cancer patients. When we looked into the biological characteristics of this humoral response, we further observed a low frequency of MAGE-A3- or MAGE-C2-specific peripheral memory B cells compared with TT-specific mBCs. Moreover, although we have identified two major regions on MAGE-C2 protein mainly recognized by MM patients, only one linear sequence was recognized by a maximal seven out of 13 seropositive patients. This means that no certain epitope could spontaneously induce common immune response in the vast majority of serologically positive patients. In one word, allo-SCT induced a break in the immune tolerance. The break still seems not sufficient for eliciting efficiency TAA-specific responses in a majority of patients.

Cancer-specific immunotherapies are used to induce or enhance a broader spectrum of the host's immune responses against TAAs. Active immunotherapy is proposed for the de novo induction, as well as the amplification, of pre-existing TAA-specific GvT responses. Monoclonal antibody therapies targeting tumor antigens are of a high specificity and were manifested to be correlated with improved survival and delayed tumor progression [143-146]. Therefore, monoclonal antibody is the most widely used form of cancer immunotherapy today. However, many oncogenic proteins, including CTAs, were manifested to be intracellularly located, and which were traditionally thought not to be accessible by monoclonal antibodies. Correspondingly, they have been considered as targets for cancer vaccines rather than for antibody-based therapy. The successful tumor Ag-specific immune response was shown to be largely dependent on $CD8^+$ and $CD4^+$ T cells [91, 147].

Even so, evidence has been accumulated that the tumor-specific antibody against intracellular TAAs is involved in multiplication of the antitumor response.

It was demonstrated that the antibody-coated antigen released by dying cells, in the form of immune complexes, could be avidly taken up, processed by dendritic cells (DCs) and further be presented to T cells. Likewise, a sufficient level of CT antigen NY-ESO-1-specific antibody was reported to determine the cross-priming of cytotoxic T cells [148-151]. It was even manifested that the antibody taken up in a living cell could bind to their intracellular targeting antigens, consequently causing apoptosis [152, 153]. The clinical trials with TAA–targeting therapeutic mAbs also showed us that such antibodies could effectively enhance antigen cross-presentation by DC to T cells *in vitro* and *in vivo*, thereby resulting in the augmentation of TA-targeted CTL generation [154, 155]. Most recently, it was reported that *in vitro* opsonization of the recombinant human (rh) MAGE-A3 protein with a MAGE-A3-specific monoclonal antibody resulted in an increased Fcγ receptor-dependent DC-uptake, which further enhanced the delivery of antigen and the induction of MAGE-A3-specific CD8+ T cells [156].

Correspondingly, the possibility of antibody therapy or vaccination targeting three intracellular proteins has been explored in mice models. A variety of tumors that expressed these intracellular proteins were clearly inhibited by their respective exogenous antibodies or by antigen-induced host antibodies [153]. Moreover, in another study, anti-NY-ESO-1 mAb was combined with anti-cancer drugs to accentuate the release of intracellular NY-ESO-1 from dying tumor cells and to help facilitate mAb access to intracellular target molecules. A strong anti-tumor effect accompanied by the augmentation of NY-ESO-1-specific effector/memory CD8+ T cells was observed [157]. These results illustrated that mAbs could capture intracellular tumor antigens such as CTAs, thus engaging in an efficient induction of CD8+ T cell responses. These preclinical research achievements greatly expanded the possibility of applying mAb for passive cancer immunotherapy.

Clinical studies revealed that protective antibody function was involved in the anti-tumor process [128]. The tumor antigen-targeting therapeutic mAbs was shown to effectively enhance antigen cross-presentation by DC to T cells, resulting in the augmentation of TA-targeting CTL generation [158, 159].

Recently, CT antigen NY-ESO-1-specific Ab and CD8+ T-cell responses were observed to be induced by nonspecific immunotherapeutic monoclonal antibodies against cytotoxic T lymphocyte antigen 4 (CTLA-4), and was correlated with a clinical benefit [160, 161]. In this study, we carried out a longitudinal survey over the MM patients with a spontaneous humoral response against MAGE-A3 and MAGE-C2. In accordance with all the basic and clinical studies mentioned above, we observed a prolonged overall survival in the patients with a humoral response against MAGE-A3 and MAGE-C2. This survival benefit proposed the function and potential application of CTAs, particularly MAGE-A3- and MAGE-C2-specific immunotherapies (even monoclonal antibody therapy) in MM patients. We also noticed that the CTA-specific response was preferentially developed in patients younger than 60 years old, which indicates that younger patients might be more responsive to CTA-specific therapy and benefit from it. On the other hand, the impact of age on the patients' survival in this study could not be completely excluded, which needs to be further clarified in future research on both patients and animal models.

In summary, our study demonstrated that the humoral responses against MAGE-A3 and MAGE-C2 primarily existed in MM patients post allo-SCT, and correlated positively with autoantibody ANAs. Although the occurrence of CTAs' specific response has been reported before, the clinical significance of autoantibody expression has rarely been reported. This observation suggests in patients post allo-SCT that active immunotherapy targeting TAAs such as CTAs could help for both the *de novo* induction and amplification of pre-existing TAA-specific responses against residual disease. Furthermore, the overall survival benefit indicates that CTA-specific antibodies could probably promote the anti-tumor process. Moreover, the potential therapeutic application of monoclonal antibodies targeting CTAs was also implicated.

6.3 MAGE-Specific Vaccine for Patients with Malignant Diseases

Cancer immunotherapy is the use of the immune system to either treat existing cancers or to prevent the development of cancer. Active immunotherapy is proposed for the de novo induction, as well as amplification, of pre-existing TAA-specific GVT responses. Tumor cells by themselves are commonly poor at initiating host-protective immunity, which is confirmed by our previous study on the spontaneous immune response against MAGE-A3 and MAGE-C2 in MM patients. Together with an adjuvant, cancer vaccine is an active cancer-specific immunotherapy. The objective of cancer vaccines is to induce or boost a broader spectrum of the host's immune responses against tumor antigens, and cause tumor regression. The recombinant protein vaccine-induced responses are unrestricted by HLA types, therefore being suitable for a larger patient population [162].

As we mentioned before, CTAs were identified as being intracellularly located and were traditionally thought not to be accessible by monoclonal antibodies. Correspondingly, they have been considered targets for cancer vaccines. MAGE-A3 vaccines have been evidenced to elicit robust immune responses in the cohort of cancer patients who received the protein together with the adjuvant [91, 159, 162-164]. The pre-existing MAGE-A3-specific T cells in cancer patients and healthy individuals have been characterized and compared with vaccine-induced T cells. High avidity $CD4^+$ T cells were selectively expanded by MAGE-A3 protein vaccination together with an adjuvant [165]. Although multiple clinical trials targeting MAGE-A3 recombinant protein-based vaccines have been studied in patients with malignancies, the quality differences between spontaneous- and vaccine-induced humoral responses were not actually evidenced.

Hence, there is a great rationale to characterize the humoral response against intracellularly located TAAs, and to reveal the quality advantage of tumor-specific vaccines with respect to humoral response. In this study, we executed the first comprehensive comparison of humoral responses against intracellular

tumor antigen MAGE-A3 induced by recombinant protein and the spontaneous response in cancer patients.

In multiple myeloma, MAGE-A3 was reported frequently expressed in 30-50% of all patients. The antibody against MAGE-A3 was not detected in MM patients pre allo-SCT, including in patients who were newly diagnosed post chemotherapy, as well as those post autologous SCT. All four detected seropositive patients belong to the group post allo-SCT. This low frequency is in accordance with previous studies in cancer patients. Allo-SCT is a potentially curative therapy for a variety of hematologic malignancies [135], while has a major impact on the immune system. Because of genetic differences between donor and recipient, mature donor lymphocytes recognize antigens on host antigen-presenting cells and initiate GvHD [131]. That is a major cause of morbidity and mortality after allo-SCT [137]. GvHD, especially its chronic form, may affect nearly any organ system. It resembles many of the clinical and laboratory features of a variety of classic autoimmune disorders such as lupus, Sjögren's syndrome and systemic sclerosis [131, 133-136], though the mechanism of this phenomenon currently remains unclear. Recently, Daikeler summarized the possible pathophysiological mechanisms based on the known data in both animals and humans [131]. The mechanisms are suggested to be: homeostatic expansion, the transfer of autoimmunity from donor to recipient, the "altered immunity" associated with GvHD and other conditions such as the conditioning regimen for HSCT. The fact that antibody responses were only detected in MM patients post allo-SCT indicated the failure of peripheral tolerance. It also indicated homeostatic expansion could favor the induction of immune responses against tumor antigens, as well as self-antigens. Furthermore, this result also indicated the possible synergistic effect of a non-specific immunotherapeutic approach on the tumor antigen-targeted therapies.

When we turned to the vaccinated group we demonstrated that MAGE-A3-specific antibodies were induced in all vaccinated patients. The result of western blot assay confirmed that the antibodies in the sera of cancer patients could really recognize not only the recombinant protein, but also the naturally produced MAGE-A3 protein derived from tumor cell lines. The result also

substantiated a feasibility of in vivo binding. Next, we asked whether the MAGE-A3 vaccine could influence the humoral responses against other tumor antigens. Among the 17 tested TAAs, antibodies against MAGE-A2 were induced in all patients treated with MAGE-A3. The humoral responses against SOX-2, P53 and AKAP4 were not affected. No new antibody against the other CT antigens and other tumor antigens was induced by the MAGE-A3 vaccine. This phenomenon indicated the MAGE-A3 vaccine to be of a high specificity to help induce the humoral response anti-targeted antigen, which could cross-react against other antigens of the MAGE family, while not affecting the total immunology level of the patients.

The generation of an antigen-specific immunologic memory requires an affinity maturation of the humoral response, and the affinity is usually reflected by the determination of avidity indices. Using a competition ELISA assay, we assessed $IC_{50,}$ which could be considered as a measure (inverse) of the avidity of antibodies in a given analyte [166, 167]. Here, the IC_{50} represented the avidity of MAGE-A3-specific antibodies induced during the entire cycle of vaccination. In the vaccinated patients we did observe a declined IC_{50}, i.e. a growing affinity during the administration of the vaccines, similar to an antigen–driven affinity maturation of the antibodies usually seen in a vaccination setting [168]. On the other hand, the recorded increase in antibody affinity supports the necessity for a repeated administration of the vaccine.

Since the protein-based ELISA and western blot could not disclose the specially targeted regions on MAGE-A3 protein, we further performed the identification of the areas containing linear B cell epitopes [149]. To address these issues, a series of synthetic 20-mer overlapping peptides spanning the sequence of the MAGE-A3 protein was used, as B cell linear epitopes typically vary from five to 20 amino acids in length. This assay was verified by identification of the linear epitopes targeted by mAb specific to MAGE-A3 and MAGE-C2. The peptide absorption assay confirms that the antibodies that recognized certain MAGE-A3 linear epitopes in the peptide ELISA assay actually constitute the anti-MAGE-A3 antibodies in the same plasma.

Compared with the spontaneous responses, the vaccine-induced antibodies recognized much more segments throughout the full length of the MAGE-A3 protein. The linear epitopes targeted by antibodies in plasma from all postvaccination patients were mainly located on the N terminal and in the region of amino acid 211 to 230. In particular, the region located on amino acids 51 to 80 and 221 to 230 seemed to be of the strongest immunogenicity for all MAGE-A3 treated patients. Interestingly, the epitope located on amino acid 51-70 was targeted by both MAGE-A3 vaccine induced Abs and spontaneous arising Abs. This epitope was also expected to be of the most immunogenicity by a B cell epitope prediction program BepiPre. This observation disclosed several distinct regions of the protein containing frequently recognized B cell epitopes. It should also be noted here that discontinuous epitopes defined by the correct 3D conformational structure of the protein might not be detected by linear epitope mapping. Therefore, they were not presented in the current study. Additionally, no common linear MAGE-A3 epitope for both humoral and cellular responses was observed in this study. Namely, no linear peptide could be singly recommended to induce both cellular and humoral responses against the MAGE-A3 protein at one time. However, the possibility of combining peptides with other MAGE-A3-specific immunotherapies could not be excluded.

For the last step, we compared the intensity of the humoral response against MAGE-A3. As expected, the titer of antibodies induced by recombinant protein is much higher than that of the spontaneously produced ones. It was demonstrated *in vitro* that a CT antigen/antibody complex was efficiently taken up by dendritic and cross-presented to T cells, and further induced strong cytotoxic T cell responses. More strikingly, this effect was optimal when polyclonal sera were used [169]. In contrast, the soluble antigen alone was only insufficiently processed, which suggested that a certain amount of antibodies are the determinant for the cross-presentation of tumor antigens. The higher titer induced by the MAGE-A3 vaccine could represent more chances to offer sufficient antibodies to form an immune complex for the antigen presentation. Nevertheless, when we estimated the Ig isotypes of MAGE-A3-specific antibodies, we illustrated antibodies with a variety of isotypes and IgG

subclasses induced by MAGE-A3 vaccine, while the spontaneous humoral responses primarily consisted of IgG2 IgG3 antibodies. Thus, we presumed that the vaccine-induced various isotypes of antibodies could more probably be recognized by Fc receptors on APCs, and be taken up and presented, which might result in an augmentation of the TAA-targeting CTL generation.

In this study, we developed an optimized memory B cell assay on the basis of ELISPOT and ELISA to characterize the Ag-specific B cells in PB. The Ag-specific B cells are normally not detectable without stimulation. We first stimulated B cells with a set of stimuli whose function was to induce the proliferation and differentiation of B cells into Ag-specific ISCs. Using this method, we could evaluate the Ag-specific B cells in various sources of human tissues such as PB, BM and tonsils. With this assay we could not only numerate microbial antigens such as TT, NP and CMV-specific B cells, but also assess the high frequency of NY-ESO-1-specific B cells in non-vaccinated MM patients post allo-SCT (our unpublished data). Here, we applied the memory B cell assay to the PBMCs from two current groups of patients. For the first time, we verified in the periphery blood of patients during vaccine therapy that there were more MAGE-A3-specific memory B cells than in those spontaneous serologically positive patients.

In summary, this study revealed the efficiency of the MAGE-A3 vaccine to induce highly frequent and specific MAGE-A3-specific B cells in PB. These B cells were responsible for high titered-antibodies that recognized various epitopes of MAGE-A3. These intensive humoral responses may synergize with the T cell response, could also represent a promising immune adjuvant therapy for proper patients with MAGE-A3 expression. Recent clinical trials demonstrated that CTLA-4 blockade enhances pre-existing immune responses to NY-ESO-1 in melanoma patients with durable objective clinical responses [160]. In light of this study, we speculate that nonspecific immunotherapeutic approaches could also have a synergistic effect on MAGE-A3 vaccination. The hypothesis should be verified by further research.

7. Conclusions

- An optimized methodology has been developed in this study. This approach is suggested to be used for the enumeration of mBCs specific to a wide variety of Ag (microbial, tumor-related, auto-antigens) in different human tissues, which could lead to significant improvements with respect to the knowledge of the biology of humoral immunity.
- Humoral responses against MAGE-A3 and MAGE-C2 could naturally arise in MM patients, particularly in MM patients post allo-SCT. Despite the frequent expression, the occurrence of a spontaneous humoral response against MAGE-A3 and MAGE-C2 was quite rare. Active immunotherapy targeting TAAs such as CTAs might help to enhance the TAA-specific GVT responses, without the elicitation of harmful GvHD.
- The overall survival benefit of the MAGE-A3- and MAGE-C2-specific humoral responses indicates CTA-specific antibodies could help promote the anti-tumor process. The potential therapeutic application of immunotherapies, especially human-derived monoclonal antibodies targeting CTAs, is implicated.
- Compared with the spontaneous response, the MAGE-A3 vaccine could more efficiently induce highly frequent and specific humoral responses with high titered-antibodies, which recognizes a variety of epitopes throughout the full sequence, in addition to the sufficient MAGE-A3-specific B cells in PB. Thus, nonspecific immunotherapeutic approaches were speculated to have a synergistic effect on MAGE-A3 vaccination. The proposed hypothesis here needs to be verified by further research.

8. References

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Selected publications

1. **Cao Y,** Gordic M, Kobold S, Lajmi N, Meyer S, Bartels K, Hildebrandt Y, Luetkens T, Ihloff AS, Kröger N, Bokemeyer C, Atanackovic D. An optimized assay for the enumeration of antigen-specific memory B cells in different compartments of the human body. J Immunol Methods. 2010 Jun 30;358(1-2):56-65.

2. **Cao Y**, Luetkens T, Kobold S, Hildebrandt Y, Gordic M, Lajmi N, Meyer S, Bartels K, Zander AR, Bokemeyer C, Kröger N, Atanackovic D. The cytokine/chemokine pattern in the bone marrow environment of multiple myeloma patients. Exp Hematol. 2010 Oct;38(10):860-7.

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4. Atanackovic D, **Cao Y**, Luetkens T, Panse J, Faltz C, Arfsten J, Bartels K, Wolschke C, Eiermann T, Zander AR, Fehse B, Bokemeyer C, Kroger N. CD4+CD25+FOXP3+ T regulatory cells reconstitute and accumulate in the one marrow of patients with multiple myeloma following allogeneic stem cell transplantation. Haematologica. 2008 Mar;93(3):423-30.

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Research paper

An optimized assay for the enumeration of antigen-specific memory B cells in different compartments of the human body

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ABSTRACT

Object: In the framework of our current study we set out to develop an optimized assay for the quantification of antigen-specific B cells in different compartments of the human body.
Methods: Mononuclear cells (MNC) derived from the peripheral blood, bone marrow (BM), or human tonsils were incubated with different combinations of stimuli. The stimulated cells and culture supernatants were then applied to IgG-ELISPOT and ELISA read-out assays and tetanus toxoid (TT)-specific B cell responses were quantified.
Results: We found that a combination of CD40L, CpG, and IL21 was optimal for the induction of TT-specific IgG-producing cells from memory B cell (mBc) precursors. This cocktail of stimuli led to a proliferation-dependent induction of $CD19^{intermediate}CD38^{high}CD138^{high}IgD^{negative}$ terminally differentiated plasma cells. Applying our optimized methodology we were also able to quantify mBc specific for cytomegalovirus and influenza virus A. Most importantly, the same method proved useful for the comparison of mBc frequencies between different compartments of the body and, accordingly, we were able to demonstrate that TT-specific mBc preferably reside within tonsillar tissue.
Conclusion: Here, we optimized an assay for the quantification of antigen-specific B cells in different human tissues demonstrating, for example, that TT-specific mBc preferably reside in human tonsils but not in the BM or the peripheral blood. We suggest that our approach can be used for the enumeration of mBc specific for a wide variety of Ag (microbial, tumor-related, auto-antigens), which will lead to significant improvements regarding our knowledge about the biology of humoral immunity.

1. Introduction

Humoral memory to an antigen (Ag) is critical for controlling infections and maintaining long-term antibody (Ab)-mediated immunity (Ahmed and Gray, 1996; McHeyzer-Williams and Ahmed, 1999). Memory B cells (mBc) have been suggested to persist for a lifetime and to maintain the plasma cell pool producing serum antibody levels over prolonged periods of time (Bernasconi et al., 2002). Persisting antigen, certain cytokines, or toll-like receptor signals may drive mBc to differentiate into long-lived plasma cells which are the source of serum antibodies. On the other hand, a reduced function of mBc may lead to impaired antigen-specific-immunity (Wasserstrom et al., 2008). Accordingly,

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human mBc play a decisive role in mediating long-term protection against a wide variety of infectious diseases.

Persistence of mBc is well documented and mBc have been suggested to reside at the sites of Ag drainage such as the marginal zones of human lymph nodes or the bone marrow (BM). Unfortunately, there is little information regarding differences in the location of certain mBc subsets as characterized by their respective target antigen. Studies have indicated that the number of mBc specific for certain antigens found in the peripheral blood often do not correlate with long-term serum antibody titers against the respective target (Hofer et al., 2006; Lanzavecchia et al., 2006; Sasaki et al., 2007). This suggests that the peripheral blood (PB) does not represent the body compartment where most of the immune globulin (Ig)-secreting cells (ISC) are located. However, it is still not clear for most Ag where else mBc specific for the respective target preferably reside and where they produce clinically relevant antibodies. This seems particularly surprising since such results would have an immediate impact on the understanding of fundamental immunological mechanisms as well as on the development and optimization of vaccination strategies, i.e. against microbial pathogens. Most likely, methodological problems in the enumeration of mBc reactive with specific Ag are the reason for our lack of knowledge regarding the differential accumulation of these cells within certain body compartments.

