Expansion of immunoglobulin autoreactive T-helper cells in multiple myeloma

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Short title: Immunoglobulin autoreactive T-helper cells in MM
Abstract

Activation and expansion of T helper (Th)-cells followed by regulation of activation, are essential to the generation of immune responses while limiting concomitant autoreactivity. In order to characterize T cells reactive towards myeloma derived monoclonal immunoglobulin (mIg), an autologous co-culture assay for single-cell analysis of mIg-responding cells was developed. When cultured with dendritic cells loaded with mIg, CD4+ Th-cells from patients with multiple myeloma (MM) showed a proliferative MHC class II-dependent response. CD8+ T cell reactivity and Th1-activation were consistently low or absent and Th2- and regulatory cytokines were expressed. The presence of such non-Th1 CD4+ T cells in peripheral blood was independent of treatment status, while the frequencies of responding cells varied between patients and reached the same order of magnitude as those measured for tetanus toxoid-specific Th memory cells. Furthermore, investigations of T cell subpopulations indicated a possible regulatory role on the mIg-responsiveness mediated by suppressive CD25^{high}FOXP3^CD4+ T cells.

It is proposed from the present results that a predominant *in vivo* activation of non-Th1 mIg-reactive CD4+ T cells constitute an Ig-dependent auto-regulatory mechanism in human MM, with possible tumour growth supporting or permissive effects.
Introduction

Multiple myeloma (MM), a malignancy of B-lineage origin, is characterized by clonal growth of transformed plasma cells in the bone marrow. The variable regions of the monoclonal immunoglobulin (mIg) are specific for the individual malignant clone, due to the mechanisms of rearrangements and mutations in the corresponding gene.\(^1,2\) While the etiology of MM remains largely unknown, interactions involving stromal cells\(^3\), as well as B- and plasma cell differentiation and growth factors such as cytokines released by T-helper type 2 (Th2) cells\(^4-7\) are believed to be implicated in the development of MM.

Studies of mouse models have provided evidence that T cells are involved in the development of plasma cell tumours.\(^8,9\) Whether T cells also play a role in human MM is not yet clear, although a number of T cell alterations associated with the disease have been described.\(^10-15\) Observations of mIg or “idiotype” reactive Th1 and CD8\(^+\) T cells in patients with MM have motivated attempts at immunotherapy based on administrations of mIg in adjuvant-like preparations or loaded in dendritic cells (DCs), mostly with a disappointing outcome.\(^15,16\) There is clearly a need for predictive and quantitative individual assessments of pre-existing antigen-specific lymphocytes, in the context of immunotherapy based on administrations of tumour antigens. Such treatment attempts will address possibly long-term antigen experienced and in vivo selected and putatively anergized T cells, a situation immunologically distinct from that encountered when vaccinating with a foreign antigen in order to induce a primary immune response by naïve lymphocytes. In addition, patients selected for inclusion in immunotherapy trials have often received prior chemotherapy, which contributes to individual variability in immune status and, thus, to unpredictability of immune responsiveness.

In attempt to identify and describe the population of mIg-reactive lymphocytes in patients with MM, we analysed responses of T cells upon interaction with autologous antigen-presenting cells consisting of homogenous and functionally synchronized dendritic cells pulsed with the autologous mIg. An obvious prerequisite for reliable determination of auto-reactivity is the absence of xeno- or allogeneic antigens in the cultures employed. Furthermore, no exogenous growth factors or cytokines were added to the co-cultures and the number of cell selection steps was minimized, in attempt to restrain in vitro biased or induced immune responses, and thereby identify an in vivo-selected T cell population. Short-term co-cultures of fresh cells collected repeatedly, together with CFSE-labelling in order to quantitate the proliferation of T cells in a mixed cell-population, were therefore employed. Isolated T cell populations were further analysed for autologous regulatory functions.
Methods

Patients and controls

Ten patients diagnosed with MM\(^{17}\) expressing an M-component of the IgG isotype were included (Supplement Table). Five patients were included at the time of diagnosis and peripheral blood samples were collected prior to initiation of therapy. Five of the patients had previously received chemotherapy, one of which remained in complete remission following an autologous stem cell transplantation four years prior to the present study. With the latter exception, repeated analyses of M-component concentrations in serum from nine of the patients included, indicated myeloma tumor progression. No laboratory findings or symptoms indicative of infections were identified. White blood cell counts, granulocytes and lymphocytes were within the normal ranges for nine of the patients at the time of blood collection (Supplement Table). Healthy age-matched volunteers served as control blood donors. The study was performed with informed consent of all blood donors and approved by the ethics committee at Karolinska hospital.

