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Pregnancy-related immune suppression leads to altered influenza vaccine recall responses

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ABSTRACT

Pregnancy is a risk factor for severe influenza infection. Despite achieving seroprotective antibody titres post immunisation fewer pregnant women experience a reduction in influenza-like illness compared to non-pregnant cohorts. This may be due to the effects that immune-modulation in pregnancy has on vaccine efficacy leading to a less favourable immunologic response.

To understand this, we investigated the antigen-specific cellular responses and leukocyte phenotype in pregnant and non-pregnant women who achieved seroprotection post immunisation. We show that pregnancy is associated with better antigen-specific inflammatory (IFN-γ) responses and an expansion of central memory T cells (Tcm) post immunisation, but low-level pregnancy-related immune regulation (HLA-G, PIBF) and associated reduced B-cell antibody maintenance (TGF-β) suggest poor immunologic responses compared to the non-pregnant.

Thus far, studies of influenza vaccine immunogenicity have focused on the induction of antibodies but understanding additional vaccine-related cellular responses is needed to fully appreciate how pregnancy impacts on vaccine effectiveness.

1. Introduction

Worldwide, influenza virus infection is a significant health problem that has an annual attack rate of 5–10% in adults and 20–30% in children [1]. In its most severe form, infection can lead to hospitalisation and even death, and these risks are particularly significant in high-risk groups that include pregnant women. Historically, influenza pandemics have been characterised by severe secondary complications particularly in pregnant women [2]. Data from observational studies have shown that the greatest risk from seasonal strains is during the third trimester of pregnancy [2]. Fortunately, immunisation has been shown to provide protection and, importantly, improve clinical outcomes [3,4]. In fact, seasonal influenza immunisation is associated with clinical benefit for both pregnant women and, by passive immunity, their newborn infants [3–5]. As a result, the World Health Organisation (WHO) recommends that all pregnant women should be immunised for influenza virus during pregnancy. One of the issues with immunisation, however, is that seasonal virus strains are always changing. To combat this problem, the WHO monitors global epidemiology of flu viruses and are thus able to provide recommendations for the forthcoming winter [6]. Currently, the WHO recommends an inactive trivalent influenza vaccine (TIV) that contains two subtypes of influenza A and one influenza B strain [6]. TIV has been shown to lead to sufficient increases in anti-viral antibodies in pregnant patients, which provide seroprotection [2]. This is clearly defined by the WHO as a 28-day post immunisation antibody titre of > 1:40 to be seroprotective [7]. Despite evidence that suggests vaccination halves the incidence of laboratory confirmed influenza infection, there is only a moderate reduction in

Abbreviations: APC, Allophycocyanin; BRC, Imperial Biomedical Research Centre; CRBC, Chicken red blood red cells; FEC, Flu/EBV/CMV; FITC, Fluorescein isothiocyanate; FoxP3, Forkhead box P3; HA, Haemagglutination; HAI, Haemagglutination inhibition; iNKT, Invariant NK T cell; MDCK, Madin-Darby canine kidney cell line; NRES, National Research Ethics Committee; NIBSC, National Institute for Biological Standards and Control; NIHR, National Institute for Health Research; PE, Phycoerythrin; Pfc, Plaque forming cells; PIBF, Progesterone induced blocking factor; PPD, Purified protein derivative; PVDF, Polyvinylidene difluoride; RDE, Receptor destroying enzyme; Tcm, Central memory T cell; Tem, Effector memory T cell; Tfh, T follicular helper; Th, T-helper; TIV, Trivalent influenza vaccine; Ttemra, Terminally differentiated effector memory T cell; TTOX, Tetanus toxoid; WHO, World Health Organisation

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influenza-like illness for the mother and neonate [8]. Therefore, the efficacy of immunisation is not entirely dependent on improved antibody titres, and other elements of the immune response must be relevant. Simply quantifying antibody titres will unfortunately fail to measure their function such as antibody-dependent cellular cytotoxicity and phagocytosis, but these attributes tend to increase in parallel with increasing antibody levels [9,10]. Whilst the importance of memory CD4 T cell responses generated during repeated *in vivo* influenza virus exposures have been established and most closely correlate to limitation of illness severity, there is limited data for the impact of CD4 and CD8 vaccine responses in pregnancy [11]. We know that immunisation leads to other immunogenic effects that potentiate anti-viral responses such as improving interferon production in pregnancy and enhancing both T and natural killer (NK) cell responses [2]. Research into vaccine efficacy have shown that the induction of antigen specific memory B and T cells are also important as they provide a regenerative pool for rapid effector responses when encountering the virus [12]. Effector mechanisms elicited by vaccines to control pathogens include: CD4 mediated Th1 responses that support B cell activation and differentiation, and cytotoxic T cell and macrophage recruitment; as well as Th2 responses to support B cell functions [12]. In addition to its effects on antibody secretion, immunisation has also been shown to enhance NK and T cell activation and poly-functionality as well as modulating pro-inflammatory cytokine production [13,14]. Therefore, Immunisation provides a strong antigenic stimulus to modulate immune responses.

In this study, we investigated the hypothesis that mothers who achieved a robust seroprotective antibody response after influenza vaccination, would, conversely, also show markedly poorer antigen-specific immune responses to influenza A when compared to non-pregnant controls. In addition, the functional differences post vaccination in immune response would be reflected in phenotypic analysis of their peripheral blood T cell, NK cell, and dendritic cell (DC) populations.

2. Materials and methods

2.1. Ethics statement

All subjects were recruited from Chelsea and Westminster Hospital, London, UK. This study was carried out in accordance with the recommendations of National Institute of Health Research (NIHR) Good Clinical Practice guidelines, and an NHS Research Ethics Committee. The protocol was approved by the National Research Ethics Service (NRES), London, UK committee as well as by Chelsea and Westminster NHS Trust, London, UK; Ref: 11/LO/0971. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

2.2. Study design

Peripheral blood was analysed using a combination of ELISpot and 9-parameter flow cytometry, in order to assess functional cellular responses and leukocyte phenotype. In addition, anti-influenza antibody titres were quantified using a haemagglutination inhibition assay. A standardised protocol was used for all samples to ensure consistency and comparability.

2.3. Study participants

Healthy pregnant patients (N = 14) were recruited from the antenatal clinic at Chelsea and Westminster Hospital prior to 33 weeks of gestation, and between October 2012 and February 2013. This enabled completion of longitudinal sampling at the desired time points prior to delivery. These patients received the influenza vaccination from their GP and were asked to provide peripheral blood samples longitudinally. The time points for longitudinal sample collection were: prior to vaccination, and four weeks and eight weeks post vaccination. These time points were chosen due to the WHO definition of protective immunity that suggests this is achieved with an increase in antibody titre four weeks post immunisation, with a 1:40 titre associated with 50% efficacy for protection [12,15]. In order to try to control for the longitudinal effects of gestation, pregnant women across a range of gestations were recruited. Patients in their third trimester of pregnancy were included as this is the most susceptible period for complications. The range was 12 + 4 to 32 + 5 weeks of gestation and the median gestation was 20 + 4 weeks. The spread of gestations of the recruited patients is shown in Table 1.

Non-pregnant female control subjects (N = 15) were recruited from maternity staff and maternity research scientists in parallel to pregnant patients. They received vaccination from occupational health or appropriately trained members of their department. Time points for sample collection were: prior to vaccination, four weeks/one-month post vaccination and eight weeks/two months post vaccination. The two month time point was chosen since few vaccine studies in pregnancy have reported vaccine responses beyond one month [2].

