

Measurement of Cellular Immunity to Influenza Vaccination in Rheumatoid Arthritis; Comparison of Three Assays

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Abstract

Objective: Monitoring of immune responses is essential in the care of immunosuppressed individuals, including rheumatic patients. Evaluation of cellular immunity is essential for confirming virus-specific effector cell functions, but it is poorly standardized, and suffers from technical limitations and inaccurate results. There is, therefore, a need for reliable techniques for assessing cell-mediated immunity. In this study we compared the cell-mediated immunity response to influenza vaccine between a population of Rheumatoid Arthritis (RA) patients and healthy subjects by three methods.

Methods: Trivalent influenza subunit vaccine was administered to 18 RA patients who were taking disease-modifying antirheumatic drugs and to 18 healthy controls. Peripheral Blood Mononuclear Cells (PBMCs) and sera were obtained immediately before and ~28 days after vaccination. Cell-mediated immunity responses to vaccination were evaluated by (1) flow cytometric analysis of IL-2/IFN- γ production in activated CD4/CD8 T-cells, (2) enzyme-linked immunosorbent assay for the analysis of IFN- γ secretion, and (3) Granzyme B activity assay. Humoral response was evaluated by the hemagglutination inhibition assay.

Results: Vaccination induced a significant increase in PBMC IFN- γ secretion and Granzyme B activity in the RA patients. Granzyme B activity also significantly increased in the controls, but there was no change in the levels of secreted IFN- γ . No group differences in the frequencies of IFN- γ /IL-2-producing activated CD4/CD8 T-cells were observed by flow cytometry. The geometric mean of hemagglutination inhibition antibody titers increased significantly for the H1N1/H3N2 influenza strains in both groups.

Conclusions: Granzyme B activity assay was the only method to detect a significant cell-mediated immunity response in both groups while significant increase in IFN- γ secretion was demonstrated only in RA patients. Flow cytometric analysis failed to show IL-2 and IFN- γ production in both groups. Currently available methods for measuring cellular responsiveness to influenza vaccination are inconsistent and limited in their ability to reflect acquired cellular immunity.

Keywords: Influenza; Inactivated influenza vaccine; Rheumatoid arthritis; Cell-mediated immunity

Introduction

Patients with autoimmune inflammatory rheumatic diseases, such as Rheumatoid Arthritis (RA), have approximately twice the risk of infectious diseases compared with the normal population [1,2]. This increased susceptibility may be attributed to both disease-associated immunoregulatory imbalances, as well as to chronic use of immunosuppressive drugs [3]. One of the key mechanistic elements that leads to the increased vulnerability to infection is the inhibitory effect of disease-modifying antirheumatic drugs (DMARDs) on lymphocyte proliferation and the associated lymphocytopenia [4]. In addition, the lymphocyte counts of DMARD-treated patients may decrease to levels comparable with those of HIV patients [4].

Influenza is a contagious respiratory illness that is considered vaccine-preventable. Influenza vaccination was shown to reduce flu-related illness, hospitalizations and deaths in healthy populations, even

when it afforded only moderate (~60%) effectiveness [5]. The European League against Rheumatism (EULAR) recommends that the administration of inactivated influenza vaccination should be strongly considered for patients with rheumatic disease [6], even those who are treated with DMARDs or tumor-necrosis factor (TNF) inhibitors (with the exclusion of the B-cell depleting agent, rituximab). In most trials upon which the recommendations were based, efficacy was assessed by humoral responsiveness, which is currently considered the gold standard for seasonal influenza vaccines. However, in the case of virus-related illnesses, such as influenza, cellular immunity is another important pathway of response, since the ability of vaccines to induce cell-mediated immunity (CMI) is believed to be important in preventing severe disease and in enabling long-term immunological memory [7].