Culture and differentiation of DCs

DCs were generated from peripheral blood precursor cells employing an adaptation of protocols described previously.\(^{18}\) In brief, peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected heparin-treated blood by density gradient centrifugation. Subsequently, 5x10\(^6\) cells/ml were cultured for adherence during 2 h, after which non-adherent cells were removed and the adherent cells cultured in AIM-V medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.2% autologous serum, 60 ng/ml of human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF, Leucomax, Sandoz, Basel, Switzerland) and 50 ng/ml of interleukin 4 (IL-4, Schering-Plough, Kenilworth, NJ, USA). On days 5 and 7 of culture, surface markers on harvested cells characteristic of DCs were analyzed by flow cytometry,\(^{18,19}\) and negativity for other lineage markers (CD3, -14, -19, and -56) was confirmed. Following pulsing (see below) of immature DCs with antigen, cells were cultured for an additional 48 h in the presence of 20 ng/ml of TNF-\(\alpha\) (R&D-systems, Minneapolis, USA) and their stage of differentiation confirmed by analysis of surface marker expression. In vitro matured DCs reached a purity of at least 90%, which was confirmed for each experiment.

Pulsing of DCs with mIg or tetanus toxoid

On the fifth day of culture, immature DCs were incubated for uptake of antigens in serum-free medium. mIgG (M-component) from the sera of patients and polyclonal IgG from healthy donors were purified on a MabTrapG-column (Pharmacia, Uppsala, Sweden) as described elsewhere.\(^{20}\) The degree of purity of the mIg fraction was assessed by polyacrylamid gel electrophoresis and the concentration of mIg by nephelometry. The mIg-preparations were tested negative for endotoxin. Tetanus toxoid (TT, Statens Serum Institut, Denmark) was obtained from SBL Vaccine (Stockholm, Sweden). The protocol for
loading of immature DCs with antigen was based on pilot experiments in which the uptake of FITC-labelled mIg at varying concentrations and for distinct periods of incubation was monitored by flow cytometry and confocal microscopy (Leica, Meyer Instruments, Houston, USA). Different concentrations of TT and mIg (log fold titrations, mIg 1-1000 µg/ml) utilized to load DCs were also assessed with respect to the kinetics of antigen-presenting functions and T cell responses in the co-culture proliferation assay described below and proliferation was also determined by incorporation of thymidine (data not shown). On the basis of these studies, immature DCs were incubated in serum-free RPMI 1640 medium for 2 hours at 37° C with TT or mIg; representative results of 3 and 100 µg/ml of the respective antigen are presented. Subsequently, cells were cultured in serum-free AIM-V medium supplemented with TNF-α (20 ng/ml) for two days prior to analysis of DC maturation markers. Flow cytometry was employed to verify that mIg did not remain on the cell surface following the pulsing and maturation of DCs.

Co-culture of CFSE-labelled PBMCs and autologous DCs

Antigen-pulsed mature DCs were irradiated (25 Gy) and seeded together with carboxyfluorescein diacetate succinimidyl ester-labelled (CFSE, Molecular probes Europe BV, Leiden, Netherlands) PBMCs at a ratio of 1:10 (10^4 or 10^5 DCs and 10^5 or 10^6 PBMCs) for co-culture in AIM-V medium. In order to assess the involvement of MHC class II in antigen-presentation, DCs were pre-incubated with mouse antibodies (5 µg/ml) against human MHC-cII (anti-human HLA-DR, BD) or with an isotype control, and then washed prior to the co-culturing. CFSE-labelled PBMCs from healthy subjects were co-cultured with DCs pulsed with allogeneic mIg purified from patients´ sera. Polyclonal IgG purified from sera of four healthy blood donors was utilized for analyses of T cell responses in co-cultures with autologous Ig-pulsed DCs derived from the respective donor. T-cell proliferation in cultures with un-pulsed DCs was also analysed. As a control for activation of T cells, anti-CD3 antibody (1 µg/ml; R&D systems, Minneapolis, USA) was added to cultures of CFSE-labelled PBMCs. When the cell yield was sufficient, the co-cultures were re-stimulated at one-week intervals by addition of mIg-pulsed mature DCs at a ratio of 1:10 (DC:lymphocyte) in AIM-V medium supplemented with 2% autologous serum and rhIL-2 (20 units/ml; Peprotech, London UK). After 1-3 such rounds of re-stimulation, the cells were analysed for intracellular cytokines and T cell surface markers by flow cytometry.

Flow cytometry and detection of cytokines

FACS analysis of cell surface markers followed standard procedures. In brief, cells were labelled with monoclonal specific antibodies (Ab) or the corresponding isotype control Ab conjugated with FITC, PE or PerCP (BD Pharmingen, San Diego, CA, USA; R&D systems, Minneapolis, USA). Supernatants from the various cultures were analyzed for TH1/Th2 cytokines utilizing the Cytometric bead array kit (BD Pharmingen). For detection of intracellular cytokines, harvested cells were incubated with Brefeldin A (Sigma, St. Louis, MO, USA) prior to fixation in paraformaldehyde and permeabilization (FACS perm,
BD) followed by surface staining with fluorochrome-conjugated Ab towards CD4, CD8 and CD3 and intracellular staining with Abs directed against IL-4 and INF-γ. For detection of intracellular FOXP3, fixed and permeabilized cells labelled with Ab towards CD4 and CD25 were incubated with anti-FOXP3 Ab or the respective isotype control Ab (eBioscience, San Diego, CA, USA). Ab-labelled cells and cytometric bead samples were acquired in a FACS Calibur flow cytometer (BD) and analyzed by Cell Quest software (BD). Concentrations of TGF-β1 were determined by an Elisa procedure according to the manufacturers instructions (OptEIA, BD).