In our current study, we aimed at developing an improved method for the enumeration of antigen-specific mBc in various compartments of the human body. To this end, we used tetanus toxoid (TT), a potent immunogen that induces persisting and protective humoral immunity, as a model antigen. In a second step, we evaluated different human tissues for the presence of antigen-specific mBc. We further correlated the antigen-specific IgG subsets produced *in vitro* by mBc from respective tissues with those in the PB to find out which body compartments these peripheral antibodies stem from.

2. Materials and methods

2.1. Patients and healthy donors

Tonsils and PB were obtained from adult patients undergoing tonsillectomy. BM and PB samples were obtained from healthy stem cell donors. Healthy subjects and patients gave informed consent in accordance with the revised version of the Declaration of Helsinki. The study protocol was approved by the local ethics committee (decision number OB-038/06).

2.2. Purification of B cells from different tissues

Tonsillar tissue was chopped into small pieces and the resulting cell suspension was washed in culture medium. Peripheral blood mononuclear cells (PBMC), BM mononuclear cells (BMMC) and mononuclear cells (MNC) from tonsils were prepared by density centrifugation on Ficoll-Hypaque. Untouched B cells and non-B cells were separated using the B Cell Isolation kit II (Miltenyi Biotech, Cologne, Germany) containing a cocktail of avidin-conjugated antibodies against a variety of non-B cells and biotin-conjugated microscopic magnetic beads.

2.3. Memory B cell assay

Briefly, 6×10^6 MNC or 3×10^5 PB-, BM- or tonsil-derived B cells mixed with 5.7×10^6 non-B cells separated from autologous PBMC were re-suspended in 2 ml RPMI 1640 medium (Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA), penicillin, streptomycin, and glutamine. Cells were seeded into 6-well plates and were incubated with combinations of 5 ng/ml polyclonal mitogens pokeweed mitogen extract (PWM; Sigma-Aldrich, St Louis, MO), 1:5000 *Staphylococcus aureus* Cowan (SAC; Sigma-Aldrich), 6 μg/ml 24-mer DNA oligonucleotide containing a phosphorothioated optimized human CpG motif (CpG 2006: 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′; DNA Technology, Risskov, Denmark), 500 ng/ml human CD40 Ligand (CD40L; GenWay Biotech, San Diego, CA), 50 ng/ml Interleukin (IL)-21 (PeproTech, Hamburg, Germany), 100 U/ml IL-2 (Roche, Mannheim, Germany), and 20 ng/ml IL-10 (Immuno-Tools, Friesoythe, Germany). Cells were cultured for 3–6 days at 37 °C with 5% CO_2.

2.4. Enumeration of antibody-secreting cells by ELISPOT

To enumerate antigen-specific immune globulin-secreting mBc, an Enzyme-Linked Immunospot Technique (ELISPOT) was used. Briefly, flat-bottomed 96-well nitrocellulose plates (Multi Screen-HA; Millipore, Bedford, USA) were coated overnight at 4 °C with 30 μl of the following Ags or monoclonal antibodies: 30 LF/ml tetanus toxoid (TT; Chiron Behring, Marburg, Germany), 2 μg/ml Glutathione-S-Transferase (GST; Abnova, Taipei, Taiwan), 4 μg/ml influenza (Flu) A virus nucleoprotein (NP; Biomol, Hamburg, Germany), 4 μg/ml Cytomegalovirus (CMV) pp28 protein (GenScript, Piscataway, USA), 2 μg/ml goat anti-human IgG-Fab antibody (Bethyl Laboratories, Montgomery, USA) in coating buffer made of carbonate–bicarbonate buffer (Sigma-Aldrich). Free protein-binding sites were blocked by incubation with 3% Albumin bovine (BSA; Biomol) at 37 °C for 2 h. PBMC ($2.5–5\times10^5$/well) were incubated in RPMI 1640 (Gibco, Paisley, United Kingdom) at 37 °C for 20 h. Plates were then washed thoroughly with water containing 0.05% Tween 20 (Sigma-Aldrich), followed by incubation with 1 μg/ml Alkaline phosphatase (ALP)-conjugated goat anti-human IgG antibody (Mabtech, Stockholm, Sweden) for 2 h at 37 °C. After washing, substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich) was added and plates were developed at room temperature for 15 min. Spots were counted using an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

2.5. Enzyme-linked Immunosorbent Assay (ELISA)

Antigen-specific IgA, IgG and IgM antibody responses were determined in plasma and culture supernatants by ELISA as described previously (Atanackovic et al., 2007). Briefly, half-area, 96-well microtiter plates (Corning Costar, Lowell, MA) were coated overnight with 25 μl/well of TT (5.7 LF/ml) or GST (1 μg/ml) or goat anti-human IgG-Fab (1 μg/ml; Bethyl Laboratories Inc., Montgomery, TX). Plates

were washed twice with PBS containing 0.05% Tween 20 and once with PBS. ELISA plates were then blocked with PBS containing 5% non-fat dry milk for 2 h at room temperature. Plasma or supernatants from cell cultures were added to the plates at 30 µl/well and were incubated at room temperature for 2 h. For the detection of antigen-specific antibody responses, alkaline phosphatase (AP)-conjugated goat anti-human IgG, IgM, IgA (Southernbiotech, Birmingham, AL), goat anti-human pan IgG and IgG1, 2, 3, 4 antibodies, (Southernbiotech) diluted 1/3000 in block buffer were added at 30 µl/well; and were incubated for 1 h at room temperature. After washing, plates were developed with substrate p-nitrophenyl phosphate (PNPP; Southernbiotech) at room temperature for 30 min. Plates were evaluated using an ELISA reader (Tecan, Männedorf, Switzerland) at a wavelength of 405 nm with a reference wavelength of 620 nm.

2.6. CFSE labelling and flow cytometry

Purified B cells or MNCs were labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and were cultured with CD40L, CpG and IL21 for 3–6 days. *In vitro*-activated cells were harvested and non-specific binding sites were blocked by pre-incubating with normal mouse IgG. Cells were then incubated at 4 °C for 20 min with fluorochrome-conjugated anti-CD19, -CD27, -CD38, -CD138, -IgD monoclonal antibody (mAb). After washing, cells were analyzed on FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences).

2.7. Statistical analysis

Since most of the data were not distributed normally, a Mann–Whitney rank sum test was performed to compare values between groups, and Spearman's rank correlation coefficient was calculated to analyze correlations between groups. For all statistical analyses, p values less than 0.05 were considered statistically significant.

3. Results

3.1. CD40L, CpG, and IL-21 represent the optimal combination of stimuli to induce TT-specific IgG-secreting B cells

Due to their relatively low frequencies, it is necessary to use polyclonal stimuli to amplify Ag-specific mBc before enumerating them in a read-out assay. In a first step, we investigated which combination of stimuli would be optimal for an efficient *in vitro* induction of IgG-secreting B cells (ISC) from PBMC. To this end, we stimulated whole PBMC from healthy donors separately with PWM, CpG 2006, IL-2, IL-10, IL-21, CD40L, and SAC. After 6 days of *in vitro* incubation with different stimuli, numbers of TT-specific ISC were determined in an ELISPOT read-out assay. In addition, TT-specific antibody produced during the *in vitro* culture was evaluated in an ELISA. We found that PWM represented the most potent inducer of TT-specific ISC from precursor B cells compared with other stimuli when used as single agents (Fig. 1a). CpG 2006 and IL-21 as single agents were also able to amply significant numbers of ISC from TT-specific mBc, however, with lower efficiency. In contrast, no TT-specific ISC were induced by IL-2, IL-10, CD40L, and SAC when used as single agents (Fig. 1a). Importantly, these results were corroborated by the ELISA, showing that IL-21, CpG, and PWM led to elevated levels of TT-specific IgG produced by ISC during the *in vitro* stimulation (Fig. 1b).

Next, we aimed at determining the most effective combination of stimuli for the induction of TT-specific ISC from mBc precursors. Incubating PBMC with combinations of 2–5 stimuli for 6 days, we observed that the combination of CD40L, CpG 2006 and IL-21 could most efficiently induce ISC from TT-specific mBc (Fig. 1c), but not IgA or IgM secreting B cells (data not shown). Although PWM, when used as a single stimulus, had shown the strongest capacity for the induction of TT-specific ISC, the addition of PWM to a combination of stimuli did not further increase the yield of TT-specific ISC (Fig. 1c). These findings were largely corroborated by observations made in the ELISA analysis of culture supernatants after *in vitro* stimulation of whole PBMC (Fig. 1d).

Previously, of all cytokines IL-2 and IL-10 have most commonly been used in memory B cell assays because IL-10 can induce the differentiation of memory B cells into ISC and IL-2 enhances the response of CD40L-stimulated B cells (Fluckiger et al., 1993; Zinkernagel et al., 1996; Tangye et al., 2003a,b). However, stimulation of a combination of CD40L/CpG 2006, with cytokine IL-21, which was identified as a very potent stimulant in our current study, led to higher numbers of TT-specific ISC compared to a combination with IL-2 or IL-10 (Fig. 1e, f). Furthermore, the addition of IL-10 and/or IL-2 to our cocktail of stimulants consisting of CD40L/CpG 2006/IL-21 did not further increase Ig secretion (data not shown).

3.2. Antibody-secreting B cells with specificities other than TT can be induced using a CD40L/CpG/IL-21 cocktail of stimuli

Having established an optimized assay for the enumeration of antigen-specific mBc, we next aimed at evaluating whether the same assay was also suitable for the analysis of B cell responses against Ag other than TT. Therefore, we applied our method to viral Ag such as CMV pp28 and influenza virus A nucleoprotein. Stimulating PBMC from healthy donors with the aforementioned combination of CD40L, CpG 2006 and IL-21 for 6 days, we clearly detected ISC specific for both antigens by ELISPOT (Fig. 2a). Corresponding IgG antibodies secreted during *in vitro* stimulation were observed in the ELISA (data not shown).

3.3. Memory B cells proliferate and differentiate into ISC upon stimulation with CD40L/CpG 2006/IL-21

It has long been known that polyclonal stimulation is necessary to induce antigen-specific ISC from memory B cells present among PBMC. However, it is unclear by which mechanisms exactly such stimuli amplify B cell signals. We used CFSE staining in order to monitor the proliferation of different B cell upon unspecific stimulation. We found that after three days, unspecific stimulation had induced division of $CD19^+$ B cells. In addition, the number of $CD138^+$ plasma cells presented in the culture had significantly increased (Fig. 3a). After 6 days, $CD138^+$ plasma cells had all divided multiple times while the proportion of cells that had undergone cell division was

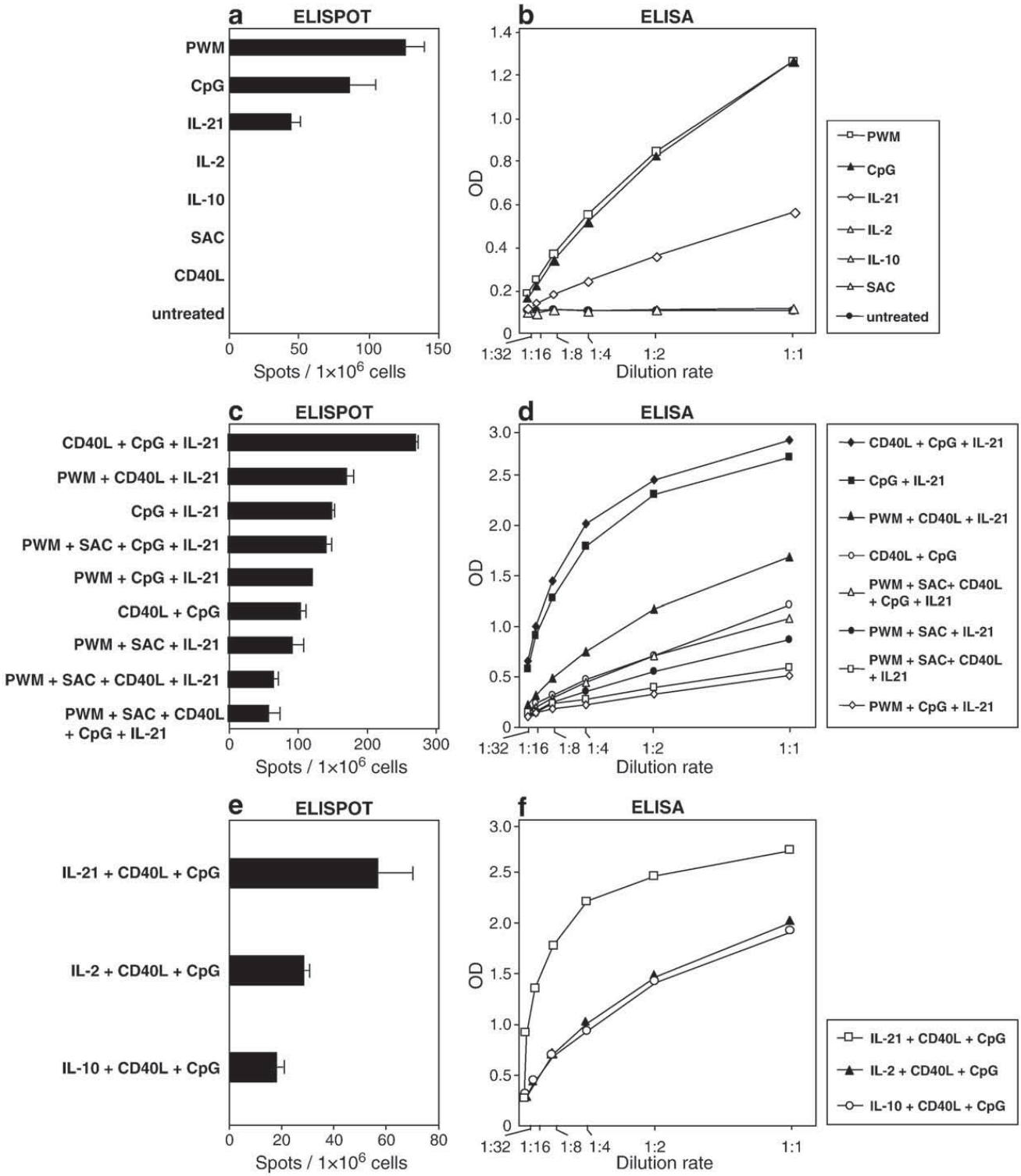

Fig. 1. A cocktail of CD40L, CpG and IL-21 represents the ideal stimulus for the amplification of antibody-secreting B cells from TT-specific mBc. (a, b) PBMC were stimulated with single agents PWM, CpG, IL-21, CD40L, and SAC for 6 days. (c, d) Alternatively, PBMC were stimulated with combinations of 2–5 stimulants for 6 days. (e, f) Finally, PBMC were stimulated with CD40L, CpG, combined with either IL-2, IL-10 or IL-21 for 6 days. TT-specific mBc precursors were detected by ELISPOT (left column) and ELISA (right column). Bars represent the mean (+SEM) number of TT-specific ISC per 1×10^6 cells for the ELISPOT and ODs of TT-specific antibody responses as measured in an ELISA.

much lower among $CD19^+$ conventional B cells. Importantly, other cell types such as T cells and monocytes had not undergone any cellular proliferation (Fig. 3a).

Further dissecting the B cell-restricted differentiation induced by stimuli CD40L/CpG 2006/IL-21, we found that after 6 days of stimulation, B cells could be divided into two distinct groups, $CD19^{high}$ and $CD19^{intermediate}$ cells. Most of the $CD19^{high}$ B cells were negative for CD27 which is a marker for memory B cells. These $CD19^{high}$ B cells had proliferated less frequently and showed almost no differentiation into

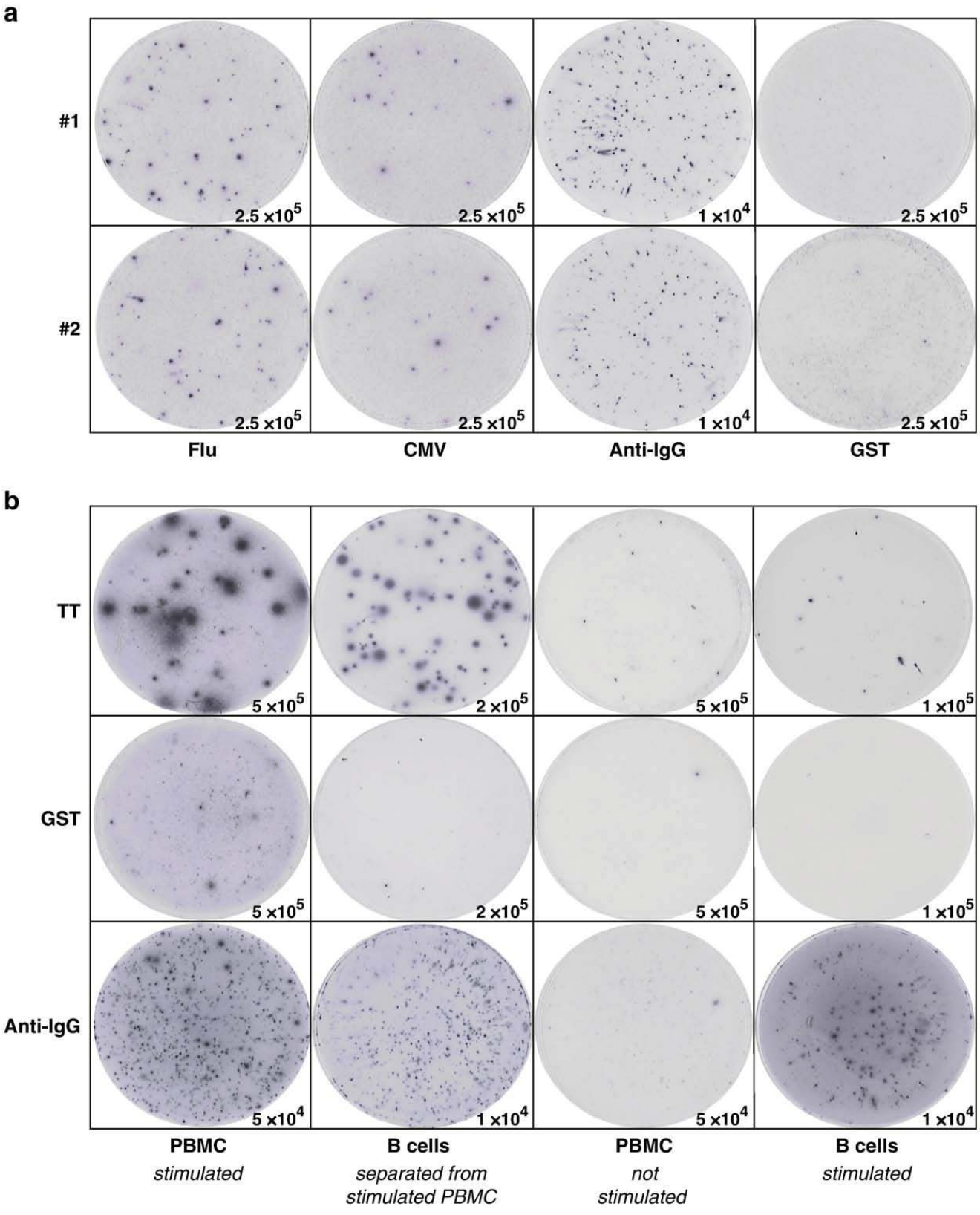

Fig. 2. Antigen-specific responses determined by ELISPOT 6 days after polyclonal stimulation. (a) Based on the analysis of TT-specific B cells, we next determined the number of influenza NP- and CMV-specific B cells among human PBMC. Experiments were performed in duplicate wells (#1 and #2). (b) For the analysis of TT-specific responses, PBMCs were cultured without stimulation or were stimulated with CD40L/CpG 2006/IL-21. After 6 days, cells were collected and applied to an ELISPOT assay. ELISPOT plates were coated with TT and anti-human IgG antibody to enumerate antigen-specific ISC and total IgG-secreting B cells, respectively, GST protein was used as negative control. In some experiments, B cells were isolated before or after polyclonal stimulation. Total B cells were also isolated before stimulation and cultured with CD40L, CpG, and IL-21 for 6 days following with ELISPOT assay.

