Vaccine 37 (2019) 3685–3693

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Altered proportions of circulating CXCR5+ helper T cells do not dampen influenza vaccine responses in children with rheumatic disease

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ARTICLE INFO

Article history:
Received 24 January 2019
Received in revised form 5 May 2019
Accepted 11 May 2019
Available online 22 May 2019

Keywords:
Influenza vaccination
Pediatric rheumatic disease
Biological therapy
Follicular helper T cells
CXCL13

ABSTRACT

Biological therapy options for the treatment of rheumatic disease target molecules that can affect the cross-talk between innate and adaptive immune responses upon vaccination. Influenza vaccination in children with rheumatic disease has been recommended, but there are only sparse data on the quality of vaccine responses from pediatric patients treated with biological therapy.

We conducted an influenza vaccine study over 3 consecutive seasons where the antibody response to TIV was evaluated in children with PRD (n = 78), including both non-treated (n = 17) and treated (with methotrexate, TNF-inhibitors with or without methotrexate, or IL-inhibitors, n = 61) children as well as healthy age-matched controls (n = 24). Peripheral B cells, T and NK cell populations, as well as CXCR5+ (follicular) helper T cells (T_{FH}) and chemokines involved in antibody responses were assessed prior to immunization in the same cohort. Data on disease duration, therapy and data on previous influenza vaccinations were retrieved.

The proportion of circulating T_{FH} cells were significantly lower in non-treated children with PRD compared to treated patients and healthy controls. The significantly lower proportion of T_{FH} cells was mirrored by a marked significant increase in CXCL13 serum level, the ligand for CXCR5, with higher levels in non-treated children with PRD compared to treated patients and healthy controls. However, the proportion of T_{FH} cells or CXCL13 level at the time of vaccination was not a predictor of the antibody response to TIV in this cohort of children. Children with PRD had an overall similar response to TIV as healthy children. Although not significant, children treated with TNF-inhibitors differed as a few children remained seronegative towards H3N2- and influenza B viruses after immunization.

Our data show that children with PRD respond to TIV as healthy children. Furthermore, plasma CXCL13 levels did not correlate to the proportion of T_{FH} cells in blood prior to immunisation, or to antibody responses following immunization.

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1. Introduction

Vaccination is a major cornerstone of medicine and numerous vaccines are included in national childhood immunization programs world-wide. Recommendations for vaccination of children with paediatric rheumatic diseases (PRD) were published by the European League Against Rheumatism (EULAR) in 2011 [1] and common childhood vaccines were recommended to be given as in healthy children. In addition, as children with PRD may be at risk for severe influenza virus infection and associated complications, it was also suggested that yearly influenza virus immunizations should be considered for children with PRD irrespective of immunosuppressive treatment modality.

PRDs are autoimmune diseases that occur before 16 years of age, and include juvenile idiopathic arthritis (JIA), juvenile dermatomyositis (JDM) and juvenile lupus erythematosus (JSLE). The introduction of disease-modifying anti-rheumatic drugs (DMARDs) with chemotherapeutic agents made a great contribution to the

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https://doi.org/10.1016/j.vaccine.2019.05.037
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quality of life for these children [2] and in more recent years biological therapy has been available. A biological therapy is defined as a medicinal product that is produced by a biological process rather than by chemical synthesis. Tumor necrosis factor inhibitors (anti-TNF) were first introduced as a therapy option for children with PRD and have also become first line therapies for certain types of diseases [3,4]. Biologicals currently used in treating autoimmune disease, including anti-CD20 antibodies, anti-TNF inhibitors, anti-IL1 inhibitors, CTLA4-immunoglobulins, and most recently anti-IL-6 receptor antibody, affect the immune system and its mediators. Several studies have evaluated biological therapy as safe and well tolerable in a majority of children [5,6]. However, these biological therapeutics target important biomolecules involved in the cross-talk between the innate and adaptive immune responses to vaccines [7,8].

The induction of protection against influenza is multifactorial and involves both innate and adaptive cellular responses as well as humoral responses mediated via neutralizing antibody (Abs) responses against viral surface proteins. The high-affinity Abs induced by trivalent inactivated influenza vaccine (TIV) are produced by terminally differentiated, long-lived plasma cells, originating from a population of B-cells that undergo germinal center reaction (GC) [9]. In addition to first line of antibody-mediated defense, memory B-cells (MBCs) account for the second line of defense. MBCs display accelerated clonal expansion and differentiation to long-lived antibody secreting plasma cells after recall responses (reviewed in Saghafian-Hedengren et al. [10]. There are several classification systems for MBC subsets, but as a bulk cell population, MBCs express CD27 (a co-stimulatory molecule and a surrogate marker for somatic mutation). Activation of MBCs leads to down-regulation of CD21 (complement receptor 2), which enables further classification of these cells as resting (CD27+CD21+) and activated (CD27+CD21low/−) MBCs [11]. The function of CD27−CD21−B cell has not been definitely described but reports suggest that these cells represent exhausted or "atypical" MBCs emerging in conditions with a chronic immune activation, including rheumatic diseases.

