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Blockage of Tumor Necrosis Factor α Versus CD80/86 Signaling on the Response to Influenza Vaccination of Rheumatoid Arthritis Patients

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Abstract

The objective of the study was to compare and characterize the response to the 2014/2015 season influenza vaccine in patients with Rheumatoid Arthritis (RA) receiving biological agents, either TNF α blockers or a T-cell activation blocker, abatacept. Sera from 88 volunteers, 37 healthy controls and 51 RA patients, were collected prior to vaccination, and 4-6 weeks and 5-6 months thereafter. RA patients were divided into two groups, 35 were being treated with TNFα blockers and 16 were being treated with abatacept. The vaccine was given intramuscularly according to the manufacturer's instructions. Antibody response to vaccination was measured by detection of vaccine-specific IgG using ELISA. Hemagglutination Inhibition Assay (HIA) was performed to access production of neutralizing antibodies following vaccination. Vaccine-specific IgG quantification by ELISA indicated a significant increase in vaccine-specific IgG from pre-vaccination to 4-6 weeks post for the anti-TNFα and control groups, but not for the abatacept group. A decrease in vaccine-specific IgG was observed between the 4-6 week and 5-6 month time-points. Although the patient numbers were low for the abatacept group, obvious differences between this group and the TNFα blockers group could be observed. The abatacept group had the highest concentration of vaccine-specific IgG in their serum. Despite this high serum concentration of vaccine-specific IgG the abatacept group did not have a high amount of neutralizing IgG as indicated by HIA, and neutralizing IgG was not elevated in the serum following vaccination. Response to the influenza vaccination for RA patients receiving TNFα blockers was comparable to the control group.

Keywords: Rheumatoid arthritis; Immunization; Influenza vaccine; Biologics; Antibodies

Abbreviations

Ab: Antibody; RA: Rheumatoid Arthritis; HIA: Hemagglutination Inhibition Assay; ELISA: Enzyme-linked Immunosorbent Assay; TNF-α: Tumor necrosis factor α; ACR: American College of Rheumatology; CTLA-4: T-Lymphocyte-associated Antigen; APC: Antigen Presenting Cell; Treg: Regulatory T-cell; Th17: Interleukin 17 T-helper cell; IL-2: Interleukin 2; STAT5: Signal Transducer and Activator of Transcription 5; QIV: Quadrivalent Influenza Vaccine; HD-TIV: High Dose-Trivalent Influenza Vaccine; BSA: Bovine Serum Albumin; PBS: Phosphate Buffered Saline; GMT: Geometric Mean Titer; BH: Benjamini Hochberg; ANOVA: Two-way Analysis of Variance; ASCs: Antibody Secreting Cells; ABCs: Antigen-specific B-Cells

Introduction

Influenza vaccination is the most effective means to prevent influenza-related morbidity and mortality, and analysis of the antibody (Ab) response to the vaccine antigens is a good measure of a person's humoral immune potential. According to the 2008 American College of Rheumatology (ACR) guidelines influenza vaccine should be given to all Rheumatoid Arthritis (RA) patients who are going to be or are already being treated with immunosuppressive drugs [1]. These guidelines are consistent with the Center for Disease Control and Prevention (CDC) recommendations. These guidelines were set in place in response to several studies demonstrating that vaccination against influenza is effective in reducing hospital admissions and deaths due to pneumonia and influenza in elderly people with chronic diseases including RA, lung disease and diabetes [2-8].

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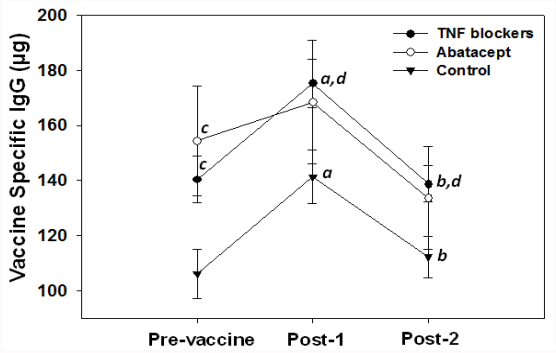

Figure 1: ELISA analysis of anti-vaccine specific IgG in the sera of untreated healthy controls (n=37), and RA patients treated with abatacept (n=16) or TNF*α* blockers (n=35). Student's *t*-test analysis with BH restrictions applied indicated a significant increase in anti-vaccine IgG concentration from pre-vaccine for TNF*α* blockers (***a***) and Controls (***a***). A significant decrease in anti-vaccine IgG concentration from Post-1 was observed for TNF*α* blockers (***b***) and controls (***b***). Baseline IgG concentrations were higher than controls for both abatacept (***c***) and TNF*α* blockers (***c***). TNF*α* blocker group IgG concentrations were higher than controls at Post-1 (***d***) and Post-2 (***d***).