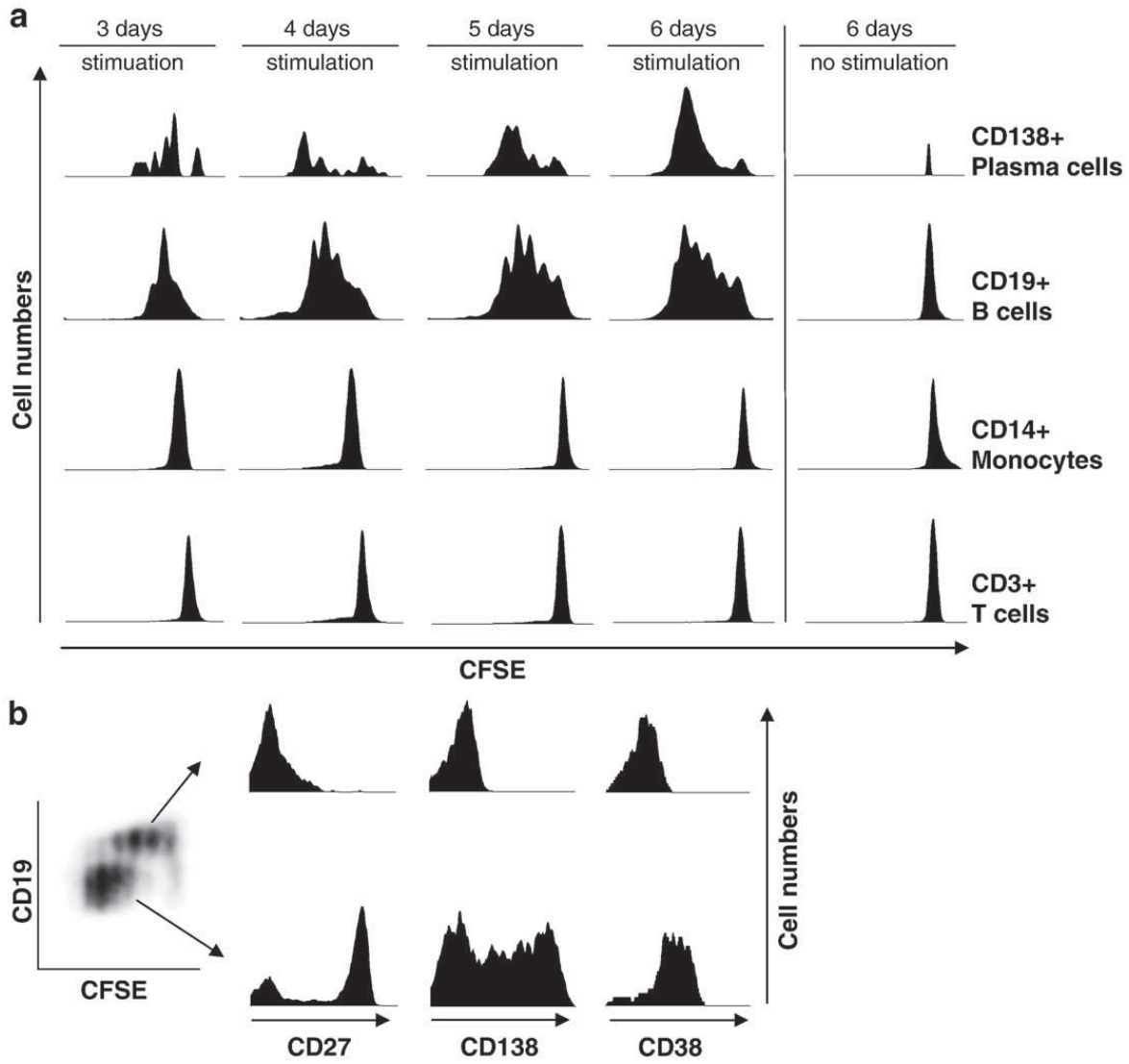

Fig. 3. Selective proliferations of human memory B lymphocyte subsets in response to CD40L, CpG 2006, and IL-21. (a) CFSE-labelled PBMCs were cultured with or without CD40L, CpG 2006, and IL-21 in human B cell medium. After 3, 4, 5 and 6 days, cells were collected. The proliferation of total B cells, plasma cells, monocytes and T cells was determined. (b) Differentiation of memory B cells to plasma cells. CFSE-labelled memory B cells were stimulated for 6 days with CD40L, CpG 2006, and IL-21, the expression of CD19, CD27, CD38 and CD138 were measured as a function of cell activation and differentiation. The cell division was evaluated in variant B cell subgroups.

$CD38^+$ or $CD138^+$ plasma cells (Fig. 3b). In contrast, the population of $CD19^{intermediate}$ B cells evidenced strong expression of the memory B cell marker CD27, had undergone several cycles of division and had developed into $CD38^+$ $CD138^+$ terminally differentiated plasma cells. Overall, these combined data suggested that the amplification of the ISC signal caused by our mixture of stimuli was most likely based on the induction of a B cell-restricted proliferation and differentiation.

3.4. IgG antibody responses observed in an ELISPOT assay are generated by TT-specific memory B cells but depend on help from non-B cells

To further confirm that TT-specific IgG responses observed after stimulation of whole PBMC were indeed generated by mBc but not by other cell types present in the culture, we isolated B cells from the culture after 6 days stimulation. When we applied these cells to our standard ELISPOT assay, we observed further amplified TT-specific IgG responses without any background signal against control protein GST (Fig. 2b). These results demonstrated that stimulated memory B cells were indeed the source of the TT-specific responses observed in our *in vitro* assays.

We also isolated B cells before stimulating them with CD40L, CpG 2006 and IL-21 for 6 days. However, applying these stimulated cells to our ELISPOT read-out assay, we observed that both numbers of detectable TT-specific B cells as well as total numbers of IgG-secreting cells were much lower than those produced by stimulated whole PBMC (Fig. 2b). These observations suggested that B cells present among total PBMC needed the help of some form of non-B cells in order to proliferate and/or fully differentiate into competent antibody-secreting cells upon unspecific

stimulation. As expected, non-B cells themselves could not be transformed into antibody-secreting cells by stimulation (data not shown).

3.5. TT-specific B cells preferably reside in human tonsils compared to the bone marrow and the peripheral blood

We had originally developed our optimized methods for the enumeration of antigen-specific B cells in order to compare numbers of ISC specific for certain Ag between different body compartments. Therefore, in a final step we aimed at evaluating numbers of TT-specific B cells within the PB, the BM, and within tonsillar tissue. To exclude a possible influence of different microenvironments containing different subsets of non-B cells, we standardized the composition of the indispensable "helper" cells by mixing a total of 3×10^5 B cells separated from PBMC, BMMC or tonsil MNCs with 5.7×10^6 B cell-depleted PBMC from the same donor. Consequently, B cells made up approximately 5% of all cells used in the culture, a proportion comparable to that usually seen among PBMC of healthy subjects. This mixture of cells was treated for 6 days with our optimized cocktail of stimuli consisting of CD40L, CpG 2006 and IL-21. Applying the stimulated cells to our ELISPOT read-out assay, we observed slightly higher numbers of TT-specific B cells in the peripheral blood than in the BM of the same individual ($p=0.23$; Fig. 4a). However, the frequency of TT-specific B cells was even further increased in tonsillar tissue than among PBMC of the same patients ($p<0.05$; Fig. 4c). These findings were again corroborated by an ELISA analysis of culture supernatants after *in vitro* stimulation of the B cell/PBMC mixture (Fig. 4b, d). In conclusion, we show here that using our optimized system, TT-specific memory B cells preferably reside in human tonsillar tissue when compared to the BM or the peripheral blood.

In order to further define which body compartments the antibody titers detected in the peripheral blood most likely stem from, we correlated the antigen-specific IgG subsets induced from mBc within respective tissues with the same IgG subsets detected in the PB plasma of the same individual. We found that the pattern of IgG subtypes produced *in vitro* by tonsillar B cells correlated significantly with the one observed in the peripheral blood in 5 out of 6 patients tested (Fig. 5a). In 4 out of 6 donors IgG subtypes produced *in vitro* by peripheral B cells correlated significantly with the IgG subtypes present in the autologous plasma (Fig. 5b). Finally, only half of the 6 donors tested showed a correlation between IgG subtypes produced *in vitro* by BM-derived B cells and the IgG subtypes present in the autologous plasma (Fig. 5c). This finding further supports our view that TT-specific antibodies are primarily produced by mBc present in tissues such as human tonsils.

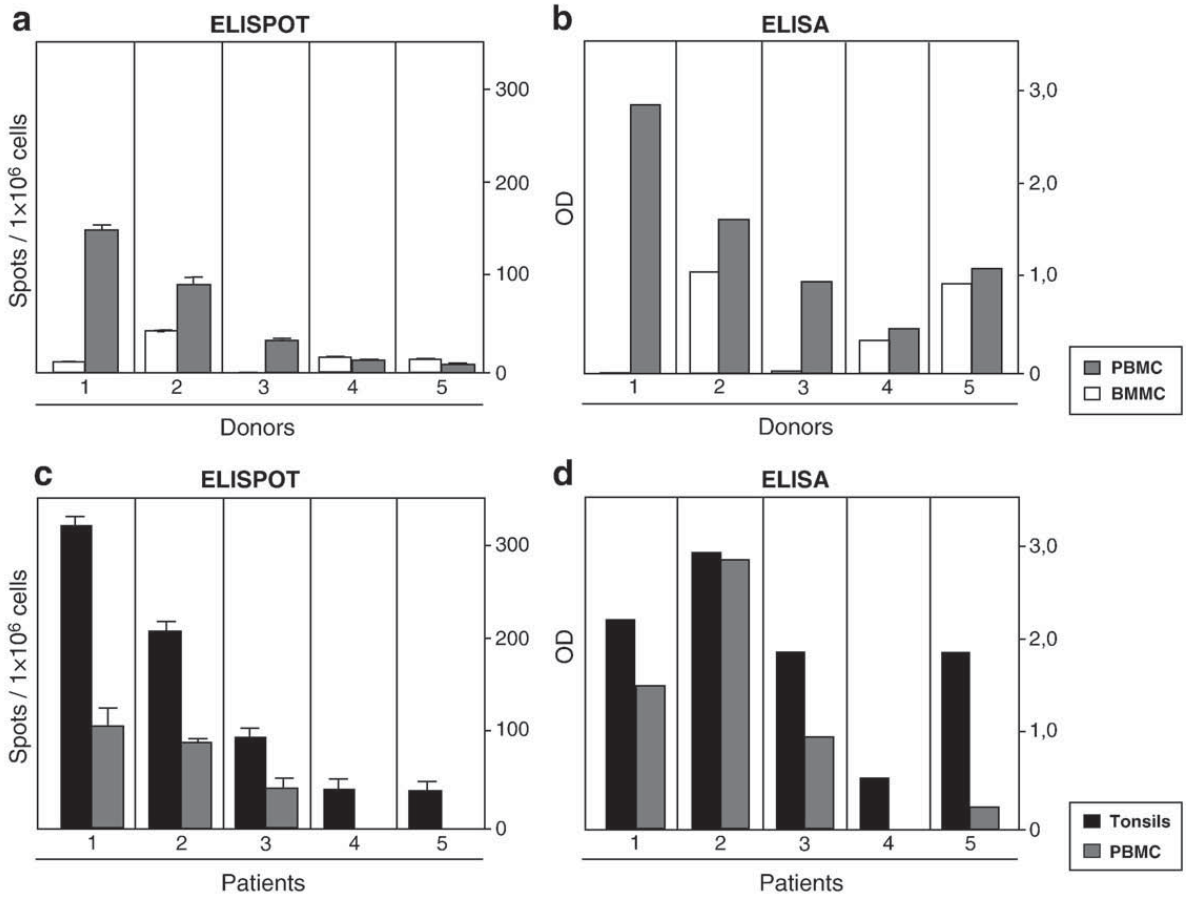

Fig. 4. Higher numbers of TT-specific mBcs in human tonsil than in the PB. 3×10^5 B cells from human PB, BM or tonsils mixed with 5.7×10^6 non-B cells from autologous PBMC were incubated with CD40L, CpG 2006 and IL-21 for 6 days. Cells and supernatants were collected and applied to ELISPOT and ELISA assays, respectively. (a, c) Bar graphs indicate mean numbers of TT-specific B cells derived from tonsils (black), peripheral blood (grey), and bone marrow (white) per total number of 1×10^6 cells. (b, d). Bar graphs indicate TT-specific IgG antibody production by B cells derived from tonsils (black), peripheral blood (grey), and bone marrow (white) in an ELISA assay.

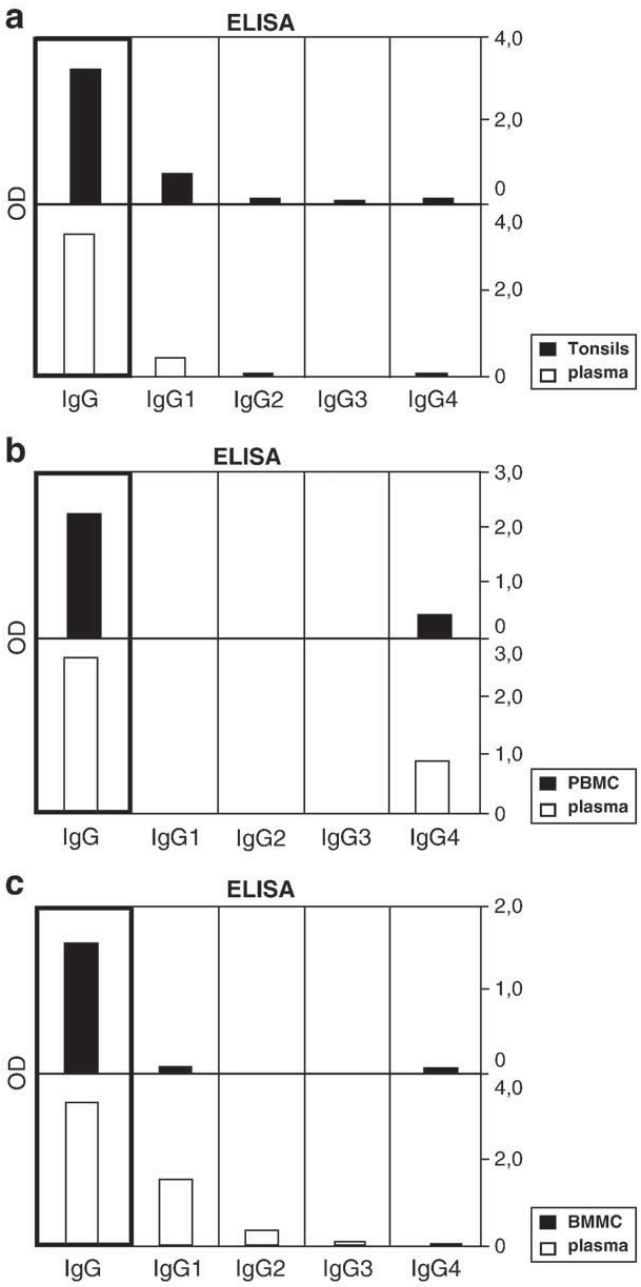

Fig. 5. TT-specific IgG subclass production in culture supernatants of stimulated B cells and plasma samples. Autologous plasma and supernatant from tonsil-, PB- and BM-derived mBc after polyclonal stimulation were analyzed for IgG subclass production using an ELISA assay. Bar graphs present TT-specific IgG1, 2, 3, 4 responses and the TT-specific total IgG responses in the supernatants of polyclonally stimulated B cells derived from tonsillar tissue MNC (a), PBMC (b) and BM (c) from representative individuals. In addition, results for PB plasma of the same subject are shown.

4. Discussion

The enumeration of antigen-specific mBc in human subjects is critical for the analysis of humoral immune responses following vaccination or infection, but has remained technically challenging due to the relatively low frequencies of these cells in the peripheral blood. In the framework of this study, we developed an improved method for the quantification of antigen-specific mBc in samples derived from different body compartments.

An effective amplification of the B cell signal before application to the read-out assay is critical for increasing the sensitivity of any given method for the quantification of Ag-specific mBc. Mitogens such as PWM as well as microbial products have previously been used to amplify mBc signals with varying success (Crotty et al., 2004). Out of all microbial products tested in our current study, we found CpG 2006 to be most effective for the induction of ISC from memory B cells when added to a cocktail of stimuli. CpG 2006 contributed significantly to the proliferation and maturation of mBc, probably by acting through Toll-like receptor 9 (TLR9), which is expressed on human memory B lymphocytes (Hornung et al., 2002; Bernasconi et al., 2003).

The binding of CD40 to CD40L (CD154) expressed on activated T lymphocytes plays a central role in B lymphocyte activation (Banchereau et al., 1994; van Kooten and Banchereau, 2000). Accordingly, T cells activated by a third-party antigen have been used for stimulating B cells in a noncognate fashion via CD40L and cytokine production (Lanzavecchia et al., 1983; Banchereau et al., 1994). Alternatively, CD40L-expressing cell lines (Tangye et al., 2003a,b; Avery et al., 2005; Muehlinghaus et al., 2005; Neron et al., 2005; Amanna and Slifka, 2006; Tuaillon et al., 2006), soluble recombinant CD40L or anti-CD40 antibody have been used for B cell stimulation (Ettinger et al., 2005; Larousserie et al., 2006; Racanelli et al., 2006; Bryant et al., 2007; Kuchen et al., 2007). We show here that in an assay for the enumeration of Ag-specific mBc, recombinant CD40L represents a critical component for the amplification of mBc signals.

Finally, cytokines such as IL-2, IL-4, and IL-10, that influence B cell development and homeostasis by regulating their proliferation and survival, have also been indicated to be useful for the induction of ISC from mBc (Konforte et al., 2009). We show here for the first time that IL-21 is an important component of any mixture of stimulants used for the induction of antigen-specific ISC from mBc. Importantly, the capacity of IL-21 to induce ISC from mBc greatly exceeded that of other cytokines such as IL-2 and IL-10. The high efficiency of IL-21 in amplifying B cell signals might be related to the fact that it seems be a surrogate for TLR signalling inducing proliferation and differentiation of human mBc into Ig-secreting plasma cells in the presence of B cell receptor and CD40 signals (Pene et al., 2004; Ettinger et al., 2005; Pene et al., 2006; Ettinger et al., 2008; Jacobi et al., 2008). Most recently, CD40L and IL-21 have been applied to converting peripheral blood memory B cells into highly proliferating, immunoglobulin-secreting B cells (Kwakkenbos et al., 2010). We believe that the introduction of this cytokine into B cell monitoring approaches will significantly facilitate the investigation of antigen-specific humoral immunity.

Previous studies have indicated that an efficient proliferation of human B cells and their subsequent differentiation into antibody-secreting cells require the combination of B cell receptor triggering, cognate T cell help, and TLR stimulation (Ruprecht and Lanzavecchia, 2006). We show here that cellular stimuli in the form of “help” from blood leukocytes are indispensable for the generation of ISC from mBc precursors. In addition to this cellular signal, the most efficient combination of soluble stimuli to induce TT-specific ISC consists of CD40L, CpG 2006 and IL-21. Importantly, we were also able to show that a CD40L/CpG 2006/IL-21-induced proliferation of memory

B cells and their differentiation into antibody-secreting plasma cells are most likely the bases of the amplified ISC signal.

Using our optimized method for the enumeration of Ag-specific B cells we were also able to quantify mBc with specificities other than TT. Thus we could demonstrate significant numbers of mBc specific for viral antigens such as CMV and influenza virus in the peripheral blood of healthy donors. Therefore, we suggest that our methodology is applicable to a wide variety of Ag, probably even Ag inducing specific mBc at a lower frequency, such as tumor antigens.

The most important goal of our current study was to develop a methodology with sufficient sensitivity and specificity to allow for the comparison of antigen-specific B cell numbers between different compartments in the body. Such comparative studies have rarely been performed, probably based on the lack of appropriate methods, and results were contradictory. Accordingly, previous findings have indicated a strong correlation between the frequency of measles- and TT-specific mBc in the peripheral blood and the respective serum antibody levels (Lanzavecchia et al., 2006). In contrast, other groups reported no significant correlation between the frequencies of circulating antigen-specific mBc and the serum titers of antigen-specific IgG (Leyendeckers et al., 1999; Rojas et al., 2008). We show here that TT-specific memory B cells preferably reside in secondary lymphoid organs like tonsillar tissue, and not in the bone marrow or the peripheral blood. We believe that the respective antibody titers, which are detectable in the periphery, primarily stem from this body compartment. It would be of major interest to investigate if the same is also the case for other types of antigens, such as tumor or auto-antigens.

In conclusion, we have developed here an optimized methodology for the enumeration of antigen-specific mBc in different compartments of the human body. We have demonstrated that the combination of CD40L, CpG2006 and IL-21 is optimal for the amplification of the ISC signal causing an expansion and differentiation of mBc. Applying our methodology to samples from healthy donors, we have shown that TT-specific mBc preferably reside in human tonsils but not in the bone marrow or the peripheral blood, and that these B cells are the primary source of TT-specific antibodies. We suggest that our approach can be used for the enumeration of mBc specific for a wide variety of Ag (microbial, tumor-related, auto-antigens) in different human tissues, which will lead to significant improvements regarding our knowledge about the biology of humoral immunity.