Another cell type found in secondary lymphoid organs are the follicular helper cells (T_{FH}), which are essential for a productive GC reaction. There is as of yet not a common view on how to phenotypically define the circulating, small sized-population of human T_{FH} cells since different research groups have used alternating combinations of surface receptors including CD45RO, PD-1 and ICOS, together with CXCR5 on CD4+ T cells to study the usefulness of T_{FH} cells as biomarkers for vaccine responses. The collective observations made so far show that blood CXCR5+CD4+ T cells contain long-lived memory cells and share functional properties with T_{FH} cell lineage in secondary lymphoid organs [12]. As a result, blood CXCR5+CD4+ T cells are currently termed peripheral memory T_{FH} cells, from now on here called T_{FH} cells. In the follicle, the cytokine IL-21 secreted from T_{FH} cells is crucial for a functional GC B cell response as it promotes the differentiation of long lived plasma cells during the GC reaction. The chemokine CXCL13 plays a pivotal role for the GC response attracting both antigen-primed B cells and T_{FH} cells to the follicle. To that end, serum CXCL13 has been suggested as a marker for GC activity [13] and previous studies have also suggested a link between circulating T_{FH} cells and influenza vaccine antibody responses [14–16].

In a study of antibody titers in children with PRD after TIV administration, children on immunosuppressive therapy (mainly oral corticosteroids and methotrexate) demonstrated similar antibody responses as healthy age-matched controls [17]. In a more recent study children with PRD showed similar antibody responses as healthy controls after TIV administration [18].

In the study described here, the primary aim was to evaluate the antibody response to TIV in children with PRD (n = 77, including both non-treated and treated) and healthy controls (n = 24) over 3 consecutive seasons. Secondly, extensive B-and T-lymphocyte phenotyping and measurements of chemokines involved in antibody responses were performed prior to influenza immunization in the same cohort.

2. Material and methods

2.1. Study population

During three influenza seasons between 2011 and 2014, patients (age 2.5–18 years) were recruited (n = 78) from the Paediatric Rheumatology Unit at Astrid Lindgren Children's hospital, Stockholm; Sweden. Twenty-two healthy age-matched controls (age 2.5–18 years) were recruited among siblings of the patients. Inclusion criteria were PRD, either without treatment (n = 17) or treatment with methotrexate (MTX), anti-TNF +/−MTX or IL-inhibitors (n = 61) (summarized in Table 1). A few patients were on low dose (2.5–5 mg) oral prednisolone for a shorter time period (maximum 2 weeks) according to current Swedish guidelines for management of PRD. Disease data was obtained from medical charts and the Swedish Paediatric Rheumatology Registry (Table 1).

Of the original number of participants enrolled, 15 controls (68%) and 61 patients (79%) completed the follow up at 3 and 10 months. In the group of children with PRD, six non-treated children, one treated with MTX and nine children on anti-TNF +/-MTX therapy did not complete the study. Study approval was obtained from the regional ethical review board in Stockholm (D-nr 2011/1576-32-2). Samples were collected after informed consent was obtained from the subjects' parents or legal guardians.

3. Immunization schedule, patient sampling and preparation of cells and serum

All subjects received intramuscular 0.5 mL of Fluarix® (GlaxoSmithKline), a seasonal inactivated trivalent influenza vaccine, at the time of inclusion. The first two seasons (2011–2012 and 2012–2013) Fluarix contained the following viral strains: A/California/7/09 (H1N1)pdm09-like virus (pandemic H1N1 2009 influenza virus), A/Perth/16/2009 (H3N2)-like virus and B/Brisbane/60/2008-like virus. The last season (2012–2013) the vaccine contained the same A/California/7/09 (H1N1)pdm09-like virus as previous seasons and two new strains: A/Victoria/361/2011 (H3N2)-like virus and a B/Wisconsin/1/2010-like virus. Blood and serum samples were obtained from all subjects prior to vaccination followed by serum samples only at 3 and 10 months post-vaccination. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by density-gradient centrifugation using Lymphoprep (Axis-Shield) and resuspended in freezing medium (90% fetal calf serum [GIBCO] with 10% DMSO [Sigma]) at −150 °C until use. Serum was isolated by centrifugation and stored at −80 °C until analysis.

3.1. Flow cytometry

For immunophenotyping of lymphocyte subsets, PBMCs were thawed and washed in complete medium (RPMI-1640, ATCC modification) supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotic-antimycotic solution (all from Life Technologies). Thereafter cell numbers were determined and the cells were rested in a 37 °C humidified incubator with 5% CO_2 for a minimum of one hour before downstream immunophenotyping. Prior to labelling with pre-titrated fluorescent antibodies, a dead cell marker (Live/Dead© probe, Life Technologies) was used to exclude non-viable

Table 1
Cohort characteristics at time of inclusion.

	Healthy controls (n = 22)	Non-treated (n = 17)	Treated (n = 61)	MTX^ (n = 14)	Anti-TNF (+/− MTX) (n = 36)	Anti-IL1/IL6 (n = 11)
Median age (range)	10.3 (2.5–18)	13.2 (4–18)	11.4 (2.5–18)	12.2 (6–16)	13 (2.5–18)	12.1 (3.5–18)
Gender (F/M)	10/12	15/2	39/22	8/6	22/14	9/2
Diagnos (n)	–	JIA*=17	JIA (52) Erythema nodusum + artheritis (1) Juvenile dermato-myositis (1) Psoriasis artheritis (4) Polyartheritis (1) Autoimmune pericarditis (1) MCTD (1)	JIA (11) Erythema nodusum + artheritis (1) Juvenile dermato-myositis (1) Psoriasis artheritis (1)	JIA (31) MCTD# (1) Psoriasis artheritis (3) Polyartheritis (1)	JIA(10) Autoimmune pericarditis (1)
Disease duration in years median (range)	–	4.5 (0.5–13)	4 (0.5–15)	1.5 (0.5–11)	4 (1–15)	6 (1–15)
Previous vaccination prior to inclusion (%)	19/22 (86%)	15 /17 (88%)	51/61 (84%)	12 /14 (86%)	28/36 (77%)	11/11 (100%