2.4. Preparation of cells and plasma

Thirty-five millilitres of peripheral blood was obtained from each subject using a Vacutainer™ system and 6 ml lithium heparin blood collection tubes (Becton Dickinson, Oxford, UK). Blood collection tubes were centrifuged and plasma for influenza antibody titres was collected, aliquoted, and stored at −80 °C until use. PBMCs from peripheral blood were prepared by density gradient centrifuge on Histopaque (Sigma-Aldrich, Dorset, UK) as described previously [16]. Cell viability was determined using a trypan blue exclusion test and samples where this was > 80% were used. For functional work, the PBMC were suspended in TCM [RPMI-1640 with Penicillin and Streptomycin (Sigma-Aldrich), at final concentrations of 100 IU/ml and 100 μg/ml, and L-glutamine (Sigma-Aldrich) at a final concentration of 2 mM], and for phenotype PBMC were suspended in Ca^{2+} and Mg^{2+} containing PBS (Sigma-Aldrich). All ELISpot and flow cytometry assays were performed on fresh samples and processing was commenced within 2 h of obtaining the peripheral blood.

Table 1
Demographic data for controls and pregnant patients. Grouped data is represented as percentages, and continuous data as mean and SD or median and IQR depending on the data distribution. Statistical analysis was undertaken using Mann-Whitney U for continuous data, and Fisher's exact test was used for grouped variables.

	Immunised controls (HC)	Immunised in pregnancy (Pr)	HC versus Pr
Ethnicity			
Caucasian	72%	71%	P = 0.8810
Black	14%	7%	
Asian	7%	7%	
Mixed	7%	14%	
Age (mean/median completed years)	25.5 IQR 24.0–29.5	33.2 SD ± 1.3	P = 0.0041
Parity			
0	86%	50%	P = 0.0828
1	14%	29%	
2	0%	21%	
Gestation at first blood test (mean/median completed weeks)		21 SD ± 7.2	
Gestational Quartiles:			
12 + 4–15 + 4		N = 5	
15 + 5–20 + 4		N = 2	
20 + 5–28 + 2		N = 4	
28 + 3–32 + 5		N = 3	

2.5. Virus propagation and titres

Live influenza viruses, which were vaccinated against in the UK during the 2012 and 2013 season using trivalent influenza vaccination, were obtained from NIBSC (NIBSC, Hertfordshire, UK). These included A/Victoria/361/2011 (H3N2) wild type virus (Cat: 11/226), A/California/7/2009 (H1N1) wild type virus (Cat: 12/174), and B/Wisconsin/1/2010 (Yagamata) wild type virus (Cat: 11/224). The above viruses were propagated using cultured Madin-Darby canine kidney cell line (MDCK, Sigma-Aldrich) and viral titres were quantified as previously described [17].

Briefly, seeded MDCK cells were washed in PBS, diluted virus stock was added to them, and these cells were incubated at 37 °C with 5% CO_2 for 1 h in a 6 well flat bottom plate. The overlay was warmed to 37 °C, 2% agarose gel (Oxoid Ltd., Thermo Fisher Scientific) was heated to 55 °C, trypsin was added to the flu overlay, and the mixture was added to each well. Once the overlay set, the plate was incubated for 3 days at 37 °C and 5% CO_2, after which point the plaques were ready for counting. The wells were stained with diluted Crystal Violet stain (Sigma-Aldrich) and the plaque-forming cells (Pfc) enumerated: A/Victoria/361/2011 wild type virus = 2.95×10^7 Pfc, A/California/7/2009 wild type virus = 2.95×10^7 Pfc, and B/Wisconsin/1/2010 wild type virus = 9.5×10^6 Pfc.

Haemagglutination Assay (HA) was used to determine virus titres and a modified version of the WHO protocol was used [18]. Twenty-five microlitres of PBS was added to every well of a 96 well 'V' bottom plate. Fifty microlitres of virus was added to the first well of each row in duplicate and two-fold serial dilutions were performed across the plate. Fifty microlitres of 0.5% chicken red blood red cells (CRBC), obtained from an abattoir and diluted in PBS, were added to each well and the plate was incubated for 1 h on ice. The resultant HA titre enabled calculation of virus dilution required for the subsequent assays. A viral dilution of 8 HA/50 μl was required. Therefore, HA titres for A/Victoria/361/2011, A/California/7/2009 and B/Wisconsin/1/2010 of 128, required dilutions of 1:16.

In addition, a UK reference strain was used to compare antibody titres against vaccine strains to account for significant antigenic variation. A HA titre of 256 was obtained for a 2009 UK pandemic swine influenza A/H1N1 strain, labelled A/England/195/2009, and so a dilution of 1:32 was subsequently calculated.

2.6. Haemagglutination inhibition assay

Plasma received receptor destroying enzyme (RDE) treatment prior to the haemagglutination inhibition assay using a modified version of the WHO protocol [18]. Briefly, 300 μl of RDE was added to 100 μl of human plasma and then incubated for 18 h at 37 °C and 5% CO_2. Prior to use, this mixture was heated to 56 °C for 60 min in order to inactivate the RDE. Thereafter, the RDE treated plasma was screened for the presence of non-specific agglutinins using a HA assay. Haemagglutination inhibition assay (HAI) was only performed on plasma that exhibited no non-specific agglutination. No samples needed to be excluded.

Twenty-five microlitres of PBS was added to rows B through to H across a 'V' bottom 96 well plate. Fifty microlitres of RDE treated plasma, diluted at a concentration of 1:10, were added to row A, and two-fold dilutions were achieved by transferring 25 μl from the preceding row into the next successive row, and the final 25 μl was discarded. The stock virus was thawed at room temperature and diluted according to the HA assay result. Twenty-five microlitres of diluted virus was added to labelled plasma containing wells and incubated for 15 min at room temperature. Twenty-five microlitres PBS was added to the last two columns in row A to act as a control. Fifty microlitres of 0.5% CRBC was added to every well and the plate was incubated until the control wells demonstrated a negative HAI.

2.7. ELISpot assay

IFN-γ, IL-10, TGF-β1 and Granzyme B ELISpot assays were performed in order to detect recall antigen/peptide specific T cell responses as previously described [16]. 1×10^5 PBMC/well were cultured in 10% (heat-inactivated) male AB plasma-RPMI (200 μl/well final volume, Sigma-Aldrich) in 96 well polyvinylidene difluoride (PVDF) backed plates (Merck Millipore, Hertfordshire, UK), that were coated with antibodies for the specific cytokines or proteases of interest (for IFN-γ, IL-10, or Granzyme B: Mabtech AB, Nacka Strand, Sweden; for TGF-β1: R&D Systems, Abingdon, UK). In duplicate wells, PBMC were stimulated with 100 μl of an antigen/peptide pool obtained from NIBSC (NIBSC, Hertfordshire, UK) or Virion-Serion (Virion-Serion, Würzburg, Germany) at the manufacturer's recommended concentrations. Stimuli included: EBV, CMV, influenza A, measles and HSV whole lysates; purified protein derivative (PPD) of *M. Tuberculosis* Tuberculin (NIBSC); purified tetanus toxoid (TTOX; NIBSC); and flu/EBV/CMV (FEC) peptide pool (NIBSC). Positive and negative controls were provided by phytohemagglutinin (PHA; 5 μg/ml, Sigma-Aldrich) and tissue culture medium (TCM) respectively. Plates were incubated at 37 °C in 5% CO_2 for 48 h. Detection of spot forming cells (SFC) required the addition of biotinylated anti IFN-γ, IL-10, Granzyme B (Mabtech AB) or TGF-β1 (R&D) and incubation, followed by the use of a concentrated streptavidin-alkaline phosphatase conjugate (Mabtech AB/R&D). Finally, a development step was carried out using a chromogen prepared from a premixed BCIP/NBT substrate kit (BioRad Laboratories Ltd., Hertfordshire, UK). SFC reading and counting was performed using an AID ELISpot reader (Oxford Biosystems Cadama, Oxfordshire, UK).