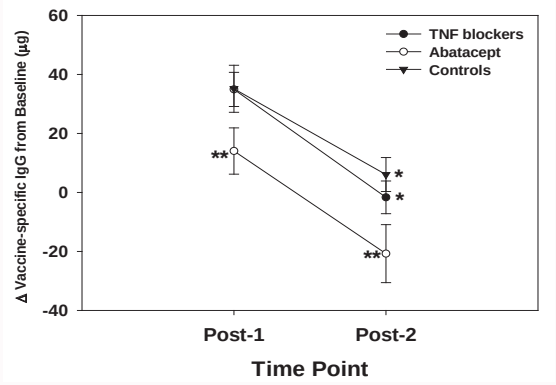

Figure 2: The differential amount of anti-vaccine IgG from the pre-vaccine (baseline) level to Post-1 and Post-2. Student's *t*-test analysis indicated a significant decrease from Post-1 to Post-2 in anti-vaccine IgG concentrations for *TNF*α* blockers and *Controls. Also, analysis showed a significant decrease in vaccine-specific IgG changes from baseline between the control group and the abatacept group **at Post-1, and Post-2. Significance at $p \leq 0.05$.

Since RA is an autoimmune disease, immunomodulatory drugs have been employed as treatment for the disease. Immunosuppressors, such as anti-tumor necrosis factor *α* (TNF*α*), biologics have been found to be effective for the treatment of RA. Many studies have been performed regarding the effectiveness of the recommended influenza vaccination for RA patients being treated with anti-TNF*α* biologics [9-15]. However, conclusions from these studies are conflicting and most studies have not measured vaccine specific antibody titers out to the end of the flu-season, 5-6 months post vaccination. Therefore, protection for individuals receiving these biologics over the duration of the flu-season is uncertain.

In the present study, the 2014/2015 influenza vaccine was administered to RA patients who were separately being treated with three different TNF*α* blockers (adalimumab (Humira), etanercept (Enbrel), or infliximab (Remicade)) or a T-cell activation blocker (abatacept). Since most TNF*α* blocker studies have not measured antibody titers out to the end of the flu-season at 6 months post vaccination, the present study includes a 5-6 month time point. Additionally, the T-cell activation blocker (abatacept) has been approved for use more recently, and its effect on the efficacy of the influenza vaccination has not been completely characterized. Therefore, the present study compares the effects of this newer biologic against the more well-known effects of the TNF*α* blockers. Abatacept consists of the extracellular domain of human cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4) and a genetically engineered fragment of the Fc region of human IgG1 [16]. Abatacept binds the CD80 and CD86 receptors present on Antigen Presenting Cells (APC) and inhibits binding to the CD28 receptor on T-cells. The binding between CD80/CD86 and CD28 is an important co-stimulatory interaction necessary for initiating T-cell proliferation and subsequent B cell responses.

Although each TNF*α* blocker employed as part of this study was designed to block binding of TNF*α* to its receptor, each has a somewhat different mode of action. Differences in the design and mode of action of each anti-TNF*α* have been reviewed [17]. Recently, another mechanism of action in RA was found for adalimumab [18]. It was reported that adalimumab binds membrane TNF*α* on monocytes and promotes the interaction between the monocytes and Treg cells. Consequently, the population of Treg cells, which are equipped to suppress Th17 cells through a mechanism involving IL-2/STAT5, was expanded. These differences in mode of action may also have an impact on the effectiveness of the influenza vaccine. Therefore, antibody responses to the vaccination were analyzed with respect to each biologic separately in addition to the group evaluation.

