Rituximab impairs IgM and IgG (subclass) responses after influenza vaccination in rheumatoid arthritis patients.

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Abstract:

Objective: Rituximab (RTX) treatment in rheumatoid arthritis (RA) patients severely hampers humoral response after influenza vaccination as determined by Haemagglutination Inhibition Assay (HI). It is not known whether HI reflects both IgM and IgG (subclass) influenza response, and whether IgM antibodies contribute to the low rate of influenza infection seen in RA patients.

Methods: 20 RA patients on methotrexate (MTX), 23 on RTX and 28 healthy controls (HC) received trivalent influenza subunit vaccination. Before, and 28 day after vaccination H1N1 and H3N2 specific antibodies were measured by HI and by IgM and IgG (subclass) ELISA. BAFF (B cell activating factor) levels were determined in serum samples before vaccination.

Results: Vaccination induced a significant increase of IgM and IgG (IgG1 and IgG3) antibodies against both strains in the HC and MTX group (all $P < 0.01$) but not in the RTX group. HI significantly correlated in all cases with IgG (IgG1) but not with IgM. In RTX late patients (RTX treatment 6-10 months before vaccination) IgG (IgG1 and IgG3) response to vaccination was restored, but not IgM response. BAFF levels were significantly increased in RA-RTX patients and correlated to total IgG levels.

Conclusion: Haemagglutination inhibition assay, used as gold standard, detects primarily IgG (IgG1) responses. IgM and IgG influenza specific antibodies increase after vaccination in HC and RA patients except in patients on RTX treatment. BAFF levels are increased in both early and late RTX treated patients, but do not correlate to influenza specific antibody response.

Introduction:

Rheumatoid arthritis (RA) patients are susceptible for many types of infection, especially after biological treatment [1-5]. One of these infections is caused by the influenza virus. Influenza consists of three RNA subtypes (A, B and C), of which Influenza A is the most frequently occurring one, and is subtyped based on the surface glycoproteins Haemagglutinin (HA) and Neuramidase (NA)[6].

Vaccination in RA patients on therapy seems to be safe [7-9] and effective even in patients on Disease Modifying Anti Rheumatic Drugs (DMARDs), prednisone or anti-TNF therapy [10]. However, rituximab (RTX) therapy hampers the humoral vaccination response to influenza measured by HI [11, 12]. RTX is a chimeric monoclonal antibody directed against the CD20 cell surface molecule that is located on B-cells. It causes B-cell depletion and thereby significantly reduces the humoral response to vaccination. Despite this reduced response rate, recurrent infection is relative low in patients on RTX compared to anti-TNF treatment[13]. This might be due to a (relatively) intact cellular immunity or an adequate IgM response. Indeed, reports on effects on cellular response to influenza vaccination are limited but until now no significant influence on cellular response was seen [14]. Most studies determine levels of influenza specific antibodies using the Haemagglutinin Inhibition assay (HI). Titers ≥40 are considered protective in healthy adults [15]. It is not known whether HI reflects both IgM and IgG response. Moreover it is not known which IgG subclasses contribute to IgG response to influenza. Measuring IgM antibodies is important to monitor early response to vaccination, and early response indicates the capacity of repopulating naïve B-cells to respond to the vaccine antigen. IgG1 and IgG3 are important immunoglobulins for complement fixation and binding to Fc receptors which could play a role in antibody dependent cellular toxicity.

One of the factors that controls B cell survival, B cell maturation and immunoglobulin class (IgG, IgA, and IgE*)* switching is BAFF (B cell activating factor), also named BLys (B lymphocyte stimulator). It has been reported that BAFF levels increase after RTX treatment in RA patients [16]. In a recent paper it was shown that RA synovial fibroblasts can produce high

levels of BAFF that induce class switch to IgA and IgG in IgD+ B cells [17]. Chen et al showed that soluble BAFF enhanced humoral immune response by elevating B lymphocyte activity of secretion of immunoglobulins in chickens that were immunized [18]. Whether BAFF levels are related to response to vaccination in RTX-treated patients is not known.

In this study we examined IgM- and IgG(subclass)- antibody response against influenza subunit measured with an Enzyme linked Immunosorbent Assay (ELISA) and compared the results to traditional HI. In addition, we analysed the relation between BAFF levels and influenza vaccination response in RA patients on MTX and RTX therapy.