Both CD4 and CD8 T cells have been reported to play roles in the clearance of primary influenza virus infections. These cells can develop into highly active effector populations that migrate to the lung in large numbers. This CMI is characterized by stimulation of T cell cytokine production [8]. Several works have demonstrated that memory T cells

may also provide long-term heterotypic protection against distinct Influenza virus [9-12]. The evaluation of CMI responses is, however, poorly standardized, requires specialized equipment and often suffers from technical limitations that eventually lead to inaccurate and inconsistent results.

We compared the CMI response to influenza vaccination as assessed by three common methods of measuring cellular immunity, flow cytometric analysis (FCA) of cytokine production, ELISA assay of IFN- γ secretion and the Granzyme B activity assay in RA patients and healthy subjects and determined the agreement between those methods. We also concomitantly evaluated these patients' humoral immune responses.

Materials and Methods

Study design

The study was approved by the institutional medical ethics committee, and written informed consent was obtained from all participants. Eighteen consecutive RA patients and 18 apparently healthy volunteers were included in the study. The RA patients were being treated with conventional DMARDs, mostly Methotrexate (MTX). All subjects were examined twice: the first time at study entry, when they were vaccinated against seasonal influenza, and the second time 4 weeks later. The venous blood samples that were taken from all participants at both visits were used for measuring the complete blood count and the erythrocyte sedimentation rate, the anti-sera were used for measuring the antibody response by the Hemagglutination Inhibition (HI) test. The peripheral blood mononuclear cells (PBMCs) in these samples were separated. Information on the use of other medications, the influenza vaccination status in the previous year and any adverse reactions to the vaccination were recorded.

Influenza vaccine

VAXIGRIP® (Inactivated Influenza Vaccine Trivalent Types A and B [Split Virion]) 2010/2011 season influenza vaccine, surface antigen, inactivated (Sanofi Pasteur SA, Lyon, France), including A/California/7/2009 (H1N1)-like virus, A/Perth/16/2009 (H3N2)-like virus and B/Brisbane/60/2008-like virus, was administered to all participants during October-December, 2010.

PBMC isolation

The PBMCs were isolated from heparinized venous blood by density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) immediately after blood was drawn. The cells were then washed with phosphate-buffered saline (PBS), resuspended at 107 cells/ml in fetal calf serum supplemented with 10% DMSO, transferred to pre-chilled 2 ml cryovials and cryopreserved in a Nalgene freezing container providing a controlled freezing rate of 1°C/min at -80°C. After 1-3 days, the cryovials were transferred to the vapor phase of a liquid nitrogen cryo tank. The cells were thawed by prompt resuspension at 1.5 × 106 cell/ml in RPMI 1640 supplemented with heat-inactivated 10% fetal calf serum, penicillin 100U/ml, streptomycin 0.1 mg/ml, and 2 mM L-glutamine (Biological Industries, Israel), then counted before further treatment.

Flow cytometric analysis (FCA)

The PBMCs were cultured at 0.16 ml/well in 96-well plates (0.5 × 106 cells/well) and stimulated with an influenza antigen mix (final concentration of 1.5 µg/ml), consisting of equal portions of β -propiolactone inactivated whole virus of H1N1 A/Brisbane/59/2007 (TGA 2008/81B), H3N2 A/Uruguay/716/2007 (A/Brisbane/10/2007-like, NIBSC 8/124) and B B/Brisbane/60/2008 (TGA 2009/82B). Cells were cultured in medium only for negative control. Stimulation with 5 µg/ml Staphylococcal enterotoxin B (SEB) (Sigma Aldrich, St. Louis, MO) served as a positive control. The PBMCs were incubated for 18 hours at 37°C 5% CO₂, and monensin (Sigma Aldrich) at a final concentration of 1 mM was added for the final 16 hours. Following incubation, the cells were washed with cold phosphate-buffered solution (PBS) blocked in 10% newborn calf serum (NBCS)/PBS, centrifuged and resuspended in blocking buffer containing phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD69, GFP-conjugated anti-CD8 and allophycocyanin (APC)-eFluor 780-conjugated anti-CD3 (eBioscience Inc., San Diego, CA) followed by 15 minutes of incubation on ice in the dark. The cells were then washed in blocking buffer and further incubated for 30 minutes in fixation/permeabilization buffer (Foxy3 Staining Buffer Set, eBioscience Inc.) at 37°C in the dark. The cells were washed three times with permeabilization buffer, centrifuged and resuspended in 10% NBCS/permeabilization buffer containing APC-conjugated anti-IFN- γ and PE-conjugated anti-IL2 (eBioscience Inc., San Diego, CA) for 30 minutes of incubation on ice in the dark. The cells were washed and analyzed on a BD FACSCanto II flow cytometer (Becton Dickinson). Lymphocytes were gated by forward and sideward scatters patterns, followed by gating of CD3+/CD8+ (Figure 1A). Among those population CD69± IFN- γ ± and CD69±IL2- γ ± quadrants were set according to the unstimulated controls.