2.8. Flow-cytometry based leukocyte phenotyping

We have previously shown that CD4 and CD8 T cell activation, proportions of effector memory T cells expression CD28, Th17 and Treg proportions and some DC and NK cell subsets, all vary during the course of pregnancy [19]. Therefore, multicolour flow cytometry was used to phenotype CD4 and CD8 T-cell subsets, NK cells, and DC proportions. PBMC were predominantly stained with murine, anti-human monoclonal antibodies according to the manufacturer's instructions. In some instances, a polyclonal rabbit antibody was used. 2×10^6 cells were stained per tube, incubated in the dark at room temperature for 30mins, washed with PBS and fixed with BD stabilizing fixative (BD Biosciences), before acquisition within 24 h. For T, and NK cells, a minimum of 100,000 events, and for DC, a minimum of 500,000 events were acquired on a 3-laser flow cytometer (BD Biosciences LSR II) and subsequently gated according to co-responding isotype controls. Analysis of flow cytometric data was performed using FlowJo version 7.65 (Tree Star Inc., Ashland, OR, USA).

T cells were identified using the following anti-human monoclonal antibodies (clones): peridinin chlorophyll protein (PerCP) Cy5.5-labelled anti-CD3 (SK7; Biolegend, London, UK); allophycocyanin (APC)-H7-conjugated anti-CD8 (SK1; Biolegend); BD Horizon V450-labelled anti-CD38 (HIT2; BD Biosciences, Oxford, UK), anti-CD127 (HIL-7R-M21; BD), and anti-CCR4 (1G1; BD); BD Horizon V500-labelled anti-HLA-DR (G46-6; BD) and anti-CD4 (RPA-T4; BD); fluorescein isothiocyanate (FITC)-labelled, anti-CD25 (2A3; BD), anti-CCR6 (53,103; R&D Systems, Abingdon, UK), and anti-PIBF (rabbit polyclonal; Biorbyt, Cambridge, UK); phycoerythrin (PE)-conjugated anti-CCR7 (150,503; R&D), anti-CCR5 (2D7; BD), anti-CCR3 (5E3; BD); APC-labelled anti-CD28 (CD28.2; BD), anti-HLA-G (87G; eBioscience), anti-CXCR3 (1C6; BD), anti-CD69 (L78; BD), and anti-HLA-DR (LN3; eBioscience); PECy7-labelled anti-CD45RA (L48; BD), and anti-CD45RO (UCHL1; BD). B cells were identified using: Qdot(R) 605-labelled anti-CD3 (UCHT1; Invitrogen, Paisley, UK) and CD19 (SJ25-C1; Invitrogen); FITC-labelled anti CD16 (3G8; BD); APC-H7-labelled anti-CD14 (MøP9; BD); and BD Horizon V450-labelled anti CD11c (B-ly6; BD)®.

NK subsets were phenotyped with: BD Horizon V450-labelled anti-

CD56 (B159; BD); FITC-labelled anti-PIBF; PE-labelled anti-iNKT (6B11; BD); APC-labelled anti-TCR-γδ (B1; BD); PE-Cy7-labelled anti-CD16 (3G8; BD); and PerCP-Cy 5.5-labelled anti-CD3 (BD).

DC of myeloid and plasmacytoid lineage, as well as HLA-G expressing tolerant variants were identified using the antibodies: Qdot® 605-labelled anti-CD3 and anti-CD19 (Invitrogen); BD Horizon V450-labelled anti-CD11c (BD); BD Horizon V500-labelled HLA-DR; FITC-labelled anti-CD16 (BD); PE-labelled anti-ILT4 (42D1; eBioscience); APC-labelled anti-HLA-G (eBioscience); PE-Cy7-labelled anti-CD83 (HB15e; BD); PerCP-Cy 5.5-labelled CD123 (&G3; BD); and APC-H7-labelled anti-CD14 (BD).

2.9. Statistical analysis

Statistical analysis was undertaken with support from a medical statistician. All data were initially tested for normality using a Shapiro Wilk test. Longitudinal analysis of vacinnated pregnant and non-pregnant controls was undertaken using mixed-effects modelling to avoid a loss of statistical power by omitting patients with incomplete data. For normally distributed data, a linear mixed effects model was used. When data did not follow a normal distribution, a generalized linear mixed effects model with gamma log-link was used. In both instances, if the main effect was significant, pairwise multiple comparisons of estimated marginal means with baseline and using sequential Bonferroni correction was performed. Data were analysed following the methods outlined by Duricki et al. and using IBM© SPSS Version 21.0 (Armonk, NY, USA) for mixed effects modelling [20].

Pregnant subjects and controls were compared using an unpaired Students t-test where the data were continuous and parametric, and for non-parametric data, Mann Whitney *U* test was used. Correlation analysis was performed to analyse the degree of association between two variables, and Pearson's correlation coefficient is shown. Statistical analysis was performed on GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

Data is presented as mean ± standard error of the mean (SEM) or median ± interquartile range (IQR) as appropriate for the distribution normality. All P-values were two-tailed and significance was defined as $P < 0.05$.

3. Results

3.1. Pregnant women produce a robust antibody response to influenza vaccination

We began by determining the efficacy of the seasonal trivalent vaccine in our pregnant cohort by measuring antibody titres to the viral strains in the vaccine. Immunisation produced robust virus specific antibody responses in both pregnant and control subjects. For most, a rise in antibody titres occurred even if the subjects already had antibody titres above 1:40, which corresponds to protective immunity (Fig. 1A, B). Some of the pregnant patients had protective immunity prior to vaccination, but the majority did not. Pre-immunisation titres in pregnancy were less than controls and although they increased after immunisation, they remained lower (not shown). It should be noted that that controls were healthcare workers and research scientists, many of whom had been immunised in previous years. Despite this, the overall fold increase achieved with vaccination was comparable in both pregnant women and controls, suggesting that the vaccine antibody response was not affected by pregnancy (Supplementary Fig. S1). Moreover, in both cohorts, immunisation increased antibody production to the 2009 H1N1 pandemic virus England/195 (Fig. 1A, B). This strain is both genetically and antigenically related to the A/California/7/2009 strain [21]. Therefore, the increase in titres to this virus may have been due to cross-reactive immunity. In addition, this effect may have been enhanced by improved memory T cell responses post vaccination. We sought to investigate the latter by measuring antigen-specific responses to influenza A as well as other viral and protein antigens and peptides known to elicit both CD4 and CD8 T cell recall responses.

3.2. Vaccination enhances antigen-specific recall responses

In order to determine if antigen specific memory T cell responses were improved post vaccination, we used the highly sensitive ELISpot assay. For an influenza specific antigenic stimulus, we chose influenza A whole lysate since both the 2009–2010 pandemic and 2011–2012 season predominantly saw influenza A strains H1N1 and H3N2 respectively [22,23] and these formed the basis for the vaccine used in the current study. In our control group, IFN-γ responses to influenza A were stable pre and post vaccination, whereas IL-10 responses increased, and TGF-β responses decreased (Fig. 2A). In this instance, the rising IL-10 antigen-specific response probably follows early T cell response, which has been shown to peak at seven days post virus exposure during *in vivo* human infection models [11]. Since TGF-β can enhance survival of central memory CD8 memory T cells whereas its blockade favours the less proliferative effector memory phenotype [24,25], the fall in peripheral TGF-β responses along with increased IL-10 post vaccination may represent increased memory T cell activity (Fig. 3A). When directly comparing controls and pregnant groups prior to vaccination, control subjects responded better to influenza A, producing more IFN-γ SFCs, and this may be due to previous vaccination history. Post vaccination, however, these responses were comparable (Supplementary Fig. S1A). Interestingly, both granzyme B (Fig. 2A) and IL-4 (not shown) responses remained stable despite the improved humeral responses seen earlier (Fig. 1). In our pregnant patients we saw a different response post vaccination, with both IFN-γ and IL-10 SFCs increased but TGF-β, granzyme B (Fig. 2B) and IL-4 (not shown) responses were all stable.

