Efficient boosting of the antiviral T cell response in B cell-depleted patients with autoimmune rheumatic diseases following influenza vaccination

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Abstract

Objectives

Booster vaccination against 2009 H1N1 influenza virus was recommended for rheumatologic patients under immunosuppressive therapy during the 2009/2010 H1N1 pandemic. In this study we assessed whether B cell depletion with Rituximab influences of the antiviral immune response in 2009 H1N1 influenza virus-vaccinated patients.

Methods

Influenza virus-specific immune responses were analysed after the first and a booster vaccination with Pandemrix™ in sixteen consecutive Rituximab-treated patients with different rheumatic autoimmune disorders. Antibody titers were determined by a haemagglutination-inhibition assay and virus-specific T cell responses were evaluated by a flow cytometry-based intracellular cytokine-secretion assay. Patients showing clinical symptoms of influenza infection were excluded from this study.

Results

Two out of seven patients with low (<10%) and four out of nine with normal (>10%) B cells developed significant antibody responses after the first vaccination. Booster vaccination led to an antibody response in one additional patient. After the first vaccination, virus-specific $CD4^+$ and $CD8^+$ T cell responses were significantly lower in patients with low B cells than in those with normal B cells. Of importance, the booster vaccination stimulated the antiviral T cell response only in patients with low B cells.

Conclusion

In the absence of a significant effect of booster vaccinations against 2009 H1N1 influenza virus on the humoral immune response in B cell-depleted patients with autoimmune rheumatic diseases, enhanced antiviral T cell responses in patients with low B cells indicate that T cells, maybe, compensate for the impaired humoral immunity in these patients.

Key words

rituximab, 2009 H1N1 influenza virus, T cell, B cell, antibodies, vaccination

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Received on January 3, 2013; accepted in revised form on March 8, 2013.

Funding: this work was supported by research grants from Roche and Novartis.

Competing interests: K. Hoschler has been Investigator of Clinical Trials sponsored by Novartis, Baxter, Sanofi Pasteur and CSL Australia; the other co-authors have declared no competing interests.

Introduction

Patients suffering from chronic autoimmune rheumatic diseases, such as rheumatoid arthritis (RA), are at major risk to develop infections (1, 2) with a two-fold increased frequency of infections in patients with RA when compared with healthy controls (3). Whether this is due to the disease, the immunosuppressive treatment, or both is still under discussion (4).

Following identification of a novel H1N1 virus and the subsequent declaration of a pandemic in summer 2009 by the World Health Organisation (5), various countries worldwide implemented countermeasures specified in their pandemic plans (*e.g.* containment measures as well as purchase and use of pandemic vaccines and virostatic drugs). Several countries launched mass vaccine programmes as soon as suitable vaccines became available. Several national societies of rheumatology and of other clinical specialties recommended two consecutive vaccinations of patients with chronic autoimmune inflammatory diseases employing the same vaccine directed against 2009 H1N1 influenza viruses.

In patients with autoimmune rheumatic diseases vaccination against influenza virus is generally safe and leads to protective humoral immune responses. Treatment with disease-modifying anti-rheumatic drugs (DMARDs), corticosteroids, or TNF antagonists does not profoundly impair the response to the vaccine (6, 7). However, Gabay *et al.* have shown that two doses of adjuvant vaccine were required to reach a similar response as detected in healthy controls (8). Rituximab (RTX) is a chimeric mouse-human monoclonal antibody selectively targeting CD20 on mature human B cells. RTX treatment leads to depletion of B cells and, subsequently, to a significant reduction of disease activity in several autoimmune rheumatic disorders such as rheumatoid arthritis (9). Immune responses after vaccination to pneumococcus or tetanus have been shown to persist despite treatment-induced B cell depletion (10). A small cohort and (11) a clinical trial has demonstrated that vaccination against H3N2 influenza led to a significant immune response in 14 patients that had formerly been treated with RTX. In contrast, vaccination against two strains of H1N1 in the same 14 patients led only to a partial immune response to one of the viral strains. The vaccination was applied 25-34 weeks after RTX treatment and B cells were only partially depleted in these patients. None of the vaccinated patients developed an influenza infection (12, 13).