Materials/methods:

Patients:

Twenty-eight healthy controls (HC), and 43 RA patients were included. All patients fulfilled the American College of Rheumatology clinical classification criteria for RA [19]. Twenty patients were on MTX (in two patients combined with other DMARDs) and 23 patients on RTX (treatment of 11 patients 4-8 weeks before vaccination and of 12 patients 6-10 months before vaccination). Data were retrieved from a previous study [12]. Patient characteristics are shown in Table 1. Mean age of RTX and MTX patients did not differ but was higher in both groups of RA patients compared to HC ($p=0.004$). Patients in the RTX-group had lower number of B-cells than patients in the MTX- and HC-group (both $p<0.001$) as a results of RTX treatment. The RTX-patients were recruited in four participating Dutch University Medical Centre's [12]. HC and patients on MTX (including some on other additional DMARD's) were recruited from the Groningen University Medical Centre. Exclusion criteria were: (i) lack of 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. The study was approved by the ethics committees of all participating centres.

Vaccine:

Trivalent subunit Influenza vaccine (Solvay Pharmaceuticals, Weesp, The Netherlands)was used and contained the following strains: A/Wisconsin/67/2005 (H3N2)-like strain, A/Solomon Islands/3/2006 (H1N1)-like strain, and B/Malaysia/ 2506/2004-like strain.

Immediately before and 28 ± 3 days after vaccination blood was drawn from patients and controls, and after centrifugation stored at -20 ^{o}C until use.

Methods:

Antibody levels against all three strains were measured before and 28 days after vaccination using HI. HI was performed with guinea pig erythrocytes following standard procedures [20] and results have previously been reported [12].

Specific anti-influenza antibodies, both IgM and IgG (subclasses), were determined by an ELISA in all samples before and after vaccination. In short, microtiter plates were coated with 1 µg/ml subunit of A/H1N1 or A/H3N2 and with $F(ab')_2$ goat anti-IgG (Jackson Immunoresearch, Newmarket, UK) for IgG standard curve or with monoclonal anti-human IgM (clone MBII, Sigma-Aldrich, Zwijndrecht, the Netherlands) for IgM standard curve. Serum samples in multiple dilutions were added and IgG, IgM, IgG_1, IgG_3, and IgG_4 standard curves were setup. Detection was performed with HRP-labelled mouse anti-human IgG (clone JDC-10), mouse anti-human IgM (clone SA-DA4), mouse anti-human IgG_3 (clone HP6050), mouse anti-human IgG_4 (clone HP6025) all from Southern Biotech (Birmingham, USA) and mouse anti-human IgG_1 (clone MH161-1, Fitzgerald, North Acton, USA) respectively, followed by color reaction with 3'3'5'5'tetramethylbenzidin (TMB) and H_2O_2. Absorbance was read at 450–575 nm in an Emax microplate reader and concentration of antibodies was calculated by SOFTmax PRO software (Molecular Devices, Sunnyvale, USA) according to standard curves included on each ELISA plate. IgG2 responses could not be detected for technical reasons.

BAFF levels were measured in baseline serum samples using BAFF quantikine ELISA (R&Dsystems, Abingdon, UK) according to manufacturers' instruction.

Statistical analysis:

Data were analysed using GraphPadPrism V5.0 (GraphPad software, San Diego, USA). Mann-Whitney rank test, Wilcoxon rank test and Spearman rank test were performed for statistical analysis. A P-value <0.05 was considered statistically significant

Results:

IgG and IgM influenza specific antibody response measured by ELISA:

Upon influenza vaccination HC had an significant increase in IgM- as well as IgG antibodies against both influenza strains (Figure 1). RA patients treated with MTX also showed a good response to influenza for both isotypes.

RA patients on RTX showed a low vaccination response compared to HC and MTX treated RA patients (Figure 1). None of the responses in the RTX group were significant.

Correlation HI and ELISA:

To evaluate to what extend HI reflects ELISA results we determined the correlation between HI and respective IgG and IgM ELISAs. IgG ELISA values correlated well with HI titers both in HC and in patient groups (Table 2). However, HI titers did not correlate with IgM levels in all samples (Table 2). There was no correlation in HC between H1N1-specific IgM antibody levels and H1N1-specific HI titers whereas in RA-MTX correlation between H3N2-specific IgM antibody levels and H3N2-specific HI titers was not significant. So lack of correlation cannot be attributed to a specific influenza strain or to MTX or RTX therapy.

