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Influenza vaccination in systemic autoimmune diseases

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Influenza Vaccination in Systemic Autoimmune Diseases

Albert Holvast

INFLUENZA VACCINATION IN SYSTEMIC AUTOIMMUNE DISEASES

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Stellingen

bij het proefschrift

Influenza vaccination in systemic autoimmune diseases

1. Influenzavaccinatie bij patiënten met systemische lupus erythematodes, de ziekte van Wegener en reumatoïde artritis is veilig. *(dit proefschrift)*

2. De antilichaamrespons en cellulaire respons op influenzavaccinatie zijn (matig) verlaagd bij patiënten met systemische lupus erythematodes. Dit is geassocieerd met het gebruik van prednison en/ of azathioprine. *(dit proefschrift)*

3. Een tweede, booster, influenzavaccinatie heeft geen toegevoegde waarde bij jaarlijks gevaccineerde patiënten met systemische lupus erythematodes en een beperkt effect indien het voorafgaand jaar geen vaccinatie plaatsvond. *(dit proefschrift)*

4. Bij patiënten met de ziekte van Wegener resulteert influenzavaccinatie in een antilichaamrespons en cellulaire respons die vergelijkbaar zijn met die van gezonde controlepersonen. *(dit proefschrift)*

5. Rituximab verhindert tot ten minste 2 maanden na de laatste gift het ontstaan van een antilichaamrespons op influenzavaccinatie bij patiënten met reumatoïde artritis; 6-10 maanden na de laatste gift treedt een matig herstel op. *(dit proefschrift)*

6. Een stelling dient in discussies een uitgangspunt te zijn, geen loopgraaf.

7. Onderzoeksconclusies zijn frequent evidence-chased in plaats van evidence-based: er wordt niet zozeer een hypothese getoetst, als wel naar paradigmatisch acceptabele en in een onderzoekslijn passende conclusies gestreefd.

8. Staand op de schouders van reuzen kan het je ook gaan duizelen.

9. Een ideaal is een richting; met een bestemming die je misschien nooit bereikt.

10. There is no short-cut for experience. *(Willem van Son)*

Groningen 2009, Bert Holvast

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RIJKSUNIVERSITEIT GRONINGEN

INFLUENZA VACCINATION IN SYSTEMIC AUTOIMMUNE DISEASES

PROEFSCHRIFT

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aan de Rijksuniversiteit Groningen
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Paranimfen:

ROGIER DONKER
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Opgedragen aan mijn pakes

Met dank aan mijn lieve ouders, broer en Maaike

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Abbreviations

ANCAs	: antineutrophil cytoplasmic antibodies
APCs	: antigen-presenting cells
BVAS	: Birmingham vasculitis activity score
Con A	: concanavalin A
CRP	: C-reactive protein
CTL	: cytotoxic T lymphocyte
DAS28	: disease activity score of 28 joints
DC	: dendritic cell
DMARDs	: disease-modifying antirheumatic drugs
ELISA	: enzyme-linked immunosorbent assay
ELISPOT	: enzyme-linked immunospot assay
ESR	: erythrocyte sedimentation rate
FCS	: fetal calf serum
GMT	: geometric mean titer
HA	: hemagglutinin
HAI test	: hemagglutination inhibition test
HI	: hemagglutination inhibition
HC	: healthy controls
IFN	: interferon
IL-2	: interleukin-2
LAIV	: live attenuated influenza vaccine
MHC	: major histocompatibility complex
MPO	: myeloperoxidase
MTX	: methotrexate
NA	: neuraminidase
NK cell	: natural killer cell
PBMCs	: peripheral blood mononuclear cells
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
PR3	: proteinase-3
RA	: rheumatoid arthritis
RTX	: rituximab
SEB	: Staphylococcal enterotoxin B
SLE	: systemic lupus erythematosus
SLEDAI	: SLE disease activity index
Th cell	: T-helper cell
TNF	: tumor necrosis factor
VAS	: visual analogue score
WG	: Wegener's granulomatosis
WIV	: whole inactivated (influenza) virus

Chapter 1

Aim and introduction to the thesis

Autoimmune diseases can be classified in diseases affecting a single organ or tissue type, called organ-specific autoimmune diseases, and diseases affecting multiple organs, called systemic autoimmune diseases. In systemic autoimmune disease, corticosteroids and immunosuppressives are frequently used to suppress disease activity. In these diseases, patients have an increased risk of infection [1-4]. Influenza is an infection with a high incidence, and in immunosuppressed patients morbidity and mortality of influenza are increased [5].

Influenza vaccination is recommended in risk groups. The World Health Organization advises to vaccinate (I) residents of institutions for elderly people and the disabled, (II) elderly, non-institutionalized individuals with chronic heart or lung diseases, metabolic or renal disease, or immunodeficiencies, (III) all individuals > 6 months of age with any of the conditions listed above, (IV) elderly individuals above a nationally defined age limit, irrespective of other risk factors, (V) other groups defined on the basis of national data and capacities, such as contacts of high-risk people, pregnant women, health-care workers and others with key functions in society, as well as children 6-23 months of age [6].

Within categories II/III, no specific diseases are mentioned. With regard to some diseases, it is uncertain whether influenza vaccination should be advised. Systemic autoimmune diseases are among the diseases for which no advice has been established. Questions remain regarding safety and immunogenicity of influenza vaccination in these diseases. Systemic autoimmune diseases can be subdivided in separate disease entities. In this thesis, we evaluate the use of influenza vaccination in three different systemic autoimmune diseases: systemic lupus erythematosus (SLE), Wegener's granulomatosis (WG) and rheumatoid arthritis (RA).

There have been concerns regarding the safety of vaccination in patients with systemic autoimmune diseases. These concerns are based on case-reports of onset of autoimmune disease following vaccination, and case-reports of relapses of established autoimmune disease following vaccination. Two theoretical mechanisms suggest vaccination may induce/ enhance autoimmunity: (I) molecular mimicry and (II) bystander activation [7]. Auto-antibodies have been reported to rise in SLE patients following influenza vaccination [8]. However, systematic studies have not shown that vaccination increases disease activity in SLE and RA patients [9,10]. In WG, a retrospective analysis showed that influenza vaccination did not induce disease activity [11], but prospective studies are lacking.

Furthermore, immunogenicity of the influenza vaccine should be evaluated in patients with systemic autoimmune diseases. In systemic autoimmune disease, both the disease itself as well as immunosuppressives may contribute to a decreased immune response to vaccination. In SLE patients, studies reporting normal antibody responses to influenza vaccination [12,13] and studies reporting a, somewhat, decreased antibody response have been published [14-18]. Immune responses to influenza vaccination have not been studied in WG. In RA, a normal immunogenicity was suggested [10], but the effect of new immunosuppressive treatments on responses has not been assessed.

Features of SLE, WG and RA

SLE, WG and RA are autoimmune diseases of unknown etiology in which genetic and environmental factors are involved. SLE is a systemic autoimmune disease with a broad range of clinical presentations. It has a prevalence of about 30-50 per 100.000 [19]. It predominantly affects women (women to men ratio 9:1), with a peak age of onset in young women between their late teens and early 40s [20]. Multiple organs can be affected by SLE; the most common pattern is a mixture of constitutional complaints with skin, musculoskeletal, mild hematologic and serologic involvement [21]. Immunologic abnormalities, especially the production of antinuclear antibodies, are a prominent feature of SLE. Its clinical course is characterized by relapsing and remitting disease activity. Depending on disease activity and affected organs, different immunosuppressive drugs may be used, such as glucocortocoids, hydroxychloroquine, azathioprine, mycophenolate mofetil, cyclophosphamide and methotrexate. B-cell depletion using rituximab (anti-CD20), in patients with disease that is resistant to other immunosuppressive therapy, is currently under investigation [20].

WG is an autoimmune inflammatory disease affecting small and medium-sized vessels, which leads to granulomatous inflammation (particularly in the airways), systemic vasculitis and glomerulonephritis. The disease is associated with the presence of antineutrophil cytoplasmic antibodies (ANCAs) directed against proteinase 3 [22-24]. In Europe, WG has an estimated prevalence of 60 per million [25]. The mean age at diagnosis is approximately 50 years, and WG is slightly more common in men. At presentation more than 90% of patients have symptoms involving the respiratory tract. Systemic symptoms are common due to systemic small vessel vasculitis, especially kidney involvement which is manifested as pauci-immune necrotizing crescentic glomerulonephritis. Often, WG starts as localized disease in the respiratory tract, followed by progression to the

generalized phase. However, for yet unknown reasons, the disease remains in the localized phase in 10-15% of patients. WG can be brought into and kept in remission with immunosuppressive drugs, such as glucocorticoids, cyclophosphamide, methotrexate and azathioprine, but relapses occur [26].

RA has a prevalence of about 1% in the industrialized world. The disease can occur at any age, but is most common among those aged 40-70 years; the incidence increases with age. It affects more women than men (women to men ratio 2.5:1). It is characterized clinically by joint pain, stiffness and swelling due to synovial inflammation and effusion. The clinical course is variable, ranging from mild arthritis to rapidly progressive multisystem inflammation with profound morbidity and mortality. Early treatment with disease-modifying antirheumatic drugs (DMARDs) is recommended; methotrexate is the drug of choice. In patients failing on this therapy, new therapies, biologic response modifiers, are used. These include anti-TNF agents (adalimumab, etanercept and infliximab), an interleukin-1 receptor antagonist (anakinra), a T-cell costimulation inhibitor (abatacept; capable of binding CD80 and CD86 on antigen presenting cells) and an anti-CD20 antibody (rituximab; depletes B cells) [27,28].

Influenza

Influenza has a high incidence, affecting approximately 5% of the adult population each year [29]. Influenza virus is a single-stranded RNA virus from the family of *Orthomyxoviridae*. In humans, three types of influenza viruses circulate: influenza A, B and C. Only types A and B cause widespread outbreaks, type C is of limited significance in humans. Influenza A viruses are classified into subtypes based on antigenic differences in the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). For HA, 16 subtypes (H1-H16) have been identified; for neuraminidase 9 subtypes (N1-N9). In aquatic birds, viruses of all HA and NA subtypes have been detected. Humans are natural hosts of subtypes H1-H3 and N1-N2 only. The influenza B virus consists of only one subtype of HA and one of NA [29].

Both HA, which has HA1 and HA2 subunits, and NA are functionally important. HA1 facilitates virus entry into host cells by binding to sialic acid receptors. HA2 mediates fusion of the viral membrane with the membrane of the endosome, induced by conformational changes in HA as a result from the mildly acidic pH in the endosomal lumen. Neuraminidase assists in the release of virions from infected cells through catalysing the cleavage of glycosidic linkages to sialic acid. Hemagglutinin is the major antigenic determinant of influenza viruses to

which neutralizing antibodies are directed; neuraminidase is the second major antigenic determinant [30].

Influenza has a characteristic epidemiological behavior which is related to antigenic variation of hemagglutinin and neuraminidase. Yearly epidemics occur, of variable burden. This is due to so-called antigenic drift: new strains evolve from circulating influenza virus strains by accumulation of point mutations in the surface glycoproteins. This process results in antigenic variants which can, partially, evade immune recognition. A second process occurs in influenza A viruses only and is called antigenic shift. Antigenic shift happens when an influenza A virus acquires a novel subtype of hemagglutinin, alone or with a novel subtype of neuraminidase, and is associated with major pandemic outbreaks [29].

Clinical manifestations and diagnosis of influenza

Influenza characteristically begins after an incubation period of one to two days with the abrupt onset of fever, headache, myalgia and malaise, accompanied by manifestations of respiratory tract illness, such as cough and sore throat. Systemic symptoms are common, due to the production of proinflammatory cytokines. Viral replication usually occurs only in the respiratory tract. However, the clinical spectrum is broad, and can range from afebrile respiratory illness similar to the common cold, to predominant systemic symptoms with relatively little clinical respiratory tract involvement. Patients with uncomplicated influenza gradually improve over two to five days [31,32]. In these cases, shedding of influenza virus declines after two days, and has an average duration of almost five days [33]. However, some people develop complicated influenza. The major complication is pneumonia, this can be a primary influenza pneumonia but is often accompanied by a secondary bacterial pneumonia, which contributes to approximately 25% of all influenza-associated deaths. Also extra-respiratory complications occur; these mainly involve increases in mortality from underlying morbidity in frail patients, such as cardiovascular pathology. In addition, spreading of the virus to other organs has been described [31,34].

Influenza can be diagnosed clinically, by viral culture, by rapid diagnostic tests, and, retrospectively, by serology. Clinical diagnosis is hampered by the occurrence of influenza-like diseases, but in case of an influenza outbreak it has a positive predictive value of 79 percent [35]. With regard to laboratory diagnosis, viral culture is the gold standard, and can be done using throat swabs, nasal washes, sputum or bronchoalveolar lavage specimens [32,36]. Culturing takes 48-72 hours, and more rapid diagnostic tests are available, which include immuno-

fluorescence assays, enzyme immunoassays, and polymerase chain reaction (PCR)-based testing Especially PCR is highly sensitive and specific, but is costly. Finally, retrospective establishment of influenza can be achieved by serology; a fourfold or greater rise in antibody titers demonstrated between serum specimens obtained during acute illness and convalescent specimens obtained 10 to 14 days later is considered diagnostic [37].

Immune response to influenza

In the immune response to influenza, both innate and adaptive mechanisms are involved. In the early stages of infection, innate immune responses control virus replication. In the innate immune response, there is no antigen-specific recognition. The innate immune response consists of various cellular (e.g. macrophages, dendritic cells and natural killer cells) and secreted components, like interferons (IFNs). Virus-infected cells produce IFN-α and IFN-β, which decrease viral reproduction in infected cells, induce protection against viral infection in surrounding cells and modulate adaptive immune responses [38]. A strong stimulus of antigen-presenting cells (APCs) like macrophages and dendritic cells is binding of viral RNA to Toll-like receptors. Upon activation, APCs produce cytokines which facilitate activation of antigen-specific adaptive responses [39]. Another role is played by natural killer (NK) cells, which may kill infected cells. During the early phase of infection, NK cells are activated through binding to major histocompatibility complex (MHC) class I-viral peptide complexes. NK cells can induce apoptotic cell death by releasing contents of their granules into the infected cell [40]. However, rapid activation of the adaptive immune response is necessary to prevent progression of the infection.

In the adaptive immune response, both B cells and T cells are involved, resulting in humoral and cellular effector mechanisms. Antibodies are primarily directed against hemagglutinin and neuraminidase and are virus-neutralizing (especially anti-hemagglutinin antibodies) as they prevent viral entry and replication in the cell. They may also result in antibody-dependent cell-mediated cytotoxicity by NK cells. Antigen-specific B cells are dependent on adequate T-cell help to switch antibody class production from IgM to IgG and to differentiate into memory B cells [41]. Next to this humoral effector mechanism, also cytotoxic T lymphocytes (CTLs) execute an important effector function. Activated antigen-specific CTLs recognize influenza-infected cells via MHC I-peptide binding, and are able to kill these cells via the action of perforins and granzymes. Both APCs and T helper cells offer signals that induce effective CTL activation and

proliferation, and the generation of CTL memory. Cell-mediated responses to influenza vaccination can be independent markers for protection from influenza infection [42,43].

The antigenic determinants of B- and T-cell responses differ. B cells have a subtype- and strain-specific response, whereas antigenic determinants of the T-cell response are more conserved across the different strains of influenza [44].

Influenza vaccines and correlates of protection

Though antiviral treatment of influenza is possible, prevention of influenza by vaccination is the most important method to reduce morbidity and mortality. First, whole inactivated virus formulations were developed. These are immunogenic, but are associated with local and systemic side effects. This led to the development of formulations of disrupted virus particles, i.e. split-virus vaccines (first licensed in the USA in 1968). Their side effect profile was better than that of whole inactivated virus; however, they have a comparatively low immunogenicity in unprimed individuals. Then, as a further refinement, formulations containing only purified hemagglutinin and neuraminidase, so-called subunit vaccines, were developed. Subunit vaccines have a similar immunogenicity profile as split-virus vaccines, but have fewer local and systemic side effects [32]. Also a live attenuated influenza vaccine (LAIV) for intranasal vaccination has been developed. This was first licensed in 2003 in the United States, and is currently awaiting approval in Europe. Importantly, the use of LAIV is contraindicated in immunocompromised hosts [45].

Subunit vaccines effectively elicit antibody responses. However, they are incapable of inducing an MHC I-restricted CTL response, as subunit vaccine antigens are presented via MHC II. Therefore, vaccinations with subunit influenza vaccine are expected to induce antibody responses and $CD4^+$ T-cell responses, but no $CD8^+$ T-cell responses [46,47]. In contrast, whole inactivated influenza virus (WIV) stimulates $CD8^+$ T cells in a MHC class I restricted way [48]; it is suggested that WIV can induce Toll-like receptor activation resulting in cross-presentation of antigens to MHC class I [49].

For antibody responses, correlates of protection have been established. A titer of ≥ 40, measured by the hemagglutination inhibition test, is considered protective in healthy adults [50]. However, especially in risk groups like the elderly, cell-mediated responses may also correlate to protection, independent from antibody titers [43]. No consensus is yet achieved with regard to assays for the evaluation cell-mediated responses and with regard to correlates of protection.

Aims and outline of the thesis

Aims of the studies presented in this thesis were to evaluate influenza vaccination in patients with systemic autoimmune diseases with regard to the following issues:

- safety
- antibody and cell-mediated responses
- influences of immunosuppressives on immune responses to influenza vaccination
- potential strategies to improve immune responses, if decreased responses to conventional vaccination are found

In **part 1** we examine influenza vaccination in SLE patients. First, we studied safety of influenza vaccination, using the SLE disease activity index, and the antibody response to influenza vaccination in a cohort of quiescent SLE patients (**chapter 2**). These topics are reviewed in **chapter 3**. Next, as cell-mediated responses are involved in the immune response to influenza, and as these can be an independent correlate of clinical protection from influenza, we studied these responses in SLE patients in a second vaccination study (**chapter 4**). As both antibody and cell-mediated responses to influenza vaccination were diminished in SLE patients, we examined booster vaccination as a strategy to enhance the immune response in SLE (**chapter 5**).

In **part 2** we study influenza vaccination in WG and RA patients. First, discussed in **chapter 6**, safety of influenza vaccination and antibody response to influenza vaccination in WG patients were evaluated. Next, cell-mediated responses to influenza vaccination were determined in WG patients (**chapter 7**). In RA patients, the antibody response to influenza vaccination was evaluated in patients using conventional disease modifying anti-rheumatic drugs versus patients on anti-CD20 treatment (**chapter 8**).

Finally, an integrated discussion of these studies is given in **chapter 9**.

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Chapter 2

Safety and efficacy of influenza vaccination in systemic lupus erythematosus patients with quiescent disease

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ABSTRACT

Aims were to assess the safety and efficacy of influenza vaccination in patients with systemic lupus erythematosus (SLE), and to evaluate the influence of immunosuppressive drugs on the immune response.

SLE patients (n = 56) and healthy controls (n = 18) were studied. All patients had quiescent disease (SLE disease activity index ≤ 5). Four patient groups were defined on the basis of their drug use: (1) no drug treatment; (2) hydroxychloroquine treatment; (3) azathioprine treatment; (4) prednisone treatment. Participants received trivalent influenza subunit vaccine during October/November 2003. Disease activity scores and side effects were recorded. Antibody titers against influenza virus were measured before and 30 days after vaccination using the hemagglutination inhibition assay.

Influenza vaccination did not result in changes in disease activity and was well tolerated. SLE patients had fewer seroconversions or fourfold titer rises for A/H1N1 ($P < 0.001$) and A/H3N2 ($P < 0.001$) than healthy controls, while for B/Hong Kong the difference was of borderline significance ($P = 0.051$). With regard to immunosuppressive treatment, fewer SLE patients using azathioprine developed fourfold titer rises against A/H3N2 ($P = 0.041$), and fewer achieved titers of ≥ 40 against A/H3N2 ($P = 0.030$) compared with the other patient groups.

Influenza vaccination in SLE patients with quiescent disease is safe but is less effective than in controls. Use of azathioprine was associated with a trend to decreased vaccination efficacy.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by relapsing and remitting disease activity. Immunosuppressive drugs are often needed to control disease activity rendering patients more susceptible for infections. Immunocompromised patients have an increased risk of morbidity and mortality following influenza infection [1]. Therefore, influenza vaccination should be considered in SLE patients. It is, however, still questionable whether vaccination might induce disease activity in patients with established autoimmune disease. A limited number of studies have been performed to establish whether influenza vaccination is safe in SLE patients [2-9]. These studies have their limitations, as most dealt with small numbers of SLE patients [3,4,6,8,9] and included patients irrespective of their level of disease activity [4,5,7-9].

Furthermore, it is unclear whether vaccination is effective in SLE patients as they are assumed to have decreased primary and secondary immune responses [10]. In addition, use of immunosuppressive drugs may further decrease the immune response following vaccination. In some studies it has been demonstrated that SLE patients display a reduced antibody response after vaccination compared to healthy adults [4,7,11]. In contrast, other studies suggested a normal vaccination efficacy [2,3,5,6]. Although it has been shown in transplantation patients that the use of drugs like corticosteroids, azathioprine, and cyclosporin decreases the antibody response after vaccination [12-16], influence of immunosuppressive drugs on efficacy of influenza vaccination in SLE patients has not been well examined.

In this study we assessed safety and efficacy of influenza vaccination and the effect of medication on vaccination efficacy in our (immunosuppressed) cohort of systemic lupus erythematosus (SLE) patients.

Methods

Patients

Patients were eligible for the study when they fulfilled at least 4 of the American College of Rheumatology criteria for SLE [17] and had quiescent disease, defined as a SLE Disease Activity Index (SLEDAI) ≤ 5 [18]. Based on predefined criteria concerning medication, patients were divided in 4 groups. Group A consisted of SLE patients who did not use immunosuppressive drugs. Patients in group B used hydroxychloroquine ≥ 400 mg/day and patients in group C used azathioprine ≥ 50 mg/day. In both groups (B and C) a stable dose of prednisone less than

10 mg/day was allowed. Finally, group D consisted of patients who used a stable dose of prednisone ≥ 10 mg/day. Stable was defined as a constant dose, unaltered for at least a period of 2 months prior to vaccination. Patients were excluded when: (1) no informed consent was given, (2) in case of pregnancy, (3) other immunosuppressive drugs than hydroxychloroquine, azathioprine, or prednisone were used. A total of 5 patients using MTX and 12 patients treated with a variety of other immunosuppressive drugs (cyclophosphamide, cyclosporine, mycophenolate mofetil) were excluded. Healthy volunteers, age and sex matched, were used as controls.

Vaccines

Influvac®, a trivalent influenza vaccine (2003-2004), was supplied by Solvay Pharmaceuticals (Weesp, The Netherlands). The vaccine contained surface-antigens (hemagglutinin and neuramidase) of viruses bred on chicken eggs, of the following strains: A/Moscow/10/99-like (A/H3N2) (A/Panama/2007/99 RESVIR-17 reass.), A/New Caledonia/20/99-like (A/H1N1) (A/New Caledonia/20/99 IVR-116 reass.), B/Hong Kong/330/2001-like (B/Shangdong/797); 15 μg hemagglutinin per virus preparation.

Procedures

Patients and controls were vaccinated with Influvac®, a subunit vaccine, in October and November 2003. SLE patients were vaccinated at a regular outpatient visit. SLEDAI was recorded for measuring disease activity. After 30 ± 3 days patients and controls were seen again during which visit SLEDAI scores were once more recorded in the patients. In addition, patients were asked to fill in a Visual Analogue Score on a scale of 0-10 (patient VAS, disease activity as experienced by the patient) during both visits. In all participants information on previous influenza vaccination was obtained and adverse effects following vaccination were recorded. Adverse effects were classified into local (itching, pain, erythema, and induration at the site of vaccination), systemic (fever, tiredness, sweating, myalgia, chills, headache, arthralgia, diarrhea, common cold like complaints), and other adverse effects.

At the time of vaccination and at the follow-up visit 10 ml blood was drawn. After sampling, serum was stored at –20° C till the end of the study.

Hemagglutination Inhibition Test (HAI test)

For quantitative detection of influenza antibodies the hemagglutination inhibition (HAI) test was used. HAI tests were performed with guinea pig erythrocytes following standard procedures [19] with slight modifications as described elsewhere [20]. Sera were tested against all three vaccine strains. The antibody response was evaluated in three ways: by assessment of a ≥ fourfold titer rise, by means of a titer rise to ≥ 40, and by the Geometric Mean Titers (GMTs). Four-fold titer rises and seroconversions are widely in use as parameters for efficacy of vaccination. Seroconversions were defined as those samples that tested negative (below 1:10) prior to vaccination, rising to at least 40 after vaccination. Titers ≥ 40 can be considered as protective in healthy adults [21], and a median titer of 28 protects 50% of healthy adult vaccinees [22].

Statistical analysis

Data were analyzed using SPSS 11 (SPSS Inc). Mann-Whitney U test, Wilcoxon Signed-Rank test, Fisher's exact test, and Kruskal-Wallis test were used where appropriate. A P-value < 0.05 was considered statistically significant.

Results

Fifty-six SLE patients and 18 healthy controls were included. Forty-three (77%) of the SLE patients had received influenza vaccination in the past compared to 4 (22%) of the healthy controls ($P < 0.001$). In accordance, more patients (34 out of 56) than controls (1 out of 17) had received influenza vaccination the year before (2002-2003; $P < 0.001$), which consisted of the same viral antigens. Patients were divided into 4 groups, based on immunosuppressive medication (**Table 1**). Medians for these various drugs were 400 mg/day of hydroxychloroquine in group B, 100 mg/day of azathioprine in group C and 10 mg/day of prednisone in group D. Baseline characteristics were equally distributed among groups. Patient groups did not differ in duration of SLE, patient VAS and SLEDAI (**Fig.** 1, $P = 0.644$) before vaccination. Within patient groups, the numbers of patients who had received influenza vaccination in the past were comparable ($P = 0.231$), however more patients in the azathioprine group had received a vaccination in the previous influenza season (2002-2003) compared to other patient groups ($P = 0.026$).

Table 1. Baseline characteristics of SLE patients and controls

Variable	No med.	HCQ	AZA	PRED	HC	*P*
Number	12	17	13	14	18	
Age Median (range)	45 (29-78)	42 (26-66)	47 (28-64)	46.5 (18-71)	40.5 (21-57)	0.518
Sex Male/ Female	4/8	1/16	1/12	0/14	4/14	0.068
Duration of disease (Yrs) Median (range)	8 (2-43)	9 (3-45)	10 (4-29)	5 (1-36)		0.730
Influenza vaccination in the past Yes/ No	8/4	1/6	12/1	2/2	4/14 #	*< 0.001*
Influenza vaccination last season Yes/ No	6/6	7/10	12/1*	9/5	1/17 #	*< 0.001*

*SLE: systemic lupus erythematosus, HCQ: hydroxychloroquine, AZA: azathioprine, PRED: prednisone, HC: healthy controls, # P < 0.001 when SLE patients are compared to healthy controls, * P = 0.026 when patients on azathioprine are compared to other patient groups*

Safety of vaccination

SLEDAI scores after vaccination did not differ significantly from scores before vaccination in any of the patient groups. However, in the azathioprine group patient VAS scores were significantly lower after vaccination. In the other patient groups no significant changes of patient VAS scores were observed (**Fig.** 1).

Concerning side effects, 3 SLE patients reported local adverse reactions, 19 reported systemic adverse reactions. One healthy control reported a local adverse reaction and 1 healthy control reported a systemic adverse reaction. The difference in systemic adverse reactions between SLE patients and controls was significant ($P = 0.02$). In particular tiredness, sweating and myalgia were reported. All adverse reactions were mild.

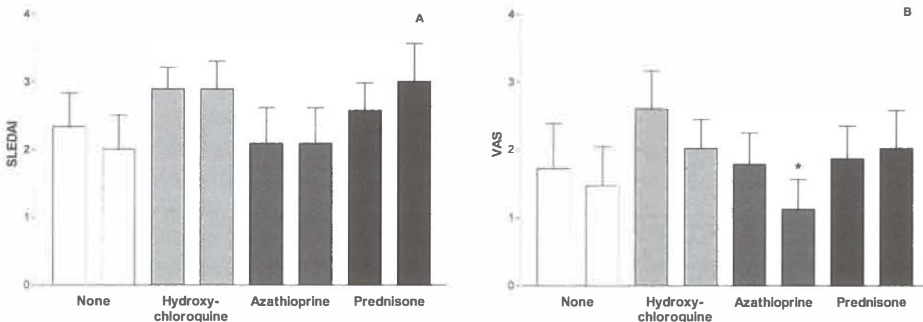

Figure 1. Influence of vaccination on disease activity in the different patient groups. *Disease activity was measured by SLEDAI (A) and patient VAS (B) depicting patient perception of disease activity. Data are presented as mean + SEM. Left bars in each couple represent results before, right bars represent data 30 days after vaccination. * P < 0.05 (Wilcoxon Signed-Rank tests).*

Efficacy of vaccination

Geometric Mean Titers (GMT) in SLE patients and in controls are shown in **Table 2**. As expected, since more SLE patients were vaccinated with the same vaccine the previous season, GMTs before vaccination were significantly higher in SLE patients compared to controls ($P < 0.001$ for A/H1N1, $P = 0.036$ for A/H3N2, and $P < 0.001$ for B/Hong Kong). In patients as well as in controls GMT increased after vaccination and did not differ significantly between both groups. However, SLE patients had less seroconversions or fourfold titer rises against A/H1N1 ($P < 0.001$) and A/H3N2 ($P = 0.001$) compared to controls, for B/Hong Kong this difference tended to be significant ($P = 0.051$; **Table 3**). Seventy-five percent of SLE patients achieved a titer after vaccination of ≥ 40 for both influenza A strains together compared to 100 percent of healthy controls ($P = 0.030$). No significant differences were found in the percentage of patients who achieved a post-vaccination titer ≥ 40 for separate influenza strains compared to healthy controls, although a trend towards a lower percentage in patients could be seen.

Table 2. Geometric mean titers to influenza

	A/H1N1 T = 0	A/H1N1 T = 28	A/H3N2 T = 0	A/H3N2 T = 28	B/HK T = 0	B/HK T = 28
SLE n = 56	32.4	142	50.0	183	16.2	64.0
HC n = 17	6.93 **	130	21.7 *	272	5.65 **	49.0

*T = 0: before vaccination, T = 28: four weeks after vaccination, B/HK: B/Hong Kong, SLE: systemic lupus erythematosus, HC: healthy controls, * $P < 0.05$, ** $P < 0.001$*

Because more SLE patients than controls had an antibody titer ≥ 40 against influenza A/H1N1 and B/Hong Kong before vaccination (**Table 3**), we assumed that this could reduce the number of patients reaching a seroconversion or fourfold increase in titer. To exclude effects of an influenza vaccination the previous season, we examined those participants who did not receive an influenza vaccination in 2002 separately. SLE patients showed significant less seroconversions or fourfold titer rises to A/H1N1 and A/H3N2 (**Table 4**).

Table 3. Seroconversions or fourfold titer rises and titers ≥ 40

	SLE n = 56	Healthy Controls N = 17	*P*-value
Seroconversion rate			
A/H1N1 s.c./4-f.	24 (43%)	16 (94%)	*< 0.001*
A/H3N2 s.c./4-f.	22 (39%)	15 (88%)	*0.001*
B/Hong Kong s.c./4-f.	23 (41%)	12 (71%)	0.051
Seroprotection rate T = 0			
A/H1N1	27 (48%)	1 (6%)	*0.001*
A/H3N2	35 (63%)	7 (41%)	0.163
B/Hong Kong	14 (25%)	0 (0%)	*0.030*
Seroprotection rate T = 28			
A/H1N1	47 (84%)	17 (100%)	0.105
A/H3N2	48 (86%)	17 (100%)	0.185
B/Hong Kong	39 (70%)	12 (71%)	1.000

Seroconversion rate: seroconversion or fourfold titer rise, T = 0: prior to vaccination, T = 28: 4 weeks after vaccination

Table 4. Response of participants with no influenza vaccination in the previous year (2002)

	SLE patients n = 22	Healthy Controls n = 16	*P*-value
Seroconversion rate			
A/H1N1	14 (64%)	16 (100%)	*0.012*
A/H3N2	10 (45%)	15 (94%)	*0.002*
B/Hong Kong	13 (59%)	12 (75%)	
Seroprotection rate T = 28			
A/H1N1	18 (82%)	16 (100%)	0.124
A/H3N2	19 (86%)	16 (100%)	0.249
B/Hong Kong	15 (68%)	12 (75%)	0.729

Effect of medication on vaccination efficacy

To evaluate the influence of immunosuppressive medication on vaccination efficacy, we compared the percentage of seroconversions or fourfold titer rises and protective titers after vaccination in patients without medication with those in patients using immunosuppressives. For this purpose we combined patient groups B-D in which immunosuppressive medication was used. This analysis showed no difference between patients without medication compared to patients using immunosuppressives in the percentage of seroconversions or fourfold titer rises

(P= 0.325 for A/H1N1, P= 0.184 for A/H3N2) nor in achievement of titers ≥ 40 (P= 0.666 for A/H1N1, P= 0.180 for A/H3N2). Next, we conducted a sub-analysis in which all patient groups were compared to each other (**Table 5**). Concerning A/H1N1 and B/Hong Kong no difference was found in the percentage of seroconversions or fourfold titer rises (P= 0.619 for A/H1N1, P= 0.316 for B/Hong Kong) nor in the achievement of titers ≥ 40 (P= 0.396 for A/H1N1, P= 0.226 for B/Hong Kong). However, concerning A/H3N2 SLE patients receiving azathioprine had less fourfold titer rises than other patient groups (P= 0.041). Furthermore, a smaller proportion of the azathioprine group achieved titers ≥ 40 against A/H3N2 (P= 0.030) compared to the other patient groups.