**T cell isolation and assessment of CD4⁺ T cell inhibitory functions**

PBMCs (stored frozen in liquid nitrogen) were stained with Abs towards CD3, CD4 and CD25 or the corresponding isotype controls (BD) and subsequently CD25^{high}CD4⁺ T cells (Tr), CD25⁻CD4⁺ T cells and CD3⁻ cells were isolated employing a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark). The threshold of CD25-fluorescence intensity for selection of CD25^{high} Tr was set above the maximum level of fluorescence emitted from CD25-labelled CD8⁺ T cells (example in Fig. 6). Assay of inhibitory functions of the Tr population isolated by cell-sorting followed published procedures. In brief, CD25⁻ CD4⁺ T cells (5-10x10³ cells/well), and irradiated (25 Gy) CD3⁻ cells (25x10³ cells/well) in the presence of anti-CD3 Ab, or mIg-pulsed DCs (25x10³ cells/well), were cultured with different numbers of Tr in 96-well plates. On the fifth day of culture, aliquots of supernatants were collected for assessment of cytokines; [³H]-thymidine was added and 16 h later cells were harvested for determination of thymidine incorporation.

**Results**

In order to assess T cell-responses towards autologous mIg or alternative antigens, PBMCs collected from the MM patients were subjected to short-term cultures in two steps. First, precursor cells were differentiated *in vitro* into dendritic cells, which were subsequently “pulsed” with antigen. Secondly, mature and antigen-loaded DCs were cultured together with freshly collected, CFSE-labelled autologous PBMCs. T cell proliferation was determined after 7 days of such co-culture, and in some cases repeated analyses were performed following re-stimulation of T cells. The regulatory functions of CD4⁺ T cells isolated by cell sorting were also examined.

**Generation of dendritic cells *in vitro***

The immature and mature DCs generated *in vitro* from adherent monocyte precursor cells exhibited phenotypic characteristics typical for their distinct stage of differentiation (methods). After “pulsing” of immature DCs with mIg or alternative antigens on the fifth day of culture followed by two additional days of differentiation in medium supplemented with TNF-α, the mature DCs displayed...
enhanced expression of MHC-clI, -II, CD80, CD83 and CD86; attenuated expression of CD64 and CD32; and no expression of CD14, in agreement with previous reports.\textsuperscript{18}

**Proliferation of CD4\textsuperscript{+} T cells co-cultured with autologous mIg-pulsed DCs**

Mature, mIg-pulsed and irradiated DCs were co-cultured at a ratio of 1:10 with autologous CFSE-labelled PBMCs. Since analyses of PBMCs from nine of the ten patients showed T cell and lymphocyte counts in comparable numbers and within normal ranges at the time of investigation, and additional selection steps of T cells led to some loss of activated cells, we chose to culture PBMCs labelled with CFSE, which allow a subsequent single cell analysis of dividing T cells by flow cytometry. Cells were cultured without any addition of exogenous growth factors, cytokines or serum. The lower limit of detection of T cell proliferation was 0.1 \%, i.e., reproducible results were obtained after 7 days of co-culture if a minimum of 1 of 1000 CFSE-labelled cells had undergone division, with a corresponding reduction in the intracellular concentration of CFSE during the culture period. The FACS analysis of CFSE-labelled T cells from patients with MM in progress, co-cultured with mIg-pulsed DCs is exemplified in Fig. 1, where the percentage of “CFSE-low” cells thus denotes the cumulative fraction of T cells that had divided during the culture period.

Analyses of T cell proliferation in such co-cultures showed that between 0.7 - 10.9 \% (with a mean + SD of 5.0 +3.6\%) of the CD4\textsuperscript{+} T cells had divided during the 7 days of co-culture with autologous mIg-pulsed DCs (Figs. 1 and 2, Supplement Table). In contrast, the CD8\textsuperscript{+} T cells, demonstrated a significantly lower degree of absence of proliferation (Fig. 2). The dominance of CD4\textsuperscript{+} versus CD8\textsuperscript{+} T cell proliferation was independent of individual variability in the ratio of CD4\textsuperscript{+} to CD8\textsuperscript{+} T cells (Supplement Table), and this pattern was also confirmed by calculating the total numbers of responding CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells per culture (Fig. 2). In the single case of MM in complete remission no significant proliferation of either the CD4\textsuperscript{+} or CD8\textsuperscript{+} T cell population was observed. Controls for Fc-mediated or non-specific effects included pulsing of DCs derived from healthy blood donors with the mIg preparations from MM patients, or purified polyclonal IgG for analyses of T-cell proliferation in cultures with Ig-pulsed DCs. A significantly lower or background degree of CD4\textsuperscript{+} T cell proliferation was measured after such cultures (Fig. 2). An occasional limited proliferation of CD4\textsuperscript{+} T cells was observed (<2\%) when the mIg preparations were assessed in cultures of non-autologous cells (Fig. 2), possibly related to alloreactivity towards allogeneic Ig determinants. The results indicated that the increased responses of CD4\textsuperscript{+} T cells from MM patients towards autologous mIg-pulsed DCs were not due to non-specific effects of Ig, a conclusion which was substantiated by MHC-blocking experiments described below.