Next, we used a number of recall antigens and peptides to see if our findings with influenza A whole lysate were due to altered memory T cell response post vaccination. In our control subjects, the increase in IL-10 SFCs we saw with influenza A was also seen in response to CMV and measles at two months, and a similar trend with HSV ($P = 0.077$) (Fig. 3A). Similarly, the fall in TGF-β responses at two months were replicated with EBV, PPD and TTOX (Fig. 3A). In addition, we saw a significant increase in IL-4 response to CMV at 2 months and a suggested increase at 1 month in response to TTOX but this decreased at 2 months (Fig. 3A). Overall, this appeared to follow the improved humeral response seen with the flu antibody titres. In pregnancy, the increase in IFN-γ and IL-10 responses to influenza A were replicated for IFN-γ with EBV and TTOX, with the latter shown to be mediated CD4 T cells [26], and for IL-10 with CMV, HSV, PPD and TTOX (Fig. 3B). IL-10 responses were fairly robust with comparable activity against virus lysates. IL-4 responses were largely muted but TGF-β responses, though not significant, appeared to suggest a fall post vaccination, which was very similar to our findings in controls (Fig. 3B). Furthermore, where Granzyme B responses in controls demonstrated no pattern, in pregnancy there was suggestion that one-month post vaccination this predominantly CD8 T cell driven recall response was increased, particularly in response to EBV and TTOX (Fig. 3A, B). Despite this, responses to FEC peptide pool, which contains CD8 epitopes, were not increased (Fig. 3A, B). When directly comparing post vaccination responses in pregnancy to controls, IFN-γ responses to TTOX (Supplementary Fig. S2A) and IL-10 responses to CMV (Supplementary Fig. S2B) were better in controls, but this was not replicated with other stimuli, and both IL-4 and granzyme B responses were largely comparable between groups. However, the fall in TGF-β SFCs produced by controls after vaccination resulted in a significantly lower response at 2 months to TTOX, EBV

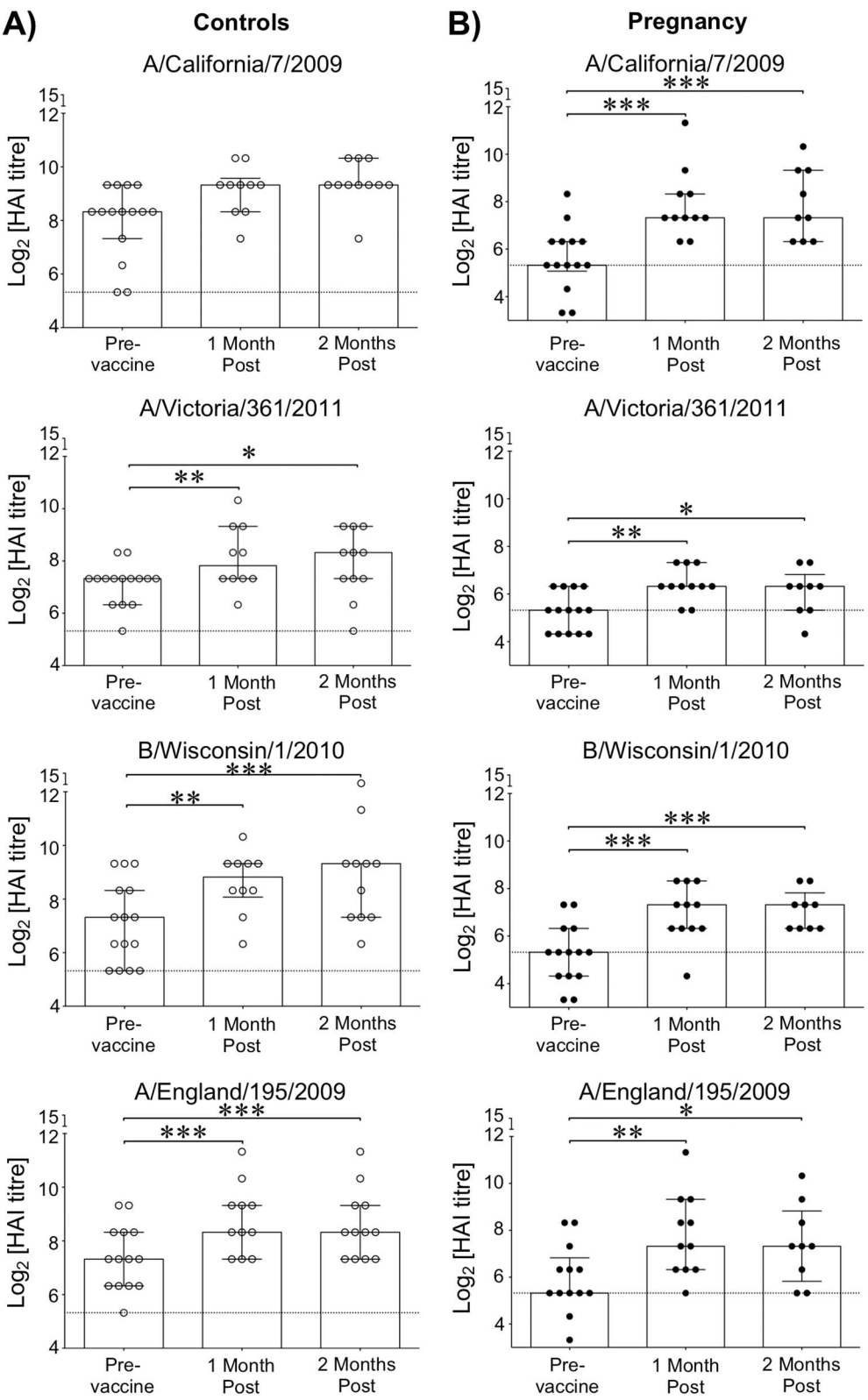

Fig. 1. Longitudinal antibody responses to seasonal influenza virus. A) Log_2 scale of HAI titres pre (N = 15), and one (N = 11) and two months (N = 12) post vaccination in controls (○). B) Log_2 scale of HAI titres pre (N = 14), and one (N = 11) and two months (N = 9) post vaccination in pregnant subjects (●). Depending on the data distribution, longitudinal data was analysed by either a a linear mixed effects model generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means to baseline and with sequential Bonferroni correction. Columns indicate mean or median, and bars SEM or IQR. P values are two tailed and significance is defined as *P < 0.05, **P < 0.01, and ***P < 0.001.

whole lysate, PPD peptide and FEC peptide pool when compared to pregnant patients (Supplementary Fig. S3). Our findings suggested that post vaccination in pregnancy there is maintenance of immune suppressive elements that may serve to expand Tcm subsets, but this is otherwise reversed in controls. We investigated how our observed changes in pregnancy were reflected in the activation of memory T cells, and immune modulation of APCs and cytotoxic NK cells.

3.3. Post-immunisation leukocyte phenotype reflects a contracted immune response

In order to broadly visualise how our ELISpot data was reflected in leukocyte phenotype we used flow cytometry to profile CD4 and CD8 T cells, and their memory subsets, and B cells as well as Tregs and progesterone sensitive NK and γδ T cells. In both controls and in pregnancy