Table 5. Influence of medication on vaccination efficacy

	No Med. n = 12	HCQ n = 17	AZA n = 13	PRED n = 14	*P*-value
Seroconversion rate					
A/H1N1	7 (58%)	7 (41%)	4 (31%)	6 (43%)	*0.619*
A/H3N2	7 (58%)	8 (47%)	1 (8%)	6 (43%)	*0.041*
B/Hong Kong	7 (58%)	8 (47%)	3 (23%)	5 (36%)	*0.316*
Seroprotection rate T = 0					
A/H1N1	6 (50%)	8 (47%)	7 (54%)	6 (43%)	*0.982*
A/H3N2	8 (67%)	11 (65%)	7 (54%)	9 (64%)	
B/Hong Kong	5 (42%)	2 (12%)	4 (31%)	3 (21%)	*0.295*
Seroprotection rate T = 28					
A/H1N1	11 (92%)	14 (82%)	9 (69%)	13 (93%)	*0.396*
A/H3N2	12 (100%)	16 (94%)	*8 (62%)*	12 (86%)	*0.030*
B/Hong Kong	11 (92%)	12 (71%)	8 (62%)	8 (57%)	*0.226*

No Med: no immunosuppressives, HCQ: hydroxychloroquine, AZA: azathioprine, PRED: prednisone

Discussion

The present study demonstrates that influenza vaccination is safe in SLE patients with quiescent disease but has decreased efficacy, in particular in patients using azathioprine. It can be argued that disease activity may increase after a longer time period than the follow-up used in this study. However, the immune response to influenza generates during the first weeks following vaccination. In case vaccination enhances established autoimmunity, this is expected to occur particularly in this period. Therefore we applied a second assessment of disease activity 4 weeks following vaccination.

We found no increase in SLE disease activity nor in patient perception of

disease activity, as measured by patient VAS, 4 weeks after influenza vaccination. This corresponds with previous studies [2-9], in which clinical and laboratory-assessed lupus disease activity did not increase following vaccination. In one study, increased disease activity was reported, though infrequent and usually mild [2]. Another study reported 1 patient (out of 11) with significantly more disease activity following vaccination [6]. Although SLE patients had more systemic side effects of influenza vaccination, these were all mild. Symptoms as tiredness, sweating and myalgia, which were considered as side effects, are common in SLE patients although these are not criteria for disease activity in SLEDAI. Whereas SLEDAI scores did not change, the symptoms mentioned above occurred in some patients following vaccination. This suggests, at the least, a temporal relationship. However, the higher frequency of side effects might be a result of a reporting bias in patients. It is known that many SLE patients with quiescent disease experience a decreased sense of well-being [23-25], which contributes to such a bias. We conclude that influenza vaccination in SLE patients appears to be safe.

Studies concerning efficacy of influenza vaccination thus far are conflicting, as some indicate normal efficacy in SLE patients [2,3,5,6] whereas others conclude that vaccination efficacy is reduced [4,7,11]. An overview is given in **Table 6**. In general, these studies contained less numbers of patients than our study, efficacy was partially analyzed and effects of previous vaccinations were not mentioned. In addition, the effects of differences in drug use were often not sufficiently taken into account. Furthermore, previous influenza vaccinations were not recorded. In summary, conflicting data can be explained by methodological differences.

Therefore, we evaluated efficacy of influenza vaccination in SLE patients in several ways. With respect to the percentage of patients who reached a seroconversion or fourfold titer rise we found that influenza vaccination is less effective for A/H1N1 and A/H3N2 in SLE patients. Accordingly, fewer SLE patients achieved a protective titer after vaccination for both influenza A strains together when compared to healthy controls, despite the fact that more patients than controls had received a vaccination consisting of the same viral antigens the year before. We suggest that the GMT in SLE patients after vaccination did not differ from controls because GMT before vaccination was higher in SLE patients which can well be accounted for by the higher rate of previous vaccination in SLE patients. The conclusion that SLE patients appear to have a decreased immune response compared to healthy controls is supported by the sub-analysis of those patients and healthy controls who did not receive an influenza vaccination the

previous season. Also in these subgroups a significantly decreased humoral response to A/H1N1 and A/H3N2 in SLE patients as compared to healthy controls was found.

Table 6. Studies dealing with efficacy of influenza vaccination in SLE patients

Study	Year	SLE (n)	Parameters	Humoral response of SLE vs HC	Effect of Immunosuppressives
1. Brodman *et al.* [5]	1978	46	GMTs, Titers ≥ 40	similar/ decreased	No significant effect of PRED, AZA or HCQ
2. Louie *et al.* [6]	1978	11	SC, GMTs	Similar	-
3. Ristow *et al.* [4]	1978	29	SC, GMTs	Trend towards decreased	No significant effect
4. Williams *et al.* [7]	1978	19	SC, GMTs, Titers ≥ 40	Decreased	PRED tended to lower responses
5. Herron *et al.* [2]	1979	20	SC, GMTs	Similar	PRED tended to lower responses
6. Kanakoudi-Tsakalidou *et al.* [3]	2001	11	SC, GMTs, Titers ≥ 40	Similar	No significant effect
7. Abu-Shakra *et al.* [11]	2002	24	SC, Titers ≥ 40	Decreased	AZA or ≥ 10 mg PRED/ day tended to lower responses

SLE: systemic lupus erythematosus, SC: seroconversion or 4-fold titer rise, GMT: geometric mean titer

Healthy controls and study design

1. 58 HC; 23 pts on PRED, mean 20 mg/ day; 3 pts on AZA, 50 mg/day; 28 pts on HCQ
2. 8 HC
3. 29 HC, matched for pre-vaccination antibody titer
4. Influenza vaccination in 19 pts and 18 HC, placebo vaccination in 21 pts and 18 HC, double blind HC were matched for pre-vaccination antibody titer.
5. 32 HC, open label study
6. Both patients and HC (5) were children
7. No HC, immunogenicity of vaccination was compared to expected immunogenicity

To evaluate whether our group of healthy controls was representative we compared the GMTs of this group with those of a healthy control group vaccinated in the course of a routine survey of the 2002-2003 Influvac vaccine (data kindly provided by Solvay Pharmaceuticals, Weesp, The Netherlands). The 2002-2003 vaccine was identical to the 2003-2004 vaccine, used in our study. A group of 17 healthy persons, age and sex matched, was compared to our group of controls. In the Solvay survey GMT of A/H1N1 increased from 7.5 to 221.4, of A/H3N2 from 16.0 to 247.2, and of B/Hong Kong from 8.2 to 90.0. The change in

GMTs was not different compared to the results obtained in the controls included in the present study (Mann-Whitney U test).Why patients showed decreased humoral responses to both influenza A strains, but not to the B/Hong Kong strain is subject of discussion. As in our study healthy controls appeared to have a decreased response to the influenza B strain, a possible explanation is that the immunogenicity of the influenza B strain was lower than the immunogenicity of the included influenza A strains. This might have caused a smaller difference in response between patients and controls, in which case the power of our study could have been too low to detect such a difference.

It is reported that the H3N2 subtype of influenza A causes more severe illness than A/H1N1 or influenza B [26], and in most seasons the prevalence of influenza A infections is higher than influenza B infections [27]. So sufficient protection to influenza A (especially A/H3N2) is clinically more relevant than sufficient protection to influenza B.

Why SLE patients have a decreased response to influenza vaccination is not entirely clear. Ioannou *et al.* demonstrated that vaccinations in SLE patients generally tend to give rise to lowered immune responses [28]. Another study showed that pneumococcal vaccination in SLE patients in general is immunogenic but that a subset of patients may remain unprotected by the currently available vaccine [29]. It is conceivable that SLE patients have an intrinsic immunological defect that results in decreased responsiveness to vaccination. The assumption of an intrinsic immune defect is supported by studies reporting decreased cellular immune responses to influenza in SLE patients [30,31].

In addition, use of immunosuppressive medication may influence the efficacy of vaccination. To assess this effect, we included patients using hydroxy-chloroquine, azathioprine and/or prednisone and analyzed data of these groups of patients separately, as there are considerable differences in pharmacological effects between these drugs. Patients using other immunosuppressives were excluded to prevent the formation of small heterogeneous subgroups. SLE patients receiving azathioprine showed a trend towards a decreased immune response against influenza A/H3N2 compared to the other patient groups. This is in concordance with the study of Abu-Shakra *et al.*, in which a trend towards a decreased immune response to influenza vaccination was observed in SLE patients who received azathioprine [11]. In renal transplant patients the use of azathioprine was reported to lower the antibody response to influenza vaccination compared to healthy controls [32] but this could not be confirmed by others [16]. Although the

number of patients included in this study is quite substantial, the subgroups (according to treatment) are quite small. Data on the effects of immunosuppressive drugs on the efficacy of the vaccination should therefore be interpreted with caution.

Twenty-five percent of SLE patients reached titers < 40 against both influenza A strains together and are not expected to be protected from influenza A infection [22]. Moreover one might expect that SLE patients experience less protection from influenza vaccination because cellular immunity also seems to be impaired after vaccination [30,31].

To improve the antibody response of immunosuppressed patients several studies have been conducted in which a second vaccination was given. In general, in immunecompromised patients an increased antibody response could not be achieved after a booster injection [33,34], although Soesman *et al.* did find an increased response in liver transplant patients [15]. Recent studies have shown that virosomal vaccines generate better cellular immune responses, and they enhance the humoral immune response following vaccination as well [35-38]. Regarding the hampered humoral and cellular immune response to influenza vaccination in SLE patients these new vaccines are of particular interest as one might expect them to improve efficacy of vaccination in SLE patients.

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Chapter 3

Influenza vaccination in systemic lupus erythematosus: safe and protective?

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ABSTRACT

Patients with systemic lupus erythematosus (SLE) show decreased immune responsiveness and are vulnerable for infectious diseases, due to the underlying disease and the frequent use of immunosuppressive drugs. Influenza has a high incidence in the population and is associated with increased morbidity and mortality in immunocompromised patients. Therefore, routine influenza vaccination of SLE patients seems indicated. However, there have been concerns about the safety of influenza vaccination in SLE as vaccination was thought to activate the autoimmune response. Safety of influenza vaccination has been studied, and, as far as SLE patients with quiescent disease are concerned, it is now generally accepted that influenza vaccination is safe.

Another point of concern is vaccine efficacy. In immunocompromised patients, the immunogenicity of vaccines may be reduced. In the immune response to influenza (vaccination) both humoral and cell-mediated responses are involved. In SLE, research on the immune response to influenza vaccination has focused on humoral immune responses, demonstrating a blunted humoral response. Future research should focus on cell-mediated immune responses as well, as these are important for clearing of influenza infection and are expected to be impaired in SLE. Because of the decreased immunogenicity of the current influenza vaccine in SLE, new influenza vaccination strategies should be explored to improve vaccination efficacy.

Introduction

Immunocompromised patients have a higher risk of complications and mortality following influenza infection [1]. Patients with systemic lupus erythematosus (SLE) may, depending on their state of disease activity and use of immunosuppressive drugs, be considered immunocompromised. Infections are a frequent cause of death in SLE patients, accounting for up to 20-55% of all deaths [2]. As influenza infection has a high incidence, with an estimated 5-20% of the general population infected annually [3], protection of disease by vaccination is a clinically relevant issue in SLE patients.

With respect to the underlying disease and its treatment, safety and efficacy of vaccines are of importance. There have been concerns about the safety of vaccination in patients with autoimmune diseases as it has been hypothesized that stimulation of the immune system via vaccination may lead to an increase in disease activity. Furthermore, SLE patients display a variety of immune dysfunctions which may influence their response to influenza vaccination. Influenza vaccine could be less immunogenic in SLE patients than in healthy adults, which may reduce the clinical efficacy of vaccination.

In this review we will discuss the safety of influenza vaccination in SLE. In addition, we will evaluate the immune response to influenza vaccination in SLE, focusing on new developments in research on cell-mediated immunity to influenza and future influenza vaccination strategies.

Safety of influenza vaccination in SLE

Vaccination of patients with autoimmune diseases has been subject to discussion for many years. It has been hypothesized that vaccination might evoke disease activity. For several reasons, this has been a matter of concern in SLE patients. First, worsening of SLE has been correlated with viral infections [4]. Secondly, there have been some case reports of increased disease activity following influenza vaccination. Following influenza vaccination, one patient (out of 20) developed a serious flare-up of pre-existing nephritis [5], and another patient (out of a series of 11 patients) displayed increased disease activity [6]. In addition, Abu-Shakra *et al.* studied levels of auto-antibodies in a cohort of 24 SLE patients. They showed that influenza vaccination may induce a transient rise in auto-antibody levels in about ten to 15% of patients [7] though this did not lead to an increase in disease activity [8].

Despite these observations, SLE patients with quiescent disease, in general, neither show an increase in clinical nor an increase in laboratory parameters of disease activity following influenza vaccination [5-15]. These studies indicate that,

although influenza vaccination in SLE may generate autoimmune phenomena, no clinically significant increase in SLE disease activity can be expected. We studied safety of influenza vaccination in a cohort of 55 SLE patients with quiescent disease. We showed that SLE Disease Activity Index (SLEDAI) scores did not increase after influenza vaccination, as studied during a follow-up of one month [13]. Therefore, influenza vaccination can be considered safe in quiescent SLE, in accordance with previous reviews on this subject [4,16].

The immune response to influenza infection and vaccination

During the early stages of influenza infection, the innate immune response restricts viral replication and spread. The innate immune response consists of various cellular and secreted components, like interferons (IFNs). IFN-α and IFN-β, produced by virus-infected cells, are known to decrease viral reproduction in infected cells and to induce protection against viral infection in surrounding cells [17]. However, the innate immune response is not sufficient to block viral spreading and clear the infection. For this, adaptive immune responses are needed. B lymphocytes, $CD4^+$ T helper lymphocytes, and $CD8^+$ cytotoxic T lymphocytes (CTLs) are all involved in the response to influenza infection (**Fig. 1**).

Anti-influenza antibodies are primarily directed against the envelope glycoproteins of the virus, which are hemagglutinin (HA) and neuramidase (NA). These antibodies, anti-HA in particular, are virus-neutralizing as they prevent viral entry and replication inside the cell. At the mucosal surfaces of the respiratory tract, secretory antibodies of the IgA class prevent infection. Circulating antibodies (IgM and IgG) diffuse to and protect the lungs. Immunological memory for B-cell responses offers protection from (re)infection with the same influenza strain [17].

B lymphocytes are dependent on adequate T helper cell responses to switch antibody class production from IgM to IgG and to differentiate into memory B lymphocytes. T helper 1 (Th1) responses stimulate both antibody production and CTLs. T helper 2 (Th2) responses stimulate the production of antibodies, but do not stimulate CTLs [18]. T helper cells also differentiate into memory T helper cells and promote the generation of memory CTLs upon resolution of influenza infection. Influenza-specific memory T cells appear to play a central role in case of an influenza reinfection [19]. T helper cells furthermore secrete cytokines like IFN-γ which may directly mediate viral clearance. It is supposed that these cytokines can have direct cytolytic effects on infected cells [20].

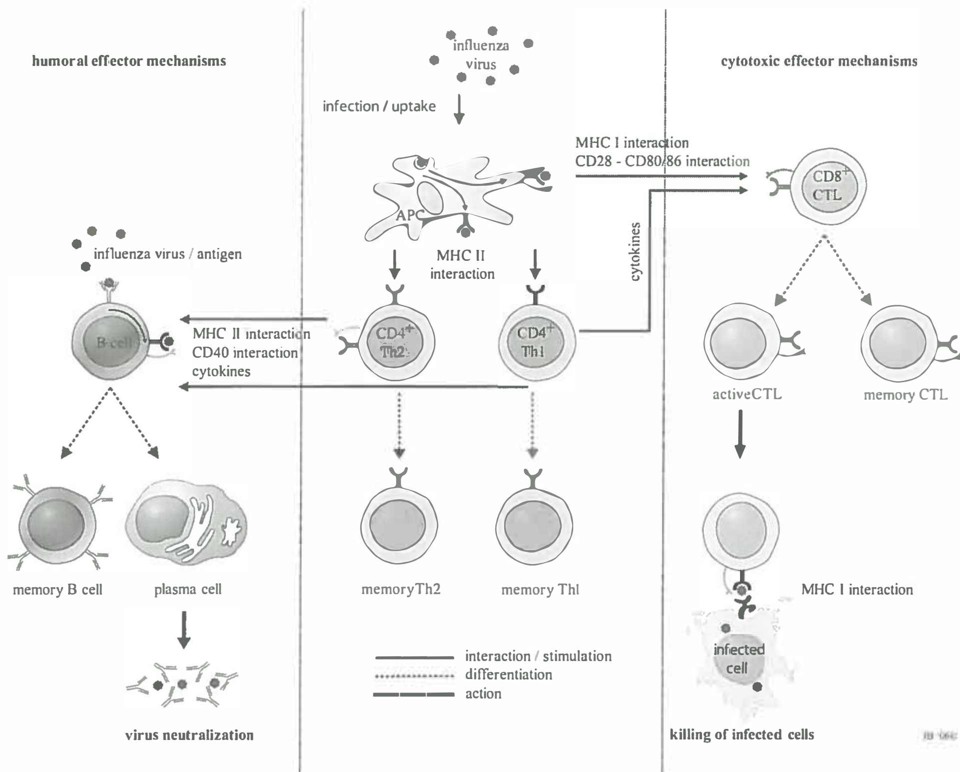

__Figure 1. Simplified scheme of the adaptive immune response to influenza infection.__ (Middle) Antigen presenting cells (APCs) are infected by influenza virus or take up influenza antigens. After being processed, influenza antigens are presented on the surface of APCs. Dendritic cells (DCs) are the most important APCs; B cells also serve as APCs. Both cell types can present influenza antigens via MHC I as well as MHC II, but only DCs can activate naïve cytotoxic T lymphocytes (CTLs). CD4⁺ T helper cells are activated by interaction with antigen presented via MHC II on APCs. Activated T helper 1 (Th1) cells stimulate B cells as well as CD8⁺ CTLs, whereas activated T helper 2 (Th2) cells only stimulate B cells. Stimulated Th1 and Th2 cells can differentiate into memory T helper cells.

__(Left: humoral effector mechanisms)__ B cells can internalize their cognate antigen through their membrane Ig, and following antigen processing they present it via MHC II. This enables Th1 and Th2 cells to recognize antigen specific B cells. Activated T helper cells can stimulate those B cells via additional interaction of co-stimulatory molecules such as the CD40 ligand on the T helper cell with CD40 on the B-cell. Furthermore, cytokines secreted by T helper cells also facilitate B-cell stimulation. The combination of direct influenza antigen stimulation and T-cell help strongly promotes B-cell proliferation, differentiation into plasma cells and generation of memory B cells. Memory B cells offer lifelong memory; plasma cells secrete large amounts of influenza neutralizing antibodies.

__(Right: cytotoxic effector mechanisms)__ CTLs recognize antigens presented via MHC I, but in order to become fully activated CTLs need further costimulating signals. DCs offer such a costimulation via CD80 or CD86, which interacts with CD28 on the T-cell. Th1 cells can stimulate CTL activation via cytokines, which promote antigen specific CTL proliferation, activation, and the generation of CTL memory. Activated CTLs recognize virus-infected cells by interacting with viral antigen presented via MHC I on the cell surface. Thereupon, CTLs induce apoptosis of the infected cell.

CTL responses are directed to virus-infected cells. Th1 responses enhance CTL activity, which is crucial in killing virus-infected cells, and, thus, in clearing viral infections, including infection by influenza [18]. This has been shown in a mouse model [21], and it is conceivable that also in humans CTLs have a protective role in influenza infections.

Most currently used influenza vaccines are inactivated formulations, consisting of either split virus or subunit antigen (isolated HA and NA) [17]. Vaccination with these vaccines can induce B lymphocyte and T helper ($CD4^+$) cell responses. However, these vaccines are poor inducers of CTL responses, as these responses require stimulation of $CD8^+$ T lymphocytes via the major histocompatibility complex (MHC) class I route.

Given the fact that B-cell and T helper cell responses are particularly important, both responses should be analyzed to evaluate the immunogenicity of currently used influenza vaccines. In humans, however, up to now evaluation of humoral responses only has been the standard.

Assessment of the humoral response to influenza vaccination has long been used to evaluate the immunogenicity of influenza vaccines and to predict clinical protection. Hemagglutination inhibition titers ≥ 40 are considered protective in healthy adults and serve as correlates of protection [22]. It has been reported that a median titer of 28 protects 50% of vaccinated healthy adults [23]. However, in elderly, titers ≥ 40 can not be considered protective. In a study in which 397 elderly were vaccinated against influenza, seventy-two (18%) participants developed laboratory confirmed influenza. Sixty per cent of these subjects had post-vaccination titers ≥ 40 and 31% had titers ≥ 640 [24]. The assessment of cell-mediated responses following influenza vaccination is not routinely performed, although some studies have shown the relevance of such an assessment. In healthy elderly persons, cell-mediated immunity has an important role in the protection against symptomatic influenza infection, independent of protection by antibodies [25].

In summary, an effective immune response to influenza infection and vaccination depends on both humoral and cell-mediated responses. In SLE patients, both arms may be disturbed, and need evaluation to give insight in efficacy of influenza vaccination.

The humoral immune response to influenza vaccination in SLE

In SLE, a diminished response to antigenic challenge, including vaccinations, has been suggested [4,16]. Thus far, studies concerning the immune response to influenza

vaccination in SLE have been conflicting, as some indicated a normal response in SLE patients [5,6,9,11,14] whereas others concluded that the immune response is reduced [10,12,13,15,26]. However, in most studies, the numbers of SLE patients were small.

In our study, 56 SLE patients with quiescent disease and 18 healthy controls received influenza vaccination. As compared to healthy controls, SLE patients showed fewer seroconversions: 43% of patients versus 94% of controls for A/H1N1, 39% of patients versus 88% of controls for A/H3N2 and 41% of patients versus 71% of controls for B/Hong Kong. Furthermore, fewer patients achieved a titer ≥ 40 to both influenza A strains (75% of patients versus 100% of controls) [13]. Although the humoral response of our SLE patients was decreased, it still fulfilled the criteria for influenza vaccine immunogenicity as agreed upon by the Committee for Proprietary Medicinal Products [27]. Therefore, the clinical relevance of such a decreased response is still unclear.

The use of immunosuppressive medication may further decrease the humoral response. The use of azathioprine is associated with a trend towards a decreased immune response to influenza vaccination [13,26]. The use of prednisone has also been reported to lower the immune response to the influenza vaccine [5,12,26].

It is clinically relevant whether vaccinated SLE patients are protected for the entire influenza season, i.e. whether they maintain protective anti-influenza titers, mounted following vaccination. This is currently unknown and is subject of investigation. Healthy adults maintain their response up to 12 weeks after vaccination [28], in immunocompromised patients, however, antibody titers tend to decline more rapidly [29].

The cell-mediated immune response to influenza vaccination in SLE

Little is known about cell-mediated immune responses to influenza vaccination in SLE patients. A diminished or disturbed T helper function has been suggested. First, in a study of 150 SLE patients a diminished T helper cell function to recall antigens, as measured by IL-2 (a Th1 cytokine) production, was present in almost half of the patients. This reduction was associated with disease activity and could not be accounted for by use of medication use [30]. Secondly, SLE patients have higher serum levels of IL-10 [31]. IL-10 is a Th2 cytokine and may suppress Th1 responses, whereas the natural response to influenza virus infection in healthy adults is Th1 skewed [32]. As T helper cells play an important role in guiding the immune response, a reduction or disturbance of their function may cause a reduced immune response to infection and vaccination.

Also CTL activity against influenza is important. However, this has not been assessed in SLE patients. In a study of 100 vaccinated institutionalized elderly, those who developed influenza illness had lower CTL activity compared with those who did not. CTL activity was determined by measuring granzyme B activity, granzyme B is an important effector molecule of stimulated CTLs. Assessments were done at vaccination, four weeks after vaccination and 12 weeks after vaccination [33].

Future influenza vaccination strategies in SLE

Recently, pneumococcal and influenza vaccination of immunocompromised patients with rheumatic diseases have been advocated [34]. As the accumulated data strongly indicate that influenza vaccination in SLE patients is safe, influenza vaccination should, indeed, be encouraged.

As discussed above, SLE patients show a diminished immune response to influenza vaccination compared to healthy adults. It has been argued that in most patients this decreased response still seems to be satisfactory [4]. However, criteria to support this conclusion were not given. It is currently unknown whether SLE patients maintain their titers following influenza vaccination for an extended period of time, which is important for protection during the whole influenza season. In addition to a hampered humoral response a blunted cell-mediated response against influenza in SLE is likely. This needs, however, further study as such a blunted response results in a suboptimal protection following vaccination.

In order to overcome a suboptimal protection, we should strive towards higher vaccine immunogenicity. One way of improving the antibody response might be a second (booster) vaccination, which has been applied in several studies. However, in general, a second vaccination did not increase the antibody response in immunocompromised patients [35,36]. Soesman, however, did find an increased response in liver transplant patients. After the first vaccination more than 68% of the liver transplant patients (n = 61) had titers ≥ 40 against all three vaccine strains, which increased to more than 80% after a second vaccination [37]. A different approach would be to use newly developed vaccines, designed for improved immunogenicity, like the virosomal influenza vaccine. Virosomal vaccines have a reconstituted membrane which incorporates influenza HA and NA antigens. The spatial organization is such that the virosome resembles a natural virus particle. A virosome does not contain influenza RNA and is, therefore, not infective [32]. It is expected that the great resemblance to the natural virus particle will improve the response to vaccination. Studies have shown that

virosomal vaccines may enhance humoral immune responses in elderly. In a study of 126 elderly subjects a virosome influenza vaccine induced up to 20% more fourfold titer rises as compared with conventional vaccines [38]. In another study of 76 elderly subjects 68.4% of subjects vaccinated with the virosome vaccine attained antibody titers of ≥ 40 to all three vaccine strains versus 38% for the subunit vaccine [39]. Furthermore virosomal vaccines may generate better T helper responses as well [32]. Importantly, virosomes can be manipulated to elicit CTL responses [40].

In summary, influenza vaccination of SLE patients should be encouraged, as influenza vaccination can be considered safe in quiescent SLE and induces reasonable immune responses. However, further evaluation of these responses, especially the cell-mediated response, is needed. New vaccination strategies appear promising to further enhance influenza vaccine immunogenicity in SLE patients, which can reduce influenza associated morbidity and mortality.

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Chapter 4

Studies of cell-mediated immune responses to influenza vaccination in systemic lupus erythematosus

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ABSTRACT

Both antibody and cell-mediated responses are involved in the defense against influenza. In systemic lupus erythematosus (SLE) patients a decreased antibody response to subunit influenza vaccine has been demonstrated. However, cell-mediated responses have not yet been assessed.

In this study, fifty-four SLE patients and healthy controls received subunit influenza vaccine. Peripheral blood mononuclear cells and sera were obtained before and one month after vaccination. Cell-mediated responses to A/H1N1 and A/H3N2 were evaluated using interferon-γ ELISpot and flow cytometry. Antibody responses were measured using the hemagglutination inhibition test.

Prior to vaccination, SLE patients had fewer interferon-γ spot-forming cells against A/H1N1 compared to controls and a lower frequency of interferon-γ^{+}CD8^{+} T cells. After vaccination, the numbers of interferon-γ spot-forming cells increased in both patients and controls, though it remained lower in patients. Also frequencies of CD4^{+} T cells producing tumor necrosis factor and interleukin-2 were lower in patients after vaccination, compared to healthy controls. As expected for a subunit vaccine, vaccination did not induce a CD8^{+} T-cell response. For A/H3N2-specific responses, results were comparable. Diminished cell-mediated responses to influenza vaccination were associated with the use of prednisone and/ or azathioprine. Patients showed a lower increase in A/H1N1-specific and A/H3N2-specific antibody titers after vaccination, as compared to controls.

In addition to a decreased antibody response, cell-mediated responses to influenza vaccination are diminished in SLE patients, which may reflect effects of the concomitant use of immunosuppressive drugs. This may render these patients more susceptible for (complicated) influenza infections.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a remitting and relapsing course. SLE patients have an increased risk of infection, due to both intrinsic disturbances of immune responses and use of immunosuppressive drugs which are often needed to control disease activity. Indeed, infection-related morbidity and mortality occur more frequently in SLE patients [1].

For influenza, infection-related morbidity and mortality are increased in immunocompromised patients [2]. As influenza infection has a high incidence, with an estimated 5-20% of the general population infected annually [3], influenza vaccination is a clinically relevant issue in SLE patients. Influenza vaccination of SLE patients is safe, as it has been shown that influenza vaccination does not induce disease activity [4]. Annual vaccination in SLE is therefore recommended [5].

In the immune response to influenza, both antibody and cell-mediated responses, comprised of $CD4^+$ and $CD8^+$ T cells, are involved. In SLE, antibody responses to influenza vaccination are diminished [6], but cell-mediated responses have not been assessed. The latter are relevant, as it has been shown that in certain groups, such as the elderly, cell-mediated responses to influenza vaccination can be a marker of clinical protection, independent from antibody responses [7]. The most frequently used vaccine formulations are split virus or subunit vaccines. With these vaccines, antigens are primarily presented via MHC II, which induces $CD4^+$ T-cell stimulation [8]. However, they are incapable of inducing MHC class I restricted $CD8^+$ T-cell responses [9]. In addition, subunit vaccines, in contrast to split virus and whole virus vaccines, do not contain any of the internal proteins that may more readily (re)activate influenza-specific $CD8^+$ T cells.

In SLE, decreased T-helper (Th) recall responses to influenza A and tetanus toxoid antigens have been reported in a subset of patients, as measured by IL-2 production upon stimulation. This decreased function could not be accounted for by the use of immunosuppressives alone, and was shown to be associated with disease activity [10]. In addition, lower levels of cell-mediated cytotoxicity against target cells infected with influenza A and B have been found in SLE patients [11].

Based on these data, we hypothesized that SLE patients have lower $CD4^+$ T-cell responses to subunit influenza vaccine and lower $CD8^+$ T-cell recall responses to influenza antigens than healthy controls. Cell-mediated responses against influenza in SLE, prior to and following vaccination, were evaluated. In addition, antibody responses were evaluated, and vaccine safety was recorded.

Methods

Study population

Patients were eligible for the study when they fulfilled at least four of the American College of Rheumatology criteria for SLE [12]. Exclusion criteria were pregnancy and the presence of an indication for yearly influenza vaccination based on concomitant disease according to international guidelines [13]. A control group of healthy individuals was included that was age and sex matched to the vaccinated SLE patients. Pregnancy was an exclusion criterion for participation as healthy control.

Study design

SLE patients and controls were included from October to December 2005. Before entry, patients were randomized in a ratio of 2:1 to receive an influenza vaccination or to serve as non-vaccinated patient control. At entry (visit 1), patients randomized for vaccination and all healthy controls were vaccinated. Patients and controls were followed up at 28 days (visit 2) and three to four months after inclusion (visit 3). PBMCs were isolated from vaccinated participants at visits 1 and 2 (see below). At each visit blood was drawn, and serum was stored at –20° C until use. Also, SLE disease activity index (SLEDAI) [14] was recorded and patients were asked to mark a visual analogue score (VAS) for disease activity on a scale of 0-10, 0 indicating no activity and 10 indicating the highest activity. Information on influenza vaccination in the previous year was obtained. Adverse effects to vaccination were recorded using a standardized questionnaire which included: itching, pain, erythema, induration at the site of vaccination, shivers, myalgia, fever, headache, nausea, arthralgia, diarrhea, use of an analgesic/ antiphlogistic drug. The study was approved by the institutional medical ethics committee, and informed consent was obtained from all participants.

Influenza vaccine

A single dose of a trivalent subunit influenza vaccine (Influvac®, 2005-2006, Solvay Pharmaceuticals, Weesp, the Netherlands), containing A/New Caledonia/ 20/99 [H1N1], A/NewYork/55/2004 [H3N2] and B/Hong Kong/330/2001, was administered intramuscularly.

Isolation, storage and thawing of PBMCs

PBMCs were isolated from heparinized venous blood by density-gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) immediately after

blood was drawn. PBMCs were frozen in RPMI 1640 (Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS), 50 μg/ml of gentamicin (Gibco, Paisley, UK) and 10% dimethylsulfoxide. PBMCs were stored in liquid nitrogen until use. Pre- and post-vaccination samples, from a SLE patient and a matched control, were simultaneously thawed and batch-processed. A minimum cell viability of > 90%, evaluated by trypan blue staining, was required. Preceding ELISpot assays, PBMCs were rested, by overnight incubation at 37° C. Cells were counted before plating, using an automated cell counter (Beckman Coulter, Fullerton, CA, USA).