ELISA assay of IFN- γ secretion from PBMCs: The PBMCs were cultured at 0.5 ml/well in 48-well plates (1.8 × 106 cells/well) and stimulated with an influenza antigen mix and SEB as described above. The secreted IFN- γ concentration was measured by an ELISA kit (Peprotech, Rehovot, Israel) according to the manufacturer's instructions.

Granzyme B activity assay: The PBMCs were cultured at 0.5 ml/well in 48-well plates (1.8 X 106 cells/well) and stimulated with an influenza antigen mix and SEB as described above. The cells were lysed in lysis buffer (150 mM NaCl, 15mM Tris, 1% Triton x100), then stored at -760C. Granzyme B activity was measured according to the protocol described by Gijzen et al. [13]. Briefly, frozen cell lysates were subjected to three freeze/thaw cycles to enable the release of Granzyme B. Recombinant Granzyme B standards (Enzo Life Sciences International, Inc., PA) and cell lysates were added in duplicate to a 96-well plate (20 µl/well). The reaction was started upon the addition of 80 µl of substrate solution containing 400 µM of Ac-IEPD-pNA substrate (Calbiochem, Darmstadt, Germany) in assay buffer (100 mM HEPES pH 7.5, 10% (w/v), sucrose, 0.1% (w/v) CHAPS, and 10 mM DTT (Sigma Aldrich, Rehovot, Israel). The plate was sealed, covered and incubated in a dark humidified chamber at 370C for 20 h. After incubation, the plate was read at 405 nm. Granzyme B units were calculated using a 4th order polynomial curve with a log(concentration)-log (absorbance) plot, and corrected for protein concentrations by the BCA protein assay (Thermo Scientific, IL).

Humoral response: The antibody response was measured by the HI test according to a standard WHO procedure as previously described [14]. The titer of an antiserum not showing any inhibition was

recorded as 1/10. Humoral response was defined as either a fourfold or greater rise in the titer of HI antibodies, or a rise from a non-protective baseline level (<1/40) to ≥ 1/40). Geometric mean titers of antibodies were calculated to assess the immunity of the whole group.

Statistical analyses: Non-parametric tests were used for the analysis since most parameters were not normally distributed (based on the Shapiro-Wilk test). Parametric tests were performed for the log transformation of the parameters. Any two groups were compared using Student's t-test, the Mann-Whitney U non-parametric test and the Wilcoxon signed rank non-parametric test as applicable. Comparisons of the proportion of responders in the patient and control groups were assessed with Fisher's exact test. Statistical analyses were carried out using the IBM SPSS statistics system for Windows, release 20.

Results

Characteristics of RA patients and healthy controls: The 36 study participants included 18 RA patients and 18 healthy controls. The male-to-female ratio was similar between the groups, while the mean age of the controls was significantly lower than that of the RA patients ($p=0.014$, Table 1). At the time of vaccination, all RA patients were being treated with at least one conventional DMARD (MTX, 10 patients, 55.5%), prednisone (4 patients, 22.2%) and Plaquenil (6 patients, 33.3%).