^ MTX; methotrexate.
* JIA; Juvenile idiopathic arthritis.
# MCTD; mixed connective tissue disease.

cells from the analysis. Cells were incubated in BD Brilliant Stain buffer with a combination of following pre-titrated mouse anti-human monoclonal antibodies CD10, CD14, CD19, CD21, CD27, CD38, IgD and IgG for B cell subset analysis. When sufficient amount of PBMCs were available T_{FH}, T and NK cell phenotyping was performed which included specific combinations of the following surface markers: CD3, CD4, CD8, CD14, CD16, CD19, CD45RO, CD56, CD57, NKG2C, CXCR5, ICOS, PD-1. Isotype-matched controls were used for the appropriate gating of $ICOS^+$ and $PD\text{-}1^+$ T_{FH} cells. A minimum of 500.000 live PBMCs were used for staining with each panel of antibodies and downstream analyses of T_{FH} cells and B cell subsets originated from a minimum of 40.000 CD3+ or 10.000 CD19+ live cells within the lymphocytes gate. All were purchased from BD Biosciences except NKG2C (RnDSystems). Due to sample limitations, not all analyses could be performed on all children (see Figs. 1 and 2 respectively).

3.2. Elisa

Commercially available ELISA kits were used for the following measurements in serum; IgG and IL-21 (both from Mabtech), and CXCL13 and sCD40L (both from RnDSystems). All experiments were performed according to instructions provided by respective manufacturer.

3.3. Hemagglutinin inhibition assay

Influenza-specific antibodies were measured using the hemagglutination inhibition (HI) assay as described previously [19]. Each serum sample was measured twice and the mean value included for analysis. Pre- (day 0) and post-vaccination (at month 3 and 10) sera were tested simultaneously. Prior to testing, each serum was treated with receptor destroying enzyme (Cholera filtrate, Sigma, Germany) to inactivate non-specific inhibitors achieving a final serum dilution of 1:10. The sera were then diluted serially twofold in V-bottom microtiter plates. The virus was adjusted to 4HA units/25 μl, which was verified by back-titration. Virus strains used were A/California/7/09 (A(H1N1)pdm09), A/Perth/16/2009, A/Victoria/361/2011 (A(H3N2), B/Brisbane/60/2008 (B-Victoria-lineage and B/Wisconsin/1/2010 (B-Yamagata-lineage). After incubation at room temperature (RT) for 30 min freshly prepared 0.5% turkey red blood cells were added, the plates were mixed for agitation followed by a further incubation at RT for 30 min. Determination of the HI titer was performed by calculation of the reciprocal of the last serum dilution which contained non-agglutinated red blood cells.

3.4. Statistics

Flow cytometry data, as well as that for the soluble factors, were analysed with Kruskal-Wallis test to compare differences between treated, non-treated and healthy children. Fisheŕs exact test was performed when analysing seroprotection rates within the three groups against TIV. A p-value <0.05 was considered significant, and these evaluations were performed with Prism v5.0f (GraphPad Software Inc). Geometric Mean Titre (GMT) was calculated by averaging the natural logarithm transformed titres, and taking the natural number e to the power of that average. Corrected natural log-transformed titres were calculated according to the method of Beyer et al. [20] i.e. by fitting the linear regression model $T_{Post} = a + b^*T_{Pre}$ and calculating $T_{Post}corrected = T_{Post} - b^*T_{Pre}$, where T_{Pre} and T_{Post} are natural log-transformed titres before and after vaccination, a denotes the model intercept and b denotes the slope coefficient. The Corrected Geometric Mean Titre (CGMT) was then calculated by taking e to the power of the average of all $T_{Post}corrected$. All computations were performed using R, version 3.5.0.