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The cytokine/chemokine pattern in the bone marrow environment of multiple myeloma patients

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Objective. **The interaction of multiple myeloma (MM) with its bone marrow (BM) microenvironment is important for the homing pattern, survival, and proliferation of malignant plasma cells. We aimed at answering the question which cytokines, chemokines, and growth factors are typically found in the BM of untreated MM patients as well as in MM patients after allogeneic stem cell transplantation (alloSCT).**

Materials and Methods. **We determined the concentrations of 34 cytokines/chemokines in the supernatants of 10 myeloma cell lines, as well as in the plasma derived from BM and peripheral blood samples of 10 newly diagnosed MM patients, 20 MM patients who had received allogeneic stem cell transplantation (alloSCT), and 20 healthy donors.**

Results. **Besides cytokines/chemokines known to be secreted by myeloma cell lines, such as interleukin-1 receptor antagonist (IL-1RA), IL-8, monocyte chemotactic protein−1 (MCP-1), macrophage inflammatory protein (MIP)−1α, MIP-1β, and MIP-3α, we also detected significant levels of epidermal growth factor (EGF), hepatocyte growth factor (HGF), IL2R, IL-12p40/p70, IL-22, interferon-γ (IFN-γ)−inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), and regulated on activation normally T-cell expressed and secreted (RANTES) in culture supernatants. The BM environment in MM patients evidenced elevated concentrations of HGF, IL-2R, IL-16, EGF, IL-1RA, IP-10, MCP-1, and monokine induced by IFN-γ. Additionally, in the BM of MM patients post alloSCT, we found selectively elevated concentration of IL-4, IL-6, IL-8, IL-12p40/p70, and eotaxin. Eotaxin levels were particularly high in patients with chronic graft-vs-host disease.**

Conclusions. **Our study demonstrates characteristic cytokine/chemokine patterns in the BM environment of MM patients before and after alloSCT. Certain factors, such as MIP-1α, MCP-1, HGF, IL-16, IP-10, and eotaxin, might not only be developed into diagnostic instruments and/or predictive biomarkers, but are also potential targets for future myeloma- or graft-vs-host disease−specific therapies.** © **2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Multiple myeloma (MM) is the second most common adult hematological malignancy. It is characterized by the expansion of a plasma cell clone that localizes to the bone marrow (BM) causing bone lesions, hypercalcemia, anemia, and proteinemia [1]. The interaction of myeloma cells with BM stroma is important for their homing pattern, survival, and proliferation [2]. Accordingly, myeloma cells and stromal cells both produce chemokines and cytokines that promote development and proliferation of MM.

Despite significant advances in the treatment of this disease, MM remains essentially incurable by conventional anti-tumor therapy [3]. Although the introduction of allogeneic stem cell transplantation (alloSCT) has resulted in higher remission rates and even occasional cures, the therapeutic improvement has been hampered by significantly increased treatment-related morbidity and mortality as a consequence of graft-vs-host-disease (GVHD) [4]. An unbalanced immune response with a cytokine dysregulation

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comparable to the one observed in autoimmune diseases [5–12] is considered a major cause of GVHD [5,6].

In this study, we aimed to answer the question of which cytokines, chemokines, and growth factors are typical components of the BM microenvironment of patients with MM before and after alloSCT. To this end, we performed a comprehensive analysis of the concentrations of 34 soluble factors in the supernatants of MM cell lines, as well as in plasma derived from BM and peripheral blood (PB) of newly diagnosed MM patients and MM patients who had received alloSCT.

Materials and methods

Patients and healthy donors

In total, 20 consecutive myeloma patients post alloSCT, 10 newly diagnosed myeloma patients, 10 healthy BM donors, and 10 healthy blood donors were studied (Table 1). Healthy subjects and myeloma patients who were admitted for treatment at the University Medical Center Hamburg-Eppendorf gave informed consent in accordance with the revised version of the Declaration of Helsinki. All patients treated with alloSCT had previously received conventional chemotherapy and autologous SCT. Patients undergoing alloSCT received pretransplant conditioning with melphalan (140 mg/m^2) and fludarabine (90−150 mg/m^2). GVHD prophylaxis consisted of antithymocyte globulin (60 mg/kg) in the case of unrelated SCT; short course methotrexate on days +1, +3, and +6; and cyclosporine A (3 mg/kg) until day +180. The study protocol had been approved by the local ethics committee (decision number OB-038/06). At the time of sample collection, 11 of the 20 patients after alloSCT showed signs of chronic GVHD. Chronic GVHD was defined according to published criteria and was diagnosed by evaluating clinical symptoms and/or biopsies from skin, oral mucosa, liver, and gut [13]. Because most samples were acquired before establishment of the International Staging System in 2005 [14], patients were staged according to the Durie-Salmon classification. Remission status was evaluated for all 20 BM samples from patients post alloSCT based on a modification of the criteria specified by the European Group for Blood and Marrow Transplantation [15].

Table 1. Clinicopathological characteristics of multiple myeloma patients

Characteristics	New MM patients	MM patients post alloSCT
Total	10	20
Age (y)		
60 or younger	6	12
Older than 60	4	8
Gender		
Male	4	11
Female	6	9
BM-infiltrating PC (%)		
0−10	0	14
10−50	6	5
51−100	4	1
Heavy chain isotype		
IgG	4	9
IgA	4	8
IgA + IgG	1	0
IgM	0	0
Light chain	1	3
Light chain isotype		
Kappa	6	13
Lambda	4	7
Initial stage (Durie-Salmon)		
I	3	4
II	1	6
III	6	10
Cytogenetics		
Deletion 13q14	4	7
No deletion 13q14	6	13
Previous treatment		
None	10	0
Chemotherapy alone	—	20
Autologous stem cell transplantation	—	20
Allogeneic stem cell transplantation	—	20

alloSCT = allogeneic stem cell transplantation; BM = bone marrow; Ig = immunoglobulin; MM = multiple myeloma; PC = plasma cell.

BM and blood samples

BM and blood samples from myeloma patients were obtained during routine diagnostic procedures. Samples obtained from consenting healthy donors were part of BM or blood donations. A routine BM smear examination was performed to control the quality of BM aspiration. Samples were immediately centrifuged at 500*g* for 10 minutes at 4°C and aliquots of plasma samples were stored at −80°C until used for analysis.

Myeloma cell lines

Myeloma cell lines EJM, KMS-12-BM, NCI-H929, RPMI-8226, U-266, OPM-2, LP-1, and IM-9 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cell lines Brown and SK-007 were provided by the New York branch of the Ludwig Institute for Cancer Research. Cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown to 80% confluency and the medium was replaced with serum-free medium for 24 hours before collection. Supernatants were collected and stored at −80°C until analysis.

Multiplex analysis of cytokines/chemokines

Concentrations of cytokines/chemokines in cell culture supernatant and plasma were analyzed in duplicates using Multiplex Bead-based Luminex Technology (Invitrogen, Carlsbad, CA, USA). Concentrations of the following 31 cytokines/chemokines were determined: epidermal growth factor (EGF), eotaxin, fibroblast growth factor−basic, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor (HGF), interferon (IFN)-α, IFN-γ, interleukin-1 receptor antagonist (IL-1RA), interleukin (IL)-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8 (chemokine [C-X-C motif] ligand 8, CXCL-8), IL-10, IL-12p40/p70, IL-13, IL-15, IL-16, IL-17, IFN-γ−inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monokine induced by IFN-γ (MIG), macrophage inflammatory proteins (MIP)-1α, MIP-1β, regulated on activation normally T-cell expressed and secreted (RANTES), tumor necrosis factor−α (TNF−α), and vascular endothelial growth factor. Measurements

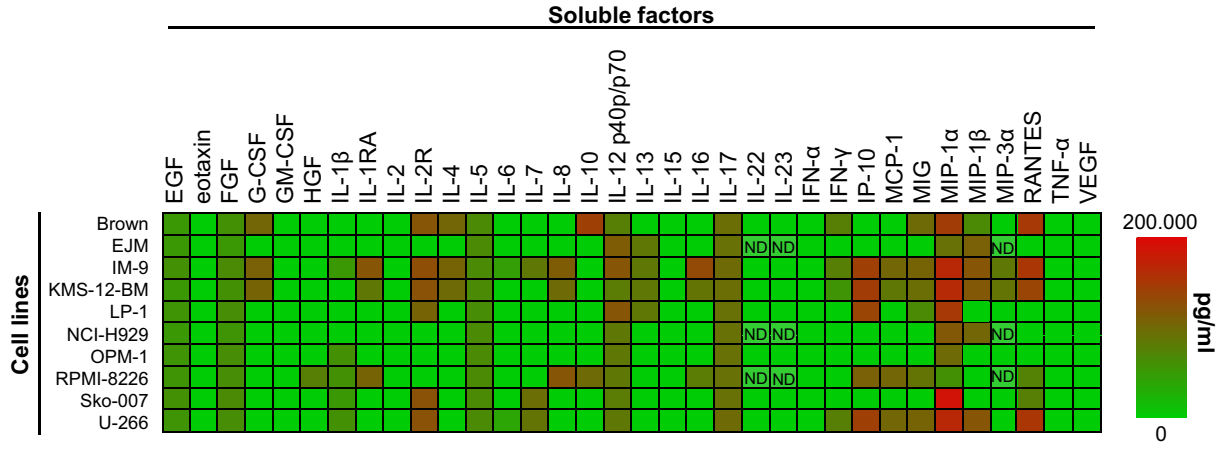

Figure 1. Cytokines, chemokines, and growth factors constitutively secreted by multiple myeloma (MM) cells. Applying a Luminex assay and enzyme-linked immunosorbent assays to supernatants of 10 different myeloma cell lines, concentration of 34 cytokines, chemokines, and growth factors were determined. Because of limited material, interleukin (IL)-21, IL-23, and macrophage inflammatory proteins (MIP)−3α could only be determined in supernatants of seven myeloma cell lines. Concentrations (pg/mL) of the given factors are indicated by fields in colors varying from dark green (undetectable) to dark red (very high concentration). ND = not determined.

were performed on a Luminex 200 analyzer (Luminex Corporation, Austin, TX, USA) and resulting data were evaluated using Xponent (Luminex Corporation) software. Lower limits of detection were in the range of 3.2 to 78.0 pg/mL.

Enzyme-linked immunosorbent assay

IL-22, IL-23, and MIP-3α (CCL20) levels in plasma and culture supernatants of MM cell lines were determined in duplicate applying Quantikine ELISAs (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Lower limits of detection were 2.7, 6.8, and 0.5 pg/mL, respectively.

Statistical analysis

To account for the nonparametric distribution of most cytokine concentrations, Mann-Whitney rank sum test was performed to compare data between patients and controls, while Spearman's rank correlation coefficient was calculated to analyze correlations between clinical characteristics of the patients and cytokine/chemokine concentrations. If a cytokine's concentration remained below the detection limit, 50% of the respective limit was used to calculate the mean cytokine concentration. For all statistical analyses, p values <0.05 were considered statistically significant.

Results

Human myeloma cell lines constitutively secrete a variety of cytokines and chemokines

In order to identify soluble factors that are produced by myeloma cells and not only by the BM environment, culture supernatants of 10 different myeloma cell lines were analyzed for concentrations of 34 soluble factors, including cytokines, chemokines, and growth factors. The cytokine secretion profile of the myeloma cells is shown in Figure 1.

Multiplex analyses demonstrated that each myeloma line secreted at least seven cytokines/chemokines. Among all soluble factors, the tumor-related inflammatory factor MIP-1α was most commonly detected, being produced at high concentrations by six cell lines and at intermediate levels by three cell lines. Other factors frequently detected at significant levels in the culture supernatants of myeloma cell lines were IL-2R, IL-12p40/p70, IL-17, IP-10, MIP-1β, and RANTES.

Cytokines/chemokines EGF, fibroblast growth factor, G-CSF, HGF, IL-1RA, IL-4, IL-5, IL-7, IL-8, IL-10, IL-16, IFN-γ, MCP-1, MIG, and MIP-3α were found less commonly and showed comparably lower concentrations. Finally, eotaxin, GM-CSF, IL-1β, IL-2, IL-6, IL-13, IL-15, IFN-α, TNF−α, and vascular endothelial growth factor remained undetectable in the supernatants of all 10 myeloma lines.

Myeloma patients show a typical pattern of soluble factors in the BM microenvironment

Because the BM is the body compartment from which myeloma originates and is where most of the tumor load resides, we next determined the concentrations of 34 cytokines/chemokines in the BM plasma of 10 newly diagnosed MM patients, 20 MM patients post alloSCT, and 10 healthy controls. Multiplex analyses revealed significantly increased levels of IL-16, IL-2R, MCP-1, HGF, IL-1RA, MIG, IP-10, and EGF in the BM microenvironment of untreated myeloma patients when compared to controls (Fig. 2). In some MM patients, elevated levels of MIP-1α and MIP-1β were observed. However, this did not reach statistical significance.

As can be expected, BM donors were younger than MM patients (median age: 36 years; range, 21−43 years) and there were more male subjects among them. However, we do not believe that these factors are likely to explain the marked differences we observed between patients and

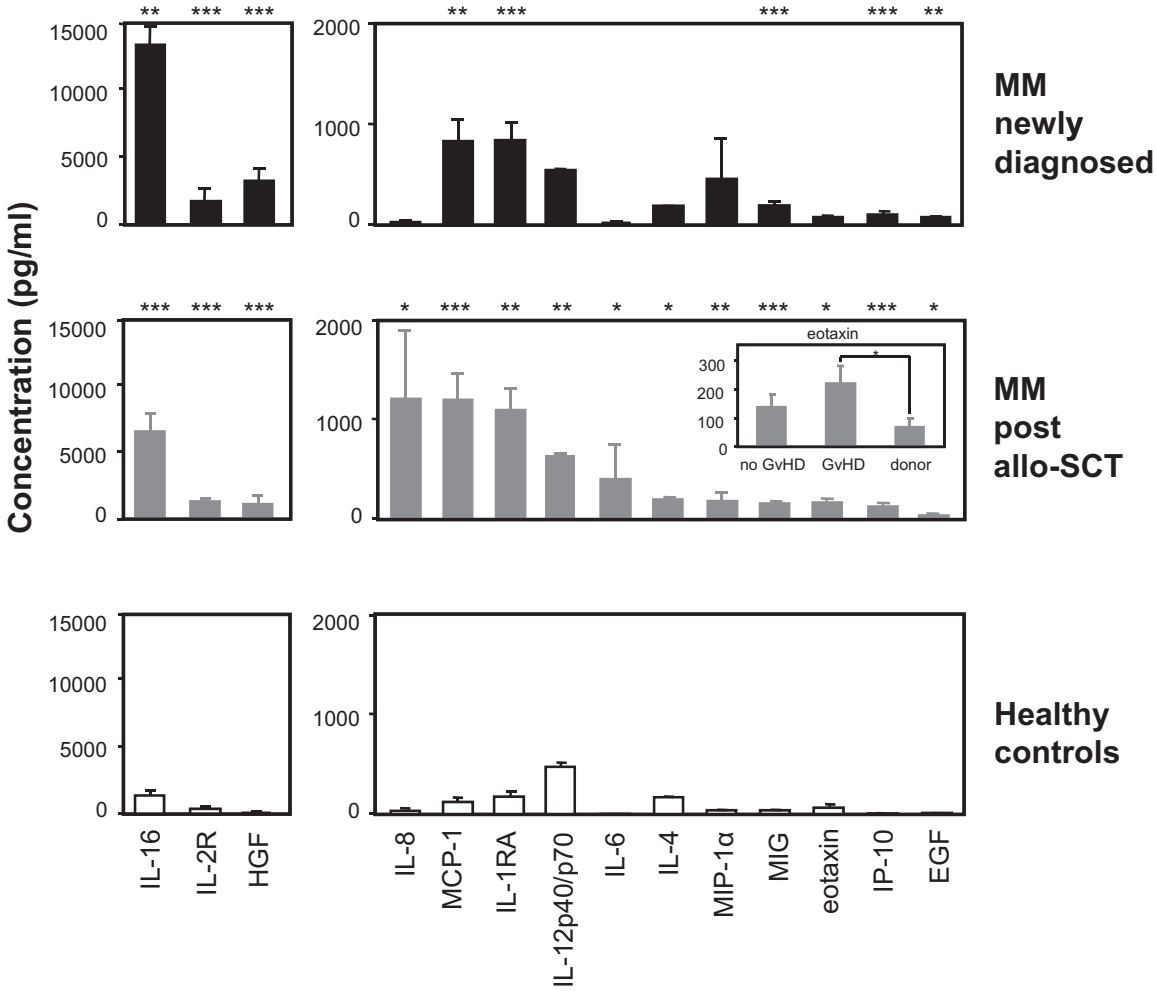

Figure 2. Cytokine/chemokine secretion in the bone marrow (BM) microenvironment of multiple myeloma (MM) patients. BM plasma samples were collected from 20 MM patients post allogeneic stem cell transplantation (alloSCT), 10 newly diagnosed MM patients, and 10 BM donors. Concentrations of 34 cytokines, chemokines, and growth factors were determined in duplicates in BM plasma applying a Luminex assay and enzyme-linked immunosorbent assays. Data are only shown for soluble factors being expressed at different concentrations in the BM of MM patients compared to normal controls. Mean concentrations (pg/mL) ± standard error of mean are shown as bar graphs for newly diagnosed MM patients (black), MM patients post alloSCT (gray), and healthy BM donors (white). Asterisks indicate statistically significant differences between the respective patient group and healthy donors (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

healthy controls regarding their BM cytokine/chemokines environment. Correlating the BM cytokine/chemokine levels with the clinical characteristics of MM patients (Table 1), we did not detect an association between the concentrations of the respective soluble factors and age, gender, heavy/light chain isotype, or cytogenetics. However, BM levels of IL-16 ($r = 0.829, p = 0.003$) and HGF ($r = 0.805, p = 0.005$) correlated positively with myeloma cell infiltration within the BM. In addition, IP-10 ($r = 0.770$, $p = 0.009$), HGF ($r = 0.645$, $p = 0.044$), and IL-6 ($r = 0.664, p = 0.036$) correlated significantly with the initial stage of disease. These findings suggest that the respective cytokines/chemokines are either directly produced by BM-residing myeloma cells or that the malignant cells promote the secretion of the soluble factors by the BM environment.

AlloSCT induces a characteristic cytokine signature in the BM of MM patients

To analyze associations between local cytokine/chemokine levels and alloSCT, we next compared results obtained from BM plasma samples of 20 MM patients post alloSCT with those observed in healthy controls. All patients after alloSCT were in partial remission and had detectable minimal residual disease in their BM. Median time since transplantation was 37 months (range, 13−71 months) and patients were in a state of full hematological recovery.

Despite the fact that the patients with alloSCT had only a minimal remaining tumor load, they showed a cytokine/chemokine signature that was, in many aspects, comparable to the one observed in untreated MM patients. Thus, as in newly diagnosed MM patients, we found increased levels of IL-16,

IL-2R, MCP-1, HGF, IL-1RA, MIG, IP-10, and EGF in the BM microenvironment of MM patients post alloSCT (Fig. 2). However, in the BM of MM patients post alloSCT, a number of additional factors were found at elevated concentrations. Selectively increased local concentrations were observed for IL-8, IL-12p40/p70, IL-6, IL-4, MIP-1α, and eotaxin (Fig. 2).

Comparing both groups of myeloma patients, we found that concentrations of IL-8 ($p < 0.05$), IL-12p40/p70 ($p < 0.01$), and eotaxin ($p < 0.05$) were significantly higher in the BM microenvironment of patients post alloSCT than in the BM of untreated MM patients (Fig. 2). In contrast, concentrations of HGF were significantly lower in the BM plasma of MM patients who had received alloSCT ($p < 0.05$) than in newly diagnosed patients.

In order to investigate possible associations between the presence of certain soluble factors in the patients' BM and the presence of chronic GVHD, we divided the post alloSCT group into nine patients with alloimmune symptoms at the time of analysis and 11 patients without active GVHD. Although IL-16, IL-2R, MCP-1, HGF, IL-1RA, MIP-1α, MIG, and IP-10 were elevated in both subgroups, we found BM concentrations of IL-8, IL-6, IL-13, and EGF to be increased only in patients without GVHD ($p < 0.05$). On the other hand, elevated concentrations of eotaxin were only found in patients with chronic GVHD ($p < 0.05$) (Fig. 2).