To further investigate whether HI is mainly determined by IgG antibodies, sera of HC (n=14) were depleted of IgM by incubation with agarose anti-IgM (Sigma-Aldrich, cat nr A9935). After IgM depletion, IgG levels (original range 31 to 158 µg/ml) had not changed (percentage compared to untreated sample: average 103.2%, SD 21.3%), while IgM levels (range untreated 3.7 to 224 µg/ml) were reduced (percentage compared to untreated sample: 14.8 %, SD 9.9%). Performing HI with IgM depleted and untreated samples showed no changes in titer, confirming that HI is mainly determined by IgG antibodies.

IgG subclass response:

Determination of IgG subclass response by ELISA showed a significant IgG1 response in HC and both patient groups for H1N1 and H3N2 after vaccination as can be seen in table 3 ($P < 0.001$). Remarkably, also the IgG1 response towards both influenza strains reached significance in RA patients on RTX. H1N1 –specific responses were seen in all groups for IgG3 (HC: $P < 0.001$, MTX: $P < 0.01$, RTX: $P < 0.05$). However, in contrast to both HC and RA-MTX patients who had an increase in IgG3 response against H3N2 (HC: $P<0.01$, MTX: $P<0.01$), the

RA-RTX group failed to reach an adequate increase in influenza specific IgG3 after vaccination. IgG4 subclass response only showed a significant increase for H1N1 in HC (P=0.02), and no increase in both patients groups.

As mentioned before total IgG levels correlated well with HI titers. This seems primarily due to the IgG1 response, which forms the largest part of IgG. IgG1 levels correlated well with HI in HC and patient groups for both influenza strains ($P < 0.05$). There was no correlation between HI levels and IgG3 anti-influenza levels in HC and RA-MTX patients, but HI titers correlated significantly with IgG3 anti-influenza levels in RA-RTX in both strains. No correlation was found between HI titers and IgG4 levels.

Early and late Rituximab treatment groups:

When patients within the RTX group were divided in patients that had received RTX 4-8 weeks before vaccination (early) and in those that had received RTX 6-10 months (late) prior to vaccination, an increase in influenza-specific IgG antibodies was observed in the 'late' RTX group only (Figure 2). In the latter group IgG to A/H1N1 increased from 48.9 ± 35.5 to 137.9 ± 127 (P=0.002) and IgG to A/H3N2 increased from 39.6 ± 32.8 to 63.1 ± 49.8 (P=0.001). The early group did not show a significant increase against either strain. In contrast, IgM response was not seen for either strain in both early and late groups (Figure 2). In the late RTX group a significant increase for IgG1 and IgG3 was found for both H1N1 (resp. P=0.037 and P=0.007) and H3N2 (resp. P=0.009 and P=0.010). The early RTX group did not show an increase in IgG1 nor in IgG3 to either influenza strain.

BAFF levels at baseline:

As expected, we found high BAFF levels in RA patients that had been treated with RTX, both in early and late groups (Figure 3A). BAFF levels in these patients were significantly increased compared to BAFF levels in HC and RA-MTX (all P<0.001). The levels were (median (range)): HC 0.66 ng/ml (0.14-1.04), RA-MTX 0.72 ng/ml (0.49-1.30, RA-RTXearly 2.56 ng/ml (1.28-4.58), RA-RTXlate 2.18 ng/ml (1.28-4.83). There was no difference between BAFF levels of early and late groups. A significant correlation was present between baseline BAFF levels and total IgG levels before (Figure 3B) and after vaccination (P<0.001 and P<0.05 respectively), but not with IgA and IgM levels when data of patients and controls were

combined. In the separate (smaller) groups these correlations lost significance. Also influenza specific IgG levels after vaccination were correlated to BAFF levels in combined groups ($P<0.05$), but not in separate groups. There was one exception: BAFF levels and IgG3 specific anti-influenza levels in RA-RTX patients were negatively correlated, $P<0.05$, both for H1N1 and H3N2.

Discussion:

Seasonal influenza vaccination evokes a good response in healthy persons and RA patients treated with MTX, as shown by a significant increase in influenza-specific IgG (IgG1 and IgG3)- and IgM- antibodies detected after vaccination. RA patients treated with RTX show a hampered response to influenza vaccination, especially for IgM antibodies to influenza.