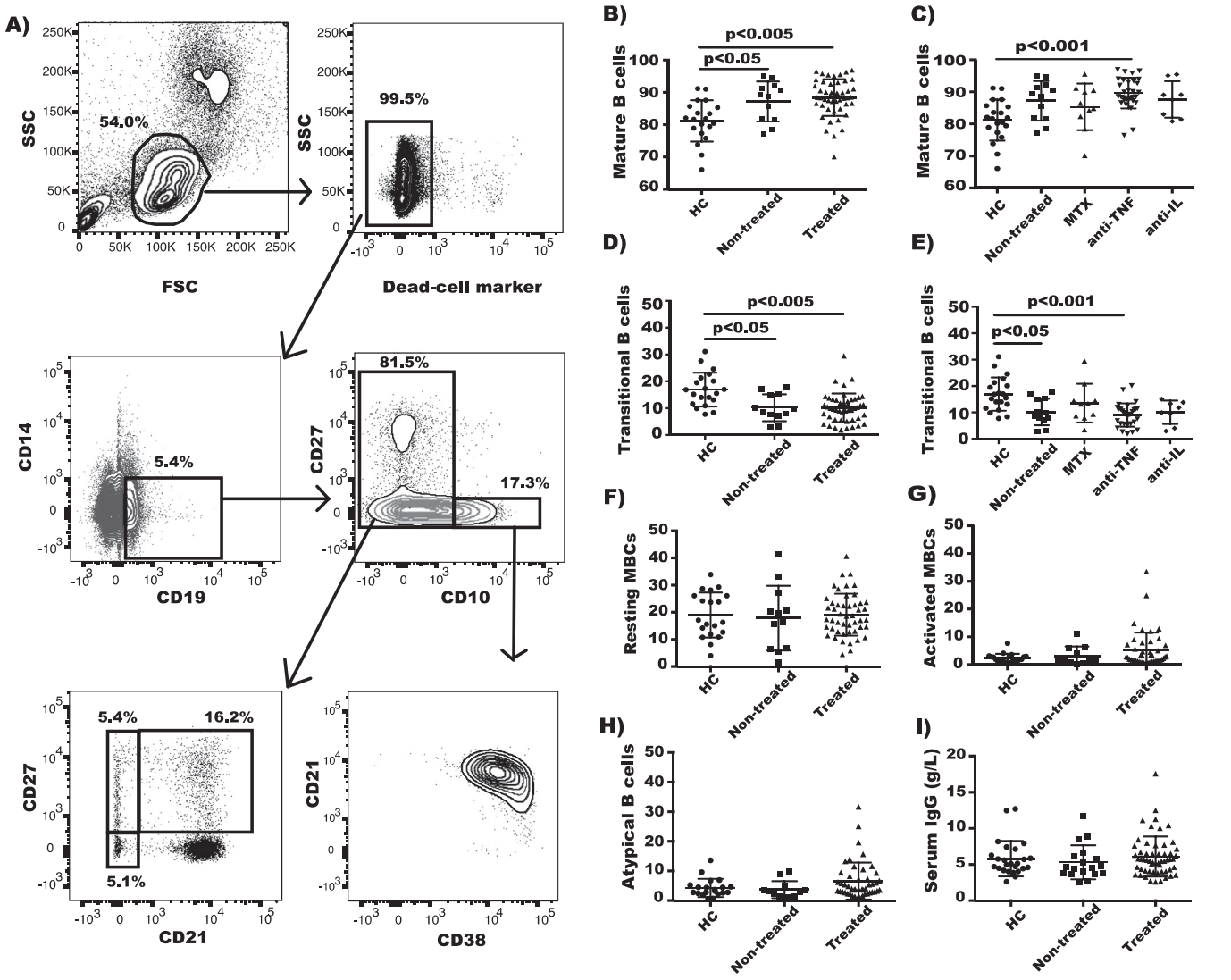

Fig. 1. Preserved B cell compartment in children. B cell populations in peripheral blood sampled from healthy controls (HC) (n = 20) and patients with paediatric rheumatic disease, non-treated (n = 12) or under treatment (n = 48). The patients were further subdivided into groups treated with either only MTX (n = 10), anti-TNF-α therapy +/− MTX (n = 30) or anti-interleukin therapy (n = 8). After gating on lymphocytes based on forward/side scatter criteria and exclusion of dead cells, monocytes (CD14+ cells) and doublets, positive expression of CD19 was used to gate B cells (A). Thereafter mature B cells were defined as CD10− cells (B, C), transitional B cells as CD10+ CD27− CD21+ CD38+ cells (D, E), resting memory B cells as CD27+ CD21+ mature B cells (F), activated memory B cells as CD27+ CD21− mature B cells (G) and atypical memory B cells as CD27− CD21− mature B cells (H). Total serum IgG measured by ELISA (I). Statistical analyses were performed using Kruskall-Wallis test.

4. Results

4.1. Clinical characteristics and previous immunisation of children with PRD

The study cohort consisted of both treated and untreated children with PRD, as well as healthy age-matched controls (Table 1). There were no significant differences in age distribution or diagnosis between the untreated or treated children. There were more girls both in the untreated and treated groups compared to healthy controls where boys dominated. Treatment with TNF-inhibitors (+/− MTX) were the most common therapy in our cohort (n = 36). Disease duration varied in all patient groups from 6 months to 15 years, also in untreated patients. The majority of patients and controls had previously received influenza immunizations according to the national vaccine registry (Vaccinera®). There were no serious adverse effects upon immunization as reported by parents (data not shown).

4.2. Altered proportions of mature and transitional B cells, but preserved B cell memory in PRD children

Before immunisation, PBMCs were collected and the B cell compartment was analysed with flow cytometry (Fig. 1). Compared to healthy controls, children with PRD, both treated and non-treated, showed elevated proportions of mature B cells (p-value <0.05 and 0.005, Fig. 1B). The higher proportion of mature B cells was highly significant in children on anti-TNF(+/− MTX) therapy following stratification of the treated group into type of treatment (p-value < 0.001, Fig. 1C). On the other hand, transitional B cells were significantly lower both in untreated and treated children compared to healthy controls (p-value < 0.05 and 0.005, Fig. 1D), which was sustained following stratification of the treated group, in which the anti-TNF (+/−MTX) treated patients reflected the reduction in transitional B cells (p-value <0.001, Fig. 1E). There were no significant differences in the frequencies of memory B cell populations between healthy children or those with PRD (p = 0.69, p = 0.2,