Distinct cytokine/chemokine gradients exist between the PB and the BM of myeloma patients

Next, we asked the question of which soluble factors would show a selective over- or underproduction in the BM compared to the same patient's PB. These analyses were performed based on the hypothesis that a BM-specific secretion pattern for certain cytokines/chemokines might point to a biologically relevant role of the given factors in the pathogenesis of MM. Second, concentration gradients of chemotactic factors from the PB into the BM or vice versa might result in the selective homing of certain cells, i.e., myeloma cells themselves or leukocyte subsets, into the respective body compartment.

In the BM plasma of healthy donors, only eotaxin (BM/PB ratio, 22:1) ($p < 0.05$), IL-8 (6:1) ($p < 0.01$), IL-16 (64:1) ($p < 0.001$), and MCP-1 (2.6:1) ($p < 0.05$) were enriched, while the vast majority of soluble factors showed equal levels in the BM and PB (Fig. 3). In newly diagnosed MM patients, we observed that IL-16 (BM/PB ratio, 425:1), HGF (145:1), and IL-1RA (17:1) (all $p < 0.01$) were highly enriched in the BM plasma compared to the patients' own PB plasma (Fig. 3).

When we analyzed MM patients post alloSCT, we were able to confirm findings in untreated patients with regard to the selective enrichment of distinct cytokines/chemokines (IL-16 (BM/PB ratio, 111:1), HGF (13:1), and IL-1RA (6:1) (all $p < 0.001$) in the patients' BM (Fig. 3). In addition to this common pattern of cytokine/chemokine gradients observed for myeloma patients independently of treatment, we were also able to define characteristic gradients only found after treatment with alloSCT. Thus, enrichment of cytokines IL-8 (20:1) ($p < 0.001$), MCP-1 (2:1) ($p < 0.01$), and IL-2R (1.3:1) ($p < 0.05$) in the BM was more pronounced in the transplanted patients (Fig. 3). In contrast, EGF (1:8) ($p < 0.01$) and eotaxin (1:12) ($p < 0.01$) were enriched in the PB compared to the BM of myeloma patients post alloSCT (Fig. 3).

Discussion

Survival and proliferation of MM is dependent on the BM microenvironment [16,17]. Accordingly, myeloma cells themselves are known to produce several cytokines and chemokines [18–31]. In addition to myeloma cells, BM stromal cells and BM-residing endothelial cells of MM patients are capable of producing a wide variety of soluble factors [27]. Importantly, many of these cytokines and chemokines have been considered promoters of tumor development and progression [12,32–38], sometimes functioning as growth factors or chemoattractants for myeloma cells, sometimes stimulating cellular adhesion. Other soluble factors seem to promote osteoclastogenesis and/or angiogenesis [38–41].

Here, we characterized the profile of cytokines/chemokines and different growth factors in myeloma cell lines and in the PB and the BM of MM patients. We were able to confirm production of IL-1RA, IL-8, MCP-1, MIP-1α, MIP-1β, and MIP-3α by MM cells, which was demonstrated previously by other assays [21,26,27]. Importantly, we also detected production of soluble factors, such as EGF, HGF, IL2R, IL-12p40/p70, IL-22, IP-10, MIG, and RANTES, which have not previously been reported to be produced by myeloma cells. Many of these soluble proteins have been isolated from tumor tissue or biological fluids of patients with solid tumors and have been suggested to promote progression of these malignancies by increasing local inflammation or directly stimulating tumor growth [42]. We demonstrate for the first time that the same factors are also produced by myeloma cells, indicating that they may play a similar role in the pathogenesis or promotion of this hematological malignancy.

Performing a comprehensive analysis of the cytokine/chemokine milieu in the BM environment of patients with MM, we were able to demonstrate elevated levels of HGF, IL-2R, and IL-16, which have previously been shown to be highly expressed in the PB of MM patients [43–46]. Moreover, we have shown here for the first time that concentrations of EGF, IL-1RA, IP-10, MCP-1, and MIG are increased in the BM of MM patients.

HGF and MCP-1 have both been suggested to represent myeloma growth factors [47–50]. In our study, increased levels of both soluble factors were observed in the BM of MM patients when compared to healthy donors. In addition, in newly diagnosed MM patients, HGF levels correlated

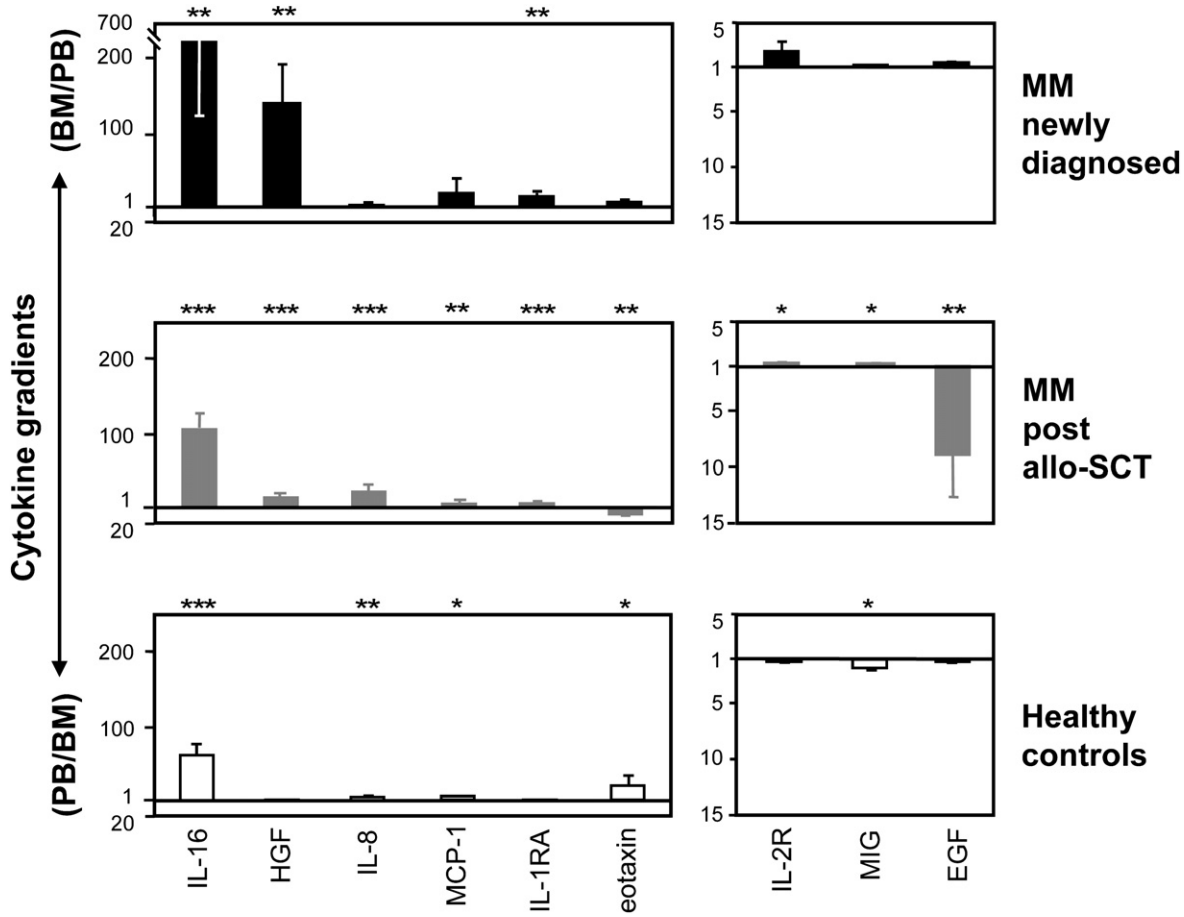

Figure 3. Cytokine/chemokine gradients between the bone marrow (BM) and the peripheral blood (PB). Plasma samples derived from the BM and the PB were obtained from 20 multiple myeloma (MM) patients post allogeneic stem cell transplantation (alloSCT), 10 newly diagnosed MM patients, and 10 PB donors. Concentrations of cytokines, chemokines, and growth factors were determined and ratios of cytokine concentrations within the BM environment vs the same patient's PB plasma were calculated. Mean ratios (BM/PB or PB/BM) are shown as bar graphs for newly diagnosed MM patients (black), MM patient post alloSCT (gray), and healthy BM donors (white). Asterisks indicate statistically significant differences between BM and PB ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

positively with the degree of BM infiltration by myeloma cells and with stage of the disease. We therefore believe that HGF and MCP-1 are both likely to promote the development and/or progression of MM and might, therefore, represent potential targets for myeloma therapy.

IL-16 is known to induce chemotaxis of $CD4^+$ T cells, monocytes, and eosinophils [45,51]. Although it has been suggested that peripheral IL-16 levels are increased in MM patients [43,45]. It has remained unclear which cell types are responsible for the IL-16 production. We show that IL-16 is secreted by myeloma cell lines and that increased IL-16 levels are present in the BM of untreated MM patients and patients post alloSCT. Moreover, we also show that a distinct concentration gradient exists for IL-16 pointing from the PB into the BM, particularly in patients post alloSCT. In addition, IL-16 levels correlated significantly with the degree of BM infiltration by myeloma cells. We, therefore, propose that IL-16 might play a significant role in the pathophysiology of MM.

IP-10 is a chemokine induced in a variety of cells in response to IFN-γ and lipopolysaccharides. The receptor of IP-10 ,CXCR3, is expressed on normal plasma cells, plasmablasts, and myeloma cells regulating chemotaxis and plasma cell migration into the BM [26,52–54]. IP-10 and its receptor CXCR3 were shown to regulate the proliferation and survival of myeloma cells [55]. We show here that IP-10 is secreted by myeloma cell lines and is increased in the BM environment of MM patients compared to healthy controls. Interestingly, BM concentrations of IP-10 correlated significantly with the stage of disease, further suggesting that IP-10 might be developed into a useful diagnostic and prognostic parameter or even a therapeutic target.

In addition to identifying potential targets for diagnostic and therapeutic measures in MM, we also aimed at describing typical cytokine patterns in the BM of MM patients post alloSCT. We found concentrations of IL-4, IL-6, IL-8, IL-12p40/p70, and eotaxin to be specifically elevated in the BM of this patient group. Furthermore, enrichment of

cytokines IL-6 and IL-8 in the BM was much more pronounced in transplanted than in untreated patients. In the case of eotaxin and EGF, on the other hand, a strong concentration gradient pointing in the direction of the PB was only present in patients post alloSCT, but not in untreated patients. Therefore, we believe that, in addition to the factors described here for the general population of MM patients, factors such as IL-8 and eotaxin might represent interesting subjects of investigation in patients who have received alloSCT.

Correlating the presence of certain soluble factors in the patients' BM with the presence of GVHD, we found that in patients post alloSCT, the increased production of eotaxin was based solely on increased levels of this cytokine in the BM of those patients suffering from GVHD. Interestingly, in patients with GVHD, the concentration of eotaxin in the PB was 22 times higher than that in the BM, and eotaxin concentrations in MM patients with GVHD were higher than in those of post alloSCT patients without GVHD. Increased expression of this chemokine has been detected previously in the main target organs of GVHD, such as skin, liver, and lung, and it might be that eotaxin is released from these tissues primarily into the peripheral blood [56,57]. This hypothesis would also be supported by our finding that eotaxin is not produced by myeloma themselves suggesting infiltrating inflammatory cells as producers of this chemokine. Accordingly, we believe that eotaxin, which is also one of the most relevant chemokines in the pathology of allergic conditions, might represent a factor with major importance for the pathophysiology of GVHD and orchestrating target organ-specific homing of inflammatory cells. Therefore, monitoring of eotaxin levels in patients with MM after alloSCT might be of high interest, and targeting eotaxin might help to inhibit development and progression of GVHD.

In conclusion, our present study indicates that distinct cytokine/chemokine patterns are present in the BM environment of patients with MM, as well as in myeloma patients post alloSCT. Our findings suggest that myeloma cells themselves contribute to the production of a number of the respective cytokines. The myeloma-related cytokines/chemokines described herein might potentially be used as diagnostic marker, predictive biomarkers, or targets for future myeloma-specific therapies. We have initiated a study with larger patient number to evaluate these selected soluble factors in a prospective fashion. Collecting further data on the pathophysiological role of the respective cytokines/chemokines will contribute to a more effective management of MM and treatment-related complications, such as GVHD, in patients after alloSCT.

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Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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Booster vaccination of cancer patients with MAGE-A3 protein reveals long-term immunological memory or tolerance depending on priming

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We previously reported results of a phase II trial in which recombinant MAGE-A3 protein was administered with or without adjuvant AS02B to 18 non-small-cell lung cancer (NSCLC) patients after tumor resection. We found that the presence of adjuvant was essential for the development of humoral and cellular responses against selected MAGE-A3 epitopes. In our current study, 14 patients that still had no evidence of disease up to 3 years after vaccination with MAGE-A3 protein with or without adjuvant received an additional four doses of MAGE-A3 protein with adjuvant AS02B. After just one boost injection, six of seven patients originally vaccinated with MAGE-A3 protein plus adjuvant reached again their peak antibody titers against MAGE-A3 attained during the first vaccination. All seven patients subsequently developed even stronger antibody responses. Furthermore, booster vaccination widened the spectrum of $CD4^+$ and $CD8^+$ T cells against various new and known MAGE-A3 epitopes. In contrast, only two of seven patients originally vaccinated with MAGE-A3 protein alone developed high-titer antibodies to MAGE-A3, and all these patients showed very limited $CD4^+$ and no $CD8^+$ T cell reactivity, despite now receiving antigen in the presence of adjuvant. Our results underscore the importance of appropriate antigen priming using an adjuvant for generating persistent B and T cell memory and allowing typical booster responses with reimmunization. In contrast, absence of adjuvant at priming compromises further immunization attempts. These data provide an immunological rationale for vaccine design in light of recently reported favorable clinical responses in NSCLC patients after vaccination with MAGE-A3 protein plus adjuvant AS02B.

antibody | $CD4^+$ T cell | $CD8^+$ T cell | immunization | non-small-cell lung cancer

Cancer/testis (CT) antigens all share a common expression pattern: They are found frequently in a large variety of human tumors but not in normal tissue, except immunoprivileged germ-line tissues (1). MAGE-A3 may well be the most commonly expressed gene among CT antigens and is detected in >50% of primary NSCLC (2, 3). A small proportion of these patients naturally develop antibody responses to MAGE-A3, indicating that this tumor antigen is capable of evoking spontaneous immune responses. Active immunotherapy holds the potential to boost such preexisting immune responses and to induce *de novo* MAGE-A3-specific immunity targeting MAGE-A3-expressing tumor cells.

MAGE-A3 vaccination using recombinant protein is likely to have several advantages when compared with vaccines consisting of commonly used "short" peptides. Such peptides are potentially presented by unprofessional antigen-presenting cells (APCs) in the absence of appropriate costimulation, notably from helper T cells, thus generating a less efficient immune response (4). In contrast, long peptides and proteins are much more likely to elicit an integrated immune response made of a variety of $CD4^+$ and $CD8^+$ T cell as well as B cell responses, after being taken up, processed, and presented by professional APCs (5, 6).

We previously reported the immunological results of a MAGE-A3 protein vaccination study in non-small-cell lung cancer (NSCLC) patients (7). Stage I/II patients without evidence of disease after resection of their MAGE-A3-expressing primary tumor received four injections at 3-week intervals of a recombinant MAGE-A3 fusion protein (MAGEA3/ProtD/His). Of 18 patients that completed the study, half received the protein alone (cohort 1) and half received the protein in the presence of AS02B, a saponin-based adjuvant containing monophosphoryl lipid A (cohort 2). By analyzing humoral immunity and T cell responses to selected MAGE-A3 peptides, we showed that vaccination with recombinant MAGE-A3 protein was able to induce antibody and $CD4^+$ T cell responses but that the presence of adjuvant AS02B was a prerequisite for the development of MAGE-A3-specific immunity.

In light of these encouraging immunological data, we sought to further define the requirements for immunological and clinical efficacy of this vaccine by exploring the initial impact of immunological adjuvant on long lasting memory responses after additional MAGE-A3 protein vaccination.

We describe here the immunological results of booster vaccination with MAGE-A3 protein. Of the 18 patients enrolled in study LUD99–010, 14 had no evidence of disease for up to 3 years after completing their original vaccine regimen. The 14 patients agreed to receive a new cycle of four tri-weekly injections of MAGE-A3 fusion protein; but this time, patients in both cohort 1 (originally vaccinated without adjuvant, $n = 7$) and cohort 2 (originally vaccinated with adjuvant, $n = 7$) received the MAGE-A3 protein in the presence of adjuvant AS02B. In addition, we evaluated a patient with pancreatic neuroendocrine cancer and a patient with pediatric osteogenic sarcoma, enrolled in compassionate single-patient protocols (SPP), who received eight consecutive injections every 3 weeks of MAGE-A3 fusion protein with AS02B adjuvant.

Author contributions: D.A., N.K.A., G.R., E.W.H., L.J.O., and S.G. designed research; D.A., Y.C., E.R., and S.G. performed research; D.A., N.K.A., C.A.F., G.R., E.W.H., C.B., L.J.O., and S.G. contributed new reagents/analytic tools; D.A., N.K.A., Y.C., E.R., G.R., C.B., L.J.O., and S.G. analyzed data; and D.A. and S.G. wrote the paper.

Conflict of interest statement: GlaxoSmithKline has licensed from the Ludwig Institute for Cancer Research certain patents relating to the compositions used in the clinical trial reported in this article. E.R., G.R., E.W.H., L.J.O., and S.G. are employees of the Ludwig Institute for Cancer Research; however, none of them are inventors of the relevant patents.

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The immunological objectives of the current study were to: (*i*) investigate the breadth of vaccine-induced B and T cell responses by using comprehensive monitoring techniques with full-length MAGE-A3 antigen; (*ii*) analyze whether MAGE-A3 protein vaccination elicited long lasting immunological memory by measuring the impact of booster vaccinations; and (*iii*) address the impact of adjuvant AS02B in the initial vaccine preparation on long-term immunity and tolerance.

Results

Antibody Responses to MAGE-A3 Vaccination. Antibody responses against the MAGE-A3 and protein D components of the fusion protein vaccine were analyzed by ELISA. To prevent false positive results due to reactivity against potential bacterial contaminants within the vaccine preparation, we analyzed serological responses against unrelated proteins made in the same bacterial vector and against MAGE-A3 protein prepared from insect cells transfected with a baculovirus vector encoding for MAGE-A3.

We now confirmed and extended our previously published results of immune responses elicited during the first cycle of vaccination with MAGE-A3 fusion protein: Of seven patients in cohort 1 who received the MAGE-A3 fusion protein in saline, only one patient (AS-08) developed an antibody response to MAGE-A3 protein after four injections (Fig. 1). Interestingly, patient EC-01, who had a preexisting antibody response to MAGE-A1, a CT antigen that shares 66% amino acid sequence homology with MAGE-A3, did not show an increase in his antibody titer against MAGE-A1 nor seroconverted to recognize MAGE-A3. None of the patients in cohort 1 reacted with bacterially derived truncated MAGE-A3$_{64–226}$, including patient AS-08 [supporting information (SI) Fig. 5*A*]. In contrast, of seven patients in cohort 2 who received one cycle of vaccination with MAGE-A3 protein plus adjuvant AS02B, all except patient AO-10 developed very strong antibody titers to both full-length baculovirus-derived MAGE-A3 (Fig. 1) and *Escherichia coli*-derived truncated MAGE-A3$_{64–226}$ (SI Fig. 5*A*).

Next, we analyzed antibody responses to MAGE-A3 elicited during the second cycle of vaccination with MAGE-A3 protein plus adjuvant AS02B in both cohorts. Only two patients in cohort 2 (SC-11 and LK-19) still had detectable MAGE-A3 antibodies before repeat vaccination (Fig. 1), whereas all other patients had no detectable MAGE-A3 antibodies after an average period without vaccination of 945 days (±34) for cohort 1 and 598 days (±82) for cohort 2. Patient AO-10, the only patient in cohort 2 who had not reacted to MAGE-A3 after the first vaccine cycle, seroconverted after the second vaccine cycle. The six remaining patients in cohort 2 reached the maximum antibody titers to MAGE-A3 attained during the first vaccination after just one new injection and subsequently developed even stronger antibody responses. These responses were typical of a booster vaccination, indicating persistence of memory B cell precursors generating recall antibody responses upon reimmunization.

