IgG antibody and TH1 immune responses to influenza vaccination negatively correlate with M-protein burden in monoclonal gammopathy of undetermined significance

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Abstract

Background: The emergence of non-malignant conditions such as monoclonal gammopathy of undetermined significance (MGUS) in the elderly may further alter their increased susceptibility to infections, however this remains poorly defined. The immune response to influenza vaccination has not been examined in MGUS, a benign plasma cell monoclonal expansion in the bone marrow that secretes elevated levels of monoclonal immunoglobulin (M-protein). Pre-existing antibody titers to infectious agents can be depressed, but the degree of immunosuppression in response to active vaccination remains unclear. Furthermore, the association of M-protein levels with impaired antigen specific response has not been examined to date in MGUS.

Methods: We investigated antibody as well as T cell responses in 19 MGUS patients and age-matched healthy controls following influenza vaccination. H1N1 and H3N2 influenza-specific IgG antibodies were measured by ELISA. The frequencies of H1N1 and H3N2 specific IFN-γ secreting cells were measured by ELISpot.

Results: Polyclonal isotype-switched immunoglobulin levels were significantly reduced in the MGUS cohort (p<0.05). At the cohort level, H1N1 and H3N2 influenza-specific IgG titers were comparable in MGUS and HCs. However, a key distinction emerged in relation to M-protein levels. MGUS patients with high M-protein had decreased influenza specific IgG titers which they failed to expand post-vaccination. Low M-protein MGUS H1N1-specific IFN-γ responses were high and comparable with HCs but showed no discernible increase post-vaccination, while cellular responses to H1N1 in high M-protein MGUS were markedly reduced.

Conclusions: Overall, MGUS showed depressed immune responses to influenza vaccination that varied strikingly with M-protein levels.

Keywords: Monoclonal gammopathy of undetermined significance, M-protein, influenza vaccination, humoral response, cellular response

Introduction

Monoclonal Gammopathy of undetermined significance (MGUS) is a pre-malignant condition that mainly affects the elderly. It is characterized by the clonal proliferation of plasma cells resulting in the observed monoclonal protein (M-protein). It is diagnosed by presence of M-protein in the serum at levels <30g/L, bone marrow plasma cell infiltration of <10% and no clinical manifestations of organ damage or the presence of a B cell malignancy. MGUS is associated with a lifelong risk of progression to multiple myeloma or other related malignancy at a rate of 1% per year [1]. Multiple myeloma is a plasma cell proliferative disorder which features a reduction in polyclonal immunoglobulin levels and more profound B cell dysfunction than MGUS. Infections are a leading cause of morbidity and mortality and their spectrum has been reported to change subsequently relative to disease activity and immunosuppressive treatment that leaves the patients immunocompromised [2]. Recent studies have shown that myeloma is almost always preceded by MGUS therefore establishing a key role for MGUS in the pathway to myeloma [3,4]. The increased susceptibility of myeloma patients to infections has been assessed extensively [5-7], however the risk of infections in MGUS has been less well studied. An increased risk of bacterial as well as viral infections has been described in a small number of studies [8-10]. MGUS also has variable consequences on the immune system. Polyclonal immunoglobulin levels are normal or reduced in MGUS but the degree of immunosuppression, if present at all, is unknown and therefore it is unclear whether patients with MGUS have compromised humoral or cellular immunity. Whether the low polyclonal immunoglobulin levels
and or high monoclonal protein concentration are associated with impaired antigen specific response has not yet been established in MGUS.

Both B and T cells are important in the immune response. Little is known of the B cell function in MGUS, yet B cell function dysregulation in myeloma is more profound and has a consequence on the immune response as the humoral response to vaccination is hampered. Disturbances in the T cell compartment may also result in impaired immune response, however these have been studied in less detail in MGUS. Absolute numbers of CD4+ and CD8+ T cells were shown to be comparable to healthy controls in MGUS [11]. Significantly increased frequencies of CD4+CD25+FoxP3+ T reg cells in MGUS as well as treated and untreated myeloma have been reported. These cells were shown to maintain their functionality as they inhibited proliferation and IFN-γ production [12,13].