**MHC class II-restriction of the mIg-associated proliferative response**

In order to examine the possible involvement of MHC class II (MHC-clII) in the response of CD4\textsuperscript{+} T cells described above, mIg-pulsed DCs were pre-incubated with blocking antibodies directed
against MHC-clII prior to co-culturing, which led to a pronounced (>65%) decrease in the proliferation of CD4+ T cells from 8 of the 9 patients with a proliferative response (Figs. 1 and 3). In one case preincubation with the isotype control antibody also interfered with the proliferation of CD4+ T cells, although to a lesser extent than the anti-MHC-clII antibodies (71% versus 95% inhibition respectively; Fig. 3, patient n° 6). Together, these results indicate that for 7 of 9 patients, MHC-clII was involved in antigen presentation to CD4+ T cells specific for mIg derived peptides.

**CD4+ and CD8+ T cell response to DCs pulsed with tetanus toxoid**

Tetanus toxoid (TT) was employed as an alternative antigen in order to evaluate the efficiency of the present co-culture model with respect to presentation of exogenous antigens to T cells. This approach might also provide an indication of memory responses of T cells from patients with MM to alternative antigens. Therefore, the proliferation of and cytokine production by T cells from six of the patients who had previously been vaccinated with TT, as well as from four vaccinated healthy volunteer blood donors, in co-culture with DCs pulsed with TT were examined.

In contrast to what was observed in cultures with DCs pulsed with mIg, both the CD4+ and CD8+ T cells from all six patients responded in culture with TT-pulsed DCs with significant increases in proliferation (with mean values of 8.0 and 4.3% and ranges of 1.8-15.1 and 1.0–9.4% for CD4+ and CD8+ T cells, respectively) which were comparable to the responses of T cells from healthy vaccinated blood donors (Fig. 4). Preincubation of TT-pulsed DCs with anti-MHC-clII-Ab prior to co-culturing, inhibited the proliferative response of CD4+ T cells from five of the six patients by more than 70% (exemplified in Fig. 1). There was no correlation found between the degree of CD4+ T cell responses towards TT and mIg.

**The pattern of Th-cytokine production**

Since analyses of T cells cultured with mIg-pulsed DCs showed a predominant Th-cell response, the levels of Th1 and Th2 cytokines in the culture supernatants were determined in order to identify a putative polarity in Th-activity. The concentrations of IFN-γ in the supernatants were consistently low or at background values (Fig. 5). This finding did not reflect an inability of the patients´ T cells to respond to TcR stimulation by producing IFN-γ (Fig. 5), since anti-CD3 ligation induced a greater than 100-fold mean increase in IFN-γ production by the same cell population (3280 pg/ml), a response comparable to that of anti-CD3 stimulated T cells from healthy control subjects (2770 pg/ml). Furthermore, the mean concentrations of IFN-γ in co-cultures of patients´ T cells with autologous DCs loaded with TT were increased (1360 pg/ml; control T cells 2305 pg/ml).

Preincubation of TT-pulsed DCs with antibodies blocking MHC-clII inhibited this increase by approximately 40%, indicating that MHC-clII-restricted Th1-cells were participating in the response to TT. The concentrations of IL-6 in co-cultures with mIg-pulsed DCs (Fig. 5) were comparable to those obtained with TT (control T cells 149 pg/ml).
The yield of cells obtained from some blood samples allowed additional cytokine analyses. Thus, re-analyses of the culture supernatants and/or detection of intracellular IL-4 and IFN-γ were performed following restimulation of lymphocytes from four of the patients, by repeated additions of autologous mIg-pulsed DCs at one-week intervals. The cytokine pattern identified was similar to that described above, i.e., the level of IFN-γ was consistently low or below the limit of detection. Intracellular IL-4 was detected in 9.7–25.6% of the restimulated CD4⁺ T cells, while the corresponding value for intracellular IFN-γ was 1.3-2.8% (data not shown). IL-6 and IL-10 concentrations increased in two of the re-stimulated cell cultures, while IFN-γ remained below detection limit upon re-stimulation (not shown). The results were thus consistent with a non-Th1 cytokine pattern upon restimulation with mIg-pulsed DCs. Furthermore, TGF-β was detected in co-cultures containing mIg-pulsed DCs.