In striking contrast, only one of seven patients from cohort 1 (DS-03) developed a high-titered antibody response to MAGE-A3 after receiving the MAGE-A3 protein vaccine plus adjuvant (Fig. 1). Another four patients (EC-01, DG-06, WS-07, and SG-09) developed low-titer antibodies against MAGE-A3 after the second vaccine cycle (mean titer 1/500), whereas patient AS-08 gradually recovered the antibody titer originally seen after the first vaccine cycle without adjuvant. In comparison with what is expected from patients receiving MAGE-A3 protein and AS02B for the first time, the immunogenicity of the vaccine and adjuvant was markedly decreased in patients previously vaccinated with protein alone (mean reciprocal titer for cohort 1 at day 85b = 1,475 vs. mean reciprocal titer for cohort 2 at day 85 = 5,890, $P = 0.003$ by t test, SI Fig. 5*B*).

It is important to note that this decreased capacity to mount

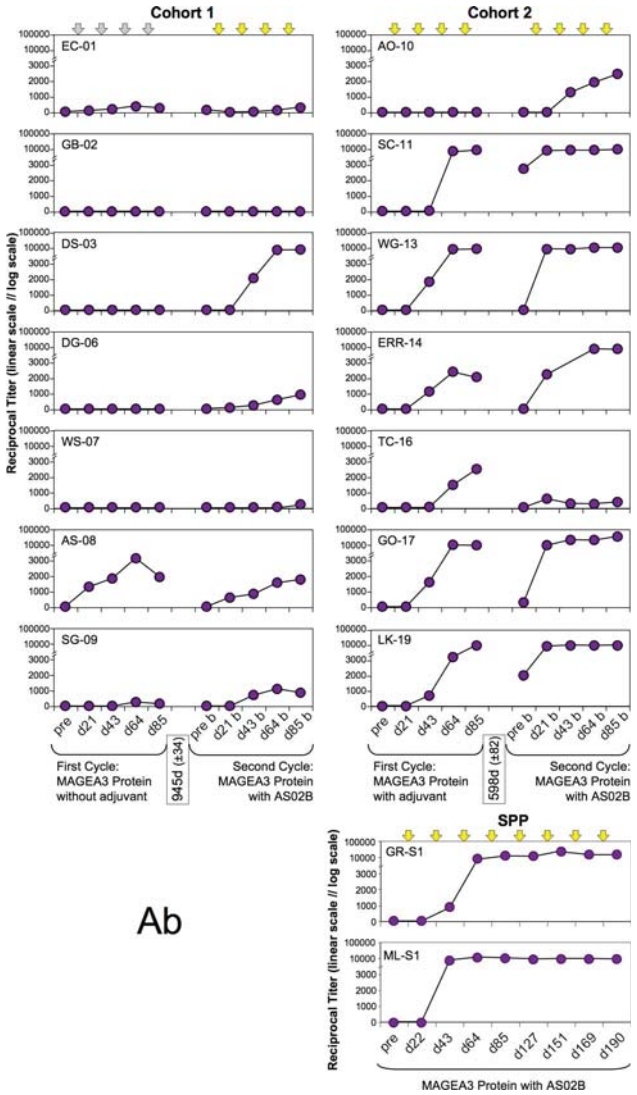


Fig. 1. Antibody responses to MAGE-A3 in vaccinated patients. Reciprocal antibody titers against baculovirus-derived MAGE-A3 protein were measured by ELISA and are shown for each time point from prestudy (pre) to day (d) 85 for both vaccine cycles in the presence (yellow arrows) or absence (gray arrows) of adjuvant. Each box represents one patient, with cohort 1 patients on the left, cohort 2 patients on the right, and SPP patients at the bottom. None of the patients developed any significant reactivity against control proteins NY-ESO-1, LAGE-1, or p53. Results are representative of at least three independent experiments.

antibody response in cohort 1 was observed only for MAGE-A3 but not for the protein D part of the vaccine (SI Fig. 5*C*). Remarkably, all patients developed high-titer antibodies against influenza protein D when receiving the vaccine with AS02B, regardless of their original cohort, indicating a higher intrinsic immunogenicity for this foreign antigen and a selective inhibition of MAGE-A3-specific immunity after vaccination in the absence of adjuvant.

The two additional patients, GR-S1 and ML-S2, who received continuous MAGE-A3 protein plus AS02B vaccination as single-patient protocols (SPP), both developed a strong antibody response to MAGE-A3 and protein D and even showed an extension of antibody reactivity to other MAGE family member sharing homology with MAGE-A3 but not to other tumor antigens such as p53 or NY-ESO-1 (Fig. 1 and SI Fig. 5*D*). Such a widening of seroreactivity specifically against other MAGE

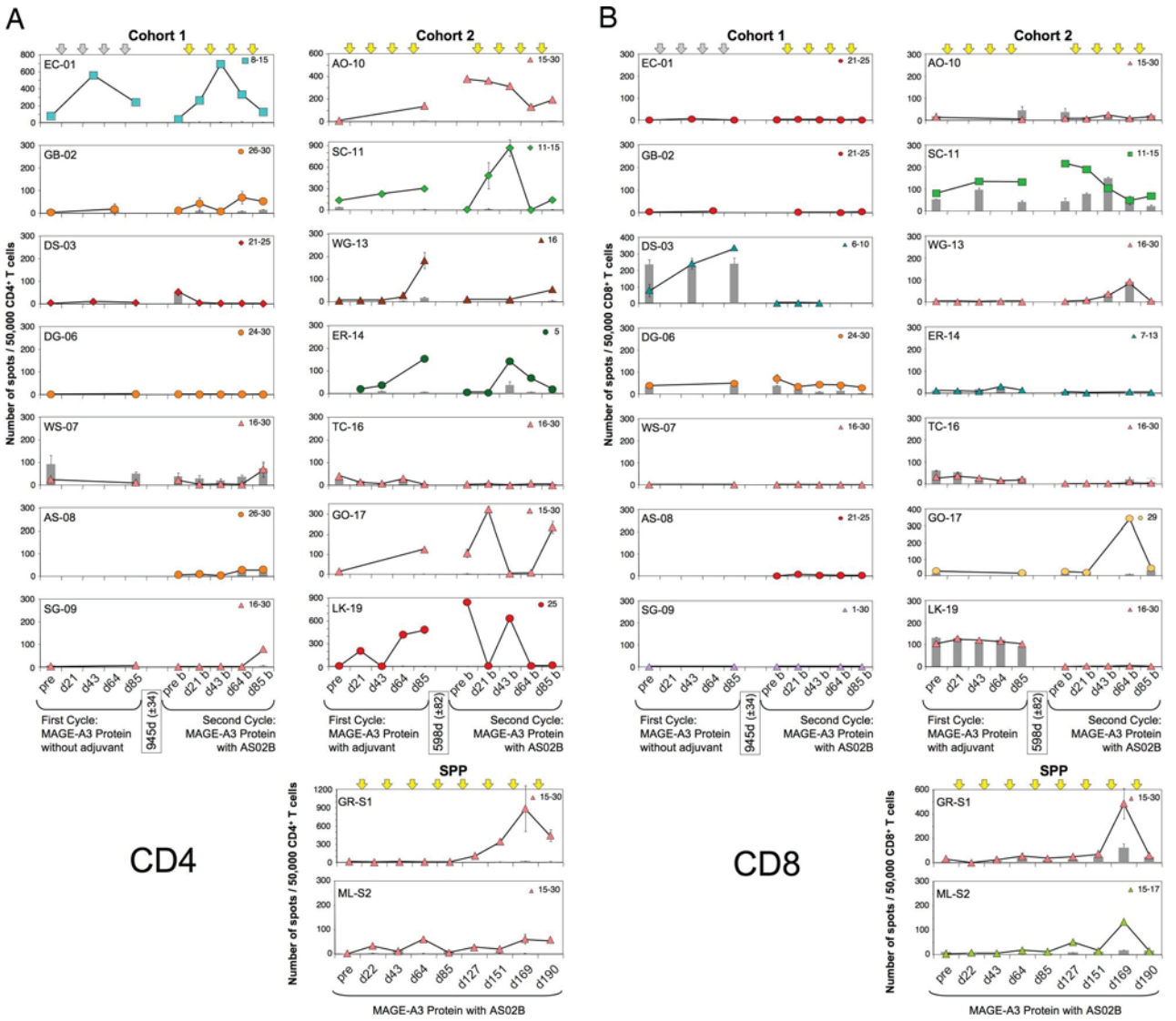


Fig. 2. CD4$^+$ and CD8$^+$ T cell response to MAGE-A3 in vaccinated patients. Mean numbers of IFN-γ producing cells of 50,000 presensitized CD4$^+$ (*A*) or CD8$^+$ (*B*) T cells were measured by ELISPOT against indicated individual or pooled MAGE-A3 long overlapping peptide(s) as indicated by color symbols and are shown for each time point from prestudy (pre) to day (d) 85 for both vaccine cycles in the presence (yellow arrows) or absence (gray arrows) of adjuvant. If detectable, responses to irrelevant peptide-pulsed targets are shown as gray bars. Each box represents one patient, with cohort 1 patients on the left, cohort 2 patients on the right, and SPP patients at the bottom. Results are representative of at least two independent experiments, and error bars represent SD of replicates.

antigens was also observed in patients SC-11 and GO-17 (data not shown).

Analyzing the isotypes of the immunoglobulins induced by vaccination, we observed a mixed profile of responses, with half of the responders generating preferentially IgG1 against MAGE-A3 and protein D, whereas the other half developed more IgG2 against both. Most patients, however, developed a strong IgG4 response against MAGE-A3 and protein D after a second cycle of vaccination, indicating that prolonged antigen exposure may favor this isotype subclass (SI Fig. 5*E*).

T Cell Responses to MAGE-A3 Vaccination. We have adapted a protocol, developed for tumor antigen NY-ESO-1 (8–10), for the monitoring of MAGE-A3-specific T cell responses. Specific T cell precursors are expanded, and T cell activity is visualized by ELISPOT. This *in vitro* sensitization protocol is not strong enough to induce *de novo* responses, and detection of specific T cell responses reflects *in vivo* priming. Compared with our previous analysis, we expanded the cellular monitoring to now include the repertoire of potential T cells to all possible MAGE-A3 epitopes in any HLA restriction context. CD4$^+$ and CD8$^+$ T cell responses were analyzed by using MAGE-A3 antigen in the form of either long overlapping peptides covering the entire sequence of MAGE-A3 or recombinant adenovirus encoding full-length MAGE-A3.

In Fig. 2*A*, a representative CD4$^+$ T cell response of each patient is shown for individual peptides or peptide pools. Similar to what was observed for antibodies, there were major differences between the two cohorts. Of seven patients from cohort 1, only patient EC-01 showed a strong CD4$^+$ T cell response against a peptide pool from MAGE-A3, which was already detectable in the first vaccine cycle and was maintained during the second cycle. It cannot be excluded that the response in this MAGE-A1 seropositive patient was due to cross-reactivity of potential preexisting CD4$^+$ T cell responses against MAGE-A1. Another two patients (SG-09 and GB-02) developed weak CD4$^+$ T cell responses to their respective MAGE-A3 peptide pool at the very end of the second vaccine cycle. There was no correlation of

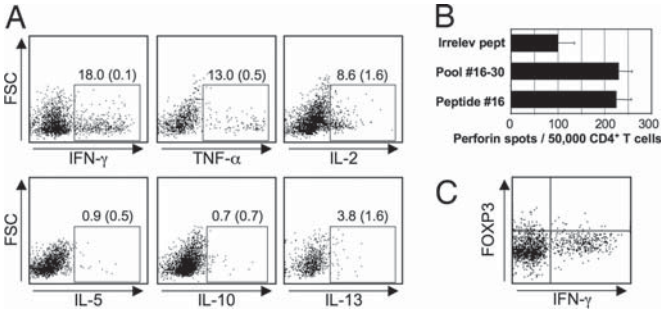


Fig. 3. Functional analyses of vaccine-induced MAGE-A3-specific CD4$^+$ T cells. (*A*) Intracellular cytokine staining of CD4$^+$ T cells of representative patient LK-19 on day 43b responding to vaccination with MAGE-A3 protein. CD4$^+$ T cells were presensitized for 14 days by using pooled MAGE-A3 peptides, and intracellular cytokine staining of Th1 (*Upper*) and Th2 (*Lower*) cytokines was performed after reexposure to single MAGE-A3 peptides. To differentiate effector from target cells, T-APC were stained beforehand by using the intracellular dye CFSE and gated out. Percentages of CD4$^+$ effector T cells expressing the given cytokine in response to MAGE-A3 peptide 141–160 are indicated. Background levels were determined by using T-APC pulsed with irrelevant peptide (irrelev pept) and are shown in parentheses. (*B*) Perforin ELISPOT assays for cytolytic activity of MAGE-A3-specific CD4$^+$ T cells. Representative results are shown for CD4$^+$ T cells obtained on day 85 from patient WG-13 responding against pooled MAGE-A3 peptides (#16–30) and single MAGE-A3 peptide 151–170 (#16). (*C*) After *in vitro* sensitization with pooled peptides, MAGE-A3-specific CD4$^+$ T cells did not coexpress Treg marker FOXP3. Representative results of intracellular costaining of IFN-γ and FOXP3 are shown for MAGE-A3 141–160-specific CD4$^+$ T cells of patient LK-19 obtained on day 43b.

CD4$^+$ T cell responses with antibody elicited against MAGE-A3 in cohort 1. None of the patients from cohort 1 developed any CD8$^+$ T cell response in either vaccine cycle (Fig. 2*B*).

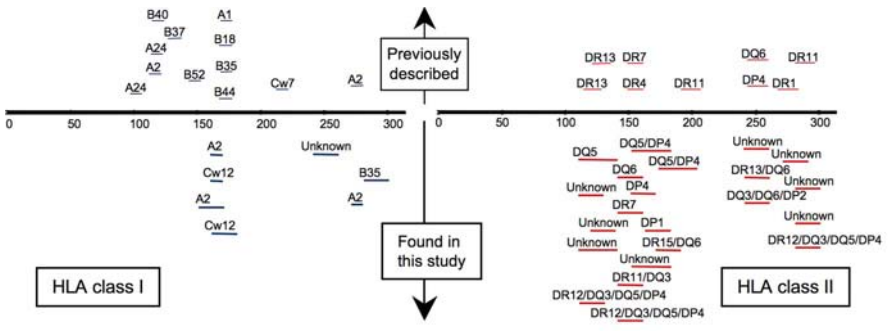
In contrast, six of seven patients in cohort 2 developed a CD4$^+$ T cell response to MAGE-A3 that could be detected in most cases after the first vaccine cycle. In all six patients, the CD4 responses could be recalled during the second vaccine cycle (Fig. 2*A*). In addition, two of seven patients (SC-11 and GO-17) in cohort 2 had CD8$^+$ T cell responses to MAGE-A3 during the second vaccine cycle (Fig. 2*B*). Interestingly, these two patients showed some of the strongest antibody titers to MAGE-A3 (Fig. 1) and serological cross-reactivity to other MAGE antigens (data not shown).

By analyzing intracellular expression of 6 different cytokines in individual responding patients, we observed, as in our previous study (7), that vaccine-induced CD4$^+$ T cells specifically produced Th1-type (IFN-γ, IL-2, TNF-α) but not Th2-type (IL-5, IL-6, IL-10) cytokines (Fig. 3*A*). We also observed cytolytic activity of MAGE-A3-specific CD4$^+$ T cells in Perforin ELISPOT assays (Fig. 3*B*). These MAGE-A3-specific CD4$^+$ T cells did not coexpress FoxP3, a marker for T regulatory cells (Tregs; Fig. 3*C*) even after *in vitro* sensitization.

Finally, the two patients that had received continuous vaccination with MAGE-A3 protein plus AS02B developed CD4$^+$ and CD8$^+$ T cell responses to MAGE-A3 peptide pools after extensive vaccination (Fig. 2 *A* and *B*). In addition, both patients developed antibodies against MAGE-3 with titers even higher than patients in cohort 2 (Fig. 1).

Mapping of Previously Uncharacterized T Cell Epitopes Naturally Processed from MAGE-A3. Fig. 2 showed the strongest representative T cell responses of each patient against single peptides or peptide pools from MAGE-A3, and these appeared to have dynamic characteristics, possibly reflecting shifting immunodominance or mobility in the localization and accessibility of T cell effectors from peripheral blood. Yet many patients developed polyclonal multiepitopic CD4$^+$ T cell responses that were mapped by testing reactivity with individual peptides. HLA restriction was assayed by using partially histocompatible targets in ELISPOT assays. Altogether, almost all patients who developed CD4$^+$ T cell responses to MAGE-A3 did so against more than one epitope. For example, patient EC-01 had a CD4$^+$ T cell response that was mapped to peptide MAGE-A3 141–160 and appeared restricted by HLA-DR07 whereas patient SC-11 CD4$^+$ T cells reacted to the same epitope but in the context of HLA-DR11 (SI Fig. 6*A*). This patient also had detectable CD4$^+$ T cell responses to MAGE-A3 peptides 111–130 and 281–300 in HLA contexts that remain to be determined (data not shown). Collectively, we were able to identify a large number of previously unknown CD4$^+$ epitopes of MAGE-A3 by analyzing vaccine-induced T cell responses in our patients after protein vaccination (Fig. 4).

We also mapped CD8$^+$ T cell responses against MAGE-A3 in patient GO-17 to peptide 281–300 restricted by HLA-B35 and in patient ML-S2 to peptide 160–169 with promiscuous HLA restriction (Fig. 4). Notably, CD8$^+$ T responses were also seen in these MAGE-A3 protein-vaccinated patients after a sensitization using adenovirus encoding full-length MAGE-A3 (SI Fig. 6*B*). This indicates that the MAGE-A3 antigen was naturally processed by antigen presenting cells and allowed the stimulation of specific precursors primed *in vivo* by the vaccine. Although no tumor cell lines with the proper HLA restriction and antigen expression could be tested for direct T cell recognition, the results with recombinant adenovirus sensitization argue in favor of a vaccine-induced repertoire capable of recognizing naturally processed MAGE-A3 antigen.



MAGE-3 peptide sequence	HLA class I potential restriction element
151-170	A*02
160-169	A*02
160-169	Cw*12
161-180	Cw*12
241-260	Unkwown
281-300	B*35

MAGE-3 peptide sequence	HLA class II potential restriction element
110-140	DQB1*05
110-140	Unknown
111-130	DRB1*12/DQB1*03/DQB1*05/DPB1*04
141-160	DQB1*06
141-160	DRB1*12/DQB1*03/DQB1*05/DPB1*04
151-170	DPB1*04

Fig. 4. Summary of T cell epitopes from MAGE-A3 previously described and found in this study. (*Left*) Epitopes in the context of HLA class I (left, blue bars) or class II (right, red bars) are shown aligned to scale along the amino acid sequence of full-length MAGE-A3 protein (black graduated bar). Whenever defined, a selection of potential HLA restriction alleles is indicated. (*Right*) Summary of HLA class I- or class II-restricted epitopes of MAGE-A3 defined by analyzing T cell responses after vaccination with full-length MAGE-A3 protein.

Discussion

The potential clinical impact of the vaccine formulation described in this article has been reported recently (11). Results from a randomized phase II placebo-controlled multicentered trial indicated efficacy in improving disease-free survival of stage II NSCLC patients vaccinated with MAGE-A3 protein plus AS02B in the adjuvant setting. Based on this finding, a phase III study has been proposed.

A detailed analysis of antibody as well as $CD4^+$ and $CD8^+$ T cell responses in patients immunized with MAGE-A3 protein is indispensable for further improving our understanding of the immunological basis of this approach and for optimizing future vaccination therapies. We showed here that vaccination with a recombinant MAGE-A3 fusion protein plus adjuvant AS02B induces consistent high-titered antibody and broad polyclonal $CD4^+$ T cell responses in patients with NSCLC. We also detected MAGE-A3-specific $CD8^+$ T cell responses in patients who developed the highest-titered broadly specific antibody responses after vaccination. So far, few trials have been conducted in which recombinant proteins were used as immunogen in cancer vaccines (12–16), and if T cell responses have been reported, they consisted mainly of $CD4^+$ T cells (17, 18). Two reports have shown that vaccination with CT antigen NY-ESO-1 as a recombinant protein, formulated either with saponin-based adjuvant ISCOMATRIX or with cholesterol-bearing hydrophobized pullulan, induced strong antibody as well as $CD4^+$ and $CD8^+$ responses in the majority of patients (19, 20). In another recent trial with NY-ESO-1 protein, $CD8^+$ T cell responses correlated with strong antibody responses in half of patients vaccinated with NY-ESO-1 protein mixed with Incomplete Freund's Adjuvant and CpG (21). In these studies and in the study reported here with MAGE-A3 protein, induction of strong antibody responses against the cancer antigen were prominent features of the vaccine. A possible role for antibodies may be to form immune complexes with the vaccine antigen and thus facilitate its cross-presentation to $CD8^+$ on MHC class I molecules, as we have shown *in vitro* (22). Additionally, inflammatory signals, i.e., mediated by Toll-like receptors, may further enhance cross-presentation (23, 24).