Influenza antigens used in assays of cell-mediated responses

β-propiolactone inactivated whole virus (WIV) of A/New Caledonia/20/99 (H1N1) and A/Hiroshima/52/2005 (H3N2) were used to stimulate PBMCs. A/Hiroshima/52/2005 is a very closely related antigenic variant of the vaccine strain A/NewYork/55/2004.

Interferon-γ (IFN-γ) ELISpot assay

Nitrocellulose plates (Nunc, Rochester, NY, USA) were coated overnight at 4° C with 50 μl anti-human IFN-γ, 15 μg/ml per well (Mabtech, Nacka Strand, Sweden). Plates were washed and blocked with culture medium (CM; RPMI supplemented with 50 μg/ml gentamicin and 10% FCS) for one hour at room temperature (RT). Subsequently, 2×10^5 PBMCs were added per well, in 200 μl, and incubated in CM at 37° C with WIV of A/H1N1 and A/H3N2, at a final concentration of 5 μg total viral protein/ml. Concanavalin A stimulation, 5 μg/ml, was used as a positive control and a negative control consisted of PBMCs in CM alone. Stimulation tests were performed in duplo. After 48 hours plates were washed with phosphate buffered saline (PBS), and 50 μl of 1 μg/ml biotinylated anti-human IFN-γ (Mabtech) was added per well for 3 hours at RT. Next, plates were washed again, and 50 μl 1:1000 streptavidin-alkaline phosphatase (Mabtech) per well was added for 1.5 hours at RT. Plates were washed and 100 μl BCIP/NBT-plus substrate (Mabtech) was added per well for 10 minutes. Finally, plates were washed with tap water. After drying, spots were counted using an automated reader (automated ELISpot video-analysis system, Sanquin, Amsterdam, The Netherlands). Results are referred to as IFN-γ spot-forming cells, as IFN-γ-producing $CD4^+$ and $CD8^+$ T cells as well as natural killer (NK) cells, following WIV stimulation, have been described [15].

Flow cytometry

For stimulations, 1.0 - 1.5 x 10^6 PBMCs were cultured in 200 μl CM, in 5 ml polypropylene round-bottom Falcon™ tubes (Becton Dickinson and Company (BD), Franklin Lakes, NJ, USA). Staphylococcal enterotoxin B, at 5 μg/ml, (SEB, Sigma-Aldrich, Saint Louis, MO, USA) was used as a positive control. WIV A/New Caledonia (H1N1) and WIV A/ Hiroshima (H3N2) were used at final concentrations of 1 μg of total viral protein/ml. WIV and negative control (medium only) cultures were incubated in the presence of 10 μg/ml anti-CD28/CD49 (BD). Cells were incubated for 18h at 37° C, the final 16h in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). Following incubation, 10 μl 40mM EDTA in PBS was added, tubes were vortexed and incubated for 10 minutes, to facilitate resuspending. Next, 2 ml FACS lysing solution (BD) was added for 10 minutes. Cells were spun down and washed in PBS-1% bovine serum albumin. Subsequently cells were permeabilized in 500 μl PERM-2 (BD) for 10 minutes in the dark in the presence of pacific blue and orange (Invitrogen, Carlsbad, CA, USA), in a different combination for each stimulus, to enable fluorescent cell barcoding [16]. PBS-20% FCS was added for 5 minutes. Cells were washed and pooled per PBMC sample. Next, anti-CD3-FITC, anti-CD4-PE-Cy7, anti-CD8-PercP, anti-CD69-APC-Cy7, anti-IFN-γ-Alexa 700, anti-tumor necrosis factor (TNF)-APC and anti-interleukin (IL)-2-PE (all from BD) were added, following the manufacturer's instruction. After incubation for 30 minutes at RT, cells were washed and immediately analyzed on a LSR II flow cytometer (BD). Data for at least 1 x 10^6 $CD3^+$ cells were collected. Using the Win-List software package (Verity Software House, Topsham ME, USA), positively and negatively stained populations were gated and Boolean gating was applied. First, lymphocytes were gated by CD3 expression and sideward scatter patterns. Next, $CD4^+$ and $CD8^+$ T-cell populations were gated as $CD4^+CD8^-$ or $CD4^-CD8^+$, respectively. Then, cells from different stimulation tubes were separated in a pacific blue/orange plot. Finally $CD69^{+/-}$ $cytokine^{+/-}$ quadrants were set for the different stimuli simultaneously, according to the negative and positive controls (**Fig. 2A**). Percentages of antigen-specific cells were expressed as the percentage of $CD69^+$ $cytokine^+$ $CD4^+$ or $CD8^+$ T cells within the total $CD4^+$ or $CD8^+$ T-cell population.

Antibody response to influenza

For quantitative detection of anti-influenza antibodies the hemagglutination inhibition (HAI) test was employed, following standard procedures [17]. Influenza A/New Caledonia/20/99 [H1N1] and A/NewYork/55/2004 [H3N2] were provided

by Solvay Pharmaceuticals (Weesp, The Netherlands). Seroconversions were defined as a fourfold rise in titer one month after vaccination, and seroprotection was defined as a titer ≥ 40. Titers < 10 (below detection level) were assigned a value of 5 for calculation purposes [18].

Statistical analysis

Data were analyzed using SPSS 14 (SPSS Inc., Chicago, IL, USA). Titers were log-transformed prior to testing of geometric mean titers. For comparisons of T-cell cytokine responses, Mann-Whitney U tests and Wilcoxon signed rank tests were used. All T-cell frequencies reported are after background subtraction of the frequency of the identically gated population of cells from the same sample stimulated without antigen. For correlations, Spearman's rho was used. Age was normally distributed and tested with Student's *t*-test. For all other variables Fisher's exact test and Mann-Whitney U test were used where appropriate. A two sided *P* value < 0.05 was considered statistically significant. No adjustments for multiple testing were made given the explorative design of the study.

Results

Patient characteristics

Eighty SLE patients gave informed consent to participate and were randomized: 54 for the vaccination group and 26 for the non-vaccination group. Two patients initially randomized for the non-vaccination group were excluded (due to pregnancy and withdrawal, respectively). Patient groups did not differ in sex, age, and medication use. More patients in the vaccination group had received an influenza vaccination the year before as compared to patients not receiving vaccination and controls (**Table 1**).

Cell-mediated responses against A/H1N1 and A/H3N2 were measured in a subset of vaccinated SLE patients (n = 38) and controls (n = 38), matched for age and sex. This subset was based on availability of a matched control and proper acquisition of PBMCs prior to and one month following vaccination. Mean age (SD) in this subgroup was 43.4 years (10.2); 24% were male.

Lower pre-vaccination cell-mediated responses to A/H1N1 and A/H3N2 in SLE patients

In ELISpot, prior to vaccination, SLE patients had fewer IFN-γ spot-forming cells against A/H1N1 and A/H3N2 as compared to controls (Fig. 1). In flow cytometry, the frequency of $CD4^+TNF^+$ T cells upon A/H1N1 stimulation was lower in SLE

patients than in controls (**Fig.** 2B). SLE patients also had a lower frequency of IFN-γ$^+$CD8$^+$ T cells upon A/H1N1 stimulation as well as lower frequencies of IFN-γ- and TNF-producing CD8$^+$ T cells upon A/H3N2 stimulation (**Fig.** 3 A + B).

Table 1. Baseline characteristics and disease parameters

		SLE patients		HC
		Non-vaccinated n = 24	Vaccinated n = 54	Vaccinated n = 54
Sex, males		2 (8.3%)	10 (18.5%)	11 (20.4%)
Age, mean (SD)		45.5 (11.5)	44.8 (13.6)	43.1 (10.9)
Influenza vaccination in previous year		9 (37.5%)	34 (63.0%) *	3 (5.6%) ***
Without immunosuppressives		5 (20.8%)	5 (9.3%)	n/a
Prednisone median (range), in users (mg/day)		10 (41.7%) 6.25 (2.5 - 15)	28 (51.9%) 5 (1.25 – 15)	n/a
Hydroxychloroquine median (range), in users (mg/day)		10 (41.7%) 400 (200 - 800)	30 (55.6%) 400 (200 – 1000)	n/a
Azathioprine median (range), in users (mg/day)		6 (25%) 87.8(50 - 125)	17 (31.5%) 125 (75 – 200)	n/a
Other immunosuppressive drugs		0 (0%)	6 (11.1%) #	n/a
SLEDAI, median (range)	t = 0	2 (0 - 8)	2 (0 - 12)	n/a
VAS, median (range)	t = 0	2.2 (0 - 5.6)	1.6 (0 - 6.6)	n/a

*SLE: systemic lupus erythematosus, HC: healthy controls, SLEDAI: systemic lupus erythematosus disease activity index, VAS: visual analogue score, n/a: not applicable, # Methotrexate; 5 patients used 15 mg per week, 1 used 25 mg per week, * P < 0.05 (vaccinated SLE patients versus non-vaccinated SLE patients), *** P < 0.001 (HC versus vaccinated SLE patients)*

Lower cell-mediated responses to A/H1N1 and A/H3N2 in SLE patients following influenza vaccination

Following vaccination, 68.4% of SLE patients and 71.1% of controls showed a rise in IFN-γ spot-forming cells against A/H1N1; for A/H3N2 60.5% of patients and 73.7% of controls showed a rise. Rises were similar in SLE patients and controls. After vaccination, the number of IFN-γ spot-forming cells remained lower in SLE patients, compared to controls (**Fig.** 1).

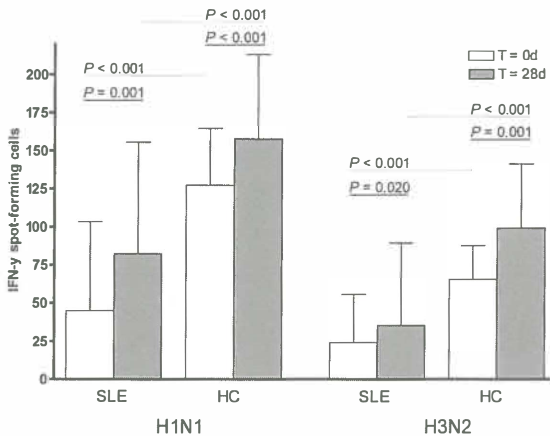

Figure 1. IFN-γ ELISpot. *ELISpot of IFN-γ spot-forming cells per 2 x 10^{5} PBMCs in systemic lupus erythematosus patients (SLE) and healthy controls (HC), in response to A/H1N1 and A/H3N2 stimulation before vaccination (T = 0 days) and four weeks after vaccination (T = 28 days). Results are corrected for responses in unstimulated cultures from the same sample. Medians and interquartile ranges are shown.*

Following vaccination, A/H1N1-specific IFN-γ-producing $CD4^+$ T cells increased in 66.7% of SLE patients and 65.7% of controls. Similarly, A/H1N1-specific TNF-producing $CD4^+$ T cells increased in 61.1% of patients and 71.4% of controls. In 71.4% of controls, also IL-2-producing $CD4^+$ T cells increased (**Fig. 2B**). For A/H3N2, 60% of SLE patients and 61.8% of controls showed an increase in $IL\text{-}2^+CD4^+$ T cells following vaccination; 73.5% of controls showed an increase in TNF^+CD4^+ T cells as well (**Fig. 2C**). So, in SLE patients the response to vaccination was restricted to a more limited cytokine profile. Moreover, SLE patients reached lower frequencies of TNF- and IL-2-producing $CD4^+$ T cells against A/H1N1 compared to controls ($P = 0.014$ and $P = 0.034$, respectively).

As was expected, neither SLE patients nor controls showed changes in percentages of A/H1N1- and A/H3N2-specific $CD8^+$ T cells upon vaccination. Accordingly, post-vaccination, similar differences in influenza-specific $CD8^+$ T cells were observed as pre-vaccination (data not shown).

Adequate responses of $CD4^+$ and $CD8^+$ T cells following SEB stimulation in SLE patients

Upon SEB stimulation, SLE patients and controls showed similar frequencies of IFN-γ-, TNF- and IL-2-producing $CD4^+$ T cells (**Fig. 4**A) and $CD8^+$ T cells (**Fig. 4B**). This indicated that T cells from SLE patients were generally capable of adequate cytokine responses.

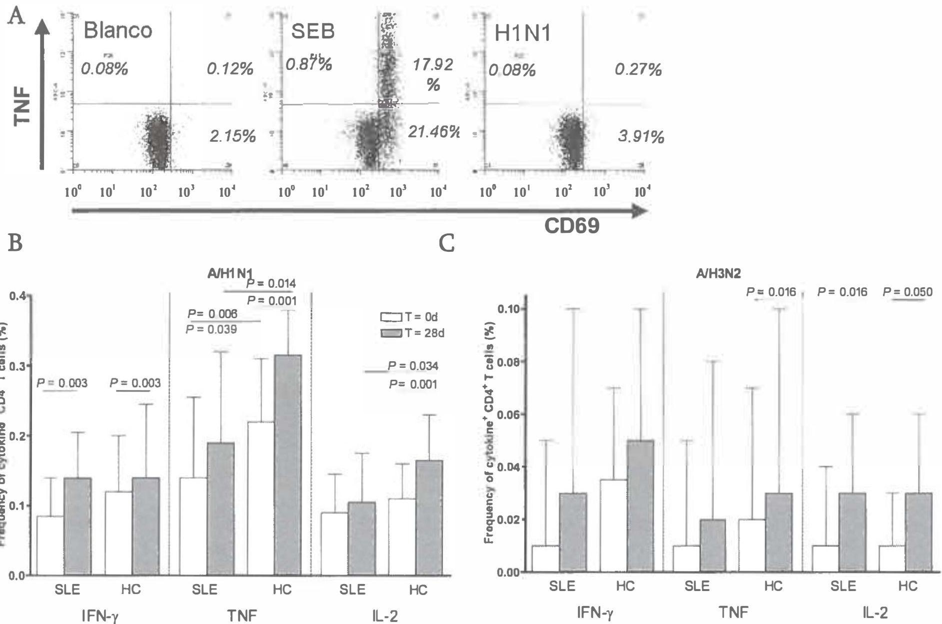

Figure 2. CD4⁺ T-cell responses against A/H1N1 and A/H3N2. (A) Representative example of gating of activated (CD69⁺) tumor necrosis factor (TNF) producing CD4⁺ T cells, in a pre-vaccination sample of a healthy control; unstimulated cells (left), stimulated with Staphylococcal enterotoxin B (SEB, middle), and stimulated with A/H1N1 (right).
Frequencies of cytokine-producing CD4⁺ T cells upon (B) A/H1N1 and (C) A/H3N2 stimulation in systemic lupus erythematosus patients (SLE) and healthy controls (HC), before vaccination (T = 0 days) and four weeks after vaccination (T = 28 days). Results are corrected for responses in unstimulated cultures from the same sample. Changes did not differ significantly between SLE patients and HC. Medians and interquartile ranges are shown.

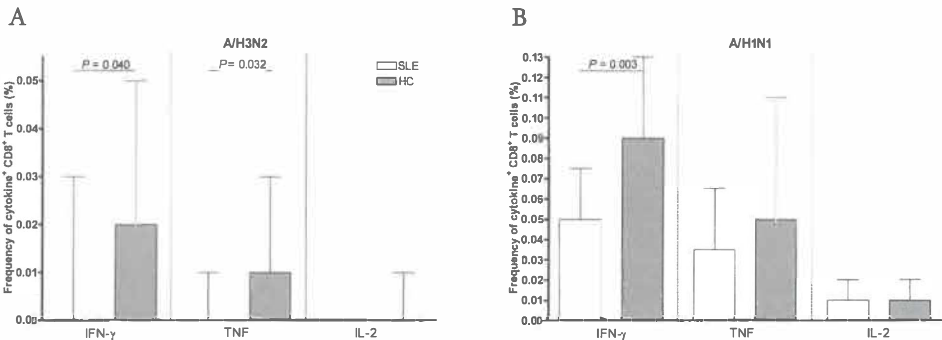

Figure 3. CD8⁺ T-cell responses against A/H1N1 and A/H3N2. Frequencies of cytokine-producing CD8⁺ T cells prior to vaccination upon (A) A/H1N1 and (B) A/H3N2 stimulation in systemic lupus erythematosus patients (SLE) and healthy controls (HC). Results are corrected for responses in unstimulated cultures from the same sample. Medians and interquartile ranges are shown; for IL-2 production following stimulation with A/H3N2 in systemic lupus erythematosus patients, both the median and the interquartile range were 0.

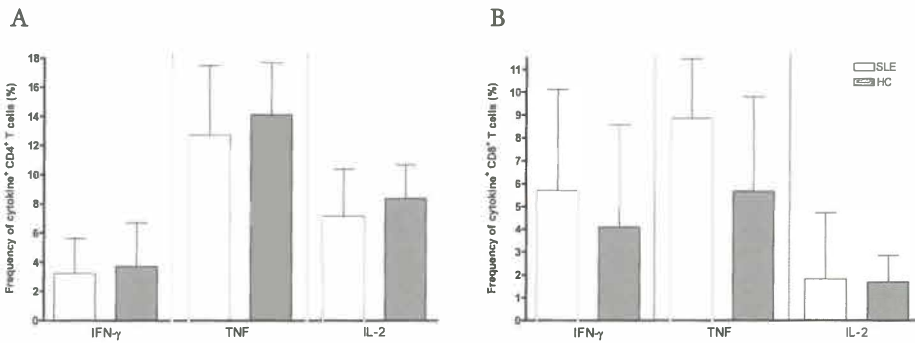

Figure 4. CD4⁺ and CD8⁺ T-cell responses to Staphylococcal enterotoxin B. Frequencies of cytokine-producing (A) CD4⁺ and (B) CD8⁺ T cells, in systemic lupus erythematosus patients (SLE) and healthy controls (HC), upon stimulation with Staphylococcal enterotoxin B in pre-vaccination samples. Results are corrected for responses in unstimulated cultures from the same sample. Medians and interquartile ranges are shown.

Lower antibody response to influenza vaccination in SLE patients

Prior to vaccination, SLE patients had a higher GMT against A/H1N1 as compared with controls. One month post-vaccination, SLE patients and controls reached comparable GMTs to each vaccine strain. However, the fold-increases following vaccination were lower in SLE patients for the A/H1N1 and A/H3N2 strains. Three to four months after vaccination, titers had decreased in both SLE patients and controls; GMTs remained comparable. SLE patients had a lower seroconversion rate for A/H1N1 than controls (P = 0.001), but for A/H3N2 seroconversion rates in SLE and controls were similar. Prior to vaccination, seroprotection rates were comparable in SLE patients and controls. One month after vaccination SLE patients had a lower seroprotection rate against the A strains compared with controls, which was significant for A/H3N2 (P = 0.032). Three to four months after vaccination seroprotection levels had dropped in SLE patients as well as controls to comparable levels (**Table 2**). Taken together, the antibody response in SLE patients was, moderately, decreased. This was further substantiated by results in serologically naïve SLE patients and controls (pre-vaccination titer < 10). For A/H1N1, 5 of 11 (46%) SLE patients showed such a seroconversion, versus 20 of 25 (80%) HC (P = 0.056); for A/H3N2 this occurred in 1 of 7 (14%) SLE patients versus 18 of 22 (82%) HC (P = 0.003). Finally, we analyzed whether immunosuppressive medication influenced antibody responses. No such influence was found (data not shown).

Table 2. Antibody response to influenza vaccination

		SLE n = 54	HC n = 54
GMT			
A/ H1N1	t = 0	18.9	10.9**
	t = 28d (f.i.)	76.5 (4.0)	98.2 (9.0***)
	t = 3-4m	51.3	62.7
A/ H3N2	t = 0	15.8	12.4
	t = 28d (f.i.)	86.4 (5.5)	138.0 (11.1**)
	t = 3-4m	55.8	76.0
Seroconversion rate, n (%)			
A/ H1N1	t = 28d	24 (44.4%)	42 (77.8%)**
A/ H3N2	t = 28d	37 (68.5%)	41 (75.9%)
Seroprotection rate, n (%)			
A/ H1N1	t = 0	15 (27.8%)	8 (14.8%)
	t = 28d	44 (81.5%)	48 (88.9%)
	t = 3-4m	36 (67.9%)	39 (72.2%)
A/ H3N2	t = 0	8 (14.8%)	9 (16.7%)
	t = 28d	41 (75.9%)	50 (92.6%)*
	t = 3-4m	37 (69.8%)	45 (83.3%)

*GMT: geometric mean titer, f.i.: fold increase, seroconversion: fourfold or greater rise in titer, seroprotection: titer ≥ 40, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$*

Changes in IFN-γ spot-forming cells following vaccination correlated with seroconversions in both SLE patients and controls

The change in IFN-γ spot-forming cells against A/H1N1, measured by ELISpot, correlated positively with seroconversion against A/H1N1 (R = 0.311, P= 0.058 for controls; R = 0.348; P = 0.032 for SLE patients; R = 0.339, P = 0.003 for all vaccinees). For A/H3N2 such a correlation was observed in controls (R = 0.318, P= 0.052), but not in SLE patients. No correlations were observed between $CD4^+$ T-cell cytokine responses and antibody responses in controls or SLE patients.

Prior vaccination did not influence cell-mediated responses, but did lower antibody responses

In a subanalysis, SLE patients (n = 13) and controls (n = 35) who were not vaccinated in the previous year, were evaluated. Groups did not differ in age; mean age (SD) was 40.2 (8.9) years in SLE patients and 44.5 (9.6) in HC (P= 0.164). In the IFN-γ ELISpot assay, SLE patients had fewer spot-forming cells

prior to vaccination against A/H1N1 (P = 0.023) and A/H3N2 (P = 0.034) than controls. After vaccination similar differences were found, though these did not reach significance (P = 0.125 for A/H1N1 and P = 0.051 for A/H3N2). Also flow cytometry results showed a tendency towards a restricted $CD4^+$ T-cell response in SLE (data not shown).

In this subanalysis no differences in antibody responses (GMTs, fold increases of GMTs, seroconversion and seroprotection rates) were found between SLE patients and HC (data not shown). In addition, a comparison was made between SLE patients who were vaccinated the previous year (n = 20) versus those who were not (n = 34). Vaccination in 2004 led to a higher pre-vaccination GMT against A/H1N1 (26.6 versus 10.5; P = 0.001) and, subsequently, lowered seroconversion rate (27% versus 75%; P= 0.001).

The use of prednisone and/ or azathioprine was associated with lower cell-mediated responses to influenza vaccination

Patients using prednisone and/ or azathioprine (PRED/AZA; n = 22) were compared to patients who did not use these drugs (n = 16). In this subanalysis, no differences were noted prior to vaccination. Following vaccination, patients on PRED/AZA had fewer IFN-γ spot-forming cells against A/H1N1 and A/H3N2 (P= 0.004 and P = 0.007, respectively) and lower frequencies of A/H1N1-specific IFN-γ-, TNF- and IL-2-producing $CD4^+$ T cells (P = 0.004, P= 0.033 and P= 0.036, respectively) as well as A/H3N2-specific IFN-γ-producing $CD4^+$ T cells (P= 0.023). No differences in $CD8^+$ T-cell responses to A/H1N1 and A/H3N2 were observed (data not shown). In patients not using prednisone and/ or azathioprine, cell-mediated responses to influenza vaccination were not significantly lower than in healthy controls (data not shown).

No increase in disease activity following influenza vaccination, but more adverse effects in SLE than in controls

Prior to inclusion (**Table 1**), and during follow-up, vaccinated and non-vaccinated patient groups did not differ in SLEDAI and VAS scores. At visit 2, median (range) SLEDAI scores were 2 (0 – 13) in vaccinated SLE patients versus 2 (0 – 8) in non-vaccinated patients and at visit 3 these were 2 (0 – 10) versus 2 (0 – 4), respectively. For VAS scores, median (range) scores at visit 2 were 1.4 (0 – 8.1) in vaccinated SLE patients versus 2.1 (0 – 7.4) in non-vaccinated patients, at visit 3 these were 1.8 (0 – 9.4) versus 2.2 (0 – 8.9) respectively. Following vaccination, SLE patients more often reported itching (18% vs. 2% in controls; P = 0.006),

erythema (24% vs. 4%; $P = 0.003$) and induration (30% vs. 11% $P = 0.026$) at the site of vaccination, and arthralgia (16% vs. 4%; $P = 0.046$). All adverse effects were mild and short-lasting.

Discussion

To our knowledge, this is the first study to evaluate cell-mediated immune responses to subunit influenza vaccine in patients with a systemic autoimmune disease. To do so, we used ELISpot and flow cytometry. ELISpot is the more sensitive assay, whereas flow cytometry allows phenotyping and detection of multiple cytokines, which offers additional information on the gamma of the response [19].

Cell-mediated recall responses to influenza were lower in SLE patients. Prior to vaccination, SLE patients had considerably fewer IFN-γ spot-forming cells than controls against both A/H1N1 and A/H3N2. $CD4^+$ T-cell responses to A/H1N1 were lower in SLE patients, which reached significance for TNF-producing $CD4^+$ T cells. Also $CD8^+$ T-cell responses were lower in SLE patients than in controls, for both A/H1N1 (IFN-γ production) and A/H3N2 (IFN-γ and TNF production).

Following influenza vaccination, cell-mediated responses to influenza remained lower in SLE patients. Although both SLE patients and controls showed an increase in IFN-γ spot-forming cells upon vaccination, for A/H1N1 as well as A/H3N2, numbers remained lower in SLE patients. SLE patients showed an increase in cytokine-producing A/H1N1-specific and A/H3N2-specific $CD4^+$ T cells following vaccination, however, this increase was restricted with respect to cytokine profile compared to controls. Moreover, SLE patients reached lower frequencies of A/H1N1-specific TNF-producing and IL-2-producing $CD4^+$ T cells after vaccination. As expected, we did not observe a change in cytokine-producing $CD8^+$ T cells following vaccination in either SLE patients or controls.

As $CD4^+$ and $CD8^+$ T-cell responses to SEB were normal in SLE patients, the decreased cell-mediated response to influenza vaccination could not be attributed to a decreased responsiveness of T lymphocytes in general. Furthermore, the observed differences in cell-mediated responses were, at least largely, independent of previous influenza vaccination status as well. The degree of influenza vaccination in the previous year was higher in SLE patients, but in a subanalysis comparing previously unvaccinated SLE patients with controls, SLE patients still showed considerably lower responses. Importantly, the use of medications played a major role, as the use of prednisone and/ or azathioprine was associated with

lower cell-mediated responses against both A/H1N1 and A/H3N2 following vaccination.

A diminished T helper cell response in SLE patients to influenza has been reported previously [10], as measured by IL-2 secretion in the supernatant of influenza-stimulated PBMCs of unvaccinated patients. We found a decreased $CD8^+$ T-cell recall response in SLE patients to influenza antigens, which is in accordance with a previous study [11]. WIV, as used in this study, is able to induce $CD8^+$ T-cell responses in vivo and to reactivate memory $CD8^+$ T cells in vitro [20]. However, WIV might be a weaker stimulus of $CD8^+$ T cells as compared to live virus, due to lower antigen presentation on MHC I.

Importantly, fewer influenza-specific PBMCs in SLE may be of clinical relevance. Recently, it was shown that numbers of spot-forming cells correlate with clinical protection from, culture-confirmed, influenza in young children [21]. These numbers may vary depending on antigen type and influenza strain, as median numbers in our assays were higher than in assays in which HA or vaccine components were used [9,21-23], and as in the present study A/H3N2-specific cell-mediated responses were lower than A/H1N1-specific responses. WIV contains core antigens in addition to surface antigens. Also, the uptake and presentation of WIV is more efficient [8]. Both factors might contribute to higher responses to WIV compared to HA or vaccine components.

SLE patients showed normal T-cell cytokine responses to SEB. Previous reports reported a normal capacity of PBMCs from SLE patients to respond to different stimuli, though diminished cell-mediated responses may be present during active disease [10,24-26]. As our cohort of SLE patients predominantly had quiescent disease, this may explain their normal responses to SEB. In addition, previous studies reported decreased proliferation of PBMCs [27-29], whereas others found normal proliferative capacity [30], or heterogeneous results [31].

Diminished cell-mediated responses to influenza vaccination in SLE patients appear to reflect, in particular, effects of immunosuppressive drugs. Effects of previous influenza vaccinations, or natural infections, could not be completely excluded. Whether intrinsic defects are involved, such as a defective antigen-presenting cell function [32,33], is uncertain.

In SLE, antibody production upon influenza vaccination is lower than in the general population [4]. In the present study, we too found lower antibody responses in SLE patients, as reflected by lower fold-increases in titers, a trend towards lower post-vaccination GMTs and fewer seroconversions in serologically naïve

SLE patients. Notably, antibody titers are the gold standard for protection and with regard to seroprotection rates, little differences were observed between SLE patients and controls. Influenza vaccination in the previous year was associated with a lower seroconversion rate to A/H1N1; both vaccines contained the same A/H1N1 strain. Effects of previous influenza vaccination on antibody responses remain subject to discussion, as some studies reported decreased antibody responses [34-36], whereas others found similar [37-39] or improved responses [40].

We evaluated relationships between antibody and cell-mediated responses, as $CD4^+$ T-cell help is necessary for antibody responses [41]. However, we did not find a correlation between $CD4^+$ T-cell responses and antibody responses using flow cytometry. We did observe a, modest, correlation in SLE patients between changes in IFN-γ spot-forming cells against A/H1N1, measured by ELISpot, upon vaccination and seroconversion to A/H1N1. This suggests that in a subset of poorer-responding patients both cell-mediated and antibody responses are affected. Possibly, no correlation between $CD4^+$ T-cell responses and antibody responses was observed due to the lower sensitivity of flow cytometry as compared to ELISpot [19].

Finally, we showed that influenza vaccination did not induce disease activity over a period of three to four months. This confirms previous studies, reviewed in [5].

Our study has some limitations. First, the sample size was relatively small and multiple comparisons were made. However, a proper power analysis was not possible as this is the first study to explore cell-mediated responses to influenza vaccination in SLE patients. Second, medication use in vaccinated SLE patients was heterogeneous. Third, more vaccinated SLE patients than controls had received an influenza vaccination in the previous year, which was of influence upon antibody responses. Fourth, there are no well-defined correlates between cell-mediated responses to influenza and the risk of influenza infection, which limits translation of our results to clinical implications. Fifth, phenotypes of cells responding in ELISpot assays are unknown. It can be speculated that NK cells are among the cells which have responded in our ELISpot assay [15].

Despite these limitations, we conclude that the combined data point towards diminished cell-mediated immune responses to influenza vaccination in a cohort of SLE patients representative for daily practice. Diminished cell-mediated responses may reflect effects of the concomitant use of immunosuppressive drugs. The antibody response to influenza vaccination is also reduced in SLE patients.

Clinicians should be aware that this combined defect might increase the morbidity and mortality due to influenza virus infection, in particular in patients on prednisone and/ or azathioprine. Therefore evaluation of clinical protection from influenza in SLE patients, following influenza vaccination, seems indicated in order to assess if more effective influenza vaccines, or vaccination strategies, are warranted.

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Chapter 5

A second, booster, influenza vaccination has limited additional effect on antibody responses in quiescent systemic lupus erythematosus

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ABSTRACT

In systemic lupus erythematosus (SLE), a decreased antibody response upon influenza vaccination has been reported. In this study, we assessed whether a booster vaccination could improve antibody responses, as determined by seroprotection rates, in SLE patients.

SLE patients (n = 52) with quiescent disease (SLE disease activity index; SLEDAI ≤ 4) and healthy controls (n = 28) received subunit influenza vaccine in October - December 2007. After four weeks, only SLE patients received a second vaccination. Sera were obtained prior to both vaccinations, and four weeks after the second vaccination. At each visit, SLE disease activity was recorded. The hemagglutination inhibition test was used to measure antibody titers. Seroprotection was defined as a titer ≥ 40.

Following the first vaccination seroprotection rates and geometric mean titers (GMTs) to each vaccine strain increased in both SLE patients and controls, to comparable levels. Seroprotection rates in SLE patients after the first vaccination were 86.5% to A/H1N1, 80.8% to A/H3N2 and 61.5% to the B strain; GMTs were 92.6, 56.2 and 39.2, respectively. Overall, the booster vaccination did not lead to a further rise of seroprotection rates and geometric mean titers in SLE patients. However, in patients not vaccinated in the previous year, GMT and seroconversion rate to A/H1N1 did rise following the booster vaccination. Both influenza vaccinations did not increase SLEDAI scores.

Additional value of a booster influenza vaccination in SLE is limited to patients who were not vaccinated in the previous year.

Introduction

Infections are a frequent cause of death in systemic lupus erythematosus (SLE) patients, accounting for up to 20 – 55% of all deaths [1]. An increased risk of infection in SLE is related to both intrinsic disturbances of immune responses and use of immunosuppressive drugs which are often needed to control disease activity.

One of the most frequent infections is influenza, with an estimated 5% of the adult population infected annually [2]. In immunocompromised patients, influenza has a higher morbidity and mortality [3]. Vaccination is considered the cornerstone for prevention of influenza related morbidity and mortality, and is recommended in immunocompromised patients [4]. As influenza vaccination does not induce disease activity in SLE, there is an increasing support for annual influenza vaccination of SLE patients [5,6].