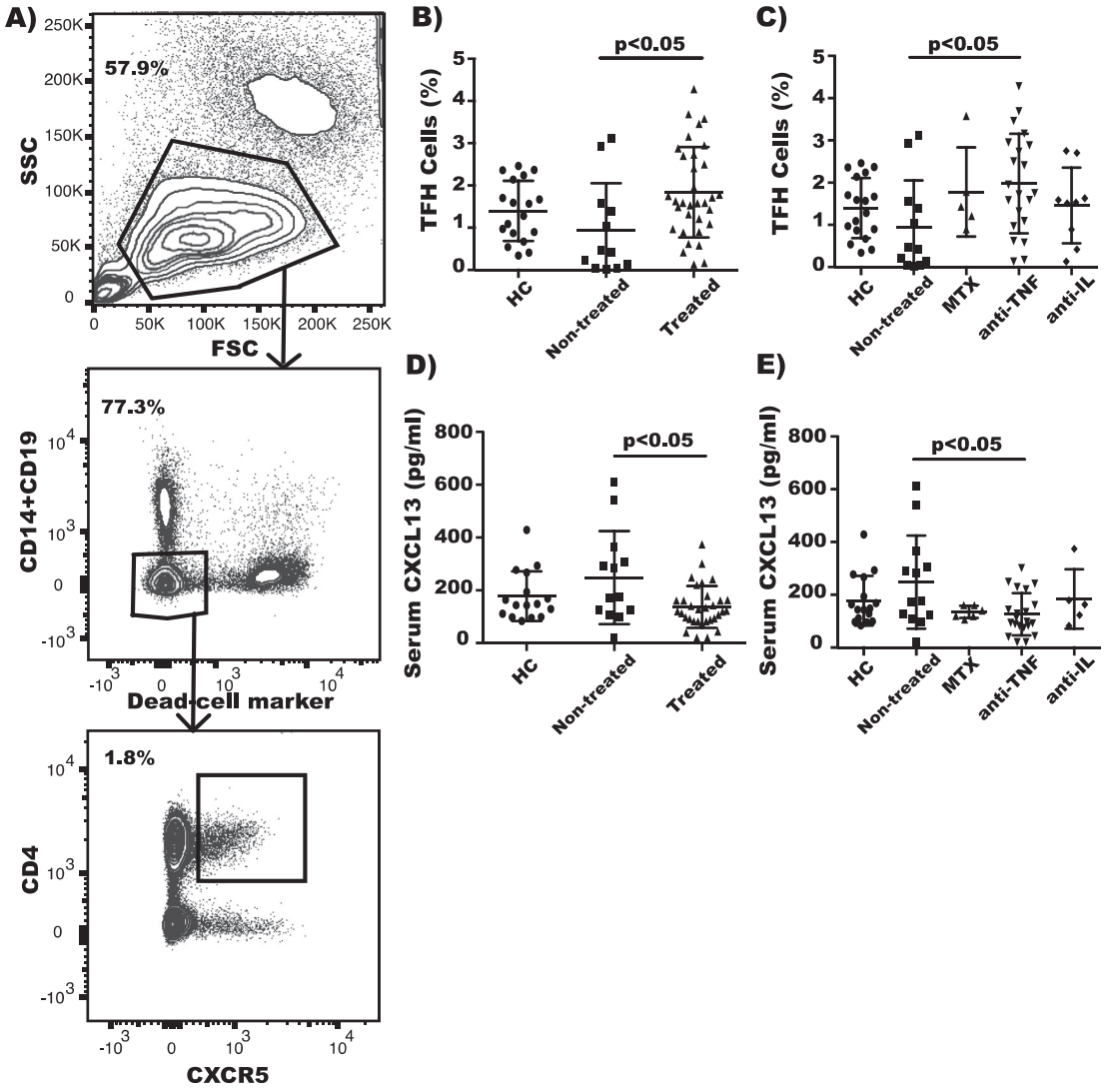

Fig. 2. Reduced frequency of peripheral T_{FH} cells in untreated children with PRD. The proportion of T_{FH} cells derived from healthy controls (HC) (n = 17) and patients with paediatric rheumatic disease, non-treated (n = 14) or under treatment (n = 28) was analysed by flow cytometry. Representative gating strategy for CD4+ CXCR5+ (T_{FH}) cells (A). Non-treated children displayed significantly lower proportion of T_{FH} cells in blood compared to HC and children on treatment (B, C). In parallel, the chemokine CXCL13 was elevated in non-treated children compared to HC and children on treatment (D, E). Statistical analyses were performed using Kruskall-Wallis test.

p = 0.09 respectively, Fig. 1F–H). Likewise, the total circulating IgG levels did not significantly differ between healthy controls or children with PRD, regardless of being on treatment or not (p-value = 0.40 Fig. 1I).

4.3. Reduced frequency of T_{FH} cells and higher CXCL13 in blood of non-treated children with PRD

We assessed the frequency of circulating T_{FH} cells as they have been shown in earlier studies to be related to potent influenza vaccine responses [14–16]. By means of bulk CD4+ T (T_H) cells, we observed no differences in their proportions between healthy children compared and those with PRD (Table 2). However, the proportion of T_{FH} cells was lower in non-treated children with PRD compared to treated children (p-value = 0.008, Fig. 2B), with most evident difference upon comparing non-treated children with those on anti-TNF (+/− MTX) after stratification of the treated group ($p < 0.05$, Fig. 2C). There were no significant differences between the groups in the proportions of CD45RO+, PD-1+ or ICOS+ T_{FH} cells and further subgrouping of T_{FH} cells for pairwise combinations of these markers generated small cell counts for reliable statistical analyses (data not shown). No differences were found for T- or NK-cell subsets (Table 2).

The serum levels of CXCL13, IL-21 and sCD40L cytokines that have been associated with efficient T_{FH} cell responses were measured. The level of CXCL13, a ligand of CXCR5, was significantly higher in untreated compared to treated children with PRD (p-value < 0.05, Fig. 2D), and the major difference was again for children on anti-TNF (+/− MTX) therapy after subgrouping based on treatment (p-value < 0.05, Fig. 2E). No differences were detected in the levels of IL-21 and sCD40L between treated and untreated children (data not shown). There were no significant correlation between the proportion of T_{FH} cells and CXCL13 in either patients or controls.