Materials and Methods

The influenza vaccine for the 2014/2015 season was obtained from Sanofi-Pasteur (Swiftwater, PA). Composition of the quadrivalent 2014/2015 season normal vaccine (QIV) was (A/California/07/2009 (H1N1), A/Texas/50/2012(H3N2), B/Massachusetts/02/2012, B/Brisbane/60/2008) with the high dose vaccine (HD-TIV) lacking the B/Brisbane/60/2008 component. The QIV vaccine contained 15*µ*g HA per each of the four viruses for a total of 60*µ*g HA for each 0.5ml dose. The HD-TIV contains 60*µ*g HA per virus strain for a total of 180*µ*g HA per 0.5ml dose. Reagents employed for the ELISA assays included anti-human IgG (IgG Fab fragment), Bovine Serum Albumin (BSA), Fish Gelatin, human IgG, anti-human IgG conjugated to peroxidase, and 3,3',5,5' Tetramethylbenzidine (TMB). All ELISA reagents were obtained from Sigma-Aldrich, St. Louis, MO. Goose erythrocytes employed for the hemagglutination assays were obtained from the animal care facility at the Wadsworth Center. ELISA plates were washed with an ELx405 Select CW plate washer from BioTek (Winooski, VT). ELISA plate results were read with an EL808 plate reader from BioTek (Winooski, VT).

Subjects and experimental design

The study included 88 volunteers: 37 were healthy age-matched controls (22 females and 15 males) and 51 were RA patients (39 females and 12 males). The RA patients were divided into groups depending on the biologic being given to them for treatment, abatacept (16) and TNF*α* blockers (35). Of the patients on tumor necrosis factor-alpha (TNF*α*) blockers, there were 8 on Humira (adalimumab), 8 on Remicade (infliximab), and 19 on Enbrel (etanercept). Sera were collected from each participant prior to the vaccination and at 4-6 weeks (Post-1) and 5-6 months (Post-2) after the vaccination. Participants were administered the QIV and HD-TIV vaccines intramuscularly according to the manufacturer's recommendation. All participants 65 and older received the HD-TIV vaccine. The study

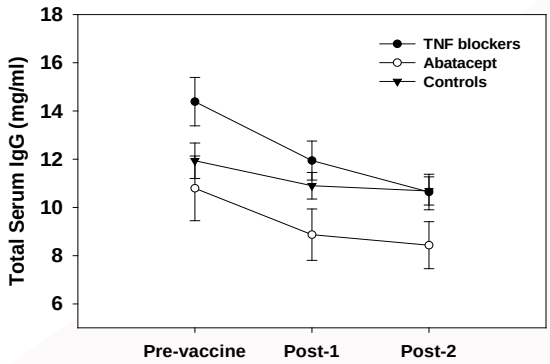

Figure 3: Total serum IgG levels before and after Flu vaccination. Sera IgG concentrations were measured by ELISA. The abatacept group had a somewhat lower serum IgG concentration than the TNFα blockers group. At the Post-1 and Post-2 time points the abatacept group had a slightly lower serum IgG than the control group as well.

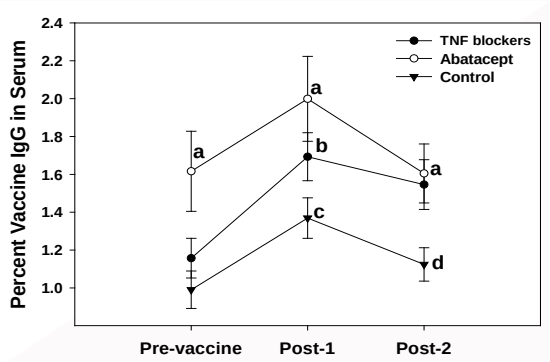

Figure 4: The percentage of serum anti-vaccine IgG based on the total serum IgG (anti-vaccine IgG/total IgG x 100). Student's *t*-test analysis with BH restrictions applied indicated that the abatacept group had a significantly higher serum vaccine-specific IgG percentage than the controls at each time point (a) pre-vaccination, Post-1, and Post-2. Significant increases in the percentage of serum vaccine-specific IgG were noted at Post-1 for the TNF*α* blockers (b) and the control group (c). The percent anti-vaccine IgG was lower in the controls than the TNF blocker group at Post-2 (d). Significance at $p \leq 0.05$.

was approved by the Bassett Health Center Internal Review Board (IRB protocol #1076) and each participant completed and signed a consent form. Vaccine-specific immunoglobulin G (IgG) Ab concentrations, total serum IgG concentrations, and Hemagglutination Inhibition (HAI) measurements were performed in a blinded fashion with only the subject ID and vaccine dose known to the analysts.