To our knowledge, the specific B and T cell responses to any influenza vaccine have so far not been analysed together in the same study of patients with autoimmune rheumatic diseases after RTX treatment. Nor are there data on the influence of RTX on both arms for the specific immune response after vaccination with H1N1 strains available. In this study, we therefore addressed the question whether vaccination with a monovalent, adjuvanted vaccine against 2009 H1N1 influenza virus (*e.g.* Pandemrix™) is protective in rheumatologic patients treated with RTX. B cell-mediated immune responses to vaccination were assessed by a haemagglutination-inhibition (HI) assay. Humoral immune responses were then correlated with individual B cell percentages and specific T cell responses to the H1N1 antigen.

Materials and methods

Patients and treatment

Appropriate informed consent was obtained from all patients included into the study, and the clinical research was conducted in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee. Sixteen consecutive patients treated in the division of rheumatology in St. Gallen were recruited. All patients had been treated in our division with RTX during the previous 1 to 36 months (mean 9.8 months, median 6.0 months) according to the standard protocol used in rheumatoid arthritis. After a premedication with 100 mg methylprednisolone, patients received two intravenous infusions of 1000 mg RTX. All patients received therapies with other immunomodulatory drugs such as methotrexate. Some patients were treated with up to 4 additional cycles of RTX prior to the last, the reference, application

Table I. Patient cohort.

	Age	Sex	Diagnosis	Rituximab (months since)	Cycles of Rituximab	Concomitant medication	Prednisolone mg/d
Patient 1	24	f	Antisynthetase syndrome	29	2	Cyclosporine A	–
Patient 2	31	f	Immunothrombopenia	7	1	Azathioprine	–
Patient 3	64	f	Overlap-syndrome	2	2	Mycophenolate mofetil	5
Patient 4	26	f	Overlap-syndrome	36	2	Methotrexate, Hydroxychloroquine	–
Patient 5	61	m	RA	2	2	Leflunomide	12.5
Patient 6	43	f	RA	14	5	Leflunomide	–
Patient 7	55	m	RA	2	1	Methotrexate	5
Patient 8	46	f	Sjögren's disease	1	1	Azathioprine	5
Patient 9	39	f	Sjögren's disease	6	2	Methotrexate	7.5
Patient 10	65	f	Sjögren's disease	20	3	Methotrexate	–
Patient 11	27	f	SLE	1	1	Azathioprine	–
Patient 12	42	f	SLE	18	2	Methotrexate	7.5
Patient 13	39	f	SLE	9	1	Azathioprine	5
Patient 14	48	f	SLE	1	4	Mycophenolate mofetil	12.5
Patient 15	61	f	SLE	4	1	Azathioprin, Hydroxychloroquine	5
Patient 16	63	f	Polyangiitis with granulomatosis	6	2	Methotrexate	10

RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; f: female; m: male.

of the drug. The patients recruited to the study suffered from antisynthetase syndrome, immunothrombocytopenia, rheumatoid arthritis, systemic lupus erythematosous, Sjögren's syndrome, overlap syndrome, and granulomatosis with polyangiitis (formerly Wegener's disease). Patient data are summarised in Table I.

Patients were recruited to this study if they had received a first intramuscular vaccination with Pandemrix™. Three to four weeks later, a booster vaccination was performed as recommended by the guidelines of several medical societies. The vaccinations consisted of 0.5 ml split virion inactivated vaccine (Pandemrix™) containing a 15 mg haemagglutinin (HA) dose of the 2009 H1N1 influenza virus antigen. Blood and serum samples were collected 4 weeks after the first and again 4 weeks after the second vaccination. Laboratory assessment of disease activity included the erythrocyte sedimentation rate and C-reactive protein (CRP) on the day of the vaccination and 4 weeks later.