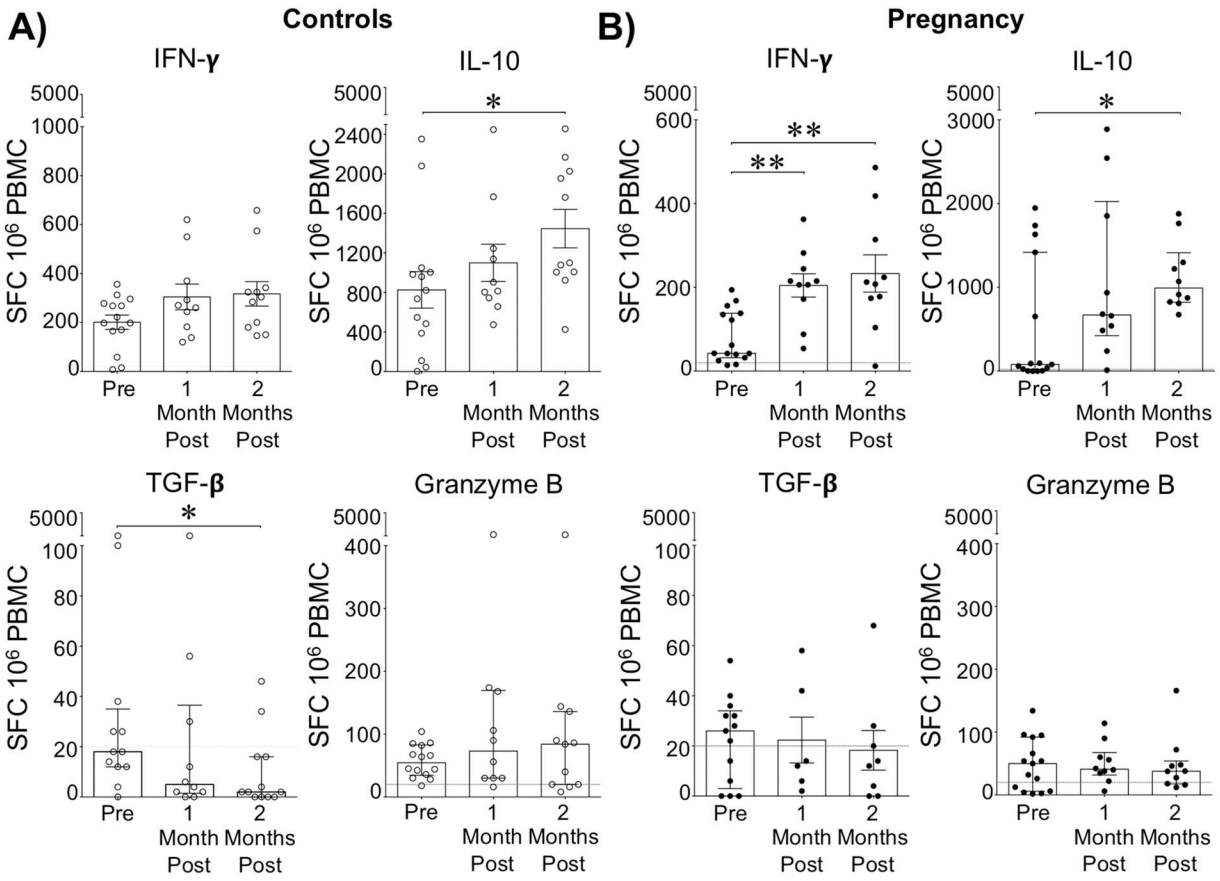

Fig. 2. Longitudinal analysis of IFN-γ, IL-10, TGF-β and Granzyme B ELISpot responses to influenza A. Analyte results are shown for A) controls, and B) pregnant patients. For IFN-γ, IL-10, and Granzyme B, sampling at time points for controls (○): pre (N = 15), and one (N = 10) and two months (N = 11) post vaccination; and in pregnancy (●): pre (N = 14), and one (N = 11) and two months (N = 9) post vaccination. For TGF-β, sampling at time points for controls (○): pre (N = 12), and one (N = 10) and two months (N = 12) post vaccination; and in pregnancy (●): pre (N = 13), and one (N = 6) and two months (N = 8) post vaccination. Depending on the data distribution, longitudinal data was analysed by either a linear mixed effects model generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means to baseline and with sequential Bonferroni correction. Columns indicate mean or median, and bars SEM or IQR. P values are two tailed and significance is defined as *$P < 0.05$ and **$P < 0.01$.

there was an increase in proportions of T cell memory subsets with controls showing greater CD4 Tem and CD8 Tcm, and in pregnancy there were greater CD4 and CD8 central memory T cells, with the former expressing increasing amounts of HLA-DR (Fig. 4). This was also particularly evident in the CD4 Ttemra subset in controls. However, it should be noted that, like most healthy individuals, proportions of these subsets were small [27] and so the surface expression of markers may have a disproportionate impact on our analysis. Nonetheless, HLA-DR expression on memory T cell effectors, in some conditions, has been shown to confer resistance to Treg mediated immune suppression [28]. Both cohorts also saw a fall in exhaustion and proapoptotic markers, PD-1 and annexin V [29], in CD4 and CD8 T cells (Fig. 4). This occurred alongside a fall in CCR5 expression on Tregs (Fig. 4), which is an important chemokine for Treg recruitment to sites of inflammation [30]. However, B cell proportions remained stable post vaccination in both cohorts and their expression of CD11c, which is thought to define a subset of atypical memory cells, remained low [31].

Beyond the above-mentioned similarities, regulation of immune responses appeared to be very different in pregnancy. Whereas in controls we also saw a fall in CD4 and CD8 T cell activation, measured by CD38 and HLA-DR expression, this was not replicated in pregnancy. Post vaccination control subjects experienced an increase in mDCs and subjectively better memory T cell proportions when compared to pregnancy cross sectionally (Fig. 5A and B), and far greater PIBF expression on cytotoxic $CD56^{hi}$ and cytokine producing $CD16^{+}CD56^{lo}$ NK cells, and γδ T cells, as well as HLA-G expression on Tregs (Fig. 5C and D). Although some of these changes were also seen in pregnancy, they were not of the same magnitude. Taken together, our observations in controls, which show greater immune regulation, depicts a contracted immune response post vaccination. However, in pregnancy there appears to be expansion of the Tcm subset post vaccination (Fig. 6A and B) and less pronounced changes in regulatory phenotypes (Figs. 4 and 5).

4. Discussion

This is the first study to investigate, in a single cohort, the adaptive and innate immune response to influenza immunisation in pregnancy. The data presented demonstrate how pregnancy achieves seroprotection post vaccination alongside a partially altered immune response. In pregnancy, immunisation still elicits a potent immune response with *ex vivo* antibody and *in vitro* antigen specific responses largely comparable to non-pregnant controls. However, unique to pregnancy, the antigen response is associated with expanded proportions of memory T cells, and a loss of or tendency for minimal immune regulation relative to non-pregnant controls.

In keeping with previous published reports, we found that virus specific antibody titres increased post immunisation in both pregnant and non-pregnant individuals [2]. Our pregnant patients had not been previously immunised and therefore their baseline antibody titres were lower than controls, many of whom had been immunised in the previous year. Vaccination also resulted in antibodies being produced

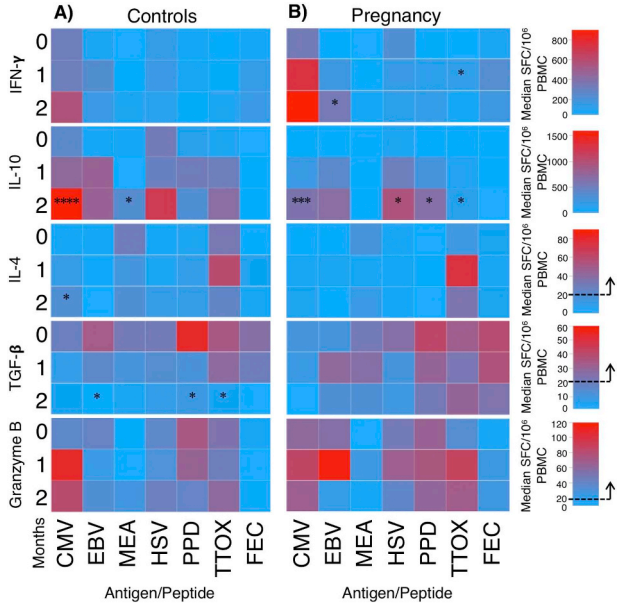

Fig. 3. Longitudinal analysis of IFN-γ, IL-10, IL4, TGF-β and Granzyme B to recall antigens and peptides. A summary heatmap of median ELISpot responses to antigens/peptides is shown for A) controls and B) pregnant patients for each analyte. The individual scales used for each cytokine is shown with the threshold of 20 SFC/10^6 PBMC indicated. The scale used for IL-10 responses to measles was 0–120 SFC/10^6 for better visualisation. The heat-map was generated and analysed using SPICE [85]. Data was analysed using a generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means to baseline and with sequential Bonferroni correction. Where differences were significant, on the heatmap, P values are indicated and defined as *P < 0.05, **P < 0.01, and ***P < 0.001.