Total serum IgG assay

Total serum IgG was determined using a standard ELISA assay. Plates were coated with 1μg/well anti-human IgG in 100μl of 0.1M carbonate buffer, pH 9.6 overnight at 4-8°C. Blocking was performed at room temperature with 5% BSA in Phosphate Buffered Saline (PBS). Sera were diluted 1:50,000 with 2% BSA in PBS and applied to the blocked and washed plate in triplicate. Standard curves on each plate were made with human IgG. Detection of bound serum IgG was performed using anti-IgG gamma chain conjugated to peroxidase. Color development was obtained with TMB substrate and the process was stopped with the addition of 1 N H_2SO_4. Plate results were read at wavelengths 450nm and 570nm. Analysis of the absorbance results was performed using KC4, v3.4 software. Results were expressed as mg IgG/ml.

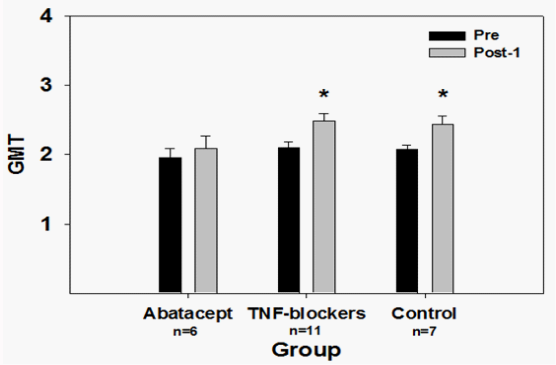

Figure 5: Hemagglutination Inhibition (HAI) assay. The individual patient's HAI titers from those receiving the HD-TIV vaccine is shown here. The plot shows Geometric Mean Titer (GMT) obtained for the Pre and Post-1 time points. As indicated the abatacept group had titers lower at Post-1 than those for the TNF*α* blockers and control groups. Significant increases in GMT between Pre and Post-1 are indicated by an asterisk,*. Significance at $p \leq 0.05$ by paired Student's *t*-test.

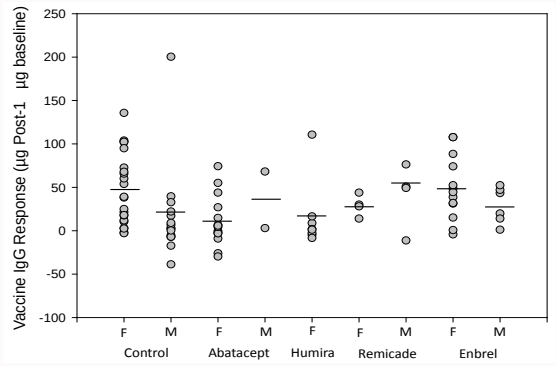

Figure 6: The individual differences in serum anti-vaccine IgG between pre-vaccination and Post-1 were plotted by sex and treatment. The results indicate that control females had a slightly higher increase in anti-vaccine IgG than control males (p=0.006 by Mann-Whitney rank sum test). Additionally, the increase in anti-vaccine IgG in the Enbrel group females was not significantly different from the control female group.

Influenza vaccine specific antibody assay

Serum IgG Ab directed toward the influenza virus vaccine was measured with a modified ELISA assay. Plates were coated with influenza vaccine (1μg HA/well) and human IgG (in duplicate wells) for the standard curve. Vaccine coating the plate was the QIV vaccine for those study subjects <65 years of age and the HD-TIV vaccine for those >65 years of age. Plates were blocked with 5% fish gelatin in PBS. Sera were diluted 1:1000 with 3% fish gelatin in PBS and applied to the blocked and washed plate in triplicate. The presence of flu-vaccine IgG was detected as indicated above for the total serum IgG.

Hemagglutination Inhibition (HAI) assay

This assay was performed in a 96-well round bottomed polypropylene plate with goose erythrocytes (50μl packed RBC+5ml PBS). Sera from the high dose vaccine group were serially diluted in the plate with PBS (1:50, 1:100, 1:200, 1:400, 1:800). An aliquot of 25μl HD-TIV vaccine diluted 1:500 in PBS was then added to all wells of the plate except those for serum only controls. Plates were shaken for 30min in a Lab-Line Instruments, Inc. (Melrose Park, NJ) shaker at setting 3. Diluted goose erythrocytes were added to each well, and the plate was again shaken for 3min. After 30min. a picture was taken of the plate to record the results.

Table 1: Descriptive characteristics of the study sample.