Haemagglutination inhibition (HI) test

The immune response against Pandremix™ was analysed using a haemagglutination inhibition (HI) test. Coded serum samples were stored at -80°C until tested. Antibody responses after the first and after the second vaccination were analysed at the Respiratory Virus Unit of the Health Protection Agency, Microbiological Services Division (Colindale, UK) according to standard methods as previously described (14, 15). Briefly, sera were pretreated with receptor destroying enzyme (RDEII, Denka Seiken Ltd., Japan) according to manufacturer's recommendation. Serum samples were tested in twofold serial dilutions in duplicate at initial dilutions of 1:8 and a final dilution of 1:1024. For the test, whole egg-grown virus (NIBRG121; generated by reverse genetics with haemagglutinin and neuraminidase genes from A/California/7/2009(H1N1) and internal genes from A/PR/8/1934(H1N1), kindly provided by the National Institute for Biological Standards and Control, Health Protection Agency, Potters Bar, UK) was used. Virus stock was diluted in Dulbecco's Phosphate Buffered Saline to obtain an antigen content equivalent to four haemagglutination units (HAU). The HI titer was determined as the highest dilution of serum that completely inhibited haemagglutination of the red blood cells (turkey blood).

Antiviral T cell responses and flow cytometry

Heparinised blood was obtained from the patients 4 weeks after the first and again 4 weeks after the second booster vaccination with Pandemrix™ . Peripheral blood mononuclear cells (PBMCs) were collected by density gradient centrifugation according to standard protocols. PBMCs of the vaccinated patients were co-incubated with 0.94 µg specific antigen (X-179A) over 8 hours. The optimal antigen concentration for re-stimulation had been determined in preliminary studies utilising PBMCs from healthy vaccinated donors. For intracellular staining, re-stimulated cells were surface-stained and fixed with cytofix-cytoperm (BD Biosciences) for 20 min. Fixed cells were incubated at 4°C for 40 minutes with permeabilisation buffer (2% FCS/0.5% Saponin/PBS) containing anti-IFN-γ or anti-IL-4 monoclonal antibodies (mAb) (BD Biosciences). Cells were surface-stained with saturating amounts of antibodies against CD3, CD4, CD8, and CD19 (all from BD Biosciences). B and T cell numbers were determined by CD3, 4, and 19 positive counts in PBMCs. Samples were analysed by flow cytometry using a FACSCanto (Becton Dickinson), data were analysed using Cell Quest and FlowJo software (Tree Star, Inc.).

Statistical methods

Non-parametric tests were used for the analysis since most variables were not normally distributed. In addition, parametric tests were performed for the log transformation of the variables. Associations between the response to vaccination, patient group and B cell

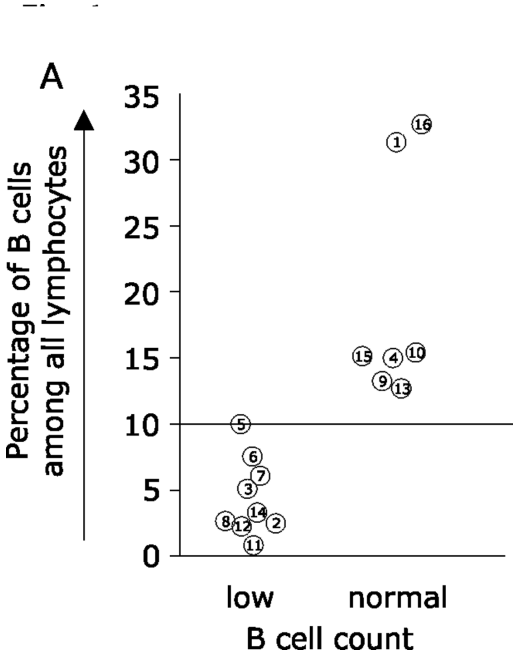
percentages were examined using the χ^2 and the Fisher exact tests.