However, several studies have reported a decreased antibody response in SLE patients. Seroprotection (titer ≥ 40) rates were lower in SLE patients than in healthy adults, which may limit clinical protection from influenza in (part of) vaccinated SLE patients [7]. Several strategies have been developed to increase antibody responses to influenza vaccination, the most important being addition of an adjuvans, administration of booster vaccinations, increase of antigen dosage in the vaccine and intradermal instead of intramuscular vaccine administration. All have been reported to have additional value in certain patient groups, as compared to conventional vaccination [8-11]. We chose to evaluate a booster vaccination in our SLE cohort, as this strategy has two advantages over the others. First, in contrast to other strategies, the safety profile of conventional subunit vaccine in SLE has been established. This is important as triggering of autoimmunity is a concern in systemic autoimmune disease. Second, this strategy would be easiest to implement within current vaccination practice.

In liver transplantation patients, an increase of the antibody response following trivalent booster vaccination has been shown [11]. Moreover, in SLE, a booster of A/H1N1 solely, one month after a first vaccination, increased GMT [12]. However, there are also patient groups in which a booster vaccination had no additional value, such as dialysis patients, bone marrow transplant recipients and severely immunocompromised HIV patients [10,13-17].

Based on previous data from our group, we hypothesized that influenza vaccination would result in a lower seroprotection rate in SLE patients [18], and that administration of a booster vaccination would increase seroprotection rate up to

the level of seroprotection reached in healthy adults after a single vaccination. To test this hypothesis, we administered a booster dose of influenza subunit vaccine to SLE patients with quiescent disease, four weeks after a first vaccination. Antibody responses were determined prior to the first and second vaccination, and four weeks after the booster vaccination.

Methods

Patients and controls

SLE patients were eligible for the study when they fulfilled at least four of the American College of Rheumatology criteria for SLE [19] and had quiescent disease, defined as a SLE disease activity index (SLEDAI [20]) $\leq$ 4. Exclusion criteria were pregnancy, malignancy and the use of prednisone > 30 mg/day. A control group of healthy individuals was included that was age and sex matched to the patients on group level. Pregnancy was an exclusion criterion for participation as healthy control.

Study design

We conducted an open, prospective, controlled study. SLE patients and controls were included from October - December 2007. At entry (t = 0), patients and controls received intramuscularly a single dose of trivalent subunit influenza vaccine (Influvac® 2007-2008, Solvay Pharmaceuticals, Weesp, the Netherlands), containing A/Solomon Islands/3/2006 [H1N1], A/Wisconsin/67/2005 [H3N2] and B/Malaysia/2506/2004. After four weeks (t = 1), patients received a second, booster, vaccination. Healthy controls were not given a booster vaccination, because this does not increase antibody responses [11,15,17,21]. Serum was obtained at t = 0 and t = 1 in patients and controls, and four weeks thereafter (t = 2) in patients alone. At each visit, the SLEDAI was recorded. Routine measures were used to determine anti-double-stranded DNA (by Farr assay) and complement C3 and C4. From all participants, information on influenza vaccination in the previous year was obtained. Adverse effects to vaccination were recorded using a standardized questionnaire which included: itching, pain, erythema, induration at the site of vaccination, shivers, myalgia, fever, headache, nausea, arthralgia, diarrhea, use of an analgesic/ antiphlogistic drug. The study was approved by the institutional medical ethics committee, and informed consent was obtained from all participants.

Antibody response to influenza

For quantitative detection of influenza antibodies the hemagglutination inhibition (HAI) test was used. HAI tests were performed in duplo with guinea pig erythrocytes following standard procedures [22] with slight modifications as described elsewhere [23]. Seroprotection was defined as a titer ≥ 40, seroconversion was defined as ≥ fourfold rise in titer; titers < 10 (below detection level) were assigned a value of 5 for calculation purposes [24].

Power and statistical analysis

Data were analyzed using SPSS 14 (SPSS Inc, Chicago, IL, USA). Titers were log-transformed prior to testing of geometric mean titers (GMTs). For testing differences in age between groups Student's t test was used. Changes of GMTs, anti-double-stranded DNA antibodies, complement C3 and C4 were tested using Wilcoxon signed-rank test; McNemar tests were used to test changes in seroprotection rates and seroconversion rates. Between groups, differences in GMTs were tested using the Mann-Whitney U test. For all other comparisons, the Chi-square test or Fisher's exact test were used, depending on the size of the expected counts. A P value < 0.05 was considered statistically significant. Based on previous results, it was hypothesized that a single vaccination would result in a 60% seroprotection rate against all three vaccine strains together [18], and that this would increase to 78% following a booster vaccination [11]. Seroprotection against all three vaccine strains together was defined as a titer ≥ 40 against each of the vaccine strains in the same serum sample. For a power of 80% at an alpha of 5% to demonstrate such a difference, 47 SLE patients had to be included. Accounting for a 10% drop-out, this number was raised to 52.

Results

Patient characteristics

Fifty-four SLE patients gave informed consent to participate, of whom one patient withdrew prior to entry and one patient was excluded due to active disease. Fifty-two patients completed the study, and their characteristics are summarized in **Table** 1. Their mean age (SD) was 45.2 (10.0) years and 17.3% were males. Seventy-one % of patients had been vaccinated against influenza in the previous year (2006). Median SLEDAI score at entry was 2, and most patients used immunosuppressives, especially prednisone, hydroxychloroquine and azathioprine. In the group of healthy controls, age and sex were comparable to those in

SLE patients. Also vaccination history was similar, as most controls had participated in the hospital's annual influenza vaccination campaign before.

Table 1. Baseline characteristics

	SLE n = 52	HC n = 28
Sex, males (%)	9 (17.3%)	6 (21.4%)
Age, mean (SD)	45.2 (10.0)	45.2 (11.3)
Influenza vaccination in previous year (2006)	37 (71.2%)	20 (71.4%)
No immunosuppressives (%)	5 (9.6%)	n/a
Prednisone median (range), in users (mg/day)	31 (59.6%) 5 (1.25 - 25)	n/a
Hydroxychloroquine median (range), in users (mg/day)	25 (48.1%) 400 (200 - 400)	n/a
Azathioprine median (range), in users (mg/day)	15 (28.8%) 125 (50 - 200)	n/a
Other immunosuppressive drugs	7 (13.5%) #	n/a
SLEDAI, median (range)	2 (0 - 4)	n/a

n/a: not applicable

 # four patients used methotrexate (one pt 10 mg/week, three pts 15 mg/wk), three patients used mycophenolate mofetil (one pt 1000 mg/day, two pts 2000 mg/day) and one patient used ciclosporin 200 mg/day (same pt also used methotrexate)

The first influenza vaccination induced comparable seroprotection rates and geometric mean titers in SLE patients and controls

Prior to vaccination, seroprotection rate against all three vaccine strains together did not differ between SLE patients (19.2%) and controls (7.1%; $P = 0.199$). Following the first vaccination, this rate tended to be higher in patients than in controls, surprisingly (51.9% versus 28.6%, respectively, $P = 0.060$). For patients, this rate was close to what was expected, but for controls it was much lower than anticipated - largely due to a low post-vaccination seroprotection rate against the B strain.

Prior to vaccination, seroprotection rates and GMTs, for each strain, were comparable in SLE patients and controls. Following the first vaccination, seroprotection rates and GMTs increased in both patients and controls. Responses to the B strain were lower as compared to those to the A strains. SLE patients reached a seroprotection rate of 86.5% for A/H1N1, 80.8% for A/H3N2 and 61.5% for the B strain. Their post-vaccination GMT was 92.6 for A/H1N1, 56.2 for A/H3N2 and 39.2 for the B strain. Controls reached comparable seroprotection

rates and GMTs (**Fig. 1**). Also fold increases in GMTs were comparable in SLE patients and controls. SLE patients showed fold increases of 2.7, 2.1 and 1.9 for strains A/H1N1, A/H3N2 and B, respectively. Controls showed fold increases in GMT of 2.7, 1.7 and 1.8 for strains A/H1N1, A/H3N2 and B, respectively.

A booster influenza vaccination did not increase seroprotection rates and geometric mean titers in SLE patients

Primary focus was the effect of booster vaccination upon seroprotection rates in SLE patients. The second vaccination did not further increase these seroprotection rates (**Fig. 1A**). Accordingly, at t = 2, the proportion of patients with seroprotection to all vaccine strains was 55.8%, demonstrating that there was no significant increase following the second vaccination. Similarly, the second vaccination did not induce a significant rise in GMTs (**Fig. 1B**).

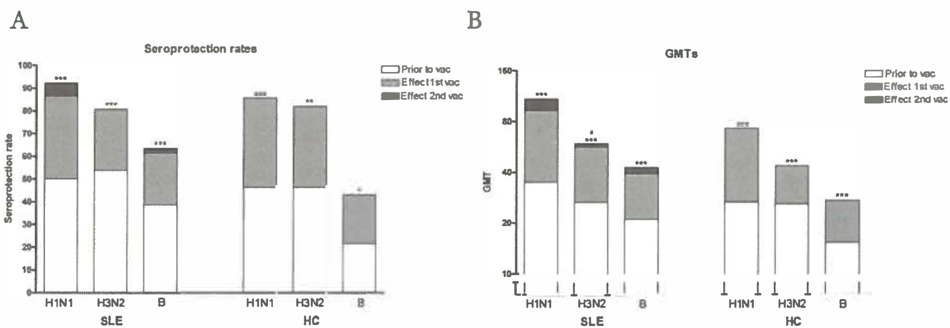

*Figure 1. Antibody responses. (A) Seroprotection rates and (B) geometric mean titers (GMTs) in systemic lupus erythematosus (SLE) patients and healthy controls (HC). Seroprotection was defined as a titer ≥ 40. * P < 0.05, ** P < 0.01, *** P < 0.001: after 1st vaccination vs. prior to vaccination # P < 0.05: SLE vs. HC, after 1st vaccination.*

Low seroconversion rates in both patients and controls

Seroconversion rates were low in both SLE patients and controls. After the first vaccination, seroconversion rates to A/H1N1 were 34.6% in SLE patients and 28.6% in controls, for A/H3N2 rates were 25.0% and 10.7% and for B rates were 19.2% and 10.7%, respectively. Following the booster vaccination (t = 2 vs. t = 1), five (9.6%) SLE patients showed a seroconversion to A/H1N1 whereas none of the patients showed a seroconversion to either A/H3N2 or the B strain. In SLE patients, when using baseline titers (t = 0) as reference, for A/H1N1, the seroconversion rate tended to be higher after the second vaccination (t = 2 vs. t = 0) than after the first vaccination (46.2% versus 34.6%; $P = 0.070$). However,

the booster vaccination did not lead to a further increase of seroconversion rates for A/H3N2 and the B strain.

Influence of previous influenza vaccinations

A large part of SLE patients (71.2%) and controls (71.4%) had received an influenza vaccination in the previous year. Vaccination in the previous year led to higher pre-vaccination seroprotection rates, which reached significance for strains A/H3N2 (P = 0.016) and B (P = 0.027) in patients, and for A/H1N1 in controls (P= 0.038, **Fig. 2A**). Accordingly, pre-vaccination GMTs were higher in previously vaccinated participants; in patients this difference was significant again for strains A/H3N2 (P = 0.001) and B (P = 0.026), and in controls for A/H1N1 (P= 0.004, **Fig. 2B**). Influenza vaccination in the previous year did not influence titers and seroprotection rates after the first vaccination, except for the B strain in controls. The post-vaccination seroprotection rate to the B strain was higher in controls not vaccinated in the previous year (75%) than in previously vaccinated controls (30%, P= 0.044, **Fig. 2 A** + B).

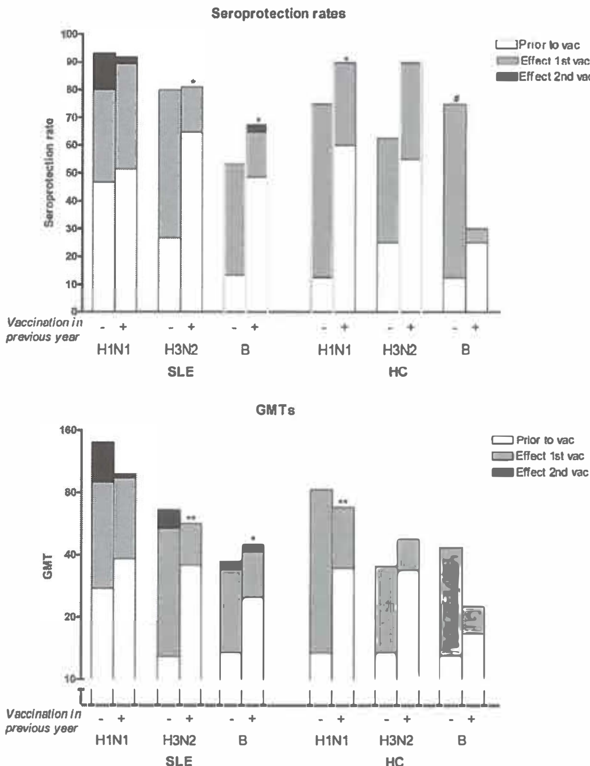

Figure 2. Effects of previous influenza vaccinations upon antibody titers.
(A) Seroprotection rates and (B) geometric mean titers (GMTs) in systemic lupus erythematosus (SLE) patients and healthy controls (HC), according to vaccination status in the previous year. Seroprotection was defined as a titer ≥ 40. For A/H1N1 in patients not vaccinated in the previous year, there was a trend towards an increase in GMT following the second vaccination (P = 0.055).
** P < 0.05, ** P < 0.01: prior to vaccination, vaccinated in the previous year versus not vaccinated in the previous year*
 # P < 0.05: After first vaccination, vaccinated in the previous year versus not vaccinated in the previous year

Higher pre-vaccination titers in patients and controls vaccinated in the previous year lowered seroconversion rates after the first vaccination. In patients, this was most pronounced for the A/H3N2 strain. Patients not vaccinated in the previous year showed a 60.0% seroconversion rate to A/H3N2, versus 10.8% in previously vaccinated patients (P = 0.001). In controls, similar differences were observed, reaching significance for the B strain. Controls not vaccinated in the previous year showed a 37.5% seroconversion rate to the B strain, versus 0% of the previously vaccinated controls (P= 0.017).

Notably, for A/H1N1, vaccinations in the previous year influenced the response to the booster vaccination. In SLE patients not vaccinated in the previous year, the booster tended to increase the GMT to A/H1N1, but not to A/H3N2 and the B strain. Following the booster vaccination, the GMT to A/H1N1 increased from 89.8 to 139.3 (P= 0.055). In previously vaccinated patients, the GMT was not influenced (**Fig. 2B**). For seroconversion rate, a similar effect was found; in SLE patients not vaccinated in previous year, the seroconversion rate increased from 46.7% to 80% (P= 0.062) but in previously vaccinated patients the seroconversion rate did not change (29.7% vs. 32.4%).

The use of prednisone and/ or azathioprine was associated with lower antibody responses to influenza vaccination

The use of immunosuppressives was heterogeneous, but stable during the duration of the study. Previous studies have reported lower antibody responses to influenza vaccination in SLE patients treated with steroids and azathioprine, but not in patients treated with hydroxychloroquine [18,25-27]. We performed a subanalysis in which patients using prednisone and/ or azathioprine (PRED/AZA; n = 28) were compared with patients using no immunosuppressives or hydroxychloro-quine only (NO-imm/HCQ; n = 17); patients using other immunosuppressive drugs then prednisone, azathioprine and hydroxychloroquine were excluded because of low numbers (n = 7). PRED/AZA patients were somewhat younger than NO-imm/HCQ patients, but the groups did not differ with regard to influenza vaccination in the preceding year. PRED/AZA patients had a lower antibody response to influenza vaccination as compared to NO-imm/HCQ patients, reflected by a lower GMT against A/H1N1 and A/H3N2 following the first vaccination, and a lower seroconversion rate against A/H1N1. The second vaccination had a slight additional effect for A/H1N1 within PRED/AZA patients (**Table 2**).

Table 2. Effects of immunosuppressive drugs on antibody responses

SLE patients		prednisone/ azathioprine n = 28			no immunosuppr./ hydroxychloroquine n = 17		
		T = 0	T = 1	T = 2	T = 0	T = 1	T = 2
Age, mean (SD)		42.1 (9.2)			48.4 (10.0)*		
Influenza vaccination in previous year (2006)		21 (75%)			12 (70.6%)		
S.P. rate	H1N1	14 (50%)	23 (82.1%)	25 (89.3%)	8 (47.1%)	16 (94.1%)	16 (94.1%)
	H3N2	10 (35.7%)	19 (67.9%)	19 (67.9%)	13 (76.5%)*	16 (94.1%)#	16 (94.1%)#
	B	11 (39.3%)	17 (60.7%)	17 (60.7%)	7 (41.2%)	11 (64.7%)	10 (58.8%)
GMT	H1N1	39.5	72.5	92.8†	32.0	130.5*	130.5
	H3N2	20.0	39.0	41.0	39.2*	78.4*	83.3*
	B	22.9	36.7	40.0	18.4	40.8	43.4
S.C. rate	H1N1	n/a	4 (14.3%)	3 (10.7%)	n/a	11 (64.7%)**	1 (5.9%)
	H3N2	n/a	5 (17.9%)	0	n/a	5 (29.4%)	0
	B	n/a	3 (10.7%)	0	n/a	5 (29.4%)	0

T = 0: prior to vaccination; T = 1: four weeks after the first vaccination; T = 2; eight weeks after the first vaccination, four weeks after the second vaccination

S.P. rate: seroprotection rate, GMT: geometric mean titer, S.C. rate: seroconversion rate, n/a: not applicable

** $P < 0.05$, ** $P < 0.01$, # $P = 0.064$: SLE patients using prednisone and/or azathioprine versus patients using no immunosuppressive drugs or hydroxychloroquine only*

† $P < 0.05$: T = 2 vs. T = 1

Disease parameters did not increase following the influenza vaccinations and adverse effects of both vaccinations were mild in SLE patients

SLEDAI scores and levels of anti-double stranded DNA antibodies did not increase following the vaccinations. Levels of C3 and C4 remained almost stable during this period; slight increases of C3 and C4 levels were observed (**Table 3**). More SLE patients (19.6%) experienced erythema after both the first and second vaccination, compared to controls (0%; $P = 0.013$). In SLE patients, adverse effects to the first and second vaccination were comparable (data not shown).

Table 3. Disease parameters

	T = 0	T = 1	T = 2
SLEDAI, median (range)	2 (0 - 4)	2.5 (0 - 8)	2 (0 - 8)
anti-dsDNA, median (range) (units/ml)	16 (<3 - 397)	18.5 (<3 - 275)	18.5 (<3 - 261)
C3, median (range) (g/l)	0.91 (0.42 - 1.42)	0.91 (0.35 - 1.45)	0.93 (0.31 - 1.45)*
C4, median (range) (g/l)	0.14 (<0.02 - 0.52)	0.15 (<0.02 - 0.52)	0.16 (0.02 - 0.50)**

T = 0: prior to vaccination; T = 1: four weeks after the first vaccination; T = 2: eight weeks after the first vaccination, four weeks after the second vaccination; Anti-dsDNA: anti-double-stranded DNA; C3, C4: complement C3 and C4
** $P < 0.05$, ** $P < 0.01$: at T = 8 weeks vs. T = 0 (prior to vaccination)*

Discussion

In SLE, a hampered antibody response to influenza vaccination has been reported in several studies [7]. As seroprotection rates are related to clinical protection from influenza, strategies to improve antibody responses are relevant in SLE. In the present study, we evaluated whether a second, booster, influenza vaccination could increase antibody titers. We did not find such an enhancing effect. Following the first vaccination, seroprotection rates and GMTs rose for each strain, but these did not rise further following the second vaccination. As an exception, there was a clear trend in the response to A/H1N1 in SLE patients who were not vaccinated in the previous year. This response did increase following the booster vaccination, in terms of GMT and seroconversion rate. The booster vaccination had mild adverse effects and did not increase SLEDAI scores.

Our findings regarding A/H1N1 in patients not vaccinated in the previous year appear to be in accordance with a previous study in SLE patients, in which boosting was performed for A/H1N1 solely and was found to increase GMT [12]. In this study, no information is presented regarding previous influenza vaccinations

but it is likely that most patients had not received an influenza vaccination before, since there was much uncertainty regarding safety of vaccination in SLE [5].

In liver transplantation patients, a trivalent booster vaccination (28 days after the first vaccination) led to higher GMTs to all vaccine strains. Furthermore, the seroprotection rate against all three strains increased from > 68% after the 1st vaccination to > 80% after the booster vaccination [11]. Also in frail elderly, a booster vaccination after 84 days increased GMTs, as detected by ELISA assay [28]. In healthy elderly, increases in seroconversion rate and GMT following a booster vaccination have been reported [21], however, the majority of studies did not find an additional effect [29,30]. Similarly, in other patient groups such as bone marrow transplant recipients [16], severely immunocompromised HIV patients [17] and dialysis patients [10,13-15], booster vaccination did not have additional value. Also in healthy adults, booster vaccination did not increase antibody responses [11,15,17,21].

Why booster vaccination did not improve the antibody response to influenza in SLE patients remains speculative. First, previous vaccinations appear to limit the effect of a booster vaccination, as reported in this study. Second, it may be argued that booster vaccination can only have effect in patients with a low (< 40) titer after a first vaccination. In this study, over 80% of patients had achieved protective titers to the A strains after the first vaccination and for these strains this may have hindered a further increase. This does not apply for the B strain as the seroprotection rate was 61.5% after the first vaccination. Nevertheless, the seroprotection rate to the B strain did not increase either following the booster vaccination.

Responses and titers to the B strain were low in both patients and controls. Generally, antibody titers to B strains are lower than titers to A strains [16,31,32]. This may be due to lower immunogenicity of the B strain as compared to the A strains, or a lower sensitivity of the HAI test. The HAI test for influenza B with whole virus particles, which is standard and was applied here, was previously found to be less sensitive than testing with influenza B virus disrupted with ether [33].

In accordance with previous reports, the use of prednisone and/ or azathioprine was associated with lower antibody responses to influenza vaccination in SLE patients [18,25-27].

As a secondary study question, we evaluated whether a booster vaccination, supposed it were effective, would increase antibody responses in SLE patients up to levels reached in healthy controls after a single vaccination. However, we did not observe differences in antibody responses between SLE patients and controls.

Patients showed similar responses as in a previous study, but the responses of controls were lower than expected [18]. Although also some previous studies did not find differences between patients and controls [26,34-36], most have shown antibody responses in SLE patients to be lower than in controls [12,18,25,27,37]. Furthermore, we found that cell-mediated responses to influenza vaccination, which correlate to clinical protection from influenza infection [38], are hampered in SLE as well (Arthritis & Rheumatism, in press). It is not clear why in the present study, SLE patients and controls did not differ in antibody responses, but several factors could be involved. First, lack of power, as the study was not powered to study this question. Second, lower immunogenicity of current vaccine strains could have restrained differences between patients and controls. Third, controls had a higher degree of previous influenza vaccinations as compared to a previous study, while they did not differ with regard to age and sex [18]. Possibly, influenza vaccinations in the preceding year hindered antibody responses [39-41], in which case previous findings of impaired responses were (partly) due to differences in vaccination history between SLE patients and controls. This implicates that actual differences between SLE patients and controls may be less than expected. In an extensive overview, Beyer *et al.* reported that especially for the B strain there is a general tendency to a lower post-vaccination GMT and seroprotection rate in previously vaccinated groups [31], as we observed in our healthy controls. It has been suggested that vaccines which are antigenically close to a prior vaccine may be partially eliminated by pre-existent cross-reactive antibodies, thus reducing the immune response [42].

Finally, the influenza vaccinations did not affect disease activity, which is in accordance with previous studies [5]. However, it has been reported previously that although SLEDAI scores remain stable after influenza vaccination, levels of auto-antibodies may transiently increase [43].

In this study, a control group of SLE patients vaccinated once was not included, which might be a limitation. Here, SLE patients functioned as their own controls with regard to the effects of the first and the booster vaccination. This increased the statistical power to detect the expected additional effect of a booster vaccination, as it enabled a matched samples analysis. This has been done previously [11,12,14-17,21,32], though indeed some studies included a patient group vaccinated once and a patient group vaccinated twice [10,13,28-30].

In summary, booster vaccination with subunit influenza vaccine had no additional value in annually vaccinated SLE patients. In this study we did not find

differences between SLE patients and controls in the antibody response to subunit influenza vaccine. However, the study was not designed and powered to detect such a difference. Therefore, we do not challenge previous studies showing decreased responses in SLE patients after influenza vaccination. As such, other strategies to improve antibody responses, mentioned earlier, should be considered. For example, the use of an MF59-adjuvanted influenza vaccine, which has a higher immunogenicity than conventional trivalent inactivated vaccine in adults with chronic diseases [8]. Another option would be to use an increased vaccine dose. In hemodialysis patients, not using immunosuppressive drugs, a booster influenza vaccination did not have an additional effect upon titers, but a single double-dose vaccine did have additional value [10]. Finally, intradermal application of conventional influenza vaccine was reported to have higher immunogenicity in elderly as compared to intramuscular vaccination [9]. Whether these strategies enhance the immune response to influenza vaccination in SLE should be studied in controlled studies.

We conclude that the positive effect of a booster influenza vaccination on antibody responses was limited to SLE patients who were not vaccinated in the previous year. These findings are restricted to patients with quiescent disease. Our results implicate that there is no additional value in offering a booster to annually vaccinated SLE patients. This is of clinical importance, as annual influenza vaccination is recommended in SLE patients. Finally, it should be noted that in SLE patients who were not vaccinated in the previous year, administration of a booster vaccination may be considered.

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Chapter 6

Wegener's granulomatosis patients show an adequate antibody response to influenza vaccination

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ABSTRACT

Wegener's granulomatosis (WG) is a systemic vasculitis characterized by relapsing and remitting disease activity. Immunosuppressive drugs are used to control disease, but increase susceptibility to infection. Therefore, influenza vaccination should be considered in WG patients. This study was performed to assess immunogenicity of influenza vaccination in WG patients.

We performed a randomized, controlled trial in WG patients with quiescent disease, defined as Birmingham Vasculitis Activity Score (BVAS) < 2. Patients were randomized to receive influenza vaccination (n = 49) or to participate as control (n = 23). In addition, healthy controls (n = 49) were vaccinated. At entry and at one and three to four months after entry, antibody responses to vaccination were determined. Furthermore, disease activity was measured (BVAS), adverse effects were recorded, and antineutrophil cytoplasmic autoantibodies (ANCA) titers were determined.

WG patients achieved high seroprotection rates to all three influenza strains, comparable to healthy controls. Only for the A/H1N1 strain patients had a lower seroconversion rate ($P = 0.002$) and geometric mean titer ($P = 0.037$) than controls. After one month, one control and one vaccinated WG patient had developed active disease. At three to four months, two additional control patients had developed active disease versus none of the vaccinated patients ($P = 0.099$). Vaccination did not influence ANCA titers. Adverse effects did not differ between patients and healthy controls.

Influenza vaccination in WG patients with quiescent disease induced a sufficient antibody response. Dutch Trial Register, NTR1130.

Introduction

Wegener's granulomatosis (WG) is a systemic vasculitis characterized by relapsing and remitting disease activity. WG patients are at risk for infections [1,2], in part related to the use of immunosuppressive drugs. Furthermore, the median age of WG patients is relatively high, which increases morbidity and mortality following infection [1].

Influenza has a very high yearly incidence, and shows considerable morbidity and mortality. Vaccination is the cornerstone for prevention of influenza. Yearly vaccination reduces the severity of influenza and related complications, and is recommended in immunocompromised patients [3]. Therefore, influenza vaccination appears indicated in WG patients. It is clinically relevant whether WG patients mount protective immune responses. However, there are no studies on the immunogenicity of influenza vaccination, nor any other vaccination, in WG. Both the disease itself and immunosuppressive drugs might hamper the response to vaccination in WG. In systemic lupus erythematosus (SLE), another systemic autoimmune disease, it has been found that the disease itself diminishes the antibody response to influenza vaccination, and that azathioprine may further decrease this response [4,5]. In solid organ transplant recipients, immunosuppressive drugs decrease responses to vaccinations as well [6].

In addition, concerns regarding vaccination-induced activation of established autoimmune diseases have to be taken into account. For SLE, prospective studies did not show an increase in disease activity following influenza vaccination [7]. For WG, prospective data are lacking. A retrospective cohort study suggests that influenza vaccination does not lead to an increase in disease activity in WG [8], though there is a case-report of a relapse of vasculitis following influenza vaccination [9].

Therefore, we performed a prospective, randomized, controlled study to evaluate the immunogenicity of influenza vaccination in WG patients with quiescent disease and to assess a possible influence of influenza vaccination on the risk of relapse.

Methods

Patients and controls

Patients eligible for the study fulfilled criteria for Wegener's granulomatosis [10] and had quiescent disease, defined as Birmingham Vasculitis Activity Score (BVAS) < 2. Exclusion criteria were BVAS ≥ 2, indication for yearly influenza

vaccination due to concomitant disease (based on international guidelines) [3], use of prednisone > 30 mg/day and/or cyclophosphamide > 100 mg/day, and pregnancy. Patients with active disease were excluded for two reasons, first, the uncertainty regarding vaccination-induced disease activation, and, secondly, expected changes in immunosuppressive medication which might influence the interpretation of the effect of influenza vaccination on disease activity and the analysis of the antibody response to vaccination. Similarly, patients using prednisone > 30 mg/day and/or cyclophosphamide > 100 mg/day were considered to have instable disease and therefore were considered ineligible. Patients were randomized to receive an influenza vaccination or to serve as patient control, in a ratio of 2:1. Randomization was stratified for influenza vaccination in the previous year and block randomization with a block length of three consecutive patients was used. A control group of healthy individuals was included; for this purpose, health care workers participating in the yearly influenza vaccination campaign were asked to participate. Exclusion criteria for participation as healthy control were the use of immunosuppressive drugs, malignancy, or pregnancy.

Study design

We conducted a prospective, randomized, open, controlled study. WG patients and controls were included from October to December 2005. At entry (visit 1), patients randomized for vaccination and all healthy controls were vaccinated. Patients and controls were seen again after one month (visit 2) and three to four months (visit 3). During all visits 10 ml blood was drawn, and serum was stored at –20° C until use. At each visit BVAS was recorded for assessing disease activity, and antineutrophil cytoplasmic autoantibodies (ANCA) titers were measured as described previously [11]. A negative titer was recorded as 0. Also, patients were asked to fill in a Visual Analogue Score on a scale of 0-10 (patient VAS, disease activity as experienced by the patient), 0 indicating no activity and 10 indicating the highest activity. From all participants information on influenza vaccination in the previous year was obtained. Adverse effects to vaccination were recorded using a standardized questionnaire which included: itching, pain, erythema, induration at the site of vaccination, shivers, myalgia, fever, headache, nausea, arthralgia, diarrhea, use of an analgesic/ antiphlogistic drug. The study was approved by the institutional medical ethics committee, and informed consent was obtained from all participants.

A single dose of a trivalent subunit influenza vaccine (Influvac®, 2005-2006, Solvay Pharmaceuticals, Weesp, the Netherlands), containing A/New Caledonia/

20/99 [H1N1], A/NewYork/55/2004 [H3N2] and B/Hong Kong/330/2001, was administered intramuscularly.

Antibody response to influenza

For quantitative detection of influenza antibodies the hemagglutination inhibition (HAI) test was used. HAI tests were performed with guinea pig erythrocytes following standard procedures [12] with slight modifications as described elsewhere [13]. Sera were tested for antibodies to all three vaccine strains. The antibody response was evaluated by serological parameters and criteria as defined by the European Agency for the Evaluation of Medicinal Products (EMEA) [14]. Serological parameters were seroprotection rates, seroconversion rates, and mean geometric increases.

Seroprotection rate is the percentage of vaccinees with titers ≥ 40, a titer which can be considered protective in healthy adults [15]. Seroconversion corresponds to a negative pre-vaccination titer (< 10) converting to a titer ≥ 40 one month post-vaccination (original definition of seroconversion) or a significant increase in antibody titer, i.e. at least a fourfold increase in titer. Mean geometric increases correspond to the fold increase in geometric mean titers (GMTs) within a study population one month after vaccination. Titers < 10 (below detection level) are assigned a value of 5 for calculation purposes [14].

Statistical analysis

Data were analyzed using SPSS 14 (SPSS Inc). Titers were log-transformed prior to testing of geometric mean titers. For testing differences in age between groups Student's t test was used. For prednisone use, azathioprine use and geometric mean titers the Mann-Whitney U test was used. For all other variables the Chi-square test or Fisher's exact test were used, depending on the size of the expected counts. A P value < 0.05 was considered statistically significant. Power analysis was based on differences in antibody response. We hypothesized that the seroprotection rate in healthy controls would be 80% per strain [14]. A 20% lower seroprotection rate in WG patients was considered clinically relevant. To give the study a power of 80% at an alpha of 5% to demonstrate such a difference if it would exist (one-sided testing), 54 WG patients and 54 healthy controls had to be vaccinated.