Influenza vaccination is considered safe and efficacious (as determined by HI) in RA patients [21] even when treated with DMARDs and on anti-TNF therapy [11, 12]. Using ELISA we were able to unravel the humoral response after influenza vaccination. A significant increase in both IgG- and IgM- influenza specific antibodies was found in HC and RA patients treated with MTX after vaccination. In line with previous results based on HI, in RA-RTX patients no increase was seen in either IgG or IgM antibodies. When patients were divided in early RTX and late RTX a significant increase was seen in IgG antibodies but not in IgM antibodies in the late group. Previously it was shown that patients in the late group (those who received RTX 6-10 months before vaccination) had a modestly restored response measured by HI [12]. Our results only show an increase in IgG antibodies In these patients. Rehnberg et al investigated vaccination response to influenza vaccine and pneumococcal polysaccharides vaccine in RA patients 6 days before (n=8) and 6 months after RTX treatment (n=11) compared to RA patients on MTX treatment (n=10) [22]. They measured cellular response on day 6 and humoral response on day 21. Formation of influenza-specific B cells was lower in post-RTX groups compared to pre-RTX group and controls, and absence of influenza-specific IgG production was observed in 55% of the post-RTX group. These data corroborate with ours in the way that in our late RTX-group in which we included patients up to 10 months after RTX we did see a modest restored IgG response. In a study by Pescovitz et al the effect of RTX on human in vivo antibody immune responses was investigated [23]. The IgG and IgM response to a neoantigen was investigated as well as anti-tetanus, diphtheria,

mumps, measles and rubella by means of ELISA. They showed that during the time of B-lymphocyte depletion, RTX recipients had a decreased antibody response to neoantigens and significantly lower titers after recall immunization. With recovery of the B-cells immune responses returned to normal. They conclude that immunization during the time of B-lymphocyte depletion, although ineffective, does not preclude a subsequent response to the antigen.

The IgG subclass ELISA demonstrated that the major part of the influenza-specific IgG response in all patient groups as well as HC consisted mainly of IgG1. In HC IgG subclass response after Influenza vaccination with an inactivated subunit vaccine has been compared to vaccination with a live attenuated vaccine [24]. In young persons an IgG1 and IgG3 response could be demonstrated but in older persons (>58 years) there was only a significant IgG1 response [24]. In another influenza vaccination study only IgG1 and IgG2 antibodies were determined. A slight IgG2 response was only seen in young children after they had been 'primed' (had previous contact) [25]. IgG2 responses remains controversial because other studies failed to detect an increase both in young children and in elderly patients. In our study the average age of the patients was above 45 which could explain why the IgG4 response was lower, especially in patient groups that are considered to be immune compromised because of disease and medication. The IgG4 response detected in HC might be explained by this influence of age as the HC were younger than the patient groups. As stated before the role of IgG4 is less clear from previous studies than IgG1 and IgG3 and the clinical consequences of the differences in IgG4 response remain to be elucidated.

BAFF levels were significantly increased in RTX treated patients, both in early and late groups. There was a significant correlation between BAFF levels and total IgG levels in combined HC and RA patients. After vaccination only IgG3 influenza antibodies were correlated to BAFF levels in RTX-RA patients, no other correlations were seen between BAFF levels and response to influenza in patients and controls. This is in accordance with a recent study in which baseline BLys/BAFF levels were found not to correlate to humoral response to influenza vaccination in SLE patients [26]. Only patients with low BAFF (BLys) levels demonstrated an increased response, like we found in our study. BAFF is expressed by a variety of innate immune cells, like dendritic cells, macrophages and neutrophils, whereas

BAFF receptors mainly are expressed by B cells [27]. Levels of BAFF appear to be critical for controlling peripheral B cell numbers and survival of autoreactive B cells, so in case of low B cell numbers like during RTX treatment, BAFF levels increase [27]. This has been reported for RA patients whose BAFF levels increased after RTX infusion and stayed elevated for at least 1-2 months [16]. In primary Sjögren's patients treated with RTX it was shown that more transitional B cells were present in reconstituted B cells population during the early recovery phase, corresponding to bone-marrow derived populations [28]. This might explain why we see no correlation between BAFF levels and response to vaccination.Our study shows that influenza specific IgG and IgM antibodies can be measured by ELISA, which has advantages over the HI method. Commercially available IgG and IgM anti-influenza type A or B ELISAs have been used in literature, but not compared to HI [29]. Another study reported on the use of an IgG ELISA using the pandemic H1N1 HA protein as a coating antigen, and they found a concordance 98.4% with HI [30]. Recent studies show the advantages of ELISA methods over other methods as being quicker and easier to automate [31, 32].