Results

B cells

A cohort of 16 patients was recruited 1 to 36 months after the last treatment with RTX for different autoimmune rheumatic diseases. Blood samples of all patients were analysed 4 weeks after a first and again 4 weeks after a second, booster vaccination against 2009 H1N1 influenza virus (Pandemrix™, table I). B cell percentages and their absolute numbers were determined in relation to the total lymphocyte numbers. Normal range of B cells is around 13.5% among in peripheral blood lymphocytes (Inghirami, Am J Pathol 1990; 136: 357–67).2 patients (no. 1 and 16) appeared to have high normal levels of B cells. Then 2 groups appear. Patients 4, 9, 10, 13, and 14 grouping around 15% and patients 2, 3, 5, 6, 7, 8, 11, 12, 14 grouping between 0 and 10% B cells (Fig. 1). For further analysis patients were grouped into 2 groups depending on their relative B cell counts: low (<10%) or normal (>10%) B cells.

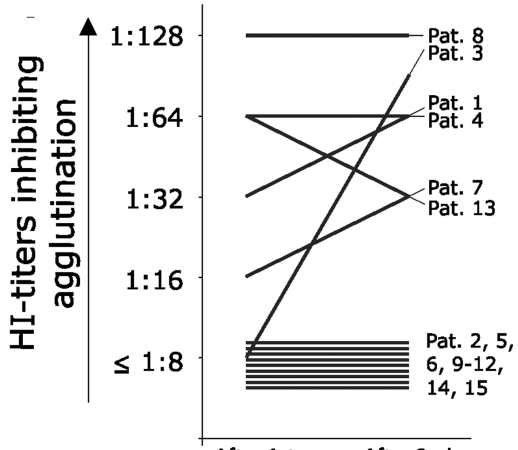
Fig. 1. B cells: PBMC were isolated by Ficoll gradient centrifugation from whole blood of rheumatologic patients treated with RTX. B cells were stained with anti-CD19 antibodies and analysed by flow cytometry. The percentages (Part **A**) and absolute numbers (Part **B**) of B cells in the lymphocyte populations defined by gating in the FSc/SSc plot are depicted for patients with low (<10%) and normal B cells (>10%), respectively. The individual patients are labelled with numbers.



Specific antibody response

Specific antibody responses after vaccination were determined using the HI assay 4 weeks after a first and again 4 weeks after a second, booster vaccination with Pandemrix™. The booster vaccination was applied at the time point of the first visit together with the first analysis. Our measurements showed significant levels of specific antibody responses (titer >1:8) to the first vaccination in only six of the sixteen patients (38%). The second, booster vaccination led to an increase (≥2 titration steps or minimum fourfold increase) of the titer of specific antibodies in one additional patient only, increasing the number of responders after the booster to 7 of 16. This patient showed a low percentages of B cells (5.04%). More than half of the patients (9/16; 56.3%) neither responded to the initial nor to the booster vaccination. When the patients were analysed in relation to their B cells, 2/9 (22.2%) and 4/7 (57.1%) patients with low and normal B cells displayed virus-neutralising antibody responses, respectively (Fig. 2, χ^2 test, p=0.32). Thus, the degree of B cell-depletion did not influence the development of the antiviral B cell response in our cohort.

Fig. 2. Specific antibody responses: antibody titers to 2009 H1N1 were determined by a haemagglutination assay. Serum was obtained from all patients 4 weeks after a first vaccination with Pandemrix™ (left part) and 4 weeks after a second "booster" vaccination (right part). Data are depicted as serial 1:2 dilutions of the haemagglutination assay.
The specific number of patients responding with an antibody titer >1:8 is shown as a table below the figure in separate for patients with low or normal B cells 4 weeks after the first and 4 weeks after the second vaccination.



B cell percentages	After 1st vaccination	After 2nd vaccination
<10% = low	2pos./7neg.	3pos./6neg.
>10% = normal	4pos./3neg.	4pos./3neg.