Results

Patient characteristics

Seventy-three WG patients gave informed consent to participate. One patient initially randomized for the control group developed active disease prior to the start of the study and was excluded. Seventy-two patients started and completed the study. Thirty-seven were male (51%), mean age (± SD) was 59 (± 14) years. Forty-nine patients were vaccinated, 23 served as patient controls (**Table 1**). Patients groups did not differ in sex and age. All patients who started the study had a BVAS of 0 at entry, except for one patient in the patient control group with a BVAS of 1 (arthralgia).

Table 1. Baseline characteristics

	WG patients		HC
	Non-vaccinated n = 23	Vaccinated n = 49	Vaccinated n = 49
Sex, males (%)	14 (61%)	23 (47%)	21 (43%)
Age, mean (range)	62 (42 - 88)	57 (27 - 87)	47 (25-63)***
Influenza vaccination in previous year (%)	13 (57%)	32 (65%)	4 (8%)***
No immunosuppressives (%)	15 (65%)	23 (47%)	-
Prednisone range, in users (mg/day)	3 (13%) 5 - 15	16 (33%) 2.5 - 12.5	-
Azathioprine range, in users (mg/day)	5 (22%) 21.4[§] - 150	19 (39%) 35.7[§] - 150	-
Other immunosuppressive drugs	3[#] (13%)	2[ξ] (4%)	-
CRP, median (range)	6 (1 - 43)	4 (1 - 110)	n.d.

*WG: Wegener's granulomatosis, HC: healthy controls, CRP: C-reactive protein, n.d.: not determined. § 3 times 50 mg per week and 5 times 50 mg per week, respectively, # three non-vaccinated patients used mycophenolate mofetil (500 mg/day; 1000 mg/day; 2000 mg/day), ξ one vaccinated patient used cyclophosphamide (25 mg/day) and one vaccinated patient used cyclosporine (150 mg/day), *** P < 0.001 (HC versus vaccinated WG patients)*

Antibody responses

Sixty-five % of vaccinated WG patients had received an influenza vaccination in the previous year, versus 8% of healthy controls ($P < 0.001$). Accordingly, WG patients had higher pre-vaccination GMTs, though these were far below the protective level of 40 (**Table 2**). Vaccination resulted in similar seroprotection rates in patients and healthy controls for all vaccine strains, one and three to four months after vaccination (**Fig. 1A**). Seroconversion rates for strains A/H3N2 and B

were similar in patients and healthy controls. For strain A/H1N1, however, the seroconversion rate was lower in patients (**Fig. 1**B). Also, WG patients had similar post-vaccination GMTs to strains A/H3N2 and B as healthy controls but a lower post-vaccination GMT to strain A/H1N1 (**Table 2**).

Table 2. Geometric mean titers

Strains		WG n = 49	HC n = 49	pv WG n = 32	n-pv WG n = 17
A/ H1N1	before vaccination	13.6	9.9	18.1	7.8**
	after 1 month (f.i.)	62.9 (4.6)	101.0 (10.2)*	49.7 (2.7)	98.1 (12.6)*
	after 3-4 months	37.5	60.7*	30.2	56.6*
A/ H3N2	before vaccination	18.9	12.0*	20.0	17.0
	after 1 month (f.i.)	122.3 (6.5)	138.9 (11.6)	114.4 (5.7)	138.7 (8.2)
	after 3-4 months	68.5	70.6	68.7	68.0
B	before vaccination	17.9	11.2**	22.0	12.0*
	after 1 month (f.i.)	68.5 (3.8)	59.4 (5.2)	62.4 (2.8)	81.6 (6.8)
	after 3-4 months	42.3	33.5	42.2	42.5

*(Left) Comparison of geometric mean titers in Wegener's granulomatosis patients (WG) versus healthy controls (HC). (Right) Comparison of geometric mean titers in WG patients who received influenza vaccination in the previous year (previously vaccinated; pv WG) versus WG patients who did not receive an influenza vaccination in the previous year (not previously vaccinated; n-pv WG). F.i.: fold increase, * $P < 0.05$, ** $P < 0.01$*

Patients vaccinated the previous year were older than patients not vaccinated in the previous year (mean 60 years vs. 52 years, $P = 0.033$), but did not differ in the use of immunosuppressives. Pre-vaccination GMTs were higher in previously vaccinated patients, however, post-vaccination GMTs were lower in previously vaccinated patients (**Table 2**); for A/H1N1 this difference was significant. Seroconversion rates were lower in previously vaccinated patients for strains A/H1N1 and B (**Fig. 1C**). No differences were observed in achieved seroprotection rates (data not shown).

Possible effects of immunosuppressive drugs on the antibody response to vaccination were evaluated. Vaccinated WG patients using immunosuppressive drugs (n = 25) were compared with WG patients not using immunosuppressives (n = 24). Groups were comparable with respect to sex ($P = 0.156$), age ($P = 0.787$) and vaccinations in the previous year ($P = 0.538$). No differences were found in seroprotection rates, seroconversion rates (**Fig. 2**), and GMTs (data not shown). The use of prednisone or azathioprine, studied separately, was of no influence on the antibody response (data not shown).

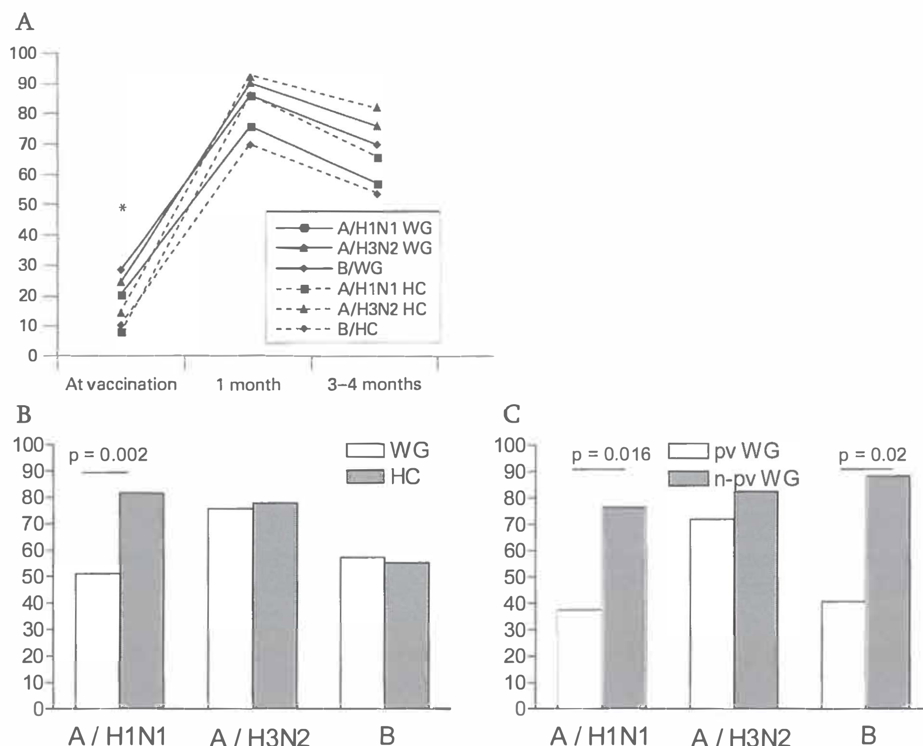

Figure 1. Antibody responses. Seroprotection (A) and seroconversion (B) rates to the vaccine strains in patients with Wegener's granulomatosis (WG) and healthy controls (HC). In (C) seroconversion rates in WG patients who received influenza vaccination in the previous year (previously vaccinated; pv WG) are compared to WG patients who did not receive an influenza vaccination in the previous year (not previously vaccinated; n-pv WG).
** P = 0.039 for the B strain; before vaccination seroprotection rate to the B strain was higher in WG patients compared to HC.*

Two possibly confounding factors were differences in age and previous influenza vaccinations. Multivariate analysis of these factors could not be performed, due to the low number of healthy controls that received an influenza vaccination in the previous year (n = 4), and the absence of healthy controls older than 63 years. Therefore, subanalyses were performed to assess the influence of these factors. First, participants were matched for age. In this subanalysis 37 WG patients and 37 healthy controls were included. Antibody responses were similar as in the whole group analysis. Here as well, a trend towards a lower response to the A/H1N1 strain was observed in WG patients, though this did not reach significance (data not shown). Next, patients (n = 17) and controls (n = 45) who were not vaccinated the previous year were compared. WG patients showed

antibody responses comparable to controls, also for the A/H1N1 strain (data not shown).

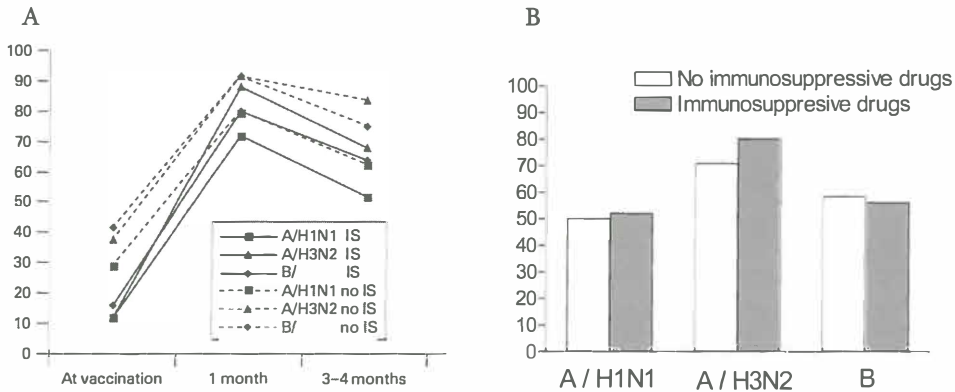

Figure 2. Influence of the use of immunosuppressive drugs on seroprotection and seroconversion rates in WG patients. Seroprotection (A) and seroconversion (B) rates to the vaccine strains in patients with Wegener's granulomatosis (WG) who used immunosuppressive drugs (IS) versus patients who did not. Seroprotection rate is the percentage of vaccinees with titers ≥ 40.

Safety parameters

Adverse effects to vaccination in vaccinated WG patients and healthy controls (**Table 3**) were comparable. Next, effects of influenza vaccination on disease activity in WG patients were evaluated. During the first month following entry, one non-vaccinated and one vaccinated WG patient developed active disease. Both patients reached complete remission after treatment with prednisone and cyclophosphamide. Two additional non-vaccinated patients developed active disease in the period up to four months after entry, versus none of the vaccinated patients (P = 0.099). At entry and during follow-up, vaccinated and non-vaccinated patients did not differ in ANCA titers, fourfold increases in ANCA titer and patient VAS scores (**Table 4**).

Discussion

To our knowledge, this is the first controlled study that evaluates immunogenicity of influenza vaccination in WG patients. In addition, we included a non-vaccinated patient group to get an indication for possible effects of influenza vaccination on WG disease activity, as vaccination-induced relapses are a concern and prospective studies of vaccinations in WG are lacking. Randomization was considered obligatory, to prevent a bias in patient groups. Because of possible disadvantageous effects of randomization for the non-vaccinated, patients with an indication for yearly influenza vaccination due to concomitant disease were excluded.

Table 3. Adverse effects to influenza vaccination

		WG n = 49	HC n = 49	*P*
Itching		3 (7.3%)	0 (0%)	0.091
Pain	No	34 (81.0%)	39 (79.6%)	0.677
	Mild	6 (14.3%)	9 (18.4%)	
	Moderate	2 (4.8%)	1 (2.0%)	
	Severe	0	0	
Erythema		4 (10.3%)	1 (2.0%)	0.166
Induration		5 (12.8%)	5 (10.2%)	0.745
Shivers		1 (2.5%)	5 (10.2%)	0.217
Myalgia		5 (12.2%)	5 (10.2%)	1.000
Fever		1 (2.5%)	0 (0%)	0.449
Headache		5 (12.5%)	5 (10.2%)	0.749
Nausea		2 (5.0%)	2 (4.1%)	1.000
Arthralgia		3 (7.5%)	1 (2.0%)	0.322
Diarrhea		1 (2.5%)	2 (4.1%)	1.000
Use of analgesic or antiphlogistic drug		3 (7.5%)	3 (6.1%)	1.000

WG: Wegener's granulomatosis, HC: healthy controls.

Antibody responses to influenza vaccination in WG patients and healthy controls were similar. First, vaccination induced high seroprotection rates in WG patients, comparable to healthy controls, which is of clinical importance. Also, seroconversion rates, which define adequate responders to vaccination, did not differ between patients and healthy controls, except for the A/H1N1 strain. Finally, results for GMTs, used to compare the magnitude of the response between

groups, were analogous to those for seroconversion rates. Importantly, seroprotection rates and GMTs to strains A/H3N2 and B were comparable between patients and healthy controls for a period of three to four months. This indicates that clinical protection from influenza infection may have been achieved for at least the larger part of the influenza season. Furthermore, influenza vaccination in WG patients fulfilled EMEA serological criteria on influenza vaccine immunogenicity. In adults (18-60 years), for each strain at least one of the following criteria should be met: seroconversion rate > 40%, mean geometric increase > 2.5, seroprotection rate > 70%. In subjects over 60, criteria are > 30%, > 2.0, > 60%, respectively [14].

Table 4. Disease parameters in WG patients

		Vac. WG n = 49	Non-vac. WG n = 23
ANCA titer, median (range)	before vaccination	40 (0 - 1280)	80 (0 - 1280)
	after 1 month	80 (0 - 1280)	160 (0 - 1280)
	after 3-4 months	40 (0 - 1280)	80 (0 - 1280)
Fourfold increase in ANCA (%)	after 1 month	5 (10.2%)	4 (17.4%)
	after 3-4 months	3 (6.2%)	2 (9.1%)
VAS, mean (SD)	before vaccination	1.9 (2.0)	2.0 (1.8)
	after 1 month	1.8 (2.2)	1.8 (2.3)
	after 3-4 months	1.9 (2.1)	1.6 (1.9)

WG: Wegener's granulomatosis. Fourfold increases in ANCA titer: as compared to ANCA titers at entry.

In transplant recipients, several immunosuppressive drugs have been shown to hamper antibody responses to vaccination [16-18]. In the present study, no effects of immunosuppressive drugs on the antibody response were found. However, the number of WG patients receiving immunosuppressive drugs was low and drug use was heterogeneous. Therefore, the conclusion that in WG patients immunosuppressive drugs have no effect on the immune response to vaccination should be made with precaution and is limited to the low doses of azathioprine and prednisone given in this study.

Immunogenicity of (influenza) vaccination in autoimmune diseases has been a subject of discussion, as it can be hypothesized that a dysregulated immune system is not able to mount normal responses to vaccination. In SLE, this seems to be the case [7], for WG these are the first data. As antibody responses were similar in WG patients and healthy controls, despite the use of immunosuppressives by

half of the patients, WG patients appear to have a normal capacity to respond to vaccine stimuli. The difference between SLE and WG may be due to a generalized immune disturbance in SLE, versus a restricted immune dysfunction in WG. This can be suspected, as SLE is characterized by numerous autoimmune features, exemplified by the generation of a broad spectrum of autoantibodies [19], whereas dysregulation in WG appears to be more specific, involving ANCA to proteinase-3 (PR3) and myeloperoxidase (MPO) [20].

The effect of repeated influenza vaccination on antibody response to vaccination was analyzed in WG patients as this may be relevant in patient care. There has been considerable discussion whether or not annually repeated influenza vaccination influences antibody responses to vaccination. Several studies reported decreased humoral responses in case of repeated vaccinations with inactivated influenza vaccines [21-23]. We too, observed a trend towards lower post-vaccination GMTs in previously vaccinated WG patients, reaching significance for the A/H1N1 strain (same vaccine strain since 2000-2001). Seroconversion rates were lower as well, except for A/H3N2, which was the only new strain in the vaccine. However, others found similar [24-26] or improved responses [27] following repeated influenza vaccinations. Smith *et al.* used mathematical modeling to explain apparently conflicting results, suggesting that vaccines which are antigenically close to a prior vaccine may be partially eliminated by pre-existent cross-reactive antibodies, thus reducing the immune response [28]. In line with this suggestion, it has been reported recently that repeated vaccination with an identical influenza strain in consecutive years leads to lower titers as compared to vaccination in a naïve individual, unrelated to the pre-vaccination titer [29].

Regarding the risk of relapse, four patients developed active disease over a period of 3.5 months, equivalent to an annual incidence of 19%, which is in accordance with the expected number of relapses [30-32]. Influenza vaccination in WG patients with quiescent disease did not influence the occurrence of relapses, and did not increase ANCA titers or patient VAS scores. ANCA titers reflect disease activity to some extent, though there are controversial data [33,34]. However, data on the risk of relapse following vaccination need to be interpreted with caution, as the study was not designed to detect such an effect, and was considerably underpowered in this respect. Furthermore, our findings are restricted to the use of a trivalent subunit vaccine for intramuscular use. Of note, the live attenuated intranasal vaccine available in some countries is contra-indicated in immunosuppressed hosts.

Although our findings seem plausible, this study has some limitations. First, the study was powered to detect a clinically relevant difference in antibody response between WG patients and healthy controls. However, the number of included patients and controls was lower than required. To our opinion this will not be of major influence on the conclusions. Apart from the A/H1N1 strain, no tendency was observed towards a lower response in WG patients, making it unlikely that a significant difference with healthy controls would have been observed if five more patients and controls had been included. Second, WG patients were older and had received an influenza vaccination in the previous year more often than healthy controls. Both factors may affect immune responses. Previous vaccination status may limit seroconversion rates to a newly administered vaccine, as may have been the case for A/H1N1 in this study. To assess whether differences in age and prior vaccination status were of influence, subanalyses were performed. When WG patients and healthy controls were matched for age, no differences were found in seroprotection rates, seroconversion rates and achieved GMTs, though a trend towards a decreased response to the A/H1N1 strain was observed in WG patients. Concerning prior vaccination status, WG patients and healthy controls who did not receive an influenza vaccination in the previous year showed similar antibody responses, for all strains. From these subanalyses it appeared that age had a minor influence, and prior vaccination status a more profound influence, at least for the antibody response to the A/H1N1 strain. Third, BVAS and VAS may have been influenced to some extent by a lack of blinding in this study; however, BVAS criteria are fairly objective.

In conclusion, we show that in WG patients with quiescent disease, subunit influenza vaccine resulted in adequate antibody responses, despite older age and use of immunosuppressive drugs, and did not appear to increase the risk of disease relapse. WG patients constitute a population in which influenza vaccination seems indicated. Though questions regarding serologic responses to repeated vaccinations may remain, annual vaccination can be considered clinically effective [35,36]. Therefore, annual influenza vaccination in WG patients with quiescent disease seems recommendable.

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Chapter 7

Cell-mediated immune responses to influenza vaccination in Wegener's granulomatosis

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ABSTRACT

Both antibody and cell-mediated immune responses are involved in the defense against influenza. Although antibody responses are unaltered in Wegener's granulomatosis (WG), the level of cell-mediated responses to vaccination could be deficient, which may increase susceptibility for influenza infection. *In vivo*-generated cell-mediated responses have not been studied previously in WG.

In this study, twenty-five WG patients and healthy controls received subunit influenza vaccine. PBMCs were obtained before and one month after vaccination. Cell-mediated responses to A/H1N1 and A/H3N2 were assessed using interferon-γ (IFN-γ) ELISpot, IFN-γ ELISA, and intracellular cytokine staining for IFN-γ, tumor necrosis factor (TNF) and interleukin-2 (IL-2).

Prior to vaccination, patients and controls showed similar recall responses to A/H1N1 and A/H3N2. Following vaccination, patients and controls showed similar levels of increase in spot-forming cells against A/H1N1 ($P = 0.009$ and $P < 0.001$, respectively) and A/H3N2 ($P = 0.011$ and $P = 0.005$, respectively). Responding cells were of similar functionality in WG and HC as they produced comparable amounts of IFN-γ. By flow cytometry, upon vaccination, proportions of cytokine-producing $CD4^+$ T cells increased in patients and controls. For A/H1N1 this increase reached significance for IFN-γ in patients and for IL-2 in controls. For A/H3N2, significant increases were observed for TNF and IL-2 in patients and for IFN-γ, TNF and IL-2 in controls. As expected with a subunit influenza vaccine, vaccination did not induce $CD8^+$ T-cell responses.

Cell-mediated responses to influenza vaccination in WG patients are comparable to those in healthy controls. Dutch Trials Register, NTR1130.

Introduction

Wegener's granulomatosis (WG) is an autoimmune inflammatory disease affecting small and medium-sized vessels, which leads to granulomatous inflammation (particularly in the airways), systemic vasculitis and glomerulonephritis. The disease is associated with the presence of antineutrophil cytoplasmic antibodies (ANCAs) directed against proteinase 3 [1]. WG patients are at risk for infections [2-4], and the median age of WG patients is relatively high. Therefore, influenza vaccination should be considered in WG.

Influenza has a high incidence as approximately 5% of adults develop symptomatic influenza each year [5]. Annual vaccination reduces the severity of influenza and related complications, and is recommended in immuno-compromised patients [6]. Development of protective immune responses to influenza following vaccination is clinically relevant in WG patients. The immune response to influenza consists of both antibody and cell-mediated responses. In WG, antibody responses to influenza vaccination appear to be similar to those in healthy controls [7,8], but cell-mediated responses have not been studied. The latter are relevant as it has been shown that in certain groups, such as the elderly and young children, cell-mediated responses to influenza vaccination can be a marker of clinical protection, independent of antibody responses [9-11].

In systemic lupus erythematosus (SLE), another systemic autoimmune disease, T-cell responses to influenza vaccination are reduced (Holvast *et al. Arthritis & Rheumatism* 2009, in press). Also in WG, the level of antigen-specific T-cell responses to vaccination may be reduced because infection rates are increased and immunological disturbances have been described, such as skewing of the $CD4^+$ T-cell pool and dysfunctional regulatory T cells [12,13]. *In vivo*-generated cell-mediated immune responses, e.g. following a vaccination, have not been studied previously in WG. We performed an explorative study on cell-mediated responses to influenza vaccination in WG using ELISpot, ELISA and flow cytometry to assess various aspects of cell-mediated responses. This study was part of a study in which also antibody responses to influenza vaccination were evaluated; these results have been published elsewhere [8].

Methods

Study population

Patients eligible for the study fulfilled criteria for Wegener's granulomatosis [14] and had quiescent disease, defined as Birmingham Vasculitis Activity Score

(BVAS) < 2. Exclusion criteria were BVAS ≥ 2, indication for yearly influenza vaccination due to concomitant disease (based on international guidelines) [6], use of prednisone > 30 mg/day and/or cyclophosphamide > 100 mg/day, and pregnancy. Patients with active disease were excluded for two reasons, first, the uncertainty regarding vaccination-induced disease activation, and, secondly, expected changes in immunosuppressive medication which might influence the interpretation of the effect of influenza vaccination on disease activity and the analysis of the immune response to vaccination. Similarly, patients using prednisone > 30 mg/day and/or cyclophosphamide > 100 mg/day were considered to have instable disease and therefore were considered ineligible. A control group of age- and sex-matched healthy individuals was included; for this purpose, health care workers participating in the yearly influenza vaccination campaign were asked to participate. Exclusion criteria for participation as healthy control were the use of immunosuppressive drugs, malignancy, or pregnancy.

Study design

WG patients and controls were included from October to December 2005 and were vaccinated intramuscularly with a single dose of a trivalent subunit influenza vaccine (Influvac®, 2005-2006, Solvay Pharmaceuticals, Weesp, the Netherlands), containing A/New Caledonia/20/99 [H1N1], A/NewYork/55/2004 [H3N2] and B/Hong Kong/330/2001. Prior to vaccination and four weeks after vaccination, peripheral blood mononuclear cells (PBMCs) were isolated. The study was approved by the institutional medical ethics committee, and informed consent was obtained from all participants.

ELISpot, ELISA and flow cytometry

Isolation, storage and thawing of PBMCs. PBMCs were isolated from heparinized venous blood by density-gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) immediately after blood was drawn. PBMCs were frozen in RPMI 1640 (Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS), 50 µg/ml of gentamicin (Gibco, Paisley, UK) and 10% dimethylsulfoxide. PBMCs were stored in liquid nitrogen until use. Pre- and post-vaccination samples, from a WG patient and a matched control, were simultaneously thawed and batch-processed. A minimum cell viability of > 90%, evaluated by trypan blue staining, was required. Preceding ELISpot assays, PBMCs were rested, by overnight incubation at 37° C. Cells were counted before plating, using an automated cell counter (Beckman Coulter, Fullerton, CA, USA).

Interferon-γ (IFN-γ) ELISpot assay. Nitrocellulose plates (Nunc, Rochester, NY, USA) were coated overnight at 4° C with 50 µl anti-human IFN-γ, 15 µg/ml per well (Mabtech, Nacka Strand, Sweden). Plates were washed and blocked with culture medium (CM; RPMI supplemented with 50 µg/ml gentamicin and 10% FCS) for one hour at room temperature (RT). Subsequently, 2 x 10^5 PBMCs were added per well, in 200 µl, and incubated in CM at 37° C with WIV of A/H1N1 and A/H3N2, at a final concentration of 5 µg total viral protein/ml. Concanavalin A (ConA) stimulation, 5 µg/ml, was used as a positive control and a negative control consisted of PBMCs in CM alone. Stimulation tests were performed in triplicate. After 48 hours plates were washed with phosphate buffered saline (PBS), and 50 µl of 1 µg/ml biotinylated anti-human IFN-γ (Mabtech) was added per well for 3 hours at RT. Next, plates were washed again, and 50 µl 1:1000 streptavidin-alkaline phosphatase (Mabtech) per well was added for 1.5 hours at RT. Plates were washed and 100 µl BCIP/NBT-plus substrate (Mabtech) was added per well for 10 minutes. Finally, plates were washed with tap water. After drying, spots were counted using an automated reader (automated ELISpot video-analysis system, Sanquin, Amsterdam, The Netherlands). Results are referred to as IFN-γ spot-forming cells, as IFN-γ-producing $CD4^+$ and $CD8^+$ T cells as well as natural killer (NK) cells, following WIV stimulation, have been described [15].

IFN-γ enzyme-linked immuno sorbent assay (ELISA). Cells were cultured in 96-well round-bottom Cellstar® plates (Greiner Bio-One, Kremsmünster, Austria), under conditions as described for ELISpot. Following stimulations, supernatants were frozen at -20° C until analysis. For IFN-γ ELISAs, samples were fourfold diluted and tested using the PeliKine Compact™ human IFN-γ ELISA kit (Sanquin, Amsterdam, the Netherlands), according to the manufacturer's instructions.

Flow cytometry. For stimulations, 1.0 - 1.5 x 10^6 PBMCs were cultured in 200 µl CM, in 5 ml polypropylene round-bottom Falcon™ tubes (Becton Dickinson and Company (BD), Franklin Lakes, NJ, USA). Staphylococcal enterotoxin B (SEB, Sigma-Aldrich, Saint Louis, MO, USA) and ConA were used as a positive control, at 5 µg/ml. WIV A/New Caledonia (H1N1) and WIV A/ Hiroshima (H3N2) were used at final concentrations of 1 µg of total viral protein/ml. WIV and negative control (medium only) cultures were incubated in the presence of 10 µg/ml

anti-CD28/CD49 (BD). Cells were incubated for 18h at 37° C, the final 16h in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich). Following incubation, 10 µl 40mM EDTA in PBS was added, tubes were vortexed and incubated for 10 minutes, to facilitate resuspending. Next, 2 ml FACS lysing solution (BD) was added for 10 minutes. Cells were spun down and washed in PBS-1% bovine serum albumin. Subsequently cells were permeabilized in 500 µl PERM-2 (BD) for 10 minutes in the dark in the presence of pacific blue and orange (Invitrogen, Carlsbad, CA, USA), in a different combination for each stimulus, to enable fluorescent cell barcoding [16]. PBS-20% FCS was added for 5 minutes. Cells were washed and pooled per PBMC sample. Next, anti-CD3-FITC, anti-CD4-PE-Cy7, anti-CD8-PercP, anti-CD69-APC-Cy7, anti-IFN-γ-Alexa 700, anti-tumor necrosis factor (TNF)-APC and anti-interleukin (IL)-2-PE (all from BD) were added, following the manufacturer's instruction. After incubation for 30 minutes at RT, cells were washed and immediately analyzed on a LSR II flow cytometer (BD). Data for at least 1 x 10^6 $CD3^+$ cells were collected.

Using the Win-List software package (Verity Software House, Topsham ME, USA), positively and negatively stained populations were gated and Boolean gating was applied. First, lymphocytes were gated by CD3 expression and sideward scatter patterns. Next, $CD4^+$ and $CD8^+$ T-cell populations were gated as $CD4^+CD8^-$ or $CD4^-CD8^+$, respectively. Then, cells from different stimulation tubes were separated in a pacific blue/orange plot. Finally, $CD69^{+/-}$ $cytokine^{+/-}$ quadrants were set for the different stimuli simultaneously, according to the negative and positive controls. Percentages of antigen-specific cells were expressed as the percentage of $CD69^+$ $cytokine^+$ $CD4^+$ or $CD8^+$ T cells within the total $CD4^+$ or $CD8^+$ T-cell population. All T-cell frequencies reported are after background subtraction of the frequency of the identically gated population of cells from the same sample stimulated without antigen.

Statistical analysis

Data were analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA). Log-transformed prior to testing were: ELISpot, ELISA, $CD4^+$/ $CD8^+$ T-cell data on SEB and ConA, and $CD8^+$ T-cell data on A/H1N1. $CD4^+$ T-cell data on A/H1N1 were root-transformed prior to testing. All variables were tested using Student's *t*-test, except for previous influenza vaccination (Fisher's exact test) and $CD4^+$ T-cell responses against A/H3N2 (Mann-Whitney U tests and Wilcoxon signed rank test). For correlations, Pearson's and Spearman's correlation coefficients were used where appropriate. A two sided P value < 0.05 was considered statistically significant.

Results

Patient characteristics

Responses were evaluated in twenty-five WG patients and age- and sex-matched controls; in one patient and one control cells were of insufficient viability, therefore 24 WG patients and controls remained for analysis. Characteristics are shown in **Table 1.** Half of the patients had received an influenza vaccination in the previous year, versus only one control ($P = 0.001$). Eleven out of 24 patients used immunosuppressives, most frequently azathioprine and prednisone. All patients were in complete remission (BVAS = 0) at entry. During follow-up, one patient developed active disease (BVAS = 5).

Table 1. Baseline characteristics and disease parameters

		WG n = 24	HC n = 24
Sex, males		13	12
Age in years, mean (SD)		50.5 (10.0)	49.4 (8.4)
Range		27 – 64	25 - 63
Influenza vaccination in previous year		12 (50%)**	1 (4.2%)
Without immunosuppressives (%)		13 (54.2%)	n/a
Duration in months, median (range)		39 (8 - 154)	n/a
With immunosuppressives (%)		11 (45.8%)	n/a
Duration in months, median (range)		4 (2 - 20)	n/a
Prednisone		8 (33.3%)	n/a
Median (range), in users (mg/day)		5.63 (2.5 – 12.5)	
Azathioprine		8 (33.3%)	n/a
Median (range), in users (mg/day)		100 (35.7§ – 150)	
Other immunosuppressive drugs		2 (8.3%)#	n/a
BVAS, median (range)	t = 0	0 (0-0)	n/a

WG: Wegener's granulomatosis, HC: healthy controls, BVAS: Birmingham Vasculitis Activity Score, n/a: not applicable, duration: length of time without immunosuppressives or length of time of stable use of current immunosuppressives

 # One patient used ciclosporin 125 mg/day and one patient used cyclophophamide 25 mg/day, both did not use any other immunosuppressive. § One patient used azathioprine 50 mg five times per week.

** $P < 0.01$

Cell-mediated immune responses

Activated ($CD69^+$) cytokine-producing T cells were quantified by flow cytometry (**Fig. 1A**). Upon SEB and ConA stimulation, WG patients and controls showed similar frequencies of IFN-γ-, TNF- and IL-2-producing $CD4^+$ T cells (**Fig. 1B**) and $CD8^+$ T cells (**Fig. 1C**). This indicated that T cells from WG patients were generally

capable of adequate cytokine responses. After assessing general responsiveness, we analyzed influenza-specific cell-mediated responses. By ELISpot, prior to vaccination, WG patients had similar numbers of IFN-γ spot-forming cells against A/H1N1 and A/H3N2 as compared to controls (**Fig. 2A**, $P = 0.632$ and $P = 0.377$, respectively). To assess functional capacity of these responding PBMCs, the total amount of IFN-γ produced upon A/H1N1 and A/H3N2 stimulation was determined. In this regard, patients and controls did not differ either (**Fig. 2B**).