against our control virus, H1N1 subtype A/England/195/2009, in both cohorts. This was not surprising as the strain is both genetically and antigenically related to the A/California/7/2009 virus and the haemagglutinin differs by only three amino acids [21]. Other reports have shown that *in vivo* infection with pandemic strains and *in vitro* PBMC cultures using influenza A virus, are able to elicit cross-reactive immunity [21,32,33]. This occurs because cell-mediated responses required for antibody production are not strain specific but are capable of targeting other influenza A subtypes [34]. This is characteristic of immunisation [35–37]. In fact, both TIV and live attenuated influenza vaccine (LAIV) have been shown to induce type 2 interferon (IFN-γ^+) expressing T and NK cells as well as memory NK cells with better recall responses [38–41]. Although pregnancy tends to favour a more potent inflammatory response to influenza A virus, with increased NK and T cell IFN-γ and MIP-1β production, other interferon responses such as IFN-α and IFN-λ responses are attenuated but improve post immunisation [13,42]. Our findings showed that influenza A antigen specific IFN-γ responses increased post immunisation in pregnancy but not in controls. However, IL-10 responses increased in both cohorts. Whereas the IFN-γ responses likely reflect improved immunogenicity that may be anti-flu or represent a pregnancy-specific inappropriate immune response as seen in lung tissue in murine models [43], the IL-10 data may represent the contraction phase of the immune response following antigen exposure and clearance and is thus seen in both cohorts. Despite this finding, expression of CD4 and CD8 T cell pro-apoptotic and exhaustion markers appeared to reduce in both groups post-vaccination. Although in murine models, apoptosis is positively associated with antibody responses [44], PD-1 expression on virus specific CD8 T cells results in dysregulated responses and increased viral titres [45], and overexpression of senescence makers on influenza specific memory CD8 T cells negatively correlates with antibody production in older patients [46]. Therefore, reduced expression of PD-1 and annexin V on CD4 and CD8 T cells, most likely reflects immunisation-related improved immune response.

Another important variation between controls and pregnant patients, was the TGF-β response to influenza A, as well as following stimulation by a number of other antigens and peptides. TGF-β is a multifunctional immunomodulatory protein that has a role in CD4 memory T cell maintenance, and also inhibits effector T cell proliferation as well as cytokine and cytolytic activity [47]. This is also partly due to the influence of TGF-β on the generation of FoxP3$^+$ Tregs [48]. This protein is also important for T follicular helper (Tfh) differentiation and in both human *in vitro* work and murine experiments, it has been shown to regulate apoptosis of Tfh cells and control T helper signalling to previously activated B-cells that produce antibody [49–51]. In controls, these responses were significantly reduced post vaccination. Similar to our findings, Wang et al have previously shown that there is a negative correlation between increasing levels of IL-10 and TGF-β after influenza vaccination, where the latter results in suppression of the antibody response [52]. However, in our study, in pregnancy, this fall in TGF-β was not replicated to the same extent. In controls, these results suggest a contracted effector and memory response, which is not unusual, particularly since TIV is not a self-replicating vaccine that provides long-standing immunity. In pregnancy, however, TGF-β is a potent mediator of immune tolerance and is generally found in higher concentrations, so the apparent lack of change in our pregnant patients may represent a sustained TGF-β response for other functions [53,54].

Memory T cells that interact with MHC molecules from a range of APC and B cells, unlike their naïve counterparts, are thought to facilitate heightened immune protection [55,56]. Ideally, IL-2 producing memory CD4 and CD8 T cells and CCR7 expression on CD8 T cells enable enhanced proliferative capacity and retention in secondary lymphoid organs to provide improved systemic protection [57]. In the current study, both CD4 and CD8 T cell CCR7$^+$ Tcm proportions were expanded in pregnancy post vaccination, whereas in controls only CD8 Tcm saw a modest increase. Alongside this expansion, our results also suggested a fall in Tem and Ttemra proportions in pregnancy, although there was also a small increase in the expression of HLA-DR on these subsets. In controls, however, HLA-DR expression on CD4 Ttemra was significantly increased. In rheumatoid arthritis, which typically improves in pregnancy, Fonseka et al have shown that effector memory HLA-DR$^+$ cells express a distinctive transcriptomic signature with Th1 and cytotoxicity-associated genes and are able to produce abundant IFN-γ and granzyme A upon stimulation [58]. In contrast to Tcm, these non-proliferative memory T cells and subsequent effector responses following stimulation are not typically required two months post immunisation. In addition, overexpression of HLA-DR on memory T cells is also associated with non-responsiveness [59,60]. This being said, Ttemra proportions are relatively small in healthy individuals but the subset has been shown to expand with increasing age and CMV seropositive status [61]. CMV serology was unknown in our cohorts but UK prevalence is reported as being 40% in adults and approximately 50% in pregnant women in most industrialised countries [62,63]. Interestingly, in controls, proportions of mature mDC expressing CD83, which are potent APC and T cell stimulators, showed an increase post vaccination. The same changes were not seen in pregnancy. In the context of influenza A, these DC confer resistance to infection and show improved anti-viral type I IFN production [64]. We have previously shown that proportions of mDC and CD83$^+$mDC do not significantly change during pregnancy [19,65]. Therefore, our findings may reflect pregnancy-specific active suppression of DC function, which may negatively impact on the vaccination related benefits of an expanded Tcm population.

The disparity between controls and pregnant patients in what would be considered a protective immunisation response was extended to other aspects of immune modulation. Although IL-10 influenza A responses improved in both groups post vaccination, and these are