	RA patients (n=18)	Healthy controls (n=18)
Age mean ± SD in years	60.4 ± 13.8	46.4 ± 18.2
Female:Male ratio	1:1.6	1:2
Duration of RA, y, median (range)	12 (2-34)	
DASb median (range)	4.4 (1.26-7.5)	
Methotrexate, n (%)	10/18 (55.5)	
Prednisone, n (%)	4/18 (22.2)	
Plaquenil, n (%)	6/18 (33.3)	
aRA, rheumatoid arthritis; bDAS, disease activity score		

Table 1: Baseline characteristics and disease parameters in RA patients and healthy control subjects.

FCA of IL-2- and IFN- γ -producing CD4+/CD8+ cells: The FCA of CD4/CD8 T cell cytokine production was performed before and 4 weeks after the influenza vaccination (Figure 1). Influenza-specific cells were defined as the percentage of CD69+/IFN- γ - and CD69+/IL-2- producing cells within the total CD8 and CD4 T cell population in the antigen-stimulated cultures subtracted by those in the negative control cultures. In general, the mean percentage of cytokine-producing T cells (CD4 and CD8) was higher among the controls compared to the patient group, however not to a level of significance. Also, there was no significant difference in the frequencies of IFN- γ /IL-2-producing activated CD4 or CD8 T-cells, either before and 4 weeks following influenza vaccination.

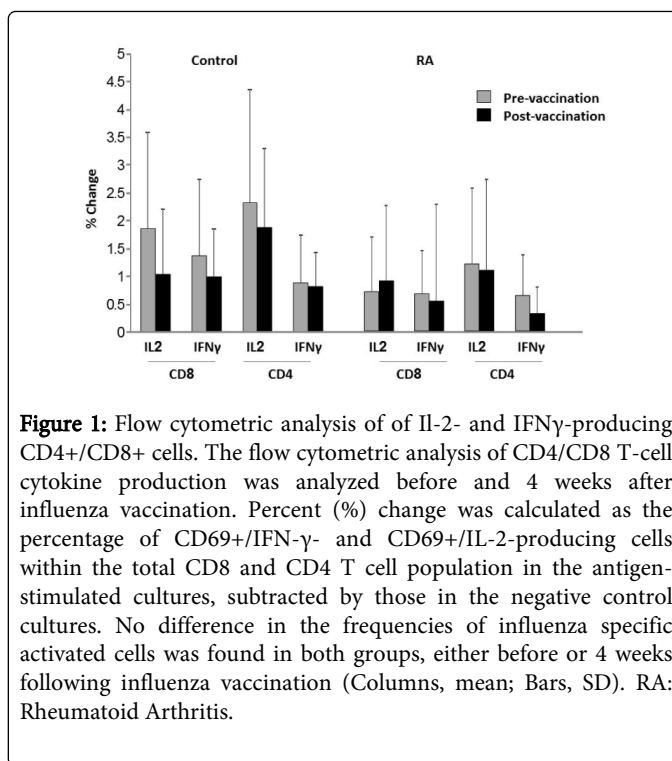


Figure 1: Flow cytometric analysis of of IL-2- and IFN γ -producing CD4+/CD8+ cells. The flow cytometric analysis of CD4/CD8 T-cell cytokine production was analyzed before and 4 weeks after influenza vaccination. Percent (%) change was calculated as the percentage of CD69+/IFN- γ - and CD69+/IL-2-producing cells within the total CD8 and CD4 T cell population in the antigen-stimulated cultures, subtracted by those in the negative control cultures. No difference in the frequencies of influenza specific activated cells was found in both groups, either before or 4 weeks following influenza vaccination (Columns, mean; Bars, SD). RA: Rheumatoid Arthritis.