	Abatacept (n=16)		TNF blockers (n=35)		Control (n=37)		Total (n=88)	
	n	%	*n*	%	*n*	%	*n*	%
Male	2	12.5	10	28.6	15	40.5	27	30.7
Female	14	87.5	25	71.4	22	59.5	61	69.3
Regular vaccine dose	10	62.5	23	65.7	27	73.0	60	68.2
High vaccine dose	6	37.5	12	34.3	10	27.0	28	31.8
No flu vaccine past 5 years)	1	6.3	2	6.5	0	0	3	3.6
History of diagnosed flu	1	6.7	4	12.9	3	8.1	8	9.6
Age	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	60.9	10.9	59.0	9.4	57.7	8.7	58.8	9.4
Duration RA (years)	13.7	6.9	13.1	11.6	-	-	-	-
Duration current RA treatment (months)	58.6	28.8	77.1	45.0	-	-	-	-

Further HAI assays were performed on all the sera samples for pre-vaccination (1:40 dilution) and Post-1 and Post-2 sera (1:160 dilution). A control well composed of diluted sera and red cells (no virus) served as a negative control for hemagglutination. Partial inhibition was indicated by a red halo surrounding a diffuse red cell pellet, while tight red cell pellets were scored as complete inhibition. Geometric Mean Titers (GMT) were calculated based on the HAI results for each study group.

Statistical analysis

The major portion of the statistical analysis of the data was performed by Student's t-test comparisons with applied Benjamini-Hochberg (BH) restriction. Other statistical methods used were two-way Analysis of Variance (ANOVA) and Mann-Whitney Rank Sum Test. Analysis of the association between Ab concentrations and RA/treatment duration for each diagnostic group was performed by Pearson's correlation. Significance was indicated by $P \leq 0.05$. Analysis was performed with SAS software (Cary, NC) and in R (https://www.r-project.org/).

Results

Population characteristics

There were 88 participants in the study of which 51 were RA patients. The RA patients were divided into two groups, those receiving TNFα blockers (Enbrel, Humira, or Remicade) and those receiving abatacept. Table 1 gives a description of the study population. The mean age did not differ between the three study groups (58.8±9.4 years). Also, the duration since diagnosis of RA did not differ significantly between the TNFα blocker and abatacept groups (13.4±9.3 years). The duration of treatment for the TNFα blocker group was somewhat longer than that for the abatacept group. Table 2 lists other medications being taken by patients in the TNF blocker and abatacept groups. The majority of subjects in the treatment groups were female. Since RA is an autoimmune disease and females are more prone to autoimmune diseases it follows that a larger proportion of the RA patients would be female [19].

Vaccine-specific IgG data reveals significant differences from controls

Determination of anti-Flu IgG concentration at each time point, pre-vaccine, Post-1, and Post-2 showed a number of differences between the various groups (Figure 1). Student t-test analysis indicated a significant increase in antibody concentration between Pre-vaccine and Post-1 for the TNFα blockers group and controls. Further, a significant decrease between Post-1 and Post-2 was noted for TNFα blockers and controls. The anti-flu IgG levels for the abatacept group did not significantly differ among any of the time points. The IgG concentrations of the TNFα blockers group were significantly higher than controls at each of the three-time points. There was a statistically significant two-way interaction of diagnostic group*time (p=0.0446). The interaction can be characterized as disordinal, with a more moderate slope between Pre-vaccination and Post-1 between the abatacept and other two groups. A graph of the change in flu-IgG concentrations from baseline to Post 1 and Post 2 for each of the groups demonstrates that the abatacept group had a significantly lower change in vaccine-specific IgG production for each of the post-vaccination time points (Figure 2). An almost identical increase in vaccine-specific IgG was obtained for the TNFα blocker and control groups at Post-1. However, the TNFα blockers group showed a steeper decline in vaccine-IgG from Post-1 to Post-2 when compared to the control group.

Total serum IgG was slightly lower for the Abatacept group

Total serum IgG at the three time-points was slightly higher for the TNFα blockers group than for the controls or the abatacept group (Figure 3). Serum IgG concentrations decreased rather sharply from baseline to the Post-2 for the TNFα blockers group. However, this decrease was not significant when BH restrictions were applied. Alternatively, the abatacept group had somewhat lower serum total IgG concentrations at each time point compared to those for the TNFα blockers and at Post-1 and Post-2 compared to the control group.