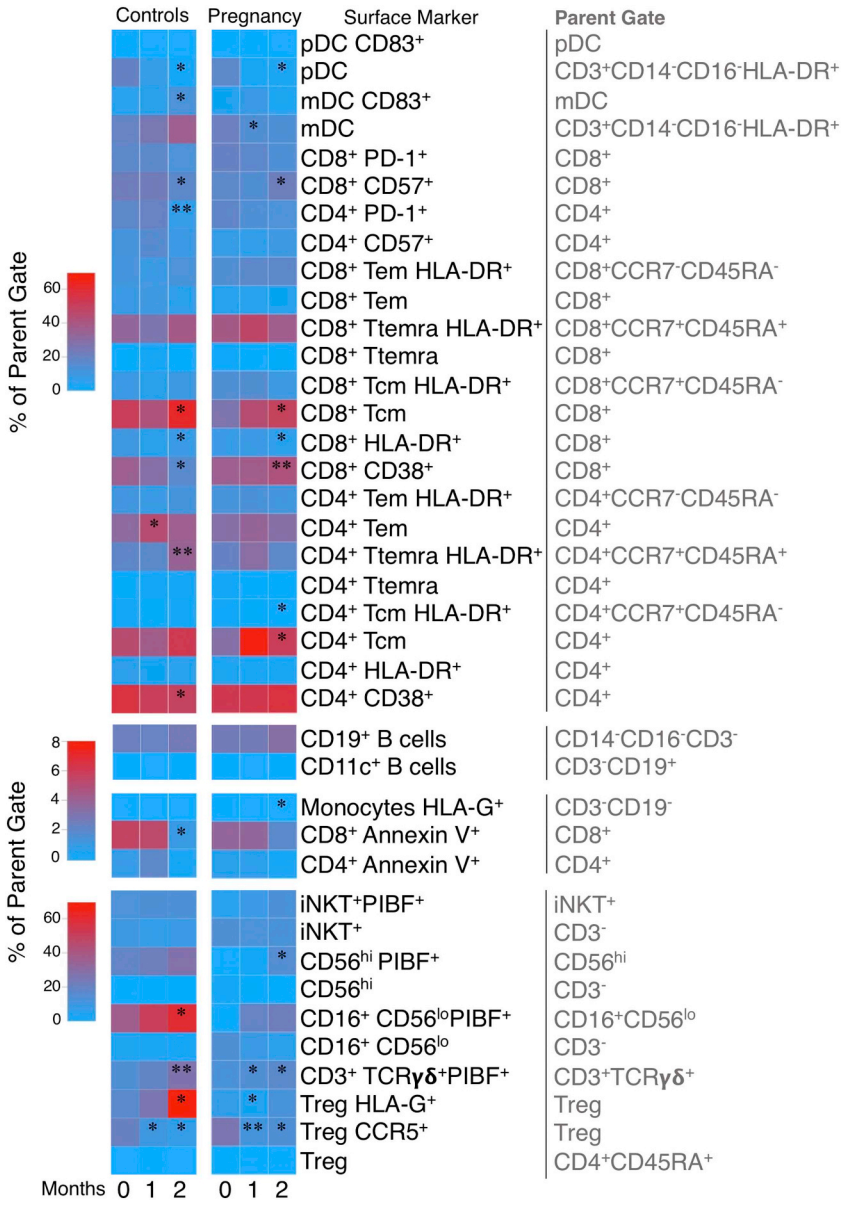

Fig. 4. Longitudinal analysis of leukocyte phenotype pre and post vaccination in controls and pregnant patients. Flow-cytometric phenotypic analysis was undertaken for unstimulated PBMC obtained from both controls and pregnant subjects. Shown is a summary heatmap of the mean/median proportion of expression of each marker/leukocyte subset, depending on the data distribution, in relation to the parent gate as indicated. Depending on the data distribution, longitudinal data was analysed by either a linear mixed effects model generalized linear mixed effects model with gamma log-link and pairwise multiple comparisons of estimated marginal means to baseline and with sequential Bonferroni correction. Where differences were significant, on the heatmap, P values are indicated and defined as *P < 0.05 and **P < 0.01.

associated with better antibody production and generation of serological memory [52,66,67], Treg proportions were stable, and in pregnancy, IL-10 producing tolerant DC were reduced [68]. However, CCR5 expression on Tregs was reduced and since CCR5 expression is required for homing and recruitment of Tregs, this may reduce migration of Tregs to the peripheral compartments without affecting suppressive function as suggested in murine studies and human HIV positive *in vivo* and *in vitro* work [30,69,70]. Post-vaccination in controls, HLA-G^+ Tregs accumulated in peripheral blood to a greater extent when compared to in pregnancy, and HLA-G expression on Tregs corresponds to a subtype that can modulate T cell responses without the need for APC [71]. This increase in Tregs with a broad immunomodulatory potential in controls is likely to reflect a contracted immune response post immunisation. Similarly, the increased expression of PIBF on $CD3^+$TCR-$\gamma\delta^+$, $CD16^+CD56^{lo}$, $CD56^{hi}$ and iNKT subsets in controls, post vaccination, probably also follow a decline in immune response, since PIBF expression is negatively associated with NK function and inflammatory T cell cytokine production [72,73]. PIBF is produced following leukocyte interaction with progesterone, which in turn is an important neuroendocrine immune modulator in pregnancy [74–77]. In fact, progesterone has been shown to be a key immune-modulator for anti-viral and cytolytic immune responses in pregnancy [19]. However, as we have previously shown, PIBF expression on NK and γδ-TCR^+ T cells does not vary a great deal across subsets with gestation in normal healthy pregnancy [19]. In non-pregnant mice, progestogen treatment protects against poor outcome during primary H1N1 infection despite reduced anti-viral antibody production and CD8 memory T cell responses [78]. However, following a secondary challenge with H3N2 influenza A, survival amongst these mice was reduced compared to controls, even though progestogen treatment did not reduce hemagglutinin stalk antibody titres [78]. It is interesting then, that in pregnancy we did not show a similar level of post immunisation immune regulation, and this may suggest that the vaccine effects on T and NK cells is less potent compared to non-pregnant controls.

Taking the approach of using global antigenic stimuli to assess cellular function gives a broad overview of vaccine effects. However, it is prone to influences from previous exposure the individual will have had including their CMV serological status, Bacillus Calmette–Guérin (BCG) vaccine history and/or tuberculosis (TB) exposure, and tetanus vaccine history, as well as the effects of gestation in the pregnant cohort. We have previously shown that IFN-γ and IL-10 responses are

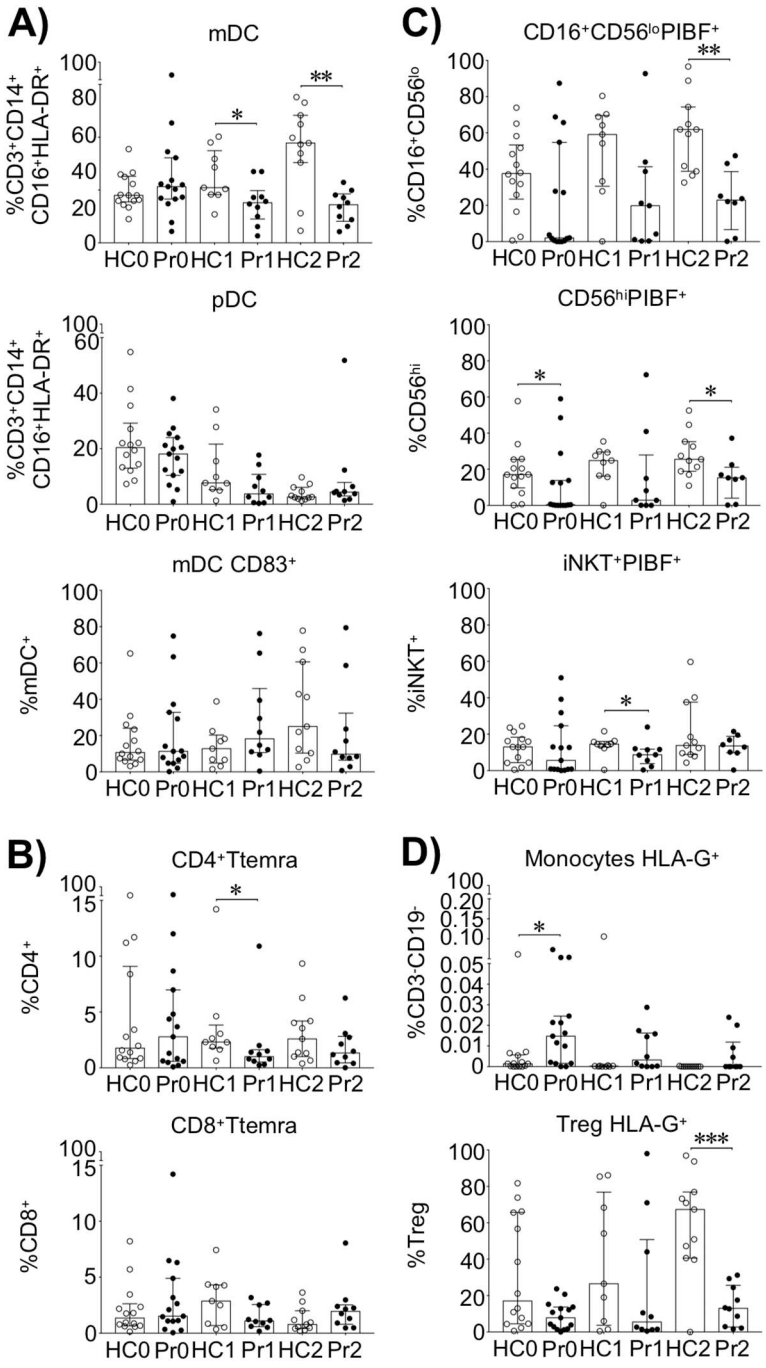

Fig. 5. Cross-sectional analysis of control and pregnant leukocyte phenotypes comparing proportions of DC, CD4 and CD8 Ttemra, NK subsets and iNKT, tolerant DC, and HLA-g$^+$ Tregs. Shown is the comparison between controls and pregnant patients across timepoints of A) mDC (CD3$^-$CD19$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD11c$^+$CD123$^-$), pDC (CD3$^-$CD19$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD11c$^+$CD123$^+$) and CD83$^+$mDC proportions; B) CD4 and CD8 Ttemra (CCR7$^-$CD45RA$^+$) proportions; PIBF expression on CD16$^+$CD56^{lo}, CD56^{hi} NK subsets and iNKT cells; and tolerant DC (CD3$^-$CD19$^-$CD11c$^+$HLA-DR$^+$CD14$^+$CD16$^+$CD123$^+$ILT4$^+$CD83$^+$HLA-g$^+$), and HLA-g$^+$ Tregs (CD4$^+$CD45RO$^+$CD25$^+$CD127^{lo}) proportions. Sampling was done at time points: pre-vaccination (HC0, N = 12; Pr0, N = 13), one-month (HC1, N = 10; Pr1, N = 6) and two months (HC2, N = 12; Pr2, N = 8) post vaccination. Cross-sectional analysis was undertaken using an unpaired Mann Whitney *U* test. Columns indicate median and bars IQR. P values are two tailed and significance is defined as *P < 0.05, **P < 0.01, and ***P < 0.001.