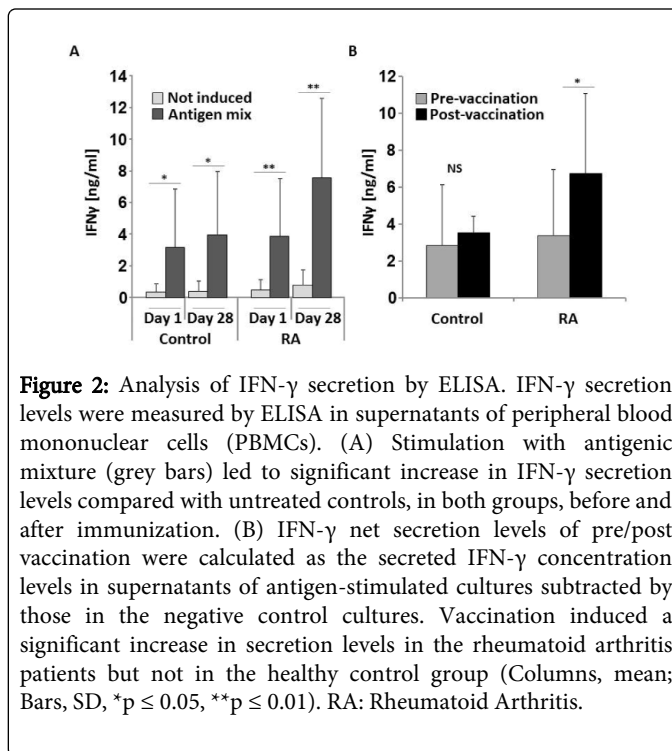


Figure 2: Analysis of IFN- γ secretion by ELISA. IFN- γ secretion levels were measured by ELISA in supernatants of peripheral blood mononuclear cells (PBMCs). (A) Stimulation with antigenic mixture (grey bars) led to significant increase in IFN- γ secretion levels compared with untreated controls, in both groups, before and after immunization. (B) IFN- γ net secretion levels of pre/post vaccination were calculated as the secreted IFN- γ concentration levels in supernatants of antigen-stimulated cultures subtracted by those in the negative control cultures. Vaccination induced a significant increase in secretion levels in the rheumatoid arthritis patients but not in the healthy control group (Columns, mean; Bars, SD, * $p \leq 0.05$, ** $p \leq 0.01$). RA: Rheumatoid Arthritis.

IFN- γ secretion from PBMCs: IFN- γ secretion levels were measured by ELISA in the supernatants of PBMCs that were stimulated with either an influenza antigen mixture or with SEB, or left untreated. Stimulation of both groups with an antigenic mix led to a significant increase in secreted IFN- γ concentrations from PBMCs compared with the untreated control secretion levels before as well as at four weeks post-vaccination (Figure 2A). However, when the pre-

vaccination net secretion levels were compared to the post-vaccination ones (defined as the IFN- γ concentrations in the stimulated cultures subtracted by those in the negative control cultures), the vaccination was found to induce a significant increase in PBMC IFN γ secretion levels only in the RA patients, and not in the healthy controls (Figure 2B).

Granzyme B response: A Granzyme B standard curve was plotted as described in the Materials and Method section, with a standard line showing a squared correlation coefficient (R^2) equal to 0.9997 in the range of 0-20 units (Figure 3A). Both groups showed a significant increase in Granzyme B activity levels in response to antigenic mixture stimulation at 4 weeks post-vaccination. Interestingly, no stimulation-induced increase in activity was observed on the day of vaccination (Figure 3B). A comparison of the pre- and post-vaccination net secretion levels (defined as the Granzyme B activity units/mg protein in the stimulated cultures subtracted by those in the negative control cultures) revealed a significant increase in Granzyme B activity in both the RA patients and healthy controls (Figure 3C).