Percentage of vaccine-specific IgG reveals further significant differences between groups

Determination of the percentage of the total IgG in the serum that was specific to the influenza vaccine revealed additional differences between the two RA groups and the controls (Figure 4). The concentration of vaccine-specific IgG in serum was highest at each time point for the abatacept group compared to the controls. Vaccine-specific IgG percentages were higher than controls for the TNFα blocker group for the Post-1 and Post-2 time points. There were no significant changes in percentage of flu-Ab between Post-1 and Post-2 for both the TNFα blockers and control groups. However, the TNFα blockers group maintained a somewhat higher percentage of vaccine-specific Ab at Post-2 than did either the abatacept or the control group.

Table 2: Number and percentage of other medications for subjects in each group.

Medication	All Subjects N (%)	Abatacept N (%)	TNF blockers N (%)	Control N (%)
Methotrexate	31 (35.2)	9 (56.3)	22 (62.9)	0
Prednisone	14 (15.9)	5 (31.3)	9 (25.7)	0
Hydroxychloroquine	2 (2.3)	2 (12.5)	0	0
Leflunomide	3 (3.4)	1 (6.3)	2 (5.7)	0
Sulfalazine	0	0	0	0

Table 3: Association between disease and treatment duration with respect to Flu-IgG[a,b].

	Post 1 (μg)	Post 2 (μg)	Ab Titer change from baseline Post 1[c]	Ab Titer change from baseline Post 2[c]
All RA Subjects Combined				
Disease duration	-0.0773	-0.0286	-0.0972	-0.0435
Current treatment duration	-0.0226	-0.0706	-0.2333	-0.2490
Abatacept				
Disease duration	0.1117	0.2709	0.0996	0.4057
Current treatment duration	-0.2269	-0.2298	0.0177	0.0416
TNF blockers				
Disease duration	-0.1735	-0.1881	-0.1274	-0.1250
Current treatment duration	0.0487	-0.0518	-0.3676*	-0.4088*

a) RA patients treated with the indicated biologics.
b) Results were obtained by Pearson's correlation.
c) The * represents significance, $p \leq 0.05$.

HIA revealed weak hemagglutination for the abatacept group ≥65 years old

Hemagglutination inhibition analysis was performed as a measure of Flu-vaccine specific anti-HA responses. All subjects in the study displayed complete inhibition at a serum dilution of 1:40 for the pre-vaccine titer. Serial serum dilutions were carried out to 1:800 for the first two time-points, pre-vaccine and Post-1, for members of the ≥65 yr group. A significant increase in the GMT between Pre and Post-1 was noted for both the TNFα blocker and control groups. However, for the abatacept group HAI titers remained near the baseline, i.e. pre-vaccination levels (Figure 5). Analysis of hemagglutination at 1/160 serum dilutions for Post-1 and Post-2 among those receiving the QIV vaccine showed mostly complete hemagglutination inhibition at this dilution and there was no significant difference from controls.

Change in vaccine-specific IgG between Pre and Post-1 for males versus females

The difference in flu-specific Ab concentration between pre-immunization and Post-1 was calculated for males and females separately and for each biologic (Figure 6). The results indicated that the female controls had slightly more of an Ab boost from the vaccination than did the males and this sex difference was consistent between the normal and high dose groups (data not shown). The TNFα blocker, Enbrel, was comparable to the controls with respect to proportionate increases in vaccine-specific IgG between females and males at Post-1.

Patient treatment duration and vaccine specific Ab is negatively correlated for TNF blockers

There was no influence of RA duration on the response to the vaccination according to analysis *via* Pearson's correlation (Table 3). The RA patients were analyzed in a combined group and individually for both the TNFα blockers and the T-cell activation blocker. There was a statistically significant negative correlation between duration of current treatment and change in vaccine-specific Ab titer from baseline at both Post-1 and Post-2 times for the TNFα blocker group ($p \leq 0.05$). This indicates that longer treatment duration was associated with a smaller increase in vaccine-specific Ab titer post-vaccination.

Discussion

Data presented in this report indicate that RA patients being treated with abatacept had a weaker response to the influenza vaccination than did the TNFα blocker and control study subjects, as shown by lower anti-Flu vaccine IgG production and lower hemagglutination inhibition compared to the 4-6 week post responses by the TNFα blockers and control subjects. Although the abatacept group had the highest serum percentage of their IgG as Ab binding to vaccine antigens in comparison tothe controls over the three time points, there was no significant increase from baseline in anti-vaccine IgG at 4-6 weeks after the vaccination. This is in contrast to the responses of those in the TNFα blocker and control groups, which displayed a relatively higher increase from baseline in anti-vaccine IgG after the vaccination at 4-6 weeks; however, 25% of the RA patients treated with abatacept (4 of 16) did display an increase equivalent to that of the controls, which suggests that additional patient demographics will need to be considered in the future. Also, the data indicated that any increase in percentage of vaccine-specific antibody produced by the abatacept group decreased over the next 5-6 months and was back to near baseline by the end of the flu-season. In contrast, the percentage of flu-vaccine specific Ab levels remained elevated over the entire influenza season for the TNFα blocker group.