Influenza has a high incidence and vaccination reduces the morbidity and mortality caused by influenza virus infection. Influenza vaccination is recommended for individuals in high-risk groups including the elderly and immunocompromised [14]. As MGUS is more prevalent with increasing age, a substantial number of the patients fall under the high-risk group of elderly individuals, so they receive seasonal influenza vaccination. Whether this vaccination is effective is unknown.

Therefore, we devised a prospective study to investigate the impact of monoclonal protein on the humoral and cell-mediated immune responses in MGUS patients following vaccination with a trivalent subunit influenza vaccine in order to analyze whether MGUS monoclonal protein burden is associated with functional deficits of the immune system. MGUS patients show suboptimal response to vaccination with M-protein concentration impacting their response.

Methods
Patients and controls
Patients that were known with MGUS at the University Medical Centre Groningen hematology department were included in the study when they fulfilled the standard diagnostic criteria for MGUS: serum M protein levels <30g/L, clonal plasma cells in bone marrow <10% and no myeloma related dysfuction or other B-cell proliferative diseases [15]. Patients were excluded if they had current infection, defined as fever in combination with clinical focal signs of infection and the need for therapeutic antibiotic treatment, influenza vaccination within the 6 months prior to the study, pregnancy or malignancy. The duration of MGUS varied between patients from 1 till 19 years since diagnosis. Persistent monoclonal proteins were detected in all MGUS patients.

For control purposes, sex and age matched healthy individuals who had no co-morbidities or malignancy, condition associated with immune dysfunction or use of immune modulating drugs were included parallel to the MGUS patients. The healthy controls were recruited from the populations visiting general physician practice for yearly vaccination. Healthy controls with monoclonal protein in their serum were excluded. All participants included gave written informed consent in accordance with the Declaration of Helsinki. The institutional medical ethics committee of University Medical Centre Groningen approved the study.

All MGUS patients and controls were vaccinated between October 2010 and January 2011. They received an intramuscular injection of the influenza vaccine (InfluvacÖ, Solvay Pharmaceuticals, Netherlands). The vaccine, a subunit preparation of licensed 2010-2011 trivalent inactivated virus, contained A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008. Information on influenza vaccination in the previous years was obtained. Local and systemic adverse reactions for up to 7 days post vaccination were recorded by participants using a standard questionnaire which included the reporting of pain, itching, induration at site of injection, fever, myalgia and flu-like symptoms. No serious adverse reactions were observed.

Evaluations
At study entry, baseline standard laboratory parameters were measured; complete blood counts including leukocyte differentiation, lymphocyte subsets (CD3+, CD4+, CD8+, CD19+ cells) and total IgM, IgA and IgG immunoglobulin levels. Normal ranges of serum immunoglobulins in our lab were defined as: 7-16 g/L IgG, 0.7-4 g/L IgA and 0.4-2.3 g/L IgM. The presence of a monoclonal immunoglobulin was determined by immunofixation.

Further venous blood samples were obtained at the day of vaccination (day 0), 7 days and 28 days post-vaccination. Serum was separated by centrifugation and stored at -20°C until further analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation of CPT vacutainer tubes (BD) drawn venous blood immediately after venipuncture. PBMCs were then frozen in RPMI 1640 (Cambrex Bioscience, Verviers, Belgium) supplemented with 10% human pooled serum, 50 µg/mL gentamicin (Gibco, Paisley, UK) and 10% dimethylsulfoxide(Merck, Germany). PBMCs were stored in liquid nitrogen until further use.