Our study does have some limitations. As mentioned before our patient and HC groups are rather small, in particular when the RTX group is additionally divided into an 'early' and 'late' subgroup. Another limitation is the age-difference in HC and patients. This might have influenced the IgG3 and IgG4 values as has been found in other studies before [24]. Another possible confounder could be the non-standardised use of additional DMARD's. In the MTX group 2 patients used additional DMARD's and most of the RTX treated patients were on MTX as well and one was on corticosteroids. In the MTX group this does not seem to influence the response.

Concluding, this study shows that Haemagglutination Inhibition assay reflects primarily IgG influenza antibodies. It also shows that RA patients treated with RTX have a hampered IgG- as well as IgM-response after influenza vaccination. Although BAFF levels are significantly increased in RTX treated patients, this does not have an effect on humoral response to vaccination. These results, in combination with data that show that RTX treatment does not (severely) interfere with cellular immunity and lack of increased infection rate in RTX-treated patients, strongly favour a central role of T-cells in the defence against influenza virus.

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JW, SvA, KRW, AdH and MB contributed to the design of the study, interpretation of the data, and drafting of the manuscript. SvA, KRW and MB contributed to the inclusion of the patients. JL and GH performed experiments, and contributed to the interpretation of the data. JW and MB wrote the manuscript. All authors read and approved the manuscript.

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The authors declare that they have no competing interests.

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Legends to the figures and tables:

Figure 1. **IgG and IgM anti-influenza response in HC, RA-MTX, and RA-RTX.** Antibodies before (open symbols) and 28 days after(filled symbols) influenza vaccination were determined against H1N1 and H3N2 subunit by ELISA in serum of 28 healthy controls (HC), 20 RA-patients treated with methotrexate (RA-MTX) and 23 RA-patients treated with rituximab (RA-RTX).

Figure 2. **IgG (subclass) and IgM anti-influenza response in RA-RTX early and RA-RTX late**. Antibodies before and 28 days after influenza vaccination were determined against H1N1 and H3N2 subunit by ELISA in serum of 23 RA-patients treated with rituximab (RA-RTX), divided into 11 RA-RTX early (RTX 4-8 weeks before vaccination) and 12 RA-RTX late (RTX 6-10 months before vaccination).

Figure 3. **BAFF baseline levels in serum of HC, RA-MTX, and RA-RTX (early, late).** A. BAFF levels (ng/ml) measured by ELISA in in serum of 28 healthy controls (HC), 20 RA-patients treated with methotrexate (RA-MTX) and 23 RA-patients treated with rituximab (RA-RTX), divided into 11 RA-RTX early (RTX 4-8 weeks before vaccination) and 12 RA-RTX late (RTX 6-10 months before vaccination). B. Correlation between baseline BAFF levels and total IgG levels in HC, RA-MTX, and RA-RTX ($r= -0.33$, $P=0.005$).

Table 1: **Baseline characteristics of RA patients, treated with rituximab (RTX) or methotrexate (MTX), and HC.**

Table 2. **Correlations between HI titers and IgG and IgM ELISA levels respectively** .Anti-influenza levels before and after vaccination were measured against H1N1 and H3N2 in HC (healthy controls), RA patients treated with methotrexate (RA-MTX) or rituximab (RA-RTX) and correlated to HI titers.

Table 3: **IgG1, IgG3, and IgG4 antibody levels to H1N1 and H3N2 before and 28 days after influenza vaccination in HC (healthy controls), RA patients treated with methotrexate (RA-MTX) or rituximab (RA-RTX).**

Table 1. Baseline characteristics of RA patients, treated with rituximab (RTX) or methotrexate (MTX), and HC.

	RA-RTX (N=23)	RA-MTX (N=20)	Healthy controls (N=28)
Age, mean± SD Years	55.5 ± 7.6	57.1 ±- 6.7	45.2 ± 11.3#
Gender, female (%)	16 (70)	11 (55)	22 (78.6)
Previous vaccination(%)	12 (52)	10 (50)	20 (71.4)
Duration R.A. median years	13.8	8.7	
MTX dosage, median (range)	17.5 (10-25) n=10	16.3 (10-25)	NA
Prednisone dosage, median (range) mg/day	8.75 (3.8 to 40) n=15	0 (0 to 0)	NA
Other DMARDs n(%)	1 (4)	2 (10)	
IgG, g/L, median (range)	9.6 (3.4-16.7)*	11.5 (8-14.9)	11.6 (7.6-18.4)
IgG1	5.9 (2.3-10.4)	6.6 (4.9-9.8)	6.3 (3.7-8.6)
IgG2	2.2 (0.7-5.5)*	2.6 (1.3-4.8)	2.9 (1-8.3)
IgG3	0.4 (0.1-0.9)	0.6 (0.2-1.2)	0.4 (0.1-1.4)
IgG4	0.2 (0-1.1)	1 (0-1.8)	0.7 (0-1.8)
IgA, g/L, median (range)	2.2 (0.4-4)	2.1 (0.8-5.3)	1.8 (0.9-5.6)
IgM, g/L, median (range)	0.98 (0.2-2.3)	1 (0.5-4.3)	0.8 (0.3-2)
Interval before vaccination (%)			
4-8 weeks after Rituximab	11 (48)		
6-10 months after Rituximab	12 (52)		