**Regulation of the activation of T-helper cells**

Since the fraction of autoreactive Th-cells that responded to autologous mIg varied among the patients (Fig. 2, Supplement Table) and the patterns of cytokines were characteristic of Th2-effector cells and compatible with the presence of regulatory T cells, we examined whether variations in the functions and/or numbers of regulatory T cells correlated to the degree of Th-response for each individual patient. For this purpose, CD25<sup>high</sup>CD4⁺ T cells (Tr) were isolated from PBMCs by cell sorting, FOXP3-expression was verified and sorted cells were co-cultured with autologous CD25<sup>CD4⁺</sup> T cells for assessment of suppressive activity. T cells from seven healthy donors provided a measure of normal suppressive functions of Tr.

For all the cell samples examined from patients with MM, more than 90% (91-97%) of the population of CD25<sup>high</sup>CD4⁺ T cells stained positively for intracellular expression of FOXP3 (Fig. 6A), thus confirming crucial markers of regulatory T cells. Moreover, most Tr expressed TGF-β on the cell surface (not shown). These Tr were functionally active, as reflected in their ability to inhibit proliferation (Fig. 6B) and suppress both Th1- and Th2-type cytokines produced by CD25<sup>CD4⁺</sup> T cells (Fig. 6C) with the exception of Tr from a single patient, which exhibited no suppression of cytokine production. In most cases, the Tr-dependent reductions in the levels of Th-cytokines were paralleled by an elevation in the concentration of TGF-β (Fig. 6C). In summary, the phenotype, inhibitory functions and a certain individual variability in the degree of suppressive effects demonstrated by Tr isolated from peripheral blood of patients with MM were comparable to those observed from control subjects (Fig. 6).

However, the frequency of such functional CD25<sup>high</sup> Tr within the population of CD4⁺ T cells or expressed in relationship to the total CD3⁺ T cell population (the latter is chosen for data presentation due to the individual variability in CD4/CD8 ratio) showed a pronounced variability among individual patients with MM. Furthermore, the frequency of functional Tr among peripheral T cells tended to be inversely correlated to the degree of the proliferative response to mIg-pulsed DCs. Thus, patients’ T cells that exhibited pronounced proliferative Th-responses at day 7 of co-culture with mIg-pulsed DCs, contained significantly decreased frequencies of Tr (<1% of CD3⁺ T cells) as compared to control T cells.
populations, and conversely, a relatively lower proliferative response to mIg-pulsed DCs was associated with normal to increased frequency of Tr (Fig. 7, top).

Such correlation resembled the observed dependency on the ratio of Tr to responder CD4\(^+\) T cells for inhibition of proliferation as shown in Fig. 6. To address whether the autologous Tr mediated inhibitory functions could target the mIg-responding Th-cells, experiments were repeated for four patients with a sufficient cell yield after the sorting procedure. mIg-pulsed DCs were cultured with autologous CD4\(^+\) T cells with increasing concentrations of Tr, as described for Fig. 6. The presence of autologous Tr decreased the proliferation of responding CD4\(^+\) T cells (Fig. 7, bottom) thus indicating a Tr-dependent regulation of the T cell response.

**Discussion**

The present study reveals a predominance of mIg-autoreactive CD4\(^+\) Th-cells exhibiting a non-Th1 phenotype in the peripheral blood of patients with progressing MM. Analysis of the differential content of CFSE in proliferating T cells, most of which had divided more than five times during 7 days of culture with antigen-presenting cells, indicated that the frequency of mIg-responding cells in the pool of peripheral CD4\(^+\) T cells ranged between 1/10\(^4\) and 1/10\(^3\). Such values are orders of magnitude above frequencies expected to be associated with the response of naïve T cells to foreign antigens and thus indicate an *in vivo* activation and expansion of specific cells, similar to what was observed for the anti-TT responses of T cells from individuals who had been vaccinated against TT. This mIg-related T cell response also shared several characteristics with the response of memory cells to TT (Fig. 1), including the kinetics of cell division, the range of response frequencies and expression of memory markers (e.g. CD45RO) by responding cells.

However, there were also major differences between the autoreactive response to mIg and the anti-TT response, including the degree of involvement of CD8\(^+\) T cells and Th1-effector cells, as might be expected given the distinct origin and molecular nature of the antigens. The general predominance of CD4\(^+\) over CD8\(^+\) T cells in responding to mIg-loaded DCs is consistent with the notions of a preferential MHC-clII-dependent pathway for exogenously loaded antigens in antigen-presenting cells; whereas antigens expressed intracellularly would mainly access the MHC-clI presentation pathway. The degree of such compartmentalization may vary for different antigens as suggested previously, and exogenous antigens internalised into DCs may also gain access to the MHC-clI pathway.\(^{22}\)

The subpopulation of peripheral T cells from vaccinated patients and healthy control subjects that responded to TT included CD8\(^+\) T cells, in addition to CD4\(^+\) T cells, an observation compatible with reports on cross-presentation of this antigen.\(^{23}\) Furthermore, both Th1- and Th2-effector T cells were
activated towards TT, as indicated by the pattern of the cytokines produced. In contrast, the levels of IFN-γ, the hallmark of Th1 function, in culture supernatants and expressed intracellularly in T cells cultured with mIg-pulsed DCs, were consistently low or below detection level. At the same time, one or several B-lineage helper cytokines including those identified as risk factors in the development of MM,4-7 were expressed. The pattern of expression of TNF-α was similar to that observed for IFN-γ (i.e. measured at low or background levels; not shown).