Antibody response was considered pos. if the detected titer was > 1:8

2009 H1N1 influenza virus-specific T cell response

The patients were assessed for the presence of IFN-γ producing 2009 H1N1 influenza virus-specific CD4 and CD8 T cells as marker of a virus-specific T cell response. After the first vaccination, the capacity of CD4 and CD8 T cells to produce IFN-γ upon stimulation with the 2009 H1N1 influenza virus-specific antigen was markedly increased (p=0.062 and p=0.068, resp.) if T cells were derived from patients with normal percentages of B cells: T cells of patients with low and normal B cells contained 0.95% (CD4)/1.61% (CD8) and 1.98% (CD4)/4.37% (CD8) 2009 H1N1 influenza virus-specific IFN-γ

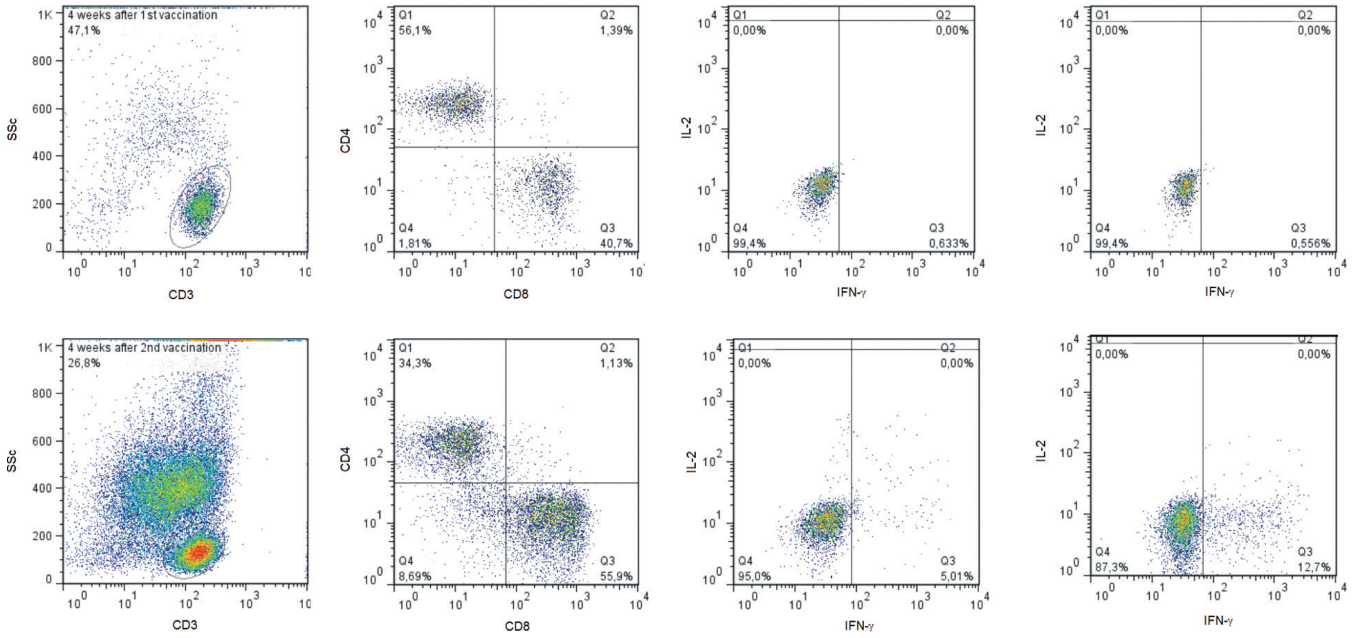


Fig. 3. 2009 H1N1-specific T cell response after primary immunisation: PBMC of (patient no. 11) were co-incubated with the H1N1v-antigen for 8 hours. Cytoplasmic IFN-γ and IL2 were stained with directly labelled mAb and analysed by flow cytometry (EPICS, Beckman Coulter, Fullerton, CA, MatMeth). Cells surfaces were stained with mAb to CD3, CD4, CD8, and CD19. PBMC were assessed 4 weeks after the initial vaccination (upper panels) and 4 weeks after the booster vaccination (lower panels). The leftmost plots depict PBMC in CD3 staining/sideward scatter. Lymphocytes were gated for further analyses. The second plots depict CD4 *vs*. CD8 staining gated on lymphocytes, The right plots display the expression of IFN-γ producing T cells for CD4 T cells (right) and CD8 cells (rightmost). Patient no. 11 belonged to those with low B cells. Abbreviation: SSc: Sidewards Scatter.