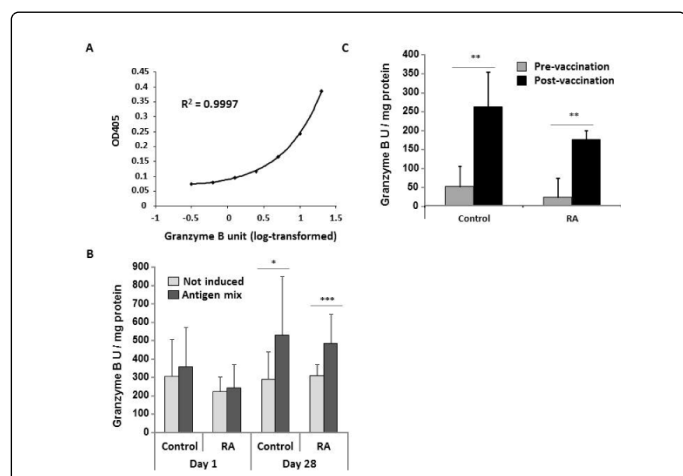


Figure 3: Granzyme B activity assay. Granzyme B activity assays were measured in cultures of peripheral blood mononuclear cells (PBMCs) using a colorimetric substrate. A. The Granzyme B standard curve was plotted as described in the Materials and Method section. B. Stimulation with antigenic mixture in both groups led to an increase in Granzyme B activity compared with untreated controls 4 weeks post-vaccination. C. Comparison of pre- and post-vaccination net secretion levels (defined as the Granzyme B activity units/mg protein in the stimulated cultures subtracted by those in the negative control cultures) revealed a significant increase in Granzyme B activity in both rheumatoid arthritis patients and healthy controls (Columns, mean; Bars, standard deviation; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p = 0.068$). RA: Rheumatoid Arthritis.

Immunogenicity of the vaccine: Both the RA patients and the controls displayed a significant increase in geometric mean titers of HI antibodies against two of the three vaccination serotypes at 4 weeks post-vaccination (Figure 4A). The geometric mean titers (GMTs) of A/California/7/2009 (H1N1) increased from 12.97 to 86.21 ($p = 0.01$) for the controls, and from 24.26 to 196.16 ($p = 0.005$) for the RA group. The GMTs of A/Perth/16/2009 (H3N2) increased from 25.9 to 149.11 ($p = 0.005$) for the controls, and from 18.18 to 211.72 ($p = 0.0004$) for the RA patients. The GMTs of the third serotype, B/Brisbane/60/2008-

like, increased for both groups (from 293.5 to 491.99 for the controls, and from 378.26 to 740.55 for the RA patients), but this increase did not reach a level of significance. There were no significant differences between the GMTs before and after vaccination between the patients and controls for each serotype examined.

Seroprotection and humoral response to vaccination: Seroprotection and response rates were calculated as described in the Materials and Methods section (Figures 4B and 4C). The pre-vaccination seroprotection rates against the B strain were high for both groups tested (94.4% and 100% for the controls and RA patients, respectively). This finding was in agreement with previous studies performed in our group [14-17].

The rate of responders to the B serotype was very low (16.67% for both groups). The response rates to H3N2 was identical for both groups (50%), while the response rate to the H1N1 was significantly higher in the patient group (77.8% Vs 38.9% for the controls, $p = 0.04$).