Measurement of anti-influenza IgG antibodies by ELISA
The levels of anti-influenza specific IgG antibodies to A/H1N1 and A/H3N2 were determined by an in-house ELISA. In short, ELISA plates (Coaster) were coated with 1 µg/ml subunit of A/H1N1 and A/H3N2 and subsequently incubated with serum samples collected at day 0, day 7 and day 28 following vaccination. The detection of influenza-specific antibodies was done with mouse anti-human IgG-HRP (Southern Biotech, USA), followed by incubation with TMB substrate (Sigma). Absorbance at 450nm was read with an Emax microplate reader and antibody concentrations calculated by SOFTmax PRO software (Molecular Devices, Sunnyvale, USA). For a standard curve, a dilution series of human IgG standard, N
protein (Siemens) was used on every plate and the antibody contents of the samples were read from the linear part of the sigmoid curve.

IFN-γ ELISpot
Pre and post vaccination PBMC samples from MGUS patients and matched healthy controls were simultaneously thawed and further analysed. Viability was evaluated by trypan blue staining and then the thawed PBMCs were incubated overnight at 37°C before proceeding with the ELISpot assay. Before plating, the cells were counted using an automated cell counter (Beckman Coulter, Fullerton, CA, USA).

96 well filtration plates (MAISPWU10, Millipore, Ireland) were coated overnight at 4°C with 100μl of 15μg/mL anti-human IFN-γ (Mabtech, Nacka Strand, Sweden). Plates were subsequently washed and blocked for 2 hours at 37°C with culture medium (RPMI 1640 supplemented with 10% fetal calf serum and 50 μg/mL gentamycin). 200,000 PBMCs were added per well in duplicates and in combination with different stimuli to make up a volume of 200μL/well and incubated at 37°C with culture medium. Antigenic stimuli to make up a volume of 200µL/well and incubated at 37°C with culture medium. Antigenic stimuli to make up a volume of 200µL/well

Concanavalin A at 2.5 µg/mL was used as a positive control while PBMCs in culture medium alone were used as a negative control. Following 48 hour incubation and washing the plates once with tap water and analysing on an ELISpot reader (automated-video-analysis system, Sanquin, Amsterdam, Netherlands).

Statistical analysis
Data were analysed with Graphpad Prism 4 (Graphpad software Inc.). Mann-Whitney U test, Wilcoxon signed rank test and Fisher’s exact test for categorical data were used as appropriate. For correlations, Spearman’s rank correlation coefficient was used. P-values < 0.05 were considered statistically significant.

Results and discussion
A total of 19 MGUS patients gave informed consent and were included; their median age (range) was 66 (49-81) and 63% were male. They also had a similar vaccination history to healthy controls, with the majority of the participants having been vaccinated in the preceding influenza season. Ninety percent of the patients were vaccinated against influenza the previous year compared to 79% of healthy controls. The study demographics are shown in (Table 1).

The concentration of M-protein varied from unquantifiable levels to 24.8g/L. M-protein was detected by immunofixation but was unquantifiable in 8 patients by serum protein electrophoresis because the concentration of the M-protein was small or the M-protein migrated in the β fraction. It has been recently shown that MGUS patients with unquantifiable M-protein are qualitatively similar to MGUS with quantifiable M-protein as they have persistent M-protein and also progress to plasma cell malignancies at a similar rate [16]. Significantly reduced relative numbers of CD19+ cells compared to the healthy controls (p=0.05). Significantly reduced serum levels of polyclonal IgG (p=0.023) and IgA (p=0.027) but not IgM (p=0.08) immunoglobulin were also observed for the MGUS cohort compared to the healthy controls. A proportion of MGUS patients (8/19; 42%) exhibited subnormal serum polyclonal IgG levels (< 7g/L) (Table 1).