NA: not applicable

# P<0.01 compared to RA-RTX and RA-MTX

* P=0.05 compared to HC

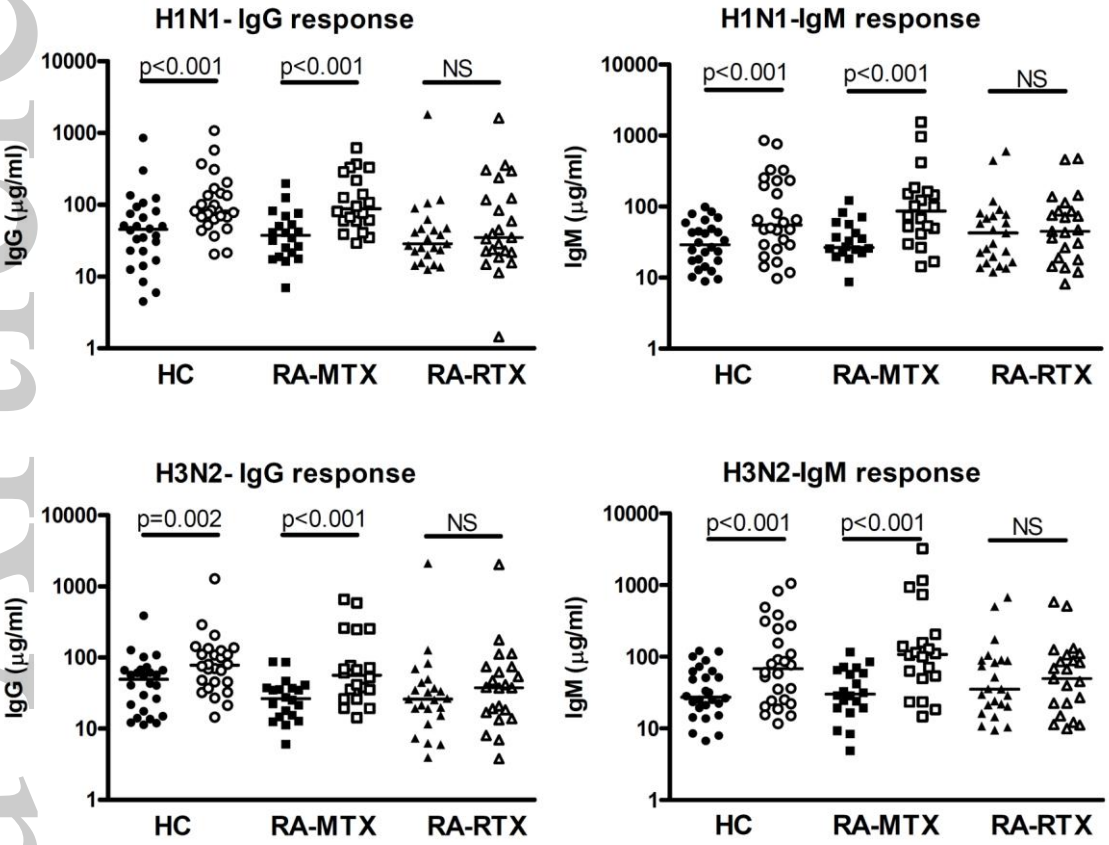
Table 2. Correlations between HI titers and IgG and IgM ELISA levels respectively.