Together, these results indicate that DCs derived from blood precursor cells of patients with MM evoke “composite” T-memory responses to non-self exogenous antigens, in contrast to the response to mIg. The observed differences are interpreted as reflecting the nature of the antigens; the processing/presentation pathways of antigen-presenting cells as well as a prior in vivo selection of the responding T cell population, rather than any major limitation associated with the cell culture model employed (including possible antigen-presenting cell dysfunction) or a generally deficient or skewed responsiveness of patients´ T cells. The capacity of the CD4+ and CD8+ T cell populations to respond with production of IFN-γ, as well as with proliferation was further confirmed by TCR-ligation experiments.

Studies of murine models have demonstrated the importance of T cells either promoting the development of plasma cell tumours,9 or mediating resistance e.g. by idiootype-specific CD4+ T cells in a transgenic model of myeloma.8 Regulatory implications of idiootype associated Th1 or CD8+ T cells in human MM have also been proposed.15 Our results, obtained by a culture method allowing for the identification of dividing cells on a single cell level, in autologous conditions with limited additions of supplemental growth factors, point to a predominant non-Th1 CD4+ T cell population reactive with mIg-pulsed DCs. The present findings suggest that in human MM mIg-associated CD4+ T cells activated in vivo may not be, at least not in a population predominant fashion, committed to a “anti-tumour” phenotype, but may include contain B lineage supporting helper cells. Possible growth-promoting effects of mIg-specific Th2-cells on clonal myeloma cells could either be indirect (e.g., by providing Th2 cytokines in the circulation and/or in the local tumour environment) or via direct interactions of specific Th-cells, the latter of which would be dependent on presentation of endogenous Ig-peptides in the context of MHC-clIII expressed on the myeloma or clonotypic precursor B cells24-26. Autologous MHC-clIII-dependent antigen-presentation of peptides derived from endogenous Ig to T cells has been described previously.27-29 Furthermore, T cells autoreactive against epitopes in the IgVh region have been proposed to regulate the production of the corresponding antibodies30-32 in addition to mediate tolerance in autoimmune disease to peptides derived from IgG.30,33

Since T cells cultured with DCs disclosed an individually variable expansion of an mIg-reactive subpopulation of Th-cells (Fig. 2) and the cultured T cell population included regulatory T cells, we examined whether variations in the functional status and/or frequencies of Tr correlated to the Th-response towards mIg for the individual patients. The autologous inhibitory functions of CD4+CD25highFOXP3+ Tr from peripheral blood of patients were similar to those of control subjects, when assessed at defined ratios (Fig. 6). The production of TGF-β in cultures with Tr present and the
expression of TGF-β on the cell surface of Tr indicate that TGF-β was a potential mediator of suppression.\textsuperscript{34}

However, pronounced variations in the frequency of such functional Tr were observed among MM patients (Fig. 7, top), and there was an inverse relationship between the numbers (relative and absolute) of functional CD25\textsuperscript{high}FOXP3\textsuperscript{+} Tr present in the total T cell population and responsiveness to mIg-pulsed DCs. Thus, T cells from patients containing a normal to high frequency of Tr exhibited a relatively attenuated proliferative response in culture, consistent with the observed concentration dependent inhibitory activity demonstrated by Tr (Fig. 6) and conversely, the proliferative T cell responses to mIg-pulsed DCs were enhanced when Tr frequencies were reduced (Fig. 7, top). Further evidence for such Tr dependent suppressive functions was obtained from co-culture experiments of mIg-pulsed DCs and isolated CD4\textsuperscript{+} T cell populations from four patients (Fig. 7, bottom), indicating a Tr-dependent regulation of the mIg-autoreactive T cell response. No such correlation between the proliferative response to TT and frequency of Tr could be found.

Since the description of CD25\textsuperscript{+} regulatory T cells by Sakaguchi et al\textsuperscript{35} Tr have been detected in association with a variety of human diseases, including cancer. Tr in peripheral blood from patients with MM were recently described as either dysfunctional\textsuperscript{36} or present in elevated frequencies and active in assays of allogeneic suppression.\textsuperscript{37} The present results are technically not comparable to these reports, since we employed sorted T cell populations instead of bulk cultures for analyses of T-regulatory functions, which were measured towards autologous and not allogeneic target CD4\textsuperscript{+} T cells, in order to analyse the regulation of autoreactive CD4\textsuperscript{+} T cells in association with MM.