stable during pregnancy but peak at delivery, whereas IL-4 and Granzyme B responses peak at approximately 34 weeks of gestation but not necessarily in response to influenza A [19]. In the current study, by using linear regression analysis of baseline ELISpot responses to influenza A and antibody titres, we show that there were no gestational changes in either response, other than the antibody titre to B/Wisconsin/1/2010 virus, which fell significantly with gestation. Previous studies have investigated mean increases in maternal HAI titres post-vaccination at different gestations of pregnancy and have not shown a benefit in vaccinating with TIV at a specific time point during pregnancy [79,80]. In a study by Katz et al the authors showed a mean increase in HAI titres in the third trimester but these differences were not statistically significant [79]. In our study, whilst baseline antiviral antibody and cell-mediated responses to influenza A were unaffected by gestation, the extent of seroprotective vaccination response at one month was influenced by the timing of vaccination. Namely, when comparing the fold increase in antibody titres at one-month post vaccination for patients recruited at < 20 weeks of gestation compared to those > 20 weeks of gestation, later gestations were associated with better antibody production against A/California/7/2009 and B/Wisconsin/1/2010 viruses. However, these numbers were small and so the data needs to be interpreted with caution. Irrespective, future flu vaccination work should control for gestation to tease out the effect this may have on vaccination responses.

Whilst comparable demographics (ethnicity and parity), standardised UK vaccination programmes, and occupational screening for healthcare workers that requires BCG status and vaccination for tetanus, will help to mitigate some of the effects of previous vaccines, they may still have impacted our findings. This was particularly evident in our controls were previous influenza exposure and vaccination led to

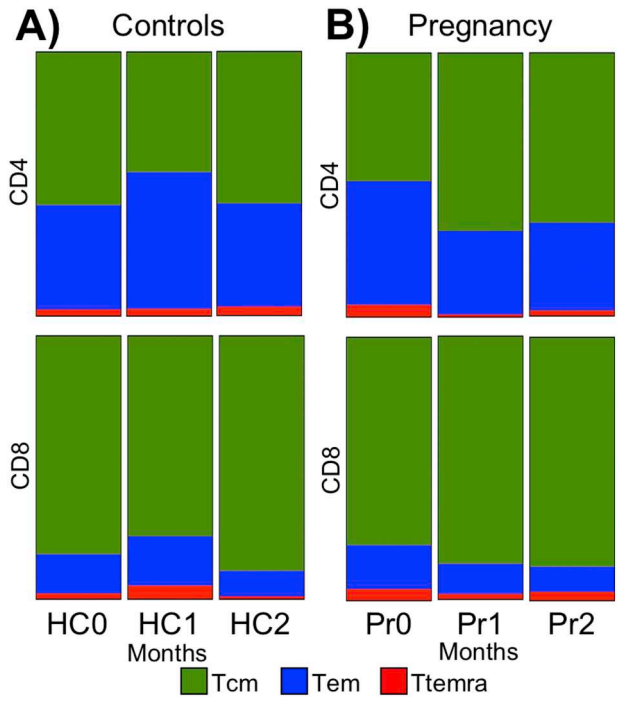

Fig. 6. Distribution of memory T cell subsets in controls and pregnant patients, pre and post immunisation. Shown are the mean/median proportions of Tcm ($CCR7^+CD45RA^+$), Tem ($CCR7^-CD45RA^-$) and Ttemra ($CCR7^-CD45RA^+$) in both cohorts, depending on the data distribution, across time points: pre-vaccination (HC0, Pr0), one-month (HC1, Pr1) and two months (HC2 , Pr2) post vaccination.

greater baseline antibody titres and IFN-γ responses. Our non-pregnant controls included both healthcare workers and research scientists who would be interacting with maternity patients and so were offered influenza vaccination. Although some of our controls were previously vaccinated, a proportion were vaccination naïve. Analysing these two subgroups, we found that baseline HAI titres for A/California/7/2009, B/Wisconsin/1/2010 (Supplementary Fig. S4) and A/England/195/2009 (not shown) in vaccination naïve controls were intermediate and not significantly different to either pregnant women or previously vaccinated non-pregnant controls; or, in the case of A/Victoria, similar to pre-vaccinated controls, but significantly higher than in pregnant women (Supplementary Fig. S4). This may be because exposure to influenza virus and the incidence of influenza infection in healthy individuals working in the healthcare setting is greater than in the general public, contributing to the quantity of pre-vaccination anti-flu antibody titres. A similar pattern of findings was observed when reviewing pre-vaccination IFN-γ ELISpot responses to influenza A whole lysate, where the number of SFC from PBMC obtained from vaccine naïve controls was not greater than observed in pregnant patients, while the response of previously vaccinated controls was greater than in pregnant patients (Supplementary Fig. S4). Previous *ex vivo* studies investigating vaccinations across two seasons has suggested that cellular responses (IFN-γ) are not further enhanced by repeat vaccination but antibody production may benefit [81,82]. Our results with both subgroups of controls support this finding. It is also possible that the difference between controls and pregnant patients is due to immune-modulation in pregnancy, which may be associated with suppression of inflammatory responses as shown previously [83]. However, cellular responses to other antigens in our patient group were not supressed, suggesting a vaccine specific effect. Therefore, future studies on the response to flu vaccination should categorise participants into flu-vaccination naive or pre-exposed groups and make comparisons of each group separately. In addition, future studies should include vaccine-specific functional experiments using virus strains found in the seasonal TIV as well as flow-cytometric assessment using intracellular cytokine staining to determine which leukocyte subsets were involved.

5. Conclusions

The ability to imprint robust CD4 and NK cell memory enables better recall responses and improves vaccine efficacy. Current measures of vaccine responsiveness rely on antibody titres, but this is ultimately of limited value and only represents a surrogate marker for clinical efficacy as it does encompass anti-viral function. During influenza infection, CD4 help is important for both antibody function and cytotoxic T lymphocyte activity. Measuring these responses in addition to antibody titres to determine vaccine efficacy is particularly important in patient groups, such as in pregnancy, where these responses are modulated. During the 2009 H1N1 outbreak, mortality and morbidity in the pregnancy population was significantly greater than the non-pregnant cohort, with the risk of death four times as high [84]. Even in the vaccinated population, pregnancy is associated with comparatively poor clinical benefit with a greater number of patients with symptoms of influenza-like illness, and hospitalisation with laboratory confirmed influenza [5,8]. Our findings suggest that influenza vaccination in pregnancy does not result in the same memory T cell and NK cell responses as non-pregnant patients. This may explain the driving force behind the severity of influenza infection in pregnancy that is associated with significantly increased maternal mortality despite vaccination strategies. That being said, vaccination is still associated with significant benefits for both mother and baby should be recommended for all pregnant women.

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Author contributions

NMS, NI, and MRJ had a substantial contribution to the conception and design of the project. NMS, NI, WSB and MRJ were responsible for the acquisition, analysis, and interpretation of the data, and drafted the work. Virus propagation and HAI assays were performed by NMS with assistance from WSB's laboratory. NMS performed the flow cytometry and ELISpot experiments. All authors contributed to revising of the manuscript and have approved the final version.

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