Monoclonal protein load correlates with influenza-specific H1N1 IgG responses
To determine vaccine-specific antibodies, we assayed the serum concentration of IgG antibodies to H1N1 and H3N2 using ELISA. Before vaccination, influenza-specific IgG titers to H1N1 and H3N2 strains were comparable and did not differ significantly in both the healthy controls and MGUS. A significant response to both the H1N1 and H3N2

### Table 1. Baseline characteristics of MGUS patients and controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Controls (n=19)</th>
<th>MGUS (n=19)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (SD)</td>
<td>63.7 (8.39)</td>
<td>65.9 (7.56)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>11/8</td>
<td>12/7</td>
<td>NS</td>
</tr>
<tr>
<td>Vaccination 2009/2010, N. (%)</td>
<td>15 (78.9)</td>
<td>17 (89.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin (g/L), median (range)</td>
<td>17 (12-20.5)</td>
<td>17.5 (15-21)</td>
<td>NS</td>
</tr>
<tr>
<td>Monoclonal protein isotype, N (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgG</td>
<td>10 (53)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgA</td>
<td>5 (26)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IgM</td>
<td>-</td>
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NS, not significant; *, significant decrease in CD19+ cells relative numbers, p<0.05; NA, not applicable.
strain was observed both in healthy controls and MGUS at day 7 and this response was also sustained at day 28 (Supplement figure S1). We examined whether the magnitude of the influenza specific IgG antibody response after vaccination was associated with the monoclonal protein size. A negative relationship was found between the level of M-protein and influenza-specific IgG titers whereby high levels of M-protein were associated with depressed influenza H1N1 specific IgG titers post-vaccination. An inverse correlation between M-protein levels and H1N1-specific IgG titers was observed at day 7 (p=0.02, spearman’s r=−0.51) and day 28 (p=0.0123, spearman’s r=−0.56) (Figure 1A).

To further assess how high M-protein concentration affects the kinetics of the influenza specific response, subjects were further stratified into two groups according to their concentration of M-protein; low M-protein and high M-protein. We used a cut-off concentration for low M-protein MGUS of <15g/L, as monoclonal protein concentrations of ≥15g/L have been described as a risk factor for progression to myeloma [15,17]. The levels of prevaccination influenza specific IgG titers were significantly lower in the high M-protein group and the antibody levels post-vaccination remained depressed in high M-protein MGUS. As a group, the low M-protein MGUS patients showed significantly increased influenza specific IgG levels to H1N1 at day 7 (p=0.002) and day 28 (p=0.002) but high M-protein MGUS did not show a significant difference in titers pre and post-vaccination (Figure 1B). Similar responses were observed for H3N2 where low serum M-protein was associated with significantly increased H3N2 specific IgG response at day 7 (p=0.0007) and day 28 (p=0.0002). On the other hand, in patients with high M-protein levels no discernible increase in H3N2 specific IgG levels at both day 7 and day 28 occurred (Figure 1B).

Next, we assessed whether having an IgG MGUS was associated with lower responses compared to other isotypes. Patients were further stratified according to whether they had IgG M-protein or non-IgG (IgA and IgM). Patients with an IgG MGUS had similar levels of H1N1 specific IgG compared to those with other MGUS isotypes. However, for the H3N2 strain, IgG MGUS patients had significantly lower titers prevaccination (p=0.0435). Even though they had a significant rise in titers at day 28 their response was lower than that in non-IgG MGUS individuals (p=0.04) (data not shown).

High M-protein levels are associated with lower numbers of IFN-γ secreting cells
Influenza specific IFN-γ secreting cells were identified in healthy control and MGUS before vaccination. Their frequencies did not differ between the healthy controls and MGUS patients for both the H1N1 and H3N2 influenza strains. A significant rise in H1N1-specific IFN-γ secreting cells was observed at day 28 in the healthy control group with healthy controls. A significantly higher fold increase in IFN-γ secreting cells was shown for healthy controls compared to the MGUS group who failed to expand the numbers of H1N1-specific IFN-γ secreting cells (p=0.035) (Supplement figure S2). Comparison of H3N2-specific IFN-γ secreting cells did not reveal any significant expansions in numbers after vaccination within and between the healthy control and MGUS groups (data not shown).