With respect to the RA patients receiving the HD-TIV vaccine, the HAI titers of the TNFα blockers showed a significant increase 4-6 weeks post vaccination, while there was no significant increase in titer for patients being treated with abatacept. The HA1 titer increase for the TNFα blocker group was comparable to the control group, whereas the abatacept group values stayed close to baseline levels. Others have also reported a reduced immune response to influenza vaccination in RA patients being treated with abatacept [20,21]. Since

a major percentage of the RA patients in this study were being treated with etanercept (Enbrel) the similarity to control responses to the vaccination in the TNF blocker group is consistent with the finding that etanercept does not alter overall global immune functions [22]. HAI titers recorded as geometric mean titers were observed to be slightly lower than those of normal healthy controls for RA patients treated with TNFα blockers after receiving the influenza vaccine for the 2005/2006, 2006/2007, and 2007/2008 seasons [12,15]. Titers were closest to that of controls for the H1N1 virus, while H3N2 and B strains had a reduced HAI titer in the RA patients. A reduction in the protective immune response to the trivalent influenza vaccine for RA patients receiving TNFα blockers was observed for the H3N2 and B components [13]. This reduced HAI response was associated with a decrease in Antibody Secreting plasma Cells (ASCs), identified by Elispot assay at 1 month and 6 months post vaccination, in the RA patients treated with TNFα blockers compared to healthy controls. The present study indicates that RA patients receiving TNFα blockers had a comparable response to the 2014/2015 season vaccine to that of the healthy controls, although the antibody titer for the TNFα blocker group did decline somewhat faster than for the controls between time-points post-1 and post-2.

Abatacept, which inhibitsT-cell activation through selectively blocking the binding of CD80/CD86 on antigen presenting cells to the CD28 on T-cells (16),was recently reported to decrease peripheral $CD4^+CD28^+$ follicular helper-like T-cells in RA patients [23]. Abatacept was also shown to decrease the number of $CD4^+FOXP3^+$, $CD4^+Helios^+$, and $CD4^+CD39^+$ Treg cells in the peripheral blood at 3 months after the start of treatment in RA patients positive for Anti-Citrullinated Protein Antibodies (ACPA) [24]. The effects of abatacept on T-cells could be most detrimental to the elderly, since it has been found that T-cells are correlated with vaccine protection in the elderly [25]. The hemagglutination results presented in this report for the subjects over 65 years of age indicated that individuals being treated with abatacept had lower HAI titers after vaccination than the TNFα blockers group, with a major percentage of the subjects having titers between 1:50 and 1:100. To obtain a good response to a pathogen or a vaccine, antigen will be presented to a T-cell through interaction with an antigen presenting cell, such as a dendritic cell through the CD80/CD86 (APC) and CD28 (T-cell) co-stimulatory interaction. If this is not allowed to occur, T-cells would not get activated and will not stimulate B-cells to produce ASCs. In the case of influenza vaccination, the influenza-specific antibody secreting population in the blood after vaccination corresponds closely to the vaccine-specific increase in serum antibody [26]. The abatacept group did not have a strong flu vaccine-specific increase in serum IgG at Post-1, which is in line with the mode of action for this biologic in that abatacept compromises the T-cell/antigen presenting cell interaction.