IgG and IgM anti-influenza levels correlated with HI titers

IgG ELISA vs HI		**H1N1**		**H3N2**	
		T=0	T=28	T=0	T=28
HC	Spearman r	0.85	0.50	0.51	0.51
	P	<0.0001	0.0091	0.0076	0.0083
RA -MTX	Spearman r	0.69	0.82	0.68	0.66
	P	0.0007	<0.0001	0.0011	0.0017
RA -RTX	Spearman r	0.51	0.56	0.71	0.78
	P	0.0129	0.005	0.0001	<0.0001
IgM ELISA vs HI		**H1N1**		**H3N2**	
		T=0	T=28	T=0	T=28
HC	Spearman r	0.19	0.12	0.51	0.49
	P	ns	ns	0.0081	0.0119
RA -MTX	Spearman r	0.71	0.5	0.32	0.32
	P	0.0004	0.0236	ns	ns
RA -RTX	Spearman r	0.6	0.54	0.51	0.62
	P	0.0025	0.0081	0.013	0.0018

Table 3. IgG1, IgG3, and IgG4 antibody levels to H1N1 and H3N2 before and 28 days after influenza vaccination in HC (healthy controls), RA patients treated with methotrexate (RA-MTX) or rituximab (RA-RTX).

		Mean +/- SD (µg/ml)	Mean +/- SD (µg/ml)	P value
HC		T=0	T=28	
IgG1	A/H1N1	50.58 ± 86.15	97.23 ± 96.67	<0.001
	A/H3N2	42.81 ± 59.84	97.04 ± 122.40	<0.001
IgG3	A/H1N1	0.348 ± 0.899	1.169 ± 2.137	<0.001
	A/H3N2	0.350 ± 0.852	0.948 ± 1.929	<0.001
IgG4	A/H1N1	0.195 ± 0.492	0.234 ± 0.610	0.022
	A/H3N2	0.520 ± 1.573	0.578 ± 1.586	ns
RA -MTX				
IgG1	A/H1N1	23.05 ± 17.99	83.00 ± 92.85	0.0001
	A/H3N2	17.30 ± 14.53	98.25 ± 169.3	0.0001
IgG3	A/H1N1	0.134 ± 0.225	0.331 ± 0.440	0.0024
	A/H3N2	0.152 ± 0.205	0.484 ± 0.625	0.0015
IgG4	A/H1N1	0.137 ± 0.217	0.221 ± .0396	ns
	A/H3N2	0.197 ± 0.297	0.256 ± 0.484	ns
RA - RTX				
IgG1	A/H1N1	25.87 ± 20.27	59.87 ± 78.32	0.0053
	A/H3N2	25.39 ± 24.37	37.35 ± 43.90	0.0379
IgG3	A/H1N1	0.215 ± 0.617	0.303 ±0.841	0.0467
	A/H3N2	0.227 ± 0.561	0.296 ± 0.663	ns
IgG4	A/H1N1	0.079 ± 0.166	0.074 ±0.150	ns
	A/H3N2	0.095 ± 0.143	0.083 ± 0.164	ns



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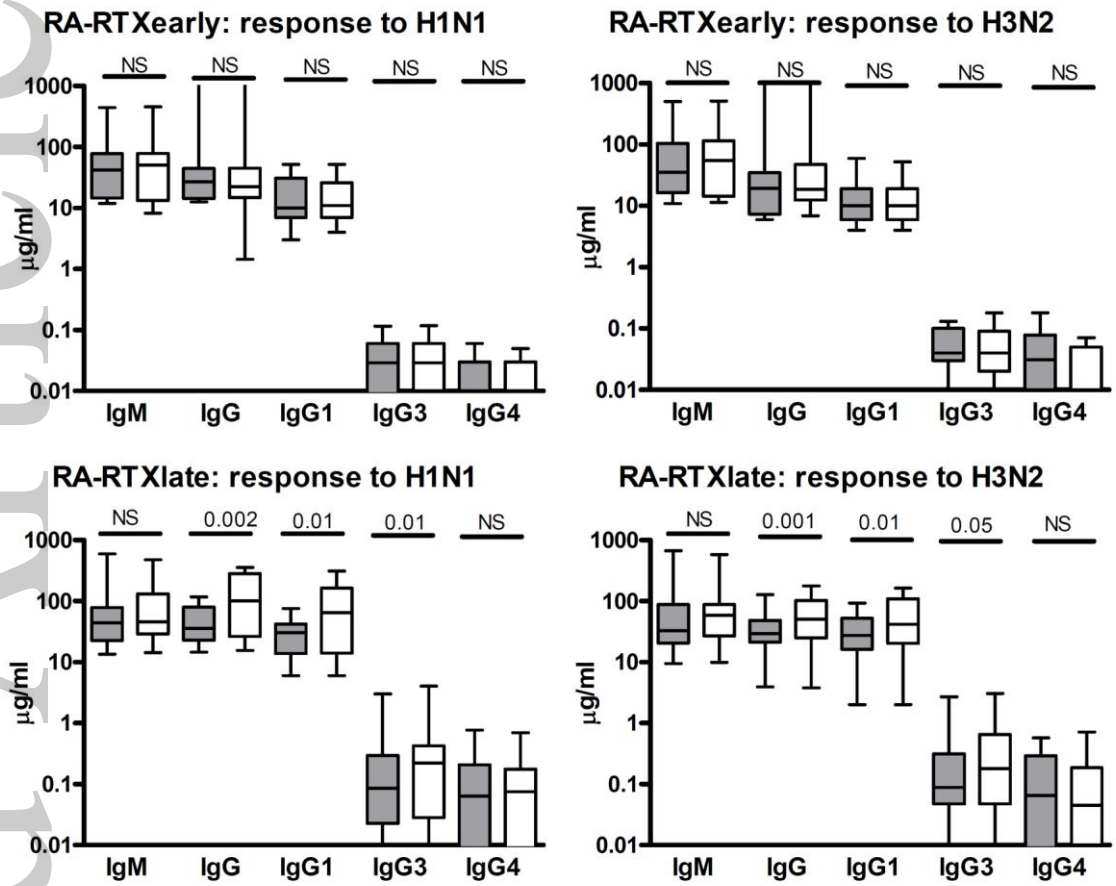