4.4. Modest response to influenza immunisation in all children

As the majority of our patients were previously immunized against influenza, the pre-immunisation titres were high for the H1N1pdm09 strain in all groups of children (Fig. 3). Lower pre-vaccine titres against the H3N2-like and influenza B viruses compared to H1N1pdm09 were found in all groups. Taking previous

Table 2
Frequency of T- and NK cell populations in peripheral blood of healthy controls and children with PRD.

NK- T cell populations (proportion of parent %, median (range))	Healthy controls (n = 17)	Non-treated (n = 14)	Treated (n = 28)	p-value[#]
Live CD3+ CD14–CD19– lymphocytes (T cells)	72 (49–90)	74.5 (50–89)	77.5 (61–90)	0.47
(Helper) CD4+ T cells	49 (31–67)	48.5 (24–70)	50 (30–62)	0.77
(Cytotoxic) CD8+ T cells	34 (21–54)	35.5 (15–49)	33 (22–57)	0.88
CD57+CD4+ T cells	1 (0–13)	1 (0–22)	2 (0–11	0.37
CD16+CD8+ T cells	1 (0–9)	0 (0–4)	2 (0–11)	0.05
CD57+CD8+ T cells	17 (6–49)	18.5 (6–71)	26 (3–58)	0.37
CD4+CD8+ T cells	0 (0–3)	0(0–7)	0.5 (0–13)	0.47
CD4+CD8+CD57+ T cells	10 (2–56)	15.5 (1–94)	20.5 (1–60)	0.56
CD4−CD8− T cells	14 (4–35)	14.5 (5–51)	13.5 (6–32)	0.85
CD4−CD8−CD57+ T cells	12 (6–51)	16 (6–59)	17.5 (7–34)	0.62
Live CD3−CD14−CD19−CD56+ lymphocytes (NK cells)	73.5 (39–92)	72 (50–83)	76 (48–90)	0.25
CD56bright NK cells	5 (2–9)	5 (2–30)	7 (1–19)	0.12
CD56dim NK cells	66 (34–89)	63 (39–90)	67 (40–92)	0.42
CD16+ NK cells	63 (38–95)	63.5 (39–90)	72(40–92)	0.90
CD57+ NK cells	40 (4–74,5)	35 (7–62)	35 (7–66)	0.90
NKG2C+ NK cells	4 (0–60)	5 (0–66)	3 (0–66)	0.72
NKG2C+ CD56dim NK cells	2 (0–59)	4 (0–66)	3 (0–66)	0.47
CD16+CD56dim NK cells	72.5 (48–96)	68.5 (38.5–93)	77 (43–93)	0.70
CD57+CD56dim NK cells	42 (5–75.5)	39 (8–64)	37 (8–67)	0.85
NKG2C+CD57+CD56dim NK cells	37 (0–75.5)	14 (0–73)	5 (0–84)	0.14

[#] Differences in cell populations among 3 groups (healthy controls, non-treated and treated patients) were analyzed by the non-parametric Kruskall-Wallis test.

immunizations into account, the follow-up samples were corrected using a linear regression model as previously described [20].

There was no significant change in H1N1pdm09 corrected GMT (cGMT) between immunisation and follow up at three months for any of the groups although the cGMT increased in the group healthy (n = 15) and treated (n = 45) children. However, cGMT thereafter significantly declined for all groups ($p < 0.05$, respectively) up to 10 months post immunisation. For H3N2, there was a significant increase in cGMT in healthy children ($p < 0.05$) after immunisation which was not detected in children with PRD. As observed for H1N1pdm09, cGMT decreased significantly during the follow up period in all groups ($p = 0.05$). There were no significant changes for the influenza B vaccine virus at three months or 10 months follow up.

Although the vaccine response in the group of children treated for PRD in general was similar to healthy and non-treated children, a few children responded poorly. In the cohort of children who completed follow up at 10 months, there were 3 un-immunized healthy children and 5 un-immunized children in the PRD group, all treated with anti-TNF inhibitors. Therefore, vaccine responses in the different treatment groups were analysed separately but there were no significant differences in relation to therapy (data not shown) or to prior immunizations. In the group of treated children without prior history of influenza immunisation, 3 out of 5 responded well and one child showed a partial response with antibodies against H1N1 only. In the healthy children without previous influenza immunisation, 2 responded well and one child, aged 2.5 years did not mount influenza-specific Abs. Although not significant, children treated with TNF-inhibitors (+/− MTX) differed as there were more seronegative children at baseline and a few children also remained seronegative for H3N2- and influenza B viruses after immunization. In addition, the T_{FH} cell proportion did not impact antibody responses (data not shown).

4.5. Better long-lasting protection against H1N1pdm09 viruses

HI titres ≥40 has been considered as a cut-off for seroprotection after influenza immunization. After 3 months post-vaccination 93–100% of children displayed levels above the cut-off for H1N1pdm09 based on the corrected titres (Fig. 3). After 10 months, 70% of healthy and treated PRD children showed HI titres ≥40 while only 45% of the non-treated children showed HI titers ≥40 ($p > 0.05$). The seroprotection rate at three months for H3N2 were much lower with only 37–53% of the children showing seroprotective titres after 3 months, and this further declined to approximately one third of children at 10 months post immunization (no differences between the groups of children however). The seroprotection rate for influenza B was the lowest, with only one third of children with titres above ≥40 at 3 months and further declining in healthy and treated PRD children (no differences between the groups of children).