In order to assess whether monoclonal protein concentration has an influence on influenza specific cell mediated responses, we computed correlations between the concentration of M-protein and the number of influenza specific IFN-γ secreting cells. High levels of M-protein were associated with lower IFN-γ responses than that those
in the MGUS cohort with low M-protein. The number of H1N1-specific IFN-γ secreting cells at day 28 was inversely correlated with monoclonal protein levels (p=0.0182) with a Spearman's rank correlation coefficient of -0.5812 (Figure 2A). No correlations were observed for the H3N2 strain. In an analysis stratified by M-protein concentration, levels ≥15 g/L were associated with significantly lower number of IFN-γ secreting cells specific for the H1N1 strain but not for H3N2. The number of IFN-γ secreting cells in high M-protein MGUS was significantly lower at day 0 (p=0.0235) and at day 28 (p=0.0092) compared to the MGUS cohort with low M-protein (<15g/L) even though low M-protein levels were not associated with a significant increase in IFN-γ secreting cells (Figure 2B).

This study shows that high M-protein levels in MGUS are associated with impaired humoral and cell-mediated response to vaccination. The immune response to influenza vaccination is influenced by factors such as age, host immune defects as well as the type of vaccine administered [18-20]. The presence of M-protein in the serum of MGUS patients is a consequence of the monoclonal expansion of plasma cells and this could possibly affect humoral and/or cellular immune response. Hypogammaglobulinaemia which is often associated with myeloma, [21] is also associated with decreased immune responses. However hypogammaglobulinaemia has been reported at a lower frequency in MGUS [22-24].

Hypogammaglobulinaemia which is often associated with myeloma, [21] is also associated with decreased immune responses. However hypogammaglobulinaemia has been reported at a lower frequency in MGUS [22-24]. The depressed humoral response seen in MGUS is partly reflected by a diminished production of polyclonal immunoglobulins as displayed by a large fraction of the patients. Our data demonstrated a decrease in polyclonal immunoglobulin levels in MGUS and hypogammaglobulinaemia in 42% of the patients. Whether such factors, together with the detected monoclonal protein load, lead to a decrease in specific antibody production has not been investigated before. Also of importance is the fact that MGUS predominantly affects the elderly in whom further age-associated changes and decline in functioning of the immune system have been described. These changes include a decrease in naïve B cell generation, decrease in class switch recombination resulting in decreased IgG production, altered balance of CD4+ T cell memory cells and decreased T cell proliferative responses [25-28].

These age-associated changes result in the consequentially observed increased risk of infection and reduced response to vaccination [29,30]. This should not bias our results as we specifically included age-matched controls.

IFN-γ production described in our study following vaccination is presumably a result of CD4+ T-cells and limited CD8+ T-cell help. Vaccination with subunit trivalent inactivated vaccine leads to Th-cell help for antibody production but no CTL responses [31]. The data from our study indicates that pre-existing influenza specific IFN-γ secreting cells can be detected before vaccination in healthy individuals as well as in MGUS. This could be because of previous encounter with the virus and also previous vaccinations. Vaccination elicited a substantial T cell response in healthy controls as a significant increase in IFN-γ secreting cells was demonstrated whereas MGUS patients failed to expand the response. This suggests that the protective role exerted by IFN-γ during secondary response to influenza virus could be affected in MGUS, and this has consequences on induction of antibody responses as well as delayed resolution of the immune response. It remains to be investigated whether the observed poor response could also be partially due to quantitative and functional defects.
in T-cells. Defects in the T-cell compartment, if present, may also contribute to the less favourable vaccination responses as T-cell dependent B-cell responses were poor.