producing cells, respectively (Fig. 3). Following the booster vaccination, T cells of patients with low and normal B cells contained 6.85% (CD4)/16.47% (CD8) and 1.57% (CD4)/2.33% (CD8) 2009 H1N1 influenza virus-specific IFN-γ producing cells, respectively (p=0.0008 and p=0.002, comparing the IFN-γ response of CD4 and CD8 cells of patients with low *vs*. normal B cell counts 4 weeks after the second vaccination, respectively). In contrast to the T cell responses in patients with low B cells, those from patients with normal B cells did not change (Fig. 4). Thus, booster vaccination against 2009 H1N1 influenza virus stimulated antiviral T cellular immunity to a higher extend in patients with a high degree of peripheral B-cell depletion.

Clinical assessment

Four weeks after the booster vaccination, patients were asked to fill out a questionnaire answering questions about clinical symptoms possibly associated with an influenza virus infection. The diagnosis of influenza was not established in any patient. However, 6 out of 8 patients from the group with low B cells reported influenza like-symptoms within the last 6 months, whereas patients from the from the group with normal B cells percentages did not report influenza like-symptoms at all (p=0.0031). Both patient groups reported other infection-associated symptoms such as sudden high fever, sore throat, or cough. In addition, 3 patients from the group with low B cells reported to have missed days at work compared to none from the group with normal B cells (χ^2 test, p=0.0906, Table II). Subsequent to the vaccination clinical flares of the autoimmune rheumatic diseases were not reported in either of the groups.

Discussion

The management of patients with chronic inflammatory autoimmune diseases is a major challenge for both, physicians and patients. While a major goal nowadays is to achieve remission, the control of other risk factors, partly created or at least enhanced by the therapy, needs to be taken into consideration. One of these risk factors in patients under immunosuppressive therapy is the occurrence of infections. Infections with influenza viruses are common in the general population with a regular yearly seasonal occurrence. Vaccination against influenza viruses is generally recommended for individuals with impaired immune function, including patients under immunosuppressive therapies with various non-biologic and biologic drugs. Some medical associations have proposed a booster vaccination for these patients while others have not (16). Only one study so far has demonstrated the effectiveness of booster vaccinations in patients under immunomodulatory therapy with RTX (8). Our observations suggest that a booster vaccination of patients treated with RTX may be beneficial if less than 10% of all lymphocytes are B cells.

While a similar antibody response in our study was detected after vaccination in the study of Oren *et al*. (12, 13), Gabay *et al*. demonstrated a higher serum protection rate of 74.6% after the first and of 85.5% after the second vaccination in patients with rheumatic diseases (8). Early after treatment with RTX, this rate was decreased tenfold. This is even less than in our study (33% serum protection rate among patients with low B cells). Based on the