$CD4^+$ T cells have been shown to also play a decisive role in antitumor responses after vaccination (25, 26). Importantly, although a main function of $CD4^+$ T cells in this setting is to provide help for the initiation, the amplification, and the maintenance of $CD8^+$ T cell responses (27, 28), $CD4^+$ T cells are also capable of activating effector cells other than $CD8^+$ cells. For instance, tumor-infiltrating cells such as eosinophils and macrophages contribute to an effective antitumor response after activation by neighboring tumor-specific $CD4^+$ T cells (29–32). We showed that vaccine-induced $CD4^+$ T cells were capable of producing the cytolytic molecule perforin and, accordingly, may have an immediate effector function against tumors, as has been shown *in vitro* (33, 34) and *in vivo* (35). Finally, IFN-γ secreted by tumor-infiltrating $CD4^+$ T cells or activated bystander cells has the potential to promote tumor recognition and elimination by up-regulating expression of MHC molecules and might also contribute to the inhibition of tumor angiogenesis (36, 37).

One major goal of every tumor vaccine approach must be to generate persisting T cell memory to guarantee continuous surveillance of tumor development and progression. However, the question of whether persistence of antigen is necessary for maintaining T cell memory is still a subject of intense debate (38). Furthermore, information on the durability of vaccine-specific T cell responses during the months and years after discontinuation of vaccination has been extremely limited. A slow decline of specific T cells has been described only in individual patients over the period of several months after repeated peptide vaccination (39–41). In our current study, we demonstrate that vaccination with the recombinant protein of a tumor antigen is capable of inducing memory T and B cell responses that persists for at least 2 years after the last application of the vaccine. Importantly, such immune responses could be boosted by a single readministration of the recombinant MAGE-A3 protein antigen leading to a rapid reactivation of MAGE-A3-specific immune responses in those patients who had been primed in the right immunological context.

We have also reported that absence of adjuvant in the vaccine during priming resulted in the failure of the vaccine to induce detectable antibody and T cell responses, an observation confirmed by others (42, 43). Our study now demonstrates that immune memory was also persistent in patients primed with MAGE-A3 protein without an inflammatory adjuvant. However, this memory effect was manifested by a profound antigen-specific tolerance that compromised further vaccination attempts to convert and rescue an effective MAGE-A3-specific immune response. Although we cannot exclude the possibility that the route of administration (i.d. versus i.m.) contributed to the induction of tolerance in patients receiving MAGE-A3 protein without adjuvant, we are convinced that the absence of adjuvant represented the critical point. Our findings, therefore, support the view that the longevity of T cells memory and its biological characteristics are irreversibly imprinted at the time of immune priming. Our data also highlight the need for the addition of an adjuvant to the initial priming phase for the tumor antigen but not for foreign proteins, such as influenza virus protein D in our fusion protein construct, suggesting that there may be an additional level of regulation or tolerance to overcome when targeting MAGE-A3-specific immunity and that the presence of noncognate immunogenic epitopes from protein D did not help establishing MAGE-A3 T cell responses.

How can future tumor vaccine approaches be further optimized? Although there are only very limited data on the effect of multiple cycles of vaccination in humans, a few studies support the idea of progressive strengthening of tumor-specific immunity over prolonged courses of vaccination (18, 20, 44). As we observed in this study with broadening of immune responses upon repeated vaccination, the application of serial vaccinations might indeed improve the immunological, and hopefully also clinical, efficacy of cancer vaccines. Another way to further improve future tumor vaccine approaches might be to counteract systemic or local immunosuppressive influences. In addition to their role in suppressing autoimmune responses, Tregs represent a main obstacle of an effective antitumor T cell response (45) and might even be induced by tumor vaccination, thus undermining a clinically relevant immune response. An ideal vaccine would generate effectors with intrinsic resistance to regulatory mechanisms, and this probably needs to occur at priming, as suggested by our data on immune imprinting. Although limited patient material kept us from performing a more detailed analysis of the role of Tregs in the immune response developed after vaccination with MAGE-A3 protein, we hypothesize that the majority of $CD4^+$ T cells induced in vaccinated patients represent conventional memory T cells. First, MAGE-A3-specific $CD4^+$ T cells were, even after *in vitro* stimulation, negative for FoxP3, the most specific Treg marker to date (46–48). Second, as in our first analysis (7), MAGE-A3-specific $CD4^+$ T cells strongly produced IL-2 and TNF-α, cytokines not associated with Tregs (49). In addition, none of the $CD4^+$ produced cytokines such as IL-10, which is found in certain Treg subtypes (50, 51).

Together, results from this study highlight critical parameter for a successful cancer vaccine, which include: use of antigen formulations consisting of recombinant proteins or long peptides, addition of a proper adjuvant for priming, generation of long lasting memory, application of serial vaccinations, and the design of effective booster strategies. In conjunction with additional measures using knowledge on important costimulatory

mechanisms to counteract immune regulation, vaccine strategies will hopefully transform into an effective weapon against human cancer.

Materials and Methods

Patients and Vaccine Composition and Administration. The vaccine containing 300 μg of a MAGE-A3 fusion protein (consisting of a His-tagged full-length MAGE-A3 protein and influenza protein D (GlaxoSmithKline), was administered in saline or AS02B (monophosphoryl lipid A and QS21; GSK) once every 3 weeks for four consecutive injections (equal to one cycle). Patients without evidence of disease after surgical resection of stage I or II NSCLC were split into two cohorts as part of protocol LUD99–010 approved by the IRBs of the Ludwig Institute for Cancer Research and of Weill Medical College of Cornell University (SI Fig. 7). Cohort 1 first received one cycle of the fusion protein alone i.d. in the absence of adjuvant, and up to 3 years later (average 945 $\pm$ 68 days) received another cycle of the fusion protein i.m. with adjuvant AS02B. Cohort 2 received two cycles of fusion protein i.m. with adjuvant AS02B spaced by up to 2 years (average 598 $\pm$ 164 days). In addition, two patients with pancreatic neuroendocrine cancer and pediatric osteogenic sarcoma, respectively, who were enrolled in IRB-approved compassionate single-patient protocols, received eight consecutive triweekly i.m. injections of MAGE-A3 protein with adjuvant AS02B. Peripheral blood mononuclear cells (PBMC) and plasma were collected and cryopreserved before each vaccination as well as 3 weeks after the last injection.

Serological Analyses Against Recombinant Proteins. Patient plasma samples were analyzed by ELISA for seroreactivity to various recombinant full-length protein antigens (baculovirus-derived MAGE-A3, *E. coli*-derived protein D, MAGE-A4, NY-ESO-1, LAGE-1, and p53), to recombinant truncated proteins (MAGE-A3 64–226, MAGE-A1 57–219), and to the vaccine fusion protein MAGEA3/ProtD/His itself as described (7) with modifications *SI Materials and Methods* for details and titer calculations).

Monitoring of $CD4^+$ and $CD8^+$ T Cell Responses. Monitoring of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells specific for MAGE-A3 was performed by ELISPOT and CYTOSPOT after a single *in vitro* presensitization as described (7–10, 52) with modifications *SI Materials and Methods* for details).

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CD4+CD25+FOXP3+ T regulatory cells reconstitute and accumulate in the bone marrow of patients with multiple myeloma following allogeneic stem cell transplantation

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ABSTRACT

Background
Very little is known about the number and function of immunosuppressive CD4+CD25+FOXP3+ T regulatory cells (Treg) in the human bone marrow and it is unclear whether bone marrow-residing Treg are capable of regenerating following allogeneic stem cell transplantation. This is particularly surprising since the bone marrow represents a major priming site for T-cell responses and Treg play important roles in the prevention of T-cell-mediated graft-versus-host disease and in promoting tumor escape from T-cell-dependent immunosurveillance.

Design and Methods
Applying flow cytometry, real-time polymerase chain reaction, and functional assays, we performed the first study on bone marrow and peripheral blood Treg in healthy donors as well as multiple myeloma patients before and after allogeneic stem cell transplantation.

Results
We found that, following the allogeneic transplantation, donor-derived CD4+CD25+FOXP3+ Treg expanded faster than conventional CD4+ T cells, leading to an accumulation of Treg in the bone marrow of transplanted patients who lack relevant thymic function. The reconstituted bone marrow-residing CD4+CD25+FOXP3+ Treg of myeloma patients after allogeneic stem cell transplantation consisted preferably of CD45RA-CCR7- memory T-cells and contained low numbers of T-cell receptor excision cycles, indicating that Treg had indeed expanded outside the thymus. Importantly, bone marrow-residing Treg of newly diagnosed and myeloma patients after allogeneic stem cell transplantation expressed high levels of transforming growth factor β and cytotoxic T-lymphocyte antigen 4, and showed a strong inhibitory function.

Conclusions
We suggest that allogeneic stem cell transplantation provides a short but significant window of opportunity for CD8+ T cells before an exuberant regeneration of immunosuppressive Treg sets in. Later after transplantation, bone marrow-residing Treg probably contribute to suppressing graft-versus-host disease but may also undermine persistent immune control of multiple myeloma.

Key words: multiple myeloma, immunology, tumor immunology, T cells, T regulatory cells, transplantation

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Introduction

Multiple myeloma is a clonal B-cell malignancy characterized by an accumulation of mature plasma cells in the bone marrow leading to bone destruction and bone marrow failure. Myeloma remains incurable and even with high-dose chemotherapy and autologous stem cell transplantation (SCT) patients show a median survival of only 3 to 5 years.[1] Following allogeneic SCT lower relapse rates have been reported, probably due to a graft-versus-myeloma effect mediated by donor lymphocytes.[2] A proportion of patients treated with allogeneic SCT even achieve molecular remission resulting in long-term freedom of disease.[3] Unfortunately, these therapeutic improvements have been hampered by a high treatment-related mortality[4] based on the fact that immune responses derived from the allogeneic graft are not strictly myeloma-specific and are, therefore, associated with immune-mediated side effects.

For more than two decades, one principal goal in the field of allogeneic SCT has been to segregate beneficial graft-versus-myeloma effects from life-threatening graft-versus-host disease (GVHD). We reasoned that induction chemotherapy might play an important role in the generation of graft-versus-myeloma effects since it has been shown that chemotherapy-induced lymphodepletion is capable of boosting antitumor immunity.[5] The eradication of the immunosuppressive influence of regulatory T cells has been proposed as one mechanism underlying this paradigm.[6]

Prevention of immune reactivity to self-antigens is primarily achieved through negative selection in the thymus, however, some autoreactive T cells escape into the periphery and several mechanisms are implemented to keep such anti-self T cells in check. It has recently become clear that peripheral tolerance is largely maintained by immunosuppressive regulatory T cells (Treg), such as $CD4^+CD25^+$ T cells, which typically co-express transcription factor forkhead box P3 (FOXP3).[7] Animal models have shown that $CD4^+CD25^+$ Treg have the potential to prevent GVHD following allogeneic SCT by inhibiting pathogenic T cells.[8] Accordingly, the numbers of Treg seem to be reduced in the peripheral blood of patients suffering from GVHD[9] and increased $CD4^+CD25^+FOXP3^+$ Treg numbers in donor-derived stem cell transplants result in a diminished risk of GVHD.[10,11]

Unfortunately, in addition to their role in suppressing autoimmune responses, Treg also represent a main obstacle to an effective anti-tumor T-cell response.[12] Patients with solid tumors have increased numbers of Treg in their peripheral blood and tumor-infiltrating Treg are associated with reduced survival in cancer patients.[12] In animal models, elimination of Treg led to increased tumor-specific immune responses[13] and to an enhanced T cell-mediated rejection of established tumors.[14] Accordingly, depletion of $CD4^+CD25^+$ Treg seems to enhance anti-tumor immunity in cancer patients.[15]

Practically nothing is known about the presence and function of Treg in the human bone marrow, especially after allogeneic SCT. This seems surprising since studies have emphasized the role of the bone marrow as a major priming site for T-cell responses[16,17] and T cells directed against solid tumors are enriched within this compartment.[18,19] Furthermore, bone marrow-residing memory T cells are involved in the control of dormant hematologic malignancies,[20] and, in patients with myeloma, such bone marrow-infiltrating lymphocytes have the potential to target myeloma cells and their precursors.[21] Based on these findings and the fact that the bone marrow represents the immediate tumor environment of myeloma, we conducted the first study focusing on marrow-residing $CD4^+CD25^+FOXP3^+$ Treg.

$CD4^+CD25^+FOXP3^+$ Treg have traditionally been thought to be generated exclusively in the thymus and there is still no definitive answer to the question whether human $CD4^+CD25^+FOXP3^+$ Treg can expand in the periphery or whether peripheral non-regulatory T cells can convert into human Treg *in vivo*.[22] In our patients, donor-derived stem cell preparations containing conventional T cells as well as $CD4^+CD25^+FOXP3^+$ Tregs were transferred into recipients who were lacking relevant thymic function. While thymic involution begins in early childhood, the thymus may retain some low-level activity during adult life in supporting T-cell differentiation.[23] High-dose chemotherapy, however, has devastating effects on the thymus[24] and GVHD in patients following allogeneic SCT further contributes to the destruction of thymic function.[25] Overall, it seems highly unlikely that the thymus has the capacity to make a significant contribution to the reconstitution of naïve T cells in elderly patients who have undergone allogeneic SCT. Any significant expansion of Treg in these patients should, therefore, be based on the proliferation of donor Tregs or on the conversion of donor-derived conventional T cells into Treg.

In this study, we performed the first systematic analysis of Treg numbers and function in the human bone marrow examining myeloma patients treated with allogeneic SCT. For comparison, Treg of healthy bone marrow donors and newly diagnosed myeloma patients were analyzed and peripheral blood Treg of the same patients were examined in parallel. The potential of Treg to reconstitute following allogeneic SCT was explored and the immunosuppressive function of marrow-derived Treg was investigated.

Design and Methods

Patients and healthy stem cell donors

Forty consecutive post-allogeneic SCT myeloma patients, 17 newly diagnosed myeloma patients, 24 healthy bone marrow donors and 15 blood donors were studied. Healthy subjects and myeloma patients, who were admitted for treatment at the University Medical Center Hamburg-Eppendorf, gave informed consent in accordance with the Declaration of Helsinki of 1975, as revised in 2000. All patients treated with allogeneic SCT had undergone previously received conventional chemotherapy and autologous SCT. Patients received pretransplant conditioning with melphalan (140 mg/m^2) and fludarabine (90-150 mg/m^2) within

a prospective trial as recently reported.[26] GVHD prophylaxis consisted of antithymocyte globulin (60 mg/kg) in the case of unrelated SCT, short course methotrexate on days +1, +3, and +6, and cyclosporine A (3 mg/kg) until day +180. The study protocol was approved by the local ethics committee (Hamburger Ärztekammer; decision number OB-038/06).

Bone marrow and blood samples

Bone marrow and blood samples from myeloma patients were obtained during routine diagnostic procedures. Samples obtained from consenting healthy donors were part of marrow or blood donations. Mononuclear cells were isolated by density gradient centrifugation and underwent immediate analysis by flow cytometry or were cell-sorted for extraction of RNA or genomic DNA.

Flow cytometry

Fresh bone marrow for flow cytometry was available from 40 post-allogeneic SCT myeloma patients, 17 newly diagnosed myeloma patients, and 15 healthy bone marrow donors. Peripheral blood was available from 18 of the post-transplant patients and 14 of the newly diagnosed patients. Mononuclear cells were stained using monoclonal antibodies to CD4, CD25, CCR7 (R&D Systems, Minneapolis, MN, USA), CD8, and CD45RA (BD Biosciences, San Jose, CA, USA) and appropriate IgG isotype controls. Co-staining of intracellular FOXP3 was performed applying anti-FOXP3 monoclonal antibody PCH101 (eBioscience, San Diego, CA, USA). Samples were analyzed using a FACSCalibur cytometer and CELLQuest software (BD Biosciences).

Purification of CD4+CD25+ Treg and inhibition assay

$CD4^+$ T cells were positively selected using magnetic beads (Dynal, Oslo, Norway). $CD4^+CD25^+$ cells were isolated from $CD4^+$ cells using anti-CD25 beads (Miltenyi Bergisch Gladbach, Germany). Autologous bone marrow or peripheral $CD4^+$ T cells (2.5×10^4) were stimulated with 5 µg/mL soluble anti-CD3 (BD Biosciences) and 0.5 µg/mL anti-CD28 (eBioscience) antibodies. As feeder cells, 5×10^5 irradiated autologous peripheral blood mononuclear cells, which had been depleted of $CD3^+$ T cells, were added. To determine the inhibitory capacity of Treg, 2.5×10^4 $CD4^+$ $CD25^+$ T cells were added to each well. Cells were co-cultured in 96-well round-bottomed plates in a final volume of 200 µL complete RPMI containing 10% human serum for 5 days. Cell proliferation was measured using the Biotrak™ cell proliferation enzyme-linked immunosorption assay system (Amersham Biosciences, Piscataway NJ, USA). Responder cells were pulsed with 10 µM bromodeoxyuridine for the last 18 hours of culture. Following fixation, peroxidase-labeled anti-bromodeoxyuridine was added and absorbance was read at 450 nm using a microtiter plate spectrophotometer (SLT Labinstruments, Salzburg, Austria).

Real-time polymerase chain reaction

T-cell subpopulations were isolated from total bone marrow mononuclear cells using a FACSAria cell sorter (BD Biosciences). Extraction of genomic DNA was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extraction of RNA and reverse transcription were performed using the RNeasy Mini Kit (Qiagen) and AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time polymerase chain reaction was performed as described previously.[27] Primer sequences for target genes and the housekeeping gene glyceraldehydephosphate dehydrogenase (*GAPDH*) are given in *Supplementary Table 1*.

Statistical analysis

The Mann-Whitney U test was used to calculate differences between groups of patients and Wilcoxon's test was applied to determine significant differences between immune parameters within the same groups of patients. Spearman's rank correlation was used to analyze correlations between patients' characteristics and immunological parameters. Results were considered statistically significant if $p<0.05$.

Results

Patients' characteristics

Reflecting the general myeloma population, newly diagnosed and post-allogeneic SCT patients showed a male predominance; the typical patient was around 60 years old and IgGκ was the most common idiotype (*Supplementary Table 2*). The only significant differences between these two groups were a lower number of bone marrow-infiltrating plasma cells ($p=0.001$) and a higher serum albumin ($p=0.003$) in the post-transplant myeloma patients.

The myeloma patients who had undergone allogeneic SCT had all received the same induction chemotherapy. At the time of analysis, all transplanted patients had fully engrafted and showed complete chimerism. The median interval between transplantation and analysis was 37 months (range, 4-110 months). Thirty-one (77.5%) of the post-allogeneic SCT patients were in complete or near-complete remission (immunofixation-positive), seven (17.5%) had suffered a relapse, and two (5.0%) showed progressive disease. Most of the transplanted patients (n=33; 82.5%) were off immunosuppressive medication and did not display any signs of GVHD. However, one patient suffered from acute GVHD (grade II) and six patients had limited (n=3) or extensive (n=3) chronic GVHD.

CD4+FOXP3+ Treg reconstitute in the bone marrow of myeloma patients after allogeneic SCT

Since it was unknown whether human bone marrow-derived Treg would show a pattern of reconstitution comparable to that observed for non-regulatory T cells, we first examined whether the time passed since transplantation was related to the number of Treg present in the bone marrow of our post-transplant myeloma patients. Plotting the percentages of bone marrow-residing $CD8^+$ T cells against

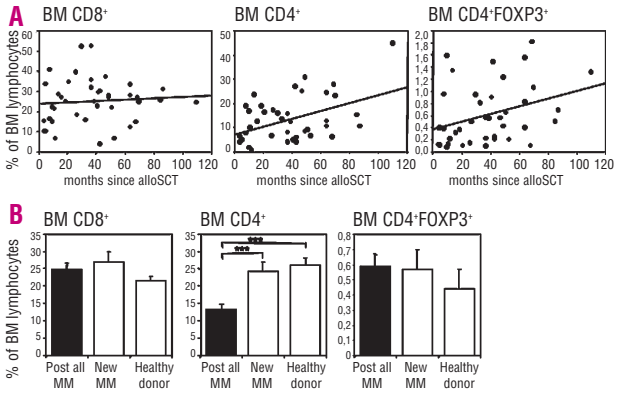

Figure 1. CD4⁺FOXP3⁺ Treg reconstitute in the bone marrow of myeloma patients after allogeneic SCT. Percentages of bone marrow-residing CD8⁺ T cells, CD4⁺ T cells, and CD4⁺FOXP3⁺ Treg were determined in 17 newly diagnosed myeloma patients (MM), in 40 myeloma patients following allogeneic SCT (Post MM) and in 15 healthy bone marrow donors using flow cytometry. Data are given as percentage of total bone marrow (BM) lymphocytes plotted against the number of months since allogeneic SCT (A). Mean percentages of each T-cell subset were also compared between groups (B). Bars show mean values ± standard error of mean. Asterisks indicate statistically significant differences between groups (*p<0.001).**

the number of months that had passed since transplantation did not show a significant association between the two parameters. In contrast, percentages of marrow-residing conventional CD4⁺ T cells correlated strongly (p=0.01, r=0.46) with the time passed since transplantation. The same was true for CD4⁺FOXP3⁺ Treg which seemed to have undergone a significant (p=0.02, r=0.37) expansion in the bone marrow of patients following allogeneic SCT (Figure 1A). Importantly, conventional T cells as well as Treg were both donor-derived, as indicated by donor-specific real-time polymerase chain reaction analysis (*data not shown*).