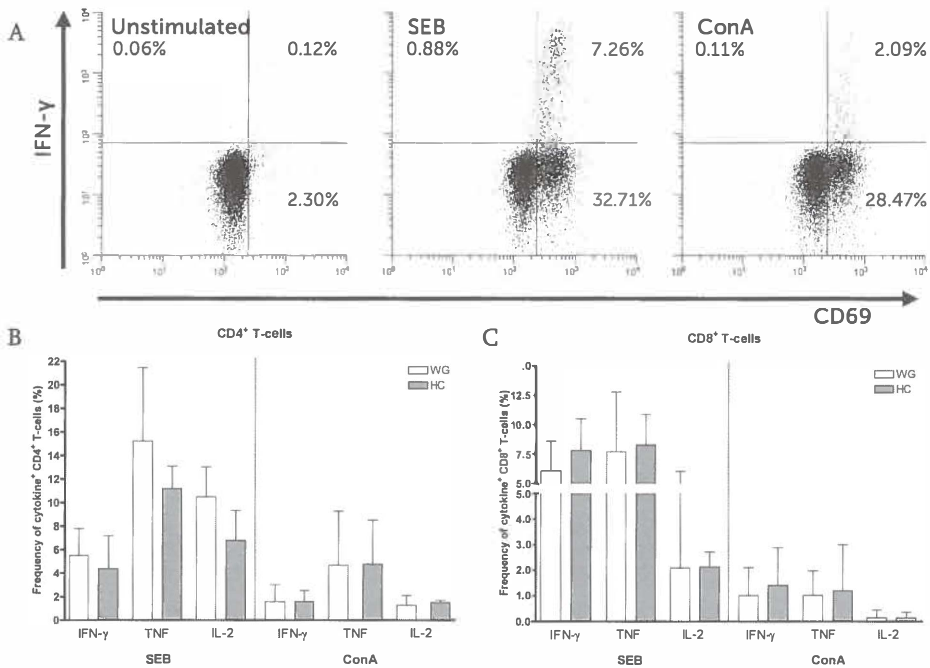

Figure 1. CD4+ and CD8+ T-cell responses against SEB and ConA. (A) Representative example of gating of activated (CD69+) interferon-γ (IFN-γ)-producing CD4+ T cells, in a pre-vaccination sample of a Wegener's granulomatosis (WG) patient; unstimulated cells (left), stimulated with Staphylococcal enterotoxin B (SEB, middle), and stimulated with concanavalin A (ConA) (right).
Frequencies of (B) cytokine-producing CD4+ and (C) CD8+ T cells upon stimulation with SEB and ConA in WG patients and healthy controls (HC). Results are corrected for responses in unstimulated cultures from the same sample. Medians and interquartile ranges are shown.

Flow cytometry was applied to get more insight in the phenotype and the cytokine pattern of responding cells (**Fig. 3A**). Pre-vaccination, the frequency of cytokine-producing CD4+ T cells against A/H1N1 did not differ between patients and controls (**Fig. 3B**). For A/H3N2, only IFN-γ-producing CD4+ T cells could be detected, at similar frequencies in patients and controls (**Fig. 3C**). Within CD8+ T cells, recall responses could only be detected for A/H1N1. Frequencies of IFN-γ-

and TNF-producing CD8+ T cells were similar in patients and controls; IL-2-producing CD8+ T cells could not be detected (**Fig. 3D**).

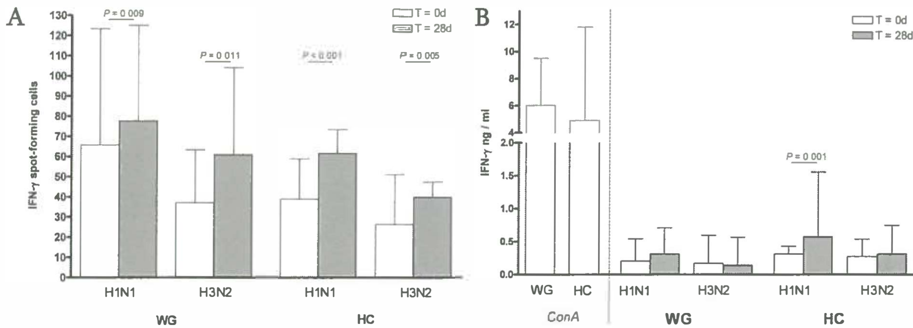

Figure 2. IFN-γ ELISpot and ELISA. (A) ELISpot of IFN-γ producing cells and (B) IFN-γ ELISA, per 2 x 10⁵ PBMCs, in Wegener's granulomatosis patients (WG) and healthy controls (HC), in response to A/H1N1 and A/H3N2 stimulation before vaccination (T = 0 days) and four weeks after vaccination (T = 28 days). For ELISA, also responses to concanavalin A (ConA) are shown. Results are corrected for responses in unstimulated cultures from the same sample. Medians and interquartile ranges are shown. In ELISA, vaccination tended to increase IFN-γ production in WG upon A/H1N1 stimulation (P = 0.068).

Following vaccination, both WG patients and controls showed a rise in IFN-γ spot-forming cells against A/H1N1 (P = 0.009 and P < 0.001, respectively) and A/H3N2 (P = 0.011 and P = 0.005, respectively). After vaccination, numbers of spot-forming cells were comparable in patients and controls (**Fig. 2A**). For A/H1N1, also the amount of IFN-γ produced, as measured in the supernatant, increased. For controls, this increase was significant (P = 0.001), for patients this was borderline significant (P = 0.068); levels of IFN-γ were similar in patients and controls. For A/H3N2, no marked increases were observed in either patients or controls (**Fig. 2B**).

By flow cytometry, for A/H1N1, increases of frequencies of cytokine-producing CD4+ T cells were modest and reached statistical significance only for IFN-γ in patients (P = 0.041) and IL-2 in controls (P = 0.018) (**Fig. 3B**). For A/H3N2, CD4+ T-cell responses were detectable following vaccination, albeit at a lower level than those against A/H1N1. In patients, frequencies of TNF- and IL-2-producing CD4+ T cells increased (P = 0.025 and P = 0.024, respectively); in controls, increases were observed for IFN-γ, TNF and IL-2 (P = 0.021, P = 0.013 and P = 0.011, respectively) (Fig. **3C**). For both A/H1N1 and A/H3N2, frequencies

of cytokine-producing CD4⁺ T cells were similar in patients and controls following vaccination. Vaccination did not induce a CD8⁺ T-cell response (data not shown).

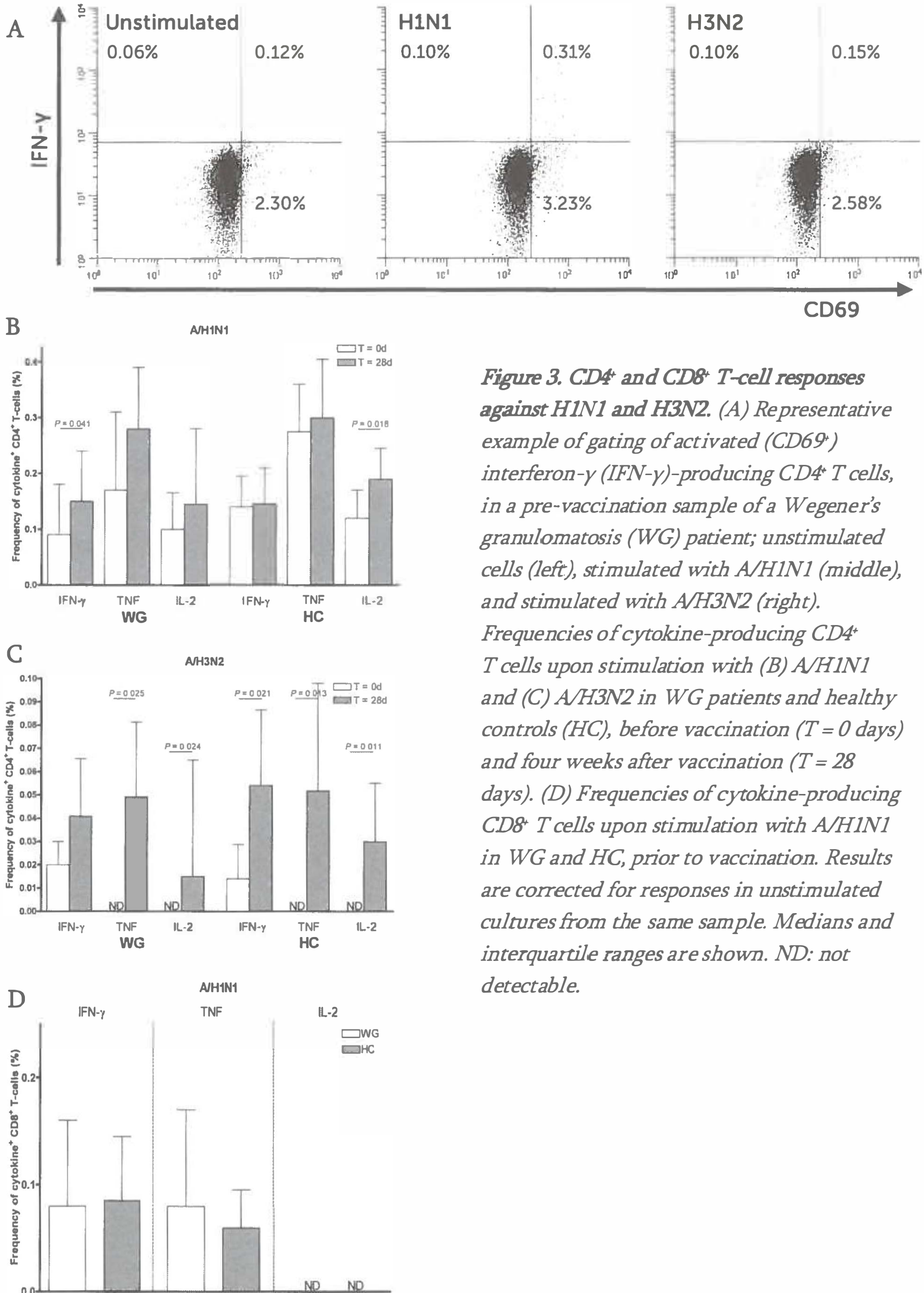

Figure 3. CD4⁺ and CD8⁺ T-cell responses against H1N1 and H3N2. *(A) Representative example of gating of activated (CD69⁺) interferon-γ (IFN-γ)-producing CD4⁺ T cells, in a pre-vaccination sample of a Wegener's granulomatosis (WG) patient; unstimulated cells (left), stimulated with A/H1N1 (middle), and stimulated with A/H3N2 (right). Frequencies of cytokine-producing CD4⁺ T cells upon stimulation with (B) A/H1N1 and (C) A/H3N2 in WG patients and healthy controls (HC), before vaccination (T = 0 days) and four weeks after vaccination (T = 28 days). (D) Frequencies of cytokine-producing CD8⁺ T cells upon stimulation with A/H1N1 in WG and HC, prior to vaccination. Results are corrected for responses in unstimulated cultures from the same sample. Medians and interquartile ranges are shown. ND: not detectable.*

Correlations

Subunit influenza vaccine is expected to induce a CD4+ T helper response [15], in ELISpot, this is reflected by changes in numbers of spot-forming cells following vaccination. Indeed, changes in numbers of spot-forming cells following vaccination correlated to changes in IFN-γ+CD4+ T-cell frequencies for A/H1N1 and A/H3N2 in controls and for A/H3N2 in patients (**Fig. 4** A + **B**). Next, we addressed correlations between antibody titers and cell-mediated responses against A/H1N1 and A/H3N2; antibody titers have been reported previously [8]. For the generation of antibody responses, help signals from CD4+ T helper cells are necessary. However, CD4+ T-cell responses did not correlate to antibody responses. In patients and controls, prior to vaccination and after vaccination, there were no correlations for A/H1N1 nor for A/H3N2 between antibody titers and CD4+ T-cell cytokine responses or IFN-γ spot-forming cells (**Fig. 4 C + D**).

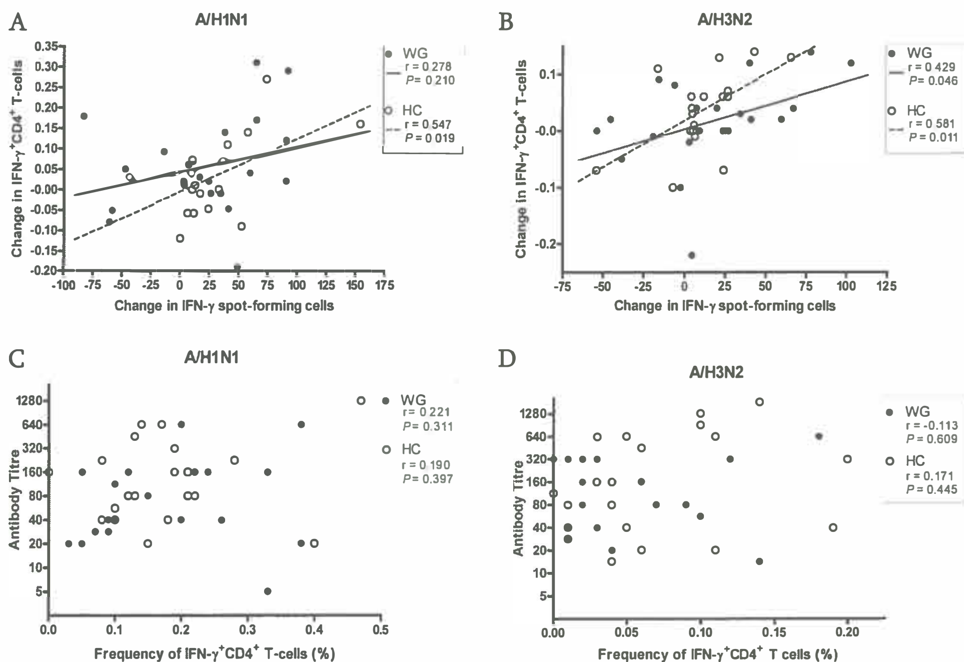

Figure 4. Correlations between responses to influenza vaccination. *Changes (after vaccination minus prior to vaccination) in IFN-γ producing PBMCs correlate to changes in CD4+IFN-γ+ T cells against A/H1N1 (A) and A/H3N2 (B), in Wegener's granulomatosis patients (WG) and healthy controls (HC). However, antigen-specific T-cell responses do not correlate to antibody titers. Shown are IFN-γ+CD4+ T cells after vaccination and antibody titers after vaccination, for A/H1N1 (C) and A/H3N2 (D).*

Influence of immunosuppressives and previous influenza vaccination

Though numbers were small, possible influences of the use of immunosuppressives and influenza vaccination in the previous year upon cell-mediated immune responses against A/H1N1 and A/H3N2 were evaluated in WG patients. When comparing WG patients without immunosuppressives (n = 13) to patients using any immunosuppressive (n = 11), no differences were found, nor a trend towards such differences. Following vaccination, patients with and without immunosuppressives had similar numbers of IFN-γ spot-forming cells against A/H1N1 and A/H3N2 (P = 0.554 and P = 0.537, respectively) and similar frequencies of A/H1N1-specific IFN-γ-, TNF- and IL-2-producing $CD4^+$ T cells (P = 0.878, P = 0.871 and P = 0.927, respectively) as well as similar frequencies of A/H3N2-specific IFN-γ-, TNF- and IL-2-producing $CD4^+$ T cells (P = 0.773, P = 0.617 and P = 0.685, respectively).

Next, we compared patients who had received an influenza vaccination in the previous year (n = 12) with patients who had not received an influenza vaccination in the previous year (n = 12). In the previous year, the A/H1N1 vaccine strain was identical. Prior to and after vaccination, previously vaccinated patients and not-previously vaccinated patients did not differ in cell-mediated immune responses (data not shown).

Discussion

To our knowledge, cell-mediated immune responses to vaccination have not been assessed in WG. We questioned whether immunological disturbances in WG would lower cell-mediated responses to influenza vaccination. In this explorative study, we did not find differences in cell-mediated immune responses to subunit influenza vaccination between WG patients and controls. This suggests that the functional capacity of the T-cell pool to respond to vaccination is intact in WG.

Cell-mediated recall responses to influenza were comparable between WG patients and controls. This is in accordance with previous studies, in which normal cytokine and proliferation responses to recall antigens in WG patients (i.a. tetanus toxoid) have been reported [17-19].

Following vaccination, the number of spot-forming cells increased in both patients and controls to similar levels. Responding cells in patients and controls appeared to be of comparable functional capacity, as the total amount of IFN-γ produced by PBMCs upon A/H1N1 and A/H3N2 stimulation was similar in patients and controls. $CD4^+$ T-cell cytokine responses to influenza vaccination

were modest, but measurable, and were observed for both A/H1N1 and A/H3N2 in patients and controls; again, levels remained similar in patients and controls. Responses were, at least largely, independent of previous vaccination status and the use of immunosuppressive drugs. Thus, functional capacity of the T-cell pool does not appear to be affected by the immunological disturbances in WG. This conclusion is restricted to patients with quiescent disease and patients receiving relatively mild immunosuppressive therapy. Active disease and/ or use of more potent immunosuppressives, such as cyclophosphamide, may result in diminished cell-mediated immune responses.

In SLE, cell-mediated responses to influenza vaccination were reduced, which was associated with the use of prednisone and/ or azathioprine (Holvast *et al. Arthritis & Rheumatism* 2009, in press). In WG, we did not observe such an effect of immunosuppressive therapy, though prednisone and azathioprine were used in comparable doses. Possibly, other, disease-related, factors are involved in SLE. However, this comparison should be made cautiously, as the number of WG patients was relatively small.

With regard to $CD8^+$ T cells, frequencies were very low and not detectable for A/H3N2. For A/H1N1, IFN-γ- and TNF-producing $CD8^+$ T-cell frequencies were recorded at similar levels in patients and controls. As expected with a subunit influenza vaccine, we did not observe a change in cytokine-producing $CD8^+$ T cells following vaccination; subunit influenza vaccine induces MHC class II restricted $CD4^+$ T-cell stimulation [20], but not MHC class I restricted $CD8^+$ T-cell responses [21].

To quantify influenza-specific cells, we used ELISpot and flow cytometry. ELISpot is the more sensitive assay, whereas flow cytometry allows phenotyping of responding cells as well as detection of multiple cytokines [22]. It has been shown that next to $CD4^+$ T cells, also $CD8^+$ T cells and a proportion of NK cells produce IFN-γ upon stimulation with influenza virus [15]. However, it is expected that subunit influenza vaccine primarily induces a $CD4^+$ T-cell response [20]. Accordingly, changes in numbers of IFN-γ spot-forming cells following vaccination correlated to changes in IFN-$\gamma^+$$CD4^+$ T-cell frequencies for A/H1N1 and A/H3N2 in controls and for A/H3N2 in patients.

Previously, we reported adequate antibody responses to influenza vaccination in WG patients [8]. As measurements of antibody and cell-mediated responses were performed within the same study, we evaluated whether they were correlated. We did not observe any correlation between antibody responses and IFN-γ spot-

forming cells or CD4+ cytokine-producing cells. This is in accordance with others [23]. Therefore, cell-mediated and antibody responses appear to be independent measures of the response to influenza vaccination, as has also been suggested in several clinical studies. In elderly, cell-mediated responses were an independent marker of clinical protection from influenza infection [10]. Similarly, in young children numbers of spot-forming cells correlated to clinical protection from culture-confirmed influenza [11]. These and other reports show the clinical relevance of cellular immune responses to influenza vaccination. However, in contrast to antibody responses, up till now no consensus regarding correlates of protection has been established for cell-mediated responses.

This study has several limitations. First, the number of patients and controls studied was relatively small. Second, the use of immunosuppressives was heterogeneous and relatively mild. Third, the proportion of participants that had received an influenza vaccination in the previous year was much higher among patients than controls. Previous influenza vaccination is known to influence antibody responses [24], however, in a subanalysis, it did not seem to influence cell-mediated responses.

In conclusion, cell-mediated responses to subunit influenza vaccination were similar in WG patients with quiescent disease and controls, despite the use of, relatively mild, immunosuppressive drugs in patients. Antibody responses to influenza vaccination are adequate as well, and no indications of detrimental effects upon disease activity have been reported. Therefore, influenza vaccination in WG patients with quiescent disease can be recommended.

This paper will be published as a Concise Communication.

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Chapter 8

Humoral responses after influenza vaccination are severely reduced in patients with rheumatoid arthritis treated with rituximab

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ABSTRACT

For rheumatoid arthritis (RA) patients yearly influenza vaccination is recommended. However, its efficacy in patients treated with rituximab (RTX) is unknown. The objective of this study was to investigate the efficacy of influenza vaccination in RA patients treated with RTX, and the duration of the possible suppression of the humoral immune response following RTX treatment. Furthermore, the effects of former influenza vaccination and safety were assessed.

23 RA patients having received RTX (11 patients 4-8 weeks after RTX, and 12 patients 6-10 months after RTX), 20 RA patients on methotrexate (MTX), and 29 healthy controls (HC) received trivalent influenza subunit vaccine. Levels of antibodies against the three vaccine strains were measured before and 28 days after vaccination using the hemagglutination inhibition assay. DAS28 was used to assess RA activity.

Following vaccination, geometric mean titers (GMT) significantly increased for all influenza strains in the MTX and HC group, but for none in the total RTX group. However, in the subgroup of patients 6-10 months after RTX a rise in GMT for A/H3N2 and A/H1N1 was demonstrated, in the absence of a recurrence of $CD19^{+}$-cells. Seroconversion and seroprotection occurred less often in RTX patients compared to MTX patients for A/H3N2 and A/H1N1, and compared to HC for A/H1N1. Previous vaccination in RTX patients led to higher pre- and post-vaccination GMT for A/H1N1 compared to not previously vaccinated RTX patients. DAS28-scores did not change after vaccination.

RTX reduces humoral responses following influenza vaccination in RA patients, with a modestly restored response 6-10 months after RTX administration. Previous influenza vaccination in RTX patients increases pre- and post-vaccination titers. RA activity was not influenced.

Introduction

Patients with rheumatoid arthritis (RA) are considered immunocompromised and at increased risk of infection [1]. Therefore, although the exact prevalence, morbidity and mortality of influenza in patients with RA are unknown, yearly influenza vaccination is recommended [2].

Influenza vaccination is safe and results in protective levels of anti-influenza antibodies in most RA patients, even when treated with prednisone, disease modifying antirheumatic drugs (DMARDs) or TNF-alpha blocking agents [3,4]. A growing group of RA patients is being treated with rituximab (RTX), depleting B cells for 6-9 months. Theoretically, humoral responses to neoantigens can not be elicited during B-cell depletion. Anti-influenza antibody response after influenza vaccination has been shown to be blunted in RA patients treated with RTX [5,6]. However, the exact level and duration of suppression of the humoral immune response, and the influence of previous influenza vaccination on antibody response after treatment with RTX remain unclear.

In order to make recommendations for the usefulness and timing of influenza vaccination in RA patients treated with RTX, we investigated humoral responses in RA patients following vaccination with trivalent subunit influenza vaccine 4-8 weeks or 6-10 months after treatment with RTX. The responses were compared with responses in RA patients treated with methotrexate (MTX) and healthy controls (HC). In addition, the influence of previous influenza vaccination on antibody response and safety of influenza vaccination were assessed.

Methods

Patients and healthy controls

Patients had to fulfill the American College of Rheumatology clinical classification criteria for RA. Two groups of RA patients were defined. One group consisted of RA patients who were treated with RTX (RTX group), either at 4-8 weeks (early-RTX subgroup) or 6-10 months (late-RTX subgroup) after treatment. RTX was administered in 2 cycles of 1000 mg IV with 100 mg methylprednisolone IV, except for one patient who instead received 4 cycles of 375 mg/m^2 based on a protocol for concomitant mixed cryoglobulinemia. The second group consisted of RA patients treated with methotrexate, at a minimum dose of 10 mg/week; the use of additional DMARDs was allowed. Health care workers served as healthy controls (HC group). Patients in the RTX group were recruited in all four

participating Dutch university medical centers. MTX patients and HC were recruited from the Groningen Medical Center. Exclusion criteria were: (i) no informed consent, (ii) age under 18, (iii) malignancy, (iv) pregnancy, (v) known allergy to or former severe reaction following vaccination with trivalent influenza subunit vaccine.

Vaccine

Trivalent influenza subunit vaccine (Influvac® 2007-2008, Solvay Pharmaceuticals, Weesp, The Netherlands), containing purified hemagglutinin and neuramidase of the following strains: A/Wisconsin/67/2005 (H3N2)-like strain, A/Solomon Islands/3/2006 (H1N1)-like strain, and B/Malaysia/506/004-like strain.

Procedures

Patients and HC received the influenza vaccine intramuscularly from October 2007 up till January 2008. Immediately before and 28 ± 3 days after vaccination blood was drawn for measurement of $CD19^{+}$-cell count, CRP, ESR, and anti-influenza antibodies. Disease Activity Score of 28 joints (DAS28) was recorded before vaccination, and 7 and 28 days afterwards. From all participants information on previous influenza vaccination was obtained, and adverse effects occurring in the first seven days post-vaccination were recorded. The study was approved by the ethics committees of all participating centers.

Hemagglutination Inhibition Assay (HI)

For the detection of influenza antibodies the hemagglutination inhibition (HI) test was used. HI assays were performed with guinea pig erythrocytes following standard procedures [7]. The following parameters for efficacy of vaccination were evaluated: geometric mean titer (GMT), fold increase in titer, ≥ 4-fold titer rise resulting in a post-vaccination level of ≥ 40 (seroconversion), and titer rise to ≥ 40 (seroprotection). HI titers ≥ 40 are generally considered to be protective in healthy adults [8].

Statistical analysis

All other data are presented as median (range), except for GMT, which is shown as mean (SD). Data were analyzed using SPSS 16.0 for Windows (SPSS, Inc.). ANOVA, Student's *t* test with Bonferroni correction, Kruskal-Wallis test, Friedman test, Wilcoxon Signed-Rank test, Mann-Whitney U test, Chi Square

test, Fisher's Exact test, and Spearman's Rank Correlation test were used where appropriate. A P value < 0.05 was considered statistically significant.

Results

Patient characteristics

Twenty-three RA patients were included in the RTX group (11 in the early-RTX group and 12 in the late-RTX group), 20 RA patients in the MTX group and 29 individuals in the HC group. The mean age in the RTX group did not differ from the MTX group ($P = 0.477$), but was higher compared to the HC group ($P = 0.004$). Patients in the RTX group had higher baseline DAS28 scores than in the MTX group ($P = 0.001$), and fewer B cells than patients in the MTX and HC group (for both $P < 0.001$) (**Table 1**).

Efficacy of influenza vaccination

As expected, the GMT for the A/H3N2- and the B-strain prior to vaccination were higher in the HC group ($P = 0.002$ and $P = 0.008$), since more HC than patients received an influenza vaccination in the season 2006/2007. GMT following vaccination increased for all three influenza strains in both the HC group (A/H3N2, $P = 0.001$; A/H1N1, $P < 0.001$; B, $P < 0.001$) and the MTX group (A/H3N2, $P < 0.001$; A/H1N1, $P < 0.001$; B, $P = 0.022$). In contrast, no significant increase in GMT after vaccination was found in the RTX group. Post-vaccination titers were higher for all three strains in the HC group and for both A strains in the MTX group than in the RTX group. The fold increase in titer was larger in the HC group for A/H1N1 ($P = 0.001$) and B ($P = 0.030$), and in the MTX group for A/H3N2 and A/H1N1 (both $P < 0.001$), than in the RTX group (**Table 2**).

GMT rose after vaccination in the late-RTX group for A/H3N2 ($P = 0.040$) and A/H1N1 ($P = 0.042$), but not in the early-RTX group, resulting in higher post-vaccination GMT (A/H3N2, $P = 0.040$; A/H1N1, $P = 0.003$; B, $P = 0.007$) and larger fold increase (A/H3N2, $P = 0.041$; A/H1N1, $P = 0.043$) in the late-RTX group, thereby indicating some recovery of the humoral immune response 6-10 months after treatment with RTX. At baseline the peripheral blood $CD19^{+}$-cell count was comparable for the early- and the late-RTX group (0 (0 - 0.01 x 10^9/l) vs. 0 (0 - 0.08 x 10^9/l), $P = 0.072$). However, 28 days after vaccination significantly more B cells were present in the late-RTX group than in the early-RTX group (0 (0 - 0) vs. 0.01 x 10^9/l (0 - 0.10 x 10^9/l), $P = 0.004$).

Table 1. Baseline characteristics

	1. RTX (n = 23)	2. MTX (n = 20)	3. HC (n = 29)	*P* value
Age (years), mean (SD)	55.5 (7.6)	57.1 (6.7)	46.5 (12.5)	*0.004* (1 vs. 3) 0.477 (1 vs. 3)
Sex (F/M), no. (%)	16/7 (70/30)	11/9 (55/45)	23/6 (79/21)	0.192
Influenza vac. '06/'07, no. (%)	12 (52)	10 (50)	21 (72)	0.195
Duration RA (years), median (range)	13.8 (1.1-40)	8.7 (0.3-21)	N/A	0.098
MTX (mg/week), median (range)	17.5* (10-25)	16.3 (10-25)	N/A	0.873
Prednisone (mg/day), median (range)	8.75† (3.8-40)	0 (0-0)	N/A	*<0.001*
DMARDs, no (%)				
azathioprine	1 (4)	-	N/A	
sulphasalazine	-	1 (5)	N/A	
leflunomide	-	1 (5)	N/A	
Interval after RTX (4-8 wk/6-10 mo), no. (%)	11/12 (48/52)	N/A	N/A	
Previous RTX cycles, no. (%)				
0	11 (48)	N/A	N/A	
1	5 (22)	N/A	N/A	
2	6 (26)	N/A	N/A	
3	0	N/A	N/A	
4	1 (4)	N/A	N/A	
CD19^{+}-cells (x 109/l), median (range)	0 (0-0.09)	0.16 (0-0.24)	0.25 (0.09-0.44)	*<0.001* (1 vs. 3) *<0.001* (1 vs. 3)

Characteristics at baseline of rheumatoid arthritis (RA) patients treated with rituximab (RTX), RA patients treated with methotrexate (MTX), and healthy controls (HC).
** n = 10; † n = 15*

Table 2. Geometric mean titers (GMT) and fold increase in GMT

		HC	MTX	RTX		
				All RTX	Early RTX	Late RTX
		n = 29	n = 20	n = 23	n = 11	n = 12
GMT, mean (SD)						
A/H3N2	pre	27.6 (2.9)*	13.9 (2.8)	13.1 (2.3)	10.0 (1.7)	16.8 (2.7)
	post	44.5 (2.2)†§	34.2 (1.9)†§	14.4 (2.5)	9.4 (2.1)	21.2 (2.6)†¥
A/H1N1	pre	27.0 (3.0)	14.6 (2.5)	15.0 (2.0)	11.3 (1.8)	19.4 (2.0)¥
	post	73.6 (2.2)†§	47.6 (2.8)†§	18.5 (2.7)	10.0 (1.6)	32.7 (2.8)†¥
B	pre	15.7 (2.6)*	7.7 (1,9)	8.9 (2.1)	6.0 (1.6)	12.6 (2.3)¥
	post	29.7 (2.5)†§	13.4 (2.5)†	10.9 (2.4)	6.6 (1.6)	17.3 (2.5)¥
Fold increase, median (range)						
A/H3N2		1.4 (-1.4 - 16)	2 (1 - 11.3)†	1 (-2 - 2)	1 (-2 - 2)	1 (-1.4 - 2)¥
A/H1N1		2 (-1.4 - 128)†	4 (1 - 16)†	1 (-2 - 8)	1 (-2 - 1.4)	1.2 (-1,3 - 8)¥
B		1.4 (-1.4 - 32)	1 (-1.4 - 16)	1 (-2 - 5.7)	1 (-1.4 - 2)	1 (-2 - 5.7)

Geometric mean titers (GMT) and fold increase in GMT for influenza A/H3N2, A/H1N1 and B, before (pre) and after (post) vaccination with trivalent influenza subunit vaccine, in healthy controls (HC), rheumatoid arthritis (RA) patients treated with methotrexate (MTX), and RA patients treated with rituximab (RTX), which were further split up in the subgroup early-RTX (4-8 weeks after RTX) and late-RTX (6-10 months after RTX).
** P < 0.05 compared to MTX and RTXgroup; † P < 0.05 compared to pre-vaccination titer; § P < 0.05 compared to RTX group; ¥ P < 0.05 compared to early-RTX group.*

Seroconversion occurred more often in the MTX group for A/H3N2 (P = 0.011) and A/H1N1 (P = 0.020) than in the RTX group. Seroconversion for any of the three influenza strains occurred in only three patients in the RTX group (all for A/H1N1), all belonging to the late-RTX group.

Seroprotection was achieved more often for A/H1N1 (P = 0.020) in the HC group and for A/H3N2 (P = 0.020) and A/H1N1 (P = 0.025) in the MTX group compared to the RTX group. The percentage of persons with a post-vaccination titer ≥ 40 irrespective of the pre-vaccination titer was higher in the HC group than in the RTX group for A/H3N2 (P < 0.001), A/H1N1 (P < 0.001) and B (P = 0.020), and for A/H3N2 (P = 0.025) and A/H1N1 (P = 0.010) in the MTX group compared to the RTX group. Seroprotection in the RTX group occurred in only six patients, whereof 5 in the late- vs. one in the early-RTX group (P = 0.108) (**Fig. 1**).