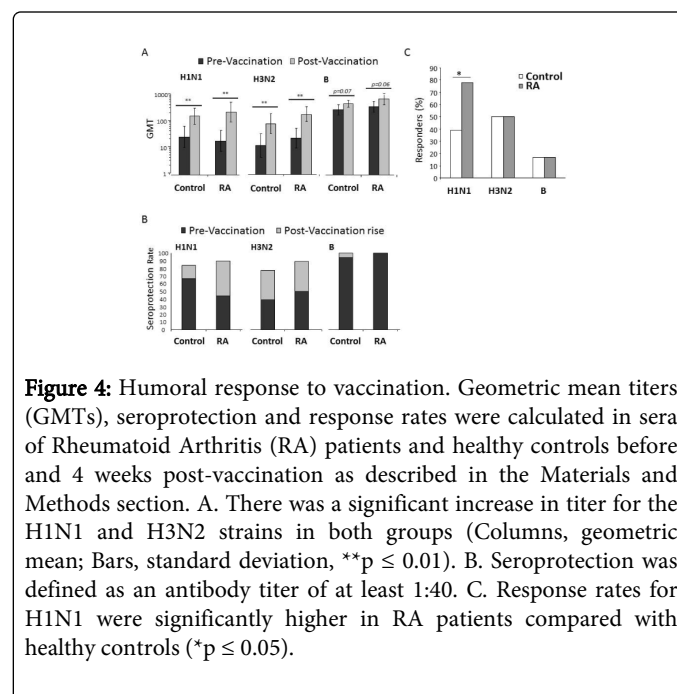


Figure 4: Humoral response to vaccination. Geometric mean titers (GMTs), seroprotection and response rates were calculated in sera of Rheumatoid Arthritis (RA) patients and healthy controls before and 4 weeks post-vaccination as described in the Materials and Methods section. A. There was a significant increase in titer for the H1N1 and H3N2 strains in both groups (Columns, geometric mean; Bars, standard deviation, ** $p \leq 0.01$). B. Seroprotection was defined as an antibody titer of at least 1:40. C. Response rates for H1N1 were significantly higher in RA patients compared with healthy controls (* $p \leq 0.05$).

Discussion

In the present study, we evaluated the cellular and humoral immune responses to seasonal influenza vaccination among patients with RA and compared them with the responses of a healthy control group. Humoral immunity was measured by the detection of influenza-specific antibody titer using the HI test pre- and post-vaccination. A significant increase in the GMT of HI was demonstrated for the H1N1 and H3N2 influenza strains in both groups, and pre-vaccination protective titers for the B influenza strain were also observed for both groups. These results confirm a preserved humoral responsiveness in rheumatic patients, as had been shown by us in earlier studies [14-17].

We compared the extent of cell-mediated immunity by using three different methodologies, FCA of IFN- γ /IL-2-producing activated CD4/CD8 T-cells, ELISA assay of IFN- γ secretion and the Granzyme-B activity assay. Our results showed no significant change in the frequencies of IFN- γ /IL-2-producing activated CD4 or CD8 T cells

before and after vaccination in both groups when we used the FCA approach. Vaccination did induce a significant increase in PBMC IFN- γ secretion, as measured by ELISA, in the RA patient group but not in the control group. In contrast, there was a significant post-vaccination increase in Granzyme B activity in both groups, suggesting that this method is probably the most sensitive of the three in the evaluation of cellular response to seasonal influenza vaccine.

Taken together, these results reveal considerable inconsistency between three currently available methods for the measurement cell-mediated immunity. Unexpectedly, FCA was the least sensitive among them. This is especially interesting since it is the one most commonly used in the clinical setting and also the most complicated one among the three since it requires expensive and complex instrumentation and demands considerable expertise to operate. Intracellular cytokine staining by FCA is one of the main assays currently used to monitor CMI responses. It is a powerful method that allows multiparametric, high-speed evaluation of unique subsets of cells that are otherwise difficult to distinguish [18]. Such detailed sub-setting of cells provides accurate analysis of immunological responses, especially when it is important to know what kind of response had been generated or in order to investigate the mechanism of the immunological response [19]. However, due to the rather low sensitivity of the technique, rare cell populations, such as antigen-specific T cells, are often not easily detected. Moreover, cytokine synthesis by cells is not constitutive, their intracellular concentrations are relatively low, and the kinetics of coexpression for different cytokines may vary. Thus, accurate detection may be difficult at relatively low frequencies of target cells [20]. Another possible explanation for the relatively low T cell response observed by FCA in immunocompromised patients is the choice of the type of vaccination. The most currently used influenza vaccines are the trivalent inactivated ones that consist of either a split virus or its surface glycoproteins, hemagglutinin and neuraminidase [21]. These vaccines induce a predominantly antibody response and lead to limited protection, especially in elderly or immunocompromised populations [22,23], presumably due to relatively poor cytotoxic T cell response. In contrast, the alternative live attenuated influenza vaccines were shown to induce a robust type 1 immunity response, characterized by IFN- γ production and T cell recruitment [24]. However, these vaccines are not recommended by most published guidelines, including the EULAR guideline, for vaccination in adult patients with autoimmune inflammatory rheumatic diseases due to risk of infection [6].