5. Discussion

In our cohort of children with PRD, the effects of different treatment modalities on lymphocyte subsets and influenza vaccine response were studied. As of today, only a few studies exist on the effects of biological therapy in children (summarized in [18]). Although there were alterations in peripheral blood B- and T-lymphocyte subsets in children with PRD, this did not have a significant impact on the influenza vaccine responses.

Previous studies in our laboratory and others [21,22], have shown that children with PRD have alterations in the peripheral B cell compartment. In the current study which included a higher number of children with PRD as well as healthy age-matched controls, both treated and non-treated children with PRD showed a reduction in transitional B cells where children on anti-TNF inhibitors (+/− MTX) were most affected compared to healthy controls. Contrary to Glaesener et al. we could not attribute the reduced frequency of transitional B cells to MTX therapy only [22]. The role of human transitional B cells, recent emigrants from the bone marrow compartment, is still to be fully elucidated in the context of autoimmune disease as well as their role in humoral immunity [23]. In adults with autoimmune disease, expansion of transitional B cells has been reported, and in that setting linked to faulty removal of B cells with autoreactive BCRs and preferential autoantibody production [23,24]. More recently, a subset of pre-naïve B cells with the capacity to produce IL-10 and thus impair CD4− T cell activation was described [25]. Whether a reduced frequency of transitional B cells in children with PRD as opposed to adults, is linked to more efficient humoral immune responses remains to be studied in larger cohorts than ours. Prior to immunization in our cohort, the peripheral memory B cell compartments was assessed and there were no differences between healthy children

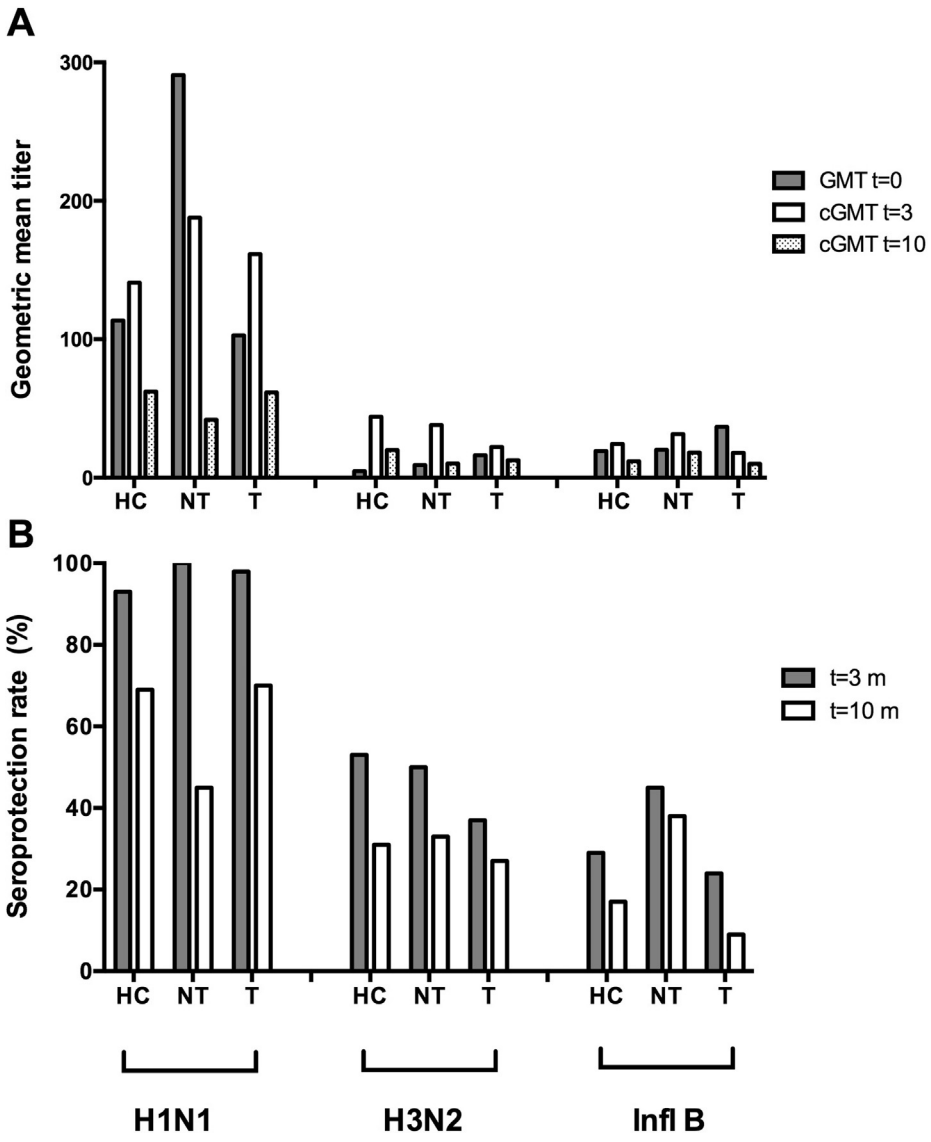

Fig. 3. Evaluation of vaccine response after TIV in children with and without PRD. (A) The participants were followed for 10 months and the influenza antibody response was evaluated in healthy (HC), non-treated (NT) and treated (T) children with PRD. Healthy children mounted a significant antibody response to H3N2 at 3 months. In all groups, there was a significant decrease in cGMT between 3 and 10 months follow up for both H1N1 and H3N2. (B) The seroprotection rate for H1N1 at 3 months were high for all groups compared to H3N2 and influenza B. Seroprotection rates declined between 3 and 10 months.

and children with PRD, which implies a preserved functional B cell memory also in treated children as previously reported [22].