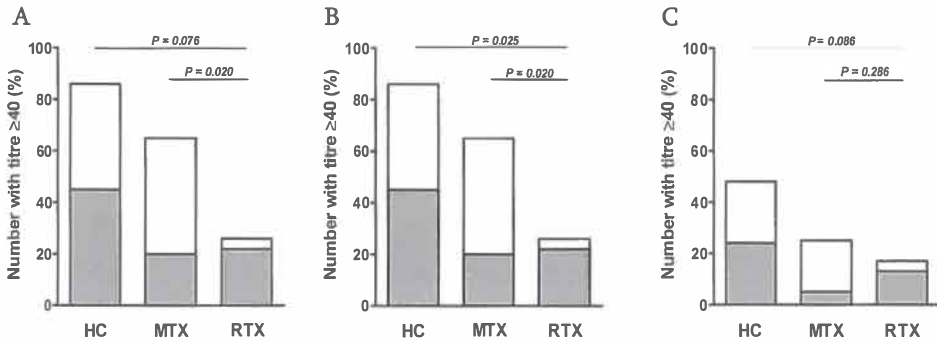

Figure 1. Number of anti-influenza titers ≥ 40. *For influenza A/H3N2 (A), A/H1N1 (B) and B (C) after vaccination with trivalent influenza subunit vaccine, in healthy controls (HC), rheumatoid arthritis (RA) patients treated with methotrexate (MTX), and RA patients treated with rituximab (RTX). Grey bars represent pre-vaccination titer ≥ 40, white bars represent post-vaccination titer ≥ 40 in patients with a pre-vaccination titer < 40 (seroprotection).*

Impact of previous vaccination

HC vaccinated the year before showed higher baseline GMT for A/H3N2 (41.8 (1.8) vs. 13.5 (2.9), *P* = 0.018) compared to previously unvaccinated HC. Conversely, the fold increase in titer following vaccination in the previously vaccinated HC was lower compared to not previously vaccinated HC for A/H3N2 and B (1 (-1.4 - 8) vs. 2.8 (1 - 16), *P* = 0.003; 1.4 (-1.4 - 8) vs. 2.8 (1 - 32), *P* = 0.023). In the MTX group higher baseline GMT in previously vaccinated patients compared to not previously vaccinated patients were shown for the A/H1N1-strain and the B-strain (31.8 (2.1) vs. 9.7 (2.7), *P*= 0.019; 10.4 (2.0) vs. 5.7 (1.6), *P*= 0.015). There was a lower fold increase in previously vaccinated MTX patients for A/H3N2, A/H1N1 and for B than in not previously vaccinated MTX patients (1.4 (1 - 4) vs. 4 (2 - 11.3), *P*= 0.003; 2 (1 - 5.7) vs. 6.7 (1 - 16), *P*= 0.018; and 1 (-1.4 - 1) vs. 3.4 (1 - 16), *P* = 0.001). Patients in the RTX group who were previously vaccinated had higher baseline, but also higher post-vaccination antibody titers against A/H1N1 than the not previously vaccinated RTX patients (19.4 (1.8) vs. 11.3 (2.0), *P*= 0.036, and 30.8 (2.6) vs. 10.7 (2.0), *P*= 0.007).

Seroconversion occurred more often for the A/H3N2-strain in not previously vaccinated MTX patients compared to previously vaccinated MTX patients (50 vs. 0%, *P*= 0.016), but not for HC and RTX patients for any of the influenza strains (data not shown).

Previously unvaccinated HC more often developed seroprotection for the influenza B-strain than previously vaccinated HC (75 vs. 9.5%, *P* = 0.001). Not

previously vaccinated MTX patients developed seroprotection for A/H3N2 and B more frequently (70 vs. 20%, $P = 0.035$; 40 vs. 0%, $P = 0.043$) compared to those vaccinated the year before. However, the number of patients with a post-vaccination titer ≥ 40, irrespective of pre-vaccination titer, did not differ between previously vaccinated and unvaccinated RTX patients (data not shown).

Correlations between B cells and vaccination responses

In the RTX group $CD19^+$ B cells tended to increase 4 weeks after vaccination from 0 (0 - 0.08 x 10^9/l) to 0 (0 - 0.10 x 10^9/l) ($P = 0.058$), due to regeneration of B cells in the late RTX subgroup: their B cells increased following vaccination from 0 (0 - 0.08 x 10^9/l) to 0.01 x 10^9/l (0 - 0.10 x 10^9/l) ($P = 0.031$), in contrast to the early-RTX subgroup (from 0 (0 - 0.01 x 10^9/l) to 0 (0 - 0), $P = 0.317$). However, in both early- and late-RTX subgroup correlations between B-cell count and pre-vaccination GMT, post-vaccination GMT, fold increase in GMT, rates of seroconversion and seroprotection were absent (data not shown).

Safety of vaccination: side effects and RA activity

There were no differences in the occurrence of side effects between the three groups. RA activity, assessed with DAS28 scores prior to, and 7 and 28 days after vaccination, was not influenced by influenza vaccination in both the MTX group (3.04 (0.77 - 5.17) vs. 2.93 (0.49 - 3.71) vs. 2.59 (1.00 - 4.22), $P = 0.287$) and the RTX group (3.95 (2.15 - 5.71) vs. 3.97 (2.15 - 6.26) vs. 4.02 (2.04 - 6.77), $P = 0.834$).

Discussion

The present study clearly shows that humoral responses to influenza subunit vaccine in RA patients receiving RTX are severely hampered as compared to RA patients on MTX and HC. This holds true for almost all outcomes. Our results are in line with those from a study in 4 RA patients evaluating humoral responses following influenza vaccination 84 days after treatment with RTX [6]. A larger study by Oren *et al.* including 14 RA patients on RTX only showed a lower GMT for influenza B and reduced rates of achieving a combined endpoint of seroconversion and seroprotection for influenza A/H3N2 in RTX patients, compared to 29 RA patients on various DMARDs and 21 HC [5]. The discrepancy between Oren's and our results might be explained by larger time span between treatment with RTX and influenza vaccination in Oren's study than in our study (18 months vs.

10 months in our late-RTX group), and only 7 patients received influenza vaccination in the first 6 month after RTX in Oren's study.

The hampered response seems temporary as a significant rise in GMT after influenza vaccination in the late RTX group was found, while no increase in GMT was present in the early-RTX group. Moreover, the only 3 cases of seroconversion in the RTX group occurred in the late-RTX-group, and out of the 6 cases of seroprotection in RTX patients 5 occurred in patients from the late-RTX group.

Although B cells are required for the development of humoral immune responses to neoantigens, and depletion of B cells following RTX would be expected to reduce humoral immune responses to neoantigens, no correlation could be demonstrated between B-cell count and the humoral responses following influenza vaccination in the three groups studied (HC, MTX and RTX). This might be attributed to insufficient sensitivity of the standard quantitative assessment of B cells (lowest measurable B-cell count being 0.01 x 10^9/l) [9]. Responders to influenza vaccination in the late RTX group already probably achieved some level of B-cell repopulation that was undetectable using standard methods. An other explanation could be that the number of B cells in lymphoid tissues, i.e. sites where vaccine-mediated immune responses are initiated, are not correctly reflected by the peripheral blood B-cell numbers: $CD19^+/CD20^-$ B cells have been shown to remain present in the bone marrow after 2 cycles of RTX in RA patients [10].

Yearly repeated influenza vaccination leads to higher pre-vaccination anti-influenza antibody titers during the following year [11] and a reduction in mortality [12]. In the current study we indeed found higher pre-vaccination GMT and lower fold increase in titer in previously vaccinated HC and MTX patients, compared to not previously vaccinated HC and MTX patients. However, previously vaccinated RTX patients had besides a higher pre-vaccination titer for A/H1N1 also a higher post-vaccination titer. Notably, peripheral blood B cells after recovery from RTX-induced B-cell depletion mainly consist of immature and naïve B cells, and low numbers of B cells remain for up to 2 years [13,14]. Our findings may therefore point to the persistence of memory B cells in other compartments than the peripheral blood that are capable of responding to the vaccine, and indicate that repeated yearly vaccination could be of additional value in achieving adequate levels of anti-influenza antibodies following influenza vaccination of RA patients treated with RTX.

Influenza vaccination was safe. Side effects between the study groups were comparable and influenza vaccination did not increase RA activity.

Finally, one should keep in mind that the correlates of protection for influenza following influenza vaccination in immunocompromised patients are not well defined. Anti-influenza titers determined by HI are considered protective when ≥ 40, and 50% of persons with a titer of 28 are estimated to be protected; however, this has only been validated in young healthy adults [8]. Moreover, cellular immune responses have been shown to be also of major importance in vaccination-mediated protection from influenza [15], and are affected by RTX as well. Since even titers < 28 might provide some level of protection, even small increases in anti-influenza titer can be of clinical relevance. Therefore the modest rise in titer in the late-RTX group might be valuable.

Our study has some limitations: (i) although this is the largest study to evaluate the response to influenza vaccination in RA patients treated with RTX, the number of patients is still relatively small. However, the results are uniform and statistical significance was reached for many parameters; (ii) HC were younger than the RA patients, and age is an important factor in influenza vaccination response [2]. Since the age of MTX patients and RTX patients was comparable, and HI titers were significantly higher in MTX patients compared to RTX patients, the difference in HI titers between HC and RTX patients was unlikely to be caused by differences in age; (iii) although the use of additional DMARDs was not standardized, most of the RTX patients using DMARDs used MTX, and only one patient was on high dose corticosteroids. In the MTX group only 2 patients used DMARDs other than MTX. Therefore we do not expect the unrestricted use of DMARDs to have influenced the study outcome. Moreover, the allowance of additional DMARDs offers the possibility to extrapolate our data to daily practice, where use of additional DMARDs is common. The difference in corticosteroid use between the MTX and RTX group will probably not have changed outcome, since even a dosage of prednisone > 7.5 mg/day has been shown not to affect the humoral response following influenza vaccination in RA patients [3,4].

In conclusion, this study shows a severely hampered humoral immune response to trivalent subunit influenza vaccine in RA patients treated with RTX, compared to RA patients on MTX and HC. Six-10 months after RTX treatment this response was slightly restored, but still reduced. Previously vaccinated RTX patients performed better for A/H3N2 and A/H1N1 compared to RTX patients

who were not previously vaccinated. We recommend yearly influenza vaccination for RA patients. For those patients who start RTX treatment pre-emptive influenza vaccination should be considered.

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Chapter 9

Summary, Discussion, Perspectives and Key Findings

Summary

In this thesis, we set out to address safety of and immune responses to influenza vaccination in patients with autoimmune diseases.

In part I, following the general introduction in **chapter 1**, three vaccination studies in patients with SLE are presented. In **chapter 2**, which discusses the results of the first vaccination study, we evaluated antibody responses to and safety of influenza vaccination in patients with quiescent SLE. We formed predefined patient groups according to use of immunosuppressives. Compared to healthy controls, SLE patients had a lower seroconversion rate to each vaccine strain. Also, a lower percentage of SLE patients achieved seroprotection against both A strains together compared to controls. In addition, the use of azathioprine was associated with a further decrease of the antibody response. Importantly, influenza vaccination did not lead to an increase in disease activity, measured as SLEDAI score.

In **chapter 3**, we extended these topics with a review of current data on influenza vaccination in patients with SLE. In general, previous studies indicate that influenza vaccination does not induce disease activity. With regard to immune responses to influenza vaccination, published data are conflicting as some studies reported diminished antibody responses, whereas others reported normal responses in SLE. In general, the antibody response to influenza vaccination appears to be modestly reduced in SLE, and conflicting data may be explained by methodological differences. Furthermore, we discussed topics for future research, such as cell-mediated immune responses and potential immune response-enhancing vaccination strategies.

Cell-mediated responses, though important in influenza, had not been studied in SLE. Therefore, the second vaccination study, discussed in **chapter 4**, focused on cell-mediated responses prior to and following influenza vaccination in a cohort of SLE patients representative for daily practice. We found that cell-mediated recall responses to influenza were lower in SLE patients compared to controls, despite the fact that more SLE patients than controls had received an influenza vaccination in the previous year. Following vaccination, responses remained lower in SLE patients. Furthermore, by flow cytometry, cell-mediated responses to vaccination appeared to be more restricted in SLE patients: for fewer cytokines increases in frequencies of antigen-specific $CD4^+$ T cells were observed. Lower responses appeared to be associated with the use of immunosuppressive medication (prednisone and/or azathioprine).

As a secondary focus, we extended our analysis of vaccine safety by including randomization between a patient group receiving vaccination versus a non-vaccination group and by prolonging the follow-up to 3-4 months. During follow-up, disease activity did not change from disease activity prior to vaccination within the vaccinated group, and there were no differences in disease activity between vaccinated patients and non-vaccinated patients at any point during follow-up.

As we had found a diminished antibody response in SLE patients, we performed a third vaccination study to evaluate the effect of a second, booster, influenza vaccination as a potential strategy to enhance immune responses (**chapter 5**). However, a booster vaccination was of limited additional value. Only in patients not vaccinated in the previous year, the response to A/H1N1 increased following the booster vaccination. Immune responses in healthy controls were lower than expected, and no differences in antibody response between patients and controls were found in this study.

In part II, we studied influenza vaccination in two other systemic autoimmune diseases, WG and RA. In WG this had not been studied previously. In **chapter 6** antibody responses to and safety of influenza vaccination in WG patients are presented. WG patients showed similar antibody responses as healthy controls. Immunosuppressive medication, most frequently prednisone (median dose in users 5 mg/day) and azathioprine (median dose in users 100 mg/day), did not appear to influence antibody responses in the doses used in this study. Influenza vaccination did not lead to a change in disease activity in vaccinated WG patients, nor did disease activity differ between vaccinated WG patients and non-vaccinated WG patients during follow-up. As part of this vaccination study, also cell-mediated responses to influenza vaccination were analyzed in a subset of WG patients and controls (**chapter 7**). Both recall responses and responses to vaccination were similar in WG patients and controls.

Finally, in **chapter 8**, we studied effects of B-cell depleting therapy (using the anti-CD20 drug rituximab) upon antibody responses to influenza vaccination in RA patients. Here, it was shown that, as expected, antibody responses are abrogated by B-cell depletion. Interestingly, in RA patients 6-10 months after rituximab treatment, antibody responses began to return. Also in RA patients, influenza vaccination did not influence disease activity.

Discussion

How do our studies relate to each other? And how could they be interpreted, in the context of other studies? We discuss these issues in two themes: influenza vaccination and disease activity, and immune responses to influenza vaccination. Immune responses to influenza vaccination are discussed with respect to antibody responses, cell-mediated responses, influence of immunosuppressives and responses to other vaccinations.

Influenza vaccination does not appear to induce disease activity in systemic autoimmune disease

In SLE, a considerable number of studies have addressed safety and immunogenicity of influenza vaccination; an overview is presented in **Table 1**. Following a publication in 1948 about the onset of fatal SLE in three nurses after multiple vaccinations with multiple vaccines (typhoid-paratyphoid, scarlet-fever streptococcus toxin and toxin-antitoxin diphtheria) [1] vaccinations were not used in SLE patients. In 1976 the United States' national influenza immunization program encouraged research groups to evaluate the safety and immunogenicity of influenza vaccination in SLE patients. A total of 125 SLE patients were vaccinated, in separate studies by five research groups. Flares were studied; no validated disease activity index was available. In all studies vaccination was well tolerated, and during follow-up serious flares occurred in 4 out of 125 vaccinated patients and in 1 of 21 control patients. No significant change in complement and autoantibody levels occurred after vaccination. Later studies applied the SLEDAI to assess disease activity. Here, in six separate studies, a total of 218 patients received influenza vaccination, of whom 162 were vaccinated in our own studies. In three studies [2,3,chapter 4], also 24, 14 and 24 non-vaccinated patients were included, respectively. Again, no detrimental effect of influenza vaccination upon disease activity was demonstrated. In our studies, we did not observe major flares (SLEDAI > 12 points [4]) and no increase in number of minor exacerbations (change in SLEDAI > 3 points [4]) following vaccination. Most studies included both patients with inactive disease and patients with active disease [2, 3, 5-10,chapter 4]; we vaccinated 6 active patients. A few studies included only patients with quiescent disease [11,chapter 2 and 5], and three studies did not state whether patients were active or inactive [12-14]. Therefore, there is now a large body of evidence supporting the safety of influenza vaccination in patients with quiescent lupus. In active lupus, influenza vaccination does not appear to aggravate disease activity; however, numbers are too small to draw final conclusions.

Table 1. Safety and immunogenicity of influenza vaccination in SLE patients

Study (chronological order)	Subjects	Follow-up (wks)	Safety	Antibody responses	Influence of drugs
Brodman *et al* [5]	46 SLE; 58 HC	8	No major flare-ups	Trend towards decrease	No significant effect of prednisone, azathioprine, or hydroxychloroquine
Louie *et al.* [12]	11 SLE; 8 HC	12	1 diffuse proliferative GN	Similar	-
Ristow *et al.* [13]	29; 29 HC	8	1 focal GN	Trend towards decrease	No significant effect
Williams *et al.* [6]	19 SLE; 21 SLE controls; 18 HC	20	1 flare in each group	Decrease	Prednisone: trend towards lower responses
Herron *et al.* [14]	20 SLE; 32 HC	16	1 major flare, 3 flares	Similar	Prednisone: trend towards lower responses
Turner-Stokes *et al.* [7]	28 SLE; 35 HC	4	No flares	Trend towards decrease	No significant effect
Kanakoudi-Tsakalidou *et al.* [11]	(children) 11 SLE; 5 HC	24	No flares	Similar	No significant effect
Abu-Shakra *et al.* [2,8,9]	24 SLE; 24 SLE controls	12	Decrease of mean SLEDAI in both SLE groups	Decrease	Prednisone ≥ 10 mg/ day or azathioprine: trend towards lower responses
Mercado *et al.* [10]	18 SLE; 18 HC (*HC not vaccinated*)	8	Decrease of mean SLEDAI	NC	No significant effect
Stojanovich [22]	23 SLE; 46 SLE controls	52	No worsening of disease activity in vac. SLE	ND	ND
Holvast *et al.* (chapter 2)	56 SLE; 18 HC	4	No increased SLEDAI	Decrease	Azathioprine: lower responses
Del Porto *et al.* [3]	14 SLE; 14 SLE controls; 10 HC	26	2 flares in vac. and 1 in non-vac. patients; no increased SLEDAI	Quite similar	
Holvast *et al.* (chapter 4)	54 SLE; 24 SLE controls; 54 HC	12-16	No increased SLEDAI	Decrease	No significant effect
Holvast *et al.* (chapter 5)	52 SLE; 28 HC	8	No increased SLEDAI	Similar	No significant effect

SLE: systemic lupus erythematosus, HC: healthy control, GN: glomerulonephritis, ND: not determined, NC: no comparison of antibody responses to those (expected) in HC. Partly adapted from Conti et al [30]*, with permission.*

In WG, there was hardly any information regarding safety and efficacy of vaccinations. A retrospective study by Stassen and colleagues, also done in the Groningen WG cohort, showed that influenza vaccination did not increase the number of relapses. On the contrary, influenza vaccination was associated with fewer relapses [15]. It may be hypothesized that influenza infection may induce relapses, and that, therefore, influenza vaccination reduces the number of relapses. The first prospective studies, from Zycinska and co-workers and from our group, did not show an increase in disease activity following vaccination, nor differences in disease activity compared to non-vaccinated patients. In both studies, patients in remission were studied [16,chapter 6]. However, as both studies were not powered to assess the safety of vaccination, this issue can not be answered conclusively.

In RA, a number of studies have been performed in which possible influences of influenza vaccination on disease activity were assessed (**Table 2**). In accordance with the findings of Stassen *et al.* in WG [15], in a retrospective analysis, RA patients who received influenza vaccination had fewer disease exacerbations as compared with non-vaccinated RA patients. During a period of 6 months, exacerbations occurred in 1 of 17 vaccinated patients and 7 of 19 non-vaccinated patients; 5 out of 7 non-vaccinated patients reported a flu-like illness in the month prior to exacerbation of the disease [17]. Prospective studies showed that influenza vaccination did not induce increased disease activity [3,7,14,17-25,chapter 8]. In some studies, a control group of non-vaccinated RA patients was included; no differences in the occurrence of disease flares were observed between vaccinated and non-vaccinated RA patients. In other studies, disease activity in vaccinated RA patients following influenza vaccination was compared to disease activity prior to vaccination. Again, no change in disease activity following influenza vaccination was shown. With regard to the inclusion of patients with inactive or active disease, several studies did not mention disease activity of included RA patients [7,14,17,19,22,23,26,27]. Other studies included both active and inactive RA patients [18,20,21,24,25,28,chapter 8]. One study included only patients with low disease activity (DAS28 < 4) [3], one study reported disease activity to be high in RA patients at time of vaccination [29]. Also in active disease, influenza vaccination was well tolerated, and did not aggravate disease activity. Therefore, influenza vaccination in RA patients can be considered safe, and this appears to apply to both active and inactive disease.

Antibody responses to influenza vaccination are somewhat reduced in SLE, but not in WG and RA

With regard to antibody responses to influenza vaccination in **SLE**, results have been somewhat controversial. From the five initial studies, three reported a (trend towards) decreased antibody response to influenza vaccination in SLE [5,6,13], whereas two reported similar responses in SLE as compared with healthy controls [12,14]. Also in later studies, some reports mentioned a (slightly) reduced antibody response in SLE [7,8,chapter 2 and 4], others reported responses comparable to those in healthy controls [3, 11,chapter 5]. We, too, had conflicting results as in two of our own studies we did find a lower antibody response in SLE, whereas we could not confirm this in our latest study. How to explain these apparently controversial results? Several factors may be involved. First, differences in the number of patients and controls included, resulting in differences in power. Second, variations in immunogenicity of vaccine strains. Third, the variable degree of previous influenza vaccinations among participants may have resulted in different responses. Fourth, as the use of immunosuppressive drugs was associated with reduced antibody responses in several studies, differences between studies with regard to drug use may have influenced results. Taken together, it appears that differences in antibody responses between SLE patients and healthy controls are only modest. Furthermore, we report in chapter 4 that SLE patients and controls showed a similar course of antibody titers over a period of 3-4 months following vaccination. We did assess whether we could enhance antibody responses in SLE patients by administering a second, booster, vaccination. However, this was of limited additional value, as titers increased only for A/H1N1, and only in patients not vaccinated the previous year [chapter 5].

In contrast to SLE, no studies were available in **WG** regarding antibody responses to influenza vaccination, or to any other vaccination. Almost concurrently, both Zycinska and we reported that antibody responses are comparable to those in healthy controls [16,chapter 6].

In **RA**, we found that patients on methotrexate and healthy controls had similar antibody responses to influenza vaccination. This is in accordance with previous reports, as generally, RA patients showed antibody responses to influenza vaccination which are close to those in healthy controls [30]. However, there is controversy whether some immunosuppressives might influence antibody responses; this is discussed below.

Table 2. Safety and immunogenicity of influenza vaccination in RA patients

Study (chronological order)	Subjects	FU (wk)	Safety	Antibody responses	Influence of drugs
Herron *et al.* [14]	17 RA; 32 HC	16	6 flares (similar to expected spontaneous rate)	Quite similar	Prednisone: trend for lower responses
Turner-Stokes *et al.* [7]	10 RA; 35 HC	4	No flares	Quite similar	No association.
Chalmers *et al.* [18]	65 RA; 61 RA controls; 64 HC	4	2 flares in vac. group, 3 in placebo group	Similar	No significant effect
Cimmino *et al.* [19]	30 RA	4	6 flares (similar to expected spontaneous rate)	ND	ND
Francioni *et al.* [25]	40 RA; 40 HC	4	No change in clinical picture	Similar	ND
Caporali *et al.* [17]	17 RA; 19 RA controls	26	1 flare in vac. RA, 7 in non-vac RA ($p < 0.05$)	ND	ND
Fomin *et al.* [20]	82 RA; 30 HC	6	No change in mean disease activity	Slightly reduced	No effect of prednisone, MTX, anti-TNF, hydroxychloroquine
Del Porto *et al.* [3]	10 RA; 10 RA controls; 10 HC	26	2 flares in vac. RA, 3 in non-vac RA; Mean DAS28 did not increase.	Quite similar	No significant effect
Stojanovich [22]	23 RA; 31 RA controls;	52	No worsening of disease activity in vac. RA	ND	ND
Kaine *et al.* [28]	208 RA (99 on anti-TNF)	4	ND	NC	MTX: trend for lower responses Anti-TNF: no effect
Kubota *et al.* [26]	27 (anti-TNFα) RA; 36 RA controls; 52 HC	4-6	ND	Similar	Anti-TNF: no effect
Kapetanovic *et al.* [27]	149 RA; 18 HC	4-6	ND	NC	Anti-TNF: lower response than for MTX
Gelinck *et al.* [29]	4 RA on anti-CD20; 19 RA on anti-TNF; 20 HC	4	ND	Quite similar in RA on anti-TNF	Anti-CD20: lower responses
Oren *et al.* [21]	14 RA on anti-CD20; 29 RA on DMARDs; 21 HC	4	No change in disease activity in both RA groups	Quite similar in RA on DMARDs	Anti-CD20: lower responses
Gelinck *et al.* [23]	64 anti-TNF (52 RA); 48 not on anti-TNF (27 RA); 18 HC	4	No deterioration of underlying disease	Similar	Anti-TNF lowers GMTs, but not protection rates
Elkayam *et al.* [24]	20 RA on anti-TNF; 23 RA controls; 17 HC	4-6	No change in change in disease activity	Similar	Anti-TNF: no effect
Van Assen *et al.* (chapter 8)	23 RA on anti-CD20; 20 RA on MTX and 29 HC	4	No change in disease activity	Similar in RA on MTX	Anti-CD20: no responses up to 6-10 months after anti-CD20

RA: rheumatoid arthritis, HC: healthy control, FU (wk): follow-up (weeks), ND: not determined, NC: no comparison of antibody responses to those (expected) in HC. Partly adapted from Conti et al [30], with permission.

Cell-mediated responses to influenza vaccination are reduced in SLE, normal in WG and unknown in RA

Cell-mediated responses have been shown to be important in the response to influenza, and may constitute (at least in certain patient categories) independent correlates of protection from influenza infection [31,32]. We determined cell-mediated responses by IFN-γ ELISpot and by intracellular cytokine-staining for IFN-γ, TNF and IL-2. Both assays determine functional responses. ELISpot is a highly sensitive assay [33], and by flow cytometry we were able to phenotype responding cells and to gain more information on the spectrum of the cytokine response. Other approaches for the evaluation of cell-mediated responses are discussed in the **Perspectives** section.

In SLE patients, cell-mediated responses against influenza were lower compared with controls, prior to influenza vaccination and following influenza vaccination. In WG, cell-mediated responses to influenza vaccination were comparable to those in healthy controls. In RA, to our knowledge, cell-mediated responses to influenza vaccination have not been studied. In general, little is known about cell-mediated responses to vaccination in systemic autoimmune diseases. The only report is a study from 1979 by Pons *et al.*, in which cell-mediated cytotoxicity was lower in SLE patients prior to and following vaccination with inactivated influenza vaccine as compared to healthy controls [34]. However, we did not observe $CD8^+$ T-cell responses to vaccination, as was expected when a subunit vaccine is used (subunit vaccine antigens are presented via MHC II [35, 36])

Cell-mediated responses appear to be affected in a quite general manner in SLE. Proliferative capacity is reported to be lower [37-39], and T-helper (Th) recall responses to influenza A and tetanus toxoid antigens have been reported to be decreased in a subset of patients, as measured by IL-2 production upon stimulation. This decreased function could not be accounted for by the use of immunosuppressives alone, and was shown to be associated with disease activity [40]. Other studies, too, found reduced cell-mediated responses during active disease [41-43]. We, however, did not find any correlation between SLEDAI scores and cell-mediated responses to influenza prior to influenza vaccination, nor between SLEDAI scores and responses after vaccination (data not shown), though this may be due to the low number of patients with active disease.

We also examined correlations between cell-mediated responses, especially $CD4^+$ T-cell responses, and antibody responses, as the generation of antibody responses to influenza vaccination requires T-cell help, offered by $CD4^+$ T-helper

cells. In our study on SLE patients, we did observe a correlation between changes in the number of IFN-γ-producing spot-forming cells upon vaccination and seroconversion rate against A/H1N1. As a subunit vaccine is expected to induce mainly a $CD4^+$ T-cell response, this indicates a relation between $CD4^+$ T-cell responses and antibody responses. However, this was not confirmed in our study on WG patients. In another study, no correlation between cell-mediated responses and antibody responses was found as well [44], indicating that cell-mediated responses are an independent measure of response to influenza vaccination.

Immunosuppressives may limit immune responses

In SLE, there have been reports that the use of azathioprine or prednisone may be associated with reduced antibody responses to influenza vaccination [6,8,14]. We, too, found that use of azathioprine was associated with a decreased antibody response [chapter 2 and 5]. Also for cell-mediated responses, we found such an effect [chapter 4].

In contrast to our observations in SLE patients, we did not find influences of immunosuppressive drugs on the response to influenza vaccination in WG. Predominantly azathioprine and prednisone were used in WG patients. Numbers of patients may have been too small to detect such influences.

In RA, rituximab has been shown to severely hamper antibody responses to influenza vaccination [21,29], as was confirmed by our own study [chapter 8]. Our results also indicate that in patients 6-10 months after rituximab treatment, the antibody response to influenza vaccination starts to restore. This may be helpful for the timing of influenza vaccination in (RA) patients treated with rituximab. With regard to influences of other immunosuppressives, tendencies to lower responses have been described for anti-TNF, methotrexate and prednisone, but these effects are controversial. For anti-TNF preparations, reports of a lower antibody response with anti-TNF treatment [23,27], and reports of a normal antibody response have been published [20,24,26,28]. For both prednisone [14] and methotrexate [28] a trend towards lower antibody responses has been reported, but this was not found in other studies [18,20,27]. Abatacept, a T-cell costimulation inhibitor, could hamper immune responses to influenza vaccination, but this has not yet been studied.

Our data on influenza vaccination are in accordance with data on other vaccinations in SLE and RA

How do our findings on influenza vaccination relate to current reports on other vaccinations in patients with established systemic autoimmune disease? With regard to safety, other vaccines do not appear to induce disease activity either. In

SLE, this has been studied for pneumococcal vaccination, haemophilus influenzae type B vaccination, tetanus toxoid vaccination, and hepatitis B vaccination [45,46]. In one study, pneumococcal vaccine, haemophilis influenzae type B vaccine and tetanus toxoid were administered simultaneously; still, disease activity was not affected [47]. Only for the polio vaccine, a relation with SLE flares has been suggested based on a retrospective analysis [48]. Other vaccines have not been studied in SLE. For WG, there have been no trials on safety and efficacy of vaccinations except for influenza. In RA, pneumococcal vaccination and hepatitis B vaccination did not induce disease activity [49,50], other vaccines have not been studied.

With regard to immunogenicity, in SLE, results with other vaccines such as pneumococcal, haemphilus influenzae B, tetanus toxoid and hepatitis B vaccines show that the majority of patients respond and reach protective titers, but a significant minority does not [46]. With influenza, we reported that the slope of decrease of antibody titers in SLE patients and controls over a period of 3-4 months following vaccination was similar. This has also been found with pneumococcal vaccination [51]. In RA, in contrast to influenza vaccination, responses upon vaccination against hepatitis B and pneumococci might be modestly hampered, though controversy exists. One controlled study suggested that the percentage of responders to pneumococcal vaccination may be lower in RA as compared to healthy controls [52], whereas another controlled study found similar responses [53]. Similarly, one uncontrolled study reported lower than expected responses to pneumococcal vaccination [54], while two other uncontrolled studies state that vaccination resulted in good responses [28,55]. For hepatitis B vaccination, in a small and uncontrolled study, responses were lower in RA than would have been expected for healthy adults [49]. With pneumococcal vaccination, methotrexate was found to lower responses [28,53,55], as has also been reported with influenza vaccination [28].

Influenza vaccination is safe in SLE, WG and RA, but there are differences regarding immune responses

Findings regarding safety of (influenza) vaccination in established systemic autoimmune diseases are similar: no prospective study has shown that vaccination may induce disease activity [56]. However, with regard to immunogenicity, our findings, and those of others, are heterogeneous. It appears that influenza vaccination results in (modestly) reduced antibody responses in SLE, at least in part of the patients, whereas influenza vaccination in WG and RA (except for

rituximab treated patients) resulted in antibody responses similar to those in healthy controls. Also cell-mediated responses were reduced in SLE, but not in WG. How to explain lower responses to influenza vaccination in SLE, but not in WG and RA?

Both intrinsic factors and immunosuppressive drugs can be incriminated. In SLE, intrinsic immune disturbances could be involved such as diminished function of antigen-presenting cells [57] or dysfunction of T cells to generate clonal expansions, or to produce cytokines upon stimulation [37-39]. In this thesis, azathioprine was found to be associated with lower antibody responses (chapter 2 and 5), and in chapter 4 we reported that the use of prednisone and/or azathioprine was associated with lower cell-mediated responses to influenza vaccination. However, in WG and RA, such effects were not observed, despite the use of comparable amounts of immunosuppressives. Third, other factors, like degree of influenza vaccination in the preceding seasons could have influenced results to some extent, but differences regarding previous influenza vaccinations between patients and controls were similar in studies on SLE and WG.

General limitations to the studies

When assessing safety by evaluating changes in disease activity following influenza vaccination on group level, it is questionable whether one should study effects on mean disease activity or on the occurrence of flares. With regard to the number of flares following influenza vaccination, all studies in SLE, WG and RA have been underpowered. It would take a large cohort of vaccinated versus non-vaccinated patients to demonstrate that influenza vaccination leads to an increase in flares. Nevertheless, the combined data of studies on this issue strongly indicate that it is unlikely that influenza vaccination induces disease flares.