Collectively, our results point to a) a predominance among peripheral T-cells of non-Th1 CD4\textsuperscript{+} T cells reactive with mIg-presenting cells; b) a negative regulation of CD4\textsuperscript{+} T cell activation including mIg-autoreactive Th-cells mediated by suppressive Tr in MM. mIg-reactive T cells of alternative phenotypes might obviously co-exist among tumour-antigen associated immune reactivities, and the resulting effector functions upon activation could be modulated by regulatory T cells, analogous to mechanisms involved in certain autoimmune disorders.

A tumour antigen associated Th2-activation, possibly insufficiently regulated by Tr, could be a risk factor in MM, a hypothesis that is compatible with the results from our studies of MM-associated IL-10 and CTLA-4 polymorphisms.\textsuperscript{38,39} Whether increased frequencies of mIg- or alternative tumour antigen-reactive non-Th1 cells are associated with tumour progression and whether the Th-populations include cells of a putative pathological importance remain to be investigated in extended studies. If such a “tumour feedback” mechanism as proposed here exists, it could have implications for developments of immunotherapies in MM and other B lineage derived tumours. mIg-directed therapies e.g., administration of DCs loaded with mIg, might be associated with a risk for a predominant activation of pre-existing circulating mIg-specific Th2-cells. Administration of DCs may in addition reinforce tolerance mechanisms, as indicated by observations of Tr expansions in peripheral blood of MM patients after injections of DCs.\textsuperscript{40} It was also reported that protein mixtures derived from myeloma cells and
internalized in DCs induce primarily regulatory T cells,\textsuperscript{41} which further emphasizes the conclusion that an understanding of the complex relationships between B-lineage tumour cells and the immune system is a prerequisite for the development of effective immunotherapies.

Acknowledgements

This study was supported by the Torsten and Ragnar Söderberg foundation, Sweden and the Karolinska Institute, Sweden. The discussions with professor G. Holm are gratefully acknowledged.

Authorship: M.O., M.A. performed research, analyzed data; A.G. collected and analyzed data, reviewed the paper; A.S. designed and performed research, analyzed and interpreted data, wrote the paper.

Conflict of Interest Disclosure: The authors declare no competing financial interests.
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Figure legends

**Figure 1. Proliferation of CD4+ T cells co-cultured with autologous DCs pulsed with mIg or tetanus toxoid.**
The percentages of proliferating cells among the total CD4+ T cell population analysed after 7 days of co-culture are indicated, as deduced from the relative numbers of CD4+ T cells with reduced intracellular concentrations of CFSE (“CFSE-low”). Middle graphs depict a decrease in proliferation of CD4+ T cells when antigen-pulsed DCs were preincubated with anti-MHC-clII antibodies (ab), in comparison with preincubation with isotype control (right graphs) or without antibody (left graphs).

**Figure 2. Proliferation of T cells co-cultured with Ig-pulsed autologous DCs.**
Immature DCs were pulsed with autologous mIg from MM patients sera (“auto-mIg MM”). DCs were subsequently subjected to differentiation in the presence of TNF-α for two days and irradiated prior to co-culture with autologous PBMCs labelled with CFSE at a ratio of 1:10 (DC:PBMC). Cells were harvested after 7 days of culture for FACS analysis of surface T cell markers and intracellular concentration of CFSE (Fig. 1). mIg preparations from patients were also utilized for pulsing of DCs derived from healthy subjects prior to cultures with autologous CFSE-labelled PBMCs (“allo-mIgCtrls”; mean values of eight different mIgs are shown). Alternatively, autologous polyclonal Ig (“auto-IgCtrls”; four controls donors included) were utilized for pulsing of DCs derived from the respective healthy subject prior to cultures with autologous CFSE-labelled PBMCs. The mean percentages (left graph) and total cell numbers/culture (right graph) of CD4+ and CD8+ T cells that had proliferated during culture (“CFSE-low”) of representative experiments are depicted. Significant differences (**) p<0.01; * p<0.05, t-test) versus the CD4+ T cell response to auto-mIg pulsed DCs of MM patients (“auto-mIg MM”) are indicated.

**Figure 3. Decreased CD4+ T cell proliferation by antibody blocking of MHC-clII.**
mIg-loaded DCs were preincubated with anti-HLA-DR or isotype control antibodies prior to co-culturing with CFSE-labelled PBMCs, as in Fig. 2. For each patient (n=9), the percentage inhibition of the CD4+ T cell proliferation by blocking (striped bars) or isotype control antibodies (white bars) are presented (example of FACS analysis in Fig. 1). In the case of n° 2, 4 and 8, the control antibody increased the percentage of proliferating cells marginally, resulting in negative values.