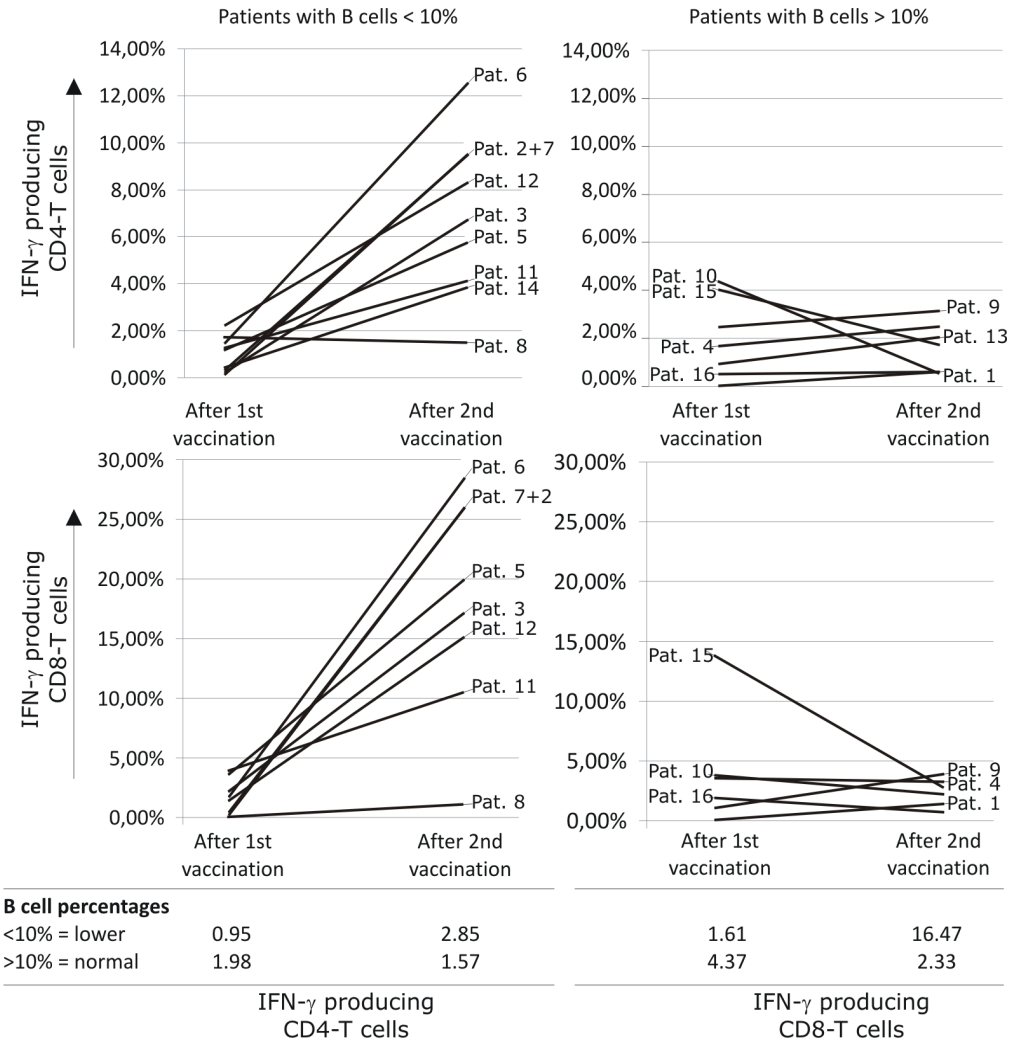


Fig. 4. 2009 H1N1-specific T cell response after primary and booster immunisation. PBMCs were co-incubated with the specific antigen for 8 h. Cells were stained for cytoplasmic IFN-γ and for surface CD3, CD4, and CD8. The percentages of IFN-γ producing T cells 4 weeks after the first and 4 weeks after the booster vaccination are shown for CD4 T cells (upper panels) and CD8 cells (lower panels). T cells of patients with low (<10%) and with normal (>10%) B cells are depicted in the left and the right panels, respectively. Individual patient identification numbers are indicated. The mean percentages of IFN-γ producing CD4 or CD8 T cells are shown as a table below the figure.

data of our cohort we conclude that booster vaccinations are not very effective with respect to the specific antibody response.