Next, we analyzed whether immune reconstitution had led to complete replenishment of the bone marrow with donor-derived Treg and conventional T cells at the median time of 37 months post-transplantation. We observed comparable frequencies of bone marrow-residing CD8⁺ T cells in post-allogeneic SCT patients, newly diagnosed patients, and healthy bone marrow donors (Figure 1B), indicating that CD8⁺ T cells had already fully reconstituted in our transplanted patients, a finding supporting earlier studies showing a very rapid expansion of CD8⁺ T cells during the first weeks following allogeneic SCT.[28] Such a rapid expansion of CD8⁺ very early after transplantation would also explain why a relation between the number of these cells and the time passed since transplantation was not detectable at our study's median follow-up time of 37 months post-transplantation.

When we analyzed percentages of bone marrow-residing CD4⁺ T cells in all three groups, we found markedly decreased numbers of these cells in the transplanted patients compared to newly diagnosed patients and healthy controls (Figure 1B), supporting previous studies showing diminished numbers of conventional CD4⁺ T cells for several years after allogeneic SCT. Surprisingly, however, this was not the case for donor-derived CD4⁺FOXP3⁺ Treg, which, in contrast to their non-regulatory CD4⁺counterpart, had already reached levels comparable to those in healthy volunteers, indicating a profound difference between the two types of CD4⁺ cells regarding their potential to expand after myeloablative therapy in these patients without significant thymic function.

Immune reconstitution results in the accumulation of CD4⁺CD25⁺FOXP3⁺ Treg in the bone marrow of myeloma after allogeneic SCT

Analyzing whether the preferential reconstitution of Treg might have led to an accumulation of these cells within the bone marrow of post-allogeneic SCT myeloma patients, we indeed observed a strong enrichment of CD4⁺FOXP3⁺ Treg in the transplanted patients compared to in newly diagnosed patients and healthy controls (Figure 2). As shown in the representative dot plot in Figure 2, we generally found only a minority of all bone marrow-resident CD25⁺ T cells to be positive for FOXP3, while nearly all FOXP3⁺ T cells simultaneously expressed CD25. In contrast to FOXP3-CD25⁺ T cells, which are probably activated T cells, these triple-positive CD4⁺CD25⁺FOXP3⁺ cells most likely represent *true* Tregs and were also strongly enriched among the CD4⁺ T cells of the post-transplant myeloma patients (Figure 2).

In accordance with the idea that Treg might contribute to the suppression of GVHD in patients after allogeneic SCT, we observed lower percentages of CD4⁺FOXP3⁺ Treg in the

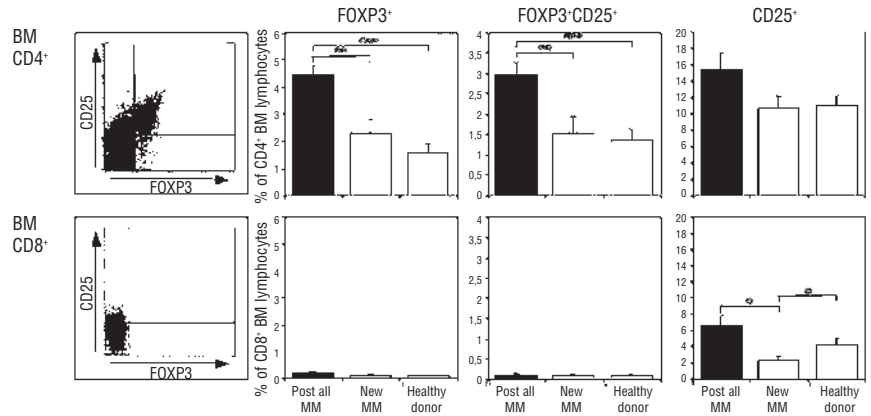

Figure 2. Accumulation of CD4⁺FOXP3⁺ Treg in the bone marrow of myeloma patients after allogeneic SCT. Percentages of bone marrow-residing CD4⁺ (upper row) and CD8⁺ (lower row) T cells expressing FOXP3 and/or CD25 were determined using flow cytometry in 40 myeloma patients following allogeneic SCT, 16 newly diagnosed myeloma patients, and 15 healthy donors. Data are given as percentages of all CD4⁺ and CD8⁺ T cells expressing the given combination of antigens. Bars show mean values + standard error of mean. Asterisks indicate statistically significant differences between groups (*p<0.05, **p<0.01, ***p<0.001).

bone marrow of our patients with GVHD than in those without GVHD (*data not shown*). However, given the small number of patients suffering from allo-immune phenomena, this finding failed to reach statistical significance (p=0.13). Interestingly, the presence of progressive disease or relapse of myeloma did not have any influence on the numbers of Treg present in the patients' bone marrow, supporting the hypothesis that bone marrow-residing Treg might contribute to the prevention of GVHD while allowing immune-mediated graft-versus-myeloma effects.

It has recently been reported that FOXP3+ Treg are also present among CD8+ T cells.[29] However, we never observed protein expression of FOXP3 in bone marrow CD8+ T cells (Figure 2B), suggesting that T cells with a regulatory function are restricted to the CD4+ subpopulation. In contrast, we did observe significant expression of CD25 on marrow-residing CD8+ T cells (Figure 2), but, as in the case of CD4+ T cells, there were no differences regarding CD25 expression between groups, supporting the idea that CD25-expressing CD4+ and CD8+ T cells mostly represented activated non-regulatory T cells.

Comparing Treg numbers in the bone marrow with those in the peripheral blood, we observed that Treg had indeed reconstituted in the periphery and that, in general, the percentages of these cells were higher in the peripheral blood than in the bone marrow (Figure 3). Importantly, however, donor-derived CD4+FOXP3+ Treg in post-allogeneic SCT myeloma patients were much more likely to remain in the bone marrow than the same cells in the control group (Figure 3), indicating a promoting effect of allogeneic SCT on Treg homing into the bone marrow compartment.

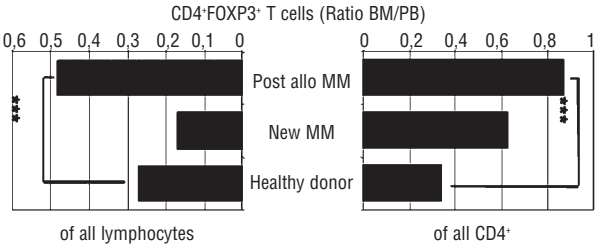

Figure 3. Ratio of bone marrow/peripheral blood CD4+FOXP3+ Treg as an indicator of Treg homing into the bone marrow compartment. Percentages of bone marrow-residing and corresponding peripheral blood CD4+FOXP3+ Treg were determined using flow cytometry in 14 patients with newly diagnosed myeloma and in 18 myeloma patients following allogeneic SCT. The ratio of bone marrow CD4+FOXP3+ Treg/peripheral blood CD4+FOXP3+ Treg was calculated as an indicator of Treg homing to the bone marrow compartment. Since peripheral blood mononuclear cells were not available from healthy bone marrow donors, peripheral blood mononuclear cells of 15 consecutive healthy blood donors were analyzed for comparison. Bars show mean ratios. Asterisks indicate statistically significant differences between groups (*p<0.001).**

Bone marrow-residing CD4+CD25+FOXP3+ Treg express high levels of transforming growth factor β (TGF-β) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) and exhibit full inhibitory function

Since a recent study indicated a diminished function of peripheral CD4+CD25+ Treg in patients with myeloma,[30] we next investigated the functional properties of the Treg in the bone marrow of our post-transplant patients. We found that bone marrow-residing Treg generally exerted a very strong inhibitory effect on the proliferation of autologous non-regulatory CD4+ T cells. In some cases, the inhibitory capacity of bone marrow Treg even seemed to be stronger in both groups of myeloma patients than in healthy donors (Figure 4A). Bone marrow CD4+CD25+ Treg from myeloma patients were as potent in suppressing the proliferation of conventional CD4+ as were CD4+CD25+ Treg derived from the peripheral blood of the same patient (*Supplementary Figure 1A*).

In order to identify some of the possible mediators of the immunosuppressive function of bone marrow Treg, we analyzed, for the first time, the expression of a number of key molecules which have been linked to the inhibitory function of these cells, in Treg residing in the human bone marrow. When we analyzed FACS-sorted bone marrow CD4+CD25+ T cells for their expression of a variety of genes using real-time polymerase chain reaction, we found that these cells were significantly enriched for Treg because only CD4+CD25+ T cells expressed high levels of FOXP3 and CD25 (*Supplementary Figure 1B*). Since it has been suggested that TGF-β1, CTLA-4, and interleukin 10 (IL-10) contribute to the immunosuppressive effects exerted by Treg *in vivo*,[22] we quantitatively analyzed the expression of these genes in Treg. While we did not observe any difference in the expression of IL-10 between CD4+CD25+FOXP3+ Treg and their CD4+CD25-FOXP3- counterparts, we found that only bone marrow Treg expressed significant levels of CTLA-4 while conventional CD4+CD25- T cells did not (*Supplementary Figure 1B*). The most striking hallmark of bone marrow-residing CD4+CD25+FOXP3+ Treg was, however, their strong expression of TGF-β1 which approached the expression levels of the *GAPDH* housekeeping gene (*Supplementary Figure 1B*). We, therefore, conclude that CTLA-4 and especially TGF-β1 might represent mediators of the immunsuppressive function of Treg residing in the bone marrow compartment.

Reconstituted bone marrow CD4+FOXP3+ Treg of post-allogeneic SCT myeloma patients represent memory T cells which have expanded outside the thymus

Finally, we investigated whether a peripheral expansion of Treg was responsible for the regeneration and the accumulation of Treg in the bone marrow compartment. T-cell receptor (TCR) diversity expressed on T-cell precursors is created in the thymus through recombination of gene segments encoding the variable parts of the TCR α and β chains. During these processes, by-products of the rearrangements are generated in the form of TCR excision circles (TREC). As these molecules are lost upon further mitotic cell division, their quantification is considered a valuable tool to measure the proliferative history of T cells and their developmental proximity to the thymus.[31]

In our current study, we investigated for the first time

TREC levels in bone marrow-residing Treg and other T-cell subpopulations in order to determine which cell types had undergone significant numbers of cell divisions in the periphery of myeloma patients following allogeneic SCT. We used $CD3^+CD4^-CD8^-$ cells as positive controls since these lymphocytes represent *early* T cells which have only recently been generated from T-cell precursors and which accumulate in the bone marrow.[32] As expected, $CD4^-CD8^-$ T cells showed very high numbers of TREC (*Supplementary Figure 2*), indicating that these T cells had indeed undergone only minimal numbers of cell divisions since they had been generated. In contrast, conventional $CD4^+$ and $CD8^+$ T cells showed very low TREC concentrations (*Supplementary Figure 2A*) based on the fact that these lymphocytes had expanded in the periphery following allogeneic SCT. Notably, we observed equally low levels of TREC in bone marrow-infiltrating $CD4^+CD25^+$ Treg (*Supplementary Figure 2A*), supporting the idea that following allogeneic SCT these cells had undergone as many peripheral cell divisions as their conventional T-cell counterparts.

The bone marrow is a site where memory T cells preferentially accumulate[33] and T cells can be divided into different memory subclasses based on their expression of CCR7.[34] Naïve and central memory T cells are CCR7-positive and home efficiently into lymphoid tissue whereas CCR7-negative effector memory T cells have the potential to migrate into peripheral sites of inflammation. Analyzing the expression of CCR7 on regular $CD4^+$ and $CD4^+FOXP3^+$ Treg we did not observe any differences between the groups (*Supplementary Figure 2B*). We suggest that this may be due to the fact that CCR7 is not only expressed on naïve T cells but also on antigen-experienced *central memory* T cells.[34] We did, however, observe a highly increased prevalence of CCR7-negative $CD8^+$ T cells in the bone marrow of all myeloma patients regardless of their treatment status (*Supplementary Figure 2B*), indicating that the presence of the disease in the bone marrow might cause a shift towards an effector phenotype among $CD8^+$ T cells.

Finally, we analyzed bone marrow-residing T cells for expression of CD45RA, which can be found on naïve T cells that have recently emigrated from the thymus but not on T cells that have evolved into memory-type T cells following release into the periphery.[35,36] While there was no difference between groups regarding $CD8^+$ T-cell expression of CD45RA, we observed a significantly reduced number of CD45RA-positive bone marrow $CD4^+$ T cells in post-allogeneic transplant patients compared to in newly diagnosed myeloma patients and healthy donors (*Supplementary Figure 2B*) demonstrating the defective thymus-dependent production of naïve $CD4^+$ T cells in these patients.

In all groups analyzed, bone marrow-residing $CD4^+FOXP3^+$ Treg were less likely to express CD45RA than their conventional $CD4^+$ counterparts. However, the expression of this marker was even further reduced following allogeneic (*Supplementary Figure 2B*), suggesting that the Treg reconstitution we had observed in the bone marrow of our patients following allogeneic SCT was indeed based on the expansion of donor-derived memory-type T cells and not on the *de novo* production of thymus-derived CD45RA-expressing Treg.

Discussion

To contribute to resolving the questions of whether in humans conventional T cells can convert into Treg and whether $CD4^+CD25^+FOXP3^+$ Treg have the capacity to proliferate and expand outside the thymus, we analyzed bone marrow-residing and peripheral Treg of post-allogeneic SCT myeloma patients who are unlikely to have relevant thymic function. In these patients, immune reconstitution following allogeneic SCT is characterized by a rapid expansion of peripheral $CD8^+$ memory T cells and a delayed recovery of $CD4^+$ T cells for several years post-transplantation.[28,37,38] Peripheral naïve T cells are generally reconstituted very late after transplantation[37,38] and, at least in the case of $CD4^+$ T cells, this regeneration of antigen-inexperienced cells is largely thymus-dependent.[39,40]

When we analyzed percentages of allogeneic $CD8^+$ T cells at a median time of 37 months after allogeneic SCT, we found that these cells had already fully reconstituted. The subset of conventional bone marrow $CD4^+$ T cells expanded during the years following allogeneic SCT although the numbers of these cells remained significantly reduced, suggesting that the patients' thymus was not capable of restoring these T cells and that peripheral expansion was not able to compensate fully for this deficit. In marked contrast, $CD4^+CD25^+FOXP3^+$ Treg, which also expanded following allogeneic SCT, were already completely reconstituted. In our patients, donor-derived stem cell preparations were transferred into recipients who had undergone myeloablative treatment and were devoid of relevant thymic function. The expansion of Treg in our patients must, therefore, have been based on the expansion of donor Treg or on the conversion of donor-derived non-regulatory T cells into $CD4^+CD25^+FOXP3^+$ Treg. In addition, we observed preferential expression of a memory phenotype by bone marrow-residing Treg. These observations further support the conclusion that the Treg expansion we observed was based on the division of memory-type Treg or on the conversion of conventional memory $CD4^+$ T cells into Treg. Finally, in conjunction with the above-mentioned findings, our observation of minimal levels of TREC in bone marrow-residing $CD4^+CCD25^+FOXP3^+$ Treg leads us to the conclusion that these Treg do indeed have the potential to expand outside the thymus as has been indicated for Treg in the peripheral blood.[11,41]

In our post-allogeneic SCT myeloma patients, Treg reconstitution resulted in an accumulation of donor-derived $CD4^+CD25^+FOXP3^+$ cells in the bone marrow. Our data suggest that one reason for this might be enhanced homing of Treg into the bone marrow compartment following allogeneic SCT where Treg might contribute to keeping GVHD under control.[42] It is not unlikely that bone marrow-residing Treg have the capacity to prevent GVHD without infiltrat-

ing the respective target organ.[43] In mice the ability of Treg to enter priming sites of pathogenic T cells within lymphatic tissue determines their ability to suppress GVHD[44] and the bone marrow compartment as a major site for T-cell priming might be an ideal place for Treg to act protectively against GVHD.

Alternatively, bone marrow-residing myeloma cells might use Treg to evade immunosurveillance. Thus, solid tumors seem to actively attract FOXP3-expressing Treg into their environment where they undermine the induction of tumor-specific immunity.[12] However, the fact that the vast majority of our post-transplant patients showed no signs of GVHD and were in complete remission despite the accumulation of fully inhibitory Treg in their bone marrow might suggest that these cells allow for a graft-versus-myeloma effect while preventing harmful allo-immunity.

Two groups have recently examined numbers and function of Treg in the peripheral blood of myeloma patients with somewhat conflicting results.[30,41] While there were important differences in the approaches of both groups, which might explain the differing results, both groups observed an increase in $CD4^+CD25^+$ T cells in the peripheral blood of myeloma patients. In addition, Prabhala *et al.* analyzed a few patients for $CD4^+FOXP3^+$ Treg showing decreased proportions of these cells in multiple myeloma. While our focus was on the bone marrow compartment and not on the peripheral blood, we did not observe any differences in the percentages of Treg in the bone marrow or in the periphery between newly diagnosed myeloma patients and healthy donors (*data not shown*). One explanation for these divergent results might be that we did not use CD25 as a Treg marker but analyzed protein expression of FOXP3. The identification of FOXP3, which is not only the most reliable marker for Treg to date but also represents a crucial developmental factor for these cells, has been a major advancement.[22] Moreover, our data suggest that CD25 cannot be used as a Treg marker for the quantitative analysis of bone marrow-residing Treg since it is also unspecifically expressed by a large number of conventional bone marrow-residing $CD4^+$ and $CD8^+$ T cells.

When we examined the function of bone marrow-derived Tregs of our post-allogeneic SCT myeloma patients, we found that $CD4^+CD25^+FOXP3^+$ Treg expressed high levels of CTLA-4 and the immunosuppressive cytokine TGF-β1 and efficiently suppressed the proliferation of conventional T cells. While this is the first study to examine Treg function in the bone marrow of myeloma patients, others observed a decreased inhibitory function of Tregs from the peripheral blood of these patients.[30] Our results are, however, in agreement with those of Beyer *et al.* who detected a normal suppressor function of $CD4^+CD25^+$ T cells in patients with myeloma.[41] As has been pointed out previously,[41] the differing findings can best be explained by differences in the experimental approaches used by the respective groups. While Prabhala *et al.* used whole peripheral blood mononuclear cells as proliferating cells, accepting variations in the composition of mononuclear cells subtypes between groups, we and others have used highly purified $CD4^+$ or $CD4^+CD25^-$ T cells as the stimulated cell fraction.

The most promising concept for active immunotherapy of myeloma might be the combination with allogeneic SCT. This setting offers the advantages of an immune system unaffected by negative influences of the tumor. Furthermore, active immunotherapy following allogeneic SCT can build on anti-tumor immunity generated as a part of graft-versus-myeloma effects.[45] We suggest that allogeneic SCT provides a short but significant window of opportunity for $CD8^+$ T cells before an exuberant regeneration of immunosuppressive Treg sets in. Future therapeutic approaches in myeloma should focus on keeping Treg under control while amplifying and shaping allogeneic SCT-induced graft-versus-myeloma effects, e.g. by applying polyvalent cancer vaccines and/or tumor-specific adoptive immunotherapy. Such approaches could help to boost and broaden a post-transplant anti-myeloma immune response and might contribute to preventing recurrences in patients with multiple myeloma.

Authorship and Disclosures

DA designed the research, analyzed the data, and wrote the paper; YC performed research, contributed vital new analytical tools, and analyzed the data; TL performed research and analyzed the data; JP, CF, JA, KB, CW, TE and ARZ performed the research; BF and CB analyzed the data; NK designed the research and analyzed the data. The authors reported no conflict of interest.

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