**Figure 4. CD4+ and CD8+ T cell proliferation in cultures with autologous DCs pulsed with tetanus toxoid.**
Immature DCs pulsed with tetanus toxoid (TT) were subjected to differentiation in the presence of TNF-α and subsequently irradiated prior to co-culture, as in the case of mIg. The proliferative response of T cells from six patients with MM (n° 1, 2, 5-8) and four healthy blood donors, all of whom had been vaccinated...
against TT, were analysed after 7 days of co-culture with TT-pulsed DCs. The mean percentages (left) and total numbers/culture (right) of dividing “CFSE-low” CD4+ and CD8+ T cells are depicted. There was no significantly different T cell response towards TT between the MM patients and control subjects. Significant differences comparing the CD4+ and CD8+ T cell proliferation in cultures with TT- versus unpulsed DCs are indicated (** p<0.01; * p<0.05, t-test).

Figure 5. Concentrations of IFN-γ and IL-6 in co-cultures with mIg- or TT-pulsed autologous DCs. Supernatants from co-cultures with mIg-, TT- and un-pulsed DCs, as well as from anti-CD3-stimulated PBMCs, were collected for analysis of cytokine concentrations by cytometric bead array. IFN-γ (left graph) and IL-6 (right graph) were measured in such cultures for eight of the patients with a proliferative response towards mIg-pulsed DCs (Fig. 2) and five of the six patients that had received a TT-vaccination (Fig. 4). The mean concentrations (+SD) are shown (Y-axis in log scale).

Figure 6. The phenotype and functions of CD25highCD4+ T cells. (A) Analysis of CD25highCD4+CD3+ T cells (Tr) for intracellular expression of FOXP3. The threshold of CD25-fluorescence intensity for the analysis gate of Tr was set above the maximum level of fluorescence emitted from CD25-labelled CD8+ T cells. For all the patient samples, more than 90% of the CD25highCD4+ T cells were FOXP3-positive (histogram). Grayshaded histogram depicts the background fluorescence intensity of the isotype control antibody. (B) Suppression of anti-CD3-induced proliferation of CD25CD4+CD3+ T cells by different relative numbers of Tr. The 100% value on the y-axis indicates the maximal anti-CD3-induced proliferation (cpm) of CD25CD4+CD3+ T cells without Tr present (1:0). The mean percentage (+SD) of the maximal response assessed at different ratios of these two CD4+ T cell subpopulations is depicted for patients (MM; n=9) and control subjects (C; n=5). The degree of Tr-dependent inhibition of proliferation was not significantly different between the groups. (C) The Tr-mediated suppression of proliferation was accompanied by decreased concentrations of Th-cytokines in the culture supernatants and in most cases, elevated levels of TGF-β. The concentrations of cytokines in cultures with Tr present (1:1) are expressed in percentage of the concentrations obtained in cultures without Tr (1:0), which is plotted for each patient (triangles) and control subjects (circles) for the respective cytokine. (Occasional increases in the concentration of TGF-β of more than 100% are plotted as 100% for graphical reasons).

Figure 7. Inverse correlation of CD25highFOXP3+CD4+ Tr frequency and CD4+ T cell proliferative response to mIg-pulsed DCs. (Top) Decreased frequencies of CD25highFOXP3+CD4+ Tr among peripheral CD3+ T-lymphocytes (x-axis) correlated with increased proliferative response to mIg-pulsed DCs for the individual MM patient (X- and Y-values for patient n° 1-10 are plotted). The mean percentage of dividing CD4+ T cells after 7 days of co-culture with mIg-pulsed DCs is shown (y-axis). Corresponding mean Tr frequency of seven
control donors was 2.1% (SD 0.9). (Bottom) Suppression of CD4+ T cell proliferative response to mIg-pulsed DCs during co-culture in the presence of autologous Tr. As in Fig. 6, the 100% value on the y-axis depicts the proliferation of CD4+ T cells without Tr present (1:0). Proliferation assessed at increasing concentration of Tr (1:0.25, 1:0.5, 1:1) is expressed in % of maximal response. The results of DC co-cultures with sorted CD4+ T cell populations from four patients are shown. Due to limited cell sorting yield of Tr, it was not possible in all experiments to assess proliferation at every ratio indicated (x-axis).
Figure 1

**mIg-pulsed DCs**

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<th>no ab</th>
<th>anti-MHC clIII</th>
<th>control ab</th>
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<td>3.6%</td>
<td>1.0%</td>
<td>3.9%</td>
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**TT-pulsed DCs**

<table>
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<th>control ab</th>
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<tr>
<td>9.2%</td>
<td>2.4%</td>
<td>10.1%</td>
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</tr>
</tbody>
</table>
Figure 6

A

B

C

Figure 6

CD3^4^25^{hi}  

Counts

% of max. proliferation

% change in concentrations of cytokines

1 : 0  1 : 0.5  1 : 1

IFN-γ  TNF-α  IL-10  IL-6  IL-4  TGF-β

91%

- MM
- C

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Figure 7

% mIg-reactive CD4+ T cells in culture

Frequency of CD25++

% of maximal proliferation

1:0 1:0.25 1:0.5 1:1
Expansion of immunoglobulin autoreactive T-helper cells in multiple myeloma

Masih Ostad, Margareta Andersson, Astrid Gruber and Anne Sundblad