Following influenza vaccination, secretion of influenza-specific antibodies leads to a rise in serum antigen-specific antibodies. [32,33] IgG antibodies are the predominant antibody class induced after vaccination and increase till they reach a peak 4 weeks post vaccination [34,32,35,36]. Our results show that the amount and, to a lesser extent, the type of M-protein have a significant influence in the development of influenza specific IgG antibodies after vaccination. High M-protein MGUS patients had lower pre-vaccination influenza-specific IgG titers which they failed to increase post-vaccination. Furthermore, increasing M-protein concentration was associated with decreased H1N1-specific IgG response in MGUS. Therefore, poor post-vaccination influenza specific IgG antibody levels observed in MGUS patients with high M-protein may predict response to influenza vaccination. This suggests that these patients are likely to be less protected by vaccination. Patients with IgG MGUS showed significantly lower H3N2-specific IgG titers both pre and post vaccination compared to healthy controls. IgG M-protein is associated with decreased polyclonal IgG production possibly due to suppression of normal B cell differentiation. This then leads to attrition of the plasma cell compartment therefore limiting the number of plasma cells specific for some antigens. In vaccination studies with myeloma patients, IgG myeloma patients had lower serological responses compared to those with IgA paraprotein or Bence Jones [37]. In line with these observations, another study showed that patients with IgA myeloma consistently responded with higher antibody titres and had higher increases in levels of antibodies after pneumococcal vaccination compared to IgG and IgM myeloma [38].

A large population-based study in MGUS by Kristinsson et al., describing an increased risk of bacterial and viral infections that included influenza also showed that higher M-protein concentrations at diagnosis were associated with higher risks of infection [8]. Our results indicate that M-protein load has a great influence on the response to vaccination. The reduced response observed in MGUS may be a consequence B cell dysregulation that results in the M-protein with high M protein being a probable marker of more profound underlying B cell dysfunction. The high M-protein levels together with the consistently observed accompanying hypogammaglobulinaemia are important factors that influence the immune responses to influenza vaccination. We postulate that the clonal expansion of plasma cells lead to decreased B cell repertoire diversity. This alteration in repertoire consequently limits the number of cells available to respond to antigenic challenge and therefore may contribute to decreased humoral response. However no repertoire analysis has been carried out in MGUS, but data in the elderly suggests that B cell diversity decreases with age and the immunoglobulin repertoire diversity correlated with antibody response and health status [39,40].

Although there were similar absolute numbers of circulating CD19+ B cells in healthy controls and MGUS patients, a decrease in the relative number of B cells was observed in MGUS and this might have contributed to the hampered responses. Other B cell subsets important in immune response and aging that include naïve and memory B cells have not been studied [41-44]. There is evidence suggesting that B cells not only function as antibody producers but also as antigen presenting cells and are required for primary T-cell expansion and differentiation [45]. This change in MGUS B cell frequencies observed in our study could also result in insufficient help for T-cells therefore being consequential of the impaired T-cell responses. Our study had some limitations in that the sample size was small and the numbers of subjects in subgroups were low when stratified for M-protein concentration as well as the type of M-protein. On the other hand, showing such significant results in the relative low number of patients studied suggest a strong effect for the high M-protein group. Larger studies are required to confirm our findings. Our MGUS cohort is a heterogeneous group, ranging from individuals with steady and low M-protein levels to those with increasing and high M-protein levels inclined towards transformation to myeloma and other B cell proliferative disorders therefore their degree of immunosuppression is likely to vary.

Conclusions
In summary, MGUS patients displayed subnormal polyclonal immunoglobulin levels. The presence of this hypogammaglobulinaemia together with M-protein load has an influence on the humoral and cellular mediated immune response to vaccination. Moreover, our results suggest that the level of M-protein may be a predictor of poor vaccination response.

Additional files

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
NB, MB and SS designed the study. MB, KW, HN and MK recruited the patients and controls. MB, JW and KW coordinated the study, ST and GH performed the laboratory work. ST performed the statistical analysis and all authors were involved in interpreting the results. ST, NB and MB wrote the paper. AH and AdH contributed materials. Final manuscript was read and approved by all the authors. The authors report no potential conflict of interest.

Publication history
Received: 14-Mar-2013 Accepted: 05-Apr-2013 Published: 09-Apr-2013
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