Measurement of secreted IFN- γ in PBMC supernatants by ELISA is another common technique routinely used to detect anti-viral T-cell response. This method is highly quantitative, it can measure a broad range of concentration, it is cost-effective and easy to operate. Indeed, our current ELISA results revealed that PBMC stimulation with an antigenic mix led to a significant increase in secreted IFN- γ levels compared with the untreated PBMCs. However, no significant changes were found when we compared the pre- and post-vaccination values in the control group. IFN- γ is a cytokine that plays a central role in promoting both innate and adaptive anti-viral immune responses by being released from Natural Killer (NK) and Natural Killer T (NKT) cells and by CD4 Th1 and CD8 cytotoxic T lymphocyte cells, respectively [25,26]. It is therefore possible that, at least in part, the measured IFN- γ response was due to NK cells, as part of an innate immunity response.

Among the three methods tested in the current study, Granzyme B activity assay was the only one to detect an influenza-specific cellular

immune response in both the RA and control groups. Granzymes are serine proteases that mediate caspase-cascade activation in virus-infected cells upon release from their effector cells, cytotoxic T cells and NK/NKT cells, and thus lead to the programmed cell death of their target [27]. The use of Granzyme B activity as a marker of cell-mediated cytotoxicity has been evaluated before, and found to be specific, robust and in correlation with traditional measures of cytolytic activity [28,29]. In combination with antibody titers, Granzyme B activity levels were shown to predict vaccine efficacy in elderly populations [13,30]. They were also found to be the main contributor to cytotoxic T cell-mediated antiviral response in mice [31].

Our study has several limitations. The study groups were not matched for age, with the healthy controls being approximately 15 years younger than RA patients ($p < 0.05$). Therefore, mismatching may have confounded our results, as such that older subjects have lower vaccine responses, but greater preexisting influenza exposure. Still, this mismatching does not influence the comparison between the different assays. A power calculation was not performed prior to the study since the variability of the assays was not known and blood samples from only 18 patients were available for the analyses. Hence, it is possible the study was underpowered to detect a difference in T cell responses by FCA or IFN- γ ELISA. Other methods that we did not assess include ELISPOT analysis, a very sensitive assay that measures the numbers of cells that respond by secreting cytokines, and real-time PCR analysis, to quantify mRNA transcripts of response genes. These assays were not included since they are time consuming, expensive and require considerable expertise to be performed accurately and reproducibly, therefore limiting their usefulness in many scenarios.

In summary, we believe this to be the first study that compared the cellular response to influenza vaccination among RA patients as measured by three different methods. The results of this study confirm previous findings on the preserved humoral immune response of RA patients to influenza vaccination. The agreement between the cellular response to the humoral response was shown only by the Granzyme B activity assay but not by the ELISA or the FCA methods we had utilized to measure cellular immune responsiveness.

Author's Contributions

NMB participated in the design of the study, carried out the experiments, participated in the analysis and interpretation of data, performed the statistical analysis and drafted the manuscript. UA participated in the design of the study, helped to carry out the experiments, participated in the analysis and interpretation of data and helped to draft the manuscript. SA participated in the design of the study and in the collection of the samples. MM and EM performed and analyzed the hemagglutination inhibition assay. DC participated in the design and coordination of the study and helped to draft the manuscript. OA conceived of the study, participated in the design and coordination of the study and helped to draft the manuscript.

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