T cells are considered to play an important role during influenza infections (17). Bingham *et al*. (18) have shown that after vaccination against H1N1 the immune protection in mice was dependent on CD4 positive but not on CD8 positive T cells or B cells. Our data of more IFN-γ producing CD4 and CD8 positive T cell in patients with normal B cell percentages suggest a direct correlation between the percentage of B cells and the induced T cell response after the initial vaccination. The data concerning T cell priming with a scarcity of B cells are controversial. Some studies have demonstrated that the priming of T cells remains unaffected (19-21), while others claim that it is impaired (22-24). T cell priming is generally considered to be induced by dendritic cells. B cells, on the other hand, are thought to play a less important role for the activation of naïve T cells (25), possibly due to the small number of specific B cells. B cells, however, can provide additional signals for the fine-tuning the T cell responses, *e.g*. via CD40-CD154 ligation (26, 27). Antigen presentation through B cells can be critical for the induction of CD4 (24, 26, 27) and CD8 T cells (28). Especially during the early immune response, 4-6 hours after antigen exposition, B cells play a role as antigen presenting (26, 29, 30) In our setting, the patients' immune systems had to develop a strategy to induce T

Table II. Clinical symptoms and sick leave.

yes or no	no. of answers, patients with B cells <10%	no. of answers, patients with B cells >10%,	χ^2-test *p*-value
Influenza-like symptoms in the last 6 months (n)			
Specific influenza symptoms (n)	6 yes / 2 no	7 no	0.0031
Sudden high fever (n)	3 yes / 4 no	1 yes / 6 no	0.2367
Sore throat (n)	4 yes / 4 no	2 yes / 5 no	0.398
Cough (n)	6 yes / 3 no	2 yes / 5 no	0.1306
Pain in the limbs (n)	7 yes / 1 no	2 yes / 5 no	0.0201
Sick leave* (n)	3 yes / 5 no	6 no	0.0906

*sick leave was defined as an absence from work for at least one full working day.

cells to a novel antigen challenge while B cell help was differently affected by the B cell depletion of RTX. We hypothesise that the priming of T cells may be altered in this group of patients as a strong T cell response in patients with low B cells is only achieved after the booster vaccination. Whether the strong T cell response observed after booster vaccination would have developed, probably with delay, without booster vaccination remains open at this point. In accordance with others, our data demonstrated a positive correlation between the B cell number and the cellular response of RTX-treated RA patients (31). The higher response of CD4 and CD8 T cells to the antigen 4 weeks after the booster vaccination in patients with low B cell numbers may be due to B cell dependent and B cell independent mechanisms for specific T cell activation.

The scenario of a low antibody and an increased T cell response has been demonstrated in elderly persons, who also have difficulties in generating a protective humoral response to influenza vaccines (32). Elderly persons are, however, able to mount a protective T cell immune response to influenza antigens (33). In analogy to these observations it seems feasible that increased specific T cell activities may develop to compensate for a lack of humoral B cell response.

Our study has some relevant limitations: Baseline levels of the T cell response and of antibodies were not known. No baseline antibody titers prior vaccination have been demonstrated in other studies (11, 13). When the pandemia became important and it's outcome was yet completely unknown the study was quickly set up. At that point we did not include a base line assessment, which could have been problematic for the ethics committee and a negative decision concerning this point could have delayed and devalued the whole study. Secondly, the population analysed was heterogeneous. Nevertheless we would not consider this undoubted disadvantage a major bias, as the proposed mechanism appeared to be a general immunological effect independent of the disease or its activity. Thirdly, the number of patients was small, always a major limitation. However, a clear effect with no outlayers was demonstrated. Even a small cohort like ours may therefore be sufficient to describe a general effect, leading to a new, interesting hypothesis for further testing. Our data cannot exclude that the boosted T cell response observed in these patients was, at least in some cases, caused by a normal immune reaction to an infection with 2009 H1N1 influenza viruses rather than by the booster vaccination.

Conclusion

Our results suggest that vaccination with the 2009 H1N1 influenza virus vaccine Pandemrix™ may be immunologically effective in rheumatologic patients previously treated with RTX. Our data demonstrate that determination of the humoral immune response via antibody levels is not sufficient to monitor the effectivity of vaccinations in patients with reduced B cell function due to B-cell depletion. Whether this is also true for the vaccination with other viral antigens, whether it extends to other populations of immunocompromised persons and whether the number of B cells may be used as a surrogate marker in the decision making for a booster vaccination needs to be established in future studies.

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