A functional GC response in secondary lymphoid tissues is crucial for effective immune response to vaccines. To this end, several parameters have been suggested as possible surrogate markers for the GC response, with peripheral T_{FH} cells and serum CXCL13 as examples [13,26]. In the participants enrolled in this study, non-treated children showed a lower proportion of T_{FH} cells compared to children on anti-TNF therapy, which is in accordance with the literature [22,27]. However, no significant differences could be detected between patients and healthy controls. The proportion of T_{FH} cells in the different groups was not mirrored by CXCL13 levels in serum and no significant correlation was found between these parameters. As T_{FH} cells express CXCR5 on the cell surface, low levels of CXCL13 (the ligand of CXCR5) together with a higher proportion of peripheral T_{FH} cells might indicate that the ligand already is bound to its receptor. Alternatively, and in contrast to a previous study showing a positive correlation between vaccine response, blood CXCL13 and ICOS+ T_{FH} cells in HIV+ patients [13], there may not be a positive relationship between blood T_{FH} cells and CXCL13 levels in our patient setting. In support of the latter, our data did not find any relationship between CXCL13 levels in serum and magnitude of antibody responses to TIV at the 3- or 10-month follow-up.

Yearly influenza vaccination in healthy children is not recommended in Sweden. There are general recommendations of yearly influenza vaccination in certain risk-groups, where patients on immunosuppressive therapies are one of them. In children with PRD, the treating physician was left with the decision to recommend TIV or not, up to 2011 when the EULAR recommendations were published [1]. The high numbers of previously influenza immunized children were therefore surprising as were the high pre-vaccine HI titers in our cohort. However, this may to some extent be explained by the high vaccine coverage noticed in the pandemic influenza A(H1N1) vaccine in Sweden during 2009–2010 where approximately 60% of the population received the vaccine [24]. In addition, the reported incidence of A(H1N1)pdm09 disease in Sweden during 2009 was also the highest in children,

and in 2010–2011 a new wave of pandemic H1N1pdm09 also affected Sweden. Interestingly, the seroprevalence of HI titers ≥40 in children 5–14 years of age rose from approximately 5% in 2007 to >80% in 2011, which is in accordance with the baseline seroprevalence in our cohort [24]. In Norway, a substantial rise in prevalence of antibodies at protective titers, from 1.8% to 65.3%, was also observed between August 2009 and January 2010 in the age group of 10–19 year-olds [28]. However, the seroprotection rate for H1N1pdm09 after vaccination with 96% was still higher than reported from a study conducted in Spain over two more recent seasons (2013/2014 and 2014/2015) [18].

In our study, the baseline overall HI titers for all influenza strains were similar between healthy children and children with PRD, treated or not. The high H1N1pdm09 titers are most likely explained by a robust memory B cell response. During our follow up of 10 months, protective titers remained high for H1N1 compared to the other strains. Also the peripheral memory B cell compartment was similar for all children which supports the hypothesis that children with PRD have a normal B cell memory as previously reported by us [10].The overall vaccine response for H3N2 and influenza B viruses in our cohort was modest using the linear regression model suggested by Beyer et al. [20] for highly immunized populations. However, the protection rates at 3 months was similar to other reports [18,29] although there were some differences in follow up time (2 vs 3 months).

It should be noted that children on TNF inhibitors differed somewhat to children in the other therapy groups both in terms of a reduced proportion of transitional B-cells and in the immune response to vaccines where a few children remained seronegative. TNF plays an important role for B cell biology; both as an activator of hematopoietic stem cells in the bone marrow and in T-cell mediated B-cell activation in the GCs [30]. To further elucidate the immunological consequences of TNF inhibitors for B cell immunity in children would require longitudinal sampling of larger cohorts of pediatric patients in conjunction with vaccine studies using both T-dependent and T-independent vaccines [31]. Some limitations should be acknowledged here. Although we initially recruited >70 children with PRD the drop-outs made the different treatment groups as well as the group of non-treated patients smaller. We also had to recruit patients over three seasons which resulted in change of vaccines. Whether any of the participants had a clinically visible infection despite immunization is unclear, as the vaccine efficacy was not evaluated over the seasons.

In summary, our results are in line with previous studies describing a similar response to TIV in children with rheumatic disease and healthy age-matched controls. Biological therapy does not significantly reduce the response to TIV. The finding of a reduced CXCR5+ TFH cell population in non-treated children with PRD is novel and warrants further studies, both in the context of autoimmunity and in the immune response to vaccines.

Acknowledgements

The authors would like to thank Mrs. Birgit Troschke and Mrs. Carmen Karstädt for their excellent technical support in performing the hemagglutinin assays and Karina Mördrup for coordinating patient recruitment.

Funding

This work was funded by Stiftelsen Frimurarna Barnahuset, an unconditional grant from Pfizer Incorporated, and the Stockholm County through the ALF agreement. The work was completed independent of the funders.

Contribution

AN conceptualized and designed the study with input from ÅL, ES and SSH. All authors contributed to patient recruitment and collection, and interpretation of clinical parameters. AV was responsible for study logistics and sampling, with input from ÅL, AN, and ES. ÅL, SSH and AN designed the experimental setup and optimized the methods. HMIS, SSH and BS performed the laboratory work. ÅL, SSH and AN reviewed all collected data, with input from all authors. SSH and AN finalized the first version of the manuscript as well as the revised version, and all authors read and approved of the final version.

Competing interests

The authors declare no conflict of interest.

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