More SLE and WG patients than controls had previously received an influenza vaccination, which hampered interpretation of antibody responses to influenza vaccination. Previous influenza vaccinations may influence antibody responses to subsequent influenza vaccinations, as was discussed in chapters 5 and 6. Most notable effects are increased pre-vaccination titers and lower seroconversion rates [58]. In addition, also previous influenza infections influence responses to subsequent influenza vaccination. However, these infections can not be assessed reliably. It would be preferable if future studies would match patients and controls with regard to their vaccination status.

In measuring cell-mediated responses to influenza vaccination, measuring response to vaccination after 28 days may be suboptimal. The kinetics of T-cell

responses are such, that the largest proliferation takes place in the first 10 days following antigen exposure [59]. However, antibody responses should be evaluated three to four weeks following vaccination [60]. Logistically, we were unable to organize analyses in patients after 10 and 28 days. We chose to do T-cell analyses after 28 days, as preliminary experiments showed that cell-mediated responses to vaccination responses were detectable at that time point, and as criteria for evaluation of antibody responses have been standardized, in contrast to cell-mediated responses.

Perspectives

With regard to future perspectives, several questions remain regarding clinical application of influenza vaccination in patients with systemic autoimmune disease, and several alternative approaches could be used to study immune responses.

Should patients with active disease receive influenza vaccination?

Influenza vaccination appears to be safe in patients with systemic autoimmune diseases, both in quiescent and active disease. However, immunogenicity of influenza vaccine in these patients might be a topic for future research. Both disease activity itself, as well as the use of immunosuppressives may limit immune responses in patients with active disease. In SLE, there is a tendency that immunogenicity is lower in patients with active disease, though this could also be related to concomitant use of immunosuppressives [13,47]. It will be difficult to form a study group with active disease that is large enough, as influenza vaccination is seasonal and prolonged inclusion is therefore not possible. A multi-center trial might be the appropriate way to address this issue.

Does influenza vaccination prevent influenza in systemic autoimmune disease?

Thus far, studies have not addressed the clinical burden (incidence, morbidity, mortality) of influenza in systemic autoimmune disease, nor the clinical efficacy of influenza vaccination. Does influenza vaccination lower the rate of laboratory-confirmed influenza, and is this clinical efficacy similar to that in healthy controls? In one study, it was reported that influenza vaccination lowers the rate of respiratory infections in patients with SLE and RA [22]. However, this was not a

randomized study, and influenza infection was not laboratory-confirmed. As there are many influenza-like diseases, it is necessary to study laboratory-confirmed influenza cases. In another small study, 14 SLE patients, 10 RA patients and 10 healthy controls were vaccinated, and tested for presence of influenza in case of symptoms suggestive for influenza. However, the study was heavily underpowered as only one case of influenza was detected. Furthermore, non-vaccinated patients and controls were not studied [3]. Notably, no study has reliably evaluated whether vaccination provides protection from infection for any vaccine in patients with a systemic autoimmune disease. As influenza has a high incidence, influenza vaccination seems the best candidate for such a study.

Alternative approaches to evaluate cell-mediated responses

Especially in SLE, in which cell-mediated responses to influenza vaccination were diminished, further aspects of cell-mediated responses are of interest. Other approaches for the evaluation of cell-mediated immunity offer different sets of information. A distinction may be made between techniques assessing the number of antigen-specific cells, and techniques assessing their functional capacity. In our studies, we evaluated functional capacity. To quantify antigen-specific cells, tetramer-based staining of lymphocytes is the most accurate technique, but this is a MHC-restricted approach. Shifting to functionality, factors which are of importance, and have not been studied thus far, are proliferative capacity, important to yield robust responses in vivo, and cytotoxic capacity, as an endpoint of functional cascades. For proliferation assays, carboxyfluorescein succinimidyl ester (CFSE) staining could be applied to quantify responses by flow cytometry, as an alternative to radioactive methods [61]. Cytotoxicity could be studied using granzyme B release assays, as a nonradioactive alternative to 51Cr-release assays using labeled influenza-infected cells [62]. Also of interest with regard to functional capacity are multifunctional T cells. These are T cells which respond to antigen-specific stimulation by secreting multiple cytokines. They have been shown to produce larger amounts of particular cytokines compared to single cytokine-producing cells, and are associated with higher degrees of protection from clinical infection [61]. The amount of cytokines produced may be measured using the mean fluorescence intensity for the applied fluorochrome in flow cytometry. To use this approach, a flow cytometric assay needs to be used which is standardized and validated on site [63].

Influenza vaccine development

Next to conventional split-virus and subunit influenza vaccines, new vaccine formulations have been developed and tested. For instance, MF-59 adjuvanted vaccines. These vaccine formulations have been shown to have a higher immunogenicity as compared to conventional influenza vaccine [64,65]. This type of vaccines is thought to yield additional value in certain risk groups, such as the elderly, in which the burden of influenza is higher and the response to influenza vaccination diminished. Whether these vaccines should be studied in patients with systemic autoimmune disease as well, is yet unknown. This does not appear to be indicated, as in this thesis we discussed that, in contrast to the elderly, a large majority of patients with systemic autoimmune diseases responds to conventional influenza vaccination. However, clinical efficacy of conventional influenza vaccination should be confirmed.

Influenza vaccination is recommended in systemic autoimmune diseases, implementation in clinical practice should be encouraged

Influenza has a high incidence, and can have considerable morbidity and mortality in risk groups. Patients with systemic autoimmune diseases are at increased risk of infection; thus influenza vaccination seems indicated. Influenza vaccination in systemic autoimmune disease does not appear to induce disease activity, and the majority of patients respond to vaccination. Therefore they should be offered annual influenza vaccination. Though this recommendation is made, implementation in clinical practice is lacking. Probably, this is due to the absence of guidelines concerning vaccinations in patients with systemic autoimmune diseases. Such a guideline should be achieved, and is currently being made by the European League Against Rheumatism. We hope that our studies may contribute to the establishment of these guidelines.

Key Findings

- Influenza vaccination in systemic autoimmune disease (SLE, WG, RA) appeared safe, as no increases in disease activity following vaccination were observed.
- Influenza vaccination in WG and RA resulted in antibody responses which were comparable to those in healthy controls; in SLE, the antibody response appeared to be modestly diminished.
- In SLE, the use of azathioprine was associated with lower antibody responses. No clear influence of prednisone and hydroxychloroquine upon antibody responses was found. For other immunosuppressive drugs, numbers were too small to make any conclusions. In WG and RA, conventional immunosuppressive treatment was not associated with diminished antibody responses. However, anti-CD20 (rituximab), as studied in RA patients, completely abrogated antibody responses to influenza vaccination in the first two months following RTX; in patients 6-10 months after RTX, there was a moderate recurrence of these antibody responses.
- Cell-mediated responses to influenza vaccination were lower in SLE as compared to healthy controls, this was associated with the use of prednisone and/ or azathioprine. In WG, cell-mediated responses to influenza vaccination were comparable to those in healthy controls.
- A second, booster, influenza vaccination had limited additional value in SLE patients not vaccinated in the previous year.

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Nederlandse Samenvatting

(voor niet-ingewijden)

Inleiding

Systemische auto-immuunziekten en griepvaccinatie

Er bestaan verschillende vormen van auto-immuunziekten, aandoeningen waarbij de afweer lichaamseigen weefsels of cellen aanvalt. Er zijn auto-immuunziekten waarbij één orgaan of weefseltype wordt aangevallen, orgaanspecifieke auto-immuunziekten, en aandoeningen waarbij meerdere organen aangevallen worden, systemische auto-immuunziekten. Om patiënten met een systemische auto-immuunziekte te behandelen, worden vaak afweeronderdrukkende medicijnen gebruikt. Zowel door de aandoening, als door afweeronderdrukkende medicijnen zijn patiënten met een systemische auto-immuunziekte meer vatbaar voor infecties dan gezonde volwassenen. Griep (influenza) is een veelvoorkomende infectie. Bij gezonde volwassenen verloopt griep meestal mild, maar bij patiënten met een verlaagde afweer is in geval van griep het risico op complicaties (bijvoorbeeld longontsteking) en sterfte verhoogd. Bij dergelijke risicogroepen wordt een jaarlijkse griepvaccinatie aanbevolen. Het lijkt dus zinnig om ook patiënten met een systemische auto-immuunziekte de griepvaccinatie aan te bieden, als onderdeel van goede patiëntenzorg. Waarom dan toch een studie hiernaar?

Er zijn twee hoofdredenen om griepvaccinatie bij deze patiëntengroepen te onderzoeken: veiligheid en werkzaamheid. Veiligheid van vaccinatie is punt van discussie bij auto-immuunziekten, omdat de mogelijkheid bestaat dat vaccinaties kunnen leiden tot het ontstaan van auto-immuunziekten, of tot verergering van activiteit van reeds bestaande auto-immuunziekten. Werkzaamheid van vaccinatie staat ook ter discussie, omdat de opgewekte bescherming door vaccinatie bij patiënten met een systemische auto-immuunziekte verlaagd zou kunnen zijn, zowel door de ziekte zelf, gekenmerkt immers door ontregeling van de afweer, als door gebruikte afweeronderdrukkende medicijnen.

Verschillende systemische auto-immuunziekten

Systemische auto-immuunziekten zijn een heterogene groep aandoeningen. Hun ontstaanswijze is niet volledig opgehelderd; er is sprake van een samenspel van erfelijke factoren en omgevingsfactoren. Ze verschillen in ontstaanswijze, symptomen, behandeling en prognose. Ook binnen een bepaalde systemische auto-immuunziekte bestaan verschillen in uiting en verloop van de ziekte. Wij hebben ons onderzoek gericht op drie systemische auto-immuunziekten: systemische lupus erythematodes, de ziekte van Wegener en reumatoïde artritis.

Systemische lupus erythematodes (SLE) komt voor bij 1 op de 2500 mensen en treft met name vrouwen. Vaak openbaart de aandoening zich bij jonge vrouwen tussen de late tienerjaren en begin veertig. Meerdere organen kunnen aangedaan zijn bij SLE; vaak is er een combinatie van symptomen van de huid, het spier- en skeletstelsel, milde afwijkingen in het bloedbeeld en meer algemene klachten zoals vermoeidheid. Nierbetrokkenheid komt ook relatief vaak voor en is een ernstigere uiting van de ziekte. Er is ontregeling van de afweer bij SLE, waardoor veel verschillende lichaamseigen structuren aangevallen kunnen worden. Het verloop van SLE wordt gekenmerkt door wisselende ziekteactiviteit, met periodes van rustige/ afwezige ziekte en opvlammingen van de ziekte. Een breed scala aan afweeronderdrukkende medicijnen wordt gebruikt om ziekteactiviteit te onderdrukken.

De **ziekte van Wegener (WG)** komt in Europa voor bij 1 op de 16.500 mensen en treft iets meer mannen dan vrouwen. WG patiënten zijn meestal ouder dan SLE patiënten, bij diagnose is hun leeftijd gemiddeld 50 jaar. Bij WG worden kleine en middelgrote vaten aangevallen, dit leidt tot ontstekingshaarden (met name in de luchtwegen), vaatontsteking, die zich op allerlei plaatsen kan voordoen, en nierontsteking. Deze ontstekingen ontstaan dus niet door infecties, maar door auto-immuunprocessen. Bij een klein deel van WG patiënten beperkt de ziekte zich tot de luchtwegen. Net als bij SLE, worden verschillende afweeronderdrukkende medicijnen gebruikt om de ziekteactiviteit te onderdrukken. Echter, opvlammingen van de ziekte komen voor.

Reumatoïde artritis (RA) komt veel voor, namelijk bij 1 op de 100 mensen; vrouwen hebben vaker RA dan mannen. RA kan op iedere leeftijd ontstaan, maar het aantal mensen bij wie de ziekte vastgesteld wordt, stijgt met de leeftijd - de grootste groep is tussen de 40 en 70 jaar. Symptomen van RA zijn vooral gerelateerd aan de kenmerkende gewrichtsontstekingen: gewrichtspijn, -stijfheid en -zwelling. Ook bij RA is er een grote variatie in het verloop van de ziekte. De behandeling met afweeronderdrukkende medicijnen is de laatste jaren sterk verbeterd, door het beschikbaar komen van middelen die specifiek op ontstekingsprocessen kunnen ingrijpen. Daarnaast is het mogelijk geworden om bij persisterende ziekteactiviteit een medicijn te gebruiken dat bepaalde afweercellen uitschakelt: rituximab. De invloed van dit middel op griepvaccinatie is onderwerp van ons onderzoek geweest.

Griep

Griep is een virusinfectie; een virus dringt cellen binnen want het heeft een gastheercel nodig om zich te kunnen voortplanten. Griep komt veel voor, ieder jaar wordt ongeveer 5% van de volwassen bevolking ziek door griep. Het griepvirus wordt verspreid via hoesten en infecteert via de luchtwegen. In landen met een gematigd klimaat, zoals Nederland, komt de griep met name voor in het late najaar en de winter. Er zijn meerdere varianten van het griepvirus die mensen kunnen infecteren. Op het moment dat iemand met een griepvirus geïnfecteerd wordt, bouwt deze persoon afweer op tegen dat bepaalde griepvirus. Deze *specifieke* afweerreactie wordt onthouden binnen het afweersysteem, en dit kan bij toekomstige blootstelling aan dezelfde virusstam bescherming tegen infectie bieden. Echter, kenmerkend voor griepvirussen is dat ze continu een klein beetje veranderen, hierdoor ontstaan varianten die nog niet eerder gezien zijn door het afweersysteem en waarvoor dus nog geen afweerreactie opgeslagen is. Dit heeft tot gevolg dat mensen meerdere keren in hun leven de griep kunnen krijgen, dat er een jaarlijkse griepgolf bestaat en dat de samenstelling van het griepvaccin jaarlijks aangepast moet worden.

De symptomen van griep ontstaan één à twee dagen na infectie met een griepvirus. Klassiek is er sprake van luchtwegklachten (hoesten, keelpijn) met plotselinge koorts, hoofdpijn, spierpijn en algehele malaise. Echter, griep houdt zich lang niet altijd aan dit klassieke beeld. Meestal verloopt griep ongecompliceerd en treedt geleidelijk herstel op na twee tot vijf dagen. Soms ontstaan complicaties, waarvan een longontsteking de belangrijkste is. Bij kwetsbare patiënten kan griep het overlijden aan onderliggend lijden, zoals hart- en vaatziekten, verhogen.

Er zijn een aantal virussen, verkoudheidsvirussen, die griepachtige ziektebeelden kunnen geven. Wanneer iemand griepachtige klachten heeft kan dus zonder verdere diagnostiek niet met zekerheid gezegd worden of er sprake is van een infectie door het griepvirus of door een ander virus dat soortgelijke klachten geeft.

De afweerrespons tegen griep

Het afweersysteem bestaat uit vele componenten, vele hiervan zijn ook bij de afweer tegen griep betrokken. Een griepvaccinatie beoogt om bepaalde onderdelen van de afweer tegen griep te stimuleren en op deze manier bescherming te bieden tegen daadwerkelijke griepinfectie. Een groot deel van dit proefschrift gaat over reacties (responsen) van het afweersysteem op griepvaccinatie.

Het afweersysteem bestaat uit een aspecifieke, aangeboren afweer en een specifieke, aangeleerde afweer. Beide zijn betrokken bij de afweer tegen griep. Het kenmerkende van de specifieke, aangeleerde afweer is dat er een 'geheugen' bestaat voor ziekteverwekkers waarmee iemand eerder in aanraking is geweest. Een griepvaccinatie heeft als doel om dit geheugen op te wekken. Vandaar dat ons onderzoek zich gericht heeft op het ontstaan van specifieke, aangeleerde afweerresponsen na griepvaccinatie. Binnen dit deel van het afweersysteem bestaan twee hoofdcategorieën celtypen: B cellen en T cellen, welke beide belangrijk zijn voor een goede afweerrespons tegen het griepvirus. B cellen maken antilichamen, dit zijn eiwitten die in het bloed kunnen circuleren en aan specifieke structuren (bijvoorbeeld van een ziekteverwekker) kunnen binden. Op deze wijze kunnen antilichamen virusdeeltjes *buiten cellen* onschadelijk maken. T cellen kunnen ook specifieke structuren herkennen: afbraakproducten die door lichaamseigen cellen gepresenteerd worden. Deze afbraakproducten kunnen afkomstig zijn van een ziekteverwekker die *in de cel* is gaan zitten, zoals een virus. In reactie hierop kan een deel van de T cellen, de cytotoxische T cellen, een dergelijke geïnfecteerde cel doden en daarmee het virus bestrijden. Een ander deel van de T cellen, T helper cellen, bieden sturing aan de afweerrespons: zij stimuleren specifieke antilichaamproductie door B cellen en stimuleren het doden van geïnfecteerde cellen door cytotoxische T cellen.

Een stijging van de productie van specifieke antilichamen door B cellen wordt een *antilichaamrespons* genoemd. Een stijging van het aantal specifieke T cellen wordt een *T-cel respons* genoemd. Binnen griepvaccinatie onderzoek geldt de antilichaamrespons als de gouden standaard: hierover is het meeste bekend, en deze kan tot op zekere hoogte gekoppeld worden aan de waarschijnlijkheid dat iemand beschermd is tegen het oplopen van griep. De T-cel respons is meer experimenteel; er is aangetoond dat deze van belang is, zeker bij bepaalde groepen zoals ouderen, echter er zijn nog geen gestandaardiseerde uitkomstmaten voor.

Onderzoeksvragen

De studies die in dit proefschrift gepresenteerd worden hadden tot doel om griepvaccinatie bij patiënten met een systemische auto-immuunziekte te evalueren, met betrekking tot de volgende kwesties:

- veiligheid van griepvaccinatie
- antilichaamrespons en T-cel respons op griepvaccinatie

- invloeden van afweeronderdrukkende medicijnen op de afweerrespons op griepvaccinatie
- mogelijke strategieën om de afweerrespons op griepvaccinatie te verbeteren, in geval van gebleken verlaagde afweerrespons op griepvaccinatie

Samenvatting van de resultaten

Na een algemene introductie in **hoofdstuk** 1, onderzochten wij in **deel 1** griepvaccinatie bij patiënten met SLE. In **hoofdstuk 2** werden de veiligheid en de antilichaamrespons beoordeeld. Griepvaccinatie gaf, gemiddeld over een groep van 56 SLE patiënten, geen stijging van de ziekteactiviteit (gescoord met een medische ziekte-index en door patiënten zelf) en geen ernstige bijwerkingen. De antilichaamrespons op griepvaccinatie was bij SLE patiënten lager dan bij gezonde controlepersonen. Wel had de meerderheid van de SLE patiënten een antilichaamrespons, en bereikten veel SLE patiënten een eindwaarde die als beschermend beschouwd wordt. We keken ook naar de invloed van de afweeronderdrukkende medicijnen azathioprine, hydroxyl-chloroquine en prednison. Binnen patiënten met SLE was het gebruik van azathioprine, een medicijn dat T cellen remt, geassocieerd met een verdere verlaging van de antilichaamrespons. Deze resultaten riepen de vraag op of de verlaging van de antilichaamrespons gepaard zou gaan met, of veroorzaakt zou worden door, een verlaagde T-cel respons. Alvorens dit te bestuderen, hebben we in **hoofdstuk 3** resultaten van eerdere studies van andere onderzoeksgroepen op een rij gezet in een literatuurbespreking. Hierbij bleek dat er nog geen studies verricht waren naar de T-cel respons op griepvaccinatie en strategieën om de afweerrespons op vaccinatie te verbeteren.

In **hoofdstuk 4** bespreken we de T-cel respons op griepvaccinatie. Hiervoor is een tweede vaccinatiestudie verricht. Zowel voor als na vaccinatie hadden patiënten met SLE minder griepspecifieke T cellen dan gezonde controlepersonen. Deze verlaging was geassocieerd met het gebruik van de afweeronderdrukkende medicijnen prednison en azathioprine. In deze studie vonden we wederom geen toename van ziekteactiviteit door griepvaccinatie en een verlaagde antilichaam-respons op griepvaccinatie bij SLE patiënten.

Omdat we in de studies van hoofdstuk 2 en 4 bij SLE patiënten een verlaagde antilichaamrespons op griepvaccinatie gevonden hadden, wilden we onderzoeken of deze respons verhoogd kon worden door een andere vaccinatiestrategie te kiezen. Het toedienen van een 2^{e} griepvaccinatie, een maand na de reguliere

vaccinatie, is hiervoor bij andere aandoeningen werkzaam gebleken. In **hoofdstuk 5** onderzochten wij of dit ook bij SLE patiënten het geval is. Een dergelijke *booster* vaccinatie bleek van beperkte toegevoegde waarde. In het algemeen was er geen sprake van een antilichaamrespons op de 2[e] griepvaccinatie, alleen bij patiënten die het voorgaande jaar geen griepvaccinatie hadden gekregen, zagen wij een antilichaamrespons op de 2[e] vaccinatie. Dit betekent dat het niet zinvol lijkt om SLE patiënten 2 vaccinaties te geven, tenzij ze het voorgaande jaar niet gevaccineerd zijn. Mogelijk dat andere strategieën om de afweerreactie op griepvaccinatie te verbeteren succesvoller zijn, zoals het toepassen van een nieuw vaccintype.

Het gebruik van prednison en/of azathioprine was geassocieerd met een lagere antilichaamrespons op griepvaccinatie, net als eerder in hoofdstuk 4 voor de T-cel respons gevonden was en in hoofdstuk 2 voor de antilichaamrespons. In deze studie hadden gezonde controlepersonen een lagere antilichaamrespons dan verwacht, we zagen hierdoor geen verschillen tussen de antilichaamrespons van SLE patiënten en die van gezonde controlepersonen. De betekenis hiervan is voer voor discussie, zo kunnen vaccinstammen van dat jaar een andere reactie gegeven hebben dan stammen gebruikt in andere jaren.

In **deel 2** onderzochten we griepvaccinatie bij patiënten met WG en RA. In **hoofdstuk 6** keken we naar de antilichaamrespons op griepvaccinatie bij WG patiënten, en de veiligheid hiervan. Dit was tot dan toe niet eerder onderzocht. De antilichaamrespons van WG patiënten was vergelijkbaar met die van gezonde controlepersonen. Afweeronderdrukkende medicatie had geen effect op de bereikte antilichaamresponsen, althans niet in de hier gebruikte doseringen. Na griepvaccinatie was er op groepsniveau geen sprake van veranderingen in ziekteactiviteit bij de gevaccineerde WG patiënten, en werd ook geen verschil gezien in ziekteactiviteit in vergelijking met een groep ongevaccineerde WG patiënten. Deze studie gaf, samen met een gelijktijdig verschenen studie van een andere onderzoeksgroep, duidelijke aanwijzingen dat griepvaccinatie bij WG patiënten veilig is en een goede effectiviteit heeft.

Als onderdeel van dezelfde vaccinatiestudie bestudeerden wij in **hoofdstuk 7** bij een deel van de WG patiënten en gezonde controlepersonen ook de T-cel respons. Bij WG zijn er verstoringen van het T-cel systeem beschreven, wat de vraag opriep of dit compartiment op vaccinatie een zelfde reactie zou vertonen bij WG patiënten als bij gezonde controlepersonen. Wij vonden zowel voor als na

vaccinatie geen verschillen in het aantal griepspecifieke T cellen bij WG patiënten en gezonde controlepersonen. Dit duidt erop dat het afweersysteem bij WG niet dermate ontregeld is dat dergelijke responsen verstoord raken. Op basis van hoofdstuk 6 en 7 lijkt griepvaccinatie bij WG patiënten dan ook zinvol.

Tot slot richtten we ons in **hoofdstuk** 8 op RA patiënten. De effecten van rituximab, een medicijn dat B-cellen (de antilichaamproducerende cellen) verwijdert, op de antilichaamrespons op griepvaccinatie worden besproken. Deze B-cel depletie leidde tot het verdwijnen van de antilichaamrespons bij patiënten die rituximab tot 2 maanden voor vaccinatie hadden gekregen. Het uitschakelen van B cellen door deze therapie is tijdelijk. Bij RA patiënten die rituximab 6-10 maanden eerder hadden gekregen, begon de antilichaamrespons terug te keren. Deze bevindingen zijn van belang bij het bepalen van adviezen over wanneer een griepvaccinatie zinvol is: vóór het geven van rituximab, of anders geruime tijd na de laatste gift van rituximab. Net als bij SLE en WG patiënten, leidde griepvaccinatie bij RA patiënten niet tot een toename van ziekteactiviteit.

Discussie

Andere studies naar vaccinaties bij SLE en RA

Ook andere onderzoeksgroepen hebben gekeken naar vaccinaties bij SLE en RA. Net als wij, vinden de meeste onderzoeksgroepen een enigszins verlaagde antilichaamrespons op griepvaccinatie bij SLE. Ook voor andere vaccinaties is dit gevonden. Bij RA lijkt de ziekte op zichzelf niet te leiden tot lagere respons op griepvaccinatie. Echter, van rituximab wordt ook door andere groepen beschreven dat het de respons op griepvaccinatie sterk beperkt.

Algemene beperkingen van de studies in dit proefschrift

Hoe de afweerreactie op vaccinatie verloopt, kan gebruikt worden als voorspelling voor daadwerkelijke bescherming tegen griep. Met name voor de antilichaam-respons zijn hier gegevens over bekend, voor de T-cel respons is dit veel minder duidelijk. In het algemeen blijft het lastig uitspraken te doen over het beschermd zijn tegen griep, als niet expliciet het ziek worden door griepbesmetting gemeten wordt. Dit vereist een grote logistieke inspanning, en was voor de studies in dit proefschrift niet haalbaar.

Veranderingen in ziekteactiviteit na griepvaccinatie kunnen op groepsniveau gemeten worden, als gemiddelde ziekteactiviteit, en op individueel niveau, als het optreden van ziekteopvlammingen. Wanneer de hoofdvraag van een studie is of

het aantal ziekteopvlammingen na vaccinatie groter is dan het aantal spontane opvlammingen bij niet-gevaccineerde patiënten, dan zijn grote aantallen patiënten met SLE, WG en RA vereist. Dit wil zeggen dat er statistisch gezien meer patiënten bestudeerd hadden moeten worden, om hierover met een redelijke zekerheid een uitspraak te kunnen doen. Een dergelijk groot cohort patiënten was in onze setting, en ook elders, niet voorhanden. Echter, de gecombineerde data van onze studies wijzen erop dat het onwaarschijnlijk is dat griepvaccinatie leidt tot ziekteopvlammingen.

In de studies in hoofdstuk 2, 4 en 6 was het relatieve aantal SLE en WG patiënten dat een griepvaccinatie in het voorgaande jaar had gekregen groter dan het relatieve aantal gezonde controlepersonen. Het is bekend dat eerdere griepvaccinaties de antilichaamrespons op griepvaccinatie kunnen beïnvloeden. Daarnaast beïnvloeden eerdere griepinfecties de antilichaamrespons op griep-vaccinatie, echter deze infecties zijn niet betrouwbaar vast te stellen. Voor toekomstige studies heeft het de voorkeur om groepen patiënten en gezonde controlepersonen samen te stellen die overeenkomen qua vaccinatie status.

Wij hebben de T-cel respons 28 dagen na griepvaccinatie gemeten. Maar de hoogte van de T-cel respons piekt rond de 10^{e} dag na vaccinatie; onze metingen hebben dus na die piek plaatsgevonden. Metingen van de antilichaamrespons dienen 3 tot 4 weken na vaccinatie gedaan te worden. Het zou dus optimaal geweest zijn wanneer wij analyses gedaan hadden na zowel 10 als 28 dagen, echter dit was logistiek niet haalbaar. We hebben ervoor gekozen om ook de T-cel respons na 28 dagen te meten, omdat verkennende experimenten lieten zien dat deze ook dan nog meetbaar was, en omdat er wel gestandaardiseerde criteria voor de antilichaamrespons zijn en niet voor de T-cel respons.

Nieuwe ontwikkelingen

Toekomstige studies zouden zich op een aantal vragen kunnen richten. De studies in dit proefschrift geven aan dat griepvaccinatie veilig lijkt te zijn bij patiënten met een systemische auto-immuunziekte, zowel bij rustige als bij actieve ziekte. Het is nog onduidelijk of griepvaccinatie ook leidt tot een goede afweerrespons bij patiënten met actieve ziekte. De ziekte zelf en afweeronderdrukkende medicijnen, gegeven tijdens actieve ziekte, zouden de afweerrespons kunnen hinderen. Om deze vraag te beantwoorden moet een aanzienlijke groep patiënten met actieve ziekte bestudeerd worden. Dit lukt niet in één ziekenhuis, hiervoor zal een studie van meerdere samenwerkende ziekenhuizen nodig zijn.

Ten tweede is nog onduidelijk of griepvaccinatie bij patiënten met een systemische auto-immuunziekte het aantal griepinfecties verlaagt, en of dit in dezelfde mate geldt als voor gezonde controlepersonen. Om deze vraag te kunnen beantwoorden moet een groot aantal gevaccineerde en ongevaccineerde patiënten en gezonde controlepersonen gevolgd worden. Hierbij zal bij verdenking van griep diagnostiek nodig zijn om te bevestigen dat het om een daadwerkelijke griepinfectie gaat.

Ten derde kan de T-cel respons op griepvaccinatie nader bestudeerd worden. Dit geldt met name voor SLE patiënten, omdat bij hen de T-cel respons verlaagd is. De testen gebruikt in onze studies richten zich met name op de functionele capaciteit van T-cellen om te reageren op stimulatie, een andere benadering is om het aantal griep-specifieke cellen te bepalen zonder direct naar functie te kijken. Ook met betrekking tot de functionele capaciteit van de T-cellen zijn er nog niet bestudeerde aspecten, zoals hun vermogen om zich te delen na stimulatie en om geïnfecteerde cellen te doden.

Ten vierde zijn er veel ontwikkelingen gaande op het gebied van griepvaccinontwikkeling. Zo worden verschillende toevoegingen, adjuvantia genoemd, getest die de afweerrespons op vaccinatie verhogen en daardoor tot een betere bescherming kunnen leiden. Deze vaccins hebben een toegevoegde waarde voor groepen waarbij griep meer complicaties en sterfte geeft, zoals ouderen, en waarbij de afweerrespons op het conventionele vaccin verlaagd is. De studies in dit proefschrift laten zien dat een grote meerderheid van de patiënten met een systemische auto-immuunziekte respondeert op conventionele vaccinatie, wat er op duidt dat een nieuw vaccintype voor hen niet nodig zal zijn. Echter, daadwerkelijke bescherming is nog niet onderzocht en te overwegen valt een beter werkzaam vaccin te testen, vooral bij SLE, omdat een deel van de SLE patiënten onvoldoende reageert.

Advies ten aanzien van griepvaccinatie bij patiënten met een systemische auto-immuunziekte

Aangezien griepvaccinatie bij patiënten met een systemische auto-immuunziekte niet tot toename van ziekteactiviteit lijkt te leiden en aangezien de meerderheid van de patiënten respondeert op vaccinatie, adviseren wij om jaarlijkse griepvaccinatie toe te passen. Dit vanwege het vele voorkomen van griep en vanwege de aanzienlijke complicaties en sterfte die de griep geeft bij risicogroepen. Deze aanbeveling wordt toenemend gegeven in de wetenschappelijke literatuur en vanuit artsenverenigingen. Toepassing in de praktijk is

echter wisselend, waarschijnlijk door het ontbreken van duidelijke richtlijnen hierover. Dergelijke richtlijnen zijn van belang en worden momenteel gemaakt door de Europese Reumatologie Vereniging (EULAR).

Hoofdbevindingen in dit proefschrift

- Griepvaccinatie bij systemische auto-immuunziekten (SLE, WG, RA) lijkt veilig te zijn.
- Griepvaccinatie bij patiënten met WG en RA resulteert in antilichaamresponsen die vergelijkbaar zijn met die in gezonde controle-personen; echter bij patiënten met SLE is deze respons enigszins verlaagd.
- Bij SLE patiënten was het gebruik van azathioprine geassocieerd met lagere antilichaamresponsen. Van overige afweerremmende medicatie werd geen duidelijk effect op de antilichaamrespons gezien, of was het aantal patiënten dat een dergelijk medicijn gebruikte te klein om hierover een betrouwbare uitspraak te kunnen doen. Bij WG en RA, leidde de conventionele afweerremmende medicatie niet tot lagere antilichaamresponsen. Het medicijn rituximab, dat B cellen uitschakelt, bestudeerd bij RA patiënten, gaf een volledige afwezigheid van de antilichaamrespons op griepvaccinatie in de eerste 2 maanden na gebruik; 6-10 maanden na gebruik was er enige terugkeer van deze respons.
- T-cel responsen op griepvaccinatie waren lager bij SLE patiënten dan bij gezonde controlepersonen, dit was sterk geassocieerd met het gebruik van prednison en/of azathioprine. Bij WG patiënten waren T-cel responsen op griepvaccinatie niet verschillend van die bij gezonde controlepersonen.
- Een tweede, *booster*, griepvaccinatie heeft alleen toegevoegde waarde bij SLE patiënten die het voorgaande jaar niet gevaccineerd zijn.

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