Although the vaccine-specific IgG boost after the vaccination for the abatacept group was not significant, the overall percentage of vaccine-specific IgG in the serum was higher than that of controls at each time point. Hemagglutination results for the abatacept group did not differ significantly at baseline from either the TNF-blocker or control groups. Since the ELISA results are simply measuring antibody to the flu-virus proteins, these results include neutralizing and non-neutralizing IgG specific to the influenza virus. It would seem then that the abatacept group sera contained more non-neutralizing IgG than the sera from the TNF-blocker and control groups. It has been reported that subjects with relatively high levels of initial flu-specific antibody show a very small increase in antibody after vaccination [27]. Moreover, this high percentage of virus specific antibody would likely be non-neutralizing, such as IgG against denatured protein, conserved viral protein regions, or virus strain HA proteins from previous vaccinations. Therefore, the high percentage of vaccine-specific IgG in the sera from the abatacept group would not mean better protection for these individuals. High flu-virus specific antibody concentrations in RA patient's sera may be due to B-cell expansion in these individuals. Sequencing of the V-gene repertoire of B cells from RA synovial tissue revealed an intensive expansion of select B cell clones [28]. It was suggested that the somatic diversification of the B cell clones could have taken place in peripheral secondary lymphoid organs. Intraclonal diversity of the V-gene repertoire within ASCs derived from these B cell clones revealed continuous activation of the B cell clones over relatively long-periods of time. In RA patients, a portion of the ASCs generated by this process could be specific to the influenza virus due to yearly influenza vaccination or infection by the virus. The ASCs and Antigen-specific B Cells (ABCs) have a long survival time and migration of these cells through peripheral tissues including the blood would result in generally elevated levels of virus specific antibody [29,30]. Long-lived memory B cells have the potential to accumulate dysfunctional organelles, such as mitochondria [31]. To maintain healthy memory B cells a functioning autophagy system is essential. In the absence of an efficient autophagy system oxidative stress and membrane lipid peroxidation would promote cell death. Perhaps, memory B cell clones generated in the RA patients may further be maintained in the patients being treated with the biologics through reduction of mediators of inflammation and oxidative stress. An evaluation of RA patients who would be good responders to abatacept revealed that individuals with a relatively high memory B-cell count had a good response to treatment with abatacept [32]. Therefore, development of memory B-cell clones in RA patients, generation of diverse plasma cell populations from these clones, and maintenance of these clones *via* decreasing oxidative stress could cause the serum flu-specific IgG to be elevated over controls in patients treated with abatacept.

The present study found that female controls had a slightly higher increase in vaccine-specific antibody at 4-6 weeks post vaccination than did the males. A higher antibody titer after the influenza vaccination in females compared to males has been reported by others [33-36]. Females presented a greater vaccine efficacy as indicated by fewer diagnosed cases of influenza viral infection. Differences in response to the influenza vaccine have been suggested to be due in part to sex steroid hormones. Many immune cells possess estrogen receptors. These cells include lymphocytes, macrophages, and dendritic cells. The involvement of estrogen receptors on innate immune system cells has recently been examined [37-39]. Estrogen-dependent activation of estrogen receptors regulates the development and function of select dendritic cell subsets. Estrogen receptor signaling promoted the development of conventional and plasmacytoid dendritic cells. In the present study, the increased antibody production by the females in the control population was not observed in the RA patients treated with abatacept. Estrogen regulation of the dendritic cell population causing increased antibody production in the females could be blocked by abatacept, since in this case the function of the dendritic cells in respect to activating T-cells would be hampered.

It was noted that total serum Ab concentrations decreased over each time point for each group. This could be due to seasonal variation in B-cells counts. It has been reported that B-cell counts are high in winter and low in summer [40]. Also, a study concerning

the safety of influenza vaccination of RA patients undergoing TNFα blocker treatment reported percentages of B-cells for RA patients and controls as decreased at the 6 month time point, which would be near the end of the flu season-in spring [15]. A study employing gene expression profiles of white blood cells during each month of the year determined that lymphocyte counts are lower in spring and early summer [41]. It is possible that this seasonal variation in B-cell counts could result in lower total serum IgG in spring compared to winter and could be the reason for the decrease in serum IgG at each time point observed in this study.

Summary

Results presented here indicate that the treatments of RA patients with TNFα blockers do not compromise the immunological response to the yearly influenza vaccination. However, vaccine-specific IgG was lower at the end of the season in this group compared to healthy controls. RA patients treated with an inhibitor of T-cell activation (abatacept) did seem to have a compromised immune response to the flu vaccine. Although response to the vaccine was low for this group, vaccination is recommended, since most subjects receiving abatacept had some small boost in IgG at 4-6 weeks post. Interestingly, subjects in both the TNFα blocker and abatacept groups had a higher percentage of flu-specific serum antibodies than did the control subjects and an argument was presented as to how this could occur. Separation of the groups into males versus females suggested that the females within the control group had a slightly higher IgG response to the vaccination than did the males. The TNFα blocker, Enbrel, showed a similar trend to the controls in this respect. Present study patient numbers are low and, therefore, the findings regarding males versus females should be considered preliminary. More studies with larger patient numbers would be able to prove or disprove the later findings regarding the influence of the biologics on sex differences in IgG production after vaccination.

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