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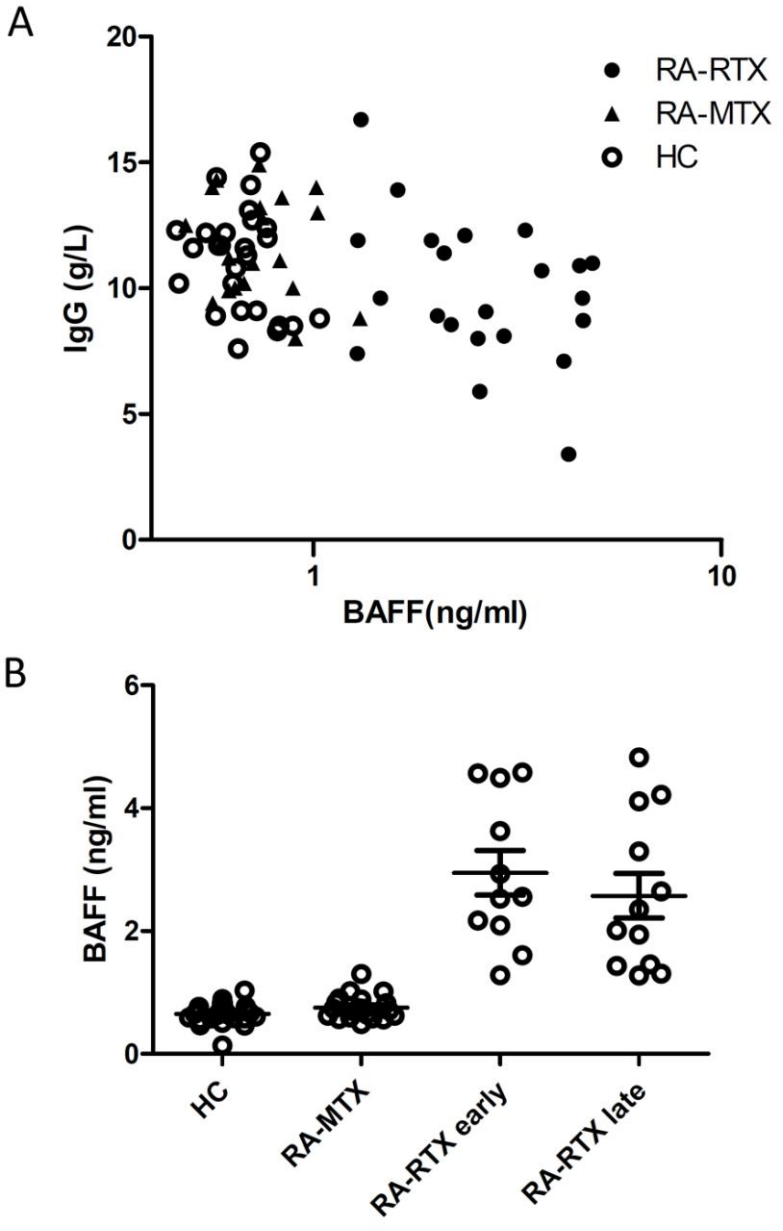

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